Cytogenetic damage of human lymphocytes treated with a phytopharmaceutical containing plant essential oils and madder root extract

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The objective of the present study was to evaluate the cytogenetic activity in human lymphocytes *in vitro* of the phytopharmaceutical Cystenal which contains extract from madder root (*Rubia tinctorum*) and various plant essential oils. Cystenal induced sister chromatid exchanges, reduced mitotic activity and replication index at almost all doses tested, and induced chromosome aberrations at highest doses (1.0 and 1.2 µl/ml). Cytotoxic and cytostatic effects were clearly dose-dependent, while for SCEs there was a peak response at a dose of 0.8 µl/ml. The clastogenicity of Cystenal most probably was due to its high toxicity.

Key words: chromosome aberrations, sister chromatid exchanges, human lymphocytes, essential oils, madder root

INTRODUCTION

Various phytopharmaceutical and plant-derived drugs from the ancient times are used for the treatment of various diseases. They are also sold to the public for traditional and unconventional medicinal purposes. At the same time, tens or even hundreds of cases of acute poisoning with herbal medicines are registered each year all over the world [1]. However, use of phytopharmaceuticals is not under regulatory control in many countries, although very little is known about their acute toxicity. Only a few papers contain data on their "latent" toxicities such as teratogenesis, carcinogenesis, and mutagenesis.

Genotoxicity of individual components of various herbal medicines has previously been studied using different methods. Different hydroxyanthraquinones were found to be genotoxic in *Salmonella typhimurium* [2, 3] and mammalian cells *in vitro* [4, 5]. However, for some compounds the results *in vitro* were not in agreement with the results *in vivo* where no genotoxicity was found [6, 7]. Some recent reports indicated that such plant components as their essential oils might be genotoxic *in vitro* as well as *in vivo* [8–10].

The objective of the present study was to evaluate cytogenetic activity in human lymphocytes *in vitro* of the phytopharmaceutical Cystenal which is legally used in Lithuania and some other Central European countries for the treatment of kidney stones. It contains extract from madder root (*Rubia tinctorum*) and various plant essential oils. Antraquinones occurring in madder root are genotoxic *in vitro* [2] and carcinogenic in rats after the treatment *in vivo* [11]. Various plant essential oils were able to induce chromosome aberrations and sister chromatid exchanges in human lymphocytes *in vitro*, and gene mutations in *Drosophila melanogaster* somatic cells *in vivo* [9, 10]. Thus, it is important to know whether Cystenal also exhibits genotoxic activity.

MATERIALS AND METHODS

Chemicals. Cystenal was purchased from a drugstore. According to manufacturer's (Galena AS, Czech Republic) specification, it contains 9.3 mg of tincture from madder root, 140 mg magnesium salicylate, 5.75 g of etheric oils (unspecified), 0.75 ml of ethanol and up to 70 ml of olive oil. All other reagents were purchased from Sigma (USA). Before the treatment of lymphocytes, one part of Cystenal was diluted with two parts of dimethyl sulfoxide (DMSO).

Cytogenetic procedures. Whole peripheral blood from healthy volunteers was grown in HEPES-buffered RPMI 1640 medium containing 12% heat-inactivated new-born calf serum, 50 µg/ml gentamycin, 2 mM α-glutamine, 10 µg/ml of 5-bromo-2'-deoxyuridine and 7.8 µg/ml phytohemagglutinin P. Cell cultures were incubated at 37 °C for 72 h. Treatment with

Cystenal was carried out 48 h after culture initiation and lasted for the period of 24 h. Since whole blood cultures display many properties common with the liver microsomal cytochrome P450 system [12], no external metabolizing enzymes were added. Colchicine (0.5 μg/ml) was present in cell cultures for the last three hours. The cells were treated with hypotonic solution (0.075M KCl) and fixed in methanol: acetic acid (3:1). Air-dried slides were differentially stained by fluorescence plus Giemsa technique, exactly as previously has been described [13].

Chromosome aberrations (CA) and sister chromatid exchanges (SCE) were scored on coded slides. No less than 100 first-mitotic division metaphases per culture were analyzed for CA and no less than 50 second-mitotic division metaphases for SCE. The cells were selected for the centromere number no less than 44, good morphology and clear staining.

The cytotoxicity of Cystenal was evaluated by means of mitotic activity (proportion of cells at mitosis). No less than 1000 cells were scored to determine mitotic activity.

Cell replicative kinetics was determined by means of replicative index ($RI = [M_1 + 2M_2 + 3M_3]/N$, where M_1, M_2, M_3 are the number of cells that have undergone one, two or three cycles of replication, respectively, and N is a total number of cells scored) exactly as described earlier [13]. RI shows the average number of times cells have divided in culture. Two hundred cells were scored to determine RI.

Statistical analysis. All statistical analyses were performed using InStat V2.02 (GraphPad Software, USA) statistical package. Statistical tests were chosen according to the nature of the data analyzed.

RESULTS AND DISCUSSION

Effects of the phytopharmaceutical Cystenal on the frequency of CA, mitotic activity, SCE and RI values in human lymphocytes *in vitro* are shown in Table. It induced SCEs, was cytotoxic and cytostatic at almost all doses tested, and induced chromosome aberrations at highest doses (1.0 and 1.2 μl/ml).

Cytotoxic and cytostatic effects were clearly dose-dependent, while for SCEs there was a peak response at a dose of 0.8 μl/ml. Such a dose dependency may be explained by the toxicity of Cystenal and the survival of less damaged cells (containing less SCEs) at higher doses. The toxicity of Cystenal may also be closely related to the induction of chromosome aberrations. Indeed, Cystenal was able to induce chromosome aberrations at high toxicity levels only (1.0 and 1.2 μl/ml) when inhibition of mitotic activity was 70% or higher. Thus, it may be classified as "high toxicity clastogen" [14], which induces chromosome aberrations by secondary mechanism associated with cytotoxicity. It was suggested [15] that such compounds do not react with DNA, are not genotoxic *in vivo* and usually are not carcinogenic. It is interesting that a good example of such "high toxicity clastogen" may be menthol which is a constituent of many essential oils [16].

Two classes of chemical compounds may be responsible for the genotoxicity of Cystenal *in vitro*: antraquinones present in extract form madder root, and monoterpenes present in plant essential oils.

Some main antraquinones in its glycoside form are present in madder roots: alizarin, rubiadin, purpurin and lucidin. It has been previously shown that alizarin is mutagenic in *Salmonella typhimurium* strain TA1537, while purpurin and lucidin were responsible for various genotoxic effects in mammalian cells [3]. However, alizarin was unable to form DNA adducts in primary rat hepatocytes [17] and purpurin showed a protective action against the bacterial mutagenicity of heterocyclic amines [18]. Nevertheless, dose-dependent increases in benign and malignant tumour formation were observed in the liver and kidneys of rats that received a diet supplemented with 1 or 10% of dried madder roots [11].

Table. **Effects of the phytopharmaceutical Cystenal on the frequency of aberrant cells, mitotic activity, sister chromatid exchange (SCE) and replication index (RI) values in human lymphocyte cultures**

Treatment	Concentration, μl/ml	Aberrant cells, % ± S.E.M.	SCE/cell ± S.E.M.	RI ± S.E.M.	Mitotic activity, % ± S.E.M.
Blank	0	0.50 ± 0.50	8.86 ± 0.48	2.40 ± 0.05	3.96 ± 0.61
DMSO	2.3	2.50 ± 1.10	8.48 ± 0.50	2.44 ± 0.05	3.14 ± 0.55
"Cystenal"	0.5	5.00 ± 1.54	14.88 ± 0.77*	1.93 ± 0.06**	1.98 ± 0.44
	0.7	4.00 ± 1.96	16.06 ± 0.71*	1.83 ± 0.06**	0.49 ± 0.22*
	0.8	4.00 ± 1.96	20.72 ± 0.85*	1.79 ± 0.06**	0.58 ± 0.24*
	1.0	7.69 ± 1.91*	16.52 ± 0.83*	1.61 ± 0.06**	0.30 ± 0.17*
	1.2	10.00 ± 3.00*	17.50 ± 0.68*	1.55 ± 0.06**	0.50 ± 0.22*

*$P < 0.05$, Student's *t*-test
** $P < 0.05$, *z*-test [12]

Another source of genotoxicity of Cystenal may be essential oils present in this drug. Different essential oils as well as their constituents have been previously reported to be genotoxic in mammalian and non-mammalian cells [8–10]. However, it was suggested that their clastogenicity may be due to a high toxicity of essential oil [9]. Thus, it is quite reliable that essential oils will be non-mutagenic *in vivo, i.e*. they will be of no real genetic risk to humans.

Our results clearly indicate that the phytopharmaceutical Cystenal is genotoxic *in vitro*. It also contains some constituents that have previously been shown genotoxic for mammalian cells *in vitro* and insect cells *in vivo*, and carcinogenic for rats. So, further genotoxicity testing of Cystenal *in vivo* or/and its carcinogenicity is obviously needed in order to confirm/reject its safety.

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CITOGENETINĖS PAŽAIDOS ŽMOGAUS LIMFOCITUOSE, *IN VITRO* PAVEIKTUOSE FITOFARMACINIU PREPARATU, TURINČIU ETERINIŲ ALIEJŲ IR DAŽINĖS RAUDĖS ŠAKNIES EKSTRAKTO

S a n t r a u k a

Tirtas fitofarmacinio preparato „Cystenal“, turinčio eterinių aliejų ir dažinės raudės ekstrakto, citogenetinis aktyvumas žmogaus limfocituose *in vitro*. Nustatyta, kad preparatas indukavo seserinių chromatidžių mainus (SCM) ir chromosomų aberacijas (CA), be to, buvo citotoksiškas ir citostatiškas. Didžiausias SCM kiekis buvo stebėtas limfocituose, paveiktuose 0,8 µl/ml preparato, o CA – 1,0 ir 1,2 µl/ml. Preparato klastogeniškumas gali būti siejamas su jo citotoksiniu veikimu.