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COMPARATIVE EPR *IN VITRO* AND *EX VIVO* SPECTROSCOPY STUDY OF THE LEVELS OF LIPID PEROXIDATION PROCESSES IN LIVERS AND KIDNEYS OF MICE AFTER TREATMENT BY NATURALLY ISOLATED ANTIOXIDANTS

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ABSTRACT

Many natural isolated extracts and fractions from different medicinal plants exhibit *in vitro* and *in vivo* antioxidant properties and can reduce oxidative stress damages induced by different xenobiotics. However, for a number of natural antioxidants it has been found that depending on doses or under certain conditions they can exert antioxidant or pro-oxidant activity.

The aim of our present experiments was - first by direct *in vitro* Electron Paramagnetic Resonance (EPR) spectroscopy to characterize the flavonoid glycoside **IBG-RA-22 (rutin),** extracted from the medicinal plant *Nothapodytes foetida*, a camptothicin-producing plant similar to *Campthotheca acuminata* and **astragaloside I** isolated from the *in vitro* transformed roots of medicinal plant *Astragalus membranaceus* and second by EPR spin-trapping technique to study *ex vivo* the behavior of these naturally isolated antioxidants. These preliminary EPR spectroscopy studies demonstrated that each antioxidant exhibited a singlet EPR signal in solution of DMSO. Moreover, at the studied doses and experimental conditions IBG-RA-22 and **astragaloside I** exhibited slight pro-oxidant properties in the livers of treated mice when compared to those of the control group of mice, while they behave as antioxidants in the kidneys of treated mice.

Keywords: Rutin, IBG-RA-22, *Nothapodytes foetida, Campthotheca acuminata, Astragalus membranaceus, cycloartane saponins,* EPR spin trapping technique, ROS production.

INTRODUCTION

Approximately 8000 naturally occurring compounds are known, which include simple phenolic, phenolic acids, lignans, coumarins, styrylpyrones, flavonoids, stilbenes, flavonolignans and tannins, (1). It is generally accepted that phenolic compounds behave as antioxidant as a result of the activity of the phenolic moiety. It is believed that one of the mechanisms of their antioxidant activity is radical scavenging *via* hydrogen atom donation. Flavonoids, a group of phenolic compounds widely occurring in the plant kingdom, are believed to be good antioxidants, and their ability to inhibit lipid oxidation has been widely investigated (2, 3). Rutin an effective flavonoid component of the traditional Chinese and Indian herbs, is one of the most commonly found flavonol glycosides identified as vitamin P with quercetin and hesperidin. Rutin has been reported to have clinically relevant functions, including antihypertensive, anti-inflammatory, antihemorrhagic activity, and ability to strengthen the capillary permeability and stabilization of platelets (4, 5). The pharmaceutical effects can be attributed in part

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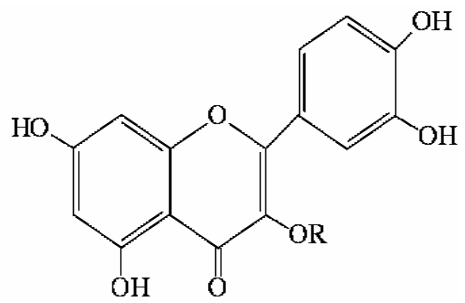
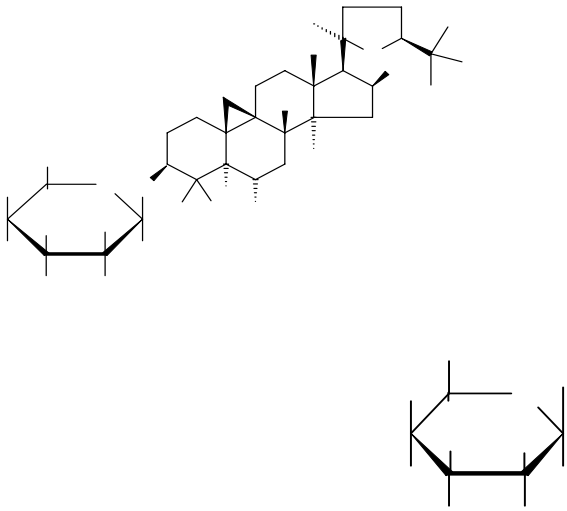
to its ability to scavenge free radicals, and has been proven to exhibit strong antioxidative and free-radical-scavenging activities (6, 7).

Astragalus membranaceus is one of the oldest and most frequently used herb for traditional medicine in several Asian countries and as a tonic it is well known to strengthen the host defense system (8, 9). The active components are γ-amnonobutiric acid (GABA), isorhamnetin, quercitin, kaemferol and polyamine. These flavonoids may have beneficial health effects because of their various biological effects, immunomodulatory activity, antioxidant properties and their inhibitory role in various stages of tumor development as shown in many studies (10, 11). Moreover, for flavonoids have been reported to exert pro-oxidant properties including the formation instead of scavenging of radicals (12).

All these above reported findings persuaded us to study by *ex vivo* EPR spin trapping technique the behavior of both naturally isolated IBG-RA-22 and astragaloside I **(Figure 1)**, in relation with their antioxidant activity.



A.



B.

Figure 1. Chemical structures of IBG-RA-22 (rutin) (A) and Astragaloside I (B)

MATERIALS AND METHODS

Plant materials and Chemicals

From *Nothapodytes foetida*, pure rutin (IBG-RA-22) was isolated and characterized by LC-MS and NMR as previously described (**Figure 1A)**, (13).

The cycloartane saponine astragaloside I - 3-O-β-(2',3'-di-O-acetyl)-D-xylopiranosyl-6-O-β-D-glucopyranosyl-cycloastragenol was isolated from *in vitro* transformed hairy roots of medicinal plant *Astragalus membranaceus*, cultivated in airlift bioreactor **(Figure 1B)**, according to Ionkova et al. (14). The spin-trapping agent, n-tert-butyl-alpha-phenylnitrone (PBN) and dimethylsulfoxide (DMSO) were purchased by Sigma Chemical Co, St. Louis, USA. All other chemicals used in this study were analytical grade.

Animals and treatment

White laboratory (Swiss albino) mice of approximately weight 20-40 g were used for the experiments. The mice were housed in polycarbonate cages in controlled conditions (12 h light/dark cycles), temperature of 18–23°C and humidity of 40–70%, with free access to tap water and standard laboratory chow. Experiments were carried out in accordance with European directive 86/609/EEC of 24.11.1986 for protection of animals used in scientific and experimental purposes. Mice were divided in two groups (5 mice in each group) and inoculated i.p. with IGB-RA-22 or astragaloside I dissolved in saline. Before treatment, every mouse was weighed and inoculated i.p. with a volume of IBG-RA-22 or astragaloside I that corresponded to a dose of 20 mg/kg for IBG-RA-22 and 8 mg/kg for astragaloside I. Control group was inoculated with solvent only. After 3 hours of the treatment all animals in the tested and control group were exsanguinated under light ether anesthezia and the livers and kidneys were collected and washed in cool saline.

Electron paramagnetic resonance (EPR) study

All EPR measurements were performed at room temperature on a X-band EMX^{micro}, spectrometer Bruker, Germany, equipped with standard Resonator. All EPR experiments were carried out in triplicate and repeated. Spectral processing was performed using Bruker WIN-EPR and Simphonia software.

1. EPR in vitro study of IBG-RA-22 and astragaloside I in solution forms

Quartz capillaries were filled with IBG-RA-22 or astragaloside I dissolved in DMSO and placed in the EPR cavity and EPR spectra were recorded at room temperature at the following EPR settings: **for IBG-RA-22**: 3529.5 G center field, 0.808 mW microwave power, 10.00 G modulation amplitude 200.00 G sweep width, a receiver gain 2x10^3, 655.36 ms time constant, 163.963 s sweep time; **for astragaloside I**: 3510.00 G center field 4.132 mW microwave power, 10.00 G modulation amplitude, 100 G sweep width, a receiver gain 2x10^3, 5242.88 ms time constant, 225.28 s sweep time.

2. Preparation of tissue homogenates

EPR study of ROS production were performed according to Shi et al., 2005 with some modifications (15). Briefly, about 0.1 g of liver or kidney tissue was homogenized for 2 min after addition 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. After centrifugation, 0.4 ml supernatant of homogenized tissue was taken in a quartz tube and stored in liquid nitrogen for EPR measurement. EPR spectra were recorded at room temperature. EPR settings were as follows: 3503.74 G center field, 20.49 mW microwave power, 0.50 G modulation amplitude, 100 G sweep width, a receiver gain 1x10^6, 327.68 ms time constant, 81.92 s sweep time.

Statistical analysis

Statistical analysis was performed with Statistica 6.1, StaSoft, Inc. and results were expressed as means ± standard error (SE). Statistical significance was determined by the Student's t-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Results from recorded EPR spectra of IBG-RA-22 and astragaloside I are presented on **Figure 2** and **Figure 3**. The free radicals detected in both studied solutions exhibited a single line EPR spectrum with a g value 2.0045 for 200 μM solution of IBG-RA-22 and g value 2.00358 for solution of astragaloside I.

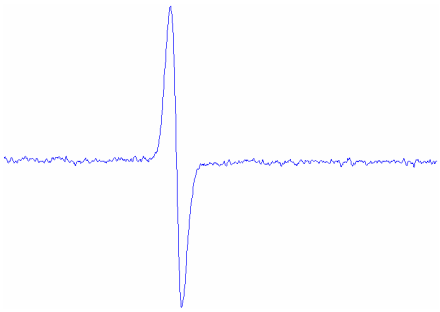


Figure 2. EPR spectrum of IBG-RA-22 in solution.

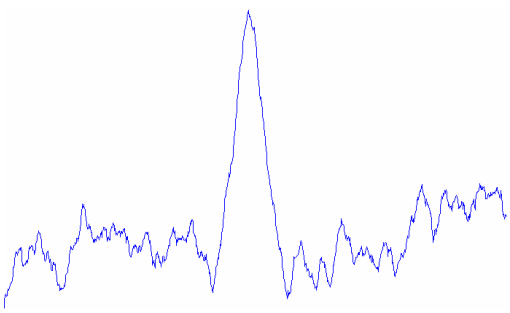


Figure 3. EPR spectrum of Astragaloside I in solution

Results from EPR *ex vivo* study on the levels of ROS production in liver and kidney tissues of tested and control mice are presented on **Figure 4, Figure 5, Figure 6 and Figure 7.** Three hours after treatment, ROS production marked by EPR spectra signals of the studied mice liver and kidney homogenates could be detected. EPR spectra of mice liver and kidney free radicals trapped by PBN exhibited six-lines (**Figure 4 and Figure 5**). The calculated hyperfine splitting constants of the spin adducts registered were: a^N = 13.93 G and a^H = 2.34 G for liver tissues and a^N = 13.94 G and a^H = 2.34 G for kidney tissues, respectively. Based on the values of their splitting constants the spin adducts were identified as PBN/$^{\cdot}OCH_3$ radicals. To confirm that the radicals trapped by PBN originated, only from the livers and kidneys of mice, additional control samples containing IGB-RA-22 or astragaloside I plus DMSO solution of PBN or only DMSO solution of PBN, were also studied but no PBN spin adducts were registered (data not shown). As is seen on **Figure 6** the levels of ROS production (calculated as double integrated plots of EPR spectra of the PBN adducts) in liver homogenates of mice treated by IBG-RA-22 or astragaloside I, were almost twice higher comparing to that of the control mice.

The levels of ROS production in kidney homogenates of the tested and control mice are presented on **Figure 7.** As is seen the ROS production in kidney tissues of mice treated by IBG-RA-22 was slightly lower than that of the controls, while in mice treated by astragaloside I it was slightly higher than that of the control mice.

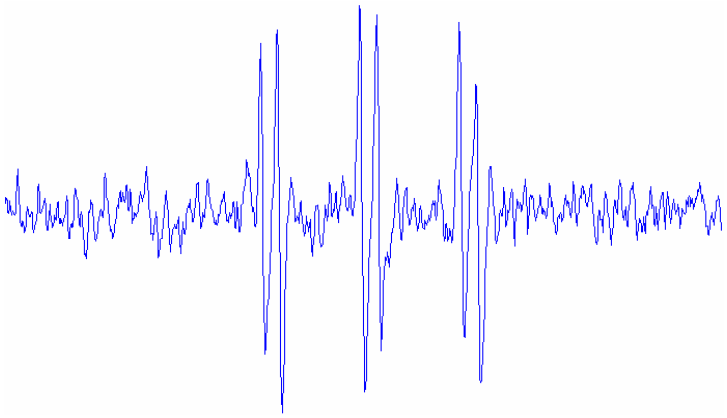


Figure 4. EPR spectrum of the PBN adduct registered in kidney homogenates of mice treated by Astragaloside I

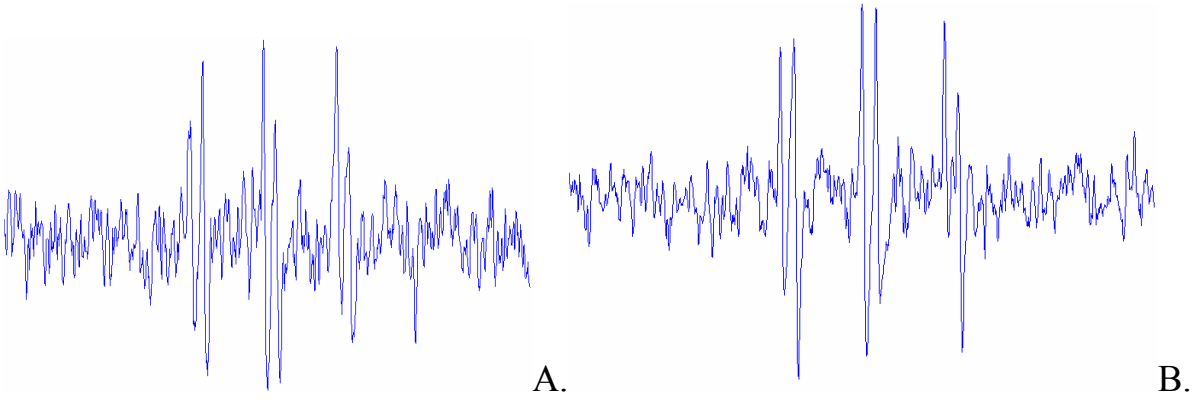


Figure 5. EPR spectrum of the PBN adduct registered in liver (A) and kidney (B) homogenates of mice treated by IBG-RA-22

DISCUSSION

For different flavonoids have been reported presences of semiquinone anion free radicals in their structures (16). Bearings in mind both: the presence of flavonoid structures in IBG-RA-22 and astragaloside I and the calculated values of the g factors of their EPR spectra, we accept that EPR signals were due to semiquinone anion free radicals presenting in

their structures. A number of studies showed that the pharmaceutical effects of rutin can be attributed in part to its ability to scavenge free radicals, exhibiting strong antioxidative and free-radical-scavenging activities. Cao and co-workers reported that despite the potential for electron sharing in its structure at high concentrations, rutin radicals start to interact with other rutin molecules, iron and oxygen to a greater extent than to quench hydroxyl radicals which results in a pro-oxidative effect instead of antioxidant ones (12).

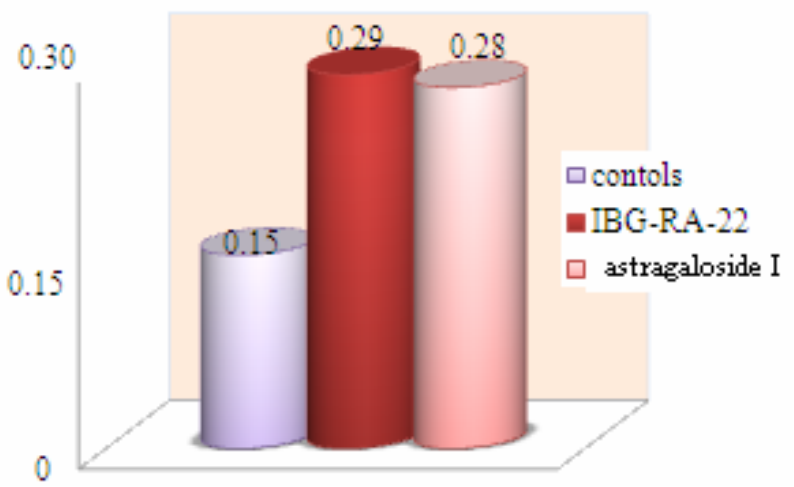


Figure 6. Levels of ROS production expressed by arbitrary units in liver homogenates of control mice and mice treated by IBG-RA-22 or Astragaloside I

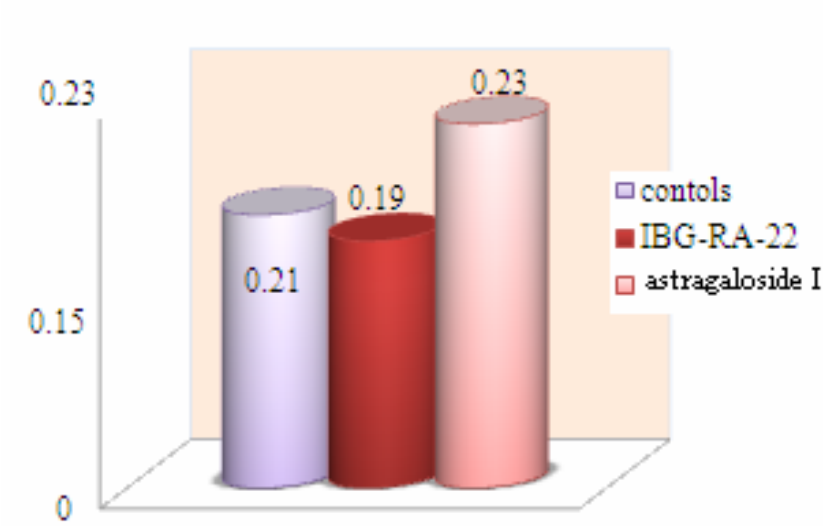


Figure 7. Levels of ROS production expressed by arbitrary units in kidney homogenates of control mice and mice treated by IBG-RA-22 or Astragaloside I

At present, the most common uses of Astragalus root in herbal medicine in the United States are as an immunostimulant to counteract the immune suppression associated with cancer chemotherapy and in the treatment of HIV infection. (17). Yin et al., reported a study on the mutagenicity of 102 raw pharmaceuticals used in Chinese traditional medicine and found that an Astragalus hot water extract given by i.p. injection produced chromosomal aberrations in the bone marrow of mice (18). At this stage there is little or no information regarding adverse reactions with Astragalus uses.

Formerly, it was demonstrated that the reaction of DMSO with $^{\cdot}OH$ produced $^{\cdot}CH_3$ radicals, and that oxidation of $^{\cdot}CH_3$ in aerobic conditions produced $^{\cdot}OCH_3$ radicals (19, 20). Since, for the present *ex vivo* EPR study the mice tissues homogenates were prepared in DMSO solution of PBN at aerobic conditions we accepted

that *in vivo* IBG-RA-22 and astragaloside I can cause generation of $^{\cdot}OH$ radicals which were trapped as final PBN/$^{\cdot}OCH_3$ radical adducts.

Based on these preliminary studies and on some of the above reported studies, we accept that at the experimental conditions used and studied concentrations, IBG-RA-22 and cycloartane saponine astragaloside I can induce ROS species that might be the reason for their pro-oxidative effect in the liver tissues of the treated mice. However, at the same experimental conditions these naturally isolated antioxidants showed as soon as antioxidant properties rather than pro-oxidant ones. We also consider that further more detailed EPR studies are necessary to be carried out in this direction.

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