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Full Length Research Paper

Exploring the anticoagulant and antiplatelet effect of the extracts of the red marine alga *Acanthophora spicifera*

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Cardiovascular diseases represent the major cause of death and morbidity in the world. An uncontrolled activation of the coagulation cascade and platelet aggregation may lead to the formation of thrombi. Antithrombotic drugs have limitations and may produce side effects, and consequently, alternative therapies have been extensively investigated. Thus, the aim of this work was to evaluate anticoagulant and antiplatelet effects of extracts (prepared in methanol, dichloromethane, ethyl acetate or acetone) of the Brazilian alga *Acanthophora spicifera* and some commercial products, biotin, myristic acid, cholesterol, β-carotene and vitamin B_{12}. Samples were tested on Prothrombin Time, Activated Partial Thromboplastin Time, Fibrinogen Coagulation and Thrombin Time, which are routinely used at clinical trials and on platelet aggregation. From the result, all extracts or products inhibited plasma coagulation as well as inhibited platelet aggregation induced by collagen or ADP. Moreover, the extracts inhibited the enzymatic activity of thrombin, tested upon a specific chromogenic for thrombin. The extracts or commercial products were devoid of toxicity, since no lyse occurred on platelets or red blood cells in the presence of them. In conclusion, the extracts of *A. spicifera* or products from the market have biotechnological potential and may be useful to develop a new class of antihemostatic drugs.

Key words: *Acanthophora spicifera*, red marine alga, antiplatelet, anticoagulant, bioprospecting, vascular diseases.

INTRODUCTION

Hemostasis balance is a physiological coordinate process of anticoagulant and coagulant events that allows blood to circulate through the cardiovascular system or to prevent bleeding after injury. Platelets as well as the coagulation cascade participate in such process, as well as they may cause vascular diseases.

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Disturbs that alter such equilibrium may result in hemorrhage or thrombosis, while the latter is one of the most important cardiovascular diseases, as ischemic attack, stroke and myocardial infarction. They lead to mortality or morbidity worldwide, mainly in western populations (Zhao et al., 2017). Thrombosis generally has medical complications to patients or recurrent hospitalization, and this fact increases healthcare costs (Furie and Furie, 2008). The current therapy to such diseases is performed by administration of anticoagulant and/or antiplatelet drugs. Heparin and coumarins are usually used as anticoagulants, but they have drawbacks, as risk of bleeding, narrow therapeutic window, non-specific binding, thrombocytopenia and/or osteoporosis (Mourão, 2015). On the other hand, in order to manage thrombosis disturbs, antiplatelet drugs may be employed. Thus, platelets are critical in thrombotic disorders. In this way, there are many drugs in the market able to impair platelet aggregation, preventing the formation of a platelet thrombus. Aspirin has been used as the drug of choice for the long-term treatment, but, it also has serious side effects, and gastric hemorrhage is the most serious complication. Other drugs, as clopidogrel and ticlopidine have limitations, as well.

Thus, the search for new anticoagulant or antiplatelet drugs without side effects, more potent and safer may constitute an important task to improve such treatments; and, natural sources may be a promising strategy. Marine organisms are composed of molecules with a chemical diversity, and some of them have application in medicine (Mourão, 2015; Cirne-Santos et al., 2018). However, their potential is not well-explored (Mourão, 2015). Previous reports showed that a diterpene isolated from the marine brown alga, *Dictyota menstrualis* as well as from other seaweeds or sponges inhibited platelet aggregation and coagulation of plasma (de Andrade et al., 2014; de Andrade et al., 2011). Seaweeds are classified as green algae (Chlorophyta), brown algae (Phaeophyta), red algae (Rhodophyta) and Blue-green algae (Cyanobacteria). Most of seaweeds are red (6,000 species) and the rest known are brown (2,000 species) or green (1,200 species).

The red algae are the oldest groups of eukaryotic algae and the largest, with about 5,000–6,000 species, and some of them have medicinal properties. *Acanthophora spicifera* species belong to rhodophyta and are widely distributed in the tropical and subtropical regions (Kilar and McLachlan, 1986), and in Brazil, they are along the entire coast (Ávila et al., 2012). There are many reports describing biological effects of *A. spicifera*, as: antioxidant, antibacterial, anti-malarial, antiviral, anti-inflammatory, and anticancer (Duraikannu et al., 2014; Duarte et al., 2004). The assessment of chemical profile of genus *Acanthophora* showed the presence of sterols, as cholesterol and derivatives (Flora and Rani, 2013), fatty acids, vitamins and carotenoids (Polat and Ozogul, 2008).

Moreover, the analysis of the extract of *A. spicifera* through GC-MS has demonstrated the presence of fatty acids and sterols as well as using mass spectrometry has revealed octanol, piperazine, benzoic acid and octadecenoic acid, as major constituents of methanolic extract (Flora and Rani, 2013). It is well known that the seaweed *A. spicifera* is rich in halogenated molecules, as terpenes, phenols and acetogeninas, whose functions are related to their survival into the marine environment (Nogueira et al., 2016), but little is known of natural chemistry of the genus *Acanthophora*. However, antihemostatic action of *A. spicifera* has not been explored. Thus, the objective of this work was to investigate antiplatelet and anticoagulant effects the crude extracts of *A. spicifera* and some commercial molecules, which are produced by this alga.

MATERIALS AND METHODS

The commercial products: biotin (Sigma-Aldrich, USA – B4501), myristic acid (Sigma-Aldrich, USA – M3128), cholesterol (Sigma-Aldrich, USA – C8667), β-carotene (Sigma-Aldrich, USA – C9750) and vitamin B_{12} (Sigma-Aldrich, USA – V2876). ADP and collagen type I from Chrono Log Corp. (Havertown, PA, USA).

Algal collection and preparation of extracts of A. spicifera

Specimens of the red marine alga *A. spicifera* were collected in May, 2013 by snorkeling at depth of 0.5-10 m, at Orla Bardot (a tribute to French actress Brigite Bardot) in the city of Armação dos Búzios (22° 05' 03" S, 41° 53' 01" W). Armação dos Búzios or just Búzios is a small balneary with a population about 30,000 people, located at 170 km from the city of Rio de Janeiro, Brazil. The fresh material was immediately taken to the laboratory and screened to remove epiphytes and associated organisms. Air-dried specimens (204 g) were exhaustive and sequential extracted with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), acetone (Me_2CO), and methanol (MeOH) at room temperature. After filtration and evaporation under reduced pressure, the extract (100 µg) was analyzed by thin-layer chromatography (TLC) eluting with 20 ml of n-hexane and ethyl acetate (7:3), which was performed with a Merck Kieselgel GF254. The spots were detected by inspection in the ultraviolet light (254 and 365 nm), and then revealed by spraying with $CeSO_4$ at 2% H_2SO_4, followed by heating at 100°C for 15 min.

Assays

Platelet aggregation

Platelet aggregation assays were carried out according to (Fuly et al., 1997), with modifications, using platelet-rich-plasma (PRP) obtained from healthy volunteer donors (experiments were approved by the Federal Fluminense University Committee for Ethical in Experimentation – CEP-UFF, CAAE: 28941314.0.0000.5243). PRP was prepared by the centrifugation of citrated (0.31%, v/v) whole blood at 25°C (340 x g for 12 min). Platelet aggregation was measured turbidimetrically using a Whole Blood Aggregometer (Model 490 2D - Chrono-Log Corporation, Pennsylvania, USA). Assays were performed at

37°C in siliconized glass cuvettes using 300 µl of PRP under stirring, and aggregation was triggered by the addition of ADP or collagen. One hundred percent (100%) of platelet aggregation was determined as the full platelet response obtained 6 min after the addition of a supramaximal concentration of the agonists (concentration that gives 70-80% of aggregation), and 0% (base line) of platelet aggregation was the light transmittance recorded of PRP alone. The effect of the extracts of *A. spicifera* was performed by incubating different concentrations of the extracts with PRP for 5 min at 37°C, and then, platelet aggregation was triggered by adding ADP (15 µM) or collagen (5 µg/mL). Inhibitory effect on platelet aggregation was expressed as the difference in the maximal responses of platelets in the presence or in the absence of the extracts of *A. spicifera*, after challenge with agonists. Control experiments were performed in the presence of saline or 0.9% (v/v) of dimethylsulfoxide (DMSO).

Plasma coagulation

Prothrombin time (PT), activated Partial Thromboplastin Time (aPTT) or Thrombin Time (TT) assays were performed according to the manufacturer's instructions (Wiener Laboratories, Rosario, Argentina). For the PT test, the extracts of *A. spicifera* were incubated with plasma (50 µl) for 10 min at 37°C, and then, 100 µl of pre-warmed thromboplastin with calcium were added to initiate coagulation.

For the APTT test, the extracts of *A. spicifera* were incubated for 10 min at 37°C with plasma plus 100 µL of the APTT reagent, cephalin and kaolin in a final volume of 200 µl, with the reaction started by the addition of $CaCl_2$ (8.3 mM, final concentration). The TT assay was performed by incubating plasma with 50 µl of the extracts of *A. spicifera*, 50 µl of saline or DMSO and incubated at 37°C for 1 min. Then, coagulation was initiated by adding 1 nM human α-thrombin (Haematologic Technologies Inc., Vermont, USA). The Fibrinogen Coagulation (FC) assay was performed by incubating the extracts of *A. spicifera* for 10 min at 37°C with 200 µl commercial fibrinogen (2 mg/ml, from Sigma Chemical Co., USA) in a final volume of 250 µl. Then, coagulation was triggered by the addition of thrombin (10 nM). For all assays, coagulation was performed on a Multichannel Coagulometer (Amelung, Model KC4A, Labcon, Germany), and coagulation time was recorded in seconds. Plasma was obtained from a pool of healthy volunteer donors and diluted in an equal volume of saline.

The *ex vivo* coagulation tests were considered, as follow: saline, DMSO (1% v/v, final concentration or commercial products (300 µg/mice) were administered into mice intravenously (i.v.). After 2 h, blood was collected by cardiac puncture, then blood was centrifuged at 1,800 g for 10 min, and plasma was transferred to plastic tubes and prothrombin time (PT) or activated partial thromboplastin time (aPTT) tests were performed. Experiments were approved by CEP-UFF, under protocol CAAE: 28941314.0.0000.5243.

Hydrolytic activity upon chromogenic substrate

Hydrolysis of the chromogenic substrate, H-D-Phe-pipecolyl-Arg-pNA.2HCl (S-2238), bought from Chromogenix (Milan, Italy) was monitored using a microplate reader (SpectraMax, Model M4, Molecular Devices, Menlo Park, CA, USA), equipped with a mixer and heating system at A405 nm. The extracts of *A. spicifera* were incubated with thrombin (40 nM, final concentration) for 10 min. at 37°C, and then, the reaction was triggered by adding S-2238 (0.5 mM, final concentration). The reaction was monitored during 20 min at 37°C. Control experiments were performed by incubating thrombin with DMSO (1% v/v, final concentration) or saline, instead of the extracts of *A. spicifera*.

In vitro cell toxicity

Human platelets were incubated with the extracts of *A. spicifera* or products, saline or DMSO (0.9% v/v) for 10 min at 37°C, then lactate dehydrogenase (LDH) activity was measured using a LDH-P UV kit (Wiener Laboratories), according to the manufacturer's instructions. Hundred percent of platelets lysis was achieved by adding water or sonicating PRP. Hemolysis was determined according to Bauer et al. (2012), with modifications. A 13% solution of human erythrocytes was incubated with *A. spicifera* or products, saline or DMSO (0.9%) for 3 h at 37°C. Then, solution was centrifuged (1,200 g for 10 min) and the hemoglobin was measured at A 578 nm using a Micro Plate Reader (SpectraMax, Model M4, Molecular Devices, California, USA). Hundred percent of hemolysis was obtained by adding 1% Triton X-100 to cells. Experiments were approved by CEP-UFF, CAAE: 28941314.0.0000.5243.

Statistical analysis

The results were expressed as the means ± standard error (SEM) obtained with the number of experiments performed indicated. The statistical significance of differences among experimental groups was evaluated using the Student t-test (p-values < 0.05 were considered statistically significant).

RESULTS AND DISCUSSION

Chemical composition

The assessment of the chemical profile of *A. spicifera* extract using gas chromatography–mass spectrometry (GC-MS) analysis has demonstrated the presence of fatty acids, and sterols (Flora and Rani, 2013; Polat and Ozogul, 2008). Furthermore, analysis of the chemical profile of genus *Acanthophora* (Rhodophyta, Ceramiales) showed the presence of sterols, as cholesterol and derivatives (Dayong et al., 2011), fatty acids (Kumari et al., 2014), vitamins and carotenoids (Aihara and Yamamoto, 1968). Nogueira et al. (2016), by ^{1}H-NMR technique, evaluated the presence of phenolic molecules in the extracts of *A. spicifera* prepared using increasing polarity solvents. In such work, the anti-HIV effect of these extracts was due to the presence of aromatic chemical structures. Here, we tested antiplatelet or anticoagulant effect of the crude extracts of the seaweed *A. spicifera* as well as the commercial available products (biotin, myristic acid, cholesterol, B-carotene and vitamin B_{12}).

Effect of the extracts of *A. spicifera* on platelet aggregation

Platelets are anucleate megakaryocyte fragments that passively circulate in the blood stream. However, after a stimulus, in an injury, they rapidly activate, interact with other platelets and with endothelium or extracellular matrix molecules to form a hemostatic plug, preventing blood loss (Qiu et al., 2015; Ruggeri, 2002; Clemetson,

2012).

Moreover, platelets participate in many other processes, as inflammation and defense from microbial infection. However, undoubtedly, their main function is to prevent hemorrhage after an injury (Clemetson, 2012). To perform such functions, platelets have three granules, alpha (α), dense (δ) and lysosomes (λ) which contain molecules (protein and non-protein) with pro- and anti-thrombotic effects (Clemetson, 2012). After activation and aggregation of platelets, they secrete their granule content in order to amplify the hemostatic system, and enhance the formation of a clot by the coagulation cascade. The events of platelet adhesion, activation, aggregation and secretion of their granules are complex and may be stimulated by several agonists, as ADP, collagen and thrombin, and involve many ligands and/or plasma membrane-receptors (Clemetson, 2012). Thus, platelets circulate into blood stream in an inactive form, and just after a stimulus, they turn into active form in order to prevent and to stop blood loss. If an imbalance occurs in both forms of platelets, hemorrhage or procoagulant disorders may occur.

Cardiovascular diseases are the main causes of death and disability worldwide, with approximately 30% of deaths (Clemetson, 2012; Roth et al., 2015). Despite improved treatments and awareness of people, the rate of these diseases rose over the last decade (Alwan et al., 2010). Moreover, diabetes, obesity, depression, bad eating habits may contribute to increase them in the near future (Alwan et al., 2010). Thus, therapies to prevent blood coagulation and to inhibit platelet functions are employed, and in most of cases with positive results. Literature has described molecules that are regularly used as drugs to treat such diseases (DeWald and Becker, 2014; Nutescu et al., 2016). However, the current treatments have some drawbacks, as limited efficacy, resistance appearance, limitations related to the routes of administration and may induce side effects as well (Choi et al., 2013; Nutescu et al., 2005; Agnelli, 2005).

Thus, researchers are looking for new molecules with anticoagulant and/or antiplatelet effects able to improve therapies, but without restrictions or limitations. Lot of molecules derived from natural sources, be it from terrestrial or marine environment, have shown antihemostatic effects (Pimentel et al., 2003; de Andrade et al., 2011; Yoon et al., 2015; Lorigooini et al., 2015). Nonetheless, as known, most products with a clinical or medical interest came from oceans, approximately 33 and 25% from sponges and algae, respectively (Kijjoa and Sawangwong, 2004; Newman and Cragg, 2012). Seaweeds produce molecules with a variety of biological and pharmacological activities (Cirne-Santos et al., 2018), and many of them had led to the development of drugs with antihemostatic action (Collins et al., 2016; Kwak, 2014; Mourão, 2015).

In fact, oceans should be taken into consideration to discover new molecules, rather than the terrestrial environment (Collins et al., 2016). Here, we investigate the ability of four extracts (prepared using solvents of different polarity: dichloromethane, ethyl acetate, acetone and methanol) of the seaweed *A. spicifera* to inhibit coagulation and platelet aggregation. As shown in Figure 1, the extracts of *A. spicifera* (100, 200 or 400 µg/ml) inhibited aggregation of human PRP induced by 15 µM ADP (Panel A) or 5 µg/ml collagen (Panel B), in a concentration-dependent manner. The inhibitory profile of all extracts was very similar, regardless the agonist tested. At the lowest concentrations, the methanol (MET) or dichloromethane (DCM) extracts inhibited less the aggregation induced by ADP or collagen, respectively.

However, at the highest concentration, such difference disappeared, and both extracts inhibited around 25% the aggregation of platelets. The ETA, ACT, MET extracts achieved similar inhibitory percentages on collagen-induced aggregation. Because of the low inhibitory percentages, IC50 values of extracts were not possible to determine. Commercial molecules, which are present in A. spicifera seaweed (Ganesan et al., 2008), were tested on platelet aggregation. As seen in Figure 2, these products inhibited ADP- or collagen-induced platelet aggregation in a concentration-dependent manner; and, different half-maximal inhibitory concentration (IC_{50}) values for the products were achieved (Table 1), ranging from 25 to 350 µM. On the other hand, IC_{50} of biotin was not able to determine, regardless the inducer tested (Table 1).

It is noteworthy that ADP or collagen does not share the same receptor and signaling pathways (Clemetson, 2012), so a difference at the percentage of inhibition can be achieved. After incubation of the extracts of *A. spicifera* (600 µg/ml) or products (2 mM) with platelets or erythrocytes, no trace of lactate dehydrogenase activity or release of hemoglobin was detected, respectively (data not shown). Thus, at such concentrations, the extracts of *A. spicifera* or products were devoid of toxicity. In general, products derived from seaweed have low cytotoxicity (Wang et al., 2007), and such assays are an important task that should be considered when the aim is to develop new drugs. Based on these results, the extracts of *A. spicifera* inhibited platelet aggregation and, therefore, could be considered to aid the development of new classes of antiplatelet agents.

Effect of the extracts of *A. spicifera* and products on coagulation

The effect of the extracts of *A. spicifera* on coagulation was investigated through the *in vitro* methods:

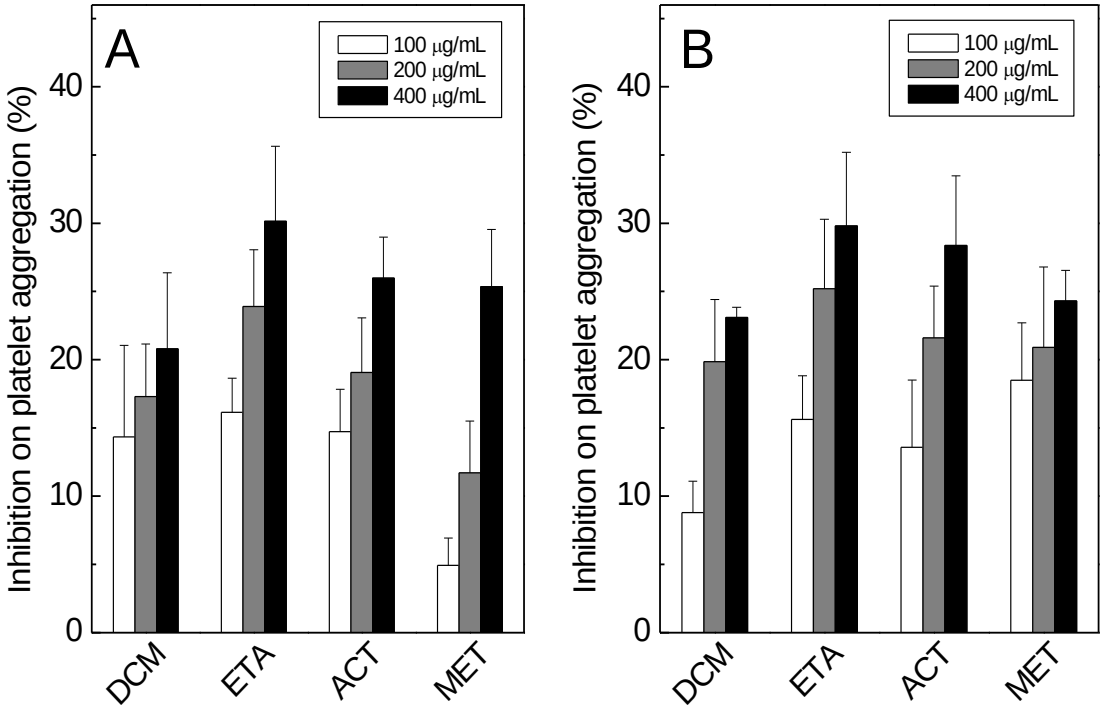

Figure 1. Effect of the extracts of *A. spicifera* on platelet aggregation. Different concentrations (100, 200 or 400 µg/ml) of the extracts of *A. spicifera* prepared in dichloromethane (DCM), ethyl acetate (ETA), acetone (ACT) or methanol (MET) were incubated with PRP for 6 min. at 37°C, and then 15 µM ADP (Panel A) or 5 µg/mL of collagen (Panel B) was added to medium to trigger aggregation, as described in methods. Results are expressed as the means ± SEM of individual experiments (n = 3).

Prothrombin Time (PT), activated Partial Thromboplastin Time (aPTT), thrombin time (TT) and Fibrinogen Coagulation (FC), and results are shown in Table 2. These methods are traditionally employed to investigate bleeding or procoagulant disorders in patients as well as to look for molecules with procoagulant or anticoagulant effect. The PT and APPT tests measure the participation of the extrinsic and intrinsic pathway of the coagulation cascade, respectively. Both tests are inexpensive, easily performed and give accurate results over possible coagulation defects.

However, the division of the coagulation cascade into intrinsic and extrinsic pathways is only a useful concept for interpreting the results of such investigation, and does not have any *in vivo* validity. The TT and FC tests do not have a clinical signification by themselves, but they are important in combination with PT or aPTT test. As seen in Table 2, most of the extracts had anticoagulant action in such tests; and, none of them had a procoagulant activity. The extracts prepared in ACT or MET did not have any effect on coagulation time in the PT test, and at the highest concentration (400 µg/ml), the DCM or ETA extracts delayed coagulation time. The extract prepared in MET did not interfere in the coagulation time at any of all methods. On the other hand, the extracts in DCM, ETA or ACT delayed the plasma coagulation time with different potencies.

Overall, it is not possible to postulate a more efficient extract to inhibit coagulation. The extract in DCM or ACT inhibited the conversion of fibrinogen into fibrin through FC or TT tests. Thus, the extracts of *A. spicifera* prolonged coagulation by interfering at the intrinsic and extrinsic pathways of the coagulation cascade, and at the activity of thrombin, as well. Based on these results, one may speculate an interaction between molecules of extracts with thrombin, leading to the inhibition of the activity of enzyme. The commercial products were tested on *ex vivo* coagulation through the PT and aPTT tests, as well. As seen in Table 3, vitamin B_{12}, b-carotene, myristic acid, and cholesterol delayed the plasma coagulation time of mice at both tests, when compared to vehicle (saline or DMSO).

For PT assay, the extracts of *A. spicifera* (100, 200 or 400 µg/ml) were incubated with plasma for 10 min at 37°C, and then thromboplastin was added to induce coagulation. For aPTT assay, the extracts of *A. spicifera* were incubated with plasma plus cephalin for 10 min at 37°C, and then, $CaCl_2$ (8.3 mM) was added to induce coagulation. For FC assay, the extracts of *A. spicifera* were incubated for 10 min at 37°C with

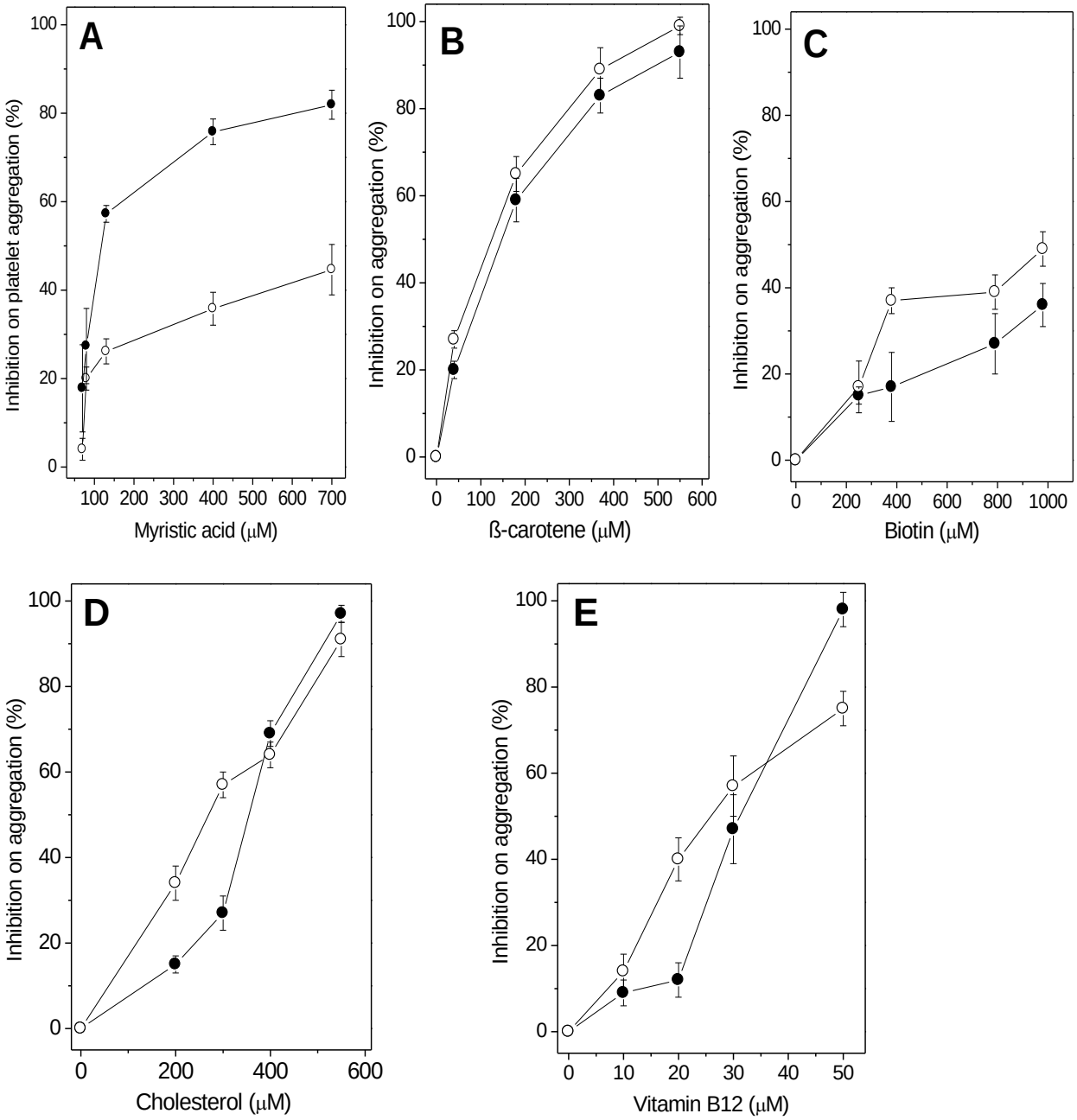

Figure 2. Effect of products on platelet aggregation. Different concentrations of commercial products myristic acid (Panel A), β –carotene (Panel B), biotin (Panel C), cholesterol (Panel D) and vitamin B_{12} (Panel E) were incubated with PRP for 6 min. at 37°C, and then 5 µg/ml of collagen (-○-) or 15 µM ADP (-●-) were added to medium to trigger aggregation, as described in methods. Results are expressed as the means ± SEM of individual experiments (n = 3).

fibrinogen (2 mg/ml), and then, thrombin (10 nM) was added to induce coagulation.

For TT assay, the extracts of *A. spicifera* were incubated with plasma, and thrombin (1 nM) was added to induce coagulation. Results are expressed as the means ± SEM of individual experiments (n = 4). *, $p < 0.05$ when compared to saline or DMSO. AS-DCM, *A. spicifera* in dichloromethane, AS-ETA, *A. spicifera* in ethyl acetate, AS-ACT *A. spicifera* in acetone, AS-MET, *A. spicifera* in methanol. Mice received a single i.v. injection of 100 µL of vehicle (saline or DMSO) or 300 µg/mice of commercial products (vitamin B_{12}, β-carotene, biotin, myristic acid, and cholesterol). After 2 h, blood was collected from mice, centrifuged and

Table 1. IC_{50} of commercial products on platelet aggregation.

Products	collagen	ADP
Myristic acid	ND	125
Carotene	135	110
Biotin	ND	ND
Cholesterol	350	270
Vitamin B_{12}	25	30

ND= not determined.

Table 2. Effect of the extracts of *A. spicifera* on coagulation.

Samples	Concentration	Coagulation time			
		PT	aPTT	FC	TT
Saline	0.15 M	21.7 ± 1	65.1 ± 13	32.6 ± 12	22.2 ± 5
DMSO	1 % v/v	23.7 ± 2	68.5 ± 12	40.4 ± 18	21.4 ± 3
AS-DCM	100	25.8 ± 1	103.4 ± 18*	82.2 ± 17	28.9 ± 4
	200	26.1 ± 2	149.0 ± 12*	93.2 ± 15	49.7 ± 1*
	400	28.9 ± 1	277.8 ± 55*	101.1 ± 10	89.4 ± 3*
AS-ETA	100	25.1 ± 1	98.4 ± 18	47.3 ± 10	22.5 ± 2
	200	27.1 ± 3	131.7 ± 67	79.6 ± 11	31.3 ± 5
	400	29.5 ± 2	147.8 ± 14*	121.3 ± 8*	63.9 ± 4*
AS-ACT	100	23.4 ± 0.9	102.7 ± 13*	72.2 ± 5	45.1 ± 4*
	200	24.4 ± 0.8	148.3 ± 14*	100.6 ± 6*	55 ± 3*
	400	25.3 ± 0.8	245.4 ± 29*	143.1 ± 8*	94 ± 2*
AS-MET	100	25.4 ± 1	74.5 ± 27	80 ± 10.9	23 ± 2.2
	200	24.4 ± 1	89.8 ± 19	67.1 ± 9.6	30 ± 3.3
	400	25.5 ± 0	96.0 ± 21	65.5 ± 15	30 ± 2.9

plasma was subjected to aPTT or PT tests, according to methods; *, $p < 0.05$ when compared to saline or DMSO.

Effect of the extracts of *A. spicifera* on hydrolysis of S-2238

S-2238 is a specific chromogenic substrate, usually employed to test the enzymatic activity of thrombin, that it is a serine protease that cleaves fibrinogen (a soluble protein) into fibrin (an insoluble protein), resulting in the formation of a firm and stable clot. Besides, thrombin can trigger platelet aggregation and activate some coagulation factors of the cascade (factors V, VIII and XI), promotes vasoconstriction, and has mitogenic effects (Coughlin, 2000). Apart from the coagulant action, thrombin has anticoagulant effect through the activation of protein C, inactivating factors V and VIII (Crawley et al., 2007; Della-Valle et al., 2007). Some of these functions of thrombin depend on its catalytic site and two other sites (the anion-binding exosites I and II), in which are positively charged, and may interact with negative surfaces, promoting the pharmacological functions of thrombin (Crawley et al., 2007; Della-Valle et al., 2007). Anticoagulants, as hirudin and analogs have been designed to allow the binding to thrombin through these charged sites (Nutescu, 2016; Huntington, 2014). As seen in Figure 3, at all concentrations of extracts (100, 200 or 400 µg/ml), inhibition of the enzymatic activity of thrombin, measured by the hydrolysis of S-2238 was achieved. Again, the methanolic extract (MET) inhibited the activity of thrombin less, and the extracts in DCM and ACT achieved the highest inhibitory percentage, close to 100%. Surprisingly, these extracts (DCM and ACT) had the highest inhibitory effect at all the coagulation tests, as well. The extract in ETA inhibited around 20% of the thrombin activity, at any of the tested concentration.

Table 3. Effect of commercial products on ex vivo plasma coagulation.

Groups	aPTT	PT
Saline	28 ± 0.7	12 ± 0.6
DMSO	29 ± 0.1	11 ± 0.1
Vitamin B12	800± 0.5	800± 0.4*
β-carotene	800 ± 0.3*	800 ± 0.4*
Biotin	55 ± 0.1	27 ± 0.1
Myristic acid	136 ± 0.1*	101 ± 0.1*
Cholesterol	800 ± 0.7*	800± 0.2

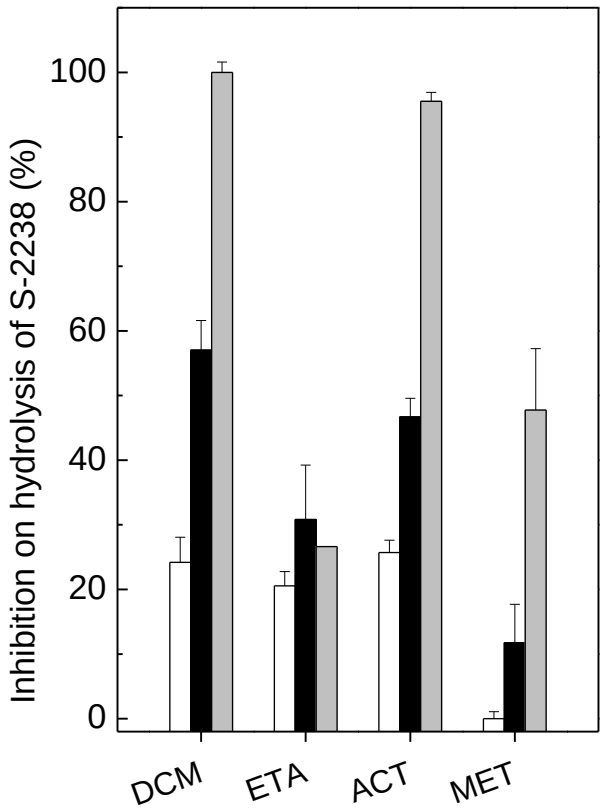

Figure 3. Effect of the extracts of *A. spicifera* on hydrolysis of S-2238. The extracts of *A. spicifera* (100, white columns; 200, black columns or 400 µg/ml, gray columns) prepared in dichloromethane (DCM), ethyl acetate (ETA), acetone (ACT) or methanol (MET) were incubated with thrombin (40 nM) for 10 min at 37°C, then reaction was initiated by adding S-2238 (0.5 mM). The reaction was monitored at A 405 nm during 20 min, and 100% of activity was obtained as the difference between reads obtained at the end and at the beginning of the reaction in the absence of extracts. Data are expressed as means ± SEM of two individual experiments (n = 4).

Conclusion

Taken together, the extracts of different polarity of *A. spicifera* and commercial products inhibited aggregation of platelets and coagulation of plasma, and, thus, they have antihemostatic properties. However, the mechanism of action on aggregation should be investigated further. This work also shows the

importance of bioprospecting studies for discovering molecules of natural sources without toxicity and easily cultivated, that could be used to develop drugs to improve antithrombotic therapy.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antibacterial potential of extracts of the roots of *Zingiber officinale* against bacterial strains commonly associated with nosocomial infections

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***Zingiber officinale,* commonly known as ginger, has been used as a medicinal plant for decades for medical and culinary purposes. This study aimed to determine the antimicrobial potential of ginger root extracts using the Kirby-Bayer agar diffusion method to compare the zones of inhibition of the extracts to those of synthetic antibiotics against five clinical bacterial pathogens (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter aerogenes* and *Klebsiella pneumoniae*). Ginger extracts showed more antimicrobial activity against the five test organisms compared to the synthetic antibiotics. The least resistant bacterium was *S. aureus* while the remaining four bacterial strains were strongly resistant to most of the antibiotics. Antimicrobial activity of ginger root extracts at various concentrations revealed that *E. coli* had the lowest concentration (1.2 mg/ml) in 20 mg/ml while the highest concentration (9.1 mg/ml) was observed for *S. aureus* in 75 mg/ml. Phytochemical screening of the ginger root extracts revealed the presence of all the tested secondary metabolites (saponins, tannin, flavonoids, glycoside, terpenoids and alkaloids). A number of phytochemicals present in ginger were identified to be possibly responsible for the antibacterial activity of ginger roots. These could be used independently or in combination with synthetic antibiotics to create more efficient antibiotics. Findings of this study can contribute to on-going research towards identifying alternative treatment of nosocomial infections that eliminate the use of antibiotics. With these findings, scientists could be employed in the health sector to create antibiotics that are not resisted by pathogens that cause infections in health care facilities.**

Key words: Antibacterial, ginger, phytochemical, zone of inhibition.

INTRODUCTION

Antibiotics are antimicrobial medicines that are used to treat and prevent bacterial infections. When used properly, antibiotics can save lives by fighting these infections. However, the misuse and abuse of these drugs has led to an increase in drug resistance which makes it difficult to successfully treat bacterial infections and control microbial pathogenicity. The misuse of antibiotics over the years has caused the development of

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Figure 1. Ginger roots used in this study.

multi drug resistance (MDR) in pathogenic bacteria. This is where the demand for natural products such as ginger comes in as alternative medical treatment for these pathogenic bacteria. Antibiotics are classified based on their modes of action such as cell wall inhibition which prevent cell wall generation, cell membrane inhibition which causes disorganization of the cell membrane and nucleic acid synthesis inhibition which block the action of DNA gyrase and topoisomerase IV, therefore inhibiting DNA replication (Verma et al., 2008).

The use of antibiotics when it is not appropriate promotes antibiotic resistance. According to the Centre for Disease Control and Prevention (2013), one-third to one-half of antibiotics use in humans is unnecessary or inappropriate. Resistance to antibiotics results in serious illness, longer recovery from disease, more-frequent or longer hospitalization, more doctor visits, and more-expensive treatments.

All natural antibiotics are plant derivatives or other natural substances that show strong antimicrobial properties and as such may be recommended by medical professionals for use as first line treatments to combat viral, bacterial, fungal, or parasitic infections. These natural alternatives work very effectively in helping patients get healthy all the while avoiding the negative side effects of conventional antibiotics. Conventional antibiotics are static; they are bound to have limitations which allow bacteria to develop mechanisms that resist their effect. On the other hand, natural plant based antibiotics such as ginger are not static and as such are able to come up with new ways of eliminating bacteria.

The use of ginger as a naturally obtained antimicrobial agent has great implications. The continuing occurrence of superbugs that are resistant to the most lethal of synthesised antibiotics is becoming a global concern. Research shows that more than 3 million people die annually from bacteria related infections (Pang et al., 1995).

Previous studies have shown the medicinal benefits of ginger extracts which warrants the need for it to be further investigated. As Gizanna et al. (2005) report, ginger has been applied to remedy ailments such as muscle conditions and arthritis. The antibacterial potential of ginger extracts is well documented in its ability to control different diseases as well as disease prevention (Rahmani et al., 2014). Ginger has been shown to have potential against some clinical isolates (Omoya and Akharaiyi, 2011) as well as some food borne bacteria (Islam et al., 2014). More studies on the antibacterial activity of ginger extracts against nosocomial pathogens are necessary in order to elucidate their antibiotic potential. This study aimed to assess resistance patterns of bacterial strains that commonly cause nosocomial infections against compounds extracted from ginger roots in order to determine the antimicrobial potential of the ginger root extracts.

MATERIALS AND METHODS

Extracts isolation and preparation

The variety of roots used in this study was Chinese ginger roots which were purchased at Spar Grocery Store in Palapye, Botswana (Figure 1). The roots were about a month's old at the time of purchase. The ginger roots (weighing around 3 kg) were thoroughly washed with distilled water, peeled and chopped into small equal fragments of about 1 cm^3. After drying, fragments were incubated in an oven at 40°C for 24 h. They were then ground into fine powder. About 10 g of the ginger powder was dissolved in 100 ml of 96% methanol. The same amount of powder was dissolved in 100 ml of distilled water which was used as a negative control. The mixtures were sonicated to break down the cell walls of the ginger roots mechanically thereby releasing their content without degrading the

Table 1. Inhibitory zones of different antibiotics on 5 different bacterial isolates.

Antibiotic	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Escherichia coli*	*Enterobacter aerogenes*	*Pseudomonas aeruginosa*
Ampicillin	R	R	R	R	R
Amoxicillin	R	R	R	R	R
Gentamycin	I	R	I	R	R
Augmentin	S	R	I	R	R
Tetracycline	R	R	R	R	R
Clindamycin	S	R	R	R	R
Erythromycin	I	R	R	R	R
Ciprofloxacin	R	R	R	R	I
Nalidixic acid	I	R	I	R	R

R=Resistant, I=intermediate, and S=Susceptible.

Table 2. Zones of inhibition (mm) of 96% methanol and distilled water extracts of ginger roots on five bacterial isolates.

Bacterial Isolate	Inhibition zone in 96% methanol (mm)	Inhibition zone in distilled water (mm)
Staphylococcus aureus	17 ± 1.53	0
Klebsiella pneumoniae	15 ± 1.37	0
Escherichia coli	20 ± 1.85	0
Enterobacter aerogenes	17 ± 1.34	0
Pseudomonas aeruginosa	21 ± 1.27	0

cells. After sonication, the mixtures were filtered with a Whatman No. 1 filter paper and the filtrates were placed in the hood for 24 h to allow evaporation to occur.

Antibiotic susceptibility testing

Five clinical isolates (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter aerogenes* and *Klebsiella pneumoniae*) were obtained from the Botswana International University of Science and Technology (BIUST) microbiology laboratory. Disk diffusion method (Collins et al., 1995) was used to determine the susceptibility of isolates to antibiotics. A 0.5 Mcfarland standard was used. Twenty four hour-old cultures were spread on prepared Mueller-Hinton Agar (MHA) plates and allowed to diffuse. Synthetic antibiotics were carefully placed on to the media with sterilized forceps. The plates were then inverted and placed in an incubator for 24 h. This allowed the determination of the presence or absence of a zone of inhibition. Isolates with the highest resistance to most of the antibiotics were selected to test against ginger root extracts.

Antibiotic sensitivity testing

The following antibiotics were used in this study; ampicillin, amoxicillin, ciprofloxacin, clindamycin, erythromycin, gentamycin, augmentin, tetracycline and nalidixic acid. Antibiotic sensitivity testing was done as described by Pierce-Hendry and Dennis (2010). Filter paper discs (7 mm in diameter) were prepared and sterilised in an autoclave. They were then placed into the ginger extracts to soak up the content. Using sterile cotton swabs, the cultures were aseptically swabbed onto sterile MHA plates. Using ethanol dipped and flamed forceps, the antibiotic discs were placed over the seeded MHA plates and sufficiently separated from each other to avoid overlapping of the inhibition zones. The plates were incubated at 37°C for 24 h and the diameter of the inhibition zones was measured. Antimicrobial activity of ginger extracts on bacterial pathogens was tested at different concentrations of 20, 50 and 75 mg/ml. For all experiments, there were three technical repeats as well as three biological repeats. Results of these were averaged.

Phytochemical tests

Phytochemical screening of the ginger root extracts was performed as described by Harborne (1998) to determine the presence of the following bioactive compounds; saponins, tannins, terpenoids, glycosides, flavonoids and alkaloids which all give ginger its antimicrobial properties.

Statistical analysis

Data was analysed and expressed as mean ± standard deviation. All tests were carried out in triplicate to improve accuracy.

RESULTS

The sensitivity of the five bacterial isolates was tested against different synthetic antibiotics. Augmentin and clindamycin both showed high antimicrobial activity against *S. aureus*. *S. aureus* was the least resistant of the five bacterial strains. The other four bacteria showed high resistance against most of the antibiotics showing that most antibiotics had low antimicrobial activity against them (Table 1).

Large zones of inhibition by methanol extracts were

Table 3. Inhibition concentrations of ginger root extracts against bacterial pathogens at different concentrations.

Multi-Drug Resistant Bacterial strain	20 mg/ml	50 mg/ml	75 mg/ml	Gentamicin	Distilled Water
Staphylococcus aureus	2.5	5.2	9.1	10	0
Klebsiella pneumoniae	2.2	4.1	7.2	12	0
Escherichia coli	1.2	3.2	5.7	13	0
Enterobacter aerogenes	1.3	2.4	4.2	9	0
Pseudomonas aeruginosa	1.5	2.7	4.8	13	0

Table 4. Phytochemical constituents of ginger extracts.

Secondary metabolite	Distilled water extract	96% Methanol extract
Saponins	- - -	+ +
Tannins	- - -	+ +
Flavonoids	- - -	+ +
Glycosides	- - -	+ + +
Terpenoids	- - -	+ + +
Alkaloids	- - -	+

+ = present at low concentration, ++ = present at high concentration, +++ = present at very high concentration, - = absent.

observed while distilled water extracts showed no inhibition zones. The highest inhibition zone was observed for *P. aeruginosa* while *K. pneumoniae* had the lowest zone of inhibition (Table 2). Table 3 shows the antimicrobial activity of the ginger root extracts tested at different concentrations. Gentamicin was used as a positive control while distilled water was used as a negative control. Inhibition concentrations ranged from 9 to 13 mg/ml for the control gentamicin. The highest recorded concentration was 9.1 mg/ml for *S. aureus* in 75 mg/ml while *E. coli* recorded the lowest inhibition concentration of 1.2 mg/ml in 20 mg/ml (Table 3).

Phytochemical screening showed that no bioactive compounds were present in distilled water extracts while all of the tested compounds were present in 96% methanol extracts with terpenoids and glycosides being present at higher concentrations than the other compounds (Table 4). Alkaloids were present at lower levels than any other compound.

DISCUSSION

This study demonstrated that methanol extracts contained compounds that showed antimicrobial properties against both the Gram positive and Gram negative isolates. Ginger extracts were shown in this study to comprise secondary metabolites such as flavonoids, alkaloids, terpenoids, tannins and saponins. This has been proved by other studies in the past (Batista et al., 1994; Barre et al., 1997). The phytochemicals tested for were those that are linked with the antimicrobial activity of the ginger roots because they serve as a defence mechanism against invasion by microorganisms (Bensky et al., 1993). Flavonoids that are present in ginger extract serve as hydroxylated phenolic substances that help plants respond to microbial infections. Saponins on the other hand have antimicrobial activity because they are able to leak some proteins and enzymes from the plant cell (Zablotowicz et al., 1996). As explained by Shimada et al. (2006), the antimicrobial property of tannins comes from their ability to bind to proline rich proteins and interfere with protein synthesis. Terpenoids, which were present in the methanol extract, interact with compounds such as vitamin A to give plants direct protection from biotic and abiotic stresses (Bensky et al., 1993).

There are some advantages associated with the use of natural sources of antibiotics such as reduced side effects, tolerance by patients, easy accessibility and the fact that most of these plant sources are renewable resources found in nature (Vermani, 2002). However, toxic effects of the ginger root extracts were not tested in this study. As such, their exact effectiveness can only be determined after toxicity tests have been done on eukaryotic cells to determine whether or not these extracts are safe for human consumption. Alternatively, extracts such as soybean oil and other oils from edible plants may be used for extraction. Studies have been conducted on the extraction of bioactive compounds present in ginger using solvents such as ethanol and methanol (Bailey-Shaw et al., 2008). Various extraction methods such as reflux, sonication and steam distillation have also been employed in ginger extraction. Because

of time limitations, the sonication method in which sound waves are applied to agitate the cellular particles of the plant was employed in this study. A frequency of >20 kHz used for extraction has high overall efficiency and high yields of the product. Thermal stability and rapid extraction time prevents the bioactive compounds from degrading (Balladin et al., 1997).

Pathogens such as *E. aerogenes* and *P. aeruginosa* used in this study have proven over the years to be resistant to many antibiotics due to the permeability barriers in their outer membrane made up of lipopolysaccharides. This allows them to colonize the surface of biofilms making them resistant to antibiotics. The resistance of these pathogens could however be reduced by antibiotic inhibitors found in plants such as ginger (Kim et al., 1995). It is possible that the maturity of the plant, the solvent used for extraction and the extraction method used to attain the secondary metabolites affected the variation of the antimicrobial activity against the test microbes.

According to Fujii (1994), morphological differences between Gram positive and negative bacteria influence the effects of the antimicrobial agents. Gram negative bacteria have influx pumps that do not allow intercellular build-up of antibacterial agents (Farzaneh and Carvalho, 2015). Therefore, there is a need to develop new antibiotics that can overcome or supress these pumps to recover the potency of antibiotics. The use of medicinal herbs such as ginger and other medicinal plants could help in combating this problem.

According to Burt (2004), the mechanisms of action of plant based drugs and their antimicrobial activity is based on the following, the antibiotic's ability to breakdown the cytotic membrane of the organism; interaction with proteins in the membrane; disintegration of the outer membrane to release LPS; destabilizing forces that leak ions into the cell; cell content coagulation and synthesis of enzymes. Complementary studies could be carried out to determine the pharmacokinetics of plant extracts, their purity as well as quantification of the bioactive compounds. Further studies could help in strengthening the potential of novel medicinal plants as cost effective agents against bacterial infections. The issue of safety of the use of herbal medicines is crucial in the public health sector because of the enormous number of people that are likely to consume the product.

It is evident from this study that ginger has antimicrobial activity against both Gram positive and negative bacteria. It is then reasonable to recommend that ginger and any other edible plant that has been proven to have antimicrobial properties be included in diet for benefits such as reducing the chances of developing bacterial infections which might help prevent frequent abuse of antibiotics. This could also help in reducing costs of treatment and development of new adverse drugs while preventing recurrent infections. It is however of paramount importance to determine the toxicity of the bioactive compounds found in every plant that is being explored for drug development by testing their side effects and pharmaceutical properties. We could not determine the *in vivo* side effects of the paper disks but for further research it is important that plants used for traditional medical practices be accompanied with thorough knowledge of the plant's bioactive compounds as well as efficacies that are backed up by scientific evidence. Following this study, toxicity studies should be carried out to check the effect of the ginger abstracts on human beings. This is to check if they are safe or not safe for use in humans as potential treatment against nosocomial infections. In conclusion, ginger roots extracts have the potential to be used to make non-synthetic antibiotics against nosocomial infections to reduce reliance on synthetic antibiotics which promote multi-drug antibiotic resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Ethnobotanical study on medicinal plants used by indigenous people in Tenta District, South Wollo, Ethiopia

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Ethnobotanical study on medicinal plants was conducted in Tenta District, South Wollo, Ethiopia from December 2016 to May 2017. Data were collected using guided field walk, semi-structured interview, group discussion and direct field observation. Data were analysed using descriptive statistics and response compared with chi-square test. The results documented 121 species of medicinal plants under 98 genera and 53 families based on local medicinal values. Of the total species recorded, Fabaceae contributed higher number (12) of medicinal plant species. Majority of plants (63.5%) were harvested from wild habitat and 16.5% from home garden. Shrubs (44.1%) were the major plant types followed by herbs (30.5%) and trees (12.7%). Pearson correlation analysis (r = 0.44) indicated that there was significant increase of knowledge about medicinal plants as age increases. According to the present study, the existence of a number of medicinal plants is an indication of the presence of ample traditional medicinal knowledge among the community but these curative medicinal plant species decline from time to time. This calls for urgent and collaborative actions to keep the balance between medicinal plants availability and their utilization by the community.

Key words: Ethnobotany, indigenous knowledge, medicinal plants, Tenta District, traditional healers.

INTRODUCTION

Medicinal plants make important contributions in the healthcare system of indigenous people as the main source of medicine for the majority of the rural populations. Plants serve as food, medicines, fuel, fodder and construction materials. They have also ritual or magical values (Abbink, 1995). They play a key role in the development of modern medicines (Pramono, 2002). Humans started to use plants for their livelihood long ago (Martin, 1995). Over centuries, indigenous people have developed their own locality specific knowledge on plant use, management and conservation (Cotton, 1996). The use of medicinal plants by indigenous people is mainly achieved through accumulation and transfer of knowledge from one generation to the next (Cunningham, 1996).

Plant diversity remains crucial for human well-being and provides a significant number of remedies required in healthcare. Medicinal plants played a pivotal role in the treatment of various problems in Ethiopia (Fekadu, 2007). For the role to be played by plant-derived products in human and livestock health care, systematic scientific investigation is vital. Due Attention was not given to

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ethnobotanical studies in the past decades in Ethiopia (Dawit et al., 2001). However, there exists an accelerated devastation of plant resources with loss of indigenous knowledge. There is a need to develop a sound research strategy and program for medicinal plants conservation, utilization and documentation including their location, existing population, places of conservation, and known traditional uses. When this documentation is achieved, it would be necessary to identify priority species for further work on characterization and data sharing through national, regional and international collaboration. Previously, no ethnobotanical study of medicinal plants was conducted in the study area. Therefore, the main objective of the study was to investigate the use and management of medicinal plants and to document the associated ethnobotanical knowledge by the indigenous people of Tenta District.

MATERIALS AND METHODS

Description of the study area

Tenta District is found in the South Wollo Zone of the Amhara Regional State, northern Ethiopia about 545 km away from the capital Addis Ababa. It is located at a latitude 11°19′N and longitude 39°15′E with average elevation of 2972 m a.s.l. (Figure 1). The average temperature of the area ranges from 4-40°C. The climate is dry where the average annual rainfall is between 700 and 1100 mm. Therefore, the rainfall is bimodal in nature with two main rainy seasons (from June to August and from February to April) (Teferi, 2013).

Reconnaissance survey

A reconnaissance survey was conducted from December 2016 to January 2017. The study was carried out in the natural forest of the district mainly in 17 kebeles (peasant associations), ten of which have forest.

Sampling design

The study was conducted in 17 kebeles of Tenta District by following rapid ethnobotanical appraisal research procedure. The selection of kebeles was made systematically based on the information gathered on the relative status of forest coverage, settlement, different agro climatic zones and availability of practitioners. Key informants were selected based on the seniority of age in the community; local residency for a period of not less than 20 years; knowledge of forest plants in the local dialect and well versed with their use(s). Current or previous experience as herbalist was preferable.

Data collection

Actual data collection was conducted from February to May 2017. A total of 96 key informants (59 males and 37 females), were purposively selected based on their age, length of stay in the area and gender. A key informant was identified from each kebele in the study area for detail discussion about the medicinal plants. A semi-structured questionnaire guided by a mix of closed and open-ended questions was employed. Using field observation, 22 plant species were collected from the study area. Medicinal plant species were also collected from natural vegetation and home garden.

Data analysis

Data were analyzed using descriptive statistics and responses compared using chi-square test. Informant consensus factor (ICF) was calculated for categories of ailments to identify the agreements of the informants on the reported cures using the formula used by Tilahun and Mirutse (2007):

ICF = nur - nt /nur -1

Where, ICF is the Informant consensus Factor, Nur is the number of use citation and Nt is the number of species used.

RESULTS

Medicinal plant resources in the study area

A total of 121 plant species from 98 genera and 53 families were recorded as having medicinal value in the study area. About 56.2% of the medicinal plants were collected from the wild and 23.9% from home gardens and the rest was from both wild and home garden. The leading family was Fabaceae with 12 species, followed by Lamiaceae with 6 species, Asteraceae, Cucurbitaceae, Euphorbiaceae and Solanaceae each with five species.

Distribution of medicinal plants in the study sites

Of the 121 medicinal plant species identified, the highest (72.7%) proportion was recorded in Yemit and the least (19%) in Gaya (Table 1). There was no significant difference (χ^2= 12, df =9, P > 0.05) between agro climatic zone and availability of medicinal plants. There was no significant difference (χ^2= 221, df =16, P > 0.05) with respect to different kebeles and the number of medicinal plants available in the study area.

In the study area, some cattle diseases were treated with various medicinal plant species. For instance, wound was treated with 12 medicinal plants. The Informant Consensus Factor (ICF) was between 0.71 and 1 which indicates the presence of high valid information in the treatment of cattle disease in the study area (Table 2).

Most human diseases are treated with different medicinal plants and the ICF was between 0.79 and 1 which indicates the agreement of information in the study area. Stomach ache and wound, are treated with 14 and 12 plant species, respectively with high ICF (Table 3).

Among the medicinal plants reported by the local people, 57.85% were used to treat human ailments whereas the least was recorded for livestock aliments (Table 4).

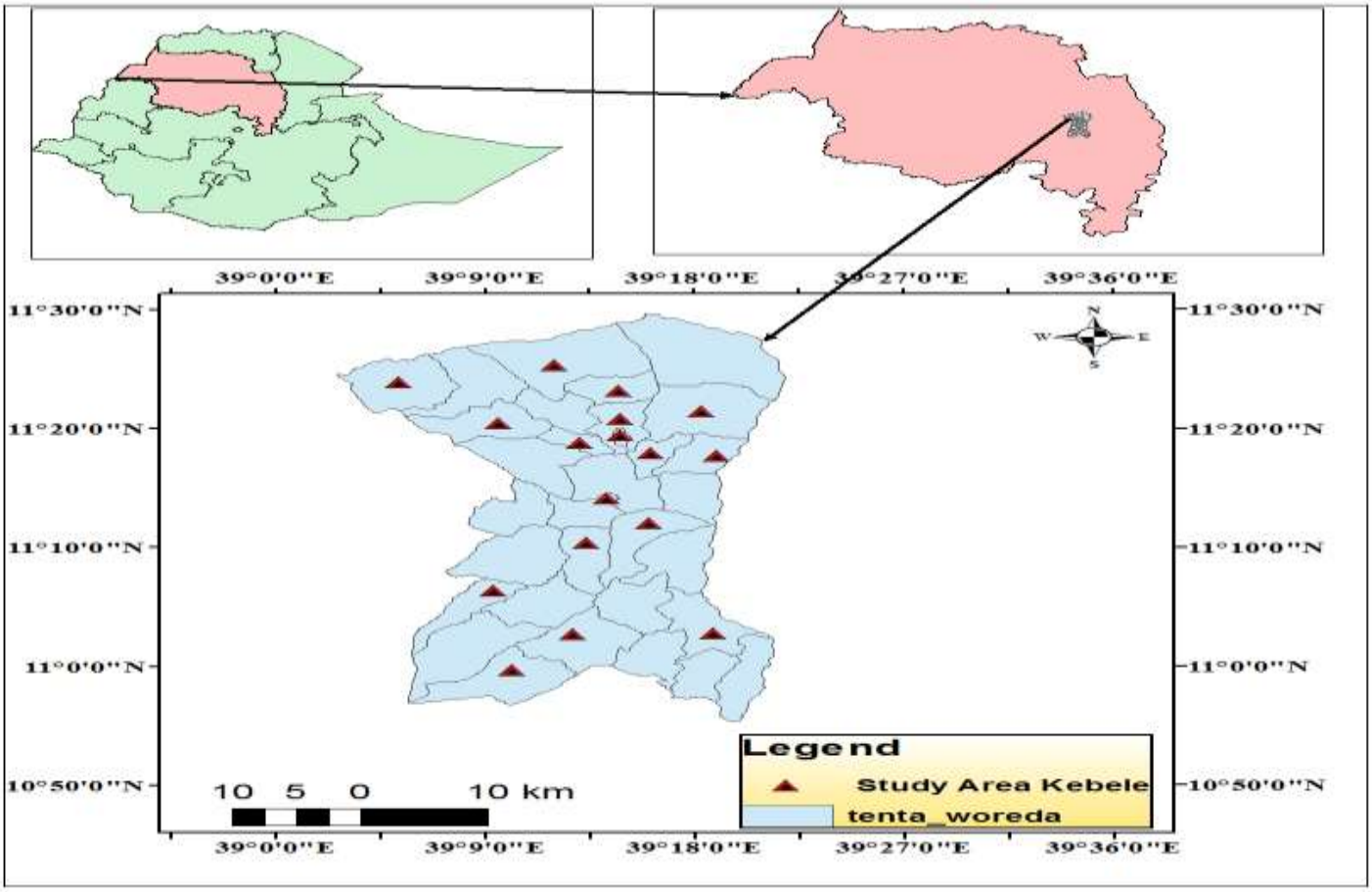

Figure 1. Map of the study area and sample sites.

Table 1. Distribution of medicinal plants in the study area.

Kebeles	Number of informants	Number of medicinal plants available
Chelemie	6	68(59.2%)
Yemit	7	88(72.7%)
Yamed	6	53(43.8%)
Tedat	6	72(59.5%)
Tenta town	6	35(28.9%)
Adjibar town	5	33(27.3%)
Billa	6	66(54.5%)
Debek	6	70(57.9%)
Yederek	7	81(66.9%)
Gafa	6	81(66.9%)
Baja	6	81(66.9%)
Sengola	4	75(62%)
Wata	6	59(48.8%)
Wortej	4	47(38.8%)
Meserbi	4	32(26.4%)
Zakunat	5	39(32.2)
Gaya	5	39(32.2)
Total	96	121(100%)

Table 2. Major livestock diseases and number of ethno veterinary plant species used treat livestock by indigenous people of Tenta District.

Disease treated	Total species	Percentage	Number of use reports	ICF value
Anthrax	1	1.53	28	1
Bloating	4	6.55	33	0.9
Coccidiosis	2	3.27	25	0.95
Cough	5	8.19	45	0.91
Diarrhoea	8	13.11	43	0.83
External parasites	3	4.91	44	0.95
Leeches	3	4.91	79	0.97
Rabies	8	13.11	39	0.71
Scabies	1	1.53	21	1
Snake poison	1	1.53	35	1
Wound	12	18.46	51	0.78
Internal parasites	3	4.91	19	0.89

Table 3. Major human diseases and number of ethno medicinal plant species used to treat human and ICF value by indigenous people of Tenta District.

Disease treated	Total species	% total	Number of use reports	ICF value
Abdominal pain	8	6.61	91	0.92
Amoeba case	3	2.48	87	0.97
Ascaris	2	1.65	53	0.98
Common cold	5	4.13	89	0.95
Cough	5	4.13	86	0.95
Dandruff	4	3.31	75	0.95
Diarrhoea	8	6.61	84	0.79
Dysentery	2	1.65	65	0.98
Eye Infection	2	1.65	63	0.98
Febrile illness	7	5.79	58	0.89
Fever	5	4.13	61	0.93
Gastritis	5	4.13	69	0.94
Head ache	5	4.13	85	0.96
Heart disease	2	1.65	35	0.97
Internal parasite	3	2.48	72	0.97
Dermal fungus	2	1.65	19	0.94
Stomach ache	14	11.57	86	0.96
Taeniasis	4	3.31	89	0.96
Wound	12	9.92	92	0.87
Lung diseases	1	0.83	39	1
Malaria	5	4.13	45	0.9

Table 4. Medicinal plants used to treat human, livestock and both human and livestock ailments

User	Number of plant species	Percentage
Human	70	57.85
Livestock	17	14.05
Both human and livestock	24	28.1
Total	121	100

Table 5. Preparation methods of the reported traditional medicinal plants.

Methods of preparation	Total preparation	%
Homogenizing in water	10	2.7
Crushing	17	11.6
Decoction	3	2.1
Squeezing	10	2.7
Chewing	11	7.5
Smoking/fumigating	16	10.9
Chopping	4	2.7
Extract with cold water	8	5.5
Concoction	4	2.7
Roasting	1	0.7
Rubbing	4	2.7
Wrapping	2	1.4
Sniffing	3	2.1
Boiling	5	3.4
Grinding	10	2.7
Heating	1	0.7
Swallowing	4	2.7
Pounding	22	15.1
Tying	8	5.5
Maceration	3	2.1
Total	146	100

Plant parts used for remedy preparation

Plant parts were prepared as medicine using fresh materials that accounted for 79%, and dried plant materials accounted for 21 %. Widely used plant parts by the people to treat human and livestock diseases include leaves, roots, barks, and stems. Majority of plant species (47.8%) were harvested for their leaves, followed by roots (14.5%), barks (9.8%), and stems (8.3%). According to informants, 91.5% of herbal preparations were from fresh plant parts followed by dried parts.

Preparation, dosage and route of administration of medicinal plants

The local communities of the area employ several methods of preparation of traditional medicines from plants. The result revealed that herbal medications were prepared differently. They often had a preference of mixing two or more medicinal plants to avoid or minimize side effect of the remedies. Most of the remedies were prepared by squeezing whereas only few species were used as medicine without being processed (Table 5).

There were different routes of administration of medicinal plants prepared by indigenous people. The main routes of administration in the study area were oral, dermal, optical, nasal, anal and sometimes mixed. Most (60.7%) medicines were administered orally followed by dermal route (15%) (Figure 2).

Indigenous knowledge transfer

A total of 43.8% of the respondents acquire medicinal plant knowledge from father/mother and least (8.3%) of the respondent obtain medicinal plant knowledge from their uncle/aunt (Table 6). There was significant difference (χ^2 =52.63, df =5, P<0.05) among respondents in transfer of indigenous knowledge about medicinal plants to the next generation.

Respondents varied (χ^2= 24.65, df =7, P <0.05) in terms of knowledge transfer about medicinal plants. Transfer of traditional knowledge from parent to elder son accounted for 25% followed by transfer to eldest daughter (17.8%) (Table 7).

DISCUSSION

According to the present study, family Fabaceae contributed the highest medicinal plant species followed by Lamiaceae and Astraceae. Similarly, other studies also showed large number of plant species from family Fabaceae used for medicinal purposes compared to other families (Abrha, 2008; Haile et al., 2008; Seyoum, 2009; Behailu, 2010).

Majority of the medicinal plants recorded in the present study were used to treat human ailments. Similarly, a study conducted in Bale Mountain National Park, Ethiopia indicated the use of large number of medicinal plants for treating human diseases than domestic animals (Haile et

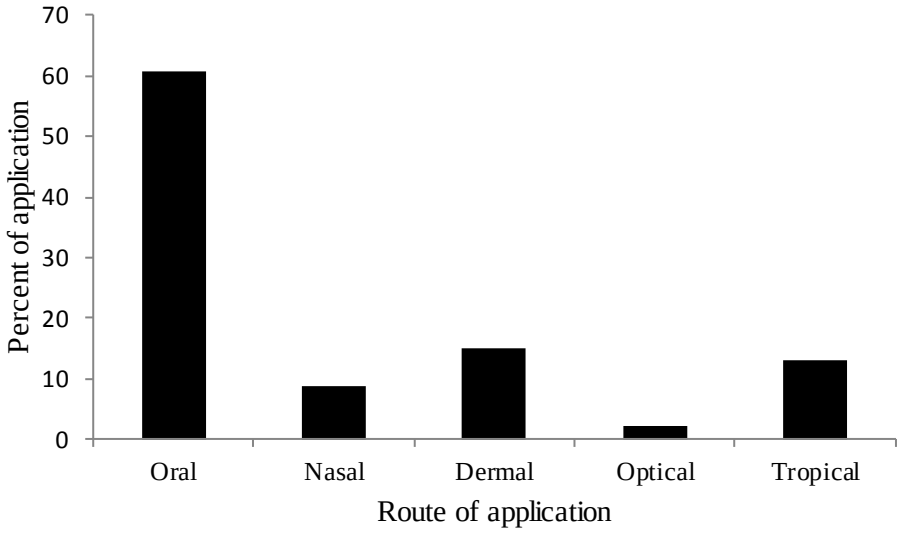

Figure 2. Route of administration.

Table 6. Sources of knowledge on the practice of traditional medicine.

Source of knowledge for	Number	Percentage
Traditional healer		
Father/ Mother	42	43.8
Wife/Husband	13	13.5
Sister/Brother	11	11.5
Uncle/Aunt	8	8.3
Neighborhood	13	13.5
Trial and error	9	9.4
Total	96	100

Table 7. Transfer of knowledge of traditional medicinal plants.

To whom knowledge is transferred to	Frequency	Percent of total
Eldest son	24	25
All children	15	15.6
Eldest daughter	18	17.8
Wife	8	8.3
Husband	7	7.3
Brother/Sister	9	9.4
Not to all	0	0
All members of the family	10	10.4
To all freely	5	5.2
Total	96	100

al., 2008).

The present study also revealed that mixing of two or more medicinal plants are common practices in remedy preparation. Similarly, according to Fisseha (2007), most traditional remedies were prepared by mixing components of two or more plants. Such practices might enhance healing capacity and minimize side effect on the patient (Mirutse, 2003). In the present study, only few remedies were made from single plant preparations. However, a study conducted in Bahir Dar Area indicated that most of

the traditional medicines were prepared single plant species (Fisseha, 2007). This might be due to difference in traditional knowledge from one area to the other. Mostly, the local people of Tenta District preferred fresh plant part for preparation of medicine. Similar findings were reported in other areas of Ethiopia (Zerihun, 2009).

The route and method of applications in the study area varied with the type of disease treated and the position where it occurred. The most common route of application found was oral followed by dermal. This might be due to the presence of wide spread internal diseases. Similar finding was also noted in other studies (Haile and Delnesaw, 2007).

Majority of the respondents were interested to transfer their traditional knowledge to their first son. This preference was associated with the perception and fear that daughters would share the knowledge with their husbands' family when they get married. Knowledge transfer to the new family was not appreciated by respondents for the purpose of keeping secret. When the first son was not considered trustworthy to keep the knowledge secretly, parents transfer to their second son or grandson. Similar finding was reported by Eskedar (2011).

In the study area, older people cited more species than the younger. As age increases experience also increases. Many studies in West African semiarid areas have reported that age has a direct bearing on knowledge of plants and plant use (Wezel and Haigis, 2000; Pare et al., 2010). Local knowledge of plants tends to accumulate with time and with continued interaction with the natural environment (Ayantunde et al., 2008). The results of this study failed to confirm the findings of Lykke et al. (2004) in five Fulani villages in the North-Sahelian area of Burkina Faso, where it was found that age was not an important determinant of plant knowledge. There was no significant difference in knowledge of plants between genders. These findings are similar to those of other studies in the Sahel of Burkina Faso (Lykke et al., 2004) and Niger (Ayantunde et al., 2008), but differ from the results of studies carried out in Niger and Mali (Wezel and Haigis, 2000).

Conclusion

The people of Tenta District are rich in indigenous knowledge in using, conserving and managing plant resources in general and medicinal plants in particular. This knowledge is transferred from elders to youngsters entirely through oral traditions and personal experiences. But this way, knowledge transmission will lead to distortion of the original knowledge or total disappearance of the practice. Human induced and natural factors are the major threats to plant species in general and to the medicinal plants in particular in the study area. Human induced threats include agricultural expansion, over grazing, deforestation, eucalyptus plantation, fire wood, medicinal plant trade, household equipment, and natural factors such as extended dry time are cited to be major threats for reduction of medicinal plants. The main threats to indigenous knowledge are caused by globalization; unwillingness of traditional healer to transfer their experience to the next generation; and increased business work; and negligence of the young generation to have the traditional knowledge. Therefore, the government has to do more in utilizing and managing different medicinal plant resources not only in Tenta District but also in the country as a whole through different activities.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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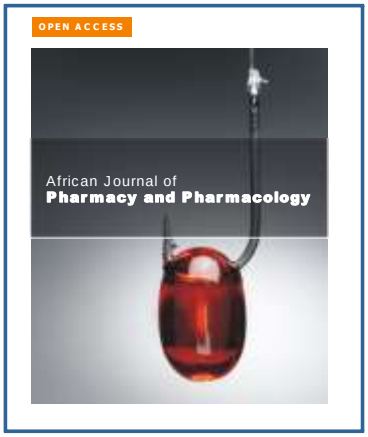

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