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MORINGA OLEIFERA LEAVES AQUEOUS EXTRACT INDUCE APOPTOSIS IN HT29 CELL LINE

Abstract

Moringa oleifera (MO) tree has been recognized for the nutritional and medicinal values, attributing anti-inflammatory, antioxidants, and antidiabetic activities of the alcoholic and/or water extracts to its various parts. Recent research has focused on therapeutic anti-cancerous effects of the water extract of MO leaves (MOE), commonly consumed as hot beverage. In-vitro cytotoxicity on esophageal, lung, and breast cancer cells were reported by MOE. The aim in this study is to investigate the anti-cancerous potential of MOE on the colon carcinoma HT29 cells. MO water extract (MOE) was prepared at concentrations (0.1%-0.5%). MOE anti-proliferative effect on HT29-cells was assessed by measuring cellular viability using trypan blue assay. MOE cytotoxicity was further examined by monitoring the change in levels of ROS (NBT assay), ATP (Luciferase assay), and LDH release (cytotoxicity assay) and on depolarization of mitochondrial membrane potential (Mito PTTMJC-1). Protective effect of N-Acetyl cysteine and catalase were assayed. Cell cycle analysis and Annexin-V/PI staining were also performed to detect cell death. Our result showed that MOE induced a dose and time-dependent decrease in viability; increase in ROS and LDH release; dissipation of mitochondrial membrane potential with concomitant decrease in ATP level. In addition, we obtained an increase in preG0 and late apoptotic events. Pretreatment with the antioxidant N-acetyl cysteine suppressed completely LDH release and restored partially and significantly cell viability while decreasing ROS level. Catalase was not protective. MOE also induced cell death with related colon cancer cell lines: Caco2, SW837, SW948, and HCT116. The induced apoptosis in HT29 cells and related CCC identified MOE with anti-cancerous therapeutic potential.

Keywords

Moringa oleifera, HT29, Colon cancer cell lines

1. INTRODUCTION

Folk Medicine utilizes phytochemicals enriched drug formulation(s) that are derived from plants and used in treatment of many ailments including cancer (Balunas & Kinghorn, 2005). World health organization has recognized the importance of medicinal plants as curative as well as potential source of therapeutic agents (World Health Organization, 1977). The interest in phytochemical derived agents have increased significantly in the recent decades. The advantage of using the complex mixture of plant extracts over synthetic drugs is their low cost and ability to target multiple sites simultaneously, overcoming thus some of the reported side effects of drugs (Gurib-Fakim, 2006). In addition, plant derived formulations may differ in composition, texture, and route of administration depending on the mode and the extracting solvent that may yield emulsions, solids, liquids or oil.

Moringa oleifera L. (MO) referred to as the Miracle tree, or “Shagara al Rauwaq” belongs to the Moringaceae family (Fahey, 2005; Goyal, et al., 2007; Anwar, et al., 2007). The National Institute of Health has highlighted the importance of MO for its nutritive and medicinal values, as well as its environmental detoxification ability (National Institutes of Health, 2008). All parts of the plant including the seeds, leaves, roots, bark, flowers and pods, are edible and consumed by humans as raw, cooked or boiled as drinks (Fuglie, 2001). Compared to other plant species, MO is richer (gram for gram) in minerals, essential amino acids, vitamins, and a wide range of phytochemicals: sitosterols, quercetin, kaempferol, glucosinolates, and isothiocyanates (Rockwood, et al., 2013; Manguro & Lemmen, 2007; Ferreira, et al., 2008). Different pharmacological and biological activities have been attributed to different parts of MO. For instance, different studies documented the anti-inflammatory (Carceres, et al., 1992), anti-tumorigenic (Elsayed, et al., 2015), and antimicrobial effects of MO seeds (Eilert, et al., 1981; Madsen et al., 1987). Other studies performed on leaves extracted with organic solvent showed hypocholesterolemic (Mehta et al., 2003), hypotensive (Gilani et al., 1994), anti-ulcerative (Pal et al., 1995), anti-diabetic (Mbikay, 2012), and platelet aggregation inhibitory (Arabshahi-Delouee et al., 2009) effects. In addition, to reported evidence in support of the possible MO role in regulating blood sugar, and treating cardiovascular disorders (Singh et al., 2009), further *in vitro* and *in vivo* studies with MO extracts demonstrated hepatoprotective (Sharifudin et al., 2013; Pari & Kumar, 2002), neuroprotective (Ganguly & Guha, 2008), immunomodulatory (Sudha et al., 2010; Mahajan & Mehta, 2010), diuretic (Carceres et al., 1992), antihypertensive (Faizi et al., 1995; Faizi et al., 1998), and anti-carcinogenic effects (Ahmed et al., 2017).

Cancer is one of the main causes of deaths worldwide (World Health Organization, 2018). Although many anti-cancerous drugs have been developed, yet the need to identify new drugs with minimal side effects remains a priority in scientific research and pharmaceutical industries. The last decade has witnessed the increase in the number of reports documenting evidence, though limited, of the anti-proliferative effect of MO. Al-Asmari et al. investigated and compared the effects of alcoholic extracts of MO bark, leaves and seeds on breast and colorectal cancerous cell lines (Al-Asmari et al., 2015). Remarkable anti-cancerous effects were shown, mediated by the decrease in cell motility and colony formation in: MDA-MB231 and HCT-8 treated with alcoholic leaves or bark extracts; while the seed extracts' exerted no effect. Another study using the alcoholic MO leaves' extract documented its cytotoxic, antioxidant and anti-proliferative effect on different cell lines: HepG2, Caco-2 and MCF-7 (Charoensin, 2014). A similar profile was also obtained with MO leaves' extract treated ovarian and prostate human cancer cell lines (Zayas-Viera & Vivas-Mejia, 2016). Although MO is widely used in traditional medicine, there is a limited number of studies on the anti-cancerous effect of water extract of MO leaves (MOE) that is highly consumed by people as tea drink. Interestingly, the toxicological evaluation of acute-oral administration of MOE showed it is safe (Awodele et al., 2012) with no mortality occurring at a maximum dose of 2000 mg/kg (Adedapo et al., 2009). Hence we opted in this study, to mimic people consumption of MO as tea beverage, (Madi et al., 2016) and examine its anti-cancerous potential. In a recent study, we showed a dose dependent decrease in viability of MOE-treated A549 cells (human lung adenocarcinoma), induced by compromised mitochondrial function, increase in ROS level and PARP -1 cleavage (Madi et al., 2016).

In the current study, we focused on examining the effect of MOE, mimicking the commonly consumed MO-tea drink by people, on colon cancer; one of the leading causes of death affecting equally males and females. More specifically, we have investigated the effectiveness and anti-cancerous potential of MOE on HT29, a model of colon cancer cell (CCC) lines.

2. MATERIALS AND METHODS

Moringa oleifera L. (MO) leaves were supplied by Salah Abdoun, agricultural engineer at “Moringa Research and Development Group Farm”, in Khartoum, Sudan. The leaves were deposited in the Post Herbarium at the American University of Beirut, under the Voucher number: “Usta 001”. Leaves were aerated and dried at room temperature in the dark, then ground, and kept in dark glass jars at 4°C. The cell line HT29, a generous gift from Dr. Ralph Abi-Habib, Department of Natural Sciences, School of Arts and Sciences at the Lebanese American University, was originally purchased from American Type Culture Collection (ATCC), Manassas, VA- USA. The following were supplied by Sigma-Aldrich, (Missouri, USA): Roswell Park Memorial Institute Medium (RPMI, cat # R7388), Dulbecco’s Modified Eagle’s Medium (DMEM cat # D5796), Heat inactivated fetal bovine serum (FBS, cat # F9665), Phosphate Buffered Saline (PBS) solution (10X, cat # D1408), and Trypsin-EDTA 1X-solution (cat # T3924), 0.4% Trypan Blue Solution (cat # T8154), antioxidants [N-acetylcysteine (NAC, cat # A9165); Catalase (cat # C3515)] and Propidium Iodide (PI, cat # P4170). Penicillin-streptomycin (PEN-Strep mixture, cat # L0022-100) was purchased from Biowest.

The ATP Bioluminescence Assay Kit HS II (cat # 11 699 709 001), Cell Proliferation Kit I for 3-(4–5 dimethyl thiazol-2yl)-2, 5 diphenyl tetrazolium bromide (MTT) assay (cat # 11 465 007 001), Cytotoxicity Detection Kit ^{PLUS} (LDH) (cat # 04 744 934001) were purchased from Roche, Mannheim- Germany. Mito PTTMJC-1 kit (cat # ab113850) and Annexin-V FITC Apoptosis Detection Kit (cat # ab14085) were purchased from Abcam (USA). P-Nitro Blue Tetrazolium Chloride (NBT, cat # 6876) was purchased from MP chemicals, CA-USA.

2.1 Preparation of *Moringa oleifera* Water Extract (MOE)

Different concentrations of MOE were prepared by soaking the pulverized leaves (0.1-0.5g) in boiled dd-H₂O (100ml) for 30 min with mild shaking. Extracts were then filtered, centrifuged and supernatant was stored as 1ml aliquots at -20°C.

Qualitative assessment of different phytochemicals was determined using the indicated tests as described in previous reports: Lead acetate test, shinoda test, honey comb test and ferric chloride test (Geetha, and Geetha, 2014); Copper acetate test (Tiwari et al., 2011); Tannic acid test and gelatin test (Neelima, et al., 2010).

2.2 Culture of Colon Cancer Cell Lines

HT29 cells were cultured in humidified CO₂ incubator at 37°C in RPMI and supplemented with fetal calf serum (10%) and Pen-Strep (0.5%). Other screened colon cancer cell lines were cultured, under the same conditions, using RPMI for HCT116 cells and DMEM for Caco2, SW837 and SW948.

2.3 Viability Assay

Viability of HT29 was determined using trypan blue exclusion assay in which dead cells stains blue whereas viable cells remain white. HT29 cells were seeded in 12 well plates (1×10⁵cells/ml) and treated for 6 and 24hours with MOE at final well concentrations varying from 0.01% to 0.05%. The % viability was determined by computing the ratio of viable cells in treated to that of control (untreated cells) multiplied by 100.

2.4 Reactive Oxygen Species (ROS) Level

HT29 cells were seeded in 96 well plates (1x10⁴/100µl) and treated with MOE at EC50 (0.03% determined by viability assay) for 3, 6, 12, and 24hours. The level of generated ROS was determined as described previously (Muñoz et al., 2000). Briefly, NBT (1mg/ml,100µl) was added to control and MOE treated HT29 cells, and incubated for 1hour. Cells were then washed with methanol (100µl/well), and left to dry at room temperature. The formed formazan crystals were solubilized by consecutive addition of KOH (2M, 120µl) and DMSO (140µl). Absorbance of the developed color was read at 630 nm using Multiskan EX ELISA reader. % ROS generated was calculated as follows:

$$\% \text{ ROS production} = 100 - \% \text{ NBT}$$

$$\% \text{ NBT reduced} = (\text{Abs of MOE treated cells} / \text{Abs of control}) \times 100$$

2.5 Lactate Dehydrogenase (LDH) Release

Cytotoxicity induced in MOE-treated HT29 cells was monitored using LDH release assay kit (ROCHE). Disturbance in the plasma membrane integrity causes the release of cytoplasmic LDH into the culture media. HT29 cells were seeded in 96 well plates (1×10^4 cells/100 μ l), and treated with MOE (0.03%) for 6 and 24 hours. The % release of cytosolic LDH into the culture media was determined by assessing the enzymatic conversion of tetrazolium salt into red formazan, compared to 3-controls: cell free control, low control (spontaneous release of LDH), and high control (maximum release of LDH). The intensity of developed color was read at 492 nm using Multiskan EX ELISA reader.

$$\% \text{ LDH released: } \frac{(\text{Abs of treated cells} - \text{Abs of low control}) \times 100}{(\text{Abs of high control} - \text{Abs of low control})}$$

2.6 Intracellular ATP Level

The intracellular ATP level was determined in both MOE-treated HT29 cells and untreated control cells using the ATP Bioluminescence Assay Kit. Cells were seeded in 12-well plate (1×10^5 cells/1 ml) and treated with MOE (0.03%) for 3, 6, 12, and 24 hours. Subsequently, cells were trypsinized, centrifuged, washed twice with 1X PBS, and lysed using the lysis reagent provided by the kit. To 50 μ l of cell lysate (transferred to white MTP plates), Luciferase enzyme (50 μ l) was added and Bioluminescence was measured using a luminometer (Fluoroscan Ascent FL).

$$\% \text{ ATP level} = \frac{\text{Bioluminescence of treated cells}}{\text{Bioluminescence control cells}} \times 100.$$

2.7 Mitochondrial Membrane Potential of MOE-treated Cells

The effect of MOE on the integrity of mitochondrial membrane in HT29 cells was assessed using Mito PTTM JC-1 assay kit. Cells were seeded (1×10^5 cells/1 ml) onto sterile glass cover slips placed in 12-well plates and treated with MOE at EC₅₀ (0.03%). Media was aspirated and cells were incubated for 15 min at room temperature with 1x Mito PTTM JC-1 (300 μ l) stain solution (provided by kit), then washed twice with buffer solution, and visualized using a fluorescent microscope (OLYMPUS; BH2-RFCA), at an excitation of 490 nm and emission of 510 nm. Images were taken by OLYMPUS DP71 camera using the DP controller (OLYMPUS, 2001-2006; 3.1.1.267) acquisition software.

2.8 Cell Cycle Progression in MOE-treated HT29 Cells

Cell cycle progression was assessed using PI staining and flow cytometry. HT29 were seeded in 100 mm petri dishes (1×10^6 cells/10 ml) and treated with MOE (0.03%) for 3, 6 and 24 hours. Cells were then trypsinized, centrifuged (1500 rpm for 5 min), washed with PBS, fixed in 70% ethanol (1d, -20°C) then incubated at 4°C (1 hour) following the consecutive addition of RNase A (100 μ l, 200 μ g/ml), PBS (350 μ l) and PI (35 μ l, 1 mg/ml). The fluorescence of the PI stained DNA was measured using Guava EasyCyte8 Flow Cytometer, collected as 10000 ungated events and the corresponding cell cycle distribution according to DNA content was determined.

2.9 Annexin-V and Propidium Iodide Staining Assay

Annexin-V and propidium iodide (PI) staining was used to determine the mode of cell death in MOE-treated HT29 cells. Briefly cells were seeded in 6-well plates (2×10^5 cells/2 ml), treated for 3, 6, and 24 hours with MOE at EC₅₀ (0.03%), trypsinized, washed with PBS, re-suspended (5 min) in Annexin-V-FITC and PI containing binding buffer, at room temperature. The Annexin-V FITC⁻/PI⁻ cell population was considered normal, whereas the Annexin-V FITC⁺/PI⁻ and Annexin-V FITC⁺/PI⁺ were indicative of early and late apoptotic cells respectively. Flow cytometry data were analyzed by the fluorescence Guava EasyCyte8 Flow Cytometer- Millipore.

2.10 Effect of Antioxidants on Viability, ROS, and LDH in MOE-treated HT29 Cells

The protective effect of NAC (5 mM) and the enzyme catalase (3.4 U/ml) on MOE-treated HT29 cells was examined. Cells were pretreated with either antioxidant, for 2 hours prior to MOE addition. Restoration of control viability, ROS, LDH levels were then determined as described before.

2.11 Effect of MOE on Related Colon Cancer Cells

Different colon cancer cell lines (HCT116, Caco2, SW837 and SW948) were treated with MOE and compared with HT29 for viability as described before.

2.12 Statistical analysis

Results are reported as the mean \pm standard error of the mean (SEM). Figure legends report on the number of determinations from the different number of experiments. Significance was determined using Microsoft Excel independent student t-test. $p < 0.05$ were considered significant. The inter-categorical statistical significance and the significance relative to the control of each parameter were also analyzed by using independent student t-test.

3. RESULTS

Qualitative phytochemical analysis of the 1% *Moringa oleifera* (MOE) water extract identified the presence of: flavonoids, saponins, di-terpenes, phenols, and alkaloids (Table 1). While tannins were not detected in the 1% extract, they were identified in water extracts greater than 5% (Data not shown).

Table 1: Phytochemical classes in MOE

Tests	Phytochemicals	1% MOE
Lead acetate Test	Flavonoids	+
Shinoda Test	Flavonoids	+
Honey Comb Test	Saponins	+
Copper Acetate Test	Diterpenes	+
FeCl ₃ Test	Phenols	+
Tannic Acid Test	Alkaloids	+
Gelatin Test	Tannins	-

3.1 MOE Decreased the Viability of HT29 Cells

MOE decreased in a dose dependent manner the viability of HT29 cells. Treating cells for 6 hours with increasing MOE concentration caused significant ($p < 0.001$) cell death (Figure 1A). Estimated EC₅₀ was obtained at 0.03% with further decrease in viability reaching 70% at 0.05%. A similar dose dependent decrease in viability profile was obtained after 24 hours reaching 98% cell death at 0.05% MOE (Figure 1A).

3.2 MOE-Treated HT29 Cells Induced LDH Release

The integrity of plasma membrane was assessed by quantifying the level of LDH released from MOE-treated (0.03%) HT29 cells into culture media. The level of released LDH increased from 8% ($p < 0.01$) to 48% ($p < 0.001$) post MO-treatment for 6 and 24 hours respectively (Figure 1B).

3.3 Time Dependent Increase in ROS Level in MOE Treated HT29 Cells

Treatment of HT29 with MOE (EC₅₀) resulted in a time dependent gradual increase in ROS level. A significant increase in ROS level of 14.7% and 53% ($p < 0.001$) was obtained at 3hours and 24hours post treatment respectively (Figure 1C).

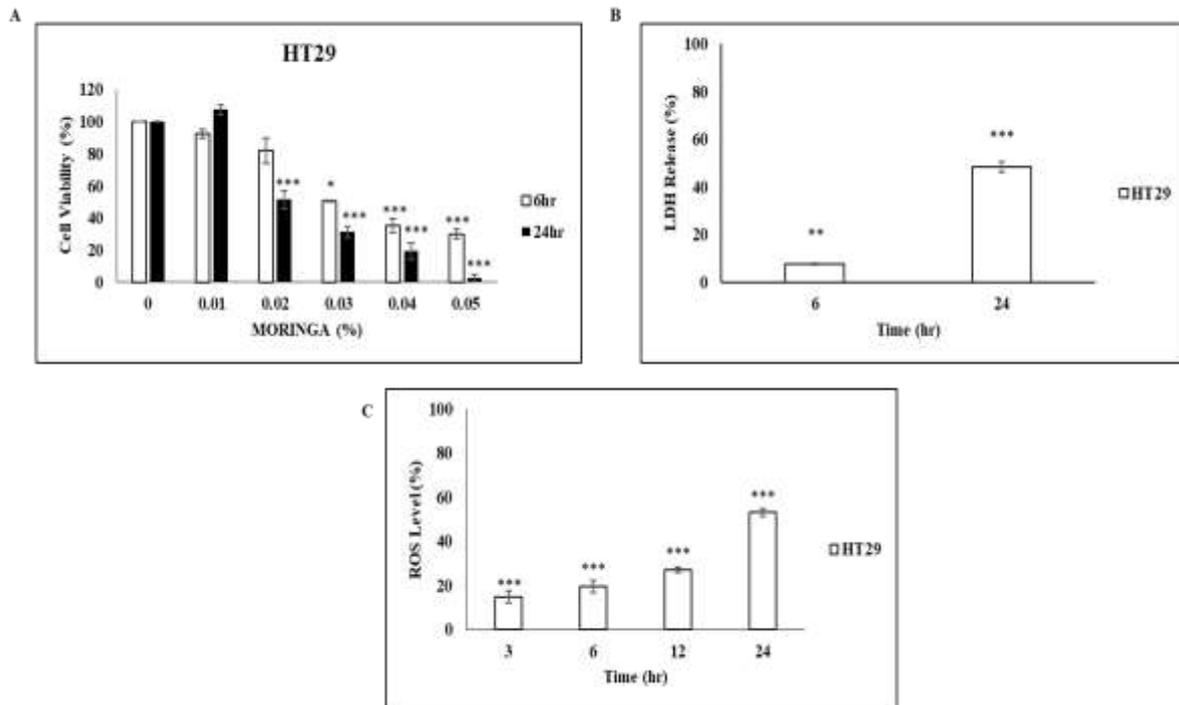


Fig. 1: Effect of MOE-treated HT29 cells on A) Viability using trypan blue exclusion assay. B) LDH released level C) Changes in ROS level with time. Data presented are the mean \pm SEM of 9 determinations of three different experiments. Asterisks on bars represent significance relative to the control, * P <0.05, ** P <0.01, and *** P <0.001 respectively.

3.4 MOE DECREASED ATP LEVEL AND DISSIPATED MITOCHONDRIAL MEMBRANE POTENTIAL IN HT29 CELLS

The effect of MOE-treated HT29 cells on mitochondrial function was assessed by monitoring changes in ATP level, and membrane potential.

MOE caused a time dependent decrease in ATP level (Figure 2A). A significant decrease of 42% (p <0.01) occurred after 6hours of treatment; with further decrease reaching 91% (p <0.001) at 24hours at EC₅₀. It is known that dissipation of mitochondrial membrane potential would decrease ATP level. To verify whether the drop in ATP level at 6hours is consequent to dissipation of membrane potential we examined the effect of MOE on membrane potential at an earlier time point. Using Mito-JC1, healthy control cells stained red orange, whereas MOE-treated HT29 cells showed increase in green stain indicating dissipation of mitochondrial membrane potential starting at 3hours (Figure 2B).

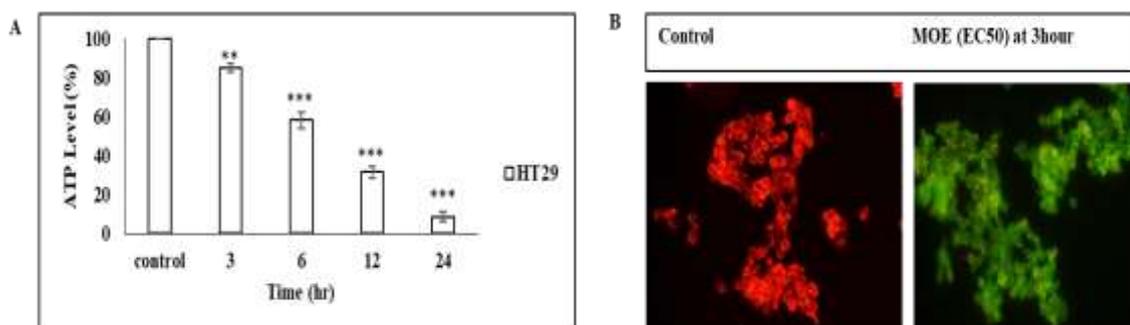


Fig.2: Effect of MOE-treated HT29 on: A) Changes in ATP level with time using ATP Bioluminescence Assay. Data presented is the mean \pm SEM of 6 determinations from three different experiments. Asterisks on bars represent significance relative to the control. ** P <0.01, and *** P <0.001 respectively. B) Mitochondrial Membrane Potential using MitoPT™ JC-1 kit; a representative image of 3 different experiments.

3.5 Cell Cycle Analysis of MOE Treated HT29 Cells

We next examined the changes in cell cycle phases in MOE treated HT29 cells. Cell phases were analyzed over different time intervals: 3, 6, and 24hours (Figure 3). Compared to control cells, MOE treated HT29, showed a significant increase (3.6 fold) with time in preG0 phase concomitant with a decrease (0.4 fold) in G2M phase. No significant fold change was detected in G0G1 or S-Phases (Figure 3C).

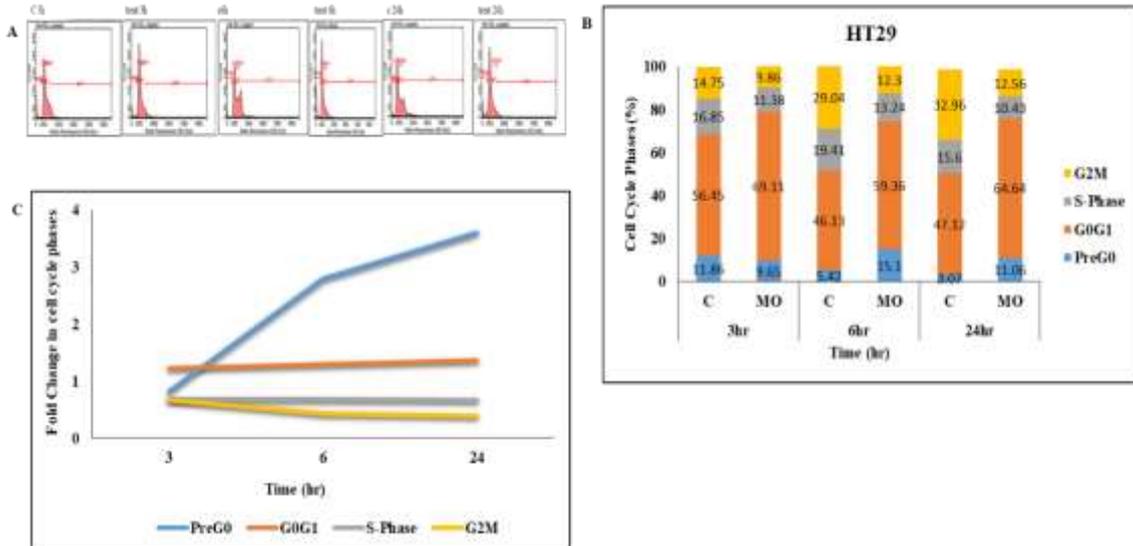


Fig.3: Distribution of cell cycle phases in MOE-treated HT29 with time using PI flow cytometry. A) Representative figure of the variation in phases with time B) Representative histogram of the effect of MOE, at different time point. C) Fold change in cell cycle phases. Data presented are the mean \pm SEM of 3 different experiments.

Next, to examine whether cell death is apoptotic or necrotic, the effect of MOE-treated HT29 was determined at 3, 6, and 24hours (Figure 4) using Annexin V-FITC PI staining. A significant increase in the percentage of cells in the late apoptosis (3 to 9-fold) was obtained, while the change of those that are undergoing necrosis was less significant (3.5 to 4.4 fold) at 3 and 24 hours respectively (Figure 4B).

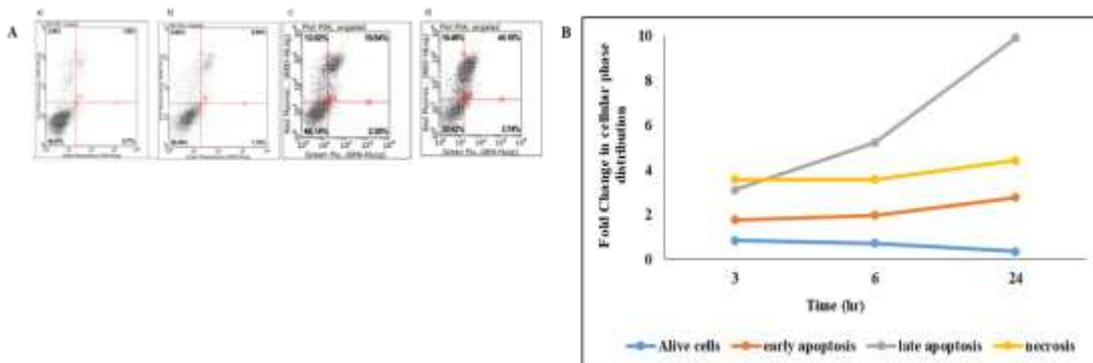


Fig.4: Apoptosis assessment in MOE-treated HT29 cells with time (3, 6 and 24hours) using Annexin-V PI: A) Representative image from one experiment. B) Fold change in cellular phase distribution in MOE treated HT29 compared to control cells at different times. Data presented is the mean \pm SEM the mean of 3 different experiments.

3.6 Protective Effect of NAC But Not Catalase

The protective effect of the antioxidants NAC and catalase in MOE-treated HT29 cells at EC₅₀ (0.03%) was investigated (Figure 5). MOE induced cytotoxicity in HT29 cells. Pretreatment with NAC increased significantly the viability (50%-75%) (Figure 5A), decreased generated ROS level (Figure 5B), and completely suppressed ($p < 0.001$) the MOE induced LDH release (from 52.8% to 2.8%) as shown in Figure 5C. Catalase on the other hand was less effective than NAC and showed variable effects on the HT29 cell line. It caused minimal increase/restoration (10%) in viability (Figure 5A), did not decrease ROS production (Figure 5B) and with no effect on LDH release (Figure 5C).

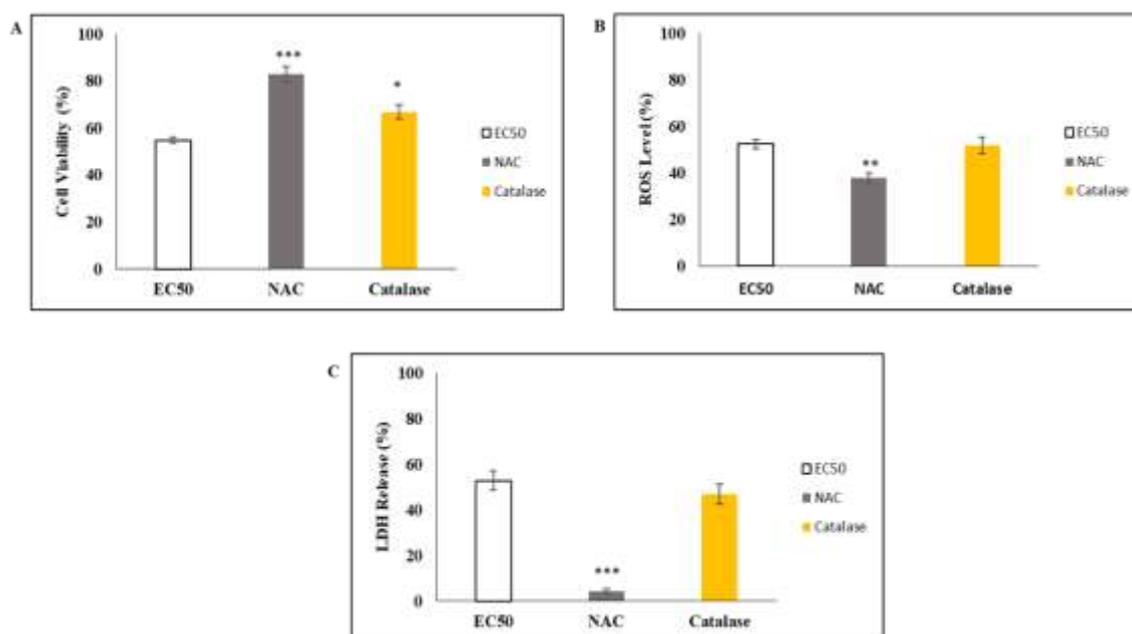


Fig.5: Effect of NAC and catalase pre-treatment on MOE-treated HT29 cells on (A) Viability, (B) ROS, (C) LDH release. Cells were pre-treated with NAC (5mM), or catalase (3.4U/ml) for 2hours, prior to MOE treatment. Data presented is the mean \pm SEM of 9 determinations from three different experiments. Asterisks on bars represent significance relative to the EC₅₀ (0.03%). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ respectively.

3.7 MOE Effect on Cell Viability of Other Colon Cancer Cell Lines

The effect of MOE on related colon cancer cell (CCC) lines was examined. Cells tested included HCT116, Caco2, SW837 and SW948. The Estimated EC₅₀ were: 0.02% HCT116 and Caco2; 0.01% for SW837 and 0.03% for SW948 (Table 2).

Table 2: Estimated EC₅₀ of MOE-treated CCC

Cell Lines	Estimated EC ₅₀
HCT116	0.02%
Caco2	0.02%
SW837	0.01%
SW948	0.03%

4. DISCUSSION

Interest in identifying novel natural drugs that are toxic to cancerous, but not to normal cells, have increased in the recent years in traditional medicine. Plants extracts have been used in the treatment of many ailments providing a source of low cost phytochemicals with effective therapeutic potential.

Moringa oleifera has been recognized for its biological activity targeting multicellular sites and therapeutic values including anti-cancer (Charoensin, 2014).

In this study, we have examined the anti-cancerous potential of MOE on HT29 cells; a model of intestinal epithelium human colon cancer cell lines (CCC) (De Luca et al., 2014). We hereby show, a dose dependent cytotoxic effect on MOE-treated HT29. MOE-induced cell death by: increasing ROS level and depolarizing in mitochondrial membrane potential (3hours), followed by a decrease in ATP level (6hours), at 24hours we obtained an increase in cell cycle preG0 phase and in late apoptotic events, with concomitant increase in LDH release (biomarker of necrotic event). Pre-treatment with anti-oxidant N-acetyl cysteine suppressed completely LDH release and significantly restored partial viability. We also tested the effect of MOE on other cell lines and they showed to be sensitive to treatment at different concentrations.

In this study we opted to use water extract of MO despite the fact that concentration of extracted compound in organic solvents are much higher. Our aim was to mimic people consumption of MO leaves as herbal tea and studying its potential anti-cancerous effect. Previous studies have reported that aqueous Moringa leaf extracts are safe at high doses (Awodele et al., 2012). A perceptible dose and time dependent increase in cell death as for 6hours was obtained in MOE-treated HT29 cells with further increase at 24hours (Figure 1A). Our results are in agreement with previous reports on HepG2 (Jung IL et al., 2015), A549 (Madi et al., 2016; Jung IL et al., 2015), HCT116 (Reda et al., 2017), and KB (Sreelatha & Padma, 2011) cancerous cell lines.

Reactive oxygen species (ROS) are one of the important regulators of cell death and survival mechanisms (Raza et al., 2017). Following treatment with MOE, we obtained an early increase in oxidative stress (ROS) occurred as of 3 hours becoming incompatible with cell viability at 24hours (Figure 1C). A major source of ROS is the electron transport chain of mitochondria. The increase in ROS following MOE-treated HT29 was accompanied by an increase in mitochondrial membrane depolarization that was further confirmed by a decrease in ATP level at 6hours (Figure 2 A & B). Further decrease in ATP level was obtained at 24hours (Figure 2A).

MOE-induced cytotoxicity however is not limited to derangement in mitochondrial membrane potential but it influenced as well the plasma membrane. A biomarker of damaged plasma membrane is associated with an increase in released lactate dehydrogenase extracellularly resulting from increased membrane permeability. In our study, LDH release was minimal after 6hours' treatment of MOE but reached maximum of 50% after 24hours (Figure 1B) indicating a possible necrotic effect. To examine whether it is necrotic or apoptotic, quantitative analysis with time showed an increase in PreG0 (Cell cycle) and late apoptotic events with no time dependent variation in necrotic effect (Annexin-V PI) (Figure 3 and Figure 4). This was further confirmed following pretreatment with NAC, a GSH precursor which suppressed completely LDH release and restored partially and significantly cell viability indicating it is apoptotic rather than necrotic (Figure 5) and that the LDH released is considered as secondary necrosis due to apoptotic cells (caspase activity measured, data not shown).

It is possible to suggest that the complete suppression of LDH release, and significant restoration of viability and ROS level) may be attributed to: a) promoting biosynthesis of intracellular GSH which decreases ROS and reverses induced cell death mechanism; b) interacting directly with thiol containing intracellular and plasma membrane bound proteins involved in the MOE induced cell death; and c) neutralizing the pro-oxidant potential of MOE components at the expense of itself getting oxidized. Regardless whether one or all of the possibilities are involved, the net result led to a protective effect against the induced cell death. On the other hand, pretreatment with catalase, a hydrogen peroxide metabolizing enzyme, exerted a minimal restoration in viability of HT29 cells (Figure 5), while had no effect on ROS and LDH levels indicating that H₂O₂ is not a major key player in the induced cytotoxicity of MOE.

5. CONCLUSION

To sum up we hereby report the potential anti-cancerous effect of MOE on HT29 cell line and related CCC. MOE-induced cell death by simultaneous increase in intracellular oxidative stress and dissipation of mitochondrial membrane potential leading to decrease of ATP level and increase in plasma membrane permeability, suppression of LDH release by NAC pretreatment favors apoptotic rather than necrotic effect (Figure 6). Both cell cycle analysis and annexin-V PI results were confirmatory the extract-induced apoptotic events occurred in HT29 cells as well as related CCC suggest therapeutic potential of MOE in preventing or treating cancer.



Fig.6: A schematic chart summarizing the apoptotic events occurring in MOE-treated HT29 colon cancer cells

6. ACKNOWLEDGMENT

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