CYTOTOXIC ACTIVITY OF PARIS QUADRIFOLIA EXTRACT AND ISOLATED SAPONIN FRACTIONS AGAINST HUMAN TUMOR CELL LINES

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Steroidal saponins isolated from many plant species belonging to Monocotyledones display potent cytotoxic activity towards many human tumor cells. We examined the cytotoxic effects of crude Paris quadrifolia extract for the first time, testing isolated saponin-rich fractions against four different human cell lines using the [(3-(4,5-dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium bromide (MTT) assay. Cytotoxic activity was tested against human promyelocytic leukemia (HL-60) cells, human cervical adenocarcinoma (HeLa) cells and human breast cancer (MDA-MB-468) cells. Human skin fibroblasts were used as non-neoplastic control cells. Our results show significant activity of the weakly water-soluble solid residue and butanolic fraction against HL-60 and HeLa cells. The solid residue exerted cytotoxicity against all tested cell lines.

Key words: In vitro cytotoxicity, MTT assay, Paris quadrifolia extract, steroidal saponins, human tumor cell lines.

INTRODUCTION

Paris quadrifolia, herb Paris, is an herbaceous perennial of the Liliaceae (Tutin, 1980) or Trilliaceae (Takhtajan, 1997) family of the class Monocotyledones. It has a long stem (10–40 cm) and a creeping fleshy underground rhizome (Tutin, 1980; Takhtajan, 1997; Gruenwald et al., 2000). It usually has four leaves, which are obovate, whorled and glabrous. The plant grows in temperate regions of Europe and Asia in shady forests and brush, especially in wet places.

The herb is used in homeopathic and traditional medicine as a remedy for headaches, neuralgia, nervous tension and migraine. It contains steroidal saponins (pennogenin glycosides), 1-dehydrotrillenogenin, ecystosterone and a kaempferol glycoside (Nohara et al., 1982; Gruenwald et al., 2000).

Saponins, a group of secondary metabolites, are widely distributed in higher plants. Depending on the nature of the aglycone, they can be classified into steroidal or triterpene groups (Oleszek, 2002).

Interest in saponins is due mainly to their pharmacological potential. It has been demonstrated that they possess haemolytic activity and exert anti-inflammatory, antifungal and antimicrobial effects (Hostettmann and Marston, 1995; Zhang et al., 1999; Chung et al., 2008). Numerous reports highlight the highly cytotoxic and anti-tumor properties of many saponins (Hostettmann and Marston, 1995). Much research indicates that extracts and steroidal saponins of Liliaceae plants have significant effects and potentially are anticancer drugs (Mimaki et al., 1995; Shao et al., 1997; Woo et al., 1998; Mimaki et al., 2001; Wang et al., 2001).

We collected P. quadrifolia, a plant containing steroidal saponins, to investigate its cytotoxic activity. The crude extract and isolated saponin fractions were tested on human promyelocytic leukemia (HL-60) cells, human cervical adenocarcinoma (HeLa) cells, human breast cancer (MDA-MB-468) cells and human skin fibroblasts. To our knowledge the cytotoxic activity of P. quadrifolia has not been investigated previously.
MATERIALS AND METHODS

PLANT MATERIAL

*Paris quadrifolia* was collected in Gdańsk (Poland). Fresh plants were dried at room temperature. A voucher specimen is deposited in the Herbarium of the Department of Biology and Pharmaceutical Botany (GDMA, Medical University, Gdańsk, Poland).

PREPARATION OF EXTRACT

Dried plant rhizomes (10 g) were incubated with distilled water for 24 h at 40°C and then extracted with ethanol (95%) for 25 h at room temperature (extracts were stirred for 1 h). After evaporation the residual extracts were combined and freeze-dried.

ISOLATION OF FRACTIONS

Lyophilized ethanolic extract of *P. quadrifolia* (1.964 g) was diluted in 12 ml water, stirred and centrifuged, after which the solid residue was washed with three portions of water (8 ml in total). The solid residue was then lyophilized, yielding 99.1 mg solid concentrate. The aqueous solution obtained (20 ml) was extracted three times with three portions of butanol saturated with water (30 ml in total). Thereafter, butanol and water residues from the organic layer were removed azeotropically on a rotary evaporator at 39°C with successive additions of petroleum ether. Traces of butanol in this concentrate were then removed in a stream of nitrogen, and 283 mg dry mass was obtained (butanol fraction). The remaining water layer from water-butanol extraction was further lyophilized, yielding 1518 mg of the substance (aqueous fraction).

TLC AND MASS SPECTROMETRY

Chromatographic separation of the butanolic and water fractions as well as the weakly water-soluble solid residue was done on thin-layer chromatography (TLC) plates (Merck GmbH, Darmstadt, Germany) using CHCl₃/MeOH/H₂O (7/3/0.5 v/v/v) as the mobile phase. The chromatograms were visualized with Liebermann-Burchard reagent (Ac₂O/ CHCl₃/H₂SO₄ at 20/50/1, v/v/v) and heated at 90°C. This procedure yielded colored spots corresponding to the presence of saponins. This further allowed the Rₜ values to be identified for most of the saponin compounds.

MALDI mass spectra of the raw extract, aqueous fraction, butanol fraction and solid residue were recorded on a BIFLEX III MALDI TOF (Bruker, Germany) mass spectrometer equipped with a nitrogen laser (λ = 337 nm) in a DHB matrix. A mixture of peptides was used as the calibration standard.

CELL CULTURE AND CYTOTOXICITY ASSAY

The HL-60 cell line was cultured in RPMI-1640 medium, the HeLa cell line was cultured in Minimum Eagle's Medium (MEM), and human skin fibroblasts and the MDA-MB-468 cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM). All media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. All cultures were maintained in a humidified atmosphere with 5% CO₂ at 37°C.

The viability of the cell lines was determined by MTT assay. Cells were seeded in 96-well microtiter plates (10⁵/ml) at 100 μl/well. After overnight incubation the cells were treated for 24 h with *P. quadrifolia* extract (diluted in water, 0–200 μg/ml) and fractions (diluted in DMSO, 0–100 μg/ml). At the end of the incubation period the medium was discarded and 100 μl MTT solution (dissolved in culture medium, 0.5 mg/ml) was added and incubated for 3 h at 37°C. The solution of formazan was made by adding 100 μl DMSO. Optical density of the formazan solution was measured at 550 nm with a plate reader (Victor, 1420 multilabel counter). Each experiment was done in triplicate in three independent trials. The IC₅₀ values were calculated from a dose-response curve and defined as the drug concentration at which cell growth was reduced to 50% of the control.

STATISTICAL ANALYSIS

The results are expressed as means ± SD of at least three independent experiments. The statistical significance of differences between the control cell line (fibroblasts) and human tumor cell lines was assessed by one-way ANOVA and Tukey's post hoc test at P<0.001.

RESULTS AND DISCUSSION

Neither the crude *P. quadrifolia* extract (0–200 μg/ml) nor the aqueous fraction exhibited cytotoxic activity towards the examined cell lines. Cytotoxic activity was observed only for the butanolic fraction and the weakly water-soluble solid residue. The latter exerted cytotoxicity towards all tested cell lines. That activity was highest against the HL-60 and HeLa cell lines, with IC₅₀ values of 13 μg/ml and 10 μg/ml, respectively (Figs. 1–4). The butanolic fraction displayed activity towards HL-60 and HeLa cells (Figs. 1, 2) and not against MDA-MB-468 cells and human skin fibroblasts (Figs. 3, 4; Tab. 1). Human skin fibroblasts were used as non-neoplastic control cells.

We found the main components of the butanolic fraction and solid residue to be steroidal saponins.
the compounds probably responsible for the action towards the tested human cell lines. The activity of the fractions and lack of action of the raw *P. quadri- folia* extract may be based on synergistic, additive or antagonistic interactions of many compounds (Huang et al., 2008).

Steroidal saponins structurally similar to the compounds of *Paris quadrifolia* have been isolated from different Liliaceae plants and investigated against human cancer cell lines (Mimaki et al., 1995; Shao et al., 1997; Woo et al., 1998; Mimaki et al., 2001; Wang et al., 2001). Mimaki et al. (2001)
examined the cytotoxic activities of steroidal saponins isolated from Liliaceae plants – *Allium narcissiflorum, Paris polyphylla* var. *chinensis* and *Lilium brownii* var. *colchesteri* – against HL-60 cells. They found relationships between saponin structure and cytotoxic activity.

Woo et al. (1998) isolated a pennogenin glycoside from *Majanthemum dilatatum*; it showed significant cytotoxic activity against human cancer cell lines – lung cancer, melanoma, ovarian cancer, stomach cancer and leukemia.

Pharmacological research on *Paris* species indicates that their isolated saponins have cytotoxic activity. The saponins from *P. polyphylla* were tested against murine leukemia (P388 and L1210) cells and human oral epithelial cancer (KB) cell line in vitro (Jun, 1989). Other work showed that polyphyllin D, a component of *P. polyphylla*, could serve as a candidate in breast cancer treatment. Treatment of two human breast carcinoma cell lines (MCF-7, MDA-MB-231) with the saponin reduced viability and induced apoptosis in a dose-dependent manner. The saponin dissipates mitochondrial membrane potential, induces down-regulation of anti-apoptotic Bcl-2 expression and induces up-regulation of pro-apoptotic Bax expression, and activates caspase-9 (Lee et al., 2005). Polyphyllin D also induces DNA fragmentation in hepatocellular carcinoma cell line HepG2 (Cheung et al., 2005).

Another saponin, formosanin-C isolated from *P. formosana*, may display anti-tumor activity (Wu et al., 1990).

The mentioned compounds isolated from *Paris* species contain a pharmaceutically important steroidal aglycone – diosgenin, which occurs in many other plants of the class Monocotyledones (Liliaceae, Dioscoreaceae). Diosgenin shows cytotoxic activities against, for example, human osteosarcoma cell line 1547 (Moalic et al., 2001), human leukemia (K562) cells (Liu et al., 2005), human melanoma (M4Beu) cells, human laryngocarcinoma (Hep-2) cells (Corbière et al., 2004) and HeLa cells (Hou et al., 2004).

We examined the cytotoxic activity of fractions isolated from whole extract of *P. quadrifolia* against different cell lines: HL-60, HeLa, MDA-MB-468 and human skin fibroblasts. The solid residue and butanolic fraction exhibited significant cytotoxic activity against HL-60 and HeLa cells. The observed selectivity of the butanolic fraction towards tumor cell lines HL-60 and HeLa provides a rationale for further isolation of saponins present in this fraction and for evaluation of their potential cytotoxic activity. The constituents of the *P. quadrifolia* fractions responsible for their cytotoxic action were not investigated in this work, but our research opens a new perspective for further investigations.

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