

Cytotoxicity and Chemical Investigation of the Aerial Parts of *Markhamia zanzibarica* (Bojer Ex Dc.) K. Schum. (Bignoniaceae)

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Abstract: The ethanolic extract of the aerial parts of *Markhamia zanzibarica* (Bignoniaceae) was tested for its cytotoxic activity against human cervical adenocarcinoma cell line (HeLa). It showed a potent effect with an IC₅₀ of 9.68 µg/ml compared to Doxorubicin (7.28 µg/ml). The fractions obtained from the successive partitioning of the total extract, were tested against the same cell line; the results revealed that *n*-hexane extract was the most potent 9.23 µg/ml, followed by ethyl acetate, butanol and chloroform fractions with IC₅₀ of 21.2, 22.4 and 49.6 µg/ml, respectively. Column chromatography led to the isolation of oleanolic acid (1), ursolic acid (2) and luteolin (3). The structures of the isolated compounds were elucidated using spectroscopic techniques.

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Keywords: *Markhamia zanzibarica*, ursolic acid, oleanolic acid, luteolin, cytotoxicity, HeLa

1. Introduction:

Triterpenes and flavonoids classes are known for their cytotoxic activity (Korkina, 2007; Moutsatsou, 2007, Narendran, *et al.*, 2014). Previous investigation on the cytotoxicity assessment of *Markhamia* genus (Narendran, *et al.*, 2014) has showed IC₅₀ of the ethanolic extract of stem bark of *Markhamia lutea* on EAC (Ehrlich Ascites Carcinoma) was 27 µg/ml. Also cytotoxic activity of the ethanolic extract of *M. tomentosa* on HeLa cancer cell lines was determined using the MTT assay where the IC₅₀ was 189.1 ± 1.76 µg/ml at 24 hours post treatment (Ibrahim, *et al.*, 2013). Therefore based on the previous studies on cytotoxic effects of *Markhamia* genus, the authors aimed to investigate the cytotoxicity of the ethanolic extract as well as its fractions on HeLa cancer cell lines.

2. Material and Methods:

General experimental procedures:

Chromatographic materials were silica gel (Merck Co., Germany) and sephadex LH-20 (Merck Co., Germany). Thin layer chromatography detection was achieved by spraying the silica gel plates (Merck Co., Germany) with *p*-anisaldehyde spraying reagent (Stahl, 1969) followed by heating at 100°C.

Melting points were determined on a Buchi melting point B-545 apparatus. Mass spectra were recorded on SHIMADZU GC/MS-QP5050A,

Software: 5000; ionization mode: EI; ionization voltage: 70ev; scan speed: 2000 amu/sec; scan interval: 0.5 sec., at the regional center for mycology and biotechnology; Al-Azhar University, Cairo, Egypt. The ¹³C-NMR and ¹H-NMR spectra were recorded on a Bruker Avance AV 400 spectrometer operating at 100 MHz (for ¹³C) and 400.13 MHz (for ¹H).

Plant material:

Plant material was collected from private garden at 169 Km Alexandria, desert road, Egypt. Samples were identified by Mrs. Therese Labib: Taxonomist at the Orman garden, Giza, and confirmed by Dr. Mohamed el Gebaly, ex-Curator of National Research Center Herbarium, Dokki, Giza. The plant material was collected on June 2012 on the flowering stage. A voucher specimen No. MZ 2012 is kept in medicinal plant herbarium in Faculty of Pharmacy, Cairo University.

Cytotoxic activity:

The potential cytotoxicity was tested by Sulphorhodamine B colorimetric assay in National Cancer Institute using cancer cell lines HeLa (cervical carcinoma cell line). HeLa cancer cell lines were grown adherently in RPMI-1640 media supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in 5% CO₂/ 95% air.

Measurement of potential cytotoxicity by Sulforhodamine B colorimetric assay (SRB assay) (Skehan, 1990):

Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 hours before treatment with the compound(s) to allow attachment of cell to the wall of the plate. Different concentrations of the compound under test (0, 5, 12.5, 25, 50 $\mu\text{g/ml}$) were added to the cell monolayer triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compound(s) for 48 hours at 37 °C and in atmosphere of 5 % CO_2 . After 48 hours, cells were fixed, washed and stained with sulforhodamine-B stain (SRB). Excess stain was washed with acetic acid and attached stain were recovered with Tris EDTA buffer. Color intensity was measured in ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified compound was added.

Extraction and isolation:

The air-dried plant material (5Kg) was defatted with *n*-hexane (3X8L) to yield *n*-hexane extract (108 gm), then the plant extracted with 95 % methanol (7X25L). Filtration and *in vacuo* concentration resulted in total alcoholic extract (460 gm), which then subjected to fractionation with solvents of increasing polarity, chloroform, ethyl acetate and *n*-butanol to yield 3 main fractions, chloroform, ethyl acetate and *n*-

butanol where their weights were 50, 45 and 114 gm, respectively. Chloroform fraction (30.5 gm) was then subjected to VLC (vacuum liquid chromatography) with gradient elution from *n*-hexane (100%) to methanol (100 %). The similar fractions were pooled together to yield three main fractions. Fraction I (1.12 gm), II (5.9 gm), III (9.2 gm). Silica gel sub-columns of fractions I and II yielded compounds (1) (100 mg) and (2) (23 mg), respectively. The ethyl acetate fraction (30.5 gm) was subjected to polyamide column with gradient elution from 100 % H_2O to 100 % methanol to yield fraction I (2.8 gm), II (5.5 gm), III (5.8 gm). Fraction I was subjected to Sub-columns of silica gel and sephadex LH-20 to isolate and purify compound (3) 150 mg.

3. Results and Discussion:

Cytotoxicity on HeLa cancer cell line (Skehan, 1990):

Using sulforhodamine-B stain (SRB) assay on HeLa cancer cell line, the cytotoxic effect showed an IC_{50} of total alcoholic extract of aerial parts of *M. zanzibarica* 9.68 $\mu\text{g/ml}$, *n*-hexane extract, the three fractions chloroform, ethyl acetate and *n*-butanol 9.23, 49.6, 21.2 and 22.4 $\mu\text{g/ml}$, respectively compared to Doxorubicin positive control 7.28 $\mu\text{g/ml}$. (Figures 1 and 2).

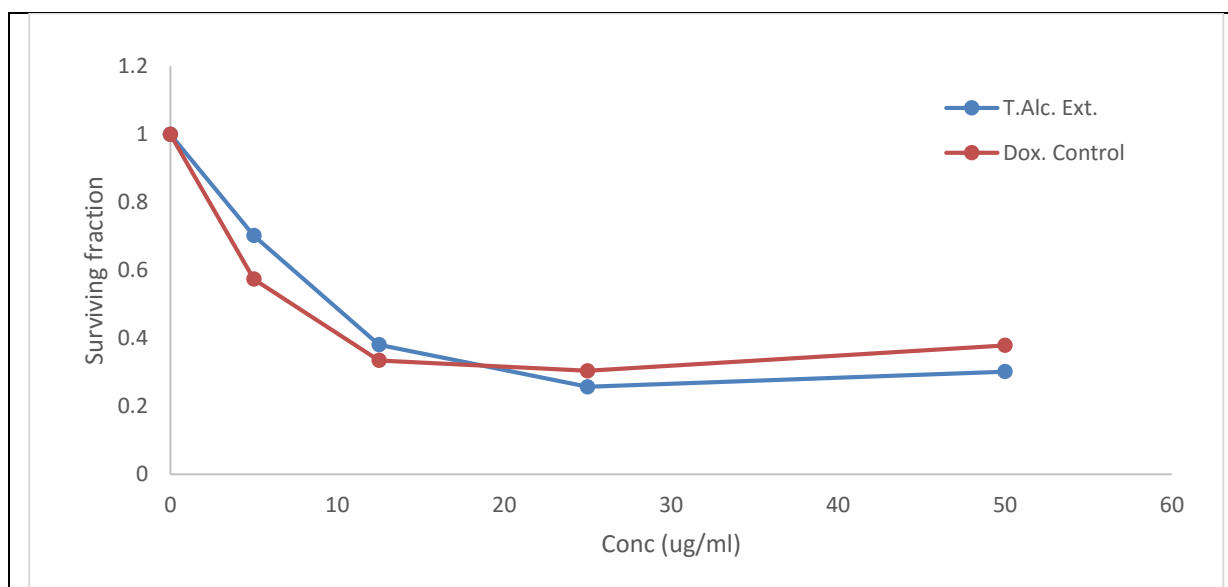


Fig (1): Cytotoxicity of total alcoholic extract of aerial parts of *M. zanzibarica* on HeLa cell line

Spectroscopic data of the isolated compounds:

Compound (1): C₃₀ H₄₈ O₃, white crystals, m.p 271-273 °C, EI-MS, m/z: 456 [M]⁺, ¹H-NMR (MeOD, 400 MHz) δ ppm: 1.30 (m, 2H, H-1), 1.50 (m, 2H, H-2), 3.00 (br.s, 1H, H-3), 1.02 (d, J=6Hz, 1H, H-5), 1.50 (m, 1H, H-6a), 1.30 (m, 1H, H-6b), 1.50,1.30 (m, each, 2H-7), 1.60 (s, 1H, H-9), 1.81 (d, J=8Hz, 2H-11), 5.16 (br.s, 1H, H-12), 1.24, 1.91 (s,m; 2H-15), 1.63 (m, 2H-16), 2.75 (br.d, J=11.2 Hz, H-18), 1.50, 1.30 (m, each, 2H-19), (1.30,1.50 (m, each, 2H-21), 1.91(m, 2H-22), 0.90

(s,3H-23), 0.88 (s,3H-24), 0.68 (s,3H-25), 0.86 (s,3H-26), 1.10 (s,3H-27), 0.72 (s,3H-29), 0.88 (s,3H-30). ¹³C-NMR (MeOD, 100 MHz) δ ppm: 39.4 (C-1), 27.7 (C-2), 77.3 (C-3), 39.6 (C-4), 55.3 (C-5), 18.5 (C-6), 33.3 (C-7), 39.8 (C-8), 47.6 (C-9), 37.1 (C-10), 23.8 (C-11), 122.0 (C-12), 144.3 (C-13), 41.8 (C-14), 28.7 (C-15), 23.4 (C-16), 46.2 (C-17), 41.3 (C-18), 45.9 (C-19), 30.9 (C-20), 33.8 (C-21), 32.9(C-22), 28.7 (C-23), 16.5 (C-24), 15.6 (C-25), 17.3 (C-26), 26.1 (C-27), 179.1 (C-28), 32.6 (C-29), 23.1 (C-30).

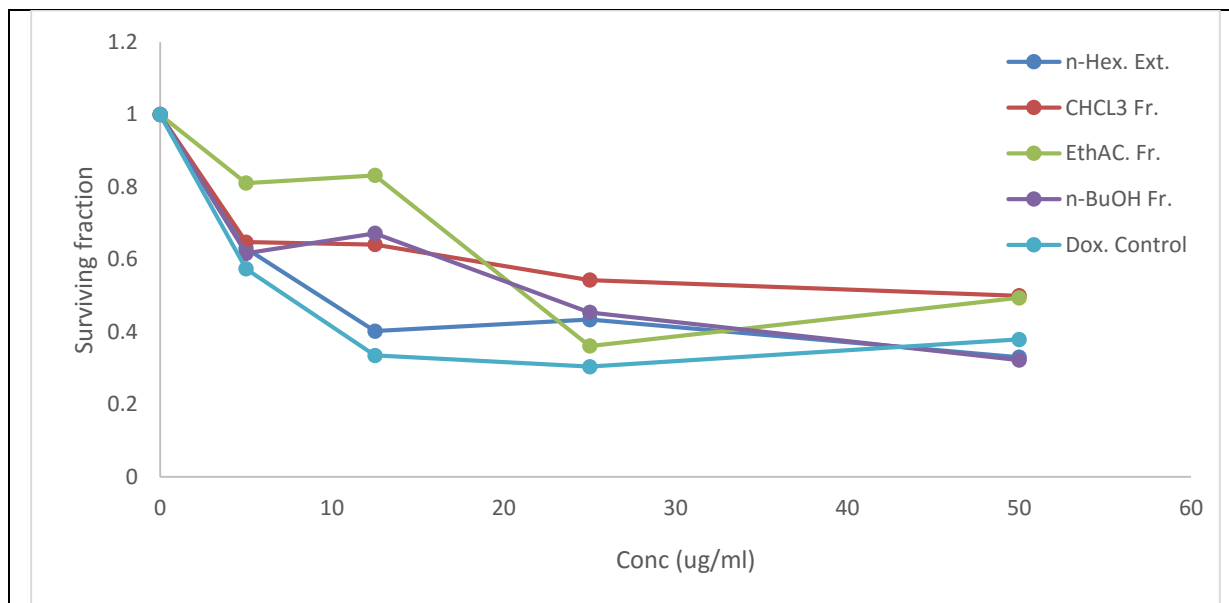


Fig (2): Cytotoxicity of total alcoholic, *n*-hexane extracts and three fractions of aerial parts of *M. zanzibarica* on HeLa cell line

Compound (2): C₃₀ H₄₈ O₃, white crystals, m.p 278-282 °C, EI/MS m/z: 456 [M]⁺. ¹H-NMR (MeOD, 400 MHz) ppm: 1.38 (m, 2H, H-1), 1.58 (m, 2H, H-2), 3.17 (dd, J=10.8Hz, 4.4 Hz, 1H, H-3), 0.83 (d, J=8 Hz, 1H, H-5), 1.58, 1.38 (m,each, 2H-6), 1.58,1.38(m, each, 2H-7), 1.50 (s, 1H, H-9), 1.95 (dd, J=8.7,3.3 Hz, 2H-11), 5.25 (br.s, 1H, H-12), 1.21, 2.06 (s, m, 2H-15), 1.67 (m, 2H-16), 2.22 (d, J=11.2 Hz, H-18), 1.58 (m, 1H, H-19), 1.1 (m, 1H, H-20), 1.38,1.58 (m, each, 2H-21), 2.06 (m, 2H-22), 0.98 (s,3H-23), 0.80 (s,3H-24), 0.997 (s,3H-25), 0.87 (s,3H-26), 1.14 (s,3H-27), 0.90 (d, J= 6.4 Hz,3H-29), 0.988 (d, J=6.8 Hz, 3H-30).

¹³C-NMR (MeOD, 100 MHz) ppm: 38.6 (C-1), 27.4 (C-2), 78.3 (C-3), 38.4 (C-4), 55.3 (C-5), 20.2 (C-6), 33.0 (C-7), 39.4 (C-8), 48.2 (C-9), 36.7 (C-10), 24.0 (C-11), 125.5 (C-12), 138.2 (C-13), 41.8 (C-14), 27.8 (C-15), 26.5 (C-16), 48.0 (C-17), 53.0 (C-18), 39.0 (C-19), 39.0 (C-20), 36.7 (C-21), 36.7 (C-22), 30.4 (C-23), 15.2 (C-24), 16.4 (C-25), 16.2 (C-26), 23.0 (C-27), 180.2 (C-28), 18.1 (C-29), 23.0 (C-30).

Compound (3): (C₁₅ H₁₀ O₆), yellow crystals, m.p. 325-328 °C, EI-MS m/z: 286 [M]⁺. ¹H-NMR (MeOD, 400 MHz) ppm: 6.42 (s, 1H, H-3), 6.10 (d, J= 1.9 Hz, 1H, H-6), 6.33 (d, J= 1.9 Hz, H-8), 7.26 (d, J= 2 Hz, 1H, H-2'), 6.80 (d, J= 9 Hz, 1H, H-5'), 7.27 (dd, J= 2, 6 Hz, 1H, H-6').

Compound (1) Fig. (3), white crystals, m.p: 271-273 °C, MS (70 e.v., EI-MS) m/z:456 [M]⁺ which revealed the molecular formula (C₃₀ H₄₈ O₃). ¹H-NMR (MeOD, 400 MHz) showed seven singlets assigned for protons of tertiary methyl groups at ppm (0.90, H-23; 0.88, H-24, 0.68, H-25; 0.86, H-26; 1.10, H-27; 0.72, H-29; 0.88, H-30), A broad doublet assigned for H-18 at ppm 2.75 (Br.d, J= 11.2 Hz, 1H), A broad signal assigned for H-3 at ppm 3.00 (Br. s, 1H), A broad signal assigned for the olefinic H-12 at ppm 5.16. ¹³C-NMR (MeOD, 100 MHz) showed two signals assigned for olefinic carbons (C-12, C-13) at 122.0 and 144.3 ppm, which suggests oleanolic skeleton. One signal characteristic for carbon attached to hydroxyl group (C-3) at 77.3

ppm and one signal characteristic for carbon attached to carboxylic group (C-28) at 179.1 ppm. The spectroscopic data were compared with previously published one (Tantangmo, *et al.*; 2010, Uddin, *et al.*, 2011; Narendran, *et al.*, 2014).

Compound (2) Fig. (3), white crystals, m.p 278-282 °C, MS (70 e.v., EI-MS) m/z: 456 [M]⁺ which revealed the molecular formula (C₃₀ H₄₈ O₃). ¹H-NMR (MeOD, 400 MHz) showed five singlets assigned for protons of tertiary methyl groups at ppm (0.80, H-24; 0.87, H-26; 0.98, H-23; 0.997, H-25; 1.14, H-27), Two doublets assigned for tertiary methyl groups (H-29 and H-30) at ppm 0.90 (d, J=6.4 Hz, 3H) and 0.988 (d, J=6.8 Hz, 3H); respectively. A doublet assigned for H-18 at ppm 2.22 (d, J= 11.2 Hz, 1H). A doublet of doublet assigned for H-3 at ppm 3.17 (dd, J=10.8, 4.4 Hz, 1H). A broad signal assigned for the olefinic H-12 at ppm 5.25. ¹³C-NMR (MeOD, 100 MHz) showed two signals assigned for olefinic carbons (C-12, C-13) at 125.5 and 138.2 ppm. One signal characteristic for carbon attached to hydroxyl group (C-3) at 78.3 ppm and one signal characteristic for carbon attached to carboxylic group (C-28) at 180.2 ppm. The spectroscopic data were compared with previously published one (Gormann, 2004; Nchu, 2009).

Compound (3) Fig. (3), yellow crystals, m.p. 325-328 °C. It gave positive tests with amyl alcohol indicating its flavonoid nature. MS (70 e. v., EI-MS) m/z: 286 [M]⁺ which revealed the molecular formula (C₁₅ H₁₀ O₆). Fragment 153.06 (100 %) represents the base peak. ¹H-NMR showed signals at ppm: Two protons, each is doublet with meta coupling at 6.10 ppm (d, J=1.9 Hz, 1H) and at 6.33 ppm (d, J=1.9 Hz, 1H) assigned for H-6 and H-8; respectively. A singlet assigned for H-3 at 6.42 ppm (s, 1H) indicating the flavonoid is flavone in nature. One proton appears as doublet with ortho coupling at 6.80 ppm (d, J=9 Hz, 1H) assigned for H-5'. A proton, appears as doublet with meta coupling at 7.26 ppm (d, J=2 Hz, 1H) assigned for H-2'. And another proton, appears as doublet of doublet at 7.27 ppm (dd, J= 2, 6 Hz, 1H) assigned for H-6'.

Also identification was confirmed with co-chromatography with authentic luteolin. The spectroscopic data were compared with previously published one. (Mabry, *et al.*, 1970; El Dib, 2014; Sofidiya, 2014).

These results encourage the authors for future investigation of cytotoxicity of the isolated pure compounds.

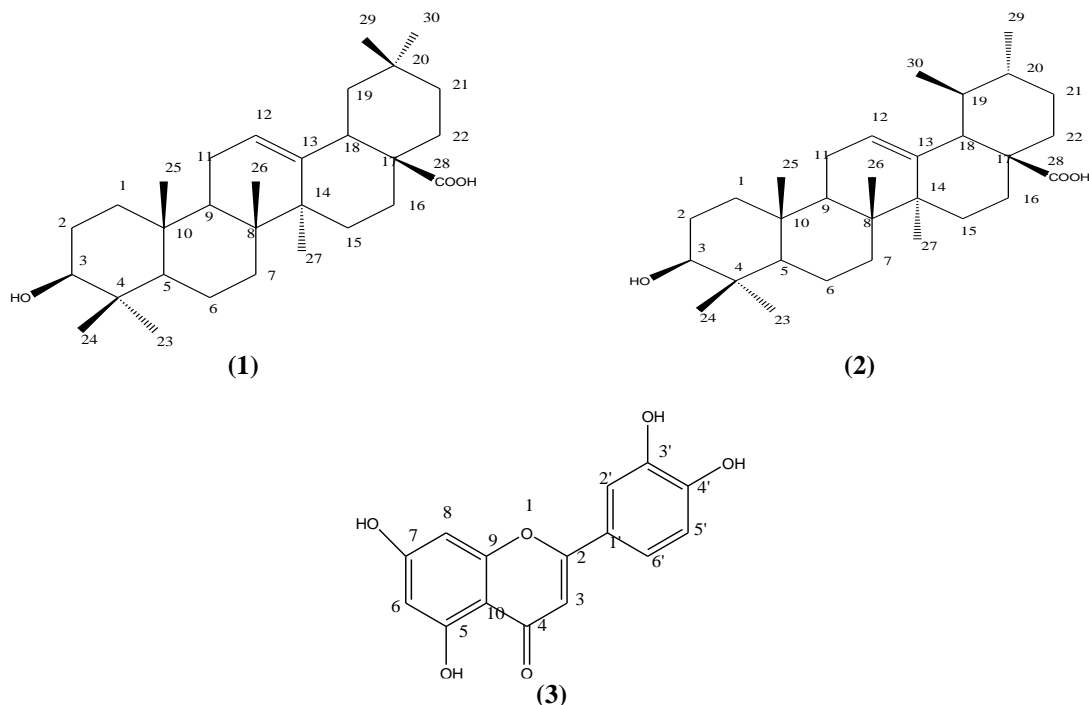


Fig. (3) Isolated compounds from the aerial parts of *M. zanzibarica*.

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