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Research Article

ANTIGENOTOXIC CAPACITY OF *PAPAVER RHOEAS* L. EXTRACT

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ABSTRACT

Application of exogenic bioactive natural plant compounds with protective, anti-mutagenic and anti-genotoxic effects against various chemical and physical agents is one of the modern approaches for reduction of the mutagenic burden of cells.

Objective: The present study aims to provide data on the cytoxic and genotoxic effects of *Papaver rhoeas* L. water leaf extract and its anti-cytotoxic and anti-genotoxic potential against the radiomimetic zeocin in two types of test-systems - *Hordeum vulgare* and human lymphocytes *in vitro*.

Methods: Mitotic index (MI) was used as an endpoint for cytotoxicity, the frequency of chromosome aberrations (MwA) and the number of induced micronuclei (MN) - as endpoints for genotoxicity/clastogenicity. Formation of aberration „hot spots" was also used as an indicator for genotoxicity in *Hordeum vulgare*.

Results: Our results show that *Papaver rhoeas* L. leaf extract has weak cytotoxic and genotoxic effects depending on the concentrations. Human lymphocytes are more sensitive than *Hordeum vulgare*. By applying two types of experimental designs with split treatment we found that *Papaver rhoeas* L. leaf extract possesses anti-cytotoxic and anti-genotoxic potential against the oxidative stress induced by the radiomimetic zeocin in both test-systems. The effect is more pronounced in schemes with experimental design 2 (with 4 hours inter-treatment time between treatments) which may indicate induction of adaptive response (AR).

Conclusion: The obtained data suggest that *Papaver rhoeas* L. leaf extract is a promising anti-mutagen/anti-clastogen with potential for phytotherapy.

Keywords: *Papaver rhoeas*; zeocin; genotoxicity; anti-mutagenesis; test-systems

INTRODUCTION

Anti-mutagenesis could be considered as a very feasible way for decreasing the negative effects of environmental genotoxins, including genotoxic carcinogens. Many studies exist about application of exogenic non-toxic bioactive natural compounds, with protective, anti-mutagenic and anti-carcinogenic effects against chemical and physical actions [1, 2, 3, 4, 5]. Medicinal plants have been extensively studied for their anti-oxidant activity and radical-scavenging activity [6, 7, 8, 9]. Anti-oxidant and anti-radical *in vitro* properties and *in vivo* topical anti-inflammatory activity of ten hydroalcoholic extracts of edible plants including poppy (*Papaver rhoeas* L. ssp.), from the Calabria region (Italy) were reported by [10]. Wild plant species may have a great potential as a source of bioactive compounds. Many of the wild plants, vegetables and fruits contain antioxidants such as polyphenols (flavonoids, tannins, catechins) and vitamins (β-carotene, vitamins C and E) [11].

Papaver rhoeas L. (family: Papaveraceae) is commonly known as 'corn poppy' and found wild in various parts of the world. *Papaver rhoeas* L. has a long history of medicinal usage. Extracts derived of this plant have been used for the treatment of a wide range of diseases including inflammation, diarrhea, sleep disorders, cough, analgesia and also the reduction of withdrawal signs of the opioid addiction. It is claimed that *Papaver rhoeas* can be useful in various conditions such as bronchitis, pneumonia and rash fever [12]. It was reported anti-ulcerogenic effect in rats [13]. The extract of *Papaver rhoeas L.* inhibits morphine tolerance in mice [14]. Natural extracts of *Papaver rhoeas* L. increase the *in vitro* developmental (IVD) competence of immature mouse oocytes [15]. The main trait of Papaveraceae is their capacity to synthesize various alkaloids.

Many studies exist about the antioxidant activities of *Papaver rhoeas* L. [16]**.** It was found that plant extract isolated from flowers of *Papaver rhoeas* L. exhibits a dose dependent free radical scavenging ability in human lymphoblastoid cell line (TK6) [17]. Antioxidant activity was determined using the DPPH assay.

Anti-oxidant properties of water (WE), ethanol (EE) and acetone (AE) extracts of corn poppy leaves were investigated [18]. Various anti-oxidant tests were used in this study: total anti-oxidant activity in linoleic acid system, DPPH scavenging activity, reducing power, chelation activity and hydrogen peroxide scavenging activity. The scavenging effects of WE and EE on DPPH radical was comparable to standard anti-oxidants such as butylated hydroxyanisole and α-tocopherol. It was demonstrated that the ethanolic extracts of *Papaver rhoeas* L. showed antimicrobial activity against the yeast *Candida albicans*, and all tested bacteria except *Bacillus subtilis* [9]. The ethanolic and water extracts exhibited strong scavenging potential against DPPH radicals - higher than 80%. Controversial data also exist. Some analysis showed that none of *Papaver rhoeas* extracts exhibited any anti-oxidant activity, either in the qualitative or quantitative DPPH assay. The poppy extract was toxic towards brine shrimps (LD_{50} = 2.4x10^{-2} mg/ml) [19]. Studies of mutagenic as well as anti-mutagenic potential are necessary to establish the safe use of plant extracts applied in folk medicine [20].

The anti-oxidant properties of natural compounds correlate frequently with their anti-mutagenic capacity. Based on its folkloric use *Papaver rhoeas* L. and the reported anti-oxidant constituents, the present study aims to provide data on the cytoxic and genotoxic effects of *Papaver rhoeas* L. leaf extract and its anti-cytotoxic and anti-genotoxic potential against the radiomimetic zeocin in two types of test-systems -*Hordeum vulgare* and human lymphocytes *in vitro*.

MATERIAL AND METHODS

Plant extract from *Papaver rhoeas* L. (PapR)

Plants *Papaver rhoeas* L. were collected around Banska Bystrica, Slovakia in May 2008. The collection of such herbs from natural medicinal plants is carried out according to the requirements of Bulgarian Medicinal Plants Act, amend., Offic. Gaz. No 66 of 26 July 2013,Chapter 3/Art. 21 (1).

Papaver rhoeas extract was prepared as follows: 60 grams of the air dried poppy leafs were cut into small pieces (2-3 cm) and transferred into a small Erlenmeyer bottle. Material from leaves was pre-extracted three times with hexane to remove chlorophyll. The plant material was then extracted four times with 100 ml of water (50°C). Water was evaporated by distillation on vacuum rotatory evaporator (20 torr, 50°C). The remaining water was removed by

azeotropic distillation with toluene [17]. Plant extract was stored at 4°C. Poppy extract was dissolved in 5 % dimethylsulphoxyde (DMSO) to prepare different work concentrations.

Mutagen

As a standard mutagen the radiomimetic zeocin (Zeo) was used, which belongs to the bleomycin's group (Cas No.: 11006-33-0).

Experimental test -systems

***Hordeum vulgare* L. (Poaceae)**

Seeds of the reconstructed karyotype of *Hordeum vulgare* MK14/2034 were used. This karyotype has been obtained as a result of the combination of two simple reciprocal translocations between chromosomes 1 and 7 and chromosomes 3 and 4 [21].

The advantage of MK14/2034 is that the reconstruction does not affect neither the centromers or the NOR regions. This fact gives the opportunity to study defined chromosome segments on the one hand, and it ensures similar sensitivity as the standard karyotype, on the other [22, 23, 24].

Human lymphocytes *in vitro*

Heparinized venous blood was obtained from peripheral venous blood of clinically healthy non-smoking donors (male and female), age between 35 – 55 years. Lymphocyte cultures containing RPMI 1640 medium (Sigma, Germany), 12 % calf serum (National center of infectious and parasitic diseases, Bulgaria), 40 mg/ml gentamycin (Pharmacia, Bulgaria) and 0.1% phytohemagglutinin PHA (Sigma, Germany) were prepared according to the standard method [25].

Endpoints

The biological activity of poppy extract and its capacity to protect DNA against the radiomimetic zeocin were evaluated based on the following endpoints:

i) for cytotoxicity - mitotic index (MI) – [26] evaluated according to the formula MI=A/1000, where A is a number of dividing cells.

ii) for genotoxicity/clastogenicity - chromosome aberrations (CA) and micronuclei (MN) [24,26, 27].

The yield of „hot spots" was evaluated in barley, which gives information about the most-sensitive chromosome segments in connection with the induction of isochromatid breaks. An adapted formula was used for comparison of the upper limit of confidence interval from the expected and observed chromatid aberrations in individual loci and evaluation of aberration „hot spots" in barley [26, 28]**.**

Experimental designs

Cytotoxicity and/or genotoxicity of poppy leaf extract

Root tip meristem cells from *Hordeum vulgare* L. were treated for 60 min with 0.25, 0.5, 0.75 and 1 mg/ml of poppy water extract (PapR).

Lymphocyte cultures (cell density $1x10^6$ cell/ml) were incubated for 60 min with 0.01, 0.05 and 0.1 mg/ml of poppy water extract (PapR).

Untreated cells were used as a negative control.

Anti-cytoxic and anti-genotoxic potential of poppy leaf extract

To study anti-cytoxic and anti-genotoxic potential of *Papaver rhoeas* L. water leaf extract we applied the most appropriate concentrations and experimental designs based on our preliminary experiments in both test-systems.

Experimental designs

i) design 1 – split treatment without any inter-treatment time between PapR and zeocin treatment;

ii) design 2 – split treatment with 4 h inter-treatment time between PapR and zeocin treatment.

Papaver rhoeas L. leaf extract was applied for 60 min with concentration of 1mg/ml for *Hordeum vulgare* and 0.1mg/ml for human lymphocytes, respectively. The radiomimetic zeocin (Zeo) was carried out with a concentration of 0.3 mg/ml (60 min) for *Hordeum vulgare* L. and 0.1 mg/ml (15 min) for human lymphocytes in the dark.

Root tip meristem cells from *Hordeum vulgare* L. were washed in distilled water after each treatment. The formation of chromosome aberrations was evaluated at 18, 21, 24, 27 and 30 h after the treatment. The seedlings were then treated with 0.025% colchicine solution saturated with α-bromonaphthaline for 2 h and fixed in ethanol: glacial acetic acid (3:1). The root tip meristems were hydrolyzed in 1N HCl at 60°C for 9 min, stained with Schiff's reagent, macerated in 4% pectinase and squashed onto slides immediately before evaluation.

The human lymphocytes were washed after each treatment in a serum-free medium and the cells were cultivated in a fresh medium RPMI 1640 with 20 % calf serum at 37°C. At the 72nd hour of cultivation 0.02% colchicine was added to the cultures, the cells were then hypotonized in 0.56 % KCl; fixed in a mixture of methanol: glacial acetic acid (3:1) and stained in 2% Giemsa.

A total of 1000 well - spread metaphases (in M_1 mitosis) of each treatment variant in both test systems were scored for the presence of chromosome aberrations (CA). MwA % ± SD was calculated. Chromatid breaks (B'), isochromatid breaks (B"), chromatid translocations (T), intercalary deletions (D), duplication-deletions (DD), dicentrics (DC) and ring chromosomes (RC) were determined.

3000 micronuclei (MN) per sample were scored for both test-systems. Colchicine treatment was omitted and the cells were directly fixed at 30 h after treatment for *Hordeum vulgare* and at 72 h after PHA stimulation for human lymphocytes. MN % ± SD was evaluated.

Statistics

The results were analyzed statistically by the χ^2 method and Fisher exact-test. The means mitotic activity (MI), micronuclei (MN) and metaphases with chromosome aberrations (MwA) after different experimental designs with split treatment were normalized [29, 30].

RESULTS

Cytotoxicity and genotoxicity of PapR leaf extract.

The mitotic activity (MI) in all treated variants was calculated as a percentage of the untreated control.

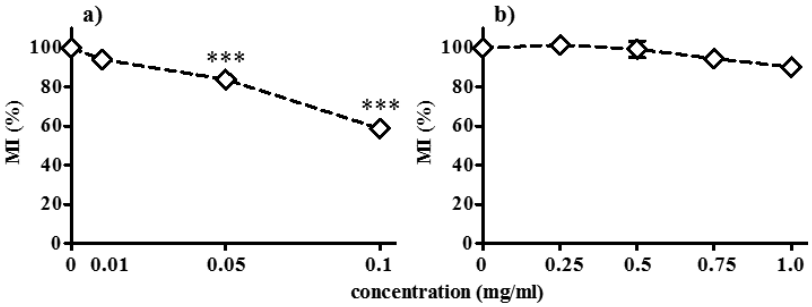

Fig. 1: mitotic activity of PapR in human lymphocytes (a) and *Hordeum vulgare* (b)

(***P<0.001)

Human lymphocytes were sensitive to PapR extract. It showed weak cytotoxic effect in concentrations of range 0.01 mg/ml and 0.05 mg/ml compared with the negative control. The most cytotoxic concentration (P<0.001) was 0.1 mg/ml, decreasing the MI to 58.7% from the value of the untreated control (100%) (Fig. 1a). The poppy extract had no or weak cytotoxic effect on *Hordeum vulgare* (MI 90.3%) applied in the concentration range from 0.25 to 1mg/ml (Fig. 1b).

No effect of DMSO on the investigated parameter was observed for both test-systems (data not shown).

Compared to the negative control PapR extract showed well expressed clastogenic activity in both test-systems clearly depending on the applied concentrations.

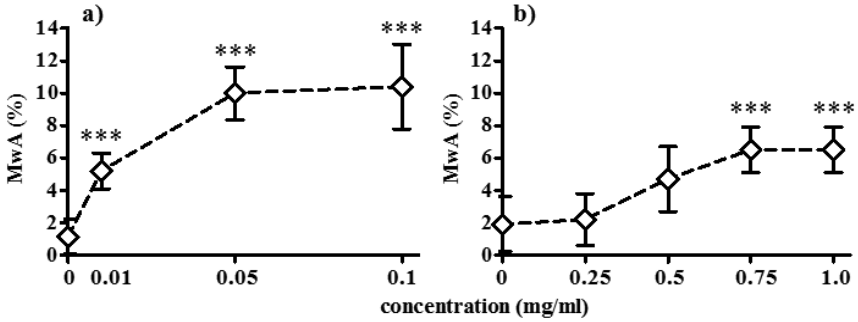

Fig. 2: chromosome aberrations induced by PapR in human lymphocytes (a) and in *Hordeum vulgare* (b)

(***P<0.001)

In lymphocytes control variants had CA 1.1%±1.1 and MN 0.4%±0.0, barley meristems had CA -1.9%±0.7 and MN - 0.13%±0.03, respectively. Chromosome aberrations increased more than 5 fold for human lymphocytes (5.2%-10.4%) and 3-fold for barley (2.2% – 6.5%) after treatment with PapR extract (Fig. 2). The induction of micronuclei was increased nearly two-fold (P<0.001) with the enhancing of the concentrations compared with the control. The frequencies of MN in lymphocytes were 1.2%-2.1% and in *H. vulgare* 0.2%-0.3%, respectively (Fig.3). Lymphocyte cultures were more sensitive to PapR than *Hordeum vulgare.*

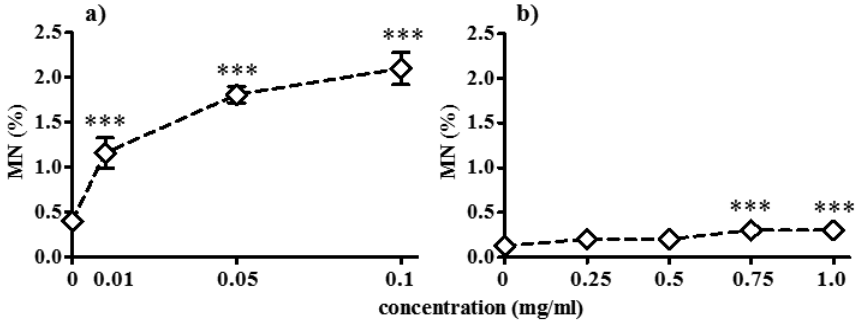

Fig. 3: micronuclei induced by PapR in human lymphocytes (a) and in *Hordeum vulgare* (b)

(***P<0.001)

PapR extract induced narrow spectrum of chromosome aberrations in both test-systems. Mainly isochromatid breaks (B"), followed by low percent of chromatid breaks (B') were observed (data not shown).

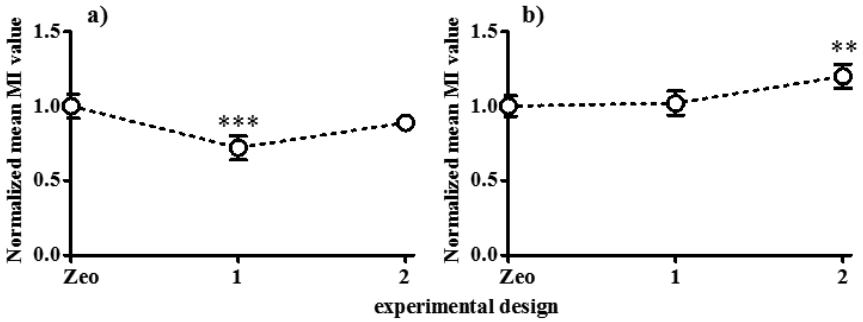

Fig. 4: mitotic activity observed after treatment using designs with split treatment with PapR extract and Zeo in human lymphocytes (a) and in *Hordeum vulgare* (b)

1. Design 1 (split treatment without any inter-treatment time)

2. Design 2 (split treatment with 4 h inter-treatment time)

The results are expressed as normalized averages.

(**P<0.01, ***P<0.001)

Anti-cytotoxic and anti-genotoxic potential of PapR leaf extract

To study the anti-cytotoxic and/or anti-genotoxic potential of PapR leaf extract against Zeo we applied two experimental designs with the most appropriate concentrations in split treatments in both test-systems.

In the test-system human lymphocytes we used concentrations of PapR that were 10-fold lower (0.1 mg/ml) than that in the plant test-system (1 mg/ml).

Mitotic activity (MI) was decreased (P<0.001) only in human lymphocytes after split treatment following experimental design 1 compared with Zeo treatment alone. (Fig.4a). In *Hordeum vulgare* MI was significantly increased (P< 0.01) when the split treatment with PapR extract was applied using design 2 (with 4 h inter-treatment time between PapR and Zeo) compared with Zeo treatment alone (Fig. 4b).

The anti-clastogenic potential of PapR leaf extract in both test-systems is demonstrated in Fig.5 and Fig. 6 based on the yield of chromosome aberrations and micronuclei for both experimental designs.

The yield of chromosome aberrations and micronuclei in human lymphocytes was decreased from 2-fold to more than 3-fold applying experimental designs of split treatment 1 and 2 compared with Zeo treatment alone (0.1 mg/ml) (Fig.5a, Fig.6a). The anti-genotoxic/anti-clastogenic effect of PapR extract in concentration 0.1 mg/ml is better pronounced (P<0.001) when design 2 of split treatment (with 4 h inter-treatment time) was used. The genotoxic effect of Zeo was decreased more than 3-fold.

The anti-clastogenic potential of PapR was well expressed in barley (Fig.5b, Fig.6b). The yield of chromosome aberrations and micromuclei was decreased (P< 0.001) from 4 to 5 - fold after applying design 2. The protective effect against Zeo damage was also well expressed when barley was treated following design 1.

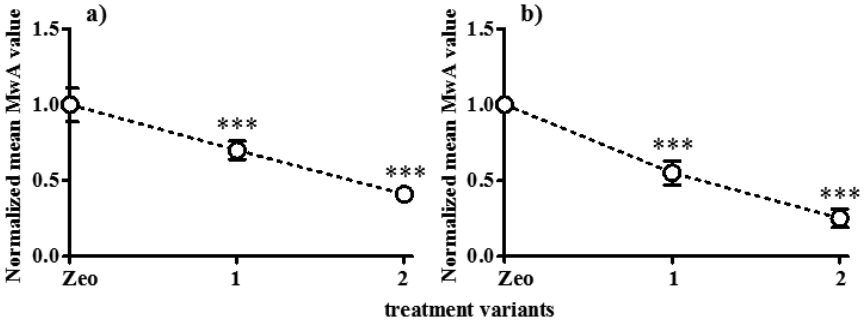

Fig. 5: anti-clastogenic potential of PapR demonstrated on the basis of the yield of chromosome aberrations (CA) after various experimental designs with split treatment with Zeo in human lymphocytes (a) and *Hordeum vulgare* (b):

1. Design 1 (split treatment without any inter-treatment time)

2. Design 2 (split treatment with 4 h inter-treatment time)

The results are expressed as normalized averages.

(***P<0.001)

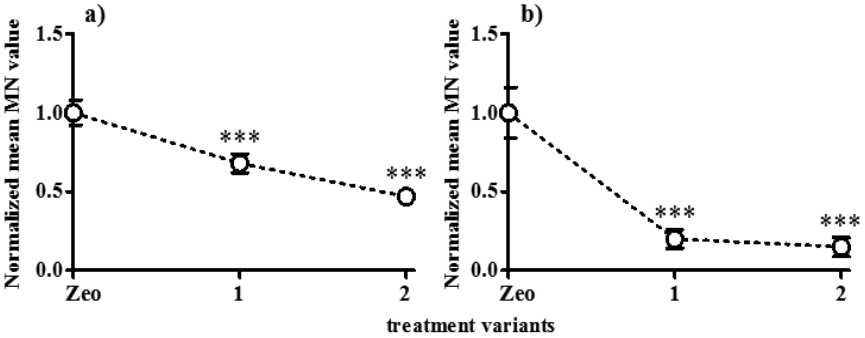

Fig. 6: anti-clastogenic potential of PapR demonstrated on the basis of the MN after various experimental designs with split treatment with Zeo in human lymphocytes (a) and *Hordeum vulgare* (b):

1. Design 1 (split treatment without any inter-treatment time)

2. Design 2 (split treatment with 4 h inter-treatment time)

The results are expressed as normalized averages.

(***P<0.001)

The "aberration hot spots" are investigated in barley chromosomes after split treatment applying the experimental designs described above. It supplies useful information about the DNA segments with higher susceptibility for induction of isochromatid breaks. Single Zeo treatment induced 11 "hot spots", whereas PapR alone only 2 (data not shown). All applied experimental designs of split treatment using PapR showed a statistical significant decrease in the yield of aberration „hot spots" compared to Zeo. Design 1 (without any inter-treatment time) → 4 „hot spots", design 2 (with 4h inter-treatment time) → 3 „hot spots". The most often observed "aberration hot spots" are shown in figure 7.

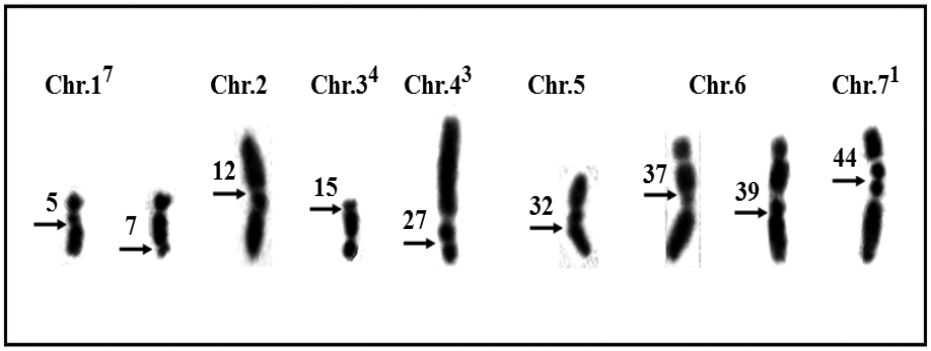

Fig. 7: isochromatid breaks (B"), displayed as "aberration hot spots" in *Hordeum vulgare*

The spectrum of induced chromosome aberrations was analyzed. It varied depending on the experimental design and test-system. The spectrum of chromosome aberrations observed after split treatment with PapR extract and Zeo and after Zeo treatment alone in human lymphocytes was more diverse than that in *Hordeum vulgare* (Fig. 8).

Split treatment applying design 1 induced not only isochromatid breaks (B") – 88.2% and chromatid breaks (B') – 3.9 % but also chromatid translocations (T) – 2%, dicentrics (DC) – 3.9% and ring chromosomes (RC) – 2%. After design 2 we found B" – 86.4%, followed by chromatid breaks (B') – 9.1 % and dicentrics (DC) – 4.5%

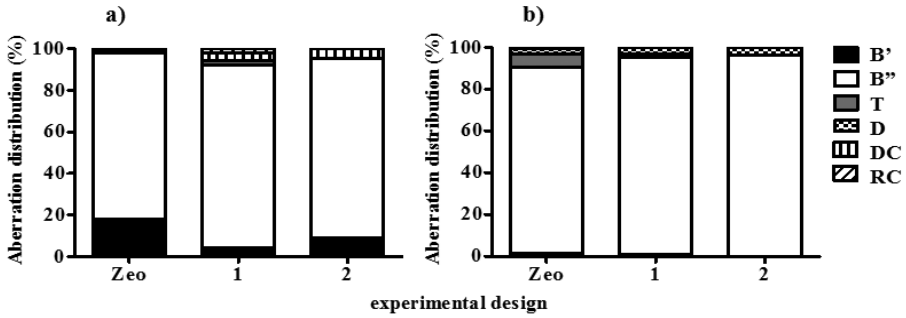

Fig. 8: spectrum of chromosome aberrations induced in human lymphocytes *in vitro* (a) and *Hordeum vulgare* (b) following various experimental designs of treatment:

1. Design 1 (split treatment without any inter-treatment time)

2. Design 2 (split treatment with 4 h inter-treatment time)

In *Hordeum vulgare* the diversity of chromosome aberrations observed after various designs with split treatment was less expressed as compared to Zeo treatment alone. Zeo induced isochromatid breaks (B") – 89.3% and chromatid breaks (B') – 1.4 %, followed by induction of chromatid translocations (T) – 6.2%, deletions (D) – 2.8% and duplication-deletions (DD) – 0.3%. After design 2 only isochromatid breaks (B") and deletions (D) were found. After design 1 we observed (B") – 92.8%, chromatid breaks (B') – 1.4 %, chromatid translocations (T) – 0.9% and deletions (D) – 4.9% (Fig.8b).

In brief PapR extract alone does not show or shows only weak cytotoxic and/or genotoxic activities compared with the negative (untreated) control clearly depending on the test-systems and the concentrations applied.

The data obtained documents the anti-cytoxic and/or anti-clastogenic potential of PapR extract against the radiomimetic Zeo in both test-systems. The effect depends on the experimental design and the concentrations used.

DISCUSSION

Reactive oxygen species (ROS) are involved in various serious diseases including cancer. Zeocin is a well known oxidative stress inducer, stimulating MDA and H_2O_2 production and as result induction of single- and double- strand breaks, and DNA base loss resulting in apurinic/apyrimidinic (AP) sites. The removal of potentially deleterious ROS through antioxidants has been suggested to be an important mediator for the protection of cells. A significant interest arises to find and investigate natural substances possessing anti-mutagenic and anti-oxidant activities against ROS inducers and to replace the synthetic compounds in food applications [31, 32]. At first it is essentially to study the safety of the natural compounds and extracts at doses of pharmacological range. Many authors report data about cytotoxic and genotoxic potential of different natural plant extracts using various endpoints [33, 34, 35]. In our present study we investigated PapR extract widely applied in folk medicine against many diseases, using markers for cytotoxicity and genotoxicity in two types of test-systems – barley and human lymphocytes *in vitro*. Different sensitivity to poppy extract was observed depending on the test-systems. Poppy extract has no, or weak cytotoxic and genotoxic effects in *Hordeum vulgare* depending on the concentrations. Human lymphocytes are more sensitive than barley. Clearly expressed cytotoxic and genotoxic effects were observed depending on the concentration. In our study the lowest concentrations used in both test-systems have no harmful effects. Our results are in conformity with the finding of other studies [17]. The researchers reported that at lower concentrations of plant extract isolated from flowers of *Papaver rhoeas* neither cytotoxic, nor genotoxic effects in TK6 cells are found, but cell proliferation is stimulated. The concentration 25 mg/ml had strong cytotoxic and genotoxic effects in TK6 cells. The authors show that the balance between beneficial and harmful effects should be always considered when choosing the effective dose. Our data are also confirmed by the study [36] where the cytotoxic property of plant extracts from twenty-three plant species of *Leguminosae* family including poppy extract were investigated using brine shrimp lethality assay.

The data present here clearly demonstrate the anti-cytotoxic and/or anti-clastogenic potential of PapR leaf extract, applied in non-cytotoxic or weak cytotoxic concentrations against the radiomimetic Zeo in split treatments. The yields of chromosome aberrations and micromuclei are decreased from 2-fold to more than 3-fold compared with Zeo treatment alone in both test-systems. The

protective effect is observed using both types of experimental designs with split treatment.

Anti-cytoxic and anti-genotoxic effects of poppy extract against Zeo are better pronounced when experimental design 2 (with 4 hour inter-treatment time between treatments) with split treatment is applied. These results are in accordance with the data where plant extracts isolated from *Gentiana asclepiadea* and *Armoracia rusticana* were used in human lymphocytes applying Comet assay [37]. The authors found that the plant extracts applied in non-toxic concentrations in split treatment with zeocin enhance the adaptive response (AR) and also decrease DNA damage caused by zeocin to more than 50%. In our experiments the yields of chromosome aberrations and also micronuclei are decreased more than 3-fold in human lymphocytes and from 4-fold to 5-fold in *Hordeum vulgare*. Probably (AR) plays an important role.

As it is known, AR could activate DNA- repair networks [38], *de novo* protein synthesis [39], antioxidants [40], molecular chaperone or epigenetic mechanisms. Many authors reported the enhanced activities of antioxidant defense system after low doses of oxidative stress [38, 41].

Some studies found that the cytotoxic and anti-clastogenic activities of poppy extract may be due to the likelihood of principle active compounds such as flavonoids and phenolic compoundsto be present in the extract [35, 42]. The phenolic compounds are often related to the antioxidant activity of plants due to their ability to adsorb and/or neutralize free radicals. Phenolic compounds are important components of poppy, and some of their pharmacological effects could be attributed to the presence of these valuable constituents. High contents of total phenolic compounds (9.73 - 19.91 mg gallic acid equivalents /g of fresh petals) and total flavonoids (7.904 - 11.45 mg quercetin equivalent/g of fresh petals) were found in different extracts of *Papaver rhoeas* L. from Southeast Serbia [43]. The presence of red pigment in the flowers of *P. rhoeas* L. originates from anthocyanins, which may act as natural antioxidants. Anti-carcinogenic and anti-oxidant activities of phenolic compounds and flavonoids are well known [17, 42, 44]. Some authors do not obtain any relationship between anti-oxidant activity and total phenolic content [10]. Extracts with high radical-scavenging and anti-oxidant activities do not show a high phenolic content. Probably anti-oxidant activities and radical-scavenging properties exhibit not only phenolic compounds but also other constituents in plant extract. Many studies show that the major compounds of the *P. rhoeas* extract are rhoeadine, rhoeadic acid [45, 46], papaveric acid [47], rhoeagenine [48] and anthocyanins [49].

The present results demonstrate the potential of leaf extract of *P. rhoeas* L. to suppress the Zeo-induced cytotoxicity and genotoxicity. PapR leaf extract make cells more resistant to oxidative stress induced by the radiomimetic Zeo. The results proposed that poppy extract could activate defence systems to overcome damage induced by radiomimetic Zeo. The protective effect is probably due to the presence of some biologically active compounds with anti-oxidant and /or chelating activities and also to induction of some other defense systems. Further biochemical analysis is necessary for the characterization of the active chemical compounds of the poppy extract including the activities of anti-oxidant enzymes.

The data obtained show that *P. rhoeas* L. leaf extract is a promising anti-clastogen and could be successfully use in further research for the purpose of the phytotherapy.

CONCLUSION

i) P. *rhoeas* L. leaf extract has weak cytotoxicity and genotoxicity in *Hordeum vulgare* and human lymphocytes depending on the concentrations used. Human lymphocytes are more sensitive than *Hordeum vulgare*.

ii) P. *rhoeas* L. leaf extract possesses anti-cytoxic and anti-genotoxic potential against oxidative stress induced by the radiomimetic zeocin in *Hordeum vulgare* and in human lymphocytes *in vitro* applying two types of experimental designs with split treatment. The effect is more pronounced in schemes with experimental design 2 (with 4 hour inter-treatment time between treatments) suggesting induction of AR.

ACKNOWLEDGMENTS

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