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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Antioxidant and anticancer activities of *Moringa oleifera* leaves

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Moringa oleifera **leaves extracted with methanol and dichloromethane were screened for antioxidant activity. The *in vitro* cancer antiproliferative and chemopreventive properties were also investigated. Radical scavenging assays with 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2´-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were used to determine the antioxidant activity. The antiproliferative assay was evaluated on three types of cancer cell lines: hepatocarcinoma (HepG2), colorectal adenocarcinoma (Caco-2) and breast adenocarcinoma (MCF-7), using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. The *in vitro* cancer chemoprevention was performed using quinone reductase (QR) induction assay on hepatoma (Hepa-1c1c7). The chemopreventive activity of the extracts was expressed as concentration to double QR activity (CD value). The methanol extract showed higher free radical scavenging activity than the dichloromethane extract (IC_{50} = 1.60±0.03 mg/ml in DPPH assay and IC_{50} = 1.02±0.06 mg/ml in ABTS assay). In the antiproliferative assay, the IC_{50} of dichloromethane extract varied from 112 to 133 µg/ml for HepG2, Caco-2 and MCF-7 cancer cells, but became more than 250 µg/ml for the methanol extract. In the chemopreventive assay, the dichloromethane extract had capacity to induce QR activity significantly (CD value = 91.36±1.26 µg/ml), while the methanol extract had no inductive effect. This study provides evidence that *M. oleifera* leaves possess antioxidant activity, as well as cytotoxic and chemopreventive properties. Therefore, it might be beneficial as a medicinal plant for alternative novel anticancer drugs and nutraceutical products.**

Key words: *Moringa oleifera*, antioxidant activity, quinone reductase, antiproliferation, cancer chemoprevention.

INTRODUCTION

Cancer is the leading cause of mortality worldwide. According to the cancer reports published by the World Health Organization (WHO) and the World Cancer Research Fund, the incidence of cancer is still increasing especially due to diet, environment and carcinogenic virus infections (WHO, 2008; World Cancer Research Fund, 2007). In hospitals, conventional drugs are commonly prescribed to cancer patients. However, due to less toxic and adverse effects of phytochemicals, the research on medicinal plants and cancer has been intensified (Johnson, 2007).

Moringa oleifera (*M. oleifera*) or drumstick is a member of Moringaceae, and it is grown extensively in many Southeast Asian countries particularly in Thailand, India, Philippines and Pakistan (Fuglie, 2001). It has long been known as a food plant in Thai cuisine and as an ingredient of Indian traditional medicine (Wutythamawech, 1997; Mishra et al., 2011). The leaves contain nutrients

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especially essential amino acids, vitamins, minerals and *β*-carotene (Sabale et al., 2008; Sharma et al., 2012). For this reason, it is used as an alternative source for nutritional supplements and growth promoters in some countries (Anwar et al., 2007). Apart from nutritional benefits, *M. oleifera* is reported to be used for the treatment of rheumatism, ascites, infection, hiccough influenza and internal abscess (Anwar et al., 2007; Mishra et al., 2011). Many recent reports on disease prevention by *M. oleifera* have been reported. The leaf extract is capable of reducing hyperglycemia and dyslipidemia (Mbikay, 2012). The ethanol extract of the leaves prevented cyclophosphamide-induced micronucleus formation and DNA damage in mice (Sathya et al., 2010). The aqueous extract enhanced hepatic glutathione restoration (Fakurazi et al., 2008). Recently, Chadamas et al. (2010) reported that tender pods decreased the for-mation of erythrocyte micronucleus in mice injected with 7,12-dimethylbenz(a)anthracene. It also demonstrated inhibitory potential against azoxymethane-induced colon carcinogenesis (Budda et al., 2011). Moreover, it has been reported that the leaf extract had potent antiproliferative activity and apoptosis inducing capacity on tumor (KB) cell line (Sreelatha et al., 2011), and it also increased the cytotoxicity of chemotherapy on pancreatic cancer cells (Berkovich et al., 2013).

To date, a variety of biological activities of parts of *M. oleifera* have been reported. Nevertheless, there are limited evidences for *M. oleifera* leaf in terms of cancer therapy and prevention. Therefore, the aim of the present study was to investigate the *in vitro* antiproliferative activity of *M. oleifera* leaf extract on three types of human cancer cell lines (HepG2, Caco-2 and MCF-7). Furthermore, the *in vitro* cancer chemoprevention was carried out using the established method, quinone reductase induction assay.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were of analytical grade. The DPPH, ABTS and Folin-Ciocalteu's phenol reagent were purchased from Merck Co. (Darmstadt, Germany). The *β*-napthoflavone, flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADP), menadione, dicoumarol, digitonin, glucose 6-phosphate, crystal violet, glucose 6-phosphate dehydrogenase (G6PD) and MTT were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The alpha-minimum essential medium (*α*-MEM), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and antibiotic-antimycotic reagent for cell culture were purchased from Invitrogen Co. (California, USA).

Plant and extraction

The leaves of *M. oleifera* were collected during November to December, 2012 from Lampang Herb Conservation, Lampang, Thailand. This plant was identified and confirmed by comparing it with voucher specimens of known identity (ID: WP2614) deposited at the Queen Sirikit Botanical Garden, Chiang Mai, Thailand. The air-dried leaves of *M. oleifera* were ground into powder and stored at 4°C until extraction. Fifteen grams of leaf powder were extracted with 350 ml of methanol. Then, the liquid extract was filtered through Whatman no. 1 filter paper. The residue was subsequently extracted with 350 ml of dichloromethane. The filtrates were evaporated and lyophilized to obtain two crude extracts: methanol and dichloromethane extracts (ME and DE). Both extracts were kept in amber glass at -20°C until use.

Determination of total phenolic and flavonoid contents

The Folin-Ciocalteu method was used to determine the amount of total phenolic compound (Singleton et al., 1999). In brief, 100 µl of each diluted extract were mixed with 2.8 ml of deionized water and 2 ml of 50% Folin Ciocalteau's phenol reagent. It was incubated for 30 min at room temperature. The absorbance of the reaction mixture was measured at 765 nanometer (nm). The total phenolic content was expressed as milligram gallic acid equivalent per gram extract (mg GAE/g extract).

The total flavonoid content was determined using the aluminum chloride colorimetric method according to Chang et al. (2002). Briefly, 100 µl of each extract were mixed with 1.5 ml of 95% ethanol, 100 µl of 10% $AlCl_3$, 100 µl of 1 M potassium acetate and 2.8 ml of deionized water. Then, the absorbance of the reaction mixture was determined at 415 nm. The total flavonoid content was expressed as milligram quercetin equivalent per gram extract (mg QE/g extract).

DPPH radical scavenging assay

The free radical scavenging activity was tested according to Mensor et al. (2001). Various concentrations of the extracts were mixed with 80 mM of DPPH in methanol. Then, the solution was incubated for 30 min at room temperature. Quercetin was used as positive control. The optical density (OD) of the solution was measured at 517 nm by a double-beam spectrophotometer. The DPPH radical scavenging activity was calculated using the equation:

$$Inhibition\ (\%) = \frac{(OD_{control} - OD_{extract})}{OD_{control}} \times 100$$

The percentage of DPPH radical scavenging activity was calculated. The 50% inhibitory concentration (IC_{50}) was expressed as the quantity of the extract necessary to react with one half of DPPH radicals.

ABTS radical cation decolorization assay

Following the published method by Re et al. (1999), the protocol for ABTS assay was slightly modified. Briefly, the ABTS radical was prepared in 2.45 mM potassium persulfate. The solution was then left for 15 min in a dark place to obtain an ABTS radical solution. This solution was subsequently diluted with ethanol before use. To the diluted solution, various concentrations of each extract (300 µl) were added. Trolox was used as positive control. After incubating for 8 h in the absence of light, the absorbance was measured at 731 nm. The percentage of ABTS radical scavenging activity was calculated using the following equation:

$$Inhibition\ (\%) = \frac{(OD_{control} - OD_{extract})}{OD_{control}} \times 100$$

The inhibitory percentage was calculated, and the IC_{50} was determined for each extract.

Cell culture

Human hepatocellular carcinoma (HepG2) (ATCC: 77400), colorectal adenocarcinoma (Caco-2) (ATCC: HBT-37) and breast adenocarcinoma (MCF-7) (ATCC: HTB-22) cell lines, and human dermal fibroblast (ATCC: PCS201012) were used in an antiproliferative assay. The cancer cell lines were cultured in 25 cm^2 culture flask using DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The human dermal fibroblast was cultured in fibroblast basal medium supplemented with FBS, glutamine, ascorbic acid, hydrocortisone and hemisuccinate. For a quinone reductase induction assay, the murine hepatoma cells designated as Hepa-1c1c7 (ATCC: CRL2026) were grown in supplemented *α*-MEM. These cells were maintained in a humidified incubator with an atmosphere comprising 5% CO_2 and 95% air at 37°C. The cells were harvested and plated either for cytotoxicity tests or subcultures when they reached 80% confluence.

Antiproliferative assay

The antiproliferation (cytotoxicity) of *M. oleifera* extracts on HepG2, Caco-2, MCF-7, Hepa-1c1c7 and fibroblast was evaluated by the MTT assay (Mosmann, 1983). The cells were plated at 1.0×10^3 cells per well in 96-well plates. Twenty four hours after plating, the cells were incubated with each extract (0 to 250 µg/ml) or cisplatin (anticancer drug) for 48 h at 37°C. The final DMSO (solvent) concentration did not exceed 0.2%. Then, 20 µl of MTT solution (5 mg/ml) were added to each well. The insoluble purple formazan crystal was dissolved in 100 µl of DMSO, and the absorbance was determined at 540 and 630 nm using a microplate reader. The percentage of cell viability was calculated. The concentration of the extract causing 50% inhibition of cancer cell growth was considered as IC_{50}.

Quinone reductase induction assay

The induction of QR activity was measured using the method established by Prochaska and Santamaria (1988), and Kang and Pezzuto (2004). The concentration of extract which allowed more than 70% Hepa-1c1c7 cell viability was used in this assay. Briefly, the cells were plated at 1×10^4 cells/well in 96-well plates, and allowed to adhere for 24 h. The cells were then treated with 0 to 100 µg/ml of each extract, *β*-naphthoflavone (QR inducer), or DMSO (solvent) for 48 h. The final concentration of DMSO did not exceed 0.2%. Duplicate plates were prepared: one for determining QR activity and one for cell density. For determining the QR activity, the cells were lysed and subsequently 200 µl of the reaction mixture was added. After 5 min, the reaction was stopped with 0.3 mM dicoumarol. The absorbance was measured at 610 nm, and the specific activity was calculated. The specific activity of QR is defined as nM/l MTT blue formazan reduced per min. The induction was calculated as the ratio of QR specific activity in the presence and absence of the extract. The concentration required to double the specific activity (CD) was determined by a curve of the ratio of QR specific activities versus concentration. In this assay, crystal violet staining was performed to determine cell density as the population of viable cells used to normalize the QR activity.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) of three-independent experiments. The analysis was performed using analysis of variance (ANOVA). The Bonferroni test with $P < 0.05$ was considered to test for a significant difference between control and treated groups.

RESULTS

Polyphenol and flavonoid contents of *M. oleifera* extracts

The polyphenol content of ME and DE were determined by Folin-Ciocalteau assay. As demonstrated in Table 1, the result showed that amounts of polyphenol were found in both extracts. ME had a higher content (216.45±4.64 mg GAE/g extract) than DE (100.12±3.70 mg GAE/g extract). In this study, the flavonoid content of *M. oleifera* extracts was determined using the aluminum chloride colorimetric method. The amount of flavonoid in ME was 65.38±2.37 mg QE/g extract and in DE 40.14±3.31 mg QE/g extract.

Free radical scavenging activity of *M. oleifera* extracts

The radical scavenging activity of *M. oleifera* extracts was evaluated using two methods including DPPH and ABTS assays. Table 2 presents the antioxidant activity of the extracts. It was found that ME exhibited the higher scavenging activity, with an IC_{50} of 1.60±0.03 mg/ml compared to DE (IC_{50} = 2.31±0.02 mg/ml). Apart from the DPPH assay, ABTS radical cation decolorization was carried out to confirm the antioxidant activity of the extracts. Both extracts showed ABTS free radical scavenging activity similar to the result of the DPPH assay. ME had a higher potential of radical scavenging (1.02±0.06 mg/ml) than DE.

Cancer cell antiproliferation of *M. oleifera* extracts

The antiproliferation was tested on HepG2, Caco-2, MCF-7 and human fibroblast cells. According to Figure 1, it was found that both extracts (0 to 250 µg/ml) contributed to similar cancer cell viability patterns. DE was more cytotoxic than ME. It showed a IC_{50} of 120.37±2.55, 112.46±3.74 and 133.58±2.47 µg/ml for HepG2, Caco-2 and MCF-7, respectively, while ME exhibited less cytotoxicity to all cancer cell lines (IC_{50} > 250 µg/ml). In addition, both extracts were tested in human fibroblast to observe their antiproliferation on normal cells. The results showed that 0 to 400 µg/ml of both extracts had no toxicity on human fibroblast (Figure 2). The cisplatin was used as positive control which was able to inhibit cancer cell proliferation. It had an IC_{50} of 13.34±1.44, 19.45±2.12 and 17.24±2.39 µM for HepG2, Caco-2 and MCF-7, respectively. According to the results, *M. oleifera* extracts not only exhibit antiproliferation on cancer cells, but also

Table 1. Total phenolic and flavonoid contents in *Moringa oleifera* extracts.

Moringa oleifera	Total phenolics (mg GAE/g extract)	Total flavonoid (mg QE/g extract)
Methanol extract	216.45±4.64	65.38±2.37
Dichloromethane extract	100.12±3.70	40.14±3.31

The values are the average of triplicate experiments, and are expressed as mean ± standard error of mean.

Table 2. Antioxidant activity of *Moringa oleifera* extracts.

Treatment	50% Inhibitory concentration	
	ABTS	DPPH
Methanol extract (mg/ml)	1.02±0.06	1.60±0.03
Dichloromethane extract (mg/ml)	3.06±0.11	2.31±0.02
Quercetin (µM)	-	0.06±0.01
Trolox (µM)	6.72±0.51	-

Antioxidant activity of *Moringa oleifera* extracts was determined by the radical scavenging activity of antioxidants against 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH). The values are the average of triplicate experiments, and are expressed as mean ± standard error of mean.

Table 3. Concentration of *Moringa oleifera* extracts to double quinone reductase activity.

Treatment	CD value
Methanol extract (µg/ml)	NI
Dichloromethane extract (µg/ml)	91.36±1.26
β-naphthoflavone (µM)	3.47±0.01

Concentration to double quinone reductase activity (CD value) is calculated and compared with negative control. The CD values are expressed as mean ± standard error of mean. *β*-naphthoflavone is used as an inducer of quinone reductase (positive control). NI = not induced

showed no cytotoxicity on normal cells.

In vitro cancer chemoprevention of *M. oleifera* extracts

Hepa-1c1c7 cells were used as *in vitro* model to measure QR activity. QR was recognized as chemopreventive enzyme. Its activity can be induced by specific inducer. In this assay, the concentration of extract, which did not interfere cell survival, was considered as tested dose. Table 3 shows the concentration of *M. oleifera* extracts to double QR activity. It was found that *β*-naphthoflavone had a CD value of 3.47±0.01 µM. Interestingly, only DE (0 to 100 µg/ml) was capable of inducing QR activity, whereas ME had no inductive effect. DE significantly increased the QR induction ratio in a dose-dependent manner ($P < 0.05$), with a CD value of 91.36±1.26 µg/ml (Figure 3).

DISCUSSION

Although *M. oleifera* has been reported for its benefits and biological activities, little is known scientifically about its antioxidant property and cancer prevention ability. Herein, *M. oleifera* extracts were evaluated for their *in vitro* antioxidant, antiproliferative and chemopreventive activities.

In the etiology of cancer, free radicals are one of the major factors necessary to cause DNA mutation, which in turn triggers the initiation stage of carcinogenesis (Johnson, 2007). Exogenous antioxidants from natural sources can improve the function of the endogenous antioxidant system which is responsible for preventing free radicals in the body (Johnson, 2004). Polyphenol is recognized as a potent antioxidant, and is found in *M. oleifera* extracts (Table 1). Recently, Charoensin and Wongpoomchai (2012) reported that the aqueous extract of *M. oleifera* leaves contained polyphenols and had DPPH radical scavenging activity. Furthermore, there are some reports which claim that *M. oleifera* leaves are rich in polyphenols and flavonoids and have antioxidant activity (Luqman et al., 2011; Santos et al., 2012). In accordance with the previous works, *M. oleifera* leaves extracted with methanol and dichloromethane also showed antioxidant activity (Table 2). The chemical analysis of *M. oleifera* extracted with methanol had shown that the major polyphenols comprised of gallic acid, quercetin and kaempferol (Sreelatha et al., 2011).

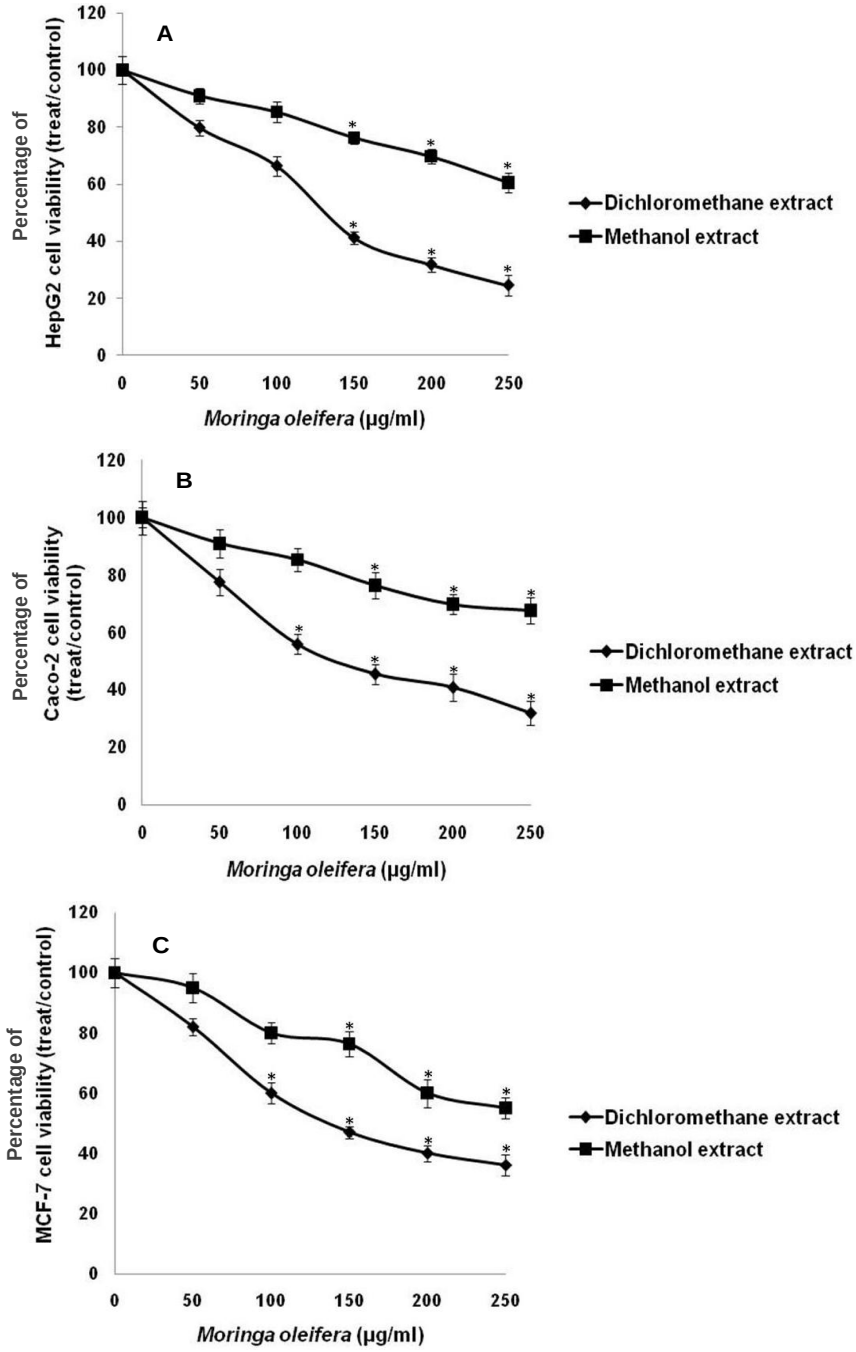

Figure 1. Effect of *Moringa oleifera* extracts on three kinds of cancer cell viability; HepG2 (A), Caco-2 (B) and MCF-7 (C). Each cancer cell type was incubated with various concentrations of extracts (0 – 250 µg/ml) for 48 h. Data are obtained from three independent experiments, and are shown as mean ± standard error of mean.
An asterisk (*) indicates significant difference ($p<0.05$) between negative control and treated groups.

Due to this reports, this might be the important evidence for ME having higher amounts of polyphenols than DE.

Accumulating reports have suggested that many naturally-occurring substances exhibit cancer chemotherapeutic effects (Khan et al., 2013). The main advantage advantage of using phytochemicals as anticancer agents is that they seem to have low adverse effects, and are more cost-effective than commercial drugs. Therefore, it is worth searching for new biologically-active phytochemicals. The present study is the first report of *M. oleifera*

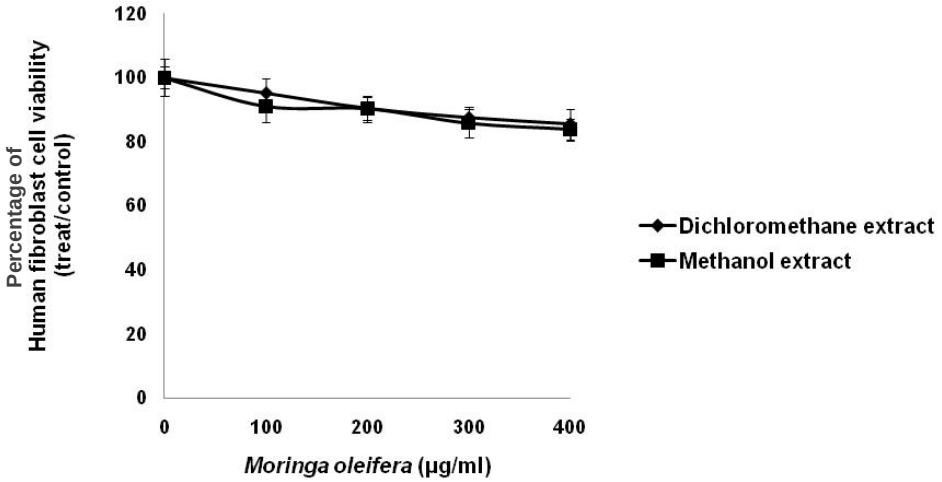

Figure 2. Cell viability of human fibroblast after treatment with *Moringa oleifera* extracts (0 – 400 µg/ml) for 48 h. Data are obtained from three independent experiments, and are shown as mean ± standard error of mean.

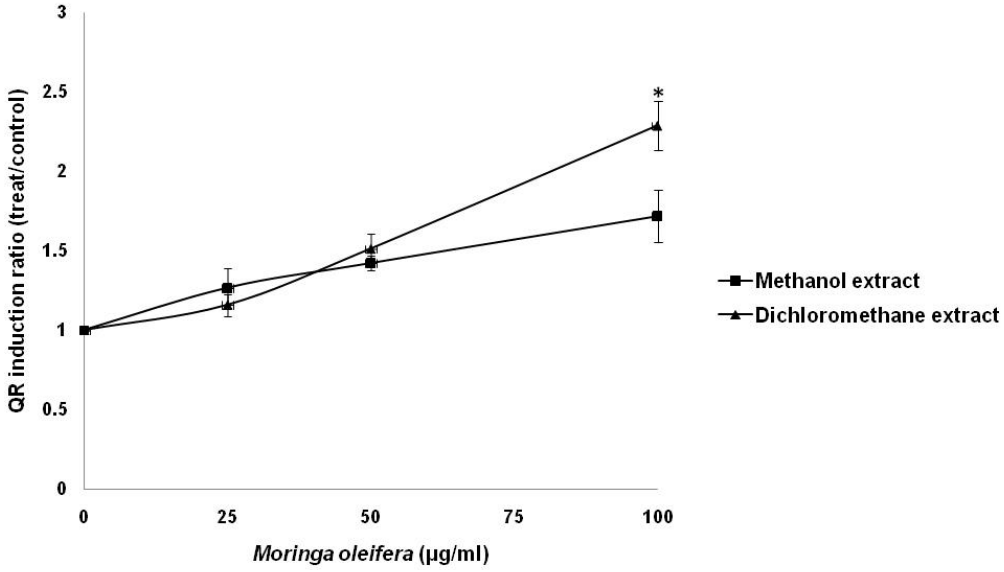

Figure 3. Quinone reductase (QR) induction by *Moringa oleifera* extracts. Hepa-1c1c7 cells were treated with 0 - 100 µg/ml of each extract, *β*-naphthoflavone (QR inducer), or DMSO (solvent) for 48 h Data are obtained from three independent experiments, and are shown as mean ± standard error of mean.
An asterisk (*) indicates significant difference of QR induction ($P < 0.05$) between negative control and treated groups.

extract regarding antiproliferations of HepG2, Caco-2 and MCF-7 cancer cells, which are not reported elsewhere. Moreover, it confirms the previous studies of cytotoxicity of *M. oleifera* extracts on human cancer cells such as pancreatic cancer cell (Panc-1) (Berkovich et al., 2013), colon cancer cells (SW480 and HCT18) (Pamok et al., 2011), and KB tumor cell (Sreelatha et al., 2011). It was found that DE had more potent cytotoxicity than ME on all cancer cell lines (Figure 1). According to the present work, each extract differently inhibited cell proliferation. This might be partly due to the differences in genotype and phenotype of cancerous cells and the active compounds in each extract. Different types of cancers have different mutational signatures (Alexandrov et al., 2013). The certain genes responsible for cell cycle and cell death are mutated in cancer cells, whereas all genes

in normal cells still remain original (Nik-Zainal et al., 2012). The mechanism underlying inhibition of cancer and normal cell proliferation is therefore determined by genetic differences which cause in both types of cells specificity and sensitivity to *M. oleifera* extract. However, the molecular mechanism by which the extract modulates cancer cell proliferation (cell cycle) and death (apoptosis) remains elusive and needs further investigation.

Although *M. oleifera* has variously biological activities, most of them rely on pod, seed and flower. As a result, it is needed to investigate more biological functions of its leaves. Since there are limited reports regarding cancer prevention, the present study aimed to evaluate chemopreventive properties. In cancer research, there are many standard methods to evaluate whether a test sample is chemopreventive (Knasmuller et al., 2002). QR or NADPH:quinone oxidoreductase 1 (NQO1) is a phase II detoxifying enzyme and catalyzes the 2-electron reduction of a broad range of chemicals especially quinones. The 2-electron reduction of quinones to hydroquinones by QR is believed to be primarily a detoxifying reaction since it bypasses the formation of the carcinogenic semiquinone and other chemicals (Cuendet et al., 2006). It also protects cells against reactive oxygen species generated by quinones and related compounds (Gerhäuser et al., 2003). Elevated QR levels correlate with prevention of *in vivo* chemical-induced carcinogenesis in the stage of initiation and promotion (Cuendet et al., 2006). Furthermore, with advantages in terms of reliability, high throughput and less-time consumption, the *in vitro* assay, particularly cell-based testing system relevant for prevention of *in vivo* carcinogenesis, has been established and used in laboratories extensively (Gerhäuser et al., 2003). For these reasons, QR is widely used as the anticarcinogenic phase II marker enzyme for evaluating cancer chemopreventive agents rather than other enzymes (Kang and Pezzuto, 2004). In the present work, *M. oleifera* extracts were assayed for their ability to induce QR activity on Hepa1c1c7 cells. It was seen that DE could induce QR activity, whereas ME had no inductive effect (Figure 3). In recent reports, it was shown that the hot water extract of *M. oleifera* leaves had high polyphenols and antioxidant activity. It also showed potent QR induction (CD value = 99.70±10.44 µg/ml) and antimutagenicity against 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide induced mutagenesis (Charoensin and Wongpoomchai, 2010, 2012). The theory of QR induction in the context of structure-activity relationship is described. The flavonoids with 2 or 3 double bonds in the C ring are crucially essential for QR induction, while the hydroxylation of the B ring is not essential (Uda et al., 1997). The mechanism by which polyphenols and flavonoids induce QR gene expression is well understood. The induction of QR gene is regulated on the transcriptional level mediated by antioxidant response element (ARE), controlled by the nuclear factor E2-related factor 2 (Nrf2) (Nguyen et al., 2009). Activation of the Nrf2/ARE pathway by polyphenols with antioxidant activity (quercetin and kaempferol), or by non-flavonoid compounds (glucosinolate and sulforaphane) is the key step of QR gene up-regulation (Uda et al., 1997; Hwang and Jeffery, 2005). This molecular mechanism leads to the increased level of QR. From the present result, it was seen that *M. oleifera* extracted with dichloromethane induced a QR activity with higher potential than that extracted with methanol. Regarding polyphenols and flavonoids, both compounds were determined in DE, with relative amounts of that of ME. This could be the major factor for increased QR activity and cancer cell antiproliferation. Apart from flavonoids, *M. oleifera* leaves have been reported to release glucosinolate compounds, when extracted with less polar solvent. 4-(alpha-l-rhamnopyranosyloxy)-benzylglucosinolate and three monoacetyl isomers were isolated from the leaves (Bennett et al., 2003). In addition, the glycosides including niaziminin A and B, and isothiocyanates were reported (Faizi et al., 1995). This is the significant reason regarding the potent QR induction as well as the cancer cell antiproliferation of DE. Hence, both effects of *M. oleifera* might arise from the flavonoids alone, or from the synergy with other compounds.

Conclusion

Conclusively, the *M. oleifera* dichloromethane extract shows high antioxidant activity, potent cancer cell antiproliferation, and induction of quinone reductase. These findings indicate the medicinal value of *M. oleifera* in terms of cancer chemotherapy and chemoprevention.

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Full Length Research Paper

Antibacterial and cytotoxic antibacterial potential of ethanol extract and fractions from *Aristolochia galeata* Mart. ex Zucc

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The aim of this investigation was to evaluate the phytochemical, antibacterial and cytotoxic activities of ethanol extract and hexane, dichlorometane, ethyl acetate and hydroethanolic fractions from *Aristolochia galeata's* rhizomes. The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) were evaluated by the broth microdilution assay to investigate the antibacterial activity of various extracts and fractions of *A. galeata* against Gram-positive and Gram-negative bacteria. The cytotoxicity of plant samples was evaluated in human cervix carcinoma cell line (HeLa) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The phytochemical study showed the presence of main secondary metabolites, steroids, flavonoids, coumarins and alkaloids. The ethanol extract and their fractions presented antimicrobial activity against Gram-positive bacteria, and *Staphylococcus aureus* was regarded the most sensitive strain with MIC of 250 µg/ml for the ethanol extract. The dichlorometane fraction showed bactericidal activity with the value of 1250 µg/ml and moderate cytotoxicity in front of the HeLa cell line tested (CC_{50} = 90 µg/ml). The results showed that *A. galeata* had effective antibacterial activity against Gram-positive bacteria and compounds extracted from *A. galeata* Mart. ex Zucc could be used as possible antimicrobials. The good antimicrobial activity and the low cytotoxicity presented by the hexane fraction can be promised for the new molecules with antibiotic activity.

Key words: *Aristolochia galeata*, antibacterial activity, minimal inhibitory concentration, cytotoxic activity, phytochemistry.

INTRODUCTION

The *Aristolochia* genus presents approximately 400 species distributed in areas from tropics to the temperate

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zones (Wu et al., 2001). *Aristolochia galeata* Mart. ex Zucc (Aristolochiaceae Family), is a native climber with a wide distribution in the Brazilian Cerrado biome, associated with road and gallery forest edges (Alves-Da-Silva et al., 2011). Some studies have indicated that many plants of *Aristolochia* genus have therapeutic properties such as analgesic, anti-diuretic, anti-inflammatory, antimicrobial, antioxidant and antiparasitic (Shafi et al., 2002; Yu et al., 2007; Pacheco et al., 2010; Papuc et al., 2010; Ahmed et al., 2010). However, many species of *Aristolochia* genus contain aristolochic acids, which can cause nephrotoxicity and mutagenicity (Kohara et al., 2002; Chen et al., 2007). Therefore, many countries have prohibited the use of phytotherapic drugs containing aristolochic acid and thus, numerous plant species with bioactive properties are no longer used, despite the possibility of separating the potentially toxic compounds (Yu et al., 2007).

Although there are no registers of antimicrobial activity of *A. galeata*, some compounds have already been isolated, and among them, stand out clerodane and labdane diterpenoids (Lopes and Bolzani, 1988). Compounds of these classes isolated from other species, present diverse biological properties, such as antiviral, antifungal and antibacterial (Salah et al., 2003; Vidal et al., 2011; Porto et al., 2012).

Studies on new therapeutic options from herbal products as antimicrobials are necessary, since bacterial infections have grown significantly, contributing to increased morbidity and mortality, especially in hospitalized and immunocompromised patients (Zhong et al., 2012; Schmitt et al., 2012; Pandey et al., 2012). Allied to the increased infections, treatment is becoming increasingly difficult in view of the notable ability of these pathogens to acquire new mechanisms of selective resistance to antibiotics (Tenover, 2006).

Therefore, in front of the lack of studies on phytochemistry, antimicrobial and cytotoxicity activity of *A. galeata* with the potential use of medicinal plants in the treatment of diseases caused by Gram-negative and -positive bacteria. The current investigation carried out a screning of ethanol extract and hexane, dichloromethane, ethyl acetate and hydroethanolic fractions of *A. galeata* against important pathogenic bacteria and evaluates its cytotoxicity potential to develop new antibacterial therapy.

MATERIALS AND METHODS

Plant and extract preparation

A. galeata rhizomes were collected in São Sebastião do Oeste, Minas Gerais, Brazil, located in coordinates -20° 14' 38.96''S and -45° 2' 14.38''W, altitude of 712 m, in August 2011. A voucher specimen (BHCB 159396) was deposited at the Instituto de Ciências Biológicas Herbarium, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. The plant material (486.95 g) was dried at 40°C, triturated and extracted by cold maceration in ethanol P.A (Vetec, Brazil) for a period of 10 days at room temperature. The extract was filtrate and concentrated in a rotary evaporator (IKA equipment, model RV10, Germany) at 40°C under reduced pressure to yield ethanol extract. The dried extract (14.28 g) was obtained after lyophilization (Liobras equipment, model K 105, Brazil). Part of this extract (4 g) was dissolved in ethanol/water (7:3) (Vetec, Brazil) and then partitioned successively with hexane, dichloromethane and ethyl acetate (Vetec, Brazil) 15 ml, three times with each solvent, resulting in 0.471, 0.783, 0.823 and 1.741 g of hexane (F1), dichloromethane (F2), ethyl acetate (F3), and hydroalcoholic (F4) fractions, respectively. The extract and fractions were screened for the presence of different phytoconstituents like saponins, tannins, alkaloids, steroids, triterpenes, coumarins and flavonoids (Wagner et al., 2001).

Bacterial strains and antimicrobial tests

The minimum inhibitory concentration (MIC) of *A. galeata* ethanol extract and their fractions were determined using a broth microdilution method as described by Clinical and Laboratory Standards Institute (CLSI, 2003) with modifications. Nine reference bacterial strains of American Type Culture Collection (ATCC) were chosen due to their ability of present multi resistant to the drugs as follows: Gram-negative *Escherichia coli* EHEC (ATCC 43895), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 27736), *Salmonella typhi* (ATCC 19430) and Gram-positive *Staphylococcus aureus* (ATCC 29213), *Streptococcus mutans* (ATCC 25175), *Staphylococcus saprophyticus* (ATCC 15305), *Staphylococcus epidermides* (ATCC 12228), *Enterococcus faecalis* (ATCC 19433), donated by the Laboratory for Reference Microorganisms of the Oswaldo Cruz Foundation, FIOCRUZ, Brazil.

The ethanol extract and fractions were dissolved in sterile dimethylsulfoxide 2% (DMSO) (Synth, Brazil) and were used in serial dilution from of 1250 until 125 µg/ml. An inoculum of 125 µl of cell culture was added to 25 µl of each concentration of samples in Mueller-Hinton broth (MH) (Himedia, India) in 96-well plates. For negative controls, wells containing MH medium or sterile DMSO 2% were used and for positive control, MH plus bacteria and the antimicrobial agent streptomycin 100 µg/ml (Sigma-Aldrich, USA) were used (growth inhibition). Plates were incubated at 35 ± 1°C for 24 h.

The MIC was assessed based on the lowest concentration of sample required to inhibit the microbial growth and was determined by measuring the absorbance at 490 nm (Powder Wave XS2, Biotec, USA). The experiments were performed in triplicate.

For assays to determine the minimum lethal concentration (MLC), aliquots of 25 µl were removed from wells without visible turbidity and placed on Agar Plate-count by a Pour-Plate Method (Costa et al., 2010). After incubation at 37°C for 24 h, colonies were counted. The concentration of sample that resulted in a growth 0.1% of initial inoculum (1.5×10^6 UFC/ml) was determined as the MLC.

Cytotoxicity analysis by the MTT assay

Human cervix carcinoma cell line (HeLa) was grew in Dulbecco's Modified Eagle Medium (DMEM) with 2% of Fetal Bovine Serum (FBS), at 37°C, 5% of CO_2 atmosphere and in 96-well microplate, until it reaches 95% of confluence. After 72 h exposure at dosages from 1000 to 0.025 µg/ml, 20 µl (2 mg/ml) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Bio Basic INC, Canada) in phosphate buffered saline (PBS) were added on each well and the plate was incubated at 37°C for 3 h. The medium was removed and 130 µl of DMSO were added and after incubation at37°C for 10 min, the absorbance was read at 540 nm in ELISA spectrophotometer (Powder Wave XS2, Biotec, USA) to determine the concentration that killed 50% of cells (CC_{50}) (Twentyman and

Table 1. Phytochemical study of ethanol extract (CE) and hexane (F1), dichlorometane (F2), ethyl acetate **(**F3) and hydroethanolic (F4) fractions from of *Aristolochia galeata*.

Plant material	Steroids/Triterpenoids	Flavonoids	Coumarins	Saponins	Alkaloids	Tanins
CE	+	+	+++	-	+	-
F1	+/-	-	+	-	+	-
F2	+++	+/-	+	-	+	-
F3	+	+++	-	-	+/-	-
F4	+/-	+/-	-	-	+/-	-

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

Luscombe, 1987). The cytotoxicity was calculated after comparing with the control (treated with 0.1% DMSO).

Statistical analyses

All tests were made in triplicate in three independent experiments. When appropriate, mean ± standard deviation were used to describe the results. The mean effective concentration CC_{50} values (concentration that reduces 50% of control group response) were determined by non-linear regression using Graph Pad Prism, 5.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Phytochemical analysis

Phytochemical analysis of *A. galeata* (Table 1) showed the presence of steroids/triterpenoids, flavonoids, coumarins and alkaloids in the ethanol extract. Majority of secondary metabolites found in the fractions ethyl acetate and dichloromethane were flavonoids and steroids/ triterpenoids, respectively. Alkaloids, coumarins and a minimal presence of steroids were detected in the hexane fraction. Saponins and tannins were not observed.

Antibacterial activity

The MIC and MLC values from crude extract of *A. galeata* are shown in Table 2. This extract showed bacteriostatic activity in Gram-positive bacteria, and *S. aureus* strain appeared to be the most sensitive bacteria with MIC of 250 µg/ml. However, no Gram-negative bacteria showed sensitivity to the extract.

Following the antibacterial evaluation of crude extracts, the fractions were tested. Like the crude extract, Gram-positive bacteria showed sensitivity (Table 2). The hexane and dichloromethane had MIC ranging from 500 to 1000 µg/ml. The most sensitive bacterial samples were *S. epidermidis* and *S. aureus*, with MIC of 500 µg/ml. The MLC was only observed for the fraction of dichlorometane of 1250 µg/ml against *S. aureus* and *S. saprohyticus*. The ethyl acetate and hydroethanolic fractions showed no bactericidal or bacteriostatic activity. The DMSO used as a negative control, showed no bacteriostatic or bactericidal activity, as expected (data not show) (Table 3).

Cytotoxicity activity

Analyzing the *A. galeata* cytotoxicity *in vitro*, the results show that the dichloromethane fraction presented moderate cytotoxic effect with CC_{50} = 90 µg/ml (Table 3). However, the crude extract and other fractions showed little cytotoxicity activity with the CC_{50} ranging between 1620 and 369 µg/ml.

DISCUSSION

Many studies have demonstrated the antibacterial activity of plants commonly used in traditional medicine (Rakholiya and Chanda, 2012; Tekwu et al., 2012, Mishra et al., 2013a. Rios and Recio (2005) suggested that MIC greater than 1 mg/ml for crude extracts or 0.1 mg/ml for isolated compounds should be avoided and proposed that activity would be very interesting in MICs of 0.1 and 0.01 mg/ml for extracts and isolated compounds, respectively. On the other hand, Fabry et al. (1998) defined active crude extracts as those having MIC values <8 mg/ml. In this study, however, MIC and MFC values of less than 1 mg/ml were considered to be of good activity.

Our results showed that the crude extracts of *A. galeata*, and their fractions presented selective antibacterial activity against Gram-positive bacteria. It is not known exactly why Gram-negative bacteria typically have lower sensitivity to components of plant extracts, but this may be related to the chemically more complex cell wall with the presence of additional membrane that can act as a selective barrier compared to the Gram-positive bacteria (Deans and Ritchie, 1987; Srinivasan et al., 2001; Nikaido, 2003).

Gram-positive bacteria belonging to the genera *Staphylococcus* and *Enterococcus*, with clinical relevance, showed sensitivity to *A. galeata*. These genera are the most frequent cause of nosocomial infections by Gram-positive strains, and the species *S. aureus,*

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MLC) (µg/ml) from *A. galeata* against gram-positive and gram-negative bacteria. Ethanol extract (CE) and hexane (F1), dichlorometane (F2), ethyl acetate (F3) and hydroethanolic (F4) fractions were evaluated.

Bacteria strain (ATCC)	CE		F1		F2		F3		F4		Streptomycin[a]	
	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC[b]
S. aureus (29 213)	250	-	750	-	500	1250	-	-	-	-	3.9	-
S. mutans (25 175)	500	-	750	-	750	-	-	-	-	-	3.9	-
E. faecalis (19 433)	500	-	1000	-	750	-	-	-	-	-	62.5	-
S. epidermides (12 228)	750	-	500	-	1000	-	-	-	-	-	3.9	-
S. saprophyticus (15 305)	750	-	750	-	750	1250	-	-	-	-	1.95	-
E. coli EHEC (43 895)	-	-	-	-	-	-	-	-	-	-	3.9	-
K. pneumoniae (27 736)	-	-	-	-	-	-	-	-	-	-	3.9	-
P. aeruginosa (27 853)	-	-	-	-	-	-	-	-	-	-	7.81	-
S. tiphi (19 430)	-	-	-	-	-	-	-	-	-	-	3.9	-

[a]Positive control; [b]Test not performed; (-) no activity.

Table 3. Effects of *A. galeata* and their fractions on HeLa cells by MTT colorimetric assay. The ethanol extract (CE) and hexane (F1), dichlorometane (F2), ethyl acetate (F3) and hydroethanolic (F4) fractions were evaluated.

Plant material	CC_{50}[a] (µg/ml)
CE	369 ± 0.06
F1	726 ± 0.05
F2	90 ± 0.08
F3	1620 ± 0.02
F4	1340 ± 0.06

[a]CC_{50} values were expressed as the mean±SD, determined from the results of MTT assay in triplicate experiments.

S. epidermidis, S. saprophyticus and *E. faecalis* are the most common in this scenario (Boneca and Chiosis, 2003; Kuroda et al., 2005). These microorganisms may acquire resistance against antibacterial drugs by a variety of mechanisms that includes drug modification or destruction, alteration of binding sites, altered metabolism, and prevention of drug entry into the cell (Tenover, 2006; Barie, 2012).

The bacterial samples analyzed presented great sensitivity to aminoglycoside streptomycin used in this study. However, many studies have reported the resistance of both Gram-positive and Gram-negative bacteria to this class of antibiotics. This fact justifies the search for new molecules with antibacterial properties (Josephson, 2006; Coutinho et al., 2010; Zhong et al., 2012).

The *Aristolochiaceae* genus has revealed its potential as antimicrobial agent. *Aristolochia* species have also been described as getting antibacterial properties against strains of medical importance (Shafi et al., 2002). The *A. esperanzae* species showed antibacterial activity against samples of *S. aureus, E. coli, S. typhimurium, B. cereus*, *C. freundii* and *L. monocytogenes* (Pacheco et al., 2010). Some species of this genus stood out due to the potential cytotoxicity of cancer cell lines (Yu et al., 2007). In our study*, A. galeata* presented low cytotoxicity in HeLa cells, except for dichloromethane fraction. The presence of large amounts of steroids/triterpenoids on the dichloromethane fraction may be related with the antimicrobial activity. These results corroborate with literature data that showed antistaphylococcal activity of terpenes (Gibbons, 2004) and antifungal of steroids (Johann et al., 2010). However, the moderate cytotoxicity shown for this fraction (CC_{50} = 90 µg/ml) may be related to compounds belonging to this class, such as sesquiterpenes, very common in species of this genus (Yu et al., 2007).

Coumarins and alkaloids were the main secondary metabolites found in the hexane fraction, which also demonstrated antibacterial activity. These classes of compounds are also described in the literature as natural antimicrobial agents (Cottiglia et al., 2001; Gibbons, 2004; Mishra et al., 2009; Mishra et al., 2013b). The hexane fraction has been promising, because besides having good antibacterial activity it also showed low cytotoxicity (CC_{50} = 0.726 mg/ml) against the cell line studied. Moreover, the hydrophobicity of this fraction determines the minimal presence of triterpenoids compounds and aristolochic acid, which is expected, because these substances have cytotoxic and nephrotoxic effects.

Conclusion

The results of the present study revealed for the first time evidence for prospect in *A. galeata* to new molecules with antibacterial activity, especially in the hexane fraction. It is relevant in front of the growing resistance that bacterial strains have shown to actual drugs.

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Full Length Research Paper

An anatomical study of medicinal species *Ajuga orientalis* L. (Lamiaceae) from Turkey

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Ajuga orientalis **(Lamiaceae), with a wide distribution area in Turkey, is a traditionally used medicine in the treatment of some skin diseases in Anatolia. The aim of this study was to determine the anatomical characteristics of the root, stem, leaf, petiole, calyx and corolla of medicinal species of *A. orientalis* in cross sections. As a result of the study, it was found out that the pith rays of the root composed 3 to 4 rowed cells and stem was quadrangular. The shape of pith cell in the stem was ovaidal-polygon. There were glandular and non-glandular hairs on the surface layers on stem, leaves, petiole, calyx and corolla. Starch particles were also detected in the cortex cells of stem. The stomata were diastatic and the leaf was bifacial. There were one big vascular bundle in the center and 4 to 5 small vascular bundles on each corner of the petiole. It was also determined that adaxial epidermis cell shapes of corolla are papillose type.**

Key words: *Ajuga orientalis*, anatomy, medicinal plant, Turkey.

INTRODUCTION

The Lamiaceae is a large family. Many species of Lamiaceae are shrubby and herbs (Heywood, 1978). The family has a cosmopolitan distribution. The Lamiaceae includes more than 250 genera and approximately 7000 species (Thorne, 1992). According to Başer (1993), Turkey is accepted as a gene center for this family. Many taxa of this family are aromatic and are often used as herb spices, folk medicines and fragrances (Werker et al., 1985). With their pleasant fragrance, many species of Lamiaceae have been used as herbal teas in Turkey. Many species are used as raw material in the cosmetic industry. Some species are traditionally used as medicinal plants (Baytop, 1984). It was reported that some *Ajuga* L. and *Salvia* L. species are cultivated as ornamental plants (Baytop, 1984; Özdemir and Şenel, 2001; Akçin et al., 2006). In addition to this, Lamiaceae has great importance due to its economical value and its variety of species.

The genus *Ajuga* belongs to Lamiaceae family and is represented in Turkey by 13 species and 22 taxa, 6 species and 1 subspecies being endemic (Davis et al., 1982, (1982 - 1988)). *Ajuga* species are used in folk medicine in different parts of the world for the treatment of rheumatism, gout, asthma, diabetes, malaria, ulcers and diarrhea and have antibacterial, antitumor, anti-feedant, and vulnerary properties (Chen et al., 1996; Ben Jannet et al., 2000). Baytop (1999) reported that some *Ajuga* species generally known as “mayasıl otu” in Turkey have been widely used in the Turkish folk medicine for their aromatic, diuretic, antipyretic, tonic, diaphoretic, astringent, bitter and homeopathic properties. *Ajuga orientalis* one of the species of *Ajuga* genus is used against some skin diseases caused by hugging (Koyuncu et al., 2010).

Although many species of Lamiaceae family are investigated anatomically (Çobanoğlu, 1988; Uysal et al., 1991; Özdemir and Altan, 2005; Aktaş et al., 2009); there is no anatomical study of *A. orientalis* in literature. Due to its importance, the study was carried out to determine the

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anatomical features of *A. orientalis.*

MATERIALS AND METHODS

A. orientalis were collected during the flowering period and natural populations in A5 Amasya (in the vicinity of Direkli village, open areas, at 1800 m, June 2012, İÖztürk Çalı - 461) which is a city in the Black Sea region of Turkey. Its taxonomical description was made according to the description given by Davis (1982).

Anatomical investigations were performed using an average of fresh specimens kept in 70% alcohol. Cross sections of root, stem, leave, petiole, calyx and corolla were taken from 30 specimens of *A. orientalis* and 50 anatomical measurements (to determine the minimum and maximum values of width-height measurements in various tissues) were conducted for each parameter. Transverse sections were made by hand using commercial razor blades and stained with Sartur reactive (Çelebioğlu and Baytop, 1949). Measurements in the sections were taken under a Leica ICC_{50} HD binocular light microscope by using a Leica Digital Camera and objectives used were ×10 and ×40. Pictures were taken with a Leica ICC_{50} HD binocular light microscope and a Leica Digital Camera.

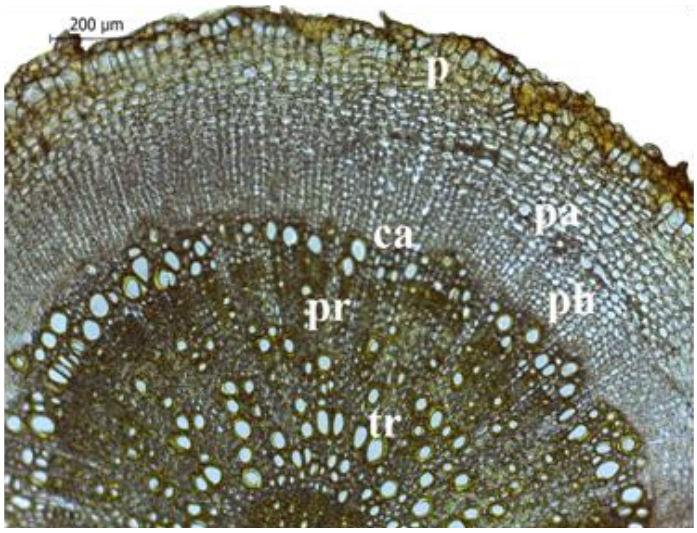


Figure 1. The cross section of root of *A. orientalis* **p:** periderm, **pa:** parenchyma cell, **ph:** phloem, **ca:** cambium, **pr:** pith rays, **tr:** trachea cell.

RESULTS

In cross-sections taken from the root, stem, leaf, petiole, calyx and corolla of *A. orientalis*, the following significant properties were observed.

In the transverse section of the root, there was a periderm which made up the outermost layer of the root of *A. orientalis* (Figure 1). The periderm had 8 to 9 layers. The dimensions of periderm cells were 15 to 60 ×15 to 57.5 µm (Table 1). Beneath the periderm, there was the multi-layered cortex, composed ovaidal and parancyhmatic cells. The cortex was 9 to 10 layers. The cambium, that composed 3 to 4 layers, was located between the xylem and the phloem. Beneath the cambium, there were xylem tissue (7.5 to 15 × 7.5 to 20 µm) composed regular trachea and tracheid cells. Xylem cells were also presented in the center, therefore the pith is not seen in the center. There were 3 to 4 layers primary pith rays between the secondary xylem cells.

A. orientalis, as a typical characteristic of the Lamiaceae, had a 4-angle stem. Its epidermis usually had one layer composed of ovoidal cells (12.5 to 42.5 × 17.5 to 50 µm). The upper surface was covered with a cuticle (1.25 to 2.5 µm). There were glandular and non-glandular hairs on the epidermis (Figure 2). Transverse section of the stem revealed 9 to 10 layered collenchyma placed on the corners. The cortex was composed of 6 to 7 layered paranchymatous cell. The dimensions of paranchymatous cells were 27.5 to 75 × 15 to 55 µm (Table 1). Starch particles were also observed in the paranchymatous cells of stem (Figure 3). There were 2 to 3 layers of sclerenchyma in the outer side of phloem. Right beneath the phloem, there was a 1 to 2 rowed cambium layer. The xylem tissue existing beneath the cambium was composed of regular trachea and tracheid cells. The vascular bundles were bigger on the corner than other parts of stem. Between the corners, there were also numerous small bundles in the *A. orientalis* stem. The vascular bundles were collateral (Figure 2). The pith was wide and consist of flat cells with intercellular spaces. There was a cavity in the centre of the pith.

In the leaves, the epidermis was single layered on upper and lower surface. There were glandular and non-glandular hairs on epidermis. The cuticle was 2.5 to 5 µm thick. Just beneath the upper epidermis cells, there were 2 to 3 rowed palisade parenchyma cells (Figure 5). The dimensions of the palisade parenchyma cells were 12.5 to 25 × 25 to 50 µm (Table 1). The 2 to 3 rowed spongy parenchyma existed beneath the palisade. The spongy parenchyma covered less space than the palisade parenchyma. The collateral vascular bundle was located in the midrib region (Figure 5). There were sclerenchyma cells in the outer side of phloem. All vascular bundles in the leaves were surrounded by bundle sheet cells. The stoma was diastic and the leaf was bifacial (Figures 4 and 5). The stoma was presented on upper and lower surfaces of the leaf (Figure 4).

Both adaxial and abaxial epidermis cells were single layered in the petiole (Figure 6). The dimensions of the adaxial epidermis cell were 15 to 45 ×17.5 to 27.5 µm, while those of the abaxial epidermis were 12.5 to 27.5 × 15 to 17.5 µm (Table 1). There were a lot of glandular and non-glandular hairs on epidermal cells which were ovoidal-rectangular shapes. Parenchymatic cortex cells were 10 to 11 layered. There was one big vascular bundle in the center and 4 to 5 small vascular bundles at each corners of petiole. The vascular bundles were surrounded by sclerenchymatic cells (Figure 6). There were

Table 1. Anatomical measurements of various tissues of *A. Orientalis*.

Parameter	Width (µm) Min.-Max.	Height (µm) Min.-Max.
Root		
Peridermis cell	15 - 60	15 - 57.5
Parenchyma cell	12.5 - 42.5	12.5 - 22.5
Trachea cell	7.5 - 15	7.5 - 20
Stem		
Cuticle	1.25 - 2.5	-
Epidermis cell	12.5 - 42.5	17.5 - 50
Parenchyma cell	27.5 - 75	15 - 55
Trachea cell	10 - 25	10 - 20
Leaf		
Cuticle	2.5 - 5	-
Adaxial epidermis cell	12.5 - 50	15 - 37.5
Abaxial epidermis cell	12.5 - 40	15 - 32.5
Palisade cell	12.5 - 25	25 - 50
Spongy cell	15 - 62.5	-
Mesophyll region	170 - 300	-
Palisade region	70 - 162.5	-
Spongy region	100 - 137.5	-
Petiole		
Adaxial epidermis cell	15 - 45	17.5 – 27.5
Abaxial epidermis cell	12.5 - 27.5	15 – 17.5
Parenchyma cell	20 - 85	17.5 – 70
Trachea cell	10 - 17.5	7.5 – 17.5
Calyx		
Adaxial cuticle	1.25 - 2.5	-
Adaxial epidermis cell	7.5 - 17.5	7.5 – 15
Abaxial cuticle	2.5 - 3.75	-
Abaxial epidermis cell	12.5 - 40	12.5 – 40
Parenchyma cell	12.5 - 27.5	12.5 – 25
Corolla		
Adaxial cuticle	1.25 - 2.5	-
Adaxial epidermis cell	7.5 - 25	10 - 37.5
Abaxial cuticle	2.5 - 3.75	-
Abaxial epidermis cell	7.5 - 32.5	12.5 - 42.5
Parenchyma cell	10 - 30	10 - 25

parenchmatic bundle sheets on the all vascular bundles. The type of vascular bundle was collateral (Figure 6). There were 2 to 3 layered collenchyma in the area between the corners.

In the calyx, the adaxial epidermis cells were smaller than abaxial epidermis cells. The dimensions of adaxial cuticle were 1.25 to 2.5 µm, whereas those of the abaxial cuticle is 2.5 to 3.75 µm (Table 1). Parenchymatic cells were flat ovoidal. There were glandular and non glandular hairs on the epidermis (Figure 7a).

In the cross-section of corolla, cuticle was present on both abaxial and adaxial epidermis cells covered by glandular and non-glandular hairs. The shapes of adaxial epidermis cells were papillose type (Figure 8). Beneath the adaxial epidermis, there were the parenchyma cells with intercellular spaces (Figure 7b). There was a vascular

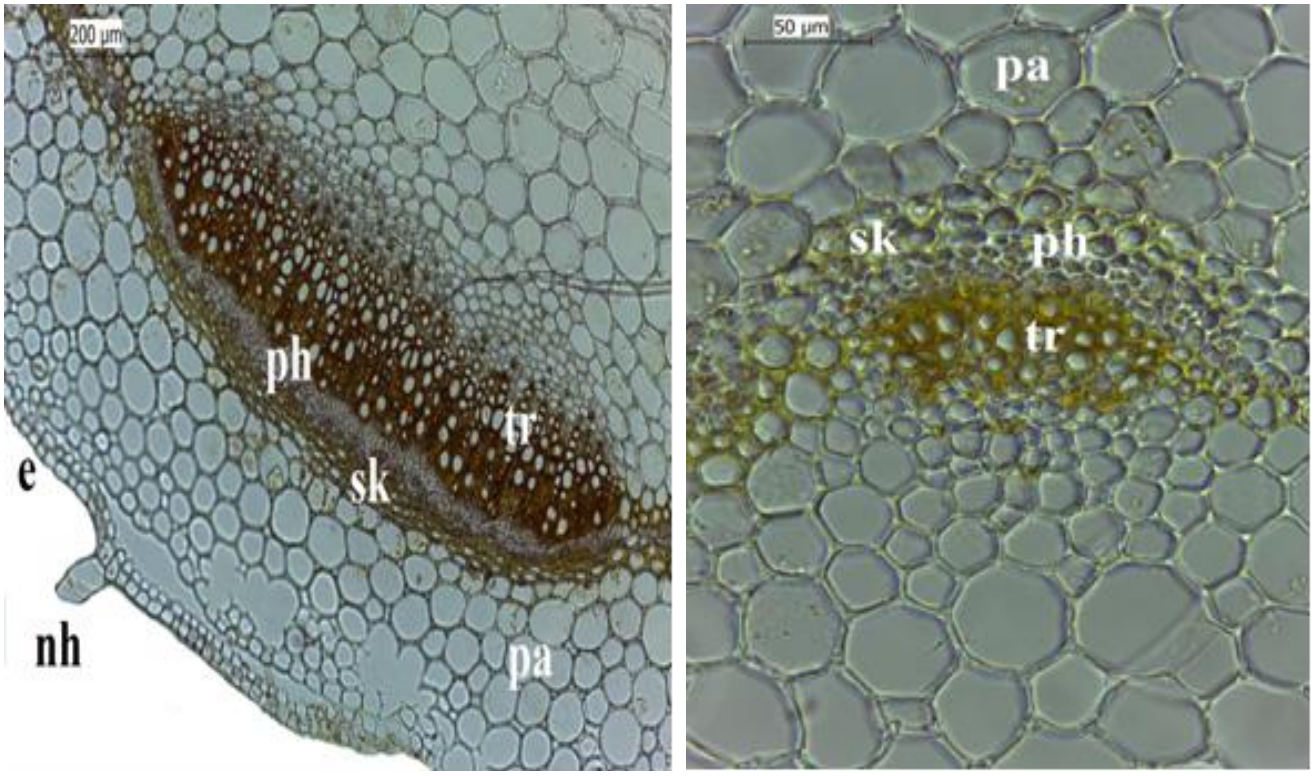


Figure 2. The cross sections of stem of *A. Orientalis*. nh: Non-glandular hair, e: epidermis, pa: parenchyma cell, sk: sclerenchyma, ph: phloem, tr: trachea cell.

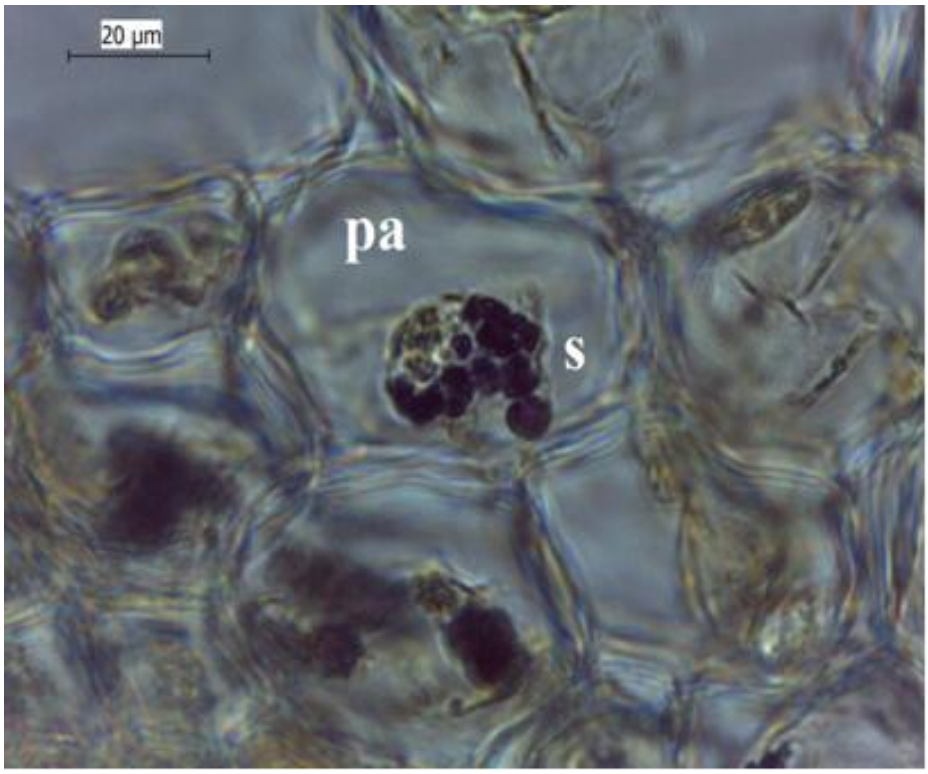


Figure 3. Starch in the parenchyma cell of stem. **s:** Starch, **pa**: parenchyma.

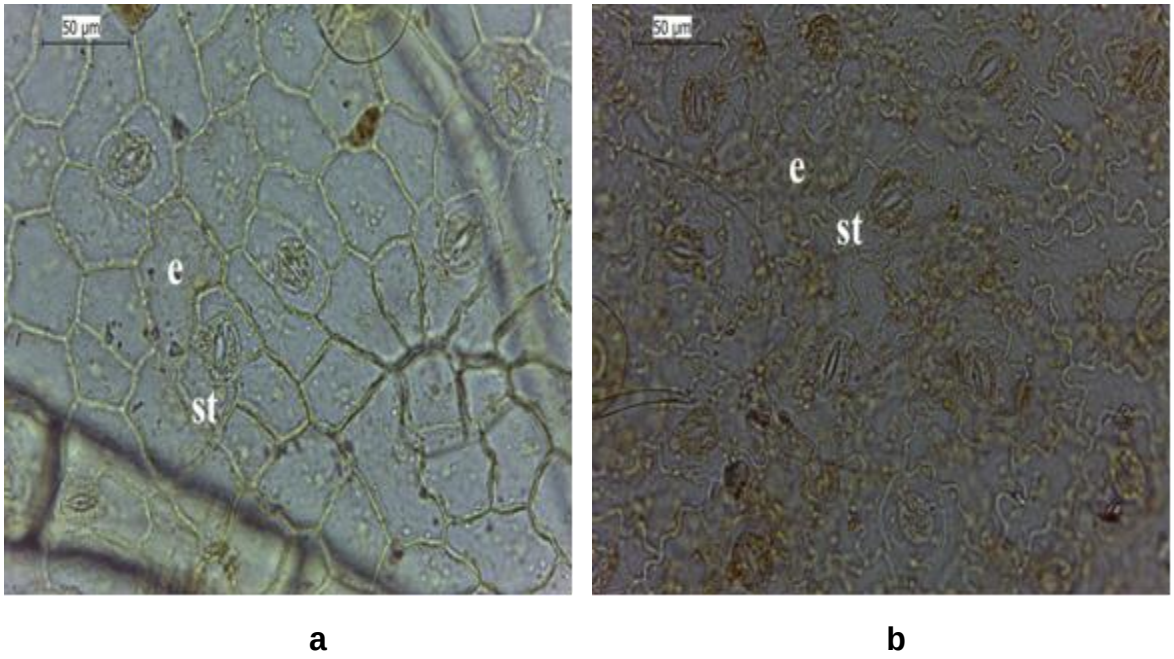


Figure 4. The surface sections of leaf of *A. Orientalis*. (a) Adaxial surface of leaf. (b) Abaxial surface of leaf. e: Epidermis cell st: stoma cell.

Figure 5. The cross sections of leaf of *A. orientalis*. gh: Glandular hair, nh:non-glandular hair, ad: adaxial epidermis cell, ab: abaxial epidermis cell, pa: parenchyma pp: palisade parenchyma, sp: spongy parenchyma, vb: vascular bundle.

bundle in the midrib.

DISCUSSION

The present study provided useful information on the anatomy of *A. orientalis*. Few studies on species *A. orientalis* had been found in literature (Sajjadi and Ghannadi, 2004; Koyuncu et al., 2010). But, the measurements and observation of anatomical characters belonging to the medicinal taxon *A. orientalis* were reported

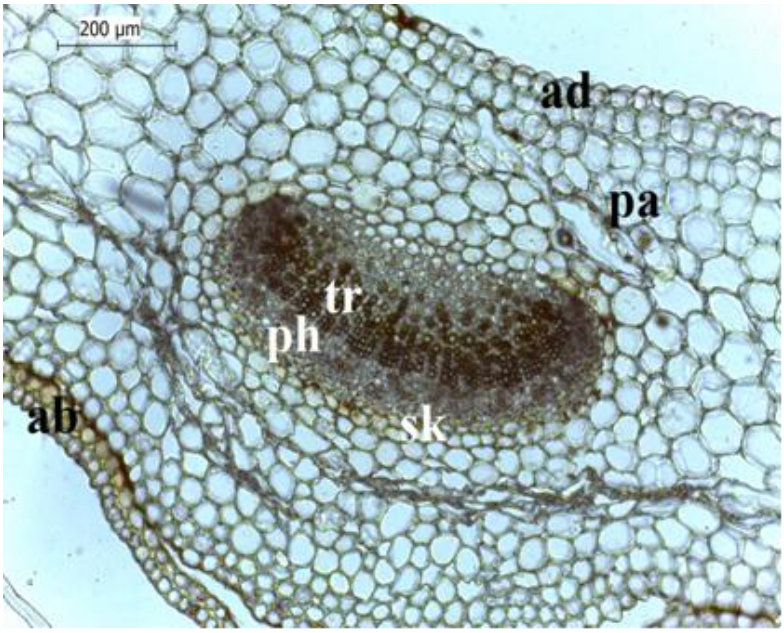
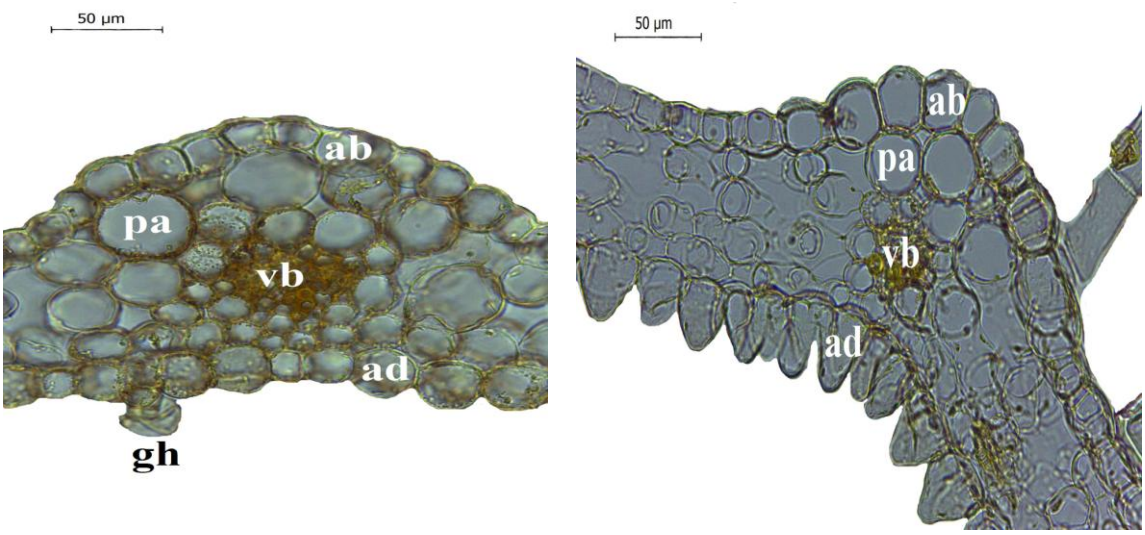


Figure 6. The cross section of petiole of *A. orientalis* ad: Adaxial epidermis cell, ab: abaxial epidermis cell, pa: parenchyma, sk: sclerenchyma, ph: phloem, tr: trachea cell.



a **b**

Figure 7. The cross sections of calyx and corolla of *A. orientalis*. (a) Calyx, (b) Corolla, gh: glandular hair, ad: adaxial epidermis cell, ab: abaxial epidermis cell, pa: parenchyma, vb: vascular bundle.

anatomical information about root anatomy of Lamiaceae family. They stated that the pith rays of roots of the family are 2 to 12 or more rowed cells. It was found that the pith rays of *A. orientalis* were composed 3 to 4 rowed cells. These findings were consistent with those of Metcalfe and Chalk (1972) and those of some studied species of Lamiaceae (Baran and Özdemir, 2006; Özkan and Soy, 2007; Baran and Özdemir, 2009). Metcalfe and Chalk (1972) also stated that the members of Lamiaceae family have quadrangular with well-defined collenchyma in the

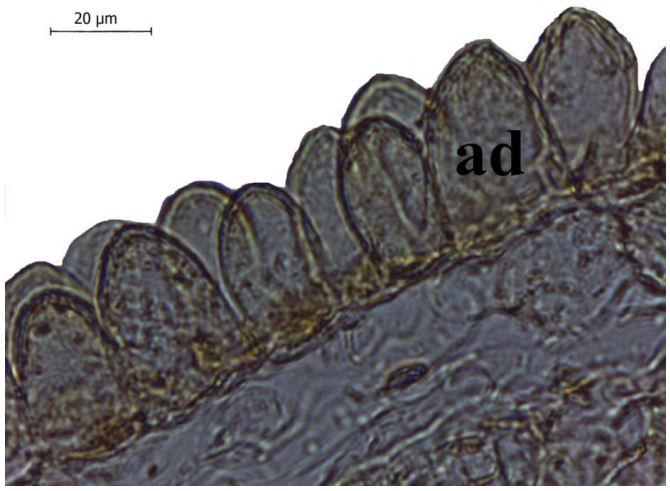


Figure 8. Cross section of corolla of *A. orientalis*; the papillose type of adaxial epidermis cells is seen. **ad:** adaxial epidermis cell.

for the first time in this paper.

Metcalfe and Chalk (1972) determined some imported four angles and scleranchymatous tissue surrounds the phloem groups of vascular bundles. In the transverse section of *A. orientalis*, the stem of this species was quadrangular with well-defined collenchyma in the four angles. It was also determined that there were 2 to 3 layers of sclerenchyma in the outer side of phloem in this study. Quadrangular stem with well-defined collenchyma in the four angles and scleranchymatous tissue surrounds the phloem groups of vascular bundles were observed in other members of Lamiaceae family (Metcalfe and Chalk, 1972; Kandemir, 2003; Baran and Özdemir, 2006; Kahraman et al., 2010). The vascular cambium located between the phloem and the xylem was 1 to 2 rowed layer in the stem of *A. orientalis*. The vascular cambium was seen in the cross-sections of other members of Lamiaceae family (Dinç and Öztürk, 2008; Baran and Özdemir, 2009). There were big vascular bundles on the corners of *A. orientalis* stem, whereas a lot of small bundles between the corners were observed in the present study. There were also starch particles in the paranchymatous cortex cells of stem. The leaf of *A. orientalis* had 2 to 3 layered palisade parenchyma and 2 to 3 layered spongy parenchyma cells. Baran and Özdemir (2009) also stated that *Lamium lycium* which is the member of Lamiaceae family has 2 to 3 layered palisade parenchyma and 2 to 3 layered spongy parenchyma cells as well. These results were parallel to the results mentioned earlier. On the other hand, the stoma type of *A. orientalis* was diasitic and the leaf was bifacial. According to Metcalfe and Chalk (1972), diasitic stoma type was the most common in Lamiaceae family. Diasitic stoma and bifacial mesophyll type were also observed on the leaf of *Ajuga chamaepitys* and *Ajuga reptans* (Akçin et al., 2006). The stomata were observed on both upper and lower surfaces of the leaf. Metcalfe and Chalk (1972) stated that the structure of the vascular bundles in the petiole of the species in the Lamiaceae could be used as a diagnostic character. The structure of petiole shows differences between genera and species. In addition to this, useful petiole anatomical characters are determined in designated taxonomical structures of some species (Shaheen, 2007; Eric et al., 2007). In the petiole of *A. orientalis*, there was one big vascular bundle in the center and 4 to 5 small vascular bundles at each corner of the petiole. Akçin et al. (2011) found that *A. reptans* has a total of nine vascular bundles: one big bundle in the middle and 4 vascular bundles at each corner. The vascular bundles of the leaf were surrounded by sclerenchymatic cells. Collenchyma in the petiole is 2 to 3 layered at each corners. In the cross-section of calyx and corolla, both adaxial and abaxial epidermis cells were covered with cuticle. In addition to this, there were a vascular bundle in the midrib of calyx and corolla. The adaxial epidermis cells of corolla were papillose type.

The most distinguishing characteristics of the species in the anatomical structure were the presence of glandular hairs on the surface layers on stem, leaves, petiole, calyx and corolla. Like other members of the Lamiaceae, *A. orientalis* had both glandular as well as non-glandular trichomes. Glandular trichomes were mainly observed on calyx and corolla, but non-glandular ones were found on the stem, leaf surface and petiole. According to Metcalfe and Chalk (1972), having glandular and non-glandular trichomes are important anatomical characters. Glandular trichomes significant taxonomic characters and act imported role for pollination in the Lamiaceae family (Navarro and El Oualidi, 2000). As a result, anatomical characters of medicinal species of *A. orientalis* were studied for the first time in this paper. According to the results, the results mentioned earlier, the anatomical features of root, stem, leaf, petiole, calyx and corolla provided useful characteristics for distinguishing species in *Ajuga* genus.

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Phytochemical and *in vitro* anti-bacterial properties of *Hibiscus sabdariffa* L (Roselle) juice

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The anti-bacterial properties of aqueous and ethanol extracts of the calyces of *Hibiscus sabdariffa* on five bacteria genera, namely, *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Shigella dysenteriae* and *Streptococcus mutans* were evaluated using agar well and disc diffusion methods. Six bioactive compounds were identified from phytochemical analysis of the juice extract of *H. sabdariffa*. Analysis revealed the presence of the following in the water and ethanol extracts: saponins (1.46%), alkaloids (0.09%), tannins (0.19%), total phenols (0.07%), flavonoids (2.41%) and glycosides (0.13%). The results revealed that both water and ethanol extracts had significant anti-bacterial effects (P ≤ 0.05) against the tested pathogenic bacteria genera with the ethanol extract of Roselle having higher anti-bacterial effects against all the pathogens. *S. aureus* and *E. coli* were the most sensitive to Roselle juice extract (47.9 and 45. 8 mm zones of inhibition, respectively). The minimum inhibitory concentration (MIC) for the ethanol extract of *H. sabdariffa* was between 0.26 and 1.03 mg/ml. The results observed in this study contribute to scientific baseline data on the anti-bacterial activities of *H. sabdariffa* juice extract.

Key words: Antimicrobial activity, minimum inhibitory concentration, pathogenic bacteria, phytochemical, *Hibiscus sabdariffa.*

INTRODUCTION

Hibiscus sabdariffa is an herbaceous annual of the family Malvaceae. The plant originated in West Africa, though it has been grown in Asia for several centuries and now has a wide distribution throughout the tropics (David and Adam, 1985). There are two main types of Roselle: *H. sabdariffa var altissima* Wester and *H. sabdariffa* var *sabdariffa*.

H. sabdariffa var *altisimma* Wester is more widely cultivated for its jute-like fibre in India, the East Indies, Nigeria and to some extent, in tropical America (Yayock et al., 1988). The flowers are yellow and calyces red or green, non fleshy, spiny and not used for food (Morton, 1987). The other distinct type of Roselle, *H. sabdariffa* var *sabdariffa* is a bushy branched sub-shrub with red or green stem and red or pale yellow inflated edible calyces. Both Roselle types grow well on fairly fertile sandy to loamy soil being usually propagated by seeds, but also growing readily from cuttings.

The increased cultivation of Roselle in many areas is centered more on its pharmaceutical, rather than its food potential. All the aforementioned ground-parts of the Roselle plant are valued in native medicine (David and Adam, 1985). Infusions of the leaves, petals and other parts are regarded as diurectic, vasorelaxative, cholerectic, febrifugal and hypotensive in folk medicine (Odigie et al., 2003; Sarr et al., 2009; Mozaffari-Khosravi et al., 2009).

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Pharmacognosists in Senegal recommend Roselle extract for lowering blood pressure (Chopra et al., 1986), while the mechanisms of blood pressure lowering effect of the calyx extract has been studied in rats (Adegunloye et al., 1996). In experiments with domestic fowl, Roselle extract decreased the rate of absorption of alcohol thereby reducing intoxication arising from alcohol consumption (Morton, 1987). As such, the extract has been found to be a favorite remedy for after-effects of drunkenness. The calyx extract is used in the treatment of debility, hypertension, dyspepsia and heart ailments. The extracts of the leaves and flowers of the Roselle plant are used internally as tonic tea for digestive and kidney functions (Bown, 1995). However, there appears to be limited information on the scientific basis for many of the acclaimed health benefits of this plant. The present study was aimed at assessing the phytochemical properties of *H. sabdariffa* var *sabdariffa* and evaluation of its antibacterial properties as a step towards providing scientific justification for its pharmaceutical uses.

MATERIALS AND METHODS

Collection and processing of samples

Dried calyces of Roselle *(H. sabdariffa*) were purchased from Kuto market in Abeokuta, South Western Nigeria. The calyces were identified by a botanist in the Department of Biological Sciences, University of Agriculture, Abeokuta, Nigeria.

Preparation of *H. sabdariffa* extracts

Extraction of juice from calyces of *H. sabdariffa* was done with water and ethanol. Replicate 100 g of dried blended calyces of Roselle were soaked in 200 ml of ethanol and distilled water separately. The beakers were covered with aluminum foil and kept at ambient temperature (29±1°C) for 12 h. The extracts were then filtered through Whatman filter paper No. 42 (125 mm) and stored at 4°C until required for analyses.

Test microorganisms

The microorganisms used in this study comprised clinical isolates of five pathogenic bacteria (*Escherichia coli, Staphylococcus aureus*, *Salmonella typhi, Shigella dysenteriae* and *Streptococcus mutans*) obtained from University College Hospital, Ibadan, Nigeria. These organisms are known to cause diseases such as diarrhoea, dysentery, oral and dental infections. Selected biochemical tests were carried out on the test cultures to confirm their identities before use. The test organisms were sub-cultured in nutrient broth (Oxoid Ltd, UK) at 37°C and stored at 4°C until required for the study. They were used for antimicrobial testing by seeding in agar plates at ca. log 7.0 CFU ml^{-1}.

Phytochemical analyses of *H. sabdariffa* extracts

Screening of *H. sabdariffa* aqueous and ethanol extracts was carried out to identify the phytochemical constituents by standard procedures (Trease and Evans, 1989; Harborne and Harborne, 1998).

Determination of saponins

Octanol (100 ml) was added to 2 g of each extract, shaken for 5 h to ensure uniform mixing before filtering through a Whatman No.1 filter paper. Twenty millilitres of 40% saturated solution of $MgCO_3$ was added to neutralize the filtrate obtained. The mixture was again filtered to obtain a clear colourless solution. To 1 ml of the clear solution 2 ml of 5% $FeCl_3$ solution was added before making up to 50 ml with distilled water. The mixture was allowed to stand for 30 min for the blood red colouration to develop. The absorbance of the samples were read with a spectrophotometer (Spectronic 20) at a wavelength of 30 nm and compared with 0 to 10 ppm standard saponin solutions. Percent saponin present in the samples was calculated as follows:

% Saponin = Absorbance of sample × Average gradient × Dilution factor/10,000

Determination of alkaloids

An amount of 2 ml of each extract was warmed with 2% H_2S0_4 for 2 min. Each extract was filtered and a few drops of Dragendorff's reagent were added. Orange red precipitation indicated the presence of alkaloids after which quantification was estimated by mixing 10 g of each sample with 20 ml of 80% alcohol. After thorough mixing, more 80 ml of 80% alcohol was added to make the mixture up to 100 ml before 1 g magnesium oxide was added. The mixture was digested in a boiling water bath for 1½ h under a reflux air condenser with occasional shaking. The residue was returned to the flask and re-digested for 30 min with 50 ml alcohol after which the alcohol was evaporated and 20 ml of hot water was added. 2 to 3 drops of 10% HCl was added and the solution was transferred into a 150 ml volumetric flask and mixed thoroughly with 5 ml each of zinc acetate and potassium ferric cyanide solutions consecutively. The mixture was allowed to stand for a 5 min before being filtered through a dry No. 1 Whatman filter paper. 10 ml of filtrate was transferred into a separator funnel and the alkaloids present were extracted by shaking vigorously with five successive 30 ml portions of chloroform. The residue obtained was dissolved in hot water and transferred into a Kjeldahl flask with the addition of 0.2 g Selenium for digestion to a colourless solution to determine the percentage nitrogen by Kjeldhal distillation method. The percentage nitrogen obtained was multiplied by dilution factor 6.26 to get percentage total alkaloid.

Alkaloid (%) = Nitrogen percentage × 6.26

Determination of tannins

An amount of 2 ml of each extract was mixed with distilled water in a separate tube and heated in a water bath. The mixture was filtered and ferric chloride reagent was added to each of the filtrates. Dark green coloration indicated the presence of tannins. To determine the amount of tannins present, 100 ml of 4:1 solvent mixture (that is, 80 ml of acetone + 20 ml of glacial acetic acid) was used to extract tannin from 2 g of each sample. After allowing the mixture to soak for 5 h, the sample was filtered using a double layered Whatman No.1 filter paper. The filtrate was made up to the 100 ml mark with distilled water and mixed thoroughly. One millilitre of sample extract was pipetted into 50 ml volumetric flask, 20 ml water was added as well as 2.5 ml Folin-Denis reagent and 10 ml of 17% Na_2CO_3 before mixing thoroughly. The mixture was made up to mark with water, mixed again and allowed to stand for 20 min. Bluish-green colouration indicated positive result for tannins. Standard tannic acid solution of range 0 to 10 ppm was treated similarly as 1 ml of sample earlier. The absorbance of the tannic

acid standard solution as well as sample was read after colour development on a spectrophotometer (Spectronic 20) at a wavelength of 760 nm. Percentage of tannin was calculated using the formula:

$$\text{Tannin (\%)} = \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{10{,}000}$$

Determination of total polyphenols

An amount of 10 g of samples were soaked in 20 ml of distilled water for 4 days. The samples were filtered and each filtrate was made up to 100 ml with distilled water. One millilitre of filtrate from each sample was measured into a test-tube, 3 ml of each 0.008 N potassium hexacyanoferrate (iii) and 0.01 N of iron (iii) chloride were added into each filtrate. The absorbance of each filtrate was read on a spectrophotometer after 10 min. The percentage total polyphenol was determined using the formula:

$$\text{Total polyphenol (\%)} = \frac{\text{Absorbance} \times \text{Average gradient} \times \text{Dilution factor}}{\text{Weight of sample}} \times 100$$

Determination of flavonoids

To 2 ml of each extract was added 2 ml of dilute sodium hydroxide and hydrochloric acid. Yellow solutions that turned colorless indicated the presence of flavonoids which were quantified by extracting 5 g of each sample with 100 ml solvent - 1% aluminum chloride solution in methanol concentrated HCl, magnesium turnins, and potassium hydroxide solution (Earnsworth et al., 1974). After extraction, absorbances of filtrates were read on a spectrophotometer at 380 mm wavelength and compared with 0.10, 0.15, 0.20, 0.25 and 0.30 ppm standards. The slope was calculated from the standard curve as follows:

$$\text{Flavonoid (\%)} = \frac{\text{Metre reading} \times \text{Slope} \times \text{Dilution factor}}{\text{Weight of sample}} \times 100$$

Determination of glycosides

Five grams of each sample were weighed into 250 ml conical flask along with 50 ml of ethanol and 20 ml of distilled water. The mixture was sonicated for 5 min before the addition of 8 ml of concentrated hydrochloric acid. It was refluxed for 3 h and cooled to room temperature. After the extraction, filtration was done using double layer of filter paper. Each extract was transferred into a two-necked 50 ml flask connected with steam generator. This was steam-distilled with saturated sodium bicarbonate solution contained in a 50 ml conical flask for 60 min. One millilitre of starch indicator was added to 20 ml of each distillate and was titrated with 0.2 m of iodine solution. The percentage glycoside was calculated thus:

$$\text{Hydrocyanide (\%)} = \frac{\text{Titre value} \times 10 \times 0.27}{1000 \times \text{weight of sample}} \times 100$$

Antimicrobial testing

The antibacterial activities of aqueous and ethanol extracts of *H. sabdariffa* were determined by paper disc and agar well diffusion methods (Omenka and Osuoha, 2000; Mahesh and Satish, 2008).

Disc diffusion method

Sterile filter paper discs (5 mm diameter) were soaked in different extracts of known concentration (20 mg/ml per disc) for 2 h. The discs were carefully placed at the centre of labeled seeded plates. The plates were incubated at 37°C for 24 h after which the diameters of the zones of inhibition were measured in millimeters with a ruler. Standard discs of the antibiotic chloramphenicol at a concentration of 10 mg served as positive antibacterial control.

Agar well diffusion method

In this method, 0.1 ml of 24 h broth culture of each of the test microorganisms containing ca. log 7.0 CFU ml^{-1} was aseptically seeded into nutrient agar plates and allowed to solidify. The dried plates were then punched with sterile cork borer (5.0 mm diameter) to make open wells. Five hundred microlitres of the test extracts were introduced into the wells before they were incubated at 37°C for 24 h. The zones of inhibition were measured and recorded as for the paper disc diffusion method.

Minimum inhibitory concentration (MIC)

Different concentrations of the extracts of the calyces of *H. sabdariffa* were prepared to obtain 12.8, 6.4 and 0.20 mg/ml. Three drops of overnight broth culture of the test organisms were inoculated into the different dilutions and incubated at 37°C for 24 h. The MIC of the concentrated extracts against each test organism was taken as the lowest concentration of the extract that inhibited the test organism.

RESULTS AND DISCUSSION

The ethanol extracts of *H. sabdriffa* had higher concentrations of bioactive metabolites than the water extracts except for alkaloids. This result agrees with the work of Glass (1991) who showed that the non-polarity of organic solvents could be responsible for the more efficient extraction of the metabolites. However, saponins were generally well extracted by either water or ethanol.

Flavonoids were found to be the most abundant bioactive agent in *H. sabdriffa* followed by saponins, while polyphenols were the least abundant (Table 1). The flavonoid constituents of *H. sabdariffa* have been reported to show good effect on peroxidase and protease activity in human blood which confirmed the benefit of Roselle as an antioxidant and anti-aging plant, in addition to indications through *in vitro* and *in vivo* studies that flavonoids hold great potential as anticancer substances (Nhung et al., 1998).

Despite the small amounts present, polyphenols extracted from *H. sabdariffa* have been found to have anti-inflammatory effects *in vitro* and *in vivo* thereby improving anti-oxidative conditions (Erl-Shyh et al., 2009). The phytochemical composition of the various extracts was comparable to previous reports of Lin et al. (2005), Olaleye (2007) and Kao et al. (2009).

Results obtained from the anti-bacterial studies presented in Table 2 showed that the calyces of *H. sabdriffa* have significant inhibitory effects on the test pathogens.

Table 1. Concentration of bioactive metabolites in extracts of *Hibiscus sabdriffa* juice.

Bioactive compound	Roselle extract	
	Water extract	Ethanol extract
Saponins (%)	1.13 ± 0.03	1.46 ± 0.02
Alkaloids (%)	0.09 ± 0.01	0.08 ± 0.01
Tannins (%)	0.07 ± 0.01	0.19 ± 0.01
Total phenols (%)	0.05 ± 0.00	0.07 ± 0.00
Flavonoids (%)	1.08 ± 0.01	2.41 ± 0.02
Glycoside (%)	0.05 ± 0.00	0.13 ± 0.01

Values are represent means of three replicates ± Standard Deviation

Table 2. Antimicrobial activities of *Hibiscus sabdriffa* juice by disc and agar well diffusion methods.

Test organism	Paper disc diffusion			Open well diffusion		
	Aqueous extract	Ethanol extract	Chloramphenicol	Aqueous extract	Ethanol extract	Chloramphenicol
Escherichia coli	41.2 ± 0.2^{b}	43.2 ± 0.2^{c}	40.4 ± 0.4^{a}	43.5 ± 0.5^{c}	45.8 ± 0.2^{d}	41.2 ± 0.2^{b}
Salmonella typhi	39.1 ± 0.3^{c}	42.0 ± 0.3^{e}	36.0 ± 0.2^{b}	40.0 ± 0.2^{d}	43.8 ± 0.2^{f}	35.1 ± 0.1^{a}
Shigella dysentariae	27.9 ± 0.3^{a}	32.8 ± 0.2^{c}	30.2 ± 0.2^{b}	35.8 ± 0.3^{d}	43.2 ± 0.2^{e}	32.9 ± 0.3^{c}
Staphylococcus aureus	43.2 ± 0.2^{c}	45.6 ± 0.6^{e}	38.9 ± 0.2^{b}	44.7 ± 0.3^{d}	47.9 ± 0.2^{f}	37.8 ± 0.3^{a}
Streptococcus mutans	26.9 ± 0.4^{c}	28.1 ± 0.1^{d}	32.1 ± 0.3^{e}	22.2 ± 0.2^{a}	23.3 ± 0.3^{b}	33.2 ± 0.2^{f}

Values are zones of inhibition measured in mm ± standard deviation. Means with the same superscript are not significantly different at 5% level of significance along the rows.

Generally, the organisms were more sensitive to alcoholic extracts than aqueous extracts. In addition, the open well diffusion method was more sensitive going by the wider inhibition zones recorded for most of the test organisms. The agar well diffusion method was used because it is known to allow better diffusion of the extracts into the medium, thus enhancing contact with the test organisms and ensuring more accurate results. This observation agrees with the work of Omenka and Osuoha (2000) who showed that open well diffusion method allows better diffusion of the extracts into the medium, thus enhancing contact with the organisms as the organisms are introduced directly into the wells. Paper disc may acts as barrier between the extract and the organism thus preventing total diffusion of active components absorbed by the discs into the medium and may be responsible for the observed differences. However, the contrary was the case for *S. mutans* which was also the least sensitive to the extracts recording the lowest inhibition zones (22.2 to 28.1 mm) for both methods (Plate 1).

The widest zone of inhibition (47.9 mm) was observed by the ethanol extract of *H. sabdariffa* against *S. aureus* in the open well diffusion method (Plate 2). This value was followed by 45.8 mm observed in the open well diffusion method for the ethanol extract of Roselle against *Escherichia coli*. A beverage containing *H. sabdariffa* L.

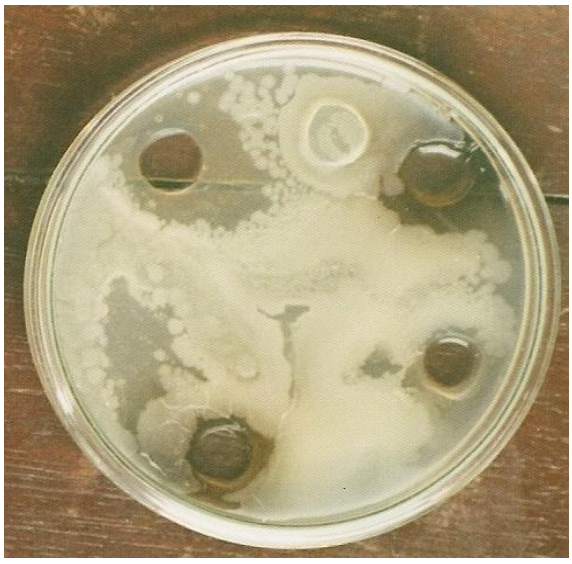

Plate 1. Inhibition of *Streptococcus mutans* by ethanol extract of *Hibiscus sabdariffa* using open well diffusion method.

was previously shown to possess bactericidal activity against *E. coli*, *Bacillus subtilis*, *S. typhi* and *K. pneumoniae* (Alian et al., 1983). An inhibitory effect of *H.*

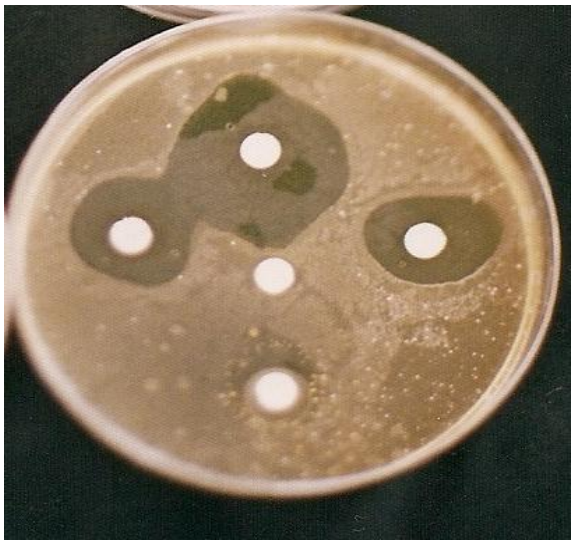

Plate 2. Inhibition of *Staphylococcus aureus* by ethanol extract of *H. sabdariffa* using disc diffusion method.

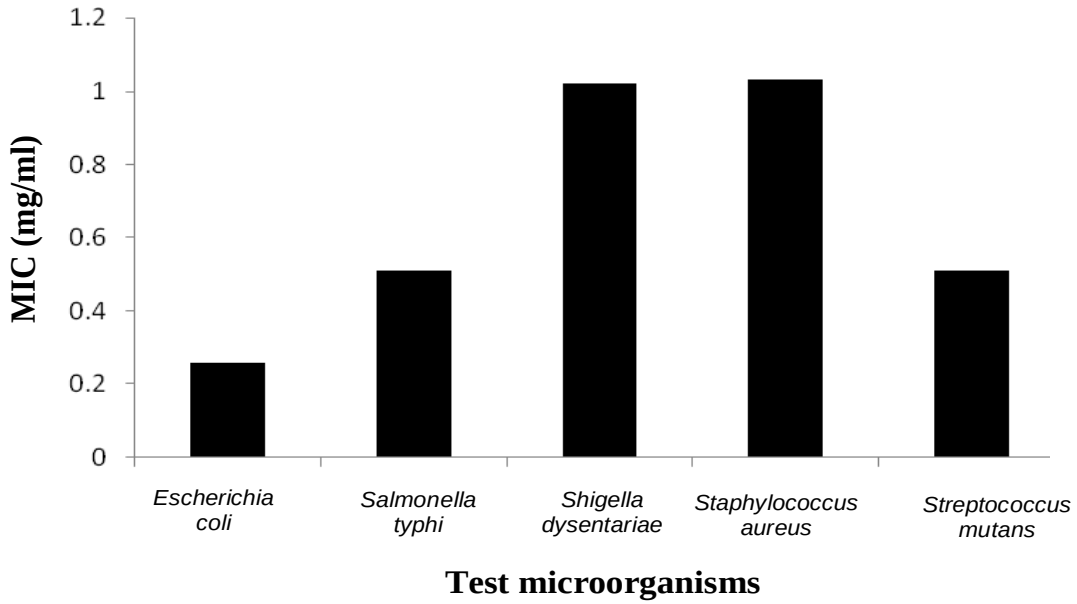

Figure 1. Minimum inhibitory concentration (mg/ml) of test bacteria by ethanol extract of Roselle.

corroborate the antibacterial effects of Roselle extracts against the test pathogens. The values obtained showed that the highest activity was recorded against *E. coli* in ethanol extracts of *H. sabdariffa* juice extract (Figure 1).

The anti-microbial activities demonstrated by the extracts of Roselle justify some of the ethno-pharmacological claims about this plant in the treatment of diseases caused by some of the test pathogens such as diarrhoea, dysentery, oral and dental infections.

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Evaluation of whitening efficacy and safety of the water extract from bamboo shavings

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This aim of this study was to evaluate the whitening efficacy of the water extract from bamboo shavings (WEBS) in malignant melanoma cells (B-16) of mice. The safety of WEBS was evaluated by the acute oral toxicity test, the repeated skin irritation test and the acute eye irritation test was also evaluated. WEBS showed strong inhibitory effects against the activity of tyrosinase in B-16 cells in a dose dependent manner, and was more potent than arbutin. The melanin content was significantly inhibited by WEBS and the cytotoxicity of WEBS was lower than that of arbutin and hydroquinone. WEBS inhibited mushroom tyrosinase and the maximum was 65.05% at 16 mg/ml with an IC_{50} of 6 mg/ml. The LD_{50} was larger than 5000 mg/kg body weight and WEBS was found to be non-toxic and non-irritating.

Key words: Water extract of bamboo shavings, whitening effect, B-16 melanoma cell, safety evaluation.

INTRODUCTION

The appearance of brown-spots on skin, as a consequence of hyperpigmentation, is a very common aesthetic problem (Pawaskar et al., 2007). This skin disorder is a consequence of melanin excess, caused by hyperactivity of melanocytes, which are the cells responsible for skin pigmentation. This disorder can be treated with cosmetically using so-called skin-whitening (also referred to as skin-bleaching) cosmetic products (Briganti et al., 2003), although the most serious cases require medical assistance. These products contain different chemicals called skin-whitening agents (Briganti et al., 2003; Balaguer et al., 2008; Cabanes et al., 1994; Petit and Piérard, 2003).

Tyrosinase is responsible for not only browning in foods, but also melanization in animals. It is important in food and cosmetics to prevent browning and over-production of melanin (Hearing and Tsukamoto, 1991). The tyrosine in skin cells is gradually oxidized into Dopa and Dopaquinone by the action of tyrosinase. This process can be influenced by various environmental factors. Dopaquinone is polymerized into Indo-5,6-quinone through a series of metabolic reaction and finally transformed into skin melanin. Hence, the function of a large number of whitening agents is to interfere with the formation of melanin by inhibiting tyrosinase activity (Yu et al., 2007; Azhar-ul-Haq et al., 2006).

Consumers are cautious about cosmetics made from chemical compounds and cosmeceuticals are preferred (Momtaz et al., 2008). Traditional skin whitening agents such as hydrogen peroxide, aminomercuric chloride and phenolic derivatives can break down melanin rapidly. They are, however, forbidden in many countries, because of the high risks of dermal corrosion, cytotoxicity, and irritability. Current skin whitening agents including arbutin, kojic acid and derivatives, vitamin C and derivatives, and licorice flavonoids are largely natural plants extracts with

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good whitening effects and minimal side effects (Yoshikane et al., 1999; Hong et al., 1999; Hansruedi et al., 1999; Curto et al., 1999; Frank et al., 2000).

During the recent years, more attention has been paid to the cosmetics market for skin whitening. There are many recent studies on skin melanin formation, whitening mechanism, whitening effect evaluation, whitening agent selection and production (Khan et al., 2006). Furthermore, various natural tyrosinase inhibitors have been reported (Passi and Nazzaro-Porro, 1981; Mayer, 1987; Khan et al., 2003). These include extracts from leaves and root barks of mulberry, which exhibited high inhibitiory effects on 3-(3,4-dihydroxypehnyl)-L-alanine (DOPA) oxidase activity (Lee et al., 2002).

Bamboo shavings (*Caulis bamfusae* in taeniam), the intermediate layer of the stems of *Bambusa tuldoides* Munro, *Sinocalamus beecheyana* var. *pubescens* P.F. Li or *Phyllostachys nigra* (Lodd.). Munro var. *henonis* (Mitf.) Stapf ex Rendle, are perennial plants of the family Gramineae (Zhang et al., 2004). In recent years, numerous bio-active components have been found in bamboo shavings including triterpenoids, saponins and sterols. The safety of the triterpenoid-rich extract (EBS) and polyphenol-rich ethanol extract (EEBS) from bamboo shavings have been evaluated (Zhang et al., 2004; Gong et al., 2010). The results show that EBS has excellent anti-fatigue, antihyperlipidemic and antihypertensive activities (Zhang et al., 2006; Jiao et al., 2007). WEBS, a water-phase extract from bamboo shavings obtained through supercritical carbon dioxide extraction, also contains numerous biologically active components such as triterpenoids, flavonoids, anthraquinones and phenolic acids. It is water soluble, yellow or light yellow in color, possesses the aroma of bamboo and is very stable for a period of two years storage.

B-16 melanoma cells are produced by mutation of normal melanocytes. Their biochemical metabolism is similar to that of normal human melanocytes especially with respect to its melanogenesis function. It is widely used in skin studies, because growing primary skin melanocytes in cell culture is difficult (Maeda and Fukuda, 1996; Qiu et al., 2000; Momtaz et al., 2008). B-16 cells were used in this study to investigate the effect of WEBS on the proliferation rate of melanocytes, and the associated tyrosinase activity, melanin content and cytotoxicity, in order to understand the whitening mechanism from the perspective of cell level.

MATERIALS AND METHODS

WEBS was prepared in our laboratory by supercritical carbon dioxide extraction from bamboo shavings and the concentration was 32 mg/ml. Arbutin (purity>98%) and hydroquinone were purchased from Shellgene Biotech Co., Ltd (Shanghai). All compounds were dissolved in DMSO with sterile filtration and stock solutions of compounds were prepared and stored in the dark at -20°C until used. Mice melanomas cell (B-16) was purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS (Nanjing). RPMI1640 medium and fetal calf serum were obtained from Gibco (US). MTT, L-DOPA, Tyrosinase, SDC and Trypsin were purchased from Sigma-aldrich (US). DMSO was purchased from Sinopharm Chemical Reagent Co., Ltd (shanghai).

Components analysis of WEBS

Total phenolic content of WEBS was determined by the Folin-Ciocalteu method (Meda et al., 2005). The total flavonoid content was determined according to the aluminum chloride colorimetric method as described by Chang et al. (2002). The triterpenoid content of WEBS was determined by colorimetry using ginsenoside as a standard and the phenol-sulfuric acid method was chosen to determine its total sugar.

Cell culture and treatment

B-16 cells were cultured and treated according to the methods described by Zhi-Ming Hu et al. (2009) with some modifications. B-16 is routinely cultured in complete RPMI1640 medium with 10% heat-inactivated fetal calf serum at 37°C with 5% CO_2 and the cell inoculums size was 3×10^6 cells/L once every 3 days.

Inhibition of mushroom tyrosinase activity

Inhibition of tyrosinase activity was determined according to the method described by Matsuda et al. (1994) with some modifications. The reaction solution was prepared and mixed according to Table 1 and heated in a water bath at 28°C. Reactions were incubated for 5 min, the absorbance value was determined at 475 nm, and the inhibition rate was [1-(A3-A4) / (A1-A2)] ×100% with PBS blank control.

B-16 cell viability assay

B16 cells (2×10^4) were seeded into each well of a 96-well plate after 0.25% trypsin dissociation. The cells were allowed to attach to the plates at 37°C for 24 h with 5% CO_2. At this point, the cultures were supplemented with WEBS at the specific concentrations in triplicate with hydroquinone and arbutin as positive controls. Complete RPMI1640 medium with appropriate compound was immediately added into the wells and left on for 48 h. The treated cells were labeled with MTT reagent for 3 h. The formazan precipitates were dissolved by DMSO and measured by absorbance at 475 nm in a ELISA reader with a reference wavelength of 650 nm (Zheng et al., 2001).

B-16 tyrosinase activity assay

For the tyrosinase activity assay, B-16 cells were seeded into each well of a six-well plate. The next day, the cells were fed with fresh medium containing the experimental compounds. Two days later, the cells were re-fed with compound-containing fresh medium again. On the 5th day of treatment, the cultures were harvested with trypsin/EDTA. Each cell suspension was centrifuged for 5 min at 1500 g, washed with cold PBS, and then solubilized in 200 ml extraction buffer. Extracts were solubilized at 4°C for 1 h, and aliquots were stored at -20°C until tyrosinase activity was assayed. (Curto et al., 1999).

Melanin content assay in B-16

For melanin content assay, B-16 cells were seeded into six-well

Table 1. Composition of reaction solution.

Item	Reaction solution (ml)			
	1	2	3	4
0.10% L-DOPA	2.0	0	2.0	0
Whitening agent solution	0	0	2.0	2.0
PBS	8.0	10.0	6.0	8.0
Tyrosinase	2.0	2.0	2.0	2.0
Total volume	12	12	12	12

plates. The next day, the cells were fed with fresh medium containing compounds incubated for 48 h. 1 ml 0.1 mol/L NaOH (containing 10% DMSO) was added after 0.25% trypsin dissociation. The absorbance at 400 nm was measured after 30 min in water bath at 80°C and the cells were counted concurrently (Nerya et al., 2003).

Determination of lactate dehydrogenase (LDH) in supernatant

LDH is an important indicator of the integrity of the cell membrane and LDH levels in cell supernatant can be determined so as to judge the cytotoxicity of the sample. 200 µl of supernatant and 0.25 ml of substrate buffer were taken and 0.05 ml of coenzyme was added with 0.05 ml of water as the blank. 0.25 ml of 2,4-dinitrophenylhydrazine was added after water bath immersion for 15 min at 37°. 2.5 ml of 0.4 mol/L NaOH was added for 3 min at room temperature and the absorbance at 400 nm was measured (Bonnekoh et al., 1990).

Changes of cell morphology

Changes were observed in cell morphology after 48 h of growth in culture with an inverted microscope.

Acute oral toxicity test

Ten male and 10 female Kunming mice whose weights between 19 and 22 g were provided by Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The temperature of the feeding room was 20 to 25°C and the relative humidity was 40 to 70%. The feed was provided by Suzhou Shuangshi Laboratory Animal Feed Science Co., Ltd.

After 16 h-fasting of animals, 10 male and 10 female mice were selected according to weight requirement. The experimental animals were treated with WEBS by one-time oral administration. After contamination, the general state was observed, weight change, toxicity symptoms, death situation, etc., of the animals for one week. Postmortem examination was carried out on dead animals and animals were killed and pathologic changes were noted.

Repeated skin irritation test

Two male and 2 female New Zealand white rabbits of common grade whose weights were from 2.5 to 2.8 kg were provided by Shanghai Shengwang Laboratory Animal Breeding Co., Ltd. The temperature of the feeding room was 19 to 26°C and the relative humidity was in the range of 40 to 70%. The feed was provided by Suzhou Shuangshi Laboratory Animal Feed Science Co., Ltd.

The hair on both sides of the spines of animals was cut 24 h before the test. The cutting area measured 3×3 cm, respectively for the left and right sides. During the test, 0.5 ml of WEBS was evenly applied on the left side with distilled water as control on the right side. The application was carried out once a day for successively 14 days. After the second day, application was conducted every day and hairs were cut, the skin reaction was observed 1 h later and the skin irritation reaction was scored according to relevant regulations in the Hygienic Standard for Cosmetics (2002) issued by the Ministry of Health. After completion of the test, the total score for 14 days, the average score for each animal in the 14 days and the average score of each animal on each day respectively, were calculated and the skin irritation intensity was graded.

Acute eye irritation test

With the slightly open left eyelid of New Zealand white rabbits of common grade, 0.1 ml of WEBS was dropped into the conjunctival sac. The upper and lower lids were closed for 1 s passively. The untreated right eye was used as control. 30 s later, physiological saline was used to wash the eye for 30 s. 1, 24, 48, and 72 h, 4 and 7 days after the eye drop. If there was no irritation reaction within 72 h, the observation was terminated. If there was an eye irritation reaction after 7 days, the observation was prolonged to 21 days. Scoring and irritation intensity grading were carried out in accordance with the Hygienic Standard for Cosmetics (2002) issued by the Ministry of Health.

RESULTS AND DISCUSSION

Components of WEBS

The pH, triterpenoid, total sugar, flavonoids and phenolic was 2.97, 3.65, 0.8, 0.69, and 0.465 mg/ml, respectively.

Effects of WEBS and arbutin on mushroom tyrosinase activity

Tyrosinase catalyzes both the hydroxylation reaction converting tyrosine into DOPA and the oxidation reaction converting DOPA into dopaquinone. This, in turn, leads to polymerization of brown pigments (Olivares et al., 2001; Jang et al., 2002). Thus, tyrosinase inhibitors can be applied to whiten the skin in cosmetics (Katagiri et al., 1998). The amount of enzyme required to increase the absorbance value by 0.001/min was defined as an enzyme activity unit and the activity was 2173.9 IU/mg. The concentration-dependent effects of WEBS, arbutin on mushroom tyrosinase inhibition are as shown in Figure 1.

Different concentrations of WEBS showed differential inhibitory effects on mushroom tyrosinase and the maximum inhibition rate was 65.05% at 16 mg/ml, that is, IC_{max} of WEBS was 16 mg/ml. Arbutin, a naturally occurring glucoside of hydroquinone, has been traditionally used in Japan to treat pigmentary disorders (Hori et al., 2004; Maeda and Fukuda, 1996). Many studies have demonstrated that arbutin is safe and less cytotoxic as compared to hydroquinone. However, its effectiveness and its depigmenting mechanism have not yet been fully characterized (Hori et al., 2004; Bang et al., 2008a, b). The

Table 2. LDH release of B-16 cells (control group 72 U/L).

Concentration (mg/ml)	LDH (U/L)		
	EBS	Arbutin	Hydroquinone
6.25	165±14	79±19	74±23
1.25	166±8	90±4	75±9
2.5	183±12	96±17	81±7
5.0	248±32	101±11	-
10.0	267±14	107±35	-
20.0	278±17	177±39	-

value for arbutin was 68.18% at 1 mg/m, The IC_{50} for WEBS and arbutin were 6 and 0.35 mg/ml, respectively. These results demonstrate that WEBS can be used as a skin-whitening agent, however, the tyrosinase-inhibiting ability was less in comparison with arbutin.

Effects of WEBS, arbutin and hydroquinone on the viabilities of B-16

Skin melanocytes can transfer melanin to the epidermal basal cells, leading to excessive darkness and local pigmentation of skin. Therefore, B-16 cell proliferation is an important indicator for judgment of the whitening effect. The effect. The effects of different concentrations of WEBS, arbutin and hydroquinone on cell viability are as shown in Figure 2.

The viability of WEBS treated cells gradually declined with increasing WEBS concentration. The viability of arbutin treated cells decreased slightly, but not to the degree observed with WEBS treatment and it was around 80%. However, the viability of hydroquinone treated cells declined drastically to 12% at the highest concentration. These results suggest that inhibiting B-16 proliferation may be the main reason that hydroquinone has striking whitening effect. WEBS could affect the quantity of melanocytes in a dose-dependent manner and it might be a possible reason for its whitening effect.

Effects of WEBS, arbutin and hydroquinone on the tyrosinase activity of B-16 cells

Most whitening products act by inhibiting tyrosinase activity or by blocking the oxidation pathway of tyrosine's generation of melanin, because tyrosine is transformed to Dopa and Dopaquinone under the action of tyrosinase. The effects of WEBS, arbutin and hydroquinone on the tyrosinase activity of B-16 cells are as shown in Figure 3.

The inhibitory effects of WEBS and arbutin on tyrosinase activity in B-16 cells was enhanced significantly with the increasing concentration. The inhibition of hydroquinone on tyrosinase in B-16 cells was relatively strong in comparison to others and was 74.9%. The results were consistent with inhibition of tyrosinase activity *in vitro*.

Effects of WEBS, arbutin and hydroquinone on the melanin of B-16 cells

Melanin is produced inside the melanocytes as a mixture of brown-black eumelanin and red-yellow phaeomelanin (Hearing and Tsukamoto, 1991). A decreased susceptibility of subjects with darker skin phototypes to UV-induced photodamage is believed to be due to the filtering ability and radical scavenging capacity of melanin (Kaidbey et al., 1979; Ezzahir, 1989; Kollias et al., 1991). The effects of WEBS, arbutin and hydroquinone on the melanin content in B-16 cells are depicted in Figure 4.

The melanin content of B-16 cells decreased with increasing concentration of WEBS, arbutin and hydroquinone, suggesting that reductions in melanin generation might underlie its whitening efficacy.

Determination of LDH in B-16 supernatant

The effects of WEBS, arbutin and hydroquinone on LDH release of B-16 cells are shown in Table 2. The level of LDH in the supernatant of B-16 cells treated with WEBS and arbutin was relatively high and very little cytotoxicity was observed. The level of LDH in cells treated with greater than 5 mg/ml hydroquinone could not be tested due to the increased cytotoxicity. It was, therefore, presumed that hydroquinone may seriously damage the cell membrane despite its strong whitening effect. Hydroquinone has been used successfully to treat many forms of epidermal hyperpigmentation, such as melasma, freckles and postinflammatory hyperpigmentation, etc. However, the biosafety of hydroquinone as a skin whitening ingredient remains controversial, although it has been in use topically for more than 50 years (Nakajima et al., 1998; Westerhof and Kooyers, 2005; Nordlund et al., 2006; Levitt, 2007).

The morphology changes of B-16 cells

The morphology of B-16 melanocytes is as shown in Figure 5. The morphology of B-16 blank cells was normal after three days in culture: the supernatant was light yellow and cells were spindle-shaped and mostly had an easily observable cell nucleus. B-16 cells treated with arbutin were round with the cell wall detached and contained irregular spheroplasts. B-16 cells treated with hydroquinone were distributed sparsely with reduced and shortened cell dendrites. It was difficult to form a mutually-maintaining reticular structure so that paracrine could not be carried out through the reticular interconnected structure to achieve common proliferation and differentiation. Concern regarding the side effects of

Table 3. Results of acute oral toxicity.

Gender	Dose (mg/kg)	Number of animals	Number of dead animals	Death rate (%)
Female mice	5000	10	0	0
Male mice	5000	10	0	0

LD_{50}: female mice; LD_{50}>5000 mg/kg; Male mice: LD_{50}>5000 mg/kg

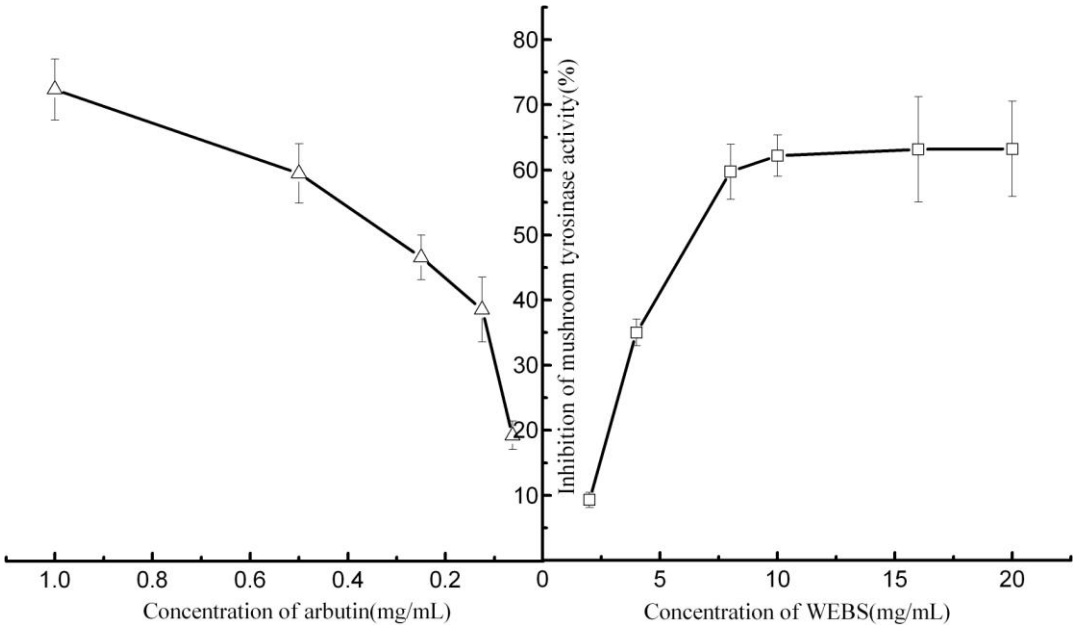

Figure 1. Effect of WEBS and arbutin on mushroom tyrosinase.

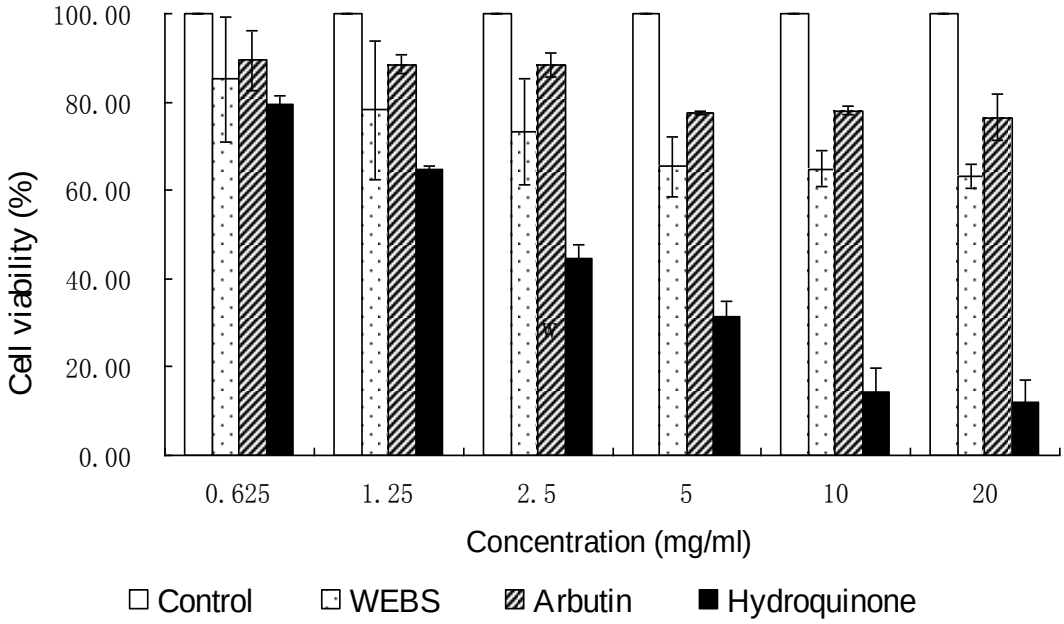

Figure 2. Effect of concentration on cell viability.

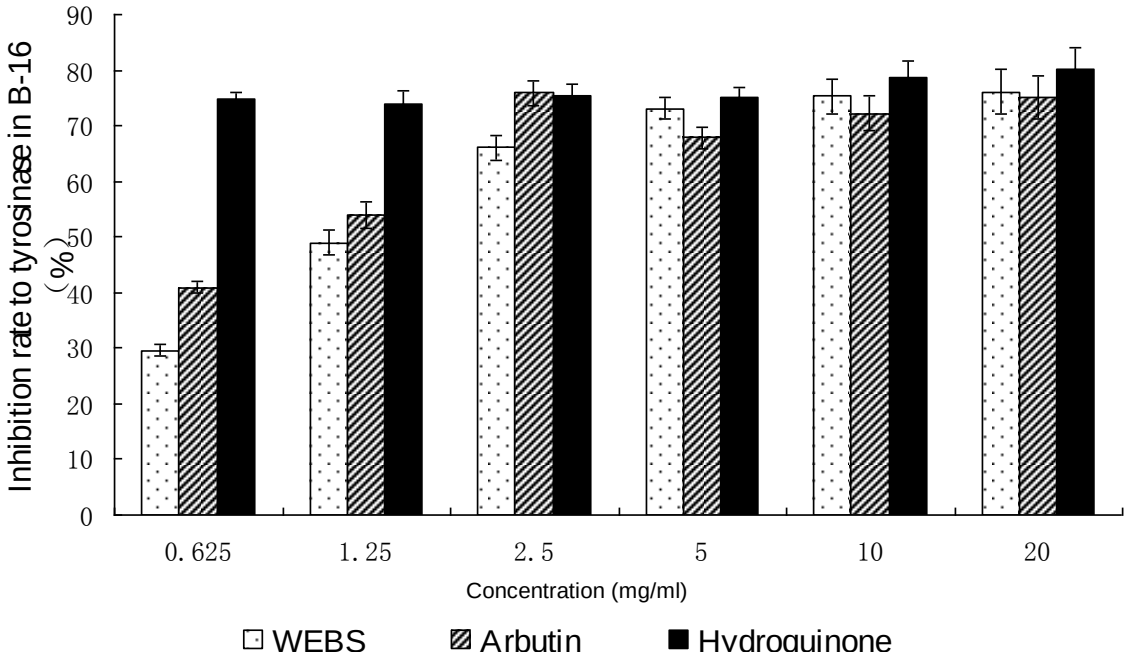

Figure 3. Effect of WEBS and positive control on tyrosinase in B-16.

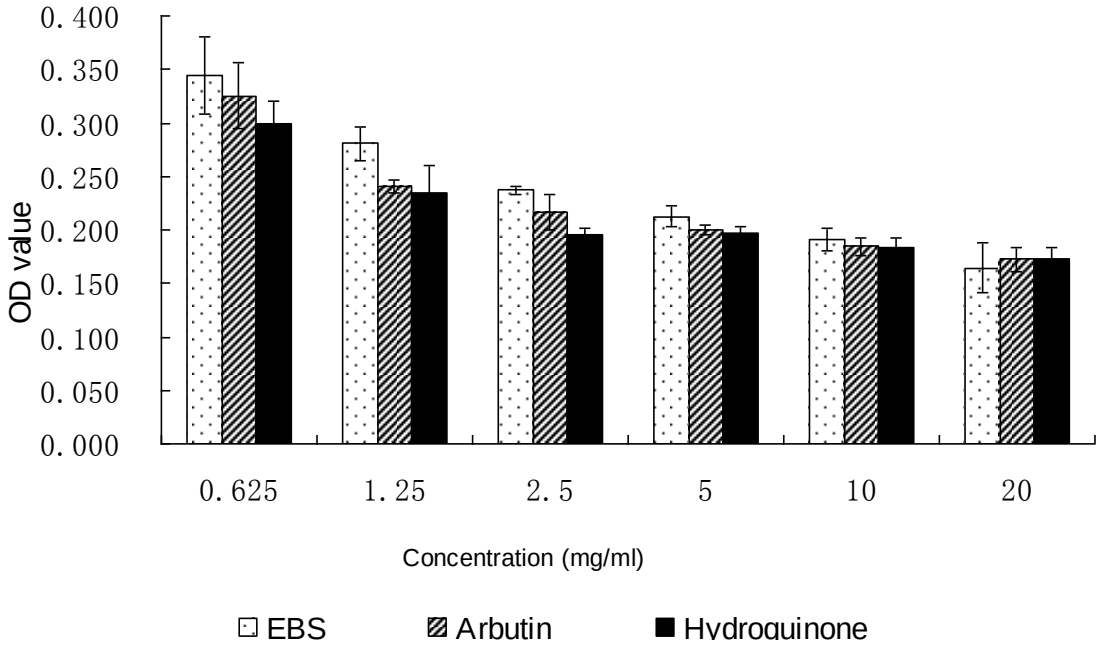

Figure 4. Effects of WEBS, arbutin and hydroquinone on the melanin of B-16 cells.

hydroquinone arose primarily, because topical application of hydroquinone may cause exogenous ochronosis, its benzene metabolites *in vivo* may cause bone marrow toxicity, carcinogenesis with excessive use (Nordlund et al., 2006; Levitt, 2007). The density of B16 cells treated with WEBS declined with less morphology damage, demonstrating that WEBS was safer than arbutin and hydroquinone.

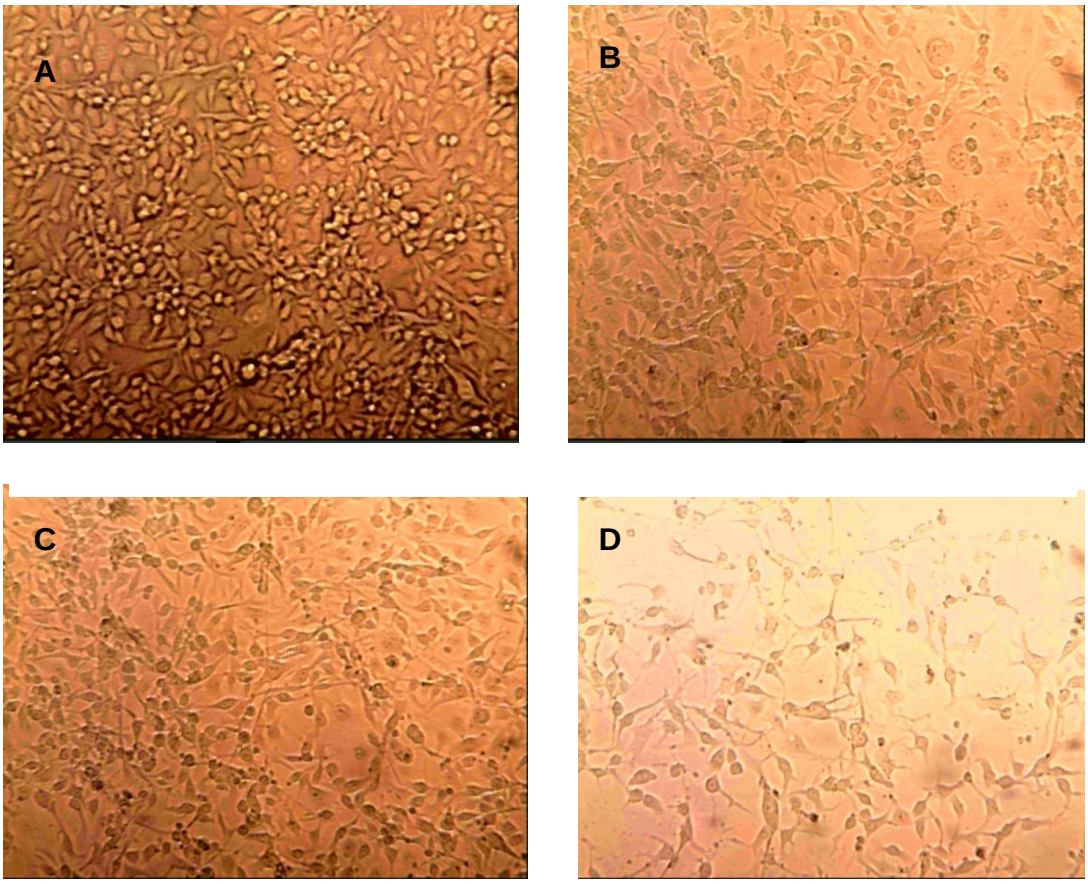

Figure 5. The morphology of B-16 melanocytes.
(A) Blank, (B) WEBS treatment (×400), (C) Arbutin treatment, (D) Hydroquinone treatment (×400).

Safety evaluation for WEBS

Acute oral toxicity test

The results of acute oral toxicity test are shown in Table 3. All animals in each group were normal with good hair gloss and no toxicity symptoms or death during the test. The LD50 of WEBS to female and male mice was both more than 5000mg/kg and classified as non-toxic.

Repeated skin irritation test

The average score for the skin irritation reaction of each animal each day was 0 in the repeated irritation test and there was, therefore, no irritation.

Acute eye irritation test

The average score of irritation to eyes of rabbits by WEBS was cornea: 0; iris: 0; conjunctiva: 0. Thus there was no irritation.

Prospect

Since biosynthesis of melanin is an enzymatic biochemical reaction with multiple steps, the inhibition of melanogenesis may be related to other enzymes besides tyrosinase. Further research is required to fully understand the effect of WEBS on other enzymes and functional factors.

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