



## Research Article

### A Comparative Study for the Effects of Laboratory and Commercially Prepared Plum Extracts on Colon-26 Adenocarcinoma Cells

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#### Abstract

##### Aim

Oxidative stress in intestine which arises from digestion and metabolism of various foods has been linked to initiation and progression of colon cancer. Fruits and vegetables contain phenolic anti-oxidants, which are non-toxic and can have significant impact on Colorectal Cancer (CRC). The objective of the present research was to investigate plum effects on colon cancer cells proliferation. We hypothesized that plum can inhibit cancer growth by preventing oxidative stress due in part to its high concentration of polyphenols.

##### Methods

We compared a commercial preparation of Plum Extract (PE60)

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with a laboratory-prepared extract of plum for their polyphenols, flavonoids, and anti-oxidation activity using established assay protocols. We then tested their anticancer effects on using colon-26 adenocarcinoma cells using a MTT cell proliferation assay.

#### Results

We found that PE60, a commercial plum extract, was rich in phenolic/flavonoids contents and significantly inhibited colon cancer cell growth. The laboratory preparation of methanol extract of plum skin when adjusted for polyphenolic contents showed similar effects to that of PE60.

#### Conclusion

Our data suggest that regular consumption of plum fruits in the form of concentrated "whole fruit juice" may be a potential strategy for preventing or treating colon cancer.

**Keywords:** Anti-oxidants; Colon-26 cells; Colorectal cancer; Plums; Polyphenols

## Introduction

The digestive tract is made up of the esophagus, stomach, and the small and large intestines. The colon is part of the body's digestive tract which processes nutrients from foods and helps pass waste material out of the body. The colon is the first part of the large intestine and is about 5 feet long. Colorectal cancer (CRC) begins when the process of normal replacement of the lining cells of the colon is deregulated. Errors in normal checks and balances that control growth during cell division transform a normal cell causing it to divide and grow uncontrollably and contribute to growth within the colon known as polyps. Polyps are precancerous lumps that grow slowly over a long period; however, the subsequent genetic mutations in these cells make them then invasive and malignant [1]. Colorectal cancers are the third most common causes of cancers in the United States in both women and men. For 2018, the American Cancer Society's estimates 97,220 new cases of colon cancer and 43,030 new cases of rectal cancer which is expected to cause about 50,630 deaths. (<https://cancerstatisticscenter.cancer.org>). Approximately 95% of all CRCs occur in people age 45 and above. According to the National Cancer Institute, the median age of individuals diagnosed with the disease is 69 years [2]. The mortality and incidence rates are 40% and 30% in men than in women, respectively even though the lifetime risks of CRC is similar in both cases (4.7% vs. 4.3%) [2].

There are several risk factors for developing CRC. It is believed that environmental factors or inherited genes or the combination of both may increase the chances of an individual to develop CRC [3]. Eating food that is high in processed or red meat (e.g., hot dogs, lamb, and beef) increases an individual's chances of initiating CRC. Broiling, grilling, frying or other techniques of cooking meat at extreme temperatures create chemicals that could also increase the risk for developing CRC [4]. Free radicals are produced during metabolism, which are highly reactive to cellular proteins, lipids, and

nucleic acid and cause cellular damage. The most sensitive of all is nucleic acids whose reaction with free radicals causes changes in genetic structures and that can progress to cancer. People who embrace a sedentary lifestyle have high chances of develop CRC whereas people involve in physical activities have a reduced risk [5]. Smoking is generally associated with lung cancer; however, is also linked with other types of cancer including CRC [6]. The prevention of CRC is generally based on the modifiable risk factors which include maintenance of reasonable degree of physical exercises, avoiding weight gain, reducing alcohol intake, avoidance of smoking, and overall healthy dietary patterns. A number of dietary agents such as consumption of fruits and vegetables can help prevent the initiation and/or progression of CRC [7].

Various dietary micronutrients such as Vitamins A, C, E, beta carotene and selenium have been proposed to have anti-carcinogenic effects, based on their anti-inflammatory or anti-oxidant properties. Anti-oxidants neutralize these free radicals and, therefore, minimize the cellular damage and thus reduce the risk of developing cancer. A plum is a delicious and juicy fruit that belongs to the genus Prunes and family Rosacea. The fruit contains assortments of healthy minerals and vitamins. They are excellent source of vitamins A, K, C, and folate. They are also good source if thiamine, riboflavin, niacin and alpha-tocopherol. The available minerals in this fruit include zinc, calcium, iron, magnesium, phosphorus, fluoride and potassium. Plums also help in supplying dietary fiber as well as other low calories to the body without any risky fats. Plums are also a rich source of bioactive polyphenol including chlorogenic acids, anthocyanins, zeaxanthin and kryptoxanthin, catechins, and quercetin which contribute to their anti-oxidation and anti-inflammatory activities and also induce powerful anti-cancer effects [8,9]. The above observations suggest that plums can be very effective in preventing cancer from different origins, including CRC. The aim of present investigation was to study the effect of plum's extract on colon cancer cells. We Hypothesize that plums because of their high content of bioactive polyphenols with anti-oxidation activity can effectively inhibit the proliferation of colon cancer cells. We used commercially available standardized preparation of plum extract named "PE60", which contained approximately 60% polyphenols to test its anti-oxidation and anti-cancer properties and compared to that of laboratory prepared extracts from plum's skin and pulp. Our data suggest that amounts of polyphenols in plum extracts are important for their anti-cancer activity in colon cancer.

## Methods and Materials

Colon-26 cell (CRL-2638) were purchased from ATCC (Manassas, VA 20110), F-12K media was purchased from Gibco (Grand island, NY14072), Fetal Bovine Serum was purchased from RAM-BIO (Missoula, Montana), antibiotics penicillin and streptomycin (BP2959) and Phosphate Buffered Saline (PBS) was purchased from Fisher (Fair lawn, New Jersey 07410). Plums (Black Baut) were purchased from a local market. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co (St. Louis, MO). A commercial preparation of Plum Extract (PE60) was from PL Thomas (Morristown, MA) and kindly gifted by Dr. Suzette Pereira (Abbott Nutrition, Columbus, OH). The 96 wells plates were purchased from COSTAR. Folin-Ciocalteu, Aluminum chloride, Diphenyl-1-Picrylhydrazyl (DPPH), quercetin, gallic

acid, trolox and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma Chemical Co. (St. Louis, MO).

## Isolation and extraction of plum fractions

The plums were washed with distilled water and then blotted dry with paper towel. Skin was peeled using a kitchen peeler. The skin was scraped to move any deposits of pulp. The skin peels were washed with distilled water to remove any remaining plum juice and then blotted with a paper towel. The pulp was cut into small pieces (about 1cm<sup>3</sup>). The seeds and presence of any skin fragments were removed from the pulp. The skin and pulp were stored at -80°C and then placed in freeze dryer. The dried skin and pulp were ground to a fine powder using a mortar and pestle with liquid nitrogen added to keep the powder frozen. The dried powder was flushed with nitrogen and stored at -80°C until used.

A known quantity (5g) of dried skin or pulp was mixed with 200ml of water or 80% methanol and placed on a shaker at room temperature overnight. The next day, the mixture was centrifuged at 2000g for 20mins using a Thermos Scientific Centrifuge (Waltham, MA) and the supernatant was collected. The methanol extract was dried in a nitrogen evaporator (Organomation Associates, Inc, Berlin, MA) and then placed in freeze dryer under vacuum over night to ensure the complete removal of residual methanol. The water extract was freeze dried. The dried extract was stored in a -20°C freezer. The dried powder of plums was dissolved in DMSO and the stock solution was prepared at 250mg/ml.

## Characterization of plum extracts

The laboratory-prepared plum extracts (skin-water extract, skin-methanol extract, pulp-methanol extract and PE60) were characterized for Total Phenolic Content (TPC), Total Flavonoids Content (TFC), anti-oxidation activity and oxygen scavenging activity as described below.

**Determination of total phenolic content:** The TPC of plum extracts was determined by using Folin-Ciocalteu method [10]. Briefly, extracts were reacted with Folin-Ciocalteu reagent (phosphopolybidic acid/phosphotungstic acid) in an alkaline condition to reduce the Folin-Ciocalteu reagent from a yellow color to a blue-green color. The intensity of blue-green color was measured at 765nm. The total phenolic content of the plum extract was calculated using a standard curve of Gallic acid and reported as Gallic acid equivalents (mg GAE/g).

**Determination of total flavonoid content:** The aluminum chloride complex-forming assay was used to determine the TFC of the extracts [11]. Briefly, extracts were reacted with Aluminum Chloride/Sodium Nitrite solution and formation of color due to binding of Aluminum to flavonoids was quantified by measuring absorption at 410nm using standard curves of quercetin. The content was calculated as quercetin equivalents (mg QE/g).

**Anti-oxidation assay:** The anti-oxidation activity of plum extracts was determined by using the DPPH method [12]. Briefly, plum extracts were added to a DPPH solution (deep purple color) and reduction of DPPH due to anti-oxidation activity was determined by measuring absorption at 515nm for a decrease in DPPH color (light purple color). Data was calculated using a standard curve of Trolox and expressed as Trolox equivalents (μmol TE/g).

**Oxygen scavenging capacity:** The oxygen scavenging activity of plum extracts were evaluated according to previously reported protocol [13]. Briefly, plum extract was added to a preformed colored ABTS\* stable free radical and a de-colorization due to anti-oxidation activity in plum extracts were measured by recording absorption at 734nm. Trolox equivalents were calculated using a standard curve and expressed in  $\mu\text{moles TE per g plum extract}$  ( $\mu\text{mol TE/g}$ ).

**Cell proliferation assay:** The colon-26 cells (CRL-2638) were cultured in RPMI media that was supplemented with 10% FBS and 1% penicillin and Streptomycin. Routine cell culturing was performed using 75mm<sup>2</sup> culture flasks, whereas cells for experimental use were cultured in a 96 well plate. The cells were incubated in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Media was changed every 3 days and cells were subculture when they became confluent. The effect of plum extracts on cell proliferation was determined using a MTT assay. Briefly, cells (10,000/well) in a 96 wells plate were incubated with plum extracts (dissolved in DMSO whose concentration did not exceed from 0.15%) in a total volume of 100 $\mu\text{l}$  for 24 or 48hr. as described above. After incubation, 20 $\mu\text{l}$  of MTT solution was added to each well and then the plate was further incubated for another 4hrs under same conditions. The purple precipitate that was formed due to reduction of MTT from mitochondrial dehydrogenases in live cells was dissolved in 200 $\mu\text{l}$  of DMSO and absorption was measured at 570nm. The control cells (without plum extract treatment) were incubated with appropriate concentration of vehicle (0.15% DMSO only). The results of cell viability are expressed as % change from control.

## Nuclear staining

After treatment with plum extracts as described above, cells were fixed with 4% paraformaldehyde for 10mins on ice. Cells were washed 3 times with ice cold PBS. Cells were then incubated with 1:100 dilution of Hoechst No. 33342 stain (10mg/mL in H<sub>2</sub>O stock solution) for 10mins. Cells were washed and dried in air. Pictures of bright field and fluorescent nuclear staining were taken using a Nikon microscope with NIS-Element software.

## Data Analysis

The data is expressed as mean  $\pm$  SD for at least 3 individual experiments. All comparisons were made by one-way ANOVA with Tukey's -HSD-post-hoc test using SPSS 20 Statistical software. Significant differences were reported at  $P < 0.05$  and indicated by "\*" where as non-significant differences were not marked.

## Results

### Characterization of plum extracts

The methanol and water extracts of plum skin, methanol extract of pulp, and water extract of PE60 were characterized for TPC, TFC, and for their anti-oxidation activity using DPPH and ABST assays. The results are as described below.

**Total phenolic content:** The data shown in figure 1 demonstrate that the commercial preparation of PE60 contained TPC in range 490-500mg/g of dry extract. The methanol extract of skin extract contained around 200mg/g of TPC where as the water of plums skin contained about 100mg of TPC/g dry weight. However, the methanol extract of plum's pulp contained the least amount of nearly 50mg TPC/g of dry weight.

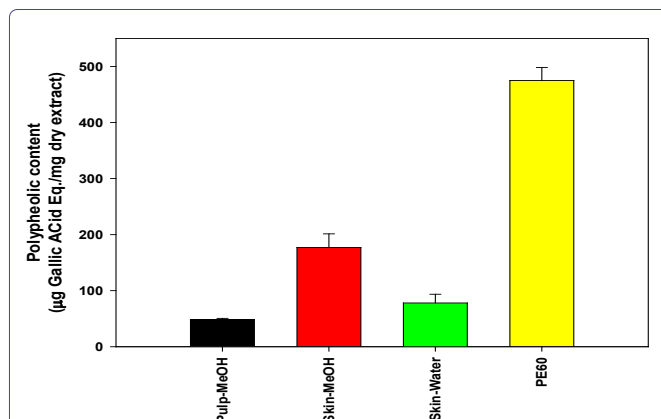


Figure 1: Determination of total phenolic content in plum extracts.

**Note:** The TPC content of water and Methanol (MeOH) extracts of plum's skin and pulp, and a Polyphenol-Enriched Fraction 60 (PE60) of plum extract were determined using Folin-Ciocalteu reagent as described in the "Methods" section. Gallic acid was used as a standard to determine TPC content. Results are expressed as mean  $\pm$  SD for at least 3 experiments.

**Total flavonoid content:** The amounts of TFC in different plum extract are shown in figure 2. The PE60 contained about 90mg of TFC/g of dry weight. The methanol and water extract of plum's skin contained similar amounts of TFC in range of 50-55mg/g of dry powder. Similar to TPC, the methanol extract of plum's pulp also contained the least amount of TFC around 10mg/g of dry weight compared to that of other preparations.

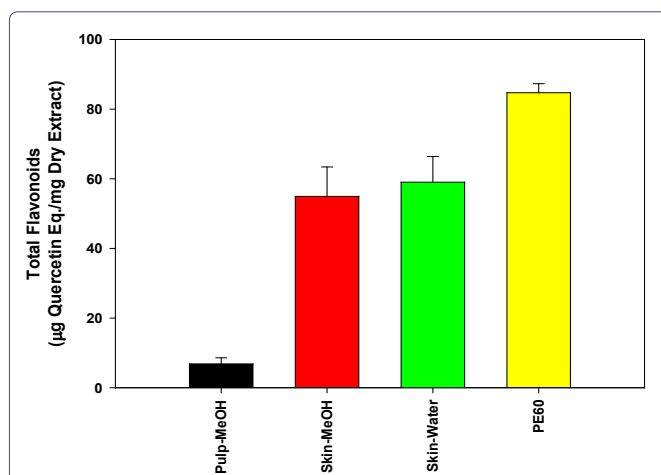


Figure 2: Determination of total flavonoid content in plum extracts.

**Note:** The TFC content of water and Methanol (MeOH) extracts of plum's skin and pulp, and a Polyphenol-Enriched Fraction 60 (PE60) of plum extract were determined using AlCl<sub>3</sub> reagent as described in the "Methods" section. Quercetin was used as a standard to determine TFC content. Results are expressed as mean  $\pm$  SD for at least 3 experiments.

**Anti-oxidation activity:** The anti-oxidation activity in different extracts of plum is shown in figure 3. The data indicates that only the commercial preparation, PE60, has the highest extent of anti-oxidation activity which represented about 4000 $\mu\text{M}$  equivalent of Trolox activity/g of dry powder. Whereas all others plum extracts have less extent of anti-oxidation activity in range of 150-200 $\mu\text{M}$  Trolox equivalent/g dry weight.

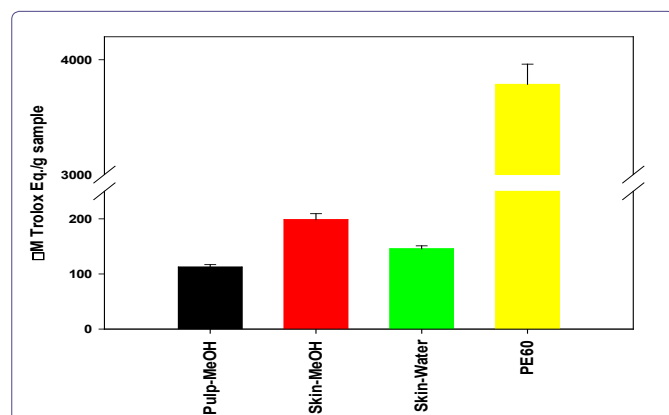


Figure 3: Anti-oxidation activity in plum extracts.

Note: The total anti-oxidation activity in water and Methanol (MeOH) extracts of plum's skin and pulp, and a Polyphenol-Enriched Fraction 60 (PE60) of plum extract was determined using a DPPH assay as described in the "Methods" section. Trolox was used to quantify the total anti-oxidation activity and data is reported as  $\mu\text{M}$  Trolox equivalent. Results are expressed as mean  $\pm$  SD for at least 3 experiments..

**Oxygen scavenging capacity:** The oxygen scavenging activity of different plum extracts is shown in figure 4. Similar to anti-oxidation activity (DPPH), it is evident from data that PE60 contained the highest capacity for scavenging free oxygen. The total oxygen scavenging activity in PE60 in water extract is  $3670\mu\text{M}$  Trolox equivalent/g dry weight, whereas the methanol extract of plum's skin contained  $904.083\mu\text{M}$  Trolox equivalent. The methanol extract of plum's pulp and water extract of plum's skin contained the least oxygen scavenging activity ( $\sim 150\mu\text{M}$  Trolox equivalent/g dry weight).

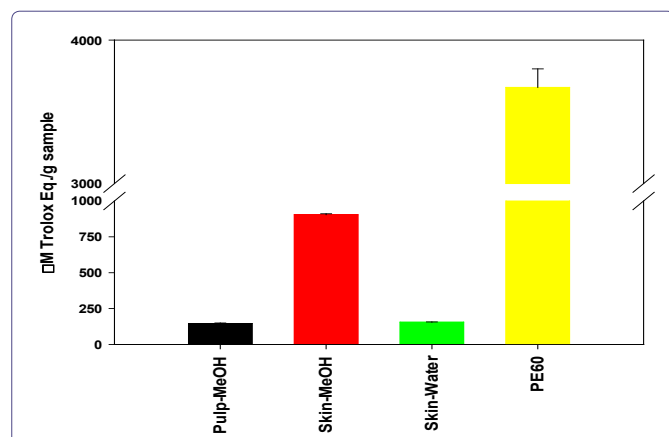


Figure 4: Oxygen scavenging activity in plum extracts.

Note: The oxygen scavenging activity in water and Methanol (MeOH) extracts of plum's skin and pulp and a Polyphenol-Enriched Fraction 60 (PE60) of plum extract were determined using an ABTS assay as described in the "Methods" section. Trolox was used to quantify the oxygen scavenging activity and data is reported as  $\mu\text{M}$  Trolox equivalent. Results are expressed as mean  $\pm$  SD for at least 3 experiments.

#### Effect of plum extract on colon-26 cancer cells viability

The results of PE60, and plum extract from skin, pulp after 24 hr treatment are shown in figure 5. After treatment with PE60, the cell viability was reduced in a dose-dependent manner reaching a signif-

icant reduction of 25% ( $P < 0.05$ ) at  $10\mu\text{g/ml}$ . The cell viability was reduced gradually on further increasing the concentration of PE60, reaching a maximum inhibition by 80% ( $P < 0.05$ ) at  $300\mu\text{g/ml}$ . The skin water or methanol extract largely did not cause any significant effect in colon cells viability except methanol extract caused a slight but significant reduction by 30% ( $P < 0.05$ ) at  $300\mu\text{g/ml}$ . Moreover, data showed that methanol extracts of plum's pulp has no significant effect at any tested concentration.

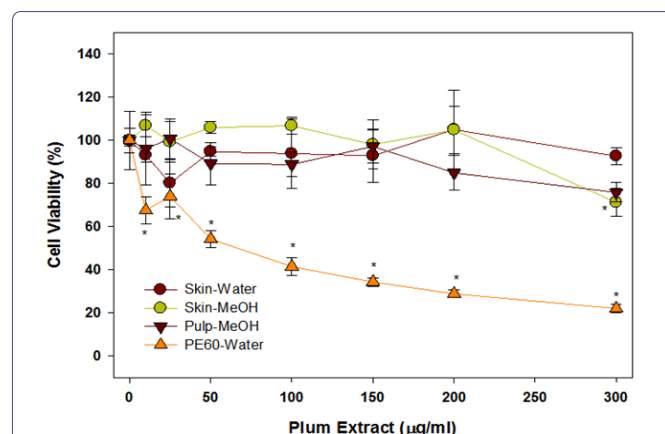


Figure 5: The effect of plum extract on colon-26 cancer cells viability after 24hrs incubation.

Note: Cells (10000/well) were grown in a 96 well plate for 24hrs in  $100\mu\text{l}$  complete media containing varying amounts of plum's water or Methanol (MeOH) extracts. The cell viability was measured by adding MTT dye as described in the "Methods". Data is calculated as % of viable cells. The data is expressed as mean  $\pm$  SD for at least 3 experiments. All comparisons were made by one-way ANOVA with Tukey's -HSD-post-hoc test using SPSS Statistics 20 software. All significant differences between control (treated with 0.15% DMSO only) and treated cells are reported at  $P < 0.05$  and indicated by "\*" where as non-significant differences are not marked.

The effect of PE60 and plum extract from skin, pulp on colon 26 cells was also tested after 48hrs of treatment. It is evident from data shown in figure 6 that the cells viability was also not affected on longer incubation at 48hr and data very much mirrors the effects observed after 24hr of incubation. The PE60 showed a dose-dependent effect whereas the water extract of plum's skin shown no significant effect on all the concentration and the methanol extract of plum's skin caused a significant reduction by 30% ( $P < 0.05$ ) at  $300\mu\text{g/ml}$ . The methanol extract of pulp has no significant effect at any tested concentration.

From these initial experiments, it was evident that the laboratory preparation of water or methanol extract of plum's skin and methanol extract of plum's pulp have no or very little effect on colon cell viability. However, the commercial preparation of Plum Extract (PE60) significantly inhibited colon-26 cell proliferation. This difference in effect between laboratory plum extracts and PE60 was due to probably because of their higher content of polyphenols in the PE60 preparation. Based on data in figure 1, further experiments were, therefore, performed using plum skin extract adjusted to their polyphenol in range that was present in PE60. However, we did not further test pulp extracts because it contained low amounts of polyphenols and adjustment of polyphenols to an equivalent levels to PE60 required a large amount of extract that was difficult to dissolve in the test volumes for desired concentrations. The effects of methanol or water



extracts of plum's skin adjusted for polyphenolic content after 24hr treatment are shown in figure 7. The data show that a concentration of polyphenols in methanol extract of plum's skin at 25µg/ml inhibited colon cell viability significantly by 55% (P<0.05). The cell viability was further decreased by 70% at 75µg/ml reaching a plateau of a maximum inhibition by 80% at 300µg/ml. The polyphenols in water extract also inhibited colon-26 cell viability but to a lesser extent than that of methanol extract. The polyphenols at 25µg/ml inhibited cell viability significantly by 45% (P<0.05); however, the cell variability remained at 45%-50% inhibition on further increasing the concentration of polyphenols up to 300µg/ml.

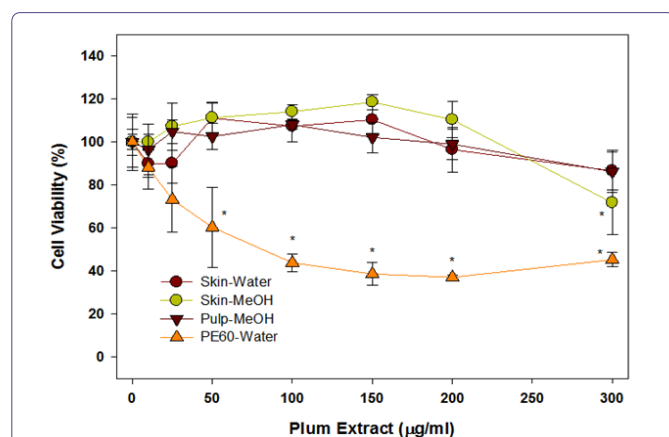


Figure 6: Effect of plum extract on colon-26 cancer cells after 48hrs of incubation.

Note: Cells (10000/well) were grown in a 96 well plate for 48hrs in 100µl complete media containing varying amounts of plum's water or Methanol (MeOH) extracts. The cell viability was measured by adding MTT dye as described in the "Methods". Data is calculated as % of viable cells. The data is expressed as mean ± SD for at least 3 experiments. All comparisons were made by one-way ANOVA with Tukey's -HSD-post-hoc test using SPSS Statistics 20 software. All significant differences between control (treated with 0.15% DMSO only) and treated cells are reported at P<0.05 and indicated by "\*" where as non-significant differences are not marked.

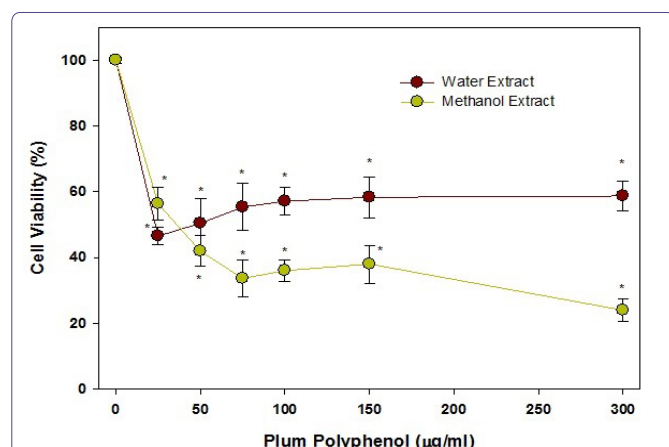


Figure 7: Effect of polyphenols in plum skin extracts on colon-26 cell viability after 24hr of incubation.

Note: Cells (10000/well) were grown in a 96 well plate for 24hrs in 100µl complete media containing variable amounts of polyphenols in plum's skin extracts. The cell viability was measured by adding MTT dye as described in the "Methods". Data is calculated as % of viable cells. The data is expressed as mean ± SD for at least 3 experiments. All comparisons were made by one-way ANOVA with Tukey's -HSD-post-hoc test using SPSS Statistics 20 software. All significant differences between control (treated with 0.15% DMSO only) and treated cells are reported at P<0.05 and indicated by "\*" where as non-significant differences are not marked.

The effects of the methanol or water extracts of plum's skin after 48hr of treatment were also assayed and are shown in figure 8. The data show that a concentration of polyphenols in methanol extract of plum's skin at 25µg/ml inhibited colon cell viability significantly by 25% (P<0.05). The cell viability was further decreased by 80% in a dose-dependent manner up to 150µg/ml and then reached a plateau. Similar to 24hr incubation, the polyphenols in water extract also inhibited colon-26 cell viability but to a lesser extent than that of methanol extract. The polyphenols at 25µg/ml inhibited cell viability significantly by 40% (P<0.05), and then caused a gradual reduction in cell viability by 50% at 300µg/ml. However, the cell viability reduced by almost 80% at 500µg/ml, which was similar to the methanolic extract.

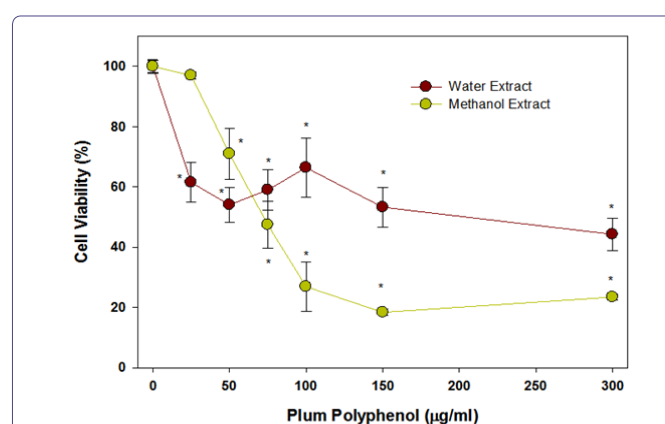


Figure 8: Effect of polyphenols in plum's skin extract on colon-26 cancer cells after 48hrs of incubation.

Note: Cells (10000/well) were grown in a 96 well plate for 48hrs in 100µl complete media. The effect of water and methanol extract on cell viability was measured by adding MTT dye as described in the "Methods". Data is calculated as % inhibition of cell growth. The data is expressed as mean ± SD for at least 3 experiments. All comparisons were made by one-way ANOVA with Tukey's -HSD-post-hoc test using SPSS Statistics 20 software. All significant differences between control (treated with 0.15% DMSO only) and treated cells are reported at P<0.05 and indicated by "\*" where as non-significant differences are not marked.

The effect of PE60 adjusted for its polyphenols contents on colon-26 cancer cells after 24hrs incubation is shown in figure 9. It is evident from the data that a significant inhibition of colon cell growth by 20% (P<0.05) was observed at 25µg/ml of polyphenols in PE60. On further increasing the polyphenol concentration in PE60, the cell viability decreased in a dose-dependent manner reaching a plateau of almost a significant 50% (P<0.05) inhibition at 75µg/ml. No further decrease in cell viability was observed on further increasing the concentration of polyphenols up to 150µg/ml.

The effect polyphenols in PE60 on colon-26 cancer cells after 48hrs incubation is shown in figure 9. The viability of colon-26 cells was effectively inhibited by polyphenols in PE60 extract on 48 incubation. The cell viability inhibited significantly by 85% (P<0.05) at 12.5µg/ml. There was only a slight improvement in inhibition and a maximum inhibition of 90% (P<0.05) was observed on further increasing the concentration over 12.5µg/ml.

### Effect of plum extracts on colon-26 cellular structure

During treatment with plum extract, it was noticed that colon-26 cells exhibited formation of large round bubble structures. Initially it

was thought that the nuclei were undergoing swelling during apoptosis. In order to confirm this, the nuclei were stained with Hoechst No. 33342. However, to our surprise, the round bubble structures were not nuclear. They appear to be vacuoles filled with water.

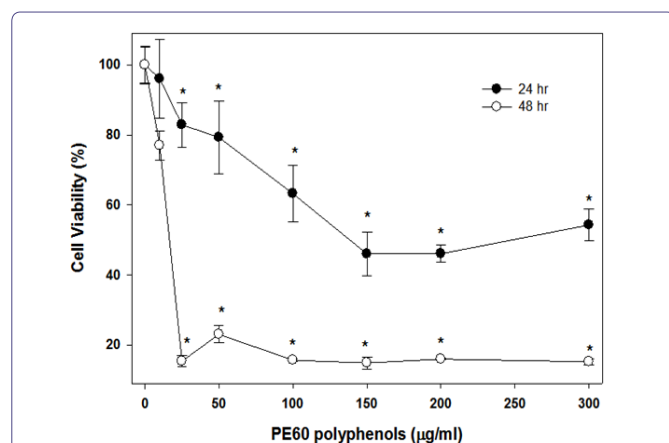


Figure 9: Effect of polyphenols in PE60 on colon-26 cancer cells.

**Note:** Cells (10000/well) were grown in a 96 well plate for 24 or 48hrs in 100µl complete media containing varying amounts of polyphenols in PE60 extract. The cell viability was measured by adding MTT dye as described in the "Methods". Data is calculated as % of viable cells and expressed as mean ± SD for at least 3 experiments. All comparisons were made by one-way ANOVA with Tukey's -HSD-post-hoc test using SPSS Statistics 20 software. All significant differences between control (treated with 0.15% DMSO only) and treated cells are reported at P<0.05 and indicated by "\*" where as non-significant differences are not marked.

cytotoxic to colon-26 cells, also caused bubble formation but to a lesser extent than that of plum skin extract, prior to undergoing cell death (Figure 11).

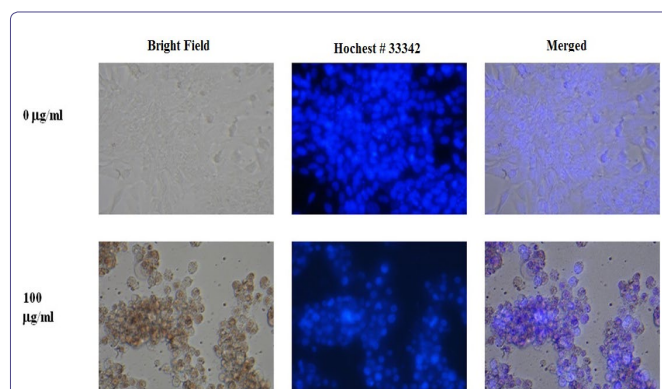


Figure 11: Effect of PE60 Extract on colon-26 Cell.

**Note:** The control (treated with 0.15% DMSO only) and treated cells (treated with PE60 for 48hr) were fixed with 4% paraformaldehyde for 10mins on ice. Cells were washed 3 times with ice cold PBS and then incubated with 1:100 dilution of Hoechst No. 33342 stain (10mg/mL in H<sub>2</sub>O stock solution) for 10mins. Pictures of nuclear staining were taken using a Nikon microscope with NIS-Element software. The Vacuoles were absent on PE60 treatment.

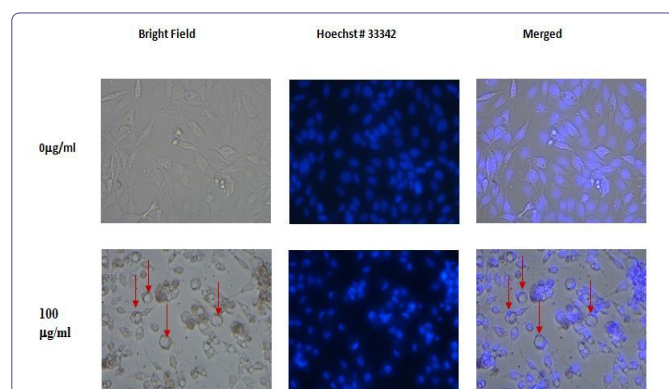


Figure 10: Effect of skin-methanol extract of plums on colon-26 Cells.

**Note:** The control (treated with 0.15% DMSO only) and treated cells (treated with methanol-extract of plum skin for 48hr) were fixed with 4% paraformaldehyde for 10mins on ice. Cells were washed 3times with ice cold PBS and then incubated with 1:100 dilution of Hoechst No. 33342 stain (10mg/mL in H<sub>2</sub>O stock solution) for 10mins. Pictures of nuclear staining were taken using a Nikon microscope with NIS-Element software. The arrow indicates the formation of vacuoles after plum extract treatment.

The methanol extract of plum skin caused bubble formation at a lower concentration of 25µg/ml (data not shown), and formed optimal circular bubbles at 100µg/ml before the cells started to die (Figure 10). In contrast, the water extract from plum skin started inducing the formation of circular bubbles at 100µg/ml (data not shown). Pulp extract was not used in this experiment because of lack of their effects on colon-26 proliferation. The PE60 extracts, which were more

## Discussion

In this study, we investigated the effect of plum extract prepared in the laboratory and compared that with PE60, a commercially standard plum extract on colon cancer cell growth. We initially characterized the phenolic and flavonoids contents and also assayed their anti-oxidation activity and compared the data of laboratory prepared plum extracts to commercially obtain standard preparation. The commercial preparation, PE60 should contain approximately 600mg of TPC in gram of the PE extracts. The TPC amount in PE60 from our analysis came out in range of 490-500mg/g of dry extract. Our data, therefore, validated the high phenolic contents in the PE60 plum extract. In our laboratory preparation of plum extracts, methanol extraction from plum skin contained about 200mg/g dry weight of TPC while the pulp only contained about 50mg/g of flavonoids dry weight. Our data show that the phenolic compounds are concentrated in skin rather than in the pulp. Our data is consistent with other studies that have also shown that phenolic compounds are mostly localized in skin of fruits [14].

Out of about 500mg of phenolic compounds, PE60 contained about 200mg of flavonoids whereas the laboratory preparation of skin contained about 50mg/g dry weight of flavonoids out of about 200mg/g of total phenolic compounds. This data suggests that plum extracts in addition to flavonoids also contained phenolic compounds of other classes. The PE60 is known to have procyanidins and chlorogenic acid classes of polyphenols. During present investigation, we did not determine the phenolic classes in the laboratory prepared extracts of plum skin and pulp.

The anti-oxidation data show that PE60 possessed a very high activity for inhibiting DPPH oxidation whereas our laboratory preparation of plum extracts, compared to that of PE60, only contained about only 24% of the anti-oxidation activity. The low anti-oxidation activity

in our laboratory preparation of plum extracts may have been due to their low content of phenolic compounds. Future experiments are planned to further validate the anti-oxidation activity of these extract in a cellular model.

In the initial experiments, PE60 inhibited the growth of colon cancer cells at about 150µg/ml whereas laboratory preparation of plum extract was not effective. It was clear that due to very low contents of polyphenols in the laboratory preparation of plum extracts, we did not find their effects on colon cancer cells. We, therefore, further tested the laboratory preparation of plum extract by adjusting their amount so that their polyphenolic contents were in the range of PE60 phenolic contents. It is evident from our results that the methanolic extract of plum skin was more effective than that of water extract. Our data showed that laboratory preparation of plum methanolic-extracts at 75µg/ml phenolic content inhibited about 70%-75% growth of colon cancer cells after 24hr. incubation. In comparison, PE60 at 75µg/ml only inhibited 40-50% colon cancer growth. This data, therefore, suggest that our laboratory preparation of plum's skin extract was more effective than PE60 when adjusted to their phenolic contents. However, there are limitations with MTT assay because drugs can interfere mitochondrial activity and, therefore, the effect of drugs may be over or under estimated [15]. During the present investigation, the effects of MTT were validated by microscopic examination and no discrepancies between two assays were noticed.

In addition to the cytotoxic effects of plum extracts, we also observed that the laboratory prepared plum extract induced a big bubble formation in colon-26 cells which was also evident when cells were treated with PE60, but to a lesser extent. Initially it was thought that the bubble structures are probably swollen nuclei. To test this possibility, cells were stained with Hoechst No. 33342 dye to stain nuclei. To our surprise, the round structures were not nuclei but appear to be vacuoles in each cell. The identity of the vacuoles is not known, but it may be water vacuoles as colon cells accumulate water in adverse situations and cause diarrhea. The data suggest that the laboratory prepared extracts may have some phenolic compounds that have caused the vacuoles formation and these compounds may be present in a lesser quantity in the PE60 extract. Further experiments are planned to isolate polyphenols from laboratory prepared extracts and PE60 and to test them head-to-head for further validation of polyphenols role in plums in preventing colon cancer. In addition, future experiments are also required to investigate if the plum extracts are active on other colon cancer cell lines as well as in *in vivo* animal models, and also to determine their effects on normal colon cells both under *in vivo* and *in vitro* conditions.

Plums have various shades of purple color. The purple pigment also has various substances such as chlorogenic acid, flavonoid and anthocyanin which work simultaneously through different signaling pathways to induce death in colon cancer cells [9]. It is interesting to note that colon cancer is also associated with high blood pressure and purple potatoes help in regulating and lowering blood pressure due to their effects on blood vessels and capillaries. Similarly, purple potatoes also have the high concentration of chlorogenic acid, which is shown to reduce colon cancer in an animal model [16]. Similar to purple potatoes, plums also have variable shades of purple pigments which are rich in chlorogenic acid [17] and chlorogenic acid has been shown to inhibit growth of human cancer cells lines [18]. During the

present investigation, no attempt was made to identify the phenolic compounds in plum extracts. Since, methanolic extract was more effective than water extract; it suggests that high molecular weight polyphenolic compounds may be responsible for the anticancer activity for colon cancer because they dissolve to a much greater extent in methanol. We found low concentration of phenolic compounds in pulp; however, the mass of pulp is far more than that of skin. It is, therefore, possible that the total amount of phenolic compounds present in the whole pulp may have considerable effect on inhibition of colon cancer growth despite the lower concentration of polyphenols. Furthermore, plum pulp is also a good source of dietary fibers. Fibers are known to produce butyric acid from digestion by gut bacteria [19]. Butyric acid has been shown to contain potent anticancer activity for colon cancer [20]. We did not test the effect of whole pulp fibers on colon cancer during present investigation.

Our study concludes that the effects of plums were dependent on their phenolic contents. Plum skin contained more concentrated phenolic compounds and greater anti-oxidation activity than pulp. Methanol extracts of plum skin was better than water extract of plum skin and effectively inhibited proliferation of colon-26 cells. The amount of plum skin is very small compare to that of pulp. In order to meet the high polyphenol intake, perhaps one need to eat a large quantity of plums, which may be a difficult task. Alternatively, the skin of plums can be removed and the active compounds can be extracted in the methanolic extract. After removing the methanol, the dried powder can be used in a tablet or capsule form to meet the high intake of plum-derived bioactive compounds for preventing colon cancer. In addition, plum extract can be used as an adjuvant supplement with current chemotherapies to better treat colon cancer patients.

In addition, future studies are required to isolate and identify the chemical nature of the active compounds. This information will help to understand the mechanism of its effect on colon cancer cells. The active compound (s) has a potential to be used as a pharmaceutical drug in the future, if found to be potent in pre-clinical or clinical studies, In conclusion, regular consumption of whole plum fruits or in the form of concentrated "whole fruit juice" may be a potential strategy for preventing colon cancer.

### Authors' Contribution

Concept, design, manuscript preparation, manuscript editing, and manuscript review: Rafat A Siddiqui, Paul Kaseloo and Sarah Witiak.

Experimental studies, data acquisition, data analysis, statistical analysis: Haiwen Li and Noura Alzahrani.

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### Conflicts of interest

There are no conflicts of interest.

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