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Research Article

Evaluation of the mutagenic potential of *Cochlospermum regium* in *Drosophila melanogaster* male germ cells

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Abstract

During the last few decades the search for medical treatments based on alternative medicine has increased significantly, making knowledge of the plants commonly used as folk medicines extremely important. The plant C*ochlospermum regium*, a member of the Cochlospermaceae found in the Brazilian *cerrado* (a type of savanna), is known to have high depurative activity and to be effective not only in treating skin problems such as pimples, boils and blotches but also in curing gastritis and ulcers. We prepared aqueous extracts using 13, 19 and 25 gL^{-1} of dried *C. regium* root and investigated these extracts for possible mutagenic effects on *Drosophila melanogaster* germ cells. Mutagenesis was assessed using the ring-X loss (RXL) test which can detect chromosome mosaicism, partial loss of the ring X chromosome and chromosome non-disjunction. Our results showed that at the concentrations tested *C. regium* extracts did not induce ring-X loss in *D. melanogaster*.

Key words: mutagenic potential, ring-X-loss, *Cochlospermum regium*, *Drosophila melanogaster*.

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Introduction

Since ancient times humans have used plants both for their prophylactic effects and for the treatment of illnesses and diseases. According to the World Health Organization (WHO) about 80% of developing countries use traditional folk medicines, 85% of which are plant extracts. The Brazilian flora has been estimated to be the largest in the world, the country being home to about 120 thousand species, of which only 1% have been studied for their phytochemical and pharmacological properties (Rizzo *et al*., 1996). Infusions of medicinal plants are widely used by Brazilians (Vicentini *et al.*, 1996) and in the central Brazilian state of Goiás a significant proportion of the population uses medicinal plants native to this region (Rizzo *et al*., 1996).

Plants produce a variety of toxic substances, some of them in significant amounts, as a defense against microorganisms (bacteria, fungi, insects and viruses) and animals. The indiscriminate medicinal use of plants, many of which are toxic, can have health risks because a dosage threshold exists for each medicinal plant as it does for synthetic pharmaceuticals (Almeida, 1993). Toxic compounds in medicinal plants may favor mutational events in somatic or germ cells, such events possibly leading to the development of somatic diseases or teratogenic, mutagenic or carcinogenic effects (D'Oliveira, 1998).

Some substances produced by medicinal plants have been studied and characterized (Salvadori *et al*., 1991; Simões *et al*., 2000; Younes *et al*., 2000; Carvalho *et al.*, 2001) but insufficient toxicological and genotoxicological studies have been done, although there has recently been growing interest in the possible toxic, genotoxic and/or mutagenic effects of those plant metabolites which are used therapeutically. These studies have included the development of rapid screening tests using bacteria, *in vitro* cell-culture assays, rodents, and the fruit fly *Drosophila melanogaster*.

The use of *D. melanogaster* as a test organism to evaluate genetic alterations offers several advantages because this fruit fly is easy to maintain in the laboratory, has a variety of genetic markers that can be used to detect mutations and there is a vast array of literature on its behavior, ecology, evolution and other biological parameters (Andrade *et al.,* 1991; Cunha, 1993). Toxicological tests which use *D. melanogaster* germ cells to evaluate genetic alterations are based on the effects of potentially toxic compounds on sex-linked recessive lethal (SLRL) mutations and loss of the ring X chromosome (ring-X loss, RXL), both of which provide important information on the risk of mutation in future generations of *D. melanogaster* and can be extrapo-

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lated to mammals with a success index of at least 80% (Spanó *et al*., 2001).

Several plants and plant infusions rich in pyrrolizidine alkaloids have been reported as having genotoxic, mutagenic, carcinogenic and teratogenic effects (Ames, 1983), some of these plants being commonly used in human diets and herbal medicines. However, many natural or synthetic anti-mutagenic substances also occur and these can modulate the effects of toxic compounds by acting as antagonists to mutagenic agents (Hayatsu *et al*., 1988).

Epidemiological studies have demonstrated a possible correlation between human dietary habits and a high frequency of certain types of cancer (Shankel *et al*., 1985), while coffee, tea and alcoholic beverages have been described as being involved in the generation of reactive oxygen species (superoxide, hydrogen peroxide and hydroxyl radicals) that can react with and damage DNA (Leitão *et al*., 1992).

In Brazil, medicinal plants are used extensively but only a few have been studied regarding their biological effects, Vicentini *et al*. (1996) having noted that it is necessary to investigate the genotoxic potential of medicinal plants in order to assess their relative beneficial or deleterious effects.

The small (1-2m) shrub *Cochlospermum regium* (Mart ex Schrank) Pilger (Cochlospermaceae), known as *algodãozinho-do-campo* in Portuguese, is native to the *cerrado*, a savanna-like area in the central plateau of Brazil. The roots of *C. regium* are extensively used in Brazilian folk (or 'popular') medicine as a cleansing agent and for the removal skin and for treating gastritis and ulcers. The work reported in this paper focused on the mutagenic effects of *C. regium* on *D. melanogaster* as assessed using the RXL test.

Material and Methods

We collected *Cochlospermum regium* from an area of *cerrado* vegetation in Silvânia, a municipality in the Brazilian state of Goiás. The *C. regium* roots were dried at 45 °C in a forced ventilation stove and ground in a fraction mill to a dry powder. Aqueous extracts were prepared by infusing the powdered root in distilled water at room-temperature (≈25 °C), extracts being prepared at concentrations of 13, 19 and 25 gL^{-1} and was tested in D. melanogaster.

The *D. melanogaster* were designed to evaluate the clastogenic activity of aqueous infusions of dried and ground *C. regium* root on metabolically inactive sperm cells (Brood 1) and metabolically active mature (Brood 2) and immature (Brood 3) spermatids.

The ring-X loss test detects mainly chromosome losses and deletions, two *D. melanogaster* lineages being used in the test; the ring-X lineage which has a sexual X chromosome in the form of a ring and the $ywsn^3$ lineage which has three genetic markers. Both lineages were maintained in 'snap-cap' bottles containing banana-agar (Marques *et al.*, 1966) at 60% relative humidity and 25 ± 1 °C.

Tests were carried out by fasting 66-hour-old ring-X males for six hours by placing them in a trip tube containing a double sheet of absorbent paper saturated with 10 mL of distilled water, after which the 72-hour-old males were removed and placed for 24 h in trip tubes containing absorbent paper saturated with 0.5 mL of one of the *C. regium* root extracts (13, 19 or 25 gL^{-1}). The same general procedure was use for positive and negative controls, except that for the positive control the root extract was replaced with a 20 mM solution of urethane. while for the negative control the extract was replaced with phosphate buffer. Treatment I - 0,013 g/mL of the extract of the plant; Treatment II - 0,019 g/mL of the extract of the plant; Treatment III - 0,025 g/mL of the extract of the plant. After being exposed to the solutions for 24 h, the males mated to produce F1 offspring by removing them from the trip tubes and placing them into tubes containing banana-agar and virgin $ywsn^3$ females where they were allowed to mate to produce Brood 1 offspring. After three days the males were transferred to fresh tubes containing banana-agar and fresh $ywsn^3$ virgin females where they mated and produced Brood 2 offspring. After a further two days the males were again transferred to fresh tubes containing banana-agar and fresh virgin $ywsn^3$ females where they mated to produce Brood 3 offspring, the males being discarded after two days. In all cases the females were discarded after that eggs were collected and a thick layer of live fermenting yeast supplemented with sucrose was added to the tubes containing the larvae every three days.

Started the emergency of the adults forms, at the tenth day, the F1 progeny were analysed during four days by entomological microscopy technic. Assessment was made possible because the sexual chromosomes of both progenitors were marked with specific visible mutations which enabled each chromosome (or segment) to be identified based on one of the 11 different possible F1 phenotypes classes. The loss of one of the phenotypic markers could occur through several mechanisms, *i.e.* deletion of a section containing a given marker (1 or 2 breaks) or the loss or gain of an entire chromosome by or non-disjunction.

The flies were divided into 11 classes, class 1-3 = ring-X loss (1 = no ring-X loss, 2 = some ring-X loss and 3 = complete ring-X loss); 4 and 8 = mosaicism; 6 and 7 = partial loss of the Y chromosome; 5, 9, 10 and 11 = non-disjunction.' or something similar]. The calculation of the frequency of complete loss of the ring X chromosome was obtained using the formula, % complete loss of the ring X chromosome = class 3 + class 4 divided by class 1 + class 3 + class 4 + class 5 + class 11. The ring-X loss test was performed according to the model proposed by Frei and Würgler (1988), and the results were classified as inconclusive, positive, faintly positive or negative.

Results

Male *D. melanogaster* fed *C. regium* extracts showed no statistically significant ring-X loss when compared to negative control *D. melanogaster* males fed phosphate buffer only (Table 1). Zijstra (1987) proposed that calculations should performed separately for the individuals in each of the different classes (Mosaicism, partial loss of the Y chromosome and non-disjunction), as compared to the total number of individuals analyzed but when this was done we again found no statistically significant differences for the extracts containing different concentrations of *C. regium* (Table 1). All these relationships were equally true for the three broods examined.

Discussion and Conclusion

Short duration tests with *D. melanogaster* allows the evaluation of the mutagenic potential of substances based on the induction of genic mutations and/or the breakage of the chromosomal elements of germinal cells, such tests being widely used in government-sponsored programs for the identification of potential genotoxic or carcinogenic industrial residues that might lead to environmental contamina-

Table 1 - Results of the ring-X loss test on male *Drosophila melanogaster* fed different concentrations of *Cochlospermum regium* extract or the mutagen urethane.

Brood number and concentration of test substance (gL^{-1})	Number of progeny with genetic changes						Percentage of progeny with complete ring-X loss
	Number of normal progeny		Type of change				
			Y chromosome loss				
	Female	Male	Complete	Partial	Mosaicism	Male non-disjunction	
BROOD 1 (3 days mating)							
C. regium extract							
0	1837	1886	9	0	5	12	1.92
13	2058	1920	33	0	3	11	1.67
19	1942	2006	30	2	3	16	1.62
25	1791	1891	25	1	2	16	1.43
Urethane							
4	1684	1782	76*	1	12*	26*	4.79*
BROOD 2 (2 days mating)							
C. regium extract							
0	1442	1574	18	0	4	11	1.50
13	1637	1650	23	1	2	14	1.50
19	1561	1721	20	0	2	9	1.32
25	1674	1753	30	3	2	12	1.75
Urethane							
4	1507	1792	50*	0	11*	28*	3.69*
BROOD 3 (2 days mating)							
C. regium extract							
0	1286	1357	23	0	2	6	1.83
13	1571	1576	17	0	2	6	1.13
19	1647	1743	21	0	3	11	1.37
25	1609	1597	20	0	2	9	1.35
Urethane							
4	1317	1345	50*	1	10*	23*	4.21*

Calculated as percentage of complete ring-X loss (class3) + mosaicism (class 4)/ no ring-X (class 1) + complete ring-X loss (class 3) + mosiciism (class 4) + non-disjunction (class 5 and 11).
*Statistically significant when compared to the negative control (0 gL^{-1} *C. regium* extract; positive control = 4 gL^{-1} urethane).

tion. The ring-X loss (RXL) test has a recognized place in toxicological genetics, where it is often used for identifying genotoxic agents. Although other chromosome alterations are also tested for in the RXL test, the main alteration tested for is the loss of the ring X chromosome. It is known that the ring X chromosome is more sensitive to the effects of ionizing radiation or chemical mutagens than normal linear chromosomes and although the basis of such sensitivity is not fully understood it is thought to involve multiple mechanisms. One mechanism thought to be involved in the complete loss of the ring X chromosome is simple chromosomic breakage or double. (Leigh, 1976; Vogel and Natarajan, 1979a, 1979b, Zimmering, 1981; Zijstra and Vogel, 1988), an alternative mechanism being the occurrence changes among sister chromatids (Racine *et al*., 1979; Würgler and Graf, 1980; Velàsques *et al*., 1986), although this type of event is rarely found in *Drosophila* (Zijstra and Vogel, 1988).

Mosaicism (classes 4 and 8) can be caused by loss of chromosomes during division (Leigh, 1976), although mosaicism can also be produced by somatic crossing-over (Stern, 1986; Leigh, 1976; Zijstra and Vogel, 1988). The partial loss of the Y chromosome (classes 6 and 7) was due to the loss of the markers for the short or long arm of this chromosome and in this case there was little doubt that the great majority of partial chromosome losses were due to chromosome breaks, although these are relatively rare in the Y chromosome as compared to the ring X chromosome. The loss of the *Bs* marker situated in the long arm of the Y chromosome is , without doubt, the result of a chromosome break, while the loss of the short arm *y*+ marker can result from breaks or point mutations. Valencia *et al.* (1984) examined the RXL test and proposed that non-disjunction results from the loss or gain of whole chromosomes.

The corrected percentage of spontaneous complete loss of the ring X chromosome obtained in our experiments varied from 1.80% to 1.94%, such variation, according to Leigh (1976), being possibly due to the influences of dietary factors and the ages of the maternal and paternal parent flies.

It is known that *D. melanogaster* post-meiotic sperm cells have no DNA repair mechanisms (Sankaranayanan and Sobels, 1976), although damage can be repaired after fertilization by maternal repair in repair-proficient oocytes (Cunha *et al.*, 1994). The fact that our results showed no statistically significant differences between *D. melanogaster* fed *C. regium* extract and those fed phosphate buffer could be due either to *C. regium* having no clastogenic activity and/or maternal repair after fertilization. It is also possible that more concentrated *C. regium* extracts would have different effects because chromosome breaks require high levels of genotoxic agents; this could be verified by producing more concentrated extracts by evaporation of the solvent and performing further experiments using the system described here.

There was no statistically significant difference between the three broods nor were any mutagenic (Brood 1 sperm without metabolic activity) or pro-mutagenic (Broods 2 (mature) and 3 (immature) spermatids with metabolic activity) effects detected. The fact that the known mutagen urethane gave positive results at concentrations comparable to (or lower than) those of the *C. regium* extracts indicates that the negative results obtained with the *C. regium* extracts were not false-negatives and supports the validity of the testing methods used.

In summary, the ring-X loss (RXL) experiments using extracts containing 13, 19 and 25 gL^{-1} of dried and powdered *C. regium* root showed no clastogenic activity and was not either directly or indirectly mutagenic as assessed by the *D. melanogaster* RXL test (capable of detecting total loss of the X ring chromosome, presence of mosaicism, partial loss of the Y chromosome and non-disjunction) nor were there any effects on metabolically inactive sperm or metabolically-active mature and immature spermatids.

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