Received: 18 March 2018 | Revised: 30 May 2018 | Accepted: 10 June 2018

DOI: 10.1002/ptr.6149

RESEARCH ARTICLE

WILEY

Induction of NAD (P)H: Quinone reductase 1 (QR1) and antioxidant activities in vitro of 'Toranja Burarama' (*Citrus maxima* [Burm.] Merr.)

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Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Numbers: 308631/2016-1 PQ, 310680/2016-6 PQ and 401409/2014-7 PVE; Fundação Estadual de Amparo à Pesquisa e Inovação do Estado do Espírito Santo, Grant/Award Numbers: FAPES–65921208/2014 and 241/2016

Toranja 'Burarama', *Citrus maxima* (Burm.) Merr. (*Citrus grandis*), is a new citrus discovered in the State of Espírito Santo, Brazil. As several varieties of citrus are known to possess antioxidant and cancer chemopreventive properties, the aim of the study was to evaluate in vitro if this Toranja possess these properties. The antioxidant activity, the potential to induce quinone reductase 1, and the influence on cell viability were measured. ESI(-)FT-ICR MS analysis was also performed and identified flavonoids, coumarins, and fatty acids in the extract. The ethyl acetate and methanolic extracts of the peels presented the highest antioxidant activity in vitro by DPPH (IC_{50} = 298.3 ± 2.6 μg/ml and 303.8 ± 0.4 μg/ml), ABTS assay (IC_{50} = 298.2 ± 6.4 μg/ml and 296.4 ± 2.5 μg/ml), and FRAP (IC_{50} = 234.6 ± 1.8 μg/ml and 398.1 ± 3.8 μg/ml). The ethyl acetate extract of the peel induced quinone reductase 1 activity in Hepa1c1c7 cells, indicating that *C. maxima* exhibited cancer chemopreventive properties.

KEYWORDS

antioxidant potential, cancer chemoprevention, citrus fruits, *Citrus maxima*, peel

1 | INTRODUCTION

Recent reviews have focused on the benefits provided for human health by the fruits of the genus *Citrus* (Ademosun et al., 2015; Castro-Vasquez et al., 2016). These plants are economically important and a source of bioactive compounds. Several studies suggest that varieties of *Citrus* grown throughout the world show antioxidant (Dulay & De Castro, 2016; Castro-Vasquez et al., 2016; Jayaprakasha, Girennavar, & Patil, 2008a; Jayaprakasha, Girennavar, & Patil, 2008b; Kelebek, 2010) and cancer chemopreventive activities (Benavente-Garcia et al., 1997).

Citrus maxima (Burm.) Merr. (Fam. Rutaceae), also known as *Citrus grandis*, is the largest species of *Citrus* and popularly known as

pummelo or shaddock (Vijaylakshmi & Radha, 2015; Wang et al., 2015). This tropical fruit originates in Southeast Asia but is also found in India, China, Japan, United States, and West Africa (Vijaylakshmi & Radha, 2015). Although the fruit is the main part consumed, some traditional uses of other plant's parts have also been reported (Vijaylakshmi & Radha, 2015).

The antioxidant potential of *C. maxima* has been already evaluated by the 2,2-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) radical assay (Jayaprakasha et al., 2008b), the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) radical-scavenging assay (Dulay & De Castro, 2016; Jayaprakasha et al., 2008b), the ferric reducing ability of plasma (FRAP) assay (Toh, Khoo, & Azrina, 2013), the oxygen radical absorbance capacity (Jayaprakasha et al., 2008b), and the trolox-equivalent antioxidant capacity (Toh et al., 2013).

Besides the antioxidant effects, *C. maxima* extracts have also exhibited antibacterial (Dulay & De Castro, 2016), algicidal (Wang et al., 2015), analgesic (Vijaylakshmi & Radha, 2015), and hypocholesterolemic activity (Vijaylakshmi & Radha, 2015).

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

Although knowledge of cancer chemoprevention is far from a complete elucidation, the induction of quinone reductase 1 (QR1) activity in murine hepatoma Hepa1c1c7 cells has been a widely employed tool to examine the potential chemopreventive activity of phytochemicals and find novel anticarcinogenic agents (Prochaska & Santamaria, 1988). Scavenging reactive oxygen species by antioxidants and enhancing carcinogen detoxification via Phase II enzymes such as QR1 induction are two important cancer chemoprevention strategies (Cuendet, Oteham, Moon, & Pezzuto, 2006).

So far, there has been limited research into the health potential of *C. maxima* cultivated in Brazil. To the extent of our knowledge, no experiments for chemoprevention have been conducted with *C. maxima*. Therefore, the aim of this study was to evaluate the cancer chemoprevention capacity through induction of nicotinamide adenine dinucleotide phosphate (NAD (P)H): QR1 in murine hepatoma cells and the antioxidant capacity of new *Citrus maxima* genotype in Brazil.

2 | MATERIALS AND METHODS

2.1 | Standards and chemicals

Flavin adenine dinucleotide disodium salt hydrate (FAD), glucose-6-phosphate (G-6P), nicotinamide adenine dinucleotide phosphate (NAD (P)H), 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, camptothecin, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH), 2,2-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), potassium persulfate, TPTZ (Fe^{3+}–2,4,6-tripyridyl-s-triazine), and quercetin were purchased from Sigma Aldrich® (St. Louis, MO, USA). All other reagents used (ammonium hydroxide [NH_4OH], sodium acetate trihydrate, glacial acetic acid, hydrochloric acid, ethanol, methanol, ethyl acetate, hexane, and acetone) were analytical grade.

2.2 | Cell lines and culture maintenance

The cell line used was murine hepatoma (Hepa 1c1c7) cells (ATCC® CRL-2026™). The cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100-IU/ml penicillin, and 100-μg/ml streptomycin at 37°C, in a humidified atmosphere containing 5% CO_2. Murine hepatoma and fibroblast cells were harvested by tripsinization with 0.05% trypsin/0.02% EDTA solution from Gibco, Grand Island, NY, USA, and mechanically removed.

2.3 | Collection and identification of plant material

Five samples of the fruits of *C. maxima* (*C. grandis*) clone Incaper 7133, of tree grafted on 'Cleopatra' mandarin, with 10 years old, were harvested in May 2015, in Experimental Farm of Sooretama, ES, Brazil. The fruits were harvested in the maturity stage, based on external color and size uniformity: oblate fruits, with a diameter of 18 cm and 1.5-kg average weight. Fruits were taxonomically identified by Flávio de Lima Alves, curator of the Genetic Bank of *Citrus*, and variety clones (INCAPER #7133) was deposited in the Genetic Bank of *Citrus* at the Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (INCAPER-www.incaper.es.gov.br), Vitória, ES, Brazil.

2.4 | Preparation of peel and juice extracts

The fruits were rinsed with tap water and dried. They were manually separated into peel (flavedo, the peripheral surface) and peeled fruits. The peels were stored at −20°C, and the peeled fruits were transformed into juice using a domestic electric squeezer. Fruit juice samples were vacuum filtered and lyophilized. The concentrates thus obtained were stored at −20°C in airtight containers until analysis. Each lyophilized juice sample (~3 g) was then extracted twice with 50 ml of methanol at 25°C for 12 hr as described by Guimarães et al. (2010). The extract was evaporated to dryness (40°C), and the dried material was dissolved in methanol (or ethanol) at a concentration of 20 mg/ml and stored at 4°C.

The peels were thawed and crushed. The crushed peels (124 g) were divided into fractions. Soxhlet extraction was performed on the fractions using hexane, ethyl acetate (EtOAc), acetone, methanol (MeOH), and methanol:water (80:20). Each fraction extracted contains 1-L final volume as described by Jayaprakasha et al. (2008a). All the extractions were performed using a Soxhlet apparatus for approximately 8 hr for each solvent. The extracts were concentrated under vacuum at 40°C in a rotary evaporator (Fisaton 801, São Paulo, Brazil) to obtain the crude extract, which was stored at −20°C until analysis.

2.5 | Determination of DPPH radical-scavenging activity

The radical scavenging activity of each fraction of the *C. maxima* peel was assessed using the radical DPPH according to Guimarães et al. (2010). Briefly, each sample of the extracts was prepared in ethanol

in serial dilutions (0.04–5 mg/ml) for the assay. Quercetin (0.2–0.001 mM) was used as control and a solution of ethanol, and DPPH was used as the reference, that is, a blank analysis. The experiment was carried out in triplicate. After 60 min in the dark at room temperature, the absorbance was determined at 515 nm. Reduction of the DPPH radical was seen by bleaching of the purple-colored solution of DPPH.

The radical-scavenging activity (RSA) was calculated according to Jayaprakasha et al. (2008b) as a percentage of the 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) and A_S is the absorbance of the solution (DPPH + sample extract or standard). The antioxidant power against the DPPH radical was expressed as the IC_{50}, as proposed by Guimarães et al. (2010).

2.6 | ABTS$^+$ radical-scavenging assay

The antioxidant activity of each of the fractions of the *C. maxima* peels was also determined by the ABTS assay according to Re et al. (1999), with modifications. A solution of ABTS$^+$ was prepared by mixing an 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 16 hr before use for the formation of the ABTS radical and then stored at 4°C until further use. Prior to use, the solution was kept at room temperature and was further diluted (1250-µl stock solution in 50-ml ethanol: water 50%) to achieve an absorbance value of 1.10 ± 0.02 at 734 nm. Ethanolic solutions of each solvent extract were prepared in serial dilutions as described for the DPPH assay. The assay was carried out in a 96-well plate, where the reaction mixture containing extract solution (30 µl) was added to the diluted ABTS radical solution (270 µl). After 6 min, absorbance was measured at 734 nm. Radical-scavenging activity (%RSA) and IC_{50} were calculated using the same equations as previously described for the DPPH assay.

2.7 | Ferric reducing antioxidant power assay (FRAP)

The FRAP assay, developed by Benzie and Strain (1996) was used to measure the antioxidant capacity of each fraction of *C. maxima* peel. Briefly, the FRAP reagent contained 2.5 ml of 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40-mM HCl plus 2.5 ml of 20-mM $FeCl_3.6H_2O$ and 25 ml of 0.3M acetate buffer (pH 3.6) and was prepared fresh. Then 270 µl of FRAP reagent was mixed with 30 µl of the test sample or ethanol (for the reagent blank) and incubated at room temperature for 10 min. After the absorbance was measured at 595 nm using a microplate reader (Molecular Devices, SpectraMax 190, USA). The experiments were carried out in triplicate, and the IC_{50} was calculated as previously described.

2.8 | Evaluation of the induction of the NAD (P)H: QR1

The activity of the quinone reductase (QR) of the phenolic fractions was assessed in 96-well plates using Hepa 1c1c7 (ATCC CRL-2026), as described by Pezzuto et al. (2005). Briefly, cells were grown to a density of 2×10^4 cells/ml in 200 µl of Dulbecco's Modified Eagle's Medium (containing 100-IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 (all Sigma, USA). After a 24-hr preincubation period, the media were renovated. The cells were incubated for an additional 48 hr. Enzyme activity was determined using an extract of *C. maxima* at a concentration of 20 µg/ml as a function of the NAD (P)H-dependent menadiol-mediated reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan. Absorption was determined at 595 nm. A total protein assay using crystal violet staining was run in parallel. The induction ratio (IR) of the QR activity represents the specific enzyme activity of agent-treated cells compared with a DMSO-treated control. The presented data are the results of three independent experiments run in duplicate. 40-Bromoflavone (CD = 53.71 nM or CD = 0.03234 µg/ml) was used as a positive control. The experiments were carried out in triplicate.

For samples with IR ≥ 2, the 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. To check dose-dependence, a serial dilution with eight different concentrations of the extracts was tested starting at 20 µg/ml. For the negative control, cells were treated with medium containing 0.5% 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 the mean ± standard deviation. CD and IC_{50} were estimated with TableCurve 2D V4 Systat Software (San Jose, CA).

2.9 | ESI(-)FT-ICR MS

For the analysis of the ethyl acetate and hexane peel extracts of *C. maxima*, 500 µl of the extract was dissolved in 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 in the negative ion mode, ESI(−), over a mass range of *m/z* 150–1,250. 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 200°C. The ion accumulation time was 0.010 s. The ESI(-)FT-ICR mass spectrum was acquired by accumulating 32 scans. The high-resolution spectra obtained ($m/\Delta m_{50\%}$ = 400,000–500,000, in which $\Delta m_{50\%}$ is the full peak width at half-maximum peak height of *m/z* 400) and a mass accuracy of <1 ppm provided the unambiguous molecular formula assignments for singly charged molecular ions. Mass spectral data were acquired and processed using Data Analysis software (Bruker Daltonics, Bremen, 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 Chemspider (www.chemspider.com) database, METLIN Metabolomics Database and PubChem (Oliveira et al., 2016).

2.10 | Statistical analysis

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

3 | RESULTS AND DISCUSSION

3.1 | Potential antioxidant of the extracts

The antioxidant potential of *Citrus* was already evaluated by several studies (Ademosun et al., 2015; Castro-Vasquez et al., 2016; Dulay & De Castro, 2016; Guimarães et al., 2010; Jayaprakasha et al., 2008a; Jayaprakasha et al., 2008b; Kelebek, 2010; Klangpetch, Phromsurin, Hannarong, Wichaphon, & Rungchang, 2016), but no study was performed with the different extracts obtained from the new *C. maxima*.

As described by Jayprakasha et al. (2008b), the use of solvents with different physicochemical properties are useful in obtaining a wide range of compounds that could be responsible for the antioxidant activity. Among the solvents used, ethyl acetate, methanol, and acetone seem to be the best for extracting antioxidants, as demonstrated by the results of the DPPH assay (Table 1), and other assays, in which the peel extracts prepared with ethyl acetate and methanol, reached significantly ($P < 0.05$) better results that the ones with pulps. These results obtained for *C. maxima* are in agreement with the ones from different species of *Citrus* (Jayaprakasha et al., 2008b; Klangpetch et al., 2016; Wang et al., 2015).

The high antioxidant activity may be related to the ability of ethyl acetate to extract polar limonoids, flavonoids, aglycones, and glucosides (Jayaprakasha et al., 2008b). For instance, Klangpetch et al. (2016) found that the ethyl acetate peel extract of kaffir lime possesses high levels polyphenols and also antioxidant and antimicrobial potential and then investigated it in prolonging the shelf life of raw chicken drumettes. As Wang et al. (2015) identified allelochemicals with algicidal activity from the ethyl acetate extracts of *Citrus* peel, including shaddock peel. Benavente-Garcia et al. (1997) reviewing about flavonoids, mentioned that several biological properties such as anticarcinogenic, cardiovascular, and antimicrobial of these substances are related to their antioxidant activity.

As presented in Table 1, in the DPPH assay, ethyl acetate, methanol, and acetone extracts presented lower IC_{50} values (298.3 ± 2.6, 303.8 ± 0.4, and 316.7 ± 7.7 μg/ml, respectively) compared with other tested extracts. As for the ABTS scavenging activity (Table 1), IC_{50} values revealed that not only ethyl acetate and methanol extracts exhibited the best antioxidant activity but also the methanol:water (80:20), with IC_{50} values of 298.2 ± 0.6, 296.4 ± 2.5, and 264.1 ± 0.4 μg/ml, respectively. And the ethyl acetate, ethanol, and acetone extracts exhibited the best ferric reducing antioxidant power determined by the FRAP assay. As demonstrated in all assays performed, the ethyl acetate extract is considered the most promising extract.

The differences shown in the antioxidant values obtained may denote a consequence of the relationship between solvent polarity and antioxidant structure and are consistent with data reported for other citrus varieties subjected to comparable extractions (Klangpetch et al., 2016). These data add valuable information to knowledge of the antioxidant capacity of toranja peels.

Most of the antioxidant compounds that have a chelating action on ABTS radicals were extracted also with methanol:water (80:20) and methanol (Table 1). 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).

As for the reduction of iron (Fe^{3+}) in FRAP assay, the most reactive extracts were ethyl acetate and ethanol in peels, a result that is consistent with the ones found by Toh et al. (2013) where it was associated that the antioxidant activity is related not only with flavonoids but also with organic acid such as ascorbic.

These results are potentially important because they imply that toranja peels are a greater source of antioxidants than the interior section (juice), which is in agreement with other studies (Ademosun et al., 2015; Toh et al., 2013). And also indicated is that the compounds involved in defense reactions of the citrus fruit are found in greater amounts in the superficial layer; therefore, the antioxidant activity should be related to the part in the fruit where protection against lipid oxidation is needed. This is consistent with a model that exhibits major differences in distribution among different tissues in the citrus fruit.

TABLE 1 In vitro antioxidant activity of *Citrus maxima* extracts determined by ferric reducing antioxidant power (FRAP), and DPPH and ABTS radical-scavenging assays

C. maxima samples		DPPH	ABTS	FRAP
Origin	Solvents used for extraction	IC_{50} (μg/ml)	IC_{50} (μg/ml)	IC_{50} (μg/ml)
Peel	Hexane	>500	455.0 ± 5.3^{a}	396.3 ± 5.1^{a}
	Ethyl acetate	298.3 ± 2.6^{a}	298.2 ± 6.4^{b}	234.6 ± 1.8^{b}
	Acetone	316.7 ± 7.7^{a}	406.5 ± 5.6^{c}	313.7 ± 0. 7^{cde}
	Ethanol	381.3 ± 8.8^{b}	381.2 ± 3.0^{d}	269.2 ± 0.7^{bde}
	Methanol	303.8 ± 0.4^{a}	296.4 ± 2.5^{be}	398.1 ± 3.8^{a}
	Methanol:water (80:20)	346.1 ± 8.6^{c}	264.1 ± 0.4^{bf}	336.7 ± 2.9^{ce}
Juice	Methanol	>500	>500	>500
Quercetin		3,3 ± 0,0	5,55 ± 0,0	3,59 ± 0,0

Note. Different letters in the same column correspond to significant differences ($P < 0.05$). Tests ($n = 3$) were performed in triplicate and expressed as the mean ± *SD*.

This is expected considering the ubiquitous citrus distribution in nature and its ability to survive under adverse conditions, including high temperatures and UV light. The results presented here prove the model. Thus, the citrus flavedo would tend to accumulate antioxidants and antimicrobial compounds 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 physiologically functional substances that give us a variety of possibilities to develop. There are several studies suggesting that the bioactive compounds in citrus peels can be used as a nutraceutical or as a functional food. According to Castro-Vasquez et al. (2016), grapefruit peel wastes are a natural source of bioactive flavonoids, mostly naringin, which could be incorporated as food ingredients or as therapeutic agents used as a part of pharmacological strategies.

The peels of citrus fruits are actually byproducts generated on a daily basis by juice extraction or the fresh-cut ready-for-consumption (minimal processing) industries, and large amounts of waste materials are generated. Domestically, citrus peels have been processed into candies and are consumed in some American homes or taken as infusion drinks in Asia (Ademosun et al., 2015). However, these byproducts of the juice extraction industry could be used as sources of natural antioxidants (Guimarães et al., 2010; Klangpetch et al., 2016).

Quercetin was used as a reference in the assays because it represents one of the major phenolic compounds found in citrus peels

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

C. maxima samples		Induction ratio[a]	% Survival	IC_{50}[b] (μg/ml)	CD[c] (μg/ml)	CI[d]
Origin	Solvents used for extraction					
Peel	Hexane	3.0 ± 0.78	95.2 ± 8.1	>20	13.13 ± 0.74	>1.5
	Ethyl acetate	2.0 ± 0.31	93.6 ± 1.8	>20	3.07 ± 1.07	>6.5
	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			

Note. Results are means ± *SD* (n = 3).

[a]Induction ratio was determined by calculating the average specific activity versus specific activity of the control.

[b]Mean value of the half-maximal inhibitory concentration of cell viability.

[c]Mean value of the concentration required to double the specific activity of quinone reductase.

[d]Chemopreventive index: ratio between IC_{50} and CD.

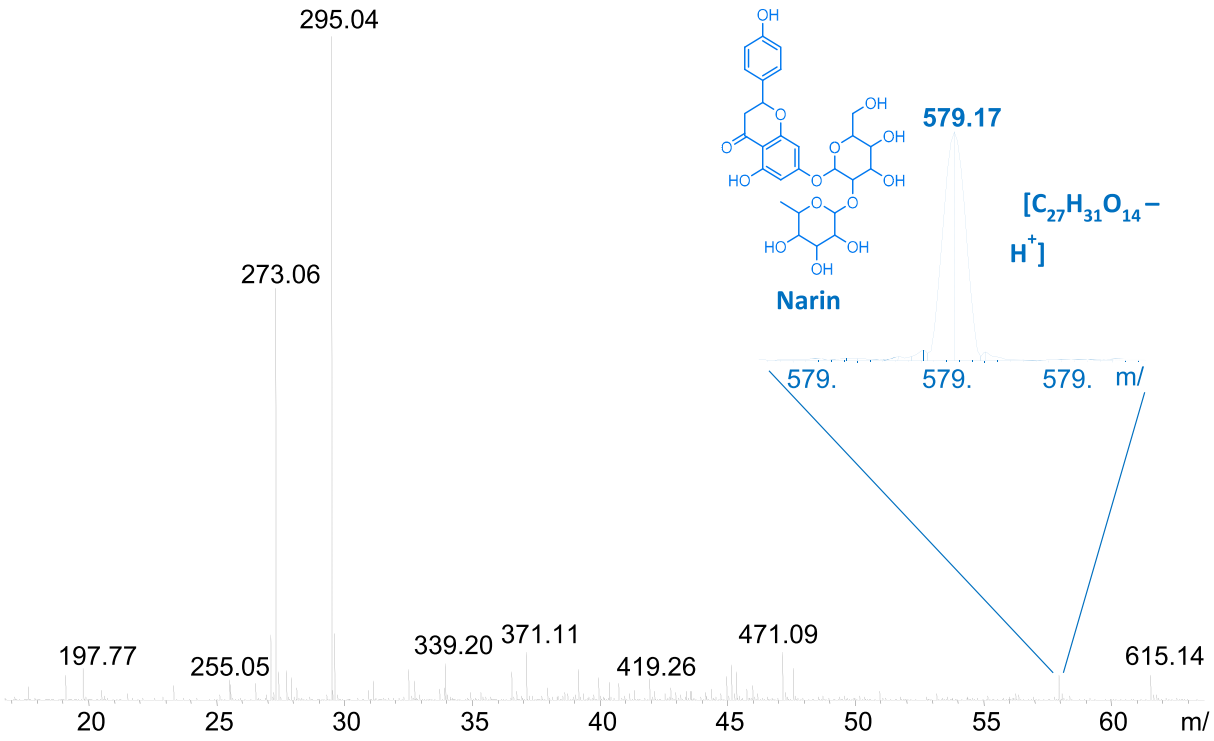

FIGURE 1 ESI(-)FT-ICR MS spectrum of the ethyl acetate extract of *Citrus maxima* peels [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Chemical species proposed from ESI(-)FT-ICR MS data for the ethyl acetate extract of *Citrus maxima* peels

$[M-H]^-$	Measured *m/z*	Theoretical *m/z*	DBE	Error (ppm)	Proposed compound	Reference
$C_7H_5O_5$	169.0145	169.0142	5	1.14	Gallic acid	Kelebek, 2010
$C_7H_{11}O_6$	191.0561	191.0561	2	0.22	Quinic acid	Touati et al., 2017
$C_7H_9O_7$	205.0354	205.0354	3	0.01	Homocitric acid (3-hydroxybutane-1,2,3-tricarboxylic acid)	—
$C_7H_{11}O_7$	207.0510	207.0510	2	0	4-O-Methyl-D-glucuronic acid	Stoddart and Jones, 1998
$C_{11}H_{11}O_5$	223.0612	223.0611	6	−0.14	Sinapinic acid	Bocco, Cuvelier, Richard, & Berset, 1998 Kelebek, 2010
$C_9H_{13}O_7$	233.0668	233.0667	3	−0.35	Trimethyl citrate	—
$C_{11}H_{11}O_7$	255.0511	255.0510	6	−0.24	Piscidic acid	Touati et al., 2017
$C_{16}H_{31}O_2$	255.2331	255.2329	1	−0.5	Palmitic acid	Nordby & Nagy, 1974
$C_{15}H_9O_5$	269.0457	269.0455	11	−0.5	Apigenin	Nogata et al., 2006 Benevente-Garcia et al., 1997
$C_{15}H_{11}O_5$	271.0613	271.0612	10	−0.27	Naringerin	Benevente-Garcia et al., 1997
$C_{16}H_{31}O_3$	271.2279	271.2279	1	−0.15	16-hydroxypalmitic acid	—
$C_{13}H_9O_7$	277.0355	277.0354	9	−0.57	(2,3,4-Trihydroxyphenyl)(3,4,5-trihydroxyphenyl) methanone ou exifone	—
$C_{18}H_{29}O_2$	277.2175	277.2173	4	−0.66	Linolenic acid	Nordby e Nagy, 1974
$C_{18}H_{31}O_2$	279.2331	279.2329	3	−0.42	Linoleic acid	Nordby e Nagy, 1974
$C_{18}H_{33}O_2$	281.2488	281.2486	2	−0.60	Oleic acid	Nordby e Nagy, 1974
$C_{13}H_{11}O_8$	295.0458	295.0459	8	0.34	3,4,5-triacetoxybenzoic acid	—
$C_{18}H_{31}O_5$	327.2180	327.2177	3	−0.86	Malyngic acid ou Aspicilin	—
$C_{15}H_{13}O_9$	337.0568	337.0565	9	−1.01	7-Hydroxycoumarin glucuronide	—
$C_{12}H_{19}O_{11}$	339.0935	339.0933	7	−0.68	beta-D-glucopyranosyl-(1->4)-D-glucono-1,5-lactone	—
$C_{17}H_{13}O_8$	345.0619	345.0616	11	−0.99	Syringetin	—
$C_{15}H_{25}O_{10}$	365.1456	365.1453	3	−0.70	3-{[3-Hydroxy-2-(hydroxymethyl)-2-methylpropanoyl]oxy}-2-({[3-hydroxy-2-(hydroxymethyl)-2-methylpropanoyl]oxy}methyl)-2-methylpropanoic acid ou Euonyminol	—
$C_{13}H_{23}O_{12}$	371.1197	371.1195	2	−0.65	(5ξ)-6-O-{(5R)-5-[(1S)-1,2-Dihydroxyethyl]-α-D-lyxopyranosyl}-α-D-xylo-hexopyranose ou 6-O-Heptopyranosyl-D-glucopyranose	—

(Continues)

TABLE 3 (Continued)

$[M-H]^-$	Measured *m/z*	Theoretical *m/z*	DBE	Error (ppm)	Proposed compound	Reference
$C_{16}H_{27}O_{10}$	379.1614	379.1610	3	−1.04	2-Methyl-3-buten-2-yl 6-O-[(2R,3R,4R)-3,4-dihydroxy-4-(hydroxymethyl)tetrahydro-2-furanyl]-β-D-glucopyranoside ou 3-Methyl-2-buten-1-yl 6-O-[(2R,3R,4R)-3,4-dihydroxy-4-(hydroxymethyl)tetrahydro-2-furanyl]-β-D-glucopyranoside ou Methyl 6-deoxy-4-O-β-D-galactofuranosyl-2,3-O-isopropylidene-α-L-mannopyranoside ou 4-O-acetyl-2,3-di-O-methyl-α-L-fucopyranosyl-(1->3)-α-L-rhamnopyranose	—
$C_{17}H_{24}NO_9$	386.1461	386.1457	6	−1.1.7	Allyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside ou Allyl (5ξ)-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-lyxo-hexopyranoside ou Allyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranoside ou (1R,2R,3R,4S)-4-[(1R)-1-Acetamido-2-acetoxyethyl]-1,2,3-cyclopentanetriyl triacetate	—
$C_{21}H_{39}O_6$	387.2756	387.2752	2	−0.99	Bis (2-butoxyethyl) azelaate ou Dioctyl bis (hydroxymethyl) malonate ou 3-O-14-Pentadecen-1-yl-D-glucopyranose	—
$C_{20}H_{15}O_9$	399.0725	399.0722	13	−0.84	Dibenzo [b,d]furan-2,3,7,8-tetrayl tetraacetate or Theaflagallin	—
$C_{17}H_{27}O_{11}$	407.1564	407.1559	4	−1.14	β-D-Fructofuranosyl 4,6-O-[(1R,2Z)-2-methyl-2-buten-1-ylidene]-α-D-glucopyranoside	—
$C_{15}H_{25}O_{13}$	413.1305	413.1301	3	−1.11	β-D-Xylopyranosyl-(1->4)-β-D-xylopyranosyl-(1->4)-D-xylopyranose	—
$C_{21}H_{39}O_8$	419.2655	419.2650	2	−1.03	2,2,3,3-Tetraethoxy-4-(nonyloxy)-4-oxobutanoate	—
$C_{16}H_{21}O_{13}$	421.0992	421.0988	6	−1.0	2-O,3-O-Bis (methoxycarbonyl)-D-glucopyranose 1,4,6-triacetate	—
$C_{23}H_{37}O_7$	425.2549	425.2545	5	−1.08	1(3)-glyceryl-Prostaglandin E2	—
$C_{23}H_{39}O_7$	427.2705	427.2702	4	−0.99	2,3-Dihydroxypropyl 9,11,15-trihydroxyprosta-5,13-dien-1-oate	—
$C_{22}H_{43}O_8$	435.2968	435.2963	1	−1.08	Hexadecanoic acid - α-D-galactopyranose	—
$C_{22}H_{17}O_{10}$	441.0832	441.0827	14	−1.13	(−)-Epicatechin-3-O-gallate ou (+)-epicatechin-3-O-gallate	Swatsitang, Tucker, Robards, & Jardine, 2000
$C_{18}H_{19}O_{13}$	443.0836	443.0831	9	−1.11		—

(Continues)

TABLE 3 (Continued)

$[M-H]^-$	Measured *m/z*	Theoretical *m/z*	DBE	Error (ppm)	Proposed compound	Reference
					Pentamethyl 3-[methoxy (oxo)acetyl]-1,4-pentadiene-1,2,3,4,5-pentacarboxylate	
$C_{21}H_{21}O_{11}$	449.1094	449.1089	11	−1.11	Flavanomarein	Venturini, Barboni, Curk, Costa, & Paolini, 2014
$C_{22}H_{43}O_9$	451.2917	451.2913	1	−1.02	Docosanal	—
$C_{17}H_{25}O_{14}$	453.1254	453.1250	5	−1.01	5-(2-Methoxy-2-oxoethyl) pentopyranosyl methyl 2-deoxy-6-(methoxycarbonyl)hex-3-ulofuranosidonate	—
$C_{19}H_{21}O_{13}$	457.0993	457.0988	9	−1.18	4-[(2-O,3-O,6-O-Triacetyl-beta-D-glucopyranosyl)oxy]-2,6-dihydroxybenzoic acid	—
$C_{24}H_{41}O_8$	457.2812	457.2807	4	−1.13	Stearyl citrate mono	—
$C_{24}H_{43}O_8$	459.2969	459.2963	3	−1.20	Fructose Oleate	—
$C_{23}H_{19}O_{11}$	471.0937	471.0933	14	−0.94	(2R,3R)-2-(3,5-Dihydroxy-4-methoxyphenyl)-5,7-dihydroxy-3,4-dihydro-2H-chromen-3-yl 3,4,5-trihydroxybenzoate ou 4'-O-methylepigallocatechin-3-O-gallate	—
$C_{21}H_{33}O_{14}$	509.1882	509.1876	5	−1.29	(1S,4aR,5R,7aS)-5-(β-D-Galactopyranosyloxy)-7-hydroxy-7-methyl-1,4a,5,6,7,7a-hexahydrocyclopenta [c]pyran-1-yl β-D-allopyranoside	—
$C_{27}H_{29}O_{14}$	577.1572	577.1563	13	−1.55	Rhoifolin ou Isorhoifolin ou Apigenin-7-rutinoside	Nogata et al., 2006 Zhang, Nan, Wang, Jiang, & Li, 2014
$C_{27}H_{31}O_{14}$	579.1726	579.1720	12	−1.15	Naringin	Nogata et al., 2006 Benevente-Garcia et al., 1997 Zhang et al., 2014
$C_{28}H_{33}O_{14}$	593.1886	593.1876	12	−1.73	Poncirin ou Neopocirin ou didymin	Nogata et al., 2006 Zhang et al., 2014
$C_{33}H_{27}O_{12}$	615.1496	615.1508	20	1.96	[(3S,4R)-3,4,5-tris[(4-hydroxybenzoyl)oxy]pentyl] 4-hydroxybenzoate	—

Note. The formula was not found in the ChemSpider database, METLIN Metabolomics Database and PubChem.

(Ademosun et al., 2015), and it has been reported that it can modulate Phase II metabolism inducing QR (Chen & Blumberg, 2008). Quercetin inhibited DPPH by 69.7 ± 2.59% at the minimum concentration tested (0.001 mM).

3.2 | Cancer chemopreventive activity of the extracts

In an attempt to determine the biological aspects of the different toranja peel extracts as a source of cancer chemopreventive substances, the effect on QR1 activity was examined. As discussed in the antioxidant evaluation, it was analyzed if the solvent chosen for extraction will affect presence of the constituents in these extracts. And to be considered a potential chemopreventive agent, the extracts should not have a cytotoxic effect, so the cytotoxicity of the extracts was evaluated (Table 2). *Citrus maxima* peels did not show cytotoxicity.

In all the extracts tested in the QR induction assay (Table 2), the IR of QR activity for the hexane and ethyl acetate extracts was higher than 2.0, and thus, these extracts were considered true activators of QR1.

A plot of the ratio of IR as a function of the extract inducer concentration allowed the determination of the extract concentration, which causes double induction (CD; Table 2). A 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 and Pezzuto (2004). CD values were determined through a dose–response assay for active extracts. Table 2 shows the effect of the hexane and ethyl acetate extracts (IR > 2) on the QR activity. These extracts presented CD values of 13.13 and 3.07 μg/ml, respectively. Therefore, ethyl acetate extracts yielded a CI higher than 6.5.

The QR in vitro assay was used as a biomarker to evaluate the ability of the extract to act as an inducer of Phase II metabolic activity. The 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). It is well established that induction of QR can offer protection against toxic and reactive chemical species (Cuendet et al., 2006). Therefore, the results presented indicate that toranja peel has a potential use for cancer prevention, and its consumption should be incentivized included as ingredient of juice, for example.

3.3 | Chemical profile of ethyl acetate and hexane peel extracts

Among the several extracts obtained by the different organic solvents, many seemed to possess chemical compounds with chemopreventive and antioxidant potential. The ethyl acetate extract was shown to have a higher CI than the hexane extract and was also the most active in scavenging radicals in all antioxidant assays. In order to better understand these biological characteristics, the chemical profile of the ethyl acetate extract was obtained by ESI(-)-FT-ICR MS analysis, as demonstrated in Figure 1. The compounds detected are reported in Table 3 and are compared with those presented in the literature. And several of the flavonoids identified in toranja are consistent with others varieties of citrus (Zhang et al., 2014).

Due to its ultrahigh mass accuracy (mass deviations lower than 1 ppm), elemental composition ($C_cH_hN_nO_oS_s$), isotopologue profile, and the double bond equivalent, ESI(-)-FT-ICR MS ensures the identification of heteroatom species as well as their degree of aromaticity, as shown in Table 3. ESI(-) promoted the detection of molecules via a deprotonation mechanism (production of $[M-H]^-$ ions). The generation of these deprotonated molecules depends on factors that include their acid-base and chemical properties, such as solubility and polarity (availability of protonation sites). However, for a complete chemical characterization, the collision-induced dissociation experiments and nuclear magnetic resonance spectroscopy analyses will be necessary for each analyte.

In an attempt to isolate a chemopreventive compound present in toranja peel, 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 QR inducer and at least partially avoid the interference of other substances present in toranja peel. Extracts prepared with ethyl acetate gave the strongest CI.

So in conclusion, the Toranja 'Burarama' peels have the potential for cancer chemoprevention, as illustrated through the induction of QR. And among all the extracts tested, the ethyl acetate extract of *C. maxima* peel presented not only antioxidant potential as also the highest CI. Those properties can be related to the several compounds detected in this extract that are also present in other *Citrus*. However, future research is needed to clarify and elucidate the mechanism of the action of these compounds. Toranja "Burarama" peel wastes appear as a promising natural source of valuable bioactive components for use in the development of new products. And health professionals and consumers should be educated regarding the benefits of this food, where an adequate intake of citrus fruit peels may induce Phase II enzymes in the body, with potential benefits in chemoprevention of cancer. Further in vivo studies are needed to determine the molecular mechanisms of antioxidant and chemopreventive activities.

ACKNOWLEDGMENTS

The Fundação Estadual de Amparo à Pesquisa e Inovação do Estado do Espírito Santo (FAPES–65921208/2014, 734/2016) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq—Grants 401409/2014-7 PVE, 308631/2016-1 PQ, and 310680/2016-6 PQ) are gratefully acknowledged for their financial support.

CONFLICT OF INTEREST

All authors declare that have no conflict of interest.

FUNDING

This study was funded by Fundação Estadual de Amparo à Pesquisa e Inovação do Estado do Espírito Santo (FAPES Grant 241/2016) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq—Grants 401409/2014-7 PVE, 308631/2016-1, and 310680/2016-6 PQ).

ETHICAL APPROVAL

This article does not report any studies with human participants or with animals performed by any of the authors.

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How to cite this article: da Silva ES, Oliveira BG, Pereira ACH, et al. Induction of NAD (P)H: Quinone reductase 1 (QR1) and antioxidant activities in vitro of 'Toranja Burarama' (*Citrus maxima* [Burm.] Merr.). *Phytotherapy Research*. 2018;1–10. https://doi.org/10.1002/ptr.6149