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IN VITRO INVESTIGATION OF THE ANTIHYPERTENSIVE EFFECT OF THE MOSS *RHODOBRYUM ONTARIENSE* (KINDB.) (KINDB.)

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The ability to modulate erythrocyte membrane fluidity, the capacity to release nitric oxide and the ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] cation scavenging activity including the total phenolic content screening of the moss *Rhodobryum ontariense* tea, a traditional Chinese herbal medicine for hypertension and a wide range of cardiovascular diseases, were conducted in vitro in this study. This medicinal tea did not affect human erythrocyte membrane fluidity, did not contain reservoir of nitric oxide to be eliminated in blood and it showed low ABTS cation scavenging activity including meager content of total phenolic compounds. The obtained results excluded in a great extent some possible mechanisms of action of *R. ontariense* tea in relation to highlighted medical conditions.

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1. Introduction

Traditional Chinese Medicine (TCM) includes a wide range of therapies, the best known including acupuncture and Chinese herbal medicine [1]. Internationally, there is growing and sustained interest in TCM. This interest is fuelled by a combination of factors including recognition of potential benefits of TCM; dissatisfaction with the traditional Western medical model; an increasing commitment to holistic care; skepticism regarding adverse drug effects, and increasing evidence for the interaction of psychological factors and outcomes of disease and treatment and consumer demand [2-4]. Patients who use TCM in Western countries report the main reason for using it is that TCM is a 'more natural' and potentially safer alternative in the treatment of chronic illness than pharmaceutical drugs or surgery. It is likely that this perception is fuelled by a view that these agents are more 'natural' than chemically engineered pharmaceuticals. Currently the use of TCM is influenced by legal restrictions, with shifts towards increasing regulation and formal recognition of TCM as a therapeutic treatment [1]. Its establishment as a mainstream therapy or increased acceptance as a therapeutic medical treatment is best achieved through evidence-based research.

Cardiovascular disorders account for 12 million deaths annually worldwide and are known to be number one group of 'killer diseases' [5]. Among them hypertension is the most common heart chronic illness which the world has been facing in last years [6]. The importance of hypertension lies in the fact that it forms one of the main risk factors for coronary heart disease, stroke, atheroslcerosis and peripheral vascular disease [7]. Reactive oxygen species (ROS) are

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directly linked to hypertension, artherosclerosis, and diabetes [8]. Antioxidant agents can treat oxidative pathologies by neutralizing ROS, chelating catalytic metals and acting as oxygen scavengers [9]. Because of the high carcinogenity of synthetic antioxidants [10], the development of effective antioxidants of natural origin is of great interest [11-13].

TCM suggests that some mosses of the genus *Rhodobryum* (Bryaceae) can cure cardiovascular diseases as crude drugs in form of medicinal tea [14]. The name for *Rhodobryum* in Yunnan Province is HuiXinCao (回心草), a name referring to the heart-healing properties of this plant; the name literally means "return-the-heart-herb" [15]. *Rhodobryum giganteum* (Schwaegr.) Par. is known to have antipyretic, diuretic and antihypertensive effect and is used for sedation, neurasthenia, psychosis, cuts, cardiopathy and expansion of heart blood vessels [16]. The same author also noted the use of *Rhodobryum roseum* (Hedw.) Limpr. as a sedative, for neurasthenia and cardiopathy. These two mosses were intensively studied in view of their chemical and bioactive components [17-20].

Pejin *et al*. [21-27] investigated the chemical composition and biological activity of the related species *Rhodobryum ontariense* (Kindb.) Kindb. for the first time. The moss *R. ontariense* lyophilised aqueous extract (100 mg/kg b.w. dissolved in 0.2 ml of saline and injected intravenously) significantly and quickly normalized arterial blood pressure and reduced pulse pressure, thus decreasing the risk for cardiovascular events in the group of spontaneously hypertensive rats [27]. The aim of this study was to put some light on the possible mechanism of antihypertensive effect of *R. ontariense* lyophilized water extract (tea) by determining its ability to modulate erythrocyte mebrane fluidity, the capacity to release nitric oxide (NO) and the ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] cation scavenging activity including its total phenolic content.

2. Experimental

2.1. Plant material

The sample of *Rhodobryum ontariense* (Kindb.) Kindb. (Bryaceae) originated from the Fraser's Hill (Malaysia, November 2010). Voucher specimen has been deposited in the Herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya (KT Yong 7635).

2.2. Extraction and isolation

Before extraction the moss was carefully inspected for contaminants: soil and other plant material were completely removed. The gametophyte tips were used for the extraction. Air-dried parts of *R. ontariense* (3 g) were ground and extracted with hot water for 30 min at room temperature. The extract (tea) was filtered and concentrated by lyophilisation to give the residue (yield, 9%) which was stored at + 4 °C for further use.

2.3. EPR measurements of order parameter as an indication of membrane fluidity

Fresh blood was obtained from four healthy volunteers between the ages of 30 and 35 years, using tubes containing 0.072 mL of 7.5% K_3EDTA as the anticoagulant per 3 mL of blood (Vacuette EDTA, Greiner Bio-One, Kremsmünster, Austria). The erythrocytes (erythrocyte membranes) were spin-labeled as described earlier [28]. Fresh blood erythrocytes were washed three times with isotonic phosphate buffered saline (PBS; NaCl 8.8 g / L, Na_2HPO4 1.2 g / L, NaH_2PO_4 0.43 g / L, pH 7.4) by centrifugation at 3500g for 10 min at 4ºC. The hematocrit for fresh blood was 40%, and all samples were adjusted to this hematocrit before incubation. The extract was added to a final concentration of 15 mg/mL and incubated for 5 min at room temperature. Ethanol solutions of the fatty acid spin-probe 5-DS (2-(3-carboxypentyl)-2-tridecyl-4,4-dimethyloxazolidine-3-oxyl; Molecular Probes, Junction City, OR, USA) were placed on the walls of tubes. The amount of DS to be used was calculated to obtain the optimal spin-label/membrane-lipid ratio of approximately 1:100. After the ethanol had evaporated, the sample was added and gently mixed. EPR spectra were recorded using a Varian E104-A EPR spectrometer (Palo Alto,

CA, USA) operating at X-band (9.1 GHz) and adjusted to the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 100 G; and scan time, 4 min; time constant 0.25 s. The temperature was controlled at 25ºC during the measurements. The order parameter (S) was calculated as shown previously with hyperfine constants taken to be Txx = Tyy = 6.1 G, Tzz = 32.4 G and was used as an indication of membrane fluidity being reversely proportional to the fluidity. The data are presented as means ± standard deviation for three separate experiments. Significances of differences were calculated using nonparametric Mann-Whitney test. Means were considered significantly different at $p < 0.05$.

2.4. EPR measurements of $^{\bullet}NO$

N-(Dithiocarboxy)sarcosine was purchased from Chemos GmbH(Dojin, Kumamoto, Japan). Fe(II)-$DTCS_2$ complex was prepared from DTCS disodium salt (60 mM) together with $FeSO_4$ (30 mM). The final concentration of the complex in samples was 20 mM. In control system the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) was added to a final concentration of 15 mM and incubated for 10 min, following which EPR recordings were conducted using parameters as in spin-trapping experiments with a difference of field centre (3340 G) and scan range (100 G). The extract was added to a final concentration of 15 mg/mL and incubated for 10 min at room temperature in the presence of Fe(II)-$DTCS_2$. The experiment was performed three times.

2.5. Determination of ABTS cation scavenging activity

Determination of ABTS cation scavenging activity was done following the ABTS method of Arnao, Cano & Acosta [29]. The reaction mixture contained 2 mM ABTS (2,2-azino-bis-ethylbenzothiazoline -6-sulfonic acid), 15 μM hydrogen peroxide and 0.25 μM horse radish peroxidase (HRP) in 50 mM phosphate buffer pH 7.5. The reactions were monitored at 730 nm (2501 PC Shimadzu, Kyoto, Japan) at 25ºC until a stable absorbance was obtained due to ABTS radical formation. Afterwards, different concentrations (0.05-0.8 mM) of ascorbic acid were added for a standard curve set-up. Adding of the extract in reaction mixture resulted in absorbance decreasing as a consequence of ABTS radical depletion. Absorbance alterations were read from standard curve and results were expressed as micrograms of ascorbic acid equivalent per milligram of the extract (μg asc mg^{-1} extract). The data are presented as means ± standard error of the three samples analysed separately.

2.6. Determination of total phenolic content

The total phenolic content in the extract was determined according to the Folin-Ciocalteu's spectrophotometric (2501 PC Shimadzu, Kyoto, Japan) procedure using ferulic acid (FA) as a standard for the calibration curve [30]. Samples were mixed with 0.25 N Folin-Ciocalteu reagents and after 3 min 0.2 M sodium carbonate solution was added and incubated for 60 min. Results were read at 724 nm and expressed as micrograms of FA equivalent per milligram of the extract (μg FA mg^{-1} extract). The data are expressed as means ± standard error of the three samples analysed separately.

3. Results

R. ontariense extract (tea) did not cause changes on the fluidity of human erythrocytes (Figure 1) and did not contain reservoir of nitric oxide to be eliminated in blood (Figure 2). Moreover, the ABTS test showed very low ABTS cation scavenging activity for this tea: 1 mg of the extract was equivalent to 1.54 ± 0.21 μg (Mean ± SE, n = 3) of ascorbic acid (Figure 3). Further, the total phenolic content in 1 mg of the extract was equivalent to 36.11 ± 2.71 μg of ferulic acid used as a standard (Figure 4).

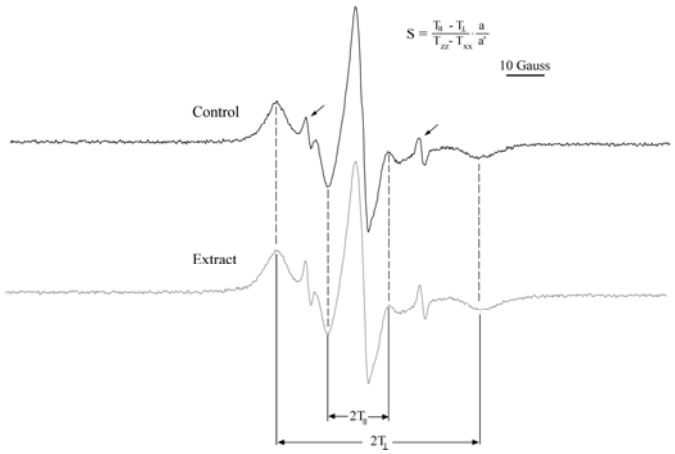

Fig. 1. The effect of Rhodobryum ontariense extract (tea) on the fluidity of human erythrocytes. S – order parameter, which is reciprocally proportional to fluidity. Dark trace – control, S = 0.736 ± 0.007; Gray trace – erythrocytes exposed to the moss extract (2 mg/mL), S = 0.743 ± 0.006. There is no statistical significance.

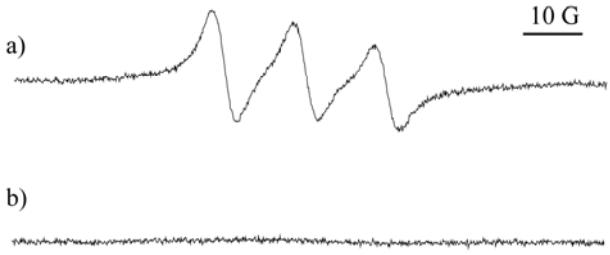

Fig. 2. The ability of Rhodobryum ontariense extract (tea) to release nitric oxide (NO). a) control (NO generating system); b) the moss extract.

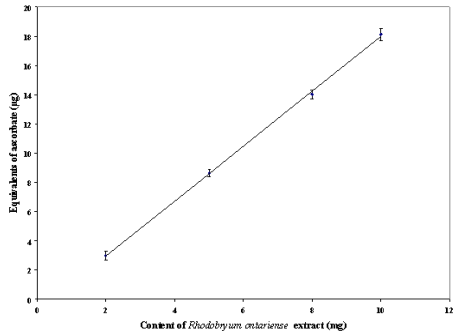

Fig. 3. The ABTS cation scavenging activity of Rhodobryum ontariense extract (tea).

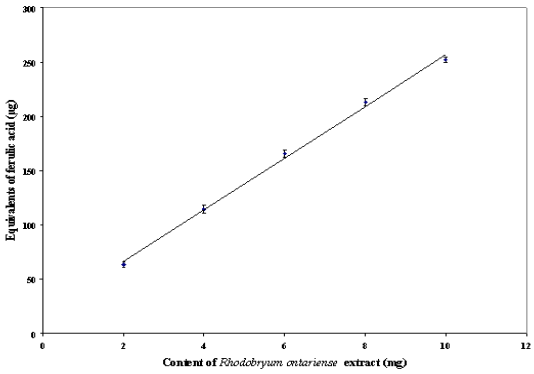

Fig. 4. The total phenolic content of Rhodobryum ontariense extract (tea).

4. Discussion

Microcirculation is a complex and integrated system in which erythrocytes represent the main definers of blood viscosity. The mechanical properties of the erythrocyte membrane, such as fluidity, seem to be of crucial importance for the ability of erythrocytes to pass through the smallest blood vessels. Some previous data have pointed out higher microviscosity of the erythrocyte membrane in spontaneously hypertensive rats in comparison to controls [31]. Hence, a link has been proposed between the alterations of mechanical properties of the erythrocyte membrane and the pathophysiology of hypertension [32]. Further investigations have confirmed this, showing that erythrocyte membrane fluidity is significantly lower in both spontaneously hypertensive rats and patients with essential hypertension than in normotensive controls [33], which led to the conclusion that decreased fluidity of the erythrocyte membrane contributes to the pathophysiology of hypertension and other cardiovascular diseases [34]. From the perspective of erythrocyte rheologic behavior, *R. ontariense* tea seems to be suitable for the use in patients with cardiovascular conditions, including essential hypertension and atherosclerosis, since it does not decrease erythrocyte membrane fluidity at physiologically relevant concentrations [35]. On the other hand, evidence suggests that nitric oxide (NO) plays a major role in regulating blood pressure and that impaired NO bioactivity is an important component of hypertension [36]. According to this study *R. ontariense* tea does not contain the reservoir of NO to be released in blood. However, the mechanism involving NO cannot be excluded since the tea components *in vivo* could act in such a way through several different modes.

The most intriguing finding of this investigation is concerned the total phenolic content of *R. ontariense* tea, suggesting that this class of compounds most probably do not have critically significant role for its observed antihypertensive effect *in vivo*. As an important category of phytochemicals, phenolic compounds have attracted more and more attention as potential agents for preventing and treating many oxidative stress-related diseases including cardiovascular one [37]. However, the shown results indicate that the moss *R. ontariense* tea does not represent a rich source of these natural antioxidants. Bearing in mind the ^{1}H NMR spectrum and characteristic TLC profile of the tea, it can be speculated that its major antioxidant constituents are saccharides as it has been the case in a recent study [38]. Indeed, *in vitro* this tea has a high hydroxyl radical (·OH) scavenging capacity (ca. 95%) which is followed by the formation of carbon dioxide radical anion ($\cdot CO_2^-$) in traces (submitted results). On the other hand, it is well known that the ·OH radical has detrimental biological activity due to its very high reactivity and can be scavenge with saccharides [39].

5. Conclusion

Generally, the obtained results excluded in a great extent some possible mechanisms of action of *R. ontariense* tea in relation to hypertension. Therefore, the investigation of its mechanism of action is going to be continued through additional *in vitro* and *in vivo* studies.

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