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ASSESSMENT OF THE GENOTOXIC ACTIVITY OF α-ASARONE AND ITS DERIVATIVES IN THE COMET ASSAY

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Abstract: In our previous paper we examined mutagenic and genotoxic activity of 4 α-asarone isomers **2**-**5** exhibiting relatively high hypolipidemic activity. In the present paper, we examined genotoxic activity of α-asarone and its isomers as the ability to damage cellular DNA, evaluated in the comet assay. Additionally, mutagenic activity of α-asarone in Ames test has been examined. The Ames test for α-asarone was carried out in accordance with the guidelines of the PN-EN ISO 10993-3 standard. Compounds **4** and **5** were found to be devoid of any genotoxic activity while maintaining their hypolipemic potential. Because mutagenic activity of compound **4** was also minor it could be considered as a candidate for further pharmacological evaluation. Genotoxic but not mutagenic activity of α-asarone has been confirmed.

Keywords: asarone, genotoxicity, mutagenicity, Ames test, comet assay

α-Asarone [**1**] is a natural substance isolated from a number of plants including *Asarum europaeum*. The compound has a strong hypolipemic activity, consisting in an increase of the HDL cholesterol fraction, and a reduction of the LDL fraction, with the total blood cholesterol level hardly being unchanged. The effect has been confirmed in animal experiments as well as in humans (1–7). At the same time, animal experiments have provided data on the carcinogenic (8, 9) and teratogenic activity (10, 11) of asarone and its derivatives. It has also been found in mice that α-asarone could reach the gonads and gametes to cause a genetic lesion which, when expressed in fertilized egg, caused premature death of the resulting embryo (12). The genotoxic activity of α-asarone has been confirmed based on sister chromatid induction tests, both in *in vitro* human leucocytes and *in vivo* murine bone marrow cells (13). The mutagenic properties of α-asarone and its oxide have been seen in the Ames test, in the presence of S9 mix fraction, for a single *Salmonella typhimurium* strain, TA100 (14, 15).

These adverse effects of asarone were the basis for the search of derivatives that would have no harmful effects for human health with unchanged hypolipemic properties.

In our previous paper, we examined mutagenic and genotoxic activity of α-asarone isomers **2**, **3**, **4** and **5** (Fig. 1) in Ames and micronucleus tests, respectively. It has been found that compounds **2** and **5** exhibited substantial mutagenic activity (with minor or no genotoxic activity), compound **3** exhibited minor mutagenic and genotoxic activity and compound **4** exhibited minor mutagenic and no genotoxic activity (16). Simultaneously, the compounds exhibited relatively high hypolipidemic potential [6, 7]. In the present paper we examined genotoxic properties of compounds **2**-**5** for the ability to damage cellular DNA, evaluated in the very sensitive comet assay (17, 18). The confirmation of the genotoxic activity of derivatives **2** and **3**, and no genotoxic activity of derivatives **4** and **5**, would allow to qualifty the latter compounds as safe and possibly useful in medicine. Additionally, mutagenic and genotoxic activity of α-asarone in Ames and comet tests, has been examined. The Ames test for α-asarone was carried out in accordance with the guideline PN-EN ISO 10993-3.

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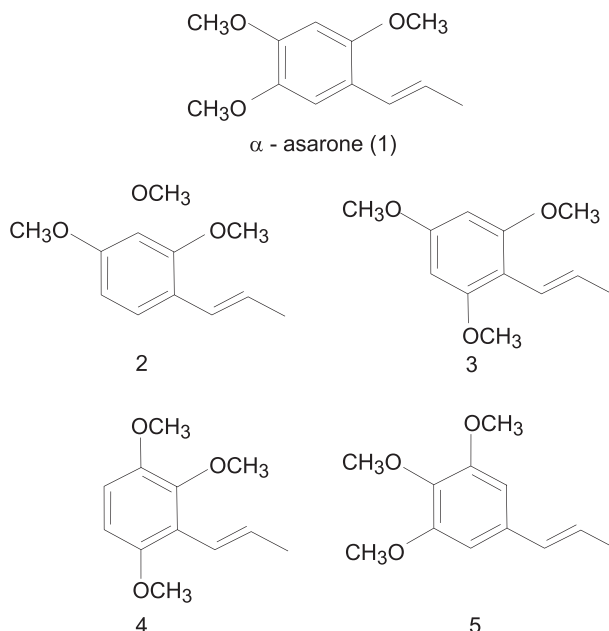

Figure 1. Structures of α-asarone and its isomers **2**, **3**, **4** and **5**.

EXPERIMENTAL

Cells

L929 line cells (murine connective tissue cells C3H/AN) were from the American Type Culture Collection (ATCC). The cells were cultured in the MEM growth medium with 10% fetal bovine serum and 1% antibiotics, and incubated at 37°C and in 5% CO_2 atmosphere.

Bacteria

Salmonella typhimurium strains: TA97, TA98, TA100, TA102 were from the CIP collection (Collection of the Pasteur Institute). These are alimentary mutants requiring the presence of histidine and biotin for growth. Bacterial strains characteristics - spontaneous reversion level, ampicillin resistance (presence of plasmid R), crystal violet sensitivity (*rfa* mutation) and UV sensitivity (*uvrB* mutation) were checked before the study according to the procedure of Maron and Ames [19].

Cell culture media

Minimal essential medium Eagle (MEM medium), fetal bovine serum (FBS), trypsin 0.5% + EDTA (Ethylenediaminetetraacetate) (Gibco); calcium and magnesium ion-free PBS (phosphate buffered saline) (IITD, Wrocław), antibiotic antimycotic (Sigma).

Bacteria culture media

Liquid medium broth (Nutrient Broth Oxoid); minimum agar medium (Bacto Agar Difco with Vogel-Bonner salts and 40% glucose); semi-liquid top agar (Bacto Agar – Difco).

Positive controls used in tests without metabolic activation (Ames test)

- 4-nitro-1,2-phenylendiamine (NPD) (Merck), concentration 20 mg/plate for strains TA97, TA98 and TA100;
- methyl methanesulfonate (MMS) (Merck), concentration 1.0 mg/plate for strain TA102.

Positive controls used in tests with metabolic activation (Ames test)

- 2-aminofluorene (2AF) (Fluka), concentration 10 mg/plate for strains TA97, TA98, TA100;
- 9-aminoacridine (Sigma), concentration 50 mg/plate for strain TA102.

Fraction S9 mix

Mutagenic properties of many chemicals only occur following metabolic activation. In the Ames

test, the tested extracts and bacterial cells were incubated in the presence of fraction S9 mix contained 10% liver homogenate from Aroclor 1254 prepreated from 8-10 week old Sprague Dawley male rats (S9) (MP Biomedicals, Inc.), which contains enzymes which are able to induce biochemical metabolism of the studied substances.

Tested substances

Doxorubicin (*Doxorubicini hydrochloride*) (Pharmacia & Upjohn); α-asarone derivatives: **2** (2,3,4-trimethoxypropenylbenzene), **3** (2,4,6-trimethoxypropenylbenzene), **4** (2,3,6-trimethoxypropenylbenzene), **5** (3,4,5-trimethoxypropenylbenzene) (Institute of Chemistry, University of Białystok). α-Asarone, (2,4,5-trimethoxypropenylbenzene) (Sigma Aldrich).

The tested substance concentrations range was limited by the cytotoxic effect and solubility in the used solvent (DMSO, Dimethyl sulfoxide) (POCh, Gliwice), and the lack of repeated precipitation in top agar.

Before the study, the IC_{50} values of each tested compound and control substances in the L929 cells were evaluated (IC_{50} is the drug concentration required to decrease cell density to 50% of the untreated culture after incubation time). The IC_{50} values of the used compounds: doxorubicin, α-asarone derivatives **2**, **3**, **4**, **5** and α-asarone were: 1.38, 387.79, 657.52, 336.18, 415.92 and 368.01 µg/mL, respectively.

Reagents

Agarose type I-A, agarose type VII-A, EDTA, ethidium bromide, Trizma base (Sigma); Triton X-100; NaCl, NaOH (POCh).

Comet assay

Sample preparation

Twenty-four-hour cell cultures were exposed for 1 h to α-asarone and its derivatives used at the concentrations of 10, 50, 100 µg/mL, and doxorubicin at the concentrations of: 0.01, 0.1, 1.0, 10.0 µg/mL (positive control); the cultures were trypsinized and the slides were prepared in agarose.

Slide preparation

The slides were covered by liquid 0.5% agarose of normal congealing point (type I-A) and left for drying. Fifty µL of the control cell suspension and the suspension of cells exposed to the tested substances were transferred to 700 µL of 1% agarose of low congealing point (type VII-A). The cell suspension density was between 10 000 and 100 000 cells/10 µL. Then, 75 µL of the suspension was placed on agarose-covered slides, covered with cover glass and cooled until the agarose layer congeals.

Testing procedure

After removing the cover glasses, the slide was placed in a lysing buffer, pH 10, composed of: 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton x-100 and NaOH until obtaining the required pH. After 24- to 48-h incubation at 4°C, the slides were rinsed with calcium and magnesium ion-free PBS at 4°C, then placed horizontally in an electrophoresis chamber, close to the anode. The chamber was previously filled with an electrophoresis buffer containing: 300 mM NaOH and 1 mM EDTA. The slides were left in the buffer for 20 min. After this time, the slides were rinsed 3 times, each time for 5 min, with a neutralizing buffer at pH 7.5 (0.4 M Tris). Then, the slides were dried, 50 µL of ethidium bromide (0.2 µg/mL) was transferred to each slide, the slides were covered with the cover glasses and placed horizontally in the slide box. The slides were stored at 4°C and evaluated within 3 days.

DNA damage assessment was performed using the LUCIA G/Comet Assay 3.5 computer software. No cell selection was performed, 90 comets were analyzed by measuring each of them. For the DNA damage degree assessment, the parameter of the relative tail moment was selected, as it was considered the most objective parameter on account of its reflecting the DNA damage degree in the control cell cultures. The test was performed using the L929 line cells which show an appropriate susceptibility to the external factors.

Doxorubicin was included in the test as positive control, due to its cell damaging activity shown in the micronucleus test (16, 21) and in the Ames test, for *Salmonella typhimurium* strains TA98 and TA100 (22).

Slide evaluation

The slides were evaluated using an Olympus BX60 microscope equipped with a fluorescent lamp. A WU filter and 200× magnification were used. Image analysis was performed using Laboratory Imaging LUCIA G/Comet Assay 3.5 software. The microcomputer statistical system for medical applications MEDISTAT version 2.1, 1992, was used for evaluation of the obtained results. The following statistical parameters were used: median, arithmetic mean, standard deviation.

Ames test

The assessment of the mutagenic effect of α-asarone was performed based on the reference Ames

test. In the study, α-asarone was examined in the concentration: 19.5, 39.1 and 78.1 µg/plate.

The test was performed according to the procedure proposed by Maron and Ames (19).

The mutagenic activity testing method using the Ames test, performed at the Biochemistry and Biopharmaceuticals Department, National Medicines Institute, has accreditation No. AB 774 of the Polish Accreditation Centre and EDQM certificate No. EDQM/MJA-032.

RESULTS AND DISCUSSION

The single cell gel electrophoresis (SCGE) technique, referred to as the comet assay, was used to evaluate the ability of α-asarone and its derivatives to induce DNA strand breaking. This assay uses the phenomenon of DNA fragmentation by the factors damaging the genetic material (20, 21), which also disintegrate the chromatin material. The

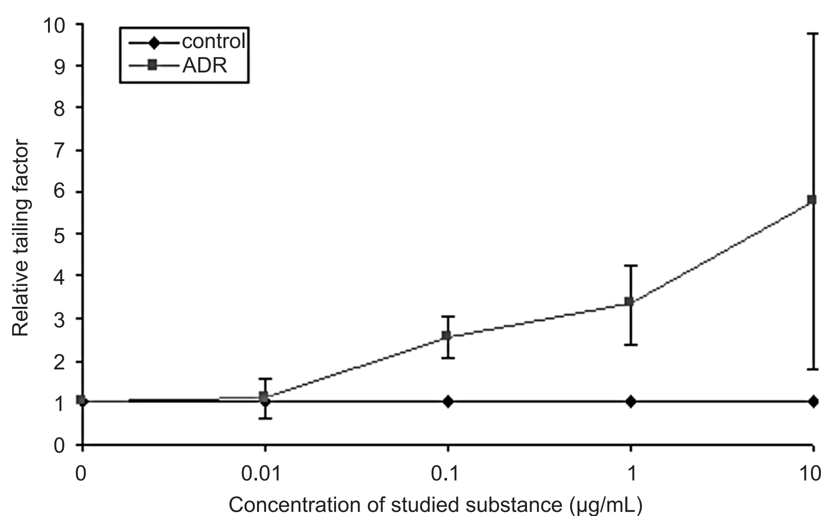

Figure 2. Relationship between the relative tail moment and doxorubicin concentration (the results show mean values from measurements of 90 comets in each of the four experiments ± SD)

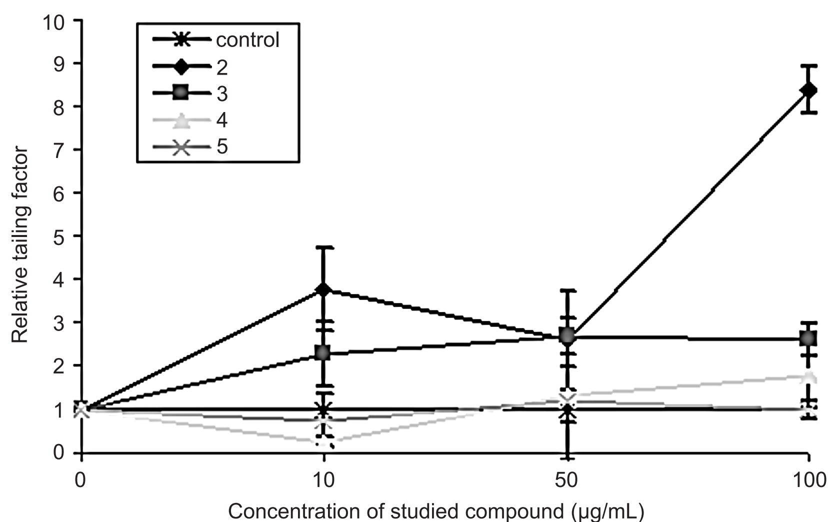

Figure 3. Relationship between the relative tail moment and tested substances, α-asarone derivatives **2**, **3**, **4** and **5** concentration (the results show mean values from measurements of 90 comets in each of the four experiments ± SD)

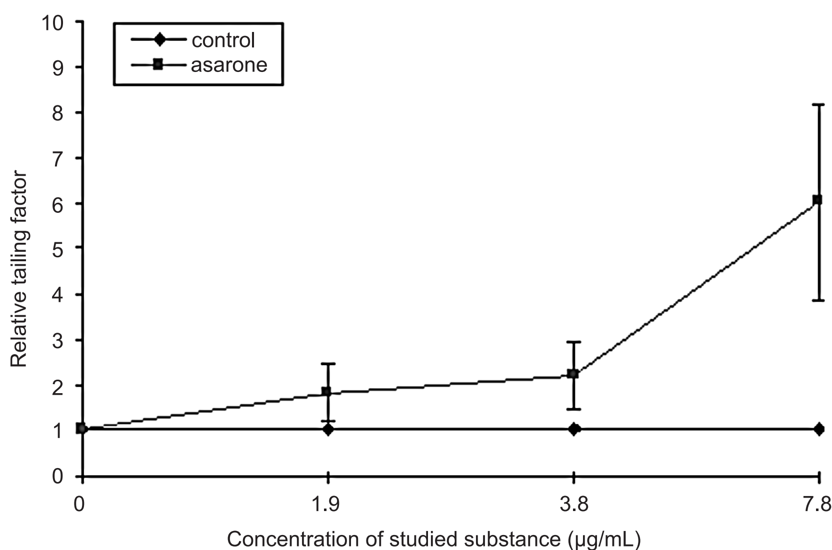

Figure 4 Relationship between the relative tail moment and α-asarone concentration (the results show mean values from measurements of 90 comets in each of the four experiments ± SD)

basic SCGE technique has been described by Östling and Johanson (17), who noticed that cells with damaged DNA, placed in an electrical field, showed migration of the damaged DNA fragments from the nucleus towards the anode. These cells, when viewed under fluorescence microscope after dying with a fluorescent stain, resembled a comet with a head and a clearly fluorescent tail. The fluorescence intensity increased with the degree of DNA damage.

The comet assay can be used to evaluate the genotoxic activity of chemical compounds and physical factors of various mechanisms of action such as: active oxygen species formation, DNA alkylation or cross-linking, resulting in DNA strand breaking. It can be used for qualitative and quantitative assessment of cellular DNA (single- and double-strand) breaks, purine and pyrimidine sites exchange, pyrimidine dimers and photodegradation products caused by UV radiation (21). For the DNA damage degree assessment, the parameter of the relative tail moment was selected, as it was considered the most objective parameter on account of its reflecting the DNA damage degree in the control cell cultures. The measure of the genotoxic activity is the degree of migration of the damaged DNA fragments outside the cell nucleus.

The results obtained in the comet assay, the relationship between the relative tail moment and the concentration of the compound used, are presented in Figures 2-4 (Fig. 2 shows the results obtained for doxorubicin – the positive control). It has been found that compounds **2** and **3** induced DNA damage in the tested cells exhibiting thus relatively high genotoxic activity. Derivatives **2** and **3** causes DNA damage (an increase in the relative tail moment value) even in the lowest concentration of 10 µg/mL, i.e., to 3.77 and 2.27, respectively (Fig. 3). For derivative **2** at 100 µg/mL the value of the relative tail moment increased to 8.39. DNA-damaging effect observed in the present study has confirmed genotoxic activity of compounds **2** and **3**, which was already observed in the micronucleus test (16). Compounds **4** and **5** – for which the value of the relative tail moment against the control was statistically insignificant – were found to be devoid of genotoxic activity in this test as well as in the micronucleus test (16).

The results of Ames test (performed in accordance with the requirements of the ISO standard) indicate the lack of mutagenic activity of α-asarone in the concentrations up to 78.1 µg/plate, both with and without the metabolic fraction (Table 1). No doubling of the revertant number was seen for any of the bacterial strains tested after exposure to α-asarone, compared to the number of spontaneous revertants in the cultures without the tested compounds. α-Asarone exhibited, however, substantial genotoxic activity, as evaluated in the comet assay.

α-Asarone genotoxic activity was manifested by an increase in the relative tail moment (Fig. 4), depending on the dose in the concentrations range

tested. The presented results are in agreement with the genotoxic effect of α-asarone observed in the micronucleus test with human lymphocyte cells and in the SOS chromotest assay (22, 23).

CONCLUSIONS

Genotoxic activity of α-asarone has been confirmed. The lack of α-asarone mutagenic activity in the Ames test is interesting in view of dominant lethal effect observed in mice by Chamorro et al. (12).

The results of the present comet assay, along with the previous results obtained using the Ames test and the micronucleus test (16), show that among the tested derivatives **2-5** compounds **4** and **5** were devoid of any genotoxic activity while maintaining its hypolipemic potential. Compound **5** exhibited, however, substantial mutagenic activity while compound **4** only minor. Compound **4** could be thus considered as a candidate for safe medicinal product and further pharmacological evaluation.

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