Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141013041312

Original Research

Safety of *Desmodium adscendens* extract on hepatocytes and renal cells. Protective effect against oxidative stress

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Received: October 09, 2014
Accepted: October 13, 2014
Published: November 28, 2014

ABSTRACT

Aim: The increased consumption of traditional medicinal plants has been driven by the notion that herbal products are safe and efficient. The purpose of this study was to evaluate the safety and the protective effect of a hydro alcoholic extract of *Desmodium adscendens* (DA) on liver (HEPG2) and kidney (LLC-PK1) cells. **Materials and Methods:** A hydro alcoholic extract of DA was used. HEPG2 or LLC-PK1 cells were treated with different does of DA, and viability test (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium [MTS]), cytotoxicity assay lactate dehydrogenase (LDH release) and study of the cell morphology were used in order to determine effects of DA on these two cells. **Results:** A viability test (MTS), a cytotoxicity assay LDH release and a study of the cell morphology revealed that pretreatment with 1 mg/ml or 10 mg/ml DA did not alter viability or LDH release in HEPG2 or LLC-PK1 cells. However, DA at the dose of 100 mg/ml significantly decreased cell viability, by about 40% ($P < 0.05$). Further, MTS studies revealed that DA 1 mg/ml or 10 mg/ml protected LLC-PK1 cells against a glucose-induced oxidative stress of 24 h ($P < 0.05$). **Conclusion:** Hence, the lowest concentrations of DA (1 mg/ml and 10 mg/ml) were safe for HEPG2 and LLC-PK1 and protective against an oxidative stress in LLC-PK1 cells. These data suggest that DA extracts used as a traditional herbal as food health supplements should be used at the lowest dosage.

KEY WORDS: Cytoprotection, *Desmodium adscendens*, HEPG2, LLC-PK1, glucose-induced stress, toxicity

INTRODUCTION

In a number of cultures, the traditional use of plants has been the mainstay of health maintenance. The effect of these plants or extracts are known for a multitude of beneficial effects, which include antibacterial, antiviral, anti-diabetic, or antioxidant, and have been the subject of numerous studies [1,2].

Desmodium adscendens (DA) (Sw.) DC. is a perennial plant from the *Fabaceae* family that commonly occurs in tropical areas of Africa, South America, Asia, Australia and Oceania. It has been used for many years because of its pharmacological properties and valued in folk medicine practices. Traditionally, DA is used as a wild vine (decoction) used in the Amazon rainforest of Peru, in South American countries and in the west coast of Africa. An aqueous extract of the leaves has been used for pain, fever and also epilepsy [3]. In Africa, DA is also frequently used to treat diseases linked to problems of smooth muscle contraction like asthma [4]. In Brazilian traditional medicine, the leaves of DA are used to treat a wide range of conditions that include gonorrhea, diarrheas, body aches, excessive urination, and ovarian inflammations. In France it is traditionally used as a food health supplement for its hepatoprotective effect since it was demonstrated that DA has a positive effect against hepatic infection *in vivo* [5]. Recently, publications on DA are focused on chemical composition of DA from Ghana and Nigeria. Based on thin layer chromatography, Pothie *et al.* [6] characterized the main compounds in DA: Flavonoids, triterpene, saponins, amines and alkaloids. Baiocchi *et al.* [7] used the high resolution mass spectrometry to quantify saponins and alkaloids. Flavonoids during an antioxidant activity-guided isolation were quantified in plant material from Africa using high performance liquid chromatography with diode array detector, mass spectrometry and multi-dimensional nuclear magnetic resonance spectroscopy [8]. This last chemical characterization identified the isovetixin-2''-0-xyloside (flavone C-glycosides)

as the main compound in an ethanol extract similar to the plant dietary supplement found in France and Belgium. The European regulation since 2006 requires for dietary supplements the characterization of all chemical compounds present in plant extracts. One pharmacovigilance case on a desmodium extract was declared to the Directorate for Competition Policy, Consumer Affairs and Fraud Control and the ANSES. An acute hepatotoxicity was associated in one woman to the consumption of this plant extract in the same time as other 5 medicine drugs (ANSES). Very few publications are available on the safety of DA, and only the acute toxicity of the plant has been evaluated in a study of potential neurological defects [9]. DA is used in France and Belgium as a food health supplement, in a liquid form issued of an alcoholic extraction, but its potential harmful effect has not been assessed to our knowledge on hepatocytes.

The purpose of this study was to evaluate the safety of a hydro-alcoholic extract of DA on hepatocytes and renal cells. The cytoprotective effect following oxidative stress on renal cells was also evaluated.

MATERIALS AND METHODS

Plant Material and Extract

The plant extract used in this study is a hydro alcoholic extract of the aerial parts of African *DA* with an extraction ratio of 1:1. 1 g of liquid extract corresponds 1 g of dry plant. This liquid extract was provided by the Nutergia Laboratory and represents the extract the concentrated liquid formula of the product sold in France and Belgium as a food dietary supplement.

Cell Culture

A pig kidney epithelial cell line (LLC PK 1) and a *Human liver hepatocellular carcinoma cell* line (HepG2) were obtained from American Type Culture Collection (Rockville, MD). They were grown in T-75 flasks in medium 199 (M 199, LLC-PK1) or Dulbeco's Modified Eafle's Medium (HepG2) supplemented with 10% fetal calf serum (Gibco) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (Sigma-Aldrich, France) at 37°C in a 5% CO_2 humidified atmosphere as we previously described [10]. When cell cultures reached about 80% confluence, cells were removed using 0.25% trypsin in ethylenediaminetetraacetic acid (Gibco) and sub-cultured into 6 or 96 well plates. At confluence, cells were serum-starved for 24 h prior to experimentation.

Viability Test

MTS is a calorimetric assay based on the ability of viable cells to convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, France) to formazan. The quantity of formazan produced, as measured by 490 nm absorbance, is directly proportional to the number of viable cells in culture. Cells were seeded in 96-well culture plates (5×10^3 cells/well). After the serum-starvation period, the medium was carefully removed and replaced by 100 μl of various concentrations of DA extract (1 mg/ml, 10 mg/ml, 100 mg/ml) for a 24 h incubation period. The cells were then incubated with 20 μl MTS tetrazolium compound for 2 h at 37°C. During the incubation, the MTS salt is metabolically reduced only by viable cells into an insoluble colored formazan, and the absorbance/optical density was read with a Victor 1420 Multilabel Counter (WALLAC, USA) at a single wavelength of 490 nm.

Lactate dehydrogenase (LDH)-Cytotoxicity Assay

15×10^4 cells/well were cultured in 6-well culture plates. The serum-starvation period was followed by 24 h of incubation with the different concentrations of DA extract, dimethyl sulfoxide (DMSO), or triton X-100. The culture supernatants were collected and the cells were lysed in a phosphate buffered saline 1X/0.1% triton solution and scraped. Then the cell suspension was sonicated for 30 s and centrifuged (1000 g, 10 min). LDH measurements were performed in supernatants and cell suspensions, using the Roche IFCC method (Roche/Hitachi Modullar P) at the University Hospital of Poitiers.

Cell Morphology

Phase contrast microscopy was conducted using an Olympus CKX 41 (Olympus, France) and Q-Capture Imaging Pro sofware Program (Olympus, Canada).

Cytoprotection

Cells were seeded at 3.10^5 cells/ml in 96-well plates, grown to 80% confluence, and synchronized by a 24 h incubation in the STARV medium at 37°C, followed by a 24 h incubation with the different concentrations of DA extract. Then cells were treated with 30 mM D-glucose for 24 h and the MTS assay was performed.

Statistical Analysis

Results are expressed as means ± standard error mean of five experiments. Statistical analysis was performed using analysis of variance (ANOVA) and then the Dunnett's post-test (GraphPad Prism®, GraphPad Software, San Diego, CA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

In this study, we assessed cell viability and LDH release after treatment of two cell lines (HepG2 and LLC PK1) with a DA extract for 24 h at three different doses (1, 10 or 100 mg/ml) and after triton ×100 treatment [Figure 1f].

Pictures of cell morphology changes are illustrated by Figure 1.1 for hepatocytes and by Figure 1.2 for renal cells. Control conditions included the vehicle DMSO, control cells without DMSO and triton for the chemical alteration of the cell shape. Figure 1.1 and 1.2 showed an alteration in cell shape only after a treatment with 100 mg/ml compared with untreated

cells and those exposed to two other doses of DA extract. This alteration of cell shape was found in the two cell lines (hepatocyte and renal cells) at the same concentrations of DA extract [Figure 1.1 and 1.2e].

Figure 2a and b shows the results of the assessment of cell viability obtained in HepG2 and LLC-PK1. The DA extract at 100 mg/ml significantly decreased ($P < 0.05$) cell viability by about 40% compared with control group with and without DMSO. The treatment of this cell line with 1 mg/ml or 10 mg/ml of the extract showed a trend to increase the cell viability between 14% and 22% although not statistically significant.

The results obtained in LLC PK1 and HepG2 cells show that the extract of DA at 100 mg/ml induced a significant decrease ($P < 0.05$) of cell viability by about 35% compared with control.

The release of LDH in the culture medium of HepG2 and LLC-PK1 after a treatment with DA extract at 100 mg/ml significantly increased cell injury ($P < 0.05$) by 7% and 16% compared with control, respectively. Treatment of cells as shown in Figure 3a and b with the DA extract at 1 mg/ml or 10 mg/ml show no significant increase in LDH release irrespective of the cell line used.

Protective Effect in LLC-PK1 Cells

After treatment with the DA extract for 24 h at different doses (1, 10 or 30 mg/ml) in LLCPK-1 cells, an oxidative stress was induced by incubating the cells for 24 h in serum-free culture medium saturated with D-glucose (30 mM), and cell viability was measured using MTS assay. The results presented in Figure 4 show that glucose-induced oxidative stress decreased significantly cell viability by about 60% ($P < 0.05$), and this decrease was prevented by pretreatment with 1 mg/ml or 10 mg/ml of DA ($P < 0.05$). In fact, at the dose of 10 mg/ml of DA extract, the observed cell viability was undistinguishable from that of control cells. Pretreament with 30 mg/ml of DA extract did not result in protection against oxidative-stress-induced decreased in cell viability.

DISCUSSION

Herbal medicines have become a popular form of therapy and patients who are self-medicated with herbs for preventive or

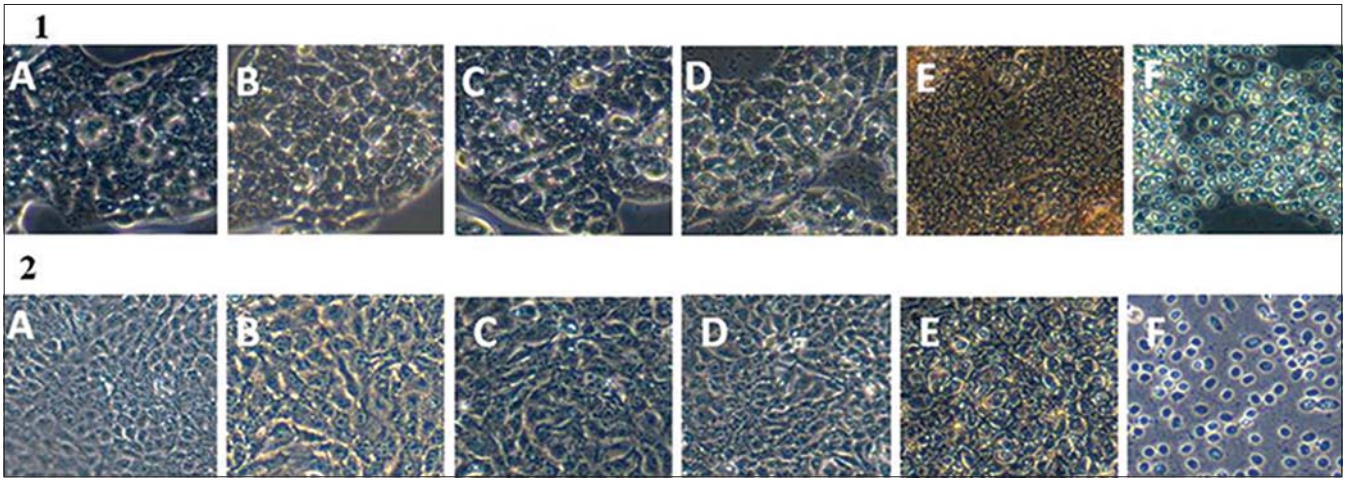

Figure 1: Morphological changes observed under light microscopy. Magnification ×10. HepG2 (1) and LLC PK1 (2), (a) dimethyl sulfoxide, (b) control, (c) 1 mg/ml desmodium extract, (d) 10 mg/ml desmodium extract, (e) 100 mg/ml desmodium extract, (f) triton ×100

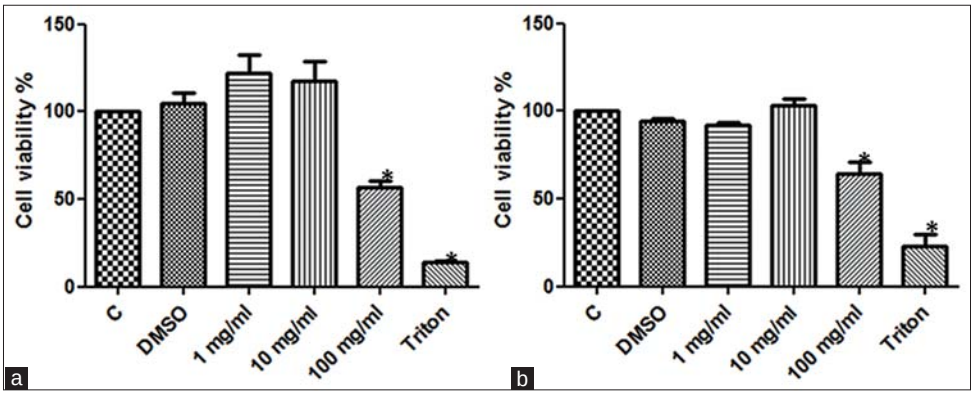

Figure 2: Effect of *Desmodium adscendens* (DA) extract on cells growth. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, (a) Cell viability of HepG2 cell line, (b) Cell viability of LLC PK1 cell line incubated for 24 h without or with DA extract 1, 10 or 100 mg/ml), dimethyl sulfoxide and triton were used as negative and positive controls respectively. Data are represented as mean ± standard error mean of six determinations. *$P < 0.05$ versus control

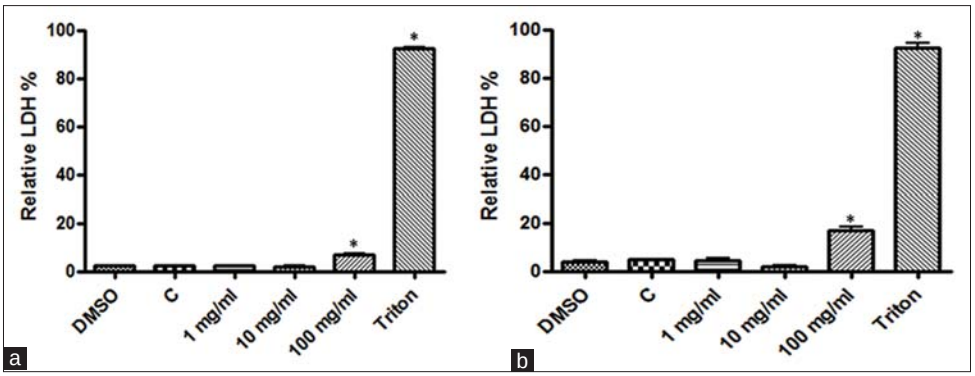

Figure 3: Effect of *Desmodium adscendens* (DA) on cell injury assessed by the lactate dehydrogenase (LDH) release, (a) LDH release of HepG2 cell line, (b) LDH release of LLC PK1 cell line incubated for 24 h without or with DA extract 1, 10 or 100 mg/ml), dimethyl sulfoxide and triton were used as negative and positive controls, respectively. Data are represented as mean ± standard error mean of six determinations. **P* < 0.05 versus control

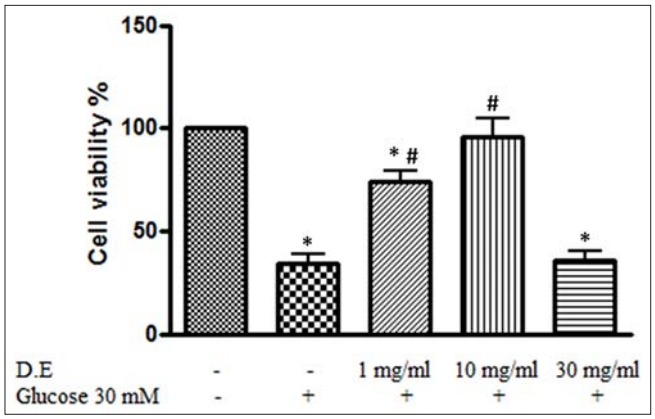

Figure 4: Protective effects of *Desmodium adscendens* (DA) extract against oxidative stress on LLC-PK1 cell line. Cell viability of LLC-PK1 cell line was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Oxidative stress was induced high glucose (30 mM) and incubated for 24 h without or with DA extract 1, 10 or 100 mg/ml). Data are represented as mean ± standard error mean of six determinations. **P* < 0.05 from control

therapeutic purposes may assume that these products are safe because they are natural. However, any product can become toxic if not used correctly [11]. Hence, the objective of this study was to evaluate the potential toxicity of a hydro-alcoholic extract of DA. We used two well-defined cells lines to appreciate potential risks of liver and renal damages [12,13]. The safety of DA extract on HepG2 and LLC-PK1 ranged from 1 mg/ml to 10 mg/ml of DA extract as suggested by the absence of changes in cell viability or cell morphology following 24 h of exposure to these concentrations. This was correlated to a lack of increase in LDH release in both cell lines.

On the other hand, our data suggest that the concentration of 100 mg/ml of DA extract significantly decreased cell viability of both HepG2 and LLC-PK1, and was associated with a significant increase of LDH release. Whether this toxicity could be attributed to the amount of alcohol used in the plant extract remains to be determined. Indeed, alcohol has been shown to increase the number of apoptotic cells [14,15] and lead to the induction of intracellular enzymes like alcohol deshydrogenase and the production reactive oxygen species (ROS) [16]. Consistent with the observed effect, ethanol cytotoxicity is dose-dependent and occurs within 24 h *in vitro* [17]. The effect of ethanol should be assessed as vehicle on our cells. The amount of ethanol in the DA extract should be measured. The dose of 100 mg/ml of DA extract is not safe despite the alcohol vehicle is known. This dosage should be used with caution.

The cytoprotective effect of this extract was also examined by using a glucose-induced oxidative stress [18,19]. Since the cytotoxicity studies discussed in the previous paragraph revealed that the dose of 100 mg/ml was not safe for the cells, a lower concentration of 30 mg/ml was used in this set of studies, in addition to the concentrations of 1 mg/ml and 10 mg/ml. Concentrations of 1 mg/ml and 10 mg/ml of DA efficiently decreased glucose-induced oxidative stress. A total recovery of cell viability at the dose of 10 mg/ml during the 24 h of exposure suggested a very efficient antioxidant activity. Hence, the concentrations of 1 mg/ml and 10 mg/ml were safe and also cytoprotective. This cytoprotective effect of low concentrations of DA against glucose-induced oxidative stress is to put in parallel with the detection of these antioxidant properties. In fact, it was shown that a hydroalcoholic extract of DA had scavenging antioxidant activities, inhibiting the ROS generation induced by H_2O_2 [20]. Cellular test, 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic) (ABTS) have shown the scavenging antioxidant effect of an hydroalcoholic extract of leaves of DA [20]. The results of the DPPH and ABTS tests, expressed in mg of vitamin C equivalents per g dry weight, were 8.47 and 12.83, respectively. ROS levels measured by flow cytometry revealed, at a concentration of 25 mg/ml, a decrease of 83% of ROS level generated by exogenous H_2O_2. In the present study, pretreatment with 30 mg/ml of DA did not provide any protection. The fact that the two lower concentrations of this study have a protective effect, contrary to the dose of 30 mg/ml, may also be ascribed to ethanol. Actually, ethanol treatment

was also shown to impair antioxidant levels *in vitro*, resulting in ROS generation and increased oxidative stress [21]. Thus, low doses are sufficient to obtain a protective effect, while at higher concentrations, oxidative stress driven by alcohol in the extract seems to annihilate the protective activity of DA. The generation of ROS by exogenous H_2O_2 was shown to be inhibited by a pre-treatment with the DA extract in a similar way to our study [9].

In conclusion, lowest concentrations (1 mg/ml and 10 mg/ml) are safe for the cells and protective against an oxidative stress. DA extracts used as a traditional medicine as food health supplements in Europe for its efficient on stress in human should be used at the lowest dosage.

ACKNOWLEDGMENTS

We are most grateful to Dr. S Sennoune (Texas Tech University) for the proofing of the manuscript and Dr. Claude Lagarde (Nutergia, France), Dr. Michel de Sarrieu (Fleurance Nature, France) and Jacques Karlson (Synadiet, France) for helpful discussions and information on the use of DA in France.

AUTHOR CONTRIBUTIONS

The author's responsibilities were as follow/MF, JMM study design and writing the manuscript; CF and MF conduct the cell culture and supplementation. All authors participated in the study and take responsibility for the content of this report. None of the authors had a personal or financial conflict of interest.

REFERENCES

1. Nergard CS, Diallo D, Inngjerdingen K, Michaelsen TE, Matsumoto T, Kiyohara H, *et al.* Medicinal use of *Cochlospermum tinctorium* in mali anti-ulcer-, radical scavenging- and immunomodulating activities of polymers in the aqueous extract of the roots. J Ethnopharmacol 2005;96:255-69.
2. Meléndez PA, Capriles VA. Antibacterial properties of tropical plants from Puerto Rico. Phytomedicine 2006;13:272-6.
3. Adjanohoun E. Contribution to ethnobotanical and floristic studies in the people's republic of Congo. Tradit Med Pharm Suppl 1988;3:428.
4. Heard O. Contribution of the study of *Desmodium adscendens*: Chemistry & pharmacology. Ph.D. France: University of Tours; 1994.
5. Gyamfi MA, Yonamine M, Aniya Y. Free-radical scavenging action of medicinal herbs from Ghana: *Thonningia sanguinea* on experimentally-induced liver injuries. Gen Pharmacol 1999;32:661-7.
6. Pothie J, Ragot J, Galand N. Planar chromatographic study of flavonoids and soyasaponins for validation of fingerprints of *Desmodium adscendens* of different origins. J Planar Chromatogr 2006;19:191-4.
7. Baiocchi C, Medana C, Giancotti V, Aigotti R, Dal Bello F, Massolino C, *et al.* Qualitative characterization of *Desmodium adscendens* constituents by high-performance liquid chromatography-diode array ultraviolet-electrospray ionization multistage mass spectrometry. Eur J Mass Spectrom (Chichester, Eng) 2013;19:1-15.
8. Zielinska-Pisklak MA, Kaliszewska D, Stolarczyk M, Kiss AK. Activity-guided isolation, identification and quantification of biologically active isomeric compounds from folk medicinal plant *Desmodium adscendens* using high performance liquid chromatography with diode array detector, mass spectrometry and multidimentional nuclear magnetic resonance spectroscopy. J Pharm Biomed Anal 2014;102C:54-63.
9. N'gouemo P, Baldy-Moulinier M, Nguemby-Bina C. Effects of an ethanolic extract of *Desmodium adscendens* on central nervous system in rodents. J Ethnopharmacol 1996;52:77-83.
10. Fares M, Armand M, Francois C, Maixent JM. ω6/ω3 Polyunsaturated fatty acid supplementations in renal cell model lead to a particular regulation through lipidome for preserved ω6/ω3 ratios. Cell Mol Biol (Noisy-le-grand) 2012;58 Suppl: OL1715-9.
11. Maixent JM. Opinion paper food supplements: The European regulation and its application in France. Thoughts on safety of food supplements. Cell Mol Biol (Noisy-le-grand) 2012;58 Suppl: OL1720-9.
12. Pinti M, Troiano L, Nasi M, Ferraresi R, Dobrucki J, Cossarizza A. Hepatoma HepG2 cells as a model for *in vitro* studies on mitochondrial toxicity of antiviral drugs: Which correlation with the patient? J Biol Regul Homeost Agents 2003;17:166-71.
13. Nilakantan V, Maenpaa C, Jia G, Roman RJ, Park F 20-HETE-mediated cytotoxicity and apoptosis in ischemic kidney epithelial cells. Am J Physiol Renal Physiol 2008;294:F562-70.
14. Valente M, Calabrese F. Liver and apoptosis. Ital J Gastroenterol Hepatol 1999;31:73-7.
15. McVicker BL, Tuma DJ, Casey CA. Effect of ethanol on pro-apoptotic mechanisms in polarized hepatic cells. World J Gastroenterol 2007;13:4960-6.
16. Castillo T, Koop DR, Kamimura S, Triadafilopoulos G, Tsukamoto H. Role of cytochrome P-450 2E1 in ethanol-, carbon tetrachloride- and iron-dependent microsomal lipid peroxidation. Hepatology 1992;16:992-6.
17. Pastorino JG, Hoek JB. Ethanol potentiates tumor necrosis factor-alpha cytotoxicity in hepatoma cells and primary rat hepatocytes by promoting induction of the mitochondrial permeability transition. Hepatology 2000;31:1141-52.
18. Yokozawa T, Kim YA, Kim HY, Okamoto T, Sei Y. Protective effect of the Chinese prescription Kangen-karyu against high glucose-induced oxidative stress in LLC-PK1 cells. J Ethnopharmacol 2007;109:113-20.
19. Pang J, Gong H, Xi C, Fan W, Dai Y, Zhang TM. Poly(ADP-ribose) polymerase 1 is involved in glucose toxicity through SIRT1 modulation in HepG2 hepatocytes. J Cell Biochem 2011;112:299-306.
20. Muanda FN, Bouayed J, Djilani A, Yao C, Soulimani R, Dicko A. Chemical composition and, cellular evaluation of the antioxidant activity of *Desmodium adscendens* leaves. Evid Based Complement Alternat Med 2011;2011:620862.
21. Katz GG, Shear NH, Malkiewicz IM, Valentino K, Neuman MG. Signaling for ethanol-induced apoptosis and repair *in vitro*. Clin Biochem 2001;34:219-27.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141006105747

Original Research

Effect of constituents from samaras of *Austroplenckia populnea* (Celastraceae) on human cancer cells

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Received: August 30, 2014
Accepted: October 06, 2014
Pubilshed: November 28, 2014

ABSTRACT

Background: Aiming the continuity of the studies of *Austroplenckia populnea*, Brazilian species of the Celastraceae family, in the present study, it was investigated the effect of crude extracts obtained with ethanol, ethyl acetate and chloroform and two purified constituents, proanthocyanidin A and 4′-*O*-methylepigallocatechin, both isolated from its samaras, on cancer cell proliferation assays. **Materials and Methods:** The human cancer cells lines MCF-7 (ductal breast carcinoma), A549 (lung cancer), HS578T (ductal breast carcinoma) and non-cancer HEK293 (embryonic kidney cells) were treated with different concentrations of extracts and constituents and the effect was observed through the acid phosphatase method. The chemical structures of the purified compounds were identified by the respective IR and ^{1}H and ^{13}C nuclear magnetic resonance spectral data. **Results:** While crude extracts from samaras of the folk medicine *A. populnea* can trigger cell proliferative effects in human cell lines, the purified compounds (proanthocyanidin A and 4′-*O*-methyl-epigallocatechin) isolated from the same extracts can have an opposite (anti-proliferative) effect. **Conclusion:** Based on the results, it was possible to suggest that extracts from samaras of *A. populnea* should be further investigated for possible cancer-promoting activities; and the active extracts can also represent a source of compounds that have anti-cancer properties.

KEY WORDS: *Austroplenckia populnea*, cancer cell proliferation, Celastraceae, samaras

INTRODUCTION

In general, a plant extract contains low concentrations of active compounds and a large number of promising but undiscovered compounds [1]. A widely observed and accepted fact about the medicinal value of plants is mainly the bioactivity of the phytocomponents present in it. Although a major effort towards of the elucidation of bioactive phytochemicals is from purification of plant materials, very few screening programs have been initiated in crude plant materials, which could yield a significant number of active extracts. Also, there is a need for developing new easy to use sensitive bioassays for screening these bioactive phytocompounds [2].

Some studies with species of the Celastraceae family show positive results for the anticancer activity [3,4]. Constituents isolated from species of the Celastraceae family such as sesquiterpenes, agarofurans and pentacyclic triterpenes present proven antitumoral properties [5-7]. Among these triterpenes, it has been reported that tingenone and pristimerin, frequently found in different species of the Celastraceae family, present potential properties against cancer cell lines [3].

Austroplenckia populnea (Reissek) Lundell is a Brazilian tropical tree that belongs to Celastraceae family. This plant, popularly known as "Mangabarana," "Marmelo-do-campo" or "Mangabeira-brava," can be found in a big tropical

ecoregion known as "Cerrado" mainly in the State of Minas Gerais, Brazil, and has been of great interest to researchers in function of its chemical constituents, and larvicidal and molluscicidal properties [8,9]. The decoct from leaves and roots of *A. populnea* have been used in traditional medicine to treat gastrointestinal disorders [10] and rheumatism [11]. During continued phytochemical investigation of extracts from leaves, stems and roots of *A. populnea* an extensive number of constituents have been isolated, finding the main compounds as different series of pentacyclic triterpenes, such as friedelanes, agarofurans and others [8,9]. When subjected to *in vitro* assays, some constituents of *A. populnea* presented properties such as antibacterial [9,12], antitrypanosomal [13], anti-inflammatory, antinociceptive [14], antiulcerogenic, analgesic [15] and male contraceptive effect [16,17]. In accordance to Monache *et al.* [18], antitumoral property was also attributed to *A. populnea*.

Considering that the bioactive property of samaras of *A. populnea* was not been reported so far and aiming to contribute to the knowledge of phytotherapeutic potential of the *A. populnea*, in the present study it was investigated the effect of crude extracts and two purified constituents from samaras on the following human cancer cell lines: MCF-7 (ductal breast carcinoma), A549 (lung cancer), HS578T (ductal breast carcinoma) and a non-cancer HEK293 (embryonic kidney cells) through acid phosphatase method.

MATERIALS AND METHODS

Plant Material and Extracts Preparation

The mature samaras of *A. populnea* were collected during the months of June and July in Nova Lima Region, Minas Gerais State, Brazil. A sample of the collected material was compared and identified with a voucher specimen (No. 10473) deposited at the Herbarium of the Museu de Historia Natural, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. The samaras were dried at room temperature and then fragmented on a knife mill. The powdered material (286.97 g) was submitted to continuous extraction process in a Soxhlet apparatus using hexane, chloroform, ethyl acetate and ethanol as solvent extractors. Afterwards, each solvent was recovered in a rotatory evaporator providing the dry hexane (SAPEH, 45.19 g), chloroform (SAPEC, 4.23 g), ethyl acetate (SAPEAE 5.83 g), and finally ethanol (SAPEE, 33.66 g) extracts.

Isolation and Structure Identification of Proanthocyanidin A and 4'-*O*-Methylepigallocatechin

To determine the principal chemical groups, each extract from samaras of *A. populnea* was analyzed following methodology suggested by Wagner *et al.* [19] and Matos [20]. Proanthocyanidin A and 4'-*O*-methylepigallocatechin were isolated from polar extract (SAPEE) of samaras through silicagel column chromatography eluted with mixtures of hexane, chloroform, ethyl acetate and methanol in order of increased polarities. These compounds were purified by preparative thin layer chromatography using mixtures of ethyl acetate and methanol.

The structure elucidation of proanthocyanidin A was based on the assignments of its infrared and ^{1}H and ^{13}C (with distortionless enhancement by polarization transfer [DEPT] experiment) nuclear magnetic resonance (NMR) spectral analysis, including comparison with the NMR data already published [21]. The compound 4'-*O*-methylepigallocatechin was identified by ^{1}H and ^{13}C NMR including 2D experiments followed by comparison with NMR spectral data previously reported [22,23].

Cell Culture and Treatment

The human tumor cells MCF-7 (ductal breast carcinoma); A549 (lung adenocarcinoma); HS578T (ductal breast carcinoma); and non-cancerous cells HEK293 (embryonic kidney cells) provide by American Type Culture Collection (Rockville, MD, USA) were maintained in Dulbecco's Modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin/streptomycin (Sigma). The cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were treated ×1 with trypsin ethylenediaminetetraacetic acid (Sigma) and washed once with phosphate buffer saline (PBS) for maintenance before transfections and cell harvesting.

The extracts were dissolved in sterile dimethylsulfoxide (DMSO) and added to the culture medium aiming to reach maximum concentration of 1% DMSO (v/v) [24]. The isolated compounds were dissolved in growth medium. The sample solutions of substances isolated from samaras of *A. populnea* were individually added to the wells (96-well microtiter plate) at four different concentrations: 125, 250, 500 and 1000 μg/mL for extracts and 50, 100, 250 and 500 μg/mL, for constituents. For each concentration of the extract or purified constituent from samaras, the assays were made in triplicate. Cells maintained in growth medium only with DMSO (1%, v/v) were used as a solvent control.

Cell Proliferation Assay

The effect of extracts and constituents from samaras of *A. populnea* on cell proliferation was evaluated through acid phosphatase method [25]. This colorimetric method is based on the conversion of substrate *p*-nitrophenyl phosphate to *p*-nitrophenol, which absorbs light at 405 nm (yellow). The amount of *p*-nitrophenol produced is proportional to the number of living cells present in the culture medium. Cells were previously plated in wells of 96 microtiter plates (100 μL of 1×10^4 cell/mL solution in each well) for 24 h. After removing the medium from microplates, the cells were rinsed twice with PBS. Freshly prepared (100 μL) of phosphatase substrate (10 mM *p*-nitrophenol phosphate [sigma] in 0.1 M sodium acetate [sigma], 0.1% triton X-100 [BDH], pH 5.5) was added to each well and the microplates

were wrapped with aluminium foil and incubated in the dark at 37°C for 2 h. An aliquot (50 μL) of NaOH (1.0 mol/L) was added to each well to stop the enzymatic reaction and followed by measuring the absorbance at 405 nm with a reference wavelength of 620 nm using a Microplater Reader (Modulus Microplate Multimode Reader for Luminescence, Fluorescence and Absorbance).

Statistics

All the experiments were conducted in triplicate. To evaluate the statistical significance, the results were analyzed by means of Student's *t*-test. Results were expressed as mean ± standard error of the mean (error bars in graphs). Differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Phytochemical Analyses

In accordance with methodology suggested by Wagner *et al.* (1984) [19] and Matos (1997) [20] were identified in the extracts isolated from samaras of A. *populnea* the presence of long chain hydrocarbons (the most part in SAPEH), triterpenes, flavonoids and catechins. This result is in agreement with previous works in which were isolated mainly pentacyclic triterpenes [9,12].

Structure Elucidation

Proanthocyanidin A and 4'-*O*-methyl epigallocatechin [Figure 1] were isolated and identified from samaras of *A. populnea*. These two compounds were previously isolated from other parts of A. *populnea* [13] and *Maytenus truncata*, another member of the Celastraceae family, by Meléndez-Salazar [26].

4'-*O*-Methylepigalocatechin [Figure 1a] was isolated as an amorphous yellowish solid (m.p. 172.0-174.0 °C). In the ^{1}H NMR spectra (CD_3OD, 400 MHz) were observed signals at d_H 6.53 (2H, s, H-2'; H-6'), d_H 5.95 (^{1}H, d, J = 2.0 Hz, H-8) and d_H 5.93 (1H, d, *J* = 2.4 Hz, H-6) associated to hydrogen of aromatic ring. This signals together those at d_H 2.85 (1H, dd, *J* = 4.2; 16.4 Hz, H-4a) and d_H 2.73 (^{1}H, dd, *J* = 4.2; 16.4 Hz, H-4b) which were attributed to hydrogen of methylene group suggested a flavane structure of the epigalocatechin type [22]. The signal at d_H 3.79 (3H, s) were attributed to methoxyl group and the signal at d_H 4.78 (^{1}H, s, H-2) and at d_H 4.19 (^{1}H, s (large), H-3) were related to carbinol hydrogen atoms. Through the ^{13}C NMR spectrum and DEPT-135 (CD_3OD, 100 MHz) were observed the signals at d_C: 79.8 (C2), 67.5 (C3), 29.3 (C4), 157.3 (C5), 96.6 (C6), 157.8 (C7), 96.1 (C8), 100.1 (C9), 158.1 (C10), 136.7 (C1'), 107.3 (C6'), 151.5 (C3' and C5'), 136.3 (C4') and 60.9 (OMe) [21-23].

Proanthocyanidin A [Figure 1b] was isolated as a brownish amorphous solid (m.p. 227.2-230.0°C). In its ^{1}H NMR spectrum (CD_3OD, 400 MHz) were observed signals at d_H 7.21, 6.72, 6.59 and at d_H 5.93 related to hydrogen of aromatic ring. The signal at d_H 3.75 was attributed to hydrogen atoms of the methoxyl group and the signal at d_H 2.89 to methylene hydrogen (H-4"). The signal at d_H 5.14 (H-3) and d_H 3.89 (H-3") were associated to methinic hydrogen neighboring to C-O and bonded to a hydroxyl group. By the ^{13}C NMR spectrum (CD_3OD, 100 MHz) and DEPT-135 were observed the signals at d_C: 77.5 (C2), 73.7 (C3), 37.4 (C4), 157.8 (C5), 97.2 (C6), 157.8 (C7), 96.3 (C8), 100.5 (C9), 156.5 (C10), 132.1 (C1'), 129.2 (C2'), 115.8 (C3' and C5'), 157.8 (C4'), 129.3 (C6'), 80.0 (C2"), 67.0 (C3"), 29.8 (C4"), 157.8 (C5"), 96.3 (C6"), 157.8 (C7"), 107.5 (C8"), 100.5 (C9"), 155.8 (C10"'), 132.1 (C1"'), 107.5 (C2"' and C6"'), 151.4 (C3"' and C5"'), 136.0 (C4"') and 60.8 (OMe) [21].

Cell Proliferation Assay

In order to evaluate the phytotherapeutic nature of samaras of *A. populnea*, three crude extracts and two purified compounds were respectively added to the culture medium of three different human tumor cells: MCF-7 (ductal breast carcinoma); A549 (lung adenocarcinoma); HS578T (ductal breast carcinoma); and in culture medium of non-cancerous HEK293 (embryonic kidney cells). These cancer cell lines were used because they are highly studied in the research involving cytotoxic analysis and could be easily used for comparison with different compounds [27]. The biological effect of the samples from samaras of A. *populnea* on cell proliferation was evaluated through the acid phosphatase method.

After 24 and 48 h of treatment, it was observed that the extracts induced an enhancement in the cell proliferation [Figure 2], and there was a significant variation within and between cell lines over time. At 24 h post-treatment of MCF-7 cells with chloroform extract, significant increase in cell proliferation was observed at concentration of 250, 500 and 1000 μg/mL and no effect with 125 μg/mL [Figure 2e]. A further increase in proliferation at 250 and 1000 μg/mL and no significant effect at 125 and 500 μg/mL were verified after 48 h incubation time [Figure 2f]. However, in relation to A549 cell line a significantly decrease in cell proliferation was observed for all concentrations (125, 250, 500 and 1000 μg/mL) of crude chloroform extract from samaras [Figure 2e]. After 24 h of the treatment, the chloroform extract only induced a small cell proliferative effect. For this reason, this type of effect was considered after 48 h incubation time [Figure 2f]. The impact of chloroform extract (250 μg/mL) after 24 h treatment, on HS578T cell proliferation, was considered significant. After 48 h incubation, it was observed that this extract induce cell proliferation at all concentration used in the experiments. In relation to HEK293 embryonic cells were observed a consistent increase in cell proliferation after 24 and 48 h of treatments, for all concentrations [Figure 2e and f]. Ethyl acetate extract induced an increase in the cell proliferation in all four cell lines and with all four concentrations, both after 24 or 48 h [Figure 2c and d]. It was also observed a significantly increased in cell proliferation induced by the four concentrations of ethanol extract (125, 250, 500 and 1000 μg/mL), after 24 or 48 h of treatment of the cells MCF-7, A549, HS578T or HEK293 [Figure 2a and b].

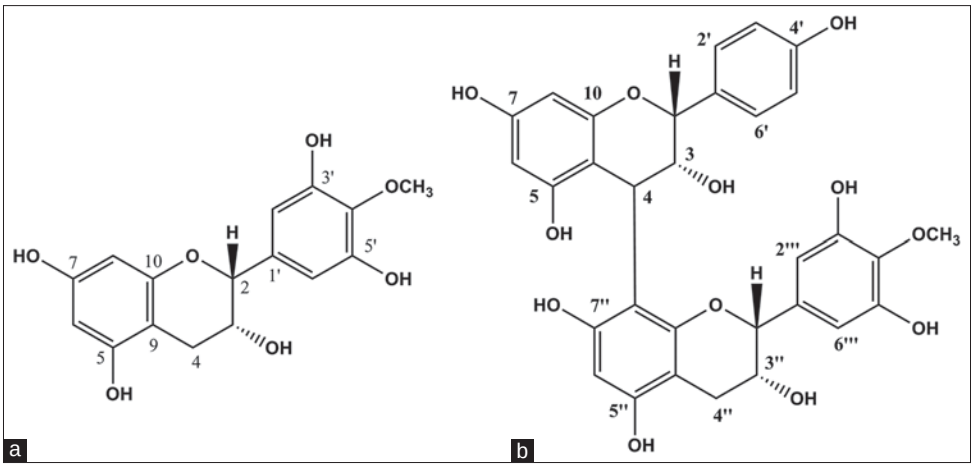

Figure 1: Chemical structures of 4'-*O*-methyl epigallocatechin (a) and proanthocyanidin A (b)

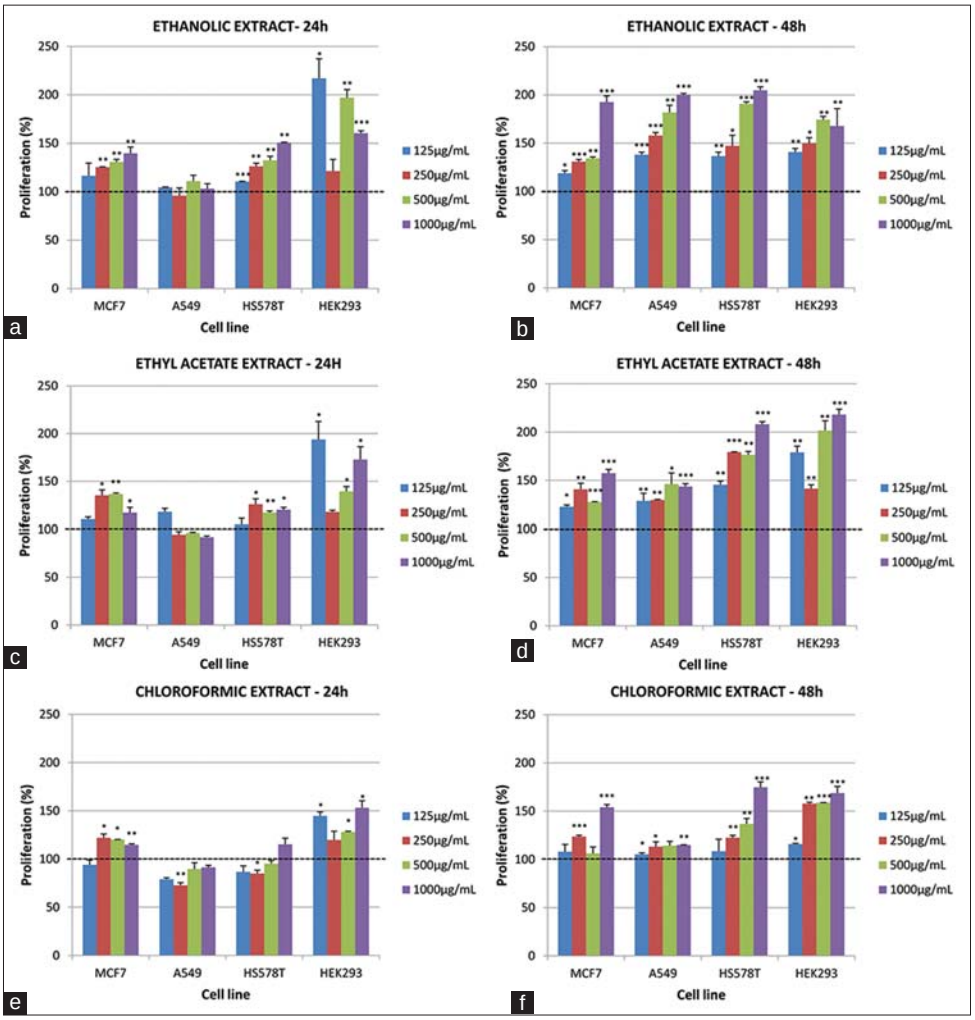

Figure 2: Impact on cell proliferation after 24 and 48 h treatment with three crude extracts isolated from samaras of *Austroplenckia populnea*, on four cell lines: MCF-7, A549, HS578T and HEK293. Treatment of chloroform extract (a) (24 h) and (b) (48 h), ethyl acetate extract (c) (24 h) and (d) (48 h), ethanol extract € (24 h) and (f) (48 h). Final median concentration of extracts was 125 µg/mL (light blue), 250 µg/mL (red), 500 µg/mL (green) and 1000 µg/mL (dark blue). Data are represented as mean relative percentage (%) and standard deviation of three replicates. Significant differences based on the control were established using Student's t-test and are represented by asterisks ($P < 0.05$: *, $P \leq 0.01$: **, $P \leq 0.001$: ***)

Two constituents isolated from samaras of *A. populnea* were also subjected to assays to determine its effect on the proliferation of the above cited cells lines. After 24 h the treatment with the proanthocyanidin A, in all concentrations,

they were not observed induction of the proliferation of cells MCF-7, A549 or HS578T. In the other hand, it was observed a significant decrease in proliferation of HEK293 cells [Figure 3a]. After 48 h incubation time, it was not observed effect on MCF-7 cells. Proanthocyanidin A at concentrations of 100 and 250 μg/mL decreased the proliferation of A549 cells [Figure 3a]. A significant decrease in HS578T cell proliferation was observed with 50, 100 and 250 μg/mL and no effect with 500 μg/mL [Figure 3a and b]. A significant increase in proliferation was observed at 48 h with HEK293 relative to 24 h treatments; however, the increase was below the control treatments. The other constituent isolated from samaras, 4'-O-methylepigallocatechin, post 24 h treatment had no significant impact on MCF-7 cell proliferation across the concentrations [Figure 3c]. Although, in A549 a significant decrease in cell proliferation at 24 h with 50 and 250 μg/mL concentration was observed and post 48 h there was further decrease in the proliferation [Figure 3c and d]. A significant decrease in proliferation of HS578T at 24 h at 100 μg/mL and further significant decrease in proliferation at 48 h at 50, 100 and 250 μg/mL was observed [Figure 3c and d]. For HEK293 cells, it was verified a significant decrease in proliferation at 24 h with 50, 100 and 250 μg/mL but increased proliferation at 48 h was observed relative to 24 h treatments but the increase was below the relative control treatments.

There was no real information on the total bioactive compounds present in samaras of this plant and the biological impact produced by each one. Thus, in the present work three crude extracts from samaras of A. *populnea* were used, obtained with ethanol, ethyl acetate and chloroform, and two purified constituents proanthocyanidin A and 4'-O-methyepigallocatechin to evaluate the impact on cell proliferation on three different human cancer cell lines MCF-7, A549, HS578T and non-cancer HEK293. It was observed from the preliminary cell proliferation data that all the three crude extracts seem to promote an increase on cell proliferation over time in all four model cell lines.

Using only crude extract, it was observed a significantly increased in cell proliferation in most of the cells used. It might reflect of an interaction of a group of compounds with different action on the cell metabolic pathway which has been acting in the growth cell regulation. The elucidation of which specific pathway in being affected by these extracts in the tumor and normal cell lines was not performed because this analysis did not focus in molecular analysis and/or gene expression analysis. The importance of the extracts in growth regulation of the normal cell lines is the main focus because parts of A. *populnea*, such as leaves and roots have been used in traditional medicine to treat ulcers, dysenteries and rheumatism. It might be dangerous for the population that make this uses because these plant parts (even in small quantity) could cause the increase of cell proliferation without cell-repair, leading to a possible increase the risk of inducing a cancer.

Conversely, the purified compounds were found to significantly decrease the cell proliferation over time in A549, HS578T and HEK293, and did not have any effect on MCF-7. Proanthocyanidin A and 4'-O-methylepigallocatechin belong to the group of flavonoids and are known for their antioxidant properties and anti-proliferative effect in cancer cell line [28,29].

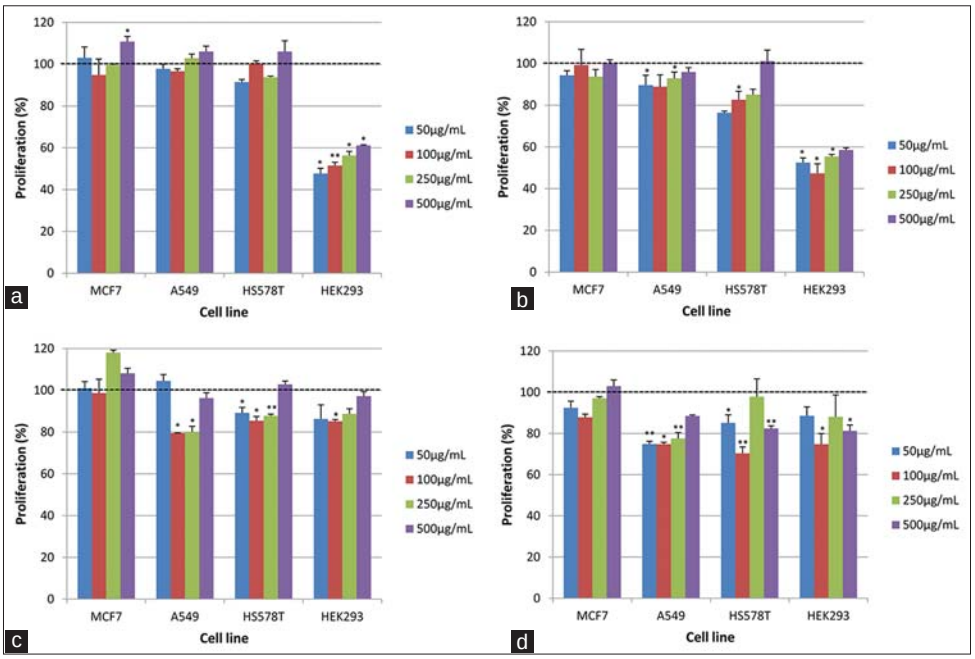

Figure 3: Impact on cell proliferation post 24 and 48 h treatment of two purified compounds from samaras of *Austroplenckia populnea* on four cell lines MCF-7, A549, HS578T and HEK293. Treatment of proanthocyanidin A (24 h) (a) and (48 h) (b), 4'-O-methylepigallocatechin (24 h) (c) and (48 h) (d). Final concentration of extracts in media was 50 µg/mL (light blue), 100 µg/mL (red), 250 µg/mL (green) and 500 µg/mL (dark blue). Data are shown as relative percentage (%) mean and one standard deviation of three replicates. Significant differences to the control were analyzed using Student's *sssss*-test and are represented by asterisks ($P < 0.05$: *, $P \leq 0.01$: **)

CONCLUSION

From the preliminary data, crude extracts from samaras of *A. populnea* could be useful in cure for various diseases as rheumatism and bones injuries because of its positive impact on cell growth, but it revokes a question of how safe it is to be taken orally. The samaras extract may contain bioactive phytocomponents that need further investigation.

ACKNOWLEDGMENT

The authors do thank the Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) for financial support.

REFERENCES

1. Rates SM. Plants as source of drugs. Toxicon 2001;39:603-13.
2. Kupchan SM, Maruyama M, Hemingway RJ, Hemingway JC, Shibuya S, Fujita T, *et al*. Eupacunin, a novel antileukemic sesquiterpene lactone from *Eupatorium cuneifolium*. J Am Chem Soc 1971;93:4914-6.
3. Gomes JP, Cardoso CR, Varanda EA, Molina J, Fernandez MF, Olea N, *et al*. Antitumoral, mutagenic and (anti) estrogenic activities of tingenone and pristimerin. Braz J Pharmacogn 2011;21:963-71.
4. Niero R, de Andrade SF, Cechinel Filho V. A review of the ethnopharmacology, phytochemistry and pharmacology of plants of the *Maytenus* genus. Curr Pharm Des 2011;17:1851-71.
5. Shirota O, Morita H, Takeya K, Itokawa H. Cytotoxic aromatic triterpenes from *Maytenus ilicifolia* and *Maytenus chuchuhuasca*. J Nat Prod 1994;57:1675-81.
6. Wang H, Tian X, Pan Y. Antitumor sesquiterpenes from *ss*. Helv Chim Acta 2003;86:3320-5.
7. Buffa W, Bolzani VD, Furlan M, Pereira SI, Pereira MA, França SC. *In vitro* propagation of *Maytenus ilicifolia* (Celastraceae) as potential source for antitumoral and antioxidant quinomethide triterpenes production. A rapid quantitative method for their analysis by reverse-phase high-performance liquid chromatography. Arkivoc 2004;6:137-46.
8. Vichnewski W, Prasad JS, Herz W. Polyhydroxyagarofuran derivatives from *Austroplenckia polpunea*. Phytochemistry 1984;23:1655-7.
9. Vieira Filho SA, Duarte LP, Paes HC, Silva GD, Sousa JR, Lanna MC. Antibacterial activity of pentacyclic triterpenes from *Austroplenckia populnea*. Acta Hortic 1999;501:199-03.
10. Correa MP, Penna LA. Dicionário de Plantas Úteis do Brasil e das Exóticas Cultivadas. Vol. 4. 6 Illustrated Vols. Rio de Janeiro: Ministério da Agricultura. Instituto Brasileiro de Desenvolvimento Florestal. 1926-1984. p. 152.
11. Gonzalez JG, delle Monache G, delle Monache F, Marini-Bettolò GB. Chuchuhuasha - A drug used in folk medicine in the Amazonian and Andean areas. A chemical study of *Maytenus laevis*. J Ethnopharmacol 1982;5:73-7.
12. Miranda RR, Duarte LP, Silva GD, Vieira Filho SA, Carvalho PB, Messas AC. Evaluation of antibacterial activity of "Mangabarana" *Austroplenckia populnea* Reissek (Celastraceae). Rev Bras Farmacogn 2009;19:370-5.
13. Duarte LP, Vieira Filho SA, Silva GDF, Pinto AS. Antitrypanosomal activity of pentacyclic triterpenes isolated from *Austroplenckia polpunea* (Celastraceae). Rev Inst Med Trop São Paulo 2002;44:109-12.
14. Andrade SF, Cardoso LG, Carvalho JC, Bastos JK. Anti-inflammatory and antinociceptive activities of extract, fractions and populnoic acid from bark wood of *Austroplenckia populnea*. J Ethnopharmacol 2007;109:464-71.
15. Seito LN, Mazaro R, Di Stasi LC. Antiulcerogenic and analgesic effects of the *Austroplenckia populnea* extracts in mice. Phytother Res 2002;16:193-6.
16. Mazaro R, Di Stasi LC, Filho SA, De Grava Kempinas W. Decrease in sperm number after treatment of rats with *Austroplenckia populnea*. Contraception 2000;62:45-50.
17. Mazaro R, Di Stasi L, De Grava Kempinas W. Effects of the hydromethanolic extract of *Austroplenckia populnea* (Celastraceae) on reproductive parameters of male rats. Contraception 2002;66:205-9.
18. Monache FD. Populnonic acid, a new triterpenic acid with friedelane carbon skeleton. Gazz Chim Ital 1972;102:636-46.
19. Wagner H, Blat S, Zgainsky EM. Plant Drug Analysis. Berlin: Springer; 1984.
20. Matos FJ. Introduction to Experimental Phytochemistry. 2nd ed. Fortaleza, Ceará: Editora UFC; 1997.
21. Weeratunga G, Bohlin L, Verpoorte R, Kumar V. Flavonoids from *Elaeodendron Balae* root Bark. Phytochemistry 1985;24:2093-5.
22. Meng X, Lee MJ, Li C, Sheng S, Zhu N, Sang S, *et al*. Formation and identification of 4'-O-methyl-(-)-epigallocatechin in humans. Drug Metab Dispos 2001;29:789-93.
23. Medeiros FA, Medeiros AA, Tavares JF, Barbosa Filho JM, Lima EO, Silva MS. Licanol, um novo flavanol, e outros constituintes de *Licania macrophylla* Benth. Quim Nova 2012;35:1179-83.
24. Rocha FD, Soares AR, Houghton PJ, Pereira RC, Kaplan MA, Teixeira VL. Potential cytotoxic activity of some Brazilian seaweeds on human melanoma cells. Phytother Res 2007;21:170-5.
25. Muniyappa MK, Dowling P, Henry M, Meleady P, Doolan P, Gammell P, *et al*. MiRNA-29a regulates the expression of numerous proteins and reduces the invasiveness and proliferation of human carcinoma cell lines. Eur J Cancer 2009;45:3104-18.
26. Meléndez-Salazar GD. *Maytenus truncata* Reissek: Phytochemical study, hystological analysis and sorptive capacity of leaves and biological evaluation of extracts. Belo Horizonte, PhD Thesis. Depto de Química, ICEx, UFMG; 2005. p. 235.
27. Liu JM, Haroun-Bouhedja F, Boisson-Vidal C. Analysis of the in vitro inhibition of mammary adenocarcinoma cell adhesion by sulphated polysaccharides. Anticancer Res 2000;20:3265-71.
28. Touriño S, Selga A, Jiménez A, Juliá L, Lozano C, Lizárraga D, *et al*. Procyanidin fractions from pine (*Pinus pinaster*) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells. J Agric Food Chem 2005;53:4728-35.
29. García-Pérez ME, Royer M, Duque-Fernandez A, Diouf PN, Stevanovic T, Pouliot R. Antioxidant, toxicological and antiproliferative properties of Canadian polyphenolic extracts on normal and psoriatic keratinocytes. J Ethnopharmacol 2010;132:251-8.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141128051842

Original Research

ScopeMed

Evaluation of probiotic potential of yeasts isolated from traditional cheeses manufactured in Serbia and Croatia

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Received: September 17, 2014
Accepted: November 28, 2014
Published: January 03, 2015

ABSTRACT

Aim: The aim of this study was to investigate the *in vitro* probiotic potential of dairy yeast isolates from artisanal cheeses manufactured in Serbia and Croatia. **Materials and Methods:** Twelve yeast strains isolated from artisanal fresh soft and white brined cheeses manufactured in Serbia and Croatia were used in the study. Survival in chemically-simulated gastrointestinal conditions, adherence to epithelial intestinal cells and proliferation of gut-associated lymphoid tissue (GALT) cells were evaluated. **Results:** The results revealed that two strains of *Kluyvereomyces lactis* ZIM 2408 and ZIM 2453 grew above one log unit (Δ log CFU/ml) in the complex colonic medium during 24 h of cultivation, while *Torulaspora delbrueckii* ZIM 2460 was the most resistant isolate in chemically-simulated conditions of gastric juice and upper intestinal tract. It was demonstrated that the strains *K. lactis* ZIM 2408 and ZIM2441 and *Saccharomyces cerevisiae* ZIM 2415 were highly adhesive to Caco-2 cells, while strains *K. lactis* ZIM 2408 and *Debaryomyces hansenii* ZIM 2415 exhibit the highest adhesion percentage to HT29-MTX cells. All strains significantly ($P < 0.0001$) decreased the proliferation of GALT cells, suggesting the possible strain-specific immunomodulatory potential of the isolates. **Conclusion:** The dairy yeast isolates exhibit strain-specific probiotic properties, particularly the strain *K. lactis* ZIM 2408, which appears to be the best probiotic candidate in terms of all three criteria. Taking into account their immunomodulatory potential, the yeast isolates could be further tested for specific probiotic applications and eventually included in functional food formulated for patients suffering from diseases associated with an increased inflammatory status.

KEY WORDS: Caco-2, HT29-MTX, GALT, host-microbe interactions, immunomodulation, *Kluyvereomyces lactis*

INTRODUCTION

The Western Balkan Countries (WBC) harbour a variety of traditional fermented foods produced by spontaneous or controlled fermentation from cow's, ewe's and goat's milk. These products feature a rich diversity of lactic acid bacteria (LAB) as part of their natural microflora, with relevant genetic, metabolic and technological features, making these bacteria

potential candidates as probiotic microorganisms [1-6]. However, the diversity and probiotic potential of yeasts in WBC artisanal dairy products is still insufficiently explored.

Probiotics, defined as 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' [7], have been demonstrated to exert health-promoting effects through various proposed mechanisms: (a) Competition with pathogenic bacteria for nutrients and binding sites in the gut epithelium, (b) inactivation of the toxins and metabolites produced by pathogens, (c) the production of antimicrobial substances which inhibit the growth of pathogenic microorganisms, (d) stimulation/modulation of the immune response, or (e) anti-carcinogenic action [8]. The beneficial effects of probiotics are shown to be strain specific, pointing to the need to use various screening systems to identify specific probiotics to treat specific disorders and symptoms [9]. Human colon tumorigenic cell lines such as Caco-2 and mucin-producing HT29-MTX are recognized as good models for elucidation of the mechanisms involved in host-microbe interactions, although they lack the complexity of the human immune system [8,10-14]. In addition, probiotics can interact with gut-associated lymphoid tissue (GALT) and bind to epithelial surface receptors, inducing humoral and cellular immune responses [15]. Furthermore, recent studies have shown that probiotics exhibit beneficial health effects by directly modulating or down-regulating the immune system through modification of the immune response in GALT, thereby preventing the symptoms of inflammatory bowel disease, allergies and asthma [16]. Hence, GALT primary cells have been suggested as an improved *in vitro* model for studying the interactions of microorganisms, because they are non-transformed, non-tumorigenic and produce mucin [17].

A number of studies have suggested that the administration of probiotics plays a role in the promotion of human health. Numerous products intended for human consumption containing live microorganisms have been declared to have probiotic activity. Although, the design of foods containing probiotics has focused primarily on *Lactobacillus* and *Bifidobacterium* [18-22], the use of yeast probiotics is limited. *Saccharomyces boulardii* is considered a probiotic and has been widely used in lyophilized form for the prevention and treatment of human gastrointestinal tract (GIT) diseases [10,23-28]. Recently, several other yeast strains belonging to the genera *Saccharomyces*, *Debaryomyces*, *Torulaspora*, *Kluyveromyces*, *Pichia*, and *Candida* have also been shown to have probiotic potential in terms of their ability to survive simulated conditions of the GIT, and to adhere to different mammalian intestinal epithelial cells [29-32].

In our previous study, we isolated and characterized 69 yeast strains from artisanal white pickled and fresh soft cheeses manufactured in Serbia and Croatia, respectively [33]. Due to the interest of the food industry in novel candidate probiotic strains, the current study was designed to select yeast isolates with probiotic potential. The aim of this study was to challenge natural yeast isolates to a chemically simulated GIT transit and to test their ability to adhere to epithelial intestinal cell (EIC) lines, as well as to induce and modulate the proliferation of GALT cells in the presence of UV-irradiated strains. Here we present the probiotic potential of 12 dairy yeast isolates.

MATERIALS AND METHODS

Yeast Strains

The twelve autochthonous yeast strains used in this study were previously isolated from traditional cheeses manufactured in Serbia and Croatia: *K. lactis* ZIM 2408 (ENA ID HE660059); ZIM 2441 (ENA ID HE799667); ZIM 2453 (ENA ID HE660074); ZIM 2456 (ENA ID HE660077); *Torulaspora delbrueckii* ZIM 2436 (ENA ID HE660081); ZIM 2458 (ENA ID HE660079); ZIM 2460 (ENA ID HE799671); *Torulaspora quercuum* ZIM 2412 (ENA ID HE660063); *Debaryomyces hansenii* ZIM 2415 (ENA ID HE799657); ZIM 2440 (ENA ID HE799666); *Galactomyces geotrichum* ZIM 2422 (ENA ID HE799659); *Saccharomyces cerevisiae* ZIM 2447 (ENA ID HF545670) [33]. The strains were either the predominant yeast species in the cheese samples [33] and/or strains isolated from the cheeses which are allowed to be added intentionally to food (qualified presumption as safe) [34]. The strains were cultivated on YPD agar (Sigma Chemical Co., St. Louis, MO, USA) at 28°C.

Survival of Yeasts in Simulated Chemical Conditions Encountered in the GIT

The ability of the yeast strains to survive in chemical conditions that simulate those encountered in the GIT was assessed. One colony of each yeast strain was resuspended in YPD medium and grown overnight at 28°C and 220 rpm (with shaking, in aerobic conditions). The cells were harvested by centrifugation (2880 g force, 5 min) and washed twice in phosphate-buffered saline (PBS) (PBS: NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.44 g/l, KH_2PO_4 0.24 g/l; pH 7.2). The final cell concentration was adjusted to 7.0 log cells/ml. The cells were further suspended in PBS solution of pH 2 with added pepsin (3 mg/ml) at 37°C for 3 h and in PBS solution of pH 7.2 with 0.3% (w/v) Oxgall at 37°C for 4 h simulating stomach and small intestine conditions, respectively. Resistance to the chemical conditions encountered in the human colon were tested in a complex colonic model growth medium (CMGM) [35] under anaerobic conditions for 24 h. After incubation, viable colony counts were determined and survival rate was expressed as means of Δlog units of duplicates.

Adhesion of Yeast Strains to Intestinal Cell Lines

The colonocyte-like cell lines Caco-2 and HT29-MTX were used to determine the adhesion ability of the yeast isolates. Caco-2 cell lines were purchased from the European Collection of Cell Cultures (ECACC No. 86010202) and HT29-MTX was kindly supplied by Dr. T. Lesuffleur (INSERM UMR S 938, Paris, France) [36]. The culture and maintenance of the cell lines were carried out following standard procedures [5] using Advanced DMEM medium (Gibco Invitrogen, Paisley, UK) for Caco-2 and HT29-MTX supplemented with heat inactivated

foetal bovine serum (5% for Caco-2, 10% for HT29-MTX), L-glutamine (2 mM) and with a mixture of antibiotics (10 U/ml penicillin, 10 μg/ml streptomycin, 50 μg/ml gentamicin). Media and reagents were purchased from PAA (Pasching, Austria). Intestinal cells were seeded in 24-well plates and cultivated until a confluent differentiated state was reached (monolayers). Yeasts were cultured for 24 h as described above and after washing twice with Dulbecco´s PBS solution (Sigma) were resuspended in the corresponding cell-line media without antibiotics at a concentration of about 10^8 CFU/ml. Cellular monolayers were also carefully washed with Dulbecco´s PBS solution (Sigma), and yeast suspensions were added at an MOI (multiplicity of infection) ratio of about 10:1 (yeast: eukaryotic cell). Adhesion experiments were carried out for 1 h at 37°C, 5% CO_2 and, afterwards, wells were gently washed to release unattached yeasts before proceeding with the lysis of cellular monolayers using 0.25% Trypsin–ethylenediaminetetraacetic acid (EDTA) solution (PAA, Pasching, Austria). Dilutions of samples, before and after adhesion, were made in PBS solution and yeast counts were performed on YPD agar plates.

The adhesion was calculated as: % CFU adhered yeasts/CFU added yeasts. Experiments were carried out in two replicated plates and in each plate two wells were used per sample.

Proliferation of GALT in the Presence of Non-viable Yeast Strains

The yeast strains were grown in the same manner as described above. Overnight yeast cultures were harvested by centrifugation, washed two times with PBS buffer and resuspended in 5 ml of the same buffer with a final cell suspension of 10^7 CFU/ml determined by plate counting. Cells were inactivated by UV light (in the UV chamber, 15 W) for 3 cycles of 30 min each. Plate counting was carried out after UV treatment to corroborate the absence of live yeasts that could be able to recover in the proper medium. UV-inactivated yeasts were then divided into single-use aliquots, frozen in liquid N_2 and stored at −80°C until use [37]. All experimental procedures and protocols conformed to institutional guidelines for the care and use of animals in research no. 2/09 (Ethical Committee of the Faculty of Pharmacy, University of Belgrade). Animal manipulations were approved by the Ethical Committee for Experimentation on Laboratory Animals of the Faculty of Pharmacy, University of Belgrade. A total number of 3 Wistar rats (healthy female adults between 6 and 8 weeks old) were purchased from the Farm of the Military Medical Academy, Belgrade. For the experiments each animal was anaesthetized with CO_2 and, once assured of the loss of corneal reflex, its intestine was excised from the jejunum to the ileocaecal junction. The whole small intestine was placed in cold Hank´s balanced salt solution (HBSS without calcium and magnesium ions, prepared according to the formula of Gibco, Invitrogen) and kept at 4°C until processing. Finally, the animals were sacrificed using the increase of CO_2 concentration. The isolation of lymphocytes from GALT (Peyer´s Patches lymphocytes and IEL) was carried out as previously described [38]. Briefly, small pieces of the cleaned small intestine were incubated with HBSS without Ca and Mg ions, with antibiotics (gentamicin 500 mg/ml [AppliChem GmbH, Darmstadt, Germany], penicillin 20 IU/ml, streptomycin 2 μg/ml [PAA], and 10 mM Hepes). Treatment with HBSS-DTT (HBSS with 2 mM DTT and 10 mM Hepes, pH 7.2) and HBSS-EDTA (HBSS with 1 mM EDTA and 1 mM Hepes, pH 7.2) was used to release the IEL lymphocyte subset. Incubation in complete RPMI medium with antibiotics (RPMI-1640 with 2 mM L-glutamine and 25 mM HEPES (PAA, Austria), 10% heat-inactivated FSB, 100 mg/mL streptomycin (Sigma) and ampicillin (Sigma) with collagenase 100 IU/ml (Gibco Invitrogen) was used to isolate the PP subset of lymphocytes. The PPL and IEL present in the supernatants were purified by Percoll (Sigma) gradient (66%-47%-25%) and then resuspended in complete RPMI medium with antibiotics.

To quantify the response of GALT to the different factors tested, 2×10^5 lymphocyte cells were incubated with UV-inactivated yeasts (at a ratio 1:5) for 4 days at 37°C in complete RPMI medium with antibiotics at 37°C with 5% CO_2. All cultures were performed in triplicate (GALT) in 96-well round-bottom microtiter plates. After 4 days of incubation, the proliferation of GALT-lymphocytes was determined with a Cell Proliferation Assay Kit (Millipore Corporation, Billerica, MA, USA) following the manufacturer's instructions. Results were compared with a negative control (lymphocytes growing in complete RPMI medium with antibiotics) to test the capability of each factor to induce GALT-proliferation.

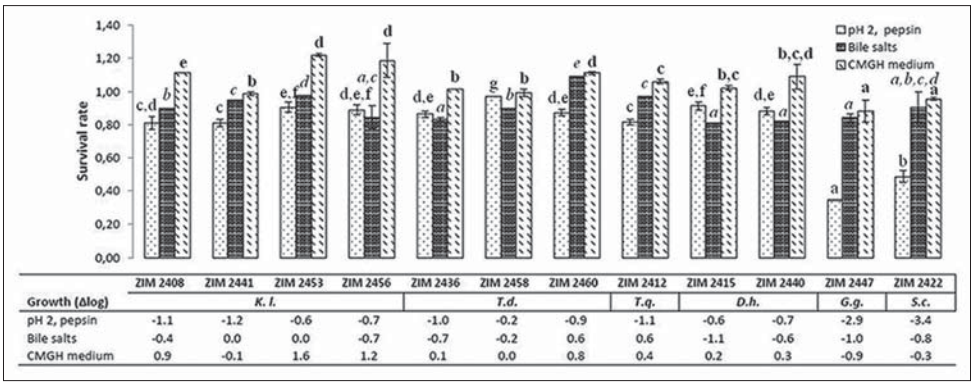
Statistical Analysis

After checking the normal distribution of the proliferation data (NORMDINST), one-way ANOVA tests were used to determine differences between each factor and the negative control. Finally, one-way ANOVA tests, together with the mean comparison test less significant difference, were used to compare the differences between the three strains. Results were represented by mean ± standard deviation or standard error. The SPSS 15.0 Statistical Software Package (SPSS Inc) was used for all determinations and the value $P < 0.05$ was considered as significant.

RESULTS

In Vitro GIT Survival of Yeast Strains

The ability of the 12 yeast strains to survive chemical conditions similar to the conditions found in the GIT was tested. The results showed that most of the strains survived moderately under simulated gastric conditions (from 81% to 97%; Δlog CFU/ml from -0.2 to 1.2) with the exception of the strains belonging to *G. geotrichum* and *S. cerevisiae* which showed a poor survival rate (35% and 49%; Δlog CFU/ml -2.9 and 3.4, respectively) [Figure 1]. Moreover, the rate of survival was either maintained or slightly decreased in the presence of bile salts for all tested strains (from 81% to 109%; Δlog from −1.1 to 0.6). Two strains of *K. lactis* ZIM 2453 and ZIM 2456 grew above one log unit (Δ log CFU/ml) in CMGM during 24-h of cultivation. Nevertheless, *T. delbrueckii* strains ZIM 2458 and



	ZIM 2408	ZIM 2441	ZIM 2453	ZIM 2456	ZIM 2436	ZIM 2458	ZIM 2460	ZIM 2412	ZIM 2415	ZIM 2440	ZIM 2447	ZIM 2422
Growth (Δlog)	*K. l.*				*T.d.*			*T.q.*	*D.h.*		*G.g.*	*S.c.*
pH 2, pepsin	-1.1	-1.2	-0.6	-0.7	-1.0	-0.2	-0.9	-1.1	-0.6	-0.7	-2.9	-3.4
Bile salts	-0.4	0.0	0.0	-0.7	-0.7	-0.2	0.6	0.6	-1.1	-0.6	-1.0	-0.8
CMGH medium	0.9	-0.1	1.6	1.2	0.1	0.0	0.8	0.4	0.2	0.3	-0.9	-0.3

Figure 1: Survival rate and Δlog CFU/ml of yeast strains under simulated stomach conditions (pH 2), in an environment simulating upper gastrointestinal tract (bile salt) and under simulated colonal conditions in a CMGM medium, *K.l.* - *Kluyveromyces lactis*; *T.d.* - *Torulaspora delbrueckii; T.q.* - *T. quercuum; D.h.* - *Debaryomyces hansenii; G.g.* - *Galactomyces geotrichum; S.c.* - *Saccharomyces cerevisiae*. Within each GIT challenge, columns that do not share the same letter are statistically different (p<0.05).

ZIM 2460 were the most resistant in simulated conditions of gastric juice and upper intestinal tract, respectively.

The Adhesion Ability of Yeast Strains to Intestinal Cell Lines

The adhesion ability to a monolayer EIC was investigated for the 12 yeast dairy strains using human cell lines (Caco-2 and HT29-MTX) carried out as per the procedure documented in Nikolic et al., 2014 [5]. The results are presented in Figure 2. As seen in Figure 2, the results demonstrate that three natural dairy yeast isolates, *K. lactis* ZIM 2408, *K. lactis* ZIM 2441 and *D. hansenii* ZIM 2415, were highly adhesive (more than 60% of added yeasts). The adhesion properties of the strains *K. lactis* ZIM 2408 and *S. cerevisiae* ZIM 2415 followed the same tendency in both intestinal cell lines, although they exhibited lower percentages of adhesion to the mucus-producing cell line HT29-MTX. An exception was strain *K. lactis* ZIM 2441 which exhibited good adhesion to the Caco-2 cell line but significantly lower adhesion to the HT29-MTX cell line ($P < 0.05$).

Proliferation of GALT in the Presence of UV Inactivated Yeasts Isolates

The proliferation indexes of GALT measured in the presence of the stimuli are presented in Figure 3. In general, the results showed that the proliferation of GALT cells was reduced in the presence of the yeast strains compared to the control (RMPI in the absence of the stimulus). Specifically, the strain *K. lactis* ZIM 2408 and three *Torulaspora* isolates (*T. delbrueckii* ZIM 2436, *T. quercuum* ZIM 2412 and *Torulaspora* sp. ZIM 2460) significantly reduced the number of GALT cells in comparison to non-treated GALT cells ($P < 0.0001$ in all cases, except for *S. cerevisiae* ZIM 2415 and *S. cerevisiae* ZIM 2440, $P < 0.001$). None of the strains increased the proliferation of GALT cells.

DISCUSSION

In recent years, an increasing number of studies have suggested that the administration of probiotics plays a role in the promotion of human health. Numerous products intended for human consumption containing live microorganisms have been declared to have probiotic activity. Various studies have confirmed that bacterial species, mostly *Lactobacillus* and *Bifidobacterium* strains, can modulate GALT responses [18,20-22]. The scientific and clinical interest in finding microorganisms with the ability to regulate intestinal immune response has increased due to the accumulating evidence that the GIT microbiota play a critical role in the initiation and prevention of inflammatory bowel diseases, allergies, eczema and various atopic diseases [9,16,39,40]. Although recent reviews indicate the health-promoting properties of yeasts, studies describing the probiotic potential of yeast strains are still limited [41,42].

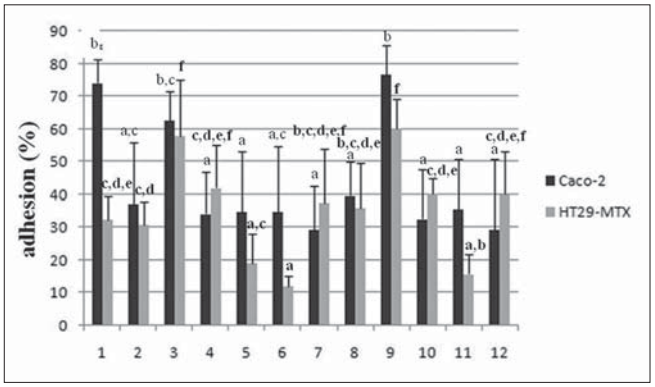


Figure 2: Adhesion of the yeasts isolates to Caco-2 and HT29-MTX cell lines. The asterisk is showing statistically different (p<0.05) adhesion in between cell lines. 1. *Kluyveromyces lactis* ZIM 2441; 2. *K. lactis* ZIM 2453; 3. *K. lactis* ZIM 2408; 4. *K. lactis* ZIM 2456; 5. *Torulaspora delbrueckii* ZIM 2458; 6. *T. delbrueckii* ZIM 2436; 7. *T. quercuum* ZIM 2412; 8. *Torulaspora* sp. ZIM 2460; 9. *Saccharomyces cerevisiae* ZIM 2415; 10. *S. cerevisiae* ZIM 2440; 11. *S. cerevisiae* ZIM 2447; 12. *Debaryomyces hansenii* ZIM 2422. The adhesion was calculated as: % CFU adhered yeasts/CFU added yeasts. Experiments were carried out in two replicated plates and in each plate two wells were used per sample. The Student's *t*-test was used for each strain to determine if the data in between the adhesion to two IEC lines are significantly different from each other, $P < 0.05$ value was considered significant. Within each IEC line, columns that do not share the same letter are statistically different ($P < 0.05$).

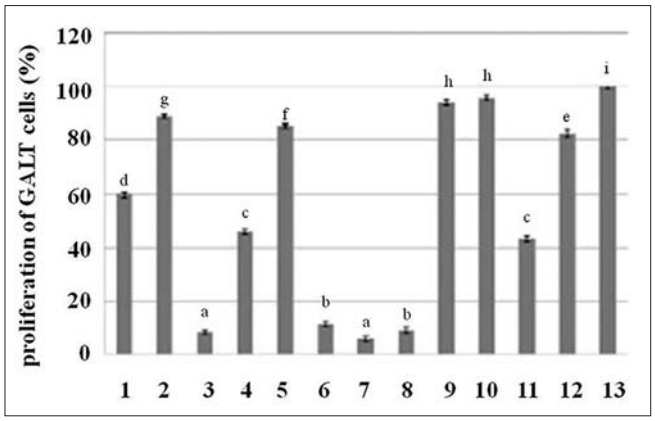


Figure 3: Proliferation of GALT cell isolated from rats, co-cultured for four days in the presence of the yeast isolates at ratio (yeast : cell line) 5:1. 1. *Kluyveromyces lactis* ZIM 2441; 2. *K. lactis* ZIM 2453; 3. *K. lactis* ZIM 2408; 4. *K. lactis* ZIM 2456; 5. *Torulaspora delbrueckii* ZIM 2458; 6. *T. delbrueckii* ZIM 2436; 7. *T. quercuum* ZIM 2412; 8. *Torulaspora* sp. ZIM 2460; 9. *Saccharomyces cerevisiae* ZIM 2415; 10. *S. cerevisiae* ZIM 2440; 11. *S. cerevisiae* ZIM 2447; 12. *Debaryomyces hansenii* ZIM 2422; 13. Control (lymphocytes without the stimuli). The Student's *t*-test was used for each strain to determine if the data in between the proliferation in the presence of yeasts and for the control to two IEC lines are significantly different from each other, $P < 0.001$ value was considered significant. Columns that do not share the same letter are statistically different ($P < 0.05$).

In this study, the probiotic potential of the natural yeast isolates originating from artisanal cheeses was evaluated in terms of their survival under simulated GIT conditions, adhesion to the intestinal epithelial cell lines Caco-2 and HT29-MTX and modulation of GALT cell proliferation. According to the FAO/WHO guidelines for the evaluation of probiotics for human food applications [7], the survival of probiotic strains in gastric and intestinal digestion is one of the desirable properties that strains with probiotic potential should present. The results obtained in this study showed high resistance of the yeast isolates to low pH conditions that could be related to an adaptation to acidic conditions in the natural environment from which the strains were isolated, similar to that reported for lactobacilli of food origin [43,44]. Interestingly, in contrast to LAB of food origin the majority of the yeast isolates proliferated in the colonic model under anaerobic conditions [5]. In general, our results confirmed that the yeasts associated with food have a rather good ability to survive under simulated GIT conditions [30, 45,46]. Nevertheless, as was demonstrated for LAB, the acid and bile resistance were more strain than species dependant properties [47]. The colonization of intestinal mucosa is another important criterion for selection of strains with probiotic potential [7], since their health-promoting effects might be partly dependent on their persistence in the intestine and adhesion to mucosal surfaces [48]. However, adhesion was shown not to be a prerequisite for probiotic yeasts in order to have inhibitory action against pathogenic bacteria [49]. Human colon tumorigenic cell lines such as Caco-2 and mucin-producing HT29-MTX are recognized as good models for elucidation of the mechanisms involved in host-microbe interactions, although they lack the complexity of the human immune system [50,51]. In general, our results showed better adhesion of the yeast strains to the Caco-2 cell lines than to HT29-MTX. The presence of the glycoprotein (mucin) layer in the HT29-MTX cell line might have hindered the availability of the cells as receptors for yeasts. The use of HT29-MTX cells could be advantageous in studying the adhesion ability of microorganisms to EIC, since they represent a mucin-secreting cell culture that expresses similar protein patterns to human intestinal epithelium [51]. On the other hand, the adhesion ability of the different bacterial strains to independent cultures of HT29-MTX cells was shown to be lower in comparison to the Caco-2 cell line [52], suggesting that various models should be used in order to study the adhesion abilities of particular strains.

The next important characteristic of potential probiotic candidates is the capacity to modulate the immune response of the host. Probiotics can interact with GALT and bind to epithelial surface receptors, inducing humoral and cellular immune responses. Furthermore, recent studies have shown that probiotics exhibit beneficial effects by directly modulating or down-regulating the immune system through modification of the immune response in GALT, preventing in that way the symptoms of inflammatory bowel disease, allergies and asthma [16]. Hence, GALT primary cells have been suggested as an improved *in vitro* model for studying the interactions of microorganisms within the host, due to the fact that they are non-transformed, non-tumorigenic and produce mucin [17]. Two main effects of probiotics on a host's immunity, demonstrated in several *in vitro* and *in vivo* studies, are strengthening the immunological barrier through the development of the innate and adaptive immune system and decreasing the immune responsiveness to unbalanced inflammatory conditions [9]. Different yeast species, many of them usually found in fermented food, such as *D. hansenii*, *T. delbrueckii*, *K. lactis*, and *S. cerevisiae*, have also shown tolerance to passage through the GIT, adhesion to intestinal Caco-2 cell lines, and immunostimulatory activity [50]. Interestingly, our results revealed the reduced proliferation of GALT cells in the presence of the yeast isolates, indicating that yeast strains isolated from artisanal cheeses have the potential to modulate the host response and to have possibly immunosuppressive activity, perhaps by up- and down-regulation of various cytokines. Similarly, the results of Romanin et al. [53] indicate that the inhibition of innate epithelial response could be a rather general property of different yeast species.

In general, the use of probiotics yeasts, such as *S. boulardii*, is shown to be safe in healthy populations and, to the best of our knowledge, no adverse effects have been reported in immunocompetent patients. However, a recent systematic review documented that probiotic products based on *S. boulardii* increase the risk of complications, such as fungemia or a rare gastrointestinal allergic reaction, in immunocompromised subjects [54]. Although rare, serious complications from probiotics (i.e., fungemia) in immunocompromised patients, or in those who had central venous catheters, highlight the need to establish the safety profile of these agents when they are used in anyone other than healthy populations.

Taking this safety issue together with the immunomodulatory potential of the yeast isolates tested in this study, they could eventually be included in functional food formulated for patients suffering diseases associated with an increased inflammatory status. The main advantage of using the probiotic yeast isolates instead of bacterial ones could be related to the prevention and treatment of antibiotic-associated diarrhea, since they are not affected by antibiotics.

CONCLUSION

The results obtained in this study demonstrate that dairy yeast isolates exhibit strain-specific probiotic potential, since they are able to survive simulated conditions of the intestinal tract, to colonize the intestine and there is a suggestion of their immunomodulatory activity. In particular, the strain *K. lactis* ZIM 2408 appears to be the best probiotic candidate studied due to its ability to survive under chemically simulated GIT conditions, its adherence to EIC and its immunosuppressive activity, and could be further investigated for specific probiotic applications. Hence, following the FAO/WHO criteria and EFSA recommendations, it is necessary to underline that the safety and health-promoting efficacy of particular yeast probiotic strains need to be further tested in pre-clinical trials.

ACKNOWLEDGEMENTS

T. Lesuffleur (INSERM U843, Paris, France) is acknowledged for the kind supply of HT29-MTX cellular line. This work was funded by the SEE-ERA-NET Plus Project ERA-195/01 (PSALAB), the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant OI 173019, and Ministry of Science, Education and Sports of the Republic of Croatia (Project 0581990 "Probiotics, prebiotics and functional starter cultures). We are grateful to Nathaniel Aaron Sprinkle, native English editor for the proofreading of the manuscript.

REFERENCES

1. Golic N, Strahinic I, Terzic-Vidojevic A, Begovic J, Nikolic M, Tolinacki M, *et al*. Molecular diversity among natural populations of *lactobacillus paracasei* and *Lactobacillus plantarum/paraplantarum* strains isolated from autochthonous dairy products. Eur Food Res Technol 2012;234:627-38.
2. Jokovic N, Nikolic M, Begovic J, Jovcic B, Savic D, Topisirovic L. A survey of the lactic acid bacteria isolated from Serbian artisanal dairy product Kajmak. Int J Food Microbiol 2008;127:305-11.
3. Lukic J, Strahinic I, Jovcic B, Filipic B, Topisirovic L, Kojic M, *et al.* Different roles for lactococcal aggregation factor and mucin binding protein in adhesion to gastrointestinal mucosa. Appl Environ Microbiol 2012;78:7993-8000.
4. Nikolic M, Jovcic B, Kojic M, Topisirovic L. Surface properties of *Lactobacillus* and *Leuconostoc* isolates from homemade cheeses showing auto-aggregation ability. Eur Food Res Technol 2010;231:925-31.
5. Nikolic M, López P, Strahinic I, Suárez A, Kojic M, Fernández-García M, *et al.* Characterisation of the exopolysaccharide (EPS)-producing *Lactobacillus paraplantarum* BGCG11 and its non-EPS producing derivative strains as potential probiotics. Int J Food Microbiol 2012;158:155-62.
6. Topisirovic L, Kojic M, Fira D, Golic N, Strahinic I, Lozo J. Potential of lactic acid bacteria isolated from specific natural niches in food production and preservation. Int J Food Microbiol 2006;112:230-5.
7. FAO, WHO. Probiotics in Food, Health and Nutritional Properties and Guidelines for Evaluation. FAO Food and Nutritional Paper; 2006. p. 85.
8. Boirivant M, Strober W. The mechanism of action of probiotics. Curr Opin Gastroenterol 2007;23:679-92.
9. Delcenserie V, Martel D, Lamoureux M, Amiot J, Boutin Y, Roy D. Immunomodulatory effects of probiotics in the intestinal tract. Curr Issues Mol Biol 2008;10:37-54.
10. Czerucka D, Dahan S, Mograbi B, Rossi B, Rampal P. *Saccharomyces boulardii* preserves the barrier function and modulates the signal transduction pathway induced in enteropathogenic Escherichia coli-infected T84 cells. Infect Immun 2000;68:5998-6004.
11. Frank CF, Hostetter MK. Cleavage of E-cadherin: A mechanism for disruption of the intestinal epithelial barrier by *Candida albicans*. Transl Res 2007;149:211-22.
12. Klingberg TD, Pedersen MH, Cencic A, Budde BB. Application of measurements of transepithelial electrical resistance of intestinal epithelial cell monolayers to evaluate probiotic activity. Appl Environ Microbiol 2005;71:7528-30.
13. Klingberg TD, Lesnik U, Arneborg N, Raspor P, Jespersen L. Comparison of *Saccharomyces cerevisiae* strains of clinical and nonclinical origin by molecular typing and determination of putative virulence traits. FEMS Yeast Res 2008;8:631-40.
14. Satish Kumar R, Kanmani P, Yuvaraj N, Paari KA, Pattukumar V, Arul V. *Lactobacillus plantarum* AS1 binds to cultured human intestinal cell line HT-29 and inhibits cell attachment by enterovirulent bacterium *Vibrio parahaemolyticus*. Lett Appl Microbiol 2011;53:481-7.
15. Hardy H, Harris J, Lyon E, Beal J, Foey AD. Probiotics, prebiotics and immunomodulation of gut mucosal defences: Homeostasis and immunopathology. Nutrients 2013;5:1869-912.
16. Ouwehand AC. Antiallergic effects of probiotics. J Nutr 2007;137:794S-7.
17. Solano-Aguilar GI, Vengroski KG, Beshah E, Lunney JK. Isolation and purification of lymphocyte subsets from gut-associated lymphoid tissue in neonatal swine. J Immunol Methods 2000;241:185-99.
18. Foligne B, Zoumpopoulou G, Dewulf J, Ben Younes A, Chareyre F, Sirard JC, *et al.* A key role of dendritic cells in probiotic functionality. PLoS One 2007;2:e313.
19. Jankovic I, Sybesma W, Phothirath P, Ananta E, Mercenier A. Application of probiotics in food products – challenges and new approaches. Curr Opin Biotechnol 2010;21:175-81.
20. Marcinkiewicz J, Ciszek M, Bobek M, Strus M, Heczko PB, Kurnyta M, *et al.* Differential inflammatory mediator response *in vitro* from murine macrophages to lactobacilli and pathogenic intestinal bacteria. Int J Exp Pathol 2007;88:155-64.
21. O'Hara AM, O'Regan P, Fanning A, O'Mahony C, Macsharry J, Lyons A, *et al.* Functional modulation of human intestinal epithelial cell responses by *Bifidobacterium* infantis and *Lactobacillus salivarius*. Immunology 2006;118:202-15.
22. Toki S, Kagaya S, Shinohara M, Wakiguchi H, Matsumoto T, Takahata Y, *et al.* Lactobacillus rhamnosus GG and Lactobacillus casei suppress *Escherichia coli*-induced chemokine expression in intestinal epithelial cells. Int Arch Allergy Immunol 2009;148:45-58.
23. Born P, Lersch C, Zimmerhackl B, Classen M. The Saccharomyces boulardii therapy of HIV-associated diarrhea. Dtsch Med Wochenschr 1993;118:765.
24. Buts JP, Dekeyser N, Stilmant C, Delem E, Smets F, Sokal E. *Saccharomyces boulardii* produces in rat small intestine a novel protein phosphatase that inhibits Escherichia coli endotoxin by dephosphorylation. Pediatr Res 2006;60:24-9.
25. McFarland LV. Meta-analysis of probiotics for the prevention of traveler's diarrhea. Travel Med Infect Dis 2007;5:97-105.
26. Sazawal S, Hiremath G, Dhingra U, Malik P, Deb S, Black RE. Efficacy of probiotics in prevention of acute diarrhoea: A meta-analysis of masked, randomised, placebo-controlled trials. Lancet Infect Dis 2006;6:374-82.
27. Surawicz CM. Probiotics, antibiotic-associated diarrhoea and *Clostridium* difficile diarrhoea in humans. Best Pract Res Clin Gastroenterol 2003;17:775-83.
28. Zanello G, Meurens F, Berri M, Chevaleyre C, Melo S, Auclair E, *et al*. *Saccharomyces cerevisiae* decreases inflammatory responses induced by F4+ enterotoxigenic Escherichia coli in porcine intestinal epithelial cells. Vet Immunol Immunopathol 2011;141:133-8.

29. Generoso SV, Viana M, Santos R, Martins FS, Machado JA, Arantes RM, *et al. Saccharomyces cerevisiae* strain UFMG 905 protects against bacterial translocation, preserves gut barrier integrity and stimulates the immune system in a murine intestinal obstruction model. Arch Microbiol 2010;192:477-84.
30. Kumura H, Tanoue Y, Tsukahara M, Tanaka T, Shimazaki K. Screening of dairy yeast strains for probiotic applications. J Dairy Sci 2004;87:4050-6.
31. Maccaferri S, Klinder A, Brigidi P, Cavina P, Costabile A. Potential probiotic *Kluyveromyces marxianus* B0399 modulates the immune response in Caco-2 cells and peripheral blood mononuclear cells and impacts the human gut microbiota in an *in vitro* colonic model system. Appl Environ Microbiol 2012;78:956-64.
32. Tiago FC, Martins FS, Rosa CA, Nardi RM, Cara DC, Nicoli JR. Physiological characterization of non-*Saccharomyces* yeasts from agro-industrial and environmental origins with possible probiotic function. World J Microbiol Biotechnol 2009;25:657-66.
33. Golic N, Cadež N, Terzic-Vidojevic A, Suranská H, Beganovic J, Lozo J, *et al.* Evaluation of lactic acid bacteria and yeast diversity in traditional white pickled and fresh soft cheeses from the mountain regions of Serbia and lowland regions of Croatia. Int J Food Microbiol 2013;166:294-300.
34. EFSA. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed. EFSA J 2012;10:1-84.
35. Macfarlane GT, Macfarlane S, Gibson GR. Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. Microb Ecol 1998;35:180-7.
36. Lesuffleur T, Porchet N, Aubert JP, Swallow D, Gum JR, Kim YS, *et al.* Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. J Cell Sci 1993;106:771-83.
37. López P, Monteserín DC, Gueimonde M, de los Reyes-Gavilán CG, Margolles A, Suárez A, *et al.* Exopolysaccharide-producing *Bifidobacterium* strains elicit different *in vitro* responses upon interaction with human cells. Food Res Int 2012;46:99-107.
38. Hidalgo-Cantabrana C, Nikolic M, López P, Suárez A, Miljkovic M, Kojic M, *et al.* Response elicited by exopolysaccharide-1 producing *Bifidobacterium animalis* subsp. *lactis* strains and their polymers upon immune cells isolated from rat blood and gut associated lymphoid tissue. Anaerobe 2014;26:24-30.
39. Nylund L, Satokari R, Nikkilä J, Rajilic-Stojanovic M, Kalliomäki M, Isolauri E, *et al.* Microarray analysis reveals marked intestinal microbiota aberrancy in infants having eczema compared to healthy children in at-risk for atopic disease. BMC Microbiol 2013;13:12.
40. Strober W, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. J Clin Invest 2007;117:514-21.
41. Fleet GH, Balia R. The public health and probiotic significance of yeasts in foods. In: Querol A, Fleet GH, editors. Yeasts in Food and Beverages. Germany: Springer; 2006. p. 381-98.
42. Moslehi-Jenabian S, Pedersen LL, Jespersen L. Beneficial effects of probiotic and food borne yeasts on human health. Nutrients 2010;2:449-73.
43. Burns P, Vinderola G, Reinheimer J, Cuesta I, de Los Reyes-Gavilán CG, Ruas-Madiedo P. Technological characterization and survival of the exopolysaccharide-producing strain Lactobacillus delbrueckii subsp. lactis 193 and its bile-resistant derivative 193 in simulated gastric and intestinal juices. J Dairy Res 2011;78:357-64.
44. Terzic-Vidojevic A, Vukasinovic M, Veljovic K, Ostojic M, Topisirovic L. Characterization of microflora in homemade semi-hard white Zlatar cheese. Int J Food Microbiol 2007;114:36-42.
45. Psomas E, Andrighetto C, Litopoulou-Tzanetaki E, Lombardi A, Tzanetakis N. Some probiotic properties of yeast isolates from infant faeces and Feta cheese. Int J Food Microbiol 2001;69:125-33.
46. Trotta F, Caldini G, Dominici L, Federici E, Tofalo R, Schirone M, *et al.* Food borne yeasts as DNA-bioprotective agents against model genotoxins. Int J Food Microbiol 2012;153:275-80.
47. Lo Curto A, Pitino I, Mandalari G, Dainty JR, Faulks RM, John Wickham MS. Survival of probiotic lactobacilli in the upper gastrointestinal tract using an *in vitro* gastric model of digestion. Food Microbiol 2011;28:1359-66.
48. Collado MC, Isolauri E, Salminen S, Sanz Y. The impact of probiotic on gut health. Curr Drug Metab 2009;10:68-78.
49. van der Aa Kühle A, Skovgaard K, Jespersen L. *In vitro* screening of probiotic properties of Saccharomyces cerevisiae var. boulardii and food-borne Saccharomyces cerevisiae strains. Int J Food Microbiol 2005;101:29-39.
50. Arroyo-López FN, Romero-Gil V, Bautista-Gallego J, Rodríguez-Gómez F, Jiménez-Díaz R, García-García P, *et al.* Potential benefits of the application of yeast starters in table olive processing. Front Microbiol 2012;5:34.
51. Lenaerts K, Bouwman FG, Lamers WH, Renes J, Mariman EC. Comparative proteomic analysis of cell lines and scrapings of the human intestinal epithelium. BMC Genomics 2007;8:91.
52. Laparra JM, Sanz Y. Comparison of *in vitro* models to study bacterial adhesion to the intestinal epithelium. Lett Appl Microbiol 2009;49:695-701.
53. Romanin D, Serradell M, González Maciel D, Lausada N, Garrote GL, Rumbo M. Down-regulation of intestinal epithelial innate response by probiotic yeasts isolated from kefir. Int J Food Microbiol 2010;140:102-8.
54. Kelesidis T, Pothoulakis C. Efficacy and safety of the probiotic *Saccharomyces boulardii* for the prevention and therapy of gastrointestinal disorders. Therap Adv Gastroenterol 2012;5:111-25.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141128032845

Original Research

Screening for hemostatic activities of popular Chinese medicinal herbs *in vitro*

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Received: October 11, 2014
Accepted: November 28, 2014
Published: January 03, 2015

ABSTRACT

Aims: This study aimed to identify new hemostyptics by assessing the coagulation enhancing activity of 114 Chinese herbal extracts *in vitro*. **Methods:** Herbs were boiled in water for 30 min, filtered and then lyophilized filtrates (10 mg/mL) were dissolved in water. Coagulation was assayed as prothrombin time (PT). Plasma diluted in saline was incubated with each extract for 5 min and then PT reagent was added, followed by $CaCl_2$ solution and the time taken to form clots was measured. Extracts that decreased coagulation time were regarded as containing active compounds. The abilities of extracts to activate Factor XII were assessed and the activated form of factor XII (XIIa) was resolved by SDS-PAGE and visualized by silver staining. **Results:** Coagulation time was obviously shortened by extracts of Alpinia Rhizome, Areca, Artemisia Leaf, Cassia Bark, Danshen Root, Ephedra Herb, Epimedium Herb, Forsythia Fruit, Great Burdock Achene, Moutan Bark, Perilla Herb, Red Paeony Root, Schizonepeta Spike, Senticosus Rhizome, Sweet Annie, Uncaria Thorn and Zanthoxylum Peel. Factor XII was obviously activated by extracts of Artemisia Leaf and Great Burdock Achene, and slightly by Perilla herb. **Conclusion:** Some popular Chinese medicinal herbs have potential as hemostatic agents and could thus be develope as new strategies for the treatment and prevention of bleeding.

KEY WORDS: Hemostatic, herbal treatment, screening

INTRODUCTION

Many Chinese medicinal herbs contain substances that promote hemostasis and when administered orally or externally, can help to support the process through which bleeding stops. The hemostatic components and mechanisms of action in some of these herbs have been investigated using the mouse bleeding model [1-5]. Hemostyptics are specific hemostatic agents that retard or stop bleeding by causing blood vessels to contract or by accelerating blood clotting when externally applied [3,6,7]. The hemostatic activities of herbal hemostyptics are often due to mechanisms such as tannin astringency [8]. Some hemostatic herbs can shorten bleeding and blood coagulation times [9], thus preventing bleeding from fragile capillaries, and they can also inhibit infection and inflammation that leads to vessel leakage and damage. We previously showed that Cat-tail pollen (*Pollen Typhae*), a traditional Chinese medicinal herb, has hemostyptic effects *in vitro* and in the mouse bleeding model *in vivo* [10]. That study also showed that the hemostyptic properties of externally applied Cat-tail pollen were attributable to the activation of intrinsic coagulation [10]. Acidic polysaccharide in pollen extract directly activates factor XII in the intrinsic coagulation cascade [10]. The present study searched for novel hemostyptics by assessing ability of 114 Chinese herbal extracts to enhance coagulation activity *in vitro*.

MATERIALS AND METHODS

Materials

Extracts from 114 Chinese medical herbs were supplied by the Cooperative Research Project at the Joint Usage/Research Center (Joint Usage/Research Center for Science-Based Natural Medicine), Institute of Natural Medicine, University of Toyama [Table 1]. Human factor XII was purchased from Hematologic Technologies Inc. (Essex Junction, Vermont, USA). Pooled normal human plasma was obtained from Axis-Shield (Oslo, Norway). The coagulation assay reagent, Thromborel S was purchased from Dade Behring (Marburg, Germany) and Sysmex (Kobe, Japan), respectively.

Preparation of Extract

Extracts were prepared by boiling 45 g of each herb in 900 mL of water for 30 min and then passed through a cotton plug.

Table 1: Chinese medicinal herbs

Acanthopanax Bark (*Acanthopanacis Cortex*)	Achyranthes Root (*Achyranthis Radix*)
Aconite Root (*Aconiti Radix*)	Acorus Gramineus Rhizome (*Acori Graminei Rhizoma*)
Alpinia Rhizome (*Alpiniae Officinari Rhizoma*)	Angelica Dahurica Root (*Angelicae Dahuricae Radix*)
Apricot Kernel (*Armeniacae Semen*)	Araria Root (*Araliae Cordatae Rhizoma*)
Areca (*Arecae Semen*)	Artemisia Capillaris Flower (*Artemisiae Capillaris Herba*)
Artemisia Leaf (*Artemisiae Folium*)	Asiasarum Root (*Asiasari Radix*)
Asparagus Tubers (*Asparagi Tuber*)	Astragalus Root (*Astragali Radix*)
Atractylodes Lancea Rhizome (*Atractylodis Lanceae Rhizoma*)	Atractylodes Rhizome (*Atractylodis Rhizoma*)
Biond Magnolia Flower (*Magnoliae Flos*)	Bitter Cardamon (*Alpiniae Fructus*)
Bupleurum Root (*Bupleuri Radix*)	Burningbush (*Dictamni Radicis Cortex*)
Cassia Bark (*Cinnamomi Cortex*)	Chinese Bugbane Rhizome (*Cimicifugae Rhizoma*)
Chinese Wolfberry Root-bark (*Lycii Cortex*)	Chrysanthemum flower (*Chrysanthemi Flos*)
Cnidium Rhizome (*Cnidii Rhizoma*)	Codonopsis Root (*Codonopsitis Radix*)
Coix Seed (*Coicis Semen*)	Common Anemarrhena Rhizome (*Anemarrhenae Rhizoma*)
Cornus fruit (*Corni Fructus*)	Corydalis Tuber (*Corydalis Tuber*)
Cyperus Rhizome (*Cyperi Rhizoma*)	Danshen Root (*Salviae Miltiorhizae Radix*)
Desertliving Cistanche (*Cistanchis Herba*)	Dried Tangerine Peel (*Citri Reticulatae Pericarpium*)
Ephedra Herb (*Ephedrae Herba*)	Epimedium Herb (*Epimedii Herba*)
Eucommia Bark (*Eucommiae Cortex*)	Evodia Fruit (*Euodiae Fructus*)
Figwortflower Picrorrhiza Rhizome (*Picrorrhizae Rhizoma*)	Forsythia Fruit (*Forsythiae Fructus*)
Fritillary Bulb (*Fritillariae Bulbus*)	Ganoderma (*Ganoderma*)
Gardenia Fruit (*Gardeniae Fructus*)	Gentiana Macrophylla Root (*Gentianae macrophyllae radix*)
Ginger (*Zingiberis Rhizoma*)	Ginseng Root (*Ginseng Radix*)
Glycyrrhiza (*Glycyrrhizae Radix*)	Great Burdock (*Achene Arctii Fructus*)
Hemp Fruit (*Cannabidis Fructus*)	Himalayan Teasel Root (*Dipsaci Radix*)
Immature Orange (*Aurantii Fructus Immaturus*)	Imperata Rhizome (*Imperatae Rhizoma*)
Japanese Angelica Root (*Angelicae Radix*)	Japanese Gentian (*Gentianae Scabrae Radix*)
Jujube (*Zizyphi Fructus*)	Leonurus Herb (*Leonuri Herba*)
Lindera Root (*Linderae Radix*)	Loquat Leaf (*Eriobotryae Folium*)
Magnolia Bark (*Magnoliae Cortex*)	Malaytea Scurfpea Fruit (*Psoraleae Semen*)
Mentha Herb (*Menthae Herba*)	Moutan Bark (*Moutan Cortex*)
Mulberry Bark (*Mori Cortex*)	Mulberry Leaf (*Mori Folium*)
Myrrha (*Myrrha*)	Notoginseng Root (*Notoginseng Radix*)
Notopterygium (*Notopterygii Rhizoma*)	Ophiopogon Tuber (*Ophiopogonis Tuber*)
Oriental Waterplantain (*Rhizome Alismatis Rhizoma*)	Panax Rhizome (*Panacis* Japonici *Rhizoma*)
Peach Kernel (*Persicae Semen*)	Peony Root (*Paeoniae Radix*)
Perilla Herb (*Perilla Herba*)	Phellodendron Bark (*Phellodendri Cortex*)
Pinellia Tuber (*Pinelliae Tuber*)	Plantago Seed (*Plantaginis Semen*)
Platycodon Root (*Platycodi Radix*)	Polygala Root (*Polygalae Radix*)
Polygonum Root (*Polygoni Multiflori Radix*)	Polyporus Sclerotium (*Polyporus*)
Poria Sclerotium (*Poria Sclerotium*)	Prepared Rehmannia Root (*Processi Rehmanniae Radix*)
Processed Ginger (*Zingiberis Processum Rhizoma*)	Pueraria Root (*Puerariae Radix*)
Red Ginseng (*Ginseng Radix Rubra*)	Red Paeony Root (*Paeoniae Radix Rubra*)
Rehmannia Root (*Rehmanniae Radix*)	Safflower (*Carthami Flos*)
Saffron (*Crocus*)	Saposhnikovia Root (*Saposhnikoviae Radix*)
Sasa Leaf (*Sasa Folium*)	Saussurea Root (*Saussureae Radix*)
Schisandra Fruit (*Schisandrae Fructus*)	Schizonepeta Spike (*Schizonepetae Spica*)
Scutellaria Root (*Scutellariae Radix*)	Senticosus Rhizome (*Senticosus Rhizome*)
Sesame (*Sesami Semen*)	Shrub Chaste Tree Fruit (*Viticis Fructus*)
Sinomenium Stem (*Sinomeni Caulis Et Rhizoma*)	Smilax Rhizome (*Smilacis Rhizoma*)
Sophora Root (*Sophorae Radix*)	Sparganium Rhizome (*Sparganii Rhizoma*)
Stemona Root (*Stemonae Radix*)	Sweet Annie (*Artemisiae* Annuae *Herba*)
Sweet Flag Root (*Acori Rhizoma*)	Swertia Herb (*Swertiae Herba*)
Tall Gastrodia Tuber (*Gastrodia elata*)	Tribulus Fruit (*Tribuli Fructus*)
Trichosanthes Root (*Trichosanthis Radix*)	Turmeric (*Curcumae Rhizoma*)
Uncaria Thorn (*Uncariae Uncis Cam Ramlus*)	Zanthoxylum Peel (*Zanthoxyli Fructus*)
Zedoary (*Zedoriae Rhizoma*)	Zizyphus Seed (*Zizyphi Semen*)

Filtrates were lyophilized and dissolved in water to a final concentration of 10 mg/mL.

Coagulation Assays

Coagulation was assayed as prothrombin time (PT). Plasma diluted five-fold with isotonic sodium chloride solution (100 μL) was incubated with extracts (50 μL) for 5 min. The PT reagent (50 μL), was added and then coagulation was started by adding 25 mmol/L $CaCl_2$. The time required to form clots was measured using a KC4A coagulometer (Amelung, Lemgo, Germany). The plasma coagulation time of the control was about 350 s. Extracts that shortened coagulation time were regarded as containing

active compounds. Extracts that shorted coagulation time to <230 s were selected. However, some herb extracts that were very turbid or contained precipitates were not further investigated.

Factor XII Activation Assay

Factor XII activation was assayed [6]. A volume of 70 μL of purified human factor XII (0.14 mg/mL) and 70 μL of extracts (10 mg/mL) were incubated for 5 h and then the mixtures were resolved by SDS-PAGE and stained with silver.

RESULTS

We determined the potentiating effect of herbal extracts on the coagulation time of the extrinsic pathway to assess hemostatic activity *in vitro*. Trace amounts of PT reagent were used to start the reaction. A shortened coagulation time was taken to indicate hemostatic activity. Extracts of Alpinia Rhizome, Areca, Artemisia Leaf, Cassia Bark, Danshen Root, Ephedra Herb, Epimedium Herb, Forsythia Fruit, Great Burdock Achene, Moutan Bark, Perilla Herb, Red Paeony Root, Schizonepeta Spike, Senticosus Rhizome, Sweet Annie,

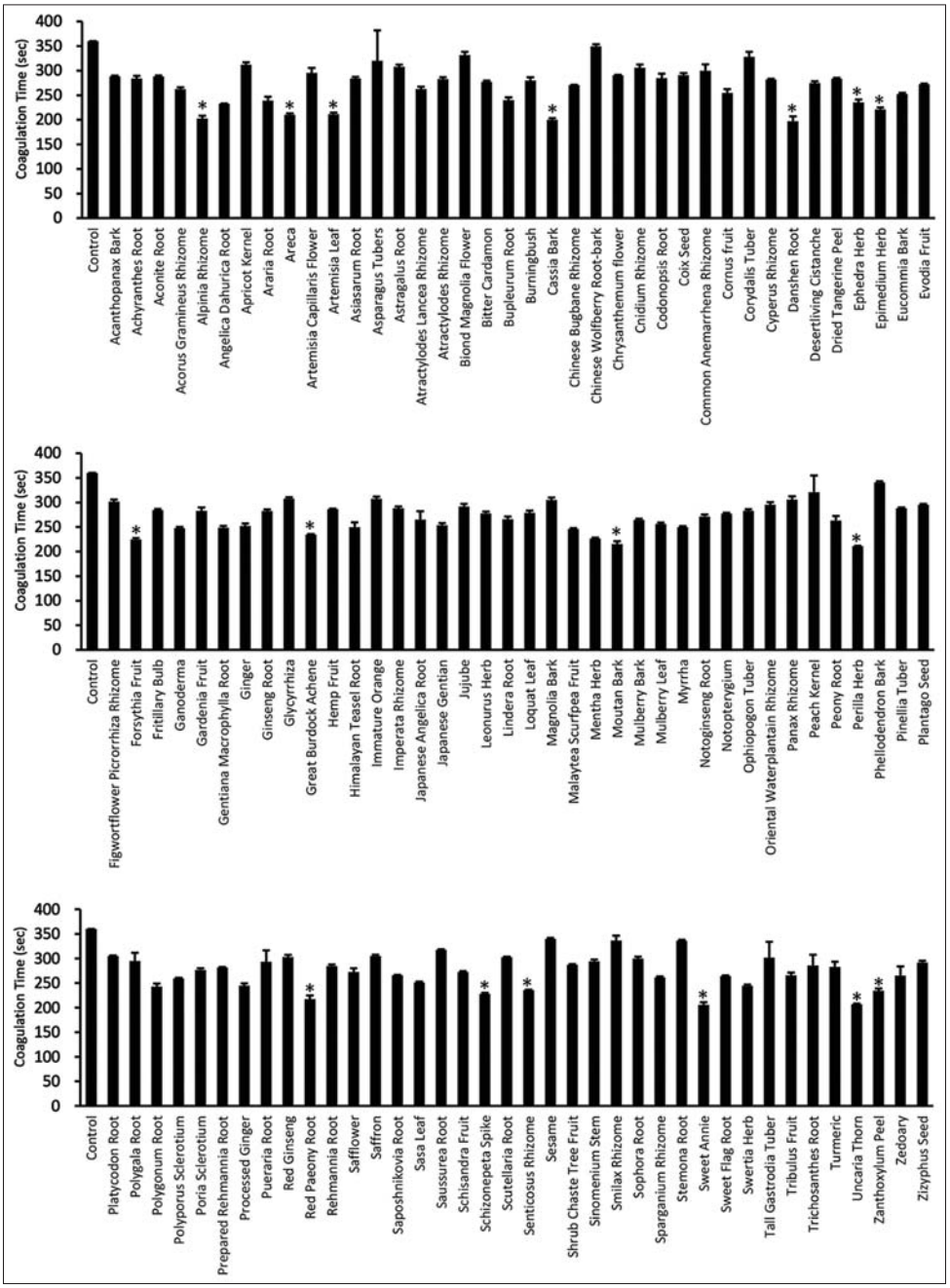

Figure 1: Effects of extracts of Chinese medicinal herbs on prothrombin time, Herbal extracts were incubated with plasma for 5 min before $CaCl_2$ solution was added and amount of time required for clot formation was measured. Data are expressed as means ± standard deviation (n = 3). *Coagulation time was under 230 s.

Uncaria Thorn and Zanthoxylum Peel shortened coagulation time [Figure 1]. We then assessed the ability of these extracts to activate factor XII. Purified human factor XII was incubated with the extracts for 5 h at 37°C and then the presence of Factor XIIa was assessed. Proteins in the mixture were resolved by SDS-PAGE and stained with silver. The light and heavy chains of factor XII were detected after adding extracts of Cat-tail pollen, Artemisia Leaf and Great Burdock Achene. A slight amount of light chain was generated by extracts of Perilla Herb [Figure 2].

DISCUSSION

The hemostatic properties of Cat-tail Pollen have been attributed to the activation of intrinsic coagulation [10,11]. Acidic polysaccharide in extracts of Cat-tail pollen directly activate factor XII in the coagulation cascade [10,11]. However, whether other hemostatic herbs such as Artemisia Leaf can activate intrinsic coagulation has remained unknown and other medicinal herbs might have as yet undiscovered hemostatic activity. Therefore, we screened active substances in 117 extracts of Chinese herbs by measuring the ability to enhance the extrinsic coagulation reaction. Extracts of Alpinia Rhizome, Areca, Artemisia Leaf, Cassia Bark, Danshen Root, Ephedra Herb, Epimedium Herb, Forsythia Fruit, Great Burdock Achene, Moutan Bark, Perilla Herb, Red Paeony Root, Schizonepeta Spike, Senticosus Rhizome, Sweet Annie, Uncaria Thorn and Zanthoxylum Peel shortened coagulation times. The ability of these extracts to activate factor XII was assessed by incubation for 1 h at 37°C, followed by resolving the reaction products by SDS-PAGE. Figure 2 shows that extracts of Artemisia Leaf and Great Burdock Achene cleaved factor XII to the light and heavy chains of factor XIIa, namely the active form of factor XII. Artemisia Leaf is a known antipyretic, insecticide, diuretic, and hemostatic agent [12]. Kneaded leaves of Artemisia Leaf have been used to treat excoriations or cuts on the skin surface. The hemostatic activity is thought to be a result of tannin astringency because mugwort contain high levels of tannin [8]. The present study found that Artemisia Leaf directly activates Factor XII. Hayakawa *et al.* reported that extracts of Artemisia Leaf leaves contain sulfated polysaccharide [13], and this probably induced the activation. However, the contribution of factor XII activation to the hemostatic activity of Artemisia Leaf is not clear. Great Burdock Achene has traditionally been used in China as an anti-inflammatory, detoxifying, or diuretic agent, and to dispel pathogenic wind-heat, promote eruption and remove toxic substances such as heavy metals [13]. The major bioactive principles in Great Burdock Achene are phenolic compounds such as lignans that have various biological properties *in vitro* and *in vivo* such as anti-cancer, antioxidant, antibacterial, antiviral anti-inflammatory and immunosuppressive activities [14-16]. However, whether Great Burdock Achene has hemostatic activity and effects on blood coagulation had not been reported. The present results showed that extracts of Great Burdock Achene and of Cat-tail pollen have essentially identical ability to activate factor XII. Negatively-charged compounds such as acidic polysaccharides in burdock fruit might contribute to the activation of factor XII. Sweet Annie and Artemisia Leaf both belong to the genus *Artemisia*. Sweet Annie (*Artemisia annua*) is a renowned antimalarial [17] and its effect on hemostasis has already been established in traditional Chinese medicine [18]. Wang *et al.* screened the hemostatic active fraction of sweet Annie using assays of plasma recalcification times and found an active fraction [19]. However, the mechanism involved in the reduction of coagulation time was not clear. Sweet Annie did not activate factor XII in the present study. Schizonepeta spike (*Schizonepeta tenuifolia*) exerts hemostatic action by promoting coagulation and inhibiting fibrinolysis [20]. Here, Schizonepeta spike shortened PT, but the activity was not dependent on factor XII activation. Extracts of Red Paeony Root also shortened blood coagulation time in the present study, which contradicted the findings of Wang and Ma, who showed that an extract of Red Paeony Root prolonged coagulation time [21]. We did not detect factor XII activation by other herbs that shorten coagulation time. These extracts might activate coagulation factors downstream of the intrinsic coagulation cascade such as factor XI and factor IX or inhibit anticoagulant proteins such as antithrombin. Our screening study showed that some popular Chinese herbs have

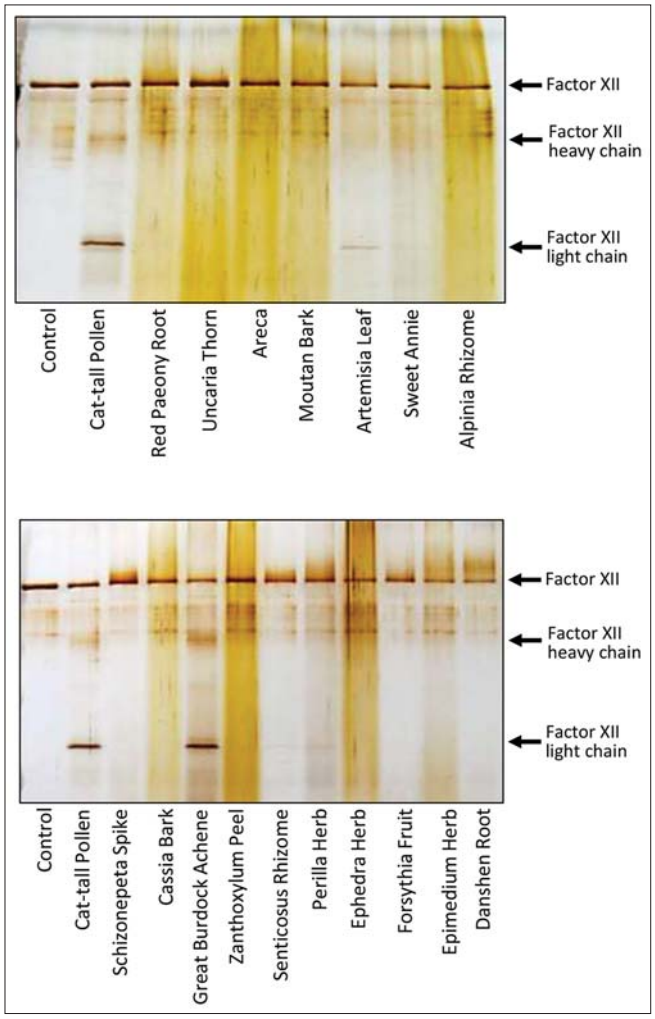

Figure 2: Analysis of factor XII activation, Chinese herbal extracts were incubated with factor XII for 5 h and then generation of activated factor XIIa was analyzed by SDS-PAGE followed by silver staining.

potential as hemostatic agents. However, to the best of our knowledge, these herbs have not been used as a treatment to preventing bleeding. The mechanisms through which these herbs shortened blood coagulation time remain also obscure. Nonetheless, these traditional herbs could be applied as a novel clinical approach to the control or prevention of bleeding.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid from the Cooperative Research Project for Joint Usage/Research Center (Joint Usage/Research Center for Science-Based Natural Medicine) Institute of Natural Medicine, University of Toyama in 2010.

REFERENCES

1. Ishida H, Umino T, Tsuji K, Kosuge T. Studies on antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. VII. On the antihemorrhagic principle in *Cirsium japonicum* DC. Chem Pharm Bull (Tokyo) 1987;35:861-4.
2. Kosuge T, Ishida H, Satoh T. Studies on antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. V. On antihemorrhagic principle in *Biota orientalis* (L.) Endl. Chem Pharm Bull (Tokyo) 1985;33:206-9.
3. Ishida H, Umino T, Tsuji K, Kosuge T. Studies on the antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. IX. On the antihemorrhagic principles in *Typha lactifolia* L. Chem Pharm Bull (Tokyo) 1988;36:4414-20.
4. Ishida H, Umino T, Tsuji K, Kosuge T. Studies on the antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. VIII. On the antihemorrhagic principle in nelumbins receptaculum. Chem Pharm Bull (Tokyo) 1988;36:4585-7.
5. Ishida H, Umino T, Tsuji K, Kosuge T. Studies on the antihemostatic substances in herbs classified as hemostatics in traditional Chinese medicine. I. On the antihemostatic principles in *Sophora japonica* L. Chem Pharm Bull (Tokyo) 1989;37:1616-8.
6. Sadiq A, Hayat MQ, Ashraf M. Ethnopharmacology of *Artemisia annua* L.: A review. In Artemisia Annua-Pharmacology and Biotechnology. Berlin, Heidelberg: Springer; 2014. p. 9-25.
7. Isler SC, Demircan S, Cakarer S, Cebi Z, Keskin C, Soluk M, *et al*. Effects of folk medicinal plant extract Ankaferd Blood Stopper on early bone healing. J Appl Oral Sci 2010;18:409-14.
8. Kimura Y, Okuda H, Okuda T, Hatano T, Agata I, Arichi S. Studies on the activities of tannins and related compounds from medicinal plants and drugs. VII. Effects of extracts of leaves of Artemisia species, and caffeic acid and chlorogenic acid on lipid metabolic injury in rats fed peroxidized oil. Chem Pharm Bull (Tokyo) 1985;33:2028-34.
9. Cordier W, Steenkamp V. Herbal remedies affecting coagulation: A review. Pharm Biol 2012;50:443-52.
10. Ohkura N, Tamura K, Tanaka A, Matsuda J, Atsumi G. Experimental study on the hemostatc activity of *Pollen Typhae*: A traditional folk medicine used by external and oral application. Blood Coagul Fibrinolysis 2011;22:631-6.
11. Ohkura N, Tauchi C, Nakayama A, Atsumi G. *Pollen Typhae* is a rapid hemostyptic. Blood Coagul Fibrinolysis 2012;23:254-5.
12. Ferreira JF, Luthria DL, Sasaki T, Heyerick A. Flavonoids from Artemisia annua L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. Molecules 2010 29;15:3135-70.
13. Chan YS, Cheng LN, Wu JH, Chan E, Kwan YW, Lee SM, *et al.* A review of the pharmacological effects of *Arctium lappa* (burdock). Inflammopharmacology 2011;19:245-54.
14. Awale S, Lu J, Kalauni SK, Kurashima Y, Tezuka Y, Kadota S, *et al.* Identification of arctigenin as an antitumor agent having the ability to eliminate the tolerance of cancer cells to nutrient starvation. Cancer Res 2006;66:1751-7.
15. Matsumoto T, Hosono-Nishiyama K, Yamada H. Antiproliferative and apoptotic effects of butyrolactone lignans from *Arctium lappa* on leukemic cells. Planta Med 2006;72:276-8.
16. Park SY, Hong SS, Han XH, Hwang JS, Lee D, Ro JS, *et al.* Lignans from *Arctium lappa* and their inhibition of LPS-induced nitric oxide production. Chem Pharm Bull (Tokyo) 2007;55:150-2.
17. Ziffer H, Highet RJ, Klayman DL. Artemisinin: An endoperoxidic antimalarial from *Artemisia annua* L. Fortschr Chem Org Naturst 1997;72:121-214.
18. Feng WY. Preliminary exploration of *Artemisia annua* L. and its praeparatum. Tibetan Med J 1978;2:82-3.
19. Wang B, Sui J, Yu Z, Zhu L. Screening the hemostatic active fraction of *Artemisia annua* L. *In-vitro*. Iran J Pharm Res 2011;10:57-62.
20. Ding AW, Wu H, Kong LD, Wang SL, Gao ZZ, Zhao MX, *et al.* Research on hemostatic mechanism of extracts from carbonized *Schizonepeta tenuifolia* Brig. Zhongguo Zhong Yao Za Zhi 1993;18:598-600, 638.
21. Wang Y, Ma R. Effect of an extract of *Paeonia lactiflora* on the blood coagulative and fibrinolytic enzymes. Zhong Xi Yi Jie He Za Zhi 1990;10:101-2, 70.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141104014605

Original Research

ScopeMed

Anti-conjunctivitis effect of fresh juice of *xGraptoveria* (Crassulaceae): A phytochemical and ethnobotanical study

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Received: September 05, 2014
Accepted: November 04, 2014
Published: November 28, 2014

ABSTRACT

Aim: The parent of x*Graptoveria*, *Graptopetalum paraguayense*, is used in Chinese folk medicine for alleviating hepatic disorders, detumescence and detoxication, lowering of blood pressure, inhibition of cancer cells, exerting diuretic effects, relieving pain and infections. No data are available regarding its anti-conjunctivitis effect. The aim of this preliminary study is to test the anti-conjunctivitis properties of x*Graptoveria* (Crassulaceae) and to identify its bioactive constituents. **Materials and Methods:** Fresh watery juice of leaves of x*Graptoveria* was extracted with *n*-butanol and the extract was analyzed using gas chromatography-mass spectrometry (GC/MS). The ethnobotanical appraisal of the anti-conjunctivitis properties of x*Graptoveria* was based on 11 interviews about the symptoms against which this plant demonstrated positive effect. **Results:** Fresh juice of x*Graptoveria* leaves applied directly to the irritated eye 2 times per day cured conjunctivitis in all reported cases. The main groups of organic compounds identified by GC/MS analysis in the fresh extracted leaf juice of x*Graptoveria* were: Alkylamines, hydroxycarboxylic acids, aliphatic and aromatic carboxylic acids, amino acids, alcohols, aromatic and aliphatic hydrocarbons. **Conclusion:** In this preliminary study, it is suggested that x*Graptoveria* exerts anti-conjunctivitis activity, through synergistic effect of different chemical compounds, most probably alkylamines and mainly hydroxycarboxylic, aliphatic, and aromatic carboxylic acids.

KEY WORDS: Alkylamines, conjunctivitis, ethnobotanical, gas chromatography-mass spectrometry, x*Graptoveria* leaf juice

INTRODUCTION

Conjunctivitis can be caused by viruses, bacteria or fungi, exposure to chemicals or irritants or long-term presence of a foreign body such as hard or rigid contact lenses [1-3].

Plants have shown considerable activity against various microbes [4-7]. It is considered that plants are a source of a wide variety of bioactive molecules that can be used for the development of new medicines with a wider spectrum of activities and with less adverse effects than those produced by the drugs currently in use [7-9].

Bulgarian folk medicine treats conjunctivitis by several plants. Most popular are species of the genus *Euphrasia*, and even their common names are related to that use "ochanka" (in Bulgarian Ochi: eyes) [10-13]. There are empirical data for a therapeutic effect of *Geumurbanum*, *Althea officinalis*, *Pimpinela saxifraga*, *Anagalis arvensis*, in cases of conjunctivitis [11-13].

Members of the Crassulaceae family are known for their antiseptic and antibacterial properties. Particularly, leaves of *Echeveria gigantean* Rose and Purpus are used for eye illness treatment [8], but there are no data about the chemical or biological studies.

The object of this study, *xGraptoveria*, is an intergeneric hybrid between *Graptopetalum paraguayense* and *Echeveria* sp. div. It is a succulent, drought-resistant perennial grown as ornamental house plant in temperate regions, as it cannot survive winter outside. The parenting taxa belong to the Crassulaceae family. They are native to Mexico and are distributed widely in tropical and subtropical countries where they are mainly cultivated as ornamental plants, but are popular in Chinese herbal medicine.

To the best of our knowledge, there is no information in the literature on the application of *G. paraguayense* juice for treatment of conjunctivitis.

The aim of this study is to test the anti-conjunctivitis properties of x*Graptoveria* (Crassulaceae) and to identify its bioactive constituents.

MATERIALS AND METHODS

xGraptoveria

We have grown x*Graptoveria* as an ornamental house plant at the Institute of Organic Chemistry with Center of Phytochemistry, Sofia, Bulgaria for more than 10 years. The vegetative reproduction of this plant can be easily induced by separating offsets and leaf cuttings. Rosettes and fine wax cover are visible external features of the leaves [Figure 1].

Ethnobotanical Study

Since its introduction in Bulgaria, x*Graptoveria* has also found its place in folk medicine. Some anecdotal data have been reported for the use of freshly obtained leaf juice for treatment of eye problems, mainly forms of conjunctivitis. We managed to collect eye healing information for x*Graptoveria* via interviewing 11 herbalists that had already been treated with the same plant. A semi-structured questionnaire [14] was constituted using the following questions: (1) What is the plant used for; (2) How is it used; (3) What part of the plant is used; (4) What is the dose used for eye treatment; (5) How long does the treatment take; (6) How many applications are needed for eye improvement?

Treatment of Volunteers with Conjunctivitis by *xGraptoveria* Leaf Juice

Volunteers

Four women and four men of age between 33 and 60, volunteered to have their eyes treated by freshly prepared *xGraptoveri*a Leaf Juice. All of them suffered from conjunctivitis as estimated by an ophthalmologist. The symptoms are described in Table 1.

Method of treatment

Fresh juice obtained directly by pressing out *xGraptoveria* leaf was immediately dropped in the irritated eye without any dilutions. Treatment of volunteers was performed in clean eyes as follows: 1 drop per eye; 2 times a day (in the morning and in the evening). The applications were done by the volunteers themselves at the Institute of Organic Chemistry with Centre of Phytochemistry, Sofia, Bulgaria.

Phytochemical Analysis of the Fresh Juice used for the Eye Treatment

Preparation of extracts of xGraptoveria

Fresh leaves of *xGraptoveria* were picked and pressed out immediately to give watery juice. The juice was then extracted with *n*-butanol. The butanol extract (xGBE) was evaporated and analyzed by gas chromatography mass spectrometry (GC/MS).

GC/MS analysis

The analysis of xGBE was performed with a Hewlett Packard 6890 GC System Plus MS 5973 (Hewlett Packard, Palo Alto, CA, USA) equipped with capillary column HP5-MS (30 cm, 0.25 mm, 0.25 mm film thickness, Agilent Technology, USA). The carrier gas was helium with flow rate 0.8 mL/min. The following temperature program was used: 100-300°C (10 min isotherm) at 5°C/min. The method of electron-impact ionization was utilized. The ion source was set at 230°C and the ionization voltage was 70 eV. Because the ion current generated depends on the characteristics of the investigated compounds and is not true quantification, GC/MS analyses do not give exact quantitative data.

Preparation of the sample for GC/MS analyses

The sample of about 5 mg of xGBE was prepared. It was silylated prior to GC/MS measuring via mixing with 75 mL of dry pyridine and 25 mL of bis(trimethylsilyl)trifluoroacetamide and heating at 80°C for 20 min.

Identification of compounds

The GC/MS identification was based on the interpretation of the mass spectral fragmentation facilitated by HP Mass Spectral Library NIST98 (Hewlett Packard, Palo Alto, CA, USA). Not all, but the main components were identified.

RESULTS

Eleven interviews were performed according to a semi-structured questionnaire (Section 2.2.) [14]. The informants were chosen

Figure 1: *xGraptoveria* (Crassulaceae)

Table 1: Conjunctivitis symptoms, diagnosis and number of applications leading to improvement and complete cure of the volunteers

Gender	Age	Symptoms	Diagnosis	Improvement	Complete cure
Female	33	Redness in the area of sclera caused by visible capillaries; pain	Allergic conjunctivitis	3	6
Female	34	Redness in the area of sclera caused by visible capillaries	Allergic conjunctivitis	3	6
Female	45	Redness in the area of sclera caused by visible capillaries; stinging and itching eyes; purulentdischarge	Bacterial conjunctivitis	2	6
Female	57	Redness in the area of sclera caused by visible capillaries	Allergic conjunctivitis	3	6
Male	34	Redness in the area of sclera caused by visible capillaries; mucoid discharge; stickyeyelids	Adenoviral conjunctivitis	2	6
Male	35	Redness in the area of sclera caused by visible capillaries; purulentdischarge; crustyeyelids	Bacterial conjunctivitis	3	6
Male	40	Redness in the area of sclera caused by visible capillaries; mucoiddischarge	Bacterial conjunctivitis	2	6
Male	60	Redness in the area of sclera caused by visible capillaries	Allergic conjunctivitis	2	6

amongst herbalists that had been treated by *xGraptoveria*. All of them gave the same answers concerning the plant and described it to have strong healing effect on human conjunctivitis. This information encouraged us to try out the effect of the plant grown in our laboratory following the treatment conditions described during the interviews (Section 2.3.2.).

Eight volunteers were diagnosed by an ophthalmologist with various types of conjunctivitis (chronic allergic, adenoviral, and bacterial conjunctivitis) [Table 1]. Redness in the area of the sclera caused by visible capillaries was the symptom observed in all cases. Allergic conjunctivitis was the case for three of the women (age 33, 34 and 57) and one of the men (age 60). Redness and pain accompanied the symptoms of the 33-old woman. Volunteers with bacterial conjunctivitis suffered from redness, mucoid or purulent discharge and crusty eyelids. The symptoms diminished the 1st day (2-3 applications) and completely disappeared the next 3 days in all described cases. The symptoms, diagnosis and number of applications leading to improvement and complete cure of the volunteers are given in Table 1.

To study the components of *xGraptoveria* leaf juice that may be responsible for the curative effect on conjunctivitis, the juice was subjected to phytochemical analysis. In order to maximize the identification of constituents, the juice was extracted with *n*-butanol and the resulting extract (xGBE) was investigated by GC/MS. This method allowed for analysis of complex mixtures as described in 2.4.2. The GC/MS chromatogram of xGBE is presented on Figure 2. The extract investigated could contain a significant number of metabolites, including some minor compounds, which cannot be identified by other methods.

The main groups of organic compounds identified by GC/MS analysis in xGBE are presented in Table 2: Alkylamines (ethylamine and butylamine), hydroxycarboxylic acids (hydroxypropenoic, hydroxybutanoic, malic, dihydroxybutanoic and methylhydroxybenzoic acids), aliphatic and aromatic carboxylic acids (malonic, pentadecanoic, oleic, hexanedioic, palmitic, stearic, benzoic, and 3-pyridinecarboxylic acids), amino acids (tryptophane), alcohols (dihydroxyethane and glycerol), aromatic and aliphatic hydrocarbons (pentamethylheptane, ethyl-dimethylbenzene and tetramethylbenzene), and sugars. Some of them (hydroxypropenoic acid, pentamethylheptane, ethyl-dimethylbenzene, butylamine, dihydroxyethane, tetramethylbenzene, hydroxybutanoic acid, dihydroxybutanoic acid and methylhydroxybenzoic acid) can exist in several isomers. However, the method did not allow for identification of the existing forms.

Table 2: GC/MS data for the main organic compounds identified in xGBE, R_t (min), TIC (%)

R_t	Compound	TIC
8.6	Ethylamine	2.3
9.0	Hydroxypropenoic acid (3-Hydroxypropenoate)	0.7
9.2	Pentamethylheptane*	0.3
11.3	Ethyldimethylbenzene*	1.1
11.8	*n*-Butylamine	19.4
12.2	Tetramethylbenzene*	1.2
12.7	Dihydroxyethane*	0.8
13.0	Hydroxybutanoic acid*	0.6
15.2	Benzoic acid	0.9
16.1	Glycerol, 3-pyridinecarboxylic acid	2.0
17.2	Malonic acid	0.7
20.2	Dihydroxybutanoic acid*	0.3
21.6	Malic acid	1.2
25.0	Methylhydroxybenzoic acid (3-methylsalicylic acid)	0.7
29.4	Pentadecanoic acid	0.4
31.6	Oleic acid	0.5
31.9	Tryptophane	1.0
32.9	Hexanedioic acid (adipic acid)	0.4
33.4	Palmitic acid	0.7
	Sugars	8.8

*Compound can exist in several isomers. Rt: Retention time, TIC: Total ion current, GC/MS: Gas chromatography-mass spectrometry, xGBE: x*Graptoveria* butanol extract

DISCUSSION

Fresh juice of *xGraptoveria* leaves was used successfully for the complete cure of various types of conjunctivitis in 8 volunteers [Table 1]. The treatment conditions were taken from the informants who had recovered from conjunctivitis by treatment with the same plant. Since this is a very preliminary study it was defined between a small set of volunteers and no clinical and pharmacological research was embarked. However, we still aimed at finding a strong motivation for further profound study leading to relevant exploitation of *xGraptoveria*. For this reason, we analyzed the phytochemical composition of the fresh juice, which was exactly the curative part used for the treatment of conjunctivitis.

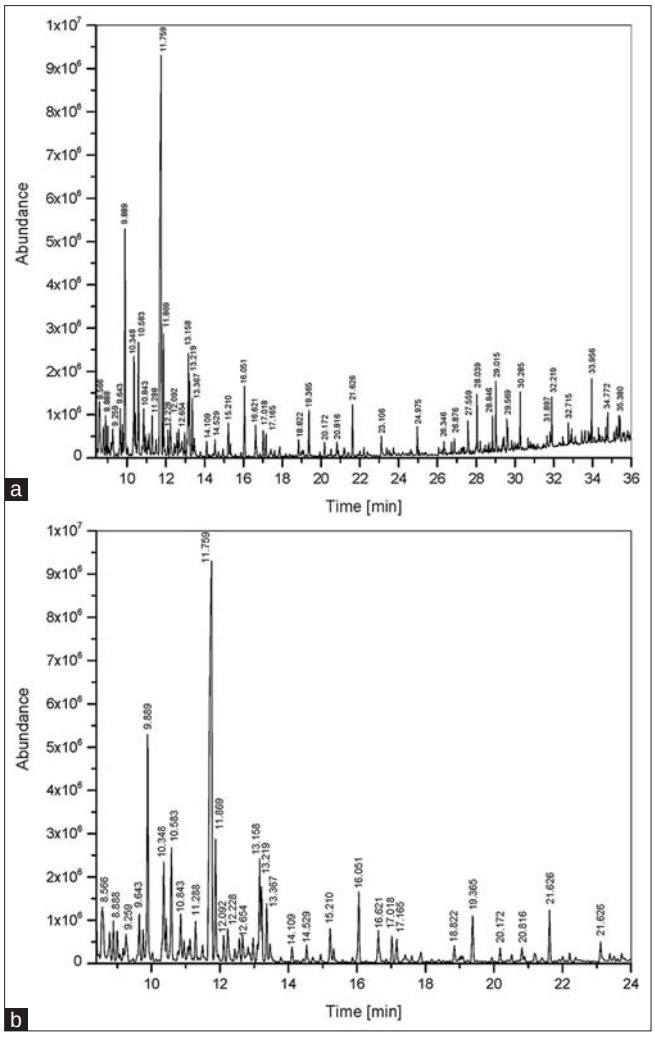

Figure 2: Gas chromatography-mass spectrometry chromatogram of *xGraptoveria* butanol extract include the identified components: (a) Retention time (R_t) = 8.6-36.0 min; (b) R_t = 8.6-24.5 min

The GC/MS analysis allowed for identification of most of the fresh juice components. However, searching the literature did not reveal any anti-conjunctivitis activity for the identified single compounds. Instead we came across some data showing interesting biological functions for these constituents that may be in relation with the observed effect.

Conjunctiva, which is the place where conjunctivitis occurs, provides a major source of immune components in the cornea. It produces the antigen immunoglobulin A that plays a critical role in mucosal immunity and also contains macrophages, neutrophilic granulocytes, mast cells, lymphocytes, and other aspects of the general mucosal immune system [15]. The macrophages play a part in modulating the T-cell immune response and mediating both the innate and acquired immune responses. Interestingly, in this relation we observed that the fresh juice of *xGraptoveria* leaves is rich of alkylamines, mostly *n*-butylamine and some ethylamine [Table 2]. Alkylamines are known immune activators. For example, *sec*-butylamine and *iso*-butylamine can activate Vγ9Vδ2 T cells in humans as a consequence of inhibition of farnesyl diphosphate synthase and the intracellular accumulation of isopentenyl pyrophosphate [16-18]. Structure analysis of several antigenic and non-antigenic alkylamines indicates that a straight or branched alkyl chain of two to five carbons with a single primary amine group as the only substituent is active, while alkylamines with one carbon or more than five carbons, or any substituent other than the primary amino group have no effect [16,19].

Presence of tryptophan in the fresh juice may be useful for influence upon the inflammatory process. Furthermore, a number of recent studies have shown a clear association between tryptophan catabolism and inflammatory reactions in a vast array of disease states [20]. The remainder of the organic acids identified in the juice possess antimicrobial activity predominantly or a combination of several biological activities. Malic acid manifests antioxidant, anti-inflammatory and antibacterial activities [21-23]. Oleic acid is active against several Gram-positive bacteria [24]. Interesting synergistic relationships were observed between some of the acids. Palmitic, pentadecanoic and oleic acids gave a mixture which was much more potent as antimicrobial agent than the single acids against 11 microorganisms [25-27]. Another example concerns oleic and linoleic acids, which were more active together against *Staphylococcus aureus* and *Micrococcus kristinae* [24,28].

After all, we decided to speculate that the healing effect of *xGraptoveria* fresh leaf juice might be due to a synergistic effect of its constituents, part of which affect the immune response while the rest act against the invading microorganisms.

CONCLUSION

This is a preliminary study on the chemical composition and anti-conjunctivitis effect of *xGraptoveria* leaf fresh juice. It is hypothesized that the effect is due to the synergistic action of the bioactive constituents – mainly alkylamines, hydroxycarboxylic, aliphatic and aromatic carboxylic acids.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Colin C. Walker, Open University, UK for help with the identification of the *xGraptoveria* plant and Dr. M. Spassova, Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia for helpful discussions.

REFERENCES

1. Sethuraman U, Kamat D. The red eye: Evaluation and management. Clin Pediatr (Phila) 2009;48:588-600.
2. Cronau H, Kankanala RR, Mauger T. Diagnosis and management of red eye in primary care. Am Fam Physician 2010;81:137-44.
3. Goodman DM, Rogers J, Livingston EH. JAMA patient page. Conjunctivitis. JAMA 2013;309:2176.
4. Vashist H, Jinda A. Antimicrobial activities of medicinal plants - Review. Int J Res Pharm Biomed Sci 2012;2:222-30.
5. Modi C, Mody S, Patel H, Dudhatra G, Kumar A, Awale M. Herbal antibacterials: A review. J Intercult Ethnopharmacol 2012;1:52-61.
6. Silva NC, Fernandes JA. Biological properties of medicinal plants: A review of their antimicrobial activity. J Venom Anim Toxins Incl Trop Dis 2010;16:402-13.

7. Ríos JL, Recio MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol 2005;100:80-4.
8. Rocha-Gracia RC, Arroyo M, Zaraín PL, Carlos BH, Romero HS, Portugal EC, *et al.* Antibacterial activity of crude extracts from Mexican plants against methicillin-resistant *Staphylococcus*. Afr J Biotechnol 2011;10:13202-18.
9. Recio MC, Rios JL, Villar A. A review of some antimicrobial compounds isolated from medicinal plants reported in the literature 1978-88. Phytother Res 1989;3:117-25.
10. Kozuharova E, Benbassat N, Napier J. New records of the remedial properties of vascular plants, some traditionally accepted as medicinal plants and some less familiar to ethnobotanists. J Phytol Balcanica 2012;18:323-33.
11. Dimkov P. Natural healing methods and living with nature (in Bulgarian). Bulgarian traditional medicine: Astrala. 2001. p. 1-3.
12. Nikolov S. Specialized Encyclopedia of medicinal plants in Bulgaria (in Bulgarian). Sofia: Publishing House "Trud"; 2006.
13. Petkov V. Contemporary phytotherapy (in Bulgarian). Sofia: Medicine and Physculture; 1982.
14. Martin GJ. Ethnobotany. London: Chapman & Hall; 1995.
15. Knop E, Knop N. The role of eye-associated lymphoid tissue in corneal immune protection. J Anat 2005;206:271-85.
16. Bukowski JF, Morita CT, Brenner MB. Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: Implications for innate immunity. Immunity 1999;11:57-65.
17. Percival SS, Bukowski JF, Milner J. Bioactive food components that enhance gammadelta T cell function may play a role in cancer prevention. J Nutr 2008;138:1-4.
18. Thompson K, Rojas-Navea J, Rogers MJ. Alkylamines cause Vgamma9Vdelta2 T-cell activation and proliferation by inhibiting the mevalonate pathway. Blood 2006;107:651-4.
19. Cao W, He W. The recognition pattern of gammadelta T cells. Front Biosci 2005;10:2676-700.
20. Moffett JR, Namboodiri MA. Tryptophan and the immune response. Immunol Cell Biol 2003;81:247-65.
21. Rathnayaka RM. Antibacterial effect of malic acid against Listeria monocytogenes, Salmonella enteritidis and *Escherichia coli* in Mango, pineapple and papaya juices. Am J Food Technol 2013;8:74-82.
22. Taylor MB, Yanaki JS, Draper DO, Shurtz JC, Coglianese M. Successful short-term and long-term treatment of melasma and postinflammatory hyperpigmentation using vitamin C with a full-face iontophoresis mask and a mandelic/malic acid skin care regimen. J Drugs Dermatol 2013;12:45-50.
23. Tang X, Liu J, Dong W, Li P, Li L, Lin C, *et al*. The cardioprotective effects of citric Acid and L-malic Acid on myocardial ischemia/reperfusion injury. Evid Based Complement Alternat Med 2013;2013:820695.
24. Dilika F, Bremner PD, Meyer JJ. Antibacterial activity of linoleic and oleic acids isolated from *Helichrysum pedunculatum*: A plant used during circumcision rites. Fitoterapia 2000;71:450-2.
25. McGaw LJ, Jäger AK, van Staden J. Isolation of antibacterial fatty acids from Schotia brachypetala. Fitoterapia 2002;73:431-3.
26. Seidel V, Taylor PW. *In vitro* activity of extracts and constituents of Pelagonium against rapidly growing mycobacteria. Int J Antimicrob Agents 2004;23:613-9.
27. Agoramoorthy G, Chandrasekaran M, Venkatesalu V, Hsu MJ. Antibacterial and antifungal activities of fatty acid methyl esters of the blind-your eye mangrove from India. Braz J Microbiol 2007;38:739-42.
28. Meyer JJ, Dilika F. Antibacterial activity of *Helichrysum pedunculatum* used in circumcision rites. J Ethnopharmacol 1996;53:51-4.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141124062203

Original Research

ScopeMed

Guaiazulene biochemical activity and cytotoxic and genotoxic effects on rat neuron and N2a neuroblastom cells

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Received: October 19, 2014
Accepted: November 24, 2014
Published: January 03, 2015

ABSTRACT

Aim: Neuroblastoma (NB)cells are often used in cancer researches such as glioblastoma cells since they have the potential of high mitotic activity, nuclear pleomorphism, and tumor necrosis. Guaiazulene (GYZ 1,4-dimethyl-7-isopropylazulene)is present in several essential oils of medicinal and aromatic plants. Many studies have reported the cytotoxic effect of GYZ; however, there are no studies that compare such effects between cancer cell lines and normal human cells after treatment with GYZ. **Materials and Methods:** In this study, we aimed to describe *in vitro* antiproliferative and/or cytotoxic properties (by 3-[4,5 dimetylthiazol -2-yl]-2,5 diphenlytetrazolium bromide [MTT] test), oxidative effects (by total antioxidant capacity [TAC] and total oxidative stress [TOS] analysis)and genotoxic damage potentials (by single cell gel electrophoresis)of GYZ. **Result:** The results indicated that GYZ have anti-proliferative activity suppressing the proliferation of neuron and N2a-NB cells at high doses. In addition, GYZ treatments at higher doses led to decreases of TAC levels and increases of TOS levels in neuron and N2a-NB cells. On the other hand, the mean values of the total scores of cells showing DNA damage were not found different from the control values. **Conclusion:** From this study, it is observed that GYZ has *in vitro* cytotoxic activity against neuron and N2a-NB cells.

KEY WORDS: Cytotoxicity, genotoxicity, guaiazulene, neuroblastoma, single cell gel electrophoresis assay, 3-(4,5 dimetylthiazol -2-yl)-2,5 diphenlytetrazolium bromide assay

INTRODUCTION

Neuroblastoma (NB) is an embryonal tumor that originates from primitive cells. It's the most common solid tumor and is responsible for 15% of all cancer-related deaths in childhood [1,2]. This tumor accounts for more than 7% of malignancies in patients fewer than 15 years of age [3,4]. NB tumors from these patients are often characterized by deregulation of many key signaling pathways regulating growth, proliferation, survival, and apoptosis, with concomitant resistance to chemotherapy [4,5]. The acquisition of multidrug resistance upon treatment with anti-cancer drugs is a common phenomenon for NBs. This is a major reason for the high frequency of fetal outcome of the disease [6]. Recently, there are strong epidemiological evidence and laboratory studies that are naturally occurring terpenes may exert cytotoxic effects against NB cells [7,8].

Terpenes are the largest group of natural substances biosynthetically derived from isoprene units [9]. Guaiazulene (1,4-dimethyl-7-isopropylazulene GYZ, Figure 1)is a bicyclic sesquiterpene derived from different plants, guaiac wood oil, *Callis intratropica* blue and *Matricaria chamomilla* L and has attracted much attention due to its beneficial biological activities [10,11]. Moreover, previous reports indicated that GYZ has antioxidant, antifungal, antimicrobial, anti-inflammatory, anti-spasmodic, anti-ulcer, antitumoral activities and relaxant properties [12-18]. Although it has been demonstrated to have interesting biological effects, GYZ has been not proven to be cytotoxic, genotoxic and antioxidant/oxidant effects on neuron and NB cell lines. Therefore, the aim of the present study was to firstly evaluate the cytotoxic/antiproliferative (3-[4,5 dimetylthiazol -2-yl]-2,5 diphenlytetrazolium bromide [MTT] assay), cytogenetic (single cell gel electrophoresis [SCGE] assay)

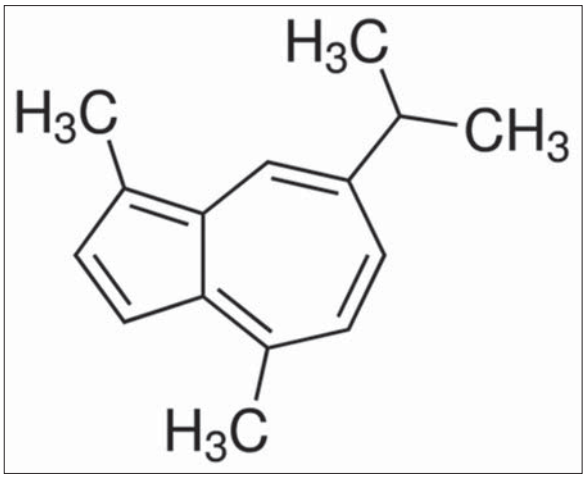

Figure 1: Chemical structure of guaiazulene

and oxidative effects (total antioxidant capacity [TAC] and total oxidative stress [TOS] analysis)of GYZ on neuron and NB cell cultures for its possible use in the complementary and alternative medicine practices.

MATERIALS AND METHODS

Test Compounds and Chemicals

GYZ (CAS 489-84-9, $C_{15}H_{18}$), Dulbecco modified eagles medium, sodium phosphate (NaH_2PO_4), potassium phosphate monobasic (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO), triton-X-100, tris, low melting point agarose, normal melting point agarose, ethidium bromide were purchased from Sigma-Aldrich® (Steinheim, Germany).

Experimental Design

Primary rat cerebral cortex neuronal cultures were prepared using rat fetuses as described previously [19]. Briefly, a total of nine new-born Sprague–Dawley rats were used in the study. The rats were decapitated by making a cervical fracture in the cervical midline, and the cerebral cortex was dissected and removed. The cerebral cortex was placed into 5 mL of Hank's balanced salt solution (HBSS, Sigma-Aldrich®, Steinheim, Germany), which had already been placed in a sterile petri dish and macromerotomy was performed with two lancets. This composition was pulled into a syringe and treated at 37°C for 25-30 min as 5 mL HBSS plus 2 mL trypsin-EDTA (0.25% trypsin- 0.02% EDTA)and chemical decomposition was achieved. 8 μL of DNase Type 1 (120 U/mL), was added to this solution, treated for 1-2 min, and centrifuged at 800 rpm for 3 min. After having thrown away the supernatant, 31.5 mL of Neurobasal® Medium (Life Technologies, Inc.)and 3.5 mL fetal calf serum (Sigma-Aldrich®, Steinheim, Germany)were added to the residue. The single cell which was obtained after physical and chemical decomposition was divided into 3.5 mL samples in each of 10 flasks coated with poly-D-lysine formerly dissolved in phosphate buffer solution. The flasks were left in the incubator including 5% CO_2 at 37°C. The flasks were then changed with a fresh medium of half of their volumes every 3 days until the cells were branched and had reached a certain maturity. Further *in vitro* experiments were performed 8 days later. This study was conducted at the Medical Experimental Research Centre in Ataturk University (Erzurum, Turkey). The Ethical Committee of Ataturk University approved the study protocol (B.30.2.ATA.0.23.85-73). We employed a cell line, N2a-NB, used widely as a model for brain cancer. The rat brain NB cell line N2a was obtained from Turkey FMD institute, Ankara, Turkey.

Treatments

GYZ was dissolved in ethanol and ethanol was evaporated to dryness at ambient temperature. GYZ was applied into cultures at concentrations of 10, 25, 50, 75, 100, 150, 200 and 400 mg/L for 24 h. The concentrations were selected according to the work of Si *et al*. [20].

MTT assay

The cells were seeded in 96-well plastic plates at a density of 1×104 cells/well and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 24 h. Cell viability assay was performed using the MTT cell proliferation kit (Cayman Chemical Company®, USA). Pure water was added in the control group. At the end of the experiment, the neurons, and N2a-NB cells were incubated with 20 μL of MTT for 30 min at 37°C. After washing the blue formazan was extracted from cells with isopropanol/formic acid (955)and was photometrically determined at 570 nm [8].

TAC and TOS analysis

The automated TAC and TOS assays were carried out by commercially available kits (Rel Assay Diagnostics, Turkey)on cell cultures of GYZ-treated cultures for 24 h [21,22].

SCGE assay

DNA damage evaluation was performed by SCGE also known as comet test assay. After the application of coverslips, the slides were allowed to gel at 4°C for 30-60 min. The coverslip was removed, and the slide was immersed in cell lysis solution (2.5 M NaCl, 0.1 M Na_2-EDTA, 1% Na-sarcosinate, 10 mM Tris-HCl (pH 10.0), 10% DMSO, and 1% triton X-100)for 1 h. and refrigerated overnight followed by alkali treatment (1 mM Na_2-EDTA and 0.3 M NaOH, pH > 12.0), electrophoresis (at 1.6 V cm−1 for 20 min, 300 mA)and neutralization (0.4 M tris, pH 7.5). The dried slides were then stained using ethidium bromide (20 lg/mL)after appropriate fixing for 10 min. The whole procedure was carried out in dim light to minimize artifact [23]. DNA damage analysis was performed at a magnification of ×100 using a fluorescence microscope (Nikon Eclips E6600, Japan)after coding the slides by one observer (Togar B.). A total of 100 cells were screened per slide. A total damage score for each slide was derived by multiplying the number of cells assigned to each grade

of damage by the numeric value of the grade and summing over all grades (giving a maximum possible score of 400, corresponding to 100 cells at grade 4).

Statistical Analysis

Statistical analysis was performed using SPSS Software (version 18.0, SPSS®, Chicago, IL, USA). For statistical analysis of obtained data Duncan's test was used. Statistical decisions were made with a significance level of 0.05.

Table 1: *In vitro* **TAC and TOS levels in cultured healthy neuron and N2a-NB cells maintained 24 h in the presence of GYZ**

Doses (μg/mL)	Healthy neuron		N2a-NB	
	TAC (mmol Trolox Equiv./L)	TOS (mmol H_2O_2 Equiv./L)	TAC (mmol Trolox Equiv./L)	TOS (mmol H_2O_2 Equiv./L)
Control	28.6±3.0	1.7±0.1	6.1±0.5	2.3±0.2
10	27.8±2.7	1.6±0.2	5.8±0.8	2.8±0.3
25	28.0±3.5	1.5±0.2	6.1±0.7	2.4±0.2
50	28.3±3.8	1.7±0.1	6.1±0.5	2.4±0.3
75	26.4±3.6	1.8±0.2	5.0±0.7*	2.6±0.2
100	26.7±2.9	1.9±0.2	4.8±0.5*	2.7±0.2*
150	21.6±2.3*	2.2±0.1*	4.1±0.6*	2.8±0.2*
200	20.7±3.0 *	2.3±0.2*	3.9±0.5*	2.9±0.2*
400	20.5±3.7*	2.5±0.1*	3.8±0.6*	2.9±0.1*

TAC: Total antioxidant capacity, TOS: Total oxidative stress, GYZ: Guaiazulene, NB: Neuroblastoma, *$P<0.05$

RESULTS

MTT assay, a colorimetric method for determining the number of viable cells in proliferation, was used to examine the inhibitory activities of GYZ on cell proliferation. Cell viability measured by MTT test after 24 h was significantly decreased in neuron, and N2a-NB cells tested at therapeutically relevant GYZ concentrations up to 150 μg/mL [Figure 2].

TAC and TOS analysis, rapid and reliable automated colorimetric assay, was used to determine the oxidative status by GYZ. As shown in Table 1, GYZ caused statistically important decreases in TAC levels at concentrations higher than 100 μg/mL in comparison with control values on rat neuron cell line. Moreover, GYZ caused statistically important increases in TOS levels at concentrations higher than 100 μg/mL in comparison with control values on rat neuron cell line. Also, 75, 100, 150, 200 and 400 μg/mL of GYZ applications caused significant decreases of TAC levels in comparison with control values on N2a-NB cell line. In contrast, the TOS levels increased at 100, 150, 200,

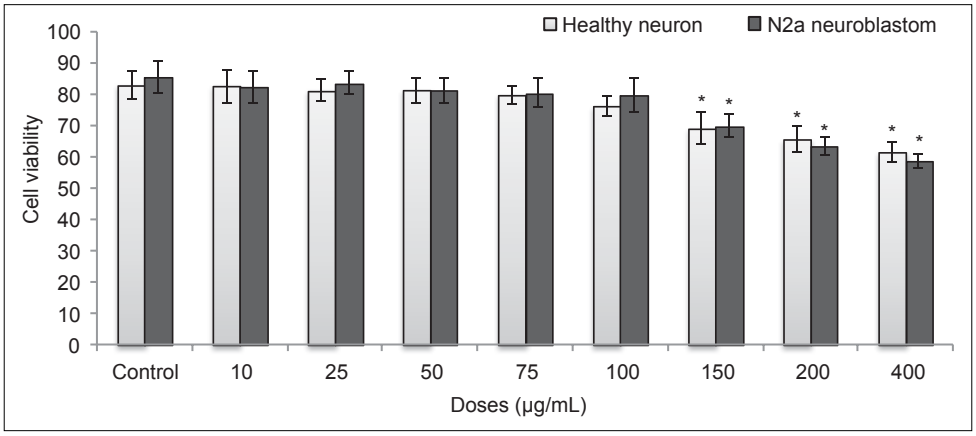

Figure 2: Cytotoxic effect of GYZ on cultured primary rat neurons and N2a NB cells. The results are given as the means ± standard deviation from six independent experiments. Compared with control, *$P < 0.05$

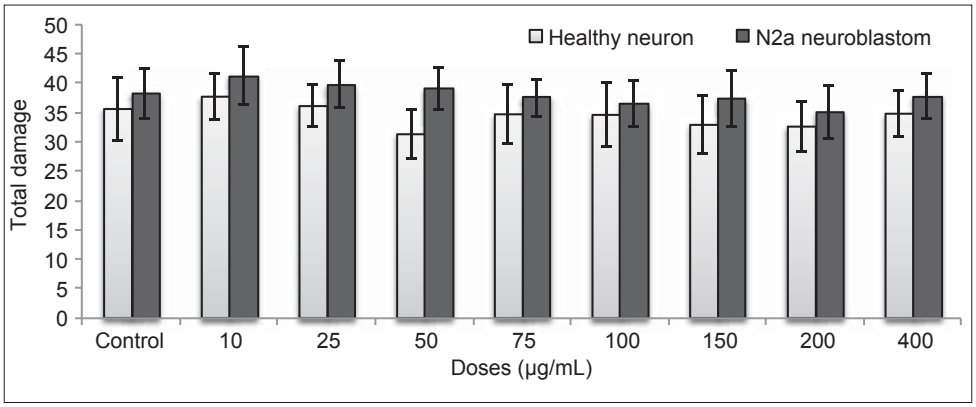

Figure 3: Effect of varying concentrations of GYZ on inducing DNA damage *in vitro* for 24 h. The results are given as the means ± standard deviation from six independent experiments. Compared with control, *$P > 0.05$

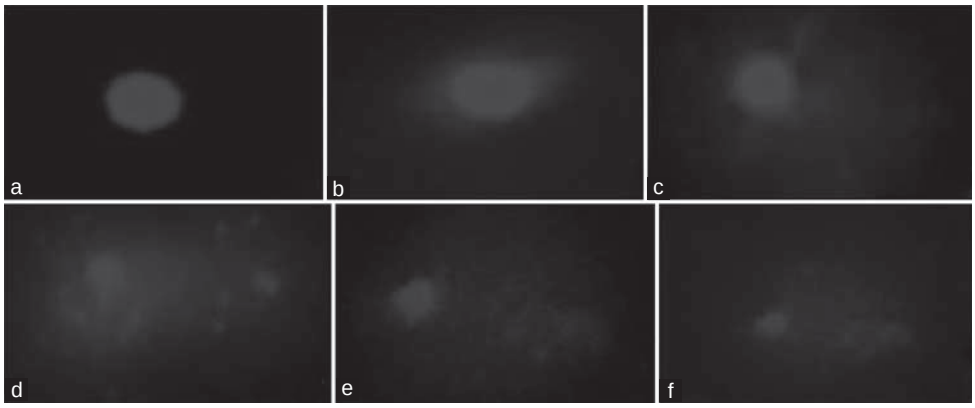

Figure 4: The scoring criteria for determining damage levels in cultured neurons (a) Class 0 (undamaged); (b) Class 1 (slightly damaged); (c) Class 3 (damaged); (d) Class 4 (highly damaged); (e) Class 5 (very highly damaged); (f) Class 6 (extremely damaged)

and 400 μg/mL concentrations of GYZ in cultured N2a-NB cells, respectively.

The induction of DNA damage in rat neuron and N2a-NB cells after exposition to GYZ was investigated using SCGE assay. The obtained results are presented as total damage score in Figure 3. None of the tested GYZ concentrations produced genotoxic effect after 24 h treatments in rat neuron and N2a-NB cells. The scoring criteria for determining DNA damage levels using comet formations in cultured neurons treated with the compounds were shown in Figure 4.

DISCUSSION

This paper describes a comprehensive *in vitro* cytotoxicity, genotoxicity and antioxidant/oxidant capacity assessments of GYZ on rat neuron and N2a-NB cells for the first time. MTT assay, based upon the ability of living cells to reduce MTT into formazan, was used to measure the cytotoxic effect of GYZ on neuron and N2a-NB cells. The results clearly demonstrate that high concentrations of GYZ induced significant cytotoxic effect on the cultured neurons and N2a-NB cell lines. These results conducted to previous studies investigated the cytotoxic effect of GYZ and azulene derivate toward gingival fibroblast, pulp cell, periodontal ligament fibroblast and human tumor cell lines including submandibular gland carcinoma, oral squamous cell carcinoma (HSC-2, HSC-3), promyelocytic leukemia (HL-60 [11,24]. Our findings are also in agreement with the results that have reported that zingiberene (a monocyclic sesquiterpene)exhibited strong antiproliferative effect against N2a-NB cell line by MTT assay [7]. In addition, Turkez *et al*. [8] demonstrated that the copaene (a tricyclic sesquiterpene), showed cytotoxic effect in N2a-NB cells. Oxidative stress is likely to be the common triggers of molecular events underlying its antiproliferative effects. The results of our study indicate that the cytotoxic activity is related to the state of pro-oxidation of GYZ.

In our study, the antioxidant/oxidant effects of GYZ were assessed in this study by the means of TAC and TOS assays. The high concentrations of GYZ caused significant increases ($P < 0.05$)of TOS levels in both cells without changing the TAC levels. In contrast to our findings, the previous studies showed that GYZ has been found to inhibit rat hepatic microsomal membrane peroxidation *in vitro* [25,26]. Similarly, the *in vitro* and *in vivo* effect of GYZ were investigated on rat hepatic cytochrome P450 (CYP)and reported that GYZ inhibited CYP1A2 activity [27]. Furthermore, Vinholes *et al*. [28] reported that GYZ showed higher scavenger capacity against DPPH *in vitro* Caco-2 cell models.

Our findings indicate GYZ is neither genotoxic nor mutagenic on healthy neurons and N2a-NB cells since the observed mean values of the total scores of cells showing DNA damage was not found significantly different from the control values on both cells. *in vitro* or *in vivo* genotoxicity of several sesquiterpenes but not GYZ in different cell types. Therefore, we discussed its genotoxic potential in comparison with other sesquiterpenes. In similar to our findings, Turkez *et al*. [19] reported that the alpha-copaene did not induce genotoxicity in cultured neuron cell lines. In addition, zingiberene did not induce genotoxic damage in cultured neuron and N2a-NB cells [7]. Furthermore, in our recently published paper, using the SCGE assay, we have demonstrated that tricyclic sesquiterpene α-copaene has neither genotoxic nor mutagenic effect neuron and N2a-NB cells [8]. In contrast to the findings, Aquino *et al*. [29] demonstrated that artesunate (a sesquiterpene lactone)induced significant DNA damage in liver cells and high doses of artesunate caused an increase in the mean number of micronucleated polychromatic erythrocytes. Likewise, Orhan *et al*. [30] reported that gossypol showed genotoxic effect in cultured mouse bone marrow cells (as shown by micronuclei index), and human lymphocyte cells (revealed by sister chromatid exchange index). These divergent results suggest the relevance of the chemical structure in the biological effect of sesquiterpenes and indicate as well the importance of using various test models to reach a valid conclusion.

CONCLUSION

In conclusion, our *in vitro* studies suggest that high concentrations of GYZ could be cytotoxic on both cells. The

efficacy of anti-cancer chemotherapy is limited by the cytotoxic effect on healthy neuron cells because of a lack of selectivity of GYZ and poor uptake of the therapeutics by N2a-NB cells.

ACKNOWLEDGMENTS

This work was supported by the Scientific and Technological Research Council of Turkey (TUBİTAK, Project Number 210T142).

REFERENCES

1. Wojtalla A, Salm F, Christiansen DG, Cremona T, Cwiek P, Shalaby T, *et al*. Novel agents targeting the IGF-1R/PI3K pathway impair cell proliferation and survival in subsets of medulloblastoma and neuroblastoma. PLoS One 2012;7:e47109.
2. Mueller WP, Coppenrath E, Pfluger T. Nuclear medicine and multimodality imaging of pediatric neuroblastoma. Pediatr Radiol 2013;43:418-27.
3. Peri A, Cellai I, Benvenuti S, Luciani P, Baglioni S, Serio M. PPARgamma in Neuroblastoma. PPAR Res 2008;2008:917815.
4. Peirce SK, Findley HW. High level MycN expression in non-MYCN amplified neuroblastoma is induced by the combination treatment nutlin-3 and doxorubicin and enhances chemosensitivity. Oncol Rep 2009;22:1443-9.
5. Li Z, Thiele CJ. Targeting Akt to increase the sensitivity of neuroblastoma to chemotherapy: lessons learned from the brain-derived neurotrophic factor/TrkB signal transduction pathway. Expert Opin Ther Targets 2007;11:1611-21.
6. Svensson K, Larsson C. A protein kinase Cbeta inhibitor attenuates multidrug resistance of neuroblastoma cells. BMC Cancer 2003;3:10.
7. Togar B, Turkez H, Tatar A, Hacimuftuoglu A, Geyikoglu F. Cytotoxicity and genotoxicity of zingiberene on different neuron cell lines *in vitro*. Cytotechnology 2014.
8. Turkez H, Togar B, Tatar A, Geyikoglu F, Hacimuftuoglu A. Cytotoxic and cytogenetic effects of α-copaene on rat neuron and N2a neuroblastoma cell lines. Biologia 2014;69:936-42.
9. Nikolić B, Mitić-Ćulafić D, Stajković-Srbinović O, Branka Vuković-Gačić B, Knežević-Vukčević J. Effect of metabolic transformation of monoterpenes on antimutagenic potential in bacterial tests. Arch Biol Sci Belgrad 2012;64:885-94.
10. O' Neal MJ, Heckelman PE, Kock CB, Roman KJ. The Merck Index. 14th ed. Whitehouse Station, NJ: Merck Research Laboratories; 2006. p. 786.
11. Fiori J, Teti G, Gotti R, Mazzotti G, Falconi M. Cytotoxic activity of guaiazulene on gingival fibroblasts and the influence of light exposure on guaiazulene-induced cell death. Toxicol In Vitro 2011;25:64-72.
12. Higa T, Sakemi S. Guaiazulene derivatives and their methods of use. US Patent No. US 4755529 A; 1988. p. 5-7.
13. Yanagisawa T, Kosakai K, Tomiyama T, Yasunami M, Takase K. Studies on anti-ulcer agents. II. Synthesis and anti-ulcer activities of 6-isopropylazulene-1-sodium sulfonate derivatives. Chem Pharm Bull (Tokyo)1990;38:3355-8.
14. Andersen FA. Thirty-seventh report of the cosmetic ingredient review expert panel on the safety assessment of cosmetic ingredients. Int J Toxicol 1999;18:59.
15. Tanaka M, Yamaki F, Saitoh M, Nakazawa T, Tanaka H, Noguchi K, et al. Relaxant action of azulene-carboxamidine derivative N1, N1-dimethyl-N2-(2-pyridilmethyl)-5-isopropyl-3,8-dimethylazulene-1-carboxamidine (HNS-32)in pig coronary artery. Pharm Pharmacol Commun 2000;6:397-404.
16. Guarrera M, Turbino L, Rebora A. The anti-inflammatory activity of azulene. J Eur Acad Dermatol Venereol 2001;15:486-7.
17. Fiori J, Gotti R, Albini A, Cavrini V. Study on the photostability of guaiazulene by high-performance liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom 2008;22:2698-706.
18. Sleha R, Mosio P, Vydrzalova M, Jantovska A, Bostikova V, Mazurova J. *In vitro* antimicrobial activities of cinnamon bark oil, anethole, carvacrol, eugenol and guaiazulene against *Mycoplasma hominis* clinical isolates. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2014;158:208-11.
19. Turkez H, Togar B, Tatar A. Tricyclic sesquiterpene alpha-copaene prevents H_2O_2-induced neurotoxicity. J Intercult Ethnopharmacol 2014;3:21-8.
20. Si CL, Shen T, Jiang YY, Wu L, Yu GJ, Ren XD, *et al*. Antioxidant properties and neuroprotective effects of isocampneoside II on hydrogen peroxide-induced oxidative injury in PC12 cells. Food Chem Toxicol 2013;59:145-52.
21. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005;38:1103-11.
22. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 2004;37:277-85.
23. Perovic A, Perovic S, Erdinger L, Hollert H. Assessment of the genotoxic potential of Lake Skadar sediments using Ames test and comet assay on the fish cell line rtl-w1. Arch Biol Sci Belgrad 2012;64:249-56.
24. Wakabayashi H, Hashiba K, Yokoyama K, Hashimoto K, Kikuchi H, Nishikawa H, *et al*. Cytotoxic activity of azulenes against human oral tumor cell lines. Anticancer Res 2003;23:4747-55.
25. Kourounakis AP, Rekka EA, Kourounakis PN. Antioxidant activity of guaiazulene and protection against paracetamol hepatotoxicity in rats. J Pharm Pharmacol 1997;49:938-42.
26. Dovolou E, Clemente M, Amiridis GS, Messinis IE, Kallitsaris A, Gutierrez-Adan A, *et al*. Effects of guaiazulene on *in vitro* bovine embryo production and on mRNA transcripts related to embryo quality. Reprod Domest Anim 2011;46:862-9.
27. Kourounakis AP, Rekka EA, Kourounakis PN. Effect of guaiazulene on some cytochrome P450 activities. Implication in the metabolic activation and hepatotoxicity of paracetamol. Arch Pharm (Weinheim) 1997;330:7-11.
28. Vinholes J, Goncalves P, Martel F, Coimbra MA, Rocha SM. Assessment of the antioxidant and antiproliferative effects of sesquiterpenic compounds in *in vitro* Caco-2 cell models. Food Chem 2014;156:204-11.
29. Aquino I, Perazzo FF, Maistro EL. Genotoxicity assessment of the antimalarial compound artesunate in somatic cells of mice. Food Chem Toxicol 2011;49:1335-9.
30. Orhan G, Ekmekci A, Menevse S. The effect of the male contraceptive agent gossypol acetic acid on mouse bone marrow cells *in vivo*: micronuclei and mitotic index. Contraception 1993;47:377-85.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141202060743

Original Research

ScopeMed

Ethnobotany, traditional knowledge and socioeconomic importance of native drink among the Oraon tribe of Malda district in India

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Received: October 11, 2014
Accepted: December 02, 2014
Published: January 03, 2015

ABSTRACT

Aim: Preparation of daily traditional drink by the indigenous tribes is a common phenomenon in India. Oraon tribes in Malda district of West Bengal, India are very much practiced in making of their own native brew, known as Chullu. Therefore, the aim of this study was to explore the whole Chullu procedure technology of the region and its socioeconomic effect on Oraon. Ethnomedicinal investigation of local plants involved in Chullu preparation was another aspect of this study. **Materials and Methods:** The present study was conducted from April 2012 to June 2013. Consecutive field surveys were performed to collect information from Chullu producers to focus the procedure technology of local brew by means of semi-structured individual interviews, informal interviews and group discussion. A semi-structured questionnaire process was also performed to obtain the information regarding the ethnic use of plant species involved in Chullu preparation. **Results:** The present study revealed that four medicinal plant species along with rice having strong local ethnomedicinal value were used to prepare this indigenous drink. Oraon prepare the brew using their unique home-made distillation process. Commercialization of this local brew represents an alternative income to develop their economic condition, especially for poor households. The index of importance value was considered to evaluate the importance, usage, and knowledge of the five studied species. **Conclusion:** It could be concluded that practices of Chullu preparation represent a bonding between ethnic knowledge and Oraon people of the province. Commercialization of Chullu may be considered as a source of alternative way of income for poor households in the region.

KEYWORDS: Alternative income, Chullu, ethnobotany, Oraon, Malda district

INTRODUCTION

Traditional liquor preparation is a common household practice among different tribal communities in India. They consume native drink (e.g., sajpani, kiad etc.) virtually in every occasion of life such as various festivals, marriage ceremony and even in funeral [1]. Almost all tribes prepare and consume traditional drink but their names, ingredients, and mode of preparation differ greatly from community to community and region to region. Rice is the main ingredient preparing these native drinks or liquors. However, the tribals use local medicinal plant parts along with rice to prepare their native liquor and believe that the beverage prepared in this process prevents headache, insomnia, body ache, urinary troubles and cholera [2-4]. The North-eastern provinces of India, colonized by native Bodo, Garo, Rabha, Karbi, Ahom, Deori, Dimasa, Kachari, etc. communities are best known for the production of household liquors [4,5]. However, there is virtually no data available regarding the traditional alcoholic beverage preparation in the sub-Himalayan non-hilly region of Bengal.

Malda, district of West Bengal in India, is characterized by its diversified historical dynasty, mango production, rich wetland, natural vegetation and its diversified ethnic groups including Santala, Oraon, Rajbanshi, Namasudre, Polia, Mundas, Malpaharias etc. [6] Amongst these tribes, Oraon community are famous in preparing quality local drink or known as Chullu from rice. In fact Chullu production is a part of their culture, identity, myths, and spiritual practices. Their own traditional way of preparing the brew with medicinally important plants distinguished them from rest of the tribal communities of the region. The recipe of brew preparation is however a secret and passed on generation after generation orally. Besides, they also make a good living by selling this native drink.

So far there is no authentic documentation of Chullu preparation technology used by Oraons' of Malda district of West Bengal, India. The plants those were used in preparing Chullu, either known medicinally or have ethnic use among Oraons'. Therefore, a semi-structured questionnaire process was also intended to focus the local use of the plants, their parts involved in medicinal practices and preparation of drugs from those plant parts. Another aspect which was considered in this study was the economic significance of Chullu production and sale. Hence, it may be a pioneer study to explore the Chullu procedure technology and its economic impact among Oraon community of the district along with ethnomedicinal value of used plant species.

MATERIALS AND METHODS

Study Area

Malda (latitude and longitude of 24°40′20″N to 25°32′08″N and 88°28′10″E to 87°45′50″E respectively), a district of West Bengal, India with a total land area of 3455.66 km^2. [6] is known as '*mango district*' for its wide array of mango variety and production. It consists of two municipalities, 15 blocks or sub-divisions and 3701 villages with a population of more than forty lakh. The district is also characterized by its diversified wetland and forest vegetation. The Adh soi wetland (beel), located at Harischandrapur-II block of the region, is one of the largest among the wetlands of the state comprising rich vegetation due to its macrophytic diversity [7]. Adina and Bhalluka forests are the two most important forest areas of the district. A few small forest areas are also scattered in Old Malda, Habibpur, Harishchandrapur and in Gajol blocks [8].

Most of ethnic communities live mainly in the four blocks of this province namely, Gazole, Bamongola, Habibpur and Old Malda comprising more than 85% of total tribal population. Hence we considered these four blocks as our study area [Figure 1] due to its noticeable Oraon population. The climate of this region is extreme because of its geographical position. The district has a hot summer (35-42°C) from March to September and a very cold winter (6-12°C) from November to February. The monsoon starts from June and continues to the mid-September and the average rainfall is approximately 1453.1 mm.

Oraon Community

Oraon community is one of the largest tribal groups in India, possessing a unique tradition and culture. In Malda district, a sizeable portion of the tribal communities are the Oraons who mostly inhabits in the remote villages [9]. They have distinctive lifestyle and are fond of festivals of various kinds like Jatrapala (one kind of play portraying colorful stories), Gambhira (a kind of play portraying the social satire, political circumstances or the life story of god and goddesses), folk songs and dances, traditional musical instruments etc. Consumption of Chullu prepared mainly from rice is common for Oraons during these occasions. Besides, they regularly consume this drink during marriage ceremony, birth of a child or even in funeral. In fact,

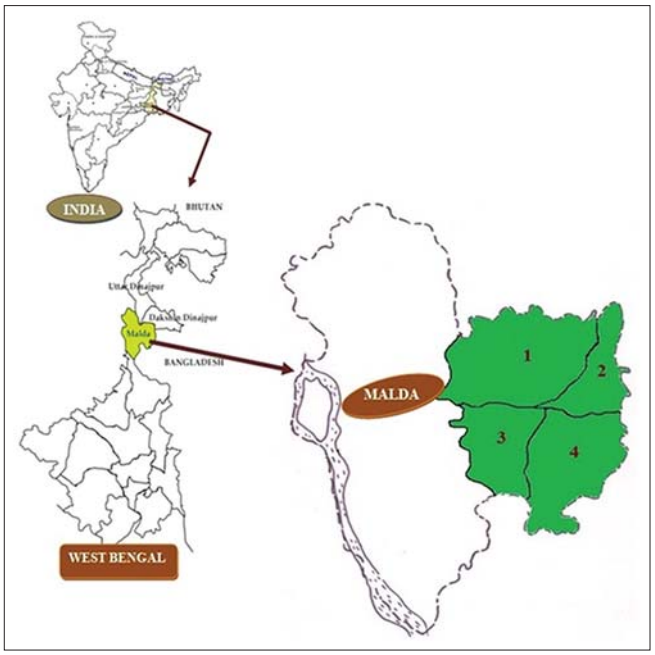


Figure 1: The map of study area (Malda district) showing four main Oraon populated zones (1: Gazole block; 2: Bamongola block; 3: Old Maldah block; 4: Habibpur block)

a section of the community makes a good living by preparing Chullu.

Data Collection

Prior to survey, several meetings were held with the community members to explain the purposes of the study being conducted and to obtain their prior informed consent (PIC). The survey was carried out among the 19 villages (Chaknagar, Haspukur, Rishipur, Bhabuk, Parameshpur, Dhumpur, Nityanandapur, Aktail, Dangapara, Lakhitur, Habibpur, Jhinjhinipukur, Pakuahut, Jagdala, Kanturka, Kenpukur, Salaidanga, Majhra, Baidyapur) of above mentioned four blocks (Gazole, Bamongola, Habibpur and Old Malda) during last year (April 2012 to June 2013). Hundreds of informants were interviewed to get the information regarding the Chullu preparation and plants and plant-parts involved therein. The sociocultural and marketing value of this drink has also been considered. The whole survey procedure involved several levels of interviewing such as semi-structured individual interviews, informal interviews, open-ended questionnaires, and group discussion with the local informants.

Total Key Informants

Hundreds of villagers of the study area were interviewed, but the information given by professional Chullu producers and persons with proven knowledge on plants involved in Chullu production were only recorded. After cross verification, the information obtained only from 201 Chullu producers based on their experiences (174 female, 27 male) of 19 villages were recorded. Among the ethnomedicinal practitioners, we found 27 healers who were involved in traditional healing practices,

gave information regarding the ethnic use of the same plants involved in Chullu production for treating ailments. To survey the economic aspect of the Chullu production, authors also interviewed 56 vendors of 19 villages.

Data Analysis

In order to evaluate the importance of the medicinal plants as per the local informants of the villages, the value of importance (IVs) index was determined [10]. IVs index measures the importance of a plant species based on how many informants cite one species as the most important one among the total number of informants (Value varies from 0 to 1). IVs= nis/n; where, nis= number of informants who consider the species to be the most important, and n= total number of informants.

RESULTS

After interviewing with 201 Chullu producers, the local beverage procedure technology by Oraon people was summarized under two main sub-legends: preparation of Chullu -starter and preparation of Chullu. It was observed that Chullu production which has great impact on Oraon people, indirectly help to uplift the village economy. Virtually, commercialization of Chullu is an alternative way of their daily livelihood. A total of four medicinal plants including *Holarrhena pubescens*, *Wattakaka volubilis*, *Ichnocarpus frutescens* and *Clerodendrum viscosum* along with rice (*Oryza sativa*) which were used in the preparation of this drink had great ethnomedicinal value in the locality.

Chullu Procedure Technology

Preparation of Chullu-starter

To prepare Chullu-starter (locally known as *modguli*), rice grains and 4 different plant parts are mixed together in a 2:1 ratios and dusted. Briefly, rice grains are taken in earthen pot and cleaned in water, followed by drying under sunlight for 1-2 days. Different plant parts like bark and leaves of *H.* pubescens, fruit and bark of *W. volubilis*, leaves of *I. frutescens* and *C. viscosum* are also cleaned well to remove dust particles and dried. Then, the plant parts along with rice grains are powdered by *dheki* (a wooden mortar with a large wooden handle). The powdered material is then sieved. The sieved material is locally known as *modgura*. Water (1/3rd of the total powder) is added to modgura to make dough and thick tablet like structures of 5-8 cm. in diameter are prepared [Figure 2]. These tablets are called modguli. The *modgulis* are kept in between two layers of straw for 4-5 days or until the pungent smell comes (the process is known as jag-dewa). Now, the starters are prepared for sundry. These modgulis are kept on clean cloth under sunlight for another 7-10 days. Finally, the sundried Chullu-starters are packaged depending upon their sizes for marketing [Figure 2].

Preparation of Chullu

To prepare Chullu, cooked rice is the main ingredient. Briefly, starters (four to five pieces for 1 kg of cooked rice) are dusted

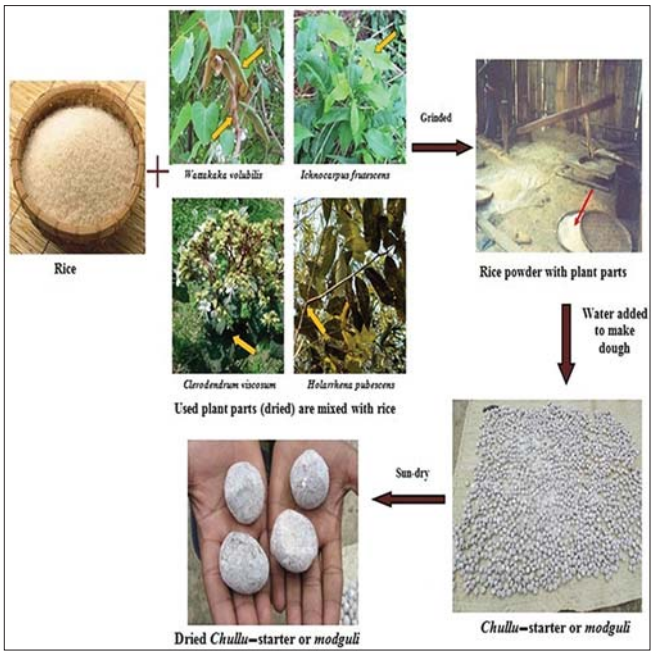


Figure 2: Preparation of Chullu-starter

and mixed properly with cooked rice and taken in an earthen pot (handi). Water (400-500 ml) is added into the pot covering the mouth with a banana leaf, followed by an earthen lid and left for fermentation. A yellowish watery juice with a strong alcoholic pungent smell comes out after 3-4 days, which is filtered with a clean cloth into another pot. This first fermented yellowish beverage is known as *hanria*. Fresh water (2-2.5 lit) is added in the same earthen pot containing fermented rice and *hanria* and left for 12-18 h [Figure 3].

Now to prepare typical Chullu, Oraon follow their unique distillation process. In this preparation, three pots are piled one above the other. The lowest pot containing the fermented rice with hanria, the middle one being an empty earthen pot with several pores (known as *jhanjhi*) at the bottom and the topmost aluminum pot filled with cold water. The air gaps between each pot are sealed with mud. A pipe is inserted and sealed with mud at the side bottom of the middle earthen pot. The entire preparatory set is then placed over earthen oven starting the heating process [Figure 3]. After heating, the vapor goes up from the lower-most pot passes through the pores of the middle pot and comes in contact with upper-most pot containing cold water. Due to cooling, the vapor condenses into water which comes down and is collected in the bottle through the pipe of the middle pot. Thus, the prepared beverage is watery in color possessing alcoholic odor and is known as Chullu or mod. Finally, this alcoholic beverage is packaged in glass-bottle for selling in the market.

Ethnomedicinal Uses of Plants Involved in Chullu

During survey, we found that the plants used in Chullu preparation had great local medicinal values. Therefore, a semi-structured questionnaire and individual interview [Figure 4] among the healers had been carried out among 19

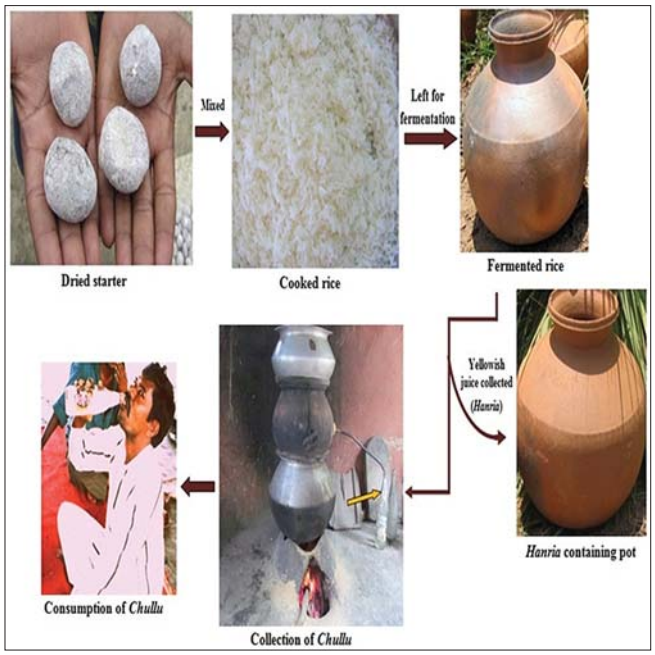
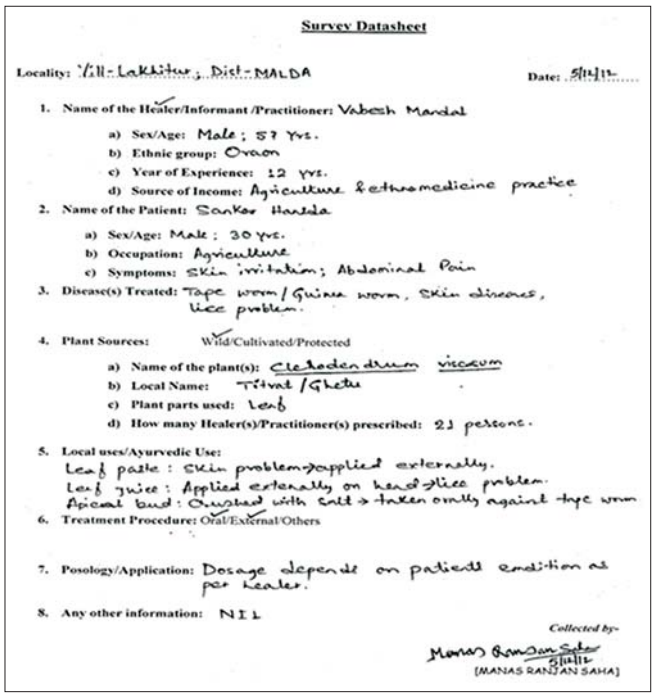


Figure 3: Preparation of Chullu



Survey Datasheet

Locality: Vill - Lakhitur ; Dist - MALDA Date: 5/11/12

1. Name of the Healer/Informant /Practitioner: Vabesh Mandal
   a) Sex/Age: Male ; 57 Yrs.
   b) Ethnic group: Oraon
   c) Year of Experience: 12 yrs.
   d) Source of Income: Agriculture & ethnomedicine practice
2. Name of the Patient: Sankar Hansda
   a) Sex/Age: Male ; 30 yrs.
   b) Occupation: Agriculture
   c) Symptoms: Skin irritation; Abdominal Pain
3. Disease(s) Treated: Tape worm / Guinea worm, Skin disease, lice problem.
4. Plant Sources: Wild/Cultivated/Protected
   a) Name of the plant(s): Clerodendrum viscosum
   b) Local Name: Tivat / Ghetu
   c) Plant parts used: Leaf
   d) How many Healer(s)/Practitioner(s) prescribed: 21 persons.
5. Local uses/Ayurvedic Use:
   Leaf paste : Skin problem→applied externally.
   Leaf juice : Applied externally on head lice problem.
   Apical bud : Crushed with salt → taken orally against tape worm
6. Treatment Procedure: Oral/External/Others
7. Posology/Application: Dosage depends on patient's condition as per healer.
8. Any other information: NIL

Collected by-
[MANAS RANJAN SAHA]

Figure 4: Questionnaire datasheet of ethnomedicinal use of plants in studied region

villages to obtain the information. After interviewing with some experienced traditional healers authors found a massive ethnomedicinal value in the region. However, as per our title concerned, we focused only on the above mentioned five plant species [Table 1] as those are used in Chullu preparation. A total of 27 traditional experienced healers were chosen after cross verification for obtaining the information regarding ethnic use of those species only.

It was observed that the herbal formulation from the bark of *H. pubescens* was prescribed by 22 healers out of 27 to treat chronic diarrhoea, chronic dysentery, urinary troubles, bleeding of piles etc. whereas *C. viscosum* was prescribed by 21 healers to treat several disorders. Similarly, *W. volubilis*, *I. frutescens*, *O. sativa* were also used in various purposes as shown in Table 1. The IVs result exhibited high IV for all the species [Table 1] establishing greater ethnic knowledge regarding plant resources in the studied area. However, the IVs value of *O. sativa* is low (0.49) in comparison to others and prescribed only by only 13 healers. The study attempts to highlight that the above mentioned five plants are most valued species in the studied region and if sustainably harvested, they could be used as an alternative livelihood strategy for poor people.

DISCUSSION

Chullu and Village Economy

The local traditional liquor, Chullu occupies a sizable portion of village economy, especially the economy of poor tribal people. The Oraon community is actively involved in production and marketing of Chullu. Though the Oraons are involved in Chullu production, sometimes non-tribal agents provide funds to the tribals to produce liquor in a large scale and collect from them to be sold in the urban areas. The starters are processed into two different packets depending upon their sizes and sold at market. Similarly, traditional Chullu is packaged in bottles to sell at beer-shop. It was found that the large packets (5-7 cm diameter each) of starter are sold @ 15 or USD 0.24 of per packet containing 8 pieces whereas small packets (2-3 cm diameter each) are sold @ 8 or USD 0.13 of per packet with 8 pieces. Hanria, the first alcoholic product during preparation of Chullu is also sold @ 5-7 or USD 0.08 to 0.11 per glass of 100 ml whereas the typical Chullu is sold @ 30 or USD 0.48 per bottle of 550 ml.

During survey, we found that though the tribals consume Chullu throughout the year, production of this drink usually at its peak in dry season like summer mainly because drying of starter is relatively easy in dry season. It was also observed that commercialization of Chullu occurred when a groups of villagers from different parts gathered in local fairs, ritual ceremonies, Jatrapalas, folk songs or dance programs or in other social activities. The other means of selling occurred when someone or a group of villagers go to the urban areas due to their personal purposes carry the native drink and sell those during their stay in urban areas. Sales also occur through the agents who directly purchase the indigenous brew. As evident from Table 2, inhabitants of Haspukur, Baidyapur, Kanturka, Dhumpur, Lakhitur, Pakuahat, Bhabuk and Habibpur villages amongst 19 are more interested than others in preparing the local brew which indirectly helps to boost up their economic condition. However, the frequency of alcohol preparation in the villages was more than 65 percent suggesting high concern to prepare the traditional liquor. Excess amount of local brew are

Table 1: List of medicinal plants investigated for Chullu preparation and their respective ethnic uses

Plant species/family/Vn	Used parts	Ethnomedicinal use	Formulation prescribed by no. of healers	IVs
H. pubescens (Buch.-Ham.) Wall. ex DC. (Apocynaceae)/ Vn Koriya/Indrajab	Bark	The bark is grinded to make powder and taken orally with milk to cure from chronic diarrhea, chronic dysentery, urinary troubles and bleeding of piles. The seeds are used as anthelmintic	22	0.81
W. volubilis (Linn.f.) Stapf. (Asclepiadaceae)/Vn Muniraj/ Barka Dabai	Leaves and roots	i) Leaves are crushed to make paste and used externally on eczema, boils and abscesses ii) Leaves are grinded to make powder and taken orally along with cow's milk in body weakness iii) Leaves are used as sex stimulant along with the healers' own ingredients iv) Roots are utilized in case of fever and jaundice	18	0.66
O. sativa L. (Poaceae)/Vn Dhan	Grain	The grains (*chal*) are soaked in water for whole night and next morning the decoction is taken orally to treat gastric problems	13	0.49
I. frutescens (L.) W.T. Aiton. (Apocynaceae)/Vn Kathmol	Root	i) Roots are used in leucorrhoea, skin diseases ii) Root decoction is used in fever and cough	19	0.70
C. viscosum Vent. (Verbenaceae)/Vn Titvat	Leaf	i) The apical bud are crushed with salt and take to prevent worm (tapeworm or guinea worm) infection and also used in liver disorders ii) Leaf paste is applied externally to prevent skin problems The leaf juice is applied on head to prevent lice	21	0.78

Vn: Vernacular name, *C. viscosum: Clerodendrum viscosum, I. frutescens: Ichnocarpus frutescens, O. sativa: Oryza sativa, W. volubilis: Wattakaka volubilis*, IV: Importance value

Table 2: Frequency of Chullu preparation in studied villages

Name of studied villages	Total no. of population studied[#]	Total number of persons preparing alcohol in village	Frequency of Chullu preparation by local people (%)
Chaknagar	345	269	77.97
Haspukur	460	375	81.52
Parameshpur	439	349	79.49
Baidyapur	521	439	84.26
Jhinjhinipukur	296	203	68.58
Aktail	345	268	77.68
Nityanandapur	211	153	72.51
Kanturka	362	301	83.14
Rishipur	209	164	78.46
Srirampur	358	277	77.37
Dhumpur	421	355	84.32
Lakhitur	379	330	87.07
Jagdala	265	211	79.62
Dangapara	119	86	72.26
Pakuahat	451	368	81.59
Salaidanga	223	162	72.64
Majhra	195	142	72.82
Bhabuk	385	324	84.15
Habibpur	535	450	84.11

[#]Total population studied=(Number of Chullu producing person+No. of non-Chullu producing person)

also produced in those areas as per the demand of agents. Hence, commercialization of local drink would be a good alternative way to the ethnic people if properly manufactured.

In selling local traditional brew, the most critical factors are the lack of proper infrastructure, proper management, communication, transportation, local market or beer-shops etc. It has been observed that some of the villages are in such remote areas that the transportation facilities are inaccessible. Therefore it becomes difficult to reach to the desired places or sometimes become detached due to some natural calamities. As a result the transportation charges become more than the production cost of Chullu. Seasonality, especially rainy season is also a great factor. Due to the presence of heavy moisture in the environment and/or inadequate sunlight, the starters don't get dried up well leading to fungal contamination. As a result, the drink prepared with those half-dried starters becomes toxicated or sometimes fatal.

Transmission of Knowledge

The knowledge of the wild medicinal plants used in various purposes are based on regular practices, oral transmission and are also influenced by several factors such as age, gender, relationship and other sociocultural factors generating variability in a particular zone [11]. The privacy of traditional medical practices in the indigenous people is a common phenomenon [12]. The use of selected plant species in Chullu preparation distinguishes the Oraon people from other communities in the studied region and it confirms that the knowledge is confined within this community. The informants reported that they always keep their medicinal plant knowledge secret. The open transfer of indigenous knowledge could only take place verbally along the family line, usually from older knowledgeable person to younger ones. The transfer of knowledge takes place hardly to the people outside the family and passed only on substantial cash payment.

CONCLUSION

Through the present survey we intended to have a detailed account of local drink production in selected regions of rural Bengal. Rural Malda district to be precise, we have also given special emphasis to the tribal community and their way of preparing local drink, Chullu. Oraon economy involved typical method of Chullu production which is indigenous to their community. We found that Chullu production, trade and marketing are a popular occupation among Oraon communities.

Any kind of traditional alcoholic drink is popular among the tribal communities and the non-tribals because of its cheap cost and high alcohol content. The tribal communities use native brew virtually in every occasion, from birth to funeral. They used several herbs and shrubs for the preparation of local drink. Some of which are ethno-botanically important. Oraons have a popular belief that the plant parts used in Chullu actually help them in combating against various ailments such as headache, insomnia etc.

Overall, it is apparent that the present survey is some of the most comprehensive one on Chullu production and marketing in Bengal and certainly the most exhaustive for rural Malda district of West Bengal.

ACKNOWLEDGMENT

The authors wish to thank the Oraon practitioners and beverage makers, especially Vabesh Mandal (57 years) of Lakhitur village, Lakhsmi Begam (36 years) of Haspukur, Kashiram Mandal (62 years) of Bhabuk, etc. who shared their knowledge in the interrogation process directly. The authors also like to express their gratitude to field assistants for their constant support. The field work study for this research was supported by the Department of Science and Technology (DST), West Bengal Government, India and UGC for the fellowship.

REFERENCES

1. Saikia B, Tag H, Das AK. Ethnobotany of foods and beverages among the rural farmers of Tai Ahom of North Lakhimpur district, Assam. Indian J Tradit Knowl 2007;6:126-32.
2. Singh PK, Singh KI. Traditional alcoholic beverage, Yu of Meitei communities of Manipur. Indian J Tradit Knowl 2006;5:184-90.
3. Samati H, Begum SS. Kiad, a popular local liquor of Pnar tribe of Jaintia hills district, Meghalaya. Indian J Tradit Knowl 2007;6:133-5.
4. Deka D, Sarma GC. Traditionally used herbs in the preparation of rice-beer by the Rabha tribe of Gopalpara district, Assam. Indian J Tradit Knowl 2010;9:459-62.
5. Das AJ, Deka SC, Miyaji T. Methodology of rice beer preparation and various plant materials used in starter culture preparation by some tribal communities of North-East India: A survey. Int Food Res J 2012;19:101-7.
6. Saha MR, Sarker D De, Kar P, Sen Gupta P, Sen A. Indigenous knowledge of plants in local healthcare management practices by tribal people of Malda district, India. J Intercult Ethnopharmacol 2014;3:179-85.
7. Chowdhury M, Das AP. Macrophytic diversity and community structure of *Adh Soi* wetland of Maldah district of Paschimbanga, India. In: Ghosh C, Das AP, editors. Recent Studies in Biodiversity and Traditional Knowledge in India. Malda, India: Gour College; 2011. p. 109-15.
8. Mitra S, Mukherjee SK. Some less known plants from Malda district of West Bengal used for the treatment of arthritis, rheumatism and gout. Int J Pharma Res Biosci 2013;2:337-45.
9. Roy SK. Tribes Education and Gender Question. New Delhi: Northern Book Centre; 2005. p. 30-4.
10. Byg A, Baslev H. Diversity and use of palms in Zahamena, eastern Madagascar. Biodivers Conserv 2001;10:951-70.
11. Lozada M, Ladio A, Weigandt M. Cultural transmission of ethnobotanical knowledge in a rural community of Northwestern Patagonia, Argentina. Econ Bot 2006;60:374-85.
12. Giday M, Asfaw Z, Elmqvist T, Woldu Z. An ethnobotanical study of medicinal plants used by the Zay people in Ethiopia. J Ethnopharmacol 2003;85:43-52.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141020064838

Original Research

ScopeMed

Antioxidant activity and phytochemical constituent of two plants used to manage foot and mouth disease in the Far North Region of Cameroon

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Received: September 18, 2014
Accepted: October 20, 2014
Published: November 28, 2014

ABSTRACT

Aim: Plants used in the Far North Region of Cameroon by livestock farmers to manage foot and mouth disease (FMD) in cattle and the phytochemical composition and antioxidant potentials of two of them (*Boscia senegalensis* [BS] and *Tapinanthus dodoneifolius* [TD]) were investigated in this study. **Materials and Methods:** Ethno veterinary data were collected from 325 livestock farmers using semi-structured interviews from September 2011 to April 2012. The 2,2-diphenyl-picrylhydrazyl radical scavenging activity and total phenolic content (TPC) were first performed with five different solvents to choose the best extract of each plant based on these two factors. To achieve our aim, the ferric iron reducing activity, hydroxyl radical scavenging activity (HRSA), free radical scavenging activity (FRSA), vitamin E and iron content were analyzed on extracts selected using current techniques. **Results:** The results showed that 12 plants of 8 different families are regularly used by farmers to manage FMD. It also demonstrated that acetone extract of TD and methanolic extract of BS are the extracts which showed the best total antioxidant activity (AA) and the best TPC. In general, TD show the best AA during the HRSA and FRSA analysis compared with BS. Similarly, TD content more phenolic compounds and tannins than BS. Both plants contain proteins, saponins, tannins, phenols, alkaloid, and polyphenols which are known to have many biological activities. **Conclusion:** These results support the AA of both plants and can justify their use by herders to treat FMD which is often followed by many secondary diseases.

KEY WORDS: Antioxidant activity, *Boscia senegalensis*, foot and mouth disease, *Tapinanthus dodoneifolius*

INTRODUCTION

In the Far North Region of Cameroon where the rate of extreme poverty is the highest in the country (41%), livestock production is one of the main activities and represents the second highest source of cash income to the rural populations after cotton [1,2]. In this region where breeding is a tradition for many native ethnic groups, infectious animal diseases including foot and mouth disease (FMD), constitute one of the mainhindrances to cattle productivity and production [3]. To treat this disease and to improve their yield, 69% of farmers use a variety of veterinary pharmaceutical medications and 25% of them use traditional medicine [4]. Herders who use veterinary drugs usually misuse them [4] and this would bring many consequences for animals and consumers [5,6].

Instead, the plants already very accessible, even used at uncertain doses are generally effective, easy to prepare and to administer, non-toxic, and little or no cost to herders [7]. They constitute a good alternative for the treatment of animals in this area. Indeed, in many countries in Africa, herbal medicines are more and more used to treat animal diseases [9,10]. Ethnobotanical documentation of medicinal plant used is generally an appropriate means of identifying potential sources of new drugs. Unfortunately, in this region of Cameroon, according to available literature, there is not yet information concerning the use of traditional medicine in animal clinics.

The aim of this study was to investigate the plants used for the treatment of FMD in cattle by livestock farmers in the Far

North Region of Cameroon and to evaluate the phytochemical composition and antioxidant potentials of both of them (*Boscia senegalensis* [BS] Lam. and *Tapinanthus dodoneifolius* [TD] [DC.] Danser).

MATERIALS AND METHODS

Study Area

A survey was conducted in the Far North Region of Cameroon [Figure 1] from September 2011 to April 2012 as previous described [4]. The region lies between 9° and 13° north and 13° and 16° east with a total area of approximately 34,263 km^2 [10,11]. The mean annual rainfall is 700 mm and the temperature ranges between 27 and 41°C [12]. This area has a semi-arid climate with a single rainy season and one dry season of 8 months [9]. Two phytogeographic zones characterize the region: Sudanian in the southern grades and Sahelian in the Logon floodplain [13]. The plants mentioned during the survey by famers were directly collected with their help and then identified by M. Hamawa, a botanist in the University of Maroua. Phytochemical screening of all the plants collected were done and a literature were found to select the best plants to study further.

Plant Material

Plant materials used in this study were collected in June 2013 from Petté (leaves of BS) and Tokombéré (twigs of TD) districts in the Diamaré and Mayo Sava Divisions respectively in Far North Region of Cameroon. TD were collected from host plant *Acacia albida*. These plants were then identified and authenticated in the National Herbarium in Yaoundé were the Voucher specimens already existed under the reference numbers 23137 SRF/Cam and 50271 HNC respectively for BS and TD. The samples were collected in the morning (around 7 am), cleaned with tap water and air dried under a shade before grinding it to powder using a desk top mill fitted with a 500 μm sieve. The dried samples were then pulverized in a mill. The powder obtained was then sieved using a sieve of mesh 500 μ in diameter. The obtained powders were then put in polyethylene plastic, sealed and stored at 4°C in a refrigerator until when they were needed for analysis. The first phytochemical screening for choosing the plants for this study was done in the Veterinary Diagnostic Laboratory (Iowa State University, US), while the remainder analysis were performed in the National Advanced School of Agro-industrial Sciences (University of Ngaoundere, Cameroon) in July and August 2013.

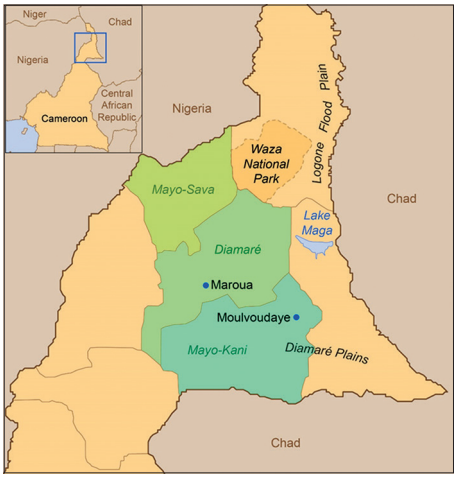

Figure 1: Study area in the Far North Region of Cameroon [4]

Determination of an Optimum Solvent Partition Procedure

An analysis was first conducted to determine the solvent that would allow us to have the best antioxidant activity (AA) and the highest quantity of total phenolic compounds for both plants. To this effect, we considered the 2,2-diphenyl-picrylhydrazyl (DPPH) radical scavenging activity of extracts as the indicator of potential AA of the extracts [14].

Extraction

Five different solvents were used including: water, 70% acetone (v/v), methanol, 80% methanol (v/v) and dichloromethane. They were used due to their properties and because they were available in our laboratory [15]. To obtain each extract, 25 g of ground plant sample were extracted by stirring with 250 mL of solvent at room temperature (around 25°C) for 24 h and filtering through Whatman #1. The volume of the extract was removed by evaporation and the lot stored in a sealed tube at 4°C until when needed. For the dried extracts (pure methanol and dichloromethane), 0.02 g of powder obtained after evaporation were mixed with 100 mL of methanol and added to obtain a solution of 0.2 μg/μL. As against the other extracts which still contained water (80% methanol extracts and 70% acetone), volumes were complemented to 100 mL with distilled water. For aqueous extracts of which the volumes obtained were >100 mL, the volume was complemented to 200 mL with distilled water.

Determination of total phenol content (TPC) of the best extracts

TPC was determined using the Folin–Ciocalteu colorimetric method [16].

DPPH radical scavenging activity

The method described by Tepe *et al.* [17] was used. The AA of each extract was represented by IC_{50} parameter (substrate concentration required to cause the loss of 50% of the initial concentration of DPPH).

Determination of AA of the Best Extracts

Ferric iron reducing activity (FIRA)

The method described by Oyaizu [18] was used for this analysis.

Hydroxyl radical scavenging acti

This was conducted according to the method of [19] with some modifications described below. In a test tube, 100 μL of extract, 500 μL of 5.6 mM 2-deoxy-D-ribose, 200 μL of a premixture of 100 μM $FeCl_3$ and 100 mM ethylenediaminetetraacetic acid (1:1 v/v) solution, 100 μL of 1.0 mM H_2O_2, and 100 μL of 1.0 mM aqueous ascorbic acid were introduced. Tubes were well vortexed and incubated at 50°C for 30 min. Thereafter, 1 mL of 2.8% trichloroacetic acid and 1 mL of 1% thiobarbituric acid were added to each tube and vortexed mixture and heated in a water bath at 50°C for 30 min. The extent of oxidation (or deoxyribose degradation) was evaluated by measuring the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control ($A_{control}$) and of the sample (A_{sample}) using equation where the controls contained all the reaction reagents except the extract or positive control substance. Mannitol was used as standard. The AA of the extracts was expressed as equivalent mannitol (mg of manniol/g of powder) according to the following equation:

$$HRSA(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Free radical-scavenging activity (FRSA) by the use of a stable ABTS radical cation

The FRSA was determined by ABTS radical cation decolorization assay modified by Re *et al.* [20]. The results were corrected for dilution and expressed in mg trolox per 100 g dry weight.

$$A.A\ (\%) = (OD_{initial} - A_{assay})/OD_{initial} \times 100$$

Determination of Total Flavonoid

The method of aluminum trichloride [21] was used to determine the total flavonoids content.

Determination of Total Tannins

This analysis was conducted following the method described by Makkar *et al.* [16].

Determination of Vitamin E

The vitamin E content was assessed by the spectrophotometric method as described by De Leenheer et al., and Milne *et al.* [22,23], with some modifications described below. Briefly, 1 g of powder was mixed with 30 mL of the solution of hexane/acetone (30/70). The mixture was successively heated to reflux for 1 h, cooled and filtered using the Whatman #1. The filtrate was washed with distilled water in a separating funnel and the lipid phase was collected in an Eppendorf.

For each sample, 0.1 g of oil were diluted in ethanol, transferred to tanks and their optical density were read at 522 nm. Trolox was used for calibration.

Determination of Iron Content

This assay was carried out according to the method of [24].

Phytochemical Screening

This analysis was performed using the best extracts selected after the previous analysis (TPC and FIRA) according to classic laboratory methods as described by [15,25-27].

Statistical Analysis

Data were presented as mean ± standard deviation. One-way analysis of variance followed by Duncan's multiple range test was performed using Statgraphics 5.0. (Windows, www.statgraphics.com) to compare this data. A probability level of 0.05 was accepted as being significant.

RESULTS

Survey Result

The results of the survey showed that 12 plants of 08 different families were used to treat FMD in the Far North Region of Cameroon. The parameters of use of each plant is summarize in Table 1. It is also important to note that the number of plants give here is less than the real number of plants used, because many farmers refused to tell us the local name of what they use. It revealed that the Mimosaceae family is the most widely used (23% or 3 of 13). The parts used are generally administered in macerated form (46% or 6 of 13) but may sometimes be given in association with salt. Administration to the animal is generally by the oral route (77% or 10 of 13). The doses are not generally defined and no undesirable secondary effects have been reported. During the survey, farmers said to use these plants alone or in combination with some elements like salt, urine of the animal that will be treated, honey, *natron*, *tchoukouri*, and *kilbou* (calcium carbonate). Among the 25% of herders who use the traditional medicine to manage FMD [4], 28%, 23%, 15% and 13% respectively use calcium carbonate, urine, salt and honey in association or not.

Selection of the Best Solvent

As tabulated in Table 2, the TPC fluctuate from 0.207 to 8.578 g/100 g diabetes mellitus (DM). For the leaves, it is the extraction with methanol which gave the highest TPC (3.539 ± 0.258 g/100 g DM) while the dichloromethane had the lowest value of TPC (0.207 ± 0.01 g/100 g DM). Instead of TD the best TPC was recorded when using methanol (8.578 ± 0.532 g/100 g DM).

As the TPC, the IC_{50} were also significantly ($P < 0.05$) varied from one solvent to another with values that range from 1.41 ± 0.50 mg/mL (methanolic extract of BS) and 357.12 ± 37.35 mg/mL (80% methanolic extract of BS). The IC_{50} of the leaves methanolic extract was significantly lower than that of the water extract (11.35 ± 1.86 mg/mL). But for TD it is the 70% acetone extract

Table 1: Overview of plants used for the treatment of cattle affected by FMD

Scientific name	Families	Local name (Fulfulde)	Parts used	Administered form	Administration route
A. nilotica	Mimosaceae	Gawari	Leave	Decoction	Oral
A. nilotica	Mimosaceae	Gabde	Fruit	Maceration	Oral
A. seyal	Mimosaceae	Bulbi	Stem back	Maceration+salt	Oral
T. dodoneifolius	Loranthaceae	Yowtéré caski	Stem	Maceration	Oral
K. senegalensis	Meliaceae	Daleehi	Stem back	Maceration+salt/gruel	Oral
B. senegalensis	Capparaceae	Buldumhi	Leave	Decoction	Oral/injection
T. aestivum L.	Gramineae	Alcamari	corn	Maceration+honey	Dermal
A. indica	Meliaceae	Ganyi	Root	Maceration	Dermal
C. limon	Rutaceae	leemon	Fruit	Juice	Oral
V. paradoxa	Sapotaceae	Kareti	Fruit	Butter	Oral
T. indica	Fabaceae	Jabbe	Fruit	Juice	Oral
Sorgho sp.	Poaceae	Gawari	Seed	Malt	Oral
Z. mays	Poaceae	Maasarji	Stem	Ash	Dermal

FMD: Foot and mouth disease, *A. nilotica: Acacia nilotica, A. seyal: Acacia seyal, T. dodoneifolius: Tapinanthus dodoneifolius, K. senegalensis: Khaya senegalensis, B. senegalensis: Boscia senegalensis, T. aestivum: Triticum aestivum, A. indica: Azadirachta indica, C. limon: Citrus limon, V. paradoxa: Vitellaria paradoxa, T. indica: Tamarindus indica, Z. mays: Zea mays*

Table 2: TPC and DPPH radical scavenging activity

Solvents	Plants				Standards	
	B. senegalensis		*T. dodoneifolius*		AC	BHT
	TPC (g/100 g DM)	IC_{50} (μg/mL)	TPC (g/100 g DM)	IC_{50} (μg/mL)	IC_{50} (μg/mL)	
Dichloromethane	0.207 ± 0.018^{c}	/	0.333 ± 0.011^{d}	/	$18.27\pm0.45^{\beta}$	$22.38\pm0.10^{\gamma}$
Methanol	3.539 ± 0.258^{a}	$1.41\pm0.50^{a\pi}$	8.578 ± 0.532^{a}	/		
80% Methano	1.412 ± 0.079^{b}	$357.12\pm37.35^{d\varphi}$	3.6015 ± 0.097^{c}	$116.29\pm3.07^{a\eta}$		
70% Acetone	0.213 ± 0.024^{c}	$282.26\pm4.39^{c\Psi}$	3.695 ± 0.103^{bc}	$84.79\pm13.11^{b\lambda}$		
Water	1.476 ± 0.027^{b}	$11.35\pm1.86^{b\rho}$	4.011 ± 0.336^{b}	$108.10\pm17.42^{b\rho}$		

Values with different letters of the French alphabet in the same column are significantly different ($P<0.05$), Mean±SEM; $n=3$, letters of the Greek alphabet represent the correlation coefficient of the standard obtained ($^{\pi}0.9824$, $^{\varphi}0.9055$, $^{\Psi}0.9901$, $^{\rho}0.8614$, $^{\eta}0.9750$, $^{\lambda}0.9342$, $^{\rho}0.9022$, $^{\beta}0.9041$, $^{\gamma}0.9595$), /Values inferior to zero, BHT: Butylated hydroxytoluene, AC: Ascorbique acid, DM: Dry matter, SEM: Standard error of mean

which had an IC_{50} (84.79 ± 13.11 mg/mL) significantly lower than that of water (108.10 ±17.42 mg/mL) ($P < 0.05$).

AA

Table 3 shows the summary of the results of AA of both plants. The reduction of ferrous ion (Fe^{3+}) to ferric ion (Fe^{2+}) is measured by the intensity of the resultant blue-green solution which absorbs at 700 nm. It shows that the reducing power of methanol extract of BS is much more pronounced (16.850 ± 1.340 mg ascorbic acid/g of powder) than that of acetone extract of TD. On a contrary the ability of leaves to stabilize the radical cation $ABTS^{0+}$ was significantly less (523.62 ± 0.86 μM Trolox/100 g DM) than the one of the acetone extract of TD (1460.10 ± 3.90 μM Trolox/100 g DM). It also came out that deoxyribose was significantly more protected by the acetone extract of TD (6.557 ± 0.017 mg mannitol/100 g DM) than methanol extract of BS (2.786 ± 0.409 mg mannitol/100 g DM). We also noted a significative difference between the different types of techniques used to evaluate the AA of the plants studied. Indeed, for both plants, the best activity was noted when using the trolox equivalent antioxidant capacity.

Some Important Elements Content of BS and TD

The levels of flavonoids measured was 305.463 ± 4.453 mg ethoxyquin (EQ) of quercetin/100 g DM for BS and 80.894 ± 1.684 mg EQ of quercetin/100 g DM for TD but this plant contained significantly more tannins (8.429±0.011 g EQ tannic acid/100 g DM) [Table 4].

Table 3: Antioxidant activities of plants

	Plants		*P* value
	B. senegalensis	*T. dodoneifolius*	
HRSA$^{\alpha}$	2.786 ± 0.409^{b}	6.557 ± 0.017^{a}	0.0001
FRSA$^{\beta}$	523.62 ± 0.86^{a}	1460.10 ± 3.90^{b}	0.0000
FIRA$^{\varphi}$	16.850 ± 1.340^{a}	0^{b}	0.0000

Values with different letters in the same row are significantly different ($P<0.05$), Mean±SEM, $n=3$, α: mg mannitol/100 g DM (0.9669); $^{\beta}$μM trolox/100 g DM (0.9904), $^{\varphi}$mg ascorbic acid/g of powder ($R^2=0.9726$), HRSA: Hydroxyl radical scavenging activity, FRSA: Free radical scavenging activity, FIRA: Ferric iron reducing activity, *B. senegalensis: Boscia senegalensis, T. dodoneifolius: Tapinanthus dodoneifolius*, SEM: Standard error of mean

Our results also showed that the methanol extract of BS contained more vitamin E (5.740 ± 0.562 mg trolox/100 g MS) and iron (0.478 ± 0.015 mg/100 g DM) than the acetone extract of TD.

Phytochemical Screening

Phytochemical analysis showed that extracts of both plants contain flavonoids, tannins, phenol, polyphenols compounds, glycosides, proteins, triterpenes and alkaloids [Table 5].

DISCUSSION

It became clear from the survey conducted that many plants are used to treat FMD. This can be explained by the fact that, FMD

Table 4: Total tannins, flavonoid, Iron and vitamins E content of *B. senegalensis* and *T. dodoneifolius*

	Plants		*P* value
	B. senegalensis	*T. dodoneifolius*	
Flavonoid[α]	305.463±4.453[a]	80.894±1.684[b]	0.0002
Tannins[β]	1.372±0.081[b]	8.429±0.011[a]	0.0000
Vitamin E[φ]	5.740±0.562[a]	0.814±0.010[b]	0.0001
Iron (mg/100 g DM)	0.478±0.015[a]	0.379±0.022[b]	0.0003
Water content (%)	6.93±0.15[a]	5.63±0.25[b]	0.0001
Ash (%)	9.99±0.20[b]	7.81±0.21[a]	0.0002

Values with different letters in the same row are significantly different ($P<0.05$); Mean±SEM; $n=3$; α: mg EQ of quercetine/100 g DM ($R^2=0.9866$), [β]g EQ tannic acid/100 g DM (0.961), [φ]mg trolox/100 g MS, [ρ]UI/g of powder, *B. senegalensis: Boscia senegalensis, T. dodoneifolius: Tapinanthus dodoneifolius*, SEM: Standard error of mean

Table 5: Phytochemical composition of the alcoholic extracts of *B. senegalensis* and *T. dodoneifolius*

Chemical compound	Plants	
	B. senegalensis	*T. dodoneifolius*
Flavonoid	-	+
Phenols	+	+
Polyphenols compound	+	+
Tannins	+	+
Alkaloid (Hager's test)	-	+
Alkaloid (Dragendoff test)	+	+
Triterpenes	+	-
Glycosides	+	-
Protein	+	+
Saponins	+	+

+: Present, -: Absent, *B. senegalensis: Boscia senegalensis, T. dodoneifolius: Tapinanthus dodoneifolius*

which is a viral disease is generally followed by many secondary diseases [28]. Thus, the plants used by farmers of our study may help on treating secondary infections or symptoms or to boost the immune system because the majority said they saw an improvement on the health of the animals treated. Indeed, one plant can be a source of different biological active compounds, this is why one plant can be used to treat many diseases [29]. The knowledge of medicinal plant use among the livestock farmers is said to have been developed gradually over a period of practical experience and are generally transmitted from one generation to another.

The results showed that for BS, it is the methanol extract that showed the best total AA and the highest amount of TPC. This could be explained by the fact that generally methanol allows the extraction of anthocyanins, flavones, simple phenols, polyphenols, etc. [30], which are groups of compounds that the quantities in a plant were correlated to the AA [31-34]. The results also show that, when TPC increases, IC_{50} increases also. This shows that for this plant, it is the phenolic compounds that are responsible for the total AA. This confirms the findings of [33]. In the case of TD there is no correlation between TPC and total AA. It is the 70% acetone extract that shows the best AA and yet it has lower TPC than methanol and water extract. This could indicate that for this species it is not only phenols that are responsible for its AA. Indeed, acetone in addition to extract phenols, can also extract flavonols [30] for which the AA is well documented [33]. In addition, being mixed with water, the acetone may also allow removal of tannins and other phenolic compounds [35].

The results showed that the parts of the plants study have a good activity against free radical in comparison with some plants already used for the FMD and its secondary diseases treatment [36,37]. It also came out that, the best activity of both plants is noted when testing the FRSA by use of a stable ABTS radical cation. This may suggest that, the best mechanism of the AA of the plant extracts is the capacity to pick up free radical and stabilize the radical cation. Therefore, both plant extracts may contain more antioxidant compounds of first class which are the compounds which convert highly reactive radicals into stable molecules [38]. They can completely stop a reaction until the radicals are fully consumed. The antioxidants of this class are phenolic compounds [39]. Hence, our extracts may contain the phenolic compounds responsible for their AA, which is also verified by the results of Table 4. These polyphenols which act via the trapping of reactive oxygen species by reduction and the formation of inert complexes with iron and copper ions are capable to protect polyunsaturated lipids against oxidation phenomenon, generators of radicals and lipid aldehydes responsible for the development of several diseases [40].

The analysis obtained revealed the high concentration of both the tannins and flavonoid contents. The beneficial effects of several flavonol glycosides and various flavonoid-rich extracts of various plants are already known to have AA [41]. These suggest that in the present study, there may also be a correlation between the rich tannins and flavonoid contents of the extract and its potent AA.

Tannins form a group of phenolics compounds resulting from polymerization of flavonoids units [42]. The tannin content of both plants can be considered higher when compared with plant materials used by herders to treat animal and human diseases [43-46]. These compounds are also known for their antioxidant and antitumor [47-49] which may help to treat the signs of FMD.

Similarly, the flavonoids which constitute the most important groups of phenols in plants [50,51] were found in high quantity in leaves. The amount of flavonoids contained in the extracts studied is very high compared to some plants used for the treatment of inter-digital lesions in animals [52]. In fact this symptom is one of the main characteristics of FMD when animals are affected. Studies made in 1986 by Selway [53] and 2 years later by Thomas [54] already show that several flavonoids have antiviral effects, this makes us think that these group of compounds may contribute in the mechanisms of recovery of health of animals affected by FMD. They are also a good antioxidants, antimicrobial, antibacterial, anti-inflammatory, antiviral, antitumor, etc. [55-59].

The amount of vitamin E and iron obtained from plant extracts are not negligible since by definition, vitamins and trace elements are needed just in few quantities in the body. In addition, vitamin E is known among all the other vitamins as

the best antioxidant with vitamin C [60]. The quantity found may contributed to improve the health of the cattle treated because it is well k that a low vitamin E status in cows reduce the immune response and likely increase the incidence of certain diseases. This prove it importance for the contribution to animal health [61]. Its AA are generally done by inhibiting lipid peroxidation in biological membranes [62]. Iron is also known to have AA [63].

The ash content ranged from 8 to 10%, which is significant and shows that our extracts could be a potential source of other minerals. These minerals, which according to several studies are however very involved in maintaining the health of the animal [64,65], could contribute for the treatment of cattle affected by FMD.

CONCLUSION

It appears form this study that 12 plants of 8 different families are regularly used by livestock farmers. Both the plants analyzed have a good AA and contain phenolic compounds, flavonoid, tannins, vitamin E and iron in non-negligible quantity. In addition, the screening shown also that they contain alkaloid, triterpenes, proteins and saponins. All these compound are known to have many biological activities. This can justify their use by herders to treat animal disease. The plants studied here can be seen as a potential source of useful drugs. Further studies on these plants are ongoing in order to isolate, identify, characterize and elucidate the structure of the bioactive compounds.

ACKNOWLEDGMENTS

This study was supported by the US. National Science Foundation (DEB-1015908) via a fellowship from the Disease Ecology and Computer Modeling Laboratory (DECML) of the Ohio State University. Authors would like to thank Drs. Armand B. Abdou, Prs. Douglas Kinghorn, Andre P. Zoli and Mark Moritz and Mr. Hamawa Youkouda for their scientific contribution and Tiedja Tomdieu and Laya Alphonse for their help for collecting data in the field.

REFERENCES

1. Ziebe R, Thys E, De Deken R.Analysis of livestock production systems on the scale of a canton: The case of Boboyo in the Far north Cameroon. Rev Elev Méd Vét Pays Trop 2005;58:159-65.
2. Cameroon. Strategy Document for Growth and Employment (ECSD). Reference Framework For The Governmental Action from 2010 to 2020. Yaoundé: National Government Publication; 2009. p. 168.
3. Yaya A. Genomic polymorphism in Mycoplasma mycoides subsp. Mycoides SC applications in molecular epidemiology and validation of a model of subcutaneous inoculation to study the virulence of the strains. PhD thesis. National Polytechnic Institute of Toulouse; 2008. p. 175.
4. Vougat NRRB, Tiédja T, Ziébé R, Foyet HS, Moritz M, Vondou L, Schrunk DE, Imerman PM, Rumbeiha WK, Garabed R.Quality and use of veterinary pharmaceuticals by cattle farmers in the Far North Region of Cameroon. [No published].
5. Babapour A, Azami L, Fartashmehr J. Overview of antibiotic residues in beef and mutton in Ardebil, North West of Iran. World Appl Sci J 2012;19:1417-22.
6. Donkor ES, Newman MJ, Tay SC, Dayie NT, Bannerman E, Olu-Taiwo M. Investigation into the risk of exposure to antibiotic residues contaminating meat and egg in Ghana. Food Control 2011;22:869-73.
7. Jabbarm A, Akhtar MS, Muhammad G, Lateef M. Possible role of ethnoveterinary medicine in poverty reduction in Pakistan: Use of botanical anthelmintics as an example. J Agric Soc Sci 2005;1:187-95.
8 Yirga G, Teferi M, GideyG, Zerabruk S. An ethnoveterinary survey of medicinal plants used to treat livestock diseases in Seharti-Samre District, Northern Ethiopia. Afr J Plant Sci 2012;6:113-9.
9. Moritz M, Ewing D, Garabed RB. On Not knowing zoonotic diseases: Pastoralists' ethnoveterinary knowledge in the far north region of Cameroon. Hum Organ 2013;72:1-11.
10. Institut Nationale de la Statistique (INS). Population et Affaires Sociales. Annuaire Statistique du Cameroun: Deuxième Partie 2010. p. 39-62.
11. MIDIMA. Diagnostic assessment 2008-2009, to update the regional director of planning and development scheme Sustainable Planning (SDRADDT) in the region of the Far North conducted in 2011. Final report (main document) in November 2009. Ministry of Economy, Planning and Development of territory; 2011. p. 36.
12. Koulaouna LB. Sustainable management of pastoral resources to face hydro-climatic constraints in dry areas: the case of the floodplain of logon (Far North Cameroon). Thesis, University of Maroua; 2013. p. 145.
13. Moritz M, Kitchey K, Saidou K. The social context of herding contracts in the far north region of Cameroon. J Mod Afr Stud 2011;49:263-85.
14. Ling LT, Palanisamy UD, Cheng HM. Prooxidant/antioxidant ratio (ProAntidex) as a better index of net free radical scavenging potential. Molecules 2010;15:7884-92.
15. Prashant T, Bimlesh K, Mandeep K, Gurpreet K, Harleen K. Phytochemical screening and extraction: A review. Int Pharm Sci 2011;1:98-106.
16. Makkar HP, Blummel M, Borowy NK, Becker K. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J Sci Food Agric 1993;61:161-5.
17. Tepe B, Daferera D, Sokmen A, Sokmen M, Polissiou M. Antimicrobial and antioxidant activities of the essential oil and various extracts of *Salvia tomentosa* Miller (Lamiaceae). Food Chem 2005;90:333-40.
18. Oyaizu M. Studies on products of browning reaction: Antioxidative activity of products of browning reaction. Jpn J Nutr 1986;40:307-15.
19. Halliwell B, Gutteridge JM, Aruoma OI. The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. Anal Biochem 1987;165:215-9.
20. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26:1231-7.
21. Yi ZB, Yu Y, Liang YZ, Zeng B. *In vitro* antioxidant and antimicrobial activities of the extract of *Pericarpium citri* reticulatae of a new citrus cultivar and its main flavonoids- LWT. Food Sci Technol 2007;4:1000-16.
22. De Leenheer AP, Nelis HJ, Lambert WE, Bauwens RM. Chromatography of fat-soluble vitamins in clinical chemistry. J Chromatogr 1988;429:3-58.
23. Milne DB, Botnen J. Retinol, alpha-tocopherol, lycopene, and alpha- and beta-carotene simultaneously determined in plasma by isocratic liquid chromatography. Clin Chem 1986;32:874-6.
24. Rodier J. L'analyse de l'eau: Chimie, Physico-Chimie, Bactériologie, Biologie. Paris. France: Dunod Technique; 1978. p. 240.
25. N'guessan K, Kadja B, Guédé NZ, Dossahoua T, Aké-Assi L. Screening phytochimique de quelques plantes médicinales ivoiriennes utilisées en pays Krobou (Agboville, Côte-d'Ivoire). Sci Nat 2009;6:1-15.
26. Harbone JB. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 2nd ed. London: Chapman and Hall; 1973. p. 49-188.
27. Ghani A. Medicinal Plants of Bangladesh. Dhaka: Asiatic Society of Bangladesh; 1998. p. 78-83.
28. Di Nardo A, Knowles NJ, Paton DJ. Combining livestock trade patterns with phylogenetics to help understand the spread of foot and mouth disease in sub-Saharan Africa, the Middle East and Southeast Asia. Rev Sci Tech 2011;30:63-85.
29. Pan L, Yong Y, Deng Y, Lantvit DD, Ninh TN, Chai H, *et al*. Isolation, structure elucidation, and biological evaluation of 16,23-epoxycucurbitacin constituents from Eleaocarpus chinensis. J Nat Prod 2012;75:444-52.

30. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12:564-82.
31. Cai Y, Luo Q, Sun M, Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci 2004;74:2157-84.
32. Shan B, Cai YZ, Brooks JD, Corke H. The *in vitro* antibacterial activity of dietary spice and medicinal herb extracts. Int J Food Microbiol 2007;117:112-9.
33. Sasikumar JM, Jinu U, Shamna R. Antioxidant activity and HPTLC analysis of *Pandanus odoratissimus* L. Root. Eur J Biol Sci 2009;1:17-22.
34. Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: Current methods and future trends. Afr J Biotechnol 2008;7:1797-806.
35. Elhefian EA, Ayman AJ, Adam MM, Omer AA, Abdul HY. A preliminary qualitative study of two common *Acacia* species in Sudan. EJ Chem 2012;9:851-6.
36. Karou D, Dicko MH, Simpore J, Traore SA. Antioxidant and antibacterial activities of polyphenols from ethnomedicinal plants of Burkina Faso. Afr J Biotechnol 2005;4:823-8.
37. Olajide O, Idowu D, Okolo S, Orishadipe A, Sunday T. Phytochemical and antioxidant properties of some Nigerian medicinal plants. Am J Sci Ind Res 2013;4:328-32.
38. Demanze C, Karliskind A. Analyses des antioxydants dans les corps gras alimentaires, n 28, APRIA. Actualités Scientifiques et Techniques en Agroindustrielles; 1980. p. 127.
39. Kortenska VD, Yanishlieva NV, Kasaikina OT, Totzeva IR, Boneva MI, Russina IF. Phenol antioxydant efficiency in various lipid substrates containing hydroxyl compounds. Eur J Lipid Sci Technol 2002;104:513-9.
40. Nkhili E. Polyphénols de l'Alimentation: Extraction, Interactions avec les ions du Fer et du Cuivre, Oxydation et Pouvoir antioxydant. Thèse de Doctorat. Université D'Avignon-Montpellier, France; 2009. p. 378.
41. Amic D, Davidovic-Amic D, Beclo D, Trinajstic N. Structure-radical scavenging activity relationships of flavonoids. Croat Chem Acta 2003;76:55-61.
42. Abdou BA, Njintang YN, Scher J, Mbofung CM. Phenolic compounds and radical scavenging potential of twenty Cameroonian spices. Agric Biol J North Am 2010;1:213-24.
43. Abdou BA, Vroumsia T, Nkouam GB, Njintang YN, Mang YD, Scher J, *et al*. Effect of solar and electric drying on the content of the phenolic compounds and antioxidant activity of three varieties of onion (*Allium cepa* L). Int J Biol Pharm Allied Sci2012;1:204-20.
44. Kabore A, Traore A, Pare S, Sawadogo BC, Kalkoumdo G, Tamboura HH, *et al*. Ethno-medicinal study of plants used in ectoparasites infections of ruminant livestock in sahelian region of Burkina Faso, West Africa. J Nat Prod Plant Resour 2012;2:611-6.
45. Edeoga HO, Okwu DE, Mbaeble BO. Phytochemical constituents of some Nigerian medicinal plants. Afr J Biotechnol 2005;4:685-8.
46. Ndip RN, Malange Tarkang AE, Mbullah SM, Luma HN, Malongue A, Ndip LM, *et al*. *In vitro* anti-*Helicobacter pylori* activity of extracts of selected medicinal plants from North West Cameroon. J Ethnopharmacol 2007;114:452-7.
47. Yoshizawa S, Horiuchi T, Fujiki H, Yoshida T, Okuda T, Sugimura T. Antitumor promoting activity of (-)-epigallocatechin gallate, the main constituent of "Tannin" in green tea. Phytother Res 1987;1:44.
48. Okuda T, Yoshida T, Hatano T. Polyphenols from Asian plants, structural diversity and antitumor and antiviral activities. In: Huang MT, Ho CT, Lee CY, editors. Phenolic Compounds in Food and Their Effects on Health II, Antioxidants and Cancer Prevention. Washington, DC, USA: American Chemical Society; 1992. p. 160-83.
49. Okuda T, Ito H. Tannins of constant structure in medicinal and food plants-hydrolyzable tannins and polyphenols related to tannins. Molecules 2011;16:2191-217.
50. Abdou BA. Contribution to the study of the development of a food based functional spices from Cameroon: Physicochemical characterization and functional. PhD thesis, University of Ngaoundere; 2009. p. 228.
51. Cíz M, Cízová H, Denev P, Kratchanova M, Slavov A, Lojek A. Different methods for control and comparison of the antioxidant properties of vegetables. Food Control 2010;21:518-23.
52. Saha P, Mazumder UK, Haldar PK, Sen SK, Naskar S. Antihyperglycemic activity of *Lagenaria siceraria* aerial parts on streptozotocin induced diabetes in rats. Diabetol Croat 2011;40:49-60.
53. Selway JW. Antiviral activity of flavones and flavans. In: Cody V, Middleton E, Harborne JB, editors. Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure Activity Relationships. New York: Alan R Liss, Inc.; 1986. p. 521-36.
54. Thomas PR, Nash GB, Dormandy JA. White cell accumulation in dependent legs of patients with venous hypertension: a possible mechanism for trophic changes in the skin. Br Med J (Clin Res Ed) 1988;296:1693-5.
55. Kim HP, Mani I, Iversen L, Ziboh VA. Effects of naturally-occurring flavonoids and biflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. Prostaglandins Leukot Essent Fatty Acids 1998;58:17-24.
56. Wang HK, Xia Y, Yang ZY, Natschke SL, Lee KH. Recent advances in the discovery and development of flavonoids and their analogues as antitumor and anti-HIV agents. Adv Exp Med Biol 1998;439:191-225.
57. Pietta PG. Flavonoids as antioxidants. J Nat Prod 2000;63:1035-42.
58. Jachak SM. Natural products: Potential source of COX inhibitors. CRIPS 2001;2:12-5.
59. Agrawal AD. Pharmacological activities of flavonoids: A review. Int J Pharm Sci Nanotechnol 2011;4:1-5.
60. Zhao B, Tham SY, Lu J, Lai MH, Lee LK, Moochhala SM. Simultaneous determination of vitamins C, E and beta-carotene in human plasma by high-performance liquid chromatography with photodiode-array detection. J Pharm Pharm Sci 2004;7:200-4.
61. Spears JW. Role of mineral and vitamin status on health of cows and calves. WCDS Adv Dairy Technol 2011;23:287-97.
62. Panfili G, Fratianni A, Irano M. Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereals. J Agric Food Chem 2003;51:3940-4.
63. Ahola JK, Engle TE, Whittier JC. Trace Minerals and the Immune System in Cattle. Western Beef Resource Committee, Cattle Producer's Library, Nutrition Section, CL315; 2010. p. 315-1-5.
64. Traore AO. Manuel D'Alimentation Animale. Paris: L'Harmattan; 2009. p. 164.
65. Smith T. Importance of Trace Minerals for Cattle Health and Fertility. Short Round Article Reprint. The Official Publication of CBR; 2011. p. 1-4.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141010122750

Original Research

Ethnomedicinal plants used for snake envenomation by folk traditional practitioners from Kallar forest region of South Western Ghats, Kerala, India

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Received: August 28, 2014
Accepted: October 10, 2014
Published: November 28, 2014

ABSTRACT

Background: The traditional medicinal systems of Indian folklore abundantly use medicinal plants or its derivatives for the treatment of snakebites. However, this traditional knowledge is on the verge of extinction, and there is an immediate necessity to conserve this oral traditional knowledge primarily by proper documentation and scientific authentication. The present ethno botanical study carried out among the folk medicine practitioners in the rural settle mental areas of Kallar forest region of southern Kerala, aims to document the folk herbal knowledge particularly for snake envenomation. **Materials and Methods:** The survey was conducted during the period of June 2012-July 2013 in the rural and forest settlement areas of Kallar in the Thiruvananthapuram district of Kerala. Direct observation and oral communications with local folk medicine practitioners in this region were adopted to collect valid information regarding the herbal formulations used to treat snake bite patients. **Results:** The study enumerates a list of 24 plant species belonging to seventeen families with anti-venomous potential. The scientific, vernacular and family names of these plants, along with the part used and their application modes are also enumerated in this communication. **Conclusions:** Plants are believed to be potent snake bite antidotes from centuries back, and knowledge about the use of plants is strictly conserved among tribes through generations without recorded data. It is the need of the hour to document these old drug formulations and is the cardinal responsibility of the scientific community to validate it and come up with new potent drug molecule for the benefit of snake bite victims.

KEY WORDS: Ethnomedicine, folk tradition, medicinal plants, snake antidote, South Western Ghats

INTRODUCTION

The folk medicinal system has a deep rooted history among rural populations in India. This unique system of knowledge has been evolved from the harmonious living of aboriginal people with nature from time immemorial. Even today the various indigenous communities and many local populations in the country rely on this century old therapeutic tradition to meet their health care and to treat many anomalies. The ayurveda medicine, a well-established medical paradigm from ancient India is believed to be emerged from folk medicine date back to over 2000 years [1]. The folk medicine uses medicinal plants abound; nearly 25,000 effective plant based formulations are believed to be in credit [2]. The art of healing practices in the folk tradition are purely based on knowledge, skills and practices over century old experience of trial and error and many times it is shrouded by religious myths and beliefs.

Death due to snake envenomation has been a major socio-economic and medical emergency of many tropical and subtropical countries with heavy rainfall and humid climate [3,4]. This is mainly because of the working habit of rural folks and also due to the increased settlements of humans in the natural habitats of snakes. The annual statistical information counts more than two lakh snake bite cases with 35,000-50,000 deaths in India [5,6]. Serotherapy is the only accepted therapy so far in modern medicine, but it is found expensive and often produces adverse side-effects in the bite victims [7]. The rural and tribals people living in a remote area greatly depend on folk medicines for the treatment of bites from any venomous creatures. Nowadays these herbal antidotes used in folk traditional medicine gained much attention by toxicological research groups worldwide for developing a new alternative, effective, cheap and less allergic venom inhibitors to counteract venom toxins [8].

Kerala lying in the southern tip of Indian subcontinent is known for its cultural diversity and also for biological diversity. Kallar-Ponmudi reserve forest is a part of South Western Ghats of Kerala and is about 45 km from state capital city, Thiruvananthapuram. Due to the varied climate and topographic conditions, the forest shows remarkable diversity of vegetation and is a great emporium of ethnobotanical wealth. A wide number of ethnobotanical surveys conducted previously in many tribal pockets and rural parts of Kerala have reported a countable number of medicinal plants with high therapeutic effect, but much larger number of folk medicine have still remained endemic to certain rural pockets [9,10]. The present study aims at conserving largely the herbal knowledge for snake envenomation used by the folk practitioners in the Kallar region of South Western Ghats, Kerala and avail it to the scientific community.

MATERIALS AND METHODS

Study Areas

The present field survey was conducted in the rural settle mental areas of Kallar forest region (08°30´ N latitude and 076°56´ E longitude) [Figure 1], which is in Thiruvananthapuram district of Kerala. The Kallar region is marvellous for evergreen forest of South Western Ghats; the nearby hill station Ponmudi is 15 km away from Kallar. The climate here is moderately hot and humid. The major tribal group inhabiting the area is Kani tribals, one of the largest tribal groups in Kerala. Ethnobotanical explorations were undertaken in rural pockets of Kallar panchayat, where the local population lives in blend with nature and have their own customs and traditions.

Data Collection

For ethnobotanical exploration, periodic field trips were undertaken in the rural and forest settle mental areas of Kallar in the Thiruvananthapuram district of Kerala. The study was conducted using a survey method during the period of June 2012-July 2013. The study randomly selected five families

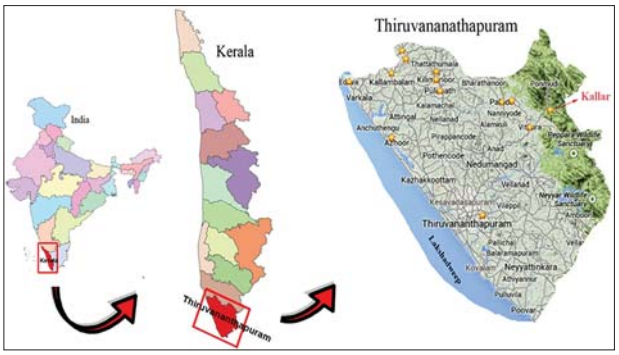

Figure 1: Map of Kallar forest region in Thiruvananthapuram district of Kerala, India

of local snake envenomation practitioners in the site, which are dispersed in and around the forest area. Either direct observations or oral communications with local practitioners were adapted to collect valid information regarding their venom envenomation treatment mode using medicinal plants. Detailed information about the use of plants in the treatment of snake venom envenomation including the parts used for the treatment, methods for the preparation of medicine, mode of administration in the patient and dose used in the treatment were collected and documented. Information collected from different local practitioners were further cross-checked and compared. A digital camera (Nikon D 3100), a global positioning system (Garmin 76 CS), a field book and a pocket lens were also used for the work. The documented plants were further authenticated by Dr. Valsala, Herbarium Curator, Department of Botany, University of Kerala, Thiruvananthapuram.

RESULTS

The present ethnobotanical survey provides information on a few plants that are used either as first aid or as an antidote for snake venom by the folk practitioners in the Kallar region of Kerala. The plants are used in different ways to treat different persons based on the physical need of the victims and also based on its local availability. The survey listed a total of 24 plant species belonging to seventeen families [Figure 2]. The documented plants arranged in Table 1 represent their botanical names, followed by the vernacular name, family name, useful part and its application mode. Application of the plant or its sap onto the bite area, chewing leaves and bark or drinking plant extracts or decoctions, nasal application of plant juice etc., are some procedures intended to neutralize snake venom activity.

DISCUSSION

The present ethnobotanical survey confirmed that the local population of the country still largely depends on medicinal herbs in and around their surroundings as the traditional tool to meet their primary health care. As they are living close to nature, the local inhabitants have acquired outstanding knowledge about the medicinal use of the regional herbal resources. However, many times medicinal practices may

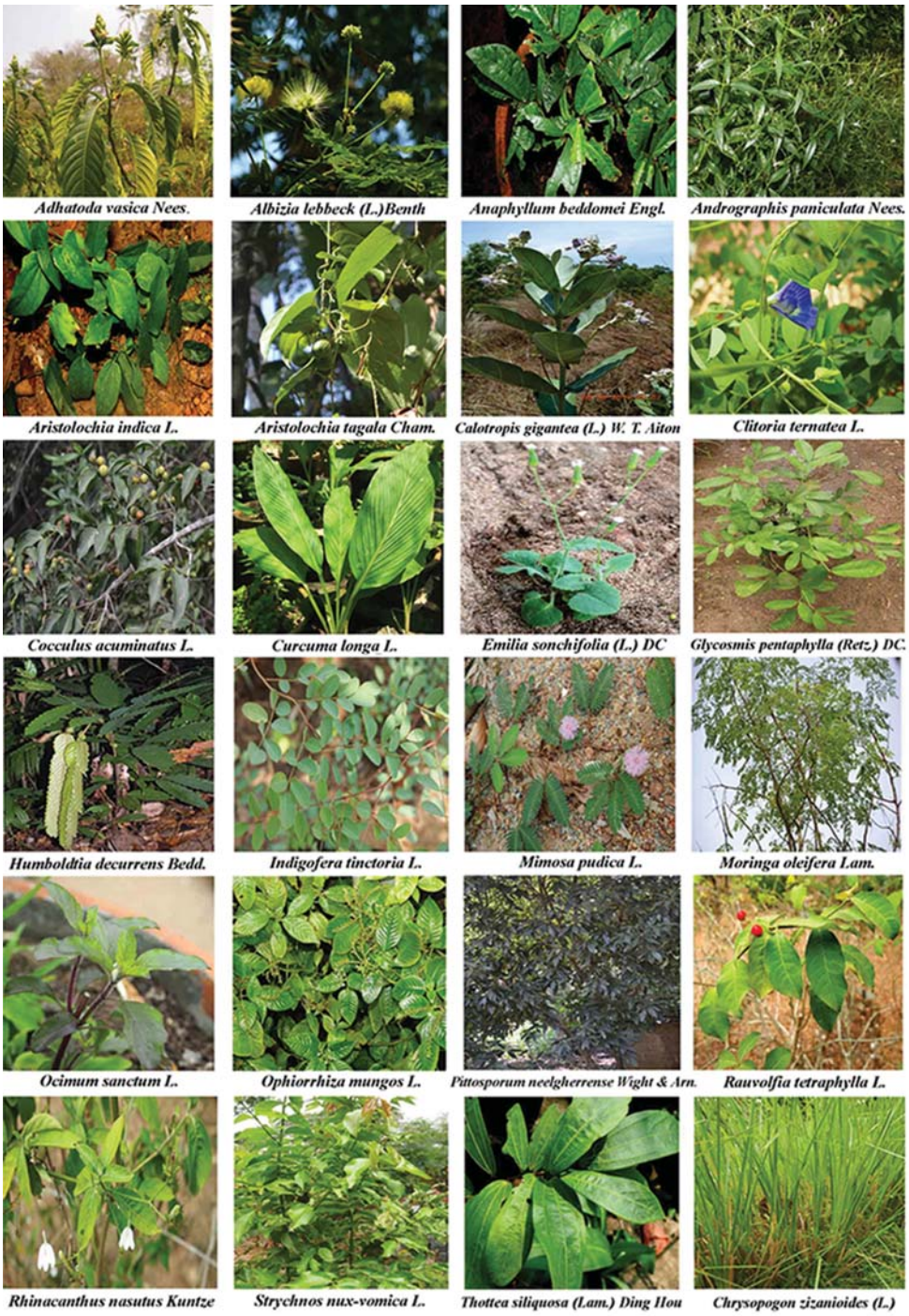

Figure 2: Anti-snake venomous plants documented from the Kallar region of South Western Ghats

incorporate or base themselves on spiritual beliefs. From the words of a folk physician, one gets venom or poison through five different routes-*damshanam** (bite), *sparshanam** (touch), *shwasanam** (breath), *snanam** (bath) and *panam** (drink) and treatment should be done carefully by monitoring the route of penetration of poison. The practitioners utilize a number of herbal antidotes to treat the patients challenged with snake venom. The healers administer the herbal combination both externally and internally to counteract the venom toxicity. The treatment practices are also influenced by mysticism and are followed by a strict dietary schedule to promote the complete cure.

CONCLUSIONS

India is one of the twelve mega-biodiversity countries in the World. Kerala, the southernmost state of India, is having rich biodiversity with a wide variety of therapeutic plants. The tropical forest of Western Ghats ranges offers the most diverse biological resources to the state. The rural people and different ethnic

Table 1: List of anti-snake venomous plants documented from Kallar region of South Western Ghats

Scientific name	Vernacular name	Family name	Useful part	Direction	Application
Adhatoda vasica Nees.	Aadalodakam	Acanthaceae	Leaf	Decoction, paste	Internal/external
Albizia lebbeck (L.) Benth	Nenmeni vaka	Mimosaceae	Root bark, bark	Paste	External
Anaphyllum beddomei Engl.	Keerikizhangu	Araceae	Rhizome	Paste	External
Andrographis paniculata Nees.	Kiriyath	Acanthaceae	Whole plant	Decoction, paste	Internal/external
Aristolochia indica L.	Cheriya arayan; Garudakodi	Aristolochiaceae	Whole plant	Decoction, paste	Oral/external
Aristolochia tagala Cham.	Valiya arayan	Aristolochiaceae	Whole plant	Decoction, paste	Oral/external
Calotropis gigantea (L.) W. T. Aiton	Erukku	Apocynaceae	Leaf and root	Paste	External
Chrysopogon zizanioides (L.) Roberty	Ramacham	Poaceae	Root	Paste	External
Clitoria ternatea L.	Sangupushpam	Fabaceae	Root	Juice	Internal
Cocculus acuminatus L.	Vally canjiram	Menispermaceae	Stem	Paste	External
Curcuma longa L.	Manjal	Zingibereaceae	Rhizome	Paste	External
Emilia sonchifolia (L.) DC	Muyal cheviyan	Asteraceae	Leaf	Decoction, paste	Oral/external
Glycosmis pentaphylla (Retz.) DC.	Panal	Rutaceae	Leaf	Decoction, paste	Oral/external
Humboldtia decurrens Bedd. Ex Oliv.	Neeruvatti	Fabaceae	Root	Paste	External
Indigofera tinctoria L.	Neela amari	Fabaceae	Root	Decoction	Oral
Mimosa pudica L.	Thottal vadi	Fabaceae	Root	Paste	External
Moringa oleifera Lam.	Moringa	Moringaceae	Root bark	Tincture	External
Ocimum sanctum L.	Krishnathulasi	Lamiaceae	Leaf, root	Juice	Oral
Ophiorrhiza mungos L.	Keeripacha	Rubiaceae	Root	Root	Oral
Pittosporum neelgherrense Wight and Arn.	Analivenga	Pittosporaceae	Bark	Decoction, paste	Oral/external
Rauvolfia tetraphylla L.	Paampumkolli	Apocynaceae	Root	Paste	External
Rhinacanthus nasutus Kuntze	Nagamulla	Acanthaceae	Leaves and roots	Paste	External
Strychnos nux-vomica L.	Kanjiram	Loganiaceae	Seeds and roots	Paste	External
Thottea siliquosa (Lam.) Ding Hou	Alpam	Aristolochiaceae	Root	Paste	External

tribal groups are the repositories of valuable herbal medicine. This knowledge treasure is strictly conserved to specific people in the community and is little shared to the outside world. This traditional knowledge has been passed verbally through generations without any written documentation [11-13]; it may hold the key to several new discoveries and wonder drugs. Global estimation accounts that nearly three fourth of the herbal formulations used worldwide were descended from the plants first used in local folk therapy [2].

In view of the number of deaths caused by snake bite, particularly where anti-venom is not readily accessible, the development of thermo-stable cheap remedies suitable for emergency treatment is important. The folk traditional practitioners have a strong faith and belief in herbal medicinal care for snake bite treatment, and they acclaim that the survival rate is high even in the advanced stage of envenomation. The use of plants against snake venom has long been identified but closer scientific attention has been given only since the last 20 years [14].

The ethnomedicinal information hopes to play a vital role in developing new scientifically validated and standardized drugs for snake bite treatment. Nowadays traditional medicine and complementary and alternative medicine are getting more attention within the context of healthcare provision and health sector reforms [15]. Moreover, it may further be mentioned that over-exploitation of some rare plant species for medicinal purpose may ultimately lead to their disappearance in the future. Therefore, attention should also be made on proper exploitation and utilization of these medicinal plants. The present study highlighted the traditional or folk knowledge of Kallar region of southern Kerala for the treatment of snakebites. The knowledge of these medicinal plants used in the regional folk traditional system has been of great importance, especially as a lead for the discovery of novel drug molecules for snake bite treatment. Among the wealth of ethno pharmacological information listed in the present paper, the characterization and scientific validation of few plants are currently in progress in our laboratory. The root extracts of *Ophiorrhiza mungos* L., the bark of *Pittosporum neelgherrense* Wight and Arn., the leaf and root extracts of *Aristolochia indica* L. and the leaf extracts of *Glycosmis pentaphylla* (Retz.) DC. are currently under study of its claim of the snake antidote activity. The promising results obtained from the root extract of *O. mungos* L. antivenomous effect against *Daboia russelii* Shawb and Nodder venom has been published [16] and further studies are currently going on to isolate the active principles and to elucidate the exact mechanism of action of root extract components with viper venom proteins.

ACKNOWLEDGMENTS

This work was funded by Kerala State Council for Science, Technology and Environment (KSCSTE), Government of Kerala, India. Department of Computational Biology and Bioinformatics and Department of Zoology under the University of Kerala are acknowledged for the facilities provided. The technical support from the Department of Botany, the University of Kerala for the authentication of plants identified from the study site is also gratefully acknowledged. We would also like to thank Mr. Ravan Schneider, Mr. Nirmal Roberts, Mr. Vinayaraj and Dr. Ayyappan and Dr. Pierre Grard for sharing the photographs of *Andrographis paniculata*, *Albizia lebbeck*, *Aristolochia tagala* and *Humboltia decurrens* respectively. Above all, the help and cooperation received from all the informants in Kallar panchayat during the data collection are gratefully acknowledged.

*The words mentioned in the text are in Sanskrit language, an ancient and classical language of India.

REFERENCES

1. Prakash JW, Raja RD, Anderson NA, Williams C, Regini GS, Bensar K, *et al*. Ethnomedicinal plants used by Kani tribes of agasthiarmalai biosphere reserve, Southern Western Ghats. Indian J Tradit Knowl 2008;7:410-3.
2. Verma S, Singh SP. Current and future status of herbal medicines. Vet World 2008;1:347-50.
3. Banerjee RN. Poisonous snakes and their venoms, symptomatology and treatment. Ahuja, editor. In: Progress in Clinical Medicine. 2nd series. India: Heinemann; 1978. p. 136-79.
4. Bhat RN. Viperine snake bite poisoning in Jammu. J Indian Med Assoc 1974;63:383-92.
5. Chippaux JP. Snake-bites: Appraisal of the global situation. Bull World Health Organ 1998;76:515-24.
6. Bawaskar HS. Snake venoms and antivenoms: Critical supply issues. J Assoc Physicians India 2004;52:11-3.
7. Meenatchisundaram S Parameswari G, Subbraj T, Michael A. Anti-venom activity of medicinal plants – A mini review. Ethnobotanical Leafl 2008;12:1218-20.
8. Gomes A, Das R, Sarkhel S, Mishra R, Mukherjee S, Bhattacharya S, *et al.* Herbs and herbal constituents active against snake bite. Indian J Exp Biol 2010;48:865-78.
9. Rajith NP, Ramachandran VS. Ethnomedicine of Kurichyas, Kannur district, Western Ghats, Kerala. Indian J Nat Prod Resour 2010;1:249-53.
10. Udayan PS, George S, Tushar KV, Balachandran I. Ethnomedicine of Malapandaram tribes of Achenkovil forest of Kollam district, Kerala. Indian J Tradit Knowl 2007;6:569-73.
11. Perumal Samy R, Ignacimuthu S, Sen A. Screening of 34 Indian medicinal plants for antibacterial properties. J Ethnopharmacol 1998;62:173-82.
12. Samy RP, Ignacimuthu S. Antibacterial activity of some folklore medicinal plants used by tribals in Western Ghats of India. J Ethnopharmacol 2000;69:63-71.
13. Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN, Ray C. Screening of Indian plants for biological activity: I. Indian J Exp Biol 1968;6:232-47.
14. Fattepur SR, Gawade SP. Preliminary screening of herbal plant extracts for anti-venom activity against common sea snake (*Enhydrina schistosa*) poisoning. Pharmacogn Mag 2004;16:56-60.
15. Kumar KJ. Fuzzy logic and modern analytical tool coupling in comprehensive evaluation of traditional and complementary medicine. Pharmacognosy 2006;2:202-3.
16. Krishnan SA, Dileepkumar R, Nair AS, Oommen OV. Studies on neutralizing effect of *Ophiorrhiza mungos* root extract against *Daboia russelii* venom. J Ethnopharmacol 2014;151:543-7.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141025103405

Original Research

ScopeMed

Cytotoxic and antimicrobial activities of substituted phenanthrenes from the roots of *Combretum adenogonium* Steud Ex A. Rich (Combretaceae)

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Received: September 25, 2014
Accepted: October 25, 2014
Published: November 28, 2014

ABSTRACT

Aim: The aim of this study was to isolate the bioactive compounds from the roots of *Combretum adenogonium* and assess for its antibacterial and cytotoxic properties. **Materials and Methods:** The extract was obtained using 20% aqueous ethanol and further subjected to fractionation with 1:1 n-butanol/water. Chromatographic analyses of the n-butanol fraction led to the isolation of compounds (1-3). The compounds (1-3) were assayed for antibacterial activities using two-fold microdilution methods and cytotoxicity using brine shrimps lethality assay.

Results: Following spectroscopic analyses the compounds were established as 2,3,8-trihydroxy-4,6-dimethoxyphenanthrene (1α) and 2,3,8-trihydroxy-4,6-dimethoxy-9,10-dihydrophenanthrene (1β). Compound 2 was derived from 2,3,8-trihydroxy-4,6-dimethoxyphenanthrene condensation with methyl acetate while Compound 3 was derived from 2,3,8-trihydroxy-4,6-dimethoxy-9,10-dihydrophenanthrene condensation with methyl propionate. These compounds (1-3) were active against *Pseudomonas aeruginosa* with minimal inhibitory concentration-value of 0.16 mg/ml. The compounds (1-3) also exhibited significant toxicity with LC_{50} (95% confidence interval [CI]) of 12.11 (7.32-20.05) μg/ml compared to standard anticancer drug, cyclophosphamide which had LC_{50} (95% CI) value of 16.37 (12.01-22.31) μg/ml. **Conclusion:** These compounds add for a novel structure that can be synthesized, further screened for *in vitro* and *in vivo* models and clinical trials in order to evaluate its potential for further development as new anticancer agent.

KEY WORDS: Antimicrobial, cytotoxicity, brine shrimps lethality test, *Combretum adenogonium*, substituted phenanthrenes

INTRODUCTION

Phenanthrenes are a class of phenolic compounds with three fused rings, which are presumably formed by oxidative coupling of the aromatic rings of stilbene precursors. A large number of differently substituted phenanthrenes are known to occur in more than 10 plant families and mainly have been isolated from orchidaceae, combretaceae, dioscoreaceae and betulaceae families [1]. Phenanthrenes and stilbenes are well-recognized as phytoalexins [2] and have been reported to possess various biological activities, including antitumor [1,3] antibacterial [4] and anti-inflammatory activities [5]. Phenanthrenes and their derivatives from the genus *Combretum* have been isolated from *Combretum hereroense*, *Combretum apiculatum*, *Combretum collinum* and *Combretum molle* [6-11]. The phenanthrenes and dihydrophenanthrenes from *Combretum caffrum* possessed good activity against murine P388 lymphocytic leukemia cell lines [3]. Structure-activity relationships of phenanthrenes and stilbenes are important in activity because substituents determine the planarity of the molecules, which is essential for drug-receptor interactions [6,12]. Although *Combretum adenogonium* is widely used in African traditional medicine, there is no existing report on the bioactive compounds from the plant that provides such pharmacological effects to humans. The current study investigated the bioactive agents from the roots of *C. adenogonium*, which led to the isolation of compounds (1-3). Herein, we report the isolation and structure elucidation of the compounds by means of nuclear magnetic resonance spectroscopy (^{1}H NMR,^{13}C NMR, DEPT 135 and DEPT 90). The signals were measured on 400 MHz spectrophotometer using tetramethylsilane as internal standard and dimethyl sulfoxide (DMSO) as NMR solvent.

MATERIALS AND METHODS

Materials

Acetone, n-Butanol, petroleum ether were purchased from Kas medics (Kas Medics ltd, Tanzania). Dichloromethane was purchased from UNILAB (UNILAB®, Nairobi, Kenya), ethanol (absolute) was bought from Fluka Chemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands) whereas DMSO was purchased from Sigma® (Poole, Dorset, UK). *Staphylococcus aureus* (NCTC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 29953) were obtained from the Department of Microbiolgy, the Muhimbili University of Health and Allied Sciences (MUHAS). Nutrient broth was purchased from Tulip Diagnostic (P) Ltd (Microxpress™, Goa, India). Iodonitrotetrazolium chloride was bought from SIGMA® (Sigma-Aldrich®, St Louis, USA). The Brine Shrimps eggs were purchased from Aquaculture innovations (Grahamstown 6140, South Africa) and sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast.

Collection and Extraction of Plant Materials

One sample of roots of *C. adenogonium* was collected from Handeni district, Tanga region, Tanzania in May, 2010. The plant was identified by Haji O. Seleman of the University of Dar es Salaam, Botany Department. Herbarium specimen (voucher specimen collection number LBM 965) is deposited in the Herbarium of the Botany Department, University of Dar es Salaam. The collected plant material was air dried, pulverized and extracted with 20% aqueous ethanol at room temperature for 24 h. The extract was dried under vacuo, followed by freeze-drying before analysis.

Isolation of Compound (1-3)

The root extract (111.20 g) was dissolved by shaking in 1:1 mixture of n-butanol and water and the two phases were separated in a separating funnel. An amount of 35.59 g of n-butanol fraction was packed into a 4.5 cm × 50 cm silica gel column and eluted successively with petroleum ether, petroleum ether/dichloromethane and dichloromethane/acetone. A total of 65 fractions were collected and analyzed using thin layer chromatogram (TLC) and each fraction which displayed similar TLC profiles was combined. Fractions 57-67 were combined, and further eluted in a small column to give semi-pure sub-fractions 11-16. These sub-fractions were combined and subjected to preparative TLC using 2% acetone/dichloromethane to yield 7.8 mg of compounds 1α and 2. These compounds which were yellowish amorphous powder, UV-positive with R_f value 0.5 in 2% acetone/dichloromethane were identified as 2,3,8-trihydroxy-4,6-dimethoxyphenanthrene (1α) and substituted phenanthrenes (2) respectively. Compounds (1β) and (3) were obtained from further purification of compounds (1α) and (2) and re-analyzed with NMR spectroscopy.

Determination of Antibacterial Activity

Antibacterial activities of the compounds (1-3) were determined against three strains of bacteria (2 Gram-negative and 1 Gram-positive) and their minimum inhibitory concentrations (MICs) were assayed through two-fold microdilution method using sterile 96-well microtitre plates [13]. Each well of the plates were first preloaded with 50 μl of the broth media followed by an addition of 50 μl of the compounds (1-3) (=0.8 mg/mL) into the first wells of each row tested. The resulting mixture were serially two-fold diluted with tryptone soya broth media (made by dissolving 7.5 g of tryptone soya broth in 250 ml of sterilized distilled water) for each case 50 μl were drawn from the first row wells and transferred into the next and subsequent row wells. The remaining 50 μl from the last row well were discarded. Thereafter, 50 μl of the bacterial suspension (0.5 McFarland standard turbidity) was added in each well. Gentamycin sulfate was used as a positive control, DMSO as a negative control while the rows with tryptone soya broth and bacteria only were used as growth controls. Both plates were incubated at 37°C for 24 h. MIC-values were determined by adding 20 μl of 0.02% p-iodonitrotetrazolium (INT) chloride dye in each well, followed by incubation for 1 h at 37°C. The MIC-values of the compounds (1-3) was read at the concentration where a marked reduction in color formation due to bacterial growth inhibition was noted.

Brine Shrimps Lethality Test (BST)

Cytotoxicities of compounds (1-3) were evaluated by using BST as previously reported [14]. Briefly, a stock solution (5 mg/ml) of the compounds was prepared by dissolving them in DMSO. Different levels of concentrations (240, 120, 80, 40, 24 and 8 μg/ml) were prepared by drawing different volumes from the stock solutions and then added into vials, each containing ten brine shrimps larvae. The volume was adjusted to 5ml with artificial sea water prepared by dissolving 3.8 g of sea salt in 1 L of distilled water. Each level of concentration was tested in duplicate. The negative control contained brine shrimps, artificial sea water and DMSO (0.6%) only. The vials were then incubated under light for 24 h. The dead larvae were counted, and the mean was subjected to analysis using Fig P computer program (Biosoft Inc., USA).

RESULTS

Compound (1α) and (2)

The ^{1}H NMR spectrum of compound 1α and 2 had three aromatic proton singlets at δ_H 9.08 (H-5, *s*), 7.08 (H-7, *s*) and 6.97 (H-1, *s*) whose corresponding ^{13}C NMR signal were δ_C 109.07 (C-5), 99.45 (C-7) and 111.63 (C-1), respectively. The ^{13}C NMR spectrum of these compounds indicated signals due to oxygenated quaternary aromatic carbons at δ_C 153.22 (C-4), 157.75 (C-6), 147.68 (C-2), 144.91 (C-3) and 122.45 (C-8) as in Fig. 1α. Two of these carbons have methoxyl substituents as established by two sharp singlets at δ_C 55.7 (4-OCH_3) and 55.31 (6-OCH3) in the DEPT 135 and DEPT 90 respectively, while the remaining were due to hydroxyl substituent's. By comparing the peaks with those reported in the literature [Tables 1 and 2], the main skeleton of compound 1α was

found to be that of phenanthrene moiety [3,15,16]. Peaks appearing at δ_C 74.49 (CH) and 79.98 (CH_2) were assigned to C-11 and C-13, respectively as in structure 2. The ^{13}C NMR spectrum also showed a peak at 176.85 that are carbon signals for carbonyl (C-12) of structure 2. Since these peaks were seen as minor signals in the ^{13}C NMR, it is likely that compound 1α was reacting with impure molecule (a) to give compound 2. Thus, compound 1α was established as a major constituent whereas the other compound 2 was in minor quantity and its peaks overlapped with those of 1α. The structure of Compound 1α was established as 2,3,8-trihydroxy-4,6-dimethoxyphenanthrene and the impure molecule (a) was identified as methyl acetate. Compound 2 was derived from 2,3,8-trihydroxy-4,6-dimethoxyphenanthrene condensation with methyl acetate.

Table 1: ^{13}C NMR data of phenanthrenes (1α)[a] and (2)[a]

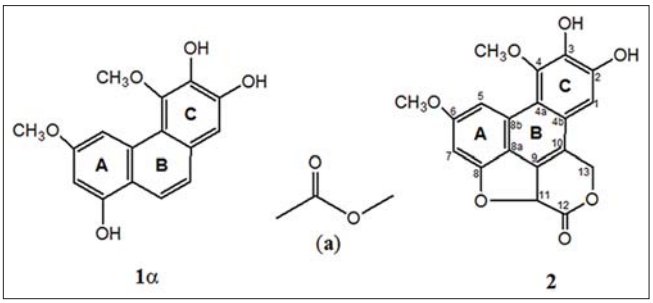
Position	Type of ^{13}C NMR	δ_C Observed (1α)	Type of ^{13}C NMR	δ_C Observed (2)	δ_C Reported [3,15]
1	CH	111.58	CH	111.58	110.42
2	C	147.68	C	147.68	147.63
3	C	144.91	C	144.91	144.96
4	C	153.22	C	153.22	153.23
4a	C	117.22	C	117.22	118.42
4b	C	110.95	C	110.95	
5	CH	109.03	CH	109.03	108.88
6	C	157.75	C	157.75	154.11
7	CH	99.41	CH	99.41	99.45
8	CH	122.45	CH	122.45	121.54
8a	C	133.74	C	133.74	134.05
8b	C	124.04	C	124.04	124.03
9	C	114.04	C	114.04	117.1
10	CH	126.22	CH	126.22	126.23
11			CH	74.49	
12	C		C	208.29	
13			CH_2	74.98	
4-OCH_3	CH_3	55.70	CH_3	55.70	60.12
6-OCH_3	CH_3	55.31	CH_3	55.31	55.77

[a]NMR data were measured on 400 MHz. The assignments were based on ^{13}C NMR, DEPT 135 and DEPT 90 experiments. TMS was used as internal standard. DMSO was used as NMR solvent. TMS: Tetramethyl silane, DMSO: Dimethyl sulfoxide

Table 2: ^{1}H NMR chemical shifts (in ppm) of phenanthrenes (1α)[a] and (2)[a]

Position	Multiplicity	δ_H Observed (1α)	δ_H Observed (2)	δ_H Reported [3,15,16]
1	*s*, 1H	6.96	6.96	6.96
5	*s*, 1H	9.07	9.07	9.07
7	*s*, 1H	7.07	7.07	6.74
10	*s*, 1H		2.11	
11	*s*, 1H		4.14	
13	*s*, 2H		3.95	
6-OCH_3	*s*, 3H	3.99	3.99	3.99
4-OCH_3	*s*, 3H	3.97	3.97	3.97

[a]NMR data were measured on 400 MHz. The assignments were based on ^{1}H NMR, ^{13}C NMR, DEPT 135 and DEPT 90 experiments. TMS was used as internal standard. DMSO was used as NMR solvent. TMS: Tetramethyl silane, DMSO: Dimethyl sulfoxide

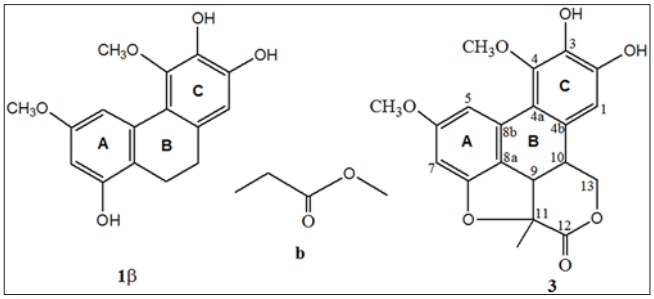


Compound (1β) and (3)

Spectra for this compound exhibited almost similar pattern as it was for compound (1α) except that it indicated many major peaks at high field region of both ^{1}H and ^{13}C NMR.

The ^{1}H NMR spectrum of compound 1β exhibited three aromatic proton singlets at δ_H 9.07s (H-5, *s*), 7.07 (H-7, *s*) and 6.96 (H-1, *s*) whose corresponding ^{13}C NMR signal appeared at δ_C 109.03 (C-5), 99.41 (C-7) and 111.58 (C-1). Further characteristic peaks in ^{13}C NMR appeared at δ_C 29.55 and 32.03 corresponding to C-9 and C-10 as it is for the dihydrophenanthrene [3]. In DEPT-135 experiment signals resonating at 29.55 and 32.03 were CH signals instead of CH_2 as commonly reported for dihydrophenanthrenes, a factor which provides evidence for presence of substituent in C-9 and C-10 as it is in structure 3. The ^{13}C NMR and DEPT 135 showed peaks at δ_C 208.29 (C-12), 68.45 (C-11) and 55.79 (C-13, 2H) which are carbon signals for carbonyl and oxygenated aliphatic carbons. These signals were assigned to C-12, C-11 and C-13 in the molecule as it is in structure 3. These peaks had low intensity indicating that, an impurity (b) was thought to have been involved in the reaction with the major compound 1β to form compound 3. The ^{13}C NMR and ^{1}H NMR signals for the mixture of major compound 1β and minor compound 3 overlapped as indicated in Tables 3 and 4. The structure of Compound 1β was established as 2,3,8-trihydroxy-4,6-dimethoxy-9,10-dihydrophenanthrene and the impure molecule (b) was identified as methyl propionate. Compound 3 was derived from 2,3,8-trihydroxy-4,6-dimethoxy-9,10-dihydrophenanthrene condensation with methyl propionate.

Since these compounds were in mixed form, altogether were subjected to an uniform bioscreening study. Cytotoxicities of



compounds (1-3) were done by using the brine shrimps lethality assay and results indicated that the compounds (1-3) had LC_{50} (95% confidence interval [CI]) of 12.11 (7.32-20.05) μg/ml. These compounds (1-3) were also tested for antimicrobial activities against three bacterial strains namely *P. aeruginosa*, *E. coli* and *S. aureus*, and they were active only for *P. aeruginosa* with MIC-value of 0.16 mg/ml.

The standard anticancer drug, cyclophosphamide used had LC_{50} (95% CI) value of 16.37 (12.01-22.31) μg/ml.

DISCUSSION

The isolated compounds (1-3) were evaluated for their cytotoxic and antimicrobial activities. The compounds (1-3) exhibited potent cytotoxic activities against BST with LC_{50} value of 12.11 μg/ml and mild antimicrobial activities against *P. aeruginosa* with MIC-value of 0.16 mg/ml. The LC_{50} value of the standard anticancer drug, cyclophosphamide was found to be 16.37 μg/ml. Potent cytotoxic phenanthrenes both *in vitro* and *in vivo* have also been reported by other researchers [19]. Stemophenanthrene isolated from *Stemona tuberosa* were found to possess moderate cytotoxic activities against four cancer cell lines [23]. Numerous studies have also reported on the significant cytotoxic properties of phenanthrenes isolated from different species against various cell lines [1]. In contrast, the current study used a rapid, reliable, inexpensive and convenient in-house brine shrimp toxicity assay. Nevertheless, it has been demonstrated that BST correlates well with cell lines studies and other biological properties [14,20]. Plants that are rich in phenanthrenes such as *Cremastra appendiculata* have a long history of use in Chinese traditional medicine for treatment of various cancers [21]. The current study also showed mild antimicrobial activities of the isolated phenanthrenes against *P. aeruginosa* with MIC-value of 0.16 mg/ml. Previous antimicrobial studies from ethanolic root extract of *C. adenogonium* showed MIC-value of 1.25 mg/ml against *P. aeruginosa* [22]. These findings indicate that antimicrobial activities of the roots might be contributed largely by the presence of phenanthrenes compound. Previous findings have reported antimicrobial activities from phenanthrenes isolated especially from orchidaceae and combretaceae family although most of them were found to possess weak to moderate antimicrobial activities against the test strains [1]. *C. adenogonium* is used by African traditional healers for treatment of leprosy, cough, syphilis, snakebite, aphrodisiac, new and chronic wounds, malaria, septic wounds and fungal infection of the scalp [22]. Thus, the antimicrobial and cytotoxic properties of phenanthrenes from *C. adenogonium* that have been reported further lends support to its application by African traditional healers for treatment of such ailments.

On the other hand, following overlapping multiplicities from the NMR experiments, literature analysis was done to determine the possible impurities and their integral peaks. Similarities were observed for the most common laboratory solvents which are CH_3COOH, CH_3COCH_3, $CH_3COOCH_2CH_3$ and D_2O. As shown in Table 2, the ^{1}H NMR spectrum of compound 2 exhibited three proton singlets at δ_H 2.11 (H-10, *s*) and at δ_H 4.14 (H-11, *s*) which correspond to signals at δ_H 2.10 (CH_3COO^-, *s*) of the acetic acid as solvent impurity and at δ_H 4.14 ($RCOOCH_2R$, *q*) of the ethyl

Table 3: ^{13}C NMR data of phenanthrene (1β)[a] and (3)[a]

Position	δ_C Observed (1β)	Type of ^{13}C NMR	δ_C Observed (3)	Type of ^{13}C NMR	δ_C Reported [3,15]
1	111.63	CH	111.63	CH	110.42
2	147.73	C	147.73	C	147.00
3	144.95	C	144.95	C	144.96
4	153.26	C	153.26	C	153.23
4a	117.26	C	117.26	C	118.00
4b	111.00	C	111.00	C	140.37
5	109.07	CH	109.07	CH	108.88
6	157.80	C	157.80	C	154.11
7	99.45	CH	99.45	CH	99.45
8	122.49	CH	122.49	CH	121.54
8a	133.78	C	133.78	C	134.05
8b	124.09	C	124.09	C	124.03
9	29.55	C	29.55	C	29.74
10	32.03	CH	32.03	CH	30.34
11			68.45	C	
12			176.85	C	
13			55.79	$-CH_2$	
14			22.02	CH_3	
4-OCH_3	55.74	CH_3	55.74	CH_3	60.12
6-OCH_3	55.34	CH_3	55.34	CH_3	55.77

[a]NMR data were measured on 400 MHz. The assignments were based on ^{13}C NMR, DEPT 135 and DEPT 90 experiments. TMS was used as internal standard. DMSO was used as NMR solvent. TMS: Tetramethyl silane, DMSO: Dimethyl sulfoxide

Table 4: ^{1}H NMR data peaks of phenanthrene (1β)[a] and (3)[a]

Position	Multiplicity	δ_H Observed (1β)	δ_H Observed (3)	δ_H Reported [3,15,17]
1	s, 1H	6.97	6.97	6.96
5	s, 1H	9.08	9.08	9.07
7	s, 1H	7.08	7.08	6.74
10	s, 1H	2.11	2.11	2.12
13	s, 2H		1.13	
6-OCH_3	s, 3H	3.99	3.99	3.99
4-OCH_3	s, 3H	3.97	3.97	3.97

[a]NMR data were measured on 400 MHz. The assignments were based on ^{1}H NMR, ^{13}C NMR, DEPT 135 and DEPT 90 experiments. TMS was used as internal standard. DMSO was used as NMR solvent. TMS: Tetramethyl silane, DMSO: Dimethyl sulfoxide

Table 5: ^{1}H NMR chemical shifts of common laboratory solvents [18][a]

Residual solvents	Proton	Multiplicity	$CDCl_3$	D_2O
Acetic acid	CH_3	*s*	2.10	2.08
Acetone	CH_3	*s*	2.17	2.22
Ethyl acetate	CH_3CO	*s*	2.05	2.07
	CH_2CH_3	*q*, 7	4.12	4.14
	CH_2CH_3	*t*, 7	1.26	1.24

[a]NMR data were measured on 400 MHz. The assignments were based on ^{1}H NMR, ^{13}C NMR, DEPT 135 and DEPT 90 experiments. TMS was used as internal standard

acetate as solvent impurity [Table 5]. The proton singlets at δ_H 2.11 (H-10, *s*) which corresponds to signals at δ_H 2.10 (CH_3COO^-, *s*) of acetic acid was also observed in compound 3 [Table 4]. In ^{13}C NMR spectrum, signal appeared at δ_C 208.29 (C-12) in compound 2 [Table 1] corresponds to δ_C 207.07 (R-CO-R) of the acetone as a solvent impurity. The ^{13}C NMR signals at δ_C 176.85 (C-12) and at δ_C 22.02 (C-14) of compound 3 corresponds to δ_C 175.99 (RCOR) of the acetone as solvent impurity and δ_C 21.03 (CH_3COO^-) of the acetic acid as solvent impurity [Table 6]. These substitution patterns observed in the molecules may have occurred during chromatographic or NMR analysis.

CONCLUSION

The preliminary cytotoxic activities exhibited by the substituted phenanthrenes add for a novel structure that can be synthesized, further screened for *in vitro* and *in vivo* models and clinical trials in order to evaluate its potential as new anticancer agent.

ACKNOWLEDGMENTS

This project was supported by grants from the Ministry of Health and Social Welfare, Tanzania, under MUHAS postgraduate research program. The authors are grateful to Prof. Dr. Luc Pieters of the Department of Pharmacognosy and Phytochemistry, Department of Pharmaceutical Sciences, University of Antwerp, Belgium for aiding on spectroscopic analysis of the isolated compounds. This manuscript is dedicated to the late Professor Zakaria H. Mbwambo for his enormous inspiration during time of his carrier at our institute.

REFERENCES

1. Kovács A, Vasas A, Hohmann J. Natural phenanthrenes and their biological activity. Phytochemistry 2008;69:1084-110.
2. Hart JH. Role of phytostilbenes in decay and disease resistance. Annu Rev Phytopathol 1981;19:1138-43.
3. Pettit GR, Singh SB, Niven ML, Schimdt JM. Cell growth inhibitory dihydrophenanthrene and phenanthrene constituents of the African tree *Combretum caffrum*. Can J Chem 1988;66:406-13.
4. Eloff JN, Famakin JO, Katerere DR. Isolation of an antibacterial stilbene from *Combretum woodii* (Combretaceae) leaves. Afr J Biotechnol 2005;4:1166-71.
5. Yang L, Qin LH, Bligh SW, Bashall A, Zhang CF, Zhang M, *et al*. A new phenanthrene with a spirolactone from *Dendrobium chrysanthum* and its anti-inflammatory activities. Bioorg Med Chem 2006;14:3496-501.
6. Katerere DR. Phytochemical and pharmacological studies of African Combretaceae. PhD Thesis. Glasgow, UK: University of Strathclyde; 2001.
7. Letcher RM, Nhamo LR. Chemical constituents of the Combretaceae. Part I. Substituted phenanthrenes and 9,10-dihydrophenanthrenes from the heartwood of *Combretum apiculatum*. J Chem Soc (C) 1971;1:3070-8.
8. Letcher RM, Nhamo LR. Chemical constituents of the Combretaceae. Part III. Substituted phenanthrenes 9, 10-dihydrophenanthrenes, and bibenzyls from the heartwood of *Combretum psidioides*. J Chem Soc Perkin I 1972;1:2941-7.
9. Letcher RM, Nhamo LR, Gumiro IT. Chemical constituents of the Combretaceae. Part II. Substituted phenanthrenes 9, 10-dihydrophenanthrenes and a substituted bibenzyl from the heartwood of *Combretum molle*. J Chem Soc Perkin I 1972;1:206-10.
10. Letcher RM, Nhamo LR. Chemical constituents of the Combretaceae. Part IV. Phenanthrene derivatives from the heartwood of *Combretum hereroense*. J Chem Soc Perkin I 1973;1:1179-81.
11. Malan E, Swinny E. Substituted bibenzyls, phenathrenes and 9,10-dihydrophenathrenes from the heartwood of *Combretum apiculatum*. Phytochemistry 1993;34:1139-42.
12. Rogers CB, Verotta L. Chemistry and biological properties of the African Combretaceae. In: Hostettman K, Chinyanganga F, Maillard M, Wolfender JL, editors. Chemistry, Biological and Pharmacological Properties of African Medicinal Plants. Harare, Zimbabwe: University of Zimbabwe Publications; 1996.
13. Eloff JN. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med 1998;64:711-3.
14. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med 1982;45:31-4.
15. Kováks A. Isolation and structure elucidation of compounds with antitumor activity from *Tamus Communis* and *Xanthium italicum*. Ph.D. thesis, Seizeg, Hungary; 2009.
16. Radwan MM, Elsohly MA, Slade D, Ahmed SA, Khan IA, Ross SA. Biologically active cannabinoids from high-potency Cannabis sativa. J Nat Prod 2009;72:906-11.
17. Pettit GR, Cragg GM, Herald DL, Schmidt JM, Lohavanijaya P. Isolation and structure of combrestatins. Can J Chem 1982;60:1374.
18. Gottlieb HE, Kotlyar V, Nudelman A. NMR Chemical Shifts of Common Laboratory Solvents as Trace Impurities. J Org Chem 1997;62:7512-5.
19. Lee YH, Park JD, Baek NI, Kim SI, Ahn BZ. *In vitro* and *in vivo* antitumoral phenanthrenes from the aerial parts of *Dendrobium nobile*. Planta Med 1995;61:178-80.
20. Mclaughlin JL. Crown gall tumors on potato discs and brine shrimp lethality: Two simple bioassays for higher plant screening and fractionation. In: Hostettmann K, editor Methods in Plant Biochemistry; Assay for Bioactivity. Vol. 6. San Diego, CA: Academic Press; 1991. p. 1-32.
21. Xue Z, Li S, Wang S, Wang Y, Yang Y, Shi J, *et al*. Mono-, Bi-, and triphenanthrenes from the tubers of *Cremastra appendiculata.* J Nat Prod 2006;69:907-13.
22. Mushi NF, Mbwambo ZH, Innocent E, Tewtrakul S. Antibacterial, anti-HIV-1 protease and cytotoxic activities of aqueous ethanolic extracts from *Combretum adenogonium* Steud. Ex A. Rich (Combretaceae). BMC Complement Altern Med 2012;12:163.
23. Khamko VA, Quang DN, Dien PH. Three new phenanthrenes, a new stilbenoid isolated from the roots of *Stemona tuberosa* Lour. and their cytotoxicity. Nat Prod Res 2013;27:2328-32.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141028022407

Original Research

ScopeMed

Acute and subchronic antihyperglycemic activities of *Bowdichia virgilioides* roots in non-diabetic and diabetic rats

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Received: October 15, 2014
Accepted: October 28, 2014
Published: November 28, 2014

ABSTRACT

Aim: The present study was undertaken to evaluate the acute and subchronic antihyperglycemic effects of methanolic extract of *Bowdichia virgilioides* root bark of *B. virgilioides* in streptozotocin (STZ)-induced diabetic rats. **Materials and Methods:** The extract (100, 250 or 500 mg/kg) was orally administered to male Wistar diabetic (STZ, 42 mg/kg i.v.) and non-diabetic rats into two main protocols: (i) subchronic experiments, where animals were treated for 21 days with *B. virgilioides* extract and the following parameters were evaluated: Body weight, fluid and food intake (determined daily), urinary glucose and urea (every 3 days) and glycemia (every 5 days). At the end of the experimental period, skeletal muscles (extensor digitorum longus [EDL] and soleus), retroperitoneal and epididymal white adipose tissues were collected and weighed; liver samples were used for the determination of the lipid and glycogen contents; (ii) acute experiments, which evaluated the alterations on fasting and post-prandial glycemia and on glucose tolerance using the oral glucose tolerance test (OGTT). **Results:** In subchronic experiments, the treatment with *B. virgilioides* extract did not change any parameter evaluated in diabetic and non-diabetic animals. On fasting and post-prandial glycemia, the extract treatment did not promote changes in the glycemia values in diabetic or non-diabetic animals. In OGTT, the treatment with 500 mg/kg *B. virgilioides* extract reduced the hyperglycemia peak after a glucose overload, when compared with non-treated diabetic animals, resulting in a lower area under curve. **Conclusion:** The results of our work indicate that *B. virgilioides* root extract promotes an acute antihyperglycemic effect in STZ-diabetic rats; this effect probably occurs through an inhibition of the intestinal glucose absorption. The continuity of the research is necessary to elucidate these possibilities.

KEY WORDS: Antihyperglycemic effect, antihyperglycemic plants, *Bowdichia virgilioides*, fabaceae, medicinal plants

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders characterized by chronic hyperglycemia in the postprandial and/or fasting states. In addition, other disturbances such as dyslipidemia, obesity and oxidative stress are commonly involved in the development of the diabetic complications that may lead to a premature mortality. These alterations are mainly caused by insulin deficiency and/or insulin resistance. Based on studies in several countries, the World Health Organization and the American Diabetes Association have estimated that there will be 285 million people worldwide with diabetes in 2010 and projected to rise to 439 million in 2030 [1], indicating a situation of "epidemic diabetes." This rise can be attributed to several

factors, such as population growth, urbanization, changing lifestyle (sedentary daily life and increased consumption of energy-rich diets) and an increased prevalence of obesity among population as it represents a main risk factor for incidence of diabetes [2-4].

The available therapeutic approach to diabetes mellitus attempts to maintain the glycemia values close to normality and is based on diet, oral hypoglycemic drugs and insulin administration, used independently or in combination [5,6]. Despite the availability of insulin and multiple classes of hypoglycemic agents, several adverse effects are observed with the use of these drugs, such as hypoglycemia, liver dysfunction, lactic acidosis and others. In addition, the excessive cost of the diabetes treatment is another disadvantage. These negative aspects in the conventional diabetes therapy stimulate the use of alternative and/or complementary medicines, for example, the treatment with herbal preparations and/or constituents. In traditional practices, plant-based medicinal products are known since ancient times and have been used to control diabetes around the world.

World ethnobotanical information about medicinal plants reports almost 800 plants used in the control of diabetes mellitus [7-9]. More recently, in addition to the pharmacological diabetic animal models and classical methodologies used to the selection of plants with antidiabetic effect, the ethnopharmacological studies have been applied a diversity of novel approaches and refined assays to elucidate the mechanism of action that explains their hypoglycemic activity. Among these, it can be highlighted: studies in cell cultures [10,11], investigation of changes in components of the insulin intracellular signaling [12,13] and the use of genetically diabetic animal models [14,15]. These studies will generate essential information useful to the advances in the development of novel antidiabetic terapy.

Bowdichia virgilioides belongs to Fabaceae family and is a plant commonly distributed in the Amazonian lowlands of Brazil, Bolivia and up north to Central America, typically in savanna fields; it is known by the popular names: *Sucupira-do-cerrado, sucupira-do-campo, angelim-amargoso, coração-de-negro*. *B. virgilioides* preparations have also been used in popular medicine for the treatment of leishmaniasis and malaria [16,17]. Almeida *et al*. [18] have demonstrated that essential oil from *B. virgilioides* leaves presented antimicrobial activity against several pathogenic microorganisms. Although the use of *B. virgilioides* to treat diabetes was previously cited by Oliveira and Saito [19], describing its popular preparation to the complementary treatment of this disease, no other studies have been carried out to confirm the beneficial effect of this plant species in biochemical or physiological parameters altered in diabetes.

Chemical studies with *B. virgilioides* have revealed the presence of alkaloids, terpenoids and benzofuran derivatives in the stem bark [20-23], volatile constituents, essential oils, flavonoids and isoflavonoids in roots [24-27], tannins in inner bark and leaves [28] and geraniol, caryophyllene and anthocyanin in fruits [29,30]. Some of these constituents have been related with the antidiabetic activity of other plant species, for example, flavonoids [31,32], terpenoids [33,34], anthocyanins [35] and gallotannins [36].

In this way, the present study was undertaken to evaluate the acute and subchronic antihyperglycemic effects of methanolic extract of *B. virgilioides* root bark of *B. virgilioides* in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Materials

Root bark of *B. virgilioides* was collected in the Poconé region (S15 47 159; W56 21 345; S 16 18 112; W 056 33 952), Mato Grosso state, Brazil in February of 2006. The plant was identified, and a voucher specimen (number 24,600) was stored at the Central Herbarium of the Federal University of Mato Grosso (UFMT).

Preparation of the *B. virgilioides* Methanolic Extract (BvMeOH)

The air-dried and ground *B. virgilioides* roots (4.85 kg) were macerated (7 days) and extracted, sequentially, with hexane (1 × 3 L/kg) and methanol (4 × 3 L/kg), respectively. The BvMeOH (350 g; 7.2%) was obtained after filtration and removal of the solvent under reduced pressure. The powdered BvMeOH was stored at 4°C and dissolved to the desired concentration prior to use.

Animals

Male Swiss-Webster mice (25-30 g) and Wistar rats (180-210 g) were housed in a room under standard laboratory conditions (12:12 hour light-dark cycle, 24±1°C) and were given free access to water and normal lab chow diet (Purina® Labina). All experiments took place between 08:00 and 10:00 a.m. The animals were managed according to the Brazilian College of Animal Experimentation and approved by the Committee for Ethics in Animal Experimental of the UFMT (no: 23108.043016/10-6).

Acute Toxicity Test

Groups of male mice received orally by gavage a single administration of BvMeOH in different concentrations: 100, 250, 500, 1000, 2000, 3000, 4000 or 5000 mg/kg. The control group received vehicle (1% Tween 80). The animals were individually observed during 0, 5, 10, 15 and 30 minutes; 1, 2, 4 and 8 h and during 1 week (one time a day) after BvMeOH or vehicle administration. The behavior alterations observed were registered in a table adapted from Malone's study [37].

Induction of Diabetes

STZ, 42 mg/kg; Sigma Aldrich, St. Louis, MO, USA dissolved in 0.01 mol/L citrate buffer (pH 4.5) was administered by a single intravenous injection in previously 16 hours fasted rats. Five days

after STZ administration, animals with post-prandial glycemia between 350 and 500 mg/mL were used in the experiments. Non-diabetic control (NC) animals received a citrate buffer injection.

Subchronic Experiments

Five days after STZ or citrate buffer injection, the animals were divided into the following groups: DC group – diabetic control (DC) rats; DT100 group – diabetic rats treated with 100 mg/kg of BvMeOH; DT250 group – diabetic rats treated with 250 mg/kg of BvMeOH; DT500 group – diabetic rats treated with 500 mg/kg of BvMeOH; DG group – diabetic rats treated with glibenclamide; DI group – diabetic rats treated with insulin; NC group – NC rats; NT100 group – non-diabetic rats treated with 100 mg/kg of BvMeOH; NT250 group – non-diabetic rats treated with 250 mg/kg of the BvMeOH and NT500 group – non-diabetic rats treated with 500 mg/kg of the BvMeOH. The extract was dissolved in 1% Tween 80. The diabetic and non-diabetic groups (except DI), received the BvMeOH and/or Tween 80 (controls) orally by gavage, once a day, during 22 days. DG group received 2mg/kg of glibenclamide at 9:00 a.m. and 6:00 p.m., from day 5 to day 21. DI received 3 U/rat of Insulin NPH – Lilly (s.c.), at 9:00 a.m. and 6:00 p.m., from day 5 to day 21.

Body weight, fluid and food intake were determined daily, urinary glucose and urea every 3 days and glycemia every 5 days. Blood samples for plasma glucose determination were collected from the tip of the tail. At the end of the experimental period, rats were anesthetized and sacrificed by decapitation, and samples of the free running blood were collected for glucose measurement. skeletal muscles (EDL and soleus), retroperitoneal and epididymal white adipose tissues were collected and weighed. Hepatic lipid and glycogen contents were determined. Urinary glucose was determined using the dinitrosalicylic acid (Sigma Aldrich, St. Louis, MO, USA) reaction [38], urinary urea by the urease reaction (Labtest® kit) and plasma glucose was measured by the glucose oxidase method (Labtest® kit). Hepatic glycogen was determined after acidic hydrolysis and titration for monomeric glucose that was quantified with anthrone reagent [39]. The liver lipid content was determined by gravimetric method after extraction as described by Folch *et al*. [40].

Oral Glucose Tolerance Test (OGTT)

The OGTT was performed in overnight fasted NC, DC and normal and diabetic 100, 250 and 500 mg/kg extract-treated animals (NT100, NT250, NT500, DT100, DT250, DT500). The control groups received 1% Tween 80. All animals received an oral load of glucose (2.5 g/kg). The BvMeOH or vehicle was administered at same time as glucose. Plasma glucose was measured in blood withdrawn from the tip of the tail, before load ($t = 0$) and after 15, 30, 45, 60, 75 and 90 min.

Fasting and Post-prandial Glycemia

To evaluate the effect of BvMeOH on fasting and post-prandial glycemia, the blood glucose level of each rat was determined at the beginning of the experiment. In the fasting glycemia determination, blood samples were collected after an overnight fasting (14 h). The same groups and proceedings were done after feeding of animals to evaluate the extract effects on post-prandial glycemia. The different doses of BvMeOH administered to normal and diabetic rats were the same as previously described.

Statistical Analysis

Data were expressed as mean ± standard error of mean. Statistical analysis was performed using the Statistic Software package (Statsoft, Tulsa, OK, USA). Bartlett's test for the homogeneity of variances was initially used to determine whether the data complied with the assumptions for parametric analysis of variance (ANOVA). The one-way ANOVA was employed to analyze the data between treated groups and their respective control groups (diabetic or non-diabetic). In the subchronic experiment the summed data of each parameter during statistical evaluation were compared. Glycemia areas under curves (AUC) were compared. Differences were considered significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

RESULTS

Acute Toxicity Study

Normal mice treated with BvMeOH in doses ranging from 100 to 500 mg/kg did not present any behavior alteration. A dose of 1000 mg/kg reduced the motility of animals 15 min after its administration. At higher doses (2000, 3000, 4000 and 5000 mg/kg), it was initially observed palpebral ptosis and subsequent somnolence that persisted until 1 h after the treatment. No deaths were observed in this assay. Based on these results the doses of 100, 250 and 500 mg/kg were chosen for the antihyperglycemic assays.

Subchronic Treatment

The subchronic treatment with BvMeOH for 21 days did not reduce the high post-prandial glycemia of diabetic rats. The other biochemical parameters evaluated (liver glycogen and lipid content, urinary glucose and urinary urea) did not also differ between diabetic groups treated or not with BvMeOH [Tables 1 and 3]. The extract did not alter the lower body weight gain of diabetic, as well as the food and liquid intake, the urinary volume [Table 2] and the weights of skeletal muscles and white adipose tissues [Table 3]. The treatment with insulin and glibenclamide improved all the parameters described above. In the same way, the BvMeOH treatment did not change any parameter in normal, non-diabetic animals (data not shown).

Acute Treatment

The BvMeOH did not alter the fasting and post-prandial glycemia values in normal and diabetic groups [Figures 1c-1f]. In OGTT, the treatment with 500 mg/kg BvMeOH reduced the hyperglycemia peak after a glucose overload when compared

Table 1: Effects of *B. virgilioides* root bark extract on plasma glucose, urinary glucose and urinary urea of non-diabetic, DC, DT100, DT250, DT500, DI, and DG groups in subchronic experiment during 21 days

Groups	1	6	11	16	21
Plasma glucose (mg/dL)					
NC	153±6	146±5	145±5	146±5	157±5
DC	482±40	555±54	519±47	489±16	585±31
DT100	468±12	437±62	394±53	458±46	614±63
DT250	461±20	382±54	404±53	429±51	476±75
DT500	511±15	597±26	564±31	520±48	475±60
DI (3 U/rat)	461±90	470±92	201±33*	208±40#	214±49#
DG (2 g/kg)	415±67	338±59	321±55*	326±59*	299±49#
Urinary glucose (mg/24 h)					
DC	8.14±0.90	9.29±1.06	8.48±1.25	9.53±1.77	10.45±0.60
DT100	8.00±1.07	8.14±1.42	9.31±1.38	8.20±0.96	9.52±0.90
DT250	8.56±0.93	9.54±1.31	9.68±0.86	10.16±0.74	10.14±1.00
DT500	9.00±1.20	10.73±0.76	7.26±0.79	6.10±1.09	6.81±1.00
DI (3 U/rat)	9.33±0.68	9.22±1.52	0.05±0.03##	0.16±0.08##	0.05±0.03##
DG (2 g/kg)	4.81±1.47	4.51±1.61	3.97±1.40#	4.92±1.67#	4.55±1.50#
Urinary urea (g/24 h)					
NC	0.43±0.03	0.42±0.05	0.59±0.04	0.63±0.06	0.74±0.03
DC	1.27±0.15	1.48±0.17	1.56±0.13	1.44±0.17	1.64±0.15
DT100	1.29±0.08	1.40±0.17	1.67±0.13	1.47±0.13	1.94±0.34
DT250	1.16±0.09	1.28±0.16	1.47±0.10	1.57±0.07	1.69±0.27
DT500	1.57±0.23	1.57±0.16	1.13±0.10	0.98±0.12	0.94±0.14
DI (3 U/rat)	1.43±0.02	1.33±0.06	1.03±0.07	1.02±0.11	0.86±0.14*
DG (2 g/kg)	0.97±0.18	1.09±0.19	1.17±0.15	1.33±0.16	1.14±0.16

B. virgilioides: Bowdichia virgilioides, NC: Non-diabetic control, DC: Diabetic control, DT100: Diabetic treated with 100 mg/kg extract, DT250: Diabetic treated with 250 mg/kg extract, DT500: Diabetic treated with 500 mg/kg extract, DI: Diabetic treated with insulin, DG: Diabetic treated with glibenclamide, SEM: Standard error of the mean, ANOVA: Analysis of variance. The values are expressed as mean±SEM of 5-8 animals. *$P<0.05$; #$P<0.01$; ##$P<0.001$ versus DC (ANOVA one-way)

Table 2: Effects of *B. virgilioides* root bark extract on body weight, food and liquid intake and urinary volume of non-diabetic, DC, DT100, DT250, DT500, DI, and DG groups in subchronic experiment during 21 days

Groups	Body weight (g)		Food intake (g)	Liquid intake (mL)	Urinary volume (mL)
	Initial	Final			
NC	198±3	318±8*	513±10*	741±17*	128±12*
DC	194±9	206±15	791±27	3194±280	2350±261
DT100	207±4	243±6	755±30	3029±332	2207±276
DT250	203±3	258±6	778±31	3203±247	2335±218
DT500	199±6	202±11	730±37	3266±198	2340±183
DI (3 U/rat)	191±7	285±17#	567±9##	1538±67#	1023±318##
DG (2 g/kg)	213±3	295±12#	630±44#	1674±332#	1670±129#

The values are expressed as mean±SEM of 5-8 animals. *$P<0.05$, #$P<0.01$, ##$P<0.001$ versus DC (ANOVA one-way). *B. virgilioides: Bowdichia virgilioides*, NC: Non-diabetic control, DC: Diabetic control, DT100: Diabetic treated with 100 mg/kg extract, DT250: Diabetic treated with 250 mg/kg extract, DT500: Diabetic treated with 500 mg/kg extract, DI: Diabetic treated with insulin, DG: Diabetic treated with glibenclamide, SEM: Standard error of the mean, ANOVA: Analysis of variance

with DC rats [Figure 1a]. Therefore, the AUC of DT500 group was significantly lower than of DC and DT250 groups, although it was higher than the AUC of NC group. The AUC of normal rats treated with BvMeOH, at any tested dose, was not different in comparison with normal, non-treated rats [Figure 1b].

DISCUSSION

The results obtained in this study showed that, although *B. virgilioides* extract did not change any parameter evaluated in diabetic rats after the subchronic experiment, the extract showed an acute antihyperglycemic effect (500 mg/kg), reducing the hyperglycemia peak when a glucose load was administered to diabetic animals.

Diabetes experimental model with STZ-induced animals is widely used for screening of compounds including natural products with different antidiabetic activities. After STZ administration, the drug is taken up by pancreatic beta cells through glucose transporter type 2 (GLUT2), promoting irreversible destruction of these cells by two main mechanisms: DNA alkylation, which promotes its fragmentation, and exposition of beta cells to reactive oxygen species damage. The final result is degranulation and/or reduction of insulin secretion [41,42]. Although insulin treatment has become one of the most important therapeutic agents to ameliorate symptoms of type I diabetes, there are continued efforts to find insulin substitutes, mainly from plant sources. Traditional herbal remedies prescription instead of conventional treatment can be justified by some of its advantages, for example, their effectiveness, no or few side-effects and relatively low cost. Therefore, several studies have focused on the comprehension of the ethnopharmacological, phytochemical and clinical importance of medicinal plants for diabetes treatment.

Studies with herbal remedies have shown that several mechanisms are involved, isolated or combined, in the promotion of its antidiabetic effect: stimulation of pancreatic cells insulin release, reduction of liver glucose production, enhancement of glucose uptake by peripheral tissues and inhibition of intestinal glucose absorption. In this way, it was

Table 3: Effects of *B. virgilioides* root bark extract on retroperitoneal, epididymal and perirenal adipose tissue weight, liver and soleus and EDL muscles weight and glycogen and lipid liver content of NC, DC, DT100, DT250, DT500, DI, and DG groups in subchronic experiment during 21 days

Groups	Retroperitoneal weight (g)	Epididymal weight (g)	Perirenal weight (g)	Liver weight (g)	Soleus weight (g)	EDL weight (g)	Liver glycogen content (mg/g)	Liver lipid content (mg/g)
NC	2.42±0.42	2.71±0.25	0.70±0.12	11.95±0.82	0.28±0.01	0.26±0.02	25.17±3.45	48.55±0.80
DC	0.51±0.21	0.91±0.18	0.10±0.04	11.58±0.94	0.23±0.02	0.19±0.02	8.82±2.77	41.24±3.02
DT100	0.42±0.18	0.99±0.19	0.09±0.03	12.85±0.76	0.22±0.02	0.20±0.02	5.92±3.29	44.68±1.49
DT250	0.48±0.09	1.16±0.09	0.13±0.04	12.68±0.36	0.22±0.01	0.20±0.01	7.47±3.66	45.96±1.80
DT500	0.46±0.23	0.92±0.16	0.09±0.03	10.85±1.03	0.18±0.01	0.17±0.01	8.32±2.61	38.87±1.90
DI (3 U/rat)	2.23±0.42$^{\#\#}$	2.54±0.68$^{\#\#}$	0.45±0.11$^{\#}$	12.85±0.73	0.24±0.01	0.25±0.01	20.12±3.02$^{\#\#}$	45.50±1.10
DG (2 g/kg)	1.22±0.41$^{\#}$	1.80±0.27$^{\#}$	0.23±0.06*	13.37±0.40	0.25±0.01	0.25±0.01	19.64±5.15$^{\#\#}$	40.03±7.03

EDL: Extensor digitorum longus, *B. virgilioides: Bowdichia virgilioides*, NC: Non-diabetic control, DC: Diabetic control, DT100: Diabetic treated with 100 mg/kg extract, DT250: Diabetic treated with 250 mg/kg extract, DT500: Diabetic treated with 500 mg/kg extract, DI: Diabetic treated with insulin, DG: Diabetic treated with glibenclamide, SEM: Standard error of the mean, ANOVA: Analysis of variance. The values are expressed as mean±SEM of 5-8 animals. *$P<0.05$, $^{\#}P<0.01$, $^{\#\#}P<0.001$ versus DC (ANOVA one-way)

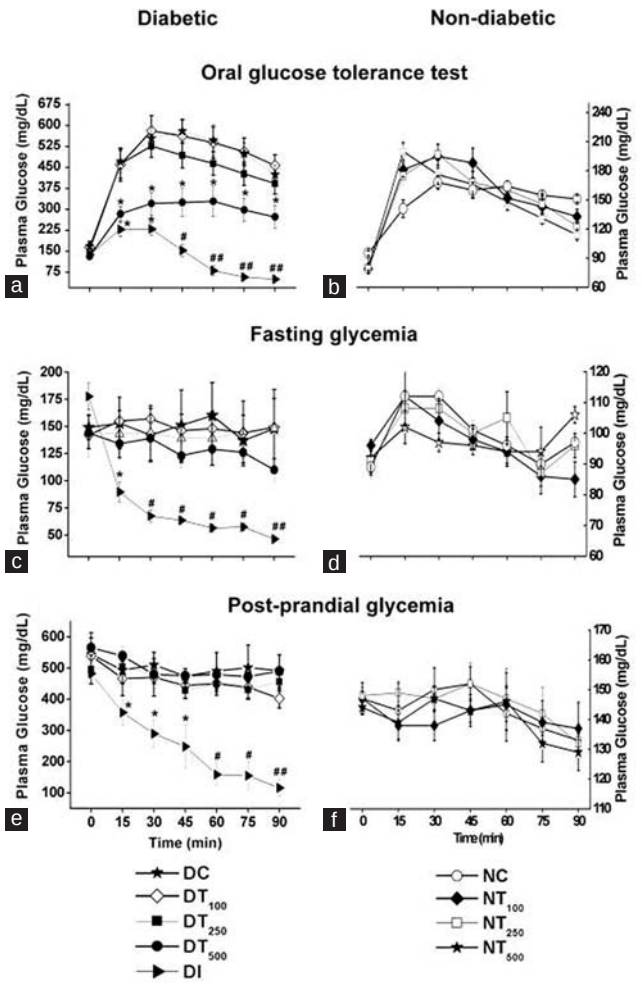

Figure 1: Effects of *Bowdichia virgilioides* root bark extract on oral glucose tolerance (a and b), fasting glycemia (c and d), and post-prandial glycemia (e and f) of diabetic control (DC) and non-diabetic control, diabetic and non-diabetic treated with 100 mg/kg extract, diabetic and non-diabetic treated with 250 mg/kg extract diabetic and non-diabetic treated with 500 mg/kg extract, diabetic treated with insulin. Values are expressed as mean ± standard error of the mean of 5-8 animals. *$P < 0.05$, $^{\#}P < 0.01$, $^{\#\#}P < 0.001$ versus DC (one-way analysis of variance)

recently demonstrated that the root bark of *Paeonia suffruticosa* presented an *in vitro* antidiabetic effect through inhibition of glucose uptake by intestinal brush border membrane vesicles and enhancement of the glucose uptake by culture fibroblasts and adipocytes cells [43]. This same study also demonstrated that peonol, an active compound isolated from non-polar fraction of *Paeonia suffruticosa* root bark, had an *in vivo* beneficial effect, with amelioration of oral glucose tolerance in diabetic rats, corroborating the study from Jung *et al.* [44] that demonstrated the antihyperglycemic effect of this plant. Many other studies showed significant improvement in the glucose tolerance of STZ-diabetic rats treated with different plant extracts, for example *Plantago ovata* husks [45], *Sclerocarya birrea* stem bark [46] and *Ipomoea aquatica* leaf stem [47]. According to these studies, the antihyperglycemic effect could be achieved, at least in part, by inhibition of intestinal glucose absorption. In this same way, the present work showed that the administration of *B. virgilioides* root extract in association with a glucose loading markedly reduced the hyperglycemia peak in diabetic rats. The absence of effects on fasting and post-prandial glycemia values and in subchronic treatment suggested that the plant extract probably exerts its effect via inhibition of the glucose intestinal absorption, and might not have effect on glucose uptake by peripheral tissues. However, changes in the glucose tolerance were not observed in normal rats treated with *B. virgilioides* extract.

Medicinal plants acting at intestinal level to promote the antihyperglycemic effect may inhibit glucose absorption at two distinct targets: (i) classical carbohydrate absorption mediated by the Na+/glucose co-transporter, and (ii) facilitative transport through GLUT2 present in the apical membrane [48]. It has been shown that insulin reduces the GLUT2 quantity in both apical and basolateral enterocyte membranes, promoting a rapid traffic of this glucose transporter away from the plasma membrane and preventing GLUT2 insertion into the apical membrane, independently of the glucose amount in the luminal intestine [49]. Furthermore, apical membrane GLUT2 is dramatically increased in an experimental diabetes model characterized by insulinopenia [50], and changes in GLUT2 quantity in the apical enterocyte membrane of

diabetic rats treated with antihyperglycemic plant extracts cannot be ruled out. In agreement with this present work, the glycemia reduction observed in diabetic rats treated with *B. virgilioides* extract could be explained, at least in part, by a decrease of the large GLUT2 number in the apical enterocyte membrane. The same effect was not observed after the treatment of normal rats, which possibly have the regulation of this glucose transporter by insulin under regular conditions. Similar results were found by Ndong *et al*. [51], which examined the effects of *Moringa oleifera* extract on the glucose tolerance in normal Wistar rats and Goto-Kakizaki diabetic rats. *Moringa oleifera* oral administration ameliorated the glycemia in both rats, but the beneficial effect promoted by plant treatment was more evident in Goto-Kakizaki diabetic rats than in normal Wistar rats. Further studies are needed to clarify the different responses between normal and diabetic rats in the antihyperglycemic effect of *B. virgilioides* extract, mainly in the GLUT2 regulation at enterocyte membrane. Finally, another mechanism by which this extract could be promoting a decrease in the glucose intestinal absorption is through enhanced intestinal motility, a mechanism that could be more investigated.

The chemical characterization of *B. virgilioides* extract indicated the presence of alkaloids, flavonoids and terpenoids (unpublished data). Since flavonoids and terpenoids are associated with antidiabetic activity of several plant species, it could be speculated that the antihyperglycemic effect of *B. virgilioides* extract is related to these compounds. Complementary studies are necessary to clarify this hypothesis.

The results of our work indicate that *B. virgilioides* root extract promotes an acute antihyperglycemic effect in STZ-diabetic rats; this effect probably occurs through an inhibition of the intestinal glucose absorption. The continuity of the research is necessary to elucidate these possibilities. Although several studies have demonstrated the antidiabetic properties of medicinal plants, the use of phytochemicals in diabetes treatment has not been validated with scientific criteria that support their substitution for the conventional therapy. This indicates that, in addition to studies showing the plant antihyperglycemic activity, other approaches are necessary to explore more profoundly these findings, which will help to consolidate the use of medicinal plants in the treatment of diabetes mellitus.

ACKNOWLEDGMENTS

We thank the technicians Air Francisco Costa, Marlene Mariano and Libério Neto for their help and Msc. Harri Lourenzi of the Plantarum Institute for identification and authentication of *Bowdichia virgilioides*. We are also grateful for the support given by Fundação de Amparo à Pesquisa do Estado de Mato Grosso (Cuiabá, Brazil), Coordenação de Aperfeiçoamento de Pessoal Superior (Brasília, Brazil), Conselho Nacional de Pesquisa Científica (CNPq) and Centro de Pesquisa do Pantanal (CPP) for financial support.

REFERENCES

1. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract 2010;87:4-14.
2. Caballero B, Rubinstein S. Environmental factors affecting nutritional status in urban areas of developing countries. Arch Latinoam Nutr 1997;47:3-8.
3. Seidell JC. Obesity, insulin resistance and diabetes – A worldwide epidemic. Br J Nutr 2000;83 Suppl 1:S5-8.
4. Misra A, Khurana L. Obesity and the metabolic syndrome in developing countries. J Clin Endocrinol Metab 2008;93:S9-30.
5. Warren RE. The stepwise approach to the management of type 2 diabetes. Diabetes Res Clin Pract 2004;65 Suppl 1:S3-8.
6. Cohen A, Horton ES. Progress in the treatment of type 2 diabetes: New pharmacologic approaches to improve glycemic control. Curr Med Res Opin 2007;23:905-17.
7. Ivorra MD, Payá M, Villar A. A review of natural products and plants as potential antidiabetic drugs. J Ethnopharmacol 1989;27:243-75.
8. Alarcon-Aguilara FJ, Roman-Ramos R, Perez-Gutierrez S, Aguilar-Contreras A, Contreras-Weber CC, Flores-Saenz JL. Study of the anti-hyperglycemic effect of plants used as antidiabetics. J Ethnopharmacol 1998;61:101-10.
9. Yeh GY, Eisenberg DM, Kaptchuk TJ, Phillips RS. Systematic review of herbs and dietary supplements for glycemic control in diabetes. Diabetes Care 2003;26:1277-94.
10. Alonso-Castro AJ, Salazar-Olivo LA. The anti-diabetic properties of *Guazuma ulmifolia* Lam are mediated by the stimulation of glucose uptake in normal and diabetic adipocytes without inducing adipogenesis. J Ethnopharmacol 2008;118:252-6.
11. Tamrakar AK, Kumar R, Sharma R, Balapure AK, Lakshmi V, Srivastava AK. Stimulatory effect of *Ceriops tagal* on hexose uptake in L6 muscle cells in culture. Nat Prod Res 2008;22:592-9.
12. Wang ZQ, Ribnicky D, Zhang XH, Raskin I, Yu Y, Cefalu WT. Bioactives of *Artemisia dracunculus* L enhance cellular insulin signaling in primary human skeletal muscle culture. Metabolism 2008;57:S58-64.
13. Baviloni PD, dos Santos MP, Aiko GM, Reis SR, Latorraca MQ, da Silva VC, *et al*. Mechanism of anti-hyperglycemic action of *Vatairea macrocarpa* (Leguminosae): Investigation in peripheral tissues. J Ethnopharmacol 2010;131:135-9.
14. Shinde J, Taldone T, Barletta M, Kunaparaju N, Hu B, Kumar S, *et al*. Alpha-glucosidase inhibitory activity of *Syzygium cumini* (Linn.) Skeels seed kernel *in vitro* and in *Goto-Kakizaki* (GK) rats. Carbohydr Res 2008;343:1278-81.
15. Vianna R, Brault A, Martineau LC, Couture R, Arnason JT, Haddad PS. *in vivo* anti-diabetic activity of the ethanolic crude extract of *Sorbus decora* C.K. Schneid. (Rosacea): A medicinal plant used by Canadian james bay cree nations to treat symptoms related to diabetes. Evid Based Complement Alternat Med 2011;2011:237941.
16. Bourdy G, DeWalt SJ, Chávez de Michel LR, Roca A, Deharo E, Muñoz V, *et al*. Medicinal plants uses of the Tacana, an Amazonian Bolivian ethnic group. J Ethnopharmacol 2000;70:87-109.
17. Deharo E, Bourdy G, Quenevo C, Muñoz V, Ruiz G, Sauvain M. A search for natural bioactive compounds in Bolivia through a multidisciplinary approach. Part V. Evaluation of the antimalarial activity of plants used by the Tacana Indians. J Ethnopharmacol 2001;77:91-8.
18. Almeida JR, Silva-Filho RN, Nunes XP, Dias CS, Pereira FO, Lima EO. Antimicrobial activity of the essential oil of *Bowdichia virgilioides*, Kunt. Braz J Pharmacol 2000;16:638-41.
19. Oliveira F, Saito ML. Some Brazilian plants employed in diabetes treatment. Braz J Pharmacol 1989;2/4:170-96.
20. Torrenegra R, Bauereiss P, Achenbach H. Homoormosanine-type alkaloids from *Bowdichia virgilioides*. Phytochem 1989;28:2219-21.
21. Marinho LC, Cunha MT, Thomas G, Barbosa-Filho JM. Constituents of *Bowdichia virgilioides*. Fitoterapia 1994;65:475-6.
22. Melo FN, Navarro VR, Silva MS, Da-Cunha EV, Barbosa-Filho JM, Braz-Filho R. Bowdenol, a new 2,3-dihydrobenzofuran constituent from *Bowdichia virgilioides*. Nat Prod Lett 2001;15:261-6.
23. Barbosa-Filho JM, Da Silva Almeida JR, De Oliveira Costa VC, Da-Cunha EV, Da Silva MS, Braz-Filho R. Bowdichine, a new diaza-adamantane alkaloid from *Bowdichia virgilioides*. J Asian Nat Prod Res 2004;6:11-7.
24. Arriaga MC, Machado MI, Gomes GA, Craveiro AA. Volatile constituents from roots of *Bowdichia virgilioides*, Kunt. J Essent Oil Res 1998;10:205-6.

25. Velozo LS, Silva BP, Silva BE, Parente JP. Constituents from the roots of *Bowdichia virgilioides*. Fitoterapia 1999;70:532-535.
26. Arriaga AM, Gomes GA, Braz-Filho R. Constituents of *Bowdichia virgilioides*. Fitoterapia 2000;71:211-2.
27. Juck DB, De Rezende LC, David JP, De Queiroz LP, David JM. Two new isoflavonoids from *Bowdichia virgilioides*. Nat Prod Res 2006;20:27-30.
28. Thomazzi SM, Silva CB, Silveira DC, Vasconcellos CL, Lira AF, Cambui EV, *et al*. Antinociceptive and anti-inflammatory activities of *Bowdichia virgilioides* (sucupira). J Ethnopharmacol 2010;127:451-6.
29. Mell CD. Interesting sources of natural dyestuffs. Text Colorist 1929;51:453-5.
30. Jorge-Neto J. Pharmacognostic study of essential oil of sucupira, *Bowdichia virgilioides*. Ver Fac Farm Odontol Araraquara 1970;4:203-4.
31. Marzouk MS, Soliman FM, Shehata IA, Rabee M, Fawzy GA. Flavonoids and biological activities of Jussiaea repens. Nat Prod Res 2007;21:436-43.
32. Sharma B, Balomajumder C, Roy P. Hypoglycemic and hypolipidemic effects of flavonoid rich extract from *Eugenia jambolana* seeds on streptozotocin induced diabetic rats. Food Chem Toxicol 2008;46:2376-83.
33. Tan MJ, Ye JM, Turner N, Hohnen-Behrens C, Ke CQ, Tang CP, *et al*. Antidiabetic activities of triterpenoids isolated from bitter melon associated with activation of the AMPK pathway. Chem Biol 2008;15:263-73.
34. Lü H, Chen J, Li WL, Ren BR, Wu JL, Kang HY, *et al*. Hypoglycemic and hypolipidemic effects of the total triterpene acid fraction from Folium Eriobotryae. J Ethnopharmacol 2009;122:486-91.
35. Grace MH, Ribnicky DM, Kuhn P, Poulev A, Logendra S, Yousef GG, *et al*. Hypoglycemic activity of a novel anthocyanin-rich formulation from lowbush blueberry, *Vaccinium angustifolium* Aiton. Phytomedicine 2009;16:406-15.
36. Klein G, Kim J, Himmeldirk K, Cao Y, Chen X. Antidiabetes and Anti-obesity Activity of Lagerstroemia speciosa. Evid Based Complement Alternat Med 2007;4:401-7.
37. Malone MH, Robichaud RC. A hippocratic screen for pure or crude drug materials. Lloydia 1962;25:320-332.
38. Summerson WH, Hank PB, Oser BL. Practical Physiological Chemistry, 12th ed. New York: Blakiston Co.; 1947.
39. Carroll NV, Longley RW, Roe JH. The determination of glycogen in liver and muscle by use of anthrone reagent. J Biol Chem 1956;220:583-93.
40. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.
41. Szkudelski T. The mechanism of alloxan and streptozotocin action in beta cells of the rat pancreas. Physiol Res 2001;50:536-46.
42. Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia 2008;51:216-26.
43. Lau CH, Chan CM, Chan YW, Lau KM, Lau TW, Lam FC, *et al*. Pharmacological investigations of the anti-diabetic effect of cortex moutan and its active component paeonol. Phytomedicine 2007;14:778-84.
44. Jung CH, Zhou S, Ding GX, Kim JH, Hong MH, Shin YC, *et al*. Antihyperglycemic activity of herb extracts on streptozotocin-induced diabetic rats. Biosci Biotechnol Biochem 2006;70:2556-9.
45. Hannan JM, Ali L, Khaleque J, Akhter M, Flatt PR, Abdel-Wahab YH. Aqueous extracts of husks of Plantago ovata reduce hyperglycaemia in type 1 and type 2 diabetes by inhibition of intestinal glucose absorption. Br J Nutr 2006;96:131-7.
46. Dimo T, Rakotonirina SV, Tan PV, Azay J, Dongo E, Kamtchouing P, *et al*. Effect of *Sclerocarya birrea* (Anacardiaceae) stem bark methylene chloride/methanol extract on streptozotocin-diabetic rats. J Ethnopharmacol 2007;110:434-8.
47. Sokeng SD, Rokeya B, Hannan JM, Junaida K, Zitech P, Ali L, *et al*. Inhibitory effect of Ipomoea aquatica extracts on glucose absorption using a perfused rat intestinal preparation. Fitoterapia 2007;78:526-9.
48. Kellett GL, Brot-Laroche E, Mace OJ, Leturque A. Sugar absorption in the intestine: The role of GLUT2. Annu Rev Nutr 2008;28:35-54.
49. Tobin V, Le Gall M, Fioramonti X, Stolarczyk E, Blazquez AG, Klein C, *et al*. Insulin internalizes GLUT2 in the enterocytes of healthy but not insulin-resistant mice. Diabetes 2008;57:555-62.
50. Burant CF, Flink S, DePaoli AM, Chen J, Lee WS, Hediger MA, *et al*. Small intestine hexose transport in experimental diabetes. Increased transporter mRNA and protein expression in enterocytes. J Clin Invest 1994;93:578-85.
51. Ndong M, Uehara M, Katsumata S, Suzuki K. Effects of oral administration of *Moringa oleifera* Lam on glucose tolerance in goto-kakizaki and wistar rats. J Clin Biochem Nutr 2007;40:229-33.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141211071136

Original Research

ScopeMed

Evaluation of the antioxidant activity and the healing action of the ethanol extract of *Calotropis procera* bark against surgical wounds

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Received: November 21, 2014
Accepted: December 11, 2014
Published: January 03, 2015

ABSTRACT

The objective of the present study was to evaluate the antioxidant potential and the wound healing effect of the ethanolic extract of the bark of *Calotropis procera*. The antioxidant study was evaluated *in vitro,* using 2,2-diphenylpicrylhydrazyl (DPPH) and deoxyribose degradation assays. Wound healing was studied using excision and incision wound on normal and dexamethasone-suppressed wound healing rodent models. Alkaloids, flavonoids, proteins and phenols were screened in the extract used whereas saponins and true tannins were absent. The extract contains only 12.5 gallic acid equivalent and 399.54 rutin equivalent. It was found to inhibit DPPH and deoxyribose oxidation (IC_{50} = 24.24 and 5.40 respectively). *In vivo* study demonstrated a significant reduction in the epithelialization time ($P < 0.001$) to 17-18 days in normal and dexamethasone treated rats following the ethanolic extract of the bark of *C. procera* application. The same extract also significantly increased the breaking strength in dexamethasone treated rats. Histological examination of incision wounds of treated group showed matured extracellular matrix, numerous fibroblasts. This study illustrated an excellent potential of the bark of *C. procera* therapy on dermal wound healing, with a tentative mechanism of action related to improved collagen deposition and reduced inflammatory reaction.

KEY WORDS: antioxidant, breaking strength, *Calotropis procera*, epithelialization, skin

Introduction

Many medicinal plants are claimed to be useful for wound healing in the cameroonian traditional system of medicine. Among these plants, *Calotropis procera* (Asclepiadaceae) originated from the Afro-Asian monsoonal regions and spread to the Indian subcontinent and subtropical America, Mascarene Islands and drier parts of Australia. The latex and the leaves are said to be valuable for skin injuries [1]. These properties are accompanied by toxic effects following oral administration or dermal contact of latex in animals. The root bark is known to promote secretion and to be useful in treating skin disease, among other ailments [2]. But the wound healing potential of the stem bark has not been evaluated yet.

The biological mechanism associated with wound healing is complex. Platelet aggregation during hemostasis liberates a number of soluble mediators, which initiate the healing process. Hemostasis is followed by an early inflammatory phase that is characterized by vasodilatation, increased capillary permeability, complement activation and polymorphonuclear and macrophage migration into the wound within three days. Macrophages are actively phagocytic, and secrete regulatory factors that are responsible for the proliferation by fibroblasts and endothelial cells (granulation tissue) around the 5th day post-injury heralds the "proliferative phase." Fibroblasts synthesize collagen and ground substance (proteoglycans and fibronectin), which support new cells, and the fragile capillary buds, which appear around this time (angiogenesis). Epithelialization requires the migration of epithelial cells across the granulation tissue, to close the epidermal defect. Collagen synthesis continues for many months after wound closure but also undergoes continual lysis, so a delicate balance exists between the two processes. This final remodeling phase, accompanied by increasing tensile strength of the wound, and a decreasing cellularity, may continue for up to a year [3]. Wound healing is currently a clinical challenge due to inconsistencies encountered in the healing processes and the financial burden [4]. Therefore, medicinal plants have generated much interest for the treatment of skin ailments as they are affordable and purportedly safe from hypersensitive reactions [5]. The present study was designed to evaluate the antioxidant potential and the wound healing effect of the ethanol extract of *C. procera* bark.

Material and Methods

Plant Material and Extraction

Leafy plants of C. procera were obtained from the city of Maroua. The identity was confirmed by plant taxonomist Todou G., from the University of Maroua. A voucher specimen was deposited at the Herbarium of the University Campus. Powdered stem bark of C. procera was extracted with ethanol (70°) at room temperature. The extract solution was filtered, and the solvent was evaporated under reduced pressure. The dark green mass obtained 4.50% yield based on dry bark was considered as the ethanol extract used in the study.

Drugs

2-deoxy-d-ribose was from Sisco Research Laboratories PVT LTD (India). Trichloroacetic acid was from Sigma (Germany). Thiobarbituric acid was from Titan Biotech LTD (India). 1, 1- diphenyl-2-picrylhydrazy (DPPH) was from Fluka (Switzerland). Ketamine (Rotexmedica-Trttau-Germany), diazepam (Renaudin-France), dexamethasome (Guangdong Medecine and Health Products I/E corp) and nylon surgical treat size 1 (Agary Pharmaceutical Ltd) were purchased from a local pharmacy store). All other chemicals were of laboratory grade and used as received.

Quantitation of Total Phenols

Total phenolics content (TPC) was measured using the Folin–Ciocalteu method [6]. A standard curve was prepared using 0.625 μg/mL - 10 μg/mL gallic acid. TPC content was expressed as gallic acid equivalent (GAE) per mg of extract.

Quantitation of Flavonoids

Flavonoids content (FC) was measured using the method described by Makkar *et al.* [7]. A standard curve was prepared using 10-100 μg/mL rutin. FC content was expressed as rutin equivalent (RE) per mg of extract.

Antioxidant Activity

All determinations were done in triplicate. The concentrations of samples that provide 50% inhibition (IC50) were obtained by interpolation from linear regression analysis.

DPPH Radical Scavenging Assay

Scavenging of the stable DPPH radical was assayed in vitro according to the method described by Obame *et al.* [8]. Various concentrations of the ethanol extract of the bark of *C. procera* (0.5, 1, 2 and 4 μg/mL) were added to a 0.5 mL solution of DPPH (0.125 mM in 95% ethanol). The mixture was shaken and allowed to stand at room temperature for 30 min, and the absorbance was measured at 517 nm in a spectrophotometer. Percent inhibition was calculated from the control. Ascorbic acid and gallic acid at doses lower than that which have been reported to act as pro-oxidant [9,10] were used as a standard compound in this assay.

Degradation of Deoxyribose (Fenton's reaction)

The ability of the ethanolic extract of the bark of *C. procera* to prevent Fe_2^+/H_2O_2-induced decomposition of deoxyribose was carried out using the method described by Hinneburg *et al.* [11]. Briefly, the reaction mixture contained 100 μL of extract dissolved in water, 500 μL of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4-NaOH buffer (50 mM, pH 7.4), 200 μL of premixed 100 μM $FeCl_3$ and 104 mM EDTA (1:1 v/v) solution, 100 μL of 1.0 mM H_2O_2 and 100 μL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50°C for 30 min.

Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to each tube. The samples were vortexed and heated in a water bath at 50°C for 30 min. The extent of oxidation was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated as follows:

$$\text{Inhibition}(\%) = \frac{\text{Absorbance of the control-Absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

Animals

All animal procedures were in strict accordance with the NIH Guide for the care and Use of Laboratory Animals. Locally bred male albino-Wistar rats weighing 150-180 *g*, fed on a standard diet and allowed water ad libitum were used. Animals were caged under laboratory environment and 12-h dark and light cycles. Standard rodent chow pellets were given ad libitum with free access to water.

Determination of *In Vivo* Cicatrizant Activity

Grouping of animals

For each model, 20 animals were divided into 4 groups of 5 rats as follow: Group 1: H_2O; Group 2: Dexamethasone i.m (DX); Group 3: C. procera (50 mg) eosinophil cationic protein (ECP); and group 4: Dexamethasone + C. procera (DX+ECP).

Excisional Wound Model

Animals were anesthetized by intramuscular injection of ketamine/diazepam (ketamine 25 mg/kg and diazepam 10 mg/kg). An area (4 cm^2) was marked using a frame and marker pen. The required area of the dorsal fur of the animals was shaved with an electric clipper. The area was sterilized by spraying with 70% ethanol. A full thickness skin (4 cm^2) was excised from the predetermined area by removing the epidermis and dermis layer with a surgical blade (0.1 mm) until the subcutaneous fat (avoiding *panniculus carnosus* and the muscle layer). Group I was applied topically with distilled water (negative control), Group II with distilled water (positive control), Groups III and IV with the plant extract (dose approximately 50 mg g/wound) every two days until the wound was completely healed. Special care was taken to avoid variation in the dose given. Animals were monitored every day. Wound diameter was recorded in vertical and horizontal planes daily, as well as epithelialization time that indicate the formation of new epithelial tissue to cover the wound. The lesions on each rat were also rated using the following parameters: (1) The presence and type of exudates, (2) erythema, (3) swelling, (4) ulceration and (5) crust formation [12]. The degree of wound healing was calculated using formula:

$$\frac{\text{Wound area on zero day-wound area on corresponding day}}{\text{wound area on zero day}} \times 100$$

The number of days for complete epithelization was noted. Wounds were considered closed (completely healed) if moist granulation tissue was no longer apparent and the wound was covered with new epithelium.

Incisional Wound Model

A 5-cm incision was made perpendicular to the axis of symmetry of the animal and the two borders of the wound were stitched together at its center, with interrupted sutures at distance of 1 cm. Treatment was started immediately, and every 48 h the compound being tested was applied to the wound. On 10th day post wounding, animals were sacrificed by chloroform overdose and wound areas from each animal were dissected carefully. Stripes of equal size (width) from one side were cut, and the line was drawn on either side, 3 mm away from the wound, for breaking strength determination. One piece of tissue was fixed in 10% formalin for histopathological examination, and the other was used to quantify the wound breaking strength (WBS).

Determination of Wound Breaking Strength

Both ends of each skin stripe were fixed with a pair of steel clip, one clip was allowed hanging on a stand and other clip with a freely suspended polyethylene bag through a string run over a pulley. It was then gradually filled with water from a polyethylene reservoir till the wound stripe was broken at the site of the wound. The amount of water required to break the wound was noted and expressed as tensile strength of the wound in grams [12]. The tensile strength was calculated according to the following equation:

$$\text{Tensile strength} = \frac{\text{Total breaking load}}{\text{cross sectional area}}$$

For preliminary screening, an activity greater than 25% is considered significantly important, and the sample is described as having positive wound-healing activity. The percentage of activity was calculated according to the following formula:

$$\text{Activity}(\%) = \frac{\text{WBSc-WBSt}}{\text{WBSc}} \times 100$$

WBSt = Average of the force necessary to open the wound of a treated mouse.

WBSc = Average of the force necessary to open the wound of an untreated mouse (control).

Histomorphological Study

Skin specimens were immediately fixed in 10% (v/v) neutral formalin until the tissues hardened. Each specimen was embedded in a paraffin block, and thin sections (5 μm) were prepared and stained with hematoxylin and eosin (HE) (for general morphological observations). Slides were examined qualitatively under a light microscope, for collagen formation, fibroblast proliferation, angiogenesis, and epithelialization.

Statistical Analysis

All data were expressed as mean ± standard deviation. Statistical analyses were evaluated by one-way ANOVA followed by Dunett test. $P < 0.05$ was regarded as significant.

Results

Phytochemical Content and Antioxidant Activity of the Ethanolic Extract of *C. procera*

In the present study, the ethanolic extract of *C. procera* have shown strong DPPH radical scavenging (IC50 28.57 μg/mL). Total phenols in the ethanol extract were found to be 12.5g GAE/g extract, and total flavonoids were found to be 399.54 g RE/g extract. The ethanolic extract showed its ability to quench the stable DPPH radical and inhibit deoxyribose degradation. Table 1 shows the percent inhibition of DPPH with *C. procera* ethanolic extract and pure antioxidant compounds at different concentrations (0.625-5 μg/mL). The activity of plant extract was between14.90 and 69.65 %. Ascorbic acid and gallic acid showed the highest radical scavenging effectiveness. Activity of ascorbic acid acid was between 81.45 % and 83.79%. That of gallic acid was between 80.37 and 82.29. Calculated IC50 were 28.57 mg/mL, 2.13 μg/mL and 2.16 μg/ml for *C. procera* ethanolic extract, ascorbic acid and gallic acid respectively.

Table 2 shows the percent inhibition of deoxyribose degradation with *C. procera* ethanolic extract and pure antioxidant compounds at different concentrations (0.5-5 μg/mL). The activity of plant extract was between 27.68 and 43.75%. Ascorbic acid and gallic acid showed the highest antioxidant effectiveness. Activity of ascorbic acid acid was between 69.19% and 89.28%. That of gallic acid was between 82.14 and 90.18. Calculated IC50 were 3.50 μg/mL, 1.66 μg/mL and 1.60 μg/mL for *C. procera* ethanolic extract, ascorbic acid, and gallic acid, respectively.

Table 1: Antiradical activity of ethanolic bark extract of *C. procera* on DPPH *in vitro*

Inhibition (%)			
Concentration (μg/mL)	Ascorbic acid	Gallic acid	*C. procera*
0.625	81.45	80.37	14.90
1.25	81.87	80.79	50.91
2.5	82.45	81.45	63.74
5	83.79	82.29	69.65

C. procera: Calotropis procera

Table 2: Antioxidant activity of ethanolic bark extract of *C. procera* on deoxyribose *in vitro*

Inhibition (%)			
Concentration (μg/mL)	Ascorbic acid	Gallic acid	*C. procera*
0.5	69.19	82.14	27.68
1	82.14	83.93	35.27
2	83.03	87.50	37.05
4	89.28	90.18	43.75

C. procera: Calotropis procera

Wound Contraction and Epithelialization Time

Results of the wound contraction rate and epithelialization time are shown in Table 3 and Figure 1 respectively. The extract-treated group demonstrated significantly higher wound contracting ability ($P < 0.001$) than the control group from day 4 post-wounding. In the extract treated, group complete healing was observed on 17th-18th day, while untreated group (control) and Dexamethasone treated animals took more than 20 days for healing of wounds.

Wound Breaking Strength

In incision wound study, tensile strength was 672.90 g/cm^2 in the

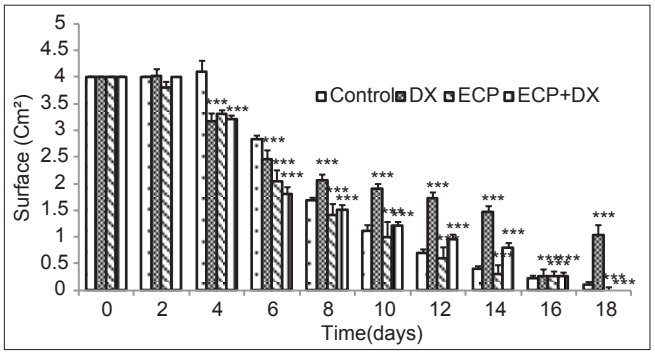

Figure 1: Effect of the ethanol extract of the stem bark of *Calotropis procera* on wound contraction in rats each value represents the mean ± standard error of mean, $n = 5$. ***$P < 0001$: Difference significant when compared to control, DX: dexamethasone, ECP: Ethanol extract of the stem bark of *Calotropis procera*, ECP+DX: Ethanol extract of the stem bark of *Calotropis procera* combined with dexamethasone

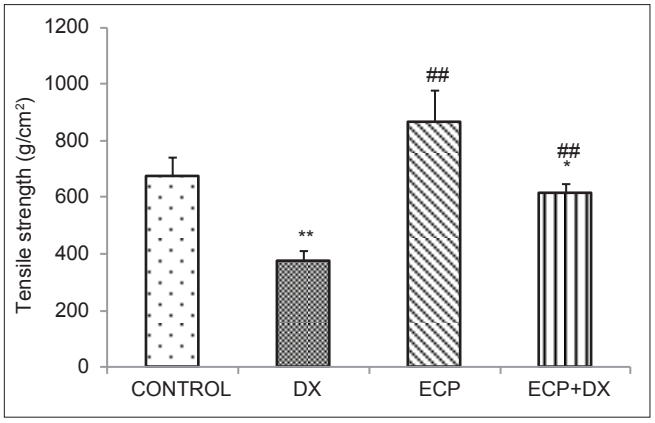

Figure 2: Effects of the ethanol extract of *Calotropis procera* on the tensile strength of rats. Each value represents the mean ± standard error of mean, n = 5. ##P<0.01: Difference significant when compared to control, DX: dexamethasone, ECP: ethanol extract of the stem bark of *Calotropis procera*, ECP+DX: ethanol extract of the stem bark of *Calotropis procera* combined with dexamethasone

Table 3: Effects of the ethanol extract of *C. procera* on epithelialization time in rats

	Control	DX	ECP	ECP+DX
Epithelialization time (days)	21.00±1.12	31.00±1.12***	17.00±1.55###	18.00±0.00###

Each value represents the mean±SEM, n=5, ***P<0.001: Difference significant when compared to control, ###P<0.001, Difference significant when compared to dexaméthasone. Dx: Dexaméthasone, ECP: Ethanol extract of the stem bark of *Calotropis procera*, ECP+DX: Ethanol extract of the stem bark of *Calotropis procera* combined with dexamethasone, SEM: Standard error of the mean, *C. procera: Calotropis procera*

control group. *C. procera* enhanced the tensile strength of 10 days old wounds as compared with wounds of the untreated group. In this study, dexamethasone decreased this force by 44.44%, while the plant extract exerted an activity of 28.41% when compared to control animals. Finally, the plant extract prevents dexamethasone inhibition on the tensile strength by 64.08 % [Figure 2].

Increase in tensile strength may be due to increase in collagen concentration per unit area and stabilization of fibers. Tensile strength was improved in all groups treated with the ethanol extract of the stem bark of *C. procera*. This may be due to promotion of collage nation and cross-linking.

Histomorphological Study

Figure 3 shows the various hitomorphological features of the skin tissue obtain with different treatments. Histological sections of scar tissue from extract-treated rats showed increased and well-organized dermis, more fibroblasts than control rats. Scar tissue sections obtained from dexamethasone treated rats revealed fewer collagen fibers and fibroblasts than all the other groups. Blood vessels of dexamethasone treated rats were dilated compared to the other groups of rats, and the epithelium layer was poorly organized.

Discussion

Despite the large number of researches, wound healing is still challenging investigators. The healing process consists of different phases including contraction, epithelialization, granulation, collage nation and scar maturation which are concurrent but independent to each other. In this study, the antioxidant activity of the ethanolic extracts was tested against two important oxygen radicals, DPPH and hydroxyl radical. Oxygen radicals are toxic waste products which produce oxidative stress during the inflammatory phase of wound healing [13]. Scavengers application to the injury site has been reported to be effective in inflammatory conditions and wound

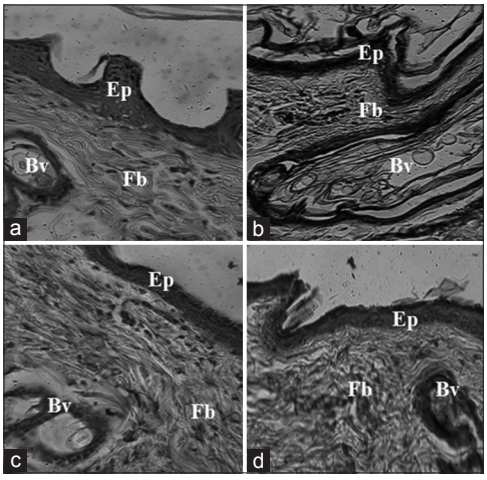

Figure 3: Histological section of control wound (a), dexamethasone (b), *Calotropis procera* (c), and dexamethasone + *Calotropis procera* (d) (HE stain, ×400). Bv: Blood vessel; Cg: collagen; Ep: epithelial cells; Fb: fibroblasts

healing [14,15]. These health effects have been traditionally attributed to the antioxidant property and is associated with the development of matured collagen fibers and fibroblasts with better angiogenesis [16,17]. Accordingly, natural accelerators of wound healing with antioxidant action are of great interest for surgery, dermatology and modern cosmetology [14].

The DPPH assay validated the free radical scavenging activity of the ethanolic extract. Oxidation products of deoxyribose by hydroxyl radical, upon heating with thiobarbituric acid under acid conditions, would yield a pink chromogen with the maximum absorbance wavelength of 532 nm. Added hydroxyl radical scavengers compete with deoxyribose for hydroxyl radicals and diminish chromogen formation [18]. Phytochemical screening of CPE revealed the absence of saponins and glycosides. Tannins were also likely absent since the potassium dichromate test was negative, even though incidental properties of tannins were indicated because the gelatin test was positive. In fact, gelatin is not only precipitated by tannins, but also by gum arabic, starch inulin and methyl gallate [19]. But, phenols, flavonoids, alkaloids, reducing sugar and proteins were detected in the extract used. The scavenging activity of the extract in this study is expected due to its phenolic content, beside other phytochemicals. In effect, it is known that the wound healing properties of plants, in most cases, are associated with their significant antioxidant activities [20]. Two models were used to assess the effect of an ethanol extract of the stem bark of *C. procera* on acute wounds in rats. Prohealing action of the extract was observed in normal and dexamethasone-induced healing delay in acute wounds. This effect was demonstrated by an increase in the rate of wound contraction and by a reduction of epithelialization time. Significant increase in tensile strength (> 25%) compared to dexamethasone was observed, which were further supported by histopathological analysis, indicating improved collagen maturation. In normal tissues, strength, integrity, and structure are provided by collagen [21]. Hence, bark extract of *C. procera* can restore skin structure and function. The molecular mechanism involved in the extract effect was not studied; nevertheless, the reduction in epithelialization time may be related to an anti-inflammatory effect of the extract during wound repair. This was supported by the presence of inflammatory signs in dexamethasone treated rats 10 days after wounding (dilated blood vessels, reduction in erythema and wound contraction 48 h after injury) [22]. Reversely, more intense granulation tissue was evident in the treated groups. Interestingly, in this study we observed that the *C. procera* administered in combination with dexamethasone at higher dose, significantly deteriorated the wound healing rather than improving it when compared with dexamethasone treatment alone, indicating complex drug-herb interaction(s) and/or other effective compound(s) responsible for the wound healing effectiveness of *C. procera* regimen. The ethanolic extract of the bark of *C. procera* also exerted its curative effect through enhancement of the tensile strength as described in this study. The healing effect observed with our extract may be due the plant action, at least partly, to its antioxidant potential, as well as the improvement of collagen deposition. Antioxidants and flavonoids have been reported to improve wound healing through increasing collagen cross-linking and then breaking strength [23].

conclusion

On the basis of the considerations obtained in the present study, the bark of *C. procera* possess considerable antioxidant property as evident from the result of antiradicalar assay. This extract demonstrated wound healing effect by accelerating wound closure and epithelialization *in vivo*. This effect may be due the antioxidant potential of the extract and may be attributed at least partly on its improvement of collagen deposition. Results obtained provided additional data for the use of the bark of *C. procera* as wound healing agent. However, to understand fully the process of wound healing by a *C. procera* extract, it is essential to study the basic cell biology, immunology and biochemistry involved in the processes of inflammation and collagen metabolism, and how these pathways are regulated.

References

1. Rasik AM, Raghubir R, Gupta A, Shukla A, Dubey MP, Srivastava S, *et al.* Healing potential of *Calotropis procera* on dermal wounds in Guinea pigs. J Ethnopharmacol 1999;68:261-6.
2. Khairnar AK, Bhamare SR, Bhamare HP. *Calotropis procera*: An ethnopharmacological update. Adv Res Pharm Biol 2012;2:142-56.
3. Bradley M, Cullum N, Nelson EA, Petticrew M, Sheldon T, Torgerson D. Systematic reviews of wound care management: (2). Dressings and topical agents used in the healing of chronic wounds. Health Technol Assess 1999;3:1-35.
4. Jones KR, Fennie K, Lenihan A. Evidence-based management of chronic wounds. Adv Skin Wound Care 2007;20:591-600.
5. Raina R, Parwez S, Verma PK, Pankaj NK. Medicinal plants and their role in wound healing. Vet Scan 2008;3:1-7.
6. Nurul AZ, Darah I, Shaida FS, Nor AS. Assessment of antioxidant activity, total phenolic content and *in vitro* toxicity of Malaysian red seaweed, *Acanthophora spicifera*. J Chem Pharm Res 2011;3:182-91.
7. Makkar HP, Becker K, Abel H, Pawelzik E. Nutrient contents, rumen protein degradability and antinutritional factors in some colour and white flowering cultivars of *Vicia faba* beans. J Sci Food Agric 1997;75:511-20.
8. Obame LC, Koudou J, Kumulungui BS, Bassolé IH, Edou P, Ouattara AS, *et al*. Antioxidant and antimicrobial activities of Canarium schweinfurthii Engl. essential oil from Centrafrican republic. Afr J Biotechnol 2007;6:2319-23.
9. Ling LT, Palanisamy UD, Cheng HM. Prooxidant/antioxidant ratio (ProAntidex) as a better index of net free radical scavenging potential. Molecules 2010;15:7884-92.
10. Yen GC, Duh PD, Tsai UL. Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. Food Chem 2002;79:307-13.
11. Hinneburg I, Dorman HJ, Hiltunen R. Antioxidant activities of extracts from selected culinary herbs and spices. Food Chem 2006;97:122-9.
12. Kumar N, Prakash D, Kumar P. Wound healing activity of *Solanum xanthocarpum* Schrad. and Wendl. fruit. Indian J Nat Prod Resour 2010;1(4):470-5.
13. Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: Molecular and cellular mechanisms. J Invest Dermatol 2007;127:514-25.
14. Basak PY, Agalar F, Gültekin F, Eroglu E, Altuntas I, Agalar C. The effect of thermal injury and melatonin on incisional wound healing. Ulus Travma Acil Cerrahi Derg 2003;9:96-101.
15. Korkina LG, Mikhal'chik E, Suprun MV, Pastore S, Dal Toso R. Molecular mechanisms underlying wound healing and anti-inflammatory properties of naturally occurring biotechnologically produced phenylpropanoid glycosides. Cell Mol Biol (Noisy-le-grand) 2007;53:84-91.
16. Hsu S. Green tea and the skin. J Am Acad Dermatol 2005;52:1049-59.
17. Lodhi S, Jain AP, Sharma VK, Singhai AK. Wound-healing effect of flavonoid-rich fraction from *Tephrosia purpurea* linn. on streptozotocin-induced diabetic rats. J Herbs Spices Med Plants 2013;19:191-205.
18. Cheng Z, Li Y, Chang W. Kinetic deoxyribose degradation assay and its application in assessing the antioxidant activities of phenolic compounds in a fenton-type reaction system. Anal Chim Acta 2003;478:129-37.
19. Atkinson E, Hazleton EO. A Qualitative Tannin Test. Biochem J 1922;16:516-7.
20. Suntar I, Akkol K, Nahar L, Sarker SD. Wound-healing and antioxidant properties: Do they co-exist in plants? Free Radic Antioxid 2012;2:2-7.
21. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci 2004;9:283-9.
22. Masoko P, Picard J, Eloff JN. The use of a rat model to evaluate the *in vivo* toxicity and wound healing activity of selected *Combretum* and *Terminalia* (Combretaceae) species extracts. Onderstepoort J Vet Res 2010;77:E1-7.
23. Seifter E, Crowley LV, Rettura G, Nakao K, Gruber C, Kan D, *et al.* Influence of vitamin A on wound healing in rats with femoral fracture. Ann Surg 1975;181:836-41.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141028021746

Original Research

ScopeMed

Acute and sub-acute oral toxicity assessment of the methanolic extract from leaves of *Hibiscus rosa-sinensis* L. in mice

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Received: October 07, 2014
Accepted: October 28, 2014
Published: November 28, 2014

ABSTRACT

Background: The leaves of *Hibiscus rosa-sinensis* L. (Malvaceae) are used for the treatment of dysentery and diarrhea, to promote draining of abscesses and as analgesic agent in the traditional medicine of Cook Islands, Haiti, Japan and Mexico. **Aim:** The present study investigated the oral acute and subacute toxicity of methanol leaf extract of *H. rosa-sinensis* in mice. **Materials and Methods:** In the acute toxicity study, a single oral dose of 2000 mg/kg of extract was given to five mice at 48 h intervals. Animals were observed individually for any clinical signs of toxicity or mortality for 14 days. In the sub-acute toxicity study, mice were treated with 400 mg/kg and 800 mg/kg doses of the extract for 14 days. The hematological and biochemical parameters and histopathology of liver and kidneys of animals were studied at the end of the experiment. **Results:** For acute treatment, the extract did not reveal any signs of toxicity or mortality in any animal, during the 14 days observation period. The LD_{50} of extract was estimated to be greater than 2000 mg/kg. In the sub-acute toxicity study, administration of 400 mg/kg and 800 mg/kg doses of extract to mice for two weeks did not reveal any marked adverse effects on hematological, biochemical parameters and histopathology of liver and kidney in the 400 mg/kg group. However, hepato-renal toxicity as evidenced by elevated levels of alanine aminotransferase, aspartate aminotransferase, total and indirect bilirubin, urea and creatinine was seen in the animals that received 800 mg/kg dose of extract for 14 days. In addition, in the same group of animals, the histological assessments of liver and kidney also showed various adverse effects viz. dilated sinusoids, apoptotic nuclei and inflammatory infiltrate inside sinusoidal capillaries in the liver, and marked the disorganization of tubules and glomeruli, and enlarged interstitial spaces in the kidney. **Conclusion:** The results of this study suggest that for traditional medicinal purpose, only a low dose of *H. rosa-sinensis* leaf extract (i.e., 400 mg/kg) should be considered as safe.

KEY WORDS: Acute toxicity, *Hibiscus rosa-sinensis* L, histopathology, Malvaceae, subacute toxicity

INTRODUCTION

The majority of the people in developing countries use various traditional herbal medicines to treat a number of diseases and ailments [1]. Although, many studies have been undertaken in the past to investigate the pharmacological potential of such remedies, however, rather little work has been done to assess the potential toxicities of such products. There is now growing evidence that many herbal medicines do cause serious toxicity to their users [2,3]. Therefore, much more scientific attention is now being given to assess the potential toxicity of herbal medicines than before.

Hibiscus rosa-sinensis L. (Malvaceae) is a perennial shrub distributed in tropical and subtropical regions of the world. In traditional medicine, the leaves of this plant are used against dysentery and diarrhea, to promote draining of abscesses, against gonorrhea and as analgesic agent in Cook Islands, Guam, Haiti, Japan and Mexico, etc. [4]. The petroleum ether extracts of the leaves and flowers of this plant have been shown to possess the antibacterial activities [5]. The mucilage from the leaf of this plant has also been shown to possess considerable anti-complementary activity [6]. In addition, Sachdewa *et al*. have reported the hypoglycemic effects of leaf extract of *H. rosa-sinensis* in rats [7]. A recent study has also shown that the leaf extract of this plant possesses significant *in vitro* and *in vivo* activity against *Hymenolepis diminuta*, a zoonotic helminth parasite [8]. Although, the pharmacological properties of *H. rosa-sinensis* leaf extract are widely known, but there is insufficient data about its potential toxicity. Therefore, the present study was undertaken to assess the acute and sub-acute oral toxicity of *H. rosa-sinensis* leaf extract in mice.

MATERIALS AND METHODS

Experimental Animals

Healthy Swiss albino mice of either sex, weighing between 25 and 30 g, were used. The animals were housed individually in acrylic cages and maintained in a standard laboratory environment. They were fed with standard rodent pellets and water ad libitum. All the experimental protocols related to use of mice were approved by the Institutional Ethics Committee (Animal Models) of North-Eastern Hill University, Shillong.

Plant Material

The leaves of *H. rosa-sinensis* were collected from North Tripura district of Tripura, India in August, 2010 and identified by a plant taxonomist. A voucher specimen (AKY-11882) of plant material has been deposited in the Parasitology and Ethnopharmacology Lab., Department of Zoology, NEHU, Shillong. The leaves were dried under shade, powdered and extracted in methanol, using a Soxhlet extractor. The final yield (w/w) of plant extract was about 18%.

Acute Toxicity Study

This study was performed as per the up-and-down-procedure of Organization for Economic Cooperation and Development (OECD) guidelines 425 [9]. A limit dose of 2000 mg/kg of extract was used involving five mice. Each mouse was treated with a single oral dose of 2000 mg/kg of extract in sequence at 48 h intervals. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h, and daily thereafter, for a total of 14 days for any clinical signs of toxicity or mortality.

Sub-acute Toxicity Study

The sub-acute toxicity study on plant extract was performed as per the OECD guidelines 407 [10], with slight modifications. Based on the findings of the acute toxicity test, two different doses of extract, i.e. 400 mg/kg (low dose) and 800 mg/kg (high dose), were selected and administrated orally daily for 14 days to two different groups of mice ($n = 10$). The third group of mice ($n = 10$) was included as a control and received only vehicle for the same duration. After extract treatments, all the experimental animals were observed daily for any abnormal clinical signs and mortality for 14 days. At the end of 14 day's observation period, the animals were anaesthetized, and their blood samples were collected through cardiac puncture with and without anticoagulant (EDTA), for hematological and biochemical studies, respectively. In hematological studies, red blood cell (RBC), white blood cell (WBC), and platelet counts, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were determined using a hematology analyzer (Nihon Kohden Celltac MEK 6410 K Cell Counter). For biochemical analysis, blood without additive was centrifuged at 3000 × *g* at 4°C for 10 min, serum was separated and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, total bilirubin, direct and indirect bilirubin, urea and creatinine were estimated using a semi-automated Biochemical Analyzer (Bayer RA-50). For histopathological studies, the liver and kidney of animals were excised and examined macroscopically. These organs were then preserved in 10% buffered formalin for histopathological examinations by standard techniques.

Statistical Analysis

Data are expressed as mean ± standard errors of the mean. Evaluations were performed using Student's *t*-test, and by one-way analysis of variance, followed by Bonferroni test. $P < 0.05$ was considered as statistically significant.

RESULTS

The acute toxicity test revealed that oral administration of a single 2000 mg/kg dose of *H. rosa-sinensis* methanol leaf extract to five mice did not brings out any signs of toxicity or mortality in treated animals during the 14 days observation period. In the subacute toxicity tests, administration of 400 and 800 mg/kg doses of *H. rosa-sinensis* leaf extract to two groups of mice did not showed any visual symptoms of toxicity or mortality in animals during the entire 14-days observation period. Furthermore, the haematological and biochemical parameters and histopathology of liver and kidneys of mice that received the 400 mg/kg dose of plant extract did not show any noticeable adverse effects [Table 1]. However, various treatment-related adverse effects were noticeable in biochemical and hematological parameters and in the histopathogy of liver and kidney of animals that received the 800 mg/kg dose of extract for 14 days [Tables 1 and 2, Figure 1]. The biochemical analysis revealed a significant increase in ALT, AST, total bilirubin, urea and creatinine in 800 mg/kg extract-treated animals [Table 1]. Whereas, the hematological analysis showed only a slight increase in the

Table 1: Effects of oral administration of *H. rosa-sinensis* methanol leaf extract for 2 weeks on biochemical parameters of mice (n=10)

Parameters	Control	Plant extract	
		400 mg/kg	800 mg/kg
ALT (U/l)	50.65±0.16	52.40±0.87	152.00±0.93**
AST (U/l)	94.83±1.04	98.07±3.40	120.30±10.47*
ALP (U/l)	154.33±1.38	152.30±2.36	180.40±1.69
Total bilirubin (mg/dl)	1.00±0.00	0.94±0.20	1.74±0.01*
Direct bilirubin (mg/dl)	0.16±0.05	0.18±0.01	0.86±0.20
Indirect bilirubin (mg/dl)	0.24±0.06	0.26±0.03	0.55±0.01*
Urea (mg/dl)	18.00±0.21	18.23±0.22	35.80±0.63**
Creatinine (mg/dl)	0.52±0.04	0.5 1±0.06	1.67±0.05*

Data are expressed as mean±SEM. *P<0.05 versus control, one-way ANOVA *post-hoc* Bonferroni test; **P<0.01 versus control, one-way ANOVA *post-hoc* Bonferroni test. SEM: Standard error of the mean, ANOVA: Analysis of variance, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, *H. rosa-sinensis: Hibiscus rosa-sinensis*

Table 2: Effects of oral administration of *H. rosa-sinensis* leaves extract for 2 weeks on hematological parameters of mice (n=10)

Parameters	Control	Plant extract	
		400 mg/kg	800 mg/kg
RBC count ($\times 10^6$ mm^{-3})	4.31±0.60	4.89±0.29	6.90±0.21*
WBC count ($\times 10^3$ mm^{-3})	6.50±0.31	6.02±0.24	6.86±0.22*
Hb (g/dl)	9.08±0.21	9.59±0.20	9.83±0.25
Mean corpuscular Hb (pg)	29.25±0.27	30.12±0.21	30.28±0.28
Mean corpuscular Hb concentration (g/dl)	31.14±0.50	30.04±0.46	31.72±0.61
Mean corpuscular volume (fl)	86.62±0.90	87.21±0.56	86.30±1.18
Platelet count ($\times 10^3$ mm^{-3})	1.20±0.10	1.50±0.17	1.90±0.18

Data are expressed as mean±SEM. *P<0.05 versus control, one-way ANOVA *post-hoc* Bonferroni test. SEM: Standard error of the mean, RBC: Red blood cell, WBC: White blood cell, Hb: Hemoglobin, *H. rosa-sinensis: Hibiscus rosa-sinensis*

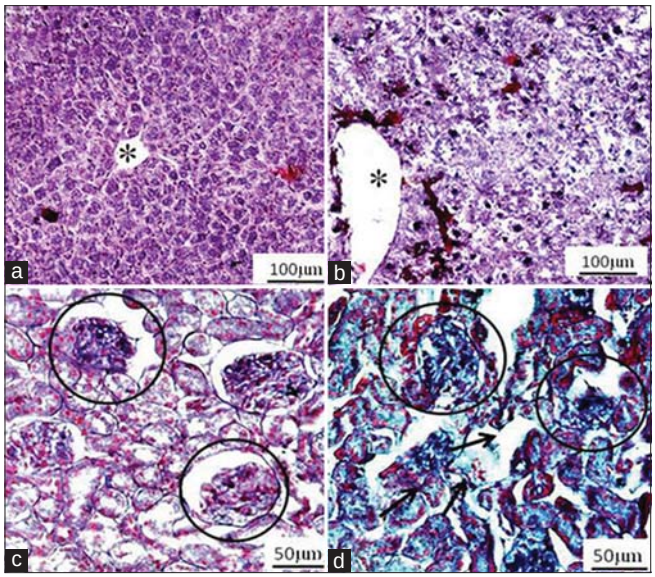

Figure 1: (a) Cross section of the liver of a control mouse, showing well-preserved liver plates and central vein (*). (b) Cross section of the liver of a mouse treated with *Hibiscus rosa-sinensis* leaf extract (800 mg/kg) by oral route for 14 days, showing dilated sinusoids and central vein (*). (c) Cross section of the kidney of a control mouse, showing conserved glomeruli (encircled areas) and tubules. (d) Cross section of the kidney of a mouse treated with *H. rosa-sinensis* leaf extract (800 mg/kg) by oral route for 14 days, showing disorganization of tubules (regular arrow) and glomerulus (encircled area) (haematoxylin-eosin)

RBC and WBC counts in the 800 mg/kg-extract treated group of animals [Table 2].

In the histopathological investigations, the microanatomy of liver and kidneys did not present any treatment-related adverse effects in the animals that received the 400 mg/kg dose of plant extract (photomicrographs not shown). However, the histological assessments of liver and kidney of animals that received the 800 mg/kg dose of extract for 14 days showed various abnormalities in tissues viz. dilated sinusoids, apoptotic nuclei and inflammatory infiltrate inside sinusoidal capillaries in the liver and disorganization of tubules and glomeruli, enlarged interstitial spaces, etc. in the kidneys, which were not present in the control group [Figure 1].

DISCUSSION

In general, the safety studies on herbal medicines have been carried out by performing acute and sub-acute toxicity tests in laboratory animals (e.g. rodents and non-human primates) [3]. In the present study, we investigated the acute and sub-acute oral toxicity of *H. rosa-sinensis* leaf extract in Swiss albino mice. The acute and subacute toxic effects differ principally from each other with respect to the amount of test agent involved and the time intervening before the effects are observed. While, the acute effects are normally observed soon after a single exposure of test agent, the sub-chronic effects are usually monitored over an extended period during which there is repeated exposure of test agent.

In the present acute toxicity study, administration of a single 2000 mg/kg dose of plant extract to five mice did not reveal any signs of toxicity or mortality in any animal during the entire observation period. Therefore, the LD_{50} of extract may be considered to be greater than 2000 mg/kg. According to the Globally Harmonized System (GHS) of Classification and Labelling of Chemicals, the substances having an LD50 value greater than 2000 mg/kg are considered as relatively safe [11]. In some related studies, the LD_{50} values of therapeutic herbal extracts have been found to be greater 2000mg/kg, and as per the GHS criterion, these extracts have been considered to be reasonably safe on acute exposure [12].

In the sub-acute toxicity study, two groups of mice were treated with 400 and 800 mg/kg doses of extract for 14 days and the effects were monitored on biochemical and hematological parameters and histopathological features of liver and kidneys of treated animals. Interestingly, none of the studied parameters showed any evidence of adverse effects in 400 mg/kg treated group of mice. However, various treatment-related adverse effects were visible in the studied parameters in the 800 mg/kg treated group of mice. For example, a significant increase was observed in ALT, AST and total bilirubin in 800 mg/kg extract-treated animals. Since, ALT is a cytoplasmic enzyme found in very high concentration in the liver, an increase of this specific enzyme suggests a possible hepatocellular damage due to the extract treatment in mice. Similar findings were reported by Rhiouani *et al*. in case of the Moroccan traditional medicinal plant, *Herniaria glabra*, where the highest dose of extract caused a significant increase in ALT [13]. Similarly, the finding of an elevated level of total bilirubin in 800 mg/kg group may be due to the retention of bile subsequent to intrahepatic or extrahepatic bile flow in treated animals [13]. In addition to these parameters, the urea and creatinine also showed a significant rise in 800 mg/kg group. According to Satyanarayana *et al*. a concomitant rise of creatinine and urea provides a positive indication towards adverse effects in kidney functions [14]. Therefore, a concurrent increase in the levels of urea and creatinine, as noticed in the present study, indicates that 800 mg/kg

dose of extract also brings out certain adverse effects on kidney functions in experimental animals. Of the various hematological parameters analyzed in this study, only some slight increase was noticeable in the RBC and WBC counts in 800-mg/kg extract treated group of mice. It is assumed that this increase in erythrocytes and leukocytes may be due to an increase in the rate of hematopoiesis. In general, in such clinical conditions, the body produces more cells to meet out the demand for more mature cells, for example in conditions such as dehydration, blood loss, etc. [15]. Therefore, it is likely that any such factors might be responsible for the rise of RBC and WBC counts in 800-mg/kg group. It may be mentioned here that in many toxicity tests, the rapid rate of renewal of hematopoiesis has been considered as a sensitive target for toxicity [15].

Another important finding of this study was this that the histological assessments of liver and kidneys of animals that received the 800 mg/kg dose of extract for 14 days showed various treatment-related toxicological changes viz. dilated sinusoids, apoptotic nuclei and inflammatory infiltrate inside sinusoidal capillaries in the liver and disorganization of tubules and glomeruli, enlarged interstitial spaces, etc. in the kidney. This observed hepato-renal toxicity of the high dose of plant extract is also supported by marked elevations of ALT, AST, creatinine, and urea in biochemical tests, which are good indicators of liver and kidney functions [14,16]. Therefore, these two findings together suggest that administration of the extract at 800 mg/kg dose for two weeks duration induces significant damage to the liver and kidneys of treated animals.

CONCLUSION

In conclusion, the acute toxicity study on *H. rosa-sinensis* leaf extract suggests that a single 2000 mg/kg limit dose of extract is devoid of any adverse effects in mice. However, the subacute toxicity study indicates that repeated intake of extract in high doses (800 mg/kg) for 14 days may cause liver and kidney toxicity in mice. Hence, this study suggests that a low dose of extract (i.e. 400 mg/kg) of this plant should be considered as safe in traditional medicinal use.

ACKNOWLEDGMENTS

This study was supported, in part, by a grant under the Departmental Special Assistance Program of the University Grants Commission (UGC), New Delhi in the Department of Zoology, NEHU, Shillong. PN was recipient of a Fellowship by the UGC, New Delhi. We thank the editor and the anonymous reviewer for their constructive comments to revise the paper.

REFERENCES

1. Liu WJ. Introduction to traditional herbal medicines and their study. In: Liu JH, editor. In: Traditional Herbal Medicine Research Methods. New Jersey: John Wiley & Sons, Inc.; 2011. p. 1-26.
2. Wojcikowski K, Johnson DW, Gobé G. Medicinal herbal extracts – renal friend or foe? Part one: The toxicities of medicinal herbs. Nephrology (Carlton) 2004;9:313-8.
3. Fennell CW, Lindsey KL, McGaw LJ, Sparg SG, Stafford GI, Elgorashi EE, *et al*. Assessing African medicinal plants for efficacy and safety: Pharmacological screening and toxicology. J Ethnopharmacol 2004;94:205-17.
4. Jadhav VM, Thorat RM, Kadam VJ, Sathe NS. Traditional medicinal uses of *Hibiscus rosa-sinensis*. J Pharm Res 2009;2:1220-2.
5. Arullappan S, Zakaria Z, Basri DF. Preliminary screening of antibacterial activity using crude extracts of *Hibiscus rosa-sinensis*. Trop Life Sci Res 2009;20:109-18.
6. Shimizu N, Tomoda M, Suzuki I, Takada K. Plant mucilages. XLIII. A representative mucilage with biological activity from the leaves of *Hibiscus rosa-sinensis*. Biol Pharm Bull 1993;16:735-9.
7. Sachdewa A, Nigam R, Khemani LD. Hypoglycemic effect of *Hibiscus rosa sinensis* L. leaf extract in glucose and streptozotocin induced hyperglycemic rats. Indian J Exp Biol 2001;39:284-6.
8. Nath P. Evaluation of anthelmintic activity of some medicinal plants used in the folk-lore medicine system of Riang tribe in Tripura. Ph.D. Thesis, Department of Zoology. Shillong: North Eastern Hill University; 2014. p. 284.
9. OECD. OECD Guidelines for the testing of chemicals, repeated dose 28-day oral toxicity study in rodents. 407. 1995. p. 8. [Adopted 1995 Jul 27].
10. OECD. OECD Guidelines for the Testing of Chemicals, Acute Oral Toxicity- up-and-down-procedure (UDP). 425. 2008. p. 27. [Adopted 2008 Oct 03].
11. Anonymous. Globally Harmonized System of Classification and Labelling of Chemicals (GHS). The Purple Book. New York, Geneva: United Nations Economic Commission for Europe; 2005. p. 60.
12. Konan NA, Bacchi EM, Lincopan N, Varela SD, Varanda EA. Acute, subacute toxicity and genotoxic effect of a hydroethanolic extract of the cashew (*Anacardium occidentale* L.). J Ethnopharmacol 2007;110:30-8.
13. Rhiouani H, El-Hilaly J, Israili ZH, Lyoussi B. Acute and sub-chronic toxicity of an aqueous extract of the leaves of *Herniaria glabra* in rodents. J Ethnopharmacol 2008;118:378-86.
14. Satyanarayana PS, Singh D, Chopra K. Quercetin, a bioflavonoid, protects against oxidative stress-related renal dysfunction by cyclosporine in rats. Methods Find Exp Clin Pharmacol 2001;23:175-81.
15. d'Yvoire MB, Bremer S, Casati S, Ceridono M, Coecke S, Corvi R, *et al.* ECVAM and new technologies for toxicity testing. In: Balls M, Combes RD, Bhogal N, editors. New Technologies for Toxicity Testing. Advances in Experimental Medicine and Biology. Vol. 745. New York: Landes Bio Science and Springer Science+Buisness Media, Inc.; 2012. p. 171.
16. Hall RL. Principles of clinical pathology for toxicology studies. In: Hayes WA, editor. Principles and Methods of Toxicology. 4th ed. Philadelphia: Taylor and Francis; 2001. p. 1001-38.

Source of Support: Nil, Conflict of Interest: None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20140929114417

Review Article

Hormesis and homeopathy: The artificial twins

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Received: September 10, 2014
Accepted: September 29, 2014
Published: November 28, 2014

ABSTRACT

Homeopathy claims a curative reaction from small doses of a substance, high doses of which cause symptoms similar to those the patient is suffering from. Hormesis is a concept of biphasic dose-response to different pharmacological and toxicological agents. According to this concept, a small dose of a noxious agent can exert a beneficial action. A hypothesis is defended here that hormesis as a general principle can be assumed only for the factors present in the natural environment thus having induced adaptation of living organisms. Generalizations of the hormesis phenomenon used in support of homeopathy are unfounded. Low-dose impacts may be associated with a higher risk in a state of organ sub-compensation or failure especially in the elderly patients. Practical recommendations should be based neither on the hormesis as a default approach nor on the postulates of homeopathy. All clinically relevant effects, hormetic or not, should be tested by the methods of evidence-based medicine.

KEY WORDS: Dose-response, homeopathy, hormesis, placebo

INTRODUCTION

Homeopathy claims a curative reaction from a small dose of a drug of which high doses cause symptoms similar to those from which the patient is suffering [1]. Homeopathy originated in 19th century, prior to the acceptance of the germ and gene bases of disease; it has never been based on scientific evidence [2]. Results of randomized trials do not provide acceptable evidence that homeopathic treatments are more effective than placebo [3,4]; although there are also contradicting statements [5]. It is known that the placebo-effect is used in homeopathy [6]. It is however possible that some empirical knowledge is successfully used in homeopathy unrelated to its axioms – "Like can be cured with like," "less is more" [7] or the memory of water [8].

Hormesis has been defined as a biphasic dose-response relationship in which the response at low doses is opposite to the effect at high doses [1]. According to this concept, a small dose of a noxious agent can exert a beneficial action. Some publications generalizing hormesis [9,10] can be cited in support of homeopathy. However, claims that homeopathy is based on hormesis create an illusion that it employs a scientific method. The difference between hormesis and homeopathy is that hormesis can be observed at low but measurable concentrations; while homeopathy claims effects of infinite dilutions, whereas the concept of memory of water [8] is used as an explanation. There is an opinion [2], shared by the author that the term hormesis should not be linked with homeopathy. If homeopaths have valuable empirical knowledge, it should be verified by the methods of evidence-based medicine. There must be no artisanal secrets in the health care. Potentially useful empirical knowledge gathered in homeopathy, alternative or complementary medicine, should be scientifically tested and discussed in the professional literature. The Journal of Intercultural Ethnopharmacology is an excellent forum for this purpose.

HORMESIS: GENERAL PRINCIPLE ONLY FOR ENVIRONMENTAL FACTORS

Among the known hormetic agents are pro-oxidants, heavy metals, heat, exercise, food restriction [11,12], and different kinds of stress [13]. Living organisms come in contact with all these factors in the natural environment, so that the hormetic effects can be explained from an evolutionary standpoint. The term "hormetins" has been used in the literature for hormesis-inducing compounds [14]. For antibiotics [12], hormetic effects develop secondarily along with the adaptation of microorganisms and development of antibiotic resistance. Another example: Thousands years' adaptation of certain human populations to ethanol resulted in detectable hormesis also for this toxic agent: Moderate alcohol consumption was reported to be associated with a reduced risk of coronary heart disease and other health benefits [15]. There has been no plausible explanation of hormesis as a default principle in the pharmacological theory [16]. Scientific foundations of some hormetic mechanisms were discussed within the framework of stress response pathways [17]. However, different kinds of stress are an integral part of the environmental impact on living organisms, who have been accordingly adapted to it. Hormesis as a general principle is conceivable only for the agents that have induced adjustment of living organisms, so that a deviation in either direction from an optimum would be

harmful. This is obviously the case for visible light, ultraviolet and ionizing radiation [18], atmospheric pressure, as well as for many chemical substances and microelements present in the environment. It is not surprising that potentially toxic heavy metals, which are present in the natural environment, act hermetically in plants [19]. There are no general reasons to expect hormetic responses for the factors absent in the natural environment. Among explanations for the hormetic effects, discussed in the literature, are an excess of repair mechanisms in response to mild damage [20] and a proposed existence of two receptor types (small quantity of high-affinity receptors and large numbers of low-affinity ones) [21,22]. Both hypotheses have not been sufficiently proven, in particular, as umbrella mechanisms for different types of agents. Moreover, some reported hormetic effects can be doubtful because of the difficulties of differentiation between low-level hormetic and placebo effects [23] questionable reliability of some data, poor study designs, etc. It should be stressed that a response to an agent usually increases with increasing concentration; in contrast, a placebo effect does not depend on concentrations, while homeopathic remedies can be extremely diluted so that the agent can be absent in the solution. For research purposes, placebo effects can be excluded in animal experiments without conditioning [24,25] and especially in plants [19], where hormetic effects can be studied.

Hormesis phenomenon was discussed in the context of homeopathy [1,9,16,26]; it was sometimes generalized and treated as a matter-of-course [9]. For example, the question: "Is hormesis likely to occur for all types of drugs?" was answered: "There are sufficient data to conclude that the hormetic dose response is common, reproducible, and a biological expectation in the vast majority of biological systems, end points measured, and chemical classes tested" [9]. The question "May drugs be acting hormetically even though the experimental data appear inconsistent with this interpretation?" was responded: "The hormesis concept establishes a biological context for some of the key 'rules' of pharmacology and toxicology" [9]. Such statements make an impression that the hormesis is a general principle. However, generalizations, according to which "hormetic-like biphasic dose responses may represent a general biological dose-response pattern or strategy" [9] have never been substantiated [27,28]. Moreover, hormesis, usually, relates to a single response, while toxic impacts can have different responses [23]. Some noxious stimuli can act synergistically with other factors, for example, on the cells with a limited or no capacity for cellular regeneration such as cardiomyocytes or neurons. It can be of particular importance in conditions when such cells are pre-damaged by ischemia so that even a mild additional damage would act according to a no-threshold dose-response pattern without hormesis. In conditions close to a functional decompensation of an organ, even minimal additional damage can be detrimental. In such conditions, which are not uncommon especially in elderly patients, the concept of hormesis can be dangerous if used in the clinical decision-making. For example, it would hardly be indicated to apply mild asphyxia in angina pectoris or small doses of ethanol in end-stage liver disease with a hope for a hormetic effect as a "general biological dose-response pattern" [9].

DISCUSSION

Considering the above, the statement: "The hormesis concept is a fundamental dose response, highly conserved, and set in an evolutionary framework" [9] is true a priori only for the factors that have induced evolutionary adjustment. If even hormesis was observed in studies of the substances that are absent in the environment such as antineoplastic, anxiolytic or anti-seizure drugs [22,29], or resveratrol (the latter was extensively discussed in the Volume 29 of Human and Experimental Toxicology, while relevance of the hormetic effects was questioned) [30], there is still no reason to conclude that "hormetic dose responses are broadly generalizable, being independent of biological model, endpoint measured, and stressor agent, and represent a basic feature of biological responsiveness to chemical and physical stressors" [29]. The publications containing generalizations of this kind [9] can be cited in support of homeopathy and placebo, in gerontology and other fields of medicine, also to endorse official registration of drugs without specific effects or efficacy not exceeding that of placebo. It can pave the way for homeopathy and placebos instead of evidence-based treatments, as inexpensive substitutes, especially for elderly patients. There are many examples of marketed compounds without scientifically demonstrated efficacy [31], in Russia often in the guise of evidence-based medications; while artificial theoretic concepts are created to promote them [32]. Promotion of unproven health schemes can be harmful especially for elderly people [33]. In the medical practice, deception is normally objectionable on the grounds that it limits autonomy and breaches trust; these grounds possibly do not apply to placebos when they are prescribed within appropriate ethical limits [34]; although there is an opinion that clinical placebo interventions are unethical and unnecessary [35]. If even placebo therapy with misinformation of a patient might be ethically acceptable in certain cases [36], it is still not a reason to publish biased information. Remarkably, it seems that some patients are influenced not only by medical advertizing, which is sometimes misleading in Russia, but also directly or indirectly by professional publications. In conditions when commercial considerations tend to replace medical ethics, some patients try to come clear with their ailments with the help of professional literature, which is their right. However, in Russia, public access to the medical literature is limited [37].

Moreover, persistence and development of spurious theoretic concepts can sooner or later result in the application of invasive procedures with questionable clinical indications [38]. For example, in the preceding article [32] a series of studies was commented that has become internationally known in 1986 after a publication in The Lancet with participation as coauthor of the health minister of that time [39]. There followed numerous publications in Russian and foreign journals continued until today [40-47] (more references are in [32]). Cultures of smooth muscle cells or macrophages were used for testing of blood atherogenicity, anti- or pro-atherogenic action of various substances. The agents were considered atherogenic if they enhanced cholesterol accumulation by the cultured cells. Drug dosages were

calculated on the basis of cell culture experiments [44]. In addition to the drugs, many natural substances were shown by the same researchers using the cell culture method to be effective against serum atherogenicity: Black elder berries, calendula and violet flowers [45], grape seeds and stems [46] etc. Extracts from 13 different mushrooms were shown to significantly lower serum atherogenicity [47]. However, as discussed in [32], the relationship between serum atherogenicity in a cell culture and atherogenesis *in vivo* must be inverse rather than direct. For example, in familial hypercholesterolemia, caused by abnormality of lipoprotein receptors, ineffective clearance of low density lipoprotein (LDL)-cholesterol from serum causes hypercholesterolemia and predisposes to atherosclerosis [48]. Up-regulation of LDL-receptors (and, correspondingly, of the LDL-cholesterol uptake by cells) is one of the paradigms to the atherosclerosis therapy [49]. Accordingly, if an agent reduces cholesterol uptake by cells *in-vitro*, it can be expected to cause serum cholesterol elevation *in-vivo*. Nevertheless, following their concept, the same researchers started applying extracorporeal apheresis through a column with immobilized LDL aimed at the "removal of non-lipid atherogenicity factor(s)" twice monthly for the period of 7-9 months [43]. Further experimentation in the same direction was recommended. The patients were men 46-59 years old with functional Class II-III angina pectoris, an angiographically documented stenosis of 2-3 coronary arteries and a normal cholesterol level. During this trial, the patients were reported to feel better, endure higher physical loads, and have heightened sexual activity [43], which could have been caused by a placebo effect. It is reasonable to assume that invasive procedures are associated with a placebo effect, which might be stronger than that of non-invasive procedures [50]. Blood apheresis is associated with certain risks [51], although severe side-effects such as shock or allergic reactions were reported to be very rare [52]. Efficiency of the apheresis in the study [43] cannot be excluded, although apheresis is usually aimed at removal of lipids and lipoproteins e.g., in patients with severe drug-resistant LDL-hypercholesterolemia or lipoprotein elevation and premature atherosclerosis [53,54]. Considering the above, indications to apheresis in [43] should be checked again.

CONCLUSION

Hormesis as a general principle has never been proven as an umbrella theoretic basis for factors that are absent in the environment. If an agent is present in the natural environment, existence of its optimal level can be assumed, which would correspond to the current environmental level or, considering that the natural selection is a slow process, to some average from the past. Low-dose impacts may be associated with a higher risk in a state of organ sub-compensation or failure especially in elderly patients. Accordingly, practical recommendations should be based neither on the hormesis as a default approach [55] nor on the "like cures like," "less is more" [7] or other postulates of homeopathy. All clinically relevant effects, including hormetic ones, should be tested by the methods of evidence-based medicine.

REFERENCES

1. Peper A. Aspects of the relationship between drug dose and drug effect. Dose Response 2009;7:172-92.
2. Moffett JR. Miasmas, germs, homeopathy and hormesis: Commentary on the relationship between homeopathy and hormesis. Hum Exp Toxicol 2010;29:539-43.
3. Ernst E. Homeopathy: What does the "best" evidence tell us? Med J Aust 2010;192:458-60.
4. Hill C, Doyon F. Review of randomized trials of homoeopathy. Rev Epidemiol Sante Publique 1990;38:139-47.
5. Fisher P. What is homeopathy? An introduction. Front Biosci (Elite Ed) 2012;4:1669-82.
6. Teixeira MZ, Guedes CH, Barreto PV, Martins MA. The placebo effect and homeopathy. Homeopathy 2010;99:119-29.
7. Ernst E. The truth about homeopathy. Br J Clin Pharmacol 2008;65:163-4.
8. Teixeira J. Can water possibly have a memory? A sceptical view. Homeopathy 2007;96:158-62.
9. Calabrese EJ, Iavicoli I, Calabrese V. Hormesis: Its impact on medicine and health. Hum Exp Toxicol 2013;32:120-52.
10. Calabrese EJ, Iavicoli I, Calabrese V. Hormesis: Why it is important to biogerontologists. Biogerontology 2012;13:215-35.
11. Marques FZ, Markus MA, Morris BJ. Hormesis as a pro-healthy aging intervention in human beings? Dose Response 2009;8:28-33.
12. López-Diazguerrero NE, González Puertos VY, Hernández-Bautista RJ, Alarcón-Aguilar A, Luna-López A, Königsberg Fainstein M. Hormesis: What doesn't kill you makes you stronger. Gac Med Mex 2013;149:438-47.
13. Le Bourg E. Hormesis, aging and longevity. Biochim Biophys Acta 2009;1790:1030-9.
14. Rattan SI. Rationale and methods of discovering hormetins as drugs for healthy ageing. Expert Opin Drug Discov 2012;7:439-48.
15. Saremi A, Arora R. The cardiovascular implications of alcohol and red wine. Am J Ther 2008;15:265-77.
16. Oberbaum M, Singer SR, Samuels N. Hormesis and homeopathy: Bridge over troubled waters. Hum Exp Toxicol 2010;29:567-71.
17. Rattan SI, Deva T. Testing the hormetic nature of homeopathic interventions through stress response pathways. Hum Exp Toxicol 2010;29:551-4.
18. Mitchel RE. The dose window for radiation-induced protective adaptive responses. Dose Response 2009;8:192-208.
19. Poschenrieder C, Cabot C, Martos S, Gallego B, Barceló J. Do toxic ions induce hormesis in plants? Plant Sci 2013;212:15-25.
20. Wiegant FA, de Poot SA, Boers-Trilles VE, Schreij AM. Hormesis and cellular quality control: A possible explanation for the molecular mechanisms that underlie the benefits of mild stress. Dose Response 2012;11:413-30.
21. Hayes DP. Adverse effects of nutritional inadequacy and excess: A hormetic model. Am J Clin Nutr 2008;88:578S-81.
22. Calabrese EJ. Hormesis and medicine. Br J Clin Pharmacol 2008;66:594-617.
23. Pickrell JA, Oehme FW. Examining the risks and benefits of replacing traditional dose-response with hormesis. Hum Exp Toxicol 2005;24:259-64.
24. McNabb CT, White MM, Harris AL, Fuchs PN. The elusive rat model of conditioned placebo analgesia. Pain 2014.
25. Haour F. Mechanisms of placebo effect and of conditioning: Neurobiological data in human and animals. Med Sci (Paris) 2005;21:315-9.
26. Bellavite P, Chirumbolo S, Marzotto M. Hormesis and its relationship with homeopathy. Hum Exp Toxicol 2010;29:573-9.
27. Mushak P. How prevalent is chemical hormesis in the natural and experimental worlds? Sci Total Environ 2013;443:573-81.
28. Jager T, Barsi A, Ducrot V. Hormesis on life-history traits: Is there such thing as a free lunch? Ecotoxicology 2013;22:263-70.
29. Calabrese EJ. Cancer biology and hormesis: Human tumor cell lines commonly display hormetic (biphasic) dose responses. Crit Rev Toxicol 2005;35:463-582.
30. Lindsay DG. Commentary on 'resveratrol commonly displays hormesis: Occurrence and biomedical significance'. Hum Exp Toxicol 2010;29:1024-5.
31. De Deyn PP, D'Hooge R. Placebos in clinical practice and research. J Med Ethics 1996;22:140-6.

32. Jargin SV. Phytoestrogens and soy products: Perspectives of application. J Intercult Ethnopharmacol 2013;2:67-72.
33. Jarvis WT. Quackery: A national scandal. Clin Chem 1992;38:1574-86.
34. Foddy B. A duty to deceive: Placebos in clinical practice. Am J Bioeth 2009;9:4-12.
35. Hróbjartsson A. Clinical placebo interventions are unethical, unnecessary, and unprofessional. J Clin Ethics 2008;19:66-9.
36. Linde K, Fässler M, Meissner K. Placebo interventions, placebo effects and clinical practice. Philos Trans R Soc Lond B Biol Sci 2011;366:1905-12.
37. Jargin SV. Limited access to the international medical literature in Russia. Wien Med Wochenschr 2012;162:272-5.
38. Jargin SV. Renal biopsy for research: An overview of Russian experience. J Interdiscip Histopathol 2014;2:88-95.
39. Chazov EI, Tertov VV, Orekhov AN, Lyakishev AA, Perova NV, Kurdanov KA, *et al*. Atherogenicity of blood serum from patients with coronary heart disease. Lancet 1986;2:595-8.
40. Sobenin IA, Chistiakov DA, Bobryshev YV, Orekhov AN. Blood atherogenicity as a target for anti-atherosclerotic therapy. Curr Pharm Des 2013;19:5954-62.
41. Orekhov AN. Direct anti-atherosclerotic therapy; development of natural anti-atherosclerotic drugs preventing cellular cholesterol retention. Curr Pharm Des 2013;19:5909-28.
42. Orekhov AN, Sobenin IA, Korneev NV, Kirichenko TV, Myasoedova VA, Melnichenko AA, *et al*. Anti-atherosclerotic therapy based on botanicals. Recent Pat Cardiovasc Drug Discov 2013;8:56-66.
43. Orekhov AN, Melnichenko AA, Sobenin IA. Approach to reduction of blood atherogenicity. Oxid Med Cell Longev 2014;2014:738679.
44. Orekhov AN, Pivovarova EM, Sobenin IA, Yakushkin VV, Tertov VV. Use of cell culture for optimisation of direct antiatherogenic therapy with verapamil. Drugs 1992;44 Suppl 1:105-10.
45. Gorchakova TV, Myasoedova VA, Sobenin IA, Orekhov AN. The reduction of serum atherogenicity by natural anti-inflammatory drugs. Abstracts of the XIV International Symposium on Atherosclerosis; 2006 June 18-22; Rome, Italy. Atheroscler Suppl 2006;7:230.
46. Korennaya VV, Myasoedova VA, Nikitina NA, Sobenin IA, Orekhov AN. Bioflavonoid-rich botanicals reduce blood serum atherogenicity in perimenopausal women. Abstracts of the XIV International Symposium on Atherosclerosis; 2006 June 18-22; Rome, Italy. Atheroscler Suppl 2006;7:444.
47. Ryong LH, Tertov VV, Vasil'ev AV, Tutel'yan VA, Orekhov AN. Antiatherogenic and antiatherosclerotic effects of mushroom extracts revealed in human aortic intima cell culture. Drug Dev Res 1989;17:109-17.
48. Marais AD. Familial hypercholesterolaemia. Clin Biochem Rev 2004;25:49-68.
49. Scharnagl H, März W. New lipid-lowering agents acting on LDL receptors. Curr Top Med Chem 2005;5:233-42.
50. Wartolowska K, Judge A, Hopewell S, Collins GS, Dean BJ, Rombach I, *et al*. Use of placebo controls in the evaluation of surgery: Systematic review. BMJ 2014;348:g3253.
51. Gilliss BM, Looney MR, Gropper MA. Reducing noninfectious risks of blood transfusion. Anesthesiology 2011;115:635-49.
52. Bambauer R, Schiel R, Latza R. Low-density lipoprotein apheresis: An overview. Ther Apher Dial 2003;7:382-90.
53. Parhofer KG. How will new medications affect the lipoprotein apheresis situation in Germany? Atheroscler Suppl 2013;14:71-2.
54. Julius U. Updates in apheresis and atherosclerotic research. Ther Apher Dial 2013;17:124.
55. Mushak P. Limits to chemical hormesis as a dose-response model in health risk assessment. Sci Total Environ 2013;443:643-9.

Source of Support: Nil, Conflict of Interest: None declared.

Review Article

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141030015241

Antidiabetic potential of some less commonly used plants in traditional medicinal systems of India and Nigeria

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Received: September 30, 2014
Accepted: October 30, 2014
Published: November 28, 2014

ABSTRACT

The incidence of diabetes mellitus continue to rise annually all over the world with India and Nigeria having recorded cases of 65.1 and 3.9 million respectively in 2013 and expected to increase by a large amount in 2035. Hyperglycemia is a pre-condition for the development of diabetic complications and is accompanied by an increase in the production of free radicals. The present available treatment option for diabetes like sulfonylurea, metformin and alpha-glucosidase are restricted by their limited actions, secondary failure rates, and side-effects; and unaffordable to the majority of the population. Hence, the need to screen for more medicinal plants with antidiabetic ability due to the fact that plants are; biodegradable, safe and cheap with fewer side-effects. In this review article, we have presented the current status of diabetes in India and Nigeria and the role of some less commonly used medicinal plants from both countries that have antidiabetic potential.

KEY WORDS: Antidiabetic plants, diabetes, hypoglycaemic activity, medicinal plants, oxidative stress

INTRODUCTION

The World Health Organization (WHO) defines diabetes mellitus (DM) as a degenerative and chronic disease that occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use insulin [1]. It is a disorder of the metabolism of carbohydrates, fats, and lipids, which is characterized by a high fasting blood sugar [2]. It manifests as chronic hyperglycemia and leads to the development of diabetes-specific micro vascular pathology in the retina, glomerulus and peripheral nerve culminating into serious complications affecting the eyes, kidneys and arteries [3,4].

WHO statistics shows that worldwide 347 million people have diabetes and 80% of diabetic deaths occur in low and middle-income countries [1]. According to the International Diabetes Federation, India is ranked second only to China in the list of top ten countries for a number of people with diabetes. [5]. In Africa, it is estimated that about 19.8 million adults have diabetes with Nigeria and South Africa having 3.9 and 2.6 million, respectively. It is estimated that by 2035, the percentage of diabetic patients in Africa would cross an alarming figure of 58% [5].

Type 2 diabetes, is the major form of diabetes accounting for 90-95% of all diabetic cases [6] and nearly half of all patients suffering from the disease are older than 65 years of age [7]. It is a complicated and divergent disease which in addition to blood sugar control requires the management of lipid parameters, blood pressure and thrombotic factors [8].

The treatment for diabetes is both difficult and tedious; it is expensive, costly and not affordable by majority of African and Asian populations [9]. The current treatments for DM include the use of insulin and synthetic drugs such as sulfonylurea, metformin, alpha-glucosidase inhibitors and thiazolidinedione's in addition to lifestyle adjustments. These synthetic drugs are valuable but restricted by their limited action, pharmacokinetic properties, secondary failure rates and accompanying side-effects like hypoglycemia, damage to liver, lactic acidosis, diarrhea, abdominal pain, weight loss and loss of appetite [7,10-12].

Due to the problems associated with the current treatments, a large percentage of diabetics resort to alternative remedies that are purported to improve glycemic control [8]. The WHO estimated that approximately 80% of the world's population rely mainly on traditional medicines for their primary health care [13]. The screening of medicinal plants for novel bioactive compounds is, therefore, an important goal for scientists. Importantly, the plant based drugs are biodegradable, safe, and cheap, having fewer side-effects, in India, China and other ancient traditional medicinal systems in the world, medicinal plants have been the major source of treatment for DM since time immemorial [14-16].

The importance of research on medicinal plants is validated by the fact that a plethora of new drugs have been developed from plants; relevant examples include cromolyn used as bronchodilator, developed from *Ammi visnaga* (L) Lamk;

galegine, from *Galega officinalis* L, which is a model for the synthesis of metformin and other bisguanidine-type antidiabetic drugs, papaverine from *Papaver somniferum* which forms the basis of cerapramil used in the treatment of hypertension, [17]. *Artemisia annua* (Quinhaosu) gave rise to artemininin, this compound and it analogs are now used as antimalarial therapy in many countries [18]. Paclitaxel (Taxol®), the most exciting plant-derived anticancer drug discovered in recent years, is derived from several key precursors (the baccatins) in the leaves of various Taxus species; *Taxus brevifolia* [19].

Although the role of natural product in new drug discovery is encouraging and has frequently resulted in development of new drugs [20], the success of drug discovery depends on evolving stringent criteria to avoid false positive drug candidates. Surfeit of information warrants proper documentation. The evaluation of the scientific efficacy of traditional systems of medicine is an area of great interest especially in developing economies where sometimes the cost of medication may be prohibitive. Excellent reviews [13,21-23] on antidiabetic plants have already been written. This current review aims to bring in focus and document the use of less commonly used antidiabetic plants on which fewer studies have been conducted. Some of these plants, albeit less researched, hold immense potential as antidiabetic therapeutic agents in India and Nigeria.

ANTIDIABETIC PLANTS USED IN NIGERIA AND INDIA

The climatic conditions in Nigeria and India support the growth and thriving of various plant species and hence the use of these plants by the poor population to ameliorate disease conditions. There are about 800 plants that may possess antidiabetic properties according to ethno botanical information [24]. Most of the current drugs available have been directly or indirectly derived from plants. An example is metformin that was derived from the plant *G. officinalis* L.

The plants with antioxidant and antidiabetic potential included in this review are *Azadirachta indica* (AI) A. Juss, *Mangifera indica* (MI) L, *Terminalia arjuna* Roxb. Ex DC, *Terminalia catappa* L, *Terminalia chebula* Retz, *Syzygium cumini* (L) Skeels, *Syzygium aromaticum* (L) Merr. and L.M. Perry, *Vernonia amygdalina* (VA) Delile and *Xylopia aethiopica* (XA) (Dunal) A. Rich.

The general botanical data, taxonomic data, distribution in the world, experimental design, compounds isolated, mechanism of action, the antidiabetic and antioxidant capability of the plants are presented below:

Mangifera indica L. (Common Name: Mango)

Mango in an important species of the family anacardiaceae and the genus *Mangifera*, it is native to South East Asia from where it spread all over the world, it is the most popular fruit in the tropical and subtropical regions of the world. It is the national fruit of India, Pakistan, Philippines and the national tree of Bangladesh [25].

The plant is widely grown in Nigeria, where in addition to the fruit consumption it is used for the treatment and management of diabetes [26]. The peel and pulp of the plant contain carotenoids, and polyphenols such as quercetin, kaempferol, gallic acid, caffeic acid, catechins, tannins, mangiferin, leucocyanidin, epiatechin, quercetin and chromogenic acid [27]. Phenolics have scavenging activity on free radicals mainly due to the presence of hydroxyl groups. Recently, Mohan *et al.* [28] isolated a compound 1, 2, 3, 4, 6-penta-O-gallolyl-β-D-glucose from the methanolic extract fraction of mango that is a potent inhibitor of 11-β-hydroxysteriod hydrogenase enzyme and ameliorates high fat diet (HFD) induced diabetes in C57BL/6 mice.

Mangiferin (1, 3, 6, 7-tetrahydroxy-xanthone-C2-β-D-glucoside) a bioactive compound isolated from MI possesses a wide range of pharmacological actions including being anticancer [29,30], antibacterial [31], anti HIV [32], antioxidants [33], and antidiabetic [34,35].

The administration of mangiferin at a dose of 10 and 20 mg/Kg body weight (i.p.) in type 1 and 2 diabetic rats for 30 days showed significant antidiabetic, hypo-lipidemic, alpha amylase and alpha-glucosidase inhibitory effect [36]. This glucoside has also been shown by Li *et al.* [37] to improve renal function of diabetic nephropathy in rats and its inhibitory effect on overexpression of transforming growth factor-β1, advanced glycation end and extracellular matrix accumulation, Polyol pathway activation, reactive oxygen species (ROS) generation and mesangial cells proliferation. Miura *et al.* [38] demonstrated that the mangiferin exerts its antidiabetic activity by decreasing the insulin resistance.

The ethanolic extracts of MI showed significant free radical scavenging activity and have cytoprotective (anti-apoptotic) effect; the leaves and fruits extract reduce the absorption of glucose in type 2 diabetes and stimulate glycogenesis in liver causing reduction in blood glucose level [39].

Vernonia amygdalina Delile (Asteraceae)

VA is a perennial shrub-like plant with green leaves growing up to 1.3-3 m high that is native to Africa, widely grown in Nigeria and West Africa. It is reported to contain phytochemicals useful in the treatment and management of certain diseases. It has been introduced into India and is now being cultivated in parts of central and eastern India [40].

VA is rich in amino acids, minerals and vitamins [41]. The decoction from the leaves is often used in the African traditional treatment for the management of diabetes, malaria, infertility, and sexually transmitted diseases [42-47]. The plant is said to have antimalarial compounds like alkaloids, tannins, and saponins [48] and also anticancer properties [49]. In comparison to other plants, VA accounted for 9.2% of medicinal plants used as an alternative medicine in central Nigeria [50]. In Nigeria, a dosage form of freeze-dried aqueous leaf extract of this plant has been developed and formulated, which is suitable for therapeutic use in the management of DM. Mostly in Nigeria, the decoction

from the leaf is often used in combination with that of other plants by traditional healers and medical practitioners to treat diabetes, fever and gastrointestinal problems [51].

The ethanolic extracts of the plant has a strong bioactive compound that has blood sugar lowering action in rats and can serve as an effective antioxidant [52], Ong *et al*. [53] showed that VA has anti-hyperglycemic effect on streptozotocin (STZ)-induced diabetic rat model and this effect is mediated through the inhibition of key hepatic G6pase, which causes an increase in expression and translocation of GLUT4 in skeletal muscles. The combined leaf extract of *A. indica* (AI) and VA ameliorates hyperglycemia and hepatic oxidative stress in diabetic rats [54] and the methanolic extract of VA has the ability to mitigate cycasin-induced oxidative damage in colonic tissues [55].

The composite decoctions of VA, *Gongronema latifolium* (Benth) and *Occimum gratissimum* (Linn) reduced the postprandial blood glucose concentrations of diabetic subjects [56]. Two flavonoids and terpenoids: Vernolide and edotides have been isolated from the VA plant. Octahydrovernodalin is the most important bitter principle in the plant [57]. Ong *et al*. [58] also isolated four main polyphenols in the ethanolic extract namely dicaffeoyl-quinic acid, chlorogenic acid, 1,5-dicaffeoyl-quinic acid and luteolin-7-O-glucosidase. Dicaffeoyl-quinic acid is the most abundant in the plant. The administration of 400 mg/Kg body weight of VA extract is found to exert most effective anti-hyperglycemic activity [59].

The two major glucose transporters that regulate glucose uptake into the tissues are GLUT1 (non-insulin responsive) and GLUT4 (insulin-responsive). While G6pase is one of the rate-limiting gluconeogenic enzymes that regulate, the synthesis of glucose and results has shown strong suppression of G6pase activity by extracts of VA [60]. VA extract was found also to protect pancreatic β-cells and the polyphenols present are responsible for this action especially dicaffeoyl-quinic acid.

Most of the traditional uses of the plant have been systematically and scientifically validated and the study of oxidative stress in diabetic rats showed that the aqueous extracts of VA decrease the levels of serum malondialdehyde an indication of the antioxidant property of the plant [60].

Xylopia aethiopica (Dunal) A. Rich

XA, also known as the African pepper or Ethiopian pepper, belongs to the family annonaceae and the genus *Xylopia*. It is a tropical, slim, tall and aromatic tree that grows up to 15-30 m. It is found in the west, central and southern Africa in humid forest zones, native to Nigeria, Ghana, Kenya, Ethiopia, Senegal and Uganda.

XA is a common ethno medicine in West Africa where it is used in the treatment of rheumatism and arthritis, cough, stomachache, bronchitis, biliousness and dysentery [61]. The fruit and vegetable have many medicinal properties and contains phytochemicals, vitamins and minerals. Phytochemicals like flavonoids are potentially anti-allergic, anti-carcinogenic, anti-viral and antioxidants, the ethanolic extract of XA was found to increase steroid hormone [62], the aqueous extract was also shown to have anti-amylase and anti-lipase activity with antioxidant potentials [63].

A poly-herbal formulation sold in Nigeria containing the following: *Stachytarpheta angustifolia, Alstonia congensis, and* XA in the ratio 3:2:1 was found to have hypoglycemic and hyperlipidemic activities [64].

Syzygium aromaticum (Linn.) Merrill and Perry (Myrtaceae) (Common Name: Cloves)

S. aromaticum (clove) belongs to the family myrtaceae and the genus *Syzygium*. Native to Indonesia, this plant can grow to a height of 8-12 m, it is an aromatic flower bud commonly used in Africa, Asia and other parts of the world for the preparation of different spicy dishes. In Nigeria most traditional medical practitioners use the fruits and cloves by boiling in water and the decoction is administered to patients for the treatment of cough, chest congestion and catarrh and the compound eugenol present in this plant is responsible for the aroma and has antioxidative and antimycotic ability [65].

A triterpenoid compound extracted from the clove plant named oleanolic acid has potent diuretic/saluretic, anti-hyperlipidemic, antioxidant and hypoglycemic effects [66], Ngubane *et al*. [67] showed that oleonolic acid exhibited anti-hyperglycemic effect in STZ-induced diabetic rats by the attenuation of the activities of glycogenic enzymes and the compound eugenol present in this plant is responsible for the aroma and has antioxidative and antimycotic ability. The oil from the extract of this plant protects experimental animals from hepato-nephrotoxicity and oxidative stress due to aflatoxins [68].

Clove bud powder (CBP) possesses high phenolic content, free radical scavenging activity and metal chelating and reducing properties, the major phenolic compounds found are Kaempferol, isoquercitrin, gallic acid, ellagic acid, and caffeic acid [69]. Dietary supplementation of CBP in type 2 diabetic rats showed anti-hyperglycemic, hepatoprotective, hypolipidemic and antioxidant activities, by suppressing oxidative stress and delaying carbohydrate digestion [70].

Oleanolic acid (3 β-hydroxy-olea-12-en-28-oic acid) and maslinic acid have been reported to modulate the activity of the intestinal glucose transporters and carbohydrate hydrolyzing enzymes thus reducing postprandial hyperglycaemia and that the ethanolic extract of this plant suppresses elevated blood glucose levels in type 2 diabetic KK-A^y mice [70].

Free and bound phenolic extract of clove bud was found to inhibit carbohydrate hydrolyzing enzymes; alpha-amylase and alpha-glucosidase in a dose-dependent manner (200-800 μg/ml) [71]. Decreasing the postprandial hyperglycemia peak is very crucial in the treatment of diabetes; there is a strong correlation between the phenolic content of clove and the enzyme inhibitory

activities and with a strong antioxidant property which is the mechanism and the basis for its anti-diabetic action [71].

Azadirachta indica A. Juss (Common Name: Neem)

AI A. Juss is a member of the Meliacea family and the genus Azadirachta. It is a fast-growing tree that can reach up to 15-20 m and can sometimes reach 40 m. The plant is native to India and adapted to sub-arid and sub-humid tropical climates. It is widely grown in India, Pakistan, Indonesia, Sri Lanka, Caribbean, Nigeria, South and Central America. It is called "Dogonyaro" in Nigeria and grown all over the country, especially in the northern region. The plant has been used in the Indian Ayurveda traditional medicine for over 2000 years for the healing of various diseases and ailments [72].

The composite leaf extract of AI and VA at 500 mg/Kg body weight ameliorates hypoglycemia and hepatic oxidative stress in STZ-induced diabetic rats [54]. AI leaves glucosamine an active component of neem leaves is responsible for immunostimulatory activity in albino mice [73].

The chloroform extract of AI administered on murine diabetic model for 21 days significantly reduced the fasting blood sugar and islet regeneration and protection properties [74]. The administration of 500 mg/kg body weight of AI leaf extract and AI bark extract was effective in improving the antioxidant status in cardiac and skeletal muscles [75]. Khosla *et al*. [76] showed that azadirachtin and nimbin are the active ingredients in AI and they have the ability to regenerate the pancreatic beta cell. Recently Tiwari *et al*. [77] showed that the administration of the composite extract of *Aegle marmelos*, AI, *Murraya koengii*, *Occimum sanctum*, and *S. cumini* at 100 mg/Kg body weight caused a significant reduction in the blood sugar level, total cholesterol, triglyceride, low-density lipoproteins and an increase in the level of high-density lipoproteins.

Syzygium cumini (L.) Skeels

This plant belongs to the family myrtaceae and the genus *Syzygium*; it is an evergreen tropical plant native to South East Asia and widely grown in Africa. The fruit of the plant is widely used in cooking as spice and condiments to add flavor to foods.

S. cumini is well-known for its antidiabetic properties; gallic acid, rutin and chlorogenic acid are the main phenolic present in this plant and the extracts of all parts of the plant is used in traditional medicine [78]. Aqueous extract is found to improve endothelial dysfunction, antioxidant, anti-inflammatory and anti-thrombic properties of adenosine deamine activity in erythrocytes [78].

A dose of 400 mg/kg body weight of aqueous seed extract of *S. cumini* has hypoglycemic, insulin sensitizing and hypo-lipidemic activity in HFD-STZ induced rats due to an increase in peroxisome proliferator-activated receptor $(PPAR)_{y}$ and $PPAR_{\alpha}$ protein expression [79]. The active fraction of *S. cumini* was found to regenerate pancreatic islets and insulin secretion in STZ-induced diabetic mice [80]. Sharma *et al*. [81] demonstrated that the aqueous extract of *S. cumini* seed when given orally to mice at a dose of 250 mg/kg body weight for 21 days effected and repaired the liver damage associated with alloxan diabetes. The extract of this plant inhibits alpha-glucosidase and alpha-amylase, which are the two enzymes responsible for the metabolism of carbohydrate, and this limits the postprandial glucose and consequently controlling diabetes [82]. The seed extract is found to act as a chemo-protective agent against *in vivo* oxidative stress and genomic damage [83].

Terminalia catappa. L.

This plant belongs to the family Combretacea and to the genus Terminalia found growing in the warmer parts of India, Asia, Africa and Australia. The tree is primarily used as an ornamental and as a shade tree; the seeds are edible like almonds. The extracts of the bark and leaves are reported to have anticancer and aphrodisiac capability [84], antioxidant and anti-inflammatory [85] and anti-malarial [86]. This may be as a result of high contents of tannins in the plant making them a good source of antioxidants [87]. Kinoshita *et al*. [88] isolated chebulagic acid and corilagin from the 50% ethanol extract of the plant with a strong free radical scavenging activity and these compounds are found to have hepato-protective and antioxidant actions, by suppressing the generation of ROS followed by the inhibition of apoptosis.

Terminalia arjuna (Roxb) Wight and Arn

This is a plant belonging to the family Combretaceae and genus Terminalia commonly called arjuna. It is a large tree found throughout the South Asia region, and it is an exotic tree in India, it can grow up to a height of 25-30 m. The bark and fruits of this plant is used in traditional Indian medicine as an anti-dysentric, anti-pyretic, astringent, cardiotonic, lithotriptic, anticoagulant, hypolipidemic and anti-microbial, the large amount of flavonoids is responsible for the antioxidant and anti-microbial properties [89]. The bark contains arjunine a lactone, arjunetin, essential oils and reducing sugars. The methanolic extract exhibited analgesic activity and acute anti-inflammatory activity [90]. The extracts of this plant have the presence of alkaloids, triterpenoids, tannins and flavonoids. Gallic acid, apigenin, luteolin, quercetin, epicatechin, ellagic acid and 1-O-galloyl glucose are some of the compounds that have been isolated from this plant [91].

A dose of 250 and 500 mg/kg body weight of *T. arjuna* extract was found to have reno-protective and antioxidant ability in isolated perfused kidneys [92]. The leaf extracts when administered at a dose of 100 and 200 mg/kg body weight orally to STZ-induced diabetic rats was found to significantly normalize blood glucose level and this is due to its antioxidant role [93]. Due to the presence of tannins, saponin, and flavonoids, the bark extract exhibited antidiabetic activity by enhancing the peripheral utilization of glucose by correcting the impaired liver and kidney glycolysis and by limiting gluconeogenic formation, an action similar to that of insulin [94]. Perveen *et al*. [95] showed that

Table 1: Summary of the selected plant species with their active component and their therapeutic effects

Plant species name	Used component	Property/effect	References
MI L.	1, 2, 3, 4, 6-penta-O-gallolyl-β-D-glucose	Inhibits 11-β-hydroxysteriod hydrogenase enzyme. antidiabetic	[23]
	Mangiferin (1, 3, 6, 7-tetrahydrox y-xanthone-C2-β-D-glucoside)	Improve renal function of diabetic nephropathy in rats. Decrease insulin resistance	[29,30,32,33]
VA Delile	Ethanolic extracts	Lowers blood sugar	[48]
	Dicaffeoyl-quinic acid	Protect pancreatic β cells	[55]
XA (Dunal) A. Rich.	Aqueous extract	Anti-amylase, anti-lipase activity with antioxidant potentials	[58]
S. aromaticum (Linn.)	Oleanolic acid	Anti-hyperlipidemic, antioxidant and hypoglycaemic	[6]
Merrill and Perry	Oleanolic acid and maslinic	reducing postprandial hyper-glycaemia	[65]
AI A. Juss	Chloroform extract	Reduced fasting blood sugar, islet regeneration	[70]
	Azadirachtin and Nimbin	Regeneration of pancreatic beta cells	[72]
S. cumini (L.) Skeels	Aqueous seed extract	Hypoglycaemic, insulin sensitising and hypo-lipidemic activity in HFD-STZ induced rats. Repaired liver damage associated with alloxan diabetes	[74,76]
T. catappa. L	Chebulagic acid and Corilagin	Antioxidants	[83]
T. arjuna (Roxb)	Leaf extracts	Normalise blood glucose levels	[88]
Wight and Arn	Ethanolic extracts	Inhibit oxidation and lipid degradation	[91]
T. chebula Retz	Methanolic extracts	Plant inhibits lipid peroxide formation and scavenge hydroxyl and superoxide radical	[94]

MI: *Mangifera indica*, VA: *Vernonia amygdalina*, XA: *Xylopia aethiopica*, AI: *Azadirachta indica, S. aromaticum: Syzygium aromaticum, S. cumini: Syzygium cumini, T. catappa: Terminalia catappa, T. arjun: Terminalia arjuna , T. chebula: Terminalia chebula*, HFD: High fat diet, STZ: Streptozotocin

the antioxidant activity of *T. arjuna* bark extract is due to the rich concentration of tannins, triterpenoid and saponins like arjunic acid, arjunolic acid, arjungenin, arjunglycosides, gallic acid, ellagic acid, oligomeric proanthocyanidins, and that the antidiabetic activity is due to the stimulation of β-cells of the pancreatic islets. The administration of *T. arjuna* ethanolic extracts at a dose of 250 mg/kg body weight per oral was found to reverse diabetic condition by inhibiting oxidation and degradation of lipids [96], and due to the fact that *T arjuna* extract has the ability to reduce postprandial hyperglycemia an important cardiovascular risk factor in type 2 diabetic patients, it has got a promising anti-hyperglycaemic and hypo-lipidemic effects in type 2 diabetics [97].

Terminalia chebula Retz. (Combretaceae)

This plant belongs to the family combretaceae, and the genus *Terminalia* found growing in the Sub-Himalayan tracts. It is a tall tree plant rising to about 15-25 m. It is a revered plant in India and has been extensively used in the Ayurveda, Unani and Homeopathic medicine. It has a beneficial effect on digestive diseases, urinary diseases, diabetes, skin, heart, irregular fevers, constipation, ulcers, vomiting, colic pain and hemorrhoids [98]. Phyto-constituents present in this plants is hydrolysable tannins like gallic acid, chebulagic acid, punicalagin, chebulamin, corilagin, neochebulini acid, ellagic acid, casuarinas and 2,3,6-tri-O-galloyl-β-D-glucose, 1,6-di-O-galloyl-D-glucose and terchebulin. The methanolic extracts from the plant inhibits lipid peroxide formation and scavenge hydroxyl and superoxide radicals [99]. The methanolic extract of *T chebula* has antioxidant, anti-inflammatory and anticancer ability and the phenolic derivatives, hydrolysable tannins and oleanane type triterpenoids are the active principles [100]. Chebulagic acid from *T. chebula* at 100 mg/kg body weight significantly reduced postprandial blood glucose levels of Sprague-Dawley rats when compared to the control group.

CONCLUSION

The present review presents the current scientific literature with respect to the antidiabetic and antioxidant potential of *AI*, *MI*, *T. arjuna*, *T. catappa*, *T. chebula*, *S. cumini*, *S. aromaticum*, VA *and* XA; summarize in Table 1. These plants are not the most popular when it comes to their use as antidiabetic plants used in traditional medicine, but yet they are widely used in some traditional medicinal system in India and Nigeria. It is hoped that further studies on these plants will target the isolation, purification and characterization of the bioactive compounds, which may lead to the discovery of potent antidiabetic drugs for the management and treatment of diabetes.

ACKNOWLEDGMENT

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REFERENCES

1. World Health Organization. Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia: Report of a WHO/IDF consultation. Geneva: World Health Organization; 2006.
2. Modak M, Dixit P, Londhe J, Ghaskadbi S, Devasagayam TP. Indian herbs and herbal drugs used for the treatment of diabetes. J Clin Biochem Nutr 2007;40:163-73.
3. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414:813-20.
4. Mehdi U, Toto RD. Anemia, diabetes, and chronic kidney disease. Diabetes Care 2009;32:1320-6.
5. Guariguata L, Nola T, Beagley J, Linnenkamp U, Jacqmain O. IDF Diabetes Atlas. 6th ed. Brussels, Belgium: International Diabetes Federation; 2013.
6. Philippe J, Raccah D. Treating type 2 diabetes: How safe are current therapeutic agents? Int J Clin Pract 2009;63:321-32.
7. Virdi J, Sivakami S, Shahani S, Suthar AC, Banavalikar MM, Biyani MK.

Antihyperglycemic effects of three extracts from *Momordica charantia*. J Ethnopharmacol 2003;88:107-11.
8. Mohammed A, Ibrahim MA, Islam MS. African medicinal plants with antidiabetic potentials: A review. Planta Med 2014;80:354-77.
9. Bosi E. Metformin – The gold standard in type 2 diabetes: What does the evidence tell us? Diabetes Obes Metab 2009;11 Suppl 2:3-8.
10. Asche CV, McAdam-Marx C, Shane-McWhorter L, Sheng X, Plauschinat CA. Association between oral antidiabetic use, adverse events and outcomes in patients with type 2 diabetes. Diabetes Obes Metab 2008;10:638-45.
11. Jacobsen IB, Henriksen JE, Beck-Nielsen H. The effect of metformin in overweight patients with type 1 diabetes and poor metabolic control. Basic Clin Pharmacol Toxicol 2009;105:145-9.
12. Jia W, Gao W, Tang L. Antidiabetic herbal drugs officially approved in China. Phytother Res 2003;17:1127-34.
13. Demain AL, Sanchez S. Microbial drug discovery: 80 years of progress. J Antibiot (Tokyo) 2009;62:5-16.
14. Rizvi SI, Mishra N. Traditional Indian medicines used for the management of diabetes mellitus. J Diabetes Res 2013;2013:712092.
15. Udayakumar R, Kasthurirengan S, Mariashibu TS, Rajesh M, Anbazhagan VR, Kim SC, *et al*. Hypoglycaemic and hypolipidaemic effects of *Withania somnifera* root and leaf extracts on alloxan-induced diabetic rats. Int J Mol Sci 2009;10:2367-82.
16. Inayat-ur-Rahman, Malik SA, Bashir M, Khan R, Iqbal M. Serum sialic acid changes in non-insulin-dependant diabetes mellitus (NIDDM) patients following bitter melon (*Momordica charantia*) and rosiglitazone (Avandia) treatment. Phytomedicine 2009;16:401-5.
17. Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. Environ Health Perspect 2001;109 Suppl 1:69-75.
18. Miller LH, Su X. Artemisinin: Discovery from the Chinese herbal garden. Cell 2011;146:855-8.
19. Kingston DG. Taxol and its analogs. In: Cragg GM, Kingston DG, Newman DJ, editors. Anticancer Agents from Natural Products. 2nd ed. Boca Raton, FI: Taylor and Francis; 2012. p. 123-75.
20. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. J Nat Prod 2007;70:461-77.
21. Tiwari BK, Pandey KB, Abidi AB, Rizvi SI. Therapeutic potential of Indian medicinal plants in diabetic condition. Ann Phytomed 2013;2:37-43.
22. Mukherjee PK, Maiti K, Mukherjee K, Houghton PJ. Leads from Indian medicinal plants with hypoglycemic potentials. J Ethnopharmacol 2006;106:1-28.
23. Grover JK, Yadav S, Vats V. Medicinal plants of India with anti-diabetic potential. J Ethnopharmacol 2002;81:81-100.
24. Loh SP, Hadira O. *In vitro* inhibitory potential of selected Malaysian plants against key enzymes involved in hyperglycemia and hypertension. Malays J Nutr 2011;17:77-86.
25. Fasoli E, Righetti PG. The peel and pulp of mango fruit: A proteomic samba. Biochim Biophys Acta 2013;1834:2539-45.
26. Etuk, EU, Bello SO, Isezuo SA, Mohammed BJ. Medicinal plants used for the treatment of diabetes mellitus in the North Western region of Nigeria. Asian J Exp Biol Sci 2010;1:55-9.
27. Ma X, Wu H, Liu L, Yao Q, Wang S, Zhan R, *et al*. Polyphenolic compounds and antioxidant properties in mango fruits. Sci Hortic 2011;129:102-7.
28. Mohan CG, Viswanatha GL, Savinay G, Rajendra CE, Halemani PD. 1,2,3,4,6 Penta-O-galloyl-β-d-glucose, a bioactivity guided isolated compound from *Mangifera indica* inhibits 11β-HSD-1 and ameliorates high fat diet-induced diabetes in C57BL/6 mice. Phytomedicine 2013;20:417-26.
29. Shoji K, Tsubaki M, Yamazoe Y, Satou T, Itoh T, Kidera Y, *et al*. Mangiferin induces apoptosis by suppressing Bcl-xL and XIAP expressions and nuclear entry of NF-κB in HL-60 cells. Arch Pharm Res 2011;34:469-75.
30. Yoshimi N, Matsunaga K, Katayama M, Yamada Y, Kuno T, Qiao Z, *et al*. The inhibitory effects of mangiferin, a naturally occurring glucosylxanthone, in bowel carcinogenesis of male F344 rats. Cancer Lett 2001;163:163-70.
31. Subbiya A, Mahalakshmi K, Pushpangadan S, Padmavathy K, Vivekanandan P, Sukumaran VG. Antibacterial efficacy of *Mangifera indica* L. kernel and *Ocimum sanctum* L. leaves against *Enterococcus faecalis* dentinal biofilm. J Conserv Dent 2013;16:454-7.
32. Wang RR, Gao YD, Ma CH, Zhang XJ, Huang CG, Huang JF, *et al*. Mangiferin, an anti-HIV-1 agent targeting protease and effective against resistant strains. Molecules 2011;16:4264-77.
33. Das J, Ghosh J, Roy A, Sil PC. Mangiferin exerts hepatoprotective activity against D-galactosamine induced acute toxicity and oxidative/nitrosative stress via Nrf2-NFκB pathways. Toxicol Appl Pharmacol 2012;260:35-47.
34. Sellamuthu PS, Muniappan BP, Perumal SM, Kandasamy M. Antihyperglycemic effects of mangiferin in STZ-induced diabetic rats. J Health Sci 2009;55:206-14.
35. Ichiki H, Miura T, Kubo M, Ishihara E, Komatsu Y, Tanigawa K, *et al*. New antidiabetic compounds, mangiferin and its glucoside. Biol Pharm Bull 1998;21:1389-90.
36. Dineshkumar B, Mitra A, Manjunatha M. Studies on the antidiabetic and hypolipidemic potentials of mangiferin (Xanthone glucoside) in STZ-induced type1 and type 2 diabetic rat models. Int J Adv Pharm Sci 2010;1:75-85.
37. Li X, Cui X, Sun X, Li X, Zhu Q, Li W. Mangiferin prevents diabetic nephropathy progression in streptozotocin-induced diabetic rats. Phytother Res 2010;24:893-9.
38. Miura T, Ichiki H, Hashimoto I, Iwamoto N, Kato M, Kubo M, *et al*. Antidiabetic activity of a xanthone compound, mangiferin. Phytomedicine 2001;8:85-7.
39. Ling LT, Yap SA, Radhakrishnan AK, Subramanian T, Cheng HM, Palanisamy UD. Standardised *Mangifera Indica* extract is an ideal antioxidant. Food Chem 2009;113:1154-9.
40. Bhattacharjee B, Lakshminarasimhan P, Bhattacharjee A, Agrawala DK, Pathak MK. *Vernonia amygdalina* Del (Asteraceae) - An African medicinal plants introduced in India. Zoo's Print 2013;28:18-20.
41. Eleyinmi AF, Sporns P, Bressler DC. Nutritional composition of *Gongro-nema latifolium* and *Vernonia amygdalina*. Nutr Food Sci 2008;38:99-109.
42. Borokini TI, Ighere DA, Clement M, Ajiboye TO, Alowonle AA. Ethno biological survey of traditional medicine practice for the treatment of piles and diabetes mellitus in Oyo State. J Med Plants Stud 2013;1:30-40.
43. Farombi EO, Owoeye O. Antioxidative and chemopreventive properties of *Vernonia amygdalina* and *Garcinia biflavonoid*. Int J Environ Res Public Health 2011;8:2533-55.
44. Gbolade AA. Inventory of antidiabetic plants in selected districts of Lagos State, Nigeria. J Ethnopharmacol 2009;121:135-9.
45. Abo KA, Fred-Jaiyesimi AA, Jaiyesimi AE. Ethnobotanical studies of medicinal plants used in the management of diabetes mellitus in South Western Nigeria. J Ethnopharmacol 2008;115:67-71.
46. Erasto P, Van de Venter M, Roux S, Grierson DS, Afolayan AJ. Effect of leaf extract of *Vernonia amygdalina* on glucose utilization in chang liver C2C12 muscle and 3T3-L1 cells. Pharm Biol 2009;47:175-81.
47. Akah PA, Ekekwe RK. Ethno pharmacology of some Asteraceae family used in Nigerian traditional medicine. Fitoterapia 1995;66:351-5.
48. Sharma MC, Sharma S. Pharmacognostic and phytochemical screening of *Vernonia amygdalina* Linn against selected bacterial strains. Middle-East J Sci Res 2010;6:440-4.
49. Wong FC, Woo CC, Hsu A, Tan BK. The anti-cancer activities of *Vernonia amygdalina* extract in human breast cancer cell lines are mediated through caspase-dependent and p53-independent pathways. PLoS One 2013;8:e78021.
50. Amira OC, Okubadejo NU. Frequency of complementary and alternative medicine utilization in hypertensive patients attending an urban tertiary care centre in Nigeria. BMC Complement Altern Med 2007;7:30.
51. Atangwho IJ, Egbung GE, Ahmad M, Yam MF, Asmawi MZ. Antioxidant versus anti-diabetic properties of leaves from *Vernonia amygdalina* Del. growing in Malaysia. Food Chem 2013;141:3428-34.
52. Atangwho IJ, Ebong PE, Eteng MU, Eyong EU, Obi AU. Effect of *Vernonia amygdalina* Del. leaf on kidney function of diabetic rats. Int J Pharmacol 2007;3:143-8.
53. Ong KW, Hsu A, Song L, Huang D, Tan BK. Polyphenols-rich *Vernonia amygdalina* shows anti-diabetic effects in streptozotocin-induced diabetic rats. J Ethnopharmacol 2011;133:598-607.
54. Akinola OB, Omotoso GO, Akinola OS, Dosumu OO, Adewoye ET. Effects of combined leaf extract of *Vernonia amygdalina* and

Azadirachta indica on hepatic morphology and hepatotoxicity markers in streptozotocin-induced diabetic rats. Zhong Xi Yi Jie He Xue Bao 2011;9:1373-9.
55. Lolodi O, Eriyamremu GE. Effect of methanolic extract of *Vernonia amygdalina* (common bitter leaf) on lipid peroxidation and antioxidant enzymes in rats exposed to cycasin. Pak J Biol Sci 2013;16:642-6.
56. Ejike CE, Awazie SO, Nwangozi PA, Godwin CD. Synergistic postprandial blood glucose modulatory properties of *Vernonia amygdalina* (Del.), *Gongronema latifolium* (Benth.) and *Occimum gratissimum* (Linn.) aqueous decoctions. J Ethnopharmacol 2013;149:111-6.
57. Yeap SK, Ho WY, Beh BK, Liang WS, Ky H, Yousr AH, *et al*. *Vernonia amygdalina*, an ethno veterinary and ethno medical used green vegetable with multiple bioactivities. J Med Plants Res 2010;4:2787-812.
58. Ong KW, Hsu A, Tan BK. Anti-diabetic and anti-lipidemic effects of chlorogenic acid are mediated by ampk activation. Biochem Pharmacol 2013;85:1341-51.
59. Ebong PE, Atangwho IJ, Eyong EU, Egbung GE. The antidiabetic efficacy of combined extracts from two continental plants: *Azadirachta indica* (A. Juss) (Neem) and *Vernonia amygdalina* (Del.) (African Bitter Leaf). Am Biochem Biotech 2008;4:239-44.
60. Eteng MU, Bassey BJ, Atangwho IJ. Biochemical indices of macrovascular complication in diabetic rat model: Compared effects or *Vernonia amygdalina*, *Catharanthus roseus* and chlopropamide. Asian J Biochem 2008;3:228-34.
61. Fleischer TC, Mensah ML, Mensah AY, Komlaga G, Gbedema SY, Skaltsa H. Antimicrobial activity of essential oils of *Xylopia aethiopica*. Afr J Tradit Complement Altern Med 2008;5:391-3.
62. Woode E, Alhassan A, Abaidoo CS. Effect of ethanolic fruit extract of *Xylopia aethiopica* on reproductive function of male rats. Int J Pharm Biomed Res 2011;2:161-5.
63. Oben J, Etoundi CB, Kuate D, Ngondi JL. Anti-amylase, anti-lipase and antioxidant effect of aqueous extracts of some Cameroonian spices. J Nat Prod 2010;3:165-71.
64. Ogbonna SO, Mbaka GO, Adekunle A, Anyika EN, Gbolade OE, Nwakakwa N. Effect of a poly-herbal formulation, Okudiabet, on alloxan-induced diabetic rats. Agric Biol J North Am 2010;1:139-45.
65. Ene AC, Atawodi SE. Ethno- medicinal survey of plants used by the Kanuri's of North-eastern Nigeria. Indian J Tradit Knowl 2012;11:640-5.
66. Somova LO, Nadar A, Rammanan P, Shode FO. Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic and ursolic acids in experimental hypertension. Phytomedicine 2003;10:115-21.
67. Ngubane PS, Masola B, Musabayane CT. The effects of *Syzygium aromaticum*-derived oleanolic acid on glycogenic enzymes in streptozotocin-induced diabetic rats. Ren Fail 2011;33:434-9.
68. Adefegha SA, Oboh G, Adefegha OM, Boligon AA, Athayde ML. Antihyperglycemic, hypolipidemic, hepatoprotective and antioxidative effects of dietary clove (*Szyzgium aromaticum*) bud powder in a high-fat diet/streptozotocin-induced diabetes rat model. J Sci Food Agric 2014;94:2726-37.
69. Khathi A, Serumula MR, Myburg RB, Van Heerden FR, Musabayane CT. Effects of *Syzygium aromaticum*-derived triterpenes on postprandial blood glucose in streptozotocin-induced diabetic rats following carbohydrate challenge. PLoS One 2013;8:e81632.
70. Kuroda M, Mimaki Y, Ohtomo T, Yamada J, Nishiyama T, Mae T, *et al*. Hypoglycemic effects of clove (*Syzygium aromaticum* flower buds) on genetically diabetic KK-Ay mice and identification of the active ingredients. J Nat Med 2012;66:394-9.
71. Adefegha SA, Oboh G. *In vitro* inhibition activity of polyphenol-rich extracts from *Syzygium aromaticum* (L.) Merr. & Perry (Clove) buds against carbohydrate hydrolyzing enzymes linked to type 2 diabetes and Fe(2+)-induced lipid peroxidation in rat pancreas. Asian Pac J Trop Biomed 2012;2:774-81.
72. Subapriya R, Nagini S. Medicinal properties of neem leaves: A review. Curr Med Chem Anticancer Agents 2005;5:149-6.
73. Kumar VS, Navaratnam V, Ramachandran S. Isolation and characterization of glucosamine from *Azadirachta indica* leaves: An evaluation of immunostimulant activity in mice. Asian Pac J Trop Biomed 2012;51:561-7.
74. Bhat M, Kothiwale SK, Tirmale AR, Bhargava SY, Joshi BN. Antidiabetic properties of *Azardiracta indica* and *Bougainvillea spectabilis*: *In vivo* studies in murine diabetes model. Evid Based Complement Alternat Med 2011;2011:561625.
75. Shailey S, Basir SF. Protective role of *Azadirachta indica* against oxidative damage in skeletal and cardiac muscle of alloxan diabetic rats. Int J Pharm Sci 2012;4:471-7.
76. Khosla P, Bhanwra S, Singh J, Seth S, Srivastava RK. A study of hypoglycaemic effects of *Azadirachta indica* (Neem) in normaland alloxan diabetic rabbits. Indian J Physiol Pharmacol 2000;44:69-74.
77. Tiwari BK, Kumar D, Abidi AB, Rizvi SI. Efficacy of composite extract from leaves and fruits of medicinal plants used in traditional diabetic therapy against oxidative stress in alloxan-induced diabetic rats. ISRN Pharmacol 2014;2014:608590.
78. De Bona KS, Bonfanti G, Bitencourt PE, Cargnelutti LO, da Silva PS, da Silva TP, *et al*. *Syzygium cumini* is more effective in preventing the increase of erythrocytic ADA activity than phenolic compounds under hyperglycemic conditions *in vitro*. J Physiol Biochem 2014;70:321-30.
79. Sharma AK, Bharti S, Kumar K, Krishnamurthy B, Bhatia J, Kumari S, *et al*. *Syzygium cumini* ameliorates insulin resistance and β-cell dysfunction via modulation of $PPAR_{\gamma}$, dyslipidemia, oxidative stress and TNF-α in type 2 diabetic rats. J Pharmacol Sci 2012;119:205-13.
80. Dusane MB, Joshi BN. Seeds of *Syzygium cumini* (L.) Skeels: Potential for islet regeneration in experimental diabetes. Zhong Xi Yi Jie He Xue Bao 2011;9:1380-7.
81. Sharma B, Siddiqui MS, Kumar SS, Ram G, Choudhary M. Liver protective effects of aqueous extracts of *Syzygium cumini* in Swiss albino mice on alloxan-induced diabetes mellitus. J Pharm Res 2013;6:853-8.
82. Shinde J, Taldone T, Barletta M, Kunaparaju N, Hu B, Kumar S, *et al*. Alpha-glucosidase inhibitory activity of *Syzygium cumini* (Linn.) Skeels seed kernel *in vitro* and in Goto-Kakizaki (GK) rats. Carbohydr Res 2008;343:1278-81.
83. Arun R, Prakash MV, Abraham SK, Premkumar K. Role of *Syzygium cumini* seed extract in the chemoprevention of *in vivo* genomic damage and oxidative stress. J Ethnopharmacol 2011;134:329-33.
84. Mininel FJ, Junior CS, Espanha LG, Rosende FA, Varanda EA, Leite CQ, *et al*. Characterization and quantification of compounds in hydro-alcoholic extract of the leaves from *Terminalia catappa* Linn. (Combrataceae) and their mutagenic activity. Evid Based Complement Alternat Med 2014;2014:11, doi: 10.1155/2014/676902.
85. Chen PS, Li JH. Chemopreventive effect of punicalagin, a novel tannin component isolated from *Terminalia catappa*, on H-ras-transformed NIH3T3 cells. Toxicol Lett 2006;163:44-53.
86. Chanda S, Rakholiya K, Nair CR. Antimicrobial activity of *Terminalia catappa* L. leaf extracts against some clinically important pathogenic microbial strains. Chin Med 2011;2:171-7.
87. Chyau CC, Tsai SY, Ko PT, Mau JL. Antioxidant properties of solvent extracts from *Terminalia catappa* leaves. Food Chem 2000;78:483-8.
88. Kinoshita S, Inoue Y, Nakama S, Ichiba T, Aniya Y. Antioxidant and hepatoprotective actions of medicinal herb, *Terminalia catappa* L. from Okinawa Island and its tannin corilagin. Phytomedicine 2007;14:755-62.
89. Mandal S, Patra A, Samanta A, Roy S, Mandal A, Mahapatra TD, *et al*. Analysis of phytochemical profile of *Terminalia arjuna* bark extract with antioxidative and antimicrobial properties. Asian Pac J Trop Biomed 2013;3:960-6.
90. Biswas M, Biswas K, Karan TK, Bhattacharya S, Gosh AK, Haldar PK. Evaluation of analgesic and anti-inflammatory activities of *Terminalia arjuna* leaf. J Phytol 2011;3:33-8.
91. Singh PP, Chauhan SM. Activity-guided isolation of antioxidants from the leaves of *Terminalia arjuna*. Nat Prod Res 2014;28:760-3.
92. Raj CD, Shabi MM, Jipnomon J, Dhevi R, Gayathri K, Subashini U, *et al*. *Terminalia arjuna*'s antioxidant effect in isolated perfused kidney. Res Pharm Sci 2012;7:181-8.
93. Biswas M, Kar B, Bhattacharya S, Kumar RB, Ghosh AK, Haldar PK. Antihyperglycemic activity and antioxidant role of *Terminalia arjuna* leaf in streptozotocin-induced diabetic rats. Pharm Biol 2011;49:335-40.
94. Ragavan B, Krishnakumari S. Antidiabetic effect of *T. arjuna* bark extract in alloxan induced diabetic rats. Indian J Clin Biochem 2006;21:123-8.
95. Perveen K, Kahn R, Siddiqui WA. Antidiabetic effects afforded by *Terminalia arjuna* in high fat-feed and STZ-induced type 2 diabetic rats. Int J Diabetes Metab 2011;19:23-33.
96. Chander R, Singh K, Khanna AK, Kaul SM, Puri A, Saxena R, *et al*.

Antidyslipidemic and antioxidant activities of different fractions of *Terminalia arjuna* stem bark. Indian J Clin Biochem 2004;19:141-8.
97. Morshed MA, Haque A, Rokeya B, Ali L. Anti-hyperglycaemic and lipid lowering effect of *Terminalia arjuna* bark extracts on STZ-induced type 2 diabetic model rats. Int J Pharm Pharm Sci 2011;3:450-4.
98. Juang LJ, Sheu SJ, Lin TC. Determination of hydrolyzable tannins in the fruit of *Terminalia chebula* Retz. by high-performance liquid chromatography and capillary electrophoresis. J Sep Sci 2004;27:718-24.
99. Bag A, Bhattacharyya SK, Chattopadhyay RR. The development of *Terminalia chebula* Retz. (Combretaceae) in clinical research. Asian Pac J Trop Biomed 2013;3:244-52.
100. Manosroi A, Jantrawut P, Ogihara E, Yamamoto A, Fukatsu M, Yasukawa K, *et al*. Biological activities of phenolic compounds and triterpenoids from the galls of *Terminalia chebula*. Chem Biodivers 2013;10:1448-63.

Source of Support: Nil, **Conflict of Interest:** None declared.

Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20141202055655

Monograph

Ethnopharmacological uses of *Antidesma madagascariense* Lam. (Euphorbiaceae)

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Received: October 07, 2014
Accepted: December 02, 2014
Published: January 03, 2015

ABSTRACT

Antidesma madagascariense Lam. is an indigenous plant of the Mascarene Islands which has interestingly shined as a promising traditional medicinal plant. The ethnobotanical uses of this plant were geared toward the treatment and management of dysentery, albumin in the urine, jaundice, fever, diabetes, skin infections, rheumatic and body aches among others. Preliminary screening of this plant coupled with a plethora of *in vitro* and *in vivo* tests have furnished scientists with documented findings that have appraised its traditional use in the treatment and management of infectious diseases. The presence of antidesmin, a commonly characterized component of *Antidesma* species, might justify the medicinal virtues of this plant. The present monograph aims at providing the botanical description, traditional uses and latest findings documented on *A. madagascariense*. Nonetheless, continued research on this plant needs to be completed in order to rationalize the use of this promising plant as a potential source of beneficial constituents for the treatment and management of human diseases and hence set up promising optimism for drug development.

KEY WORDS: *Antidesma madagascariense,* antidesmin, ethnopharmacology, Mascarene Islands

INTRODUCTION

Antidesma madagascariense Lam., (Euphorbiaceae) is a tropical plant whose genus contains 170 species. It is indigenous to the Mascarene region in the Western Indian Ocean as well as to Madagascar [1,2]. The different species are adapted to survive in warm and tropical regions, for instance, Asia and Oceania, but also East Africa, which has a deep-rooted tradition to the use of these medicinal plants. Commonly known as "Bois bigaignon bâtard" and "Bois bigayon" in Mauritius, it is widely distributed in humid forests but can also be seen in Reunion Island in dense thickets of medium altitude (100-1600 m) [1,3,4]. Other familiar vernacular names include 'Bois de cabri', 'Bois de cabri blanc', 'Bois de gaulette blanc' and 'Bois d'oiseaux' in Reunion island [4]. The genus Antidesma is derived from the Greek words 'anti-against' and 'thema- band' and refers to a tree-like species which provides bast fibers for making rope. The epithet 'madagascariensis' demonstrates the origin of the species or to where it is widespread [1].

BOTANICAL DESCRIPTION

A. *madagascariense* is a genus of dioecious shrub or low tree, often little branching and stunted, with a slightly fissured brownish gray bark [Figure 1] reaching 5 m height [2,4,5]. The genus, formerly grouped in the *Phyllanthoideae* family now belong to the Euphorbiaceae family due to the elongated U-shaped connective of the anthers that is the most notable character of this family [2]. This plant is characterized by the ample variations in the size, texture and shape of the leaves, which can be coriaceous to papery, often oval to elliptic, 4-10 cm long and 3-5 cm wide, the margins entirely or slightly sinuous. The presence of domatia is very visible in the axils of primary nerves. This tree bears minute greenish or red flowers of different sexes [1,3]. Often abundant in clusters of small berries, the ovoid dark red fruits which turn into shiny purple-black when ripe, are more or less flattened and 6-7 mm long [1,4,5].

ETHNOPHARMACOLOGICAL USES

A. *madagascariense* is an indigenous and an endemic plant that has always been used in folkloric medicine among the local population of the Mascarene Islands for the treatment and management of various ailments. A decoction of the leaves of A. *madagascariense* has been traditionally used to treat dysentery [3,6]. The decoction obtained after boiling 10 leaves of A. *madagascariense* in 1 L of water can regularly be consumed for the treatment of albumin in the urine [3]. Furthermore, the leaves and barks have been reported to possess diuretic,

Figure 1: *Antidesma madagascariense*. (a) Whole plant, (b) bark/stems, (c) fruits and (d) leaves

astringent, as well as febrifuge properties, and also used diabetes management [3,6]. A bath in the leaf decoction has been reported to alleviate skin infections, rheumatic and body aches. The leaves of A. *madagascariense* mixed with those of *Aphloia theiformis* (Flacourtiaceae) and *Toddalia asiatica* (Rutaceae) are used to treat jaundice [1]. Interestingly, A. *madagascariense* can be used to treat edema in pregnancy and can also be employed in the case of stroke depending on the type of decoction which is prepared [Table 1] [3].

BIOLOGICAL ACTIVITIES OF SELECTED CONSTITUENTS

Preliminary phytochemical screening of the leaves of A. *madagascariense* indicates the presence of phenols, tannins, alkaloids, flavonoids, cyanogenetic heterosides as well as leucoanthocyanins, sterols and saponins [1,7,8]. Interestingly, A. *madagascariense* has also been found to contain triterpenes and hydrolysable tannins, carpusin, and a dimer - antidesmin [Figure 2], a common constituent also characterised in other *Antidesma* species [9,10]. The bacteriostatic and bactericidal properties of A. *madagascariense*, validated from the local folk medicine, can be attributed to the presence of tannins in the plant. Furthermore, the aqueous and methanol extracts of the leaves demonstrated their molluscicidal properties against species of *Biomphalaria* and *Bulinus* and antifungal properties against *Cladosporium cucumerinum* [1,3]. Different fractions of the leaves and stems (water, methanol, chloroform and hexane) of A. *madagascariense* were previously reported to have significant inhibitory effects on Gram-positive bacteria *Staphylococcus aureus*, Gram-negative bacteria *Pseudomonas aeruginosa* and the fungus *Aspergillus niger* showing their potent antimicrobial activities [6,11]. The ability of the methanol stem extracts of A. *madagascariense* to exhibit contractile properties on rat ileal smooth muscles coupled with the recent antioxidant and antimicrobial findings on A. *madagascariense*

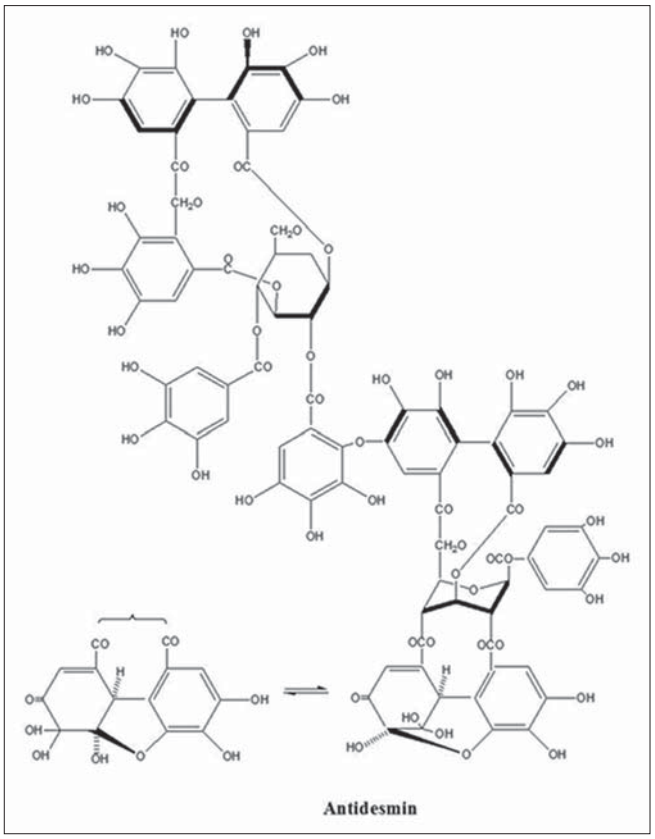

Figure 2: Antidesmin- a common constituent in all Antidesma species [10]

Table 1: Decoction preparation needed for the treatment of stroke and edema in pregnancy [3]

Decoction of 10 leaves of *A. madagascariense* with:
A small quantities of *Coix lacryma-jobi* roots
15 leaves of *A. theiformis*
15 cm bark of *E. laurifolium*
0.5 cm root of *R. mucronata*
3 entire plants of *B. pilosa*
10 leaves and vines of 'Betel sauvage' (*Piper* sp.)

A. madagascariense: Antidesma madagascariense, A. theiformis: Aphloia theiformis, E. laurifoliu: Erythroxylum laurifolium, R. mucronata: Rhizophora mucronata, B. pilosa: Bidens pilosa

validate its ethnobotanical use in the effective treatment of dysentery [1,6,7].

PHARMACOLOGICAL STUDIES

Antimicrobial Activities

The different crude extracts of A. *madagascariense* exhibited potent antimicrobial activity which was found to increase with increasing polarity. The methanol leaves extracts of A. *madagascariense* had potent inhibitory effects against *Enterococcus faecalis* (minimum inhibitory concentration [MIC] = 60 μg/ml), *S. aureus* (MIC = 500 μg/ml), Methicillin-resistant *S. aureus* (MRSA) (MIC = 250 μg/ml) and *Candida albicans* (MIC = 500 μg/ml) [7].

Antioxidant Activities

The antioxidant potential was validated in several *in vitro* assays carried out on different crude extracts and fractions of leaves of A. *madagascariense* with IC_{50} values ranging from 3.94-87.05 μg/ml, 3.18-13.26 μg/ml and 6.29-25.24 μg/ml for the 2,2-diphenyl-1-picrylhydrazine (DPPH), superoxide (SO) and nitric oxide (NO) radical scavenging assays respectively. While the *n*-butanol extracts of A. *madagascariense* had the most potent antioxidant activity for DPPH (% radical scavenging potential [RSP] = 93.68 ± 8.69%) and NO (% RSP = 65.56 ± 7.56%) assays, ethyl acetate extracts had a high percentage RSP (99.53 ± 7.53 %) in SO assay [12]. Similarly, a concentration of 0.5 mg/ml of methanol A. *madagascariense* leaves extracts were able to scavenge 70.6 ± 2.2% and 64.5 ± 1.8% of hydroxyl and hypochlorous acid radicals correspondingly, thus validating the antioxidant properties of A. *madagascariense* [7].

Antiglycation Activities

Antiglycation activities were confirmed in the ethylacetate (96.63 ± 10.36%), methanol (86.35 ± 5.65) and *n*-butanol (84.65 ± 6.35%) fractions of A. *madagascariense* and this was comparable to the anti-glycation drug aminoguanidine ($P < 0.05$). Nevertheless, A. *madagascariense* extracts were found to have no activity against mitochondrial respiration in a MTT cytotoxicity assay ($P > 0.05$) [12]. The efficacy of A. *madagascariense* extracts in managing diabetes was assessed through the inhibition of key carbohydrate hydrolyzing enzymes. All the extracts exhibited variable inhibitory effects on α-amylase activity ($P < 0.05$) with ethylacetate fraction having the best inhibitory effect (IC_{50} = 61.52 ± 11.09 μg/ml) which was lower than acarbose (IC_{50} = 75.86 ± 8.16 μg/ml). Moreover, active fractions of A. *madagascariense* were found to inhibit significantly ($P < 0.05$) amylase activity in mouse plasma from 7.80% to 49.37%. α-glucosidase activity was significantly inhibited by A. *madagascariense* extracts with IC_{50} values ranging from 19.70 ± 2.87 μg/ml to 44.92 ± 5.67 μg/ml, which was comparable to the drug 1-deoxynojirimycin.

The repressive capacity of A. *madagascariense* extracts to increase blood glucose concentration in mice was investigated by *in vivo* studies in glycogen-loaded mice. Ethyl acetate fraction was found to be more potent with glucose-lowering properties (−59.4%) comparable to acarbose (−55.1%) [8]. Moreover, *in vivo* studies of A. *madagascariense* on rat everted intestinal sacs indicated that aqueous extract A. *madagascariense* significantly ($P < 0.05$) enhanced the uptake of D-glucose and fluid transport. It was also noted that the concentration of above 0.375 mg/ml of the extract was needed to enhance mucosal disappearance, gut wall content and serosal appearance of fluid ($P < 0.05$). However, L-tyrosine and K^+ transport was not significantly enhanced to the contrary of Na^+. Therefore, the ability of A. *madagascariense* extracts to promote the transport of glucose, fluid and Na^+ across rat everted intestinal sacs might be attributed to the presence of bioactive phytochemicals, for instance, flavonoids, alkaloids, leucoanthocyanins, phenols and saponins, in A. *madagascariense* leaves, which have possibly interact with the Na^+/glucose co-transporter in the enterocytes [13].

IMMUNOMODULATORY PROPERTIES

The *in vitro* immunomodulatory property of A. *madagascariense* showed that extracts were able to modulate significantly ($P < 0.05$) the immune response of phagocytes and monocytes at different steps. At a concentration of 100 μg/ml, the inhibitory activity of crude methanol A. *madagascariense* extracts on whole blood phagocytes for reactive oxygen species (ROS) production was 94.2%. It was also suggested that A. *madagascariense* directly inhibited a final common biochemical target such as NADPH oxidase enzyme or scavenge ROS since it did not affect a specific transductional pathway [14].

Endophytic Fungi from *A. madagascariense*

Finally, due to its eminent and documented pharmacological activities, A. *madagascariense* was recently selected for the screening of endophytic fungi. The endophytic and saprobic fungi recovered from the living and dead leaves of A. *madagascariense* revealed that they were closely related to *Aspergillus*, *Guignardia*, *Fusarium*, *Penicillium*, *Pestalotiopsis* and *Trichoderma* genera. Phylogenetic analysis of the DNA extracts of these fungi successfully demonstrated that they belong to five different fungal lineages (Hypocreaceae, Trichocomaceae, Nectriaceae, Xylariaceae, and Botryosphaeriaceae) [15].

CONCLUSION

This monograph has attempted to throw into the limelight some of the ethnobotanical uses of the plant A. *madagascariense* which has been documented in the traditional Mauritian folklore as a promising plant possessing various biological activities. *In vitro* and *in vivo* studies conducted so far on A. *madagascariense* extracts revealed its potent antimicrobial, antioxidant, anti-diabetic as well as its immunomodulatory properties. However, continuing research needs to be performed in order to validate the potency of this plant as a good candidate for pharmacological action and thus appraise its traditional uses.

REFERENCES

1. Gurib-Fakim A, Brendler T. Medicinal and Aromatic Plants of Indian Ocean Islands: Madagascar, Comoros, Seychelles and Mascarenes. Stuttgart, Germany: Medpharm Sci Publication; 2004.
2. Hoffman P. The genus *Antidesma* (Euphorbiaceae) in madagascar and comoros Islands. Kew Bull 1999;54:877-85.
3. Gurib-Fakim A, Guého J, Bissoondoyal MD. Medicinal Plants of Mauritius, Tome 2 (Cucurbitaceae-Oxalidaceae). Rose-Hill, Mauritius: Indian Ocean Edition; 1996.
4. Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), 2008. *Antidesma madagascariense* Lam. Available from: http://arbres-reunion.cirad.fr/especes/euphorbiaceae/antidesma_madagascariense_lam. [Last accessed on 2014 Sep 27].
5. Gurib-Fakim A. Trees of the World, 2013. [In press].
6. Narod FB, Gurib-Fakim A, Subratty AH. Biological investigations into

Antidesma madagascariense Lam. (Euphorbiaceae), *Faujasiopsis flexuosa* (Lam.) C. Jeffrey (Asteraceae), *Toddalia asiatica* (L.) Lam. and *Vepris lanceolata* (Lam.) G. Don (Rutaceae). J Cell Mol Biol 2004;3:15-21.
7. Rangasamy O. The search for anti-infectives from Mauritian flora. PHD Thesis. University of Mauritius; 2014.
8. Mahomoodally MF, Subratty AH, Gurib-Fakim A, Choudhary MI, Khan SN. Traditional medicinal herbs and food plants have the potential to inhibit key carbohydrate hydrolyzing enzymes *in vitro* and reduce postprandial blood glucose peaks *in vivo*. Sci World J 2012;1-9.
9. Gurib-Fakim, A. An illustrated guide to the flora of Mauritius and the Indian Ocean islands. Baie du Tombeau, Mauritius: Caractere Ltée; 2003.
10. Gurib-Fakim, A. Medicinal Plants of Mauritius and of the World. Mauritius: Caractere Ltée; 2007.
11. Mahomoodally MF, Gurib-Fakim A, Subratty AH. Antimicrobial activities and phytochemical profiles of endemic medicinal plants of Mauritius. Pharm Biol 2005;43:237-42.
12. Mahomoodally FM, Subratty AH, Gurib-Fakim A, Choudhary MI. Antioxidant, antiglycation and cytotoxicity evaluation of selected medicinal plants of the Mascarene Islands. BMC Complement Altern Med 2012;12:165.
13. Mahomoodally MF, Fakim AG, Subratty AH. Stimulatory effects of Antidesma madagascariense on D-glucose, L-tyrosine, fluid and electrolyte transport across rat everted intestine, comparable to insulin action *in vitro*. Br J Biomed Sci 2006;63:12-7.
14. Mahomoodally F, Mesaik A, Choudhary MI, Subratty AH, Gurib-Fakim A. *In vitro* modulation of oxidative burst via release of reactive oxygen species from immune cells by extracts of selected tropical medicinal herbs and food plants. Asian Pac J Trop Med 2012;5:440-7.
15. Jeewon R, Ittoo J, Mahadeb D, Jaufeerally-Fakim Y, Wang H, Liu A. DNA based identification and phylogenic characterization of endophytic and saprobic fungi from *Antidesma madagascariense,* a medicinal plant in Mauritius. J Mycol 2013;2013:1-11.

Source of Support: Nil, Conflict of Interest: None declared.