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Full Length Research Paper

Traditional Tongan treatments for infections: Bioassays and ethnobotanical leads for activity

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An ethnobotanical survey of Tongan pharmacopoeia, conducted through semi-structured interviews with healers, informed the selection of twenty-six plants for antimicrobial bioassays. The parts of the plants recommended by the healers were collected and extractions were made with methanol and hexane in various concentrations, and screened against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* using microbial inhibition assay. Further, minimum inhibitory concentrations (MICs) of *Syzygium corynocarpum* (A.Gray) Müll. Stuttg. were determined against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) using extractions from both the healer-recommended plant part—young leaves—and two other plant parts—mature leaves and bark—to test the hypothesis of greater antimicrobial activity in the traditionally used part. Cytotoxicity of the extractions was determined by trypan blue assay on human lymphocytes. The microbial inhibition assays yielded six species that inhibited the growth of *S. aureus*, *E. coli* and/or *C. albicans* at rates of 40% or above compared to the controls. The only species that inhibited all three microbes at above 40% was *S. corynocarpum*. Subsequent testing on *S. corynocarpum* revealed that both the methanol and hexane extractions of young leaves inhibited *S. aureus* (MIC 125 and 500 µg/ml respectively) and MRSA (MIC 250 and 500 µg/ml respectively), while only the methanol extract of the bark showed inhibition against both microbes (MIC = 250 µg/ml), and the extractions from the mature leaves showed no activity at the concentrations tested. An additional assay with extractions of *S. corynocarpum* on human lymphocytes suggests no cytotoxicity compared to control cells. These results support the traditional use of several of the tested plants. In particular *S. corynocarpum*, which has not previously been studied for antimicrobial activity, showed greater activity by the traditionally used part, emphasizing the importance of documenting specific plant parts used during ethnobotanical interviews.

Key words: Ethnobotany, antimicrobial, Tonga, *Syzygium corynocarpum*, minimum inhibitory concentration (MIC).

INTRODUCTION

Historically, the Kingdom of Tonga in the South Pacific Ocean has been the geographical crossroads of

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Polynesian trade, a cultural history that lends itself to advancing and perpetuating diverse ethnobotanical knowledge (George, 1989). While the ethnopharmacopoeia of Tonga has been recorded to some extent (Bloomfield, 1986; Croft and Tu'ipulotu, 1980; George, 1989; Weiner, 1971; Whistler, 1991a, b; World Health Organization (WHO), 1998; Yuncker, 1959), to our knowledge this study represents the only publication to date that discusses plants used by Tongans specifically to treat infections and provides experimental evidence of the antimicrobial properties. Phytochemicals have been a valuable source of antimicrobial compounds (Cragg et al., 1999; Hong-Wei, 2011). The misuse and overuse of antimicrobial drugs over the past 60 years has led to the emergence of resistant microbial strains that are increasingly difficult to treat (Center for Disease Control (CDC), 2013; Levy, 2002) and has prompted investigations that seek to discover new ways to combat infectious microbes.

In this study, we report on the traditional uses of 26 species of plants used by Tongan healers to treat skin and mouth infections and the results from bioassays of microbial growth-inhibiting properties of the healer-recommended parts of each of these plants. While several plants showed positive antimicrobial activity, *Syzygium corynocarpum* (A.Gray) Müll. Stuttg. showed the most activity and is a plant species for which no previous study of antimicrobial activity has been published. Therefore *S. corynocarpum* was selected for further screening to determine minimum inhibitory concentrations (MICs) against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA). Because previous research notes different phytochemistry and different bioactivity of different plant parts (Chavan and Gaikwad, 2013), in this further screening, extracts from various plant parts of *S. corynocarpum* were assayed to test the hypothesis of greater antimicrobial activity in the traditionally used part. Cytotoxicity of the extracts of *S. corynocarpum* was also evaluated.

METHODOLOGY

Ethnobotanical interviews

Ethnobotanical interviews were conducted during the summer of 2002 on the Tongatapu, Vava'u and Ha'apai island groups. With pre-informed consent and according to Institutional Review Board (IRB) standards, twenty-nine healers were questioned in semi-structured interviews in the Tongan language regarding plant selection and plant preparation techniques used to treat various skin and mouth infections. Healers were mostly females from 20 to 90 years of age, with the age range distributed fairly evenly with eight healers aged 20 to 40, ten healers between 40 and 60 years of age, and 11 healers at least 60 years old or older.

Plant materials

Plant collection in 2002 for percent inhibition bioassays (conducted in 2003) was based on the specific part used by informants (for example, young leaves, leaves, bark, fruit). Samples of plant parts for each referenced species were preserved in 100% ethanol. The local Tongans, as well as botanical publications (Whistler, 1991b, 1992a; WHO, 1998) aided Ryan Huish in plant identification. Voucher herbarium specimens were collected and filed in the Stanley L. Welch Herbarium (BRY) at Brigham Young University, (see Table 1). In the summer of 2011, an additional three samples of *S. corynocarpum* (young leaves, mature leaves, and bark) were collected from Tongatapu, Tonga, for minimum inhibitory concentrations (MICs) and cytotoxicity assays (conducted in 2012). The plant was identified by Ryan Huish and the associated voucher specimens are stored in the Hollins Herbarium (vouchers RH193, RH194, RH195). The accepted Latin names of all plants included in this study were verified in July, 2014 (The Plant List, 2013), and the family assignments followed the Angiosperm Phylogeny Group III system (APGIII) (Stevens, 2014).

Plant extractions

Both methanol (organic polar) and hexane (organic nonpolar) extracts were taken from each of the collected plant species using methods as described in Cates et al. (2013). Five grams of plant tissue were ground in liquid nitrogen and then extracted three times by grinding in 33 ml 50/50 methanol/methylene chloride. Extracts were then filtered through cheesecloth and fast grade qualitative filter paper (VWR, grade 415). Roto-evaporation at temperatures below 45°C removed the solvents. The exudate was then dissolved in methanol (10 ml), poured into a 30 ml separatory funnel and washed twice with 7 ml of hexane. The methanol and hexane fractions were then separated, air dried in three-dram vials, and based on the dry extract weight, made into a final concentration of 8 mg/ml using dimethyl sulfoxide (DMSO), and then stored in a -80°C freezer. Following the initial microbial inhibition assay, the 2011 samples of *S. corynocarpum* were extracted for further testing following methods adapted from Shrestha et al. (2014). Five grams of each plant tissue (young leaves, mature leaves and bark) were ground separately in liquid nitrogen and then extracted sequentially with hexanes and methanol. Sample extracts were dried using nitrogen gas to reduce oxidation. Finally, the extracts were dissolved in 100% DMSO to a final concentration of 8 mg/ml and stored at -20°C.

Microbial cultures

Microorganisms used in the microbial inhibition assay were provided by Dr. Rex G. Cates, Brigham Young University, Provo, UT. The bacterial cultures *S. aureus* (ATCC 12600) and *E. coli* (ATCC 25922) were grown on tryptic soy agar (TSA) then prepared and maintained in tryptic soy broth (TSB). *Candida albicans* (ATCC 90028) cultures were grown on sabouraud dextrose agar (SDA) and maintained in sabouraud dextrose broth (SDB). For the subsequent testing of *S. corynocarpum* through microwell broth dilution assay, *S. aureus* and Methicillin-resistant *S. aureus* COL (MRSA) were obtained and cultured in Mueller Hinton broth. The culture of MRSA was provided by Dr. Bryan Wilkinson, University of Illinois, IL.

Percent inhibition assay

Microbial percent inhibition assay was performed using methods as found in Cates et al. (2013) using flat-bottomed 96-well microtitre plates (Becton Dickinson, Franklin Lakes, NJ). Stock solutions of the extracts were first pipetted into the 96-well plates in triplicate. Extracts were buffered by adding 25 µl of 10 mM phosphate buffered saline (PBS, pH 7.2) for *S. aureus* and *E. coli* and 10 mM

Table 1. Tongan medicinal plants used to treat infections with frequency of reference by informants.

Scientific name	Family	Tongan name	V#[a]	Part[b]	Freq[c]	Traditional use
Achyranthes aspera L.	Amaranthaceae	Tamatama	52	L	8	Thrush, wounds, ringworm
Aleurites moluccanus (L.) Willd.	Euphorbiaceae	Tuitui	87	B	2	Mouth infection
				N	1	Boils
Allophylus cobbe (L.) Raeusch.	Sapindaceae	Tava	78	YL	2	Skin infection
				B	3	Skin infection, boils
Casuarina equisetifolia L.	Casuarinaceae	Toa	98	B	1	Mouth infection, induce vomiting
Centella asiatica (L.) Urb.	Apiaceae	Tono	69	L	4	Rash, boils
Cestrum nocturnum L.	Solanaceae	Laukaupo'uli	53	L	5	Wounds
Curcuma longa L.	Zingiberaceae	Enga	99	R	2	Skin infection
Euodia hortensis Forst.	Rutaceae	Uhi	50	L	5	Mouth infection, fungal rash, boils
Glochidion ramiflorum J.R. Forst. & G. Forst.	Phyllanthaceae	Malolo/Masikoka	95	L	10	Mouth infection, wounds, boils
Hibiscus rosa-sinensis L.	Malvaceae	Kaute-tonga	68	L	1	Ringworm
Hoya australis R. Br. ex Traill	Apocynaceae	Laumatolu	56	L	1	Skin infection
Jatropha curcas L.	Euphorbiaceae	Fiki	81	L	6	Wounds, bleeding
Macropiper puberulum Benth.	Piperaceae	Kavakava'ulie	94	L	5	Skin infection
Morinda citrifolia L.	Rubiaceae	Nonu	90	L	11	General infection
				B	2	Sore throat with fever, cough, boils
				YF	6	Sores on tongue
Oxalis corniculata L.	Oxalidaceae	Kihikihi	88	L	2	Inflammation and gum swelling
Pandanus sp.	Pandanaceae	Fa	97	AR	3	Skin infection, rash
Phymatosorus scolopendria (Burm f.) Pichi-Serm.	Polypodiaceae	Laufale	96	L	4	Skin infection, fungal rash
Premna serratifolia L.	Lamiaceae	Volavalo	70	L	3	Rash
				B	1	Skin infection
Senna alata (L.) Roxb.	Fabaceae	Te'elango	51	L	9	Fungal infection
Solanum viride Forst f. ex Spreng.	Solanaceae	Polo Tonga	76	L	2	Infected wounds, rashes
Syzygium corynocarpum (A. Gray) C. Mueller	Myrtaceae	Hehea	89	YL	11	Mouth or skin infection
Syzygium malaccense (L.) Merr. & Perry	Myrtaceae	Fekika	93	L	6	Mouth infection
Tarenna sambucina (Forst f.) Durand ex Drake	Rubiaceae	Manonu	73	L	1	Sore throat
				B	1	Skin infection
Vigna marina (Burm.) Merr.	Fabaceae	Lautolu (Tahi)	57	L	4	Red mouth with loss of appetite, fungal infection
Vitex trifolia L.	Lamiaceae	Lalatahi (Lai lala)	55	L	5	Throat infection, itchy skin rash
Zingiber zerumbet (L.) Roscoe ex Smith	Zingiberaceae	Angoango	100	R	5	Thrush

[a]Parts: L, leaves; B, bark; N, nut; R, rhizome, YF, young fruit, AR, aerial root, YL, young leaves. [b]V#: "voucher specimen number from collector RH" (stored in Brigham Young University Herbarium). [c]Freq: frequency of reference by informants.

succinic acid buffer solution (SBS, pH 5.6) for *C. albicans*. After buffering, randomized control wells were assigned and then 175 µl of prepared inoculum was pipetted into each of the remaining 96 wells in randomized groupings. The final concentrations of the extracts in the 96 well plates were 1000, 500, and 250 µg/ml for methanol extractions and 500, 250, and 125 µg/ml for hexanes extractions. Each concentration was replicated three times with triplicate in each run. An automated spectrophotometric plate reader (CERES UV900 HDi) was used to take optical density measurements at 600 nm; these were made before and after 24 h of incubation (37°C) to determine percent inhibition rates, based on the percent of microbial growth inhibition compared to the control.

Minimum inhibitory concentration (MIC) assay

Based on activity displayed in the initial microbial inhibition assay, *S. corynocarpum* was selected for further testing to determine its MIC against *S. aureus* following the protocol of Shrestha et al. (2014). Inoculum was prepared by incubating single colonies of *S. aureus* and MRSA, separately, in 10 ml of Mueller Hinton Broth at 37°C for 24 hours. Serial dilutions of *S. corynocarpum* extracts were prepared with concentrations ranging from 500–62.5 µg/ml in a 24-well plate. Four µl of the cultures were added to individual wells. Then, 100 µl of each extract concentration was transferred to a 96-well plate in triplicate. Gentamicin in concentrations equivalent to plant extracts were used as a positive control and a vehicle control was run using DMSO in the same concentrations as were contained in the plant extracts (not exceeding 6.5%). Following 24 hours of incubation at 37°C, 60 µl of *p*-iodonitrotetrazolium violet (INT; Sigma-Aldrich, St. Louis, MO) was added to each well. Living bacteria reduces the INT dye and changes the color of the solution from colorless to pink. The concentration at which there was no reduction of INT represents the MIC value (Mann and Markham, 1998).

Cytotoxicity assay

To evaluate the cytotoxicity of the *S. corynocarpum* extracts against normal cells, we measured their effect on the viability of human lymphocytes using trypan blue assay. Blood was obtained from healthy donors with prior written consent. Lymphocytes were separated from whole blood using Lymphoprep (Stemcell Technology, BC, Canada). The lymphocytes were stimulated to divide by treating with phytohaemagglutinin (PHA) for 48 h. After 48 h, cells were counted and seeded in a new plate with one million cells per ml. “Control cells” remained untreated, and the test cells were treated with various concentrations (500 to 62.5 ug/ml) of the extracts. After 24 h of treatment, extract-treated and control cells were stained with trypan blue and counted under microscope. Cells stained with trypan blue were considered dead; cells not stained were considered live. Viability of cells was calculated as No. of live cells/No. of total cells × 100.

RESULTS AND DISCUSSION

Ethnobotanical interviews

Twenty-six plants referenced by healers were tallied for frequency of citation and specificity of use against various infections (Table 1). Plants were used singly or in combinations of up to 8 plants. Of the 132 references of plant use by healers, 92 references were for leaves (19 species), 13 for young leaves (2 species), 10 for bark (6 species), 7 for rhizome (2 species), 6 for young fruit (1 species), 3 for aerial root (1 species), and 1 for nut. Preparations to treat skin infections were generally applied topically, while treatments for mouth infections were mostly taken internally with initial topical application. Plants were prepared most frequently by crushing to extract juice, and then used directly or with water as the most common solvent, but other referenced solvents included lemon juice (1), coconut milk (3), seawater (1), or coconut oil (1). Treatment preparations also infrequently cited the use of heat through the use of boiling (2), hot water (1), or heating over fire (1). The species most frequently cited by healers were *Morinda citrifolia* L. and *S. corynocarpum*. A high frequency of citation indicates cultural significance and may also reflect their effectiveness and/or availability (Heinrich et al., 2009). *M. citrifolia* has many documented ethnobotanical uses (Whistler, 1991b, 1992b) and is also a very prevalent species in villages and agriculture allotments. The frequency of citation for *M. citrifolia* may likely reflect its prevalence and high cultural value, although the results of our bioassays do not detect any apparent antimicrobial activity (Table 2). *S. corynocarpum* was the next most frequently cited species; however, it is becoming an increasingly rare species (CBD, 2009). Therefore, this frequency of citation may more likely stem from its effectiveness rather than availability, as seen in the inhibition rates of our bioassays (Table 2). Three other species with high frequencies of citation are *Achyranthes aspera* L., *Glochidion ramiflorum* J.R. Forst. & G. Forst., and *Senna alata* (L.) Roxb. but these did not show highly positive activity in bioassays.

Percent inhibition

Of the 26 species tested, six showed microbial inhibition at 40% or higher against one or more pathogenic microbes as compared to the controls (Table 2). The methanol extract of the bark of *Allophylus cobbe* (L.) Raeusch. (Sapindaceae) showed 49% inhibition of *S. aureus* at the highest tested concentration. The methanol extracts from the young leaves were also active, displaying 72 and 69% inhibition of *C. albicans* at the two highest concentrations. Islam et al. (2012) have shown that methanol extracts of *A. cobbe* were active against *C. albicans* and ethanol extracts were active against *S. aureus*. Chavan and Gaikwad (2013) also found the mature leaves had higher inhibition than young leaves against *S. aureus*.

Both methanol and hexane extractions of *Casuarina equisetifolia* L. (Casuarinaceae) bark showed activity against *S. aureus* at a concentration of 500 µg/ml with respective inhibition values of 44 and 43%. Neither methanol nor hexane extracts were notably active against *E. coli* and *C. albicans*. A previous investigation found inhibition of *S. aureus* and *E. coli* by the ethanol extracts

Table 2. Percent inhibition of methanol (polar) and hexanes (non-polar) extracts (reported in various concentrations of µg/ml) from Tongan medicinal plants against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*.

Plant species	Part[a]	*Staphylococcus aureus*						*Escherichia coli*						*Candida albicans*					
		Methanol			Hexanes			Methanol			Hexanes			Methanol			Hexanes		
		1000	500	250	500	250	125	1000	500	250	500	250	125	1000	500	250	500	250	125
Achyranthes aspera	L	-	-	-	7	12	12	-	20	-	-	-	-	6	5	3	8	6	4
Aleurites moluccanus	B	26	15	7	9	6	1	-	-	-	-	-	-	3	1	-	4	4	-
	N	-	-	-	-	-	-	-	-	-	-	-	-	3	3	-	5	4	1
Allophylus cobbe	YL	8	-	-	-	-	10	33	-	-	-	-	-	**72**	**69**	10	-	-	-
	B	49	37	22	11	3	-	10	16	-	-	-	-	4	4	-	5	2	-
Casuarina equisetifolia	B	39	44	30	43	27	22	11	-	-	3	-	-	5	5	4	2	-	-
Centella asiatica	L	-	-	-	-	-	-	-	-	-	-	-	-	6	7	2	7	6	3
Cestrum nocturnum	L	-	6	14	10	5	14	-	-	-	-	-	-	**97**	**97**	**97**	10	9	1
Curcuma longa	R	4	1	-	1	-	-	-	-	-	4	-	-	1	-	5	1	1	-
Euodia hortensis	L	5	-	-	22	14	4	-	-	-	-	-	-	10	6	3	9	7	3
Glochidion ramiflorum	L	20	20	12	9	9	7	-	-	-	-	-	-	5	4	-	5	4	1
Hibiscus rosa-	L	-	-	-	-	-	-	-	-	-	-	-	-	10	5	-	4	3	1
Sinensis Hoya australis	L	-	-	-	3	-	-	-	-	-	-	-	-	12	5	4	3	-	-
Jatropha curcas	L	26	2	-	-	-	-	-	-	-	1	-	-	5	4	0	11	7	4
Macropiper puberulum	L	13	6	3	17	9	4	-	-	-	5	-	4	19	11	6	21	12	8
Morinda citrifolia	L	-	-	-	-	-	-	-	-	-	5	-	4	19	11	6	21	12	8
	B	16	11	1	-	-	-	-	-	-	-	-	-	23	20	15	14	10	3
	YF	10	2	-	-	-	-	-	-	-	-	-	-	6	5	-	11	7	4
Oxalis corniculata	L	18	6	3	-	-	-	-	-	-	-	-	-	8	8	2	8	5	2
Pandanus sp.	AR	-	8	1	8	3	-	-	-	-	-	-	-	4	3	-	9	5	1
Phymatosorus scolopendria	L	9	7	5	2	-	-	-	-	-	-	-	-	3	6	1	4	-	1
Premna serratifolia	L	6	-	-	59	46	34	-	-	-	5	-	-	5	4	2	20	13	8
Senna alata	L	-	-	-	34	20	5	2	6	-	-	-	-	9	6	1	4	0	-
Solanum viride	L	6	14	8	-	-	-	-	-	-	-	-	-	14	8	3	5	-	-
Syzygium corynocarpum	YL	93	70	55	43	9	-	53	41	27	23	-	-	**79**	**78**	**75**	**76**	**67**	-
Syzygium malaccense	L	40	26	18	21	13	5	15	-	-	-	-	-	1	**-**	**-**	2	2	-
Tarenna sambucina	L	7	-	-	25	32	15	-	-	-	1	-	-	9	5	-	10	7	3
	B	-	-	-	-	11	-	-	4	-	-	-	-	2	-	-	4	1	-
Vigna. marina	L	-	-	-	-	-	-	-	2	2	-	-	-	5	3	1	4	4	-
Vitex trifolia	L	-	-	-	-	-	-	-	-	-	-	-	-	8	4	3	16	10	1
Zingiber zerumbet	R	3	-	-	-	-	-	-	-	-	-	-	-	17	5	2	4	2	-

[a]Parts: L, leaves; B, bark; N, nut; R, rhizome; YF, young fruit; AR, aerial root; YL, young leaves

Table 3. Minimum inhibitory concentration (MIC) values (µg/ml) of different extracts of *Syzygium corynocarpum* against *Staphylococcus aureus* and MRSA.

Plant part	*S. aureus*		MRSA	
	Methanol	Hexane	Methanol	Hexane
Young leaves	125	500	250	500
Mature leaves	-	-	-	-
Bark	250	-	250	-

of the aerial parts of *C. equisetifolia*, but no activity against *C. albicans* (Ramanathan et al., 2012). However, Gumgumjee and Abdulrahaman (2012) found that the ethanol extract of the mature leaves did inhibit *C. albicans*.

Among the top referenced plants by Tongan healers (Table 1), the methanol extraction of the leaves of *Cestrum nocturnum* L. (Solanaceae) showed strong activity against *C. albicans*, displaying inhibition rates of 97% at all three concentrations. Previously, Barnabas and Nagarajan (1988) and Chatterjee (1968) evaluated the phytochemical constituents of *C. nocturnum* and found flavonol glycosides, alkaloids and steroidal saponins, some of which exhibited antifungal and antibacterial activity. In our examination, *C. nocturnum* actually showed negligible antibacterial activity, but excellent antifungal properties. Other studies have examined the antimicrobial activity of various parts of this plant (Khan et al., 2011; Prasad et al., 2013), although only Khan et al. (2011) evaluated the plant against *C. albicans*. They observed only a 65% inhibition of *C. albicans*, using *n*-butanol and aqueous fractions (separately) of the crude methanol extract of the whole plant (Khan et al., 2011).

Hexane extracts of *Premna serratifolia* L. (Lamiaceae) leaves showed 59 and 46% inhibition of *S. aureus* at the highest two concentrations, but no noteworthy activity against *C. albicans* and *E. coli.* Our results are somewhat consistent with the findings of Singh (2011), who examined ethanol extracts of various plant parts to reveal that leaves were least effective against *E. coli*, while root callus was the most effective against *E. coli*. Previous work on the bark, wood, and roots of *P. serratifolia* also showed some antimicrobial activity (Rajendran, 2010; Rajendran and Basha, 2010). The methanol extracts of the leaves of *Syzygium malaccense* (L.) Merr. & Perry (Myrtaceae) showed 40% inhibition of *S. aureus* at the highest concentration, but negligible activity with respect to *C. albicans* or *E. coli.*

In the microbial inhibition assay, *S. corynocarpum* was the only plant that displayed inhibition rates above 40% for all three microbes. “Furthermore, there has been no published research on the antimicrobial activity of this species.” The methanol extraction of the young leaves of *S. corynocarpum* showed the highest inhibition rates against *S. aureus*, at 93%. Although antistaphylococcal activity decreased with lower concentrations, the inhibition level of the methanol extract was 55% even at the lowest tested concentration. The hexane extraction of the young leaves at the highest tested concentration, inhibited 43% of *S. aureus* growth. *S. corynocarpum* showed 53 and 41% inhibition of *E. coli* by the methanol extracts at the highest two concentrations, respectively. *S. corynocarpum* also showed fairly constant inhibition rates of 79, 78 and 75% of *C. albicans* at all three concentrations by methanol extracts and 76 and 67% inhibition of *C. albicans* by hexane extracts. For ethnobotanical context, *S. corynocarpum* was one of the plants most referenced by Tongan traditional healers for mouth and skin infections including thrush, which is caused by *C. albicans*.

Minimum inhibitory concentration (MIC)

The MIC values for hexane and methanol extractions of the different parts of *S. corynocarpum* against *S. aureus* and MRSA are reported in Table 3. Both methanol and hexane extractions of the young leaves showed activity against *S. aureus* (MIC = 125 and 500 µg/ml, respectively) and MRSA (MIC = 250 and 500 µg/ml, respectively) while the extractions of mature leaves showed no activity at the tested concentrations. The MIC values for the methanol extraction of the bark showed activity against both *S. aureus* and MRSA (MIC = 250 µg/ml) while hexane extraction of the bark showed no activity in the tested concentrations. MIC values for gentamicin against *S. aureus* and MRSA were 3.5 and 10 µg/ml, respectively. It is logical to presume that the MIC values for *S. corynocarpum* would be much lower once the active compound(s) are isolated and tested and would thus be more comparable to the positive control. The vehicle control (DMSO) did not show any activity against these bacterial strains. The greater activity shown by the traditionally used plant part-young leaves-over the non-traditional parts underscores the sophistication and value of traditional plant lore and emphasizes the importance of recording details during ethnobotanical interviews. Leaves in general were by far the most common plant part cited by Tongan healers to treat infection. For *S. corynocarpum*, Whistler documents the medicinal use of the leaves as well as of the bark, but he

does not report specific use of the young leaves (Whistler 1991b; 1992a; 1992b). However, every informant who referenced *S. corynocarpum* did specify the use of its young leaves, despite mature leaves being more abundant on the plant. The young leaves of *S. corynocarpum* are more tender and have a deep maroon-purple color while the mature leaves are dark green. This distinct coloration may reflect differences in bioactive compounds present. In addition, the young leaves of plants are generally more vulnerable and therefore may logically contain more defensive secondary metabolites to protect against herbivory or pathogens (Freeman and Beattie, 2008). Our results support the Tongan ethnobotanical use of the young leaves against infection, and indicate that the bark, as reported by Whistler, may be effective in traditional remedies as well.

Cytotoxicity

In order to evaluate the cytotoxicity of the active crude extracts of *S. corynocarpum* on normal cells, we measured their effects on the viability of human lymphocytes. The viability of human lymphocytes treated with each concentration of the extracts did not vary from the viability of control cells. After 24 h of treatment, the viability of human lymphocytes were >90% in all the treated and control samples suggesting no cytotoxicity and positive potential for further testing. In considering future research and use of *S. corynocarpum*, it is important to note that the Convention on Biological Diversity's Tonga report has listed the conservation status of *S. corynocarpum* as vulnerable (CBD 2009). Future involvement should implement precautions to preserve the species and thus its cultural use.

Conclusion

This antimicrobial study of 26 ethnobotanically-selected Tongan plants has shown at least 40% inhibition of microbial growth by six traditionally used plants: *A. cobbi*, *C. equisitifolia*, *C. nocturnum*, *P. serratifolia*, *S. corynocarpum* and *S. malaccense*. While the bioassay results offer evidence of bioactivity in some of the ethnomedicinal plants, a poor performance in an antimicrobial bioassay completed according to standard protocol cannot entirely refute biological activity. Healers use varied solvents, physical manipulation and combinations of plants in order to prepare a traditional treatment for *in vivo* use, which may potentiate chemical reactions that cannot be reproduced according to standard *in vitro* methodology-based bioassay protocol. Further, there are other complex mechanisms involved with the infection process, such as quorum sensing (Quave et al., 2011) or biofilm formation (Quave et al., 2012; Talekar et al., 2014), which may not be elucidated by the bioassays conducted here. Further testing of various plant parts of of *S. corynocarpum* against *S. aureus* and MRSA supported the hypothesis of greater activity by the traditionally used part, emphasizing the importance of documenting specific plant parts used during ethnobotanical interviews. Extracts of *S. corynocarpum* showed no cytotoxicity to normal human cells, suggesting positive potential for further study. Ongoing research is underway to isolate the biologically-active compound(s) through bioassay-guided fractionations.

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Conflict of Interest

Authors have not declared any conflict of interest.

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Effects of aqueous extract of *Waltheria indica* leaves on blood profile of male albino rats

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This study was to investigate the effect of oral administration of aqueous extract of *Waltheria indica* leaf on the blood profile of male albino rats. The extract showed significantly ($p < 0.01$) different increase of red blood and white blood cell counts values at 400 and 800 mg/kg doses. However, the extract showed insignificant difference in packed cell volume, mean corpuscular volume, mean corpuscular haemoglobin and haemoglobin concentrations between the control and the treated groups. Result also showed significant increase ($p < 0.01$) in the values of alanine and aspartate aminotransferases (ALT and AST), blood urea nitrogen (BUN) and creatinine at 800 and 2000 mg/kg doses. *W. indica* leaf extract also induced periportal cellular infiltration and diffuse hydropic degeneration of the hepatocytes in the liver. The study emphasized the cautious use of *W. indica* leaf as it may be hepatotoxic at high doses.

Key words: *Waltheria indica* leaf, blood profile, liver, rats.

INTRODUCTION

Traditionally, plants are used in treatment of diseases in different parts of the world (Hostettman et al., 2000) and their use contribute significantly to primary health care delivery (Holetz et al., 2002). Herbal medicines still remain the mainstay of about 75 to 80% of the whole population in developing countries, for primary health care because of cultural acceptability (Parekh and Chanda, 2006). Each culture or community within an area, whether large or small, has its own ethnobotanical perspective which differs from one another. Plants are regarded as invaluable sources of pharmaceutical products (Olalde, 2005). While traditional healers are still consulted in Nigeria as a first choice due to the fact that traditional medicine blends readily into the socio-cultural life of the people (Kela and Kufeji, 1995), healing from diseases are gotten from plants by other countries of black Africa (Grierson and Afolayan, 1999; Anani et al., 2000). Sofowora (1984) defined medicinal plant as any plant in which one or more of its parts contain substance(s) that can be used for therapeutic purpose or as precursors for pharmaceutical synthesis. The use of plant and animal parts in medicine have since been widely documented in the records of ancient China, India and Egypt, and practice was based on series of "trial and error", which could not be substantiated by proven scientific theories. However, these practices have produced results of proven efficacies compared to the conventional modern medicine (Chopra et al., 1956). *Waltheria indica* L.,

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also known as sleepy morning (Burkill, 2000), belongs to the family Sterculiaceae. It is widespread in West Africa (Akobunda and Agyakwa, 1998). Locally, the plant is called 'hankufah' in Hausa and 'korikodi' in Yoruba (Hutchinson and Dalziel, 1958; Irvine, 1961). The uses of the plant are diverse; the plant has been used as an infusion or decoction where febrifugal, purgative, emollient, tonic, analgesic and astringent action is sought (Burkill, 2000). It is used in Northern Nigeria by the Hausas for the treatment of skin diseases, as an aphrodisiac and as children's medicine at birth and during teething (Mohammed et al., 2007). In the Fulani community, the aqueous extract of the root is used in relieving aches and pains during the 'Sharo' festival. Among the Yoruba, the aqueous extract of the root and stem are used in treating syphilis, internal haemorrhage, and as a restorative after the labours of farming activity (Mohammed et al., 2007).

The trypanocidal, antibacterial, anti-inflammatory, analgesic and haematinic properties of *W. indica* plant have been reported (Oladiji et al., 2005; Bala et al., 2010; Olajuyigbe et al., 2011). However, there is the need to ascertain the safety of this plant because of their wide usage. Therefore, the aim of this study is to investigate the effect of aqueous extract of *W. indica* leaves on haematological and serum biochemical parameters of male albino rats.

MATERIALS AND METHODS

Experimental animals

Thirty six healthy white male adult albino rats (100 to 190 g) obtained from the Animal House, Faculty of Veterinary Medicine, University of Ibadan, Nigeria, were used for the study. The rats were fed with rat cubes (Ladokun Feeds Limited, Ibadan, Nigeria) and water *ad libitum*. During the study, the rats were kept at the Experimental Animal House of the Department of Veterinary Physiology, Biochemistry and Pharmacology, University of Ibadan, Ibadan. Animals were acclimated to their new environment for two weeks before the commencement of the experiment. All experimental protocols were in compliance with University of Ibadan Ethics Committee on Research in Animals as well as internationally accepted principles for laboratory animal use and care.

Plant

The *W. indica* plants were obtained from a farm land at Moniya in Akinyele Area Council of Ibadan, Oyo state, Nigeria and identified at the Herbarium, Department of Botany, University of Ibadan with voucher number UIH-22371.

Extract preparation

The leaves of the plant were separated from the whole plant and air dried at room temperature for two weeks. A total of 200 g of the ground powder was soaked in 1 L of distilled water for 24 h at room temperature. The mixture was filtered into conical flask with Whatman filter paper. The filtrate was concentrated *in vacuo* using a rotary evaporator at 40°C to produce a gel-like extract, which weighed 43 g (21.5% yield). Appropriate concentration of the extract extract was then subsequently made by dilution with distilled water into graded doses and administered to the rats.

Experimental design

Thirty six male albino rats were randomly divided into six groups (n = 6), labeled A to F where group A served as the control while the animals in the groups B, C, D, E and F served as the treated group. The treated groups were then orally administered with 200, 400, 800, 1600 and 2000 mg/kg body weight of the extract, respectively for 21 days.

Acute toxicity study

The acute toxicity study of aqueous extract of *W. indica* was determined according to the method of Adedapo et al. (2009). Rats that have been fasted for 16 h were randomly divided into six groups of six per group. Graded doses of the extract (200, 400, 800, 1600, 2000 mg/kg p.o) corresponding to groups B, C, D, E and F were separately administered to the rats in each of the 'test' groups by means of bulbed gavage needle. Meanwhile for the control group (group A) was orally administered with distilled water (3 ml/kg) only. All the animals were then allowed free access to food and water and observed for 48 h, for any signs of toxicity. The numbers of deaths within this period were recorded.

Collection of blood and serum samples

Blood samples were collected through the orbital sinus from diethyl ether anaesthetized albino rats into heparinised bottles for haematological studies. Meanwhile, for non-heparinised bottles, the blood samples were allowed to clot. The blood samples were centrifuged at 3000 rpm for 10 min and the serum was separated from the clot and transferred into clean bottles for biochemical analysis.

Haematological and serum biochemical studies

The packed cell volume (PCV) and haemoglobin concentration were determined by conventional method (Duncan et al., 1994). Erythrocyte count was determined by the haematocytometry method as described by Jain (1986). Total white blood cell (WBC) counts were made in a haemocytometer using the WBC diluting fluid and differential leucocytes counts were made by counting the different types of WBC from Giemsa stained slides viewed from each of the 30 fields of oil immersion objective of a microscope (Coles, 1989). Erythrocyte indices including mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were determined from the values obtained from red blood cells (RBC) count, haemoglobin concentration and PCV values (Duncan et al., 1994). Total protein was measured using biuret reaction, while albumin was measured by colorimetric estimation using Sigma diagnostic reagent (Sigma Diagnostic, UK.), which contained bromocresol green (BCG). Meanwhile, globulin was obtained from difference total protein and albumin. AST and ALT were determined using a photoelectric colorimeter as described by Duncan et al. (1994). However, serum urea and creatinine levels were determined using photoelectric colorimeter as described by Coles (1989).

Histopathology

All the animals from each of the treated groups B, C, D, E, F and the control were sacrificed 24 h after their respective daily doses. The rats were thereafter quickly dissected to remove the liver and then

Table 1. Acute toxicity study in rats after 48h of administration with aqueous extract of *W. indica.*

Group	Dose (mg/kg)	No of dead rats	Toxic signs observed
Control	Distilled water	0	No toxic changes observed
B	200	0	No toxic changes observed
C	400	0	No toxic changes observed
D	800	0	No toxic changes observed
E	1600	0	No toxic changes observed
F	2000	0	Slight diarrhoea

Table 2. Effect of graded doses of the aqueous extract of *W. indica* on haematological parameters of rats (n = 6).

Parameter	Control	B (200 m/kg)	C (400 m/kg)	D (800 m/kg)	E (1600 m/kg)	F (2000 m/kg)
RBC (× 10^{12}/L)	7.80±0.09	8.11±0.19	8.87±0.04**	8.90±0.27**	7.63±0.11	7.50±0.14
PCV (%)	47.0±0.45	48.0±0.71	49.6±0.51	48.6±0.40	46.2±0.92	45.0±1.27
Hb (g/L)	15.3±0.07	15.7±0.09	16.3±0.11	15.9±0.23	15.3±0.28	15.5±0.94
MCHC (%)	32.48±0.27	32.74±0.35	32.96±0.31	32.84±0.24	33.22±0.95	34.46±2.09
MCH (pg)	19.58±0.27	19.40±0.42	18.42±0.12	18.00±0.62	20.10±0.39	20.70±1.33
MCV (fl)	60.24±0.70	59.30±1.10	55.90±0.50	54.80±1.75	60.54±1.07	59.96±0.72
WBC(× 10^{9}/L)	9.80±0.01	9.70±0.13	10.50±0.20**	11.00±0.10**	11.20±0.15**	9.50±0.19
Eosinophil (%)	2.2±0.20 (0.22±0.019)[a]	2.0±0.32 (0.19±0.033)[a]	2.2±0.49 (0.23±0.053)[a]	2.2±0.73 (0.24±0.081)[a]	2.4±0.4 (0.27±0.048)[a]	2.0±0.55 (0.19±0.053)[a]
Lymphocytes (%)	65.6±1.29 (6.4±0.12)[a]	67.2±1.07 (6.5±0.10)[a]	70.0±0.84 (7.4±0.21)[a]	71.4±1.97 (7.8±0.26)[a]	73.0±1.70* (8.2±0.15)[a]	73.2±2.22* (6.9±0.21)[a]
Neutrophils (%)	22.8±2.31 (2.2±0.23)[a]	30.4±1.17* (2.9±0.15)[a]	31.8±2.42* (3.3±0.25)[a]**	25.8±1.11 (2.8±0.11)[a]	25.4±1.72 (2.8±0.19)[a]	22.8±2.18 (2.2±0.22)[a]
Monocyte (%)	2.6±0.25 (0.25±0.019)[a]	1.6±0.25 (0.15±0.023)[a]	3.8±0.37 (0.40±0.046)[a]*	1.2±0.20 (0.13±0.022)[a]	2.2±0.50 (0.25±0.058)[a]	2.0±0.32 (0.19±0.033)[a]
Platelets (µl)	69000±8983	111400±20951	103600±10414	119200±8291	111600±15095	109800±13800

Results are reported as mean±standard error of mean (S.E.M) and analyzed using the one-way analysis of variance (one-way ANOVA) and Duncan Multiple range Tests (n=6). Superscripted items indicate significant values (* P< 0.05, ** P<0.01). [a]absolute counts of the differential in (× 10^{9}/L).

liver and then transferred into 10% buffered formalin. The organs were dehydrated in ethanol (70 to 100%), cleared in xylene and embedded in paraffin. Tissue sections were examined under a light microscope after staining with haematoxylin and eosin (H and E) (Culling, 1963; Lillie, 1965).

Statistical analysis

The data obtained from the experiment were presented as mean ± standard error of mean (SEM) and analyzed using the one-way analysis of variance (one-way ANOVA). The group means were separated by Duncan Multiple range Tests at 95% confidence interval using GraphPadInstat® software.

RESULTS

Acute toxicity study

Result showed that no mortality was observed in the treated and untreated groups (Table 1). From the observations, there was slight diarrhoea at the higher dose of 2000 mg/kg.

Effect of the aqueous extract of *Waltheria indica* on haematological parameters of the rats

Result in Table 2 showed the effect of graded doses of *W. indica* on haematological parameters of rats. The extract doses of 400 and 800 mg/kg b.w (Groups C and D) caused significantly increase ($P < 0.01$) in RBC counts. The extract dose of 400, 800 and 1600 mg/kg b.w (groups C, D and E) also showed significant increased ($P < 0.01$) in white blood cell counts. The lymphocyte

Table 3. Effects of the aqueous extract of *W. indica* on serum biochemical parameters of rats (n=6).

Parameter	A	B (200 mg/kg)	C (400 mg/kg)	D (800 mg/kg)	E (1600 mg/kg)	F (2000 mg/kg)
Total protein (g/dl)	8.30±0.10	8.04±0.17	7.49±0.16	7.66±0.09*	7.72±0.06*	7.52±0.21**
Albumin (g/dl)	5.04±0.09	4.42±0.25	4.42±0.17	4.44±0.16	4.58±0.10	4.08±0.36*
Globulin (g/dl)	3.28±0.05	3.64±0.09	3.52±0.14	3.22±0.11	3.20±0.09	3.30±0.08
AG ratio	1.56±0.09	1.18±0.10*	1.22±0.07	1.40±0.11	1.38±0.07	1.26±0.11
AST (U/L)	40.20±0.37	42.8±0.37**	44.0±0.89**	45.2±1.02**	45.6±0.51**	47.40±0.68**
ALT (U/L)	28.2±0.58	29.6±0.93	30.8±1.11	32.0±0.55**	31.6±0.40*	33.4±0.40**
ALP (U/L)	93.8±2.78	89.4±4.40	80.4±1.33	118.8±3.39**	83.8±6.56	98.2±5.27
BUN (mg/dl)	13.2±0.37	15.6±0.24**	15.4±0.51**	14.6±0.40	15.8±0.38**	16.6±0.25**
Creatinine (mg/dl)	0.54±0.06	0.82±0.07*	0.92±0.08**	1.00±0.06**	0.80±0.03	0.90±0.11**

Results are reported as mean± standard error of mean (SEM) and analyzed using the one-way analysis of variance (one-way ANOVA) and Duncan Multiple range Tests (n=6). Superscripted items indicate significant values (*P< 0.05, **P<0.01).

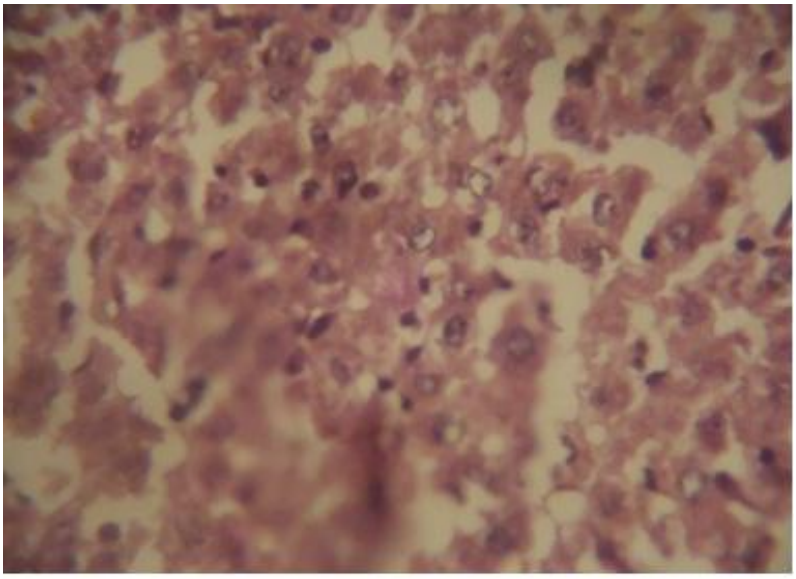

Figure 1. Liver of the control showing no visible lesion. M ×100 H&E

count was also significantly increased ($P < 0.05$) for the groups E and F. In addition, the neutrophils counts for the groups B and C were significantly increased ($P < 0.05$) compared to the control group. There were no significant difference in PCV, Hb count, MCV, MCHC, MCH and platelets counts between the treated groups (group B, C, D, E and F) and the control group (group A).

Effects of the aqueous extract of *Waltheria indica* on serum biochemical parameters of the rats

The result of the effects of the graded doses of *W. indica* on serum biochemical parameters was presented in Table 3. There was significant decrease in total protein for the groups D, E ($P < 0.05$) and F ($P < 0.01$) compared to the control (group A). Furthermore, there was a significant ($P < 0.05$) decrease in albumin for group F compared to the control. Level in AST showed significantly increase ($P < 0.01$) in the treated groups (B to F) compared to the control. More so, the level of BUN showed significant increase ($P < 0.01$) in the treated groups (B, C and F) compared to the control. Nevertheless, the levels of ALP was significantly increased ($P < 0.01$) for group D. The level of creatinine was significantly increased ($P < 0.01$) for group B, C, D and F compared to the control. However, there were no significant difference in globulin between the control (A) and treated groups (B to F).

Histopathological effects

Histopathological evaluation of rats treated with aqueous leave extract of *W. indica* is presented in Figures 1 to 4. No visible lesion was observed in the liver (Figure 1, showed

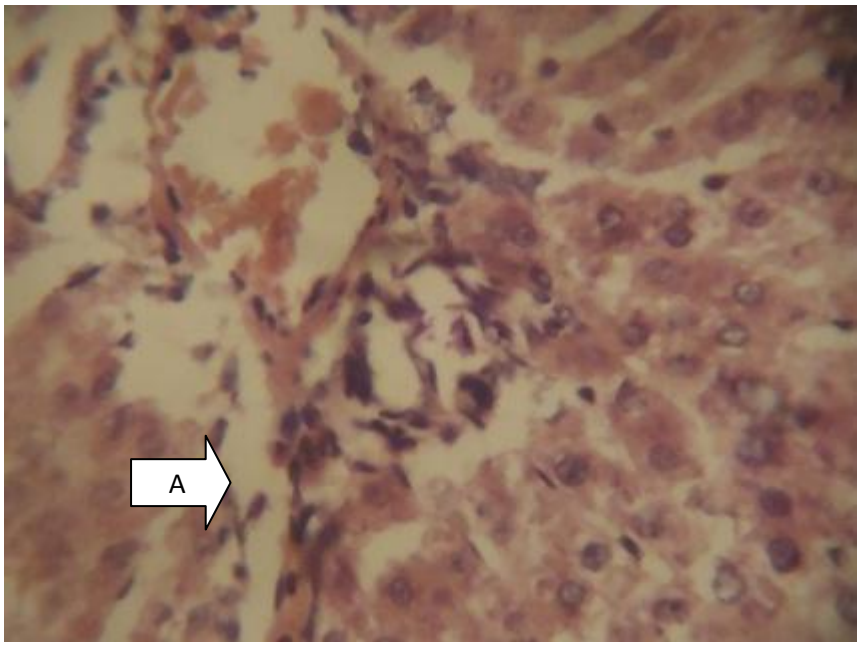

Figure 2. Liver of group B (200 mg/kg) showing periportal cellular infiltration A, by mononuclear cells. M ×100 H&E.

showed by an arrow) of the control group. Periportal cellular infiltration by mononuclear cells was observed in the liver of group B (Figure 2, showed by an arrow A) and group E (Figure 4, showed by an arrow D). The liver of group C (400 mg/kg b.w) has diffuse hydropic degeneration of hepatocytes (Figure 3, showed by an arrow C).

DISCUSSION

There was no mortality observed except for the slight diarrhoea that was observed at the 2000 mg/kg b.w dose. This shows that *W. indica* has wide safety margin. This finding is supported by the result of the haematological effect of *W. indica* leaves in albino rats. There were no significant changes observed in the values of PCV, haemoglobin, MCH, MCHC and MCV between the treated groups and the control group. The extract caused significant increase in red blood cell counts at 400 and 800 mg/kg doses. This could be due to the presence of iron and proteins in *W. indica* plant as reported by Oladiji et al. (2005) and iron is a major component of red blood cell. This observation is in agreement with the ethnobotanical use of this plant in treatment of anaemia (Gbadamosi et al., 2012).

The oral administration of aqueous extract of *W. indica* leaves also caused significant increase in white blood cell counts. The increase in neutrophils and lymphocytes accounted for the increase in white blood cell counts. This observation of increase in the white blood cells counts by this plant extracts shows that the principal function of phagocytes which is to defend against microorganisms by ingesting and destroying them, thus contributing to cellular inflammatory processes will be enhanced (Paul, 1993; Swenson and Reece, 1993; Adedapo et al., 2005). This is attested to by the antibacterial activity of this plant (Olajuyigbe et al., 2011; Mongalo et al., 2012). The increase in white blood cell counts may be due to the presence of cardiac glycosides in this plant extract. It has been demonstrated that plants with high composition of cardiac glycosides has been found to inhibit microbial growth and is capable of protection against microbial infection, thereby increasing the white blood cell counts (Gonzolel and Mather, 1982).

However, we observed from the results that the extract caused significant reduction in total protein at doses 800, 1600 and 2000 mg/kg b.w (groups D, E and F, respectively). This reduction showed that the plant inhibit protein biosynthesis. Serum albumin is a good criterion to assess the function and secretory capacity of the liver (Yakubu et al., 2005). This may indicate hepatic toxicity of this extract at higher doses. Aminotransferases (ALT and AST) are produced in the liver and are good markers of damage to liver cells but not necessarily the severity of the damage (Rej, 1989). They are normally present at low levels in the blood, however, if the liver cells are damaged, it would be expected that some of the enzymes leak into the blood and increase in levels. Increase in serum level of AST and ALT as observed in this study may reflect damage to liver cells. Increase serum ALT is known to occur in liver disease and it has been used as a tool for measuring hepatic necrosis (Bush, 1991).

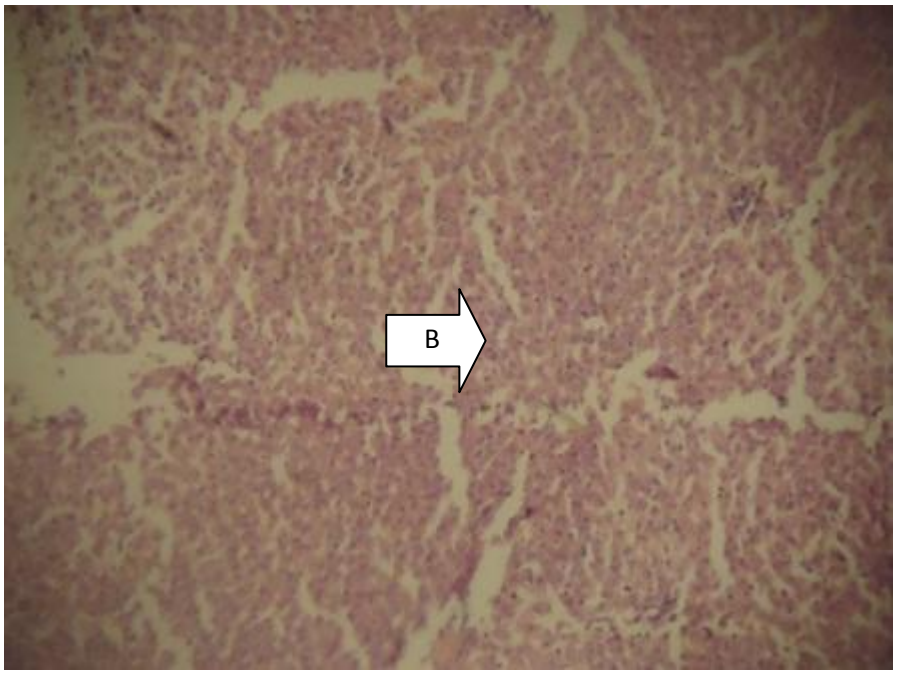

Figure 3. Liver of group C showing diffuse hydropic degeneration, B of hepatocytes. M ×100 H&E.

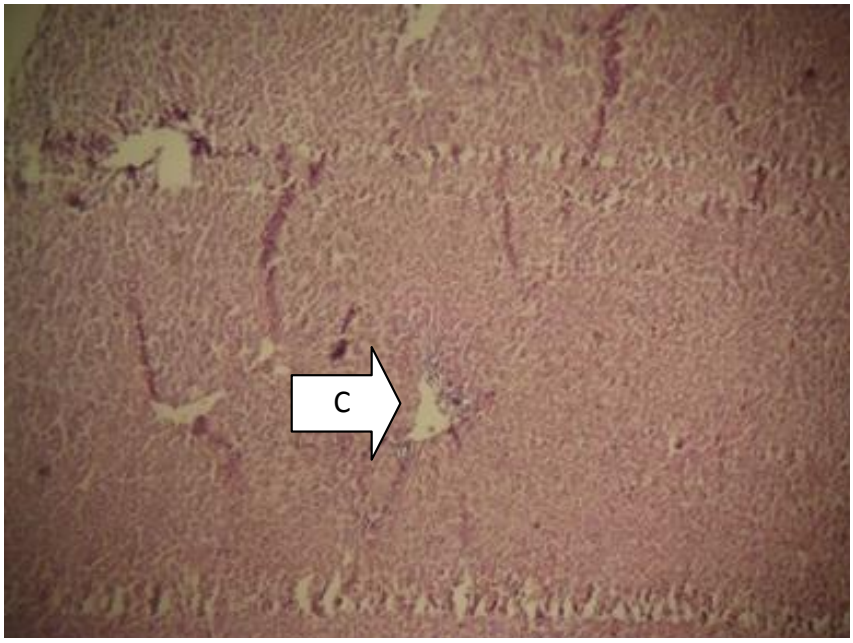

Figure 4. Liver of group E showing periportal cellular infiltration C, by mononuclear cells. M ×100 H&E.

The result obtained in this study is in agreement with findings of Ajibade et al. (2011) who also reported increase levels of ALT and AST by methanolic seed extract of *Moringa oleifera*. Urea is one of the non-protein nitrogenous substances that accumulate in the plasma when renal excretion is reduced. The causes of increased blood urea levels include: high protein diet, intestinal haemorrhage, dehydration, severe haemorrhage and

shock among others. Urea level could be decreased due to the following: liver failure, low protein diet, anabolic steroids, diabetes insipidus, etc. (Bush, 1991). The increased blood urea nitrogen level observed in this study may be due to the high protein content of *W. indica* plant. Gbadamosi et al. (2012) reported that *W. indica* contained 11.9% crude protein. This increased urea level may point to renal dysfunction which resulted in reduced urea excretion.

Creatinine is measured primarily to assess kidney function. A rise in blood creatinine is observed only with marked damage to functioning nephrons. The plasma level of creatinine is independent of protein ingestion, water intake, rate of urine production and exercise. Since its rate of production is constant, elevation of plasma creatinine is indicative of under-excretion, suggesting kidney damage (Gross et al., 2005). The increase level of creatinine observed in this study shows that the extract may cause renal dysfunction.

The study concludes that excessive use of aqueous extract of *W. indica* leaves can be hepatotoxic; therefore caution should be applied to the use of *W. indica* leaves despite its numerous medicinal values.

Conflict of Interest

The author(s) declared that there is no conflict of interest as regards this paper.

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