DE GRUYTER
OPEN

herba polonica
Vol. 59 No. 4 2013
DOI: 10.2478/hepo-2013-0022

EXPERIMENTAL PAPER

Antiviral and cytotoxic activities of anthraquinones isolated from *Cassia roxburghii* Linn. leaves

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Summary

The cytotoxic activity of petroleum ether extract of the leaves of *Cassia roxburghii* Linn. against HCT-116 and MCF-7 cell lines resulted with IC_{50}=34.9 and 38.04 μg/ml, respectively, while against HepG-2 showed no activity. A bioassay guided-fractionation approach was conducted to isolate and identify the active cytotoxic principles. Further chromatographic separation and purification of the petroleum ether extract resulted in the isolation of two anthraquinones identified as aloe-emodin acetate and aloe-emodin, along with stigmasterol, β-sitosterol and palmitic acid. The structure elucidation of isolated compounds was

performend using 1D, 2D-NMR and HR-MS. Furthermore, the cytotoxicity of aloe-emodin acetate and aloe-emodin were evaluated and resulted with IC_{50} = 153.30 and 70.02 μg/ml against HCT-116 and with 93.20 and 53.20 μg/ml against MCF-7, respectively, while against HepG-2 showed no activity. Moreover, the antiviral activity of the two isolated anthraquinones was tested against influenza virus-A, and resulted with IC_{50} = 10.23 as well as 2.00 and with CC_{50} = 1.32 and 0.47 μg/ml, respectively.

Key words: *Cassia roxburghii Linn., Fabaceae/Leguminosae, anthraquinones, antiviral activity, cytotoxic activity, SAR*

INTRODUCTION

Cassia roxburghii Linn., commonly known as Ceylon senna (red cassia), belongs to the family *Fabaceae/Leguminosae* (subfamily *Caesalpiniaceae*) it is a fairly large "shower" tree with feather like pinnately compound leaves and twigs covered with a dense carpet of fine and soft hair [1]. *C. roxburghii* Linn. has been of keen interest in phytochemical and pharmacological research due to their excellent medicinal values. Although in traditional medicine *C. roxburghii* Linn. have been well known for their laxative and purgative properties and for the treatment of skin diseases [2], also is has been reported that the MeOH extract of its seed protect liver against toxic effects caused by ethanol-CCl_4 combination [3], there is now an increasing body of scientific evidence demonstrating that the plants possess many other beneficial properties.

The members of *Cassia* species are rich sources of polyphenols, anthraquinone derivatives, flavonoids and polysaccharides [4, 5]. They have been found to exhibit anti-inflammatory [6], antioxidant [7], hypoglycaemic [8], antiplasmodial [9] larvicidal [10], antimutagenic and anticancer activities [11]. They are also widely used for the treatment of wounds [12], skin diseases such as ringworm, scabies and eczema, gastro-intestinal disorders like ulcers [13, 14], and jaundice [15].

The phytochemical studies of *Cassia* species have provided some biochemical basis for their ethnopharmacological uses in the treatment and prevention of various diseases and disorders which are confirmed to be related to their contents of hydroxyanthraquinone [16, 17]. Hydroxyanthraquinones are the active principles in a large number of plant-derived drugs and fungi as the toxic metabolites and colouring matters [18]. Hydroxyanthraquinones are present in plant extracts as pharmacologically inactive glycosides but are thought to be activated by glycosidic cleavage *in vivo* by microorganisms in the intestinal flora [19].

The aims of the presented study were to review the phytochemical constituents of the petroleum ether extract of *C. roxburghii* Linn. searching for the anthraquinones, accompanied by an evaluation of the cytotoxicity and antiviral activity of the isolates, and then highlights their potentials as candidates for new drugs that may be valuable in the treatment and prevention of human and livestock diseases.

MATERIALS AND METHODS

Plant material

C. roxburghii Linn. leaves were collected in December 2006 from El-Orman Botanical Garden, Giza, Egypt. The plant was identified by Mrs. Terase Labib, Head of the Taxonomist at El-Orman Botanical Garden. A voucher specimen (No. C-5-63) was kept at the Herbarium of El-Orman Botanical Garden. The plant materials were dried and grounded into fine powder and kept for further analysis.

Extraction procedure

The air-dried powdered leaves (250 g) of *C. roxburghii* Linn. were subjected to extraction using petroleum ether (60–80ºC) at room temperature using percolation method. TLC examination of the petrol extract carried out on pre-coated silica gel plates 60 F_{254} (Merck), and developed with toluene-ethyl acetate (8:2), revealed the presence of two yellow spots (365 nm), which upon spraying with 5% or 10% ethanolic KOH (Bornträger reaction) showed red spots in visible and red fluorescence spots in UV-365 nm [20]. Then, the obtained petrol extract was washed several times with MeOH (solvent-solvent fractionation) to completely remove all polar contaminants and then the resultant fraction was evaporated under reduced pressure till complete dryness, to afford (17 g, 6.8%) a green semi-solid, which was subjected to CC on silica gel (200 mesh). Fractions of 200 ml were collected. Each was monitored by TLC sprayed with vanillin sulfuric acid [21], and similar fractions were combined together, the column was successively eluted first with 100% petrol (60–80), then with gradient increase of polarity with ethyl acetate (0.5, 1, 2, 3 and 5%). Fraction 1 eluted with 100% petroleum ether gave waxy hydrocarbons (discarded), 0.5% EtOAc (fractions 2–8) contained mainly fatty acid (palmitic acid) (grey and greyish-blue on TLC), 1% and 1.5% EtOAc (fractions 9–20) contained mainly mixtures of steroidal (purple) and/or terpenoidal compounds (dark-blue) as appeared on TLC and sprayed with vanillin-sulfuric acid, which were then rechromatographed over silica gel (200 mesh) afforded white needles of stigmasterol and β-sitosterol. Fractions (22–31) eluted with 2–3% EtOAc gave an anthraquinone-containing orange semi-solid material, which was rechromatographed over silica gel (200 mesh) eluted first with 100% petrol (fractions 1–3), 2% EtOAc (fractions 4–7), and 3% EtOAc (fractions 8–12). Fractions 1–3 yielded negligible amounts of material and were not subjected to further investigation. Fractions 4–7 were combined on the basis of identical TLC behaviour, and on concentration furnished an orange solid (1.5 mg) crystallized form MeOH to yield compound (1) as an orange powder. Fractions 8–12 were combined on the basis of identical

TLC behaviour, and concentrated to afford an orange solid (7 mg) which was crystallized form EtOH to yield compound (2) as an orange powder. Petroleum ether, EtOH and MeOH solvents used for the extraction and purification processess were of AR grade.

General experimental procedures

IR: spectra were obtained (KBr-Disk) using JASCO FT/IR-410 spectrophotometers. UV: JASCO V-530. UV/VIS spectrophotometer – Jasco EMC-418 was used. NMR: 1D-spectra were obtained using a pulse sequence supplied from JEOL JNM-AL-400 MHz NMR spectrometer for (^{1}H, ^{13}C-NMR, DEPT-45, -90, -135 and ^{1}H-^{1}H COSY) in DMSO-d_6. 2D-spectra (HSQC and HMBC) were obtained using a pulse sequence supplied from Varian Gemini VNMR-500 MHz NMR spectrometer. Chemical shifts were given in values (ppm) relative to trimethylsilane (TMS) as an internal reference. High-resolution MALDI-MS: High resolution mass spectra were obtained on JEOL JMS-700N for electron ionization or on JEOL JMS-T100 TD for electrospray ionization, using α-Cyano-4-hydroxycinnamic acid (CHCA) as a matrix (*m*/z 189.17).

Cell culture

HepG-2 (human hepatocellular liver carcinoma) HCT-116 (human colon carcinoma) and MCF-7 (human breast carcinoma) were generously provided by Prof. Stig Linder, Karolinska Institutet, Stockholm, Sweden. All cells were maintained in RPMI 1640 (Lonza Biowahittkar) medium. All media were supplemented with 1% antibiotic-antimycotic mixture (10 000 U/ml potassium penicillin, 10 000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B and 1% l-glutamine. All antibiotics and l-glutamine were purchased from Biowest, France.

MTT cytotoxicity assay

The cell viability was investigated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. MTT was used to determine the effect of plant extract and the isolated compounds under study on the viability of tumor cells [22]. All preceding steps were carried out in sterile laminar airflow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). All incubations were done at 37ºC in 5% CO_2 incubator in a humidified atmosphere (Sheldon, TC2323, Cornelius, OR, USA). Cells were seeded onto 96-well micro-titer plastic plates at a concentration of $10x10^3$ and allowed to adhere for 24 h. Media were aspirated and a fresh medium (without serum) was added to cells with various concentrations of plant extract, and isolated anthraquinones (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 μg/ml) and incubated for 48 h. For negative control, media were added instead of the plant extract. Positive control was prepared by adding

100 μg/ml of *Annona cherimolia* extract which is known as a strong cytotoxic natural agent that results in 90–100% cell lethality when used under the same conditions [23]. Then the absorbance was measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%. The percentage of change in viability was calculated according to the formula: (absorbance of extract/absorbance of negative control) –1) x 100.

A probit analysis was carried for IC_{50} determination using SPSS 11 program.

Assay for antiviral activity

Cells and viruses

Mardin-Darby canine kidney cells (MDCK) were grown in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (unless otherwise stated) at 37°C in a 5% CO_2 incubator. Original virus solution: influenza virus A/WSN/33 (3.72×10^7 $TCID_{50}$/ml), with 100TCID50/well infection.

Cytopathic effect inhibition assay

MDCK cells were seeded (100 cells/well = 3.0×10^4 cells/well) in 96-well plates and cultured in MEM/10% FBS for 2 days at 37ºC to >90% confluence. 40 μl of test sample (4% solution, 2 fold dilutions) were added to 960 μl of MEM (-), and then from the mixture 120 μl/well were added to the each well of 96-wells. Then cells were washed with FBS and infected with approximately 50 plaque forming units (PFU) of influenza virus (1000$TCID_{50}$/ml). 100 μl aliquot of the cell suspension was added to each well of a 96-well flat-bottomed microtitre tray containing 100 μl of various concentrations of the test sample. After three days of incubation at 37°C in 5% CO_2, the number of viable cells was determined by MTT method [24]. The cytotoxicity of each compound was evaluated in parallel with antiviral activity, which was based on the viability of mock-infected cells, as monitored by MTT method. The 50% antiviral effective dose (EC_{50}) and the 50% cytotoxic dose (CC_{50}) of sample were determined. The absorbances were determined with Tecan Infinite® 200 PRO Modular Microplate Readers at a test wavelength of 560 nm.

Statistical analysis

The results obtained in all analyses were expressed in mean ±SD (standard deviation). The levels of statistical significance ($p<0.05$) were calculated based on one-way ANOVA test for comparisons among means.

RESULTS AND DISCUSSION

The cytotoxic activity of petroleum ether extract of leaves of *Cassia roxburghii* Linn. revealed promising cytotoxicity against HCT-116 and MCF-7 cell lines, with IC_{50} = 34.9 and 38.04 μg/ml. Further chromatography over silica gel column chromatography resulted in the isolation of two anthraquinone; aloe-emodin acetate (1) and aloe-emodin (2) [18, 25], along with stigmasterol, β-sitosterol and palmitic acid.

Compound 1 was obtained as an orange powder, and its molecular formula $C_{17}H_{12}O_6$ was established from the quasi-molecular ion peaks at *m/z* 314.07904 $[M+2H]^+$ and 335.05261 $[M+Na]^+$ (calcd 314.07849 and 355.05316 for $C_{17}H_{14}O_6$ and $C_{17}H_{12}O_6Na$ respectively) as determined by HRMALDI-TOF/MS analysis. UV λ_{max} (MeOH) of compound (1) exhibited absorption maxima at 225, 255, 274, 285 and 435 nm suggested an anthraquinone as a basic structure [25, 26]. This was supported by IR absorption at 3500 cm^{-1}, 1680 cm^{-1} and 1635 cm^{-1} due to phenolic hydroxyl, conjugated carbonyl and chelated quinone carbonyl respectively [27], these data along with two ^{13}C-NMR signals which appeared at δc 181.299 and 191.595 indicated that compound (1) could be an anthraquinone. ^{1}H-NMR spectrum of compound (1) showed the presence of a strong singlet signal at δ_H 2.14 (3H, *s*, COCH_3-12) suggesting the presence of an acetoxy group which was confirmed by the characteristic signals appeared in the ^{13}C-NMR spectrum at δ_C 20.700 (CO$\underline{CH_3}$) and δ_C 170.129 ($\underline{CO}CH_3$) and confirmed by HSQC and HMBC correlations. Further analysis of ^{1}H-NMR spectrum revealed the presence of methylene group at δ_H 5.22 (2H, br*s*, CH_2-11) which broadened due to long range coupling with aromatic protons [28]. Furthermore, the ^{1}H-NMR and COSY spectra revealed the presence of a number of contiguous cross peaks characterizing the two sets of doublet signals which appeared at δ_H 7.41 (1H, *dd*, *J*=1.2 and 6.8 Hz, H-7) and at δ_H 7.74 (1H, *dd*, *J*=1.2 and 6.8 Hz, H-5) corresponding to the *meta*- and *ortho*-couplings of the aromatic protons (Ring-A) respectively, in addition to a triplet signal that appeared at δ_H 7.83 (1H, *t*, *J*=6.8 Hz) corresponding to H-6 of Ring-A due to the ortho-coupling with both of H-5 and H-7, together with two sets of *meta*-coupled signals that appeared at δ_H 7.36 (1H, *d*, *J*=1.2 Hz, H-2) and δ_H 7.70 (1H, *d*, *J*=1.2 Hz, H-4) corresponding to the aromatic protons of Ring-B. Signals that appeared at δ_H 11.92 and 11.93 (1H, *s*, D_2O exchangeable) were assignable to C-1 and C-8 H-bonded hydroxyl protons, respectively [18]. ^{13}C-NMR, DEPT-135 and HSQC spectra revealed the presence of one methyl (δ_C 20.700), one methylene (δ_C 64.225), twelve (five methine and seven quaternary) aromatic or olefinic carbons (δ_C 114.500 – 161.406), an ester carbonyl (δ_C 170.129) and two chelated quinone carbonyls (δ_C 181.299 and 191.595) which gave an overall suggestion that compound (1) could be an anthraquinone [18]. The HMBC experiment was conducted to get the long rang correlations (^2J and ^3J) and revealed the presence of three cross peaks that represent the correlations of the methylene group at δ_H 5.22 with

δ_C 146.472 (C-3), 122.145 (C-2) and 117.779 (C-4) confirming the attachment of methylene group to C-3 of Ring-B. Furthermore, the methylene group at δ_H 5.22 and the acetoxy methyl at δ_H 2.14 (CO*CH*$_3$) exhibited a long rang correlation with δ_C 170.129 (*CO*CH$_3$) which confirming the existence of the acetoxy group to be directly attached to the methylene group at δ_H 5.22. Therefore, the structure of compound (1) was elucidated as depicted in (Figure 1) and assigned to 1,8-dihydroxy-3-[(acetyloxy)methyl]-9,10-anthracenedione aloe-emodin acetate which is isolated for the first time from this genus and for the third time from nature [28, 29].

Quinones are widely distributed in plants, bacteria, and animals and have been used as traditional dying agents. Anthraquinone derivatives were reported to have anti-tumor activities, and they are clinically used as anticancer agents [30, 31].

Our study was conducted to investigate the cytotoxicity of the isolated constituents from *C. roxburghii* Linn. against HepG-2, HCT-116 and MCF-7 cell lines, based on bioassay guided fractionation. The preliminary results revealed that the petrol extract of *C. roxburghii* Linn. exhibited IC_{50}=34.9 and 38.04 μg/ml against HCT-116 and MCF-7, and resulted with no activity against HepG-2 which suggests some selectivity. This prompted us to follow up the fractionation process searching for the active constituents, which resulted with the isolation of two anthraquinones (1 and 2) along with stigmasterol, β-sitosterol and palmitic acid.

The cytotoxicity of isolated compounds was evaluated against HepG-2, HCT-116 and MCF-7 cell lines (tab. 1). Obtained results revealed that both of aloe-emodin acetate and aloe-emodin were inactive against HepG-2, while cytotoxicity of aloe-emodin (2) was stronger than that of acetyl aloe-emodin (1) with IC_{50}=70.02 and 153.30 μg/ml against HCT-116 and with 53.20 and 93.20 μg/ml against MCF-7, respectively.

Table 1.

The cytotoxicity and anti-influenza virus-A of the petroleum ether of *C. roxburghii* Linn. and the isolated compounds

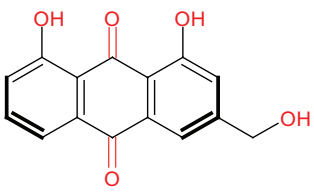
Sample	Cytotoxicity (IC_{50} μg/ml)			Anti-Influenza Virus-A	
	HCT-116	MCF-7	HepG-2	IC_{50}	CC_{50}
Petroleum ether	34.9	38.04	0	ND	ND
β-Sitosterol	58.4	73.2	49.4	ND	ND
Stigmasterol	44.1	54.3	75.8	ND	ND
Palmitic acid	97.4	67.7	0	ND	ND
Aloe-emodin acetate	153.3	93.2	0	10.23	1.32
Aloe-emodin	70.02	53.2	0	2.00	0.47

IC_{50}: 50% inhibition concentration
CC_{50}: 50% cytotoxic concentration
ND: not determined (insufficient sample concentration)

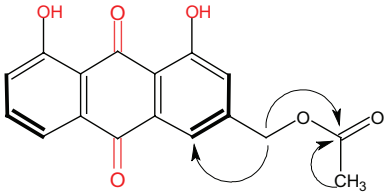
The antiviral activity (tab. 1) of aloe-emodin acetate and aloe-emodin was tested using MTT method against influenza virus-A, the obtained results revealed that aloe-emodin showed more potency than acetyl aloe-emodin with inhibition dose concentration (IC_{50}) of 2.00 and 10.23 μg/ml respectively, while the cytotoxic concentration (CC_{50}) of 0.47 and 1.32 μg/ml, respectively.

It seems that the observed activity of the isolated anthraquinones (1 and 2) can be attributed to the number of the hydroxy groups in the anthraquinone nucleus which seemed to play an important role in the degree of cell growth inhibition. In brief, the effectiveness of anthraquinones with two or three hydroxy groups was higher than of those with no hydroxy groups such as 9,10-dioxoanthracene [32]. However, the number of hydroxyl substituents of anthraquinones seems to be less influential on the antimutagenicity than the position of the hydroxyl substituent itself. Interestingly, intramolecular hydrogen bonding could occur in anthraquinones leading to more potency [33], this appeared clearly in our study (fig. 1) as both of aloe-emodin (2) and its acetate derivative (1) have two OH groups at 1,8-positions of the anthraquinone skeleton, but aloe-emodin has one more OH group gave it more cytotoxicity and more antiviral activity. Furthermore, Edenharder et al., reported that carbonyl function within the anthraquinone skeleton (fig. 1) is a prerequisite for their antimutagenicity and genotoxicity, which mediated by non-covalent DNA-binding [34, 35]. Moreover, it was established that cytochrome P-450 substrates containing fused aromatic and heteroaromatic rings would give rise to overall molecular planarity with relatively small molecular depth. This implies that the binding sites of cytochromes P-450 contain a number of hydrophobic aromatic amino acid residues orientated so as to allow the occupation by similar substrates containing co-planar aromatic rings as in the anthraquinone skeleton e.g. compounds (1 and 2) [36]. In addition, the binding sites of the enzyme contain hydrophilic amino acid residues capable of hydrogen bonding to carbonyl moieties [37]. This seems to illustrate why anthraquinone with two free carbonyl groups was the most potent extracellular domain (ECD) inhibitor than others. However, the sulfonyl and carboxyl groups possessed electron-attracting capability. The electronic factor may play a role in the interaction between the carbonyl groups of anthraquinones (1 and 2) and the hydrophilic amino acid of cytochrome P-450. In addition, an additional methyl group (in our study we have 3-acetyloxymethyl (1) and 3-hydroxymethyl (2)) at the C-3 position enhanced the inhibition; wherase a carboxy1 group at the C-3 position (rhein) reduced the antimutagenic activity more than 20-fold. This result also demonstrates that the anthraquinone containing the electron-repelling substituent had a higher antimutagenic activity than that of the electron-attracting substituent [33]. Furthermore, the cytotoxic activity of anthraquinones (1 and 2) can be attributed to their ability to form a redox cycle with their semiquinone radicals, leading to the formation of reactive oxygen species that include superoxide anion radical, hydrogen peroxide, and hydroxyl radical [38-40]. The generation of ROS could contribute to cytotoxic properties of parent compounds, and may contribute to mitochondrial damage,

reduction of mitochondrial transmembrane potential, release of cytochrome C and Smac, and subsequent caspase activation and apoptosis [41]. Redox cycling can cause lipid peroxidation, consumption of reducing equivalents, oxidation of DNA, and DNA strand breaks. Further, ROS can activate a number of signaling pathways, including those of protein kinase C and RAS [42].



aloe-emodin



aloe-emodin acetate

Figure 1.

The structural features of the isolated anthraquinones with high multifunctional activities. Selected HMBC correlations are indicated by (→), and $^{1}H-^{1}H$ COSY correlations are indicated by bold bonds (–).

ACKNOWLEDGEMENT

The authors are grateful to School of Pharmaceutical Sciences, Nagasaki University, Nagasaki, Japan, for providing facilities for instrumental analysis and also for performing the antiviral activity. This was done through a research grant for Dr. Magdy M. D. Mohammed, and it was fully funded by Heiwa Nakajima Foundation-Japan.

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AKTYWNOŚĆ ANTYWIRUSOWA I CYTOTOKSYCZNA ANTRACHINONÓW WYIZOLOWANYCH Z LIŚCI *CASSIA ROXBURGHII* LINN.

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Streszczenie

Aktywność cytotoksyczna wyciągu benzynowego z liści *Cassia roxburghii* Linn. przeciwko liniom komórkowym HCT-116 i MCF-7 wynosiła odpowiednio IC_{50}=34.9 i 38,04 μg/ml, natomiast nie wykazano aktywności przeciwko HepG-2. Przeprowadzono biologiczne frakcjonowanie w celu wyizolowania i identyfikacji aktywnych związków o działaniu cytotoksycznym. Późniejsza separacja chromatograficzna i oczyszczenie wyciągu benzynowego zaowocowało wyizolowaniem dwóch antrachinonów oznaczonych jako octan aloe-emodyny i aloe-emodyna, a także stigmasterolu, β-sitosterolu i kwasu palmitynowego. Określenie struktury wyizolowanych składników przeprowadzono przy użyciu 1D, 2D-NMR i HR-MS. Określono także cytotoksyczność octanu aloe-emodyny i aloe-emodyny, której wartość wyniosła odpowiednio IC_{50}=153,30 i 70,02 μg/ml przeciwko HCT-116 oraz odpowiednio 93,20 i 53,20 μg/ml przeciwko MCF-7. Nie wykazano aktywności przeciwko HepG-2. Testowano też aktywność przeciwwirusową dwóch wyizolowanych antrachinonów przeciwko wirusowi grypy typu A. Wyniosła ona odpowiednio IC_{50}=10,23 oraz 2,00 przy CC_{50}=1,32 i 0,47 μg/ml.

Słowa kluczowe: *Cassia roxburghii Linn., Fabaceae/Leguminosae, antrachinony, aktywność antywirusowa, aktywność cytotoksyczna, SAR*