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Screening of cytoprotector against methotrexate-induced cytogenotoxicity from bioactive phytochemicals by umu test combined with micronucleus Assay

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To develop new cytoprotector to reduce the risk of second cancers caused by methotrexate-induced cytogenotoxicity, cytoprotective effects of ten kinds of phytochemicals and their combinations were evaluated by umu test combined with micronucleus assay. It is demonstrated that allicin, proanthocyanidins, polyphenols, eleutherosides and isoflavones owned higher antimutagenic activities than other phytochemicals. At the highest dose tested, the MTX genetoxicity was inhibited by 34.03%~67.12%. Of all the bioactive phytochemical combinations, the combination of grape seed proanthocyanidins and eleutherosides from siberian ginseng as well as green tea polyphenols and eleutherosides showed stronger antimutagenic effects, the inhibition rate of methotrexate-induced genotoxicity separately reached 74.7 ± 6.5 % and 71.8 ± 4.7%. The Kunming mice treated by MTX along with bioactive phytochemicals combinations showed significant reduction in micronucleus induction and sperm abnormality rate compared to MTX treated control groups without cytoprotector candidates ($p<0.01$). Moreover, obvious increases in thymus and spleen indices were observed in cytoprotector candidates treated groups. The results indicated that bioactive phytochemicals combinations owned potentials to be used as new cytoprotectors, besides umu test was the same effective assay to evaluate antimutagenic potential of phytochemicals as Ames test.

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Title Page

Title: Screening of cytoprotector against methotrexate-induced cytogenotoxicity from bioactive phytochemicals by umu test combined with micronucleus Assay

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Abstract

To develop new cytoprotector to reduce the risk of second cancers caused by methotrexate-induced cytogenotoxicity, cytoprotective effects of ten kinds of phytochemicals and their combinations were evaluated by umu test combined with micronucleus assay. It is demonstrated that allicin, proanthocyanidins, polyphenols, eleutherosides and isoflavones owned higher antimutagenic activities than other phytochemicals. At the highest dose tested, the MTX genetoxicity was inhibited by 34.03%~67.12%. Of all the bioactive phytochemical combinations, the combination of grape seed proanthocyanidins and eleutherosides from siberian ginseng as well as green tea polyphenols and eleutherosides showed stronger antimutagenic effects, the inhibition rate of methotrexate-induced genotoxicity separately reached 74.7 ± 6.5 % and 71.8 ± 4.7%. The Kunming mice treated by MTX along with bioactive phytochemicals combinations showed significant reduction in micronucleus induction and sperm abnormality rate compared to MTX treated control groups without cytoprotector candidates ($p<0.01$). Moreover, obvious increases in thymus and spleen indices were observed in cytoprotector candidates treated groups. The results indicated that bioactive phytochemicals combinations owned potentials to be used as new cytoprotectors, besides umu test was the same effective assay to evaluate antimutagenic potential of phytochemicals as Ames test.

Key words: cytoprotector; methotrexate; phytochemical; umu test

Introduction

Methotrexate (MTX) is one of the most intensively investigated and effective chemotherapeutic agents by inhibiting dihydrofolate reductase, resulting in depletion of tetrahydrofolate. However, the genotoxic effects of MTX have been reported in the somatic cells employing chromosome aberration and micronucleus test as the end points of evaluation (Choudhury et al. 2004). A present study has confirmed that MTX induced cytotoxic and genotoxic effects in the germ cells (Padmanabhan et al. 2008). Furthermore, MTX-treated RA patients have an increasing incidence of melanoma, non-Hodgkin's lymphoma, and lung cancer (Buchbinder et al. 2008). And, MTX chemotherapy for gestational trophoblastic tumors and acute lymphoblastic leukemia really increases the risk of second tumors (Schmiegelow et al. 2009). Thus, the development of efficient protective agents which could reduce the risk of second cancers caused MTX has attracted more and more attention.

In recent years，a number of protective compounds have been investigated as cytoprotectors to defend normal cells from the damage induced by MTX. Horie et al. reported that aged garlic extract could protect IEC-6 cells from the MTX-induced intestinal damage (Horie et al. 2006). Verschaeve et al. found that apricot and β-carotene treatment could alleviate the impairment of oxidative stress and ameliorate MTX-induced intestine damage (Verschaeve & Van Staden 2008). Chang et al. showed that MTX-induced apoptosis of IEC-6 cells could be repressed by the pre-treatment of lutein (Chang et al. 2013). Daggulli et al. also observed that carvacrol significantly reduced deleterious effects of MTX on testicular tissue (Daggulli et al. 2014). Contrasting with garlic, apricot, *Origanum onites L*. panax ginseng and American ginseng, cytoprotective effect of siberian ginseng extract has been reported rarely. Moreover, most of the previous studies are focused on the cytoprotection of individual plant extracts or combined with other agents, such as β-carotene and quercetin. Studies on the protective potentials of various bioactive phytochemicals, such as siberian ginseng eleutherosides, chrysanthemum chlorogenic acid, ginger root gingerols, grape seed proanthocyanidins, green tea polyphenols and so on, especially plant extract combinations have received less attention. To develop more efficient protective agent against methotrexate-induced cytogenotoxicity, cytoprotective activity of ten different extracts and some extracts combinations were evaluated in vivo and in vitro tests.

Materials and methods

Materials

S. typhimurium TA1535/pSK1002 was kindly provided by Dr. Yoshimitsu Oda (Osaka Prefectural Institute of Public Health, Osaka, Japan). 4-NQO was used as a positive control. DMSO served as control and solvent. All bioactive phytochemicals were purchased from Changsha Active Ingredients Group Inc (China). Kunming specific pathogen-free mice (4–6 weeks old, average body weight 19 ± 2 g) were provided by the Henan Laboratory Animal Center. License number: SCXK (Yu) 2005-0001. The present study was conducted in accordance with the principles outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Umu test

The umu test was performed according to the method of Oda (Oda et al., 1985). The induction units (IU) of β-galactosidase activity, was calculated according to the following equation:

$IU=1000(A_{420}–1.75\times A_{550})/25\times 0.1\times A_{600}$.

Induction ratio (R) was calculated according to the following equation:

R=sample IU/control IU.

Assay of bone marrow micronucleus and indices of thymus, spleens in mice

Thirty - two mice were randomly divided into four groups with eight each group (4 males and 4 females). The combination of bioactive phytochemicals was prepared by dissolving the two bioactive phytochemicals in DMSO, later diluted it with distilled water to an effective component concentration of 50mg L^{-1} for each bioactive phytochemical. The combination of bioactive phytochemicals was administered one week prior to the MTX exposure. Treatment group Ⅰ: mice were given a combination of green tea polyphenols and eleutherosides from Siberian ginseng (0.2ml/10g·W, i.g. once daily) for 15 days, and a single dose of MTX (2mg kg^{-1}, i.p. once daily) was added on the 8th day. Treatment group Ⅱ: mice were given a combination of grape seed proanthocyanidins and eleutherosides from Siberian ginseng for 15 days, and MTX was administered on the 8th day in a similar manner.

Model group: animals received distilled water instead of bioactive phytochemicals combinations for 15 days and the same MTX protocol applied to this group on the 8th day. Control group: mice were given distilled water through 15 days and physiological saline instead of MTX was administered on the 8th day in a similar manner. Twelve hours after the final doses, the animals were euthanized by cervical dislocation.

The micronucleus assay was performed according to the method of Schmid (Schmid, 1975). Femurs were removed from the animals, bone marrow extracted with fetal calf serum and maintained at 37°C. The material was homogenized, transferred to a centrifuge tube and centrifuged at 1,000× g for 5 minutes. The supernatant was discarded and samples were prepared with the remaining cells. Then, samples were allowed to air dry and 24 hours later they were fixed in absolute methanol during 5 minutes. After staining with 2% Giemsa stain diluted with distilled water. Slides were coded and from dry slides about 1500 tinge blue coloured polychromatic erythrocytes were scanned from each animal, and the incidence of micronuclei in polychromatic (PCEs) and normochromatic (NCEs) erythrocytes was counted and the PCEs/NCEs ratios were scored.

The thymus and spleen indices were assayed according to the method (Zhang et al., 2003) and calculated as follows:

thymus or spleen index = thymus or spleen weight/body weight × 1000.

Sperm Deformity Test in mice

Twenty-four male mice were randomly divided into four groups with six each group. MTX and the combination of bioactive phytochemicals were administered into the body by using the above-mentioned methods. Twelve hours after the final doses, the mice were sacrificed by cervical dislocation to get the bilateral epididymis. Sperm deformity test was carried out according to the following method of Wyrobek et al. Two sperm suspensions were prepared from the caudal of each testis by mincing the caudal in physiological saline (Wyrobek et al., 1983). The sperm was spread on a slide glass and stained with 1% Eosine Y for 45 min after which the slides were air dried. 1000 sperm cells of mice were assessed for morphological abnormalities under oil immersion at × 1,000 magnification. Sperm head morphology was scored under the category of normal, sperm without hook, amorphous head, banana head and triangular head.

Statistical analysis

Values are presented as means ± standard deviation (SD).The data were analyzed for statistical significance using t-test (SPSS 13.0 for Windows). A p value of less than 0.05 was deemed as significant.

Results

Screening of cytoprotector derived bioactive phytochemicals based on SOS/umu test

Effects of ten different plant extracts on umu gene expression in *S. almonella typhimurium* TA1535/pSK1002 exposed to MTX were shown in Table 1. It was demonstrated that allicin, proanthocyanidins, polyphenols, eleutherosides and isoflavones showed stronger antimutagenic activities than other five kinds of plant extracts. At the highest dose tested, the MTX genetoxicity was inhibited by 34.03%~67.12%. Antimutagenic potential of plant extract combinations were illustrated in Fig.1 Of all the plant extract combinations, the combination of grape seed proanthocyanidins and eleutherosides from siberian ginseng as well as green tea polyphenols and siberian ginseng extract exhibited higher cytoprotective activity. The inhibition rate of methotrexate-induced genotoxicity separately reached 74.7 ± 6.5 % and 71.8 ± 4.7%. Thus, the both combinations of plant extracts were selected as cell protective agent candidates, and the cytoprotective effects of candidates would be subsequently assessed by micronucleus test and sperm malformation test in vitro.

Evaluation of the antimutagenic potentials of cytoprotector candidates by micronucleus Test

The micronucleus assay is internationally recognized as the standard method to detect mutagenicity of chemicals. In order to assess the protective effects of the candidate cytoprotectors against the genotoxicity caused by MTX, micronucleus assay was involved in the next trial. With administration of the candidate plant extract combinations, the data of bone marrow polychromatic erythrocyte micronucleus test in mice exposed to MTX were presented in Table 2. Whether male or female, there were statistically significant increases (p<0.01) in the frequency of micronucleated polychromatic erythrocytes and ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) in model group and control group. But, the treatment of the candidate cytoprotectors markedly

decreased the incidence of mice bone marrow micronucleus, and improved the ratio of PEC and NEC. Furthermore, there were significant difference between treatment groups and model group (p<0.01). In terms of the inhibition of micronucleus formation, the combination of grape seed proanthocyanidins and eleutherosides from siberian ginseng was superior to the combination of green tea polyphenols and eleutherosides. Meanwhile, the two treatment groups did not cause differences between male and female mice. It could be considered that the two combinations had a marked effect on suppression of MTX-induced micronuclei in mice bone marrow cells.

Influence of cytoprotector candidates on reproductive toxicity induced by MTX

To investigate whether the combinations of bioactive phytochemicals treatment could strengthen or weaken the reproductive toxicity induced by MTX, sperm tests had been adopted. The method is one reliable and easy way to detect reproductive toxicity. In this study, the kinds of abnormal sperm in any group were mainly no hooks, amorphous, bananas and triangular head heads. As could be seen from Table 3, the incidence of mouse sperm head deformity of treatment groups had no significant difference from that of the control (p>0.05). Significant differences could be observed between the treatment groups and model groups (p< 0.01). The summary of no hooks and amorphism heads in model accounted up to 90% of the total sperm head morphology, significantly higher than that of control group and treatment group. The results illustrated that the two combinations of bioactive phytochemicals may alleviate the reproductive toxicity caused by MTX.

Influence of cytoprotector candidates on immune organ indices

As cytoprotector candidates, the effect of two combinations of bioactive phytochemicals on thymus and spleen indices of mice exposed to MTX was shown in Table 4. The thymus and spleen indices were similar between control and the treatment groups in both male and female mice. MTX exposure reduced thymus and spleen indices of mice. Kawai reported that MTX could markedly decrease white blood cells, thymic and splenic lymphocytes at dose ≥ 5 mg/kg (Kawai, 2014). But, there was a significant difference between the treatment plus control group and the model group (p< 0.01). The combination of proanthocyanidins and eleutherosides obviously diminished the effects of MTX exposure on indices of thymus and spleens in mice. And, the index increase of spleens in the treatment of proanthocyanidins and eleutherosides combination was higher than that of polyphenols and eleutherosides combination administration. Our findings implied that the two combinations of bioactive phytochemicals not only could affect the immune organ indices of experimental animal Kunming mice, but also could effectively relieve the immune toxicity caused by MTX.

Discussion

The effective estimating for antimutagenic properties of bioactive phytochemicals includes the bacterial gene mutation assay (Ames test (Verschaeve and Van Staden, 2008; Horn and Vargas, 2003), mammalian cell culture benzo(a)pyrene metabolism assay (Cassady et al., 1988), the mammalian cell gene mutation assay (Mersch-Sundermann et al., 2004), and the in vitro micronucleus assay (Serpeloni et al., 2008). Compared with animal-cell-based systems, microbe-based assays for screening cytoprotectors present several advantages, such as the simplicity of the procedures, the relatively short time needed to obtain results, and cost-effective. Nevertheless, except for Ames test, there are few reports in the literature describing the studies of evaluation for antimutagenic properties of bioactive phytochemicals by umu test so far. In this study, we selected a short term bacterial test systems, umu test, to evaluate antimutagenic potential of plant extracts. According to above results, it was deduced that the umu test own the ability to assess antimutagenic activity of plant extracts. Moreover, based on comparison of umu test results (486 chemicals) with Ames test (274 compounds) as well as rodent carcinogenicity data (179 compounds), Reifferscheid and Heil found good agreement between umu test and Ames test results (Reifferscheid & Heil 1996). Thus umu test could be developed an effective high-throughput screening assay to evaluate antimutagenic potential of phytochemicals same as Ames test and comet assay.

Natural plant medicine is a very important resource to develop new cytoprotectors. Cytoprotective effect of many bioactive phytochemicals has been proved in the past several decades. For example, Isoflavones from soybean seeds have showed antimutagenic activity in *S. typhimurium* TA1535/pSK1002 and TA100 (Miyazawa et al., 1999). And then garlic extract has been proved that it could reduce apoptotic cell injury induced by MTX. It should be noted that garlic was most popular supplement in U.S. households (Amagase et al., 2001). Meanwhile, polyphenols from green tea (Chinese Gunpowder and Japanese Sencha) exhibited high antimutagenic activity in the Ames test as well as in *S. cerevisiae* D7 test. Moreover, in the peripheral blood lymphocytes method reduced number of abberant

cells as well as decreased number of chromosome breaks was observed using both green tea extracts (Bunkova et al., 2005). What's more, grape seed extract played a role in attenuating the genotoxicity induced by cisplatin (Attia et al., 2008). Contrasting with allicin, proanthocyanidins, polyphenols and isoflavones, cytoprotective effect of eleutherosides from siberian ginseng has been reported rarely. Our results showed that single eleutherosides and its combinations with proanthocyanidins, polyphenols had strong antimutagenic effect. Previous research found that water extract of Siberian ginseng showed significant antioxidant activity and protective effect against oxidative DNA damage induced by H_2O_2 (Park et al., 2006). Moreover, the methanol extract of root, stem, and leaf of *Eleutherococcous senticosus* showed inhibitory effects on the mutagenicity induced by 2-AF or Trp-P-1 in *S. typhimurium* TA98 (Park et al., 2002). It was considered that the detrimental effects of MTX was partly due to its direct toxic action by increasing reactive oxygen species production, although the exact mechanisms of methotrexate-induced toxicity had not yet been elucidated to date (Oktem, 2006). Therefore, we inferred that the cytoprotector candidates may play a key role in attenuating the methotrexate-induced cytogenotoxicity due to their antimutagenic and antioxidant activity.

In addition, the previous study had proved that MTX could bring out the reproductive toxicity (Padmanabhan et al., 2008). MTX treatment significantly reduced the sperm count and increased the occurrence of sperm head abnormalities. However, the administration of phytochemicals combinations to Kunming specific pathogen-free mice presented clearly decreases in sperm abnormality rate in the case of MTX exposure. Akram et al (2012) ever reported that American ginseng extract treatment exhibited therapeutic effects on sperm parameters in rats treated with Cyclophosphamide (CP), which is an antineoplastic agent and immunosuppressive medicine in the treatment of various types of tumors, and autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis (Tripathi and Jena, 2009). Moreover, ginseng has been demonstrated to have a cytoprotective effects against these toxins, in which administration of *Panax ginseng* extract is reported to markedly decrease the 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced pathological and genotoxical damages in rat testes (Lee et al., 2007). Recent studies had confirmed that green tea and soybean extracts showed protective effects against reproductive toxicity induced by CP. Fahmy et al (2014) found a significant decrease in the percentage of sperm abnormalities in orally administrated soybean extracts. Zanchi et al (2015) demonstrated that polyphenols improved CP-induced damage on reproductive system, and its effect is probably due to high concentrations of catechins and antioxidant activity. According to the above results, it can be deduced that the combination of grape seed proanthocyanidins and siberian ginseng eleutherosides as well as green tea polyphenols and eleutherosides was able to weaken the reproductive toxicity induced by MTX.

Conclusions

In this study, we developed an effective screening assay to evaluate antimutagenic potential of bioactive phytochemicals based on umu assay coupled with micronucleus test. Allicin, proanthocyanidins, polyphenols, eleutherosides and isoflavones showed higher antimutagenic activity than other phytochemicals. Moreover, of all the bioactive phytochemicals combinations, the combination of proanthocyanidins and eleutherosides as well as polyphenols and eleutherosides showed higher cytoprotective effects. In the case of MTX exposure, the administration of the two combinations of bioactive phytochemicals to Kunming mice presented clearly decreases in micronucleus induction and sperm abnormality rate. The results implied that phytochemicals combination of proanthocyanidins, eleutherosides and polyphenols could be used as new cytoprotectors.

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299 Table 1. Effects of bioactive phytochemicals on *umu* gene expression by MTX (50 μg ml^{-1}) in *Salmonella typhimurium* TA1535/pSK1002

Phytochemicals	Extracts Effective Components Concentration (mg ml^{-1})	R	Inhibition (%)	Phytochemicals	Extracts Effective Components Concentration (mg ml^{-1})	R	Inhibition (%)
Control	0	9.52±0.82					
Chlorogenic Acid (Chrysanthemum)	0.0001	9.11±0.58	4.31	Proanthocyanidins (Grape seed)	0.001	9.33±0.48	2.00
	0.001	9.02±0.65	5.25		0.01	8.73±0.69	8.30
	0.01	8.9±0.49	6.51		0.1	7.57±0.52**	20.48
	0.05	9.33±0.51	2.00		1	6.50±0.53**	31.72
	0.25	8.67±0.57	8.93		5	5.76±0.61**	39.50
Allicin (Garlic)	0.001	7.33±0.54**	23.00	Polyphenols (Green tea)	0.01	9.16±0.50	3.78
	0.01	6.23±0.62**	34.56		0.1	8.02±0.55**	15.76
	0.05	5.85±0.66**	38.55		1	6.78±0.46**	28.78
	0.25	5.36±0.54**	43.70		10	5.52±0.52**	42.02
	1.25	4.84±0.71**	49.16		50	4.23±0.66**	55.57
Gingerols (Ginger root)	0.001	9.27±0.52	2.63	Polysaccharides (Reishi mushroom)	0.001	10.21±0.62	-
	0.01	9.81±0.66	-		0.01	9.35±0.56	1.79
	0.1	8.98±0.54	5.67		0.1	8.79±0.48	7.67
	1	9.04±0.59	5.04		1	8.32±0.49*	12.61
	5	8.86±0.58	6.93		5	7.06±0.57**	25.84
Ginkgo flavone (Ginkgo leaf)	0.01	8.95±0.53	5.99	Eleutherosides (Siberian Ginseng root)	0.001	8.29±0.76*	12.92
	0.1	9.43±0.43	0.95		0.01	7.43±0.71**	21.95
	1	9.21±0.49	3.26		0.1	4.86±0.60**	48.95
	5	8.58±0.58	9.87		0.5	3.93±0.77**	58.72
	25	7.54±0.60**	20.80		1.5	3.13±0.87**	67.12
Ginsenosides (Ginseng root)	0.01	9.76±0.48	-	Isoflavones (Soybean)	0.001	8.91±0.72	6.41
	0.1	9.82±0.57	-		0.01	8.19±0.70*	13.97
	0.5	8.59±0.55	9.77		0.1	7.43±0.57**	21.95
	2.5	8.76±0.49	7.98		1	6.85±0.55**	28.05
	12.5	8.97±0.56	5.78		5	6.28±0.77**	34.03

300

301 Note: Inhibition(%)=100×($R_{control}$-R_{sample})/ $R_{control}$; $^{*}p<0.05$, $^{**}p<0.01$.

302

303 Table 2 Effect of cytoprotector candidates on incidence of micronucleated polychromatic erythrocytes in bone marrow cells and thymus and spleen indices of
304 mice treated with Methotrexate

Groups	Number of mice treated/sex	Total number of PCE observed	Number of PCE with MN	Total number of MN	Average micronucleus rate per 1000 PCE±S.D.	PCEs/NCEs
Control	4 F	6246	16	16	2.56±0.52	0.92±0.13
	4 M	6098	25	27	4.10±0.60	0.89±0.18
Model	4 F	6158	109	115	17.68±3.20$^{\Delta}$	0.65±0.21$^{\Delta}$
	4 M	6212	124	131	19.97±3.50$^{\Delta\Delta}$	0.59±0.14$^{\Delta}$
Treatment Ⅰ	4 F	6176	79	82	12.83±2.71 **	0.78±0.17 **
	4 M	6138	81	86	13.22±2.78**	0.75±0.23 **
Treatment Ⅱ	4 F	6143	58	63	9.42±2.16 $^{**\#\#}$	0.81±0.15 **
	4M	6112	65	69	10.67±2.36$^{**\#\#}$	0.79±0.22 **

305 Note: 1) Control Group = saline; Model Group = MTX +normal saline; Treatment Group Ⅰ = MTX + combination of polyphenols and eleutherosides; Treatment
306 Group Ⅱ = MTX + combination of proanthocyanidins and eleutherosides. 2)F = female mice; M = male mice; 3)$^{\Delta\Delta}P$<0.01 vs control. 4) $^{**}P$<0.01 vs Model
307 Group. 5) $^{\#\#}P$<0.01 vs Treatment Ⅰ.
308

309 Table 3 Effect of cytoprotector candidates on sperm head abnormalities of male Kunmin mice after consecutive 7

310 days of MTX treatment

Groups	Number of mice treated	Total number of sperm observed	Sperms with abnormal head	Incidence of sperm head abnormalities	Constituent of different sperma with abnormal head morphology %			
					Lack hook	Amorphous	Banana-like	triangular
Control	6	6000	128	2.13±0.45	37.5	49.2	8.6	4.7
Model	6	6000	283	4.72±0.67$^{\Delta}$	42.4	50.9	5.3	1.4
Treatment	6	6000	163	2.74±0.51**	39.9	47.2	9.8	3.1
Treatment	6	6000	156	2.60±0.38**	36.5	48.8	10.2	4.5

311 Note: 1) Control Group = saline; Model Group = MTX +normal saline; Treatment Group Ⅰ = MTX + combination of
312 polyphenols and eleutherosides; Treatment Group Ⅱ = MTX + combination of proanthocyanidins and eleutherosides.
313 2)$^{\Delta\Delta}P$<0.01 vs control. 3) $^{**}P$<0.01 vs Model Group.
314
315

316 Table 4 Effect of cytoprotector candidates on thymus and spleen indices of mice exposed to Methotrexate

Groups	Number of mice treated /sex	Thymus index (mg g^{-1})	Spleen index (mg g^{-1})
Control	4 F	3.27±0.65	4.41±0.53
	4 M	3.18±0.83	4.63±0.61
Model	4 F	1.36±0.77△△	2.25±0.46△△
	4 M	1.54±0.58△△	2.12±0.57△△
Treatment Ⅰ	4 F	2.12±0.48**	3.85±0.54**
	4 M	2.27±0.52**	4.08±0.38**
Treatment Ⅱ	4 F	3.08±0.79**	3.98±0.63**
	4M	2.96±0.66**	4.24±0.85**

317

318 Note: 1) Control Group = saline; Model Group = MTX +normal saline; Treatment Group Ⅰ = MTX + combination of
319 polyphenols and eleutherosides; Treatment Group Ⅱ = MTX + combination of proanthocyanidins and eleutherosides. 2)F
320 = female mice; M = male mice; 3)△△P<0.01 vs control. 4) **P<0.01 vs Model Group.

Figure 1. Effects of phytochemical combinations on umu gene expression in S. typhimurium TA1535/pSK1002 exposed to MTX (50 mg L-1).

Each expermental condition was independently repeated three times. Values shown are mean ± SD of three replicate experiments. A: Garlic allicin, B: Grape seed proanthocyanidins, C: Green tea polyphenols, D: Siberian ginseng Eleutherosides, E: Soybean isoflavones, AB: Garlic allicin + Grape seed proanthocyanidins; AC: Garlic allicin + Green tea polyphenols, AD: Garlic allicin + Siberian ginseng eleutherosides, AE: Allicin + Soybean isoflavones, BC: Grape seed proanthocyanidins + Green tea polyphenols, BD: Grape seed proanthocyanidins + Siberian ginseng eleutherosides, BE: Grape seed proanthocyanidins + Soybean isoflavones, CD: Green tea polyphenols + Siberian ginseng eleutherosides, CE: Green tea polyphenols + Soybean isoflavones, DE: Siberian ginseng eleutherosides + Soybean isoflavones. The concentration of each bioactive phytochemical was 1mg ml-1. **$p<0.01$ as compared to control, a: $p<0.01$ as compared to D, b: $p<0.05$ as compared to D.