UNIVERSIDADE JOSÉ DO ROSÁRIO VELLANO - UNIFENAS
LUIZ SILVA SOUZA

AUSÊNCIA DE GENOTOXIDADE E REDUÇÃO DOS EFEITOS GENOTÓXICOS INDUZIDOS POR DOX DA TINTURA DE SEMENTES DE *Helianthus annuus* Linné (GIRASSOL) REVELADAS PELO ENSAIO DO MICRONÚCLEO

Alfenas-MG
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Orientador: Marcelo Fabiano Gomes Boriollo, BSB, MSc, PhD.

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Autor: Luiz Silva Souza

Orientador: Prof. Dr. Marcelo Fabiano Gomes Boriollo, BSB, MSc, PhD.

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“Um pouco de ciência nos afasta de Deus.
Muito, nos aproxima.”

Louis Pasteur

RESUMO

SOUZA, Luiz Silva. **Ausência de genotoxidade e redução dos efeitos genotóxicos induzidos por DOX da tintura de sementes de *Helianthus annuus* Linné (girassol) reveladas pelo ensaio do micronúcleo**. 121 f. Dissertação (Mestrado em Ciência Animal) - UNIFENAS, Alfenas, 2012.

A efetividade terapêutica potencial das sementes de *H. annuus* L. tem sido demonstrada (alívio dos sintomas da asma, efeitos da proteção gástrica, cicatrização, propriedades anti-inflamatórias e antimicrobianas), em adição à sua importante fonte de nutrientes. Esta pesquisa avaliou a mutagenicidade e anti-mutagenicidade do óleo e da tintura de sementes de *H. annuus* L. usando o ensaio do micronúcleo em medula óssea de camundongos. A associação sobre os efeitos genotóxicos induzidos pela doxorrubicina (DOX) também foi analisada. Grupos experimentais foram avaliados após 24-48h de tratamento com N-Nitroso-N-ethylurea (NUE) e DOX (controles positivos), NaCl (controle negativo), tintura (250-2.000 $mg.Kg^{-1}$ de THALS) e duas fontes de óleo de girassol (250-2.000 $mg.Kg^{-1}$ de POHALS e FOHALS). Ensaios anti-mutagênicos foram realizados usando os controles positives associados com a tintura e óleos de girassol, separadamente. A frequência de eritrócitos policromáticos micronucleados (MNPCEs) foi estatisticamente diferente ($p < 0,05$) entre (*i*) os tratamentos controles positives e negativo, (*ii*) controles positivos e tratamento com THALS e (*iii*) tratamento com THALS e sua associação aos controles positivos. Contudo, uma leve genotoxicidade foi observada na associação THALS+DOX. Ambas as fontes de óleos (FOHALS e POHALS) revelaram resultados similares, cujas frequências de MNPCEs foram consistentes com aqueles observados no controle negativo. Diferenças significativas também ocorreram entre os tratamentos de óleos e suas associações com os controles positivos. A ausência de genotoxicidade (clastogenia/aneugenia) da THALS, POHALS e FOHALS pode ser inferida independentemente da dose, tempo (exceto para FOHALS) e gênero de camundongo (exceto para POHALS e FOHALS). Efeitos anti-genotóxicos moderados de THALS sugerem um potencial mecanismo ligeiramente protetor sobre os efeitos genotóxicos induzidos pela DOX.

Palavras-chave: Medula óssea, *Helianthus annuus* L. (girassol), ensaio do micronúcleo, roedores, tintura e óleo.

ABSTRACT

SOUZA, Luiz Silva. **Nongenotoxic effects and a reduction of the DOX-induced genotoxic effects of *Helianthus annuus* Linné (sunflower) seeds revealed by micronucleus assays in mouse bone marrow**. 121 f. Dissertação (Mestrado em Ciência Animal) - UNIFENAS, Alfenas, 2012.

In addition to being an important source of nutrients, the potential therapeutic effectiveness of *H. annuus* L. seeds has been previously demonstrated; the beneficial properties of *H. annuus* L. seeds include an ability to relieve asthmatic symptoms, protective effects on the stomach, benefits to healing, and anti-inflammatory and anti-microbial properties. This research evaluated the mutagenicity and anti-mutagenicity of oil and tincture of *H. annuus* L. seeds using the micronucleus assay in bone marrow of mice. The interaction between these preparations and the genotoxic effects of doxorubicin (DOX) was also analyzed. Experimental groups were evaluated after 24-48 h of treatment with N-Nitroso-N-ethylurea (NEU) and DOX (positive controls), NaCl (a negative control), a sunflower tincture (250-2,000 mg.Kg-1 of THALS) and two sources of sunflower oils (250-2,000 mg.Kg-1 of POHALS and FOHALS). Anti-mutagenic assays were carried out using the sunflower tincture and oils separately and in combination with these controls. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) was significantly different ($p < 0.05$) between (*i*) the positive and negative control treatments, (*ii*) the positive controls and animals treated with THALS and (*iii*) animals treated with THALS and and THALS combined with the positive controls. However, a slight genotoxicity was observed in the animals treated with the combination of THALS+DOX. Both sources of oils (FOHALS and POHALS) revealed similar results; in these groups, the frequencies of MNPCEs were similar to those observed in negative controls. Statistically significant differences were also observed between the sunflower oil treatments and their associated positive controls. There was no genotoxicity (clastogenicity/aneugenicity) observed in THALS, POHALS and FOHALS regardless of the dose, time (except FOHALS) and gender of mouse (except POHALS and FOHALS). The moderate anti-genotoxic effects of THALS suggest a potential slight protective mechanism against DOX-induced genotoxic effects.

Keywords: bone marrow, *Helianthus annuus* L. (sunflower), micronucleus assay, rodents, tincture and oil.

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LISTA DE ABREVIATURAS

°C	Graus Celsius
ANOVA	Análise da variância *one-way*
BFB	*Breakage-fusion-bridge*
cDNA	*Complementary desoxirribonucleic acid*
CEMIB	Centro Multidisciplinar para Investigação Biológica
CEPEAU	Comitê de Ética em Pesquisa Envolvendo Animais da UNIFENAS
COBEA	Colégio Brasileiro de Experimentação Animal
DNA	*Desoxirribonucleic acid*
DOX	Cloridrato de doxorrubicina
DSBs	*Double-strand breaks*
E	Eutanásia
FISH	*Fluorescent in situ hybridization*
FOHALS	*Food oil of seeds of H. annuuss L.*
g	Gramas
H. annuus L.	*Helianthus annus Linné*
HepG2	*Human liver hepatocellular carcinoma cell line*
HUVEC	*Human umbilical vein endothelial cells*
Kg	Quilogramas
µL	Microlitros
mL	Mililitros
mM	Milimolar
MN	*Micronucleus*
MNPCEs	*Micronucleated polychromatic erythrocytes*
MUFA	*Monounsaturated fatty acid*
n	Nível de ploidia
n	Número de camundongos
NaCl	*Sodium chloride*
NBUD	*Nuclear budding*
NCE	*Normochromatic erythrocyte*

NPB	*Nucleoplasmic bridge*
NSL	*Non-saponifiable lipids*
NEU	*N-Nitroso-N-ethylurea*
PCE	*Polychromatic erythrocyte*
POHALS	*Pharmaceutical oil of seeds of H. annuus L.*
PUFA	*Polyunsaturated fatty acid*
SSBs	*Single-strand breaks*
T	Tratamento
THALS	*Tincture of seeds of H. annuus L.*
TPA	*12-O-tetradecanoylphorbol-13-acetate*
Unib:SW	Camundongos heterogenéticos *Swiss albinus*

SUMÁRIO

1. INTRODUÇÃO

Pela primeira vez, a presente pesquisa avaliou os efeitos genotóxicos (clastogênia e/ou aneugênia) da tintura de sementes de girassol empregando o ensaio do MN em PCE da medula óssea de camundongos *Swiss albinus*. Duas diferentes fontes de óleo de girassol também foram inseridas no presente ensaio. Em adição, a ação dessa tintura e dos respectivos óleos de girassol sobre os efeitos genotóxicos induzidos pela DOX foi também estudada. A inexistência de genotoxicidade da THALS, do POHALS e do FOHALS, independentemente da dose, do tempo (exceto ao FOHALS o qual demonstrou um aumento na proporção de MNPCEs em 48h) e do gênero (exceto ao POHALS ou FOHALS os quais demonstraram um aumento na proporção de MNPCEs ao gênero masculino ou gênero feminino, respectivamente), tem sido sugerida na presente pesquisa científica. Elevada genotoxicidade do DOX também foi observada a partir de uma dosagem acima daquela administrada em humanos, em comparação àquela genotoxicidade do controle positivo NUE usado no ensaio do MN, independentemente do tempo e do gênero do animal.

Entretanto, indícios dos efeitos anti-genotóxicos da THALS quando associado ao tratamento quimioterápico do DOX foram observados, o que sugere uma parcialidade dos efeitos genotóxicos induzidos pela DOX na medula óssea de camundongos. Tais evidências não foram encontradas nos tratamentos de associação POHALS e DOX ou FOHALS e DOX e, consequentemente, qualquer mecanismo protetor contra os efeitos genotóxicos induzidos por DOX não pode ser inferido. A relação PCE/NCE frente ao tratamento com THALS revelou diferenças entre as menores e maiores concentrações testadas, sugerindo toxicidade sistêmica moderada dose-dependente. Essa toxicidade também foi observada no tratamento com o DOX, independentemente do tempo e do gênero do animal. Contudo, uma discreta redução dessa toxicidade foi observada na associação de THALS e DOX, fato este que poderia ser explicado pela própria existência de toxicidade da THALS na sua concentração máxima apenas. A relação PCE/NCE observada no tratamento com o POHALS revelou diferenças entre a maior e as demais concentrações testadas, cujas relações PCEs/NCEs foram intermediárias aos controles$^{+/-}$ do ensaio, sugerindo uma leve-moderada toxicidade sistêmica do POHALS. A associação do POHALS e DOX não evidenciou uma relação PCE/NCE compatível ao ensaio mutagênico do POHALS ou ao controle negativo do ensaio, sugerindo uma proteção nula contra os efeitos tóxicos induzidos pela DOX. A relação PCE/NCE do FOHALS revelou diferenças entre as menores e maiores concentrações testadas, contudo mostrou compatibilidade com a relação PCE/NCE dos controles^{+}, apontando para a

toxicidade sistêmica do FOHALS (principalmente a partir de 500 mg.Kg^{-1}). Similarmente ao POHALS, a associação do FOHALS e DOX não evidenciou uma relação PCE/NCE compatível ao controle negativo do ensaio, sugerindo mais uma vez ausência de proteção contra os efeitos tóxicos induzidos pela DOX.

Outros estudos envolvendo a genotoxicidade de extratos e óleos de sementes de *H. annuus* L. deveriam ser conduzidos [e.g., teste de mutagenicidade com *Salmonella typhimurium* (teste de Ames) como indicador de carcinogenicidade em potencial para mamíferos, teste de mutação gênica em células de mamíferos (*mouse lymphoma assay*), testes citogenéticos *in vitro* e aneuploidia, teste do micronúcleo em células do micronúcleo *in vitro*, hibridação *in sito* fluorescente (FISH) e sua aplicação à mutagênese, teste do cometa para a detecção de dano no DNA e reparo em células individualizadas, genômica funcional e proteômica em mutagênese (cDNA arrays, microarrays analyses), entre outros], a fim de caracterizar os seus potenciais efeitos e mecanismos de ação genotóxicos e anti-genotóxicos e, principalmente, para o estabelecimento de limites ao consumo humano, de potenciais riscos à saúde humana ou, até mesmo, para implementação racional de estratégias quimiopreventivas.

2. OBJETIVOS E JUSTIFICATIVAS

Apesar de outras pesquisas suportarem a efetividade do potencial terapêutico do óleo (ozonizado) ou extrato aquoso de sementes de girassol (*H. annuus* L.), como por exemplo, alívio dos sintomas asmáticos (HEO et al., 2008), efeitos de proteção gástrica (CARDOSO et al., 2004; RICARDO et al., 2007), propriedades cicatrizantes (RODRIGUES et al., 2004), anti-inflamatória (ZAMORA et al., 2006; AKIHISA et al., 1996; PLOHMANN et al., 1997) e anti-microbiana (MITSCHERG et al., 1983; MENÉNDEZ et al., 2002; RODRIGUES et al., 2004; CARDOSO et al., 2004), um número limitado de investigações visando o conhecimento dos efeitos genotóxicos e mutagênicos tem sido observado até o presente momento (ANTONIA et al., 1998; INDART et al., 2007; ROJAS-MOLINA et al., 2005). Objetivando contribuir com as informações sobre o potencial genotóxico de fitoterápicos e alimentos, a presente pesquisa avaliou os efeitos mutagênicos de duas fontes de óleo e da tintura de sementes de *H. annuus* L. (girassol) usando o ensaio do micronúcleo *in vivo* na medula óssea de camundongos. A ação da concentração máxima permitida do *H. annuus* L. (óleos e tintura) sobre os efeitos genotóxicos induzidos pelo DOX (cloridrato de doxorrubicina) também foi estudada (i.e., avaliação anti-mutagênica).

3. REVISÃO DE LITERATURA

O girassol cultivado (*Helianthus annuus* L.) é uma das 67 espécies do gênero *Helianthus*. Definida como uma planta dicotiledônea e um membro da Família *Compositae* (Asteraceae), tendo uma flor típica composta (HEISER, 1976). A cabeça do girassol ou inflorescência consiste de 700-8000 flores, dependendo do cultivar (LUSAS, 1985). Espécies diplóides, tetraplóides e hexaplóides são conhecidas (FICK, 1989). O girassol cultivado contém 34 cromossomos (2n = 34). O nome do gênero do girassol é derivado do Grego *helios* ("sol") e *anthos* ("flor"). A semente do girassol é chamada um "aquênio" pelos botânicos, definido como um fruto seco, simples, germinado único com a semente aderida à parede interna em apenas um ponto. O aquênio consiste de um endosperma de semente (muitas vezes chamada de caroço, semente descascada ou carnes pelos processadores de óleo) e um pericarpo aderente (casco ou concha), o qual é a parede do fruto (LUSAS, 1985). A proporção de casco e caroço nas sementes de girassol varia consideravelmente (SALUNKHE et al., 1992). Tipos de sementes não oleoginosas de girassol contém mais casco (47%) do que tipos de sementes oleoginosas (20-30%). A composição das sementes é marcadamente afetada pela variedade de girassol (SALUNKHE et al., 1992; EARLE et al., 1968). Não obstante, as faixas de composição das sementes descascadas de girassol (%, peso seco) mostraram-se de acordo com o seguinte (GONZÁLEZ-PÉREZ & VEREIJKEN, 2007): proteína $^{20,4–40,0}$; peptídeos, aminoácidos e outros nitrogênios não proteicos $^{1–13\%}$; carboidratos $^{4–10\%}$; lipídeos $^{47–65\%}$; ácidos graxos (palmítico $^{5–7\%}$; esteárico $^{2–6\%}$; araquídico $^{0,0–0,3\%}$; oléico $^{15–37\%}$; linoléico $^{51–73\%}$; linolênico $^{<0,3\%}$); tocoferol $^{0,07\%}$; carotenóides $^{0,01–0,02\%}$; vitamina B1 $^{0,002\%}$; ácido clorogênico $^{0,5–2,4\%}$; 'acido quínico $^{0,12–0,25\%}$; ácido caféico $^{0,05–0,29\%}$; minerais totais $^{3–4\%}$; potássio $^{0,67–0,75\%}$; fósforo $^{0,60–0,94\%}$; enxofre $^{0,26–0,32\%}$; magnésio $^{0,35–0,41\%}$; cálcio $^{0,08–0,10\%}$; sódio $^{0,02\%}$ (LUSAS, 1985; SALUNKHE et al., 1992; EARLE et al., 1968; GHEYASUDDIN et al., 1970; GASSMANN, 1983; GONZÁLEZ-PÉREZ et al., 2002; ROBERTSON, 1975; FRANQUART SEED COMPANY, 2002, WAN et al., 1979; SCHWENKE & RAAB, 1973; BAU et al., 1983; SABIR et al., 1974; BEROT & BRIFFAUD, 1983; MADHUSUDHAN et al., 1986).

Tocoferóis são excelentes antioxidantes naturais que protegem os óleos contra a rancidez oxidativa. A forma α tem a maior atividade biológica de vitamina E, enquanto que a forma γ tem sido reportada para ter a maior atividade antioxidante (WARNER et al., 1990). Os esteróis encontrados no óleo de girassol incluem β-sitosterol, stigmasterol, campesterol, δ-5-avenasterol, e δ-7-stigmasterol (TROST, 1989; ITO et al., 1973). Esteróis de plantas são

apenas absorvidos minimamente pelos humanos, e sua ingestão parece inibir o colesterol intestinal e a absorção do ácido biliar (DUPONT, 1980). Muitos traços de metais em óleo refinado, desodorizado e branqueado são removidos durante o processamento. Esse fato é particularmente importante visto que o cobre e o ferro reduzem enormemente a estabilidade oxidativa do óleo (COWAN, 1966). Outros metais tais como chumbo e cádmio são motivos de preocupação devido à sua toxicidade e sua suposta ligação à doença cardíaca coronariana e hipertensão (BIERENBAUM et al., 1975).

Óleo de girassol contém altos níveis de PUFA (69% - *polyunsaturated fatty acid* – PUFA) com proporções de gordura poli-insaturada:gordura saturada de aproximadamente 6,4. Meydani e colaboradores (1991) mencionaram que os estudos sobre carcinogenicidade de PUFA em modelos animais geralmente suportam a visão documentada no reporte do *Committee of Diet, Nutrition, and Cancer* (1982) sobre o relacionamento positivo entre incidência de câncer, gordura na dieta, e o papel de dietas altas em conteúdo de ácido linoléico, a proporção de tumorigênese experimental, e o desenvolvimento de tumores espontâneos principalmente em camundongos e ratos fêmeas. Entretanto, dados de diversos estudos humanos investigando o risco, a incidência, ou a progressão de câncer da mama e cólon indicaram que o PUFA não aumenta o risco e pode ser negativamente associado com esses cânceres (MEYDANI et al., 1991). Esses autores mencionaram que dos 48 estudos investigando a gordura na dieta e o câncer de mama ou cólon (internacional, coorte de caso controle, população especial em risco, ou progressão da doença), 19 reportaram sobre o PUFA (PUFA, gordura vegetal, ou ácido linoléico). Na maioria desses estudos, nenhuma associação com a ingestão de PUFA foi reportado. Entretanto, estudos fora desses grupos reportaram uma associação entre a ingestão de PUFA e câncer: um estudo reportou uma associação positiva com PUFA (HEMS, 1978) e quatro estudos reportaram uma associação negativa entre PUFA e câncer de cólon ou mama (MECKEOW-EYSSEN & BRIGHT-SEE, 1984; KAIZER et al., 1989; TUYNS et al., 1988; VERREAULT et al., 1988), muito embora os projetos dos estudos não fossem comparáveis (MEYDANI et al., 1991). Em outra pesquisa envolvendo grupos de camundongos glabros Skh:HR-1, Reeve e colaboradores (1996) mostraram uma resposta fotocarcinogênica de elevada gravidade quando o conteúdo poli-insaturado da gordura na dieta mista (óleo de algodão saturado hidrogenado) foi aumentada, quer seja medida como incidência de tumor, multiplicidade de tumor, progressão de tumores benignos para carcinoma de células escamosas, ou redução da sobrevida.

Pesquisas envolvendo plantas medicinais e seus extratos vêm crescendo na assistência à saúde em função de sua fácil aceitabilidade, disponibilidade e baixo custo. Considerável

parcela da população mundial utiliza a medicina popular para seus cuidados primários em relação à saúde, e se presume que a maior parte dessa terapia tradicional envolve o uso de extratos de plantas ou seus princípios biologicamente ativos (FARNSWORTH et al., 1985; KAUR et al., 2005). No Brasil, cerca de 8% das 100.000 espécies vegetais catalogadas foram estudadas quanto as suas propriedades químicas, e estima-se que apenas 1.100 espécies tenham sido avaliadas quanto às suas propriedades terapêuticas (GARCIA et al., 1996). O panorama brasileiro na área farmacêutica revela um gasto de aproximadamente dois a três bilhões de dólares por ano na importação de matérias-primas empregadas na produção de medicamentos. Ainda, tal panorama mostra que 84% dos fármacos consumidos no país provêm da importação e 78-80% dos fármacos produzidos provêm das empresas multinacionais (BERMUDEZ, 1995; MIGUEL & MIGUEL, 1999), índices que justificam a busca de alternativas para superar a dependência externa por parte da indústria químico-farmacêutica brasileira.

Compostos biologicamente ativos têm sido reconhecidos quanto as suas propriedades farmacológicas, contudo vários desses compostos não puderam ser introduzidos em terapêutica devido às suas propriedades toxicológicas, carcinogênicas e mutagênicas (AMES, 1983; KONSTANTOPOULOU et al., 1992; TAVARES, 1996). No desenvolvimento de novos fármacos, as análises dos ensaios de genotoxicidade representam considerável peso, visto que a maioria das indústrias farmacêuticas delibera o processamento de um novo agente terapêutico com base também nos dados de genotoxicidade *in vitro* e *in vivo* (PURVES et al., 1995). Nesse contexto, os ensaios para avaliação da atividade mutagênica das plantas usadas pela população bem como suas substâncias isoladas, são necessários e importantes para estabelecer medidas de controle no uso indiscriminado. Além disso, é preciso esclarecer os mecanismos e as condições que mediaram o efeito biológico, antes que as plantas sejam consideradas como agentes terapêuticos (VARANDA, 2006).

Os efeitos genotóxicos de um agente mutagênico potencial dependem do seu alvo celular. Alguns compostos químicos necessitam ser metabolizados antes de adquirir sua capacidade mutagênica (MATEUCA et al., 2006). Agentes mutagênicos podem induzir alterações genômicas atingindo direta e/ou indiretamente a molécula de DNA, ou ligando-se às proteínas envolvidas na manutenção da integridade genômica (KIRSCH-VOLDERS et al., 2003). As consequências das interações entre os agentes mutagênicos e os seus alvos podem conduzir a diferentes tipos de danos no DNA (aductos de DNA, sítios alcalinos lábeis, rupturas dos filamentos) e mutações que vão desde alterações nucleotídicas simples (mutações gênicas) até alterações cromossômicas estruturais (mutações cromossômicas) ou numéricas

(mutações genômicas). Finalmente, o destino celular é então determinado pelas várias lesões provocadas sobre o genoma e a capacidade celular intrínseca de reparo ou processos de eliminação por apoptose (DECORDIER et al., 2002).

Tanto quanto os estudos de genotoxicidade estão preocupados, o ensaio do micronúcleo *in vivo* (*micronucleus* – MN) em medula óssea de roedores desempenha um papel crucial à bateria de testes que objetivam a identificação de riscos por agentes mutagênicos (MATEUCA et al., 2006), especialmente à avaliação de riscos mutagênicos em que permite a consideração de fatores metabólicos *in vivo*, da farmacocinética e dos processos de reparo do DNA, embora estes possam variar entre as espécies, entre tecidos e entre os mecanismos genéticos (OECD, 1997a,b; RIBEIRO et al., 2003). Uma vez que o eritroblasto da medula óssea se desenvolve em um eritrócito policromático (*polychromatic erythrocyte* – PCE) (i.e., célula originada após a extrusão do núcleo principal), qualquer micronúcleo que tenha sido formado pode permanecer atrás, de outro modo, no citoplasma anucleado. Deste modo, a frequência de eritrócitos policromáticos micronucleados (*micronucleated polychromatic erythrocytes* – MNPCEs) tem sido o principal *endpoint*. Por outro lado, a mensuração de MNPCEs em sangue periférico tem sido igualmente aceitável em qualquer espécie na qual a inabilidade do baço remover eritrócitos micronucleados tem sido demonstrada, ou a qual tem mostrado uma adequada sensibilidade para detectar agentes que causam aberrações cromossômicas numéricas ou estruturais. Os resultados positivos (i.e., um aumento na frequência de eritrócitos policromáticos micronucleados em animais tratados) indicam que uma substância induz a formação de micronúcleos, os quais surgem como um resultado de danos cromossômicos ou danos ao aparato mitótico nos eritroblastos das espécies teste, enquanto que os resultados negativos indicam que, sob as condições experimentais, a substância teste não induz a produção de micronúcleos nos eritrócitos imaturos das espécies teste. Todavia, o número de eritrócitos normocromáticos (*normochromatic erythrocyte* – NCE) no sangue periférico, que contém micronúcleos entre um dado número de eritrócitos maduros, pode também ser usado como *endpoint* deste ensaio (OECD, 1997c; RIBEIRO et al., 2003). Além disso, o conhecimento dos efeitos genotóxicos induzidos por fitoterápicos e alimentos empregando o ensaio do MN *in vivo* em mamíferos tem sido o anseio de vários grupos de pesquisadores (CHANDRASEKARAN et al., 2011; SILVA et al., 2011; INDART et al., 2007; VENKATESH et al., 2007; ALVES et al., 2013).

Micronúcleos (MNs) são corpos extranucleares pequenos que surgem durante a divisão celular a partir de fragmentos cromossômicos / cromossômicos acêntricos ou cromossomos / cromátides totais que se atrasam na anáfase e não são incluídos nos núcleos

filhos durante a telófase (FENECH & MORLEY, 1985). Micronúcleos abrigando fragmentos cromossômicos podem resultar de quebras do DNA de fita dupla, conversão de quebras de fita simples (*single-strand breaks* – SSBs) em quebras de fitas duplas (*double-strand breaks* – DSBs) após a replicação celular, ou inibição de síntese de DNA. O reparo equivocado de duas quebras cromossômicas pode conduzir a um rearranjo cromossômico assimétrico produzindo um cromossomo dicêntrico e um fragmento acêntrico. Frequentemente, os centrômeros de cromossomos dicêntricos são puxados para polos opostos das células durante a anáfase resultando na formação de uma ponte nucleoplásmica (*nucleoplasmic bridge* – NPB) entre os núcleos filhos e um fragmento acêntrico que fica atrasado para formar um MN (FENECH et al., 2005; THOMAS et al., 2003). Micronúcleos abrigando cromossomos totais são essencialmente formados a partir de defeitos na maquinaria de segregação cromossômica tais como deficiências nos genes que controlam o ciclo celular, falha do fuso mitótico, cinetócoro, ou outras partes do aparato mitótico ou por danos até subestruturas cromossômicas, rompimento mecânico (ALBERTINI et al., 2000) e hipometilação do DNA centromérico (FENECH et al., 2005). Micronúcleos podem também surgir por amplificação gênica via ciclos de quebra-fusão-ponte (*breakage-fusion-bridge* – BFB), quando o DNA amplificado está seletivamente localizado em locais específicos da periferia do núcleo e eliminado via brotamento nuclear (*nuclear budding* – NBUD) durante a fase S do ciclo celular (FENECH, 2002). O destino do MN após a sua formação na célula micronucleada é pobremente entendido. Seu destino pós-mitótico inclui: (*i*) eliminação da célula micronucleada como uma consequência do apoptose (DECORDIER et al., 2002), (*ii*) expulsão da célula (quando o DNA dentro do MN não é esperado para ser funcional ou capaz de replicação devido a ausência dos componentes citoplasmáticos necessários); (*iii*) reincorporação dentro do núcleo principal (quando o cromossomo reincorporado pode ser indistinguível daqueles do núcleo principal e pode retomar a atividade biológica normal); (*iv*) retenção dentro do citoplasma da célula como uma entidade extra-nuclear (quando o MN pode completar um ou mais rodadas de replicação cromossômica/DNA) (LEACH & JACKSON-COOK, 2004).

4. MATERIAL E MÉTODOS

4.1. Fitoterápicos

Tintura e óleo de sementes de girassol foram adquiridos comercialmente e armazenados de acordo com as recomendações do fabricante [tintura de sementes de *H. annuus* L. (THALS): YOD Comércio de Produtos Naturais Ltda., ref. # 544606; óleo alimentício de sementes de *H. annuus* L. (FOHALS): Cargil Agrícola S.A.; óleo farmacêutico de sementes de *H. annuus* L. (POHALS): Farmácia Alfenense de Manipulação Ltda.]. A tintura foi submetida ao processo de extração alcoólico em equipamento Evaporador Rotativo (Marconi Equipamentos para Laboratório Ltda., mod. MA-120) e suas densidades de massa e relativa foram determinadas pelo método de picnômetro (FARMACOPÉIA BRASILEIRA ANVISA/FIOCRUZ, 2010).

4.2. Sistema-Teste *in vivo*

Camundongos heterogenéticos *Swiss albinus* (Unib:SW) adultos jovens (entre 7 e 12 semanas – período púbere), machos e fêmeas, com massa corporal entre 30 g e 40 g (i.e., a variação de peso entre os animais, para cada sexo, não deverá exceder a ± 20% da massa média) e saudáveis, provenientes do Centro Multidisciplinar para Investigação Biológica, Área da Ciência Animal de Laboratório, UNICAMP (CEMIB, http://www.cemib.unicamp.br), foram utilizados no teste do micronúcleo em eritrócitos da medula óssea (CHORILLI et al., 2007; CSGMT, 1986).

Os animais foram mantidos em grupos do mesmo sexo, em caixas de polipropileno, em ambiente climatizado a 22 °C ± 3 °C, umidade relativa do ar igual a 50% ± 20%, e em ciclos de luminosidade de 12 horas (i.e., 12 horas claro/12 horas escuro). Estes foram tratados com ração comercial Labina Purina® (Nestlé Purina Petcare Company) e água *ad libitum*, e aclimatados às condições do laboratório por 7 dias (período experimental) antes da realização do experimento. Ao final do período experimental, cada animal foi pesado e, de acordo com o peso, recebeu tratamento de 100 µL do líquido (NaCl 150 mM em água para injeção; THALS pós-processo de extração alcoólica; FOHALS; POHALS; NUE; DOX) para cada 10 g de massa corpórea.

Cada animal foi devidamente identificado na cauda (marcações numéricas) a fim de assegurar a continuidade dos registros e das interpretações ao longo do estudo (OECD, 1997c). Após o tratamento experimental, os animais foram submetidos à eutanásia por inalação de dióxido de carbono em câmaras de acrílico adaptadas (*Report of the American Veterinary Medical Association Panel Euthanasia, 2000*). O material contaminado foi acondicionado em sacos de plásticos duplos e armazenado em locais específicos até o momento de sua incineração, conforme as normas de segurança e saúde da instituição. Esta pesquisa foi conduzida de acordo com a Declaração Universal dos Direitos do Animal (UNESCO, 1978), os Princípios Éticos na Experimentação Animal (Colégio Brasileiro de Experimentação Animal – COBEA), a Lei de Crimes Ambientais Brasileira (*Lei N^{o} 9.605 de 12 de Fevereiro de 1998*), as Normas Brasileiras para a Prática Didático-Científica da Vivisseção de Animais (*Lei N^{o} 6.638 de 8 de maio de 1979*), e aprovação do Comitê de Ética em Pesquisa Envolvendo Animais da UNIFENAS (CEPEAU protocolo no. 04A/2008).

4.3. Grupos experimentais

Grupos experimentais (i.e., cada grupo constituído por 3 machos e 3 fêmeas) foram avaliados após 24 e 48 horas de único tratamento administrado por gavagem (grupos experimentais do ensaio mutagênico: 7-16, 21-30 e 35-44; grupos experimentais do ensaio anti-mutagênico: 17-20, 31-34 e 45-48), conforme metodologia estabelecida previamente (OECD, 1997c). Grupos controles negativos (1 e 2) e positivos (3, 4, 5 e 6) também foram incluídos mediante único tratamento administrado de modo intraperitoneal (Figura 1).

4.4. Processamento da medula óssea

A análise do micronúcleo em eritrócitos da medula óssea de camundongos foi realizada 24 e 48 horas após o tratamento, empregando-se a metodologia descrita previamente (SCHMID, 1976; ZAMBRANO et al., 1982). Logo após a eutanásia, os fêmures foram cirúrgica e assepticamente removidos e os animais apropriadamente descartados. Cada fêmur foi seccionado na extremidade proximal e o conteúdo do canal medular foi lavado com 1,5 mL de solução de NaCl 150mM e transferido para um tubo de centrífuga de 15 mL.

Esse material foi ressuspendido com o auxílio de uma pipeta Pasteur a fim de assegurar uma distribuição ao acaso das células da medula óssea. Então, a suspensão foi centrifugada a 1.000 r.p.m. (Centrífuga de Bancada Microprocessada Mod. NT 810, Nova

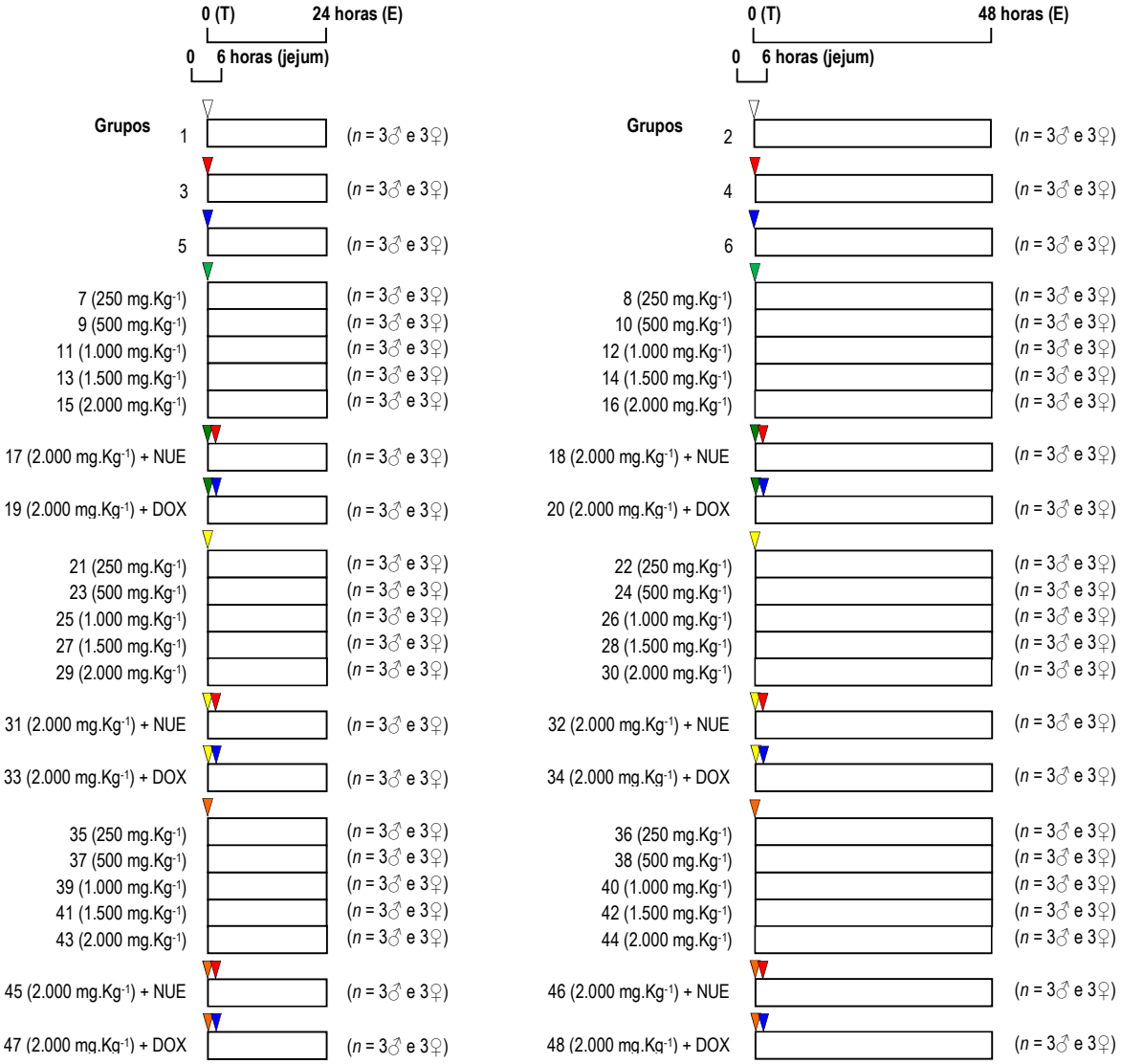
Técnica Ind. Com. Equipamentos p/ Laboratório Ltda., Piracicaba, SP, Brasil) por 5 minutos. O sedimento resultante foi ressuspendido em 500 μL de solução de NaCI 150mM adicionado de formol 4% e o sobrenadante descartado. As lâminas foram preparadas por meio de esfregaço (2 lâminas por animal), secas a temperatura ambiente por 24 horas, e coradas em cuba de coloração contendo corante de Leishman eosina-azul de metileno [corante puro por 3 min, seguido pelo corante diluído em água destilada (1:6) por 15 min], a fim de diferenciar eritrócito policromático (PCE) de eritrócito normocromático (NCE).

Eritrócitos policromáticos (PCEs) foram observados em aumento de 1.000 × empregando-se microscopia óptica (Nikon Eclipse E-200), contados (2.000 eritrócitos policromáticos anucleados *per* animal), com o auxílio de um contador de células digital (Contador Diferencial CCS02, Kacil Indústria e Comércio Ltda., PE, Brasil), e fotografados (Câmera Digital 8.1 Megapixels DC FWL 150). O número de eritrócitos policromáticos (PCEs), o número e a frequência de eritrócitos policromáticos micronucleados (MNPCEs), e a relação entre eritrócitos policromáticos e normocromáticos (PCE/NCE) foram relatados.

4.5. Análise estatística

Os dados obtidos no ensaio do micronúcleo foram submetidos à análise da variância *one-way* (ANOVA), em delineamento inteiramente casualizado no esquema fatorial 10 × 2 × 2 (tratamento × sexo × tempo), e comparação de médias por meio do teste de Tukey (α = 0,05) empregando o sistema computacional SAS® versão 9.2.

Figura 1. Protocolo experimental para o ensaio mutagênico e anti-mutagênico da tintura e óleo de sementes de *H. annuus* L. (girassol).



▽ NaCl 150 mM

▼ *N-Nitroso-N-ethylurea* 50 mg.Kg^{-1} – NUE (Sigma N8509)

▼ Cloridrato de doxorrubicina 5 mg.Kg^{-1} – DOX (Eurofarma Labs. Ltda., ref. # L83608)

▼ Tintura de sementes de *H. annuus* L. (THALS $^{\text{Tincture of seeds of } H.\ annuus \text{ L.}}$)

▼ Óleo farmacêutico de sementes de *H. annuus* L. (POHALS $^{\text{Pharmaceutical oil of seeds of } H.\ annuus \text{ L.}}$)

▼ Óleo alimentício de sementes de *H. annuuss* L. (FOHALS $^{\text{Food oil of seeds of } H.\ annuuss \text{ L.}}$)

T (Tratamento)

E (Eutanásia)

n (Número de camundongos)

5. RESULTADOS E DISCUSSÃO

Pesquisas envolvendo plantas medicinais agregam importantes informações para as novas descobertas e o desenvolvimento de potenciais quimioterápicos, além de auxiliar o estabelecimento de medidas seguras para o seu uso popular ou adequadas posologias (ELGORASHI et al., 2003; ARORA et al., 2005). *H. annuus* L. tem sido considerado uma importante fonte de óleo natural durante séculos e tem sido usado como um medicamento preventivo contra a diurese, diarreia, e várias doenças inflamatórias (LEWI et al., 2006), no alívio dos sintomas asmáticos (HEO et al., 2008), efeitos de proteção gástrica (CARDOSO et al., 2004; RICARDO et al., 2007), propriedades cicatrizantes (RODRIGUES et al., 2004), ação anti-inflamatória (ZAMORA et al., 2006; AKIHISA et al., 1996; PLOHMANN et al., 1997) e anti-microbiana (MITSCHERG et al., 1983; MENÉNDEZ et al., 2002; RODRIGUES et al., 2004; CARDOSO et al., 2004).

Entretanto, investigações visando o conhecimento dos efeitos genotóxicos e mutagênicos de *H. annuus* L. foram alvos de poucas pesquisas (ANTONIA et al., 1998; INDART et al., 2007; ROJAS-MOLINA et al., 2005), fato este que nos impulsionou avaliar os efeitos nocivos, sob a óptica mutagênica e anti-mutagênica (i.e., clastogenicidade e/ou aneugenicidade) da tintura e do óleo de sementes de *H. annuus* L. usando o ensaio do MN *in vivo*. O ensaio do MN tem sido usado como um *endpoint* citogenético eficaz para a avaliação de danos cromossômicos induzidos por agentes químicos mutagênicos e carcinogênicos (YAMAMOTO & KIKUCHI, 1980; HEDDLE et al., 1981; HEDDLE et al., 1983). A formação do MN e a incidência de aberrações cromossômicas foram fortemente correlacionadas (HEDDLE et al., 1983; ALMASSY et al., 1987). Entre as várias técnicas usadas para detector os efeitos genéticos e genotóxicos, o ensaio do MN tem sido considerado simples, barato, menos oneroso e de fácil e conveniente aplicação, particularmente em estudos genotóxicos (HEDDLE et al., 1983; SCHMID, 1975).

As análises estatísticas obtidas a partir dos números e das frequências de MNPCEs e da relação PCE/NCE na medula óssea de camundongos, para cada um dos tratamentos controles (positivos e negativos), experimentais mutagênicos e anti-mutagênicos da THALS, mostraram inexistência de diferenças significativas ($p < 0,05$) entre os tempos (24 e 48 horas) ou os gêneros (masculino e feminino). Entretanto, as análises obtidas a partir dos números e das frequências de PCEMNs revelaram diferenças estatísticas ($p < 0,05$) entre os três tratamentos controle (i.e., NaCl $^{grupo\ Tukey\ C\ e\ C'}$, NUE $^{grupo\ Tukey\ B\ e\ B'}$ e DOX $^{grupo\ Tukey\ A\ e\ A'}$). Do mesmo modo, essas diferenças foram observadas entre os tratamentos controles positivos e

experimentais mutagênicos (i.e., NUE *grupo Tukey B e B'*, DOX *grupo Tukey A e A'* e 250-2.000 mg.Kg^{-1} of THALS *grupo Tukey C e C'*), ou ainda, entre os tratamentos mutagênicos (i.e., 250-2.000 mg.Kg^{-1} of THALS *grupo Tukey C e C'*) e anti-mutagênicos (i.e., NUE + THALS *grupo Tukey B e B'* e DOX + THALS *grupo Tukey B e B'*) (Tabela 1). Esses resultados apontaram para as seguintes sugestões: (*i*) inexistência de genotoxicidade (mecanismos clastogênicos e/ou aneugênicos) da THALS, independentemente da dose de administração fitoterápica (250-2.000 mg.Kg^{-1}), do tempo de tratamento (24 e 48 horas) e do gênero do animal (masculino e feminino), conforme suportados pelos resultados das alterações genéticas espontâneas observadas nos tratamentos controles negativos (NaCl 150 mM); (*ii*) a existência de genotoxicidade (clastogenia e/ou aneugenia) elevada do DOX sob dosagem de 5 mg.Kg^{-1} [i.e., dosagem acima daquela administrada de modo quimioterapêutico em humanos: de 0,55 a 1,25 mg.Kg^{-1} considerando indivíduos de 90 e 40 Kg (50 mg.ampola^{-1}), respectivamente; de 0,11 a 0,25 mg.Kg^{-1} considerando indivíduos de 90 e 40 Kg (10 mg.ampola^{-1}), respectivamente], cujas frequências de MNPCEs foram expressivamente acima daquelas observadas no controle positivo NUE (50 mg.Kg^{-1}) do ensaio do MN, independentemente do tempo de tratamento (24 e 48 horas) e do gênero do animal (masculino e feminino); (*iii*) indícios dos efeitos anti-genotóxicos (anti-clastogenia e/ou anti-aneugenia) da THALS (2.000 mg.Kg^{-1}) quando associado com o tratamento quimioterápico do DOX (5 mg.Kg^{-1}). Portanto, há uma proteção parcial contra os efeitos genotóxicos induzidos pela DOX na medula óssea de camundongos, muito embora o efeito genotóxico observado nessa associação tenha sido compatível com aquele observado no NUE apenas.

Em adição, as análises obtidas a partir da relação PCE/NCE mostraram diferenças estatísticas ($p < 0,05$) entre os controles positivos e negativos (i.e., NaCl *grupo Tukey A''*, NUE *grupo Tukey CD''* e DOX *grupo Tukey D''*). Do mesmo modo, essas diferenças foram observadas entre as menores e maiores concentrações da THALS administradas nos tratamentos experimentais mutagênicos (i.e., 250 *grupo Tukey B''*, 500 *grupo Tukey BC''*, 1.000-1.500 *grupo Tukey BCD''* e 2.000 mg.Kg^{-1} *grupo Tukey CD''*), sendo observadas toxicidades parcialmente intermediárias àquelas dos respectivos controles positivos e negativo. Quanto aos ensaios anti-mutagênicos, padrões estatísticos similares da relação PCE/NCE foram encontrados entre as concentrações de THALS usadas no ensaio mutagênico e a associação da THALS + DOX *grupo Tukey BC''*, ou ainda, entre as concentrações de THALS do ensaio mutagênico e a associação da THALS + NUE *grupo Tukey BCD''*. Esses resultados reforçam a hipótese de possíveis toxicidade sistêmica dose-dependente da THALS, especialmente a partir da concentração de 500 mg.Kg^{-1} (conforme suportado pela observação da toxicidade do NUE), bem como a existência de

toxicidade sistêmica do DOX sob dosagem de 5 mg.Kg^{-1}, independentemente do tempo de tratamento (24 e 48 horas) e do gênero do animal (masculino e feminino). Contudo, há uma moderada e discreta redução dessa toxicidade quando o tratamento do DOX está associado à THALS, conforme suportado pela observação da toxicidade do DOX apenas. A relação PCE/NCE indicativa de toxicidade nessa associação (DOX + THALS) poderia também ser explicada pela existência de toxicidade da THALS associada a uma concentração de 2.000 mg.Kg^{-1}, fato este não reprodutível no tratamento mutagênico da menor concentração de THALS (250 mg.Kg^{-1}). Contudo, uma possível ausência ou maior redução da toxicidade dessa associação (DOX + THALS), empregando doses ≤ 250 mg.Kg^{-1}, permanece uma intrigante questão a ser explorada.

Pela primeira vez, a presente pesquisa deposita informações acerca dos efeitos mutagênicos e anti-mutagênicos da THALS, muito embora sem qualquer inferência da sua composição fitoquímica. Entretanto, pesquisas sobre os aspectos fitoquímicos e farmacológicos de plantas *Compositae* têm demonstrado que alguns esteróis (KASAHARA et al., 1994) e alcano-6,8-dióis (AKIHISA et al., 1994; YASUKAWA et al., 1996), isolados das flores de *Carthamus tinctorius*, e alguns triterpenos incluindo Ψ-taraxasterol e taraxasterol, das florese de *C. tinctorius*, *C. morifolium* e *H. annuus* L. (OINUMA, 1994), exibiram considerável atividade contra o edema inflamatório de orelha de camundongos induzido por 12-*O*-tetradecanoilforbol-13-acetato (*12-O-tetradecanoylphorbol-13-acetate* – TPA) (KASAHARA et al., 1994; YASUKAWA et al., 1996; OINUMA et al., 1994) e a promoção do tumour em pele de camundongo (KASAHARA et al., 1994; YASUKAWA et al., 1996). O isolamento de helianol das flores tubulares de *H. annuus* L. e sua caracterização como *3,4 - seco - 19(10→9)abeo - 8α,9β,10α - eupha - 4,24 - dien - 3 - ol* foi posteriormente relatada (AKIHISA et al., 1996a). Em outro estudo, a distribuição de helianol e outros compostos (taraxasterol, Ψ-taraxasterol, α-amirin, β-amirin, lupeol, taraxerol, cicloartenol, 24-metilenocicloartanol, tirucalla-7,24-dienol e dammaradienol) nas frações alcoólicas de triterpeno separadas dos lipídeos não-saponificável (*non-saponifiable lipids* – NSL) do extrato de metanol de 11 flores tabulare e 9 flores ligulados provenientes de 15 espécies de plantas *Compositae*, incluindo *H. annuus* L., foram também descritas. Tais alcóois triterpenos mostraram marcada atividade inibitória (dose inibitória de 50% foi de 0,1-0,8 mg *per* orelha) de acordo com seus efeitos inibitórios demonstrados sobre a inflamação induzida por 12-*O*-tetradecanoilforbol-13-acetato (*12-O-tetradecanoylphorbol-13-acetate*) em camundongos, especialmente a inibição mediada por helianol (0,1 mg *per* orelha) e oito outros triterpenos

(taraxerol, β-amirin, cicloartenol, α-amirin, lupeol, 24-metilenocicloartanol, Ψ-taraxasterol e taraxasterol), os quais mostraram níveis comparáveis aqueles da indometacina e, adicionalmente, foram sugeridos para serem potentes agentes anti-tumoures (AKIHISA et al., 1996b).

Levando-se em consideração o POHALS, as análises estatísticas obtidas a partir dos números e freqüências de MNPCEs e da relação PCE/NCE mostraram inexistência de diferenças significativas ($p < 0,05$) entre os tempos (24 e 48 horas), para cada um dos tratamentos experimentais mutagênicos e anti-mutagênicos. Contudo, tais diferenças ($p < 0.05$) ocorreram entre os gêneros dos camundongos (masculino e feminino). Entre os tratamentos controles positivos e experimentais mutagênicos ou controle negativo (i.e., NUE *grupo Tukey B e B'* e DOX *grupo Tukey A e A' versus* 250-2.000 mg.Kg^{-1} do POHALS e NaCl *grupo Tukey C and C'*), ou ainda, entre os tratamentos mutagênicos do POHALS (i.e., 250-2.000 mg.Kg^{-1} *grupo Tukey C e C'*) e anti-mutagênicos (i.e., NUE + POHALS *grupo Tukey A e A'* e DOX + POHALS *grupo Tukey A e A'*), as diferenças estatísticas foram observadas ($p < 0,05$) (Tabela 2). Esses resultados apontam para as seguintes sugestões: (*i*) inexistência de genotoxicidade (mecanismos clastogênicos e/ou aneugênicos) do POHALS, independentemente da dose de administração fitoterápica (250-2.000 mg.Kg^{-1}) e do tempo de tratamento (24 e 48 horas), conforme suportados pelos resultados das alterações genéticas espontâneas observadas no tratamento do controle negativo (NaCl 150mM). Apesar dessa ausência de efeitos genotóxicos, diferenças na freqüência de MNPCEs podem ser encontradas entre os gêneros (machos e fêmeas), sendo o gênero feminino aquele que apresentou o menor índice MNPCEs em todos os tratamentos experimentais mutagênicos; (*ii*) inexistência dos efeitos anti-genotóxicos (anti-clastogenia e/ou anti-aneugenia) do POHALS (2.000 mg.Kg^{-1}) quando associado ao tratamento quimioterápico do DOX (5 mg.Kg^{-1}) e, portanto, ausência de proteção dos efeitos genotóxicos induzidos pela DOX na medula óssea de camundongos.

As análises obtidas a partir da relação PCE/NCE mostraram diferenças estatísticas ($p < 0,05$) entre a maior e as demais concentrações do POHALS administradas nos tratamentos experimentais mutagênicos (i.e., 250-1.500 mg.Kg^{-1} *grupo Tukey B''* e 2.000 mg.Kg^{-1} *grupo Tukey BC''*), as quais também revelaram uma relação PCE/NCE intermediária entre os controles positivos e negativo (i.e., NaCl *grupo Tukey A''*, NUE *grupo Tukey CD''* e DOX *grupo Tukey D''*). Quanto aos ensaios anti-mutagênicos, a relação PCE/NCE mostrou-se similar entre os controles positivos e a associação POHALS + DOX *grupo Tukey CD''/D''*, ou ainda, entre os controles positivos e a associação POHALS + NUE *grupo Tukey D''*. Esses resultados apontam uma leve

toxicidade sistêmica do POHALS, independentemente da dose terapêutica administrada. Ainda, toxicidade sistêmica evidente do DOX sob dosagem de 5 mg.Kg^{-1}, independentemente da concomitante associação com a administração do POHALS e, portanto, ausência de proteção contra os efeitos genotóxicos induzidos pela DOX na medula óssea de camundongos.

A continuação do ensaio do micronúcleo empregando-se outra fonte de óleo de girassol (i.e., óleo industrializado para fins alimentícios – FOHALS) revelou um resultado estatisticamente similar àquele encontrado no POHALS, exceto pelo fato da existência de diferenças significativas ($p < 0,05$) nos números e freqüências de MNPCEs entre os tempos de tratamento. As análises estatísticas obtidas a partir da relação PCE/NCE, para cada um dos tratamentos experimentais mutagênicos e anti-mutagênicos, mantiveram a inexistência de diferenças significativas ($p < 0,05$) entre os tempos (24 e 48 horas) e a existência de tais diferenças entre os gêneros dos camundongos (masculino e feminino). Da mesma forma, as análises obtidas a partir dos números e freqüências de MNPCEs revelaram diferenças estatísticas ($p < 0,05$) entre os tratamentos controle positivos (i.e., NUE *grupo Tukey B e B'* e DOX *grupo Tukey A e A'*) e experimentais mutagênicos ou controle negativo (i.e., 250-2.000 mg.Kg^{-1} do FOHALS e NaCl *grupo Tukey C e C*), ou ainda, entre os tratamentos mutagênicos (i.e., 250-2.000 mg.Kg^{-1} FOHALS *grupo Tukey C e C'*) e anti-mutagênicos (i.e., NUE + FOHALS *grupo Tukey A e A'* e DOX + FOHALS *grupo Tukey A e A'*) (Tabela 3). Esses resultados apontam para as seguintes sugestões: (*i*) inexistência de genotoxicidade (mecanismos clastogênicos e/ou aneugênicos) do FOHALS, independentemente da dose de administração fitoterápica (250-2.000 mg.Kg^{-1}). Contudo, diferenças nas freqüências de MNPCEs podem ser encontradas entre os tempos de tratamento (24 e 48 horas) e os gêneros (machos e fêmeos), haja vista o aumento na proporção de MNPCEs no tempo de 48h e no gênero feminino para a maioria das concentrações do tratamento experimental mutagênico (250-1.500 mg.Kg^{-1}). Tal hipótese foi fortemente suportada pelos resultados das alterações genéticas espontâneas observadas no tratamento controle negativo (NaCl 150 mM); (*ii*) inexistência dos efeitos anti-genotóxicos (anti-clastogenia e/ou anti-aneugenia) do FOHALS (2.000 mg.Kg^{-1}) quando associado ao tratamento quimioterápico do DOX (5 mg.Kg^{-1}) e, portanto, ausência de proteção contra os efeitos genotóxicos induzidos pelo DOX na medula óssea de camundongos.

Ainda, as análises obtidas a partir da relação PCE/NCE revelaram diferenças estatísticas ($p < 0,05$) entre as menores e maiores concentrações do FOHALS administradas nos tratamentos experimentais mutagênicos (i.e., 250 mg.Kg^{-1} *grupo Tukey B''*, 500 mg.Kg^{-1} *grupo*

grupo Tukey BC'' e 1.000-2.000 mg.Kg^{-1} *grupo Tukey C''*), as quais também revelaram uma relação PCE/NCE compatível com àquelas observadas nos controles positivos (i.e., NUE *grupo Tukey C''* e DOX *grupo Tukey BC''*). Quanto aos ensaios anti-mutagênicos (i.e., FOHALS + DOX *grupo Tukey C''* e FOHALS + NUE *grupo Tukey C''*), a relação PCE/NCE mostrou padrões estatisticamente similares aos controles positivos (i.e., NUE *grupo Tukey C''* e DOX *grupo Tukey BC''*) e os ensaios mutagênicos (exceto na concentração de 250 mg.Kg^{-1} de FOHALS *grupo Tukey B''*). Esses achados apontam para a existência de toxicidade sistêmica do FOHALS, considerando dosagens ≥ 500 mg.Kg^{-1}. Ainda, toxicidade sistêmica evidente do DOX sob dosagem de 5 mg.Kg^{-1}, independentemente da administração concomitante do FOHALS e, portanto, confirmando mais uma vez a ausência da proteção contra os efeitos genotóxicos induzidos pelo DOX na medula óssea de camundongos.

Estudo genotóxico do azeite de girassol e azeite de girassol ozonizado (dose limite de 2 g.kg^{-1}.d^{-1} baseando-se na evidência de toxicidade dos estudos subcrônicos por via intragástrica do produto) foi feito usando o ensaio MN na medula óssea de camundongos (linhagem Cenp: NMRI de ambos os gêneros) (MONTERO et al., 1998). Neste estudo, os tratamentos com o óleo de girassol não provocaram danos citotóxicos nas linhagens eritrocitárias estudadas, conforme relatado nas análises de relação PCE/NCE, os quais não corroboram com os nossos achados a partir dos óleos farmacêutico e industrializado. Contudo, esta pesquisa mantém a hipótese de que nenhum efeito clastogênico ocorre na medula óssea dos animais tratados com o óleo de girassol sob as condições experimentais (MONTERO et al., 1998).

Outros estudos têm investigado a adequação de diferentes óleos vegetais para a dieta humana, reportando sobre reduções na genotoxicidade e/ou potencialização do câncer pelo óleo de gergilim (SALERNO & SMITH, 1991), óleo de girassol (COGNAULT et al., 2000), óleo de perilla e palma (NAKAYAMA et al., 1993), óleos de oliva, girassol, amendoim, milho e soja (LA VECCHIA et al., 1995), óleo de semente de linho (RAO et al., 2000), e óleo de coco (BURNS et al., 1978), entre outros. Os ácidos graxos, um principal componente dos óleos vegetais, também tem sido estudados em relação ao seu possível papel na modulação da genotoxicidade e carcinogenicidade. A atividade genotóxica de óleos vegetais [óleos de sementes de gergelim, girassol, germe de trigo, linho, soja e ambos óleos de oliva extra-virgem de primeira classe e baixo grau (refinado)] consumidos por humanos foram também testados pelo teste de recombinação e mutação somática de *Drosophila* (*Drosophila melanogaster* SMART assay) (ROJAS-MOLINA et al., 2005). Óleo de linho produziu a resposta mais forte, enquanto que os óleos de gergelim, germe de trigo e soja mostraram

alguma atividade genotóxica. Óleos de girassol e de oliva de baixo grau forneceram resultados inconclusivos ou diagnósticos biológicos negativos, possivelmente devido a baixa concentraçao de PUFAs, mesmo como produtos refinados, e o óleo de oliva extra-virgem foi claramente não-genotóxico. Argumenta-se que a genotoxiciddade é provavelmente devido a composiçao do ácido graxo dos óleos, os quais após peroxidação podem formar adutos de DNA especifícos. Tais resultados foram em geral concordantes com evidências dos estudos epidemiológicos e experimentais sumarizados por Bartsch et al. (1999): que n-PUFAs estão relacionados a produção de dado oxidativo ao DNA; que uma alta ingestão de n-6 PUFAs está envolvida com alguns tipos de cânceres; e que n-9 MUFAs (*monounsaturated fatty acid* – MUFA) e n-3 PUFAs podem ter um papel na prevenção do câncer. Adicionalmente, foi também sugerido que as concentrações relativas de ácido linolénico C18:3 n-3 de cadeia curta, ácido linoléico C18:2 n-6, e polifenóis são os principais fatores responsáveis pela genotoxicidade dos óleos de cozinha no ensaio SMART (ROJAS-MOLINA et al., 2005). Apesar da existência dessas informações, dados contraditórios ou inconclusivos foram encotrado na literatura. Por exemplo, um estudo encontrou que o ácido linoléico (C18:2 n-6 PUFA) suprimiu a proliferação de células cancerígenas (BOOVENS et al., 1984), enquanto que outros estudos indicam um efeito promotor sobre a carcinogênese (APPEL et al., 1994; JOHANNING & LIN, 1995; NEWCOMER et al., 2001). O ácido oléico (C18:1, n-9 MUFA), um promotor da prolifereação de células cancerígenas (BOOVENS et al., 1984), tem sido reportado para ser um agente anti-mutagênico e anti-cancerígeno efetivo (SIEGEL et al., 1987; IWADO et al., 1991). O ácido linolênico (C18:3 *short-chain* n-3 PUFA) mostrou atividade anti-cancerígena em alguns estudos (BOOVENS et al., 1984; BÉGIN et al., 1985), porém promoveu câncer em outros estudos (RAMON et al., 2000; NEWCOMER et al., 2001). Compostos fenólicos, um outro constituinte importante dos óleos vegetais, estão presentes na fase lipídica não-saponificável. Os fenólicos estão envolvidos em ambos os processos extra- e intra-cellulares, incluindo mecanismos citosólicos de desintoxicação, ativação de enzima microssomal, e a eliminação de radicais livres (DEFLORA & RAMEL, 1988; VISIOLI et al., 1998). As evidências indicam que os polifenóis podem inibir a mutagenicidade de genotoxicantes (SANTOS et al., 1999; WEISBURGER, 2000) e funcionam como agentes anti-câncer (KATIYAR et al., 1997).

Os efeitos citotóxicos e clastogênicos do óleo de girassol aquecido foram estudados respectivamente em linfócitos, hepatócitos (HepG2) e em células endoteliais da veia umbilical humana (*human umbilical vein endothelial cells* – HUVEC) (INDART et al., 2007). Em linfócitos incubados com extrato aquoso de óleo de girassol aquecido contendo 0,075 ou 0,15

μM de substâncias que reagem com tiobarbitúrico (esse extrato teve um alto conteúdo de aldeídos polares), a taxa de quebras cromossômicas foi de 18,4% e 23,1%, comparado com 8,7% e 6,6%, ou 8,1% e 9.2%, respectivamente em linfócitos incubados com o mesmo volume de um extrato aquoso de óleo não-aquecido ou água destilada. Em células HepG2 ou HUVEC, as propriedades citotóxicas do óleo de girassol aquecido foram dose dependentes, iniciando a citotoxicidade em concentrações tão baixa quanto 0,25 μM. Em contrapartida, o mesmo volume do óleo não-aquecido ou da água destilada foi não-tóxico para essas células. Os resultados obtidos mostraram que um extrato aquoso obtido a partir do óleo aquecido é clastogênico e, em altas doses, citotóxico. Esses dados sugeriram que o extrato aquoso, obtido de óleos culinários submetidos ao estresse térmico, com um alto conteúdo de aldeídos é clastogênico e citotóxico. Especula-se que a ingestão de guandes quantidades desses produtos pode ser também relevante à saúde humana, especialmente naquelas doenças secundárias às quebras cromossômicas tais como determinadas malformações congênitas e certos tipos de cânceres. Este último fato pode ser reforçado pelos resultados previamente demonstrados em que a administração de óleo de girassol estressado termicamente aos ratos é teratogênico (INDART et al., 2002). Compostos de aldeídos mostraram capacidades de serem absorvidos do intestino para a circulação (JENKINSON et al., 1999; GROOTVELD et al., 2001) e induzir diferentes classes de danos tais como a geração de células espumosas a partir de macrófados, devido a elevada expressão do receptor necrófago, CD36 (VIANA et al., 2005), ou a depleção de diferentes antioxidantes tal como a glutatinona *in vivo* (GROOTVELD et al., 1999, 2001).

A genotoxicidade de drogas anti-câncer é de especial interesse devido ao risco de indução de neoplasias secundárias. Pacientes submetidos aos tratamentos com agentes quimioterápicos para mieloma múltiplo, doença de Hodgkin, e tumores ovarianos estão em risco para o desenvolvimento de tumores secundários após a contemplação do tratamento (CANELLOS et al., 1975; CASCIATO & SCOTT, 1979; FERGUSON & PEARSON, 1996). O uso de agentes citoprotetores representa um método alternativo para a redução da toxicidade radioterapêutica e quimioterapêutica em tecidos normais (HOSPERS et al., 1999), prevenindo assim o risco dos efeitos potencialmente genotóxicos tal como a formação de tumor secundário (FERGUSON & PEARSON, 1996). Para aqueles agentes, cuja dose máxima tolerada é limitada inicialmente pela mielossupressão, várias estratégias estão sendo avaliadas para permitir o escalonamento da dose, na esperança de obter melhores resultados clínicos com os medicamentos quimioterapêuticos atualmente disponíveis (VENKATESH et al., 2007).

A doxorrubicina (DOX) é um importante agente anti-câncer entre as antraciclinas. Essa droga é considerada um componente importante de vários regimes quimioterapêutico para o carcinoma de mama e o carcinoma de pequenas células de pulmão. Durante a metástase do carcinoma da tireóide, a DOX é provavelmente o melhor agente disponível (VENKATESH et al., 2007). A DOX é também um importante agente para o tratamento bem sucedido da doença de Hodgkin e linfomas de não- Hodgkin (YOUNG et al., 1981). Uma relação dose-resposta para a DOX em vários regimes curativos tem sido mostrada, e doses diminuídas resultam taxas de sobrevivência inferior (HITCHCOCK-BRYAN et al., 1986), enquanto que um aumento na dose de DOX é limitada devido à grave cardiotoxicidade, um principal problema na aplicação clínica de DOX. Existe um amplo corpo de evidência para mostrar que o alvo celular dominante da DOX é o DNA (MYERS, 1998). As antraciclinas são classificadas como inibidores da topoisomerase II, porque elas estabilizam eficazmente o complexo de clivagem dessa enzima (e.g., Topoisomerase II desempenha um importante papel na segregação de partes recentemente replicadas de cromossomos entrelaçados (HOLM et al., 1985; UEMURA & YANAGIDA, 1986), condensação e descondensação dos cromossomos, e superenrolamento do DNA intracelular (UEMURA et al., 1987; YANAGIDA & STERNGLANZ, 1990). A DNA topoisomerase II catalisa a quebra e a reunião de ambas as fitas de DNA, relaxa a torção superhelicoidal, e concatena ou desconcatena o DNA circular. A DNA topoisomerase II realiza essas transformações topológicas transportando um segmento de DNA de fita dupla através de uma quebra de fita dupla transitória mediado pela enzima num outro DNA (LIU et al., 1980)). O efeito genotóxico mais prontamente detectável de agentes que interagem com a topoisomerase em células somáticas é a formação de um complexo ternário consistindo de droga, estabilizando assim a quebra do DNA de fita dupla (ANDERSON & BERGER, 1994). A DOX é um potente inibidor da DNA topoisomerase II, fato este que estabiliza a quebra do DNA fita dupla e não permite a reunião delas, conduzindo à morte celular (KAUFMANN, 1989; DEL BINO et al., 1991; TREVINO et al., 2004). Essas quebras de DNA fita dupla podem subseqüentemente conduzir às quebras cromossômicas que finalmente conduzirão à produção de MN, com subseqüente divisão celular (NATARAJAN et al., 1986). A persistência de lesões de DNA em células tratadas com DOX tem sido confirmada pelo ensaio do MN. A incidência de micronúcleos mostra uma resposta á dose em triagem de larga escala para clastógenos suspeitos (HEDDLE et al., 1983). A atividade anti-tumor das antraciclinas é consistente com sua habilidade para interagir com o DNA celular, e essa interação não-covalente pode inibir a função de DNA ligases, helicases, e outroas proteínas dependentes de

DNA, em adição a topoisomerase II. Contudo, seus efeitos celulares estão de modo algum limitados a esses tipos de inibição enzimática. A DOX exibe uma ampla variedade de efeitos celulares, e é provável que nenhum único mecanismo de ação seja responsável pelo total de respostas clínicas e celulares observadas (MYERS, 1998; WEISS, 2006). A DOX forma um complexo estável com o ferro férrico, o qual reage com o oxigênio, formando ânions de superóxido, peróxido de hidrogênio e radicais hidroxilas. DOX é conhecida por causar quebras de DNA dupla fita, rearranjos cromossômicos e eventos mutacionais, e é um potente carcinógeno (ZWEIER, 1984). DOX tem sido reportado para induzir a formação de MN, cromatídio e aberrações cromossômicas: quebras de DNA fita simples e dupla *in vitro* e *in vivo* (BEAN et al., 1992; AL-HARBI, 1993; AL-SHABANAH, 1993; DELVAEYE et al., 1993; JAGETIA & NAYAK, 1996; JAGETIA & NAYAK, 2000; JAGETIA & ARUNA, 2000; SHAN et al., 1996; DHAWAN et al., 2003). A principal toxicidade aguda induzida pela DOX é a supressão da medula óssea, e a utilidade clínica em longo prazo é limitada por uma cardiotoxicidade crônica irreversível dependente de dose cumulativa, que se manifesta como insuficiência cardíaca congestiva ou cardiomiopatia (VAN ACKER et al., 1995; VAN ACKER et al., 2000).

Portanto, é essencial rastrear novos agentes farmacológicos que podem proteger as células normais contra a toxicidade cumulativa induzida pela DOX. As plantas, em virtude de sua ampla utilidade em medicina tradicional, são menos tóxicas e tem atraído à atenção de pesquisadores em torno do mundo no recente passado. As plantas contêm muitos compostos, e provavelmente essas possam vir a proporcionar melhores efeitos protetores do que uma única molécula (VIDHYA & DEVRAJ, 1999). A presença de muitas moléculas em plantas pode ser vantajosa, já que algumas delas podem neutralizar a toxicidade de outras, e como um resultado, o efeito líquido pode ser benéfico para o propósito terapêutico. Por exemplo, o efeito de várias concentrações (200, 250, 300, 350 e 400 mg/kg de massa corpórea) de *Aegle marmelos* sobre os efeitos genotóxicos induzidos por DOX na medula óssea de camundongos foi estudado (VENKATESH et al., 2007). O tratamento de camundongos com diferentes concentrações de DOX (5, 10, ou 15 $mg.kg^{-1}$ de massa corpórea) resultou em uma elevação dependente de dose na freqüência de MNPCE bem como NCE na medula óssea, e foi acompanhado por um declínio dependente de dose da DOX na relação PCE/NCE. O tratamento oral de camundongos com *Aegle marmelos*, uma vez ao dia durante cinco dias consecutivos antes do tratamento da DOX, significativamente reduziu a freqüência de micronúcleos induzidos pela DOX acompanhado por uma significante elevação na relação PCE/NCE em todos os momentos de pontuação. Esse efeito quimioprotetor observado pode

ser devido à soma total de interações entre diferentes ingredientes dessa mistura complexa. O grau de proteção dependeria da interação de componentes isoladamente ou em conjunto com o agente genotóxico. Os mecanismos de ação plausíveis de *Aegle marmelos* na proteção contra o insulto genômico induzido por DOX foram a eliminação de $O_2^{\bullet-}$ e $^{\bullet}OH$ e outros radicais livres, aumento da capacidade antioxidante, restauração da atividade de topoisomerase II e a inibição da formação do complexo ferro-DOX (VENKATESH et al., 2007). Outro estudo foi realizado para avaliar o potencial genotóxico do extrato hidroalcoólico da folha de *Copaifera lansdorffii* Desf. e sua influência sobre a genotoxidade induzida pelo agente quimioterapêutico DOX usando o ensaio do MN em sangue periférico de camundongos *Swiss*. Tais resultados demonstraram que *C. lansdorffii* Desf. não foi genotóxico e, ainda, em animais tratados com *C. lansdorffii* Desf. e DOX, o número de MN foi significativamente reduzido quando comparado aos animais que receberão apenas DOX. A atividade antioxidante putativa de um ou mais compostos ativos de *C. lansdorffii* Desf., entre eles dois principais flavonóides heterosídeos (quercitrina e afzelina), podem explicar o efeito dessa planta sobre a genotoxidade de DOX (ALVES et al., 2013).

A relação PCE/NCE é um indicador da aceleração ou inibição de eritropoiese. Essa relação tem sido reportada para variar com o tempo de pontuação, e o declínio contínuo na relação PCE/NCE pode ser devido à inibição da divisão celular, morte de eritroblastos, remoção de células danificadas e/ou diluição do conjunto de células existentes com células recentemente formadas (VENKATESH et al., 2007). Vários mecanismos podem ter sido responsáveis pela citotoxicidade da DOX e a indução de micronúcleos (GEWIRTZ, 1999), incluindo a intercalação de DOX no DNA celular (PAINTER, 1978; KIYOMIYA et al., 2001), estabilização do complexo DNA-topoisomerase II (POMMIER et al., 1985; GUANO et al., 1999), toxicidade mediada por radicais livres causado pela ciclagem redox do radical semiquinona (BACHUR et al., 1979) ou formação de espécies de oxigênio reativo pelo complexo ferro-DOX (MYERS, 1998; ELIOT et al., 1984; KONOREV et al., 1999). Por outro lado, outros agentes químicos tais como o captopril e a desferrioxamina (AL-HARBI, 1993; AL-SHABANAH, 1993), β-caroteno e vitaminas A, C e E (LU et al., 1996; GULKAC et al., 2004; COSTA & NEPOMUCENO, 2006), tiol N-acetilcisteína, probucol, lovastatina e flavonóides hidrofílicos, tais como rutina e luteolina (D'AGOSTINI et al., 1998; AL-GHARABLY, 1996; BARDELEBEN et al., 2002; SADZUKA et al., 1997) têm sido reportados para reduzir os MNs / genotoxicidade / toxicidade induzidos por DOX em camundongos. Ainda, os defensores da medicina herbal sempre afirmam que as misturas são melhores do que as substâncias químicas puras, porque dezenas de compostos biologicamente

ativos em plantas trabalham juntos para produzir um efeito maior do que qualquer uma substância química por si mesma (MACKENZIE, 2001).

Tabela 1. Incidência de PCEMNs e relação de PCE/NCE em medula óssea de camundongos *Swiss albinus* (machos e fêmeas) após tratamentos (24 e 48 horas) controles$^{+/-}$ (NaCl, NUE e DOX), experimental mutagênico (THALS) e anti-mutagênico (THALS + NUE e THALS + DOX). Análise estatística de variância *one-way* (ANOVA), em delineamento inteiramente casualizado no esquema fatorial 10 × 2 × 2 (tratamento × sexo × tempo), e comparação de médias por meio do teste de Tukey (α = 0,05) empregando o sistema computacional SAS® versão 9.2.

Tratamento	Número de PCEs analisados		PCEMN				PCE/NCE		NCE (*n*)	
	24h	48h	24h (*n*)	48h (*n*)	24h (%)	48h (%)	24h	48h	24h	48h
NaCl 150 mM										
Σ ♀	6276	6281	27	28	0,43	0,45	261,50	330,58	24	19
Σ ♂	6208	6260	28	35	0,45	0,56	67,48	156,50	92	40
Σ	12484	12541	55 ^{C}	63 ^{C}	0,44 $^{C'}$	0,50 $^{C'}$	107,62 $^{A''}$	212,56 $^{A''}$	116	59
N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀	6034	6055	85	101	1,41	1,67	1,17	3,47	5166	1745
Σ ♂	6057	5984	194	109	3,20	1,82	1,04	0,96	5843	6216
Σ	12091	12039	279 ^{B}	210 ^{B}	2,31 $^{B'}$	1,74 $^{B'}$	1,10 $^{D''}$	1,51 $^{D''}$	11009	7961
Cloridrato de doxorrubicina (5 mg.Kg^{-1})										
Σ ♀	6253	6186	179	149	2,86	2,41	5,01	28,91	1247	214
Σ ♂	6212	6203	155	181	2,50	2,92	70,59	31,49	88	197
Σ	12465	12389	334 ^{A}	330 ^{A}	2,68 $^{A'}$	2,66 $^{A'}$	9,34 $^{CD''}$	30,14 $^{CD''}$	1335	411
Tintura de sementes de H. annuus L. (THALS: girassol)										
Σ ♀	6383	6341	27	31	0,42	0,49	99,73	132,10	64	48
Σ ♂	6146	6197	26	25	0,42	0,40	109,75	140,84	56	44
Σ $_{250\ mg.kg^{-1}}$	12529	12538	53 ^{C}	56 ^{C}	0,42 $^{C'}$	0,45 $^{C'}$	104,41 $^{B''}$	136,28 $^{B''}$	120	92
Σ ♀	6236	6252	37	40	0,59	0,64	86,61	105,97	72	59
Σ ♂	6217	6223	40	29	0,64	0,47	74,90	141,43	83	44
Σ $_{500\ mg.kg^{-1}}$	12453	12475	77 ^{C}	69 ^{C}	0,62 $^{C'}$	0,55 $^{C'}$	80,34 $^{BC''}$	121,12 $^{BC''}$	155	103
Σ ♀	6228	6302	38	45	0,61	0,71	54,63	82,92	114	76
Σ ♂	6244	6256	42	29	0,67	0,46	66,43	68,00	94	92
Σ $_{1.000\ mg.kg^{-1}}$	12472	12558	80 ^{C}	74 ^{C}	0,64 $^{C'}$	0,59 $^{C'}$	59,78 $^{BCD''}$	74,75 $^{BCD''}$	208	168
Σ ♀	6144	6324	37	46	0,60	0,73	52,51	64,53	117	98
Σ ♂	6206	6203	44	35	0,71	0,56	43,10	60,22	144	103
Σ $_{1.500\ mg.kg^{-1}}$	12350	12527	81 ^{C}	81 ^{C}	0,66 $^{C'}$	0,65 $^{C'}$	47,36 $^{BCD''}$	62,32 $^{BCD''}$	261	201
Σ ♀	6186	6182	47	48	0,76	0,78	39,65	52,39	156	118
Σ ♂	6256	6170	48	37	0,77	0,60	40,62	47,46	154	130
Σ $_{2.000\ mg.kg^{-1}}$	12442	12352	95 ^{C}	85 ^{C}	0,76 $^{C'}$	0,69 $^{C'}$	40,14 $^{CD''}$	49,81 $^{CD''}$	310	248
Tintura de sementes de H. annuus L. (THALS: 2.000 mg.kg^{-1}) + N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀	6223	6207	89	92	1,43	1,48	80,82	15,79	77	393
Σ ♂	6229	6228	103	109	1,65	1,75	87,73	86,50	71	72
Σ $_{2.000\ mg.kg^{-1}}$	12452	12435	192 ^{B}	201 ^{B}	1,54 $^{B'}$	1,62 $^{B'}$	84,14 $^{BCD''}$	26,74 $^{BCD''}$	148	465
Tintura de sementes de H. annuus L. (THALS: 2.000 mg.kg^{-1}) + Cloridrato de doxorrubicina (5 mg.Kg^{-1})										
Σ ♀	6223	6236	106	103	1,70	1,65	80,82	97,44	77	64
Σ ♂	6251	6248	97	96	1,55	1,54	127,57	120,15	49	52
Σ $_{2.000\ mg.kg^{-1}}$	12474	12484	203 ^{B}	199 ^{B}	1,63 $^{B'}$	1,59 $^{B'}$	99,00 $^{BC''}$	107,62 $^{BC''}$	126	116

As letras A, B, C e D correspondem à escala padronizada de Tukey (letras idênticas não são significativamente diferentes. $p < 0,05$).

Tabela 2. Incidência de PCEMNs e relação de PCE/NCE em medula óssea de camundongos *Swiss albinus* (machos e fêmeas) após tratamentos (24 e 48 horas) controles$^{+/-}$ (NaCl, NUE e DOX), experimental mutagênico (POHALS) e anti-mutagênico (NUE + POHALS e POHALS + DOX). Análise estatística de variância *one-way* (ANOVA), em delineamento inteiramente casualizado no esquema fatorial 10 × 2 × 2 (tratamento × sexo × tempo), e comparação de médias por meio do teste de Tukey (α = 0,05) empregando o sistema computacional SAS® versão 9.2.

Tratamento	Número de PCEs analisados		PCEMNs				PCE/NCE		NCE	
	24h	48h	24h (*n*)	48h (*n*)	24h (%)	48h (%)	24h	48h	24h (*n*)	48h (*n*)
NaCl 150 mM										
Σ ♀	6276	6281	27	28	0,43	0,45	261,50	330,58	24	19
Σ ♂	6208	6260	28	35	0,45	0,56	67,48	156,50	92	40
Σ	12484	12541	55 ^{C}	63 ^{C}	0,44 $^{C'}$	0,50 $^{C'}$	107,62 $^{A''}$	212,56 $^{A''}$	116	59
N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀	6034	6055	85	101	1,41	1,67	1,17	3,47	5166	1745
Σ ♂	6057	5984	194	109	3,20	1,82	1,04	0,96	5843	6216
Σ	12091	12039	279 ^{B}	210 ^{B}	2,31 $^{B'}$	1,74 $^{B'}$	1,10 $^{D''}$	1,51 $^{D''}$	11009	7961
Cloridrato de doxorrubicina (5 mg.Kg^{-1})										
Σ ♀	6253	6186	179	149	2,86	2,41	5,01	28,91	1247	214
Σ ♂	6212	6203	155	181	2,50	2,92	70,59	31,49	88	197
Σ	12465	12389	334 ^{A}	330 ^{A}	2,68 $^{A'}$	2,66 $^{A'}$	9,34 $^{CD''}$	30,14 $^{CD''}$	1335	411
Óleo farmacêutico de sementes de H. annuus L. (POHALS: girassol)										
Σ ♀ ^{B}	6257	6263	22	23	0,35	0,37	184,03	202,03	34	31
Σ ♂ ^{A}	6256	6254	26	40	0,42	0,64	142,18	152,54	44	41
Σ $_{250\ mg.kg^{-1}}$	12513	12517	48 ^{C}	63 ^{C}	0,38 $^{C'}$	0,50 $^{C'}$	160,46 $^{B''}$	173,85 $^{B''}$	78	72
Σ ♀ ^{B}	6102	6251	23	24	0,38	0,38	179,47	195,34	34	32
Σ ♂ ^{A}	6198	6210	44	45	0,71	0,72	134,74	135,00	46	46
Σ $_{500\ mg.kg^{-1}}$	12300	12461	67 ^{C}	69 ^{C}	0,54 $^{C'}$	0,55 $^{C'}$	153,75 $^{B''}$	159,76 $^{B''}$	80	78
Σ ♀ ^{B}	6262	6257	35	36	0,56	0,58	173,94	195,53	36	32
Σ ♂ ^{A}	6242	6249	52	53	0,83	0,85	132,81	135,85	47	46
Σ $_{1.000\ mg.kg^{-1}}$	12504	12506	87 ^{C}	89 ^{C}	0,69 $^{C'}$	0,71 $^{C'}$	150,83 $^{B''}$	160,33 $^{B''}$	83	78
Σ ♀ ^{B}	6266	6266	37	38	0,59	0,61	164,89	184,29	38	34
Σ ♂ ^{A}	6253	6236	56	58	0,90	0,93	107,81	119,92	58	52
Σ $_{1.500\ mg.kg^{-1}}$	12519	12502	93 ^{C}	96 ^{C}	0,74 $^{C'}$	0,77 $^{C'}$	130,29 $^{B''}$	145,37 $^{B''}$	96	86
Σ ♀ ^{B}	6264	6263	43	48	0,69	0,77	145,67	169,27	43	37
Σ ♂ ^{A}	6204	6238	57	62	0,92	0,99	64,63	69,31	96	90
Σ $_{2.000\ mg.kg^{-1}}$	12468	12501	100 ^{C}	110 ^{C}	0,80 $^{C'}$	0,88 $^{C'}$	89,70 $^{BC''}$	98,43 $^{BC''}$	139	127
Óleo farmacêutico de sementes de H. annuus L. (POHALS: 2.000 mg.kg^{-1}) + N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀ ^{B}	6121	6129	182	176	2,97	2,87	2,57	2,06	2379	2971
Σ ♂ ^{A}	6057	6139	149	160	2,46	2,61	1,99	2,98	3043	2061
Σ $_{2.000\ mg.kg^{-1}}$	12178	12268	331 ^{A}	336 ^{A}	2,72 $^{A'}$	2,74 $^{A'}$	2,25 $^{D''}$	2,44 $^{D''}$	5422	5032
Óleo farmacêutico de sementes de H. annuus L. (POHALS: 2.000 mg.kg^{-1}) + Cloridrato de doxorrubicina (5 mg.Kg^{-1})										
Σ ♀ ^{B}	6120	6247	179	160	2,92	2,56	2,05	2,77	2980	2253
Σ ♂ ^{A}	6194	6075	173	155	2,79	2,55	1,93	3,33	3206	1825
Σ $_{2.000\ mg.kg^{-1}}$	12314	12322	352 ^{A}	315 ^{A}	2,86 $^{A'}$	2,56 $^{A'}$	1,99 $^{D''}$	3,02 $^{D''}$	6186	4078

As letras A, B, C e D correspondem à escala padronizada de Tukey (letras idênticas não são significativamente diferentes. $p < 0,05$).

Tabela 3. Incidência de PCEMNs e relação de PCE/NCE em medula óssea de camundongos *Swiss albinus* (machos e fêmeas) após tratamentos (24 e 48 horas) controles$^{+/-}$ (NaCl, NUE e DOX), experimental mutagênico (FOHALS) e anti-mutagênico (FOHALS + NUE e FOHALS + DOX). Análise estatística de variância *one-way* (ANOVA), em delineamento inteiramente casualizado no esquema fatorial 10 × 2 × 2 (tratamento × sexo × tempo), e comparação de médias por meio do teste de Tukey (α = 0,05) empregando o sistema computacional SAS® versão 9.2.

Tratamento	Número de PCEs analisados		PCEMNs				PCE/NCE		NCE	
	24h	48h	24h (*n*) ^{A}	48h (*n*) ^{B}	24h (%) ^{A}	48h (%) ^{B}	24h	48h	24h (*n*)	48h (*n*)
NaCl 150 mM										
Σ ♀	6276	6281	27	28	0,43	0,45	261,50	330,58	24	19
Σ ♂	6208	6260	28	35	0,45	0,56	67,48	156,50	92	40
Σ	12484	12541	55 ^{C}	63 ^{C}	0,44 $^{C'}$	0,50 $^{C'}$	107,62 $^{A''}$	212,56 $^{A''}$	116	59
N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀	6034	6055	85	101	1,41	1,67	1,17	3,47	5166	1745
Σ ♂	6057	5984	194	109	3,20	1,82	1,04	0,96	5843	6216
Σ	12091	12039	279 ^{B}	210 ^{B}	2,31 $^{B'}$	1,74 $^{B'}$	1,10 $^{C''}$	1,51 $^{C''}$	11009	7961
Cloridrato de doxorrubicina (5 mg.Kg^{-1})										
Σ ♀	6253	6186	179	149	2,86	2,41	5,01	28,91	1247	214
Σ ♂	6212	6203	155	181	2,50	2,92	70,59	31,49	88	197
Σ	12465	12389	334 ^{A}	330 ^{A}	2,68 $^{A'}$	2,66 $^{A'}$	9,34 $^{BC''}$	30,14 $^{BC'}$	1335	411
Óleo alimentício de sementes de H. annuus L. (FOHALS: girassol)										
Σ ♀ ^{A}	6425	6108	27	30	0,42	0,49	146,02	244,32	44	25
Σ ♂ ^{B}	6204	6213	22	23	0,35	0,37	39,02	28,37	159	219
Σ $_{250\ mg.kg^{-1}}$	12629	12321	49 ^{C}	53 ^{C}	0,39 $^{C'}$	0,43 $^{C'}$	62,21 $^{B''}$	50,50 $^{B''}$	203	244
Σ ♀ ^{A}	6031	6175	27	35	0,45	0,57	26,69	89,49	226	69
Σ ♂ ^{B}	6153	6227	23	35	0,37	0,56	20,79	21,70	296	287
Σ $_{500\ mg.kg^{-1}}$	12184	12402	50 ^{C}	70 ^{C}	0,41 $^{C'}$	0,56 $^{C'}$	23,34 $^{BC''}$	34,84 $^{BC''}$	522	356
Σ ♀ ^{A}	6359	6231	30	38	0,47	0,61	23,64	27,57	269	226
Σ ♂ ^{B}	6141	6181	26	38	0,42	0,61	17,85	15,08	344	410
Σ $_{1.000\ mg.kg^{-1}}$	12500	12412	56 ^{C}	76 ^{C}	0,45 $^{C'}$	0,61 $^{C'}$	20,39 $^{C''}$	19,52 $^{C''}$	613	636
Σ ♀ ^{A}	6174	6174	30	39	0,49	0,63	14,00	7,80	441	792
Σ ♂ ^{B}	6156	6303	29	45	0,47	0,71	14,62	12,68	421	497
Σ $_{1.500\ mg.kg^{-1}}$	12330	12477	59 ^{C}	84 ^{C}	0,48 $^{C'}$	0,67 $^{C'}$	14,30 $^{C''}$	9,68 $^{C''}$	862	1289
Σ ♀ ^{A}	6256	6042	33	41	0,53	0,68	10,88	7,04	575	858
Σ ♂ ^{B}	6179	6490	44	47	0,71	0,72	13,82	9,64	447	673
Σ $_{2.000\ mg.kg^{-1}}$	12435	12532	77 ^{C}	88 ^{C}	0,62 $^{C'}$	0,70 $^{C'}$	12,17 $^{C''}$	8,19 $^{C''}$	1022	1531
Óleo alimentício de sementes de H. annuus L. (FOHALS: 2.000 mg.kg^{-1}) + N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀ ^{A}	6108	6125	148	155	2,42	2,53	2,66	2,58	2292	2375
Σ ♂ ^{B}	6083	6112	149	205	2,45	3,35	2,02	2,79	3017	2188
Σ $_{2.000\ mg.kg^{-1}}$	12191	12237	297 ^{A}	360 ^{A}	2,44 $^{A'}$	2,94 $^{A'}$	2,30 $^{C''}$	2,68 $^{C''}$	5309	4563
Óleo alimentício de sementes de H. annuus L. (FOHALS: 2.000 mg.kg^{-1}) + cloridrato de doxorrubicina (5 mg.Kg^{-1})										
Σ ♀ ^{A}	6111	6115	165	157	2,70	2,57	3,85	5,64	1589	1085
Σ ♂ ^{B}	6197	6161	212	191	3,42	3,10	2,58	2,10	2403	2939
Σ $_{2.000\ mg.kg^{-1}}$	12308	12276	377 ^{A}	348 ^{A}	3,06 $^{A'}$	2,83 $^{A'}$	3,08 $^{C''}$	3,05 $^{C''}$	3992	4024

As letras A, B e C correspondem à escala padronizada de Tukey (letras idênticas não são significativamente diferentes. $p < 0,05$).

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7. ANEXOS

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DECLARAÇÃO DE REVISÃO ORTOGRÁFICA

Declaro que a Dissertação de Mestrado, do curso de pós-graduação *Stricto Sensu* em Ciência Animal, da Universidade José do Rosário Vellano – UNIFENAS, intitulada **"Ausência de genotoxidade e redução dos efeitos genotóxicos induzidos por dox da tintura de sementes de *Helianthus annuus* linné (girassol) reveladas pelo ensaio do micronúcleo"**, e desenvolvida pelo aluno Luiz Silva de Souza, sob orientação do Prof. Dr. Marcelo Fabiano Gomes Boriollo, passou pelo processo de edição e correção ortográfica.

Campo Belo, 25 de julho de 2013.

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COMITÊ DE ÉTICA EM PESQUISA

PARECER N.º04A/2008

O COMITÊ DE ÉTICA EM PESQUISA – CEP, da UNIFENAS, Setor de Experimentação Animal, tendo analisado, nesta data, o protocolo do projeto de pesquisa intitulado, **Avaliação da mutagenicidade e antimutagenicidade dos extratos hidroalcoólicos de fitoterápicos usando os ensaios do micronúcleo e do cometa em medula óssea e sangue periférico de roedores *in vivo***, de autoria do Prof. Dr. Marcelo Fabiano Gomes Boriollo, resolveu enquadrá-lo na categoria de aprovado para fins de início da pesquisa.

Alfenas, 25 de Abril de 2008.

Profa. Helena Engel Velano
Coordenadora do CEP

COMITÊ DE ÉTICA EM PESQUISA - unifenas

8. ARTIGO CIENTÍFICO

Nongenotoxic effects and a reduction of the DOX-induced genotoxic effects of *Helianthus annuus* Linné (sunflower) seeds revealed by micronucleus assays in mouse bone marrow

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Keywords:

bone marrow / *Helianthus annuus* L. (sunflower) / micronucleus assay / rodents / tincture and oil

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Abstract

Scope: This research evaluated the mutagenicity and anti-mutagenicity of oil and tincture of *H. annuus* L. seeds using the micronucleus assay in bone marrow of mice. The interaction between these preparations and the genotoxic effects of doxorubicin (DOX) was also analysed.

Methods and results: Experimental groups were evaluated after 24-48h of treatment with NEU (N-Nitroso-N-ethylurea) and DOX (positive controls), NaCl, a sunflower tincture (THALS) and two sources of sunflower oils (POHALS and FOHALS). Anti-mutagenic assays were carried out using the sunflower tincture and oils separately and in combination with these controls. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) was significantly different between (*i*) the positive controls and animals treated with THALS and (*ii*) animals treated with THALS and THALS combined with the positive controls. However, a slight mutagenicity was observed in the animals treated with the combination of THALS+DOX. Both sources of oils revealed similar results; in these groups, the frequencies of MNPCEs were similar to those observed in negative controls.

Conclusion: There was no mutagenicity in THALS, POHALS and FOHALS regardless of the dose, time (except FOHALS) and gender (except POHALS and FOHALS). The moderate anti-mutagenic effects of THALS suggest a potential slight protective mechanism against DOX-induced genotoxic effects.

1 Introduction

The cultivated sunflower (*Helianthus annuus* L.) is one of 67 species in the genus *Helianthus*. It is a dicotyledonous plant and a member of the *Compositae* (*Asteraceae*) family, having a typical composite flower [1]. The inflorescence or sunflower head consists of 700–8000 flowers, depending on the cultivar [2]. Diploid, tetraploid and hexaploid species are known [3]. The cultivated sunflower contains 34 chromosomes ($2n = 34$). The genus name for sunflower is derived from the Greek Helios ('sun') and anthos ('flower'). Sunflower seeds are called 'achenes' by botanists and are defined as a dry, simple, one-seeded fruit with the seed attached to the inner wall at only one point. An achene consists of a seed endosperm (often called a kernel, dehulled seed or meat by oil processors) and an adhering pericarp (hull or shell), which is the wall of the fruit [2]. The relative proportion of the hull and kernel in a sunflower seed varies considerably [4]. Non-oilseed sunflower types contain more hull (47%) than oilseed types (20–30%). The composition of the seed is markedly affected by the sunflower variety [4, 5]. Nevertheless, the composition ranges of sunflower dehulled seeds (on a percentage dry weight basis) is as follows [6]: protein $^{20.4–40.0}$; peptides, amino acids and other non-protein nitrogen $^{1–13\%}$; carbohydrates $^{4–10\%}$; lipids $^{47–65\%}$; fatty acids (palmitic acid $^{5–7\%}$, atearic $^{2–6\%}$; arachidic acid $^{0.0–0.3\%}$, oleic acid $^{15–37\%}$; linoleic acid $^{51–73\%}$, and linolenic acid $^{<0.3\%}$); tocopherol $^{0.07\%}$; carotenoids $^{0.01–0.02\%}$; vitamin B1 $^{0.002\%}$; chlorogenic acid (CGA) $^{0.5–2.4\%}$; quinic acid (QA) $^{0.12–0.25\%}$; caffeic acid (CA) $^{0.05–0.29\%}$; total minerals $^{3–4\%}$; potassium $^{0.67–0.75\%}$; phosphorus $^{0.60–0.94\%}$; sulphur $^{0.26–0.32\%}$; magnesium $^{0.35–0.41\%}$; calcium $^{0.08–0.10\%}$; and sodium $^{0.02\%}$ [2, 4, 5, 7–16].

Tocopherols are excellent natural antioxidants that protect oils against oxidative rancidity. The α form has the highest biological vitamin E activity, and the γ form has been reported to have the highest antioxidant activity [17]. The sterols found in sunflower oils include β-sitosterol, stigmasterol, campesterol, δ-5-avenasterol, and δ-7-stigmasterol [18, 19]. Plant sterols are only minimally absorbed by humans, and their ingestion appears to inhibit intestinal cholesterol and bile acid absorption [20]. Most trace metals in refined, bleached and deodorized sunflower seed oil are removed during processing. It is particularly important that copper and iron be removed because these metals greatly reduce the oxidative stability of the oil [21]. Other metals, such as lead and cadmium, are of particular concern due to their toxicity and their supposed link to coronary heart disease and hypertension [22].

Sunflower oil contains high levels of PUFA (69% - polyunsaturated fatty acid) with polyunsaturated fat: saturated fat ratios of approximately 6.4. Meydani and collaborators

(1991) [23] reported that the studies of the carcinogenicity of PUFA in animal models generally support the view documented in the report of the Committee of Diet, Nutrition, and Cancer [24]; this committee proposed positive relationship between cancer incidence, dietary fat, a diet high in linoleic acid content, the promotion of experimental tumorigenesis, and the development of spontaneous tumours, primarily in female mice and rats. Nevertheless, data from diverse human studies investigating risk, incidence, or progression of cancer of the breast and colon indicated that PUFA does not increase risk and may in fact be negatively associated with these cancers [23]. These authors state that 19 of the 48 studies investigating dietary fat and breast or colon cancer (international, cohort case-control, special population at risk, or disease progression) reported data on PUFA (PUFA, vegetable fat, or linoleic acid). In most of these studies, no association between PUFA intake and cancer was reported. However, some studies have reported an association between PUFA intake and cancer; one study reported a positive association with PUFA [25] and four studies reported a negative association between PUFA and colon or breast cancer [26–29], although the designs of these studies were not directly comparable [23]. In another study involving groups of Skh:HR-1 hairless mice, Reeve and collaborators [30] demonstrated a photocarcinogenic response of increasing severity as the polyunsaturated content of the mixed dietary fat (hydrogenated saturated cottonseed oil) was increased, whether the severity was measured as tumour incidence, tumour multiplicity, progression of benign tumours to squamous cell carcinoma, or reduced survival.

Research involving medicinal plants and their extracts has been on the rise in health care due to its easy acceptability, availability and low cost. A considerable portion of the world population uses traditional medicine for their primary health care, and it is presumed that most of this traditional therapy involves the use of plant extracts or their biologically active components [31, 32]. Only approximately 8% of the 100,000 species catalogued in Brazil have been characterized for their chemical properties, and it is estimated that only 1,100 species have been evaluated for therapeutic purposes [33]. A Brazilian pharmaceutical survey reveals an expense of approximately two to three billion dollars a year on import of raw materials used in the production of medicines. Still, this survey shows that 84 % of medicines consumed in the country come from imports and 78-80% of the drugs produced come from multinational companies [34, 35]; these statistics justify the search for alternatives to overcome the international dependence of the Brazilian chemical-pharmaceutical industry.

Biologically active compounds have been recognized for their pharmacological properties, but many of these compounds cannot be introduced as therapeutic agents due to

their toxicological, carcinogenic and mutagenic properties [36–38]. In drug development, the analysis of genotoxicity assays represent a considerable effort, as most pharmaceutical organizations evaluate a new therapeutic agent based on *in vitro* and *in vivo* data genotoxic [39]. In this context, tests to evaluate the mutagenic activity of the plants used by the population as well as their isolated compounds are necessary and important for establishing control measures in widespread use. Furthermore, it is necessary to clarify the mechanisms and conditions that mediate the proposed biological effect before plants are considered as therapeutic agents [40].

The genotoxic effects of a potential mutagen depend on its cellular target(s). Some chemicals need to be metabolized before acquiring their mutagenic capacity [41]. Mutagens can induce genomic changes by directly or indirectly targeting DNA or by binding to proteins involved in the maintenance of genome integrity [42]. The consequences of mutagen-target interactions may lead to different types of DNA damage (DNA adducts, alkali-labile sites, strand breaks) and mutations ranging from single nucleotide changes (gene mutations) to structural (chromosome mutations) or numerical chromosome changes (genome mutations). The fate of the cell is ultimately determined by whether the various lesions inflicted on the genome are repaired or eliminated by apoptosis [43].

As far as genotoxicity studies are concerned, the *in vivo* micronucleus (MN) assay in rodent bone marrow plays a crucial role in the test battery aimed at identifying hazardous mutagens [41]; this assay is especially suited to assessing mutagenic hazards because it allows consideration of multiple factors, such as *in vivo* metabolism, pharmacokinetics and DNA repair processes, even though these processes vary among species, among tissues and among genetic endpoints [44–46]. Because a bone marrow erythroblast develops into a polychromatic erythrocyte (PCE) (i.e., cell generated by the extrusion of its main nucleus), any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Thus the frequency of micronucleated polychromatic erythrocytes (MNPCEs) has been the principal endpoint for MN assays. Furthermore, the measurement of MNPCEs in peripheral blood has been equally amenable to any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated, or which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. Positive results (i.e., an increase in the frequency of micronucleated polychromatic erythrocytes in treated animals) indicate that a substance causes the formation of micronuclei, which are the result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species, whereas negative results indicate that, under

the test conditions, the test substance does not cause the formation of micronuclei in the immature erythrocytes of the test species. Nevertheless, the number of normochromatic erythrocytes (NCE) in the peripheral blood that contain micronuclei among a given number of mature erythrocytes can also be used as the endpoint of this assay [46, 47]. In addition, understanding the genotoxic effects induced by phytotherapeutics by and foods by employing the mammalian *in vivo* MN assay has been the goal of several researchers groups [48–52].

Micronuclei (MN) are small, extranuclear bodies that arise in dividing cells from acentric chromosome fragments, chromatid fragments, whole chromosomes, or whole chromatids that lag behind in anaphase and are not included in the daughter nuclei in telophase [53]. Micronuclei harboring chromosomal fragments may result from direct double-strand DNA breakage, conversion of single-strand breaks (SSBs) into double-strand breaks (DSBs) after cell replication, or the inhibition of DNA synthesis. The misrepair of two chromosome breaks may lead to an asymmetrical chromosome rearrangement, producing a dicentric chromosome and an acentric fragment. Frequently, the centromeres of the dicentric chromosomes are pulled to opposite poles of the cells at anaphase; this results in the formation of a nucleoplasmic bridge (NPB) between the daughter nuclei and an acentric fragment that lags behind to form a MN [54, 55]. Micronuclei harboring whole chromosomes are primarily formed from defects in the chromosome segregation machinery, such as deficiencies in the cell cycle-controlling genes; the failure of the mitotic spindle, kinetochore, or other parts of the mitotic apparatus; or by damage to chromosomal substructures, mechanical disruption [56] and hypomethylation of centromeric DNA [54]. Micronuclei can also arise by gene amplification via breakage-fusion-bridge (BFB) cycles where amplified DNA is selectively localized to specific sites at the periphery of the nucleus and eliminated via nuclear budding (NBUD) during the S-phase of the cell cycle [57]. The fate of MN after their formation in the micronucleated cell is poorly understood. Their post-mitotic fate includes: (*i*) elimination of the micronucleated cell by apoptosis [43], (*ii*) expulsion from the cell (when the DNA within the MN is not expected to be functional or capable of replication due to the absence of the necessary cytoplasmic components); (*iii*) reincorporation into the main nucleus (after which the reincorporated chromosome may be indistinguishable from those of the main nucleus and may resume normal biological activity); or (*iv*) retention within the cell's cytoplasm as an extra-nuclear entity (after which MN may complete one or more rounds of DNA and chromosome replication) [58].

Despite others studies supporting the potential therapeutic effectiveness of oil (ozonised) or aqueous extract of sunflower seeds (*H. annuus* L.), including the relief of

asthma symptoms [59], protective effects against gastric lesions [60, 61], cicatrizing properties [62], and anti-inflammatory [63–65] and anti-microbial properties [60, 62, 66, 67], a limited number of investigations aimed at understanding the genotoxic and mutagenic effects have been carried out so far [50, 68, 69]. To contribute to the information on the genotoxic potential of herbal extracts and food, the present study evaluated the mutagenic effects of two sources of oil and tincture of *H. annuus* L. (sunflower) seeds using *in vivo* micronucleus assays in mouse bone marrow. The effect of the maximum permissible concentration of *H. annuus* L. (oils and tincture) on the doxorubicin (DOX)-induced genotoxic effects in mice bone marrow was also studied (i.e., an evaluation of the antimutagenic properties of these preparations).

2 Material and Methods

2.1 Phytotherapeutics

Tincture and oil of sunflower seeds were purchased commercially and stored according to the manufacturer's recommendations [tincture of *H. annuus* L. seeds (THALS): Yod Comércio de Produtos Naturais Ltda., cat. # 544606, Campinas, SP, Brazil; food oil of *H. annuus* L. seeds (FOHALS): Agricultural Cargill S.A., Mairinque, SP, Brazil; pharmaceutical oil of *H. annuus* L. seeds (POHALS): Farmácia de Manipulação Alfenense Ltda., Alfenas, MG, Brazil]. The tincture was subjected to an alcoholic extraction process in a Rotary Evaporator (Marconi Equipamentos para Laboratórios Ltda., mod. MA-120, Piracicaba, SP, Brazil) and their mass and relative densities were determined by the pycnometer method [70].

2.2 System - Test *in vivo*

Healthy, heterogeneous, young adult male and female *Swiss albinus* (Unib: SW) mice (between 7 and 12 weeks - pubescent period), with a body weight between 30 g and 40 g (i.e., the variation weight between the animals, for each sex, should not exceed the ± 20% of medium mass) were provided by CEMIB (*Centro Multidisciplinar para Investigação Biológica na Área* da *Ciência em Animais de Laboratório* – UNICAMP;

http://www.cemib.unicamp.br), and erythrocytes from the bone marrow of these mice were used in the micronucleus assay [71, 72].

The animals were kept in groups of the same sex, in polypropylene boxes, in an air-conditioned environment to 22° C ± 3° C, with relative air humidity of 50% ± 20%, and with 12-hour day-night cycles (i.e., 12h light and 12h dark). These were fed with Purina® Labina commercial rations (Nestlé Purina Pet Care Company) and water *ad libitum*, and acclimated to laboratory conditions for 7 days (a trial period) before the execution of the experiment. At the end of the trial period, each animal was weighed and, according to the weight, received 100 μL of the indicated liquid [150 mM NaCl in water for injection, THALS (administered after alcoholic extraction), FOHALS, POHALS, NEU, or DOX] for every 10 g of body mass.

Each animal was properly identified by its tail using numerical markings to ensure the continuity of the records and of the interpretations throughout the study [47]. After the experimental treatment, the animals were euthanized by inhalation of carbon dioxide in adapted acrylic chambers (as described in the Report of the American Veterinary Medical Association Panel on Euthanasia, 2000). The contaminated material was packaged in double plastic bags and stored in specific locations until the moment of its incineration, in accordance with the safety and health standards of the institution. This research was conducted in accordance with the Universal Declaration of Animal Rights [73], the ethical principles in Animal experimentation (*Colégio Brasileiro de Experimentação Animal* – COBEA), the Brazilian Environmental Crimes Law (*Lei N^o 9.605 de 12 de Fevereiro de 1998*), the Brazilian standards for Didactic-Scientific Practice of Vivisection of Animals (*Lei N^o 6.638 de 8 de Maio de 1979*), and with the approval of the Committee of Ethics in Research Involving Animals of UNIFENAS (CEPEAU Protocol No. 04A/2008).

2.3 Experimental Groups

Experimental groups (consisting of 3 males and 3 females each) were assessed after 24 and 48 hours of single treatment administered by gavage (experimental mutagenic test groups: 7-16, 21-30 and 35-44; anti-mutagenic test experimental groups: 17-20, 31-34 and 45-48), according to the previously established methodology [47]. Negative control groups (1 and 2) and positive control groups (3, 4, 5 and 6) were also included in single treatments administered intraperitoneally (Figure 1).

2.4 Processing the bone marrow

Micronucleus assays using erythrocytes from the bone marrow of mice were performed 24 and 48 hours after treatment, using the methodology described previously [74, 75]. Shortly after euthanasia, the femora were surgically and aseptically removed, and the animals appropriately discarded. Each femur was sectioned at the proximal end and the contents of the spinal canal were washed with 1.5 mL of 150 mM NaCl solution and transferred to a 15mL centrifuge tube.

This material was resuspended with a Pasteur pipette to ensure a random distribution of bone marrow cells. The suspension was then centrifuged at 1,000 rpm (Centrífuga de Bancada Microprocessada, Mod. NT 810, Nova Técnica Ind. e Com. de Equip. para Laboratório Ltda., Piracicaba, SP, Brazil) for 5 minutes. The resulting sediment was resuspended in 500 μL of a 150 mM solution of NaCI then 4% formol was added and the supernatant was discarded. The slides were prepared by smearing (2 blades per animal), dried at room temperature for 24 h and stained in coloration tub containing Leishman's eosin methylene blue dye [pure dye for 3 min, followed by diluted dye in distilled water (1:6) for 15 min] to differentiate polychromatic erythrocyte (PCE) from monochromatic erythrocyte (NCE).

Polychromatic erythrocytes (PCEs) were observed at a magnification of 1000× using optical microscopy (Nikon Eclipse E-200), counted (2000 polychromatic erythrocytes anucleated per animal) with the aid of a digital cell counter (Contador Diferencial CCS02, Kacil Indústria e Comércio Ltda., PE, Brasil Contador Diferencial CCS02, Kacil Indústria e Comércio Ltda., PE, Brazil) and photographed using an 8.1 Megapixel Digital Camera (DC FWL 150). The number of polychromatic erythrocytes (PCEs), the number and frequency of micronucleated polychromatic erythrocytes (MNPCEs), and the ratio of polychromatic erythrocytes and monochromatic (PCE/NCE) were reported.

2.5 Statistical Analysis

The data obtained in the micronucleus assay were submitted to *one-way* analysis of variance (ANOVA), using a factorial scheme of $10 \times 2 \times 2$ (treatment × gender × time), and medium comparison with Tukey's test ($\alpha = 0.05$) using SAS® version9.2 computer software.

3 Results and Discussion

Researches involving medicinal plants contribute important information to the new discoveries and the development of potential chemotherapeutics and help establish safe measures for the popular use or appropriate dosage [76, 77]. *H. annuus* L. has been considered an important source of natural oil for centuries and has been used as a preventive medicine against diuresis, diarrhoea, and various inflammatory diseases [78], and has also been used for the relief of asthmatic symptoms [59], gastric protection [60, 61], its healing properties [62], anti-inflammatory action [63–65] and anti-microbial properties [60, 62, 66, 67]. However, studies aimed at understanding the mutagenic and genotoxic effects of *H. annuus* L. were subject of comparatively little research [50, 68, 69], which drove us to evaluate the harmful mutagenic and anti-mutagenic properties (i.e., clastogenicity and/or aneugenicity) of oil and tincture of *H. annuus* L. seeds using the MN assay *in vivo*. The MN assay is an effective cytogenetic end point in assessing the damage induced by chemical mutagens and carcinogens [79–81]. The formation of micronuclei and the incidence of chromosomal aberrations are highly correlated [81, 82]. Among the various techniques used to detect genetic and genotoxic effects, the MN test is simple, inexpensive, and less cumbersome than other methods and allows convenient and easy application; this is particularly true for genotoxic studies [81, 83].

The numbers and frequencies of MNPCEs and the PCE/NCE ratio in the bone marrow of mice were analyzed statistically for each one of the treatments: positive and negative controls, mutagenic assay and anti-mutagenic assays. Animals treated with THALS showed no significant differences ($p < 0.05$) between treatment times (24 h and 48 h) and genders. However, the analysis of the MNPCEs numbers and frequencies revealed significant differences ($p < 0.05$) between the three control treatments (i.e., NaCl $^{Tukey\ groups\ C\ and\ C'}$, NEU $^{Tukey\ groups\ B\ and\ B'}$ and DOX $^{Tukey\ groups\ A\ and\ A'}$). Likewise, these differences were observed between the positive controls and experimental mutagenic treatments (i.e., NEU $^{Tukey\ groups\ B\ and\ B'}$, DOX $^{Tukey\ groups\ A\ and\ A'}$ and 250-2,000 mg.Kg^{-1} of THALS $^{Tukey\ groups\ C\ and\ C'}$) and between the mutagenic treatments (i.e., 250-2.000 mg.Kg^{-1} of THALS $^{Tukey\ groups\ C\ and\ C'}$) and anti-mutagenic assays (i.e., NEU + THALS $^{Tukey\ groups\ B\ and\ B'}$ and DOX + THALS $^{Tukey\ groups\ B\ and\ B'}$) (Table 1). These results suggest the following: (*i*) There is no genotoxicity (clastogenicity or aneugenicity) of THALS, regardless of the dose of phytotherapic administration (250-2.000 mg. Kg^{-1}), the treatment time (24 h and 48 h) or the gender of the animal (male and female), and this is supported by the observation of spontaneous genetic changes in the negative

control groups (150mM NaCl); (*ii*) There is a high genotoxicity (clastogenicity or aneugenicity) in DOX-treated animals given a dose of 5 $mg.Kg^{-1}$ [i.e., dosage above that which would be administered chemotherapeutically in humans: 0.55 to 1.25 $mg.Kg^{-1}$ considering individuals of 90 and 40 Kg (50 $mg.ampula^{-1}$), respectively; from 0.11 to 0.25 $mg.Kg^{-1}$ considering individuals of 90 and 40 Kg (10 $mg.ampula^{-1}$), respectively], whose MNPCE frequencies were significantly above those observed in the positive NEU control (50 $mg.Kg^{-1}$) MN assay, regardless of the treatment time (24 h and 48 h) and the gender of the animal; (*iii*) There is evidence of anti-genotoxic effects (anti-clastogeny and/or anti-aneugeny) when THALS (2,000 $mg.Kg^{-1}$) is administered in combination with the chemotherapy agent DOX (5 $mg.Kg^{-1}$). Therefore, THALS provides a partial protection against the genotoxic effects induced by DOX in the bone marrow of mice, although the genotoxic effect observed in this treatment combination has is similar to that observed in NEU-treated animals.

In addition, the analysis obtained from the PCE/NCE ratio showed significant differences ($p < 0.05$) between the positive and negative controls (i.e., NaCl $^{Tukey\ group\ A''}$, NEU $^{Tukey\ group\ CD''}$ and DOX $^{Tukey\ group\ D''}$). In the same way, these differences were observed between the lowest and highest concentrations of THALS administered in the experimental mutagen treatments (i.e., 250 $^{Tukey\ group\ B''}$, 500 $^{Tukey\ group\ BC''}$, 1.000-1.500 $^{Tukey\ group\ BCD''}$ and 2.000 $mg.Kg^{-1}$ $^{Tukey\ group\ CD''}$ THALS), and the observed toxicities were partially intermediate to those observed in the positive and negative controls. In the anti-mutagenic tests, similar statistical patterns of PCE/NCE were found between the THALS concentrations used in the mutagenic and the association of THALS + DOX $^{Tukey\ group\ BC''}$, or even, between the concentrations of mutagenic test from THALS and the association THALS + NEU $^{Tukey\ group\ BCD''}$. These results reinforce the hypothesis that there is a potential systemic dose-dependent toxicity of THALS, especially above the concentration of 500 $mg.Kg^{-1}$ (as supported by the observation of the NEU toxicity) and a systemic toxicity of DOX under dosage of 5 $mg.Kg^{-1}$, regardless of the treatment time (24 h and 48 h) and gender of the animal. However, there is a moderate and slight reduction of toxicity when DOX treatment is combined with THALS, as supported by the observation of toxicity with DOX alone. The PCE/NCE ratio is indicative of toxicity in this association (DOX + THALS) could also be explained by the existence of toxicity of THALS that is associated with doses of 2,000 $mg.Kg^{-1}$, a fact that is not reproducible in the lowest concentration of mutagen treatment THALS (250 $mg.Kg^{-1}$). However, a possible absence of or greater reduction of the toxicity of this combination (DOX + THALS) placing doses ≤ 250 $mg.Kg^{-1}$, remains an intriguing question to be explored.

For the first time, this research has provided information on the mutagenic and anti-mutagenic effects of THALS, although without any inference of its phytochemical composition. Research on the phytochemical and pharmacological aspects of *Compositae* plants have demonstrated that some sterols [84] and alkane-diols-6.8 [85, 86] isolated from the flowers of *Carthamus tinctorius* and Ψ-taraxasterol triterpenes and taraxasterol from the flowers of *C. tinctorius*, *C. morifolium* and *H. annuus* L. [87] exhibited considerable activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammatory ear-oedema in mice [84, 86, 87] and tumour promotion in mouse skin [84, 86].

The isolation of helianol from the tabular flowers of *H. annuus* L. and the characterization of 3.4-seco-19(10→9)*abeo* - 8α,9β,10α eupha-4.24-dien-3-ol was previously reported [88]. In another study, the distribution of helianol and other compounds was also describe; these compounds (including taraxasterol, Ψ-taraxasterol, α-β-amyrin, amyrin, lupeol, taraxerol, cycloartenol, 24-methylenecycloartanol, tirucalla-7.24-dienol and dammaradienol.) were identified in the triterpene alcohol fractions separated from the non-saponifiable lipids (NSL) of the methanol extracts of 11 tabular flowers and 9 ligulate flowers from 15 species of *Compositae* plants, including *H. annuus* L. Such triterpene alcohols showed marked inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation in mice (50% inhibitory dose was 0.1-0.8 mg *per* ear); helianol was an especially potent inhibition (0.1 mg *per* ear), and eight other triterpenes (taraxerol, β-amyrin, cycloartenol, lupeol, α-amyrin, 24-methylenecycloartanol, Ψ-taraxasterol and taraxasterol) showed levels comparable to that of indomethacin and were proposed to be potent anti-tumour agents [64].

In animals treated with POHALS, the statistical analysis obtained from the numbers and frequencies of MNPCEs and the PCE/NCE ratios showed no significant differences ($p < 0.05$) between the treatment times (24 h and 48 h), each of the experimental mutagenic treatments and the anti-mutagenic assay. However, there were significant differences ($p < 0.05$) between the male and female mice in the positive controls, experimental mutagenic treatments, and negative control (i.e., NEU $^{Tukey\ groups\ B\ and\ B'}$ and DOX $^{Tukey\ groups\ A\ and\ A'}$ *versus* 250-2.000 mg.Kg^{-1} of POHALS and NaCl $^{Tukey\ groups\ C\ and\ C'}$); statistically significant differences ($p < 0.05$) were even observed between the mutagenic treatments of POHALS (i.e., 250-2.000 mg.Kg^{-1} $^{Tukey\ groups\ C\ and\ C'}$) and similar treatments in the anti-mutagenic assays (i.e., NEU + POHALS $^{Tukey\ groups\ A\ and\ A'}$ and DOX + POHALS $^{Tukey\ groups\ A\ and\ A'}$) (Table 2). These results point to the following suggestions: (*i*) There is no genotoxicity (clastogenicity

or aneugenicity) associated with POHALS, regardless of the dose of phytotherapic administration (250-2,000 mg.Kg^{-1}) and the treatment time (24 h and 48 h), as supported by the observation of spontaneous genetic changes in the negative control treatment (150mM NaCl). Despite this absence of genotoxic effects, the differences in the frequency of MNPCEs can be observed between the genders, with the female mice presented the lowest MNPCE index in all experimental mutagenic treatments; (*ii*) There are no anti-genotoxic effects (anti-clastogeny and/or anti-aneugeny) of POHALS (2,000 mg.Kg^{-1}) when combined with the chemotherapeutic agent DOX (5 mg.Kg^{-1}) and, therefore, there is no protection from the genotoxic effects induced by DOX in the bone marrow of mice.

The analysis obtained from the PCE/NCE ratio showed statistically significant differences ($p < 0.05$) between the highest concentration and the other lower concentrations of POHALS administered in the experimental mutagenic treatments (i.e., 250-1.500 mg.Kg^{-1} *Tukey group B''* and 2.000 mg.Kg^{-1} *Tukey group BC''*), which also revealed an intermediate PCE/NCE ratio between the positive and negative controls (i.e., NaCl *Tukey group A''*, NEU *Tukey group CD''* and DOX *Tukey group D''*). In the anti-mutagenic tests, the PCE/NCE ratio was similar between the positive controls and the combination of POHALS + DOX *Tukey group CD''/D''* and even between the positive controls and the combination of POHALS + NEU *Tukey group D''*. These results indicate a slight systemic toxicity of POHALS, regardless of the therapeutic dose administered. There systemic toxicity of DOX was still evident at the dosage of 5 mg.Kg^{-1}, regardless of concomitant administration of POHALS; therefore, POHALS offers no protection from the genotoxic effects induced by DOX in the bone marrow of mice.

Applying the micronucleus test using to source of sunflower oil (i.e., industrialized food for oil – FOHALS) revealed statistically similar results to those found in POHALS-treated animals, except there were of significant differences ($p < 0.05$) in the numbers and frequencies of MNPCEs between the times of treatment. The statistical analysis of the PCE/NCE ratio of each of the experimental mutagenic treatments and anti-mutagenic assays indicated no significant differences ($p < 0.05$) between the treatment times (24 h and 48 h) and supported the observed differences between the genders of mice. In the same way, the analysis obtained from the numbers and frequencies of MNPCEs revealed significant differences ($p < 0.05$) between the positive control treatments (i.e., NEU *Tukey groups B and B'* and DOX *Tukey groups A and A'*) and experimental mutagenic or negative controls (i.e., 250-2.000 mg.Kg^{-1} of FOHALS and NaCl *Tukey groups C and C*); differences were also observed between the mutagenic treatments (i.e., 250-2.000 mg.Kg^{-1} FOHALS *Tukey groups C and C'*) and in the anti-

mutagenic assays (i.e., NEU + FOHALS $^{Tukey\ groups\ A\ and\ A'}$ and DOX + FOHALS $^{Tukey\ groups\ A\ and\ A'}$) (Table 3). These results point to the following suggestions: (*i*) There is no genotoxicity (clastogenicity and/or aneugenicity) associated with FOHALS, regardless of the dose of phytotherapic administration (250-2,000 mg.Kg^{-1}). However, there are differences in the frequencies of MNPCEs between the treatment times (24 h and 48 h) and genders; the greatest proportion of MNPCEs is observed after 48 h in the female mice for FOHALS treatment concentrations (250-1,500 mg.Kg^{-1}). This hypothesis was strongly supported by the observation of spontaneous genetic changes in the negative control treatment (150 mm NaCl); (*ii*) There are no anti-genotoxic effects (anti-clastogenic and/or anti-aneugenic) of FOHALS (2,000 mg.Kg^{-1}) when combined with DOX (5 mg.Kg^{-1}), and therefore FOHALS offers no protection from the genotoxic effects induced by DOX in the bone marrow of mice.

The analysis of the PCE/NCE ratio showed statistically significant differences ($p < 0.05$) between the animals treated with lowest and the highest concentrations of FOHALS administered in the experimental mutagenic treatments (i.e., 250 mg.Kg^{-1} $^{Tukey\ group\ B''}$, 500 mg.Kg^{-1} $^{Tukey\ group\ BC''}$ and 1,000-2,000 mg.Kg^{-1} $^{Tukey\ group\ C''}$); the mice treated with the highest concentration of FOHALS also had a PCE/NCE ratio similar to those observed in the positive controls (i.e., NEU $^{Tukey\ group\ C''}$ and DOX $^{Tukey\ group\ BC''}$). In the anti-mutagenic tests (i.e., FOHALS + DOX $^{Tukey\ group\ C''}$ and FOHALS + NEU $^{Tukey\ group\ C''}$), the PCE/NCE ratio showed statistically similar patterns to the positive controls (i.e., NEU $^{Tukey\ group\ C''}$ and DOX $^{Tukey\ group\ BC''}$) and mutagenic tests (except at a concentration of 250 mg.Kg^{-1} of FOHALS $^{Tukey\ group\ B''}$). These findings point to the existence of a systemic toxicity of FOHALS at doses ≥ 500 mg.Kg^{-1}. This also indicates a systemic toxicity of DOX under the dose of 5 mg.Kg^{-1}, regardless of concomitant administration of FOHALS; these data confirmed that FOHALS does not offer protection from genotoxic effects induced by DOX in the bone marrow of mice.

Genotoxic studies of sunflower oil and olive oil sunflower ozonized (at a dose limit of 2 g.kg^{-1}.d^{-1}, based on evidence of toxicity from subchronic studies via intragastric administration of the product) were previously carried out using the MN assay in the bone marrow of mice using male and female Cenp: NMRI mice [89]. In this study, the treatment with sunflower oil did not cause cytotoxic damage to erithrocytes, as reported in the analyses of the PCE/NCE ratio, which does not corroborate with our findings from the pharmaceutical and industrial oils. However, this research proposes the hypothesis that no clastogenic effect occurs in the bone marrow of animals treated with the sunflower oil under experimental conditions [89].

Other studies have investigated the suitability of different vegetable oils for the human diet, reporting reductions in genotoxicity and cancer potentiation by sesame oil [90], sunflower oil [91], perilla and palm oil [92], olive, sunflower, peanut, corn, and soy oils [93], flax seed oil [94], and coconut oil [95], among others. The possible role of fatty acids, a main component of vegetable oils, in modulating genotoxicity and carcinogenicity has also been studied. The genotoxic activity of vegetable oils [seed oils of sesame, sunflower, wheat germ, flax, and soy oil, and both first-class extra-virgin and low-grade (refined) olive oil] consumed by humans were also tested in a *Drosophila* somatic mutation and recombination test (the *Drosophila melanogaster* SMART assay) [69]. Flax oil produced the strongest response, while sesame, wheat germ, and soy oil showed some genotoxic activity. Sunflower oil and the low-grade olive oil gave inconclusive results or negative biological diagnoses, possibly due to lower concentrations of PUFAs, even as refined products, and extra-virgin olive oil was clearly not genotoxic. It has been argued that the genotoxicity of an oil is most likely due to the fatty acid composition of the oil, which after peroxidation can form specific DNA-adducts. Such results were in general agreement with evidence from experimental and epidemiological studies summarized by Bartsch and collaborators (1999) [96]: n-PUFAs are related to the generation of oxidative DNA damage, a high intake of n-6 PUFAs is implicated in some types of cancers, and n-9 MUFAs and n-3 PUFAs may have a role in cancer prevention. Additionally, it was suggested that the relative concentrations of short-chain C18:3 n-3 linolenic acid, C18:2 n-6 linoleic acid, and polyphenols are the major factors responsible for the genotoxicity of cooking oils in the SMART assay [69]. Despite the existence of this information, contradictory or inconclusive data were found in the literature. For instance, one study reported that linoleic acid (C18:2 n-6 PUFA) suppressed cancer cell proliferation [97], while other studies indicated an enhancing effect on carcinogenesis [98–100]. Oleic acid (C18:1, n-9 MUFA), a promoter of cancer cell proliferation [97], has also been reported to be an effective anticancer and antimutagenic agent [101, 102]. Linolenic acid (C18:3 short-chain n-3 PUFA) had anticancer activity in some studies [97, 103], but promoted cancer in other studies [104, 100]. Phenolic compounds, another important constituent of vegetable oils, are present in the unsaponifiable lipid phase. Phenolics are involved in both extra- and intracellular processes, inducing cytosolic detoxifying mechanisms, microsomal enzyme activation, and the scavenging of free radicals [105, 106]. Evidence indicates that polyphenols can inhibit the mutagenicity of genotoxic agents [107, 108] and function as anticancer agents [109].

The clastogenic and cytotoxic effects from heated sunflower oil were studied in lymphocytes, hepatocytes (HepG2) and in human umbilical vein endothelial cells (HUVEC) [50]. In lymphocytes incubated with water extract of heated sunflower oil containing 0.075 or 0.15 μM of thiobarbituric acid-reactive substances (this extract has a high content in polar aldehydes), the rate of chromosomal breakage was 18.4% and 23.1%, compared to 8.7% and 6.6%, or 8.1% and 9.2%, respectively in lymphocytes incubated with the same volume of a water extract from non-heated oil or distilled water. In HepG2 or HUVEC cells, the cytotoxic properties of heated sunflower oil were dose dependent, and the cytotoxicity occurred at concentrations as low as 0.25 μM. In contrast, the same volume of non-heated oil or distilled water was non-toxic for these cells. The results show that a water extract obtained from heated oil is clastogenic and, in higher doses, cytotoxic. These data also suggested that a water extract, obtained from culinary oils submitted to heat stress, with a high content of aldehydes is clastogenic. It was speculated that the ingestion of large amounts of these products may also impact human health, especially in those diseases secondary to chromosomal breakage such as certain congenital malformations and certain types of cancer. This last fact can be corroborated by previous reports indicating that the administration of thermally stressed sunflower oil to rats is teratogenic [110]. Aldehyde compounds can be absorbed from the gut into the circulation [111, 112] and induce different types of damage such as the generation of foam cells from macrophages due to the increased expression of the scavenger receptor, CD36 [113] or the depletion of different antioxidants such as glutathione *in vivo* [112, 114].

The genotoxicity of anticancer drugs is of special interest because of the risk of inducing secondary malignancies. Patients undergoing treatment with chemotherapeutic agents for multiple myeloma, Hodgkin disease, and ovarian tumours are at risk for the development of secondary tumours after the completion of treatment [115–117]. The use of cytoprotective agents represents an alternative method for reducing radiotherapeutic and chemotherapeutic toxicity in normal tissues [118], thus reducing the risk of potentially genotoxic effects such as secondary tumour formation [117]. For those agents whose maximum tolerated dose is limited primarily by myelosuppression, several strategies are being evaluated to permit dose escalation in the hope of obtaining better clinical results with the currently available chemotherapeutic drugs [51].

Doxorubicin (DOX) is an important anthracyclines anticancer agent. It is a valuable component of various chemotherapeutic regimens for breast carcinoma and small-cell lung carcinoma. In metastatic thyroid carcinoma, DOX is most likely the best available agent [51].

DOX is also an important agent for the successful treatment of Hodgkin disease and non-Hodgkin lymphomas [119]. A clear dose-response relation for DOX in several curative regimens has been shown, and decreased doses result in inferior survival rates [120], whereas an increase in DOX dose is limited because of severe cardiotoxicity, a major problem in the clinical application of DOX. There is a large body of evidence to show that the dominant cellular target of DOX is DNA [121]. The anthracyclines are classified as topoisomerase II inhibitors because they effectively stabilize the cleavage complex of this enzyme. Topoisomerase II plays an important roles in the segregation of newly replicated parts of intertwined chromosomes [122, 123], the condensation and decondensation of chromosomes, and the supercoiling of intracellular DNA [124, 125]. DNA topoisomerase II catalyzes the breakage and rejoining of both DNA strands, relaxes the superhelical twist, and catenates or decatenates circular DNA. DNA topoisomerase II performs these topological transformations by transporting one double stranded DNA segment through an enzyme-mediated transient double-strand break in another. The several DNA strands are rejoined after completion of the above process [126]. The most readily detectable genotoxic effect of topoisomerase-targeting agents in somatic cells is the formation of a ternary complex containing the drug, thus stabilizing the DNA double-strand breaks [127]. DOX is a potent DNA topoisomerase II inhibitor, and it stabilizes DNA double-strand breaks and does not allow them to rejoin, leading to cell death [128–130]. These DNA double-strand breaks may subsequently lead to chromosomal breaks that finally may lead to the production of MN following cell division [131]. The persistence of DNA lesions in DOX-treated cells has been confirmed by MN assay. The incidence of micronuclei showed a dose response in large-scale screening for suspected clastogens [81]. The antitumor activity of anthracyclines is consistent with their ability to interact with cellular DNA, and this noncovalent interaction can inhibit the function of DNA ligases, helicases, and other DNA-dependent proteins, in addition to topoisomerase II. However, their cellular effects are by no means limited to this type of enzymatic inhibition. DOX exhibits a wide range of cellular effects, and it is likely that no single mechanism of action will account for all of the observed clinical and cellular responses [121, 132]. DOX forms a stable complex with ferric iron, which reacts with oxygen, forming superoxide anions, hydrogen peroxide, and hydroxyl radicals. DOX is known to cause single-strand DNA breaks, chromosomal rearrangements, and mutational events, and it is a potent carcinogen [133]. DOX has been reported to induce micronuclei, chromatid and chromosome aberrations, and DNA single- and double-strand breaks *in vitro* and *in vivo* [134–142]. The major acute toxicity induced by DOX is bone marrow suppression, and the long-term clinical usefulness is

limited by a cumulative dose-dependent irreversible chronic cardiotoxicity, which manifests itself as congestive heart failure or cardiomyopathy [143, 144].

Therefore, it is essential to screen for newer pharmacological agents that can protect the normal cells against DOX-induced cumulative toxicity. Many plants that have been widely used in traditional medicine are less toxic than pharmaceutical agents and have recently attracted the attention of researchers around the world. Plants contain many compounds, and it is likely that these can provide better protective effects than a single molecule [145]. The presence of many molecules in plants may be advantageous, as some of them may counteract the toxicity of others, and as a result, the net effect may be beneficial for therapeutic purposes. For example, the effect of various concentrations (200, 250, 300, 350, and 400 mg/kg body weight) of *Aegle marmelos* on the doxorubicin (DOX)-induced genotoxic effects in mice bone marrow was studied [51]. Treatment of mice with different concentrations of DOX (5, 10, or 15 $mg.kg^{-1}$ body weight) resulted in a dose-dependent elevation in the frequency of micronucleated polychromatic and normochromatic erythrocytes in mouse bone marrow, and it was accompanied by a DOX dose-dependent decline in the PCE/NCE ratio. The treatment of mice with *Aegle marmelos*, orally once daily for 5 consecutive days before DOX treatment, significantly reduced the frequency of DOX-induced micronuclei and significant increased the PCE/NCE ratio at all scoring times. This observed chemoprotective effect may be due to the sum total of interaction between different ingredients of this complex mixture. The degree of protection may depend on the interaction of components individually or collectively with the genotoxic agent. The plausible mechanisms of action of *Aegle marmelos* in protecting against DOX-induced genomic insult were scavenging of $O_2^{\bullet-}$ and $^{\bullet}OH$ and other free radicals, increase in antioxidant status, restoration of topoisomerase II activity, and inhibition of the formation of DOX-iron complex [51]. Another study was undertaken to evaluate the genotoxic potential of *Copaifera lansdorffii* Desf. leaf hydroalcoholic extract and its influence on the genotoxicity induced by chemotherapeutic agent doxorubicin (DOX) using the *Swiss* mouse peripheral blood micronucleus test. The results of this study demonstrated that *C. lansdorffii* Desf. was not itself genotoxic and that in animals treated with *C. lansdorffii* Desf. and DOX, the number of micronuclei was significantly decreased compared to animals receiving DOX alone. The putative antioxidant activity of one or more of the active compounds of *C. lansdorffii* Desf., including two major flavonoid heterosides (quercitrin and afzelin), may explain the effect of this plant on DOX genotoxicity [52].

The PCE/NCE ratio is an indicator of the acceleration or inhibition of erythropoiesis. The PCE/NCE ratio has been reported to vary with scoring time, and a continuous decline in the PCE/NCE ratio may be due to the inhibition of cell division, the killing of erythroblasts, the removal of damaged cells, or dilution of the existing cell pool with newly formed cells [51]. Several mechanisms may have been responsible for the cytotoxicity of DOX and micronucleus induction [146], including the intercalation of DOX into cellular DNA [147, 148], the stabilization of the topoisomerase II–DNA complex [149, 150], free radical–mediated toxicity caused by redox cycling of the semiquinone radical [151], or the formation of reactive oxygen species by the DOX-iron complex [121, 152, 153]. On the other hand, other chemicals such as captopril and desferrioxamine [135, 136], β-carotene and vitamins A, C, and E [154–156], thiol N-acetylcysteine, probucol, lovastatin, and hydrophilic flavonoids such as rutin and luteolin [157–160] have also been reported to reduce DOX-induced MN, genotoxicty, and citotoxicity. However, proponents of herbal medicine always claim that mixtures are better than pure chemicals because the dozens of biologically active compounds in plants work together to produce a greater effect than any one chemical on its own [161].

4 Concluding Remarks

In conclusion, this research evaluated the genotoxic effects (clastogeny and aneugeny) of a sunflower seed tincture using the MN assay in PCE in bone marrow of *Swiss albinus* mice for the first time. Two different sources of sunflower oil were also examined in this study. In addition, the interaction between sunflower seeds tincture and oils and the genotoxic effects induced by DOX was also studied. This study observed an absence of genotoxicity of THALS, POHALS and FOHALS, regardless of dose, time (except FOHALS, which showed an increase in the proportion of MNPCEs in 48 h) and gender (except POHALS, which showed an increase in the proportion of MNPCEs in the male gender, and FOHALS, which showed an increase in the proportion of MNPCEs in the female gender). A high genotoxicity of DOX was also observed from in animals treated with a dose greater than that administered to humans, compared to the genotoxicity of the positive control NEU used in the MN assay, regardless of the treatment time and gender of the animal.

However, evidence of the anti-genotoxic effects of THALS when combined with DOX was observed; this suggests that the genotoxic effects induced by DOX in the bone marrow of mice are not uniform. Such evidence was not observed in the treatments associated with

POHALS and DOX or FOHALS and DOX, and consequently, any protective effects of these two preparations against the genotoxic effects of DOX cannot be inferred. The PCE/NCE ratio in the animals treated with THALS revealed differences between the lowest and the highest concentrations tested, suggesting a moderate systemic dose-dependent toxicity. This toxicity was also observed in the animals treated with DOX, regardless of the treatment time and gender of the animal. However, a slight reduction in that toxicity was observed when animals were treated with a combination of THALS and DOX, which may be explained by the existence of THALS toxicity at the maximum concentration. The PCE/NCE ratio observed after treatment with POHALS revealed differences between the higher and lower POHALS concentrations tested; the PCEs/NCE ratios of POHALS-treated animals were intermediate to positive and negative controls, suggesting a light to moderate systemic toxicity of POHALS. The animals that were treated with a combination of POHALS and DOX did not show a PCE/NCE ratio compatible to the mutagenic assay of POHALS or the negative control assay, suggesting a null protection against toxic effects induced by DOX. The PCE/NCE ratio of FOHALS revealed differences between the lowest and the highest concentrations tested, however showed compatibility with the PCE/NCE of controls^{+}, pointing to the systemic toxicity of FOHALS (mainly from the 500 mg.Kg^{-1} treatment group). Similarly to the POHALS data, the combination of FOHALS and DOX did not show a PCE/NCE ratio compatible to the negative control test, suggesting once again the absence of protection against the toxic effects induced by DOX.

Finally, other studies involving the genotoxicity of *H. annuus* L. seed extracts and oils should be conducted [including mutagenicity assays with *Salmonella typhimurium* test (Ames test) as an indicator of potential carcinogenicity to mammals, gene mutation test in mammalian cells (mouse lymphoma assay), cytogenetic and aneuploidy tests *in vitro*, micronucleus test in cultured cells *in vitro*, fluorescent *in situ* hybridization (FISH) test for mutagenesis, comet test to detect of DNA damage and repair in individual cells, and functional genomic and proteomic tests for mutagenesis (cDNA microarrays and other array analyses)], to characterize the potential effects and genotoxic and anti-genotoxic mechanisms and, importantly, for the establishment of limits for human consumption, the delineation of potential risks to human health, and for rational strategies for implementing chemo-preventive measures.

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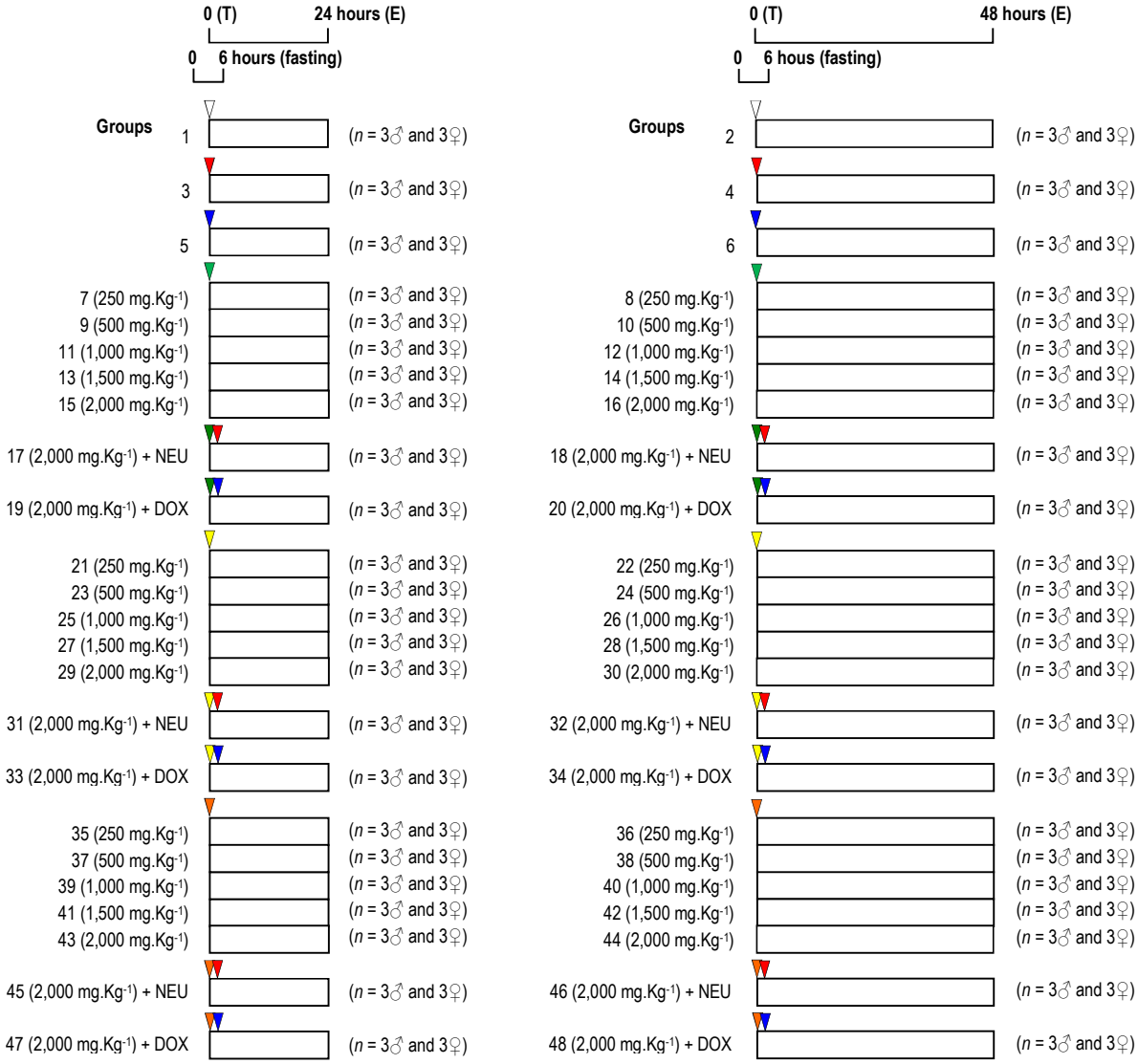
Figure 1. Experimental protocol for the mutagenic and anti-mutagenic assays using tincture and oils of *H. annuus* L. (sunflower) seeds.



▽ 150 mM NaCl

▼ N-Nitroso-N-ethylurea 50 $mg.Kg^{-1}$ – NEU (Sigma N8509)

▼ Doxorubicin Hydrochloride 5 $mg.Kg^{-1}$ – DOX (Eurofarma Laboratórios Ltda., ref. # L83608)

▼ Tincture of *H. annuus* L. seeds (THALS)

▼ Pharmaceutical oil of *H. annuus* L. seeds (POHALS)

▼ Food oil of *H. annuus* L. seeds (FOHALS)

T (Treatment)

E (Euthanasia)

n (number of mice)

Table 1. The incidence of MNPCEs and PCE/NCE ratio in bone marrow of male and female *Swiss albinus* mice after testing for 24 h and 48 h. Shown are data from the positive and negative controls (NaCl, NEU and DOX), an evaluation of the mutagenicity of THALS, and an evaluation of the anti-mutagenicity of THALS (THALS + NEU and THALS + DOX). *One-way* statistical analysis of variance (ANOVA), in a factorial scheme 10 × 2 × 2 (treatment × gender × time) evaluated Tukey's test (at a significance level of 5%) using SAS® software (version 9.2).

Treatment	Number of PCEs analyzed		PCEMN				PCE/NCE		NCE (*n*)	
	24h	48h	24h (*n*)	48h (*n*)	24h (%)	48h (%)	24h	48h	24h	48h
150 mM NaCl										
Σ ♀	6276	6281	27	28	0,43	0,45	261,50	330,58	24	19
Σ ♂	6208	6260	28	35	0,45	0,56	67,48	156,50	92	40
Σ	12484	12541	55 ^{C}	63 ^{C}	0,44 $^{C'}$	0,50 $^{C'}$	107,62 $^{A''}$	212,56 $^{A''}$	116	59
N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀	6034	6055	85	101	1,41	1,67	1,17	3,47	5166	1745
Σ ♂	6057	5984	194	109	3,20	1,82	1,04	0,96	5843	6216
Σ	12091	12039	279 ^{B}	210 ^{B}	2,31 $^{B'}$	1,74 $^{B'}$	1,10 $^{D''}$	1,51 $^{D''}$	11009	7961
Doxorubicin hydrochloride (5 mg.Kg^{-1})										
Σ ♀	6253	6186	179	149	2,86	2,41	5,01	28,91	1247	214
Σ ♂	6212	6203	155	181	2,50	2,92	70,59	31,49	88	197
Σ	12465	12389	334 ^{A}	330 ^{A}	2,68 $^{A'}$	2,66 $^{A'}$	9,34 $^{CD''}$	30,14 $^{CD''}$	1335	411
Tincture of H. annuus L. seeds (sunflower)										
Σ ♀	6383	6341	27	31	0,42	0,49	99,73	132,10	64	48
Σ ♂	6146	6197	26	25	0,42	0,40	109,75	140,84	56	44
Σ $_{250\ mg.kg^{-1}}$	12529	12538	53 ^{C}	56 ^{C}	0,42 $^{C'}$	0,45 $^{C'}$	104,41 $^{B''}$	136,28 $^{B''}$	120	92
Σ ♀	6236	6252	37	40	0,59	0,64	86,61	105,97	72	59
Σ ♂	6217	6223	40	29	0,64	0,47	74,90	141,43	83	44
Σ $_{500\ mg.kg^{-1}}$	12453	12475	77 ^{C}	69 ^{C}	0,62 $^{C'}$	0,55 $^{C'}$	80,34 $^{BC''}$	121,12 $^{BC''}$	155	103
Σ ♀	6228	6302	38	45	0,61	0,71	54,63	82,92	114	76
Σ ♂	6244	6256	42	29	0,67	0,46	66,43	68,00	94	92
Σ $_{1,000\ mg.kg^{-1}}$	12472	12558	80 ^{C}	74 ^{C}	0,64 $^{C'}$	0,59 $^{C'}$	59,78 $^{BCD''}$	74,75 $^{BCD''}$	208	168
Σ ♀	6144	6324	37	46	0,60	0,73	52,51	64,53	117	98
Σ ♂	6206	6203	44	35	0,71	0,56	43,10	60,22	144	103
Σ $_{1,500\ mg.kg^{-1}}$	12350	12527	81 ^{C}	81 ^{C}	0,66 $^{C'}$	0,65 $^{C'}$	47,36 $^{BCD''}$	62,32 $^{BCD''}$	261	201
Σ ♀	6186	6182	47	48	0,76	0,78	39,65	52,39	156	118
Σ ♂	6256	6170	48	37	0,77	0,60	40,62	47,46	154	130
Σ $_{2,000\ mg.kg^{-1}}$	12442	12352	95 ^{C}	85 ^{C}	0,76 $^{C'}$	0,69 $^{C'}$	40,14 $^{CD''}$	49,81 $^{CD''}$	310	248
Tincture of H. annuus L. seeds (2,000 mg.kg^{-1}) + N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀	6223	6207	89	92	1,43	1,48	80,82	15,79	77	393
Σ ♂	6229	6228	103	109	1,65	1,75	87,73	86,50	71	72
Σ $_{2,000\ mg.kg^{-1}}$	12452	12435	192 ^{B}	201 ^{B}	1,54 $^{B'}$	1,62 $^{B'}$	84,14 $^{BCD''}$	26,74 $^{BCD''}$	148	465
Tincture of H. annuus L. seeds (2,000 mg.kg^{-1}) + Doxorubicin hydrochloride (5 mg.Kg^{-1})										
Σ ♀	6223	6236	106	103	1,70	1,65	80,82	97,44	77	64
Σ ♂	6251	6248	97	96	1,55	1,54	127,57	120,15	49	52
Σ $_{2,000\ mg.kg^{-1}}$	12474	12484	203 ^{B}	199 ^{B}	1,63 $^{B'}$	1,59 $^{B'}$	99,00 $^{BC''}$	107,62 $^{BC''}$	126	116

The letters A, B, C and D correspond to Tukey's studentized range (the same letter are not significantly different. $p < 0.05$).

Table 2. The incidence of MNPCEs and PCE/NCE ratio in bone marrow of male and female *Swiss albinus* mice after testing for 24 h and 48 h. Shown are data from the positive and negative controls (NaCl, NEU and DOX), an evaluation of the mutagenicity of POHALS, an evaluation of the anti-mutagenicity of POHALS (POHALS + NEU and POHALS + DOX). *One-way* statistical analysis of variance (ANOVA), in a factorial scheme of 10 × 2 × 2 (treatment × gender × time), evaluated by Tukey's test (at a significance level of 5%) using SAS® software (version 9.2).

Treatment	Number of PCEs analyzed		PCEMNs				PCE/NCE		NCE	
	24h	48h	24h (*n*)	48h (*n*)	24h (%)	48h (%)	24h	48h	24h (*n*)	48h (*n*)
150 mM NaCl										
Σ ♀	6276	6281	27	28	0,43	0,45	261,50	330,58	24	19
Σ ♂	6208	6260	28	35	0,45	0,56	67,48	156,50	92	40
Σ	12484	12541	55 ^{C}	63 ^{C}	0,44 $^{C'}$	0,50 $^{C'}$	107,62 $^{A''}$	212,56 $^{A''}$	116	59
N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀	6034	6055	85	101	1,41	1,67	1,17	3,47	5166	1745
Σ ♂	6057	5984	194	109	3,20	1,82	1,04	0,96	5843	6216
Σ	12091	12039	279 ^{B}	210 ^{B}	2,31 $^{B'}$	1,74 $^{B'}$	1,10 $^{D''}$	1,51 $^{D''}$	11009	7961
Doxorubicin hydrochloride (5 mg.Kg^{-1})										
Σ ♀	6253	6186	179	149	2,86	2,41	5,01	28,91	1247	214
Σ ♂	6212	6203	155	181	2,50	2,92	70,59	31,49	88	197
Σ	12465	12389	334 ^{A}	330 ^{A}	2,68 $^{A'}$	2,66 $^{A'}$	9,34 $^{CD''}$	30,14 $^{CD''}$	1335	411
Pharmaceutical oil of H. annuus L. seeds (sunflower)										
Σ ♀ ^{B}	6257	6263	22	23	0,35	0,37	184,03	202,03	34	31
Σ ♂ ^{A}	6256	6254	26	40	0,42	0,64	142,18	152,54	44	41
Σ $_{250\ mg.kg^{-1}}$	12513	12517	48 ^{C}	63 ^{C}	0,38 $^{C'}$	0,50 $^{C'}$	160,46 $^{B''}$	173,85 $^{B''}$	78	72
Σ ♀ ^{B}	6102	6251	23	24	0,38	0,38	179,47	195,34	34	32
Σ ♂ ^{A}	6198	6210	44	45	0,71	0,72	134,74	135,00	46	46
Σ $_{500\ mg.kg^{-1}}$	12300	12461	67 ^{C}	69 ^{C}	0,54 $^{C'}$	0,55 $^{C'}$	153,75 $^{B''}$	159,76 $^{B''}$	80	78
Σ ♀ ^{B}	6262	6257	35	36	0,56	0,58	173,94	195,53	36	32
Σ ♂ ^{A}	6242	6249	52	53	0,83	0,85	132,81	135,85	47	46
Σ $_{1,000\ mg.kg^{-1}}$	12504	12506	87 ^{C}	89 ^{C}	0,69 $^{C'}$	0,71 $^{C'}$	150,83 $^{B''}$	160,33 $^{B''}$	83	78
Σ ♀ ^{B}	6266	6266	37	38	0,59	0,61	164,89	184,29	38	34
Σ ♂ ^{A}	6253	6236	56	58	0,90	0,93	107,81	119,92	58	52
Σ $_{1,500\ mg.kg^{-1}}$	12519	12502	93 ^{C}	96 ^{C}	0,74 $^{C'}$	0,77 $^{C'}$	130,29 $^{B''}$	145,37 $^{B''}$	96	86
Σ ♀ ^{B}	6264	6263	43	48	0,69	0,77	145,67	169,27	43	37
Σ ♂ ^{A}	6204	6238	57	62	0,92	0,99	64,63	69,31	96	90
Σ $_{2,000\ mg.kg^{-1}}$	12468	12501	100 ^{C}	110 ^{C}	0,80 $^{C'}$	0,88 $^{C'}$	89,70 $^{BC''}$	98,43 $^{BC''}$	139	127
Pharmaceutical oil of H. annuus L. seeds (2,000 mg.kg^{-1}) + N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀ ^{B}	6121	6129	182	176	2,97	2,87	2,57	2,06	2379	2971
Σ ♂ ^{A}	6057	6139	149	160	2,46	2,61	1,99	2,98	3043	2061
Σ $_{2,000\ mg.kg^{-1}}$	12178	12268	331 ^{A}	336 ^{A}	2,72 $^{A'}$	2,74 $^{A'}$	2,25 $^{D''}$	2,44 $^{D''}$	5422	5032
Pharmaceutical oil of H. annuus L. seeds (2,000 mg.kg^{-1}) + Doxorubicin hydrochloride (5 mg.Kg^{-1})										
Σ ♀ ^{B}	6120	6247	179	160	2,92	2,56	2,05	2,77	2980	2253
Σ ♂ ^{A}	6194	6075	173	155	2,79	2,55	1,93	3,33	3206	1825
Σ $_{2,000\ mg.kg^{-1}}$	12314	12322	352 ^{A}	315 ^{A}	2,86 $^{A'}$	2,56 $^{A'}$	1,99 $^{D''}$	3,02 $^{D''}$	6186	4078

The letters A, B, C and D correspond to Tukey's studentized range (the same letter are not significantly different. $p < 0.05$).

Table 3. The incidence of MNPCEs and PCE/NCE ratio in bone marrow of male and female *Swiss albinus* mice after testing for 24 h and 48 h. Shown are data from the positive and negative controls (NaCl, NEU and DOX), an evaluation of the mutagenicity of FOHALS, an evaluation of the anti-mutagenicity of FOHALS (FOHALS + NEU and FOHALS + DOX). *One-way* statistical analysis of variance (ANOVA), in factorial scheme of 10 × 2 × 2 (treatment × gender × time), evaluated by Tukey's test (at a significance level of 5%) using SAS® software (version 9.2).

Control	Number of PCEs analyzed		PCEMNs				PCE/NCE		NCE	
Treatment	24h	48h	24h (*n*) ^{A}	48h (*n*) ^{B}	24h (%) ^{A}	48h (%) ^{B}	24h	48h	24h (*n*)	48h (*n*)
150 mM NaCl										
Σ ♀	6276	6281	27	28	0,43	0,45	261,50	330,58	24	19
Σ ♂	6208	6260	28	35	0,45	0,56	67,48	156,50	92	40
Σ	12484	12541	55 ^{C}	63 ^{C}	0,44 $^{C'}$	0,50 $^{C'}$	107,62 $^{A''}$	212,56 $^{A''}$	116	59
N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀	6034	6055	85	101	1,41	1,67	1,17	3,47	5166	1745
Σ ♂	6057	5984	194	109	3,20	1,82	1,04	0,96	5843	6216
Σ	12091	12039	279 ^{B}	210 ^{B}	2,31 $^{B'}$	1,74 $^{B'}$	1,10 $^{C''}$	1,51 $^{C''}$	11009	7961
Doxorubicin hydrochloride (5 mg.Kg^{-1})										
Σ ♀	6253	6186	179	149	2,86	2,41	5,01	28,91	1247	214
Σ ♂	6212	6203	155	181	2,50	2,92	70,59	31,49	88	197
Σ	12465	12389	334 ^{A}	330 ^{A}	2,68 $^{A'}$	2,66 $^{A'}$	9,34 $^{BC''}$	30,14 $^{BC'}$	1335	411
Food oil of H. annuus L. seeds (sunflower)										
Σ ♀ ^{A}	6425	6108	27	30	0,42	0,49	146,02	244,32	44	25
Σ ♂ ^{B}	6204	6213	22	23	0,35	0,37	39,02	28,37	159	219
Σ $_{250\ mg.kg}^{-1}$	12629	12321	49 ^{C}	53 ^{C}	0,39 $^{C'}$	0,43 $^{C'}$	62,21 $^{B''}$	50,50 $^{B''}$	203	244
Σ ♀ ^{A}	6031	6175	27	35	0,45	0,57	26,69	89,49	226	69
Σ ♂ ^{B}	6153	6227	23	35	0,37	0,56	20,79	21,70	296	287
Σ $_{500\ mg.kg}^{-1}$	12184	12402	50 ^{C}	70 ^{C}	0,41 $^{C'}$	0,56 $^{C'}$	23,34 $^{BC''}$	34,84 $^{BC''}$	522	356
Σ ♀ ^{A}	6359	6231	30	38	0,47	0,61	23,64	27,57	269	226
Σ ♂ ^{B}	6141	6181	26	38	0,42	0,61	17,85	15,08	344	410
Σ $_{1,000\ mg.kg}^{-1}$	12500	12412	56 ^{C}	76 ^{C}	0,45 $^{C'}$	0,61 $^{C'}$	20,39 $^{C''}$	19,52 $^{C''}$	613	636
Σ ♀ ^{A}	6174	6174	30	39	0,49	0,63	14,00	7,80	441	792
Σ ♂ ^{B}	6156	6303	29	45	0,47	0,71	14,62	12,68	421	497
Σ $_{1,500\ mg.kg}^{-1}$	12330	12477	59 ^{C}	84 ^{C}	0,48 $^{C'}$	0,67 $^{C'}$	14,30 $^{C''}$	9,68 $^{C''}$	862	1289
Σ ♀ ^{A}	6256	6042	33	41	0,53	0,68	10,88	7,04	575	858
Σ ♂ ^{B}	6179	6490	44	47	0,71	0,72	13,82	9,64	447	673
Σ $_{2,000\ mg.kg}^{-1}$	12435	12532	77 ^{C}	88 ^{C}	0,62 $^{C'}$	0,70 $^{C'}$	12,17 $^{C''}$	8,19 $^{C''}$	1022	1531
Food oil of H. annuus L. seeds (2,000 mg.kg^{-1}) + N-Nitroso-N-ethylurea (50 mg.Kg^{-1})										
Σ ♀ ^{A}	6108	6125	148	155	2,42	2,53	2,66	2,58	2292	2375
Σ ♂ ^{B}	6083	6112	149	205	2,45	3,35	2,02	2,79	3017	2188
Σ $_{2,000\ mg.kg}^{-1}$	12191	12237	297 ^{A}	360 ^{A}	2,44 $^{A'}$	2,94 $^{A'}$	2,30 $^{C''}$	2,68 $^{C''}$	5309	4563
Food oil of H. annuus L. seeds (2,000 mg.kg^{-1}) + Doxorubicin hydrochloride (5 mg.Kg^{-1})										
Σ ♀ ^{A}	6111	6115	165	157	2,70	2,57	3,85	5,64	1589	1085
Σ ♂ ^{B}	6197	6161	212	191	3,42	3,10	2,58	2,10	2403	2939
Σ $_{2,000\ mg.kg}^{-1}$	12308	12276	377 ^{A}	348 ^{A}	3,06 $^{A'}$	2,83 $^{A'}$	3,08 $^{C''}$	3,05 $^{C''}$	3992	4024

The letters A, B and C correspond to Tukey's studentized range (the same letter are not significantly different. $p < 0.05$).

9. SUBMISSÃO DE ARTIGO

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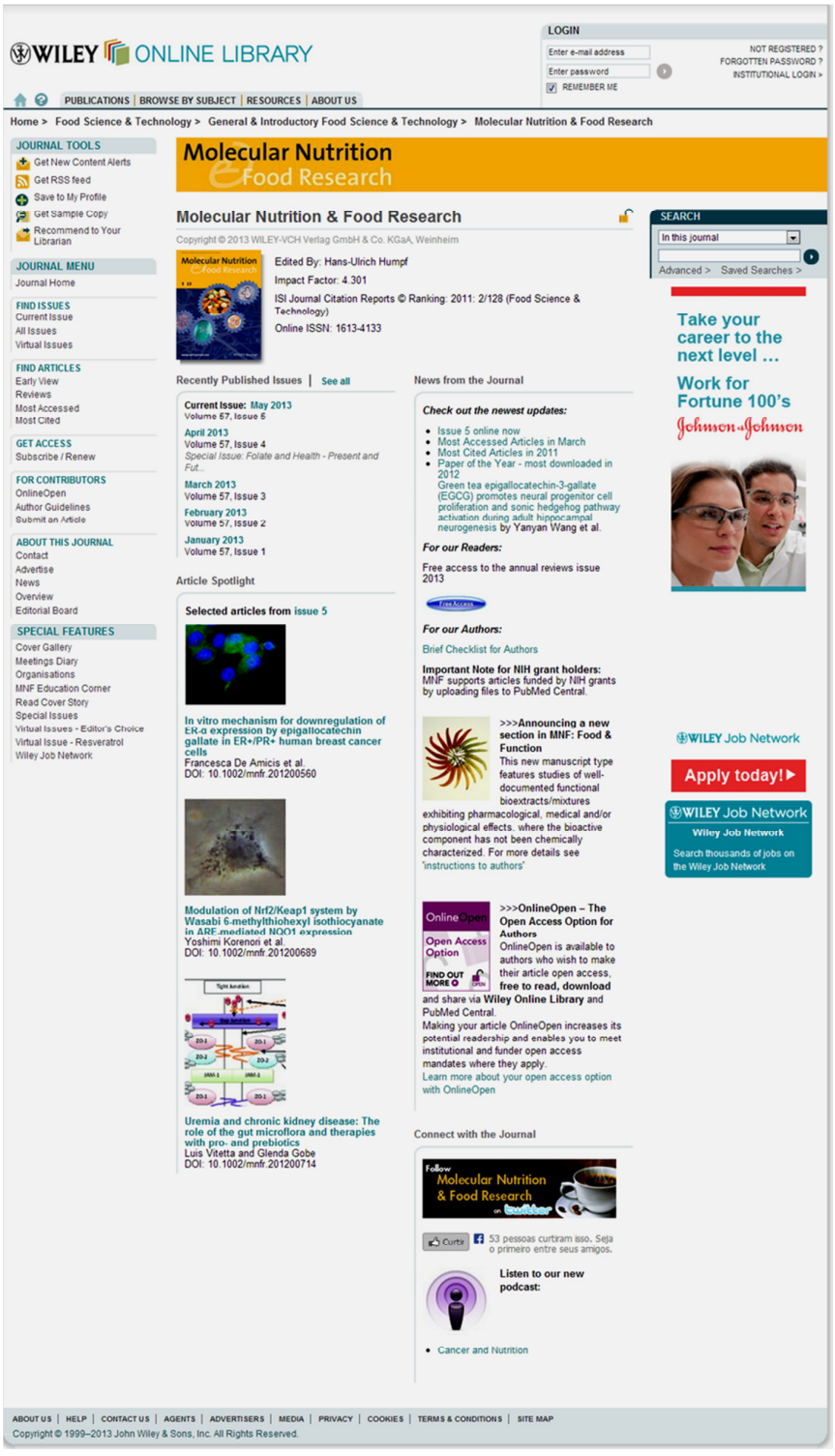


Jul 23, 2013

To **Molecular Nutrition & Food Research** Editorial Office

Dear Editor-in-Chief,

Hans-Ulrich Humpf (Münster, Germany)

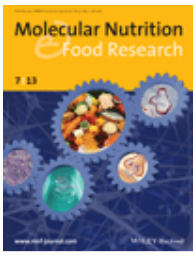
We would like to submit our manuscript entitled **"Nongenotoxic effects and a reduction of the DOX-induced genotoxic effects of *Helianthus annuus* Linné (sunflower) seeds revealed by micronucleus assays in mouse bone marrow"** for your consideration. We assure you that the content is original and not presently under consideration for publication elsewhere. The manuscript has been reviewed and agreed upon by all authors and is solely the work of the following researchers: **Boriollo MFG, Souza LS, Resende MR, Silva TA, Oliveira NMS, Braoios MCCR, Fiorini FE**. We can assure you that the text has been carefully revised and formatted according to Author Guidelines (MNFR).

We thank you in advance for your consideration and hope that the manuscript is now suitable for publication in Molecular Nutrition & Food Research.

Our most sincere regards,

Prof. Dr. Marcelo Fabiano Gomes Boriollo, BSB, MSc, PhD.

Molecular Nutrition & Food Research



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Last update: April 10, 2013
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Instructions to authors

Molecular Nutrition & Food Research
April 2013

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1 Aims and scope
Molecular Nutrition & Food Research (MNF) is a primary research journal devoted to health, safety and all aspects of molecular nutrition such as nutritional biochemistry, nutrigenomics and metabolomics aiming to link the information arising from the related disciplines Bioactivity & Safety, Immunology, Microbiology and Chemistry.

MNF is published in 12 issues *per* year, including regular issues as well as topical issues. Four categories of scientific contributions are accepted for publication:
(i) research articles,
(ii) reviews,
(iii) educational papers, and
(iv) food & function articles.

⇒ **Introducing 'Food & Function' - a new section in MNF:**
Manuscripts in which the individual components responsible for any biological activity have not been chemically characterized (e.g. animal studies with an uncharacterized extract of fruits) will not be accepted as full research articles. However, in these cases,authors may submit their manuscript in a shortened form for the new section "Food & Function". In this section, concise contributions describing the functional effects of food without a detailed characterization of the bioactive components will be considered for publication (for further details see Section 4 - Types of contributions).

Our Early View online publication is updated weekly and enables papers to be available online and citable before going into print.

2 General terms of publication
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MNF offers a web-based manuscript submission and peer review system. This service guarantees fast and safe submission of manuscripts and rapid assessment. Using this system is obligatory, conventional submission of manuscripts is not accepted.

3.1 General remarks

To submit your manuscript online, please proceed along the following steps:

- Prepare your manuscript and illustrations in the appropriate format, according to the instructions given below (see Sections 4 to 9). Please also make sure that your paper conforms with the scientific and style instructions of **MNF** as given herein. Links for English language assistance also provided here.
- If you have not already done so, create an account for yourself in the system at the submission site, http://mc.manuscriptcentral.com/mnf/ by clicking on the "Create Account" button.
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3.2 Electronic manuscripts

Please follow the instructions in Section 5 "Organization of manuscripts" when preparing the electronic version of the manuscript and ensure that data are given in the order and the correct style for the journal.

- Main text (incl. front material) as well as figure legends and tables (in this order) should be given in one file, preferably saved in .doc or .rtf format - Word 2007 or older, doc(x).
- Data should be typed unjustified, without hyphenation except for compound words. Use carriage returns only to end headings and paragraphs; spacing will be introduced by the typesetter.
- Do not use the space bar to make indents; where these are required (e. g. tables) use the TAB key.
- If working in Word for Windows, please create special characters using **Insert**/**Symbol**.
- Figures should preferably be in TIFF, EPS, PPT or the original format. See section 5.9 for details.

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3.3 Revised manuscripts

In revised manuscripts the areas containing the major required changes should be marked and the script color changed. The file(s) with the changes visible on screen should be submitted to the online procedure. Upon acceptance of the manuscript the final uploaded version will be taken as the basis for copy editing and the subsequent production process.

4 Types of contributions

Three types of scientific contributions are considered for publication:

(i) **Research articles** describing complete investigations. Unsolicited research articles should not exceed 6500 words in total; this includes references, figure legends and tables. Papers of up to 7 printed pages will be published free of charge; for papers exceeding that length a **page charge** (see the journals For Authors page) will be levied. Please note that the length of an article depends greatly on the type of figures and tables provided. Manuscipts must not have been published previously, except in the form of a preliminary communication.

(ii) **Reviews** providing an overview on the current research in a specific field. Review articles should not exceed 8500 words in total including references, figure legends and tables. Review articles of up to 15 printed pages will be published free of charge; for papers exceeding that length a **page charge** (see the journals For Authors page) will be levied.

(iii) **Educational papers** describing and/or explaining a method or technique used in food and nutrition research. They should be written in continuous style with headings (not numbered). An educational paper may be supplemented by multimedia material (e. g. animations or video sequences) which will be only available online.

(iv) **Food & Function articles** describing studies of well-documented functional bioextracts/mixtures exhibiting pharmacological, medical and/or physiological effects, where the bioactive component has not been chemically

characterized. However, the work reported has be supported by animal and/or human studies. Research based solely on cell culture will not be considered.
They should be written in a concise and continuous style without subheadings with a maximum of 2500 words (including references as well as figure and table legends) and three display elements (figures and tables). For an example of this type of article format click here. Longer articles will not be accepted for this category. Any additional material pertinent to the study should be provided as Supporting Information online only. This includes e.g any detailed Materials & Methods description. Authors submitting in this category should please make sure that they select 'Food & Function' as article type during submission.

Reviews and educational papers will normally be invited by the Editors. Authors wishing to submit a review or an educational paper should send a brief outline of its contents to the Editor-in-Chief (schreier@pzlc.uni-wuerzburg.de) before the manuscript is drafted.

5 Organization of manuscripts
Manuscripts must be typewritten with double spacing (including references, tables, legends, *etc.*).

5.1 Contents of first page of manuscript (all types of contributions)

The first page of the manuscript should contain only the following:

1) Title of the paper containing only the keywords pertaining to the subject matter. Standard abbreviations may be used in the title.
2) Full names (including first name) of the authors and the name of the institute. If the publication originates from several institutes the affiliations of all authors should be clearly stated by using superscript numbers after the name and before the institute.
3) Name (and title) and full postal address of the author to whom all correspondence (including galley proofs) is to be sent. E-mail and fax number must be included to speed up communication.
4) A list of abbreviations used in the paper excluding standard abbreviations (see list of "Standard Abbreviations", Section 9).
5) Keywords (max. 5, in alphabetical order).

5.2 Abstract (all types of contributions)

The second page of the manuscript should contain the abstract only. For research articles it should be structured as follows:

Scope
Methods and results
Conclusion (focus on nutritional relevance)

The abstract must be self-explanatory and intelligible without reference to the text. It should not exceed 200 words. Abbreviations, but not standard abbreviations, must be written in full when first used.

5.3 Division into sections (research articles only)

Manuscripts should be divided into the following sections:

"1 Introduction": containing a description of the problem under investigation and a brief survey of the existing literature on the subject.
"2 Materials and methods": for special materials and equipment, the manufacturer's name and location should be provided.
"3 Results"
"4 Discussion"
"5 References"

Sections 3 and 4 may be combined and should then be followed by a short section entitled "Concluding remarks". Subdivisions of sections should be indicated by subheadings.

5.4 References (all types of contributions)

References should be numbered sequentially in the order in which they are cited in the text. The numbers should be set in brackets, thus [2, 18]. References are to be collected in numerical order at the end of the manuscript under the heading "References"; they should also be typed with double spacing throughout. Papers with multiple authors should be limited to listing five authors. Where there are more than five authors, the first four should be listed, followed by *et al*. Please include the title of the manuscript in full, followed by a full stop. Journal names

should be abbreviated according to the practice of PubMed. The abbreviated journal name and the volume number should be in italics. Please note the following examples.

Journals:
[1] Keppler, K., Hein, E.-M., Humpf, H.-U., Metabolism of quercetin and rutin by the pig caecal microflora prepared by freeze-preservation. *Mol. Nutr. Food Res.* 2006, *50*, 686-695.

Other serial publications such as "*Advances in Food and Nutrition Research*" should be cited in the same manner as journals.

Books:
[2] Eisenbrand, G., Dayan, A. D., Elias, P. S., Grunow,W., Schlatter, J. (Eds.), *Carcinogenic and Anticarcinogenic Factors in Food*, Wiley-VCH Verlag,Weinheim 2003.

Chapter in a book:
[3] Geis, A., in: Heller, K. J. (Ed.), *Genetically Engineered Food - Methods and Detection*, Wiley-VCH Verlag, Weinheim 2003, pp. 100-118.

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5.5 Acknowledgements

Acknowledgements as well as information regarding funding sources should be provided on a separate page and will appear at the end of the text (before the "References").

5.6 Conflict of interest statement

Authors are responsible for disclosing all and any financial and personal relationships between themselves and others that might bias their work. To prevent ambiguity, authors must state explicitly whether potential conflicts do or do not exist. Should such a conflict of interest exist, a statement to that effect must be included in a separate paragraph following on from the acknowledgements section detailing - for each author - the nature of the conflict. Even if there is none, this should also be stated. This is a mandatory requirement for all articles.

5.7 Tables

Tables with suitable captions at the top and numbered with Arabic numerals should be collected at the end of the text on separate sheets (one page *per* table). Column headings should be kept as brief as possible and indicate units (in parentheses). Footnotes to tables should be indicated with a), b), c) *etc*. and typed on the same page as the table.

5.8 Supporting information

Extensive tables should be published online as supporting information. This material will not be typeset so authors should prepare it in the final form in which it should appear (no further editing will be done). Also for this reason there will be no galley proofs of this material. Supporting information will be made freely available on the web (similar to the table of contents and the article abstracts). Authors are permitted to place this material on their homepages when they are setting up a link to the full-text version of the article in Wiley Onine LIbrary.

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Type	Resolution
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- Crop, or scale, figures to the size intended for publication; no enlargement or reduction should be necessary. Otherwise figures should be submitted in a format which can be reduced to a width of 50-80 mm or 120-170 mm, with symbols and labels to a height of 2.0 mm (after reduction) and a minimum line weight of 0.3 pt for black lines.
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Abbreviations are hindrances to a reader working in a field other than that of the author, and to abstractors. Therefore, their use should be restricted to a minimum. Abbreviations should be introduced only when repeatedly used. Abbreviations used only in a table or a figure may be defined in the legend. Standard abbreviations may be used in the title and keywords. If nonstandard abbreviations are used in the Abstract they should be defined in the Abstract, in the list of abbreviations of the manuscript, as well as when first used in the body of the paper.

Section 9 at the end of these instructions contains the list of standard abbreviations which may be used without definition in the articles published in **MNF**.

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8 Reporting specific data
8.1 Chemical structures

Structures should be produced with the use of a drawing program such as ChemDraw.
Structure drawing preferences are as follows:
As drawing settings select:

chain angle 120°
bond spacing 18% of width

fixed length 14.4 points (0.508 cm, 0.2 in.)
bold width 2.0 points (0.071 cm, 0.0278 in.)
line width 0.6 point (0.021 cm, 0.0084 in.)
margin width 1.6 points (0.056 cm, 0.0222 in.)
hash spacing 2.5 points (0.088 cm, 0.0347 in.)
As text setting select: font, Arial or Helvetica; size, 10 pt.
Under the preferences choose: units, points; tolerances, 3 pixels.
Under page setup choose: paper, US Letter; scale, 100%.

Using the ChemDraw ruler or appropriate margin settings, create structure blocks, schemes, and equations having maximum widths of 11.3 cm (one-column format) or 23.6 cm (two-column format). Note: if the foregoing preferences are selected as cm values, the ChemDraw ruler is calibrated in cm. Also note that a standard sheet of paper is only 21.6 cm wide, so all graphics submitted in two-column format must be prepared and printed in landscape mode.
Use boldface type for compound numbers but not for atom labels or captions.
Authors using other drawing packages should, as far as possible, modify their program's parameters to reflect the above guidelines.

8.2 Physical and other data

It is important that novel compounds, either synthetic or isolated/produced from natural sources, be characterized completely and unambiguously. Supporting data normally include physical form, melting point (if solid), UV/IR spectra, if appropriate, ^{1}H and ^{13}C NMR, mass spectral data, and optical rotations or CD information (when compounds have chiral centers).

Reports on flavor constituents should conform to the recommendations made by the International Organization 5 of the Flavor Industry (IOFI). Thus, substances must be identified using the latest analytical techniques. In general, any particular substance must have its identity confirmed by at least two methods; that means, in practice, comparison of chromatographic and spectroscopic data (which may include GC, MS, IR, and NMR) with those of an authentic sample. If only one method has been applied, the identification has to be labeled as "tentative": This is also valid in case of identification performed only by comparison of literature data.

Equations should be numbered consecutively and referred to the text; *e.g.* defined as in Eq. (1).

Physical data should be quoted with decimal points (e. g. 25.8 Jk^{-1} mol^{-1}), and arranged as follows where possible - but in any event in the same order within the manuscript (when measurement conditions remain unchanged they need only be mentioned once, for instance in the column headings): m.p./b.p. 20°C; $[\alpha]_D^{20}$ = -13.5 (*c* = 0.2 in acetone) ^{1}H NMR (200 MHz, [D_8]THF, 25°C, TMS): δ = 1.3 (q, ^{3}J (H,H) = 8 Hz, 2 H; CH_2), 0.9 ppm (t, ^{3}J (H,H) = 8 Hz, 3 H; CH_3); IR(Nujol): $\tilde{\nu}$ = 1790 cm^{-1} (C=O); UV/Vis (*n*-hexane): λ_{max} (ε) = 320 (5000), 270 nm (12000); MS (70 eV): *m/z* (%): 108 (20) [M^+], 107 (60) [M^+ -H], 91 (100) [$C_7H_7^+$]. Plane angles in products of units can have either ° or deg as the unit.

Nomenclature, symbols, and units: The rules and recommendations of the International Union of Pure and Applied Chemistry (IUPAC), the International Union of Biochemistry (IUB), and the International Union of Pure and Applied Physics (IUPAP) should be adhered to.

8.3 Nucleotide and protein sequences

New nucleotide data must be submitted and deposited in the DDBJ/EMBL/GenBank databases and an accession number obtained before the paper can be accepted for publication. Submission to any one of the three collaborating databanks is sufficient to ensure data entry in all. The accession number should be included in the manuscript, e. g. as a footnote on the title page: ‚Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number(s) -'. If requested the database will withhold release of data until publication. The most convenient method for submitting sequence data is by the World Wide Web:

EMBL *via* Webin:
http://www.ebi.ac.uk/embl/Submission/webin.html
GenBank *via* Bankit:
http://www.ncbi.nlm.nih.gov/BankIt/
DDBJ *via* Sakura:
http://sakura.ddbj.nig.ac.jp/

Alternatively, the stand-alone submission tool ‚Sequin' is available from the EBI at http://www.ebi.ac.uk/Sequin and from NCBI at http://www.ncbi.nlm.nih.gov/Sequin/.

For special types of submissions (e. g. genomes, bulk submissions *etc.*) additional submission systems are available from the above sites.

Database contact information:

EMBL: EMBL Nucleotide Sequence Submissions
European Bioinformatics Institute
Wellcome Trust Genome
Campus , Hinxton, Cambridge CB10 1SD, UK
Tel.: +44 1223 494400; fax: +44 1223 494472
E-mail: datasubs@ebi.ac.uk
http://www.ebi.ac.uk

GenBank: National Center for Biotechnology
Information
National Library of Medicine,
Bldg. 38A, Rm 8 N-803
Bethesda, MD 20894, USA
Tel.: +1 301 496 2475; fax: +1 301 480 9241
E-mail: info@ncbi.nlm.nih.gov
http://www.ncbi.nlm.nih.gov

DDBJ: Center for Information Biology and
DNA Data Bank of Japan
National Institute of Genetics, 111 Yata,
Mishima, Shizuoka 411-8540, Japan
Tel.: +81 559 81 6853; fax: +81 559 81 6849
E-mail: ddbj@ddbj.nig.ac.jp
http://www.ddbj.nig.ac.jp

Protein sequences which have been determined by direct sequencing must be submitted to Swiss-Prot at the EMBL Outstation - The European Bioinformatics Institute. Please note that we do not provide accession numbers, **in advance**, for protein sequences that are the result of translation of nucleic acid sequences. These translations will automatically be forwarded to us from the EMBL nucleotide database and are assigned Swiss-Prot accession numbers on incorporation into TrEMBL.

Results from characterization experiments should also be submitted to Swiss-Prot at the EBI. This can include such information as function, subcellular location, subunit *etc*.
Contact information:

Swiss-Prot: Swiss-Prot submissions,
European Bioinformatics Institute
Wellcome Trust Genome Campus, Hinxton
Cambridge, CB10 1SD, UK
Tel.: +44 1223 494400; fax: +44 1223 494472
E-mail: datasubs@ebi.ac.uk (for sequence submissions); update@ebi.ac.uk (for characterization information)
http://www.ebi.ac.uk

9 Standard abbreviations

The abbreviations as listed below may be used without definition in the articles published in **MNF**. Please refer to Section 5.14 for the correct usage of abbreviations in **MNF**.

A	absorbance
ACN	acetonitrile
A/D	analog to digital converter
amu	atomic mass unit
API	atmospheric pressure ionization
BMI	body mass index
bp	base pairs
BSA	bovine serum albumin
CBB	Coomassie Brilliant Blue
CE	capillary electrophoresis
CEC	capillary electrochromatography
CFE	continuous flowelectrophoresis
CID	collision-induced dissociation
cpm	counts *per* minute

CV	coefficient of variation
CZE	capillary zone electrophoresis
1-D	one-dimensional
2-D	two-dimensional
Da	dalton (molecular mass)
DAD	diode-array detection (or diodearray detector)
2-DE	two-dimensional gel electrophoresis
DMEM	Dulbecco's modified Eagle medium
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
dsDNA	double-stranded DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EOF	electroosmotic flow
ER	endoplasmic reticulum
ESI	electrospray ionization
FAB	fast atomic bombardment
FAME	fatty acid methyl esters
FITC	fluorescein isothiocyanate
GC	gas chromatography
GMO	genetically modified organism
HDL	high density lipoprotein
HEPES	*N*-(2-hydroxyethyl)piperazine-2'-(2-ethane-sulfonic acid)
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
HSA	human serum albumin
HTML	hypertext mark-up language
id	inside diameter
IEF	isoelectric focusing
Ig	immunoglobulin
IL	interleukin
IFN	interferon
IT	ion trap
kbp	kilobase pairs
kDa	kilodalton (molecular mass)
LC	liquid chromatography
LDL	low denisty lipoprotein
LOD	limit of detection
LOQ	limit of quantitation
LPS	lipopolysaccharide
mAb	monoclonal antibody
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
Mbp	megabase pairs
MHC	major histocompatibility complex
MOPS	3-(*N*-morpholino)propanesulfonic acid
M_r	relative molecular mass (dimensionless)
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MUFA	monounsaturated fatty acid
m/z	mass-to-charge ratio
NMR	nuclear magnetic resonance
od	outside diameter
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
p*I*	isoelectric point
PMSF	phenylmethylsulfonyl fluoride
PMT	photomultiplier tube
ppm	parts *per* million
PTFE	polytetrafluoroethylene
PUFA	polyunsaturated fatty acid
PVP	polyvinylpyrrolidone

RIA	radioimmunoassay
RNA	ribonucleic acid
RP	reversed phase
rpm	rotations *per* minute
RSD	relative standard deviation
RT-PCR	reverse transcriptase-PCR
SCFA	short chain fatty acid
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SIM	selected ion monitoring
S/N	signal-to-noise ratio
SPE	solid-phase extraction
ssDNA	single-stranded DNA
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIC	total ion current
TLC	thin-layer chromatography
TOF	time of flight
Tris	tris(hydroxymethyl)aminomethane
URL	uniform resource locator
Vh	volt x hours
VLDL	very low density lipoprotein