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MALE REPROTOXICITY ASSOCIATED WITH *SOPHORA JAPONICA* TREATMENT: EVALUATION OF CELLULAR AND MOLECULAR EVENTS *IN VITRO*

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Toxicological studies are urgently needed to confirm the safety of *Sophora japonica* extracts for clinical use. In particular, in addition to pharmacy and pharmacology, phytotherapy, herbal medication, *Sophora japonica* extracts are widely used as biologically active supplements. Scientfic data suggests that some of *Sophora japonica* extract components have very comprehensive biological effects. In the present study, our hypothesis assumed the potential reprotoxicity of *Sophora japonica* extract in respect to mouse germ cells (GC-1 spg, GC-2 spd) *in vitro*. Specifically, we were interested in the high-performance liquid chromatography (HPLC) identification of extract components and its stress-related effects on cellular and biochemical features, such as mitochondrial metabolism, cell cycle progression, oxidative stress balance and micronuclei formation. The results indicate that *Sophora japonica* extract induces oxidative/nitrosative stress-mediated impairment of the mechanism for free radicals scavenging, which may provoke genotoxic events in germ cells, by cell cycle arrest and micronuclei formation. Therefore, the interplay between reactive oxygen species (ROS)/reactive nitrogen species (RNS) and antioxidant system is critical for normal testicular function maintenance in the their environment. The specific pathways and mechanisms involved in the reprotoxicity of *Sophora japonica* need to be further investigated.

K e y w o r d s : *Sophora japonica, mouse germ cells, reprotoxicity, oxidative stress, cell cycle arrest, fertility reduction, high-performance liquid chromatography*

INTRODUCTION

Natural products derived from plants are a potential source of new drugs. For this reason, they should be devoided of side effects on the human body functions. The studies on toxicity mediated by preparations consisting *Sophora japonica* are relevant due to the fact that this plant is commonly used in phytotherapy and cosmetology. Literature data on safety aspects of *Sophora japonica* is very limited, so further research is required (1, 2).

Sophora japonica L. is a popular species almost all over Europe. The dry flowers (Huaihua or Flos Sophorae) and the flower buds (Huaimi or Flos Sophorae Immaturus) are commonly used in European and Chinese Pharmacopeia. Flavonoids from its buds and fruits have been used as a hemostatic agent in traditional Chinese medicine (3). Approximately 153 chemical compounds have been isolated from the leaves, branches, flowers, buds, pericarps of *Sophora japonica*. Among them, several flavonoids and isoflavonoids triterpenes, alkaloids, polysaccharides, amino acids comprise the active constituents of *Sophora japonica*, which exhibit a wide range of *in vitro* and *in vivo* biological activities (3-5). Modern pharmacological studies showed that active components and/or crude extracts of *Sophora japonica* exhibit some pharmacological properties, such as cardiovascular effects as well as anti-inflammatory, antiosteoporotic, antioxidant, antitumor, antibacterial, antiviral, hemostatic, and antiatherosclerotic effects (6-10). Most of these effects are consistent with those observed for *Sophora japonica* in popular medicine. Furthermore, pharmacological and clinical practices revealed that it has also antifertility activities (11).

Antitumor properties of the biologically active substances of *Sophora japonica* were widely studied on various cell lines *in vitro* (7, 12-18). One of the components quercetin, isolated from *Flos Sophorae* can significantly inhibit the growth of MCF-7 and CNE2 cells in a dose- and time-dependent manner (16). In addition, three isoflavonoids, namely genistein, sophoricoside, and genistein, isolated from fruits of *Sophora japonica* at concentration 100 mg/mL, show positive antitumor activity with a high rate of growth inhibition (3).

The main aim of the present study was to investigate the toxic effects of *Sophora japonica* extract on two mouse spermatogenesis pathway cell lines. The experiment was based on the *in vitro* model of the meiosis so we have chosen two cell lines at different stages: 1) spermatogonia germ cells GC-1 spg and 2) spermatocyte cells GC-2 spd. We focused on the potential male fertility reduction as a cause of *Sophora japonica* extract activity. We evaluated the effect of *Sophora japonica* extract on mitochondria status, oxidative stress balance, cell cycle progression and micronuclei formation. Also, HPCL extract separation and β-glucan measurement was performed to supplement our data.

MATERIALS AND METHODS

Sophora japonica extract preparation

Ethanol extract was prepared by infusion of air-dry raw material - dried fruit *Sophora japonica* (FITO SVIT, Ukraine, trademark Naturalis). The ratio of the extracted substance to extractant was 1:5 (air-dry mass: volume, g:mL). *Sophora japonica* fruit was finely chopped into small pieces. The steep took place for 14 days at a temperature of 20 – 22°C in the dark. The prepared extract was sterilized using nylon membrane filters (pore size 0.45 μm). The extract was stored in tightly sealed dark containers in a refrigerator at 4°C for later use. For experiments, cells were treated with 1 – 3% *Sophora japonica* extract prepared by direct dilution in cell culture medium.

Preparation of high-performance liquid chromatography fractions

The extract was separated into a fraction by high-performance liquid chromatography (HPLC) method. A fraction with large amount of compounds from retention fime 12 – 22 min was collected for analysis. The HPLC fraction obtained in this way was intended for further research.

High-performance liquid chromatography-ultraviolet-charged aerosol detector method

Measurement of chlorogenic and caffeic acid in *Sophora japonica* extract. The chromatographic separation was completed on a 250 mm × 4.6 mm HypersilGold C18 column with particle size 5 μm (Thermo Scientific), the operating temperature was maintained at 30°C. Ultraviolet (UV) spectra were acquired over the range of 195 – 350 nm. The LC mobile phase consisted of 0.5% formic acid aqueous solution (A) and acetonitrile (B), which were combined in a gradient program: 0 – 10 min, linear gradient from 40 to 50% B; 10 – 15 min, linear gradient from 50 to 60% B, isocratic at 60% B. The flow rate was 1.0 mL/min and 10 μl was injected volume. The standard mixture solution of caffeic acid and chlorogenic acid was prepared to final concentration 50 μg/mL, 100 μg/mL, of each analyte respectively.

Measurement of fructose, saccharose, and glucose in Sophora japonica extract

Samples were analyzed by HPLC coupled to a corona detector (HPLC-CAD). The HPLC equipment consisted of an integrated Dionex system. Data were analyzed using Chromelen Software (Dionex). The chromatographic separation was achieved with a Sugar-D column (4.6 × 250 mm, 5 mm, Cosmosil) operating at 35°C. The mobile phase was acetonitrile/deionized water, 75:25 (v/v) at a flow rate of 1 mL/min. Sugars identification was made by comparing the relative retention times of sample peaks with standards. Five external standard substances were used including fructose, saccharose, and glucose. Quantification was made by the external standard method and the results were expressed in mg per mL of solution.

Determination of (1→ 3),(1→ 4)-β-D-glucan

The content of (1→ 3),(1→ 4)-β-D-glucan was determined using β-glucan assay kit (Megazyme) according to manufacturer's protocol. The amount of β-glucan was expressed as mg mL^{-1} of extract. In the case of positive control, barley flour was used.

Cell culture

Mouse spermatogonia germ cells GC-1 spg (ATCC CRL-2035) and mouse spermatocyte cells GC-2 spd (ATCC CRL-2196) were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cell lines were grown in DMEM with 4.5 g/l glucose and 1 mM sodium pyruvate (without L-glutamine), supplemented with 10% FBS and antibiotic mix solution (100 U/mL penicillin, 0.1 mg/mL streptomycin, 29.2 mg/mL L-glutamine). The mouse cell cultures were maintained at 37°C in a humidified atmosphere in the presence of 5% CO$_2$. Cultures were passaged at approximately 90% confluence by trypsinization and replated at a constant density of 1.0×10^3/cm^2 for all experimental procedures. After 24 h, the cells were treated with *Sophora japonica* extract, left 48 h unless otherwise mentioned and then analyzed. All treatments were performed at least in triplicate.

Cell metabolic activity assay

The antiproliferative activity of plant extract was measured using MTT assay. MTT solution was added to GC-1 spg and GC-2 spd cell culture medium at a final concentration of 5 mg/mL. After 4 hours incubation at 37°C, formed blue crystals were dissolved in DMSO. The absorbance of reduced MTT was assayed at 595 and 620 nm (measurement and reference wavelengths, respectively). The results are presented as %, while readings for non-treated cells (negative control) are considered as 100%.

Oxidative stress

Superoxide (O$_2^{\bullet-}$) and nitric oxide (NO) generations were measured as the efflux of derivatives of dihydroethidium (DHE probe, final concentration 5 μM) and 4-amino-5 methylamino-2',7'- difluorofluorescein diacetate (DAF-FM probe, final concentration 5 μM), respectively according to the manufacturer's instructions. Detection and quantification of O$_2^{\bullet-}$ and NO concentrations were performed using InCell Analyzer 2000 and presented as relative fluorescence units (RFU). A minimum of 1000 cells was counted in each sample.

Quantification of reduced glutathione

Quantitative measurement of reduced glutathione was estimated using fluorescent dye (Thiol Tracker Violet, final concentration 5 μM) as described in manufacturer protocol. Digital images were captured with InCell Analyzer 2000, the fluorescence intensity of the stained cells was measured with InCell Analyzer analysis module and at least 1000 cellular events were counted. Results are presented as relative fluorescence units (RFU).

Cell cycle profile analysis

The GC-1 spg and GC-2 spd cells were treated with a selected concentration of *Sophora japonica* extract for 48 hours. Then, cells were washed once in cold PBS buffer and stained with Hoechst 33342 solution (final concentration 1 μg/mL) for 10 min. The cell cycle was profiled using DNA Cell cycle plug-in from ImageJ software on fluorescent microphotographs taken with the use of InCell Analyzer 2000. Results are presented as % of cells in each of G0/G1, S and G2/M phases. A minimum of 1000 cells was counted in each sample.

Micronuclei formation

Measurement of chromosomal DNA damage in cells in response to the genotoxic compound was estimated using the InCell Analyzer 2000 micronuclei formation analysis module.

The analysis was performed on digital images taken after cell cycle assessment. A minimum of 1000 cells was automatically counted in each sample improving a statistical reliability of the results.

Statistical analysis

The analysis of the results was performed using GraphPad Prism ver. 6.0. Unless otherwise stated, results represent the mean ± SD from at least three independent experiments. Statistical significance was assessed by one-way analysis of variance (ANOVA) with Dunnett multiple comparison tests. A P-value of < 0.05 was considered as statistically significant.

RESULTS

Phytochemistry of Sophora japonica

At the beginning, we identified the concentration of chlorogenic acid (115 ± 2.82 mg/L) and caffeic acid (0.86 ± 0.069 mg/L) in the *Sophora japonica* extract using HPLC-DAD metod (*Fig. 1*). Simultaneously, concentrations of glucose, fructose, sucrose in the extract was 4.68 ± 0.04; 11.54 ± 0.07; 0.27 ± 0.001 mg/mL, respectively (*Fig. 2*). Then we determined the concentration of β-glucan in *Sophora japonica* extract which was 0.93 ± 0.01 mg/mL.

Metabolic activity

We next evaluated the effect of *Sophora japonica* extract on GC-1 spg and GC-2 spd cells in terms of mitochondrial activity changes. We observed dose-dependent decrease in metabolic activity of cells linked with increasing concentration of substance of interest used. The highest decrease in cellular activity was observed in the case of 3% of the *Sophora japonica* extract. Obtained results were comparable between both cell lines (GC-1 spg: 1% extract = 6.2%; 2 % extract = 21.8%; 3 % extract = 41.7%; GC-2 spd: 1% extract = 7.8%; 2% extract =

16.6%; 3% extract = 26.3%). In the case of HPLC fraction, we noted the reduction of metabolic activity only in GC-2 spd cells. Furthermore, HPLC fraction was characterized by ~5% increase in metabolic activity of cells and its decrease at higher concentrations (*Fig. 3*). We were interested in the toxicity of *Sophora japonica* plants, so we decided to choose 3% extract concentration for further analysis.

Oxidative and nitrosative stress balance

As expected, 48 hours after *Sophora japonica* treatment, we observed ROS overproduction in both cell line tested. In detail, the level of superoxide in GC-1 spg cells was increased by 1.6% and 2.7%, after treatment with *Sophora japonica* extract and its HPLC fraction, respectively. On the other hand, the GC-2 spd cell line responded with 2.3 and 7.4% increase in superoxide generation after incubation with the *Sophora japonica* extract and its HPLC fraction, respectively (*Fig. 4a-4b*). Interestingly, only HPLC fraction affected NO overproduction when compared to non-treated cells (*Fig. 4c-4d*). We observed also a slight fluctuation of reduced glutathione level in GC-1 spg and GC-2 spd cells after 48 hours, but results did not turn statistically significant (Fig. *4e-4f*).

Cell cycle profile

Next, we controlled the effect of *Sophora japonica* extract on the cell cycle distribution of both cell line tested. We noted that the proportion of G0/G1 phase was 62.29 ± 3.29% (GC-1 spg) and 61.00 ± 1.18% (GC-2 spd) after 48 h treatment. The number of cells in S phase increased by 3% (from 14.1 in controls to 17 in *Sophora japonica* extract), while in G2/M phase decreased by 10% (from ~32 in controls to ~21 in *Sophora japonica* extract). HPLC fraction did not significantly affect GC-1 spg cell cycle profile (*Fig. 5a*).

On the other hand, in terms of GC-2 spd cells, the effects of HPLC fraction on cells were similar to 3% *Sophora japonica* extract: percentage of cells in the G0/G1 phase increased by 9.1%, S phase 14.8 ± 0.67 % (control - 14.6 ± 0.67) and G2/M phase was reduced by 9.3% (*Fig. 5b*).
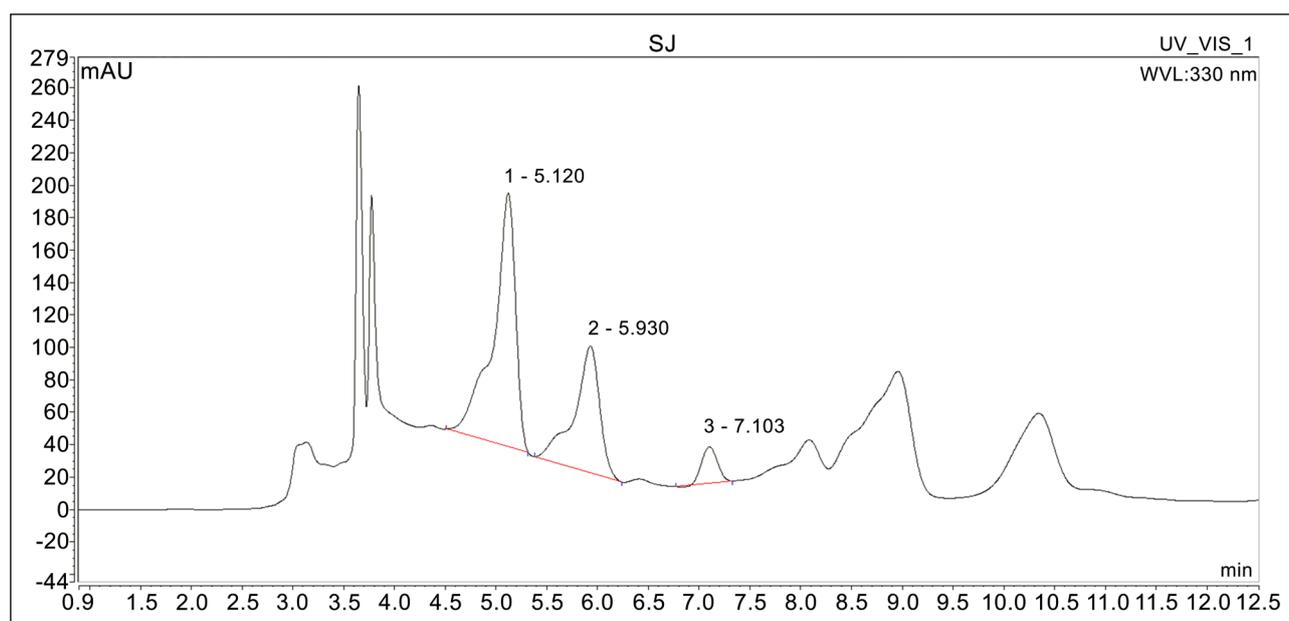

Fig. 1. HPLC chromatogram of *Sophora japonica* extract: chlorogenic acid: 5.120 min; caffeine: 5.930 min; caffeic acid: 7.103 min. Quantification was made by the external standard method and the results were expressed in mg per mL of solution.
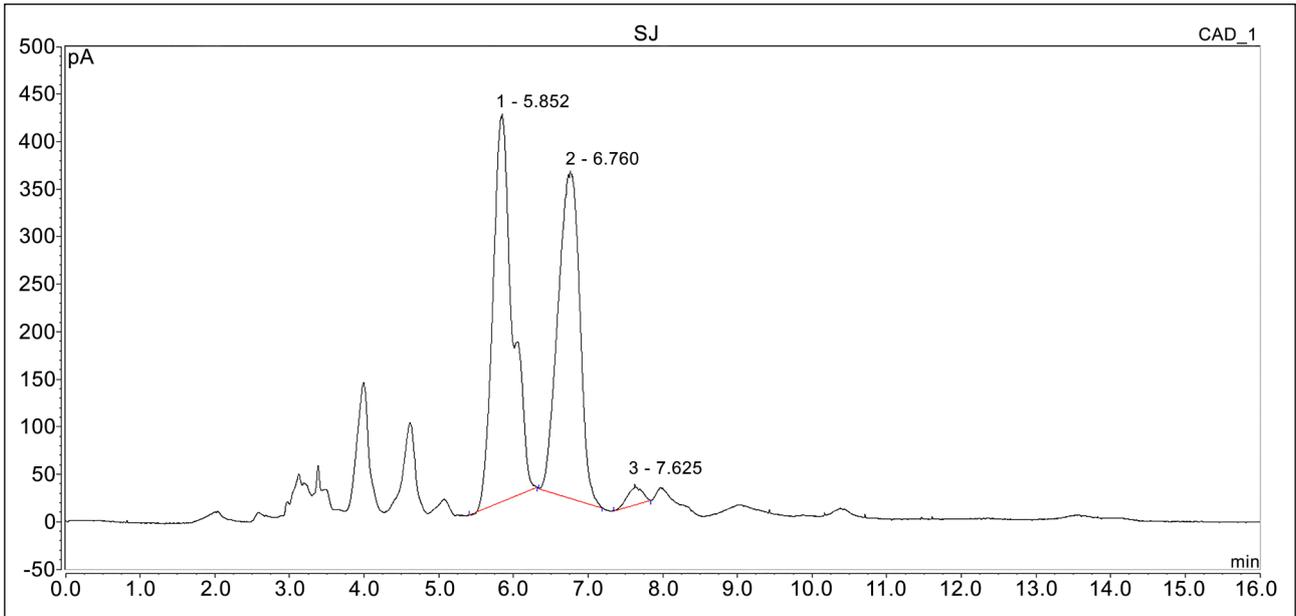
Fig. 2. HPLC chromatogram of *Sophora japonica* extract: fructose: 5.852 min; glucose: 6.760 min; saccharose: 7.626 min. Quantification was made by the external standard method and the results were expressed in mg per mL of solution.
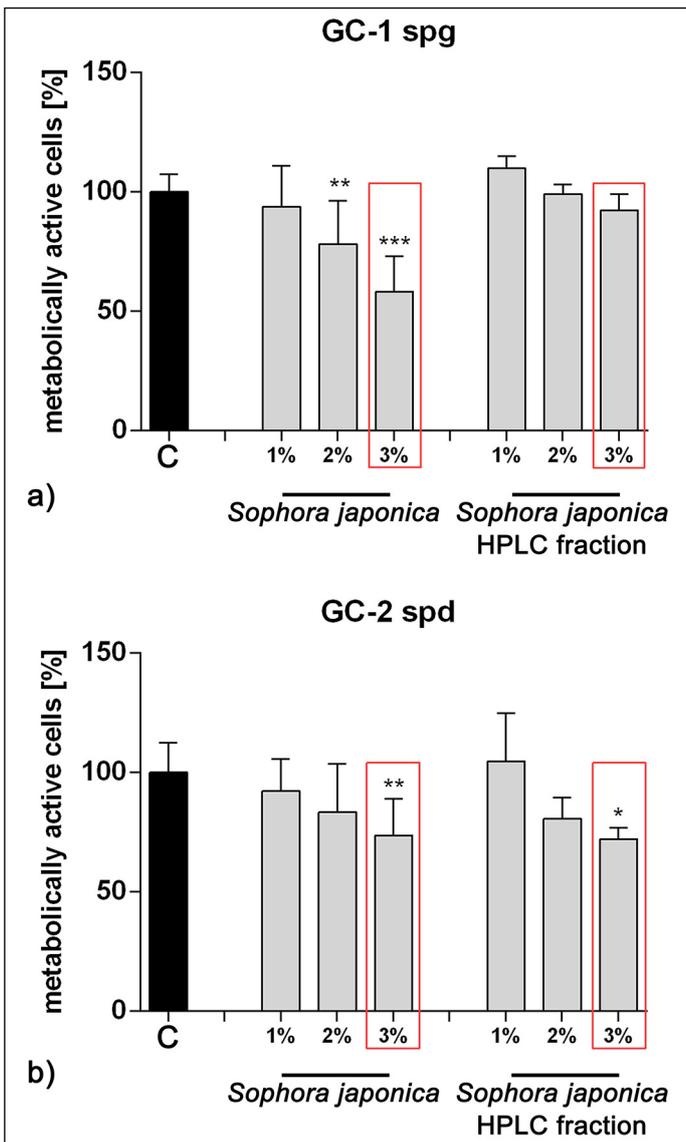

Fig. 3. Sophora japonica extract and its HPLC fraction (1 – 3%) induce changes in the mitochondrial activity of GC-1 spg cells (*a*) and GC-2 spd cells (*b*). Differences between control and test samples were assessed with one-way ANOVA and Dunnett's a post hoc test. A P-value of < 0.05 was considered as statistically significant between groups. Bars indicate SD, n = 3; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; no indication, no statistical significance.
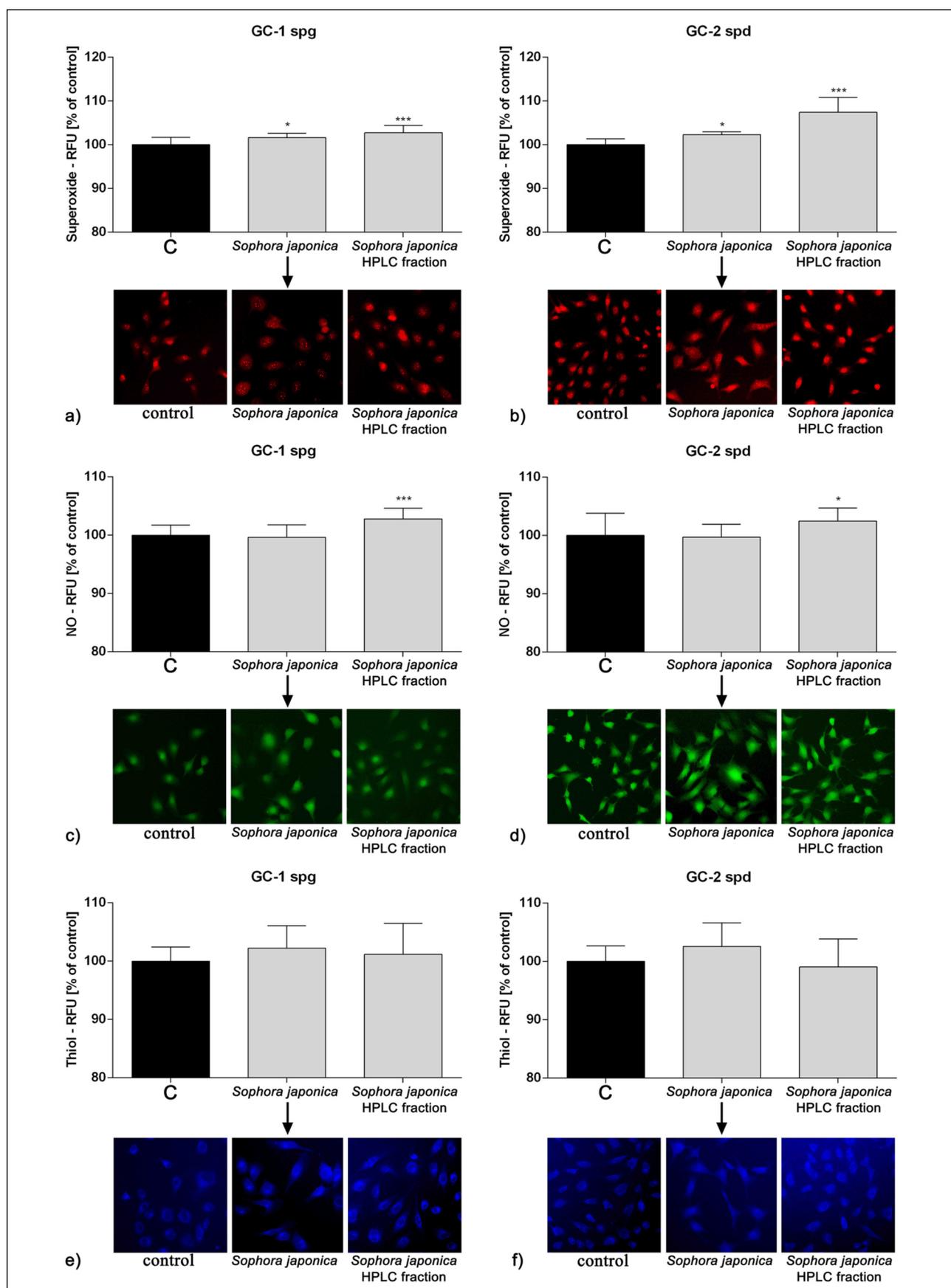
Fig. 4. Effects of *Sophora japonica* extract on spermatogenic cells in terms of superoxide (*a, b*), nitric oxide (*c*, *d*) and Thiol (*e*, *f*) production as relative fluorescence units (RFU). Representative images are shown. Differences between control and test samples were assessed with one-way ANOVA and Dunnett's a post hoc test. A P-value of < 0.05 was considered as statistically significant between groups. Bars indicate SD, n = 3; ***P < 0.001; *P < 0.05; no indication, no statistical significance.
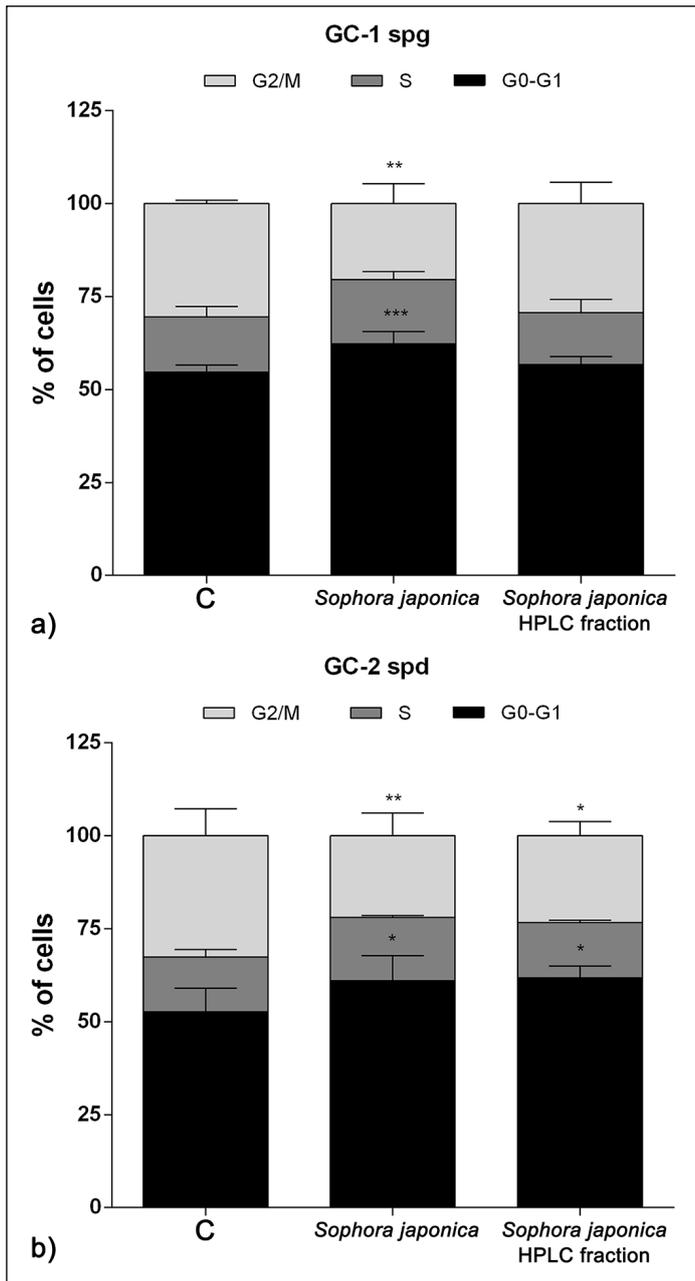
Fig. 5. Effects of *Sophora japonica* extract on the cell cycle distribution in GC-1 spg (*a*) and GC-2 spd cells (*b*). Differences between control and test samples were assessed with one-way ANOVA and Dunnett's a *post hoc* test. A P-value of < 0.05 was considered as statistically significant between groups. Bars indicate SD, n = 3; ***P < 0.001; *P < 0.05; no indication, no statistical significance.

Micronuclei in genotoxicity assessment

Oxidative stress may directly induce DNA damage. For this reason, we decided to investigate the frequency of micronuclei formation (MN) caused by *Sophora japonica* treatment. Percentage of MN in GC-1 spg cells increased to 55.5% while in GC-2 spd cells to 17.7%, which was more by 45, 8 and 15% than in the corresponding controls. On the other hand HPLC fraction caused an increased MN formation up to 14.4% in GC-1 spg and in 6.5% in GC-2 spd, which is 4.7 and 3.5% more in relation to control (*Fig. 6a-6b*).

DISCUSSION

Information on the side effects and safety evaluations for *Sophora japonica* are limited, although this plant is frequently used. Literature data provides, many questions about molecular mechanisms of inhibition of proliferation and apoptosis of tumor cells using flavonoids, alkaloids and other biologically active substances of plant origin. *Sophora japonica* (or its components) can modulate various molecular pathways of tumor development due to the entire spectrum of antitumor biologically active substances: flavonoids and isoflavonoids triterpenes, alkaloids, polysaccharides, amino acids (14, 16). Several *in vivo* and *in vitro* studies have demonstrated the significant inhibitory effect of *Sophora japonica* and its individual components on cancer (14). Oral administration of ethanol extracts of *Flos Sophorae* (50, 100, and 200 mg/kg) to sarcoma 180 (S180) tumor-bearing mice once a day for 21 days significantly inhibited tumor growth by 29%. These extracts also increase the levels of superoxide dismutase and IL-2 and decrease the levels of malondialdehyde, TNF-α, vascular endothelial growth factor, basic fibroblast growth factor, and matrix metalloproteinase in the serum of tumor-bearing mice. *Flos Sophorae* has been also shown to inhibit the proliferation of S180 cells *in vitro* in a dose-
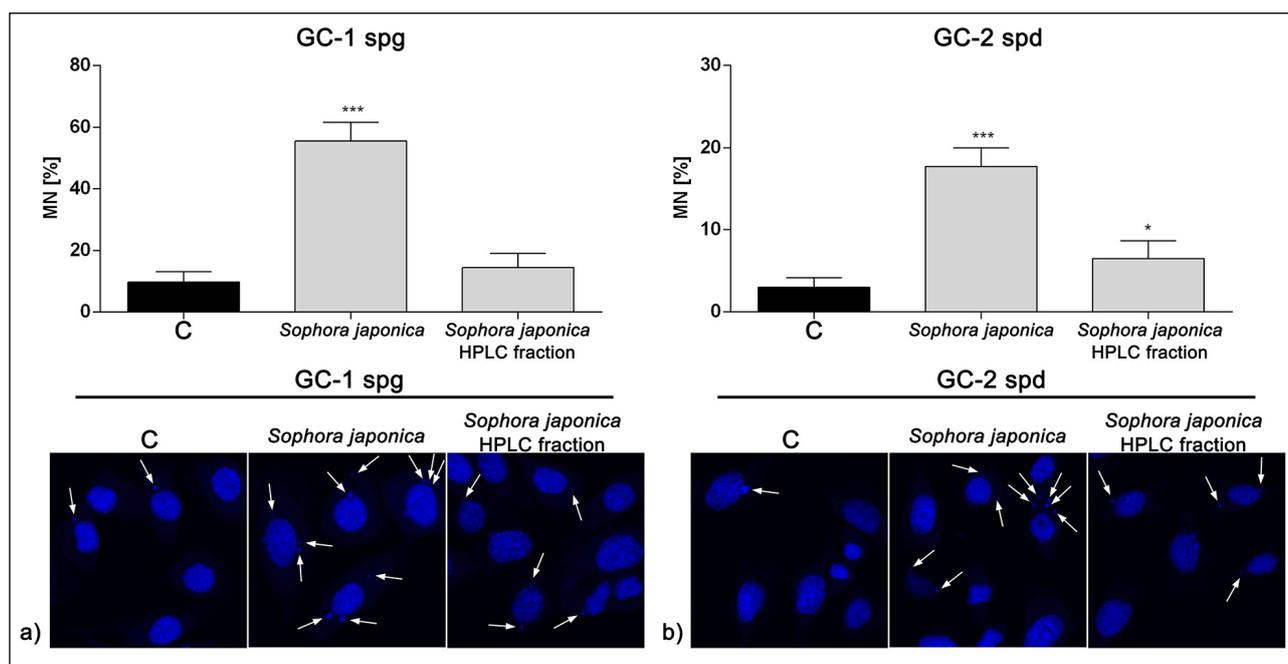
Fig. 6. Effects of *Sophora japonica* extract on micronuclei (MN) formation in GC-1 spg (*a*) and GC-2 spd (*b*) cells. Representative images are shown. Differences between control and test samples were assessed with one-way ANOVA and Dunnett's a post hoc test. A P-value of < 0.05 was considered as statistically significant between groups. Bars indicate SD, n = 3; ***P < 0.001; *P < 0.05; no indication, no statistical significance.

dependent manner (3, 19). The substances responsible for this effect may be phytochemicals such as flavonoids and isoflavonoids mentioned earlier (14).

In this study at the beginning, we identified the content of chlorogenic and caffeic acid as well as glucose, fructose and sucrose in *Sophora japonica* extract using the HPLC method. It is very possible that these polyphenolic compounds, like flavonoids, determine the antioxidant properties of extract tested. Furthermore, they play an immunological role. Our results obtained confirmation in others studies (20).

Further, we showed the presence of β-glucan in *Sophora japonica* extract. Antitumor activity of β-glucans have been extensively studied in fungi and may be responsible for the significant immunomodulatory activity (21).

Next, our results of the MTT assay indicate differences in the metabolic activity of GC-1 spg and GC-2 spd cell lines due to the different concentrations of the *Sophora japonica* extract and its HPLC fractions. Inhibition of cell metabolic activity was dose-dependent. The GC-1 spg cell line was characterized by a higher sensitivity to *Sophora japonica* extract, while GC-2 spd cells less responded by reducing their metabolic activity compared to control cells. The influence of 3% HPLC fraction extract on GC-1 spg was even less harmful. Such a difference in cell metabolic activity may indicate the various content of biologically active substances in the extract and HPLC fractions, while the difference in the results of cell lines tested may result from a different cell types and their function. Literature data supports the inhibitory effect of *Sophora japonica* extract on the cell metabolic activity *in vitro*. The cytotoxic properties of *Sophora japonica* can be also directly linked to alkaloids (17, 22, 23).

The reason of observed metabolic activity decrease can be oxidative stress as the first line of cellular response to the external factors. Recently, several reports have suggested that oxidative stress is a complex mechanism rather than a simple imbalance between the production and elimination of ROS. Oxidants and free radicals are continuously produced in living organisms with endogenous and external sources such as oxygen and nitric oxide. An increase in the normal redox state of a cell causes toxic effects that may lead to cell and tissue damage (16, 24). Also, the oxidative stress is considered to be one of the main factors of infertility and this is why maintaining prooxidant-antioxidant balance parameters such as: melatonin, advanced oxidation protein products (AOPPs) and total antioxidant capacity (TAC) is crucial for the preservation of male fertility (25).

Our studies have revealed significant oxidative stress of cells in the action of *Sophora japonica* extract. The mechanism of toxicity was less related to the production of free radicals and the appearance of oxidative stress compared to HPLC fraction where it was more pronounced. These results may be related to a slight decrease in Thiol pool (reduced glutathione) which could mean an impairment of the mechanism for free radicals scavenging. Undoubtedly mitochondria are the most sensitive structures to ROS. Moreover, there is a direct correlation between ROS levels, AMP-activated protein kinase activity (AMPK) and mitochondrial network. The AMPK activity is essential for the expression of genes involved in the ROS detoxification and the regulation of cellular energy homeostasis. As a consequence, ROS overproduction may exert deleterious effects on the mitochondrial activity and thus induce cytotoxicity through oxidative stress and AMPK dysregulation pathways (26). These data correlate with the results of MTT assay. Others also reported that *Sophora japonica* may activate antioxidants mechanism as the first line of defense against free radical damage (27).

Based on the results so far, we were wondering what could lead to an imbalance in the antioxidant systems of the cells treated. Thus, we evaluated the cell cycle progression resulting from genotoxic events. It is known that under the influence of some chemo-preparations, tumor cells die, mainly through apoptosis and an increase in the number of cells in the G1 phase. As an example, doxorubicin causes the cell cycle arrest in the G2/M phase (28, 29).

The results of our research indicated the cell cycle arrest in G0/G1 phase in response to *Sophora japonica* treatment due to the decrease in the number of cells in the G2/M phase. A similar effect was noted on HPLC fraction relatively to GC-2 spd. Results obtained in cell cycle distribution analysis were correlated with MTT assay. Literature data provides evidence for cell cycle arrest of Hep-2 cells in G0/G1 phase by alkaloid from *Sophora japonica* oxymatrine. Furthermore, the cells in the G0/G1 phase were significantly increased while cells in the S phase were reduced with increasing concentrations of oxymatrine, which suggests that the apoptosis of Hep-2 cells induced by oxymatrine may occur in the G0/G1 phase (30). Flavones and flavonols exert cytotoxic effects by causing G2/M arrest and inducing apoptosis. (18, 31-33).

The marker of genotoxicity is the emergence of a micronuclei appereence. The genotoxic effect of *S. japonica* flavonoids was manifested by the induction of DNA damage, mutations, micronuclei formation both, *in vitro* and *in vivo*. In addition, the mechanism of the genotoxicity of flavonoids is related to the formation of ROS (34, 35). An increase in the percentage of cells in the G0/G1 cell cycle indicates a toxic effect that the cell reacts to and is mainly accompanied by cell death through apoptosis.

Finally, the formation of the micronucleus testifies the genotoxicity of the test compound, the decrease in cell viability, which is a genetic instability marker. We have investigated that the percentage of micronuclei in *GC-1 spg* and *GC-2 spd* cells was 45.8% and 15%, respectively related to control cells. Data provide evidence which components of the *Sophora japonica* extract may by responsible for the observed genotoxic effects (36). Data suggest, that mechanism of genotoxicity of flavonoids, alkaloids are associated with their influence on the topoisomerase II, 5-reductase enzyme inhibition, suppressing the NF-κB, the Akt and MAPK signaling pathways, or the formation of free radicals of oxygen from quinone derivatives (3). Alkaloids may induce the formation of micronuclei or cause mutations in DNA. Furthermore, cytotoxic effects of flavonoids on breast and prostate cancer cells are highly related to the expression of hormone receptors (14).

In conclusion, spermatogonia stem cells have the unique ability for reproduction and production of mature sperm in the testicles. These cells multiply after puberty and commence spermatogenesis. Exposure to *Sophora japonica* affects the quality of male reproductive function. The results indicate that the *Sophora japonica* extract should be used with caution for men, since it can cause genotoxic events on spermatocytes and spermatogenesis, by cell cycle arrest in G0/G1 phase and micronuclei formation. The specific pathways and mechanisms involved in the testicular genotoxicity of *Sophora japonica* need to be investigated.

Consequently, the decrease in viability of GC-1 spg and GC-2 spd cells, cell cycle arrest in G0/G1 phase followed by an increase in micronuclei formation were a significant genotoxic effect of *Sophora japonica*. Our results clearly indicate that the sensitivity of germ cells to Sophora japonica extract is closely related to the stage of their differentiation and metabolic activity. In order to preserve reproductive potential, it is necessary to improve techniques to protect cells from the harmful effects of *Sophora japonica*. This effect can be simultaneously predicted by the outcome of its exposure to the tumor cells. Therefore, before creating phytotherapeutic preparations based on *Sophora japonica* biologically active supplements, it is crucial to adjust the dose of the drug and conditions for its use.

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