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Full Length Research Paper

Canvassing of biological attributes by *In vitro* screening of *Canarium bengalense*

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Various Hoang family remedies known as Dai-Thien-Nuong, utilize *Canarium bengalense* Roxb. (Family: *Burseraceae*) as a major component for the treatment of tumor and liver damage. This study was performed using the crude methanol extract of *C. bengalense* Roxb. bark and its petroleum ether, carbon tetrachloride, dichloromethane and aqueous soluble fractions for antioxidant, cytotoxic, thrombolytic, membrane stabilizing and antimicrobial activities. The antioxidant activity was assessed by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay. The cytotoxic and thrombolytic activities were determined using vincristine sulphate and *streptokinase* as standards, respectively. Heat and hypotonic solution-induced conditions were induced to determine membrane stabilizing activity. Disc diffusion method was used to determine the antimicrobial activity of the test samples. Among all the test samples, the crude methanol extract showed the highest free radical scavenging activity (IC_{50} = 50.62±0.26 µg/ml). In cytotoxic activity assay, the pet-ether soluble fraction revealed the highest cytotoxic potentials (LC_{50} = 8.96 ± 0.64 µg/ml). The same sample showed 31.08±0.12% of clot lysis in thrombolytic activity assay. In membrane stabilizing activity assay, the dichloromethane soluble fraction showed 60.94 ± 0.31% inhibition of heat induced haemolysis of RBC and this finding was found to be more significant than that of acetyl salicylic acid (42.12±0.38%) used as standard in this assay. Phytocomponents responsible for the observed activities should be isolated for new drug development.

Key words: *Canarium bengalense* Roxb, total phenolic content, cytotoxicity, thrombolytic activity, membrane stabilizing activity.

INTRODUCTION

Many medicines used nowadays are plant derived. Some of these medicines are directly isolated from their natural sources and used as it is. Others are modified using different drug designing techniques to maximize the

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desired effects, improve potency and minimize the side effects. Reports of World Health Organization (WHO) indicate that a huge percentage of global population (about 80%) prefer traditional medicines for their primary health care (Kabir et al., 2015). It is necessary to screen the medicinal plants, isolate and modify the active components and develop new medicines.

The local name of *C. bengalense* Roxb. (Family: Burseraceae) is Dhuna Rata or East Indian Copal. The native origin of the plant is not known but the plant is abundant in Vietnam, China, Laos, Myanmar, Thailand and India-Assam (Wu et al., 2008). Extracts from leaf and root is used in bronchitis, leprosy, jaundice, cough and asthma. Anti-inflammatory, antiseptic and anti-asthmatic activities were demonstrated by leaf and bark extracts (Le et al., 2012). The species has been reported to be useful in skin rashes and snake bite (Sarkar et al., 2017). A new flavone glycoside and six known compounds with cytoprotective properties have been isolated from the stem bark of *C. bengalense.* This might be the reason of the traditional use of this plant in tumor and liver damage (Le et al., 2012).

It is important to investigate the medicinal plants of Bangladesh for prominent biological activities (Sharmin et al., 2017; 2018). *C. bengalense* is abundant in Bangladesh and very few experiments were carried out using the bark of the plant. Therefore, antioxidant, cytotoxic, thrombolytic, membrane stabilizing and antimicrobial activity assays were conducted using the crude methanol extract of *C. bengalense* bark extract as well as its organic and aqueous soluble fractions.

MATERIALS AND METHODS

Plant materials

C. bengalense bark was collected from Mirpur botanical garden, Dhaka, Bangladesh. A voucher specimen (DACB 47558) for this collection has been maintained in Bangladesh National Herbarium for future reference.

Small pieces of the bark were sun dried for several days. Then the bark pieces were oven dried for 24 h for better grinding. High capacity grinding machine was used to powder the dried bark. 500 g powdered material was soaked in 3.0 l of methanol in a clean, ambered color reagent bottle (5.0 l) for 10 days. The bottle was occasionally shaken and stirred. Then the plant material was filtered, and the filtrate was evaporated to dryness using a rotary evaporator at 40°C and 50 r.p.m to give the crude methanol extract. The concentrated methanol extract (5 g) was fractionated by modified Kupchan partition protocol (Van Wagenen et al., 1993) and the petroleum ether (PESF, 0.8 g), carbon tetrachloride (CTCSF, 1.7 g), dichloromethane (DCMSF, 1.3 g) and aqueous (AQSF, 0.7 g) soluble materials were obtained and refrigerated until further use.

Drugs and chemicals

Beacon Pharmaceutical Ltd provided *Streptokinase*. All other drugs, reagents and solvents were obtained from Sigma-Aldrich, Munich, Germany.

Total phenolic content

The method developed by Harbertson and Spayd (2006) was used to determine the total phenolic content of the test samples.

Antioxidant activity

The antioxidant activity of the test samples was determined using BHT and ascorbic acid as reference standards (Brand-Williams et al., 1995).

Brine shrimp lethality bioassay

The cytotoxic potential of the plant samples was determined using single day in vivo assay. The test samples were assayed using *Artemia salina*. Vincristine sulphate was used as the reference standard (Meyer et al., 1982). The lethality of brine shrimp nauplii was used in this assay to determine cytotoxic activity.

Thrombolytic activity

The thrombolytic activity was determined following the method developed by Prasad et al. (2007). *Streptokinase* was used as positive control.

Membrane stabilizing activity

The ability of the extract and fractionates to inhibit heat and hypotonic solution induced haemolysis of human erythrocytes was assessed following the method developed by Omale et al. (2008).

Antimicrobial screening

Disc diffusion method was used to investigate the antimicrobial potential of the crude extract and its aqueous and organic soluble fractionates by observing their ability to generate zone of inhibition (Bayer et al., 1966).

Statistical analysis

Three replicates of each sample were used for statistical analysis and all of the values are expressed as the mean ± standard deviation (SD). The results were evaluated by a two-tailed non-parametric pair t-test. $P < 0.05$ was considered statistically significant.

RESULTS

The present study was undertaken to assess the antioxidant, cytotoxic, thrombolytic, membrane stabilizing and antimicrobial activities of the crude methanol extract of *C. bengalense* and its organic and aqueous soluble materials.

Table 1. Total phenolic content, antioxidant and cytotoxic activities of *Canarium bengalense*.

Samples/ Standard	Total phenolic content (mg of GAE/g of dried extract)	Free radical scavenging activity IC_{50} (µg/ml)	Brine shrimp lethality bioassay LC_{50} (µg/ml)
ME	14.31 ± 0.23	50.62 ± 0.26	18.37 ± 0.38
PESF	10.75 ± 0.41	59.42 ± 0.63	8.96 ± 0.64
CTCSF	9.44 ± 0.16	514.65 ± 0.10	25.12 ± 0.61
DCMSF	4.31 ± 0.25	361.35 ± 0.21	27.00 ± 0.22
AQSF	2.75 ± 0.11	266.74 ± 0.46	54.27 ± 0.32
Vincristine sulfate	-	-	0.45 ± 0.04
BHT	-	20.93 ± 0.54	-
Ascorbic acid	-	3.63 ± 0.21	-

ME= Crude methanol extract; PESF= Pet-ether Soluble Fraction; CTCSF= Carbon tetrachloride soluble fraction; DCMSF= Dichloromethane soluble fraction; AQSF= Aqueous soluble fraction; GAE= Gallic acid equivalent; BHT= Butylated hydroxytoluene.

Table 2. Thrombolytic and membrane stabilizing activities of *Canarium bengalense* bark extract and soluble fractions.

Samples/Standard	% of lysis of RBCs	% Inhibition of haemolysis	
		Heat induced	Hypotonic solution induced
ME	20.69 ± 0.28	9.29 ± 0.19	9.76 ± 0.06
PESF	31.08 ± 0.12	46.50 ± 0.48	4.28 ± 0.32
CTCSF	27.58 ± 0.55	33.12 ± 0.11	12.93 ± 0.17
DCMSF	21.96 ± 0.23	60.94 ± 0.31	14.85 ± 0.22
AQSF	16.35 ± 0.21	17.21 ± 0.25	49.17 ± 0.31
Water	3.79 ± 0.55	-	-
Streptokinase	65.01 ± 0.36	-	-
Acetyl salicylic acid	-	42.12 ± 0.38	72.00

ME= Crude methanol extract; PESF= Pet-ether Soluble Fraction; CTCSF= Carbon tetrachloride soluble fraction; DCMSF= Dichloromethane soluble fraction; AQSF= Aqueous soluble fraction.

Different test samples of *C. bengalense* demonstrated presence of phenolic components within the range of 2.75 to 14.31 mg of GAE/g of sample. Among the test samples, the crude methanol extract showed the highest value of phenolic content (14.31 ± 0.23 mg of GAE/g of sample). In free radical scavenging activity assay, the highest free radical scavenging activity was given by the crude methanol extract (IC_{50} = 50.62 ± 0.26 µg/ml) followed by pet-ether soluble fraction (IC_{50} = 59.42 ± 0.63 µg/ml), as compared to ascorbic acid and BHT exhibiting IC_{50} values of 3.63 µg/ml and 20.93 µg/ml, respectively (Table 1).

In case of brine shrimp lethality bioassay, among all the test samples of the bark of *C. Bengalense*, the highest cytotoxic activity was given by the pet-ether soluble fraction (LC_{50} = 8.96 ± 0.64 µg/ml) followed by the crude methanol extract (LC_{50} = 18.37 ± 0.38 µg/ml) as shown in Table 1.

In thromboltic activity assay, the pet-ether soluble and the carbon tetrachloride soluble fractions showed 31.08 ± 0.12% and 27.58 ± 0.55% of clot lysis, respectively as compared to 65.01% clot lysis by the standard *streptokinase* (Table 2).

At concentration 1.0 mg/ml, the test samples of *C. bengalense* protected the haemolysis of RBCs induced by heat and hypotonic solution as compared to the standard acetyl salicylic acid (0.10 mg/ml). The dichloromethane soluble fraction showed 60.94 ± 0.31% inhibition of heat induced haemolysis which is found to be more significant than acetyl salicylic acid (42.12 %). On the other hand, the aqueous soluble fraction inhibited 49.17 ± 0.31 % hypotonic solution-induced haemolysis of RBCs as compared to 72.00% by acetyl salicylic acid (Table 2).

The antimicrobial activity of *C. bengalense* test samples was evaluated against gram positive and gram negative bacteria and the results were compared with standard antibiotic, ciprofloxacin. Among the test samples of *C. bengalense*, only the carbon tetrachloride soluble fraction and dichloromethane soluble fraction revealed antimicrobial activity with zone of inhibition ranging from 7.0 to 9.0 mm. The highest zone of inhibition (9.0 mm)

Table 3. Antimicrobial activity of test samples of *Canarium bengalense*.

Test microorganisms	Diameter of zone of inhibition (mm)		
	CTCSF	DCMSF	Ciprofloxacin (30 µg/disc)
Bacillus cereus	8.0 ± 0.14	7.0 ± 0.31	45.0 ± 2.01
B. megaterium	8.0 ± 0.42	7.0 ± 0.21	42.0 ± 1.17
B. subtilis	7.0 ± 0.33	-	42.0 ± 0.73
Staphylococcus aureus	7.0 ± 0.55	-	42.0 ± 0.23
Sarcina lutea	8.0 ± 0.24	-	42.0 ± 0.56
Escherichia coli	7.0 ± 0.13	8.0 ± 0.45	42.0 ± 0.43
Pseudomonas aeruginosa	7.0 ± 0.32	7.0 ± 0.31	42.0 ± 1.11
Salmonella typhi	9.0 ± 0.51	-	42.0 ± 1.11
S. paratyphi	8.0 ± 0.12	7.0 ± 0.22	47.0 ± 2.33

CTCSF= Carbon tetrachloride soluble fraction; DCMSF= Dichloromethane soluble fraction.

was showed against *Salmonella typhi* by the carbon tetrachloride soluble fraction (Table 3).

DISCUSSION

A few phyto-components like sabinene, caryophyllene and α-humulene have been previously isolated from *C. bengalense* (Thang et al., 2004). Among them sabinene has been reported to possess antioxidant activity (Quiroga et al., 2015). Again, β- caryophyllene from the essential oil of *Aquilaria crassna*, demonstrated significant antioxidant potential (Dahham et al., 2015). Therefore, these phytocomponents might have contributed to the observed antioxidant activity of the species under investigation (Table 1).

In Vietnam, *C. bengalense* has been used as an ingredient for making remedies of cancer and liver damage for a long period of time (Le et al., 2012). β-caryophyllene has been found to show anti-cancer activity against colorectal cancer cells (Dahham et al., 2015). Therefore, this phyto-component might be held responsible for the observed cytotoxic potential of the test samples (Table 1). In case of thrombolytic activity assay, the findings may help in the development of new cardiovascular drugs using the bark of *C. bengalense* (Table 2).

There are lots of similarities between human red blood cell membranes with that of lysosome. Hence, the membrane stabilizing activity can be correlated to anti-inflammatory effect (Mounnissamy et al., 2008). Sabinene has been reported to exhibit strong anti-inflammatory activity by inhibiting nitric oxide production in lipopolysaccharide and interferon gamma-triggered macrophages (Valente et al., 2013). 1% sabinene was observed to inhibit lens protein-induced inflammation in rabbit's eye (Quan-Sheng et al., 1993). Therefore, this phytocomponent might be considered responsible for the observed membrane stabilizing activity.

In support of the observed antimicrobial activity, it can be stated that sabinene, β-caryophyllene and α - humulene have been found to exhibit significant antimicrobial activity (Arunkumar et al., 2014; Dahham et al., 2015; Rahman et al., 2016). Therefore, the presence of these compounds in the species under investigation might be the reason of observed antimicrobial activity (Table 3).

Conclusion

Mankind has been fighting against many diseases such as cancer, heart diseases and neurodegenerative diseases for many years. Some medicines like antibiotics that are found to be useful now may not be found to be effective in future due to development of resistance. Therefore, mankind is continuously searching for better and more potent medicines with fewer side effects. In this investigation, the test samples of *C. bengalense* showed significant cytotoxic and membrane stabilizing potentials. The plant should be further analyzed for the identification of the compounds responsible for the observed activities.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Tolerance and observance of methotrexate in the treatment of rheumatoid arthritis at the university Hospital Bogodogo, Burkina Faso

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This study is aimed at assessing tolerance and observance of the patients with RA (rheumatoid arthritis) treated by methotrexate at University Hospital Bogodogo, in Burkina Faso. A cross-sectional study was conducted from July to September 2015 in outpatients with rheumatoid arthritis monitored in rheumatology service. Data were obtained from interview, by reviewing medical cards of the patients and consultation report forms. In total, 205 patients with RA were included. Among them, 135 were treated with methotrexate (65.9%). All patients treated with methotrexate took folic acid tablet weekly. The duration of RA evolution varied from 1 to 360 months with a mean of 48 month. The mean duration of treatment with methotrexate was 30.2 months. Among the patients treated with methotrexate, 28.8% observed adverse reactions, 53.3% declare taking their medication without break during the last month. Discomfort (8.9%) and nausea (5.9%) were the most frequent adverse effects. Stock out of methotrexate in community pharmacy (10.4%) and adverse effect (5.9%) were the main reasons of patient's inobservance. Methotrexate is always the cornerstone of RA treatment. This study confirms the good tolerance of methotrexate and difficulty of observance with this treatment.

Key words: Rheumatoid arthritis, observance, methotrexate, tolerance, Burkina Faso.

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory, chronic and autoimmune pathology. Its prevalence in the world varies from 0.4 to 1% according to the gender, race or ethnicity, period and regions (Gabriel, 2001; Igor et al., 2015). However, data about prevalence of RA are limited for the Sub-Saharan African region (Igor et al., 2015). Its

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Table 1. Distribution of patients with rheumatoid arthritis according to the anti-rheumatism drug used (n=205).

Anti-rheumatism drug	Number of patients (%)
Methotrexate only	132 (64.4)
Methotrexate + Hydroxy chloroquine	2 (0.9)
Methotrexate + Hydroxy chloroquine + Sulfasalazine	1 (0.5)
Hydroxy chloroquine	67 (32.7)
Sulfasalazine	3 (1.5)

hospital prevalence was estimated in African countries such as Togo (0.45%) (Houzou et al., 2013) and Burkina Faso (2.18%) (Ouedraogo et al., 2014). Because of the high cost of medical and pharmaceutical care with the use of most recent medicines, RA is a health public issue. Moreover, patients with RA cannot be very active in economic system. Immunosuppressive drugs are the base of the RA treatment and aims to improve symptoms prevent complications and improve quality of life. Considering the literature, methotrexate is the immunosuppressive drug most frequently prescribed (Sany et al., 2004; Weinblatt et al., 2013; Shinde et al., 2014). Patients using methotrexate are highly exposed to its adverse effects of two main factors: cytotoxic effect and prolonged duration of treatment. Inobservance to treatment is associated with relapse or persistence of RA. The previous studies about observance with methotrexate showed that the majority of patients have not a good adherence (Contreras Yanez et al., 2010; Bluett et al., 2015). About 40% patients are non-adherent to methotrexate (Bluett et al., 2019). Several studies were performed about RA in Burkina Faso (Ouedraogo et al., 2017; 2016; Yameogo, 2016). However, up until now, there was no research concerning adverse effects and observance with methotrexate in Burkina Faso. This work aimed to assess tolerance and observance of the patients with RA treated by methotrexate at university hospital Bogodogo.

METHODOLOGY

Population

A cross-sectional study was performed in patients that visited rheumatology service of the hospital Bogodogo from February to May 2017. The patients were included according to the following criteria: having RA agreeing with the American College of Rheumatology (ACR) and European League against Rheumatism Collaborative initiative (EULAR) classification for rheumatoid arthritis, diagnosis carried on by a physician, patient treated with methotrexate. Patient not having medical report form was not selected.

Variables

The survey form included the following variables:

(1) Demographic characteristics: age, sex, residence.
(2) Clinical data: antecedents, duration of RA.
(3) Therapeutic data: immunosuppressive drugs including methotrexate and concomitant drugs.
(4) Tolerance: adverse reaction notified in the medical report.
(5) Adherence with methotrexate: discontinuity or temporary break of treatment with methotrexate, reasons of these breaks.

Data collection

Data were collected from June to July 2017. A resident pharmacy student has reviewed the medical dossiers of the patients and interviewed the patients to complete information during their medical visits.

Adherence includes two components: observance and persistence to treatment (Dossa et al., 2015). Only observance with methotrexate was measured on base of the patient's statement. The question to assess observance of the patient was: "During the last month, did you already miss a dose of your treatment with methotrexate?"

To assess adverse reaction of methotrexate, French method of causality assessment applied to individual cases of pharmacovigilance was used (Arimone et al., 2011). The score of intrinsic imputation ranged from I_0 (the lowest score) to I_6 (the highest score) according to the causality level.

Data analysis

Data were registered with Epi-Info 7. Patient was considered "observant" when he responded "No" at the question to assess observance. Statistical analyses were performed by calculated prevalence of patient's characteristics, methotrexate adverse effects and observance.

Ethical considerations

The head of the rheumatology service and the director of Hospital Bogodogo agreed the protocol before the starting data collection. Patients gave their consent for the interview.

RESULTS

Characteristics of the patients

In total, 205 patients with RA were followed in the rheumatology service and 135 (65.9%) patients were treated with methotrexate. All patients treated with methotrexate took folic acid tablet weekly. The distribution of patients with RA according to the anti rheumatism drug is showed in the Table 1.

Table 2. Distribution of patients with rheumatoid arthritis treated with methotrexate according to socio-demographic characteristics and medical antecedent (n=135).

Socio-demographic characteristics		
Characteristics		**Frequency (%)**
Sex	Female	73.3
	Male	26.7
Age group	10-20	3.8
	20-30	16.1
	30-40	22.5
	40-50	21.2
	50-60	19.9
	60-70	10.6
	70-80	5.9
Residence	Urban area	92.8
	Rural area	7.2
Medical antecedent	Gastrointestinal ulcer	10.4
	Hypertension	9.6
	Diabetes	3.7
	Sinus inflammation	2.2
	Abortion	2.2
	Sore throat	1.5
	Asthma	1.5
	Smoking	1.5
	HIV	0,7
	Sickle cells disease	0.7
	Alcoholism	0.7

The Table 2 shows the distribution of patients according to the socio-demographic, clinical and therapeutic characteristics. The duration of RA evolution varied from 1 to 360 months with a mean of 48 month. The mean duration of treatment with methotrexate was 30.2 months.

Tolerance of the methotrexate

Among the patients treated with methotrexate, 59 adverse reactions were observed in 28.8% of them. The Table 3 indicates the distribution of adverse reactions cases according to the score of causality assessment. All the adverse reactions were previously described in the drug reference monograph Martindale (Sweetman, 2011).

Adherence to treatment with methotrexate

Among the 135 patients treated with methotrexate, 53.3% declare taking their medication without break during the last month, 24.4% did temporary one or several breaks of their treatment during the last month. The reasons of inobservance that patients declared are listed in the Table 4. However, some patients missed their medical visit (22.2%). Their observance could not be measured.

DISCUSSION

This study has some limits. Firstly, it has been conducted in patients visiting hospital and results cannot be applied to the global population of Burkina Faso. Secondly, the observance of patients that didn't come at medical visits is not measured. In last, as any cross-sectional study using previous reported data, data about patients are missing in some medical reports.

Methotrexate was used in the majority of patients of our study (65.9%). Andia et al. (2016), found 60% in a study at Niamey National Hospital. In despite of the marketing

Table 3. Distribution of adverse reaction in patients treated with methotrexate according to the score of causality assessment (n=135).

Adverse reaction	Number of cases (%)	Score of causality assessment
Discomfort	12 (8.9)	I_4
Nausea	8 (5.9)	I_4
Vomiting	6 (4.4)	I_4
Allergy	5 (3.7)	I_4
Cough	5 (3.7)	I_4
Stomach pain	4 (2.9)	I_4
Vertigo	4 (2.9)	I_4
Diarrhea	2 (1.5)	I_4
Edema	2 (1.5)	I_4
Anemia	3 (2.2)	I_4
Transaminases augmented	3 (2.2)	I_4
Interstitial lung disease	3 (2.2)	I_4
	1 (0.7)	I_6

Table 4. Reasons declared by patients to justify their inobservance with methotrexate treatment (n=135).

Reason	Frequency (%)
Stock out in community pharmacy	10.4
Adverse effect	5.9
Financial incapacity	3.7
Patient doesn't understand importance of treatment	2.9
Patient desires pregnancy	2.2
Patient travelling	1.5
Surgery of the eye	0.7

of biotherapies, methotrexate is again the main and first intention drug for RA treatment. Its good tolerance, efficacy and affordable cost make it a gold standard for RA medication.

Moreover, the ratio of sex was 0.36 for males. Women are the most affected by RA. The literature confirms high frequency of RA among women (Silman et al., 2002; Slimani et al., 2014; Andia et al., 2016). Patients had a mean age of 44.4 with age minimum and maximum of 10 and 77, respectively. Previous study found mean age of 45.9 in Niamey (Andia et al., 2016), 50.1 in Algeria (Slimani et al., 2014). Regarding the age range affected, RA can occur any age with a tendency in the 25 to 50 years (Le Loët et al., 2006; Slimani et al., 2014, Andia et al., 2016).

The majority of patients lived in urban area (92.8%). Lekpa et al. (2018) found 79.4% living in Dakar. RA was more frequent in urban population than rural individuals. Many environmental factors such as lifestyle would be associated (Hayem, 2002).

The score of imputation is higher than 4 for all adverse reactions. However, adverse effects were observed in one-third of the patient (28.8%). Serious adverse effects were uncommon. These results confirm the good tolerance of methotrexate described in literature. The low frequency of anemia could be related to folic acid used concomitantly with methotrexate.

Finally, the results show that more of the half of patients was inobservant. Patients more frequently cited stock out in community pharmacy and adverse effects of methotrexate to justify their inobservance. There are also uncommon reasons in patients treated with methotrexate: desire of pregnancy and travelling. According to the literature, the main reason of treatment break was adverse effects (WHO, 2003).

Conclusion

This is the first study assessing the patient's tolerance and observance with methotrexate in Burkina Faso. The results corroborate data in literature suggesting in the one hand, that methotrexate has a good tolerance and in the other hand, that observance with methotrexate is

inadequate for most patients. Therapeutic education and strengthening methotrexate supply chain should be undertaken to improve the care of patients with RA in Burkina Faso.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Use of medicinal plants for the treatment of gastric ulcer in some parts of Southwestern Nigeria

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Ulcer is erosion in the lining of the stomach or duodenum. It is caused by disruption of the gastric mucosal defense and repair systems. There has been much interest in natural medicines derived from traditional knowledge of pharmacological properties of plants recently. The major aim of this study was to conduct an ethnobotanical survey of plants used in the treatment of Gastric ulcers in some local government areas of Ibadan, South-West, Nigeria. Five local government areas which have prominent traditional medical practitioners and herbal markets were visited. Ninety-two plants belonging to forty-five different families were recorded. The prominent plants' families recorded include; Apocynaceae, Loranthaceae, and Lamiaceae. Some of the most frequently used medicinal plants mentioned by the respondents are: *Sphenocentrum jollyanum* Pierre, *Euadenia trifoliolata* (Sch. &Thon.) Oliv., *Khaya ivorensis* A. Chev., *Lonchocarpus cyanescens* Benth, and *Kigelia africana* (Lam.) Benth. Most of the herbs were prescribed together with other plants and recipes. Modes of administration were mostly concoctions and decoctions. Cultivation and proper documentation of some of the plants which may become endangered over time is therefore encouraged. Most of the identified plants have been used regularly by the traditional medical practitioners and the efficacies have been proven.

Key words: Gastric ulcer, ethnobotanical survey, medicinal plants, traditional medicine, South-west Nigeria.

INTRODUCTION

Traditional medicines entail the frequent use of medicinal plants in the management of gastric disorders (Schmeda-Hirschman and Yesilada, 2005). Several experimental studies have revealed the beneficial effects of herbal and plant extracts in the prevention of gastric injury. These studies have shown the three main functions of medicinal plants such as cytoprotective, antisecretory and antioxidant activities either alone or combined; they are responsible for protection against gastric mucosa (Al-Mofleh, 2010; Salami et al., 2014). Plant materials have been a main source of natural therapeutic remedies and are used in the treatment of various infectious diseases in many developing countries (Beverly and Sudarsanam, 2011; Dike et al., 2012). Medicinal plants have confirmed

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their contribution to the treatment of diseases, such as malaria, diabetes, HIV/AIDS, mental disorders, sickle-cell anaemia (Elujoba et al., 2005) and microbial infections (Okigbo and Mbajuka-Nyoku, 2005).

Ulcer can be defined as erosion in the lining of the stomach or duodenum and is caused by the disruptions of the gastric mucosal defense and repair systems (Al-Mofleh, 2010). Gastric ulcer is formed in the stomach. The drugs used in the treatment of ulcer include proton pump inhibitors, receptor blockers, and drugs affecting the mucosal barrier and acting on the central nervous system. The modern approach used to control gastric ulceration is to inhibit gastric acid secretion, promote gastro protection, block apoptosis and stimulate epithelial cell proliferation for effective healing (Bandyopadhyay et al., 2000). Hence, conventional medicine treats ulcer by proton pump inhibitors (PPI), H_2 receptor antagonist, antacids and antibiotics for *Helicobacter pylori*. However, there are reports of adverse effects and relapse in the long run. This makes people to find alternative medications. Furthermore, many of these drugs do not achieve all the valuable necessities (Dharmani and Palit, 2006). The clinical evaluation of these drugs showed development of tolerance and incidence of relapse and side effects thus making their efficacy debatable. This has been the rationale for the development of new, safe anti-ulcer drugs. Herbal drugs can lead to the development of such anti-ulcer drugs because these drugs are considered safe due to their natural ingredients. Recently, focus on plant research has increased all over the world and a large body of evidence has been collected to show huge potential of medicinal floras used in several traditional systems of medicine. There has been much attention given to natural medicines derived from the traditional knowledge of the pharmacological properties of plants recently. Herbal medicine is fast emerging as a substitute treatment to available synthetic drugs for ulcer management possibly due to lower costs, fewer adverse effects, availability and perceived effectiveness. Many tropical herbs have been scientifically reported to possess effective anti-ulcer activity (Goulart and Sela, 2005; Singh et al., 2008). Medicinal plants and dietary nutrients have been shown to possess gastro-protective activity (Kath and Gupta, 2006; Malairajan et al., 2007; Siti et al., 2009).

Kumar et al. (2011) reported some plants used for the treatment of peptic ulcer disease (PUD) in India. Some of these plants include *Amomum subulatum*, *Scoparia dulcis*, *Jasminum grandiflorum*, *Davilla rugosa*, *Kielmeyera coriacea*, *Larrea divaricata*, *Qualer grandiflora*, *Mammea americana*, *Anacardium occidentale*, *Ocimum sanctum*, and *Azadirachta indica*. Previous works have been conducted on the anti-ulcer activities of medicinal plants including Bhajoni et al. (2016) on *A. indica*; Bello et al. (2016) on *Cassia sieberiana;* Balogun et al. (2013) on *Nauclea latifolia* among others. However, there is little or no documentation of medicinal plants used for the treatment of gastric ulcer in Southwestern Nigeria. This documentation will serve as reference to researchers and traditional medical practitioners alike. Nigeria is known to be one of the most important countries in West Africa and is richly blessed with great diversity of medicinal plants. However, some of these medicinal plants are becoming threatened and endangered (Sonibare and Abegunde, 2012). The present study therefore aims to document the various medicinal plants used in treating gastric ulcer in Southwestern Nigeria.

MATERIALS AND METHODS

Study area

The local government areas visited for the survey include: Ibadan South/West LGA, Akinyele LGA, Oluyole LGA, Ibadan North East LGA, and Egbeda LGA. The locality map is as shown in Figure 1. Ibadan South/West includes Molete and Bode market, Akinyele LGA includes Ojoo, Idi-ose, Moniya, and Ajibode, Oluyole LGA includes Idi Ayunre and Cocoa Research Institute of Nigeria (CRIN), Ibadan North-East LGA includes Oje market while Egbeda LGA includes Gbagi market, Alakia market. The five LGAs are part of the 11 LGAs of Ibadan, Oyo State with latitude 7°22′N and longitude 3°55′E. The region, being South West has tropical climate with two distinct seasons: dry and wet. The dry season is usually between November and February. Rainfall occurs throughout the year with an average annual rainfall of 250 cm^3 while dry season is usually between November and February. The areas still have villages with little or no access to modern health care and thereby rely on traditionalists and TMPs for solutions to their health challenges. Most of the natives interviewed are Yoruba and their occupations include herb selling and trading. Some of the places are rural areas which are not well developed, while some of the areas are moderately urbanized as shown in Figure 1.

Informed consent

The people visited include traditional medicine practitioners (TMP), herb sellers, and the elderly who have knowledge of medicinal plants used in the treatment of various ailments including gastric ulcer. Informed consent was obtained orally from all participants including TMPs, herb sellers and the elderly before inception of the interview.

Questionnaire administration and data collection

The survey was conducted between August 2015 and February 2016 where focused group discussion method was used at Traditional Medical Practitioners' meeting which usually take place fortnightly. The association of TMPs at Oluyole Local Government of Ibadan comprises both males and females; twelve members were present on the day of the interview. Herbs markets were mostly visited in the local governments and the herb sellers who were all women were interviewed. There were brief interruptions during the interview as customers visit them to buy herbs.

Semi-structured questionnaire and oral interview were used to obtain the relevant information and, in few cases, incentives were

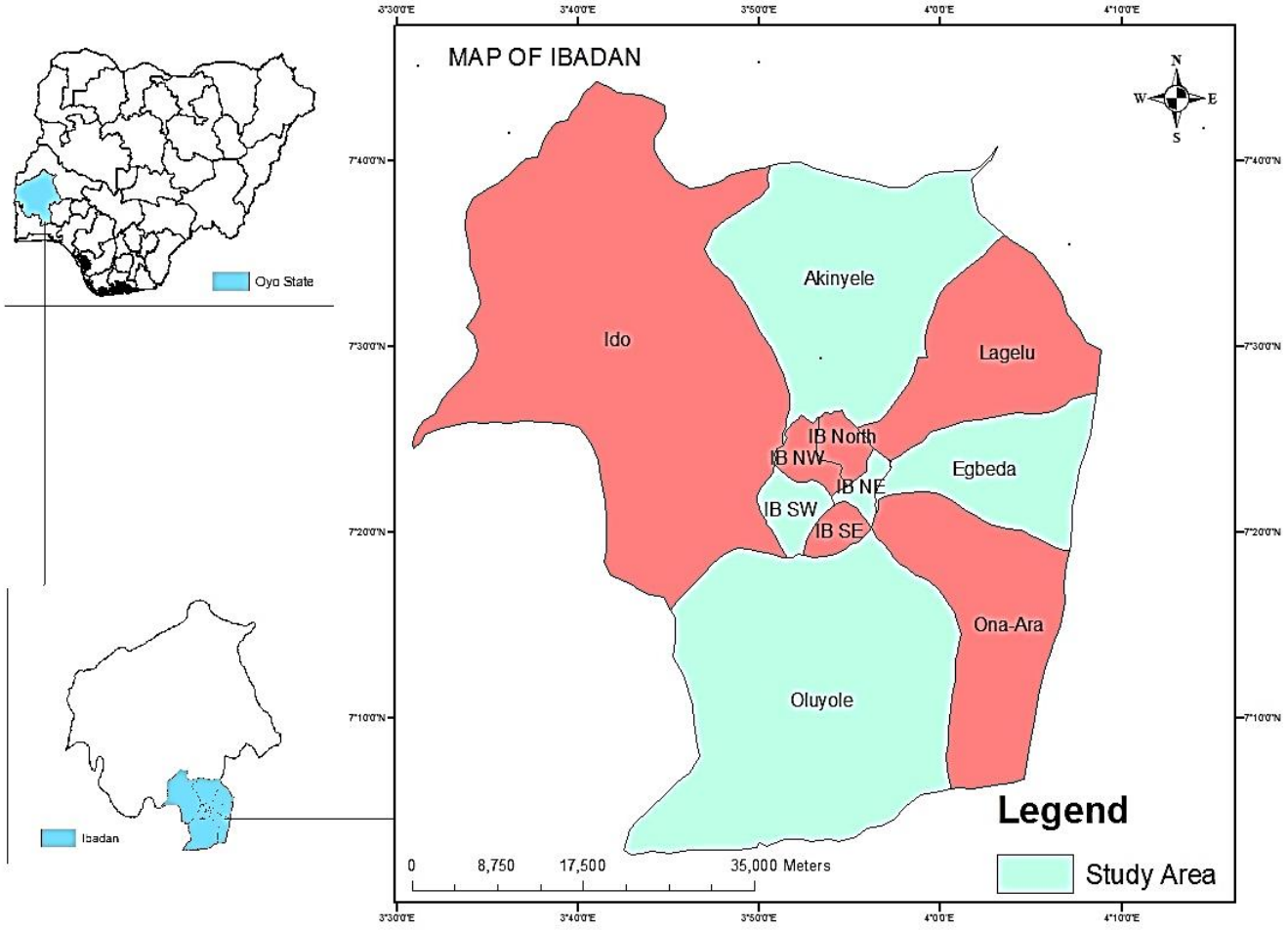


Figure 1. Map of Ibadan showing the local government areas visited for the survey.

given to unwilling respondents. Some of the questions asked included: the local name and scientific name of the medicinal plants used in the treatment of gastric ulcer, mode of administration of the plant, side effects of the mentioned plant, other method(s) of treatment apart from herbs, etc. Demographic information such as name, age, sex, religion, marital status, education, and occupation of the respondents was obtained. The vernacular names of the plants were given in most cases because most of the respondents are not learned; textbooks and journals were consulted to confirm the botanical names (Gbile, 1984; Burkill, 1995). Fidelity level was employed using the method of Friedman et al. (1986) which shows the percentage of respondents claiming the use of each plant species for the same major purpose. This was calculated as:

$$FL\ (\%) = (Np / N) \times 100$$

where Np = Number of respondents claiming a specific use for each of the plants mentioned; N = Total number of respondents using the species for any purpose.

The top mentioned plants were collected, identified, and authenticated. Voucher specimens of the plants were deposited at Forest Herbarium Ibadan (FHI) with their respective FHI numbers. Isolated compounds from the plants were reported.

RESULTS

Demographic data

Seventy-two respondents participated in the survey and were not secretive about their knowledge of the medicinal properties of the plant species. The responses were encouraging throughout the survey. The population of the respondents is made up of herb sellers (72.3%), TMPs (16.6%), and the elderly (11.1%). Their ages were between 25 and 70 years. All the respondents were Nigerians from the Yoruba ethnic group with 23.7% practicing Christianity, 34.7% practicing traditional religion and 41.6% practicing Islamic religion. The age group 41-60 had the highest percentage (50%) while the majority of the respondents were females (69.4%). Most of the respondents practice Islam (41.6%), followed by traditional religion (34.7%), then Christianity (23.7%) (Figures 2 to 5).

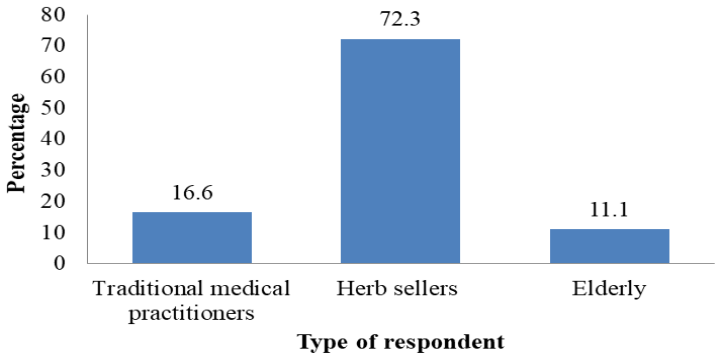


Figure 2. Type of respondent (%).

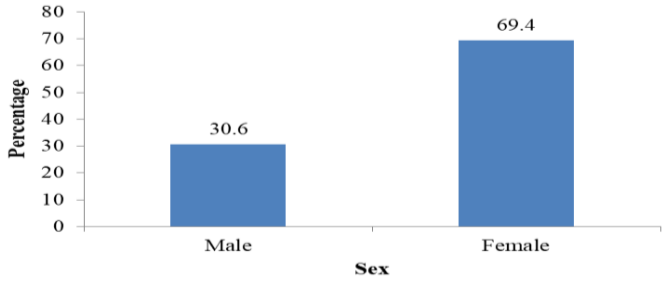


Figure 3. Sex of respondent (%).

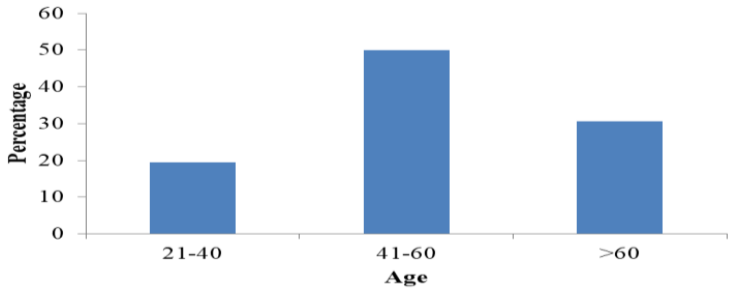


Figure 4. Age of respondents (%).

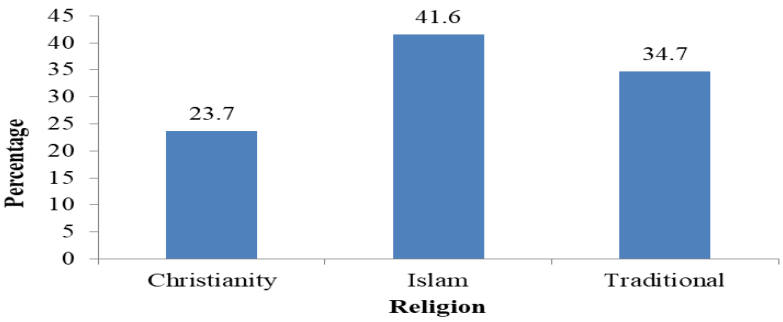


Figure 5. Religion of respondents (%).

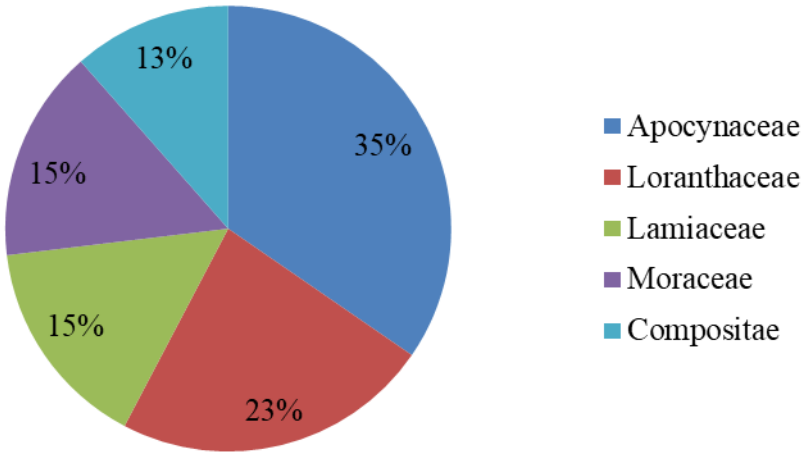


Figure 6. Distribution of plant species according to family (%).

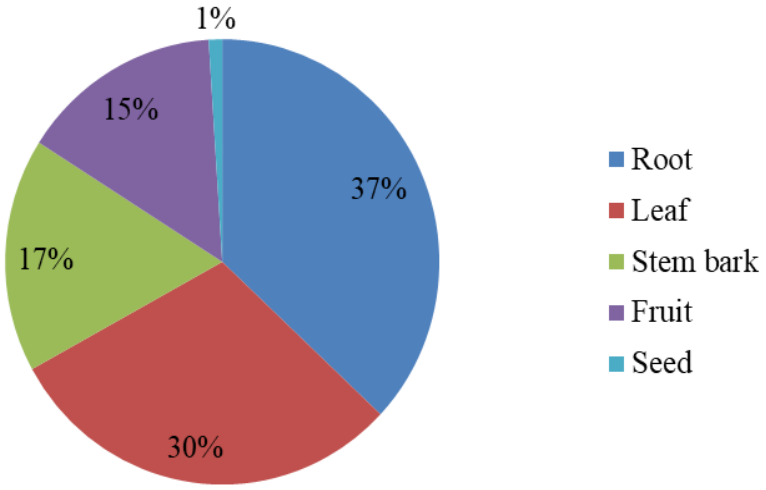


Figure 7. Plant parts used in the treatment of gastric ulcer (%).

Plant information and their families

A total of 92 plant species belonging to 45 different families were identified (Table 1). The table shows the list of identified plant species, families, local names, plant parts used, frequency of occurrence, and fidelity level. The most prominent among these plant families are the Apocynaceae, Loranthaceae and Lamiaceae with nine, six, and five species, respectively. Other plant families include Compositae and Moraceae with four species each (Figure 6). The plants' roots and leaves are mostly used for herb preparation while the seeds are least used (Figure 7). Many of these plant species are also used for treating wounds and diabetes. Most of the respondents confirmed regular supply of their plant remedies from the forest. Knowledge of herbal treatment was mainly inherited; few of the informants went for the training. Some of the mentioned plants include *Ageratum conyzoides* L., *Aloe barbadensis* L., *Alstonia scholaris* (L.) R. Br., *Artocarpus altilis* (Parkinson) Fosberg, *Artocarpus integrifolia* Auct, *Aspilia africana* (Persoon) C.D. Adams, *Bacopa monniera* L., *Benincasa hispida* Thunb., *Bryophyllum pinnatum* (Lam.) Oken, *Carica papaya* L., *Ceiba pentandra* (L.) Gaertn., *Centella asiatica* L., *Citandra cymulosa* Benth, *Citrus aurantium* L. among others (Table 1).

Many of these plants are obtained from the forest, while a few are collected from the garden around the house.

Method of preparation

The herbal remedies can be prepared either from dry

Table 1. Medicinal plants used for the treatment of gastric ulcer

S/N	Botanical name	Family	Local names	Part(s) used	Frequency of occurrence	Fidelity Level (%)
1	*Ageratum conyzoides* L.	Compositae	Imi-esu	Leaf	4	5.6
2	*Aloe barbadensis* L.	Liliaceae	Eti-erin	Root	5	6.9
3	*Aloe barteri* L.	Liliaceae	Eti-erin	Leaf	5	6.9
4	*Alstonia boonei* (L.)R.	Apocynaceae	Ahun	Whole plant	6	8.3
5	*Ananas comosus* (L.) Merr.	Bromeliaceae	Penapu ibile	Fruit	1	1.4
6	*Artocarpus altilis* (Parkinson)	Moraceae	Berefuutu	Leaf	2	2.8
7	*Artocarpus integrifolia* auct.	Moraceae	Tapoun	Root	2	2.8
8	*Asparagus racemosus* Willd.	Liliaceae	Aluki	Root	4	5.6
9	*Aspilia africana* (Persoon) C.D. Adams	Compositae	Yonyon-agbute	Leaf	1	1.4
10	*Bacopa monniera* L.	Scrophulariaceae	Awenu	Fruit	1	1.4
11	*Benincasa hispida* Thunb	Cucurbitaceae	Abua	Fruit	1	1.4
12	*Bryophyllum pinnatum* (Lam.) Oken	Crassulaceae	Abamoda	Leaf	1	1.4
13	*Byrsocarpus coccineus Schumach & Thonn.*	Connaraceae	Amuje	Stem bark	3	4.2
14	*Carica papaya* L.	*Carica papaya* L.	Ibepe	Seed, fruit	5	6.9
15	*Ceiba pentandra* (L.) Gaertn.	Malvaceae	Araba	Leaf	5	6.9
16	*Centella asiatica* L.	Apiaceae	Tutugbo	Fruit	1	1.4
17	*Citandra cymulosa* Benth	Mimosaceae	Aagba	Root	1	1.4
18	*Citrus aurantium* L.	Rutaceae	Jaganyin	Leaf	4	5.6
19	*Citrus medica* L.	Rutaceae	Osan wewe	Leaf, Root	6	8.3
20	*Citrus sinensis* L.	Rutaceae	Jaganyin	Leaf, Root	6	8.3
21	*Clitandra orientals Hall.*	Apocynaceae	Aagba	Root	4	5.6
22	*Clitandra togolana Hall.*	Apocynaceae	Aagba	Root	3	4.2
23	*Cocos nucifera L.*	Arecaceae		Fruit	2	2.8
24	*Cruda klainei Pierre ex De Wild.*	Caesalpinaceae	Afomo	Leaf	6	8.3
25	*Curculigo pilosa*	Hypoxidaceae	Epakun	Seed	25	34.7
26	*Dalbergia lactea Vatke.*	Papilionaceae	Ojiji	Root	8	11.1
27	*Desmodium gangeticum (L.) DC.*	Papilionaceae	Emo	Root	12	16.7
28	*Detarium microcarpum Guill. & Perr.*	Caesalpinaceae	Arira	Stem bark	12	16.7
29	*Emblica officinalis Gaertn*	Euphorbiaceae	Ojiji	Leaf	8	11.1
30	*Englerina goborensis (Engl.) Balle*	Loranthaceae	Afomo	Leaf	12	16.7
31	*Englerina lecardii (Engl.) Balle*	Loranthaceae	Afomo	Leaf	9	12.5
32	*Entada gigas L.*	Mimosaceae	Aagba	Root	28	38.9
33	*Euadenia trifoliolata (Sch. &Thon.) Oliv.*	Capparaceae	Logbokiya	Leaf	26	36.1
34	*Ficus arnottiana Miq.*	Moraceae	Obata	Fruit	7	9.7
35	*Ficus exasperata Vahl.*	Moraceae	Ipin	Root	7	9.7

Table 1. Contd.

36	*Floscopa africana (P. Beauv) C.B. Clarke*	Commelinaceae	Toronini	Root	11	15.3
37	*Fluerya aestuans L.*	Urticaceae	Iranje	Leaf	12	16.7
38	*Garcinia cambogia L.*	Clusiaceae	Okuta	Fruit	10	13.9
39	*Globimetula braunii (Engl.) Danser*	Loranthaceae	Afomo	Leaf	12	16.7
40	*Hedranthera barteri (Hook. F.) Pichon*	Apocynaceae	Oko aja	Root	6	8.3
41	*Hemidesmus indicus (L.) R. Br.*	Apocynaceae	Ogbe akuko	Root, leaf	7	9.7
42	*Hoslundia opposite Vahl*	Lamiaceae	Efinrin odan	Leaf	9	12.5
43	*Hunteria umbellate K. Schum*	Apocynaceae	Erin	Root	12	16.7
44	*Khaya ivorensis A. Chev.*	Meliaceae	Ogano	Stem bark	28	38.9
45	*Kielmeyera coriaceae Mart. & Zucc.*	Caricaceae	Emo	Stem bark	9	12.5
46	*Kigelia africana (Lam.) Benth.*	Bignoniaceae	Pandoro	Root, stem bark, fruit	26	36.1
47	*Lagenaria siceraria (Mol.) Standl*	Cucurbitaceae	Igba	Stem bark	3	4.2
48	*Lonchocarpus cyanescens benth*	Papilionaceae	Elu	Leaf	22	30.6
49	*Macaranga barteri Mull. Arg*	Euphorbiaceae	Agbaasa	Root	10	13.9
50	*Markhamia tomentosa (Benth.) K. schum*	Bignoniaceae	Oruru	Root, Bark	9	12.5
51	*Microdesmis puberula Hook. F. ex Planch.*	Pandaceae	Aringo	Leaf	12	6.7
52	*Morinda citrifolia L.*	Rubiaceae	Oruwo	Fruit, Root	18	25.0
53	*Motandra guineensis (Thonn.) A.D.C.*	Apocynaceae	Aagba	Root	21	29.2
54	*Musa paradisiaca L.*	Musaceae	Ogede agbagba	Fruit	23	31.9
55	*Musa sapientum L.*	Musaceae	Omini	Fruit	21	29.2
56	*Ocimum basilicum L.*	Lamiaceae	Efinrin aja	Leaf	18	25
57	*Ocimum sanctum L.*	Lamiaceae	Efinrin aja	All parts	16	22.2
58	*Oxytenanthera abyssinica (A. Rich.) Munro*	Poaceae	Aparun	Root	13	18.1
59	*Parkia biglobosa Jacque Benth.*	Mimosaceae	Igba	Stem bark	22	30.6
60	*Parquetina nigrescens Afzel*	Asclepiadaceae	Ogbo	Root, Leaf	18	25
61	*Peperomia pellucida (L.) Kunth*	Piperaceae	Erin	Root	12	16.7
62	*Persea Americana Mill.*	Lauraceae	Oguro	Leaf	12	16.7
63	*Phragmanthera capitate (Sprengel) S.Balle*	Loranthaceae	Afomo	Leaf	5	6.9
64	*Phragmanthera kamerunensis (Engl.) Balle*	Loranthaceae	Afomo	Leaf	7	9.7
65	*Picralima nitida Thellung*	Apocynaceae	Erin	Root	9	12.5
66	*Piper guineense Schumach. & Thonn.*	Piperaceae	Iyere	Root	11	15.3
67	*Piper nigrum L.*	Piperaceae	Iyere	Fruit	12	16.7
68	*Plectranthus amboinicus (Lour.) Spreng*	Lamiaceae	Aringo	Whole plant	6	8.3
69	*Plumbago zeylanica L.*	Plumbaginaceae	Inabiri	Root	7	9.7
70	*Pseudocedrella kotschyi (Schweinf.) Harms*	Meliaceae	Emigbegiri	Stem bark	29	40.3
71	*Pycnanthus cingolensis (Welw.) Warb.*	Myristicaceae	Akomu	Stem bark	12	16.7

Table 1. Contd.

72	*Pyrus communis L.*	Rosaceae	-	Leaf	5	6.9
73	*Rauvolfia vomitoria Afzel.*	Apocynaceae	Asofeyeje	Root, leaf	4	5.6
74	*Ricinodendron heudelotii (Hutch. & E.A. Bruce)*	Euphorbiaceae	Erinmodo	Stem bark	7	9.7
75	*Ritchiea capparoides (Andrews) Britten*	Capparaceae	Capparaceae	Leaf	6	8.3
76	*Sarcocephalus latifolius Smith Bruce*	Rubiaceae	Egbesi	Stem bark, root	5	6.9
77	*Securidaca longepedunculata Fres.*	Polygalaceae	Ipeta	Root	14	19.4
78	*Spathodea campanulata Beauv.*	Bignoniaceae	Ruuru	Root, stem bark	3	4.2
79	*Spondias mombin L.*	Anacardiaceae	Iyeye	Stem bark	14	9.4
80	*Sphenocentrum jollyanum Pierre*	Menispermaceae	Akerejupon	Root, fruit	30	41.7
81	*Staudtia stipitata Warb.*	Myristicaceae	Amuje	Stem bark	4	5.6
82	*Talinum triangulare (Jacq.) Willd*	Portulacaceae	Gbure	Leaf	8	11.1
83	*Tapinanthus buntingii (Sprague) Danser*	Loranthaceae	Afomo	Leaf	7	9.7
84	*Terminalia pallida Brandis*	Combretaceae	Idi	Whole plant	14	19.4
85	*Terminalia superba Engl. & Diels.*	Combretaceae	Afara	Stem bark	12	16.7
86	*Urena lobata Linn.*	Malvaceae	Efore loka	Leaf	15	20.8
87	*Uvaria afzelii Sc. Elliot*	Annonaceae	Gbogbonise	Root	30	41.7
88	*Uvaria chamae P. Beauv*	Annonaceae	Eruju	Root	30	41.7
89	*Vernonia amygdalina Delile*	Compositae	Ewuro jije	Root	18	25
90	*Vernonia odoensis Delile*	Compositae	Ewuro oko	Root	12	16.7
91	*Vitellaria paradoxa C.F Gaertn.*	Sapotaceae	Emiyemi	Stem bark	16	22.2
92	*Zingiber officinale Rosc.*	Zingerberaceae	Atale	Leaf	14	19.4

plants from markets or freshly collected samples from the forest, around homes or home gardens. However, the respondents confirmed that both forms of plant materials are efficient in the preparation of the herbal remedies except in some cases where freshly collected samples are preferred. The main method of preparation given is decoction (boiling in water). Others are infusion and concoction. Preference was given to the method of decoction. The time required for boiling varies and is dependent on plant parts or nature of plant. The preparation is to be taken orally in all cases.

Enumeration of species

The herb sellers mentioned some plants which should be prepared as concoction; *Citrus medica* L. leaves and roots, *Citrus sinensis* L. leaves and roots, *Citrus medica* var. *acida* leaves and roots. Some herb sellers also mentioned that the fruit and root of *Sphenocentrum jollyanum* Pierre should be ground into powder and drunk with pap or water. Root of *Vernonia amygdalina* Delile should be cooked with water (decoction) and allowed to cool before drinking. Other recipes such as honey and raw egg are usually used with *Ananas comosus* (L.) Merr. fruit. Root of *Uvaria afzelii* Sc. Elliot and stem bark of *Parkia biglobosa* Jacque Benth. are boiled together with water and taken orally on a daily basis. Some medicinal plant species are used in combination when boiled with water; these include stem barks of *Vitellaria paradoxa* C. F. Gaertn., *Khaya ivorensis* A. Chev., and *Pseudocederela kotshyi* (Schweinf). Harms. fruits and roots of *Morinda citrifolia* L., roots and fruits of *S. jollyanum* Pierre, stem barks of *Detarium microcarpum* Guill. & Perr., *Staudtia stipitata* Warb., *Kigelia africana* (Lam.) Benth. and *Sarcocephalus latifolius* Smith Bruce. A particular

Table 2. Authentication of selected plants.

Plant sample	Voucher Specimen no.
Curculigo pilosa	FHI 109816
Entada gigas	FHI 110507
Euadenia trifoliolata	FHI 110522
Kigelia africana	FHI 110520
Sphenocentrum jollyanum	FHI 110510
Uvaria chamae	FHI 110508
Vitellaria paradoxa	FHI 109816

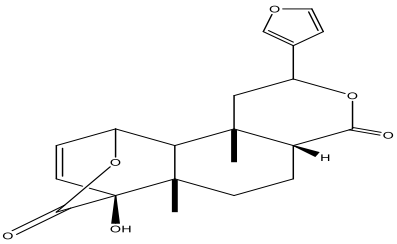
Identification and authentication of most mentioned plants

The voucher specimens of the most mentioned plants with their respective FHI numbers are presented in Table 2 with some of the reported isolated compounds (Figure 8). Moody et al. (2005) isolated columbin and isocolumbin from Sphenocentrum jollyanum seeds. Catechin and epicatechin were isolated from Vitellaria paradoxa stem bark (Emmanuel et al., 2016), while Valls et al. (2006) isolated pilosidine and Piloside A from Curculigo pilosa rhizomes. Uvaretin and isouvaretin were isolated from Uvaria chamae (Hufford and Lasswell, 1976), while Lazare et al. (2015) isolated lupeol and β sitosterol from Kigelia africana (Figure 8).

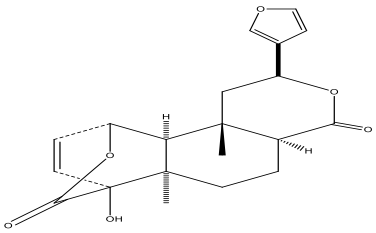
DISCUSSION

The ethnobotanical survey on medicinal plants with anti-ulcer activities was conducted to access information about the uses of plants in traditional medicine. A total of 72 respondents were accessed who were assisted to complete the questionnaire. Respondents were located in five different local governments in Ibadan, South West Nigeria: Ibadan South/West LGA, Akinyele LGA, Oluyole LGA, Ibadan North East LGA, and Egbeda LGA. Majority of the respondents were herb sellers (52 respondents), while 12 of the respondents were Traditional Medical Practitioners TMPs (12 respondents), and 8 of the respondents were elderly. All respondents belonged to the native Yoruba tribe and they speak Yoruba dialect fluently. The findings from this survey suggest that most of the plant families have prospects and thus can be explored for drug development scientifically. The family Apocynaceae has a comparatively high incidence in the list thus may contain useful species that can be further explored as sources of anti-ulcer drugs. The results also showed that majority of the respondents are familiar with the use of certain species such as *Entada gigas*, *K. africana*, *Curculigo pilosa*, *K. ivorensis*, *Lonchocarpus cyanescens*, *P. biglobosa*, *S. jollyanum*, *Musa sapientum*, *Uvaria chamae*, *U. afzelii*, *P. kotschyi*, and *Euadenia trifoliolata* in the treatment of gastric ulcer. This was inferred from the frequency of occurrence and fidelity level of the plant species. This result also revealed that various parts of the plants especially the leaves, stem bark, roots, and fruits but rarely the whole plants have been used in the treatment of the disease. Investigations on the plant parts used and the mode of preparation and administration revealed that water was the main medium for all the medicinal preparations irrespective of the plant part(s) or combinations used. The drug was administered along with honey or hot pap. Most of the respondents claimed there are usually no side effects in the use of the medicinal plants. Many scientific researchers have reported the anti-ulcer activities of some of the plants identified in this study. Chilaka et al. (2010) and Orole et. al. (2013) worked on *U. chamae* and *K. africana,* respectively. Ghangale et al. (2009) also evaluated the aqueous extract of *O.sanctum* for its anti-ulcer activity against methanol induced ulcer in Wistar rats. The investigation revealed that *O. sanctum* exhibited significant antiulcer activity by enhancing antioxidant potential of gastric mucosa thereby reducing mucosal damage. Chioma and Raymond (2013) also worked on the anti-ulcerogenic activity of the methanol extract of *C. pentandra* stem bark on indomethacin and ethanol-induced ulcers in rats. The results showed that *C. pentandra* possess ulcer protective properties against experimentally induced ulcers and validates its traditional use in the treatment of stomach pain and ulcer. Kayode et al. (2015) also evaluated the anti-ulcer activities of *Securidaca longepedunculata* and *Luffa cylindrica* in Wistar rats using ethanol-induced gastric ulcer. The study demonstrates that the leaf extracts of the two plants are possible potent gastro-protective and anti-ulcer agents, thus providing evidences that may justify their ethno-medicinal uses as anti-ulcer agents.

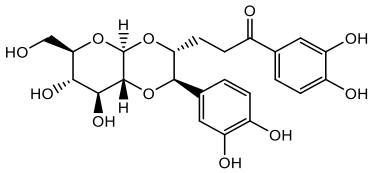
The survey showed that majority of the plant species used for the treatment of gastric ulcer is sourced from the wild. It has been reported that although, medicinal plants are necessary in deciding a programme of action for primary health care, most of the traditional medical practitioners have not cultivated the habit of conservation techniques. Most of these genetic resources are largely



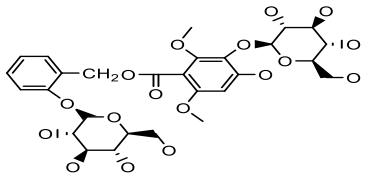
Columbin from *Sphenocentrum jollyanum* (Moody et al., 2005)



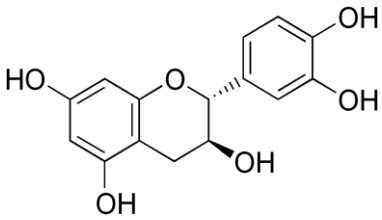
Isocolumbin from *Sphenocentrum jollyanum* (Moody et al., 2005)



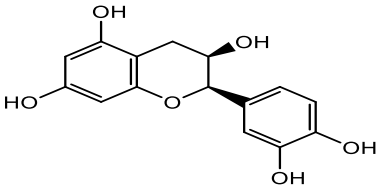
Pilosidine from *Curculigo pilosa* (Valls et al., 2006)



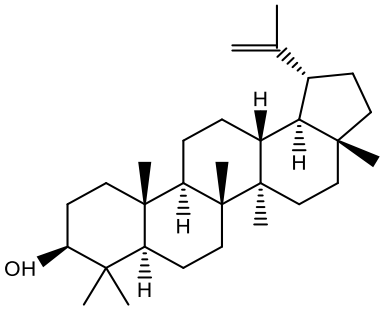
Piloside A from *Curculigo pilosa* (Valls et al., 2006)



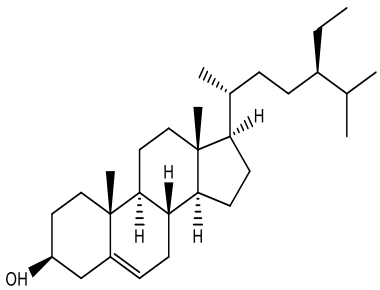
Catechin from *Vitellaria paradoxa* (Emmanuel et al., 2016)



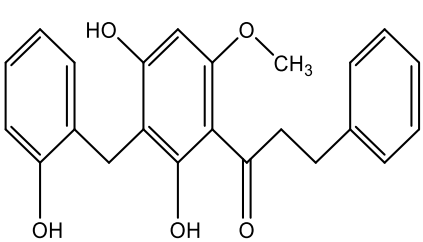
Epicatechin from *Vitellaria paradoxa* (Emmanuel et al., 2016)



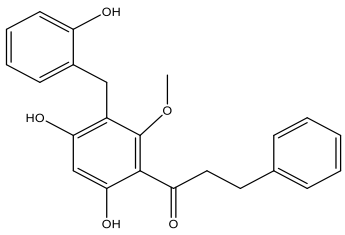
Lupeol from *Kigelia africana* (Lazare et al., 2015)



β-Sitosterol from *Kigelia africana* (Lazare et al., 2015)



Uvaretin from *Uvaria chamae* (Hufford and Lasswell, 1976)



Iso-uvaretin from *Uvaria chamae* (Hufford and Lasswell, 1976)

Figure 8. Isolated compounds from selected plants used in the treatment of gastric ulcer.

undocumented for now and the indigenous knowledge of their relevance is steadily being lost due to unsustain and continuous harvesting of plants from the wild. Reports of unsustainable harvesting of various medicinal plants in different communities in Africa and other continents of the world are quite enormous (Soladoye et al., 2005). The case was not different in the areas visited in Ibadan during the survey as some of the herb sellers and Traditional Medical Practitioners (TMPs) indicated the problem they encounter in finding some of the herbs at localities where they previously existed. This might be an indication of the fast disappearance of these plants which may eventually lead to extinction of these important species if urgent measures are not taken.

An estimate of 70 to 80% of people worldwide relies mostly on traditional, largely herbal medicine to meet their primary healthcare needs (Pei, 2001). Unfortunately, there are many cases of unsustainable harvesting of various medicinal plants in different communities in Africa and other continents of the world. The use of important medicinal plants in a sustainable way must be ensured in order to have a considerable long term effect on the environment, health care and economy (Wong et al., 2001). Therefore, encouraging cultivation and proper documentation of these plants is essential to prevent them from being endangered and going into extinction. Based on the information on the local uses of these medicinal plants, biological activities and subsequent isolation of the biologically active compounds from the plants can be carried out which will thus form the basis for future drug discovery from these indigenous medicinal plants. Bioactive compounds have been previously isolated from some of the mentioned plants. Moody et al. (2006) isolated columbin and isocolumbin from *S. jollyanum* seeds. Catechin and epicatechin were isolated from *V. paradoxa* stem bark (Emmanuel et al., 2016), while Valls et al. (2006) isolated pilosidine and Piloside A from *Curculigo pilosa* rhizomes. Conservation of the traditional knowledge of these medicinal plants is greatly advised for future reference. Research is ongoing in the laboratory on the gastroprotective activity of selected plants using indomethacin induced gastric ulcer in Wister rats. Bioactive compounds which could serve as lead compounds will be isolated, characterised, and elucidated in addition to the existing bioactive compounds from medicinal plants.

Conclusion

This study revealed ninety-two medicinal plants used in the treatment of gastric ulcer in five local government areas in Ibadan, Southwestern part of Nigeria. This report provides relevant information on indigenous knowledge on plants used for the treatment of gastric ulcer in the study area. The documentation apart from broadening the existing knowledge on the plants used could also serve as a guide for further work on the reported plants for the treatment of gastric ulcer. The study plays an important role in the documentation and conservation of traditional knowledge of plants for present and future use.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effects of methanolic tuber extract of *Cyperus esculentus* Linn (tiger nuts) on sub-acute liver damage in albino rats

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This study evaluated the effects of *Cyperus esculentus* methanolic tuber extract (CEME) on carbon tetrachloride (CCl_4)-induced sub-acute liver damage in albino rats. Dried tubers of *C. esculentus* were pulverized and extracted by cold maceration, using 80% methanol. Thirty albino rats, randomly assigned to 6 groups (A–F) of 5 each were used for the study. Sub-acute liver damage was induced in Groups A-E rats using intra-peritoneal injections of CCl_4. Group A was treated with distilled water placebo, while Groups B, C and D were treated with 200, 400 and 800 mg/kg CEME, respectively. Group E was treated with 100 mg/kg Silymarin and Group F was also given distilled water placebo. Treatment was done orally for 15 days, after which hepatocellular integrity and liver function were evaluated. Results showed that treatment with CEME (at all doses) led to significantly lower ($p<0.05$) serum alanine aminotransferase and aspartate aminotransferase activities, bilirubin levels and relative liver weight of the CEME-treated groups, when compared to Group A rats. It was concluded that administration of CEME as used in the study led to significant protection of hepatocellular integrity, enhancement of hepatic excretion of bilirubin and amelioration of CCl_4-induced inflammatory enlargement of the liver.

Key words: *Cyperus esculentus,* methanolic tuber extract, hepatoprotection, liver damage.

INTRODUCTION

The liver is the largest solid organ in the body (Kuntz and Kuntz, 2006; Saukonnen et al., 2006). It is involved in the metabolism of numerous substances (including bilirubins, pophyrins, bile acids, amino acids and proteins,

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carbohydrates, lipids and lipoproteins, hormones and vitamins), biotransformation, detoxification and acid-base balance (Kuntz and Kuntz, 2006). Its multi-various functions constantly expose it to injury that may lead to different forms of liver disorders/diseases (Ihedioha, 2005; Saukonnen et al., 2006). Liver diseases have a worldwide distribution, and are a major cause of morbidity and mortality globally (Blachier et al., 2012; Lozano et al., 2012; Nwokediuko et al., 2013; Sarin and Maiwal, 2018). Diseases of the liver have been ranked the fifth most common cause of death and the second leading cause of mortality amongst all digestive diseases (Williams, 2006; Lozano et al., 2012; Rehm et al., 2013; Sarin and Maiwal, 2018). Toxic liver diseases constitute a large proportion of liver disorders/diseases, and its occurrence has been steadily increasing over the years (Suk and Kim, 2012; Rehm et al., 2013; Nwokediuko et al., 2013).

Plants constitute a large part of traditional medicines and continue to provide mankind with therapeutic remedies and novel drug leads (Gurib-Fakim, 2006; Newman and Cragg, 2012). Despite the availability of modern medicines, medicinal plants are commonly used in developing countries to meet most primary health care needs, and many people in developed countries also patronize medicinal plants-based alternative and complementary therapies (WHO, 1999; Veeresham, 2012). However, most plant species that are traditionally used as medicines have not been scientifically evaluated for efficacy and possible orthodox medical applications (WHO, 1999; Gurib-Fakim, 2006; Atanosov et al., 2015).

Cyperus esculentus is a grass-like plant in the family *Cyperaceae* (De Vries, 1991; Takhatajah, 1992; Coskuner et al., 2002; Dhouha et al., 2016). It is commonly known as chufa (in Spanish) (Oderinde and Tairu, 1988; Dhouha et al., 2016), other names include tiger nut, earth nut, yellow nut sedge, ground nut and rush nut (Oderinde and Tairu, 1988; Umerie et al., 1997; Coskuner et al., 2002; Oladele and Aina, 2007; Arafat et al., 2009; Sanchez-Zapata et al., 2012; Dhouha et al., 2016). Tiger nut tubers are freely growing and are eaten uncooked in their natural form (Ejoh et al., 2006). Three varieties of tiger nut tubers are available; they are yellow, black and brown varieties (Umerie et al., 1997; Okafor et al., 2003; Belewu and Abodunrin, 2006; Oladele and Aina, 2007; Arafat et al., 2009), are widely distributed in Europe and Africa including Nigeria where they are widely consumed uncooked (Oderinde and Tairu, 1988; Omode et al., 1995; Ejoh et al., 2006; Dhouha et al., 2016). Tiger nut tubers are rich in starch, fats, sugars, proteins, oleic acid, and vitamins B, C and E (Temple et al., 1990; Omode et al., 1995; Okwu, 2005; Belewu and Belewu, 2007; Dhouha et al., 2016). It has been also reported that they are rich in minerals such as phosphorous, potassium, calcium, magnesium and iron (Temple et al., 1990; Omode et al., 1995; Belewu and Belewu, 2007; Oladele and Aina, 2007; Arafat et al., 2009; Dhouha et al., 2016). Their antioxidant capacity is known to be relatively high because reports from previous studies have shown that they contain considerable amounts of water-soluble flavonoids and glycosides which are known natural antioxidants (Temple et al., 1990; Eteshola and Oraedu, 1996; Pietta, 2000; Oloyede et al., 2014).

For many years, the tubers of *Cyperus* species have been used traditionally as remedy for several diseases including hepatotoxicity (Mehta et al., 1999; Hassanein et al., 2011) and as antioxidative agent (Satoh et al., 2004). Although many researchers have worked on *C. esculentus* tubers, the tubers are not well utilized due to limited information on their medicinal potential and nutritional benefits (Rita, 2009; Adejuyitan, 2011; Ukwuru and Ogbodo, 2011; Oyedepo and Odoje 2014). Ameen et al. (1999) reported that oral administration of oily extracts of *C. esculentus* tuber significantly protected against carbon tetrachloride (CCl_4)-induced hepatic damage in male albino rats. Oyedepo and Odoje (2014) reported that administration of tiger nut flour in varying percentages in rat pellets for 21 days preceding CCl_4 administration exhibited a potential hepatoprotective activity against CCl_4-induced hepatotoxicity in male Wister albino rats. Also, recent report by Onuoha et al. (2017) showed that oral administration of tiger nut based nutri-milk to rats preventively ameliorated acetaminophen-induced hepatotoxicity. There is no information in available literature on the effects of methanolic tuber extract of *C. esculentus* on sub-acute liver damage, hence the present study which evaluated the effects of methanolic tuber extract of *C. esculentus* Linn on CCl_4-induced sub-acute liver damage in albino rats.

MATERIALS AND METHODS

Drugs, chemicals, clinical chemistry assay kits and equipment

Methanol, carbon tetrachloride (CCl_4) and silymarin were obtained from Sigma-Aldrich, St. Louis, Missouri, USA. Thiopentone sodium was obtained from Chandra Bhagat Pharma Pvt., Ltd., Mumbai, India. The assay kits for clinical biochemistry evaluation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities, and levels of total proteins, total cholesterol and albumin in serum were procured from Quimica Clinica Aplicada (QCA), Spain. The test kit for the assay of total bilirubin in serum was procured from Randox Laboratories Ltd, County Antrim, United Kingdom. All the clinical chemistry determinations were done using the CHEM5V3® Semi-automated Clinical Chemistry Analyzer (Erba Diagnostics, Manheim, Germany). Other routine reagents and chemicals used for the study were of analytical grade.

Collection of tiger nut tubers, identification and preparation of extract

Fresh yellow tubers of *C. esculentus* (Figure 1) were collected from

Figure 1. Picture showing yellow and fleshy tubers of *C. esculentus* Linn (tiger nuts).

Nsukka in Enugu State, Nigeria, in March 2016. The tubers were identified by a Botanist (Mr. A.O. Ozioko) at the Department of Botany, University of Nigeria, Nsukka. They were washed, allowed to dry under shade, and ground into powder. Eighty percent (80%) methanol was added to 1000 g of the powdered tubers and the resulting mixture was shaken intermittently at 2 h interval for 48 h. After that period, the mixture was filtered and concentrated to dryness using a rotary evaporator (Buchi, Switzerland), and referred to as *C. esculentus* methanol tuber extract (CEME).

Experimental animals

Thirty adult male albino rats (*Rattus norvegicus*) weighing between 200-250 g, were used for the study. They were kept in clean cages in the laboratory Animal House of the Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka, at room temperature of 23- 29°C, and acclimatized for 2 weeks before the commencement of the study. The albino rats were fed commercial rat pellets; product of Grand Cereals Nig. Ltd, Jos, Nigeria, and clean drinking water *ad libitum.* The ethical *g*uidelines governing the use of animals for laboratory experiment were strictly adhered to (Zimmermann, 1983; Ward and Elsea, 1997). The protocol for the laboratory animal study was approved by the Faculty of Veterinary Medicine Experimental Animal Ethics Committee, University of Nigeria, Nsukka.

Evaluation of the effects of CEME on CCl_4-induced sub-acute liver damage

The albino rats were randomly assigned to six groups (A – F) of five rats per group. Sub-acute liver damage was induced in rats in Groups A – E by intra-peritoneal injection of 1 ml/kg body weight CCl_4 in equal volume of olive oil (50 % volume/volume) on day 0, and after every 72 h (3 days) for 12 days (Singh et al., 2012). Group A rats were given 10 ml/kg distilled water as placebo (negative control), Groups B, C and D rats were treated with 200, 400 and 800 mg/kg CEME respectively, Group E rats were treated with 100 mg/kg Silymarin (a known hepatoprotective drug) as positive control, Group F rats were given 10 ml/kg distilled water as placebo (normal control). The treatment with CEME and Silymarin started a day after the initial CCl_4 injection and was done orally twice daily for 15 days. On day 15, blood samples for clinical chemistry assay were collected from the albino rats using the orbital technique (Bolliger and Everds, 2010).

The blood samples were allowed to stand at room temperature for 45 min to clot, they were then centrifuged at 3000 revolutions per minute for ten minutes using a table centrifuge (Jenalab Medical, England). The serum was harvested and used immediately for serum biochemistry assay following standard procedures.

The serum ALT and AST activities were determined using the QCA ALT and AST test kits, based on the Reitman and Frankel colorimetric method (Reitman and Frankel, 1957; Colville, 2002). The ALT in the serum sample and standard catalyzed the reaction of L-alanine and alpha-ketoglutaric acid to form pyruvic acid and L-glutamic acid, while the AST catalyzed the reaction of L-aspartic acid with alpha- ketoglutaric acid to form oxaloacetic acid and L-glutamic acid. These ketonic acids produced were reacted with 2-4, dinitrophenyl hydrazine to form a corresponding coloured hydrazone. The optical density of the coloured hydrazone was then measured at 505 nm wavelength using the semi-automated analyzer, and ALT/AST activity quantified. Serum ALP activity was assayed using the QCA alkaline phosphatase test kit, which is based on the phenolphthalein monophosphate method (Klein et al, 1960; Colville, 2002). In the method, alkaline phosphatase in the

Table 1. The ALT, AST and ALP activities of rat groups* given sub-acute toxic doses of CCl_4 and treated with varied doses of CEME.

Group	Means ± standard error		
	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Group A	130.22±30.87[a]	177.23±34.39[a]	262.64±16.22[a]
Group B	71.33±26.55 [b]	89.38±9.95[b]	225.59±10.64[ab]
Group C	73.57±18.71[b]	111.31±7.59[b]	228.82±15.95[ab]
Group D	51.25±7.18 [b]	96.44±8.47[b]	192.02±32.51[ab]
Group E	58.49 ±10.41[b]	110.17±11.59[b]	182.30±20.79[b]
Group F	30.13±2.00[b]	54.09±1.10[c]	187.70±23.68[b]

[a, b, c] Different alphabetical superscripts in a column indicate significant differences between the groups, $P < 0.05$. * Group treatments: Group A – CCl_4 + distilled water placebo; Group B - CCl_4 + 200 mg/kg CEME; Group C - CCl_4 + 400 mg/kg CEME; Group D - CCl_4 + 800 mg/kg CEME; Group E - CCl_4 + 100 mg/kg Silymarin; Group F - Distilled water placebo only.

serum and a standard (containing 30 IU/L alkaline phosphatase) hydrolyzed a colourless substrate of phenolphthalein monophosphate giving rise to phosphoric acid and phenolphthalein which at alkaline pH turned into a pink colour. The optical density of the pink coloured solution was measured at 546 nm wavelength using the semi-automated analyzer, and the alkaline phosphatase activity quantified.

Serum total protein levels were determined using the QCA total protein test kit based on the direct Biuret method (Lubran, 1978; Johnson, 2008). This procedure involved a reaction of the proteins in the serum samples and a standard (containing 5 g/dl of proteins) with copper ions in the Biuret reagent in an alkaline medium, which resulted in the formation of a stable coloured complex. The optical density of the coloured complex was measured at 546 nm wavelength using the semi-automated analyzer, and the serum total protein quantified. The serum albumin was assayed using the QCA albumin test kit, which is based on the bromocresol green method (Doumas and Peters, 1997; Johnson, 2008). This procedure involved the reaction of the albumin in the serum samples and standard (containing 5 g/dl of albumin) with bromocresol reagent at acid pH to form a coloured complex. The optical density of the coloured complex was measured at 630 nm wavelength using the semi-automated analyzer, and serum albumin level quantified. The globulin levels were calculated by subtracting the serum albumin levels from the total protein levels (Johnson, 2008). The serum total cholesterol levels were determined using the QCA total cholesterol test kit, which is based on the enzymatic colorimetric method (Allain et al., 1974; Rifai et al., 2008). In this procedure, total cholesterol in the serum samples and a standard (containing 200 mg/dl of cholesterol) was enzymatically hydrolyzed by cholesterol esterase and further oxidized by cholesterol oxidase contained in the QCA total cholesterol working reagent. The reactions led to formation of a coloured quinonic derivative. The optical density of the coloured quinonic solution was measured at 505 nm wavelength using the semi-automated analyzer, and total serum cholesterol quantified. The serum total bilirubin levels in the serum samples were assayed using the Randox® bilirubin test kit (Randox Laboratories Ltd, County Antrim, United Kingdom), which is based on the Jendrassik and Grof method (Doumas et al., 1973; Higgins et al., 2008). In this determination, the serum samples were reacted with diazotized sulfanilic acid in the presence of caffeine to produce an azopigment, and their optical densities were measured at 578 nm using the semi-automated analyzer, and the total serum bilirubin quantified. After blood sample collection, the rats were sacrificed by euthanizing them with intra-peritoneal injection of 250 mg/kg thiopentone sodium and confirmatory exsanguinations (AVMA, 2013). The liver of each rat was carefully dissected and weighed, and the relative liver weight was calculated.

Data analysis

The clinical biochemistry data were subjected to one way analysis of variance (ANOVA) using the SPSS software (version 16). The least significant difference procedure was used to separate variant means, post-hoc. Probability less than 0.05 was accepted as significant, and a summary of the results were presented as means ± standard error, in tables.

RESULTS

Plant extraction

Plant extraction yielded golden brown coloured CEME, which was soluble in water with percentage yield of 25.43% weight/weight.

Effects of CEME on CCl_4-induced sub-acute liver damage

The mean serum ALT activity of the Group A rats was more than four times that of Group F rats, while that of Groups B, C, D and E rats were about 1.5 times to 2 times that of Group F rats (Table 1). The serum ALT activity of the Groups B, C, D and E rats were significantly lower ($P < 0.05$) than that of Group A, and were not significantly different ($P > 0.05$) from that of Group F rats (Table 1). The mean serum AST activity of the Group A rats was more than 3 times that of Group F rats, while that of Groups B, C, D and E were about double that of Group F (Table 1). The serum AST of Groups B, C, D and E were significantly lower ($P < 0.05$) than that of Group A, and were also significantly higher ($P < 0.05$) than that of Group F rats (Table 1). The mean serum ALP activity of the Group A rats was significantly

Table 2. Levels of serum proteins in rat groups* given sub-acute toxic doses of CCl_4 and treated with varied doses of CEME.

Group	Means ± standard error		
	Total proteins (g/dl)	Albumins (g /dl)	Globulins (g/dl)
Group A	5.87±0.20[a]	2.92±0.30[a]	2.95±0.33[a]
Group B	5.95±0.31[a]	3.31±0.29[ab]	2.64±0.23[a]
Group C	6.37±0.30[ab]	3.60±0.20[ab]	2.77±0.20[a]
Group D	6.47±0.06[ab]	3.45±0.16[ab]	3.01±0.17[a]
Group E	6.79±0.36[b]	3.53±0.27[ab]	3.26±0.22[a]
Group F	6.73±0.24[b]	3.72±0.15[b]	3.02±0.12[a]

[a, b, c] Different alphabetical superscripts in a column indicate significant differences between the groups, $P < 0.05$. * Group treatments: Group A – CCl_4 + distilled water placebo; Group B - CCl_4 + 200 mg/kg CEME; Group C - CCl_4 + 400 mg/kg CEME; Group D - CCl_4 + 800 mg/kg CEME; Group E - CCl_4 + 100mg/kg Silymarin; Group F - Distilled water placebo only.

Table 3. Serum levels of total cholesterol and bilirubin, and the relative liver weight of rat groups* given sub-acute toxic doses of CCl_4 and treated with varied doses of CEME.

Group	Means ± standard error		
	Total cholesterol (mg/dl)	Total Bilirubin (mg/dl)	Relative liver weight
Group A	89.54±10.42[a]	2.58±0.10[a]	4.23 ±0.14[a]
Group B	86.42±7.51[a]	2.24±0.03[b]	4.05 ± 0.14[ab]
Group C	81.95±5.18[a]	2.36±0.12[ab]	3.70 ± 0.15[b]
Group D	84.54±6.96[a]	2.17±0.10[b]	3.78 ± 0.05[b]
Group E	87.38±5.35[a]	2.29±0.12[ab]	3.74 ± 0.13[b]
Group F	73.60±5.07[a]	1.46±0.06[c]	3.31 ± 0.09[c]

[a, b, c] Different alphabetical superscripts in a column indicate significant differences between the groups, $P < 0.05$. * Group treatments: Group A – CCl_4 + distilled water placebo; Group B - CCl_4 + 200 mg/kg CEME; Group C - CCl_4 + 400 mg/kg CEME; Group D - CCl_4 + 800 mg/kg CEME; Group E - CCl_4 + 100mg/kg Silymarin; Group F - Distilled water placebo only.

higher ($P < 0.05$) than that of Groups E and F, but there were no significant differences ($P > 0.05$) between the serum ALP activity of the treated groups (B, C and D) and all other groups (Table 1).

The mean serum total protein levels of the rats in Groups A and B were significantly lower ($P < 0.05$) than that of rats in Groups E and F, but there were no significant differences ($P > 0.05$) between the serum total proteins of rats in Groups C, D and other rat groups (Table 2). The mean serum albumin levels of rats in group A were significantly lower ($P < 0.05$) than that of rats in Group F, but there were no significant differences ($P > 0.05$) between the serum albumin levels of rats in Groups B, C, D and E and all other groups (Table 2). There were no significant variations ($P > 0.05$) between the serum globulin (Table 2) and total cholesterol (Table 3) levels of rats in all the groups. The mean serum total bilirubin levels of the Group A rats was significantly ($P < 0.05$) higher than that of Groups B, D and F, while that of rats in Group F was significantly lower ($P < 0.05$) than that of all other groups (Table 3). The relative liver weight of the Group A rats was significantly higher ($P < 0.05$) than that of rats in Groups C, D, E and F, while that of rats in Group F was significantly lower ($P < 0.05$) than that of rats in all other groups (Table 3).

DISCUSSION

The comparatively higher serum enzyme activity of ALT, AST and ALP in all the groups that were given CCl_4 shows that CCl_4 induced liver damage by altering the integrity of the hepatocytes and affecting liver function adversely (Mukherjee, 2003; Kim et al., 2010). The alteration of serum ALT, AST and ALP activity levels implied that CCl_4 caused damage to the liver hepatocytes. Elevation in serum transaminases (AST and ALT) is a biomarker of hepatocellular necrosis and hepatotoxicity (Friedman et al., 1996). The elevated levels of ALT, AST and ALP as recorded in this study validates the reports of Nishigaki et al. (1992), Ameen et al. (1999), Raja et al. (2007), Shafaq et al. (2009) and Oyedepo and Odoje (2014), who also reported elevated levels of these hepatic enzymes in serum of albino rats

given hepatotoxic doses of CCl_4.

The administration of CEME (at all doses) protected liver function of the rats whose livers were damaged with CCl_4 by protecting their hepatocellular integrity, and in this case CEME administered at 800 mg/kg, compared favourably with Silymarin (a standard hepatoprotective drug). The ability of administered CEME to protect hepatocellular integrity as recorded in this study agrees with the reports of Ameen et al. (1999), Farok et al. (2011) and Onuoha et al. (2017) who reported significant decreases in ALT and AST in rats treated with oily extracts of *C. esculentus* tuber, aqueous extract of *C. esculentus* tuber, and tiger nut milk, respectively. Oyedepo and Odoje (2014) reported a marked decline in ALT, AST and ALP in rats pretreated with varying percentages of tiger nut floor in rat pellets for 21 days preceding CCl_4 administration. *In vitro* studies on the hepatoprotective activity of solvent-free microwave extract of *C. esculentus* also showed that the IC_{50} of the essential oil content of *C. esculentus* tubers on monolayers of rat hepatocytes was > 1000 µg/ml, and exhibited hepatoprotection at 18.5 µg/ml (Hassanein et al., 2011). The ability of CEME to protect hepatocellular integrity in this present study may be due to hepatocyte membrane stabilization by active phytochemicals like flavonoids and water soluble glycosides which are reported constituents of *C. esculentus* tubers (Temple et al. 1990; Eteshola and Oraedu, 1996; Oloyede et al., 2014), and which are well known natural antioxidants (Temple et al., 1990; Pietta, 2000; Satoh et al., 2004; Oloyede et al., 2014). Since free radicals play important role in CCl_4-induced liver damage, it is believed that compounds that neutralize such radicals may have hepatoprotective properties. Other natural products that possess antioxidant properties have also been reported to protect against CCl_4-induced hepatotoxicity (Hsiao et al., 2003).

The depletion of serum total proteins and albumins in rats that were given CCl_4 is also an indication of liver dysfunction associated with CCl_4 administration (Navarro and Senior, 2006). Serum albumin is the major protein in the blood synthesized by the liver. It is a clinically useful marker of hepatic synthetic function (Friedman et al., 1996). The administration of CCl_4 in this study adversely affected hepatic synthesis of albumins, and treatment with CEME led to slight elevation of serum total protein and albumin of the treated rats. Chukwuma et al. (2010) and Hwang (2004) reported increases in the level of serum total proteins and albumins in rats treated with aqueous extract of *C. esculentus* tuber. Protein synthesis stimulation has been recognized as a hepatoprotective mechanism. It helps to accelerate the process of regeneration and the production of replacement liver cells (Rip et al, 1985; Tadeusz et al., 2001).

The lack of significant variations in the serum globulin, and total cholesterol levels among the groups shows that CCl_4 administration and CEME treatment had no effects on these parameters. The significantly lower serum bilirubin of rats in groups B and D suggests that treatment with 200 and 800 mg/kg enhanced hepatic excretion of bilirubin which was adversely affected by CCl_4 administration. Bilirubin is a diagnostic marker of liver and blood disorders, it is the end product of the breakdown of haemoglobin (Singh et al., 2011). Damage to the liver cells causes impairment of bilirubin excretion, thus causing accumulation of bilirubin in the blood and extracellular fluid (Singh et al., 2011). The effect of CEME administration on serum total bilirubin in this present study agrees with the reports of Amani et al. (2012) on extracts of a related plant, *C. alternifolius*. The higher relative liver weight of rats in groups A and B shows that the liver was enlarged as a result of inflammation due to damage to the liver cells caused by CCl_4 (Bukhsh et al., 2014), and the results obtained from this study showed that treatment with the CEME at 400 and 800 mg/kg, and Silymarin at 100 mg/kg was able to significantly reduce the inflammatory enlargement which was induced by CCl_4 administration.

Conclusion

From the results obtained from this study, it was concluded that the administration of *C. esculentus* methanolic tuber extract to albino rats whose livers were experimentally damaged with CCl_4, protected hepatocellular integrity, enhanced hepatic bilirubin excretion and ameliorated inflammatory enlargement of the liver, and its hepatoprotective effects compared effectively with a standard hepatoprotective drug (Silymarin). These findings imply that *C. esculentus* methanolic tuber extract is hepatoprotective against carbon tetrachloride-induced liver damage.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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24 h pre-incubation of EA.hy926 cells with angiotensin II regulates insulin-dependent activation of eNOS in a concentration-dependent manner

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The rennin-angiotensin II system (RAS) and the insulin-PI3kinase signalling pathways cross-interact with important physiological and pathophysiological consequences for cells and the whole organism. Here, the effect of 24 h pre-incubation of EA.hy926 with two different concentrations of angiotensin II, on insulin-mediated activation of the PI3kinase-AKT-eNOS signalling was investigated. Quiescent EA.hy926 cells were treated with insulin (100 nM, 30 min) following 24 h pre-treatment with or without either 0.1 or 1 µM of angiotensin II. Cell lysates were immunoblotted for phospho AKT Ser-473, phospho eNOS Ser-1177 and normalized with β-actin. Homogenates of EA.hy926 treated with insulin in the presence or absence of 1 µM angiotensin II, were also subjected to nitric oxide synthase (NOS) activity assay using titrated arginine as substrate. To exclude cytotoxicity of the 1 µM angiotensin II concentration, Trypan blue cell viability assay as well as the microscopic examination of unstained and DAPI/Phalloidin stained EA.hy926 cells were undertaken. Insulin resulted in about 2 fold increase in phospho-eNOS Ser-1177 and 8 fold increase in phospho-AKT Ser-473 levels in treated compared to untreated cells. A 2 fold increase was observed in the NOS activity of insulin-treated and untreated cells preincubated with Ang II. 24 h pre-treatment with 0.1 µM Ang II did not significantly interfere with the responses to insulin but the 1 µM Ang II pre-treated EA.hy926 showed significant attenuated insulin induced phosphorylation of eNOS Ser-1177 and AKT Ser-473, alongside impaired NOS activity. The Ang II-treated cells showed normal nuclei and cytoskeletal architecture. Angiotensin II concentration-dependently regulated basal and insulin-mediated PI3Kinase-AKT-eNOS signalling in cultured endothelial cells.

Key words: 24 h pre-incubation, angiotensin II, regulates, insulin-PI3Kinase-AKT-eNOS signalling, concentration-dependently.

INTRODUCTION

Just like skeletal muscle cells, adipocytes and hepatocytes, endothelial cells are important targets for insulin action

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(Kubota et al., 2011). Endothelial cells increase their nitric oxide (NO) output in response to insulin through the calcium-independent PI3kinase mediated enhancement of endothelial NO synthase (NOS III) activity (Muniyappa et al., 2011). The released NO dissolves in the aqueous phase of the circulating blood and diffuses to reach vascular smooth muscle cells resulting in smooth muscle relaxation and vasodilation (Zhao et al., 2015). Insulin binds to and activate its receptor, to recruit and activate the insulin receptor substrate (IRS)-PI3-Kinase-AKT signalling upstream of eNOS (Lizcano et al., 2002). Endothelial dependent vasodilation is vital to the regulation of vascular tone and blood pressure (Coretti et al., 2002; Yunn et al., 2016). NO also possesses anti-atherosclerotic, anti-inflammatory and anti-coagulative functions as well as being vital for vasculogenesis (Brasier et al., 2004; Ahiuwalia et al., 2004). In chronic disorders such as type II diabetes mellitus, obesity, dyslipidemias, dysmetabolic syndrome, cardiac failure and coronary heart diseases, insulin-target cells are less responsive to insulin (Greenfield and Campbell, 2004; Nolan et al., 2011).

The earliest manifestation of endothelial resistance to insulin constitute endothelial dysfunction, characterised mainly by impaired activation of the Insulin-PI3kinase-AKT-eNOS signalling in a setting of either normo or hyperinsulinaemia (Wheatcroft et al., 2003; Calles-Escandon and Cipolia, 2001; Cefalu, 2001). The consequential impaired NO-dependent vasodilation leads to heightened risk of cardiovascular morbidities and mortalities (Mombouli and Vanhoutte, 1999; Tangvarasittichai, 2015; Abbasi et al., 2016; Haffner et al., 2000). The blood concentration of Ang II is elevated in insulin-resistant states as a result of the activation of the rennin aniotensin aldosterone system (RAAS) (Surapongchai et al., 2017; Mehta and Griendling, 2007). Inhibition of Ang II action with either Ang II type-1 receptor antagonists or angiotensin converting enzyme inhibitors (ACEI) could restore insulin-sensitivity in hypertensives and type II diabetic patients (Pavo et al., 2003; Shiuchi et al., 2004). Apart from Ang II, other pro-inflammatory cytokines including TNF-alpha and IL-6 are elevated and implicated in the pathogenesis of insulin resistance (Bastard et al., 2006; Shoeison et al., 2006). These peptide hormones and cytokines provide useful tools for the *in vitro* induction of insulin resistance in cultured target cells (Bastard et al., 2006; Shoeison et al., 2006; Andreozzi et al., 2004).

Pre-treatment of skeletal muscle cells with Ang II induced insulin-resistance manifested as impaired insulin-mediated glucose transporter 4 (GLUT 4) translocation from intracellular storage sites to the plasma membrane and intracellular glucose uptake (Wei et al., 2008). Acute pre-treatment of cultured endothelial cells (ECs) with Ang II impaired insulin-PI3Kinase-AKT-eNOS signalling via the induction of serine phosphorylation of IRS resulting in impeded tyrosine Phosphorylation needed for IRS dependent PI3K activation (Andreozzi et al., 2004). Here, the effect of 24 h pre-incubation of confluent monolayer of EA.hy926 cells with two different concentrations of Ang II to determine any concentration-dependent regulation of the insulin-PI3K-eNOS signalling was investigated.

METHODOLOGY

Materials

EA.hy926 cells (ATCC, USA), monoclonal rabbit phospho AKT-Ser 473, phospho eNOS Ser-1177 antibodies, polyclonal secondary goat anti-rabbit, and anti-mouse antibodies were from cell signalling technology. Dubelcco's Modified Eagles Medium (DMEM), fetal bovine serum, penicillin, streptomycin, angiotensin II, polyclonal mouse β-Actin primary antibody, sodium dodecyl sulfate (SDS), polyacrylamide, Trypan blue dye, diamidino-2-phenylindole dihydrochloride (DAPI), rhodamine-phalloidin, insulin, methanol, sodium orthnovanidate, betaglycerophosphate, protease inhibitor cocktail, ammonium persulfate TEMED, nicotinamide adenine dinucleotide phosphate (NADPH), scintillation fluid, bovine serum albumin, Tween-20, polyvinylidene difluoride (PVDF) membrane, glycine, sodium chloride, sodium EDTA, Trypan blue dye, PBS, glycine, tertrahydrobiopterine (BH4) and Triton X-100 were from Sigma Aldrich. ECL solution A and B, NOS activity assay kit (Caymans), and titrated arginine were from Cambridge Bioscience. Tris HCl and sodium deoxycholate were from Fishers Scientific. Bradford ultra protein assay kit was from Expedeon protein solutions.

Cell culture

Cells were cultured and maintained in DMEM containing 5 mM glucose supplemented with 10% foetal bovine serum, 1 µM BH4, 100 U/ml of penicillin and 100 µg/ml of streptomycin. They were used for experiments when they became 80 to 100% confluent in either 10 c or 6 cm corning cell culture dishes. They first had their culture media replaced with serum free DMEM conditioned with or without either 0.1 or 1 µM Ang II for 24 h in the incubator (air 95%, O_2 5% and humidified). At the end of the Ang II pre-incubation period, the cells were treated with or without insulin (100 nM, 30 min). Phenol red free DMEM was used as starvation and treatment media in the NOS activity experiments.

Western blot

The cells were rinsed with ice cold wash buffer (1X PBS, Na_2VO_3, EDTA) and lysed in 200 µl of RIPA lysis buffer (50 mM Tris HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% w/v SDS, 200 µM Na_2VO_4, 1 mM lytic cocktail) on ice for 10 min, harvested with the aid of a plastic cell scrapper and transferred into 0.5 ml Eppendorf. The lysate was clarified by micro centrifugation (10000 g, 10 min) before the proteins in 100 µl were resolved by SDS polyacrylamide gel electrophoresis (4% stacking gel, pH 6.8; 8.5% resolving gel, pH 8.0), in a running buffer (190 mm glycine, 25 mM Tris, 0.1% SDS). Resolved gel proteins were transferred to PVDF membrane by passing a constant current of 100 mA for 4 h through the transfer buffer (190 mM glycine, 25 mM Tris, 20% methanol). The protein blots were subsequently incubated with polyclonal primary antibodies to β-actin (1:10000), phospho AKT Ser-473 (1:1000) and phospho eNOS Ser-1177 (1:1000) for 1 h each before they were washed and probed with polyclonal goat anti-mouse (1:5000) and anti-rabbit (1:1000) for 1 h

each. Following repeat final washes, the immunoblots were incubated with ECL chemilumniscence solution A and B. Detection of protein bands was with the aid of Chemidoc (Biorad). Protein quantification was with Imagelab software (Biorad).

Cell culture

Cells were cultured and maintained in DMEM containing 5 mM glucose supplemented with 10% foetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin. They were used for experiments when they became 80 to100% confluent in either 10 or 6 cm corning cell culture dishes. They first had their culture media replaced with serum free DMEM conditioned with or without either 0.1 or 1 µM Ang II for 24 h in the incubator (air 95%, O_2 5% and humidified). At the end of the Ang II pre-incubation period, the cells were treated with or without insulin (100 nM, 30 min). Phenol red free DMEM was used as starvation and treatment media in the NOS activity experiments.

Western blot

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NOS activity assay

Treated and untreated confluent cells in 10 cm corning cell culture dishes were rinsed with ice cold wash buffer (1X PBS, 1 mM EDTA) and then harvested with a cell scrapper in 1 ml of same buffer. They were subsequently pelleted by centrifugation (10000 g, 5 min) and the wash buffer discarded before homogenizing in 50 µl homogenization buffer by sonicating for 20 min on ice. Splashed homogenate on the sides of the microfuge tubes were recovered by repeating the spin for 1 min before pipetting up and down to disperse, and the protein content of the supernatant was determined (Bradford Ultra Protein Assay). The protein concentrations of all samples were standardized to 5 mg/ml or more. The NOS activities of the homogenates were measured by the rate of conversion of titrated arginine to titrated citrulline according to the assay kit manufacturer's instruction.

Cell viability assay

Confluent EA.hy926 cells were serum starved for 24 h in DMEM with or without 1 µM Ang II conditioning before they were detached by trypsinization and re-suspended in 1 ml of PBS. 50 µl of each cell suspension was incubated with equal volume of 4% Trypan blue on ice for 5 min (Strober, 2001; Altman et al., 1993). Thereafter, 20 µl of the dispersed cell-dye mixture was counted with a haemocytometer and the number of dead cells (dark-stained from dye retention) and total number of cells were counted for 10 squares. The percentage of cell viability was subsequently calculated from:

$$\text{Cell viability (\%)} = \frac{\text{Total number of live cells}}{\text{Total number of cells}} \times 100$$

Microscopy

Normal and Ang II induced insulin-resistant cultured EA.hy926 cells were first examined under the light microscope (x40 magnification). Thereafter, EA.hy926 cells grown over glass cover slips were fixed in 4% paraformaldehyde and stained with DAPI (1:20000 dilution of 2 mg/ml stock) and Rhodamine conjugated Phalloidin (1:100 methanol dilution of 0.5 mg/µl DMSO stock), to view their actin cytoskeleton using the Snapshot Widefield fluorescence microscope. Image capturing was with the Coolsnap camera at x20 magnification.

Statistical analyses

Results are expressed as mean ± standard error mean (SEM) of 3 or more independent experiments and statistical analyses was with graph pad prism 7.0 using either the student's t test or one way analysis of variance (ANOVA) with Tukey's multiple comparison tests. Level of statistical significance was set at P values less than 0.05.

RESULTS

Initial assessment of EA.hy926 cell responses to insulin treatment (Figure 1A to D) showed that the level of phosphorylated AKT Ser-473 was 780.20 ± 134.00% of untreated cells (n=11) while phosphorylated eNOS Ser-1177 level was 205.90 ± 33.15% of untreated cells (n=14). The NOS activity in insulin-treated cells was 363.30 ± 60.68% of untreated cells (n=5). The level of phosphorylated eNOS Ser-1177 in insulin-treated EA.hy926 following their pre-incubation with either 0.1 or 1 µM Ang II for 24 h were 151.54 ± 12.91% and 90.58 ± 20.23% of their respective treatment-matched control, compared to 166.99 ± 23.07% obtained in insulin-treated normal cells (Figure 2A). Insulin-induced NOS activity in Ang II pre-treated cells was 66.47 ± 7.82% of control compared to 181.90 ± 11.25% in non-Ang II pre-treated cells (Figure 2B). The basal level of phosphorylated eNOS Ser-1177 in EA.hy926 cells pre-treated with Ang II (0.1 or 1 µM Ang II, 24 h) were 120.36 ± 6.30 and 171.38 ± 21.44% of untreated cells, respectively (Figure 3A). Basal NOS activity was also increased following 24 h 1 µM Ang II pre-treatment (220.70 ± 26.73% of untreated cells (Figure 3B). Microscopic examination of 1 µM Ang II treated cells and untreated cells showed normal histology (Figure 4A and B) and actin cytoskeleton (Figure 4C and D). The percentages of cell viability assessed using the

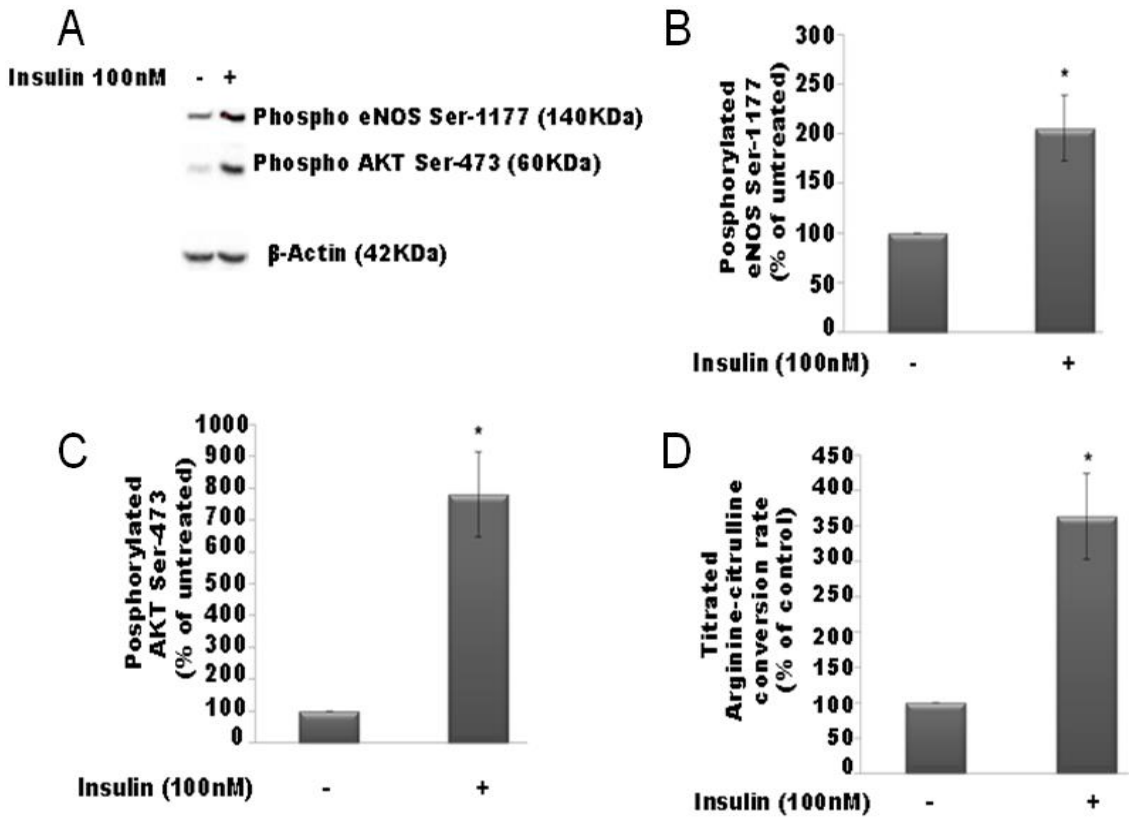

Figure 1. EA.hy926 cell responses to insulin. The effect of treating quiescent EA.hy926 cells with insulin (100 nM, 30 min). (**A**) Representative immunoblot of phosphorylated eNOS Ser-1177, phosphorylated AKT Ser-473 and β-actin of insulin treated and untreated EA.hy926 cells. (**B**) Densitometry of phospho eNOS Ser-1177 shows that insulin resulted in 2 fold increase in the mean level of phosphorylation of eNOS at Ser-1177 (*P< 0.05, Student's t test). Data are mean ± SEM of 14 separate experiments. (**C**) Densitometry of phospho AKT Ser-473. Insulin treatment resulted in 8 fold increase in the mean level of phosphorylation of AKT at Ser-473 relative to untreated controls (*P<0.05, Student's t test). Data are mean ± SEM of 11 different experiments. (**D**) NOS activity of EA.hy926 cell homogenates measured by the rate of conversion of titrated Arginine to citrulline expressed a percentage of untreated control. Insulin resulted in 4 fold increase in the NOS activity of treated EA.hy926 cells compared to untreated controls (*P<0.05, Student's t test). Data represent mean ± SEM of 5 separate experiments.

Trypan blue dye exclusion assay were 97.55 ± 1.22 and 99.11 ± 0.03 in untreated and 1 µM Ang II-treated EA.hy926 cells, respectively (Figure 5).

DISCUSSION

Treatment of serum-starved EA.hy926 cells with insulin (100 nM, 30 min), resulted in significant activation of their PI3K-AKT-eNOS pathway, evident from the increased levels of AKT phosphorylated at Ser-473 and eNOS phosphorylated at Ser-1177 on immunoblots of treated EA.hy926 cell lysates as compared to untreated controls (Figure 1A, B and C). The observations here are in line with previously published data (Montagnani et al., 2001; Repetto et al., 2006; Xiao et al., 2015). The increased level of insulin-mediated eNOS phosphorylation observed in immunoblots was further corroborated by the observation of significantly higher NOS activity of insulin-treated EA.hy926 cells compared to untreated controls (Figure 1D), in line with previously reported data where resveratrol induced eNOS phosphorylation at Ser-1177 correlated with enhanced NOS radioactivity (Notas et al., 2006). Taken together, both results suggest enhanced NO output from EA.hy926 cells following insulin stimulation. *In-vivo*, NO produced by endothelial cells dissolves in the aqueous phase of the blood and diffuses to influence vascular smooth muscle cells (VSMCs) by activating their guanylyl cyclase with consequent activation of myosin light chain dephosphorylase, which dephosphorylates the myosin light chains, resulting in the dissociation of the myosin light chain heads from actin culminating in smooth muscle relaxation (Ling et al., 2015; Sausbier et al., 2000; Somiyo and Somiyo, 2003; Lincoln et al., 2001). Dysregulation of this endothelial NO-dependent vasodilation plays important pathogenic

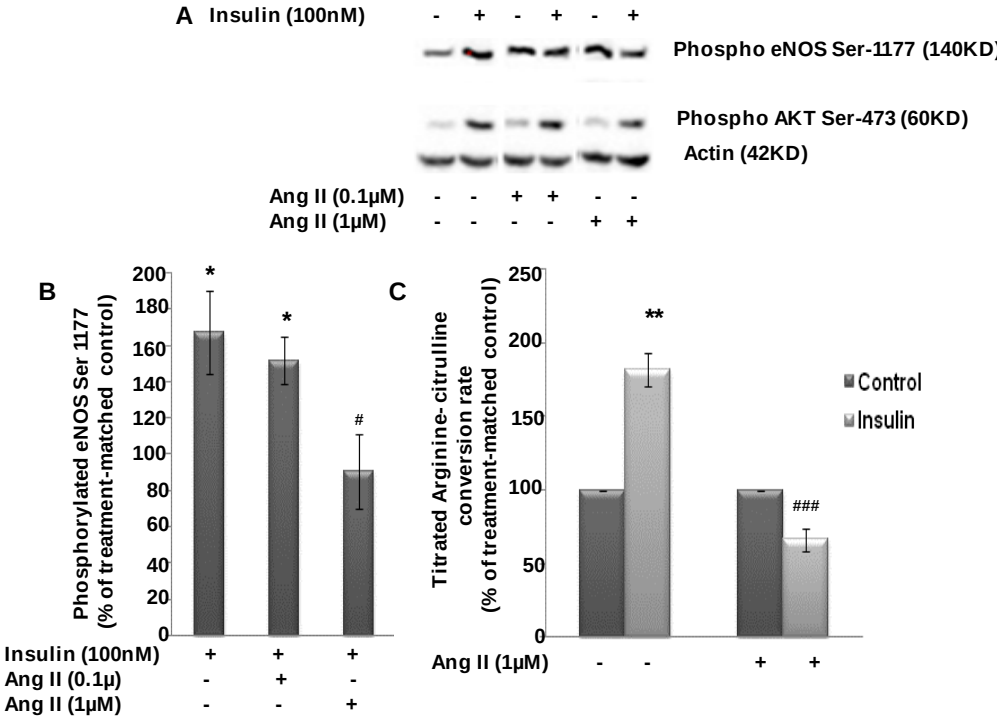

Figure 2. Insulin responses of EA.hy926 cells pre-treated with two different concentrations of Ang II. (A) Representative immunoblot of insulin-treated and untreated EA.hy926 cell lysates showing phosphorylated eNOS Ser-1177, phosphorylated AKT Ser-473 and β- actin. (B) Densitometry of phosphorylated eNOS Ser-1177 expressed as percent of treatment-matched control. Insulin resulted in significant increase in the levels of phosphorylation of eNOS at Ser 1177 in Ang II-untreated and 0.1μM Ang II pre-treated EA.hy926 cells but the response in 1μM Ang II pre-treated EA.hy926 cells were significantly impaired relative to untreated cells. The results represent the mean ± SEM of 3-4 separate experiments. (C) NOS activity of insulin treated and untreated EA.hy926 cells in the presence or absence Ang II pre-treatment (1μM, 24 h). The insulin mediated increase in the NOS activity was significantly impaired in the presence of Ang II pre-treatment (**P<0.01 vs. treatment-matched control, $^{###}$P<0.01 vs. insulin treated in the absence of Ang II, One way ANOVA, Tukey's multiple comparison). Data represent mean ± SEM of 3 separate experiments

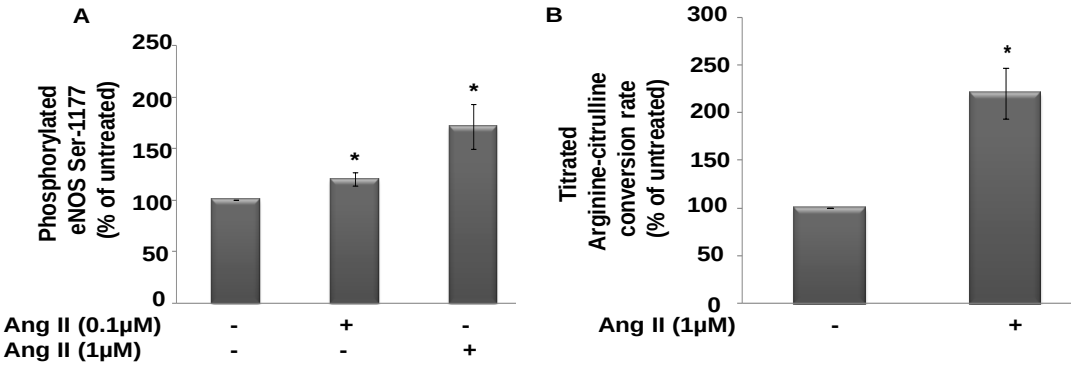

Figure 3. Basal eNOS activation is enhanced following 24 h Ang II pre-treatment of EA.hy926 cells. (**A**) Densitometry of anti-phospho eNOS Ser-1177 immunoblot of EA.hy926 pre-treated with or without Ang II (0.1 μM or 1 μM, 24 h). The level of phosphorylated eNOS Ser-1177 was significantly higher in Ang II pre-treated cells (120 ± 6.0% and 171 ± 21% of untreated for 0.1 and 1 μM respectively) compared to untreated (*P<0.05 versus untreated, Student's t test. Data represents mean ± SEM of 3-4 independent experiments. (**B**) NOS activity of EA.hy926 in the presence or absence of Ang II pre-treatment (1 μM, 24 h). NOS activity was higher in the 1 μM Ang II pre-treated cells (221 ± 26.73% of untreated (*P<0.05, Student's t test). Data represents mean ± SEM of 4 independent experiments.

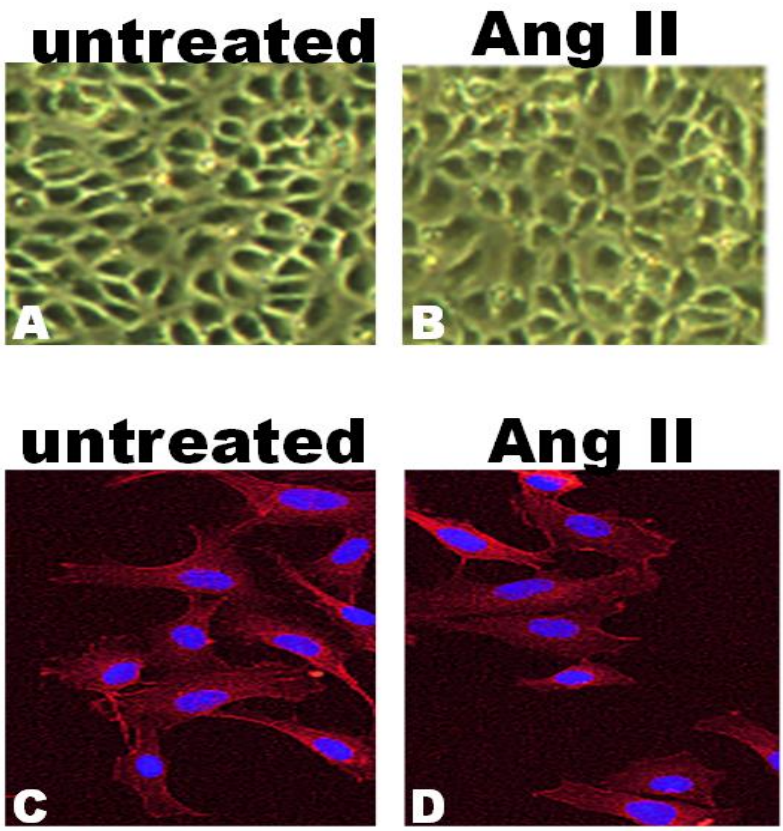

Figure 4. Effect of high Angiotensin II concentration on morphology of EA.hy926 cells. Top panel represents light photomicrograph of EA.hy926 cells (A) in the absence and (B) presence of Ang II (1 µM, 24 h). Ang II pre-treated cells remained adherent to their cell culture dishes and showed no morphological distortions relative to untreated cells. Bottom represents fluorescence photomicrograph of EA.hy926 cells (C) in the absence of Ang II and (D) presence of Ang II (1 µM, 24 h). There were no observable distortions in the architecture of the nuclei and actin cytoskeleton in Ang II pre-treated cells relative to untreated cells.

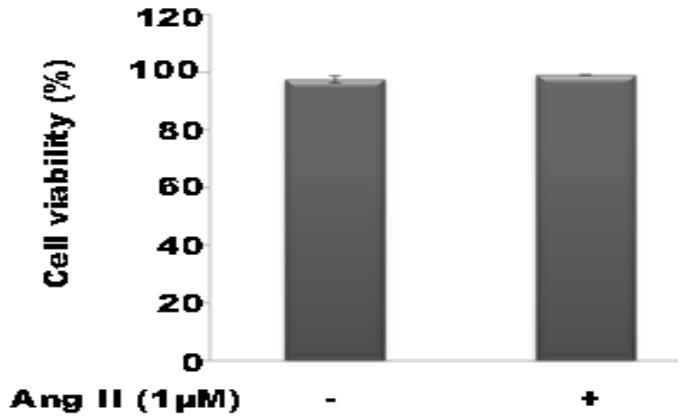

Figure 5. Effects of Ang II on the Viability of EA.hy926 cells. Trypan blue dye exclusion assay of EA.hy926 cells pre-treated with or without Ang II (1 µM, 24 h). Ang II pre-treatment did not affect the cell viability ($P>0.05$, Student's t test. The result represents the mean ± SEM of 3 separate experiments.

role in essential hypertension and insulin-resistant states such as obesity and type II diabetes mellitus (Schalkwijk and Stehouwer, 2005; Fontes-Guerra et al., 2015; Prieto et al., 2014; Tangvarasittichai, 2015). Hence, impaired NO-dependent vasodilation is a common feature in cardiovascular morbidities associated with these disease phenotypes (Giles et al., 2012; Mohan et al., 2010). Besides its role in regulating vascular tone, the anti-coagulating, anti-inflammatory and anti-apoptotic functions of NO produced by endothelial cells are important for maintenance of cardiovascular health (Ahiuwalia et al., 2004; Lamb and Goldstein, 2008; Randomski et al., 1990; Esmon and Esmon, 2011). The results from the Western blot experiments showing how 24 h Ang II (either 0.1 or 1 µM) pre-incubation of EA.hy926 differentially affected their response to insulin-treatment (100 nM, 30 min) were revealed because of the contrasting outcomes (Figure 2A and B). With the 0.1 µM Ang II pre-treatment prior to insulin stimulation, the eNOS Ser-1177 and AKT Ser-473 phosphorylation responses were not significantly altered relative to cells treated with only insulin. On the contrary, EA.hy926 cells pre-incubated with the 1 µM Ang II concentration for 24 h prior to stimulating with insulin, showed diminished insulin-mediated phosphorylation of AKT at Ser-473 and eNOS at Ser-1177 compared to none-Ang II treated cells. One previous study suggested that pre-incubation of primary HUVECs with 0.1 µM Ang II for a shorter time duration of 30 min prior to stimulation with insulin, impaired the insulin-PI3kinase signalling response via a different mechanism involving negative regulation of ins-PI3K-AKT signalling (Andreozzi et al., 2004), but no record can be found describing the effects of 24 h Ang II pre-incubation of EA.hy926 on insulin signalling via this pathway. Another study which reported the Ang II induced differential insulin action in rat skeletal muscles was conducted *in vivo* (Surapongchai et al., 2017). Although it suggested that a lower concentration of 100 ng/ml was more effective than 500 ng/ml at impairing insulin action, the findings does not invalidate the results of this *in vitro* study as the investigators did not determine the equilibrium Ang II concentration at the tissue level, prior to homogenization. The 1 µM concentration of Ang II had been previously reported to induce apoptosis in cultured HUVECs using detection by flow cytometry (Xiao et al., 2015) or the DNA fragmentation test (Dimmeler et al., 1997), an effect that was linked to attenuated endothelial NO production in both studies. Even though the former study also reported a differential proapototic effects following 24 h pre-incubation of HbmECs with 0.1 and 1 µM Ang II concentrations, it failed to extend the evaluation to any concentration-dependent effect in terms of changes in the levels of phosphorylated AKT Ser-473, phosphorylated eNOS Ser-1177 nor NO output of basal and agonist treated cells like in the present study, as the 0.1 µM concentration was adopted in the subsequent parts of the report. Contrary to the aforementioned proapoptotic studies, this study has not observed any significant alteration in the percentage cell viability of dish-adherent EA.hy926 post 24 h incubation with the 1 µM Ang II, using the Trypan blue viability assay technique (Figure 5). Although this dye exclusion viability assay is a less sensitive assay than ELISA and flow cytometry used in both previously reported studies. The Ang II pre-treated cells appeared normal under the light microscope; they neither showed significant distortion in their nuclei nor actin-cytoskeletal architecture compared to untreated cells (Figure 4C and D). Taken together, these data seem to suggest that EA.hy926 cells well-tolerated 24 h pre-incubation with the 1 µM Ang II concentration and this concentration seems to be more effective at modelling insulin-resistance in cultured ECs than the 0.1 µM concentration used in earlier *in vitro* acute drug exposure experiment (Andreozzi et al., 2004). Although it would seem to be in line with a recent study involving a different agonist from insulin (Xiao et al., 2015; Li et al., 2016), the observation in both studies differ in several aspects. While in the present study basal phosphorylated AKT Ser-473, phosphorylated eNOS Ser-1177 and NOS activity increased following 24 h pre-treatment with Ang II in a concentration dependent manner (Figure 3). Xiao et al. (2015) reported it diminished as the concentration of Ang II was increased. The observation in this study is novel as it highlights the significant contribution of accentuated basal NOS activation to the attenuated response to insulin by Ang II pre-treated EA.hy926 cells, when the data are normalized as ratios of treatment-matched controls.

The mechanisms underlying the contrasting outcomes of pre-treatment with the two different concentrations of Ang II on EA.hy926 response to insulin as reported in this study were not elucidated, but several possibilities could be inferred. Firstly, endothelial cells have been reported to produce angiotensin converting enzyme 2 (ACE 2), capable of degrading Ang II *in vitro* (Lovren et al., 2008). It is possible that with the smaller concentration of 0.1 µM, most of the added Ang II is degraded from the starvation medium, in the course of the 24 h pre-incubation of the cells. On the contrary, in cells treated with 1 µM Ang II, the effect was sustainable throughout the pre-incubation period due to the availability of some intact Ang II in the medium at the point of stimulation with insulin, despite the degradative action of the endothelial ACE 2. To exclude this as a possible underlying mechanism behind the Ang II-induced differential response of EA.hy926 cells to insulin, further work would have to be carried out to measure the residual Ang II concentration in the starvation medium at the end of the 24 h incubation period. ACE 2 degrades Ang II to yield Ang (1-7) which can activate the PI3K-AKT-eNOS signalling (Yang et al., 2012; Shi et al., 2015; Xiao et al., 2015). More Ang(1-7) could have been formed with the higher concentration of 1 µM Ang II, resulting in the relatively higher basal phosphorylated eNOS Ser-1177 levels and NOS activity in 1 µM Ang II pre-treated cells as compared to untreated and 0.1 µM Ang II pre-treated

cells (Figure 2A and B).

Another possible mechanism underlying the contrasting outcome seen here with the two different pre-treatment concentrations of Ang II could be differential expression of the agonist receptor subtypes. To exclude this, it would be necessary to experimentally compare AT1 and AT2 receptor proteins expression by EA.hy926 cells incubated under the two different Ang II concentrations. While relative AT1R predominance will favour an impaired insulin-response, a relatively predominant expression of AT2R could either enhance or impair insulin response depending on whether the data is normalized as a ratio of treatment-matched controls or not. This hypothesis could be further investigated by assessing changes in the receptor subtype expression under both treatment conditions at both protein and gene levels. Thirdly, it is also possible that differences in the extent of activation of NADPH oxidase system by Ang II in the two different conditioned starvation media, prior to stimulating with insulin, contributed to the different experimental outcomes. ROS are known to activate other serine/ threonine kinase pathways such as JUNK and MAPK that negatively regulate the insulin-PI3Kinase signalling (Andreozzi et al., 2004). Another interesting observation in this study was the fact that the basal eNOS Ser-1177 phosphorylation was higher in the Ang II induced insulin-resistant EA.hy926 versus none Ang II-treated controls (Figure 3A). This response could be reactive and could support the hypothesis of differential expression of the Ang II receptor subtypes, as Ang II acting via AT2R could induce eNOS Ser-1177 phosphorylation via the Bradykinin activation pathway (Yayama et al., 2006) or ACE 2 mediated production of Ang (1-7) from Ang II concentration dependently (Tassone et al., 2013; Li et al., 2016; Xiao et al., 2015). Incremental changes in basal phosphorylation of eNOS at Ser 1177 in the 1 µM Ang II compared to 0.1 µM pre-treated cells were observed. The incremental trend seen in the Ang II induced eNOS phosphorylation may be due to differences in either the residual Ang II or generated Ang (1-7) concentration following ACE 2 degradative action. Similar observation was made with the NOS activity of 1 µM Ang II pre-treated cells being significantly higher than basal activity (Figure 3B).

In conclusion, while 24 h pre-incubation of EA.hy926 with 1 µM concentration of Ang II significantly attenuated both the insulin-mediated phosphorylation of eNOS and NOS activity, the 0.1 µM concentration was not so effective in this study. The reactive enhancement of basal eNOS activation following 24 h pre-incubation with Ang II is thought to have made important contributions to the observed impaired response to insulin by EA.hy926 cells, when the data are expressed as a percentage of treatment-matched controls, as in the present study. The cells also tolerated the 1 µM Ang II concentration well enough, suggesting a suitable *in vitro* cell culture model of endothelial insulin-resistance.

Investigating the relative expression of the AT1 and AT2 receptor protein as well as the quantification of the residual Ang II and Ang (1-7) generated following the degradative actions of ACE 2, in the cell starvation media at the end of the 24 h incubation period, could further validate this limited study.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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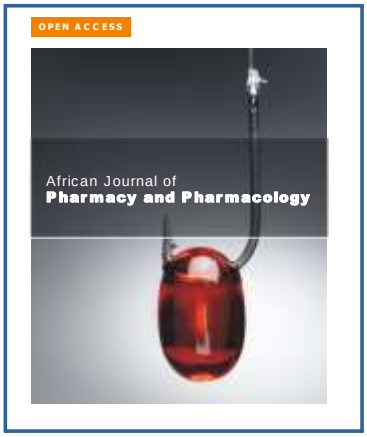

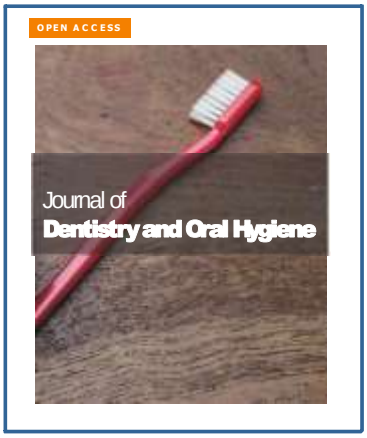

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