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Review Article

Studying genotoxic and antimutagenic effects of plant extracts in *Drosophila* test systems

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ABSTRACT: To gain more information on biological effects of plants, particularly herbs used in human medicine and diet, *in vitro* and *in vivo* methods have been developed to predict their genotoxicity and/or antigenotoxicity in various test systems. The sex-linked recessive lethal (SLRL) and somatic mutations and recombination (SMART) tests are *in vivo* assays on *D. melanogaster* that have been used to test both mutagenic and antigenotoxic effects of extracts from numerous plant species used worldwide. The similarity of metabolic pathways between *Drosophila* and mammals and the ability to activate promutagens make the results of these tests widely applicable. Besides, *Drosophila* presents significant orthology with human genes that control cancers, which makes the assays on *Drosophila* reliable and informative for extrapolations onto humans.

Keywords: plant extracts, medicinal herbs, *Drosophila*, genotoxicity, antimutagenicity, diet

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INTRODUCTION

Detecting genotoxic effects of various compounds and their mixtures in different components of environment has called for constant surveys in pharmacy and biology. An array of standard *in vitro* and *in vivo* genotoxicity test systems has been established on different organisms, such as bacteria, yeast, *Drosophila* and mammals (Venitt & Parry 1984; Kirkland 1990). A genotoxic effect can result from a large variety of possible injuries to genetic material in cells, from single-strand DNA breakages to chromosomal changes, and it is of major importance in genotoxicity testing to develop methods that can reliably, with sufficient sensitivity, detect either such a vast array of damage or a general cellular response. No single test can detect the effect of every substance, and the concept of a battery of tests has therefore been implemented in many regulatory guidelines (Aardema & MacGregor 2002; Billinton *et al.* 2008). Antigenotoxic and antimutagenic effects of substances are possible to test using the same test protocols as for genotoxicity testing (Simić *et al.* 1998; Weisburger 2001). This can reveal the capability of substances for preventing or repairing damage to the genetic material in a cell.

Drosophila protocols for testing genotoxicity. To minimise the use of higher animals in toxicological research, tests with the fruit fly have been developed. *Drosophila melanogaster* has been used in a number of studies on genotoxicity, as well as antigenotoxicity, of various compounds and mixtures. Particularly valuable are *in vivo* tests for detecting somatic or germinative mutations (de G Mitchell & Combes 1984; Fujie & Fujikawa 1996). The similarity of metabolic pathways between *Drosophila* and mammals and the ability to activate promutagens make the results of these tests widely applicable. *Drosophila* presents an orthology of 68% of the human genes that control cancers, which makes the assays on *Drosophila* reliable and informative for extrapolations onto humans (Rubin *et al.* 2000; Lindsley & Zimm 2012).

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The sex-linked recessive lethal (SLRL) assay is an *in vivo D. melanogaster* test based on characteristics of sex-linked inheritance, and it detects gene mutations, small deletions or certain types of chromosome aberrations that are lethal in hemizygous and homozygous conditions before the adult stage (Wurgler & Graf 1985). The estimated number of all loci on the X chromosome that can mutate to recessive lethals is up to 800, which makes the evaluation of test results reliable. The SLRL test of genotoxicity has been chosen for the purpose of particular research, i.e., if a compound had a known effect on fertility or some reproductive stage. Mutagens vary widely in their potency for inducing mutations, and the sensitivity in detecting their effects is equally variable. The SLRL test provides information about the effect of agents at three male germ-line stages that differ in their sensitivity to potential mutagens and promutagens. According to the SLRL test procedure, flies are exposed on successive days, which provides information about effects of the tested agents on germinative material at the postmeiotic (spermatozoa and spermatids), meiotic (spermatocytes) and premeiotic (spermatogonia) stages. According to the literature, any toxic effect should be most severe at the meiotic stage, and it is expected that the antimutagenic effects would vary accordingly (Vogel 1984). This makes the SLRL protocol suitable for studying both mutagenic and antimutagenic effects on germinative cells. The results obtained for example in Stamenkovic-Radak *et al.* (2005) show that spermatocytes were the most sensitive to the mutagenic activity of methyl methanesulphonate (MMS), and that the strongest antimutagenic effect of the royal jelly used in that study was expressed at this stage as well. In a recent study that compared genotoxic effects of acute administration of a *Cotinus coggygria* methanol extract in an SLRL test and *in vivo* comet assay in rats, this traditional medicinal plant showed a strong genotoxic effect in both test systems under particular experimental conditions (Matic *et al.* 2011), which illustrates the suitability of *Drosophila* test systems for extrapolation to mammals and humans.

Among the most frequently employed *in vivo* genotoxicity assays developed in *Drosophila melanogaster*, the somatic mutation recombination test (SMART) can be used to detect a wide spectrum of genetic changes, such as point mutations, deletions, some types of chromosomal aberrations, mitotic recombinations and gene conversions (Idaomar *et al.* 2002; Munerato *et al.* 2005; Carmona *et al.* 2011). The flexibility of the test protocol, as well as the possibility of acute, chronic or combined treatments with several agents, make this test valuable in investigating various biologically active mutagens, promutagens and antimutagens (Heres-Pulido *et al.* 2004; Cakir & Sarikaya 2005; Costa & Nepomuceno 2006). The wing spot test is based on detection of mutations and recombinations resulting from heterozygosity loss during embryonic development, which is phenotypically expressed on wings of the adult fly (Graf *et al.* 1984; Graf *et al.* 1998). Antigenotoxic effects of various compounds have also been tested in *Drosophila*, mainly using somatic mutation tests (Nakano *et al.* 1994; Graf *et al.*1996; Rizki *et al.* 2001). The SMART test was used to detect antimutagens for the first time by Negishi *et al.* (1989), and this was followed by studies employing the same protocol to test antigenotoxicity of either single compounds or mixtures (Olvera *et al.* 1995; El Hamss *et al.* 1999; Santos *et al.* 1999; Lehmann *et al.* 2000; Abraham 2001). Somatic assays expose a large portion of mitotically growing cells in imaginal discs of the larvae. If a genetic alteration occurs in one of these imaginal disc cells, this alteration will be present in all the descendant cells and will form a clone of mutant cells. If the alteration causes a visible change in the phenotype, the mutant cell clone can be detected as a spot of mutant cells on the body surface in adult flies. Recently, the comet assay has also been adapted to be used *in vivo* in *D. melanogaster*, and studies with the combined tests give comprehensive results (Gaivão & Sierra 2014).

Genotoxicity and antigenotoxicity of plant extracts in Drosophila Assays. Green plants, in general, contain mutagenic and carcinogenic substances in different ratios (Sandermann 1988; Veleminsky & Gichner 1988; Plewa & Wagner 1993), but there is little information about their biological effects. Herbal medicines have a long history of use in prevention and treatment of diseases (Williamson 2003), but in contrast with conventional drug research and development, the toxicity of traditional herbal medicines is not often evaluated. In the post-genome era, several *in silico*, *in vitro* and *in vivo* approaches and methods could be applied to predict genotoxicity and teratogenicity of herbal medicinal products (Ouedraogo *et al.* 2012).

Many plants also have antimutagenic and/or anticlastogenic properties (Mitscher *et al.* 1996), and many traditionally used medicinal herbs can inhibit genotoxic effects as well. Studying antimutagens from herbs and spices in everyday diet is important because of their possible application in dietary prevention of cancers and other somatic mutation-related diseases (Hayatsu 1988; Ferguson 1994; Craig 1999). Studies have been conducted that have enabled identification of a large number of genes repressed or stimulated by a specific herbal compound (Zhou *et al.* 2004). One of the mechanisms unfolds via scavenging the reactive oxygen species (ROS) that may contribute to DNA damage and inhibition of repair mechanisms in a cell (Allen & Tresini 2000).

The SMART test protocol has been employed to test both genotoxic and antigenotoxic effects of extracts from different plant species used worldwide (El Hamss

et al. 2003; Romero-Jimenez *et al*. 2005). In a study of Romero Jimenez *et al*. (2005), the genotoxic activity of six commonly used medicinal herbs (*Matricaria chamomilla* L., *Tilia cordata* Miller, *Mentha piperita* L., *Mentha pulegium* L., *Uncaria tomentosa* (Willd. ex Roem. & Schult.) DC. and *Valeriana officinalis* L.) and their potential antigenotoxic effect were evaluated using the *D. melanogaster* SMART assay and hydrogen peroxide as an oxidative mutagen. The obtained results indicate the ability of hydrogen peroxide to induce somatic mutations and mitotic recombinations. All herbal infusions used in that study have been shown to be strong desmutagens against hydrogen peroxide. The results of inhibition obtained for *M. chamomilla*, *T. cordata*, *M. piperita*, *M. pulegium* and *V. officinalis* can be explained by attributing it to synergism between their phenolic contents and hydrogen peroxide due to the known ability of phenols to scavenge reactive oxygen species.

It has been demonstrated that juices from several vegetables, spices and herbs protect against certain carcinogens through their antioxidant capacity. Carotenoids, vitamine E and plant fibres have been implicated as anticarcenogenic agents (Hayatsu *et al*. 1988). In a study of Sortibran *et al*. (2015), the genotoxicities of celery (*Apium graveolens* L.), coriander (*Coriandrum sativum* L.), epazote (*Chenopodium ambrosioides* L.), parsley (*Petroselinum crispum* (Miller) A.W. Hill) and watercress (*Nasturtium officinale* (L.) R. Br.) were evaluated in a somatic *D. melanogaster* mutation and recombination test using crosses with regular and high levels of metabolising cytochrome P450 enzymes and 4-nitroquinoline (4NQO) as a carcinogen. The antioxidant strength of plant extracts analysed was dependent on the concentration, and a direct relationship was observed between the concentration of the vegetable extracts and radical-scavenging activity. The authors did not detect any correlation of the previously reported flavonoid content of the vegetables and plant extracts (Yang *et al*. 2008) with the observed antioxidant activity.

Sage is one of the plants reported to show antioxidative activity (Baricevic & Bartol 2000). Many species of this genus, including *Salvia officinalis* L., are used in traditional medicine in the form of herbal tisane or etheric oil. Its biochemical properties (the presence of tannin, triterpenoids, flavonoids, estrogenic substances, saponins and volatile oil, together with vitamin C and A in fresh leaves) add to the therapeutic effects of sage. Dried leaves are commonly used as a culinary spice for flavouring and seasoning. We studied the antimutagenic potential of *S. officinalis* by means of the somatic mutation and recombination test on *D. melanogaster* (Patenković *et al*. 2009). Methyl methanesulphonate was used as the mutagen, and different types of treatment were performed: short acute treatment with sage infusion or MMS, longer (chronic) treatment with sage solution or MMS and two combined treatments, i.e., short treatment with sage followed by longer treatment with MMS and *vice versa*. The sage infusion used in our experiments showed a clear antimutagenic effect, reducing the frequency of mutations induced by MMS. An inhibitory effect of sage tea was obtained and confirmed when pre- or post-treatments with the mutagen were used. The results indicate that although sage in this regime decreases the number of mutational events, it is not efficient enough in the case of a 2-h sage pre-treatment. Antioxidant activity and suppression of metabolic activation are possible mechanisms through which sage, or some of its components, acts as a desmutagen. Similar results, under conditions of the same experimental protocol, were obtained with an aqueous fruit extract of fennel *Foeniculum vulgare* Mill. (Amkiss *et al.* 2013). In addition to this, the same mutagen (MMS) and same protocol of the SMART test in *D. melanogaster* were used to study the mutagenic and antimutagenic effects of yellow gentian *Gentiana lutea* L. (Patenković *et al*. 2013). The results showed that an aqueous infusion of *G. lutea* was not genotoxic in somatic cells of *D. melanogaster* at a concentration of 25 mg/mL, under either chronic or acute treatment. However, results obtained with co- and post-treatments showed that gentian enhanced the frequency of mutant clones above the values obtained with MMS alone, by 22.64 and 27.13%, respectively, suggesting a synergism of gentian with MMS.

Different, although scarce, data on genotoxic potential were obtained when oil plant extracts were used instead of infusions. The essential oil of *M. piperita* was shown to be genotoxic in the SMART test (Lazutka *et al*. 2001). In contrast, the essential oil of *M. pulegium* was not genotoxic in the same assay (Franzios *et al.* 1997; Karpouhtsis *et al.* 1998). Idaomar *et al*. (2002) found by the SMART test that *Helichrysum italicum* (Roth) G. Don fil., *Ledum groenlandicum* Oeder (= *Rhododendron groenlandicum* (Oeder) Kron & Judd) and *Ravensara aromatica* Sonn. (= *Cinnamomum camphora* van der Werff) essential oils and their mixture reduce the frequency of urethane-induced mutations in *D. melanogaster*. In another study, Mezzoug *et al*. (2007) showed in the same system that *Origanum compactum* Benth. essential oil and some of its sub-fractions and constituents are antimutagenic against the indirect-acting mutagen urethane, and also against the direct-acting mutagen methyl methanesulphonate.

Essential oils extracted from three medicinal plants (Idaomar *et al.* 2002) showed toxic and antimutagenic effects in the wing spot test when used as a mixture. Both the antimutagenic effect and toxicity are possibly a result of their interaction with the cytochrome P-450 activation system. Furthermore, an antigenotoxic effect against spontaneous mutations, in regard to the total of the spots recorded, was obtained after a 2-h

treatment. This indicates that sage tea components can significantly reduce the number of somatic mutations. Such an effect is absent after prolonged exposure to sage tea, which is attributable to some toxic effects of chronic exposure. A similar effect was obtained with bell pepper, which weakly increased the frequency of spontaneous mutations (El Hamms 2003) and content of ascorbic acid in co-treatments in a study of Kaya *et al.* (2002).

The discrepancy of results obtained with infusions as opposed to oil extracts could be attributed to differences between the tests, i.e., the comet assay vs. the somatic mutation assay, or to biology of the organism used if the test is *in vivo*. Results obtained in bacteria and yeast test systems showed that *Salvia offcinalis* and its major components, thuyone, 1,8-cineole, camphor and limonene, inhibit UVC-induced mutagenesis in *Salmonella typhimurium*, *Escherichia coli* and *Saccharomyces cerevisiae* (Knežević-Vukčević *et al.* 2005; Vuković-Gačić *et al.* 2006). However, the antimutagenic potential of *S. officinalis* in our experiments with the *D. melanogaster* SMART system was assayed in the form of a tea infusion while that of *Gentiana luetea* was assayed as an aqueous solution rather than the essential oil, since the use of herbal infusions is quite common in the human diet, and in *Drosophila* tests the odour of oils in certain concentrations can be repellent for flies, potentially causing a bias in the results.

The differences observed in biological activities of plant extracts in oils and infusions could also be attributed to qualitative and quantitative differences in their capacities for transport through cell membranes (Plewa & Wagner 1993). In that regard, if only common compounds present in both solutions are taken into account, oils must be more active than infusions because the former are more concentrated during processing (for a review see Bakkali *et al.* 2008).

Synergistic effect of different components in plant extracts. Comparison of the genotoxicity of essential oils in different test systems shows that different compounds present in essential oils are responsible for genotoxicity in tests. Genotoxic properties of essential oils extracted from dill (*Anthum graveolens* L.), peppermint (*M. piperita*) and pine (*Pinus sylvestris* L.) needles were studied using chromosome aberrations and sister chromatid exchange (SCE) tests in human lymphocytes *in vitro* and the *D. melanogaster* SMART test *in vivo* (Lazutka *et al.* 2001). Essential oils from dill herb and seeds are similarly active in human lymphocytes, but very different in the SMART test in *Drosophila*. Furthermore, the composition of essential oils differs significantly even within the same taxon, depending on many factors, such as genetic background, climate, soil, growing conditions, etc. (Muller-Riebau *et al.* 1997). Despite increasing research on flora, species of higher plants have only partly been chemically and pharmacologically investigated. Chemical composition affects bioactivity as well, which makes genetic risks complex. One possible solution is to identify genotoxicity of individual compounds present in an essential oil mixture, and extrapolate to human exposure. Genetic engineering methods are attractive as a way to eliminate or reduce genotoxic compounds, i.e., engineering of peppermint to contain relatively more menthol and less menthone (Lange & Croteau 1999) may reduce the genotoxicity of peppermint essential oil.

Consumption of vegetables, spices and herbs in the human diet has been associated with healthy nourishment and is considered almost completely safe, although most of them are complex mixtures that could also contain mutagenic and carcinogenic chemical compounds. Genotoxic and/or antigenotoxic effects depend on the various compounds present in their extracts. The fact that a lower concentration of *Cotinus* extract was not genotoxic in an alkaline comet assay (Matic *et al.* 2011) suggests possible antigenotoxic activity of polyphenolic constituents. Partial chemical analysis of a methanol extract of *Cotinus coggygria* Scop. showed flavonoids, tannins and phenolic compounds to be the main components (Stanic *et al.* 2009), and dominant compounds in the ethyl acetate partition of *C. coggygria* were disulfuretin, sulfuretin, sulfurein, gallic acid, methyl gallate and pentagalloyl glucose (Westenburg *et al.* 2000). It has been suggested that polyphenolic compounds produce anticarcinogenic effects, and gallic acid and its derivatives are biologically active and have been reported to be free radical scavengers (Kawada *et al.* 2001; Sohi *et al.* 2003). Flavonoids are naturally occurring molecules with antioxidant, cytoprotective and anti-inflammatory activity. Tannins, as one such class of compounds, are suspected of possessing protective properties. Fedeli *et al.* (2004) showed that they are capable of protecting against DNA breakage at low concentrations, although at high levels they can be genotoxic.

It has been indicated that some components in plant extracts or a combination of them can be genotoxic to *D. melanogaster*, leading to ambiguous conclusions. In a study by Sohni & Kale (1997), the SLRL test was done to evaluate the mutagenicity of *Combretum erythrophyllum* (Burch.) Sond., a tree highly valued in traditional medicine in southern Africa. It was observed that the aqueous extract caused mutations in the meiotic stage of *D. melanogaster*. Because the authors did not isolate the active therapeutic ingredient, the agent responsible for this effect is still speculative. Extracts from *Plantago major* L., used in treatments of many diseases around the world, have produced contradictory results in toxicity tests (Samuelsen 2000). In a study of Pimenta & Nepomuceno (2005), the *Drosophila* somatic mutation and recombination test was used to investigate genotoxic and antigenotoxic properties of an aqueous extract of *P. major*; under their experimental conditions, it was

genotoxic, indicating that recombination was a major response. The chemical composition of *P. major* is very complex. Some reports suggest that certain constituents of *P. major* contain toxic agents, such as oxalic acid, nitrates and erucic acid (Guil *et al*. 1997). In a study on *Drosophila* , the SMART test was performed with an aqueous fruit extract of fennel *F. vulgare* (Amkiss *et al*. 2013), whose fruit is known to contain rich phenolic compounds. Investigation of the mutagenicity, antimutagenicity and anticarcinogenicity of fennel and some of its components indicated that trans-anethole, the main component, is not mutagenic in several test systems (Shahat *et al*. 2011). In addition, pre-treatment with trans-anethole and eugenol even in high doses led to significant antigenotoxic effects against several strong mutagens (Abraham 2001). The inhibitory effect detected in that study can be attributed to a wide range of constituents of the plant, including trans-anethole, estragole, fenchone, sesquiterpenoids, coumarins and the studied polyphenolics.

The compound 4-nitroquinoline 1-oxide (4NQO) was used to study the effect of chlorophyll in *Drosophila* (Negishi *et al*. 1997). The administration of both the mutagen and chlorophyll to flies was by simultaneous feeding, similar to human diet conditions. The results showed significant inhibitory activity of chlorophyll extract from spinach toward somatic mutations. The chlorophyll samples were given to *Drosophila* orally, together with the mutagen, which is closer to the real setting for humans than the bacterial assay. A possible mechanism of inhibition is disturbance of the mutagenic activity of 4NQO through metabolic enzymes, which makes the results of *Drosophila* genotoxicity and antimutagenicity tests applicable to humans.

Since numerous plants, herbs and spices are widely used in the human diet and particularly in traditional medicine, it is important that systematic studies be conducted in order to identify their principal chemical components and characterise their individual and mixed mutagenic and/or antimutagenic effects in relevant test systems on different organisms. The genomic approach offers a powerful tool for defining and predicting the pharmaco-toxicological activities of medicinal herbs. The rapid progress in "omics" technologies creates a great opportunity to compare the underlying mechanisms and effects of various compounds in different test systems and create a sound basis for better hazard identification and for more relevant genotoxicity assessment.

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Botanica SERBICA

REZIME

Ispitivanje genotoksičnog i antimutagenog efekta biljnih ekstrakata u test sistemima na *Drosophila*

Marina Stamenković-Radak i Marko Andjelković

Da bi se dobile informacije o biološkim efektima biljaka, posebno lekovitog bilja koje je u širokoj upotrebi u medicini i ishrani, metode *in vitro* i *in vivo* su razvijene radi procene njihovog genotoksičnog i/ili antimutagenog efekta u različitim test sistemima. Test za polno vezane recesivne mutacije i test za somatske mutacije i rekombinacije su *in vivo* eseji na *Drosophila melanogaster* koji se koriste za testiranje kako genotoksičnog tako i mutagenog efekta ekstrakata biljnih vrsta korišćenih širom sveta. Sličnost metaboličkih puteva *Drosophila* i sisara, kao i sposobnost aktiviranja promutagena, čine rezultate ovih testova široko primenljivim. Osim toga, genom *Drosophila* poseduje značajnu ortologiju sa genima čoveka koji su uključeni u kontrolu kancera, što testove na *Drosophila* čini pouzdanim i informativnim u ekstrapoliranju rezultata.

Ključne reči: ekstrakti biljaka, lekovito bilje, *Drosophila*, genotoksičnost, antimitagenost, ishrana