

Antigenotoxic properties of extracts of representatives of the genus *Monarda* introduced in the Urals

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Received 12 April 2024; revised 04 October 2025

Due to the fact that many antitumor drugs have genotoxicity, the aim of the study was to evaluate the antigenotoxic properties of extracts of the genus *Monarda* representatives using the *Drosophila melanogaster* model organism as an example. High IDC (Index of DNA-Comets) characterizes 0, 1% extracts of *M. fistulosa* var. *fistulosa* and *M. fistulosa* var. *menthifolia* when used both individually and together with etoposide. Whereas the extract of *Monarda fistulosa* var. *media* shows antigenotoxic properties by reducing the IDC and showing a unidirectional hypoexpressive effect relative to the *sqh* gene. Thus, the extract of *Monarda fistulosa* var. *media* is the most suitable protector against the effects of the anticancer drug etoposide.

Keywords: *Drosophila*, Etoposide, Comet-assay, Mosaicism, Expression

The prevalence of oncological diseases around the world leads to a variety of approaches to their treatment. A search and testing of newly synthesized medicinal substances, as well as various additional biologically active components, is underway. As the latter, extracts, essential oils and collections of medicinal plants are studied. Among medicinal plants there are variants both as an independent medicine and as a protector for use according to a certain scheme with antitumor drugs already used in practice to damage to genetic material at different levels of organization, anticancer drugs can cause changes in gene activity, for example, cisplatin provokes the expression of endonuclease (EndoG), which in turn can act as a factor contributing to the appearance of lymphomas¹. A decrease in the genetic activity of nalidixic acid has been described during pretreatment of *E. coli* strain cells with greater celandine juice². Also, in addition to the desmutagenic effect, medicinal plants can also demonstrate bio-antimutagenic one, characterised by participation in the processes of metabolism, detoxification and repair of damaged DNA. Similar effects were found for

British inula (*Inula britannica* L. family Compositae), and chamomile³⁻⁶.

Representatives of the genus *Monarda* are characterised by various effects: antibacterial, antioxidant, anti-age, etc.⁷⁻¹³. Such properties are due to certain biologically active substances in their composition, such as flavonoids, monoterpene phenols, thymol, carvacrol¹⁴. An increase in the efficiency of repair systems due to the antioxidant effect contributes to a decrease in genetic activity. Anticancer drugs, in particular etoposide, which is an inhibitor of topoisomerase II, have pronounced genotoxicity both at the level of chromosomal aberrations and at the level of DNA damage¹⁵.

Drosophila melanogaster Meig. has been used as a model in the study of fundamental mechanisms underlying such biological processes as individual development¹⁶⁻¹⁷, development and functioning of the nervous system¹⁸, and innate immune responses¹⁹⁻²⁴. *Drosophila* has also become a useful genetic tool for studying human diseases, such as rare Mendelian diseases²⁵⁻²⁶, neurodegenerative disorders²⁷⁻²⁸, and cancer²⁹⁻³⁰. Additionally, one of the important selection criteria is the presence of a small genome and high gene homology with vertebrates³¹⁻³².

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Hypothesis: choose an extract that protects against side effects but does not alter the main cytotoxic effect of the anticancer drug etoposide. The use of a nutrient substrate with a variable composition makes it possible to test extracts of medicinal plants by per os³³. In this connection, the aim of the study was to evaluate the antigenotoxic properties of extracts of *M. didyma*, *M. fistulosa* var. *fistulosa*, *M. fistulosa* var. *media* and *M. fistulosa* var. *menthifolia* on the example of *D. melanogaster*, using the antitumor drug etoposide.

Materials and Methods

Study subject and preparation of extracts

We used the following laboratory lines of *D. melanogaster*: Canton-S (Normal, wild type, BDSC_64349), yellow (I: 0,0), white singed 3 (I: sn 21,0, w 15,0), da-Gal4 (P{da-GAL4.w-}3), BDSC_7194 (w[*]; P{w[+mC]=sqh-EYFP-Mito}3).

The selected lines were kept on fresh nutrient medium at +24 °C and 65% humidity. Plants of 2020 were used, grown in the Botanical Garden of the Ural Branch of the Russian Academy of Sciences from collection seeds (Russia, Sverdlovsk Region, and Yekaterinburg). *Monarda* grass was harvested in the flowering phase at the end of July, 2020. Plants of these species have been under the conditions of introduction for 2 years, successfully passed the adaptation period and were not treated with growth factors during cultivation.

For the preparation of extracts, the aerial parts of the plants were used. Extraction with 70% ethanol was carried out in the ratio: dry raw material and extraction agent 1:10 (1 g/10 mL). Drugs tested during the work: etoposide (Veropharm, Russia) and extracts of four representatives of the genus *Monarda*. Experimental groups of individuals were grown on the following nutrient media: control, etoposide 0,664 mg/mL, extract solution of *M. fistulosa* var. *menthifolia*/*M. fistulosa* var. *media*/*M. fistulosa* var. *fistulosa*/*M. didyma* 0,1%, as well as an extract together with etoposide (concentration of the antitumor drug was determined based on the lethality rate of the individuals previously)³⁴.

SMART (Somatic Mutation And Recombination Test)

We crossed females from the yellow lines (yellow body colour, the yellow gene is localised on the X chromosome) and white singed 3 males (white eyes and scorched bristles on the body, the genes are also localised on the X chromosome), placing them on the

test medium for no more than 72 hours (24 °C, 65% humidity). F₁ hybrid females of the wild phenotype (brown-grey body, straight bristles and red eyes) were analysed. The appearance of bristles atypical for the normal phenotype in colour and shape was recorded. The area containing such a bristle was registered in the table as a single spot y (yellow) or sn (singed) or a double spot y sn.

DNA comet assay

To assess DNA damage in the third instar larval enterocytes, an alkaline variant of the DNA comet assay was used, which allows for the determination of single-strand DNA breaks in cells³⁴. The cell material was prepared by repeatedly puncturing the individuals and then homogenizing them. A fluorescence microscope was used to visualise and rank the DNA comets (Carl Zeiss Axio Imager M2, Germany).

EYFP system

We used tissue-nonspecific Gal4 - daughterless (females) no later than 6 hours after departure from the puparium and males from the spaghetti squash - EYFP - Mito line (sqh). This gene is involved in the processes of morphogenesis, proliferation, and regulation of signalling. w[*]; P{w[+mC]=sqh-EYFP-Mito}3 is a *Drosophila* strain in which UAS regulatory sequences direct the expression of a mitochondrial import sequence under the control of the *sqh* gene, which encodes the regulatory light chain of non-muscle myosin type 2. Hybrid offspring (not older than 7 days) grown on the tested media were analysed for changes in the fluorescence intensity in the abdominal region in females and males (Fig. 1C, D). We used this driver system because it initiates the general expression of the *spaghetti squash* target gene in all tissues and organs, and allows us to evaluate the change in the main effect of the cytotoxic drug etoposide by changing the level of oxidative stress and the activity of the apoptotic and anti-apoptotic gene cascade. Photofixation was performed on a Leica DM500 B microscope. Then, the change in the green spectrum was measured using the ImageJ program.

Statistical analysis

The χ^2 criterion with Yates's correction was used to assess the frequency of mosaicism. The expression level of the *sqh* gene was assessed using the nonparametric Kruskal-Wallis test. The data analysis in the DNA-comet test was performed using 2-way ANOVA (Tukey's multiple comparisons test).

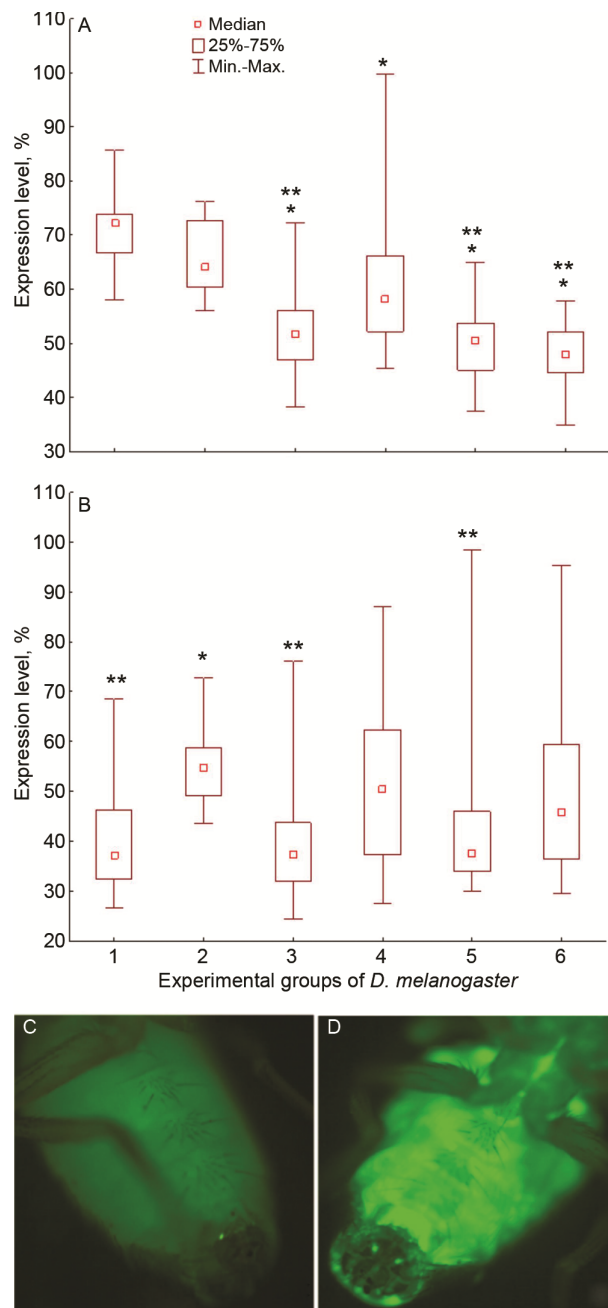


Fig. 1 — Expression level of females (A) and males (B) of experimental groups (1 – control, 2 – Etoposide 0.664 mg/mL, 3 – Extract *M. fistulosa* var. *media*, 4 – Extract *M. fistulosa* var. *fistulosa*, 5 – Extract *M. fistulosa* var. *menthifolia*, 6 – Extract *M. didyma*); An example of EYFP low (C) and highly intensive expression (D). The hybrid heterozygote flies *da/sqh*. The values were compared with each other using Kruskal-Wallis test. [* $, P < 0.05$ compared to the group of control flies; ** $, P < 0.05$ compared to the group of flies with etoposide]

Statistica 13.0 and GraphPad Prism 8.0 software was used for statistical processing, and ImageJ was used to measure fluorescence intensity.

Results

When accounting for mosaicism in the SMART test, none of the four extracts tested showed any genotoxic manifestation. Singed type spots appeared more frequently in the experimental group grown on a nutrient medium supplemented with *M. fistulosa* var. *fistulosa* extract (0.1%), but similar fluctuations in values also remain within the control group. According to Table 1, the combined use of extracts with the antitumor drug etoposide at the tested concentrations resulted in a decrease in the frequency of mosaicism. Also, for etoposide, the appearance of aberrant phenotypes was recorded, which do not occur when combined with extracts. A similar antigenotoxic effect was previously described for the *Prunella grandiflora* extract L³⁵.

Using the system EYFP, we determined the multidirectional effect of the use of extracts in almost all experimental groups relative to females and males. For all experimental groups of females, hypoeexpression of the *sqh* gene (decreased activity) was revealed. Abdominal luminescence did not decrease below 58.02% in the control sample, while in the experimental groups, when using *M. didyma*, the lower limit was 34. 81%. The interval of luminescence is expanded to maximum when individuals are cultivated on a nutrient medium with the introduction of *M. fistulosa* var. *fistulosa* extract and the average value is closer to the value of the etoposide group than in other groups grown on extracts, and it is in this group that the maximum upper limit of the interval was observed, namely – 99.77% (Fig. 1A). The smallest range of interval in the expression value was recorded in the group grown on a nutrient medium with the introduction of *M. fistulosa* var. *media* (38.26-72.29).

In turn, overexpression of the *sqh* gene was recorded in males. Above all is the lower limit of the expression in the experimental group exposed to – 43.57%. The only group of males in which hypoeexpression was found, like females, were individuals grown on a nutrient medium supplemented with an extract of 0.1% *M. fistulosa* var. *media*. When exposed to other monarda extracts, the upper limit of the interval was higher (*M. fistulosa* var. *fistulosa* – 87.11, *M. fistulosa* var. *menthifolia* – 98.33, *M. didyma* – 95.21) than in the case of using the *M. fistulosa* var. *media* – 76.14% (Fig. 1B).

This is the first study to provide data regarding the effects of extracts from *M. fistulosa* var. *media*,

Table 1 — Accounting for somatic mosaicism on *Drosophila melanogaster* with yellow and singed markers

Groups	Summary	Number of individuals with mutant spots					χ^2	<i>P</i>
		y	sn	y sn	Other mutant phenotypes	%		
Control	573	1	2	0	0	0.52	-	-
Etoposide (0.664 mg/mL)	833	1	12	0	9	4.59	7.55	0.006
Extract <i>M. fistulosa</i> var. <i>menthifolia</i> 0.1%	406	0	2	0	1	0.405	<0.0001	0.992
Extract <i>M. fistulosa</i> var. <i>fistulosa</i> 0.1%	205	0	2	0	0	0.9756	0.03	0.86
Extract <i>M. fistulosa</i> var. <i>media</i> 0.1%	407	0	3	0	0	0.424	<0.0001	0.995
Extract <i>M. didyma</i> 1%	381	0	4	0	1	1.0499	0.9	0.344
Extract <i>M. fistulosa</i> var. <i>menthifolia</i> 0.1% + Etoposide	272	0	2	0	0	0.7353	0.01	0.916
Extract <i>M. fistulosa</i> var. <i>fistulosa</i> 0.1%+Etoposide	514	1	4	0	2	1.474	0.7	0.404
Extract <i>M. fistulosa</i> var. <i>media</i> 0.1%+Etoposide	322	0	3	0	0	0.9317	0.08	0.771
Extract <i>M. didyma</i> 0.1%+Etoposide	422	0	6	0	0	1.4218	1.3	0.254
Extragent 0.1% (EtOH 70%)	242		3			1.2397	0.42	0.519

M. didyma, *M. fistulosa* var. *fistulosa*, *M. fistulosa* var. *menthifolia* on DNA repair *in vivo* after induction of DNA damage by etoposide influence on drosophila model.

The comet assay was used to examine the influence of *M. fistulosa* var. *media*, *M. didyma*, *M. fistulosa* var. *fistulosa*, *M. fistulosa* var. *menthifolia* extracts on DNA damage. We analysed the number of cells of flies with DNA damage and the index of DNA comets (IDC). The results of the studies are presented in Fig. 2A & 2B.

According to the data obtained, both plant extracts (*M. fistulosa* var. *fistulosa* and *M. fistulosa* var. *menthifolia*) adversely affect the studied parameters of DNA damage in drosophila, but *M. fistulosa* var. *fistulosa* shows a more pronounced negative effect than the other extracts. Significant differences were found compared with the etoposide group (*M. fistulosa* var. *fistulosa*) increases the number of cells with DNA damage by almost 30% [$P < 0.001$; IDC = 2.38]. Between extracts, the extract from *M. fistulosa* var. *fistulosa* turned out to be more aggressive in this case ($P < 0.05$). Cultivation of *D. melanogaster* with plant extracts from *M. fistulosa* var. *media* and *M. didyma* exhibits lower IDC values compared to the other two extracts (Fig. 2A). At the same time, supplementation of *Drosophila* with *M. didyma*, but not with *M. fistulosa* var. *media*, is associated with an increase in single-stranded

DNA breaks in cells (Fig. 2B) to compare with the control group. Significant differences were found compared with the etoposide group (*M. fistulosa* var. *media* decreases the number of cells with DNA damage more than 32% [$P < 0.05$; IDC = 1.13], and *M. didyma* reduces the number of cells with DNA damage by about 42% [$P < 0.05$; IDC = 0.92]).

Discussion

According to the results obtained, not all representatives of the genus *Monarda* are characterised by the absence of genotoxic manifestations under the conditions of introduction. At the same time, the greatest effect was observed at the level of DNA damage, while the number of realised somatic mutations and recombinations remained at the level of the control sample. The combined use of extracts with the anticancer drug etoposide reduces the genetic activity of the latter in the SMART analysis. This effect is important for minimising secondary tumor formation after chemotherapy treatment. But despite such effects demonstrated by representatives of the Lamiaceae family, other manifestations of the genetic activity of the drug are possible³⁶. The DNA comet-assay is an important component of such testing, often the antigenotoxic properties of the extract are observed precisely at the level of the degree of DNA damage³⁷. According to our results, out of all the extracts, two

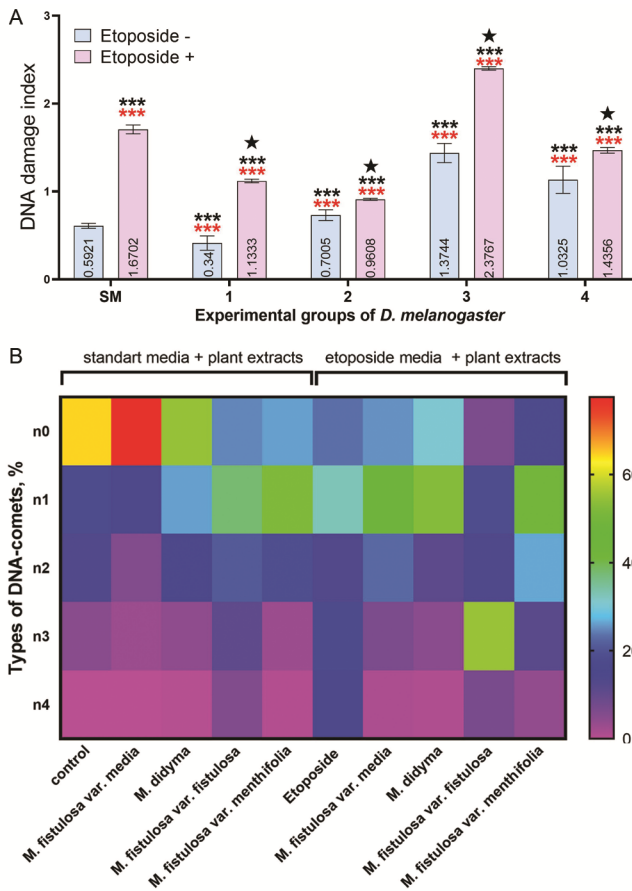


Fig. 2 — Influence of plant extracts 1 - *M. fistulosa* var. *media*, 2 - *M. didyma*, 3 - *M. fistulosa* var. *fistulosa*, 4 - *M. fistulosa* var. *menthifolia* on DNA stability of *D. melanogaster*; (A) The DNA damage index was calculated using the formula $(0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4) / \Sigma$, where n_0 - n_4 is the number of DNA comets of each type; Σ is the sum of the analysed DNA comets. The data was presented as columns (mean \pm standard deviation). [$*$, $P < 0.05$ compared to the group of control flies; red $*$, $P < 0.05$ compared to the group of flies with etoposide]; (B) The frequency of different types of DNA comets in experimental groups

are characterised by high IDC values when used together with etoposide, namely extract of *M. fistulosa* var. *fistulosa* and *M. fistulosa* var. *menthifolia*. At the same time, the *M. fistulosa* var. *fistulosa* extract together with etoposide demonstrates the degree of DNA damage much more than the anticancer drug alone. A similar negative effect on DNA was described for carvacrol, which is one of the most abundant biochemical constituents of monards³⁸. According to the data of Bolotnik *et al.* the content of flavonoids in *M. fistulosa* var. *fistulosa* in inflorescences is 3 times higher, and in leaves 1.5 times higher than in *Monarda fistulosa* var. *media*³⁹ and biochemical analysis of flavonoid content was carried out in three variation of *M. fistulosa*: *Monarda*

fistulosa var. *fistulosa* (0.012%), *Monarda fistulosa* var. *media* (0.015%) and *Monarda fistulosa* var. *menthifolia* (0.018%). Both in samples grown in the Moscow region and in Belarus, carvacrol is the dominant component of the essential oil of *M. fistulosa* var. *fistulosa*⁴⁰.

A rather high toxic potential of the *M. fistulosa* var. *fistulosa* extract in the form of a tincture on outbred white rats was also shown, and therefore it was assigned to class III of acute toxic substances⁴¹.

Active participation in the destabilisation of the expression of the *sqh* gene, the described toxic and genotoxic manifestations, the effect of synergy with the antitumor drug etoposide makes it inappropriate to use the extract of *M. fistulosa* var. *fistulosa* and *M. fistulosa* var. *menthifolia* at 0.1% concentration for biomedical purposes. The *sqh* gene product and myosin function in cellular processes such as cytokinesis and promote tissue morphogenesis. It is an ortholog of the human *MYL9* gene, which is associated with the development of familial hypertrophic cardiomyopathy. Mutations in the *sqh* gene lead to tau-mediated mitochondrial elongation, which causes neurotoxicity. A possible decrease in the applied concentration will lead to a less pronounced manifestation of the above effects. As for the extract of *M. didyma*, in addition to a multidirectional effect in changing the expression of the *sqh* gene, it does not demonstrate pronounced negative effects. However, the literature describes its insecticidal properties that affect the fertility and viability of *D. suzukii* individuals, which suggests its more detailed testing to minimise risks⁴².

The most promising in terms of further research and the manifestation of antigenotoxic properties is the *M. fistulosa* var. *fistulosa* var. *media* extract, which is characterised by a stable hypoexpressive effect on the *sqh* gene, the lowest IDC among the tested representatives of the genus *Monarda*, and the frequency of mosaicism within the control group.

Conclusions

According to the SMART results, with the combined use of etoposide and the tested extracts of representatives of the genus *Monarda*, the frequency of somatic mosaicism is reduced to the values of the control group. Individuals grown with the combined use of etoposide and *M. fistulosa* var. *fistulosa* extract are characterised by the maximum IDC. All tested extracts exceeded the IDC of the control group, except for the extract of *M. fistulosa* var. *media*,

which is characterised by a decrease in IDC. Also, the *M. fistulosa* var. *media* extract exhibits a unidirectional change in the expression of the *sqh* gene in *D. melanogaster* individuals of both genders.

Funding statement

The research funding from Ural Federal University Program of Development within the Priority-2030 Program is gratefully acknowledged.

Acknowledgements

The authors would like to thank the staff of the Institute of Cytology and Genetics of the Russian Academy of Sciences and the Laboratory of Geroprotective and Radioprotective Technologies of the Institute of Biology, Komi Science Centre, Ural Branch, Russian Academy of Sciences for providing *Drosophila melanogaster* lines used in the work.

Conflict of interest

No conflict of interest was reported by the authors.

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