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ANTICANCER ACTIVITY OF ETHANOL AND POLYPHENOL EXTRACTS OF *PORTULACA QUADRIFIDA* LINN. ON HUMAN COLON CANCER CELL LINES

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ABSTRACT

Portulaca quadrifida Linn. (Portulacaceae) is traditionally used for the treatment against various ailments in tropical and subtropical parts of India without any scientific knowledge. Polyphenols, present in different fruits and vegetables, have retained attention in recent years. In the present study the effects of ethanol and polyphenol extracts of *Portulaca quadrifida* Linn. on the proliferation of Human colon cancer HT-29 and a normal L-6 cell lines were investigated by MTT assay and Trypan blue dye exclusion assay followed by DNA fragmentation assay. Total polyphenol content of both the extracts was determined by FCR method. Both ethanol and Polyphenol extracts efficiently decreased the proliferation of HT-29 cell lines. In addition the polyphenol extract exhibited fragmentation of DNA in HT-29 cell lines efficiently. Our results confirm that both extracts exhibited significant effect against HT-29 cell lines and are found less effective against normal L-6 cell lines indicating the cancer specific effect of *Portulaca quadrifida Linn*.

KEY WORDS: ***Portulaca quadrifida*, anticancer activity, Polyphenols, Trypan blue, DNA fragmentation.**

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INTRODUCTION

Cancer remains one of the most dreaded diseases causing an astonishingly high death rate, second only to cardiac arrest. Around one quarter of deaths in the US are accounted for by Cancer. According to the American Cancer Society, on an average, 559,312 people die of the disease each year despite tremendous efforts to find methods of control and cure. Thus, not surprisingly, every fourth citizen of a developed country will be stricken sometime during his/ her life and approximately 400 new incidents emerge per 100 000 people annually[1]. The statistics released by WHO in 2008 and GLOBOCAN indicate that there is a high likelihood of developing countries approaching the same incident rates of cancer as developed ones, because of life style changes, average age of the population, tobacco usage, etc. The fact that conventional and newly emerging treatment procedures like chemotherapy, catalytic therapy, photodynamic therapy and radiotherapy have not succeeded in reverting the outcome of the disease to any drastic extent, has made researchers investigate alternative treatment options. The extensive repertoire of traditional medicinal knowledge systems from various parts of the world are being re-investigated for their healing properties[2].

In recent years, more interest has been paid to protect foods and human beings against oxidative damage caused by free radicals like hydroxyl, peroxyl, and superoxide radicals. One possible solution is to explore the potential antioxidant and anticancer properties of plant extracts or isolated products of plant origin[3]. There are number of alternative medicine systems based on traditional theories and philosophy that have originated in specific geographical areas and evolved over the years. The most widely practiced of these include the *Unani, Ayurveda and Sidha* and the *Chinese* system of medicine that have originated from the Arab, India and China respectively. These medicinal systems have identified more than 700 individual herbal extracts as well as several drug preparations which claim to treat and/or prevent several diseases including cancer. It is well known that many polyphenol compounds, such as phenolic acids, flavonoids, anthocyanidins and tannins, which possess remarkable antioxidant and anticancer activities, are rich in plant materials. Some studies have shown the positive correlation of the increased dietary intake of natural antioxidants with the reduced coronary heart disease and cancer mortality, as well as with longer life expectancy[4, 5]. Moreover, many polyphenol compounds have shown many health-benefiting bioactive properties, such as antioxidant, anticancer, antiviral, antiinflammatory activities, and an ability to inhibit human platelet aggregation[6]. In addition, efforts have been spent studying the bioavailability of polyphenols in humans. Polyphenols are absorbed and have been detected in animal and human plasma, including phenolic acids, flavanones, quercetin, proanthocyanidins, and anthocyanins[7].

Colorectal cancer is the second leading cause of cancer death in North America and Europe and the fourth most common form of cancer worldwide[8]. Many researchers are focusing on the health benefits of phytochemicals in relation to colon cancer. Higher levels of phenolic compounds have been detected in animal intestine than in other tissues after oral supplementation. Phenolic compounds could inhibit colon cancer development in human intestines before they are absorbed and are detected in the plasma[9, 10].

Portulaca quadrifida Linn. (Portulacaceae) is a small diffused, succulent, annual herb found throughout the tropical parts of India. It is used as a vegetable and also used for various curative purposes. It is said to be useful in asthma, cough, urinary discharges, inflammations and ulcers. A poultice of the plant is applied in abdominal complaints, erysipelas and hemorrhoids[11]. *Portulaca quadrifida* has been reported to possess

antifungal activity against *Aspergillus fumigates* and *Candida albicans*[12]. Our earlier studies report phytochemical composition of *Portulaca quadrifida*[13]. In spite of its rich phytochemical composition and wide traditional use against various ailments the plant has not been studied for its anticancer activity. Therefore, this study was proposed to investigate the anticancer activity of ethanol and polyphenol extracts of *Portulaca quadrifida* Linn. This could be the first documentation of *Portulaca quadrifida* Linn. for its anticancer activity.

MATERIALS AND METHODS

Collection of plant material

Fresh whole plant material of *Portulaca quadrifida* Linn. was collected from the local fields of Gulbarga. The plant specimen was identified and authenticated by Prof. Y.N. Seetaram Department of Botany, Gulbarga University, Gulbarga. A voucher specimen (No. HGUG -906) is preserved in the herbarium of Department of Botany, Gulbarga University, Gulbarga.

Chemicals and reagents

3-(4, 5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Folin-ciocalteu reagent (FCR), Trypan blue, Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco, USA, trypsin was obtained from Sigma Aldrich Co, St Louis, USA. EDTA and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India. All other chemicals used were of analytical grade.

Cell lines and Culture medium

Human colon cancer cell line, HT-29 and L6 cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. It is a suspension culture and stock cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 μg/mL) and amphotericin B (5 μg/mL) in a humidified atmosphere of 5% CO_2 at 37 ºC until confluent. The confluent cell suspension was centrifuged at 2000 rpm for 10 min and the cell pellet was resuspended in fresh medium. The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 micro titer plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of ethanolic extract

The defatted powdered plant material of *Portulaca quadrifida* Linn. was exhaustively extracted with ethanol in a soxhlet apparatus for atleast 10 h. The solvent was removed by evaporation under reduced pressure. The dried semisolid ethanol extract of *Portulaca quadrifida* Linn. (EEPQ) was kept in a refrigerator till further analysis.

Extraction of polyphenols

The extraction of polyphenols was carried out according to the method suggested by Hertog et al[14]. The extraction was carried out with 3% formic acid in methanol. Ground plant material of *Portulaca quadrifida* Linn. (500mg) was accurately weighed. 25 ml of 3% formic acid in methanol was added, and the sample was stirred for 10 min. Clear supernatant was collected after centrifugation (4000 rpm, 10 min). The procedure was repeated for four times with 25 mL of solvent until color was no longer released. Supernatants were combined and taken to dryness using a rotary evaporator at 40 ºC. The remaining phenolic concentrate was redissolved in 5 mL of methanol. Extract was kept in the freezer (-18 ºC) until analysis. A 7 mL portion of acidified (hydrochloric acid; 2 M) 60% aqueous methanol was added to the sample and placed in an oven at 90 ºC for 90 min exactly. Tube was allowed to cool, and supernatant was filtered through a 0.45 μm filter. The polyphenol extract of *Portulaca quadrifida* Linn. (PEPQ) was kept frozen until further analysis.

Determination of total phenol content

Total polyphenol content (TPC) was determined according to the Folin-Ciocalteu procedure[15]. Deionized water (1.8 mL) was added to 0.2 mL of sample appropriately diluted. Folin-Ciocalteu reagent (0.2 mL) was then added, and tubes were shaken vigorously. After 3 min, sodium carbonate solution (0.4 mL of 35% w/v) was added, along with 1.4 mL of deionized water. Reaction mixture was mixed well and left in the dark for 1 h. Absorbance values at 725 nm were converted to gallic acid equivalents (GAE) by using gallic acid as a reference compound.

MTT cytotoxicity assay

Anticancer activity of EEPQ and PEPQ was carried out by MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide; Sisco, India] cytotoxicity assay according to the method described by Zhao et al[16] with few modifications. In brief, the cells were seeded into the wells of a 96-well micro titer plate (Axygen, California) at $2x10^3$ cells per well with 100 µL DMEM growth medium and then incubated for 24 h at 37 ºC under 5% CO_2 in a humidified atmosphere . Later, the medium was removed while fresh growth medium supplemented with varying concentrations of EEPQ or PEPQ was added. After 48 h of incubation at 37 ºC under 5% CO_2, the medium was removed and 20 µL MTT reagent was added. After incubation for 4 h at 37 ºC, the MTT reagent was removed before adding 100 µL DMSO to each well and gently shaken. The absorbance was then determined by ELISA reader (Spetfax, Chennai, India) at 492nm. Control wells received only the media without the tested samples. Inhibition of cell growth by the test extracts was calculated as % anticancer activity as follows,

% anticancer activity = (Ac – As/Ac) x 100, where Ac and As refer to the absorbance of control and the sample, respectively.

Trypan blue dye exclusion assay

The Trypan blue dye exclusion assay was performed as described by Scheck et al[17] with few modifications. The cells were plated in 96-well micro titer plates at $2x10^3$ cells per well with 100 µL DMEM growth medium and incubated for 24 h at 37 ºC under 5% CO_2 in a humidified atmosphere. After 24 h the medium was removed and replaced with fresh medium plus 10% FBS and supplemented with varying concentrations of EEPQ or PEPQ. Cells were harvested 48 h after treatment by digestion with 0.25% trypsin – EDTA solution at 37 ºC for 2-3 minutes. Control wells received only the media without the tested samples. The cells were stained with trypan blue and live cells were enumerated in order to determine % growth inhibition. The experiments were done in triplicates.

DNA fragmentation studies

HT-29 cells (3×10^6 /ml) were seeded into 60 mm Petri dishes and incubated at 37 ºC with 5% CO_2 atmosphere for 24 h. The cells were washed with medium and were treated with PEPQ (210 µg/mL) and incubated at 37 ºC, 5% CO_2 for 24 h As the incubation time ended, the chromosomal DNA of cancer cells was prepared with Roche apoptotic DNA ladder kit. Briefly, cells were harvested and lysed with lysis buffer for 10 min. Then the samples were mixed with isopropanol before passing through the filter and washed. The DNA was eluted from the filter and treated with RNAse at 37 ºC for 30 min before loading onto 1% agarose gel electrophoresis and run at 50 V/cm for 3 h. The gel was visualized under UV transilluminator and photographed.

Statistical analysis

Results were calculated as mean ± S.E.M. where appropriate, one-way ANOVA followed by Post hoc Dunnet's t-test was employed. Values $P<0.05$ were considered significant, using Instat @ Graph Pad Software.

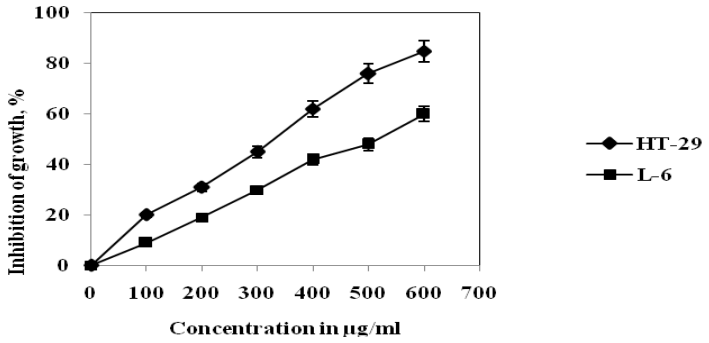
RESULTS AND DISCUSSION

Total phenolic contents of EEPQ and PEPQ were found to be 31.3 ± 2.4 and 49.2 ± 2.6 mg

of GAE/g of the extract. Two main Polyphenols, quercetin and rutin, were identified in the PEPQ by HPLC analysis (Data not shown).

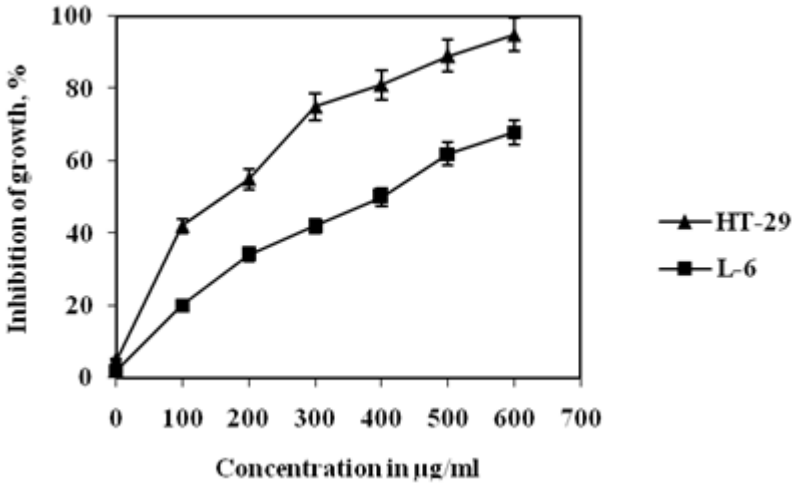
Recently, considerable attention has been focused on identifying naturally occurring substances capable of inhibiting, retarding and preventing cancer. A wide array of phenolic substances, particularly those present in dietary and medicinal plants, have been reported to possess these activities. It has been demonstrated that polyphenols inhibit the proliferation of cultured mammalian cells, including colon carcinoma, hepatoma[18], vascular smooth muscle cells[19], lung carcinoma[20], breast carcinoma[21], mouse epidermal cells[22], melanoma, prostate[23] and leukemic cells[24].

Figure 1
Growth inhibition of human colon cancer cell line HT-29 and normal cell line L-6, by EEPQ in MTT assay.



The results are mean ± SEM from triplicate experiments.

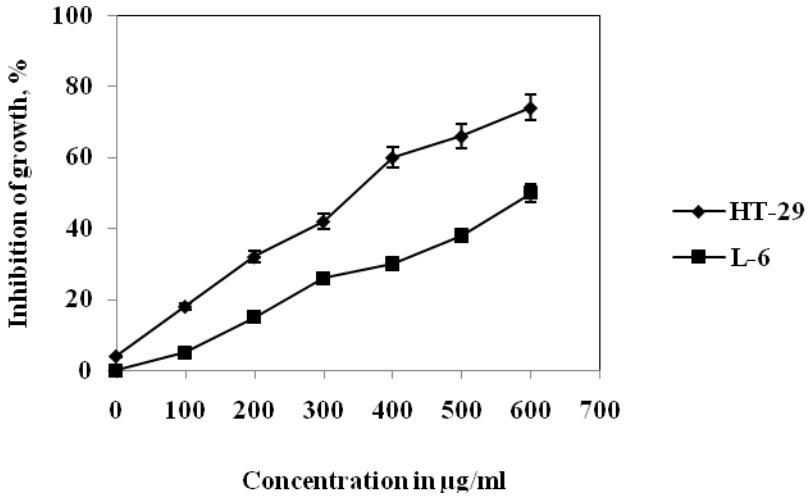
Figure 2
Growth inhibition of human colon cancer cell line HT-29 and normal cell line L-6, By PEPQ in MTT assay.



The results are mean ± SEM from triplicate experiments.

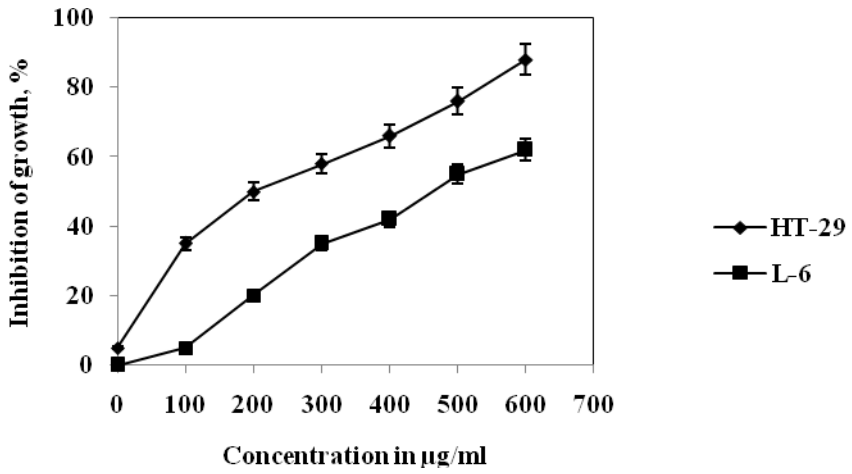
The effect of EEPQ and PEPQ on proliferation of human colon cancer cell lines, HT-29 and normal cell lines, L-6 was investigated using MTT assay. In the MTT assay, a mitochondrial enzyme in living cells, succinate dehydrogenase, cleaves the tetrazolium ring and converts the MTT to an insoluble purple formazan and the amount of formazan produced is directly proportional to the number of viable cells[25]. Figure-1 illustrates the effect of EEPQ on the proliferation of HT-29 and L-6 cell lines and Figure-2 illustrates the effect of PEPQ on the proliferation of HT-29 and L-6 cell lines. The effect of PEPQ on the proliferation of HT-29 cell lines (IC_{50}: 160 ± 1.32 µg/mL) was significant ($p<0.05$) when compared with its effect on normal L-6 cell lines (IC_{50}: 400 ± 1.72 µg/mL). EEPQ also exhibited significant reduction in proliferation of HT-29 cell lines (IC_{50}: 322 ± 1.56 µg/mL) and less effect on proliferation of normal L-6 cell lines (IC_{50}: 515 ± 1.97 µg/mL). However the effect of EEPQ was found less significant when compared with PEPQ on the proliferation of both HT-29 and L-6 cell lines.

The cytotoxicity of EEPQ and PEPQ on HT-29 and L-6 cell lines was further confirmed by Trypan blue dye exclusion assay. The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay was based on the assumption that the dead cells will take the dye and viable cells won't[26]. HT-29 and L-6 cell lines were treated with varying concentrations of EEPQ and PEPQ. Both extracts exhibited significant toxicity against HT-29 cell lines. The IC_{50} values for EEPQ and PEPQ against HT-29 cell line were found to be 340 ± 1.62 µg/mL and 200 ± 1.39 µg/mL (Figure-3 and Figure-4). The effect of both extracts on L-6 cell line was insignificant when compared with HT-29 cell line. The IC_{50} values of EEPQ and PEPQ against L-6 cell lines were found to be 600 ± 1.98 µg/mL and 460 ± 1.76 µg/mL respectively. However the effect of EEPQ was found less significant when compared with PEPQ on the proliferation of both HT-29 and L-6 cell lines.



The results are mean ± SEM from triplicate experiments.

Figure 3
Growth inhibition of human colon cancer cell line HT-29 and normal cell line L-6, by EEPQ in Trypan blue assay.



The results are mean ± SEM from triplicate experiments

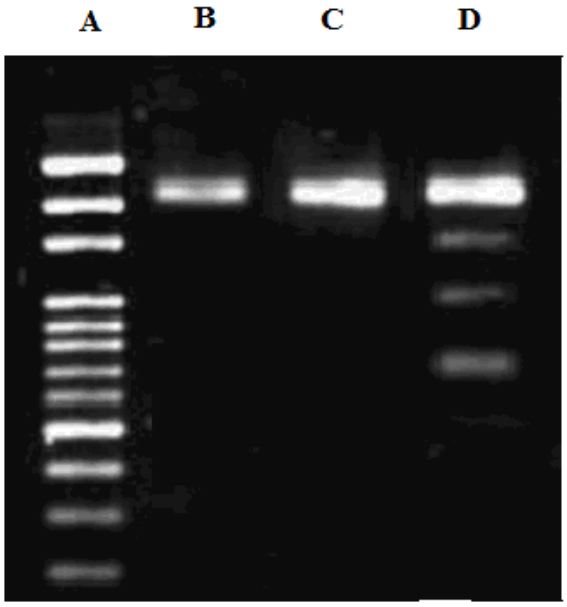
Figure 4
Growth inhibition of human colon cancer cell line HT-29 and normal cell line L-6, by PEPQ in trypan blue assay.

High intake of fruits and vegetables is believed to be beneficial to human health. Fruit, vegetables and some beverages, are particularly rich in dietary polyphenols. Various studies have suggested (but not proven) that dietary polyphenols may protect against cardiovasucalar diseases, neurodegenerative diseases and some forms of cancer. Dietary polyphenols may exert their anticancer effects through several possible mechanisms, such as removal of carcinogenic agents, modulation of cancer cell signaling and antioxidant enzymatic activities, and induction of apoptosis as well as cell cycle arrest[27]. The study was further progressed to investigate the mechanism of anticancer activity of EEPQ and PEPQ. HT-29 cells were treated with respective IC_{50} concentrations of EEPQ and PEPQ to determine whether they induce HT-29 cell death via apoptosis or by necrosis. HT-29 cells treated with PEPQ showed active apoptosis after 24 h of incubation. However, apoptosis was not significant in case of cells treated with EEPQ. Apoptosis was confirmed by DNA fragmentation on 1% agarose gel electrophoresis (Figure -5). The difference in results of EEPQ and PEPQ could be due to their total polyphenol content. These results suggest that PEPQ inhibit the proliferation of cancer cell lines by inducing apoptosis.

The mechanism of action of many anticancer drugs is based on their ability to induce apoptosis[28, 29]. Apoptosis is a form of programmed cell death characterized by cytoplasmic condensation, plasma membrane blebbing and nuclear pycnosis, leading to nuclear DNA breakdown into multiples of ~200 bp oligonucleosomal size fragments. The detection of apoptosis in cultured cells relies heavily on techniques involving the extraction of nuclear DNA and characterization of such oligonucleosomal ladders by gel electrophoresis. More attention is paid to the ability of drugs to induce apoptosis in the process of evaluation of anti-tumor agent's effectiveness. The therapeutic application of apoptosis is currently being considered as a model for the development of anti-tumor drugs[30]. As the cell's intrinsic cell death program, apoptosis plays a key role in growth control of cells and tissue homeostasis and consequently, an imbalance or inactivation of important pathways can result in tumor formation and progression[31]. Furthermore,

cytotoxic therapies in anticancer treatment, *e.g.* chemotherapy, γ-irradiation, immunotherapy or suicide gene therapy, are mainly dependent on the function of cell apoptosis[32, 33] and many tumors develop different escape mechanisms that subsequently result in drug resistance[34]. Therefore, the induction and recovery of the apoptotic response in tumor cells are relevant steps in anticancer treatment.

Figure 5
DNA fragmentation pattern in HT-29 cell line on 1% agarose gel electrophoresis



Lane A DNA ladder, Lane B HT-29 cells (Untreated), Lane C HT-29 cells treated with EEPQ, Lane D HT-29 cells treated with PEPQ.

Agents that suppress the proliferation of malignant cells by inducing apoptosis may represent a useful mechanistic approach to both cancer chemoprevention and chemotherapy. While many anticancer agents have been developed, unfavorable side effects and resistance are serious problems[35]. Thus, there is growing interest in the use of plant materials for the treatment of various cancers and the development of safer and more effective therapeutic agents[36]. An ideal anticancer drug should specifically target the malignant cells while non-malignant cells in the body should not be impaired. While this is unfortunately rarely achieved, the present data demonstrates that the effect of EEPQ and PEPQ on the proliferation of tumor cells is significant and the effect is weak in case of normal cells. These findings suggested a tumor-specific effect of both the extracts.

Evidence suggests that polyphenol compounds such as tannins and resveratrol are able to traverse cell membranes and may enter the cytoplasmic or nuclear space. The question of bioavailability of polyphenols in mammalian systems also needs to be addressed. Relatively recent work by Asensi et al[37] with resveratrol indicates that it may have a relatively low bioavailability due to its biotransformation and rapid elimination. Further investigation is necessary to determine the bioavailability of EEPQ and PEPQ.

CONCLUSION

In conclusion, our results suggest that EEPQ and PEPQ can inhibit colon cancer cell

population growth, without impairing normal cells. In addition PEPQ induce apoptosis in colon cancer cell lines. However EEPQ failed to induce apoptosis in cancer cells. The cytotoxic properties of polyphenol extract could be explained, in part by the presence of quercetin and rutin. Dietary intakes of *Portulaca quadrifida* Linn. may have the potential to reduce colon cancer risk. However, further studies aimed at determining the anticancer properties of the other major constituents of ethanol and polyphenol extracts of *Portulaca quadrifida* Linn. as well as identifying the unknown compounds of both extracts, are necessary to understand their bioactivity completely.

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