

Full Length Research Paper

Evidence for an *in vitro* anticoagulant and antithrombotic activity in *Tulbaghia violacea*

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The aim of this study was to investigate the *in vitro* antithrombotic and anticoagulant properties in *Tulbaghia violacea*. The bulb and leaf extracts of *T. violacea* and garlic showed that *T. violacea* exhibited antithrombotic activities which were higher than those found in garlic. The IC₂₀₀ values for the leaf and the bulb extract were 0.4 and 0.3 mg/ml, respectively for the TT assay. The IC₅₀ value was 1.73 mg/ml for the bulb extract of the *T. violacea*. No IC₅₀ was obtained for the leaf extract of *T. violacea*. IC₂₀₀ and IC₅₀ values could not be determined for the garlic extract. Seasonal studies were also conducted and indicated that the activity obtained for the aqueous extract was lost during the winter season. Throughout the course of this study it was observed that *T. violacea* exhibited biological activities which were comparable to garlic. These results indicate that *T. violacea* can be used as an alternative to garlic and that it may contribute to pharmaceutical applications and informal health services.

Key words: Anticoagulant, antithrombotic, garlic, thrombin inhibition, *Tulbaghia violacea*.

INTRODUCTION

A great deal of research has been completed and published on the anticoagulant and antithrombotic activities of garlic (*Allium sativum*). The beneficial effects of garlic include lowering of plasma cholesterol, decrease of fibrinogen, coupled with increased fibrinolytic activity, and inhibition of platelet activity (Agriga and Seki, 2006; Bordia, 1978; Mohammad and Woodward, 1986; Rivlin, 2006; Srivastava, 1986). The therapeutic actions of garlic and its constituents have been well documented. These include antithrombotic and anticancer effects (Mohammad and Woodward, 1986; Srivastava, 1986; Makheja and Bailey, 1990). These beneficial effects result in the improvement of blood fluidity. However, as garlic significantly enhances fibrinolytic activity, it is theoretically

possible that its over-activity could cause platelets to aggregate through the release of fibrinogen degradation products (FDP) because it has been reported that excessive fibrinolysis is associated with the release of FDP (Bordia, 1978). Both aqueous and oil extracts of garlic have been shown to inhibit platelet aggregation *in vitro* and *in vivo* (Bordia et al., 1996; Makheja and Bailey, 1990; Fukao et al., 2007). The inhibitory effect of processed garlic on human platelet aggregation has been known since 1978 (Lawson et al., 1992). According to Mohammad and Woodward (1986), when an aqueous extract of garlic was added to platelet rich plasma (PRP), platelet aggregation was inhibited. These findings clearly showed that garlic has anticoagulant properties.

Tulbaghia violacea (*T. violacea*), a plant that has been postulated to have similar activities as garlic however, has not been extensively assessed scientifically in terms of its biological activities. Only a few publications have reported on the biological activities of *T. violacea*, as compared to garlic.

T. violacea is commonly known as wild garlic, wilde knoffel (Afrikaans), isihaqa (Zulu) or itswele lomlambo (Xhosa). *T. violacea* is indigenous to the Eastern Cape region of South Africa and is widely used as a herbal remedy for various ailments, with leaves and bulbs the most commonly used. Its medicinal uses include treat-

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Abbreviations: AGE, aged garlic extract; DADS, diallyl disulphide; DAS, diallyl sulphide; DATS, diallyl trisulphide; DMBA, 7,12-dimethylbenz (a) anthracene; DMSO, dimethyl sulphoxide; IC₂₀₀, doubling of the clotting time; IC₅₀, 50% inhibition; TT, thrombin-induced clotting time; TXB₂, thromboxane B₂; SAC, S-allyl ethylcysteine; SAMC, S-allyl methylcysteine; *T. violacea*, *Tulbaghia violacea*.

ment for fever and colds, asthma, tuberculosis, and stomach problems. The leaves of the plant are used to treat oesophageal cancer and may also be eaten as vegetables. The plant is also used as a snake repellent. In spite of its usefulness, adverse effects and fatalities have been reported following treatments with extracts of *T. violacea*. These include gastroenteritis, abdominal pain, acute inflammation and sloughing of the intestinal mucosa, contraction of the pupils and subdued reaction to stimuli (Mac Donald et al., 2004; van Wyk et al., 1997; van Wyk and Gericke, 2000).

None of the activities of *T. violacea* listed above have to date, been tested *in vitro*. In literature it is postulated that *T. violacea* may have similar biological activities as garlic since they belong to the same family (both belonging to the Order Asparagales, Family Alliaceae) and both have the characteristic sulphur smell of garlic. It is postulated that the plants may have similar secondary metabolites and that the activity might be due to compounds similar to those found in garlic (van Wyk et al., 1997; van Wyk and Gericke, 2000; Burton, 1990; Thamburan et al., 2006; Jacobsen et al., 1968; Bungu et al., 2006).

The known biological activities of *T. violacea* include antibacterial and antihypertensive effects (Motsei et al., 2003). Gaidamashvili and Van Staden (2001) found *T. violacea* to have antibacterial activities against *Staphylococcus aureus* and *Bacillus subtilis*. Duncan et al. (1999) utilizing the angiotensin converting enzyme assay, screened for antihypertensive properties. *T. violacea* leaves had inhibition levels which were above 50% (aqueous extract gave 72% inhibition, ethanol extract exhibited 61% inhibition), while *T. violacea* roots gave 49 and 27% inhibition for aqueous and ethanol extracts, respectively, with 25 µg plant extract.

Traditional medicine has gained popularity over the past 20 years and this is backed by epidemiological evidence (Rahman, 2003; Kingston and Newman, 2002; Mans et al., 2000; Farnsworth, 1984). The medicinal properties of garlic have been attributed to sulphur compounds present in these extracts (Agarwal, 1996; Hirsh et al., 2000; Knowles and Milner, 2001; Sundaram and Milner, 1993, 1996). These sulphur compounds are formed when alliinase, an enzyme present in intact garlic and all other plants belonging to the *Allium* species, reacts with alliin when the plant is crushed.

Sulphur compounds and alliinase have been isolated from *T. violacea* (Burton, 1990; Kubek et al., 2002). However, in different plants belonging to the *Allium* species alliinase has been found to differ in physical and kinetic properties although they catalyse the same reactions, i.e. the hydrolysis of S-1-propenyl, S-propyl, and S-methyl cysteine sulphoxides. Several results have been reported on the isolation and characterization of this enzyme in *Allium* species, including *Allium sativum*, *Allium cepa*, *Allium odorum*, *Allium bakerii* and *T. violacea*. According to these reports the pH optimum of alliinase from garlic

and *T. violacea* is 6.5 and for onion, 8.5. The enzyme from garlic has a relative molecular weight of 85 000 and consists of two subunits of relative molecular weight 42 000 each (Nock and Mazelis, 1986, 1987). The presence of three unidentified sulphoxide amino acids has been reported in *T. violacea* (Jacobsen et al., 1968). These observations show that there are similarities between *T. violacea* and garlic and therefore it might be possible that these plants have similar active compounds (Jansen et al., 1989; Jacobsen et al., 1968).

In this study, the antithrombotic and anticoagulant activities of *T. violacea* were investigated, using the thrombin-induced clotting time (TT) and the thrombin assay, to provide evidence that *T. violacea* can be used as an alternative to garlic. This would benefit traditional healers within the South African context where traditional healers commonly use *T. violacea* for treating their patients for various ailments.

MATERIALS AND METHODS

Preparation of methanol and aqueous extracts

Fresh plant material, collected early in the morning, was washed gently under running water to remove dust and soil and separated into leaves and bulbs, which were extracted immediately. Aqueous and methanol extracts of garlic bulbs, *T. violacea* leaves and *T. violacea* bulbs were prepared separately. Plant parts (10 g) were chopped and homogenized in a blender with 50 ml of either saline (0.9% (w/v) NaCl) or methanol at 4°C. The crude extracts were incubated at 37°C for 15 min, followed by centrifugation at 1500 g for 10 min at 4°C (Mohammad and Woodward, 1986). The supernatant was filtered using Whatman No 1 filter paper to remove residual plant material. The aqueous extracts were tested immediately except where stated otherwise. The methanol extracts were dried under vacuum and stored at 4°C in the dark. On the day of the assay the dried extracts were re-dissolved in DMSO and diluted with saline to yield a final DMSO concentration of 0.5% (v/v) for anticoagulant activities.

Preparation of infusions

Boiling water (50 ml) was added to 2.5 g of chopped plant material. The mixture was left to stand overnight, and then filtered through Whatman No 1 filter paper. The filtrate was then tested for biological activity.

Assays for determining the anticoagulant and antithrombotic activity

Thrombin - induced clotting time assay (TT)

This assay measures the prolongation of thrombin generation. When human plasma is incubated with a compound which inhibits blood coagulation, and thrombin is added to initiate coagulation, the time taken for the clot formation will be prolonged as compared to the control (no inhibitor added). In this assay, 200 µl of human plasma (pre-incubated at 37°C for 5 min before use) was incubated with 50 µl of inhibitor (or buffer for control) for 5 min at 37°C. One hundred µl of bovine thrombin (2.5 U/ml, Sigma) was added to initiate the reaction. The time for clot formation was recorded (Dong

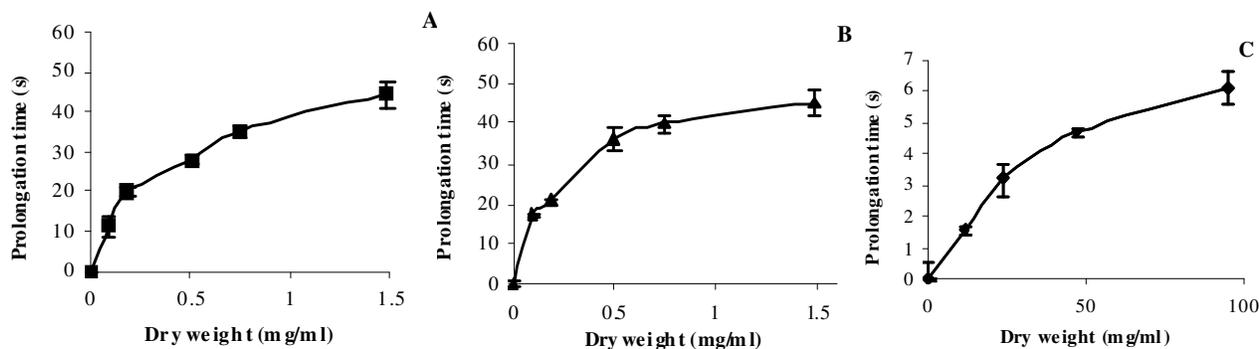


Figure 1. Prolongation of clot formation by aqueous extracts of (A) *T. violacea* bulbs; (B) *T. violacea* leaves; and (C) garlic bulbs (mean \pm SD, $n=3$).

Table 1. Prolongation of clot formation by infusions of *T. violacea* bulbs, *T. violacea* leaves and garlic bulbs (mean \pm SD, $n=3$).

Compound	Dry weight (mg/ml)	Prolongation time (s)
<i>T. violacea</i> bulbs	2	17 \pm 5
<i>T. violacea</i> leaves	2	12 \pm 2
Garlic bulbs	2	3 \pm 2
Garlic bulbs	20	18 \pm 1

et al., 1998). Heparin was used as a positive control. Results are expressed as a prolongation time, relative to the control, or as a clotting time and the control is indicated in the graph.

Thrombin inhibition

A modified method of Rob et al. (1997) and Nakajomi and Ajisaka (1990) was used to measure thrombin inhibition. The method uses a chromogenic substrate specific for thrombin, S2238 (α -Phe-L-Pipecolyl-L-Arg-*p*-nitroanilide) [Chromogenix]. Bovine thrombin (10 μ l, 30 U/ml) was incubated with each extract (50 μ l) at room temperature for 5 min. The substrate (190 μ l, 0.75 mM) was then added to initiate the reaction. The change in absorbance was measured at 410 nm every 10 s for 190 s.

Ethical clearance

All experiments undertaken in this study were sanctioned and authorised by the Human Ethics Committee of the Nelson Mandela Metropolitan University, conforming to the guidelines of the Declaration of Helsinki and Tokyo for humans, and was approved by the Research Directorate of the institution.

RESULTS AND DISCUSSION

A comparison of the *in vitro* activity of various extractions of *T. violacea* and garlic

To validate the traditional use of *T. violacea*, initial experiments compared the anticoagulant activity of infusions of

T. violacea bulbs and leaves and garlic bulbs to their aqueous extraction. A comparison between the anticoagulant and antithrombotic activities of infusions and aqueous extracts of *T. violacea* bulbs and leaves, and garlic bulbs was performed. Two assays, namely the thrombin assay and the prolongation of the thrombin-induced clotting time (TT) assay, were employed.

Thrombin-induced clotting-time

It was noted that at 0.5 mg/ml the aqueous extracts of the bulbs (Figure 1A) and leaves (Figure 1B) had prolonged the formation of thrombin clot by 28 ± 1 and 36 ± 2 s, respectively. To confirm this, the IC_{200} values were calculated and were 0.4 mg/ml for the bulbs and 0.3 mg/ml for the leaves. However no prolongation of thrombin clot formation was observed for the aqueous extracts of the garlic bulbs at 0.5 and at 45 mg/ml garlic had a prolongation time of only 4.5 ± 1 s, with no IC_{200} value being obtained (Figure 1C).

All three infusions did prolong the TT, however, none of them could double the clotting time (IC_{200} not reached) at the concentrations tested (2 mg/ml for *T. violacea* leaves and bulbs and 2 and 20 mg/ml for garlic bulbs). These results are summarized in Table 1, where it can be seen that infusions of *T. violacea* bulbs were more active than the infusions of the *T. violacea* leaves and garlic bulbs, as was the case with aqueous extracts (Figure 1). The relatively low activities of the infused extracts as compared to the aqueous extracts could be due to incomplete extraction during the preparation of infusions, and/or the possible effect of the boiling water on the active compound(s). The effect of heat on the plant activities will be discussed later.

Thrombin inhibition

In addition to the TT, aqueous extracts of *T. violacea* bulbs and leaves and garlic bulbs were compared for thrombin inhibition. The results are shown in Figure 2 and

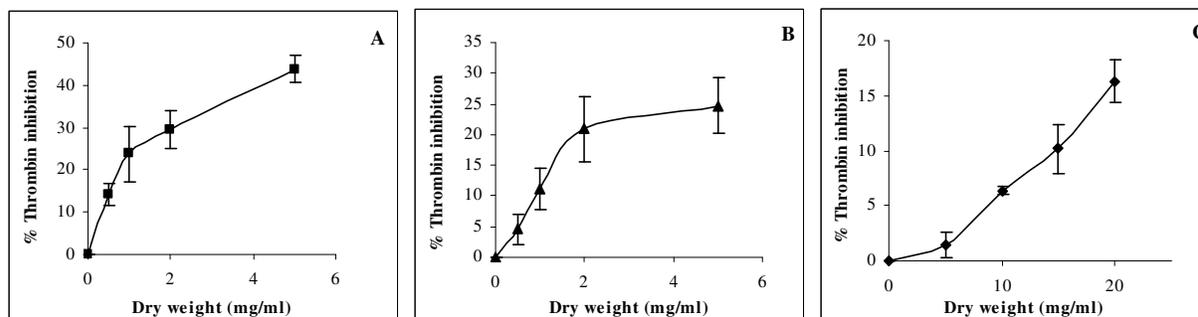


Figure 2. Thrombin inhibition by aqueous extracts of (A) *T. violacea* bulbs; (B) *T. violacea* leaves; and (C), garlic bulbs (mean \pm SD, n = 3).

indicate that all three extracts inhibited thrombin, however, none of them reached the IC_{50} value. The garlic bulbs again showed less activity as compared to the *T. violacea* bulbs and leaves. During the TT the *T. violacea* leaves showed more activity than the *T. violacea* bulbs (Figure 1), but for the thrombin assay the bulbs of *T. violacea* had a higher inhibition than the leaves (Figures 2 A and B). At 5 mg/ml the *T. violacea* bulbs, leaves and garlic bulbs had inhibited thrombin by 43.8 ± 3.2 , 24.7 ± 4.6 and $1.4 \pm 1.1\%$, respectively.

In literature, anticoagulant activities are reported among garlic's health benefits. In a study by Makheja and Bailey (1990), antiplatelet activities of garlic and onion were compared. It was found that the total antiplatelet activity which was present in garlic was five times more potent than onion (17250 and antiplatelet units per 100g fresh weight for garlic and onion, respectively). It was therefore expected that garlic would exhibit more activity when compared to *T. violacea*. However, as was seen in Figures 1 and 2, this was not the case. Some researchers have observed a lower than expected effect for garlic's anticoagulant activities. For example, Rahman and Billington (2000) measured platelet aggregation in response to adenosine diphosphate (ADP) after dietary supplementation with 5 ml of aged garlic extract (AGE) for 13 weeks. They found that the rate of ADP-induced aggregation was not significantly affected by ingestion of AGE.

In order to explain these differences, Rahman and Billington (2000) reported that the problem in investigating the health promoting effects of garlic is that many different preparations and doses have been used. These preparations (pressed garlic juice, garlic oil, dry powder, liquid extracts, dried extracts and oil macerates) have compositions which vary widely.

The effect of heat on anticoagulant and antithrombotic activity of *T. violacea*

It was speculated that heat may affect the inhibitory activity of the extracts. This question arose as infusions

(which are prepared by soaking the plant in boiled water), had negligible inhibitory activity compared to the aqueous extract. Half of the extracts were boiled for 30 min in a water bath, while the remainder were not boiled. Figure 3A depicts the thrombin inhibition curves of boiled and unboiled bulb extracts respectively. It was found that both the boiled and the unboiled extracts inhibited thrombin.

The difference between the two extracts was 7% at 10 mg/ml; un-boiled extract inhibited thrombin by $34.9 \pm 3.5\%$ and boiled extract inhibited thrombin by $27.8 \pm 2.2\%$ (Figure 3A). Boiled and un-boiled extracts of garlic, *T. violacea* leaves and bulb were tested for prolongation of the TT. The results are shown in Figure 3B. The thrombin assay results (bulbs only) indicated that boiling the extracts did not significantly decrease the anticoagulant activity.

These results suggest that boiling the extract does not destroy the compounds responsible for the anticoagulant activity of the plant. These findings are in agreement with that of Chutani and Bordia (1981). These authors observed that both raw and fried garlic significantly enhanced fibrinolytic activity by 72 and 63%, respectively. The raw garlic had higher activity than the fried one, which is similar to what was observed in this study. However, Bordia et al. (1978) reported that boiled garlic extract had very little effect on decreasing the thromboxane B_2 (TXB_2) levels in the serum of rats compared to raw garlic extract (control, 65.0 ± 2.8 ; raw garlic, 13.8 ± 2.1 ; boiled garlic, 56.3 ± 14.2). Song and Milner (2001) clarified this when they found that raw garlic reduced 7, 12-dimethylbenz (a) anthracene (DMBA)-induced DNA adduct formation by 64% (DMBA-the presence of induced adducts are an early biomarker to cancer risk), garlic heated for 30 s in a microwave oven did not influence the degree of protection, garlic crushed before heating in the microwave oven for 30 s resulted in 62% reduction in adduct formation. However, with only 10% remaining alliinase activity, crushed or uncrushed garlic which was heated in a microwave oven for 60 s completely blocked the protection offered by garlic with no alliinase activity remaining. These findings are reported to be due to the loss of alliinase activity. Alliinase is a ther-

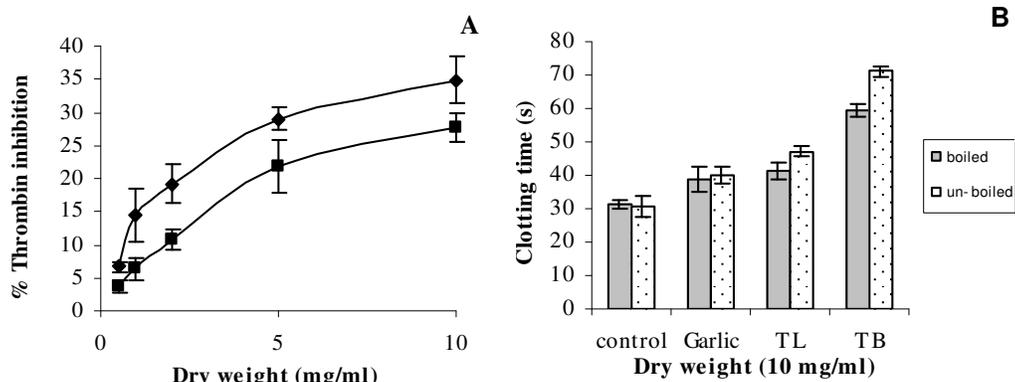


Figure 3. Comparative anticoagulant activities between boiled and un-boiled extracts. (A) % Thrombin inhibition in boiled (■) and un-boiled (◆) *T. violacea* bulb extracts. (B) Prolongation of thrombin induced clotting by garlic, *T. violacea* leaves (TL) and bulb (TB) extracts (mean \pm SD, n = 3) (Bungu et al., 2006).

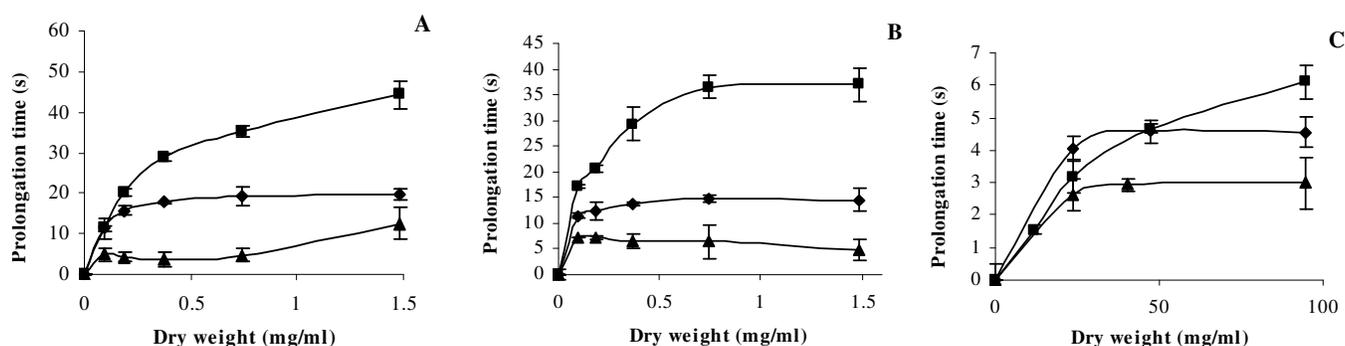


Figure 4. Comparative thrombin-induced clotting time for aqueous extracts of *T. violacea* (A) bulbs, (B) leaves and (C) garlic bulbs over time. Extracts were prepared on the same day and stored at 4°C in the dark and re-tested after 1 and 2 days. Fresh extract -■-; 1 day old extract -◆- and 2 days old extract -▲- (mean \pm SD, n=3).

molabile glycoprotein. It was possible that the remaining 10% alliinase activity was enough to convert alliin to active compounds in the garlic that was micro-waved for 30 s. Based on these contradictory reports on garlic's biological activities, this study reports that heating the *T. violacea* extract does not decrease the plant's anticoagulant activity significantly.

By measuring and comparing the thrombin inhibition and TT it was found that both the plants exhibited anticoagulant activities and that *T. violacea* has a higher activity than garlic. It was found that the inhibitory activity could not be attributed to a protein; suggesting that sulphur compounds could be responsible for the activity as reported in literature.

Time studies on the stability of activity

Thrombin-induced clotting time

A time study was conducted, measuring the effect of *T. violacea* bulb and leaf extracts on TT over a period of

seven days. This was conducted to determine how stable the active compounds were and if the activity could indeed be attributed to sulphur compounds present in the extract. This was not completed for *T. violacea* methanol extracts as they were dried immediately after extraction. A garlic extract was tested as a control, based on the findings of Mohammad and Woodward (1986). They reported that an aqueous extract of garlic could be kept for up to seven days at -20°C without losing platelet aggregation activity. In this study however, no activity was found after the extract was left at -20°C for one day. The extracts were bubbled with carbon dioxide to displace oxygen, again no inhibitory activity was obtained after a day at -20°C. The extracts were then stored at -80°C and at 4°C. Only at 4°C was it found that the activity decreased with time in all three extracts and it was completely lost after day 2 (Figure 4 A-C). The fresh extract displayed IC₂₀₀ values of 0.4 mg/ml for the bulbs and 0.3 mg/ml for the leaves, respectively. The extracts seemed to be unstable at -80°C, as the activity was not retained after one day of storage (results not shown) relative to the freshly prepared extracts.

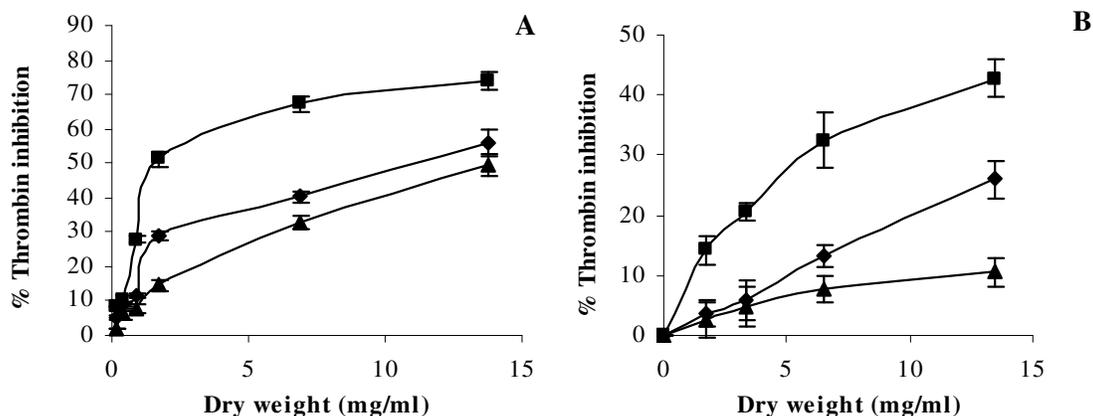


Figure 5. Thrombin inhibition by aqueous extracts of *T. violacea* (A) bulbs and (B) leaves over time. Extracts were prepared on the same day, stored at 4°C in the dark and tested again after 1 and 2 days. Fresh extract -■-; 1 day old extract -◆- and 2 days old extract -▲- (mean ± SD, n=3).

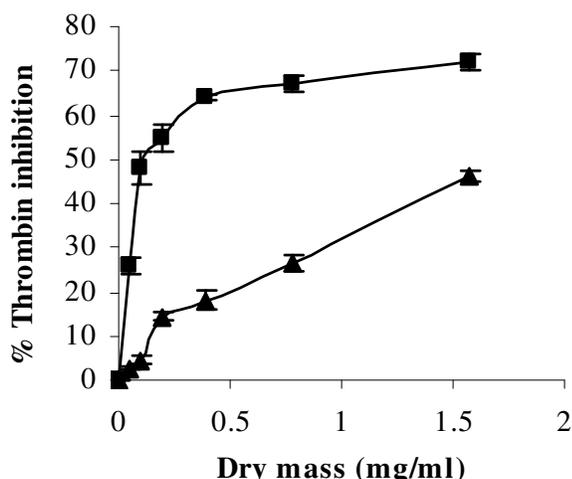


Figure 6. Comparative thrombin inhibition by methanol extracts of *T. violacea* bulbs -■- and leaves -▲- (mean ± SD, n=3).

Thrombin inhibition

The thrombin inhibition of methanol extracts of *T. violacea* bulbs and leaves was determined to be higher than the aqueous extracts (Figures 5 and 6). They displayed an IC_{50} value of 0.21 and 1.57 mg/ml for the bulbs and the leaves, respectively (Figure 6). The aqueous bulb extract displayed an IC_{50} value of 1.725 mg/ml (Figure 5A) whereas the leaf extract did not reach an IC_{50} value (Figure 5B). The methanol bulb extract was 8.2 fold more active than the aqueous bulb extract.

The stability of aqueous extracts of *T. violacea* bulbs and leaves during storage at 4°C was tested for thrombin inhibition over time. The inhibitory activity had decreased four fold after two days (Figure 5) when compared to the freshly prepared extracts. The methanol extracts showed consistent results during storage at 4°C; five month old extracts retained inhibitory activity equivalent to when the

Table 2. Seasonal variation in the effect of aqueous extracts of *T. violacea* bulbs and leaves on thrombin – induced clotting time.

Month	Bulbs IC_{200} (mg/ml)	Leaves IC_{200} (mg/ml)
Mar	0.40 ± 0.03	0.30 ± 0.02
Apr	0.29 ± 0.01	0.10 ± 0.02
May, Jun, Jul	NI	NI
Aug, Sep, Oct	NS	NS
Nov	0.50 ± 0.03	0.20 ± 0.05

NI: no inhibition.

NS: no significant inhibition (IC_{200} values not reached). (Mean ± SD, n=3).

extracts were freshly tested.

Seasonal variation on the stability of activity

Thrombin-induced clotting time

The effect of aqueous extracts of *T. violacea* bulbs and leaves on thrombin-induced clotting time was measured over a period of nine months (from March to November).

Plants were harvested and extracted once a month and the results obtained showed that the activity of the plant varied throughout the time period tested (Table 2). The activity was lost during winter (May, June and July). No variation in the activity of the methanol extracts was found over this time period (results not shown).

Thrombin inhibition

To establish whether the thrombin inhibition activity also varied during the course of the time period tested, plants were harvested, extracted and tested every month from

Table 3. Seasonal variation in thrombin inhibition of methanol and fresh aqueous extracts of *T. violacea* bulbs at 1 mg/ml.

Month	Aqueous	Methanol
May	+	+++
June	-	+++
July	-	+++
Aug	+++	+++
Sept	+++	+++
Oct	+++	+++
Nov	+++	+++

- : No inhibition (0-19% inhibition).
 +: 20-49% inhibition.
 ++: 50-69% inhibition.
 +++: 70-80% inhibition.

May to November. Table 3 illustrates that the activities of the aqueous extracts were affected during winter months (May, June and July). The methanol extracts maintained a constant level of activity throughout the time period tested. These results indicate that the area of origin, the time of the year (season) and the method of extraction all have an effect on the activity of the plant.

The present study investigated the *in vitro* anticoagulant properties of *T. violacea* and provides valuable insight to the traditional use of this plant within South Africa. The results reflect that *T. violacea* has anticoagulant and antithrombotic activities *in vitro*. However, for its medicinal use it should be noted that the activity is dependent on several factors i.e. a suitable extraction method (an infusion, aqueous or organic), the season of the year when the plant is harvested for use, and an appropriate method of storage.

T. violacea has been suggested to have similar biological activities and active compounds to garlic, where these properties are ascribed to organosulphur compounds such as diallyl disulphide (DADS), diallyl sulphide (DAS), diallyl trisulphide (DATS), S-allyl ethylcysteine (SAC), S-allyl methylcysteine (SAMC) and ajoene. To date nothing has been reported on the antithrombotic and anticoagulant activities of *T. violacea*, although the presence of three unidentified sulphoxide amino acids has been reported in *T. violacea* (Jacobsen et al., 1968). These observations show that there are similarities between *T. violacea* and garlic which warrants further investigation.

The fact that *T. violacea* exhibits both anticancer (Bungu et al., 2006) and anticoagulant activity is of great significance. Studies *in vitro* and *in vivo* show that cancer cells interfere with the blood coagulation system. In light of this, a compound with anticancer activities is more attractive as a therapeutic agent if it also exhibits mild anticoagulant activities.

Throughout the course of this study it was seen that *T. violacea* exhibits biological activities which are compara-

ble to garlic. These results indicate that *T. violacea* can be used as an alternative to garlic and that it may contribute to pharmaceutical applications and informal health services. Future work on the anticoagulant and anti-cancer biological activities of *T. violacea* should focus on the oxidation state of the sulphur compounds and how this influences the biological activity observed.

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