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ARTICLES

Full Length Research Paper

Phytochemical, nutritional and anti-nutritional properties of leaves, stems bark and roots of trees used in popular medicine for the treatment of malaria in South Eastern Nigeria

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Environmental factors are known to affect genes in various ways. They are affecting the gene products including the production of both primary and secondary metabolites, hence the need to assess the phytochemical and nutritional compositions of *Morinda lucida* Benth and *Alstonia boonei* De Wild growing in Nsukka, Enugu state, south eastern Nigeria. The qualitative analyses of the plant parts showed the presence of all the tested phytochemicals in various levels ranging from trace (+) to very heavily present (++++). The quantitative estimates showed significant variations in their values in leaves, stems bark and roots in some phytochemical components. Results of the proximate also showed the contents of protein were 2.46 - 17.69 and 1.45-2.1; fats 0.49-1.88 and 0.84- 1.4; carbohydrates 68.76- 81.63 and 17.81- 31.38; and ash 2.33-2.9 and 0.78- 2.75 in *M. lucida* and *A. Boonei* plant parts, respectively. Vitamins A, E and K and anti-nutritional factors, phytate and oxalate also varied in the leaves, stems bark and roots. The results showed that these medicinal plants grown in Nsukka, a Derived Savanna Agro-Ecology, are rich in phytochemicals and proximate nutrients. The rich presence of these phytochemicals and other nutritive values supports the use of the different parts of these plants in ethno-medicine and equally creates the possibility for their use in drug formulation.

Key words: *Alstonia boonei,* ethno-medicine, *Morinda lucida,* phytochemicals, Nsukka.

INTRODUCTION

Alstonia boonei De wild belonging to the family Apocynaceae is a tree commonly called pattern wood or stool wood (Aigbokhan, 2014). The local names of this plant in South Eastern Nigeria are Egbu, Egun, or Egbe.

This plant species is primarily a timber; however, the natives of South Eastern Nigeria, use virtually all the parts of this plant - leaves, stems bark and roots, for medicinal purposes (Aigbokhan, 2014).

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However, *A. boonei*, is not used only in South Eastern Nigeria, but there are various reports of its use in ethno-medicine across Africa, especially in countries where it is not considered a sacred plant (Terashima, 2003; Betti, 2004; Alshawsh et al., 2009; Tepongning et al., 2011). *A. boonei* is used widely in the prevention and treatment of malaria among the indigenous population of South Eastern Nigeria. Many authors have reported its use in the treatment of diverse ailments like fever, intestinal helminthes, rheumatism, hypertension and diarrhea (Terashima, 2003; Adomi, 2008; Bello et al., 2009). *Morinda lucida* (Benth), on the other hand, belongs to the family of Rubiaceae with common names as Indian mulberry, Brimstone tree and Hog tree apple (Aigbokhan, 2014). This family is known to be widely used in traditional medicine. Its primary use in South Eastern Nigeria is as a medicinal plant. The vernacular names among the Igbos are Ogere, Huka, Ezeogu and Njisi (Aigbokhan, 2014). *M. lucida*, like many other medicinal plants, is a multipurpose medicinal plant. It is reported to be used in the treatment of yellow fever, malaria, hypertension, dysentery, stomach ache, ulcer, gonorrhea and leprosy (Adesida and Adesogan, 1972; Makinde and Obih, 1985; Tona et al., 1999). Medicinal plants and their rich phytochemical components have diverse anti-bacterial and anti-plasmodium activities on humans and other organisms. Reported phytochemicals in plants include alkaloids, tannins, flavoids, phenolics and many others. Phytochemicals associated with *A. boonei* and *M. lucida* include alkaloids, tannins, saponins, glycosides, flavonoids and triterpenoids (Kucero et al., 1972; Ayiku, 1992; Fasola and Egunyomi, 2005; Afolabi et al., 2007). There are several alkaloids of which echitamine and echitamidine compounds are the major ones that have been isolated from *A. boonei* stems bark (Adotey et al., 2012). Medicinal plants have nutritional functions; these nutrients are essential for the physiological activities of the human body. Such nutrients are carbohydrates, proteins, fats, mineral elements, and vitamins and even dietary fiber. All these play important role in satisfying human needs for energy and life processes (Hoffman et al., 1998; Dingman, 2002). Several researchers have worked on the phytochemicals, proximate and/or vitamins and anti nutritional components of medicinal plants. Several studies have shown that plant and plant products under the control of multiple genes are affected by the environment in diverse ways (Kucero et al., 1972; Ayiku, 1992; Fasola and Egunyomi, 2005; Afolabi et al., 2007). Environmental stresses could trigger defense or adaptive mechanisms resulting in the accumulation of a wide range of antioxidants in some environments more than the others (Hare et al., 1999; Hasegawa et al., 2000).

Antioxidants are used by plants as defense mechanism against several forms biotic and abiotic stresses. This implies that changes in a particular environment and/or plants growing in different environments could accumulate various levels of these secondary metabolites.

It has been reported that plants respond to environmental stresses such as high or low temperatures, drought, salts, UVB/light and acclimatize to these conditions in varying degrees (Hare et al., 1999; Hasegawa et al., 2000; Tattini et al., 2004; Myungmin, 2008).Environmental conditions that induce stress on plants affect the concentrations of antioxidants as they are produced to serve as plant defense mechanism. Some researchers have reported that high temperature induces phytochemical accumulation in several plant species as a defense strategy (Lefsrud et al., 2005). So even though several researchers had reported their findings on the chemical properties of *M. lucida* and *A. boonei* in some parts of Nigeria and other countries, there is also need to study the phytochemicals, nutritional and anti-nutritional properties of these multipurpose medicinal plants growing in Nsukka. The aim of this research is therefore, to study the phytochemicals, proximate and anti-nutrient compositions of *M. lucida* and *A. boonei*; and the vitamin composition of *M. lucida*.

MATERIALS AND METHODS

Collection and identification of plant samples

The plant parts (leaves, stems bark and roots) of *Morinda lucida* were collected at Zoological garden, Department of Zoology and Environmental Biology while *A. boonei* plant parts were collected from the Botanical garden, Department of Plant Science and Biotechnology, both at the University of Nigeria, Nsukka (UNN). Nsukka is in a Derived Savanna Agro-Ecology located at latitude 06° 52' N longitude 07° 24' E and altitude 442 m above sea level. The plant specimens were identified and authenticated at Department of Plant Science and Biotechnology, UNN and Bioresources Development and Conservative Program (BDCP), Nsukka, Enugu state.

Processing of plant extracts

The plant materials were dried at room temperature. The dried plant parts were ground to powder with a milling machine in the laboratory and the powdered samples were stored in separate air tight containers.

Extraction process

Hot water extract of the sample was used for the analyses. The process was performed by soaking 50 g of the powdered sample in 500 ml of distilled water and boiling for about 10 min, thereafter, it was double filtered with cheese cloth. The filtrate was allowed to cool before drying in hot-air oven.

Chemical analyses of the samples

The phytochemicals, proximate, vitamin and antinutrient analyses were determined using standard methods (AOCC, 2002; Edeoga et al., 2005). The qualitative phytochemical composition was determined before quantitative analysis. The phytochemical analyses were conducted for steroids, alkaloids, terpenoids, tannins, saponins, flavonoids, phenols, hydrogen cyanide and

Table 1. Qualitative phytochemical analyses of the leaves, stems bark and roots of *Morinda lucida* and *Alstonia boonei*

Phytochemical	*Morinda lucida*			*Alstonia boonei*		
Phytochemicals	Leaves	Bark	Roots	Leaves	Bark	Roots
Steroids	++	++	+	++	+	++
Alkaloids	+++	++	+	++	++	+++
Terpenoids	+	++	+	++	+	+++
Tannins	+++	++	+	+	++	+++
Saponins	+	+	+	+	+	++
Flavonoids	+++	++	++	++	++	+++
Phenols	++++	+++	++	++	+	++
HNC	+	+	++	+	+	+
Glycosides	++	+	+	+++	+++	+++

++++ = very heavily present, +++ = heavily present, ++ = present, + = trace.

glycosides in each of the plant samples – leaves, stems bark and roots. The proximate analysis was done for proteins, fats, fiber, moisture, ash and carbohydrates, and for vitamins A, E and K. The anti-nutritional factors determined were phytate and oxalate.

Data analyses

The data collected from the replicated plant samples for the quantitative phytochemicals, proximate, vitamin and antinutrient analyses were used in the analyses of variance using GENSTAT package (Genstat release 7.22 DE) (GENSTAT 2009). The means values were tested for significance and separated using Fisher's least significant difference (F-LSD) only when the F-ratio is significant (Obi, 2002).

RESULTS

The qualitative analyses showed strongly present, present and trace amount of the different phytochemicals in both *M. lucida* and *A. boonei* plant parts (Table 1). The presence of alkaloids, tannins, glycosides, terpenoids, saponins, phenols, hydrogen cyanide, flavoniods and steroids were observed in both *M. lucida* and *A. boonei* plant parts. The quantitative analyses showed significant variations in most of the phytochemical constituents of plant parts at FLSD $P \leq 0.05$ (Table 2). Equally the tannins content in the leaves of *M. lucida* (1.49 mg/100 g) varied significantly from the quantity in the stems bark (1.37 mg/100 g) and roots (0.47 mg/100 g). The tannins contents in *A. boonei* were much lower than the values obtained in *M. lucida*. The leaves, stems bark and roots of *M. lucida* contained higher values in terpenoids, alkaloids, steroids and phenols, while *A. boonei* leaves, stems bark and roots had higher values in hydrogen cyanide, saponins and glycosides. The different parts of these two medicinal plants are rich in phytochemical constituents. The proximate analysis showed that these plant parts contain nutritive components such as fats, proteins and carbohydrates at diverse levels (Tables 3

and 4). The protein contents ranged from 2.46 to 17.69 and 1.45 to 2.81 in *M. lucida* and *A. boonei* plant parts, respectively (Tables 3 and 4). The fiber contents were higher in *A. boonei* roots, stems bark and leaves. It was observed that *M. lucida* which had higher carbohydrate contents also had lower moisture content.The vitamin contents of *M. lucida* showed higher retinol (vitamin A) in the leaves than in the stems bark and roots ranging from 17.56 in the leaves to 1.25 in the roots (Table 5). Equally, vitamin K was higher in the leaves (2.61 to 0.62), while the values of vitamin E did not vary significantly in the leaves and bark; however, both varied significantly from the roots (Table 5). Table 6 shows the anti-nutrient compositions of the plant parts. Different levels of phytate and oxalate were observed in *M. lucida* and *A. boonei* plant parts as shown in the table. Phytate was significantly higher in the leaves of both *M. lucida* and *A. boonei* than in the other parts. The values obtained for oxalate were also significantly higher in the leaves of both plants ranging from 2.43 to 2.11 and 0.33 to 14 in *M. lucida* and *A. boonei*, respectively.

DISCUSSION

The qualitative screening of these medicinal plants from this ecological zone showed rich presence of bio-active phytochemicals while the detailed quantitative analyses revealed the actual values in the different plants parts; thus serving as a reference to their use in ethno-medicine among the people of South Eastern Nigeria. Plant phytochemicals have antibiotic, antiviral, anti-plasmodial and anti-parasitic properties as has been reported in several studies (Oliver-Bever, 1986; Adomi, 2008; Ene et al., 2008; Alshawsh et al., 2009). These results corroborate some previous studies on the phytochemical screening of *Morinda lucida* in other ecological zones, which also revealed the presence of alkaloids and flavonoids (Adeyemi et al., 2014), tannins, alkaloids,

Table 2. Phytochemical quantitative analyses of leaves, stems bark and roots of *Morinda lucida* and *Alstonia boonei*

Medicinal plants	Sample	Tannins (mg/100 g)	HNC (mg/g)	Flavonoids (mg/100 g)	Terpenoids (mg/100 g)	Alkaloids (mg/100 g)	Saponins (mg/g)	Steroids (mg/g)	Phenols (mg/100 g)	Glycosides (mg/g)
Morinda lucida	Leaves	1.49[a] ± 0.003	0.16[a] ± 0.002	0.28[a] ± 0.003	2.31[b] ± 0.009	2.64[b] ± 0.008	0.06[a] ± 0.004	1.16[a] ± 0.003	4.84[a] ± 0.003	0.43[a] ± 0.004
	Bark	1.37[b] ± 0.004	0.15[b] ± 0.003	0.08[b] ± 0.006	3.55[a] ± 0.011	3.01[a] ± 0.005	0.06[a] ± 0.004	1.16[a] ± 0.003	4.84[a] ± 0.003	0.43[a] ± 0.004
	Roots	0.47[c] ± 0.003	0.14[c] ± 0.002	0.08[b] ± 0.003	1.95[c] ± 0.003	0.25[c] ± 0.003	0.02[b] ± 0.006	0.44[b] ± 0.004	1.05[b] ± 0.004	0.14[b] ± 0.004
	LSD (P ≤ 0.05)	0.003	0.002	0.003	0.007	0.005	0.004	0.003	0.003	0.003
Alstonia boonei	Leaves	0.366[b]±0.004	1.994[b]±0.002	0.785[a]±0.004	0.426[a]±0.003	0.125[a]±0.003	1.375[a]±0.005	0.195[a]±0.004	0.064[a]±0.0003	1.375[b]±0.004
	Bark	0.375[a]±0.002	1.094[c]±0.004	0.075[b]±0.005	0.227[b]±0.002	0.026[c]±0.0046	1.254[b]±0.004	0.034[c]±0.004	0.041[b]±0.0006	1.394[a]±0.002
	Roots	0.036[c]±0.002	0.375[a]±0.002	0.004[c]±0.0003	0.075[c]±0.004	0.035[b]±0.0020	0.375[c]±0.003	0.035[b]±0.005	0.005[c]±0.0005	1.076[c]±0.002
	LSD (P ≤ 0.05)	0.002	0.0027	0.0029	0.0024	0.0028	0.0035	0.0033	0.0004	0.0022

HNC= Hydrogen cyanide. * Values = mean ± Standard Deviation, Values followed by different letters are significantly different from one another.

Table 3. Range and mean of the proximate composition (%) of the leaves, stems bark and roots of *Morinda lucida*.

Medicinal plant	Plant part	Fats		Proteins		Moisture		Fiber		Ash		Carbohydrates	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Morinda lucida	Leaves	1.825-2.0	1.88[a]±0.058	17.43-18.2	17.69[a]±0.26	6.92-7.0	6.93[a]±0.35	2.3-3.0	2.9[c]±0.05	2.907-2.94	2.9[a]±0.002	68.09-69.6	68.76[b]±0.67
	Bark	0.643-0.70	0.66[b]±0.19	4.371-4.378	4.38[b]±0.002	5.3-5.485	5.43[b]±0.6	5.928-6.0	5.96[b]±0.13	2.33-2.3388	2.33[c]±0.002	80.4-81.25	80.9[a]5±0.28
	Roots	0.41-0.50	0.49[c]±0.008	2.452-2.459	2.46[c]±0.002	4.2-5.73	4.9[b]±0.45	6.77-7.2	6.96[a]±0.13	2.422-2.56	2.47[b]±0.05	80.6-82.16	81.63[a]±0.052
	LSD (P≤ 0.05)	-	0.12	-	0.51	-	0.9	-	0.27	-	0.09	-	1.78

Values = mean ± Standard Deviation, Values followed by different letters are significantly different from one another.

Table 4. Proximate composition (%) of the leaves, stems bark and roots of *Alstonia boonei*.

Medicinal plant	Plant part	Fats	Proteins	Moisture	Fiber	Ash	Carbohydrates
Alstonia boonei	Leaves	0.844[b] ± 0.0036	2.106[b] ± 0.005	67.254[a] ± 0.004	7.153[c] ± 0.003	2.155[b] ± 0.002	20.488[b] ± 0.008
	Bark	0.404[c] ± 0.004	2.811[a] ± 0.008	67.055[b] ± 0.0045	9.164[b] ± 0.004	2.754[a] ± 0.004	17.814[c] ± 0.015
	Roots	1.414[a] ± 0.0038	1.45[c] ± 0.005	52.515[c] ± 0.0032	12.454[a]±0.004	0.785[c] ± 0.003	31.387[a] ± 0.012
	LSD (P≤ 0.05)	0.0031	0.0047	0.0031	0.0028	0.0022	0.0099

Values = mean ± Standard Deviation, Values followed by different letters are significantly different from one another.

Table 5. Vitamin contents of leaves, stems bark and roots of *Morinda lucida.*

Sample	Vitamin A	Vitamin E	Vitamin K
Leaves	$17.56^a \pm 0.002$	$1.57^a \pm 0.002$	$2.61^a \pm 0.006$
Bark	$6.64^b \pm 0.006$	$1.54^b \pm 0.003$	$1.57^b \pm 0.005$
Roots	$1.25^c \pm 0.004$	$0.96^c \pm 0.006$	$0.62^c \pm 0.005$
LSD (P ≤ 0.05)	0.003	0.004	0.004

Values = mean ± Standard Deviation, Values followed by different letters are significantly different from one another.

Table 6. Anti-nutrient composition of leaves, stems bark and roots of *Morinda lucida* and *Alstonia boonei*

Medicinal Plant	Sample	Phytate (mg/100 g)	Oxalate (%)
Morinda lucida	Leaves	$3.65^a \pm 0.006$	$2.43^a \pm 0.003$
	Bark	$1.89^b \pm 0.003$	$2.12^b \pm 0.003$
	Roots	$1.87^b \pm 0.005$	$2.11^b \pm 0.004$
	LSD(P ≤ 0.05)	0.003	0.004
Alstonia boonei	Leaves	$1.345^a \pm 0.0042$	$0.334^a \pm 0.0045$
	Bark	$0.408^b \pm 0.0056$	$0.311^b \pm 0.005$
	Roots	$0.157^c \pm 0.0021$	$0.144^c \pm 0.0025$
	LSD(P ≤ 0.05)	0.0034	0.003

L.S.D (P ≤ 0.05), Values = mean ± Standard Deviation, Values followed by different letters are significantly different from one another.

flavonoids and glycosides (Ajaiyeoba et al., 2006). Flavonoids, which are present in both medicinal plant parts are effective as free radial scavengers as has been observed in several studies (Del-Rio et al., 1994; Selah et al., 1995; Okwu, 2004). Flavonoids have also anti-allergic, anti-inflammatory, anti-viral, anti-proliferative and anti-carcinogenic properties (Middleton and Kandaswami, 1993), hence, the importance of their presence in these medicinal plants.

These plant parts are rich in phenolic compounds which have been reported as one of the largest and most ubiquitous groups of plant metabolites (Singh et al., 2007). They possess several biological properties, some of which are antiapoptosis, antiaging, anticarcinogen, antiinflammation, antiatherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities (Han et al., 2007). Several studies have described the antioxidant properties of medicinal plants which are rich in phenolic compounds. Phenolic compounds are generally known to have antioxidant properties. Some of the characteristics of saponins include formation of foams in aqueous solutions, range from haemolytic activity to cholesterol binding properties and bitterness (Sodipo et al., 2000; Okwu, 2004). Plant parts have saponins, thus increasing their efficacy as drugs making them capable of boosting immune system

(Okwu, 2004).The presence of steroids in these plant parts also makes them useful against cerebral malaria, thus, confirming their effectiveness as anti- plasmodial agents as reported by David et al. (2004). Several functions of steroids in living organisms range from acting as haemolytic activity, cholesterol binding and anti bacterial properties (Sodipo et al., 2000; Okwu, 2004; Raguel, 2007). The presence of steroids in *Morinda lucida,* however, is in disagreements with the report of Saganuwan et al. (2009) which showed that steroids were absent in this plant. This contradiction may be due to environmental interactions between organisms and their environment. As a result of these interactions, the environment may have influence on cellular productions via their actions on the genes. Thus, the production of any phytochemical, steroids in this case may depend on the combinations of necessary defense mechanism of the plant based on the prevalent environmental stresses. Alkaloids are the most essential of all phytochemicals, it is abundant in all the plant parts. They have a wide range of activities of which anti-parasitic properties are one of them (Louw et al., 2002; Abu et al., 2014). The presence of these phytochemicals supports the use of these two indigenous medicinal plants *A. boonei* and *M. lucida* in the herbal treatment of malaria, and beyond this the rich presence of varied active ingredients in *A. boonei* and *M. lucida* justifies their wide usage in the herbal treatment

of other ailments in South Eastern Nigeria.

Equally, the nutritional components of the plants showed moderate quantities of proximate compounds – carbohydrates, protein, fat, fiber, ash and high moisture content. Malaria patients tend to lose appetite and these herbal drugs in addition to their bioactive phytochemicals against the parasites also serve as sources of energy, protein, mineral elements and vitamins to the patients. All these contribute to the quick recovery of the body cells. The crude fiber contents of most samples were quite high. Fiber helps to absorb excess water in the colon, retain a good amount of moisture in the fecal matter and can offer protection against conditions like hemorrhoids, colon cancer, chronic constipation and rectal fissures (Jimaima et al., 2003). Diabetes affects about 10% of Nigerian adults (Global Nutrition Report, 2014) and diets high in fiber is generally efficient in the management of plasma glucose concentration in diabetic individuals. The low fats and carbohydrates composition of *A. boonei* leaves confirms the fact that vegetables are relatively low in calories and fats (Jayaraj et al., 2008). The observed high protein and carbohydrate values of *M. lucida* leaves could be attributed to its low moisture content which made the nutrients to be more concentrated. The vitamin composition of the samples were appreciable showing that they can alleviate the problem of 'hidden hunger' (micronutrient deficiencies) in the patients by contributing to daily vitamin intake. Micronutrient deficiencies are becoming widespread especially in those countries in the developing part of the globe. Vitamins A and E are powerful antioxidants and could be effective in combating degenerative diseases like atherosclerosis (Nwanjo, 2005). An inherent malaria sign/symptom is the depletion of red blood cells. Boosting the intake and absorption of iron and other nutrients necessary for blood formation is therefore very necessary. Vitamin A improves iron status probably by reducing the levels of infection, improving production and proliferation of red blood cells in the bone marrow, increasing the absorption of iron from food in the intestine and mobilization from body stores (Nnam, 2011). The appreciable ash values of the samples show that they are good sources of minerals and vitamins which are essential for day to day metabolic regulation of the body. This suggests that these plants are not only of high medicinal value but also of high nutritional value and studies have shown that nutritional and medicinal properties of plants are as a result of the interaction between their phytochemical and nutrient constituents (Nnam et al., 2012).

Conclusion

The different secondary metabolites responsible for the enthno-medicinal properties of these plant parts are alkaloids, tannins, saponins, terpenoids etc. *M. lucida* and *A. boonei* in combination with other potential anti-malarial plants may therefore be possible sources for the discovery of new chemotherapeutic agents in targeting of *P. falciparum* and other related parasites causing malaria. The plant parts have additional nutritional values because of the proximate and vitamins contents, hence these plants may also supply needed macro and micro-nutrients to the patients.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Assessment of thrombolytic, membrane stabilizing potential and total phenolic content of *Typha elephantina* Roxb.

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In this study, the crude methanolic extracts of whole palnt of *Typha elephantina* Roxb. were directed for screening of thrombolytic activity, membrane stabilizing activity, and total phenolic content. Human erythrocytes were taken for the analysis of both thrombolytic and membrane stabilizing activities and here streptokinase (SK) and acetyl salicylic acid (ASA) were used as standard for both tests, respectively. The methanolic extract of whole plant demonstrated high level of thrombolytic activity at the concentration of 4 mg/ml (33.33% of clot lysis) among various concentrations such as 2, 4, 6, 8, 7 and 10 mg/ml. On the other hand, the methanolic extract at different concentrations (1, 3, 5, 7 and 9 mg/ml), dose dependently inhibited the % of haemolysis of red blood cell (RBC) in case of heat induced condition and in case of hyponotic solution induced haemolysis, the methanolic extract just protected the RBC membrane. The total phenolic content (TPC) determination was investigated as a part of antioxidant assay. For the TPC determination, Folin-Ciocalteu method was used and the total phenolic content (126.33±4.33) of methanolic extract of *T. elephantina* was expressed by milligram of gallic acid equivalent to per gram of extract.

Key words: *Typha elephantina* Roxb., thrombolytic activity, membrane stabilizing activity, total phenolic content.

INTRODUCTION

Medicinal plants are defined as the plants which are beneficent for the recuperation of various diseases (Shahriar et al., 2014). In recent time, approximately 30% of the pharmaceuticals are produced from the plants present worldwide (Khan et al., 2010). Atherothrombotic diseases are characterized by the presence of serious impacts of thrombus in blood vessel (Mannan et al., 2011). By adhering to the destructed areas of endothelial surface, thrombocytes exert a key role in the development of atherothrombosis. In thrombosis activated activated platelets form platelets to platelets bonds, also bind to leucocytes and thus bringing them into a

convoluted process of plaque generation and growth. Plasmin, a neutral fibrinolytic agent causes clot lysis by breaking down the fbinogen and fibrin contained in a clot. Additional plasminogen can easily convert to plasmin by streptokinase which forms a 1:1 stochiometric complex with plasminogen (Chowdhury et al., 2011). Moreover, phlorotannin, isolated from marine brown algae, have a singular trait in acceleration of dissolution of intravascular blood clot, via inhibition of antiplasmin (Prasad et al., 2007).

Generally, several oxidative damages and related inflammatory actions are accelerated by free radicals generated within the body. The red blood cell (RBC) membrane assimilates to lysosomal membrane, so that the action of drug to stabilize RBC membrane could be anticipated to the membrane stabilizing activity (Islam et al., 2015). The most frequently used therapeutic agents against oxidation and inflammation are NSAIDS which illicit their action by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membranes (Debnath et al., 2013). As various adverse conditions such as intestinal side effects, mucosal erosion, etc., are associated with NSAIDs, researchers have concentrated to trace medicinal plants in order to find new anti-inflammatory agents with reduced side effects (Islam et al., 2015).

In Bangladesh, most of the people living in far-off hilly regions and also the country is enriched with a potent diversity of medicinal plants disseminate over the forests, crop fields, roadsides gardens and water lands (Shahriar et al., 2014). It is therefore necessary to conduct broad spectrum evaluation of the local flora exploited in traditional medicines for various biological activities and therefore ultimately leading to the new drug development (Chowdhury et al., 2011). In account of this, our concentration has been focused particularly on *Typha elphantina* Roxb. belonging to the family Typhaceae, a bush like small plant which locally known as Hogal. The plant grows plenty in the Sundarban forest as well as in other low lying areas of Sylhet, Chittagong in beels and haors (Khair, 2014). *T. elephantina* Roxb. widely scattered across northern Africa and southern Asia. It is defined as native in many countries all over the world such as Algeria, Egypt, Libya, Uzbekistan, Palestine, Israel, Saudi Arabia, Assam, Bangladesh, India, Bhutan, Nepal, Pakistan, Burma, etc..

It is cooling and aphrodisiac in nature; used in splenic enlargement, burning sensation, and leprosy. The root-stock has stringent and diuretic properties, also useful in case of dysentery, gonorrhoea and measles and the ripe fruits and soft and woolly floss of male spikes are used as medicated absorbent to wounds and ulcers in emergency cases (Rahman et al., 2014). Several chromatographic and spectroscopic analysis carried out on fruit extract of this plant revealed the presence of four chemical constituents named pentacosane, 1-triacontarol, β-sitosterol, β-sitostery-3-O-β glycopyranoside. These four chemical constituents are supposed to exert various

pharmacological activity such as anti-inflammatory, anti-pyretic, anti-tumor activities, etc., (Ruangrungsi et al., 1987). On the other hand, a previous investigation also provides evidence that *T. elephantina* Roxb. possesses analgesic, cytotoxic and anthemintic properties (Bulbul et al., 2013; Rahman et al., 2014). As part of our perpetual investigations on medicinal plants of Bangladesh, the methanolic extract of whole plant of *T. elephantina* Roxb. was studied for the thrombolytic activity, membrane stabilizing activity and also for the antioxidant property in terms of total phenolic content.

METHODOLOGY

Plant collection and preparation of plant materials

The whole plant was collected from NSTU campus, Sonapur, Noakhali, Chittagong during January 2015. Plant sample of *T. elephantina* Roxb. was identified by Mostaq Ahmed, Assistant professor, Department of Botany, Noakhali Government College, Noakhali, Bangladesh, where its voucher specimen (No. 025) was deposited. The plant parts were sun dried for 10 days and then ground by using high capacity grinding machine to produce coarse powder.

Preparation of extract

Powder (600 g) whole plant was soaked in 4.5 L methanol in a desiccator through occasional shaking and stirring. After 15 days, the solvent was removed and filtration was carried out by using sterile cotton and Whatman filter paper no. 1 (Sargent, Welch, USA). Then, rotary evaporation was carried out to concentrate the filtrate and was kept in room temperature in fresh and clean air for obtaining a brownish mass.

Streptokinase

Streptokinase that was commercially available in lyophilized stac vial (incepta pharmaceutical Ltd) of 15,00,000 I.U. and was collected and 5 ml sterile distilled water was added and mixed properly to produce a suspension. It was then used as a stock from which 100 μl (30,000 I.U) was drawn *in vitro* thrombolysis.

Thrombolytic activity

A method developed by Prasad et al. (2006) was used for the assessment of *in vitro* thrombolytic activity of *T. elephantina* extract using streptokinase (SK) as positive control with minor modifications. According to this method, healthy volunteers (n=3) were selected for collecting 5 ml venous blood and then transferred to five distinct pre weighed sterile micro centrifuge tube (1 ml/tube). These five tubes were then incubated for 45 min at 37°C. After clot formation, serum was completely aspirated out from the tubes without hampering the clot formed. Again each tube having clot was weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each pre weighed clot containing micro centrifuge tube, 100 μl of methanolic extract at several concentrations (2, 4, 6, 8 and 100 mg/ml) suspended overnight were added. As a positive and negative control, 100 μl of streptokinase and 100 μl of sterilized distilled water were separately kept in control tubes, respectively. All tubes were incubated again

for 90 min at 37°C and observed for clot lysis. Finally, the differences in weight taken before and after clot lysis were expressed as percentage of clot lysis following the under beneath equation.

% of clot lysis = (wt. of released clot/clot wt.) × 100

W2 - W3 / W2 × 100

where W2 is the weight of clot after 45 min incubation (g) and W3 is the weight of lysed clot after 90 min incubation (g).

Membrane stabilizing activity

The methanolic extract of *T. elephantina* Roxb. was examined by using hypotonic solution induced and heat induced erythrocyte haemolysis method developed by Omale and Okafor (2008).

Collection of blood sample

For this study, 2 ml of venous blood was collected from each of the healthy male volunteers of Bangladesh aged between 20 and 23 years having no record of taking oral contraceptive or anticoagulant therapy and free from diseases (using a protocol approved by Institutional Ethics Committee). The collected RBCs were kept in a test tube with an anticoagulant EDTA under standard conditions (temperature 23 ± 2°C and relative humidity 55±10%).

Preparation of erythrocyte suspension

The collected blood containing EDTA was centrifuged for 10 min at 3000 g and being washed for three times with isotonic solution (0.9% NaCl). The volume of saline was measured and reconstituted as a 40% (v/v) suspension with isotonic buffer solution (pH 7.4) which contained 1 L distilled water: $NaH_2PO_4.2H_2O$, 0.26 g; Na_2HPO_4, 1.15 g; NaCl, 9 g (10 mM sodium phosphate buffer). Thus, the suspension finally collected was the stock erythrocyte (RBC) suspension.

Hypotonic solution-induced haemolysis

The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffer saline (pH 7.4) containing either the different concentrations of methanolic extract (1, 3, 5, 7 and 9 mg/ml) or acetyl salicylic acid (0.10 mg/ml). Acetyl salicylic acid was used as a reference standard. After the mixtures were subjected to incubation for 10 min at room temperature, followed by subsequent centrifugation for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm using UV spectrophotometer (Biswas et al., 2013). The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

% Inhibition of haemolysis = 100 × {(X_1- X_2) / X_1}

where X_1 is the optical density of hypotonic-buffered saline solution alone (control) and X_2 is the optical density of test sample in hypotonic solution.

Heat induced hemolysis

Aliquots (5 ml) of the isotonic buffer, containing different concentrations (1, 3, 5, 7 and 9 mg/ml) of extract of the plant were put into two duplicate sets of centrifuge tubes .The vehicle, in the exact amount, was added to another tube as control. Erythrocyte suspension (30 µL) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54°C for 20 min in a water bath. The other pair was maintained at 0-5°C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm using UV spectrometer (Biswas et al., 2013).

The percentage inhibition or acceleration of hemolysis in tests and was calculated using the following equation:

% Inhibition of haemolysis = 100 × {(X_2- X_1) / (X_3-X_1}

where X_1 is the test sample unheated, X_2 is the test sample heated and X_3 is the control sample heated.

Total phenolic content

Total phenolic content of *T. elephantina* Roxb. was determined with Folin-Ciocaltu reagent using gallic acid as standard. Gallic acid at concentrations of 6.25, 12.5, 25, 50 and 100 mg/ml and concentration of 2 mg/ml of plant extract were also prepared in ethanol.

Then 0.5 ml of extract solution, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of Na_2CO_3 (7.5% w/v) solution were mixed for analysis. The mixture was incubated for 20 min at room temperature and then the absorbance was measured at 760 nm by UV-spectrophotometer (UV-1800, Shimadzu, Japan). By using the standard curve (Raju et al., 2013) prepared from gallic acid solution with different concentration, the total phenols content of the sample was measured. The phenolic contents of the sample were expressed as mg of gallic acid equivalent (GAE)/g of the extract.

Statistical analysis

Data was expressed as mean ± standard error of mean (SEM).

RESULTS

The present study was an attempt to determine the thrombolytic, membrane stabilizing properties and total phenolic content of methanolic extract of *T. elephantina* Roxb. and the results have been circumscribed in Tables 1, 2 and 3, respectively.

Thrombolytic activity

As a part of searching drugs having capability to facilitate blood clot lysis from natural sources, the extractives of *T. elephantina* was examined for thrombolytic potential and the results are presented in Table 1. 100 µl SK, which was used as positive control (30,000 I.U.) exerted 40.13 ± 0.39% clot lysis activity. On the other hand, as negative control, distilled water showed a negligible percentage of lysis of clot (2.56 ± 0.44%). The plant extract at different concentrations such as 2, 4, 6, 8 and 10 mg/ml demonstrated mild to moderate clot lysis activity where in

Table 1. Effect of different concentrations of the methanolic extract of *Typha elephantina* and the controls on *in vitro* clot lysis.

Sample	Concentrations (mg/ml)	% of clot lysis
Sample 1	2	18.75 ± 0.94
Sample 2	4	33.33 ± 1.06
Sample 3	6	10.79 ± 1.19
Sample 4	8	8.23 ± 1.12
Sample 5	10	7.20 ± 0.41
SK	-	40.13± 0.39
Blank	-	2.56 ± 0.44

Data represented as Mean ± SEM (n=3); SK: Streptokinase; SEM: standard error of mean.

Table 2. Effect of methanolic extract of *Typha elephantina* in hypotonic solution induced haemolysis of erythrocyte membrane.

Treatment	Concentrations (mg/ml)	Optical density of samples in hypotonic solution	% Inhibition of haemolysis
Control	1	3.974± 0.011	-
Sample 1	1	3.075 ±0.055	22.62
Sample 2	3	3.135 ±0.038	21.11
Sample 3	5	3.169 ±0.025	20.25
Sample 4	7	3.216 ±0.014	19.07
Sample 5	9	3.347 ±0.11	15.77
ASA	0.10	1.032 ±1.00	74.01

Data presented as Mean±SEM (n=3); ASA: acetyl salisylic acid; SEM: standard error of mean.

Table 3. Effect of methanolic extract of *Typha elephantina* on heat induced hemolysis of erythrocyte membrane.

Treatment	Concentrations (mg/ml)	OD of sample		% of inhibition of haemolysis
		Heated	Unheated	
Control	1	2.032±0.020	-	-
Sample 1	1	0.868± 0.011	0.603±0.012	18.54
Sample 2	3	0.995± 0.010	0.643±0.024	25.34
Sample 3	5	1.183± 0.064	0.630±0.105	39.00
Sample 4	7	1.382± 0.172	0.741± 0.157	45.26
Sample 5	9	1.382± 0.172	0.741± 0.157	49.65
ASA	0.10	0.672±0.014	0.296± 0.016	75.81

n=3; OD: Optical density; SEM: standard error of mean. Data expressed as Mean ± SEM.

case of 4 mg/ml, the highest clot lysis effect, that is, 33.33 ± 1.06% was achieved. Here also, the % of clot lysis by this plant extract was found to be dose independent and Figure 1 shows the percentage of clot lysis for different concentrations of the methanolic extract, positive control and negative control.

Membrane stabilizing activity

The crude methanolic extract also assayed for membrane stabilizing activity and the results were summarized in Tables 2 and 3 for hypotonic and heat induced haemolysis conditions. In case of hypotonic induced haemolysis, the methanolic extract at all the doses (1, 3, 5, 7, and 9 mg/ml) protected the human erythrocyte membrane. On the other hand, during heat induced haemolysis, all the aforementioned concentrations showed 18.54, 25.34, 39.00, 45.26, and 49.65% inhibition of lysis of RBC membrane, respectively. This inhibition of haemolysis was found to be dose dependent, increasing with increased concentration of the extract and was comparable with that obtained for ASA. On both cases, ASA (0.1 mg/ml) was used as standard and thus
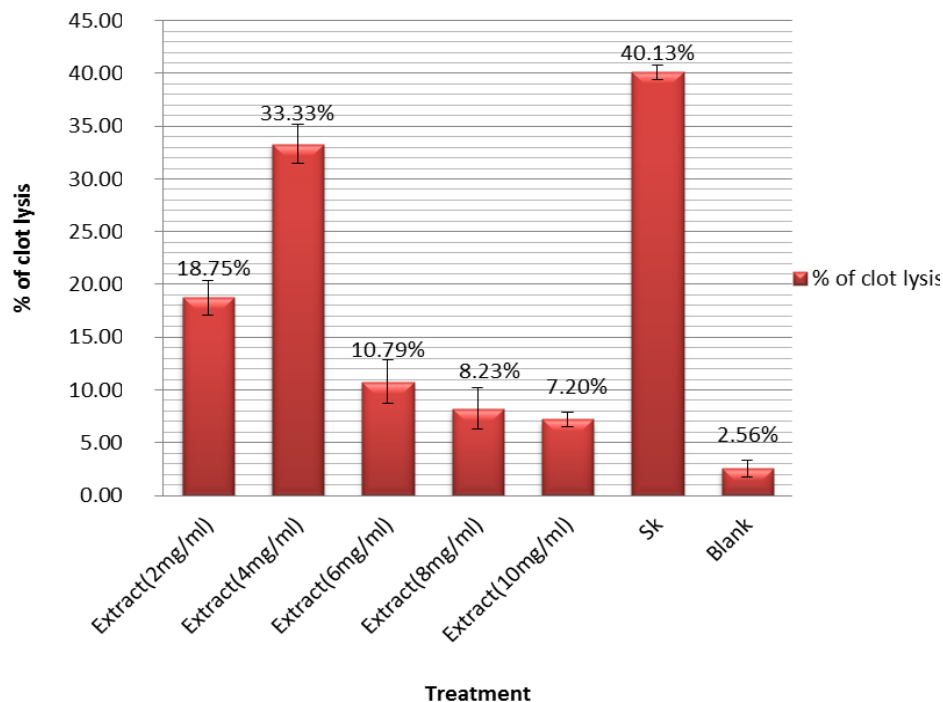
Figure 1. Thrombolytic activity of *Typha elephantina* at different concentrations.

Table 4. Total phenolic content determination of methanolic extract of *Typha elephantina* Roxb.

Sample	S/N	Absorbance of the sample	Absorbance	Total phenolic content (mg of GAE/g) of extract
Methanol extract	1	0.805	0.840±0.025	126.33±4.33
	2	0.827		
	3	0.889		

Data presented as Mean± SEM (n=3); SEM: Standard error of mean.

protected the RBC membrane by inhibiting lysis by 74.01 and 75.81%, respectively. Figure 2 exhibited the total phenomenon regarding membrane stabilizing activity in case of both hypnotic and heat induced haemolysis.

Total phenolic content

Table 4 shows the total phenolic content of the *T. elephantina* extract. The result is reported as gallic acid equivalents and it was 126.33 ± 4.33 mg of GAE/g of extract. Thus, the result manifested that plant extract has good antioxidant activity.

DISCUSSION

Various researchers have conducted several studies to find out the herbs and natural foods having thrombolytic effect and there is also evidence that, coronary events and stroke can be prevented by consuming such foods (Khan et al., 2010). The clots already formed in the blood can be dissolved by various thrombolytic agent; but these drugs are not free from adverse reactions and can be responsible for serious life threatening consequences (Mannan et al., 2011). An extensively used thrombolytic agent known as streptokinase plays seminal role in converting additional plasminogen to plasmin. But adverse complications such as bleeding and embolism are related with this agent and lead the researchers to conduct research work in order to discover novel sources of herbs and natural foods having thrombolytic effect with minimal side effects (Bhowmick et al., 2014). As a part of this research work, we tried to search out whether the methanolic extract of whole plant possesses clot lysis potentiality or not. The comparison study between positive and negative control clearly revealed that clot lysis did not take place when water was added to the clot.
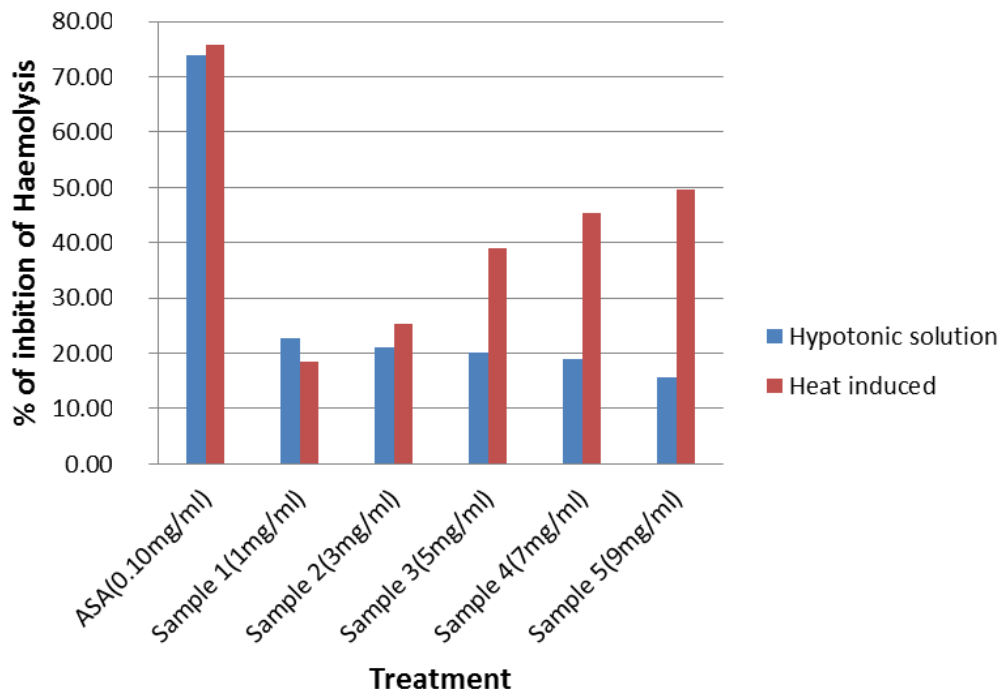
Figure 2. Effect of different concentration of *Typha elephantina* on hypnotic and heat induced haemolysis.

This prominent result encouraged us to compare five different concentrations of the test sample in same way with negative control and thus showed antithrombotic effect in a dose independent manner. It was also observed from the results that the sample in concentration 4 mg/ml exhibited the highest clot lysis activity (33.33±1.06%) among the five concentrations. In an investigation carried out by Umesh et al. (2014) on *T. angustifolia* L. leaves extract, another species of Typhaceae family also provide evidence about the presence of thrombolytic properties in this family species. Since phytochemical analysis revealed the presence of flavonoids, tannin, phenols, saponin, alkaloid in the crude extract of *T. elephantina* Roxb (Rahman et al., 2014) and it could be prognosticated that mainly tannin, saponin and alkaloid phytochemicals may confirm its clot lysis activity.

In this study, the membrane stabilizing activity of this plant at different concentrations was also investigated. Various lysozomal enzymes and hydrolytic components are excreted by phagocytes during inflammation period. These chemical agents account for damages to surrounding organelles and tissues (Dewan et al., 2013). As RBCs membranes are injured when exposed to various detrimental substances such as hypotonic medium, heat, etc., that is why hypotonic solution and heat induced haemolysis of erythrocyte membrane was chosen as an assessment for membrane stabilizing activity (Bhowmick et al., 2014). Earlier investigation has asserted that various herbal preparations rich in flavonoids and other phenolic compounds are capable of stabilizing the erythrocyte membrane and exert anti-inflammatory activity (Sadique et al., 1989). Their anti-inflammatory activities are namely due to their inhibitory effect on enzymes related to the production of the inflammatory mediators and metabolism of arachidonic acid (Metowogo et al., 2008). The results of this study displayed that, methanolic extract of *T. elephantina* Roxb. at different concentrations protected the RBC membrane against lysis induced by hypotonic solution and heat. As articulated earlier about the presence of phytochemicals such as flavonoids and other phenolic compounds in this plant, these phytochemicals may be liable for their membrane stabilizing activity by either preventing the release of phospholipase or by inhibiting cyclo-oxygenases which are denoted as the crucial catalytic elements in inflammatory pathway.

On the other hand, surplus production of reactive oxygen species play significant role to the development of hazardous tissue damage with variety of pathological process like ischaemia, inflammation, atherosclerosis and thrombosis (Diaz et al., 1997). The lack of balance between peroxidants and antioxidants play crucial role for the development of atherosclerosis (Khan et al., 1998). An investigation conducted in previous time has revealed that plant flavonoids instigate potent anti-inflammatory and anti-oxidant properties (Middleton et al., 1992). This paper also delivered us about the amount of total phenolic content as a part of the antioxidant test.

Conclusions

These findings conclude that, the methanolic extract of *T. elephantina* Roxb. have potentiality for thrombolytic and membrane stabilizing activities. The total phenolic content (126.33 ± 4.33 mg of GAE/g of extract) is also in close conformity to the presence of antioxidant property in this plant. Therefore, the plant demands further systemic, chemical and biological investigations to determine and isolate the active principles.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Sub-chronic toxicity of the leaf aqueous extract of *Bidenspilosa* Linn (Asteraceae) in male and female rats

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Bidens pilosa is used empirically for the treatment of various illnesses among which are: jaundice, conjunctivitis, cataract, malaria, ulcers and hypertension. This study is aimed at evaluating the sub-chronic toxicity of the leaf aqueous extracts of *B. pilosa*. The effects of the administration of the aqueous extracts of *B. pilosa* on physical (body weight, relative weight of organs), biochemical and histological parameters were studied in male and female albino Wistar rats. Daily doses of extract (100, 500 and 1000 mg/kg) and distilled water were administered orally for 4 weeks; during which signs of toxicity were checked. At the end of the experiments, the rats were sacrificed, their blood and organs collected for biochemical, histological and haematological analyses. No death was recorded. At the end of the treatment, there were no significant (P>0.05) variations of the body weight gain and the relative weights of detoxification organs (liver and kidney). The analysis of biochemical parameters showed a significant (p<0.001) variation of serum levels of ALT and creatinin. Histological analysis of liver and kidney, revealed no modification. The analysis of haematological parameters showed no significant difference between males and females, overall. However, when considering each sex in particular, it was observed that in males red blood cell distribution width-coefficient variation (RDWcv) significantly increased at the doses of 500 mg/kg (P=0.02) and 1000 mg/kg (P=0.04) (vs. control). Unlike in males, white blood cells (WBC) in females decreased significantly at 500 (P=0.01) and 1000 mg/kg (P=0.01) (vs. control). It can be concluded that *Bidenspilosa* has a relatively low toxic effects in both sexes, with some gender differences in the haematological results.

Key words: *Bidenspilosa*, Asteraceae, sub-chronic toxicity, rat.

INTRODUCTION

Bidens pilosa is an Asteraceae, a family of plants with high importance contributing to medicinal species worldwide. The plant is indigenous to the Amazon rainforest and other tropical areas of South America,

Africa, the Caribbean and the Philippines (Ezeonwumelu et al., 2011). It is empirically used, as a decoction, for the treatment of various illnesses: in Cameroon for example, it is used for the management of hypertension (Dimo et al., 1999), in Taiwan for anti-oedemic and anti-inflammatory activity (Duke, 1997). Difficult access to conventional drugs in low/middle income countries, like Cameroon, favours the use of medicinal plants for the management of cardiovascular diseases and oxidative stress and *B. pilosa* has been extensively studied for those properties (Dimo et al., 1999, 2002; Chiang et al., 2004). In addition, the plant is used for curing many other diseases in and out of Cameroon (Dimo et al., 2002; Ezeonwumelu et al., 2011).

There are some variations in the level of activity of the different species of Bidens, although the general properties appear similar (Brandao et al., 1997; Andrade-Neto et al., 2004). These variations can be accounted for by the fact that, *B. pilosa* leaf aqueous extract contains different active principles responsible for it effects, as demonstrated by some researchers (Brandao et al., 2004; Chiang et al., 2004; Ezeonwumelu et al., 2011). However, it is very important to evaluate the safety margin of the plant, although it is popularly used in Africa in its aqueous form; which is also the most widely studied from the literature review. Despite the pertinent toxicological studies by Cárdenas et al. (2006) with a single dose of 1000 mg/kg, Ezeonwumelu et al. (2011) whose work only involved males and the preliminary study of its acute toxicity by Longo et al. (2008), there is insufficient information on the toxicity of this aqueous extract, especially when comparing both sexes. Therefore, this study aimed to evaluate the sub-chronic toxicity and important haematological, histological and biochemical effects of aqueous extract of *B. pilosa* in male and female rats.

MATERIALS AND METHODS

Preparation of plant extract

The *B. pilosa* plant was harvested in the Centre region of Cameroon in July 2010, early in the morning. It was identified by a botanist from Cameroon National Herbarium and a voucher specimen number 65112/HNC of the plant was deposited. The leaves were dried under shade and ground into fine powder. Decoction method of extraction was used: 670 g of the powder was weighed into an empty clean beaker and 4.5 L of distilled water was added and boiled for 5 min. It was cooled at room temperature and filtered using first a clean cotton cloth and then Whatmann No. 4 filter paper plugged in a funnel. The filtrate was poured into clean dry silver dishes, placed in oven and dehydrated to dryness at 60°C. The yield was 16.7%.

Laboratory animal acquisition and handling

Wistar rats were bred at the Animal House in the High Teacher Training School of the University of Yaounde I. Forty healthy young adult Wistar rats (2.5-3.5 months old), both sexes in equal number, weighing between 120 and 160 g were obtained and housed two per cage for 1 week to allow for acclimation. The animals' cages were lined with saw dust, at room temperature with adequate ventilation, under a naturally illuminated environment with 12 h of light and 12 h darkness. They were fed with standard chow. They had access to clean drinking water *ad libitum*. The animal experiments were conducted according to the USA National Institute of Health Guide for the care and use of laboratory animals (NIH, 1996) and ethical guidelines for investigation of experimental pain in animals (Zimmerman, 1993).

Sub-chronic toxicity test

Organization for Economic Cooperation and Development (OECD) Test Guidelines (TG) that describe short-term repeated-dose toxicity testing: Repeated Dose 28-day Oral Toxicity Study in Rodents (TG407) was used for the study (OECD, 2006). The forty acclimated healthy Wistar rats, the two sexes being in equal number, were weighed and grouped randomly into eight groups (n = 5) as follows: A low dose (100 mg/kg), a medium dose (500 mg/kg) and a high dose (1000 mg/kg, both sexes); the control group comprised of five rats of either sex, to which distilled water (10 mL/kg) was administered. They were given food thirty minutes after administration of the extract by gavage. Body weights of the animals were taken daily for 28 days before administration of the extracts. The end-of-week weights were used to plot the curve of the body weight variation. The rats were observed daily to detect differences in appearance, discoloured fur, diarrhoea, bloody stool and constipation, loss of appetite and thirst and lack of interest in the environment. After 28 days, all animals survived and were allowed to fast overnight before the sacrifice. To minimize pain and stress in animals, they were anaesthetized with pentobarbital (50 mg/kg, IP). About 3 mL of blood was first collected with the aid of a syringe for full blood count; this was followed by cervical dislocation allowing optimal collection of the rest of blood for biochemical parameters analysis. For the latter, blood samples were collected from the animal into non-heparinized vacutainers. Organs were removed, weighed and absolute and relative organ weights determined, and then organs were preserved separately for each animal in 10% neutral buffered formalin to prevent tissue autolysis and kept for histological analysis. Relative organ weight was determined using the following formula:

Relative organ weight = [Absolute organ weight (g) / Body weight of rat on sacrifice day (g)] x 100

Biochemical analysis

After the collection of about 3 mL of blood to be used for blood parameter analysis, the rest blood collected into non-heparinized tubes was allowed to clot for 30 min and then centrifuged at 3000 rpm for 10 min. The serum was separated and analysed, using the URIT- 810 Chemistry Analyzer for HDL-cholesterol, total cholesterol, triglycerides and proteins and some enzymes: alanine aminotransferase (ALT, MAK052 Sigma ALT Activity Assay, Germany), aspartate aminotransferase (AST, MAK055 Sigma AST Activity Assay Kit, Germany) and creatinine (MAK080 Sigma Creatinine Assay Kit, Germany) The readings were done at wavelength 450 nm and manufacturer's instructions were strictly followed.

Histological study

Preserved tissues were histologically analysed at the laboratory of Animal Physiology of the University of Yaounde I. Tissues (liver and

kidney) were processed with microtome (ErnstLeitzWetzlar GMBH 530497 No. 537, Germany) and automated tissue processor (USA), embedded, placed on a slide and stained with Haematoxylin and Eosin stain (HE) (Ganter and Jolles, 1970). The tissues were observed under light microscope for changes in structure and the pictures taken with digital camera (Kodak, USA) attached to the eyepiece of the light microscope.

Haematological analysis

With the aid of a syringe, blood (about 3 mL) was collected from both sexes of animal in a 5 mL heparinised and properly labelled tubes, then shaken gently to allow it homogenize. A blood count was ran using the Full Blood Count machine, URIT 3300 following the manufacturer's instruction, to automatically get the following indices: red blood cell distribution width-coefficient variation (RDWcv), haemoglobin (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), haematocrit (HCT), red blood cell (RBC) and platelet (PLT).

Statistical analysis

Data are expressed as mean values±standard error of mean (SEM) and group data comparisons of test and control means were evaluated by ANOVA test while comparison between sexes was done by the paired T-test using SPSS version 20.0. Significance was set at $p < 0.05$.

RESULTS

No death or considerable change of behaviour was noted in treated rats as compared to the control. Increases in bodyweights of rats were observed within each group of rats from days zero to 28. However, no significant variation of the mean % body weight gain was observed when comparing males and females, or comparatively with their controls (Figure 1).

Likewise, in either sex, no significant variation of the relative weight of liver and kidney was observed after 4 weeks of treatment. In males, the values for liver were 1.9, 1.9, 2.1 and 2.3% while in females, they were 2.4, 1.9, 2.0 and 1.6% in control, 100, 500 and 1000 mg/kg, respectively (P>0.05). With regards to the kidney, the relative weights were 2.1, 2.9, 2.1 and 2.8% in males while in females, they were 2.0, 2.5, 2.2 and 2.4% in the control, 100, 500 and 1000 mg/kg, respectively (P>0.05).

An increase of the level of HDL-cholesterol was noted in both sexes of rats, as compared to their respective controls. In males, the HDL- cholesterol levels were 32.04 ± 2.97, 21.16 ± 3.75, 22.62 ± 1.08 mg/dL, respectively. This corresponded to increases of 129.67, 51.68 and 62.15% for doses of 100, 500 and 1000 mg/kg, as compared to the control (14.01 ± 2.29 mg/dL). This increases were significant at the doses of 100 (*p<0.01*) and 1000 mg/kg (*p<0.001*). In females, changes observed in the levels of HDL-cholesterol were from 15.26 ± 1.64 to 23.53 ± 2.34, 20.26 ± 0.93 and 22.40 ± 2.39 mg/dL, corresponding to significant increases

($p < 0.05$) of 54.19, 32.76 and 46.78% for the doses of 100, 500 and 1000 mg/kg respectively (Figure 2).

Counterwise, as shown in Figure 3, there was a decrease of the level of ALT in both sexes of rat, as compared to their respective controls. In males, the ALT levels were 3.23 ± 2.22, 3.88 ± 0.64 and 3.88 ± 0.64 IU/L, respectively. This corresponded to decreases of 40.51, 28.54 and 28.54% for doses 100, 500 and 1000 mg/kg, as compared to the control (5.43 ± 0.66 IU/L). The decrease was significant only at the dose of 100 mg/kg ($p < 0.001$). In females, changes observed in the levels of ALT were from 4.65 ± 0.74 to 3.23 ± 2.22, 3.23 ± 2.22 and 3.88 ± 0.64 IU/L, corresponding to decreases of 30.53, 30.53 and 16.55% for the doses of 100, 500 and 1000 mg/kg, respectively. These decreases were significant at the doses of 100 ($p < 0.05$) and 500 mg/kg ($p < 0.001$).

Administration of *B. pilosa* extract for four weeks caused a decrease of the level of AST, at the lowest doses, in both sexes of rat, as compared to their respective controls. In males, AST levels were 2.52 ± 0.28, 4.65 ± 0.67, 4.71 ± 0.30 IU/L, respectively. This corresponded to a significant decrease of 34.88% ($p < 0.001$), and non-significant increase of 20.15 and 21.70% for doses 100, 500 and 1000 mg/kg, as compared to the control (3.87 ± 0.46 IU/L). In females, non-significant decreases of 30.53, 30.53 and increase of 16.55% were found in the levels of AST after treatment with the doses of 100, 500 and 1000 mg/kg, respectively (Figure 4).

As concerns proteins, from a value of 4.75 ± 0.2 mg/mL, protein levels increased to 5.08 ± 0.15, 5.66 ± 0.15, 5.29 ± 0.48 mg/mL, in males. These corresponded to increases of 6.94, 19.15 and 11.36% for the doses 100, 500 and 1000 mg/kg of *B. pilosa* extract. The increase at the dose of 500 mg/kg was significant ($p < 0.05$). In females in the contrary, no significant variation was observed. Increases were 4.20, 20.26 and 0.38% at the above doses, respectively (Table 1).

Creatinine levels decreased by 15.55 ($p < 0.05$), 18.88 ($p < 0.05$) and 3.61% ($p > 0.05$) in males while in females significant ($p < 0.01$) decreases of 20.32, 20.32 and 25.47% were observed at the respective doses of 100, 500 and 1000 mg/kg (Table 1).

After four weeks of treatment with the plant extract (100, 500 and 1000 mg/kg), no injury or inflammation of the liver or the kidney was observed. The figures 5 and 6 display the cross sectional areas of the kidney and liver, respectively.

As shown in Table 2, the paired sample T-test analysis of haematological parameters did not reveal any significant difference between males and females, overall. However, for RDWcv in males, there was a significant increase at the doses of 500 (P=0.02) and 1000 mg/kg (P=0.04) (vs. control). Unlike in males, WBC in females decreased significantly at 500 (P=0.01) and 1000 mg/kg (P=0.01) (vs. control).
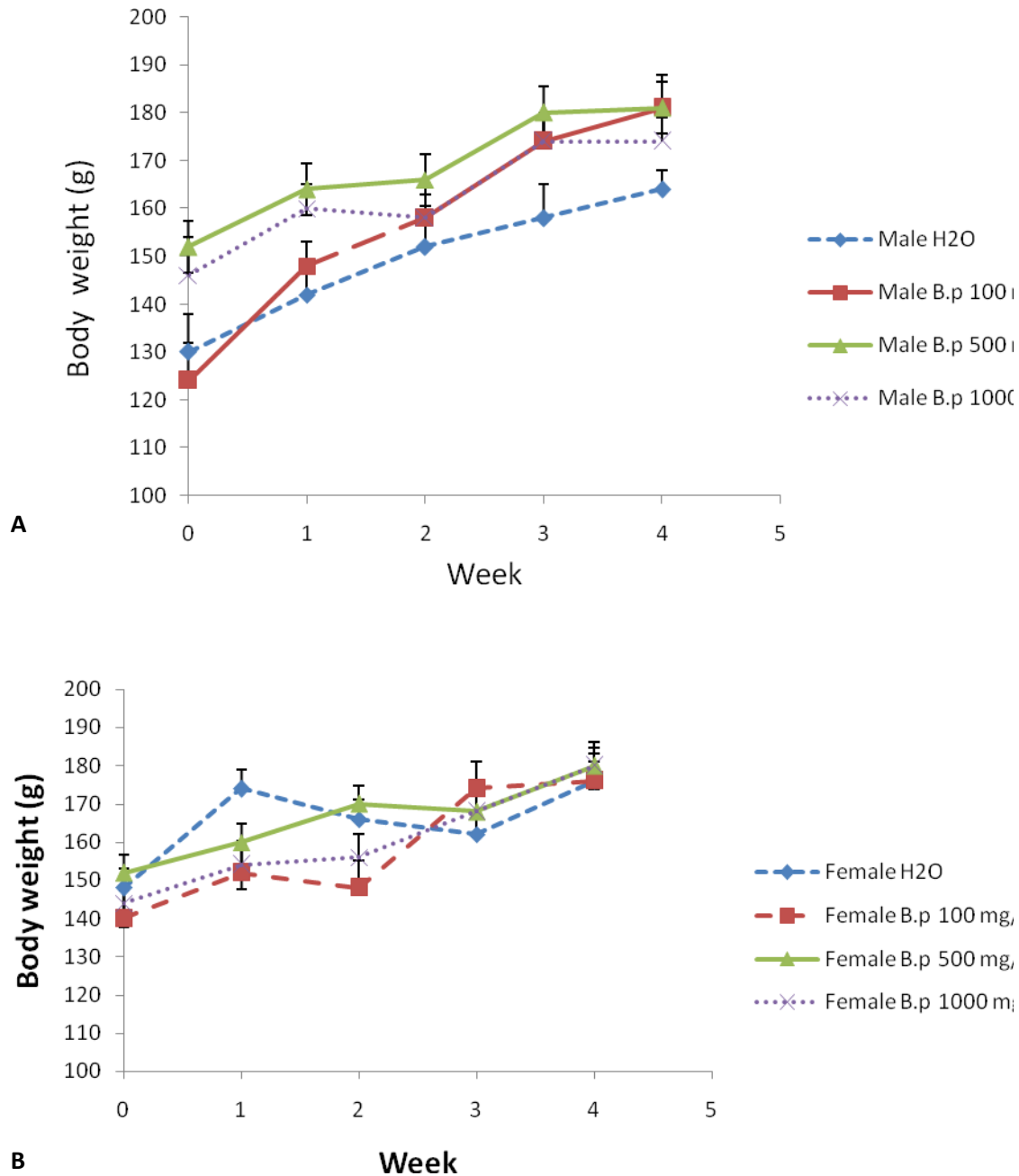
Figure 1. Variation of body weight during sub-chronic treatment with *B. pilosa*. A: males, B: females, each point represents the mean ± SEM, n = 5.

DISCUSSION

The toxicity study of *B. pilosa* did not elicit any mortality throughout the duration of the experiment, thus showing relatively high safety index in rats. Increases in body weights of rats were observed within each group of rats from days 0 to 28. The observed increases in body weight could be attributed to the nutritive components in their chow, as they were well fed and surely assimilated well their food. Since no significant difference was observed between controls (receiving H_2O) and test animals (receiving *B. pilosa*), the increase in body weight cannot be attributed to the components in the plant as revealed by Duke (1997). However, there was a general reduction in mean percentage body weight gain of rats administered with *B. pilosa* extract as compared to controls. Analyses of various species of *Bidens* have been conducted in several countries. Although, there is
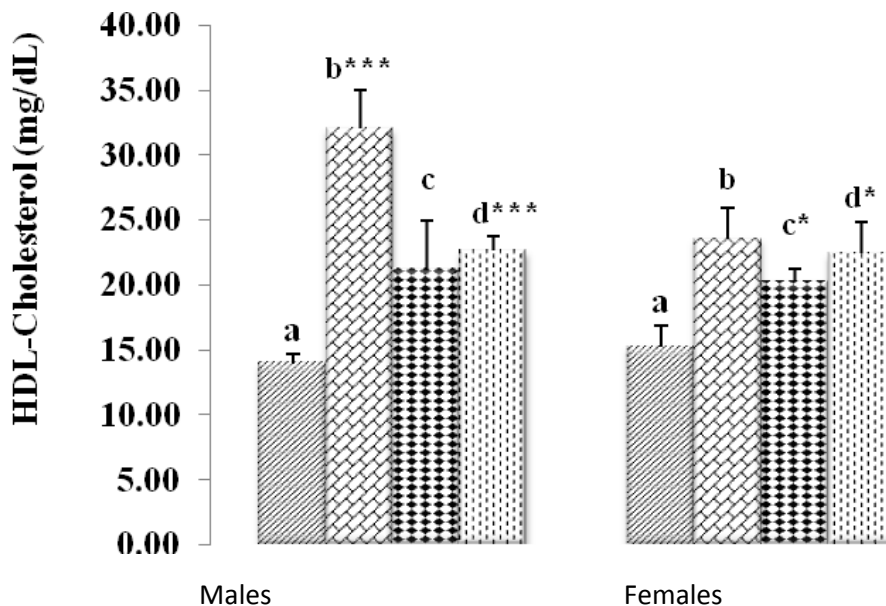
Figure 2. Effects of aqueous extract of *B. pilosa* leaf on the level of HDL-cholesterol after 4 weeks of treatment. Each bar represents the mean ± SEM, n = 5. *p<0.05, ***p<0.001, Significant difference with respect to control (distilled water). a, Distilled water; b, *B. pilosa* (100 mg/kg); c, *B. pilosa* (500 mg/kg); d, *B. pilosa* (1000 mg/kg)

Figure 3. Effects of aqueous extract of *B. pilosa* leaf on the levels of ALT after 4 weeks of treatment. Each bar represents the mean ± SEM, n = 5. ***p<0.001, Significant difference with respect to control (distilled water). a, distilled water; b, *B. pilosa* (100 mg/kg); c, *B. pilosa* (500 mg/kg); d, *B. pilosa* (1000 mg/kg)
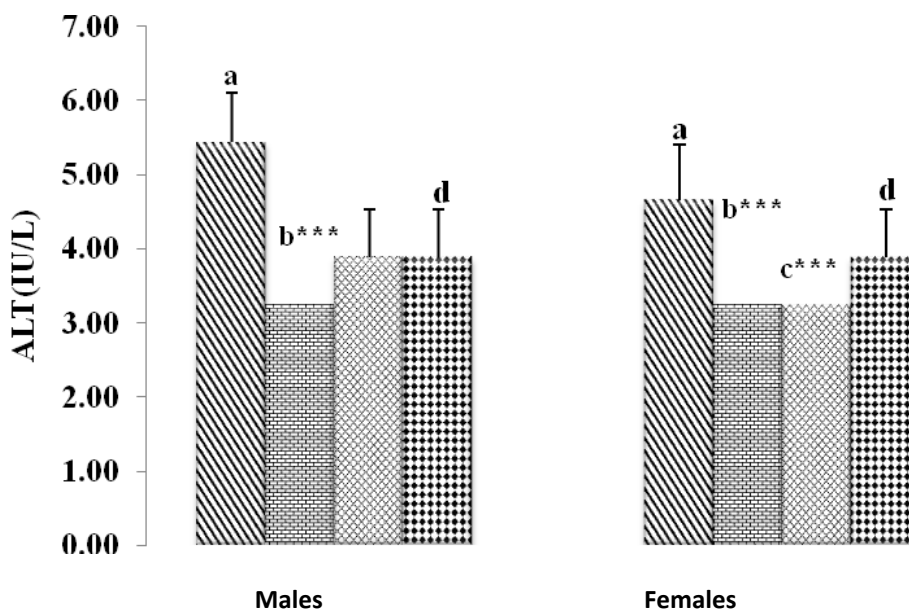
some variation in the level of activity of the different species of *Bidens* probably due to different levels of active constituents, the general property appears similar (Andrade-Neto et al., 2004). These phytochemical components have been found to be responsible for the various medicinal activities of *B. pilosa* including
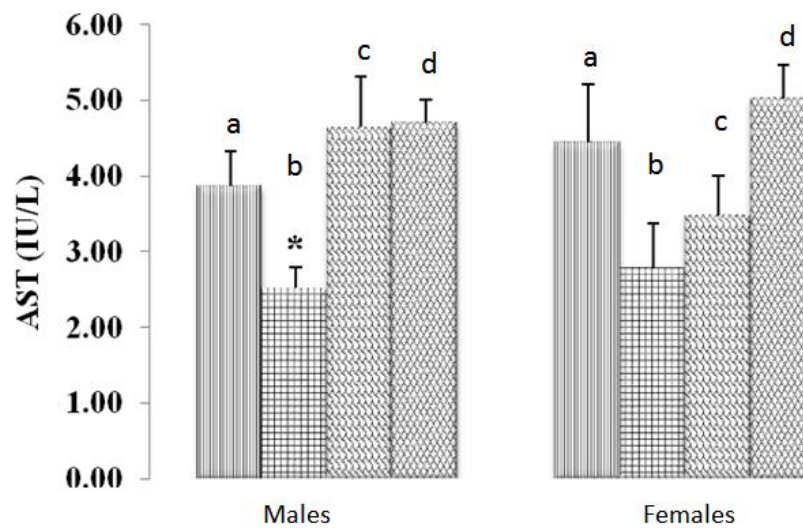
Figure 4. Effects of aqueous extract of *B. pilosa* leaf on the level of AST after 4 weeks of treatment. Each bar represents the mean ± SEM, n = 5. *p<0.05, Significant difference with respect to control (distilled water). a, distilled water; b, *B. pilosa* (100 mg/kg); c, *B. pilosa* (500 mg/kg); d, *B. pilosa* (1000 mg/kg).
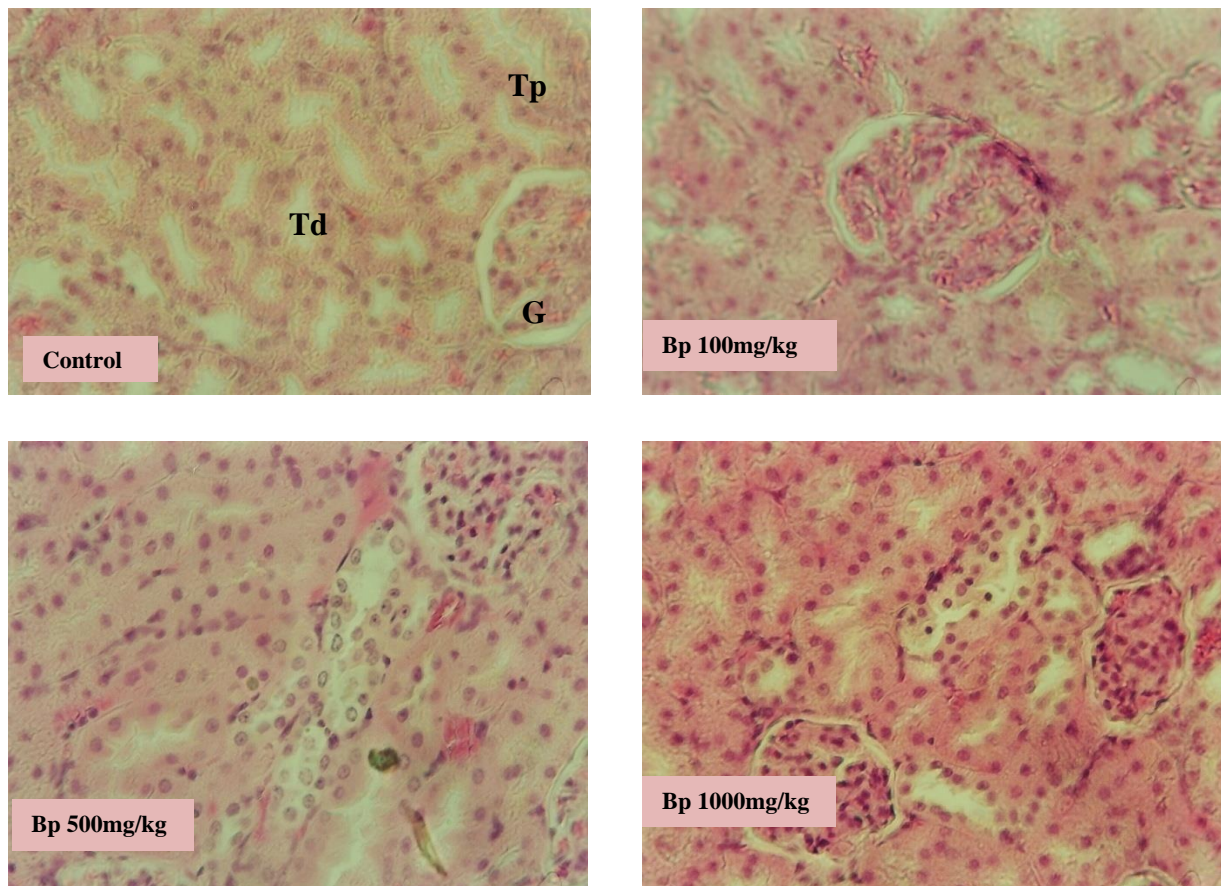

Figure 5. Light micrographs of kidney histological sections (HE x 400). G: glomerule; DCT: distal convoluted tube; PCT: proximal convoluted tube; Bp: *Bidenspilosa* extract.

Table 1. Effects of *B. pilosa* on serum proteins and creatinine in male and female rats.

Dose of *B. pilosa* extract	100 mg/kg	500 mg/kg	1000 mg/kg	100 mg/kg	500 mg/kg	1000 mg/kg
Effect	Increase in serum proteins			Decrease in creatinine		
Males	6.94% (p>0.05)	19.15% (p<0.05)	11.36% (p>0.05)	15.55% (p<0.05)	18.88% (p<0.05)	3.61% (p>0.05)
Females	4.20% (p>0.05)	20.26% (p>0.05)	0.38% (p>0.05)	20.32%(p<0.01)	20.32%(p<0.01)	25.47%(p<0.01)

P<0.05, P<0.01 significant difference between tests and control groups

Table 2. Haematological parameters.

Blood parameter	*B. pilosa* (500 mg/kg)		*B. pilosa* (1000 mg/kg)		Control (distilled water)	
	f	m	f	m	f	m
	AV (SD)	AV (SD)	AV (SD)	AV (SD)	AV (SD)	AV (SD)
Platelet count(x10^9/L)	201.50 (79.90)	204.50 (40.31)	21.67(8.39)	304 (141.42)	80 (1.41)	177.50 (103.9)
Haemoglobin (g/dL)	14.45 (0.63)	15.70 (1.84)	15.37(1.35)	11.65(4.45)	14.10 (0.1)	11.95 (1.77)
Red Blood Cells (x10^{12}/L)	7.39 (0.03)	8.55 (.93)	7.87 (0.80)	5.89 (2.23)	7.27 (0.07)	5.81 (0.23)
White blood cells (x10^3/μL)	9.45 (1.63)*	8.80 (0.14)	9.90 (0.44)*	8.20 (3.68)	14.05 (0.07)	8.80 (0.04)
Haematocrit (%)	44.85(2.33)	48.90 (4.81)	46.67 (4.10)	36.20 (14.00)	47.7 (0.42)	37.50 (5.80)
Mean cell volume (fL)	60.70 (3.39)	59.85 (3.04)	63.23 (0.61)	61.45 (0.49)	65.85 (0.21)	62.25 (4.03)
Mean cell Hb concentration (g/dL)	32.20 (0.28)	32.6 (0.57)	30.90 (1.80)	32.20 (0.14)	29.15 (0.21)	31.50 (0.71)
Mean cell Haemoglobin (pg)	19.50 (0.99)	19.10 (0.28)	19.50 (1.23)	19.75 (0.07)	19.35 (0.21)	19.20 (0.28)
(RDWcv) (%)	15.25 (0.64)	15.65 (0.64)*	14.7 (1.56)	15.1 (0.14)*	15.35 (0.07)	13.56 (0.08)

*P<0.05 significant difference between tests and control groups, f = female, m=male, AV (SD) =average (standard deviation), red blood cell distribution width coefficient variation = RDWcv

antimalarial activity due to the presence of acetylene and flavonoids (Brandao et al., 2004), chemo protective activities of ethyl acetate and butanolic fractions (Chiang et al., 2004; Suzigan et al., 2009), anticancer properties by photoactivated polyacetylenes (Hou et al., 1989; Geissberger and Sequin, 1991; Sundararajan et al., 2006), anti-inflammatory properties (Pereira et al., 1999) among others.

The mean relative organ weights of rats did not vary significantly in either sex. Thus, the plant extract had no observable influence on those relative organ weights of the liver and kidney. These data demonstrate that, it might not be proper to conclude that *B. pilosa*, at the used doses, is capable within four weeks to affect either the detoxifying organs' weights or the body weight. The increase of body weight observed in all animal groups after four weeks can be explained by the normal growth process (Otimenyin et al., 2010), as the animals were still relatively young at the start of the experiment. These results are globally similar to those of Lakmichi et al. (2010) while studying the toxicological profile of hydro ethanolic extract of *Corrigiola telephiifolia* (Corryophyllaceae) in rat. Assuming that, a decrease of the relative weights of detoxifying organs is a marker of the harmful effect of a toxicant (Witthawaskul et al., 2003), this results demonstrate that, aqueous extract of the leaves of *B. pilosa* shall have very low toxic effect or

none. This is in line with the findings from the study of acute toxicity of the plant by Longo et al. (2008).

Plasma protein level increased significantly only in males and at the dose of 500 mg/kg. Creatinine levels generally significantly decreased in both sexes. Creatinine is a biomarker of the kidney function (Mbaka et al., 2010). Injury of the kidney could have caused an increase of the level of creatinine. While the increase of protein level at the single dose of 500 mg/kg and decrease of creatinine levels at several doses (100-1000 mg/kg) are difficult to explain, one can at least say without risk of mistake that, the plant extract did not affect the renal function. This was confirmed by the histological architecture of kidney that did not change after treatment with *B. pilosa* extract.

Likewise, the aqueous extract of *B. pilosa* seems to have a protective effect on the liver. In effect, histological analysis of the liver also did not show any noticeable modification of the architecture of the liver of treated rats as compared to the controls. Treatment with aqueous extract of *B. pilosa* extract revealed significant decreases in the levels of ALT (at 100 mg/kg in males, 100 and 500 mg/kg in females), when compared with the control. The level of AST decreased significantly when animals (males) were treated with 100 mg/kg *of B. pilosa*; proving that only lowest doses of extract caused a significant effect on the liver. The two most important transaminases
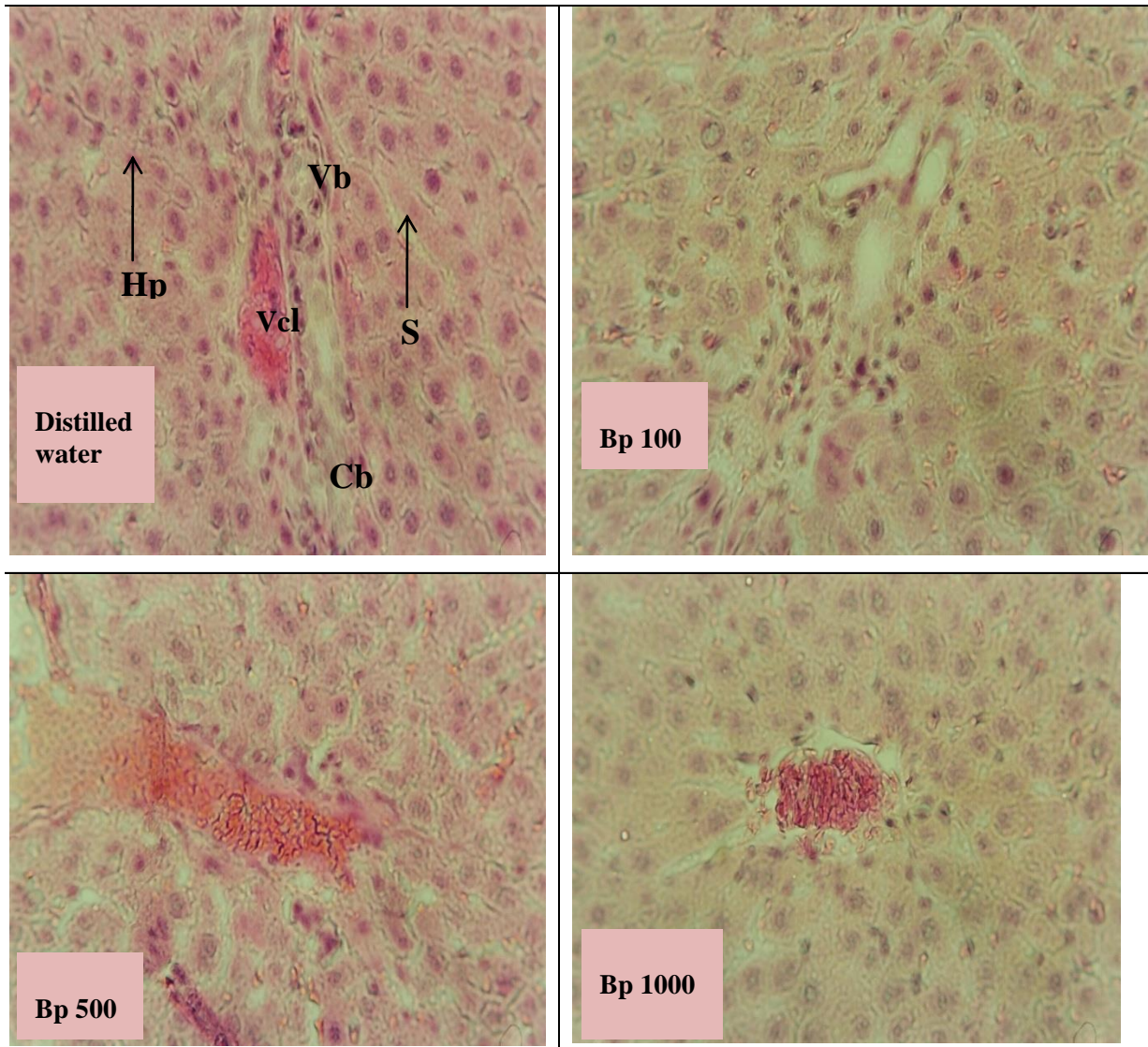
Figure 6. Micro section of Liver from control and treated rats (HEX40). Cb: Bile duct ; Vb: bile vessel ; Vcl: centro-lobularvein; Hp: hepatocyte, S: sinusoid. No difference was observed between the control and treated rats

are aspartate amino transferase (AST) and alanine amino transferase (ALT). They are present in high concentrations in liver and muscles. An increase of ALT level is a strong indicator of liver disease (Hartwell and Schwartz, 2009). These results reinforce the idea that, *B. pilosa* might have hepatoprotective activity. Such activity of the plant was actually demonstrated by other researchers (Suzigan et al., 2009; Li-Ping et al., 2008; Chin et al., 1996), meanwhile, they opposed to the findings of Ezeonwumelu et al. (2011). But according to the latter, there is a likelihood that, reversible damages might be caused by short term administration of *B. nitens* aqueous extract.

Lipid profiles were also evaluated during this study. Prevention of coronary diseases requires low triglycerides,

LDL- cholesterol, associated with high HDL-cholesterol (Cerisier et al., 2004). As compared to the controls, it was found that, 28-day treatment of rats with the extract induced significant increase of HDL-cholesterol. At the dose of 100 and 1000 (in males) and 500 and 1000 mg/kg (in females), significant increases were recorded. These results are similar to those of Adewole and Ojewole (2008) who reported on the protective effect of *Annona muricata* on the lipid profile and the oxidative stress of hepatocytes in streptozotocine diabetic rats.

Males displayed significant increases of their RDW_cv in test groups as compared to control, but their mean corpuscular volume remained similar with control. Processes which are known to cause elevation of the RDW in the face of normocytic or microcytic indices

include iron deficiency, red blood cell fragmentation, certain hemoglobinopathies, myelofibrosis and sidero-blasticanemia; of which the most frequent is iron deficiency (Morgan and Peck, 1988). Elevated RDW aids differentiating between uncomplicated iron deficiency anemia (elevated RDW, normal to low MCV). Elevated RDW and normal MCV can be associated with any of the following conditions: 1)-Early iron, vitamin B12, or folate deficiency; 2)-Dimorphic anemia (for example, iron and folate deficiency); 3)-Sickle cell disease; 4)-Chronic liver disease or 5)-Myelodysplastic syndrome (Mates, 2004).

A significant decrease of white blood cells (WBC) was observed exclusively in females. WBC is naturally involved in the defence mechanisms of the body. Therefore, females could be considered more vulnerable to the plant extract, with regards to their immune capacity.

Conclusion

It can be concluded from this study that, the aqueous extract of the leaves of *B. pilosa* has a relatively good margin of safety in both sexes, but haematological indices like RDW or WBC were differently affected in males and females. It will be worth carrying out, an important study on this plant to determine its long term effect in animals.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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