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Chemopreventive Potential of Sunflower Seeds in a Human Colon Cancer Cell Line

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ABSTRACT

Sunflower Seed (SS) (*Helianthus annuus* L.) is an oil seed crop that is a good source of protein. The objective of this study was to investigate the chemopreventive potential of SS extracts (defatted and whole) through determination of phytochemical content, antioxidative potential and cytotoxic effects on Caco-2 cells. Phytochemical (total phenolic, total flavonoid) content, Trolox Equivalent Antioxidant Capacity (TEAC), free radical-scavenging ability of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Potential (FRAP) were determined according to standard protocol. Lactate dehydrogenase (LDH) release (cytotoxicity assay) was used to measure *in vitro* cell damage of Caco-2 colon cancer cells. Activity of cellular detoxification and antioxidant enzymes was determined with selected concentrations of SS extracts (25, 50 and 100 µg mL^{-1}). Apoptotic activity was assessed through the activity of Caspase-3 and DNA fragmentation ELISA. Anti-inflammatory activity was assessed through Cox-2. Total phenolic content was 62.76±7.37 mg GAE/100 g and total flavonoid content of SS extracts was 26.49 mg CE/100 g. Antioxidant potential: TEAC was 858.83±97.7 µmol TE/100 g, DPPH radical scavenging (IC$_{50}$ value) was 8 mg mL^{-1} and FRAP was 14.13±1.25 mmol/Fe^{+2}/g for SS extracts. The LDH release (%), cytotoxicity, after 12 and 24 h incubation with SS extracts was 39.8 and 53.1% at 25 µg mL^{-1}. Caspase-3 activity was 18.60±0.19, Cox-2 was 0.28 U mL^{-1} and DNA fragmentation ELISA was 0.47 EF at 24 h incubation of extract at 100 µg mL^{-1}. The results for the antioxidant and detoxification enzymes yielded were: GST (0.0017±0.001 nmol min^{-1} mL^{-1}), CAT 24.1±0.13 U mg^{-1}, GPx 30.4±0 nmol min^{-1} mL^{-1}, SOD 313.3±3.98 U mL^{-1} and GSH 13.7± nmol mL^{-1}. Results of this study revealed high phenolic and flavonoid content and suggested cytotoxic and antioxidative potential of SS extracts as a chemopreventive agent.

Key words: Phytochemicals, colorectal cancer, cancer prevention, cytotoxic, sunflower seeds, antioxidant potential

INTRODUCTION

Helianthus annuus L. (sunflower) belongs to the Asteraceae plant family and is native to North America (Chandler *et al.*, 1986). As a good source of vitamin E, sunflower seeds are excellent sources of energy and are often utilized for livestock feed (Mapiye *et al.*, 2013). In the United States, production of sunflower seeds is for the development of oil and also for their use as toppings in salads and snacks.

Phytochemicals such as flavonoids and polyphenols are present in sunflower seeds (Nadeem *et al.*, 2011). Phytochemicals provide functional benefits such as cancer blocking agents by scavenging free radicals that induce phase II enzymes (Glutathione-S-Transferase (GST), uridine diphosphate (UDP)-glucuronyltransferase and DNA repair). Sunflower seeds exhibit antioxidant and detoxifying properties (Stoner *et al.*, 1997). Free radicals are Reactive Oxygen Species (ROS) and can also be reactive nitrogen, chlorine, bromine and oxygen species (Halliwell, 2006). The ROS can be found in all living organisms that utilize oxygen (Sharma, 2013).

Phytochemicals may inhibit the progression of cancer (Kelloff *et al.*, 2000). Phytochemicals are added to various foods to enhance antioxidant activity and many combinations of phytochemicals are added as they work synergistically to provide biological effects to consumer (Nadal-Serrano *et al.*, 2013). The presence of tocopherols and tocotrienols (vitamin E) enables sunflower seeds to have free radical scavaging ability in lipid peroxidation (Sattler *et al.*, 2004). The three stages of lipid peroxidation are initiation, propagation and termination. During the propagation phase, tocopherol can function as an electron donator thus stabilizing the unstable molecule and further leading to the termination of lipid peroxidation (Schneider, 2009).

Colon cancer is an increasing problem that may be prevented through dietary changes. Originating in the digestive tract, the tumor cells metastasize and are able to invade the colon. The mechanisms that cause colon cancer vary, however, the genesis of colon neoplasms is affected by genetic factors. For example, medelian tumor syndrome increases the risk of developing colorectal adenomas (Lamlum *et al.*, 2000) and other factors such as mutations, overexpression of enzymes and chromosomal abnormalities are linked to cause adenoma formation (Gustin and Brenner, 2002). Little is known about the anti-cancer properties of Sunflower Seeds (SS) because further *in vitro* and *in vivo* studies are needed in order to understand the mechanisms in disease prevention (Russo, 2007). Most *in vitro* studies conducted on SS focused on its toxicological effects and very few studies were on anti-cancer properties of SS (Bradford and Awad, 2010; Fernandez-Blanco *et al.*, 2015; Al-Jumaily *et al.*, 2013). The aim of this study was to determine the phytochemical content, antioxidant potential and *in vitro* effects of sunflower seed extracts on human colon cancer Caco-2 cell line.

MATERIALS AND METHODS

Sample preparation: Solvents utilized in the experiment were of pure analytical grade and obtained from the Sigma Chemical Company (St Louis, MO.) and the Fisher Scientific Company (Suwanne, GA). Experiments were performed using organic Sunflower Seeds (SS) purchased from a local health food store in Huntsville, Alabama, US, ground Sunflower Seeds (SS) (453 g) were defatted for 8 h using soxhlet extractor and refluxed with n-hexane (200 mL). The SS samples were evaporated at 40°C using a rotary evaporator (Safe-aire, Fisher Hamilton, Gaithersburg, MD). Methanol (80%) was used for the final SS extracts value of 10 mL and stored at -20°C until further analysis (Weisz *et al.*, 2009; Seneviratne *et al.*, 2009).

Determination of total phenolic and flavonoid content: The Folin Ciocalteau method as outlined by Singleton *et al.* (1999) and expressed as mg GAE/100 g dry weight was used for the determination of total phenolic content. Total flavonoid colorimetric assay as described by Marinova *et al.* (2005) with modifications and expressed as Catechin Equivalents mg/100 g dry weight was used for the determination of flavonoid content.

2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability: Free radical scavenging activity was determined as outlined in the methods by Brand-Williams *et al.* (1995). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) concentration mixture (0.1 mM) was incubated with sample in the dark and the absorbance was measured at 517 nm for 30 min time intervals using a microplate reader (Synergy HT, Bio Tek Instruments, Winooski, VT, US). The DPPH percentage of inhibition was determined using the following calculation:

$$\text{Inhibition of DPPH (\%)} = \frac{\text{Abs control-Abs sample}}{\text{Abs control}} \times 100 \qquad (1)$$

Ferric Reducing Antioxidant Power (FRAP): The reducing power was determined with modifications using the method (Benzie and Strain, 1999) and the results for the Ferric Reducing Antioxidant Power (FRAP) was expressed as mmol Fe^{2+}/g dry weight.

Trolox Equivalent Antioxidant Capacity (TEAC): Trolox Equivalent Antioxidant Capacity (TEAC) was measured using the methods described by Zulueta *et al.* (2009) with modifications. Results were expressed as µmol TE/100 g and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at a concentration of 1 mM served as the stock standard.

Oxygen Radical Absorbance Capacity (ORAC): Oxygen Radical Absorbance Capacity (ORAC) assay was performed (Zulueta *et al.*, 2009) with modifications. The AAPH radical (2, 2-azinobis (2-amidinopropane) dichloride) was used to generate the radical species. The assay was conducted for 60 min at 37°C in a microplate reader (Synergy HT, Bio Tek Instruments, Winooski, VT, US). The following formula was used to calculate the ORAC activity:

$$\text{AUC} = 1+\text{RFU1/RFU0}+\text{RFU2/RFU0}+\text{RFU3/RFU0}+\ldots\ldots+\text{RFU60/RFU0} \qquad (2)$$

where, AUC is the area under the curve and RFU_0 is the relative fluorescence value at time zero.

Cell culture: The Caco-2 cells (American Type Culture Collection (ATCC, Manassas, VA) were seeded in a 24 well culture plate and incubated at 37°C and 5% CO_2. Cells were maintained in a mixture of Dulbecco's Modified Eagle Media (DMEM) with 10% Fetal Bovine Serum (FBS), until the development of a monolayer.

Lactate dehydrogenase (LDH) assay: Lactate dehydrogenase (LDH) released by cell damage of Caco-2 (colon cancer) cells was determined by incubation for 12 and 24 h with varying concentrations of SS extracts (25-400 µg mL^{-1}) prepared with fresh DMEM serum free media. Cells were also treated with 80% methanol in DMEM serum free media to serve as control (0 µg mL^{-1}).

Enzyme assays: Enzymatic activity was measured spectrophotometrically for Glutathione-S-Transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) and also quantitatively measured reduced glutathione (GSH) at respective wavelengths using the methods outlined in the Cayman's Assay Kits (Cayman Chemical Company, Ann Arbor, MI, US) (Griffith, 1985; Habig *et al.*, 1974; Jaskot *et al.*, 1983; Folhe and Otting, 1984; Johansson and Borg, 1988).

Cyclooxygenase (Cox-2): Cyclooxygenase was determined by measuring the oxidation of tetramethly-p-phenylenediamine (TMPD) at 590 nm (Kulmacz and Lands, 1983).

Apoptotic activity: Caspase-3 activity was determined colorimetrically (405 nm) in Caco-2 cells treated with SS extracts. Cellular DNA Fragmentation ELISA was measured spectrophotometrically (450 and 690 nm) according to manufacturer instructions (Promega Corporation, Madison, WI).

Statistical analysis: Analysis of data was reported as ±SEM and analyzed using the SAS 9.1 software. Determination of significant differences among treatment groups and means were determined using ANOVA and Tukey's standardized range test. The significance level was established at $p \le 0.05$.

RESULTS

Total phenolic and total flavonoid content: Chemical analysis of the SS extracts revealed the presence of total phenolics and flavonoid compounds (Table 1). A temperature affects was seen between the four extraction methods. A significantly ($p<0.05$) high phenolic content was observed in SS defatted-25°C extracted sample. The results showed that the removal of lipid content present in sunflower seeds, increases the extractability of phenolic compounds and other constituents present in sunflower seeds. In addition, a temperature affect was observed at a higher phenolic content in whole-25°C and defatted-25°C SS sample. There was no significant difference found in flavonoid content among defatted-25°C, defatted-170°C and whole-170°C. Highest Total Phenolic Content (TFC) content was seen in defatted-25°C (Table 1), revealing the increased extractability of TFC with removal of lipid content.

Antioxidant activity: Antioxidant activity of SS extracts was determined utilizing the DPPH. Results showed that low concentrations (8-10 mg mL^{-1}) of SS extracts were able to scavenge DPPH radical by 50% and peeked at a concentration of 20 mg mL^{-1} (Fig. 1 and Table 2).

Results obtained from the TEAC assay reported a higher ABTS radical scavenging ability in the SS extracts defatted-170°C (Table 3). The TEAC of SS was significantly ($p<0.05$) higher in defatted-170°C (858.83 μmol TE/100 g) in comparison to whole-25°C (629.06 μmol TE/100 g), whole-170°C (736.43±88.98 μmol TE/100 g) and defatted-170°C (496.21 μmol TE/100 g).

Table 1: Total phenolic and flavonoid content of sunflower seed extracts

Sunflower seeds	Total phenolic content (mg GAE/100 g)	Total flavonoids content (mg CE/100 g)
Whole-25°C	48.39±2.32[b]	8.24±0.67[b]
Whole-170°C	46.99±3.27[b]	24.71±0.23[a]
Defatted-25°C	62.76±3.37[a]	26.49±1.19[a]
Defatted-170°C	36.74±2.41[c]	23.91±1.53[a]

Values are Means±SEM, n = 3. [abc]Values not sharing common superscript are significantly different ($p<0.05$) by Tukey's studentized range test. Whole and defatted: Extraction at temperatures of 25 and 170°C, GAE: Gallic acid equivalents, CE: Catechin equivalents

Table 2: DPPH in sunflower seed

DPPH IC$_{50}$ (mg mL^{-1})

Treatments	Sunflower seeds
Whole-25°C	8
Whole-170°C	9
Defatted-25°C	10
Defatted-170°C	9

Whole and defatted: Extraction at temperatures of 25 and 170°C, DPPH: 2, 2-Diphenyl-1-picrylhydrazyl
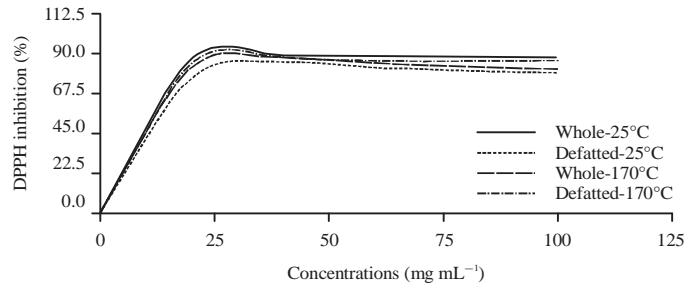
Fig. 1: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of sunflower seed extracts
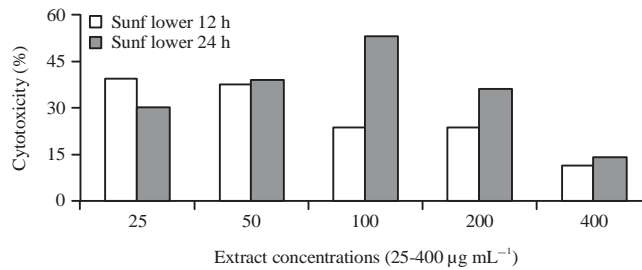

Fig. 2: Lactate dehydrogenase release (cytotoxicity) of Caco-2 cells treated with sunflower seed extracts for 12 and 24 h

Table 3: Antioxidant potential of sunflower seed extracts

Sunflower seed	TEAC (µmol TE/100 g)	FRAP (mmol/Fe^{+2}/g)	ORAC (µM TE/g)
Whole-25°C	629.06±8.35[c]	24.08±3.93[a]	493.30±0.605[c]
Whole-170°C	736.43±8.98[b]	14.13±1.25[b]	29576.15±37.70[a]
Defatted-25°C	496.21±5.01[d]	6.82±2.99[c]	513.57±1.59[c]
Defatted-170°C	858.83±12.756[a]	11.44±5.99[b]	12532.10±42.1[b]

[abc]Values are Means±SEM, (n = 3) not sharing common superscript are significantly different (p<0.05) by Tukey's studentized range test. Whole and Defatted: Extraction at temperatures of 25 and 170°C, FRAP: Ferric reducing antioxidant power, Fe^{2+}: Ferrous ion, ORAC: Oxygen radical absorbance capacity assay, TEAC: Trolox equivalent antioxidant capacity

The reducing ability of SS extracts as determined by FRAP yielded a higher value in whole-25°C compared to the other extraction methods. Results showed that the reducing ability of SS extracts is increased when the cellular matrix of the sunflower seeds are subjected to a temperature of 25°C. The ORAC assay expressed as (µM TE/g) showed the significantly higher value of ORAC was in whole-170°C SS extract.

Lactate dehydrogenase (cytotoxicity (%)): To determine the cytotoxicity effects of SS extracts on colon cancer cells, Caco-2 cells were incubated for 12 and 24 h with varying concentrations (25-400 µg mL^{-1}) of SS extracts (Fig. 2). After a 12 h incubation period, sunflower seeds were able to induce the highest percent cellular cytotoxicity at a low concentration of 25 µg mL^{-1} in Caco-2 cells. With an increase in concentration there was a decrease in lactate dehydrogenase release. After a 24 h incubation period, sunflower seeds were able to induce the highest lactate dehydrogenase release in Caco-2 cells at a concentration of 100 µg mL^{-1}. With a 24 h incubation period that by increase in concentration of SS extracts from 25-100 µg mL^{-1} also increased in percentage of cellular cytotoxicity.
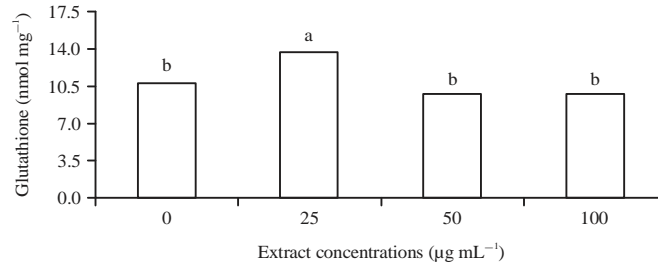
Fig. 3: Glutathione (GSH) levels of Caco-2 cells treated with sunflower seed extracts for 24 h

Table 4: Caspase-3 activity in Caco-2 cells treated with sunflower seed extracts

Concentrations (μg mL^{-1})	Sunflower seed extracts
0	1.00±0.03[c]
25	1.38±0.07[b]
50	1.56±0.02[b]
100	2.19±0.09[a]

Values are Mean ±SD, n = 3. [abc]Values not sharing common superscript are significantly different (p<0.05) using Tukey's studentized range test

In cells treated with SS extracts, percent cytotoxicity (Fig. 2) for 12 h ranged from 11.5-39.8%. With an increase in concentration, there was a decrease in LDH release. Highest LDH release was seen at a concentration of 25 μg mL^{-1}. At this concentration, the SS extracts were able to affect the cellular integrity of the Caco-2 colon cancer cells by inducing cellular cytotoxicity. At concentrations higher than 25 μg mL^{-1}, there was a decrease in the release of LDH from the cytosol in Caco-2 colon cancer cells (23.7-11.5). The LDH (cytotoxicity %) in Caco-2 cells treated with SS extracts for 24 h ranged from a low of 13.9 to a high of 53.1%. Highest LDH (cytotoxicity %) in Caco-2 cells treated with SS extracts, for 24 h was at a concentration of 100 μg mL^{-1}. With an increase in concentration of 25-100 μg mL^{-1}, LDH release increased from 30.1-53.1%, after which there was a decrease (39.2%) in LDH.

Enzymatic activities: In cells treated with SS extracts, there were significant differences in reduced glutathione content in Caco-2 colon cancer cells (Fig. 3 and 4). The highest GSH content was observed in cells treated at 25 μg mL^{-1}. Concentrations of 50 and 100 μg mL^{-1} were also not statistically different for GST activity. The GST activity was lower in cells treated compared with 0 (10.7) to 25 μg mL^{-1} (13.7 nmol mL^{-1}). There was a higher GST activity in cells treated with a concentration compared of 50 (9.85) to 100 μg mL^{-1} (9.79 nmol mL^{-1}). The GPx activity of SS was highest at a concentration of 50 μg mL^{-1} (30.4 nmol min^{-1} mL^{-1}). There were significant differences in GPx activity in cells treated with concentrations of 0, 25 and 100 μg mL^{-1}. The SOD activity was different in cells treated with defatted concentrations. Highest SOD activity was seen at 50 μg mL^{-1} (313.3 U mL^{-1}). The CAT activity in Caco-2 cells was significantly higher in control cells (0 μg mL^{-1}) (24.1 U mg^{-1}). With increasing concentration of SS extracts, there was a decrease in CAT activity. The lowest CAT activity was observed in cells treated at a concentration of 100 μg mL^{-1} (18.0 U mg^{-1}).

Cellular DNA fragmentation ELISA and caspase-3 and Cox-2 activities: Caspase-3 (Table 4) activity was significantly higher in cells treated with SS extracts at a concentration of 100 μg mL^{-1} (18.60±0.190). With an increase in concentration, there was an increase in caspase-3
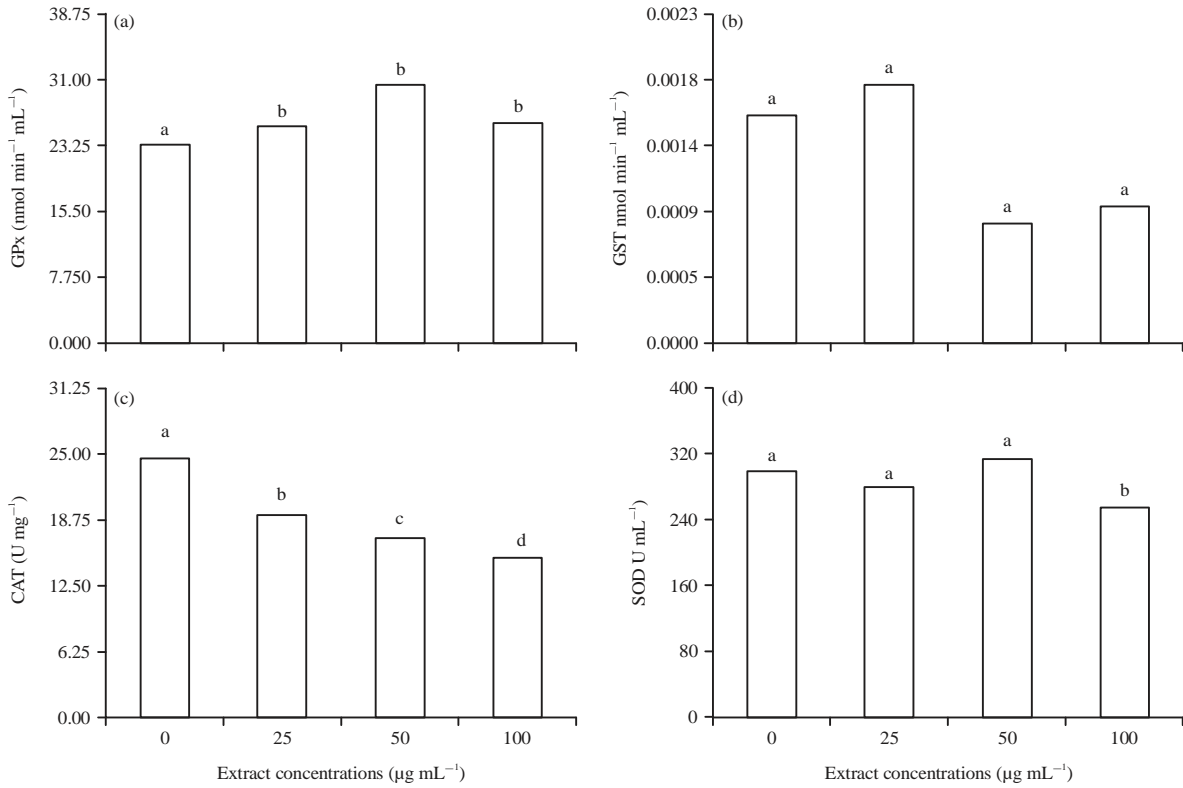
Fig. 4(a-d): Enzymatic activity of antioxidant and detoxification enzymes (a) Glutathione peroxidase (Gpx), (b) Glutathione-s-transferase (GST), (c) Catalase (CAT) and (d) Superoxide dismutase (SOD) in Caco-2 cells treated with sunflower seed extracts for 24 h
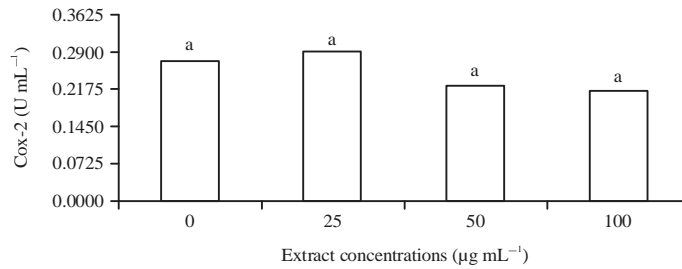

Fig. 5: Cyclooxygenase (Cox-2) activity of Caco-2 cells treated with sunflower seed extracts for 24 h

activity when compared to the control. In Caco-2 cells treated at a concentration of 25 µg mL^{-1}, an increase was seen in Cox-2 activity (Fig. 5) compared to the control (0.27 U mL^{-1}). There were no significant differences among cells treated with different extract concentrations Cox-2 activity. DNA fragmentation of Caco-2 cells (Fig. 6) treated with SS extracts was highest at a concentration of 100 µg mL^{-1}, however, there was a slight increase at 50 µg mL^{-1} (0.46 EF) and 100 µg mL^{-1} (0.47 EF).
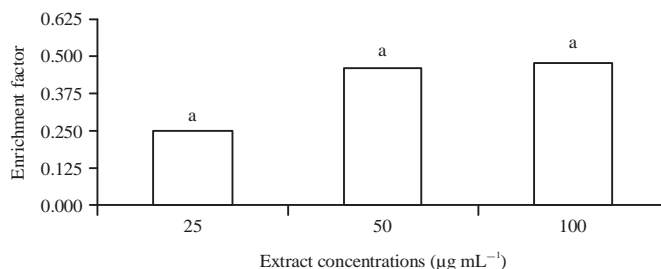
Fig. 6: Cellular DNA fragmentation ELISA of Caco-2 cells treated with sunflower seed extracts for 24 h

DISCUSSION

Phenolic acids (resveratrol), flavonoids (genistein), vitamins (tocopherols) and carotenoids (lycopene) are active dietary chemoprevention compounds (Russo, 2007). Research on antioxidants, as a form of chemoprevention is increasing (Bagchi *et al.*, 2014; Singh, 2014; Klotz, 2014; Danaraddi *et al.*, 2014). Results of the study reveal the presence of phenolic and flavonoid content (Table 1) with increased antioxidant potential (Fig. 1, Table 3). The use of a higher temperature appeared to affect the extractability of the phenolic and flavonoid compounds, thus increasing the scavenging ability of the SS extracts in defatted-170°C and whole-170°C compared to defatted-25°C and whole-25°C. In the presence of heat, chemical bonds are readily broken (Berg *et al.*, 2002). In addition, heat affected the cellular matrix of the sunflower seeds allowing for the release of phenolic and flavonoid compounds along with other phytochemicals that may contribute to the antioxidant potential of SS extracts. Sunflower seed oil, when utilized in cooking, can withstand high temperatures of 232°C while still remaining stable. Vitamin E, a powerful antioxidant is present in sunflower seeds and olive oil, which can contribute to its oxidative stability (Velasco and Dobarganes, 2002).

The results show that the whole-170°C and defatted-170°C SS extracts values yielded the highest ORAC among extract treatments. The higher temperature possibly allowed for the release of phenolic and flavonoid compounds along with other constitutes like vitamin E present in sunflower seeds. These finding are similar to the findings of White *et al.* (2009), who reported an increase in flavonoid content of cranberry pomace with an increase in extraction temperature. There results also revealed higher ORAC values of cranberry extracts (170 and 190°C) compared to whole cranberries.

The results (Fig. 1) showed that all extraction methods were able to inhibit the DPPH radical. The defatted-25°C extracts revealed elevated DPPH radical savaging ability with an IC_{50} of 10 µg mL^{-1}, which may be attributed to the highest total phenolic and flavonoid content in that extract.

Natural plant products (sesame oil and sunflower oil) are reported to have substantial anti-tumor effects in both *in vitro* and *in vivo* models (Kapadia *et al.*, 2002). In the present study the human colon cancer cell line Caco-2 was used to determine the *in vitro* effects of sunflower seed extracts. The SS extracts (25 µg mL^{-1}) were able to affect the cellular integrity of the Caco-2 colon cancer cells by inducing cellular cytotoxicity. With an increase in concentration, there was an increase in caspase-3 activity when compared to the control. Caspase-3 is an effector caspase that is associated with program cell death and is activated by initiator caspases that are associated with program cell death (Porter and Janicke, 1999).

The results from this study can serve as a consideration for further combination chemotherapy studies. Chang *et al*. (2012) conducted an *in vitro* study on the colon cancer cell line DLD-1 with the specific knockout gene GRP78 that plays a role in the development of colon cancer. They concluded that by utilizing the chemotherapy drug epirubicin in conjunction with two antioxidants, dithio-threitol (DTT) and Propyl Gallate (PG) in knockdown mice resulted in cellular apoptosis. The sunflower seed extracts could enable the food industry to explore the use of SS in functional food product development.

CONCLUSION

The temperature utilized for the extraction of phenolics and flavonoids greatly affected the extractability. Alternative processing methods of both whole and defatted may be utilized in the food industry in order to enhance and achieve the desired phytochemical content of SS extracts for use as a nutraceutical. Sunflower seed extracts being a natural source of antioxidants is supported by the antioxidant potential revealed by results obtained from TEAC, FRAP, ORAC and DPPH. The increased activity in select antioxidant and detoxification enzyme activities suggests that SS extracts may act as antioxidant and detoxifying agents. Further *in vivo* studies are needed to determine the anti-cancer effect of SS extracts on colon cancer in animal models.

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