Medical Oncology (2018) 35:54
https://doi.org/10.1007/s12032-018-1114-7

ORIGINAL PAPER

Protective effects of purple carrot extract (*Daucus carota*) against rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide

Glaucia Resende Soares[1] · Carolina Foot Gomes de Moura[1] · Marcelo Jose Dias Silva[2] · Wagner Vilegas[2] · Aline Boveto Santamarina[1] · Luciana Pellegrini Pisani[1] · Debora Estadella[1] · Daniel Araki Ribeiro[1]

Received: 20 February 2018 / Accepted: 7 March 2018 / Published online: 15 March 2018

Abstract
The aim of this study was to evaluate the chemopreventive potential of purple carrot extract following rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide (4NQO). For this purpose, histopathological analysis, proliferative status, antioxidant activity and inflammatory status were investigated in this setting. A total of 20 male rats were distributed into four groups as follows ($n=5$ per group): Group 1—free access to water and commercial diet for 12 weeks; Group 2—received 4NQO at 50 ppm dose in drinking water daily and commercial diet for 12 weeks; Group 3—free access to water and received diet supplemented with purple carrot extract (0.1 g/kg) for 12 weeks; and Group 4—received 4NQO at 50 ppm dose in drinking water daily and diet supplemented with purple carrot extract (0.1 g/kg) for 12 weeks. Histopathological analysis revealed that animals treated with purple carrot extract reduced the oral lesions such as dysplasia and squamous cell carcinoma. Animals with oral pre-neoplastic lesions and treated with purple carrot extract decreased ki-67 and 8-OHdG immunoexpression. Moreover, pNFκBp50 and MyD88 protein expressions were decreased after purple carrot treatment associated or not with 4NQO exposure. SOD-Mn mRNA levels increased with treatment with purple carrot extract as well. In conclusion, our results demonstrated that purple carrot extract was able to protect oral lesions induced by 4NQO in Wistar rats as a result of antioxidant activity, anti-inflammatory potential and antiproliferative and antimutagenic actions.

Keywords Oral cancer · Rat · 4-Nitroquinoline 1-oxide · Purple carrot · Chemopreventive studies

Introduction

Head and neck squamous cell carcinoma is ranked the fifth most diagnosed type of cancer. It affects around 600,000 patients *per year* worldwide being characterized by phenotypic, etiological, biological and clinical heterogeneity [1–3]. Oral cancer is multifactorial disease closely related to lifestyle such as tabagism and high consumption of alcoholic beverages, nutritional deficiencies and chronic environmental pollutants exposure [4, 5]. Administration of 4-nitroquinoline 1-oxide (4NQO) in drinking water has been reported as a suitable model for studying oral carcinogenesis process phase by phase because the chemical agent induces a whole spectrum of neoplastic lesions in the rat tongue mucosa [6]. Herein, several chemopreventive agents, both naturally occurring and synthetic compounds, have been studied against 4NQO-induced oral carcinogenesis model [7].

Gollücke et al. [8] reviewed the recent studies dealing with the use of polyphenols against carcinogenesis. Purple carrots (*Daucus carota*) contain high amounts of anthocyanins in their flesh taproots [9]. It is commonly used as a natural food colorant in candies, ice cream and beverages. To date, purple carrot has been used as a natural dye in the food industry, replacing artificial ones [10]. Besides the use as a natural additive, the consumption of purple carrots has increased in Western Europe, due to the relevant contribution of polyphenols from the diet [11]. Recently, Algarra et al. [12] reported in vitro antioxidant potential and polyphenol content of red carrots in Spain. The extracts contained between 1.9 and 4.9 g/kg of polyphenols, equivalent to the content found in grape juice, being the antioxidant activity of purple carrot higher than yellow carrot.

✉ Daniel Araki Ribeiro
daribeiro@unifesp.br

[1] Department of Biosciences, Federal University of São Paulo (UNIFESP), Rua Silva Jardim, 136, Vila Mathias, Santos, SP 11050-020, Brazil

[2] Sao Paulo State University (UNESP), Campus Litoral Paulista, São Vicente, SP, Brazil

To the best of our knowledge, the nutraceutical potential of purple carrot is not yet fully established. In vitro studies have demonstrated the ability of purple carrot extract to inhibit the proliferation of some tumor cell lines, as well as to promote antioxidant activity [13, 14]. In 2010, Poudyal et al. [15] were pioneers in testing purple carrot juice for preventing cardiovascular diseases. The authors verified that purple carrot juice improved some biochemical parameters, such as dyslipidemia, glucose tolerance, hypertension, among others, after induction of metabolic syndrome in rats. The effects were attributed to phenolic compounds, since rats treated with β-carotene did not have the same biological effects in the same experimental design [15].

As a result and because of the lack of scientific evidence, the aim of this study was to evaluate the chemopreventive potential of purple carrot extract following rat tongue carcinogenesis induced by 4NQO. For this purpose, histopathological analysis, proliferative status, antioxidant activity and inflammatory status were investigated in this setting.

Materials and methods

Animals and experimental design

All experimental protocols involving animals were made in accordance with that described in the Guiding Principles for the Use of Laboratory Animals. The study was approved by the Animal Committee of Federal University of São Paulo, UNIFESP (number 1969230415). Male Wistar rats (8-week-old, weighing approximately ~ 250 g) were obtained from Development Center of Experimental Models for Medicine and Biology (CEDEME), Federal University of São Paulo, SP, Brazil. They were maintained under controlled conditions of temperature (24 ± 2 °C), light–dark periods of 12 h.

A total of 20 male rats were distributed into four groups as follows ($n = 5$ per group): Group 1 (CTRL)—free access to water and commercial diet (Nuvital™, PR, Brazil) for 12 weeks; Group 2 (4NQO CTRL)—received 4NQO at 50 ppm dose in drinking water daily and commercial diet (Nuvital™, PR, Brazil) for 12 weeks; Group 3 (Carrot CTRL)—free access to water and received diet supplemented with purple carrot extract (0.1 g/kg) (Christian Hansen™, SP, Brazil) for 12 weeks; Group 4 (4NQO + carrot)—received 4NQO at 50 ppm dose in drinking water daily and diet supplemented with purple carrot extract (0.1 g/kg) (Christian Hansen™, SP, Brazil) for 12 weeks.

The experimental period was established in order to evaluate the putative nutraceutical effects of purple carrot extract during experimental carcinogenesis as described elsewhere [16–18]. The daily dose was calculated to provide the equivalent to humans, i.e., 2 g polyphenols/day, taking into consideration the rat metabolism (twice faster than humans). This amount was reported by the American Dietetic Association (ADA) as sufficient to promote beneficial health effects [19]. This value corresponds to 400 mg/L of purple carrot extract [19]. All animals were checked daily for behavior and general health conditions. Body mass was recorded weekly. At the end of the experimental period (12 weeks), all animals were anesthetized with inhalational anesthetic halothane (Tanohalo™, Cristália™, Brazil) and euthanized for tissue collection.

Chemical analysis

Sample preparation

The purple carrot extract (5 mg, Christian Hansen™, SP, Brazil) was dissolved in methanol 10 mL (LC–MS grade, Sigma-Aldrich™, Taufkirchen, Germany), and the mixture was centrifuged for 5 min at 3000×*g*. The supernatant was filtered through a nylon membrane disk 22.25 mm diameter, 0.22 µm pore size (Flow Supply™, Cotia, SP, Brazil).

Characterization of purple carrot extract by electrospray ionization mass spectrometry (ESI-MSn)

ESI-MSn data were collected in the negative and positive ion modes with a Fleet LCQ Plus ion-trap instrument from Thermo Scientific (ThermoScientific™, Bremen, Germany). The capillary voltage was set at − 20 kV, the spray voltage at − 5 kV and the tube lens offset at 100 V, sheath gas (nitrogen) flow rate at 80 (arbitrary units) and auxiliary gas flow rate at 5 (arbitrary units). Data were acquired in MS1 and MSn scanning modes. The diluted solution was then directly infused into the ESI source at a flow rate of 10 mL/min via a microsyringe pump. The mass analyzer was set to scan along a m/z range of 100–2000. ESI-MS/MS experiments were carried out by selection of a specific ion by Q1 and then submitting this to collision-induced dissociation with Ar in a collision chamber. The capillary temperature was 275 °C. Xcalibur 2.2 Software (ThermoScientific™, Bremen, Germany) was used for data analysis. The degree of confidence for this method is 99%. Comparison of the observed MS1 and MSn spectra with those found in the literature was the main tool for identification of polyphenols.

Determination of total polyphenols

Total polyphenol contents were determined according to the method described by Singleton [20] and Ghasemzadeh et al. [21] with some modifications. The Folin–Ciocalteu reagent (10 mL) and the sample (10 mL MeOH = [100 µg/mL]) were mixed, and the reaction stopped after exactly 3 min. Then, Na_2CO_3 (5 mL = [50 µg/mL]) was added, and the solution homogenized and placed in the dark for 120 min at room

temperature in 96-well plates. Absorbance was measured at 750 nm (in triplicate) using a UV–Vis spectrophotometer (BioTek model Epoch). A standard curve was created using gallic acid (Sigma-Aldrich Chemical Co., St. Louis, MO) as the standard substance with a concentration range of 6.25–200.0 µg/mL. Results were expressed in mg of gallic acid equivalent per mL of sample (mg GAE/mL).

Histopathological analysis

After completing the experimental period, the tongues were longitudinally bisected for histopathological examination. The tissues were fixed in 10% buffered formalin (Merck™, Darmstadt, Germany), embedded in paraffin blocks and stained with hematoxylin and eosin (H.E., Merck™, Darmstadt, Germany). Histopathological analyses of the tongue sections were evaluated by light microscopy. The presence of hyperplasia, dysplasia and carcinoma per animal was observed.

Immunohistochemistry

Tongue sections of 4 µm were deparaffinized in xylene and rehydrated in graded ethanol (99.5%) and pre-treated in a microwave with 10 mM citric acid buffer (pH 6, 0.1 M citric acid—Synth™, São Paulo, Brazil; 0.1 M sodium citrate—Synth™, São Paulo, Brazil) for three cycles of 5 min each for antigen retrieval. They were pre-incubated with 0.3% hydrogen peroxide (Synth™, São Paulo, Brazil) for inactivation of endogenous peroxidase and then blocked with 5% normal goat serum for 30 min. The specimens were then incubated with anti-8-hydroxy-20-deoxyguanosine (8-OHdG, Santa Cruz Biotechnologies Inc™, MO, USA) at 1:100 dilution or anti-ki-67 (Biocare™, Concord, CA, USA) at 1:150 dilution, at 4 °C, overnight. This was followed by two washes in PBS and further incubation with a biotinylated secondary antibody (Biocare™, Concord, CA, USA), diluted 1:100 in PBS for 1 h. The sections were washed twice with PBS followed by the application of preformed avidin–biotin complex (Biocare™, Concord, CA, USA) for 1 h. The bound complexes were visualized by the application of a 0.05% solution of DAB (3,3-diaminobenzidine; Sigma™, St. Louis, MO, USA). The specimens incubated with 8-OHdG were counterstained with hematoxylin (Sigma™, St. Louis, MO, USA), and those specimens incubated with ki-67 were counterstained with fast green (Merck™, Darmstadt, Germany). Sections stained using immunohistochemistry were analyzed for the percentages of immunopositive cells in control (normal tissue) and "hot-spot" areas, i.e., hyperplasia, dysplasia and carcinoma. For this purpose, multiple representative fields in posterior region of tongue (four to six) from each slide were counted at 400× magnification. These values were used as labeling indices.

Micronucleus test

The micronucleus test was performed in bone marrow, according to Ribeiro et al. [22]. For this purpose, femoral bones were collected and stored in sodium chloride 0.9%. The proximal epiphyses of the bones were removed and 1 mL of fetal bovine serum (FBS; Cultilab™, Campinas, São Paulo, Brazil) was injected into the medullar canal and a smear on glass slides was performed with the suspension formed by the bone marrow and fetal bone serum. After drying the slides, they were stained with Giemsa (Merck™, Darmstadt, Germany). One thousand polychromatic erythrocytes cells were analyzed per animal. Slides were scored blindly using a light microscope with a 100x immersion objective.

Western blotting analysis

Tongues were homogenized in lyses buffer containing 100 mM Tris–HCl (pH 7.5), 1% Triton X-100, 10% sodium dodecyl sulfate (SDS), 10 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mg aprotinin from bovine lung/mL. The sample homogenate was centrifuged at 20,800×*g* for 40 min at 4 °C, and the supernatant was collected. The total protein concentration was measured with Bradford Reagent (LGC Laboratories, Inc).

Protein samples were electrophoretically separated in 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The 1% bovine serum albumin solution was used to block the membranes overnight at room temperature. The membranes were incubated overnight with the following primary antibodies: pNFκBp50 (sc-101744) purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The MyD88 (ab2064), TRAF6 (ab33915) and GADPH (ab128915) antibodies were obtained from Abcam (Cambridge, UK). Membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies for 1 h at room temperature.

The bands were visualized with enhanced chemiluminescence scanned at UVITec (Cambridge, UK) after adding the ECL reagent (Bio-Rad Laboratories, Inc). The intensities of each band sample were quantified by ImageJ software (ImageJ, National Institute of Health, Maryland, USA). While performing calculations of each band obtained for analysis of proteins of interest for this study were normalized using β-actin levels of the respective membrane.

Real-time PCR

Tongue tissue was homogenized using cold Trizol Reagent (Invitrogen™, Carlsbad, CA, USA) according to the

manufacturer's instructions. Total RNA isolated was determined using a NanoDrop spectrophotometer (ThermoScientific™, Madison, WI, USA). The samples were treated with DNAse (Invitrogen™, Carlsbad, CA, USA), and then, cDNA synthesis was performed using reverse transcriptase (Invitrogen™, Carlsbad, CA, USA). Real-time PCR was performed in 7500 Fast Real-Time PCR System (Applied Biosystems™, Foster City, CA, USA) using the SYBR Green Kit (Applied Biosystems™, Foster City, CA, USA). The primers sequence is listed in Table 1.

PCRs were performed in triplicate samples and submitted to 40 cycles of 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. An amplification efficiency curve using different cDNA dilutions was also performed for each gene tested. To normalize the data for the control and experimental groups, arbitrary units were calculated as: arbitrary unit $= 2^{-\Delta\Delta C_T}$, and $\Delta\Delta C_T =$ sample $\Delta C_T -$ control ΔC_T, where C_T is the threshold cycle.

Table 1 Primers sequence to qPCR analysis

Gene		5′-sequence-3′
GAPDH	Forward	CAACTCCCTCAAGATTGTCAGCAA
	Reverse	GGCATGGACTGTGGTCATGA
SOD/Mn	Forward	AACATTAACGCGCAGATCA
	Reverse	AATATGTCCCCCACCATTGA
SOD/CuZn	Forward	CCAGTGCAGGACCTCATTTT
	Reverse	CCTTTCCAGCAGTCACATTG
Caspase 3	Forward	AGCGGATTCCTGAGAGAGTG
	Reverse	GAGAATCGAACGGCAATAGG

Statistical analysis

All the data were expressed as mean ± standard deviation (SD). Kruskal–Wallis nonparametric test followed by Dunn's posttest was applied to histopathological analysis, western blotting and micronucleus test. One-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison post hoc test was used to immunohistochemistry and real-time PCR. The statistical analysis was performed using GraphPad Prism™ 6.0 program. $p < 0.05$ was considered to be significant.

Results

Chemical analysis

Total polyphenols content showed a concentration of 330.6 ± 2.9 mg GAE/mL of purple carrot extract. The fingerprint of purple carrot extract (Christian Hansen™, SP, Brazil) was analyzed by ESI-MS in the negative and positive ion modes in order to identify what polyphenols are present in the sample (Figs. 1 and 2). A total of 12 constituents were identified with collision energy ramp from 25 to 30% and comparisons with fragmentation profiles (Table 2). Several polyphenolic compounds were identified such as ascorbic acid, citric acid, catechin, peonidin, caffeoylquinic acid, caffeoyl *N*-tryptophan, procyanidin dimer type B, peonidin 3-xylosylgalactoside, peonidin 3-xylosylglucosylgalactoside, cyanidin-3-(2″xylopiranose-6″-6‴-feruloylglucopyranose)-galactopyranose, peonidin 3-caffeoyl-*p*-hydroxybenzoyl-sophoroside-5-glucoside and cyanidin 3′-(6″,6″-dicaffeoylsophoroside)-5-glucoside.

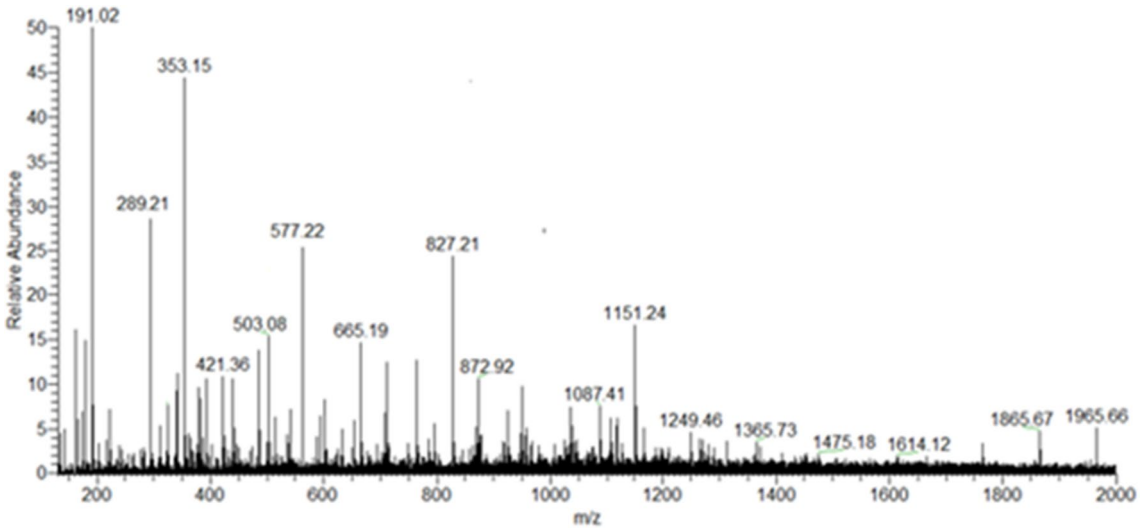

Fig. 1 Direct flow injection ESI-MS fingerprint spectra in full scan, obtained in negative ion mode of purple carrot extract

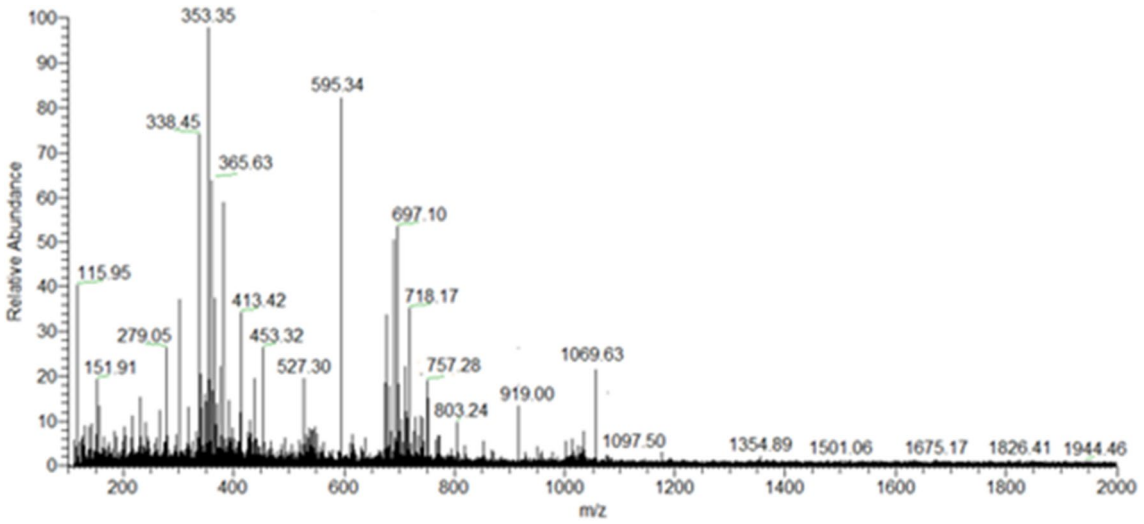

Fig. 2 Direct flow injection ESI-MS fingerprint spectra in full scan, obtained in positive ion mode of purple carrot extract

Table 2 Compounds identified from purple carrot extract

	Compound	$[M+H]^+/[M+H]^-$	MS/MS ions
1	Ascorbic acid	175 $[M+H]^-$	115
2	Citric acid	191 $[M+H]^-$	87
3	Catechin	289 $[M+H]^-$	245
4	Peonidin	301	287, 286, 268, 258, 230
5	Caffeoylquinic acid	353 $[M+H]^-$	191
6	Caffeoyl *N*-tryptophan	365	203
7	Procyanidin dimer type B	577 $[M+H]^-$	425, 451, 407, 559, 289
8	Peonidin 3-xylosylgalactoside	595	302, 301, 286, 258
9	Peonidin 3-xylosylglucosylgalactoside	757	301, 286, 268
10	Cyanidin-3-(2″xylopiranose-6″-6‴-feruloylglucopyranose)-galactopyranose)	919	287, 355, 554, 625
11	Peonidin 3-caffeoyl-*p*-hydroxybenzoyl-sophoroside-5-glucoside	1069	463, 907
12	Cyanidin 3′-(6″,6″-dicaffeoylsophoroside)-5-glucoside	1097	449, 629, 935

Clinical findings

Neither complications nor behavioral changes were observed during the experimental design. No statistically significant differences ($p > 0.05$) were observed in final body weight and weight gain for all groups evaluated. No animals died during the experiment. Such findings are shown in Table 3.

Histopathological analysis

Neither histopathological changes were observed in oral epithelial cells in the control group (G1) nor in the group treated with purple carrot extract only (G3) (Fig. 3a). The carcinogen 4NQO was effective for inducing oral lesions in the cancer group. The lesions were microscopically diagnosed as dysplasia and squamous cell carcinoma (Fig. 3b, c). In this group, a total of two specimens had dysplasia and three animals presented squamous cell carcinoma. A decrease in the total number of oral lesions was observed in the group treated with purple carrot extract following oral carcinogenesis (G4) when compared with Group 2. Such lesions were diagnosed as dysplasia (three animals) and hyperplasia (two animals). The numerical data are demonstrated in Table 4.

Immunohistochemistry

8-OHdG immunoexpression was detected in the nucleus or cytoplasm of rat tongue mucosa. Weak immunoreactivity for 8-OHdG was detected in the negative control group (G1) (Fig. 4a). The immunoreactivity was restricted to some

Table 3 Means and standard deviation of final body weight and weight gain, feed intake and water intake of rats treated with 4NQO and diet supplemented with purple carrot extract

	CTRL	4NQO CTRL	Purple carrot	4NQO + carrot
Daily water (mL)	184.8 ± 16.4	188.7 ± 11	164.3 ± 26.4	189.5 ± 16.2
Daily food (g)	120.4 ± 7.1	119 ± 7.4	116.3 ± 8	124.8 ± 8.4
Final body weight (g)	328 ± 13.1	406.7 ± 26.9	345.6 ± 45.1	384.6 ± 93.4
Weight gain (g)	116 ± 29.8	142.7 ± 47.6	102 ± 31.5	143.8 ± 57.8

$p > 0.05$

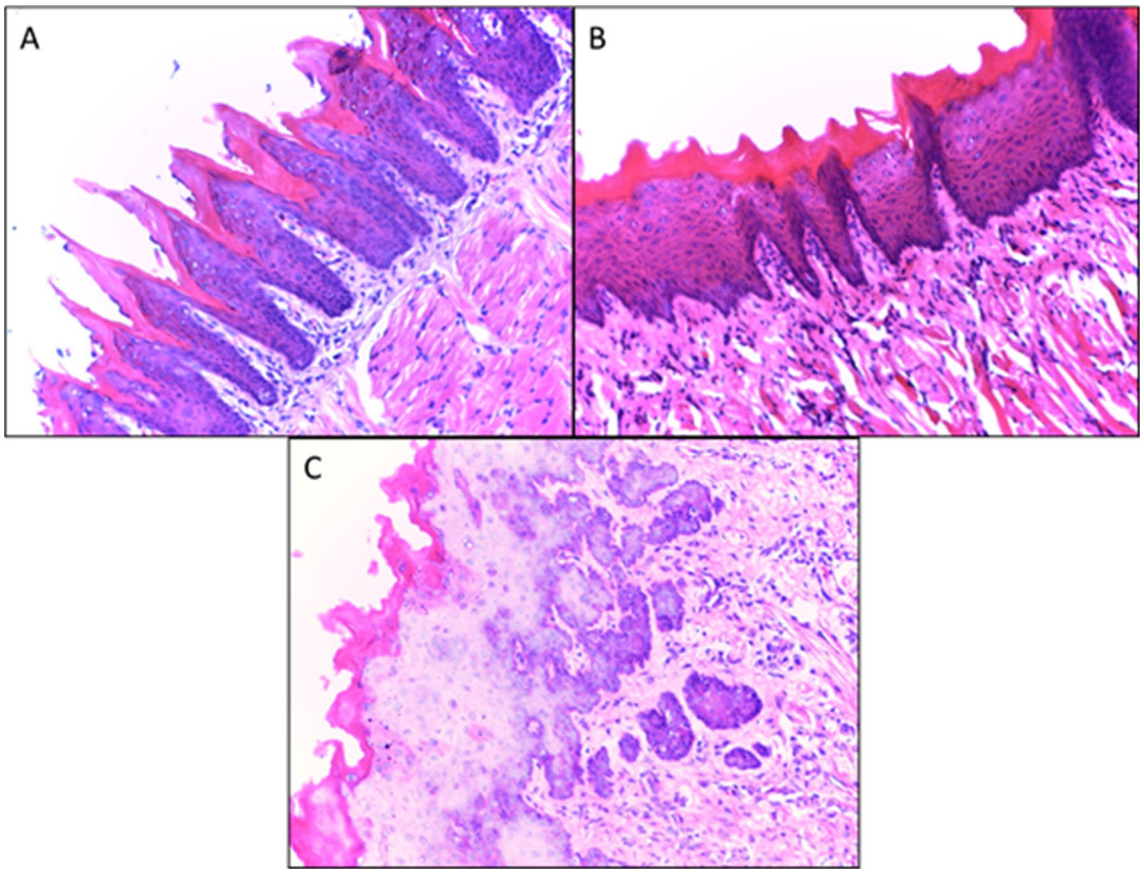

Fig. 3 Photomicrographs from rat tongue mucosa following 4NQO-induced oral carcinogenesis model treated with purple carrot extract. **a** Normal tongue epithelium, **b** dysplastic lesion, **c** oral squamous cell carcinoma. H.E. stain, ×40 magnification

Table 4 Incidence of histopathological lesions in tongue of rats in the 4-nitroquinoline 1-oxide (4NQO) model for oral carcinogenesis and treated with purple carrot extract

Groups ($n = 5$)	Normal (score 0)	Hyperplasia (score 1)	Dysplasia (score 2)	Carcinoma (score 3)
CTRL (G1)	5	0	0	0
4-NQO CTRL (G2)	0	0	2	3
Carrot CTRL (G3)	5	0	0	0
4-NQO + carrot (G4)*	0	2	3	0

*$p < 0.05$ when compared by G2 (4NQO)

cells of superficial layer of oral epithelium. The immunoexpression of this immunomarker was considered negative for this group. In the group exposed to 4NQO, most of tongue cells were positive for 8-OHdG expression (Fig. 4b). The immunoexpression was observed to all layers of oral epithelium indistinctly. Purple carrot extract was able to reduce the 8-OHdG immunoexpression when compared to 4NQO group (G2), being statistically significant differences ($p < 0.05$) between groups (Fig. 4c, d). The numerical results can be better visualized in Fig. 5.

Ki-67 immunoexpression was detected in the nucleus of oral mucosa cells. In the CTRL group, ki-67

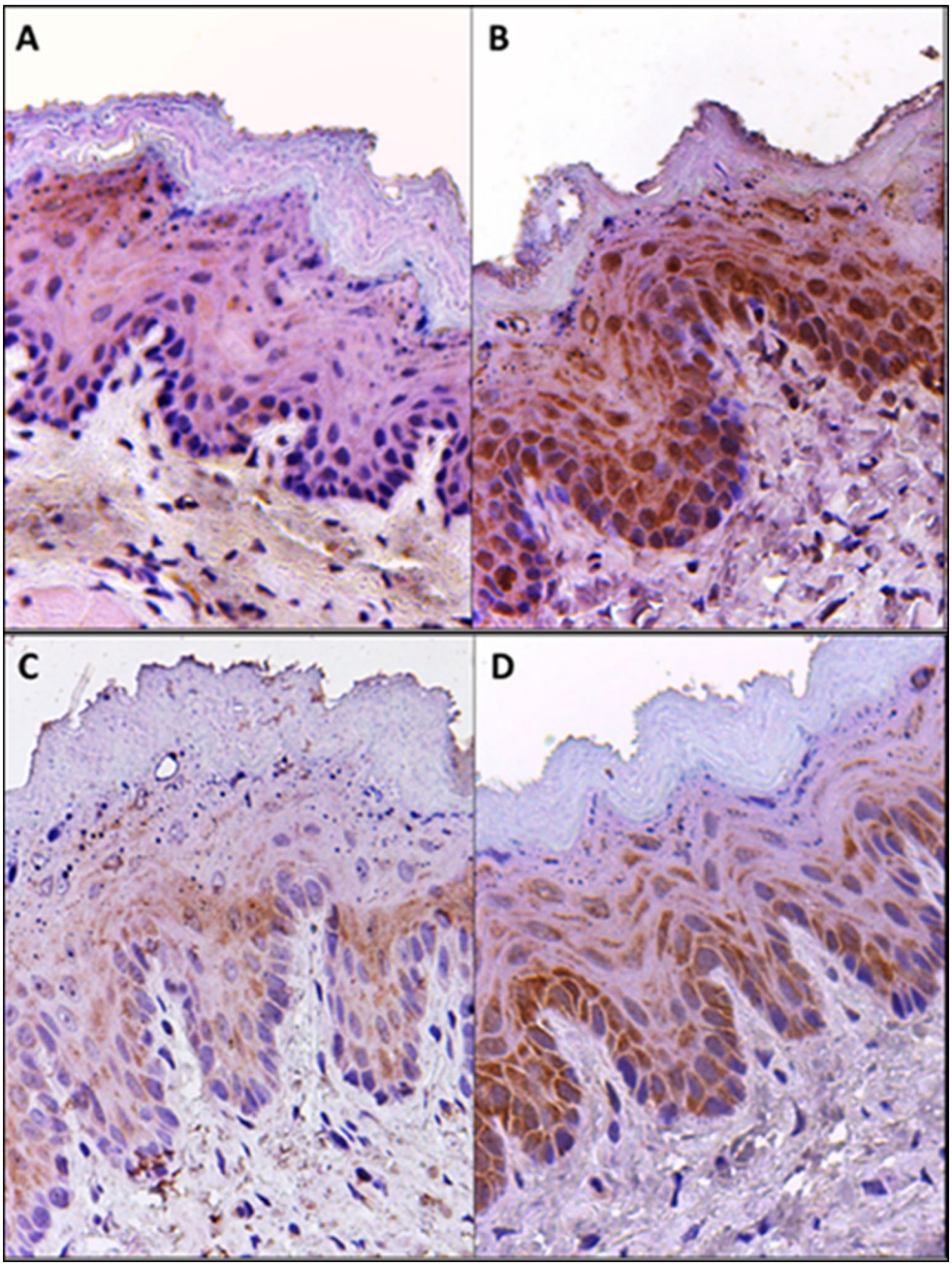

Fig. 4 Immunohistochemistry for 8-OHdG following rat tongue carcinogenesis induced by 4NQO. Immunopositive cells were detected either nucleus or cytoplasm of oral epithelium with brown stain. **a** CTRL; **b** 4-NQO CTRL; **c** purple carrot extract CTRL; **d** 4-NQO + purple carrot extract. Avidin–biotin complex method counterstained by Harris hematoxylin. ×40 magnification

immunoexpression was detected in the basal layer of oral mucosa (Fig. 6a). The groups exposed to 4NQO revealed increased Ki-67 immunoexpression when compared to the CTRL group (Fig. 6b, c). Diet supplementation with purple carrot extract decreased ki-67 immunoexpression when compared to 4NQO CTRL group (G2). Numerical results are demonstrated in Table 5.

Micronucleus test

Micronucleus test was performed in bone marrow to evaluate a possible antimutagenic effect of purple carrot extract following experimental oral carcinogenesis induced by to 4NQO. In this assay, 4NQO was mutagenic in bone marrow cells as depicted by high number of micronucleated

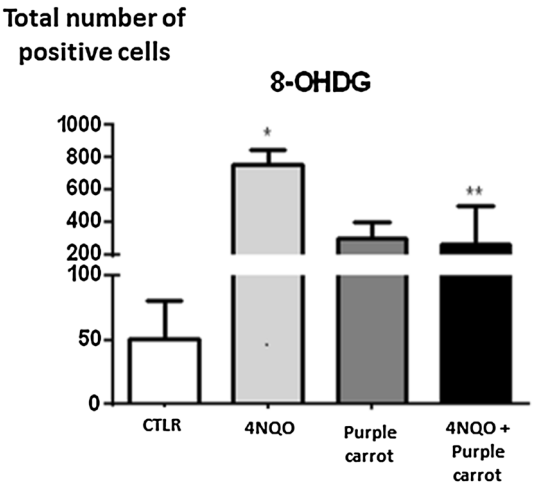

Fig. 5 Total number of 8-OHdG-immunopositive cells following rat tongue carcinogenesis induced by 4NQO treated with purple carrot extract. Results are expressed as mean ± SD. *$p < 0.05$ when compared to negative control (CTRL). **$p < 0.05$ when compared to 4NQO CTRL

cells in this group. The number of micronuclei in animals exposed to 4NQO and treated with purple carrot extract (G4) was decreased when compared with 4NQO group (G2) (Fig. 7b). Such data showed statistically significant differences between groups ($p < 0.05$). These data are summarized in Fig. 7. Control group (G1) and purple carrot extract group (G3) do not induce micronuclei significantly being the results similar to that found in CTRL (control) (Fig. 7a).

Western blotting

In this study, it was observed that protein expression of pNFκBp50 in rat tongue cells of control and 4NQO groups was increased when compared to the groups treated with purple carrot extract. Statistically significant differences ($p < 0.05$) were noticed to the purple carrot groups and respective controls. It seems that purple carrot extract promotes downregulation of this inflammatory marker in tongue cells of rats. The same results were obtained to MyD88, since statistically significant differences ($p < 0.05$) were noticed to the rats treated with purple carrot extract when compared to controls (negative control and 4NQO groups). TRAF 6 did not show statistically significant differences ($p > 0.05$) among groups. Such findings are summarized in Fig. 8.

Real-time PCR

The antioxidant enzymes manganese superoxide dismutase (CuZn-SOD) and copper–zinc superoxide dismutase (Mn-SOD) were analyzed by real-time polymerase chain reaction (qPCR). Mn-SOD nRNA levels increased after treatment with purple carrot extract for both groups associated with induction of oral cancer (G3 and G4) (Fig. 9a). However, the treatment with purple carrot extract decreases CuZn-SOD mRNA levels in oral mucosa cells, being statistically significant differences ($p < 0.05$) when compared to control group (Fig. 9). Other groups (cancer or cancer and treated with purple carrot extract) did not show any differences when compared to respective controls. Such findings are detailed in Fig. 9b.

When caspase 3 was evaluated in this setting, interesting findings were obtained to the pro-apoptotic marker. When animals were exposed to 4NQO, mRNA caspase 3 level decreased when compared to control group. No remarkable differences were noticed to groups treated with purple carrot extract with or without concomitant 4NQO exposure. Such findings are shown in Fig. 10.

Discussion

The protective effect of food rich in antioxidants using oral carcinogenesis model is described by several studies. To date, vitamin C is the antioxidant agent most investigated, followed by grape extract and garlic [23, 24]. In general, polyphenols have multifunctional benefits to human health [25]. It has been suggested that about 7–31% of all cancer worldwide could be reduced by diets containing high amounts of fruits and vegetables [26]. According to Ding et al. [27], the consumption of polyphenols may modulate several biological events closely associated with the development of oral cancer.

Previous studies conducted by our research group have demonstrated nutraceutic activity of purple carrot extract for preventing tissue degeneration, genotoxicity and oxidative stress induced by cadmium exposure in multiple organs of Wistar rats [9]. In view of these promising results, the aim of this study was to evaluate the protective effects of purple carrot extract following rat tongue carcinogenesis induced by 4NQO. For this purpose, histopathological analysis, cell proliferation, apoptosis and oxidative stress and inflammatory status were used in this study.

Regarding clinical findings, no statistically significant differences were noticed to water and food consumption as well as weight gain after conducting the experimental design. These data show that neither purple carrot extract consumption nor 4NQO exposure changed the rat basal metabolism. Poudyal et al. [16] have showed that the ingestion of purple carrot juice reduced abdominal fat deposition improving glucose tolerance and lipid status after 8 weeks of supplementation in rats suffering metabolic syndrome. Purple carrot was also described as a new strategy to ameliorate cholesterol levels [28].

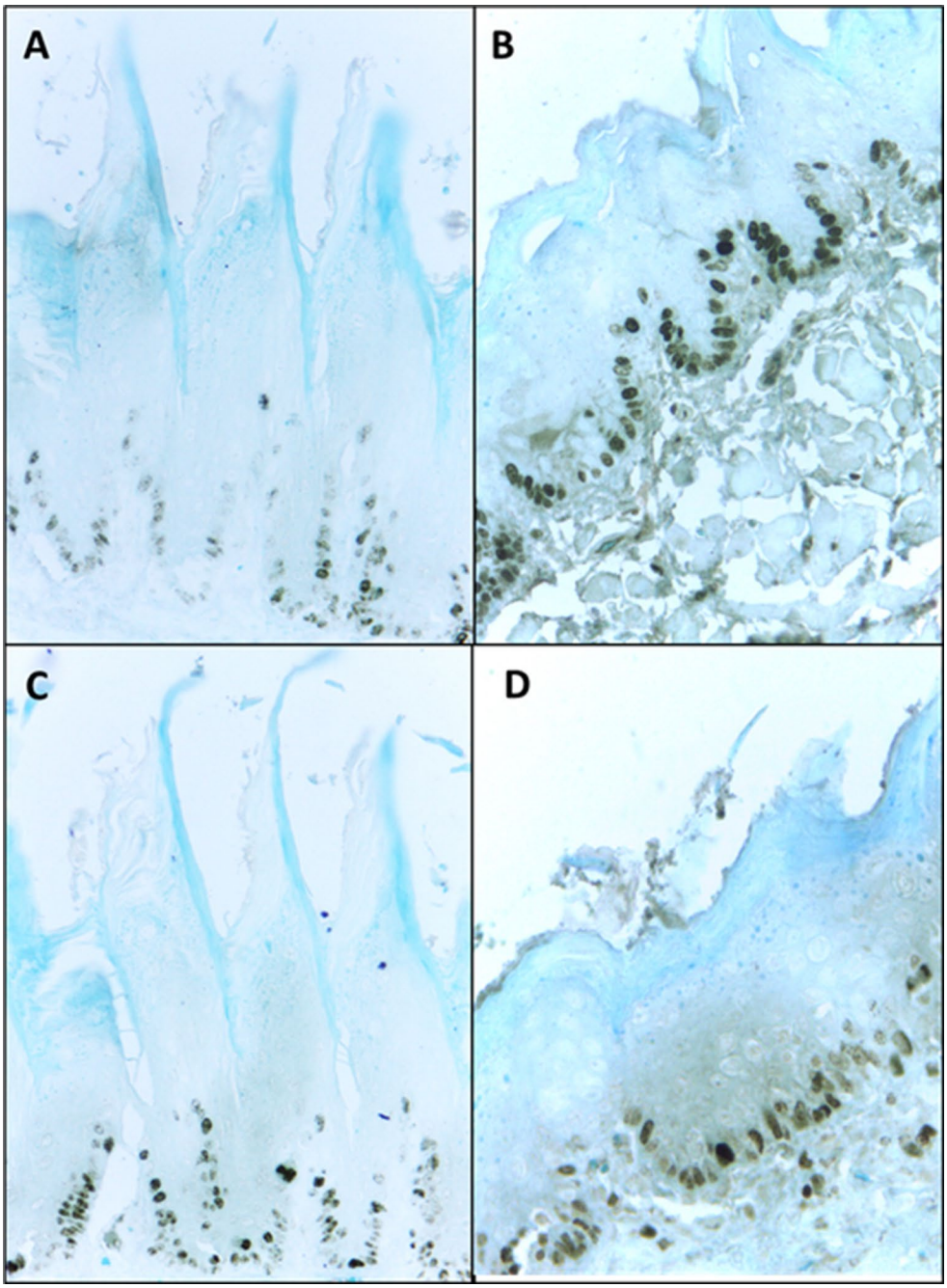

Fig. 6 Immunohistochemistry for 8-OHdG following rat tongue carcinogenesis induced by 4NQO. **a** CTRL; **b** 4-NQO CTRL; **c** purple carrot extract CTRL; **d** 4-NQO + purple carrot extract. ABC method counterstained by Harris hematoxylin. ×40 magnification

Table 5 Ki-67 immunoexpression in tongue of rats in the 4-nitroquinoline 1-oxide (4NQO) a model for oral carcinogenesis and treated with purple carrot extract

Groups ($n = 5$)	0–20%	> 20–40%	> 40–60%	> 60–80%	> 80–100%
CTRL (G1)	5	0	0	0	0
4-NQO CTRL (G2)	0	2	1	2	0
Carrot CTRL (G3)	5	0	0	0	0
4-NQO + carrot* (G4)	2	2	1	0	0

*$p < 0.05$ when compared with 4NQO CTRL group (G2)

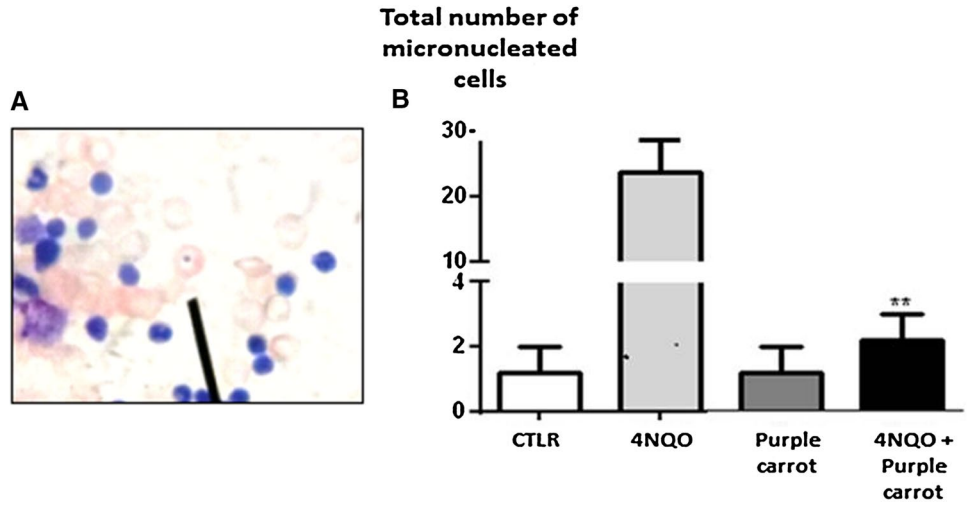

Fig. 7 Micronucleus data following rat tongue carcinogenesis induced by 4NQO. **a** micronucleated cell (arrow). **b** Total number of micronucleated cells in rats treated with purple carrot extract. Results are expressed as mean + SD. *$p < 0.05$ when compared to CTRL (control group. **$p < 0.05$ when compared to 4NQO CTRL

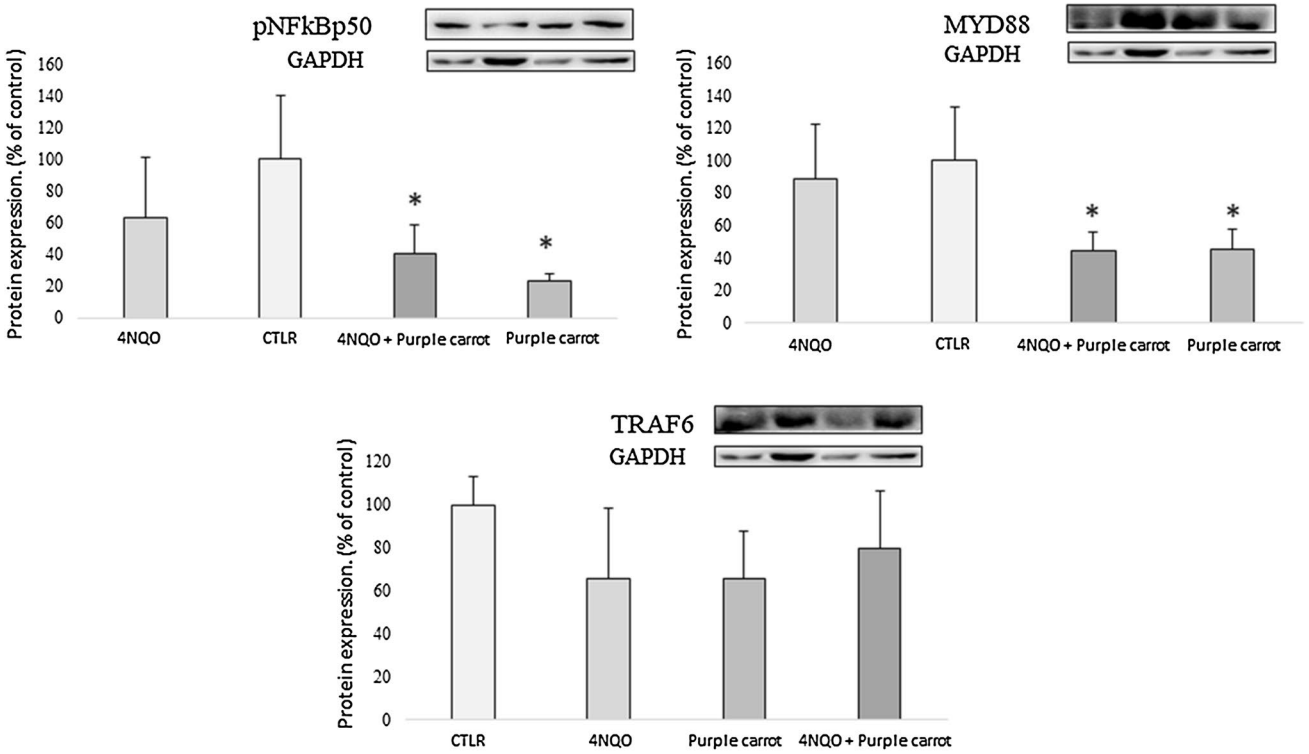

Fig. 8 Protein expression of pNFκBp50, MYD88 and TRAF 6 following oral carcinogenesis in rats treated with purple carrot extract. Results are expressed as mean + SD. *$p < 0.05$ compared to the respective control (4NQO or CTRL)

It has been described that purple carrot extract contains more than 40 phenolic acids; being anthocyanidins and chlorogenic acid as the major antioxidant compounds [29]. In this study, a chemical characterization of purple carrot extract was conducted in order to detect the main bioactive compounds. The following bioactive substances of biological interest was found by ESI-MSn: ascorbic acid, citric acid, catechin, peonidin, caffeoylquinic acid, caffeoyl *N*-tryptophan, procyanidin dimer type B, peonidin 3-xylosylgalactoside, peonidin 3-xylosylglucosylgalactoside, cyanidin-3-(2″xylopiranose-6″-6‴-feruloylglucopyranose)-galactopyranose, peonidin 3-caffeoyl-*p*-hydroxybenzoyl-sophoroside-5-glucoside and cyanidin 3′-(6″,6″-dicaffeoylsophoroside)-5-glucoside. Similar studies also identified these compounds in purple carrot extracts [30, 31]. Such bioactive substances are known to be effective in chemopreventive studies, such as catechin, peonidin, caffeine and cyanidin. In fact, the scientific literature

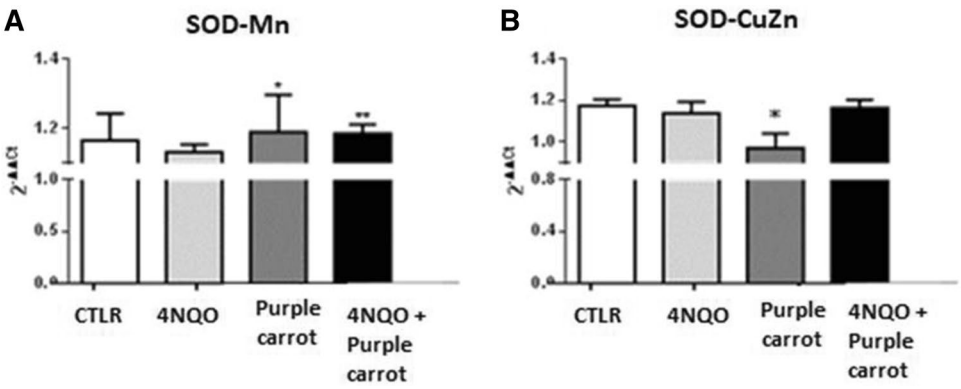

Fig. 9 Real-time PCR from SOD-Mn and SOD-CuZn following rat tongue carcinogenesis induced by 4NQO and treated with purple carrot extract. $*p < 0.05$ when compared to CTRL (control group). $**p < 0.05$ when compared to 4NQO CTRL

shows that purple carrot is a great source of cyanidin derivatives with substitution patterns for acylated or non-acylated complex sugars [32]. Acylated compounds display higher antioxidant activity than non-acylated forms [33]. As a result of its composition, the biological activity of purple carrot extract is in the position of the glycosylation, since 3,5-glycosylation pattern in anthocyanidins suggests a lower biological activity when compared to glycosylation in position [34].

Cell cycle control is essential for cell function as far as tissue homeostasis in living organisms. This process is essential for preventing oral carcinogenesis. Ki-67 expression is directly correlated with lower differentiation of tumors, suggesting that the biomarker could contribute to the understanding of the biological behavior of cancer process and prognosis [35]. Previous study conducted by our research group has demonstrated the ability of antioxidant compounds in modulating the experimental oral carcinogenesis by blocking the proliferative activity of tongue epithelium cells induced by grape juice concentrate [17]. Other authors

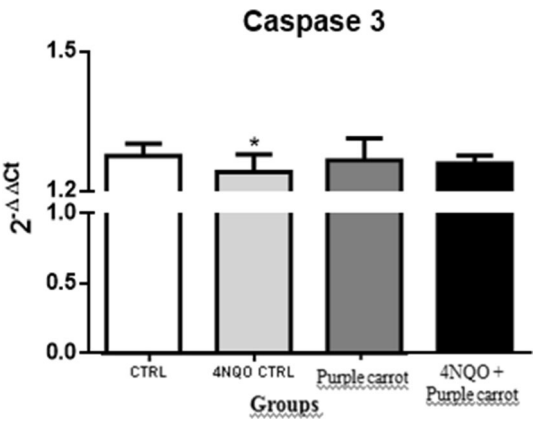

Fig. 10 Real-time PCR from caspase 3 following rat tongue carcinogenesis induced by 4NQO and treated with purple carrot extract. $*p < 0.05$ when compared to CTRL (control group). $**p < 0.05$ when compared to 4NQO CTRL

have revealed that Chinese herbal mixture (antitumor B) is relevant for inhibiting the proliferation of epithelial cells in rat oral pre-neoplastic lesions, such as hyperplasia and dysplasia [28]. The influence of purple carrot extract on proliferative activity of neoplastic cells was also described in vitro study conducted by Netzel et al. [13]. The authors reported the ability of the extract to inhibit the proliferation of colorectal adenocarcinoma (HT-29) and promyelocytic leukemia (HL-60) cells by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Our results demonstrated that purple carrot extract decreases Ki-67 immunoexpression following tongue carcinogenesis induced by 4NQO in rats treated with purple carrot extract. Taken as a whole, we assumed that purple carrot extract modulates the proliferative activity of initiated cells during oral cancer development in the promotion phase of chemical carcinogenesis.

Dietary antioxidant phytochemicals protect cells from genotoxic agents by decreasing DNA damage, reducing risk of mutation in the initiation phase of carcinogenesis [36]. DNA damage can be measured by a variety of analytical techniques [37]. Oxidative DNA damage promoted by reactive oxygen species can be evaluated by micronucleus test as well as by immunohistochemistry analysis using 8-OHdG, a promising biomarker of oxidative DNA damage [38, 39]. Micronucleus is a consequence of chromosome breakage or loss during mitosis induced by aneugenic or clastogenic agents [40]. The incidence of micronucleus is an indicator for genetic instability, a contributing factor for carcinogenesis [41]. In the present work, the incidence of micronucleated cells in bone narrow cells decreased in the group that received purple carrot when compared to 4NQO group. Such results are in agreement with others [42–44].

8-OHdG is induced by guanine oxidation due to the interaction of oxygen-free radicals with nucleobases of the DNA strand; this product is pro-mutagenic and a potential marker of carcinogenesis [45]. Several studies demonstrated that 8-OHdG level is increased in various human cancers and in animal experimental models [46, 47]. In this study, a significant number of 8-OHdG-positive cells were noticed to

animals treated with purple carrot extract following experimental oral carcinogenesis. Devi et al. [48] reported that anthocyanins act as an antioxidant agent reducing free radicals and preventing oxidative damage to DNA from hydroxyl radical. Cho et al. [49] found that the antioxidant effects of anthocyanins include enhancing antioxidant enzyme activity and increasing mRNA expression of these enzymes. Moreover, other phenolic compounds present in purple carrot extract were reported as protective agents against oxidative stress in rats [50, 51].

Oxidative damage can be prevented by inducing different antioxidant responses and re-establishing or maintaining redox homeostasis [52]. Redox homeostasis is a complex endogenous antioxidant defense system, comprised by endogenous antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and non-enzymatic compounds like glutathione, proteins and others [53]. The overproduction of ROS metabolites during oxidative stress can inhibit the activity of antioxidant enzymes, such as SOD-Mn and SOD-CuZn [54]. Since antioxidant agents can protect cells from the harmful effects promoted by reactive oxygen species, they have been extensively investigated [55]. In the present study, it was observed that purple carrot extract increased the expression of SOD-Mn mRNA levels in both groups that received diet associated with the purple carrot extract. These data were consistent with as previously reported in cadmium intoxication model in Wistar rats [9]. By comparison, some studies evaluated the extract of a medicinal herb from India called *Melastoma Malabathricum* Linn (MM) carcinogenesis model and luteolin (LUT) a natural polyphenolic flavone present in various fruits and vegetables using murine model of liver carcinogenesis [56]. It was reported that both MM and LUT extracts act as antioxidant agents by re-establishing the activity of the enzymes as a result of increasing the expression of SOD-Mn. Nevertheless, antioxidants from grape juice did not alter the expression of SOD-Mn or SOD-CuZn following oral carcinogenesis induced by 4NQO [17]. Therefore, these data support the hypothesis that purple carrot extract exerts a potent antioxidant activity on oral mucosa cells following rat tongue carcinogenesis induced by 4NQO by up-regulating SOD-Mn mRNA levels.

Toll-like receptor (TLRs) are a family of type I transmembrane receptors composed by 13 types in rodents. They are present in different cellular types such as epithelial and immune cells [57]. The TLR4 signaling pathway involves phosphorylation and ubiquitination closely associated with inflammatory host response [57]. In particular, TLR4 dimerizes into two different signaling pathways after activation, one myeloid differentiation factor 88 (MyD88)-dependent pathway and one MyD88-independent pathway. In the dependent pathway, MyD88 is recruited to activate phosphorylation of interleukin receptor-associated kinases IRAK1 and IRAK4, which in turn activates the tumor necrosis-associated factor TRAF-6 adapter protein. TRAF-6 forms a complex with enzymes involved in the ubiquitination process, activating transforming growth factor beta-activated kinase 1, which then phosphorylates the inhibitor kinase complex IKKβ, triggering the decoupling of NFκB in NFκBp50 and NFκBp65 dimers through degradation of its inhibitory protein IκB [57]. Therefore, NFκB translocates into the nucleus and controls the expression of an array of inflammatory cytokine genes [58]. Our results demonstrated that purple carrot extract decreased the expression of NFκBp50 and MyD88 in rats during the development of oral cancer. Purple carrot extract per se also decreased the expression levels of these proteins. Such findings are completely new and therefore difficult to discuss. Independently of this scenario, purple carrot extract exerts an anti-inflammatory potential as a result of down-regulating NFκBp50 and MyD88 proteins expression. This led to us to conclude that the chemopreventive activity of purple carrot extract is due to the anti-inflammatory potential through modulating Toll-like signaling pathway. However, this is an area that warrants further investigation since the approach has not been addressed so far.

Reduced expression of pro-apoptotic caspases has been reported in a variety of cancers [59]. Heshiki et al. [60] reported the intense expression of cleaved caspase-3 in tumor tissue samples from patients with squamous cell carcinoma of the head and neck using immunohistochemistry. In this work, we showed that 4NQO reduced the caspase-3 levels, and purple carrot extract was not able to alter the expression of this apoptotic biomarker. It seems that the chemopreventive action exerted by purple carrot extract is not linked to the mechanisms of cellular death, such as apoptosis. Further studies are welcomed to elucidate the issue.

In conclusion, our results demonstrated that purple carrot extract was able to protect oral carcinogenicity lesions induced by 4NQO in Wistar rats as a result of antioxidant activity, anti-inflammatory potential and antiproliferative and antimutagenic actions in Wistar rats.

Acknowledgements GRS and CFGM are recipients of CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) fellowship. DAR and WV are recipients of CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnologico) fellowship. MJDS is a recipient of FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) fellowship.

Compliance with ethical standards

Conflict of interest None declared.

References

1. Network CGA. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015;517(7536):576–82.
2. Dayyani F, Etzel CJ, Liu M, Ho C, Lippman SM, Tsao ASRM. Research-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC). Head Neck Oncol. 2010;2:15.
3. Parkin DM, Whelan SL, Ferlay J, Storm H. Cancer incidence in five continents, Cancer base no. 7, vols I–III. Lyon: IARC Scientific Publications; 2005.
4. Chuang AY, Chuang TC, Chang S, Zhou S, Begum S, Westra WH, Ha PK, Koch WM, Califano JA. Presence of HPV DNA in convalescent salivary rinses is an adverse prognostic marker in head and neck squamous cell carcinoma. Oral Oncol. 2008;44:915–9.
5. Gillison ML, Shah KV. Human papillomavirus-associated head and neck squamous cell carcinoma, mounting evidence for an etiologic role for human papillomavirus in a subset of head and neck cancers. Curr Opin Oncol. 2001;13:183–8.
6. Tanaka T, Kawabata K, Kakumoto M, Matsunaga K, Mori H, Murakami A, Kuki W, Takahashi Y, Satoh K, Hara A, Maeda M, Ota T, Odashima S, Koshimizu K, Ohigashi H. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis by citrus auraptene in rats. Carcinogenesis. 1998;19:425.
7. Tanaka T, Ishigamori R. Understanding carcinogenesis for fighting oral cancer. J Oncol. 2011;60:3740.
8. Gollücke APB, Aguiar O Jr, Barbisan LF, Ribeiro DA. Use of polyphenols against carcinogenesis, putative molecules mechanisms of action using in vitro and in vivo test systems. J Med Food. 2013;16(3):1–7.
9. Claudio SR, Gollucke AP, Yamamura H, Morais DR, Bataglion GA, Eberlin MN, Peres RC, Oshima CT, Ribeiro DA. Purple carrot extract protects against cadmium intoxication in multiple organs of rats, genotoxicity, oxidative stress and tissue morphology analyses. J Trace Elem Med Biol. 2016;33:37–47.
10. Turker N, Aksay S, Ekiz HI. Effect of storage temperature on the stability of anthocyanins of a fermented black carrot (*Daucus carota* var. L.) beverage, shalgam. J Agric Food Chem. 2004;52(12):3807–13.
11. Singh DP, Beloy J, Mclnerney JK, Day L. Impact of boron, calcium and genetic factors on vitamin C, carotenoids, phenolic acids, anthocyanins and antioxidant capacity of carrots (*Daucus carota*). Food Chem. 2011;132:1161–70.
12. Algarra M, Fernandes A, Mateus N, Freitas V, da Silva JCGE, Casado J. Anthocyanin profile and antioxidant capacity of black carrots (*Daucus carota* L. ssp. sativus var. atrorubens Alef.) from Cuevas Bajas, Spain. J Food Compos and Anal. 2014;33:71–6.
13. Netzel M, Netzel G, Kammerer DR, Schieber A, Carle R, Simons L, Bitsch I, Bitsch R, Konczak I. Cancer cell antiproliferation activity and metabolism of black carrot anthocyanins. Innov Food Sci Emerg Technol. 2007;8:365–72.
14. Sevimli-Gur C, Cetin B, Akay S, Gulce-Iz S, Yesil-Celiktas O. Extracts from black carrot tissue culture as potent anticancer agents. Plant Foods Hum Nutr. 2013;68(3):293–8.
15. Poudyal H, Panchal S, Brown L. Comparison of purple carrot juice and β-carotene in a high-carbohydrate, high-fat diet-fed rat model of the metabolic syndrome. Br J Nutr. 2010;104(9):1322–32.
16. Fracalossi AC, Comparini L, Funabashi K, Godoy C, Iwamura ES, Nascimento FD, Nader HB, Oshima CT, Ribeiro DA. Ras gene mutation is not related to tumour invasion during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. J Oral Pathol Med. 2011;40(4):325–33.
17. de Jesus GP, Ribeiro FA, de Moura CF, Gollucke AP, Oshima CT, Ribeiro DA. Anti-tumor activity of grape juice concentrate in the rat tongue two-stage initiation-promotion protocol induced by 4-nitroquinoline 1-oxide. Toxicol Mech Methods. 2014;24(4):276–83.
18. Ribeiro DA, Kitakawa D, Domingues MA, Cabral LA, Marques ME, Salvadori DM. Survivin and inducible nitric oxide synthase production during 4NQO-induced rat tongue carcinogenesis, a possible relationship. Exp Mol Pathol. 2007;83(1):131–7.
19. ADA, American Dietetic Association. Position of the American Dietetic Association, functional foods. J Am Diet Assoc. 2004;104:814–26.
20. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidant by means of Folin–Ciocalteu reagent. Method Enzymol. 1999;299:155–78.
21. Ghasemzadeh A. Antioxidant activities, total phenolics and flavonoids content in two varieties of Malaysia young ginger (*Zingiber officinale* Roscoe). Molecules. 2010;15:4324–33.
22. Ribeiro DA, Grilli DG, Salvadori DM. Genomic instability in blood cells is able to predict the oral cancer risk, an experimental study in rats. J Mol Histol. 2008;39(5):481–6.
23. Balasenthil S, Ramachandran CR, Nagini S. Prevention of 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis by garlic. Fitoterapia. 2001;72(5):524–31.
24. Kandarkar SV, Sawant SS, Reade PC. Ultrastructural changes to the palatal mucosa of rats following the application of 4-nitroquinoline-1-oxide (4NQO) and vitamin C. Oral Oncol. 1998;34(4):247–52.
25. Li X, Zhang Y, Yuan Y, Sun Y, Qin Y, Deng Z, Li H. Protective effects of selenium, vitamin E, and purple carrot anthocyanins on D-galactose-induced oxidative damage in blood, liver, heart and kidney rats. Biol Trace Elem Res. 2016;173(2):433–42.
26. World Cancer Research Fund/American Institute for Cancer Research. Food, nutrition, physical activity, and the prevention of cancer, a global perspective. Washington DC: AICR; 2007.
27. Ding Y, Yao H, Yao Y, Fai LY, Zhang Z. Protection of dietary polyphenols against oral cancer. Nutrients. 2013;5(6):2173–91.
28. Wright OR, Netzel GA, Sakzewski AR. A randomized, double-blind, placebo-controlled trial of the effect of dried purple carrot on body mass, lipids, blood pressure, body composition, and inflammatory markers in overweight and obese adults, the QUENCH trial. Can J Physiol Pharmacol. 2013;91(6):480–8.
29. Metzger BT, Barnes DM, Reed JD. Purple carrot (*Daucus carota* L.) polyacetylenes decrease lipopolysaccharide-induced expression of inflammatory proteins in macrophage and endothelial cells. J Agric Food Chem. 2008;56(10):3554–60.
30. Charron CS, Kurilich AC, Clevidence BA, Simon PW, Harrison DJ, Britz SJ, Baer DJ, Novotny JA. Bioavailability of anthocyanins from purple carrot juice, effects of acylation and plant matrix. J Agric Food Chem. 2009;57(4):1226–30.
31. Olejnik A, Rychlik J, Kidoń M, Czapski J, Kowalska K, Juzwa W, Olkowicz M, Dembczyński R, Moyer MP. Antioxidant effects of gastrointestinal digested purple carrot extract on the human cells of colonic mucosa. Food Chem. 2016;190:1069–77.
32. Stintzing FC, Stintzing AS, Carle R, Frei B, Wrolstad RE. Color and antioxidant properties of cyanidin-based anthocyanin pigments. J Agric Food Chem. 2002;50:6172–81.
33. Konczak I, Terahara N, Yoshimoto M, Nakatani M, Yamakawa O. Regulating the composition of anthocyanins and phenolic acids in a sweet potato cell culture towards production of polyphenolic complex with enhanced physiological activity. Trends Food Sci Tech. 2005;16:377–88.
34. Jing P, Bomser JA, Schwartz SJ, He J, Magnuson BA, Giusti MM. Structure-function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. J Agric Food Chem. 2008;56(20):9391–8.

35. Bôas DS, Takiya CM, Coelho-Sampaio TL, Monção-Ribeiro LC, Ramos EA, Cabral MG, dos Santos JN. Immunohistochemical detection of Ki-67 is not associated with tumor-infiltrating macrophages and cyclooxygenase-2 in oral squamous cell carcinoma. J Oral Pathol Med. 2010;39(7):565–70.
36. Caputo F, Vegliante R, Ghibelli L. Redox modulation of the DNA damage response. Biochem Pharmacol. 2012;84(10):1292–306.
37. Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. Free radical-induced damage to DNA, mechanisms and measurement. Free Radic Biol Med. 2002;32(11):1102–15.
38. Araldi RP, de Melo TC, Mendes TB, de Sá Júnior PL, Nozima BH, Ito ET, de Carvalho RF, de Souza EB, de Cassia SR. Using the comet and micronucleus assays for genotoxicity studies, a review. Biomed Pharmacother. 2015;72:74–82.
39. Collins AR. Measuring oxidative damage to DNA and its repair with the comet assay. Biochim Biophys Acta. 2014;1840(2):794–800.
40. Torres-Bugarín O, Macriz Romero N, Ramos Ibarra ML, Flores-García A, Valdez Aburto P, Zavala-Cerna MG. Genotoxic effect in autoimmune diseases evaluated by the micronucleus test assay, our experience and literature review. Biomed Res Int. 2015;19:4031.
41. Kirsch-Volders M, Plas G, Elhajouji A, Lukamowicz M, Gonzalez L, Vande Loock K, Decordier I. The in vitro MN assay in 2011, origin and fate, biological significance, protocols, high throughput methodologies and toxicological relevance. Arch Toxicol. 2015;85(8):873–99.
42. Batista ÂG, Ferrari AS, da Cunha DC, da Silva JK, Cazarin CB, Correa LC, Prado MA, Carvalho-Silva LB, Esteves EA, Maróstica Júnior MR. Polyphenols, antioxidants, and antimutagenic effects of *Copaifera langsdorffii* fruit. Food Chem. 2016;197(Pt B):1153–9.
43. Horta RN, Kahl VF, Sarmento Mda S, Nunes MF, Porto CR, Andrade VM, Ferraz Ade B, Silva JD. Protective effects of acerola juice on genotoxicity induced by iron in vivo. Genet Mol Biol. 2016;39(1):122–8.
44. de Moura CF, Ribeiro FA, de Jesus GP, da Silva VH, Oshima CT, Gollücke AP, Aguiar O Jr, Ribeiro DA. Antimutagenic and antigenotoxic potential of grape juice concentrate in blood and liver of rats exposed to cadmium. Environ Sci Pollut Res Int. 2014;21(22):13118–26.
45. Valavanidis A, Vlachogianni T, Fiotakis C. 8-Hydroxy-2′-deoxyguanosine (8-OHdG), a critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2009;27:120–39.
46. Tanaka H, Fujita N, Sugimoto R, Urawa N, Horiike S, Kobayashi Y, Iwasa M, Ma N, Kawanishi S, Watanabe S, Kaito M, Takei Y. Hepatic oxidative DNA damage is associated with increased risk for hepatocellular carcinoma in chronic hepatitis C. Br J Cancer. 2008;98:580–6.
47. Agha-Hosseini F, Mirzaii-Dizgah I, Farmanbar N, Abdollahi M. Oxidative stress status and DNA damage in saliva of human subjects with oral lichen planus and oral squamous cell carcinoma. J Oral Pathol Med. 2012;41(10):736–40.
48. Devi PS, Kumar MS, Das SM. DNA damage protecting activity and free radical scavenging activity of anthocyanins from Red Sorghum (*Sorghum bicolor*) Bran. Biotechnol Res Int. 2012;25:8787.
49. Cho J, Kang JS, Long PH, Jing J, Back Y, Chung KS. Antioxidant and memory enhancing effects of purple sweet potato anthocyanin and cordyceps mushroom extract. Arch Pharm Res. 2003;26(10):821–5.
50. Coskun O, Kanter M, Korkmaz A, Oter S. Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and beta-cell damage in rat pancreas. Pharmacol Res. 2005;51(2):117–23.
51. Zhang ZF, Fan SH, Zheng YL, Lu J, Wu DM, Shan Q, Hu B. Purple sweet potato color attenuates oxidative stress and inflammatory response induced by D-galactose in mouse liver. Food Chem Toxicol. 2009;47(2):496–501.
52. Espinosa-Diez C, Miguel V, Mennerich D, Kietzmann T, Sánchez-Pérez P, Cadenas S, Lamas S. Antioxidant responses and cellular adjustments to oxidative stress. Redox Biol. 2015;6:183–97.
53. Pisoschi AM, Pop A. The role of antioxidants in the chemistry of oxidative stress, a review. Eur J Med Chem. 2015;97:55–74.
54. Verma A, Bhatt PC, Kaithwas G, Sethi N, Rashid M, Singh Y, Rahman M, Al-Abbasi F, Anwar F, Kumar V. Chemomodulatory effect *Melastoma malabathricum* Linn against chemically induced renal carcinogenesis rats via attenuation of inflammation, oxidative stress, and early markers of tumor expansion. Inflammopharmacology. 2016;24(5):233–51.
55. Hamid AA, Aiyelaagbe OO, Usman LA, Ameen OM, Lawal A. Antioxidants, its medicinal and pharmacological applications. Afr J Pure and Appl Chem. 2010;4(8):142–51.
56. Zhang Q, Yang J, Wang J. Modulatory effect of luteolin on redox homeostasis and inflammatory cytokines in a mouse model of liver cancer. Oncol Lett. 2016;12(6):4767–72.
57. Morais CA, de Rosso VV, Estadella D, Pisani LP. Anthocyanins as inflammatory modulators and the role of the gut microbiota. J Nutr Biochem. 2016;33:1–7.
58. Takeda K, Akira S. Toll-like receptors. Curr Protoc Immunol. 2015;109:1–10.
59. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol. 2013;5(4):a008656.
60. Heshiki W, Tomihara K, Yamazaki M, Arai N, Nakamori K, Noguchi M. Constitutive activation of caspase-3 in non-apoptotic oral squamous cell carcinoma cells. J Cancer Sci Ther. 2015;7:2014.