

RESEARCH ARTICLE

In Vitro Studies on Phytochemical Content, Antioxidant, Anticancer, Immunomodulatory, and Antigenotoxic Activities of Lemon, Grapefruit, and Mandarin Citrus Peels

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Abstract

Background: In recent years, there has been considerable research on recycling of agro-industrial waste for production of bioactive compounds. The food processing industry produces large amounts of citrus peels that may be an inexpensive source of useful agents. **Objective:** The present work aimed to explore the phytochemical content, antioxidant, anticancer, antiproliferation, and antigenotoxic activities of lemon, grapefruit, and mandarin peels. **Materials and Methods:** Peels were extracted using 98% ethanol and the three crude extracts were assessed for their total polyphenol content (TPC), total flavonoid content (TFC), and antioxidant activity using DPPH (1, 1-diphenyl-2-picrylhydrazyl). Their cytotoxic and mitogenic proliferation activities were also studied in human leukemia HL-60 cells and mouse splenocytes by CCK-8 assay. In addition, genotoxic/ antigenotoxic activity was explored in mouse splenocytes using chromosomal aberrations (CAs) assay. **Results:** Lemon peels had the highest of TPC followed by grapefruit and mandarin. In contrast, mandarin peels contained the highest of TFC followed by lemon and grapefruit peels. Among the extracts, lemon peel possessed the strongest antioxidant activity as indicated by the highest DPPH radical scavenging, the lowest effective concentration 50% ($EC_{50} = 42.97 \mu\text{g extract/mL}$), and the highest Trolox equivalent antioxidant capacity ($TEAC=0.157$). Mandarin peel exhibited moderate cytotoxic activity ($IC_{50} = 77.8 \mu\text{g/mL}$) against HL-60 cells, whereas grapefruit and lemon peels were ineffective anti-leukemia. Further, citrus peels possessed immunostimulation activity via augmentation of proliferation of mouse splenocytes (T-lymphocytes). Citrus extracts exerted non-cytotoxic, and antigenotoxic activities through remarkable reduction of CAs induced by cisplatin in mouse splenocytes for 24 h. **Conclusions:** The phytochemical constituents of the citrus peels may exert biological activities including anticancer, immunostimulation and antigenotoxic potential.

Keywords: Antioxidant assay - chromosomal aberration assay - cell viability - HL-60 cells - in vitro mouse splenocytes

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Introduction

Oxidative stress is defined as a disturbance between the formation of reactive oxygen species (ROS, pro-oxidants) and their elimination by antioxidant defenses mechanism. This disturbance is the primary cause of several health problems such as cancer, heart diseases, aging and neurodegenerative diseases (Alfadda and Sallam, 2012). ROS are produced from endogenous (internal) and / or exogenous (external) sources. Endogenous ROS is generated from physical and mental stress, restriction in blood supply to the tissues, microbial infection, cancer, and aging. Exogenous ROS is produced from radiation, environmental pollutions, pharmaceuticals and industrial chemicals (Klaunig et al., 2010). ROS can damage macromolecules (DNA, protein, lipid), leading to alternations in genetic material that may cause cancer (Reuter et al., 2010). In this sense, exploring the

antimutagenic/anticancer compounds from plant sources that are capable of repairing genomic changes are given a vast significance to protect human beings from human health problems (Roleira et al., 2015).

Citrus fruits are one of the most popular food crops in the world for their nutritional and therapeutic values. As per recent information in 2013, the world production of citrus fruits reached 135 million tons that harvested over 9.6 million hectares. Egyptian citrus fruits contributed to 4.09 million tons of the world production that harvested over 1.75 million hectares in 2013. The mandarin, lemon, and limes, and grapefruit are represented 28.6, 15.1 and 8.4 million tons of global production, respectively (FAOSTAT, 2015). Large quantities of citrus peel wastes are generated during citrus-juice processing industry. The citrus by-products are considered a low-priced source of bioactive compounds that can be economically exploited to boost the national income in the industrial area (Liu et

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al., 2012). Polymethoxyflavones (PMFs) are a class of novel flavonoids containing two or more methoxy groups that are entirely found in citrus peels, mainly orange peels (Wang et al., 2008). Chemopreventive activity of PMFs against the incidence of cancer was reported in vivo and in vitro systems (Fan et al., 2007; Lee et al., 2011; Wang et al., 2014).

The phytochemical content and antioxidant activity of citrus peels were evaluated in various regions of the world. However, little information is available on the antioxidant activity and bioactive content of Egyptian citrus species that cultivated under organic agriculture. Also, there has been very restricted data into cytotoxic, immunomodulatory and antigenotoxic activities of the citrus peels in particular lemon, mandarin and grapefruit peels. Therefore, the present work was designed to explore the following endpoints. (1) total polyphenol and flavonoid content; (2) antioxidant capacity by DPPH; (3) in vitro cytotoxic effect in human leukemia HL-60 cells and mouse splenocytes; (4) mitogenic proliferation response in mouse splenocytes; (5) chromosomal aberrations (CAs) in vitro mouse splenocytes.

Materials and Methods

Chemicals and Regents

Folin-ciocalteu reagent (FCR); chlorogenic acid (CA); 2, 2-diphenyl-2-picrylhydrazyl (DPPH), sodium nitrate (NaNO_3); sodium carbonate (Na_2CO_3); aluminum chloride (AlCl_3); dimethyl sulfoxide (DMSO); cisplatin (CDDP); penicillin and streptomycin were obtained from Nacalai Tesque (Kyoto-Japan). Concanavalin (Con A); colchicine; 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox); (+)-Catechin were purchased from Sigma-Aldrich (St Louis, MO, United States). Cell counting kit (CCK-8) assay, fetal bovine serum (FBS), RPMI-1640 medium were supplied from Dojindo Laboratories (Kumamoto, Japan), Biowest (Nuaille, France) and Wako Chemicals Industries (Osaka, Japan) respectively.

Extraction process

Eureka lemon (*Citrus limon*), grapefruit (*Citrus paradise*) and Baladi mandarin (*Citrus reticulata*) were collected from Research and Production Station of National Research Centre, El-Nubaria district, El-Behira Governorate, Egypt. The citrus trees were planted in sandy soil under organic agricultural conditions. The citrus peels were dried and ground into powder by electric a mill. The dried peels were submerged into 98% ethanol (EtOH) in round bottom flask equipped with a condenser at room temperature. The extraction procedures were repeated four times and the liquid extracts were collected, filtrated and evaporated using vacuum rotary evaporator at 40°C, giving a brown oily residue. The three extracts had a pleasant odor owing to presence part of the essential oil in the infusions.

Phytochemical Content

Determination of Total Polyphenol Content (TPC)

TPC was determined using FCR method as described by Herald et al. (2012), and chlorogenic acid (CA) is used

as reference standard. Briefly, the extracts were dissolved in DMSO to prepare different concentrations ranging from 0.156- 20 mg/mL. Aliquot of the diluted extract (25 μl) was mixed with 125 μl of FCR (1:10 diluted with Milli-Q water) and 125 μl of Na_2CO_3 (10% w/v) in a well of the 96 well flat-bottomed microplate. The mixture was kept in the dark at room temperature with intermittent shaking for 10 min. The mixture reaction (200 μl) was transferred into a new well of a 96 well microplate and allowed to stand for 5 min. The absorbance of the mixture was measured using microplate reader (SH-1000, Corona Electronics, Ibaraki, Tokyo, Japan) at 600 nm versus the blank. The blank sample had the same mixture but FCR was replaced with Milli-Q water. A calibration curve of CA was prepared under the same conditions as described above in the range from 0.0625 to 2 mg/mL. The amount of TPC was expressed as mg CA equivalent per mg of the sample (mg CAE/mg sample) through the calibration curve of CA.

Determination of Total Flavonoid Content (TFC)

TFC was determined using the AlCl_3 colorimetric method as described by Herald et al. (2012), and catechin was used as the reference standard. Briefly, the diluted extract (25 μl) was mixed with Milli-Q water (125 μl) and 5% NaNO_2 (7.5 μl). The mixture reaction was allowed to stand for 6 min followed by addition 10% AlCl_3 solution (15 μl). The reaction was left to stand for 5 min before the addition 1 N NaOH (50 μl) and Milli-Q water (275 μl). Then, an aliquot of the reaction mixture (200 μl /well) was transferred into 96 well microplate. The absorbance of the mixture was measured at 510 nm versus the same mixture which containing Milli-Q water (15 μl) instead of AlCl_3 as a blank. A calibration curve of (+) catechin was prepared under the same conditions as described above in the range from 0.0156 to 1 mg/mL. The amount of TFC was expressed as mg (+) catechin equivalent per mg of the sample (mg CE/mg sample) through the calibration curve of (+)catechin.

Determination of antioxidant activity using DPPH

DPPH radical scavenging activity was determined according to the procedures described by Herald et al. (2012). Briefly, 10 μl of the diluted extract at different concentrations (2.5-1000 $\mu\text{g}/\text{mL}$) 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 (final concentration was 0.5 mM in 100% EtOH). The mixture was shaken vigorously and kept at room temperature for 30 min. Subsequently, the mixture reaction (200 μl /well) was transferred into a 96 well microplate. The optical density (OD) of the mixture was determined at 517 nm against the blank which containing 50 μl of 100% EtOH as a substitute of DPPH. The inhibition of the DPPH radical scavenging was calculated according to the following formula: DPPH radical scavenging activity (%) = $[1 - (\text{OD of sample} - \text{OD of blank}) / (\text{OD of control} - \text{OD of blank})] \times 100$.

EC_{50} value ($\mu\text{g extract}/\text{mL}$) is the effective concentration of the plant extract able to scavenge 50% of DPPH radical. Trolox is used as a standard antioxidant reference to convert the DPPH scavenging capacity of each sample to

the Trolox equivalent antioxidant capacity (TEAC). Trolox was prepared by the same procedures described above in the concentration ranging from 2.5 to 1000 µg/mL. TEAC value was calculated as the ratio between EC₅₀ of Trolox (µg/mL) and EC₅₀ of extract (µg/mL).

Human cell culture and Treatment

The human promyelocytic leukemia HL-60 cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL) and streptomycin sulfate (100 mg/mL). The cells were placed in 100 mm-diameter disposable Petri-dish and maintained in an incubator (5% CO₂ and 90 % humidity) at 37°C. The cell passage was performed twice a week to keep the cell viability and exponential cell growth. The extracts were dissolved in DMSO as a stock solution (50 mg/mL) and diluted in RPMI-1640 medium to prepared different concentrations ranging from 0.5 to 500 µg/mL. The maximum final concentration of DMSO in the medium was less in 1%.

Isolation of mouse splenocytes

Mouse splenocytes were prepared as described previously (Ko and Joo, 2010). Briefly, the spleen was excised from an adult male mouse (3-months-old inbred Swiss strain, National Research Centre, Dokki, Cairo, Egypt) and transferred into a sterile cell strainer (40µm) over a petri-dish containing the RPMI-1640 medium. The spleen was gently crushed through the sieve using the plunger end of the syringe. The resulting cells were centrifuged at 1000 rpm for 10 min at room temperature. The cell pellets were resuspended in 1mL of erythrocyte lysis buffer (144 mM NH₄Cl, 1.7 mM Tris Base, pH 7.2) at room temperature for 5 min. Then, 9 mL of phosphate buffer (PBS) were added to stop the lysis followed by centrifugation at 1000 rpm for 5 min. The cell pellets were washed twice with PBS (0.14 M NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄, 1.76 mM KH₂PO₄, and pH 7.2). The cells were resuspended in complete medium (RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL) and streptomycin sulfate (100 mg/mL) and counted using a hemocytometer. The experimental animals were conducted under the guidelines for the Care and Use of Laboratory Animals in Scientific Research approved by ethical committee at National Research Centre.

In vitro cytotoxicity/viability assay

The cell viability was determined colorimetrically with CCK-8 as described previously (Diab et al., 2015). Cytotoxicity of citrus peel extracts was evaluated in two types of cells, human leukemia HL-60 cells, and primary murine splenocytes. HL-60 cells (50 × 10³ cell/100 µl/well in a 96 well plate) were grown in medium containing different concentrations of extracts (0.5-500 µg/mL) for 24 h.

Mouse splenocytes (1 × 10⁵ cell/100 µl/well in a 96 well plate) were grown in complete medium supplemented with different concentration of plant extracts (20-500 µg/mL) in the absence of

Con A for 48 h.

After the end of incubation, a volume of 20 µl of CCK-8 was added per well, and the plate was incubated in the CO₂ incubator for 3 h. CCK-8 contains the tetrazolium salt WST-8 [2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] which reduced by mitochondrial dehydrogenases to generate a water-soluble yellow-color product (formazan). The quantity of formazan is directly proportional to the number of living cells (Tsukatani et al., 2011). The samples absorbance was measured at wavelength 450 nm against blank which containing medium only. The cell viability was calculated according to the following formula: (%) = (OD of sample - OD of blank) / (OD of control - OD of blank) × 100.

In vitro mitogen proliferation assay

Proliferation response of mouse splenocytes to mitogen was determined using the CCK-8 colorimetric assay. Mouse primary splenocytes are composed of about 90% of lymphocytes (50 % B cells and 45% T cells) and up to 10% other immune cells such as macrophage and dendritic cells (Małaczewska et al., 2016). Con A is a plant mitogen for T-lymphocytes. Mouse splenocytes (3 × 10⁵ cell/100 µl/well in a 96 well plate) were grown in complete mediums supplemented by plant extracts (50-500 µg/mL) in the presence or absence of Con A (5 µg/mL). After 48 h, 20 µl of CCK-8 was added per well, and the plate was further incubated for 3 h. The absorbance of the samples was measured at 450 nm using a microplate reader. The immunoproliferation was expressed as stimulation index according to the following formula: Stimulation index = O.D. of Con A- stimulated cells / O.D. of non-stimulated cells (Krifa et al., 2014).

Assessment of genotoxicity and antigenotoxicity

Chromosomal aberration assay

Mouse splenocytes were cultured at a density of 5 × 10⁶ cell/mL in 60 mm petri dish. Following 24 h of incubation, the cells were treated with plant extracts alone (100 µg/mL), CDDP alone (10 µg/mL) or combination of both (plant extract+ CDDP) for 24 h. A negative control (non-treated cells) was also evaluated. Two hours before harvest, the cells incubated with colchicine (200 µg/mL) to arrest the cells at the metaphase stage. The cells were harvested by centrifugation at 1500 rpm for 10 min at room temperature. The cell pellets were resuspended in hypotonic solution (0.075 M KCl) for 20 min at 37°C followed by centrifugation at 1500 rpm for 10 min. The cell pellets were fixed with a freshly prepared ice-cold fixative (3:1 volume of methanol: glacial acetic acid) followed by centrifugation for at 1500 rpm for 5 min. The cell pellets were washed twice with the fixative, and the drops of cells suspension were dropped onto a clean microscopic slide. After drying, the slides were stained with 10% Giemsa in phosphate buffer (0.06 M Na₂HPO₄ and 0.06 M KH₂PO₄, pH 6.8) for 10 min, washed with distilled water, air-dried. At least 500 well-spread metaphases were analyzed per concentration under a light microscope at 2000X magnification for chromosomal aberration. The reduction rate (%) was calculated according the following equation: Reduction

(%) = [number of aberrant cells in (A) - number of the aberrant cells in (B)] / number of the aberrant cells in (A) - number of the aberrant cells in (C) × 100 (Diab and Elshafey 2011). Where (A) represents positive control (cells treated with CDDP alone); (B) represents culture treated with plant extract + CDDP; (C) represent negative control (non-treated cells)

Data Analysis

The experiments were replicated at least three times with quadruplicate wells in each concentration. The results were computerized and analyzed using Statistical Package for Social Science (SPSS Inc, version 17, Chicago, IL, USA). One-way analysis of variance (ANOVA) and Duncan's New Multiple-range test with a confidence interval of 95% were used to determine the differences among the means. The P value <0.05 were considered as statistically significant. Cell viability and proliferation data were subjected to Student's t-test for independent samples with equal or unequal variances. Linear regression analysis calculated both EC₅₀ (median effective concentration) and IC₅₀ (median inhibitory concentration). The Pearson correlation coefficients (r) were conducted between DPPH, TPC, and TFC.

Results and Discussion

Determination of TPC and TFC

FCR is a mixture of phosphomolybdate and phosphotungstate that commonly used for quantification of polyphenol compounds in plant extracts. This assay is widely used for its simplicity, popularity, low-cost, and reproductively. In this reaction, the movement of a single electron from polyphenol was caused a reduction of FCR to produce blue colored phosphotungstic-phosphomolybdenum complex. However, quantification of TPC interferes with any oxidation substances in plant sample that react with FC reagent in an inhibitory, additive or enhancing manner (Singleton et al., 1999; Cicco et al., 2009).

Data presented in Table (1) illustrated that, among the citrus extracts, the lemon peel possessed the highest level of TPC (142.63±18.74 µg CAE/mg extract) that represented 2.3 and 2.6-fold increases compared with the values for grapefruit and mandarin peel respectively. It was reported that the citrus peels are characterized by the high-level content of polyphenol antioxidants compared to their segment and seeds (Abeysinghe et al., 2007; Guimarães et al., 2010). The accumulation of polyphenol compounds in the outer epidermal cells was contributed in protection the plant from UV damage, pathogenic microorganisms and insect attacks (Mierziak et al., 2014).

The aluminum chloride colorimetric assay is widely used for quantification of TFC in plant extracts. In this reaction, the presence of NaNO₂ in alkaline medium resulted in nitration the aromatic ring that has a catechol group with its 3 or 4 positions unsubstituted or sterically hindrance. The reaction between aluminum chloride and carbonyl and hydroxyl groups of flavones and flavonols produce a yellow stable complex which turned instantly to a red-color complex after addition of NaOH (Peğal and

Pyrzynska, 2014).

As shown in Table (1), the order of TFC was arranged in descending order as follows: mandarin peel > lemon peel ≥ grapefruit peel. Similarity, the variations in TPC and TFC were recorded in 21 varieties of 11 citrus peels. Among all of them, lemon peel had the highest level of TPC (1882±65 µg of gallic acid/g extract) and the lowest amount of TFC. While tangelo and mandarin peels (5615±93µg/g extract and 5237±68µg /g extract, respectively) possessed the highest level of TFC which expressed in µg quercetin/g extract (Ramful et al., 2010). The diversity in TPC and TFC are related to agriculture factors such as nitrogen and nutrient supply, mulching, irrigation, light exposure, temperature, cultural methods, and fruit ripening level (Ghasemi et al., 2009; Ramful et al., 2010; Oboh and Ademosun, 2012). Moreover, this variation is associated with the extraction procedures such as the solvent type and its concentration, solvent/solid ratio, extraction time and temperature, and pH value (Li et al., 2006). According to Hegazy and Ibrahim (2012), alcoholic solvents (ethanol and methanol) are the superior solvents for extraction of polyphenol/flavonoids compounds from orange peels among the other solvents such as hexane, acetone, dichloromethane, and ethylacetate. In fact, that the ethanol have hydrophilic (OH group), and hydrophobic (hydrocarbon portion) ends that have a propensity for extraction polar and non-polar compounds respectively. Hence, the addition of water increases the polarity of ethanol which reflected its high affinity to extract polyphenols from high, mid, and low ends of polarity (Spigno et al., 2007).

Determination of antioxidant activity by DPPH

The stable radical DPPH molecule is characterized by the presence of an odd, unpaired electron in its outer orbital which responsible for the visible dark purple. In this reaction, DPPH molecule is reduced by hydrogen-donating antioxidant compounds and became stable, non-radical (diamagnetic) molecule and decolorized to yellow-colored diphenyl-picrylhydrazine (Kedare and Singh, 2011). The major characteristics of DPPH are its simplicity, rapidity, and accuracy. However, many factors can be influenced on the DPPH assay, for example, the interaction between antioxidants, reaction time and interference compounds (Kedare and Singh, 2011). Indeed, the antioxidant compounds can be classified as be hydrophilic (water-soluble), hydrophobic (lipid-soluble) and bound (insoluble) to cell walls that cannot react with DPPH. Hydrophilic and hydrophobic antioxidant compounds react with DPPH at different rates, and the reaction will not reach the finishing point in a reasonable reaction time. For this reason, the amount of plant sample necessary to react with one-half of the DPPH is selected as an endpoint for quantification the antioxidant activity (Kedare and Singh, 2011).

The present study showed that, the three extracts were differently exhibited DPPH antiradical activity in a concentration-dependent manner (Figure 1). As shown in Table (1), Trolox was potently radical scavenging activity with the minor EC₅₀ value (6.5 µg extract/mL) as compared with the citrus peel extracts. Lemon peel extract had the

higher DPPH radical scavenger, lower EC_{50} (42.97 μg extract/mL) and higher TEAC value indicating its greater antioxidant activity. Whereas, grapefruit peel extract possessed the lower DPPH, higher EC_{50} values (>1000 μg extract/mL) and lower TEAC values reflecting its lower antioxidant activity. It is known that, the plant extract, having superior antioxidant activity, is characterized by its greater antiradical activity with lower EC_{50} and higher TEAC values (Fernandes de Oliveira et al., 2012).

Pearson's correlation analysis

Table (2) shows the interrelationship between TPC, TFC and antioxidant activity for citrus peels. The present study recorded positive correlation between TPC and TFC of three extracts verified that flavonoids represented the primary fraction of polyphenol compounds. Indeed,

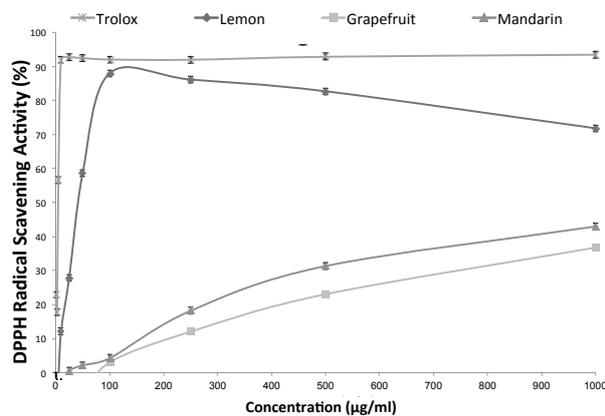


Figure 1. Antiradical Activity of Citrus Peel Extract and Trolox. Data are represented as mean % \pm SD

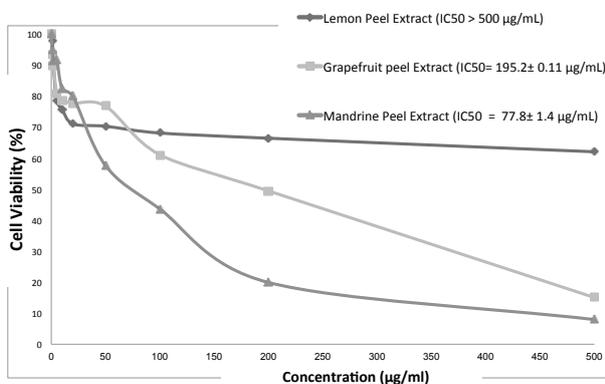


Figure 2. Cytotoxic Activity of Citrus Peel Extracts Against Human Leukemia Promyelocytic HL-60 Cells. The cells (50×10^4 cells/mL), grown in a 96-well plate, were incubated with different concentrations of citrus peels for 24h. Then, CCK-8 (20 μL) were added to the cells for 3 h. Data are represented as mean % \pm SD

phenolic compounds consist of simple phenols (phenolic acid) and complex phenol (polyphenols), depending on the number of phenol subunits attached to it. Simple phenols are low-molecular weight compounds that include only one phenol subunit. Polyphenol compounds are intermediate (flavonoids) or high (condensed tannins, lignans, and stilbenes) molecular weight compounds having more than one phenol subunit in their chemical conFigureuration (Landete, 2012).

Interestingly, the negative relationships between TPC and DPPH for the citrus peel extracts were shown in Table (2). This relationship attributed to two possible reasons. Firstly, FCR reacts with both phenolic and non-phenolic compounds such as vitamin C, lipid, amino acids (Georgé et al., 2005). Secondly, the high concentration of ethanol (98%) is inadequate to release hydrophilic phenolic compounds which responsible for antioxidant activity (Naczka and Shahidi, 2006).

Further, the negative correlations between TFC and DPPH for the three extracts were reported in Table (2). Similar findings reported negative correlations between TPC and DPPH for orange (*Citrus sinensis* L) peel extract and its fractions (Diab et al., 2015). This negative

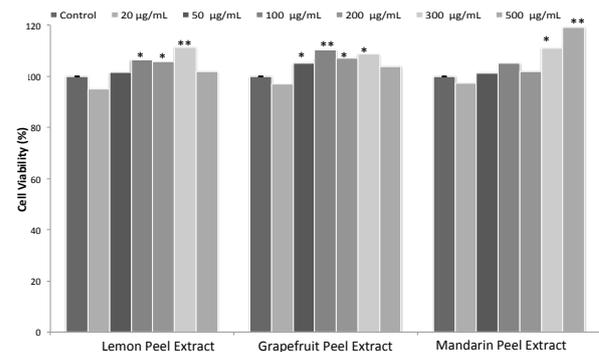


Figure 3. Effect of Citrus Peel Extracts on cell viability in Non-stimulated Primary Mouse Splenocytes. The cells (1×10^6 cells/mL), grown in a 96-well plate, were incubated with different concentrations of citrus peels for 48 h. thereafter, the cells were incubated with 20 μL of CCK-8 for 3 h. Data are represented as mean % \pm SD. * $P < 0.05$, ** $P < 0.01$ compared to control culture

Table 2. Pearson's Correlation Coefficients between TPC, TFC and DPPH Assays of Citrus Peels

Correlation	Lemon Peel Extract	Grapefruit Peel Extract	Mandarin Peel Extract
TPC & TFC	0.963**	0.609	0.514
TPC & DPPH	-0.829*	-0.664	-0.858
TFC & DPPH	-0.703	-0.997*	-0.882

*Significant at $p < 0.05$; **Significant at $p < 0.01$

Table 1. Measurement of Phytochemical Content and Antiradical Scavenging Activity of Citrus Peels

Samples	TPC	TFC	Antioxidant activity	
	(μg CAE/mg extract)	(μg CE/mg extract)	EC_{50} value (μg extract/ml)	TEAC value
Lemon Peel Extract	142.63 \pm 18.74 ^a	16.24 \pm 5.28 ^a	42.967	0.157
Grapefruit Peel Extract	59.68 \pm 30.75 ^b	15.17 \pm 1.64 ^a	>1000	0.005
Mandarin Peel Extract	52.83 \pm 11.32 ^b	19.36 \pm 1.79 ^{ab}	>1000	0.006
Trolox (Standard)	-----	-----	6.5	-----

The Data having different superscript letters in each column are significantly different from one another as calculated by ANOVA (Duncan's test, ($p < 0.05$))

Table 3. Protective Activity of Citrus Peel Extracts against Cisplatin-induced Chromosomal Aberrations in Mouse splenocytes

Treatment	Gap	Br/Frag	Del	M. A	Including gaps		Excluding gaps		
	%	%	%	%	Mean% ± S.E	R(%)	Mean %±S.E	R(%)	
Control Treatments									
Negative (non-treatment)	1.6	2	0.2	----	3.80 ± 0.58 ^a		2.20 ± 0.37 ^a		
Positive (CDDP,10 µg/mL)	4	10.4	2.2	0.8	17.40 ± 0.51 ^f		13.40 ± 0.51 ^e		
Lemon Peel Extract (LPE)									
LPE (100 µg/mL)	0.4	2.8	0.2	0.2	3.60 ± 0.40 ^a		3.20 ± 0.37 ^a		
LPE (100 µg/mL)+CDDP	1.8	4.6	1	0.6	8.00 ± 0.55 ^b	69.1	6.20 ± 0.66 ^{bc}	62.2	
LPE (50 µg/mL)+ CDDP	3.8	9	0.2	----	13.00 ± 1.38 ^e	32.4	9.20 ± 0.86 ^d	37.5	
Grapefruit Peel Extract (GPE)									
GPE (100 µg/mL)	1.2	2.6	0.2	----	4.00 ± 0.77 ^a		2.80±0.80 ^a		
GPE (100 µg/mL)+CDDP	1.8	4	1	0.6	7.40 ± 1.50 ^b	73.5	5.60±0.93 ^b	69.6	
GPE (50 µg)+CDDP	3.4	6	1.8	0.4	11.60 ± 0.68 ^{dc}	42.6	8.20±0.66 ^{cd}	46.4	
Mandarin Peel Extract(MPE)									
MPE (100 µg/mL)	0.4	2	0.8	----	3.20 ± 0.73 ^a		2.80 ± 0.58 ^a		
MPE (100 µg/mL)+CDDP	0.8	5	0.8	----	6.60 ± 0.40 ^b	79.4	5.80 ± 0.66 ^b	69.8	
MPE (50 µg/mL)+CDDP	1.2	7.8	0.8	0.4	10.20 ± 1.28 ^{cd}	52.9	9.00 ± 1.18 ^d	39.2	

Total 500 metaphases were examined per each treatment; Data are expressed as mean%± S.E; Br=Break; Fra= Fragment; Del= Deletion; M.A metaphases with more than one type of aberrations; R = Reduction rate; The Data having different superscript letters in each column are significantly different from one another as calculated by ANOVA (Duncan' test, p<0.05)

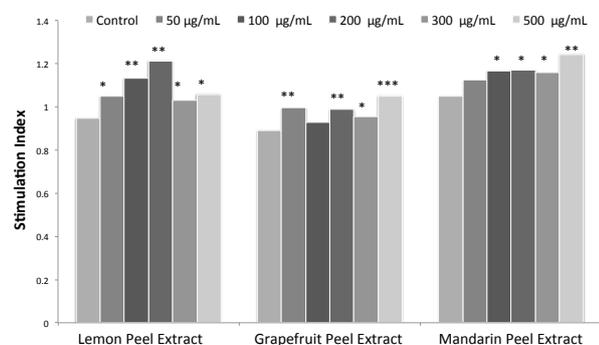


Figure 4. Effect of Citrus Peel Extracts on Proliferation of Mouse Splenocytes. The cells (3×10^6 cells/mL), grown in a 96-well plate, were incubated with different concentrations of citrus peels with and without Con A (5 µg/mL) for 48 h. thereafter, the cells were incubated with 20 µL of CCK-8 for 3 h. Data are represented as mean % ± SD. * P<0.05; ** P<0.01 compared to control culture

correlation may be due to synergistic or antagonistic interaction among the bioactive compounds in the crude extracts (Wang et al., 2011). It was found that chemical con Figureuration of flavonoids are closely associated with their antioxidant activity (Heim et al., 2002). For example, the presence of catechol (ortho-dihydroxy) structure in the B-ring provide the flavonoid with highly antiradical scavenging activity (Bors et al., 1990). Further, the presence of the 2, 3- unsaturation in combining with a 4-oxo function participates in electron transferring from the B-ring to C-ring (Rice-Evans, 2001).

In vitro cytotoxic assay

A-Human leukemia HL-60 cells

The majority of chemotherapy drugs is not only cytotoxic to the cancer cells but also is toxic to healthy cells and has immune suppressive side effects. Therefore, the discovery of novel compounds that possess not only cytotoxic activity against cancer cells but also non-toxic to healthy cells and modulating the immune response has

become an important goal of research in the biomedical sciences (Sak, 2012). The present study showed that, all the tested extracts were clearly decreased the cell viability in a concentration-dependent manner in HL-60 cells (Figure 2). According to Atjanasuppat et al. (2009), the cytotoxic activity of the extracts has classified into four groups according to their IC_{50} value. Those are active extract ($\leq 20 \mu\text{g/ml}$), moderately active extract ($>20-100 \mu\text{g/ml}$), weakly active extract ($>100-1000 \mu\text{g/ml}$), inactive extract ($>1000 \mu\text{g/ml}$). In view of that mandarin peel had a moderate anticancer activity ($IC_{50} = 77.8 \pm 1.4 \mu\text{g/mL}$). While grapefruit peel ($IC_{50} = 195.2 \pm 0.11 \mu\text{g/mL}$) and lemon peel ($IC_{50} > 500 \mu\text{g/mL}$) exhibited a weak cytotoxic toward HL-60 cells. The anticancer activity of citrus peels was reported either in the form of single molecules as or as a mixture of molecules (Manassero et al., 2013; Wang et al., 2014; Rawson et al., 2014). For example, the essential oils of lemon and grapefruit peel exhibited moderated to weak cytotoxicity toward human prostate (PC-3), lung (A549) and breast (MCF-7) tumor cell lines (Zu et al., 2010). Moreover, ethanolic extract from orange peel and its fractions exhibited a weak to moderate cytotoxic activity toward HL-60 cells (Diab et al., 2015).

B-In vitro mouse splenocytes cytotoxicity

The *in vitro* cytotoxicity can predict the level of acute toxicity (oral and intervenous) in animal studies. Subsequently, the number of animals can be reduced for *in vivo* toxicity assay (Ukelis et al., 2008). As depicted in Figure (3), negative controls of all extracts have a cell viability of 100%. In non-stimulated mouse splenocytes, all extracts were increased cell viability in a concentration-independent manner, indicating their potential non-cytotoxic and proliferative activities. Cell viability reached its maximum after treatment the splenocytes with lemon, grapefruit, and mandarin peel extracts at concentrations 300, 200, and 500 µg/mL, respectively. Interestingly, insignificant reductions in cell viability were observed after treatment the splenocytes with 20 µg/mL of lemon

(95%), grapefruit (96%), and mandarin (97%) peels. This data indicated that the lowest concentration did not induce cytotoxic and proliferative activities in mouse splenocytes. The obtained results did not permit to measure median lethal concentration (LC_{50}), indicating that citrus peels had LC_{50} values $> 1000 \mu\text{g/mL}$. This data are in line with the finding of Hosseinimehr et al. (2003) who found that bitter orange peel was non-toxic in male mouse at the dose 1000 mg/kg.

In vitro mouse splenocytes proliferation assay

Exploration the natural products that stimulate or suppress lymphocyte proliferation response is considered the rapidly growing area of autoimmunity, inflammation and cancer immunotherapy (Duong et al., 2011). Lymphocytes are key effector cells of humoral and cell-mediated immune responses mediated by B and T lymphocytes, respectively (Kawai et al., 2006). Several assays have been applied for measuring the growth pattern of murine lymphocytes. The mitogenic activity has used lipopolysaccharide and Con A as immunostimulant agents for B and T-lymphocytes, respectively (Colic et al., 2002; Ang et al., 2014). This activity represents an early stage of the immune response and has evaluated as the first screening of immunomodulatory activity (de Melo et al., 2011). In the present study, the incubation of mouse splenocytes (T-lymphocytes) with citrus extracts in the presence or absence of Con A were used as a model for their vital role in cellular mediated immunity (Duong et al., 2011). As shown in Figure (4), the three extracts increased the proliferation of mouse splenocytes in a concentration-independent manner compared to control cultures. Lemon peel extract exerted a significant immunostimulation activity at all tested concentrations that reached its highest level after treatment splenocytes with the concentration 200 $\mu\text{g/mL}$. Interestingly, grapefruit and mandarin peels had insignificant increase in the stimulation index at a low concentrations 100 and 50 $\mu\text{g/mL}$, respectively. The maximum proliferation index was recorded for grapefruit and mandarin at the concentration 500 $\mu\text{g/mL}$ ($p < 0.01$). These data suggested that citrus peels had mitogenic activity and stimulated the proliferation of T-lymphocytes response to Con A through their bioactive compounds of the extract. Similar results obtained by Tanaka et al. (1999) reported that auraptene isolated from citrus peel (*Citrus natsudaidai Hayata*), have been exerted an augmentation activity on mouse splenocytes stimulating response to Con A. Immunomodulatory properties of plants are closely associated to polyphenol compounds. Polyphenols containing OH group at R2 position (e.g., caffeic acid and chlorogenic acid) have been possessed lesser potent immunostimulating activity than polyphenol containing other groups at the same position (Chiang et al., 2003; Cuevas et al., 2013). The immunostimulation by plant extracts is believed to be a promising way to prevent and cure disease (Kumar et al., 2012).

It is noteworthy that, the citrus peels exhibited a weak to moderate cytotoxic/antiproliferative activity against human leukemia HL-60 cells (cells undergoing mitosis). As well, citrus peels exerted their potential non-cytotoxic and proliferative effects toward non-

stimulated mouse splenocytes (cells in resting stage of mitosis) or stimulated cells (cells undergoing mitosis). This means that, polyphenol compounds are not only toxic to cancerous cells, but also are non-toxic or are less toxic to normal cells.

In vitro chromosomal aberrations (CAs) assay

CAs assay has used as a marker for DNA damage of cancer risk in humans. Mouse splenocytes are the most sensitive indicator of genetic damage in both in-vivo and in-vitro model (Steiblen et al., 2005). In the present study, genotoxic effect of citrus peels was examined at the concentration 100 $\mu\text{g/mL}$ in mouse splenocytes for 24 h. The results showed that the tested extracts did not induce a dramatic increase in the frequency of structural CAs compared to the negative and positive control cultures. These data indicated their non-genotoxicity and supported their potential uses as chemopreventive agents. Similarity, the methanol extract of citrus peel (*Citrus aurantium var. amara*) cannot induce micronuclei in mouse bone marrow cells at the doses of 200 and 400 mg/kg (Hosseinimehr et al., 2003; Hosseinimehr and Karami, 2005). Further, the essential oil of bitter orange did not possess genotoxic activity at the doses from 0.1% to 0.5% in the Drosophila wing spot test (Demir et al., 2009).

Table (3) shows the antigenotoxic activity of the tested citrus peels at two concentration (50, 100 $\mu\text{g/mL}$) against chemotherapeutic agent CDDP. It found that CDDP was induced a remarkable increase in the occurrence of CAs, mainly breaks/fragments (10.4%) compared with control culture (2.0%). This implied that CDDP exerts its genotoxic activity by reacting with the N7- position of purine base in the DNA molecule which forms DNA adducts (Eastman, 1999; Tanida et al., 2012). The intrastrand and interstrand cross-linked DNA adducts interfered with DNA replication and transcription causing DNA damage and inhibiting cell proliferation (Eastman, 1999; Attia, 2010). Further studies demonstrated that the antitumor activity of CDDP is correlated to its genotoxic activity on tumor cells. Besides, the toxicity of CDDP in the healthy cells is associated with the production of ROS (Basu and Krishnamurthy, 2010; Attia, 2010)

The protective activity of the citrus extracts was examined by simultaneous treatment of extracts (50, 100 $\mu\text{g/mL}$) and CDDP (10 $\mu\text{g/mL}$) to mouse splenocytes. Data showed that the citrus peels were drastically reduced the frequency of CAs induced by CDDP for 24 h. The reduction rate ranged from 32.4% to 69.1% for the lemon peel, from 42.6% to 73.5% for the grapefruit peel and from 52.9% to 79.4% for the mandarin peel. Similar results showed that bitter orange peel extract exhibited antimutagenic activity against cyclophosphamide and radiation in mouse bone marrow cells (Hosseinimehr and Karami, 2005; Hosseinimehr et al., 2003). Further, bitter orange (*Citrus aurantium*) peel oil decreased somatic mutation in the wings of *Drosophila melanogaster* induced by potassium dichromate, cobalt chloride, ethyl methane-sulfonate and N-ethyl-N-nitrosourea (Demir et al., 2009). Further, citrus flavonoid (naringin) inhibited mutagenesis in *Salmonella typhimurium* strain TA100 NR- induced by N-methyl-N'-nitro-N-nitrosoguanidine

(Francis et al., 1989).

These data suggested that citrus peels exert their antigenotoxicity in a desmutagenic manner. Indeed, antimutagens classified into desmutagens or biomutagens. Desmutagenic compounds exert their action extracellularly to inactivate the mutagenic chemicals before they attack DNA molecule mutagens. Demutagenic agents are frequently administered before or simultaneous with the mutagenic agents. In the other hand, biomutagens are administered after mutagenic compounds and act intracellularly by inhibition the fixation of mutations (De Flora and Ferguson 2005; Słoczyńska et al., 2014). Taken together, the bioactive compounds presented in citrus peels exert their anticancer activity through the antimutagenic mechanism. Earlier reports describing citrus peels as powerful antioxidants provide protection against diverse mutagens through elimination ROS generated from mutagens and prevent mutation-related diseases in human (Alfadda and Sallam, 2012; Wang et al., 2014; Rawson et al., 2014; Diab et al., 2015)

In conclusion, this study exhibited a weak to moderate antitumor activity of the tested citrus peels in HL-60 cells. The same extracts increased the cell viability and stimulation index of mouse splenocytes in absence or presence of Con A indicating their non-cytotoxicity and immunostimulation activity. The three citrus extracts exerted their antimutagenic activity through reduction of CAs induced by CDDP in mouse splenocytes in a desmutagenic manner (simultaneous treatment). Further biological experiments are required for a better understanding of the mechanistic studies of citrus peels as chemopreventive agents. Extra antioxidant assays and quantification of phytochemical content (such as saponins, tannins) are needed for increased knowledge about the antioxidant mechanism of crude extracts and their fractionations and the isolated pure compounds.

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