Pharmaceutical Biology

http://informahealthcare.com/phb
ISSN 1388-0209 print/ISSN 1744-5116 online
Editor-in-Chief: John M. Pezzuto
Pharm Biol, Early Online: 1–4
 DOI: 10.3109/13880209.2014.950386

informa
healthcare

SHORT COMMUNICATION

Antimutagenic and antioxidant activity of a protein fraction from aerial parts of *Urtica dioica*

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Abstract

Context: *Urtica dioica* L. (Urticaceae), stinging nettle, has been employed as a folklore remedy for a wide spectrum of ailments, including urinary disorders, prostatic hyperplasia, and liver diseases. It has been also used traditionally for cancer treatment.

Object: To evaluate the potential chemopreventive properties of a protein fraction from the aerial part of *Urtica dioica* (namely $UDHL_{30}$).

Materials and methods: $UDHL_{30}$ has been tested for the antimutagenic activity in bacteria (50–800 μg/plate; Ames test by the preincubation method) and for the cytotoxicity on human hepatoma HepG2 cells (0.06–2 mg/mL; 24 and 48 h incubation). Moreover, the antioxidant activity of $UDHL_{30}$ (0.1–1200 μg/mL; ABTS and superoxide-radical scavenger assays) was evaluated as potential protective mechanisms.

Results: $UDHL_{30}$ was not cytotoxic on HepG2 cells up to 2 mg/mL; conversely, it exhibited a strong antimutagenic activity against the mutagen 2-aminoanthracene (2AA) in all strains tested (maximum inhibition of 56, 78, and 61% in TA98, TA100, and WP2uvrA strains, respectively, at 800 μg/plate). In addition, a remarkable scavenging activity against ABTS radical and superoxide anion (IC_{50} values of 19.9 ± 1.0 μg/mL and 75.3 ± 0.9 μg/mL, respectively) was produced.

Discussion and conclusions: $UDHL_{30}$ possesses antimutagenic and radical scavenging properties. Being 2AA a pro-carcinogenic agent, we hypothesize that the antimutagenicity of $UDHL_{30}$ can be due to the inhibition of CYP450-isoenzymes, involved in the mutagen bioactivation. The radical scavenger ability could contribute to 2AA-antimutagenicity. These data encourage further studies in order to better define the potential usefulness of $UDHL_{30}$ in chemoprevention.

Keywords

Antimutagenicity, chemoprevention, stinging nettle

History

Received 8 January 2014
Revised 11 June 2014
Accepted 27 July 2014
Published online 4 December 2014

Introduction

Chemoprevention is defined as a strategy for preventing, suppressing, or reversing the carcinogenic progression by using natural, synthetic, or biologic chemical agents. Many phytochemicals have received a special attention as new promising chemopreventive agents. Some of them were found to act as antimutagenic agents during initiation, by preventing carcinogen induced DNA-injuries (e.g., by inhibiting mutagen uptake, by blocking the pro-carcinogen bioactivation, by protecting cells from mutagen-induced oxidative stress, etc.), or by promoting DNA-damage repair (Kada & Shimoi, 1987). Moreover, blocking the proliferation of transformed cells has been found to be a possible mechanism for inhibiting cancer development and progression (Sun and Yang, 2010). Curcumin, epigallocatechin gallate, resveratrol, and sulforaphane are examples of natural compounds possessing interesting chemopreventive properties (Shu et al., 2010).

Urtica dioica L. (Urticaceae), stinging nettle, is a very common plant used, since the ancient time, as a traditional remedy for many complaints, including urinary disorders, prostatic hyperplasia, diabetes mellitus, and liver diseases (Chrubasik et al., 2007). Moreover, leaves and seeds are widely used in folk medicine as herbal medicines by oncologic patients (Gözüm et al., 2007; Kav et al., 2008). Lignans, phenolic compounds, and sterols have been highlighted to be mainly involved in the antioxidant, antinflammatory, and protective effects of the plant. In addition, a rare agglutinin isolated from *U. dioica* rhizhomes (M_w 8.5 kDa) has been shown to possess *in vitro* antiproliferative properties (Chrubasik et al., 2007). In line with the traditional use of stinging nettle, in the present study, a protein fraction (namely $UDHL_{30}$), isolated from aerial parts of *U. dioica* was evaluated for the potential chemopreventive properties. Initially, the antimutagenicity of $UDHL_{30}$, in terms of ability to inhibit the genotoxic effects induced by known mutagens,

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was evaluated in the bacterial reverse mutation assay (Ames test). An exogenous metabolic activator was also added to the system, in order to highlight a possible ability of the fraction to block the mutagen cytochrome P450-based bioactivation. Furthermore, taking into account that different extracts of *U. dioica* exhibited hepatoprotective and anticancer effects (Özkol et al., 2012), the ability of $UDHL_{30}$ to inhibit the growth of human hepatocellular carcinoma cells (HepG2) was tested. Finally, the antioxidant activity, particularly the ability of neutralizing both synthetic and oxygen reactive species (ROS), was studied as a potential protective mechanism.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO). S9 fraction was obtained from Moltox (Molecular Toxicology, Boone, NC) and prepared as a mixture according to Di Sotto et al. (2013).

Plant material

Urtica dioica dried aerial parts (origin: Lublin region, Poland; collection stage: ripening; harvest time: August 2011) were purchased from the manufacturer Herbar Sp. z o.o. (Milejow, Poland). The specimen was taxonomically identified at the Department of Pharmacognosy from Lithuanian University of Health Sciences. A voucher specimen (no. 3-77-1) was deposited in the Herbarium of Lithuanian University of Health Sciences.

$UDHL_{30}$ protein fraction

Extraction of the protein fraction from dried aerial parts of *U. dioica* was carried out by separation with ammonium sulfate precipitation and detection by isoelectric focusing in polyacrylamide gel (Savickiene et al., 2012). The method is widely used for the initial separation of crude extracts because it is easy, inexpensive, and reveals proteins on the basis of their solubility. $UDHL_{30}$ fraction contained a protein amount of 0.41 ± 0.02 mg/g (referred to raw material) and two peptides, whose molecular weights were 30 kDA and 120 kDA, respectively. The first peptide (about 0.1%) has been hypothesized to be a lectin, since lectin size is from 29 kDA to 34 kDa; in support, it also possessed specific hemagglutination activity (0.2 ± 0.02 mg/mg protein) (Savickiene et al., 2012).

Antimutagenic activity

The antimutagenicity of $UDHL_{30}$ (50–800 μg/plate; 1:2 dilution factor) was evaluated in the bacterial reverse mutation assay (Ames test), according to Di Sotto et al. (2013). Various strains, including *Salmonella typhimurium* TA98 and TA100, and *Escherichia coli* WP2uvrA (Research Toxicological Centre, Pomezia, Italy), were used. To perform the experiments, strains were cultivated up to a concentration of 1×10^9 bacteria/mL (Di Sotto et al., 2013). The exogenous metabolic activator S9 was prepared as a mixture according to Di Sotto et al. (2013). The antimutagenicity of $UDHL_{30}$ was evaluated against the mutagens 2-nitrofluorene (2NF; 6 μg/plate for TA98 without S9), sodium azide (SA; 5 μg/plate for TA100 without S9), methyl methanesulfonate (MMS; 1700 μg/plate for WP2uvrA without S9), and 2-aminoanthracene (2AA; 5 μg/plate for TA98 and TA100 and 25 μg/plate for WP2uvrA with S9). Mutagen concentrations were chosen from the linear part of the concentration–response curve of mutagens, as they induced a submaximal mutagenic effect (about 70%). Control plates containing the mutagen (100% of mutagenic activity) or the vehicle alone (DMSO 2% v/v; lack of mutagenic activity) were also included. Experiments were repeated at least twice and each concentration was tested in triplicate. Percentage inhibition of the mutagen-induced revertant colonies was calculated as previously described (Di Sotto et al., 2013). According to Di Sotto et al. (2013), the antimutagenicity was reported to be strong (inhibition higher than 40%), moderate (inhibition in the range of 25–40%), and weak or null (inhibition lower than 25%).

Antiproliferative activity

HepG2 cells (American Type Culture Collection, Milan, Italy) were grown under standard conditions (37°C and 5% CO_2) in DMEM-Ham's F12 supplemented with 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in 75 cm^2 flasks, and subcultured every 4 d, renewing growth medium every 2–3 d. Different concentrations of $UDHL_{30}$ (0.06–2 mg/mL, 1:2 dilution factor) were tested for mitochondrial cytotoxicity by the MTT tetrazolium salt colorimetric assay (Vitalone et al., 2011). A vehicle control (corresponding to 100% cell viability) and a standard cytotoxic agent (doxorubicin, 3 μg/mL) were also included.

Antioxidant activity

Antioxidant activity of $UDHL_{30}$ (0.1–1200 μg/mL; from 1:2 to 1:5 dilution factor) was evaluated in term of ABTS and superoxide-radical scavenger activity, according to Di Sotto et al. (2013). The experiments were performed in 96-multiwell plates and the absorbance was determined by a microplate reader (Epoch Microplate Spectrophotometer, Biotek, Shoreline, WA). Each concentration was tested in triplicate and each experiment was repeated at least twice. For each test, negative (vehicle; lack of antioxidant activity) and positive controls (standard antioxidant agent trolox) were included. The efficacy of the radical scavenger activity was expressed as Trolox equivalent antioxidant capacity (TEAC). At this purpose, a calibration curve of TEAC was built for both assays, and analyzed by linear regression.

Statistical analysis

All values were expressed as mean ± SE. The one-way analysis of variance (one-way ANOVA), followed by Dunnett's Multiple Comparison Post Test, was used to verify the significance of a positive response; $p < 0.05$ was considered statistically significant. Statistical analysis was performed with GraphPad Prism™ 4.0 software (GraphPad Software, Inc., San Diego, CA). The concentration–response curves were constructed using the ''Hill equation'': $E = E_{max}/[1 + (10^{LogEC50}/A)^{HillSlope}]$, where E is the effect at a given concentration of agonist, E_{max} is the maximal effect, EC_{50} is the concentration that produces a 50% response, A is the

Table 1. Effect of the protein fraction from *Urtica dioica* herb ($UDHL_{30}$) on the number of revertant colonies mutagen-induced in *Salmonella typhimurium* TA98 and TA100 and in *Escherichia coli* WP2*uvr*A in the absence of the metabolic activator S9 (mean ± SEM, $n = 6$).

		Number of revertant colonies		
	[μg/plate]	TA98 -S9	TA100 -S9	WP2*uvr*A -S9
$UDHL_{30}$	50	117.8 ± 1.5	617.6 ± 10.9	135.5 ± 5.7
	100	119.7 ± 2.9	619.8 ± 18.2	131.1 ± 1.9
	200	118.4 ± 3.2	529.8 ± 6.5	133.7 ± 3.9
	400	119.4 ± 1.1	531.4 ± 9.5	148.3 ± 5.2
	800	119.1 ± 1.2	576.0 ± 9.2	139.3 ± 8.6
Vehicle		44.7 ± 1.3[a§]	123.0 ± 4.3[b§]	7.3 ± 1.1[b§]
Mutagen		149.4 ± 2.3[c]	683.9 ± 24.2[d]	148.7 ± 2.2[e]

[a]DMSO 50 μL.
[b]H_2O 25 μL + DMSO 25 μL.
[c]2-Nitrofluorene (6 μg/plate).
[d]Sodium azide (5 μg/plate).
[e]Methyl methane sulfonate (1700 μg/plate).
[§]A significant difference between mutagen and vehicle ($p < 0.01$; Anova + Dunnett's Multiple Comparison Post Test).

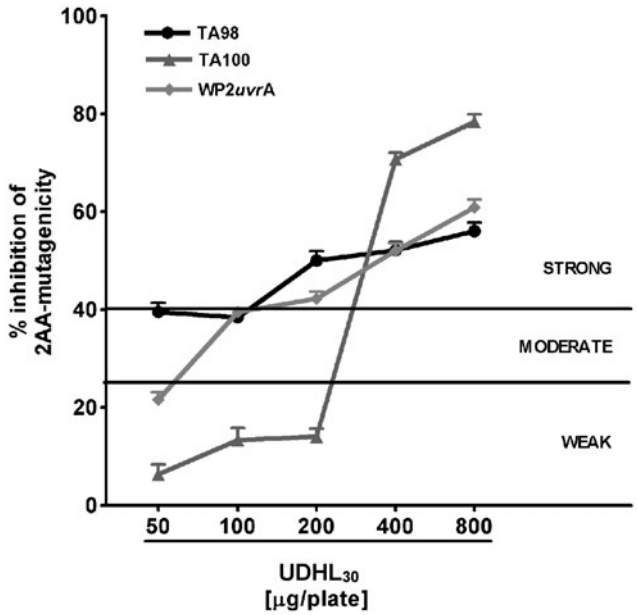

Figure 1. Antimutagenic activity of the protein fraction from *Urtica dioica* herb ($UDHL_{30}$) against 2-aminoanthracene (2AA) in *Salmonella typhimurium* TA98, TA100, and in *Escherichia coli* WP2*uvr*A strains in the presence of the metabolic activator S9. Values are expressed as mean ± SEM ($n = 6$). Strong: inhibition > 40%; moderate: inhibition between 25% and 40%; weak: inhibition < 25%.

agonist concentration in molar, HillSlope is the slope of the agonist curve.

Results and discussion

$UDHL_{30}$ (0.06–2 mg/mL, 1:2 dilution factor) did not display significant reduction in HepG2 viability, up to the maximum concentration tested after both 24 h and 48 h treatment. The treatment with doxorubicin (3 μg/mL) produced a 62.3 ± 2.4% reduction of cell viability, with respect to the vehicle control (data not shown). These results allow us to hypothesize that other compounds contained in the aerial parts of stinging nettle are involved in the beneficial effects on liver tissue. Also, a weak sensitivity of hepatocellular carcinoma cells to the treatment with $UDHL_{30}$ could be hypothesized. In contrast, an aqueous extract of *U. dioica* leaf (although it was not phytochemically characterized) has been shown to induce antiproliferative effects on breast cancer cells, so suggesting a potential usefulness of the plant in breast cancer chemotherapy (Fattahi et al., 2013).

When tested in the Ames test, $UDHL_{30}$ induced neither cytotoxic nor mutagenic effects up to 800 μg/plate (corresponding to 302 μg/mL); in addition, it produced no significant antimutagenic effects against 2NF, SA, and MMS (Table 1). Conversely, $UDHL_{30}$ (50–800 μg/plate; dilution factor 1:2) reduced the number of revertant colonies induced by 2AA, in a statistically significant and concentration-dependent manner in all strains tested (Figure 1). According to Di Sotto et al. (2013), the antimutagenicity was strong and reached, at 800 μg/plate, a 56, 78, and 61% inhibition in TA98, TA100, and WP2uvrA strains, respectively. 2AA is a pro-carcinogen requiring a CYP450-mediated bioactivation to carcinogenic species. Its major reactive derivative is an *N*-hydroxyarylamine, which acts as a DNA-intercalating agent, so forming a covalent adduct (Di Sotto et al., 2013; Murata & Kawanishi, 2011). Taking into account that the 2AA-inhibition was produced in all strains and with a concentration-dependent trend, we hypothesized that $UDHL_{30}$ can inhibit the CYP450 isoenzymes, responsible for 2AA-bioactivation. Accordingly, an aqueous extract from nettle leaf (although it was not phytochemically characterized) was shown to significantly inhibit the mutagenicity of sodium azide and acrylic amide in the presence of S9 (Darsanaki et al., 2012).

$UDHL_{30}$ (0.1–640 μg/mL) exerted a remarkable scavenger activity against both the ABTS· radical and the superoxide anion. The inhibition was concentration dependent and reached the maximal value (about 90% scavenger activity) at 120 and 400 μg/mL (Figure 2A and B). The IC_{50} values were 19.9 ± 1.0 μg/mL (slope = 1.45 ± 0.14; $R^2 = 0.998$) and 75.3 ± 0.9 μg/mL (slope = 2.69 ± 0.11; $R^2 = 0.999$), corresponding to 2.46 ± 0.2 μg/mL and 309.6 ± 6.1 μg/mL TEAC. Therefore, the potency of $UDHL_{30}$ for scavenging the superoxide anion was higher than Trolox. Taking into account that the 2AA-mediated genotoxic damage is also due to the release of reactive oxygen species (ROS) (Murata & Kawanishi, 2011), the scavenger activity of $UDHL_{30}$, particularly against the superoxide anion, could contribute to its antimutagenicity.

Conclusion

In the present paper, for the first time, we found that a protein fraction from *U. dioica* aerial parts (namely $UDHL_{30}$) possessed interesting antimutagenic and radical scavenger properties. The antimutagenicity was highlighted against 2AA, which represents one of the main carcinogenic health hazards associated to occupational exposure and dietary intake, being frequently formed at high temperatures during cooking and frying of meat and fish, or during the incomplete combustion of organic compounds (Diggs et al., 2011).

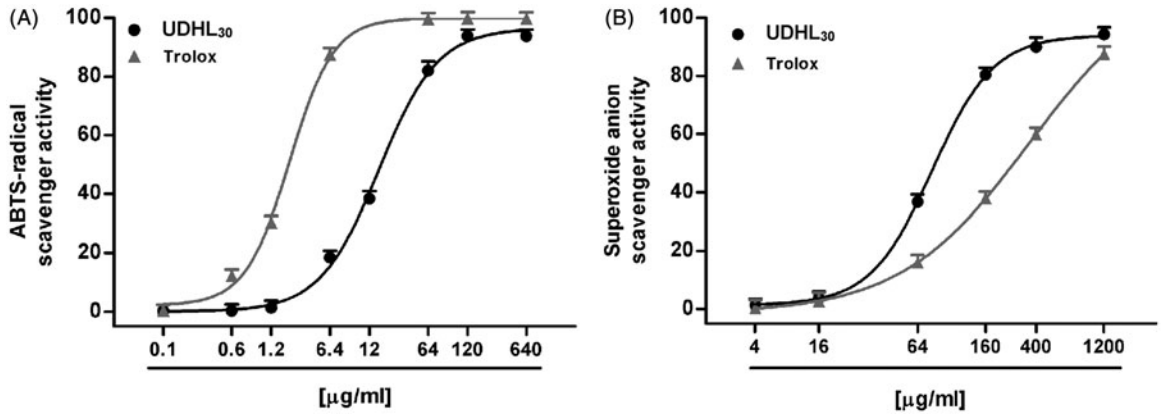

Figure 2. Concentration–response curve for the scavenger activity of the protein fraction from *Urtica dioica* herb ($UDHL_{30}$) against the ABTS radical (A) and the superoxide anion (B). Points represent the mean ± SEM ($n = 6$).

The present results encourage further studies in order to better define the potential usefulness of $UDHL_{30}$ in chemoprevention, particularly against DNA-damages induced by environmental and dietary carcinogens.

Declaration of interest

The authors declare no conflict of interest. This work is supported by ''Enrico and Enrica Sovena'' Foundation.

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