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Research article

Phytochemical, cytotoxicity and antioxidant investigation of *Cassia alata* leaves growing in Egypt

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Key words: *Cassia alata* leaf, antioxidant, anthraquinones, fatty acids, unsaponifiable matter, cytotoxicity.

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Abstract

Leaf extracts of *Cassia alata* L traditionally used for treatment of a variety of diseases. Chloroform fraction of leaves was evaluated for its potential antitumor properties *in vitro*. MTT assay was used to examine the cytotoxic effect on three human cancer cell lines namely HepG2, MDA-MB-231 and Caco2. Chloroform fraction showed remarkable cytotoxicity against HepG2 cells with IC_{50} = 37.4 µg/ml at exposure time 48 h. This observation was confirmed by morphological investigation. The fraction exhibited weak anti-proliferative effect on Caco2 and MDA-MB-231 cells (8.2% and 11.6% respectively), with IC_{50} values >100 µg/ml. DPPH free radical scavenging activity of the fraction (100 µg/ml) revealed weak antioxidant activity (7.8%). Further bioassay-guided fractionation of the cytotoxic fraction led to the isolation and characterization of three anthraquinones (rhein, aloe-emodin and emodin). On the other hand, n-hexane fraction was saponified into unsaponifiable and saponifiable matters. GC/MS of the fatty acid methyl esters revealed that the major fatty acids were palmitic (37.02%), linolenic (24.27%), linoleic (15.22%) and stearic (14.18%) acid which composed 90.69% of the total fatty acids. GC/MS analysis of the unsaponifiable matter showed that phytol was the major constituent (74.59%) and β-sitosterol (17.45%) was the second abundant one. The cytotoxicity profile might represent new promising compounds with potential for development as an anticancer drug with low or no toxicity to non-cancer cells.

Introduction

Cassia alata (L.) Roxb., (syn. *Senna alata* L) family Fabaceae. It is widely distributed in the tropical countries including Egypt [1]. Chemical constituents of *C. alata* leaves include emodin [2], chrysoeriol, kaempferol, quercetin, 5,7,4'-trihydroflavanone, kaempferol-3-*O*-β-D-glucopyranoside, kaempferol-3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside, fatty acids, hydrotetratriacontane, n-dotriacontanol, n-triacontanol [3], aloe-emodin, rhein methyl ester, aloe-emodin-O-β-glucoside, kaempferol [4]. Kaempferol [5,6], Kaempferol-3-*O*-gentiobioside [7], 5, 7-dihydroxy-3', 4'- dimethoxy flavone and 2, 5, 7, 4' tetrahydroxy Isoflavone [8,9], propelargonidins, is a condensed tannins [10] and volatile oil [11].
C. alata leaf extract has been reported to have various pharmacological activities including antibacterial [12,13], cytotoxicity [14,15], anti-inflammatory [16], antidiabetic [17], antihepatotoxic and hepatoprotective effects [18,19], antiseptic [20], antiviral [21] and exhibited strong DPPH radical scavenging activities [22].

Natural plant compounds are highly varied in structure. However, there is an increase attention on extracts and biologically active compounds isolated from plant species used in herbal medicine, due to the fewer side effects. Medicinal plants are playing an important role as anticancer agents and it is significant that many of currently used anticancer agents are derived from natural source such as plants [23].
The present study is focused in isolation and chemical identification of unsaponifiable matter, fatty acid and anthraquinones, in addition to the antioxoidant and cytotoxicity activities of chloroform fraction of *Cassia alata.*

Experimental

Material and Methods

Chemicals

Folin–Ciocalteu reagent (Sigma Chemical Co., St. Louis, Mo., USA). All solvents and chemicals were HPLC grade, Fisher Scientific, USA. Silica gel 60 mesh (Merck, Germany) and Sephadex LH-20 (Sigma, USA) for

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column chromatography. TLC silica gel F_{254} or polyamide aluminum sheets 20 x 20 cm and HPTLC Silica gel F_{254} aluminum sheets 20 x 20 cm (Merck, Germany).
Dulbecco's modified Eagle's medium (DMEM), Trypsin-Versene (EDTA) Mix, Dulbecco's Phosphate-Buffered Saline (DPBS), L-Glutamine and penicillin/streptomycin stock from Lonza Verviers SPRL, Belgium. Fetal Bovine Serum (FBS; SeraLab, UK), Doxorubicin (trade name: Adriamycin; Pfizer company, Australia), and DPPH (Sigma, Germany). MTT dye (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and Di-methyl sulfoxide (DMSO) from SERVA Electrophoresis GmbH, Germany.

Apparatus and Instruments

Nuclear magnetic resonance (NMR) spectra were measured on Bruker AV-400 spectrometer operating at a frequency of 400 MHz using DMSO as solvent at room temperature with tetramethylsilane (TMS) as an internal standard. Electron ionization (EI) mass spectra (EI-MS) were carried out on a THERMO Scientific Corp.; USA), mass spectrometer 70 eV. FLUOstar Optima microplate reader (BMG labtech, Germany) and inverted microscope (Olympus CK2, Japan).

Gas Chromatography/Mass Spectrometry (GC/MS) analysis of unsaponifiable matter

GC-MS analysis of unsaponifiable matter was carried out using gas chromatography-mass spectrometry instrument stands with the following specifications Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp.; USA), coupled with a thermo mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TG-5MS column (30 m x 0.25 mm i.d.; 0.25 μm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 ml/min and a split ratio of 1:10 using the following temperature program: 50 ˚C for 3 min; rising at 5 ˚C /min to 300 ˚C and held for 20 min. The injector and detector were held at 280 ˚C. Diluted samples (1:10 hexane, v/v) of 0.2 μl of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450. Most of the compounds were identified using two different analytical methods: mass spectra (authentic chemicals, Wiley spectral library collection and NSIT library) and by comparison with previous references and data.

Gas chromatographic-mass spectrometry (GC-MS) analysis for fatty acid methyl esters

GC-MS analysis of the fatty acid methyl esters was carried out using gas chromatography-mass spectrometry instrument stands with the following specifications. Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp.; USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a PR-5MS column (30 m x 0.25 mm i.d.; 0.25 m film thickness). Analyses were carried out using helium as the carrier gas at a flow rate of 1.0 ml/min at a split ratio of 1:10 and the following temperature program: 50 ˚C for 3 min; rising at 4.0 °C/min to 260 ˚C and held for 6 min; rising at 6 ˚C /min to 300 ˚C and held for 1 min. The injector and detector were held at 200 ˚C. Diluted samples (1:10 hexane, v/v) of 0.2 μl of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450. Most of the compounds were identified using two different analytical methods: mass spectra (authentic chemicals, Wiley spectral library collection and NSIT library) and by comparison with previous data.

Plant materials

Cassia alata (L.) Roxb., leaves were collected from Faculty of Agriculture Farm, Cairo University and were kindly identified by, Orman Botanical garden, Giza and Dr. Mona Marzok, Taxonomist in the Herbarium of National Research Centre (NRC), Cairo, Egypt. A voucher specimen was deposited at the Herbarium of the NRC, Cairo, Egypt.

Extraction

Fresh leaves were dried at room temperature in shad and grinded to fine powder**.** Air-dried, powdered (2 kg) were extracted by methanol at room temperature and concentrated under reduced pressure at 40°C. The residue of methanolic extract (22.57 %) was suspended in water with assistance of ultrasonic water bath and partitioned successively with hexane (3.6%), chloroform (3.33%), ethyl acetate (0.74%), n-butanol (4.63%) and aqueous-soluble fractions (10.27%). All extracts were concentrated till dryness in rotor vapor at 40 ˚C. The obtained extracts were stored at -20 ˚C until used.

Isolation and identification of saponifiable and unsaponifiable matter of n-hexane extract

About 2g of n-hexane fraction were saponified with alcoholic KOH 10% (ethanol 95%) and the residue was fractionated into unsaponifiable and saponifiable matters according to the method of Farag *et al.* [24], as well as, the fatty acids were liberated by acidification of the saponifiable matter, extracted with ether, dried over anhydrous Na_2So_4 and concentrated under reduced pressure. The fatty acid methyl esters were prepared according to the method adopting by Kinsella [25]. Fatty acid methyl esters and unsaponifiable matter were analyzed by GC/MS and were identified on the basis of

fragmentation pattern of mass spectra data and a library database [Wiley (Wiley Institute, Los Angeles, CA), NIST (National Institute of Technology, Los Angeles, CA)] and by comparison with previous data. Quantitative determination was carried out on the basis of peak area measurements of the GC/MS chromatograms.

Isolation and characterization of anthraquinones

Chloroform crude fraction was subjected to silica gel (mesh 60) column chromatography (CC) and was successively eluted gradient with a mixture of solvents of increasing polarity made up n-hexane: ethyl acetate (100%, up to 25:75 v/v), then $CHCl_3$: ethyl acetate (100%, up to 70:30 v/v) yielding 98 fractions (20 ml) and the fractions were monitored by TLC silica gel F_{254} sheets (Merck) using different solvent systems as follows: toluene: ethyl acetate (8:2 v/v) and n-hexane: ethyl acetate (70:30 v/v), $CHCl_3$: ethyl acetate (9:1 v/v). Visualization was carried out using *p*-anisaldehyde - sulphuric acid reagent heated at 110°C and alcoholic KOH (10%). Similar fractions of the same R_f (TLC) in different solvent systems were combined and pooled together, to give 18 major fractions.

F1 to F5 corresponding to the n-hexane (100%) and F6 to F8 corresponding hexane: ethyl acetate (95:5 v/v) were fatty acids and lipids. F9 to F11 corresponding the n-hexane: ethyl acetate (90:10), F12 corresponding to the n-hexane: ethyl acetate (75:25 v/v). F13 corresponding the n-hexane: ethyl acetate (50:50), F14 corresponding to the n-hexane: ethyl acetate (25:75 v/v), F15 corresponding to the $CHCl_3$: ethyl acetate (90:10 v/v), F16 corresponding the $CHCl_3$: ethyl acetate (80:20 v/v), F17 and F18 corresponding to the $CHCl_3$: ethyl acetate (70:30).

F9 to F18 were checked for the presence of anthraquinones using HPTLC silica gel F_{254} and TLC polyamide and the presence of yellow spots which upon spraying with 10% ethanolic KOH (Bornträger reaction) showed red spots in visible and red fluorescence spots in UV-365 nm indicated the presence of anthraquinones [26].

This was finally F9, combined of F10 and F11, F12 and F13 were separated by HPTLC silica gel F_{254} and TLC polyamide using $CHCl_3$: ethyl acetate (9:1 v/v) as the mobile phase. The bands were visualized and detected under UV light at 254 and 365 nm then scraped off from TLC plates, eluted from the silica with absolute methanol, and filtered. The isolated compound(s) appeared as yellow spots which upon spraying with 10% ethanolic KOH (Bornträger reaction) showed red spots in visible and red fluorescence spots in UV-365 nm. The purity of the isolated compounds was confirmed by TLC using various solvent systems. Three different pure anthraquinone compounds were obtained; each pure compound was applied to a Sephadex LH-20 column consequently and then submitted to purification on a reversed-phase SPE cartridge C18 column previously activated by elution of methanol.

Cell culture

Three different types of human cancer cells: triple negative breast cancer cells (MDA-MB-231), hepatocellular carcinoma cells (HepG2) and colon adenocarcinoma cells (Caco2) were purchased from vacsera, Egypt and maintained as monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% L-glutamine 200 mM and 1% penicillin/streptomycin stock. Monolayer was passage at 70-90% confluence using trypsin-EDTA solution. All cell incubations were maintained at humidified CO_2 incubator with 5% CO_2 at 37°C.

Cytotoxicity activity (MTT assay)

The cytotoxicity in the present study is based essentially on previous report of Mosmann [27] with some modification. Briefly, HepG2, MDA-MB-231 (8000 cells/well) or Caco2 (5000 cells/well) cells were seeded onto 96-well plates in a total volume of 200 µl and left overnight to form a semi-confluent monolayer. Cell monolayers were treated in octuplates with vehicle (DMSO, 0.1% v/v) or test sample (100 µg/ml) for an exposure time of 48 h. Doxorubicin hydrochloride was used as cytotoxic reference drug. At the end of exposure, 30 µl/well of MTT solution in DPBS (5 mg/ml) were added to all wells and left to incubate for 90 min. The formation of formazan crystals was visually confirmed using phase contract inverted microscope (Olympus CK2, Japan). DMSO (100 µl/well) was added to dissolve the formazan crystals with shaking for 10 min after which the absorbance was read at 540 nm against no cell blanks on a FLUOstar Optima microplate reader (BMG labtech, Germany). The percentage of cell death was calculated using the following formula:

$$\%\ \text{cell death} = 100 - [\frac{At - Ab}{Ac - Ab} \times 100]$$

Whereas, At: Absorbance of treated cells, Ab: Absorbance of blank (medium only without cells) and Ac: Absorbance of control.

Dose-response experiment was performed on sample producing ≥ 50% loss of cell viability using five serial 2-fold dilutions (100, 50, 25, 12.5 and 6.25 µg/ml). IC_{50} values were calculated using the dose response curve fit to non-linear regression correlation using Graph Pad Prism® V6.0 software.

Morphological assessment

Assessment of morphological changes of cells following treatments was performed using phase contrast inverted microscope (Olympus CK2, Japan) and

photomicrographs were taken with digital eyepiece camera (Total magnification is 150×).

Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The method used in the present study is based essentially on previous reports of Braca *et al.* and Nara *et al.* [28,29] with some modification. Briefly, chloroform fraction was prepared in DMSO as 10× stock solution (1 mg/ml). Stock solution (20 µl/well) was dispensed in triplicates onto 96-well plates (flat-bottomed, Greiner bio one, Belgium). The assay was started with the addition of DPPH reagent (0.004% wt/v in methanol, 180µl/well) to give a final concentration of the sample as 100 µg/ml. Appropriate blank was prepared using the solvent only in addition to the same amount of DPPH reagent to get rid of any inherent solvent activity. Negative control was also run in parallel to correct for any non-DPPH absorbance by colored fraction at the test wavelength. The plate was immediately shaken for 30 seconds and incubated in the dark for 30 min at room temperature. The remaining DPPH was measured at 540 nm on a FLUOstar Optima microplate reader (BMG labtech, Germany). Quercetin dihydrate was used as a positive control. DPPH radical scavenging activity was calculated using the equation below.

$$\% \text{ DPPH Scavenging Activity} = 100 \times \frac{Ab - As}{Ab}$$

Whereas, Ab: Absorbance of blank and As: Absorbance of sample. Absorbance of blank and sample are the averages of triplicates determinations of the corrected readings of blank and sample at 540 nm, respectively.

Results and Discussion

Fatty acids composition of *Cassia alata* leaves

Fatty acids composition of leaves is presented in Table 1. The major fatty acids were palmitic (37.02%), linolenic (24.27%), linoleic (15.22%) and stearic (14.18%) acid and composed 90.69% of the total fatty acids. The fatty acid palmitic represented the predominate one. The major unsaturated fatty acids consist mainly of linolenic, linoleic followed by oleic acid and linolenic acid represented the most abundant unsaturated one. The total fatty acids are characterized by much higher proportion of saturated fatty acids (54.76%) followed by unsaturated fatty which represented 45.24% of the total fatty acids. Oleic (5.04%) and myristic acid (2.65%) were present in minor level while the other identified fatty acids methyl esters were less than 1%.

Table 1. Fatty acids composition of *C. alata* leaves

Fatty acids	RT	Relative Percentage
Tetradecanoic acid, methyl ester (Myristic acid) C14:0	30.89	2.65
Hexadecanoic acid, methyl ester (Palmitic acid) C16:0	38.13	37.02
Hexadecanoic acid, 14-methyl, methyl ester	41.72	0.91
2-Hexadecenoic acid, methyl ester	42.40	0.71
Octadecanoic acid, methyl ester (Stearic acid) C18:0	44.91	14.18
9-Octadecenoic acid (Oleic acid) C18:1	45.38	5.04
9,12-Octadecadienoic acid methyl ester (Linoleic acid) C18:2	46.69	15.22
9,12,15-Octadecatrienoic acid, methyl ester, (α Linolenic acid) 18:3	48.42	24.27
Mono unsaturated fatty acids		5.75
Poly unsaturated fatty acids		39.49
Unsaturated fatty acids		45.24
Saturated fatty acids		54.76

RT: retention time by min

Unsaponifiable matter composition of *Cassia alata* leaves

The data of the GC/MS analysis of the unsaponifiable matter revealed the presence of 23 compounds from which 14 major compounds accounted (99.02%) were identified whereas the minor compounds represented (0.98%) and couldn't be identified. Results in table 2 shows that phytol $C_{20}H_{40}O$ (3,7,11,15-tetramethylhexadec-2-en-1-ol) acyclic diterpene alcohol represented the major constituents (74.59%) and β-sitosterol $C_{29}H_{50}O$ (17.45%) was the second major one. Among the phytosterols namely stigmasterol $C_{29}H_{48}O$ was identified in minor level and constituted 4.13%. So phytosterol accounted 21.58% of the total unsaponifiable fraction. On the other hand, fatty acid, volatile monoterpene and hydrocarbones were identified in traces levels. Phytol was found to be major constituent of unsaponifiable matter of *Cassia alata* leaves. In this concern, several studies addressed on its biological activity [30,31].

Anthraquinones

TLC purification of F9 resulted in the isolation of compound I (22 mg) in pure form. Purification based on TLC result to give compound II (15 mg) from combined F10 and F11. Compound III was obtained in pure form from combined F12 and F13 (10 mg). Identification of the isolated pure compound was based on spectrometric analyses ^{1}HNMR, EI-MS and by comparison with previous data (Figure 1).

Table 2. GC/MS analysis of unsaponifiable matter of *C. alata* leaves

Compound	RT	Relative percentage
Limonene	10.43	0.06
6-Dodecanone	32.47	0.30
8,11,14 Eicosatrienoic acid,	33.73	0.08
Uk	37.84	0.05
Phytol	38.04	74.59
Uk	43.54	0.18
Oleic acid	44.70	0.16
trans -Farnesol	49.34	0.32
Uk	49.89	0.09
Uk	50.43	0.05
Behenic alcohol	50.70	0.10
Uk	53.42	0.36
1-Heptatriacotanol	53.88	0.25
Uk	54.20	0.08
Citronellic acid	54.45	0.23
Uk	54.79	0.09
Uk	55.13	0.04
Nerolidol	55.57	1.20
Stigmasterol	55.99	4.13
1-Heptatriacotanol	56.21	0.05
β-sitosterol (Stigmast-5en-3-ol)	57.19	17.45
Palmitoleic acid	57.44	0.10
Uk	57.69	0.04

Uk = unknown, RT: retention time by min

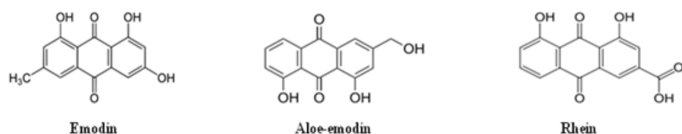

Figure 1. Chemical structures of isolated anthrquinones

Compound I was obtained from F9 as orange-yellow amorphous powder. ^{1}H-NMR spectrum revealed the presence of a sharp singlet signal appeared at δ_H 2.42 (3H, *s*, CH_3), two sets of double of doublets appeared at δ_H 6.54 and 7.10 (each, 1H, J = 2.4 Hz) corresponding to H-7 and H-5 respectively, moreover, at δ_H 7.18 and 7.52 (each, 1H, J = 1.2 Hz) corresponding to H-2 and H-4 respectively. EI-MS: m/z 269,3 m/z 241.2 [M − 28], 225. The identity of the isolate was achieved by analysis of ^{1}HNMR and mass spectrum, and comparison data with literature [32–34] and data confirmed the compound as emodin $C_{15}H_{10}O_5$ (4,5,7-trihydroxy-2-methyl anthraquinone).

Emodin has previously reported as anticancer [35] and was isolated from *C. alata* leaves [2,36], it has been found in a wide variety of plant species belonging to the genus *Cassia* [37,38].

Compound II was obtained from F10 and F11 as orange-yellow amorphous powder, the compound gives positive Borntrager's test which indicates its anthraquinone nature. ^{1}H-NMR spectrum revealed the presence of a methylene group at δ_H 4.63 (2H, br *s*, CH_2-11) which broadened due to long range coupling with aromatic protons [39]. Furthermore, two sets of broad signals appeared at δ_H 7.29 (1H, br *s*, H-2) and δ_H 7.69 (1H, br *s*, H-4) corresponding to the aromatic protons of ring-B. Moreover, two sets of doublets appeared at δ_H 7.37 (1H, br *d*, J = 8.4 Hz, H-7) and at δ_H 7.71 (1H, br *d*, J = 8.4 Hz, H-5) corresponding to the *ortho*-coupled aromatic protons (Ring-A) respectively, in addition to a triplet signal appeared at δ_H 7.80 (1H, *t*, J = 8.4 Hz) corresponding to H-6 of (ring-A) due to the *ortho*-coupling with both of H-5 & H-7, ESI-MS: m/z 269.98, m/z 240.2 [M − 28]. Hence, and according to the above data the compound can be identified as aloe-emodin and through comparison with NMR and MS spectra of published data [33,40,41].

Generally aloe-emodin has previously reported as anticancer [42–44]. It has been reported to exhibit an anticancer activity on neuroectodermal tumors, lung squamous cell carcinoma, and hepatoma cells [42,45–47] and was isolated from *C. alata* leaves [36,48,49] . It has been found in a wide variety of plant species belonging to the genus [44].

Compound III was obtained from F12 and F13 as orange-yellow amorphous powder and the compound gives positive Borntrager's test which indicates its anthraquinone nature. Rhein (1,8-dihydroxyanthraquinone-3-carboxylic acid) was structurally identified as one compound out using MS and ^{1}H NMR spectra as follows: EI-MS: m/z 284, 267, 256, 239, 228, 211, 183, 155, 142, 126. It showed the molecular ion at m/z 284, which was corresponding to the molecular formula $C_{15}H_8O_6$ of rhein. The ^{1}H NMR spectrum revealed five aromatic protons of which two were broad singlets due to *meta* coupling. ^{1}H NMR (400 MHz, DMSO): 11.9 (1H,brs, C1–OH), 11.5 (1H,brs, C8–OH), 8.13 (1H,brs, C2–H), 7.40 (1H, d, J = 8.5 Hz, C5–H), 7.73 (1H, m, C6–H), 7.75 (1H,br s, C4–H), 7.81(1H, d, J = 8.0 Hz, C7–H). EI-MS m/z 284.31 $(M-1)^-$, rhein is characterized by decarboxylation that is molecular ions resulting in [M-COOH] m/z 239. The ions at m/z 253 and 267 were also observed in the mass spectrum of rhein. The structure identification of rhein was performed by ^{1}HNMR, MS analysis and with comparison published data [33,50,51].

The anticancer activity of rhein has previously reported [51–53] and was isolated from *C. alata* leaves [49,54,55].

Cytotoxicity activity (MTT assay)

The evaluation of the anti-proliferative activity of the chloroform fraction of *C. alata* leaves against the proliferation of three different cancer cell lines at a final concentration of 100 µg/ml was illustrated in Figure 2. The pre-screen revealed that the chloroform fraction has high anti-proliferative effect on HepG2 cells only with

100% loss of cell viability relative to solvent control. It exhibited weak anti-proliferative effect on Caco2 and MDA-MB-231 cells with 8.2% and 11.6% loss of cell viability relative to solvent control, respectively. The assay was further investigated against HepG2 cells only in a dose-response experiment for IC_{50} determination, whereas, IC_{50} values of chloroform fraction against Caco2 and MDA-MB-231 cells were considered to be >100 µg/ml.

Figure 3 displays the dose-dependent effects of chloroform fraction on HepG2 cells to reveal the IC_{50}. The IC_{50} of chloroform fraction was 37.4 µg/ml and doxorubicin was tested as positive control and gave IC_{50} of 1.4 µg/ml (Table 3). This effect has stimulated us to ongoing work to isolate and identify the active ingredients of the fraction. This led to the isolation of valuable compounds in the light of their ability to inhibit HepG2 cell proliferation. Three anthraquinones were isolated and identified from the fraction, these anthraquinones composition may be responsible for the cytotoxic activity against HepG2 cancer cells. The cytotoxic effects (e.g., cell death) may be attributed to the arrest of the cell cycle and the induction of apoptosis in cancer cells or the fraction could effectively inhibit the uptake of glucose in tumor cells, caused changes in membrane-associated functions and led to cell death.

Table 3. IC_{50} of chloroform fraction of *C. alata* leaves and doxorubicin on the proliferation of HepG2 cell line, as tested by MTT reduction assay

Sample	IC_{50} (µg/ml)
Chloroform fraction	37.4
Doxorubicin	1.4

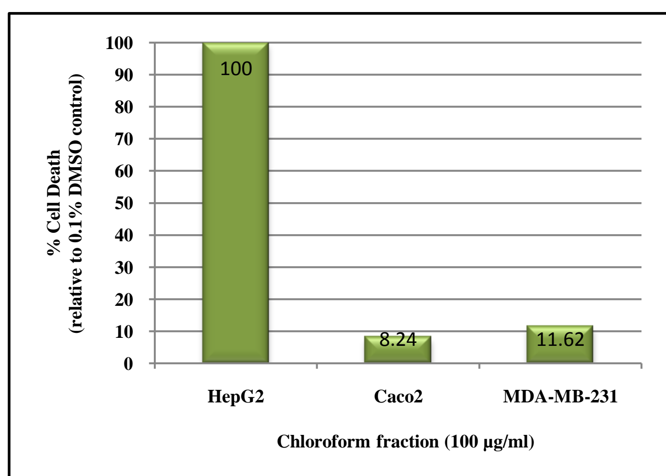

Figure 2. Pre-screening of chloroform fraction of *C. alata* leaves against the proliferation of three different cancer cell lines at 100 µg/ml, as tested by MTT reduction assay.

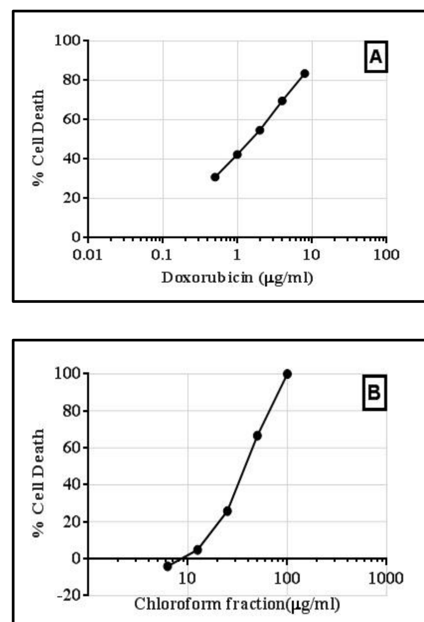

Figure 3. Dose-response curves showing the effect of doxorubicin (A) and chloroform fraction of *C. alata* leaves (B) against the proliferation of HepG2 cells as tested by MTT reduction assay

Several other studies have dealt with the cytotoxic activity evaluation of *C. alata* leaves extracts. Hexane fraction of *C. alata* leaves exhibited cytotoxic activity against parental A549 (lung cancer cells) and OV2008 (ovarian cancer cells) cell lines [56,57], also the hexane fraction and f6l (a mixture of polyunsaturated fatty acid esters) exerted a cytotoxic effect on MCF-7 (breast carcinoma cells), T24 (bladder carcinoma cells) and Col 2 (colorectal carcinoma cells) cell lines in a dose dependent manner, but were not effective against A549 (non-small cell lung adenocarcinoma) and SK-BR-3 (breast carcinoma cells) cell lines [14]. On the other hand, petroleum ether extract possessed anticancer activity against HCT-15 (colon carcinoma) and Hep2 (Human cervix carcinoma) cell lines [15]. From other species, petroleum ether extract of *C. roxburghii* Linn. leaves exhibited cytotoxic activity against HCT-116 (colon carcinoma cells) and MCF-7 (breast carcinoma cells) cell lines while against HepG-2 cell line showed no activity [44].

The petroleum ether extract of *C. fistula* seeds significantly induced antitumor activity against HeLa cell line [58]. The *n*-hexane extract of *Senna gardneri* and the ethyl ether extracts of *Senna splendida* showed cytotoxic effect against human colon and human glioblastoma cell lines [59]. Six bioactive compounds from *Cassia italica*, showed anticancer properties using Ehrlich ascites carcinoma cell line and Hepatoma cell (HepG2) cell line. The identified compounds showed variable antioxidant activities [60]. Three anthraquinones compounds (emodin, aloe-emodin and Chrysophanol) isolated from *C. garrettiana* heart wood showed

appreciable antitumor activity against HT-29 (human colon adenocarcinoma), HeLa (human cervical adenocarcinoma), KB (oral cavity cancer) and MCF-7 cell lines [61].

Microscopic examination of chloroform fraction of *C. alata* leaves against HepG2 cell line

Morphological assessment of control/treated cells was performed using phase contrast microscopy equipped with digital camera after 48 h exposure time. Morphological signs of toxicity included cell rounding, shrinking and loss of contact with neighboring cells and the substratum of the wells (cell floating). These morphological features have led to the loss of monolayer integrity in comparison to the control (0.1% DMSO) as shown in Figure 4 and 5.

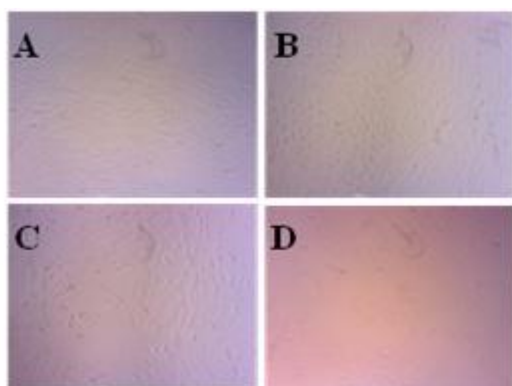

Figure 4. Photomicrographs morphology of HepG2 cell monolayers treated with either vehicle (0.1% DMSO-A) or chloroform fraction of *C. alata* leaves at 25 μg/ml (B), 50 μg/ml (C) and 100 μg/ml (D). The total magnification is 150X

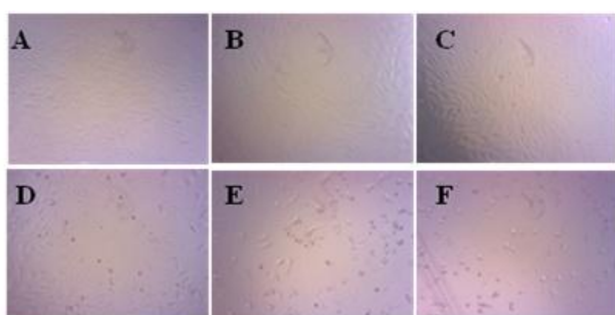

Figure 5. Photomicrographs morphology of HepG2 cell monolayers treated with either vehicle (0.1% DMSO-A) or doxorubicin at 0.5 μg/ml (B), 0.99 μg/ml (C), 1.99 μg/ml (D), 3.97 μg/ml (E) and 7.95 μg/ml (F). The total magnification is 150X

Antioxidant activity (DPPH radical scavenging assay)

The pre-screen of antioxidant activity revealed that chloroform fraction showed weak antioxidant activity with 7.8% DPPH scavenging activity. These results revealed that may be the absence of antioxidant compounds. Previous studies have dealt with antioxidant activity of *cassia alata* leaves [22,62,63], in addition indole alkaloid of *cassia alata* leaves exhibited strong antioxidant potential [64].

Conclusion

Chloroform fraction of *Cassia alata* L leaves were evaluated for its potential antitumor properties *in vitro*. The fraction showed remarkable cytotoxicity against HepG2 cells, this observation was confirmed by morphological investigation. However, the fraction exhibited weak anti-proliferative effect on Caco2 and MDA-MB-231 cells and exhibited weak antioxidant activity. The anticancer activity against HepG2 cells is possibly due to anthraquinones content. Further bioassay-guided fractionation of the cytotoxic fraction led to the isolation of three anthraquinones (rhein, emodin and aloe-emodin) all known to have useful bioactivities including anticancer activities. On the other hand, the fatty acids and the unsaponifiable matter were fractionated and identified by GC/MS. These findings show the importance of screening *Cassia alata* as medicinal plant for various cancers cell lines and the responsible active compounds. The cytotoxicity profile might represent a new promising extract with potential for development as an anticancer drug against HepG2 cells with low or no toxicity to non-cancer cells. Further work is certainly needed to develop and evaluate the anthraquinones compounds as anticancer.

Conflict of Interests

The authors declare that there is no conflict of interest.

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