UNIVERSIDADE VILA VELHA-ES

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

INDUÇÃO DE NAD(P)H:QUINONA REDUTASE E ATIVIDADE ANTIOXIDANTE *in vitro* DE TORANJA BURARAMA (*Citrus maxima* (Burm.) Merr)

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Dissertação apresentada a Universidade Vila Velha, como pré-requisito do Programa de Pós-graduação em Ciências Farmacêuticas, para a obtenção do grau de Mestre em Ciências Farmacêuticas.

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Aprovada em 31 de agosto de 2016,

Banca Examinadora:

Profª. Drª. Denise Coutinho Endringer – UVV

Profª. Drª. Elaine Cristina Viana- UVV

Profª. Drª. June Ferreira Maia – UVV

(Orientadora)

Dedico esta dissertação à minha mãe Arlete, que sempre fez tudo por mim e não mede esforços para realizar meus sonhos, à minha avó Delorme, que com muito amor ajudou na minha criação, e ao meu marido Romeri que me acompanhou nessa jornada e é meu maior incentivador.

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Isaías 41:20

“Pois todas as coisas foram criadas por ele, e tudo existe por meio dele e para ele. Glória a Deus para sempre! Amém!”

Romanos 11:36

SUMÁRIO

RESUMO

SILVA, Elysiane Senna da, Universidade Vila Velha - ES, agosto de 2016. **Indução de NAD(P)H: quinona redutase e atividade antioxidante *in vitro* de Toranja Burarama (*Citrus maxima* (Burm.) Merr)**. Orientadora: Profª. Drª. June Ferreira Maia.

Diferentes variedades de frutas cítricas possuem propriedades antioxidantes e de quimioprevenção do câncer. Toranja Burarama (*Citrus maxima* (Burm.) Merr) é uma variedade de fruta cítrica cultivada no Estado do Espírito Santo, Brasil. O objetivo deste trabalho foi avaliar o potencial antioxidante e de quimioprevenção do câncer desses frutos, em extratos de solventes orgânicos das cascas e do suco por ensaios *in vitro*. A atividade antioxidante de cada extrato foi medida pela capacidade do extrato de eliminar os radicais livres nos ensaios colorimétricos 2,2-difenil-1-picrylhidrazyl radical (DPPH) e 2,2-azino-bis [3-etilbenzotiazolina-6-sulfónico] (ABTS). As atividades dos extratos para a indução de nicotinamida-adenina-dinucleótido (fosfato) [NAD (P) H: quinona reductase] (QR) foram avaliadas em células murinos Hepa1c1c7. Para os extratos que apresentaram maior razão de indução (IR) de QR, a viabilidade celular foi medida utilizando-se 3- (4,5-dimetiltiazol-2-il) -2,5-difenil tetrazólio (MTT) e o extrato que apresentou maior índice quimiopreventivo (IC) foi selecionado para posterior análise por ESI (-)FT-ICR MS. Os extratos do flavedo mostraram propriedades biológicas superiores. Os resultados da atividade antioxidante foram expressos como concentração de extrato (µg/mL) requerida para reduzir a quantidade original de radicais livres em 50% (IC_{50}). O extrato de acetato de etila das cascas da Toranja apresentou maior atividade antioxidante *in vitro* na eliminação de radicais DPPH (IC_{50} = 298,3 µg/mL), entre hexano, acetona, etanol, metanol, metanol: água (80:20) e suco. Para o ensaio ABTS o extrato metanólico apresentou a maior atividade antioxidante (IC_{50} = 296,4 µg/mL). O extrato de acetato de etila induziu atividade de QR de uma forma dose dependente (0,4 a 8,0 mg/mL) com um aumento de 3,0 vezes do IR e IC> 6,5. Este extrato não foi citotóxico para a linhagem celular fibroblastos L929. Ácido quínico, ácido ftálico, 16-hydroxypalmitic e ácidos graxos linoleico, ácido benzóico e ácido propanóico foram identificados no extrato. Em resumo, o extrato acetato de etila das cascas de *Citrus maxima* exibiu potencial efeito de quimioprevenção do câncer pela indução da quinona redutase *in vitro*.

Palavras-chave: *Citrus maxima*, frutas cítricas, quimioprevenção do câncer, potencial antioxidante, casca.

ABSTRACT

SILVA, Elysiane Senna da, Universidade Vila Velha - ES, agosto de 2016. **Induction of NAD(P)H:quinone reductase and antioxidant activities *in vitro* of Toranja Burarama (*Citrus maxima* (Burm.) Merr)** Adviser: Profª. Drª. June Ferreira Maia

Different varieties of citrus possess antioxidant and cancer chemopreventive properties. Toranja Burarama (*Citrus maxima* (Burm.) Merr) is a variety of citrus grown in the State of Espírito Santo, Brazil. The aim of this research was to evaluate the antioxidant and cancer chemoprevention potential of these fruits in organic solvents extracts from the peels and juice by *in vitro* assays. The antioxidant activity of each extract was measured by the extract's ability to scavenge free radicals in 2,2-diphenyl-1-picrylhidrazyl radicals (DPPH) and 2,2-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) colorimetric assays. The activities of the solvent extracts for the induction of nicotinamide adenine dinucleotide (phosphate) [NAD(P)H:quinone reductase] (QR) were examined in murine Hepa1c1c7 cells. For extracts that presented higher induction ratio (IR) of QR, cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and the extract that presented highest chemopreventive index (CI) was selected for further ESI(-)FT-ICR MS analysis. Extracts from the flavedo section showed higher biological properties. Antioxidant activity results were expressed as extract concentration (µg/mL) required to reduce the original amount of free radicals by 50% (IC_{50}). The ethyl acetate extract of the toranja's peels presented the highest antioxidant activity *in vitro* scavenging DPPH radicals (IC_{50} = 298.3 µg/mL), among the hexane, acetone, ethanol, methanol, methanol:water (80:20) and juice. For the ABTS assay the methanol extract presented the highest antioxidant activity (IC_{50} = 296.4 µg/mL). The ethyl acetate extract induced QR activity in a dose-dependent manner (0.4 to 8.0 mg/mL), with a 3.0 fold increase IR and CI > 6.5. This extract was not cytotoxic to L929 cell line fibroblasts. Quinic acid, phthalic acid, 16-hydroxypalmitic and linoleic fatty acids, benzoic acid and propanoic acid were identified in extract. In summary, ethyl acetate extract of *Citrus maxima* peels exhibited potential cancer chemopreventive by its *in vitro* induction effect on quinone reductase.

Keywords: *Citrus maxima*, citrus fruits, cancer chemoprevention, antioxidant potential, peel.

Artigo científico

Induction of NAD(P)H:quinone reductase and antioxidant activities *in vitro* of Toranja Burarama (*Citrus maxima* (Burm.) Merr)

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Abstract

Toranja Burarama (*Citrus maxima* (Burm.) Merr) is a variety of citrus grown in the State of Espírito Santo, Brazil. We evaluated its antioxidant and quinone reductase (QR) induction potential by *in vitro* assays. Cell viability and ESI(-) FT-ICR MS analysis also were performed. Peel's extract induced QR activity in Hepa1c1c7 cells with a 3.0 fold increase induction ratio (IR) and Chemopreventive Index (CI) > 6.5. *Citrus maxima* peels exhibited cancer chemopreventive properties.

Keywords: *Citrus maxima*, citrus fruits, cancer chemoprevention, antioxidant potential, peel.

Introduction

Recent review has focused on the benefits provided by Citrus, either in their juices, peels, seeds or leaves for human health (Lv et al., 2015). These plants are important both economically and as a source of in bioactive compounds. Several studies have suggested that varieties of citrus grown throughout the world show antioxidant (Barreca et al., 2016; Castro-Vasquez et al., 2016; Oikeh et al., 2016; Xi et al., 2014; Karoui & Marzouk, 2013; Oboh & Ademosum, 2012; Jayaprakasha et al., 2008a; Jayaprakasha et al., 2008b; Álvarez-González et al., 2004) and cancer chemopreventive activities (Rawson et al., 2014; Perez et al., 2010; Poulose et al., 2006).

Citrus maxima (J. Burm.) Merr (Genus Citrus, Fam. Rutaceae) is the largest of all Citrus (Oyedepo, 2012). Several investigators have proposed that this tropical fruit plant species, also known as *C*. *grandis*, is originated from South East Asia but is also found in India, China, Japan, Indonesia, United States of America, Philippines, Thailand, West African Countries (Barrion et al., 2014; Buachan et al., 2014; Susandarini et al., 2013; Oyedepo, 2012). It is known as pummelo or shaddock (Wang et al., 2015; Oyedepo, 2012).

The antioxidant potential of *C*. *maxima* was evaluated using different assays, for example, ABTS radical assay (Oboh & Ademosun, 2012; Jayaprakasha et al., 2008b), DPPH radical-scavenging activity (Barrion et al., 2014; Xi et al., 2014; Oboh & Ademosun, 2012; Jayaprakasha et al., 2008b; Mokbel et al., 2006); FRAP assay (Buachan et al., 2014; Xi et al., 2014; Toh et al., 2013), oxygen radical absorbance capacity (ORAC) (Jayaprakasha et al., 2008b) and Trolox-equivalent antioxidant capacity (TEAC) (Toh et al., 2013).

Besides antioxidant effects, *C*. *maxima* extracts have also exhibited anti-inflammatory (Shivananda et al., 2013; KunduSen et al., 2011b), antibacterial (Barrion et al., 2014; Mokbel et al., 2006), algicidal (Wang et al., 2015), hepatoprotective (Chowdhury et al., 2015), anti-hypertensive (Oboh & Ademosun, 2011), cardioprotective (Buachan et al., 2014), and anti-diabetes effects (Oboh & Ademosun, 2011). *C*. *maxima* leaves exhibited significant antitumor activity (KunduSen et al., 2011a). In general, consumption of this fruit exhibited health benefits in terms of prevention of diseases caused by oxidative stress (Oyedepo, 2012). A review (Vijaylakshmi & Radha, 2015) presented also some others pharmacological activities of this plant.

Cancer chemoprevention may be achieved by the strategy of use compounds to enhance endogenous mechanisms that reduce the risk of cancer development through reversion, suppression, or prevention of the process of carcinogenesis (Watteenberg, 1985).

Although knowledge of cancer chemoprevention is far from complete, the induction of QR activity in murine hepatoma Hepa1c1c7 cells has been widely employed as a tool to examine the potential chemopreventive activity of phytochemicals. Scavenging reactive oxygen species by antioxidants and enhancing carcinogen detoxification via phase II enzymes such as quinone reductase (QR) induction are two important cancer chemoprevention strategies (Dinkova-Kostova &

Talalay, 2010; Cuendet et al., 2006). *In vitro* screening assays for antioxidants and QR inducers, have been developed for the discovery of novel natural product anticarcinogens (Prochaska et al., 1992; Prochaska & Santamaria, 1987).

There has been limited research into the health potential of *Citrus maxima* cultivated in the State of Espírito Santo, Brazil. Therefore, the goal of this research was to evaluate the cancer chemoprevention capacity through induction of NAD(P)H:quinone reductase in murine hepatoma cells. To the extent of our knowledge, no such experiments have been conducted with *C*. *maxima*. Investigation of the antioxidant capacity of *Citrus maxima* cultivar in Brazil also was done.

Materials and Methods

Reagent

Flavin adenine dinucleotide disodium salt hydrate (FAD), glucose-6-fosfato (G-6P), nicotinamide adenine dinucleotide phosphate (NADP), bovine serum albumin (BSA), glucose-6-phosphate dehydrogenase (G6PD), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), menadione, Tween 20, Tris-HCl, digitonin, sodium dodecyl sulfate (SDS), crystal violet, 4'-bromoflavone, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhidrazyl radicals (DPPH), 2,2-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), potassium persulfate, TPTZ (Fe^{+3} – 2,4,6-tripyridil-S-triazine), quercetin, were purchased from Sigma Aldrich® Chemical Co, St. Luis, MO, USA. All other reagents used (NH_4OH, sodium acetate trihydrate, acetic acid glacial, chloridric acid, ethanol, methanol, ethyl acetate, hexane, and acetone) were of analytical grade and were obtained from various commercial sources.

Cell lines and culture maintenance

Murine hepatoma (Hepa 1c1c7) cells (American Type Culture Collection - ATCC® CRL-2026™) and Tissue adipose fibroblasts NCTC clone 929 [L cell, L-929, derivative of Strain L] (ATCC® CCL-1™) (Cell Line Service, Rio de Janeiro, Brazil) were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/ml streptomycin at 37°C, in a incubator containing 5% CO_2 for 24 h. Once cells reached a monolayer, with greater than 70 - 80% confluence, DMEM medium from the flask was discarded. Cells were rinsed with 10 mL of Phosphate-Buffered Saline and detached with trypsin for 10 min at 37ºC. Confluence was determined using Trypan blue (Sigma) dye exclusion and the cells were diluted in DMEM to 1.10^4 cells/mL.

Collection and identification of plant material

Fruits of *Citrus maxima* (Burm.) Merr. (Family Rutaceae), commonly called in Brazil as Toranja Burarama, were harvested, at the commercial maturity stage, based on external color and size uniformity, in Sooretama, ES, Southeastern Brazil. Fruits were taxonomically identified by the Botanist Flávio de Lima Alves and a representative sample (INCAPER #7133) was deposited in the Genetic Bank of Citrus at the Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (INCAPER), Vitória, ES, Brazil. The oblate fruits, 18 cm diameter and 1.5 kg average weight, were harvested in May 2015. The investigation was carried out on 5 samples.

Preparation of peel and juice extracts

Fruits were rinsed with tap water and dried with paper towels in the laboratory. Fruits were divided into peel (flavedo, the peripheral surface) and peeled fruits. Peels were removed manually and stored at -20°C until further use. Peeled fruits were cut into halves and juice was obtained using an electric domestic squeezer. Fruit juice samples

were vacuum filtered, then lyophilized and the concentrates obtained were stored at -20ºC in airtight containers until used for analysis. Lyophilized juice sample (~3g) were extracted with 50 mL of methanol at 25ºC for 12 h as described at Guimarães et al. (2010). The extract was filtered and the residue extracted with one additional 50 mL portion of methanol. The extract evaporated to dryness (40 ºC) and dried material was dissolved in methanol (or ethanol) at a concentration of 20 mg/mL, and stored at 4ºC for further use.

Peels, after thawing, were shredded (using a blend). Peels fractions were extracted with five different solvents as described in Jayaprakasha et al. (2008a). All the extractions were done using a Soxhlet apparatus, for approximately 8h each, separately. Peels (124 g) were successively, exhaustively, extracted, with hexane, ethyl acetate (EtOAc), acetone, methanol (MeOH) and methanol:water (80:20), 1 L each. After, the extracts were cooled, concentrated, separately, under vacuum at 40°C in a rotary evaporator (Fisaton 801, São Paulo, Brazil) to obtain a semisolid material which was then used to obtain the crude extract. Stock solutions of each extract with three different concentrations: 100 mg extract/mL DMSO (for the MTT assay), 20 mg extract/mL DMSO (for the antioxidant assays), and 4 mg extract/mL DMSO (for the Quinone Reductase induction assay) were prepared and kept at -20ºC prior to being used.

Determination of DPPH radical scavenging activity

Radical scavenging activity of each selected solvent fractions of *Citrus maxima* peels was assessed using the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Guimarães et al., 2010). Samples were prepared using the stock DMSO solution (20 mg/mL) and ethanolic solutions of each solvent extract were prepared in serial dilutions (from 0.04 - 5 mg/mL) and used for the assay. Quercetin was used as control. Briefly, in microplate 96-wells the reaction mixture containing 30 µL extract solution and 270 µL

aqueous ethanolic solution (80:20 v/v) containing DPPH radicals ($6x10^{-5}$ mol/L) was added in each one of the wells. The experiment was carried out in triplicates. The mixture was left to stand for 60 min in the dark at room temperature. Using a blank containing ethanol and DPPH radicals as reference, absorbance was determined at 515 nm. Reduction of the DPPH radical was seen by bleaching of the purple-colored solution of DPPH, therefore lower absorbance values of the reaction mixture indicates higher free radical scavenging activity.

The radical scavenging activity (RSA) was calculated according to Jayaprakasha et al. (2008b) as a percentage of DPPH discoloration using the equation: % RSA = $[(A_{DPPH} - A_S)/(A_{DPPH})] \times 100$, where A_{DPPH} is the absorbance of the DPPH solution (DPPH radical + ethanol); A_S is the absorbance of the solution (DPPH radical + sample extract or standard). Antioxidant power against DPPH free radical was expressed as IC_{50} which is the extract concentration (µg/mL) required to reduce the original amount of free radicals by 50% and was calculated as proposed from Guimarães et al. (2010).

$ABTS^{\cdot+}$ Radical scavenging assay

The method used was the ABTS (radical cation 2,2-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]) decolorization assay as proposed by Re et al. (1999), with modifications. The assay is based on the ability of an antioxidant compound to quench the $ABTS^{\cdot+}$ relative to that of a reference antioxidant. A solution of $ABTS^{\cdot+}$ (radical cation) was prepared by mixing ABTS stock aqueous solution (7 mM) with 2,45 mM potassium persulfate (final concentration). The mixture was maintained in the dark, at room temperature, for 16h before use for the formation of ABTS radical. Then the radical cation solution was stored at 4ºC until further use. Before use, this solution needs to return to room temperature and is further diluted (1250 µL stock solution in 50 mL ethanol:water 50%) to achieve an absorbance value of 1.10 $\pm$ 0,02 at 734 nm. Ethanolic solutions of each solvent extract were prepared in serial dilutions (from 0.04 -

5 mg/mL) as described for the DPPH assay. The assay was carried out using 96 well plates, and the reaction mixture containing extract solution (30 µL) and diluted ABTS radical solution (270 µL) was added in each one of the wells. After 6 minutes, absorbance was measured, by means of a UV-vis spectrophotometer at 734 nm. Radical scavenging activity (% RSA) and EC_{50} were calculated using the same equations as described previously for the DPPH assay.

Evaluation of quinone reductase (QR) induction activity

The potential QR-inducing activity of the *C*. *maxima* peels extracts and juice was assessed by the NADPH-generating system, coupling the oxidation of menadiol to the reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] producing reduced MTT, a insoluble formazan dye as described previously (Prochaska & Santamaria, 1988).

Stock solutions of the extracts were prepared at a concentration of 4 mg extract/mL in 100% DMSO. A working solution was made taking 20 µL from stock solution and adding 180 µL of sterile PBS. Then, the concentration of 0.4 mg/mL in DMSO and 90% PBS was obtained.

Enzyme activity was assessed using murine hepatoma Hepa-1c1c7 cells. Cells were seeded in two 96-well plates (one for quinone reductase and the other for protein determination) at a density of $1x10^4$ cells/mL in 200 µL of α-MEM supplemented with 10% FBS. Cells were grown for 24 h at 37ºC in incubator with CO_2. Old media was decanted after 24 h incubation, and 190 µL fresh media and 10 µL of 10% DMSO in PBS (containing test samples) were added to each well. Cells were incubated for an additional 48 h period at 37ºC in incubator with CO_2. For QR assay, the medium was decanted and cells were lysed with 50 µL of 0,8% digitonin and 2 mM EDTA solution (pH 7.8) were added to each well, except blank. Then, cells were incubated at 37ºC for 10 min in incubator. Plates were agitated for 10 min at room temperature. After that,

every well (except blank), were added with 200 µL of reaction mixture. Thus, for 2 plates a stock solution (50 mL) was prepared as described: cold water (46.6 mL), BSA (33.3 mg), MTT (15 mg), 0.5 M Tris-HCl pH = 7,4 (2.5 mL), 1.5% Tween 20 (0.33 mL), 7.5 mM FAD (0.033 mL), 50 mM NADP (0.03 mL), 150 mM Glucose-6-phosphate (0.33 mL), 50 mM menadione (50 µL), Glucose-6-phosphate dehydrogenase (G6PDH) (100 units). Enzyme and menadione were added just before the mixture was dispensed into plate. G6PDH was dissolved using reaction mixture. The plate was shaken at room temperature until the blue color became apparent. At least 5 min later the plates were placed into a microplate reader (Molecular Devises, Spectra Max 190, USA) and optical density was estimated at 595 nm. The DMSO control OD (optical density) was 0.4.

Considering that a potential chemopreventive agent cannot be cytotoxic, a % of survival was calculated as: % Survival = [Abs sample/average Abs negative control] x 100. Total QR activity was determined as [Abs($_{SAMPLE}$) x 3247]/5.

Specific acitivity (SA) is defined as nmol of MTT blue formazan formed per mg protein per min (Kang & Pezzuto, 2004). Since *C*. *maxima* extracts, being tested as QR inductors, may reduce the rate of cell growth, it is desirable to relate the QR activity to the percentage of viable cells (amount of protein) in each microtiter well. Thus, the amount of protein was determined by staining with crystal violet in an identical set of test plates. Media was decanted and 200 µL of 0.2% crystal violet solution in 2% ethanol were added to each well, including blank and incubated for 10 min at room temperature. Crystal violet was decanted and plate rinsed for 2 min (until blue dye was not seen) with tap water and completely dried in a hood. After drying, 200 µL of 0.5% SDS in 50% ethanol was added to each well and incubated for 5 – 10 min at room temperature on shaker to solubilize the bounded dye completely. The plates were scanned at 595 nm into a microplate reader Molecular Devises, Spectra Max 190, USA) (OD for control was around 1.0).

Induction ratio (IR) of QR activity = [specific enzyme activity of agent-treated group]/[average of specific enzyme activity of DMSO control group] (Kang & Pezzuto, 2004). For samples with IR $\geq$ 2, extract was considered active. A plot of the ratio of IR as a function of extract inducer concentration permits the determination of the concentration which causes double induction (CD). CD values were determined through a dose response assay for active extracts. In order to check dose-dependence, a serial dilution with eight different concentrations of the extracts was made (beginning with 0.4 mg/mL). For the negative control, cells were treated with medium containing 10% DMSO, and 4'-Bromoflavone was used as the positive control. According to Song et al. (1999), 4'-Bromoflavone induces QR activity in cultured hepatoma 1c1c7 cells (CD = 10 nM). A chemopreventive index (CI) = IC_{50}/CD was also calculated where IC_{50} = half-maximal inhibitory concentration of cell viability. Assays were performed in triplicate and expressed as mean $\pm$ standard deviation. CD and IC_{50} were estimated with TableCurve 2D V4 Systat Software (San Jose, CA).

Evaluation of *in vitro* cytotoxicity

Cell viability was measured by the ability of viable mitochondria to reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT; M5655; Sigma, USA) to the blue formazan product, a colorimetric assay described by Mosmann (1983).

Stock solutions of the extracts were prepared at a concentration of 100 mg extract.mL^{-1} in 100% DMSO. A working solution was made taking 3 µL from stock solution and adding 297 µL of DMEM and preparing a serial dilution obtaining concentrations (1, 10, 100 and 1000 µg/mL).

Briefly, the non-cancerous L929 fibroblasts were seeded into 96-well plates at seeding density of 1 x 10^4 cells.mL^{-1} and cultured for 24 h in DMEM. After incubation period, 50 µL of test solutions (in the presence or absence of increasing concentrations

of the solvent extracts) were added to each well. Cells were incubated for an additional 24 h period at 37ºC in incubator with CO_2. Camptothecin (CPT) was used as positive control. After 24 h incubation, old media was aspirated and 100 µl of MTT (5 $mg.mL^{-1}$) in DMEM medium was added to each well and the cells were incubated at 5% CO_2 chamber for 2 h. Following the incubation period, the MTT solution was aspirated and 100 µL of dimethyl sulfoxide (DMSO) was added to each well in order to solubilize the violet formazan cystals entrapped in viable cells. The absorbance of each 96-well plate was measured at 595 nm using a microplate reader (Molecular Devises, Spectra Max 190, USA). The mitochondrial function was then calculated, as a percentage of the control group (without any extract), considered as 100%. The experiments were carried out in triplicate. Percent of survival was calculated as % survival = [Abs_{SAMPLE}/average $Abs_{NEGATIVE\ CONTROL}$] x 100 and IC_{50} (half-maximal inhibitory concentration of cell viability) was determined.

ESI (-) FT-ICR MS

For the analysis of the tehyl acetate peels extract of *C. maxima*, 500 µL of the extract were dissolved with 500 µL of methanol. The methanolic solution was basified with 4 µL of the NH_4OH (Vetec Fine Chemicals Ltda, Brazil). The mass spectrometer (model 9.4 T Solarix, Bruker Daltonics, Bremen, Germany) was set to operate on negative ion mode, ESI (-), over a mass range of *m/z* 150–1250. The ESI source conditions were as follows: a nebulizer gas pressure of 1.4 bar, a capillary voltage of 3.8 kV, and a transfer capillary temperature of 200ºC. Ions time accumulation was of 0.010 s. ESI(-)FT-ICR mass spectrum was acquired by accumulating 32 scans. Spectra obtained with high resolution (16M) and a mass accuracy of <1 ppm provided the unambiguous molecular formula assignments for singly charged molecular ions. Mass spectra data were acquired and processed using Data Analysis software (Bruker Daltonics, Brenen, Germany) and the elemental compositions of the compounds were determined by

measuring the *m/z* values. The proposed structures for each formula were assigned using the Chemspider (www.chemspider.com) database.

Statistics

Statistical analyses were performed using GraphPad software (San Diego, CA, 176 USA). Data were expressed as mean and standard deviation and statistical comparisons were carried out using one way analysis of variance (ANOVA) followed by Tukey post-test. The level of significance was identified at $p<0.05$.

Results

Potential antioxidant of the extracts

In this work we performed two different *in vitro* assays, DPPH$^{\cdot}$ and ABTS$^{\cdot+}$, in order to evaluate the antioxidant potential of *C*. *maxima*, after extraction with organic solvents, such as hexane, ethyl acetate, acetone, ethanol, methanol and methanol:water (80:20). Solvents with different physicochemical properties were chosen in order to obtain a wide range of compounds that could be responsible for the antioxidant activity. In this perspective, we chose the methodology described in Jayprakasha et al. (2008 b).

DPPH assay measures the electron donation ability of the sample, thus scavenging DPPH radicals. The change in absorbance of DPPH radicals indicates the antioxidant potential of the extracts. *Citrus maxima* presented a higher DPPH value in the peels extracts than its juice methanol extract, at the concentrations studied. Actually, extracts quenched DPPH in a dose-dependent manner, regardless the solvent used for extraction. Thus, the results showed increase in the radical scavenging activities of all extracts, as the concentration increased from 125 to 500

µg/mL. Antioxidant activities of the peels extracts were significantly ($p < 0,05$) higher than those of the juice methanol extract.

The results of antioxidant activities in the DPPH and ABTS assays were expressed as IC_{50} [concentration (µg/mL) of extract required to scavenge 50% of the free radicals], thus a lower EC_{50} value indicates more potent antioxidant activity. As showed in Table 1, for the DPPH assay, ethyl acetate, methanol and acetone extracts presented lower EC_{50} values (298.3 µg/mL, 303.8 µg/mL and 316.7 µg/mL) respectively.

For the ABTS assay (Table 1), IC_{50} revealed that the methanol:water (80:20) extracts presented significantly ($p < 0.05$) lower IC_{50} values (264.1 µg/mL), followed by methanol and ethyl acetate extracts, 296.4 µg/mL and 298.2 µg/mL, respectively.

Table 1. *In vitro* antioxidant activity of *Citrus maxima* extracts determined by DPPH radical scavenging activity and ABTS radical scavenging assay.

Citrus maxima samples		DPPH	ABTS
Origin	Solvents used for extraction	IC_{50} (µg/mL)	IC_{50} (µg/mL)
Peel	Hexane	> 500	455,0 ± 5,38[a]
	Ethyl acetate	298,3 ± 2,66[a]	298,2 ± 6,46[b]
	Acetone	316,7 ± 7,74[a]	430,8 ± 17,37[a]
	Ethanol	381,3 ± 8.85[b]	381,2 ± 3,07[c]
	Methanol	303,8 ± 0,46[a]	296,4 ± 2,52[b]
	Methanol Water (80:20)	346,1 ± 8,62[c]	264,1 ± 0,46[d]
Juice	Methanol	> 500	> 500

Different letters in the same column correspond to significant differences ($p < 0.05$). Tests (n = 3) were performed in triplicate and expressed as mean ± SD.
$IC_{50,}$ the inhibitory concentration (µg/mL) required to reduce 50% the original amount of free radicals.

Cancer chemopreventive activity of the extracts

In addition to the antioxidant aspect of the toranja's peels extracts, this work attempted to better understand the biological aspects of these extracts as source of cancer chemopreventive substances. The effect on QR activity may be dependent of the solvent used for extraction, a observation that further illustrates the different constituents of these extracts. Considering that a potential chemopreventive extract cannot be cytotoxic, the percentage of cells survival was evaluated (Table 2).

All the extracts from *C. maxima* peels and juice were evaluated by the quinone reductase induction assay. As demonstrated in Table 2, the Induction Ratio (IR) of QR activity for the hexane and ethyl acetate extracts were higher than 2.0.

Table 2. Induction of quinone reductase in Hepa1c1c7 cells by *Citrus maxima* extracts.

Citrus maxima samples			
Origin	Solvents used for extraction	Induction ratio[a]	% Survival
Peel	Hexane	3.0 ± 0.78	95,2 ± 8,1
	Ethyl acetate	2.0 ± 0.31	93,6 ± 1,8
	Acetone	1.5 ± 0.3	84,1 ± 16,7
	Ethanol	1.4 ± 0.07	83,9 ± 5,8
	Methanol	1.3 ± 0.14	90,6 ± 9,6
	Methanol Water (80:20)	1.2 ± 0.01	95,9 ± 9,2
Juice	Methanol	0.71 ± 0.08	109,4 ± 7,89

Results are means ± SD (n = 3).
[a]Induction ratio of specific activity of the quinone reductase.

For samples with IR $\geq$ 2.0, extract was considered active. A plot of the ratio of IR as a function of extract inducer concentration allowed the determination of the extract concentration which causes double induction (CD) (Table 3). A chemopreventive index (CI) was then obtained by dividing the IC_{50} (concentration for 50% inhibition of cell viability) values by the respective CD values as indicated by Kang & Pezzuto (2004). CD values were determined through a dose response assay for active extracts. Table 3 shows the effect of hexane and ethyl acetate extracts (IR > 2) on QR activity. These extracts presented CD of 13.13 $\mu g.mL^{-1}$ and 3.07 $\mu g.mL^{-1}$, respectively. Therefore, ethyl acetate extracts yielded a CI higher than 6.5.

Table 3. Effect of *Citrus maxima* extracts on quinone reductase activity in Hepa1c1c7 cells.

Citrus maxima samples		IC_{50}[a]	CD[b]	CI[c]
Origin	Solvents used for extraction	(µg/mL)	(µg/mL)	
Peel	Hexane	>20	13.13 ± 0.74	>1,5
	Ethyl acetate	>20	3.07 ± 1.07	>6,5

Results are means ± SD (n = 3).
[a]Mean value of the half-maximal inhibitory concentration off cell viability.
[b]Mean value of the concentration required to double the specific activity of quinone reductase.
[c]Chemopreventive index: ratio between IC_{50} and CD.

Ethyl acetate and hexane peel extracts did not exhibit *in vitro* cytotoxicity

In an attempt to clarify if the extracts that presented induction ratio (IR) $\geq$ 2.0, did not affect cell viability, a methodology using L929 fibroblasts was performed in order to measure the ability of viable mitochondria to reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT). Results were presented as IC_{50} (µg/mL), for the hexane extract (525.01 ± 8.20 µg/mL) and for the ethyl acetate extract (435.05 ± 0.63

µg/mL). Thus, high concentrations of both extracts are required for 50% inhibition of cell viability.

Chemical profile of ethyl acetate peel extract

Several organic solvents were used to extract chemical compounds with chemopreventive and antioxidant potential. Ethyl acetate extract was shown to have higher chemopreventive index (CI) than hexane extract and also, was one the most active in scavenging radicals both in DPPH and ABTS assays. In order to better understand these biological characteristics, the ethyl acetate extract was submitted for chemical ionization at its chemical profile was investigated by ESI-FT-ICR mass spectra in ESI(-) electron ionization mode and by comparison to compounds described in the literature. Results from this analysis are presented in Table 4.

Table 4. Chemical species proposed from ESI(-)FT-ICR MS data for the ethyl acetate extract of *Citrus maxima* peels.

Supposedly formula	**M/Z measured**	**Proposed Structure**	**M/Z theoretical**
$C_7H_{12}O_6$	191,05607	D-(−)-Quinic acid	192.167
$C_9H_{14}O_7$	233,06676	Trimethyl citrate	234.203
$C_{11}H_{12}O_7$	255,05109	3,4,5-Trimethoxyphthalic acid	256.209
$C_{16}H_{32}O_3$	271,22791	16-hydroxypalmitic acid	272.423
$C_{11}H_{14}O_8$	273,06153	4-Oxo-4H-pyran-3-yl β-D-glucopyranoside	274.224
$C_5H_9N_9O_5$	274,06502	*	*****
$C_{13}H_{10}O_7$	277,03554	(2,3,4-Trihydroxyphenyl)(3,4,5-trihydroxyphenyl)methanone	278.214
$C_{18}H_{32}O_2$	279,23307	Linoleic acid	280.445
$C_{13}H_{12}O_8$	295,04584	3,4,5-triacetoxybenzoic acid	296.230
$C_7H_7N_9O_5$	296,04942	*	*****
$C_9H_{24}N_6O_6$	311,16880	*	*****

Supposedly formula	M/Z measured	Proposed Structure	M/Z theoretical
$C_{10}H_{26}N_6O_6$	325,18451	*	*
$C_{11}H_{20}O_{11}$	327,09346	6-{[2,4,5-Trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl]oxy}tetrahydro-2H-pyran-2,3,4,5-tetrol	328.270
$C_{11}H_{28}N_6O_6$	339,20015	*	*
$C_{15}H_{26}O_{10}$	365,14558	3-{[3-Hydroxy-2-(hydroxymethyl)-2-methylpropanoyl]oxy}-2-({[3-hydroxy-2-(hydroxymethyl)-2-methylpropanoyl]oxy}methyl)-2-methylpropanoic acid	366.361
$C_{13}H_{24}O_{12}$	371,11974	(5ξ)-6-O-{(5R)-5-[(1S)-1,2-Dihydroxyethyl]-α-D-lyxopyranosyl}-α-D-xylo-hexopyranose	372.323
$C_5H_{12}N_{16}O_6$	391,10561	*	*
$C_{20}H_{16}O_9$	399,07249	Dibenzo[b,d]furan-2,3,7,8-tetrayl tetraacetate	400.336
$C_{21}H_{40}O_7$	403,27055	1,4,11,14,17,20,23-Heptaoxaspiro[4.23]octacosane	404.538
$C_{17}H_{28}O_{11}$	407,15635	β-D-Fructofuranosyl 4,6-O-[(1R,2Z)-2-methyl-2-buten-1-ylidene]-α-D-glucopyranoside	408.398
$C_{12}H_{40}O_8$	409,26547	*	*

* The formula was not found in the ChemSpider database.

Discussion

A large number of investigators (Barreca et al., 2016; Castro-Vazquez et al., 2016; Klangpetch et al., 2016; Oikeh et al., 2016; Ademosun et al., 2015; Barrion et al., 2014; Park et al., 2014; Singh et al., 2014; Xi et al., 2014; Karoui & Marzouk, 2013; Hegazy & Ibrahium, 2012; Oboh & Ademosun, 2012; Guimarães et al., 2010; Jayaprakasha et al., 2008a; Jayaprakasha et al., 2008b; Mokbel et al., 2006) have used DPPH assay in order to evaluate antioxidant potential of Citrus. Factors contributing for such preference for this assay include the fact that it is an accurate,

inexpensive and a rapid method for assessing antioxidant activity (Alam et al., 2013; Karoui & Marzouk, 2013; Scherer & Godoy, 2009; Sharma & Bhat, 2009).

Ethyl acetate, methanol and acetone seem to be the best solvents to extract antioxidants, as measured by the DPPH assay. Activity of peel extracts prepared with ethyl acetate and methanol, reached up to 75% of inhibition. Our results are according to Mokbel et al. (2006) that determined antioxidant activities of crude extracts from Buntan (*C. grandis* Osbeck) fruit peel. These authors found DPPH scavenging activities of 24.1% for hexane fraction and 72.8% for the ethyl acetate fraction and 68.3% for the methanolic fraction. Xi et al. (2014) studying antioxidant activities of Chinese local pummelo varieties found that the DPPH values of the pummelos and grapefruits varied from 35.93% to 55.53% in peels and from 29.31% to 58.13% in pulps. The majority of peels had a higher DPPH value than their pulps.

In the present work ethyl acetate peel extract exhibited higher free radical scavenging activity at all concentrations tested. Ethyl acetate is used for extraction of polar limonoids and flavonoids aglycones and glucosides (Jayaprakasha et al., 2008b). These results corroborate with many studies which also found the highest antioxidant activity of citrus peels on ethyl acetate extract, using different citrus plants (Klangpetch et al., 2016; Wang et al., 2015; Jayaprakasha et al., 2008b). For instance, Klangpetch et al. (2016), evaluating the bioactivity of various Citrus varieties, found that the most effective as an antioxidant fraction was the ethyl acetate peel extract of kaffir lime; which they then investigated in prolonging the shelf life of raw chicken drummettes. Also Wang et al. (2015) identified the allelochemicals from the ethyl acetate extracts of citrus peel, including shaddock peel, and showed their algicidal activities. The results presented in the present work suggest that the ethyl acetate extract could be successfully used as an antioxidant source of *Citrus maxima* peels.

Quercetin (0.2 - 0.001 mM) was used as a control antioxidant, since it represents one of the phenolic compounds found in significantly high concentrations in citrus peels (Ademosun et al., 2015) and has been reported that it can modulate phase II metabolism inducing quinone reductase (QR) (Chen & Blumberg, 2008), in part via an impact on signal transduction pathways that affect the antioxidant-response element (ARE) (Tanigawa et al., 2007). This standard presented percentage inhibition of the DPPH radical (69.7 $\pm$ 2.59) at the minimum concentration tested (0.001 mM).

The differences showed in DPPH values (Table 1) may denote a consequence of the relationship between solvent polarity and antioxidant structure, and they are consistent with data reported for other citrus varieties subjected to comparable extractions (Klangpetch et al., 2016; Park et al., 2014). This data add valuable information to knowledge on the antioxidant capacity of toranja peels.

Most of the antioxidant compounds that have action chelating ABTS radicals were extracted with methanol:water (80:20). The addition of 20% water to methanol enhanced the extracting power of antioxidant activity. MeOH and MeOH:water (80:20) may be used for the extraction of medium polar and polar compounds such as aglycones and glucosides of flavonoids, limonoids, ascorbic acid and sugars (Jayaprakasha et al., 2008a).

These results are of potential importance because they infer that toranja peels are major sources of antioxidants than the interior section (juice). Such observations are in agreement with the literature (Ademosun et al., 2015; Zhang et al., 2014; Toh et al., 2013; Oboh & Ademosun, 2012), where the antioxidant capacity in peels of citrus fruits was higher than in their pulps. This indicates that compounds thought to be involved in defense reactions of the citrus fruit are found in greater amounts in its superficial layer. Therefore, the antioxidant activity should be related to the place in the fruit where protection against lipid oxidation is needed. This is expected considering the ubiquitous citrus distribution in nature and its ability to survive under adverse

conditions, including high temperatures and UV light. This is consistent with a model that exhibit major differences in distribution within different tissue in the citrus fruit. The results presented here fit well such model. Thus, citrus flavedo would tend to accumulate antioxidants and antimicrobial compounds with attributes to help protect internal tissues of the fruit itself against oxidation, resistance to microorganisms and insect pests.

Citrus peel is clearly a useful resource containing important physiological functional substances that add a variety of developing possibility to us. There are various studies suggesting that bioactive compounds in citrus peels can be used as a nutraceutical or as a functional food. For example, Kang (2014) demonstrated that citrus peel extracts were effective as anti-inflammatory by inhibition of LPS-induced cytokines secretion in macrophage. According to Castro-Vasquez et al. (2016), grapefruit peel wastes are a natural source of bioactive flavonoids, mostly naringin, that could be incorporated as food ingredients or as therapeutic agents being part of pharmacological strategies.

Actually, peels of citrus fruits are by-products generated in daily life or by the juice extraction or the fresh-cut ready for consumption (minimally processing) industries and generate large amounts of waste materials. Domestically citrus peels have been processed into candies and consumed by some Americans homes, or are taken as infusion drinks in Asia (Ademosun et al., 2105). However, these by-products of the juice extraction industry could be used as source of natural antioxidants (Klangpetch et al., 2016; Xi et al., 2014; Park et al., 2014; Hegazy & Ibrahium, 2012; Oboh & Ademosun, 2011; Ayala-Zavala et al., 2010; Guimarães et al., 2010).

Quinone reductase *in vitro* assay was used as a biomarker to evaluate the extract ability to act as an inducer of phase II metabolic activity. Induction of QR activity in murine hepatoma Hepa1c1c7 cells is a well defined and important tool for the

screening of novel phytochemicals with chemopreventive potential (Kang & Pezzuto, 2004; Prochaska & Santamaria, 1988).

While mass spectrometry normally cannot provide sufficient information for a complete structure assignment of the compounds, the m/z values and molecular formulas may be detected, when employing chemical ionization techniques.

In an attempt to isolate a chemopreventive compound present in toranja's peels, it should be expected that the compound may occur in low concentrations and even as a complex mixture. Thus, our first objective was to select an organic solvent fraction that could effectively extract the quinone reductase inducer, and at least partially avoid the interference of other substances present in toranja's peels. Extracts prepared with ethyl acetate gave the strongest chemopreventive index.

Conclusion

Toranja Burarama peels have the potential for cancer chemoprevention and this was illustrated trough induction of quinone reductase. The ethyl acetate extract of *Citrus maxima* peels presented antioxidant potential and the highest chemopreventive index. We have shown that quinic acid, phthalic acid, 16-hydroxypalmitic, linoleic fatty acids, benzoic acid and propanoic acid were detected in this extract. Future research needs to purify and elucidate action mechanism of the compounds and if it is made from an individual compound or the existence of possible synergism, if any, among the bioactive compounds. Toranja Burarama peels wastes would appear as a promising natural source of valuable bioactive components for use in the development of new products. Health professionals and consumers should be educated regarding the benefits of this food. It is possible that, an adequate intake of citrus fruit peels may induce phase II enzymes in the body, with potential benefits in chemoprevention of cancer. *C*. *maxima* peels did not show cytotoxicity in both cell lines evaluated.

However, further studies into action mechanisms i*n vivo* safety will be needed, through research with experimental animals and dose effect.

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Conflict of Interest

The authors disclosed no conflict of interest.

References

Ademosun AO, Oboh G, Passamonti S, Tramer F, Ziberna L, Boligon A A, Athayde, ML. 2015. Phenolics from grapefruit peels inhibit HMG-CoA reductase and angiotensin-I converting enzyme and show antioxidative properties in endothelial EA.Hy 926 cells. Food Sci Hum Well 4:80–85.

Alam MN, Bristi NJ, Rafiquzzaman M. 2013. Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. Saudi Pharm J 21:143-152.

Álvarez-González I, Madrigal-Bujaidar E, Martino-Roaro L, Espinosa-Aguirre JJ. 2004. Antigenotoxic and antioxidant effect of grapefruit juice in mice treated with daunorubicin. Tox Lett 152:203–211.

Ayala-Zavala JF, Rosas-Domínguez C, Vega-Vega V, González-Aguilar GA. 2010. Antioxidant enrichment and antimicrobial protection of fresh-cuts fruits using their own byproducts: Looking for integral exploitation. J Food Sci 75:R175–R181.

Barrion ASA, Hurtada WA, Papa IA, Zulayvar TO. 2014. Phytochemical composition, antioxidant and antibacterial properties of pummelo (*Citrus maxima* (Burm.)) Merr. against *Escherichia* coli and *Salmonella typhimurium*. Food Nutr Sci 5:749–758.

Barreca D, Gattuso G, Laganã G, Leuzzi U, Bellocco E. 2016. C- and O-glycosyl flavonoids in Sanguinello and Tarocco blood Orange (*Citrus sinensis* (L.) Osbeck) juice: Identification and influence on antioxidant properties and acetylcholinesterase activity. Food Chem 196:619–627.

Buachan P, Chularojmontri L, Wattanapitayakul SK. 2014. Selected activities of *Citrus maxima* Merr. fruits on human endothelial cells: enhancing cell migration and delaying cellular aging. Nutrients 6:1618–1634.

Castro-Vasquez L, Alañón ME, Rodríguez-Robledo V, Pérez-Coello MS, Hermosín-Gutierrez I, Díaz-Maroto MC, Jordán J, Galindo MF, Arroyo-Jiménez MM. 2016. Bioactive flavonoids, antioxidant behavior, and cytoprotective effects of dried grapefruit peels (*Citrus paradise* Macf.). Oxid Med Cell Longev [Online] Available at: http://dx.doi.org/10.1155/2016/8915729. Accessed on 23 July 2016

Chen C-YO, Blumberg JB. 2008. *In vitro* activity of almond skin polyphenols for scavenging free radicals and inducing quinone reductase. J Agric Food Chem 56:4427–4434.

Chowdhury MRH, Sagor MAT, Tabassum N, Potol MA, Hossain H, Alam MA. Supplementation of *Citrus maxima* peel powder prevented oxidative stress, fibrosis, and hepatic damage in carbon tetrachloride (CCl_4) treated rats. 2015. Evid Based Compl Altern Med Longev [Online] Available at: http://dx.doi.org/10.1155/2015/598179. Accessed on 23 July 2016

Cuendet M, Oteham CP, Moon RC, Pezzuto JM. Quinone reductase induction as a biomarker for cancer chemoprevention. 2006. J Nat Prod 69:460–463.

De Long MJ, Prochaska HJ, Talalay P. 1986. Induction of NAD(P)H:quinone reductase in murine hepatoma cells by phenolic antioxidants, azo dyes, and other chemoprotectors: A model system for the study of anticarcinogens. Proc Natl Acad Sci Med Sci 83:787–791.

Dinkova-Kostova AT, Talalay P. 2010. NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), 1 multifuncional antioxidant enzyme and exceptionally versatile cytoprotector. Arch Bioch Bioph 501:116-123.

Guimarães R, Barros L, Barreira JCM, Sousa MJ, Carvalho AM, Ferreira ICFR. 2010. Targeting excessive free radicals with peels and juices of citrus fruits: Grapefruit, lemon, lime and orange. Food Chem Tox 48:99–106.

Hegazy AE, Ibrahium MI. 2012. Antioxidant activities of orange peel extracts. World Appl Sci J 18:684–688.

Jayaprakasha GK, Girennavar B, Patil BS. 2008a. Radical scavenging activities of Rio Red grapefruits and Sour orange fruit extracts in different *in vitro* model systems. Biores Tech 99:4484–4494.

Jayaprakasha GK., Girennavar B, Patil BS. 2008b. Antioxidant capacity of pummelo and navel oranges: Extraction efficiency of solvents in sequence. Food Sci Tech 41:376–384.

Kang K. 2014. Citrus peel extract inhibits LPS-induced cytokines secretion in macrophage. Intern J Bio-Sci Bio-Tech 6:11-20.

Kang YH, Pezzuto JM. 2004. Induction of quinone reductase as a primary screen for natural product anticarcinogens. Meth Enzym 382:380–414.

Karoui IJ, Marzouk B. 2013. Characterization of bioactive compounds in Tunisian bitter orange (*Citrus aurantium* L.) peel and juice and determination of their antioxidant activities. BioMed Res Intern [Online] Available at: http://dx.doi.org/10.1155/2013/345415. Accessed on 15 July 2016

Klangpetch W, Phromsurin K, Hannarong K, Wichaphon J, Rungchang S. 2016. Antibacterial and antioxidant effects of tropical citrus peel extracts to improve the shelf life of raw chicken drumettes. Intern Food Res J 23:700–707.

KunduSen S, Gupta M, Mazumder UK, Haldar PK, Saha P, Bala A. 2011a. Antitumor activity of *Citrus maxima* (Burm.) Merr. leaves in Ehrlich's ascites carcinoma cell-treated mice. Intern Sch Res Network ISRN Pharm [Online] Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3195351/ Accessed on 15 July 2016

KunduSen S, Gupta M, Mazumder UK, Haldar PK, Saha P, Bhattacharya S, Naskar S, Panda SP. 2011b. Exploration of anti-inflammatory potential of *Citrus limetta* Risso and *Citrus maxima* (J. Burm.) Merr. Pharm 1:702–709.

Lv X, Zhao S, Ning Z, Zeng H, Shu Y, Tao O, Xiao C, Lu C, Liu Y. 2015. Citrus fruit as a treasure trove of active natural metabolites that potentially provide benefits for human health. Chem Central J 9:68.

Mokbel MS, Watanabe Y, Hashinaga F, Suganuma T. 2006. Purification of the antioxidant and antimicrobial substance of ethyl acetate extracts from Buntan (*Citrus grandis* Osbeck) fruit peel. Pakistan J Bio Sci 9:145–150.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 65:55-63.

Oboh G, Ademosun AO. 2012. Characterization of the antioxidant properties of phenolic extracts from some citrus peels. J Food Sci Tech 49:729-736.

Oboh G, Ademosun AO. 2011. Shaddock peels (*Citrus maxima*) phenolic extracts inhibit α-amylase, α-glucosidase and angiotensin I-converting enzyme activities: A nutraceutical approach to diabetes management. Diab Met Synd Clin Res Rev 5:148–152.

Oikeh EI, Omoregie ES, Oviasogie FE, Oriakhi K. 2016. Phytochemical, antimicrobial, and antioxidant activities of different citrus juice concentrates. Food Sci Nutr 4:103–109.

Oyedepo TA. 2012. Antioxidant potential of *Citrus maxima* fruit juice in rats. Glob Adv Res J Med Med Sci 1:122–126.

Park J, Lee M, Park E. 2014. Antioxidant activity of orange flesh and peel extracted with various solvents. Prev Nutr Food Sci 19:291-298.

Perez JL, Jayaprakasha GK, Cadena A, Martinez E, Ahmad H, Patil BS. 2010. *In vivo* induction of phase II detoxifying enzymes, glutathione transferase and quinone reductase by citrus triterpenoids. BMC Compl Altern Med 10:51.

Poulose SM, Harris ED, Patil BS. 2006. Antiproliferative effects of Citrus limonoids against human neuroblastoma and colonic adenocarcinoma cells. Nutr Cancer 56:103–112.

Prochaska HJ, Santamaria AB, Talalay P. 1992. Rapid detection of inducers of enzymes that protect against carcinogens. Proc. Natl. Acad. Sci. 89:2394–2398.

Prochaska HJ, Santamaria AB. 1988. Direct measurement of NAD(P)H: quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. Anal Bioch 169:328–336.

Rawson NE, Ho C, Li S. 2014. Efficacious anti-cancer property of flavonoids from citrus peels. Food Sci Hum Well 3:104–109.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad Bio Med 26:1231–1237.

Scherer R, Godoy HT. 2009. Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. Food Chem 112:654–658.

Sharma OP, Bhat TK. 2009. DPPH antioxidant assay revisited. Food Chem 113:1202-1205.

Shivananda A, Muralidhara Rao D, Jayaveera KN. 2013. Analgesic and anti-inflammatory activities of *Citrus maxima* (J. Burm) Merr in animal models. Res J Pharm Bio Chem Sci 4:1800–1810.

Singh J, Sood S, Muthuraman A. 2014. *In vitro* evaluation of bioactive compounds, anti-oxidant, lipid peroxidation and lipoxigenase inhibitory potential of *Citrus karna* L. peel extract. J Food Sci Tech 51:67–74.

Song LL, Kosmeder II JW, Lee SK, Gerhäuser C, Lantvit D, Moon RC, Moriarty RM, Pezzuto JM. 1999. Cancer chemopreventive activity mediated by 4'-bromoflavone, a potent inducer of Phase II detoxification enzymes. Cancer Res 59:578–585.

Susandarini R, Subandiyah S, Rugayah, Daryono BS, Nugroho LH. 2013. Assessment of taxonomic affinity of Indonesian pummelo (*Citrus maxima* (Burm.) Merr.) based on morphological characters. Amer J Agric Bio Sci 8:182-190.

Tanigawa S, Fujii M, Hou D. 2007. Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. Free Rad Bio Med 42:1690–1703.

Toh JJ, Khoo HE, Azrina A. 2013. Comparison of antioxidant properties of pomelo [*Citrus grandis* (L) Osbeck] varieties. Intern Food Res J 20:1661–1668.

Vijaylakshmi P, Radha R. 2015. An overview: *Citrus maxima*. The J Phytopharm 4:263–267.

Xi W, Fang B, Zhao Q, Jiao B, Zhou Z. 2014. Flavonoid composition and antioxidant activities of Chinese local pummelo (*Citrus grandis* Osbeck.) varieties. Food Chem 161:30-238.

Zhang M, Nan H, Wang Y, Jiang X, Li Z. 2014. Comparison of flavonoid compounds in the flavedo and juice of two pummelo cultivars (*Citrus grandis* L. Osbeck) from different cultivation regions in China. Molecules 19:17314–17328.

Wang H, Kang H, Zhang L, Cheng S, Liu H, Liu H, Sun B. 2015. Composition of ethyl acetate extracts from three plant materials (shaddock peel, pomegranate peel,

pomegranate seed) and their algicidal activities. Pol J Environ Stud 24:1803–1807.

WATTENBERG, L. W. 1985. Chemoprevention of cancer. Cancer Research. 45:1-8.

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