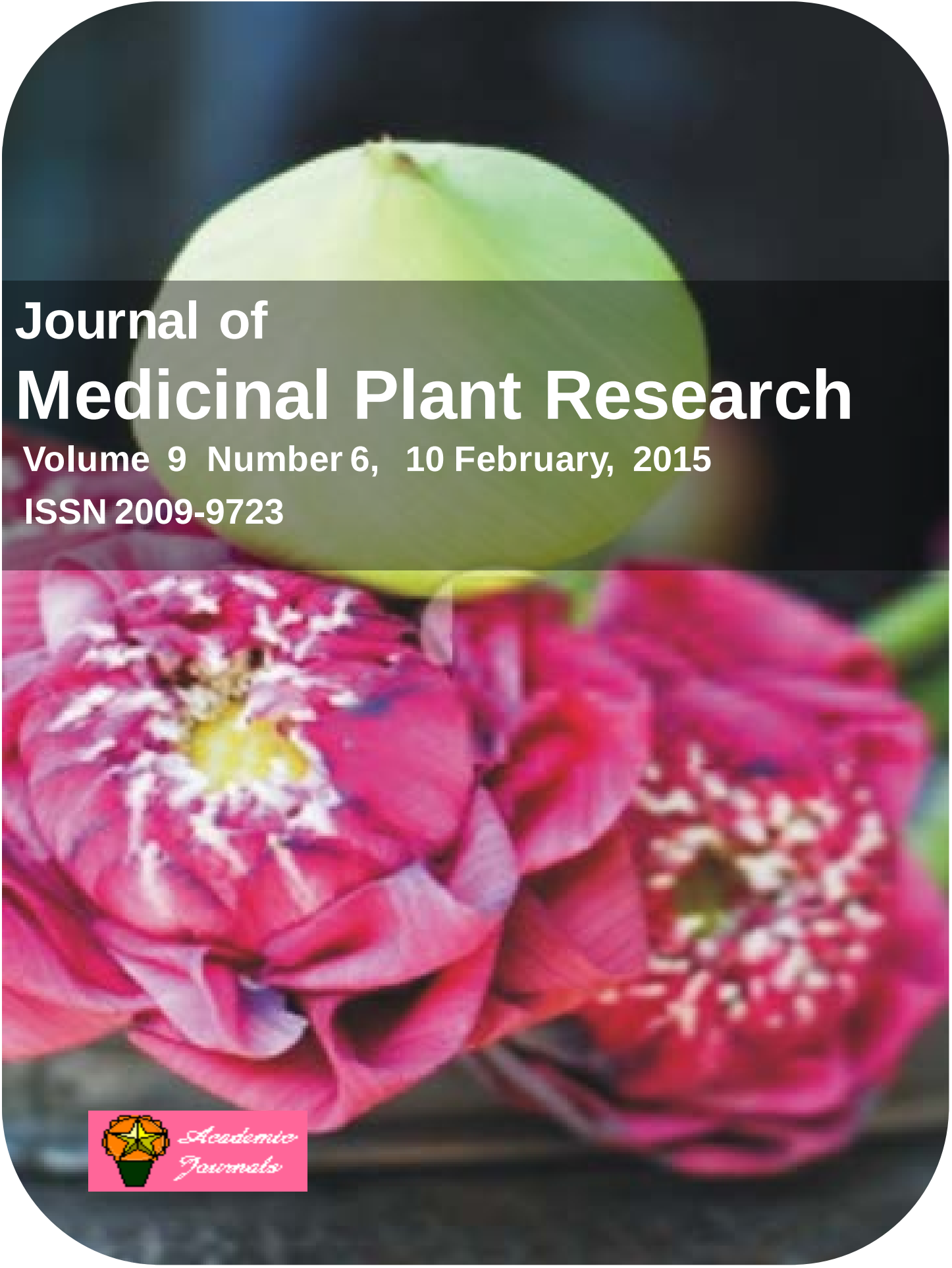

Journal of Medicinal Plant Research

Volume 9 Number 6, 10 February, 2015
ISSN 2009-9723

Academic Journals

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academicJournals

Vol. 9(6), pp. 159-168, 10 February, 2015
DOI: 10.5897/JMPR2014.5513
Article Number: 560141550863
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Comparative studies between the chemical constituents and biological properties of the extracts from the leaves and barks of *Myracrodruon urundeuva* Fr. All.

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Received 7 October, 2014; Accepted 6 February, 2015

The present study describes the comparation between the chemical constituents and biological properties of leaves and barks of *Myracrodruon urundeuva* for proposing the medicinal use of the plant from leaves, avoiding the degradation by remotion of the barks. The results of chromatographic profile of the hydroalcoholic extracts and fractions of leaves and barks showed similarities in the composition of the phytoconstituents, with variation for some of them that are in greater quantity of the bark extracts. The hydroalcoholics extracts from leaves (HELMu) and barks (HEBMu) exhibited promising 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (IC_{50} = 4.8 and 7.0 µg/ml, respectively), comparable to gallic acid (IC_{50} = 4.8 µg/ml). No significant cytotoxicity was observed in the concentrations evaluated (IC_{50} > 400 µg/ml). The HEBMu exhibited greater nociceptive activity than HELMu, however HELMu was more active against oral pathogens. This study provides support for the use of the leaves in traditional medicine instead of bark.

Key words: Anacardiaceae, phytochemical and chromatographic profile, biological activities, sustainable use.

INTRODUCTION

Myracrodruon urundeuva Fr. All. is commonly used in northeastern Brazil because of its high medicinal power (Goes et al., 2005). It is popularly known as aroeira-do-cerrado, aroeira-do-sertão, aroeira-preta, urundeuva, among others (Indian Prairie Educational Foundation (IPEF), 2012; Viana et al., 1995). It is used to treat ulcers

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and lesions of the skin and mucous membranes, against infections of the respiratory, digestive and genitourinary systems (Berger et al., 2007; Santos et al., 2007; Viana et al., 1995). Furthermore, studies have observed antifungal, anti-inflammatory, antiulcerogenic, antihistamine, antibradicinina and analgesic effects coupled with the absence of toxic and teratogenic effects of bark extracts (Botelho et al., 2007; Carvalho, 1994; Moraes et al., 2005; Naruzawa and Papa, 2011). Despite such great importance, the extractive exploration, due to the excellent physical, chemical and biological properties, generated losses of genetic material and committed to conservation of existing population (Pacheco, 2006). With consolidated properties and uses, it necessary to provide alternative sustainable use of the remaining populations. Therefore, this study aims to determine similarities between the phytochemical profile, chromatographic and biological potential of leaves and bark of *M. urundeuva*, to propose the use of leaves as a more sustainable alternative to medicinal use of this species.

MATERIALS AND METHODS

Plant material and extraction procedure

M. urundeuva leaves and barks were collected in Glaucilândia (Minas Gerais, Brazil) and identified by Prof. Dr. Rubens Manoel dos Santos in Unimontes (Universidade Estadual de Montes Claros), Minas Gerais. A voucher specimen was deposited in the Unimontes herbarium under number 3534. The leaves and barks were dried naturally in the shade for 96 h, pulverized, stored in paper bags, and refrigerated at -10°C. The hydroalcoholic extracts of the leaves (HELMu) and bark (HEBMu) were prepared by maceration exhaustive method in which 100 g of powdered vegetable material were placed in 1000 ml of ethanol/water (7:3 v/v). The extracts were stored in the dark at room temperature for 7 days, with occasional shaking. The extracts were filtered, evaporated and refrigerated at -10°C. This extraction process was repeated with the residues for another three times (HELMu yield 24.3%. w/w and HEBMu yield 23.9% w/w). The solid extracts were partitioned with hexane, dichlorometane, ethyl acetate and isobutanol to yield hexane, dichlorometane, ethyl acetate and isobutanol fractions. The solvents were evaporated under reduced pressure below 40°C.

Phytochemical analysis

Plant materials were screened for the presence of alkaloids, saponins, tannins, total phenols, anthraquinones, flavonoids, cardiotonic glycosides and sterols using the methods previously described by Harborne (1999).

Chromatographic profiles

Chromatographic plates of silica gel G 60 F_{254} (TLC) were used to determine the chromatographic profiles of extracts and fractions from the leaves and barks of *M. urundeuva*. Mixtures of hexane/ethyl acetate in proportions of 8:2 to 2:8 were used as mobile phase. Plates were observed in UV light at a wavelength of 254 nm and then reveled with vanillin sulfuric solution. The retention factors (Rf) were used as a comparison measure. The comparation between high performance liquid chromatography (HPLC) profiles was performed in waters liquid chromatography using a Phenomenex-C18 (250 × 4.6 mm, 10 µm) analytical column and a diode array detector. The partitioned extracts were solubilized at 500 µg/ml using a gradient system with water and methanol, starting with 0% of organic phase up to 100% (variation 10% every 2.0 min).

Antioxidant assay

To measure the antioxidant activity of the extracts (HELMu and HEBMu, 0.5 to 10 µg/ml) and standards (gallic acid and ethanol), the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was carried out according to the procedure described previously (Alvarenga et al., 2013), with slight modifications. Briefly, the DPPH radical scavenging activity was measured in triplicate, in a reaction mixture containing 0.1 ml of 1 mM DPPH radical solution, 0.8 ml of ethanol 99%, and 0.1 ml of extract (in methanol). The same mixture was used for the standards. The solution was rapidly mixed, and the scavenging capacity was measured spectrophotometrically by monitoring the decrease in the absorbance at 517 nm. The antioxidant activity was expressed as IC_{50}, which was defined as the concentration of the extracts required to inhibit the formation of DPPH radicals by 50%.

Toxicity to mammalian cells

The LLC-MK2 fibroblast cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 5% of inactivated fetal calf serum, and maintained at 37°C in 5% CO_2. A cell suspension was seeded at a concentration of 1×10^6 cells/ml in a 96-well microplate containing RPMI 1640 medium. Thereafter, the cells were treated with HELMu and HEBMu at different concentrations (6.25, 12.5, 25, 50, 100, 200, and 400 µg/ml). The plates were incubated at 37°C for 24 h, and the biological activity was evaluated by using the MTT colorimetric method [MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in a microplate reader at 515 nm. RPMI 1640 medium plus dimethyl sulphoxide (DMSO) and RPMI 1640 medium were used as positive and negative controls, respectively (Twentyman and Luscombe, 1987). All the experiments were performed in triplicate. The percentage of cell viability was determined by the formula:

$$\% \text{ cell viability} = 1 - [(Y - N) / (N - P)] \times 100$$

Where Y = absorbance of wells containing cells and HELMu or HEBMu at different concentrations; N = negative control; P = positive control.

Antimicrobial activity

Antibacterial sensitivity testing using disc diffusion method

The agar diffusion method using paper discs was performed according to procedures described by the National Committee for Clinical Laboratory Standards (NCCLS) (2003) against *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 15922) and *Shigella flexneri* (ATCC 12022). Sterile paper discs were impregnated with equal volume (50 µl) of each crude extracts (250 mg/ml). After drying, they were placed on Mueller Hinton Agar plates inoculated with suspensions of the test strains.
The antibiotics chloramphenicol 30 µg and gentamicin 120 µg and solution of 5% Tween 80 in saline were used as controls. The

Table 1. TLC chromatographic profile of fractions obtained from HELMu and HEBMu.

Extracts/fractions		Retention factor							
A	Leaves	0.90	0.84	-	-	0.53	-	0.18	-
	Barks	-	-	0.71	0.63	-	0.32	-	-
B	Leaves	0.82	0.72	0.67	0.56	0.33	0.27	-	0.08
	Barks	0.82	0.72	0.67	0.56	-	0.27	0.15	0.08
C	Leaves	0.58	-	0.21	-	-	-	-	-
	Barks	-	0.26	-	-	-	-	-	-
D	Leaves	0.90	0.74	-	-	-	-	-	-
	Barks	0.90	0.74	-	-	-	-	-	-

A: hexane fraction; B: dichlorometane fraction; C: ethyl acetate faction; D: isobutanol fraction obtained from of HELMu and HEBMu

plates were incubated for 24 h at 35 ± 2°C. After this period, the inhibition zones were measured in millimeters. All procedures were performed in triplicate. Inhibition zones with diameter less than 12 mm were considered as having low antibacterial activity. Diameters between 12 and 16 mm were considered moderately active, and those with > 16 mm were considered highly active (Indu et al., 2006).

Antibacterial activity by microdilution minimum inhibitory concentration (MIC) assay methods

The antimicrobial activity of the HELMu and HEBMu was examined by the broth microdilution method against strains of oral bacteria *Streptococcus salivarius* (ATCC 7073), *Streptococcus oralis* (*Streptococcus sanguis*) (ATCC 10557), *Streptococcus mutans* (ATCC 25175) and *Lactobacillus rhamnosus* (ATCC 9595) and strains of disc diffusion test. Minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) were determined according to the NCCLS (2005). All tests were performed in Mueller Hinton Broth (Valgas et al., 2007). Overnight broth cultures of each strain were prepared at a final concentration of 1.5×10^8 CFU/ml for bacteria in a 96-well microtiter plate (Oliveira et al., 2006). Extracts were investigated in the ranges 10 to 500 µg/ml to oral bacteria and 0.12 to 250 mg/ml for other microorganisms. Resazurin was added to the culture medium as a growth indicator (0.02%). Microbial growth was determined after incubation at 37°C for 24 h. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth. MBC is defined as the lowest concentration of the extract at which inoculated microorganisms are completely killed. To determine the MBC, broth was taken from each well (100 µl) and inoculated in Mueller Hinton Agar for 24 h at 37°C (Palomino et al., 2002). All determinations were performed in triplicate: two positive growth controls were included (Dalirsani et al., 2011).

Preliminary analgesic activity: acetic acid-induced writhing in mice

The assay was performed as described by Koster et al. (1959) using albino mice Swiss. The nociceptive effect caused by injection of acetic acid was detected by observing abdominal writhing associated with stretching of the whole body. Animals were treated with different doses of HELMu and HEBMu (100, 300, and 500 mg/kg, *per or*), saline, or indomethacin (5 mg/kg), which was used as a reference compound. Thirty minutes after treatment, all the animals received 0.6% acetic acid i.p.; 10 min later, the number of abdominal constrictions was recorded for 20 min, by visual observation of the animals. The experiment was performed by protocol number 001/2012/CEUA according to Colégio Brasileiro de Experimentação Animal (COBEA) and approved by Comissão de Ética no Uso de Animais (CEUA) of Engineering College of UNESP/ Ilha Solteira.

Data analysis

All determinations were realized in triplicate. The results were statistically analyzed by analysis of variance (ANOVA) followed by the Dunnett or Tukey test, with significant differences being considered if $p < 0.05$. All values are presented as mean ± standard error of the mean (SEM). The level of significance used in analysis of the data was less than 0.05 ($p < 0.05$).

RESULTS

Phytochemical study

The phytochemical study not detected alkaloids or cardiotonic glycosides, but detected the presence of antraquinones in low concentrations in both HELMu and HEBMu. Flavonoids and steroids are present in moderate concentrations, while tannins and saponins are in high concentrations in both extracts.

Chromatographic profile

Were performed analysis by thin layer chromatography (TLC) and HPLC of the hexane (A), dichlorometane (B), ethyl acetate (C) and isobutanol (D) fraction obtained from of HELMu and HEBMu. It was possible to verify by TLC (Table 1) similarities in relation to compounds mainly the majoritarian. The analysis of TLC (Table 1) and HPLC

Table 2. Antimicrobial activity of HELMu and HEBMu by diffusion disc method (inhibition zones and SD in mm).

Extract (250 mg/ml)	Inhibition zones ± SD				
	S. aureus	***E. faecalis***	***E. coli***	***P. mirabilis***	***S. flexneri***
HELMu	12.0 ± 1.00	9.3 ± 0.58	NI	9.5 ± 0.50	15.7 ± 0.58
HEBMu	NI	NI	NI	NI	NI
Chloramphenicol	21.1 ± 1.00	20.2 ± 0.30	NI	NI	NI
Gentamicin	NI	NI	26.3 ± 0.58	26.3 ± 4.0	24.0 ± 1.00

NI- no inhibition.

(Figures 1 and 2) showed good similarities of compounds; however the proportions in the fractions are different. In the fraction A of the leaves the broad peak in 0.66 min can be attributed to the volatile (essential oil) compounds that did not occur in the barks, the other peaks are present in both chromatogram but in lower proportions in the leaves. The chromatographic profile of the fractions B and D are very similar, varying only in the area of some peaks, which are higher in the fractions of the extracts from bark. However, the chromatograms of fraction C differ because peaks appear in the range of 4 to 6 min to HEBMu that are not present in HELMu.

Antioxidant assay

DPPH is a free radical compound that is widely used to test the free radical scavenging ability of various samples. Antioxidants interacting with DPPH, they can either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character (Ramirez-Mares and De Mejia, 2003). The color of the reaction changes from purple to yellow, which can be quantified by the change in the absorbance at 517 nm. The HELMu and HEBMu were capable of scavenging DPPH radicals in a concentration-dependent manner. The HELMu displayed IC_{50} = 4.8 µg/ml and HEBMu IC_{50} = 7.0 µg/ml whereas the value of IC_{50} found for gallic acid was 4.8 µg/ml. These results show that DPPH radical scavenging activity of the HELMu is comparable to the gallic acid, indicating that their compounds may contribute to neutralize the oxidant agents produced during pain and inflammatory states. The DPPH radical scavenging activity of the HEBMu, though lower than gallic acid, is very satisfactory.

Toxicity to mammalian cells

The cultures of LLC-MK2 mammalian fibroblast cells were treated with HELMu and HEBMu (separately) at concentrations of 6.2, 12.5, 25, 50, 100, 200 and 400 µg/ml for 24 h, in triplicate. The viability of the cultures was determined by establishing a relation between the absorbance values obtained in the treated and untreated (control) groups, as shown in Figure 3. No significant cytotoxicity was observed in the concentrations evaluated after 24 h of treatment, only control 25% DMSO display significant toxicity with $p < 0.05$. These results showed that both extracts no present toxicity at concentration evaluated with $IC_{50} > 400$ µg/ml.

Antimicrobial activity

Using the diffusion method was possible to observe that HELMu showed moderate activity on *S. aureus* and *S. flexneri* with inhibition zone of 12.0 and 15.7 mm, respectively, and the HEBMu did not display activity against any of the strains tested (Table 2). Antibiotics used as control showed inhibition zones within the standards established by the NCCLS (2003). In the microdilution test (Table 3) HELMu showed for this same microorganisms MIC ≥ 62.5 and 125 mg/ml on *S. aureus* and *S. flexneri,* respectively. For HEBMu, MIC or MBC could not be determined for these microorganisms because antimicrobial activity in the agar diffusion test was not observed. In relation to oral bacteria, the HELMu presented results more significant (MIC ≤ 200 µg/ml and MBC ≤ 300 µg/ml against *S. oralis*) than HEBMu. MIC and MBC of the HEBMu were not determinate for *S. salivarius* and *S. mutans.*

Analgesic activity

Acetic acid-induced writhing in mice

HELMu showed inhibition percentage lower (42.3, 65.8 and 55.4 at doses of 100, 300 and 500 mg/kg, respectively) than HEBMu that presented significant peripheral analgesic activity at all the evaluated doses (Figure 4). The inhibition percentage of the number of writhing of acetic acid-induced writhing in mice for HEBMu was 84.0, 85.9 and 74.7% at doses of 100, 300 and 500 mg/kg, respectively. The HEBMu at doses 100 and 300 mg/kg presented significant analgesic activity with 84.0 and 85.9% ($p < 0.0001$) inhibition of acetic acid writhing compared to control, but the effect was lower than produced by indomethacin (99.0%). The inhibitory

Table 3. Minimal inhibitory concentration (MIC in µg/mL) and Minimum Bactericidal Concentration (MBC in µg/mL) of extracts and positive controls.

Microorganism	HELMu (µg/ml)		HEBMu (µg/ml)		Control MIC (µg/ml)
	MIC	MBC	MIC	MBC	
S. salivarius	≤ 300	≤ 400	ND	ND	0.5^{Chlorh}
S. oralis	≤ 200	≤ 300	≤ 400	≤ 400	8^{Chlorh}
S. mutans	≤ 400	≤ 500	ND	ND	0.5^{Chlorh}
L. rhamnosus	≤ 400	≤ 400	≤ 400	≤ 400	10^{Chlorh}
S. aureus	≤ 67.5	≤ 125	ND	ND	0.5^{Chlorh}
E. faecalis	≤ 67.5	≤ 125	ND	ND	0.5^{Chlor}
P. mirabilis	≤ 125	≤ 250	ND	ND	0.5^{Gent}
S. flexneri	≤ 125	≤ 250	ND	ND	20^{Gent}

ND – Not determined. The positive control: Chlorh: chlorhexidine, Gent: Gentamicin.

effect of the extract is not dose-dependent: increasing the extract dose from 300 to 500 mg/kg which reduces the inhibition percentage from 85.9 to 74.7% with HEBMu and 65.8 to 55.4 with HELMu.

DISCUSSION

Preliminary phytochemical screening and chromatographic profile showed the presence of several chemical compounds classes in the hydroalcoholic extracts suggesting great similarity in chemical composition between leaves and bark of *M. urundeuva*. Despite the similarity of the chromatographic profiles between HEBMu and HELMu, the fraction C of HEBMu showed peaks in the range of 4 to 6 min that did not appear in the fraction C of HELMu. The presence of this compounds in the HEBMu can have relation with the higher analgesic activity presented by bark. In chromatographic study of *Vernonia polifanthes*, for example, using a mobile phase containing 90% methanol was possible to observe the presence of several flavonoids such as protocatechuic acid and para-hydroxy-benzoic acid with retention times of 4.58 and 6.37 min, respectively (Pereira, 2010).

Moreover, the fraction chromatograms were very similar to the profiles obtained by Viana et al. (1995) for alcoholic and hydroalcoholic extracts of *M. urundeuva*. These presented peaks between 1 and 6 min, suggesting the presence of tannins and other phenolic compounds. The similarities as well as the chemical variations in the production of secondary metabolites have been the subject of several studies of this species (Da Silva et al., 2013). The variation in the secondary metabolites is associated at climate, water and nutrients availability, UV radiation and attack of pathogens which are factors to which the plant is daily exposed (Falkemberg et al., 2003; Gobbo-Neto and Lopes, 2007).

The phytochemical and chromatographic studies did not detect any alkaloids and cardiac glycosides but identified high concentrations of saponins, flavonoids and tannins. The presence of saponins and flavonoids can be directly related to peripheral analgesia exibited by HEBMu and HELMu (Mutalik et al., 2003; Zayachkivska et al., 2005; Owoyele et al., 2008). The classification of antinociceptive drugs is usually based on their mechanism of action; these drugs can act on either the peripheral nervous system or the central nervous system (Planas et al., 2000). The acetic acid writhing test is a standard sensitive test for both opioid and non-opioid analgesics (Steranka et al., 1987; Habib and Waheed, 2013; Wang et al., 2013). When animals are intraperitoneally injected with acetic acid, a painful reaction and acute inflammation emerge in the peritoneal area. The stimulation of peritoneal nociceptors is indirect and occurs with the release of endogenous substances, which stimulate nervous endings (Berkenkopf and Weichman, 1988; Gyires and Torna, 1984). In this study, HEBMu inhibited significantly the number of writhing responses in mice intraperitoneally injected with acetic acid in dose 100 and 300 mg/kg, showing a significant anti-nociceptive activity. However, this writhing test alone does not substantiate the effect of HEBMu (Srinivasan et al., 2003). The peripheral analgesic activity displayed by both extracts is probably mediated via the inhibition of peripheral mediators which could be related with the antioxidant effects observed *in vitro* in the DPPH model.

The DPHH model demonstrated that HELMu and HEBMu exerted significant antioxidant activity with IC_{50} = 4.8 and 7.0 µg/ml, respectively. Similar results were found by Machado et al. (2014) for *M. urundeuva* with the same method, wherein found IC_{50} < 4.0 µg/ml for alcoholic extracts, emphasizing the strong antioxidant activity of this specie. Recent studies have shown that free radicals are responsible for mechanisms that cause inflammation and pain, and that high levels of reactive oxygen species (ROS) can induce pain. ROS are also involved in persistent pain, including neuropathic and inflammatory pain generated from many redox processes

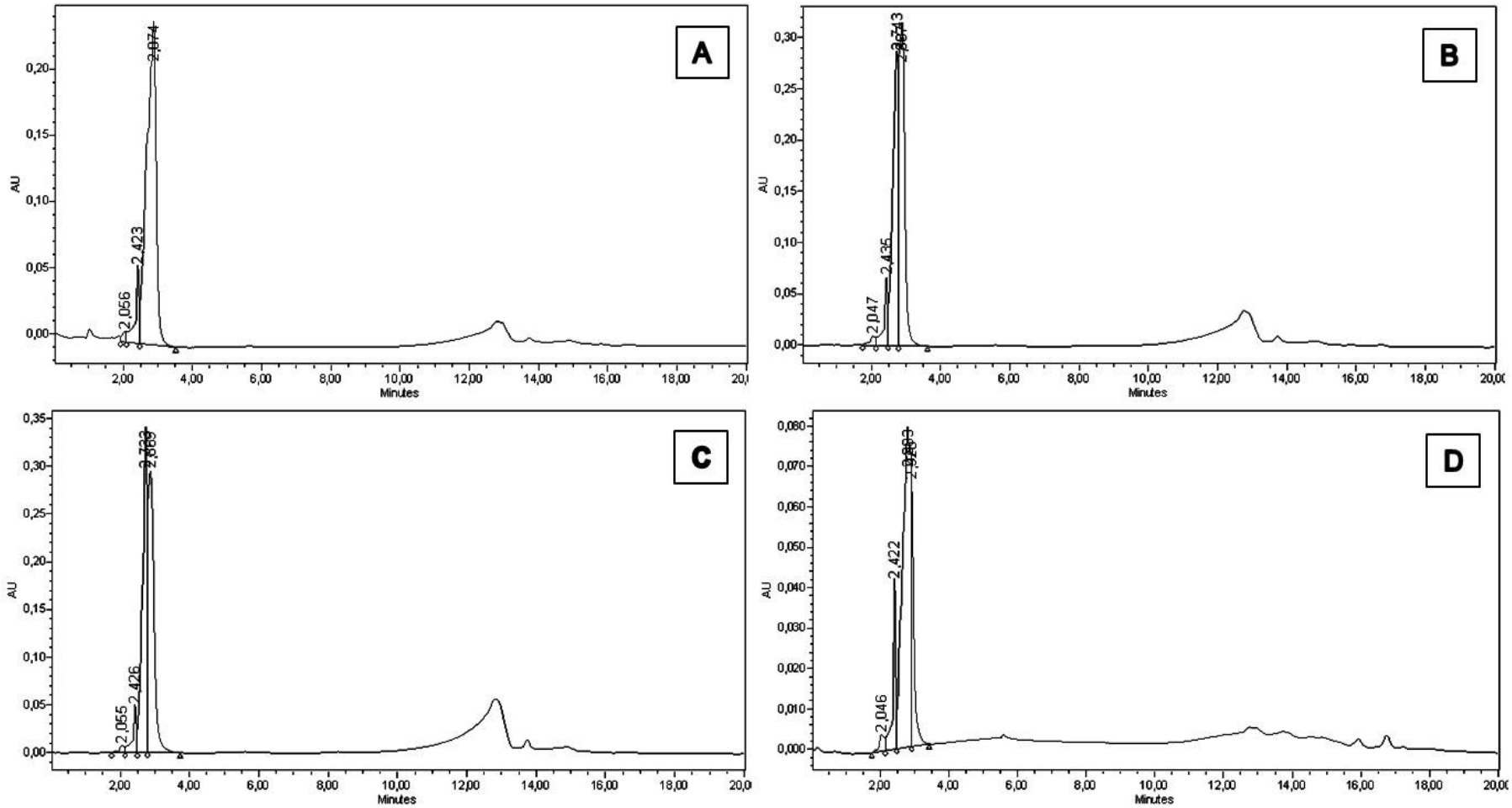

Figure 1. Chromatographic profile of the fractions of HELMu: hexane (A), dichlorometane (B), ethyl acetate (C) and isobutanol (D) frations.

and are known to be major free radicals in the human body (Fidanboylu et al., 2011; Kumar, 2011). Antioxidants substances have the ability to scavenge these free radicals and thus prevent intracellular oxidative damage that results in pain (Valko et al., 2007). The activity antioxidant displayed by extracts can be related with presence of phenolic compounds that possess the capacity to neutralize free radicals (DPP▪) (Silva et al., 2007) and that differences in the structure or concentration of this compounds in the leaves and barks determine the difference obtained between the values of antioxidant activity of HELMu and HEBMu.

In the antimicrobial evaluation the HELMu proved be more efficient than HEBMu in disk diffusion and microdilution test. Only the HELMu inhibited growth of *S. aureus, E. faecalis*, *P. mirabilis*

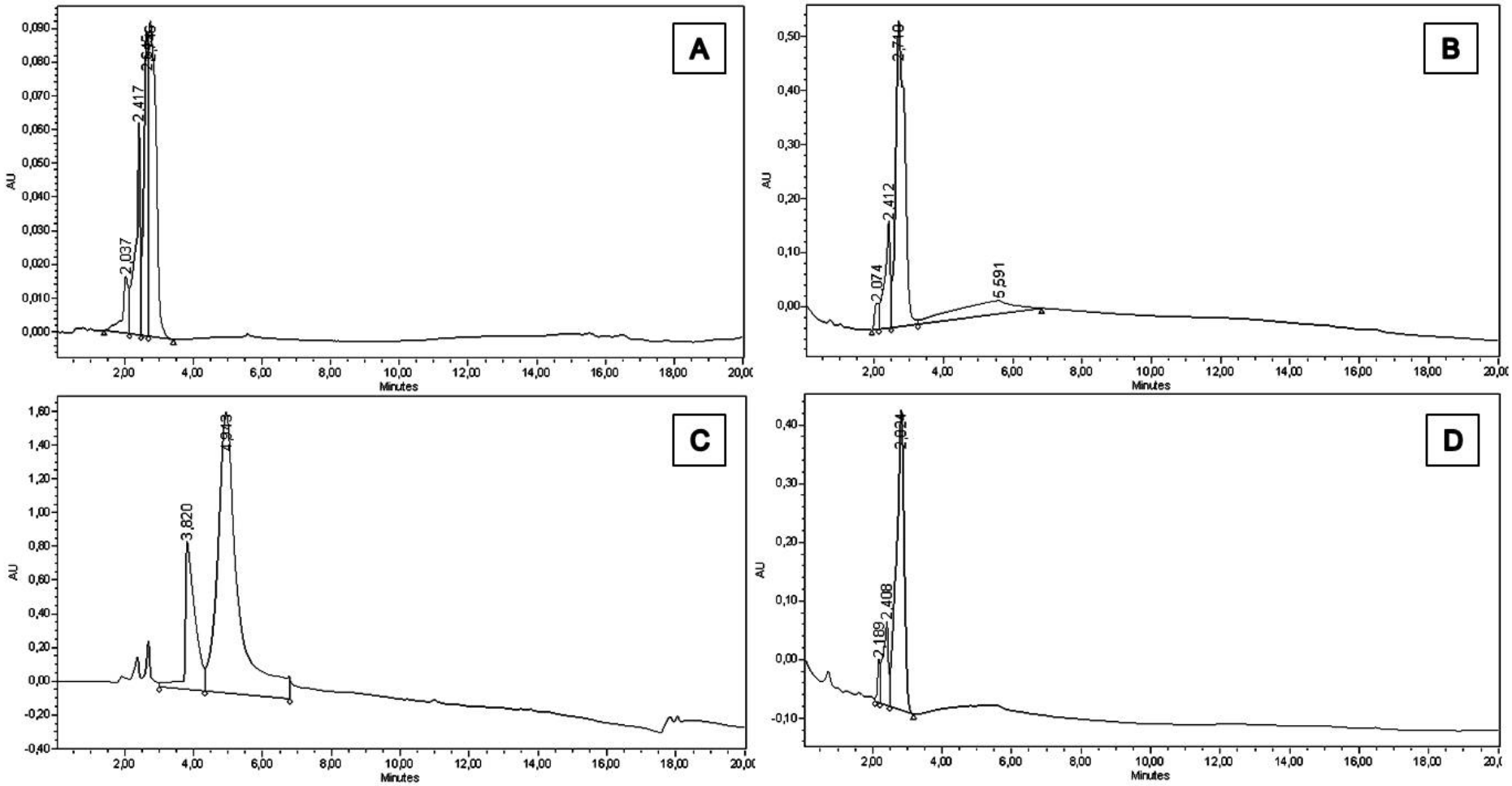

Figure 2. Chromatographic profile of the fractions of HEBMu: hexane (A), dichlorometane (B), ethyl acetate (C) and isobutanol (D) fractions.

and *S. flexneri*. In relation to oral bacteria, the HELMu was more active with lower values of MIC and BMC (MIC ≤ 200 µg/ml and MBC ≤ 300 µg/ml against *S. oralis*) than the HEBMu that was not active against *S. salivarius* and *S. mutans*. The higher activity of HELMu can be associated with the presence of essential oils in the leaves in conjunction with saponnins and flavonoids (Petti and Scully, 2009; Orhana et al., 2010; Maatalah et al., 2012; Rapper et al., 2013

The toxicity of HELMu and HEBMu was evaluated using MTT assays on LLC-MK2 mammalian fibroblast cells. This test provides important information on the toxic effect of chemical compounds in direct contact with the cell culture. Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. When

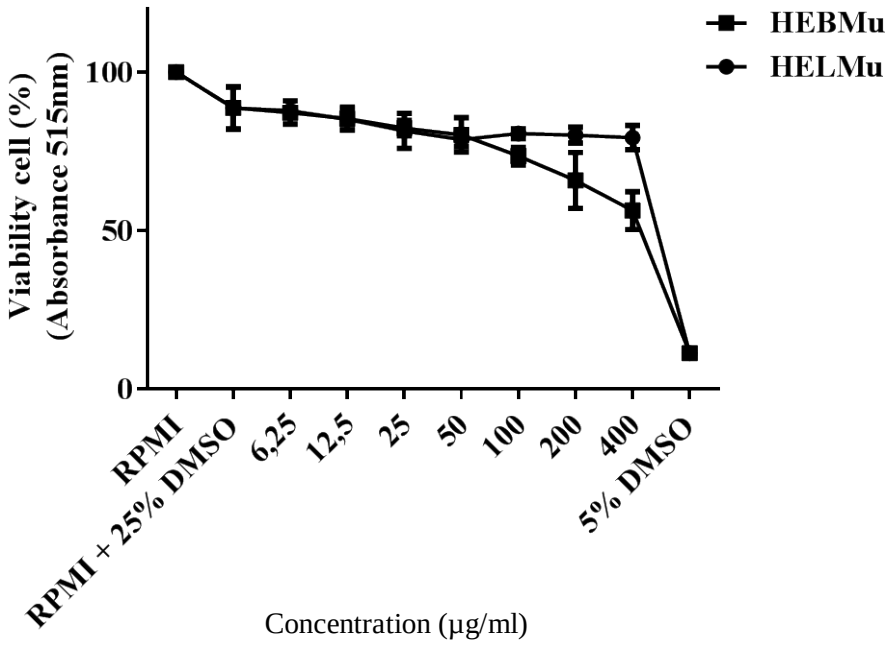

Figure 3. Effects of HELMu and HEBMu on the viability of LLC-MK2 mammalian fibroblast cells. The percentage cell viability was determined using MTT assay after 24 h treatment with the indicated concentrations.
Values are expressed by mean ± S.D. *p<0.05 vs. control indicates statistically significant differences (ANOVA followed by Tukey's Tests)

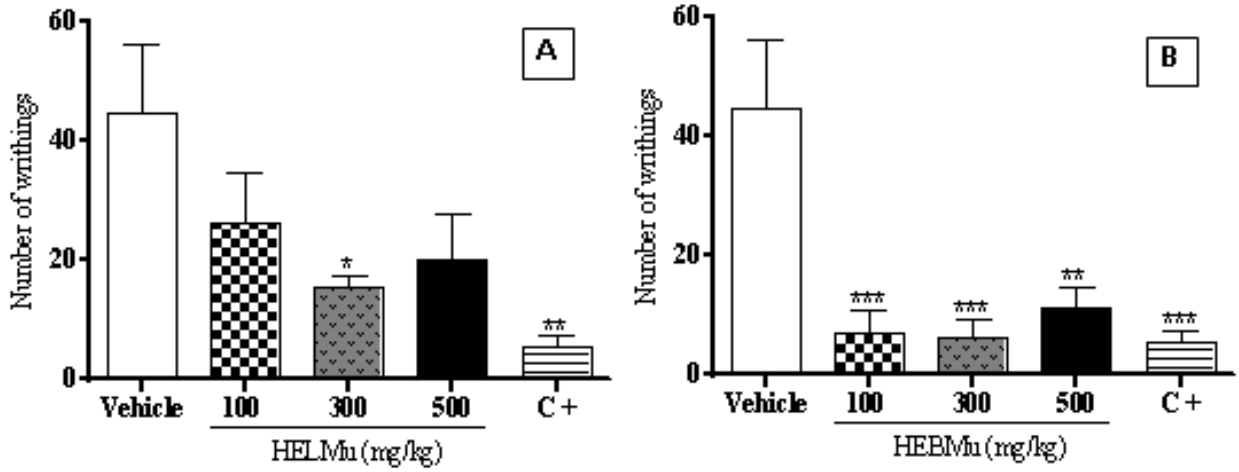

Figure 4. Effects of HELMu (A) and HEBMu (B) on the acetic acid-induced writhing assay. Vehicle (control), HELMu and HEBMu (100, 300 e 500 mg/kg), indomethacin (C+, 5mg/kg) were administered i.p. 0.5 h before acetic acid injection. Each column represents mean + SEM (n = 6). *p<0.01**p<0.001***p<0.0001 (ANOVA followed by Tukey's test).

When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood, but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT (Marshall et al., 1995). It is one of the most commonly used tests for determining cytotoxicity of drugs (Riss et al., 2013). In this assay, both extracts no present toxicity at concentration evaluated with IC_{50} > 400 µg/ml, which is an estimated value, since the test was conducted up to 400 µg/ml showing that the actual value is greater.

In conclusion, HELMu exhibits strong antioxidant activity, moderated antimicrobial activity and moderated

nociceptive effect, and no toxicity, supporting the use of the leaves in traditional medicine instead of the bark.

Conflict of interest

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Banco do Nordeste do Brasil (BNB), Conselho de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Pró-Reitorias de Pesquisa e Pós-Graduação da Universidade Estadual de Montes Claros and Pró-Reitorias de Pesquisa e Pós-Graduação da Universidade Estadual Paulista for financial support and fellowships.

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Vol. 9(6), pp. 169-178, 10 February, 2015
DOI: 10.5897/JMPR2014.5644
Article Number: xxxxxxxx
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

The effect of growth regulators on two different *in vitro*-cultured explants of *Carapa guianensis*

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Received 10 October, 2014; Accepted 14 January, 2015

***Carapa guianensis* Aubl. (Meliaceae), known locally as andiroba, is a multi-use species from Amazonia. Andiroba oil is considered an important natural product in the Brazilian market, and international demand is increasing due to its cosmetic and pharmaceutical potential. *C. guianensis* trees produce seed irregularly over different harvest periods, leading to inconsistent oil production and difficulties with supply. No management plans or protocols have been developed for *in vitro* or clonal production of *Carapa* seedlings and the maintenance of genetic resources. The objective of this study was to assess the effect of growth regulators on explants (young leaves, old leaves and apical buds). Explants consisting of leaf segments 1 cm on a side were cultivated in MS medium with and without growth regulators. Evaluation was based on fresh and dry weight of the explants after 20 days. In the media with 2,4-dichlorophenoxyacetic acid (5, 15, 35 or 45 µM), changes were observed in weight and explant appearance (callus). Bud breakage and development of shoots were achieved using 5 µM of 6-benzylaminopurine. Overall, the results showed that 2,4-dichlorophenoxyacetic acid stimulates callus formation on andiroba foliar explants, while 6-benzylaminopurine was superior to thidiazuron for the initial development of shoots.**

Key words: Growth regulators, *Carapa guianensis*, *in vitro*, tissue culture, organogenesis.

INTRODUCTION

Carapa guianensis Aubl. (Melicaceae), commonly known as andiroba, is a neotropical tree distributed throughout South and Central America, as well as the Caribbean Islands (Cloutier et al., 2007). It is a multi-use species, the main product being seed oil used for medicinal purposes due to its significant limonoid content (Mendonça and Ferraz, 2007; Henriques and Penido, 2014). The bio-oils obtained from *C. guiannesis*

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seed have physical and chemical properties that make them acceptable renewable diesel fuels (Iha et al., 2014). Andiroba oil is used as an insect repellent (Freire et al., 2006) and in the manufacture of cosmetics, due to its high level of unsaturated triacylglycerols (TAG) (Cabral et al., 2013). Additionally, its wood is valued for the construction of buildings and furniture (Guariguata et al., 2002), and cultivated andiroba trees have the potential to recover degraded land.

The exploitation of *C. guianensis* is inevitable and is intensifying, mainly in central Amazonia where seed extraction leads to population reduction, as seed dispersal is the main reproductive mode. Evidence indicates that the size of felled logs has been decreasing for decades (Fortini and Zarin, 2011).

Andiroba trees produce seed irregularly over different harvest periods (Tonini et al., 2008). This variability, which does not allow continuous oil production, generates management difficulties, resulting in periods with low seed and oil production (Frankie et al., 1974; McHargue and Hartshorn, 1983). Industries that use *C. guianensis* need a constant source of homogeneous raw plant material.

Plant regeneration by tissue culture, through either organogenesis or somatic embryogenesis, is a prerequisite for potential clone propagation, genetic transformation and *in vitro* preservation for germplasm from timber trees, including andiroba (Handley, 1995; Park et al., 1998; Minocha and Jain, 2000). Clone propagation in aseptic conditions is an alternative method of propagation for some medicinal plant species with large-scale production issues, accelerating the conventional propagation process and producing genetically identical plants (Zhou and Wu, 2006).

This study evaluated the effects of growth regulators on *C. guianensis* foliar explants and apical buds, with a view toward shoot induction and providing guidelines for optimizing andiroba cultivation.

MATERIALS AND METHODS

Plant

Seeds of *C. guianensis* Aublet (andiroba) were collected in the city of Rio de Janeiro (Jardim Botânico do Rio de Janeiro) beneath identified parent trees, with previous authorization from the institution. Seeds, weighing around 20 to 30 g were washed, soaked in water for 24 h, placed in 200 ml plastic bottles with equal volumes of sterile soil fertilized with plant humus, and watered twice a week. Three kinds of explants were used: (a) young leaf explants (less than 1 week old), when they were pink-colored; (b) old leaf explants, when each leaf was at most 2 weeks old and green; and (c) shoot apical buds. The younger leaves for explants were simply cut into three parts (apex, middle and base). Leaf fragments (1 cm^2) were obtained from the older green leaves. Apical buds were collected from the same seedlings when the apical segment was still green and soft.

Surface sterilization of explants

The surfaces of the explants from young and old leaves were sterilized with a 50% (v/v) commercial bleach solution for 1.5 min and then washed three times for 1 min each in sterile distilled water. Surface-sterilized explants were placed with their adaxial or abaxial surfaces firmly in contact with the medium in culture flasks.

The apical bud explants were surface-sterilized with a 50% (v/v) commercial bleach solution, for 2 min, followed by a quick dip in 70% (v/v) ethanol solution, and then washed 3 times with sterile distilled water. The exposed ends of the explant were trimmed aseptically and then inoculated on the medium.

Tissue culture

To assay the effectiveness of growth regulators (GR), surface-sterilized leaf explants were inoculated on sterile MS medium (Murashige and Skoog, 1962). The medium was supplemented with 30 g/L sucrose, 7 g/L agar and vitamins, with or without growth regulators (MS 0) (Macedo et al., 1999).

To determine if foliar explants would respond to medium supplemented with one GR at a time, the following GRs were used: indoleacetic acid (IAA) (1, 5, 15, 35 or 45 µM), 6-benzylaminopurine (BA) (1, 5, 15, 35 or 45 µM), 2,4-dichlorophenoxyacetic acid (2,4-D) (1, 5, 15, 35 or 45 µM), and thidiazuron (TDZ) (0.5, 1 or 5 µM) (Figure 1).

Combinations of BA and IAA (1 + 1; 1 + 5 or 5 + 5 µM) were also tested. The plant material was observed for 2 months. For each treatment, 12 explants were used, and the experiments were repeated three times. The explants were placed on the medium with either the abaxial or the adaxial surface turned up. In order to determine if callus obtained from foliar explants would undergo indirect organogenesis, callus explants were subcultivated on control, IAA, BA and TDZ media. Then, calli developed on the lowest and the highest (5 and 45 µM) 2,4-D medium concentrations were subcultivated (Figure 1). Explants cultured for 4 weeks in 2,4-D supplemented medium with callus formation were transferred to fresh MS 0 medium or to medium supplemented with IAA (1 or 5 µM), BA (1 or 5 µM), 2,4-D (1 or 5 µM) or TDZ (0.5, 1 or 5 µM). Explants alive after 4 weeks were subcultivated on MS 0 supplemented with 3 g/L charcoal (Figure 1).

For bud growth experiments, the explants were inoculated in MS medium with IAA, BA, TDZ (1 or 5 µM) or no GR, and with or without 3 g/L charcoal (Figure 1). After 4 weeks, the bud explants were transferred to MS medium supplemented with cytokinins and auxins, in an attempt to achieve organogenesis: IAA (0.5, 1 or 5 µM), BA (0.5, 1 or 5 µM), TDZ (0.5, 1 or 5 µM) and combinations of IAA and BA (1 + 5 µM; 5 + 1 µM; 1 + 1 µM) (Fig. 1). Assays performed on a small number of samples with higher concentrations of IAA and BA (15, 35 or 45 µM) and a combination of IAA and BA (5 + 5 µM) produced brown and dry explants. Therefore, these GR combinations were not tested for bud explants.

All experiments were performed in a climate-controlled room equipped with white fluorescent lamps (Osram F20T12/CW) (approximately 20 µmol m^{-2} s^{-1} photosynthetically active radiation, PAR). For all treatments, a 16-h photoperiod was used. Cultures were maintained at 25±1°C.

Assessment of leaf explant development

After the culture periods detailed above, the effect of each treatment was evaluated by dry and fresh explant mass. For dry weight, the leaf explants were individually oven-dried in aluminum

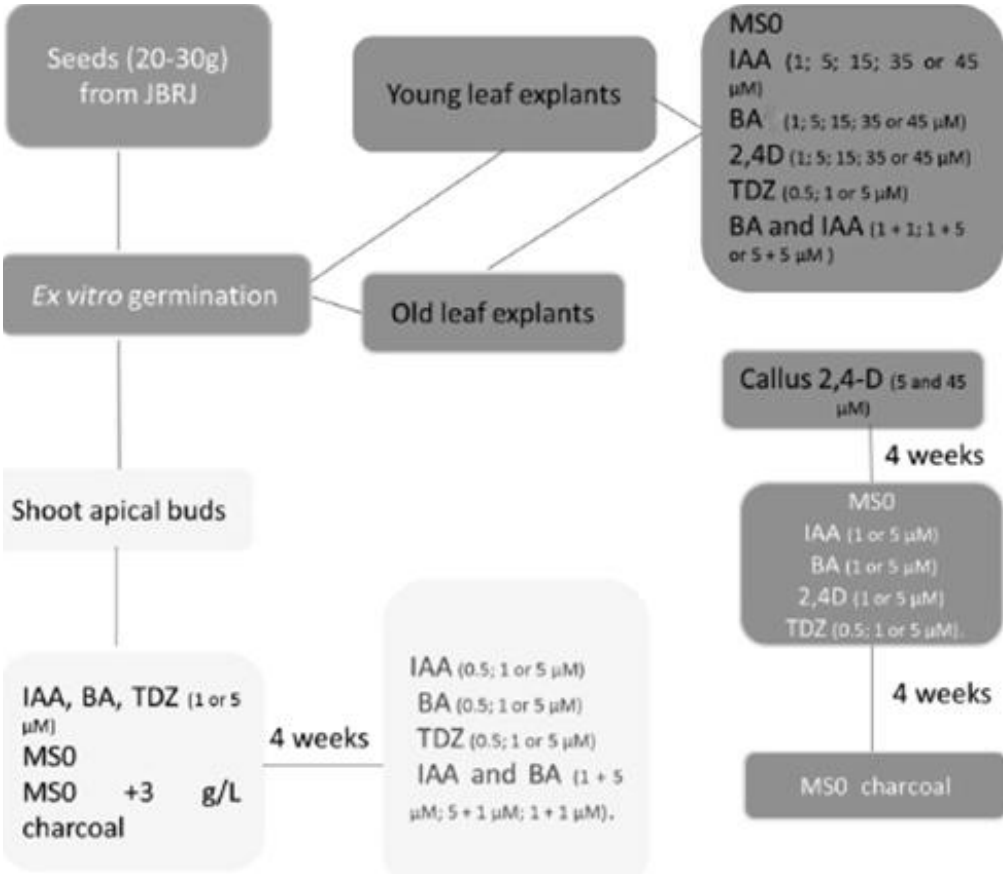

Figure 1. sequence of assays

vessels at 40°C to constant mass and then weighed. To measure fresh weight, the material was removed from the culture flasks and immediately weighed to prevent dehydration.

Assessment of apical bud explants development

After the culture period, the height of explants and leaf length were measured weekly with a ruler. Fresh weight was determined immediately after each explant was removed from the culture flask after 8 weeks of culture; the dry weight was determined after the explant was oven-dried at 40°C to constant mass.

Statistics

The results were analyzed by Analysis of Variance (ANOVA), followed by Tukey's test with a significance level set at $\alpha = 0.05$, using Statistica 7 software for Windows. Means ± standard error (SE) are presented.

RESULTS

A method for organogenesis, either direct or indirect, was developed. Indirect organogenesis involves the production of organs by callus stage, whilst direct organogenesis is related to the formation of organs directly on the surface of cultured intact explants (Us-Camas et al., 2014). The purpose of this study was to produce *in vitro* shoots as an alternative to sexual propagation for *C. guianensis*. Sexual propagation is limited by the tendency of andiroba seeds to lose their power of germination soon after harvest, as a result of dehydration. Micropropagation of selected phenotypes of *C. guianensis* is also desirable since propagation by seed yields high levels of genetic variability, a limiting factor for its commercial use.

Old leaf explants

No callus formation was observed from old leaf-tissue explants cultured in media supplemented with IAA, BA, TDZ or IAA-BA combinations (Figure 2). However, friable whitish callus was formed in all explants cultured in 2,4-D media, except the medium supplemented with 1 µM 2,4-

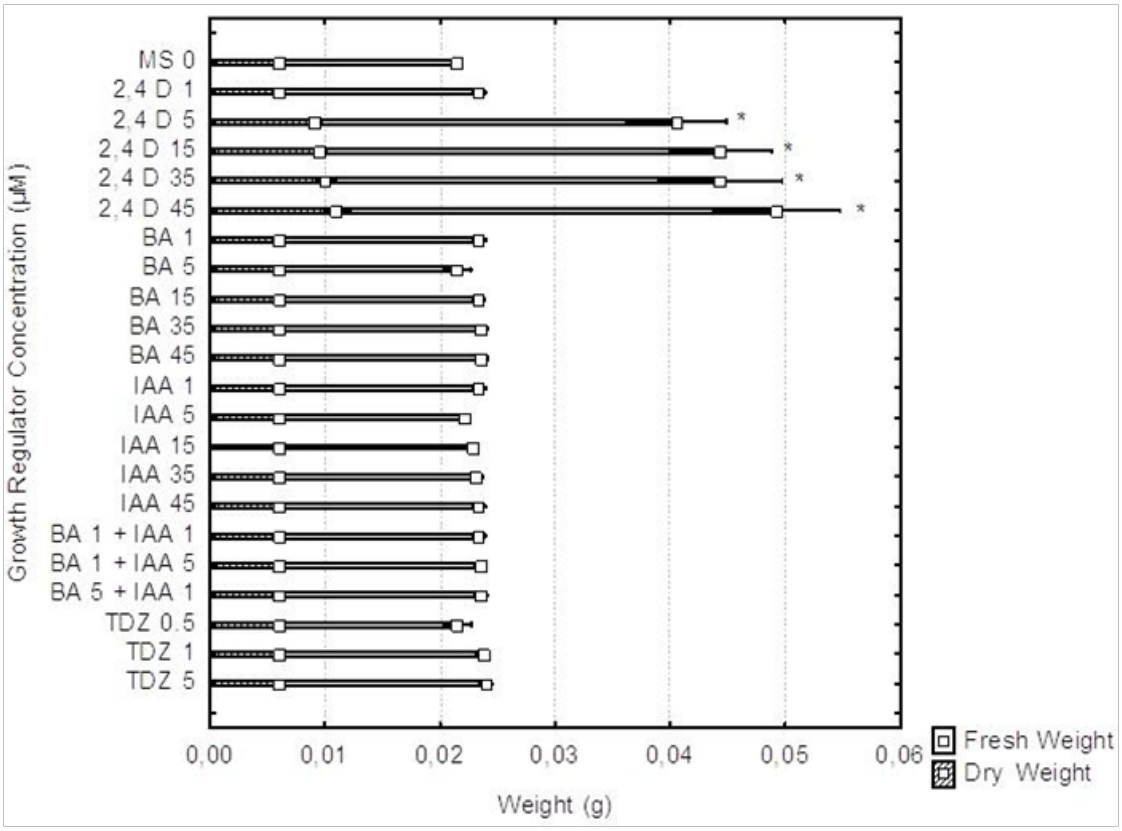

Figure 2. Fresh and dry weight of explants from old leaves of *Carapa guianensis* cultured.

D (Figures 2 and 3). After 4 weeks of observation, a significant difference, according to Tukey's test, in explant fresh and dry weight was observed when comparing explants cultured in 2,4-D supplemented medium with others cultured in MS 0 or with IAA, BA, TDZ or IAA-BA supplemented medium (Figure 2). No difference was observed between the orientations of explants (abaxial or adaxial surface turned up) in differently supplemented MS medium or MS 0 for 4 weeks.

After calluses were obtained on 2,4-D supplemented medium, the effects of different subculture medium conditions on callus development were investigated (Figure 1). Therefore, calluses obtained from old leaf explants, after 4 weeks of culture in 2,4-D medium (5 or 45 µM of 2,4-D), were transferred to fresh medium. Calluses obtained with 5 µM of 2,4-D and then transferred to medium with 1 or 5 µM of 2,4-D or 0.5, 1 or 5 µM of TDZ survived and showed increase in callus mass (Figures 4 and 5.). These calli acquired a brighter green color (Figure 5). The calli that were subcultivated on MS 0 or MS supplemented with IAA or BA did not survive after 4 weeks. These explants turned brown and became dry. Calli subcultivated on 2,4-D and TDZ that showed no or very few signs of brown parts after 4 weeks were transferred to MS 0 supplemented with 3 g/L of charcoal (Figure 1). Only a few calli (30%) that came from 1 µM 2,4-D produced very small thin roots after 4 weeks (Figure 6). The calli that came from TDZ in all concentrations merely maintained their green color.

Callus obtained with 45 µM of 2,4-D and then transferred to medium with 1 or 5 µM of 2,4-D or 0.5, 1 or 5 µM of TDZ also showed increases in callus mass (Figure 7). The calli that were transferred to MS 0 or MS supplemented with IAA or BA did not survive after 4 weeks. These explants turned brown and became dry, as shown subsequently. Rhizogenesis was not observed in any calli that were first subjected to 2,4-D 45 µM supplemented medium and then transferred to MS 0 supplemented with charcoal, as observed with the callus from 2,4-D. They merely maintained the green callus mass.

Young leaf explants

Young, pink-colored leaf explants were tested to compare the results obtained using old leaf explants, and to

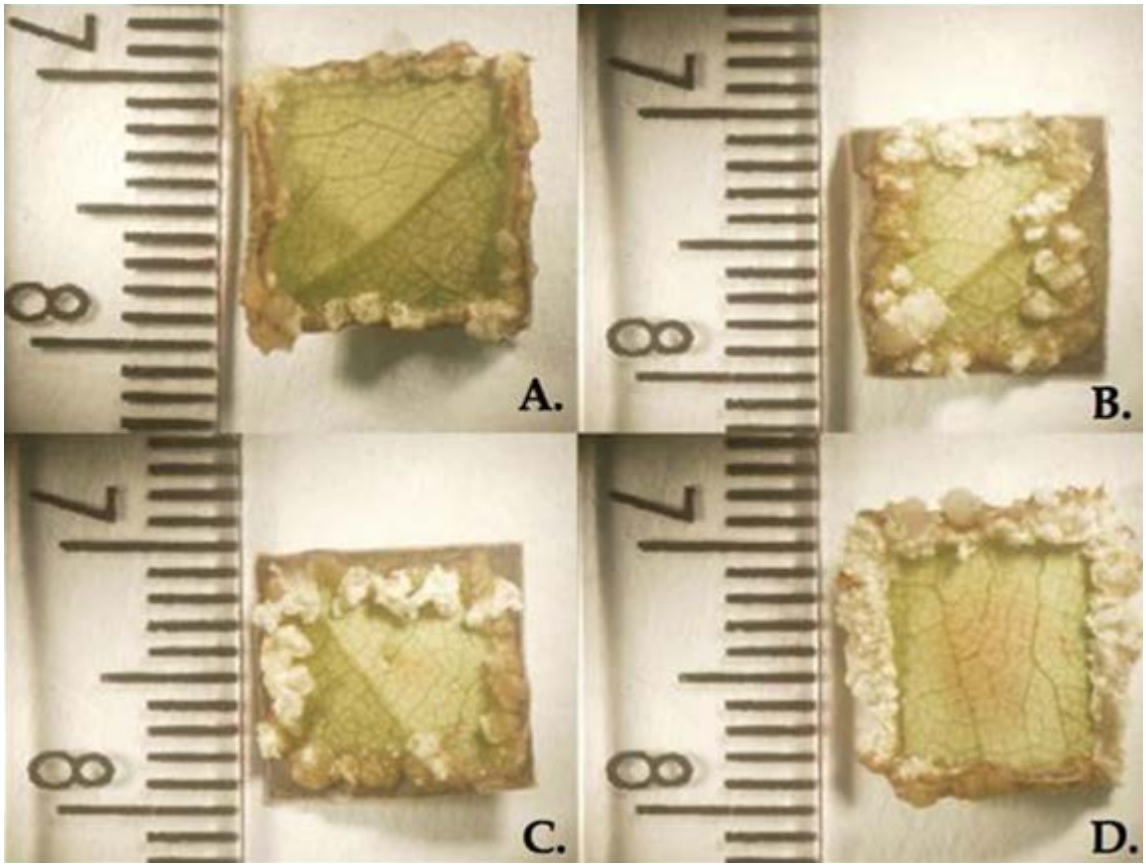

Figure. 3 Four-week-old old leaf explants of *Carapa guianensis* on medium with different concentrations of 2,4-D. (A) 5 µM; (B) 15 µM; (C) 35 µM; (D) 45 µM

determine if they would respond to GR better than older leaves from the same plants (Figure 1). However, these explants did not survive more than 4 weeks on any culture medium. After the first week of incubation in all media, explants turned from pink, to a pale yellow to green, and then to brown. There was no difference in response among the three parts of the leaf (apex, middle and base).

Apical shoot bud explants

Apical bud explants were cultured in MS 0 and with cytokinins and auxins to check their development (Figure 1). Bud breakage was 100% successful only in MS 0 supplemented with charcoal, but shoots did not grow longer than 1 cm. A mean of 3 to 4 small shoots were obtained per bud explant (Figure 8). On the other media, no bud explant development was observed.

Shoots developed only on BA supplemented medium (Figure 8), after the initial growth on MS 0. On the other GR supplemented media, the explants did not develop and the shoots maintained the size that they had reached on the first medium (MS 0). No signs of rooting were observed. When the medium was not supplemented with 3 g/L of charcoal, all the explants turned brown and died. The plantlets reached 2.55 ± 0.36 cm in height (mean ± standard error) at the end of 2 months. The leaves reached 3.66 ± 0.38 cm in length (mean ± standard error).

DISCUSSION

The present results for callus culture contrast with those obtained by Da Costa Nunes et al. (2002) and Rocha and Quoirin (2004). Using a cotyledonary node culture, Da Costa Nunes et al. (2002) found that callus formation in *Cedrela fissilis* Vell., a woody species of Meliaceae, occurred with the growth regulators naphthalene acetic acid (NAA) and BA. These authors obtained the largest increase of fresh weight in treatments with combinations

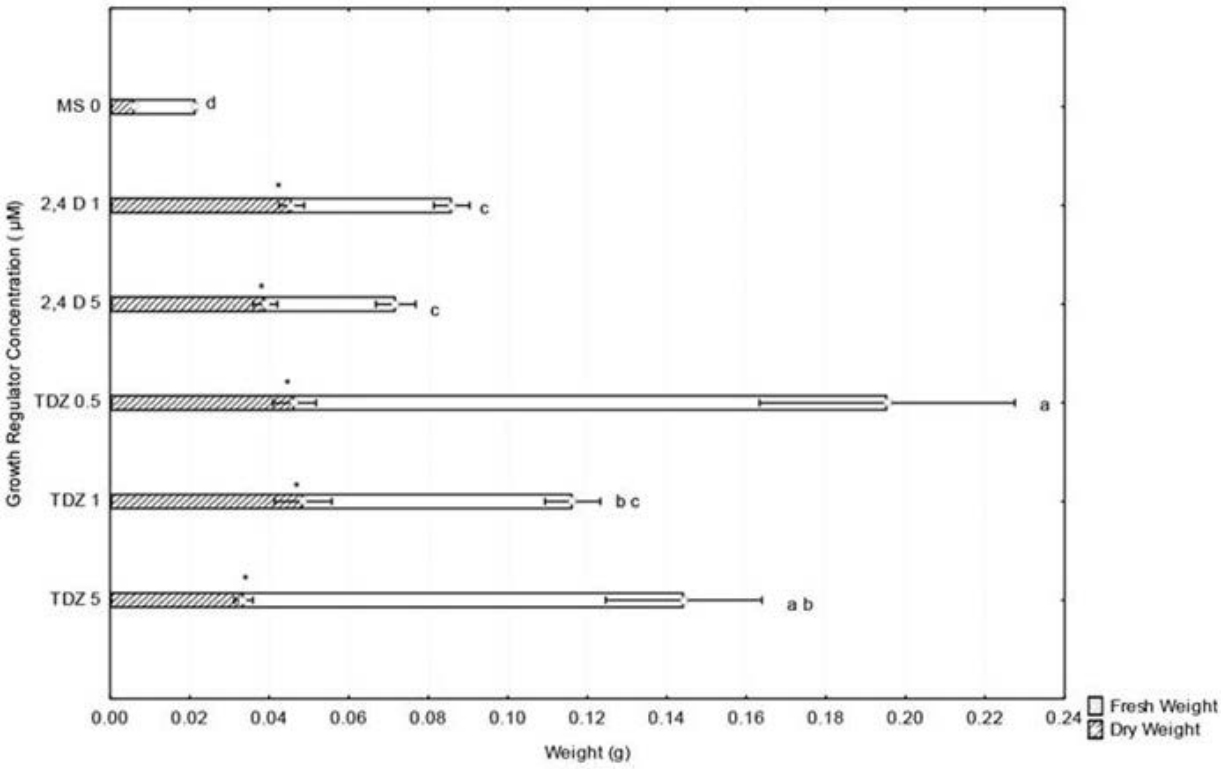

Figure. 4 Fresh and dry weight of *Carapa guianensis* callus obtained in 5 µM of 2,4-D supplemented medium and subcultivated on MS0; 1 or 5 µM of 2,4-D or 0.5, 1 or 5 µM of TDZ supplemented medium. Data represent mean values per treatment, and bars indicate SE of 36 explants/medium. Mean values with the same letter or * are not significantly different based on ANOVA followed by Tukey's test at $P \leq 0.05$.

of 6-BA at 1.25, 2.5 and 5.0 µM with 2.5, 1.25 to 5.0 or 5.0 µM of NAA, respectively. Rocha and Quoirin (2004) observed callus formation in mahogany (*Swietenia macrophylla* King), using BA. However, Vila et al. (2009) reported that 2,4-D induces callus formation in *C. fissilis* and somatic embryos were formed after 6 months, reducing the concentration of GR in the medium. In this present study, although the entire plant did not regenerate, there was a morphogenic response with the appearance of roots. In general, the absence or reduction of plant growth regulators led to the development and differentiation of somatic embryos or their conversion into plantlets (Merkle, 1995; Hu et al., 2008; Kumar et al., 2008; Yang et al., 2008).

In the present study, *C. guianensis* leaf explants developed *in vitro* in different 2,4-D concentrations and showed callus formation. In agreement with our results, Vila et al. (2007) noted that callus mass in zygotic embryo cultures of *Melia azedarach* L. (Meliaceae) was induced by high concentrations of 2,4-D and Picloran. To differentiate embryos from calli originating from hypocotyls or immature cotyledons in *Azadirachta indica* A. juss. (Su et al., 1997), it was necessary to use medium supplemented with IAA. Thus, it is evident that callus production in different woody species of Meliaceae is induced by different growth regulators, in varied concentrations. Furthermore, the type of auxins and cytokinins used in the culture media was shown to strongly influence callus formation.

Cytokinins, principally BA, have been reported to be a positive influence to break dormancy from buds and increase its development, as seen on Husain and Anis (2009), where MS medium with 5 µM of BA was the best condition for multiple shoots growth and the increase of length. BA is naturally present in plant tissues, plus its stability in comparison to other cytokinins (Letham and Palni, 1983), may be an explanation for the better response from explants using BA.

In the present work, rhizogenesis was only obtained in callus and not in plantlets. Rhizogenesis was observed when GR was reduced in MS 0 medium. These results agree with that of Basto et al. (2012). It was not possible in the same medium to develop roots from plantlets. A different response was also observed by Da Costa et al.

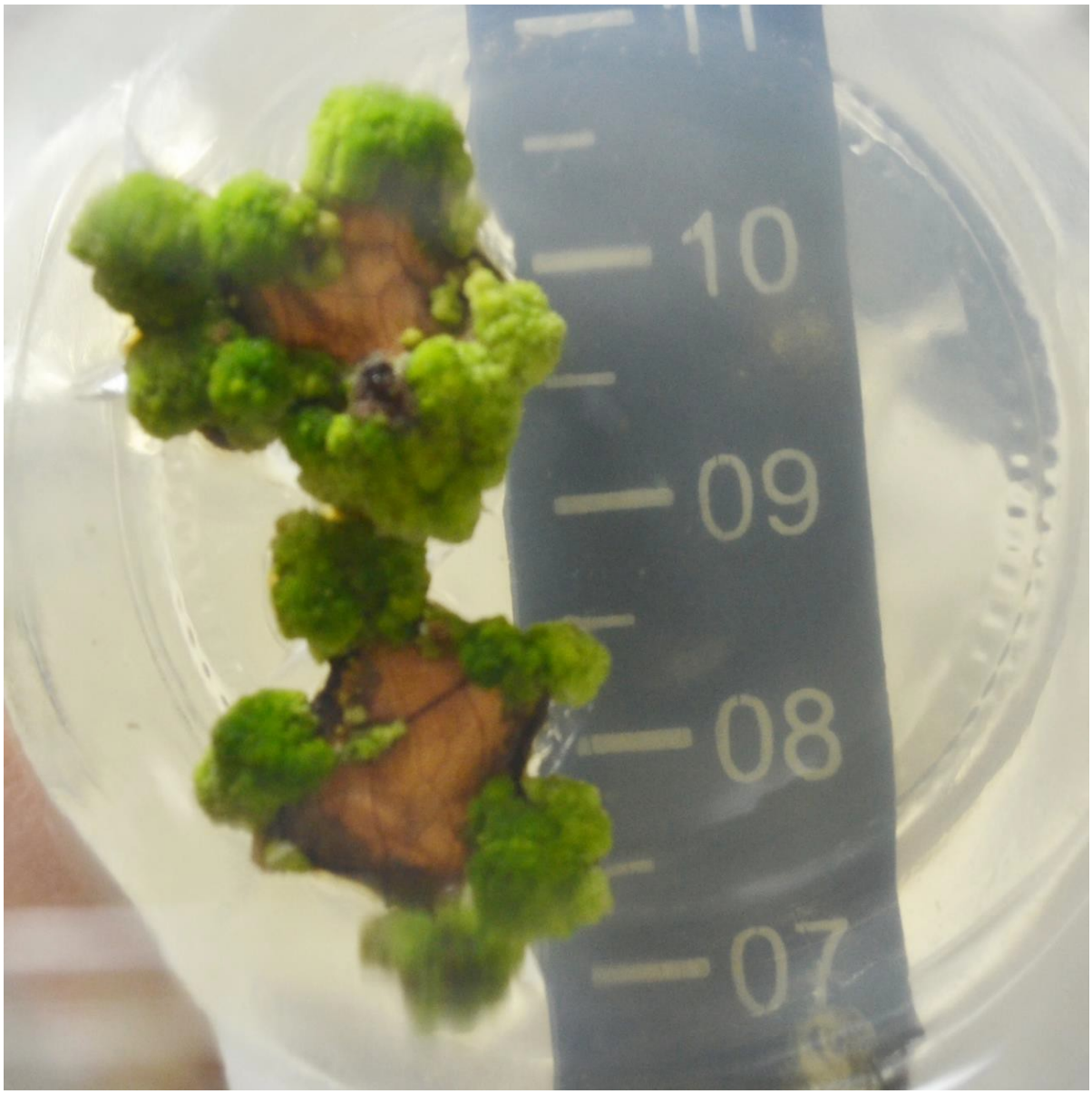

Figure. 5 Two-month-old callus of *Carapa guianensis* first obtained in 5 µM of 2,4-D- supplemented medium and then subcultivated on 0.5 µM of TDZ-supplemented medium. Ruler in cm.

(2002), who reported rooting rates of over 87% of *C. fissilis* node cuttings without growth regulators, and with Millán-Orozco et al. (2011) regarding *C. odorata* shoots from seeds germinated *in vitro.*

The success of *in vitro* regeneration relies on the rooting percentage and survival of the plantlets in field conditions. Future studies can focus on achieving rhizogenesis by using media with indole-3- butyric acid (IBA). The IBA improved rooting efficiency and the superiority of IBA in rhizogenesis was also envisaged by other workers (Chiruvella et al., 2011). Rooted plantlets with 4 to 6 fully expanded leaflets will be transferred into plastic cups containing sterilized soil, sand and water to acclimatization tests.

In conclusion, although the induction of callus in *C. guianensis* has been achieved and bud breakage was inducted, further research is required to confirm the efficiency of embryogenic tissue or bud induction. However, the protocol described here may be suitable for clonal propagation and genetic transformation of *C.*

Figure. 6 Roots on callus of Carapa guianensis subcultivated a second time on MS0 supplemented with 3 g/L of charcoal (red arrow indicates root).

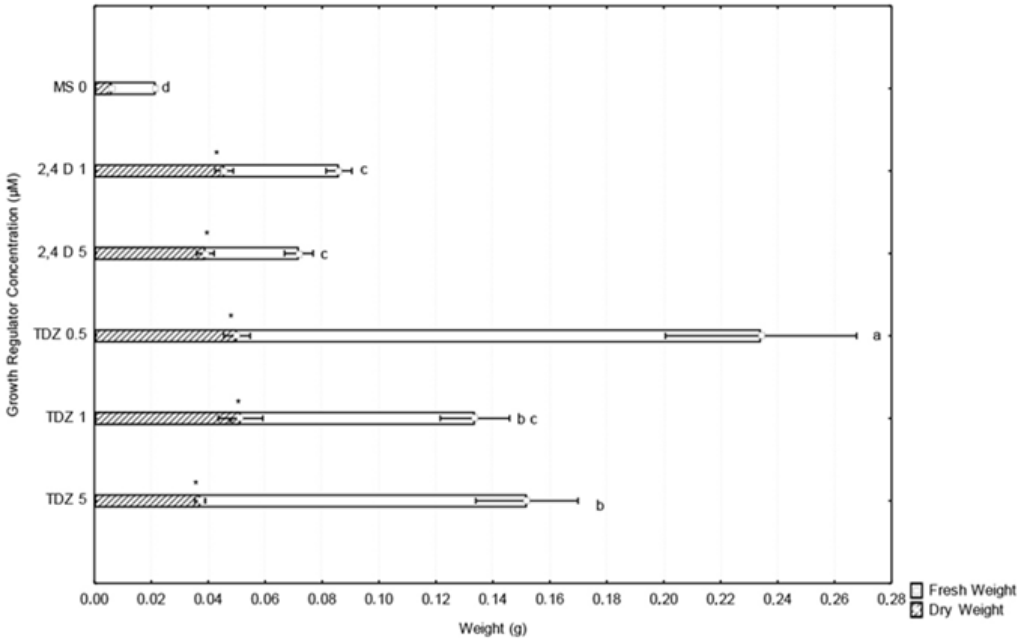

Figure. 7 Fresh and dry weight of *Carapa guianensis* callus obtained in 45 µM of 2,4-D supplemented medium and subcultivated on MS0; 1 or 5 µM of 2,4-D or 0.5, 1 or 5 µM of TDZ supplemented medium. Data represent mean values per treatment, and bars indicate SE from 36 explants/medium. Mean values with the same letter or * are not significantly different based on ANOVA followed by Tukey's test at P≤ 0.05.

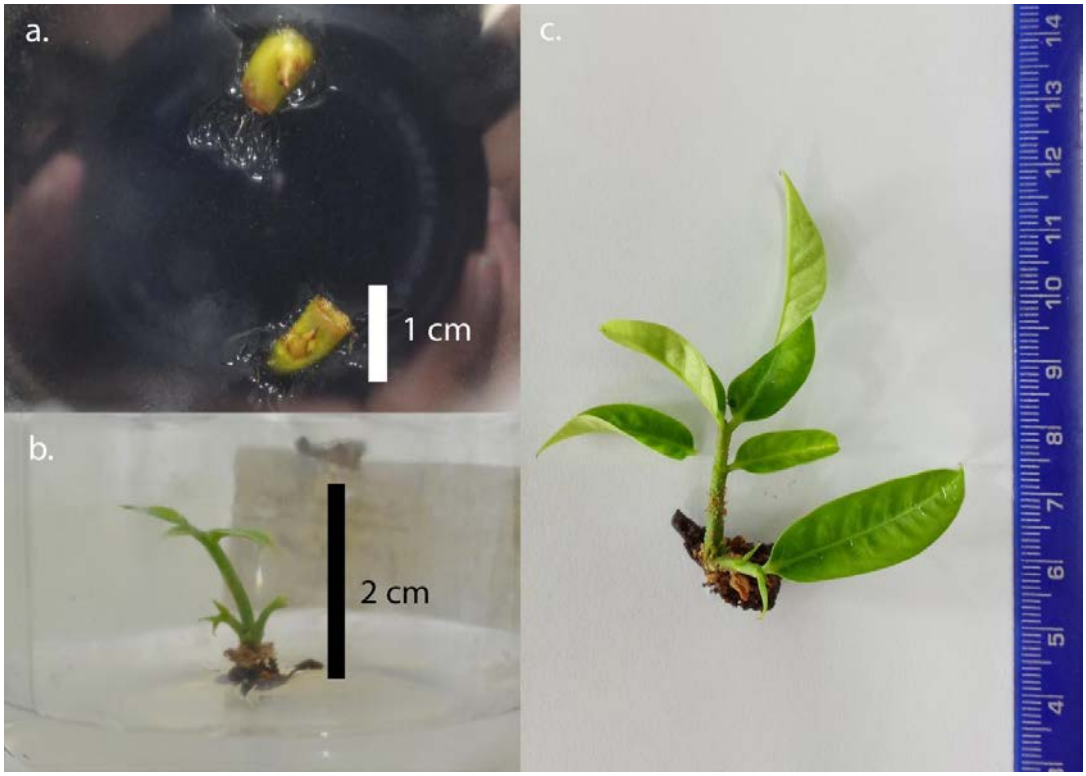

Fig. 8 In vitro establishment of plantlets of *Carapa guianensis* (a) Apical buds on MS0 medium supplemented with 3 g/L charcoal, (b) Plantlet after 4 weeks of subculture on MS supplemented with 5 µM of BA, (c) Plantlet after 8 weeks on MS supplemented with 5 µM of BA.

guianensis.

ACKNOWLEDGEMENTS

The authors are indebted to the Universidade Federal do Estado do Rio de Janeiro (UNIRIO) and to the Instituto de Pesquisas Jardim Botânico do Rio de Janeiro.

Competing interests

We have no conflicting or competing financial interests.

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Vol. 9(6), pp. 179-192, 10 February, 2015
DOI: 10.5897/JMPR2014.5685
Article Number: 915206C50871
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Ethnobotanical survey and *in vitro* antiplasmodial activity of medicinal plants used to treat malaria in Kagera and Lindi regions, Tanzania

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Received 10 November, 2014; Accepted 5 February, 2015

Tanzania has over 12,000 plant species, some of which are endemic and have potential to yield useful medicines. This study seeks to document such plants used as traditional medicines for treatment of malaria in Kagera region of northwestern Tanzania and Lindi region in south eastern Tanzania. The study also reports on the antiplasmodial activity against chloroquine-resistant *Plasmodium falciparum* (Dd2) strain of some of the documented plants using the parasite lactate dehydrogenase method. A total of 108 plant species, among which the families Compositae (14; 12.96%), Fabaceae (12; 11.11%), Euphorbiaceae (8; 7.41%), Melastomataceae (6; 5.56%) and Myrtaceae (4; 3.70%) were documented. Sixteen (16; 44.4%) of 36 extracts from 31 plant species that were tested inhibited malaria parasites growth by more than 50%. *Bersema abyssinica* stem bark extract was the most active with 86.67% inhibition rate followed by *Bridelia micrantha* stem bark extract with 71.87% inhibition rate. These results confirm the potential for plants used in traditional medicine to yield active antimalarial compounds. Further *in vitro* and *in vivo* screening supported by bioassay-guided isolation of active compounds from plants showing good safety margin is suggested.

Key words: Ethnobotanical survey, medicinal plants, malaria, treatment, *in vitro* antiplasmodial, Tanzania.

INTRODUCTION

Human malaria is caused by five *Plasmodium* species namely; *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi,* but *Plasmodium falciparum* is the most widespread and virulent species (World Health

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Organization (WHO), 2013; Cox-sigh and Singh, 2008). Malaria in Tanzania is mainly caused by *P. falciparum* with *Anopheles gambiae* complex being the main vector United States Agency for International Development (USAID, 2014). Tanzania has high malaria prevalence and it is among six African countries that have many reported cases of malaria, with an estimated 10 to 12 million cases and 60,000 to 80,000 malaria-related deaths per year (USAID, 2014; WHO, 2012). Although the Tanzania HIV/AIDS and Malaria Indicator Survey (2011/2012) reported a decrease in the prevalence of malaria in Tanzania, the trend remains unchanged. Prevalence is still high in rural areas and the Lake Victoria zone as compared to other parts of the country Tanzania Commission for AIDS (TACAIDS, 2013).

Malaria is a curable disease that is treated by both modern drugs and herbal medicines (Kinung'hi et al., 2010; Gessler et al., 1995). However, the emergence of *P. falciparum* strains resistant to almost all classes of antimalarial drugs dictates that efforts be increased to develop new antimalarial drug candidates (Dondorp et al., 2009; Wongsrichanalai et al., 2002). Since most antimalarial drugs that are currently being used like quinine and artemisinin derivatives originate from traditionally used medicinal plants (Wells, 2011), this source has a great potential to provide new antimalarial molecules.

Tanzania is estimated to have over 12,000 higher plant species, of which 10% are used for medicinal purposes, and may yield active antimalarial compounds (Mahunnah et al., 2012). Previous reports confirm that some of these plants are used in traditional medicine for treatment of malaria (Gessler et al., 1995), and malaria is leading among diseases that are popularly treated with medicinal plants (Moshi et al., 2009; Mahunnah, 1987). Some of these plants have been reported in previous studies (Moshi et al., 2009; Gessler et al., 1995; Mahunnah, 1987), but many have not been documented and only a few have been tested for antimalarial activity. Therefore, this study reports plant species used for treatment of malaria in Kagera and Lindi regions of Tanzania and results of some of the plants that were screened for *in vitro* antiplasmodial activity.

MATERIALS AND METHODS

Documentation, identification and collection of the medicinal plants used for treatment of malaria

Disease-specific ethnobotanical survey was conducted in six villages in Kagera region (North-west of Tanzania) and one village in Lindi region (South east of Tanzania). In Kagera region the study was conducted in November, 2012 in Buzi Bukombe, Buzi Kishura and Kwamkenge villages in Bukoba rural district, Buleza village in Muleba disctrict, Rwambaizi village in Karagwe district, and Kashozi village in Misenyi district. The study in Lindi region was conducted in July, 2012 in Mchakama village located in Kilwa district. Information was collected from well known and experienced traditional healers and herbalists who were informants in a previous ethnomedical study (Moshi et al., 2009). Before collecting information all the participants were informed about the study objectives and agreed to participate by signing an informed consent form. Vernacular names of the plants, part(s) used, method for preparation, route of administration and possible signs of toxicity were documented. Preliminary identification was done by a Botanist, Mr. Haji. O. Selemani, in the field and further authenticated in the Herbarium. Voucher specimens are deposited in the Herbaria at Muhimbili University of Health and Allied Sciences and at the Botany Department, University of Dar es Salaam. The selection of the plants to be tested for antimalarial activity was based on absence in the literature of previous antimalarial testing, reported antimalarial use in other countries or emphasis made by the traditional healers regarding efficacy for malaria treatment. This study received ethical clearance (Ref. No. MU/DRP/AEC/Vol.XIII/01st August 2011) from the Muhimbili University of Health and Allied Sciences Institutional Review Board.

Preparation of extracts

Dry powdered plant materials were macerated in 80% ethanol, at room temperature, for 24 h and then filtered through cotton wool to separate the liquid crude extracts from the solid materials. The solid plant materials were macerated again in the same solvent for another 24 h and the extracts obtained from the first and the second extractions were mixed before drying. The liquid crude extracts were concentrated under *vacuo* by using Heldolph® rotary evaporator (Heldolph instruments GmbH, Schwabach, Germany) to obtain viscous extracts which were further dried by using a freeze drier (Edwards High Vacuum International, Crawley Sussex, England).

In vitro antiplasmodial screening

Malaria parasites

Blood stage chloroquine-resistant *P. falciparum* Dd2 strains (*Pf* Dd2; MRA-156 deposited by TE Wellems, Lot# 59443398) were used. The parasites were donated to the University of Buea in Cameroon by BEI-resources (MR4/ATCC® Manassas, VA, USA).

Malaria culture medium

RPMI-1640 (Lot# RNBC 8874) culture medium with L-glutamine and 20 mM HEPES (Sigma®, Steinheim, Germany) was used.

In vitro culture of malaria parasites

P. falciparum Dd2 laboratory strains were maintained in culture according to the method of Trager and Jensen (Trager and Jensen, 1976) with modifications (Zofou et al., 2013). The parasites were grown in O^+ red blood cells (RBCs) maintained in complete malaria culture medium composed of RPMI-1640 medium supplemented with 2 mg/ml $NaHCO_3$, 10 µg/ml hypoxanthine, 2 mg/ml glucose, 1% albumax II as source of proteins, lipids and 10 µg/ml gentamicin. The cultures were incubated at 5% CO_2, 5% O_2, 90%

N_2 in a humidified incubator set at 37°C (SHEL LAB® Sheldon Mfg Inc, OR, USA). All materials were purchased from SIGMA (Sigma®, Steinheim, Germany) except Albumax II (GIBCO™, Invitrogen) and gentamicin (ROTEX MEDICA, Trittau - Germany) which were supplied locally in Cameroon.

Preparation of plant extracts and standard drug

Stock solutions of 400 µg/ml for each crude extract was prepared by first dissolving 4 mg of crude extract into 100 µl dimethyl sulfoxide (Sigma®) followed by addition of RPMI-1640 medium to 10 ml. Artemether (Sigma®) was first dissolved in dimethyl sulfoxide and then diluted with RPMI-1640 medium to 5 µg/ml. All solutions were sterilized by using 0.22 µm syringe-adapted filters (Corning®, NY, USA) and stored at 4°C until use.

In vitro antiplasmodial activity assay

In vitro antiplasmodial activity was assessed using the parasite lactate dehydrogenase (pLDH) assay (Makler et al., 1993). Non-synchronized 1% parasitized red blood cells (pRBCs) at 2% haematocrit (hct) in 96 well cell culture plates (Costar®, Corning, NY, USA) were incubated in triplicates with 100 µg/ml crude extracts or with 1.25 µg/ml artemether. Wells with parasitized cells but without extract or drug served as negative controls (100% parasite growth) and wells without parasitized cells but with red blood cells only at 2% hct served as blank controls. The plates were incubated for 48 h at 37°C in a humidified incubator set at 5% CO_2, 5% O_2, 90% N_2. After incubation for 48 h parasite growth was confirmed by the aid of a light/UV fluorescence microscope (TENSION®, China) with Acridine orange filter (λmax Abs = 490 nm; λmax Em = 525 nm) and a counting device (Lennox Grain Analysis NG NG21, SPI®, USA) before the plates were frozen overnight at -20°C. In the pLDH assay the plates were thawed at room temperature to hemolyse red blood cells, and the 10 µL of malaria culture were incubated with 50 µL Malstat solutions and 12.5 µL nitroblue tetrazolium/phenazine ethosulfate for 1 h in darkness. Parasite growth was determined by measuring the activity of pLDH enzymes at 650 nm using a microplate reader (Emax-Molecular Devices Corporation, California, USA) and the optical density (OD) values obtained were used to calculate antiplasmodial activity. The average OD value of the blank control (2%hct RBC only) was subtracted from all OD values. The antiplasmodial activity was expressed as percentage inhibition rate of parasites growth.

$$\%\text{Growth inhibition rate (\%IR)} = \frac{(\text{OD negative control} - \text{OD treated})}{\text{OD negative control}} \times 100$$

RESULTS

Documentation and identification of medicinal plants used for treatment of malaria in Kagera and Lindi regions, Tanzania

A total of 108 plants species distributed into 41 plant families were documented and identified in six villages in Tanzania (Table 1). Fourteen plant species (12.96%) belonged to the family Compositae, 12 plant spp (11.11%) belonged to Fabaceae, 8 plant spp (7.41%) belonged to Euphorbiaceae, 6 plant spp (5.56%) belonged to Melastomataceae and 4 plant spp (3.70%) belonged to Myrtaceae. The families Anacardiaceae, Graminae, Labiatae, Meliaceae and Rutaceae each had 3 plant spp (2.78%) while other families were represented by 2 or 1 plant spp (Figure 1). The reported medicinal plants were identified as trees (37%), herbaceous plants (34%), shrubs (20%), climbers (5%), grass (2%) and wood climbers (2%) as shown in Figure 2. In addition, all the reported medicinal plants are administered orally, mostly as decoctions. Boiling was the most common method of preparation (Table 1). Leaves were the most used part of the plants, representing 46% of all plant parts reported followed by stem bark (19%), aerial parts (15%), roots (8%), whole plants (4%), seeds (4%), fruits (3%) and whole stem (1%) as shown in Figure 3.

In vitro antiplasmodial activity of the extracts

The results reported in Table 2 show that 16 (44.4%) out of the 36 extracts of 31 plant species that were tested inhibited the growth of the chloroquine-resistant Dd2 malaria parasite strains by more than 50%. The extract of *Bersema abyssinica* stem barks was the most active with 86.67% inhibition rate followed by the extract of *Bridelia micrantha* stem barks which inhibited parasite growth by 71.87%. The ethanol extracts of *Anthocleista grandiflora* stem barks*, Funtumia Africana* stem bark and leaves, and extracts from leaves of *Vernonia glabra, Ipomoea rubens*, *Pycnanthus angolensis*, *Eriobotrya japonica* and *Oxyanthus speciosus* were the least active with growth inhibition rate of less than 30% against the chloroquine-resistant Dd2 strains (Table 2).

DISCUSSION

The results of the current study support results of previous ethnobotanical studies done in Tanzania and outside Tanzania. In previous studies *Abrus precatorius*, *Adansonia digitata, Azadirachta indica*, *Cassia*

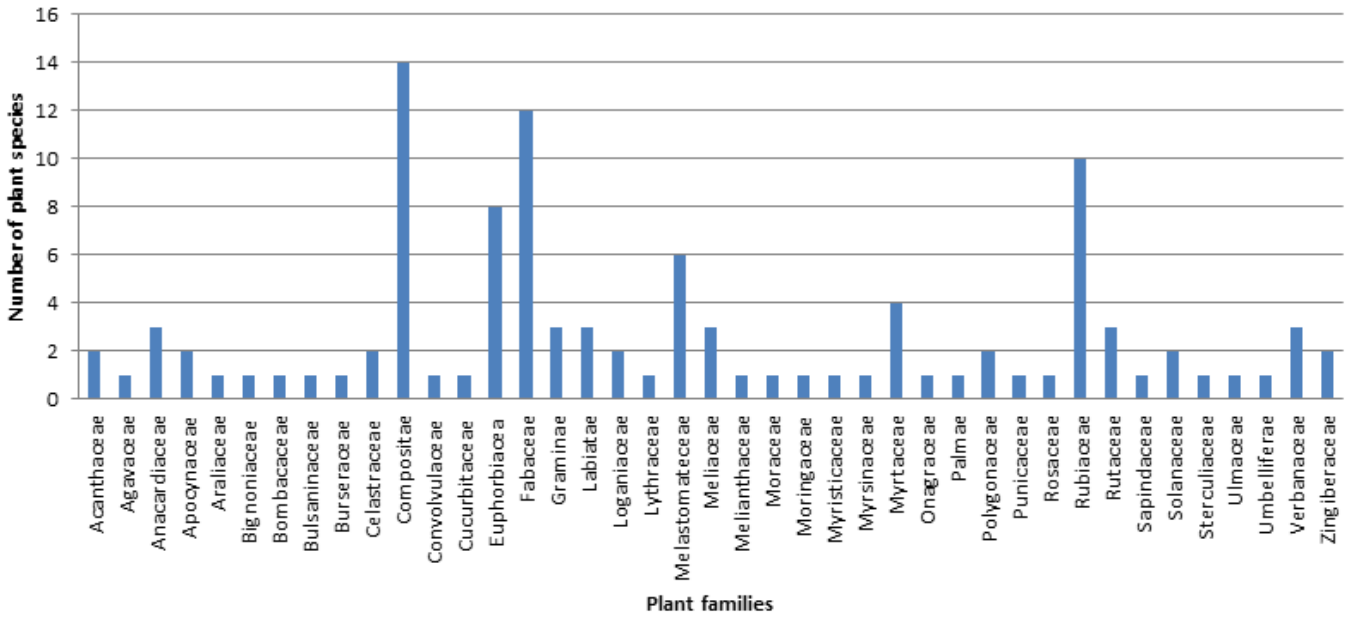

Figure 1. Distribution of plant species into different families

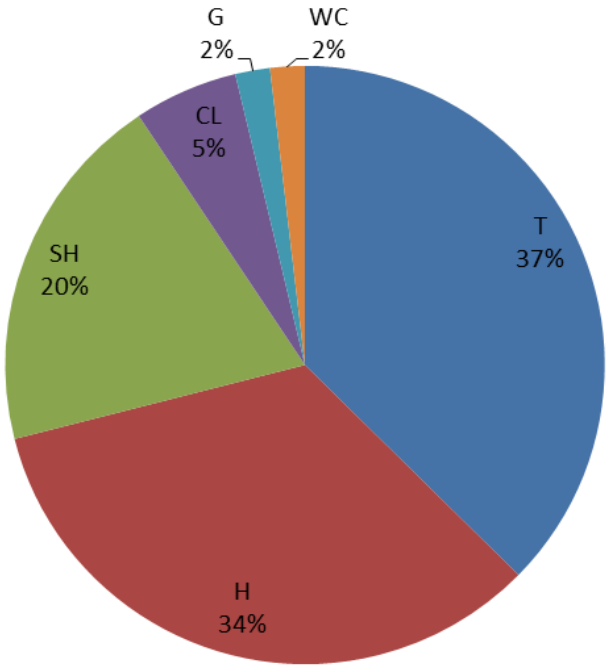

Figure 2. Percentage use of different types of plants. WC = wood climber, G = grass, SH = shrub, CL = climber, T = tree, H = herbs

didymobotrya, *Dombeya shupangae, Ethrina sacleuxii*, *Lantana camara*, *Mangifera indica*, *Maytenus senegalensis*, *Momordica foetida*, *Parinari excelsa*, *Pseudospondias microcarpa*, *Psidium guajava*, *Syzygium*

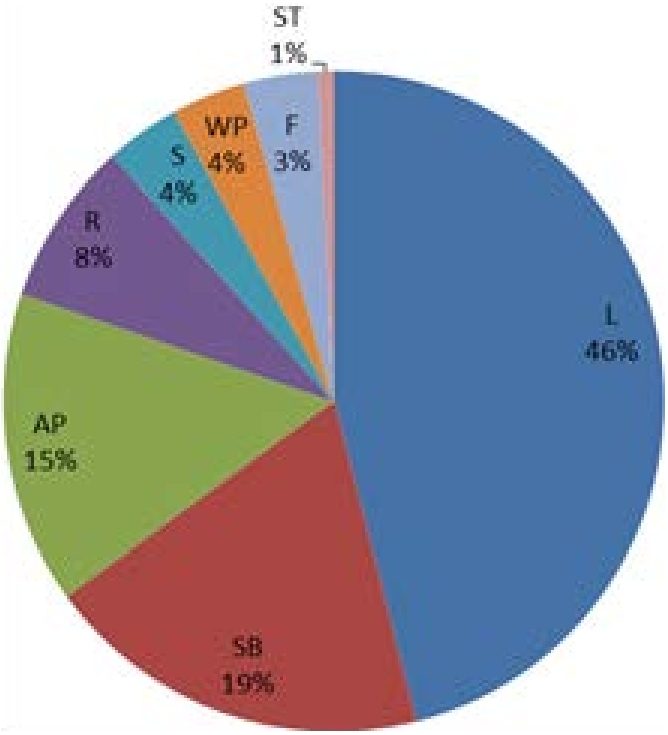

Figure 3. Percentage use of different plant parts. L = leaves, F = fruits, S = seeds, WP = whole plant, R = root, SB = stem bark, ST = whole stem, AP = aerial part.

cordatum, *Todalia asiatica*, *Vangueria infausta*, *Vernonia amygdalina*, and *Zanthoxylum chalybeum* were reported to be used in the treatment of malaria in Tanzania and some of them have shown good *in vitro* antimalarial activity against multi-drug resistant *P. falciparum* K1 malaria parasites (Amri et al., 2012; Augustino et al., 2011; Gessler et al., 1994; Weenen et al., 1990). Similarly, *Erythrina abyssinica*, *Markhamia lutea*, *Teclea nobilis*, *Adansonia digitata*, *Lantana camara*, *Azadirachta indica*, *Zynthoxylum chalybeum, Maytenus senegalensis*, *Vernonia amygdalina*, *Momordica foetida*, *Mangifera indica*, *Moringa oleifera*, *Leonotis nepetifolia*, *Maesa lanceolata, Psidium guajava, Funtumia africana, Canna indica, Cymbopogon citratus* and *Pycnanthus angolensis* are used in traditional medicine for malaria treatment in Kenya, Uganda, Cameroon and Nigeria (Lacroix et al., 2011; Nguta et al., 2010; Tabuti et al., 2008; Titanji et al., 2008; Katuura et al., 2007; Odugbemi et al., 2007).

It is notable that some of the reported plants belong to the families Compositae (13%), Euphorbiaceae (7.4%), Fabaceae (11.1%), and Rubiaceae (9.2%) which are known to contain chemical compounds with good antimalarial properties (Ntie-Kang et al., 2014; Batista et al., 2009). The study has provided useful information that supports traditional healers' claims for antimalarial activity and earlier observations that plants used in traditional medicine are a potential source of new antimalarial lead compounds (Onguéné et al., 2013; Bero et al., 2009).

All the extracts tested for *in vitro* antiplasmodial activity at 100 µg/ml inhibited the growth of malaria parasites to different percentages. *Bersama abyssinica, Bridelia micrantha, Canarium schweinfurthii* and *Antiaris toxicaria* stem bark extracts; *Aspilia natalensis*, *Aspilia mossambicensis* and *Desmodium salicifolium* aerial part extracts; *Maesa lanceolata* and *Rhytignia obscura* leaf extracts; *Pycnanthus angolensis* fruit and *Hallea rubrostipulata* root extracts inhibited parasite growth by more than 60%. The ethanol extract of *B. abyssinica* was the most active with 86.67% inhibition rate against Dd2. In a previous study Kassa et al. (1996) reported that the ethanol extract of *B. abyssinica* stem bark exhibited good *in vitro* antimalarial activity against *P. falciparum* tine-FAC-2/ Ethiopia with IC_{50} = 11 µg/ml. Similarly, a study done in Cameroon by Ngemenya et al. (2005) showed that the methanol extract of *B. abyssinica* leaves exhibited good *in vitro* antiplasmodial activity with an IC_{50} of 2.7 µg/ml. Meanwhile, the chloroform extract of *M. lanceolata* was reported to exhibit very good antiplasmodial

Table 1. Medicinal plants used traditionally in the treatment of malaria in Kagera and Lindi regions, Tanzania

Family	Plant species	Vernacular name	Nature	Part (s) used	Preparation	Voucher number
Acanthaceae	*Acanthus pubescens* (Oliv.) Vatke	Amatoju	SH	R, L	Decoction	RN 01
	Thunbergia alata (Sims)	Wankula	CL	WP	Decoction	RN 02
Agavaceae	*Dracaena steudneri* Engl.	Omugorogoro	T	SB	Decoction	RN 03
Anacardiaceae	*Pseudospondias microcarpa* (A. Rich) Engl	Omuziru	T	L	Decoction	RN 04
	Rhus vulgaris Meikle	Omukanja	SH	R, L, F	Root and leaf Decoction. Ripe fruits eaten	RN 05
	Mangifera indica L.	Omnembe, Mwembe	T	SB	Decoction	RN 06
Apocynaceae	*Funtumia africana* (Benth) Staff	Mwezamaino, Omwelamaino	T	L	Decoction	RN 07
	Holarrhena pubescens (Huch – Ham) G.Don	Nalupande	SH	R	Decoction	4665
Araliaceae	*Schefflera goetzenii* Harms	Olugogome	T	SB, L	Decoction	RN 08
Bignoniaceae	*Markhamia lutea* (Benth) K. Schum	Omushambya	T	SB	Decoction	RN 09
Bombacaceae	*Adansonia digitata* L.	Mbuyu	T	L, F	Leaves are eaten like vegetables. Powder from dry fruits used to make juice	RN10
Bulsaninaceae	*Impatiens gomphophylla* Bak.f	Olwita mkole	H	L	Decoction	RN 11
Burseraceae	*Canarium schweinfurthii* Engl.	Omubafu wa kike/muubani wa kike	T	SB, L	Decoction	RN 12
Celastraceae	*Salacia lovettii* N. Hallé & B. Mathew	Omzindabikaka	T	SB, L	Decoction	RN 13
	Maytenus senegalensis (Lam.) Exel	Omunyaburiko	T	SB, L	Decoction	RN14
Compositae	*Bidens schimperi* Sch. Bip ex Walp	Orwongwa	H	AP	Fresh aerial parts pounded then mixed with clean water, taken orally.	RN 15
	Aspilia mossambicensis (Oliv.) Wild	Eshurwa rusharila, Esisa	H	AP, WP	Fresh aerial parts pounded then mixed with clean water, taken orally.	RN 16
	Gynura scandens O. Hoffm	Ekizimya mulilo	CL	L	Fresh leaves squeezed to get juice. Juice taken orally	RN17
	Vernonia colorata (Wild.) Drake	Ekishura	SH	L	Decoction	RN 18

Table 1. Cont'd

	Vernonia amygdalina Delile	Omubilizi	T	L	Decoction	RN 19
	Crassocephalum mannii (Hook.f) Milne-Redh	Omugango	T	SB, L	Decoction	Voucher not collected
	Crassocephalum vitellinum (Benth) S. Moore	Ekishenda	H	AP, L	Decoction or fresh leaves squeezed to get juice, taken orally.	RN 20
	Aspilia pluriseta (Schweinf)	Lusharila eshurwa	H	AP	Decoction	RN 21
	Bidens pillosa L.	Akakururа	H	AP	Decoction	RN22
	Aspilia natalensis (Sond) Wild	Kanyamoisa	H	L	Decoction. Fresh leaves squeezed and liquid obtained applied in the nose	RN 23
	Melanthera scandens (Schum &Thonn) Roberty	Omlela	H	L	Decoction	RN 24
	Guizotia scabra (Vis.) Chiov	Echihongosheija	H	L	Decoction	RN 25
	Senecio spp	Ekikarabwe	H	L	Decoction	Voucher not collected
	Vernonia glabra (Steetz) Vatke	Msangusangu	H	L	Decoction	4664
Convolvulaceae	*Ipomoea rubens* Choisy	Kataba	CL	L	Decoction	RN 26
Cucurbitaceae	*Momordica foetida* Schum.	Orwihula	SH	WP	Decoction	RN 27
	Alchornea cordifolia (Schum & Thonn) Müell. Arg	Omujululuzi	SH	L	Decoction or young fresh leaves pounded then mixed with water, taken orally	RN 28
	Sapium ellipticum (Hochst.) Pax	Omushasha	T	L, SB	Decoction of leaves or stem bark. Fresh leaves can be used to prepare cold infusion	RN 29
Euphorbiace	*Phyllanthus nummulariifolius* Poir	Karungi	H	AP	Decoction	RN 30
	Ricinodendron heudelotii (Baill) Pax	Kabaka njagala	SH	L	Decoction	RN 31
	Croton macrostachyus Dell	Omwowa	T	SB	Decoction	RN 32
	Bridelia micrantha (Hochst.) Bail	Omushamako	T	R,SB	Decoction	RN 33
	Acalypha indica L.	Obweya	H	L, S	Decoction	RN 34
	Canna indica L.	Maruru	H	S	Seeds grounded, powder used to make warm infusion.	RN 35
	Erythrina abyssinica D.C	Omulinzi	T	SB	Decoction	RN 36
Fabaceae	*Cassia didymobotrya* Fress	Omulembelembe	SH	L	Decoction	RN 37
	Desmodium salicifolium (Poir) DC	Batengeliange/Omukongoranwa	H	AP, L	Decoction	RN 38

Table 1. Cont'd.

	Tephrosia aequilata Bak	Endalabugazi	H	WP	Decoction	Voucher not collected
	Kotschya africana Endl.	Ekyangwe ekiango	H	AP	Decoction	RN 39
	Eriosema parviflorum E. Mey	Mshelere	H	L	Decoction or fresh leaves squeezed to get juice, taken orally	RN 40
	Dalbergia malangensis E.P Sousa	Omugorora	WC	L	Decoction	RN 41
	Macrotyloma axillare (E. Mey) Verdc	Akaihabukuru	CL	AP	Decoction	RN 42
	Indigofera arrecta A. Rich	Omusoroka	H	AP	Decoction or aerial parts pounded then mixed with water, taken orally.	RN 43
	Abrus precatorius L.	Karigoligo	CL	L	Fresh leaves pounded then mixed with water, taken orally.	RN 44
	Erythrina schliebenii Harms	Mlindimila	T	SB	Decoction	4661
	Erythrina sacleuxii Hua	Mlindimila	T	SB	Decoction	4662
Graminae	*Pennisetum purpureum* Schum	Olutete	G	L	Decoction	RN 45
	Vossia cuspidata (Roxb) Grift	Ekishararago	G	L	Decoction	RN 46
	Cymbopogon citratus L.	Mchaichai	G	L	Hot infusion	RN 47
Labiatae	*Platostoma africanum P.* Beauv.	Nyanjaeyera	SH	AP	Dry powder used to make warm infusion	RN 48
	Leonotis nepaetifolia (L.) R. Br	Ekitatelante	H	L	Decoction	RN 49
	Ocimum kilimandscharicum Gürke	Kaswagara	H	S	Powder from dry seeds used to make warm water infusion	RN 50
Loganiaceae	*Anthocleista grandiflora* Gilg	Mgabaigana	T	L, R, SB	Decoction	RN 51
	Strychnos spinosa Lam.	Orurema	SH	L	Dry powder used to make warm infusion or decoction bathed to children	Voucher not collected
Lythraceae	*Lawsonia inermis* L.	Eina	H	L, S	Leaf decoction. Powdered seeds used to make warm infusion	RN 52
Melastomataceae	*Dissotis rotundifolia* (Sm) Triana	Obwehehe/Obwee	H	AP	Decoction or fresh aerial parts pounded then mixed with clean water, taken orally	RN 53
	Melastomastrum capitatum (Vahl) A. & R. Fern)	Katuntun	H	AP	Fresh aerial parts pounded then mixed with clean water. Dry aerial parts used to prepare warm infusion	RN 54

Table 1. Cont'd.

	Dissotis melleri Hook.f.	Ekituntun/Etuntun	H	AP	Aerial parts pounded then mixed with water, taken orally	RN 55
	Melastomastrum segregatum (Benth) A.& R Fern.	Eitulu	H	AP	Decoction or cold infusion.	RN 56
	Dissotis brazzae Cogn	Bulitulo	H	AP	Decoction	RN 57
	Pilea holstii Engl.	Omufura/Eimyo	SH	L	Fresh leaves squeezed then mixed with water, taken orally.	RN 58
	Trichilia emetica Vahl.	Omushunguti, Mushunguti	T	SB, L	Decoction	RN 59
Meliaceae	*Pseudobersama mosssambicensis* (Sim) Verdc	Omusiibi	T	SB, L	Decoction	Voucher not collected
	Azadirachta indica A. Juss.	Mwarobaini	T	L	Decoction	RN 60
Melianthaceae	*Bersama abyssinica*	Omujalya	SH	R, SB, L	Decoction	RN 61
Moraceae	*Antiaris toxicaria* (Pers) Lesch	Omujuju	T	SB, L	Decoction	RN 62
Moringaceae	*Moringa oleifera* (Lam.)	Mlonge	T	L	Decoction. Dry powder used to make warm infusion	RN 63
Myristicaceae	*Pycnanthus angolensis* (Welw.) Warb	Omunonoba	T	SB	Decoction	RN 64
Myrsinaceae	*Maesa lanceolata* Forsk	Omuzilanyama/ Omuhanga	T	RB, SB, L	Decoction	RN 65
	Syzygium guineense (Willd.) DC	Omuchwezi	T	L	Decoction	RN 66
	Syzygium cordatum Krause	Omugege	SH	SB, L	Fresh leaves or stem barks grounded then mixed clean with water, taken orally	RN 67
Myrtaceae	*Syzygium cumini* (L.) Skeels	Mzambarau	T	SB, L, F	Decoction of stem bark or leaf. Ripe fresh fruits eaten	RN 68
	Psidium guajava L.	Mpera	T	L	Fresh leaves pounded then mixed with clean water, used orally	RN 69
Onagraceae	*Ludwigia octovalvis* (Jacq.) Haven *ssp. brevisepala* (Brenan) P.H. Raven	Wejunge	H	L	Decoction	RN 70
Palmae	*Raphia farinifera* (Gaertn) Hyl.	Omubobo	T	ST, R	Decoction	Voucher not collected

Table 1. Cont'd.

Polygonaceae	*Polygonum senegalense* Meisn	Kinyanyanja	H	AP	Decoction or aerial parts pounded then mixed with water, taken orally.	RN 71
	Rumex abyssinica	Akanulilizi	H	AP	Aerial parts pounded then mixed with water, taken orally	RN 72
Punicaceae	*Punica granatum* L.	Omukomamanga	T	F	Outer part of the fruits dried then powdered. Powder used to make warm infusion	RN 73
Rosaceae	*Eriobotrya japonica* (Thunb.) Lindl	Musharazi/Omusharazi	T	R, SB, L	Root decoction. Powdered dry stem barks and leaves used to prepare warm infusion. Fresh fruits eaten.	RN 74
Rubiaceae	*Spermacoce princeae* (K. Schum) Verdc	Ekaiza nkoju	H	AP	Decoction	RN 75
	Psydrax parviflora (afzel) Bridson ssp. *Rubrocostata* (Robyn) Bridson	Omushangati	T	SB, L	Decoction of SB or leaves. Fresh leaves squeezed to get juice.	RN 76
	Tricalysia coriacea (Benth.)Hiern	Omushekera	SH	L, F	Fresh leaves pounded then mixed with clean water or fresh leaves boiled	RN 77
	Vangueria infausta Burch	Mtugunda, Amabungo	SH	L	Decoction	Voucher not collected
	Rytigynia obscura Robyns	Omulokola/Lulokola	SH	L	Decoction	RN 78
	Oxyanthus speciosus DC	Omwanikibira	T	L	Decoction	RN 79
	Pentas bussei (K. Krause)	Rusharila kibira	H	AP	Decoction	RN 80
	Pavetta lynesii Bridson	Orwingula, Omuingula	SH	L	Decoction	RN 81
	Chassalia umbraticola Vatke	Mwataibare	SH	SB, L	Decoction	RN 82
	Hallea rubrostipulata (K. Schum) J.F. Leny	Mchunguchugu	T	SB, R	Decoction	4663
Rutaceae	*Zanthoxylum chalybeum* Engl.	Omutaregwairungu	T	SB	Decoction	RN 83
	Toddalia asiatica (L.) Lam	Orukwatango	CL	L	Decoction	Voucher not collected
	Teclea nobilis Delile	Omuzo	T	R	Decoction	RN 84
Sapindaceae	*Lecaniodiscus fraxinifolius* Bak	Omwasha	T	L	Decoction	Voucher not collected
Solanacaeae	*Physalis peruviana* L.	Kitutun kikubwa	H	L	Fresh leaves pounded then mixed with water, used orally	RN 85

Table 1. Cont'd.

	Datura stramonium L.	Ekitaigwa, Amalulu	SH	S	Decoction	RN 86
Sterculiaceae	*Dombeya shupangae* (K. Schum)	Omutangarara, Mtangarara	T	L	Decoction	RN 87
Ulmaceae	*Trema orientalis* Bullock	Omuhuwe	T	SB, L	Dry powder used to prepare warm infusion	Voucher not collected
Umbelliferae	*Centella asiatica* (L.) Urb	Mbatama	H	WP	Decoction	RN 88
Verbenacea	*Clerodendrum cephalanthum* Oliv	Ekishekesheke	WC	L	Decoction	RN 89
	Lantana camara L.	Lukulata	SH	L	Decoction	RN 90
	Lantana trifolia L.	Omuhuchi	SH	AP	Decoction	RN 91
Zingiberaceae	*Aframomum angustifolium* (Sonn.) K. Schum	Orushasha	SH	L	Cold infusion	Voucher not collected
	Costus afer Ker-Gawl	Ekigagi	H	R, AP	Decoction or eaten raw	RN 92

Plant part: R = root, ST = Stem, SB = Stem bark, AP = Aerial parts, L = Leaves, F = Fruits, S = Seeds, WP = Whole plant. Nature of the plant: SH = Shrub, H = Herb, T = Tree, CL = Climber, WC = Wood climber, G = Grass

Table 2. *In vitro* antiplasmodial activity of 80% ethanol crude extracts at 100 µg/ml against *P. falciparum* Dd2 strains.

Plant Family	Plant species	Part tested	Percentage growth inhibition rate (% IR) of crude extracts at 100 µg/ml on *P. falciparum* Dd2 strain
Acanthaceae	*Acanthus pubescens* (Oliv.)	R	41.50 ± 6.32
Apocynaceae	*Funtumia africana* (Benth) Staff	SB	17.51 ± 8.07
	Funtumia africana (Benth) Staff	L	14.21 ± 2.74
Burseraceae	*Canarium schweinfurthii* Engl.	SB	61.94 ± 15.61
Celastraceae	*Salacia lovetii* N. Halle & B. Mathew	L	32.35 ± 3.50
Compositae	*Guizotia scabra* (Vis.) Chiov	WP	49.09 ± 0.03
	Aspilia mosambicensis (Oliv.) Wild	AP	69.34 ± 7.05
	Aspilia natalensis (Sond) Wild	AP	65.23 ± 0.25
	Vernonia glabra (Steetz) Vatke	L	12.44 ± 1.18
Convolvulaceae	*Ipomoea rubens* Choisy	L	27.61 ± 1.83

Table 2. Cont'd.

Euphorbiaceae	*Bridelia micrantha* (Hochst.) Bail	SB	71.87 ± 1.53
	Phyllanthus nummulariifolius Poir	WP	38.88 ± 7.83
	Phyllanthus nummulariifolius Poir	WP	51.31 ± 12.84[a]
Fabaceae	*Erythrina schliebenii* Harms	SB	39.86 ± 13.97
	Dalbergia malangensis E.P Sousa	L	39.78 ± 7.88
	Dalbergia malangensis E.P Sousa	ST	32.37 ± 8.49
	Macrotyloma axillare (E. Mey) Verdc	AP	33.21 ± 1.37
	Desmodium salicifolium (Poir) DC	AP	68.41 ± 13.33
	Erythrina sacleuxii Hua	SB	42.08 ± 5.49
Labiatae	*Leonotis nepaetifolia* (L.) R. Br	AP	54.43 ± 9.07
Loganiaceae	*Anthocleista grandiflora* Gilg	SB	9.18 ± 6.77
Melastomataceae	*Melastomatrum capitatum* (Vahl) A. & R. Fern)	AP	39.06 ± 3.47
	Dissotis brazzae Cogn	AP	52.31 ± 0.55
	Dissotis rotundifolia (Sm) Triana	AP	33.64 ± 0.44
Moraceae	*Antiaris toxicaria* (Pers) Lesch	L	34.72 ± 6.25
	Antiaris toxicaria (Pers) Lesch	SB	61.18 ± 2.02
Melianthaceae	*Bersama abyssinica*	SB	86.67 ± 11.32
Myristicaceae	*Pycnanthus angolensis* (Welw.) Warb	F	65.43 ± 9.62
	Pycnanthus angolensis (Welw.) Warb	SB	40.63 ± 8.10
	Pycnanthus angolensis (Welw.) Warb	L	28.63 ± 5.07
Myrsinaceae	*Maesa lanceolata* Forsk	L	53.46 ± 1.86
Myrtaceae	*Syzygium cordatum* Krause	SB	55.46 ± 13.43
Rosaceae	*Eriobotrya japonica* (Thunb.) Lindl	L	20.52 ± 3.35
Rubiaceae	*Hallea rubrostipulata* (K. Schum) J.F.Leny	R	64.54 ± 7.56
	Hallea rubrostipulata (K. Schum) J.F.Leny	SB	53.22 ± 5.58
	Pentas bussei (K. Krause)	AP	59.92 ± 4.41
	Oxyanthus speciosus DC	L	29.19 ± 9.66
	Rhytignia obscura Robyns	L	22.35 ± 5.42
	Artemether (1.25 µg/ml)		91.98 ± 10.46

WP= whole plant; L= leaves; SB= stem bark; ST= stem; AP= aerial parts (stem plus leaves); R= root; F= fruits [a]= aqueous extract

activity with IC50 = 1.6 µg/ml against P. falciparum clinical isolates (Katuura et al., 2007). Most plants tested in this study showed low parasite growth inhibition rate. It is not easy to identify the specific reasons for low activity but factors such as the solvent used for extraction, the method of preparation, storage conditions, and variation in the active constituents due to seasonal or geographical and model of testing may also reduce the efficacy of the extract (Weeneen et al., 1990). Furthermore, Chhabra et al. (1993) reported that preparations of medicinal plants can be used orally, rubbed into scarification, inhaled as fumes, splashed on the eyes, poured into the wound or sniffed. In this study we found that all preparations were administered orally in the form of decoction (boiled water extracts), infusion (hot water extract), juice or taken as raw fruits. In the oral route, the bioactive molecules are exposed to various barriers and enzyme systems before reaching the systemic circulation. This causes some bioactive molecules to be modified by metabolism thus, either enhance or reduce their antiplasmodial activity suggesting that the antiplasmodial activity of metabolically activated compounds may not be evident in *in vitro* assays.

Conclusion

This study reported 108 medicinal plants that are used in the traditional medicine for treatment of malaria and fevers in Kagera and Lindi regions of Tanzania. *In vitro* assays revealed substantial antiplasmodial activities of 15 plants out of 31 plant species tested. Although questionnaire based evidence suggested that decoctions from these plants were not acutely toxic, further toxicity testing will be required to establish their safety profile. Meanwhile these findings support the use of these plants for the traditional treatment of malaria. Further *in vitro* and *in vivo* screening supported by bioassay-guided isolation of active compounds of plants showing good safety margin are suggested.

Conflict of interests

The authors declare that they have no competing interests

ACKNOWLEDGEMENTS

This work was financed by Sida through MUHAS capacity building grants. Authors are very grateful to Sida for the financial support. We are grateful to Mr. Mohamedi Ngalanga, Mr. Didas Ngemera, Mr. Dominic Mushwahili, Mr. Buchadi Tibikunda and Mr. Papianus Rwechungura for showing us the medicinal plants traditionally used for treatment of malaria in their areas. We would like to extend our gratitude to Mr. Haji.O. Selemani (Botanist) for identification of the documented medicinal plants. Extraction of the plant extracts was done at the Institute of Traditional Medicine in Tanzania whereas *in vitro* antimalarial study was done at the Research Foundation in Tropical Diseases and Environment, and at the Biotechnology Unit, University of Buea in Cameroon. We are grateful to MR4/BEI-resources (Manassas, VA, USA) for donating malaria parasites used for this work.

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Vol. 9(6), pp. 193-198, 10 February, 2015
DOI: 10.5897/JMPR2014.5456
Article Number: 26136B850879
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Antioxidant evaluations of polar and non-polar fractions of *Cajanus cajan* seeds

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Received 23 April, 2014; Accepted 11 November, 2014

***Cajanus cajan* (L) Millsp., named Arhar in Hindi and Pigeon pea in English, is an important grain crop of rain-fed agriculture in semi-arid tropics, belonging to family Leguminosae. It is a food and a forage crop, rich in proteins with amino acids like methionine, lysine and tryptophan. Extensive studies on chemical composition of *C. cajan* have been done during last few decades. This article presents an overview of phytochemicals as a source of natural antioxidants in *C. cajan* seed extracts. The antioxidant potentials of the different fractions, that is, *n*-hexane, ethyl acetate, chloroform, methanol, *n*-butanol and aqueous, of *C. cajan* seeds were screened by using $ABTS^{\cdot+}$ assays, ferric reducing antioxidant power (FRAP) assay, total phenolic contents determination (TPC), total flavonoid contents (TFC) and metal chelating activity. Aqueous extract shows the highest trolox equivalent antioxidant capacity (TEAC) value, 140.69±0.34mM in $ABTS^{\cdot+}$ assays and the highest total phenolic content (TPC) value, 927.5±0.8 gallic acid equivalent (GAE). Hexane soluble fraction showed the highest percentage of metal chelating activity, that is, 79.0±0.5% bound iron and the highest FRAP value 49.08±0.55 g/ml, whereas *n*-butanol soluble fraction indicated the highest total flavonoid contents as 1691.1±0.2 mg/g QE.**

Key words: Leguminosae, *Cajanus cajan*, antioxidant potential, $ABTS^{\cdot+}$ assay, ferric reducing antioxidant power (FRAP) assay, total phenolic contents, total flavonoid contents, metal chelating activity.

INTRODUCTION

Human beings have been using plants for medicinal purposes throughout the history and even now are extensively using plant materials for preparing phyto-pharmaceuticals. Plants produce a vast array of secondary metabolites as a defense against environmental stress while same compounds can be used for pest control and healing wounds or injuries (Pal et al., 2006, 2007, 2011). The role of medicinal plants in disease prevention and control is due to the presence of antioxidant properties of their polyphenolic constituents, namely, flavones, flavonoids, isoflavones, anthocyanin, lignans, coumarin, catechins and isocatechins (Aqil et al., 2006) that are amphipathic in nature (Abbasi et al., 2010). These compounds are commonly found in both edible and nonedible plants and have multiple applications in food, cosmetics and pharmaceutical industries (Kahkonen et al., 1999). Free radicals such as hydroxyl radicals $(OH)^{\cdot}$ and superoxide anion $(O_2)^{\cdot-}$, reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), are produced

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as normal products of cellular metabolism (Young and Woodsid, 2001). Mitochondrion is the chief site of metabolism. Rapid production of free radicals can lead to oxidative damage to biomolecules including proteins, lipids, purines, pyrimidine bases and sugar moiety of nucleic acid (Valko et al., 2007). Free radicals contribute to more than hundred disorders in human beings including atherosclerosis, arthritis and ischemia, reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (Kumpulainen and Salonen, 1999).

Due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress cause free radicals depletion of immune system, change in expression and induce abnormal proteins (Halliwell, 1994). Synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone and gallic acid esters have been suspected to produce negative health effects. Moreover, these show low solubility hence their application is strongly restricted and there is a trend to substitute them with naturally occurring antioxidants (Branen, 1975).

This study was conducted to evaluate the antioxidant activities of *C. cajan* seed extract from different polarities. Because of the stability of radical cations under acidic, basic, neutral or in buffer solutions, assays are carried out under different reaction conditions. The early history of modern medicine contains description of plant derived phytochemicals, many of which are still in use (Pal et al., 2009, 2005; Mohammad et al., 2009).

C. cajan is a perennial plant, belonging to an important family Leguminoseae/Fabaceae. Other common names are red gram, Congo pea and no eye pea (Wu et al., 2009). The cultivation of Arhar goes back at least 3000 years. The center of origin is most likely Asia, from where it travelled to East Africa and then to America. This family is the third largest family amongst flowering plants. It is an erect, branched, hairy shrub, 1 to 2 m high. It is a multipurpose plant as its seeds are extensively used as dal. It is rich in proteins.

In India, its leaves are used for rearing silkworms, green pods are used as vegetable, husk. Green leaves and tops are used as fodder and green manure (Ambasta, 2004). Plants belonging to this family have high medicinal values. Infusion of leaves of *C. cajan* is used for curing anemia, hepatitis and diabetes, urinary infections and yellow fever (Kamboj, 2000). It is used as pain reliever in traditional Chinese medicine and also as sedative (Ahsan and Aslam, 2009).

In this article, we report the *in vitro* antioxidant capacities of organic fractions and aqueous extracts of *C. cajan*. In order to discover potential sources of natural antioxidants, various estimation methods like ABTS radical cation scavenging activity, FRAP assay, total phenolic contents (TPC) and total flavonoid contents (TFC) methods were followed as against conventionally used standards (Pall et al., 2011).

MATERIALS AND METHODS

Plant collection

Pigeon pea seeds were collected from the local market of Faisalabad, Pakistan in April, 2012 and were identified by Taxonomist at Department of Botany, GC University Lahore, Pakistan. Voucher specimen was deposited (Voucher No GC.Bot.Herb.1395) in the same place.

Extraction and fractionation of plant

2.0 kg of seeds were shade dried ground into fine powder and exhaustively extracted with methanol (2 L×3) at room temperature. Three extracts were mixed and concentrated to obtain greenish yellow semi-solid methanolic extract. It was dissolved in about (1.5 L) distilled water and treated with *n*-hexane (1.5 L) to yield non polar fraction along with chloroform (1.5 L), ethyl acetate (1.5 L), and *n*-butanol (1.5 L) to obtain polar fractions. Organic fractions and aqueous fraction was then concentrated on a rotary evaporator.

Chemicals and standards

2,4,6-tripyridyl-s-triazine (TPTZ), Trolox, gallic acid, Follin Ciocalteu reagent and butylated hydroxytoluene were obtained from Sigma Chemical Company Ltd. (USA) and organic solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol), sulphuric acid, sodium phosphate ammonium molybdate, ferric chloride and ferrous chloride from Merck (Pvt.) Ltd. (Germany).

Phytochemical analysis of the plant extracts

Qualitative tests for the investigation of phytochemical components like terpenes, phenols, saponins, flavonoids, alkaloids, tannins, sugars and cardiac glycosides in *C. cajan* were detected by using the methods reported by Sofowora, Trease and Evans (Prieto et al., 1999; Sofowora, 1996; Treas and Evans, 2009). The presence of a particular secondary metabolite was estimated from the intensity of color.

Test for terpenes (Salkowski test)

The presence of terpenes in *C. cajan* was confirmed by performing two tests. In the first test, spots of different extracts was placed on the TLC card and it was then sprayed with ceric sulphate solution followed by heating up to 105°C in order to develop the spots on a TLC plate heater. The presence of terpenes was indicated by the appearance of brown colored spots on the card. In the second test, 2 ml of chloroform were added to 0.5 g of each of the fraction in a test tube followed by careful addition of concentrated H_2SO_4. Appearance of reddish brown color suggested the presence of terpenes (Salkowski Test).

Tests for phenols

To 0.5 g of each of plant fraction, neutral solution of $FeCl_3$ was added; appearance of bluish-green color indicated the presence of phenols.

Tests for saponins

Saponins can be detected by vigorous shaking of 0.5 g of plant fraction with 0.5 ml distilled water; development of persistent froth

indicated the presence of saponins. Formation of an emulsion on shaking after the addition of few drops of olive oil further confirmed their presence.

Tests for flavonoids

Two methods were applied for the determination of flavonoids. In the first method, 10% of ammonia solution was added to 5.0 ml of each plant fraction followed by 1.0 ml of concentrated H_2SO_4. Appearance of yellow coloration which disappeared on standing indicated the presence of flavonoids. In the second method, appearance of persistent yellow color on addition of 1% aluminum chloride to different plant fractions indicated the presence of flavonoids.

Tests for alkaloids

Alkaloids were tested for their presence by conventional methods. Spots of different fractions were placed on a piece of TLC card followed by drying and spraying with Dragon Dorff's reagent. Development of reddish-brown color indicated the presence of alkaloid. Also addition of Mayer's reagent to a small portion of each fraction, resulted in cream colour precipitates, confirmed the presence of alkaloids.

Test for tannins

Tannins were indicated by boiling 0.5 g of each fraction in 10 ml of distilled water. As after filtration few drops of 1% $FeCl_3$ solution were added and appearance of brownish-green or bluish-black coloration confirmed the presence of tannins.

Tests for reducing sugars

To 0.5 ml of the sample fraction, 0.5 ml of Fehling solution A and B were added; reaction mixture was strongly heated for about 1 min. Appearance of the red precipitates confirmed the presence of reducing sugars.

Tests for cardiac glycosides

A violet or brown colored ring formation showed the presence of cardiac glycosides when 1 ml of glacial acetic acid was added along with a drop of $FeCl_3$ followed by the addition of 1.0 ml of concentrated sulphuric acid.

Antioxidant assays

The following assays were performed to determine the antioxidant activity. Results were recorded as shown in Table 1.

$ABTS^{+}$ Assay

Total antioxidant activity was measured in terms of Trolox Equivalent Antioxidant capacity (TEAC) method described by Re et al. (1999). 7 mM solution of ABTS was prepared in double distilled water with 2.45 mM potassium persulphate to generate $ABTS^{+}$. $ABTS^{+}$ solution was diluted with TBS buffer at pH 7.4 to obtain an absorbance of 0.70±0.02 at 734 nm. 10 µl of sample was added to 2.29 ml of diluted solution of $ABTS^{+}$. Absorbance reading was taken exactly after 1 min of initial mixing up to 6 min at 30°C. The percentage inhibition of absorbance at 734 nm was determined using the formula:

Age inhibition (at 734nm) (%) = $(1-A_f/A^0) \times 100$

A^o is the absorbance of ABTS radical cation and A_f is the absorbance after sample addition.

Ferric reducing antioxidant power

Twenty five milliliters of 300 mM acetate buffer with pH 3.5 and 2.5 ml of 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3$ (2.5 ml) were mixed to prepare the FRAP reagent. To 100 ml of each sample, 3 ml of FRAP reagent and 300 µl of distilled water was added and the absorbance was taken at 593 nm. FRAP values were calculated from standard curve of Iron (II) Sulphate (Benzie and Strain, 1996).

Total phenolics contents

Forty microliters from each of the sample were mixed with 3.16 ml of distilled water and 200 µl of 0.2 N Follin Ciocalteu reagent. 600 µl of super saturated sodium carbonate (75 g/L) was added and solution turned blue after 8 min. Absorbance was measured at 765 nm after incubation with intermittent shaking at 40°C for 30 min. Total phenolic contents were calculated from the standard curve expressed as mg/g equivalents of gallic acid (GAE) (Slinkard and Singleton, 1997).

Total flavonoid content determinations

TFC contents of various fractions were determined by the standard method (Dewanto et al., 2002). 0.25 ml of plant sample and quercetin standard solution was mixed with 1250 µl of distilled water in a test tube with addition of 75 µl of $NaNO_2$ (0.5 M), after 5 min. 0.5 ml of 1 M NaOH solution was added and volume was raised to 2.5 ml by adding distilled water. Absorbance was measured at 510 nm. TFC contents were determined from the standard curve expressed as milligrams of quercetein equivalents per gram of sample.

Metal chelating activity

The reaction mixture was prepared by adding 100 ml of sample in 0.05 ml of 2.0 mM $FeSO_4$ and 0.2 ml of 5 mM ferrozine and total volume was made up to 4 ml with double distilled ethanol, after 10 min absorbance was noted at 562 nm (Dinis et al., 1994). Percentage of bound iron was calculated using the formula in terms of EDTA standard.

Bound iron (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$

RESULTS

Phytochemical screening to find out the biologically active constituents confirmed the presence of terpenoids, phenols, saponins, flavonoids, alkaloids and reducing sugars. Results revealed that chloroform, ethyl acetate, *n*-butanol, *n*-hexane soluble fractions, methanolic and aqueous extracts were rich in terpenes, phenolics, flavonoids, saponins and reducing sugars with traces of

Table 1. Phytochemical screening results of *Cajanus cajan*.

Phytochemical	Methanolic fraction	n-hexane fraction	Chloroform fraction	Ethyl acetate fraction	*n*-butanol fraction	Aqueous fraction
Terpenes	++++	++	+++	+++	+++	++++
Phenols	+++	++	+++	+	+++	++++
Saponins	++	+++	++	+	+	-
Flavonoids	++	+++	+++	+++	++++	+
Alkaloids	+	++	+++	-	-	-
Tannins	+	+++	-	-	+++	-
Red. Sugrs	-	+	+++	++++	+	++
Card.glyc	-	-	-	-	-	-

Red. Sugrs = Reducing sugars, Card. glyc = Cardiac glycosides, Fr. = Fraction. ++++ Very strong; +++ Strong; ++ Medium; + Poor presence; - Absence. Experiments repeated three times for each treatment. Classification was based on observation of color intensity and amount of precipitates.

Table 2. Antioxidant potential of different fractions.

Plant sample	ABTS	FRAP value TE	TPC	FC	Metal chelating activity
	(TEAC M) ±S.E.M.	(µM/mL) ±S.E.M.	(GAE mg/g of extract) ± S.E.M.	(mg/g QE) ± S.E.M.	Bound iron (%)
Crude methanolic extract	109.07±0.2	49.08±0.5	510.12±0.2	968.27±0.1	79.00
n-hexane soluble fraction	89.54±0.4	11.92±0.2	320.01±0.1	1075.50±0.7	44.30
Chloroform soluble fraction	109.00±0.3	19.12±0.1	475.0±0.3	1441.0 ±0.3	09.26
Ethyl acetate soluble fraction	89.52±0.3	16.60±0.5	282.50±0.6	1387.50±0.2	15.46
n-butanol soluble fraction	103.00±0.1	09.08±0.2	430.80±0.4	1691.10±0.2	85.40
Aqueous extract	140.69±0.3	44.08±0.1	927.50±0.8	252.80±0.4	15.86

tannins. Considerable amounts of alkaloids were detected in chloroform fraction but cardiac glycosides were totally absent. Crude methanolic extract was found to contain tannins, flavonoids, terpenoids, phenols, saponins and alkaloids. Aqueous fraction contained good amounts of terpenoids and phenols along with reducing sugars, but lacked in tannins, saponins, alkaloids and cardiac glycosides.

Antioxidant evaluations were performed by using the conventional methods. $ABTS^{+}$ scavenging activities assay was calibrated by using an alpha Tocopherol analog 'Trolox' as standard. The results of $ABTS^{+}$ were expressed in terms of TEAC values as shown in Table 2. TEAC is a measure of effective antioxidant activity of the substance and stands for "Trolox equivalent antioxidant capacity". High TEAC value representing high $ABTS^{+}$ scavenging shows a greater antioxidant potential of the sample. Aqueous fraction showed the highest activity with methanolic and chloroform fractions showing slightly less activity. *n*-butanol fraction indicated greater activities and *n*-hexane and ethyl acetate fractions showed moderate activities. The TEAC values showed the following trend: aqueous extract > methanol > chloroform > *n*-butanol > ethyl acetate = *n*-hexane.

Ferric reducing antioxidant power assay involved the reduction of ferric tripyridyltriazine $Fe(III)\text{-}(TPTZ)_2Cl_3$ (pale yellow in color) to blue coloured ferrous Fe (II)-$(TPTZ)_2Cl_2$ complex at 593 nm absorbance on reacting with an antioxidant. Results were expressed in FRAP units. A higher value referred to a high reducing power as shown in Table 2. The absorbance reading was taken just 6 min interval after mixing TPTZ to the sample. FRAP value was the highest for aqueous extract, slightly less in ethyl acetate fraction but *n*-hexane and chloroform fraction showed moderate activities. Phenolic contents of the plant materials were suggested to correlate directly with antioxidant activities and also play an important role in stabilizing lipid oxidation. Order of reactivity is found to be methanol > aqueous fraction > chloroform > ethyl acetate > *n*-hexane > *n*-butanol.

The aqueous fraction was found to have the highest phenolic contents while ethyl acetate extract had the lowest phenolic contents relative to butylated hydroxy-toluene taken as a reference standard having total antioxidant activity 0.928±0.09. The most powerful antioxidants are phenolic compounds having hydroxyl group in their structures and they can be detected by TPC assay. Results as shown in Table 2 represent the

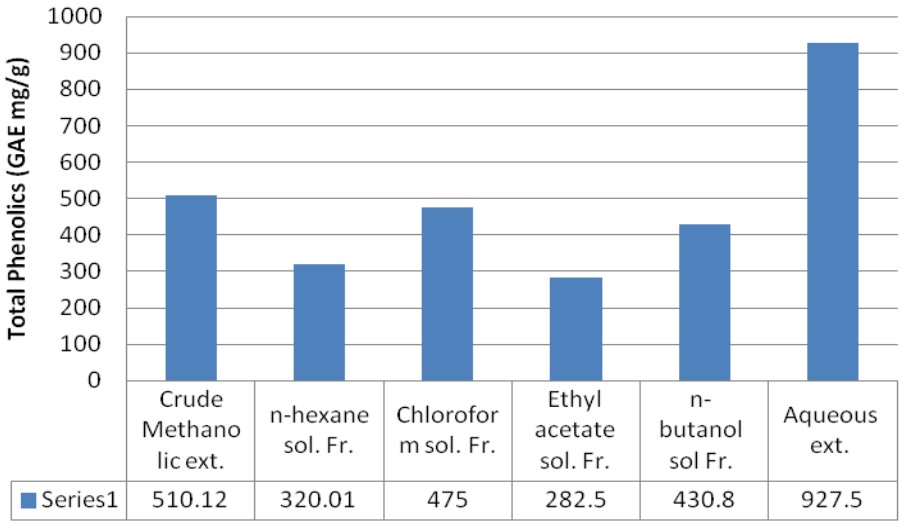

Figure 1. Total Phenolic Contents in *C. cajan*. Ext.: Extract; sol.: Soluble; Fr.: Fraction.

total phenolics in different fractions found in the order: aqueous fraction > methanolic extract > chloroform > *n*-butanol > *n*- hexane > ethyl acetate.

The principle for the determination of total flavonoids is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-4 or C-5 hydoxyl group of flavones or flavonoles. In addition, aluminum chloride forms acid labile complexes with the ortho-dihydroxyl group in the A- or B-ring of flavonoids with peak absorption at 400 nm. Table 2 represents the total flavonoid contents and observed order of reactivity of fractions: *n*-butanol > chloroform > ethyl acetate > *n*-hexane > methanolic extract > aqueous fraction.

Metal ion chelating activity results were expressed in percentage of bound iron. Iron (II) formed a colored complex with ferrozine which can be determined at 562 nm. *n*-butanol fraction had shown a maximum value of percentage bound iron. The other fractions show activity in order *n*-butanol > methanolic extract > n-hexane > aqueous extract > ethyl acetate > chloroform.

DISCUSSION

Results of ABTŚ+ assays were expressed in terms of TEAC values, that is, measure of effective antioxidant activity of a substance expressed as Trolox equivalent antioxidant capacity (Dinis et al., 1994). ABTŚ+ assays result revealed that aqueous extract and chloroform fraction of *C. cajan* has good antioxidant activity having 140.69 and 109.07 TEAC, respectively. Both extracts are rich in phenol concentration but maximum in aqueous extract, while chloroform extract contain fair amount of flavonoids and saponins as well and probably responsible for these activities.

FRAP assays results are significant in case of methanolic extract 49.08µM/ml and aqueous extract 44.08 µM/ml and insignificant for n-hexane fraction 11.92 µM/ml. Methanolic and aqueous extracts of the plant under consideration are equally important. Total phenolic contents are suggested to correlate directly with antioxidant activities as polyphenolic compounds play an important role in stabilizing lipid peroxidation. Phenolic compounds are the powerful antioxidants having hydroxyl group in their structures (Ivanova et al., 2005). On the basis of results presented in Table 2, total phenolic contents are excellent in aqueous fraction of the said plant material having a value of 927.5±0.8 GAE mg/g. Results are also expressed in Figure 1. So the seeds of *C. cajan* are a good source of phenolic compounds.

Flavonoids are recognized as potent antioxidants due to their phenolic hydroxyl group constitution. They can delay or inhibit oxidation process of lipids by inhibiting the initiation or propagation of oxidative chain reactions known as primary antioxidants. Total flavonoid contents of different polar and non-polar fractions ranges from 1691 to 1075 for n-butanol and n-hexane fractions, respectively. These results are an evidence for a good yield of total flavonoid contents of the seeds of *C. cajan*.

Iron is known to generate free radicals through the Fenton and Haber-Weiss reaction. Metal ion chelating activity of an antioxidant molecule prevents oxy-radical generation. As a result, oxidative damage also reduces the concentration of the catalyzing transition metal. High percentage of bound iron exhibited by n-butanolic fraction and methanolic extracts of *C. cajan* seeds suggested the high antioxidant potentials.

Conclusion

Screening of polar and nonpolar seed fractions of *C. cajan* revealed the presence of a beautiful aggregation of phytochemicals which are responsible for the importance

of this plant as a good source of natural antioxidants.

It was concluded that ethyl acetate soluble fraction, methanolic and aqueous extracts are rich in terpenes, phenols, flavonoids, tannins, saponins and cardiac glycosides. Ethyl acetate soluble fraction showed good activity due to the presence of many bioactive compounds whereas methanol soluble fraction showed moderate to good activity showing that these fractions are also valuable sources of bioactive compounds as well as good antioxidants. Alkaloids are also present in appreciable amounts and extracted.

Oxidative stress is understood a major cause of developing a number of diseases like Alzheimer's disease (Nunomura et al., 2006), Parkinson's disease (Wood-Kaczmar et al., 2006), rheumatoid arthritis (Hitchon and El-Gabalawy, 2004) and neuro degenaration in motor neuron disease (Cookson and Shaw, 1999). Fortunately, seeds are the edible part of plant, it is the easiest and cheapest way to obtain the natural antioxidants by taking them as a dietary component. Due to the presence of antioxidants properties in their constituents, this plant may play an important role in disease prevention and control without any side effects and toxicity as compared to synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone and gallic acid esters have been suspected to produce negative health effects (Branen, 1975). Moreover, low solubility of synthetic antioxidants restricted their application. The use of Pigeon pea seeds in our food plan may play an important role in disease prevention and control because of its valuable antioxidants properties.

Conflict of Interest

Authors have not declared any conflict of interest.

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