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Research Article

Pectin and Isolated Betalains from *Opuntia dillenii* (Ker-Gawl) Haw. Fruit Exerts Antiproliferative Activity by DNA Damage Induced Apoptosis

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ABSTRACT

In India, nearly three million patients are suffering from Cancer. There is an alarming increase in new cancer cases and every year ~ 4.5 million people die from cancer in the world. In recent years there is a trend to adopt botanical therapy that uses many different plant constituents as medicine. One plant may be able to address many problems simultaneously by stimulating the immune system to help fight off cancer cells. There appears to be exceptional and growing public enthusiasm for botanical or "herbal" medicines, especially amongst cancer patients. In present study, we studied the *in vitro* anticancer properties of various fractions of cactus *Opuntia dillenii* (Ker-Gawl) Haw.employing *Erlich* ascites carcinoma (EAC) cell lines. The EAC cells when treated with fractions of *O. dillenii* showed apoptosis that was further confirmed by fluorescent and confocal microscopy. In addition, Cellular DNA content was determined by Flow cytometric analysis, wherein pigment treated cells exhibited 78.88 % apoptosis while pulp and pectin treated cells showed 39 and 38% apoptosis respectively. Tunnel assay was carried out to detect extensive DNA degradation in late stages of apoptosis. Apoptosis was observed in all fractions; with pigment having very good activity. The data obtained suggests that pigment from *O. dillenii* fruit may be a promising agent for chemoprevention and further studies with other cell lines and animal models would help in obtaining a new drug for cancer treatment.

Keywords: Apoptosis, Anti-cancer, Betalains, Chemo-prevention

INTRODUCTION

In India, the total cancer cases are likely to rise to 1,148,757 by 2020 (Takiar *et al*., 2010).Chemo preventive agents' development is slow and inefficient while natural products are more effective and less toxic, and are required to reach the objective of cancer prevention. Although hundreds of metabolites have been isolated, only a few new drugs have been approved (Newman *et al*., 2007, Butler, 2005)[5,27]. Medical benefits from plants have been identified for centuries. Also, herbs and natural products have been shown to be lacking in much of the toxicity compared to that is observed in synthetic chemicals, thus, escalating their demand for long term preventive approaches. The development of effective and safe agents for prevention and treatment of cancer remains slow, inefficient and costly (Zou *et al*., 2005)[34]. Several species of cactus pear plants (family - Cactaceae) have become prevalent in semi-arid regions of the world. About 1500 species of cactus belong to genus *Opuntia* and many of them produce edible and highly flavoured fruits. In the light of global desertification and declining water resources, *Opuntia* is gaining importance as an effective food production system including both the vegetative and fruit parts. In addition, ancient people have used *Opuntia* cladode and fruits for their medicinal properties (Cornett, 2000, Knishinsky, 1971; Abou-Elella *et al*., 2014)[1,10,21]. In recent years, there has been a surge in interest among the scientific community with respect to *Opuntia* nutritional and health-promoting benefits. The nutraceutical benefits of *Opuntia* fruits are believed to stem from their antioxidant properties related to ascorbic acid, phenolics including flavonoids and a mixture of yellow betaxanthin and red betacyanin pigments (Jana S *et al*., 2012, Madrigal-Santillán *et al*., 2013)[19,24]. Several *in vitro* studies have shown that phenolic compounds in fruits and vegetables have antiproliferative effect (Percival *et al*., 2006). *Opuntia dillenii* (Ker-Gawl) Haw, commonly identified as pear bush, prickly pear, mal rachette or tuna, is a succulent shrub growing in semi-desert regions in the tropics and subtropics (Ahmed *et al*., 2005)[2]. *O. dillenii* is used in folk medicine or by herbal healers in many countries, for instance in India it is called as "Kanthari" or "Nagphana" (Gupta *et al*., 2002)[16], Canary Islands (Perfumi *et al*., 1996)[29] and mexico (Cornett, 2000, Knishinsky, 1971)[10,21], where it is used in the treatment of diabetes (Perez de Paz., 1988), gastric ulcers,

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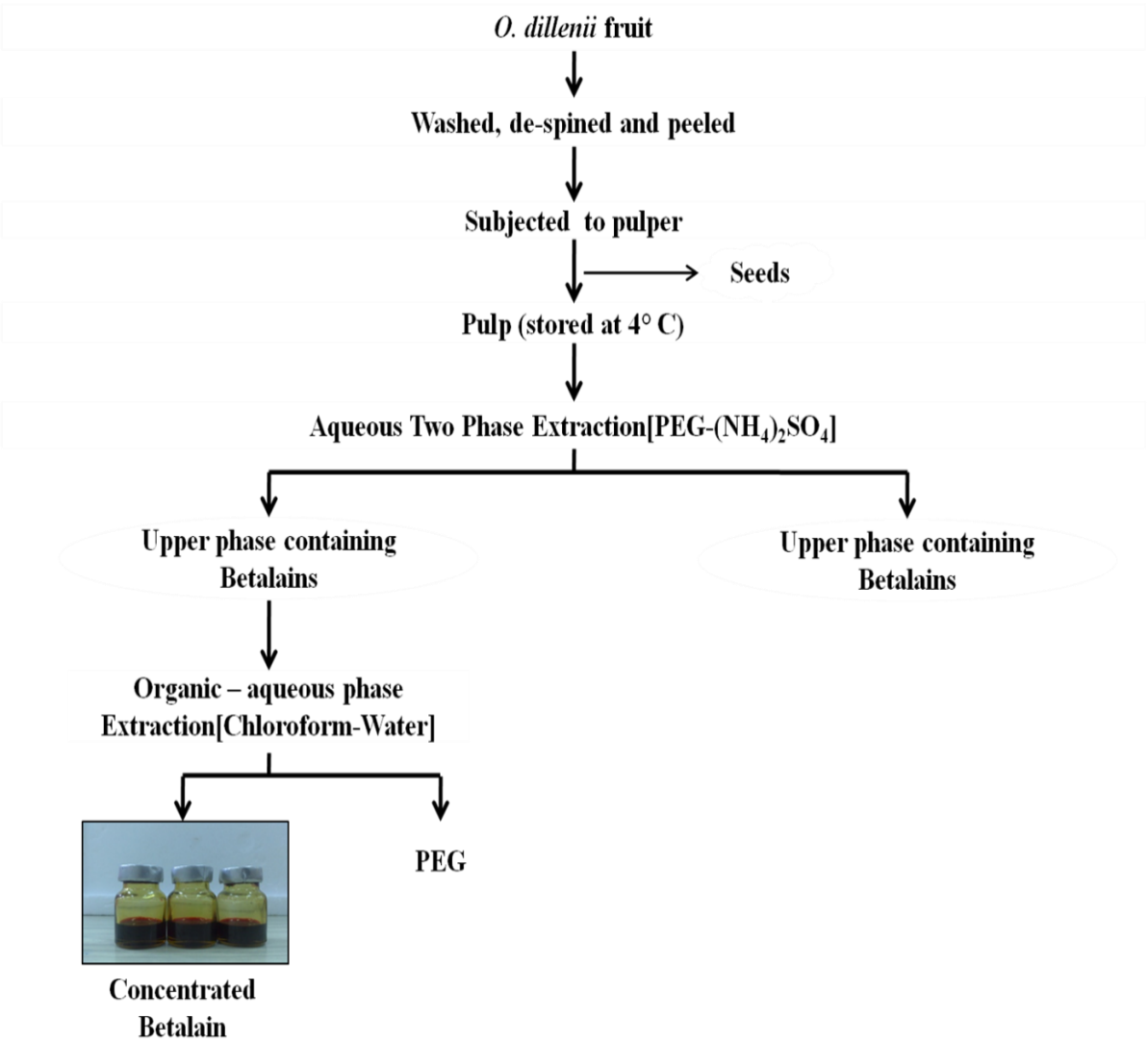


Figure 1: Schematic representation of the steps involved in the isolation of betalain from *O. dillenii* fruit pulp by aqueous two phase extraction method.

inflammation (Parket *et al*., 2001), etc. Fruit of this plant has been reported to have analgesic (Lorodel Rio & Perez-Santana, 1999)[28] and anti-hyperglycemic (Perfumi *et al*., 1996)[29] effects. The prickly pears are reported to be consumed fresh, after desiccation in sun, in marmalades or used as a colouring agent while "nopals" are consumed in Mexican regions as a constituent of salads (Chang *et al*., 2008, Diaz Medina *et al*., 2007)[7,11]. The methanolic extracts of fruit of *O. dillenii* are reported to possess notable antioxidant activity and inhibitory effect on low-density lipoprotein peroxidation (Chang *et al*., 2008)[7]. Different polysaccharides isolated from aqueous extract of *O. dillenii*, is also reported to exhibit potent immunomodulatory activity, inducing production of ROS, nitric oxide and pro-inflammatory cytokines like tumour necrosis factor α (TNF α) and interleukin 6 (IL 6) (Chauhan *et al*., 2010)[8]. The seeds of *O. dillenii* may contribute to higher antioxidant activity because of high concentrations of polyphenols, flavonoids and unsaturated fatty acids (Chang *et al*., 2008)[7]. Besides nutritional and medicinal properties, *Opuntia* have several commercial applications for example betalain, a water-soluble nitrogen-containing pigment found in high concentrations in cactus pear plants (Castellar *et al*., 2003, Diaz *et al*., 2007)[6,11] can be used as natural food colouring agent. Cactus pear extracts have anti-cancer activity; however the active component(s) have not been identified. Cactus pear extracts can be easily used as dietary supplements as it has no toxic effects (Zou *et al*, 2005)[34]. Here we attempt to describe the *in vitro* anti cancer properties of the plant *O. dillenii* on *Erlich* ascites carcinoma (EAC) cell lines.

MATERIALS AND METHODS

Cell culture and cell lines

Erlich ascites carcinoma cell line and NIH3T3 cell line were obtained from NCCS, Pune, India. Cells were cultured in DMEM (M/S Sigma-Aldrich, USA) containing inorganic salts, essential amino acids, vitamins, D-glucose, Pyruvic acid and L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (M/S Sigma-Aldrich, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin at 37ºC in 5% CO_2.

Cactus (O. dillenii) pear

The cactus plant found growing in and around Mandya District (Kunthi hills) about 45 km from Mysore was collected for its fruits. Based on the taxonomic criteria, the cactus was identified to be *O. dillenii* Haw.

O. dillenii pear represents the fruit of cactus which in developmental stages is green in colour, after maturity and ripening, turns violet in colour with spines on surface. The fruit has a thick skin containing pectin protecting violet colour pulp that is sweetish, with embedded seeds.

Extraction of pectin from cactus pear peel

O. dillenii pear fruits, after washing and removal of spines, were cut into halves; pulp was scooped along with seeds. The halved fruit peels were subjected to drying in a hot air oven (53 ± 2°C). The dried peel with residual moisture of ~5 % was then powdered in Apex mill (M/s Cadmach Machinery Co Ltd. Germany). The powdered peel was kept in polypropylene airtight containers till use. The pectin was extracted from defatted peel powder. Defatted peel was then suspended in distilled water to separate mucilage. The residue was subjected to enzymatic degradation to obtain starch and protein free sample and further extracted in acidic medium on hot water bath to obtain pectin. The sample was cooled and filtered using muslin cloth. The pectin was precipitatedby suspending thefiltrate in alcohol (Happi *et al*., 2008)[18]. The galacturonic acid content in the isolated pectin was estimated by MHDP method (Blumenkrantz *et al*., 1973)[3]. To 0.2 ml of the sample, 1.2 ml of sulphuric acid was added. Using crushed ice, the tubes were refrigerated. The mixture was shaken in a vortex mixer and the tubes heated in a water bath at 100°C for 5 min. After cooling in a water-ice bath, with the addition of 20 µl of the m-hydroxy diphenyl reagent, the tubes were shaken and within 5 min absorbance were recorded at 520 nm using Thermo Helios alpha spectrophotometer, Germany.

Extraction of pigment (Betalain) from O. dillenii pear pulp

The fresh, mature, reddish purple fruits (cactus pear) were taken for the extraction of pulp. Fruits were washed in tap water, de-spined manually and peeled. Seeds were separated from the pulp by subjecting peeled fruits to pulper. The obtained pulp was used for extracting the pigment. Pigments (Betalains) were extracted from cactus pear pulp by aqueous two phase extraction method for pigment isolation from beetroot used by Chetana *et al*., 2007 with slight modifications. Briefly, the pre-determined quantities of Polyethylene glycol (PEG 6000) and ammonium sulphate, cactus pear pulp was added and mixed for equilibration. Further, allowed for phase separation for 4-5 h. After the separation of two phases, the pigment rich upper phase was further subjected to aqueous–organic phase of chloroform and water, to remove PEG. The aqueous layer containing pigment was concentrated in flash evaporator at 35±2°C.The isolated pigment was characterized by subjecting to LC-MS (Elena *et al*., 2008)[12].

Cell growth assay

The cells were suspended in a 96-well plate (Corning Sigma-Aldrich, USA) at a density of 2×10^4 cells per well. After 48 h, they were treated with various concentrations of pigment, pectin and pulp separately, for 48 h. Next, the cells were treated with 5 mg/mL MTT in the growth medium for 4 h at 37°C. Cell viability was evaluated by comparison with a control culture (assumed to be 100% viable), measuring the intensity of the blue colour (OD at 590 nm) using a multi-well reader (Varioskan Flash multimode Plate reader, Thermo Scientific, USA).

Effect of O. dillenii fractions on normal cells

To test whether pigment, pectin and pulp induced similar cell death in normal cells, epithelial cell line NIH3T3 was tested. Cells ($5 \text{ X } 10^3$ per well) were seeded into 96-well culture plates in 200µL of the medium at 37°C with 5% CO_2. After 48 h, the supernatant was removed and new media containing various fractions of *O. dillenii* were added separately to the attached cells, incubated for another 48 h. Then the cells were subjected to MTT assay as above.

Morphological observation of cells treated with pectin, mucilage and pigment

The morphological changes of the EAC cells treated with pectin, pulp and pigment were observed with fluorescence and confocal microscopes. For fluorescence microscopy, the cells were suspended in a 96-well plate (Corning Sigma-Aldrich,USA) at a density of 2×10^4 cells per well. After 48 h of growth, they were treated with various concentrations of pigment, pectin and pulp separately, for 48 h. Then the medium was removed, the cells were washed with sterile PBS and then stained with a mixture of Ethidium bromide: acrydine orange at 0.9:1 ratio. The cells were observed under fluorescence microscope for staining after 30 min. For confocal microscopy, poly L-lysine coatedsterile glass cover slips were placed in 6 well plates and cells were seeded on to the coverslip. These cells were treated with pectin, pulp and pigment at4 to 400µM/L. The cover glass nearly full of cell on its surface (by 48h) was taken for staining with mixture of Ethidium bromide:acrydine orange at 0.9:1 ratio.

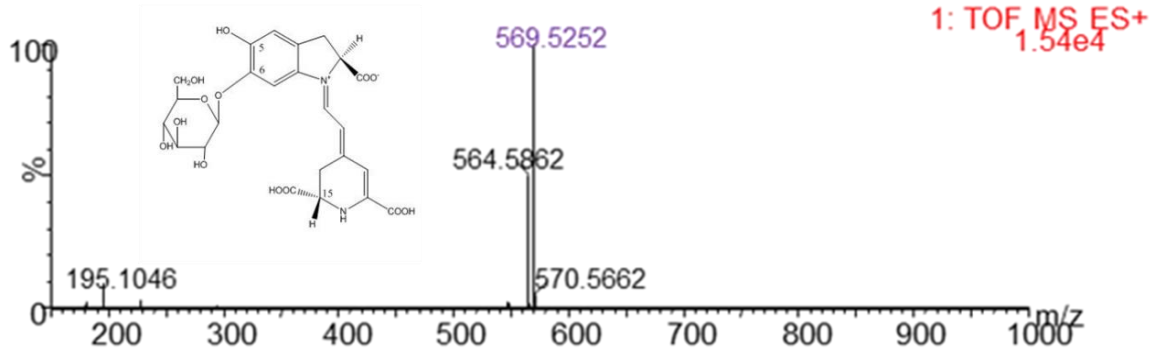
Detection of Apoptosis in Cultured Cells by FACS

Apoptotic cells were detected using FITC-conjugated Annexin-V and propidium iodide (PI) (M/S. SigmaCo., USA). Cells were washed twice with cold PBS and re-suspended in Annexin-V binding buffer (10mM HEPES, 140mM NaCl and 5mM $CaCl_2$) at a concentration of $1X10^6$ cells/mL. Then single suspension of $1X10^6$ EAC cells was prepared in a 5 mL culture tube according to the instructions of the kit in which 5µL Annexin-V-FITC at 10ug/mL and 10µL propidium iodide at 10ug/mL were added. Then the tube was gently vortexed and incubated for 15 min at room temperature in the dark. Binding buffer (400µL) was then added to each tube and the cells were analyzed by flow cytometry (Flow check, Beckman Coulter, USA).

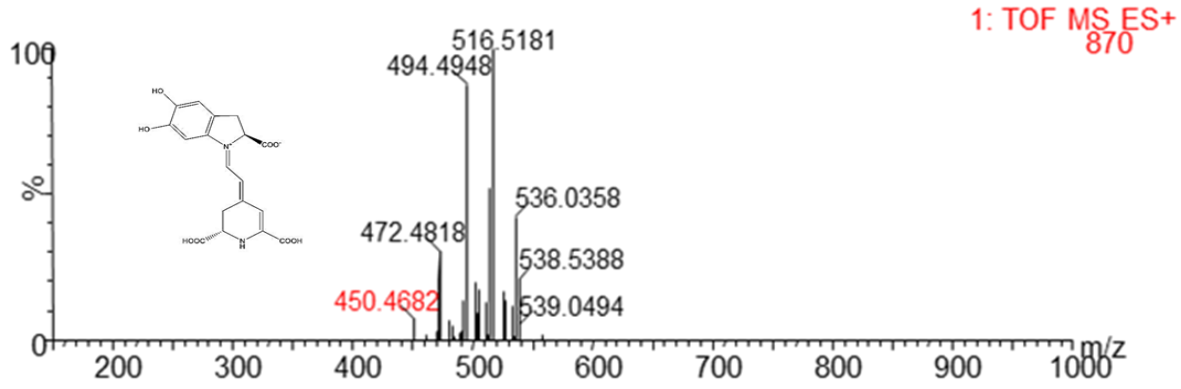
Haemolysis assay

Human whole blood samples (2–3 mL) were centrifuged 1000 X *g* for 10 min and the pellets were washed once with PBS, once with HKR buffer (pH7.4) re-suspended in HKR buffer to 4% erythrocytes, and 50µL was transferred to a 1.5-mL tube with 950µL of pigment, pectin and pulp or 0.1% Triton X-100 in HRK buffer to disrupt the RBC membrane. After 30 min at 37ºC with rotation, tubes were

Betanin (551 + H_2O = 569)



Betacyanin (449)



Betaxanthin (337)

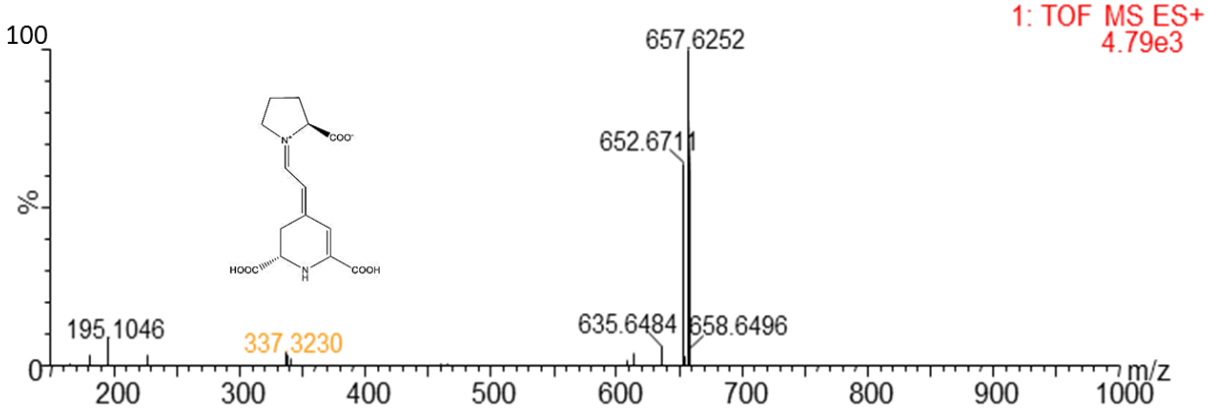


Figure 2: LC MS profile of isolated betalains from cactus pear

centrifuged for 2 min at 1000 X *g*, 300µL of supernatants were transferred to a 96-well plate, and absorbance was recorded at 540 nm.

TUNEL assay

The TUNEL assay was used to detect apoptotic cells. This assay utilizes terminal-deoxynucleotide-transferase (TdT) containing FITC labelled dUTP on the 3'-OH ends of fragmented DNA (TdT-mediated dUTP Nick-End Labeling assay or TUNEL). Cells that incorporatedfluorophore become positive for fragmented DNA would appear as bright green in their nuclei. The Kit instruction was followed (Life Technologies, Bangalore). Briefly, cells were grown to about 50 - 75% confluency on coverslips in culture media. After 48 h of pectin and pigment (0 - 400µM) exposure, the coverslips were removed from the media, washed twice with PBS and the cells were fixed in freshly prepared 2% formalin in PBS at 4°C. The cells were permeabilized in PBS containing 0.2% Triton X-100 for 5 min on ice and washed with PBS. For the coverslips to be tested (by the TUNEL procedure) were placed at room temperature and incubated for 5 min in the equilibration buffer (supplied by the manufacturer). After incubation, this solution was removed and a solution containing equilibration buffer (90µl), nucleotide mix (10µl) and TdT enzyme solution (2µl) was added, incubated in dark at 37ºC for 60 min. The reaction was terminated by washing with 2X SSC followed by a wash in PBS. The coverslips were then incubated with primary antibody (Anti-BrdU mouse monoclonal antibody PRB-1, Alexa Fluor 488 conjugate) for 30 min at room

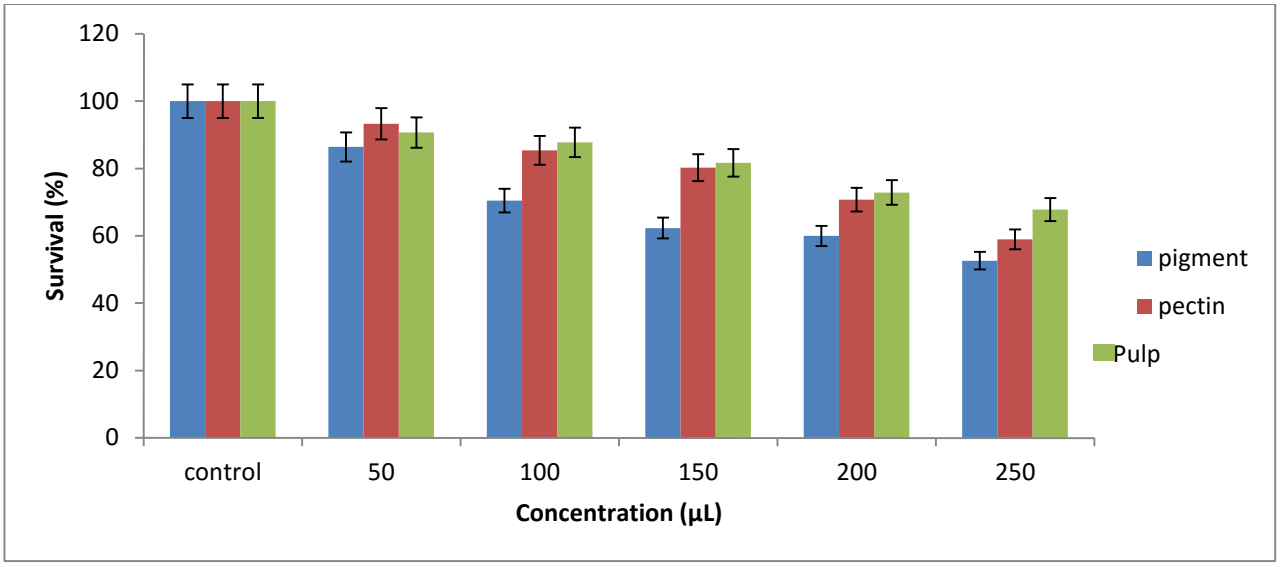
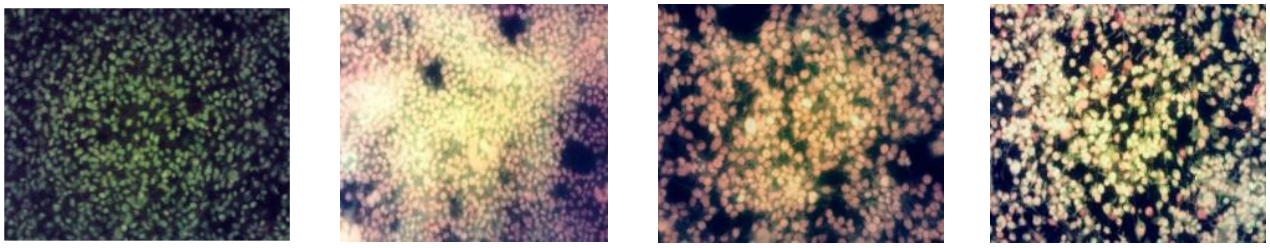
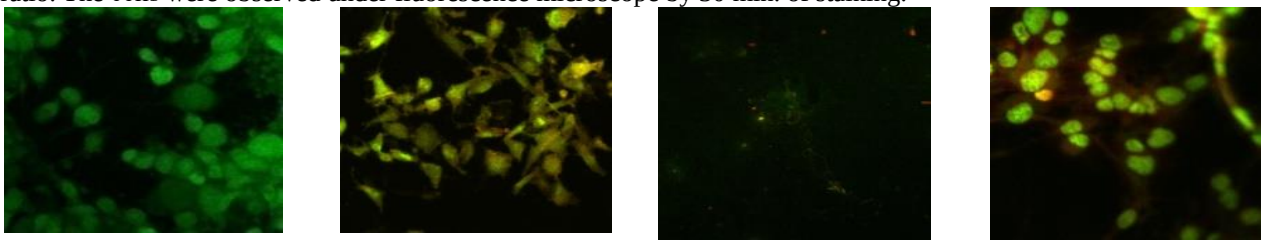


Figure 3: Cytotoxicity of pectin, pulp and betacyanin on growth of *Erlich* Ascites Carcinoma cells
The cells were suspended in a 96-well plate (Corning Sigma-Aldrich) at a density of 2×10^4 cells per well. After 48 h, they were treated with various concentrations of pigment, pectin and pulp separately, for 48 h. Next, the cells were treated with 5 mg/mL MTT in the growth medium for 4 h at 37°C. Cell viability was evaluated by comparison with a control culture (assumed to be 100% viable), measuring the intensity of the blue color (OD at 590 nm) using a multi-well reader (Varioskan Flash multimode Plate reader. Thermo Scientific)



Control Pulp Pigment Pectin

Figure 4: Fluorescence microscopic analysis of effect of pectin, pulp and betalain on EAC cell lines
The cells were suspended in a 96-well plate (Corning Sigma-Aldrich) at a density of 2×10^4 cells per well. After 48 h of growth, they were treated with fractions at various concentrations, separately, for 48 h. Then the medium was removed, the cells were washed with sterile PBS and then stained with a mixture of Ethidium bromide:acrydine orange at 0.9: 1 ratio. The cells were observed under fluorescence microscope by 30 min. of staining.



Control Pulp Pigment Pectin

Figure 5: Confocal microscopic analysis of effect of pectin, pulp and betalain on EAC cell lines
Poly L-lysine coated sterile glass coverslips, were placed in 6 well plates and cells were seeded on to the coverslip. These cells were treated with pectin, pulp and betacyaninat4 to 400µM/L. The cover glass nearly full of cell on its surface (by 48 h) was taken for staining with mixture of Ethidium bromide:acrydine orange at 0.9:1 ratio.

temperature away from sunlight. Then 0.5µL of the propidium iodide/RNaseA staining buffer was added. The cells were incubated for an additional 30 min at room temperature protecting from light during the incubation.
The coverslips were then mounted on glass slides for confocal microscopy.

Statistical Analysis

All measured data were presented as mean ± SD. The differences among groups were analyzed using the one-way ANOVA by SPSS12.0 statistical software. Statistical significance was defined as $P<0.05$.

RESULTS

Isolation and characterization of pectin and pigment

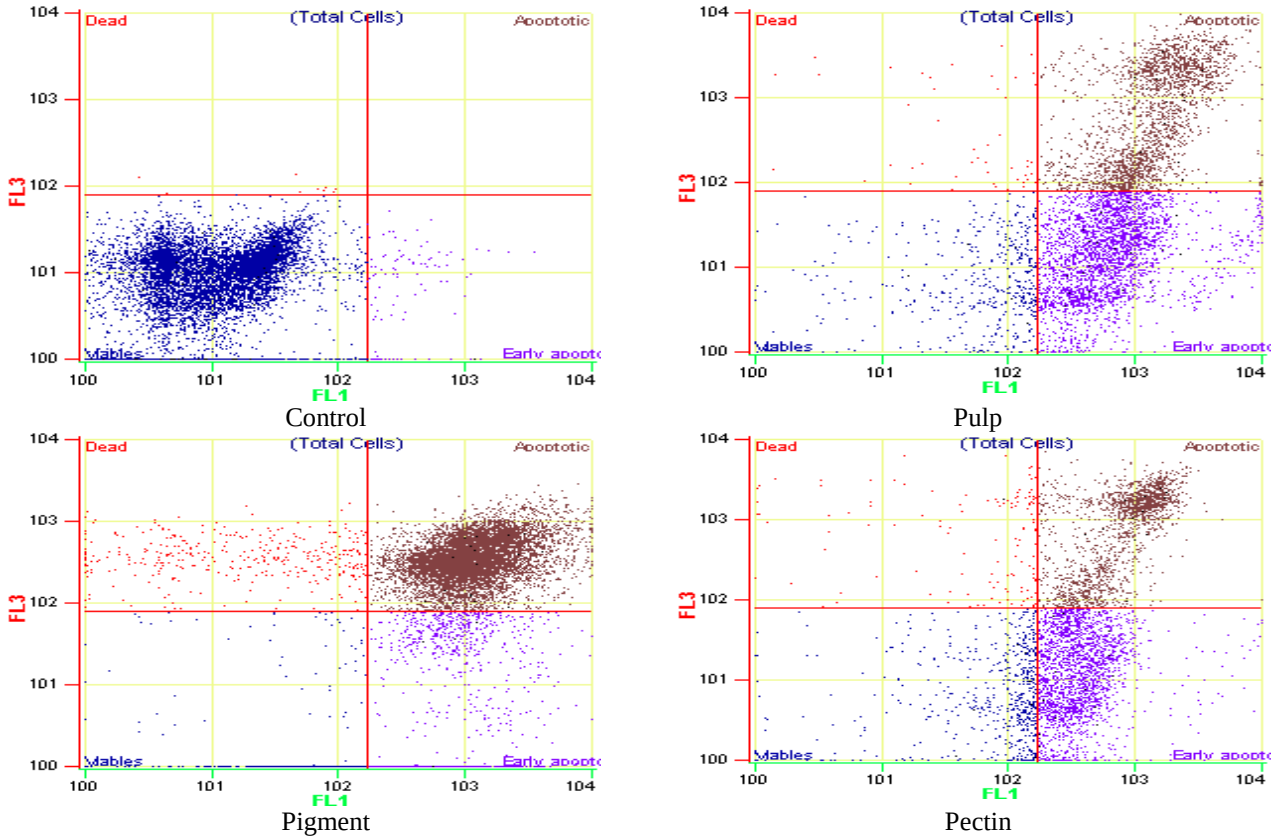


Figure 6: Flow cytometric determination and graphical representation of apoptosis on EAC cells

The yield of pectin isolated from the cactus pear skin was ~17%.The isolated pectin was confirmed by estimating galacturonic acid content by MHDP method .The yield of pectin with 74% galacturonic acid was obtained. The visible absorption spectra of the isolated betalain (Fig 1) was recorded from 240 to 800 nm, where, it showed a major absorption peak at 535 nm indicating the presence of red pigment betacyanin as the major compound in the cactus fruit pulp. LC MS analysis, at absorption maxima at 535nm, clearly indicated that the pigment was betacyanin (Fig 2).

Pectin, pulp and pigment induced cell proliferation inhibition and apoptosis of EAC cells in vitro

We evaluated the cytotoxic activity of fractions of cactus on EAC cell lines by MTT assay. The results indicated that the treatment with pigment, pectin and pulp induced dose–dependent cytotoxicity (Fig 3). Pigment showed more activity in lowering cell viability followed by pectin and pulp. We found significant cytotoxicity at the250µL (275µg) concentration (P<0.05) of betacyanin. However, the cytotoxicity decreased after 48 h (data not shown). The IC_{50} of betacyanin (concentration that induces 50% inhibition of cell growth) was 71.9µg/ml for EAC at 48 h. These data suggest that EAC cells were susceptible to betacyanin. Pectin also showed significant cytotoxicity at the concentration of 2.5 mg.

Characterization of the EAC cell death by pigment, pectin and pulp

To explore whether apoptosis played important role in the cytotoxicity of pigment, pectin and pulp, EAC cells were treated separately with pigment, pectin and pulp for 48 h. As shown in Fig 5, the outcome of the Annexin V/PI detection showed that betacyanin increased the percentage of apoptosis cells in EAC cells (P<0.05) while the induction of apoptosis by pectin and pulp was low. Flow cytometric results re-confirmed the results of fluorescent (Fig 4) and confocal microscopy (Fig 6). The percent of apoptosis recorded was 78.88,39 and 38% in pigment, pulp and pectin treated cells respectively (Table 1). The pigment, betacyanin of the cactus *O. dillenii* was better in inducing apoptosis of EAC cells than the pectin and pulp.

Effect of pigment, pectin and pulp on normal cells

The pigment, pectin and pulp when tested on normal cell line NIH3T3, marginal death of cells was observed. In the case of pulp treatment, the death of cells was more compared to pectin and pigment (Fig 7). This indicated that the fractions of O. dillenii taken for study were not toxic on normal cells.

TUNEL assay

Terminal deoxynucleotidyltransferase (TdT) dUTP Nick-End Labeling (TUNEL) assay has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis (Kyrylkova et al, 2012)[22]. DNA breaks induced by pectin and pigment of O. dillenii exposed a large number of 3´-hydroxyl ends. These hydroxyl groups can then serve as starting points for terminal deoxynucleotidyltransferase (TdT), which adds deoxyribonucleotides in a template-independent fashion. Addition of the deoxythymidine analog 5-bromo-2´-deoxyuridine 5´-triphosphate (BrdUTP) to the TdT reaction serves to label the break sites. Once incorporated into the DNA, BrdU can be detected by an anti-BrdU antibody using standard immune histochemical techniques. The Alexa Fluor® 488 dye produces

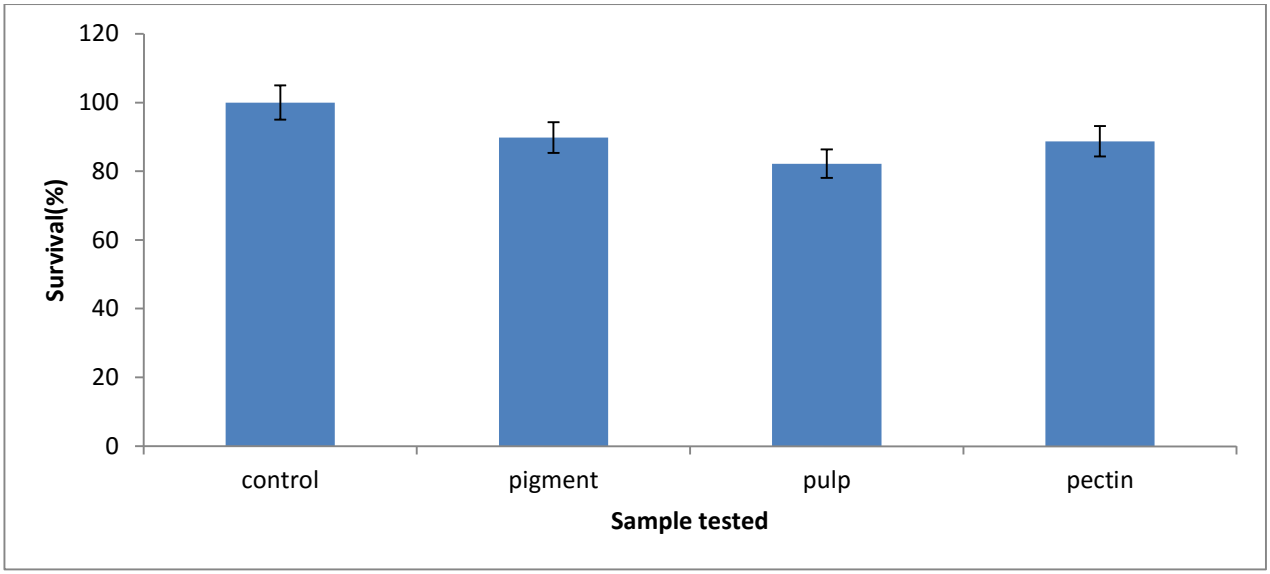


Figure 7: Effect of *O. dillenii* fractions on normal cells

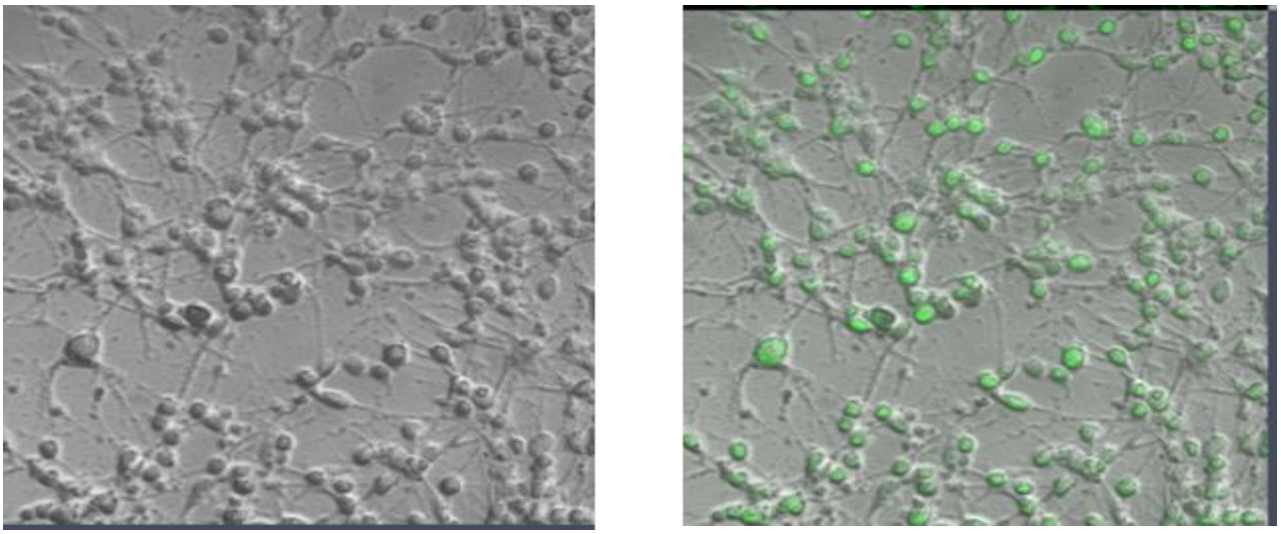
fluorescent conjugates that are brighter and more photos stable. Propidium iodide is included to determine the total cellular DNA content. TUNEL assay confirmed apoptosis due to DNA damage (Fig 8).

Haemolytic assay

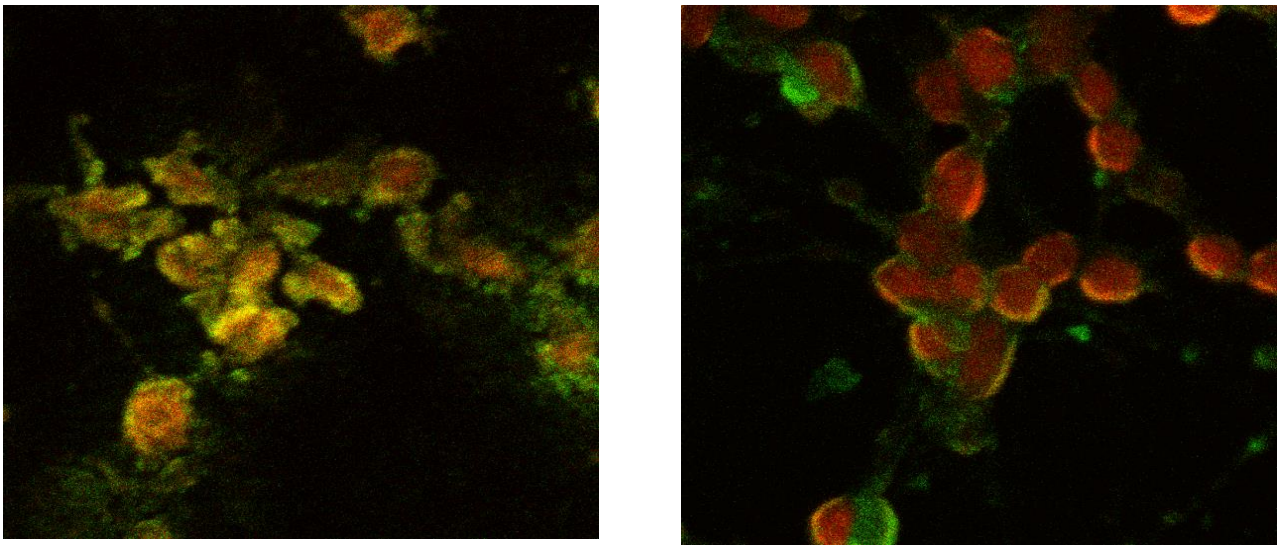
The % haemolysis for control and samples are represented in Fig 9. The results indicated that all samples exhibited less than 5% hemolytic activity and did not affect the morphology of the erythrocytes and had no cytotoxic effects.

DISCUSSION

Increased consumption of fruit and vegetables is associated with prevention of various diseases and the oxidative damage, which is an important etiologic risk factor for cancer and heart diseases (Zou et al., 2005)[34]. The plant families of the order Caryophyllales, including the Cactaceae synthesises betalains, the characteristic red-violet and yellow, nitrogen-containing pigment, by thephenylpropanoid metabolic pathway (Mabry *et al*., 1980)[23]. Betalains are the most characteristic substances of *O. dillenii*, along with α-pyrones, e.g., opuntiol and some derivatives (Qiu *et al*., 2002, 2003, 2007), and several phytosterols, one among them is the opuntisterol (C29- 5β-sterol) and its glucoside, opuntisteroside (Jiang *et al*., 2006)[20]. *O. dillenii* fruit pulp, an unusual source of taurine (244 mg/L) (data not shown), a non standard amino acid of plant origin; known to have several health benefits like bile acid conjugation (Mizushima *et al*., 1996)[25], detoxification (Nakashima *et al*., 1982)[26], membrane stabilization, osmo-regulation, and modulation of cellular calcium levels (Birdsall, 1998)[4]. *O. dillenii* cladode and fruits are palatable, attractively coloured with edible seeds, which are known tohave many health-promoting components (Cornett, 2000, Knishinsky, 1971)[10,21].In a recent study, Gupta (2012)[17] has isolated Betanin from Cactaceae family, which is the key anti-cancer agent against human chronic myeloid cancer cell line, and also inhibits cervical and bladder cancer. The juicy and purple flesh encloses edible seeds, and both constituents contain low molecular health-promoting substances in relatively high amounts besides fibre (Medina *et al*., 2007). The fruits of *Opuntia* species (prickly pears) rich in betanin and isobetanin are considered a better source of food colorants than the presently used red beets (*Beta vulgaris* L.) (Stintzing *et al*., 2001, Moreno *et al*., 2008)[32]. Therefore, the pulp, red pigment and pectin occurring in *O. dillenii* isolated in this study were checked for their pro-apoptotic activities. The pigments which is mainly composed of betalain (betacyanin and betaxanthin) (Fig 1 and 2) and pectin induced apoptosis in EAC cell lines in a concentration dependent manner with pigment having maximum activity which was confirmed with Annexin-V-FITC flow cytometric analysis (Fig 6). Sreekanth *et al*., (2007)[31] have also demonstrated that betalains have apoptotic activity against human chronic myeloid leukemia cell line (K562) in a concentration dependent manner while, Feugang *et al*., (2010)[13] have reported inhibitory effects of aqueous extract of cactus pear on cancer cell growth via accumulation of intracellular ROS, which may activate a series of reactions resulting in apoptosis. Hence to know the trigger for apoptosis, TUNEL assay was carried out, which revealed that the apoptosis is due to DNA damage (Fig 8). For pigment to be used as a chemo-preventive agent it should not have any effect on the normal cells and hence it was subjected for haemolytic assay where all the three fractions of *O. dillenii* did not show any harmful effects (Fig 9). “Let food be your medicine”- as rightly said by Hippocrates 2,500 years ago, cancer chemoprevention with strategies using foods and medicinal herbs has been regarded as one of the most perceptible fields for cancer control (Sreekanth *et al*., 2007)[31]. The cactus pear extracts tested in this study could be such a candidate in cancer prevention for both normal and high-risk populations and prevention of recurrence of cancers. Due to the fact that cactus is a wasteland crop and does not require agronomic care; it makes a prime candidate as a chemopreventive herbal therapeutic. Moreover, the safety of plant/ food-derived products, it holds promise for long-term use.



Control EAC cells



Pectin treated EAC cells

Pigment treated EAC cells

Figure 8: Induction of apoptosis analysed by TUNEL assay

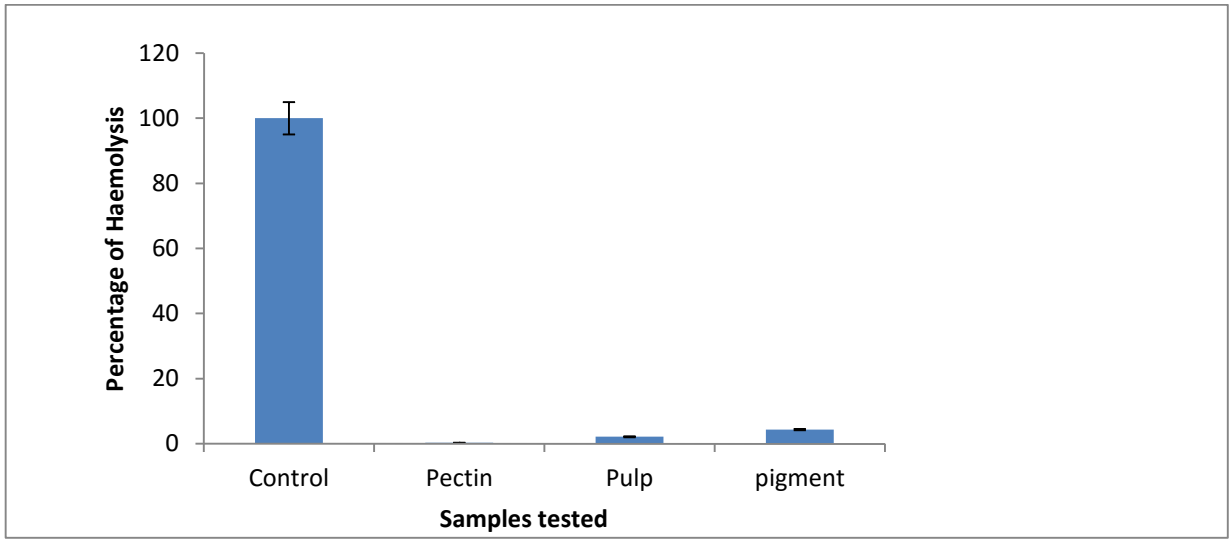


Figure 9: Haemolytic assay of *O. dillenii* fractions

ACKNOWLEDGEMENTS

Table 1: Flow cytometry of pectin, pulp and betalain (pigment) treated EAC cells

Region	Control	Pulp	Pigment	Pectin
Dead	0.09%	0.90%	3.90%	2.98%
Apoptic	0.00%	39.49%	78.88%	38.42%
Viables	98.92%	8.66%	4.59%	13.25%
Early apoptic	0.99%	50.95%	12.53%	45.35%

The authors are grateful to Director, CSIR-CFTRI, Mysuru. This study was funded by Department of Biotechnology, Govt. of India, New Delhi.

CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this manuscript.

Abbrevations

MTT -3-(4, 5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS-Phosphate buffer saline; FACS-Fluorescence-activated cell sorting; HKR-HEPES–Krebs–Ringer; FITC -Fluorescein isothiocyanate; SSC- saline-sodium citrate; MHDP- m-hydroxydiphenyl

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