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Inhibition of Proliferation of K-562 Human Blood Cancer Cell Due To Opuntia Elatior Fruit Extract

Chirag U. Narayankar[1], M. Mane[2], R. Patil[3], A. Magdum M. D. Satpute[4], S. Gaikwad[5] and D. K. Gaikwad[6]

[1, 2, 3, 4, 5, 6]*(Department of Botany, Shivaji University Kolhapur416004, Maharashtra India)*

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Abstract: *The ethanolic extract of Opuntia elatior was studied for anticancer potential against human blood cancer cell line K-562. The cytotoxicity of ethanolic extract was analyzed, using MTT assay and flow cytometric analysis. It was noticed that IC50 value of Opunria elatior curde extract was 74.32 µl/ml showing its cytotoxicity in the human blood cancer cell line by using MTT assay. The flow cytometry evaluation exhibits cell arrested in G2/M and S phases. Cell arrested due to Opuntia elatior extract shows higher than positive control Cisplatin. Thus Opuntia elatior fruit extract might be used as potent source of anticancerous compound against human blood cancer.*

Keywords*: Opuntia elatior, MTT, Flow cytometry, LC-MS/MS*

I. Introduction

Cancer begins to become one of the leading causes of death worldwide, and only substantial steps have been taken in reducing the morbidity and mortality of such a disease (Aziz et al., 2016). According to the American Cancer Society, the overall number of related deaths in 2007 was 7.6 million due to cancer. In 2050, there are expected to be 27 million additional cancer cases and 17.5 million deaths from cancer throughout the globe (Thun et al., 2009). Plants have such a long track record of use in treatment for cancer and it's also notable that more than 60 per cent of the anti- cancer drugs commonly obtained from natural sources.

Opuntia is a broad genus with succulent shrubs, indigenous to new world, but commonly cultivated in the dryer parts of the world because of their unique appearance and attractive flower. According to El-Mostafa et. al., (2014), Opuntia extracts have been used as food and medicinal purposes,and their clinical value has subsequently been shown by in vitro and in vivo scientific studies (Hail, 2005). In addition to food, Opuntia ficus-indica is being used to treat diabetes, whooping cough, prostate problems, dentistry, rheumatism, nosebleed, and central Mexico (El- Mostafa et al., 2014). Hence in the present study it was thought worthwhile to evaluate the anticancerous potential of O. elatior fruits.

II. Material And Methods

For MTT assay - K562 (Leukemia) cell line, RPMI Medium 1640 (Cat No-11875-093), Antibiotic – Antimycotic 100X solution (Thermo fisher Scientific) **-** Cat No-15240062. Using graph Pad Prism Version 5.1 and calculated the IC 50 of compounds.

For Flow cytometry analysis - Cell line – K562, 70 % ethanol (in DI water), Phosphate-buffered saline (PBS), PI/RNase staining solution, BD Biosciences (Catalog no. 550825), Cytomics FC500 Flow cytometer, Beckman Coulter, USA, Analysis software – FlowJo X 10.0.7

For LC-MS/MS – QTOF/6500 series Q-TOF B.05.01 (B5125.3) LC-MS instrument. Precursor ions were selected in Q1 with an isolation width of 2 D and fragmented in the collision cell, applying a slope of collision energies in the range 5–45 eV. nitrogen was used as Collision gas and product ions were identified with a collision RF of 150/400 Vpp, transfer time of 70 ms, pre-pulse storage of 5 ms, pulse frequency of 10 kHz, and spectra rate of 1.5 Hz for collision-induced dissociation (CID) of in-source fragment ions, with the in-source CID energy increased from 0 to 100 V. Accurate mass spectra were acquired in the m/z range of 50–1000 at an acquisition rate of 2 spectra per seconds.

Extraction of plant material: The fruits of Opuntia elatior used in this study were collected from the dryer region of Solapur district. The fruits of Opuntia elatior were extracted by continuous extraction in Soxhlet apparatus for 12 hr., using ethanol (40 - 45 oC boiling range) as a solvent (Horwitz, 1980). The extraction the solvent was evaporated, using rotary evaporator and the powder obtained was dried over anhydrous sodium sulphate and stored -4oC for further analysis. The various concentration of ethanolic extracts were prepared for the further analysis.

MTT assay- The cells were seeded at a density of approximately 5×103cells/well in a 96-well flat-bottom micro plate and maintained at 370C in 95% humidity and 5% CO2 for overnight. Different concentration (400, 200, 100, 50, 25, 12.5µl/mL) of samples was treated. The cells were incubated for another 48 hours. The cells in well were washed twice with phosphate buffer solution, and 20 µL of the MTT staining solution (5mg/ml in phosphate buffer solution) was added to each well and plate was incubated at 370C. After 4h, 100 µL of di- methyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and absorbance was recorded with a 570 nm using micro plate reader (Ghagane et al., 2017 and Bhat et al., 2018).

Surviving cells (%) = Mean OD of test compound /Mean OD of Negative control ×100.

For Flow cytometry analysis – Cells were seeded into 6-well plates and incubated at 37°C for 24 hours. Cells were treated with the IC 50 concentration of the 64 % alcoholic Opuntia elatior fruit extract for 16 hours, trypsinized and taken into 15ml tubes. Cells were washed with 1X DPBS, fixed in chilled 70% Ethanol (- 20oC), washed with 1X DPBS twice, resuspended in 400 µl PI-RNase solution per million cells and taken into 1.5ml tubes. Samples were mixed well and analyzed by Cytomics FC500 Flow cytometer, Beckman Coulter, USA (Pozarowski and Darzynkiewicz, 2004).

LC-MS/MS Analysis: The alcoholic extract Opuntia elatior of fruit was studied with the help of 6200 series TOF/6500 series Q-TOF B.05.01 (B5125.3) LC-MS instrument. Precursor ions were selected in Q1 with an isolation width of 2 D and fragmented in the collision cell, applying a slope of collision energies in the range 5–45 eV. nitrogen is used as Collision gas and product ions were identified with a collision RF of 150/400 Vpp, transfer time of 70 ms, pre-pulse storage of 5 ms, pulse frequency of 10 kHz, and spectra rate of 1.5 Hz for collision-induced dissociation (CID) of in- source fragment ions, with the in-source CID energy increased from 0 to 100 V. Accurate mass spectra were acquired in the m/z range of 50–1000 at an acquisition rate of 2 spectra per seconds. Internal calibration was carried using signals at m/z 121.0509 (protonated purine) and 922.0098 (protonated hexakis (1H,1H,3H-tetrauoropropoxy) phosphazine) in positive mode. Both raw HPLC–QTOFMS (Agilent 6540 UHD QTOF LC-MS) full single MS and MS/MS data, and for data mining based on molecular formulae estimations and fragment patterns was processed with Mass Hunter Workstation software (Qualitative Analysis). Using the algorithm employed for full single MS data, ions with identical elution profiles and related m/z values (representing different isotopes of the same compound) were extracted by molecular features extraction (MFEs).

III. Result

Cell Viability: The in vitro Cytotoxicity of alcoholic fruit extract of O. elatior (DP) was measured using an MTT assay. The 12.5 to 400 µl/ml concentrations of DP for 48 hour were found to be cytotoxic, and cell viability was found 95.381, 72.690, 59.400, 45.786, 20.259, and 15.073 at 12.50, 25.00, 50.00, 100.00, 200.00, and 400.00 µl/ml. Concentration 12.50 µl/ml and lower concentration of DP did not causes any effect on the viability of K-562 cells and the IC 50 value of extract was 74.13 µl/ml. (Fig. 1). Effect in vitro cytotoxicity of alcoholic fruit extract of O. elatior (DP). Were screened against K562 human cancer cell line and viability of tumor cell was confirmed by MTT assay. The alcoholic extract O. elatior on cell viability against K 562 cancer cell line was concentration dependent.

Flow Cytometry analysis: The flow cytometry analysis of untreated, positive control and O. elatior (OP), indicate that percentage of cell in different phase of cell cycle as compared to untreated cell showed inhibition of cell in the G0/G1 phase due to Opuntia elatior fruit extract. It is followed by and increase DNA damage cell Sub G1 population due to the Opuntia elatior extract the proliferating cell population G2/M was decreased significantly. (Table 1 and 2) (Fig.1, 2).

LC-MS/MS analysis-The various bioactive compounds detected by LC-MS/MS analysis mainly exhibits alkaloids, flavonoids, amino acid, plant hormone in fruit extract of Opuntia elatior. (Table. 3) (Fig. 5). The aqueous alcoholic extract were identified among this Sulfabenzamide is sulfonamide group, Arecoline and Chlorpromazine is alkaloid, Kinetin is amino purines group, Leucine is amino acid, Eseroline is pyrroloindole, Rhamentin is flavonoid, Bilirubin is normal catabolite cell pathway.

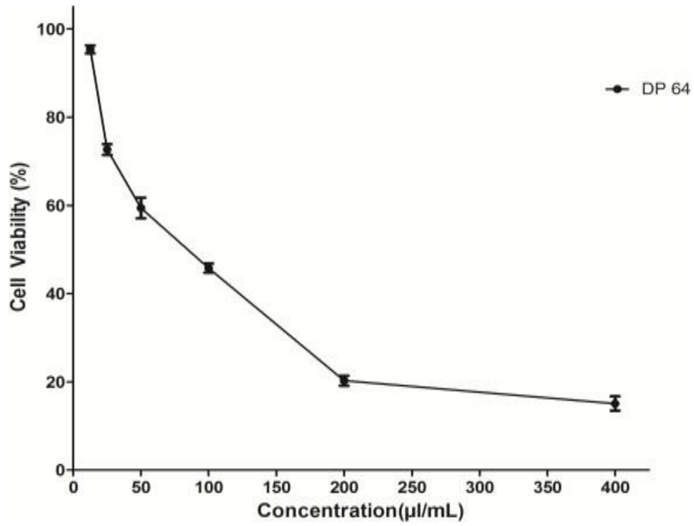
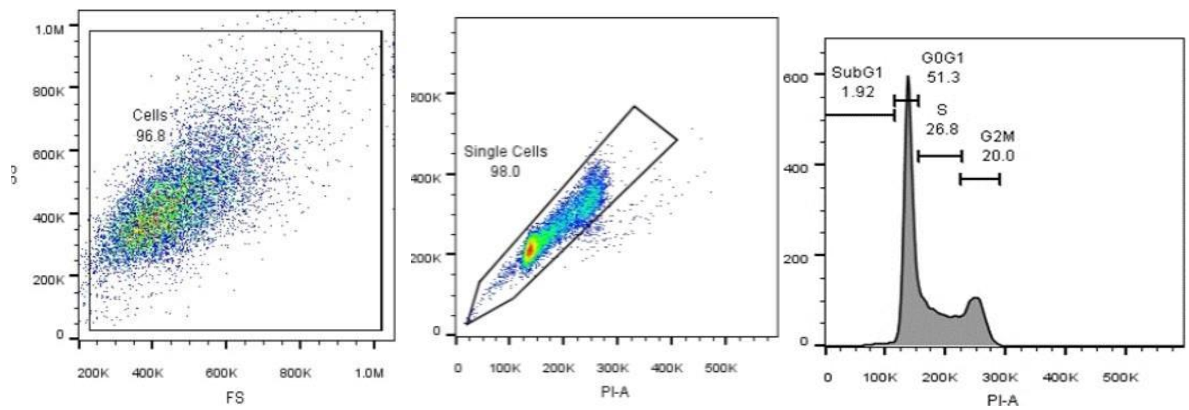
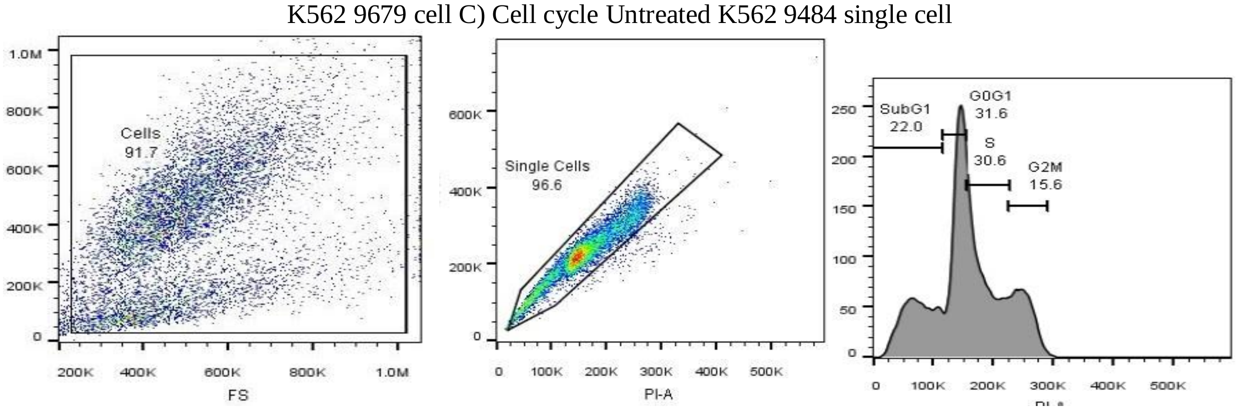


Figure no. 1: Cytotoxicity Assessment by MTT assay in K-562 cells following exposure of various concentrations of Alcoholic fruit extract of Opuntia elatior



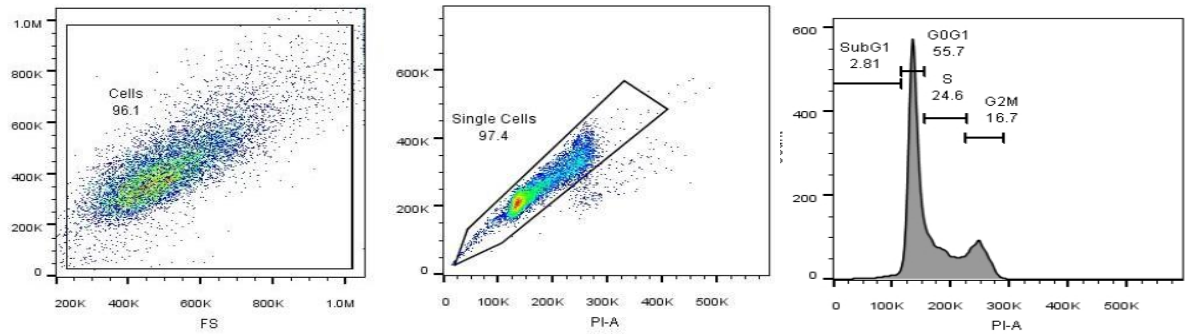
A B C

Figure no. 2: A) Flow cytometric analysis of untreated cell K562 10000 cell (Ungated) B) Cell cycle untreated K562 9679 cell C) Cell cycle Untreated K562 9484 single cell



A B C

Figure no. 3: Flow cytometric analysis of Cisplatin A) Cell cycle Cisplatin K562 10000 cell (Ungated) B) cell cycle Cisplatin K562 9170 cell C) Cell cycle Cisplatin K562 single cell



A B C

Figure no. 4: Flow cytometry analysis of O. elatior fruit extract A) cell cycle sample DP K562 10000 cell (ungated) B) Cell cycle DP K562 9608 cell C) Cell cycle sample DP K562 single cell

Table no.1: Cell arrested in different phases of cell cycle by using Flow cytometric analysis. (Untreated sample, Positive control Cisplatin, DP sample)

Phase of cell cycle	Untreated sample		Positive control Cisplatin		DP Sample	
	Count of cell	Frequency of %	Count of cell	Frequency of %	Count of cell	Frequency of %
Single cell	9484	98.0	8859	96.6	9358	97.4
Sub G1	182	1.92	1953	22.0	263	2.81
GO/G1	4866	51.3	2796	31.6	5215	55.7
S	2566	26.8	2711	30.6	2305	24.6
G2/M	1894	20.0	1380	15.6	1562	16.7

Table no. 2: Effect of Opuntia elatior fruit extraction different phases of cell cycle K562 human blood cancer cell line

Sr.No.	Sample Name	% of cells in different phases of cell cycle			
		SubG1 (Damaged DNA)	G0/G1	S	G2/M
1	Untreated	1.92	51.3	26.8	20.0
2	Positive control (Cisplatin) – 20 μg/mL	22.0	31.6	30.6	15.6
3	Sample DP – 74.13 μL/mL	2.81	55.7	24.6	16.7

LC-MS/MS analysis:

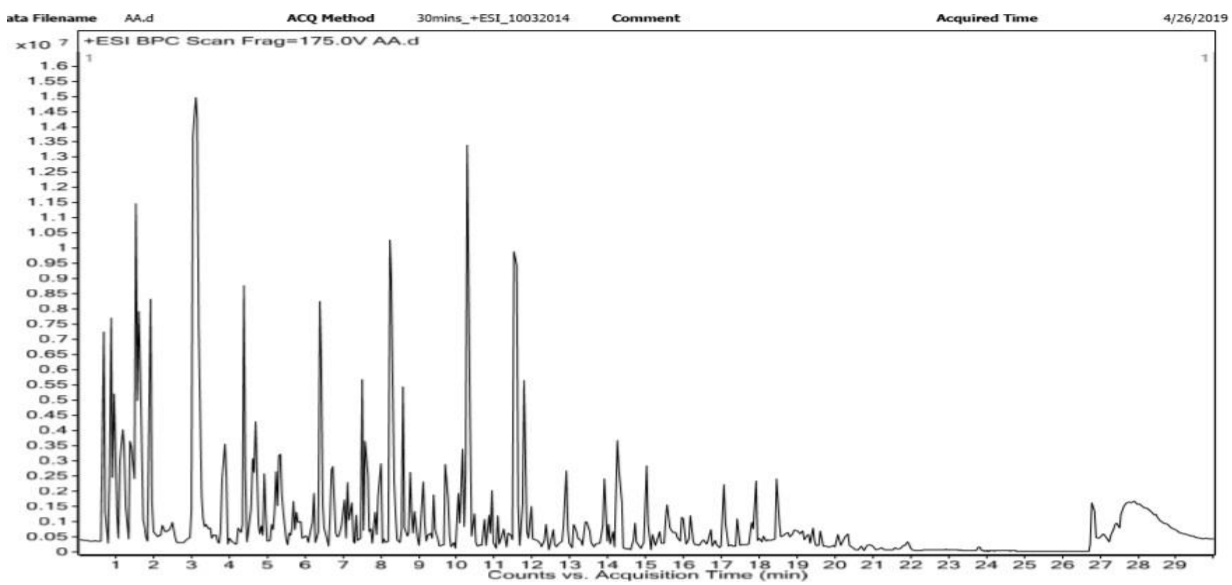


Figure no. 5: LC-MS/MS chromatogram of alcoholic extract of Opuntia elatior

The various bioactive compounds detected by LC-MS/MS analysis mainly exhibits alkaloids, flavonoids, amino acid, plant hormone in fruit extract of Opuntia elatior. (Table. 3) (Fig. 5). The aqueous alcoholic extract were identified among this Sulfabenzamide is sulfonamide group, Arecoline and Chlorpromazine is alkaloid, Kinetin is amino purines group, Leucine is amino acid, Eseroline is pyrroloindole, Rhamentin is flavonoid, Bilirubin is normal catabolite cell pathway .

The compound Sulfabenzamide exhibits colorectal, kidney and cervical cancer inhibitor properties (Gupta et al., 1988, and Lee et al., 2007). Arecoline reported from Areca catechu also found to be inhibiting Breast cancer, blood cancer and oral cancer inhibitor properties (Tsai et al., 2008, and Chen and Chang, 2011, Lin et al., 2015). Kinetin reported from Nicotiana tabacum also found to be inhibiting Breast cancer cell (Mehrzad and Rajabi, 2011). Leucine reported from Craterostigma plantagineum found inhibiting liver, kidney, urinary bladder and breast tumor xenograft Chinese hamster inhibitor properties (Gong et al., 2015, Kato et al., 1979, Zhao et al., 2006, Tobey and Ley, 1971 and Xiao et al., 2016). Chlorpromazine reported from Hyptis martiusii inhibits colorectal, Kidney, cervical cancer inhibitor properties (Lee et al., 2007, and Gupta et al., 1988). Eseroline reported from Desmostachya bipinnata shows, inhibiting Myeloma cancer inhibitor properties (Rickardson et al., 2006). Rhamentin reported from Pisum sativum and Vicia faba found to be inhibiting Ehrlich ascites cancer, breast cancer inhibitor properties (Ertekin et al., 2016, and Lanet al., 2018). Bilirubin reported Strelitzia Nicolai found to be inhibiting Colon cancer, gastric cancer inhibitor properties (Keshavan et al., 2004, and Rao et al., 2006).

Table no. 3: Analysis of bioactive compounds in Opuntia elatior extract by LC-MS/MS

Name	Formula	Mass	RT	Anticancer Properties	Reference
Sulfabenzamide	C13 H12 N2 O3 S	276.0556	0.672	breast cancer cell line T-47D	Mohammadpour et al., 2012.
Arecoline	C8 H13 N O2	155.0957	0.962	Breast cell line p53. Human Leukemia Cell line K 562. Human Oral Cancer Cell line T28	Tsai et al., 2008. Chen and Chang, 2011. Lin et al., 2015
Kinetin	C10 H9 N5 O	215.0788	1.051	Breast cancer cell line MCF-7	Mehrzad and Rajabi, 2011
Leucine	C6 H13 N O2	131.094	1.089	Liver cancer cell line Hep G2 and Kidney cancer cell line A549.human urinary bladder cell line T24. MARY-X human breast tumor xenograft. Chinese hamster cells line CHO (Ovary cancer). Breast cancer cell line MDA-MB-231 and MCF-7	Gong et al., 2013. Kato et al., 1979. Zhao et al., 2006. Tobey and Ley, 1971. Xiao et al., 2016.
Chlorpromazine	C17 H19 Cl N2 S	318.0949	3.668	colorectal cancer cell line HCT116 and Kidney cancer cell line A549. Human cervical cell line HeLa	Lee et al., 2007. Gupta et al., 1988.
Eseroline	C13 H18 N2 O	218.1414	5.524	Myeloma cell line RPMI 8226	Rickardson et al., 2006.
Rhamentin	C16 H12 O7	316.0577	5.915	Ehrlich's ascites carcinoma cell line (EAC). Breast cancer cell line MCF-7	Ertekin et al., 2016 Lan et al., 2018.
Bilirubin	C33 H36 N4 O6	584.2634	20.104	Colon cell lines HCT15, HCT116, SW480 and Lo Vo. Human gastric cancer cell line TMK-1 cell line	Keshavan et al., 2004. Rao et al., 2006.

IV. Discussion

The development of resistance to the standard chemotherapeutic anticancer drug is a major problem which is developed due to the active efflux anticancer drug ATP binding site (ABC) superior family of drug transporter (Baguley, 2010). The various mechanism for the cell death caused by cytotoxicity compound are different on the basis of cancer cell line. The Cisplatin mostly induces apoptosis the number of cancer cell (Siddik, 2003 and Watanabe et al., 2008). While Lim et al., (2010) indicated that Cisplatin induced necrosis HepG2 cell could be switched over to apoptosis in response to Ursodeoxycholic acid. The various compounds were identified from Opuntia elatior extract which are reported for various bioactivites by other researchers showed such as Sulfabenzamide showed anticancerous activity on breast cancer (Mohammadpour et al., 2012). Arecoline was cytotoxic effect on Breast, Leukemia, Oral cancer (Tsai et al., 2008, Chen and Chang, 2011 and Lin et al., 2015). Kinetin showed anticancerous activity on breast cancer (Mehrzad and Rajabi, 2011). Leucine displayed anticancerous activity on liver, kidney, human urinary bladder, ovary, breast cancer (Gong et al., 2015, Kato et al., 1979, Zhao et al., 2006, Tobey and Ley, 1971 and Xiao et al., 2016.), Chlorpromazine against colorectal, kidney, cervical cancer (Lee et al., 2007 and Gupta et al., 1988), Eseroline showed anticancer activity on Myeloma cell line (Rickardson et al., 2006), Rhamentin exhibited against on Ehrlich's ascites carcinoma, Breast cancer (Ertekin et al., 2016 and Lan et al., 2018). Bilirubin against Colon, gastric cancer (Keshavan et al., 2004 and Rao et al., 2006). Also the role of arecoline in peripheral blood mononuclear cell human kertinocyes in response to regulation inflammatory process has been reported by several researcher (Hsu et al., 2001 and Jeng et al., 2003). This alkaloid shows immunosapration by inducing apoptosis of lymphocytes (Selvan and Rao, 1993). The studies of Chan and Chang, (2011) indicate that the tumor necrosis factor (TNF) are TNF2 play an important role in arecoline death cell and concluded that the reduction in proliferation of arecoline treated cell was mediated through TNRF2 pathway as a molecular mechanism responsible for arecoline induced immuno separation. In the present study the alcoholic fruit extract O. elatior exhibits anticancerous cytotoxic activity On K-562 human leukemia cell line. However further studies on the mechanism of action of DP extracts risk and benefits on the plant based bioactive molecule is under process.

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