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Antioxidant and antigenotoxic potential of *Ramaria largentii* Marr & D. E. Stuntz, a wild edible mushroom collected from Northeast Romania

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ABSTRACT

Ramaria largentii Marr & D. E. Stuntz (orange coral mushroom) is a wild edible mushroom whose chemical composition and bioactivity have not been investigated. Herein, we present a study on the phenolic constituents, antioxidant and antigenotoxic effects of a hydromethanolic extract of the fruiting bodies. Total phenolic content, estimated by Folin-Ciocalteu assay, was found to be 42.33 ± 0.18 mg GAE/g. Protocatechuic and vanillic acids were detected by HPLC-DAD-ESI-MS. The extract showed good free radical scavenging and reducing capacities (EC_{50} = 64.3 ± 0.2 and 61.54 ± 0.46 μg/mL, respectively). In normal Vero cells, the extract (100, 200 and 300 μg/mL) showed no genotoxic potential and moreover, almost completely protected DNA against H_2O_2-induced damage (2.09–7.91% tail DNA) (24 and 48 h pre-treatment). Taken together, the results of our study show that *Ramaria largentii* extract is devoid of genotoxicity and has a remarkable DNA protective activity against H_2O_2-induced damage.

1. Introduction

From ancient times edible mushrooms have been used both as food and medicine. Besides their attractive taste and aroma, edible mushrooms have an important nutritional value since they are rich in proteins (15.2–80.93% dry weight), carbohydrates (35–70% dry weight), vitamins (B_1, B_2, B_{12}, C, D, E, β-carotene), minerals (6–10.5% dry weight, mainly K, P, Mg, Ca, Cu, Fe, Zn) and low in fats. Edible mushrooms are a good source of essential amino acids (arginine, aspartic and glutamic acids) and dietary fibers (β-glucans, chitin). With regard to the fat content, palmitic, oleic and linoleic acids are the most abundant. Edible mushrooms also contain bioactive secondary metabolites such as phenolic compounds, sterols and triterpenes (Guillamón et al., 2010). These nutrients and metabolites are responsible for the biological activities reported for edible mushrooms (antioxidant, anti-inflammatory, hypoglycemic, hypocholesterolemic, antihypertensive, antitumor, immunomodulatory, hepatoprotective, antibiotic and antiviral effects) (Boa, 2004; Cheung, 2010; Guillamón et al., 2010).

Oxidative stress is involved in aging but also in pathological conditions such as cancer, cardiovascular disease, ischemic/reperfusion injury, rheumatoid arthritis, diabetes and neurological disorders (Valko et al., 2007). There is a consistent body of evidence from epidemiological and interventional human trials that antioxidant-rich foods and antioxidant supplementation reduce the risk and incidence of many chronic diseases (Ferreira et al., 2009). The antioxidant potential of edible mushrooms has been extensively investigated in both *in vitro* and *in vivo* models. Various extracts of edible mushrooms were found to scavenge reactive oxygen species (superoxide anion radical, hydroxyl radical, H_2O_2) and nitric oxide, chelate pro-oxidant transition metal ions, reduce lipid peroxidation, increase the levels of non-enzymatic antioxidants (vitamins C and E, glutathione), stimulate antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase,

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glutathione reductase) and inhibit xanthine oxidase (Ferreira et al., 2009; Jia et al., 2009; Kozarski et al., 2015).

DNA oxidation leads to gene mutations and chromosomal aberrations which play an important role in the development of cancer and other severe disorders (neuromuscular, neurodegenerative, cardiovascular, metabolic and immune disorders) (Jackson and Bartek, 2009). Human body is permanently exposed to genotoxic agents that cause DNA oxidation (UV light, nicotine in tobacco smoke, pollutants in water and foods such as aflatoxin B1 and ochratoxin A, food processing by-products such as benzo(a)pyrene and nitrosamines). Oxidative stress-induced DNA damage might also arise during anticancer chemotherapy and radiotherapy (Jackson and Bartek, 2009; Luca et al., 2016). Therefore, identification of new potential antigenotoxic agents, efficient and safe, is of great interest. The ability of several edible mushroom extracts but also mushroom constituents to protect DNA against genotoxic agents has been investigated in different cell lines (Burkitt's lymphoma cells, Chinese hamster lung fibroblastic V79 cells, human laryngeal epidermoid carcinoma HEp2 cells, human hepatoma HepG2 cells, human lymphocytes). Extracts of *Agaricus bisporus*, *Ganoderma lucidum*, *Inonotus obliquus, Agrocybe cylindracea, Agaricus blazei*, *Lentinula edodes* (shiitake), *Lactarius vellereus* afforded DNA protection in cells exposed to different genotoxicants such as H_2O_2, hydroxyl radicals (Roupas et al., 2012; Shi et al., 2002b), methyl methanesulfonate (Guterres et al., 2005; Miyaji et al., 2004), cyclophosphamide (Delmanto et al., 2001) and 2-amino-3-methylimidazo(4,5-f)quinoline (Mlinarič et al., 2004).

The genus *Ramaria* comprises approx. 300 species distributed worldwide; some species are edible whereas others (*R. formosa*, *R. pallida*) are mildly poisonous causing nausea, vomiting and diarrhea (Łuszczyński, 2009; Nasim et al., 2008). Edible mushrooms of the genus *Ramaria* have been poorly investigated. Few studies reported only on the constituents and biological activities of *R. aurea* (Khatua et al., 2015), *R. flava* (Gursoy et al., 2010; Liu et al., 2013) and *R. botrytis* (Barros et al., 2009). *R. largentii* (orange coral mushroom) is an edible species usually living in habitats with conifers (Ouzouni et al., 2009). No studies have investigated the chemical constituents and biological effects of this species. Only the heavy metal accumulation in the fruiting bodies was reported (Ouzouni et al., 2009; Rieder et al., 2011). In the present work, a hydromethanolic extract of the fruiting bodies was investigated regarding its phenolic profile, antioxidant and antigenotoxic potential.

2. Material and methods

2.1. Chemicals

Gallic, caffeic, protocatechuic and vanillic acids, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium ferricyanide, ferric chloride, sodium carbonate, sodium hydroxide, linoleic acid, formic acid, dimethyl sulfoxide (DMSO), sodium lauryl sarcosinate, normal and low melting point agarose were purchased from Sigma-Aldrich (Steinheim, Germany). Ethylenediaminetetraacetic acid (EDTA), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid monosodium salt (ferrozine), H_2O_2 and lipoxidase from soybean were supplied by Fluka (Steinheim, Germany). Folin-Ciocalteu's phenol reagent, ferrous chloride and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were from Merck (Darmstadt, Germany). Ethidium bromide was obtained from Carl Roth (Karlsruhe, Germany). Trichloroacetic acid and potassium persulfate were from Riedel-de Haën (Seelze, Germany). Dulbecco's Modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum, streptomycin and penicillin were from Biochrom AG (Berlin, Germany). All other solvents and reagents were of analytical grade. Ultrapure water was obtained using an Ultra Pure Water System type Ultra Clear TWF UV (SG Water, Barsbüttel, Germany).

2.2. Mushroom species

Fruiting bodies of *R. largentii* Marr & D. E. Stuntz (Gomphaceae) were collected in Poiana Stampei (Northeast of Romania) in September 2013. Authentication was made at the Laboratory of Mycology and Phytopathology, Faculty of Biology, Alexandru Ioan Cuza University of Iasi, Romania. Voucher specimens were deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Grigore T. Popa University of Medicine and Pharmacy Iasi, Romania.

The fruiting bodies were cleaned without washing, freeze-dried and powdered.

2.3. Extraction

Dried and powdered fruiting bodies (50 g) were extracted with 96% ethanol as previously described (Zavastin et al., 2015b). The residue was further extracted twice, each time with 500 mL of methanol:water (1:1, v/v) under stirring at 350 rpm for 3 h at room temperature in dark condition. The hydromethanolic extracts were pooled together, evaporated to dryness under reduced pressure at 40 °C and stored at −18 °C.

2.4. Estimation of total phenolic content

Total phenolic content was estimated by Folin-Ciocalteu assay (Wangensteen et al., 2004). In brief, the extract (25 mg/mL, 40 μL) was mixed with ultrapure water (3.16 mL) and Folin-Ciocalteu reagent (200 μL). After 5 min, 20% sodium carbonate (600 μL) was added. The reaction mixture was allowed to stand for 2 h at room temperature. The absorbance was measured at 765 nm. The phenolic content was expressed as mg of gallic acid equivalents (GAE)/g of extract.

2.5. RP-HPLC-DAD-ESI-MS analysis of phenolic compounds

The analysis of phenolic compounds was performed by reversed-phase high performance liquid chromatography (RP-HPLC) using an Agilent 1200 Series HPLC system with diode array detector (DAD) coupled to an Agilent 6520 accurate-mass quadrupole time-of-flight (Q-TOF) mass spectrometer equipped with an electrospray ionization (ESI) source. The separation was carried out on a 250 × 4.6 mm, 5 μm Hypersil ODS C18 column (Thermo Scientific). The mobile phase consisted of acetonitrile (A) and water with 0.1% formic acid (B). A gradient elution varying from 0% to 100% A over 90 min was applied. The injection volume was 20 μL. The flow rate and detection wavelength were set to 0.5 mL/min and 280 nm, respectively. The mass spectrometric detection was performed in the negative ion mode (capillary voltage −4.0 kV, skimmer voltage −68 V, drying gas flow rate 7 L/min, drying gas temperature 235 °C, nebulizer pressure 25 psig). Masses were scanned from 80 to 2800 amu in steps of 0.3 amu. MassHunter Workstation software was used for data processing. Phenolic compounds were identified by comparing their retention times, UV and ESI-MS spectra with those of authentic standards.

2.6. ABTS radical cation scavenging assay

ABTS radical cation was generated according to Re et al. (1999) by incubating ABTS (7 mM) with potassium persulfate (2.45 mM) for 12–16 h in dark followed by dilution with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm (equilibration at 30 °C).

Different concentrations of extract (3.12–25 mg/mL, 20 μL) were mixed with ABTS radical cation solution (1.98 mL). The absorbance of the mixture was measured at 734 nm after 6 min of reaction at 30 °C (Re et al., 1999). Caffeic acid (62.5 μg/mL-0.5 mg/mL) was used as positive control. ABTS radical cation scavenging activity (%) was calculated using the formula: $[(A_{control} - A_{sample})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of ABTS radical cation solution and A_{sample} is the absorbance in the presence of extract/caffeic acid. The results were expressed as EC_{50} values (concentration of sample producing 50% activity) calculated by linear interpolation between values above and below 50% activity.

2.7. Reducing power assay

Reducing power was evaluated as described by Ferreira et al. (2007) with some modifications. Briefly, the reaction mixture containing the extract (1–60 mg/mL, 50 μL), 1% potassium ferricyanide (1.25 mL) and 0.2 M phosphate buffer, pH 6.6 (1.2 mL) was incubated at 50 °C for 20 min. After addition of 10% trichloroacetic acid (1.25 mL) followed by centrifugation (3000 rpm, 10 min), the upper layer (1.5 mL) was mixed with ultrapure water (1.25 mL) and 0.1% ferric chloride (0.25 mL). The absorbance at 700 nm was measured after 90 s. Caffeic acid (0.05–0.4 mg/mL) was the positive control. The results were expressed as EC_{50} values (concentration of sample giving an absorbance of 0.5) (Ferreira et al., 2007).

2.8. Ferrous ion chelating assay

Ferrous ion chelating ability was assessed according to the method described by Tung et al. (2009) with slight modifications. The extract (3.12–50 mg/mL, 0.4 mL) was mixed with 2 mM ferrous chloride (40 μL), 5 mM ferrozine (80 μL) and ethanol (1.65 mL). The absorbance of the mixture was measured at 562 nm after 10 min of reaction. EDTA (12.5 μg/mL-0.1 mg/mL) was used as positive control. Ferrous ion chelating activity (%) was calculated using the formula: $[(A_{control} - A_{sample})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance in the presence of extract/EDTA. The results were expressed as EC_{50} values calculated as described in 2.6.

2.9. 15-Lipoxygenase inhibition assay

The assay was performed as described by Wangensteen et al. (2004) with minor changes (Cretu et al., 2013). The extract (50 mg/mL, 50 μL) was mixed with 0.2 M borate buffer, pH 9 (0.9 mL) and 15-lipoxygenase from soybeans (lipoxidase) (10,000 U/mL in borate buffer, 50 μL). After incubation at 25 °C for 10 min, a solution of linoleic acid in borate buffer (final concentration: 134 μM, 2 mL) was added. The absorbance of the mixture was recorded at 234 nm for 90 s. Caffeic acid (0.5–5 mg/mL) was the positive control. 15-Lipoxygenase inhibition (%) was calculated using the absorbances of the control and samples (extract/caffeic acid) after 30 and 90 s reaction time: $[(A_{control,90} - A_{control,30}) - (A_{sample,90} - A_{sample,30})/(A_{control,90} - A_{control,30})] \times 100$. The results were expressed as EC_{50} values calculated as described in 2.6.

2.10. Cell culture

Vero cells (ATCC CCL 81) were cultivated in DMEM, supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 IU/mL penicillin. The cells were seeded at a density of 3.5×10^4 cells/well in 24-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and maintained overnight at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air (Binder CB 150 incubator, Tuttlingen, Germany). The cells were used in further experiments (cell viability and antigenotoxicity assays) when reaching the confluence in the monolayer stage.

2.11. Cell viability assay

MTT assay (Mosmann, 1983) was used to evaluate cell viability. After reaching the confluence in the monolayer stage, the growth medium was removed, the cells were washed with PBS, resuspended in fresh medium supplemented with different concentrations of extract (50, 100, 200 and 300 μg/mL) and incubated for 24 and 48 h at 37 °C. The cells treated with ultrapure water (solvent used to dissolve the extract) served as sham control. After incubation, the medium was discarded and the cells were washed with PBS and covered with fresh medium (100 μL). MTT (10 μL, 5 mg/mL) was added to each well followed by a further incubation for 3 h at 37 °C. The formazan dye was solubilized in DMSO (300 μL/well) and quantified at 540 nm. The results were expressed as % viability.

2.12. Antigenotoxicity/genotoxicity assay

Antigenotoxic potential was evaluated by alkaline single-cell gel electrophoresis assay (Comet assay) as described earlier (Olive and Banath, 2006; Reis et al., 2016). After incubation of Vero cells with *R. largentii* extract (100, 200 and 300 μg/mL) or corresponding volume of ultrapure water (sham control) for 24 and 48 h (as described in 2.11), the medium was discarded and Vero cells were washed with PBS and treated with H_2O_2 (50 μM) as genotoxicant for 30 min. Controls treated only with H_2O_2 (50 μM) or extract (100, 200 and 300 μg/mL) were also included. Afterwards, the cells were detached by trypsinization and submitted to Comet assay. For each treatment, the cell suspension (200 μL, aprox. 40,000 cells) was mixed with 1% low melting agarose (1000 μL) at 37 °C and quickly poured onto 1% normal melting agarose precoated slides. After agarose gelation, the slides were immersed in freshly prepared cold lysis solution (1.2 M NaCl, 100 mM Na_2EDTA, 0.1% sodium lauryl sarcosinate, 0.26 M NaOH, pH > 13) and lysed overnight at 4 °C under dark conditions. The slides were further washed with electrophoresis solution (0.03 M NaOH, 2 mM Na_2EDTA, pH ≈ 12.3) for three times at room temperature and placed in a horizontal gel electrophoresis tank very close to the anode. The electrophoresis was carried out at 0.6 V/cm for 25 min. The procedure was performed under dimmed light to prevent additional DNA damage. After electrophoresis, the slides were rinsed with ultrapure water. Comets were visualized by ethidium bromide staining (20 μg/mL, 30 s) using a fluorescence microscope (Nikon Eclipse 600, Japan). Comet scoring was performed by OpenComet plugin for ImageJ. The results were expressed as % tail DNA.

2.13. Statistical analysis

Folin-Ciocalteu and antioxidant assays were performed in triplicate and the results were expressed as mean ± standard deviation. In case of cell viability and antigenotoxicity assays, five replicates were performed for each concentration, the results being expressed as mean ± standard error of mean. The statistical evaluation was done using the *t*-test. Values of $p < 0.001$, $p < 0.01$ and $p < 0.05$ were considered statistically significant.

3. Results and discussion

To the best of our knowledge, the chemistry and bioactivity of *R. largentii* have not been investigated so far. There are very few studies on other edible *Ramaria* species. Liu et al. (2013) reported the antitumor activity of *R. flava* ethanol extract against human breast, lung and gastric cancer cell lines (MDA-MB-231, NCI-H520

and BGC-803, respectively). Antioxidant activity (free radical scavenging, reducing and ferrous ion chelating effects) was reported for extracts obtained from *R. flava* (Gursoy et al., 2010; Liu et al., 2013) and *R. aurea* (Khatua et al., 2015). Taking into consideration the bioactivity reported for other *Ramaria* species, we initiated a study aiming to investigate the chemical composition and biological activities of *R. largentii*. The present work reports on the phenolic compounds, antioxidant and antigenotoxic effects of the hydromethanolic extract of *R. largentii* fruiting bodies.

3.1. Phenolic content and profile of R. largentii extract

Total phenolic content of *R. largentii* extract, evaluated by Folin-Ciocalteu method, was found to be 42.33 ± 0.18 mg GAE/g. This value is higher than the ones reported for extracts and fractions of other edible *Ramaria* species (*R. botrytis*, *R. flava, R. aurea*) (6.66–22.14 mg GAE/g) (Barros et al., 2009; Gursoy et al., 2010; Khatua et al., 2015; Liu et al., 2013) but lower than the phenolic content determined in the water fraction of *R. flava* ethanolic extract (61.01 mg GAE/g) (Liu et al., 2013). In addition, protocatechuic and vanillic acids were detected in *R. largentii* extract by comparing their retention times, MS and UV data with those of commercial standards (protocatechuic acid: retention time 34.3 min, $[M-H]^-$ at *m/z* 153, 260 nm; vanillic acid: retention time 51.1 min, $[M-H]^-$ at *m/z* 153, 260 nm) (Fig. 1). Both phenolic acids are reported for the first time in *R. largentii*. Protocatechuic acid was also reported in *R. botrytis* by Barros et al. (2009). Other phenolic acids detected in *Ramaria* species are *p*-hydroxybenzoic acid (in *R. botrytis*) (Barros et al., 2009), caffeic, cinnamic and gallic acids (in *R. aurea*) (Khatua et al., 2015). Liu et al. (2013) reported flavonoids (quercetin, chrysin and pinocembrin) in *R. flava*. However, the presence of flavonoids in mushrooms is a controversial issue. Gil-Ramírez et al. (2016) reported that mushrooms cannot synthesize flavonoids because they lack chalcone synthase and chalcone isomerase, enzymes involved in the biosynthesis of flavonoids. Besides, mushrooms do not absorb flavonoids from the substrates they grow on. Therefore, the authors concluded that the contamination of mushrooms and lack of specificity of spectrophotometric quantification methods might have caused the positive results in flavonoid identification and quantification assays (Gil-Ramírez et al., 2016). For many edible mushrooms (*Lentinus edodes*, *Pleurotus ostreatus*, *Agrocybe aegerita*, *Flammulina velutipes*, *Volvariella volvacea*), the antioxidant effects were found to correlate with the phenolic contents in the tested extracts (Cheung, 2010; Guillamón et al., 2010). Overall, it seems that phenolic acids can be useful taxonomic markers within the genus *Ramaria*. Besides, they have diverse biological effects (antioxidant, antimicrobial, antitumor) and were reported to contribute to the health benefits of edible mushrooms (Heleno et al., 2015; Reis et al., 2016).

3.2. Antioxidant activity of R. largentii extract

Several assays were performed to assess the antioxidant potential of *R. largentii* extract.

3.2.1. ABTS radical cation scavenging activity

ABTS assay evaluates the free radical scavenging activity due to hydrogen-donating ability; hydrogen-donating antioxidants convert the blue-green ABTS radical cation to its uncolored non-radical form (Re et al., 1999). *R. largentii* extract scavenged ABTS radical cation in a dose-dependent manner. The scavenging activity was found to be 93.53 ± 0.03% at 250 μg/mL. According to the EC_{50} values (Table 1), caffeic acid showed higher scavenging activity than *R. largentii* extract. There are few studies on the free radical scavenging activity of other *Ramaria* species but these studies assessed the scavenging abilities towards DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and some reactive oxygen species such as hydroxyl radical and superoxide anion radical. *R. flava* ethanolic extract and its fractions scavenged DPPH and hydroxyl radicals with IC_{50} values of 5.86–46.80 and 18.08–116.55 μg/mL, respectively (Liu et al., 2013) whereas *R. flava* methanolic extract scavenged DPPH radical by 94.78% at 12 mg/mL (Gursoy et al., 2010). A polyphenol-rich extract of *R. aurea* showed DPPH and superoxide radicals scavenging effects (EC_{50} = 0.384 and 0.283 mg/mL, respectively) (Khatua et al., 2015). In light of these literature data, *R. largentii* extract seems to be a reasonably efficient free radical scavenger.

3.2.2. Reducing power

Electron donation is also involved in free radical scavenging (Leopoldini et al., 2011). The reducing power assay evaluates the electron-donating capacity of antioxidants, namely the capacity to reduce the ferric cyanide complex (Fe^{3+}) (yellow) to ferrous cyanide form (Fe^{2+}) (green to blue) (Ferreira et al., 2007). The reducing power of *R. largentii* extract increased from 0.07 at 7.27 μg/mL to 1.11 at 145.45 μg/mL. In the same assay, the reducing power of

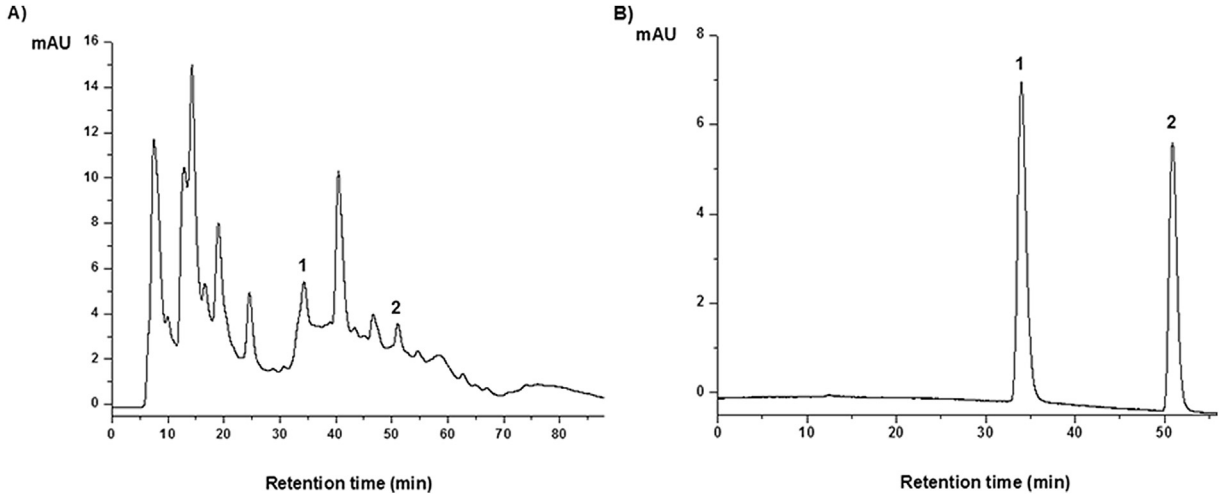

Fig. 1. RP-HPLC-UV trace (280 nm) of *Ramaria largentii* extract (A) and standard mixture (B) (1-protocatechuic acid, 2-vanillic acid).

Table 1
Antioxidant activities of *Ramaria largentii* extract.

Extract/Positive control	EC_{50} (μg/mL)			
	ABTS scavenging activity	Reducing power	Ferrous ion chelation	15-Lipoxygenase inhibition
R. largentii extract	64.3 ± 0.2	61.54 ± 0.46	2497 ± 6	>833.34
caffeic acid	2.1 ± 0.0	1.96 ± 0.02	–	29.1 ± 0.3
EDTA	–	–	6.32 ± 0.04	–

caffeic acid, the positive control, ranged from 0.07 at 0.33 μg/mL to 0.67 at 2.67 μg/mL. Overall, *R. largentii* extract showed a weaker reducing power than caffeic acid (Table 1). Reducing abilities were reported for other *Ramaria* species. At 20 mg/mL, the methanolic extract of *R. flava* exhibited a reducing power of 1.915 (Gursoy et al., 2010). An EC_{50} value of 1.025 mg/mL was reported for a polyphenol-rich extract of *R. aurea* (Khatua et al., 2015). According to these results, it is obvious that *R. largentii* extract has a high efficacy as reducing agent.

3.2.3. Ferrous ion chelation

Ferrous ions are involved in the generation of hydroxyl radicals *via* Fenton-type reactions. Hydroxyl radicals generated in close vicinity of cell membranes attack the unsaturated fatty acids of the membrane phospholipids thus promoting lipid peroxidation. When generated close to cell mitochondria or nucleus, hydroxyl radicals cause DNA oxidation leading to gene mutations (Klaunig et al., 2011). Therefore, chelation of ferrous ions plays an important role in reducing oxidative stress. This assay evaluates the ability of metal chelators to interfere with the formation of ferrous ion-ferrozine complex (violet) (Tung et al., 2009). The ferrous ion chelating activity of *R. largentii* extract varied from 3.04 ± 0.33% at 0.92 mg/mL to 97.61 ± 0.15% at 9.22 mg/mL, being considerably lower than that determined for EDTA (Table 1) and other *Ramaria* extracts. At only 2 mg/mL, *R. flava* methanolic extract almost completely chelated ferrous ions (96.75 ± 0.28%) (Gursoy et al., 2010). The polyphenol-rich extract of *R. aurea* chelated ferrous ions with an EC_{50} value of 0.95 mg/mL (Khatua et al., 2015). Although a direct comparison of these activities with our results is difficult due to different experimental protocols used in the ferrous ion chelation assay, it is obvious that *R. largentii* extract is not an efficient metal chelator.

3.2.4. 15-Lipoxygenase inhibition

Human lipoxygenases, enzymes catalyzing the peroxidation of 1,4-diene-type polyunsaturated fatty acids, play important roles in many diseases and therefore they represent important therapeutic targets. 15-Lipoxygenase is involved in asthma, cardiovascular disease and stroke (Rai et al., 2010). As far as our literature survey could ascertain, numerous studies attempted to identify potent 15-lipoxygenase inhibitors of both natural and synthetic origin. The 15-lipoxygenase inhibitory potential of *R. largentii* extract was evaluated by assessing its ability to reduce the generation of peroxidation products with conjugated double bonds and consequently, the increase in absorbance at 234 nm (Wangensteen et al., 2004). Lipoxygenase type-1 isolated from soybeans is widely used as a model for human lipoxygenases due to both structural and functional similarities (Serpen and Gökmen, 2007). In our study, at a concentration of 833.34 μg/mL, the 15-lipoxygenase inhibitory activity of *R. largentii* extract was 46.82 ± 0.17%. Caffeic acid exhibited higher enzyme inhibition (98.21 ± 0.43% at 83.34 μg/mL). It is obvious that *R. largentii* extract is a weak 15-lipoxygenase inhibitor (Table 1). The capacity of other *Ramaria* species to inhibit 15-lipoxygenase has not been studied yet. In previous investigations, we determined higher 15-lipoxygenase inhibitory effects for *Armillaria mellea* and *Lactarius salmonicolor* ethanolic extracts (EC_{50} = 290.93 ± 2.05 and 226.67 ± 0.95 μg/mL, respectively) (Zavastin et al., 2015a, 2015b).

3.3. Cytotoxic potential of R. largentii extract

In vitro toxicity screening on normal cells is the first stage of a toxicological evaluation and also the starting point for the assessment of a potential *in vivo* toxicity (Frión-Herrera et al., 2014). To the best of our knowledge, there are no previous reports on the potential toxicity of *R. largentii* and other edible *Ramaria* species. This study investigated the toxicity of *R. largentii* extract against Vero cell line. Vero (normal African green monkey kidney epithelial) cells are often used as normal cells in cytotoxicity screening (Frión-Herrera et al., 2014). Cell viability was assessed by a standard MTT assay based on the ability of mitochondrial dehydrogenases in living cells to reduce MTT (yellow) to a water insoluble formazan (violet-blue) (Stockert et al., 2012). *R. largentii* extract (50–300 μg/mL) did not decrease significantly the viability of Vero cells after 24 and 48 h treatment. Slight reductions in Vero cell viability (8.19 and 9.72%) were determined after 48 h treatment with high concentrations of extract (200 and 300 μg extract/mL, respectively) (Fig. 2). Overall, it may be concluded that *R. largentii* extract in concentrations of 50–300 μg/mL is non-toxic to normal Vero cells. Extracts of other edible mushroom species (*Agaricus blazei*, *Grifola frondosa*, *Hericium erinaceus*) were toxic to Chinese hamster fibroblast cells (V79-4) (reduction of cell viability to 38–77.8% after 24 h treatment with extracts in a concentration of 2 mg/mL) (Nur Shahirah et al., 2014).

3.4. Antigenotoxic/genotoxic potential of R. largentii extract

As human body is constantly exposed to genotoxic agents, antioxidants that are safe and effective in protecting DNA provide considerable health benefits. The ability of *R. largentii* extract to protect DNA against oxidative stress-induced damage was evaluated by alkaline single-cell gel electrophoresis assay (Comet assay). This assay enables the detection of DNA damage (single- or double-strand breaks, alkali-labile sites) (Anderson and Laubenthal, 2013). H_2O_2 was used as genotoxic agent; its genotoxicity is based on the ability to react with reduced transition metals (iron, copper) and generate hydroxyl radicals that cause DNA lesions consisting in strand breaks, oxidized bases and abasic sites. Hydroxyl radicals are also responsible for other severe lesions causing loss of cell viability such as membrane lipid peroxidation and protein carbonylation (Horváthová et al., 2009; Kang et al., 2014). Within this assay, the extent of DNA damage is indicated by the comet tail whereas intact DNA concentrates in the comet head (Liao et al., 2009). Exposure of Vero cells to H_2O_2 (50 μM, 30 min) caused a significant increase in DNA damage in comparison with sham control. *R. largentii* extract afforded a remarkable protection against H_2O_2-induced DNA damage. After 24 and 48 h pre-treatment with *R. largentii* extract (100, 200 and 300 μg/mL), DNA damage was almost completely prevented by all tested concentrations. It is worth mentioning that the antigenotoxic activity was not dependent on the incubation time. For each extract concentration, the genoprotective effects

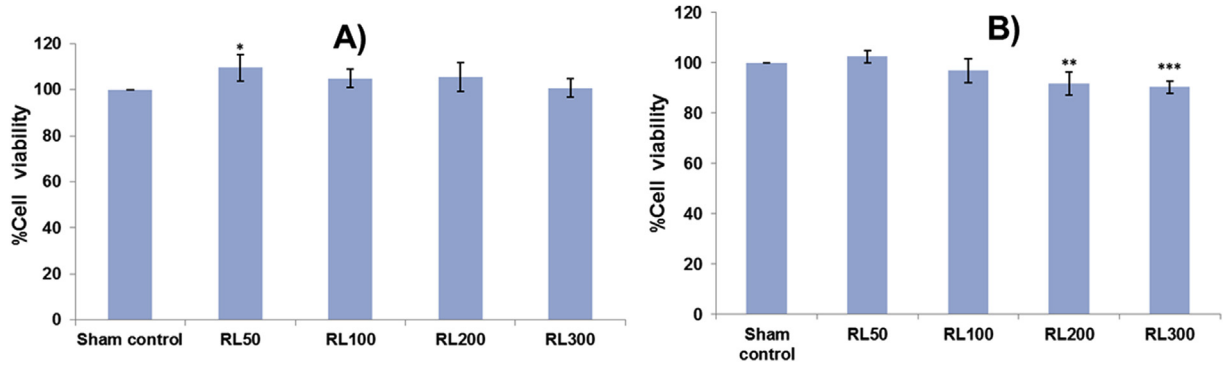

Fig. 2. Viability of Vero cells after 24 (A) and 48 h (B) treatment with *Ramaria largentii* extract (50, 100, 200 and 300 μg/mL). Results represent the mean ± SE of five independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 when comparing the effects of *Ramaria largentii* extract with sham control (*t*-test).

after 24 h pre-treatment were comparable with those detected after 48 h pre-treatment (Figs. 3 and 4). The genoprotective activity of *R. largentii* extract against H_2O_2-induced damage can be ascribed, in part, to its phenolic constituents. As *R. largentii* extract proved to be a weak ferrous ion chelator, it is more than likely it does not reduce ferrous ion-mediated conversion of H_2O_2 into hydroxyl radicals. Most probably, the extract decreases oxidative stress-induced DNA damage by a direct scavenging of H_2O_2 and other intracellular reactive oxygen species (hydroxyl radical) generated by H_2O_2. In addition, indirect mechanisms such as up-regulation of cellular enzymatic (catalases, glutathione peroxidases) and non-enzymatic systems (reduced glutathione) that detoxify H_2O_2 (Kang et al., 2014; Winterbourn and Hampton, 2008) might also be involved in genoprotection. As *R. largentii* extract showed a total phenolic content of only 4.23% (42.33 mg/g), antigenotoxic effects cannot be ascribed solely to its phenolic compounds; other constituents and possible synergistic interactions among constituents might contribute to the antigenotoxic effects of *R. largentii* extract. However, our literature survey revealed cell-based studies on the genoprotective potential of other edible mushroom species but not *Ramaria* species. At a concentration of 0.5 mg/mL, cold (20 °C) and hot (100 °C) aqueous extracts of *Agaricus bisporus* and *Ganoderma lucidum* fruiting bodies, respectively afforded almost complete protection against H_2O_2-induced DNA damage in Raji cells (Burkitt's lymphoma, ATCC CCL-86) (Shi et al., 2002b). Organic extracts of *Agaricus blazei* reduced the micronuclei frequency in Chinese hamster lung V79 cells exposed to methyl methanesulfonate while the antigenotoxic activity of aqueous extracts was controversial (Guterres et al., 2005). DNA damage induced by methyl methanesulfonate (single-strand breaks caused by alkylation and further depurination) in human laryngeal epidermoid carcinoma HEp-2 cells was reduced by the aqueous extracts of *Lentinula edodes* obtained at 22 ± 2 and 4 °C (concentrations of 1 and 0.5 mg/mL, respectively) (Miyaji et al., 2004). The methanolic extract of *Lactarius vellereus* (500 μg/mL) caused 70% reduction in DNA damage in human hepatoma HepG2 cells exposed to 2-amino-3-methylimidazo(4,5-f)quinolone (carcinogen heterocyclic amine generated during cooking meat) (Mlinarič et al., 2004). In addition, the antigenotoxic and antimutagenic effects of *Agaricus blazei* and *Lentinula edodes* were confirmed by *in vivo* studies (Delmanto et al., 2001; Lima et al., 2001). For these two mushroom species, differences in genoprotection among different strains were reported (Guterres et al., 2005; Lima et al., 2001). Other extracts isolated from *Lentinula edodes*, *Flammulina velutipes*, *Auricularia auricula*, *Pleurotus sajor-caju*, *Volvariella volvacea* and *Hypsizygus marmoreus* showed no DNA protective activity against H_2O_2-induced damage in Raji cells (Shi et al., 2002b). However, the mechanisms underlying the genoprotective effects of edible mushrooms still need to be elucidated. Only a few genoprotective constituents were identified in mushroom species. The antigenotoxic activity of *Agaricus bisporus* was associated with tyrosinase identified in the fruiting bodies; the enzyme converts tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) which is further oxidized to dopaquinone and other oxidation products responsible for an

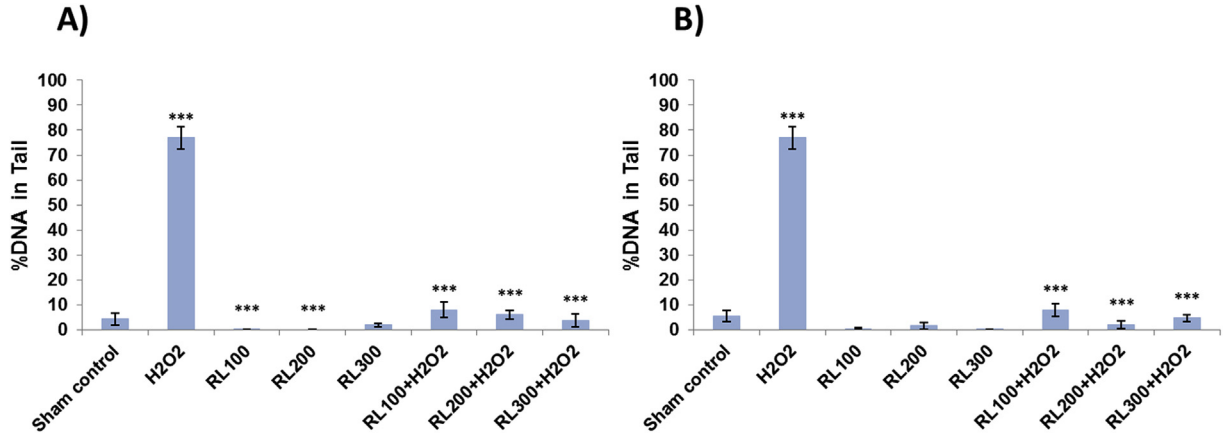

Fig. 3. % Tail DNA in Vero cells after 24 (A) and 48 h (B) pre-treatment with *Ramaria largentii* extract (100, 200 and 300 μg/mL). Results represent the mean ± SE of five independent experiments. ***p < 0.001 when comparing the effects of *Ramaria largentii* extract with H_2O_2-treated cells and H_2O_2-treated cells with sham control (*t*-test).

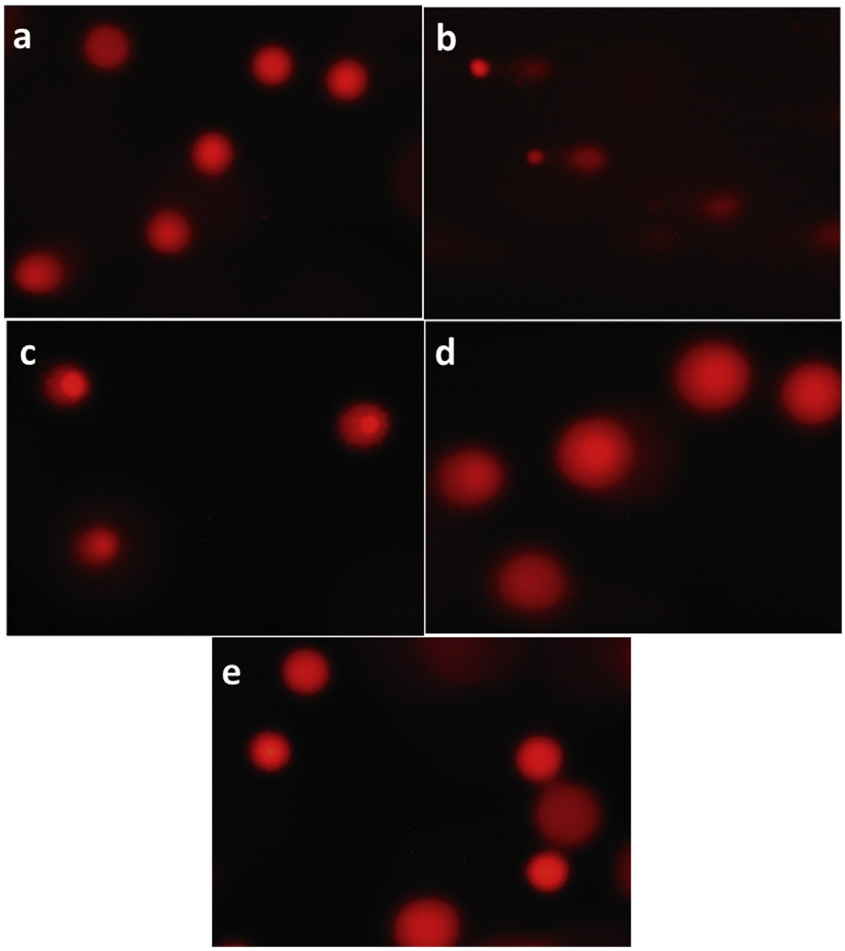

Fig. 4. Photomicrographs of comet length in Vero cells: (a) normal, (b) exposed to H_2O_2 (50 μM, 30 min), exposed to H_2O_2 (50 μM, 30 min) after 48 h pre-treatment with *Ramaria largentii* extract: (c) 100 μg/mL, (d) 200 μg/mL, (e) 300 μg/mL.

enhancement of the cellular antioxidant defense (up-regulation of γ-glutamylcysteine synthetase with an increase in glutathione level, up-regulation of NAD(P)H: quinone oxidoreductase) (Shi et al., 2002a). A water-soluble polysaccharide and β-glucan were reported to be involved in the antigenotoxic activity of *Ganoderma lucidum* and *Agaricus blazei* (or *A. brasiliensis*), respectively (Angeli et al., 2006, 2009; Roupas et al., 2012). Protective mechanisms of *A. blazei* β-glucan against DNA damage induced by different genotoxicants were elucidated: inhibition of enzymes involved in the activation of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (cooked food mutagen) and benzo(a)pyrene (environmental carcinogen), inactivation of reactive oxygen species generated during benzo(a) pyrene activation, direct binding to benzo(a)pyrene and inhibition of its penetration into the cell (Angeli et al., 2006, 2009). The capacity of *A. blazei* organic extracts to stimulate DNA repair mechanisms was attributed to linoleic acid (Guterres et al., 2005). Other mushroom constituents (phenolic compounds, triterpenes, carotenoids, vitamins C and E) are known to have DNA protective effects (Aissa et al., 2012; Ayed-Boussema et al., 2013; Ferguson et al., 2005; Ramos et al., 2008; Siddique et al., 2009) and therefore they might contribute to the antigenotoxic potential of certain mushrooms.

Although edible mushrooms are considered to be devoid of toxicity and safe, recent investigations on their genotoxic potential showed that some of them cause DNA damage. Extracts of *Agaricus blazei*, *Lactarius vellereus*, *Grifola frondosa*, *Hericium erinaceus* showed no genotoxic effects in cell-based assays (Bellini et al., 2006; Guterres et al., 2005; Mlinarič et al., 2004; Nur Shahirah et al., 2014). With respect to *Agaricus blazei*, the *n*-butanolic extract proved to be genotoxic only in HTC rat hepatoma cells. As these are drug-metabolizing cells, the genotoxicity of *n*-butanolic extract was most probably due to the generation of genotoxic metabolites (Bellini et al., 2006). The hot aqueous extract of *Ganoderma* did not show *in vivo* genotoxic effects; no DNA lesions were detected in mouse lymphocytes after acute and subchronic oral administration of *Ganoderma* extract (Chiu et al., 2000). Obviously, the genotoxic potential of edible mushrooms should be assessed to confirm the safety for human consumption. In the present study, *R. largentii* extract treatment alone (24 and 48 h incubation with 100, 200 and 300 μg/mL) had no genotoxic effects. The comet parameters for assessment of DNA damage (% tail DNA) were comparable with those of sham controls (Figs. 3 and 5).

The lack of genotoxicity and the remarkable antigenotoxic activity of *R. largentii* extract in normal Vero cells encourage further studies on identification of antigenotoxic constituents and elucidation of the mechanisms underlying their antigenotoxic effects.

4. Conclusions

This study is the first report on the chemical composition and bioactivity of *Ramaria largentii*. Protocatechuic and vanillic acids were detected in the hydromethanolic extract of the fruiting bodies. The extract showed good free radical scavenging and reducing capacities and excellent DNA protective effects against

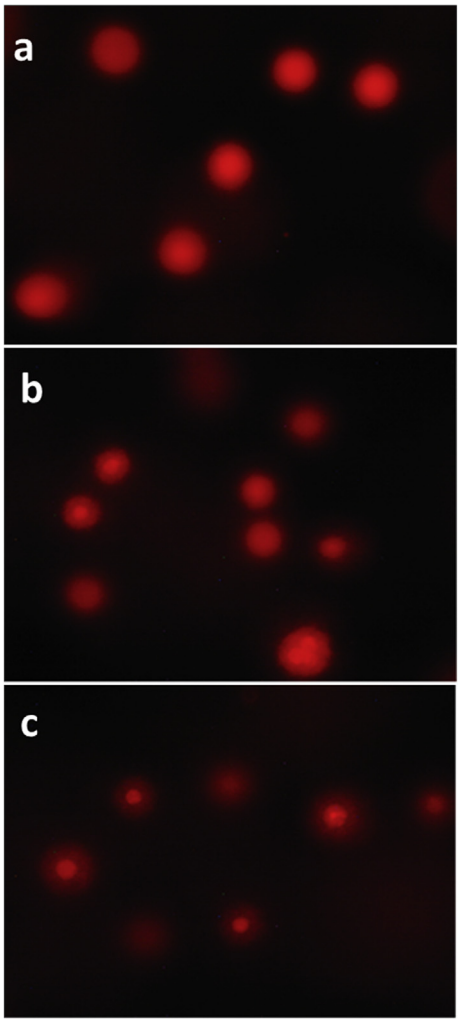

Fig. 5. Photomicrographs of comet length in Vero cells: (a) normal, after 48 h treatment with *Ramaria largentii* extract: (b) 200 μg/mL, (c) 300 μg/mL.

H_2O_2-induced damage. In addition, this study clearly revealed that *Ramaria largentii* extract lacks genotoxic potential. According to these results, *Ramaria largentii* extract might afford a certain level of protection against oxidative stress and oxidative stress-induced DNA damage and therefore might have potential health benefits. Further *in vivo* studies are needed to confirm this assumption. Identification of antigenotoxic constituents in *Ramaria largentii* and elucidation of the mechanisms underlying their antigenotoxic potential are of great interest. Other chemical constituents and *in vivo* bioactivities should be further investigated.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.02.006.

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