RESEARCH ARTICLE

In Vitro Antioxidant and Antiproliferative Activities of Novel Orange Peel Extract and It's Fractions on Leukemia HL-60 Cells

Kawthar AE Diab[1,3]*, Reham Ezzat Shafik[2], Shin Yasuda[3]

Abstract

In the present work, novel orange peel was extracted with 100%EtOH (ethanol) and fractionated into four fractions namely F1, F2, F3, F4 which were eluted from paper chromatographs using 100%EtOH, 80%EtOH, 50%EtOH and pure water respectively. The crude extract and its four fractions were evaluated for their total polyphenol content (TPC), total flavonoid content (TFC) and radical scavenging activity using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. Their cytotoxic activity using WST assay and DNA damage by agarose gel electrophoresis were also evaluated in a human leukemia HL-60 cell line. The findings revealed that F4 had the highest TPC followed by crude extract, F2, F3 and F1. However, the crude extract had the highest TFC followed by F4, F3, F2, and F1. Depending on the values of EC_{50} and trolox equivalent antioxidant capacity, F4 possessed the strongest antioxidant activity while F1 and F2 displayed weak antioxidant activity. Further, incubation HL-60 cells with extract/fractions for 24h caused an inhibition of cell viability in a concentration-dependent manner. F3 and F4 exhibited a high antiproliferative activity with a narrow range of IC_{50} values (45.9 - 48.9μg/ml). Crude extract exhibited the weakest antiproliferative activity with an IC_{50} value of 314.89 μg/ml. Analysis of DNA fragmentation displayed DNA degradation in the form of a smear-type pattern upon agarose gel after incubation of HL-60 cells with F3 and F4 for 6 h. Overall, F3 and F4 appear to be good sources of phytochemicals with antioxidant and potential anticancer activities.

Keywords: Novel orange peel - total polyphenol content - total flavonoid content - DPPH assay - HL-60 cells - CCK-8

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Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and '*westernized*' diets (Jemal et al., 2011). Leukaemia is a type of blood cancer manifested by the failure of cell death of abnormal white blood cells, or inability of hematopoietic cells to differentiate into functional mature white blood cells. Induction of differentiation or cell death in immature hematopoietic cells has been applied for leukemia prevention or therapy (Petrie et al., 2009). The National Cancer Institute estimates that in the United States 52380 individuals will be diagnosed with some form of leukemia in the year 2014, and thus, leukemia represents about 3% of all new cancer cases (NCI, 2014).

In recent years, great attention has been paid to the recovery of bioactive compounds from agro-industrial waste due to their ability to promote benefit for human health. These compounds are useful as beneficial food constituents, food flavours, antioxidant, cosmetics, or drug adjuvant (Yang et al., 2011). As per recent information, the world production of citrus is 128.9 million tons during 2011 (FAO, 2011). In 2010, 5.8 million tons of citrus fruit was produced in Egypt contained 2.58 million tons of orange (FAO, 2011). A large portion of this production is addressed to the industrial extraction of citrus juice which leads to huge amounts of residues, including seeds, peel and segment membranes. Peels represent between 50 to 65%of total weight of the fruits and remain as the primary by-product. In Egypt, major quantities of the peels are not further processed and produced serious environmental pollution (Siles et al., 2010).

Citrus flavonoids have been found to have health-

[1]*Genetics and Cytology Department, National Research Centre,* [2]*Chemistry of Tanning Materials and Leather Technology Department, National Research Centre, Dokki, Cairo, Egypt,* [3]*Laboratory of Food Functional Chemistry, School of Agriculture, Tokai University, Aso, Kumamoto, Japan *For correspondence: kawthar_diab@yahoo.com*

related properties including antioxidant, anticancer (Lai et al., 2013, Im et al., 2014), anti-mutagenic (Hosseinimehr and Karami, 2005; Demir et al., 2009), anti-allergic, anti-inflammatory and antimicrobial (Hamdan et al., 2013) activities. Additionally, citrus peels have been used as source materials for animal feed, fiber production and fuel production (Bampidis and Robinson, 2006; Lashkari and Taghizadeh, 2013). Conventional treatment chemotherapy could cause adverse and toxic side effects on normal cells while curing cancer and therefore fails to control the disease. The alternative solution for the harmful effects of synthetic agents is the use of natural plants, which provide outstanding contribution to modern therapeutics (Sultana et al., 2014). Leukemia HL-60 cell line, derived from a single patient with acute promyelocytic leukemia, provides a unique in vitro model system for studying chemotherapeutic agents as novel anti-cancer drugs (Li et al., 2007). The current study aimed to evaluate the total polyphenol content, total flavonoid content, antioxidant activity of crude extract and four fractions of orange peels. Additionally, their antiproliferative activity and DNA fragmentation assay were also evaluated in human leukemia HL-60 cells.

Materials and Methods

Chemicals and reagents

Folin-Ciocalteu reagent, chlorogenic acid (CA); 2, 2-diphenyl-2-picrylhydrazyl (DPPH); sodium nitrate ($NaNO^2$); sodium biocarbonate (Na_2CO_3); aluminum chloride ($AlCl^3$); dimethyl sulfoxide (DMSO) and streptomycin were purchased from Nacalai Tesque (Kyoto-Japan). (+)- Catechin and 6- hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (St Louis, MO, United States). Cell counting kit (CCK-8) assay, fetal bovine serum (FBS), RPMI-1640 medium were obtained from Dojindo Laboratories (Kumamoto, Japan), Biowest (Nuaillé, France) and Wako Chemicals Industries (Osaka, Japan) respectively.

Analytical Instruments

NMR experiments were recorded on Jeol spectrometer (Kyoto, Japan) in DMSO-d6 1H (500 MHz) and 13C (125 MHz) NMR; UV absorption spectra were measured on Shimadzu model-UV-240 (Kyoto, Japan); Column chromatography (CC) was performed using polyamide 6S (Riedel-De-Haen, German) and Sephadex LH-20 (Pharmacia) ; Two dimensional paper chromatography (TDPC) and paper preparative chromatography (PPC) were carried out on Whatman No. 1 and 3 MM paper, respectively, using the following solvent systems: (1) BAW (n-BuOH/AcOH/H2O, 6:1:2); (2) H_2O; (3) AcOH/H_2O (15:85).

Extraction and fractionation

Navel oranges (*Citrus sinensis L*) were cultivated under organic farming conditions in the Research and Production Station of National Research Centre, El-Nubaria district, El-Behira Governorate, Egypt. The soil of the station was classified as a sandy soil. The orange trees were eight years old. The orange peels were dried and ground into powder by electric a mill. Dry peels (300 g) were extracted with 100% EtOH (ethanol) and then evaporated under vacuum pressure at 55°C to yield viscous residue (250g). The concentrated EtOH extract was fractionated by PPC on Whatmann 3MM paper using solvent system (1) to yield four fractions namely F1, F2, F3 and F4 which were eluted from the paper using 100% EtOH; 80 %EtOH; 50% EtOH and H_2O respectively.

Determination of Total Polyphonol Content (TPC)

The microplate TPC method was based on the 96-well microplate Folin-Ciocalteu assay described by Al-Duais et al. (2009) with some modification. Briefly, the sample was dissolved in DMSO to prepare different concentrations ranging from 0.156 to 20 mg/ml. A total 25 μl of the diluted sample was mixed with 125 μl of 10% Folin-Ciocalteu reagent (v/v) and 125 μl of 10 % Na_2CO_3 (w/v) in a well of 96-well plate. The reaction mixture was kept in the dark at room temperature for 10 min with intermittent shaking. The absorbance of mixture was measured at 600 nm using microplate reader (SH-1000, Corona Electronics, Ibaraki, Japan) against blank solution which was prepared by same procedures described above except that Folin-Ciocalteu reagent was substituted by water. A calibration curve of chlorogenic acid (CA) was prepared under the same conditions as described above in the concentration ranging from 0.0625 to 2 mg/ml. The amount of TPC was expressed as mg CA equivalent (mg CAE)/mg sample through the calibration curve of CA. All experiments were repeated at least three times with quadruplicate wells in each experiment.

Determination of total flavonoid content (TFC)

The microplate TFC method was based on the 96-well microplate $AlCl^3$ method described by Herald et al. (2012), using catechin as the reference standard. A total 25 μl of the diluted sample in DMSO was mixed with 125 μl of Milli-Q water and 7.5 μl of 5% $NaNO_2$ for 6 min. Then 15 μl of 10% $AlCl^3$ was added and stood for 5 min followed by addition of 50 μl of 1M NaOH and 275 μl of Milli-Q-water to the mixture. The absorbance of the mixture was measured at 510 nm using microplate reader versus blank which prepared in similar way by replacing $AlCl^3$ with Milli-Q-water (15μl). The calibration curve of (+) catechin was prepared by the same procedures described above in the concentration range (0.0156-1 mg/ml). The amount of TFC is expressed in mg catechin equivalent per mg of sample (mg CE/mg sample).

Determination of antiradical activity by DPPH

DPPH radical scavenging method based on the 96-well microplate was determined according the procedures described by Herald et al. (2012) with some modification. A total of 10 μl of diluted sample in DMSO was mixed with 90 μl of 70% EtOH, 100 μl of 0.1M sodium acetate buffer (pH 5.5) and 50 μl of DPPH solution (0.5 mM in 100% EtOH). After 30 min from shaking at room temperature, the absorbance of the mixture reaction was measured at 517 nm using microplate reading versus blank which replaced 50 μl of DPPH with 50 μl of 100%EtOH

in the mixture reaction. Radical scavenging activity (RSA) was estimated as follows: RSA (%) = [1 - (OD of sample - OD of blank)/ (OD of control - OD of blank)] × 100. The antioxidant activity was expressed as EC_{50} (μg/ml) which is the effective concentration of extract required to inhibit a 50% of RSA. Trolox, a water-soluble analogue of vitamin E, was used as standard antioxidant reference suggested by Alam et al. (2013) to convert the inhibition capability of each sample to the trolox equivalent antioxidant capacity (TEAC). TEAC was calculated as the ratio between EC_{50} of trolox (μg/ml) and EC_{50} of extract (μg/ml).

Cell culture and treatment

Human leukemia HL-60 cell line was obtained as gift from Dr. Shin Yasuda (Tokai University, Kumamoto, Japan). The cells were maintained by twice weekly passage in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin sulfate. The cells were placed in 60 mm-diameter Petri-dish to grow in suspension culture at 37°C in a 5% CO_2 incubator with 90% humidity. The plant samples were dissolved in DMSO (<1%) as 50 mg /ml stock solution and were further diluted in culture medium before use to achieve final concentrations ranging from 1.0 to 500 μg/ml.

Cell viability and cytotoxicity assay

The cytotoxicity was measured by a quantitative colorimetric assay with CCK-8 based on the novel highly water-soluble tetrazolium salt, WST-8 [2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Berridge et al., 2005). Briefly, HL-60 cells (50 $\times 10^3$/100μl/well) were seeded together with various concentrations of plant samples into a 96-well plate. After 24 h, 20 μl of CCK-8 was added per well and the plate was incubated at 37°C in a 5% CO_2 incubator for an additional 3 h. WST-8 is reduced by dehydrogenase in the cells to produce a yellow-color formazan dye , which soluble in tissue culture media. The amount of formazan dye is directly proportional to the number of living cells and is measured at optical density of 450 nm using a 96-well microplate reader. The cell viability (%) = (OD of sample– OD of blank)/(OD of control - OD density of blank) × 100.

Analysis DNA damage by agarose gel electrophoresis

DNA damage was estimated by electrophoresis of genomic DNA from HL-60 cells as described previously (Tan et al., 2004). In brief, HL-60 cells (1×10^6 cells/ml) were seeded in a 30-mm tissue culture Petri-dish and treated with F3 and F4 at concentrations 100, 200 and 500 μg/ml for 6 h. The cells were harvested by centrifugation at 1000 rpm for 10 min at 4°C. The cells washed twice with 1 ml of PBS containing 20 mM EDTA. The cell pellets were lysed in 250 μl lysis buffer (100 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0 and 5% Triton X-100) containing 400 μg/ml DNase-free RNase at 37°C for 90 min followed by incubation with 20 μl of proteinase K (200 μg/ml) at 50°C. After incubation for 2 h, 100 μl of mixture of PCI (Phenol: chloroform: isoamyl alcohol in the ratio of 25:24:1) was added prior centrifugation at 10, 000 rpm for 5 min at 4°C. The upper aqueous layer containing DNA was transferred into new eppendorf tube and 100 μl of chloroform: isoamyl alcohol (24:1) was added followed by centrifugation at 10, 000 rpm for 5 min at 4°C. DNA was precipitated from the upper layer by addition of 100 μl isopropanol overnight at -20°C and then centrifuged at 10, 000 rpm for 10 min at 4°C. The pellets were washed, dried, dissolved in 12 μl of Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.4) mixed, in loading dye and electrophoresed in 1.8% agarose at 50V for 1.5 h in Tris borate EDTA buffer (TBE, 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH. 8.0). Agarose gel was stained with ethidium bromide (0.5 mg/ml in TBE) and was visualized under ultraviolet transillumination and the image was taken by gel documentation system (Bio-Rad).

Data analysis

Data were reported as means±standard deviation of three replicate determinations. Statistical analysis was performed with Statistical Package for Social Science (SPSS Inc, version 17, Chicago, IL, USA). Mean values were compared by one way analysis of variance (ANOVA) with Duncan's multiple range method. The results were considered statistically significant at p value less than 0.05. EC_{50} (median effective concentration) and IC_{50} (median inhibitory concentration) were determined using linear regression analysis. The Pearson correlation coefficients (r) were conducted between DPPH, TPC and TFC.

Results

Identification and characterization of flavonoids compounds

The 100%EtOH crude extract was fractionated by PPC on 3MM paper and eluted by solvent system(1) to obtain 4 bands or fractions. These bands were further fractionated using subcolumns or paper chromatography to give rise to 12 pure compounds as follows:

F1: The brown material which was obtained after evaporating the solvent (100% EtOH) was subjected to TDPC which revealed the presence of three major compounds. These three compounds were isolated and purified by applying on a Sephadex LH-20 column and eluted gradually with 25% EtOH, 50% EtOH, 75% EtOH, and final with 100% EtOH. These compounds were found to possess flavonoid nature on the basis of their Rf-values, colour properties and UV spectral data (Markham and Geiger, 1994) which were similar to quercetogetin (1), tangeretin (2), nobiletin (3).

F2: A brown viscous material was obtained after evaporating the solvent (80% EtOH). TDPC of F2 proved to contain mainly two major spots of flavonoid nature. The two compounds were separated by applying on a Sephadex LH-20 column using H^2O/EtOH as eluents. The two compounds were identified as scutellarein (4), sinensetin (5) according to their Rf-values, colour properties and UV spectral data (Markham and Geiger, 1994).

F3: TDPC of F3 which eluted with 50% EtOH revealed the presence of five main compounds possess flavonoid nature. F3 was concentrated and subjected to PPC using solvent system (3), which five bands were

separated, collected, eluted, and dried which afforded five pure compounds. The five compounds were identified as eriocitrin (6), narirutin (7), hesperidin (8), diosmin (9), rutin (10) by Cobalt phthalocyanine (COPC) using flavonoid markers as reference markers under UV light (Harborne et al., 1973).

F4: This fraction was eluted with pure H_2O. After evaporating the solvent under reduced pressure, it was subjected to TDPC which revealed the presence of two major compounds. These two compounds were isolated and purified by applying on a Sephadex LH-20 column and eluted with H_2O/EtOH to isolate each compound into pure form. The Rf-values, colour properties and UV spectral data (Markham and Geiger, 1994) indicated that the compounds were narirutin 4'-O-glucoside (11) 6, 8, -di-C-glucosylapigenin (12). The structures of 12 compounds were confirmed through 1H and 13^C -NMR spectral data (Agrawal et al., 1989; Markham and Geiger, 1994).

Determination of TPC and TFC

TPC was determined according to the Folin-Ciocalteau method which is based in the reduction of phosphomolybdic-phosphotungstic acid (Folin) reagent to a blue-colored complex in an alkaline solution (Cicco et al., 2009). The amounts of TPC determined in this way are not absolute measurements of the amounts of phenolic compounds but are in fact based on their chemical reducing capacity relative to an equivalent reducing capacity of standard reference such as chlorogenic (Rover and Brown, 2013). The TPC and TFC values for crude extract and its four different ethanol concentration fractions (100% EtOH, 80% EtOH, 50% EtOH and H^2O) were recorded in Table (1). In case of TPC, the highest TPC was found in F4 (80.46±12.31 μg CAE/mg extract) followed by crude extract (46.06±12.81 μg CAE/mg extract), F2 (43.84±5.63 μg CAEg/mg extract), F3 (35.21±3.32 μg CAE/mg extract) and F1 (24.61±2.64 μg CAEg/mg extract) in the decreasing order. These data suggested that fractionation of the crude extract concentrated the polyphenol compounds in F4 which contain highly water-soluble polyphenol compounds. Indeed, crude phenolic extracts contain complex mixtures of some classes of phenols, which are selectively soluble in the different solvents. The solubility of phenolics is governed by their chemical nature which may vary from simple to highly polymerized substance in different quantities, as well as the polarity of the solvents used (Dai and Mumper, 2010). Structurally, phenolic compounds have at least one aromatic ring with one or more hydroxyl groups and divided into 10 different classes depending on their basic chemical structure. Within these classes phenolic acids and flavonoids represent 30 and 60%, respectively of the total polyphenols intake with the Mediterranean diet. There is a possibility of interaction of phenolics with other plant components such as carbohydrates and proteins that may lead to the formation of complexes that may be quite insoluble (Heim et al., 2002, Tripoli et al., 2007). Likewise, solvent polarity play role in increasing phenolic solubility. Phenolic compounds are polar compounds with usually extracted with polar solvent such as ethanol and water and their combination (Garcia-Salas et al.., 2010). Addition of water to organic solvent usually creates a more polar medium which cause plant material to swell thereby allowing the solvent to penetrate more easily in the solid matrix and increase extractability of polyphenols (Spigno et al., 2007).

In the case of TFC, the crude extract was the richest one (31.43±9.62 μg CAE/mg extract) compared with its fractions. TFC was in the descending order as follows: crude extract>F4> F3>F2> F1. Our findings are similar to those obtained by Hegazy and Ibrahim (2012) who reported that ethanol extract of orange peel contains the highest content of TPC and TFC compared with other solvents such as dichloromethane, acetone, hexane and ethyl acetate. Likewise, TPC and TFC of orange peel extract reported in literature showed a great variation (Ghasemi et al., 2009; Hegazy and Ibrahium, 2012). This is may be attributed to geographical location of plants;

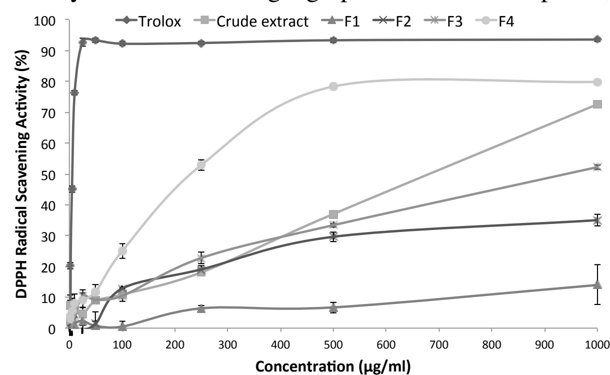

Figure 1. Antiradical Activity of Orange Peel Extract and its Fractions in DPPH Assay

Table 1. Determination of Total Phenolic and Flavonoid Content and Radical Scavenging Activity Capacity in Orange Peel Extract and its Fractions

Sample	TPC (μg CAE/mg extract)	TFC (μg CE/mg extract)	Antioxidant activity EC^{50} value (μg extract/ml)	TEAC value
Peel extract	46.06±12.81 [b]	31.43±9.62 [d]	693.714	0.01
F1	24.61±2.64 [a]	4.67±2.02 [a]	>1000	<0.006
F2	43.84±5.63 [b]	5.18±0.41 [ab]	>1000	<0.006
F3	35.21±3.32 [ab]	7.73±0.99 [bc]	950.22	0.007
F4	80.46±12.31 [c]	9.32±1.55 [c]	234.62	0.029
Trolox	------	-------	6.7	----------

*The experiment was performed in quadruplicate wells and repeated three times with similar results. The data were expressed as mean± SD. Values within the same column follow by different superscript letters are significantly different from one another ($p<0.05$) as determined by ANOVA followed by Duncan's multiple comparison test

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method of extraction used; extraction power of the solvent used and its chemical nature (organic or aqueous); degree of polymerization and interaction of these compounds with each other (Ghasemi et al., 2009; Oboh and Ademosun, 2012).

Determination of antiradical activity

Antiradical power of the plant samples was measured in term of hydrogen-donating ability using DPPH which is a stable, nitrogen-centered free radical and produces deep purple colour in ethanol solution. The principle of this assay is based on ability of an antioxidant to quench DPPH free radicals due to the power of hydrogen of donating ability and convert them from purple colour to a colourless/bleached product, DPPH-H (i.e., 2, 2-diphenyl-1-hydrazine or a substituted analogous hydrazine), resulting in a decrease in the absorbance of DPPH at 517 nm (Pisoschi et al., 2009). As shown in Figure (1), the extract/fractions were capable of scavenging DPPH radicals in a concentration dependent manner. It is very important to point out that a low EC_{50} value reflects a high antioxidant activity of the extracts, since the concentration necessary to inhibit the radical oxidation in 50% is low (Table 1, Figure1). Based on %RSA and EC_{50} values determined, F4 had the strongest antioxidant activity (236.62 μg extract/ml) followed by crude extract (693.71 μg extract/ml) and F3 (950.22 μg extract/ml). F1 and F2 exhibited the lowest antiradical activity more than 1000 μg extract/ml (Table 1). Positive control (Trolox) exhibited the highest antioxidant activity (EC_{50}=6.7 μg /ml) compared to the extract/fractions. TEAC value represents a comparative antioxidant activity measure of bioactive compounds in the extract with respect to a water soluble form of α-tocopherol (Trolox). It is a worth noting that, the higher %RSA, lower EC_{50} value and the higher TEAC value are reflected the higher antioxidant activity of the extracts (Oliveira et al., 2012). As shown in Table (1), F4 had the higher TEAC and the lower EC_{50} followed by crude extract followed by F3, while F1 and F2 were exhibited the lowest TEAC values and highest EC_{50} indicating antioxidant efficacy of extract/fraction attributed to influence of solvent polarity on the extractability of phenolics (Park et al., 2014). This implied that, the superiority of the F4 is probably due to its high polarity which allows the accumulation of a variety of polar antioxidant compounds. In contrast, the Inferiority of F1 and F2 are attributed to their lower polarities which allow the accumulation of non-polar polyphenol. This view was supported by the fact that, polar solvents are suitable for extraction of polar phenolic compounds like H_2O and EtOH. Indeed, EtOH tends to be good solvent for polar and non-polar compounds because it contains both a highly polar -OH group and a non-polar hydrocarbon portion. Consequently, lower concentration of ethanol is suitable for extraction of polar polyphenol compounds and higher concentration of ethanol is suitable for extraction of non-polar polyphenol compounds (Kotz et al., 2011). By increasing the proportion of water, the polarity of the ethanol also increases. When this is achieved, the aqueous ethanol system is able to extract phenolic substances from both ends of the polarity range (highest polarity substances and low polarity substances), as well as those of moderate polarity (Spigno et al., 2007).

The correlations between TPC, TFC and DPPH

The Pearson's correlation coefficients among TPC, TFC and DPPH were statistically analyzed and presented in Table (2). Positive significant correlation was observed between TPC and TFC for crude extract (r=0.905, p<0.05) attributed to flavonoids represented major part of the polyphenol. All flavonoids described in Citrus sp. can be classified into these groups: flavanones, flavones, flavonols, polymethoxyflavones and anthocyanins (Nogata et al., 2006). The major flavonoids in orange peel are flavanone glycosides such as eriocitrin (6), narirutin (7), hesperidin(8) and narirutin 4'-O-glucoside (11); flavones glycosides such as diosmin (9) and rutin (10) ; C-glycosylated flavones such as 6, 8, -di-C-glucosylapigenin (12). Polymethoxyflavones aglycones such as quercetogetin (1), tangeretin (2), nobiletin (3), scutellarein (4) and sinensetin (5) were distributed widely in orange peel (Tripoli et al., 2007).

Negative correlations were observed between TPC and DPPH assays of crude extract and its four fractions (Table 2). There are three possible explanations for this negative correlation. First, chemical structure of polyphenols, namely certain structure features such as number and position of phenolic hydroxyl groups, have a significant influence on radical scavenging activity (Jeong et al., 2007). Second, some nonphenolic compounds react with Folin-Ciocalteu reagent but not effective as free radical scavengers. Citrus peels are important source of antioxidant such as tannin, saponin, limonene, ascorbic acid, carotenoid, some essential mineral (Barros et al., 2012).

Table 2. Pearson's Correlation Coefficient between TPC, TFC and DPPH Scavenging Activity for Orange Peel Extract and its Fractions

Correlation	Peel extract	F1	F2	F3	F4
TPC & TFC	0.905*	-0.97	0.994	-0.999*	0.596
TPC &DPPH	-0.829*	-0.97	-0.867	-0.631	-0.995
TFC &DPPH	-0.703	-1.00**	-0.916	0.592	-0.674

*Correlation is significant at p<0.05; ** Correlation is significant at p<0.01

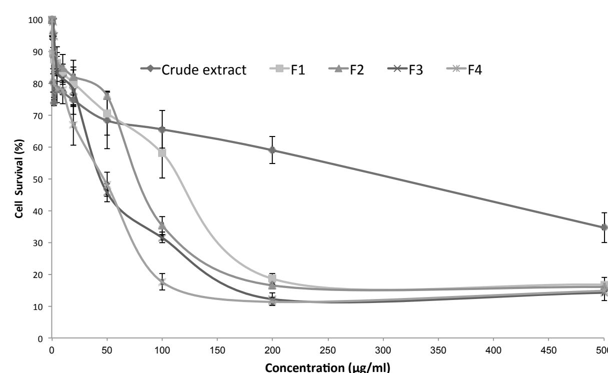

Figure 2. Cell Viability of HL-60 cells treated with crude extract and four fractions of orange peel for 24 h

A positive correlation was observed between TFC and DPPH assay for F3 (r=0.592) suggesting the intensity of antioxidant activity of flavonoids is markedly influenced by the number and position of hydroxyl groups on the B and A rings, and by the extent of conjugation between the B and C rings (Amic et al., 2007; Justino et al., 2009). According to literature evidence, the strongest antiradical activity determined using DPPH was observed for eriocitrin (6) which have two hydroxy groups bound to the B ring in ortho position in relation to each other. When one hydroxy group in the B ring of the flavonoids luteolin-7-O-rutinoside or eriocitrin was replaced by the methoxy one giving, respectively, diosmin (9) and hesperidin (8) the antiradical activity was weaker (Sroka et al., 2005). Flavonoid rutin (10) contributes the most to the total antioxidant activity since it possesses a catecholic B-ring, double bond at the 2, 3 position of the C ring, conjugated with the 4-oxo group (Barreca et al., 2013).

Negative correlations were observed between TFC and DPPH assay for crude extract, F1, F2 and F4. It seem that the losing OH group in ortho C3, 4, OH group in C3, oxo function in C4 and double bond at C2 -C3 which would influence scavenging capacity (Woodman et al., 2005; Wolfe and Liu, 2008). Recently, tangeretin (2) and nobiletin (3) did not scavenge the DPPH due to lacking of free hydroxyl groups (Yoon et al., 2011; Barreca et al., 2013). Moreover, the negative correlation may be attributed to synergistic and antagonistic interaction among the bioactive compounds (Wang et al., 2011).

Table 3. Antiproliferative Activity of Orange Peel Extract and its Fractions Against HL-60 Cell Line

Sample	IC_{50} (μg/ml)
Crude extract	314.89±0.99
F1	115.27±4.65
F2	98.93±10.1
F3	45.9±4.35
F4	48.9 ±1.94

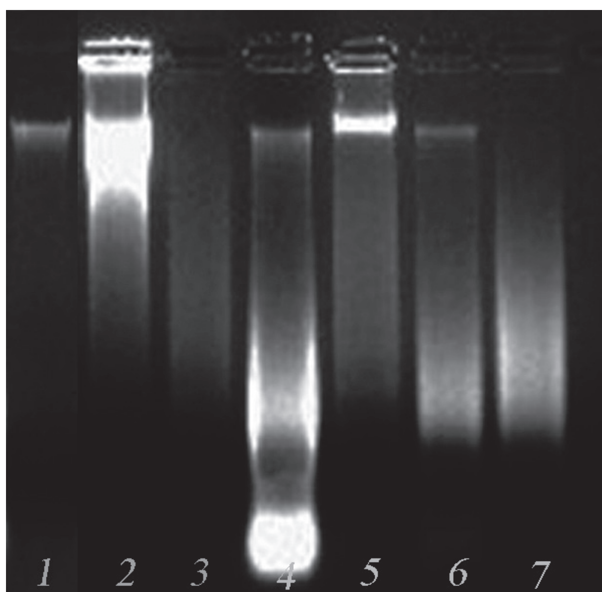

Figure 3. Agarose Gel Electrophoresis Demonstrating DNA Degradation in the form of Smear Pattern in the HL-60 Cells Treated with 100, 200 and 500 μg/ml of F3 and F4 for 6 h. Lane 1 (untreated cells), lanes 2 -4(100, 200, 500 μg/ml of F3), lanes 5-7 (100 , 200, 500 μg/ml of F4).

Cell viability assay

As shown in Figure (2) incubation HL-60 cells with extract/fractions at different concentrations (in the range of 1-500 μg/ml) for 24 h reduced the number of viable cells in a concentration-dependent manner. The susceptibility of cells to the extract exposure was characterized by IC_{50} values (Table 3). The antiproliferative potency of the extract/fractions in descending order was as follows: F3 > F4 > F2 > F1 > crude extract. According to Atjanasuppat et al. (2009), the antiproliferative activities of the extracts were categorized according to the values of IC_{50} into four groups: active (≤20μg/ml), moderately active (>20-100 μg/ml), weakly active (>100-1000 μg/ml), inactive (>1000 μg/ml). In the US National Cancer Institute plant screening program, a crude extract is generally considered to have in vitro cytotoxic activity if the IC_{50} value in cancer cells is ≤30μg/ml (Boik et al., 2001). Based on this criterion, F3 and F4 exhibited a moderate cytotoxic activity with narrow range of IC_{50} value (45.9 - 48.9μg/ml), while crude extract possessed the highest IC_{50} value (314.89 μg/ml) and the weakest cytotoxic activity against HL-60 cells. These data are in line with previous studies that reported that the citrus flavonoids exhibited their anticancer activity either in a form of individual compounds or in crude extract (Weng and Yen, 2012). For example, flavonoid glycosides such as narirutin (7), hesperidin (8) and diosmin (9) decreased cell viability of DU145 prostate cancer cells (Lewinska et al., 2015). Tangeretin (2) and nobiletin (3) inhibited proliferation of breast cancer (Chen et al., 2014), leukemia (Hsiao et al., 2014), gastric carcinoma (Dong et al., 2014) and lung carcinoma (Park et al., 2012; Charoensinphon et al., 2013). It was reported that the saturations of the A-ring of citrus flavonoids with methoxy groups positively correlated with increased biological activity. Thus compounds containing only five methoxy groups such as sinensetin (5) and tangeretin (2) had a weak antiproliferative activity against HL-60 cells (Li et al., 2007).

DNA fragmentation assay

Cancer cells frequently possess apoptotic defects. The induction of apoptosis is a primary mechanism of many anticancer agents to kill cancerous cells. Apoptosis is a programmed cell death and a highly organized physiological mechanism to destroy injured or abnormal cells. Apoptotic cells exhibit remarkable morphology features and characteristic molecular expression (Elmore, 2007). According to IC_{50} values, F3 and F4 displayed moderate antiproliferative activity and therefore they were chosen for DNA fragmentation to further understand the mechanistic pathway of apoptosis. HL-60 cells were incubated with F3 and F4 at the higher concentrations 100, 200, 500μg/ml. After 6 h incubation, the cellular DNA was isolated from HL-60 cells and subjected to 1.8% agarose gel electrophoresis. It was observed that the F3 and F4 induced random DNA degradation in the form of smear upon agarose. Similar results obtained by

Yumnam et al. (2014) who observed that no DNA ladder formation in the liver cancer cells HepG2 treated with hesperidin suggesting its ability to induce cell death by caspase independent apoptotic pathway (paraptosis). In fact, the detection of DNA smear, rather than of discrete internucleosomal DNA fragmentation, upon agarose gel electrophoresis suggest, possibly, that the mode of cell death was necrosis or a caspase-independent apoptotic pathway (Constantinou et al., 2009). The use of natural products as therapeutic agents against cancer has become very popular in the recent years considering the toxicity of chemotherapeutics. Natural products are considered to be safe and also reduce the mutagenicity in normal cells (Cragg et al., 2009). The cytostatic activity of plant extract on cancer cells is often much better than effect of their isolated active biological compounds, due to a complex interplay of the composite mixture of compounds present in the whole plant (additive/synergistic and/or antagonistic) rather than constituent single agents alone (Katiyar et al., 2012). In conclusion, citrus flavonoids inhibited cell proliferation of HL-60 via induction necrosis cell death. Further investigations are needed to anticancer mechanism of these compounds in HL-60 cells.

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