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Changes in chromosome structure, mitotic activity and nuclear DNA content from cells of Allium Test induced by bark water extract of *Uncaria tomentosa* (Willd.) DC

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Abstract

The influence of water extract of *Uncaria tomentosa* (Willd.) DC bark on the meristematic cells of the root tips of *Allium cepa* L., e.g. cells of Allium Test, was investigated. The experiment was carried out in two variants: (1) continuous incubation at different concentrations (2, 4, 8 and 16 mg/ml) of the extract for 3, 6, 12, 24, 48 and 72 h; and (2) 24-h incubation in three concentrations of the extract (4, 8 or 16 mg/ml), followed by post-incubation in distilled water for 3, 6, 12, 24 and 48 h. During the continuous incubation, the mitotic activity was reduced (2 and 4 mg/ml) or totally inhibited (8 and 16 mg/ml), depending on the concentration of the extract. All the concentrations resulted in gradual reduction of the mitotic activity. In the concentration of 2 mg/ml, the mitotic activity reached its lowest value after 12 h (2 mg/ml) and after 24 h in 4 mg/ml, followed by spontaneous intensification of divisions during further incubation. Instead, in higher concentrations of the extracts (8 and 16 mg/ml), the mitotic activity was totally inhibited within 24 h and did not resume even after 72 h. Incubation caused changes in the phase index, mainly as an increase in the number of prophases. After 24 h of incubation, in all phases, condensation and contraction of chromosomes were observed.

During post-incubation, divisions resumed in all concentrations, reaching even higher values than the control. Cytometric analysis showed that the extract caused inhibition of the cell cycle at the border between gap_2 and beginning of mitosis (G_2/M).

Keywords: *Uncaria tomentosa*; Allium Test; Activity of mitosis; Chromosome condensation and chromosome contraction; Blockade of G_2/M cycle; Nuclear DNA content

1. Introduction

Uncaria tomentosa (Rubiaceae) water extracts were use in folk medicine (De Jong et al., 1999; Heitzman et al., 2005; Keplinger, 1982; Reinhard, 1997; Reinhard, 1999). Their use is raising more and more interest worldwide, mainly connected with the phytotherapy of cancer. Apart from alkaloids, which intensively affect the metabolism of live organisms, *Uncaria tomentosa* has been found to contain numerous glycosides of quinovic acid (Aquino et al., 1989; Cerri et al., 1988), triterpenes such as ursolic and oleanolic acid (Aquino et al., 1997) and numerous sterols (Senatore et al., 1989) as well as polyphenolic and uncarine acids (Lee et al., 2000; Wirth and Wagner, 1997). The results obtained so far indicate that the compounds isolated from *Uncaria tomentosa* accelerate phagocytosis (Wagner et al., 1985), show anti-inflammatory activity (Aguilar et al., 2002; Aquino et al., 1991; Krowicka et al., 1998; Reinhard, 1997; Sandoval-Chacon et al., 1998; Senatore et al., 1989), antimutagenic action (Keplinger et al., 1999; Sheng et al., 2000), antiviral activity (Keplinger et al., 1999) and contraceptive action (Salazar and Jayme, 1998). It has also been shown that the extracts have a cytoprotective effect against factors inducing oxidative stress in the human body (Deschmarchelier et al., 1997; Sandoval et al., 2000). They act as immunostimulators, as evidenced by raised

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production of interleukin-1 and -6 in macrophages of alveoli (Lemaire et al., 1999). However, the most important fact is the antiproliferative effect of these extracts (Åkesson et al., 2003). They induce delayed-type apoptosis and, depending on concentration, strongly inhibited proliferation in vitro of human cancer cells: HL-60 leukemia, lymphoma line (Raji) from B cells transformed with the EBV virus (Sheng et al., 1998), as well as a breast cancer cell line (Riva et al., 2001). Simultaneously, the same preparation applied to rats increased leukocytosis in vivo, stimulated in vitro the proliferation of healthy lymphocytes isolated from the animals, and also induced higher leukocytosis in healthy humans (Sheng et al., 2000; Wurm et al., 1998). Additional toxicological research carried out on experimental animals showed that *Uncaria tomentosa* extracts were not toxic (Santa-Maria et al., 1997; Sheng et al., 2000). These results are very promising since they indicate that the extracts have a selective antimitotic effect only with respect to damaged cells.

In order to study the mechanism and broad range of action of the water extracts of *Uncaria tomentosa* bark, the objective of this work was to determine the influence of the extract on meristematic cells of root tips of *Allium cepa* L. This model root system of plant cells is commonly used as a test for investigating environmental pollution factors, toxicity of chemical compounds and evaluating potential anticancer properties (Keightley et al., 1996; Kupidłowska et al., 1994; Kuraś and Malinowska, 1978; Majewska et al., 2003; Podbielkowska et al., 1981, 1995). It has been used since 1938 (Levan, 1938). It is very comfortable as it is easy to make preparations of onion roots. They contain rather homogenous meristematic cells, having only 16 chromosomes, which are very long, well visible and get stained easily. The test is a fast and inexpensive method, allowing the investigation of universal mechanisms for meristematic plant cells and extrapolation on animal cells. The comparison of results obtained with animal and human test systems to those obtained using a model plant system (*Allium*) could bring additional information (mainly cariological) on the biological activity of *Uncaria tomentosa* extracts and could contribute to explaining the mechanisms of their action on cells.

Our work dealt with the influence of different concentrations of the bark extract on cell morphology and the intensity of cell divisions. Flow cytophotometry was useful for evaluating the relative amount of DNA in the interphase nuclei (Otto, 1990) and ratio of G_1 and G_2 during mitotic cycle under treatment. We have used these methods in more complex investigations on the influence of the *Uncaria tomentosa* extract on human cancer cells in vitro (not published). The aim of this work was therefore to find concentrations of water extract of *Uncaria tomentosa* bark which inhibited mitoses and to investigate the fate of the cells when they were removed from the extract and transferred into water for post-incubation, to find out if the changes were reversible. Changes in structure of the interphase chromatin were analyzed, and phases of mitosis as well as chromosomal aberrations counted, which would indicate possible dangerous effects of treatment. Flow cytometry was used to investigate changes in the G_2 to G_1 ratio. Chromosomal changes, in particular mitotic phases, whose elevation would indicate possible dangerous effects of the treatment, were counted.

2. Material and methods

2.1. Preparation of the extracts

Bark of *Uncaria tomentosa* originated from Laborations Induquimica, Lima, Peru was supplied by A-Z Medica Company, Gdańsk. The voucher material is deposited at the Laboratory of Phytochemistry, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland. A dry sample of ground bark (1 g) was added to 10 ml of Milli-Q water at room temperature and mixed in a shaker for 12 h. The extract was then sonicated (4 impulses × 1 min.) (Sonics and Materials Inc., Vibra Cell) and centrifuged (17,092 × *g* for 20 min.). The supernatant was used to prepare an extract, containing 80 mg/ml of soluble substances, which was then used as a stock to prepare the solutions of all concentrations used in the experiments.

2.2. HPLC-fingerprint analysis of alkaloids

To 625 μl of stock solution of extract containing 50 mg of soluble substances, 15 ml 2% sulphuric acid solution were added and sonified for 15 min in an ultrasonic bath (Bandelin Sonorex RK 103H). The mixture was then centrifuged at 17,092 *g* for 10 min and extracted three times with 10 ml ethylacetate. Next, the aqueous phase was separated and adjusted to pH 10 with 10% NH_4OH and extracted three times with 10 ml of ethylacetate each. The organic extracts were combined, evaporated to dryness and the residue dissolved in 1 ml of methanol. To 100 μl of methanol solution corresponding to 5 mg of dry mass of water extract, 50 μl of caffeine (1 mg/ml) were added as internal standard. Next, this solution was adjusted with methanol to a volume of 500 μl.

The qualitative and quantitative content of alkaloids was determined by the HPLC fingerprint analysis [HPLC: L-7100 Intelligent Pump (Merck-Hitachi), L-7200 Autosampler (Merck-Hitachi), L-7450 Diode Array Detector (Merck-Hitachi); Software: D-7000 Chromatography Data Station Software Version 4.0; Column: LiChrospher® 100 RP-18 (250 mm × 4 mm, Merck); Precolumn: LiChrospher® 100 RP-18 (4 mm × 4 mm, Merck); solvents: A, phosphate buffer solution (10 mM, pH, 6.6), B, methanol: acetonitrile (1:1); gradient: (60% A and 40% B) to (30% A and 70% B); injection 10 μl of sample; time: 35 min; washing: 20% solvent A and 80% solvent B; temp: 21 °C; flow rate: 1.0 ml/min.; detection: 245 nm] (Sheng et al., 2000; Stuppner et al., 1992). The results of this analysis are presented in Fig. 1 and Table 1.

2.3. Allium cepa Test

Root tips of onion (*Allium cepa* L. var. Dawidowska). e.g. cells of *Allium cepa* Test (Fiskesjö, 1985) were used for the experiments. The roots were grown in distilled water in 250-ml Erlenmeyer flasks under laboratory conditions. After reaching a length of 3 cm (±0.5 cm), the roots were treated with the extracts, 2, 4, 8 or 16 mg/ml. The treatment (incubation) of roots was carried out in two variants. The first variant was a continuous