

UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
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**EFEITOS DO ÓLEO ESSENCIAL DE *Cymbopogon citratus* STAPF
(CAPIM-LIMÃO) SOBRE O PROCESSO DE CARCINOGENESE
QUÍMICA EM FÊMEAS BALB/C**

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“Metade de mim agora é assim
De um lado a poesia, o verbo, a saudade
Do outro a luta, a força e a coragem pra chegar no fim
E o fim é belo e incerto... depende de como você vê
O novo, o credo, a fé que você deposita em você e só.”

Fernando Anitelli

“Tem que sambar o tango, tomar o tombo
Aceitar o tapa que a mentira tampa
Manter o tom de tolerância
Tolerando o chato que não se cansa.” (Trevis)

“Pra dilatarmos a alma
Temos que nos desfazer
Pra nos tornarmos imortais
A gente tem que aprender a morrer
Com aquilo que fomos
E aquilo que somos nós.” (Fernando Anitelli)

“Há um tempo em que é preciso abandonar as roupas usadas, que já têm a forma do nosso corpo, e esquecer os nossos caminhos, que nos levam sempre aos mesmos lugares. É o tempo da travessia... e se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos.” (Fernando Pessoa)

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Capítulo I

Introdução

1- Introdução

1.1- Carcinogênese química

O câncer é uma doença crônico-degenerativa responsável por aproximadamente 12% das causas de morte no mundo (Guerra et al., 2005). Sua elevada taxa de morbidade/mortalidade se dá pelo aumento gradual ocorrido na industrialização, o que levou as pessoas a modificarem seus padrões alimentares, e pela redução nas taxas de mortalidade em geral, com aumento da expectativa de vida e envelhecimento populacional, afetando a prevalência de doenças crônico-degenerativas (como o câncer, doenças cardiovasculares, diabetes tipo 2, doença de Alzheimer e outros agravos relacionados ao envelhecimento e à obesidade) (Albala et al., 2001; Waters, 2001; Guerra et al., 2005).

Alguns estudos mostram as neoplasias de próstata, pulmão, estômago, esôfago, cólon e reto como as mais frequentes nos homens brasileiros, enquanto nas mulheres as neoplasias de mama, colo uterino, pulmão, estômago, cólon e reto prevalecem (Guerra et al., 2005). Quanto à mortalidade, tumores de pulmão, próstata, estômago, esôfago, boca e faringe são prevalentes em homens, e os de mama, pulmão, colo de útero, estômago, cólon e reto são prevalentes em mulheres (WHO, 2002). Esses dados populacionais impulsionam os pesquisadores a tentarem entender melhor a evolução dessa doença para descobrir suas causas e/ou relacioná-la com fatores de risco, e a procurarem novos métodos de prevenção e tratamento.

O processo de carcinogênese química ocorre através de múltiplas etapas denominadas de iniciação, promoção, progressão e manifestação (Dragan et al., 1991; Pitot, 1993, 2001). A iniciação e promoção estão bem estabelecidas experimentalmente. Entende-se por iniciação a interação de um agente químico ou físico com o DNA da célula-alvo. É um processo irreversível, porque altera permanentemente a estrutura do DNA, através de ligações covalentes, distorção ou quebra da molécula (Dragan et al., 1991; Pitot, 1993, 2001). A promoção se dá quando tal alteração é expressa, ocorrendo o aparecimento de lesões focais de células alteradas ou lesões pré-neoplásicas (Dragan et al., 1991; Pitot, 1993, 2001). Essas lesões apresentam importantes modificações em níveis tanto moleculares quanto morfológicos, incluindo genes que controlam a proliferação celular, diferenciação e apoptose (Cordon-Cardo, 1998; Ray et al., 2007). Quando as lesões adquirem características de malignidade tem-se a fase de progressão (Pitot, 1993, 2001), na qual são observadas alterações no genoma, relacionadas à proliferação celular/apoptose, invasividade, capacidade de produzir metástases e determinadas alterações bioquímicas, fornecendo substrato

biológico para a manifestação clínica do câncer (Rodrigues e De Camargo, 1999; Robbins e Cotran, 2005).

Os agentes químicos começaram a serem reconhecidos como carcinogênicos para o homem no século XVIII, observando-se os altos índices de câncer de pele de escroto em limpadores de chaminé e de carcinogênese nasal pelo uso de rapé. Com isso, no século XX, desenvolveram-se modelos animais de carcinogênese química para o estudo da biologia tumoral e detecção de substâncias com potencial cancerígeno (Rodrigues e De Camargo, 1999; Robbins e Cotran, 2005).

Os cancerígenos químicos podem atuar nas diferentes etapas da carcinogênese. Os agentes iniciadores podem ser enquadrados em duas grandes categorias: 1) compostos de ação direta, que não exigem biotransformação para exercer sua carcinogenicidade e 2) compostos de ação indireta, ou pré-cancerígenos, que exigem conversão metabólica para produzir cancerígenos finais com caráter nucleofílico, como, por exemplo, radicais de oxigênio, que são capazes de levar a lesões oxidativas e transformar as células-alvo (Preston e Williams, 2005). As células iniciadas, com alterações permanentes em seu DNA, passam a se proliferar em ritmo diferente das outras células normais, adquirem novas mutações e respondem de maneira mais efetiva aos compostos promotores, ou não genotóxicos (Preston e Williams, 2005).

De forma geral, os modelos *in vivo* de carcinogênese química experimental requerem longo tempo de acompanhamento dos animais para observação de neoplasias, tornando-os dispendiosos e de execução laboriosa. Por esta razão, existe grande interesse na padronização de ensaios mais rápidos, que permitam detectar alterações que precedem o aparecimento das neoplasias (De Camargo et al., 1999). Essas alterações, designadas biomarcadores, estão associadas a estágios iniciais do processo de carcinogênese e podem ser identificadas em nível molecular, celular ou morfológico (Lippman et al., 1990; Pereira et al., 1994; Ward e Henderson, 1996; Femia e Caderni, 2008). Como exemplo podemos citar as alterações estruturais precoces no cólon, mama e bexiga de roedores. Na mama podem ser encontradas as displasias e hiperplasias epiteliais (Cardiff e Wellings, 1999), na bexiga, as hiperplasias simples e as hiperplasias papilíferas/nodulares (Cohen, 1998, 2002) e na mucosa do cólon, as criptas aberrantes, que podem ser identificadas como únicas ou na forma de focos de criptas aberrantes (FCAs) (Femia e Caderni, 2008).

Porém a maioria dos estudos na área de carcinogênese é limitada, pois são utilizados modelos que induzem lesões preneoplásicas e/ou neoplásicas em apenas um órgão, o que

pode gerar, por exemplo, “resultados falso-negativos” quanto aos efeitos de determinado composto sobre a carcinogênese. Portanto é possível que o efeito inibitório ou promotor de uma substância química ou grupos de compostos seja mais bem analisado em mais de um órgão-alvo (Ito e Imaida 1992; Ito et al., 2000; Tsuda et al., 1996) sendo, portanto, os modelos experimentais de múltiplos-órgãos mais adequados para a análise de substâncias quimiopreventivas (Tsuda et al., 1996).

1.2- Carcinogênese mamária

O câncer de mama é a neoplasia mais freqüente em mulheres e sua etiologia não é totalmente conhecida (Parkin et al., 2001). Como uma doença altamente heterogênea, é representada por neoplasias que possuem história natural diversa, histologia complexa e resposta variável às terapias convencionais (quimioterapia, braquioterapia e radioterapia) (Parkin et al., 2001).

Suas taxas de mortalidade e morbidade vêm aumentando gradualmente na maioria das “sociedades industrializadas” nas últimas décadas e aumentado abruptamente em mulheres mais jovens desde o ano de 2002 (Jemal et al., 2002; Althuis et al., 2005; Bouchardey et al., 2007). Pelo pouco conhecimento de sua causa e das razões desse aumento, uma prevenção efetiva ainda é difícil de ser traçada, porém alguns fatores de risco são bem estabelecidos, tais como a exposição à radiação (Cutuli et al., 2001; Andrieu et al., 2006), ao tabaco (Band et al., 2002; Ha et al., 2007), á cancerígenos químicos, poluentes ambientais, praguicidas, drogas e luz ultravioleta (Perera, 1997). Estima-se que 50% dos casos de câncer de mama possam ser atribuídos a fatores de risco que incluem idade, estilo de vida e história familiar e reprodutiva (Wolf e Weston, 1997; Snedeker, 2001).

Por razões práticas, a maioria dos estudos experimentais de carcinogênese mamária é conduzida em roedores devido à baixa freqüência de tumores espontâneos observados nesses animais em estudos de longa duração (Anderson, 1992; Russo e Russo, 1996). Tumores experimentais induzidos pela administração de cancerígenos químicos constituem ferramentas úteis para o entendimento das múltiplas etapas da carcinogênese mamária (Anderson, 1992; Tornqvist et al., 1994), bem como testes de potencial cancerígeno de agentes químicos ambientais (Medina et al., 1981; Grubbs et al., 1985; Welsch, 1987) e estudos preventivos e terapêuticos da carcinogênese mamária (Russo et al., 1990; Russo e Russo, 1994). As neoplasias mamárias quimicamente induzidas são, em geral, carcinomas hormônio-dependentes. A incidência, multiplicidade e tipos de tumores mamários são influenciados pela idade, tempo de exposição ao cancerígeno, história reprodutiva, desregulação endócrina, dieta

e outros fatores que alteram o desenvolvimento e o grau de diferenciação da glândula mamária (Russo e Russo, 1994). As substâncias químicas mais utilizadas nos modelos experimentais de indução da carcinogênese mamária em fêmeas de ratos e camundongos são a 7,12-dimetilbenz(a)antraceno (DMBA) e a N-metil-N-nitrosourea (MNU). A exposição de ratas jovens a esses cancerígenos antes da primeira prenhez aumenta a susceptibilidade das glândulas mamárias à iniciação do câncer (Russo et al., 1992).

1.3- Carcinogênese de bexiga urinária

No ano de 1999, as neoplasias de bexiga constituíam um dos quatro tipos de câncer mais frequentes no homem no mundo, precedida por câncer de próstata, pulmão e câncer colorretal (Netto Jr., 1999). Mais de 90% dessas neoplasias são representadas pelos carcinomas de células transicionais, 6% pelos carcinomas epidermóides, e os demais por adenocarcinomas e tumores de origem não epitelial (Rischmann, 2000; Gabriel et al., 2007).

A taxa de mortalidade por câncer de bexiga varia em diferentes países e em diferentes regiões de um mesmo país. As taxas mais elevadas são notificadas na Dinamarca, Reino Unido, Bélgica e Itália, e as mais baixas, no Japão, Singapura e Venezuela (Paneau et al., 1992). Estimativas de 2006 mostraram mais de 105 mil novos casos de câncer de bexiga e aproximadamente 36 mil mortes por essa doença apenas na Europa (Ferlay et al., 2007), o que indica a importância deste tipo de câncer como uma causa de mortalidade em humanos.

As neoplasias uroteliais de bexiga urinária humana, em especial, têm uma alta taxa de recorrência após remoção cirúrgica e tratamento, na dependência do tipo de câncer – se superficial ou invasivo (Rischmann, 2000), o que demanda preocupação e acompanhamento médico periódico (Negri e La Vecchia, 2001; Rabbani et al., 2001; Patton et al., 2002). Deste modo, medidas preventivas e terapêuticas como mudança do estilo de vida, a adoção de dietas pobres em gorduras, além do uso de quimioterápicos, radioterápicos, BCG e procedimentos invasivos como cirurgia aberta e ressecção endoscópica, estão sendo propostas (Vena et al., 1992).

O urotélio, epitélio de transição, reveste o trato urinário inferior se estendendo da pelve renal, passando pelos ureteres e bexiga urinária até a uretra. Tumores que acometem este tipo de epitélio são designados de tumores de células transicionais (uroteliais), embora possam se desenvolver tumores glandulares e de células escamosas (Cohen, 2002). No homem as neoplasias de células transicionais da bexiga são divididas em duas categorias (Epstein et al., 1998): 1) neoplasias papilíferas bem diferenciadas de baixo grau, com pouca capacidade para invasão e para desenvolvimento de metástases e 2) neoplasias de alto grau

que progridem de uma superfície displásica e carcinoma *in situ* a lesões invasivas com metástases em linfonodos locais e em sítios distantes. Frequentemente essas neoplasias apresentam áreas com diferenciação escamosa ou glandular, ou mesmo, áreas com células indiferenciadas.

Em roedores, em especial ratos e camundongos, as neoplasias uroteliais têm sua origem como hiperplasias simples, passando às hiperplasias nodulares e papilíferas, papilomas (lesões benignas), carcinomas de alto grau não invasivos e, finalmente, neoplasias invasivas (Cohen, 1998, 2002). Em camundongos as neoplasias são altamente malignas (lesões carcinomatosas e displásicas de alto grau) e, frequentemente, se metastatizam para linfonodos regionais, pulmão e eventualmente para outros sítios. Biologicamente estas neoplasias apresentam mutações no gene p53 e em outros genes relacionados; diferentemente das neoplasias uroteliais em ratos que apresentam baixa frequência de mutações no gene p53 e raramente metastatizam. Desta forma o modelo de carcinogênese urotelial em ratos relembra as neoplasias uroteliais papilíferas de baixo grau, enquanto o modelo em camundongos relembra as neoplasias altamente malignas e de alto grau em humanos (Cohen, 1998, 2002).

As substâncias mais utilizadas em modelos de carcinogênese química de bexiga são a N-butil-N-(4-hidroxibutil)nitrosamina (BBN), a N-(4-(5-nitro-2-furil)-2-tiazolil)formamida (FANT) e a N-metil-N-nitrosuréia (MNU) (Gabriel et al., 2007). Doses relativamente baixas destes agentes interagem com o DNA e induzindo mutações (gênicas ou cromossômicas), caracterizando a etapa de iniciação da carcinogênese. Entretanto, a princípio essas alterações genotípicas não são exteriorizadas fenotipicamente (Cohen e Ellwein, 1991).

Nos modelos de carcinogênese urotelial envolvendo as etapas de promoção e progressão em roedores, as alterações seqüenciais uroteliais que ocorrem durante o processo da carcinogênese são fenotipicamente observáveis: hiperplasia simples (HS), correspondente ao aumento do número de camadas de células do urotélio e hiperplasia papilífera ou nodular (HPN) que pode ser exofítica, endofítica ou mista. Muitas vezes estas lesões se associam a alterações displásicas. O desenvolvimento destas lesões está diretamente relacionado à dose e à duração do estímulo carcinogênico, e estas podem evoluir, transformando-se em papilomas e carcinomas, ou serem revertidas, restabelecendo-se, assim, o aspecto normal do urotélio (Cohen, 1983, 1998).

1.4- Carcinogênese de cólon

De acordo com estudos epidemiológicos o câncer de cólon está entre os cinco mais incidentes tanto em homens quanto em mulheres no Brasil (Guerra et al., 2005) e no mundo (WHO, 2002). Existem várias pesquisas que relacionam fatores como a dieta e a exposição ocupacional a agentes químicos com o aumento da incidência de câncer de cólon (Medrado-Faria et al., 2001; Ferguson et al., 2004).

Experimentalmente, os principais cancerígenos utilizados em estudos de carcinogênese química em murinos são a 1,2-dimetilhidrazina (DMH) e os seus metabólitos, o azoximetano (AOM) e a metilazoximetanol (MAM) (Cardeni et al., 1995; Sequeira et al., 2000; Femia e Caderni, 2008; Rosenberg et al., 2008). Esses modelos são reprodutíveis para diversas linhagens de ratos e camundongos e as lesões neoplásicas que se desenvolvem a princípio são altamente similares aos carcinomas colorretais não familiares (“esporádicos”), propiciando estudos de quimioprevenção para esse tipo de lesão. Além dos modelos citados acima, existem muitos outros cancerígenos que podem ser utilizados no estudo de neoplasias de cólon em roedores, tais como a 2-amino-1-metil-6-fenilimidazo[4,5-*b*]piridina (PhIP), 2-amino-3-metilimidazo[4,5-*f*]quinolina (IQ), 3,2'-dimetil-4-aminofenil (DMAB), *N*-metil-*N*'-nitro-*N*-nitrosoguanidina (MNNG) e a *N*-metil-*N*-nitrosuréia (MNU) (Rosenberg et al., 2008).

Esses agentes inicialmente causam alterações nas células epiteliais das criptas colônicas, tornando essas criptas de tamanho aumentado, de aspecto mais corado, com suas aberturas menos circulares (tendendo a serem alongadas, elípticas ou tortuosas), sendo chamadas de criptas aberrantes (CA). Essas CA encontram-se isoladas ou agrupadas em focos, chamados focos de criptas aberrantes (FCA) (Bird, 1995; Bird e Good, 2000; Rodrigues et al., 2002).

Acredita-se que as criptas aberrantes sejam expressões morfológicas das alterações da proliferação e diferenciação celular que ocorrem devido a alterações da zona proliferativa das criptas durante o processo de carcinogênese do cólon (Fenoglio-Preiser e Noffsinger, 1999; Rosenberg et al., 2008). De fato, na mucosa do cólon de animais tratados com cancerígenos químicos as criptas aberrantes são revestidas por células epiteliais com diferentes graus de maturação celular e displasia (Bird, 1995, Rosenberg et al., 2008). Há trabalhos que demonstram nos focos de criptas aberrantes índices de proliferação celular maiores que os da mucosa normal (Polyak et al., 1996; Shpitz et al., 1997; Fenoglio-Preiser e Noffsinger, 1999) e que existem diferenças de susceptibilidade no desenvolvimento dessas lesões entre diferentes linhagens de ratos e camundongos (Rosenberg e Liu, 1995).

1.5- Quimioprevenção do câncer

A quimioprevenção do câncer pode ser definida como a prevenção, inibição ou reversão do processo de carcinogênese, pela administração de substâncias químicas naturais ou sintéticas (Stoner et al., 1997; De Flora e Ferguson, 2005). As substâncias quimiopreventivas podem ser classificadas em três categorias, de acordo com seus efeitos nas diferentes fases da carcinogênese: 1) inibidor da formação do cancerígeno; 2) bloqueador do metabólito ativo e, 3) supressor do desenvolvimento tumoral (Wattenberg, 1985). Entretanto, é difícil a classificação precisa dos agentes quimioprotetores, visto que os mecanismos de ação da maioria dos compostos são desconhecidos, e muitos deles atuam sobre o processo de carcinogênese através de diferentes mecanismos (Stoner et al., 1997).

De fato, existem diferenças nas incidências de câncer quando são consideradas diferentes regiões do planeta, e muitas destas estão diretamente relacionadas aos hábitos de vida, exposição ocupacional, condições sócio-econômicas e de alimentação (Doll e Peto, 1981; Kligerman, 2002; Cervi et al., 2005). Pela sua natureza de múltiplos estágios, há a possibilidade de ações quimiopreventivas com compostos que atuam em mecanismos alvo-específicos, envolvidos na iniciação, promoção ou progressão do câncer (Greenwald e Kelloff, 1996; De Flora e Ferguson, 2005). Atualmente, o foco da quimioprevenção vem ganhando espaço na literatura científica em virtude do grande número de trabalhos científicos publicados acerca de diferentes produtos ou moléculas naturais em diferentes ensaios *in vitro* e *in vivo* (Greenwald e Kelloff, 1996; Ames, 2004; Ferguson et al., 2004; Femia e Caderni, 2008).

Existem vários estudos relacionando o consumo de frutas, cereais, vegetais e chás (fibras, retinóides, fenóis, inibidores de proteases, indoles, isocianatos, polifenóis, entre outros) (Potter e Steinmetz, 1996; Ferguson et al., 2004; Aggarwal and Shishodia, 2006) e micronutrientes (Omenn, 1996; Reddy, 1996; Ames, 2004) e a quimioprevenção do câncer. Os resultados desses estudos estimulam futuras pesquisas com o objetivo de identificar quais compostos dos elementos de nossa alimentação são responsáveis por tais reduções (Riboli et al., 1996; De Flora e Ferguson, 2005).

Inicialmente a maioria dos estudos epidemiológicos relacionando dieta e câncer foi realizada para identificar componentes da dieta associados à alta incidência de certos tipos de câncer, como a de colorretal e mama em países desenvolvidos e a incidência de câncer de estômago e esôfago em alguns países em desenvolvimento (Riboli et al., 1996). Esses resultados sugeriram que a intervenção no processo de carcinogênese, seja bloqueando a ação

dos fatores causais, seja atuando na identificação de fatores moduladores, é de fundamental importância na estratégia preventiva contra esta doença (Riboli et al., 1996).

1.6- Capim-limão

O Capim-limão, *Cymbopogon citratus* Stapf, popularmente conhecido como capim-santo, capim cidrão, chá-de-estrada, erva-cidreira e capim-cheiroso, é uma planta originária do sudoeste asiático e pertencente à família das *Poaceae* (*Graminea*). Perene e de médio porte, constitui-se de touceiras compactas e grandes, com distribuição por todo o globo em zonas tropicais e de savana, e não suportam regiões muito frias, sujeitas à geada. O Brasil é um dos países onde essa planta está perfeitamente aclimatada, em suas regiões tropicais (Negrelle e Gomes, 2007).

Possui odor característico exalado pelas folhas, hastes e rizomas, devido à presença, em especial, do composto citral em seu óleo essencial (OE), uma mistura de 2 isômeros, geranial e neral (Ferreira e Fonteles., 1989). Além do citral, o OE de capim-limão possui outros compostos ativos, sendo que os principais são o β -mirceno e o geraniol. O principal composto, citral, é responsável por aproximadamente 40 a 80% do total do óleo, enquanto o β -mirceno e o geraniol compõem a maior parte da porcentagem restante (Schaneberg e Khan, 2002; Barbosa et al., 2008).

O chá de suas folhas é utilizado popularmente no Brasil como antiespasmódico, analgésico, anti-inflamatório, antipirético, diurético e sedativo (Carlini et al., 1986) e seu óleo essencial é amplamente utilizado pelas indústrias de perfumes e cosméticos (Ferreira e Fonteles, 1989). Vários são os estudos descritos na literatura sobre as atividades do capim-limão, e diferentes são as formas de extração de seus compostos potencialmente ativos: óleo essencial (Blanco et al., 2007; Da Silva et al., 2008), extrato etanólico (Puatachokchai et al., 2002; Pereira et al., 2008), metanólico (Tapia et al., 2007) e até decocções e infusões (Cheel et al., 2005), sendo administrados geralmente pela via intragástrica ou através da dieta.

Algumas atividades farmacológicas do óleo essencial do capim-limão ou de seus compostos têm sido descritas na literatura. Por exemplo, Blanco e colaboradores (2007) e do Vale e colaboradores (2002) descrevem os efeitos do OE ou de seus compostos citral e β -mirceno no sistema nervoso central em camundongos Swiss machos, através da análise comportamental, enquanto Viana e colaboradores (2000) descrevem os efeitos anti-noceptivos do óleo essencial também nessa linhagem de camundongos. O óleo essencial também

apresenta atividade antifúngica contra diferentes espécies de *Candida* (Da Silva et al., 2008) e atividade antibacteriana, principalmente devido à presença do citral (Onawunmi et al., 1984).

O extrato etanólico de capim-limão não se mostrou mutagênico em *Salmonella typhimurium*, inibiu a mutagenicidade causada por vários mutágenos nessa mesma linhagem de bactérias (Vinitketkumnuen et al., 1994) e reduziu a mortalidade de *Escherichia coli* induzidas por dano oxidativo pelo SnCl_2 (Melo et al., 2001). Esse extrato também inibiu a formação de aberrações cromossômicas em linfócitos humanos expostos a mitomicina C (Meevatee et al., 1993), inibiu a formação de micronúcleos em ratos expostos a ciclofosfamida, retardou o crescimento tumoral e diminuiu o número de metástases em camundongos transplantados com células de fibrosarcoma 180 (Suaeyun et al., 1997). Além disso, os extratos etanólicos, metanólicos, decocções ou infusões apresentaram efeitos antioxidantes, reduzindo a peroxidação lipídica (analisado através da inibição da produção de TBARS) em cérebro de ratos, induzida pelo sulfato de ferro, nitroprussiato de sódio ou ácido 3-nitropropiónico (Pereira et al., 2008) ou em hemáceas, induzida por t-butil hidroperóxido (Cheel et al., 2005). Além disso, apresenta atividade de “*Free Radical Scavenging*”, através da captura do radical livre DPPH (1,1-difenil-2-picril-hidrazil) (Cheel et al., 2005; Tapia et al., 2007; Pereira et al., 2008) ou do anion superóxido (Cheel et al., 2005). O extrato de capim-limão aumentou a atividade da glutatona S-transferase em intestino de camundongos (Lam e Zheng, 1991), reduziu o dano oxidativo (8-OHdG) em hepatócitos de ratos F344 tratados com dietilnitrosamina (DEN) (Puatanachokchai et al., 2002) e a formação de adutos de DNA (7-meG e O⁶-meG) no cólon de ratos F344 induzida pelo AOM (Suaeyun et al., 1997).

Em experimentos de carcinogênese química, os efeitos do extrato etanólico do capim-limão foram observados através da redução de focos GST-P positivos em fígado de ratos F344 iniciados pela DEN (Puatanachokchai et al., 2002) e na incidência e/ou multiplicidade de focos de criptas aberrantes em ratos F344, com o capim-limão administrado durante as fases de iniciação e promoção da carcinogênese de cólon pelo AOM (Suaeyun et al., 1997).

Recentemente, Fandohan e colaboradores (2008) descreveram a atividade tóxica aguda e subaguda do óleo essencial de capim-limão. Nos experimentos de toxicidade aguda, ratos Wistar que receberam o OE na dose de até 1500 mg/kg e não demonstraram qualquer anormalidade, enquanto, a partir de 2000 mg/kg, foram observadas algumas anormalidades comportamentais, e histológicas no fígado e estômago. A partir de 3000 mg/kg, foi observada alta taxa de mortalidade. Em estudos subagudos (14 dias de administração do óleo), os animais que receberam até 500 mg/kg de OE apresentaram comportamento normal, enquanto

anormalidades no comportamento foram observadas a partir de 1000 mg/kg e, na dose de 1500 mg/kg, os animais morreram em até quatro dias.

1.6.1- Compostos majoritários isolados do óleo essencial do capim-limão

O citral, principal composto majoritário no óleo essencial de capim-limão, apresenta rápida eliminação pela urina: aproximadamente 50% de seu total ingerido é eliminado em 24 horas (Diliberto et al., 1990).

Existem vários trabalhos contraditórios sobre as atividades biológicas do citral. Enquanto alguns estudos mostram efeitos citotóxicos desse composto, medidos através do aumento da expressão da proteína p53 algumas horas após sua administração em células de cultura (Duerksen-Hughes et al., 1999), outros demonstram ausência de mutagenicidade através do teste de Ames (Gomes-Carneiro et al., 1998) ou mesmo atividade anti-clastogênica (através da redução na formação de micronúcleos, induzidos por cloreto de níquel em camundongos) e antioxidante, através da captura de superóxidos (Rabbani et al., 2006). Iersel e colaboradores (1996) descreveram a ausência de efeitos do citral na inibição da atividade de glutathione-S-transferase em células de melanoma humano, enquanto, Nakamura e colaboradores (2003) demonstraram que o citral (em especial o isômero geranial) induziu a atividade de GSTP1 em hepatócitos RL34 *in vitro*, e também demonstraram que essa substância atua como inibidor de peroxidação lipídica em pele de camundongos induzida pela TPA (12-*O*-tetradecanoilforbol-13-acetato).

O citral mostrou efeitos inibitórios na promoção de tumores de pele em camundongos Sencar iniciados por DMBA (Connor, 1991), e induziu a expressão de caspase-3 em várias células câncer hematopoiético (Dudai et al., 2005). A literatura também descreve efeitos anti-inflamatórios desse composto, através da inibição da produção de óxido nítrico (Lee et al., 2008) e, juntamente com o geraniol, descreve a inibição da atividade de CYP2B6 microsomal em células hepáticas, indicando esses compostos possuem atividade quimiopreventiva, por alterarem a biodisponibilidade de drogas metabolizadas por essa enzima do citocromo P450 (Seo et al., 2008). Porém alguns estudos demonstram efeitos tóxicos de altas doses de citral, tais como mortalidade, perda de peso corpóreo, opacidade ocular, apnéia, entre outros (Gaworski et al., 1992; Ress et al., 2003).

Outro composto majoritário do óleo essencial de capim-limão, o geraniol, apresentou ausência de genotoxicidade nos testes de Ames e de micronúcleo de medula óssea de camundongos Swiss (Doppalapudi et al., 2007). Inúmeros estudos demonstram a participação desse composto na inibição da proliferação celular. Carnesecchi e seus colaboradores (2001)

observaram efeito citostático (acúmulo células na fase G0/G1) do geraniol contra a proliferação de células neoplásicas de cólon CaCo-2, o que também foi observado em trabalhos com outras linhagens celulares de adenocarcinoma pancreático PaCa-2 e BxPC-3 (Wiseman et al., 2007) e de células neoplásicas mamárias MCF-7 (Duncan et al., 2004). Talvez um dos mecanismos primordiais desse processo de inibição tenha sido explicado por Polo e de Bravo (2006), que demonstraram que o geraniol afetou o metabolismo do mevalonato e de lipídios em células Hep G2 *in vitro*, o que é primordial para o sucesso da proliferação celular.

Da mesma forma, existem trabalhos *in vivo* demonstrando os papéis do geraniol em roedores. O geraniol administrado em conjunto ou não com 5-FU (5-fluorouracila) em células tumorais TC118, e estas posteriormente inoculadas em camundongos Swiss *nude*, inibiu o crescimento desses tumores e aumentou a sobrevida dos animais (Carnesecchi et al., 2004). Vale ressaltar também o papel desempenhado pelo geraniol inibindo o crescimento de células de hepatoma Morris 7777 transplantadas em ratos Buffalo machos e de melanoma B16 em camundongos C57BL fêmeas (Yu et al., 1995) e aumentando a sobrevida de camundongos vindos do cruzamento de camundongos C57BL com DBA/2 F, aos quais foram transplantadas células de leucemia P388 (Shoff et al., 1991).

O geraniol também reduziu a área relativa de focos GST-P positivos em fígado de ratos, quimicamente induzidos através do modelo do hepatócito resistente, bem como reduziu o tamanho, a proliferação celular e aumentou os índices apoptóticos nesses focos (Ong et al., 2006). Por fim, também foi observada atividade antioxidante do geraniol, juntamente com o citral, através da captura de radicais livres (DPPH) (Choi et al., 2000).

O β -mirceno por sua vez não apresentou atividade mutagênica, assim como os outros compostos majoritários, avaliado através do teste de Ames (Gomes-Carneiro et al., 2005; Mitić-Ćulafić et al., 2009), troca de cromátides irmãs ou avaliação de aberrações cromossômicas (Kauderer et al., 1991; Zamith et al., 1993). Pelo contrário, quando administrado juntamente com a ciclofosfamida, aflatoxina B1 ou *t*-BOOH, inibiu a ação desses mutágenos, ou seja, reduziu a frequência de trocas de cromátides irmãs e de aberrações cromossômicas (Kauderer et al., 1991; Röscheisen et al., 1993; Mitić-Ćulafić et al., 2009). Além disso, exerceu atividade antioxidante através da redução da peroxidação lipídica (Mitić-Ćulafić et al., 2009).

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Objetivos

3- Objetivos

Com base em todos os dados presentes na literatura, nossa hipótese de trabalho era a que o óleo essencial do capim-limão poderia exercer atividade protetora contra a carcinogênese química, bloqueando a etapa de iniciação ou revertendo o processo carcinogênico. Para testar nossa hipótese, os objetivos do presente trabalho foram: 1) avaliar os efeitos do óleo essencial do capim-limão sobre os danos de DNA leucócitos de sangue periférico e as taxas de proliferação celular e apoptose na mama, bexiga e cólon em fêmeas Balb/C iniciadas com a MNU e 2) avaliar os efeitos do referido óleo essencial sobre o desenvolvimento de lesões pré-neoplásicas nos órgãos anteriormente citados utilizando um modelo de indução química pela DMBA, DMH e BBN (modelo DDB).

Capítulo II

Article

Lemongrass (*Cymbopogon citratus* STAPF) essential oil effects on urinary bladder, mammary gland and colon initiation and promotion early carcinogenesis phase of Balb/C mice§

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Abstract

Lemongrass (*Cymbopogon citratus* STAPF) has been described as a potential chemopreventive agent. Thus, the present study objectives were evaluate the protective effects of oral treatment with lemongrass essential oil (LGEO) on carcinogenesis initiation phase with N-methyl-N-nitrosurea (MNU) and post-initiation carcinogenesis phase in multiple-organs model, through 7,12-dimethylbenz(a)anthracene (DMBA), 1,2-dimethylhydrazine (DMH), and N-buthyl-N-(4-hidroxibuthyl)nitrosamine (BBN) administrations in Balb/C female mice. The animals were distributed into 2 experimental protocols. Experiment 1: the animals were allocated into 3 experimental groups: G1A group (negative control), G2A group (treated with LGEO 500 mg/kg b.wt., i.g., during 5 weeks and, at the end of the 3rd and 5th weeks, received one 30 mg/kg MNU i.p. application) and G3A group (treated with the LGEO vehicle, and MNU at the end of the 3rd and 5th weeks). After 4 hours of each MNU application, blood samples were collected to perform the comet assay, and, at the end of the 5th week, all animals were euthanatized and the urinary bladder, mammary glands and colon were collected for histological analysis, apoptosis and cellular proliferation counting. Experiment 2: the animals were allocated into 3 experimental groups: G1B group (positive control, DDB-treated animals), initiated with DMBA (5x1 mg i.g. applications), DMH (4x30 mg/kg s.c. applications) and BBN (8x7.5 mg/kg i.g. applications) and treated with the LGEO vehicle, and G2B and G3B groups, similarly DDB-treated, and treated with 125 mg/kg or 500 mg/kg LGEO respectively (5x/week during 6 weeks). At the end of the experimental period, all animals were euthanatized and urinary bladder, mammary glands and colon were collected for preneoplastic and neoplastic lesions analysis. The LGEO treatment reduced DNA damage in peripheral blood leukocytes as well as mammary gland cellular proliferation index. In addition, LGEO treatment altered urinary bladder, mammary gland and colonic apoptotic indexes on MNU-initiated female mice. However, LGEO treatment did not alter preneoplastic lesions incidence on urinary bladder, mammary gland and colon nor alter neoplastic lesions incidence on DDB-treated urinary bladder and mammary glands of the animals. These results suggest that LGEO oral treatment presented anti-genotoxic effect as well as protective effects only on MNU initial alterations of Balb/C female mice.

Key words: Lemongrass, anti-genotoxicity, chemoprevention, multiple-organs carcinogenesis, carcinogenesis initiation/promotion

Introduction

Chemical carcinogenesis is a multistep and complex process that includes initiation, promotion and progression stages (Pitot, 2001; Loeb and Harris, 2008). The carcinogenesis first stage, tumor initiation, involves normal target-cells exposure to chemical or physical agents that cause permanent changes in the DNA structure/function, such covalent links, distortions or molecule-break. After one cell proliferation cycle, these initiated cells begin responding to their microenvironment in a different way, what grant them a proliferative advantage over the surrounding normal cells (Pitot, 2001; Loeb and Harris, 2008). In second stage, tumor promotion, these initiated cells start proliferating in a disorganized way (clonal expansion) to a greater extent than the surrounding normal cells, and the additional genetic damage probability in the expanding population (putative preneoplastic lesions formation) is enhanced (Pitot, 2001; Loeb and Harris, 2008). Putative preneoplastic lesions (PNL) have been considered as potential surrogate endpoint biomarkers for the potential chemopreventive identification of agents capable of inhibiting or suppressing the chemical carcinogenesis different stages in rodents (De Camargo et al., 1999). Chemically-induced PNL have been well characterized mainly in murine liver, urinary bladder, colon, prostate and mammary gland (Russo et al., 1992; Pitot et al., 1996; Pylkkänen et al., 1996; Gabriel et al., 2007; Rosenberg et al., 2008). These lesions present important changes on molecular and morphologic levels, including genes that control the cell proliferation, differentiation and apoptosis (Cordon-Cardo, 1998; Cohen, 2002; Gournay et al., 2002; Ray et al, 2007; Rosenberg et al., 2008).

Cancer chemoprevention is defined as the carcinogenesis process prevention, inhibition or reversion through natural or synthetic chemicals administration (Stoner et al., 1997; De Flora and Ferguson, 2005). Many components from medicinal or dietary plants have been identified as potential chemopreventive agents capable of inhibiting DNA damage, and even retarding or reversing the carcinogenesis process (Ferguson et al., 2004; Aggarwal and Shishodia, 2006). Furthermore, there are various studies that associate the fruit, cereal, vegetables and teas dietary intake with a lower risk for several human cancers development (Ferguson et al., 2004; Khan et al., 2008). Therefore, these findings stimulate future research to detect the potential chemopreventive agents in cancer prevention.

Lemongrass (*Cymbopogon citratus* STAPF) leaves have been widely consumed as infusions in Brazilian folk medicine in the ailments treatment and as anti-spasmodic, analgesic, anti-inflammatory, antipyretic, diuretic and sedative (Negrelle and Gomes, 2007). Various lemongrass pharmacological activities using lemongrass essential oil (LGEO) have

been described (Blanco et al., 2007; Da Silva et al., 2008). However, few studies have investigated the *Cymbopogon citratus* antimutagenic and anticarcinogenic potential activities.

Lemongrass extracts or their specific compounds (i.e. citral, β -myrcene and geraniol) have shown antimutagenic activity in different in vitro and in vivo systems (Melo et al., 2001; Rabbani et al., 2006) and various studies point to antioxidant (Choi et al., 2000; Cheel et al., 2005; Rabbani et al., 2006; Tapia et al., 2007; Pereira et al., 2008; Mitić-Ćulafić et al., 2009) activities of them. Moreover, geraniol has been found reducing the proliferative activity of Caco-2 human colon and MCF-7 human cancer cells lines (Carnesecchi et al., 2001; Duncan et al., 2004). In addition, the lemongrass ethanolic extract orally given to male Fischer 344 rats inhibited the colonic aberrant crypt foci (ACF) and of GST-P-positive foci development induced by azoxymethane and diethylnitrosamine, respectively (Suaeyun et al., 1997; Puatachokchai et al., 2002).

There is no information available about the LGEO chemopreventive potential on preneoplastic/neoplastic lesions development in mice chemical carcinogenesis. Thus, the present study was designed to evaluate the LGEO protective effects, when administered before and during initiation with the carcinogen N-methyl-N-nitrosurea (MNU) period, on female Balb/C mice primary DNA damage in peripheral blood leukocytes, and on urinary bladder, colon and mammary gland epithelial cells proliferation/apoptosis rates. In addition, the present study was aimed to evaluate the LGEO chemopreventive effects, when administered in the post-initiation period of mammary gland, colon and urinary bladder carcinogenesis, through a mouse two-stage model in female Balb/C mice, initiated with the carcinogens 7,12-dimethylbenz(a)anthracene (DMBA), 1,2-dimethylhydrazine (DMH), and N-buthyl-N-(4-hidroxiethyl)nitrosamine (BBN) (DDB model).

Material and Methods

Lemongrass essential oil extraction

Lemongrass leaves (*Cymbopogon citratus* STAPF) were collected from the medicinal plants garden (Lageado Farm, UNESP, Botucatu-SP, Brazil) and a voucher specimen (#23031) was deposited in the *Irina D. Gemtchujnirov* – BOTU herbarium. The lemongrass essential oil (LGEO) was extracted from fresh lemongrass leaves through hidrodestillation using a Clevenger apparatus during 3 hours of boiling. The LGEO resultant was stored at 4°C in a dark recipient up to the moment of use.

The chromatographic oil profile of each extraction was acquired in silica plates (Sigma-Aldrich®, USA), using hexane plus ethyl acetate, or toluene plus ethyl acetate, as mobile phases. LGEO samples or its main compounds (citral, geraniol and β -myrcene) were diluted in dichloromethane and applied in the plate through capillary tubes. Then, the plates were put into saturated cubes by the elluent, following the compounds separation through affinity; plates were reveled by sulfuric anisaldehyde. Finally, the plates were heated to a temperature of 100°C for 5 minutes and immediately photographed. To avoid differences in LGEO constitution among the extractions, the essential oils acquired on each extraction were mixed, and then used for the purposes of this study. Then, the essential oil mixture was analyzed by gas chromatography coupled with mass spectrometry (GC/MS) according to the following conditions: injection of 1 μ l of the solution (1 μ l of LGEO and 1 mL of ethyl acetate); silica capillary column: DB-5 (30m x 0.25mm x 0.25 μ m), electron impact: 70 eV, utilizing helium at 1.0 ml/min as carrier gas, injector temperature: 240°C, detector temperature: 230 °C, temperature program: 60°C to 240°C, 3 °C/min. The compounds identification was made through substance mass spectrum comparison with the database of the GC/MS (NIST 62.lib), literature and retention index (McLafferty and Stauffer, 1989; Adams, 2001).

Experimental protocol

The University's Ethics Committee for Animal Research approved the protocol used in this study (Protocol n° 595). Seventy eight female Balb/C mice were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP Campinas-SP, Brazil) and were housed in polypropylene cages covered with metallic grids in a room maintained at a 22 \pm 2 °C, 55 \pm 10% humidity and with a 12-hr light-dark cycle. The animals were fed with commercial Purina chow (LABINA, Paulínia-SP, Brazil) and water *ad libitum* for a 2-week acclimation period. Then, the mice were distributed into two experiments.

Experiment 1: This study was performed with the aim of investigating the LGEO treatment modifying effects on the mouse carcinogenesis initiation stage induced by MNU, which is an ultimate carcinogen, and it was used in order to decrease the possible metabolic enzyme induction confounding effects by the LGEO. The animals were allocated into three experimental groups (10 mice/group): G1A group (negative control) received i.g. Tween 20 administrations (2.0% - LGEO vehicle, 5 days/week, during 5 weeks) and two i.p. NaCl injections (0.9% - MNU vehicle, at the end of the 3rd and 5th weeks); G2A group (test group) received i.g. LGEO administrations (500 mg/kg - 5 days/week during 5 weeks) and two i.p. MNU injections (30 mg/kg, Sigma Chemical Co., St. Louis MO), at the end of the 3rd and 5th treatment weeks; G3A group (positive control) received the LGEO vehicle and the two i.p. MNU injections at the end of the 3rd and 5th weeks. Four hours after each MNU or NaCl treatments, peripheral blood samples were collected from the periorbital vein plexus with the purpose of performing the comet assay (Bidinotto et al., 2006). All animals were euthanized at the end of the 5th week.

Experiment 2: This study was performed with the aim of investigating the LGEO treatment modifying effects on the mice carcinogenesis promotion stage using a multiple-organ bioassay, with preneoplastic lesions detection as the endpoint. The animals were randomly allocated into three experimental groups (16 mice/group): G1B group (positive control) received DMBA (Sigma, 5x 1 mg/mouse/week, i.g., during the first to fifth experiment weeks) (Quing et al., 1997), DMH (Sigma, 4x 30 mg/kg b.wt., s.c, twice a week during the 3rd and 4th experiment weeks) (Ziliotto et al., 2008) and BBN (Tokyo Kasei Industries Co. - Tokyo, Japan, 8x 7.5 mg/mouse/week, i.g., during the 6th to 13th weeks) (Moon et al., 1993) and the LGEO vehicle during six weeks; G2B and G3B groups (test groups) received the regimen carcinogens cited upward and LGEO (125 mg/kg or 500 mg/kg b.wt. respectively, 5x/week, i.g., during the 15th to 20th weeks). All the animals were euthanized at the end of the 20th week.

Clinical examinations were performed daily and detailed physical examinations were accomplished weekly, whereas food and water consumptions were recorded twice a week. The animals were fasted overnight and sacrificed by exsanguination under sodium pentobarbital anesthesia (45 mg/kg p.c.).

Comet assay – Experiment 1

Peripheral blood samples were collected from all animals 4 hours after MNU or vehicle treatments. The Single-Cell Gel Electrophoresis assay (comet assay) was performed

under alkaline conditions according to a previously described protocol (Tice et al., 2000). Briefly, 5 µl of peripheral blood were mixed with 120 µl of 0.5% low-melting-point agarose (Invitrogen, Carlsband, CA, EUA) at 37°C and layered onto conventional microscope slides, precoated with 1.5% normal-melting-point agarose (Invitrogen). The slides were placed overnight into a cold freshly-prepared lysing solution (1% Triton X-100, 2.5 mM NaCl, 0.1 mM Na₂EDTA, 10 mM Tris with 10% dimethylsulfoxide, pH 10.0) and, then, into a horizontal electrophoresis apparatus containing alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na₂EDTA, pH>13) at 4°C for 20 min. Using the same buffer, electrophoresis was performed at 25 V and 300 mA for 20 min. After the electrophoresis, the slides were washed twice in a neutralizing buffer for 5 min (0.4 M Tris-HCl, pH 7.5), fixed in absolute alcohol for 5 min, air-dried, and stored under room temperature.

Just before analysis, the slides were stained with 50 µl (20 µg/ml) ethidium bromide and examined at 400x magnification in an epi-illuminated fluorescence microscope connected to an image analysis system (Comet II; Perspective Instruments, Suffolk, UK). Coded slides were blindly scored, and 50 nucleoids were randomly analyzed for each animal (25 cells per slide from two slides per animal). Tail intensity (the DNA percentage in the comet tail) and tail moment (product of DNA density in the tail and the average distance of DNA migration in the tail) were used to measure the DNA damage extent. Comet images with a “cloudy” appearance or with a very small head and tail like a balloon were excluded from the analysis (Hartmann and Speit, 1997).

Tissues processing and analysis

At the end of the 5th and 20th weeks (experiments 1 and 2, respectively), blood samples were collected through cardiac puncture for biochemical analysis (transaminases and creatinine levels) and, after necropsy, the inguinal mammary glands, thymus, spleen, liver, kidneys, colon and urinary bladder were removed for posterior histological processing and analysis. Thymus, liver and spleen were weighed and representative samples were fixed into 10% buffered formalin for 24 h.

The inguinal mammary glands fat pads were dissected off the skin and spread onto glass slide. The tissues were fixed at room temperature in Carnoy's solution (6:3:1, v:v:v, ethanol, chloroform and glacial acetic acid) for 4 hours, rehydrated in ethanol in gradual dilution and stained, overnight, with 0.2% carmine and 0.5% aluminum potassium sulfate. Additionally, the glands were rinsed in water and dehydrated in absolute alcohol, cleared in

xylene and permanently mounted. Whole mounts images were captured and analyzed using a Leica Qwin Soft Imaging System (Wetzlar, Germany) and then, the glands were processed for paraffin embedding. The urinary bladder was also removed, inflated by 10% phosphate-buffered formalin, cut in 4-6 strips and processed for paraffin embedding, and the entire colon was longitudinally opened, rinsed in saline and flat fixed in 10% buffered formalin for 24 h. Colons were stained with 5% methylene blue in PBS for 10 min for aberrant crypt foci (ACF) detection and counting (Bird and Good, 2000; Rodrigues et al., 2002) and then, proximal, middle and distal colon samples were cut into serial strips and processed for paraffin embedding.

The paraffin blocks of all organs were cut in 5- μ m-thick sections and stained with hematoxylin–eosin (HE) for histological analysis. Since proliferative and/or preneoplastic lesions were detected in the MNU-treated animals mammary gland (ductal hyperplasia), colon (aberrant crypt foci) and urinary bladder (simple/nodular hyperplasia) in the Experiment 1, these target-organs were selected for proliferation cellular nuclear antigen (PCNA) immunohistochemical analysis, as well as for apoptosis index analysis, in order to evaluate the LGEO protective effects in early initiation phase, induced by MNU. Preneoplastic and/or neoplastic lesions were analyzed in the Experiment 2 in order to evaluate the LGEO protective effects in early promotion stage.

Immunohistochemical staining for PCNA was performed using the streptoavidin-biotin peroxidase method, with modifications proposed by Fung et al. (1992). Briefly, deparaffinized 5- μ m-thick sections were submitted to antigenic recuperation by citric acid 0.1M (2 cycles of 5 min on microwave oven) and were treated with 3% hydrogen peroxide for 10 min, nonfat milk for 60 min, anti-PCNA antibody (1:100 dilution, Dako, Glostrup, Denmark) conjugated with anti-mouse antibody (1:200 dilution, Dako) overnight and streptoavidin-biotin peroxidase solution (1:50 dilution) for 45 min (Vector laboratory, Burlingame, CA). Chromogen color development was accomplished with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and counterstaining was performed with Harris' hematoxylin.

The cell proliferation and apoptosis indexes were expressed as the percentage of PCNA-positive cells (PCNA LI%) and apoptotic cells/apoptotic bodies (AI%) among the counted cells total number, respectively. The morphological criteria for the apoptotic cells identification was used as has been previously described (Levin et al., 1999).

Statistical analysis

Data of body weight, food consumption, preneoplastic lesions incidence and multiplicity, comet assay, cell proliferation and apoptosis indexes were compared among the three experimental groups of each experiment using the ANOVA or Kruskal-Wallis tests or Chi-Square test. The significance level adopted was $P < 0.05$.

Results

Lemongrass essential oil extraction

Chromatographic profile data from each collected sample showed the three main LGEO compounds presence described in the literature (Schaneberg and Khan, 2002; Barbosa et al., 2008) (data not shown). For this reason, all the samples acquired on each extraction were mixed, and figure 1 shows the GC/MS chromatogram of this mixture and each compound relative percentage. The lemongrass major compounds citral (neral and geranial), geraniol and β -myrcene were found representing 99.07% of the total oil, and the percentage remaining was found as the compounds 6-methyl-5-hepten-2-one and 2-undecanone.

General findings

Nine animals died during the experimental period: one mouse from the G1A group, three mice from G2A group, and five mice from G3B group. Toxicity of treatment with carcinogens or accidental small volumes aspiration of Tween 20 (vehicle) or LGEO was considered responsible for these deaths.

In the Experiment 1, no significant variation in food or water consumption was observed during the 5-weeks experimental period (data not shown). However, in the Experiment 2, a lower food consumption was observed in DDB/LGEO-treated groups (3.37 ± 0.27 and 3.39 ± 0.39 g/mouse/day for G2B and G3B groups, respectively) when compared to the DDB-treated group (3.76 ± 0.38 g/mouse/day for G1B) ($P < 0.05$) whereas water consumption was not altered during the 20-weeks experimental period.

Final body weight, spleen and thymus relative weights, and transaminases and creatinine levels data were similar among all the experimental groups in both experiments (data not shown). However, in the Experiment 1 an increase in the kidney and liver absolute and relative weights were observed in the MNU/LGEO-treated group (G2A) ($P < 0.05$) when compared to the other groups (Table 1). Moreover, in the Experiment 2 an increase in liver absolute and relative weights was found in the DDB/LGEO 500 mg/kg-treated group (G3B) in comparison to DDB-treated group (G4) ($P < 0.001$) (Table 1). Microvesicular steatosis was observed in the lemongrass 500 mg/kg-treated mice's liver in both experiments.

Carcinogen-induced proliferative/preneoplastic lesions were detected in inguinal mammary glands (ductal hyperplasia), colon (aberrant crypt foci) and urinary bladder (simple and nodular hyperplasia) in both experiments. Furthermore, urothelial transitional cell

carcinomas (TCC) and mammary adenocarcinomas were detected in DDB-treated mice, treated or not with LGEO (Experiment 2).

Genotoxic and antigenotoxic analysis – Experiment 1

Figure 2 shows the values obtained from comet analysis using tail moment and tail intensity parameters. Firstly, significant DNA damage increase in peripheral blood leukocytes was observed in the MNU-treated groups (G2A and G3A) when compared with the non-treated group (G1A) at the two moments (3rd and 5th weeks). However, a significant antigenotoxic effect of LGEO was observed in mice treated with MNU only at the 3rd week blood collect ($P < 0.001$).

Cell proliferation and apoptosis analysis – Experiment 1

Figure 3 shows PCNA-labeling (PCNA LI%) and apoptosis (AI%) indexes data in the female Balb/C mice selected target-organs at the end of the 5th week. Cell proliferation rate was higher in colonic epithelial cells (but not in ductal mammary epithelial cells) of the MNU-initiated groups (G2A and G3A), when compared to the negative control group (G1A) ($P < 0.05$). In addition, apoptosis rates were higher in colonic epithelial and urothelial cells of the MNU-initiated groups (G2A and G3A), but lower in epithelial mammary cells, when compared to the negative control group (G1A) ($P < 0.001$).

The treatment of the MNU-initiated mice with LGEO (500 mg/kg) reduced the cell proliferation rates in epithelial mammary cells ($P < 0.05$) and apoptosis in colonic epithelial and urothelial cells ($P < 0.01$ and $P < 0.05$, respectively), when compared to the MNU-treated group (G2A vs. G3A). Furthermore, LGEO treatment increased the apoptosis rates in the mammary gland (G2A vs. G3A, $P < 0.05$).

Preneoplastic and neoplastic incidence and multiplicity – Experiment 2

Table 2 shows the preneoplastic lesions incidence on urinary bladder, colon and mammary gland and neoplastic lesions incidence on urinary bladder and mammary gland of DDB-treated female Balb/C mice. The post-initiation treatment with LGEO did not alter the preneoplastic/neoplastic lesions incidence or multiplicity (data not shown) induced by the carcinogens in the specific target-organs.

Discussion

The GC/MS analysis data showed citral, β -myrcene and geraniol presence as the main constituents in LGEO samples used in this study, as previously described (Schaneberg and Khan, 2002, Barbosa et al., 2008). Fandohan and colleagues (2008) evaluated the LGEO toxicity in acute (single dose) and subacute (14 days of experiment) administrations in male and female Wistar rats. Stomach and livers histological analysis showed no relevant changes at 5-1500 mg/kg b.wt. of LGEO in subacute administration, and important changes (stomach mucosa atrophy, important inflammatory cells infiltration on stomach and liver, and necrosis and granulomas formation on liver) were found increasing the dose level to 3000 mg/kg. In our study, LGEO treatment increased kidneys and/or liver absolute and relative weights without changing transaminases and creatinine serum levels. In fact, treatment of the animals with a lipidic-nature compound (as LGEO) results in steatosis in specific organs, mainly on liver (main metabolism organ) and on kidneys (main excretion organ of citral – responsible for almost 70% of LGEO) (Diliberto et al., 1990). It is important to evaluate the long-term mice exposition to LGEO in different doses, and the different organs important changes caused by it in order to evaluate the safety of an eventual chronic LGEO treatment.

Previous results have pointed to an antioxidant activity of crude lemongrass extracts or their specific compounds (citral, β -myrcene, and geraniol) in different in vivo and in vitro systems (Choi et al., 2000; Cheel et al., 2005; Rabbani et al., 2006; Tapia et al., 2007; Pereira et al., 2008; Mitić-Ćulafić et al., 2009). Besides its ability to act as an antioxidant, inhibiting the destructive effects of reactive oxygen species, lemongrass extracts have showed potential in vitro antigenotoxic/antimutagenic and in vivo anticarcinogenicity activities (Vinitketkumnuen et al., 1994; Suaeyun et al., 1997; Melo et al., 2001; Puatanachokchai et al., 2002).

MNU has been used as a potent mutagen and a usual carcinogen for chemically-induced rodent mammary gland, colon and urinary bladder carcinogenesis (Thompson and Singh, 2000; Gabriel et al., 2007; Rosenberg et al., 2008). In addition, the MNU challenge increased the primary DNA damage values in peripheral blood leukocytes in both moments of analysis, similarly to results found in our previous study (Bidinotto et al., 2006) and the concomitant treatment with LGEO (500 mg/kg) reduced the primary DNA damage in the first MNU application. In fact, lemongrass or their specific compounds have shown antigenotoxic and/or antimutagenic activities in different in vitro and in vivo systems (Vinitketkumnuen et al., 1994; Melo et al., 2001; Ong et al., 2006; Rabbani et al., 2006), and have not shown any genotoxic or clastogenic effects (Kauderer et al., 1991; Zamith et al., 1993; Gomes-Carneiro

et al., 1998; Carnesecchi et al., 2001; Akinboro and Bakare, 2007; Doppalapudi et al., 2007). Beyond the antioxidant effect (that potentially reduces free radical-induced DNA damage) our study showed that LGEO can protect against the lymphocytes DNA damage through alkylation induced by MNU.

There are some studies of lemongrass anti-carcinogenic effects in chemically-induced model in rodents. Lemongrass extracts have showed chemopreventive effects by inhibiting colon and liver preneoplastic lesions development in rats (Suaeyun et al., 1997; Puatanachokchai et al., 2002). Dietary lemongrass extract 6-week-treatment (0.2 mg/kg, 0.6 mg/kg and 1.8 mg/kg in basal diet) reduced the GSTP-positive lesions number and the oxidative damage levels on liver (assessed by 8-hydroxydeoxyguanosine levels) in male F344 rats initiated with diethylnitrosamine, without alter the cellular proliferation indexes in surrounding tissue and into GST-P-positive foci (Puatanachokchai et al., 2002). Furthermore, dietary lemongrass ethanolic extract treatment (0.5 and 5 mg/kg in basal diet during 5 weeks) before and during azoxymethane (AOM)-initiation phase or 0.5 mg/kg on basal diet during 12 weeks in promotion phase reduced the ACF incidence (in both experiments) and, in the initiation protocol, reduced colonic DNA adducts levels (N^7 -methylguanine, and O^6 -methylguanine) as well as fecal β -glucuronidase activity (responsible for toxic and carcinogenic substances generation) activity in male F344 rats and oxidative damage induced by *tert*-butyl hydroperoxide in vitro (Suaeyun et al., 1997). In our study we evaluated the lemongrass essential oil role on preneoplastic lesions formation and growth, cellular proliferation and apoptosis in urinary bladder, colon and mammary gland.

Recently, Shilkaitis and colleagues (2000) described the MNU action mechanism in mammary carcinogenesis initiation phase in female Sprague–Dawley rats. Similarly, our results showed that MNU administration decreased apoptotic indexes but did not alter the cell proliferation rates on female Balb/C mammary gland. In contrast, MNU treatment resulted in cell proliferation and apoptosis increase on the colon and in apoptosis on the urinary bladder. The differential responses of mouse mammary gland, colon and urinary bladder to MNU administration could be associated to differences in the organs microenvironment and/or its cells metabolism (Singer and Esslmann, 1991). In addition, the oral treatment with LGEO reduced cell proliferation indexes on mammary gland and apoptotic indexes in the urinary bladder and colonic mucosa of MNU-initiated mice. In contrast, it augmented the apoptotic index in its mammary gland. These results showed that the oral LGEO treatment exerted protective effects against the alkylation-induced apoptotic modifications in the target-organs and, as a consequence, the treatment could exert a protective effect against early mutagenesis

and carcinogenesis phase induced by MNU. Thus, an effective influence of LGEO treatment in the mouse carcinogenesis initiation stage induced by MNU should continue to be investigated, mainly in medium-term bioassays.

The 6-week post-initiation treatment with LGEO did not reduce the preneoplastic and neoplastic lesions (incidence and multiplicity) development in mammary gland, colon and urinary bladder in the DDB model. In fact, studies have shown that lemongrass extract and its essential oil major compounds (i.e. citral, β -myrcene and geraniol) exerted antioxidant effects by scavenging free radical and reducing lipidic peroxidation (Choi et al., 2000; Cheel et al., 2005; Rabbani et al., 2006; Tapia et al., 2007; Pereira et al., 2008; Mitić-Ćulafić et al., 2009.), and reduced DNA adducts formation (Suaeyun et al., 1997). The oxidative stress is potentially deleterious to cells and associated with many diseases progression, including cancer (Hwang and Kim, 2007), and as lemongrass extracts or essential oil have antioxidant effects, it could exert a effective anti-promoting action in a long-term treatment regimen or exert a protective effect in a mouse chemical carcinogenesis late phase (therapeutic effects).

In conclusion, the results indicate that the lemongrass essential oil treatment modulated the early response induced by the direct mutagen MNU (initiation stage) but did not inhibit the preneoplastic/neoplastic lesions development (post-initiation stage) induced by the carcinogens DMBA/DMH/BBN in female Balb/C mice. Therefore, LGEO acted as an anti-initiating agent but not presented any suppressing effect on the promotion stage in a medium-term mouse multi-organ carcinogenesis model in the present experimental conditions. Further studies are warranted with this essential oil in order to evaluate the protective mechanisms, well-characterize its role on carcinogenesis chemoprevention, its administration safety, and the LGEO availability to be eventually chosen as a potential chemopreventive agent.

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Legends of the figures

Figure 1 – Chromatogram of the essential oil given to the animals showing the presence of 6 peaks: (1) 6-methyl-5-hepten-2-one, (2) β -myrcene, (3) neral, (4) geraniol, (5) geranial and (6) 2-undecanone. The lemongrass essential oil specific compounds percentage were 0.64%, 16.5%, 29.83%, 1.28%, 51.46%, and 0.29%, respectively.

Figure 2 – Data of tail moment (A and C) and tail intensity (B and D) obtained from comet assay in peripheral blood leukocytes obtained after four hours of mutagen administration. G1A= non-treated mice; G2A= MNU plus LGEO-treated mice and G3A= MNU-treated mice. MNU= N-methyl-N-nitrosurea (2x 30 mg/kg b.wt., 3rd and 5th weeks) and LGEO= lemongrass essential oil (500 mg/kg, 5x/week during 5 weeks). *Statistically different from G1A group; ** Statistically different from G3A group (P<0.001).

Figure 3 – Data of PCNA-labeling (PCNA LI%) and apoptosis indexes (AI%) in mammary epithelial cells (A and B, respectively), colonic epithelial cells (C and D, respectively) and urothelial cells (E – apoptosis index). ¹G1A= non-treated mice; G2A= MNU plus LGEO-treated mice and G3A= MNU-treated mice. MNU= N-methyl-N-nitrosurea (2x 30 mg/kg b.wt., weeks 3 and 5) and LGEO= lemongrass essential oil (500 mg/kg, 5x/week during 5 weeks). *Statistically different from G1A group, **Statistically different from G3A group (P<0.05, P<0.01 or P<0.001 – see results section).

Figures and Tables

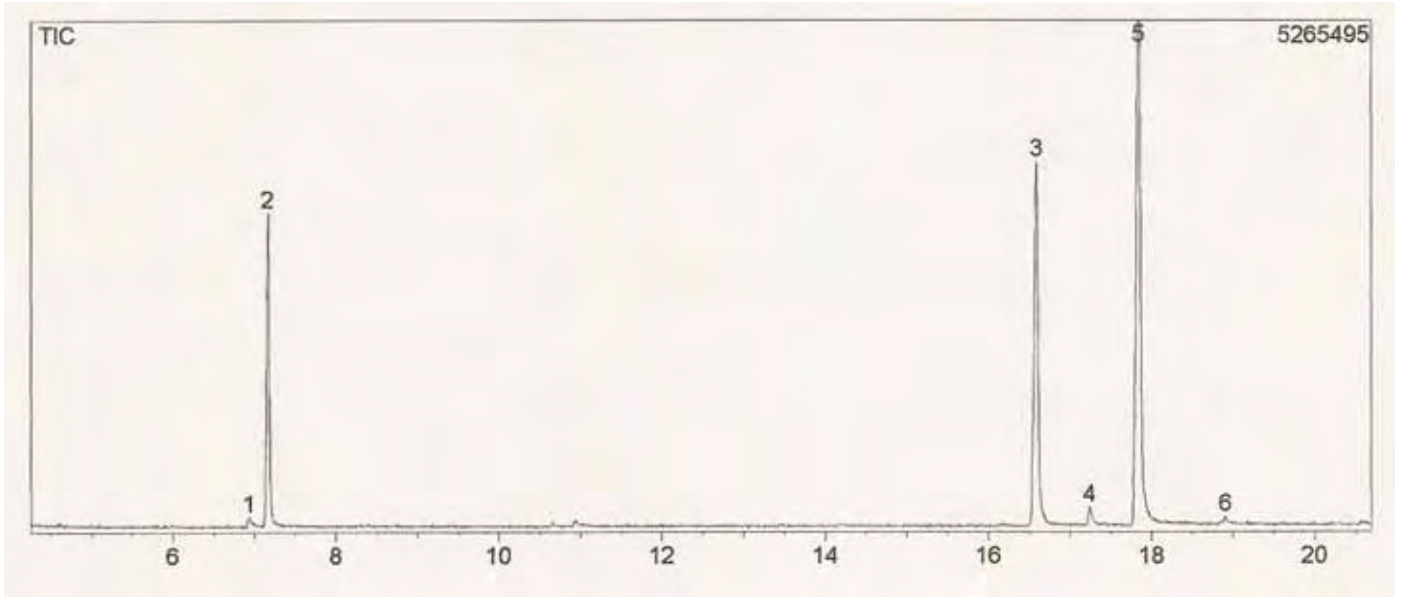
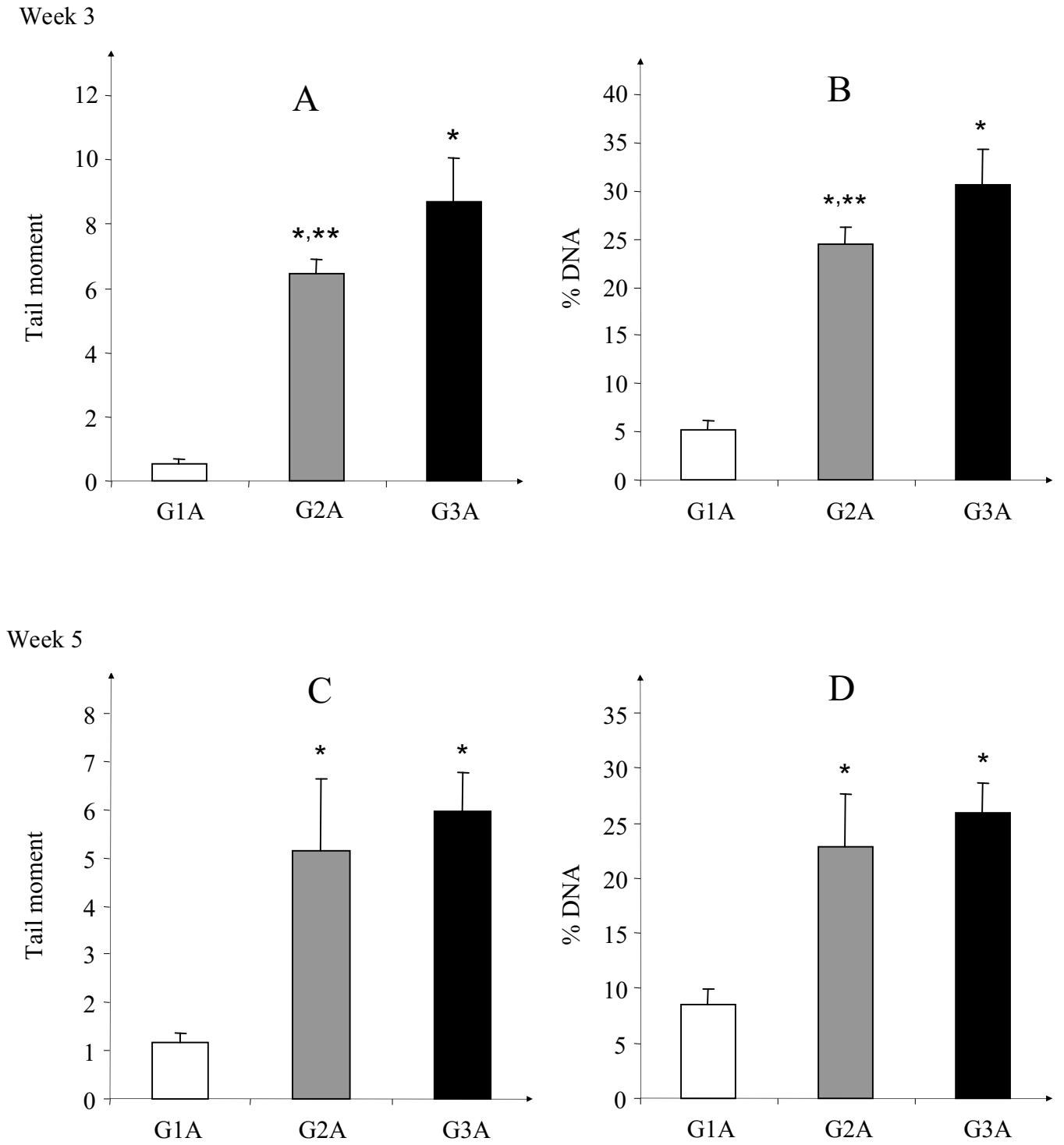


Figure 1

**Figure 2**

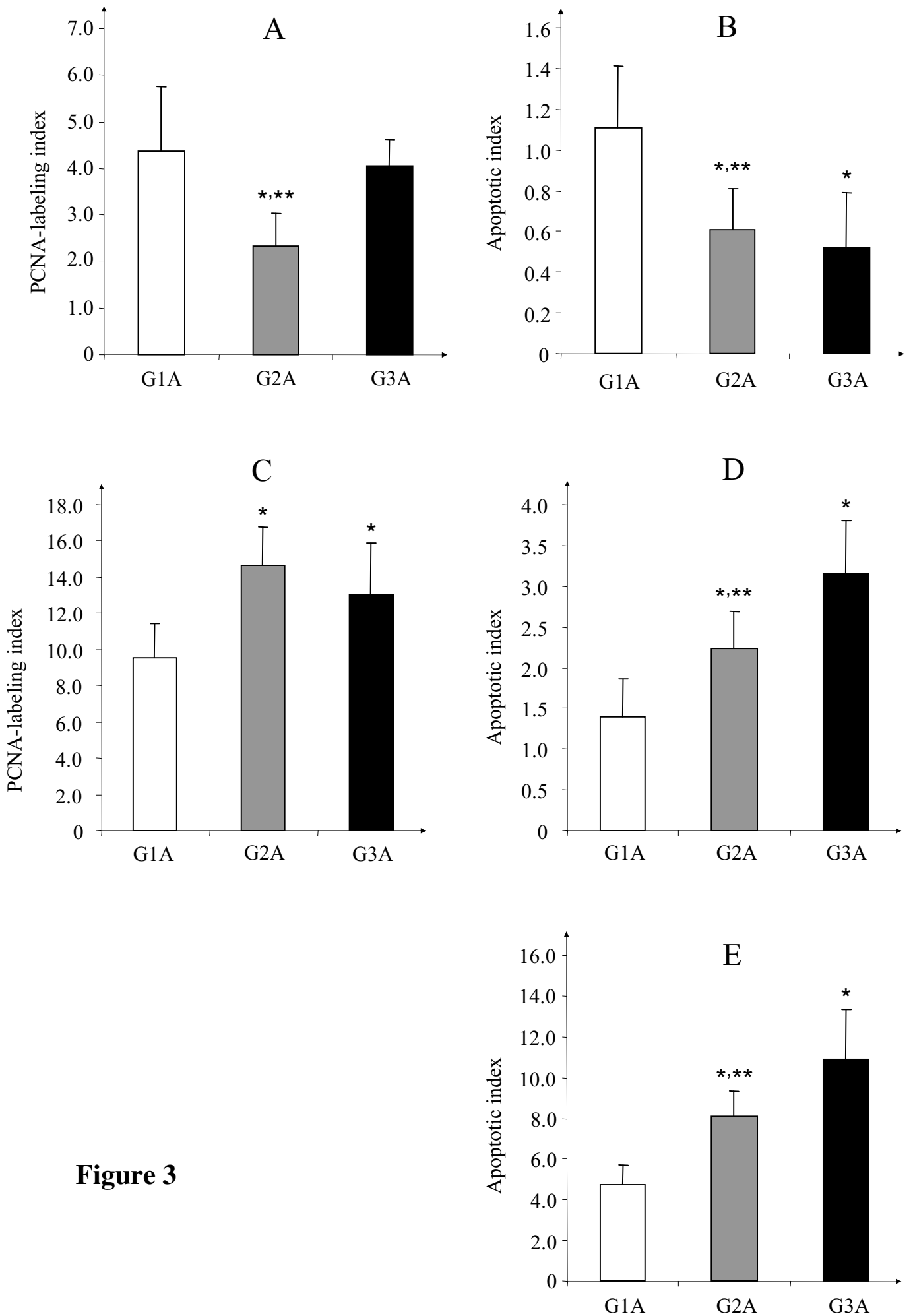
**Figure 3**

Table 1 – Absolute and relative weight of kidneys and liver

Groups/Treatment ²	N ³	Absolute weight ¹				Relative weight			
		Kidney		Liver		Kidney		Liver	
		R	L	R	L	R	L	R	L
<i>Experiment 1</i>									
(G1A) Control	09	0.15±0.01	0.15±0.01	0.99±0.10	0.66±0.04	0.66±0.04	0.66±0.04	4.33±0.51	4.33±0.51
(G2A) MNU/LGEO 500 mg/kg	07	0.18±0.02*	0.17±0.02*	1.23±0.15***	0.77±0.05***	0.77±0.05***	0.73±0.04***	5.24±0.50***	5.24±0.50***
(G3A) MNU	10	0.16±0.01	0.16±0.01	0.97±0.14	0.65±0.02	0.65±0.02	0.62±0.02	3.81±0.40	3.81±0.40
<i>Experiment 2</i>									
(G1B) DDB	16	0.18±0.04	0.17±0.03	0.93±0.09	0.80±0.15	0.80±0.15	0.74±0.14	4.13±0.21	4.13±0.21
(G2B) DDB/LGEO 125 mg/kg	16	0.18±0.06	0.16±0.02	0.97±0.14	0.83±0.20	0.83±0.20	0.74±0.12	4.55±0.45	4.55±0.45
(G3B) DDB/LGEO 500 mg/kg	11	0.19±0.05	0.18±0.03	1.11±0.15****	0.84±0.18	0.84±0.18	0.79±0.13	4.96±0.41****	4.96±0.41****

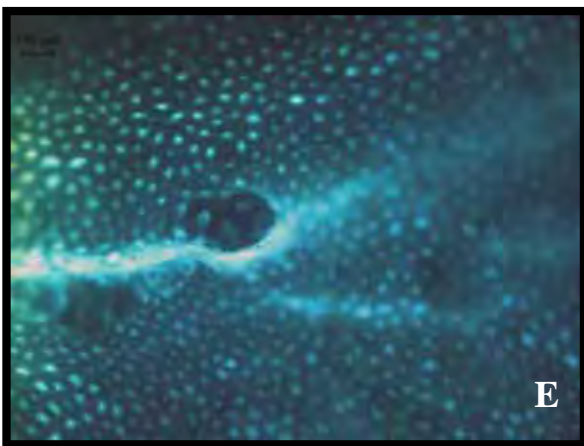
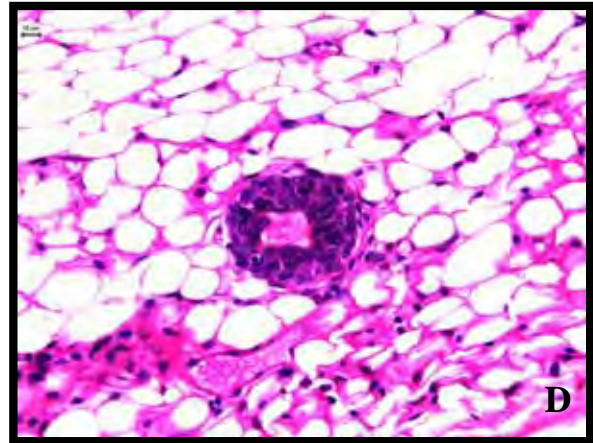
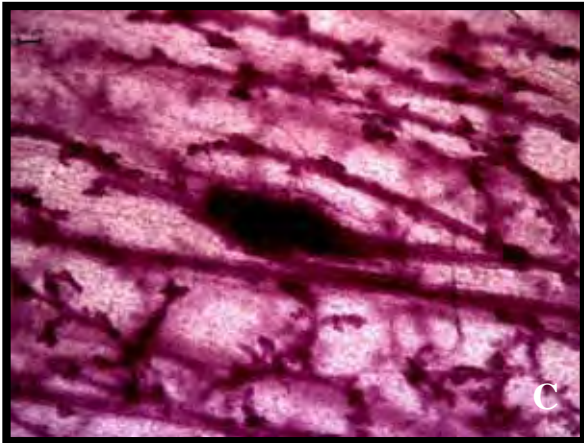
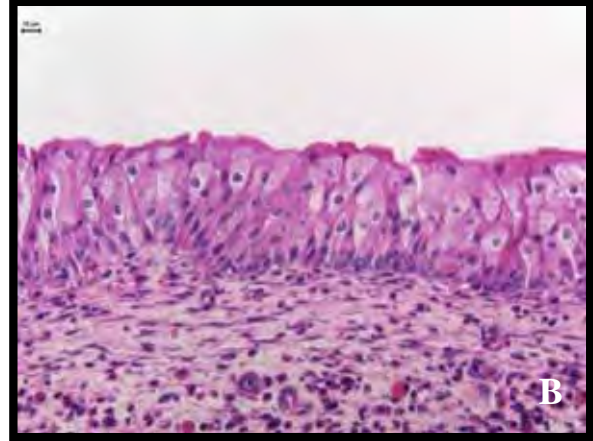
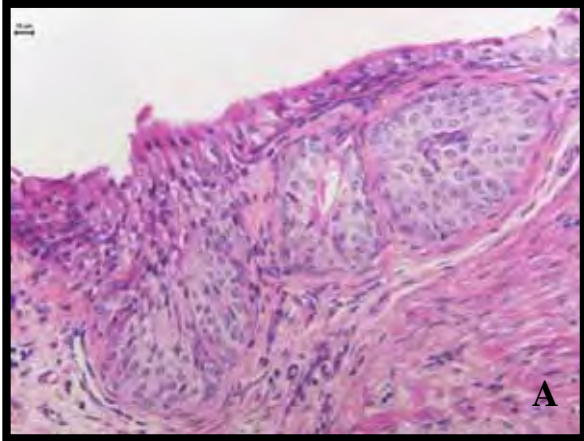
Data are expressed in mean±SD. ¹R= right kidney, L= left kidney; ²MNU= N-methyl-N-nitrosurea, 30 mg/kg, i.p.;), LGEO= lemongrass essential oil; DDB= 7,12-dimethylbenz(a)anthracene (DMBA, 1 mg/animal, i.g., five applications, one per week), 1,2-dimethylhydrazine (DMH, 30 mg/kg, s.c., four applications, two at 3rd and 2 at 4th weeks), N-buthyl-N-(4-hydroxibuthyl)nitrosamine (BBN= 7,5 mg/animal, i.g., one weekly application between the 6th and 13th weeks; ³Number of animals per group. *Statistically different from G1A or G1B group (p<0.05 or p<0.01), **Statistically different from G3A group (p<0.01), ***Statistically different from G2B group (p<0.001).

Table 2 – Preneoplastic and neoplastic lesions incidence on the urinary bladder, colon and breast of the Protocol 2

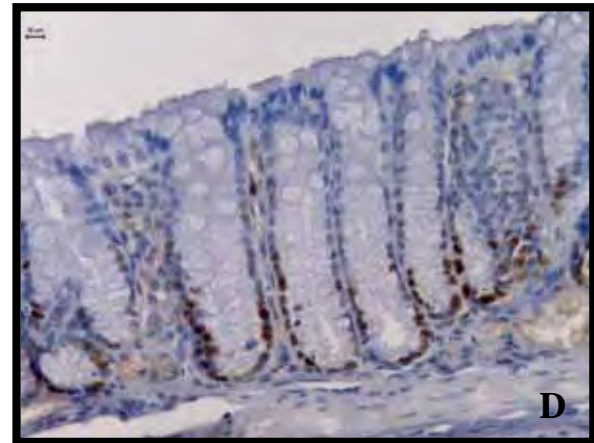
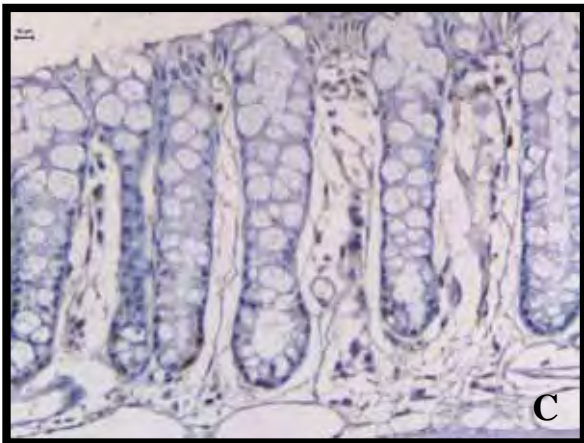
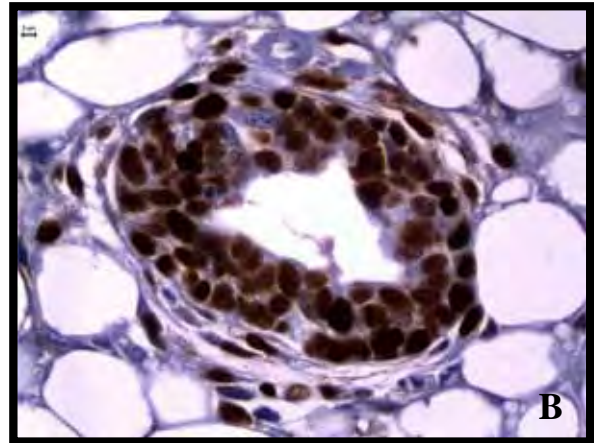
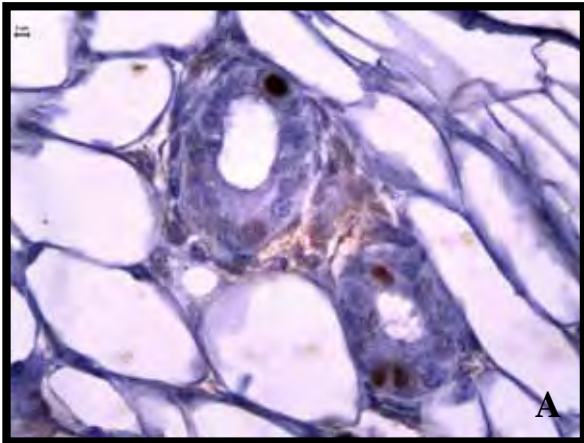
Groups/Treatment ²	N ³	Lesions ¹					
		Preneoplastic			Neoplastic		
		Urinary bladder	Colon	Mammary gland	Urinary bladder	Mammary gland	Mammary gland
(G1B) DDB	16	14 (87.5)	16 (100)	15 (93.7)	2 (12.5)	5 (31.3)	
(G2B) DDB + LGEO 125 mg/kg	16	16 (100)	16 (100)	13 (81.3)	2 (12.5)	4 (25)	
(G3B) DDB + LGEO 500 mg/kg	11	11 (100)	11 (100)	10 (90.9)	4 (36.4)	1 (9)	

Data are expressed in number of animals (percentage); ¹Urinary bladder preneoplastic lesions consist in simple and nodular hyperplasia, colon preneoplastic lesions consist in aberrant crypt foci, mammary gland preneoplastic lesions consist in ductal hyperplasia, urinary bladder lesions consist in tumor of transitional cells and mammary gland neoplastic lesions consist in adenocarcinoma; ²DDB= 7,12-dimethylbenz(a)anthracene (DMBA, 1 mg/animal, i.g., five applications, one per week), 1,2-dimethylhydrazine (DMH, 30 mg/kg, s.c., four applications, two at 3rd and 2 at 4th weeks), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN= 7.5 mg/animal, i.g., one weekly application between the 6th and 13th weeks), LGEO= lemongrass essential oil (5 weekly applications, i.g., between the 14th and 20th weeks); ³Number of animals per group.

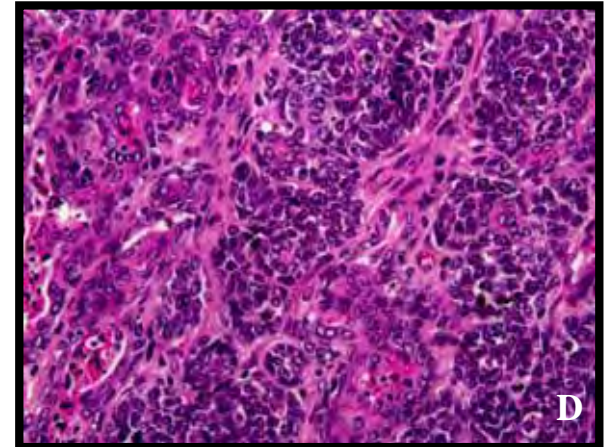
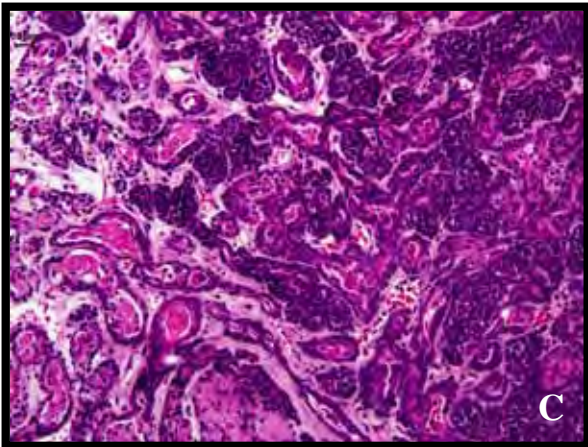
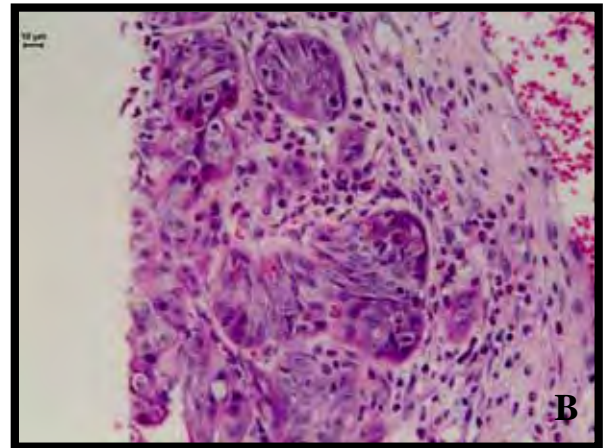
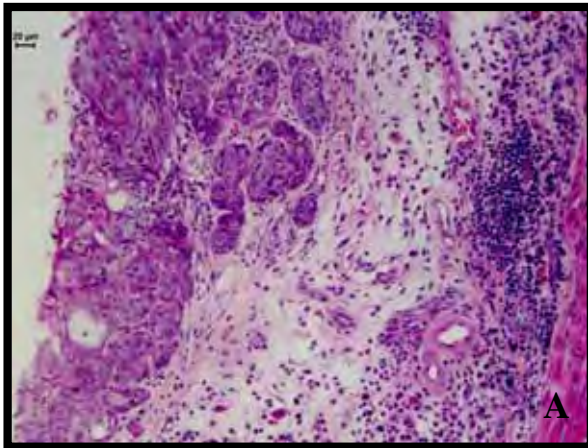
Anexos



Attachment 1 – (A-E) Preneoplastic lesions of (A and B) urinary bladder (nodular hyperplasia and simple hyperplasia, respectively), (C and D) mammary gland (ductal hyperplasia whole mount and ductal hyperplasia histological preparation, respectively) and (E) colon (aberrant crypt foci). Stained by H&E (A, B, D), carmine red (C) and methylene blue (E).



Attachment 2 – PCNA-positive cells in (A and B) normal and hyperplastic mammary gland duct, respectively, and (C and D) normal and hyperproliferative colon crypt epithelium, respectively. Counterstained by Harris' Hematoxylin



Attachment 3 –Neoplastic lesions of urinary bladder (transitional cell carcinoma) low (A) and high (B) magnification and mammary gland (adenocarcinoma) low (C) and high (D) magnification of DDB-treated female mice. Stained by H&E.