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ANTICANCER ACTIVITY OF METHANOL FRUIT EXTRACT OF *OPUNTIA FICUS INDICA* AGAINST CERVICAL CANCER USING HELA CELL LINE

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ABSTRACT

Opuntia ficus-indica, commonly referred to as prickly pear is a dicotyledonous angiosperm plant. In this study, we investigated the anticancer activities of methanolic fruit extract of *Opuntia ficus indica* in cervical cancer (HeLa) cells. The effects of the methanolic fruit extract of *Opuntia ficus indica* on the cell growth and apoptosis in HeLa were analysed by the production of reactive oxygen species (ROS), the level of mitochondrial membrane potential ($\Delta\Psi m$), DNA damage (Comet) and apoptotic morphological changes. The results indicated that the methanolic fruit extract of *Opuntia ficus indica* induce apoptosis as evident by loss of cell viability, enhanced ROS, alteration in mitochondrial membrane potential due to changes in lipid peroxidation, and increased DNA damage in HeLa cells. The results of the present study suggest that methanolic fruit extract of *Opuntia ficus indica* might be useful as a potential antitumor agent.

KEYWORDS: **HeLa, *Opuntia ficus indica*, Lipid peroxidation, apoptosis, Oxidative stress, anticancer activity.**

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INTRODUCTION

Cervical cancer (CC) is the second most common malignancy among women worldwide. It was estimated approximately 528,000 cases and 266,000 deaths in 2012 and The American Cancer Society's estimates for cervical cancer in the United States for 2016 are about 12,990 new cases of invasive cervical cancer will be diagnosed and about 4,120 women will die from cervical cancer. About 70% cervical cancer occur in developing countries. It is due to the abnormal growth of cells that are typically no symptoms in early stage and later symptoms which abnormal vaginal bleeding, discharge, and itching, and pelvic pain[1]. In recent years, plant extracts have longer been a fertile source of cure for cancer[2]. Chemotherapy, being a major treatment modality used for the control of advanced stages of malignancies and after metastasis, exhibits severe toxicity on normal tissues[3]. Plant-derived products have been used for treating various diseases of human beings and animals since time immemorial. They maintain the health and vitality of individuals, and also cure diseases, including cancer without causing toxicity. More than 50% of all modern drugs in clinical use are of natural products, many of which have the ability to control cancer cells. *Opuntia ficus indica* is one of the medicinal plants which have cured many diseases[4] and conditions, including diabetes, hypertension, hypercholesterolemic, rheumatic pain, gastric mucosa diseases and asthma. It is commonly called in Tamilnadu Chapathi balli[5]. It is a member of the Cactaceae[6] family and widely distributed in many countries such as Mexico, America and Africa[7]. An *opuntia* fruit has highly medicinal value and were established to display many pharmacological properties such as antiulcerogenic[8], neuroprotective[9], antioxidant[10], hepatoprotective[11] and anticancer[12] activities. Cervical cancer is a cancer arising from the cervix. It is due to the abnormal growth of cells that have the ability to invade or spread to other parts of the body. The use of prickly pear *Opuntia ficus indica* fruit is suggested for their beneficial and therapeutic properties[13]. Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species. ROS that may induce DNA fragmentation and apoptotic cell death in cancer cells. *Opuntia ficus indica* fruit is a traditionally used in folk medicine and it has been predicted to act as an anticancer agent. Hence, the present study, focused on the anticancer activity of methanolic fruit extract of *Opuntia ficus indica* on cervical cancer using HeLa cell line.

MATERIALS AND METHODS

2.1. Culturing cells

This study was carried out in HeLa cell line. The cell line was obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were grown as monolayer in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FCS, 200 mM L-glutamine, and 10,000U/ml penicillin, 10 mg/ml streptomycin at 37^0C in 5% CO_2 atmosphere. Stocks were maintained in 25 cm^2 tissue culture flasks. After cell numbers were counted, cells were seeded at 5 x 10^4 cells per well in 24-well plates. Cells were harvested by trypsinization.

2.2. Cell treatments

The HeLa cells were treated with methanolic fruit extract of *Opuntia ficus indica* in different concentrations and incubated at 37^0C in 5% CO_2 incubator. After 24-h incubation, the cells were harvested by trypsinisation for further experiments.

Group I : Control (HeLa cells untreated)
Group II : HeLa cells + methanol fruit extract (20µg/ml)
Group III : HeLa cells + methanol fruit extract (30µg/ml)
Group IV : HeLa cells + methanol fruit extract (40µg/ml)

2.3. Chemicals

Thiobarbituric acid (TBA), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-7-diacetyl dichlorofluorescein (DCFH-DA), Rhodamine 123 (Rh-123), heat-inactivated fetal calf serum (FCS), minimum essential medium (MEM), glutamine, penicillin–streptomycin, EDTA, and trypsin were purchased from Sigma Chemicals Co., St. Louis, USA.

2.4. Cell proliferation assay

Effect of methanol fruit extract of *Opuntia ficus indica* on the growth and proliferation of HeLa cells was determined by MTT assay based on the detection of mitochondrial dehydrogenase activity in healthy cells following the method of Mosmann [14]. Cells were seeded in 96-well plates at a density of 5x10^3cells/well in a final volume of 100 µl with DMEM an incubated up to 24 h. The cells were treated with 5-50 µM concentration of methanol fruit extract of *Opuntia ficus indica*. After 24 h, the cells were incubated with 100 µl of MTT solution (1mg/ml PBS) for 2 h at 37 $^{\circ}$C in CO_2 incubator. The medium was removed and added 100 µl of DMSO at 37 $^{\circ}$C for 1 h in the dark to dissolve the formazan crystals. The plate was read at 595 nm in a Readwell touch, ELISA plate analyser (Robonic, India).

2.5. Measurement of intracellular ROS in cells by spectrofluorimetric and fluorescence microscopic methods

After incubation of the cells with different concentrations of methanolic fruit extract of *Opuntia ficus indica* for 24 h, fluorescent dye DCFH-DA was added to the cells which were then kept in incubator for 30 min. Then the cells were washed with PBS to remove the excess dye. Intracellular ROS was measured by using a non fluorescent probe, DCFH-DA that can penetrate into the intracellular matrix of cells where it is oxidized by ROS to fluorescent dichlorofluorescein (DCF)[15]. Fluorescent measurements were made with excitation and emission filters set at 485 ± 10 and 530 ± 12.5 nm, respectively. Fluorescence microscopic images were taken using blue filter (450–490 nm).

2.6. Biochemical assays

Cells were treated with methanol fruit extract of *Opuntia ficus indica* and harvested by trypsinization. The collected cell pellet was suspended in PBS. The suspension was used for biochemical estimations. Lipid peroxidation was estimated following the method of thiobarbituric acid (TBARS) reaction[16], Superoxide dismutase (SOD) was estimated following the method of [17], Catalase (CAT) was estimated following the method

of [18], Glutathione peroxidase (GPx) was estimated following the method of [19], Reduced glutathione (GSH) was estimated following the method of [20].

2.7. Mitochondrial membrane potential detected by the Rhodamine-123 staining method

Mitochondrial membrane potential *(ΔΨM)* was assessed by Rhodamine-123 (Rh-123) lipophilic cationic dye[21]. Cells were cultured in 6 wells plate ($1x10^6$ cells/2 ml/well) and treated with methanol fruit extract of *Opuntia ficus indica*. After the 24 h treatment, the cells were incubated with Rhodamine-123 fluorescence dye for 30 min in the CO2 incubator and washed slowly for twice with PBS. The *(ΔΨM)* was evaluated qualitatively under a Floid cell imaging station (Invitrogen, USA) and photograph. Furthermore, cells were trypsinaized and fluorescence intensity was measured at 485/530 nm under Spectrofluorometer (Schimadzu, USA).

2.8. Apoptotic morphological changes by acridine orange–ethidium bromide dual staining method

Acridine orange (AO) and ethidium bromide (EBr) staining were used to detect apoptotic cells affirmation[22]. The control and methanol fruit extract of *Opuntia ficus indica* treated HeLa cells were seeded in 6-well plate ($3x10^4$/well) and incubated in CO2 incubator for 24 h. The cells were fixed in methanol:glacial acetic acid (3:1) for 30 min at RT. The cells were washed in PBS, and stained with 1:1 ratio of AO/EBr. Stained cells were immediately washed with PBS and viewed under a floid cell imaging station (Invitrogen, USA) and their digitized images were captured. The number of cells showing features of apoptosis was counted as a function of the total number of cells present in the field.

2.9. Assessment DNA damage by comet assay (Single gel electrophoresis)

The effect of methanol fruit extract of *Opuntia ficus indica* on DNA damage was determined by alkaline single cell electrophoresis (Comet) assay[23]. HeLa cells were treated with methanol fruit extract of *Opuntia ficus indica* (20, 30 and 40μM) for 24 h. Untreated cells were used as a control. Cells were collected by trypsinized, and a cell suspension was prepared in 1X phosphate-buffered saline (PBS). Then, 10 μl of cell suspension was mixed with 60 μl of 0.5% low-melting point agarose. The mixture was added to glass cavity slides and the agarose was allowed to solidify in the dark at 4°C for 45 min and then the slides were immersed freshly prepared ice cold lysis solution (10 mM Tris, 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10% DMSO) for 1hr in dark place at 4°C. After lysis, the slides were washed with distilled water, transferred to an horizontal gel-electrophoresis unit, covered with freshly prepared alkaline electrophoresis buffer (500 mM EDTA, 200 mM NaOH [pH 13]), left for unwinding of DNA for 30 min, and electrophoresis unit was performed at field strength of 22V and 200mA for 20 min. The slides were washed with dH_2O 2-3 times, fixed with 70% chilled ethanol and stained with propidium iodide (0.5μg/mL) for 15 min in dark place for exposure of DNA damage. The images were captured using an epifluorescent microscope at a 40X objective (Nikon, Eclipse TS100, Japan) equipped with an excitation filter of 510-560 nm and a barrier filter of 590 nm; with a digital camera (Nikon 4500 coolpix, Japan). One to two hundred comets on duplicated slides were analyzed. Comet image were analysis by CASP software.

2.10. Statistical analysis

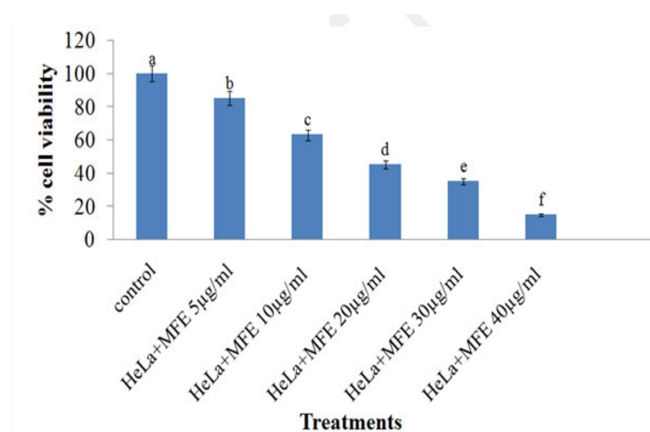
Each experiment was performed in triplicate. The results were expressed as the mean ± SD. The statistical analyses were performed using SPSS 11.0 software package. Statistical variances were assessed using ANOVA. Significant differences ($p < 0.05$) between the means were identified by Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

A large number of plants used in traditional medicines have been shown to possess anti-cancer activity. Over 3000 species of different medicinal plants have been reported to have anticancer properties[24]. In this study, the anticancer effect of methanol fruit extract of *Opuntia ficus indica* in human cervical carcinoma cell line *in vitro*. Methanol fruit extract of *Opuntia ficus indica* treatment (24h- incubation) significantly decreased percentage of cell viability in HeLa cell line. Figure 1 shows the changes in the percentage of cell viability in control and methanol fruit extract of *Opuntia ficus indica* -treated cells.

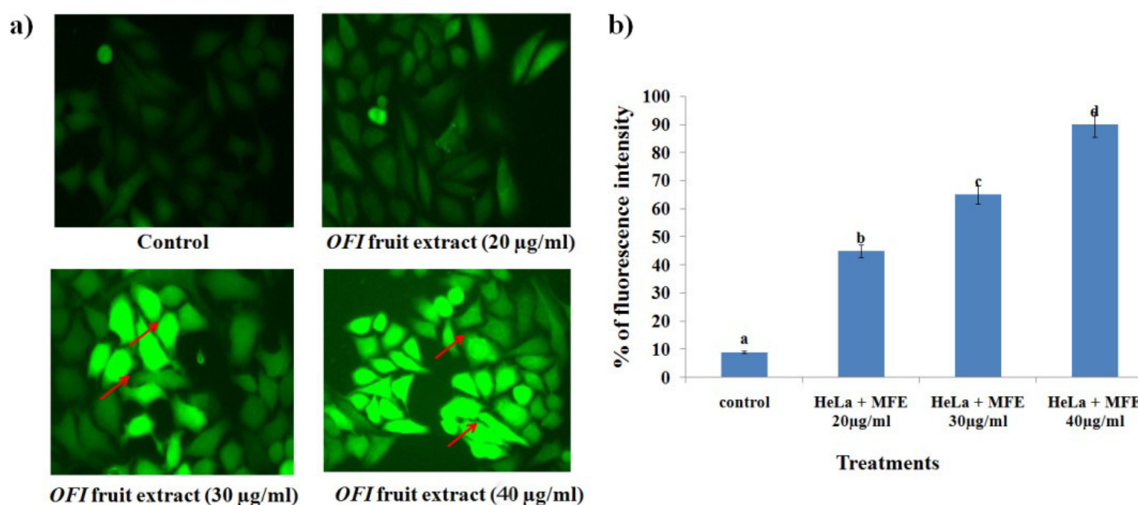
Figure1

Effect of methanol fruit extract on cell proliferation was determined by MTT assay.



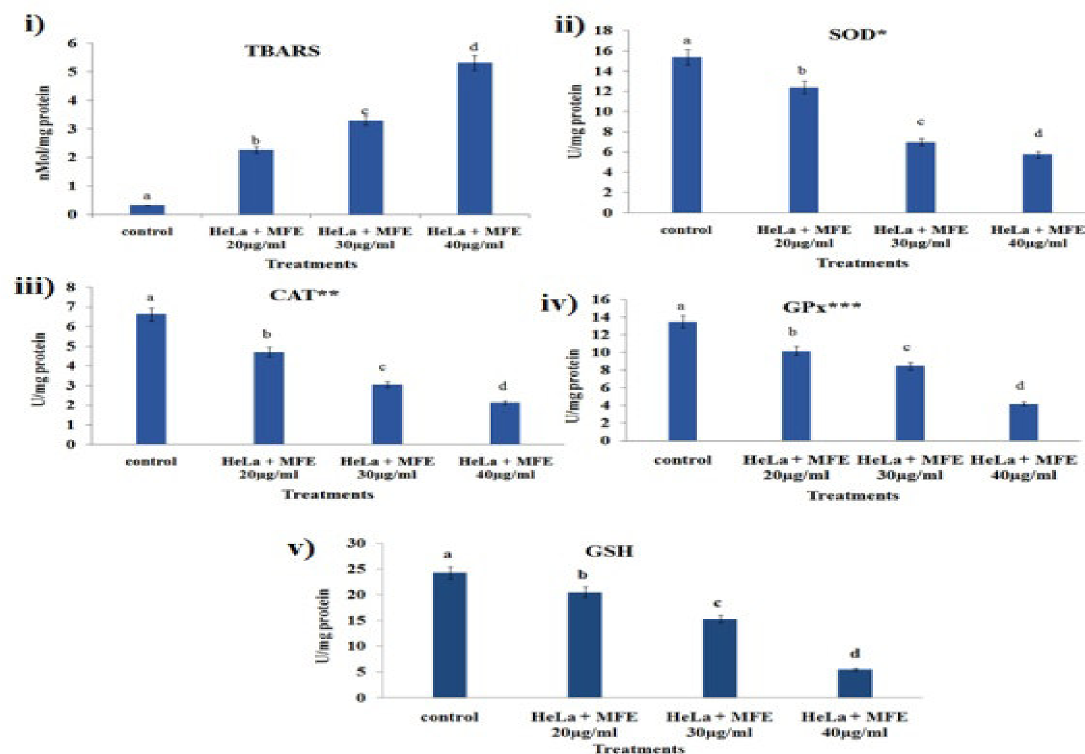
This suggested that *Opuntia ficus indica* methanol fruit extract treatment was able to inhibit the growth cancer cells during incubation. However, high concentrations (20, 30 and 40 µg/ml) of methanol fruit extract of *Opuntia ficus indica* treatment show significant cytotoxicity. It was found that 40µg/ml of *Opuntia ficus indica* could greatly inhibit the cell growth. Hence, the result indicates that concentration of phytochemicals play a role for cytotoxicity. Probably it higher concentration, methanol fruit extract of *Opuntia ficus indica* exhibits prooxidant property. This pro oxidant property might distrupt mitochondrial dehydrogenase activity. Mitochondrial activity of cancer cells may be influenced by methanol fruit extract of *Opuntia ficus indica*, and this might be the reason for the increased cytotoxicity observed in methanol fruit extract of *Opuntia ficus indica* -treated cells. In earlier report, confirmed that the methanolic flower extracts of *Opuntia dillenii* was evaluated using MTT assay. It can be used as a potential anticancer agent[25]. Cactus pear fruit extract inhibits the proliferation of skin and lung cancer. According to the American National Cancer Institute (NCI), the criteria of cytotoxic activity for the crude extracts is an IC50 < 30 µg/ml[26]. Previous study[27] demonstrated that methanolic crude extract of *Phaseohus vulgaris L* have inhibitory effect in cell proliferation of HeLa cells. Furthermore, Brewer *et al*[28] concluded that Arizona prickly pear cactus effectively inhibited cell growth in several different immortalized and cancer cell cultures *in vitro* and suppressed tumor growth. Hence, it has been reported that the extracts of fruits and stems of cactus exhibit an, anti-tumor activity[29].Methanol fruit extract of *Opuntia ficus indica* treatment caused a rapid increase of intracellular ROS in HeLa cells (Fig. 2a, b).

Figure 2
Effect of methanol fruit extract generates ROS level in HeLa cells.



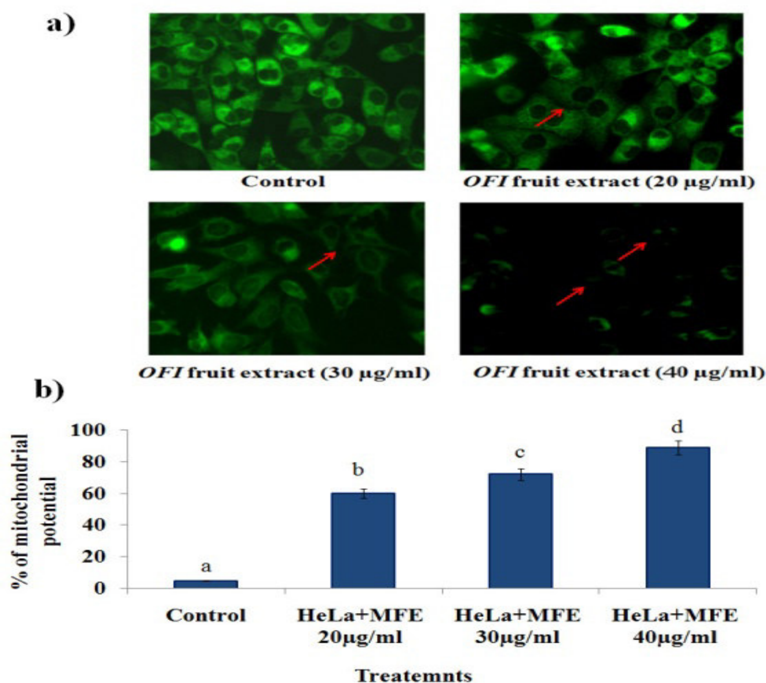
A significant increase in the ROS levels in 40 µg/ml of methanol fruit extract of *Opuntia ficus indica* -treated cells was further noticed. Many cancer cell lines associated with increased production of ROS[30]. However, the increase of ROS generation in cancer cells can further stimulate cell proliferation, cause DNA and after mutations, and promote genetic instability and the emergence of drug resistant cells[31]. Further, peroxidases present in the cancer cells catalyze a one electron oxidation of phenols to form phenoxyl radicals which, in turn, rapidly oxidize NADH to NAD•. Then this NAD radical reduces O_2 to O_2•-. This might be the reason for the increased ROS generation during methanol fruit extract of *Opuntia ficus indica* treatment. The significant increase in lipid peroxidation indices in methanol fruit extract of *Opuntia ficus indica* -treated cancer cells (Fig. 3) was observed.

Figure 3
Effect of methanol fruit extract on the enzymatic and non enzymatic activities of SOD, CAT, GPx, GSH and Lipid peroxidation in HeLa cancer cells.



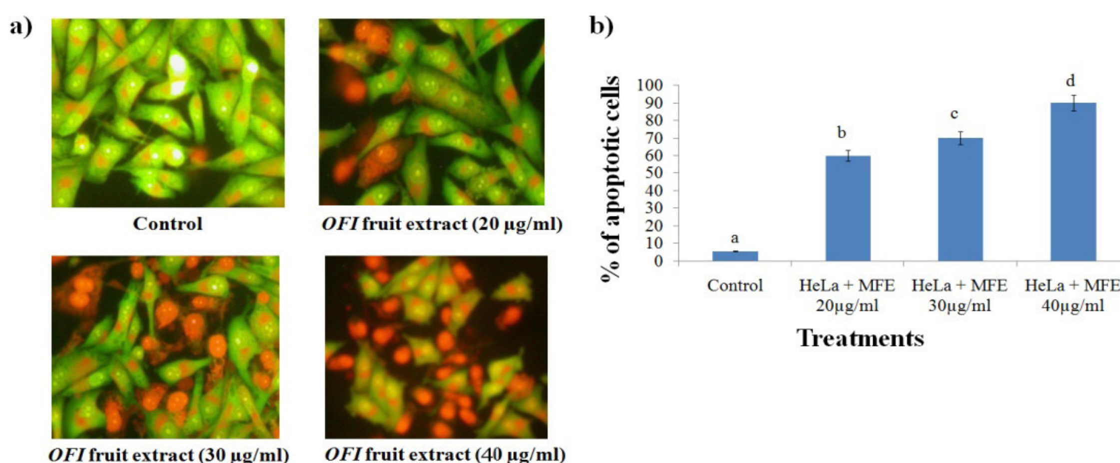
Methanol fruit extract of *Opuntia ficus indica* treatment increased the levels of lipid peroxidation in HeLa cells. Among all the concentrations (20, 30, and 40 µg/ml) tested, 40 µg/ml of methanol fruit extract of *Opuntia ficus indica* showed maximum levels of TBARS. Antioxidative effects of *Opuntia* fruit extract on lipid peroxidation inhibition in oils[32]. Activities of enzymatic antioxidants such as SOD, CAT, and GPx were depicted in Fig.3 (a-b). Methanol fruit extract of *Opuntia ficus indica* (20, 30, and 40 µg/ml) treatment significantly decreased the activities of SOD, CAT, and GPx in HeLa cells. Among all the doses tested, 40 µg/ml of methanol fruit extract of *Opuntia ficus indica* significantly decreased enzymatic activities when compared with other doses. Many studies suggested that antioxidant enzymes are critical in protecting against tumor-promoting agents. Interestingly, cell malignancy or transformation is often accompanied by a decrease in activity of antioxidant enzymes like SOD, CAT, and GPx, which increases the cell sensitivity to prooxidant compounds[33]. In this study, the authors observed decreased activities of antioxidant enzymes, i.e., SOD, CAT, and GPx in methanol fruit extract of *Opuntia ficus indica* treated cancer cells. Sankaran mirunalini et al[34] observed decreased activities of antioxidant enzymes, SOD, CAT, and GPx in *Pergularia daemia* methanol extract treated cancer cells. In this study, the effect of methanol fruit extract of *Opuntia ficus indica* on reduced glutathione levels in methanol fruit extract of *Opuntia ficus indica* -treated cancer cells was examined. Levels of GSH in control and methanol fruit extract of *Opuntia ficus indica* -treated cells were depicted in Fig. 3a. The treatment of methanol fruit extract of *Opuntia ficus indica* (20, 30 and 40 µg/ml) decreased GSH levels in HeLa cells. Among all the doses tested, 40 µg/ml of methanol fruit extract of *Opuntia ficus indica* significantly decreased GSH levels. They have also noticed prominent decrease of GSH levels in cancer cells treated with methanol fruit extract of *Opuntia ficus indica*. Our studies showed that the extracts induced apoptosis in this model of cancer cell line in a time and concentration-dependent fashion[35]. Previous studies have shown that phytochemicals depleted intracellular antioxidants, thereby induced cancer cell death. GSH, a physiological antioxidant protects cells from oxidative stress-induced apoptosis[36]. Cells are protected from undergoing apoptosis by GSH, an antioxidant and a decrease in intracellular levels of GSH is associated with enhanced susceptibility to apoptosis[37]. Changes in mitochondrial membrane potential in control and methanol fruit extract of *Opuntia ficus indica* -treated cells were depicted in Fig. 4a.

Figure 4
Effect of methanol fruit extract on mitochondrial membrane potential in HeLa cell line.



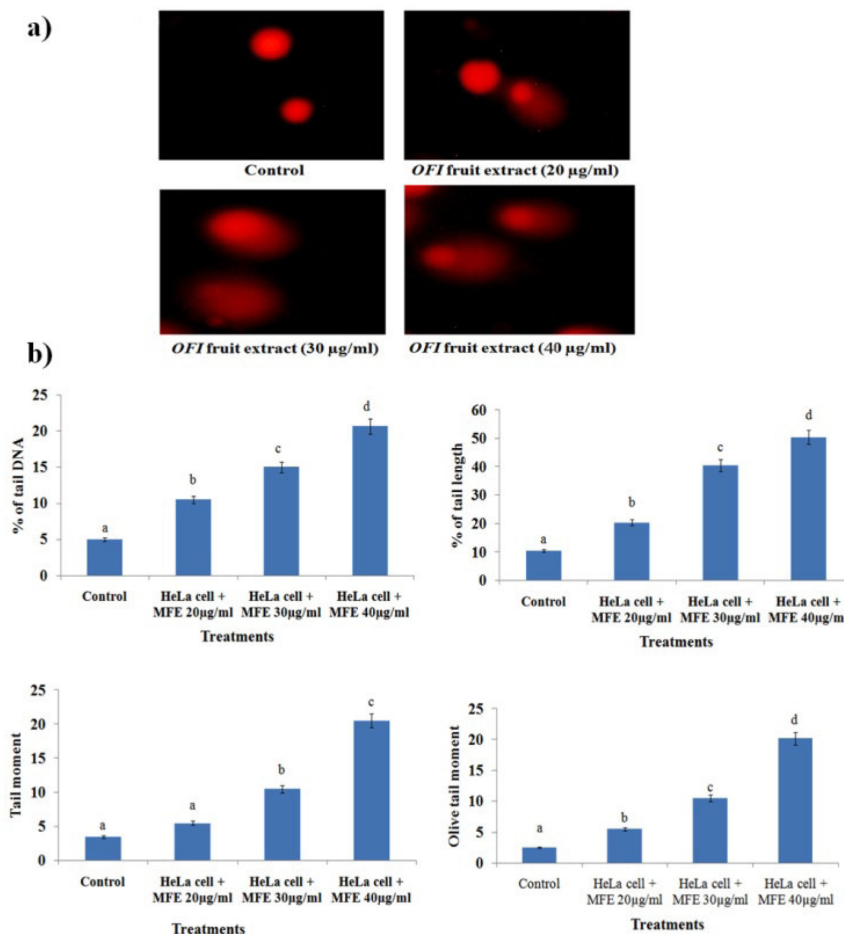
Methanol fruit extract of *Opuntia ficus indica* treatment significantly increased mitochondrial depolarization in HeLa cells. Among all the doses tested, 40 µg/ml of methanol fruit extract of *Opuntia ficus indica* showed high level of mitochondrial depolarization in HeLa cells. We observed changes in the mitochondrial membrane potential in methanol fruit extract of *Opuntia ficus indica* treated and control cells. Mitochondria are one of the most important organelles in regulating cell death as well as a marker in apoptosis[38]. We observed that the mitochondrial membrane potential has been altered during methanol fruit extract of *Opuntia ficus indica* treatment this shows that the mitochondrial membrane potential plays a role in methanol fruit extract of *Opuntia ficus indica* induced cell death. Apoptosis has been shown to play an important role in determining cellular cytotoxicity[39]. Apoptosis has been shown to be a significant mode of cell death after cytotoxic drug treatment[40].Figure 5(a) shows the effect of methanol fruit extract of *Opuntia ficus indica* on apoptotic morphological changes. Figure 5(b) shows the percentage of apoptosis. We observed 85, 72, and 68% apoptotic cells in 40, 30, and 20µg/ml of methanol fruit extract of *Opuntia ficus indica* -treated cells, respectively.

Figure 5
Effect of methanol fruit extract on Apoptotic Morphological changes in HeLa cell line.



We observed that methanol fruit extract of *Opuntia ficus indica* pretreatment significantly increased apoptotic morphological changes in HeLa cells. The increased ROS levels and subsequent oxidative DNA damage might be the reason for the increased apoptotic morphological changes in the methanol fruit extract of *Opuntia ficus indica* -treated cells. DNA is an important molecular target for tumor cell killing[41]. Figure 6 shows the photographs of comet assay.

Figure 6
Effect of methanol fruit extract of Opuntia ficus indica on DNA damage in HeLa cell line.



Control cells showed largely non-fragmented DNA Fig.6a. We observed different grades of DNA damage in methanol fruit extract of *Opuntia ficus indica* -treated cells Fig. 6(a-b). 40 µg/ml of methanol fruit extract of *Opuntia ficus indica* treatment showed highly fragmented DNA Fig.a. Digital images were further analyzed using CASP software that allows quantitative measurements of various comet assay end-points, in particular, the mean average of tail length, percentage of DNA in the tail, olive tail moment, and tail moment Fig.6b. Such endpoints are the most accepted parameters for assessing DNA damage. Methanol fruit extract of *Opuntia ficus indica* (20, 30, and 40 µg/ ml) treatment significantly increased tail length, percentage of DNA tail, olive tail moment, and tail moment in HeLa. Among all the doses tested, 40 µg/ml of methanol fruit extract of *Opuntia ficus indica* showed maximum tail length (60%), DNA tail (40%), olive tail moment (20%), and tail moment (22%). The giloe has been reported to be cytotoxic in HeLa cells and this cytotoxicity effect was due to its ability to induce DNA damage[42]. Previously, phytochemicals have been reported to induce DNA damage in cervical cancer cells by reactive oxygen species generation[43]. This might be the reason for the increased oxidative DNA damage (% tail DNA, % tail length, tail movement and olive tail movement) observed in methanol fruit extract of *Opuntia ficus indica* treated cancer cells. Subhabrata Paul *et al*[44] said that these three traditionally important plants are capable of inducing cell death in the cervical cancer cells and cause a significant amount of DNA damage to them. C33A cell line which is HPV-ve, showed maximum DNA damage, as is evident by the comet assay and also showed DNA laddering in all the extract treated sets, indicates maximum sensitivity to the plant extracts.

CONCLUSION

It can be concluded that methanol fruit extract of *Opuntia ficus indica* inhibits cell proliferation in HeLa cells through ROS dependant mitochondrial mediated apoptosis as evidenced by elevation of ROS generation resulting in loss of mitochondrial membrane potential, oxidative DNA damage. Therefore, methanol fruit extract of *Opuntia ficus indica* could be considered as an anticancer agent.

CONFLICT OF INTEREST

I have no conflict of interest.

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