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Evaluation of genotoxic and antigenotoxic activities of resveratrol in the wing spot test of *Drosophila*

Eylem Eroğlu Doğan[1*], Elif Yeşilada[2]

[1]*Department of Primary Education, Abant Izzet Baysal University, Bolu, Turkey*

[2]*Department of Medical Biology and Genetics, Inonu University, Malatya, Turkey*

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Abstract

Resveratrol (RES) is a well-known polyphenolic compound found in plants and has received much interest due to its numerous biological activities. In this study, the potential genotoxic activity of resveratrol (RES) and its antigenotoxic properties against the mutagenic agents cyclophosphamide (CP), mitomycin C (MMC) and *N*-methyl-*N*-nitrosourea (MNU) were investigated using the standard (ST) cross of the wing spot test in *Drosophila melanogaster*. It was shown that five different concentrations of RES (0.1, 0.2, 0.5, 0.75, 1 mM) employed had no significant effect on spots frequencies indicating a lack of genotoxic activity; while CP (0.5, 1, 2 mM), MMC (0.025, 0.05, 0.1 mM) and MNU (0.5, 1, 1.5 mM) treatments gave positive results for all types of spots, indicating a strong genotoxic activity. The simultaneous administration of CP (1 mM), MMC (0.05 mM) and MNU (1 mM) with RES (0.2, 0.5, 1 mM) led to considerable alterations of the frequencies of CP, MMC and MNU-induced wing spots with the total mutant clones showing reduction between 16.25% and 55.25%. The data clearly indicate a protective role of RES against CP, MMC, MNU-induced genotoxicity.

* **Corresponding Author:** Eylem Eroğlu Doğan ✉ eylemedogan@gmail.com

Introduction

Resveratrol (RES; *trans*-3,4´,5-trihydroxystilbene) is a naturally occurring phenolic compound found in many natural foods, especially high amount in grapes and other plants. RES has received considerable interest not only for its usefulness in "French Paradox" as a phytoestrogen agent (Kopp, 1998), but also for its biological functions such as inhibition of lipid peroxidation in membranes, scavenger of free radicals, protection against carcinogens and mutagens. Although there are many reports available on the various beneficial effects of RES in different test systems (Langová *et al.,* 2005; Schmitt *et al.,* 2002; Turkez and Sisman, 2012), there is only one report, recently published on the antigenotoxic effects of RES in the wing spot test of *Drosophila* (Turna *et al.,* 2014). This indicates that more experimentation is required to increase our knowledge of the antigenotoxic action of RES in the *Drosophila* test systems against different genotoxic agents.

Characteristics such as short life span, large number of offspring, a well-known anatomical situation, a wide variety of mutants, and extensive genetic homology to mammals make *Drosophila* a suitable model organism for genetic studies (Jeibmann and Paulus, 2009; Medina *et al.,* 2015). The wing somatic mutation and recombination test (SMART) of *Drosphila melanogaster* is based on the loss of heterozygosity (LOH) for two recessive markers *mwh* (3-0.3) and *flr*^{3} (3-38.8) (Graf *et al.,* 1984). SMART detects a wide spectrum of genetic changes including point mutations, deletions, chromosomal aberrations, and mitotic recombinations (Graf *et al.,* 1984; Graf *et al.,* 1998). This assay has been proved to be a powerful tool to evaluate both the genotoxic and antigenotoxic properties of different natural compounds (Idaomar *et al.,* 2002).

In the present study, therefore, SMART assay was used to evaluate the genotoxic activity of RES. To investigate its antigenotoxic effects, we have performed co-treatments with the three well-known mutagens, namely *N*-nitroso-*N*-methylurea (MNU), mitomycin C (MMC), and cyclophosphamide (CP). MNU is a strong direct-acting alkylating agent and acts with nucleophillic nitrogen, oxygen atoms in bases and DNA phosphate groups thereby inducing the genotoxic effect (Verma *et al.,* 2012). MMC is also an alkylating agent that forms monomeric adducts which bind to DNA leads to genotoxic effects such as inducing chromosomal aberrations (Sontakke and Fulzele, 2009). CP is an antineoplastic and immunosuppressive agent, metabolized to active alkylating metabolites that ultimately cause DNA damage in the form of base substitutions and chromosomal aberrations (Anderson *et al.,* 1995). These genotoxic agents were selected since they are potent inducers of mutation and recombination in the SMART assay (Rodriguez Arnaiz *et al.,* 1996; Rincon *et al.,* 1998; Spano *et al.,* 2001).

Materials and methods

Chemicals

Commercially available compounds were used in the study. MNU, (CAS Number: 684-93-5) was obtained from Sigma, MMC was (CAS number: 50-07-7) provided from Serva, and CP (CAS Number: 6055-19-2) was obtained from MP company. RES (CAS number: 501-36-0) and ethanol (CAS number: 64-17-5) were obtained from Fluka. Before use, MNU, MMC and CP were dissolved in distilled water (DW). Distilled water was used as a negative control.

Since RES is insoluble in DW as indicated in the literature (Chen *et al.,* 2013), stock solutions of RES (molecular weight 228.2) were prepared in ethanol, and further diluted to 0.2, 0.5 and 1 mM concentrations with DW. The effective ethanol concentration in the highest RES dose (1 mM) was calculated as being % 6.4 (v/v). This concentration of ethanol was tested for its possible toxic and genotoxic effects in the *Drosophila* wing spot test, and used as a negative control for RES groups in genotoxicity studies. The chemicals and stock solutions were kept at +4 ^{o}C, and all the test solutions were always prepared immediately before use. Faure's solution, which was used for mounting the insects' wings, was prepared according to the literature (Graf *et al.,* 1984).

Strains

Two different strains of *Drosophila melanogaster* carrying genetic markers located both on the left arm of chromosome 3 were used: (1) the '*multiple wing hairs*' (*mwh*, 3-0.3) and (2) the *flare*^{3} (*flr*^{3}, 3-38.8) strain. More detailed information on the genetic symbols and descriptions are given by Lindsley and Zimm (1992). These strains were cultured in bottles with standard medium for *Drosophila*, at a temperature of 25±1 ^{o}C and a relative humidity of ~60%.

The wing spot test

The wing spot test was carried out according to the protocol by Graf *et al.* (1984). Stock *flr*^{3} virgin females were mated with stock *mwh* males, called the standard (ST) cross that produces phenotypically wild-type wings, marker-heterozygous flies *(mwh flr*$^{3+}$*/mwh*$^+$*flr*^{3}) and balancer-heterozygous flies (*mwh flr*$^{3+}$*/ mwh*$^+$*TM3 BdS*), with phenotypically serrate wings. The flies completed development under optimal laboratory conditions at 25 ± 1^{o}C, 60% humidity and in darkness. Eggs from ST cross were collected for 8 h periods. Three day old heterozygous larvae were cleaned from feeding medium with a 17% NaCl solution. Then thirty larvae in each tube containing 0.5 g of *Drosophila* Instant Medium (Carolina Biological Supply, Burlington, NC, USA) with 2.5 ml of the freshly prepared different concentrations of test solutions were transferred to feed for the remainder of their larval life (~48 h), pupated and hatched as adult flies. Each treatment was done in duplicate. All adult flies were removed from the treatment vials and marker-heterozygous wings (*mwh flr*$^+$*/mwh*$^+$ *flr*^{3}), reflecting both mutation and recombination events, were mounted on slides with Faure's solution. Both dorsal and ventral sides of the wings were scored under an optical microscope at 400X magnification for the presence of the spots. The sizes of the spots in a number of cells were recorded, and the spots were classified as small single spots of either *mwh* or *flr*^{3} phenotypes, large single spots, and *mwh-flr*^{3} twin spots. Single spots are produced by mitotic recombination, mutation and chromosomal aberration. Twin spots can result from mitotic recombination between the proximal marker *flr*^{3} and the centromere of chromosome 3. In the wings of balancer-heterozygous flies (*mwh*/TM3 wings) only *mwh* single spots, due to mutational events, can be found because recombination is suppressed in cells with the multiply inverted TM3 balancer chromosome (Graf *et al.,* 1984; Graf *et al.,* 1998).

Statistical analysis

For the evaluation of the genotoxic effect, the frequency of small single, large single or twin spots, and the total frequency of spots per fly for each treatment were compared pair-wise (i.e., DW versus ethanol, DW versus genotoxic agent, ethanol versus RES, and positive control (genotoxin) alone versus genotoxin plus RES) using the conditional binomial test of Kastenbaum and Bowman (1970) at the 5% significance level. A multiple decision procedure was used to decide whether the result was positive, negative or inconclusive (Frei and Wurgler, 1988). MICROSTA program was used in the statistical analysis of the data collected in the wing spot assay (Alaraby *et al.,* 2015). The percentage of genotoxicity inhibition in combined treatments was calculated based on the control-corrected frequency of clone formation per 10^5 cells as follows: [(genotoxin alone – genotoxin plus RES/genotoxin alone) × 100] (Abraham, 1994). Inhibition percentage indicates that reduced genotoxic activity with an antigenotoxic compound. The survival rate expresses the toxicity by larvae that survived to adulthood. Statistical analysis of survival rates was performed using Chi-square test for ratios for independent samples.

Results and discussion

In the present study, we aimed to investigate genotoxicity and antigenotoxicity of a naturally occurring polyphenol phytoalexin, RES, which has attracted much interest because of its beneficial potentials for human health. For this purpose, larvae from the ST cross of the *Drosophila* SMART assay were exposed to DW, ethanol, mutagens (positive controls), RES, and mutagens plus RES.

The study was done in two parts using ST cross flies.

In the first part, toxic and genotoxic effects of control, ethanol, RES, CP, MNU and MMC groups were investigated and in the second part, antigenotoxicity experiments were carried out. Summaries of the results obtained are given in Table 1 and 2 respectively. These results refer to the concentrations tested for each of the compounds evaluated, and show the frequency of total spots observed, classified as small single, large single, and twin spots.

Table 1. Results of genotoxic potential of ethanol, RES, CP, MNU and MMC with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) progeny of the standart cross (ST).

Treatments	Survival rate (%)	No. of wings	Spots per wing (number of spots) statistical diagnosis[a]			
			Small single spots (1–2 cells; m =2)	Large single spots (>2 cells; m =5)	Twin spots (m=5)	Total spots(m =2)
Distilled Water	98	100	0.14(14)	0.04(4)	0.02(2)	0.20(20)
Ethanol 6.4 %	90	65	0.17(11) i	0.09(6)i	0.03(2) i	0.29(19) i
Resveratrol (mM)						
0.1	90	70	0.09(6) -	0.03(2) -	0.00(0) i	0.11(8) -
0.2	88	75	0.11(8) -	0.01(1) -	0.03(2) i	0.15(11) -
0.5	81	75	0.11(9) -	0.03(2) -	0.01(1) i	0.16(12) -
0.75	79	66	0.14(9) -	0.05(3) -	0.03(2) i	0.21(14) -
1.0	75	40	0.15(6) i	0.08(3) i	0.03(1) i	0.25(10) i
MNU (mM)						
0.5	96	100	4.90 (490) +	4.61(461) +	1.45(145)+	10.96(1096)+
1	95	100	5.70(570) +	5.05(505) +	1.50(150)+	12.25(1225)+
1.5	88	110	7.14 (785) +	6.35 (698) +	3.15 (347) +	16.60 (1830) +
CP (mM)						
0.5	72*	66	1.70(112) +	0.64(42) +	0.09(6) +	2.42(160) +
1	67*	75	3.16(237) +	0.84(63) +	0.20(15) +	4.20(315) +
2	41*	43	5.14(221) +	1.30(56) +	0.26(11) +	6.70(288) +
MMC (mM)						
0.025	64*	80	4.15(332) +	4.73(379) +	2.80(224)+	11.69 (935)+
0.05	60*	72	6.53(471) +	6.91(498) +	3.53(254)+	16.98(1223)+
0.1	46*	70	7.01(491) +	7.81(547) +	4.74(332)+	19.57(1370)+

[a] Statistical diagnosis according to Frei and Würgler (1988): +, positive; –, negative; i, inconclusive, m, multiplication factor for the evaluation of results significantly negative. Probability levels: $\alpha = \beta = 0.05$. One-sided statistical test; survival statistics (Chi-square test). Asterisk indicates significant difference at $p<0.05$.

Genotoxicity studies

Stock solutions of RES prepared with ethanol (% 6.4 by v/v) and diluted to desired concentrations with DW. This concentration of ethanol was tested for its toxic and genotoxic effects. Ethanol treatment slightly decreased the survival compared with the DW treatment and the result was not statistically significant (Table 1). Therefore, it can be deduced that the ethanol concentration used did not cause a significant toxicity for *Drosophila* larvae. This result is in line with the results presented by Karan *et al.* (1999) and Kaya *et al.* (2002).

Genotoxicity results are given on three categories of small single spots, large single spots, and twin spots as well as the total of spots in Table 1. The single spots were assumed to be due to gene mutation, chromosomal deletion, nondisjunction, or mitotic recombination. The twin spots were assumed to be the products of the mitotic recombination (Graf *et al.,* 1984). The frequency of total spots in DW (0.20) is in good agreement with the normal background range observed in our laboratory (Table 1) and is not significantly different from previous results reported

by other authors (Sarikaya and Yuksel, 2008; Demir *et al.,* 2009). We have found that frequency of total spots (0.29) induced by ethanol was statistically inconclusive (Table 1). This result is comparable with the result previously obtained by Kaya *et al.* (2002).

All concentrations of alkylating agents used in this study generally caused a decrease in survival. Each mutagen except MNU exerted a concentration-dependent inhibitory effect on survival, and the least survival ratios were observed with CP and MMC (Table 1). Among the three agents employed in the study, MMC was found to be the most toxic agent to *Drosophila* larvae. Podratz *et al.* (2011) have reported that survival in CP treated flies decreased in a dose dependent manner. Similarly, Spano *et al.* (2001) have tested different concentrations of CP in ST and HB crosess, and found that the highest concentration (5.0 mM) was toxic. On the other hand, RES showed an interesting but insignificant result, i.e. decreasing the survival ratio more than MNU with increasing concentration. Ethanol usage may have contributed to this observed decrease in the survival ratio. Overall, the results in Table 1 indicates that CP and MMC were toxic, whereas MNU and RES were not toxic to *Drosophila* larvae in the tested concentrations.

Table 2. Results of antigenotoxic potential of RES against CP, MNU and MMC induced genotoxicity with the the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) progeny of the standart cross (ST).

Treatments (mM)		Survival rate (%)	No. of wings	Spots per wing (number of spots) statistical diagnosis[a]				% Inhibition[b]
				Small single spots (1–2 cells; m =2)	Large single spots (>2 cells; m =5)	Twin spots (m=5)	Total spots (m =2)	
DW		98	100	0.14(14)	0.04(4)	0.02(2)	0.20(20)	
CP	RES							
1	0	67	75	3.16(237)+	0.84(63) +	0.20(15)+	4.20(315) +	
1	0.2	79*	80	2.83(226) -	0.60(48) -	0.12(10)-	3.55(284) -	16.25
1	0.5	75	80	2.03(162) -	0.32(26) -	0.10(8) -	2.45(196) -	43.75
1	1	74	80	2.12(170) -	0.23(18) -	0.14(11) -	2.49(199) -	39.60
MNU	RES							
1	0	95	100	5.70(570)+	5.05(505)+	1.50(150)+	12.25(1225)+	
1	0.2	84	80	4.13(330) -	4.01(321) -	0.49(39) -	8.63(690) -	30.08
1	0.5	85	80	3.46(277) -	3.21(257) -	0.32(26) -	7.00(560) -	43.57
1	1	82	80	3.40(272) -	2.51(201) -	0.45(36) -	6.36(509) -	48.86
MMC	RES							
0.05	0	60	72	6.53(471)+	6.91(498) +	3.53(254)+	16.98(1223)+	
0.05	0.2	76	80	5.21(417) -	4.92(394) -	2.46(197) -	12.60(1008)-	26.13
0.05	0.5	80*	80	4.07(326) -	4.11(329) -	2.07(166) -	10.26(821) -	40.05
0.05	1	85*	80	3.04(243) -	2.95(236) -	1.72(138) -	7.71(617) -	55.25

[a] Statistical diagnosis according to Frei and Würgler (1988): +, positive; –, negative; i, inconclusive, m, multiplication factor for the evaluation of results significantly negative. Probability levels: $\alpha = \beta = 0.05$. One-sided statistical test; survival statistics (Chi-square test). Asterisk indicates significant difference at $p<0.05$.

[b] Calculated as [(Genotoxin alone - Genotoxin +RES)/Genotoxin)] x 100, according to Abraham (1994).

Alkylating agents interact with different types of macromolecules such as DNA, either directly or after metabolic activation (Rodriguez-Arnaiz *et al.,* 1996). There are various studies that report that CP, MNU and MMC tests positive in several genotoxicity assays causing DNA damage in the form of base substitutions, deletions, chromosomal loss, and inducing micronucleus formation (Inouye *et al.,* 1988; Schimenti *et al.,* 1997; Rincon *et al.,* 1998). In the study, the number of spots per wing in all types of

spots of CP, MNU and MMC groups have significantly increased compared to the DW control (Table 1). Based on the frequency of total spots induced, the rank order for the genotoxicity of the mutagens was MMC>MNU>CP. Compared to the other mutagens, MMC showed a stronger genotoxic effect even at very low concentrations. Because these three mutagens produced statistically significant induction of all categories of spots, they were used as positive controls in our study to elucidate the antigenotoxic properties of RES.

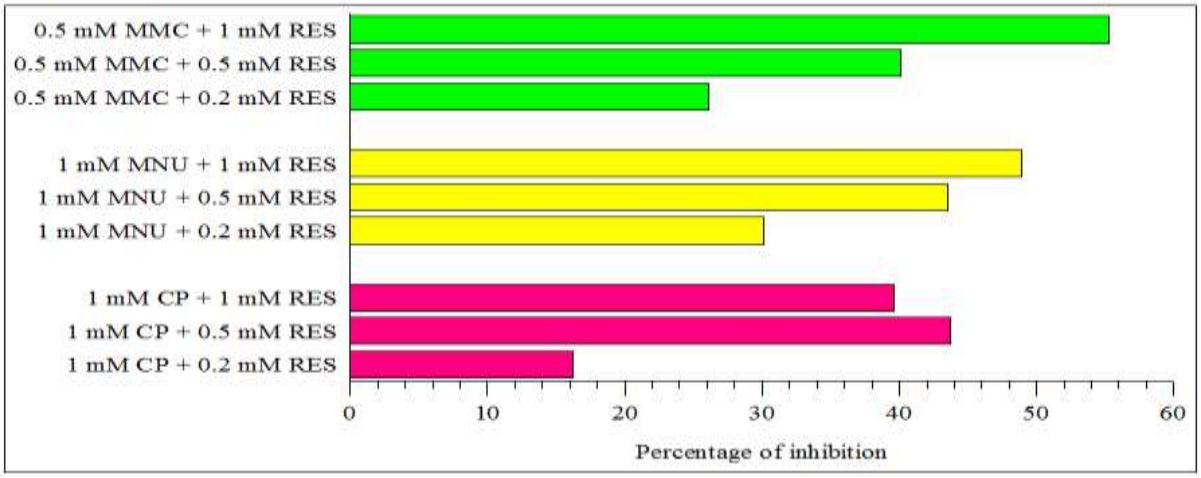

Fig. 1. Protective effects of resveratrol against CP, MNU and MMC induced genotoxicity in the wing spot test of *Drosophila melanogaster*.

The possible genotoxic effects of RES was investigated at a wide dose range (0.1 to 1 mM) in the SMART assay, and it was found that RES did not induce any types of spots significantly. Therefore, RES by itself was not genotoxic at all the concentrations tested (Table 1). Similarly, Turna *et al.* (2014) have reported that RES did not induce significant increases in the frequency of mutant spots at any of the three concentrations tested as compared to control values. However, RES has been reported genotoxic in some other test systems (Schmitt *et al.,* 2002; Fukuhara *et al.,* 2008).

The genotoxicity of RES has been attributed to the scavenging of tyrosyl free radicals in the R2 subunit of ribonucleotide reductase that catalyzes the rate-limiting step of de novo DNA synthesis (Fukuhara *et al.,* 2008). On the other hand, Stagos *et al.* (2007) have reported that RES was not genotoxic alone in human blood lymphocytes. Similarly, Turkez and Sisman (2012) have reported that the mean SCEs per cell and CA per cell rates were not changed by RES applications as compared to control values.

Antigenotoxicity studies

Antigenotoxic activity of RES was investigated using co-treatment with CP, MNU and MMC. All the combined treatments were performed by using one fixed concentration of the genotoxic agent (1 mM CP, 1 mM MNU and 0.05 mM MMC) with three different concentrations of RES (0.2 mM, 0.5 mM and 1 mM), and the results are shown in Table 2. The fixed concentrations of the genotoxic agents were chosen according to the results presented in Table 1. It is clear from the table that all the mutagens are genotoxic at all the tested concentrations. Therefore, we decided to use the moderate concentration of each mutagen in the co-treatment experiments. Survival rates when exposed to selected concentrations of mutagens alone and mutagens in association with RES were also given in Table 2.

In comparison to only mutagen administrations (CP and MMC), there is a net increase in survival rates, however, the amount of increase in survival was gradually decreased with increasing concentration of RES in CP+RES co-treatment experiments, indicating that increasing the RES concentration from 0.2 mM

to 1 mM may lead to a less toxic effect on ST cross larvae (Table 2). Thus, 0.2 mM RES concentration seems to be optimum in CP+RES co-treatment experiments. In case of co-treatment with MMC, however, survival rates were increased with applied concentrations of RES. Survival rates significantly increased in (1 mM CP + 0.2 mM RES), (0.05 mM MMC + 0.5 mM RES) and (0.05 mM MMC + 1 mM RES) co-treatment experiments.

RES generally increased the survival rates by decreasing the possible toxic effects of CP and MMC. The literature does not report any study on the effect of RES plus genotoxin administration on survival rates of *Drosophila* larvae. However, it was reported in a study that at relatively higher concentrations (≥1 mM), RES inhibited apoptosis of the mouse primary hepatocytes and increased cell viability in a dose-dependent manner, and particularly recovered the survival rate of the hepatocytes from 28% to nearly 100% by administration of 5 mM of RES (Wang *et al.,* 2012). On the other hand, in case of co-treatment with MNU, RES unexpectedly reduced the survival rates from 95% to 82%. However, these results were not found statistically significant compared to the results of the positive control group (1 mM MNU), indicating that RES did not induce any significant toxicity in MNU+RES co-treatment groups.

Co-treatments of different concentrations of RES in combination with CP, MNU and MMC led to a statistically significant reduction in all categories of mutant spots observed when compared with the genotoxins alone. The treatment of 1 mM CP in combination with 0.5 mM RES decreased the frequencies of small single and twin spots more than the highest RES concentration (1 mM) used. On the other hand, in case of RES+MNU and RES+MMC co-treatment groups, the highest concentration of RES (1 mM) caused a greater decrease in frequencies of small single, large single and total spots. These results indicate that the protective effect of RES is proportional to the concentrations applied in RES+MNU and RES+MMC co-treatment groups, and these dose–response relationships can be attributed to its antigenotoxic activity (Turkez and Sisman, 2012). It was also found that RES inhibited the genotoxicity induced by CP, MNU and MMC depending on its concentration used from low to moderate percentages, almost 44%, 49% and 55% of the induced spots respectively (Fig. 1). Similarly, RES has been reported to exhibit antigenotoxic activities against gentotoxic damage caused by ethyl methane sulfonate and potassium dichromate in the *Drosophila* wing SMART assay (Turna *et al.,* 2014). Together, our results led us to a conclude that RES acted as an antigenotoxic agent *in vivo* against CP, MNU and MMC by possibly preventing point mutations, deletions, recombinations or chromosomal aberrations in the wing spot assay of *D. melanogaster*. The antigenotoxic activity of RES against these mutagens can be explained by its regulatory effects on antioxidant enzyme system (Khan *et al.,* 2013), scavenging the free radicals and reactive oxygen species that can be derived from the metabolism of mutagens (Li *et al.,* 2006) and reducing interaction of these mutagens with DNA or increasing the cellular repair capacity (Berni *et al.,* 2012).

Conclusion

The current study showed that under the present experimental conditions ethanol and RES were neither toxic nor genotoxic, MNU was not toxic but genotoxic, and CP and MMC were both toxic and genotoxic at the tested concentrations in the *Drosophila* wing spot assay. RES did not induce any significant toxicity in the combined treatment groups, and generally increased the survival rates by decreasing the toxic effects of the alkylating agents CP, MNU and MMC. Moreover, RES was protective against the genotoxic effects of these substances by possibly preventing their mutagenic effects through different mechanisms. Further studies are necessary to clarify the mechanisms of antigenotoxic action of RES.

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