Cytotoxic and Apoptotic Effects of the Bark of Two Common Mango (Mangifera indica) Varieties from Sri Lanka on Breast and Ovarian Cancer Cells

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Authors’ contributions

Author MKE carried out laboratory experiments, analysed data and wrote the manuscript draft. Author KHT designed the study, supervised bioassays and revised the manuscript. Author SRS assisted with bioassays, preparation of plant extracts, data analysis and revised manuscript. Author IT supervised bioassays, data analysis and revised manuscript. Author EDS supervised plant extraction and revised manuscript. All authors read and approved final manuscript.

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ABSTRACT

Aims: The present study was planned to evaluate cytotoxic and apoptotic properties of the bark of two common mango varieties (Mangifera indica L.) grown in Sri Lanka [Rata Amba (RA) and Karthakolomban (KA)] in MCF-7 (ER positive breast cancer), MDA-MB-231 (triple negative breast cancer), SKOV-3 (ovarian epithelial cancer) cancer cell lines and normal mammary epithelial cells (MCF-10A).

Place and Duration of the Study: At the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo between 1st of February 2015 to April 2015.

Methodology: Cancer cells and normal cells were treated with organic extracts (hexane, chloroform, ethyl acetate and methanol) of RA and KA bark and cytotoxic effects were evaluated by SRB assay. Free radical scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) was also tested.
for active extracts. Furthermore, apoptotic effects of cytotoxic extracts were analysed by caspase 3 and 7 activation, DNA fragmentation, acridine orange/ethidium bromide (AO/EB) and Hoechst staining.

**Results:** Of the four solvent extracts used, only the methanol extract showed anti-proliferative effects against all three cancer cell lines in a dose-dependent manner. Cytotoxicity of the methanol extract of RA was higher (MCF-7 IC\(_{50}\) - 81.1 µg/mL, MDA-MB-231 IC\(_{50}\) - 91.5 µg/mL and SKOV-3 IC\(_{50}\) - 71.5 µg/mL) compared to that of the methanol extract of KA (MCF-7 IC\(_{50}\) - 123.9 µg/mL, MDA-MB-231 IC\(_{50}\) - 111.2 µg/mL and SKOV-3 IC\(_{50}\) - 137.2 µg/mL). Both the methanol extracts showed less cytotoxicity to normal mammary epithelial cells [IC\(_{50}\) - 255.6 µg/mL (RA) and IC\(_{50}\) - 615.6 µg/mL (KA)]. Methanol extracts also exhibited strong free radical scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH). Furthermore, methanol extract showed apoptotic effect against all tested cancer cell lines.

**Conclusion:** Overall findings of this study suggest that methanol extracts of the bark of two common mango varieties tested exhibit cytotoxicity through induction of apoptosis through caspase dependent mechanisms.

**Keywords:** Mangifera indica; cytotoxicity; apoptosis; breast cancer; ovarian cancer.

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1. **INTRODUCTION**

Mango (*Mangifera indica*, family: Anacardiaceae) is considered as one of the most popular fruit crops in Asian countries including Sri Lanka and India [1]. Almost all parts of the mango tree (fruits, bark, seeds and flowers) are used in traditional medicine to treat diseases such as diabetes, asthma, gastric disorders, dysentery and mouth sores [2]. It has been reported that each part of a mango tree (leaves, flowers, bark, fruits and roots, etc) contains valuable nutrients with different health promoting properties [3]. Due to these health promoting properties, there is a good demand for the use of mango in the world. Though mango fruit flesh is the highly consumed part of a mango tree, peel and bark also have received much attention due to presence of various bioactive compounds [4]. In a recent research, ethanolic extract of mango fruit peel and flesh were found to exert anti-cancer properties on some cancer cell lines [5]. Abdullah et al. [6,7] have also shown induction of apoptosis in MCF-7 and MDA-MB231 breast cancer cells by ethanolic extract of *M. indica* kernel through oxidative stress mechanisms.

*Mangifera zeylanica* is a plant endemic to Sri Lanka (endemic Sri Lankan mango). It is commonly known as ‘Etamba’, and bears edible fruits. Its bark has been used in traditional Ayurvedic medicine to treat some cancers. It was reported previously that the hexane extract of the bark of *Mangifera zeylanica* exert anti-cancer properties in breast and ovarian cancer cells and radical scavenging activity [8]. However, the bark of Sri Lankan common mango varieties have not been investigated for its potential cytotoxic and apoptotic properties on breast and ovarian cancer cells. Therefore, in the present study we assessed possible cytotoxic and apoptotic properties of the bark of two common mango varieties of *M. indica* grown in Sri Lanka, commonly known as Rata Amba and Karthakolomban.

2. **MATERIALS AND METHODS**

2.1 **Chemicals and Cell Culture Reagents**

All the cell lines and cell culture reagents used in this study were purchased from the American type cell culture (ATCC), Manassas, USA. Chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified.

2.2 **Plant Collection and Sample Preparation**

Bark of the two mango varieties, Rata Amba (RA) and Kartha Kolomban Amba (KA) (1 Kg each) was collected from home gardens from Ahangama (Galle District, Sri Lanka). Plants were identified by the Botanist at the Bandaranayake Memorial Ayurvedic Research Institute (BMARI), Sri Lanka. Voucher specimens were deposited at the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka.

2.3 **Extraction and Preparation of the Plant Extracts**

Dried barks were pulverized and then extracted to hexane, chloroform, ethyl acetate and methanol sequentially (3 times with each solvent
at room temperature). All the resulting extracts were filtered and concentrated using a rotary evaporator under reduced pressure at 40°C. Resulting residues were dissolved in dimethyl sulfoxide (DMSO) (D8418, Sigma) to prepare stock solutions for biological assays.

2.4 Free Radical Scavenging Activity

Free radical scavenging activity of the methanol extracts of the two mango varieties were assessed by DPPH assay with slight modifications [9]. Briefly, different concentrations of the methanol extracts (50 µL) were added to 60 µL of DPPH (D9132, Sigma) solution (2 mg/mL) and 90 µL of methanol. Plates were incubated in the dark for 10 min. and absorbance of treated (A) and untreated (B) wells was read at 517 nm (Synergy™ HT Multi-Mode Microplate Reader; Bio- Tek, Winooski, VT). Percentage radical scavenging ability was calculated according to the formula (A_{control} – B_{sample}/A_{control} × 100). Ascorbic acid (A 4403, Sigma) was used as the positive control.

2.5 Cell Culture

Three human cancer cell lines (MCF-7, ER positive breast cancer cells (ATCC, HTB-22™); MDA-MB-231, triple negative breast cancer cells (ATCC, HTB-26™) and SKOV-3, ovarian epithelial cancer cells (ATCC, HTB-77™) and normal mammary epithelial cells MCF -10A (ATCC® CRL-10317) were cultured in ATCC recommended medium containing 10% (v/v) fetal bovine serum (ATCC, 30-2020), streptomycin (S9137-Sigma, 0.1 mg/mL), penicillin (P 3032-Sigma, 100 U/mL) and insulin (I 6634-Sigma, 0.01 mg/ml). All the cancer cells except triple negative breast cancer cells were maintained in a humidified incubator at 37°C with 5% CO₂. The latter was maintained without CO₂.

2.6 Cell Viability Assays

Cancer cells were trypsinized, plated in cell culture treated 96 well plates (5 x 10³ cells/ well) and incubated for 24 h. After incubation, cancer cells were treated with different concentrations (25, 50, 100, 200 and 400 µg/mL) of the solvent extracts. Sulforhodamine B (SRB) assay was performed after 24 h to assess cancer cell viability. Briefly, cells were washed three times with Phosphate Buffer Saline (PBS) and fixed in the wells using 50 µL of ice-cold 50% trichloroacetic acid (T 6399-Sigma). Then the cells were washed with running tap water five times and 100 µL of 0.4% Sulforhodamine B (SRB) solution (230162-Sigma) was added to each well and incubated for 15 min at room temperature. SRB stain was then discarded and cells were washed with 1% acetic acid five times to remove any remaining unbound stain. Tris-base solution (200 µL) was added to each well and absorbance was read at 540 nm (Synergy™ HT Multi-Mode Microplate Reader) after shaking for one hour on a plate shaker. Percentage cell viability was calculated according to the formula: (mean of control group – mean of treated group/control group x 100%). Negative controls contained only DMSO and medium whereas Paclitaxel (T 7402-Sigma) was used as the positive control.

2.7 Caspase 3 and 7 Activities

Caspase 3 and 7 expression was determined using ApoTox-Glo™ triplex assay (Promega, G6321) as per manufacture’s instructions. Three cancer cell lines were treated with the methanol extracts from two mango varieties (50 and 100 µg/mL) and caspase expression determined at 24 h incubation was compared with untreated controls.

2.8 DNA Fragmentation

Cancer cells (2x10⁵ cells/ mL) treated for 24 h with 600 µg/mL of the methanol extracts were harvested by trypsinization and centrifugation. Cell pellets were incubated with freshly prepared lysis buffer (5 mMTris–HCl- pH 8, 1 M NaCl and 5 mM EDTA - pH 8, 0.5% SDS, proteinase K (200 µg/mL) and RNase A (200 µg/mL) with continuous inverting and vortexing at 55°C. DNA was extracted using phenol–chloroform–isoamyl alcohol (25: 24: 1), precipitated with ice-cold isopropanol and electrophoresed (2500 ng) on a 2.0% of agarose gel stained with ethidium bromide.

2.9 Cell Morphology and nuclear Fragmentation Analysis by Acridine Orange/ethidium Bromide (AO/EB) and Hoechst Staining

Cell morphology and nuclear fragmentation were assessed by acridine orange/ethidium bromide (AO/EB) and Hoechst staining. Cells which were 70-80% confluent were harvested by trypsinization, Cancer cells were seeded in
a 24 well cell culture plates on cell culture treated cover slips (5x10^4 cells/well) and treated with 25, 50, 100, 200 and 400 µg/mL of the methanol extracts. After 24 h, cells were fixed with 4% formaldehyde and AO/EB solution (10-20 µL) and Hoechst stain (0.005 mg/mL, 10 µL) were added. Stained cells were observed under a fluorescence microscope (OLYMPUS Co., Tokyo, BX51TRF, Japan).

2.10 Statistical Analysis

Results are expressed as mean±SD of the means of three independent experiments. One way ANOVA with Tukeypost test was used to compare groups and p ≤ 0.05 was considered as statistically significant. GraphPad Prism 5 (San Diego, California, USA) was used for statistical analyses.

3. RESULTS AND DISCUSSION

3.1 DPPH free Radical Scavenging Activity

Free radicals are believed to play an important role in various pathological manifestations. Antioxidants can protect cells from free radicals and can protect them from oxidative damages [10]. Mango bark, leaves and pulp are known to exhibit several biological activities including anti-oxidant and anti-inflammatory activities [11-13]. Results in this study demonstrate that methanol extracts from RA and KA showed DPPH free radical scavenging activity with IC_{50} values of 10.3 µg/mL and 14.3 µg/mL respectively. This indicates that these two extracts may contain different compounds with potential free radical scavenging activity.

3.2 Evaluation of Cytotoxicity of Extracts

Out of the four solvent extracts from the two mango varieties tested, only the methanol extracts of RA and KA showed cytotoxicity to all three cancer cells in a dose dependent manner after 24 h incubation. However, methanol extract of RA showed a higher cytotoxicity to all three cancer cell lines (MCF-7 IC_{50} - 81.1 µg/mL, MDA-MB-231 IC_{50} - 91.5 µg/mL and SKOV-3 IC_{50} - 71.5 µg/mL) compared to the methanol extract of KA (MCF-7 IC_{50} - 123.9 µg/mL, MDA-MB-231 IC_{50} - 111.2 µg/mL and SKOV-3 IC_{50} - 137.2 µg/mL) (Fig. 1). Contrariwise, both the methanol extracts (RA and KA) showed less cytotoxicity to normal mammary epithelial cells (IC_{50} - 255.6 µg/mL (RA) and IC_{50} - 615.6 µg/mL (KA)).

3.3 Apoptosis Related Studies (Caspase 3 and 7 Activities, DNA fragmentation and Acridine Orange/Ethidium Bromide (AO/EB) and Hoechst Staining)

Apoptosis is considered as the basic process of life of a multicellular organism that maintains homeostasis in organs and tissues [14]. Biochemical processes involved in apoptosis result in membrane blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation [15]. Caspases play a vital role in apoptosis. There are two types of caspases: initiator caspases and effector caspases, and they link with the extrinsic and intrinsic pathways of apoptosis [16]. Caspase 3 and 7 activity significantly (p<0.001) increased in MDA-MB-231 and SKOV-3 cells in time and dose dependent manner (24 h) after treatment with the methanol extracts of RA and KA. Caspase 7 was activated significantly in MCF-7 cells after treatment with the two methanol extracts (Fig. 2).

![Fig. 1. Cytotoxic activity of methanolic extracts of Mangifera indica. Cytotoxic activity of methanolic extract of [A] RA (Rata Amba) [B] and KA (Kartha Kolomban Amba) in MDA-MB-231, SKOV-3 and MCF-7 cancer cell lines](image)
Three cancer cell lines treated with the methanol extracts from RA and KA showed accumulation of fragmented DNA, showing an apoptotic pattern with no evidence of DNA fragmentation in the respective untreated controls (Fig. 3). However, a clear DNA fragmentation was not visible in MDA-MB-231 cells treated with RA.

Cancer cells exposed to the methanol extract when stained with AO/EB and Hoechst showed main morphological features associated with apoptosis such as condensation and fragmentation of nucleus, changes in the size and the shape of cells when compared to the untreated controls (Fig. 4).

Characteristic features of apoptosis such as DNA fragmentation, nuclear fragmentation, caspase 3 and 7 activation (except MCF-7 cells) were clearly detected in all three cancer cell lines after treatment with the methanol extracts of RA and KA. These effects of the methanol extracts suggest that _M. indica_ exhibits its cytotoxic properties via apoptosis, through caspase dependent mechanism in all three cancer cell lines studied. We have previously reported caspase dependant apoptosis in response to the hexane extract of the bark of Sri Lankan endemic mango (_M. zeylanica_) in SKOV-3 cells and MDA-MB-231 cells and caspase independent apoptosis in MCF-7 cells.

**Fig. 2. Caspase 3 and 7 activation [MDA-MB-231 and SKOV-3 cells (caspase 3 and 7) and MCF-7 cells (caspase 7)] in three cancer cell lines after treatment with methanolic extracts. [A] activation of caspase 3 and 7 by the methanolic extract of RA [B] activation of caspase 3 and 7 by the methanolic extract of KA

***p<0.001, *p<0.05 compared to controls. SD values are shown by error bars

**Fig. 3. DNA fragmentation analysis. Three cancer cells were treated with indicated concentrations of the two methanolic extracts (RA and KA, 600 µg/mL) for 24 h
Fig. 4. Acridine orange/ethidium bromide (AO/EB) and Hoechst staining. Three cancer cell lines (MCF-7, MDA-MB-231 and SKOV-3) after treatment with the two methanolic extracts (RA and KA, 400 µg/mL) for 24 h followed by staining with Acridine orange/ethidium bromide (AO/EB) and Hoechst. A1, B1, C1, A2, B2, C2 – control cells; a, c, e, g, i and k – treated with 400 µg/mL of RA; b, d, f, h, j and l – treated with 400 µg/mL of KA. As the yellow arrows indicate in Hoechst stained cells, fragmented and condensed cancer cell nucleus could be observed in RA and KA treated cells, while the nucleus of untreated control cells was regular in shape. Yellow arrows in AO/EB stained cells indicate altered cell morphology and condensed cell nucleus, whereas untreated control cells do not show such changes.

4. CONCLUSIONS

Results of the present study show that the bark of two common mango varieties (RA and KA) tested contain phytochemicals with cytotoxic/apoptic and free radical scavenging properties. Taken together with our previous studies on Sri Lankan endemic mango, the active compounds in the bark of the common mango varieties tested are likely to be different from active compounds in the endemic mango as the latter were found in the hexane extract.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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