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Genotoxicity of Selected Chinese Medicinal Plants, *Elephantopus Scaber*, *Glycyrrhiza Uralensis* and *Salvia Miltiorrhiza* on *Allium Cepa* Assay

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Abstract

The genotoxic effects of leaf and root extracts of *Elephantopus scaber*, root extracts of *Salvia miltiorrhiza* and *Glycyrrhiza uralensis* on the mitotic cells in *Allium cepa* root tips were studied. The crude extracts of 1 µg/ml, 50 µg/ml, 500 µg/ml and 1000 µg/ml were tested on root meristems of *A. cepa*. Quercetin was used as positive control and distilled water as negative control. The result showed that mitotic index decreased as the concentrations of crude extracts increased. The increase of the genotoxic effect corresponds to a decrease of mitotic activity. A dose-dependent increase of chromosome aberrations was observed. Abnormalities scored were stickiness, c-mitosis, bridges and vagrant chromosomes. Result of this study confirmed that the methanol leaf extracts of *E.scaber* exerted significant genotoxic effects followed by methanol root extracts of *S. miltiorrhiza* at 1000 µg/ml respectively.

Keywords: Genotoxicity; *Allium cepa*; Quercetin; Mitotic index; Chromosome aberrations

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Introduction

Worldwide, herbal medicine has become one of the most common forms of alternative therapy. The popularity of herbal medicines is associated with their easy access, therapeutic efficacy, relatively low cost, and assumed absence of toxic side effects. In Malaysia, Chinese herbal medicine is gaining attention particularly in Chinese community. Herb that may be safe in small doses may become dangerous in higher doses. The risk of overdose is higher in herbal preparations than conventional medicines due to the product variability. There may be problems arise due to the lack of adequate regulations, the pharmacological complexity of herbal products, and the lack of studies on the pharmacology and toxicity of the compounds [1].

Elephantopus scaber is a common herb of tropical countries commonly known as 'Tutup bumi'. It is known for its medicinal properties and was reported to possess antimicrobial activity [2]. Recently, Daisy et al. [3] remarked on *E. scaber* to possess anti-inflammatory and antitumour activities in animal models. *Glycyrrhiza uralensis* is a perennial herb known as the Chinese licorice. Licorice has long been valued for therapeutic use for fevers, liver ailments, dyspepsia, gastric ulcers, asthma, bronchitis, Addison's disease and rheumatoid arthritis and has been used as a laxative, antitussive and expectorant [4]. *Salvia miltiorrhiza* is an annual sage plant and among the most popular medicinal herbs. It is a hardy perennial growing to 80 cm, with toothed oval leaves and clusters of purple flowers [5]. It is commonly used either on its own or in combination with other herbs based on the concepts of traditional Chinese medicine.

Recent studies have shown that long-term exposures to herbal products might be associated with increases in the rates of morbidity and mortality. In addition to systemic toxicity, the possible genotoxicity of herbal products has been investigated in recent years. The aim of this study was to contribute to a better understanding of the genotoxic effect of of leaf and root extracts of *E. scaber*, root extracts of *S. miltiorrhiza* and *G. uralensis* using the *in vitro* mutagenicity bioassay on mitotic cells in *A. cepa* root tips.

Materials and Methods

Plant source

Fresh plant samples of *E. scaber* were collected on August 2009 from home garden, Bukit

Table 1: Types and frequencies of chromosome aberrations in treatments of different concentrations.

Treatments Concentration (µg/ml)	Mitotic Index	Chromosome aberrations				
		Stickiness	Bridges	C-mitosis	Vagrant	% of aberration cells
E. scaber Leaf (Methanol)						
1	0.41 ± 0.017	1	3	0	0	0.44
50	0.40 ± 0.021	2	4	0	0	0.67
500	0.27 ± 0.039	30	16	0	7	3.67
1,000	0.26 ± 0.045	48	24	0	13	9.44
E. scaber Leaf (Water)						
1	0.47 ± 0.022	0	1	0	1	0.22
50	0.46 ± 0.032	1	0	0	1	0.22
500	0.44 ± 0.019	3	5	1	0	1.00
1,000	0.44 ± 0.030	9	9	0	3	2.33
E. scaber Root (Methanol)						
1	0.44 ± 0.017	0	1	0	0	0.11
50	0.43 ± 0.017	1	2	0	0	0.33
500	0.40 ± 0.021	4	3	0	0	0.78
1,000	0.31 ± 0.014	5	6	0	1	1.33
E. scaber Root (Water)						
1	0.47 ± 0.024	2	1	0	0	0.33
50	0.45 ± 0.018	1	4	0	0	0.56
500	0.44 ± 0.014	6	7	0	1	1.56
1,000	0.43 ± 0.017	8	11	0	2	2.33
G. uralensis Root (Methanol)						
1	0.44 ± 0.016	0	1	0	1	0.22
50	0.42 ± 0.015	3	4	0	0	0.78
500	0.40 ± 0.023	5	8	0	1	1.56
1,000	0.15 ± 0.013	11	2	0	3	1.78
G. uralensis Root (Water)						
1	0.49 ± 0.025	1	4	0	0	0.44
50	0.47 ± 0.022	2	4	0	0	0.67
500	0.45 ± 0.016	3	7	0	1	1.22
1,000	0.38 ± 0.019	12	17	0	3	3.56
S. miltiorrhiza Root (Methanol)						
1	0.41 ± 0.018	4	3	0	0	0.78
50	0.38 ± 0.014	8	7	0	1	1.78
500	0.31 ± 0.013	9	7	1	0	1.89
1,000	0.19 ± 0.009	29	1	2	1	3.67
S. miltiorrhiza Root (Water)						
1	0.46 ± 0.023	2	3	0	1	0.67
50	0.43 ± 0.024	2	6	1	0	1.00
500	0.39 ± 0.025	5	6	0	1	1.33
1,000	0.32 ± 0.014	14	12	4	5	3.88
Quercetin						
1	0.32 ± 0.019	1	2	0	0	0.33
50	0.29 ± 0.023	1	7	0	2	1.00
500	0.24 ± 0.015	7	16	0	3	2.88
1,000	0.20 ± 0.014	22	18	1	8	5.44

Mertajam, Pulau Pinang. Samples of *S. miltiorrhiza* and *G. uralensis* were bought from a Chinese herbal shop in Kulim.

Plant extraction

The leaves and roots of *E. scaber* were dried under shed for about two weeks. *S. miliorrhiza* and *G. uralensis* were already in dried condition. The dried leaves and roots of *E. scaber* and dried roots of *S. miliorrhiza* and *G. uralensis* were extracted with methanol and water for 48 hours respectively. The extracts were filtered using filter papers. All the crude extracts were then evaporated to dryness using rotary-evaporator; BUCHI (model R-210).

Allium Cepa Assay

Pre-treatment

A. cepa bulbs were grown in tap water at room temperature for 2 to3 days. When the roots were 2cm to 4 cm in length, the bulbs were treated with different concentrations of the crude extracts (1, µg/ml 50, 500 µg/ml, 1,000 µg/ml). Another set of plants was placed in quercetin (1 µg/ml, 50 µg/ml, 500 µg/ml, 1,000 µg/ml) as positive controls while for the negative control, a set of *A. cepa* was growing in water. The solutions were changed daily and after 48 hours, root tips from each bulb was harvested, fixed in Carnoy's fixative (1:3 acetic acid: alcohol) for 24 hours. It was then stored in 70% alcohol [6].

Slides preparation

Preparation of slides was carried out as described by Sharma and Sharma [7]. After pre-treatment, the root tips were washed a few times with distilled water. They were hydrolyzed with 1N HCl at 60°C to 70°C for 5 minutes. After hydrolysis, the roots were washed. Then, about 1mm to 2 mm of the root tips were cut and placed on the slide. A small drop of aceto-orcein was dropped on the root tip and wait for 2 min. The root tip was then squashed with metal rod and another small drop of aceto-orcein was added and waited for another 2 min. The cover slip was carefully lowered on to avoid air bubbles and the sides of the slides were sealed with clear fingernail polish. The experiment was replicated 3 times. Thus, nine slides were prepared for each treatment.

Observation of specimens

The slides were observed under the light microscope at 400x and 630x magnification. The Leica Zeiss Light microscope with digital camera and Leica QWin software was used in order to get the clear image of the chromosome aberrations. Photomicrographs were made and 100 cells per slide were analysed. The mitotic index was determined by the examination and counting of cells in mitotic phases from among 100 cells per slide. Calculation of mitotic index is as follows

Mitotic index= Number of cells in mitosis/Total number of cells

Statistical Data Analysis

Data obtained from the mitotic index calculation were analysed using Analysis of Variance Technique (ANOVA) at significant level of $p<0.05$ using SPSS Program Version 17. Duncan's multiple range test was performed to determine the significant differences between treatments ($p<0.05$). The results of analysis were useful to recognize the difference between the numbers of abnormal chromosome cells treated by various concentrations of crude extracts from sample plants.

Results

Table 1 represents the effect of aqueous and methanol extracts

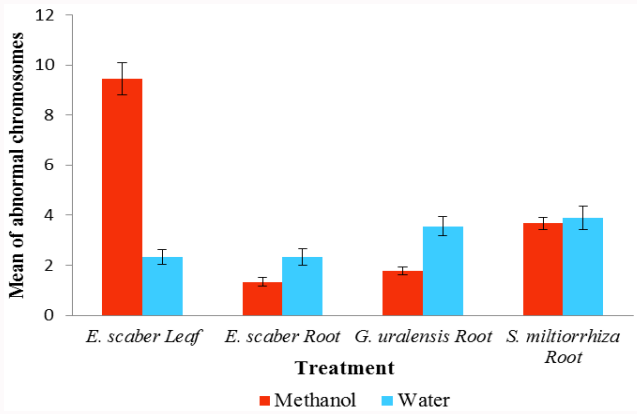

Figure 1: Mean of abnormal chromosomes following methanol and water extracts of treatments at 1,000 µg/ml.

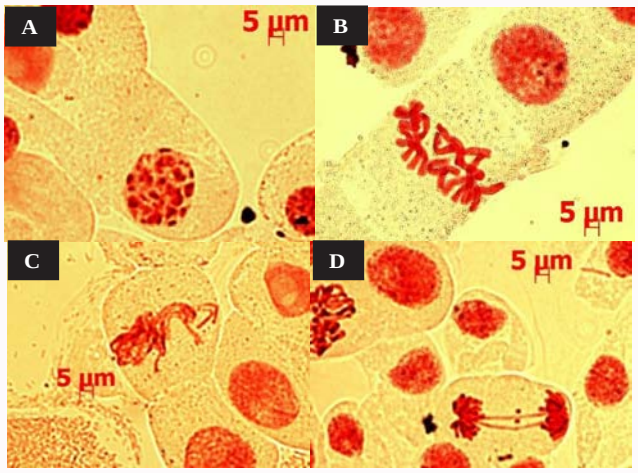

Figure 2: Chromosome aberrations observed (A): Stickiness, (B): C-mitosis, (C): Vagrant, (D): Chromosome bridges (Magnification: 630x).

of *E. scaber*, *G. uralensis* and *S. miltiorrhiza* on mitotic index. A concentration dependent decrease of mitotic index was observed in both methanol and water extracts. As shown in Table 1, the mitotic index for *E. scaber* methanol leaf crude extract decreased significantly at 500µg/ml and 1000 µg/ml. The mitotic indexes were 0.27 and 0.26 respectively as compared to mitotic index at 1 µg/ml and 50 µg/ml which were 0.41 and 0.40 respectively. Mitotic index for *G. uralensis* methanol root crude extract decreased obviously at 1000 µg/ml with mitotic index of 0.15 (Table 1). For water extract of leaf and root *E. scaber*, the mitotic index decreased slightly between the rate of 2.17% and 4.55%. For water extract of *G. uralensis,* the same result obtained but mitotic index decreased significantly at 1000 µg/ml. For water extract of *S. miltiorrhiza,* the mitotic index decreased at the same rate. Overall, the mitotic indexes in treated cells were lower compared to the distilled water (negative control) which was 0.51. Quercetin was used as positive control and as expected, the mitotic index inhibition was concentration dependent.

Chromosome aberrations observed in this study were stickiness, bridges, c-mitosis and vagrant chromosomes (Figure 1 and 2). Most of the aberrations observed were in metaphase and anaphase. The types and proportions of the chromosome aberrations induced by treatments were shown in Table 1. A high frequency of chromosome aberrations were scored at 500 µg/ml and 1000 µg/ml in every phase of mitosis in methanol leaf extracts of *E. scaber.* However, little

aberrations were observed in water leaf extracts of *E. scaber*. For water root extracts of *G. uralensis*, there was a high frequency of abnormal cells in anaphase stage at 1000 µg/ml. Most of the abnormal chromosomes observed were anaphase bridges. Both methanol and water root extracts of *S. miltiorrhiza* showed dose-dependent increase in the frequency of chromosome aberrations. Stickiness was mostly observed in methanol root extract of *S. miltiorrhiza* followed by bridges.

Discussion

The genotoxic effects of leaf and root extracts of *E. scaber*, root extracts of *S. miltiorrhiza* and *G. uralensis* on the mitotic cells in *A. cepa* root tips were studied. *A. cepa* assay enabled the assessment of different genetic endpoints. *A. cepa* root tips were very useful in this testing because the root tips were often the first to be exposed to chemicals in the soil and water [8]. In this study, two genetic endpoints were analyzed which consist of mitotic index and chromosome aberrations. Mitotic index was characterized by the total number of dividing cells in cell cycle. The decrease in the mitotic index of *A. cepa* meristematic cells can be considered as a reliable method to determine the presence of cytotoxic agents [9]. Several types of chromosome aberrations were considered in the different phases of cell division (prophase, metaphase, anaphase and telophase) to evaluate chromosomal abnormalities. According to Rank and Nielsen [10], chromosome aberrations analysis not only allowed estimation of genotoxic effects, but also enabled evaluation of their clastogenic and aneugenic actions.

Mitotic index, which reflected the frequency of mitotic cells and hence the cytotoxicity of crude extracts treatments, were analyzed. The cells of *A. cepa* root tips after treatment using methanol and water extracts of *E. scaber, G. uralensis* and *S. miltiorrhiza* showed decreased mitotic indexes. This may be due to abnormal conditions of the cells after induced by the treatments. The abnormalities of chromosomes could be due to the blockage of DNA synthesis or inhibition of spindle formation. The reduction of the mitotic index might be explained either as being due to the obstruction of the onset of prophase, the arrest of one or more mitotic phases, or the slowing of the rate of cell progression through mitosis [11].

In this study, the cytotoxic effects of crude extracts were observed at tested concentrations with a significant relationship between toxicity and increasing plant crude extracts concentrations. However, the level of toxicity with increasing concentration varied in accordance with the plant species. The mitotic activity of *E. scaber* leaf methanol extract was significantly decreased at high concentrations. The most cytotoxic concentrations were 500 µg/ml and 1000 µg/ml leading to a nearly two fold decreased in mitotic index. Thus, the result indicated that treatment with high concentration showed a lethal effect as the mitotic activity dropped below 0.30. Genotoxic agents have the potential to interact with DNA and may cause DNA damage.

There was a drastic reduction in mitotic index for methanol root extract of *G. uralensis*. The result indicated that treatment with concentration of 1000 µg/ml showed an absence of dividing cells as the mitotic activity dropped below 20%. The reduction in the number of dividing cells at tested concentrations suggested that crude extracts of these plants had mitodepressive effect on the cell division of *A. cepa*. Mitodepressive effects of some plant extracts, being the ability to block the synthesis of DNA and nucleus proteins had earlier been reported [12,13]. They may not even allow the initiation of their biosynthesis. This action occurring in the interphase nucleus could influence the ultimate structure of the chromosome during cell division and caused reduction of number in other stages [14].

Quercetin was used as positive control in this study. Quercetin demonstrated appropriate effect where dose dependent decrease of mitotic index was obtained. Quercetin is a genotoxic chemical, where positive results have been consistently reported in numerous in vitro mutagenicity and genotoxicity assays.

Chromosome aberrations provided important information and may be considered an efficient test to investigate the genotoxic potential of the treatments analyzed [15]. Aberrations were classified as chromosome bridges or fragments, which were signs of clastogenic effects caused by chromosome breaks, and vagrant chromosomes and c-metaphases, which increased the risk for aneuploidy [16]. The chromosome aberrations observed at all concentrations of the medicinal plants were stickiness, bridges, C-mitosis and vagrant chromosomes. These aberrations were due to the effect of these selected medicinal plants on the spindle formation and thus resulted in cell division disturbances. Some of the physiological aberrations that were commonly observed in this study were stickiness (Plate 2). A remarkable correlation between the frequencies of stickiness in prophase and metaphase cells and the bridges in anaphase and telophase cells, produced in *Allium*, was observed. This supports the hypothesis that stickiness may result from improper folding of chromosome fibers which makes the chromatids connected by means of subchromatid bridges [17,18]. However, Mercykutty and Stephen [12] reported that this stickiness may be interpreted as a result of depolymerisation of DNA, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fibre units of chromatids and the stripping of the protein covering of DNA in chromosomes. According to Fiskesjo [19], sticky chromosomes indicated a highly toxic, irreversible effect, probably leading to cell death.

Another remarkable abnormality was bridges. Chromosome bridges commonly occurred during anaphase and telophase. The bridges noticed in the cells were probably formed by breakage and fusion of chromatids or subchromatids [20]. According to Kabarity et al. [21], Chromosome bridges may be caused by stickiness of chromosomes which made their separation and free movements complete and thus they remained connected by bridges. A low frequency of c-mitosis and vagrant chromosomes was also observed. Their presence may be attributed to the failure of the spindle apparatus to organize and function in a normal way. At low concentration (1 µg/ml and 50 µg/ml), most aberrations observed were c-mitosis. Similar observations have been made by other workers where c-mitosis was regarded as indicative of a weak toxic effect which may be reversible [19]. However, these changes may induce the formation of polyploid cells when not reversed [8]. Vagrant chromosomes that were not organized to a specific stage of the mitotic division were also observed. This abnormality may be caused by unequal distribution of chromosomes with paired chromatids in which resulted from no disjunction of chromatids in anaphase. Vagrant chromosomes were weak c-mitotic effect indicating risk of aneuploidy [19].

The toxicity in the selected medicinal plants was identified after analyzed the mitotic index and mean of abnormal chromosomes in each treatments which were influenced by the concentration of crude extracts. At 1000 µg/ml, most treatments showed significant difference of mean of abnormal chromosomes with 1 µg/ml, 50 µg/ml and 500 µg/ml. Hence, toxicity of treatments was analyzed following Figure 1.

The toxicity effect was high in leaf of *E. scaber* than root of *E. scaber*. The observation was found in methanol extract of *E. scaber*. There were more occurrences of abnormal chromosomes in leaf part as the mean was higher, 9.44 compared to root part, 1.33. Besides that, the mitotic index decreased to 0.26 in methanol leaf extracts of *E. scaber*, lower than mitotic index in methanol root extracts of *E. scaber* which stated 0.31, both at 1000 µg/ml. As have been reported in previous study by De Silva, et al. [22], methanol extract *E. scaber* was found to contain lupeol, stigmasterol and a new germacranolide dilactone 11,13dihydrodeoxyelephantopin. The compounds in leaf of *E. scaber* pose a risk of toxicity as both alcohol and chloroform extracts of *E. scaber* have been reported to contain cytotoxic germacranolide-type sesquiterpene lactones [23].

For *G. uralensis* and *S. miltiorrhiza*, only the root parts were examined for toxicity due to scarcity to obtain the leaves and stem from the plants. In this study, root of *S. miltiorrhiza* in methanol extract was found more toxic than root of *G. uralensis* in methanol extract. This was proven as the mean of abnormal chromosomes were 3.67 in the former and 1.33 in the later. However, mitotic index in *G. uralensis* root was slightly lower than *S. miltiorrhiza* at 1000 µg/ml. This may be due to an inhibitory effect of the component on cell division and hindered the passage of affected cells into the mitotic cycle. *S. miltiorrhiza* mainly contains triterpenes such as tanshinoneII_A, and polyphenolics such as salvianolic acid B [24]. *G. uralensis* exhibited low toxicity on *A. cepa* root tips. This is accordance with majority of bacterial genotoxicity studies have reported an absence of genotoxic effects from licorice extracts [4].

Conclusion

There were no data available regarding the genotoxic effects of the selected medicinal plants in the literature. However, the results gained from this study led to the conclusion that methanol leaf extracts of *E. scaber* exerted significant genotoxic effects followed by methanol root extracts of *S. miltiorrhiza*. Lowest toxicity level was observed in methanol root extracts of *E. scaber*. Further studies to determine carcinogenicity potential of *E. scaber* are necessary to obtain a more comprehensive genotoxic assessment. Thus, *in vivo* study is recommended to be done to ascertain these findings from *in vitro* assay.

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