



SERVIÇO PÚBLICO FEDERAL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE UBERLÂNDIA



PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

**ESTUDO COMPARATIVO DOS EFEITOS MODULADORES DA (-)-CUBEBINA
SOBRE A MUTAGENICIDADE / RECOMBINOGENICIDADE INDUZIDA POR
DIFERENTES AGENTES QUÍMICOS**

Aluno: Alexandre Azenha Alves de Rezende

Orientador: Prof. Dr. Mário Antônio Spanó

Co-Orientadora: Profa. Dra. Denise Crispim Tavares

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**“O segredo da felicidade é fazer do seu
dever, o seu prazer”.
Ulysses Guimarães**

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RESUMO

(-)-Cubebina (CUB) é uma lignana dibenzilbutirolactólica isolada a partir de sementes secas de *Piper cubeba* L. (Piperaceae). CUB possui atividades anti-inflamatória, analgésica e antimicrobiana. Neste trabalho foram avaliadas a mutagenicidade e recombinogenicidade de diferentes concentrações (0,25; 0,5; 1,0; 2,0 ou 4 mM) de CUB, isoladamente ou em combinação com doxorrubicina (DXR - 0,2 mM), usando os cruzamentos padrão (ST) e de alta capacidade de bioativação metabólica (HB), do Teste para Detecção de Mutação e Recombinação Somática (Somatic Mutation and Recombination Test – SMART) em células de asas de *Drosophila melanogaster*. Os resultados observados em ambos os cruzamentos foram muito semelhantes. CUB não induziu mutação ou recombinação. A DXR, usada como controle positivo e também para avaliação antimutagênica da CUB, é um agente antineoplásico, inibidor da topoisomerase, que pode induzir quebras de cadeias simples e duplas, intercalar ao DNA, e gerar radicais livres. A recombinação é o principal efeito observado em células somáticas de asas de *D. melanogaster* quando tratadas com DXR. Nas associações (CUB + DXR), nas concentrações mais baixas, CUB reduziu significativamente as frequências de manchas mutantes induzidas pela DXR. No entanto, em concentrações mais elevadas, CUB potencializou os efeitos da DXR, elevando a frequência de manchas mutantes, e devido à citotoxicidade, houve redução nas concentrações mais elevadas. Os tratamentos combinados reduziram apenas as frequências de mutação induzidas pela DXR, mas não afetaram as frequências de recombinação. Para a avaliação da interferência da CUB sobre os danos cromossômicos (efeitos clastogênicos e aneugênicos) induzidos por DXR (2 mM), foram utilizados fibroblastos de pulmão de hamster chinês (células V79). Para este fim, as frequências de micronúcleos (MN) em 6.000 células binucleadas por tratamento foram utilizadas com parâmetro de análise. As concentrações escolhidas (2, 4, 8 e 16 µM) foram obtidas após avaliação do índice de divisão nuclear (3.000 células por tratamento), utilizado para mensurar o potencial citotóxico. Não houve diferença significativa na indução de MN observada entre os grupos tratados com as diferentes concentrações de CUB, quando comparado com o controle negativo ($P > 0,05$). Os resultados

obtidos indicam ausência de efeitos mutagênicos da CUB nas concentrações utilizadas. Os resultados também mostraram que CUB reduziu significativamente a frequência de MN induzida por DXR, com uma redução média de 63,88%. Para avaliação da interferência da CUB com o sistema enzimático de detoxificação citocromo P450 (CYP), o etil carbamato (uretano - URE) foi associado à CUB. O URE é um agente promutagênico e procarcinogênico e sua metabolização via CYP induz significativamente o aparecimento de manchas mutantes em *D. melanogaster*. As versões ST e HB do SMART foram utilizadas para estimar os efeitos antimutagênicos e antirecombinogênicos da CUB (0,25; 0,5; 1,0; 2,0 ou 4,0 mM) associados ao URE (10 mM). Os dados observados nos indivíduos MH do cruzamento ST, mostraram que efeitos inibitórios significativos foram encontrados somente em concentrações de 1,0 e 2,0 mM, enquanto que no cruzamento HB, todas as concentrações de CUB inibiram estatisticamente os danos de DNA induzidos pelo URE. Em concentrações mais baixas, as frequências de recombinação são reduzidas, porém, nas concentrações mais elevadas, as frequências de recombinação aumentam. Em conclusão, os resultados obtidos sugerem que, dependendo da concentração, CUB pode atuar como um agente sequestrador de radicais livres (baixas concentrações); ou como um pró-oxidante (concentrações elevadas), quando interage com o sistema enzimático que catalisa a detoxicação metabólica da DXR ou URE; ou ainda como indutor de reparo de DNA por recombinação. Estes estudos devem colaborar com a melhor compreensão dos mecanismos de ação da CUB e, conseqüentemente, com a produção de medicamentos eficazes, seguros e de baixa toxicidade.

ABSTRACT

(-)-Cubebin (CUB) is dibenzylbutyrolactolic lignan isolated from dry seeds of *Piper cubeba* L. (Piperaceae). CUB possesses anti-inflammatory, analgesic and antimicrobial activities. In the present study were examined the mutagenicity and recombinogenicity of different concentrations (0.25; 0.5; 1.0; 2.0 ou 4.0 mM) of CUB alone or in combination with DXR (DXR – 0.2 mM) using standard (ST) and high bioactivation (HB) crosses of the wing Somatic Mutation And Recombination Test (SMART) in *Drosophila melanogaster*. The results from both crosses were rather similar. CUB alone did not induce mutation or recombination. Doxorubicin (DXR) used as a positive control and also to antimutagenic evaluation of CUB, is a chemotherapeutic agent that inhibits topoisomerase inducing single- and double-strand breaks, intercalate into the DNA and generate oxygen free radicals. Recombination is the major effect observed in wing somatic cells of *D. melanogaster* when treated with DXR. When associated (CUB + DXR), at lower concentrations, CUB significantly reduced the frequencies of mutant spots induced by DXR. However, increasing the concentrations, CUB potentiated the effects of DXR, raising the frequencies of mutant spots and at higher concentrations, due to toxicity, the frequencies of mutant spots reduced. The combined treatments reduced DXR mutagenicity but did not affect DXR recombinogenicity. To the assessment of the interference of CUB on the chromosomal damage (clastogenic and aneugenic effects) induced by DXR (2 μ M), were used lung fibroblasts of Chinese hamster (cells V79). For this purpose, the frequency of micronuclei (MN) in 6000 binucleated cells/treatment was used as a parameter of analysis. The chosen concentrations (2, 4, 8 or 16 μ M) were obtained after evaluation of the nuclear division index (3000 cells/treatment), used to evaluated cytotoxicity potential. No significant difference in the induction of MN was observed between the groups treated with the different concentrations of CUB and the negative control ($P>0.05$). There was no significant difference on MN induction observed between groups treated with different concentrations of CUB when compared to negative control ($P> 0,05$). These findings indicate the absence of mutagenic effects of CUB at the concentrations tested. The results also showed that CUB significantly reduced the frequency of MN induced by DXR, with a mean reduction

of 63.88%. In order to evaluate the interference of CUB on the enzymatic detoxification complex cytochrome P450 (CYP), ethyl carbamate (urethane – URE) was associated with CUB. URE have been proved to be a promutagen and procarcinogenic substance due to its metabolization through CYP increases significantly the frequencies of mutant spots in *D. melanogaster*. The ST and HB versions of the SMART were used to estimate the antimutagenic / antirecombinogenic effects of CUB (0.25; 0.5; 1.0; 2.0 or 4.0 mM) associated to URE (10 mM). The data obtained showed that in the MH individuals from the ST cross, significant inhibitory effect was found only at concentrations of 1.0 and 2.0 mM, while in HB cross all concentrations of CUB statistically inhibit URE-induced DNA damage. At lower concentrations, the recombination level decreases, but at highest concentration the recombination level increases. In conclusion, our data suggest that depending on the concentration, CUB may act as a free radical scavenger at low concentrations; a pro-oxidant at higher concentrations, when it interacts with the enzymatic system that catalyzes the metabolic detoxification of DXR or URE; and/or an inducer of recombinational DNA repair. These studies should collaborate with the better understanding of mechanisms of action of CUB and, consequently, to the production of effective and safe drugs with low toxicity.

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APRESENTAÇÃO

Apresentação

As plantas têm sido usadas, ao longo da história, como fonte primária de alimento, combustível e remédio. Na área da Fitoquímica, as plantas medicinais são caracterizadas bioquimicamente devido aos seus compostos bioativos, que podem ser identificados, isolados e caracterizados quimicamente. Com base no emprego de determinadas plantas na medicina popular e em pesquisas científicas, os produtos naturais têm desempenhado um importante papel na descoberta de novas substâncias, provendo estruturas químicas que podem ser utilizadas como potencial fonte medicamentosa. Muitos produtos isolados de plantas têm mostrado promissor efeito terapêutico. Entre as plantas estudadas para este fim estão as da Família Piperaceae. O gênero *Piper* possui mais de 700 espécies distribuídas em ambos os hemisférios. Os estudos fitoquímicos de extratos de Piperaceae têm demonstrado a presença de várias moléculas bioativas, tais como amidas, ácido benzoico, cromonas, fenilpropanóides, terpenos, alcaloides e lignanas.

Piper cubeba Linn., popularmente conhecida no Brasil como *pimenta de Java*, é distribuída mundialmente em regiões tropicais e subtropicais, e possui a seguinte classificação científica:

Reino: Plantae
Divisão: Angiosperma
Classe: Magnoliopsida
Ordem: Piperales
Família: Piperaceae
Gênero: *Piper*
Espécie: *P. cubeba*

Os estudos fitoquímicos de extratos de *P. cubeba* demonstram a presença de terpenos, alcaloides e lignanas.

Estudos recentes com *P. cubeba* têm sido conduzidos com o objetivo de investigar as atividades biológicas de extratos puros, assim como de compostos

isolados, tais como a (-)-cubebina (CUB), uma lignana dibenzilbutirolactólica isolada de sementes secas de *P. cubeba*.

Este trabalho, enquanto pesquisa básica, teve como finalidade contribuir com informações sobre as atividades biológicas da CUB, por meio da avaliação *in vivo* dos efeitos mutagênicos/recombinogênicos da CUB isoladamente; e os efeitos antimutagênicos/antirecombinogênicos da CUB associada ao cloridrato de doxorrubicina (DXR) e ao etilcarbamato (URE), utilizando como ferramenta de trabalho o teste para detecção de mutação e recombinação somática (*Somatic Mutation and Recombination Test* - SMART) em *Drosophila melanogaster*; assim como a avaliação dos efeitos clastogênicos /aneugênicos da CUB isoladamente; e os efeitos anticlastogênicos/antianeugênicos da CUB associada ao DXR, por meio do Teste de Micronúcleos (MNT) em fibroblastos de pulmão (células V79) de hamster chinês cultivados *in vitro*; e o teste para avaliação da atividade antioxidante *in vitro*, por meio do ensaio com 2,2-difenil-1-picrilidrazila (DPPH) (Figura 1).

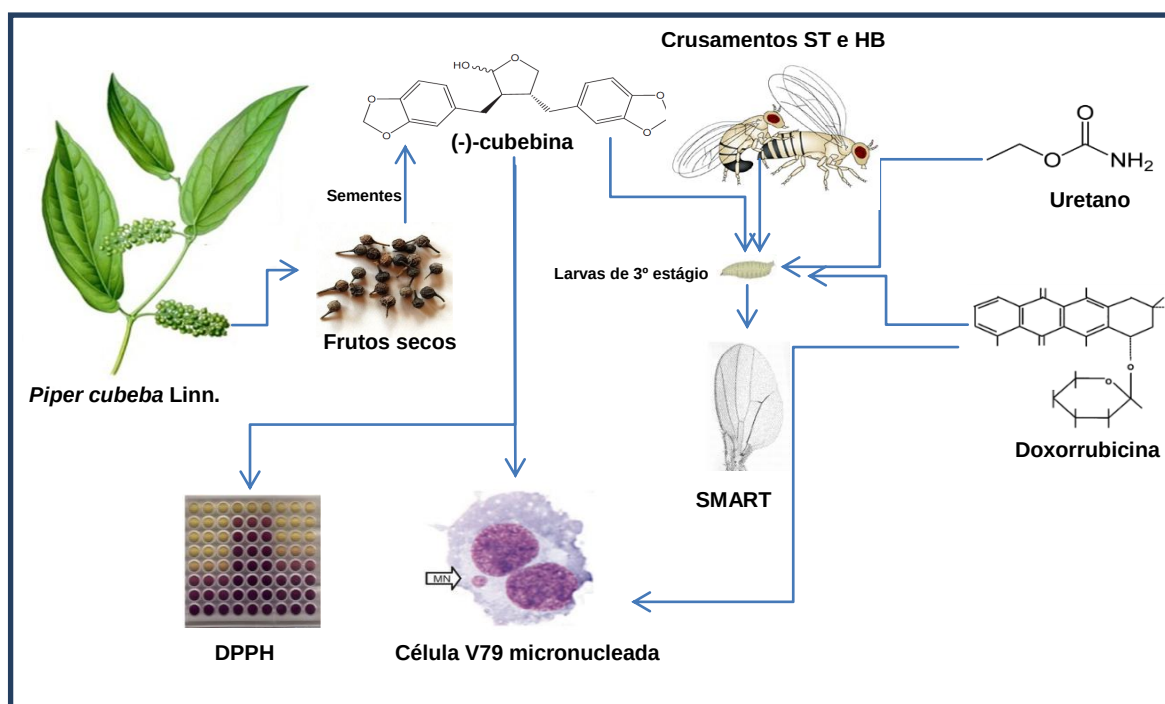


Figura 1. Diagrama dos experimentos realizados com CUB isoladamente e em associação com o cloridrato de doxorrubicina (DXR) e ao etilcarbamato (URE), empregando os testes SMART (*D. melanogaster*), MNT [fibroblastos de pulmão (células V79) de hamster chinês] e DPPH.

A CUB foi obtida pelos Profs. Drs. Márcio L. A. e Silva, da Universidade de Franca (UNIFRAN) – Franca (SP); Wilson R. Cunha (UNIFRAN); e Jairo K. Bastos, da Universidade de São Paulo (USP - RP) - Ribeirão Preto (SP), gentilmente cedida pela Profa. Dra. Denise Crispim Tavares (UNIFRAN).

As linhagens mutantes de *D. melanogaster* foram fornecidas pelo Dr. Ulrich Graf, do *Institute of Toxicology ETH and University of Zurich*, Schwerzenbach, Suíça. Os testes para detecção de mutação e recombinação somática foram realizados no Laboratório de Mutagênese da Universidade Federal de Uberlândia (UFU) – Uberlândia (MG).

As linhagens de células V79 (fibroblastos de pulmão de hamster chinês) foram gentilmente cedidas pela Profa. Dra. Ilce Mara de Syllos Cólus, da Universidade Estadual de Londrina (UEL) - Londrina (PR). Os testes para detecção de micronúcleos em células binucleadas da linhagem V79 foram realizados na UNIFRAN, sob a orientação da Profa. Dra. Denise Crispim Tavares.

A avaliação da atividade antioxidante da CUB *in vitro*, utilizando o ensaio com DPPH, foi realizada no Laboratório de Bioquímica e Biologia Molecular do INGEB – UFU, coordenado pelo Prof. Dr. Foued Salmen Espindola.

A apresentação deste trabalho foi dividida nos seguintes capítulos:

CAPÍTULO 1 – Fundamentação teórica que apresenta uma revisão geral da literatura, com informações sobre a *Piper cubeba* Linn., metabolismo secundário e (-)-cubebina; os agentes químicos utilizados (DXR e URE) e os testes utilizados (SMART, MNT e DPPH).

CAPÍTULO 2 – Apresenta o artigo “The effect of the dibenzylbutyrolactolic lignan (-)-cubebin on doxorubicin mutagenicity and recombinogenicity in wing somatic cells of *Drosophila melanogaster*”, publicado no periódico Food and Chemical Toxicology, volume 49 (6): 1235-1241, 2011. Fator de impacto = 2,999 (JCR 2011). Doi: 10.1016/j.fct.2011.03.001.

CAPÍTULO 3 – Apresenta o artigo “Comparative study of the modulatory effects of (-)-cubebin on the mutagenicity / recombinogenicity induced by different chemical

agents” a ser submetido para publicação no periódico *Environmental and Molecular Mutagenesis*. Fator de impacto = 3,709 (JCR 2011).

OBJETIVOS

Utilizando a (-)-cubebina (CUB), uma lignana dibenzilbutirolactólica isolada de sementes secas de *P. cubeba*, este estudo teve como objetivos:

1. Avaliar, por meio dos cruzamentos Padrão (ST) e de Alta Capacidade de Bioativação Metabólica (HB), do Somatic Mutation and Recombination Test (SMART) de asas de *Drosophila melanogaster* :

1.1.Os efeitos mutagênicos e recombinogênicos da CUB;

1.2.Os efeitos antimutagênicos e antirecombinogênicos da CUB em associação com o agente antineoplásico cloridrato de doxorrubicina (DXR);

1.3.Os efeitos antimutagênicos e antirecombinogênicos da CUB em associação com o etilcarbamato (URE);

2. Avaliar, por meio do Teste de Micronúcleos (MNT) em fibroblastos de pulmão de hamster chinês (células V79):

2.1.Os efeitos clastogênicos e/ou aneugênicos da CUB;

2.2.Os efeitos anticlastogênicos e/ou antianeugênicos da CUB em associação com DXR;

3. Avaliar a atividade antioxidante da CUB *in vitro*, por meio do ensaio com 2,2-difenil-1-picrilidrazila (DPPH).

CAPÍTULO 1

FUNDAMENTAÇÃO TEÓRICA

1. *Piper cubeba* Linn. (Piperales, Piperaceae)

A Ordem Piperales agrupa as Famílias Aristolochiaceae, Saururaceae, Hydnoraceae, Lactoridaceae e Piperaceae (Smith e Stockey, 2007).

A Família Piperaceae, conhecida como a Família das pimentas (Dodson, Dyer *et al.*, 2000), está classificada entre as mais primitivas Angiospermas, sendo considerada um fóssil vivo (Jaramillo, Manos *et al.*, 2004) e possui quatro Gêneros: Ottonia, Sarchorhachis, Peperomia e Piper (Jaramillo e Manos, 2001; Jaramillo, Manos *et al.*, 2004). Os dois últimos são considerados os mais expressivos, somando cerca de 2000 espécies (Jaramillo, Manos *et al.*, 2004; Quijano-Abril, Callejas-Posada *et al.*, 2006; Wanke, Samain *et al.*, 2006).

O Gênero Piper possui distribuição pantropical (**Figura 1**), com maior número de espécies ocorrendo na região neotropical (Jaramillo e Manos, 2001). Este Gênero possui grande potencial econômico e comercial, sendo a pimenta-do-reino (*Piper nigrum* Linn.) a espécie mais conhecida e estudada (Srinivasan, 2007).

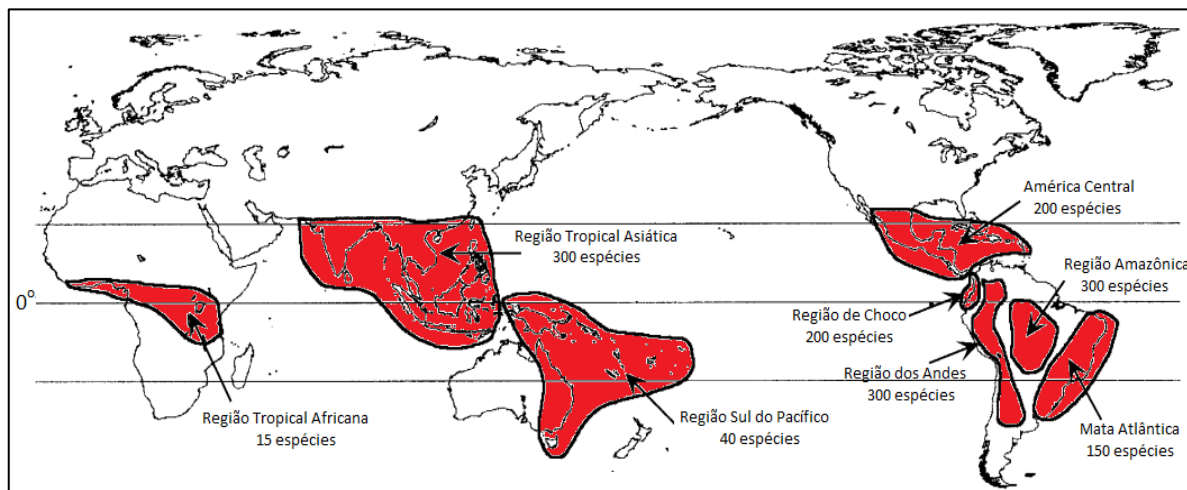


Figura 1. Distribuição geográfica de espécies do Gênero Piper (Fonte: Jaramillo e Manos, 2001, com modificações).

Algumas espécies pertencentes ao Gênero Piper são utilizadas na Medicina Ayurvedica por causa de suas propriedades medicinais e com base no uso etnofarmacológico (Prasad, Kumar *et al.*, 2005).

Piper cubeba L. (Piperales, Piperaceae), conhecida no Brasil como Pimenta-de-Java, é uma planta nativa das Ilhas de Java e Sumatra. Possui o caule perene, liso, vertical, com folhas inteiras, lisas, coriáceas e nervadas (**Figura 2A**). O fruto é marrom-acinzentado, pequeno, arredondado, enrugado e duro (**Figura 2B**) (Reshmi, Sathya *et al.*, 2010). Esta pimenta além de ser usada como tempero, é utilizada na medicina popular desde a Idade Média, principalmente na Europa, Marrocos, Índia e Indonésia (Maistro, Natel *et al.*, 2011), no tratamento de diversas enfermidades como dores abdominais, asma, diarreia e sífilis (Sastroamidjojo, 1997 *apud* Usia, Watabe *et al.*, 2005)

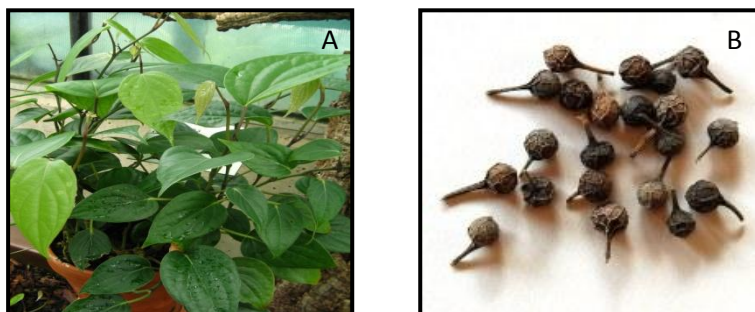


Figura 2. (A) *P. cubeba* (Fonte: <http://www.realmagick.com/piper-cubeba>); (B) Frutos secos de *P. cubeba* (Fonte: http://www.chilicult.com/hot/en/piper_cubeba.html, Acesso em: 02 de janeiro de 2012).

Pesquisas com extratos de *P. cubeba* demonstraram atividade antiinflamatória (Choi e Hwang, 2003; Yam, Schaab *et al.*, 2008), antialérgica (Choi e Hwang, 2003), antioxidante (Choi e Hwang, 2005; Nahak e Sahu, 2011), antimicrobiana (Silva, Coimbra *et al.*, 2007), genotóxica (Junqueira, Perazzo *et al.*, 2007), antineoplásica (Yam, Kreuter *et al.*, 2008), moluscida (Pandey e Singh, 2009).

Estudos fitoquímicos com extratos de plantas da Família Piperaceae tem mostrado grande diversidade de metabólitos secundários com marcantes atividades biológicas. Compostos como amidas, ácidos benzóicos, fenilpropanóides, cromonas, alcalóides, terpenos e lignanas já foram isolados (Vanin, Ramos *et al.*, 2008), sendo os três últimos, os únicos encontrados em *P. cubeba* (Elfahmi, Ruslan *et al.*, 2007).

2. Metabolismo secundário e (-)-cubebina

O metabolismo secundário de plantas pode ser definido como conjunto de processos metabólicos que originam compostos que não possuem uma distribuição universal nos vegetais, por não serem necessários a todos (Dixon, 2001).

Diferente do primário, o metabolismo secundário não é essencial para o desenvolvimento do vegetal, mas é imprescindível para a sobrevivência de uma espécie dentro de um ecossistema, viabilizando a adaptação do indivíduo no ambiente, respondendo pelas relações e interações entre planta-ambiente, tais como atração de polinizadores e proteção contra predadores (Taiz e Zeiger, 2004).

Existem três classes de metabólitos secundários: terpenos, alcalóides e compostos fenólicos (Fumagali, Gonçalves *et al.*, 2008). Os compostos fenólicos e polifenólicos têm chamado a atenção dos pesquisadores devido ao seu potencial terapêutico (Ninfali, Mea *et al.*, 2005; Saleem, Kim *et al.*, 2005).

Os polifenóis de maior abundância em plantas são os flavonóides, estilbenos e lignanas (Ramos, 2007). As lignanas são um grupo de ampla distribuição natural de compostos fenólicos em plantas (Smeds, Eklund *et al.*, 2007). Podem ser obtidas a partir da fenilalanina (**Figura 3**) e suas estruturas são derivadas da dimerização de unidades fenilpropanóides (C6-C3) (Suzuki e Umezawa, 2007).

De acordo com Elfahmi, Ruslan *et al.*, 2007, já foram isoladas vinte e quatro lignanas de *P. cubeba* (**Figura 4**).

A (-)-cubebina, uma lignana dibenzilbultiolactólica, pode ser encontrada em diversas Famílias, como Aristolochiaceae (De Pascoli, Nascimento *et al.*, 2006), Rutaceae (Bastos, Carvalho *et al.*, 2001), Myristicaceae (Blumenthal, Da Silva *et al.*, 1997) e Piperaceae (Saraiva, Vega *et al.*, 2007). Esta lignana foi inicialmente isolada do extrato hexânico de folhas de *Zanthoxylum naranjillo* Griseb (Bastos, Gottlieb *et al.*, 1996) e recentemente, obtida de sementes secas de *P. cubeba* (De Souza, Da Silva *et al.*, 2005). No entanto, esta lignana já foi isolada e identificada há mais de um século (Stecher, Finkel *et al.*, 1960).

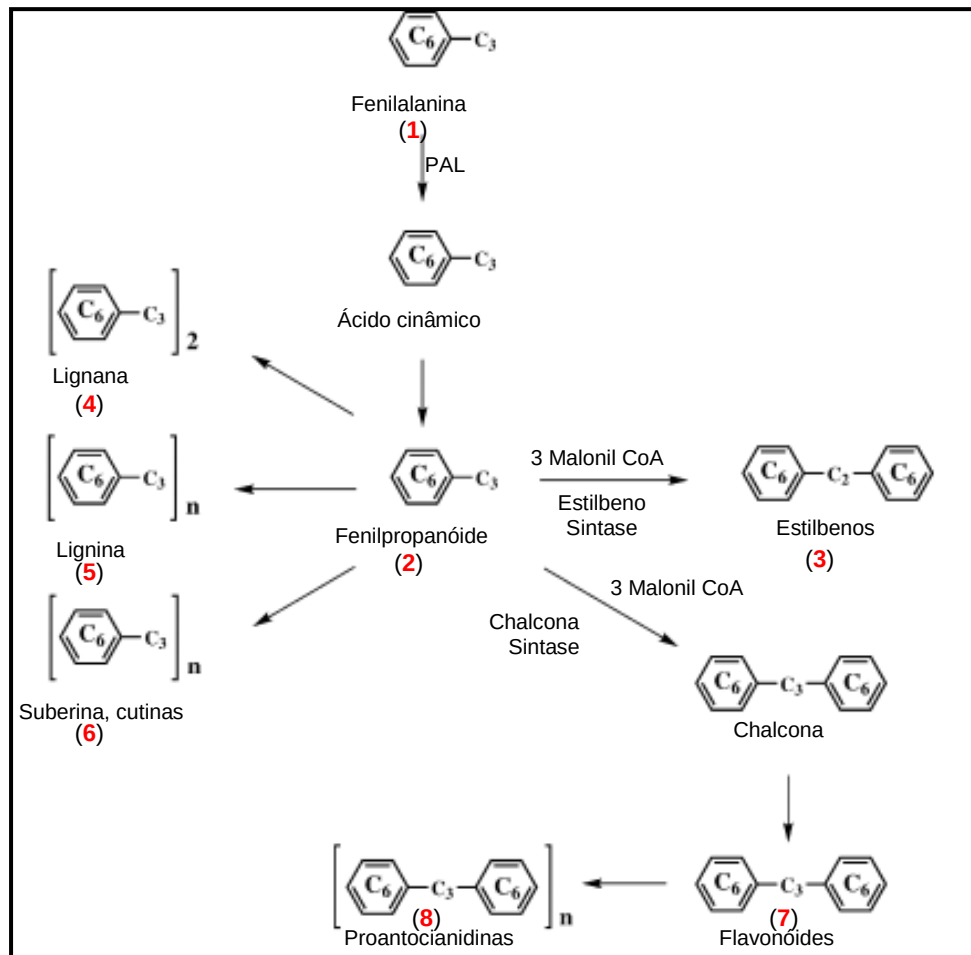


Figura 3. Produção de fenilpropanóide (2), estilbenos (3), lignanas (4), ligninas (5), suberinas e cutinas (6), flavonóides (7) e taninos (8), a partir da fenilalanina (1) (Fonte: Naczki e Shahidi, 2004, com modificações).

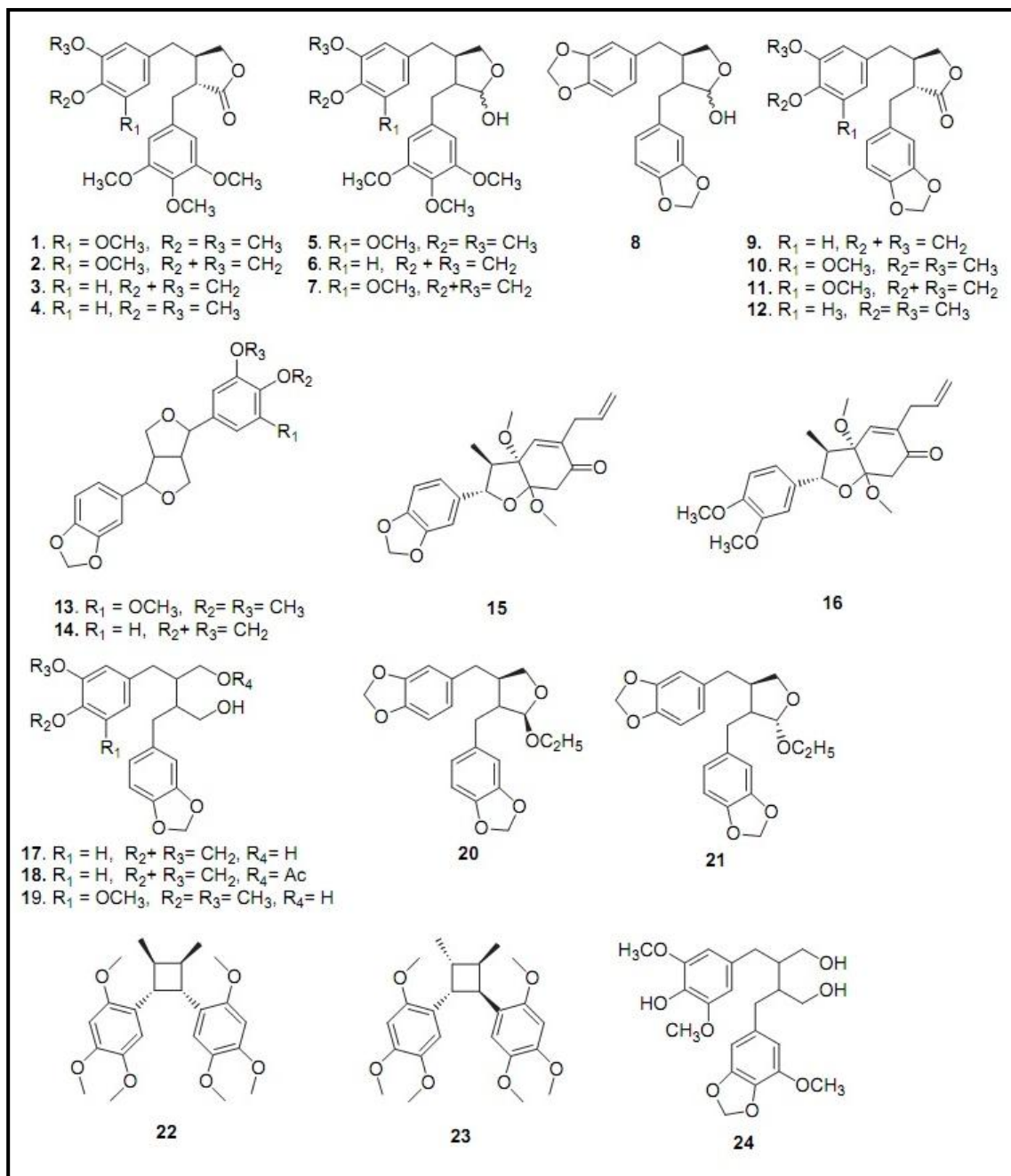


Figura 4. Lignanas extraídas de *P. cubeba*: cubebinolide (1), cubebinone (2), yatein (3), thujaplicatin trimethylether (4), cubebin (5), clusin (6), 5-methoxyclusin (7), cubebin (8), hinokin (9), isoyatein (10), 50-methoxyhinokin (11), 2-(300,400-methylenedioxy-benzyl)-3-(30,40-dimethoxybenzyl) butyrolactone (12), aschantin (13), sesamin (14), kadsurin A (15), piperenone (16) dihydrocubebin (17), hemi-arensin (18), dihydroclusin (19), b-O-ethylcubebin (20), a-O-ethylcubebin (21), heterotropin (22), magnosalin (23), 4-hydroxycubebinone (24) (Fonte: Elfahmi, Ruslan et al., 2007).

A estrutura química da (-)-cubebina (**Figura 5**), extraída de sementes de *P. cubeba*, foi definida por meio de Ressonância Magnética Nuclear ^1H (^1H NMR) e do infra-vermelho (IR) (De Souza, Da Silva *et al.*, 2005).

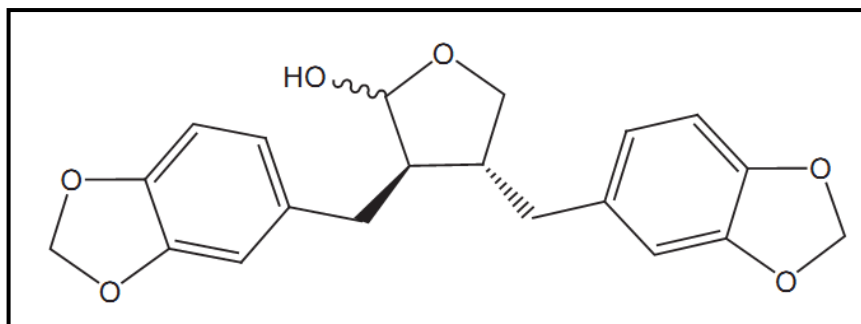


Figura 5: Estrutura química da (-)-cubebina (Fonte: De Rezende, e Silva *et al.*, 2011)

Nos últimos anos, vários estudos têm sido realizados para elucidar o potencial biológico dessa lignana. Já foram comprovadas atividades antiinflamatórias (Bastos, Carvalho *et al.*, 2001; Da Silva, De Souza *et al.*, 2005), analgésicas (Bastos, Carvalho *et al.*, 2001; Da Silva, De Souza *et al.*, 2005), antimicrobianas (Silva, Coimbra *et al.*, 2007; Silva, Martins *et al.*, 2009) e genotóxicas (Junqueira, Perazzo *et al.*, 2007; Maistro, Natel *et al.*, 2011), antimutagênicas e antirecombinogênicas (De Rezende, e Silva *et al.*, 2011).

3. Cloridrato de Doxorrubicina

Uma das linhas de pesquisa do Laboratório de Mutagênese da Universidade Federal de Uberlândia é a avaliação do potencial antimutagênico e antirecombonogênico de compostos naturais frente aos danos induzidos pelo quimioterápico adriamicina (CAS 23214–92-8) também conhecido como cloridrato de doxorrubicina (DXR). DXR é um antibiótico antineoplásico da classe das antraciclinas, isolado de culturas de *Streptomyces peucetius*, variedade *caesius* (Ashikawa, Shishodia *et al.*, 2004). Embora a principal fonte de DXR seja por meio da biossíntese microbiana, a sua obtenção é possível por meios sintéticos (Singal, Li *et al.*, 2000).

A DXR surgiu como um quimioterápico promissor em 1960 (Singal e Iliskovic, 1998) e ainda é utilizado no tratamento de diversos tumores sólidos, tais como os de mama, ovário, pulmão, leucemia aguda e em menor proporção, em tumores ginecológicos (Da Rocha, Lopes *et al.*, 2001; Minotti, Menna *et al.*, 2004; Smith, Cornelius *et al.*, 2010).

Estruturalmente, a DXR (**Figura 6**) possui um núcleo antraciclínico formado por 4 anéis antraquinonas cíclicos ligados a um amino-açúcar (daunosamina) por uma ligação glicosídica (Xu, Tang *et al.*, 2001). Devido à sua estrutura singular, a DXR é altamente lipofílica e sua meia-vida é relativamente longa no organismo (Singal, Li *et al.*, 2000).

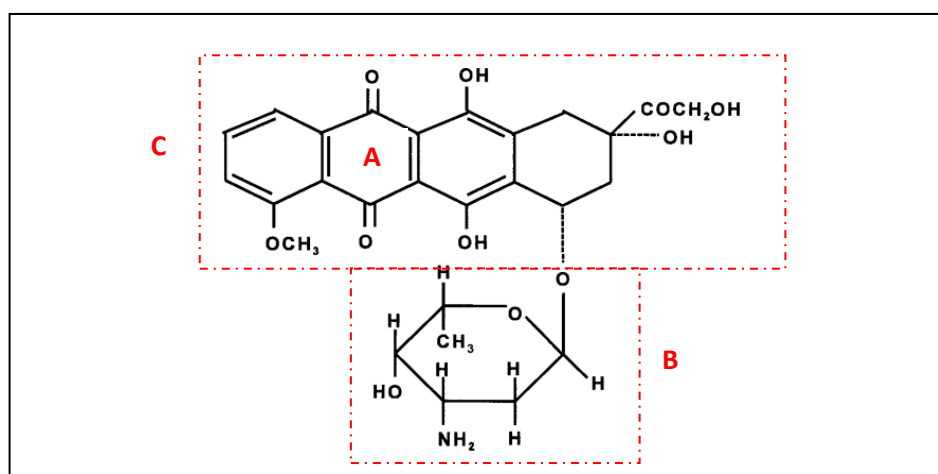


Figura 6. Estrutura química da doxorrubicina. Identificados o anel quinona (A), o amino-açúcar (B), e a porção quinona (C) (Fonte: Singal, Li *et al.*, 2000, com modificações)

A metabolização da DXR ocorre principalmente no fígado. Após a administração, os níveis plasmáticos desse quimioterápico diminuem, com uma meia-vida terminal de 20 a 48 horas. Aproximadamente 40% da dose administrada é recuperada na bile ou fezes em 5 dias; 5% a 12% desse fármaco e seus metabólitos, como o 13-OH-doxorrubicinol, aparecem na urina durante o mesmo período (Fu, 2003).

A DXR é ativa durante todo o ciclo celular, incluindo a interfase. Os tecidos de rápida proliferação são os mais sensíveis aos seus efeitos antiproliferativos (Charrois e Allen, 2004), sendo a inibição da síntese e da transcrição do DNA um dos principais mecanismos de ação (Smith, Cornelius *et al.*, 2010).

Apesar de eficiente, o uso da DXR é limitado. Já foram relatados diversos efeitos colaterais, tanto agudos como crônicos, relacionados à sua administração (Granados-Principal, Quiles *et al.*, 2010). Assim, os mecanismos de ação, interação com os diversos tipos de tumores e bem como a sua forma de apresentação, vêm sendo extensamente estudados (Ewer e Lippman, 2005; Smith, Cornelius *et al.*, 2010; Upadhyay, Mishra *et al.*, 2011).

A administração de DXR, assim como de outras antraciclinas utilizadas na quimioterapia, pode levar a distúrbios cardíacos (Jones, Swanton *et al.*, 2006; Zhu, Kukreja *et al.*, 2011). Embora ainda não esteja totalmente elucidada (Jones, Swanton *et al.*, 2006), a cardiotoxicidade induzida por DXR é atribuída à geração de radicais livres, sendo que esses radicais podem causar lipoperoxidação, acarretando alterações na integridade da membrana celular, além de alterar a função mitocondrial, o que também favorece a síntese de radicais livres (Zhou, Palmeira *et al.*, 2001).

Existem dois mecanismos fundamentais de ação da DXR: indução de danos ao DNA e produção de radicais livres. Outras vias de ação (**Figura 7**) vêm sendo estudadas e maiores informações podem ser obtidas na revisão feita por Granados-Principal, Quiles *et al.* (2010).

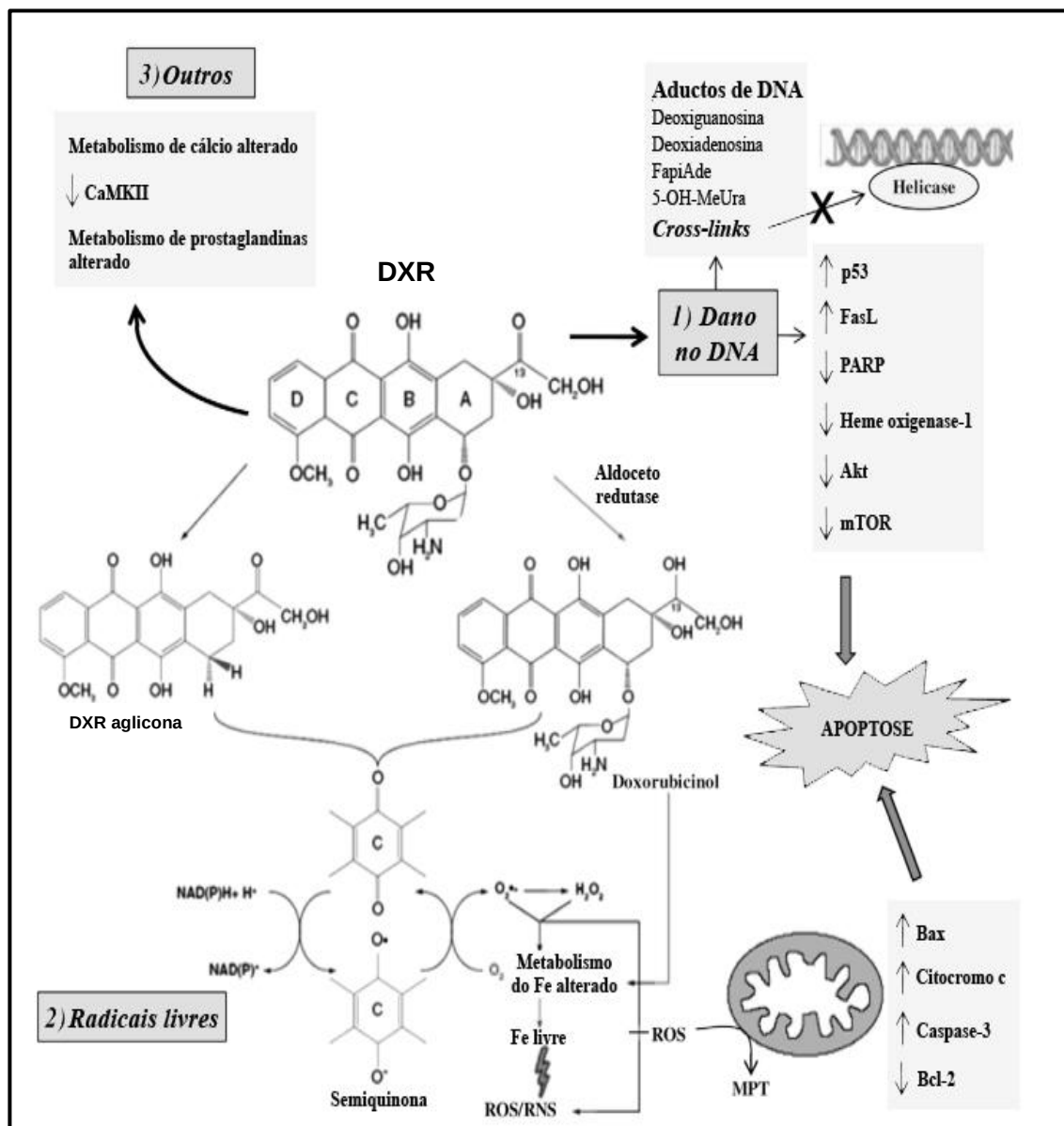


Figura 7. Mecanismos de ação da DXR (Fonte: Granados-Principal, Quiles *et al.*, 2010, com modificações).

Um dos mecanismos propostos seria a interação da DXR com a enzima topoisomerase II, a qual controla a topologia das regiões super espiralizadas do DNA, ligando-se e causando a abertura da dupla fita (Islaih, Halstead *et al.*, 2005). Sendo assim, os tratamentos com DXR impedem a re-ligação das duplas fitas seccionadas pela topoisomerase II, causando lesão permanente à molécula do DNA (Minotti, Menna *et al.*, 2004; Filyak, Filyak *et al.*, 2007). Pode atuar como agente intercalante (Ashley e Poulton, 2009), levando à formação de aductos no DNA (Spencer, Bilardi *et al.*, 2008), podendo acarretar troca de cromátides-irmãs e aberrações cromossômicas (Gulkac, Akpinar *et al.*, 2004).

Existe ainda um mecanismo de ação que envolve a biotransformação do anel A do núcleo antraciclínico (**Figura 6**). A redução de um elétron deste anel leva à formação do radical semiquinona, o qual é relativamente estável sob condições anaeróbicas. Porém, em condições aeróbicas, o elétron desemparelhado é doado ao oxigênio (O₂) formando o radical superóxido (Land, Mukherjee *et al.*, 1983; Mukherjee, Land *et al.*, 1989). O radical semiquinona pode exercer efeito tóxico direto, ou participar de reações de óxido-redução, e envolver enzimas como a NADH desidrogenase, xantina oxidase e citocromo P450 redutase (Gewirtz, 1999; Ramji, Lee *et al.*, 2003; Navarro, Busnadiego *et al.*, 2006) ou os radicais livres podem ser gerados por mecanismos não enzimáticos, em reações mediadas pelo ferro (Quiles, Huertas *et al.*, 2002). Granados-Principal, Quiles *et al.* (2010) apresentam uma revisão detalhada dos mecanismos de ação da DXR.

Em *D. melanogaster*, os danos induzidos por DXR são reparados, preferencialmente, por recombinação homóloga (Lehmann, Franco *et al.*, 2003; Valadares, Graf *et al.*, 2008; De Rezende, Graf *et al.*, 2009; De Rezende, E Silva *et al.*, 2011).

Muitos trabalhos têm avaliado a ação de compostos naturais com a finalidade de minimizar os danos induzidos pela DXR em células somáticas de *D. melanogaster* (Fragiorgi, Spanó *et al.*, 2007; Pereira, Antunes *et al.*, 2008; Valadares, Graf *et al.*, 2008; De Rezende, Graf *et al.*, 2009; Sousa, Rezende *et al.*, 2009; De Rezende, E Silva *et al.*, 2011; Felicio, Silva *et al.*, 2011), demonstrando a influência de enzimas envolvidas no processo de detoxificação de agentes xenobióticos.

4. Citocromo P450 e Etil Carbamato

Os organismos vivos, de uma maneira geral, desde as bactérias até os seres humanos, incluindo plantas, apresentam respostas coordenadas frente à exposição aos xenobióticos, induzindo enzimas e transportadores que facilitam a detoxificação (Misra, Horner *et al.*, 2011), transformando esses compostos em substâncias menos nocivas e facilitando sua excreção (Xu, Li *et al.*, 2005).

Este sistema de proteção é composto por enzimas de detoxificação que podem ser classificadas em três grupos (fases):

1. **Enzimas de Fase I** - são as mais abundantes e medeiam reações de hidrólise, redução e oxidação.
2. **Enzimas de Fase II** - atuam diretamente sobre os subprodutos tóxicos, oriundos das reações mediadas pelas enzimas de Fase I. Neste grupo estão incluídas as enzimas glutathione S-transferases (GSTs), UDP-glucuronosiltransferase (UGTs) e carboxil-transferases. GSTs e UGTs são capazes de adicionar grupos volumosos aos compostos tóxicos, aumentando sua solubilidade em água, ao passo que as carboxil-transferases catalisam reações de hidrólise de compostos que possuem grupamentos éster.
3. **Enzimas de Fase III** - consistem em um grupo conhecido como *ATP-binding cassette* (ABC) e outras enzimas transmembrânicas, que possuem a função de exportar compostos tóxicos para o exterior da célula (Misra, Horner *et al.*, 2011).

O sistema enzimático mais importante na catálise das reações de Fase I é o sistema citocromo P450 (CYP). Esta é uma grande Família de hemoproteínas capazes de catalisar a oxidação de uma grande diversidade de moléculas orgânicas, bem como na biossíntese de lipídeos, esteróides, vitaminas e outros metabólitos naturais das células (Chefson e Auclair, 2006).

A superfamília de CYP é constituída de Famílias e sub-Famílias, que são classificadas de acordo com a estrutura, propriedades físico-químicas e afinidade com substrato (Zhang, Dai *et al.*, 2012).

Até 2011, já foram nomeadas 7232 CYP e identificados 896 pseudogenes de, pelo menos, 85 espécies de eucariotos (incluindo vertebrados, invertebrados, protistas, fungos e plantas) e 20 de procariotos (El-Merhibi, Ngo *et al.*, 2011). Assim como outros animais, os insetos podem regular a expressão de genes relacionados aos processos de detoxificação (Misra, Horner *et al.*, 2011).

Comparado a humanos e plantas, pouco se conhece sobre as funções das diferentes CYP de insetos. A única exceção é o envolvimento dessas enzimas na biossíntese do hormônio 20-hidroxiecdisona (Chung, Sztal *et al.*, 2009).

Os estudos geralmente abordam as diferentes respostas adaptativas de insetos frente à pressão seletiva do meio, ou, ainda, como se tornaram resistentes a compostos tóxicos presentes no ambiente (Perry, Batterham *et al.*, 2011).

A linhagem mutante de *D. melanogaster* “ORR; flare-3” (Frölich e Würigler, 1989), utilizada no SMART (Graf e van Schaik, 1992), possui níveis elevados das enzimas Cyp6A2 e Cyp6G1 (Daborn, Boundy *et al.*, 2001), sendo uma importante ferramenta para detecção de agentes mutagênicos de ação indireta, que necessitam de ativação metabólica via CYP (Frölich e Würigler, 1989; Graf e van Schaik, 1992).

Durante o processo de biotransformação, agentes xenobióticos podem ser inativados ou, menos frequentemente, gerar compostos com efeitos mais nocivos (Misra, Horner *et al.*, 2011), como é o caso do etilcarbamato (**Figura 8**), um composto mutagênico em *D. melanogaster*, que tem sua ação potencializada por meio da metabolização das CYP (Dogan, Yesilada *et al.*, 2005; Fragiorgi, Spanó *et al.*, 2007).

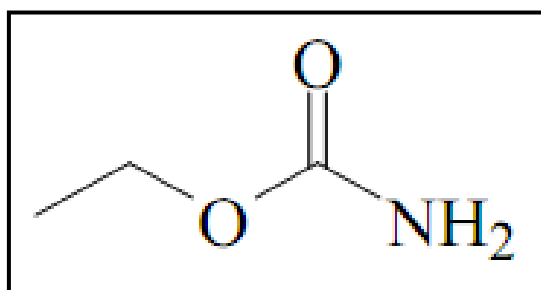


Figura 8. Estrutura química do etilcarbamato (Fonte: EFSA, 2007).

O etilcarbamato (CAS 51-79-6), também conhecido como uretano (URE), é o éster etílico do ácido carbâmico, utilizado como co-solvente de pesticidas, podendo ser também encontrado como produto intermediário durante a fabricação de bebidas alcoólicas fermentadas e na indústria têxtil (Labanca e Glória, 2008; Weber e Sharypov, 2009).

Embora haja uma extensa literatura demonstrando a ação mutagênica e carcinogênica do URE, apenas em 1985 a sua detecção e quantificação tornou-se assunto de saúde pública, quando autoridades canadenses detectaram elevadas concentrações em bebidas alcoólicas (EFSA, 2007).

No passado, URE foi utilizado como hipnótico para humanos e como anestésico para animais de laboratório (Frölich e Würigler, 1990). Atualmente, o URE, ou mesmo outros carbamatos (fenil, metil ou butil-carbamato), possuem aplicações apenas em pesquisas (Gotor, 1999).

Com base em dados epidemiológicos, em 2007 o URE foi reclassificado pela *World Health Organization's International Agency for Research on Cancer* (IARC, passando de “potencial carcinógeno” (Grupo 2B), para “provável carcinógeno” (Grupo 2A) para humanos (IARC, 2007; FAO/WHO, 2007).

Estudos têm comprovado que o URE é genotóxico e carcinogênico para ratos, camundongos, hamsters, macacos (Beland, Benson *et al.*, 2005), sendo as principais vias de absorção o trato gastro-intestinal e pele (Cha, Gu *et al.*, 2000). Em testes de toxicidade com ratos, URE causou aumento (dose dependente) de carcinomas no fígado, pulmões, pele, estômago, e de tumores do ovário e da glândula mamária (Beland, Benson *et al.*, 2005) além de ser mutagênico (Donovan e Smith, 2008) e clastogênico (Sotomayor e Collins, 1990).

O processo de eliminação do URE envolve três passos principais: hidrólise, N-hidroxilação ou C-hidroxilação e oxidação da cadeia lateral. As reações de hidrólise são mediadas por esterases, levando à produção de etanol, dióxido de carbono e amônia. Já as reações de N-hidroxilação, C-hidroxilação e oxidação da cadeia lateral, mediadas por CYP, formam durante este processo: N-hidroxicarbamato, α -hidroxi-etilcarbamato e vinil-carbamato, respectivamente (EFSA, 2007). A **Figura 9** demonstra as principais vias de metabolização do URE.

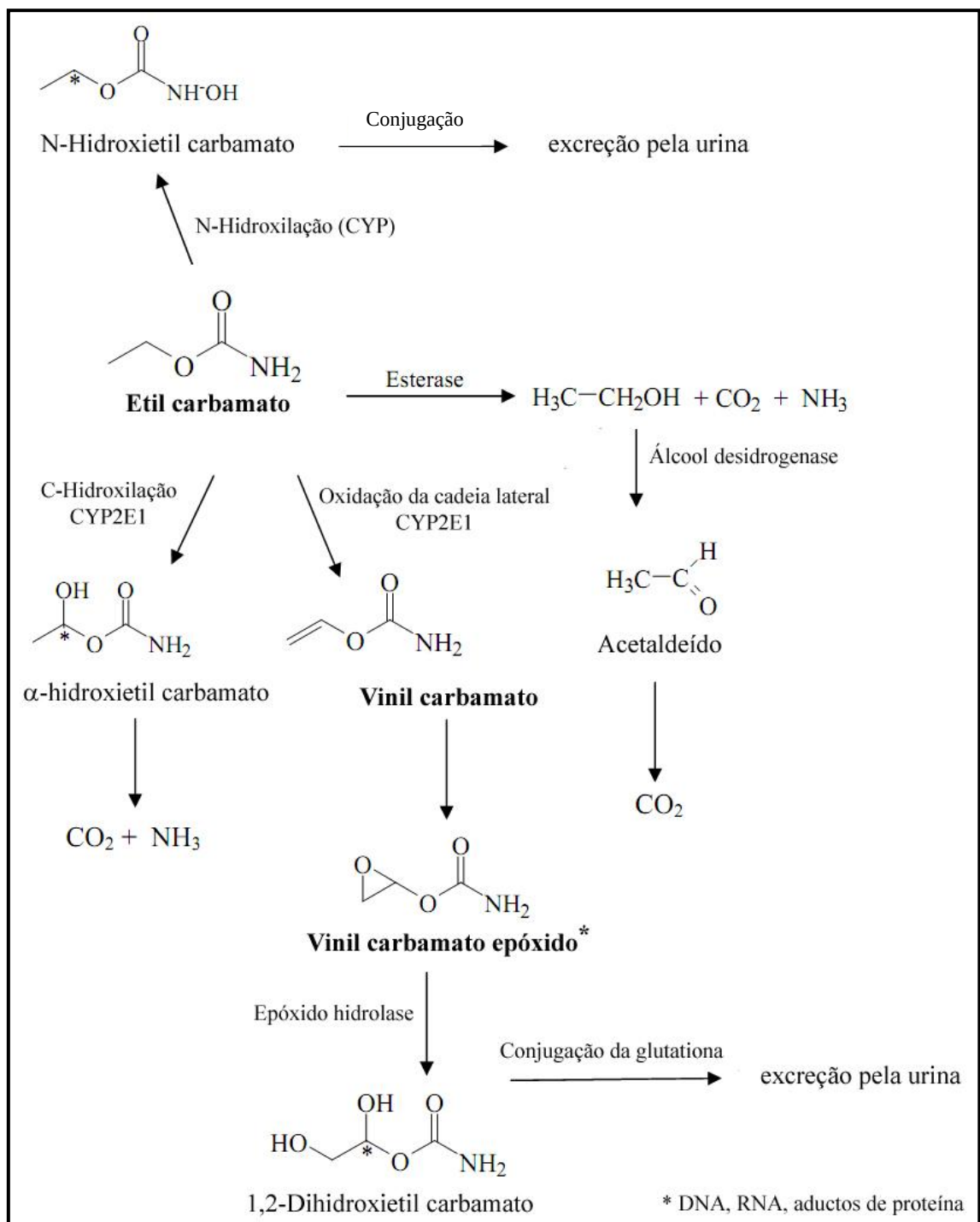


Figura 9. Principais vias de metabolização do URE (Fonte: EFSA, 2007, com modificações).

Vinil-carbamato (VC) é considerado o metabólito responsável pelo potencial carcinogênico do URE, principalmente se, por reação de epoxidação, for convertido em vinil-carbamato-epóxido, uma vez que este é capaz de se ligar covalentemente aos ácidos nucleicos (DNA e RNA) e proteínas (JOINT FAO/WHO, 2006).

Estudos demonstraram o potencial mutagênico do VC, em diversos órgãos, incluindo pulmões, fígado e glândulas mamárias (Hernandez, Slob *et al.*, 2011), demonstrando o papel das CYP na sua produção a partir do URE.

Existem evidências de que VC também seja formado mediante a participação de CYP de *D. melanogaster*, uma vez que a frequência de mutações aumenta significativamente no cruzamento de alta ativação metabólica (HB), o qual possui níveis elevados de CYP (Frölich e Würgler, 1990; Fragiorgis, Rezende *et al.*, 2008).

5. Somatic Mutation And Recombination Test - SMART

Ao longo do desenvolvimento ocorrem lesões no DNA (mutações), causadas por erros durante as divisões celulares ou por exposição a agentes químicos, físicos ou biológicos (Frank, 2010). Aliado a isso, o envelhecimento celular acarreta a diminuição da capacidade de reparo do organismo, possibilitando a fixação das mutações (Kim, Jung *et al.*, 2011).

Essas lesões podem afetar processos vitais como a duplicação e a transcrição gênica, além de causar mutações e aberrações cromossômicas, fenômenos esses que podem levar ao desenvolvimento de câncer e à morte celular. Pelo fato de causarem lesões no material genético, e potencialmente gerarem tumores em seres humanos, esses agentes são normalmente conhecidos como genotóxicos (Costa e Menk, 2000). Dessa maneira, pode-se evidenciar uma correlação entre eventos mutagênicos e a carcinogenicidade (Frank, 2010).

A utilização de bioensaios para o monitoramento da atividade biológica de extratos, frações e compostos isolados de plantas tem sido frequentemente incorporada à pesquisa fitoquímica (Noldin, Filho Cechinel *et al.*, 2003).

Nos últimos anos, é possível evidenciar um esforço mundial para reduzir a utilização de animais superiores nas pesquisas toxicológicas/genéticas, principalmente no que se diz respeito ao desenvolvimento de modelos alternativos *in vivo* (Siddique, Chowdhuri *et al.*, 2005).

A *Drosophila melanogaster* (**Figura 10**) é um inseto modelo muito bem estabelecido, que tem sido utilizado extensivamente em estudos genéticos e de biologia do desenvolvimento (Jennings, 2011; Kim, Jung *et al.*, 2011).

Existem inúmeras vantagens em se trabalhar com modelos experimentais envolvendo *Drosophila*, ao invés de vertebrados (Jennings, 2011). A *D. melanogaster* é um organismo eucarioto com tempo de geração curto, prole numerosa, baixo custo e de fácil manutenção em laboratório. Além disso, as características morfológicas e genéticas são amplamente conhecidas (Graf, Spanó *et al.*, 1996).

Após o sequenciamento do genoma de *D. melanogaster*, foi verificada homologia com cerca de 77% dos genes relacionados às doenças em humanos

(Reiter, Potocki *et al.*, 2001). Além disso, é possível constatar alta conservação evolutiva, que pode ser corroborada por dados obtidos a partir de análises proteômicas (Tickoo e Russell, 2002).

Tais descobertas, somadas aos mais de 100 anos de contribuição deste organismo à pesquisa genética, colocam a *Drosophila* como um modelo promissor não apenas para o estudo de doenças humanas, mas também para a obtenção de drogas que possam ser efetivamente utilizadas no tratamento de diversas doenças (Lehmann, Vilar Kde *et al.*, 2004; Jennings, 2011).

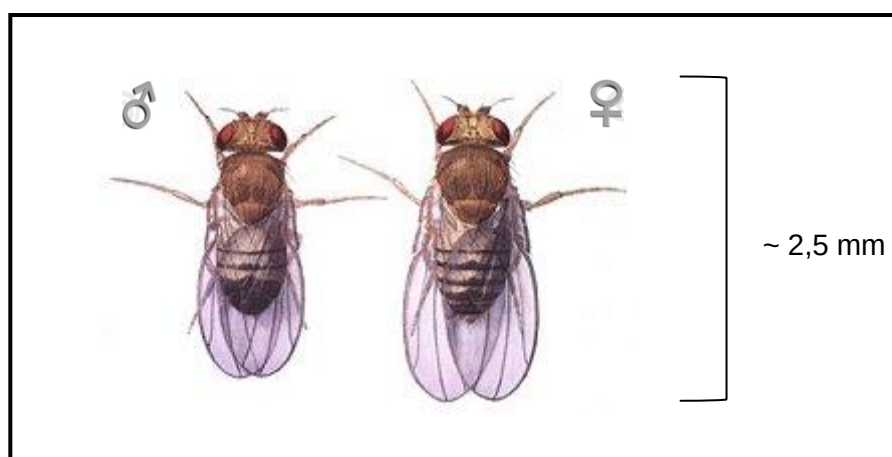


Figura 10. Representação de indivíduos adultos de *Drosophila melanogaster* (Fonte: <http://aqualandpetsplus.com/Live%20F212.jpg>, Acesso em: 06 de janeiro de 2012).

O Teste para Detecção de Mutação e Recombinação Somática (*Somatic Mutation and Recombination Test*) em células de asa de *D. melanogaster* (SMART) foi desenvolvido por Graf, Würigler *et al.*, 1984 e aprimorado por Graf e van Schaik, 1992, tem sido empregado na avaliação mutagênica e antimutagênica de misturas complexas (Laohavechvanich, Kangsadalampai *et al.*, 2006; Silva, Costa-Cruz *et al.*, 2006; Pantaleão, Alcantara *et al.*, 2007; Pereira, Antunes *et al.*, 2008; Silva, Sousa *et al.*, 2008; Valadares, Graf *et al.*, 2008; Patenkovic, Stamenkovic-Radak *et al.*, 2009; Sousa, Rezende *et al.*, 2009; Anter, Campos-Sanchez *et al.*, 2010; Yuksel, Sarikaya *et al.*, 2010; Akmoutsou, Mademtzoglou *et al.*, 2011; Felicio, Silva *et al.*, 2011) e compostos isolados (De Rezende, Graf *et al.*, 2009; Arossi, Dihl *et al.*, 2010; Danesi, Bellagamba *et al.*, 2010; Vázquez-Gómez, Sanchez-Santos *et al.*, 2010; De Rezende, Silva *et al.*, 2011; Gabriel, Dihl

et al., 2011; Palermo e Mudry, 2011; Sotibran, Ordaz-Tellez et al., 2011) por ser sensível a agentes mutagênicos, clastogênicos e/ou recombinogênicos (Graf, Würigler et al., 1984).

Este teste é realizado por meio de cruzamentos experimentais, utilizando três linhagens portadoras dos marcadores recessivos das células das asas: *multiple wing hairs* (*mwh*, 3-0,3) e *flare* (*flr*³, 3-38,8). No SMART são utilizadas três linhagens:

1) linhagem “*multiple wing hairs*” (*mwh*) com constituição genética *y; mwh jv*. O marcador *mwh*, presente no cromossomo 3, encontra-se em posição mais distal em relação ao centrômero (3-0,3). Quando expresso, o fenótipo do pêlo selvagem da asa (**Figura 11A**) é modificado, originando pêlos múltiplos (**Figura 11B**), além disso, a borda da asa é lisa.

2) linhagem “*flare-3*” (*flr*³), com constituição genética *flr*³ / *In(3LR)TM3, ri p^p sep I(3)89Aa bx^{34e} e Bd^S*. O marcador *flr*³, presente no cromossomo 3, encontra-se em posição mais proximal em relação ao centrômero (3-38,8). Quando expresso, o fenótipo do pêlo da asa também é modificado, originando pêlos que se assemelham a uma chama de vela (**Figura 12A**). Devido à sua letalidade em homozigose, este alelo é mantido graças à presença de um balanceador cromossômico (*TM3, Bd^S*). A borda da asa desses indivíduos é serrilhada, devido à presença do marcador *Bd^S*.

3) linhagem “*ORR; flare-3*” (*ORR; flare-3*), com constituição genética *ORR; flr*³ / *In(3LR)TM3, ri p^p sep I(3)89Aa bx^{34e} e Bd^S*. Os indivíduos pertencentes à essa linhagem possuem os mesmos marcadores que os da linhagem “*flare-3*”. Entretanto, linhagem “*ORR; flare-3*” é portadora de genes de expressão elevada de enzimas do complexo citocromo P450, localizados nos cromossomos 1 e 2, provenientes da linhagem “*Oregon R*”, naturalmente resistentes ao DDT (Graf, Würigler et al., 1984; Graf e van Schaik, 1992).

Com estas linhagens são realizados dois diferentes cruzamentos (**Figura 13**):

1) cruzamento padrão (ST – *standard cross*) entre machos “*mwh*” e fêmeas virgens “*flr*³” (Graf, Würigler et al., 1984; Graf, Frei et al., 1989);

2) cruzamento de alta bioativação (HB - *high bioactivation cross*) entre machos “*mwh*” e fêmeas virgens “*ORR; flr*³” (Graf e van Schaik, 1992).

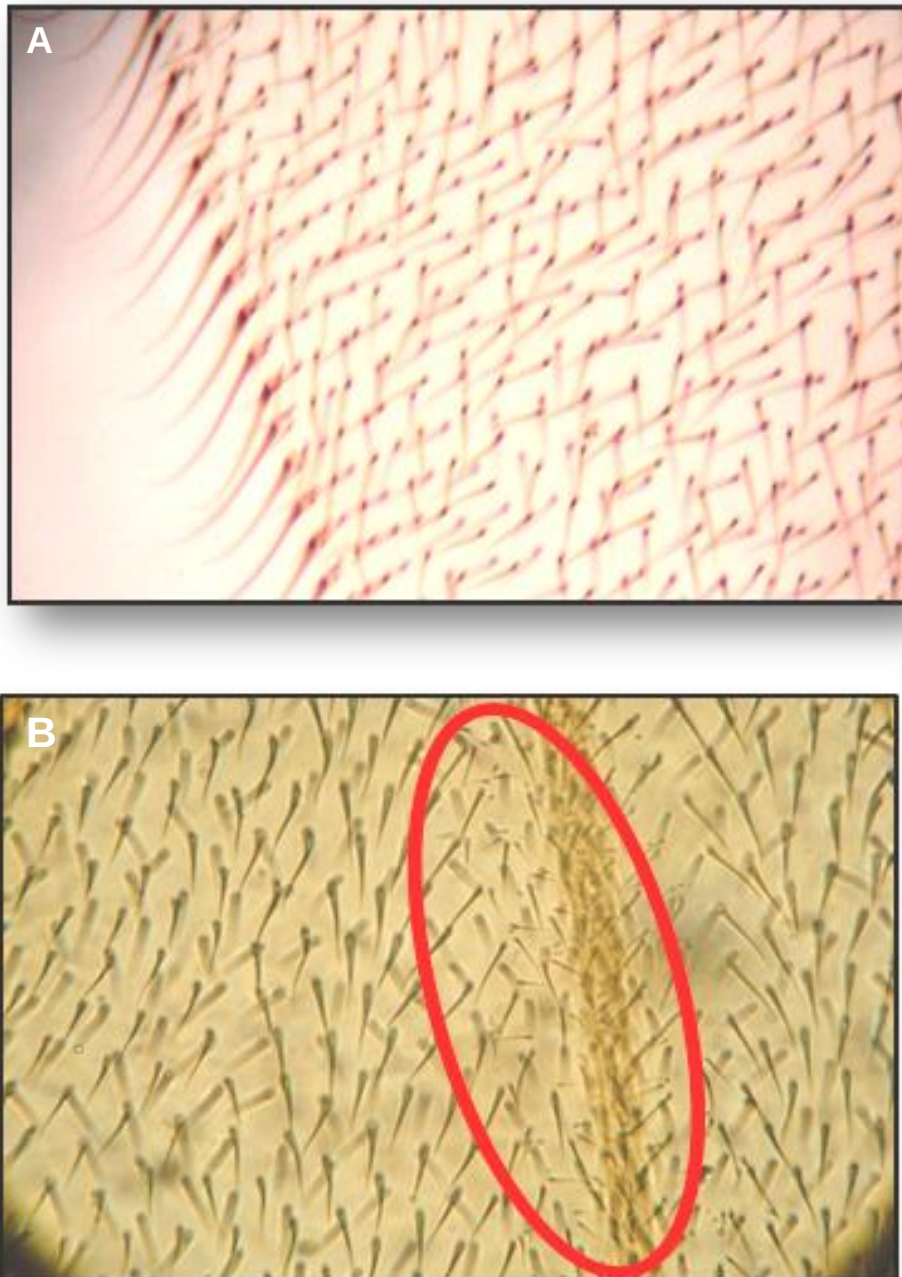


Figura 11. Fotomicrografias de asas de *D. melanogaster* obtidas em microscópio óptico de luz (aumento de 400X) demonstrando: A) pêlos selvagens; B) mancha simples do tipo *mwh*.

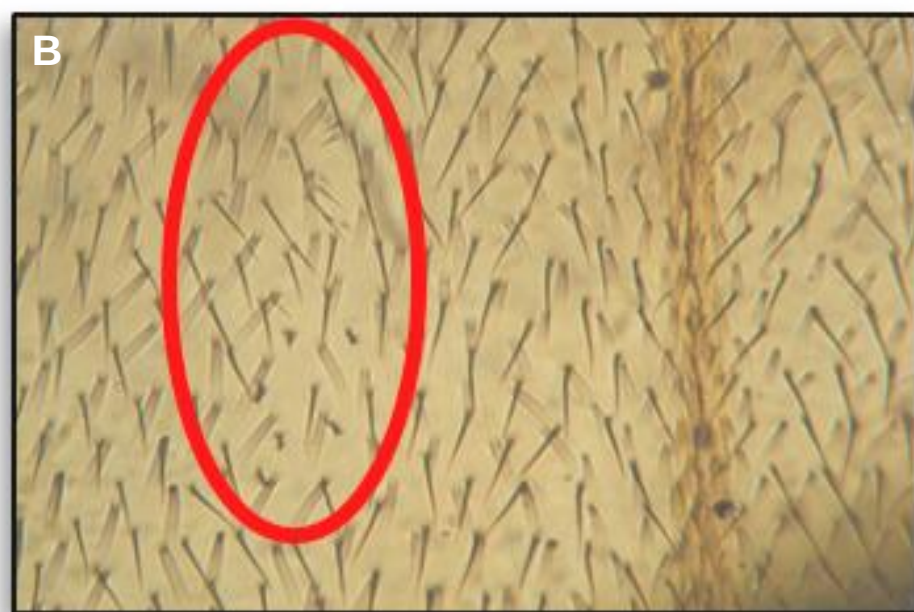
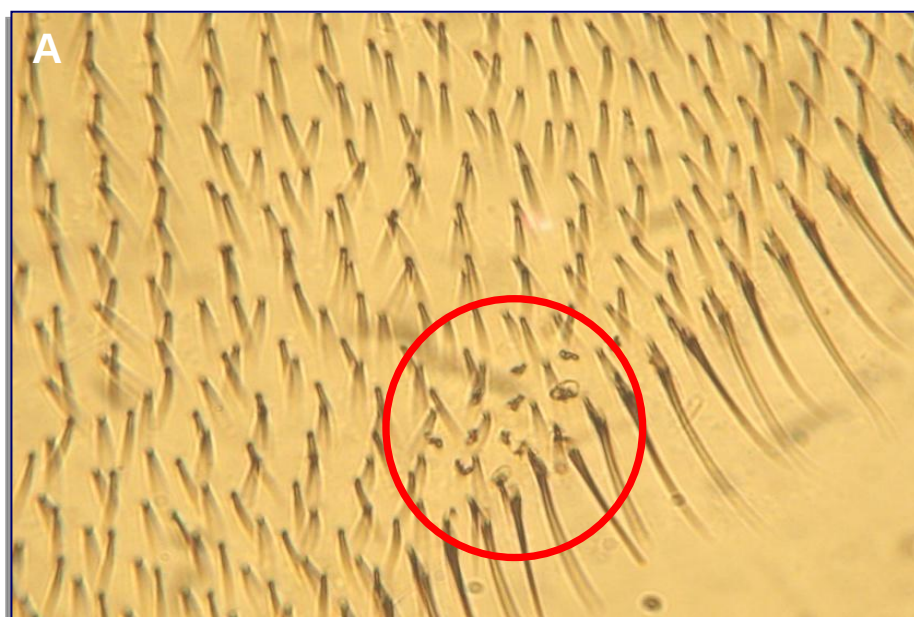


Figura 12. Fotomicrografias de asas de *D. melanogaster* obtidas em microscópio óptico de luz (aumento de 400X) demonstrando: A) mancha simples do tipo *flare*; B) mancha gêmea.

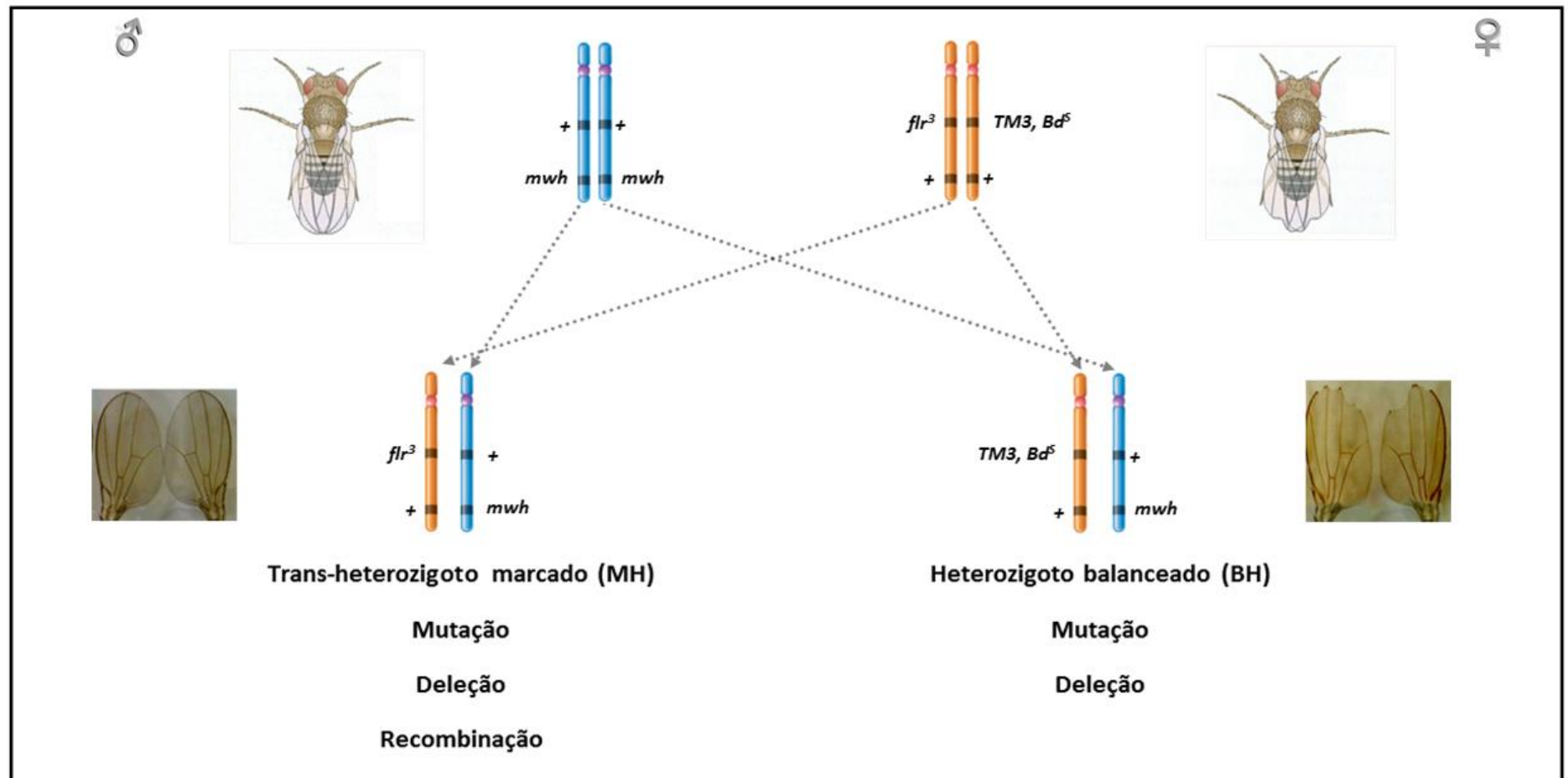


Figura 13. Representação de cruzamento realizado com as linhagens de *D. melanogaster* e o aspecto fenotípico dos descendentes gerados, assim como os tipos de eventos mutacionais identificados.

O cruzamento padrão é útil na detecção de agentes genotóxicos diretos, enquanto o cruzamento de alta bioativação é útil na detecção de agentes genotóxicos indiretos, ou promutágenos, que necessitam de ativação metabólica para induzir efeitos genotóxicos (Frölich e Würigler, 1989; Graf e van Schaik, 1992; Saner, Weibel *et al.*, 1996).

Ambos os cruzamentos produzem dois tipos de progênie: 1) Trans-heterozigoto marcado (*mwh* + / + *flr*³) (MH), que é distinguido pela borda lisa da asa; 2) Heterozigoto balanceado (*mwh* + / + *TM3, Bd*^S) (BH), com borda de asa serrilhada (**Figura 13**).

O SMART de asa em *D. melanogaster* fundamenta-se na premissa de que durante o desenvolvimento, células dos discos imaginais das asas proliferam mitoticamente até o ponto em que se diferenciam, durante a metamorfose, em estruturas que originam as asas das moscas adultas. Existem dois genes marcadores que determinam padrões fenotípicos para os pêlos das asas: pêlos múltiplos (*mwh*, 3-0.3) e pêlos cujo formato lembram uma “chama de vela”, do inglês *flare* (*flr*³, 3-38.8) (Graf, Würigler *et al.*, 1984).

Por meio de tratamentos com compostos mutagênicos, a perda de heterozigose, nos discos imaginais, é induzida, levando à formação de clones de células mutantes (manchas), os quais se expressam ao final da diferenciação celular (**Figuras 11 e 12**). Durante a análise da superfície dorsal e ventral das asas, são considerados o número e o tipo de manchas encontradas, fornecendo dados quantitativos e qualitativos, permitindo a elucidação do evento mutagênico induzido (Spanó e Graf, 1998).

Nos adultos emergentes MH, as manchas mutantes aparecem como manchas simples, apresentando o fenótipo “*mwh*” ou “*flare*”, ou como manchas gêmeas, mostrando áreas adjacentes “*mwh*” e “*flare*”. Manchas simples são consideradas aquelas que expressam apenas um dos genes mutantes, *flr*³ ou *mwh* e podem ser classificadas como simples pequenas (manchas com uma ou duas células) ou simples grandes (manchas com mais de duas células). O aparecimento desses pêlos está relacionado à mutação, aberração cromossômica (deleção), não-disjunção mitótica bem como a eventos recombinacionais (apenas mancha simples do tipo *mwh*). O aparecimento de manchas gêmeas ocorre exclusivamente por recombinação (Graf, Würigler *et al.*, 1984).

Nos indivíduos BH as manchas mutantes aparecem apenas como manchas simples do tipo “*mwh*”, produzidas por mutação, aberração cromossômica (deleção) ou não-disjunção (Spanó e Graf, 1998). Nestes indivíduos os eventos recombinacionais são suprimidos, pois o cromossomo balanceador (TM^3 , Bd^S), apresenta múltiplas inversões, o que impede o desenvolvimento de células que sofram eventuais recombinações somáticas (Graf, Würgler *et al.*, 1984; Guzmán-Rincón e Graf, 1995).

Representações demonstrando os diferentes mecanismos genéticos (mutação, deleção, e recombinação) responsáveis pelo aparecimento de manchas mutantes simples e gêmeas, bem como detalhes das linhagens utilizadas, podem ser vistos no **Anexo I**.

6. Teste do Micronúcleo

Existe uma forte relação entre danos no DNA e o desenvolvimento de diversas doenças crônicas (Jackson e Bartek, 2009). Tradicionalmente, os danos a cromossomos são estudados por meio da análise e quantificação de aberrações cromossômicas em células metafásicas. Porém, devido à complexidade de análise e detecção das aberrações, e ainda, por problemas operacionais como a perda de cromossomos ou mesmo sobreposição dos mesmos durante as preparações para obtenção de células em metáfase, foi necessário o desenvolvimento de técnicas mais simples para mensurar danos cromossômicos (Fenech, 2000).

Há mais de um século, micronúcleos (MNs) já eram descritos no citoplasma de eritrócitos, sendo chamados por Howell de “fragmentos de material nuclear” e por Jolly de “corpúsculos intraglobulares”. Dessa maneira, estas estruturas ficaram conhecidas como corpúsculos de Howell-Jolly. Anos depois, em outro tipo celular, após a exposição à radiação, estruturas similares foram descritas e receberam a denominação de fragmentos nucleares ou MNs (Kirsch-Volders, Sofuni *et al.* 2003).

Fragmentos cromossômicos que não possuem centrômero não podem ligar-se às fibras cinetocóricas e, conseqüentemente, não migram com os demais cromossomos (durante a anáfase) para os pólos opostos da célula. Na telófase, a membrana nuclear é reconstituída ao redor do conjunto de cromossomos e os fragmentos acêntricos (ou mesmo cromossomos inteiros), que não estavam ligados ao fuso mitótico, não se integram aos novos núcleos. Assim, esses fragmentos formam um pequeno núcleo individual (**Figura 14**) que pode ser detectado em células interfásicas como um pequeno corpúsculo arredondado de cromatina, separado do núcleo principal, denominado micronúcleo (MN) (Fenech, 2000; 2006; 2007).

O aumento na frequência de células micronucleadas é um biomarcador de efeitos genotóxicos, que pode refletir a exposição a agentes clastogênicos e aneugênicos (Albertini, Anderson *et al.*, 2000), possibilitando dimensionar os danos citogenéticos induzidos por qualquer agente químico (Rao, Shanbhoge *et al.*, 2006).

O Teste do MN *in vitro* (TMN) é utilizado como uma das primeiras baterias de testes para o desenvolvimento de fármacos (Garriott, Phelps *et al.*, 2002). Basicamente, consiste em submeter culturas celulares à exposição de substâncias-teste, com ou sem fonte exógena de sistema de ativação metabólica. Após a exposição, as células multiplicam-se por tempo determinado, permitindo que os MNs formados durante a divisão celular sejam observados. Estas células são fixadas, coradas e posteriormente analisadas de acordo com a presença e quantidade de MNs, por meio de microscopia óptica de luz (OECD 487, 2004).

O TMN é de relevância em estudos de Toxicologia Genética e Ambiental, tendo em vista que detecta mutações cromossômicas, podendo ser considerado como marcador precoce para carcinogênese (Bonassi, Neri *et al.*, 2003; Bonassi, Znaor *et al.*, 2007).

Para a realização do TMN, diferentes tipos celulares podem ser empregados, como de vegetais, humanos e de outros organismos. O principal requisito é que estas células sejam capazes de se dividir, ou ainda que a divisão seja passível de indução, porém este processo deve ser conhecido e possível de controle (Fenech, 2000).

Uma das linhagens comumente utilizadas para a realização do TMN é a linhagem V79 (fibroblastos de pulmão de hamster chinês), pois possuem características interessantes aos ensaios de Toxicologia Genética, como: fácil cultivo e manutenção em laboratório, e cariótipo estável. Além disso, mesmo estocadas em nitrogênio líquido, estas células não têm suas características afetadas, não comprometendo os ensaios (Bradley, Bhuyan *et al.*, 1981).

Em 1985, Fenech e Morley propuseram o bloqueio da citocinese (mas não da cariocinese), utilizando citocalasina-B (Ct-B), um inibidor da polimerização dos filamentos de actina, necessários para a formação do anel de microfilamentos, que induzem a contração do citoplasma entre os núcleos das células irmãs durante a citocinese (Fenech e Morley, 1985). Assim, com o bloqueio da citocinese, observa-se um acúmulo de células binucleadas (**Figura 14**). A análise dessas células, além de permitir a comparação da frequência de danos cromossômicos entre populações celulares, permite ainda o estudo da cinética do

mecanismo que leva a formação dos MNs (Fenech, 2000; 2006; 2007; Kirsch-Volders *et al.*, 2003).

Dependendo da concentração de Ct-B utilizada, a mesma não previne a formação e tão pouco induz a formação de MNs, não interferindo, assim, na avaliação do potencial genotóxico e antigenotóxico (Garriot *et al.*, 2002).

Os MNs nas células são uma mistura de formas transcrpcionais ativas e inativas. No entanto, na maioria dos casos, estes apresentam níveis indetectáveis de atividade transcricional. Sendo assim, genes contidos nos micronúcleos inativos têm a sua atividade funcional perdida (Hoffelder, Luo *et al.*, 2004).

O destino dos MNs, após a sua formação, é pouco conhecido. No entanto, após a mitose já foram registrados: eliminação (apoptose) de células micronucleadas, reincorporação ao núcleo principal (podendo resultar em atividade biológica normal) além de ser retido no citoplasma da célula como uma entidade extranuclear (Mateuca, Lombaert *et al.*, 2006).

Para avaliação do potencial mutagênico ou antimutagênico são realizados três ensaios independentes (n=3). Para cada tratamento são confeccionadas duas lâminas. As lâminas são analisadas em teste cego, utilizando-se microscópio óptico de luz, com objetiva de imersão (aumento final de 1000 X). Um total de 2.000 células binucleadas (por tratamento) deve ser analisado, registrando-se o total de MNs. As células analisadas são distribuídas de acordo com a quantidade de MNs: 0, 1, 2 ou mais. Para quantificação da frequência de MNs, apenas células binucleadas com núcleos intactos, com tamanhos aproximadamente iguais, mesmo padrão de coloração e dentro do limite citoplasmático, com membrana nuclear intacta, e célula claramente distinguível das células adjacentes, são analisadas (Fenech, 2000).

Para avaliar a citotoxicidade é calculado o Índice de divisão nuclear (IDN). Para tanto, são analisadas 1000 células viáveis (com citoplasma bem preservado) para cada tratamento, totalizando 3000 células. Para o cálculo do IDN utiliza-se a seguinte fórmula: $IDN = [M1 + 2(M2) + 3(M3) + 4(M4)] / N$, onde M1-M4, representam os números de células com 1, 2, 3 e 4 núcleos, respectivamente, e N o número total de células analisadas (Fenech, 2000; Eastmond e Tucker, 1989).

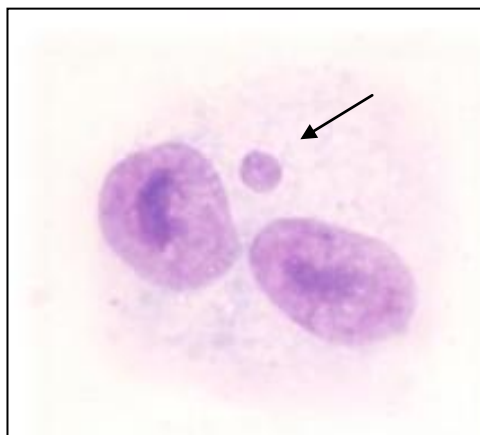


Figura 14. Fotomicrografia óptica de uma célula binucleada de pulmão de hamster Chinês (V79) micronucleada (coloração por Giemsa; analisado com aumento 1000x).

7. Ensaio antioxidante – DPPH

Radicais livres desempenham um papel importante no dano oxidativo dos sistemas biológicos (Scalbert, Manach *et al.*, 2005). Portanto, é necessário avaliar a capacidade antioxidante de compostos isolados ou de extratos brutos (Dawidowicz, Wianowska *et al.*, 2012).

Diversas metodologias têm sido utilizadas para determinar a atividade antioxidante *in vitro*, e entre elas, o ensaio espectrofotométrico empregando o radical 2,2-difenil-1-picrilidrazila (DPPH $\cdot$), que apesar de não ser biologicamente relevante, é frequentemente utilizado (Dawidowicz, Wianowska, *et al.*, 2012).

No método do DPPH (Brand-Williams, Cuvelier *et al.*, 1995), a estabilização do radical DPPH $\cdot$ é acompanhada pelo monitoramento da diminuição da absorbância a 517 nm, que ocorre devido à sua reação com algum antioxidante.

O DPPH é um radical livre, estável à temperatura ambiente, que produz uma coloração violeta em solução (**Figura 15**). Na presença de uma molécula de antioxidante, este radical é reduzido, modificando a cor da solução, passando de violeta para amarela (Deng, Cheng *et al.*, 2011). A utilização dessa metodologia fornece, de forma rápida e fácil, parâmetros confiáveis para avaliação da atividade antioxidante (Abdel-Razek, El-Shami *et al.*, 2011).

A determinação da capacidade antioxidante é determinada de acordo com a equação:

Capacidade antioxidante (%) = $[(A_0 - A_1) / A_0 \times 100]$. Onde A_0 e A_1 correspondem à absorbância a 517 nm do radical (DPPH $\cdot$), na ausência e na presença do antioxidante, respectivamente (Mishra, Ojha *et al.*, 2012).

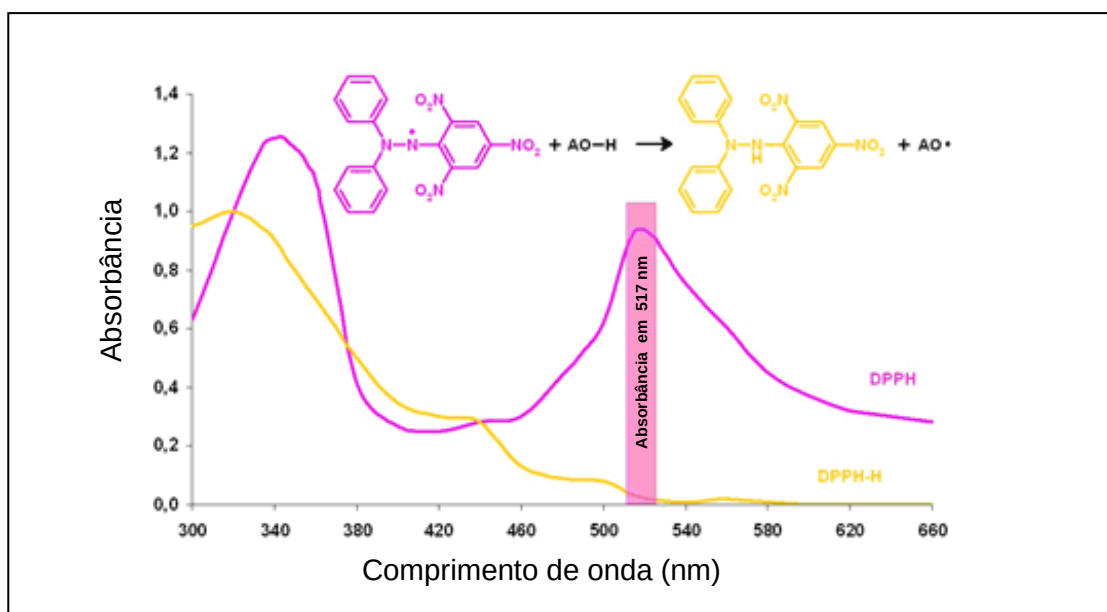


Figura 15. Esquema da estabilização do radical 2,2-difenil-1-picrilhidrazila (DPPH•) e a cinética da mudança de cor da solução mediante diminuição da absorbância a 517 nm devido à estabilização do mesmo (Fonte: <http://www.baltic-analytics.de/index.php?id=40>, Acessado em 18 de junho de 2012 – com modificações).

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CAPÍTULO 2

THE EFFECT OF THE DIBENZYL BUTYROLACTOLIC LIGNAN
(-)-CUBEBIN ON DOXORUBICIN MUTAGENICITY AND
RECOMBINOGENICITY IN WING SOMATIC CELLS OF
DROSOPHILA MELANOGASTER

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(ANEXO II)

The effect of the dibenzylbutyrolactolic lignan (-)-cubebin on doxorubicin mutagenicity and recombinogenicity in wing somatic cells of *Drosophila melanogaster*

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Abstract

The dibenzylbutyrolactolic lignan (-)-cubebin was isolated from dry seeds of *Piper cubeba* L. (Piperaceae). (-)-Cubebin possesses anti-inflammatory, analgesic and antimicrobial activities. Doxorubicin (DXR) is a topoisomerase-interactive agent that may induce single- and double-strand breaks, intercalate into the DNA and generate oxygen free radicals. Here, we examine the mutagenicity and recombinogenicity of different concentrations of (-)-cubebin alone or in combination with DXR using standard (ST) and high bioactivation (HB) crosses of the wing somatic mutation and recombination test in *Drosophila melanogaster*. The results from both crosses were rather similar. (-)-Cubebin alone did not induce mutation or recombination. At lower concentrations, (-)-cubebin statistically reduced the frequencies of DXR-induced mutant spots. At higher concentrations, however, (-)-cubebin was found to potentiate the effects of DXR, leading to either an increase in the production of mutant spots or a reduction, due to toxicity. These results suggest that depending on the concentration, (-)-cubebin may interact with the enzymatic system that catalyzes the metabolic detoxification of DXR, inhibiting the activity of mitochondrial complex I and thereby scavenging free radicals. Recombination was found to be the major effect of the treatments with DXR alone. The combined treatments reduced DXR mutagenicity but did not affect DXR recombinogenicity.

Key words: Somatic Mutation and Recombination Test; SMART; toxicity; CYP6A2.

1. Introduction

Piper cubeba Linn. (Piperaceae) is a pepper plant that is widely distributed in tropical and subtropical regions and is popularly known as *pimenta de Java* (in Brazil), *kemukus* (in Indonesia) or cubeb pepper. Its fruit is used as a spice or in folk medicine for the treatment of various diseases, such as dysentery and gonorrhea (Junqueira et al., 2007; Usia et al., 2006). It is known that extracts from *P. cubeba* have anti-inflammatory, anti-type IV allergic, antineoplastic, antileishmanial molluscicidal and genotoxic activities (Bodiwala et al., 2007; Choi and Hwang, 2003; Junqueira et al., 2007; Pandey and Singh, 2009; Yam et al., 2008).

Lignans are a class of secondary plant metabolites that are produced by the oxidative dimerization of two phenylpropanoid units. Interest in lignans and their synthetic derivatives has grown due to their applications for cancer chemotherapy and their various other pharmacological effects (Saleem et al., 2005). Here, (-)-cubebin, a dibenzylbutyrolactolic lignan, was isolated from the dry seeds of *Piper cubeba* Linn. This extract is known to possess anti-inflammatory, analgesic and antimicrobial activities (Bastos et al., 2001; da Silva et al., 2005; Silva et al., 2007; Silva et al., 2009). Although some (-)-cubebin derivative compounds ((-)-hinokinin; (-)-*O*-benzyl cubebin; (-)-*O*-(*N,N*-dimethylamino-ethyl)-cubebin) inhibit the free amastigote forms of *Trypanosoma cruzi*, the natural (-)-cubebin molecule itself, which is used as the starting compound to obtain the evaluated dibenzylbutyrolactolic derivatives, does not affect the growth of trypomastigote forms of *T. cruzi* (de Souza et al., 2005). (-)-Cubebin and derivatives isolated from *Piper cubeba* were found to significantly inhibit cytochrome P450 (CYP3A4) (Usia et al., 2006; Usia et al., 2005) and the NADoxidase activity of mitochondrial complex I (Saraiva et al., 2009).

The anthracycline antibiotic Doxorubicin (DXR), a drug that targets topoisomerase II (Top2), is one of the most effective anticancer drugs used in the clinic (Islaih et al., 2005; Lyu et al., 2007). This drug may induce mutations by intercalating into the DNA (Spencer et al., 2008) or by inducing the formation of oxygen free radicals (Navarro et al., 2006), single- and double-strand DNA breaks

(Lyu et al., 2007) and somatic recombination (de Rezende et al., 2009; Lehmann et al., 2003; Sousa et al., 2009; Valadares et al., 2008).

The wing somatic mutation and recombination test (SMART) using *Drosophila melanogaster* was developed to detect the loss of heterozygosity of suitable gene markers that have detectable phenotypes that are expressed on the wings. This assay is an efficient and quick method for quantitating the recombinogenic and mutagenic potential of chemical and physical agents (Graf et al., 1996; Spanó et al., 2001). For this purpose, two crosses are typically used: the standard (ST) cross (Graf et al., 1989) and a high bioactivation (HB) cross (Graf and van Schaik, 1992). The ST cross uses strains carrying basal levels of the metabolizing cytochrome P450 enzyme (CYP6A2) and is used to detect direct-acting genotoxins. The HB cross uses strains with high levels of CYP6A2 and is used to detect indirect-acting genotoxins that exert their genotoxic activity only when metabolized.

Several reports have demonstrated the probability of pharmacokinetic interaction between natural compounds and herbal products with conventional drugs when they are administered simultaneously (Usia et al., 2006). Here, we examine the mutagenicity and recombinogenicity of the dibenzylbutyrolactolic lignan (-)-cubebin when administered alone or simultaneously with DXR.

2. Materials and methods

2.1 Chemical compounds and media

(-)-Cubebin was isolated from the seeds of *Piper cubeba* L. as previously described (Silva *et al.*, 2007). Doxorubicin (DXR) (Rubidox[®] - Laboratório Químico Farmacêutico Bergamo Ltda., Taboão da Serra, SP, Brazil) (CAS 23214-92-8) was obtained from the Hospital de Clínicas da Universidade Federal de Uberlândia, MG, Brazil. Ultrapure water (18.2 Ω X) was obtained from a MilliQ system (Millipore, Vimodrone, Milan, Italy). (-)-Cubebin was dissolved in a mixture of 1% Tween 80 (Fluka, Buchs, Switzerland) and 3% ethanol (Neon, São Paulo, Brazil) in ultrapure water. DXR was dissolved in ultrapure water. The solutions were always prepared immediately before use. As an alternative medium, instant mashed potato flakes (Yoki[®] Alimentos S. A., São Bernardo do Campo, SP, Brazil) were used. The structural formula of (-)-cubebin is shown in Figure 1.

2.2 The Somatic Mutation and Recombination Test (SMART)

Two crosses were carried out to produce the experimental larval progeny. The first was the standard (ST) cross, in which virgin females of strain *flr³/In(3LR)TM3, ri p^p sep l(3)89Aa bx^{34e} e Bd^S* were crossed with *mwh/mwh* males (Graf *et al.*, 1984; Graf *et al.*, 1989). The second was the high bioactivation (HB) cross, in which virgin females of strain *ORR/ORR; flr³/In(3LR)TM3, ri p^p sep l(3)89Aa bx^{34e} e Bd^S* were crossed with *mwh/mwh* males (Graf and van Schaik, 1992). Each cross produced marker-heterozygous (MH) flies (*mwh flr⁺ / mwh⁺ flr³*) with normal wings and balancer-heterozygous (BH) flies (*mwh flr⁺ / mwh⁺ TM3, Bd^S*) with serrated wings. From both crosses, eggs were collected for 8 h in culture glass bottles with an agar-agar base (4% w/v) topped with a thick layer of live baker's yeast supplemented with sucrose. After 72 h ($\pm$ 4 h), third instar larvae were washed out of the bottles with ultrapure water (MilliQ system) and collected in a stainless steel strainer. For chronic feeding, two sets of vials for each cross were prepared with 1.5 g of mashed potato flakes (Yoki[®] Alimentos S.A., Brazil) and 5 ml of a solution containing (-)-cubebin alone (at a final concentration of 0.25, 0.5, 1.0, 2.0 or 4.0 mM) or with DXR (0.2 mM). Negative (1% Tween-80 and 3% ethanol in distilled water) and positive (DXR 0.2 mM) controls were included in

both experiments. The experiments were conducted at $25 \pm 1^{\circ}\text{C}$ and approximately 60% humidity. The hatched flies were stored in 70% (v/v) ethanol. The wings were removed and mounted on slides with Faure's solution (gum arabic 30 g, glycerol 20 ml, chloral hydrate 50 g and distilled water 50 ml) and analyzed under a compound microscope at 400x magnification.

2.3 Statistical analysis

For each treated series, 50 flies of both sexes were scored. The multiple-decision procedure (Frei and Würgler, 1988, 1995) was used to analyze the data, resulting in three different diagnoses: negative, positive or inconclusive. The frequency of each type of spot (small single, large single or twin) and the total frequency of spots per fly for each treatment were compared pair-wise (i.e., negative control versus (-)-cubebin; positive control (DXR) alone versus DXR plus (-)-cubebin) according to Kastenbaum and Bowman (Kastenbaum and Bowman, 1970) with $p = 0.05$ (Frei and Würgler, 1988, 1995). All inconclusive and weak results were analyzed with the non-parametric U-test of Mann, Whitney and Wilcoxon ($\alpha = \beta = 0.05$, one sided) (Frei and Würgler, 1995). Based on the clone induction frequency per 10^5 cells, the recombinogenic activity was calculated as follows. Frequency of mutation (FM) = frequency of clones in BH flies/frequency of clones in MH flies. Frequency of recombination (FR) = $1 - \text{FM}$. Frequencies of total spots (FT) = total spots observed in MH flies (considering mwh and flr³ spots)/number of flies (Santos et al., 1999; Sinigaglia et al., 2006; Sinigaglia et al., 2004). Based on the control-corrected spot frequencies per 10^5 cells, the percentage of (-)-cubebin inhibition was calculated as: $(\text{DXR alone} - (-)\text{-cubebin plus DXR}) / \text{DXR alone} \times 100$ (Abraham, 1994). Statistical comparisons of survival rates were made with the Chi-squared test for ratios of independent samples.

3. Results

Third instar larvae of *D. melanogaster* obtained from both (ST and HB) crosses were fed chronically (approximately 48 h) with (-)-cubebin (0.25, 0.5, 1.0, 2.0 or 4.0 mM) alone or in combination with DXR (0.2 mM). Each treatment was done in duplicate. The data were pooled after verifying that there were no significant differences between repetitions. The concentrations were chosen based on a dose response test, for which the survival rates of flies are given in Table 1.

At concentrations below 2.0 mM, (-)-Cubebin was not found to be toxic. Thus, we found it particularly important to evaluate the modulatory effects of low concentrations on DNA damage induced by DXR in somatic cells of *D. melanogaster*. The highest concentration (4.0 mM) of (-)-cubebin was found to be toxic in the ST cross, when administered alone or combination with DXR. A significant ($p < 0.05$) decrease in survival rates relative to the negative control group (ultrapure water) was observed. This concentration was also found to be toxic in the HB cross when administered with DXR. Although 4.0 mM (-)-cubebin alone significantly decreases the survival rates, this concentration was also tested in association with DXR.

The results of the ST cross of the SMART assays using *D. melanogaster* are depicted in Table 2. In the MH individuals, (-)-cubebin alone did not show any genotoxicity at the doses used. The DXR treatment, however, induced positive results for all categories of spots when compared to the negative control ($p < 0.05$). Lower concentrations of (-)-Cubebin (0.25, 0.5 or 1.0 mM) when administered with DXR (0.2 mM) were found to inhibit DXR-induced DNA damage (54.66, 26.21 and 26.84%, respectively, $p < 0.05$). The simultaneous administration of DXR with 2 mM (-)-cubebin (2.0 mM), however, was found to significantly increase the number of DXR-induced wing spots (by 94.30%). The highest concentration of (-)-cubebin (4.0 mM) was found to reduce the frequency of DXR-induced spots, but this difference was not significant.

The wings of the BH flies resulting from the simultaneous application of both drugs were also mounted and scored. This procedure enabled us to quantify the contribution of mutagenic and recombinogenic events to the final genotoxicity observed (Santos et al., 1999; Sinigaglia et al., 2004).

In the BH individuals of the ST cross, DXR (0.2 mM) induced a significant increase in the mutant spot frequency relative to the negative control ($p < 0.05$). In the combined treatments, only 0.5 mM (-)-cubebin significantly reduced ($p < 0.05$) the total number of spots induced by DXR. The other concentrations of (-)-cubebin (0.25, 1.0, 2.0 and 4.0 mM) did not affect DXR mutagenicity.

Based on the clone induction frequency per 10^5 cells, we compared the number of observed spots in the MH and BH flies and quantified the contribution (%) of mutation and recombination to the total number of observed spots. We found that the induced spots were mainly due to recombination. We also found that (-)-cubebin does not affect the recombinogenic activity of DXR (Figure 2).

The results of the HB cross of the SMART assays with *D. melanogaster* are summarized in Table 3. The results obtained with the MH individuals of the HB cross treated with (-)-cubebin alone were negative ($p > 0.05$) at all tested concentrations. DXR statistically increased all categories of spots when compared to the negative control ($p < 0.05$). When administered with DXR, all concentrations of (-)-cubebin were found to statistically inhibit DXR-induced DNA damage ($p < 0.05$), although the inhibition have not been dose-related.

In the BH individuals, DXR induced a significant increase in mutant spots ($p < 0.05$), relative to the negative control. Compared to DXR alone, the treatments of 0.25, 1.0 or 4.0 mM (-)-cubebin administered with DXR produced significantly fewer mutant spots, while 0.5 and 2.0 mM (-)-cubebin had no effect on the production of mutant spots.

By comparing the number of observed spots in the MH flies and BH flies, we once again found that the induced spots were mainly due to recombination. We found that (-)-cubebin displays only anti-mutagenic activity and does not interfere with DXR recombinogenicity (Figure 2).

4. Discussion

The mutagenic and/or recombinogenic potential of (-)-cubebin, alone and in combination with the chemotherapeutic agent DXR, was assayed using two crosses (ST and HB) of the wing Somatic Mutation and Recombination Test (SMART) in *D. melanogaster*. We obtained similar data in both crosses and found that (-)-cubebin alone does not modify the frequency of spontaneous mutant spots in this test system.

The reference mutagen DXR significantly increased all categories of spots. Previous studies using the SMART assay have shown that the major mutational contribution of DXR is its ability to induce recombination (de Rezende et al., 2009; Lehmann et al., 2003; Sousa et al., 2009; Valadares et al., 2008). DXR is a chemotherapeutic agent that induces single- and double-stranded DNA breaks, which are processed by recombinational DNA repair pathways. DNA double-strand breaks (DSBs) are a serious threat to the cell and can lead to chromosomal aberration, mutation and cancer. DSBs in human cells are repaired via non-homologous DNA end joining and homologous recombination repair pathways (Poplawski, 2010). Homologous recombination can result in a loss of heterozygosity or genetic rearrangements. Some of these genetic alterations can be correlated with the manifestation of recessive heritable diseases and may play a primary role in carcinogenesis. More likely, however, they are probably involved in secondary and subsequent steps of carcinogenesis that reveal recessive oncogenic mutations (Bishop and Schiestl, 2002, 2003).

There is an increasing amount of evidence to suggest that cancer and other mutation-related diseases can be prevented not only by limiting exposure to recognized risk factors but also with the intake of protective factors and by modulating the defense mechanisms of the host organism. This strategy, referred to as chemoprevention, can be pursued either with suitable pharmacological agents and/or by dietary factors (Ferguson et al., 2005). Here, we examined the mutagenicity and recombinogenicity of the dibenzylbutyrolactolic lignan (-)-cubebin extracted from seeds of *Pipper cubeba* L. when administered alone or simultaneously with the pharmacological agent DXR.

The association of (-)-cubebin with DXR in the ST cross was found to produce different results depending on the concentration used. At lower

concentrations (0.25, 0.5 or 1.0 mM) (-)-cubebin significantly reduces the frequency of DXR-induced mutant spots. At 2.0 mM, (-)-Cubebin strongly increases DXR-induced mutagenicity and recombinogenicity, affecting all types of spots (small single, large single and twin spots). The magnitude of the comutagenicity was found to be considerable, leading to an enhancement of 94.30%. At 4.0 mM, (-)-Cubebin slightly reduces the frequency of DXR-induced DNA damage, although this reduction was not found to be statistically significant. Given the significant decrease in survival rates that was observed in flies treated with 4.0 mM (-)-cubebin (Table 1), we think this reduction in DXR-induced DNA damage can be attributed to cytotoxicity, rather than a protective effect of (-)-cubebin.

In the HB cross, all concentrations of (-)-cubebin were found to significantly reduce the frequency of DXR-induced mutant spots. However, given the significant decrease in survival rates observed in the ST cross (Table 1), we conclude that the reduction in DXR-induced mutant spots that can be observed upon treatment with 0.2 or 0.4 mM (-)-cubebin in the HB cross is due to cytotoxicity.

Similar data have been generated for (-)-hinokinin (HK), a dibenzylbutyrolactolic lignan that is obtained by from (-)-cubebin, using the micronucleus (MN) test in V79 Chinese hamster lung fibroblasts to assay the effect on DXR clastogenicity. At lower concentrations, HK significantly protects against DXR-induced MN formation. This effect is thought to be due to of the ability of HK to quench reactive oxygen species. At higher concentrations, HK potentiates DXR-induced clastogenicity, suggesting that higher doses of HK may increase the oxidative stress generated by DXR (Resende et al., 2010).

The cytochrome P450 (CYP) superfamily is the most important drug-metabolizing enzyme group that metabolically activates promutagens (Takiguchi et al., 2010). Previous studies have shown that an absence of mutagenicity may result from the metabolic deactivation of a compound. For example, nor-nitrogen mustard (NNM) was found to be non-mutagenic when fed to adult flies in a sex-linked recessive lethal test. In combination with 1-phenylimidazole (PhI), an inhibitor of cytochrome P-450, sufficient quantities of the mutagen was able to reach the gonads and produce significant genetic damage. These data suggest that NNM is deactivated by this family of CYP (Zijlstra and Vogel, 1988). It has

been proposed that a similar mechanism can explain the significant potentiating action of tannic acid when administered simultaneously with either of the alkylating agents methylmethanesulfonate or nitrogen mustard in a SMART assay in *D. melanogaster* (Lehmann et al., 2000).

Previous studies have shown that MeOH- and EtOAc-soluble fractions of *Piper cubeba* significantly inhibit the CYP3A4-mediated metabolism of (*N*-methyl-¹⁴C)erythromycin (Usia et al., 2006). Thus, it is possible that (-)-cubebin acts by interacting with the enzyme systems that catalyze the metabolic detoxification of DXR in *D. melanogaster*. Such an interaction could explain the high frequency of mutant spots observed at 2.0 mM (ST cross) and the cytotoxic effect observed at 2.0 mM (HB cross) and 4.0 mM (in both ST and HB crosses).

Mitochondria have also been often implicated in cell death. (-)-Cubebin and its derivatives typically inhibit the mitochondrial complex I, as demonstrated by the inhibition of glutamate/malate-supported state 3 respiration of mitochondria and the NADH oxidase activity of submitochondrial particles (Saraiva et al., 2009). The inhibition of mitochondrial complex I is another possible mechanism to explain the toxicity observed at high (-)-cubebin concentrations.

In the *Drosophila* SMART assay (-)-cubebin significantly alters the generation of DXR-induced wing spots, which are the result of somatic mutation and mitotic recombination. Our data, in combination with the data from previous studies using different compounds, allow us to suggest that (-)-cubebin may act as a free radical scavenger at low concentrations and a pro-oxidant at higher concentrations when it interacts with the enzymatic system that catalyzes the metabolic detoxification of DXR. Alternatively, (-)-cubebin may inhibit mitochondrial complex I, leading to cell death.

In this study, we showed that (-)-cubebin can positively or negatively influence DXR-induced mutagenicity, depending on the concentration. With the long-term goal of developing chemoprevention strategies, future work will focus on investigating the conditions under which (-)-cubebin can prevent genome damage, its mechanisms of action and its pharmacokinetic interaction with conventional drugs.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 1. Survival rates upon exposure to different concentrations of (-)-cubebin alone or in combination with doxorubicin relative to control groups (ultrapure water and doxorubicin) in the wing somatic mutation and recombination test in *D. melanogaster*

Compounds		Standard cross		High bioactivation cross	
DXR	Cubebin	Survival	<i>p</i> -value	Survival	<i>p</i> -value
(mM)	(mM)	(%)		(%)	
0	0	95		95	
0	0.25	90	>0.05	90	>0.05
0	0.5	100	>0.05	90	>0.05
0	1.0	95	>0.05	90	>0.05
0	2.0	85	>0.05	90	>0.05
0	4.0	60	<0.05	90	>0.05
0.2	0	95	>0.05	95	>0.05
0.2	0.25	85	>0.05	80	>0.05
0.2	0.5	85	>0.05	80	>0.05
0.2	1.0	85	>0.05	80	>0.05
0.2	2.0	75	>0.05	70	>0.05
0.2	4.0	50	<0.05	65	<0.05

Statistical comparisons of survival rates were made with the Chi-squared test for ratios of independent samples.

Table 2. Summary of results obtained with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the standard cross (ST) after chronic treatment of larvae with (-)-cubebin and Doxorubicin (DXR)

Genotypes and treatments		Number of flies	Spots per fly (number of spots) statistical diagnosis ^a				Spots with mwh clone ^c	Frequency of clone formation/10 ⁵ cells per cell division ^d		Recombination (%)	Inhibition ^e (↓) or induction ^e (↑) (%)
			Small single spots (1-2 cells) ^b	Large single spots (>2 cells) ^b	Twin spots	Total spots		Observed	Control Corrected		
DXR (mM)	(-)-Cubebin (mM)										
<i>mwh/flr³</i>											
0	0	50	0.32 (16)	0.06 (03)	0.02 (01)	0.40 (20)	20	0.82			
0	0.25	50	0.16 (08)-	0.00 (00)-	0.00 (00)i	0.16 (08)-	08	0.33	-0.49		
0	0.50	50	0.16 (08)-	0.00 (00)-	0.00 (00)i	0.16 (08)-	08	0.33	-0.49		
0	1.00	50	0.14 (07)-	0.04 (02)i	0.00 (00)i	0.18 (09)-	09	0.37	-0.45		
0	2.00	50	0.16 (08)-	0.08 (04)i	0.02 (01)i	0.26 (13)-	13	0.53	-0.29		
0	4.00	50	0.22 (11)-	0.02 (01)i	0.00 (00)i	0.24 (12)-	12	0.49	-0.33		
0.20	0	50	2.78 (139)+	2.38 (119)+	2.66 (133)+	7.72 (386)+	371	15.20	14.38	91.90	
0.20	0.25	50	1.20 (60)*	0.92 (46)*	1.56 (78)*	3.68 (184)*	179	7.34	6.52	86.02	54.66 ↓
0.20	0.50	50	1.52 (76)*	1.58 (79)*	2.56 (128)	5.66 (283)*	279	11.43	10.61	94.27	26.21 ↓
0.20	1.00	50	1.40 (70)*	1.46 (73)*	2.80 (140)	5.66 (283)*	277	11.34	10.52	88.10	26.84 ↓
0.20	2.00	50	3.42 (171)*	4.08 (204)*	6.92 (346)*	14.42 (721)*	702	28.76	27.94	94.59	94.30 ↑
0.20	4.00	50	2.62 (131)	2.04 (102)	2.72 (136)	7.38 (369)	354	14.20	13.38	94.10	06.94
<i>mwh/TM3</i>											
0	0	50	0.26 (13)	0.00 (00)	†	0.26 (13)	13	0.53			
0.20	0	50	0.50 (25)+	0.10 (05)+		0.60 (30)+	30	1.23	0.70		
0.20	0.25	50	0.40 (20)	0.10 (05)		0.50 (25)	25	1.01	0.48		
0.20	0.50	50	0.08 (04)*	0.24 (12)		0.32 (16)*	16	0.64	0.11		
0.20	1.00	50	0.40 (20)	0.26 (13)*		0.66 (33)	33	1.34	0.81		
0.20	2.00	50	0.66 (33)	0.10 (05)		0.76 (38)	38	1.56	1.03		
0.20	4.00	50	0.42 (21)	0.00 (00)*		0.42 (21)	21	0.85	0.32		

Marker-trans-heterozygous flies (*mwh/flr³*) and balancer-heterozygous flies (*mwh/TM3*) were evaluated.

^aStatistical diagnoses according to Frei and Würgler (1988; 1995). *U*-test, two sided; probability levels: -, negative; +, positive; i, inconclusive; P≤0.05 vs. untreated control; *, P≤ 0.05 vs. DXR only

^bIncluding rare *flr³* single spots.

^cConsidering *mwh* clones from *mwh* single and twin spots.

^dFrequency of clone formation: clones/flies/48,800 cells (without size correction).

^eCalculated as [(DXR alone – DXR + (-)-cubebin) / DXR] x 100, according to Abraham (1994).

^f Balancer chromosome *TM3* does not carry the *flr³* mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.

Table 3. Summary of results obtained with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the high bioactivation cross (HB) after chronic treatment of larvae with (-)-cubebin and Doxorubicin (DXR)

Genotypes and treatments		Number of flies	Spots per fly (number of spots) statistical diagnosis ^a				Spots with mwh clone ^c	Frequency of clone formation/10 ⁵ cells per cell division ^d		Recombination (%)	Inhibition ^e (%)
DXR (mM)	(-)-Cubebin (mM)		Small single spots (1-2 cells) ^b	Large single spots (>2 cells) ^b	Twin spots	Total spots		Observed	Control Corrected		
<i>mwh/flr³</i>											
0	0	50	0.38 (19)	0.08 (04)	0.08 (04)	0.54 (27)	27	1.11			
0	0.25	50	0.28 (14)-	0.08 (04)i	0.02 (01)-	0.38 (19)-	19	0.78	-0.33		
0	0.50	50	0.26 (13)-	0.02 (01)-	0.04 (02)i	0.32 (16)-	16	0.66	-0.45		
0	1.00	50	0.40 (20)-	0.08 (04)i	0.00 (00)-	0.48 (24)-	24	0.98	-0.13		
0	2.00	50	0.40 (20)-	0.04 (02)i	0.00 (00)-	0.44 (22)-	22	0.90	-0.21		
0	4.00	50	0.44 (22)i	0.16 (08)i	0.02 (01)-	0.62 (31)-	30	1.22	0.10		
0.20	0	50	2.80 (140)+	2.78 (139)+	3.20 (160)+	8.78 (439)+	420	17.21	16.10	90.89	
0.20	0.25	50	1.74 (87)*	1.50 (75)*	1.66 (83)*	4.90 (245)*	245	10.04	8.93	92.62	44.54
0.20	0.50	50	2.32 (116)	1.46 (73)*	2.30 (115)*	6.08 (304)*	304	12.46	11.35	87.38	29.50
0.20	1.00	50	2.54 (127)	2.18 (109)*	2.64 (132)	7.36 (368)*	363	14.88	13.77	93.11	14.47
0.20	2.00	50	2.28 (114)	1.64 (82)*	2.16 (108)*	6.08 (304)*	301	12.34	11.23	88.48	30.25
0.20	4.00	50	2.20 (110)*	1.20 (60)*	2.34 (117)*	5.74 (287)*	285	11.67	10.56	89.79	34.41
<i>mwh/TM3</i>											
0	0	50	0.26 (13)	0.00 (00)		0.26 (13)	13	0.53			
0.20	0	50	0.50 (25)+	0.30 (15)+		0.80 (40)+	40	1.64	1.11		
0.20	0.25	50	0.50 (25)	0.00 (05)		0.50 (25)*	25	1.02	0.49		
0.20	0.50	50	0.70 (35)	0.00 (00)		0.70 (35)	35	1.42	0.89		
0.20	1.00	50	0.50 (25)	0.00 (00)*		0.66 (25)*	25	1.02	0.49		
0.20	2.00	50	0.66 (33)	0.10 (05)*		0.76 (38)	38	1.56	1.03		
0.20	4.00	50	0.42 (21)	0.00 (00)*		0.42 (21)*	21	0.85	0.32		

Marker-trans-heterozygous flies (*mwh/flr³*) and balancer-heterozygous flies (*mwh/TM3*) were evaluated.

^aStatistical diagnoses according to Frei and Würgler (1988; 1995). *U*-test, two sided; probability levels: -, negative; +, positive; i, inconclusive; $P \leq 0.05$ vs. untreated control; *, $P \leq 0.05$ vs. DXR only

^bIncluding rare *flr³* single spots.

^cConsidering *mwh* clones from *mwh* single and twin spots.

^dFrequency of clone formation: clones/flies/48,800 cells (without size correction).

^eCalculated as $[(\text{DXR alone} - \text{DXR} + (-)\text{-cubebin}) / \text{DXR}] \times 100$, according to Abraham (1994).

^f Balancer chromosome *TM3* does not carry the *flr³* mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.

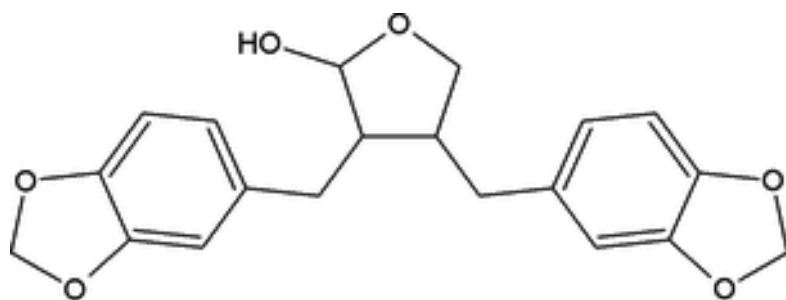


Fig. 1. Structural formula of (-)-cubebin.

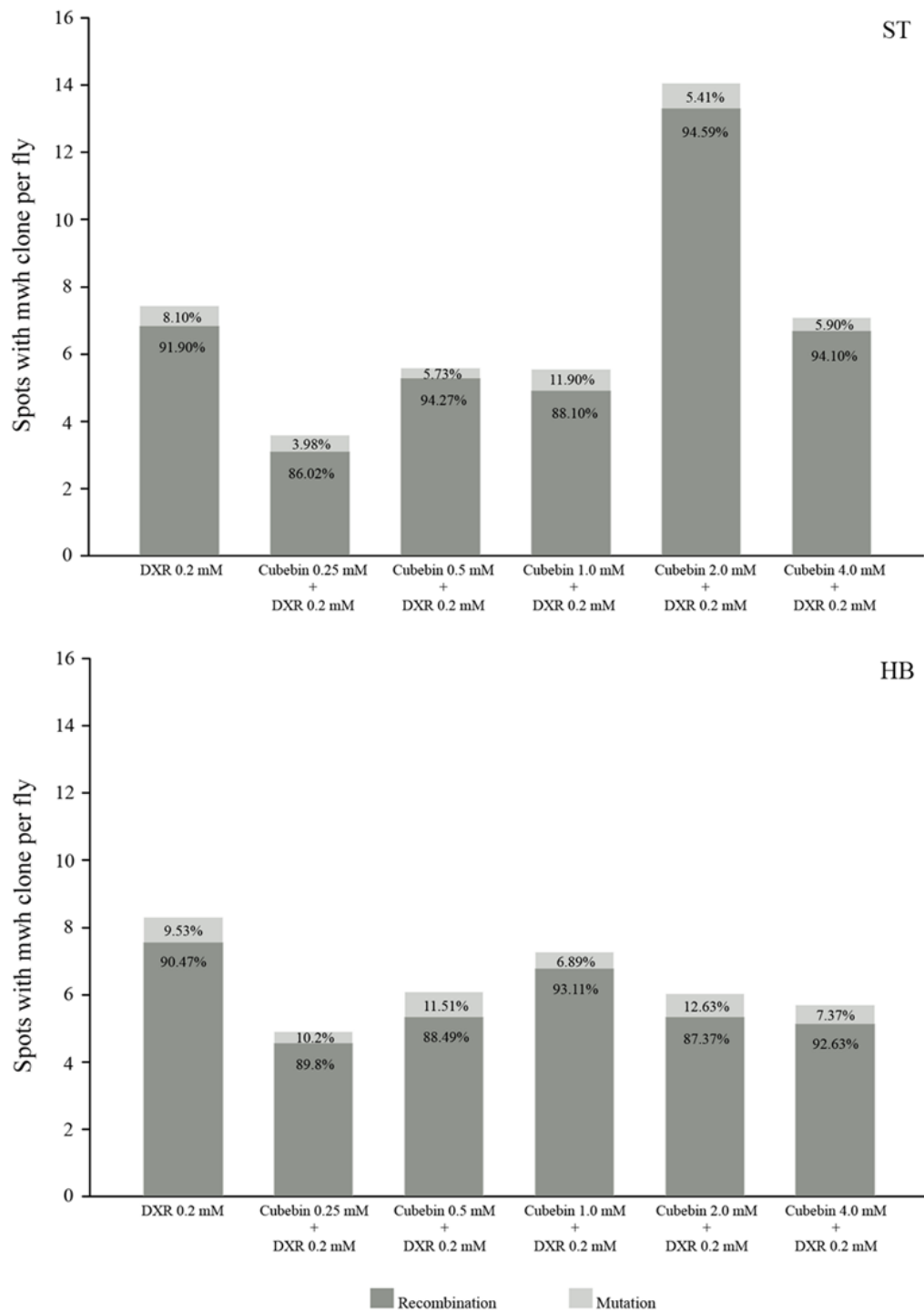


Fig. 2. Contribution of recombination and mutation (in percentage) to total mwh wing spot induction observed in MH individuals from the ST and HB crosses treated with DXR alone and in combination with different concentrations of (-)-cubebin.

CAPÍTULO 3

COMPARATIVE STUDY OF THE MODULATORY EFFECTS OF (-)-CUBEBIN ON
THE MUTAGENICITY / RECOMBINOGENICITY INDUCED BY DIFFERENT
CHEMICAL AGENTS

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COMPARATIVE STUDY OF THE MODULATORY EFFECTS OF (-)-CUBEBIN ON
THE MUTAGENICITY / RECOMBINOGENICITY INDUCED BY DIFFERENT
CHEMICAL AGENTS

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ABSTRACT

(-)-Cubebin (CUB) is a dibenzylbutyrolactolic lignan isolated from dry seeds of *Piper cubeba*. This lignan has activity against amastigotes of *Trypanosoma cruzi*, besides other activities, such as: bacteriostatic, analgesic, anti-inflammatory and antioxidant. It becomes relevant to assess its genotoxic potential and influence on the chromosomal damage induced by doxorubicin (DXR; 2 μ M) in lung fibroblasts of Chinese hamster (cells V79); and by urethane (URE; 10 mM) in somatic cells of *Drosophila melanogaster*. For this, the frequency of micronuclei (MN) in 6000 binucleated cells/treatment was used as a parameter of analysis. The nuclear division index, obtained from the analysis of 3000 cells/treatment, was used to evaluate cytotoxicity. The chosen concentrations were 2, 4, 8 and 16 μ M. No significant difference in the induction of MN was observed between the groups treated with the different concentrations of CUB and the negative control ($P>0.05$). These findings indicate the absence of a genotoxic effect of CUB at the concentrations tested. The results also showed that CUB significantly reduced the frequency of MN induced by DXR, with a mean reduction of 63.88%. In a previous study, our research group demonstrated the absence of mutagenic effects of CUB through the wing Somatic Mutation and Recombination Test (SMART) in *Drosophila*. In the present study, we used the standard and high bioactivation versions of the SMART to estimate the antigenotoxic effects of CUB (0.25; 0.5; 1.0; 2.0 or 4.0 mM) associated to URE (10 mM). The data obtained showed that in the MH individuals from the ST cross, significant inhibitory effect was found only at concentrations of 1.0 and 2.0 mM, while in HB cross all concentrations of CUB statistically inhibit URE-induced DNA damage. At lower concentrations, the recombination level decreases, but at highest concentration the recombination level increases. Our data, and previous studies, suggest that depending on the concentration, CUB may act as a free radical scavenger at low concentrations; a pro-oxidant at higher concentrations, when it interacts with the enzymatic system that catalyzes the metabolic detoxification of DXR or URE; and/or an inducer of recombinational DNA repair. These studies should collaborate with the better understanding of mechanisms of action of CUB and, consequently, to the production of effective and safe drugs with low toxicity.

Keywords: Doxorubicin; *Drosophila melanogaster*; Micronucleus; Somatic Mutation And Recombination Test; Urethane; V79 cells.

Introduction

Plants have been used throughout history as the primary source of food, fuel, and medicine [van der Kooy et al., 2009]. In the area of phytochemistry, medicinal plants are being biochemically characterized due to their possible bioactive compounds, which can be identified, isolated and subjected to detailed structural characterization [Petronilho et al., 2012]. Based in traditional use and scientific researches, natural products have been playing an important role in the drug discovery, providing novel structures that can be used as potential drugs [Newman, Cragg, 2012]. Many compounds isolated from plants have shown a promising effect in therapeutics. Among the plants investigated to date, those of the Family Piperaceae show great therapeutic potential [Reshmi et al., 2010].

The genus *Piper* has over 700 species distributed in both hemispheres, and some species are used in Ayurvedic medicine because of its medicinal properties [Prasad et al., 2005]. Phytochemical studies of extracts of Piperaceae have shown the presence of several bioactive molecules such as amides, benzoic acids, chromenes, phenylpropanoids, terpenes, alkaloids and lignans [Vanin et al., 2008]. *Piper cubeba* Linn. (Piperales, Piperaceae), popularly known as *pimenta de Java* (in Brazil), *kemukus* (in Indonesia), cubeb or tailed pepper, is worldwide distributed in tropical and subtropical regions [De Rezende et al., 2011]. Phytochemical studies of extracts of *P. cubeba* have shown the presence of terpenes, alkaloids and lignans [Elfahmi et al., 2007]. In comparison to other species of this genus, *P. cubeba* has received less attention so far, but the essential oil and lignans have been more intensively investigated since *P. cubeba* accumulates both groups of compounds in relatively high amounts [Elfahmi et al., 2007].

Recent studies with *P. cubeba* have been conducted in order to investigate the biological activities of crude extracts [Silva et al., 2007; Pandey et al., 2009], essential oils [Magalhães et al., 2012], as well as isolated compounds such as (-)-cubebin (CUB), a dibenzylbutyrolactolic lignan [Silva et al., 2005] isolated from the dry seeds of *P. cubeba*, known to possess a broad range of biological activities [Bastos et al., 2001; Silva et al., 2005; Silva et al., 2007; Silva et al., 2009; Aboul-Enein et al., 2011; De Rezende et al., 2011; Maistro et al., 2011]; and its semi-synthetic derivative (-)-hinokinin (HK) (Medola et al., 2007; Silva et al., 2007;

Resende et al., 2010). The interest in lignans, such as CUB and HK, has grown due to such biological effects [Saleem et al., 2005].

Recently, our research group demonstrated the absence of mutagenic effects of CUB and its protective effects against Doxorubicin-induced mutation assayed through the wing Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster* [De Rezende et al., 2011]. The absence of mutagenicity and the antimutagenic potentials of HK were also evaluated in micronucleated polychromatic erythrocytes (MNPCE) in peripheral blood of Wistar rats [Medola et al., 2007], and in *in vitro* micronucleus test (MNT) using Chinese hamster lung fibroblast V79 cells [Resende et al., 2010].

Micronuclei (MN) are derived from chromosomal fragments and whole chromosomes lagging behind in anaphase. So, the MNT can be used to show both clastogenic and aneugenic effects [Fenech, 2007].

The Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster* is a short-term *in vivo* assay for the detection of mutagenic and recombinagenic activity of chemicals [Graf et al., 1984; Graf et al., 1989; Graf, van Schaick, 1992]. The test measures loss of heterozygosity (LOH) induction, which may occur through point mutation, chromosomal aberrations, and mitotic recombination in two sections of chromosome 3 of *D. melanogaster*. The SMART has been successfully used to assess the mutagenic/antimutagenic effects of isolated compounds [De Rezende et al., 2009; Vázquez-Gómez et al., 2010; De Rezende et al., 2011; Sotibran et al., 2011].

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method is considered as one of the standard and easy colorimetric methods for assessment of free radical scavenging potential of an antioxidant molecule. DPPH is a stable radical in solution and appears purple color absorbing at 515-517 nm in methanol. Accepting a hydrogen (H) atom from the scavenger molecule i.e. antioxidant, resulting into reduction of DPPH[•] to DPPH₂, the purple color changes to yellow [Mishra, 2012].

In the present study, the *in vitro* MNT in binucleated Chinese hamster lung fibroblast cells (V79) was used as the test system to assess the mutagenic and the anti-mutagenic potentials of CUB against DXR-induced mutagenicity. We also investigate the protective effects of CUB against Urethane-induced mutation and recombination through SMART.

Materials and Methods

(-)-Cubebin

The CUB used in this study was kindly provided by Márcio Luis Andrade e Silva PhD of the Research Group on Natural Products, University of Franca (UNIFRAN, São Paulo, Brazil). Additional informations about CUB isolation and purification can be found in Silva et al. [2005].

Cells and culture conditions

Chinese hamster lung fibroblasts (V79) cells were kindly supplied by Ilce Mara de Syllos Cólus PhD (Universidade Estadual de Londrina, Paraná, Brazil). Cells were maintained as monolayer in plastic culture flasks (25 cm²) in HAM-F10 (Sigma-Aldrich) and DMEM (Sigma-Aldrich) (1:1) medium supplemented with 10% fetal bovine serum (Nutricell), antibiotics (0.01 mg/mL streptomycin, CAS:3810-74-0, and 0.005 mg/mL penicillin, CAS:113-98-4; Sigma-Aldrich), and 2.38 mg/mL Hepes (CAS:7365-45-8; Sigma-Aldrich), at 37°C in a BOD-type chamber (Model: 347CD, FANEM Ltda., São Paulo, Brazil). The average cell cycle time was 12 h under these conditions. The micronucleus assay was performed using cells grown in 5.0 mL culture medium.

Maintenance conditions of *Drosophila melanogaster* strains

Originally, the *D. melanogaster* stocks were kindly donated by Dr. Ulrich Graf of the Physiology and Animal Husbandry, Institute of Animal Sciences (University of Zurich, Schwerzenbach, Switzerland). Three strains of *D. melanogaster*: 1) the *multiple wing hairs*: *y; mwh j*; 2) the *flare-3*: *flr³/In(3LR)TM3, ri p^psep l(3)89Aa bx^{34e} e Bd^S*; and 3) the *ORR*: *flare-3: ORR; flr³/In(3LR)TM3, ri p^psep l(3)89Aa bx^{34e} e Bd^S*, were maintained in glass vials filled with a maintenance medium (banana, sucrose, yeast and methylparaben) under light/dark cycle (12:12), 25 ± 1°C and approximately 60% humidity in a BOD-type chamber (Model: SL224, SOLAB– Equipamentos para Laboratórios Ltda., São Paulo, Brazil).

Mutagenicity Tests

Cytokinesis-block micronucleus test in V79 cells

In order to determine the concentrations of CUB, the clonogenic efficiency assay was performed. The concentrations tested ranged from 2 to 2048 μM and the chosen concentrations were 2.0; 4.0; 8.0 or 16.0 μM , using cytotoxicity as a criterion [Franken et al., 2006].

For the experiments, 10^6 cells were seeded into tissue-culture flasks, incubated for two cycles (24 h) in complete HAM-F10/DMEM medium, washed with phosphate-buffered saline (PBS, pH 7.4), and then treated with each concentration of (-)-cubebin alone or in combination with the mutagen DXR (0.5 $\mu\text{g/mL}$) for 3 h in serum-free medium. At the end of this period, the cells were washed twice with PBS, fresh serum-supplemented medium containing 3 $\mu\text{g/mL}$ cytochalasin-B (CAS: 14930-96-2; Sigma-Aldrich) was added, and the cells were incubated for an additional 17 h. At harvest time, the cells were rinsed twice with 5 mL PBS, trypsinized using 0.025% trypsin-EDTA and centrifuged for 5 min at 900 rpm. The pellet was hypotonized in 1% sodium citrate plus one drop of 1% formaldehyde and then carefully homogenized with a Pasteur pipette. This cell suspension was centrifuged under the same conditions. The pellet was resuspended in methanol:acetic acid (3:1) and again homogenized with a Pasteur pipette. Fixed cells were then transferred to slides and stained with 5% Giemsa.

A total of 6,000 binucleated cells were scored per treatment, thus 2000/treatment/repetition. The criteria employed for the analysis of micronucleus and binucleated cells were established by Fenech [2000].

The nuclear division index (NDI) was determined for 3000 cells analyzed per treatment, for a total of 1000 cells per repetition. Cells with well-preserved cytoplasm containing 1-4 nuclei were scored. The NDI was calculated according to Eastmond and Tucker (1989) using the following formula:

$$\text{NDI} = \frac{[M1 + 2(M2) + 3(M3) + 4(M4)]}{N}$$

where M1-M4 are the number of cells with 1, 2, 3 and 4 nuclei, respectively, and N is the total number of viable cells.

DNA damage was induced using the chemotherapeutic agent doxorubicin (DXR; CAS 23214-92-8, Pharmacia Brasil Ltda., Brazil) dissolved in distilled water immediately before treatment. The final concentration in the MNT was 0.5 µg/mL. This dose was determined in pilot experiments. Positive (DXR) and negative controls were also included in the analysis. All assays described here were repeated independently three times to ensure reproducibility.

Calculation of the percent reduction in DNA damage

The percent reduction in DXR-induced damage by CUB was calculated as the number of cells with damage observed in the treatment with the DNA damage-inducing agent (DXR) minus the number of cells with damage observed in the antigenotoxicity treatment x 100, divided by the number of cells with damage observed in the treatment with the DNA damage-inducing agent minus the number of cells with damage in the control [Waters et al., 1990].

Statistical analysis

The results were evaluated by analysis of variance (ANOVA) and the Tukey test at $P < 0.05$, with the experimental criterion being the significance of the response in relation to the negative control in the genotoxicity assay, and in relation to the positive control when the antigenotoxicity of (-)-cubebin was determined based on its capacity to reduce DNA damage induced by DXR.

Somatic Mutation and Recombination Test - SMART

Crosses

The absence of mutagenic effects of CUB was previously observed by our research group using the *Drosophila* wing SMART [De Rezende et al., 2011].

Based on these data, the antigenotoxic effects of (-)-cubebin on urethane-induced somatic mutations in *D. melanogaster* were also assessed by SMART. Two crosses were carried out to produce the experimental larval progeny: 1) Standard (ST) cross (virgin females of strain *flr³/In(3LR)TM3, ri p^o sep l(3)89Aa bx^{34e} e Bd^S* crossed with *mwh/mwh* males) [Graf et al., 1984; Graf et al., 1989]; and 2) High bioactivation (HB) cross (virgin females of strain *ORR/ORR; flr³/In(3LR)TM3, ri p^o sep l(3)89Aa bx^{34e} e Bd^S* crossed with *mwh/mwh* males) [Graf,

van Schaik, 1992]. The latter cross is highly sensitive to promutagens and procarcinogens because of the increased level of cytochrome P450 present in the *ORR/ORR; flr³* strain [Hällström, Blanck, 1985] and [Saner et al., 1996]. Both crosses produce two types of progeny, which can be distinguished phenotypically by the *Bd^S* marker: (i) marker-heterozygous (MH) flies (*mwh +/+ flr³*), with phenotypically wild-type wings; and (ii) balancer-heterozygous (BH) flies (*mwh/TM3, Bd^S*), with phenotypically serrate wings. Additional information on these and related strains can be found elsewhere [Dapkus, Merrel, 1977; Hällström, Blanck, 1985; Graf et al., 1989; Graf and van Schaik, 1992; Saner et al., 1996].

Experimental procedures

Eggs, from both crosses, were collected for 8 h in culture glass bottles with an agar-agar base (4% w/v) topped with a thick layer of live baker's yeast supplemented with sucrose. After 72 h ($\pm$ 4 h), third instar larvae were washed out of the bottles with ultrapure water (MilliQ system) and collected in a stainless steel strainer. For chronic feeding, four sets of vials, of each cross, were prepared with 1.5 g of mashed potato flakes (Yoki[®] Alimentos S.A., Brazil) and 5 ml of a solution containing a final concentration of 0.25; 0.5; 1.0; 2.0 or 4.0 mM of (-)-cubebin in association with urethane (URE - CAS: 51-79-6; Fluka AG, Switzerland) 10mM. (-)-Cubebin was dissolved in a mixture of 1% Tween 80 (Fluka, Buchs, Switzerland) and 3% ethanol (Neon, São Paulo, Brazil) in ultrapure water. To calculate the survival rates upon exposure, the larvae were counted before the distribution in two series of those vials, and in other two sets of vials, the hatched flies were stored in 70% (v/v) ethanol. Negative (1% Tween-80 and 3% ethanol in distilled water) and URE as positive control, were included in both experiments. The experiments were conducted at $25 \pm 1^{\circ}\text{C}$ and approximately 60% humidity.

The wings were removed and mounted on slides with Faure's solution (gum arabic 30 g, glycerol 20 ml, chloral hydrate 50 g and distilled water 50 ml) and analyzed under a compound microscope at 40x magnification. Two types of spots can be observed on the wings of MH flies: (i) Single spots (*mwh* or *flr³*) resulting from deletions, point mutations, specific chromosome aberrations, or from recombination between the two marker genes; (ii) Twin spots (*mwh* and *flr³*) produced by mitotic

recombination between the proximal marker *flr*³ and the centromere of chromosome 3. On BH flies only *mwh* single spots can be recovered. They should reflect somatic point mutation and chromosome aberration, because recombinational events are suppressed in inversion–heterozygous cells with the multiply inverted *TM3* balancer chromosome. The wings of BH flies were mounted and analysed whenever a positive response was obtained in the MH progeny. A comparison of the results obtained from MH and BH flies was used to quantify the recombinagenic potential of the tested substance and also the protective effect on urethane-induced somatic mutations [Graf et al., 1984; Abraham, 1994; Guzmán-Rincón, Graf, 1995].

Data evaluation and statistical analysis

The wings of 40 flies from each treated series were scored, including controls. For data assessment, the multiple-decision procedure [Frei, Würgler, 1988; Frei, Würgler, 1995] was used, resulting in three different diagnoses: negative, positive or inconclusive. The frequency of each type of spot (small single, large single or twin) and the total frequency of spots per fly for each treatment were compared pair-wise (i.e. positive control (URE) alone versus URE plus (-)-cubebin) according to Kastenbaum and Bowman (1970) with $p = 0.05$ [Frei, Würgler, 1988; Frei, Würgler, 1995]. All inconclusive and weak results were analyzed with the non-parametric U-test of Mann, Whitney and Wilcoxon ($\alpha = \beta = 0.05$, one sided) in order to exclude false positives [Frei, Würgler, 1995]. To determine the recombinagenic activity, the frequency of *mwh* clones on the MH wings (*mwh* single spots plus *mwh* clones of twin spots) was compared with the frequency of *mwh* clones on the balancer-heterozygous wings. Taking into account that the *TM3* chromosome inhibits homologous recombination, the difference in *mwh* clone frequency being a direct measure of the proportion of mitotic recombination [Frei et al., 1992]. Statistical comparisons of survival rates were made with the Chi-squared test for ratios of independent samples.

DPPH Radical Scavenging Capacity Assay

The antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (CAS: 1898-66-4, Sigma–Aldrich Brasil Ltda., Brazil) method. The radical scavenging assay was adapted from Blois (1958). As positive control, butylated-hydroxyl-toluene (BHT) (CAS: 128-38-0, Sigma–Aldrich Brasil

Ltda., Brazil) was used at final concentration of 4.5mM. The DPPH methanolic solution was freshly prepared at final concentration of 0.1 mM. Samples containing different final concentrations of (-)-cubebin (4.0; 2.0; 1.0; 0.5; 0.25 or 0.125 mM) were also prepared using methanol. For antioxidant evaluation, 950 µl of DPPH solution was mixed with 50 µl of each sample of (-)-cubebin or BHT. As negative control was used 50 µl of methanol added to the DPPH solution. The mixtures were incubated for 20 min in the dark at 30°C. CUB was allowed to react with the stable radical (DPPH) in order to evaluate its free radical scavenging capacity. Then, 300 µl of each sample was transferred to a 96 well microplate (in duplicate). The decrease in absorbance (A) was monitored at 517 nm using a microplate reader (Versamax, Molecular Devices, Sunnyvale, CA). DPPH[•] radical scavenging capacity was calculated using the following equation:

$$\text{DPPH}^{\bullet} \text{ scavenging effects (\%)} = [(A_0 - A_1)/A_0 \times 100]$$

Where A_0 and A_1 correspond to the absorbances at 517 nm of the radical DPPH[•] in the absence and presence of antioxidant respectively [Mishra et al., 2012].

Results

MNT in V79 cells

According to the clonogenic efficiency assay, concentrations up to 16.0 μM of CUB were cytotoxic.

The frequencies of MN in V79 cells treated with different concentrations (2.0; 4.0; 8.0 or 16.0 μM) of CUB alone or in combination with DXR (0.5 $\mu\text{g/mL}$) are shown in **Table 1** and **Figure 1**. No significant differences in the induction of MN was observed between the groups treated with the different concentrations of CUB and the negative control ($P>0.05$). These findings indicate the absence of genotoxic effects of CUB at the concentrations tested.

The results also showed that CUB significantly reduced (63.88%) the frequency of MN induced by DXR at the concentration of 2 μM . Although concentrations up to 2.0 μM did not significantly reduce the frequency of MN induced by DXR the rate of observed reduction range from 23.68 to 25.78% (**Table 1**).

Table 1 also shows the means Nuclear Division Index (NDI) and the respective standard deviation (SD) obtained for all treated groups. No significant differences were observed in relation to the total number of MN induced in any treatment group when compared to the negative control, demonstrating the absence of cytotoxicity of the different treatments under the present experimental conditions.

Somatic Mutation and Recombination Test – SMART

The concentrations of CUB were chosen, based on previous studies [De Rezende et al., 2011]. The survival rates upon chronic treatment of CUB in combination with URE (10 mM) are given in **Table 2**. In the ST cross, the two highest concentrations (2.0 and 4.0 mM) of CUB associated with URE were toxic, showing significant ($P<0.05$) decrease of survival rates of flies when compared to the negative control group (1% Tween-80 and 3% ethanol in distilled water). Otherwise, in the HB cross, only the highest concentration (4.0 mM) of CUB in combination with URE (10 mM) has shown to be toxic, significantly decreasing ($P<0.05$) the survival rates when compared to the negative control group.

The anti-mutagenic and anti-recombinogenic potentials of CUB when associated with URE, were assayed using two crosses (ST and HB) of the wing Somatic Mutation and Recombination Test (SMART) in *D. melanogaster* (**Tables 3 and 4**). As expected, the reference mutagen (URE) induced positive results for all categories of spots, in both crosses (ST and HB), when compared to the negative control.

The results of the ST cross of the SMART assay are summarized in **Table 3**. In the MH individuals from the ST cross, although all concentrations of CUB were able to decrease the total of mutant spots, significant inhibitory effect was found only at concentrations of 1.0 and 2.0 mM (inhibition of 48.89% and 39.71% respectively).

In order to quantify the contribution of mutagenic and recombinogenic events to the total spots observed in MH flies, the wings of the BH flies treated with 1.0 or 2.0 mM of URE were also mounted and scored. In the BH individuals of the ST cross, URE (10 mM) significantly increased the mutant spot frequency compared to the negative control. CUB (1.0 or 2.0 mM) significantly reduced the total number of spots induced by URE. We verified that the induction of spots by URE was mainly due to somatic point mutation, chromosome aberration or non-disjunction than recombination, since only 9.72% was due to recombination. Nevertheless, when URE was associated with CUB (1.0 or 2.0 mM), the recombinogenicity level increases, respectively, to 15.21% and 43.13% (**Table 3**).

The results of the HB cross of the SMART assay are depicted in **Table 4**. URE statistically increased all categories of spots when compared to the negative control. The results obtained with the MH individuals of the HB cross treated with (-)-cubebin associated with URE, showed that all concentrations of CUB were found to statistically inhibit ($P < 0.05$) URE-induced DNA damage. Analysing the BH individuals, depending on the concentration of CUB, the pattern of spots is modified. At first, 32.46% of the total URE-induced mutant spots are due to recombination. Once associated to CUB, at lower concentrations 0.25, 0.5 or 1.0 mM, the recombination level decreases to 25.46; 24.10 and 15.11% respectively, but at highest concentration (4.0 mM), the recombination level increases to 63.00%.

DPPH Radical Scavenging Assay

In the present study, the DPPH free radical scavenging potential of different concentrations of CUB was measured and the results of our investigations are shown in **Figure 2**. Antioxidant activity across the five CUB concentrations (0.125; 0.25; 0.5; 1.0; 2.0 or 4.0 mM) ranged from 10.2% to 80.01%. The strongest activity is related to the highest concentration.

Discussion

Preparations of medicinal plants are widely used in human therapy. Some plants synthesize toxic chemicals, apparently as a primary defense mechanism (Marques et al., 2012). Due to the biological activities of these compounds and the increasing use of traditional herbal medicines around the world, evaluation of its genotoxic/mutagenic potential is essential [Newman et al., 2003; Ouedraogo et al., 2012].

Lignans are a class of secondary plant metabolites. The growing interest in lignans and their synthetic derivatives is due to applications in cancer chemotherapy and various other pharmacological effects [Saleem et al., 2005]. Here, we examined the genotoxicity and antigenotoxicity potentials of the CUB, a dibenzylbutyrolactolic lignan extracted from dry seeds of *P. cubeba* L., when administered alone or simultaneously with the pharmacological agent DXR, using the *in vitro* MNT as a biological end point, and the antimutagenicity/antirecombinagenicity of CUB associated with URE, using the SMART in *Drosophila*.

The *in vitro* MNT is currently considered appropriate for detection of both aneugens (spindle poisons) and clastogens (chromosomal damage) when used together with other genotoxicity tests in a standard battery. Shows simplicity of scoring, is widely applicable in different cell types, is internationally validated, has potential for automation and is predictive for cancer. The cytokinesis-block micronucleus assay (CBMN) allows assessment of nucleoplasmic bridges, nuclear buds, cell division inhibition, necrosis and apoptosis and in combination with FISH using centromeric probes, the mechanistic origin of the MN. Therefore, the CBMN test can be considered as a “cytome” assay covering chromosome instability, mitotic dysfunction, cell proliferation and cell death [Kirsch-Volders et al., 2011].

DXR, used as positive control, is a chemotherapeutic agent that has several modes of action, such as inducing DNA single- and double-stranded breaks (Poplawski et al., 2010), inhibiting DNA topoisomerase II (Barcelos et al., 2009; Islaih et al., 2005) and DNA or RNA synthesis in mammalian cells in culture, besides that can also be metabolically activated via free radical mechanism, increasing the oxygen free radicals generation (Navarro et al., 2006). According to

our results, CUB alone was not genotoxic at the concentrations tested and only the highest concentration of CUB was found to be effective in preventing clastogenic damage caused by DXR, with 63.88% reduction. The results obtained are similar to previous study (Resende et al., 2010) that evaluated the genotoxicity and the protective effects of (-)-hinokinin (HK) on methyl-methanesulfonate (MMS) and DXR- induced MN on V79 cells. HK is a semi-synthetic derivative of CUB (Silva et al., 2005) that, at lower concentrations, exerts protective effects on DXR-induced MN. In the other hand, at higher concentrations, HK acted as an enhancer of DXR-induced clastogenicity, probably increasing the oxidative stress generated by DXR (Resende et al., 2010).

Similar results were also obtained by our research group. We evaluated the interference of CUB on the frequency of mutant spots induced by DXR using the wing Somatic Mutation and Recombination Test. This *in vivo* bioassay is very sensitive and can determine genotoxicity of chemical agents by loss of heterozygosity of the cell markers. This loss of heterozygosity produces single spots (small and large) by point mutation, deletion or non-disjunction and twin spots, produced exclusively by mitotic recombination (Graf et al., 1984).

In the *Drosophila* SMART assay, DXR-induced wing spots are mainly due to recombination (Lehman et al., 2000; Rezende et al., 2009; De Rezende et al., 2011). CUB significantly alters the generation of DXR-induced wing spots, but CUB does not interfered on DXR recombinogenicity (De Rezende et al., 2011).

In the present study, the influence of CUB on URE-induced spots was also evaluated. We used URE as reference mutagen based on its genotoxic potential in *Drosophila*, with a clear dependence on metabolic activation (Frölich and Würigler, 1990). The frequencies of all types of spots determined with the HB cross are clearly higher than the corresponding frequencies in the ST cross. The metabolic pathway most probably involves cytochrome P-450-dependent enzyme activities (Frölich and Würigler, 1990; Silva et al., 2008), so it explains the drastic increase in the frequency of total mutant spots on individuals from the HB cross (frequency of total spots = 7.70) compared with those from ST cross (frequency of total spots = 1.80). The HB cross is highly sensitive to procarcinogens and promutagens because the strain *ORR; flr³/ln(3LR)TM3, ri ppsep 1(3) 89Aa bx34ee Bd^S* carries chromosomes 1 and 2 from a DDT-resistant Oregon R(R) line, which is

characterized by high levels of CYPs (Graf and Van Schaik, 1992). The statistically significant induction of twin spots indicates that URE is capable to induce recombination in both crosses. Nevertheless, the main mode of action of URE is due to mutational events than mitotic recombination. In the ST cross the contribution of recombinogenicity to the total of mutant spots was 9.72% (Table 3) and in the HB cross is 32.46% (Table 4).

In both crosses, depending on the concentration, CUB associated with URE changes the mutational and recombinational frequencies, depicting a modulatory effect on URE-induced spots. Interestingly, in the HB cross, CUB in association with URE is anti-recombinogenic at lower concentrations (0.25; 0.5 or 1.0 mM), but at higher concentrations (2.0 or 4.0 mM) significantly increases the frequency of recombination (Table 4 and Figure 2).

A chemical substance with protective potential may be effective by means of: 1) directly interacting with the clastogenic agent; 2) affecting its metabolism; 3) reducing its direct interaction with DNA; 4) increasing or decreasing cytotoxicity; 5) increasing the repair capacity of the cells [Berni et al., 2012]. Given the significant increase in recombinogenic contribution observed to the total of spots, the reduction of total spots associated with the decreased survival rates at higher concentrations (both crosses), lead us to hypothesize that these effects can be related to DNA repair (recombinational DNA repair) instead of protective effect of CUB. Similar results were also found by our research group, when CUB associated with DXR was assessed by SMART [De Rezende et al., 2011]. According to Adams et al. (2003) and LaRocque et al. (2007), homologous recombination (HR), plays an important role in repair of DNA damage in *Drosophila* and is the predominant repair pathway that occurs during S and G2 phases in mammalian systems [Liang et al., 1998; Johnson and Jasin, 2001; Rothkamm et al., 2003], however there is a clear correlation between HR and loss of heterozygosity or genetic rearrangements, leading to cancer and other chronic diseases [Bishop and Schiestl, 2002, 2003].

Many epidemiological and experimental studies have shown the effect of diet on health; thus, the relation between the consumption of certain foods and a reduced risk of some chronic diseases, including cancer, is becoming obvious [Ramos, 2007].

In fact, most of the chemicals which possess a protective effect are antioxidants, therefore their effects must be related to the number of induced primary DNA lesions [Berni et al., 2012]. In the past few years, a number of reports have focused on polyphenols and their health-related properties [Ramos, 2007].

Lignans, as CUB, are a naturally occurring plant polyphenols and due to their chemical characteristics own important biological properties such as antioxidant [Landete, 2012]. In order to better understand the mechanisms underlying the modulatory effect of CUB, the antioxidant capacity of CUB was confirmed by its capacity to reduce the DPPH radical. The DPPH in methanol produces a deep purple coloured solution with an absorption peak at 517 nm, which disappears with the presence of the radical scavenger [Deng et al., 2011]. The use of this method provides an easy and rapid way to evaluate antioxidants [Abdel-Razeck et al., 2011].

The radical scavenging potential of CUB at concentration of 5 mM was already assessed by DPPH method and the results were slightly different. The solvent used was DMSO instead of ethanol and the antioxidant capacity of CUB was measured by electron paramagnetic resonance. According to previous studies, even in a higher concentration, CUB showed lower antioxidant activity [Aboul – Enein et al., 2011]. The influence of the solvent, deprotonation of the phenolic group and time of incubation has also been outlined as important points that can influence the results [Mishra et al., 2012]. Regarding the antioxidant activity of lignans, HK was also evaluated and exerted a significant antioxidant effect on parasite mitochondria [Medola et al., 2007].

Several natural products commonly used in traditional medicines can potentially influence the bioavailability and pharmacokinetics of a broad range of medicines by inhibiting cytochrome P450 (CYP450) drug metabolism [Mukherjee et al., 2011]. *P. cubeba* extracts, CUB and its derivatives, like HK, were found to significantly inhibit CYP3A4 [Usia et al., 2005; Usia et al., 2006] and the NADH oxidase activity of mitochondrial complex I [Saraiva et al., 2009]. CYP450 inhibition or induction is probably the most common mechanism for the pharmacokinetic interactions of herbs and drugs. When CYP450 enzymes are inhibited, the concentration of drugs in plasma and tissues increases, leading to

toxicity, while induction of these enzymes results in reduced drug concentration [Mukherjee et al., 2011].

In conclusion, the co-treatments were a combination of oxidants and CUB and the reduction of mutation rate observed that, according to Sinigaglia et al. [2006], can be classified as a desmutagenic effect. The antimutagenic activity exerted by CUB is not entirely clear, being attributed to antioxidant activity and suppression of metabolic activation. Taken together, our data suggest that depending on the concentration, CUB may interact with the enzymatic system that catalyzes the metabolic detoxification of URE, inhibiting the activity of mitochondrial complex I, thereby scavenging free radicals, and that CUB may also modulate the metabolic activation of URE, inhibiting the formation of DNA-binding metabolites.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 1. Frequency of micronuclei (MNs) and nuclear division index (NDI) observed in V79 cells treated with different concentrations of (-)-cubebin and/or DXR and respective controls

Treatments (μ M)	Frequency of MNs	NDI	Reduction (%)
	Mean $\pm$ SD	Mean $\pm$ SD	
Negative Control	2.83 $\pm$ 1.16	1.75 $\pm$ 0.04	
DMSO Control	5.33 $\pm$ 2.66	1.74 $\pm$ 0.04	
2.0	4.17 $\pm$ 2.14	1.73 $\pm$ 0.02	
4.0	4.83 $\pm$ 0.75	1.71 $\pm$ 0.04	
8.0	7.33 $\pm$ 3.08	1.73 $\pm$ 0.03	
16.0	6.67 $\pm$ 1.63	1.76 $\pm$ 0.04	
DXR	19.00 $\pm$ 7.24 ^a	1.69 $\pm$ 0.08	
DXR + DMSO	19.83 $\pm$ 6.91 ^a	1.78 $\pm$ 0.03	
2.0 + DXR	8.67 $\pm$ 2.34 ^b	1.68 $\pm$ 0.07	63.88
4.0 + DXR	14.83 $\pm$ 2.14 ^a	1.72 $\pm$ 0.08	25.78
8.0 + DXR	15.00 $\pm$ 3.16 ^a	1.65 $\pm$ 0.09	24.73
16.0 + DXR	15.17 $\pm$ 3.25 ^a	1.70 $\pm$ 0.09	23.68

DMSO = Dimethylsulfoxide (12.5 μ L); DXR = Doxorubicin (0.5 μ g/mL).

6.000 binucleated cells were analyzed per treatment group.

^aSignificantly different from control group ($P < 0.05$).

^bSignificantly different from DXR group ($P < 0.05$).

Table 2. Survival rates upon exposure to different concentrations of (-)-cubebin in combination with urethane (URE) relative to control group (1% Tween-80 and 3% ethanol in distilled water) in the wing Somatic Mutation And Recombination Test in *D. melanogaster*

Treatments		Standard cross		High bioactivation cross	
URE	Cubebin	Survival	<i>p</i> -value	Survival	<i>p</i> -value
(mM)	(mM)	(%)		(%)	
0	0	95		95	
10	0	90	>0.05	85	>0.05
10	0.25	90	>0.05	85	>0.05
10	0.5	80	>0.05	80	>0.05
10	1.0	80	>0.05	85	>0.05
10	2.0	60	<0.05	70	>0.05
10	4.0	55	<0.05	50	<0.05

Statistical comparisons of survival rates were made by using Chi-square test for ratios for independent samples.

Table 3. Summary of results obtained with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the standard cross (ST) after chronic treatment of after chronic treatment of larvae with (-)-cubebin and urethane (URE)

Genotypes and treatments		Number of flies	Spots per fly (number of spots) statistical diagnosis ^a				Spots with mwh clone ^c	Frequency of clone formation/10 ⁵ cells per cell division ^d		Recombination (%)	Inhibition ^e (%)
URE (mM)	(-)-cubebin (mM)		Small single spots (1-2 cells) ^b	Large single spots (>2 cells) ^b	Twin spots	Total spots		Observed	Control Corrected		
<i>mwh/flr</i> ³											
0	0	40	0.45 (18)	0.00 (00)	0.00 (00)	0.45 (18)	18	0.97			
10	0	40	1.33 (53) +	0.30 (12) +	0.18 (07) +	1.80 (72) +	72	3.69	2.72	9.72	
10	0.25	40	1.65 (66)	0.10 (04) *	0.00 (00) *	1.75 (70)	70	3.59	2.62		03.68
10	0.50	40	1.63 (65)	0.00 (00) *	0.00 (00) *	1.63 (65)	65	3.33	2.36		13.24
10	1.00	40	1.15 (46)	0.00 (00) *	0.00 (00) *	1.15 (46) *	46	2.36	1.39	15.21	48.89
10	2.00	40	1.28 (51) *	0.00 (00) *	0.00 (00) *	1.28 (51) *	51	2.61	1.64	43.13	39.71
10	4.00	40	1.60 (64)	0.00 (00) *	0.00 (00) *	1.60 (64)	64	3.28	2.31		15.07
<i>mwh/TM3</i>											
0	0	40	0.45 (18)	0.03 (01)	^f	0.48 (19)	19	0.97			
10	0	40	1.60 (64)+	0.30 (01) i		1.63 (65) +	65	3.33	2.36		
10	1.00	40	0.98 (39)*	0.00 (00)		0.98 (39) *	39	1.99	1.02		
10	2.00	40	0.73 (29)*	0.00 (00)		0.73 (29) *	29	1.48	0.51		

Marker-trans-heterozygous flies (*mwh/flr*³) and balancer-heterozygous flies (*mwh/TM3*) were evaluated.

*P ≤ 0.05 vs. URE only.

^aStatistical diagnoses according to Frei and Würzler (1988; 1995). *U*-test, two sided; probability levels: -, negative; +, positive; i, inconclusive; P ≤ 0.05 vs. untreated control;

^bIncluding rare *flr*³ single spots.

^cConsidering *mwh* clones from *mwh* single and twin spots.

^dFrequency of clone formation: clones/flies/48,800 cells (without size correction).

^eCalculated as{(URE alone – URE + (-)-cubebin) /URE} x 100, according to Abraham (1994).

^fBalancer chromosome *TM3* does not carry the *flr*³ mutation and recombination is suppressed, due to the multiply inverted region in these chromosome.

Table 4. Summary of results obtained with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the high bioactivation cross (HB) after chronic treatment of after chronic treatment of larvae with (-)-cubebin and urethane (URE)

Genotypes and treatments		Number of flies	Spots per fly (number of spots) statistical diagnosis ^a				Spots with mwh clone ^c	Frequency of clone formation/10 ⁵ cells per cell division ^d		Recombination (%)	Inhibition ^e (%)
			Small single spots (1-2 cells) ^b	Large single spots (>2 cells) ^b	Twin spots	Total spots		Observed	Control Corrected		
URE (mM)	(-)-cubebin (mM)										
<i>mwh/flr³</i>											
0	0	40	0.58 (23)	0.00 (00)	0.00 (00)	0.58 (23)	23	01.18			
10	0	40	5.65 (226) +	1.85 (74) +	0.20 (08) +	7.70 (308) +	304	15.78	14.60	32.46	
10	0.25	40	5.00 (200)	0.30 (12) *	0.20 (08)	5.50 (220) *	220	11.27	10.09	25.46	28.57
10	0.50	40	4.90 (196)	0.60 (24) *	0.10 (04)	5.60 (224) *	224	11.48	10.30	24.10	27.27
10	1.00	40	4.10 (164)	0.20 (08) *	0.00 (00) *	4.30 (172) *	168	08.81	07.63	15.11	45.45
10	2.00	40	3.83 (153) *	0.00 (00) *	0.18 (07)	4.00 (160) *	160	08.19	07.01	33.12	48.05
10	4.00	40	2.00 (80) *	0.50 (20) *	0.00 (00) *	2.50 (100) *	100	05.12	03.94	63.00	67.53
<i>mwh/TM3</i>											
0	0	40	0.60 (24)	0.05 (02)	†	0.65 (26)	26	01.33			
10	0	40	5.00 (200) +	0.20 (08) i		5.20 (208) +	208	10.66	09.33		
10	0.25	40	4.10 (164) *	0.00 (00)*		4.10 (164) *	164	08.40	07.07		
10	0.50	40	4.25 (170) *	0.00 (00)*		4.25 (170) *	170	08.71	07.38		
10	1.00	40	3.65 (146)	0.00 (00)*		3.65 (146)*	146	07.48	06.15		
10	2.00	40	2.68 (107) *	0.00 (00)*		2.68 (107) *	107	05.50	04.17		
10	4.00	40	0.83 (33) *	0.10 (04)		0.93 (37) *	37	01.89	00.56		

Marker-trans-heterozygous flies (*mwh/flr³*) and balancer-heterozygous flies (*mwh/TM3*) were evaluated.

*P ≤ 0.05 vs. URE only.

^aStatistical diagnoses according to Frei and Würzler (1988; 1995). U-test, two sided; probability levels: -, negative; +, positive; i, inconclusive; P ≤ 0.05 vs. untreated control;

^bIncluding rare *flr³* single spots.

^cConsidering *mwh* clones from *mwh* single and twin spots.

^dFrequency of clone formation: clones/fly/48,800 cells (without size correction).

^eCalculated as {(URE alone – URE + (-)-cubebin) / URE} × 100, according to Abraham (1994).

^fBalancer chromosome *TM3* does not carry the *flr³* mutation and recombination is suppressed, due to the multiply inverted region in these chromosome.

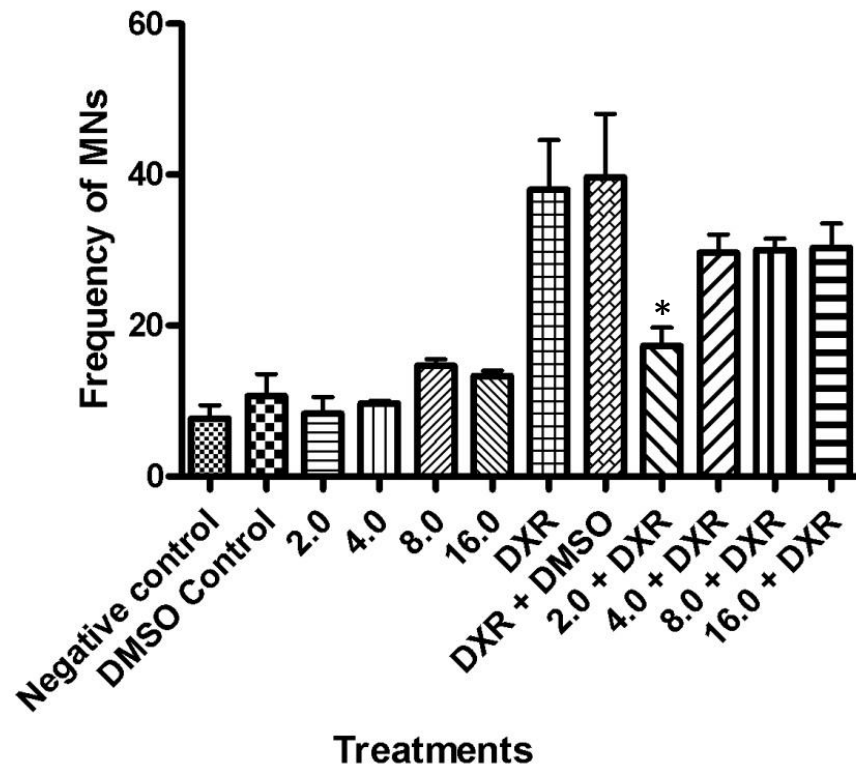


Figure 1. Frequency of micronuclei (MNs/1000 binucleated cells) observed in V79 cells treated with different concentrations of (-)-cubebin and/or DXR, and respective controls.

* Significantly different of DXR ($P < 0.05$).

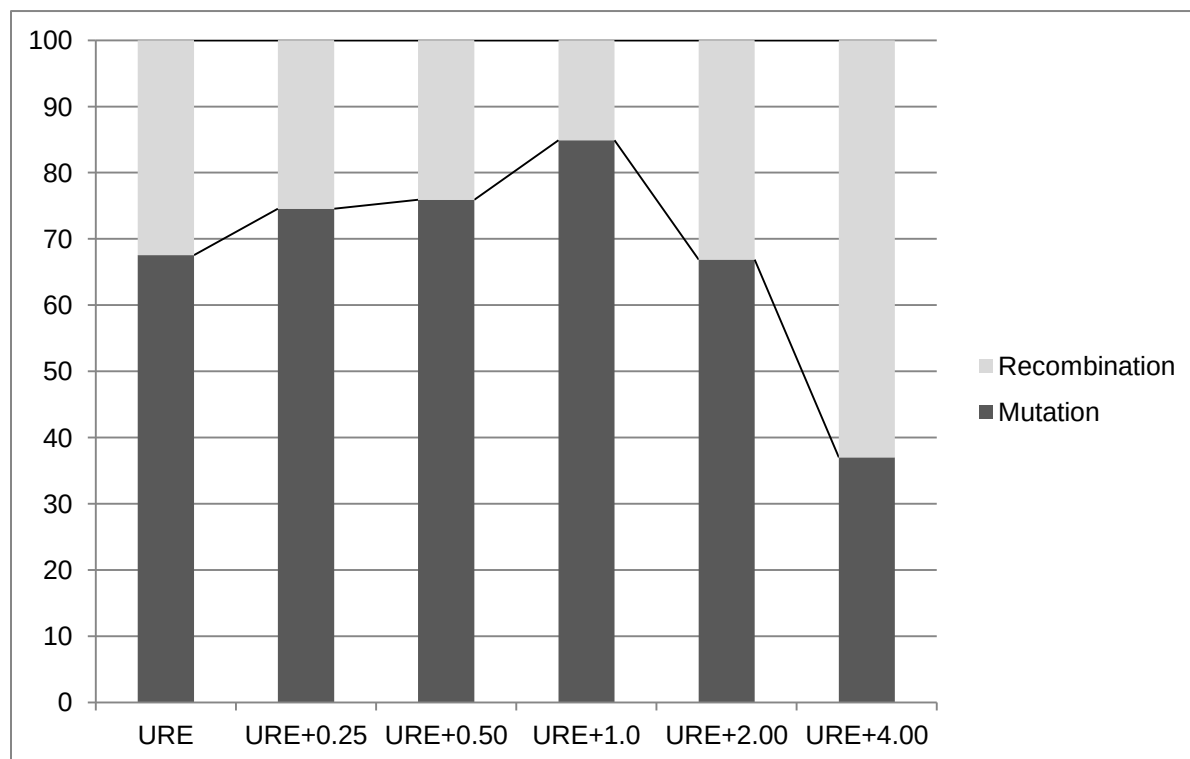


Figure 2. Contribution of mutation and recombination (%) to total mutant spot frequencies observed in MH individuals from the HB cross.

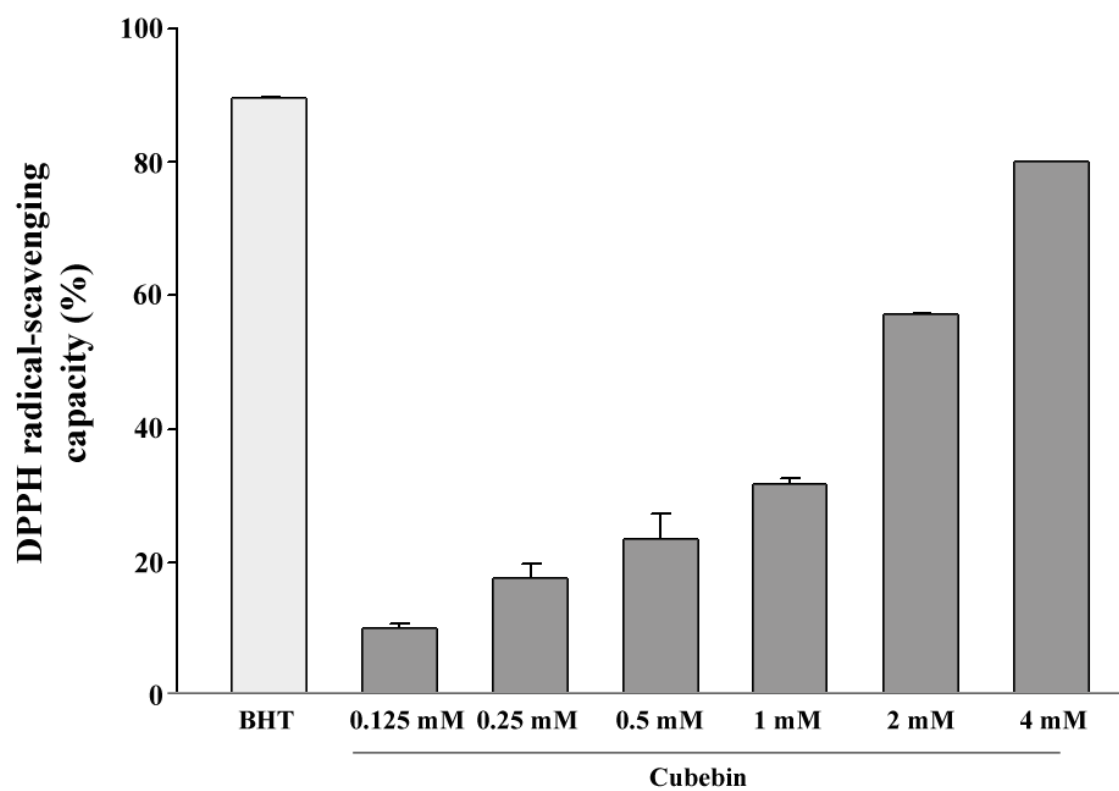


Figure 3. DPPH radical-scavenging capacity (%) of (-)-cubebin. BHT was used as positive control.

ANEXO I

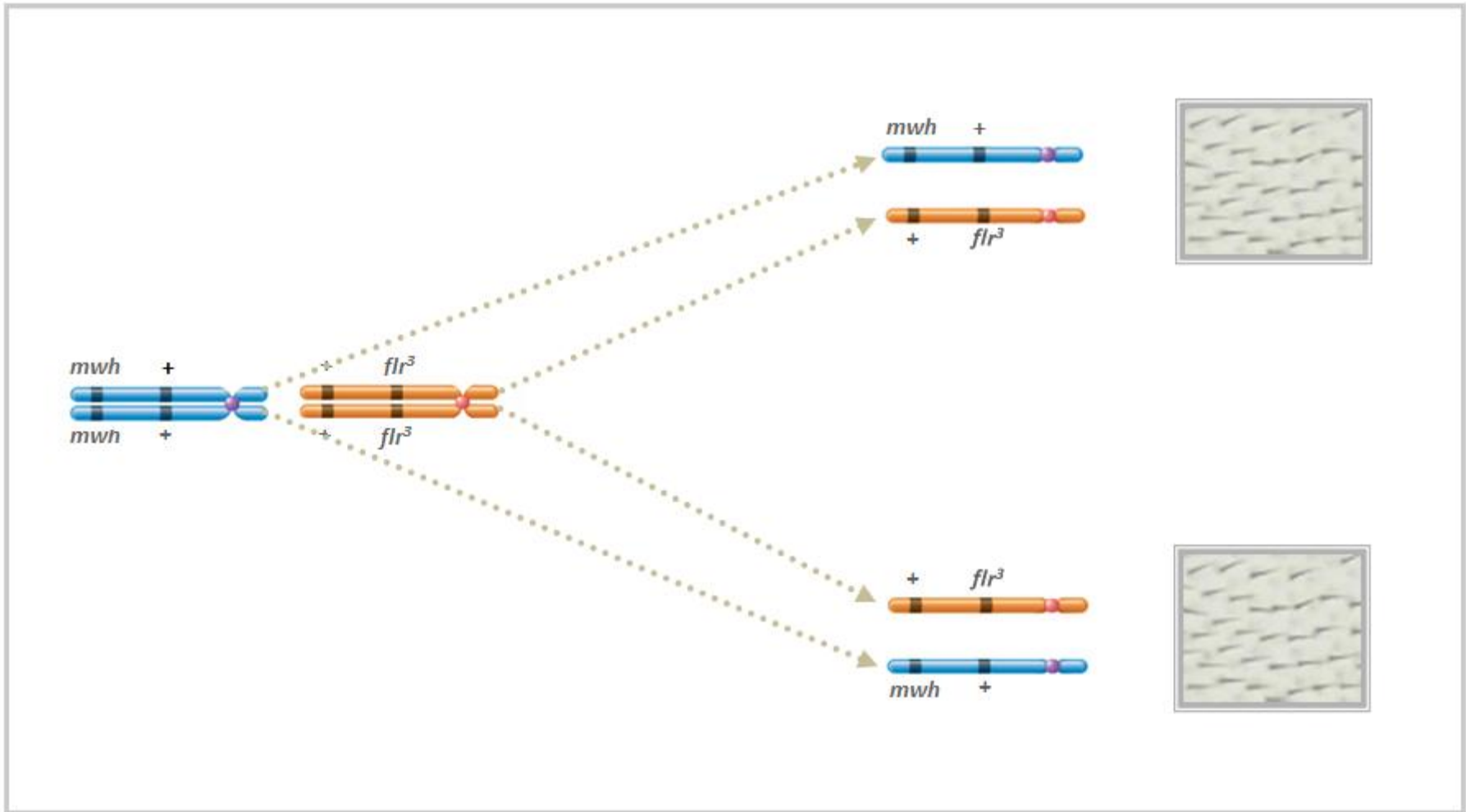


Figura 1. Esquema de divisão mitótica normal em células primordiais dos discos imaginais de asas de *D. melanogaster* (adaptado de Graf, Würigler *et al.*, 1984).

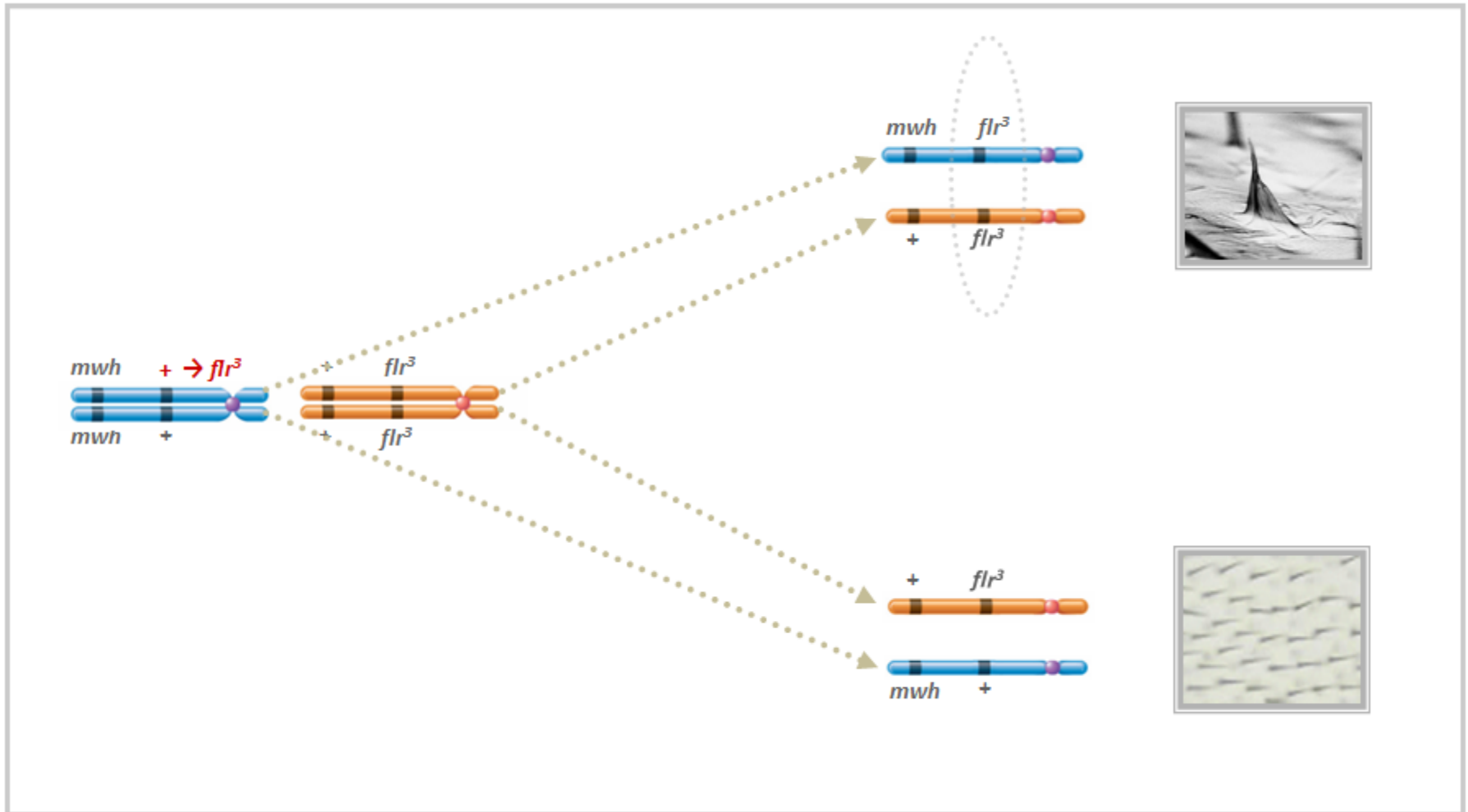


Figura 2. Esquema de divisão mitótica com ocorrência de mutação em células primordiais dos discos imaginais de asas de *D. melanogaster* (adaptado de Graf, Würdler *et al.*, 1984).

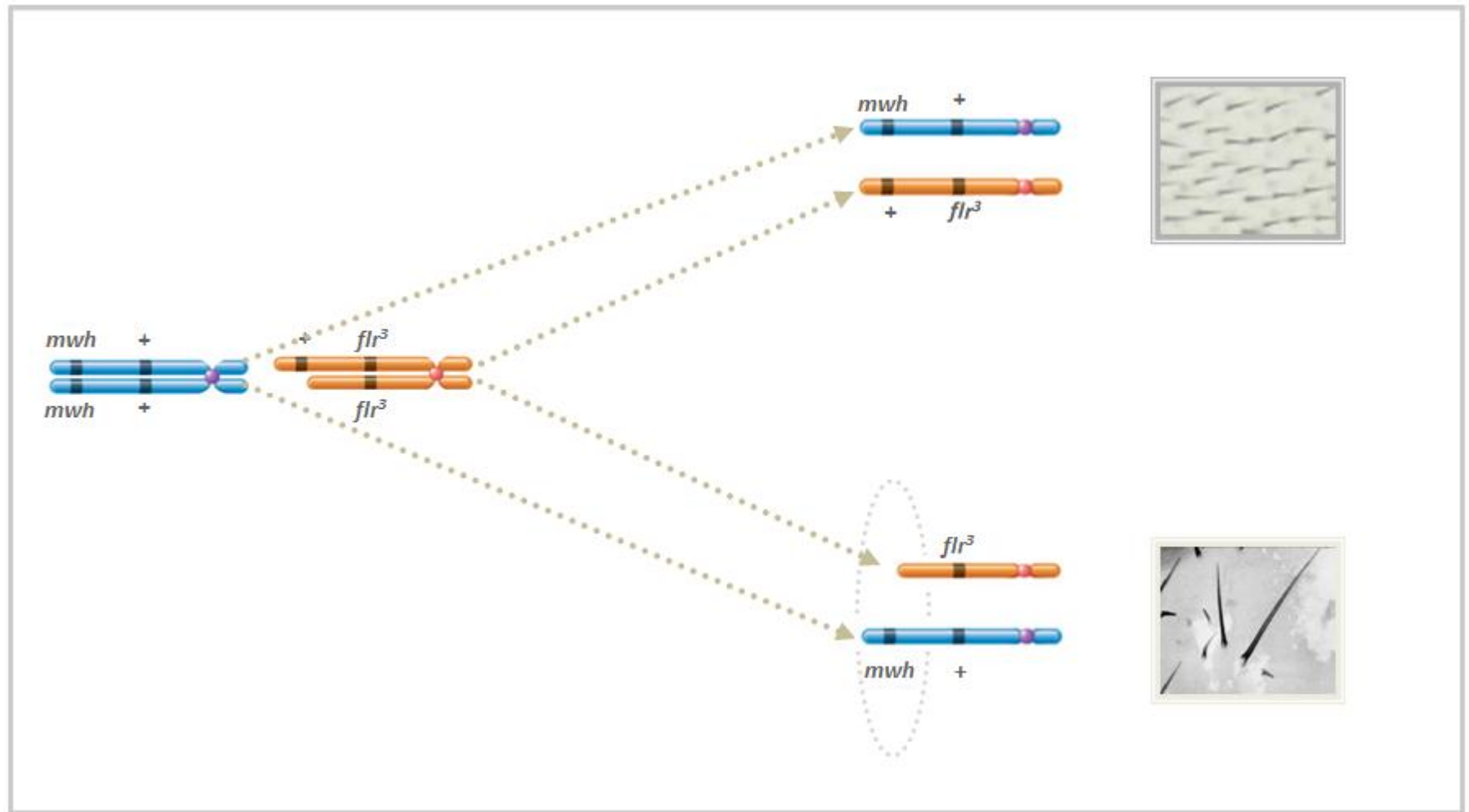


Figura 3. Esquema de divisão mitótica com ocorrência de deleção terminal em células primordiais dos discos imaginais de asas de *D. melanogaster* (adaptado de Graf, Würdler *et al.*, 1984).

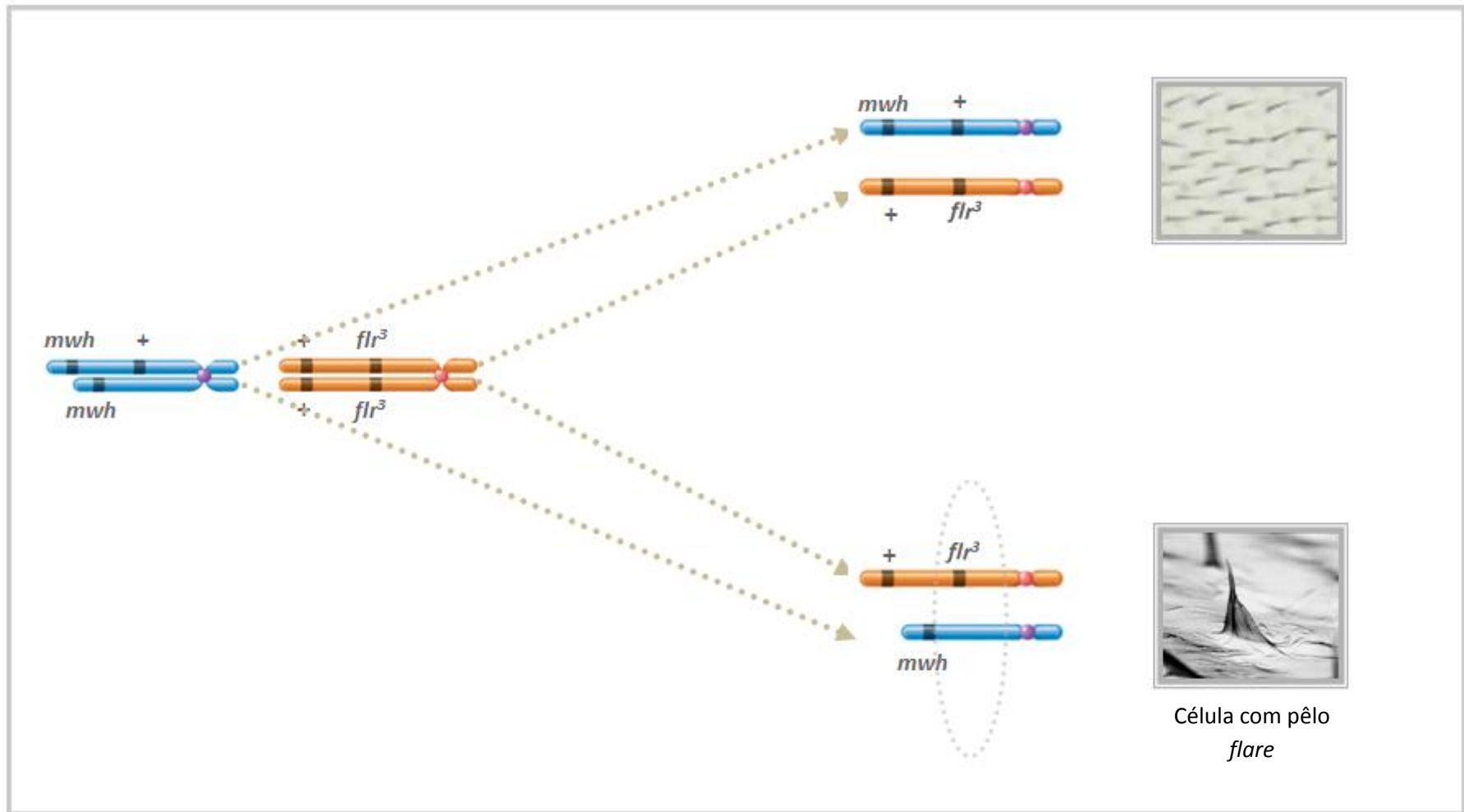


Figura 4. Esquema de divisão mitótica com ocorrência de deleção intersticial em células primordiais dos discos imaginais de asas de *D. melanogaster* (adaptado de Graf, Würigler *et al.*, 1984).

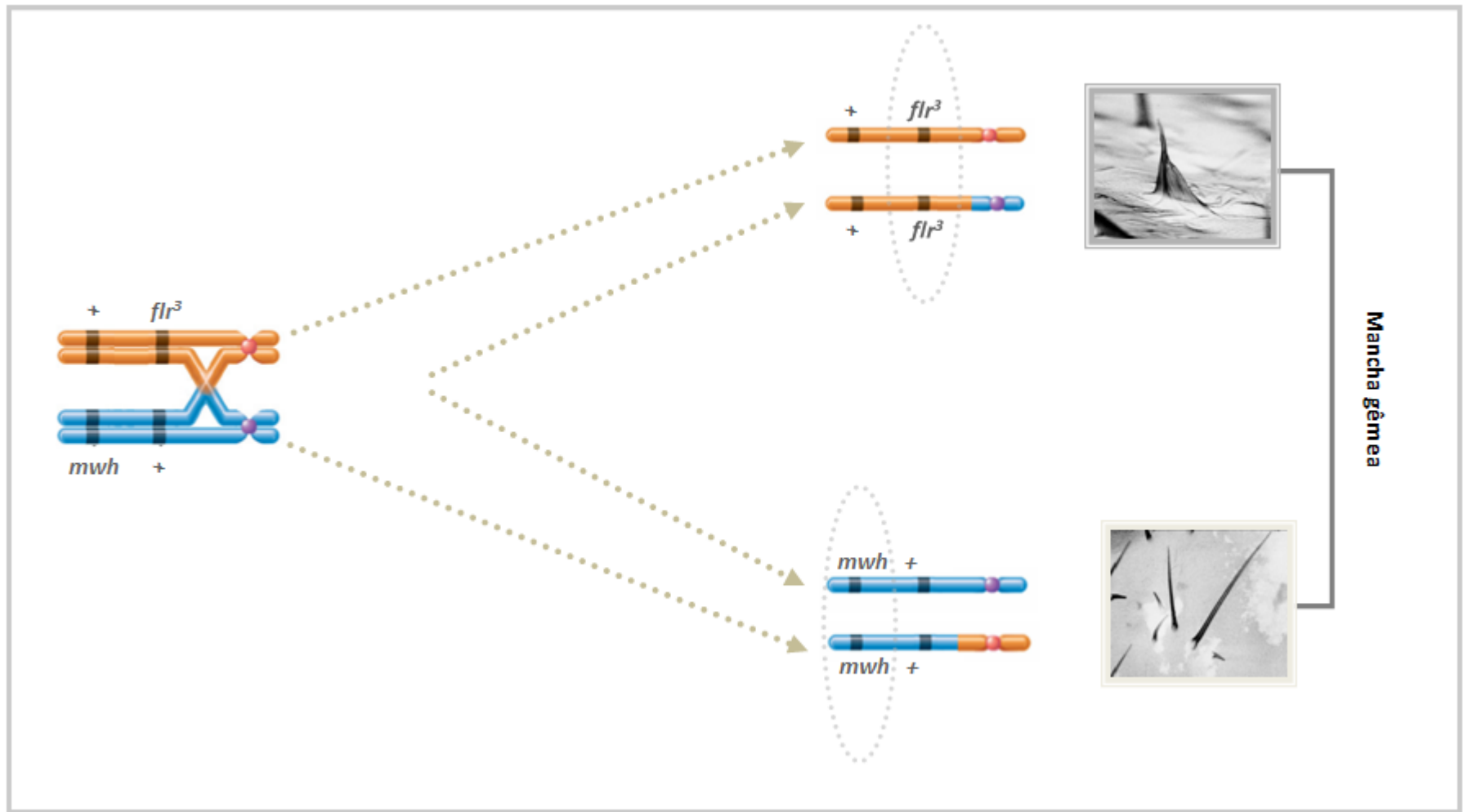


Figura 5. Esquema de divisão mitótica com ocorrência de recombinação entre o centrômero e o locus *flare* em células primordiais dos discos imaginais de asas de *D. melanogaster* (adaptado de Graf, Würigler *et al.*, 1984).

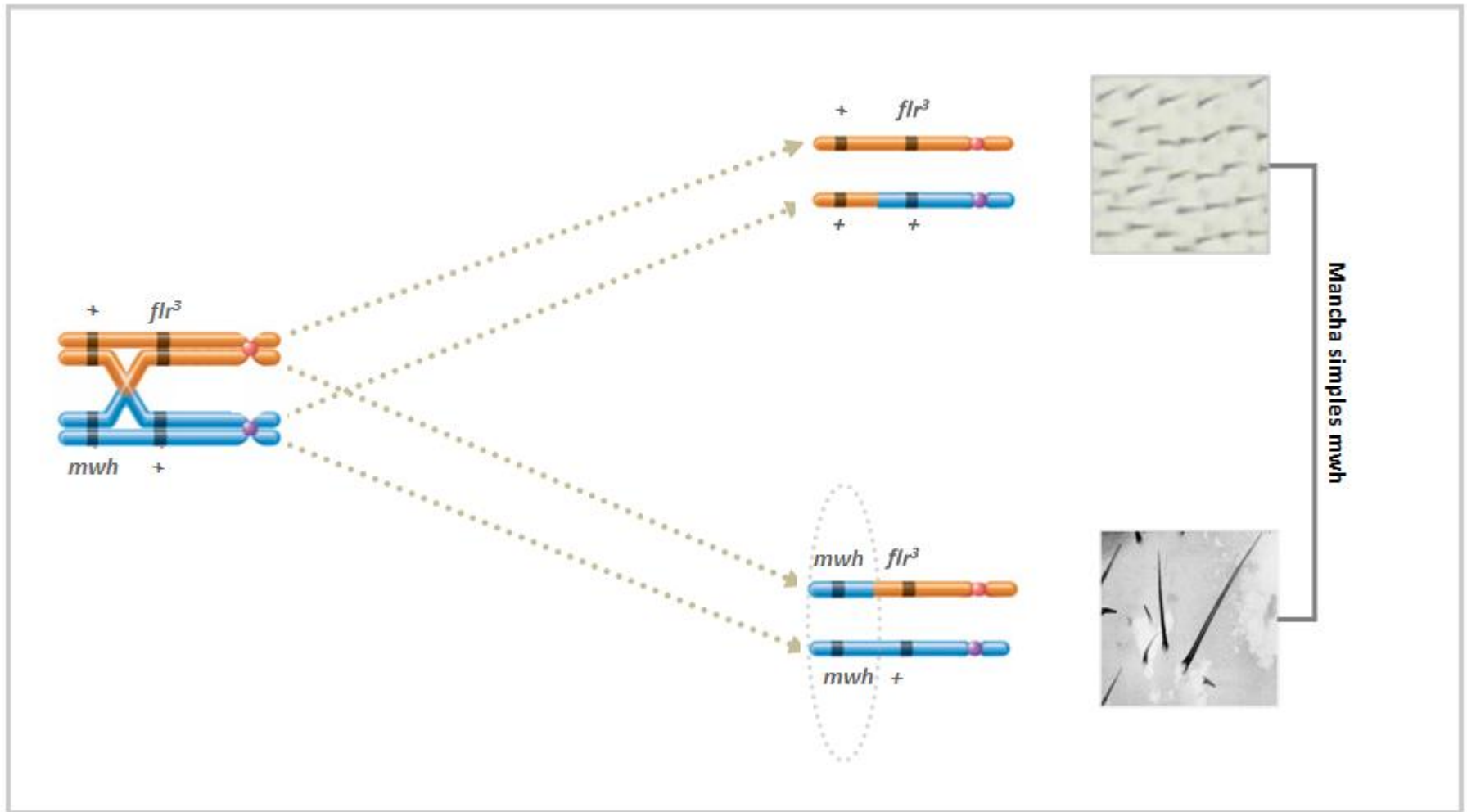


Figura 6. Esquema de divisão mitótica com ocorrência de recombinação entre os locus *mwh* e o locus *flare* em células primordiais dos discos imaginais de asas de *D. melanogaster* (adaptado de Graf, Würigler et al., 1984).

ANEXO II



Contents lists available at ScienceDirect

Food and Chemical Toxicology

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The effect of the dibenzylbutyrolactolic lignan (–)-cubebin on doxorubicin mutagenicity and recombinogenicity in wing somatic cells of *Drosophila melanogaster*

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ABSTRACT

The dibenzylbutyrolactolic lignan (–)-cubebin was isolated from dry seeds of *Piper cubeba* L. (Piperaceae). (–)-Cubebin possesses anti-inflammatory, analgesic and antimicrobial activities. Doxorubicin (DXR) is a topoisomerase-interactive agent that may induce single- and double-strand breaks, intercalate into the DNA and generate oxygen free radicals. Here, we examine the mutagenicity and recombinogenicity of different concentrations of (–)-cubebin alone or in combination with DXR using standard (ST) and high bio-activation (HB) crosses of the wing Somatic Mutation And Recombination Test in *Drosophila melanogaster*. The results from both crosses were rather similar. (–)-Cubebin alone did not induce mutation or recombination. At lower concentrations, (–)-cubebin statistically reduced the frequencies of DXR-induced mutant spots. At higher concentrations, however, (–)-cubebin was found to potentiate the effects of DXR, leading to either an increase in the production of mutant spots or a reduction, due to toxicity. These results suggest that depending on the concentration, (–)-cubebin may interact with the enzymatic system that catalyzes the metabolic detoxification of DXR, inhibiting the activity of mitochondrial complex I and thereby scavenging free radicals. Recombination was found to be the major effect of the treatments with DXR alone. The combined treatments reduced DXR mutagenicity but did not affect DXR recombinogenicity.

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1. Introduction

Piper cubeba Linn. (Piperaceae) is a pepper plant that is widely distributed in tropical and subtropical regions and is popularly known as *pimenta de Java* (in Brazil), *kemukus* (in Indonesia) or cubeb pepper. It is known that extracts from *P. cubeba* have anti-inflammatory (Bastos et al., 2001; Choi and Hwang, 2003; Yam et al., 2008a), anti-type IV allergic (Choi and Hwang, 2003), antileishmanial (Bodiwala et al., 2007), genotoxic (Junqueira et al., 2007), antineoplastic (Yam et al., 2008b), and molluscicidal activities (Pandey and Singh, 2009).

Lignans are a class of secondary plant metabolites that are produced by the oxidative dimerization of two phenylpropanoid units.

Interest in lignans and their synthetic derivatives has grown due to their applications for cancer chemotherapy and their various other pharmacological effects (Saleem et al., 2005). Here, (–)-cubebin, a dibenzylbutyrolactolic lignan, was isolated from the dry seeds of *P. cubeba* L. This lignan is known to possess anti-inflammatory (Bastos et al., 2001; da Silva et al., 2005), analgesic (da Silva et al., 2005), and antimicrobial activities (Silva et al., 2007, 2009). Although some (–)-cubebin derivative compounds ((–)-hinokinin; (–)-O-benzyl cubebin; (–)-O-(N,N-dimethylamino-ethyl)-cubebin) inhibit the free amastigote forms of *Trypanosoma cruzi*, the natural (–)-cubebin molecule itself, which is used as the starting compound to obtain the evaluated dibenzylbutyrolactolic derivatives, does not affect the growth of trypomastigote forms of *T. cruzi* (de Souza et al., 2005). (–)-Cubebin and derivatives isolated from *P. cubeba* were found to significantly inhibit cytochrome P450 (CYP3A4) (Usia et al., 2005, 2006) and the NADH oxidase activity of mitochondrial complex I (Saraiva et al., 2009).

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