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Further *in vitro* evaluation of antiradical activity of the moss *Rhodobryum ontariense* tea using EPR and fluorescence spectroscopy

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Abstract – The moss *Rhodobryum ontariense* tea has been claimed by Traditional Chinese medicine for its beneficial effects in the treatment of wide range of cardiovascular diseases including hypertension. The antiradical activity of *R. ontariense* tea (*i.e.* its lyophilised water extract) was evaluated by electron paramagnetic resonance spectroscopy/against superoxide anion ($\bullet O_2^-$), nitric-oxide (•NO), 2,2-diphenyl-1-picrylhydrazyl (•DPPH), carbon-dioxide anion ($\bullet CO_2^-$) and methoxy ($\bullet CH_2OH$) radicals/ and fluorescence spectroscopy/against hydroxyl (•OH) radicals/ in *in vitro* conditions. It reduced the production of majority of radical species tested, but in a varying degree. The most activity was observed against •OH, •DPPH and $\bullet CO_2^-$ radicals (95 ± 10%, 78 ± 4% and 67 ± 5%, respectively). In addition, the extract was shown to be active against $\bullet CH_2OH$ and •NO radicals (55 ± 8% and 45 ± 8%, respectively). On the contrary, no any antiradical activity was observed against $\bullet O_2^-$ radicals. According to the experimental data obtained, *R. ontariense* tea can be considered as a promising resource of natural products with antiradical activity and may be of some interest to research in heart disease.

DPPH / EPR / Fluorescence spectroscopy / Nitric-oxide radical / *Rhodobryum ontariense* / ROS

INTRODUCTION

Traditional Chinese medicine suggests that mosses of *Rhodobryum* species can cure hearth disorders as crude drugs (Ding, 1982). Preliminary analyses of the moss *Rhodobryum ontariense* chemical constituents have indicated presence of volatiles, carbohydrates including fructooligosaccharides (FOS; namely, 1-kestose, nystose and 1^F-ß-fructofuranosylnystose) and fatty acids (Pejin *et al.*, 2011; Pejin, Sabovljević, Tesevic & Vajs, 2012; Pejin *et al.*, 2012). In addition, the bioacivity screening related to cardiovascular diseases has been recently done, both *in vitro* and *in vivo*, suggesting therapeutic potential of this herbal medicine

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(Pejin *et al.*, 2011; Pejin, Kien-Thai & Spasojevic, 2012). Indeed, systolic, diastolic and mean arterial pressure as well as cardiac output were significantly lowered in the group of spontaneously hypertensive rats (n = 7) treated intravenously with lyophilised water extract of *R. ontariense* (100 mg/kg b.w. dissolved in 0.2 ml of saline).

The cardiovascular diseases represent an heterogeneous group of disorders that affects the heart and blood vessels. The diseases are characterised by angina pectoris, hypertension, congestive heart failure, acute myocardial infarction (heart attacks), stroke and arrhythmia. There is now considerable biochemical, physiological and pharmacological data to support a connection between free radical reactions and cardiovascular tissue injury. Actually, these disease conditions are directly or indirectly related to oxidative damages and share common mechanisms of molecular and cellular damages (Mimić-Oka *et al.*, 1999).

This study aimed to investigate *in vitro R. ontariense* tea extract as a potential resource of natural products with antiradical activity against superoxide anion ($\bullet O_2^-$), nitric oxide (•NO), 2,2-diphenyl-1-picrylhydrazyl (•DPPH), carbon dioxide anion ($\bullet CO_2^-$) and methoxy ($\bullet CH_2OH$) radicals (measured by electron paramagnetic resonance spectroscopy – EPR) and hydroxyl (•OH) radicals (measured by fluorescence spectroscopy – FS).

MATERIAL AND METHODS

General

All chemicals were used as received from Sigma-Aldrich (analytical grade or higher) without any further purification, except spin-trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) which was purchased from Enzo Life Sciences and purified according to the procedure proposed by Jackson, Liu K.J, Liu M. & Timminis (2002). Solutions were prepared using deionised water of resistivity not less than 18.2 MΩ.cm.

EPR spectra were recorded using a Varian E104-A X-band EPR spectrometer at room temperature (22°C). The spectra were processed by EW software (Scientific Software). Conditions for spectral recording of DEPMPO spin-trap adducts were: scan range 200 G, microwave frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW and time constant 0.032 s. In measurements of •NO and •DPPH radicals, parameters used were: scan range 100 G, microwave frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW and time constant 0.032 s.

Plant material

The sample of *Rhodobryum ontariense* (Kindb.) Kindb. (Bryaceae) originated from the Fraser's Hill (Malaysia, August 2012). Voucher specimen has been deposited in the Herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya (KT Yong 7635).

Extraction

Before extraction the moss was carefully inspected for contaminants: soil and plant material were completely removed. The gametophyte tips were used for the extraction. Air-dried parts of *Rhodobryum ontariense* (5 g) were ground and extracted with boiling water for 30 min. The extract (tea) was filtered and concentrated by lyophilisation to give the residue (the yield, 10%) which was stored at + 4°C for further use.

Electron paramagnetic resonance measurements

Superoxide anion radicals ($\bullet O_2^-$) were generated using riboflavin-light reaction (Savić & Mojović, 2012). Starting mixture containing 0.05 mM riboflavin, 4 mM DTPA and spin-trap DEPMPO was continuously flowed with gaseous oxygen. Finally, UV lamp (130 W) was applied for 30 s.

Nitric oxide radicals (•NO) generated from KNO_2 were detected using $Fe(DTCS)_2$ complex which was prepared by DTCS disodium salt in concentration of 60 mM with 30 mM $FeSO_4$ (Katayama *et al.*, 2001). The final concentration of the complex in sample was 20 mM.

2,2-diphenyl-1-picrylhydrazyl (•DPPH) forms stable radical which is widely used for examination of antioxidative activity (Gođevac *et al.*, 2008). Methanol solution of DPPH was examined without and after addition of phytol. The final concentration of 0.6 mM methanol solution of DPPH in sample was 0.02 mM.

Carbon dioxide anion radicals ($\bullet CO_2^-$) were generated in reaction mixture composed of 20 mM DTPA, 0.2 mM $FeSO_4$, 0.2 mM H_2O_2 and 15 mM sodium formate in cacodylate buffered solution (pH = 7.1) (Savić & Mojović, 2012).

Methoxy radicals ($\bullet CH_2OH$) were generated in reaction mixture composed of 20 mM DTPA, 0.2 mM $FeSO_4$, 0.2 mM H_2O_2 and 10% v/v of methanol in cacodylate buffered solution (pH = 7.1) (Savić & Mojović, 2012).

The measurements were performed in duplicate. In all systems tested the final concentration of *Rhodobryum ontariense* tea extract was 1 mg/mL, while the incubation time was 10 min as previously published (Savić & Mojović, 2012).

Fluorescence measurements

Anti-hydroxyl radical activity of *Rhodobryum ontariense* tea extract was determined in standard Fenton reaction (1.0 mM $FeSO_4$, 2.0 mM H_2O_2) and detected using the fluorescence probe APF (2-[6-(4-amino)phenoxy-3H-xanten-3-on-9-yl] benzoic acid). Fluorescence spectra in time domain (kinetics mode) were collected using Fluorolog-3 spectrofluorimeter (Jobin Yvon Horiba, Paris, France). Measurements were performed in well stirred and tempered quartz cuvette controlled by Peltier element (1 cm optical path length, 25°C). The slits on the excitation and emission beams were fixed to 5 nm in order to eliminate noise and compensate potential changes in fluorescence spectra shape caused by addition of antioxidant substances. Total duration of experiment was 600 s, with integration time of 1 s and excitation/emission wavelengths 500/520 nm (Gomes *et al.*, 2005).

Kinetic profile of APF probe represents linear function of time:

$$\mathbf{A\ (t) = at + A_0}$$

where is **A(t)** the amplitude of fluorescence emission in given moment of time; **a** the parameter which determines the slope of line; $\mathbf{A_0}$ the starting intensity of fluorescence emission.

In the presence of •OH radicals fluorescence emission intensity increases in time due to releasing of quencher from APF (Setsukinai *et al.*, 2003). The measurements were performed in duplicate.

Statistical analysis

All mathematical computations were performed in MatWorks MatLab 2010. The error in EPR measurements is expressed as the absolute one, while the error in FS measurements is estimated on 95% confidence interval for kinetic curve obtained.

RESULTS AND DISCUSSION

The antiradical activity of the moss *Rhodobryum ontariense* tea (*i.e.* its lyophilised water extract) was determined *in vitro* by EPR and FS measurements.

The production of •OH radicals in standard Fenton reaction was highly reduced (95 ± 10%), measured by FS. This result is in good agreement with the previous one (94 ± 1%) obtained by EPR using the spin-trap DEPMPO and confirms antiradical potential of the sample examined against the most reactive oxygen species (Pejin, Kien-Thai & Spasojevic, 2012).

Rhodobryum ontariense extract also showed a significant activity against •DPPH and $\bullet CO_2^-$ radicals (78 ± 4% and 67 ± 5%, respectively), measured by EPR. Although not relevant to physiological conditions, •DPPH test is quite frequently used in food research for determination of antioxidative capacities of various samples (Anesini *et al.*, 2008). In addition, the extract was observed to be moderately active against $\bullet CH_2OH$ and •NO radicals (55 ± 8% and 45 ± 8%, respectively), measured by EPR. From the biological point of view it is noteworthy that $\bullet CH_2OH$ radicals in reaction with the extract triggered chain of radical reactions that produced harmful carbon-centered radicals. On the contrary, no any antiradical activity was observed against $\bullet O_2^-$ radicals, measured by EPR (Fig. 1, Table 1).

Table 1. Percent of free radical production in generator reactions after the incubation with the moss *Rhodobryum ontariense* extract

Radical adduct	*Free radical production (%)*	*Absolute error (%)*
DEPMPO $\bullet O_2^-$	100	± 5
NO-Fe(DTCS)$_2$	56	± 8
•DPPH	22	± 4
DEPMPO $\bullet CO_2^-$	33	± 5
$\bullet CH_2OH$	45	± 8

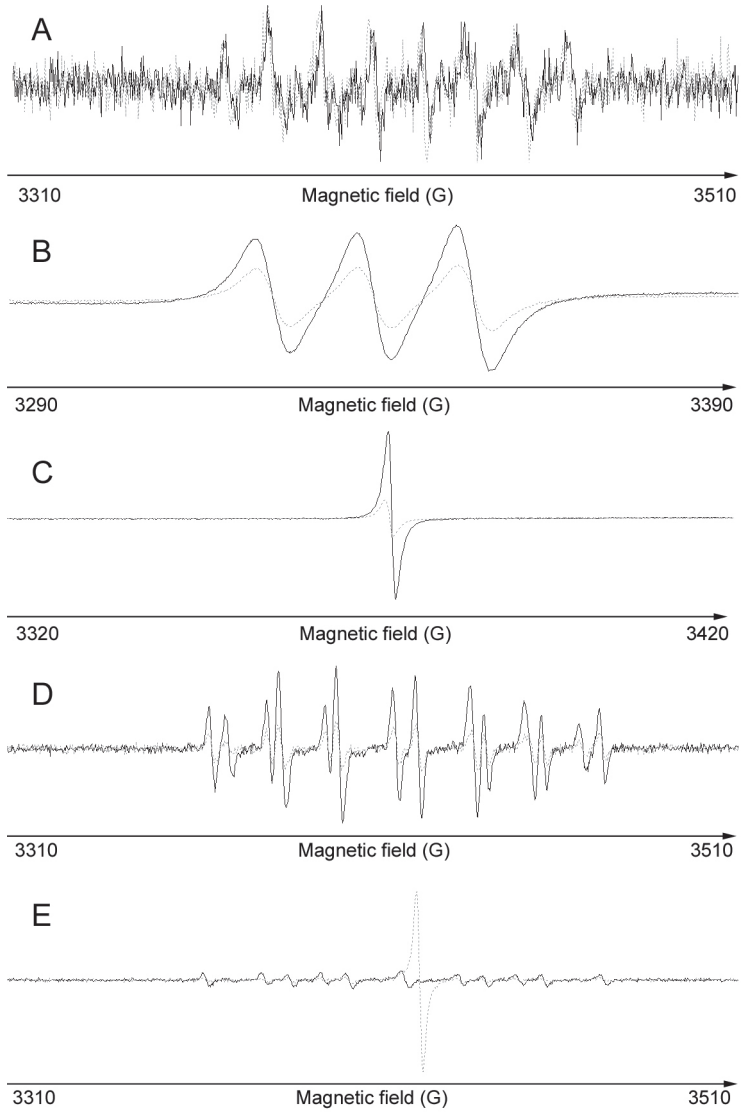

Fig. 1. EPR spectra of free radical production in generator reactions before (solid line) and after the incubation with the moss *Rhodobryum ontariense* extract (dash line). A – DEPMPO $\bullet O_2^-$ radical adduct; B – NO-Fe(DTCS)$_2$ complex radical; C – •DPPH radical; D – DEPMPO $\bullet CO_2^-$ radical adduct; E – DEPMPO $\bullet CH_2OH$ radical adduct.

Rhodobryum ontariense tea extract has been classified as rich in different types of carbohydrates by means of its spectroscopic data and typical chromatographic profile (Pejin, Sabovljević, Tesevic & Vajs, 2012; Pejin *et al.*, 2012) and poor in total phenolic compounds (Pejin *et al.*, 2012). Therefore, its saccharide constituents may be suspected as one of the scavengers of free radicals

tested, primarily of •OH radicals which is potentially the most harmful one. Indeed, these radicals are well known as highly reactive and short-lived (10^{-9} s). It has been shown that •OH radicals can be scavenged with phosphorylated fructose (Spasojević *et al.*, 2009). Hernandez-Marin & Martínez (2012) have performed a theoretical analysis of free radical scavenging properties of carbohydrates selected showing that hydrogen atom transfer from hydrocarbons investigated to •OH radical represents an exergonic process, whereas the reaction with $\bullet O_2^-$ radical is endergonic. Consequently, only anti-hydroxyl radical activity may be expected for these compounds. On the other hand, a significant anti-hydroxyl radical potential of the fructooligosaccharides 1-kestose and nystose has been recently observed (72% and 78% by EPR & 69% and 74% by FS, respectively) in *in vitro* conditions, indicating the nystose to be a more active natural product. In addition, the computational results have confirmed that nystose follows the same pattern previously shown for 1-kestose, *i.e.* that carbohydrates can react with •OH radicals (Pejin *et al.*, 2013). It is well known that FOS belong to cardioprotective nutraceuticals, so this study may be of some interest to research in heart disease.

Cardiac myocytes – under normal physiologic conditions – exhibit substantial vitality and tolerance to brief periods of ischemia. The coronary blood supply provides these normally aerobic cells with oxygen and energy containing molecules and affords the removal of waste products, such as carbon dioxide, hydrogen ion, and lactate, among others. However, when stressed by coronary underperfusion of sufficient length and/or magnitude, myocytes cannot maintain their normal physiologic milieu (Hoffman, Timothy, Poston & Erik, 2004). $\bullet CO_2^-$ radicals may be harmful in pathophysiological conditions and bioactives of natural origin with good scavenging activity against these radicals are worthy of study.

Nitric oxide is an important mediator of cellular and molecular events which impacts the pathophysiology of myocardial ischemia (Bredt & Snyder, 1994). The activity observed against •NO radicals cannot be treated as insignificant and may prove to be higher under *in vivo* conditions.

To the best of our knowledge, this is the first report of *in vitro* antiradical activity of the moss *R. ontariense* tea extract against majority of free radicals tested. The advantage of using EPR spin-trap DEPMPO lies in its sensitivity and capability for distinguishing different types of free radicals.

This study in fact provides preliminary results and a possible mechanism to support the claims of Traditional Chinese medicine using the moss *Rhodobryum* to successfully treat cardiovascular diseases (Ding, 1982). Indeed, anti-hydroxyl radical activity is an important factor in the description of antioxidant capacity of any natural product with beneficial health effects. However, more studies should be undertaken for the better understanding of the uses of *R. ontariense* tea in medicine, particularly for cardiovascular diseases – the major cause of death worldwide, according to the World Health Organization (WHO, 2013). Therefore, both chemical and biological studies of the extract examined are in progress in our labs.

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