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Full Length Research Paper

Chemical composition, antioxidant activity and toxicity evaluation of essential oil of *Tulbaghia violacea* Harv.

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Essential oil from the rhizomes of *Tulbaghia violacea* of the family Alliaceae was obtained by hydro-distillation using an all-glass Clevenger-type apparatus. *In vitro* antioxidant activities of the oil at various concentrations were assessed using 2, 2-diphenyl-1-picryl hydrazyl (DPPH), nitric oxide scavenging, reducing power and lipid peroxidation inhibition assay. The results were compared with butylated hydroxytoluene (BHT) and ascorbic acid. Brine shrimp lethality test was used to determine cytotoxicity of the oil. Gas chromatography/mass spectrophotometer (GC/MS) analyses of oil revealed 7 polysulfides with a pungent garlic-like odor. The principal constituents were dimethy disulfide, dimethy trisulfide (methyl methylthio), methyl, 2,4-dithiapentane (11.35%) and (methylthio) acetic acid, 2-(methylthio) ethanol, 3-(methylthio)- and propanenitrile (7.20%). The essential oil demonstrated moderate radical scavenging activities. Although, their EC_{50} value was lower than those of the BHT and ascorbic acid, the value was close to those reported for other Alliaceae family. The LC_{50} value of 12.59 µg/ml obtained showed that the essential oil of *T. violacea* was toxic. The implication of the toxicity of the oil is discussed.

Key words: *Tulbaghia violacea,* antioxidant, essential oils*,* gas chromatography/ gas chromatography-mass spectrophotometer (GC/GC-MS), toxicity.

INTRODUCTION

Essential oils are made up of different volatile compounds and aromatic oily liquid obtained from different plant parts (Amal et al., 2010). The composition of essential oils often varies between species (Mishra and Dubey, 1994) and it determines the organoleptic properties and biological activity of the oil (Misharina et al., 2009). The study of individual components of different essential oils has shown that many terpenes containing oils possess antiradical and antioxidant activity (Misharina et al., 2009). When essential oil is isolated from plants, they are not usually extracted as chemically pure substances, but as mixtures of many compounds, like monoterpenes and sesquiterpines which are mainly hydrocarbon (Amal et al., 2010). Essential oils and extracts have been used for many thousands of years, especially in food preservation, pharmaceuticals, alternative medicine and natural therapies (Imelouane et al., 2009). It has been well established that some plant essential oils exhibit antimicrobial properties against bacterial pathogens (Koba et al., 2009; Prabuseenivasan et al., 2006). Many plants have been employed in the management and treatment of diseases. One of such plants is *Tulbaghia violacea* Harv. It was recently reported that the extract of rhizomes of *T. violacea* is commonly employed in the management of heart diseases in Nkonkobi municipality Eastern Cape of South Africa (Olorunnisola et al., 2011).

T. violacea is commonly known as wild garlic, wilde knoffel (Afrikaans), isihaqa (Zulu) or itswele lomlambo (Xhosa). It is indigenous to the Eastern Cape region of South Africa and is widely used as an herbal remedy for various ailments. Its leaves and bulbs are the most commonly used. *T. violacea* has been reported to have various medicinal applications which include treatment for fever and colds, asthma, tuberculosis, stomach problems and oesophageal cancer (Bungu et al., 2008).

The plant is also used as a snake repellent (van Wyk and Gericke, 2000). In spite of the traditional medicinal claims on the use of *T. violacea,* there is dearth of

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information on the pharmacological activity its essential oil. This study was designed to determine the chemical constituents of the essential oil from the rhizomes of *T. violacea* using hydro- distillation techniques and also to assess it antioxidant activities and toxicity using brine shrimp lethality test which is a bench top bioassay for elementary cytotoxicity study.

MATERIALS AND METHODS

Plant materials

Fresh rhizome of *T. violacea* was collected in April from Alice, Eastern Cape, South Africa, and authenticated by Prof. D.S Grierson of Botany Department, University of Fort Hare. Voucher specimen (Sin 2010/2) was deposited at the Giffen Herbarium.

Extraction of essential oils

Rhizomes were hydro-distilled for 3 h in a Clevenger-type apparatus in accordance with the British pharmacopoeia specifications (1980). The essential oil was collected and analyzed immediately.

GC-MS analyses of the oil

GC-MS analyses of the oil was carried out using Hewlett-Packard HP 5973 mass spectrometer interfaced with an HP-6890 gas chromatograph with an HP5 column. The following conditions were used: Initial temperature 70%, maximum temperature 325°C, equilibration time 3 min, ramp 4°C/min, final temperature 240°C; inlet: split less, initial temperature 220°C, pressure 8.27 psi, purge flow 30 ml/min, purge time 0.02 min, gas type helium; column: capillary, 30 m × 0.25 mm i.d., film thickness 0.25 µm, initial flow 0.7 ml/min, average velocity 32 cm/s; MS: EI method at 70 eV.

Identification of components

The individual constituents of the oil were identified by matching their mass spectra and retention indices with those of Wiley 275 library (Wiley, New York) in computer library (Kovats 1958; Adams, 1995; Joulain et al., 2001; Joulain and Konig, 1998). The yield of each component was calculated per gram of the plant material, while the composition was calculated from the summation of the peak areas of the total oil composition. The whole experiment was replicated thrice.

Antioxidant activity

DPPH radical scavenging activity

The method of Liyana-Pathiranan and Shahidi (2005) was used for the determination of scavenging activity of free radical in the essential oil. A solution of 0.135 mM 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) in methanol was prepared. 1.0 ml of this solution was mixed with 1.0 ml of oil prepared in methanol containing 0.1 to 0.5 mg/ml of the oil and standard drugs (BHT and ascorbic acid). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorption of the mixture was measured spectrophotometrically at 517 nm. The actual decrease in absorption was measured against that of the control. All test and analysis were run in triplicates and the results obtained were averaged. The activities were also determined as a function of their % Inhibition which was calculated using the formula;

% scavenging activity = [(Ac - As) / Ac] × 100

Ac = Absorbance of the control; As = Absorbance of the sample.

Nitric oxide-scavenging activity

Nitric oxide scavenging activity was measured spectrophotometrically (Govindarajan et al., 2003). Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with different concentrations of the extract (100 to 500 µg/ml) was prepared in methanol and incubated at 25°C for 30 min. A control without the test compound but with an equivalent amount of methanol was taken. After 30 min, 1.5 ml of the incubated solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylene diamine dihydrochloride was measured at 546 nm and percentage scavenging activity was measured with reference to standard. BHT vitamin C was used as a positive control.

Lipid peroxidation and thiobarbituric acid reactions

A modified thiobarbituric acid reactive species (TBARS) assay (Ohkowa et al., 1979) was used to measure the lipid peroxide formed using egg yolk homogenate as lipid rich media (Ruberto et al., 2000). Egg homogenate (0.5 ml of 10%, v/v) and 0.1 ml of the oil extract were added to a test tube and made up to 1 ml with distilled water; 0.05 ml of $FeSO_4$ (0.07 M) was added to induce lipid peroxidation and the mixture incubated for 30 min. Then, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate were added and the resulting mixture was vortexed and then heated at 95°C for 60 min. After cooling, 5.0 ml of butan-1-ol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Inhibition of lipid peroxidation percent by the oil extract was calculated as:

[(1-E)/C] × 100

where C is the absorbance value of the fully oxidized control; E is the absorbance in presence of extract.

Reducing power of the extract

The reducing power of the oil was determined according to the method of Yen and Chen (Ebrahimzadeh et al., 2008a; Nabavi et al., 2008a, b). 2.5 ml of extract (100 to 500 µg/ml) in water were mixed with a phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K_3 Fe $(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased in reducing power. Vitamin C was used as a positive control.

Brine shrimp lethality test

Shrimp eggs were allowed to hatch and mature as nauplii in two

Table 1. Compounds obtained from GC/GC-MS analysis of *T. violacea* rhizome essential oil.

S/N	Retention time (min)	Chemical composition	Area (%)
1	21.94	Dimethy trisulfide,	0.57
2	26.77	Dimethy disulfide, methyl (methylthio) meth 2,4-dithiapentane	11.35
3	33.92	(Methylthio) acetic acid	2.58
4	38.00	(Methylthio) acetic acid, 2-(methylthiol) ethanol, propanitrile, 3-(methylthio)-	7.20
5	44.99	2,4-dithiapntane,bis-(methlythio), disulfide	0.78
		Total (%)	22.48

days in a hatching tank filled with seawater. The free-swimming nauplii were attracted by a light to a compartment from which they could be collected for the assay proper. Vials containing 2.5 to 20 µg ml^{-1} samples were prepared by dissolving the oils in dimethyl sulfoxide (DMSO) and transferring the solution to each vial. The solvent was evaporated at room temperature and seawater was added to achieve the correct concentration. 15 shrimps were added to three vials for each dose via a disposable pipette. The number of deaths out of 15 shrimps per dose was recorded after 24 h and LC_{50} values obtained from the best-fit line slope. The control solution consisted of 15 nauplii in the artificial seawater. For acceptable readings; the LC_{50} for the toxicant should fall within 27 to 35 µg ml^{-1} range (Sam et al., 1988).

Statistical analysis

Statistical analysis was carried out with Statistical Package for Social Sciences (SPSS). The data was expressed as the mean ± standard deviation and a probability of less than 0.05 ($p<0.05$) was considered to be statistically significant. Graph was drawn using Microsoft Office excel, 2007 software.

RESULTS AND DISCUSSION

GC-MS analysis of the essential oil

Essential oils from freshly collected rhizome of *T. violacea* were obtained by hydro-distillation. The yield of 250 g hydro-distillation was 0.05% (v/w). A light yellowish oily liquid with a pungent garlic-like odor was produced. The result of GC-MS analysis of the oil shows that dimethy disulfide, methyl (Methylthio) methyl sulfide and 2, 4-dithiapentane (11.35% peak area) (Methylthio) acetic acid, 2-(Methylthio) ethanol and (3-methylthio) - (7.20%) are main components, while trisulfide are minor component (0.57% peak area) (Table 1). The results (Table 1) obtained from the analysis of the oil compared favorably well with compounds identified earlier from methanolic extract of the rhizomes using ion-exchange chromatography technique (Kubec et al., 1999) and from garlic oil (Munchberg et al., 2007; Pino et al., 1991). It was believed that the polysilfide obtained are 'second generation' of biologically active sulfur species, formed by decomposition of non-volatile sulfur compounds called S-alkylcysteine sulfoxides or allicin and are responsible for the characteristic flavour and biological properties of the plant (Kubec et al., 2002; Ute et al., 2007). Diallyltetrasulfide and higher polysulfides such as the diaallylpenta-, hex- and hepta sulfide reported in garlic oil extracts was not found in oil extract of *T. violacea* rhizome. The absence of these polysulfides may be due to differences in processing procedure, time of collections, species and variation in techniques of extraction and analysis (Pino et al., 1991; Kubec et al., 2002; Ute et al., 2007). GC-MS protocol was not particularly sensitive to nitrogen compounds (Amy et al., 2000). The single nitrogen compound revealed by the GC-MS analysis, propanenitrile (7.20% peak area), was described by Ellison (1999) as having an unpleasant odor.

Antioxidant activity

It is well documented that free radicals are involved in the etiopathogenesis of numerous chronic diseases such as atherosclerosis, hypertension and coronary heart disease (Kadir et al., 2011). Thus, the ability of plant extracts to scavenge free radicals is an important property.

The antioxidant activity of essential oil of rhizomes *T. violacea* was assessed by using DPPH, nitric oxide free radical-scavenging, reducing power and lipid peroxidation inhibition assay. DDPH free radical scavenging assay is considered as a simple and very fast method for determining antioxidant activity. The effect of antioxidant on DPPH radical scavenging was due to their hydrogen donating ability or radical scavenging activity. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen donating antioxidant (Viuda- Martos et al., 2010).

The result of DPPH radical scavenging activities (% inhibition) of the oil at various concentrations showed a concentration dependent activity pattern (Figure 1). As shown, it is clear that as concentration increased, the scavenging effect also increased, reaching as high as 89.2 ± 1.5% at 0.5 mg/ml. This value was very close to the activity of synthetic antioxidants BHT (92.4 ± 3.20%) and vitamin C (98.1 ± 1.30%) at the same concentration. The results obtained for the calculated fifty percent effective inhibition concentration of the oil extract (EC_{50} = 83.0 µg/ml) correspond favourably well with what has been

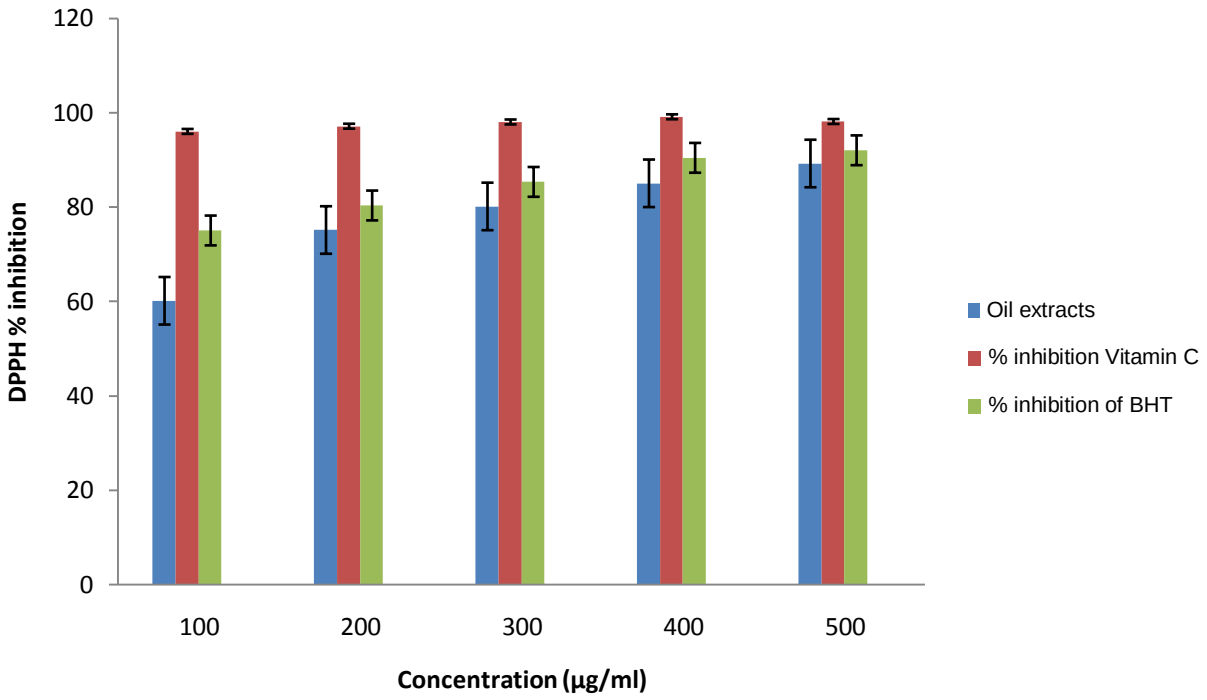

Figure 1. Scavenging effects of oil extracts from rhizomes of *T. violacea*, vitamin C and BHT on DPPH.

been reported for other family of Alliaceae such *Petroselinum sativum* (Parsley) herb oil (EC_{50} = 82.1 µg/ml), *Cuminum cyminum* (Cumin) oil (EC_{50} = 81.5 µg/ml) and *Allium cepa L.* (Onion) oil (EC_{50} = 80.0 µg/ml) at the same concentrations (Shalaby et al., 2011). However, the oil extract of rhizome of *T. violacea* demonstrate lower EC_{50} value when compared with vitamin C (EC_{50} = 52.0 µg/ml) and BHT (EC_{50} = 67.0 µg/ml) reference drugs. These values revealed that the antioxidant activity of *T. violacea* rhizome oil was still less active than butylated hydroxytoluene (BHT) and vitamin C. It was observed that the antioxidant activity of the oil extract is higher than for their individual components and this might be as a result of synergetic effects of multi component of oil (Misharina et al., 2009).

Nitric oxide scavenging activity

Nitric oxide has been implicated in inflammatory and pathogenesis of various human diseases such as cancer and cardiovascular diseases, Hence, nitric oxide scavenging capacity of extracts may help to arrest the chain of reactions initiated by excess generation of nitric oxide (NO) that are detrimental to the human health (Raushanar et al., 2009). In this study, we demonstrate that the oil extract significantly inhibited NO production from sodium nitroprusside in an aqueous solution at physiological pH and reacts with oxygen in the reaction to form nitrite. The extracts inhibit nitrite formation by directly competing with oxygen in the reaction with nitric oxide and other nitrogen oxides such as NO_3, and N_2O_3 (Osamuyimen et al., 2011). The extract showed strong concentration dependant inhibitory activities with highest percentage inhibition of nitric oxide at 0.5 mg/ml (Figure 2). Though, the oil extract demonstrated a significant inhibitory activity against nitric oxide radical, its 50% effective inhibition concentration (EC_{50} = 180 µg/ml) was comparably lower than what was obtained for garlic oil (IC_{50} = 50 µg/ml) (Reena and Kapil, 2011) and the reference drugs BHT (EC = 115 µg/ml) and ascorbic acid (EC = 134 µg/ml) (Table 2). The nitric oxide inhibiting ability of the oil extract could support the use of the plant in the treatment of oxidative induce ailments such as cardiovascular diseases.

Lipid peroxidation

Reacting oxygen species are known to cross react with lipid constituents of the cell membranes causing changes in fluidity and permeability (Nigam and Schewe, 2000), DNA mutation (Russo et al., 2001) and lipid peroxidation. The effect of these delirious reactions is the cause of most human diseases. The generation of lipid peroxidase in egg yolk lipids when incubated in the presence of ferrous sulphate with subsequent formation of malonodialdehyde (MDA) and other aldehydes that form pink chromogen (Kosugi et al., 1987) was strongly inhibited in concentration dependant manner (Figure 3) by

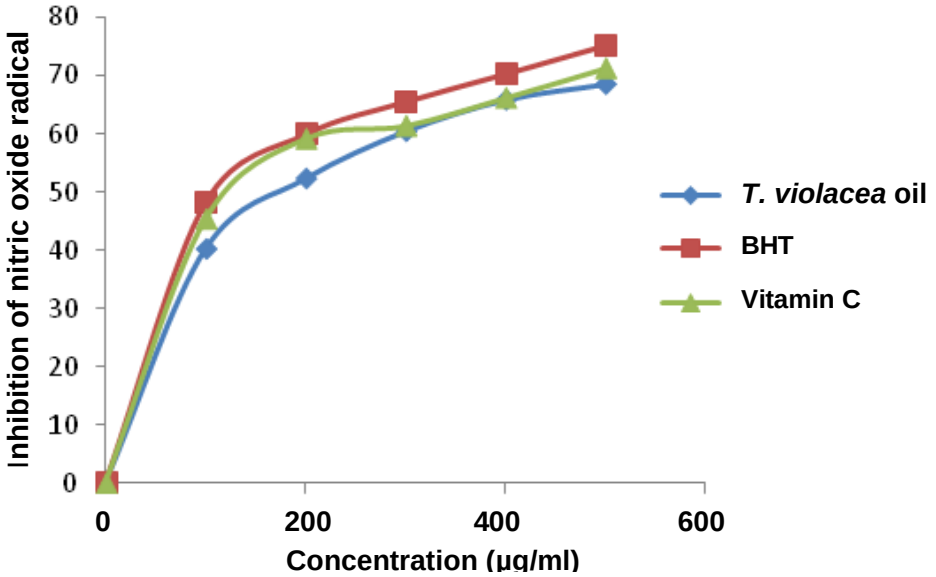

Figure 2. Scavenging effects of oil extracts from rhizomes of *T. violacea*, vitamin C and BHT on nitric oxide radical.

Table 2. Comparison of 50% effective inhibitory concentration for nitric oxide and lipid peroxidation of oil extract of rhizomes of *T. violacea*.

Extract	Nitric oxide inhibition EC_{50} (µg/ml)	LP inhibition EC_{50} (µg/ml)
OTV	180.0	192.3
BHT	115.0	138
AA	134.0	100

OTV, oil extract of *T. violacea*. BHT, butylated hydroxytoluene; AA, ascorbic acid. LP inhibition EC_{50}, concentration of oil extract for 50% inhibition of lipid peroxidation.

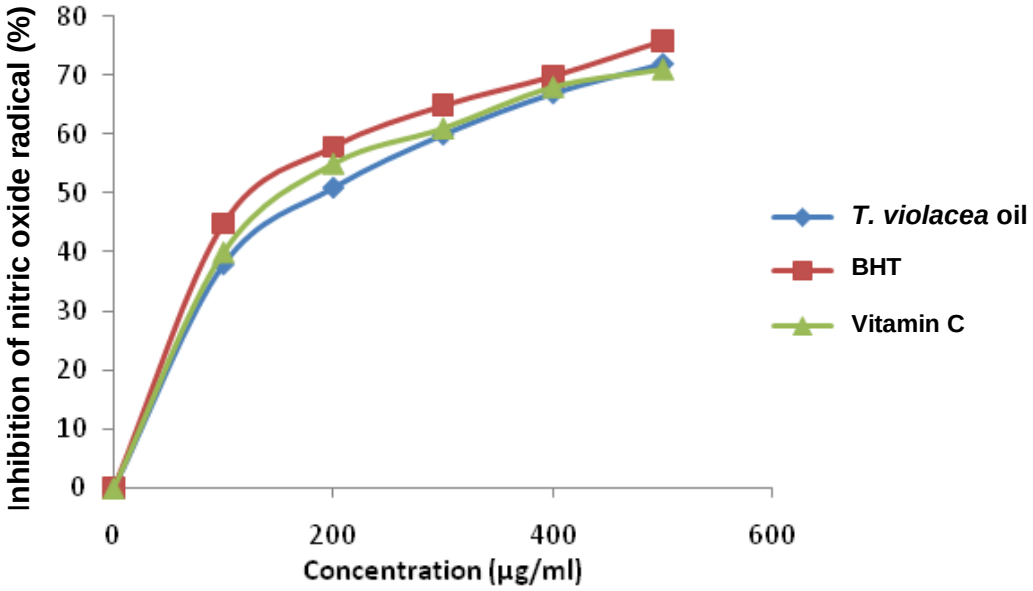

Figure 3. Scavenging effects of oil extracts from rhizomes of *T. violacea*, vitamin C and BHT on lipid peroxidation.

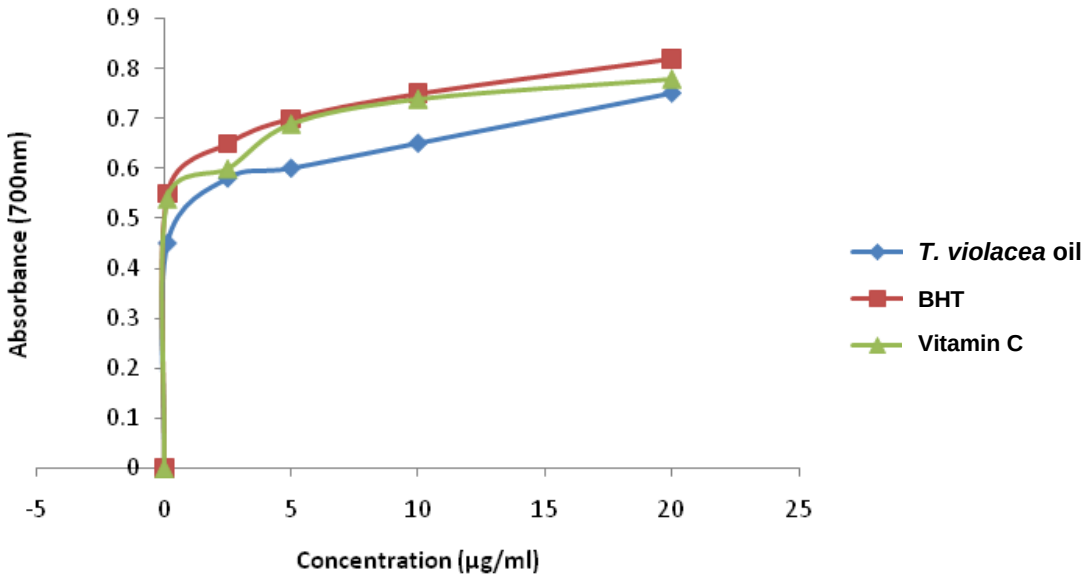

Figure 4. Ferric reducing power of oil extracts from rhizomes of *T violacea*, vitamin C and BHT.

by oil extract of *T. violacea* with EC_{50} value of 192.3 µg/ml (Table 2). This value was much lower than what was reported for methanol extract of *Mucuna pruriens* (Yerra et al., 2005) however, the value is higher when compared with the standard (BHT and AA) (Table 2). The results of the investigations revealed that oil extract of rhizome of *T. violacea* had potent lipid peroxidation inhibition activity. Although, phytochemical evaluation was not assessed in this study, other group of workers who has work on other members of Alliaceae species (onion and garlic) has reported that the observed inhibition of lipid peroxidation and radical scavenging activities might be due to the phenolic contents (Nuutila et al., 2003).

Reducing power of the extract

The antioxidant potentials of the oil extract was estimated from their ability to reduce Fe^{3+} to Fe^{2+}. This was observed observed from yellow colour of the test solution that changed to various shades of green and blue depending on the concentration of the plant extracts. The reducing power of the oil of *T. violacea* and the reference compounds increased with increasing concentration (Figure 4). In addition, the reducing value of the oil extract was significantly lower than that of BHT and ascorbic acids, used as reference compounds in this study (Figure 4). As the concentration of the oil extract increase the reducing power assay absorbance also increased. This observation follow similar trend reported for garlic oil extracts (Reena and Kapil, 2011). The reducing properties of the oil might be due to the presence of reductones (Saha et al., 2008). From the previous results and discussion it can be concluded that the oil extract of rhizome of *T. violacea* possesses the potent antioxidant substances which may be responsible for its anti-inflammatory and chemoprotective mechanism as well as justify the basis of using this plant's extract as folkloric remedies.

RESULTS AND DISCUSSION

Cytotoxic activity in brine shrimps bioassay

Brine shrimp lethality assay is frequently used as model system to measure cytotoxic effects of variety of toxic substances and plant extracts against brine shrimps nauplii (Morshed et al., 2011). The method provides a simple and inexpensive screening test for cytotoxic compounds and possesses the advantages of requiring only small amounts (0.6 mg) of compounds for investigation.

The LC_{50} (12. 59 µg/ml) value (Figure 5) obtained in these studies showed that the oil extract of rhizome of *T. violacea* was cytotoxic and this toxicity is concentration dependant (Table 2). It was observed that all the nauplii survive at the lowest concentration (2.5 µg/ml) Table 3. This significant lethality of the oil extracts (LC_{50} values less than 100 µg/ml) against brine shrimps nauplii might be due to the presence of polysulfides which has be implicated as cytotoxic agents with potential anticancer, antimicrobial and antifungal activities (Münchberg et al., 2007; Amwar et al., 2008).

The present study indicates that the essential oil of rhizome of *T. violacea* exhibit interesting biological activities such as antioxidant and cytototoxic effect and

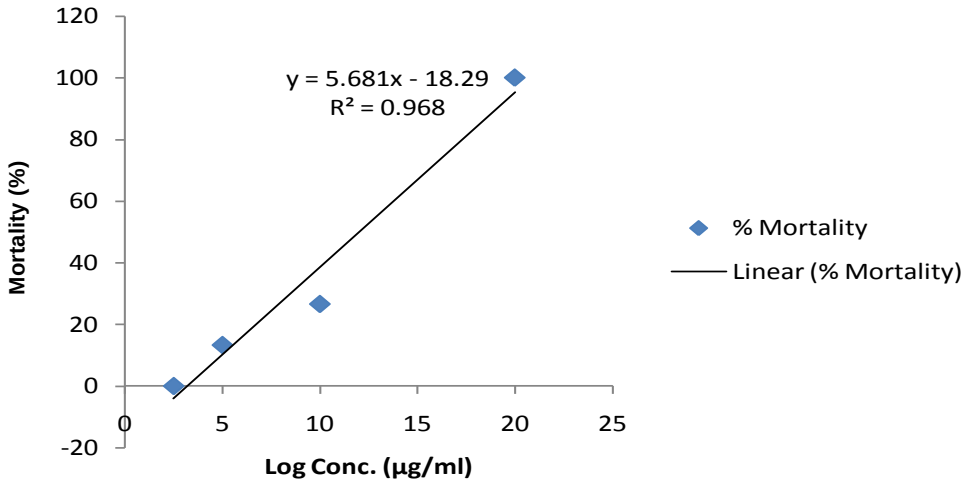

Figure 5. Determination of LC_{50} of essential oil of rhizome of *T. violaceae* against brine shrimps nauplii.

Table 3. Brime shrimp lethality test of the essential oil of *T. violacea* rhizome.

Conc. (µg/ml)	Essential oil of *T. violacea* rhizome		
	Average number of survivors	Average number of dead	Mortality (%)
2.5	15.0 ±0.00	15.0 ± 0.00	0
5.0	13.0 ± 0.00	2.0 ± 0.00	13.3
10.0	11.0 ± 0.00	4.0.0 ± 0.00	26.6
20	0	0	100
control	15	0	0
LC_{50} = 12.59 µg/ml			

Data were expressed as mean ± SD.

may serve as alternative natural source of anticancer, and antibiotic and antimicrobial agents.

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