ANTIHYPERGLYCEMIC, ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECTS OF AQUEOUS EXTRACT OF MISTLETOE (*Cladocolea loniceroides*) IN STZ-INDUCED DIABETIC MICE

Frida Monserrat Hosanna Paredes Ruiz[&], Angeles Fortis Barrera[††], Edith Ponce Alquicira[&], Julio César Almanza Pérez[††], Rubén Román Ramos[††], Jorge Soriano Santos[&]*

[&]Departamento de Biotecnología; [††]Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, Av. San Rafael Atlixco, No. 186, Col. Vicentina, Ap. P. 55-535, Deleg. Iztapalapa, CP 09340, Mexico City, Mexico.

***Corresponding author's e-mail:**   Jorge Soriano-Santos jss@xanum.uam.mx

Abstract

Background: Inhibition of carbohydrate hydrolyzing enzymes, such as α-amylase and α-glucosidase, is a key element in the regulation of diabetes mellitus (DM). The purpose of this work was to study the inhibition of carbohydrate hydrolyzing enzymes, and the antihyperglycemic activity of aqueous extract of *Cladocolea (C. loniceroides)* in streptozotocin (STZ)-induced diabetic mice.
Materials and Methods: The inhibitory activities of *C. loniceroides* aqueous extract on α-amylase and α-glucosidase were investigated *in vitro*. Glucose tolerance test was performed in normoglycemic (NG) mice which were fed with starch or sucrose. The effect of mistletoe aqueous extract (ME) was measured in (STZ)-induced diabetic mice. On day 35 of the treatment, the effect of decreasing oxidative stress (lipid peroxidation, glutathione redox state, GPx and GR specific activities, cytokines and aminotransferases analysis) was assessed.
Results: ME showed a competitive mode of inhibition for the carbohydrate hydrolyzing enzymes (CHE). The maximum antihyperglycemic activity in mice was observed for the unripe fruit aqueous extract (UFAE) for α-amylase and stem aqueous extract (SAE); for α-glucosidase due to the glycemic response reduction by 23% or 35%, respectively. UFAE decreased malondialdehyde (MDA) 1.76 times; GSH/GSSG ratio was mantained (3.08 ± 0.66); GPx activity was reduced (24%); IL-6 was reduced (18%) and the concentration of TNF-α (37%) was leveled with respect to the (STZ)-induced diabetic mice; ALT and AST (liver transaminases) levels were nearly the same compared with those found in the NG mice.
Conclusion: UFAE of *C. loniceroides* exhibited the highest antidiabetic activity in (STZ)-induced diabetic mice.

Key words: Diabetes mellitus, *Cladocolea loniceroides*, antihyperglycemic, antidiabetic, oxidative stress and α-glucosidase.

Abbreviations: CHE: Carbohydrate hydrolyzing enzymes: *C.*: *Cladocolea :* STZ: Streptozotocin:NG: Normoglycemic: ME: Mistletoe extracts: UFAE: Unripe fruit aqueous extract: RFAE: Aqueous extract of ripe fruit: SAE: Stem aqueous extract: LAE: Aqueous extract of leaves: AE: Aqueous extracts:T2D: Type 2 diabetes: GPx: Glutathione peroxidase GR: Glutathione reductase: GSH: Glutathione reduced: GSSG: Glutathione oxidized. AST =Aspartate transaminase: ALT = Alanine aminotransferase.

Introduction

Type-2 diabetes (T2D) mellitus is a chronic degenerative disease that is characterized by a relative or absolute lack of insulin, resulting in hyperglycemia. Recent statisitics show its worldwide prevalence, with a 90% of occurrence mainly in 40 to 59 year-old adults (Sandoval, 2012). Besides that, the prevalence rate is in constant increase and it is characterized by a fasting glycemia greater than 126 mg/mL (Association, 2014). On the other hand, the deterioration in the antioxidant system also has a role on the decline in the clinical state of the patient. Oxidative stress in patients with T2D causes oxidation of macromolecules and nucleic acids which occur in cell membranes (Matsudda and Shimomura, 2013; Bullon et al., 2014; Rochette et al., 2014). Particularly, some literature point to an alteration of antioxidant enzymes such as glutathione peroxidase (GPx) and glutathione reductase (GR), which affect the concentration of glutathione in its reduced form (GSH) and oxidized form (GSSG) (Díaz-Flores et al., 2012). Immunologic and inflammatory mechanisms have a role

in T2D. The main cytokines involved in the pathogenesis are interleukins (IL-1 and and IL-6) and tumor necrosis factor-alpha (TNF-α). Recent studies have demonstrated that inflammation, specifically inflammatory cytokines, are determinant on the development of microvascular diabetic complications, including neuropathy, retinopathy, and nephropathy (Roman-Ramos et al., 2012). In the treatment of T2D, oral hypoglycemic agents like sulfonylureas, meglitinides, thiazolidines, D-phenylalanine and α-glucosidases inhibitors are used in addition to insulin treatment, along with diet and exercise. However, due to inhibitors of α-glucosidase such as acarbose (a pseudotetrasaccharide and inhibitor of α-glucosidase and pancreatic α-amylase with antihyperglycemic activity), excessive inhibition of α-amylase causes side effects such as abdominal pain, diarrhea, flatulence and an increase in liver enzymes, as a consequence of an abnormal bacterial fermentation of undigested carbohydrates in the colon (Alejandro-Espinosa et al., 2013). Drawbacks associated with existing synthetic oral hypoglycemic agents have prompted continued search for alternative agents from plant sources. Consequently, some plants have been used as sources for new antioxidant and antidiabetic agents because of their traditional uses (Adaramoye et al., 2012).

Several studies have reported that over 400 plants have been used in the treatment of large number of diseases, including diabetes (Lepzem et al. 2007). In particular, polyphenols have the ability to modulate blood glucose levels. Recent research has shown that phenolic compounds have the potential to inhibit CHE such as α-amylase and α-glucosidase in the digestive organs, and thus**,** might play a role in the management of T2D (Striegel et al., 2015). In addition, beneficial effects of antioxidants in diabetes include protection of pancreatic β-cells**,** which are vulnerable to glucose toxicity (Lepzem & Togun, 2017). Some parasitic plants such as the mistletoe *Viscum (V.)* or *album coloratum* which belongs to Santalaceae family, have been shown to possess antidiabetic activity with such study, we are also trying to find out a plausible utility of the mistletoe to prevent environmental deterioration of Xochimilco site with mechanism that are critical in the regulation of insulin secretion (kim et al, 2014).

Cladocolea (C. loniceroides) is a mistletoe which belongs to the Loranthaceae family. *C. loniceroides* is a killling pest for ahuejote trees**,** and it decreases the natural value of the borough of Xochimilco as a natural habitat within Mexico City. Xochimilco has been declared a world heritage site by the United Nations Educational Scientific and Cultural Organization (UNESCO). Previously, Serrano-Maldonado et al. (2011) have studied the use of *C. loniceroides* as a source of polyphenols with a potential of cytotoxic activity on breast cancer cell-lines. Nevertheless, no reports of antidiabetic properties of *C. loniceroides* are available in the literature. Therefore, the aim of this work centers on the assessment of antidiabetic activity of aqueous extract of *C. loniceroides* in (STZ)-induced diabetic mice, as well as the role and influence of the extract in oxidative stress and inflammation. ALT and AST.

Materials and Methods
Chemical and reagents

The following chemicals were obtained from Sigma-Aldrich (Germany): α-glucosidase type I from Baker Yeast (EC 3.2.1.20), resveratrol, porcine pancreas α-amylase (EC 3.2.1.1, type VI), p-nitrophenyl-α-D-glucopyranose (pNPG; N-1377), 3,5-dinitrosalicylic acid (DNS), streptozotocin, NADPH, Glutathione disulfide, L-glutathione reduced, glutathione reductase and, glutathione peroxidase. Water-solvable starch was purchased from Meyer and acarbose from Glucobay Bayer, Mexico. Serum cytokine levels were quantified using an ELISA kit purchased from Pierce Protein Research Products (Thermo Fisher Scientific, Illinois, USA). Reflotron Test Strips forwith mechanisms that are critical in the regulation of insulin secretion. Such plants may decrease oxidative stress and may also increase insulin secretion and improve glycemic control.

Plant material and aqueous extraction

C. loniceroides (van Tieghemen) Kujit (Loranthaceae) was collected from infested *S. bonplandiana* trees in the area of Xochimilco, Mexico (19 °, 14'N, 99 ° 05 'O, altitude 2273 m) in February, 2013. The identification (URN: catalog: IBUNAM: MEXU: PA1053501) was performed by Dr. David Sebastián Gernandt from the Institute of Biology at the National Autonomous University of Mexico (UNAM). Plant material (stems, leaves, ripe and unripe fruits) was separated and used individually. Samples were dried at room temperature to constant weight; they were milled using an Udy mill (Udy Corporation Fort Collins, Co. USA) until a 420 µm mesh flour was obtained and stored at 5°C for further analysis.

Dried plant material was divided into two batches to process them by two different methods. The first batch was divided into three parts: samples (of 10 g) were macerated with distilled water (solvent:solid ratio of 10:1) with constant stirring at room temperature (20 ± 2 °C) for 12, 24 and 48 h, respectively. The second batch of samples was also split into three parts in exactly the same proportion as aforementioned; then a decoction was prepared at 95 ± 2 °C for 30, 60 and 90 min, respectively. Lastly, all the samples were filtered through Whatman no. 1 filter paper (Whatman International Ltd., Maidstone, U.K.). Filtration was lyophilized for 24 h (Scient-18N Freeze dryer, Shanghai, China).

In vitro assays
Carbohydrate hydrolyzing enzyme inhibitory activity

The α-amylase and α-glucosidase inhibitory activities were measured according to Worthington (1993a and 1993b). Acarbose and resveratrol were used as reference drugs. The percentage of inhibitory activity was calculated for all the samples as:

$$\% \ Inhibition = \frac{Ac - Ae}{Ac} \ X \ 100$$

Where**:** Ae is the sample absorbance and Ac the absorbance control without sample. Results were expressed by its half maximal inhibitory concentration (IC_{50}) value, which is defined as the sample concentration (mg/L) required to inhibit 50% of the enzyme activity.

Inhibition kinetic of enzymes

The lyophilized aqueous extracts (AE) were tested to determine the kinetic parameters of α-amylase and α-glucosidase enzyme inhibition. The activities were measured by increasing substrate concentrations by the presence/absence of sampling of lyophilized AE of *C. loniceroides*. α–amylase activity was quantified by measuring the maltose equivalents released from corn starch at 540 nm (Rubilar et al. 2011). α–glucosidase activity was quantified by assessing the *p*-nitrophenol equivalents released by pNPG at 400 nm (Jaiswal et al. 2012). The Michaelis–Menten constant (K_m), maximum enzyme reaction rate (V_{max}), and inhibition mode of aqueous extracts of *C. loniceroides,* were obtained by Lineweaver–Burk plots.

Experimental animal, ethics statement and treatment

Male mice strain CD1 of 4-6 weeks old (Charles River) with 35-40 g body weight were supplied by Universidad Autónoma Metropolitana, Campus Iztapalapa (UAM-I). This project was supported by Secretaría de Ciencia, Tecnología e Innovación with the project PINV11-13, contract ICYTDF/295/2014. The handling of laboratory animals was performed in agreement with the statutes of the CICUAL (Institutional Committee for the Care and Use of the Animals) based in the international and national rules established in the "Official Mexican Rule" for the care and use of the laboratory animals" [NOM-062-ZOO-1999]. Mice were individually housed on a 12 h:12 h light-dark cycle (6 AM lights on and 6 PM lights off). The laboratory temperature was 22 ± 1°C and the humidity was 20.5 ± 3.0%. Prior to the experiments, mice were fed with standard food for 1 week in order to adapt them to laboratory conditons. Food and water were available *ad libitum*.

Twelve hours before the experiments, they were fasted overnight, water was always available. Sixty-five mice were used for the study, they were divided into 13 groups, each consisting of 5 animals to assess the inhibition of CHE of the plant aqueous extracts as well as their effect on (STZ)-induced diabetic mice. The fasting blood glucose levels of all the mice were determined before the start of the experiment. Mice were divided into the following groups:

Carbohydrate hydrolyzing enzymes inhibitory activity

Group 1:   Normoglycemic (NG) control. Received only vehicle (0.5% carboxymethylcellulose).
Group 2:   NG reference. Acarbose was given at a dose of 100 mg/kg.
Group 3.   NG reference. Resveratrol was given at a dose of 30 mg/kg
Group 4:   NG. Aqueous extract of ripe fruit (RFAE) was given at a dose of 300 mg/kg.
Group 5:   NG. Aqueous extract of unripe fruit (UFAE) was given at a dose of 300 mg/kg.
Group 6:   NG. Aqueous extract of leaves (LAE) was given at a dose of 300 mg/kg.
Group 7:   NG. Aqueous extract of stem (SAE) was given at a dose of 300 mg/kg.

Blood glucose concentration was determined after 30 min when the mice had been intragastrically administered with the vehicle, acarbose or plant aqueous extracts. Afterwards, an oral carbohydrate tolerance test was performed as follows: mice were intragastric administered with soluble corn starch (2 g/kg) or sucrose (4 g/kg). Finally, the blood glucose was assessed at 0.5, 1, 1.5 and 2 h to obtain the glucose curve. Blood samples were collected from the tail tip at the defined times and determined using an Accu-Chek® system (Roche).

Mistletoe aqueous extracts effect on (STZ)-induced diabetic mice

Moderate diabetes was induced by two intraperitoneal injections of STZ (40 mg/kg body weight (b.w.)) freshly dissolved in a citrate buffer (100 mM, pH 4.5), in non-fasted mice on two consecutive days (Soriano-Santos et al. 2015). Blood samples were collected from the tip of the tail at the defined times, the fasting blood glucose levels were determined as previously described. Mice were considered diabetic when the fasting blood glucose level was ≥200 mg/dL.

Five-week subacute study daily, all treatments were administered intragastrically.

Group 1:   NG control. Received isotonic saline solution (4 mg/kg), once a day throughout 35 days.

Group 2:   STZ-induced diabetes control. Received isotonic saline solution (4 mg/kg), once a day throughout 35 days.

Group 3:   STZ-induced diabetic reference: Acarbose was given once a day throughout 35 days at a dose of 100 mg/kg.

Group 4:   STZ-induced diabetic reference: Resveratrol was given once a day throughout 35 days at a dose of 30 mg/kg.

Group 5:   STZ-induced diabetes. Aqueous extract of SAE was given once a day, by oral gavage procedure, throughout 35 days at a dose of 300 mg/kg.

 Group 6:   STZ-induced diabetes. UFAE was given once a day, by of oral gavage procedure, throughout 35 days at a dose of 300 mg/kg.

Lipid peroxidation

The 2-thiobarbituric acid reactive substances (TBARS) were measured using the procedure described by Jentzsch et al. (1996). An increase of MDA is linked to a rising of lipid peroxidation. Absorbance was measured at 535 nm in butanolic phase. MDA was used as a standard (0–20 mM).

Glutathione redox state assessment

The GSH redox system is essential to reduce oxidative stress. GSH, a radical scavenger, is converted into oxidized glutathione through glutathione peroxidase, and it is converted back to GSH by glutathione reductase. Measurements of GSH, GSSG and its related enzymatic reactions are important to assess the redox and antioxidant status. The animals were perfused with a phosphate-buffered saline (PBS) solution (0.15 M potassium phosphate, 0.9% NaCl, pH 7.4) through the abdominal aorta to remove residual blood elements. Fragments of liver were removed, washed in cold saline solution and stored at -70 °C for further use. GSH and GSSG measuerments were carried out according the method of Diaz-Flores et al. (2012).

GPx and GR specific activities

Liver fragments were homogenized (10% w/v) in PBS 0.1 M, pH 7.5 using a Polytron PT1200 and were centrifuged at 15 000 x g per 30 min. Supernatants were used for GPx and GR evaluation. GR activity was measured according to the method reported by Beutler (1969) and the protocol published by Lawrence and Burk (1976) was used for GPx. Both assessments were evaluated on NADPH production.

Cytokines analysis

After treatment, the animals were anesthetized using pentobarbital 25 mg/kg, the blood was collected from the orbital plexus in heparinized tubes, plasma was separated wafter 30 min of recollection, using a refrigerated centrifuge for a further estimation of cytokines (IL-6, IL-10 and TNF-α) analysis. Serum cytokine levels were quantified using an ELISA which was purchased from Pierce Protein Research Products (Thermo Fisher Scientific, Illinois, USA) to analyze IL-10, IL-6 and TNF-α.

Aminotransferases analysis

Quantifications of total aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were performed with a Roche Reflotron Plus Chemical System Analyzer (Woodley Equipment Company Ltd, Horwich, UK) and Reflotron Test Strips for ALT and AST using the blood samples that were collected from the tip of tail on the 35th day after the treatment had been completed.

Statistical analysis

The obtained data were analyzed by the Prism program Version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as the mean $\pm$ standard deviation. To determine statistically significant differences between groups, an ANOVA (one way) was followed by Turkey or Duncan post-hoc test; $p < 0.05$ was considered statistically significant.

Results
Carbohydrate hydrolyzing enzymes inhibitory activity

The α-amylase inhibitory activity from different aqueous extracts are displayed in Table 1. The UFAE and RFAE prepared by decoction for 30 min from *C. loniceroides* showed the lowest IC_{50} (µg/mL) values (1.73 ± 0.11 and 5.85 ± 0.05, respectively) of α-amylase inhibitory activity. Acarbose and resveratrol were used as experimental control. Their IC_{50} values = 7.1µg/mL and 111 - 120 µg/mL (Miao et al., 2014), respectively, were of the same order of magnitude as of the samples. On the other hand, the SAE and LAE did not show *α*-amylase inhibitory activity, regardless of the method. Table 1 also shows the inhibitory activity of α-glucosidase of AE obtained by decoction or maceration of *C. loniceroides*. The extracts with better α-glucosidase inhibitory activity were those obtained after 30 min of decoction from stem (IC_{50} = 14.71 ± 0.43 µg/mL) and leaves (IC_{50} = 37.92 ± 4.83 µg/mL). α-glucosidase inhibitory activity of acarbose (IC_{50} = 31 µg/mL) and resveratrol (IC_{50} = 1350 µg/mL, (Zhang et al., 2017)) is also of the same order of magnitude, with similar values to those afforded by SAE and LAE.

Table 1: Inhibitory activity of aqueous extracts from *C. loniceroides* obtained by decoction or maceration on α-amylase and α-glucosidase.

	Decoction			Maceration		
	30 min	60 min	90 min	12 h	24 h	48 h
	IC_{50} α-amylase (µg/mL)					
LAE	*	*	*	*	*	*
SAE	*	*	*	*	*	*
UFAE	1.73 ±0.11[a]	4.95 ±0.07[a]	3.25 ±1.17[a,b]	8.14 ±0.42[a]	14.78 ±2.56[b]	40.25 ±0.55[c]
RFAE	5.85 ±0.05[a]	6.69 ±0.18[a]	9.52 ±0.31[b]	6.63 ±0.50[a]	23.96 ±0.01[b]	52.85 ±1.92[c]
Acarbosa	7.1					
Resveratrol	111-120 (Zhang et al., 2017)					
	IC_{50} α-glucosidase (µg/mL)					
LAE	37.92 ±4.83[a]	75.25 ±3.67[b,c]	117.49 ±1.12[c]	178.92 ±13.02[b]	95.78 ±2.24[a,b]	158.95 ±6.25[b]
SAE	14.71 ±0.43[a]	82.51 ±1.88[b,c]	114.50 ±7.21[c]	56.90 ±0.66[a]	53.69 ±2.1[a]	100.02 ±0.56[a]
UFAE	159.96 ±21.19[a,b]	116.40 ±5.23[a]	170.07 ±0.95[b]	195.78 ±6.93[a]	469.65 ±4.35[b]	543.65 ±4.03[b]
RFAE	168.95 ±11.24[a]	307.60 ±17.39[b]	386.15 ±34.86[c]	1030.75 ±4.35[a]	680.87 ±55.74[b]	1030.75 ±30.36[c]
Acarbosa	31					
Resveratrol	1350 (Miao et al., 2014)					

Values are means ±SD (n=5), means in same row with different superscripts are significantly different (p<0.05).
* There is no inhibitory activity.

Inhibition kinetic of enzymes

Inhibition kinetics parameters were assessed. The RFAE and UFAE obtained by decoction at 30 min showed a competitive mode of inhibition for α-amylase (Fig 1a and 1b, respectively), unlike that of the RFAE, a non-competitive mode for α-glucosidase (Fig 1c). On the other hand, UFAE obtained by decoction at 30 min showed a competitive inhibition for the CHE (Fig 1d). Finally, the LAE and SAE obtained by decoction at 30 min only had a competitive inhibition activity against α-glucosidase (Fig. 1e and 1f; respectibly). The acarbose and resveratrol that were used as a control displayed a competitive mode of inhibition for both enzymes.
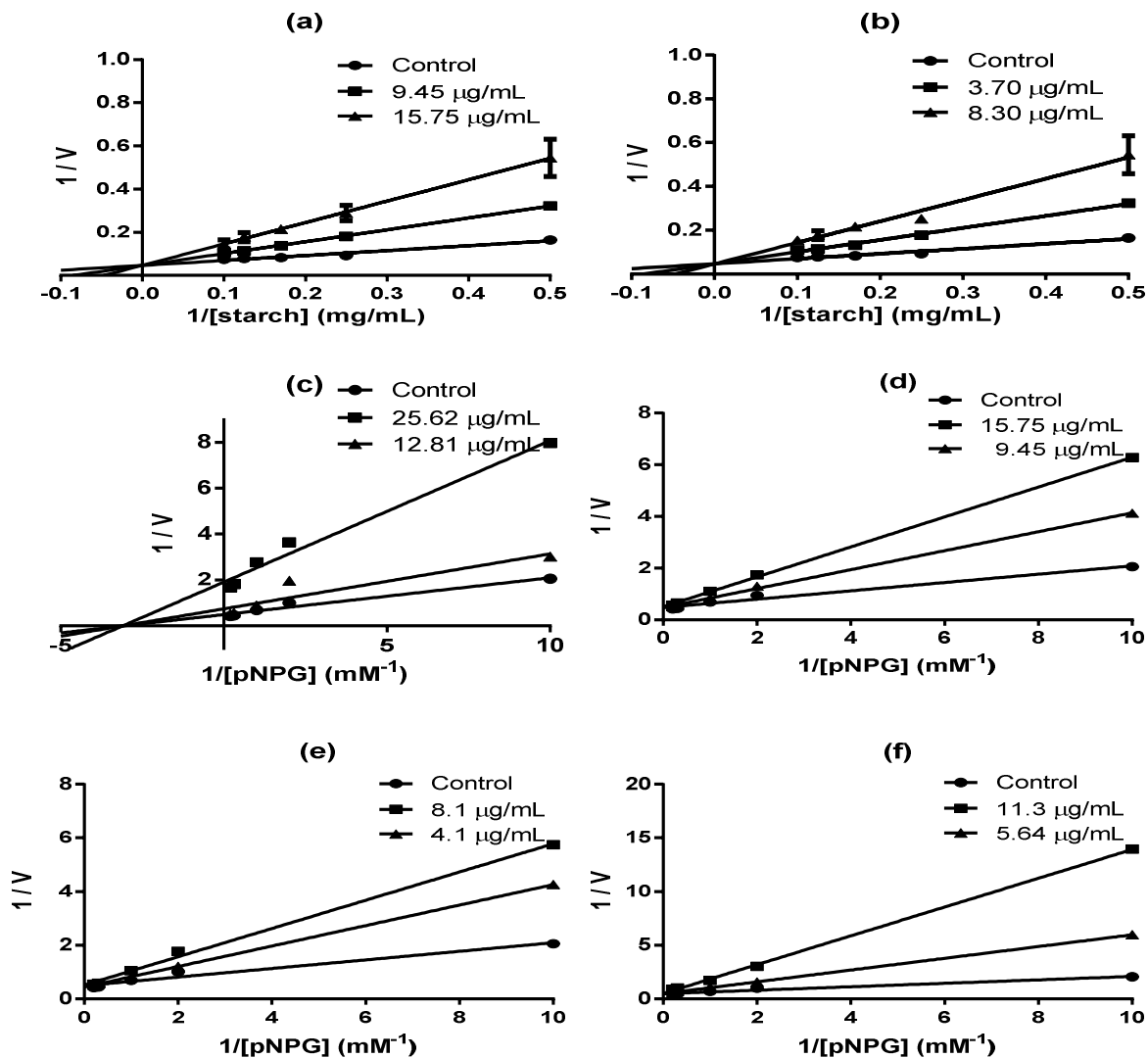
Figure 1: Lineweaver-Burk plot of the effect of aqueous extracts of *C. loniceroides* on the hydrolysis reaction catalyzed by α-amylase for (a) RFAE. (b) UFAE. And by α-glucosidase for (c) RFAE. (d) UFAE. (e) LAE. (f) SAE. All the extracts were obtained by decoction for 30 min. Each plot shows the different concentrations of aqueous extract which were evaluated. Table 2 shows the kinetic parameters from different plant aqueous extracts obtained by decoction at 30 min. Since this is a competitive inhibition mode, except for the inhibition of α-glucosidase by RFAE, Vmax value is roughly the same (21 mg/min and 2 μM/min for α-amylase and α-glucosidase, respectively) when a zero order kinetics is reached. Thus, the comparison is difficult for the inhibition kinetic parameters of α-amilasa and α-glucosidase as obtained of several plant aqueous extracts because there is no standardized way to express these kinetic values. In fact, different inhibitory activity values of CHE have been reported, nevertheless the method has not been accurately described.

Table 2: Kinetics of α-amylase and α-glucosidase inhibition by different aqueous extracts as obtained of *C. loniceroides*.

	α-amylase					α-glucosidase				
	Extract (mg/mL)	Km (mg/mL)	Vmax (μg/mL/min)	Ki' (μg/mL)	Inhibition	Extract (mg/mL)	Km (μM)	Vmax (μM/min)	Ki' (μg/mL)	Inhibition
Without extract	0	4.3	21.2	-		0	326.45	2	-	
RFAE 30 min	3.7	12.9	21.2	2.4	Competitive	0.75	323.3	1.3	0.36	Non-competitive
	8.3	20.6	20.8			1.5	343.5	0.5		
UFAE 30 min	1.25	13.9	21.5	0.71	Competitive	0.37	641.9	2.1	0.23	Competitive
	2.5	23.1	21.3			0.63	2429.7	1.9		
LAE 30 min	-	-	-	-	-	0.075	957.2	2.3	0.06	Competitive
						0.15	1199.9	2.1		
SAE 30 min	-	-	-	-	-	0.075	1123.5	2	0.01	Competitive
						0.15	2698.5	2		

- There is no inhibitory activity.

Glucose tolerance test

The starch intake of 2 g/kg bw (Fig. 2a) or the sucrose intake of 4 g/kg bw (Fig. 2b) was administered for the glucose tolerance test. Both assays resulted in a rapid and significant increase in glycemia (88% and 253% for starch or sucrose, respectively) in the NG mice which were used as controls. Both trials showed that the source of carbohydrate does affect the inhibition of CHE due to the AE. On the starch tolerance test, the best antihyperglycemic result was observed on the group treated with UFAE of *C. loniceroides* obtained after 30 min of decoction (Fig. 2a). This extract reduced the glycemic response because of the α-amylase inhibition, by 23% when compared to the group that was only given the starch. This effect was significant ($p<0.05$) and the behavior was similar to that of acarbose and resveratrol. Therefore, the UFAE that inhibited α-amylase was chosen to observe the antidiabetic effect in (STZ)-induced diabetic mice for a period of 35 days. As for the sucrose tolerance test (Fig. 2b), all AEs obtained at 30 min reduced the hyperglycemia of NG mice because of the inhibition of α-glucosidase on the sucrose hydrolysis. The SAE of *C. loniceroides* afforded the largest reduction of hyperglycemia by 35%. This figure was even higher than that of acarbose (13%) or resveratrol (11.4%). After 60 min of treatment, all of the extracts presented the same antihyperglycemic effect without any significant differences ($p > 0.05$). Thus, the SAE was chosen to evaluate the antidiabetic effect in (STZ)-induced diabetic mice for 35 days. Fig. 2c shows the antidiabetic effect of SAE and UFAE on (STZ)-induced diabetic mice. At day 35, UFAE diminished glycemia of (STZ)-induced diabetic mice similarly to that observed for acarbose and resveratrol.
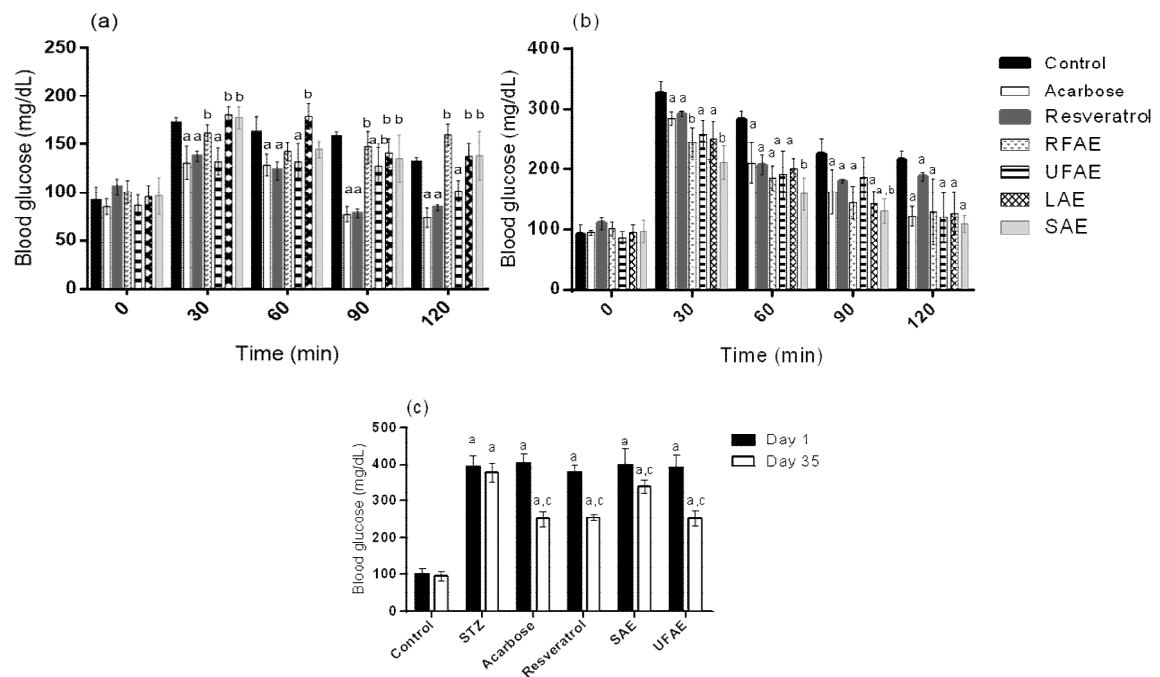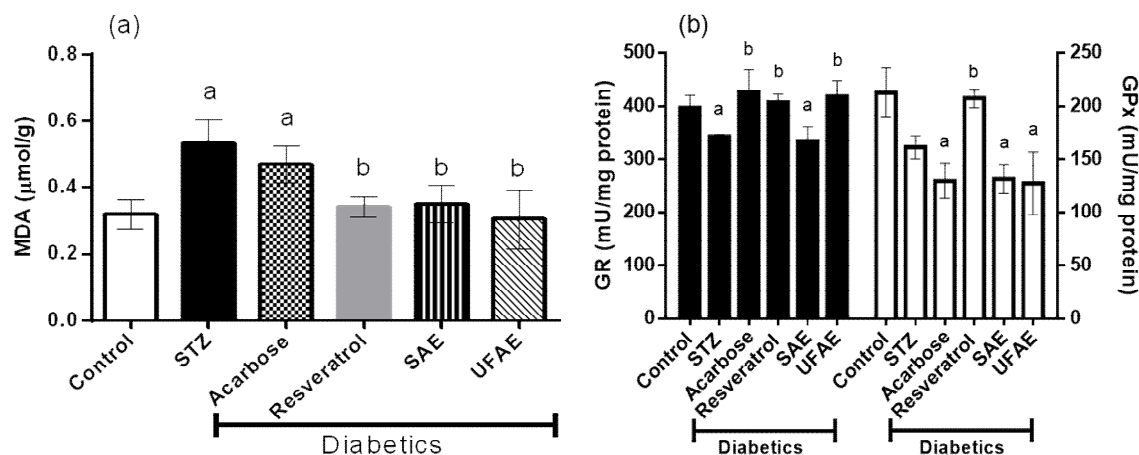

Figure 2: Effect of *C. loniceroides* aqueous extracts on glycemia in normoglycemic mice and STZ-induced diabetic mice. (a) Postprandial blood glucose levels of normoglycemic mice during a starch tolerance test. (b) Postprandial blood glucose levels of normoglycemic mice during a sucrose tolerance test. (c) The blood glucose levels were measured at the beginning and end of the treatment in STZ-diabetic mice (35 d). Values are presented as the mean±S.D. for n=5 mice. Data were analyzed by ANOVA and post-hoc Duncan test.
[a] denote significant difference compared to the control group; [b] statistically significant compared to the acarbose group ($p<0.05$); [c] statistically significant ($p<0.05$) compared to STZ-induced diabetic mice group.

Five-week sub-acute study
Lipid peroxidation

The lipid peroxidation in the different mice groups was evaluated by the TBARS, measured mainly as MDA in samples obtained from liver homogenate (Fig. 3a). The MDA concentration in TBARS increased 1.65 fold in the (STZ)-induced diabetic mice when compared to the NG mice. The MDA concentrations decreased in the mice groups treated with SAE (1.5 times) or UFAE (1.76 times) aqueous extracts obtained by decoction of *C. loniceroides* at 30 min. These MDA levels were similar to those found in the NG mice and the resveratrol group. Finally, the group of mice that was administered with acarbose produced nearly as much TBARS as the (STZ)-induced diabetic mice.

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Figure 3: Effect of different *C. loniceroides* aqueous extracts, after a five-week subacute daily dosing, assessed in liver of STZ-induced diabetic mice. (a) MDA concentration and (b) GR and GPx activities. Values are presented as the mean±S.D. for n=5 mice. [a] Statistically significant ($p < 0.05$) compared to the normoglycemic mice control group. [b] Statistically significant ($p < 0.05$) compared to STZ-induced diabetic mice group.

Effect of *C. loniceroides* on glutathione redox state and GPX and GR

Table 3 shows not only the changes in total pool of glutathione, but also the different forms of glutathione found in the liver homogenate of (STZ)-induced diabetic mice treated with SAE or UFAE. The oxidative stress was observed because the GSH concentration decreased (30%), whereas the GSSG increased (66%) significantly ($p < 0.05$) relative to the NG mice. The GSH/GSSG ratio of the (STZ)-induced diabetic mice was the lowest, 2.5 times lower than that of the NG mice. When the UFAE was administered, it was observed that the GSH/GSSG ratio (3.08 ± 0.66) was maintained due to no significant difference found with the control group ($p < 0.05$). It even maintained the ratio in a more efficient way than the acarbose or resveratrol group, used as positive controls. Similarly, the total pool of glutathione (GSH+GSSG = 324.47 ± 5.07 µM) of the mice group administered with UFAE increased, possibly due to a rise in GSH production regarding the NG mice. Figure 3b shows the effect of the aqueous extracts of *C. loniceroides* on the antioxidant enzymes. The UFAE also showed a significant effect ($p < 0.05$) because it reduced the production of GPx, but increased the production of GR, similarly to that observed for resveratrol, with regard to the (STZ)-induced diabetic mice.

Table 3: Effect of different aqueous extracts of *C. loniceroides* on the glutathione pool in liver of STZ-induced diabetic mice.

	Groups	Liver
GSH (µM)	Control	213.93 ±13.81
	STZ	148.93 ±9.98[c]
	Acarbose	148.21 ±3.51[c]
	Resveratrol	186.12 ±5.81[b,c]
	SAE	152.2 ±5.23[c]
	UFAE	244.89 ±14.94[b,c]
GSSG (µM)	Control	74.05 ±10.18
	STZ	123.59 ±5.39[c]
	Acarbose	53.43 ±3.27[a]
	Resveratrol	83.97 ±5.83[b]
	SAE	78.63 ±18.07[b]
	UFAE	79.58 ±9.97[b]
GSH/GSSG	Control	2.94 ±0.57
	STZ	1.17 ±0.10[c]
	Acarbose	2.48 ±0.10[a]
	Resveratrol	2.22 ±0.14[a]
	SAE	2.25 ±0.53[a]
	UFAE	3.08 ±0.66[a]
GSH+GSSG (µM)	Control	287.97 ±14.14
	STZ	272.52 ±10.38
	Acarbose	201.64 ±6.76[a,c]
	Resveratrol	270.09 ±10.07[d]
	SAE	230.83 ±19.01[b,c]
	UFAE	324.47 ±5.07[b,c]

[a] $p < 0.05$ compared to STZ-induced diabetic mice group; [b] $p < 0.05$ compared to the STZ-induced diabetic mice group and acarbose; [c] $p < 0.05$ compared to the normoglycemic mice control group; [d] $p < 0.05$ compared to the acarbose group.

Effect of *C. loniceroides* extracts on cytokines

The serum pro-inflamatory markers, IL-6 and TNF-α, in (STZ)-induced diabetic mice increased when compared to those of the NG mice (p<0.05). On the other hand, the level of anti-inflammatory marker IL-10 decreased in (STZ)-induced diabetic mice (Figs. 4a, 4b and 4c). The SAE and UFAE of *C. loniceroides* as well as acarbose and resveratrol were administered orally to mice and all of them decreased IL-6 levels, thus maintaining near-normal IL-6 levels as in the NG mice (Fig. 4a). Similarly, these extracts decreased the concentration of TNF-α with respect to the (STZ)-induced diabetic mice used as control (Fig. 4c), although a reduction of this concentration did not reach the level exhibited by NG mice. No extract of *C. loniceroides* had an influence on IL-10 cytokine levels, whose level remained close to that of (STZ)-induced diabetic mice (p<0.05; Fig. 4b). Just resveratrol group could elevate IL-10 regarding (STZ)-induced diabetic mice.
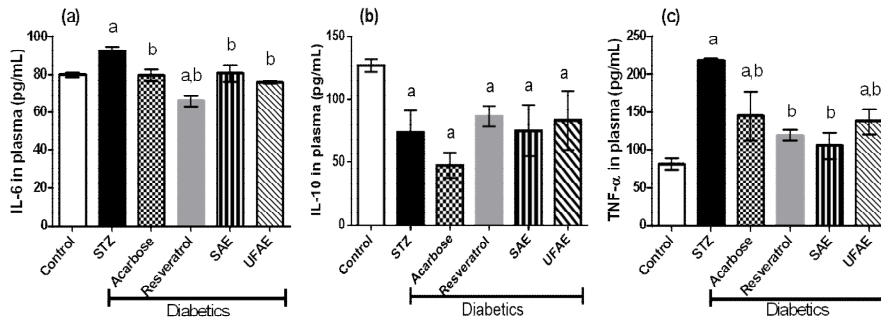
Figure 4: Effects of different *C. loniceroides* aqueous extracts on serum pro-inflamatory (IL-6 and TNF-α) and anti-inflamatory (IL-10) cytokines, after a five-week subacute daily dosing in STZ-induced diabetic mice. (a) IL-6, (b) IL-10 and (c) TNF-α. Mean±S.D. (n=5). [a] Statistically significant (p<0.05) compared to the normoglycemic mice control group. [b] Statistically significant (p<0.05) compared to STZ-induced diabetic mice group.

Liver transaminases

Figures 5a and 5b show the effect of *C. loiceroides* extracts on hepatic markers (ALT and AST) in (STZ)-induced diabetic mice. They were administered with SAE and UFAE and they maintained nearly the same ALT level as the NG mice. This level also remained the same as in (STZ)-induced diabetic mice. However, administration of acarbose did induce an increase in the serum ALT while resveratrol reduced it (p<0.05). On the other hand, AST levels in (STZ)-induced diabetic mice also increased as a consequence of diabetes. Administration of acarbose increased the serum AST level, which was similar to that of diabetic mice, but resveratrol maintained it nearly to NG mice. In contrast, the administration of SAE and UFAE for 35 days restored the level of AST to a level similar to that of the NG mice (p <0.05.).
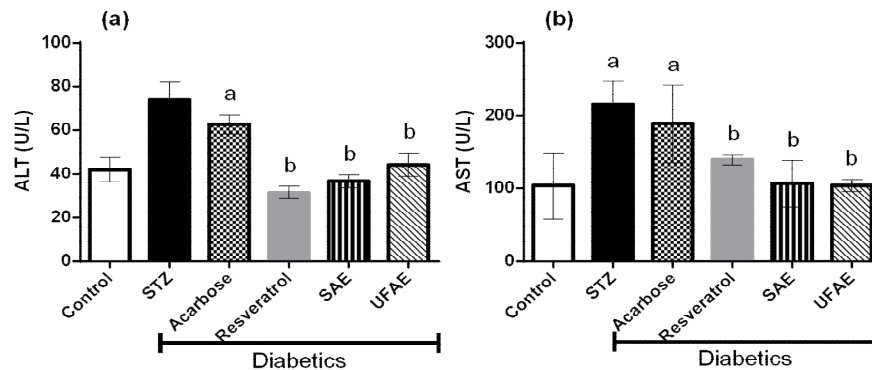
Figure 5: Effects of different *C. loniceroides* aqueous extracts on transaminases, after a five-week subacute daily dosing, assessed as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations in the liver of STZ-induced diabetic mice. (a) ALT and (b) AST. [a] Statistically significant (p<0.05) compared to the normoglycemic mice control group. [b] Statistically significant (p<0.05) compared to STZ-induced diabetic mice group.

Discussion

In diabetes disease, oxidative stress plays an important role in the development of insulin resistance and its effects (Evans et al. 2005; Verdile et al. 2015). Therefore, antioxidants can be used to manage diabetes due to their biological properties. Treatment with polyphenols could enhance the effectiveness of diabetes management. *C. loniceroides* is a

source of polyphenols, especially when the fruit is unriped. Several biological and beneficial health effects have been demonstrated by phenolic compounds in plants. There is evidence that these compounds modulate carbohydrates hydrolysis by inhibition of the enzymes α-amylase and α-glucosidase (McDougall et al., 2005). Within the Loranthaceae family, mistletoes containing large numbers of polyphenols, have already been reported, and also have antidiabetic activity *in vivo* and *in vitro* (Osadebe et al 2004, Osadebe et al., 2010). The UFAE of *C. loniceroides* showed remarkable competitive inhibition of α-glucosidase and α-amylase. Consequently, in an acute study, antidiabetic effect *in vivo* is observed by inhibiting the hydrolysis of starch, possibly by the polyphenols action that has been reported (Serrano-Maldonado et al. 2011). Also, in the subacute study, a decrease in hyperglycemia was observed in mice treated with *C. loniceroides* compared with the (STZ)-induced diabetic mice group. Then, carbohydrate hydrolyzing enzymes inhibitors may be an attractive therapeutic modality in diabetic patients (Jaiswal et al. 2012).

The group of (STZ)-induced diabetic mice showed an increase in the levels of MDA and AST, but GSH/GSSG ratio, GR and GPx activity decreases. The GSH/GSSG ratio is inversely related to oxidative stress and it is often used as a sensitive index of oxidative stress *in vivo* (Díaz-Flores et al. 2012). The alterations in GR and GPx activity produce changes in the redox state (Al-Dallen et al. 2004). Then, there is an increase in oxidative stress in the (STZ)-induced diabetic mice group, which could cause chronic hyperglycemia, that is responsible for oxidative stress because of an excessive ROS production from auto-oxidation of glucose, glycated proteins, and glycation of antoxidative enzymes which impair their capacity to detoxify the free radicals (Martín-Gallán et al. 2003).

The inhibition of intracellular ROS formation would serve as a therapeutic strategy to prevent oxidative stress in diabetes. Several studies have demonstrated that antioxidants like vitamin C, vitamin E, and polyphenols can reduce oxidative stress and lipid peroxidation in T2D patients and animals (Jaiswal et al. 2012). Therefore, in the present study, in addition to evaluating the antihyperglycemic activity of *C. loniceroides*, the mistletoe extracts effect on the oxidative stress was determined. In the five-week subacute daily dosing, the state redox was improved because the GSH/GSSG ratio and GR activity significantly increased with respect to the group of (STZ)-induced diabetic mice, the values were similar to the NG mice. It could be an indirect indicator of ROS reduction. As a result, lipid peroxidation decreased, this was determined by the levels of MDA. On the other hand, when the polyphenols exert their antioxidant action they reduce ROS (Rice-Evans et al. 1997; Martinez and Moreno 2000). Accordingly, the mistletoe study should be proposed in the characterization of phenolic compounds that could have the antioxidant effect. Thus, SAE and UFAE of *C. loniceroides* treatment could be an alternative to decrease or prevent oxidative stress in diabetes mellitus.

In previous studies, it was observed in the (STZ)-induced diabetic mice an inflamatory state due to a decrease in the concentration of IL-10, which triggered the production of IL-6 and TNF-α. The TNF-α stimulates hyperlipidemia and hepatic lipogenesis simultaneously reducing the sensitivity to insulin in muscle tissues and finally the necrosis of target organs (Khanra et al. 2015). In the present study, this inflammatory state is observed in STZ-diabetic mice. As mentioned before, hyperglycemia induces oxidative stress, and also causes an inflammatory state (Rosado Pérez and Mendoza Núñez 2007). The ROS generated by hyperglycemia induce the activation of NF-κB, which is an activating factor that regulates the expression of different inflammatory cytokines. At the same time, inflammation and oxidative stress can cause liver damage (Mittal et al. 2014) as observed in diabetic mice, through the determination of transaminases (AST y ALT).

After the administration of SAE and UFAE of *C. loniceroides* in (STZ)-diabetic mice, the concentration of IL-6 and TNF-α was reduced with respect to the group of diabetic mice. However, the concentration of IL- 10 was not improved in the diabetic group. The decrease in inflammatory cytokines could be explained by the antioxidant effect of the extracts. The polyphenols contained in the extracts can also inhibit NF-κB activation by decreasing ROS (Bhattacharya and Sil 2018). Therefore, they inhibit the expression of cytokines like TNFα and IL-6, as well as the decrease of transaminases such an AST and ALT.

In order to develop and construct knowledge about the antidiabetic activity of *C. loniceroides*, further studies should be performed to confirm whether this mistletoe may display a similar antidiabetic mechanism as the one found in other medicinal plants.

Conclusion

The UFAE of *C. loniceroides* showed an antihyperglycemic effect through the inhibition of carbohydrate hydrolyzing enzymes. Besides having this activity, further study can be interesting for the treatment of diabetes due to its effects on oxidative stress and its anti-inflammatory activity. These effects *C. loniceroides* could be due to its high polyphenol composition. However, more research is needed to confirm and evaluate these effects at the *in vivo* and clinical levels.

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THE ANTI-PROLIFERATIVE AND ANTIOXIDANT ACTIVITY OF FOUR INDIGENOUS SOUTH AFRICAN PLANTS.

Sunelle Rademan[a], Preethi G. Anantharaju[b], SubbaRao V. Madhunapantula[b] and Namrita Lall[a]

[a]Department of Plant and Soil Sciences, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, 0002, South Africa. [b] Center of Excellence in Molecular Biology and Regenerative Medicine, Department of Biochemistry, JSS Medical College, JSS Academy of Higher Education & Research, Bannimantapa, Sri Shivarathreeshwara Nagar, Mysore, 570 015, Karnataka, India.

Corresponding Author's E-mail: Namrita.Lall@up.ac.za.

Abstract

Background: Cancer is a major cause of death worldwide. Limitations of current cancer therapies necessitate the search for new anticancer drugs. Plants represent an immeasurable source of bioactive compounds for drug discovery. The objective of this study was to assess the anti-proliferative and antioxidant potential of four indigenous South African plants commonly used in traditional medicine.

Materials and Methods: The anti-proliferative activity of the plant extracts were assessed by the 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide (XTT) assay on A431; HaCat; HeLa; MCF-7 and UCT-Mel 1 cells and sulforhodamine-B (SRB) assay on HCT-116 and HCT-15 cell lines. Antioxidant activity was determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO) and superoxide scavenging assays.

Results: Three of the plant extracts (*Combretum molle*fruit, *Euclea crispa* subsp. *crispa* leaves and stems and *Sideroxylon inerme* leaves and stems showed anti-proliferative activity on the A431 cells with IC_{50}values ranging between 26.9 - 46.7 µg/ml. The *Euclea crispa* subsp. *crispa* extract also showed anti-proliferative activity on the MCF-7 cell line (45.7 µg/ml). All of the plant extracts (*Combretum molle* leaves and fruit, *Euclea crispa* subsp. *crispa* leaves and stems, *Sideroxylon inerme* leaves and stems and *Terminalia prunioides* leaves and stems) showed DPPH scavenging activity with IC_{50} values ranging from 1.8 µg/ml to 11.5 µg/ml.

Conclusion: These results indicate that the active extracts of *Combretum molle*, *Euclea crispa* subsp. *Crispa* and *Sideroxylon inerme* warrant further investigation to determine the mechanism of anti-proliferative activity against cancerous cells.   These plant extracts also show potential for further evaluation in the prevention and treatment of cancer.

Key words: South African plants, Traditional medicine, Anti-proliferative activity, Antioxidant activity.

Abbreviations: ATCC: American Type Culture Collection; **CANSA**: Cancer Association of South Africa; **DMEM**: Dulbecco's Modified Eagles Medium; **DMSO**: Dimethyl sulfoxide; **DNA**: Deoxyribonucleic acid; **DPPH**: 2, 2-diphenyl-1-picrylhydrazyl; **EMEM**: Eagle's Minimum Essential Medium; **FBS**: Fetal Bovine Serum; **IC$_{50}$**: Fifty percent inhibitory concentration; **MD**: Maryland; **NaOH**: Sodium hydroxide; **NBT**: Nitrotetrazolium Blue chloride; **NCCS**: National Centre for Cell Science; **NCI**: National Cancer Institute; **NO**: Nitric oxide; **ROS**: Reactive Oxygen Species; **RSA**: Republic of South Africa; **SD**: Standard deviation; **SRB**: sulforhodamine-B; **USA**: United States of America; **WHO**: World Health Organization; **XTT**: 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide.

Introduction

Cancer is one of the major causes of death due to non-communicable diseases world- wide.
In 2012 alone, cancer was identified as the causal agent of more than 8.2 million deaths. In addition, the incidence and mortality rates have shown an increasing trend in Africa, Asia and Central and South America. According to the World

Health Organization report, seventy percent of cancer deaths have occurred in these countries (WHO, 2014). In South Africa, more than 100,000 cases are reported each year. The six most prevalent cancers found among South African men are prostate cancer, lung cancer, colorectal cancer, esophageal cancer, Kaposi sarcoma and cancers of which the site of origin within the body is not known. The six most prevalent cancers found among South African women are breast cancer, cervical cancer, colorectal cancer, Kaposi sarcoma, melanoma and cancers of which the site of origin within the body is not known (CANSA, 2013).

Conventional cancer treatment involves various aspects to either treat the disease itself or the symptoms of the disease or both. In general, surgical removal of cancerous tissue, radiotherapy and chemotherapy are employed for the treatment of cancers. Limitations of current therapies including adverse side effects and lowered efficacy due to drug resistance, which warrants the search for new drugs (NCI cancer treatment research, 2017).

Many anticancer agents in use today, originate from natural resources such as animals, microorganisms and plants (Nobili et al., 2009). Herbal medicine forms a big part of many traditional medicine systems. The knowledge of these traditional medicine systems have provided key information for the discovery of anticancer agents from plants. Traditional medicine further forms a very important source of affordable and readily accessible health care system for most people in developing countries (Falkenberg et al., 2002).

Antioxidants are compounds that scavenge reactive oxygen species (ROS). Since ROS can cause DNA damage, which can lead to cancer development; antioxidants are believed to possess chemopreventive abilities. Well known plant derived antioxidants include 'quercetin'; 'resveratrol'; 'curcumin' and 'catechins'. Many epidemiology studies have shown that cancer incidence is low in countries with high levels of antioxidant rich plant consumption and as such employing antioxidants as a nutritional supplement might aid in cancer prevention (Borek, 1997; NCI, 2014).

The plants (*Combretum molle* R. Br. Ex G. Don, *Euclea crispa* subsp. *Crispa* (Thunb.) Gürke, *Sideroxylon inerme* L. and *Terminalia prunioides* M.A. Lawson) selected for this study have been used traditionally as anticancer agents and the available data ascribed anticancer properties to the compounds isolated from these plants. Table 1 gives the traditional usage; compounds isolated and reported anticancer activity of the plants.

Materials and methods
Materials

The A431, HeLa, and MCF-7 cell lines were obtained from American Type Tissue Collection (ATCC), MD, USA, whereas the HCT-116 and HCT-15 cell lines were obtained from NCCS Pune, India. Prof Davids, University of Cape Town, Cape Town, RSA, kindly donated the UCT-Mel 1 and HaCat cell lines. Foetal bovine serum (FBS) and antibiotics were purchased from Separations (Pty) Ltd. (Randburg, Johannesburg, RSA). The XTT cell proliferation kit II, sulforhodamine-B (SRB), DPPH, ascorbic acid, Griess reagent, sodium nitroprusside, Nitrotetrazolium Blue chloride (NBT), sodium hydroxide (NaOH), quercetin and all other materials were of analytical grade and were acquired from Sigma-Aldrich (Missouri, USA).

Plant collection and identification

All plant materials were collected during 2013 in Nelspruit, Mpumalanga. The plants were identified by the HGJW Schweickerdt Herbarium at the University of Pretoria and given herbarium specimen (PRU) numbers. *Combretum molle* R. Br. Ex G. Don leaves and fruit (PRU 120569), *Euclea crispa* subsp. *Crispa* (Thunb.) Gürke leaves, stems (PRU 120536), *Sideroxylon inerme* L. leaves, stems (PRU 120537), and *Terminalia prunioides* M.A. Lawson leaves and stems (PRU 120508).

Extraction of plants

The dried aerial parts of the plant were mechanically ground to a fine powder. The powdered plant material of each plant was extracted with ethanol for 48h and thereafter for another 24h using fresh solvent. A Buchner funnel was used to filter the solutes and which was subsequently evaporated by a vacuum rotary evaporator. The percentage yield of the plant extracts were calculated for the formula:

$$\% \text{ Yield} = \frac{\text{Extract weight (g)}}{\text{Powdered weight (g)}} \times 100$$

Cell culturing

The human epidermoid carcinoma (A431), metastatic melanoma (UCT-Mel 1), colorectal carcinoma (HCT-116 and HCT-15) and keratinocytes (HaCat) cell lines were maintained in culture flasks containing Dulbecco's Modified Eagles Medium (DMEM). The human breast adenocarcinoma (MCF-7), and cervix adenocarcinoma (HeLa) cell lines were maintained in Eagle's Minimum Essential Medium (EMEM). The complete media for all the cell lines were comprised of

the respective media supplemented with 10 % FBS and 1 % antibiotics. The antibiotic mixture consisted of 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 μg/L fungizone. All of the cell lines were grown at standard growth conditions (37°C in a humidified incubator set at 5% CO_2) and sub-cultured when they had reached 100 % confluence.

Cell proliferation assay
XTT cell proliferation kit II

The anti-proliferative activity of the samples was measured by the XTT method using the Cell Proliferation Kit II (Sigma-Aldrich, Missouri, USA). The assay was performed according to the method by Zheng et al., 2001.1 $\times$ 10^4 cells were seeded (in 100 μl) in a 96-well microtiter plate and incubated for 24h at standard growth conditions to allow for cell attachment. The plant extracts were assessed at concentrations ranging from 3.1μg/ml – 400 μg/ml. The vehicle control wells were exposed to 2% DMSO. Actinomycin D (concentrations ranging between 3.91×10^{-4} μg/ml - 0.05 μg/ml) was used as positive control. Plant extracts in medium without cells were used as blank colour controls. The microtiter plates were incubated for 72h. Subsequently, 50 μl of XTT reagent (0.3 mg/ml) was added and the plate was further incubated for another 2 h. A multi-well plate reader (BIO-TEK Power-Wave XS) was used to measure the absorbance of the colour complex at 490 nm with a reference wavelength set at 690 nm.

Sulforhodamine-B assay (SRB) assay

The anti-cancer activity was measured according to the method by Madhunapantula et al., 2008. In brief,100 μl of HCT-116 and HCT-15 cells were plated in a 96-well plate at a density of 0.5 $\times$ 10^4 cells/ml. After 48h incubation at standard growth conditions, the cells were exposed to the extracts at concentrations ranging between 0-200 μg/mL for 72h.

The SRB assay was performed as specified by Skehan et al., 1990 to determine the cell viability. Experimentally, cells were fixed in 1/4th volume of cold 50% (w/v) TCA at 4°C for 1 h. Thereafter, the media was decanted and the wells washed with water (200 μl $\times$ 4 times) to remove any remaining TCA and serum proteins. The plates were dried and then incubated with 100 μl 0.4 % SRB for 30 min to stain the cellular proteins. Quick washing with 1 % acetic acid (200 μl$\times$ 4 times) removed any unbound SRB while, the bound SRB was solubilized in 10.0mM Tris base solution (100 μl/well). A multimode plate reader was used to measure the absorbance at a wavelength of 490 nm.

Antioxidant assays
DPPH

The DPPH scavenging activity of the extracts were measured following the method by Du Toit et al., 2001. The plant extracts and positive control, vitamin C, were evaluated at concentrations ranging from 0.78 μg/ml to 100 μg/ml. DPPH ethanolic solution (0.04 M) was added to each sample well, whereas distilled water was added to the negative color control wells. The plates were incubated at room temperature in the dark for 30 minutes. Following the incubation period, the absorbance was measured using a BIO-TEK Power-Wave XS multiplate reader at a wavelength of 515 nm.

Nitric oxide

The nitric oxide scavenging potential of the samples was determined by utilizing sodium nitroprusside as a nitric oxide generator and Greiss reagent as the detector. The method by Mayur et al., 2010 was followed to determine the scavenging activity of the samples. The samples and Vitamin C, positive control, were evaluated at concentrations ranging from 15.6 μg/ml to 2000 μg/ml. Sodium nitroprusside solution (0.01 M) was added to each well and incubated for 90 min, in light at room temperature. Subsequently, Griess reagent solution (1:1) was added to each well. For the negative color controls, distilled water was added instead of Griess reagent. The absorbance values of the samples were read using a multi-well plate reader (BIO-TEK Power-Wave XS) set at a wavelength of 546 nm.

Superoxide

The method by Hyland et al., 1983 was used to determine the superoxide scavenging activity of the extracts, which, involves the use of alkaline DMSO to generate superoxide anions. In short, 100 μl of alkaline DMSO (5 mM NaOH) was added to all the wells of a 96-well microtiter plate. Serial dilutions were made to final concentrations which ranged from 3.90625 μg/ml – 500 μg/ml. Next, 10 μl of NBT (10 mg NBT, 10 ml DMSO) was added to all the sample wells, while10 μl of DMSO was added to the color control wells. The absorbance of the plates were read using a multi-well plate reader (BIO-TEK Power-Wave XS) set at a wavelength of 560 nm.

Table 1: Traditional usage, the compounds isolated and biological activity tested of the plants selected for the study.

Plant	Plant part	Traditional usage	Anticancer activity reported	Compounds isolated	Reference
Combretum molle R. Br. Ex G. Don	Fruit	Aid in childbirth.	-	-	Watt & Breyer-Brandwijk, 1962
	Leaves	Wound dressing; antidiarrheal; anthelminthic; dropsy; chest complaints; and as an aid in childbirth.	-	-	Drummond & Coates-Palgrave, 1973; Haerdi, 1964; Kokwaro, 1976; Kerharo, 1974
	Stem bark	Angina and stomach problems.	-	-	Kerharo, 1974; Watt & Breyer-Brandwijk, 1962
	Roots	Wound dressing; hookworm; snakebites; leprosy; general body swellings; fever; stomach pains; constipation; sterility and abortion.	-	-	Drummond & Coates-Palgrave, 1973; Watt & Breyer-Brandwijk, 1962; Kokwaro, 1976; Chhabra et al., 1989
	Stem	-	Cytotoxicity	-	Fyhrquist et al. 2006
	Leaves	-	Cytotoxicity	-	Fyhrquist et al. 2006
	Leaves	-	Anti-inflammatory	-	McGaw et al., 2001
	-	-	-	Combretene A; Combretene B	Bahar et al. 2004
	-	-	-	β-D-glucopyranosyl $2\alpha,3\beta,6\beta$-trihydroxy-23-galloylolean-12-en-28-oate; Combregenin; Arjungenin; Arjunglucoside I; Combreglucoside	Kemvoufo et al. 2008
	-	-	-	Sericoside; Arjunglucoside II; Punicalin	Asres et al. 2001
	-	-	-	Mollic acid; Mollic acid 3ß-O-glucoside; Mollic acid 3ß-O-arabinoside; Mollic acid 3ß-O-Xyloside	Pegel & Rogers, 1985
	-	-	-	2,6-dihydroxy-2,3,6-	Kovács et al. 2008

				trimethoxyphenanthrene; 3,6-dihydroxy-2,4,7-trimethoxyphenanthrene; 2,6-dihydroxy-4,7-trimethoxy-9,10-dihydrophenanthrene; 6,7-dihydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene	
	-	-	-	3,4-dihydroxy-4,5-dimethoxybibenzyl	Letcher et al. 1972
Euclea crispa subsp. *crispa* (Thunb.) Gürke	Roots	Coughs	-	-	Maroyi, 2013
	Unspecified parts	Melanoma skin cancer	-	-	Gramham et al. 2000
	Leaves	-	-	Hyperoside; quercitrin; epicatechin; (+)- catechins; gallocatechin	Pretorius et al. 2003
	Root bark	-	-	Lupeol; botulin; oleanolic acid	Sibanda et al. 1992
Sideroxylon inerme L.	Bark	Skin hyperpigmentation; gall sickness in stock and red water in cattle	Antioxidant activity; cytotoxicity	Epigallocatechin gallate; procyanidin B1.	Momtaza et al. 2008;
	Stem bark	Emetic	-	-	Chhabra et al. 1993
	Roots	Conjunctivitis; hernia; coughs; and paralysis	-	-	Chhabra et al. 1993
	Bark	Tonics to calves and goats	-	Cinnamic acid, kaempferol and leucanthocyanidins	Hutchings et al., 1996
Terminalia prunioides M.A. Lawson	Unspecified parts	Fungal infections	-	-	Fyhrquist, 2007
		Skin diseases	-	-	Neuwinger, 1996

Statistical analysis

A minimum of three experimental repeats were performed and each experiment was performed in triplicate to calculate the fifty percent inhibitory concentrations (IC_{50}) of the samples. One-way Anova was used to evaluate the significant difference between the plant extracts and the positive controls for the cell proliferation and antioxidant assays. The IC_{50} values and one-way Anova analysis (Turkey method) were done by using GraphPad prism 4 software.

Results and Discussion
Plant extraction yield

The ground plant material was extracted with ethanol and the percentage yield for each sample was calculated. The plant material weight and yield percentage results are given in table 2. The leaves extract from *C. molle* had the highest percentage yield (41%), whereas the *C. molle* fruit extract had the lowest percentage yield (6.5%). This finding could indicate high variability in the chemical composition of the different plant parts of the *C. molle* tree. *E. crispa* subsp. *crispa* had the second highest percentage yield (20%) followed by *S. inerme* (18%) and *T. prunioides* (14%). It would have been expected that the plant with the highest powdered material weight, *E. crispa* subsp. *crispa*, would also yield the highest percentage yield. Although ethanol is considered a more polar solvent, it does have the ability to extract non-polar compounds to a certain extent. Some of the plants might very well contain more non-polar compounds than polar compounds, which could denote why the weight of the powdered material is not directly proportional to the percentage yield among different plant species.

Table 2: Weights and percentage yield results for the plant extracts.

Plant	Powdered weight (g)	Extract weight (g)	% Yield
Combretum molle R. Br. Ex G. Don (leaves)	22.4	9.2	41
Combretum molle R. Br. Ex G. Don (fruits)	25.6	1.7	6.5
Euclea crispa subsp. *crispa* (Thunb.) Gürke (leaves and stems)	43.3	8.6	20
Sideroxylon inerme L. (leaves and stems)	13.3	2.4	18
Terminalia prunioides M.A. Lawson (leaves and stems)	22.1	3.2	14

Anti-proliferative activity

The anti-proliferative activity of the plant extracts was evaluated against the A431, HCT-116. HCT-15; HeLa; MCF-7 and UCT-Mel 1 cancerous cell lines. In addition, the anti-proliferative activity of these samples was evaluated against a normal phenotype cell line, the HaCat cell line. The XTT colorimetric assay was used to evaluate the anti-proliferative activity of the plant extracts against a range of cell lines. Mitochondrial dehydrogenase, an enzyme present in viable cells, reduces the yellow coloured water soluble form of XTT to an orange coloured insoluble formazan product (ATCC XTT Cell proliferation assay kit instruction manual, 2011). The results are given in table 3 as IC_{50} values, which denotes the concentration at which fifty percent of the cell proliferation and growth of the cells inhibited. Overall, the plant extracts showed the highest anti-proliferative activity on the A431 human epidermoid carcinoma cell line, with the *C. molle* fruit extract having activity with a low IC_{50} value of 23.2 µg/ml. The *C. molle* fruit extract has also shown to have noteworthy inhibitory effects on the growth and proliferation of some of the other cancerous cell lines including the HeLa, MCF-7 and UCT-Mel 1 cell lines with IC_{50} values found to be ranging from 48.7 to 51.3 µg/ml. Although these results show the *in vitro* potential, of the *C. molle* fruit extract to inhibit the growth and proliferation of cancerous cells, the low IC_{50} value of 45.9 µg/ml obtained for the normal HaCat cell line indicates that the extract might be more toxic towards the normal cells than those cancerous cells.

The leaf and fruit extracts of *C. molle* showed growth inhibitory effects on the HCT- 15 human Duke's type C (lymph node metastasis) (Frederiksen et al., 2003), colorectal adenocarcinoma at low IC_{50} values of 14.9 µg/ml and 24.2 µg/ml, respectively. This finding is curious when considering that no activity was found for the leaf and fruit extracts of *C. molle* on the HCT-116 which is also a human colorectal carcinoma cell line (Duke's type D- liver metastasis) (Ahmed et al., 2013). A study by Ahmed et al., 2013 showed that there are some gene mutation variant differences among the HCT-15 and HCT-116 cell lines. The HCT-15 cell line has gene mutation variants E545K and D549N for the PI3KCA gene, whereas the HCT-116 cell line showed a H4107R gene mutation variant. The HCT-15 cell line also had a S241F gene mutation variant for the TP53 gene while the HCT-116 cell line had no mutation variants for

this gene, displaying the wild type variant. The findings of the study showed that there are differences between cancerous cell lines even though the cell lines originate from the same type of tissue and disease. Therefore, it might be a possibility to find different activities of a particular sample on different cell lines originating from the same type of tissue and disease.

Table 3: Effect of the extracts on the cell proliferation of various cell lines after 72h treatment.

Treatment	IC$_{50}$ (µg/ml) ± SD						
	A431	HaCat	HCT-15	HCT-116	HeLa	MCF-7	UCT-Mel 1
Combretum molle R. Br. Ex G. Don Fruit extract	23.2 ± 0.8***	45.9 ± 7.0***	24.2 ± 0.028***	>200	48.7 ± 8.0***	50.4 ± 0.6***	51.3 ± 0.1***
Combretum molle R. Br. Ex G. Don Leaf extract	68.6 ± 4.0***	104.3 ± 0.3***	14.9 ± 0.054***	>200	>400	71.8 ± 1.0***	112.2 ± 0.8***
Euclea crispa subsp. *crispa* (Thunb.) Gürke	41.8 ± 0.4***	167.2 ± 4.0***	125.0 ± 0.034***	148.5 ± 8.9***	100.3 ± 6.0***	45.7 ± 7.0***	70.9 ± 3.0***
Sideroxylon inerme L.	46.7 ± 2.0	119.2 ± 0.8	N/D	137.2 ± 3.0***	>400	93.1 ± 6.0***	90.1 ± 3.0***
Terminalia prunioides M.A. Lawson	158.6 ± 0.05***	>400	N/D	>200	>400	140.6 ± 7.0***	140.4 ± 8.0***
Actinomycin D[a]	0.28 ± 0.018	0.6 ± 1.8 × 10^2	-	-	2.2 × 10^3 ± 5.0	1.7 × 10^3 ± 5.0	2.7 × 10^2 ± 4 × 10^4
Oxaliplatin[b]			41 ± 4.2	15.40 ± 5.2			

**P-value < 0.01; ***P-value < 0.001
A431: Human epidermoid carcinoma cell line
HaCat: Human keratinocyte cell line
HCT-116: Human colorectal carcinoma cell line
HeLa: Human cervical adenocarcinoma cell line
MCF-7: Human breast adenocarcinoma cell line
UCT-Mel 1: Human pigmented malignant melanoma cell line
IC$_{50}$: Fifty percent inhibitory concentration
SD: Standard deviation
N/D: Not determined
[a]: Positive control for the A431; HaCat; HeLa; MCF-7 and UCT-Mel 1 cell lines
[b]: Positive control for the HCT-15 and HCT-116 cell line.

The *E. crispa* extract and the *S. inerme* extract showed promising activity on the A431 cellline with IC$_{50}$ values of 41.8 µg/ml and 46.7 µg/ml, respectively. The *E. crispa* extract also showed anti-proliferative activity on the MCF-7 breast adenocarcinoma cell line with a low IC$_{50}$ value of 45.7µg/ml. Given that the IC$_{50}$ value found for the *E. crispa* extract on the HaCat cell line is high (167.2 µg/ml), it therefore indicates a much better safety margin than the *C. molle* fruit extract. As with *the E. crispa* extract, the *S. inerme* extract was found to have a relatively good safety margin due to its high IC$_{50}$ value of 119.2 µg/ml found against the HaCat cell line, when considering its activity on the A431 cell line. The *C. molle* leaf extract high to low anti-proliferative activity on the HCT-15, A431, MCF-7, HaCat and UCT-Mel 1 cell lines, while no activity was found against the HCT-116 or HeLa cell lines, up to the highest concentrations evaluated. A study by Fyhrquist et al., 2006 showed that a methanol leaf extract of *C. molle* had growth inhibitory activity on the T24 bladder cancer cell line with an IC$_{50}$ value of 27.7 µg/ml. The research by Fyhrquist et al., 2006 also indicated that a methanol root extract of *C. molle* had similar activity to the methanolic leaf extract of *C. molle* on the T24 bladder cancer cell line, while only moderate growth inhibitory activity was found for both of the extracts against the HeLa and MCF-7 cell lines.

Antioxidant activity

The antioxidant potential of the extracts was determined by evaluating their capacity to scavenge the DPPH free radical, the nitric oxide reactive nitrogen species and the superoxide reactive oxygen species. The IC$_{50}$ values are shown in table 4 and the dose-response curves of the extracts for the DPPH and nitric oxide antioxidant assays are given in figure 1 and 2, respectively. All the plant extracts tested in this study showed scavenging activity for the DPPH radical at low concentrations and scavenging activity of the nitric oxide reactive nitrogen species and the superoxide reactive oxygen species at high concentrations. Since these three molecules are different from one another, it was expected that each plant

extract would react differently to each radical. The *Terminalia prunioides* (1.8 µg/ml)and *Combretum molle* (1.9 µg/ml)leaf extracts have shown exceptional DPPH radical scavenging activity as compared with the positive control, vitamin C (1.9 µg/ml). A study by Masoko & Elof (2007) have shown that some of the extracts of *Combretum molle* and *Terminalia prunioides* do have DPPH scavenging activity. This study employed the use of a qualitative DPPH assay in which the leaf acetone extract showed strong DPPH scavenging activity where as the methanol extract of *Terminalia prunioides* and the acetone and methanol leaf extracts of *Combretum molle* has moderate DPPH scavenging activity. In the case of both plants, the hexane and dichloromethane leaf extracts showed no DPPH scavenging activity. The present study also showed that the ethanolic fruit extract of *Combretum molle* possess DPPH scavenging activity at a low IC_{50} value of 5.1 µg/ml.

Table 4: The antioxidant activity of plant extracts for the DPPH, nitric oxide and superoxide scavenging assays.

| Sample	IC_{50} (µg/ml) ± SD		
	DPPH	Nitric oxide	Superoxide
Combretum molle R. Br. Ex G. Don Fruit extract	5.1±0.05***	180.3±1.2***	166.7±1.5***
Combretum molle R. Br. Ex G. Don Leaf extract	1.9±0.006	77.46±0.3***	124.4±3.9***
Euclea crispa subsp. *crispa* (Thunb.) Gürke	2.5±0.02***	99.92±0.9***	164.6±13.2***
Sideroxylon inerme L.	11.5±0.04***	131.5±0.4***	115.6±15.6***
Terminalia prunioides M.A. Lawson	1.8±0.007**	86.13±0.2***	135.9±10.5***
Vitamin C[a] Quercetin[b]	1.9±0.005	62.74±0.9	17.35±2.8

**P-value < 0.01; ***P-value < 0.001
DPPH: 2,2-diphenyl-1-picrylhydrazyl
IC_{50}: Fifty percent inhibitory concentration
SD: Standard deviation
[a]: Positive control for the DPPH and Nitric oxide scavenging assays
[b]: Positive control for the Superoxide scavenging assay
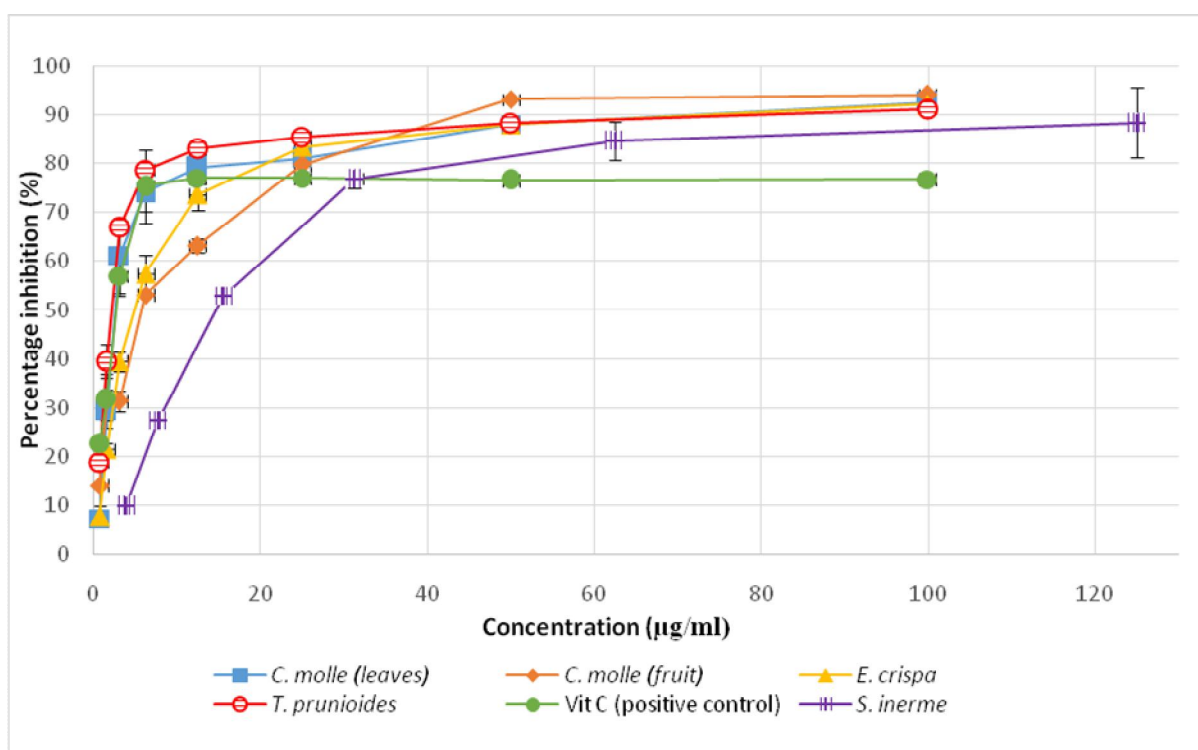
Figure 1: The dose-response curves of the inhibition of DPPH free radicals by the ethanolic plant extracts, *C. molle* (leaves); *C. molle* (fruit); *E. crispa*; *S. inerme*; and *T. prunioides*, and the positive control, Vitamin C.
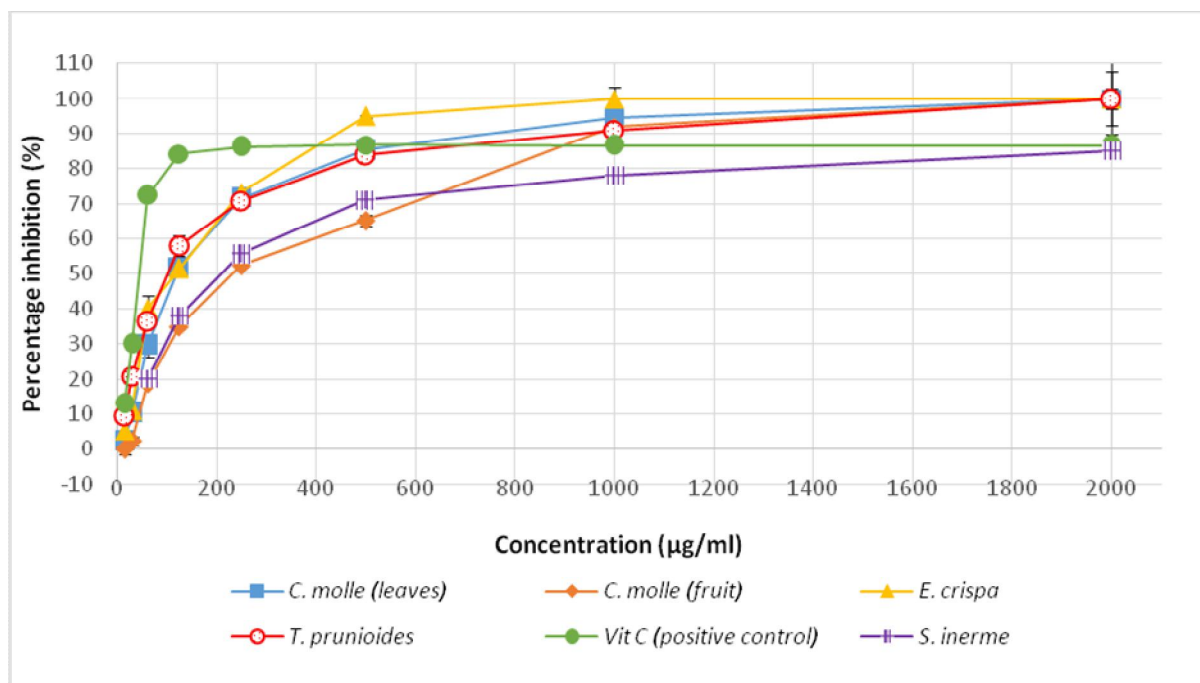
Figure 2: The dose-response curves of the inhibition of Nitric oxide reactive nitric species by the ethanolic plant extracts, *C. molle* (leaves); *C. molle* (fruit); *E. crispa*; *S. inerme*; and *T. prunioides*, and the positive control, Vitamin C.

The *Euclea crispa* extract also showed DPPH scavenging activity at an IC_{50} value of 2.5 µg/ml. Although this result compares well to a study by Shahid (2012) in which the IC_{50} values for DPPH scavenging activity from various extracts of *Euclea crispa* were shown to range from 0.84 µg/ml to 4.7 µg/ml, another study by Shahid (2012) indicated an IC_{50} value of 134.46 µg/ml for a hexane extract of *Euclea crispa*. The *Sideroxylon inerme* extract showed the activity at the highest concentration among the plant evaluated with anIC_{50}value of 11.5 µg/ml, though a study conducted by Momtaz et al., 2008 obtained an IC_{50} value of 1.54 µg/ml for the methanolic bark extract of *Sideroxylon inerme*. Differences in results obtained between research studies could be attributed to the use of different plant parts and solvents. Although the extracts did not show scavenging activity in the nitric oxide and superoxide assays at low concentrations, the extracts did indicate to have scavenging potentials comparable to that of Vitamin C for the DPPH free radical. This activity found in this study suggests that further research on the antioxidant activity of these extracts should be conducted to provide a basis for their possible use as chemopreventive agents.

Conclusion

This study was conducted to evaluate the *in vitro* anti-cancer and chemopreventive potential of four indigenous South African plants commonly used in traditional medicine. The results indicated that three of the plant extracts, *Combretum molle* fruit extract; *Euclea crispa* subsp. *crispa* and *Sideroxylon inerme*, had anti-proliferative activity on the A431 cell line at low concentrations. The leaf and fruit extracts of *Combretum molle* were observed to have potent growth inhibitory activity on the HCT-15 cell line. The *Euclea crispa* subsp. *crispa* extract showed anti-proliferative activity on the MCF-7 cell line at a low concentration. All the extracts showed antioxidant potential by scavenging of the DPPH free radical. As such, this study provides the initial evidence of the potential of these extracts as anti-proliferative agents of cancerous cells and their possible chemopreventive activity via their antioxidant properties.

Conflict of interest: Authors declare that this research presents no conflict of interests.

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APHRODISIAC EFFECTS OF METHANOLIC LEAF EXTRACT OF *PSEUDOPANAX ARBOREUS* (ARALIACEAE) (L.F. PHILLIPSON) IN NORMAL MALE RATS

*Egbe B. Besong[1], Ateufack G.[3], Albert Kamanyi[3] and Aurelien F.A. Moumbock[2]

[1]Department of Zoology and Animal Physiology; Faculty of Science; University of Buea; Cameroon;
[2]Department of Chemistry; Faculty of Science; University of Buea; Cameroon;
[3]Animal Physiology and Phytopharmacology Laboratory; Department of Animal Biology; Faculty of Science; University of Dschang; Cameroon

*Corresponding Author's: E-mail: egbe.ben@ubuea.cm

Abstract

Background: The leaves of *Pseudopanax arboreus* have been used traditionally for decades as aphrodisiac without scientific investigation. In this study, the effects of methanolic leaf extract of *P. arboreus* were evaluated on sexual behavior of normal male rats.

Materials and Methods: Twenty-eight adult male rats were randomly grouped into 4 groups of 7 rats each. Rats in group 1 were treated with 10 ml/kg body weight distilled water, group 2 rats received 6mg/kg body weight Viagra™, while the rats in groups 3 and 4 were given 46.5 mg and 93mg/kg body weight respectively of the methanolic extract of the leaves of *P. arboreus*. Female rats were made receptive by ovariectomy and subsequent hormonal treatment. Sexual behavior parameters were monitored on days 1, 7, 14 and 21 by pairing each male rat to a receptive female. Relative weight of sex organs and hormonal (FSH, LH and testosterone) profile were also determined.

Results: Doses of 46.5 mg/kg and 93 mg/kg, the extract significantly increased the mount and intromission frequencies, penile licking and relative weight of sex organs and enhanced testosterone production; and significantly reduced mount and intromission latencies, mean intromission interval, when compared to the distilled water group. The 93 mg/kg body weight dose prolonged ejaculation latency and reduced post-ejaculatory interval. However, the reference drug, Viagra™ proved more efficient than the extract.

Conclusion: The methanolic extract of the leaves of *P. arboreus* possesses aphrodisiac properties which may be due to the actions of bioactive compounds present in the extract.

Keywords: *Pseudopanax arboreus*; sexual behavior; methanolic extract; aphrodisiac.

Abbreviations: **DW:** distilled water: **ME:** methanolic extract: **ME1:** methanolic extract dose 1: **ME2:** methanolic extract dose 2: **P.:** *Pseudopanax:* **ML:** mount latency: **MF:** mount frequency: **IL:** intromission latency: **IF:** intromission frequency: **EL:** ejaculation latency: **PEI:** post ejaculatory interval: **MII:** mean intromission interval: **ICE:** inter-copulatory efficiency: **PL:** penile licking: **FSH:** follicle stimulating hormone: **LH:** luteinizing hormone: **NO:** nitric oxide: **eNO:** endothelial nitric oxide: **spp**: species: **CNS:** central nervous system : **ED**: erectile dysfunction: **MSD:** male sexual dysfunction : **W.H.O.**: World Health Organization: **ELIZA**: Enzyme-Linked Immuno-absorbent Assay: **SEM**: Standard Error of Mean: **µg**: micrograms : **kg**: kilograms: **ml**: millilitres : **mIU**: micro International Units : **ng**: nanograms : **UB-IACUC**: University of Buea Institutional Animal Care and Use Committee: **OECD:** Organization for Economic Development and Corporation: **s:** seconds.

Introduction

Sexual relationships are among the most important social and biological relationships in human life; and sexual health is an important component of an individual's quality of life and well-being (WHO, 2002). One of the main aims of marriage is procreation (reproduction) to ensure the continuity of an individual's lineage and, more importantly, for sexual fulfillment of both partners. For life to continue, an organism must reproduce itself before it dies (Yakubu et al., 2007).

In humans, reproduction is initiated by the mating of a male with a female in sexual intercourse which facilitates the coming together of sperm and egg for the purpose of fertilization (Fullick, 1994). In order to have a normal sexual intercourse and sexual fulfillment in males, the male sexual organs (the copulatory organ, that is, the penis) and factors relating to erection must function normally. The recurrent or repeated inability of the male to perform a satisfactory sexual function or any disorder that interferes with his full sexual response cycle is termed male sexual dysfunction (MSD) (Yakubu et al., 2007; Yakubu and Akanji , 2011). MSD is an important contributor of male infertility with about 30-50% of infertility cases attributed to problems with males alone (Ekwere et al., 2007). Apart from other underlying causes, MSD is a pathology that occurs naturally as age advances (Moreira et al., 2006; Wattanathorn et al., 2012). The prevalence of sexual dysfunction is still increasing. Also, despite advances in modern and orthodox medicines, its effective control by drugs or adjuvant therapies is affected by drug efficacy and safety, as well as cost (Jian et al., 2012). Continuous search for potent anti-MSD agents is, therefore, needed to develop new, safe and effective formulae for the treatment of MSD.

One of the approaches for the management of MSD is by the use of aphrodisiacs. An aphrodisiac can, therefore, be described as any substance that enhances sexual drive and/or sexual pleasure. Aphrodisiac can also be viewed as any food, drug, scent or device that can arouse or increase sexual drive or libido (Rosen and Ashton, 1993). Plants constitute an important source of medicines and play a key role in the health of a greater portion of the world's population. The use of plants or their products to treat sexual disorders or improve on sexual performance has a long history in most countries, and their investigations in animals have proven that they are effective in improving sexual desire and sexual behavior in male animals. Some of these include: *Mondia whitei* (Watcho et al., 2007), *Massularia accuminata* (Yakubu et al., 2008; Yakubu and Akanji, 2011), *Fadioga agrestris* (Yakubu et al., 2008), *Ficus capensis* (Njoku-Oji et al., 2015), *Garcinia kola* (Ralebona et al., 2012), *Arctium lappa* (Jian et al., 2012), *Monsonia angustifolia* (Fouche et al., 2015), *Caesalpinia bonduc* (Gbangkoto et al., 2015), *Moringa oleifera* (Thawatchai et al., 2012), and so forth.

Pseudopanax arboreus, commonly called "Five Finger" in Cameroon, is a New Zealand native tree belonging to the family Araliaceae. The family from tropical area origin is present in cooler climates too. They are found in the Americas, Eurasia, Africa (including Manyu Division, Cameroon), Australia, New Zealand, New Caledonia, and Pacific Islands. The Family Araliaceae is closely related to the Family Apiaceae and Family Pittosporaceae (Plunkett et al., 1997). Members of these Families have been shown to possess aphrodisiac potentials. Also, some Araliaceae contain essential oils, are resinous and heterophyllous. Members of the Family include "devil's walking-stick," (*Aralia spinosa*), the "devil's club", (*Oplopanax horridus*), *Hedera* spp. (e.g. *Hedera helix);* and herbs such as ginseng *Panax* spp., a native of Korea and used as medicinal herb. Apart from the folk claim that *P. arboreus* possesses sex stimulating potentials, there is no scientific investigation to support or refute this indigenous claim. Leaves of *P. arboreus* (Araliaceae) have been considered as aphrodisiac by the people of manyu division (Cameroon). For a long time, they have been employed traditionally by the indigenous males to ensure endurance during sexual activity, and to improve on their sexual performance. In a previous study (Egbe et al., 2017), we evaluated the effects of its leaf-aqueous extract on the sexual behavior of normal male rats. As a consequence, the present study was designed to investigate the effects of methanolic leaf extract of *P. arboreus* on sexual behavior of normal male rats.

Materials and Methods
Plant material

Fresh leaves of *P. arboreus* were harvested from the tropical rainforest of Mamfe, under the guide of a local traditional practitioner and poacher, who confirmed the plant's identity based on its local vernacular name. A full branch and an attached flower of the plant were carefully preserved in a local newspaper and taken to the National Herbarium in Yaounde for authentication, where a voucher number 2734/SFRK (YA) of the specimen was given. Meanwhile, the fresh leaves were chopped into smaller pieces, air-dried under shade for about two months and ground using an electric grinder. The ground powder (300g) was added to 3000 ml of methanol, kept for 72 hours with mechanical agitation. It was thereafter filtered using the Laboratory test sieve (ENDOCOTTS LTD, ENGLAND) of 38 μm aperture. This was followed by solvent evaporation in a rotavapor under reduced pressure to yield 47.54 g of paste giving a 15.85% yield of extraction. Part of the extract was submitted to the Department of Chemistry, University of Buea for phytochemical screening. Meanwhile, administrative doses were determined following the traditional practitioner's directives and screening tests.

Chemicals

Products used in this study included sildenafil citrate (Viagra™) (Pfizer Inc, USA), diclofenac, estradiol and progesterone (Sigma Chemicals, USA) as well as bioassay kits for FSH (DRG Diagnostics, Germany), LH (DRG Diagnostics, Germany) and testosterone (Omega Diagnostics LTD, Scotland, UK), which were all purchased from BIOPHARCAM Douala, Cameroon. All Chemicals and reagents were transported and stored under the recommended conditions until used.

Animals

Animals used were Wistar strain rats of either sex, raised in the Animal House of the Department of Zoology and Animal Physiology, Faculty of Science, University of Buea, Cameroon, under standard conditions of temperature, humidity and a 12H light/12H dark cycle. They were given free access to water and laboratory diet.

Ovariectomy and induction of estrus in females

This was done according to the methodology described by Cariton (1986) and in our previous study (Egbe et al., 2017). Briefly, a total of 30 females rats obtained from the Animal House of the Department of Zoology and Animal Physiology, Faculty of Science of the University of Buea, Cameroon, were starved for 24 hours and prepared for surgical operation. They were weighed and given an intra-peritoneal injection of 0.02 ml/100 g (equivalent to 10 mg/kg body weight) diazepam, followed by 0.01 ml/100 g (equivalent to 5mg/kg body weight) ketamine, as anaesthesia; the two injections were separated by 5 minutes interval and after the onset of anaesthesia, the right and left dorsal lumbar of each female rat was shaved and the exposed skin prepared for aseptic surgery (97% alcohol wipe). This was followed by incising the dorsal flank to penetrate the abdominal cavity and attain each ovary. The par-ovarian fatty tissue was identified and retracted and the exposed ovary and its associated oviduct severed, after making a ligature at the anterior zone to prevent bleeding. The peritoneum and skin were then stitched followed by an intramuscular injection of 0.2 ml of penicillin-G to prevent any post-surgical infection, and oral administration of diclofenac capsule at the dose of 30 mg/kg as analgesia.

About 14 days following surgery and complete healing of the wounds, each ovariectomised female rat was given 66.67 µg of estradiol benzoate solution subcutaneously to bring them to estrus. This was followed 48 hours later by another subcutaneous injection of 600 µg progesterone solution. The progesterone was administered 6 hours before pairing each female rat with a sexually-experienced, normal (non-experimental) male rat. Only those rats exhibiting good sexual receptivity (solicitation behaviour and lordosis in response to mount) and no rejection behaviour were employed in the experiment (Ratnasooriya and Dharmasiri, 2000; Watcho et al., 2007).

Experimental design
Animal grouping and extract administration

A total of 28 adult Wistar male rats obtained from our breed and weighing 180-200 g each were randomly divided into 4 groups of 7 rats each. Rats in group 1 (control) were administered 10 ml/kg body weight distilled water; group 2 received 6 mg/kg body weight Viagra™ (standard drug); while those in groups 3 and 4 were given 46.5 and 93 mg/kg body weight respectively of the leaf-methanolic extract of *P. arboreus*. Each male rat was housed in a separate polypropylene cage.

Mating behavior test

This was done following the method described by Agmo (1997), and modified by Watcho et al. (2007), and as in our previous study (Egbe et al., 2017). Briefly, 30 minutes after the administration of the test substance, an estrous female was introduced into respective cages and observed for mating performance. Observations were conducted in the dark phase (as from 20:00 local time) of the light-dark cycle under dim light and very quiet conditions. Treatment lasted for 21 days and observations were done on days 1, 7, 14 and 21. Each test session was considered ended when Mount latency (ML) and Post Ejaculatory Interval (PEI) was 20 minutes.

The following performance parameters were assessed

Mount (when the male rat raised the forelimbs and gripped the female followed by the movement of its pelvic region towards the vagina of the female rat aimed at introducing his penis into the female's vagina); Intromission (the thrusting of the pelvic region of the male rat into pelvic region of the female followed by the penetration of the erect penis into the female's vagina); penile licking (when the male bent and licked the penis without mounting or intromission; and ejaculation (when the male griped the female with the latter raising its snout in an upward direction). In rats, this often comes after a series of successive mounts and intromissions. From these parameters, the following indices were determined and/or calculated: Mount latency (ML) (the time interval from the introduction of the female into the cage until the first mount); Mount Frequency (MF) (the total number of mounts preceding ejaculation); Intromission latency (IL) (the time interval from the introduction of the female into the cage until the first intromission); Intromission frequency (IF) (the number of intromissions preceding an ejaculation); Ejaculation latency (EL) (the time from the first intromission to ejaculation); Post-Ejaculatory Interval (PEI) (the time interval between an ejaculation and the next first mount); and Mean Intromission Interval (MII) or Inter-Copulatory Efficiency (ICE) (computed as: MII (ICE)= ejaculation latency/intromission frequency) (Jian et al., 2012; Fouche et al., 2015).

Assessment of relative weight of sex organs

On day 22 following beginning of treatment, all treated groups were starved, sacrificed under ethyl-ether as anesthesia, dissected, and the following organs were isolated: testes, epididymis, vas deferens, prostate, seminal vesicle and the penis. Each isolated tissue/organ was rinsed thoroughly and wiped with clean absorbent paper, carefully freed from all connective tissues, and then weighed using an electronic balance (NVT 1601/1, OHAUS Corporation, USA). Their individual weights were then expressed as a percentage of the total body weight.

Hormonal profile assessment

Alongside the sex organs, blood was collected using a 5-ml syringe through cardiac puncture and immediately transferred into heparinized test-tubes. The blood was kept for 24 hours after which the supernatant was collected and put into test-tubes. It was then centrifuged for 15 minutes at 2500 rpm. At the end of this, the supernatant was again collected. Plasma concentrations of FSH, LH and testosterone were determined using enzymatic kits and standardised reagents, and following the protocol prescribed by the manufacturer. In each case, a blank solution was prepared to help calibrate or standardise the ELIZA reader (Tietz, 1995; Egbe et al., 2017).

Ethical considerations

The experimental animals were handled in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines for testing chemicals 423 and 425 [OECD, 2008 (a and b)]. The research protocol was approved by the University of Buea Institutional Animal Care and Use Committee (UB-IACUC) on the 30th of May 2018 and an ethical clearance number (UB-IACUC No 003/2018) was given.

Statistical Analysis

Values were expressed as Means (±SEM). Mean values were calculated for each animal and quantitative comparison between groups established from these means. One way Analysis of Variance (ANOVA) followed by Duncan test using SPSS for windows version 20.0 was done to test for significance level at $P<0.05$.

Results
Effects of leaf-methanolic extract of *Pseudopanax arboreus* on sexual behaviour of male rats
Effects on Mount (ML) and Intromission (IL) Latencies

Treatment of normal male rats with the leaf-methanolic extract of *P. arboreus* at 46.5 mg/kg and 93 mg/kg doses (ME1 and ME2, respectively) resulted in significant decreases in mount (ML) and intromission (IL) latencies, compared to the distilled water-treated (control) animals (Table 1).

Table 1: Effects of leaf-methanolic extract (ME) of *P. arboreus* on Mount (ML) and Intromission (IL) Latencies.

Parameter	Day	Treatment			
		DW	Viagra™	ME1	ME2
ML (s)	1	109.20±27.17[ad]	81.80±9.52[bd]	92.20± 11.45[cd]	92.60 ±18.90[cd]
	7	96± 30.80[ad]	77.80 ±12.81[ad]	83 ±15.65[ad]	82.80± 6.98[ad]
	14	82.40±57.74[ae]	75 ±13.85[ad]	79.40 ±16.30[ae]	76.0±8.17[ae]
	21	77.51 ±33.14[ae]	70.4 ±14.01[ae]	76.20 ±8.87[ae]	73.4± 30.8[ae]
IL (s)	1	121.20± 24.64[ad]	87.80± 8.26[bd]	99.20 ±11.61[cd]	97.60± 17.63[cd]
	7	106.33 ±27.48[ae]	86± 14.47[bd]	89.40 ±9.84[bd]	88.60 ±7.37[bd]
	14	94.17 ±48.07[ae]	85.60± 15.93[ad]	87 ±16.75[ad]	86.80± 12.01[ad]
	21	85.2 ±13.44[af]	71.4 ±32.82[ae]	79.6± 9.83[ae]	77.4± 9.93[ae]

Values are presented as Means (±SEM); DW: distilled water; ME1: leaf-methanolic extract dose1 (46.5 mg/kg); ME2: leaf-methanolic extract dose 2 (93 mg/kg); s: seconds; On the same row, values with same letter (a-c) are not significantly different; on the same row, values with different letters are significantly different; on the same column, values with the same letters (d-f) are not significantly different; on the same column, values with different letters are significantly different; $P<0.05$.

Effects on Mount (MF) and Intromission Frequencies (IF)

Like with the ML and IL, subjection of normal male rats to a 21-day treatment with the leaf-methanolic extract of *P. arboreus* at either dose (ME1 or ME2) resulted in an increase in mount frequency (MF) with significant (p<0.05) values obtained on day 21 of treatment, compared to the control animals; whereas both extract failed to induce significant (p<0.05) effects on intromission frequency (IF) throughout the treatment period compared to the control rats (Table 2).

Table 2: Effects of leaf-methanolic extract (ME) of *P. arboreus* on mount (MF) and intromission frequencies (IF) of normal male rats.

Parameter	Day	Treatment			
		DW	Viagra™	ME1	ME2
MF	1	10.55± 4.32[ad]	20.1± 4.12[bd]	12.85± 2.8[ad]	10.81± 6.67[ad]
	7	11.90 ±8.1[ad]	21.05± 2.6[cd]	13.95± 5.1[ad]	12± 3.48[ad]
	14	13.55± 3.51[ad]	22.25± 3.1[cd]	18.35± 6.66[cd]	14.3± 9.87[ad]
	21	14.15± 5.02[ad]	24.60± 2.98[cd]	20.05± 5.24[bce]	17.95± 6.36[be]
IF	1	10.15± 3.51[ad]	16.65± 3.72[bd]	11.35± 2.69[ad]	10.2± 6.1[ad]
	7	11.05± 7.02[ad]	18.15 ±2.80[bd]	12.45 ± 3.80[ad]	10.75± 2.21[ad]
	14	11.85± 2.63[ad]	19.8± 3.15[cd]	13.5± 5.97[ad]	11.35± 6.93[ad]
	21	12.7± 3.57[ad]	22.25± 3.19[cd]	16.5± 4.67[bd]	13.65 ±4.39[ad]

Values are presented as Means (±SEM); DW: distilled water; ME1: leaf-methanolic extract dose1 (46.5 mg/kg); ME2: leaf-methanolic extract dose 2 (93 mg/kg); s: seconds; On the same row, values with same letter (a-c) are not significantly different; on the same row, values with different letters are significantly different; on the same column, values with the same letters (d-f) are not significantly different; on the same column, values with different letters are significantly different (p<0.05).

Effects on Ejaculation latency (EL) and Post ejaculatory interval (PEI)

Treatment of normal male rats with the leaf-methanolic extract of *P. arboreus* at the doses of 46.5 mg/kg and 93 mg/kg (ME1 and ME2 respectively) induced contrasting effects on both EL and PEI. Rats treated with the ME1 dose witnessed a significant (p<0.05) incease in EL and a significant decrease in PEI from day 1 (613.35 ±194.76) through to day 21 (659.44± 220.82) of treatment compared to distilled water-treated (control) group; whereas those that received the ME2 dose showed a significant increase in EL (606.60± 192.83) on day 1, and (465.45± 264.73) on day 21; and a significant decrease in PEI (512.6± 336.62) on day 1, and (351.21± 173.44) compared to the distilled water-treated animals (Table 3).

Table 3: Effects of leaf-methanolic extract of *P. arboreus* on EL and PEI of normal male rats.

Parameter	Day	Treatment			
		DW	Viagra™	ME1	ME2
EL (s)	1	501.55 ±100.92[ad]	548.45 ± 168.78[abd]	613.35 ±194.76[cd]	606.60± 192.83[cd]
	7	486.65 ±135.13[ad]	578.13 ± 140.68[bd]	628.75± 189.17[cd]	577.45± 264.73[bd]
	14	477.82 ±200.92[ad]	648.23± 155.37[be]	647.31 ±194.76[bd]	526.20± 192.83[ae]
	21	435.71 ±105.13[ae]	686.41± 165.38[be]	659.44± 220.82[bd]	465.45± 264.73[af]
PEI (s)	1	380.8 ±282.86[bd]	280.95 ±175.82[ad]	512.6± 336.62[cd]	288.5± 191.6[ad]
	7	215.85±117.05[ad]	251.95± 164.1[ad]	468.15±175.3[cd]	389.9±165.44[be]
	14	369.65± 152.67[bd]	249.40± 113.3[ad]	416.4± 274.99[ce]	453.85± 330.49[cf]
	21	372.75± 168.09[bd]	237.1± 157.86[ad]	351.21± 173.44[bf]	476± 185.1[cf]

Values are presented as Means (±SEM); DW: distilled water; ME1: leaf-methanolic extract dose1 (46.5 mg/kg); ME2: leaf-methanolic extract dose 2 (93 mg/kg); s: seconds; On the same row, values with same letter (a-c) are not significantly different; on the same row, values with different letters are significantly different; on the same column, values with the same letters (d-f) are not significantly different; on the same column, values with different letters are significantly different; P<0.05.

Effects on Penile licking (PL) and Mean intromission interval (MII)

Following treatment of normal male rats with either dose of the leaf-methanolic extract of *P. arboreus,* there was a non-significant (p<0.05) increase in PL induced by both doses from day 1 (3.5±1.05 and 4.75±1.37) to day 21 (4.75± 1.92 and 5.70± 1.98) (for ME1 and ME2 respectively), compared to the control animals. Similarly, a non-significant decrease in MII was observed from day 1 (45.38±13.89 and 45.73± 14.02) to day 21 (34.33± 7.09 and 37.24± 8.47), respectively for animals receiving ME1 and ME2, compared to the control group which showed a non-significant increase in this parameter.

Table 4: Effects of leaf-methanolic extract (ME) of *P. arboreus* on PL and MII of normal male rats.

Parameter	Day	Treatment			
		DW	Viagra™	ME1	ME2
PL	1	2.55± 1.93[a]	5.55± 1.70[a]	4.75±1.37[a]	3.50± 1.05[a]
	7	3.3±1.13[a]	5.90± 1.12[a]	5.20± 1.32[a]	3.75± 2.20[a]
	14	3.35± 1.23[a]	6.25± 2.33[a]	5.45± 1.19[a]	4.55±1.57[a]
	21	3.85± 1.04[a]	7.30±2.15[b]	5.70± 1.98[a]	4.75± 1.92[a]
MII (s)	1	49.28± 15.73[a]	37.05±11.62[a]	45.38±13.89[a]	45.73± 14.02[a]
	7	49.99±20.33[a]	36.26± 29.08[a]	40.06± 11.16[a]	38.53±19.37[a]
	14	53.49± 22.71[a]	34.33± 7.09[a]	34.35± 15.75[a]	38.29±16.99[a]
	21	55.32± 29.3[a]	23.96± 18.43[a]	34.33± 7.09[a]	37.24± 8.47[a]

Values are presented as Means (±SEM); DW: distilled water; ME1: leaf-methanolic extract dose1 (46.5 mg/kg; ME2: leaf-methanolic extract dose 2 (93 mg/kg); s: seconds; On the same row, values with same letter (a-c) are not significantly different; on the same row, values with different letters are significantly different; on the same column, values with the same letters (d-f) are not significantly different; on the same column, values with different letters are significantly different; p<0.05.

Effects of leaf-methanolic extract of *P. arboreus* on hormonal profile of normal male rats.

Plasma concentrations of the male reproductive hormone, testosterone, increased significantly (p<0.05) in animals that received either dose of the leaf-methanolic extract of *P. arboreus* compared to the control animals. However, greater values were recorded in animals that received the standard drug (Viagra™) than in the extract-treated animals (Table 5). Meanwhile, effects of both doses of the extract on FSH and LH plasma levels were non-significant, compared to the control group.

Table 5: Effects of leaf-methanolic extract of *P. arboreus* on plasma concentrations of FSH, LH and Testoste**rone**

Hormone	Treatment			
	DW	Viagra™	ME1	ME2
FSH (mIU/ml)	2.31 ± 0.72^a	3.33 ± 0.15^a	2.57 ± 0.84^a	2.01 ± 0.63^a
LH(mIU/ml)	1.77 ± 0.21^a	2.94 ± 0.32^a	2.11 ± 0.66^a	1.46 ± 0.58^a
Testosterone (ng/ml)	2.71 ± 0.66^a	4.68 ± 0.93^b	4.04 ± 1.02^b	4.16 ± 0.24^b

Values are presented as Means (±SEM); DW: distilled water; ME1: leaf-methanolic extract dose 1 (46.5 mg/kg); ME2: leaf-methanolic extract dose2 (93 mg/kg); FSH: follicle stimulating hormone; LH: luteinizing hormone; on the same row, values with the same letter are not significantly different; on the same row, values with different letters are significantly different; p<0.05.

Effects of leaf-methanolic extract of *P. arboreus* on relative weight (% of body weight) of reproductive organs of normal male rats

Compared to the control group (Table 6), treatment of normal male rats with the leaf-methanolic extract of *P. arboreus* at either dose for a 21-day period provoked a non-significant (p>0.05) increase in the relative weight of reproductive organs: testes, epididymis, vas deferens, seminal vesicles, prostate and penis.

Table 6: Effects of leaf-methanolic extract of *P. arboreus* on relative weights of the reproductive organs of normal male rats.

Organ	Treatment			
	DW	Viagra™	ME1	ME2
Testes	1.32 ± 0.01^a	1.59 ± 0.14^a	1.62 ± 0.08^a	1.57 ± 0.04^a
Epididymis	0.480 ± 0.02	0.557 ± 0.06	0.516 ± 0.04	0.573 ± 0.23
Vas deferens	0.09 ± 0.03^a	0.11 ± 0.03^a	0.11 ± 0.01^a	0.12 ± 0.04^a
Sem. vesicle	0.32 ± 0.10^a	0.49 ± 0.08^b	0.41 ± 0.06^a	0.34 ± 0.11^a
Prostate	0.13 ± 0.05^a	0.27 ± 0.05^b	0.15 ± 0.01^a	0.16 ± 0.06^a
Penis	0.14 ± 0.02^a	0.23 ± 0.04^b	0.18 ± 0.03^a	0.18 ± 0.04^a

Values are presented as Means (±SEM); DW: distilled water; ME1: leaf-methanolic extract dose 1 (46.5 mg/kg); ME2: leaf-methanolic extract dose2 (93 mg/kg); on the same row, values with the same letter are not significantly different; on the same row, values with different letters are significantly different; p<0.05.

Discussion

To the best of our knowledge, there is no scientific report on the aphrodisiac effects of the leaf extract of *Pseudopanax arboreus* in the literature. The present study was, therefore, designed to scientifically investigate the folk use of *Pseudopanax arboreus* as a sex stimulant. Overall, the leaf-methanolic extract of *P. arboreus* enhanced the sexual activity of male rats compared to the control rats, although its effects were less than those of Viagra™. There is no doubt that this drug is a standard sexual enhancer and the study confirms its wide use in treating most male sexual dysfunctions, especially erectile dysfunction (ED).

Phytochemical screening of the methanolic leaf extract of *P. arboreus* reveals the presence of alkaloids, flavonoids, saponins, steroids, tannins and triterpenoids. Studies in laboratory animals such as rats have attributed the sexual stimulant activity to many components of plant extracts as the possible bioactive agents increasing endogenous testosterone level and enhancing male sexual behavior. These include steroids and steroidal saponins, which may act as intermediaries in the steroidal pathway of androgen production. Saponins can bind to hormone receptors, resulting in conformational changes that can induce the physiological functions of the hormone; or can bind to hormones receptors

involved in the synthetic-pathway of the said hormones and as a consequence, promote their production (Drewes et al., 2003; Gauthaman et al., 2008). Also, flavonoids have been involved in altering androgen levels and may also be responsible for enhancing male sexual behavior either by promoting testosterone synthesis or inhibiting its metabolic degradation (Ratnasooriya and Fernando, 2008).

Both doses of the extract induced significant decreases in ML and IL, and increases in MF and IF, compared to the distilled water-treated control group. MF and IF are regarded as indicators of libido or sexual desire; while ML and IL are also regarded as indicators of sexual arousal. Mount Frequency and Intromission Frequency are useful indices of vigor, libido and potency. While the number of mount (MF) reflects sexual motivation, increase in the number of intromission (IF) shows the efficiency of erection, penile orientation and the ease by which ejaculatory reflexes are activated (Agmo, 1997). The decreases in ML and IL and increases in MF and IF noted following administration of the leaf-methanolic extract of *P. arboreus* at both doses throughout the treatment period indicate that libido and arousal were enhanced by both doses of the plant extract (Tajuddin et al., 2004; Mbongue et al., 2005). At the 96 mg/kg body weight dose, the extract prolonged EL and increased PEI; effects that are similar to those of Viagra™. Prolonged ejaculation is an indicator of prolonged coitus. PEI is regarded as an indicator of potency, libido and potential to recover from exhaustion after the first ejaculatory series. Increment of PEI is a reflection of the improvement of erectile function and the ability to perform better copulation (Thakur et al., 2009). These increased values of PEI are indicators that *P. arboreus* has potential for use in erectile disorders, and further evaluation of *in vivo* effects on endothelial nitric oxide (eNO) production could provide insight towards the mechanism of action of *P. arboreus* on penile erection (Bivalacqua et al., 2007). This data clearly supports our findings in the previous study (Egbe et al., 2017) and thus further confirms the use of this plant in folk medicine.

PL and MII (ICE) are important indices for evaluating the effect of drug administration on erectile function (Thakur and Dixit, 2007). Both doses of the plant's – leaf methanolic extract induced an increase in PL and a decrease in MII (ICE) throughout the treatment period, compared to the distilled water-treated group, though to a lesser degree than the Viagra™-treated group. This further shows that the leaf-methanolic extract of *P. arboreus* increases sexual potency. The prolonged EL noticed with the 96 mg/kg dose, and the increased penile erection (PL) noticed with both doses suggest involvement of NO in the intervention (Du and Hull, 1999). Gingseng saponin has been shown to enhance libido and copulatory performance by acting directly on the CNS and gonadal tissues (Murphy and Lee, 2002), and evidence indicates its potential to facilitate penile erection by directly inducing the vasodilation and relaxation of the penile corpora cavernosa via an NO-dependent mechanism (Chen and Lee, 1995), including arginase inhibition (Corine et al., 2015). The improvement in sexual function observed in this study might, therefore, be as a result of the presence of such compounds in the leaf-methanolic extract of *P. arboreus*. However, further studies are necessary to identify the exact active constituent(s) responsible for the sexual function improvement activities, and the mechanisms through which these activities could be mediated.

There was an increase in plasma testosterone levels in rats treated with both doses of the extract, compared to the distilled water-treated, control rats. This major male androgen is synthesized and secreted by the Leydig cells of the testis under the influence of LH (Luteinizing Hormone), a gonadotrophin. Unfortunately, plasma levels of both LH and FSH were less significant; which means some phyto-constituents of the plant's leaves extract could mimic the role of LH to stimulate the Leydig cells. In the regulation of copulatory behavior, testosterone has been associated with an increase in sexual behavior (Mills et al., 1996; Murphy et al., 1998). Also, for normal sexual activity, penile tumescence and rigidity in addition to accessory muscles that help in improving penile rigidity and ejaculation, have been reported to be testosterone-dependent (Gauthaman et al., 2002). Furthermore, according to Hull et al. (1999) and Putnam et al. (2001), testosterone may enhance sexual behavior by increasing dopamine release in the medial preoptic area of the hypothalamus, and potentiating NO (nitric oxide) neurotransmission.

The effects of the extract on relative weight of reproductive and accessory organs, though non-significant, are a reflection of the plasma concentrations of testosterone recorded. Testosterone has been reported to be useful for the histo-morphometric development and maintenance of the testes, and ultimately, the biochemical process of sperm production (Njoku-Oji et al., 2012). The increase in the relative weight of these organs noticed in the extract-treated rats could, therefore, be attributed to the action of testosterone on them. The enhancements in the weights of accessory sexual organs of male rats are usually associated with androgenic activity and anabolic function. Androgens can stimulate the growth of accessory sexual organs (e.g., testis, seminal vesicles and prostate) and increase their weights (Chauhan et al., 2009). If certain drugs or natural compounds can increase the weights of accessory sexual organs, they are considered to possess androgenic properties (Luo et al., 2006).

Many bioactive components of plant extracts also exhibit aphrodisiac potentials by acting directly on the CNS to modulate the action of neurotransmitters and gonadal tissues in males, or through vasodilation and the generation of NO, which can also change sexual behavior. Independently of NO, alkaloids and saponins are known to increase dilation of blood vessels in the sexual organs (Zamble et al., 2008); through ROCK II enzyme (Rho-Kinase II) inhibition, hence, relaxation of smooth muscles of corpus cavernosum (Sumanta et al., 2012; Sumanta et al., 2013) and through inhibition of soluble epoxide hydrolase (sEH) (Jang et al., 2015). The results of this study suggest that the methanolic extract of the leaves of *P. arboreus* may be a potential new agent for the clinical management of MSD.

Conclusion

Overall, this study showed that the methanolic extract of the leaves of *P. arboreus* could enhance sexual behavior and performance in male rats and possess some anabolic potential. These actions could be as a result of its androgenic effects and other pathways through the actions of bioactive compounds such as alkaloids, flavonoids, saponins, steroids, tannins and triterpenoids present in the extract. The present laboratory findings support the folkloric use of this plant as an aphrodisiac by the people of Manyu Division in Cameroon.

Declaration of conflict of interest: The authors declare that this work has no conflict of interest with other people or organization.

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DUKKU AND BAEKAM SPRINGWATER INHIBIT THE UREASE ACTIVITY OF *HELICOBACTER PYLORI*

SungSook Choi,[1] HaeKyung Lee,[2] JinWoo Kim,[3] JiHan Yu,[4] HyunHo Choi,[4] HyungKeun Kim,[4] SangWoo Kim,[4] and Hiun Suk Chae[4*]

[1]College of Pharmacy, Sahmyook University, Seoul, Korea; [2]Department of Laboratory Medicine Uijongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea; [3]Department of Dermatology, Uijongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea; [4] Department of Internal Medicine Uijongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea.

[*]**Corresponding author's E-Mail:** sschoi@syu.ac.kr

Abstract

Background: Springwater (SW) contains many kinds of minerals such as sodium, potassium and copper. These metallic ions may influence the activity of metallo-enzymes such as urease via competitive inhibition. In this study, we investigated the effect of SW on the inhibition of Ni-containing urease activity, which is essential for the colonization of *Helicobacter pylori* (*H. pylori*) in the human stomach.
Materials and Methods: We studied the growth inhibition of *H. pylori* by SW. We evaluated ammonia production to detect urease activity and performed western blot analysis of UreA and UreB for enzyme production.
Results*:* SW had no significant effect on bacterial growth. Western blot analysis also showed that SW did not affect the translation of UreA and UreB, but it significantly reduced the urease activities of the Jack bean as well as that of *H. pylori* from 50 to 75%.
Conclusion: These results might indicate that the consumption of SW may prevent the colonization of *H. pylori* and ameliorate the toxic effect on gastric mucosa via the inhibition of urease activity.

Keywords: Springwater, *H. pylori*, Urease activity, Western blot.

Abbreviations: SW: Spring water, *H. pylori*: *Helicobacter pylori*, KCTC: Korean Culture Collection, BHI: Brain Heart Infusion, DS: Dukku spring, BS: Baekam spring, DSW: Dukku spring water, BSW: Baekam spring water.

Introduction

Helicobacter pylori (*H. pylori*) is a microaerobic, gram-negative bacilli that infects more than 70% of Koreans over the age of 40. *H. pylori* is closely related to upper gastrointestinal disorders such as chronic gastritis, peptic ulcer and gastric cancer (Kim et al., 2001).   Many kinds of virulence factors, such as urease and adhesion, have been widely studied for their involvement in the colonization of bacteria, and these have been a target of drug development (Dunn, 1993; Graham and Yamaoka, 2000; Dunn, 1993). In particular, urease is a very important factor for survival in the strongly acidic environment of the human gastric mucosa, because it degrades urea to ammonia and carbon-dioxide (CO_2) (Marshall et al., 1990). The ammonia-producing ability of *H. pylori* urease is about 100 times greater than that of the *Proteus* species. Moreover, the Km value of *H. pylori* urease is 10 to 20 times lower than that of *Proteus* sp., which means that the affinity of *H. pylori* urease to urea is very strong even at very low concentrations of urea (Mobley et al., 1991; Osaki et al., 2008). The antral mucosa, the pH-sensing region of the stomach, secretes gastrin, which is neutralized by the ammonia produced by urease and then damages the phospholipids of gastric epithelial cells by activating bacterial phospholipase. Therefore, the inhibition of *H. pylori* urease activity might have been considered a target of *H. pylori* treatment (Khan et al., 2004; Kosikowska and Berlick, 2011; Yu et al., 2015). For example, natural products such as biscoumarine inhibit the function of urease activity (Khan et al., 2004; Lodhi et al., 2014), and metal ions can also inhibit urease function via competitive inhibition with the nickel in the enzyme (Zaborska et al., 2004). A typical metal ion, silver ion, exerts an inhibitory function by interacting with the SH group of the enzyme. SW is an abundant source of minerals, but only a few reports have focused on its effect on *H. pylori* and urease.
    Drinking of SW has been known to increase blood flow in the gastric mucosa, to inhibit gastric emptying, and to

change the secretion of gastric juices, depending on the type of SW (Petrakova, 2001; Bertoni et al., 2002;). Sodium bicarbonate SW has been used to neutralize gastric acid and inhibit gastric cramps. Carbonate SW is known to increase the gastric mucosal blood flow, and is used in chronic gastritis (Bertoni et al., 2002). However, studies on how SW effects *H. pylori* have been rare. The present study was performed to investigate the effect of SW on the growth of *H. pylori* and urease activity.

Materials and Methods
Bacterial strain

A standard strain of *H. pylori 26*695 was purchased from the Korean Culture Type Collection (KCTC, Taejeon, Korea) for use as a test. *H. pylori* was cultured at 37 °C in a microaerobic (5% O_2, 10% CO_2 and 85% N_2 gas) atmosphere that is a standard for brain–heart infusion (BHI) (Difco, Detroit, MI) with 7% laked horse blood (Oxoid, Cambridge, CB5 8BZ, UK), 0.4% isovitalex (BBL, Sparks, MD, USA), vancomycin (6 μg/ml), amphotericin B (8 μg/ml) and trimethoprim (5 μg/ml). In the case of a liquid medium, 7% horse serum (Sigma Co. St. Louis, USA) was used instead of blood.

Springwater and Hydrochemical analysis of spring water

From the Dukku (DS, Buk-myeon, Uljin gun, province of Gyeongsangbuk-do, South Korea) and Baekam (BS, Onjeong-myeon. Uljin gun, province of Gyeongsangbuk-do, South Korea), samples were gathered every two weeks. These waters were filter sterilized (0.45 μm cellulose membrane) and kept at 4 °C for 2 weeks. Hydrochemical analysis of both types of springwater was performed according to a method established by Lee (Lee et al., 2014).

Inhibitory activity of SW on the growth of *H. pylori*

BHI liquid media were prepared using the Dukku (BHIDSW) and Baekam SW (BHIBSW) instead of distilled water. We inoculated the BHIDSW, BHIBSW and normal BHI (prepared with distilled water) with 10^8 cells/mL of *H. pylori*, followed by incubation for 3 days. After incubation, the cells were washed with PBS and then serially diluted tenfold with PBS and spotted onto BHI. Colony formations were counted after 3 days of incubation.

Inhibition of Jack bean urease by spring water

Jack bean urease (Sigma-U7752) purchased from Sigma-Aldrich (Sigma. Co. St. Louis, USA) was used for enzyme assay. 15 μl of Jack bean urease (1 μg/ml in 0.1M phosphate buffer, pH 7.4) was added to 1 ml of three different 10% urea solutions [10% urea solution was made using distilled water, Dukku spring water (DSW) and Baekam spring water (BSW), respectively] followed by incubation at room temperature for 90 min. During incubation, the produced ammonia was spectrophotometrically assayed using a Hitachi 7600 (Tokyo, Japan) at 30 min intervals. PBS was used as a control instead of a 10% urea solution.

Inhibition of *H. pylori* urease using spring water

To test the inhibitory activity of *H. pylori* urease, *H. pylori* was incubated in three different BHI media (normal BHI, BHIDSW and BHIBSW). Then, each liquid medium was inoculated with 10^8 cells/ml of *H. pylori* and after 3 days of incubation, the cells were harvested by centrifugation. The supernatant and the cell pellets were used as respective enzyme sources. The cell pellets were then suspended in PBS (the volume of the PBS was the same as the volume of the culture broth), and were then disrupted by 30 s bursts from a Sonifier at 30 W for a 50% cycle followed by centrifugation at 4℃, and the supernatant was used as an enzyme solution. The same volume of 10% urea solution and either culture broth (supernatant) or sonicated supernatant were incubated at room temperature for 90 min. During incubation, the produced ammonia was spectrophotometrically assayed using a Hitachi 7600 (Tokyo, Japan) at 30 min intervals. PBS was used as the control instead of the 10% urea solution.

Effect of pH on the urease enzyme activity

To confirm the effect of pH on the urease activity, urease assay was conducted in two different pH system (pH 7.4 and pH 9.0). 15 μl of Jack bean urease (1 μg/ml in 0.1M phosphate buffer, pH 7.4) was added to 1 ml of two different 0.5 M urea solutions (0.5 M urea solution were prepared with pH 7.4 and 9.0 PBS) by incubation at room temperature for 90 min. During incubation, the produced ammonia was spectrophotometrically assayed using a Hitachi 7600 (Tokyo, Japan) in 30 min intervals. PBS was used as a control instead of a urea solution.

Western blot analysis of *H. pylori* urease

To test the effect of SW on the synthesis of urease, western blot analysis was conducted to detect urease A and B

subunits. Anti-rabbit urease α(B-234) (sc-21016, 1:1000; SantaCruz Biotechnology, Inc., Santa Cruz, CA, USA) and Anti-rabbit urease β(B-300) (sc-22742, 1:1000; SantaCruz Biotechnology, Inc., Santa Cruz, CA, USA) were used as the primary antibodies, and Anti-mouse *H. pylori* OMP (6631) (sc-69935, 1:1000; SantaCruz Biotechnology, Inc., Santa Cruz, CA,USA) was used as the control protein. Goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (170-6515, 1:2000; Bio-Rad) and Goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate (170-6516,1;2000; Bio-Rad) were used as secondary antibodies.

After 3 days of incubating *H. pylori* in three different BHI media (normal BHI, BHIDSW and BHIBSW), *H. pylori* cells were harvested. The cells were suspended in 1 mL of PRO-PREP™ protein Extraction Solution (17081, iNtRON Biotechnology, Korea), and incubated for 30 min at 4 °C. After centrifugation at 12,000 rpm for 30 min at 4 °C, the concentrations of protein in the supernatants were determined via a BCA Protein Assay Kit (#23227, Pierce, Rockford, IL). Forty μg of total protein were electrophoresed on 8-12% SDS-PAGE and transferred to a nitrocellulose membrane (Protran BA85, Whatman/ GE Healthcare, PA, USA) via Transfer System (Bio-Rad, CA, USA). Nonspecific binding was inhibited by incubation in 5% non-fat dry milk [TBST (0.05% Tween-20 in TBS)]. After overnight incubation at 4 °C in a TBST solution (0.05% Tween-20 in TBS), the membrane was washed three times with TBST solution then incubated with horse radish peroxidase (HRP), conjugated goat anti-rabbit IgG (170-6515, 1:2000; Bio-Rad), and anti-mouse (170-6516,1;2000; Bio-Rad) for 1 hr and analyzed using highly cooled CCD cameras (AE9300-Ez-CaptureMG) and a densitograph software CS analyzer (ver3.0) (ATTO, Tokyo, Japan).

Results
Hydrochemical analysis of Dukku and Baekam springwater

The pH of the Dukku SW (DSW) was 8.90, and that of the Baekam SW (BSW) was 9.54. Both SW samples contained Si, Na, K, AL, Ca, Mg, F, Cl, S, Sr, and carbonate ion (Table 1).

Table 1: Hydrochemical analysis of Dukku and Baekam springwater

	DSW	BSW
Temperature (^{o}C)	30.5	41.6
pH	8.90	9.54
TDS (mg/L)	144	144
Alkalinity (mg/L)	1.36	1.54
Si (mg/L)	14.23	20.48
Na (mg/L)	106.4	45.4
K (mg/L)	0.10	0.07
Al (mg/L)	0.17	0.08
Ca (mg/L)	3.06	1.83
Mg (mg/L)	Nd	Nd
Fe (mg/L)	Nd	Nd
Zn (mg/L)	Nd	Nd
Ba (mg/L)	Nd	Nd
F (mg/L)	13.63	3.23
Cl (mg/L)	5.07	8.71
Br (mg/L)	Nd	Nd
NO_3^- (mg/L)	0.32	Nd
SO_4^{2-} (mg/L)	6.23	15.72
HCO_3^- (mg/L)	82.97	93.95
Sr (mg/L)	0.03	0.08

The effect of springwater on the growth of *H. pylori*

To test the effect of the growth inhibition by the SW, *H. pylori* cells were incubated in normal BHI, BHIDSW and BHIBSW, respectively. As shown in Table 2, there were no significant differences in growth, depending on the media. Therefore, SW showed no inhibitory activity against proliferation of *H. pylori*.

Table 2: Inhibitory effects of Dukku and Baekam Springwater against *H. pylori* Proliferation.

Log$_{10}$ CFU/mL (mean±SD, n=3)		
Control	BHIDSW[a]	BHIBSW[b]
8.23 ± 0.83	8. 2.1 ± 0.23	8.11 ± 0.12

a: BHIDSW, BHI media prepared with Dukku spring water
b: BHIBSW, BHI media prepared with Baekam spring water
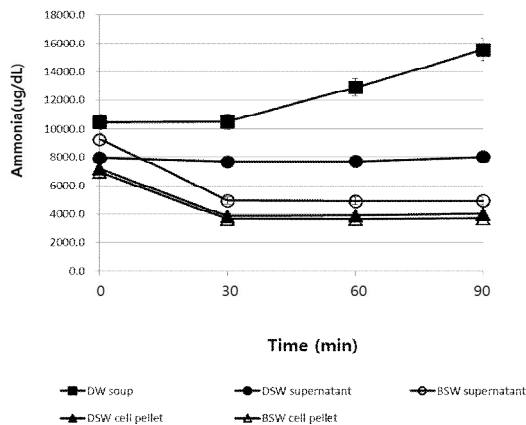
Inhibition of Jack bean Urease by springwater

To test the inhibition of Jack bean urease activity by two different samples of SW, urease assays were performed. In the presence of two samples of SW, 50% of urease activity was decreased. In the control group, the concentration of ammonia that was produced by Jack bean urease was 45,000 μg/ml, but in the presence of the two samples of SW, the concentration of ammonia produced by jack bean urease was only 20,000 μg /ml (Fig. 1).
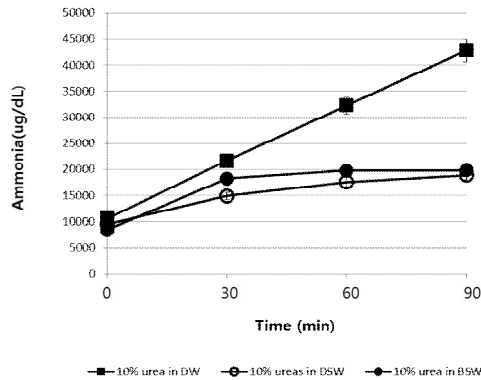
Figure 1: Inhibition of Jack bean urease by Dukku Springwater and Baekam Springwater.
DSW: Dukku Springwater, BSW: Baekam Springwater

Inhibition of *H. pylori* urease by springwater

To determine the inhibitory effect of two samples of SW against *H. pylori* urease, *H. pylori* was cultured in media prepared with BHIDSW and with BHIBSW. Both the culture broth and the cell pellets showed inhibitory activity against *H. pylori* urease. In the case of the control, the concentration of ammonia after 90 min was 16,000 μg/dl. In the case of the cell pellet, however, the concentration of ammonia after 90 min was 4,000 μg/dl. This result shows that the production of ammonia was decreased by about 75% in the presence of SW (Fig. 2).


Figure 2: Inhibition of *Helicobacter pylori* urease by Dukku Springwater and Baekam Springwater.
DW: BHI prepared with Distilled water, DSW: BHI prepared with Dukku Springwater, BSW: BHI prepared with Baekam Springwater
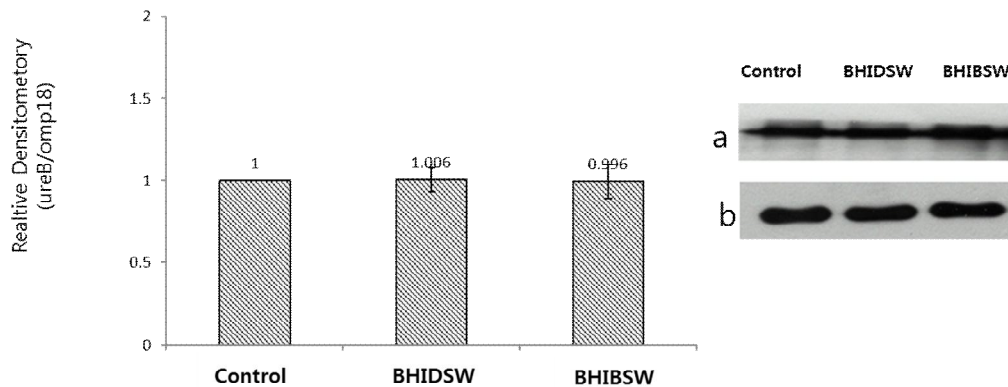
Western blot analysis of *H. pylori* urease

Western blot analysis was performed to determine the influence of SW on the synthesis of UreA and UreB

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proteins. Western blot analysis showed that similar amounts of UreA and UreB were synthesized, depending on the culture medium prepared with SW (Figs. 3, 4).
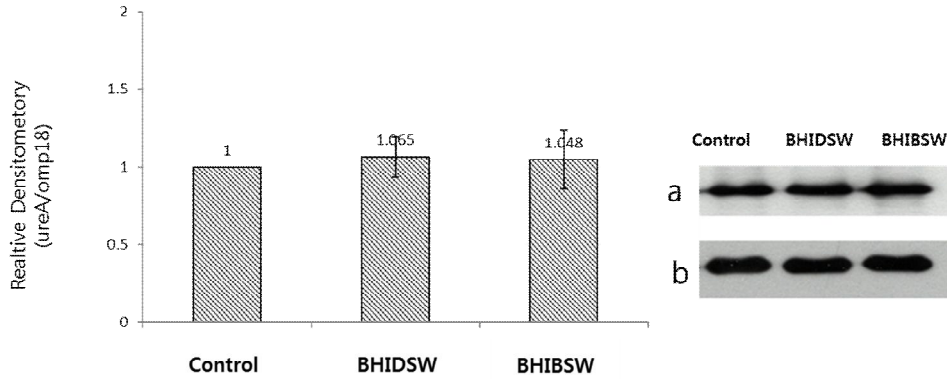
Fig. 3 Western blot analysis of *Helicobacter pylori* urease A subunit
Control: Cells were cultured in normal BHI media; BHIDSW: cells were cultured in BHI media prepared with Dukku spring water; BHIBSW: cells were cultured in BHI media prepared with Baekam spring water. a;omp18, b; ureA

Fig. 4 Western blot analysis of *Helicobacter pylori* urease B subunit
Control: Cells were cultured in normal BHI media; BHIDSW: cells were cultured in BHI media prepared with Dukku spring water; BHIBSW: cells were cultured in BHI media prepared with Baekam spring water.a;opm18, b; ureB

Discussion

Urease is a virulence factor that is necessary for the colonization of *H. pylori*, which makes it a major target for drug development. To survive in a strong acidic environment, it is necessary for *H. pylori* to degrade urea via urease to produce the ammonia that will neutralize the acidic environment (Marshall et al., 1990). Therefore, the urease-producing ability of *H. pylori* is very important for colonization. By mainly locating in the antrum, *H. pylori* weakens the pH sensing in the antrum and degrades the phospholipids of the host epithelial cells via the ammonia produced by urease (Zhang et al., 2005). An alternative to antibiotic remedies, the inhibition of *H. pylori* urease that is described above can solve the problem of gastric mucosal damage that is caused by the colonization of *H. pylori*. Many recent studies have focused on the inhibitors of urease, and the aim of the present study was to investigate the effect of SW on the urease activity of *H. pylori*. When we tested the antibacterial activity of SW against *H. pylori*, there was no significant difference in the numbers of bacteria between media made of spring water and normal culture medium (Table 1). The inhibition of *H. pylori* and Jack bean urease by SW, however, recorded maximum reductions of 75 and 50%, respectively (Figs. 1, 2). According to the hydro-chemical analysis of spring waters, they have alkaline pH (8.90 and 9.54). Therefore, it is necessary to confirm this inhibitory activity may attribute to pH effect. However, when we

tested the pH effect, there were no differences in enzyme activity between pH 7.4 and pH 9.0 (data not shown). Future *in vivo* and human study will be necessary to confirm the effect of SW on the urease activity of *H. pylori* inhabiting gastric mucosa. There have been few reports about the effects of *H. pylori* by SW, particularly with respect to the urease enzyme. Based on our data, it is conceivable that the ordinary consumption of SW may prevent the colonization of *H. pylori* by inhibiting urease in the human stomach.

Urease is a metalloenzyme containing nickel ion (Ni) essential for its activity (Benanti and Chivers 2009). Urease without Ni shows no activity and many metal ions, including copper, competitively inhibit the entry of Ni into protein (Hausinger, 1987; Todd and Hausinger, 1989; Park and Hausinger, 1996; Amtul et al., 2002). A large amount of Si dissolved in both the samples of SW might have inhibited the enzyme activity by competitively binding to the binding site of Ni (Kind et al., 1954; Amin et al., 2012). Some amount of Si is also dissolved in two SW (Table 1). And many other types of metal ions such as Ag, Hg, Mn, Pd, Co and Cd, are also known to inhibit urease activity (Kumar and Kayastha, 2010; Amin et al., 2010; Amin et al., 2012; Dong et al., 2012; Dong et al., 2013;). As described above, the two samples of SW contained various mineral ions (Table1). The exact mechanism of urease activity inhibition by the SW used in this study is still unknown whether Si or other metal ions in the main one for contributing action.

In future, it is necessary to investigate the exact mechanism and to establish the main one that plays a role in the SW. According to western blot analysis, the enzyme production of *H. pylori* was not inhibited, but its function was affected because the band intensities of UreA and UreB from different media showed similar patterns (Fig. 3 and 4).

Up to now, drinking of SW has been used to treat gastritis, peptic ulcer and functional disorder, because SW can increase blood flow, increase or inhibit gastric juices, depending on the type of SW, and inhibit gastric emptying (Petrakova, 2001; Bertoni et al., 2002;). Sodium bicarbonate spring water is usually recommended for the treatment of gastric disorders because it can neutralize gastric acid and inhibit stomach cramps. Carbon dioxide SW, which is known to increase gastric mucosal blood flow, has been used to treat chronic gastritis (Razumov et al., 2009). However, it has never been known whether these therapeutic effects of SW are related to an inhibition of the bacterial urease activity of *H. pylori* or not. Moreover, whether this effect is due simply to the effects of various minerals or to urease inhibition is still unknown, and further study is necessary. In addition, the SW used in this study has been ingested for many years by local residents living in proximity to the hot spring. Therefore, it is necessary to conduct a population-based study about the rate of *H. pylori* infection and urease activity among the people drinking this SW.

In conclusion, this study used SW that contains various metal ions and is thought to be effective for the treatment of various upper gastrointestinal disorders by inhibiting urease, which is one of the virulence factors of *H. pylori*.

Authors' contributions

Designed the study: HK Lee, JW Kim and HS Chae.
Carried the experiments: SS Choi, HK Kim, JH Yu, HH Choi, HK Kim and SW Kim.
Wrote the manuscript: SS Choi, JH Yu, HH Choi and HS Chae.
All authors read and approved the manuscript.

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