Antimicrobial and antimutagenic activities of extracts from different organs of *Echinacea angustifolia* DC (*Asteracea*)

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Summary
Some *Echinacea* species are well known for their beneficial effect on human health and immune system. In Europe, the most selling products are juice preparations or alcoholic tinctures derived from either the aerial or the underground parts of *Echinacea purpurea*, and from the roots of *E. angustifolia* or *E. pallida*. In our study, biological activity of ethanol extracts from different organs of *E. angustifolia* (herba, radix and rhizome) was compared. The best antimicrobial and antimutagenic activities were from the radix extract. On the other hand, only this extract increased frequency of mutations leading to ciprofloxacin resistance in *Salmonella* Typhimurium in all tested concentrations. For that reason, simultaneous use of *Echinacea* preparations and fluoroquinolone antibiotics, such as ciprofloxacin, should not be recommended.

Keywords
antimutagenicity; resistance; *Echinacea angustifolia*; antimicrobial activity

The treatment of microbial diseases still remains an important and challenging worldwide problem. Even though a large spectrum of antibiotics and chemotherapeutics are available for the medical treatment, the increasing antibiotic resistance to these compounds is the reason for searching new natural antimicrobially active products [1]. For centuries, plants have been a valuable source of natural products for prevention and treatment of many diseases. In particular in the last decade, we can see more intensive studies of natural therapies and gradually increased use of plant compounds for pharmaceutical purposes. The genus *Echinacea* is used most frequently for preventing or treating many bacterial, fungal and viral infections [2]. Since *Echinacea angustifolia, E. pallida* and *E. purpurea* are used commercially, these three species have received the overwhelming majority of scientific attention and much data exist regarding their genetics, phytochemistry, immunomodulatory activity and efficacy in clinical settings [3]. However, the mechanism of antimicrobial activity of *Echinacea* extracts remains unclear. Some studies reported that the antifungal activity is connected to the damage of fungal cell walls, which may explain the utility of this phytomedicine in mycosis treatment. Several classes of purported medicinal compounds have been identified from this genus, but current data suggest that no single molecule or class of molecules is responsible for activities of *Echinacea* [3]. Known bioactive molecules include alkamides, cichoric acid, caffeic acid derivatives and glycoproteins/polysaccharides. Bioavailability studies suggest that these most likely account for the majority of the observed immunomodulatory effects [4, 5]. It is evident that most actions are directed towards the stimulation of the non-specific immune system. Therefore, *Echinacea* seems to be effective not in a specific way but more generally in the enhancement of the unspecific first line defense system of our body. The imunomodulation by *Echinacea* extracts is

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achieved by multiple mechanisms e.g. induction of cytokines by macrophage, stimulation of fibroblasts, monocytes, natural killer cells activity and fagocytal activation. The stimulation of fagocytosis was activated by polysaccharides, which enhance the interleukin-10 levels and interferon-α. Some studies suggest that the healing power of *Echinacea* extracts consists in the activation of immune system and that the extracts do not have the primary antimicrobial or antiviral activity [6, 7]. In our study, we have investigated the antimicrobial and antimutagenic activity of ethanolic extracts isolated from different organs (rhizome, radix and herba) of *Echinacea angustifolia* grown in Slovakia. The positive or negative effects of studied extracts on arising of direct mutations leading to antibiotic resistance was also evaluated.

MATERIALS AND METHODS

Plant extracts preparation

The plant sort of *Echinacea angustifolia* DC (*Asteracea*) grown under multifactor cultivation condition was taken into the experiments. Both underground and above-ground parts of the plant sample, i.e. radix, herba and rhizome were examined. Plant samples were collected from the locality of its cultivation (Nitra, Slovakia). Extracts were prepared according to European Pharmacopoeia [8].

Bacterial and fungal strains

Mycobacterium smegmatis (Collection of Microorganisms of Department of Microbiology, Faculty of Natural Science, Comenius University, Bratislava, Slovakia), *Staphylococcus aureus* CCM 3953, *Staphylococcus epidermidis* CCM 4418, *Escherichia coli* CCM 3988 and *Salmonella* Typhimurium CCM 4763 (Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic), *Alternaria alternata, Aspergillus fumigatus* (Collection of Microorganisms of Department of Biochemistry and Microbiology, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovakia), *Microsporum gypseum, Trichophyton terrestre* (Laboratory of Medical Mycology, Postgraduate Medical Institute, Bratislava, Slovakia) were used for testing antibacterial and antifungal activities. Bacterial strains *Salmonella* Typhimurium TA 98 (CCM 3811) and TA100 (CCM 3812) (obtained from Czech Collection of Microorganisms) were used in the bacterial mutagenicity test. Subcultures were prepared separately in Petri dishes containing appropriate agar medium (Mueller-Hinton broth (MHB) for bacteria) Sabouraud agar (SGA) for fungi) and incubated at 30 °C for 48 h (bacteria) or at 25 °C for 96 h (filamentous fungi).

Determination of antibacterial and antifungal activities

The antibacterial activity of *Echinacea* extracts was evaluated by a microsuspension assay in 96-well microplates [9] with some modifications. MHB was inoculated with an overnight culture of bacteria (final cells density in MHB was $10^4 \cdot ml^{-1}$). All experiments were carried out in three groups. A volume of 198 ml of inoculated MHB was added to 2 μl of *Echinacea* extract at a final concentration 0.3–10 $mg \cdot ml^{-1}$. Prepared microplates were incubated with shaking at 37 °C for 8 h. The bacterial growth was quantified spectrophotometricaly by ELx808 Absorbance Microplate Reader (BioTek Instruments, Winooski, Vermont, USA) at 630 nm, measured until confluent growth. The antimicrobial effect was characterized by the IC_{50} (inhibitory concentration of a derivative which inhibits growth by 50%) and *MIC* (minimal inhibitory concentration of a derivative which inhibits microbial growth by 100%) values, which were read from toxicity curves. Ampicillin ($3 mg \cdot l^{-1}$) and gentamicin ($1 mg \cdot ml^{-1}$) were applied as control standards for antibacterial efficacy. The efficacy of prepared extracts on filamentous fungi was observed by macro-dilution technique [10] on solidified agar medium SGA during static cultivation. A volume of 5 ml of SGA was added to 50 ml of *Echinacea* extract. Prepared agar plates with *Echinacea* extracts (final concentration ranging from 0.3 $mg \cdot ml^{-1}$ to 10 $mg \cdot ml^{-1}$) were inoculated with a conidial suspension in 0.01% Tween 80 (Biolife, Milan, Italy), 1500 conidia per plate. The fungal growth was continuously measured as a diameter of the growing colony. Finally, the antimicrobial activity was characterized by *MIC* values. Amphotericin B ($2 mg \cdot l^{-1}$) was used as a control standard.

Mutagenicity assessment

Assessment of mutagenicity or antimutagenicity was performed using classical plate incorporation method [11] without metabolic activation using *Salmonella* Typhimurium TA98 and TA100. A positive response was defined as a reproducible, two-fold increase of revertants with dose-response relationship. As positive mutagens, 3-(5-nitro-2-furyl) acrylic acid (NFAA, Slovakofarma, Hlohovec, Slovakia) and 2-nitrofluorene (NF, Sigma-Aldrich, Steinheim, Germany) were used. The results from Ames test represent the mean of three separate experiments, which were statistically evaluated using the Student's *t*-test.

Determination of mutation frequencies leading to antibiotic resistance

Effects of extracts on the development of ciprofloxacin resistance were studied by a method described in our previous work [12]. The frequency of mutations leading to ciprofloxacin resistance (resistance index *RI*) was expressed as a mean number of resistant cells divided by the total number of viable cells per culture. Data shown in this study represent the mean of three independent experiments; each experiment was made in five replicate determinations and statistically evaluated by Student's *t*-test.

RESULTS AND DISCUSSION

Radix extract had the highest antimicrobial activity both against bacteria and filamentous fungi. Radix extract moderated only the growth of *Staph. aureus, Staph. epidermidis* and *E. coli* (Fig. 1A, 1B, 1C). Extracts from radix and rhizome inhibited the growth of *M. smegmatis* with a bacteriostatic effect on growth (Fig. 1D). Diluted extracts had only a moderate effect on the growth of *M. smegmatis*. No growth inhibition of all tested bacteria was observed at the extract from herba. In the presence of this extract, stimulation of growth of all tested bacteria was observed, compared with the control. All extracts had lower antimicrobial activity than the standard clinically relevant antimicrobial agents. Ampicillin at a concentration of 3 mg·l^{-1} inhibited the growth of *Staph. aureus* and *Staph. epidermidis. E. coli* was 100% inhibited by gentamicin at a concentration of 1 mg·ml^{-1}. Finally, 100% growth inhibition of *M. smegmatis* was observed in the presence of streptomycine at 1 mg·ml^{-1}.

Numerous clinical trials have been carried out on *Echinacea* preparations. It appears that the extracts shorten the duration and severity of up-

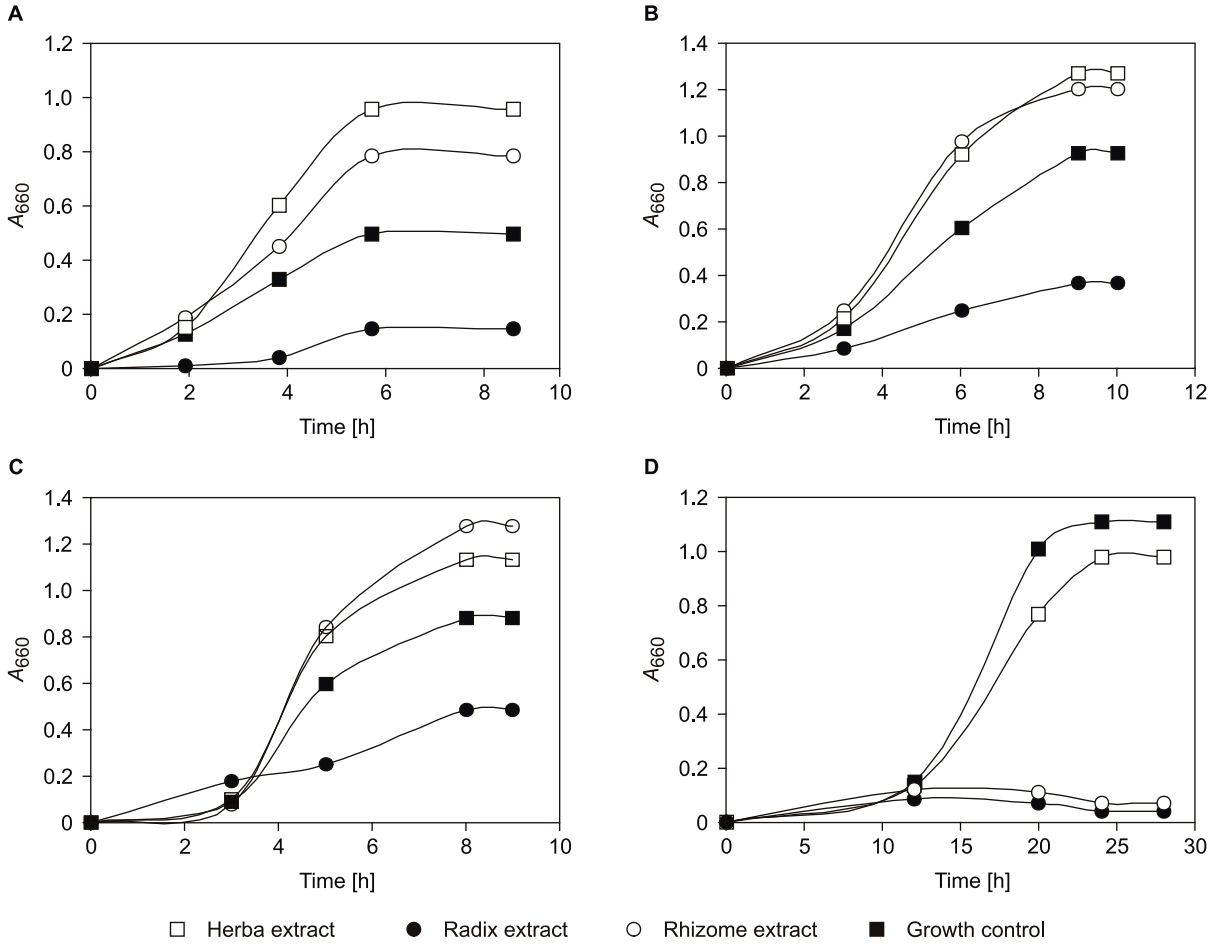

Fig. 1. Growth inhibition of bacteria by herbal extracts.

A – *E. coli*; B – *Staph. aureus;* C – *Staph. epidermidis;* D – *Mycobacterium smegmatis*.
Herba extract concentration was 10 mg·ml^{-1}; radix extract with *Mycobacterium smegmatis* (10 mg·ml^{-1}), rhizome extract with *Staph. aureus* (10 mg·ml^{-1}).

per respiratory infections when given as soon as symptoms become evident. It seems that *Echinacea* extracts work through modulation of immune mechanisms. It is clear that no single agent or class of agents is solely responsible for all of the imunomodulation effects [7, 13]. Instead, *Echinacea*-derived alkylamides, caffeic acid derivatives (cichoric, chlorogenic and cafeolytartaric acids) compounds, glycoproteins (many diverse forms) and polysaccharides (arabinogalactans, fructofuranosides and heteroxylans) all appear to be active and contributory to immunostimulatory (phagocyte-enhancing) effects [13, 14]. Therefore, the antimicrobial in vitro effect of tested extracts is not so evident and significant.

The *Echinacea* extracts from herba and rhizome had no effect on the growth of tested filamentous fungi. MERALLI et al. [15] studied and compared root extracts from three species of *Echinacea* (*E. purpurea, E. pallida* var. angustifolia *E. pallida* var. pallida). Their results showed that radix extract from *E. angustifolia* had a significant antifungal activity. Our results confirm this fact, as the radix extract did significantly affect the growth of dermatophytes *M. gypseum* and *T. terrestre*. The fungal growth was inhibited (100%) by 1 mg·ml^{-1} of this extract and showed a lethal effect on the spores (Tab. 1). Extracts inhibited the growth of *A. nidulans* weakly, only the effect of the radix extract could be noticed (Tab. 1). Our results indicate that only the root extract of *E. angustifolia* showed antibacterial and antifungal activity in vitro. These findings agree with the previous information that alcoholic tinctures of *E. pallida* and *E. purpurea* roots are beneficial and useful as adjutants in the therapy of microbial infection [13]. The antifungal activity of the radix extract was significantly lower compared to an antifungal standard amphotericin B (Tab. 1).

Concern about the safety of *Echinacea* extract and the potential hazard of its application has emerged when CAILLET et al. [16] indicated that a commercial *Echinacea* product (Echinaforce, A. Vogel/Bioforce, Roggwil, Switzerland) had mutagenic effect on *S.* Typhimurium strain TA 1535/pSK1002. Mutagenic or antimutagenic effect was determined by a plate-incorporation test with a tester strain *Salmonella* Typhimurium TA 98. No genotoxic effect was detected in any *Echinacea* extract (Fig. 2). Ethanol and/or methanol at different concentrations in water are used to extract phenols from plant materials [17]. In our previous study [18] we indicated that phenolic acids possess antimutagenic effect. According to this fact, antimutagenic potential of the extracts was examined using positive mutagens 2-nitrofluorene (NF) and 3-(5-nitro-2-furyl) acrylic acid (NFAA). Doses of 10 μg per plate of NFAA and 1 μg per plate of NF were chosen for the antimutagenicity studies since these doses were not toxic and induced revertants in *Salmonella* Typhimurium TA98. In selecting the concentration of the tested extracts, we used the amounts where cell viability was found to be above 90%. Herba extract (Fig. 2A) showed no inhibitory effect on the mutagenicity neither of NFAA nor of NF. The positive control of the mutagen in each case was considered as 100% mutagenicity. Extract from rhizome was the only successful in inhibiting the mutagenicity of NFAA by more than 50% at the basic

Tab. 1. Fungal growth in the presence of ethanolic *Echinacea* extracts.

Echinacea extracts [mg·ml^{-1}]		*Aspergillus fumigatus*	*Trichophyton terrestre*	*Microsporum gypseum*
Herbal extract	10	100%	100%	100%
	1	100%	100%	100%
	0.5	100%	100%	100%
	0.3	100%	100%	100%
Radix extract	10	0%	0%	0%
	1	50%	0%	0%
	0.5	80%	50%	10%
	0.3	100%	100%	100%
Rhizome extract	10	100%	100%	100%
	1	100%	100%	100%
	0.5	100%	100%	100%
	0.3	100%	100%	100%
Amphotericin B	2×10^{-3}	0%	0%	0%

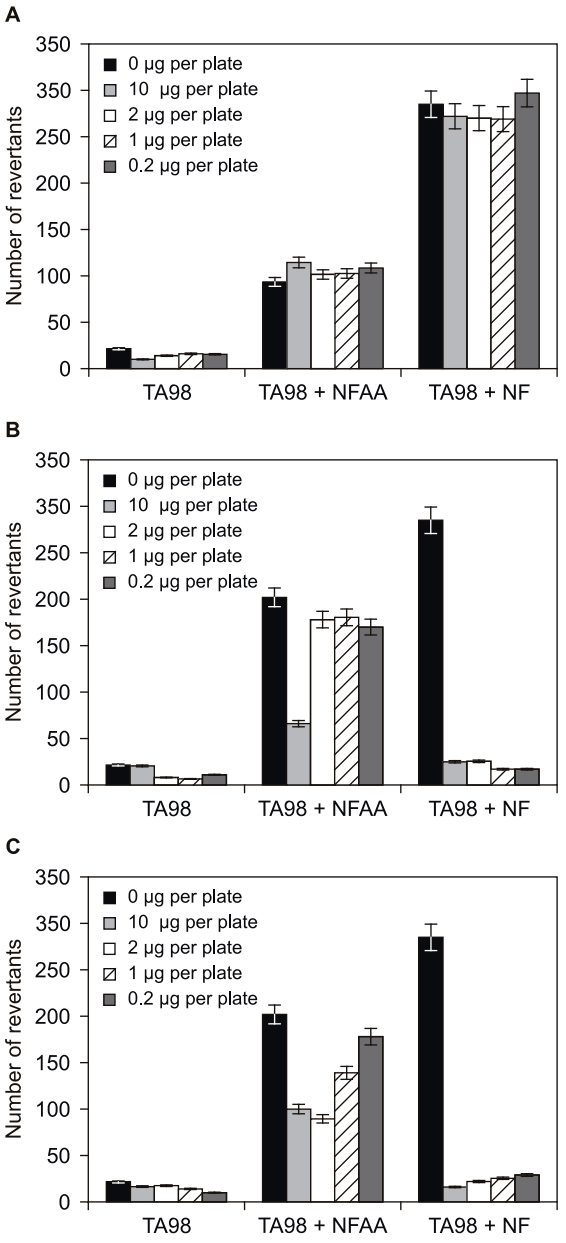

Fig. 2. Effect of ethanolic extracts from *Echinacea angustifolia* on the number of revertants induced by NFAA and NF in *S.* Typhimurium TA98.

A – herba extract, B – radix extract, C – rhizome extract. NFAA – 10 mg per plate, NF – 1 mg per plate.

(10 mg per plate) and 5-fold diluted concentration (2 mg per plate), Fig. 2C. With a decreasing concentration of the extract, this activity disappeared. Although the total phenols and caffeic acid derivatives contents in different plant parts are in the descending order: flowers > leaves > stems > roots [19], our results showed the best antimutagenic in root extract. The effect of NF was reduced almost to the level of spontaneous revertants by the radix (Fig. 2B) as well as by the rhizome extracts (Fig. 2C) in all studied concentrations. Some studies [20, 21] explain that this antimutagenic effect could be connected with the antioxidant activity of these compounds.

Antibiotic resistance represents a huge health and socio-economic problem. A new approach consists in the prevention of the development of mutations leading to antibiotic resistance using antimutagenic agents. Whereas tested extracts from *E. angustifolia* have shown antimutagenic effect consequently their effect on the development of spontaneous mutations leading to ciprofloxacin resistance in *S.* Typhimurium was studied. All undiluted extracts (10 mg per plate) increased the mutation frequency, while the radix extract acted like this in all tested concentrations (Fig. 3). This fact is of concern since the majority of commercial *Echinacea* products are root extracts. In our work [12] we showed that the enhanced content of some phenolic acids such gallic, caffeic, cinnamic or ferrulic, increased the mutation frequency leading to ciprofloxacin resistance in *S.* Typhimurium. So this negative effect of the tested extracts on antibiotic resistance could be possibly connected with the enhanced contents of the mentioned phenolics in this part of herb. However, herba and rhizome extract in lower concentrations (2 mg per plate and 0.2 mg per plate) reduced the resistance index even to a half value of the spontaneous mutation frequency. This result is a little bit surprising but is difficult to explain at the current status of knowledge, as little is known about the relationship between the antibiotic and the plant extract, and data on the in vivo activity of the extract are not available.

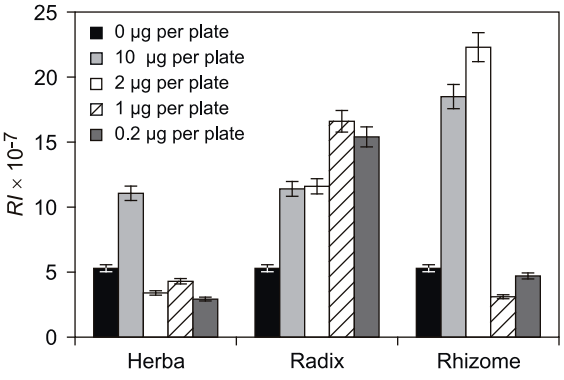

Fig. 3. Effect of ethanolic extracts from *Echinacea angustifolia* on the development of spontaneous mutations leading to ciprofloxacin resistance in *S.* Typhimurium.

CONCLUSIONS

Extracts of *Echinacea*, "alcoholic tinctures" from roots, are the widely used herbal medicines for their immunomodulative effect. In this work, we have shown the difference in the biological activity of three different extracts from *E. angustifolia*. The highest antimicrobial and antimutagenic effects were sustained in case of the radix extract. A contrario sensu, this extract increased the frequency of mutations leading to ciprofloxacin resistance. For that reason, simultaneous use of *Echinacea* preparations and fluoroquinolone antibiotics, such as ciprofloxacin, should not be recommended.

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Received 13 July 2012; revised 1 October 2012; accepted 4 October 2012.