



# Extracts of *Portulaca oleracea* L. growing in Kashmir Valley exert apoptosis mediated antiproliferative effects and inhibit migration and invasion of oral cancer cells

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Received: 10 May 2021 / Revised: 07 September 2021/ Accepted: 08 September 2021

**ABSTRACT:** The main aim of this research was to investigate the antiproliferative effects of *P. oleracea* L. extracts against oral cancer cells and the underlying mechanism of action. The effects of extracts on SCC-9 cell proliferation and colony generation were monitored by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and clonogenic assay, respectively. Morphological features were studied by phase-contrast microscopy. Apoptotic studies were carried out through AO/EB (Acridine Orange/Ethidium Bromide) staining and western blotting assay. Cell migration and invasion abilities of SCC-9 cells were studied by transwell assay. The results indicated that aqueous, ethanolic and hexane extracts all showed significant ( $P < 0.05$ ) proliferation inhibition against SCC-9 cells. However, a significant  $IC_{50}$  value was determined in case of ethanolic extract that is 52  $\mu\text{g/ml}$ . The number of colonies reduced remarkably post extract treatment. Treated cells showed disturbed morphological features pointing towards apoptotic cell death. It was observed that ethanolic extract caused nuclear disintegration and membrane damage indicated apoptotic cell death, which was further supported by western blotting revealing increased expression of Bax and decreased Bcl-2 expression in treated SCC-9 cells. Furthermore, ethanolic extract significantly blocked the potency of SCC-9 cell to migrate and invade. In conclusion, the results showed that *P. oleracea* L. possess strong antiproliferative effects against oral cancer cells SCC-9 mediated via apoptosis induction. Moreover, migration and invasion of SCC-9 cells was also inhibited significantly. Therefore, our research could prove beneficial in oral cancer research and treatment and also could help in finding lead drug candidates from *P. oleracea*.

**KEYWORDS:** Oral cancer; *Portulaca oleracea*; apoptosis; proliferation; cell migration; cell invasion.

## 1. INTRODUCTION

Plants, the most diverse living things on the surface of the earth, have assisted human beings in achieving different livelihood demands since time immemorial [1]. Plants have been a rich resource for food, shelter, medicine, and types of equipment. Medicinal plants are a group of diverse plant species utilized in the treatment of several human disorders. Medicinal plants have endowed human beings with several bioactive and pharmacologically important phytochemicals that are currently a part of modern day pharmaceuticals and some are in later-stage clinical trials. More than 70% of chemopreventives that have recently entered clinical trials for cancer therapy belong to natural products or designed on them [2].

*Portulaca oleracea* L. (Figure 1) is a very important medicinal plant growing across the globe [3]. This plant belongs to the family of Portulacaceae and is commonly called as “Ma-Chi-Xian” in China and “purslane” in English [4]. Kashmir Valley harbours a huge diversity of medicinal plants reported with remarkable contribution in pharmaceutical industry [5]. *Portulaca oleracea* L. is known as “Nunnar” in Kashmir Valley and is widely distributed as a weed in agricultural crops mainly apple fields. The indigenous people of Kashmir Valley cook aerial parts of the plant and consume it as a vegetable with rice. *Portulaca oleracea* is salty taste. The plant is considered as very healthy to eat and an immune booster in action. It is a warm-climate, herbaceous succulent annual plant with a cosmopolitan distribution. The plant is an active constituent of various traditional medicine system prescriptions like Ayurveda, Traditional Chinese Medicine and other folk medicines used locally in India especially in Kashmir Valley [6]. *Portulaca oleracea* has been identified with a huge diversity of chemical constituents responsible for its high medicinal

**How to cite this article:** Khursheed A, Jain V. Extracts of *Portulaca oleracea* L. growing in Kashmir Valley exert apoptosis mediated anticancer effects and inhibit migration and invasion of oral cancer cells. J Res Pharm. 2022; 26(1): 154-162.

importance including terpenoids, fatty acids, polysaccharides, flavonoids, alkaloids, minerals, sterols, vitamins and proteins [7-9]. The broad spectrum of pharmacological efficacies of this plant are already known including antiulcerogenic, anti-inflammatory, antimicrobial, neuroprotective, antidiabetic as well as strong anticancer activity [9-11]. However, molecular mechanism for its anticancer effects is yet to be fully explored.

Oral cancer is a malignant health disorder associated with huge mortality rates worldwide. In terms of occurrence, oral cancer ranks as sixth most frequent cancer across the globe [12]. India is one of the leading countries contributing to the global cancer burden. It is estimated that India contributes one-third to the overall global oral cancer burden [13]. India ranks second in the world in terms of occurrence and mortality associated to oral cancer. Of different subtypes, oral squamous cell carcinoma is the most dominant form of oral cancer often detectable in a pre-clinical phase [14]. The most important risk factors contributing to the development of oral cancer are tobacco abuse via chewing, smoke and smokeless tobacco, unhygienic oral cavity, alcohol abuse and long term exposure to viral infections [15]. Dearth of knowledge, disparities in environmental exposure, and behavioral risk factors indicate extensive deviations in the global occurrence and upsurges in the mortality rate associated with oral cancer.

Herein, the current research was designed to evaluate the antiproliferative effects of *P. oleracea* extracts against the oral cancer cells keeping in view the lethality and increasing oral cancer burden. Further, we tried to evaluate the mechanism of action underlying the antiproliferative effects of the extract and also investigated its effects on cell migration and cell invasion of SCC-9 cells.



Figure 1. The plant *Portulaca oleracea*.

## 2. RESULTS AND DISCUSSION

Cancer is a global health issue without any potential treatment strategy which could completely cure this lethal malignancy. Unfortunately, the oral cancer incidences are up surging at an alarming pace due to high tobacco and alcohol consumption, worldwide. Despite recent advances made in the field of oncology and cancer management strategies, the overall survival rates remain very low. The development of efficient therapeutics and management strategies are the need of the hour for eliminating oral carcinoma. Natural products have always assisted us human beings in achieving our pharmaceutical or livelihood goals [16]. The current research investigation was undertaken to evaluate the antiproliferative effects of different *P. oleracea* extracts against oral cancer SCC-9 cells.

### 2.1. Proliferation inhibition of SCC-9 cells by different *P. oleracea* extracts

Plants have a tremendous potential to manufacture phytochemicals having broad spectrum of biological applications [17]. The extracts isolated from medicinal plants have shown promising medicinal effects and have remained a pool of different bioactive phytochemicals responsible for these medicinal activities. Plant extracts have been extensively studied for their antiproliferative property against a wide range of human cancers [18]. *P. oleracea* is a medicinally important and phytochemically rich species of medicinal plants. Different solvent extracts of this plant have been tested against cancer cells like lung, liver, and breast cancer cells, but no study involved SCC-9 cells with mechanism studies.

Herein, we for the first time investigated the antiproliferative effects of aqueous, ethanolic and hexane extracts of *P. oleracea* against SCC-9 cells by performing MTT assay. The SCC-9 cells were exposed to changing concentrations of aqueous, ethanolic and hexane extracts viz 0, 40, 80, 160 and 320  $\mu\text{g/ml}$  for 48h, followed by the cell proliferation assessment. All the extracts showed promising antiproliferative effects but the strongest activity was revealed by ethanolic extract followed by aqueous and hexane extracts, respectively ( $P < 0.05$ ). Ethanolic extract treated cells showed significantly ( $P < 0.05$ ) decrease in proliferation in a concentration-reliant manner and at higher extract concentration (160  $\mu\text{g/ml}$ ) the viable cells reduced to 10% (Figure 2).

The  $\text{IC}_{50}$  value for ethanolic extract was determined and comes out to be 52  $\mu\text{g/ml}$ . The aqueous and hexane extracts showed a high  $\text{IC}_{50}$  values of 140  $\mu\text{g/ml}$  and 223  $\mu\text{g/ml}$ , respectively. Additionally, the ethanolic extract exposure of SCC-9 cells showed tremendous potential and efficacies to inhibit colony generation in the target cells (Figure 3). Therefore, it was concluded that ethanolic extract not only inhibited the proliferation but also inhibited colonies formation by SCC-9 cells.

The aqueous and hexane extracts showed antiproliferative effects but strongest  $\text{IC}_{50}$  value was noticed for ethanolic extract. Hence, further mechanism studies were carried out using ethanolic extract treatment against SCC-9 cells.

## 2.2. Morphological modifications and apoptotic cell death induced by ethanolic extract in SCC-9 cells

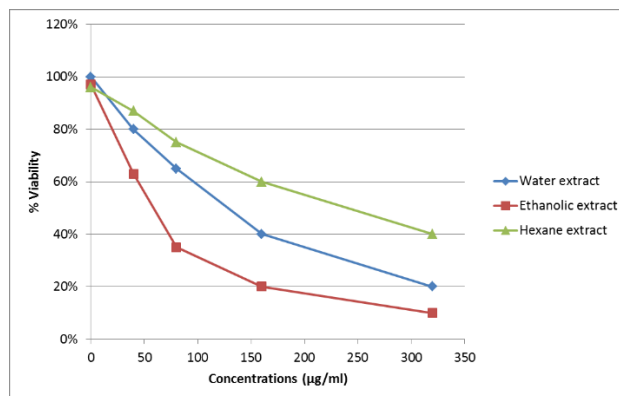
In multicellular organisms like humans, apoptosis is a natural process that regulates the normal homeostasis of cells, tissues, and organs [19]. The normal homeostasis is maintained by the elimination or disintegration of injured, malfunctioning, damaged and cancer cells. Apoptosis occurs naturally in a programmed manner, therefore it is often referred as type-I programmed cell death. Other naturally reserved death mechanisms include autophagy and necrosis [20]. The over activation of apoptosis results in tissue/cell damage, while its suppression is responsible for pathogenesis of several diseases including cancer. Therefore, a balance between cell death and living is pivotal to maintain normal cell/tissue health. The cancer cells often show decreased apoptosis, making them a prime target for therapies that activate apoptosis [21].

Medicinal plants have been found rich in phytochemical constituents that trigger apoptosis in cancer cells [20]. Earlier studies have demonstrated significant anticancer effects for *P. oleracea*, but no study has reported antiproliferative effects via apoptosis in SCC-9 cells. Herein, ethanolic extract of *P. oleracea* treated SCC-9 cells were analysed for morphological features pre and post treatment using phase-contrast-inverted microscopy. Results showed normal cellular morphology in pre-treatment cells while apparent and significant alterations were seen in the morphology and structure of treated SCC-9 cells like cell flouting, appearance of reduced density and round detached cells (Figure 4). These results suggest that SCC-9 cells undergo apoptosis related cell death after extract treatment. Apoptosis was investigated in SCC-9 cells by using acridine orange/ethidium bromide (AO/EB) staining. The pretreatment group showed normal green fluorescence indicative of regular cell morphology and no apoptotic cell population was seen. Compared to the pretreatment (control) group, the treatment group showed red fluorescent cells, which indicated apoptotic cells, and this red fluorescence increased with increasing plant ethanol extract concentrations (Figure 5). Therefore, AO/EB staining showed enhanced apoptotic percentage in treatment group indicating that the extract induced antiproliferative effects are mediated via apoptosis. The Western blotting assay further confirmed these results, showing that treated cells expressed higher Bax levels and lower Bcl-2 levels. (Figure 6). The Bax is a proapoptotic protein and Bcl-2 is an apoptosis suppressor protein.

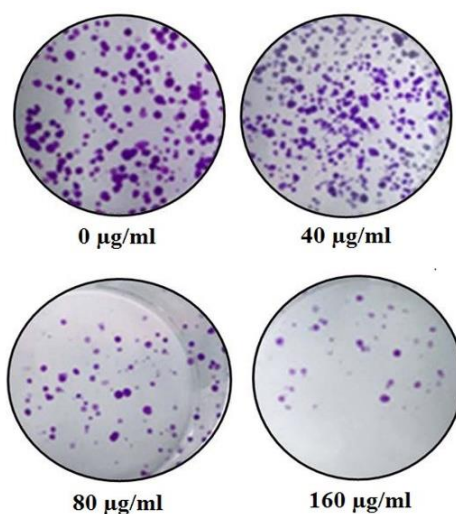
Therefore, the above morphological, fluorescence and molecular studies confirm that the antiproliferative effects of ethanolic extract of *P. oleracea* are mediated via triggering of apoptosis.

## 2.3. *P. oleracea* extract inhibited migration and invasion of SCC-9 cells

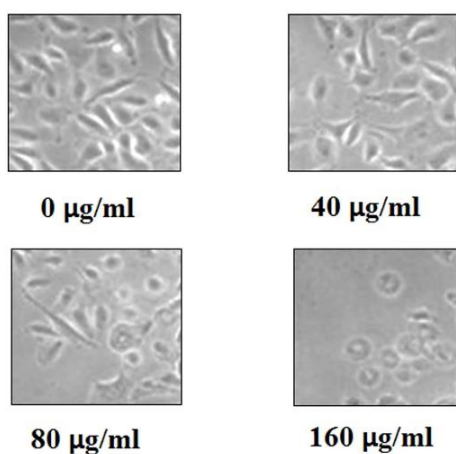
Migration and invasion are the two lethal features of malignant cancer cells contributing to metastatic disease [22]. The cancer metastasis is far more dangerous than non-metastatic as it can migrate and invade the neighboring cells, tissues or even distant regions in the body [23]. Hence, suppression of migration and invasion of metastatic cancer cells results in arresting of their metastatic feature and distant disease spread. Plants have been reported with tremendous potential of manufacturing phytochemicals that have anti-metastatic property against different human cancer cells [24].



**Figure 2.** The results of MTT assay designating the antiproliferative activity of different extracts of *P. oleracea* against the SCC-9 cells. Results depicted that all the extracts show remarkable antiproliferative activity while highest activity was reported for the ethanolic extract. All the extract concentrations were experimented in triplicate replicates and data was revealed as mean  $\pm$  SD.  $P < 0.05$ .

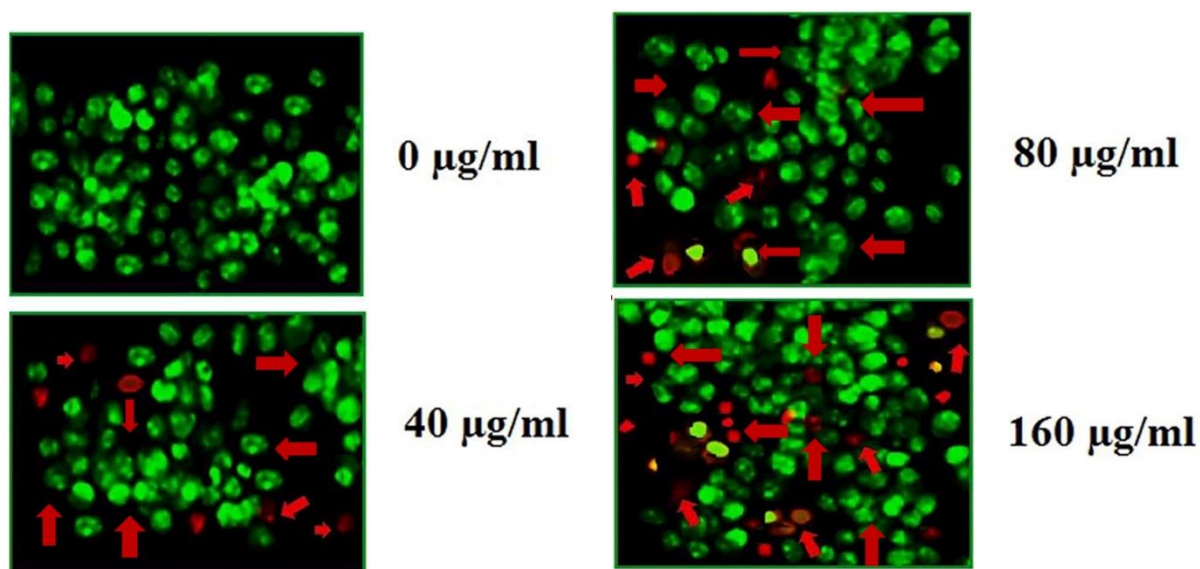


**Figure 3.** Clonogenic assay results showing the SCC-9 cell colonies in treatment and control group of ethanolic extract of *P. oleracea*. The figure clearly indicates reduced number of cell colonies in treatment groups.

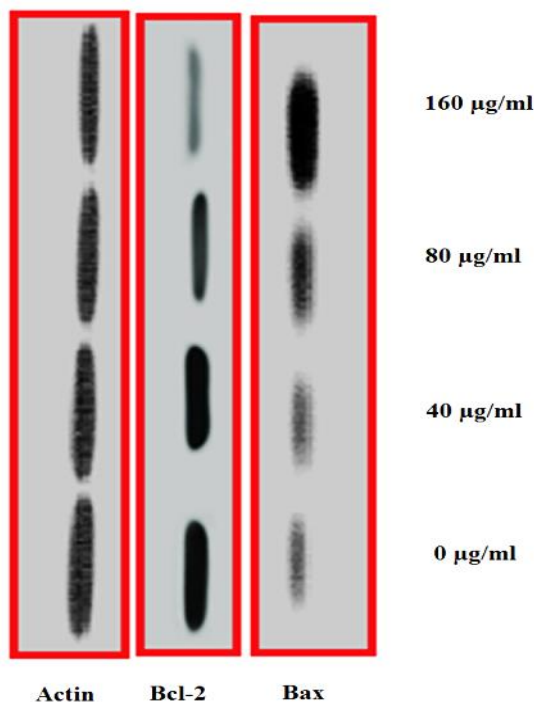


**Figure 4.** Phase contrast inverted microscopy indicating the morphological features of *P. oleracea* extract treated SCC-9 cells and controls. Results showed normal morphology for controls while disturbed morphology showing loss in cell integrity, turbidity and density was seen in treated cells.

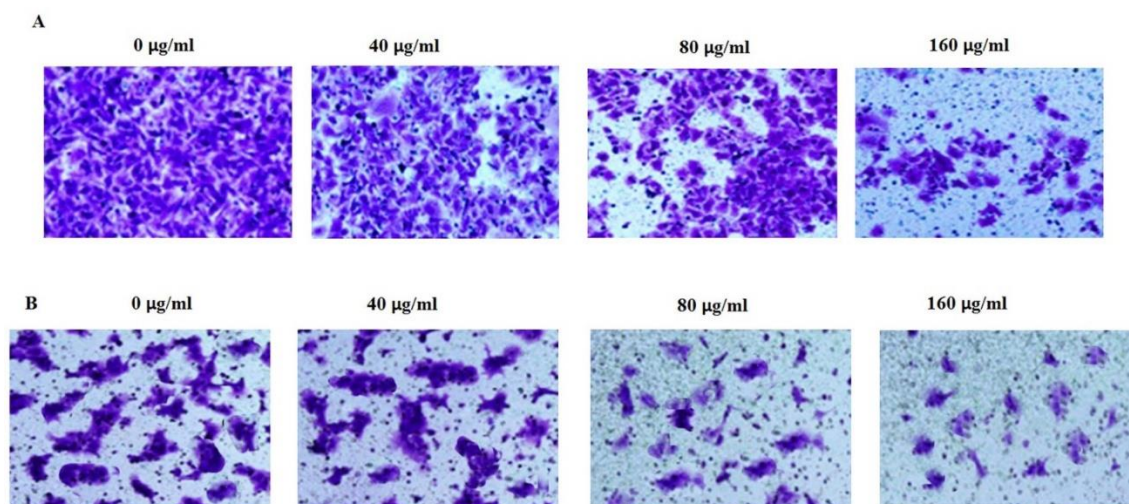
Herein, we evaluated the anti-migration and anti-invasion effects of ethanolic extract of *P. oleracea* against the metastatic oral cancer SCC-9 cells via transwell chambers assay. Results showed that control group showed significantly higher number of migrated and invaded cells. The number of migrated and invaded cells, as indicated in Figure 7A and B, were significantly lower as compared to that of control group.



**Figure 5.** The Acridine Orange/ Ethidium Bromide (AO/EB) staining assay results showing apoptotic cells in treatment and control groups of SCC-9 cells. The arrows point at red-fluorescence indicating apoptotic cells and their number increased with increase in extract concentration as indicated.



**Figure 6.** Western blotting showing the expressions of proapoptotic Bax and antiapoptotic Bcl-2 proteins in SCC-9 cells pre and post extract treatment.



**Figure 7.** Transwell chambers assay. A) Migration in treated and control SCC-9 cells. B) Invasion in SCC-9 cells pre and post *P. oleracea* ethanolic extract exposure at indicated concentrations.

Hence, these results show that the ethanolic extract of *P. oleracea* possess remarkable tendency to inhibit the migration and invasion propensities of SCC-9 oral cancer cells.

### 3. CONCLUSION

In conclusion, taken together the results showed that aqueous, ethanolic and hexane extracts of *Portulaca oleracea* L. possess remarkable antiproliferative activity against the oral cancer SCC-9 cells. Ethanolic extract showed highest inhibition, therefore further mechanism studies were carried out using this extract. Results revealed that ethanolic extract inhibited the proliferation, colony generation and induced significant proapoptotic morphological changes in SCC-9 cells. Ethanolic extract induced proapoptotic antiproliferative effects against SCC-9 cells and also inhabited their potency to migrate and invade. Therefore, the plant *Portulaca oleracea* growing in Kashmir Valley shows remarkable antiproliferative effects against SCC-9 cells. We recommend further phytochemical studies to identify active constituents responsible for strong anticancer activity.

### 4. MATERIALS AND METHODS

#### 4.1. Collection of plant material

The plant material of *P. oleracea* L. was collected from apple orchards of Central Kashmir's Chadoora area (33.9451° N, 74.7966° E, elevation of 1,577 m), a tehsil in district Budgam, Jammu and Kashmir. The plant was authenticated by Prof. Kanchan Yadav (Department of Botany, Madhyanchal Professional University, Bhopal, Madhya Pradesh) and a specimen was submitted in the Department of Botany, Madhyanchal Professional University bearing voucher specimen no. MPU-B-101. The aerial plant parts including flowers, stem and leaves were collected in month of September and cleaned with tap water. The aerial parts of the plant were shade dried for 3 weeks at a temperature of about 29-30 °C. These parts were cut into small pieces manually and stored in airtight plastic bags to avoid exposure till further use.

#### 4.2. Preparation of the extracts

The dried aerial parts of *P. oleracea* were incubated at 50 °C for 1 h, in order to remove all the retained moisture. Then the plant material was grinded thoroughly till a fine powder is obtained. The powder was suspended in the appropriate solvents chosen in the study (20% w/v): water, ethanol (95%), and hexane (70%). For aqueous, ethanolic, and hexane extractions, suspensions were placed under refluxing fitted with magnetic stirrer at 20 °C for 48 h. Aqueous extract was prepared by boiling the water and plant powder mixture for 3 h. All the suspensions were allowed to settle down and the liquid phase was passed through filtration through Whatman filter paper no. 1. The filtrate in each case was allowed to dehydrate at 70 °C with a rotatory evaporator. The final crude extracts were prepared by the mixing each crude extract with dimethyl sulfoxide (DMSO) to obtain a final concentration of 10 mg/mL. These extracts were then stored at 4 °C until further use.

### 4.3. Chemicals, reagents, cell culture and conditions

All the chemicals and reagents associated to this study were of laboratory grade and provided by the Department of Pharmacy, Madhyanchal Professional University, Bhopal, M.P, India. The SCC-9 malignant oral cancer cell line was obtained from ATCC. The SCC-9 cells were preserved and maintained in RPMI-1640 medium containing antibiotics (penicillin and streptomycin) under humid environment within a 5% CO<sub>2</sub> incubator at a temperature of 37 °C.

### 4.4. Proliferation assessment

The effects of extracts (aqueous, ethanolic and hexane) of *P. oleracea* on proliferation of SCC-9 cells were examined using MTT assay. Concisely, SCC-9 cells were plated to 96-well plates maintaining a density of  $1.3 \times 10^3$  cells/well. Then cells were exposed to aqueous, ethanolic and hexane extracts of concentrations viz 0, 40, 80, 160 and 320 µg/ml, for 48 h at 37 °C. Following treatment, SCC-9 cells were washed in PBS followed by addition of MTT solution. After the addition of MTT, the formazan crystals start to appear in the solution due to the reduction by viable cell components. Therefore, the formazan crystals evolved will be directly proportional to the number of proliferating cells post extract treatments. These formazan crystals were finally dissolved in DMSO and optical density was monitored using a microplate reader. IC<sub>50</sub> value was determined by using the concentration at which 50% of proliferation inhibition was witnessed in SCC-9 cells.

### 4.5. Clonogenic assay

To check the effects of ethanolic extract on clonogenic propensity of SCC-9 cells, we performed clonogenic assay. In brief, the 6-well plates were seeded with about 120-140 cells and left untouched overnight on incubation to let them adhere. After the accomplishment of incubation time, cells were exposed to various concentrations of ethanolic extract of *Portulaca oleracea* viz 0, 40, 80 and 160 µg/ml. Post treatment, cells were decanted of medium and replaced with fresh one and placed under incubation with no disturbance, except replacing medium after 4 days, for 12 days. Finally, the cells colonies were stained using crystal violet and pictured and numbered under a light microscope.

### 4.6. Phase contrast microscopy

SCC-9 cells were placed with a density of  $1.5 \times 10^5$  cells/well in 6-well plates and precultured for 24 h. After preculturing, cells were exposed to different concentrations of ethanolic extract viz 0, 40, 80 and 160 µg/ml and left on incubation at 37 °C for 24 h. Afterwards, cells were loaded to a phase contrast inverted microscope (OLYMPUS, Japan) for morphological assessments.

### 4.7. AO/EB staining assay

AO/EB staining assay was executed to verify the proapoptotic effects of ethanolic extract of *P. oleracea*. In brief, cells were cultured with a concentration of  $1.5 \times 10^5$  cells/well of 24-well plates. Each well plate received different concentration of ethanolic extract viz 0, 40, 80 and 160 µg/ml and cultured for 24 h at 37°C in 5% CO<sub>2</sub>. Then cells were washed two times in PBS followed by the addition of 10 µL of acridine orange/ethidium bromide (AO/EB) and left on incubation in dark for 20 min. Finally, the apoptotic features of SCC-9 cells were detected by using fluorescence microscope (OLYMPUS, Tokyo, Japan).

### 4.8. Transwell assay

Transwell chambers assay was executed to monitor the effects of ethanolic extract on migration and invasion of the oral cancer SCC-9 cells. In brief, the upper transwell chambers were placed with 600 µL of RPMI-1640 medium and  $1.5 \times 10^5$  SCC-9 cells. Lower transwells were placed with medium bearing 10% fetal bovine serum. Afterwards, different concentrations of the ethanolic extract were added to the upper transwells viz 0, 40, 80 and 160 µg/ml and left on incubation for 24 h. After the accomplishment of incubation period, the non-migrated SCC-9 cells were cleaned off using a cotton swab while migrated cells subjected to fixation with 4% formalin followed by staining using 0.1% of crystal violet. The staining last for 20 min and then migrated cells were numbered and pictured using microscopy at a magnification of 200X.

### 4.9. Western blotting

The extract treated SCC-9 cells were evaluated for proapoptotic and antiapoptotic protein expressions by western blotting. Treated cells (0-160 µg/ml) were lysed using RIPA buffer and lysates were collected for

protein quantification using BCA assay. Equal amounts of 40 µg proteins were separated on SDS-PAGE electrophoretically at 100 V. These proteins were then blotted to PVDF membranes followed by blocking of the membranes with 5% skimmed milk for half an hour. Post blocking, membranes were subjected to overnight primary antibodies treatment at 4°C using anti-Bax (catalog no. sc-7480; 1:1,000 dilutions) and anti-Bcl-2 (catalog no. sc-7382; 1:1,000 dilutions) (Santa Cruz Biotechnology, Inc., Dallas, United States). Then the primary antibody treatment was followed by secondary antibodies treatment using HRP conjugated goat anti-mouse IgG (catalog no. ab205719; 1:3,000 dilutions; Abcam., Waltham, MA, United States) secondary antibodies for 4 h in absence of light. The primary and secondary antibody treatments were performed to determine the relative protein concentrations. Actin protein served as standard control in western blot study. Finally, the protein signals were detected using ChemiDoc detection system (Bio-Rad, Hercules, CA, United States).

#### 4.10. Statistical analysis

All the individual experimental procedures were repeated in triplicates for each concentration and the data obtained was represented as mean ± SD. The data was analysed by performing one way ANOVA followed by student's t-test using SPSS software version 15.0. The statistically significant figure was taken as P < 0.05.

**Acknowledgements:** Authors are highly thankful to the Department of Pharmacy and Department of Chemistry, Madhyanchal Professional University for providing Lab space and other necessary equipment's and other components involved in this study.

**Author contributions:** Concept - A.K., V.J.; Design - A.K., V.J.; Supervision - V.J.; Resources -A.K.; Materials - A.K; Data Collection and/or Processing - A.K.; Analysis and/or Interpretation - V.J., A.K.; Literature Search - A.K.; Writing - A.K; Critical Reviews - A.K., V.J.

**Conflict of interest statement:** The authors declared no conflict of interest.

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