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Full Length Research Paper

Hepatoprotective effect of *Amaranthus hypochondriacus* seed extract on sodium arsenite-induced toxicity in male Wistar rats

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The hepatoprotective effect and antioxidant activity of ethanol extract of *Amaranthus hypochondriacus* seed (ESAH) were evaluated in sodium arsenite (SA) treated rats. Animals were divided into eight groups. The first group was used as control, group II was administered SA (2.5 mg/kg body weight). Groups III, IV and V were administered different doses of ESAH (100, 200 or 300 mg/kg b.w., respectively); groups VI, VII and VIII were administered ESAH at 100, 200, 300 mg/kg b.w. plus 2.5 mg SA/kg; respectively. Rats were orally administered different doses of ESAH daily for fourteen days. The SA was administered on the 7th and 14th day. Results showed that sodium arsenite-induced elevation of alanine aminotransferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) levels were substantially reduced by pre-treatment with 200 and 300 mg/kg b.w. of ESAH. Histopathological observations also showed hepatic protective activity of ESAH. Sodium arsenite increased the concentrations of malondialdehyde (MDA), hydrogen peroxide and reduced the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and increased frequency of micronucleated polychromatic erythrocytes. Pre-treatment with 200 and 300 mg/kg of ESAH reduced the levels of MDA and H_2O_2; increased the activities of antioxidant enzymes (SOD, CAT, GPx) and reduced frequency of induction of micronucleated polychromatic erythrocytes (mPCEs) in bone marrow cells of the rats. Treatment with SA decreased body weight and increased relative liver weight compared to control and ESAH. Thus ESAH mitigates arsenic-induced hepatotoxicity and oxidative damage in rats.

Key words: *Amaranthus hypochondriacus*, antioxidant enzymes, hepatoprotective effect, sodium arsenite.

INTRODUCTION

Arsenic occupies number one position of hazardous substances in the Comprehensive Environmental Response Compensation and Liability Act (CERCLA), Agency for Toxic Substances and Disease Registry

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(ATSDR, 2007). Environmental and occupational exposure to arsenite has been linked with a range of harmful effects in both experimental animals and humans leading to various ailments and dysfunctions of vital organs such as liver, kidney and lung (Chowdhury et al., 2001; Odunola et al., 2011; Tseng et al., 2002; Waalkes et al., 2003). Exposure to arsenic and its compounds through underground waters has been associated with lesions of the skin, certain forms of cancers (Chiou et al., 2001; Jayanthika et al., 2001) and other health effects such as neurological disorders, damaged cognitive development in children (Parvez et al., 2006; Valentine, 1994) and immunotoxicity (Patrick, 2003). Sodium arsenite is widely used as a component of herbicides, fungicides, insecticides and algaecides, and in the manufacture of arsenical soap (Chen et al., 2006; Cullen, 2008). Incidence of arsenic contamination posing serious threat to public health has been reported in various countries like India, Poland, Argentina, Bangladesh, Chile, China, India, USA, and Taiwan where it occurs in drinking water above the permissible upper limit of 10 ppm (Chatterjee et al., 1995; Das et al., 1995; Frost et al., 1993; IARC, 2004). Several studies have demonstrated that liver is the most important target organ for arsenic toxicity (Guha, 2005; Hughes et al., 2003). The liver regulates several important metabolic functions and the hepatic injury is associated with distortion of these metabolic functions (Wolf, 1999).

Plants are one of the most important resources for human foods and medicines. There has been strong evidence that intake of plant dietary factors improve health, as well as prevent diseases (Jian, 2007). Consumption of whole grain has been associated with reduced risk of chronic diseases (Bonaccio et al., 2012; Jones and Engleson, 2010; Lillioja et al., 2013). Other studies have demonstrated the protective role of diets high in grain against cancer (Nicodemus et al., 2001) and diabetes (Meyer et al., 2000). Antioxidant activity, mediation of hormones and enhancement of the immune system are the plausible physiological mechanisms health benefits exerted by grain phytochemicals (Lupton and Meacher, 1998). Substantial amount of antioxidants have also been discovered in grain-based cereal products (Baublis et al., 2000; Emmons et al., 1999; Handelman et al., 1999).

It has been proposed that the antioxidants found in grain-based foods may actually contribute to health by lowering and/or alleviating the occurrence of aging-related diseases such as cardiovascular disease, neurodegenerative disease and some kinds of cancer (Miller et al., 2002).

Amaranth (*Amaranthus hypochondriacus,* family: Amaranthaceae) is an underutilized pseudo cereal that has promising potential as an excellent source of nutrients and bioactive compounds (Escudero et al., 1999; Muyonga et al., 2008). Amaranth contains significant amounts of bioactive components such as protease inhibitors, phytosterols, squalene, amino acids, flavonoids and polyphenols (Berghofer and Schoenlechner, 2002; Taylor and Parker, 2002). Amaranth contains lysine, methionine and tryptophan that help prevent cold sores, reduce disease infection and boost the body's immune system (Bressani, 1989). Hepatoprotective activity of whole plant extract of *Amaranthus spinosus* (Zeashan et al., 2008) and *Amaranthus viridis* (Kumar et al., 2011) in experimental animals has been reported. The present study was aimed at evaluating the potential protective effects of ethanol extract of *A. hypochondriacus* seed against sodium arsenite-induced toxicity in liver of male rats.

MATERIALS AND METHODS

Chemicals

Sodium arsenite ($NaAsO_2$) was purchased from Sigma Chemical Company, St. Louis MO, USA. Sodium arsenite was orally administered to rats at a dose of 2.5 mg/kg b.w. corresponding to 1/10th of the LD_{50} sodium arsenite (Preston et al., 1987). Kits for alkaline phosphatase, aspartate amino transferase (AST), alanine amino transferase (ALT), and gamma glutamyl transferase (GGT) were obtained from Randox Laboratories Ltd, Crumlin, UK. Colchicine, giemsa stain and all other chemicals were of analytical grade and purchased from Sigma Chemical or BDH Chemical Ltd, Poole, England.

Source of plant material and preparation of the extracts

A. hypochondriacus (accession number PI590991) seeds were obtained from United States Department of Agriculture, Research Station (USDA-ARS) North Central Regional Plant Introduction Station (NCRPIS) in Ames, USA and planted at the experimental field of National Horticultural Research Institute (NIHORT), Ibadan, Nigeria, in June, 2010. At maturity, seeds were harvested, dried, milled into flour and extracted in 80% ethanol using a Soxhlet apparatus. The extract was concentrated at reduced temperature (40°C) using a rotary evaporator (Buchi, USA). The resulting molten extract was further lyophilized using a freeze dryer (Freezone 4.5, Labconco, USA) at high vacuum (133×10^{-3} mBar) with a yield of 3.91% (w/w). The dried extract was stored in air tight amber bottle and kept at -20°C.

Animals

Forty eight male albino rats weighing 120 to 150 g were used in this experiment. They were kept in departmental animal house in well cross ventilated room at 27 ± 2°C and 12 h light/12 h dark cycle. Animals were given commercial pellets (Ladokun Livestock Feeds, Ibadan, Nigeria) and water *ad libitum*. After two weeks of acclimatization, animals were randomly divided into eight treatment groups of six animals each. Group I received only distilled water and was used as control. Group II received 2.5 mg/kg b.w. of sodium arsenite (SA). Groups III, IV and V were administered ethanol extract of *A. hypochondriacus* seed (ESAH) at 100, 200 and 300 mg/kg body weight, respectively. Groups VI, VII and VIII were administered ESAH at 100, 200, 300 mg/kg body weight, respectively; plus SA at 2.5 mg/kg b.w. Rats were orally administered their respective doses of ESAH daily for 14 days, while SA was administered on the 7th and 14th day. At the end of

the experiment, body weights of rats were recorded.

Collection of samples

Animals were sacrificed by cervical dislocation 24 h after administration of the last dose of sodium arsenite following standard rules laid down by the University of Ibadan Ethics Committee on the treatment of experimental animals. Two hours prior to the sacrifice, the animals were intraperitoneally injected with 0.04% colchicine. Blood samples were collected by cardiac puncture and liver samples were harvested, rinsed in ice cold 1.15% KCl solution and weighed. The blood and liver samples were used for biochemical analyses and histopathological examination. Femoral bone marrow from each animal was collected and used for micronucleus assay.

Sample preparation for the assays

The blood was allowed to clot; serum was separated by centrifugation at 3000 × g for 10 min and used for the estimation of serum enzymes. Alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) activities were assayed using standard diagnostic kits from Randox Laboratories Ltd., UK. Femoral bone marrow cells were prepared according to the method of Salamone et al. (1980). The fixed cells on the mounted slides were scored under light microscope to detect the presence of micronucleated polychromatic erythrocytes (mPCEs), using tally counter to make scoring easier. Livers were weighed, minced and homogenized in four volume 0.01 M phosphate buffer (pH 7.4) using a Potter-Elvegin homogenizer. The homogenate was centrifuged at 20,000 × g for 20 min at 4°C using an ultracentrifuge (HERMLE LABNET Z 323K). The resulting supernatant was used for the assay of antioxidant parameters and protein content.

Evaluation of antioxidant parameters

Assessment of lipid peroxidation

Extent of lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) as described by Varshney and Kale (1990).

Determination of superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined by the method previously described by Misra and Fridovich (1972). The ability of superoxide dismutase to inhibit the auto-oxidation of adrenaline (epinephrine) in an alkaline medium (pH 10.2) to adrenochrome makes this reaction a basis for a simple assay for this dismutase. Epinephrine was added to the assay mixture containing tissue supernatant and the change in extinction coefficient was followed at 480 nm in a spectrophotometer.

Determination of catalase (CAT) activity

Catalase activity was determined according to the method of Aebi (1984). The method involves spectrophotometric monitoring the rate at which H_2O_2 is decomposed by the enzyme catalase at 240 nm.

Determination of glutathione peroxidase (GPX) activity

Hepatic GPx activity was determined spectrophotometrically according to the method of Rotruck et al. (1973). The absorbance was read at 412 nm against a blank.

Determination of hydrogen peroxide (H_2O_2) level

The level of hydrogen peroxide generated was determined as described by the method of Wolf (1994). This is based on the principle of ferrous oxidation with xylenol orange reagent. The colour development was measured spectrophotometrically at 560 nm.

Protein estimation

The protein content of the liver homogenate was determined according to the method Lowry et al (1951) using bovine serum albumin as a standard.

Histopathological studies

Liver tissues were fixed in 10% neutral buffered formalin. These tissues were processed for histopathological examination using a routine paraffin-wax embedding method. Sections of about 5 µm thickness were stained with haematoxylin-eosin dye. Photomicrographs of the stained tissue sections prepared on slides were taken with a camera attached to the compound light Nikon microscope at × 400 magnification.

Statistical analysis

Data were analysed by statistical program for the social sciences (SPSS) version 17 (SPSS, Chicago, IL, USA) and presented as mean ± standard deviation (S.D). Duncan's multiple range tests was used to compare the significance of differences at $p<0.05$ significance level.

RESULTS

Effect of treatment on body weight and relative liver weight

Results indicated that treatment with the three different doses of *A. hypochondriacus* seed extracts (groups III, IV and V) did not cause any significant changes in body and relative liver weights (Table 1). However, a significant ($p<0.05$) decrease in the percentage body weight and increase in relative liver weight was observed in rats treated with SA compared to control group. Pre-treatment with ESAH at 100, 200 and 300 mg/kg b.w. before administration of SA (groups VI, VII and VIII) did not cause any significant changes in body weight and relative liver weight when compared with control.

Effect of ethanol extract of *A. hypochondriacus* seed on sodium arsenite-induced micronucleated polychromatic erythrocytes (mPCEs)

Treatment with sodium arsenite induced significantly ($p<0.05$) formation of micronuclei in the polychromatic

Table 1. Percentage change in body weight of experimental animals administered ethanol extract of *A. hypochondriacus* seed (ESAH), mean liver weight and percentage mean relative liver weight.

Group	Treatment	Mean body weight change (%)	Mean liver weight (g)	Mean relative liver weight (%)
I	Distilled water alone	36.00[a]	6.61±1.45	3.68±0.84[b]
II	[#]SA alone	20.00[b]	10.51±2.21	6.42±2.06[a]
III	100 mg/kg ESAH	26.67[a]	7.46±0.42	3.93±0.29[b]
IV	200 mg/kg ESAH	30.00[a]	6.99±2.52	3.65±1.60[b]
V	300 mg/kg ESAH	26.67[a]	8.51±3.27	4.57±2.03[b]
VI	100 mg/kg ESAH + SA	30.00[a]	7.22±2.28	3.69±1.09[b]
VII	200 mg/kg ESAH + SA	30.00[a]	7.16±0.83	3.58±0.41[b]
VIII	300 mg/kg ESAH + SA	28.33[b]	6.46±1.03	3.32±0.92[b]

[#]SA = sodium arsenite. Values are expressed as means ± standard deviation; n = 6 for each treatment. Mean values within a column with same superscript letter are not significantly different, while different superscripts denote significant difference ($p<0.05$).

erythrocytes of the rat bone marrow cells and the degree of induction was about 2.3-fold compared to control (Table 2). Pre-treatment with ESAH at the dose of 300 mg/kg b.w. before administration of SA resulted in a significant ($p<0.05$) decrease in the induction of mPCEs to the value close to control group.

Effect of ethanol extract of *A. hypochondriacus* seed on sodium arsenite-induced changes in serum enzyme activities

Hepatic injury induced by SA caused significant changes of marker enzymes as ALT and AST activities increased significantly ($p<0.05$) compared to control group (Table 3). The changes in marker enzymes of pre-treated groups at 100, 200, 300 mg/kg doses of ESAH before administration of SA (groups VI, VII and VIII) as ALT 14.12 ($p<0.05$), 12.62 ($p<0.05$, 9.18 ($p<0.05$), respectively and AST 26.90 ($p<0.05$), 24.30 ($p<0.05$), 23.30 ($p<0.05$), respectively, are significantly different compared to SA treated group ALT (16.74) and AST (54.61). Maximum protection was observed in marker enzyme at the dose of 300 mg/kg b.w. of ESAH close to the value of control - ALT (9.39) and AST (38.17). In the case of ALP there was no significant difference observed between tested groups. The increased activity of GGT (3.47) induced by SA (group II) was significantly different from the level observed when rats were administered 300 mg/kg b.w. ESAH (group V).

Effect of ethanol extract of *A. hypochondriacus* seed on sodium arsenite-induced changes in antioxidant enzyme activity in liver

Treatment with SA caused a significant ($p<0.05$) decrease in the activities of SOD, CAT and GPx in liver tissue compared to control (Table 4). Treatment with ESAH alone at the dose of 200 and 300 mg/kg (groups IV and V) caused significant ($p<0.05$) increase in the activities of SOD and CAT compared to SA-treated rats. The GPx activity significantly ($p<0.05$) increased in treatment with 300 mg/kg ESAH alone (group V), when compared with SA-treated group. The CAT and GPx activities had significantly increased in ESAH pre-treated groups at the dose of 200 and 300 mg/kg (groups VII and VIII). The activity of GPx also increased significantly ($p<0.05$) at the dose of 100 mg/kg (group VI) when compared to SA group. The significant change as CAT 99.20 ($p<0.05$), 117.63 ($p<0.05$) and GPx 473.08 ($p<0.05$), 483.80 ($p<0.05$) ameliorated the effect of SA-induced toxicity as CAT 71.38 ($p<0.05$) and GPx 398.45 ($p<0.05$) close to control CAT (123.83), GPx (539.75). In the different dose levels of ESAH, 300 mg/kg showed maximum protection.

Effect of seed extracts of *A. hypochondriacus* on sodium arsenite-induced changes in hepatic lipid peroxidation and hydrogen peroxides

The results showed significant ($p<0.05$) elevation in the levels of MDA (11.15) and hydrogen peroxides (41.42) in SA intoxicated rats when compared to control, MDA (4.71) and H_2O_2 (29.87); respectively (Table 4). Treatment with ESAH alone at doses of 100, 200 and 300 mg/kg (groups III, IV and V) caused significant ($p<0.05$) decrease in the levels of MDA and H_2O_2 compared to control. Pre-treatment with ESAH at doses of 100 and 200 mg/kg before administration of SA (groups VI and VII) did not cause any significant reduction in the levels of MDA and H_2O_2 when compared with control. Pre-treatment with 300 mg/kg ESAH before administration of SA (Group VIII) as MDA (4.40), H_2O_2

Table 2. Induction of micronucleated polychromatic erythrocytes (mPCEs) in rat bone marrow cells after exposure to ethanol extract of *A. hypochrondriacus* seed (ESAH) and/or sodium arsenite.

Groups	Number of mPCE/1000
Control	4.43±0.32^{d}
#SA only	10.24±0.43^{a}
100 mg/kg ESAH	5.74±0.70^{c}
200 mg/kg ESAH	4.93±0.36^{cd}
300 mg/kg ESAH	4.22±0.44^{d}
100 mg/kg ESAH + SA	6.84±0.39^{b}
200 mg/kg ESAH + SA	5.53±0.45^{c}
300 mg/kg ESAH + SA	4.96±0.20^{cd}

#SA = sodium arsenite. Values are expressed as means ± standard deviation; n = 6 for each treatment. Mean values within a column with same superscript letters are not significantly different, while different superscripts denote significant difference ($p<0.05$).

(31.66) significantly ($p<0.05$) reduced the elevation of SA-induced lipid peroxidation to a level close to control as MDA (4.71) and H_2O_2 (29.87).

Histopathological observations

Results of the histopathological examination (Figure 1) of liver sections in control animals (group I) showed central vein surrounded by hepatic cord of cells, while SA treated rats liver section (group II) showed severe hepatic necrosis, with periportal cellular infiltration by mononuclear cells. Treatment with the three different doses of ESAH alone (groups III, IV and V) animals showed normal liver architecture similar to that observed in control group. Groups VI, VII and VIII animals pre-treated with different doses of the seed extracts before SA administration showed absence of necrosis and mild inflammatory collections around central vein. Normal liver architecture was observed in animals pre-treated with ESAH at 300 mg/kg b.w. (group VIII). This demonstrated hepatoprotective potential of the seed extract at a higher dose of 300 mg/kg b.w. compared to treatment with 100 and 200 mg ESAH/kg body weight.

DISCUSSION

Arsenite exerts its cellular toxicity by binding to sulfhydryl groups which results in enzyme inhibition (El-Demerdash et al., 2009). Arsenic toxicity involves oxidative damage in organs (Izquierdo-Vega et al., 2006) mainly by interaction of arsenic with protein thiols that are central components of redox-sensitive proteins in redox signalling and control pathways (Hansen et al., 2006). Sodium arsenite has been shown to decrease glutathione levels and increase lipid peroxidation in liver, kidney and heart (Ramos et al., 1995). In addition elevation in the level of serum enzymes (ALT, ALP, AST and GGT) has emerged as an index of a liver lesion (Zeashan et al., 2008).

The observed reduction in the mean body weight of experimental rats treated with sodium arsenite when compared with control group in this study is in agreement with result of El-Demerdash et al. (2009) and Bashir et al. (2006). The weight loss in rats exposed to arsenic has been associated with the induction of diabetes mellitus by the arsenite (Rahman et al., 1998). Kaltreider et al. (2001) demonstrated that exposure to low levels of arsenic altered hormonal function in the glucocorticoid system. The glucocorticoid hormones play an important role in glucose regulation as well as carbohydrate, lipid and protein metabolism. Dysfunction in the glucocorticoid system has been linked to weight loss/gain; this may explain the loss in weight observed in this study. The increase in relative liver weight in the rats treated with sodium arsenite alone when compared with control group is in agreement with previous findings in rats and mice (Gbadegesin and Odunola, 2010; Jana et al., 2006; Odunola et al., 2011; Yousef et al., 2008). Sodium arsenite intoxication has been shown to compromise the integrity of the liver in mouse, rat, fish and goat (Sharma et al., 2009; Yousef et al., 2008). The results of this study showed that sodium arsenite significantly ($p<0.05$) induced the formation of micronuclei in the polychromatic erythrocytes of the rat bone marrow cells. The induction by sodium arsenite led to about 2.3 times the mean number of mPCEs formation in the bone marrow cells of control group. Pre-treatment with ESAH at 100, 200 or 300 mg/kg before SA administration (Groups VI, VII and

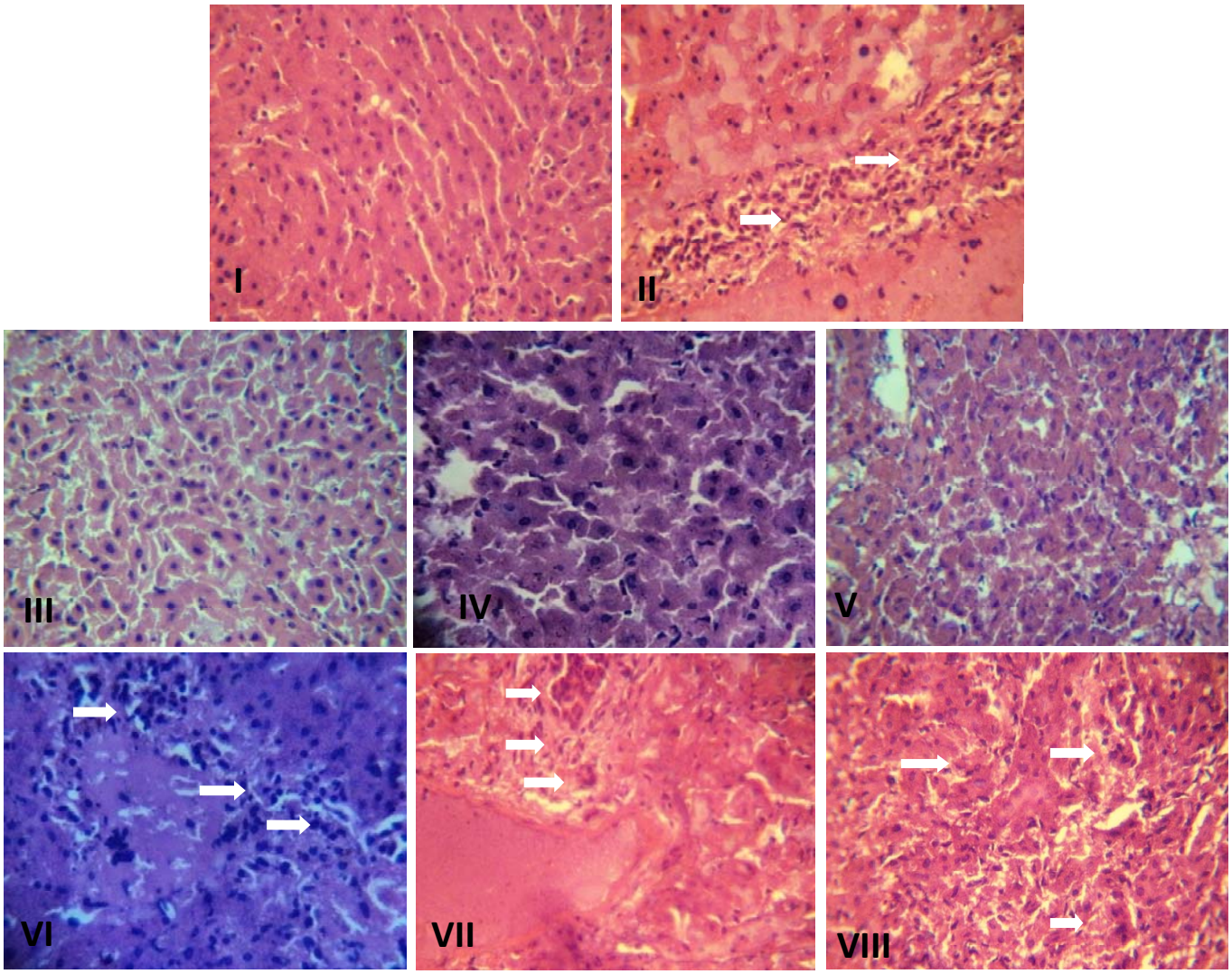

Figure 1. Results of the histopathological examination of liver sections. (I) Liver section of control rats showing normal architecture (× 400); (II) Liver section of rats treated with sodium arsenite (2.5 mg/kg b.w.) showing severe hepatic necrosis, periportal cellular infiltration by mononuclear cells (× 400); (III) Liver section of rat treated with 100mg/kg b.w. of *A. hypochondriacus* seed extract showing no visible lesions (× 400); (IV and V) Liver section of rat treated with 200 and 300 mg/kg *A. hypochondriacus* seed extract respectively, showing no visible lesions, cytoplasm and nucleus are intact and well differentiated (× 400); (VI) Liver section of rat treated with 100mg/kg *A. hypochondriacus* seed extract and 2.5 mg/kg b.w. sodium arsenite. Inflammation changes were observed, with mild hepatic degeneration (× 400); (VII) Liver section of rat treated with 200 mg/kg *A. hypochondriacus* seed extract and 2.5 mg/kg b.w. sodium arsenite. Cellular infiltration around the portal area is mild (× 400); (VIII) Liver section of rat treated with 300 mg/kg *A. hypochondriacus* seed extract and 2.5 mg/kg b.w. sodium arsenite showing very mild hepatic degeneration (×400).

VIII) significantly ($p<0.05$) reduced the frequency of mPCEs formation in bone marrow cells when compared with group treated with SA alone. Das et al. (1993) reported a similar decrease in the degree of mPCEs formation in mice pre-treated with garlic extracts before sodium arsenite challenge.

The increased activities of serum marker enzymes (AST, ALT, ALP and GGT) are conventional indicators of liver injury (Achliya et al., 2004; Thabrew et al., 1987). Yadav and Dixit (2003) reported the ability of a drug to reduce the injurious effects or to restore the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin, as the index of its protective effects. The present study revealed a significant ($p<0.05$) increase in the level of ALT and AST on administration of sodium arsenite, this may be due to the increased permeability of the plasma membrane indicating considerable hepatocellular damage. Similar results of elevated serum enzymes were earlier reported (Mallick et al., 2003; Odunola et al., 2011). The serum marker enzymes are mainly intracellular ones which mean that any damage to the hepatocytes could lead to their

Table 3. Effect of ethanol extract of *A. hypochondriacus* seed (ESAH) on serum alanine amino transaminase (ALT), aspartate amino transaminase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) in rats treated with sodium arsenite.

Group	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII
Treatment	Distilled water	#SA alone	100 mg ESAH	200 mg ESAH	300 mg ESAH	100 mg ESAH +SA	200 mg ESAH+ SA	300 mg ESAH+ SA
ALP(U/L)	66.93±10.42^{a}	71.76±19.52^{a}	62.10±5.28^{a}	50.37±15.51^{a}	43.47±16.77^{a}	69.69±4.14^{a}	53.82±17.24^{a}	49.68±9.82^{a}
ALT (U/L)	9.39±1.32^{d}	16.74±1.04^{a}	10.35±0.94^{cd}	10.58±2.26^{cd}	8.76±2.21^{d}	14.12±0.80^{b}	12.62±0.33^{bc}	9.18±0.23^{d}
AST (U/L)	38.17±3.61^{b}	54.61±11.94^{a}	38.96±3.60^{b}	33.13±1.45^{bc}	32.44±1.23^{bc}	26.90±2.89^{c}	24.30±2.18^{c}	23.30±2.35^{c}
GGT (U/L)	1.74±0.67^{ab}	3.47±0.95^{a}	2.32±1.64^{ab}	1.74±0.67^{ab}	1.16±0.00^{b}	3.18±0.58^{ab}	2.61±1.11^{ab}	1.74±0.67^{ab}

#SA = sodium arsenite; Values are expressed as means ± standard deviation; n = 6 for each treatment; Mean values within a row with same superscript letter are not significantly different, while different superscript denote significant difference ($p<0.05$).

Table 4. Changes in the activities of SOD, CAT, GPx, MDA, H_2O_2 and protein in liver of male rats treated with ethanol extract of *A. hypochondriacus* seed (ESAH) and/or sodium arsenite.

Treatment	Distilled water	#SA alone	100 mg/kg ESAH	200 mg/kg ESAH	300 mg/kg ESAH	100 mg/kg ESAH+SA	200 mg/kg ESAH+SA	300 mg/kg ESAH+SA
	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII
SOD	4.22±0.40^{a}	2.82±0.33^{dc}	3.32±0.47^{bc}	3.52±0.35^{b}	3.55±0.26^{b}	2.39±0.11^{d}	3.12±0.21^{bc}	3.31±0.47^{bc}
CAT	123.83±20.50^{a}	71.38±8.33^{e}	71.95±4.81^{de}	86.05±6.99^{cd}	110.05±5.34^{ab}	80.99±11.69^{e}	99.20±1.08^{bc}	117.63±7.55^{a}
GPX	539.75±55.70^{a}	398.45±31.25^{cd}	361.10±44.15^{d}	447.05±56.90^{bc}	482.29±55.61^{ab}	451.89±15.23^{bc}	473.08±24.02^{b}	483.80±24.99^{ab}
MDA	4.71±1.48^{c}	11.15±0.85^{a}	4.52±0.55^{c}	4.38±0.62^{c}	4.15±0.75^{c}	6.54±0.46^{b}	5.86±0.53^{b}	4.40±0.40^{c}
H_2O_2	29.87±6.70^{c}	41.42±5.89^{a}	21.85±2.57^{d}	27.33±2.36^{c}	29.39±4.80^{c}	36.38±1.33^{b}	34.19±2.04^{b}	31.66±1.37^{c}
Protein	0.66±0.06^{a}	0.60±0.05^{ab}	0.50±0.02^{cd}	0.53±0.04^{bc}	0.54±0.05^{bc}	0.44±0.05^{e}	0.49±0.02^{cde}	0.43±0.04^{e}

#SA = sodium arsenite; Values are expressed as means ± standard deviation; n = 6 for each treatment. Mean values within a column with same superscript letter are not significantly different, while different superscripts denote significant difference ($p<0.05$). Superoxide dismutase (SOD) activity is expressed as units/mg protein; catalase (CAT) activity as µmol H_2O_2 consumed/mg protein; glutathione peroxidase (GPx) as units/mg protein; malondiadehyde (MDA) as units/g tissue × 10^{-6}; Hydrogen peroxide (H_2O_2) as µmol/L.

release into the plasma leading to the observed increase in plasma activities. Hepatocellular necrosis leads to elevation of the serum marker enzymes, which are released from liver cells into blood (Ashok et al., 2001). In addition, the increased GGT activity of rats exposed to SA observed in this study is indicative of oxidative stress and cytogenetic damage (Lee et al., 2006). Pre-treatment with ESAH at the different dose levels of 100, 200 and 300 mg/kg (Groups VI, VII and VIII) attenuated the increased levels of the serum enzymes produced by SA and caused a subsequent recovery towards normalization.

It has been reported that one of the principal causes of SA induced liver injury is formation of lipid peroxides by free radical derivatives of SA (Manna et al., 2007). The body has an effective defence mechanism to prevent and neutralize the free radical induced damage. This is proficient by a set of endogenous antioxidant enzymes such as SOD, CAT, GST and GPx. These enzymes constitute the first line of cellular antioxidant defence and provide a mutually supportive team of defence against ROS (Amresh et al., 2007a). The reduced activities of SOD, CAT and GPx observed in this study points out hepatic damage in the rats administered with SA, and is probably due to exhaustion of these enzymes to scavenge excessively-generated superoxide and hydrogen peroxides, respectively; in tumour cells

(Manoharan et al., 2006). The reduced activity of SOD, CAT and GPx of rats treated with SA is in agreement with previous results (Bashir et al., 2006; Gopalkrishnan and Rao, 2006; Wu et al., 2001). Reduced activities of SOD, CAT and GPx in erythrocytes have been reported in cancer (Balasenthil et al., 2000). Reduced activity of CAT after exposure to sodium arsenite in this study could be correlated to increased generation of hydrogen peroxide. Similar observations were reported by El- Demerdash et al. (2009), during treatment of experimental rats with sodium arsenite. The decreased SOD activity in the liver suggests that accumulation of superoxide anion radical might be responsible for increased lipid peroxidation following arsenic treatment. Free radicals are produced by arsenic treatment (Yamanaka et al., 1990), by the reaction of molecular oxygen with dimethylarsine, a metabolite of dimethyl arsenic acid. Pre-treatment with 100, 200 and 300 mg/kg b.w. of *A. hypochondriacus* seed extract increased the activities of SOD, CAT and GPx in a dose dependent manner. This observation is similar to report of El-Demerdash et al., (2009) which showed that treatment with *Curcumin* extract improved the levels of liver SOD and CAT activity in rats.

Free radical-induced lipid peroxidation is regarded as one of the basic mechanism of cellular damage and therefore, the extent of tissue damage can be monitored by measuring the concentration of plasma or serum lipid peroxides (Selvendiran and Sakthisekaran, 2004). In this study, elevation of lipid peroxidation and hydrogen peroxides in the liver of rats treated with SA suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defence mechanisms to prevent the formation of excessive free radicals (Amresh et al., 2007b). Increase in plasma lipid peroxides in DMBA-painted animals has also been observed and was due to overproduction and diffusion from the damaged erythrocyte membranes and some other host tissues such as the liver (Manoharan et al., 2006). Increased plasma lipid peroxidation has been reported in several types of cancer patients (Ozdemirler et al., 1998). Pre-treatment with 100, 200 and 300 mg/kg ESAH significantly reduced the elevated levels of MDA and H_2O_2 caused by SA; restoring towards normalization. Similar observation was reported in CCl_4-induced toxicity in rats and administration of different doses of ethanol extracts of *Amaranthus spinosus* significantly prevented the heave in levels of MDA and H_2O_2 (Zeashan et al., 2008). It is likely that the mechanism of hepatoprotection of extract of *A. hypochondriacus seed* may be due to its antioxidant activity.

Conclusion

From this study, *A. hypochondriacus* seed extract seems to exhibit hepatoprotective effects and mitigates sodium arsenite-induced oxidative damage in rats. The ESAH has shown dose dependent activity as 300 mg/kg b.w. has greater activity which is comparable with the control group.

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Conflict of interest statement

The authors declare that there is no conflict of interests.

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Full Length Research Paper

Synergistic activity from *Hymenaea courbaril* L. and *Stryphnodendron adstringens* (Mart.) Coville against multidrug-resistant bacteria strains

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Infections caused by multidrug-resistant bacteria are a problem of public health, turning the search for natural products an alternative to antibiotics of great importance. The aim of this study was to investigate the *in vitro* antimicrobial activities of *Hymenaea courbaril* and *Stryphnodendron adstringens* against bacterial clinical isolates. The crude extracts of both vegetal species in study showed bacteriostatic activity against almost all bacteria evaluated, with minimum inhibitory concentration (MIC) ranging from 125 to 1250 µg/ml. The bacteriostatic activity was observed in all the fractions of *H. courbaril* and *S. adstringens* against at least one bacterial strain, except in the fraction dichloromethane of *S. adstringens*. In regard to bactericidal activity, *H. courbaril* was active only against *E. faecalis*, and *S. adstringens* showed activity against all bacterial strains, except to *Enterococcus faecalis*. The combination of extracts showed potent synergistic antimicrobial activity, with MIC values of 31.25 µg/ml against *Acinetobacter baumannii*, *Escherichia coli* and *Staphylococcus aureus*. *S. adstringens* were considered less cytotoxic compared to *H. courbaril* and the half-maximum cytotoxic concentration (CC_{50}) resulting from the combination of the two plants was 0.0082 ± 3.19 mg/ml. The results showed for the first time the synergic antibacterial activities of *H. courbaril* and *S. adstringens* against resistant bacteria, suggesting their potential use to development of new drugs.

Key words: *Hymenaea courbaril*, *Stryphnodendron adstringens*, antimicrobial activity, synergism, multidrug-resistant bacteria.

INTRODUCTION

The overuse of antibiotics and consequent selective pressure is thought to be the most important factor contributing to the increasing occurrence of resistance to antibiotics, which represents a public health issues worldwide (Ang et al., 2004). Moreover, over last decade, there has been dramatic reduction in the number of

pharmaceutical companies developing new antimicrobial agents (Boucher et al., 2009). In front of the challenge of searching for therapeutic tools that combat bacterial resistance, plants, especially those with ethnopharmacological uses, have been the main sources for the early discovery of new drugs, since the plant biological diversity is a source of a wide range of bioactive molecules, acting by different mechanisms (Chin et al., 2006). Thus, plant extracts can be used as sources of new drugs or antimicrobial compounds, which are of great importance since the emergence of resistant strains makes difficult the treatment of infections (Alviano and Alviano, 2009).

The Fabaceae vegetable family presents more than 490 species of medicinal plants, including *Hymenaea courbaril* and *Stryphnodendron adstringens*, which are used in folk medicine (Gao et al., 2010). *H. courbaril* L. is used in popular medicine as, fluidificant and expectorant, astringent, anti-diarrheal, anti mycotic, and anti-inflammatory (Correia et al., 2008). Martins et al. (2010) described the antibacterial activity of crude ethanol extracts of the bark and pulp of mealy from *H. courbaril* and the best results were obtained with minimum inhibitory concentration (MIC) of 350 µg/ml against clinical isolates of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

The *S. adstringens* (Mart) Coville is employed in folk culture in the form of a decoction or infusion as an astringent, anti-diarrheal, antimicrobial and hypoglycemic agent for the treatment of gynecological problems and healing wounds (Ishida et al., 2009). Studies showed antimicrobial activity in the extract obtained from the bark of this plant against *S. aureus, E. coli*, *P. aeruginosa* and *S. epidermidis* (Audi et al., 2004; Souza et al., 2007). There are some data on antimicrobial activity and synergy between extracts of *H. courbaril* and *S. adstringens* since the promising potential use of medicinal plants in treatment of diseases. So, the current investigation carried out the antimicrobial activity from ethanol extract and fractions obtained from barks of *H. courbaril* and *S. adstringens* and their synergism was evaluated against six bacteria of clinical interest, to prospective new antibacterial therapy.

MATERIALS AND METHODS

Plant collection

The barks from *H. courbaril* (BHCB 159,399) and *S. adstringens* (BHCB 159,400) were collected in the city of São Sebastião do Oeste, Minas Gerais, Brazil, in August, 2011. The voucher specimens were deposited at the Herbário do Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil.

Plant extract and fractions preparation and phytochemical screening

The plants material were dried at 40°C and triturated. The material (1000 g) was extracted by cold maceration in 4.0 L of ethanol P.A (Vetec, Brazil) for a period of 10 days at room temperature (25 ± 2°C) for 10 days. After it was filtrated with Whatman filter paper and concentrated in a rotary evaporator (IKA equipment, model RV10) at 40°C under reduced pressure to yield ethanol extract. The dried crude extract was obtained after lyophilization. Part of this extract (5.0 g) was dissolved in ethanol/water (7:3) and then partitioned successively with hexane (C_6H_{14}), dichloromethane (CH_2Cl_2) and ethyl acetate (AcOEt) (100 ml, 3 times with each solvent), resulting in hexane (F1), dichloromethane (F2), ethyl acetate (F3), and hydroethanol (F4) fractions, respectively (Araújo et al., 2013). The extracts and fractions were maintained in the dark and refrigerated at 4°C. They were solubilized in dimethylsulfoxide (DMSO) 2% v/v. The extract and fractions were screened qualitatively for the presence of different classes of natural products such as alkaloids, steroids, triterpenoids, coumarins and flavonoids by thin-layer chromatography (TLC) (Wagner et al., 1996). The analysis was performed on Merck silica gel 60 F254 aluminum plates. Other tests described by Matos (2000) were carried out to determine the presence of tannins and saponins.

Microorganisms and stock conditions

Six clinical isolates provided by Hospital São João de Deus, Divinópolis, Minas Gerais, Brazil, were used in antibacterial tests: *Acinetobacter baumannii* 7810, *Klebsiella pneumoniae* 7845, *P. aeruginosa* 530, *E. coli* 3004, *S. aureus* 8066 and *E. faecalis* 3110. The origin of strains was performed from urine, except for *A. baumannii* and *S. aureus,* obtained from tracheal secretions and exudates of injury, respectively. The resistance profile was performed by the automated system of identification and antibiogram (VITEK2 compact, bioMérieux): aminoglycosides, β-lactams, fluorquinolones, polymyxins, carbapenems, fosfomycin, nitrofurans, glycylcyclines and sulfonamides. Bacteria were stored in nutrient broth with 10% glycerol at freezer -80°C and subsequently activated in nutrient broth at 37°C for 24 h for use in assays. This study was approved by Ethics Committee of Hospital São João de Deus, Divinópolis, Minas Gerais, Brazil (Protocol: 186/2011).

Minimum inhibitory concentrations (MIC) and minimal lethal concentration (MLC) assays

The MICs were determined using the broth microdilution method, with modifications from standards recommended according to the Clinical and Laboratory Standards Institute (CLSI, 2003). The crude extracts and fractions were diluted in DMSO at concentrations 1250, 1000, 750, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 µg/ml. Bacterial were cultured on Mueller-Hinton agar and following bacterial growth, a standardized bacterial suspension equivalent to 0.5 McFarland was used. Subsequently, 50 µl of this solution were diluted in Mueller-Hinton broth (MHB) to a concentration of approximately 5×10^5 CFU/ml. An inoculum of 125 µl was added to

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Table 1. Phytochemical study of crude extract (CE) and hexane (F1), dichloromethane (F2), ethyl acetate (F3) and hydroethanol (F4) fractions from *H. courbaril* and *S. adstringens.*

Metabolites	*H. courbaril*					*S. adstringens*				
	CE	F1	F2	F3	F4	CE	F1	F2	F3	F4
Steroids/Triterpenoids	+/-	+/-	+++	-	+++	-	-	+/-	-	+/-
Flavonoids	+++	-	-	-	-	+++	++	++	+++	++
Coumarins	+/-	+/-	+++	++	+/-	+/-	+/-	+/-	+/-	++
Saponins	-	-	-	-	-	-	-	-	-	-
Alkaloids	+++	+/-	+++	+++	+++	++	-	+/-	++	+/-
Tanins	+/-	+/-	+/-	-	+/-	++	+/-	+/-	-	+++

(-) absence, (+/-) minimal presence, (+), (++) and (+++) grading presence

25 µl of each sample concentration plant in 100 µl of MHB in 96-well microplates. After incubation for 24 h, turbidity of the broth in the wells was observed. MIC was defined as the lowest concentration of the extract at which no visible growth could be detected. All assays were performed in triplicate and repeated three times in independent experiments. Sterile 2% DMSO was used as negative vehicle control and a Streptomycin/Penicillin solution (Sigma-Aldrich, USA) as positive control of inhibition. Following incubation of MICs plates, the minimal lethal concentration (MLC) were determined by removal of 25 µl from wells without visible turbidity and transferred to Mueller Hinton agar by a Spread-Plate method. The lowest concentration that resulted in absence of bacterial growth was determined as the MLC.

Synergy testing by microdilution checkerboard

The synergistic effects were assessed by the checkerboard test as previously described by Lee et al. (2012), with adaptations. Samples of crude extract of the species studied were serially diluted in concentrations ranging from 1.95 to 125 µg/ml. Subsequently, solutions of the same concentration were combined in a 1:1 ratio to evaluate the antimicrobial effect resulting from the interaction of *H. courbaril* and *S. adstringens*. The fractional inhibitory concentration index (FIC index) is the sum of the FICs of each of the drugs, which in turn is defined as the MIC of each drug when it is used in combination divided by the MIC of the drug when it is used alone. All experiments were independently repeated three times. Values of FIC index less than or equal to 0.50 were considered to be indicative of a synergic effect. Values ranging from 0.51 to 1.00 indicated an additive effect, values from 1.01 to 2.00 were considered as indifferent and values above 2.00 indicated an antagonist effect.

Cell culture and cytotoxicity analysis by the MTT assay

Vero cells (ATCC CCL-81) were cultured in Dulbecco's modified eagle medium (DMEM) with 2% of fetal bovine serum (FBS), at 37°C, 5% of CO_2 atmosphere, until reach 95% of confluence. Cytotoxicity of crude extracts and fractions (1000 to 0.025 µg/ml) was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Merck solution 2 mg/ml in phosphate buffered saline (PBS) (Twentyman and Luscombe, 1987). Each sample was assayed in three replicates.

Statistical analyses

All tests were made in triplicate in three independent experiments. When appropriate, mean ± standard deviation were used to describe the results. The half-maximum cytotoxic concentration (CC_{50}) was determined by non-linear regression using GraphPad Prism, 5.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Phytochemical screening

The phytochemical analysis of *H. courbaril* revealed the presence of alkaloids, coumarins, flavonoids, steroids/triterpenoids and tannins in ethanol crude extract (Table 1). Alkaloids and coumarins are present in all fractions. Steroids/triterpenoids and tannins also were found in hexane, dichloromethane and hydroethanol fractions. Saponins were absent in the samples. On the other hand, the phytochemical analysis of *S. adstringens* revealed the presence of alkaloids, coumarins, flavonoids and tannins in ethanol crude extract (Table 1). Coumarins and flavonoids are present in all fractions. Alkaloids, steroids/ triterpenoids and tannins were found in dichloromethane and hydroethanol fractions. Alkaloids also present ethyl acetate fraction and tannins in hexane fraction. Saponins were absent in the samples.

Resistance profile of clinical isolates

Table 2 shows the profile resistance of the clinical isolates to different antibiotics classes. The profile revealed by antibiogram showed that the *E. coli* 3004 was the strain that has greater resistance to antibiotics, followed by *K. pneumoniae* 7845, *P. aeruginosa* 530, *A. baumannii* 7810, *E. faecalis* 3110 and *S. aureus* 8066.

Antimicrobial activity

The crude extract of *H. courbaril* displayed bacteriostatic activity against all bacteria, except *P. aeruginosa* 530 (Table 3). The MIC values found for *E. faecalis* 3110, *E. coli* 3004, *S. aureus* 8066, *A. baumannii* 7810 and *K.*

Table 2. Resistance profile of clinical isolates in front of different classes of antibiotics used in medical clinic.

Antibiotics	Clinical isolates					
	***A. baumannii* 7810**	***K. pneumoniae* 7845**	***P. aeruginosa* 530**	***E. coli* 3004**	***S. aureus* 8066**	***E. faecalis* 3110**
β-lactam						
Amoxicillin/Clavulanic acid	-	R	R	S	-	-
Ampicilin	R	R	R	S	-	R
Ampicilin/Clavulanic acid	-	-	-	S	-	
Ampicilin/Sulbactan	R	-	-	-	-	R
Aztreonam	R	R	R	S	-	R
Cefepime	R	R	R	R	-	-
Cephalothin	R	R	R	R	-	-
Ceftriaxona	-	-	-	R	-	-
Cefotaxime	R	R	R	R	S	-
Ceftazidime	R	R	I	R	-	-
Imipenem	R	-	-	R	-	-
Ertapenem	-	I	-	R	-	-
Meropenem	R	S	R	S	-	-
Piperaciclin/Tazobactam	R	R	R	R	-	-
Aminoglycosides						
Amikacin	I	S	S	-	-	-
Gentamicin	R	R	R	R	-	I
Fluorquinolones						
Nalidixic acid	-	R	R	R	-	-
Levofloxacin	-	R	R	R	-	S
Norfloxacin	-	-	R	R	R	R
Ciprofloxacin	R	R	R	R	R	R
Polymyxin						
Colistina	S	-	-	-	-	-
Nitrofurane						
Nitrofurantoin	-	R	-	R	-	-
Fosfomicin	-	-	-	R	-	-
Glycylcycline						
Tigecycline	S	-	-	S	S	-
Sulfonamides	-	R	R	R	-	S
Trimethoprim/Sulfametoxazole	-	-	-	-	S	-

S = sensivite; R = resistant; I = intermediate

pneumoniae 7845 were of 125, 250, 500, 750 and 1000 µg/ml, respectively. The bactericidal effect of *H. courbaril* was observed only for *E. faecalis* 3110, being the value of MLC found of 1250 µg/ml. The *S. adstringens* crude extract showed bacteriostatic activity in all bacterial tested (Table 3) with MIC values ranging from 250 to 1000 µg/ml. The best MIC, 250 µg/ml was obtained from *S. aureus* 8066 and the worst, 1000 µg/ml, from *E. faecalis* 3110. The MIC obtained to *A. baumannii* 7810 and *E. coli* 3004 was 500 µg/ml and to *K. pneumoniae* 7845 and *P. aeruginosa* 530 was 750 µg/ml. The bactericidal activity was obtained for all evaluated bacterial, except to *E. faecalis* 3110. The MLC value obtained to *A. baumannii* 7810 was 1000 and 1250 µg/ml to the others strains. The bacteriostatic activity was observed in all the fractions of *H. courbaril* against at

Table 3. Minimum inhibitory concentration (MIC) (µg/ml) and minimum lethal concentration (MLC) (µg/ml) of crude extracts of *S. adstringens* and *H. courbaril* against clinical isolates.

Bacteria	*S. adstringens*		*H. courbaril*		MIC Penicillin-Streptomycin solution
	MIC	MLC	MIC	MLC	
A. baumannii	500	1000	750	-	31.25
K. pneumoniae	750	1250	1000	-	31.25
P. aeruginosa	750	1250	-	-	31.25
E. coli	500	1250	250	-	7.81
S. aureus	250	1250	500	-	31.25
E. *faecalis*	1000	-	125	1250	31.25

(-) = absence of activity

least two bacterial species (Table 4). The MIC values ranged from 125 to 1000 µg/ml, being the smallest against *S. aureus* 8066 in the ethyl acetate (F3) fraction and against *E. faecalis* 3110 in fractions hexane (F1), ethyl acetate (F3) and hydroethanol (F4). The bactericidal effect was observed against *E. faecalis* 3110 in all the fractions tested and against *S. aureus* 8066 in ethyl acetate (F3).

S. adstringens exhibited greater bacteriostatic activity in hexane (F1) and ethyl acetate (F3) with MIC values ranging from 250 to 1250 µg/ml. The hexane (F1) fraction was active against all bacteria evaluated and ethyl acetate (F3) just not demonstrated activity against *E. faecalis* 3110. No effect was observed for dichlorometane (F2) and the hydroethanol (F4) was active only against *E. faecalis* 3110. The bactericidal effect was observed in the hexane (F1) fraction against *E. coli* 3004 and on ethyl acetate (F3) fraction against *K. pneumoniae* 7845, *E. coli* 3004 and *S. aureus* 8066. The results of the combined effect of crude extracts can be observed in Table 5. The combination of extracts exhibited antibacterial activity potential, with MIC value of 31.25 µg/ml against *A. baumannii* 7810, *E. coli* 3004 and *S. aureus* 8066, indicating interaction of the type synergistic between the extracts (FIC index < 0.9).

Cytotoxicity analysis by the MTT assay

The evaluation of cytotoxic effects of crude extracts and fractions were conducted by mitochondrial reduction technique through the MTT reagent (data not show). The CC_{50} of the hydroethanol crude extract of *H. courbaril* was 4.33 ± 3.4 mg/ml. The more cytotoxic fraction derived from this extract was the hexane with CC_{50} of 3.37 ± 2.25 mg/ml. The Vero cell line showed low sensitivity to hydroethanol fraction, where the CC_{50} observed was 1.67 ± 3.5 mg/ml. The CC_{50} resulting from the combination of two plant species under study was 0.0082 ± 3.19 mg/ml. The hydroethanol crude extract of *S. adstringens* presented CC_{50} of 0.094 ± 3.3 mg/ml. The most cytotoxic fraction of this species was the hexane with CC_{50} of 0.016 ± 5.2 mg/ml. On the other hand, the sample that showed least cytotoxicity against cell line tested was the ethyl acetate fraction with CC_{50} of 0.234 ± 1.3 mg/ml.

DISCUSSION

Extracts with MIC less than 100 µg/ml, the antimicrobial activity is good, from 100 to 500 µg/ml the antimicrobial activity is moderate, from 500 to 1000 µg/ml the antimicrobial activity is weak, and over 1000 µg/ml the extract is considered inactive (Holetz et al., 2002; Aleixo et al., 2014). In this work, it was necessary to use a mix of streptomycin and penicillin as controls because some bacteria isolated from clinical samples showed resistance to one of these antibiotics when used individually.

There are different approaches to cure and control the infection caused by the multidrug-resistant (MDR) strains bacteria, one of which is by isolation of active phytochemicals that can help to prevent the spread of infection. Bacteria presented in this study showed resistance to different antibiotics classes, which makes them important models to mimic the infections that have been occurred in hospitals. Another method is to formulate new synergistic combinations using active phytochemicals that have antimicrobial properties. In this work, the synergistic effect of the crude extracts of *H. courbaril* and *S. adstringens* showed a reduction of MIC value (<100 µg/ml) in three of the four tested microorganisms (Table 5). Such synergistic combinations may result in increased therapeutic effects and reduce the chances of toxicity dose-dependent (Boucher and Tam, 2006).

The results showed by fractions of *H. courbaril* were more heterogeneous as compared with those of *S. adstringens,* however with a lower number of bacteria. The hexane and ethyl acetate fractions of *S. adstringens* were most active. These were active against the bacterial strains that showed a profile of multi-resistance to several classes of antibiotics, indicating that the mechanisms of action of antibacterial substance are able to overcome

Table 4. Minimum inhibitory concentration (MIC) (µg/ml) and minimum lethal concentration (MLC) (µg/ml) from fractions hexane (F1), dichloromethane (F2), ethyl acetate (F3) and hydroethanol (F4) derived from crude extracts of *S. adstringens* and *H. courbaril* against clinical isolates.

Bacteria	Fractions derived from crude extracts															
	S. adstringens								*H. courbaril*							
	F1		F2		F3		F4		F1		F2		F3		F4	
	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
A. baumannii	750	-	-	-	250	-	-	-	-	-	-	-	-	-	-	-
k. pneumoniae	1250	-	-	-	750	1000	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	1000	-	-	-	750	-	-	-	-	-	-	-	-	-	-	-
E. coli	500	1250	-	-	750	1000	-	-	750	-	750	-	500	-	-	-
S. aureus	1250	-	-	-	250	1250	-	-	-	-	1000	-	125	1250	250	-
E. *faecalis*	1250	-	-	-	-	-	500	-	125	1250	500	1250	125	1250	125	1250

(-) = absence of activity.

Table 5. Effect resulting from combination of crude extracts of *S. adstringens* and *H. courbaril* against clinical isolates.

Bacteria	MIC in combination (µg/ml)	FIC Sa	FIC Hc	FIC index	Interaction
A. baumannii	31.25	0.06	0.12	0.18 < 0.9	sinergic
K. pneumoniae	-	-	-	-	-
P. aeruginosa	nt	-	-	-	-
E. coli	31.25	0.02	0.02	0.05 < 0.9	sinergic
S. aureus	31.25	0.12	0.06	0.18 < 0.9	sinergic
E. *faecalis*	125	0.12	1.0	1.12	additive

(-) = absence of activity. nt = not tested. FIC Sa = FIC *S. adstringens*. FIC Hc = FIC *H. courbaril.* FIC index = sinergic (≤ 0.5), additive, (0.5 to 1.0) and antagonistic (≥ 4.0)

the various barriers of resistance.

Several classes of secondary metabolites are present in the extracts and the fractions of *H. courbaril* and *S. adstringens,* such as alkaloids, coumarins, flavonoids, steroids/triterpenoids and tannins. Cecílio et al. (2012) also observed the presence of coumarins, flavonoids, triterpenoids and tannins in ethanol extract of *H. courbaril* and flavonoids, triterpenoids and tannins in ethanol extract of *S. adstringens*, corroborating with results showed in this study. Tannins are known for antimicrobial properties, acting by different mechanisms (Scalbert, 1991). Triterpenoids from *Callicarpa farinosa* showed antimicrobial activities against different strains of *S. aureus*, with MIC ranging from 2 to 512 µg/ml (Chung et al., 2014). Flavonoids have been reported to possess antimicrobial activity against a wide range of pathogens as flavonoids from *Dorstenia* species that showed activity against methicillin-resistant *S. aureus* (MRSA) strains with MICs values ranged between 0.5 to 128 µg/ml (Dzoyem et al., 2013). Coumarins from *Angelica lucida* showed antimicrobial activity (Widelski et al., 2009). Alkaloids isolated from *Litsea cubeba* presented antibacterial activity against *S. aureus* (Zhang et al., 2012). The results of the antibacterial activity of liquid-liquid fractions showed that *S. adstringens* presented the greater antibacterial effect in hexane fractions and ethyl acetate,

suggesting that the metabolites responsible for this activity are present in these fractions.

Regarding *H. courbaril*, all fractions were active, indicating that this species has a greater diversity of secondary metabolites with antimicrobial activity. Furthermore, a higher number of active fractions of *H. courbaril* against tested Gram positive bacteria was observed. This fact may be due to composition of bacterial wall cell, where the lipopolysaccharide outer membrane that Gram negative bacteria have, restricts the diffusion of hydrophobic compounds, which could lead to greater resistance to antimicrobial substances (Biswas et al., 2013; Tajkarimi et al., 2010). Usually, Gram negative bacteria are more resistant to plant-derived antimicrobials compared to Gram positive bacteria (Biswas et al., 2013; Vlietinck et al., 1995).

Considering that the compounds with intermediate polarity of *S. adstringens* (ethyl acetate) were effective, with lower MIC (Table 4), compared with the most nonpolar compounds (hexane), this may indicate that there are effective components that act on the membranes of microorganisms or affect any transport mechanism. Although less effective, the same fractions (hexane and ethyl acetate) for the species *H. courbaril* also had the same trend as for the antimicrobial effect. This indicates first that the active principle is preserved within the family Fabaceae, which corroborates previous results (Máximo et al., 2006).

This work provides the first reports of potent antimicrobial activity resulting from the combination of the two vegetal species, *H. courbaril* and *S. adstringens* against multi-resistant Gram negative and Gram positive bacterial strains. These results encourage additional studies of extract and fractions from the barks of *H. courbaril* and *S. adstringens* for isolation of the bioactive compounds with antibacterial potential.

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Conflict of interest

The authors do not have any conflicts of interest.

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