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Biological evaluation of phytoconstituents from *Markhamia tomentosa* ethanolic leaf extract

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ABSTRACT

Phytochemicals are increasingly being used in cancer treatment due to affordability and potential anticancer effect with minimal adverse reactions compared to chemotherapy. *Markhamia tomentosa* is a medicinal plant used traditionally to treat cancer. In this study, the antiproliferative compounds from *M. tomentosa* were isolated using bioactivity-guided approach and characterized using various spectroscopic techniques. Through bioassay-guided fractionation of the crude ethanolic leaf extract, the dichloromethane (Mdf) and ethyl-acetate (Mef) fractions exhibited potent cytotoxicity activity against HeLa cells with IC_{50} values of 83.26 and 104.5 μg/ml respectively in the MTT assay. Trypan blue assay and cell cycle analysis showed that Mdf fraction demonstrated cytotoxic effect with more extensive cell death and induced G_0/G_1 phase cell cycle arrest with concomitant decrease in S phase. Mef fraction showed reduced percentage of stained dead cells as compared to Mdf fraction and induced G_2/M phase with increase in the size of sub-G_1 phase, corresponding to apoptosis. From the isolation and purification of Mdf and Mef fractions by repeated column chromatography, followed by identification by 1D and 2D NMR spectroscopy and by comparison of the NMR data with values reported in literature, sitosterol **1**, mollic acid **2**, phytol **3** and oleanolic acid **4** were isolated for the first time from *M. tomentosa*. Mollic acid **2** exhibited more potent cytotoxic activity compared to compounds **1**, **3** and **4**. The results from these findings suggest that mollic acid **2** isolated from Mef which exhibited apoptotic cell death may be responsible for the earlier reported apoptosis induction capability of *M. tomentosa* against cervical cancer cell line HeLa.

1. Introduction

Several bioactive compounds, particularly the secondary metabolites isolated from medicinal plants have potential therapeutic effects on a myriad of diseases (Ghani, 2003). Based on ethnopharmacological data which provides a significantly increased chance of finding active plants rather than a random approach, many pharmaceutical drugs have been discovered by screening natural products from plants (Reddy et al., 2012). The increasing cost and undesirable side effects of conventional treatments have encouraged people to depend more on folk medicine. Many herbal remedies have long been used as anticancer agents. Emerging evidence has demonstrated that the antineoplastic effects of phytochemicals from natural products may affect cells by various mechanisms, including prevention initiation and promotion of carcinogenesis, or apoptosis induction (Gupta et al., 2001). Identification of the activators of apoptosis may provide more effective strategies for cancer therapy (Wong and Abdul Kadir, 2012). In addition, medicinal plants used as cancer chemopreventive or therapeutic agents produce minimal adverse effects and anti-multidrug resistance (Wu et al., 2002).

Markhamia tomentosa (Benth.) K. Schum ex. Engl. is a tree in the Bignoniaceae family. Preliminary phytochemical investigations of the leaves of *M. tomentosa* showed the presence of major classes of bioactive compounds such as terpenes, steroids, saponins and flavonoids (Ugbabe et al., 2010). Pentacylic triterpenoids including pomolic acid, oleanolic acid, tormentic acid and β-sitosterol have been reportedly isolated from the stem bark of *Markhamia tomentosa* (Tantangmo et al., 2010). Ajugol, tormentic acid, carnasol and oxopomolic acid were identified by LC-ESI-MS analysis in the ethyl acetate fraction of the leaf extract of *M. tomentosa* (Sofidiya et al., 2014).

Previously, we have demonstrated the cytotoxic effect of the crude extract of the leaves of *Markhamia tomentosa* against brine shrimp larvae and HeLa cervical cancer cells, chromosomal aberrations induction on *Allium* root cells and non-toxic effect in the liver and kidney function parameters in rats (Ibrahim et al., 2013, 2014, 2016).

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In this study, the isolation and characterization of the bioactive compounds of *Markhamia tomentosa* as well as antiproliferative activity of the isolated compounds are discussed.

2. Materials and methods

2.1. General experimental procedure

The 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR spectroscopy were recorded on a Bruker Avance^{111} 400 spectrometer in deuterated chloroform ($CDCl_3$) and methanol (CD_3OD) at room temperature with tetramethylsilane (TMS) as internal standard. The designations of ^{13}C spectra were done by analysis of DEPT (90 and 135) spectra to determine primary, secondary and tertiary carbons, by the use of 2D techniques and confirmed by comparison of chemical shifts with data in literature (Mahato and Kundu, 1994; Lacroix et al., 2009). A Perkin Elmer Spectrum 100 Fourier transform infrared (FT-IR) spectrometer with Universal attenuated total reflection (ATR) sampling accessory was used to obtain the Infrared (IR) spectra. GC–MS data of analysed samples were recorded on an Agilent gas chromatograph (GC) coupled to inert mass selective detector (MSD) operated in the electron impact (EI) mode with ionization energy of 70 eV. The GC-MSD apparatus was equipped with a DB-5SIL MS (30 m × 0.25 mm i,d., 0.25 μm film thickness) fused silica capillary column and helium - at a flow rate of 2 ml/min was used as the carrier gas.

2.2. Plant collection and extraction

Fresh leaves of *Markhamia tomentosa* (Benth) K. Schum ex Engl. collected from Oke-Igbo, Ondo state, Nigeria in February 2015 were used in the taxonomic identification and authentication in the Herbarium of Department of Botany and Microbiology, University of Lagos. A herbarium specimen was deposited and a voucher number LUH 5535 was obtained. The collected leaves were air dried at room temperature (23 °C ± 2 °C) for two days and pulverised in a mechanical grinder. The powdered leaf material (5 kg) was macerated in 50 L of absolute ethanol for 72 h at room temperature. The resulting ethanol extract was filtered through a Whatman filter paper and evaporated to dryness on a rotatory evaporator (Buchi, Switzerland) at 40 °C to yield 13.31% (*w*/w). The dried, deep brown crude extract (128.98 g) was dissolved in 100 ml of water and partitioned with solvents of increasing polarity: hexane (HEX; 3 × 500 ml), dichloromethane (DCM; 4 × 500 ml), ethylacetate (EtOAc; 3 × 500 ml) and *n*-butanol (BuOH; 2 × 500 ml). The resulting fractions were evaporated to dryness using a rotary evaporator (40 °C) to afford the Hex (Mhf; 19.56 g), DCM (Mdf; 53.07 g), EtOAc (Mef; 9.80 g) BuOH (Mbf; 6.27 g) and aqueous (Maf; 14.97 g) residues.

2.3. Cytotoxicity assay

The cytotoxic effect of the crude extract (Mcr), solvent fractions (Mhf, Mdf, Mef, Mbf and Maf) and isolated compounds from *M. tomentosa* in HeLa cells was investigated using the MTT assay as earlier described by Ibrahim et al. (2013). The cells were treated with the compounds in the final concentrations ranging from 25 to 100 μM for 48 h. IC_{50} values for the fractions and compounds were calculated from dose–response curves using GraphPad Prism 4 software package.

2.4. Evaluation of cell viability

Viability of cells was determined by the trypan blue dye exclusion (TBE) method and analysed by flow cytometry 48 h after initiation. Cells were treated with 100 μg/ml Mef and 80 μg/ml Mdf. Spent tissue culture media containing detached cells, and all wash solutions, were combined and added to the cells obtained from trypsinisation of the remaining adherent monolayer. After centrifugation, the cells were stained with trypan blue (0.4%) and immediately analysed by flow cytometry on a Beckman-Coulter FC500.A minimum of 10,000 events were recorded per sample in FL3 (red fluorescence), using a 488 nm Argon ion laser for excitation.

2.5. Cell cycle analysis

As earlier described by Ibrahim et al. (2013), cell cycle analysis of Mef (100 μg/ml) and Mdf (80 μg/ml) fractions was performed using the Coulter® DNA Prep™ Reagents Kit (Beckman Coulter CA, USA).

2.6. Isolation by column chromatography (CC)

The Mef and Mdf fractions were subjected to column chromatography (silica gel 60; 0.040–0.063 mm; Merck, Germany) on suitably sized columns and the collected fractions were monitored by TLC (Merck 20 cm × 20 cm silica gel 60 F254 aluminium plates) which was analyzed under UV (254 and 366 nm) and visualized as the plates were sprayed with a 10% sulphuric acid in methanol solution and heated for 30 s.

For the Mef fraction (7.46 g), a gradient elution of hexane: ethylacetate was used on a 4 cm diameter column starting with 10% EtOAc in Hex as eluent. This was followed by a gradient of increasing EtOAc percentage, and finally with 100% MeOH. The collected fractions (100 ml each) were combined based on TLC analysis to afford four main fractions E- 1 to E- 4. Fraction E- 2 (280 mg) was re-chromatographed over silica gel in a 2 cm column with Hex:EtOAc (9:1) as solvent system to yield sitosterol **1** (10 mg). Fraction E- 4 (1.23 g) was purified by silica gel CC with EtOAc:MeOH gradient (9.5:0.5) to yield a colourless amorphous solid: mollic acid **2** (230 mg).

Similarly, 30.5 g of Mdf fraction was separated by CC using Hex: DCM and DCM: EtOAc gradient starting with 100% Hex stepped to 50 and 100% DCM. This was followed by 20 and 40% increase of EtOAc every 250 ml to afford seven main fractions D- 1 to D- 7. With Hex:DCM (1:9), yellow thick viscous oil, phytol **3** (850 mg) was obtained as fraction D- 4. Fraction D- 6 (5.13 g) was re-chromatographed over a silica gel column with a Hex:EtOAc step gradient as eluent to give six subfractions d- 1 to d- 6. Sub fraction d- 4 (770 mg) was purified further by silica gel CC eluted with Hex:EtOAC (9:1) gradient to afford oleanolic acid **4** (90 mg).

3. Results and discussion

This present study is a continuation of our previous study that demonstrated the cytotoxic activity of *Markhamia tomentosa* leaf extract against HeLa (cervical cancer) cells through the induction of intrinsic pathway of apoptosis (Ibrahim et al., 2013).

Table 1
Cytotoxic activity of extract, fractions and isolated compounds isolated from the leaves of *M. tomentosa*.

Fractions/compounds	HeLa cells IC_{50} μg/m (μM)
Crude extract	182.0 ± 8.9 (ND)
Hexane	>250
Dichloromethane	83.26 ± 13.8 (ND)
Ethyl-acetate	104.50 ± 5.1 (ND)
n-Butanol	>250
Aqueous	>500
Sitosterol **1**	>100
Mollic acid **2**	34.74 ± 5.4 (73.93 ± 11.5)
Phytol **3**	>100
Oleanolic acid **4**	>100
Melphalan	40 μM ± 2.3

ND: Not determined.