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## Zaleyia decandra exerts anti-cancer activity in HCT-116 cancer cells by impairing mitochondrial activity

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### Abstract

An attempt has been made to investigate the role of *Zaleyia decandra* on the survival of HCT colon cancer cells *in vitro*. The cell viability and apoptosis were assessed using MTT assay and acridine orange/ethidium bromide staining, respectively. Mitochondrial membrane permeability was assessed using rhodamine-123 staining. The activities of caspases were evaluated using ELISA kits. Treatment with *Z. decandra* extract increased the production of ROS in HCT cells and impaired the mitochondrial activity as evidenced by decreased reduction of tetrazolium salt and enhanced mitochondrial membrane permeability. The onset of apoptosis was evident in cells treated with the extract in acridine orange/ethidium bromide staining and was associated with elevated levels of initiator and effector caspases in the conditioned medium. In conclusion, *Z. decandra* treatment promoted the apoptosis of HCT colon cancer cells.

### Introduction

Globally 1.8 million new cases are identified as colorectal cancer and 881,000 deaths occurred in 2018 (Bray et al., 2018).

The treatment employed to date includes metoclopramide, domperidone, haloperidol, ondansetron, chlorpromazine, diphenhydramine, prochlorperazine, olanzapine and dexamethasone (Berger et al., 2016). However, usage of these drugs is associated with various adverse effects (Munker et al., 2018). The immune therapy molecule like cetuximab is also associated with skin rashes (Giuliani and Marzola, 2013; Baas et al., 2018). Collectively these observations indicate the necessity of identifying a drug with a few or no adverse effect.

Various herbs were suggested as anti-cancer drug in the treatment of colorectal cancer which include *Coptidis rhizoma* and *Scutellariae radix* (Fukutake et al., 1998), *Cissus quadrangularis* (Jainu et al., 2010), huangqin

decoction (Chen et al., 2016), *Panax ginseng* and *Veratrum nigrum* (Kee et al., 2018), *Solanum incanum* (Al-Emam et al., 2018) and grape seed (Zhang et al., 2019).

Previous studies carried out in this laboratory revealed the anti-diabetic potential of ethanolic extract of *Zaleyia decandra* in alloxan-treated Wistar rats (Meenakshi et al., 2010). *Z. decandra* was prescribed for inflammatory diseases including hepatitis, asthma and orchitis (Warrier et al., 1994). The hepatoprotective activity exerted by *Trianthema decandra* (*Z. decandra*) in carbon tetrachloride-induced liver damage indicating less or no toxic effects (Sengottuvelu et al., 2008). In total, 25 phytochemicals were reported in *n*-hexane fraction of *Z. decandra* from which 6-octadecenoic acid has cancer preventive property and *n*-hexadecanoic acid is known as an anti-inflammatory compound (Malarvizhi et al., 2015). The unsaturated fatty acid oleic acid was isolated from the ethanolic extract of *Z. decandra* (Malarvizhi et al., 2016) and found to exert anti-cancer activity through a variety of mechanisms (Carrillo et al., 2012). The



present study is aimed at delineating the anti-cancer activity of *Z. decandra* in HCT-116 colon cancer cells through MTT assay, mitochondrial membrane permeability assessment, induction of ROS, apoptosis and caspase activities.

## Materials and Methods

### Cancer cell culture

The HCT-116 colon cancer cells were procured from the National Center for Cell Science, Pune, India, and sustained in Dulbecco's modified Eagles medium, containing 10% fetal bovine serum and 1% antibiotic

and antimycotic solution [penicillin (100 units/mL), streptomycin (100 mg/mL) and amphotericin (25 µg/mL)]. The HCT-116 cells were cultured in a CO<sub>2</sub> incubator at 37°C. The cells were harvested using trypsin-EDTA and subcultured. All standard culture reagents were procured from the Invitrogen (USA). A stock solution of 10 mg/mL of *Z. decandra* was prepared in DMSO and sterile-filtered using 0.22 µm filter on the day of experiment. The extract was diluted to the required concentration (final concentration of DMSO not exceeding 0.05%).

### Preparation of ethanolic extract

The roots (100 g) of *Z. decandra* were washed with

### Box 1: MTT Assay

#### Principle

The viable cells have NAD(P)H-dependent oxidoreductase which reduces the yellow colored MTT reagent to deep purple colored formazan (an insoluble crystal). The darker the solution is due to the greater number of viable and metabolically active cells.

#### Requirements

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide); Plateshaker; Singla channel pipette (0.001-1 mL); Multichannel pipette (0.01-0.3); Class 2B hood Benchtop centrifuge; Multiwell plate reader (Biotek, Microplate Reader ELx800™); Incubator with 5% CO<sub>2</sub> at 37°C; Microplates-96 well; round or flat bottom well; Magnetic stirrer; Filter (0.22 mm); Methanol, ethanol, and dimethyl sulfoxide; Formazan; HCT-116 cells (NCCS, Pune, India); Dulbecco's modified Eagle's medium culture (serum-free low sugar)

#### Procedure

##### Preparation of MTT solution

*Step 1:* Dissolve MTT powder (500 mg) in phosphate buffer solution (5% solution; 10 mL)

*Step 2:* Use a magnetic stirrer to stir the solution for approximately 1 hour in the dark

*Step 3:* Use a filter (0.22 mm) to sterilize the solution and store in 10-mL aliquot at -20°C

*Step 4:* Unused MTT may be frozen and reused

##### Incubation of cells

Day 1

*Step 1:* Add HCT-116 cells into the 5 mL Dulbecco's modified Eagle's medium culture

*Step 2:* Centrifuge the medium in a sterile falcon tube (15 mL) at 500 rpm for 5 min

*Step 3:* Remove the media and resuspend cells to culture medium (1.0 mL)

*Step 4:* Count and record cells per mL aseptically

*Step 5:* Dilute the cells to 10<sup>6</sup> cells/mL. Use the Dulbecco's modified Eagle's medium to dilute cells

*Step 6:* Add 100 µL of cells (10<sup>5</sup> total cells) into each well and incubate for 24 hours

Day 3

*Step 1:* When removing the media, do it carefully in order to avoid variation in data

*Step 2:* Final volume should be 100 µL/well

*Step 3:* Treat the cells with a) no drug (untreated control); *Z. decandra* at indicated concentrations (0-250 µg/mL at indicated time points).

*Step 4:* Cells are plated in triplicate to minimize the variation in results. Incubate the cells for 48 hours

Day 6

*Step 1:* Remove the medium and wash cells with phosphate buffer solution

*Step 2:* Add 100 µL of 0.5 mg/mL MTT to each well. All should be done aseptically

*Step 3:* Use a plateshaker to shake the plate for 5 min (slowly increasing the shaking speed: maximum 900 shakes/min)

*Step 4:* Then incubate the plate in a CO<sub>2</sub> incubator for another 4 hours at 37°C, depending on the cell type until the intracellular formazan crystals are visible under a microscope

*Step 5:* Dimethyl sulfoxide (5%, 100 µL) is added to each well and resuspend until all crystals have been dissolved

*Step 6:* Using a multichannel pipette to mix each well thoroughly

*Step 7:* Measure the absorbance at 595 nm using the microplate reader

#### Precautions

MTT is toxic and harmful. There may be irritation of the skin and eye. Therefore, use equipment for personal protection.

MTT is light sensitive. The bottle containing MTT solution should be covered with aluminum foil to protect from light.

#### Absorbance values

The absorbance readings should be within 0.75 and 1.25. If the reading is too low, then increase the number of cells plated or the incubation time, and make sure that the cell culture condition is appropriate.

#### References

Slater et al., 1963; Gunaseelan et al., 2017

#### References (video)

Wang et al., 2018

distilled water, shade dried and powdered by a grinder. The powdered sample (30 g) was then extracted with ethanol (1: 5 ratio). The extract was concentrated at 40° C under reduced pressure using a rotary evaporator (Supervac, India) and it was stored in an air tight container (4°C) for future use.

#### ROS generation assay

A non-fluorescent probe (DCFH-DA) was used to measure the intracellular ROS generation, which readily entered the cytoplasm of cells. The probe was hydrolyzed by esterase to form dichlorofluorescein (DCF), which interacts with ROS and emits fluorescence. The intensity of the fluorescence corresponds to ROS generation (Jesudason et al., 2008).  $1 \times 10^6$  cells/well were seeded in 6-well plate and treated with *Z. decandra* extract at 100 µg/mL concentration. Then, the cells were incubated with the probe for 10 min at 37°C. The amount of ROS generated was calculated by determining the fluorescent intensities at 485 and 530 nm.

#### Assessment of mitochondrial membrane potential

The mitochondrial membrane potential ( $\Delta\psi$ M) was assessed using rhodamine-123 (Johnson et al., 1981). The cells were cultured in 6-well plate ( $1 \times 10^6$  cells/well), and they reacted with *Z. decandra*. The cells were further incubated with rhodamine-123 dye for 30 min. The mitochondrial membrane permeability was assessed using a fluid cell imaging station (Invitrogen, USA). Subsequently, the cells were detached using trypsin and subjected to fluorescence intensity analysis at 485 nm (excitation) and 530 nm (emission) using spectrofluorometer (Shimadzu, USA). The fluorescence image was captured and the representative images were used for comparison. To quantitate the data, the fluorescence was measured spectrofluorometrically.

#### Determination of Caspase-3 and 9

The levels of initiator and executor caspases were determined in the spent media following the manufacturer's directions using a colorimeter (R & D systems, USA). The harvested cells were lysed using chilled 50 µL lysis

buffer and spun at 10,000 × g for 60 sec. Then, the supernatant was incubated with buffered substrate for caspase-3 and 9 and placed at 37°C for 2 hours. The released pNA (p-nitroaniline) was assessed colorimetrically at 405 nm.

#### Apoptotic morphological changes

The cultured cells were fixed in 6-well plate ( $3 \times 10^4$ /well) and incubated with glacial acetic acid/methanol (1:3) for 30 min at 4°C. This was followed by washing the cells with phosphate buffer solution and subsequently staining with acridine orange/ethidium bromide for 30 min at 37°C. The stained cells were subjected to washing to remove nonspecific stains with phosphate buffer solution and visualized using fluorescence microscopy (Invitrogen, USA). The HCT-116 cells stained with ethidium bromide is indicated by arrow marks (Karthikeyan et al., 2011).

#### Statistical analyses

The experiments were conducted in triplicates and the results obtained were expressed as the mean ± standard deviation. Significance was calculated using one-way ANOVA and student 't' test.  $P < 0.05$  indicated a significant difference.

## Results

#### Cytotoxicity of ethanolic extract on HCT-116 cells

Based on the MTT assay result *Z. decandra* was found to have cytotoxic effect against HCT-116 cells. Its  $IC_{50}$  value was 96.8 µg/mL (Figure 1).

#### Measurement of intracellular ROS generation

The intracellular generation of ROS measured using DFC-DA florescent assay indicated the excessive generation of ROS in the cells treated with *Z. decandra* extract (100 µg/mL) as evidenced by enhanced fluorescence (Figure 2).

#### Mitochondrial membrane potential (MMP)

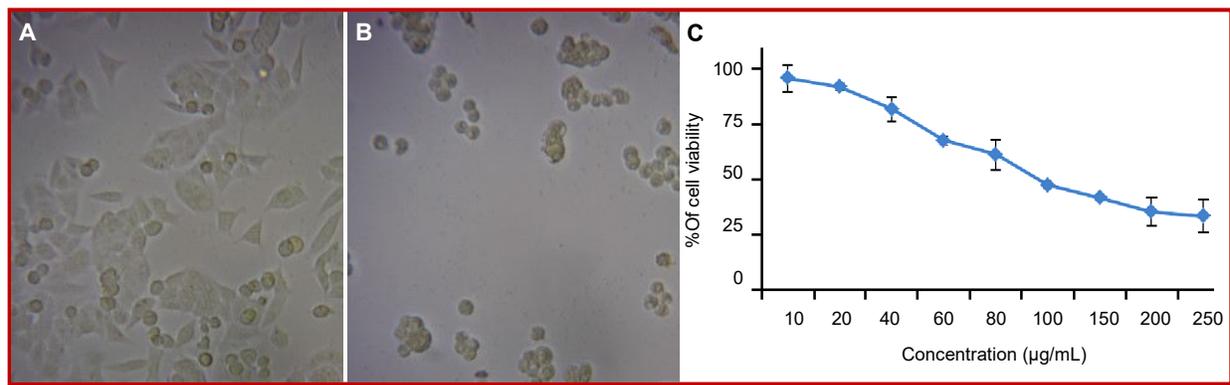


Figure 1: Photomicrographs of the control (A), *Z. decandra* (100 µg/mL)-treated cells (B) and cytotoxicity of *Z. decandra* on HCT-116 cells (C)

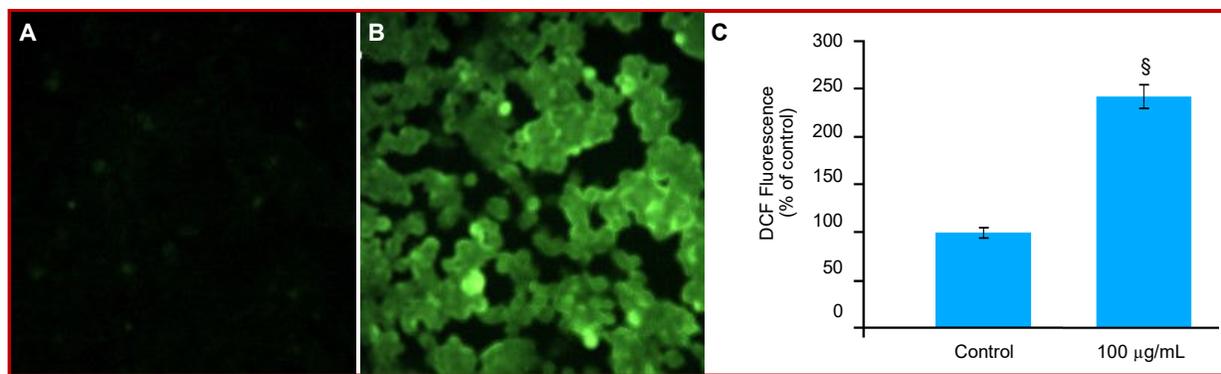


Figure 2: The fluorescence gradient in control (A) and *Z. decandra*-treated cells (B). Levels of intracellular ROS were determined using DCF-DA (C), which is membrane present and acted on by esterase to DCF which reacts to give fluorescence. Fluorescence intensity was measured using spectrofluorometer at 485 and 530 nm. Results are represented as average values of three independent experiments; § indicates statistical significance ( $p < 0.05$ )

The treated cells displayed a drastic reduction in the rhodamin binding indicating reduced mitochondrial activity (Figure 3).

#### Determination of Caspase-3 and 9

A significant increase in the level of caspase 3 and 9 was evident after treatment with *Z. decandra* indicating that the apoptotic effect of *Z. decandra* was possibly mediated through caspase 3 and 9 (Figure 4).

#### Determination of apoptotic morphological changes

To address the functional relevance of enhanced ROS production in HCT-116 cells, acridine orange/ethidium bromide staining was performed which indicated the presence of ethidium bromide uptake in cells treated with *Z. decandra* (Figure 5).

## Discussion

ROS generation is one of the most important strategies adopted by many therapies including radiation. Targeting ROS using antioxidants such as EGCG has been

reported to reduce cancer cell survival (Muthusami et al., 2013). The paradoxical role of ROS is well established in tumor proliferation and suppression. NADPH oxidase is considered an important source known to generate ROS. This enzyme is present abundantly in mitochondria. The increase in the generation of ROS in *Z. decandra* treated cells indicate that the phytoconstituents present in the extract alone or in combination could have targeted NADPH oxidase while exerting anti-proliferative effects.

This contention is further strengthened by MTT assay which evaluates the mitochondrial dehydrogenase activity. Taken together it is presumed that *Z. decandra* activates NADPH oxidases could inhibit dehydrogenases leading to augmented production of free radicals and reduce cell viability. In addition, *Z. decandra* also upregulated the levels of initiator caspase and effector caspase indicating the onset of apoptosis. It is pertinent to link the relationship between enhanced ROS generation and induction of apoptosis. Many of the pharmaceutical drugs increase the production of ROS and reduce the viability of cells; it will be interesting to

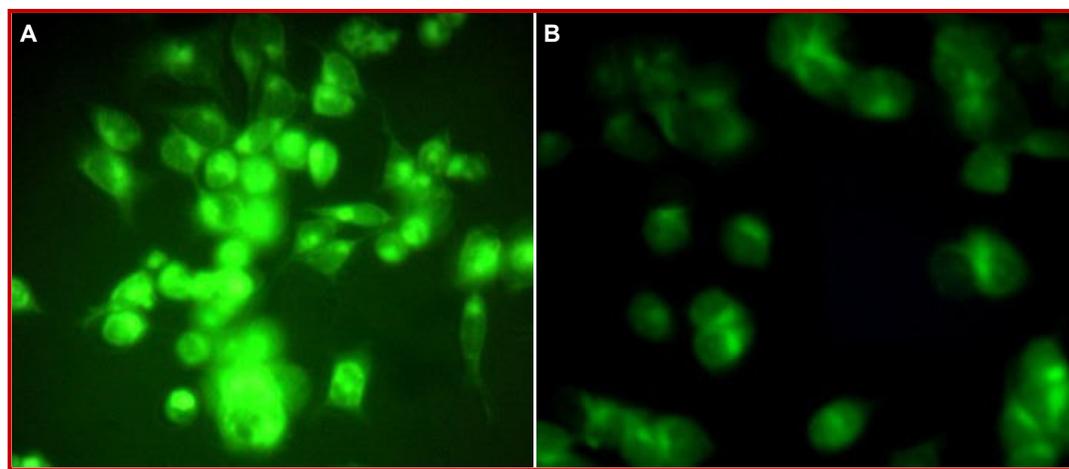


Figure 3: Permeability in *Z. decandra*-treated cells (B) when compared with control cells (A) using rhodamine-123 dye

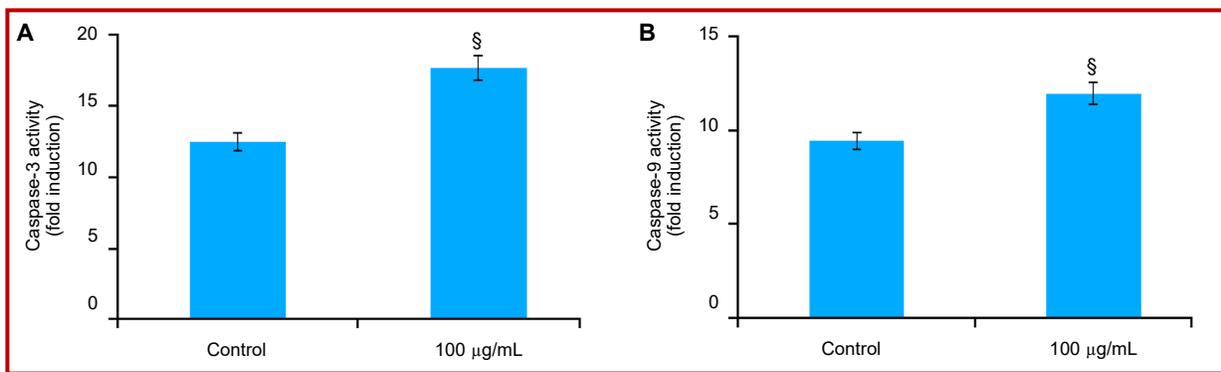


Figure 4: Effect of *Z. decandra* on the levels of initiator and effector caspases [caspase 3 (A) and caspase 9 (B)] in the conditional medium were evaluated and represented. Results are represented as average values of three independent experiments; § indicates statistical significance ( $p < 0.05$ ). Significance was calculated using one-way ANOVA and student 't' test.

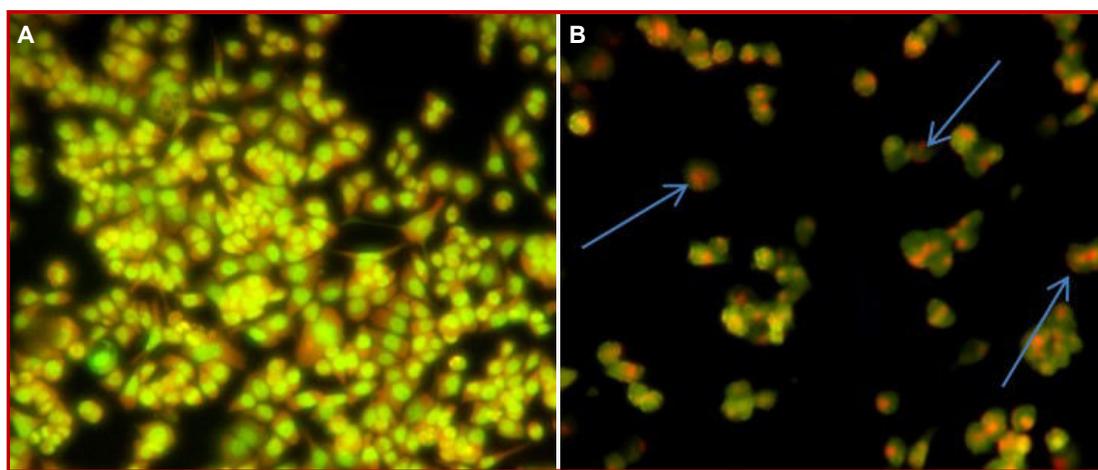


Figure 5: Stains the acridine orange/ethidium bromide staining in control (A) and *Z. decandra*-treated (B) HCT-116 cells. Arrow marks indicate the DNA damage as evidenced by nuclear staining with ethidium bromide

find out the cellular mechanisms behind the increased ROS levels in *Z. decandra* treated cells. The phytoconstituents of *Z. decandra* could have also attenuated antioxidant enzymes such as SOD, Gpx and Trx in addition to xanthine oxidase. A number of treatment regimens such as radiation, hypericin, cisplatin and cordycepin are known to produce ROS and induce cellular death/ senescence (Seong et al., 2016). Further studies are required to know the role of *Z. decandra* on the enzymatic activity in HCT-116 Colon cancer cells *in vitro* to arrive at a definitive conclusion. However, the present study clearly supports the anticancer properties of *Z. decandra* and promises to be an alternative therapeutic/ preventive source to conventional treatments as the toxicity studies carried out using animal model systems show no significant difference. The high levels of ROS generated by *Z. decandra* might have contributed to the reduced mitochondrial membrane potential. A number of studies report an inverse correlation between accumulation ROS and mitochondrial membrane permeability (Looi et al., 2013). The present study reports the efficacy of *Z.*

*decandra* in inducing cellular apoptosis and the involvement of ROS generation, alteration in the MMP and enhanced induction of caspases. Many of the mitogenic signaling to increase the cancer cell proliferation is mediated by PI3K/Akt signaling. ROS regulates PI3 kinase signaling and inhibits the activation of Akt, a key protein required for the survival of cells, and an increase in ROS generation by *Z. decandra* is also associated with reduced viability indicating the possible regulatory role of phytoconstituents of *Z. decandra* on PI3 kinase and Akt signaling. The increased activities of caspase in *Z. decandra* treated cells indicate effective initiation and execution of apoptosis. Taken together the enhanced ROS generation and uptake of ethidium bromide indicate the onset of apoptosis in cells treated with *Z. decandra* to delineate effect of *Z. decandra*. ROS induced DNA damage by *Z. decandra* in colon cancer cell is evident as stained by acridine orange/ethidium bromide, which could probably be mediated by ROS induced inhibition of PARP-1 by the constituents present in *Z. decandra*.

## Conclusion

This study reports the efficacy of *Z. decandra* in inducing apoptosis through ROS generation and altering mitochondrial activity in HCT-116 colon cancer cells.

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## Conflict of Interest

Authors declare no conflict of interest.

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