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Peanut peel extract inhibits colorectal cell lines proliferation by apoptotic mechanism

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ABSTRACT

The non-toxic anticancer agents are targeted by researchers in the biomedical field around the world. Natural phytochemicals have been potentially identified in several medicinal plants for cancers therapy. Apoptosis is a programmed cell death of great interest to study mechanism of cancer treatment. This study aimed to investigate the antioxidants activities of the peanut peels extract (PPE) and its possible apoptotic effects on colorectal cancer cell lines (CaCO2). Phytochemicals composition was determined by evaluating the total phenolics, flavonoids, total antioxidant capacities and DPPH scavenging activities. Then, the anticancer activity was evaluated by determining the inhibitory concentrations that kill 50% (IC_{50}) of CaCO2 using MTT assay. The apoptotic pathway of the extract was proved by gene expression analysis using real time polymerase chain reaction (RT-PCR) of Bax, caspase 3 and Bcl-2 genes. The results showed that PPE exhibited potent antioxidant activities and induced apoptosis in human CaCO2 cells by up-regulating Bax and caspase 3 and down-regulating Bcl_2 genes. The peanut peel extract could inhibit CaCO2 cellular proliferation due to their phytochemical contents by targeting apoptosis that inhibit colon cancer growth.

1. Introduction

Cancer is the second leading cause of death worldwide. Carcinomas could invade the surrounding tissues and metastasis to the lymph nodes and other areas of the body. The most common forms of cancer in this group are breast, prostate, lung, and colon cancer (Ahmedin et al., 2009). Diet, physical inactivity, and obesity are related to up to 30–35% of cancer deaths (Kushi et al., 2006). Some specific foods are linked to specific cancers. A high-salt diet is linked to gastric cancer (Park et al., 2011). Environmental, as used by cancer researchers, means any because that is not inherited genetically, such as lifestyle, economic and behavioral factors and not merely pollution. Colorectal cancer (CRC) is the third most common cancer worldwide. The incidence of CRC is higher in countries in the developed world, where it is the second most common cancer. CRC has a leading position in malignant cancer-related morbidity and mortality. The risk factors for colorectal cancer are hereditary genetic predisposition, age, ulcerative colitis and other colon inflammatory diseases, diets high in meat and fat or low in selenium, Lynch syndrome, smoking, and others (Goldberg et al., 2007). Colon cancers most commonly occur in the large intestine. The predominant localization of CRC is rectum (50–60%) and sigmoid colon (15–25%). Tumors of epithelial origin are the most frequent, among them adenocarcinoma is ubiquitous. Among dietary constituents implicating in colorectal carcinogenesis, consumption of vegetables and fruits has a beneficial effect (Mathers, 2002). It has been reported that consumption of pulses is inversely related with colon cancer mortality of males in 38 countries. Previous studies showed that components, such as folic acid, protease inhibitors, phytosterols (PS), saponins, inositol hexaphosphate (phytic acid, PA), isoflavones and resveratrol in grains, nuts and seeds may have anti-carcinogenic effects. As a good source of PS, PA and resveratrol, peanuts have been thought to have an effect against carcinogenesis (Awad et al., 2002). The suppression effect of PS, PA and resveratrol on colon cancer has been supported *in vitro* and *in vivo* (Tessitore et al., 2000). Whether the laboratory findings are relevant to the hypothetically protective effect of peanuts on cancers remains unexplored. Peanuts are a common food in the world and in Taiwan as well. The

association between peanut consumption and colorectal cancer has not been well studied.

Nuts have been shown to have anti-inflammatory effects by affecting the production of prostaglandins and cytokines and antioxidative effects by decreasing lipid peroxidation and protecting against oxidative DNA damage (Grosso et al., 2015; Grosso and Estruch, 2016). These protective effects could be due to some components of nuts, including polyphenols, fibers, vitamins, and minerals. In some studies, nut consumption has been associated with decreased total and cancer-specific mortalities (van den and Schouten, 2015; Eslamparast et al., 2017). 50% of the peanuts consumed in the United States take the form of peanut butter, a previous study reported the association of peanut butter with colorectal cancer incidence in the Nurses' Health Study (Yang et al., 2016). In the present study, the antioxidants activities of peanuts peel extract was assessed by analyzing their phytochemicals content and the anticancer efficacy of the extract on the human CaCO2 cell lines was evaluated.

2. Materials and Methods

2.1. Plant materials and extraction

Peanuts seeds were purchased from the Carfour hyper in Tanta City, Egypt. The plant materials were identified and authenticated by taxonomists at Botany Department, Faculty of Science, Tanta University. Peanuts peels were separated and collected from the whole seeds, air dried at room temperature and then grounded into powder using electrical mixer. Fifty grams of the grinded plants were extracted in 500 ml of 70% ethanol. Samples were filtered and crude extracts were used for the assessment of antioxidants and anticancer properties.

2.٢. Phytochemicals analysis

Total phenolic content of PDSE was evaluated and expressed as (mg) of gallic acid equivalents (GAE) per (g) of extract using GAE calibration curve using the Folin-Ciocalteau reagent according to the methods of Singleton et al. (1999). Total flavonoid content was determined according to the methods of Zhishen et al. (1999), using the aluminum chloride colorimetric method expressed as (mg) quercetin equivalent per gram of extract from a calibration curve of quercetin. Method that described by Prieto et al. (1999) was used to determine the total antioxidant capacities (TAC) that expressed as ascorbic acid equivalent. DPPH free radical scavenging capacity was evaluated spectrophotometrically as described by to Blois et al. (1999). The scavenging activity on the DPPH radical was expressed as inhibition percentage that equal [(AC –AS)/(AC)] ×100.

2.3. Cell culture and CaCo-2 cell lines

The human colorectal cancer cell line CaCo-2 cells were obtained from VACSERA Tissue Culture Unit (Cairo, Egypt). The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin and 2% L-glutamine and centrifuged at 1500 rpm for 5 minutes. Cell pellets were re-suspended in culture medium and cultured at 37 ˚C for 3 days under 5% CO_2, 95% air for stabilization. Culture renewal in the desired dilution into new flasks was done when the cells reached 70-80% confluence for 3-5 days. Logarithmically growing cells were used for all the experiments.

2.4. Cytotoxicity analysis by MTT assay

The MTT assay protocol was used to check the cytotoxic effects of the extracts on CaCo-2 cells. On the day of the viability assay, the medium was removed, and fresh medium was added. The extract was diluted with saline to different concentrations (5-100 µg/ml) and applied in triplicate to the 70–80% confluent CaCo-2 cells, incubated at 37 ˚C and 5% CO_2 for 24 hours and then, 10 µl of MTT solution was added to each well. This was followed by incubation for 4 h at 37 ˚C. The MTT solution was eliminated, and the purple formazan crystals produced were dissolved with 100 µl of DMSO. Tamoxifen (Tam) was used as a positive standard. The absorbance at 570 nm was read on an enzyme-linked immunosorbent assay reader (StatFax-2100, Awareness Technology, Inc.). The proportion of surviving cells was calculated as follows: Proportion of surviving cells = (optical density (OD) of the treated sample − OD of blank)/(OD of control − OD of blank) × 100%. The concentration of the extracts that inhibit 50% of cells (IC_{50}) was calculated from the sigmoidal curve.

2.5. Gene expression analysis by real-time quantitative polymerase chain reaction

The total RNA was extracted from the CaCo-2 cells with RNeasy Mini kit according to the manufacturer's protocol. After the determination of the RNA purity and concentration, complementary DNA was synthesized from 4 µg of the total RNA (per sample) with Quant script reverse transcriptase. The isolated complementary DNA was amplified with 2X Maxima SYBR Green/ROX qPCR Master Mix according to the manufacturer's protocol (Thermo Scientific, # K0221) and gene-specific primers. The reaction volume and qPCR thermal conditions were applied. At the end of the last cycle, the temperature was increased from 60 to 95 ˚C to produce a melt curve. The relative change in gene expression was represented as fold change with critical threshold quantities and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6. Statistical analysis

Data obtained from each experiment were expressed as mean ± S.D. Statistical differences between the experimental groups were assessed using one-way ANOVA, p Values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Phytochemical contents of PPE

Phytochemical constituents including total phenolic, total flavonoids, total antioxidant capacity (TAC) and DPPH radical scavenging activities were determined in the extract aqueous ethanolic of peanut peels. The results showed that this extract exhibited potential phytochemicals content. The level of total phenolic compounds represented 1186 ± 5.85 µg gallic acid equivalents (GAE) per ml of extract. Total flavonoids content in the PPE was 196 ± 2.41 µg quercetin equivalent/ml extract. The TAC using phosphomolybedate method was 87 ± 3.25 µg /ml. PPE was determined to be active in free radicals scavenging properties, the data of the present study denoted that the percentage of DPPH free radical scavenging activity caused by Peanut peels extracts represented 68 % and their IC_{50} value was 55 mg/ml (Table 1).

Table 1. Phytochemical analysis of PPE

Phytochemical analysis	PPE
Total phenolic (µg/ml)	1186 ± 5.85
Total flavonoids (µg/ml)	196 ± 2.41
TAC (µg/ml)	87 ± 3.25
DPPH scavenging%	68 %
IC_{50} of DPPH (mg/ml)	55

3.2. Cytotoxic effect of PPE against colorectal cancer cell lines

The colon cancer cell line (CaCo-2) was treated with different concentrations of the PPE. Their cytotoxic and anti-proliferative effects were determined based on the ability of colorectal cancer cell lines to reduce MTT producing the formazan dye. As shown in figure 1, the results revealed that PPE exhibited selective cytotoxicity in the colon cancer cell lines with a potential cytotoxic effect to a minimum concentration killing 50 % of colon cancer cell (IC_{50}) 30.50 µg/ml.

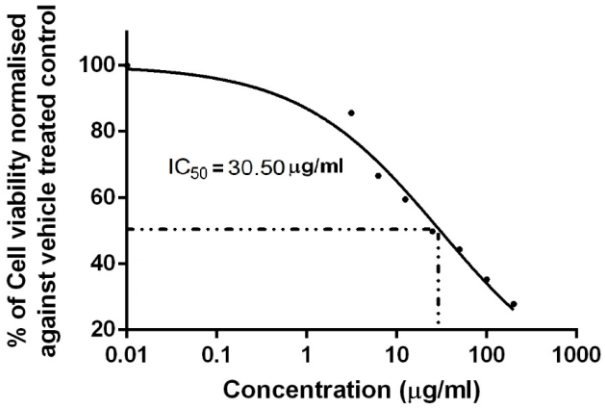

Figure 1. Effect of peanut seed coats extract on the viability of colorectal cancer cell line (CaCO2).

3.3. Apoptotic effect of PPE against colorectal cancer cell lines

Real time PCR was used to detect the relative expression of apoptosis related genes, Bax and caspace-3, and anti-apoptosis gene, Bcl-2, that reflects the changes in transcription levels of these genes in CaCo-2 cells after administration of the extract under the study for 24 hours. Throughout the whole real time PCR experiment, the housekeeping gene encoding GAPDH was used as an internal reference for normalization. The expression level of the target gene in control CaCo-2 cells was considered the baseline. Data obtained from the qPCR showed a significant ($p \leq 0.05$) up-regulation in the mRNA expression level of the apoptotic gene, Bax, in CaCo-2 cells following treatment of the PPE. A significant up-regulation was reported 3.5-folds of change (Figure 2). Moreover, the

expression level of the apoptotic gene, caspase-3, was up-regulated after treatment with the sweet red pepper extract for 24 hours with folds of change 5.6 times, when compared with control CaCo-2 cells without treatments (Figure 3). However, the mRNA expression level of the anti-apoptotic gene, Bcl-2 was down-regulated in the CaCo-2 cells due to PPE extract exposure with expression level (0.35) as compared to control group which showed fold of change 1 (Figure 4).

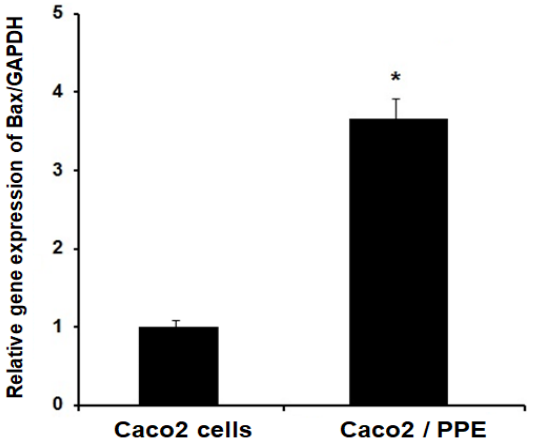

Figure 2. Real-time quantitative PCR analysis showing the relative mRNA expression of Bax/GAPDH genes in CaCo-2 cells after 24 h of sweet red pepper extract treatment .* Significant change ($p \leq 0.05$).

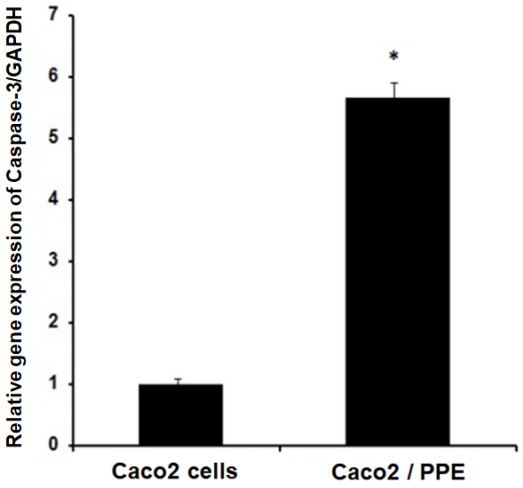

Figure 3. Real-time quantitative PCR analysis showing the relative mRNA expression of Caspase-3/GAPDH genes in CaCo-2 cells after 24 h of sweet red pepper extract treatment .* Significant change ($p \leq 0.05$).

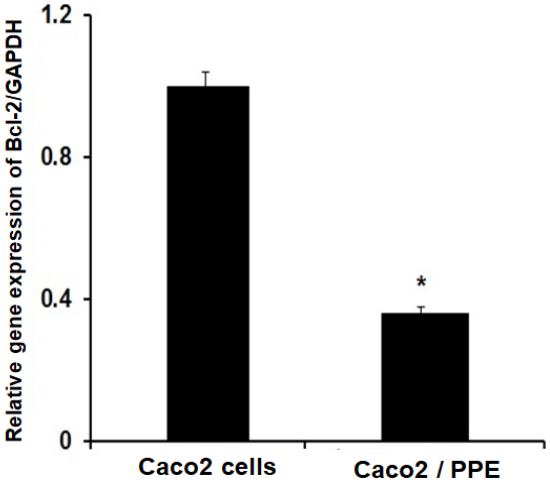

Figure 4. Real-time quantitative PCR analysis showing the relative mRNA expression of Bcl-2/GAPDH genes in CaCo-2 cells after 24 h of sweet red pepper extract treatment .* Significant change ($p \leq 0.05$).

4. Discussion

Colorectal cancer is a complex disease caused by multiple cells signaling disruptions, its treatment is depending on using radiotherapy, chemotherapy, and surgery (Schmoll et al., 2012). Chemotherapeutics can lead to cancer cell death by inducing DNA damage or initiating multiple signaling pathways, including cell cycle arrest, inhibition of global translation, DNA repair. However, the effects of cytotoxicity, drug resistance and adverse reactions are the main problems associated with chemotherapy (Woods and Turchi, 2013).

Due to the capability of natural products derived from plants to promote apoptosis and cell cycle arrest without toxic effect on the healthy cells, these natural compounds can be used for effective cancer prevention and treatment (Shahneh et al., 2014). These natural constituents including alkaloids, polysaccharides, polyphenols and diterpenoid have become promising sources of new anticancer drugs, and approximately 50% of the currently used anticancer drugs have been derived from natural products (Newman and Cragg, 2016). Interestingly, peanut is an important crop grown worldwide. Commercially it is used mainly for oil production but apart from oil, the by-product of peanut contains many other functional compounds like proteins, fibers, polyphenols, antioxidants, vitamins, and minerals which can be added as a functional ingredient into many processed foods (Arya et al., 2016). Therefore, in the present study, the phytochemical contents in the sweet red pepper extract including total phenolic, total flavonoid,

total antioxidant capacity and free radicals scavenging activity of DPPH were evaluated. The antioxidant activities of flavonoids which are beneficial to human health by scavenging harmful radicals have been addressed in the extract under the present study based on quercetin equivalent. The DPPH radicals are well known in the model system to evaluate the scavenging properties of numerous natural products. The scavenging activity of the prepared extract against DPPH radicals showed promising percentage. Our data were in accordance with the previous studies reported the phytochemical constituents in peanuts (Arya et al., 2016). Previous study reported that the peanut skins as a waste product of the peanut processing industry showed significant sources of the polyphenolic compounds that are noted for their bioactivity (Dean, 2020).

Antioxidant potential activity and cytotoxicity effects of different parts of peanuts (Gaafar et al., 2015). The present study was extended to evaluate the *in vitro* anti-proliferative effect of sweet red pepper extract against the colon cancer cell lines and the obtained results revealed that the inhibitory concentration that kills 50 % (IC_{50}) of cells for broccoli was low (25 µg/ml) indicating that this extract has a powerful anticancer efficacy. Our results were in line with previous study revealed the cytotoxic and genotoxic activities of peanuts (Awad et al., 2000; Gaafar et al., 2015. Studies of cytotoxicity, cytoprotection, and interaction with reactive oxygen species (ROS) of peanuts have been reported by Yanina et al. (2020).

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