

RESEARCH ARTICLE

Cytotoxicity of Methanol and Acetone Root Bark Extracts of *Moringa concanensis* against A549, Hep-G2 and HT-29 Cell Lines

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Abstract

This study assessed the effect of the crude methanol and acetone root bark extracts of *Moringa concanensis* at different concentrations (10, 30, 50, 70, 90, 110 μ M) on A549, Hep-G2 and HT-29 cell lines using MTT assay. The present findings showed that *M. concanensis* methanol extracts induced cytotoxicity on Hep-G2 cells. Moreover, *M. concanensis* methanol extract induced apoptosis in Hep-G2 cells involves a marked increase in generation of reactive oxygen species, caspase 3 and 9 activation, a reduction of antioxidant enzyme activities such as SOD, CAT, GST, GSH and mitochondrial membrane potential (MMP).

Keywords: *Moringa concanensis*, root bark extracts, cytotoxicity, apoptosis, caspase.

Introduction

Moringa concanensis, a small tree that resembles *M. oleifera* grows wild in India namely Rajasthan, Madhya Pradesh, Gujarat, Maharashtra, Goa, Andhra Pradesh and Tamil Nadu. The whole plant parts of the tree are used in the treatment of ascites, rheumatism, venomous bites and painful swellings (Kirtikar and Basu, 2001). The seeds of *M. concanensis* contain protein 30.07%, fiber 6.00%, moisture 5.88% and ash 9.00% respectively. Plants are the richest bioresource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Bioactive compounds from plants possess properties like anti-cancer, anti-hypertension, antihypoglycaemia, antioxidants and antimicrobial components (Surveswaran *et al.*, 2007). Twenty types of human ailments may be cured using *M. concanensis* with simple preparations. The leaves and barks of *M. concanensis* are the most potent part of the plant for medicinal use. It is used for treatment of skin tumor, tiredness, reduce blood pressure, aphrodisiac, jaundice, eyecare, diabetes and bloat (Anbazhakan *et al.*, 2000). Cancer is one of the main causes of death all over the world. WHO estimated that 84 million people would die of cancer between 2005 and 2015 (Danhier *et al.*, 2010). Accordingly, much effort has been made to develop various approaches to reduce the threat caused by cancer. Natural products offer a valuable source of compounds with a wide variety of biological activities and chemical structures and provide important prototypes for the development of novel drugs. It has been estimated that 20-25% of medicines are derived from natural products. The plant constituents able to kill cancer cells exhibit a very large range of structural types, for e.g. alkaloids, coumarins, diterpenes, flavonoids, tannins, lignans, monoterpenes, steroids and triterpenes.

Cell-based assays are becoming an increasingly important part of the preclinical pharmaceutical discovery and validation process, as researchers directly study the effects of chemical compounds upon a wide variety of cell types. This study aimed to assess the cytotoxic effect of the crude methanol and acetone extracts of *Moringa concanensis* on A549 (human lung adenocarcinoma), Hep-G2 (hepatocellular carcinoma) and HT-29 (human colon carcinoma) cell lines.

Materials and methods

Collection of plant materials: The root barks of *Moringa concanensis* were collected from the Villamuthur village, Perambalur Taluk, Tamil Nadu. The plant materials were dried under shade condition and after optimum drying; the bark materials were coarsely powdered and stored in well closed containers for further laboratory analysis.

Solvent extraction: After defatting by petroleum ether, the root bark samples of *Moringa concanensis* (15 g) were extracted by stirring with 105 mL of methanol:H₂O (80:20) at 25°C for 48 h and filtered through Whatman No. 4 filter paper. The residues were re-extracted with an additional 75 mL of methanol for 3 h. The solvent of the combined extract was evaporated under low temperature at 40°C in incubator respectively. The remaining residues, after methanol extraction and air drying, were extracted by stirring with 105 mL of 70:30 (acetone:H₂O) (v/v) at 25°C for 48 h and filtered through Whatman No. 4 filter paper. The solvents for the extract was evaporated under low temperature at 40°C in an incubator (NSW make, New Delhi) and used for further *in vitro* analysis.

Cell Culture and maintenance: A549, Hep-G2, HT-29 cell lines were procured from National centre for cell science, Pune, India. The cells were grown in DMEM medium

supplemented with 10% fetal bovine serum and antibiotics 100 units/mL penicillin, 30 µg/mL streptomycin and 20 µg/mL gentamycin at 37°C in a CO₂ incubator with 5% CO₂. Cells at 80% confluency were used for all the assays.

Cell viability assay: MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to check cell viability and to evaluate the anticancer activity of the extract (Mossmann *et al.*, 1983). Cells were trypsinised, counted and seeded into 96 well microtitre plates with a cell count of 10⁴ cells/well. The cells were allowed to attach for 1 h and treated with different concentrations of *M. concanensis* root bark acetone and methanol extracts (10, 30, 50, 70, 90 and 110 µM) and incubated in a CO₂ incubator for 48 h in different cell lines A549, Hep-G2 and HT-29. After incubation, 20 µL of MTT (5 mg/mL) was added and incubated in the CO₂ incubator 37°C for 5 h. After 5 h, 100 µL of DMSO was added to dissolve the purple formazon crystals and read at 570 nm using a micro count plate reader (Bio-Tek Instruments). Percentage viability was calculated by taking viability of the control cells as 100%. From the graphs IC₅₀ values for different cancer cell lines were determined.

Reactive oxygen species generation: Cells were seeded at a density of 1x10⁶ cells/well and allowed for 2 h attachment. After that, the cells were treated with 20 µL of DCF-DA (5 mg/mL) for 60 min. The media was refreshed and added with 50, 70 and 90 µM of *M. concanensis* root bark extracts and incubated for 48 h. After treatment schedule, the cells were washed with Phosphate buffered saline (PBS), trypsinized and fluorescence intensity was read at excitation 480 nm and emission wavelength of 520 nm. The intensity of control was set to 100% (Royall and Ischiropoulos, 1993).

Superoxide dismutase activity: SOD activity of the root bark extracts was determined as described by Sun *et al.* (1988). This assay is mainly based on the reduction of nitrobluetetrazolium (NBT). One unit of SOD activity is the amount required for 50% inhibition of NBT reduction. The SOD activity is expressed as U/mg of protein.

Catalase activity: Catalase activity of the root bark extracts was determined according to Aebi (1974). The reaction mixture contained tissue homogenate and 30 mM H₂O₂ in a 50 mM phosphate buffer pH 7.0. The activity was estimated by decrease in absorbance of H₂O₂ at 240 nm.

Glutathione-S-Transferase activity: The reaction between 1-chloro-2,4-dinitro benzene (CDNB) and reduced glutathione results in formation of dinitrophenylthioether which is measured at 340 nm (Habig *et al.*, 1974). One unit of enzyme activity is the amount of enzyme producing 1 mmol of CDNB-GSH conjugate/min.

Glutathione Peroxidase activity: It was performed as described by Paglia and Valentine (1967). The oxidized glutathione is reduced by glutathione reductase and NADPH. The oxidation of NADPH to NADP⁺ is measured by a decrease in absorbance at 340 nm and expressed as U/mg of protein.

Mitochondrial membrane potential (Δψ_m): Cells were seeded at a density of 1x10⁶ cells/well and allowed for 2 h attachment. The cells were treated with different concentrations (50, 70 and 90 µM) of *M. concanensis* root bark extracts for 48 h. After treatments, cells were washed with PBS and treated with Dihexyloxacarbocyanine iodide and incubated for 60 min and fluorescence intensity was recorded at excitation wavelength of 488 nm and emission wavelength at 500 nm (Marchetti *et al.*, 1996a;b).

Caspase activity: Cells were seeded at a density of 1x10⁶ cells/well and allowed for 2 h attachment. The cells were treated with different concentrations (50, 70 and 90 µM) of *M. concanensis* root bark extracts for 48 h. The cell were lysed and analyzed for caspase 3, 8 and 9 activities (R and D systems) at 405 nm.

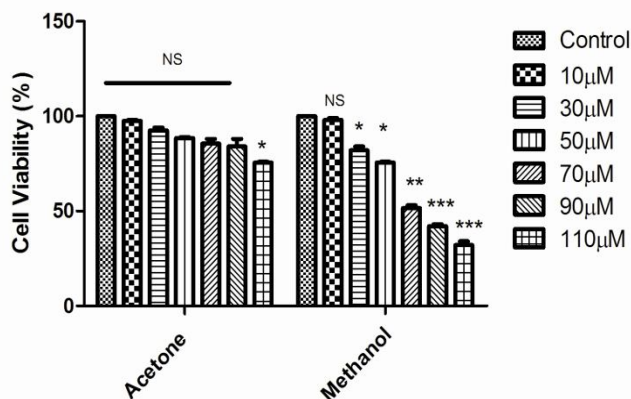
Statistical analysis: The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All the experiments were performed thrice in triplicates to ensure reproducibility. The statistical significance was set to *p<0.05, **p<0.01, ***p<0.001 when compared to control group.

Results

Effect of *M. concanensis* root bark extracts on A459, Hep-G2 and HT-29 cell lines: We found that the extract does not showed anticancer effect against HT-29 and A549 cell line. Compared to acetone, methanolic extract of *M. concanensis* root bark showed dose dependent cytotoxicity namely 10 µM (98%), 30 µM (82%), 50 µM (75.5%), 70 µM (51.5%), 90 µM (42%), 110 µM (32%) against liver cancer cell line Hep-G2 compared to control cells (no treatment) (Figs. 1-3). The IC₅₀ value was found to be 70 µM. So, methanolic extracts at 50, 70 and 90 µM were used to identify the detailed mechanism of cell death in Hep-G2 cell line.

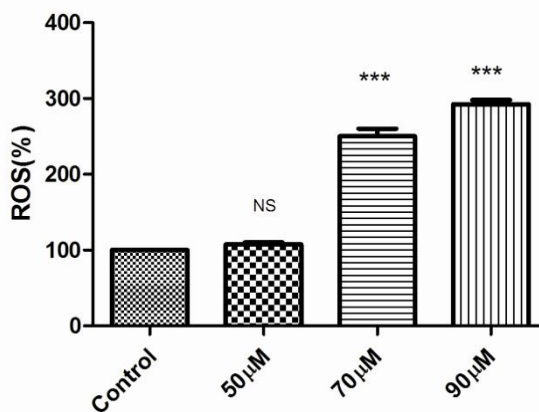
Reactive oxygen species generation in Hep-G2 cells: Reactive oxygen species are key players in oxidative stress. Increased levels of ROS are indicative for oxidative stress which in turn mediates cell death. Root bark extracts at different concentrations significantly increased the ROS levels compared to control. Methanol extract at 50, 70 and 90 µM concentrations showed 107.5, 250 and 292.5% compared to control (100%) (Fig. 4).

Fig. 1. Induced cell death in Hep-G2 cell line using *M. concanensis*.



Cell viability was determined by MTT assay. Results were expressed as % cell viability compared to control. Results are given as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, when compared to control group. NS—non significant compared to control (One way ANOVA followed by Tukey's multiple comparison).

Fig. 4. Effect of *M. concanensis* in ROS generation.



The results are expressed as ROS generated (%) when compared to control. Results are given as the mean \pm SEM. *** $p < 0.001$, when compared to control group.

Fig. 2. Induced cell death in A549 cell line using *M. concanensis*.

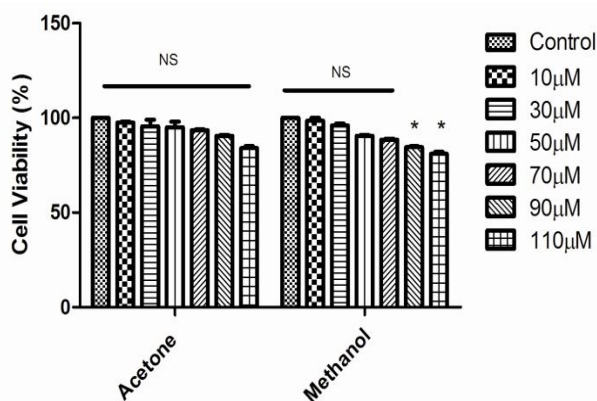


Fig. 3. Induced cell death in HT-29 cell line using *M. concanensis*.

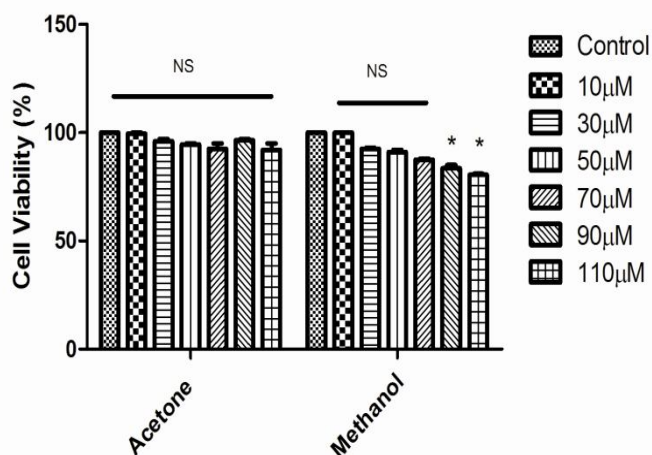
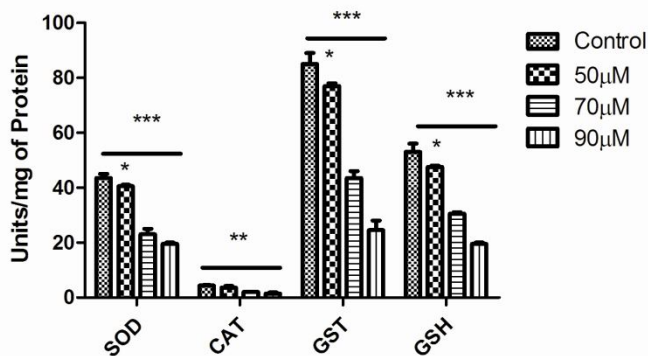


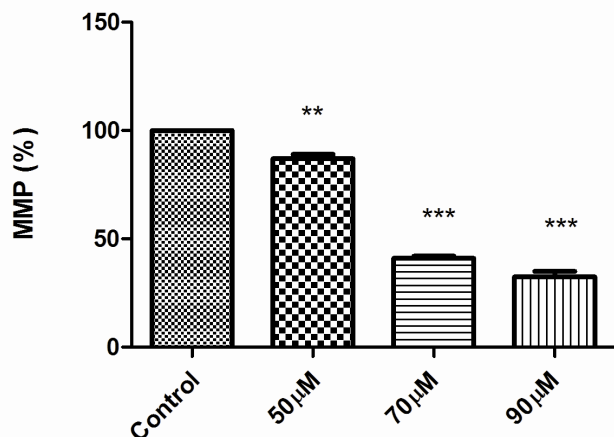
Fig. 5. Effect of *M. concanensis* on antioxidant status in Hep-G2 cell line.



Antioxidant enzyme activities: Antioxidant enzymes are involved to maintain the oxidant balance in the cells. Increased oxidant levels leads to depletion of antioxidant enzymes. In order to identify the antioxidant and oxidant balance we determined the antioxidant enzyme activities (SOD, GST, GSH, CAT) in the Hep-G2 cells treated with different concentrations of *M. concanensis* root bark extracts. We found dose dependent significant decrease in antioxidant enzyme activities in the cells treated with 50, 70 and 90 μM concentration of the extracts compared to that of control cells (Fig. 5).

Moringa concanensis induced apoptosis in Hep-G2 cells: The mitochondrial membrane potential levels in the cells treated with different concentrations of *M. concanensis* extracts were evaluated. Figure 4 shows that *M. concanensis* methanol extract at 70 μM showed dramatic loss of mitochondrial membrane potential (40%) compared to control cells (100%). Further, at 90 μM , the membrane potential decreased to 30%. These results show that *M. concanensis* extract induced dose dependent significant decline in membrane potential (Fig. 6).

Fig. 6. Reduction of mitochondrial membrane potential by *M. concanensis*.

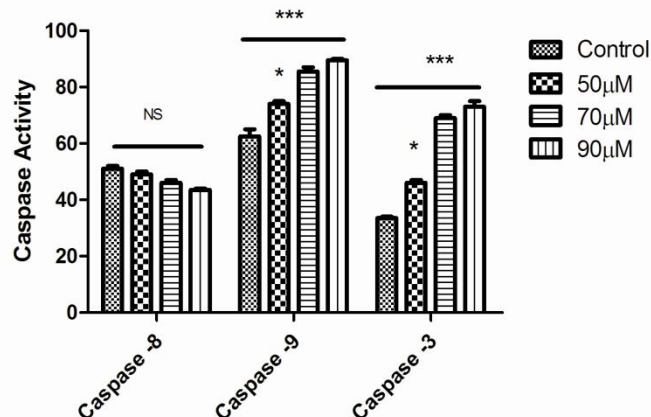


Caspase activities: Next we determined caspase 8, 9 and 3 expressions to evaluate apoptosis induced by *M. concanensis* extract in Hep-G2 cells. Methanolic extract of *M. concanensis* significantly increased the caspase 9 and caspase 3 activities in dose dependent manner compared to control cells. However *M. concanensis* extract did not up regulate caspase 8 activity. These results show that *M. concanensis* extract might induce intrinsic pathway of apoptosis through decreasing mitochondrial membrane potential and increasing caspase 9 and 3 activities (Fig. 7).

Discussion

In the present study, we found that *M. concanensis* plant showed anticancer activity against Hep-G2, liver cancer cell line while there was no effect against other cancers including lung and colon cancer. Methanolic *M. concanensis* extract induced dose dependent cell death with IC₅₀ value of 70 μM in Hep-G2 cells. Our present findings are similar to Balamurugan *et al.* (2014) who have reported on potential anticancer effect of *M. concanensis* crude extracts against Hep-G2 cells. Further studying the detailed mechanism of cell death, it was noted that *M. concanensis* extract increased the reactive oxygen species content. ROS generation in small amounts during normal cellular processes involve in cellular signaling while increased oxidative stress are important contributors in cancer development (Visconti and Grieco, 2009). Oxidative stress is caused by imbalance in the oxidant to cellular defence mechanism leading to depleted antioxidant status. *Moringa concanensis* extract dose dependently increased the ROS levels compared to the control cells. Various antioxidant enzymes such as superoxide dismutase, catalase and glutathione-s-transferase were significantly down regulated in dose dependent manner on treatment with methanolic root bark extract of *M. concanensis*. Reactive oxygen species are linked to apoptotic induction (Reuter *et al.*, 2010).

Fig. 7. Up regulation of caspase 9 and caspase 3 expressions by *M. concanensis*.



Apoptosis is a programmed cell death which is coordinately regulated by various initiation and executioner caspases (McIlwain *et al.*, 2013). We found that *M. concanensis* significantly caused dose dependent loss of mitochondrial membrane potential compared to that of control cells. Mitochondria are regulators of calcium homeostasis and involve in redox signaling. Mitochondrial dysfunction leads to increased ROS generation with dissipation in mitochondrial membrane potential and activation of various oncogenic signals (Wen *et al.*, 2013).

Caspases are endoproteases which are involved in regulating cell death mechanisms. Deregulated caspase activation leads to initiation and progression in cancer. Thus, activation of caspase expressions are involved apoptotic regulation and induction of cell death (McIlwain *et al.*, 2013). *Moringa concanensis* caused dose dependent loss of membrane potential with activation of intrinsic pathway of apoptotic proteins such as caspase 9 and caspase 3 expressions. In conclusion *M. concanensis* has a potent anticancer property against liver cancer by generating reactive oxygen species and apoptosis induction.

Conclusion

This is the first time that acetone and methanolic extracts of *M. concanensis* root bark samples have been screened against A549 (human lung adenocarcinoma), Hep-G2 (hepatocellular carcinoma) and HT-29 (human colon carcinoma) cell lines. The data obtained in this study suggest that *M. concanensis* may contain potentially toxic compounds. This study provides an important basis for further investigation into the isolation, characterization and mechanism of cytotoxic compounds. Thus, these plants could be used as a source for new lead structures in drug design to combat cancer.

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