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Short communication

# Evaluation of the antioxidants activities of four Slovene medicinal plant species by traditional and novel biosensory assays

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ABSTRACT

We investigated the antioxidant activity of methanolic and water extracts of Slovene accessions of four medicinal plant species (*Salvia officinalis*, *Achillea millefolium*, *Origanum vulgare* subsp. *vulgare* and *Gentiana lutea*). Their free radical-scavenging activity against the DPPH· free radical was studied with a spectrophotometric assay, while their biological activity with the help of a laboratory-made biosensor based on immobilized fibroblast cells (assay duration: 3 min). The observed antioxidant activity of the extracts from the four investigated medicinal plant species was dependent on both the solvent used for extraction and the assay method (conventional or biosensor-based). Independently from the assay method and the solvent used for extraction, the lowest scavenging activity was observed in root extracts of *G. lutea*. Treatment of the immobilized cells with the plant extracts resulted in an increase of the cell membrane potential (membrane hyperpolarization), possibly due to the reduction of membrane damage due to oxidation. The novel cell biosensor could be utilized as a rapid, high throughput tool for screening the antioxidant properties of plant-derived compounds.



## 1. Introduction

The scientific interest in identifying novel natural antioxidants for use in foods has considerably increased in recent years, in part because the use of synthetic antioxidants as food additives is limited by specific regulations in various countries, established on the basis of their safety. Intensive research has been carried out either to characterize the antioxidant properties of extracts from several plant materials and/or to isolate and identify the compounds responsible for those activities [1]. It is usually necessary to evaluate the free radical-scavenging activity of herbal extracts, because they represent the common form of usage of the plant in medicine as well as in food technology. Often, differential antioxidant activities of a herbal extract are determined against different free radical species, such as the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH·) the bleaching rate of which is monitored at a characteristic wavelength in the presence of the sample extract [2].

Biosensors are being increasingly used for the determination of antioxidant activities in plant extracts (see [3,4] for review). Essentially, most biosensor-based antioxidant capacity bioassays should provide information on the effect of the assayed sample on a target detector system, such as a particular molecule, a whole cell or even an organism. In this context, they differentiate themselves from biosensor technologies dedicated to the detection of a certain antioxidant or to the measurement of inhibition of the oxidation of a substrate by a certain free radical. Representative of such techniques, particularly for evaluating the antioxidant capacity of phenolic compounds, are the studies of Campanella et al. [5], who developed an electrochemical SOD sensor for the determination of superoxide in both aqueous and non-aqueous extracts of plants and phytotherapeutic products. Other methods employed enzymes such as horseradish peroxidase [6], laccase [7,8] and cytochrome *c* [9] or nucleotides [10] as biorecognition elements, usually coupled with an amperometric technique. On the contrary, very few rapid methods assay the actual response of biological systems to various antioxidants, especially complex mixtures derived from natural sources. Frog melanophores were used in order to detect the antioxidant hormone melatonin [11]. In a different approach, a biosensor was developed based on mammalian cells immobilized in an alginate matrix [12]. Changes in the free radical concentration affecting the cell membrane triggered changes to the cell membrane potential that were measured by appropriate microelectrodes.

In the present study we investigated the antioxidant activity of methanolic and water extracts of Slovene accessions of four medicinal plant species (*Salvia officinalis*, *Achillea millefolium*, *Origanum vulgare* subsp. *vulgare* and *Gentiana lutea*). Their free radical-scavenging activity against DPPH· was studied with a spectrophotometric assay, while their biological activity was determined with the help of a laboratory-made biosensor based on

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fibroblast cells, as described above [12]. In this way, we were able to evaluate both the chemical (DPPH·) and the biological antioxidant activity of the extracts, as well as compare the two methods.

## 2. Materials and methods

### 2.1. Sampling and preparation of herbal samples for biochemical analyses

The following plant material has been used: sage—*Salvia officinalis* L. (Slovene Genebank for medicinal and aromatic plants accession number 13/6—Petrinje, Slovenia), yarrow—*A. millefolium* L. (official herb sample from Slovene pharmacy), wild oregano—*O. vulgare* subsp. *vulgare* L. (Slovene Genebank for medicinal and aromatic plants accession number 9/6—Stari vrh nad Škofja loko, Slovenia), yellow gentian—*G. lutea* L. (Slovene Genebank for medicinal and aromatic plants accession number 84/1—Nanos, Slovenia).

Harvested (cut or erradicated) and cleaned plant material was dried in paper bags at 40 °C. The dry material was grinded in mortar and stored at room temperature in dark, air-free vessels until testing for antioxidative activity.

### 2.2. Preparation of plant extracts

500 mg of grinded plant material were put into 10 mL centrifuge tubes and 5 mL of extraction solvent (methanol, water or n-hexane) was added. Centrifuge tubes with plant material and solvent were put into the Ultrasound bath (Iskra PIO Sonis 4) for 5 min. After that the extracts were centrifugated (centrifuge Tehtnica Centric 322A, 4200 rpm, 5 min). Supernatant was filtered through cellulose acetate filter (0.45 μm membrane filter Sartorius AG) and stored in sample vessels until analysis. Overall extraction procedure was done at room temperature. Extraction of individual plant material was done in four replications.

### 2.3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity

The radical-scavenging activity in our study was evaluated by a standard spectrophotometric assay using the DPPH· radical in a 96-well microplate [13]. A 100 μL of sample extract and 100 μL of 0.04% methanolic DPPH· solution were added to each well. The plate was covered with aluminum foil and left to stand at room temperature for 30 min. The reduction in absorbance was measured at 520 nm with a spectrophometer (MRX Dynex Technologies, VA, USA).

The free radical-scavenging activity was calculated according to the following equation (1) [17]:

$$\%\,\text{inhibition} = \frac{(\text{solvent absorbance} - \text{sample absorbance})}{\text{solvent absorbance}} \times 100 \quad (1)$$

### 2.4. Assay of biological activity with a cell-based biosensor

Cell biosensors were constructed as described previously [18]. Vero fibroblast cells were mixed with 2 mL of 4% (w/v) sodium alginate solution and then the mixture was added drop wise, by means of a 22 G syringe, in 0.8 M $CaCl_2$. Each of the resulting calcium alginate beads had an approximate diameter of 2 mm and contained approximately $40 \times 10^3$ cells.

The biosensor was connected to an electrode made from 80% Cu, electrochemically coated with an Ag/AgCl layer and having a diameter of 0.75 mm. A cell free bead was attached to the reference electrode. Electrodes were connected to the recording device, which comprised the PMD-1608FS A/D card (Measurement Computing, Middleboro, MA). The software responsible for the recording of the signal and processing of data was InstaCal (Measurement Computing).

For each assay, the sensor system, comprising of the beads attached to the working and the reference electrode, was immersed into the sample solution (170 μL), which consisted of 20 μL of plant extract and 150 μL of 50 mM PBS solution (pH 7.4) Control solutions did not contain plant extracts.

The response of each sensor was estimated by recording the absolute maximum change of the sensor potential from 0 s to 180 s (achievement of stable response of the biosensor). The final value of the sensor potential at the end of each assay was considered as the numerical value of each response.

The biosensor was calibrated against increasing $K^+$ concentrations in Kreb's–Hepes solution. The biosensor responded in a linear pattern against increasing $K^+$ concentrations (from 20 to 144 mM), which indicated an increasing hyperpolarization of the membranes of the immobilized Vero cells (analytical results not presented). The biosensor was also calibrated against the synthetic antioxidant butylated hydroxyanisole (BHA), demonstrating a concentration-depended response, which increased by 21 or 41 mV against 0.1 or 1 mM BHA, respectively.

### 2.5. Statistical analyses

Experiments were set-up in a completely randomized design and each experiment was repeated four times. In each application, a set of five biosensors was tested against each individual sample.

## 3. Results and discussion

The antioxidant activity of four Slovene medicinal plant species was evaluated in two ways: chemically, against the stable DPPH· free radical; and biologically, by investigating their effect on the cell membrane function. From previous studies [12,14] it is known that the biosensor measures changes of the cell membrane potential, due to changes in the electrolyte flux through the membrane. These can be ascribed to various factors, including membrane lipid oxidation, receptor/ion channel activation and cell toxicity effects. It has also been shown that, upon immobilization in a calcium alginate gel, fibroblast cells experience significant oxidative stress, including the oxidation of cell membranes and changes in the flux of calcium ions [15]. Therefore, we assumed that any antioxidant activity of the investigated plant extracts would lead to an avoidance or reduction of the oxidation of the membrane of the immobilized cells, possibly leading to increased membrane integrity and, subsequently, membrane potential (membrane hyperpolarization).

The observed antioxidant activity of the extracts from the four investigated medicinal plant species depended on both the solvent used for extraction and the evaluation method (conventional or biosensor-based). Water extracts from the leaves of *A. millefolium* and *S. officinalis* demonstrated the highest free DPPH· scavenging activity (determined with the spectrophotometric assay), while the lowest activity was observed in root extracts of *G. lutea* (Fig. 1A). A roughly similar pattern was observed in the effect of plant extracts on the function of fibroblast cell membrane, although there were differences between the two methods: using the biosensor assay, leaf extracts from *A. millefolium*, *O. vulgare* and *G. lutea* demonstrated a considerably lower activity than *S. officinalis*. Therefore, no absolute correlation was observed between the results derived by the two different methods.

Methanolic extracts of *O. vulgare*, *A. millefolium* and *S. officinalis* exhibited an essentially equal free DPPH· scavenging activity,

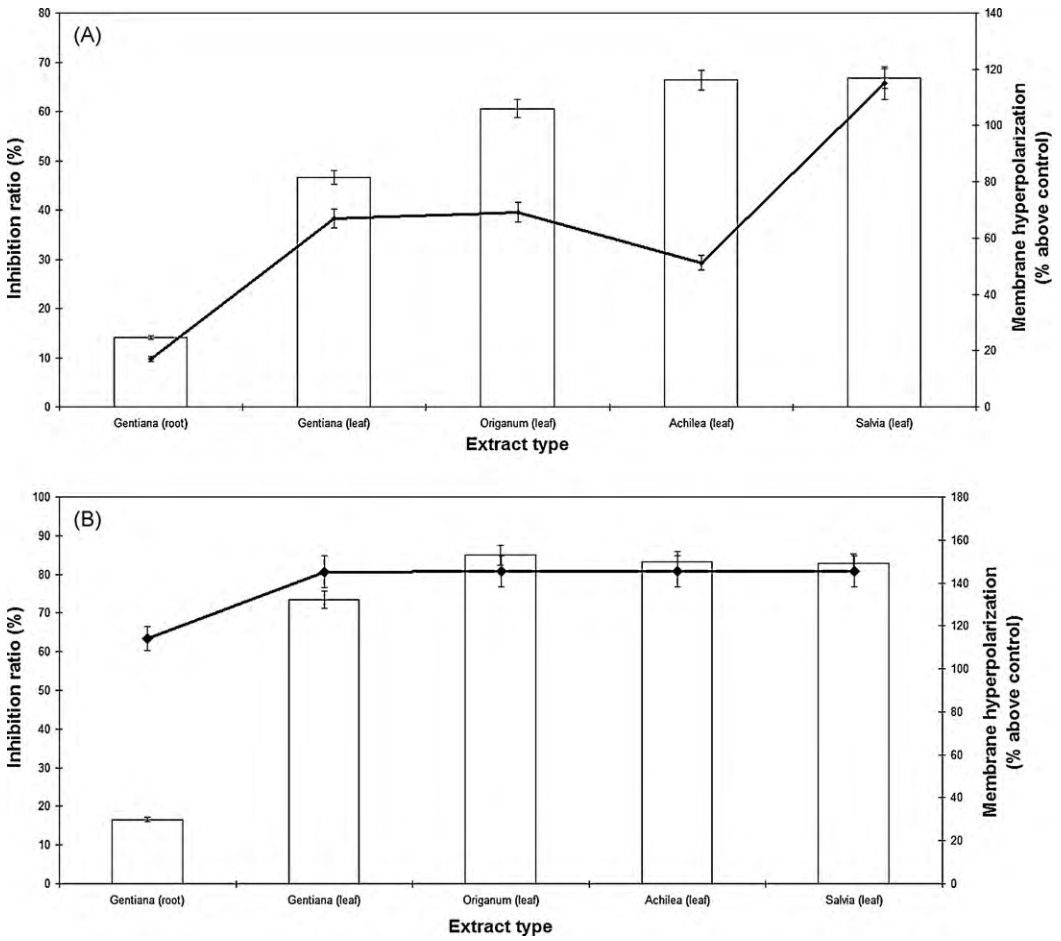


**Fig. 1.** Comparison of antioxidative activities expressed as capability of scavenging of free DPPH· (columns) or protecting the cell membrane against oxidation, expressed as the increase of the cell membrane potential relative to extract-free control (line), of (A) *water* extracts and (B) *methanolic* extracts of yellow gentian (*Gentiana lutea*) roots, yellow gentian (*G. lutea*) leaves, sage (*Salvia officinalis*) leaves, yarrow (*Achillea millefolium*) leaves and oregano (*Origanum vulgare* subsp. *vulgare*) leaves. Error bars represent standard errors of the average value of all replications with each sample.

which was the highest among the investigated species (Fig. 1B). The same pattern was observed using the biosensor method, with the exception that leaf-derived extracts of *G. lutea* produced an essentially similar effect to the other three plant species. Independently from the determination method, the lowest scavenging activity was observed in root extracts of *G. lutea*. This is in agreement with the previously reported differences in free radical-scavenging properties between leaves and roots of *G. lutea* [2].

In general, methanolic plant extracts demonstrated a higher DPPH· scavenging activity than water extracts. It was not possible to compare the two methods in an absolute manner, since the DPPH· assay is a chemical assay for antioxidant activity and does not depend on any cellular function, as in the case of the cell-based sensor. In addition, plant extracts may have affected cell function though additional, non-antioxidant effects, such as terpene-induced toxicity or interaction with membrane-based receptors [16]. This does not limit the applicability of the biosensor method, since it is desired to assay for total biological effects of the plant extracts, especially those associated with the preservation of cell membrane function; however it is not possible to determine individual antioxidants or antioxidant properties (e.g. against superoxide).

The antioxidant activities exhibited by methanolic extracts may be due to the presence of phenolic substances, such as rosmarinic acid, other phenolic acids and flavonoids or diterpenes (*S. officinalis*, *O. vulgare*) [17,18], sesquiterpenes, sesquiterpene lactones and triterpenes (*A. millefolium*) [19] and secoiridoids, xanthones and flavonoids (*G. lutea*) [2]. Water extracts, on the other hand, contain mainly polar compounds with antioxidant properties, such as flavonoids and glycosides [20].

The present study is a preliminary investigation of the potential use of the novel biosensor as a rapid screening tool of the total antioxidant effect of both water- and organic plant extracts. The sensor is less prone to errors due to the optical properties of the extracts, a considerable disadvantage of colorimetric/spectrophotometric assays [3,4,12]. An additional advantage is the use of very small sample volumes. The biosensor could be utilized as a highly reproducible, rapid and high throughput screening tool to the increasing interest of modern food science to the antioxidant properties of plant-derived compounds and additives. Further experiments are necessary in order to determine the selectivity of the assay, in particular the possible interference by non-antioxidant constituents of the plant extracts. One way to screen-out such effects is to assay for the antioxidant activity against a specific free radical (e.g. superoxide anion), the elimination of which will not be due to non-antioxidant plant moieties. In other words, the sensor could measure the protection of the immobilized cell membrane from the free radical(s) added to the assay solution, therefore complementing the information provided by other analytical methods.

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