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Screening of *Amorpha fruticosa* and *Ailanthus altissima* extracts for genotoxicity/antigenotoxicity, mutagenicity/antimutagenicity and carcinogenicity/anticarcinogenicity

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Abstract

The aim of the present study was to evaluate the potential genotoxic/antigenotoxic, mutagenic/antimutagenic, and carcinogenic/anticarcinogenic effect of *Amorpha fruticosa* (AF) fruit, *Ailanthus altissima* bark hexane (AAEH) and methanol (AAEM) extracts on a model system Saccharomyces cerevisiae.

Plants were identified and extracted by Ekaterina Kozuharova. Three concentrations of each extract were tested – 10, 100 and 1000 μg/ml. In vitro pro-oxidant/antioxidant activities were evaluated by DPPH and DNA topology assay. The potential genotoxic/antigenotoxic, mutagenic/antimutagenic and carcinogenic/anticarcinogenic effects were revealed in vivo by: Zimmermman's test on Saccharomyces cerevisiae diploid strain D7ts1, and Ty1 retrotransposition test on S. cerevisiae haploid strain 551. Zeocin was used as a positive control.

Based on the in vitro antioxidant activity the extracts could be arranged as follows: AF>AAEM>AAEH. AAEH possessed moderate oxidative potential. No genotoxic and mutagenic capacity was obtained in vivo for extracts tested. The levels of total aberrants, convertants and revertants were comparable with the control ones. No Ty1 retrotransposition was induced by extracts treatment. Further, the extracts possessed well-expressed antigenotoxic, antimutagenic and anticarcinogenic activity. Significant reduction of the total aberrants, reverse point mutations and Ty1 retrotransposition was obtained. Only the AF extract was found to reduce the levels of zeocin-induced mitotic gene conversion.

The three extracts did not possess any genotoxic, mutagenic and carcinogenic effect on Saccharomyces cerevisiae. Based on their protective activity, they can be arranged as follows: AF>AAEM>AAEH which corresponds well with their phytochemical composition. Further experiments could provide more detailed information concerning the mode of action of extracts, as well as their main constituents.

Keywords
Ailanthus altissima, *Amorpha fruticose*, carcinogenic/anticarcinogenic, genotoxicity/antigenotoxicity, mutagenic/antimutagenic effect

Introduction

Invasive plant species are considered to be one of the main reasons for biodiversity loss (Luís et al. 2012; Weidlich et al. 2020; Dyderski and Jagodziński 2021). Their distribution to new areas has led to massive extinction of plant and animal species in the last few years (Panjković et al. 2021; Szumańska et al. 2021). Two alien plant species posing an increasing threat in Bulgaria are *Amorpha fruticosa* and *Ailanthus altissima.* Both are characterized by high tolerance to various habitat conditions and aggressive invasion due to the lack of suitable herbivores to control their populations (DAISIE 2009; Monaco 2014; Global Invasive Species Database 2019).

A. fruticosa L. (Fabaceae), known as false indigo, false indigo-bush, and bastard indigobush is a shrub native to North America (Wilbur 1975; USDA NRCS 2009). The plant has a high quantity of isoflavonoids, rotenoids and prenylated stilbenoids. Among the prenylated stilbenoids, the group of amorfrutins is quite diverse (Kozuharova et al. 2017).

A. altissima (Mill.) Swingle (Simaroubaceae), known as the tree of heaven is native in China. It was introduced in Europe and North America around the end of the 18th century (Luís et al. 2012; Andonova et al. 2021). It contains alkaloids, terpenoids and aliphatic volatiles (Kundu and Laskar 2010). The phytochemical composition is reviewed by Kožuharova et al. (2014). The phytochemical analysis of the bark reveals the presence of more than 221 compounds such as alkaloids, quassinoids, phenylpropanoids, triterpenoids, volatile oils, and other compounds (Li et al. 2021).

Both plants are used in traditional medicine. *A. altissima* is often applied for the treatment of asthma, epilepsy, spermatorrhea, bleeding, ascariasis, cold, gastric (dysentery) and ophthalmic diseases, etc. (Luís et al. 2012; Kozuharova et al. 2020; Li et al. 2021). The ethnobotanical application of *A. fruticosa* is related to the treatment of stomach pain, intestinal worms, eczema, neuralgia, and rheumatism (discussed in Kozuharova et al. 2017).

It the present work we hypothesized that *A. fruticosa* fruit extract and *A. altissima* bark extract would be safe and could decrease the zeocin-induced mutagenic, recombinogenic and carcinogenic effects on *Saccharomyces cerevisiae* model organism. As these plant species are very invasive growing almost unrestrictedly, they can provide abundant and cheap resources of bioactive compounds. Their pharmacological application

may lead to excessive harvesting and thus, a decrease in their populations as a strategy for the protection of native plant habitats. Both plants are promising candidates for the pharmacology. Even though, data in literature point out that the toxicity evaluation of the plant extracts is scarce (Kozuharova et al. 2017; Li et al. 2021).

Thus, the aim of the present study was to evaluate the potential genotoxic/antigenotoxic, mutagenic/antimutagenic and carcinogenic/anticarcinogenic effect of *A. fruticosa* fruit extract (AF) and *A. altissima* bark hexane (AAEH) and methanol (AAEM) extracts on *Saccharomyces cerevisiae*.

Materials and methods

Fruits of *Amorpha fruticosa* were collected in October 2018 from a location near Pasarel village, Sofia district. Stem bark of *Ajlanthus altissima* was collected in September 2018 from a location in Sofia, Bulgaria. The plant materials were dried at room temperature, then pulverized and sieved. The fruits of *A. fruticosa* were macerated with chloroform to remove the lipophilic compounds (both the fixed and the essential oils), and then the material was dried and extracted by percolation with 70% methanol. The solvent was evaporated on a rotary evaporator; then the extract was lyophilized and named AF. The stem bark of *A. altissima* was macerated with hexane to produce the lipophilic extract, which was dried *in vacuo* and named AAEH. The resulting defatted substance was percolated with 70% methanol to obtain the hydrophilic extract, which was concentrated, lyophilized and named AAEM.

DPPH radical scavenging activity

The DPPH assay, based on a color reduction of DPPH hydrate from purple to yellow, was applied as described in Todorova et al. (2015). The radical scavenging activity is presented as concentration inhibiting 50% of the DPPH radicals. Ascorbic acid was used as a standard.

DNA topology assay

DNA topology assay was applied according to Todorova et al. (2015). The transformation of supercoiled pBR322 DNA to a relaxed circular form was photographed with UV transillumination using G:BOX (Syngene). The relative quantity of supercoiled DNA was calculated using ImageJ software.

Treatment of Saccharomyces cerevisiae cells

Stock solutions of *A. altissima* hexane (AAEH) and methanol (AAEM) extracts dissolved in 0.1% Tween 20 and *A. fruticosa* (AF) extract dissolved in sterile MQ water were prepared prior to the experiments. Cell suspensions (1×10^7 cells/ml) to the end of the ex-

ponential and the beginning of stationary growth phase were pre-treated with three concentrations – 10, 100 and 1000 μg/ml of AAEH, AAEM and AF extracts for 30 min at optimal conditions (30 °C, 200 rpm). Cells were then washed and after that treated with 100 μg/ml Zeocin for 1 min. Single treatment with Zeocin was used as a positive control. After these procedures, cells were harvested, washed and prepared for further work.

Mutagenicity/antimutagenicity test

Zimmerman's test was applied on *Saccharomyces cerevisiae* strain D7ts1 as described in Todorova et al. (2015); Todorova et al. (2017). The following endpoints were evaluated: cell survival for genotoxic/antigenotoxic and mitotic gene conversion, reverse mutations and mitotic crossingover – for mutagenic/antimutagenic effects.

Carcinogenicity/anticarcinogenicity test

The Ty1 retrotransposition test applied for *in vivo* detection of carcinogenic effect was used as described by Pesheva et al. (2005) using *S. cerevisiae* strain 551 as a tester strain. A "fold increase" higher than two compared to the control, is considered as a positive response of the Ty1 transposition test.

Statistical analysis

The statistical analysis includes an application of One-way ANOVA with Bonferroni's *post hoc* test. $P<0.05$ was accepted as the lowest level of statistical significance. Concentrations inducing 50% inhibition of the cell growth (IC_{50} values) were calculated using non-linear regression analysis (GraphPad Prizm5 Software).

Results

Preliminary chemical analysis reveals differences among the chemical composition of the extracts: AF fruit extract is rich of flavonoids and stilbenoids (amorfrutins A and B) as it was described previously by (Kozuharova et al. 2017); flavonoids are typical for the extract of AAEM and terpenoids (sterols) for AAEH that corresponds well to the data already published by us (Kožuharova et al. 2014).

Antioxidant potential

Slight to moderate radical scavenging activity of the extracts in comparison with the standard control ascorbic acid was obtained. Based on DPPH assay (Fig. 1), the AF possesses the best radical scavenging activity calculated as IC_{50}=63.71 μg/mL, followed by the AAEM with IC_{50}=696.12 μg/mL. The AAEH show the lowest radical scavenging potential (IC_{50}= 1396.97 μg/mL). The IC_{50} of the ascorbic acid was calculated as 15.94 μg/mL.

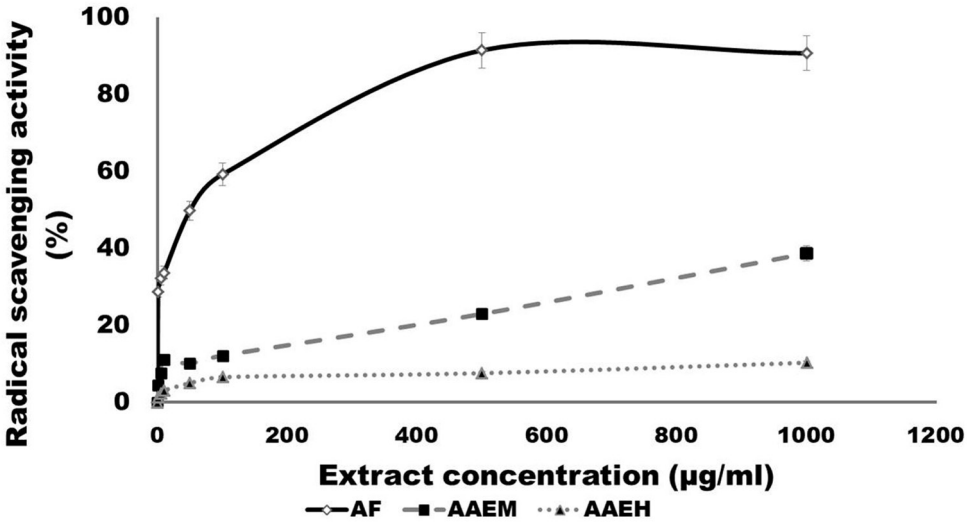

Figure 1. Radical scavenging activity (%) of *Amorpha fruticosa*, *Ailanthus altissima* methanolic and hexane extract. Data are presented mean values from at least three independent experiments.

To evaluate the oxidative potential of the extracts DNA topology assay was performed. This assay provides information not only about the oxidative/antioxidant but also on the DNA damaging/protective effect of the tested extracts. Based on the calculated relative quantity of supercoiled DNA the extracts could be arranged as follows: AF>AAEM>AAEH (Fig. 2). The hexane extract was shown to possess moderate oxidative potential.

Comparing antioxidant properties of the extracts, the moderate protection of AAEH was detected depending on the concentration – 500 and 1000 µg/mL. AF and AAEM did not show good antioxidant properties towards the hydroxyl anions (Fig. 3).

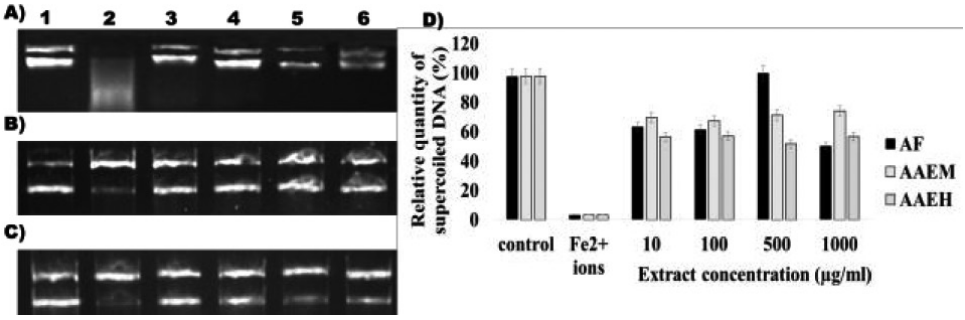

Figure 2. Agarose gel electrophoresis for studying possible DNA damaging effect. Agarose gel electrophoretic patterns of plasmid DNA treated with *A. fruticosa* L. **A** *Ailanthus altissima* methanolic **B** and hexane **C** extract in the absence of Fe^{3+} ions (0.08 mM): lane 1 – DNA control; lane 2 – Fe^{2+} ions control; lane 3 – 10 µg/mL extract; lane 4 – 100 µg/mL extract; lane 5 – 500 µg/mL extract; lane 6 – 1000 µg/mL extract **D** Densitometrical estimation of the relative quantity of supercoiled DNA.

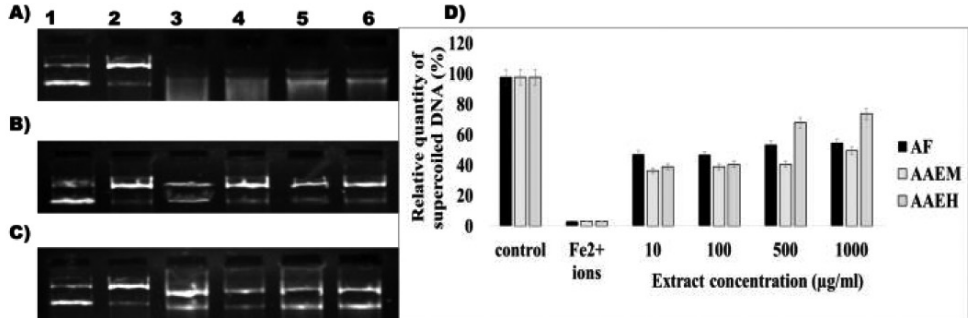

Figure 3. Agarose gel electrophoresis for studying possible DNA protective effect against Fe^{2+} ions. Agarose gel electrophoretic patterns of plasmid DNA treated with *A. fruticosa* L. **A** *Ailanthus altissima* methanolic **B** and hexane **C** extract in the presence of Fe^{3+} ions (0.08 mM): lane 1 – DNA control; lane 2 – Fe^{2+} ions control; lane 3 – Fe^{2+} ions and 10 µg/mL extract; lane 4 – Fe^{2+} ions and 100 µg/mL extract; lane 5 – Fe^{2+} ions and 500 µg/mL extract; lane 6 - Fe^{2+} ions and 1000 µg/mL extract **D** Densitometrical estimation of the relative quantity of supercoiled DNA.

Mutagenicity/antimutagenicity

The survival after the treatments was comparable with the negative control – untreated cells. No effect was obtained regarding the genetic events – convertant and revertant frequencies as well as total aberrants (Table 1). The three extracts did not possess genotoxic and mutagenic properties at the studied concentrations.

Concerning the antimutagenic properties, an increase in the cell survival in comparison with the positive control was measured after all the treatments. Significant reduction of the reverse mutations to levels comparable with that in un-

Table 1. Frequency of gene conversion in *trp5* locus, reversion in *ilv1-92* allele and mitotic crossing-over in *ade2* locus after single treatment of *S.cerevisiae* D7ts1 with 10, 100 and 1000 µg/ml AF, AAEM and AAEH. Zeocin was used as a positive control.

	Extract concentration (µg/ml)	Zeocin (µg/ml)	Survival (%)	Gene conversion/ 10^5 cells	Reversion/ 10^6 cells	Total aberrants (%)
	0	0	100	1.02±0.01	0.003±0.0002	0.437±0.021***
	0	100	32.99±2.75***	4.30±0.7***	0.038±0.0005***	2.331±0.667***
AF	10	0	99.62±1.21***	1.05±0.05***	0.002±0.00009***	0.576±0.150***
AF	100	0	99.05±3.93***	1.07±0.01***	0.002±0.0001***	0.741±0.163***
AF	1000	0	99.97±1.64***	1.07±0.07***	0.001±0.00004***	0.507±0.013***
AAEM	10	0	99.41±2.13***	1.15±0.05***	0.002±0.00005***	0.498±0.130***
AAEM	100	0	99.37±3.01***	1.13±0.03***	0.003±0.00005***	0.501±0.021***
AAEM	1000	0	98.31±1.59***	1.16±0.07***	0.002±0.00009***	0.542±0.043**
AAEH	10	0	96.47±1.98***	1.90±0.08***	0.004±0.00006***	0.602±0.046**
AAEH	100	0	89.11±2.04***	1.60±0.05***	0.004±0.00002***	0.689±0.016**
AAEH	1000	0	97.14±2.43***	2.02±0.04***	0.006±0.00001***	0.802±0.112**

Frequencies are means ± SEM, *n*=4. The significance of differences between positive control (Zeo) and treatment with various extracts' concentrations were calculated by ANOVA with a post-hoc test Bonferroni's Multiple Comparison Test (**P<0.01; ***P < 0.001).

Table 2. Frequency of gene conversion in *trp5* locus, reversion in *ilv1-92* allele and mitotic crossing-over in *ade2* locus after pre-treatment of *S. cerevisiae* D7ts1 with 10, 100 and 1000 μg/ml AF, AAEM or AAEH followed by treatment with 100 μg/ml Zeocin.

	Extract concentration (μg/ml)	Zeocin (μg/ml)	Survival (%)	Gene conversion/ 10^5 cells	Reversion/ 10^6 cells	Total aberrants (%)
	0	0	100	0.52±0.01	0.003±0.0002	0.437±0.021
	0	100	32.99±2.75***	4.30±0.70***	0.038±0.0005***	2.331±0.667***
AF	10	100	94.93±4.08***	0.75±0.50 ***	0.005±0.0009***	0.56±0.16**
AF	100	100	77.54±1.51***	0.81±0.10 ***	0.006±0.0002***	0.74±0.13**
AF	1000	100	71.01±9.07***	0.76±0.09 ***	0.005±0.0004***	0.50±0.03**
AAEM	10	100	87.68±1.46***	2.34±0.84 ns	0.021±0.0006***	1.51±0.035 ns
AAEM	100	100	76.83±2.41***	3.20±0.57 ns	0.005±0.00014***	0.57±0.056 **
AAEM	1000	100	58.06±2.11***	2.84±0.39 ns	0.006±0.00057***	0.76±0.03 **
AAEH	10	100	77.71±3.65***	6.93±0.79*	0.014±0.0035***	1.13±0.078 *
AAEH	100	100	71.55±4.59***	3.89±0.53 ns	0.031±0.0046 ns	0.92±0.09 **
AAEH	1000	100	84.60±1.24***	2.90±0.67 ns	0.032±0.0086 ns	0.85±0.05 **

Frequencies are means ± SEM, *n*=4. The significance of differences between positive control (Zeo) and treatment with various extracts' concentrations was calculated by ANOVA with a post-hoc test Bonferroni's Multiple Comparison Test (NS: nonsignificant; *P<0.05; (**P<0.01; ***P < 0.001).

treated control was obtained after the pre-treatment with AF and AAEM without concentration's effect. Around 2.5-fold lower levels were measured after pretreatment with 10 μg/ml AAEH (Table 2). AF at all the concentrations decreased the zeocin-induced mitotic gene conversion. No effect on this genetic event was obtained after pre-treatment with AAEM, while a potentiation of the zeocin recombinogenicity was observed after pretreatment with 10 μg/ml AAEH. The percent of total aberrants after the pre-treatments was also lower than that measured after single zeocin treatment.

Carcinogenic/anticarcinogenic potential

Data revealed that single treatment with 1000 μg/ml AF possesses slight dose-dependent genotoxic effect, reducing the cell survival of strain 551 to 78% (Fig. 4A). None of the other extracts affected the cell survival. On the other side, pre-treatment results in around 2-fold increased cell survival for all the concentrations of the extract in comparison with the cell survival after single zeocin treatment (Fig. 4A). No dose-dependent enhancement of cell survival is observed. The Ty1 retrotransposition events are also found. Our results clearly indicate that none of the tested concentrations of AF, AAEM and AAEH can induce Ty1 retrotransposition in *Saccharomyces cerevisiae*. These data suggest no carcinogenic properties of the extracts.

Further, well expressed anti-carcinogenic activity, measured as a reduction of the transposition rate to levels comparable with the negative control is defined when pretreatment with concentrations of AF and AAEH is applied (Fig. 4B). The only concentration that cannot protect cells from damaging action of Zeocin is the lowest concentration of AAEM – 10 μg/ml.

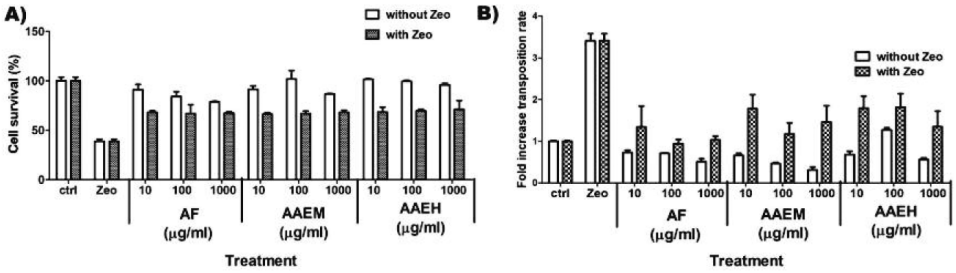

Figure 4. Cell survival **A** and Ty1 retrotransposition rates **B** of *Saccharomyces cerevisiae* strain 551 after treatment with 10, 100 and 1000 µg/ml AF, AAEM and AAEH with or without Zeocin. Where no error bars are evident, errors are equal or less than the symbols.

Discussion

Slight to moderate antioxidant activity *in vitro* of extracts is identified. Such activity of *Amorpha fruticosa* does not correspond to data published by Zheleva-Dimitrova (2013) and Ivanescu et al. (2019). The variation in the radical scavenging activity could be explained by different phytochemical composition based on the geographical origin of plant, variations in the plant extraction and the methodology, etc. Consistence between DNA topology assay results and those obtained by DPPH is found. Moderate oxidative potential leading to single-strand pDNA damage is found for hexane extract.

Our *in vivo* experiments were performed on *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* has been chosen as a model system for human cell due to the similarities in main stress response pathways (discussed in Todorova et al. 2015). Moreover, experiments on yeasts could be a valuable tool when taking into consideration the Directive 2010/63/EU. This directive is aiming to anchor firmly the „Principle of the Three Rs" – To Replace, Reduce and Refine" the use of animals for experimental and scientific purpose in the EU Member States. According to the Annex (47) there is a need to develop new methods alternative to animal testing and proposed to validation in the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) (http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam). Additionally, experiments on diploid and haploid yeast cells allow obtaining new fundamental information concerning the potential lethal effect of various chemical and physical agents and genetic instability (Evstratova et al. 2018).

The results obtained by us reveal that the three extracts do not affect the cell survival, the mitotic gene conversion in *trp5* locus, reversion in *ilv1-92* allele, mitotic crossing-over in *ade2* locus and Ty1 retrotransposition. From our point of knowledge this is the first finding that *Amorpha fruticosa* fruit extract and *Ailanthus altissima* bark extracts are not genotoxic, mutagenic and carcinogenic in our test-system.

Our research has been extended in order to evaluate the possible antigenotoxic, antimutagenic and anticarcinogenic potential of the extracts against the action of

the radiomimetic Zeocin. Zeocin was chosen as a damaging agent due to several reasons: it is a radiomimetic, member of the bleomycin family of antibiotics, that damages DNA in a way similar to that of ionizing radiation; possesses pro-oxidative capacity (Chankova et al. 2013; Todorova et al. 2015), mutagenic, and carcinogenic effect in *Saccharomyces cerevisiae* (Todorova et al. 2015), clastogenic, DNA damaging, and genotoxic effects in microalgae, higher plants, and human lymphocyte cell culture (Chankova et al. 2007; Dimova et al. 2009; Kopaskova et al. 2011; Gateva et al. 2015).

In this study it was demonstrated that pre-treatment with extracts could protect cells from the genotoxic action of Zeocin measured as cell survival. No relation between the cell survival and pre-treatment concentrations was identified. Concerning the antimutagenic capacity of extracts the specificity of their action was obvious. Significant reduction of the total aberrants was obtained after the treatment with the extracts. The only extract reducing the mitotic gene conversion was AF. No effect was observed after the pre-treatment with AAEM. A significant increase in the levels of this genetic event was measured when pre-treatment with 10 μg/ml AAEH which is in accordance with another study where sterols are reported to potentiate the activity of another member of the zeocin family – bleomycin (Hoffmann et al. 2011). Such differences in the activity could be related to the phytochemical content of the extracts. Isoflavonoids are the major constituents of AF extract while AAEH is characterized by the predominance of phytosterols. Flavonoids are already known to possess good antimutagenic properties. Based on the available literature and the present results it could be suggested that AF with flavonoinds as main constituents may protect yeast cells from zeocin-induced mitotic gene conversion and crossing over by activation of HR repair and modulation of chromatin structure.

On the other side, significant amelioration of the reverse point mutations and Ty1 retrotransposition was observed. It is well known that the antimutagenic and anticarcinogenic properties could be related to significant antioxidant activity or to activation of DNA repair processes. As in our *in vitro* experiments evidence was provided for mild to moderate antioxidant activity of extracts tested, it could be suggested that in this case the reduction of the genetic events is not related to the antioxidant potential. Having in mind that the reverse mutation frequency is used for measurement of error prone recombination (Mitchel and Morrison 1986), we could speculate that the potential mechanism of action of the extracts may be an activation of protective enzymes independent of those required for HR.

From our point of knowledge for the first time it was shown by us that *Amorpha fruticosa* fruit extract and *Ailanthus altissima* bark extracts possess no genotoxic, mutagenic and carcinogenic capacity on a model system *Saccharomyces cerevisiae*.

Based on their protective activity, they can be arranged as follows: AF>AAEM>AAEH that corresponds well with their phytochemical composition. Further experiments could provide more detailed information concerning the mode of action of extracts, as well as their main constituents.

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