

# In vitro evaluation of cytotoxicity of n-hexane extract from *Alnus sieboldiana* male flowers on VERO and HEK293 cell lines

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## Abstract

**Introduction and objective.** *Alnus sieboldiana* (Betulaceae) is a warm temperate tree distributed in highland areas along the Pacific shores of central Japan. The aim of the study was to evaluate the cytotoxic activity of fractions obtained from n-hexane extract from male flowers of *Alnus sieboldiana* towards VERO and HEK293 cell lines.

**Materials and methods.** Cytotoxicity was estimated using the MTT method. The following cell cultures were used: Vero (ECACC No. 84113001) and HEK293 (ATCC No. CRL-1573).

**Results.** The results indicate that purified n-hexane fraction (F1) showed lowest cytotoxicity among tested fractions, both towards VERO and HEK293 cell lines, with EC<sub>50</sub> values of 145 and 154 µg/ml, respectively. The highest cytotoxicity (EC<sub>50</sub>=23, EC<sub>5</sub>=10 µg/ml) was observed for fraction F3 on HEK293 cell line. Fractions F2, F3 and F4 showed higher cytotoxicity on HEK293 than on VERO cell line with EC<sub>50</sub> values of 46, 23, 40 and 73, 44, 48 µg/ml, respectively. Fractions F3 and F4 showed similar toxicity on VERO cell line (EC<sub>50</sub>=44, EC<sub>5</sub>=35 µg/ml and EC<sub>50</sub>=48, EC<sub>5</sub>=31 µg/ml, respectively), whereas F3 was significantly more toxic than F4 on HEK293 cell line (EC<sub>50</sub>=23, EC<sub>5</sub>=10 µg/ml and EC<sub>50</sub>=40, EC<sub>5</sub>=25 µg/ml, respectively).

**Conclusions.** The human HEK293 cell line was more sensitive to tested fractions with the exception of F1. Studies on cytotoxicity of *A. sieboldiana* flowers on VERO and HEK293 cell lines may prove to be useful in the assessment of non-cytotoxic concentration of fractions that will be used during future research on the biological activity of this plant. EtOAc fraction (F3) showed potent cytotoxic activity, especially on HEK293, and therefore should be further analyzed using appropriate cell lines for potential anticancer properties.

## Key words

*Alnus sieboldiana*, cytotoxicity tests, VERO cells, HEK293 cells, MTT tetrazolium

## INTRODUCTION

Genus *Alnus* (Betulaceae) consists of about 35 species of deciduous trees and shrubs. Plants belonging to genus *Alnus* are widespread in the northern hemisphere since temperate climate is most suitable for them. They can be found in various habitats including sand dunes, swamps, volcanic soils, coastal areas and mountain forests [1,2]. Plants belonging to the genus *Alnus* are used in traditional folk medicine of many countries, e.g. in treatment of various diseases including cancer, and as astringent, cathartic, emetic, febrifuge, galactagogue, hemostatic, parasiticide, skin tonic and vermifuge [2]. *Alnus sieboldiana* (Betulaceae) is a warm temperate tree distributed in highland areas along the Pacific shores in central Japan [1].

Various types of plant secondary metabolites, including terpenoids (e.g. secodammarane-type triterpenoids), flavonoids, diarylheptanoids, phenols, steroids, tannins were isolated from plants belonging to the genus *Alnus* [1,2]. Asakawa et al. isolated yashabushiketol and its dihydroderivative, belonging to 1,7-diarylheptanoids, from buds of *Alnus sieboldiana* [3]. Other important compounds

isolated from *Alnus sieboldiana* are: galangin, strictinin, pedunculagin, stachyurin, casuarinin, stenophyllanin A, pinocembrin, alnustic acid and its derivatives, alnusiin, tellimagrandin, yashabushidiol A and B, yashabushiketodiol and yashabushitriol [1–7].

Data concerning studies on biological activities of *Alnus sieboldiana* is very limited; nevertheless, compounds isolated from other *Alnus* species, also present in *A. sieboldiana*, proved to possess potent activities. Diarylheptanoids isolated from *Alnus hirsuta* suppressed the overproduction of nitric oxide (NO) and displayed inhibitory activity against NF-κB activation and TNF-α production [8,9]. Diarylheptanoids from the bark of *Alnus glutinosa* showed pronounced effect in decreasing DNA damage in human lymphocytes. Oregonin, platyphylloside, hirsutanonol, (5S)-1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-5-O-β-D-glucopyranosyl-heptan-3-one, and (5S)-1,7-bis-(3,4-dihydroxyphenyl)-5-O-β-D-[6-(3,4-dimethoxycinnamoyl)glucopyranosyl]-heptan-3-one at a concentration of 1 µg/ml decreased the frequency of micronuclei by 52.8%, 43.8%, 63.6%, 44.4%, and 56.0%, respectively, exerting a much stronger effect than the synthetic protector amifostin (17.2%, c=1 µg/ml) [10]. Diarylheptanoids isolated from the bark of *Alnus hirsuta* exhibited strong antioxidative activity and inhibited the production of nitric oxide and reactive oxygen species and the expression of proinflammatory molecules, such

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as inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-induced macrophages [11]. Platyphyllone and other diarylheptanoids from the bark of *Alnus japonica* exhibited potent anti-influenza activity against KBNP-0028 (H9N2), compared with the positive control, approved antiviral drug zanamivir [12]. Triterpenoids and flavonoids isolated from leaves of *Alnus firma* were found to inhibit HIV-1 virus replication [13].

Studies on galangin, isolated from the leaves of *Alnus sieboldiana*, showed significant inhibition of TNF- $\alpha$  gene expression in A549 cells ( $IC_{50}=94 \mu\text{M}$ ), revealing its potential application in cancer prevention [1].

Botanicals such as herbal products and nutraceuticals are often regarded as low risk since they have been used by human throughout history. However, some of them may reveal a very strong and even toxic activity in humans, which especially refers to extracts, concentrates or pure compounds obtained from plants. For this reason, it seems very important to conduct screening tests to assess both the beneficial effects and the toxicity of plant materials [14].

**Objectives:** The aim of the study was to evaluate the cytotoxicity of n-hexane extract from male flowers of *Alnus sieboldiana*. The extract was subjected to silica gel column chromatography with the use of series of eluents: n-hexane, n-hexane-EtOAc (1:1, v/v), ethyl acetate and methanol, successively. This procedure produced 4 fractions (F1, F2, F3 and F4) whose cytotoxicity was further analyzed, using *in vitro* technique, on VERO and HEK293 cell lines. Cytotoxicity was estimated using the MTT method.

## MATERIALS AND METHODS

**Plant material.** Male flowers of *Alnus sieboldiana* were collected on 2 March 2008 in Osakatoge, Kagawa Prefecture (Shikoku Island, Japan), and identified by Yoshinori Asakawa. A voucher specimen (AS\_F080302) was deposited in the herbarium of Tokushima Bunri University, Japan.

**Preparation of extracts.** The air-dried flowers were ground mechanically and then extracted (macerated) with n-hexane for 2 months. After filtration and evaporation under vacuum n-hexane, the extract was received. 25 g of the extract was absorbed on silica gel (230–400 mesh), then loaded in an open column and eluted with 500 ml each of n-hexane, n-hexane-EtOAc (1:1, v/v), ethyl acetate and methanol, successively. Each fraction was evaporated under vacuum and weighed. 12.5 g of n-hexane, 7.9 g of n-hexane-EtOAc (1:1, v/v), 0.93 g of EtOAc, and 0.65 g of MeOH fractions were obtained.

Stock solutions of extracts for *in vitro* tests were obtained by dissolving samples in DMSO at the concentration of 60 mg/ml, followed by filtration through syringe filters (pore diameter 0.2  $\mu\text{m}$ ). Stock solutions were further diluted in culture media to obtain concentrations necessary for the experiments.

**Cell culture.** The following cell cultures were used: Vero (ECACC No. 84113001 – obtained from the kidney of a normal adult African Green monkey), and HEK293 (ATCC No. CRL-1573, from a human embryonic kidney). The media in the culture, Dulbecco's Modified Eagle Medium (DMEM) and Minimum Essential Medium Eagle (MEM, Sigma),

were supplemented with 10% foetal bovine serum (FBS, SIGMA), penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (Polfa Tarchomin, Poland). Media used for evaluation of cytotoxicity contained 2% serum only. All experiments with cell cultures were carried out at 37°C in a 5% CO<sub>2</sub> atmosphere (Lab-Line incubator, USA).

**Cytotoxicity assay.** Cytotoxicity of tested compounds was estimated using the MTT method. Microculture tetrazolium assay (also referred to as mitochondrial reduction assay), is a colorimetric assay used to evaluate cell vitality. The principle of MTT is based on the ability of succinate dehydrogenase enzymes present in mitochondria of viable cells to reduce the yellow water soluble substrate 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble, purple formazan product. The Product of the reaction is further dissolved using SDS/DMF solution [20% SDS in 50% DMF: a 40% solution of sodium dodecyl sulphate (SDS) was initially prepared in phosphate buffered saline (PBS); this solution was diluted by half with dimethylformamide (DMF)] and after 24 h the absorbance was measured spectrophotometrically. Since this process may occur only in viable, metabolically active cells, the level of activity is the measure of viability.

The monolayer was trypsinized and the cells were seeded in 96-well plates at the density of  $3 \times 10^4$  cells/well (100  $\mu\text{l}$ /well) in a culture medium containing 10% FBS. Following 24-h incubation and attachment, the cells were incubated for 72 h with different concentrations of plant extracts (1.03–2,100  $\mu\text{g}/\text{ml}$ ) in culture media containing 2% FBS. The series of dilution used for assessment of cytotoxicity was as follows: 1:1; 2:1; 4:2; 8:3; 16:5; 32:9; 65:7; 131:3; 262.5; 525; 1,050 and 2,100  $\mu\text{g}/\text{ml}$ . Simultaneously, the cytotoxicity of DMSO in the concentrations present in dilutions of stock solutions was evaluated. This was achieved by preparing series of dilutions of DMSO in the culture media containing 2% FBS. Dilutions were prepared with the use of the same volumes of DMSO as volumes of stock solutions used to prepare dilutions of the tested samples. Due to this approach, the quantity of DMSO in the control dilution was the same as that present in the dilution of stock solutions, and allowed objective assessment of the cytotoxicity of tested samples. Control cells were supplemented only with a medium containing a 2% addition of FBS. All samples were incubated at 37°C at an atmosphere of 5% CO<sub>2</sub>. After 72h all culture media were removed from the plates, the cells were washed with PBS, and 100  $\mu\text{l}$  of the cell media containing 10% of MTT solution (5 mg/ml) was added to each well, after which the plates were incubated for the next 4 h at 37°C. Then, 100  $\mu\text{l}$  SDS/DMF solution per well was added and after an overnight incubation the absorbance was read. Absorbance was measured at 540 and 620 nm using a microplate reader (Epoch, BioTek Instruments, Inc., USA). Data assessment was performed with the use of Gen5 software (ver. 2.01.14, BioTek Instruments). The assessment of cytotoxicity was based on a comparison with untreated cells and expressed as EC<sub>50</sub> (concentration of the sample required to inhibit 50% of cell proliferation) and EC<sub>5</sub> (concentration inhibiting 5% of cell proliferation), calculated from the dose–response curve (curve fit – nonlinear regression, 4 parameters). The values were presented as means of triplicate analyses. This technique was previously used to evaluate the cytotoxicity of *Mutellina purpurea* L. extracts and proved to produce fast and reproducible results [14].

## RESULTS

In the presented study, fractions obtained from n-hexane extract of *A. sieboldiana* flowers were evaluated for their cytotoxicity on VERO (monkey, African green, kidney) and HEK293 (human, embryonic kidney) cell lines based on MTT assay. The  $EC_{50}$  values are presented in Table 1.

**Table 1.** The  $EC_{50}$  values of tested fractions

Fraction	VERO ( $EC_{50} \pm SD$ )	HEK293 ( $EC_{50} \pm SD$ )
F1	145 $\pm$ 3.2	154 $\pm$ 21
F2	73 $\pm$ 12.2	46 $\pm$ 1.8
F3	44 $\pm$ 2.9	23 $\pm$ 3.5
F4	48 $\pm$ 5	40 $\pm$ 2

SD – standard deviation. Values are presented in mean  $\pm$  SD (n=3)

The highest cytotoxicity ( $EC_{50}=23$ ,  $EC_5=10$   $\mu$ g/ml) was observed for fraction F3 on HEK293 cell line. Fractions F2, F3 and F4 showed higher cytotoxicity on HEK293 than on VERO cell line with  $EC_{50}$  values of 46, 23, 40 and 73, 44, 48  $\mu$ g/ml, respectively. Table 2 shows concentrations of tested fractions that inhibited 5% of cell proliferation. Similar to  $EC_{50}$ , the lowest value of  $EC_5$  was noted for F3 on HEK293, proving the highest toxicity among all fractions.

**Table 2.** The  $EC_5$  values of tested fractions

Fraction	VERO ( $EC_5 \pm SD$ )	HEK293 ( $EC_5 \pm SD$ )
F1	65 $\pm$ 11.1	79 $\pm$ 19
F2	39 $\pm$ 8	25 $\pm$ 4.1
F3	35 $\pm$ 3.7	10 $\pm$ 1.41
F4	31 $\pm$ 2.4	25 $\pm$ 2.2

SD – standard deviation. Values are presented in mean  $\pm$  SD (n=3).

## DISCUSSION

Because of the ethical and scientific concerns regarding the use of animals, many *in vitro* methods for animal toxicity testing have been developed, validated and gained regulatory acceptance as an alternative to whole animal tests. These alternative methods have been developed and validated using the Reduction, Replacement and Refinement (3Rs) approach. One of the most commonly used approaches for *in vitro* cytotoxicity assessment utilizes various continuous cell lines [15]. The presented study was designed to define the cytotoxicity of fractions obtained from flowers of *Alnus sieboldiana*. VERO, a mammalian cell line established from the kidney of the African green monkey (*Cercopithecus aethiops*) recommended for screening chemical toxicity *in vitro* [16], and HEK293, a human cell line established from the human embryonic kidney were used. A non-polar solvent (i.e. n-hexane) was used to obtain the extract, and thus, the observed cytotoxicity can be attributed to non-polar compounds, e.g. diarylheptanoids, which are widespread in genus *Alnus*.

The diarylheptanoids isolated from the bark from *Alnus japonica* showed cytotoxic activities on B16 mouse melanoma and SNU-1 human stomachic adenocarcinoma cells. Platyphylloside showed the most potent cytotoxic

activity, especially on B16 ( $EC_{50}=7,02$   $\mu$ M). Moreover, it was the only one of the tested compounds that revealed cytotoxic effect on SNU-354 (hepatocellular carcinoma) and SNU-C4 (colorectal adenocarcinoma) with  $EC_{50}=48,53$  and  $EC_{50}=45,17$   $\mu$ M, respectively. The highest activity on SNU-1 was noted for 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-O- $\beta$ -D-glucopyranosyl(1 $\rightarrow$ 3)- $\beta$ -D-xylopyranoside with  $EC_{50}=27,96$   $\mu$ M [17]. Hirsutanone, ((E)-1,7-bis(3,4-dihydroxyphenyl)hept-4-en-3-one), a diarylheptanoid found in *A. japonica*, was also isolated from *Viscum cruciatum* and showed potent anticancer properties against TK-10 cells (human renal adenocarcinoma), MCF-7 cells (human breast adenocarcinoma) and UACC-62 cells (human melanoma), with the  $EC_{50}$  values of 6.8, 1.9 and 4.8 mg/ml, respectively.

Etoposide was used as a positive control ( $EC_{50}$  values for TK-10 cells, MCF-7 cells and UACC-62 cells were 8.1, 0.33 and 0.97 mg/ml, respectively) [18]. The mechanism of cytotoxic activity of diarylheptanoids seems to be based on the ability to induce cell cycle arrest and apoptosis. Studies performed by Tian et al. proved that 1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)-4E-en-3-heptanone induces S phase arrest and apoptosis via up regulation of ATF3 and stabilization of p53 in SH-SY5Y cell line [19]. Multiple articles denote that diarylheptanoids from the genus *Alnus*, as well as from *Zingiber officinale*, *Alpinia officinarum* and *Viscum cruciatum*, are cytotoxic agents against many cancer cell lines. Similar to curcumin, which is a well-known diarylheptanoid, they may prove to be useful not only in cancer chemoprevention but also in chemotherapy [18,20,21,22].

Apart from diarylheptanoids, cytotoxic activity of *A. sieboldiana* may also be attributed to the presence of flavonoids, especially galangin (3,5,7-trihydroxyflavone) belonging to flavonols. Galangin has been reported to possess antioxidative and radical scavenging activities, as well as antimutagenic and anticlastogenic effects. Furthermore, anti-proliferation activity of galangin has been reported in various cancer cell lines. Studies performed by Zhang et al. showed that galangin significantly decreased cell viability of B16F10 melanoma cells and induced cell apoptosis [23]. Ludwiczuk et al. isolated galangin from EtOAc soluble fraction of methanol extract from *Alnus sieboldiana* leaves. Galangin slightly affected the viability of human lung cancer cells (A549), with  $EC_{50}$  value of 78  $\mu$ M [1].

Stević et al. studied antioxidant, cytotoxic and antimicrobial properties of leaves and cons of *Alnus incana* and *Alnus viridis*. The cytotoxicity evaluation conducted on HeLa cell line showed that the cons methanol extracts of both *Alnus incana* and *Alnus viridis* possessed higher cytotoxicity –  $IC_{50}$  values 39.9  $\mu$ g/ml and 47.4  $\mu$ g/ml, than leaves methanol extracts – 68.5  $\mu$ g/ml and 55.5  $\mu$ g/ml, respectively [24]. Despite using different cell lines,  $EC_{50}$  values obtained for the F4 fraction (eluted with MeOH) in the presented study correspond with those given by Stević et al. for cons methanol extracts of both *Alnus incana* and *Alnus viridis*.

Bioactivity guided isolation of anticancer constituents from the leaves of *Alnus sieboldiana* performed by Ludwiczuk et al. involved the assessment of cytotoxicity of n-hexane extract and different fractions obtained after isolation, on human non-small-cell lung carcinoma cell line A549 [1]. Crude n-hexane extract slightly affected the viability of human lung cancer cells ( $EC_{50}=925$   $\mu$ g/ml). The n-hexane extract was subjected to activity-guided silica gel column

chromatography with the use of a series of eluents: n-hexane, n-hexane–EtOAc (4:1, v/v), n-hexane–EtOAc (1:1, v/v) and ethyl acetate, successively. Fractions (H1–H5) obtained from n-hexane extract were characterized by much stronger activity in comparison to the crude extract. The most active fraction was H5 ( $EC_{50} = 128 \mu\text{g/ml}$ ) [1].

The authors of the presented study used a similar approach to fractionate n-hexane extract from flowers of *Alnus sieboldiana*, and have also observed gradual increase in the cytotoxicity of subsequent fractions, with the exception of F4 which was less cytotoxic than F3.

## CONCLUSIONS

The human HEK293 cell line was more sensitive to tested fractions with the exception of F1 (n-hexane). Fractions F3 (EtOAc) and F4 (MeOH) showed similar toxicity on the VERO cell line ( $EC_{50} = 44$ ,  $EC_5 = 35 \mu\text{g/ml}$  and  $EC_{50} = 48$ ,  $EC_5 = 31 \mu\text{g/ml}$ , respectively), whereas F3 was significantly more toxic than F4 on HEK293 cell line ( $EC_{50} = 23$ ,  $EC_5 = 10 \mu\text{g/ml}$  and  $EC_{50} = 40$ ,  $EC_5 = 25 \mu\text{g/ml}$ , respectively).

Data concerning the biological activity of extracts and compounds isolated from flowers of *A. sieboldiana*, is very limited; however, based on the obtained results it can be indicated that it may possess interesting biological activities. The studies of cytotoxicity of *A. sieboldiana* flowers on the VERO and HEK293 cell lines may prove to be useful in the assessment of non-cytotoxic concentrations of fractions that will be used during future studies on the biological activity of this plant. Since literature data suggests antiviral properties of diarylheptanoids from different plants, future studies should focus on antiviral activity of *A. sieboldiana*. EtOAc fraction (F3) showed potent cytotoxic activity, especially on HEK293, and should therefore be further analyzed with the use of appropriate cell lines for potential anticancer properties.

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