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MECANISMOS DE AÇÃO DA PRÓPOLIS NA MODULAÇÃO DE DANOS QUIMICAMENTE INDUZIDOS NO DNA

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ÍNDICE

ABSTRACT

Propolis is a complex mixture of plant resins, bee wax, essential oils and pollen. The specific chemical compounds of Brazilian propolis have allowed the identification of its plant source by comparative chemical analysis (infrared measurements) and taxonomic tests. These compounds have been identified in the exudate of Baccharis dracunculifolia leaves and are specific of Brazilian propolis, being even found at similar concentrations, demonstrating that Baccharis is the main plant source of propolis. Thus, by possessing highly complex and variable chemical composition of propolis (more than 300 components have been identified), which is intimately related to the ecology of the flora of each region visited by the bees, propolis is a natural remedy that has been used since ancient times. It is widely recommended by phytotherapists due to its anti-inflammatory, hepatoprotective, antimicrobial, and antioxidant properties. Also, propolis is used in cosmetic products and as a food constituent. Thus, the therapeutic effect of propolis on some human diseases claimed by folk medicine has raised the interest in the understanding of the biological properties of this substance related to its chemical composition. In view of the prominent therapeutic potential of propolis and the small number of studies regarding its mechanisms of action, the aim of the present investigation was to evaluate the possible antigenotoxic and antimutagenic effects of a propolis fractions on Chinese hamster ovary (CHO) cells and células de hapatobastoma humano (HepG2) by comet assay and cytokinesis-block micronucleus assay (CBMN). Treatments with propolis, at concentrations 25, 50, and 100 μg/ml, were done prior to, simultaneously and post- treatment with mutagens (direct- and indirect-acting) with different mode of action. Under these conditions, by comet assay, our data clearly showed the capability of propolis compounds to protect against chemically-induced DNA damage. The CBMN data showed the capability of propolis compounds to protect against chemically-induced chromosome damage. However, it also was seen a mutagenic effect of propolis fractions in CHO and HepG2 cells when treated with H2O2. These chemopreventive activities were not dependent of the treatment protocol nor of the concentration used. Moreover, this protective effect cannot be attributed only to the identified compounds, since others remain to be identified in each of the fraction.

RESUMO

Propolis é uma mistura complexa composta de resina, extratos vegetais, cera, óleos essências (10%), pólen (5%) e outras substâncias (5%). Alguns estudos têm mostrado, no entanto, que a composição da própolis pode variar de acordo com a flora e região onde é coletada e permitido a identificação de sua origem vegetal por análise comparativa química e taxonômica testes. Esses compostos foram identificados no exsudado das folhas da *Baccharis dracunculifolia* e são específicos da própolis brasileira, sendo encontrado em concentrações semelhantes, demonstrando que a *Baccharis ssp* é a principal fonte para composição da própolis. Assim, por possuir uma complexa e altamente variável composição química (cerca de 300 compostos já foram identificados), a qual está intimamente relacionada com a ecologia da flora de cada região visitada pelas abelhas, a própolis tem sido utilizada como medicamento natural desde os tempos antigos. Estudos têm comprovado sua ação benéfica à saúde devido a sua atividade antiinflamatória, hepatoprotetora, antimicrobiana, além de propriedades antioxidantes. Além disso, o própolis é utilizada em cosméticos bem como na alimentação. Assim, o efeito terapêutico da própolis sobre algumas doenças, devido ao seu uso popular como medicamento, tem suscitado o interesse na compreensão das propriedades biológicas desta substância relacionadando-as com sua composição química. Em vista desse potencial terapêutico proeminente da própolis e o pequeno número de estudos sobre os seus mecanismos de ação, o objetivo do presente estudo foi avaliar o possível potencial antigenotóxico e antimutagênico das frações de própolis em células de ovário de hamster chinês (CHO) e células de hepatobastoma humano (HepG2) pelo ensaio cometa e teste do micronúcleo com boqueio da citocinese (CBMN). Tratamentos com a própolis, nas concentrações de 25, 50, e 100 μg/ml, foram realizados anterior (pré), simultâneo e posterior (pós) à utilização de agentes genotóxicos/mutagênicos (de ação direta e indireta) com diferentes modos de ação de danos ao DNA. Sob essas condições, pelo ensaio cometa, nossos resultados mostraram claramente a capacidade das frações da própolis em proteger contra danos induzido quimicamente ao DNA. Os resultados do CBMN, similarmente, demonstraram a capacidade das frações da própolis em proteger contra danos cromossômicos induzido quimicamente. No entanto, também foi visto um efeito mutagénico das frações de própolis em células CHO e HepG2 quando tratadas com H_2O_2. Essa atividade quimioprotetora não foi dependentes do protocolo de tratamento nem da concentração utilizados. Além disso, este efeito protetor não pode ser atribuído aos compostos aqui identificados, uma vez que outros compostos presentes nas frações, ainda necessitam ser identificados e avaliados.

I - INTRODUÇÃO

I.1 Considerações iniciais

Vários estudos têm sido realizados objetivando a identificação de agentes que causam danos no DNA (agentes genotóxicos e mutagênicos) e também de compostos antimutagênicos, ou seja, aqueles que atuam na proteção da molécula, modulando a mutagênese. É sabido que alterações estruturais no material genético, como as quebras de fita simples e dupla, *crosslinks* e pareamento errôneo das bases púricas e pirimidínicas, podem ser induzidas por agentes químicos, físicos e biológicos ou ter origem "espontânea" (Boer & Hoejimakers, 2000). Microscopicamente, tais alterações podem interferir em mecanismos como a regulação do ciclo celular, apoptose, transcrição e duplicação do DNA; macroscopicamente, relacionam-se à gênese de várias doenças, efeitos teratogênicos e desordens herdáveis (Boer & Hoejimakers, 2000).

O termo composto "antimutagênico" foi originalmente usado por Novick e Szilard em 1952, para descrever os agentes que reduziam a freqüência de mutação espontânea ou induzida, independentemente dos mecanismos envolvidos, mantendo os níveis toleráveis do ponto de vista evolucionário (Von Borstel et al., 1996). Atualmente, os estudos de quimioprevenção de danos no DNA tornaram-se alternativa importante para a prevenção de doenças relacionadas a mutações, como por exemplo, o câncer, uma vez que o uso de estratégias que envolvem a redução ou eliminação da exposição humana a alguns fatores de risco não é sempre possível (Ribeiro et al., 2006).

As primeiras pesquisas buscando a identificação de compostos com atividade protetora contra a mutagênese data da metade do século 20. Hoje, centenas de compostos com

atividade antimutagênica e anticarcinogênica já foram identificados, mostrando que é possível desenvolver estratégias para prevenir ou proteger contra a indução de danos genéticos e doenças relacionadas. Esta hipótese é suportada por estudos epidemiológicos, que mostraram que 20%-60% de todos os cânceres estão relacionados à dieta (Doll & Peto, 1981; Doll, 1992). Knasmüller et al. (2002) estimaram que nos últimos dez anos aproximadamente 25.000 artigos foram publicados na área de antimutagênese e anticarcinogênese, e que mais de 80% dos agentes estudados são de origem vegetal e utilizados na dieta ou com propósitos medicinais.

I.2 - Própolis

Própolis (do grego, *pro* – em defesa - + *polis* - cidade) é o nome genérico utilizado para designar a resina coletada pelas abelhas a partir de várias espécies de plantas (Chemid, 1996). Essa resina é "mastigada", misturada às enzimas salivares da abelha, parcialmente digerida e misturada à cera (Ghisalberti, 1979; Marcucci, 1995). Por ter característica adesiva, a própolis é utilizada para selar eventuais aberturas na colméia, para proteção contra microorganismos e como substância embalsamadora que recobre as carcaças dos invasores, não permitindo que ocorra contaminação em decorrência de sua decomposição (Brumfitt et al., 1990; Bonheví & Coll, 1994; Higashi & Castro, 1994, Salantino, 2005). Seu uso pelo homem data de 300 anos a.C., época em que a própolis era utilizada como remédio, cosmético, para embalsamar mortos (Monti et al., 1983) e como agente cicatrizante (Ghisalberti, 1979). Ainda hoje esta resina é utilizada com propósitos medicinais em vários países, incluindo aqueles da região sudeste da Europa (Bankova, 2005)

A própolis, em geral, é composta de resina (50%), extratos vegetais (10%), cera (30%), óleos essências (10%), pólen (5%) e outras substâncias (5%) (Burdock, 1998). Dentre esses compostos sabe-se da presença de ácidos graxos e fenólicos (incluindo ácido cinâmico e cafeico) e seus ésteres, aldeídos aromáticos, álcoois, sesquiterpenos, naftalenos e flavanóides. Alguns estudos têm mostrado, no entanto, que a composição da própolis pode variar de acordo com a flora e região onde é coletada (Bankova et al., 1992, 1998; Garcia-Viguera et al., 1993; Tomas-Barberanet et al., 1993; Chi et al., 1996). No Brasil, devido a enorme extensão territorial e diversidade de sua flora, foi elaborado um mapa com os tipos de própolis coletadas nas diferentes regiões e sua respectiva composição química. Nesse estudo, foram listados dois grandes grupos e um subgrupo de compostos ricos em prenilatos e coniferaldeídos, embora, no Rio Grande do Sul, tenha sido identificada uma própolis com altas concentrações de flavonóides, fato raro em território brasileiro (AMOSTRAS DE SAÚDE, 2003). De modo geral, a própolis de zonas tropicais como o Brasil, onde a principal fonte de coleta das abelhas é o *Baccharis ssp* (alecrim do campo), o principal componente são os prenilatos (Bankova et al., 1999). Já em zonas temperadas, onde o *Populus ssp,* é a principal fonte, a própolis possui como componente principal os flavonóides.

A diversidade de compostos encontrados na própolis pode também ser devida ao modo de extração dos seus componentes, cujo fracionamento é dificultado por sua complexa composição. A maneira usual para extração dos componentes é a utilização de solventes alcoólicos, embora outros solventes sejam também utilizados. Como conseqüência, mais de 300 constituintes já foram identificados (Greenaway et al., 1991; Marcucci, 1995), embora suas funções sejam ainda desconhecidas. Desta forma, vários estudos vêm sendo conduzidos com o objetivo de avaliar as possíveis ações biológicas dos diferentes extratos e

frações da própolis. As atividades bactericida e fungicida, por exemplo, estão associadas ao predomínio dos flavonóides, flavonas, ácidos fenólicos e seus ésteres, que é comum em própolis de origem européia. No entanto, em própolis brasileiras, esses mesmos efeitos são creditados à composição rica em ácidos prenilatos p-comáricos e diterpenos (Bankova, 2005).

Os compostos derivados do ácido cafeíco, como o éster fenetil do ácido cafeíco (CAPE) e o benzil cafeato, também despertam interesse por estarem relacionados a atividades terapêuticas como efeitos antitumorais e antioxidantes (Bankova et al., 1983; Amoros et al., 1992; Marcucci, 1995; Matsushige et al., 1995). Além disso, há estudos mostrando a atividade antimutagênica (Cizmarick & Lahitova, 1998; Varanda et al., 1999) e anticarcinogênica da própolis (Rao et al., 1992; Frenkel et al., 1993; Rao et al.,1993; 1995; Matsuno et al., 1997; Kimoto et al., 1998), e sugerindo que estas estariam relacionadas à ação antioxidante dos flavonóides (Krol et al., 1990; Scheller et al., 1990; Volpert & Elstner, 1993; Pascual et al., 1994), ou à modulação do metabolismo do ácido araquidônico, induzindo apoptose e atuando na imunopotencialização contra células tumorais através do aumento da razão de células T CD4/CD8 (Kimoto et al., 1998).

Russo et al. (2006) mostraram que a ação antioxidante da própolis protege o DNA de espermatozóides humanos contra danos induzidos pelo benzo[a]pireno e por espécies reativas de oxigênio, podendo interferir sobre a fertilidade; estudos em animais experimentais evidenciaram que o extrato etanólico da própolis reduz em 50% as aberrações cromossômicas induzidas por radiações gama (Montoro, et al., 2005). Além disso, pesquisa utilizando células de linhagem leucêmica (HL-60) evidenciou que compostos da própolis (ácidos cinâmicos) inibem o crescimento celular por mecanismos de indução de apoptose (Akao et al., 2003); em células de carcinoma de cólon, KB e Caco-2, e

de próstata, DU-145, também foi demonstrado que a própolis é capaz de inibir o crescimento celular (Russo et al., 2004). Chen et al. (2004) utilizando células de melanoma humano demonstraram que a própolis induz apoptose por ativação de caspases, Bid (proteína pró-apoptótica) e citocromo C.

Portanto, devido à sua composição química e por ser um produto natural de fácil acesso, a própolis tem recebido especial atenção dentre os compostos com potencial antimutagênico e anticarcinogênico.

I.3 Testes *in vitro* e a identificação de compostos antimutagênicos

I.3.1 – Linhagens celulares

Os testes *in vitro* vêm sendo extensivamente utilizados tanto para identificar agentes mutagênicos como antimutagênicos. Os ensaios que utilizam células de mamíferos têm se mostrado bastante eficazes para estudos sobre os mecanismos envolvidos na atividade quimioprotetora, já que tais células, diferentemente das procarióticas, reproduzem algumas das situações observadas *in vivo*, como por exemplo, os sistemas de reparo do DNA e do metabolismo de compostos xenobióticos (Waters et al., 1996). Além de se evitar o uso de animais de experimentação, dentre os aspectos positivos da utilização de ensaios *in vitro* em estudos de antimutagênese, estão as possibilidades de se testar maior número de compostos em menor período de tempo e de se utilizar linhagens celulares com diferentes características e com variações nos protocolos de tratamento.

A escolha do protocolo experimental, embora dependa do tipo de agente utilizado e das características e sensibilidade das células empregadas, é uma ferramenta útil na identificação de mecanismos envolvidos no processo de antimutagênese. Nos tratamentos em que o agente quimioprotetor é utilizado simultaneamente ao mutágeno, pode-se, por

exemplo, avaliar se o primeiro atuaria diretamente sobre o xenobiótico impedindo que este vá para o interior da célula e atinja o DNA; no caso de pré-tratamentos, o antimutágeno que supostamente já estaria no interior da célula, poderia atuar sobre o sistema de metabolização celular ou, ainda, ligando-se ao mutágeno e impedindo sua interação com o DNA; quando o quimioprotetor é usado após o tratamento com o mutágeno, pode-se avaliar, por exemplo, sua atividade sobre os mecanismos de reparo de lesões no DNA (Kuroda et al., 1992).

O uso de linhagens celulares com a capacidade de metabolizar compostos sem a necessidade da utilização de sistemas exógenos de metabolização (por exemplo, S9), reduz alguns dos riscos atribuídos a este processo, como a possível atividade pró-mutagênica dos componentes da fração S9 e a desativação do antimutágeno (Gebhart, 1992; Kuroda et al., 1992). As células da linhagem HepG2, isoladas de hepatoblastoma humano (Aden et al., 1979), mantêm as enzimas de fase I e II que participam da ativação e detoxificação de mutágenos reativos com o DNA (Uhl et al., 2000), podendo, portanto, detectar com maior fidelidade os efeitos de substâncias que necessitam ser metabolizadas para exercer suas atividades genotóxicas ou mutagênicas. Por isso, essas células vêm sendo bastante utilizadas nos estudos de toxicogenética, podendo ser úteis para a detecção de diferentes tipos de indicadores de danos, como por exemplo: citotoxicidade, micronúcleo, troca entre cromátides irmãs e aberrações cromossômicas (Natarajan et al., 1991; Salvadori et al., 1993; Uhl et al., 2000; Knasmuller et al., 2002; Majer et al., 2005).

Por outro lado, linhagens de células sem a capacidade de metabolização, como é o caso das células de ovário de hamster chinês (CHO), são também freqüentemente utilizadas em estudos de mutagênese devido sua facilidade de crescimento e manipulação, além do pequeno número de cromossomos e da menor variabilidade genética (Fiore et al., 2002;

Aardema et al., 2006). As células CHO derivam de uma linhagem de células epiteliais obtidas por biopsia do ovário de um hamster chinês adulto, e seu cultivo teve início em 1957, na Faculdade de Medicina do Colorado -Denver -EUA (Puck et al., 1958).

I.3.2 Agentes mutagênicos e genotóxicos

Uma das vantagens do uso de sitemas *in vitro* para a identificação de agentes antimutagênicos, é a possibilidade da avaliação mais rápida da ação do quimioprotetor sobre diversos mutágenos com diferentes mecanismos de indução de danos no DNA. No presente estudo, o efeito protetor da própolis foi avaliado sobre danos induzidos por compostos reconhecidamente genotóxicos e mutagênicos, que atuam por diferentes mecanismos de ação e que necessitam ou não ser metabolizados a formas ativas para exercerem suas atividades.

O peróxido de hidrogênio (H_2O_2), por exemplo, é um composto que reage diretamente com o DNA por meio de espécies de oxigênio altamente reativas, que induzem quebras de fita simples ou dupla (SSBs e DSBs) e ainda podem causar alterações nas bases púricas e pirimídicas do DNA. Mais de 20 tipos de danos de base já foram identificados após o estresse oxidativo, sendo o 7,8-dihidro-8-oxoguanina (8-oxo-G) o mais comum em bases purínicas e a formação da timina glicol (Tg) o mais freqüente em bases pirimidínicas (Slupphaug et al., 2003).

O metil metano sulfonado (MMS) por sua vez, é um agente alquilante do DNA, que tem a capacidade de transformar a guanina em 7-metilguanina e a adenina em 3-metilguanina, causando, respectivamente, pareamento errôneo de bases e bloqueio da replicação do DNA. É importante ressaltar que os danos causados por agentes alquilantes

são em geral e predominantemente reparados pelo sistema de reparo por excisão de bases (Lundim et al., 2005).

Outra substância com elevada ação mutagênica e carcinogênica, e ainda bastante utilizada em estudos de antimutagênese e anticarcinogênese, é óxido de nitroquinolina (4NQO), que é um composto eletrofílico que mimetiza a ação da radiação UV. A 4NQO forma aductos de DNA que levam a vários tipos de lesões, como quebras de fita simples, formação de dímeros de pirimidina, formação de sítios abásicos e oxidação de bases. Contudo, tais lesões podem ser rapidamente reparadas pelo sistema de excisão de bases ou nucleotídeos (Mambo et al., 2003). Embora a 4NQO necessite ser metabolizada a 8-hidroxideoxiguanosina para exercer o seu potencial mutagênico por meio de danos oxidativos (Arima et al., 2006), a mesma também é utilizada como mutágeno de ação direta, por não precisar de biotransformação hepática para originar mutágenos (Gomes-Carneiro et al., 2006).

A dietilnitrosamina (DEN) é um potente carcinógeno genotóxico utilizado como agente iniciador em protocolos de hepatocarcinogênese em roedores. Após a bioativação por isoenzimas (CYP2E1) do citocromo P450, o composto torna-se altamente reativo com o DNA, formando, de maneira proporcional à dose, aductos pró-mutagênicos como o O6-etil-dioxi-guanosina e o O4- e O6-etil-dioxi-timidina (Verna et al., 1996).

I.4 Teste do Micronúcleo

O teste do micronúcleo é um método citogenético amplamente utilizado para avaliação de danos cromossômicos *in vivo* e *in vitro*, é simples, rápido e capaz de detectar, além de quebras, perdas cromossômicas (aneuploidia). Os micronúcleos (MN) são pequenas massas nucleares separadas do núcleo principal e formadas durante a telófase da mitose ou meiose,

resultantes de fragmentos cromossômicos acêntricos ou de cromossomos inteiros que não foram incluídos no núcleo principal.

O teste do micronúcleo foi originalmente padronizado para células de medula óssea de roedores *in vivo*, e sua aplicação em sistemas *in vitro* esbarrava em problemas como a dificuldade no reconhecimento das células que haviam passado por um ciclo de divisão, etapa sem a qual não há a produção de micronúcleo. Com base na descoberta de Carter (1967), que monstrava que células de camundongo, *in vitro*, tinham sua citocinese inibida pela citocalasina B (CtB) sem que houvesse bloqueio da mitose, Fenech & Morley (1985) passaram a utilizar esse composto para marcar as células que passaram por um ciclo de divisão, reduzindo, assim, a limitação do uso do teste do micronúcleo para os estudos *in vitro*. O uso da CtB resulta na formação de células binucleadas a partir daquelas que passaram por um ciclo de divisão, independente do da proporção e grau de sincronia (Figura 1).

Atualmente, o teste do micronúcleo com citocalasina vem sendo amplamente utilizado em estudos *in vivo* (cultura de linfócitos para o monitoramento genotóxico de populações), *in vitro* (avaliação do potencial mutagênico/antimutagênico de agentes químicos e seus possíveis mecanismos de ação) e *ex-vivo* (radiosensibilidade individual), em diferentes tipos celulares para identificação de parâmetros que refletem instabilidade genômica (Fenech et al., 2005).

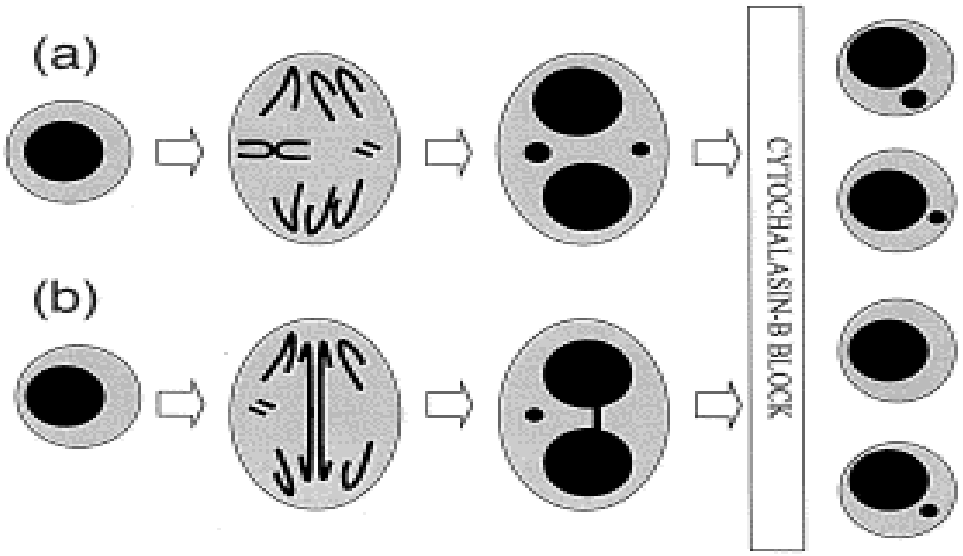

Figura 1. Modelo esquemático da formação e visualização do micronúcleo em ensaios utilizando a citocalasina B para bloqueio da citocinese.

I.5 Teste do Cometa

Rydberg & Johanson (1978), utilizando técnicas bioquímicas, foram os primeiros pesquisadores a quantificar diretamente os danos no DNA de células individualizadas. Mais tarde, Ostling & Johanson (1984) introduziram modificações na metodologia, em que células individualizadas, embebidas em agarose, eram colocadas sobre uma lâmina de microscópio, lisadas por detergentes em altas concentrações de sais e expostas a microeletroforese. As células com freqüência aumentada de quebras de cadeia do DNA apresentavam maior migração da molécula para o ânodo, permitindo a visualização de uma "cauda", após coloração com pigmento fluorescente (brometo de etídio). Devido a essa aparência, a imagem resultante foi chamada de "cometa", e levou Olive (1989) a sugerir o nome *comet assay* (teste do cometa) para identificar a técnica, também conhecida por *Single Cell Gel Electrophoresis assay (SCGE)* (Figura 2). Com a finalidade de aumentar a

sensibilidade do teste para a detecção de diferentes tipos de danos, Singh et al. (1988) e Tice et al. (1991) introduziram modificações que oferecem maior especificidade para detectar quebras de fita simples, dupla e sítios alcali-lábeis no DNA.

Comparado a outros testes de genotoxicidade, o teste do cometa apresenta vantagens como: maior sensibilidade para detectar baixos níveis de danos no DNA, uso de pequena amostra de células (não necessitando células em proliferação), avaliação de células individuais, simplicidade, baixo custo, rapidez para a obtenção dos resultados e flexibilidade, uma vez que pode ser usado para qualquer população de células, necessitando, apenas, de células viáveis. Tais vantagens têm favorecido o uso do teste do cometa em biomonitoramento humano e ambiental, em avaliação do sistema de reparo do DNA e em estudos de quimioprevenção de doenças relacionadas a lesões genotóxicas (Fairbain et al., 1995; Pool-Zobel et al., 1996; Hambly et al., 1997; Anderson et al., 1998; Moller et al., 2006).

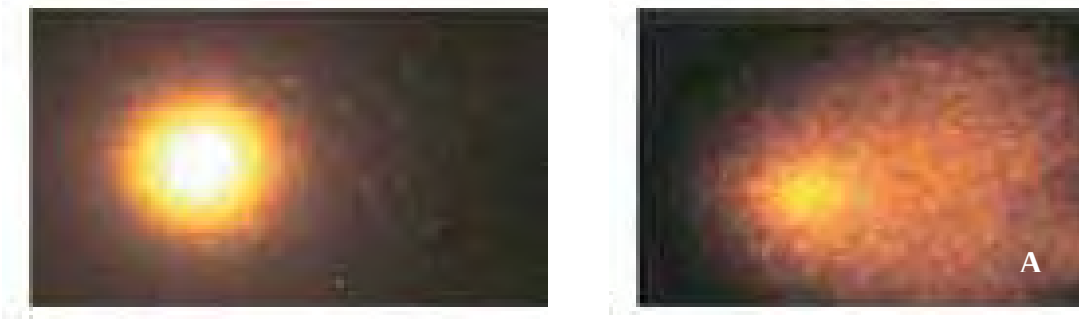

Figura 2. Fotomicrografia de células sem danos (A) e com danos (B) no DNA, detectados pelo teste do cometa (aumento de 400 X)

II - OBJETIVOS

Com base em informações existentes na literatura e em estudos prévios realizados por nosso grupo de pesquisa, que mostram o potencial antimutagênico e anticarcinogênico da própolis, o presente estudo teve como foco principal identificar qual ou quais componentes da resina são os responsáveis por sua ação quimioprotetora sobre danos quimicamente induzidos no DNA, e quais são os mecanismos envolvidos em tal atividade. Para tanto, os objetivos específicos do estudo foram:

- isolar e identificar componentes presentes no extrato aquoso de própolis (EAP);
- avaliar o efeito quimioprotetor de frações isoladas do EAP sobre compostos mutagênicos com diferentes mecanismos de ação (MMS; 4NQO, H_2O_2 e DEN);
- avaliar o potencial antigenotóxico (teste do cometa) e antimutagênico (teste do micronúcleo) das frações isoladas em dois diferentes sistemas-teste *in vitro* (HepG2 - células metabolizadoras, e CHO – não metabolizadoras);
- avaliar diferentes protocolos de tratamento com as frações isoladas do EAP (antes, simultaneamente ou após o tratamento com o mutágeno) para a prevenção de danos no DNA.

BRAZILIAN PROPOLIS PREVENTS CHEMICALLY-INDUCED DNA DAMAGE IN VITRO.

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Short running: Brazilian Propolis and DNA Damage.

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Para submissão a: *Toxicoly in vitro*

ABSTRACT

The use of natural products or their active components for prevention and/or treatment of chronic diseases is especially based on traditional medicine from various ethnic societies and on epidemiological data of dietary habits and disease patterns. Crude extracts of propolis have been long used as folk medicine, but, today, they have gained more popularity as medicine, with antibacterial, antiviral, anti inflammatory, and antioxidant activities. Mammal cell lines with or without capacity of metabolizing xenobiotics in the absence of exogenous systems have been widely used to identify chemical antimutagens and to elucidate their mechanisms of action. Thus, the aim of the present study was to evaluate the genotoxic and antigenotoxic effects of isolated fractions from an aqueous extract of Brazilian propolis using the comet assay in two cell lines - Chinese hamster ovary (CHO) and humam hepatoma (HepG2) cells. Treatments with propolis, at concentrations 25, 50, and 100 μg/ml, were done prior to, simultaneously and post- treatment with different mutagens. Under these conditions, our data clearly showed the capability of propolis compounds to protect against chemically-induced DNA damage. This chemopreventive activity was not dependent of the treatment protocol nor of the concentration used. Moreover, this protective effect cannot be attributed only to the identified compounds, since others remain to be identified in each of the fraction.

INTRODUCTION

The practice of disease prevention is the most cost effective way for improving human health. Therefore, different approaches have been actively used. The best approach for decreasing cancer incidence, for example, is the removal of environmental carcinogens and mutagens, although exposure to such compounds is sometimes unavoidable. Thus, the intake of substances that inhibit mutagenesis and carcinogenesis becomes one of several methods for preventing degenerative disease. In fact, various researchers have reported the antimutagenic and anticarcinogenic effect of many natural compounds (Salvadori et al. 1996; Ribeiro & Salvadori, 2003; Liu et al., 2004; Nabekura et al., 2005; Saito et al., 2006). Based on these findings, attention has been devoted to identify chemopreventive agents or develop food formula with components that could decrease or prevent mutations and the incidence of cancer.

Propolis is a complex mixture of plant resins, pollen, essential oils and bee wax. The chemical composition of this resinous material is variable and intimately related to the ecoflora of the region visited by the honeybees. In Brazil, bees collected resins mainly from *Baccharis dracunculifolia* buds (Greenaway et al., 1987; Marcucci and Bankova, 1999). Therefore, because of the plant source, in South America, specially in Brazil, propolis seems to be completely different from the other parts of the world.

Recently, propolis has gained increased popularity as a natural medicine, and it has been widely recommended by phytotherapists because of its anti-inflammatory (Marcucci, 1995), hepatoprotective (Said et al., 2001; Liu et al., 2004), antimicrobial (Orsi et al., 2005), and antioxidant properties (Shimazawa et al., 2005). The antitumour activities of propolis have been well documented by various investigators throughout the years. Matsuno (1997) has reported the cytotoxicity of a clerodane-type diterpene isolated from Brazilian propolis on human hepatocellular carcinoma HuH13 cells; Takai et al. (1996) have observed that the anticancer drug 5-fluorouracil is more effective in mice bearing Ehrlich carcinoma when administered in combination with water-soluble parts of propolis. Recently, we have detected an inhibition of 1,2 dimethylhydrazine (DMH)-induced colon aberrant crypt foci development in rats treated with a hydroalcoholic extract of propolis (Bazo et al., 2002). Alves de Lima et al. (2005) have also showed that an aqueous fraction of Brazilian propolis was effective in protecting against DNA damage induced by DMH in colon cells of Wistar rats.

The therapeutic effects of propolis on some human diseases have raised the interest on the biological properties of its isolated components. Therefore, the aim of the present study was to evaluate the antigenotoxic effect of isolated fractions from an aqueous extract of Brazilian propolis in Chinese hamster ovary (CHO) and humam hepatoma (HepG2) cell lines using DNA lesions as depicted by the comet assay as endpoint.

MATERIALS AND METHODS

Propolis fractions

Propolis, produced by Apis mellifera L., was collected at the Chaves Farm (Itapecerica, State of Minas Gerais, Brazil). An aqueous extract was prepared as previously described by Alves de Lima et al. (2005), frozen and lyophilized. Latter, this powder was ressuspended in 10% methanol (V/V), introduced into a Sephadex LH-20 (G&E) column (40x200mm) previously washed with distillated water, and the three fractions obtained (F1, F2, and F3) were lyophilized and analyzed by High Performance Liquid Chromatography (HPLC - Merck-Hitachi, Germany equipped with a pump model L-6200 and a diode array detector L-3000). Separations were achieved on a Lichrochart 125-4 column (Merck, Darmstadt, Germany; RP-18, 12.5 x 0.4 cm, 5 mm particle size) using water, formic acid (95:5, v/v) and methanol. The elution was carried out in a linear gradient and a flow rate of 1 mL min-1. The detection was monitored at 280 nm and compounds were identified using standards as references. For data analysis, the Merck-Hitachi D-6000 (Chromatography Data Station - DAD Manager) was used.

Chemicals

The mutagens methyl methanesulphonate (MMS - 80 µg/mL; Sigma - USA), 4-nitroquinoline 1-oxide (4NQO - 0.01 uM; Sigma - USA), n-nitrosodiethylamine (DEN – 50 mM; Sigma - USA), and hydrogen peroxide (H_2O_2 – 0.6 mM; Merck - USA) were used as positive controls. These compounds, with different mechanisms of mutagenicity, were dissolved into Ham´s F10 or DMEM medium, just before use. Their doses, as well as the doses of the propolis fractions were determined according to the literature and preliminary experiments.

Cell lines and culture conditions

Chinese hamster ovary cells (CHO K-1) and human hepatoma cells (HepG2) were grown to confluence in 94 mm plastic Petri dishes, at 37^{o}C and 5% CO_2 atmosphere, using, respectively, Ham´s F10 and DMEM medium supplemented with 10% fetal calf serum (Cultilab - Brazil) and antibiotics (penicillin 100 U/ml and streptomycin 0.1 mg/mL). Confluent cells were detached with 0.15% trypsin (Cultilab - Brazil), centrifuged at 180 *g* for 5 minutes, and 0.25 x 10^6 cells/well were seeded in 24-

well plates, for the comet assay. Cell viability was checked using trypan blue after all the treatments and it was always more than 75% of viability (data not showed).

Comet (Single Cell Gel Electrophoresis) Assay – Experimental design

Each protocol was performed in triplicate to ensure reproducibility.

- *Pre-treatment*: twenty-four hours after seeding ($0.25x10^6$ cells), medium was removed and cells treated with one of the three fractions of propolis at concentrations of 25, 50, and 100 μg/ml, for 24 hour. Then, cells were washed with PBS, and treated with 4NQO, H_2O_2, or MMS (CHO) for 5 minutes or with DEN (HepG2) for 1h. Further, cells were washed twice with PBS, tripsinized, centrifuged at 180 g for 3 min, and ressuspended into 100 μL fresh medium.
- *Simultaneous treatment*: twenty-four hours after seeding ($0.25x10^6$ cells), medium was removed and cells were simultaneously treated with one of the fractions and with mutagens MMS, 4NQO, or H_2O_2 for 5 minutes, or DEN for 1h. Then, cells were washed twice with PBS, tripsinized, centrifuged at 180 g for 3 min and ressuspended into 100 μL fresh medium.
- *Post-treatment*: twenty-four hours after seeding ($0.25x10^6$ cells), medium was removed and cells treated with MMS, 4NQO, H_2O_2, for 5 minutes, or DEN for 1h. Then, cells were washed with PBS and treated with one of the three fractions of propolis for 24h. After this period, cells were washed twice with PBS, tripsinized, centrifuged at 180 g for 3 min, and ressuspended into 100 μL fresh medium.

The comet assay was performed according to Singh et al. (1988) and Tice et al. (1991). Briefly, a volume of 10 μl of the cell suspension was added into 100 μl of 0.5% low-melting point agarose at 37°C, layered onto a precoated slide with 1.5% regular agarose and covered with a coverslip. After agarose solidification, the coverslip was removed and the slides immersed into a lysis solution (2.5M NaCl, 100mM EDTA, 10 mM Tris-HCL buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for about 24 hours. Then, slides were washed with PBS, placed in a horizontal electrophoresis chamber and left immersed in alkaline solution (300 mM NaOH, 1mM EDTA, pH 13) for 20 min. Electrophoresis was carried out for 20 minutes, at 25V and 300mA. After electrophoresis, slides were neutralized with 0.4 M Tris-HCl (pH 7.5) solution, fixed with 100% ethanol, and stored under refrigeration until analysis. After stained with ethidium bromide (20 mg/ml H_2Od), 50 randomly selected cells per culture (plate) were examined at 200 X magnification in a fluorescence microscope, using an

automated image analysis system (Comet Assay II, Perceptive Instruments, Suffolk, UK). The parameter selected as an indicator of DNA damage was tail moment (product of tail DNA/total DNA by the tail center of gravity, in arbitrary units).

Statistical Analysis

The level of DNA damage (tail moment) was assessed using the Kruskal-Wallis test followed by the post-hoc Dunn's test. p value < 0.05 was considered for statistical significance.

RESULTS

No cytotoxicity was observed in HepG2 and CHO cells treated with one of the three fractions of propolis (50 to 1000 μg/ml) for periods of 3 to 24h (data not showed).

Figure 1 shows the chemical profile of the three fractions of propolis. The conditions used to isolated fractions from propolis allowed to identify only few chemicals in each fraction: F1, quinic acid (191 M/z); F2, quinic acid (191M/z); F3, benzoic acid (121 M/z), cinnamic acid (149 M/z), and quinic acid (191 M/z).

Tables I, II, and III show the level of DNA damage (tail moment) in CHO cells treated with propolis (F1, F2, and F3) and one of the three mutagens (H_2O_2, MMS, or 4NQO). Data show that the three fractions at concentration of 25 μg/ml reduced DNA damage caused by H_2O_2 and 4NQO in the three protocols used (pre-, simultaneous, and post-treatments). For MMS, no protective effect was detected. Contrarily, it was observed an increased level of damage when F1 and F3 were used after to MMS (Table I). F1, F2, and F3, at concentration of 50 μg/ml, significantly reduced DNA damage induced by H_2O_2 in simultaneous and post-treatments, but only F3 had this effect in the pre-treatment protocol. Similar results were obtained for 4NQO when all of the fractions showed protective activity, except F2 in the post-treatment protocol (Table II). F1 and F2 were capable to reduce MMS-induced DNA damage, however, only when used after the mutagen (post-treatment). When the fractions, at concentration of 100 μg/ml, were tested against H_2O_2- and 4NQO all of them showed antigenotoxic activity in the three treatment schedules. However, the three fractions, at different protocol conditions, increased DNA damage induced by MMS. F1 and F3 increased MMS genotoxicity in pre- and simultaneous treatment, while F2 higher DNA damage in post-treatment (Table III).

The results achieved from propolis treatments in HepG2 cells are showed in Tables IV, V, and VI. At the three concentrations and treatment protocols used, F1, F2, and F3, significantly reduced DNA damage induced by H_2O_2 and DEN. However, these fractions showed different activity on 4NQO-induced genotoxicity. When used before the mutagen, F1 (50 μg/ml; Table V) and F3 (100 μg/ml; Table VI) reduced DNA damage, but F2 and F3 at 25 μg/ml (Table V), and F1 at 100 μg/ml (Table VI) increased the damage. In simultaneous and post-treatment schedules, the three fractions had protective effect, although under different concentrations (Tables IV, V, and VI).

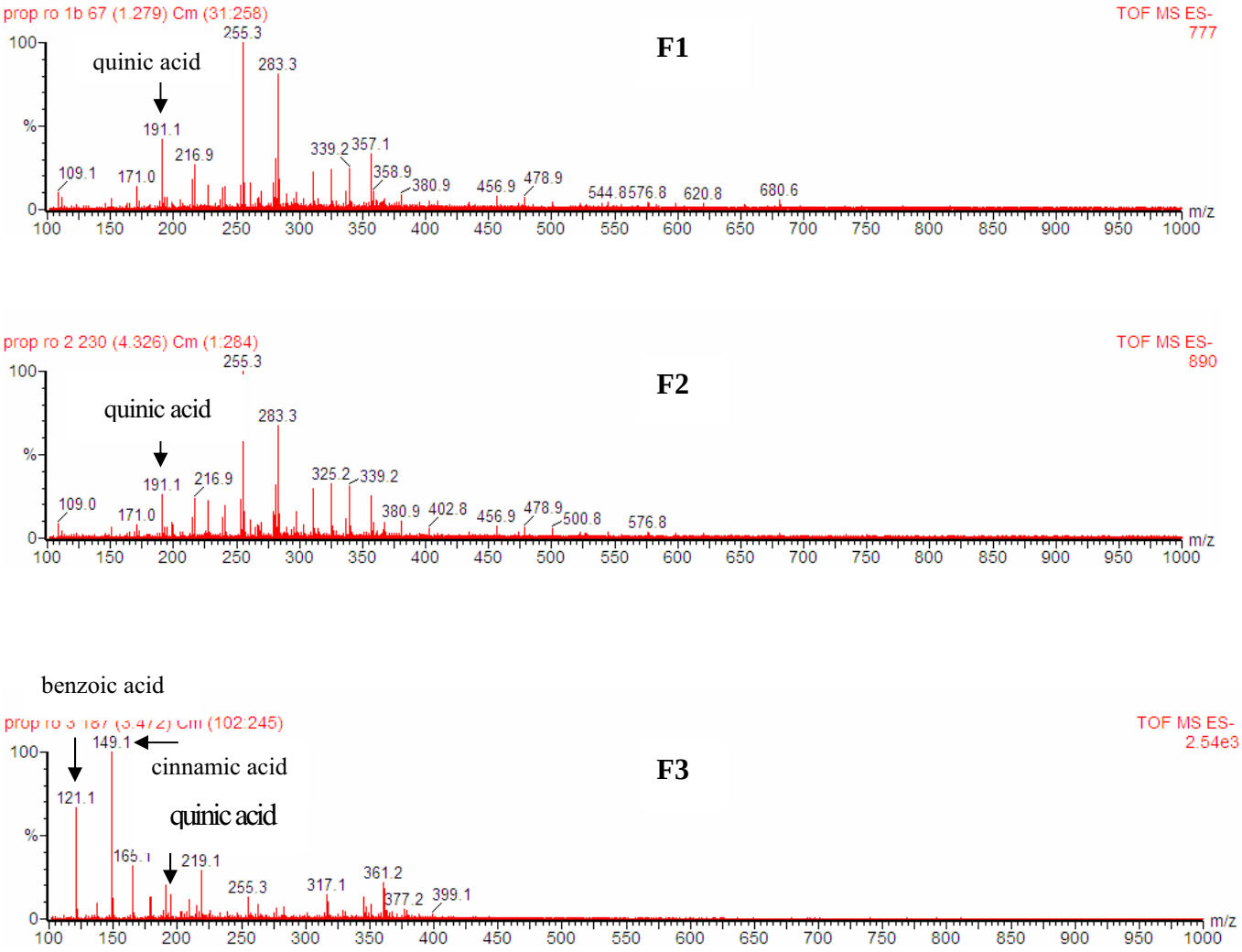

Figure 1. Chemical profile of the three isolated fractions (F1, F2, and F3) of Brazilian propolis identified by high performance liquid chromatographic (HPLC).

Table I. DNA damage (tail moment) in CHO cells exposed to three mutagens and three fractions of propolis (F1, F2, and F3, at 25 µg/ml) at different treatment protocols.

Mutagens and treatment protocols	Tail moment (Md ± SD)				
	C-	C+	F1	F2	F3
H_2O_2					
Pre-	0.13 ± 0.44	8.32 ± 2.77	5.60 ± 3.10*	6.92 ± 3.73*	4.96 ± 3.12*
Simultaneous	0.13 ± 0.44	8.32 ± 2.77	3.12 ± 2.42*	3.50 ± 2.31*	3.08 ± 2.31*
Post-	0.16 ± 0.73	2.51 ± 2.55	0.70 ± 1.90*	0.76 ± 1.78*	0.62 ± 1.15*
MMS					
Pre-	0.15 ± 0.57	8.53 ± 4.86	9.89 ± 5.18	8.30 ± 4.16	7.51 ± 3.52
Simultaneous	0.15 ± 0.57	8.53 ± 4.86	8.39 ± 4.05	8.51 ± 4.29	9.72 ± 4.23
Post-	0.22 ± 0.40	3.46 ± 3.44	4.62 ± 2.89*	4.67 ± 2.37	5.04 ± 2.81*
4NQO					
Pre-	0.13 ± 0.59	4.58 ± 4.30	1.45 ± 2.79*	3.25 ± 3.60*	1.99 ± 3.02*
Simultaneous	0.13 ± 0.59	4.58 ± 4.30	1.42 ± 3.65*	2.89 ± 4.42*	2.84 ± 5.20*
Post-	0.19 ± 0.56	2.43 ±1.79	0.50 ±1.33*	1.17 ± 1.70*	0.93 ± 1.19*

C -: negative control (vehicle control); C+: positive control (only the mutagen); H_2O_2 (0.6 mM); MMS - methyl methanesulphonate (80 µg/mL); 4NQO - 4-nitroquinoline 1-oxide (0.01 uM); pre-, simultaneous, and post: treatment schedules. * $p < 0.05$.

Table II. DNA damage (tail moment) in CHO cells exposed to three mutagens and three fractions of propolis (F1, F2, and F3, at 50 µg/ml) at three treatment protocols.

Mutagens and treatment protocols	Tail moment (Md ± SD)				
	C-	C+	F1	F2	F3
H_2O_2					
Pre-	0.07 ± 0.53	7.98 ± 3.59	6.73 ± 3.97	8.05 ± 3.07	4.48 ± 2.89*
Simultaneous	0.07 ± 0.53	7.98 ± 3.59	4.70 ± 2.78*	5.36 ± 2.46*	4.61 ± 2.29*
Post-	0.05 ± 0.33	2.26 ± 1.59	1.08 ± 1.31*	0.92 ± 1.46*	0.86 ± 1.36*
MMS					
Pre-	0.16 ± 0.61	9.47 ± 4.55	10.23 ± 3.64	9.94 ± 3.30	10.18 ± 3.29
Simultaneous	0.16 ± 0.61	9.47 ± 4.55	9.94 ± 3.09	8.97 ± 2.95	9.61 ± 2.50
Post-	0.07 ± 0.32	4.60 ± 2.12	3.77 ± 2.61*	3.88 ± 1.93*	4.77 ± 1.94
4NQO					
Pre-	0.16 ± 0.56	2.26 ± 3.51	1.18 ± 2.25*	1.13 ± 2.60*	0.64 ± 2.50*
Simultaneous	0.16 ± 0.56	2.26 ± 3.51	0.70 ± 1.72*	2.09 ± 2.72*	1.61 ± 2.81*
Post-	0.05 ± 0.28	1.28 ±1.27	0.86 ±1.13*	1.21 ± 1.62	0.75 ± 1.11*

C -: negative control (vehicle control); C+: positive control (only the mutagen); H_2O_2 (0.6 mM); MMS - methyl methanesulphonate (80 µg/mL); 4NQO - 4-nitroquinoline 1-oxide (0.01 uM); pre-, simultaneous, and post: treatment schedules. * $p < 0.05$.

Table III. DNA damage (tail moment) in CHO cells exposed to three mutagens and three fractions of propolis (F1, F2, and F3, at 100 µg/ml) at three treatment protocols.

Mutagens and treatment protocols	Tail moment (Md ± SD)				
	C-	C+	F1	F2	F3
H_2O_2					
Pre-	0.19 ± 0.56	5.36 ± 3.43	2.66 ± 2.55*	3.55 ± 2.44*	2.47 ± 2.26*
Simultaneous	0.19 ± 0.56	5.36 ± 3.43	3.72 ± 2.22*	3.87 ± 2.32*	3.85 ± 2.27*
Post-	0.04 ± 0.47	2.49 ± 1.42	1.69 ± 1.96*	0.79 ± 1.02*	1.15 ± 1.63*
MMS					
Pre-	0.16 ± 0.71	7.28 ± 6.14	10.49 ± 5.69*	8.84 ± 5.29*	9.84 ± 6.03*
Simultaneous	0.16 ± 0.71	7.28 ± 6.14	9.75 ± 4.25*	8.66 ± 4.92*	8.96 ± 4.37*
Post-	0.08 ± 0.71	8.03 ± 4.78	7.60 ± 3.09	10.42 ± 3.70*	7.95 ± 4.64
4NQO					
Pre-	0.18 ± 0.49	8.03 ± 3.55	6.23 ± 3.89*	6.70 ± 4.65*	8.21 ± 4.47
Simultaneous	0.18 ± 0.49	8.03 ± 3.55	5.50 ± 4.21*	5.77 ± 4.98*	6.53 ± 4.73*
Post-	0.22 ± 0.36	3.22 ± 2.30	1.92 ±1.81*	1.49 ± 1.63*	1.58 ± 1.51*

C -: negative control (vehicle control); C+: positive control (only the mutagen); H_2O_2 (0.6 mM); MMS - methyl methanesulphonate (80 µg/mL); 4NQO - 4-nitroquinoline 1-oxide (0.01 uM); pre-, simultaneous, and post: treatment schedules. * $p < 0.05$.

Table IV. DNA damage (tail moment) in HepG2 cells exposed to three mutagens and three fractions of propolis (F1, F2, and F3, at 25 µg/ml) at three treatment protocols.

Mutagens and treatment protocols	Tail moment (Md ± SD)				
	C-	C+	F1	F2	F3
H_2O_2					
Pre-	0.19 ± 0.39	3.51 ± 4.68	0.50 ± 2.30*	1.02 ± 4.39*	1.09 ± 2.28*
Simultaneous	0.19 ± 0.39	3.51 ± 4.68	1.08 ± 3.52*	1.00 ± 5.28*	0.79 ± 3.29*
Post-	0.05 ± 0.47	1.45 ± 1.70	0.64 ± 4.20*	0.62 ± 2.39*	0.44 ± 2.25*
DEN					
Pre-	0.08 ± 0.26	3.23 ± 5.26	0.33 ± 4.93*	0.49 ± 3.68*	0.47 ± 1.88*
Simultaneous	0.08 ± 0.26	3.23 ± 5.26	1.16 ± 4.37*	0.33 ± 2.99*	0.31 ± 5.39*
Post-	0.02 ± 0.25	1.10 ± 4.48	1.09 ± 4.00	0.49 ± 2.24*	0.71 ± 2.19*
4NQO					
Pre-	0.14 ± 0.63	17.14 ± 9.37	20.65 ± 8.95	22.94 ± 6.13*	22.22 ± 5.71*
Simultaneous	0.14 ± 0.63	17.14 ± 9.37	3.82 ± 7.17*	3.59 ± 9.05*	4.41 ± 8.40*
Post-	0.05 ± 0.30	1.03 ± 3.29	1.03 ± 2.52	0.64 ± 2.43*	0.14 ± 3.27*

C -: negative control (vehicle control); C+: positive control (only the mutagen); H_2O_2 (0.6 mM); DEN – diethyl-nitrosamine (50 mM); 4NQO - 4-nitroquinoline 1-oxide (0.01 uM); pre-, simultaneous, and post: treatment schedules. * $p < 0.05$.

Table V. DNA damage (tail moment) in HepG2 cells exposed to three mutagens and three fractions of propolis (F1, F2, and F3, at 50 µg/ml) at three treatment protocols.

Mutagens and treatment protocols	Tail moment (Md ± SD)				
	C-	C+	F1	F2	F3
H_2O_2					
Pre-	0.17 ± 0.38	2.67 ± 2.86	1.02 ± 2.58*	0.84 ± 2.14*	1.07 ± 2.13*
Simultaneous	0.17 ± 0.38	2.67 ± 2.86	0.71 ± 1.71*	1.17 ± 2.70*	0.98 ± 3.45*
Post-	0.10 ± 0.34	2.30 ± 2.50	0.96 ± 1.97*	0.93 ± 1.84*	0.75 ± 2.87*
DEN					
Pre-	0.10 ± 0.45	2.99 ± 2.25	1.50 ± 2.87*	1.45 ± 2.03*	1.39 ± 3.45*
Simultaneous	0.10 ± 0.45	2.99 ± 2.25	1.10 ± 3.42*	0.92 ± 2.05*	0.57 ± 2.06*
Post-	0.16 ± 0.46	2.78 ± 3.47	0.79 ± 1.93*	1.08 ± 2.26*	1.21 ± 2.15*
4NQO					
Pre-	0.18 ± 0.69	5.18 ± 4.92	3.85 ± 5.80*	4.79 ± 4.42*	4.48 ± 3.59*
Simultaneous	0.18 ± 0.69	5.18 ± 4.92	3.22 ± 5.52*	4.66 ± 4.59	4.98 ± 4.86
Post-	0.08 ± 0.44	3.07 ± 4.94	1.22 ± 3.65*	1.77 ± 5.93*	1.52 ± 5.87*

C -: negative control (vehicle control); C+: positive control (only the mutagen); H_2O_2 (0.6 mM); DEN – diethyl-nitrosamine (50 mM); 4NQO - 4-nitroquinoline 1-oxide (0.01 uM); pre-, simultaneous, and post: treatment schedules. * $p < 0.05$.

Table VI. DNA damage (tail moment) in HepG2 cells exposed to three mutagens and three fractions of propolis (F1, F2, and F3, at 100 µg/ml) at three treatment protocols.

Mutagens and treatment protocols	Tail moment (Md ± SD)				
	C-	C+	F1	F2	F3
H_2O_2					
Pre-	0.20 ± 0.49	13.92 ± 10.15	6.88 ± 9.81*	3.61 ± 7.63*	3.83 ± 6.57*
Simultaneous	0.20 ± 0.49	13.92 ± 10.15	2.18 ± 8.03*	2.80 ± 5.70*	1.78 ± 7.66*
Post-	0.36 ± 0.72	5.93 ± 8.97	1.49 ± 5.01*	1.07 ± 4.21*	0.99 ± 6.36*
DEN					
Pre-	0.26 ± 0.50	8.75 ± 9.03	1.08 ± 4.24*	0.68 ± 4.20*	0.87 ± 3.67*
Simultaneous	0.26 ± 0.50	8.75 ± 9.03	1.00 ± 5.77*	1.03 ± 4.87*	1.29 ± 5.50*
Post-	0.22 ± 0.57	6.13 ± 7.88	1.42 ± 5.61*	0.71 ± 4.40*	0.80 ± 5.15*
4NQO					
Pre-	0.68 ± 1.63	14.35 ± 10.98	22.69 ± 12.44*	14.41 ± 10.06	10.40 ± 8.68
Simultaneous	0.68 ± 1.63	14.35 ± 10.98	6.60 ± 10.03*	5.18 ± 9.87*	5.68 ± 9.61*
Post-	0.32 ± 0.57	6.22 ± 7.49	1.97 ± 5.98*	1.30 ± 4.06*	1.36 ± 5.33*

C -: negative control (vehicle control); C+: positive control (only the mutagen); H_2O_2 (0.6 mM); DEN – diethyl-nitrosamine (50 mM); 4NQO - 4-nitroquinoline 1-oxide (0.01 uM); pre-, simultaneous, and post: treatment schedules. * $p < 0.05$.

DISCUSSION

In recent years, propolis has gained popularity both as medicine and as food to improve health and prevent disease. Because of its broad spectrum of biological activities and use, there is a renewed interest on its chemical composition and actions (Banskota et al., 2001). Chemical analyses have identified at least 300 compounds in propolis, including fatty and phenolic acids and esters, flavonoids, terpenes, aromatic aldehydes, alcohols, sesquiterpenes, β-steroids and naphthalene (De Castro, 2001). However, in despite of many studies have proven the biological and pharmacological effects of propolis and its components, little is known about the pathways by which this honeybee product exert its activities. Thus, in order to better understand the mechanisms evolved in the protective effect of propolis on chemically-induced DNA damage, an aqueous extract was fractioned in three different solutions (F1, F2, and F3) in order to identify the main component responsible for the antigenotoxic acitivity. Nevertheless, at this moment, only few compounds could be chemically characterized in each fraction.

The chemopreventive effect of those fractions was tested in metabolically (HepG2) and no-metabolically (CHO) competent cell lines treated with direct- and indirect-acting mutagens and under different treatment protocols. Our results clearly showed the effectiveness of the Brazilian propolis in reducing H_2O_2 genotoxicity in both cell lines. It is well known that hydrogen peroxide is able to directly interacting with DNA through high reactive oxygen and hydroxyl radicals, causing damage such as single (SSBs) and double (DSBs) strand breaks (Ratnam et al., 2006, Valko et al., 2006). Various studies have reported the high antioxidant activity of propolis and some of its components (Scheller et al., 1990; Pascual et al., 1994). Banskota et al. (2000) evaluating some samples from different countries have observed a strong free radical scavenging ability of the Brazilian propolis, including water extracts from green propolis. Matsushige et al. (1996) have observed similar results with propolis from Brazil. Other authors have also showed the ability of propolis in reducing chemically-induced DNA damage, suggesting that this effect could be related with the antioxidant compounds present in this honeybee resin (Cizmarick and Lahitova, 1998; Varanda et al., 1999). Heo et al. (2001) have described that polyphenolic compounds from propolis are capable to module some enzyme activities and to suppress alkylating and radiomimetic chemical genotoxicity. Previously, Scheller et al. (1989) have detected a chemopreventive effect of an ethanolic extract of propolis against gamma radiation in

mice. Based on these findings, we suggest that the compounds (benzoic, cinnamic and quinic acids) present in our propolis fractions could have acted directly on the reactive oxygen species avoiding oxidative-DNA damage.

Similarly, in the present study we detected a protective effect of propolis fractions on 4NQO-induced genotoxicity. This compound is a powerful mutagen and carcinogen that directly and indirectly induces DNA oxidative lesions (Arima et al., 2006) and DNA adducts (Kim et al., 2006), respectively. Arima et al. (2006) have showed that 4NQO leads to formation of H_2O_2, and superoxide and hydroxil radicals. Probably, the protective effect observed, especially in CHO cells, could be attributed to the same mechanisms described before, i.e., the antioxidant activity of the propolis components. However, in HepG2 cells the results were conflicting. While the three fractions reduced 4NQO-induced DNA damage at simultaneous and post-treatment schedules, they had different behavior when administered before the mutagen. At lower concentration all of the fractions increased, instead of reducing, the levels of DNA damage. Same result was observed for the highest concentration of F1, while F3 had a protective activity. It is known that in some conditions antioxidant agents may also have pro-oxidant activity. Data from literature show that some propolis compounds may have both pro-oxidant and antioxidant properties (Kobayashi et al., 2001). Galati et al. (2002) have described the pro-oxidative activity of quercetin in erythrocytes, while some flavonoids (luteolin, chrysin, apigenin, naringenin) beside pro-oxidative characteristics also showed antioxidant effect.

Recently, Ferguson (2001) has showed mechanistic evidence indicating that some compounds can both induce and prevent damage. Polyphenols may generate reactive secondary radicals during a recycling process. Furthermore, phenoxyl radicals produced by some phenolic compounds in the course of radical scavenging are capable of oxidizing both proteins and lipids (Kagan and Tyurina, 1998). Sahu & Gray (1997) have suggested that hydroxyl radicals produced close to the DNA backbone could induce direct site-specific strand breaks that are insensitive to the free radical scavengers. Studies have shown that catalytic concentrations of flavonoids with a phenol B ring (e.g., apigenin, naringenin), upon oxidation by peroxidase, formed phenoxyl radicals that catalyzed GSH and generated ROS (Chan et al., 1999 and Galati et al., 2001). Thus, we could suppose that the fractions added to the HepG2 cell culture before 4NQO could have generated radicals that induced DNA damage.

In order to evaluate whether propolis may play a role on activation/detoxification of genotoxins, we used a metabolically competent cell line (HepG2) treated with indirect-acting mutagen. Many carcinogens and also mutagens including DEN, need to be activated by phase I enzymes to induce DNA damage. These enzymes catalyze oxidative reactions to produce oxidized metabolites that are then detoxified by phase II metabolizing enzymes into forms that are relatively inert and more easily excreted. There is considerable evidence that induction of phase II detoxification enzymes can modulate the threshold for chemical carcinogenesis, increasing cellular resistance to carcinogen exposure (Waladkhani and Clemens, 1998). Some evidences have showed that harmful substances may accumulate in the body when sulfotransferase (phase II enzyme) activity is inhibited (Weinshiboum, 1986). Indeed, numerous *in vivo* and in *vitro* studies have demonstraded that naturally occurring polyphenols may exert anticarcinogenic and cytoprotective activities against diverse chemical carcinogens and mutagens via induction of phase II detoxification enzymes (Wilkinson and Clapper, 1997). Various polyphenol or flavone compounds, natural and synthetic, produce activation of phase II enzymes activity. Such agents have been classified as monofunctional (phase II) or bifunctional inducers with capacity to increase both phase I and phase II enzymes (Prochaska et al., 1992). Yeh et al. (2005) have demonstrated that phenolic acids increase the level of phase II metabolizing enzymes in HepG2, suggesting that this compounds could be important in detoxification pathways.

Ours results showed that the three propolis fractions, in all of the treatment protocols used, were able to reduce DEN-induced DNA damage in HepG2 cells. Since these fractions have phenolic acids in their composition we may suppose that they could have acted on phase II enzymes and increased DEN detoxification. However, we cannot discard the possibility that propolis has acted as a free radical scavenging, blocking DEN metabolites to interact with DNA.

In conclusion, our data clearly showed the capability of propolis compounds to protect against chemically-induced DNA damage. However, this chemopreventive activity was not dependent of the treatment protocol nor of the concentration used. Moreover, this protective effect cannot be attributed only to the identified propolis compounds, since others remain to be identified in each of the fractions. For being a complex mixture, this resin has various composites that act alone or together by different mechanisms, which can lead to contradictory effects. Therefore, before

establishing a chemopreventive strategy using this honeybee product, it is necessary to better understand under which conditions propolis may promote health and prevent genome damage.

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THE ANTIMUTAGENIC POTENTIAL OF ISOLATED FRACTIONS OF PROPOLIS EVALUATED IN TWO CELL LINES: CHO AND HEPG2

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Key words: Chemoprevention, DNA damage, Micronucleus test, Propolis.

Short running: Propolis reduces DNA damage

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Para submissão a: *Mutation Research*

ABSTRACT

The use of natural products or their active components for prevention and/or treatment of chronic diseases are especially based on traditional medicine from various ethnic societies and on epidemiological data of dietary habits and disease patterns. Crude extracts of propolis have been long used as folk medicine, but, today, they have gained more popularity as medicine, due to its antibacterial, antiviral, anti inflammatory, and antioxidant activities. In view of the great therapeutic interest and the small number of studies regarding the mechanism of action, the present study aimed to evaluate the mutagenic and antimutagenic effects of propolis in two mammals cell lines: HepG2 and CHO, with (HepG2) and without (CHO) capacity of metabolizing xenobiotics in the absence of exogenous systems, respectively. Treatments with propolis, at concentrations of 25 and 100 μg, were done prior-, simultaneously and post-treatments with different mutagens. Under such conditions, our data showed the capability of propolis compounds to protect against chemically-induced chromosome damage, although no relationship was detected between this chemopreventive activity and the doses or treatment protocol. On the other hand, it was also observed the ability of the propolis fractions to increase H_2O_2-mutagenicity in both cell lines. In conclusion, other studies must be conducted before propolis is recommended as an chemopreventive agent against DNA damage or genetic-related disease.

INTRODUCTION

After a decade of researches, scientists are only beginning to understand how and when chemopreventive agents can be used to protect humans from genetic-related diseases. The choice of which agent or combination of agents to use is enormous since over a thousand of possible chemopreventive compounds have been identified. To address this problem, various tests, with different end-points and biomarkers, have been carried out to identify biological changes that can be used as quick screening for the protective ability of different substances (Rosin, 1993, Timbrell, 1998). Over the years, many experimental studies have generated basic understanding of the diverse mechanisms by which chemopreventive compounds act to suppress mutagenesis or tumorigenesis (De Flora & Ramel, 1988, David and Milner, 2004, Howells et al., 2007). Scientists have tried to classify potential chemopreventive agents by their expected mode of action and, therefore, lists have been made of antioxidant scavengers, anti-inflammatory agents, inhibitors of cell proliferation, hormone antagonists, inducers of cell differentiation, modifiers of oncogene expression, and other.

Because of their antioxidant activity, propolis, a complex mixture of plant resins, bee wax, essential oils and pollen, has received special attention. This resinous has a highly complex and variable chemical composition, which is intimately related to the ecology of the flora of the region visited by the bees. At present, about 300 components, mainly phenolic compounds, have been identified. Most of them belong to three main chemical groups: flavonoids, and aromatic acids, and esters, and their concentrations vary according to the region where propolis has been collected (Simões et al., 2004). Specific compounds present in the Brazilian propolis have allowed the identification of the plant source by comparative chemical analysis (infrared measurements) and taxonomic tests. Flavonoids identified in the exudate of *Baccharis dracunculifolia* leaves, have demonstrated that this plant is the main source of the propolis produced in the south region of the Minas Gerais State in Brazil (Park et al., 2004).

Used as natural medicine since ancient times, propolis is widely recommended by phytotherapists due to its anti-inflammatory (Marcucci, 1995), hepatoprotective (Said et al., 2001; Liu et al., 2004), antimicrobial (Orsi et al., 2005), and antioxidant properties (Shimazawa et al., 2005). Previous studies from our laboratory have showed the ability of propolis in reducing or inhibiting chemically-induced DNA damage and

preneoplasic lesions. Bazo et al. (2002) have observed a reduction of aberrant crypt multiplicity (pre-neoplasic lesions) in distal colon of Wistar rats when a hydroalcoholic extract of propolis was administered after the colon carcinogen 1,2-dimethylhydrazine; Alves de Lima et al. (2005) assessing the protective effect of an aqueous extract of propolis have also detected the capability of this extract in reducing primary DNA lesions induce by DNA 1,2 dimethylhydrazine in the Wistar rats colon cells.

Therefore, in view of the prominent therapeutic potential of propolis and the small number of studies regarding its mechanisms of action, the present investigation aimed to evaluate the antimutagenic effects of isolated fractions from an aqueous extract of Brazilian propolis. The experiments were designed trying to identify the compounds responsible by the protective activity against chemically-induced DNA damage, as depicted by the cytokinesis-blocked micronucleus (CBMN) assay in Chinese hamster ovary (CHO; non drug-metabolizing cells) and human hepatoma (HepG2; drug-metabolizing cells) cell lines.

MATERIALS AND METHODS

Isolated fractions of propolis

Propolis, produced by *Apis mellifera* L., was collected at Chaves Farm in Itapecerica – MG - Brazil. An aqueous extract was prepared as previously described by Alves de Lima et al. (2005), frozen, and lyophilized. Latter, the powder was ressuspended in 10% methanol (V/V), and put into a *Sephadex* LH-20 (G&E) column (40x200mm) previously washed with distillated water. The three fractions obtained (F1, F2, and F3) were lyophilized and analyzed by High Performance Liquid Chromatography (HPLC - equipped with a pump model L-6200 and a diode array detector - L-3000, Merck-Hitachi, Germany). Separations were achieved on a Lichrochart 125-4 column (Merck, Darmstadt, Germany) (RP-18, 12.5 x 0.4 cm, 5 mm particle size) using water, formic acid (95:5, v/v) (solvent A) and methanol (solvent B). The elution was carried out with a linear gradient and a flow rate of 1 mL min-1. The detection was monitored at 280 nm and compounds were identified using standards as references. For data analysis, the Merck-Hitachi D-6000 (Chromatography Data Station - DAD Manager) was used. Figure 1 shows the chemical profile of the three isolated fractions of propolis.

Chemicals

The mutagens methyl methanesulphonate (MMS - 80 µg/mL; Sigma - USA), 4-nitroquinoline 1-oxide (4NQO - 0.01 uM; Sigma - USA), n-nitrosodiethylamine (DEN – 50 mM; Sigma - USA), and hydrogen peroxide (H_2O_2 – 0.6 mM; Merck - USA) were used as positive controls. These compounds were dissolved into Ham´s F10 or DMEM medium, just before use. The doses of mutagens, as well as of the propolis fractions were based on literature, or determined in preliminary experiments.

Cell lines and culture conditions

Chinese hamster ovary cells (CHO K-1) and human hepatoma cells (HepG2) were grown until confluence in 94 mm plastic Petri dishes, at 37°C and 5% CO_2 atmosphere, using Ham´s F10 and DMEM mediums, respectively, supplemented with 10% fetal calf serum (Cultilab - Brazil) and antibiotics (penicillin 100 U/ml and streptomycin 0.1 mg/mL). Confluent cells were detached with 0.15% trypsin (Cultilab -

Brazil) for 5 minutes, and cell was counted using Newbauer chamber. After the experimental treatments, cell viability was checked using trypan blue.

The cytokinesis-block micronucleus (CBMN) assay – Experimental designs

- *Pre-treatment*: twenty-four hours after seeding (10^6 cells), medium was removed and cells were treated with one of the three fractions of propolis, at concentrations of 25 and 100 μg/ml, for 24 hour. Then, cells were washed with PBS, and treated with MMS, 4NQO, or H_2O_2 (CHO), for 5 minutes, or with DEN (HepG2) for 1h. Further, cells were washed twice with PBS and incubated into a fresh medium containing cytochalasin B (final concentration 3 ug/ml) for 24 hours.
- *Simultaneous treatment*: twenty-four hours after seeding (10^6 cells), medium was removed and cells were simultaneously treated with one of the three fractions of propolis and with each mutagen (MMS, 4NQO, or H_2O_2, for 5 minutes, or DEN for 1h). Then, cells were washed twice with PBS, and incubated for 24 hours into a fresh medium containing cytochalasin B.
- *Post-treatment*: twenty-four hours after seeding (10^6 cells), medium was removed and cells treated with the mutagens MMS, 4NQO, or H_2O_2, for 5 minutes, or DEN for 1h. After washing with PBS, cells were treated again with the isolated fractions of propolis for 24h. Then, cells were washed twice with PBS, and incubated for 24 hours into a fresh medium containing cytochalasin B.

Each protocol was performed in triplicate to ensure reproducibility. Cells were harvested and treated with cold hypotonic KCl solution (0.075 M), centrifuged (800 rpm, 8 min) and fixed with acetic acid/methanol (1:3) solution (three-times). Air-dried slides were prepared and stained with 3% Giemsa solution. 1000 binucleated cells (BNC) were analyzed for each test protocol.

Statistical Analysis

The frequencies of micronucleated cells were analyzed by using the Chi-Square test. p value < 0.05 was considered for statistical significance.

RESULTS

At this moment, the protocols used allowed to identify only few compounds in the fractions isolated from the Brazilian propolis: in F1, quinic acid (191 M/z); in F2, quinic acid (191M/z); in F3, benzoic acid (121 M/z), cinnamic acid (149 M/z), and quinic acid (191 M/z) (Figure 1). No cytotoxicity was observed in HepG2 and CHO cells after 3 to 24h treatments with one of these three fractions at dose of 50 to 1000 µg/ml. Cell viability was always higher the 85% (data not showed).

Tables I and II show the frequencies of HepG2 micronucleated cells (MNC). No protective effect was detected in pre-, simultaneous and post- treatment protocols with one of the three fractions of propolis at dose of 25 µg/ml (Table I). At 100 µg/ml F2 reduced 4NQO-induced MNC in the post-treatment protocol. However, contrarily, the three fractions increased the frequency of MNC induced by H_2O_2 mainly in pre- (F1, F2, and F3), but also in post-treatment protocol (F2) (Table II).

In CHO cells, data showed a protective effect of propolis when F2 and F3 (25 µg/ml) were used before H_2O_2. Differently, at simultaneous treatment these same fractions increased the frequency of MNC induced by this mutagen (Table III). For MMS, the three fractions prevent micronucleus formation in different protocols. Similarly, F1, F2, and F3 also reduced the frequency of 4NQO-induced MNC, but only in simultaneous and post-treatment protocols (Table III). When the higher dose (100 µg/ml) of the fractions was tested, the chemopreventive activity was better observed. For MMS and 4NQO, the three fractions were able to reduce the frequency of MNC, although increased frequency was detected when F2 was used after MMS. For H_2O_2, F1 and F3 showed protective activity in pre- and post-treatment protocols, respectively (Table IV).

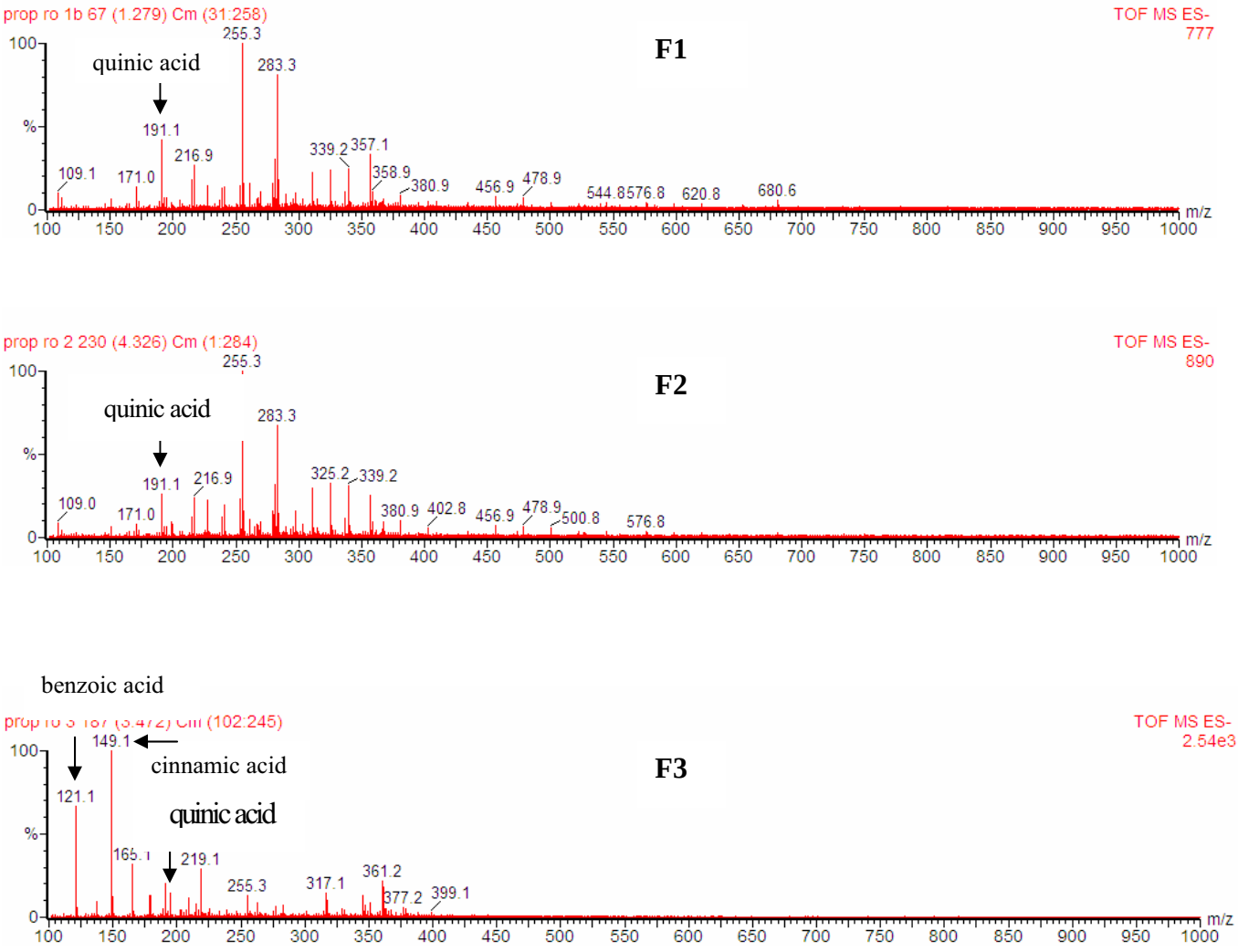

Figure 1. Chemical profile of the three isolated fractions (F1, F2, and F3) of Brazilian propolis identified by high performance liquid chromatographic (HPLC).

Table I. Frequencies of HepG2 micronucleated cells (MNC) exposed to indirect-acting mutagens and three fractions of propolis (F1, F2, and F3) at dose of 25 µg/ml.

Mutagens and treatment protocols	MNC (X±SD)				
	C-	C+	F1	F2	F3
H_2O_2					
Pre-	8.00±1.00	26.33±6.03	27.66±6.11	22.00±7.50	19.00±3.00
Simultaneous	4.33±1.53	10.33±0.60	11.33±2.31	10.33±4.04	10.66±2.33
Post-	7.00±1.00	11.66±1.15	10.00±1.00	9.66±2.08	10.33±2.08
DEN					
Pre-	5.66±1.53	11.33±3.05	9.00±2.00	9.33±2.08	10.66±1.15
Simultaneous	8.00±1.00	19.00±2.00	13.00±4.00	13.00±4.00	13.00±4.00
Post-	5.33±1.53	10.66±1.15	12.00±2.00	9.00±3.00	7.00±1.73
4NQO					
Pre-	3.33±0.57	11.00±1.73	10.00±4.00	7.33±2.31	6.66±2.31
Simultaneous	3.00±1.00	11.33±1.52	9.66±2.08	9.66±1.15	11.66±2.88
Post-	3.66±0.57	9.66±0.58	12.00±1.73	12.00±2.00	9.33±3.21

C -: negative control (vehicle control); C+: positive control (only the mutagen); H_2O_2 (0.6 mM); DEN – diethyl-nitrosamine (50 mM); 4NQO - 4-nitroquinoline 1-oxide (0.01 uM); pre-, simultaneous, and post: treatment schedules. * $p < 0.05$ (compared to C+).

Table II. Frequencies of HepG2 micronucleated cells (MNC) exposed to indirect-acting mutagens and three fractions of propolis (F1, F2, and F3) at dose of 100 µg/ml.

Mutagens and treatment protocols	MNC (X±SD)				
	C-	C+	F1	F2	F3
H_2O_2					
Pre-	4.66±0.57	15.33±0.57	23.33±1.53*	24.66±9.50*	25.00±1.00*
Simultaneous	9.66±0.57	21.33±1.15	20.00±4.00	20.00±2.00	14.66±4.62
Post-	7.33±0.57	15.33±2.51	20.00±4.00	24.66±3.00*	14.33±6.80
DEN					
Pre-	7.00±1.73	17.33±5.03	14.66±4.16	18.66±4.16	17.33±5.03
Simultaneous	6.33±0.57	16.66±2.08	14.00±2.64	9.33±7.57	15.33±3.05
Post-	5.33±1.52	10.66±1.15	12.00±2.00	9.00±3.00	7.00±1.73
4NQO					
Pre-	6.66±1.15	18.00±3.46	18.66±6.43	13.00±3.60	14.00±2.00
Simultaneous	6.66±1.53	16.66±3.05	13.66±5.50	22.66±4.16	11.33±1.15
Post-	7.33±0.57	18.66±5.03	20.66±6.11	11.66±7.23*	21.336.11

C -: negative control (vehicle control); C+: positive control (only the mutagen); H_2O_2 (0.6 mM); DEN – diethyl-nitrosamine (50 mM); 4NQO - 4-nitroquinoline 1-oxide (0.01 uM); pre-, simultaneous, and post: treatment schedules. * $p < 0.05$ (compared to C+).

Table III. Frequencies of CHO micronucleated cells (MNC) exposed to direct-acting mutagens and three fractions of propolis (F1, F2, and F3) at dose of 25 µg/ml.

Mutagens and treatment protocols	MNC (X±SD)				
	C-	C+	F1	F2	F3
H_2O_2					
Pre-	0.66±0.57	8.33±1.15	8.33±1.15[a]	6.66±0.57*[b]	6.00±1.00*[b]
Simultaneous	0.66±0.57	5.00±0.00	5.33±0.57[a]	6.33±1.53*[b]	6.33±0.57*[b]
Post-	1.33±1.15	7.00±1.00	7.33±0.57	8.00±1.00	7.33±1.15
MMS					
Pre-	1.66±1.53	15.00±0.00	13.00±2.00*	15.00±1.00	15.33±2.51
Simultaneous	1.33±0.57	11.00±0.00	11.66±2.51	10.66±1.15	8.66±0.57*
Post-	1.33±0.57	14.33±1.52	14.33±0.57[a]	12.00±1.00*[b]	12.00±1.00*[b]
4NQO					
Pre-	1.33±0.57	9.33±0.57	8.33±0.57	9.00±1.73	10.00±1.00
Simultaneous	1.66±1.15	10.33±0.57	7.33±0.57*[a]	7.33±0.57*[a]	6.66±1.53*[a]
Post-	1.00±0.00	8.33±0.57	4.66±0.57*[a]	4.33±0.57*[a]	5.00±1.00*[a]

C -: negative control (vehicle control); C+: positive control (only the mutagen); H_2O_2 (0.6 mM); MMS - methyl methanesulphonate (80 µg/mL); 4NQO - 4-nitroquinoline 1-oxide (0.01 uM); pre-, simultaneous, and post: treatment schedules. * $p < 0.05$ (compared to C+). Different letters indicate statistically significant difference between fractions.

Table IV. Frequencies of CHO micronucleated cells (MNC) exposed to direct-acting mutagens and three fractions of propolis (F1, F2, and F3) at dose of 100 µg/ml.

Mutagens and treatment protocols	MNC (X±SD)				
	C-	C+	F1	F2	F3
H_2O_2					
Pre-	1.00±1.00	5.33±1.53	4.66±0.57	5.33±0.57	4.00±1.00*
Simultaneous	1.66±0.57	6.00±1.00	4.00±1.00*	5.00±1.00	5.66±0.57
Post-	1.33±1.15	6.33±2.08	7.00±1.73	5.66±0.57	5.66±0.57
MMS					
Pre-	0.66±0.57	8.00±0.00	7.66±0.57	7.00±1.00	6.33±0.57*
Simultaneous	1.66±0.57	12.00±1.00	10.33±0.57*[a]	9.66±0.57*[a]	9.66±1.53*[a]
Post-	1.66±1.57	13.33±0.57	11.00±1.73*[a]	16.00±1.00*[b]	11.33±0.57*[a]
4NQO					
Pre-	1.33±0.57	6.66±0.57	6.00±1.00[a]	5.00±1.00*[b]	5.00±1.00*[b]
Simultaneous	1.00±0.00	6.66±0.57	6.00±1.00[a]	5.33±0.57*[b]	4.33±0.57*[b]
Post-	1.66±0.57	8.33±2.08	5.33±1.52*[a]	4.33±0.57*[a]	5.00±1.00*[a]

C -: negative control (vehicle control); C+: positive control (only the mutagen); H_2O_2 (0.6 mM); MMS - methyl methanesulphonate (80 µg/mL); 4NQO - 4-nitroquinoline 1-oxide (0.01 uM); pre-, simultaneous, and post: treatment schedules. * $p < 0.05$ (compared to C+). Different letters indicate statistically significant difference between fractions.

DISCUSSION

In the present study we focused on the effect of three propolis aqueous fractions on chemically-induced chromosome damage in two cell lines. The first was a non drug-metabolizing model (CHO-K1), and the second, a drug-metabolizing cell line (HepG2). The CBMN assay was used to evaluate the frequency of chromosome alterations. This assay was developed at first as a method for detecting chromosome damage in cells exposed to mutagenic agents (Fenech and Morley, 1985). However, this test became also a prime candidate to be used as an intermediate marker of chemopreventive activity, because of the events that cause the formation of MN through chromosomal damage, or aneuploidogenic events (Fenech et al., 1999 and Fenech, 2002).

In the first experimental series, with HepG2 cells and the three mutagens (4NQO, DEN and H_2O_2), we observed an antimutagenic effect only when F2 (100 μg/ml) was used after treatment with 4NQO (Table II). Contrarily, when the protective activity of propolis fractions were tested on H_2O_2 mutagenicity, they increased the frequency of MNC, mainly when used before the mutagen. Previously, Tavares et al. (2006) have already observed the ability of a propolis ethanolic extract (100 μg/ml) to increase DNA damage caused by doxorubicin *in vitro*. These discrepant results can be firstly explained by the complex composition of propolis.

Although, numerous articles and reviews have described the protective effect of propolis and its chemical components (Bazo et al., 2002; Alves de Lima et al., 2005; Sforcin, 2007), they have also showed that some of the compounds may have both pro- and antioxidant properties (Kobayashi et al., 2001). Sahu & Gray (1997) have suggested that hydroxyl radicals produced close to the DNA backbone could induce direct site-specific strand breaks that are insensitive to the free radical scavengers. Recently, quercetin (flavonoid found in propolis) has been shown to covalently bind to cellular DNA and protein in human intestinal Caco-2 cells and hepatic HepG2 cells (Walle et al., 2003). Thus, although it is well known that quercetin as an antioxidant flavonoid will affect the status of ROS in cells, several researchers have shown that the opposite occurs as well, specifically that ROS will metabolize certain flavonoids to species that covalently interact with key target macromolecules (Galati and O´Brien, 2004). So phenolic antioxidants compounds can be both pro-oxidative and antioxidative which suggests that flavonoids/phenolics diet could be potentially more harmful than a beneficial to health (Decker, 1997). Other studies have shown that catalytic

concentrations of flavonoids, with a phenol B ring (e.g., apigenin, naringenin), upon oxidation by peroxidase form phenoxyl radicals that catalyzed GSH and generat reactive oxygen species (Chan et al., 1999; Galati et al., 2001). Knasmüller et al. (1998; 2004) have correlated some compounds found in propolis, like chrysin and coumarin, with an increased frequency of micronucleus (MN). These authors have observed that low doses of those flavonoids are able to inhibit MN in HepG2 cells, while higher doses cause increases in the frequencies of micronucleus. In fact, flavonoids may present antimutagenic activity mainly due to their ability to scavenge free radicals (De Flora, 1998), although their pro-oxidant activities can cause damage to the genetic material (Halliwell et al., 2005).

Similarly, in CHO cells the fractions of propolis induced as much decrease as increase of chemically-induced DNA damage. The use of these two cell lines (CHO and HepG2), as well as of direct- and indirect-acting mutagens and different treatment protocols have allowed to better understand under which conditions a compound has the ability to prevent mutagenesis. For 4NQO, especially in CHO cells, the three fractions had a protective activity, independently of the dose used. 4NQO is a powerful mutagen and carcinogen that direct and indirectly induces DNA oxidative lesions (Arima et al., 2006) and DNA adducts (Kim et al., 2006), respectively. For MMS, a monofunctional alkylating agents that directly alkylate both the nitrogen and oxygen atoms of DNA bases and the oxygen moieties of the phosphate backbone (Fatur et al., 2003), the three fractions reduced DNA damage, but depending on the dose and the treatment protocol used. Perhaps, their protective effect can be attributed to the scavenging ability of the propolis components and/or to their capacity of interfering on the control of various cellular pathways, such as apoptosis (Chen et al., 1996 and Aso et al., 2004) and cell cycle (Li et al., 2007). According to Kuroda et al. (1992), those antimutagens which are effective when administered simultaneously with the mutagen may act by chemically or enzimatically inactivating mutagens, or inhibiting the activity of promutagens. On the other hand, when the chemopreventive activity occurs in post-treatment protocols, i.e., when the test-compound is administered after the mutagen, it could have acted repairing the induced DNA damage (Kuroda et al., 1992).

It has been described that flavonoids may interfere in several steps of the malignant tumors development, including protecting DNA from oxidative damage, inhibiting carcinogen activation, and activating carcinogen detoxifying systems (Galati, 2000). In the present study no fractions of propolis had the ability to interfere on the

frequency DEN-induced micronucleus in HepG2 cells. Since DEN is an indirect-acting agent that needs to be enzimatically activated to become mutagen, we could believe that the compounds present in the three fractions had no capacity to modulate these metabolic pathways. Therefore, taken together, our data suggest that those propolis fractions could have acted mainly linking and inhibiting mutagens to penetrate in the cell (simultaneous treatment) or acting on DNA-repair system (post-treatment).

In conclusion, the present results showed the potential of propolis compounds to protect against chemically-induced chromosomal damage. However, this chemopreventive activity can not be directly attribute to a specific treatment protocol nor to one of the concentration used or even to an identified propolis component, since others remain to be identified in each of the fraction. For being a complex mixture, this resin has various compounds that act alone or together by different mechanisms, which can lead to contradictory effects. Together to others data in literature our results strongly demonstrated that propolis has characteristics of a "Janus" compound, a term coined to designate substances that behave as genotoxic or antigenotoxic agents depending on the experimental conditions used (von Borstel and Higgins, 1998). Therefore, before establishing a chemopreventive strategy using this honeybee product, it is necessary to better know under which conditions propolis may promotes health and prevents genome damage.

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V - DISCUSSÃO GERAL

Um dos paradigmas das pesquisas em antimutagênese era o fato de que a metodologia empregada para a detecção de mutágenos poderia ser igualmente utilizada para detecção de agentes antimutagênicos. No entanto, esse conceito foi considerado equivocado, pois importantes mecanismos de proteção do DNA não estão presentes em muitos dos modelos experimentais convencionais (Knasmüller et al., 2002). Hoje, estudos sobre a quimioproteção de danos no DNA utilizando cultura de células de mamíferos vêm sendo freqüentemente realizados, já que esse sistema apresenta vantagens de análise e entendimento dos mecanismos de ação, e permitem a utilização de diferentes protocolos de tratamento.

Ao longo dos anos inúmeros pesquisadores têm voltado a atenção para os extratos de plantas buscando a identificação de compostos quimioprotetores. Nesse sentido, a própolis tem se mostrado uma alternativa interessante, pois vários estudos já mostraram sua ação benéfica e a de vários de seus componentes. No entanto, os mecanismos pelos quais esses compostos exercem atividade protetora necessitam, ainda, ser investigados. Assim, o presente estudo objetivou avaliar os mecanismos envolvidos na ação protetora da própolis. Para tal, foram utilizadas linhagens celulares possuidoras ou não de sistemas enzimáticos capazes de metabolizar compostos xenobióticos, visando, com isso, avaliar a ação da própolis sobre mutágenos que necessitam ou não serem ativados para reagir com o DNA. Além disso, foram utilizados dois diferentes marcadores genéticos na tentativa de verificar a ação protetora da própolis sobre lesões primárias no DNA (genotóxicas) e sobre danos cromossômicos (micronúcleo).

Várias técnicas vêm sendo empregadas para a detecção de danos no DNA. Testes de genotoxicidade, como o teste do cometa, e de mutagenicidade, como o teste do micronúcleo, possibilitam a comparação de dois parâmetros que podem fornecer

indícios de danos primários no DNA e mutações, respectivamente (Kassie et al., 2000). Por apresentar inúmeras vantagens, o teste do cometa tem sido usado para a identificação de compostos genotóxicos e antigenotóxicos em vários sistemas-teste. O teste do cometa não é utilizado para detectar mutações, mas sim lesões primárias no DNA que, depois de fixadas (não reparadas) podem resultar em mutações. Vale destacar que, diferente das mutações, as lesões detectadas nesse ensaio são passíveis de correção. Assim sendo, o teste do cometa pode trazer informações importantes sobre a cinética e o tipo de lesão reparada, embora não possibilite inferir a fidedignidade do processo de reparo (Gontijo, 2003). O teste do micronúcleo, por sua vez, é bastante utilizado para a detecção lesões (mutações) após um ciclo celular, e que não reparadas pelos mecanismos apropriados, geram alterações cromossômicas estruturais. Essas alterações podem ser causadas por agentes clastogênicos (que induzem quebras cromossômicas) ou agentes aneugênicos (que induzem aneuploidia ou segregação cromossômica anormal) (Hayashi et al, 1994).

Os agentes utilizados no estudo, H_2O_2, 4NQO, MMS e DEN atuam lesando o DNA por meio de mecanismos conhecidos como danos oxidativos, adutos e oxidação, alquilações e adutos, respectivamente. Essas lesões levam as quebras de fita simples e dupla, além de alterações de bases do DNA que podem resultar em quebra da molécula (clastogenicidade) e posterior formação de MN. Conhecendo os mecanismos pelos quais tais mutágenos atuam lesando o DNA pode-se sugerir modos de ação pelos quais a própolis exerceria seus efeitos biológicos.

Na parte inicial deste estudo, o teste do cometa foi utilizado para avaliação do potencial antigenotóxico da própolis sobre os compostos de ação direta, MMS, H_2O_2 e 4NQO, em células CHO. Os resultados mostraram o efeito protetor das frações sobre os mutágenos que induzem danos oxidativos no DNA (4NQO e H_2O_2) (Arima et al., 2006; Ratman et al., 2006). A literatura tem mostrado que uma das principais vias da ação

protetora da própolis é sua atividade antioxidante (Pascual et al., 1994, Matsuno, 1997, Chen at al., 2004). Ao utilizarmos o MMS (agente alquilante) como agente genotóxico, observamos, na maior e menor concentrações das frações, um aumento das lesões no DNA. No entanto, ao utilizarmos esse mesmo mutágeno no teste do micronúcleo observamos uma redução de células micronucleadas nas duas concentrações testadas, independente do protocolo usado em células CHO.

Já é conhecido que a própolis e vários de seus componentes possuem atividade antioxidante e seqüestradora de radicais livre, inibindo a formação de adutos no DNA e mutação (Le Bon et al., 1995, Jeng et al., 2000, Lui et al., 2004, Shimazawa et al., 2005). Tais atividades poderiam explicar a ação protetora da própolis sobre o H_2O_2 e 4NQO nos tratamentos pré e simultâneo, seqüestrando os radicais eletrofílicos gerados por esses mutágenos e conseqüente indução de danos no DNA, reduzindo, assim, as lesões primárias e posterior na formação de MN. Ao utilizarmos o MMS (agente alquilante), notamos, pelo teste do cometa, um aumento dos danos ao DNA quando as células foram também expostas a menor e maior concentrações das frações, sugerindo que compostos da própolis poderiam atuar potencializando o mutágeno. Contudo, no teste do micronúcleo observamos diminuição na freqüência de células micronucleadas, quando usado o mesmo mutágeno. Pode-se especular que os danos gerados pelo MMS em combinação com as frações da própolis, inicialmente detectados pelo teste do cometa, eram danos reversíveis que não progrediriam para a formação de MN, i.e, podiam ser reparados não resultando em mutações detectáveis pelo teste do MN. É sabido que todas as células possuem eficientes sistemas de reparo que removem lesões no DNA. Contudo esse sistema é passível de falhas, permitindo a fixação de mutações que podem ser deletérias ao organismo (Agnez-Lima et al., 2003). Bases metiladas por agentes como MMS, por exemplo, são eficientemente reparadas por BER (reparo por excisão de bases) ou por DNA metiltransferases (Lindahl e Wood, 1999). Ludin et al

(2005) avaliaram mecanismos de reparo do DNA em linhagens CHO expostas ao MMS e concluíram que as lesões induzidas por este mutágeno eram reparadas por mecanismo de BER.

Nossos resultados com células HepG2 mostraram que os danos no DNA induzidos pelos mutágenos H_2O_2, DEN e 4NQO e detectados pelo teste do cometa foram diminuídos independente da fração de própolis, concentração e protocolo utilizados. Em um estudo de revisão, Knasmüller et al. (1998) listaram uma série de enzimas com atividade metabolizadora (fase I e II) em HepG2. Assim, a HepG2 tem tido ampla utilização nos estudos de quimioproteção por possuírem importantes enzimas de detoxificação de xenobióticos, como as superóxido desmutases dependente de Mn e Cu/Zn, catalases, glutationas peroxidase e redutase (Guiterrez-Ruiz et al., 2001, Bianchi et al., 2002, Murakami et al., 2002). Por outro lado, as células HepG2 também possuem eficientes mecanismos de reparo de danos no DNA. Assim, pode-se também supor que as frações de própolis além de atuar ativando o sistema metabolizador de xenobióticos atuariam sobre o sistema de reparo, diminuindo os danos causados ao DNA. Siess et al. (1996) avaliando o efeito protetor de flavanóides e da própolis em enzimas de fase I e II observaram o aumento da atividade de enzimas detoxificadoras de xenobióticos (entre elas a glutationa transferase, presente na HepG2). Enzimas da família P450 também foram induzidas juntamente com as de fase I e II pela própolis. Le Bon et al. (1992) demonstraram a inibição da formação de adutos no DNA por meio da inibição de enzimas da família P450 pela própolis. Jeng et al. (2000) avaliando o potencial antimutagênico da própolis contra agentes de ação direta (4-nitro-O-fenilenediamina e 1-nitropireno) e indireta (2-amino-3-metilimidazo[4,5-f]quinolina e benzo[a]pireno) observaram a redução do potencial mutagênico pela própolis através de mecanismos como inibição das enzimas P450, interação e inativação dos metabólitos ativos gerados por esses mutágenos. Esses mecanismos também poderiam explicar a redução dos

danos ao DNA induzidos pela DEN pelas frações de própolis em todos os protocolos e concentrações. Sabe-se que a DEN lesa o DNA por meio de sua biotransformação e formação de metabólitos ativos pelas enzimas da família P450 (Verna et al., 1996). A inibição dessas enzimas poderia também inibir a formação dos metabólitos ativos, diminuindo, assim, suas ações.

Contudo, no teste do micronúcleo não foi observado efeito protetor das frações da própolis sobre os danos induzidos pelo H_2O_2, pelo contrário, foi detectada uma ação potencializadora de lesões nas maiores concentrações. Conforme discutido anteriormente, a própolis e seus compostos também possuem ação pro-oxidante (Halliwell et al., 2005 e Tavares et al., 2006). Por ter em sua composição várias substâncias com ação ainda não conhecida, e também pelo fato de não terem sido identificados e quantificados todos os componentes das frações testadas, não pode ser descartada a ação pro-oxidante das frações sobre o H_2O_2.

Finalizando, o presente estudo mostrou o potencial antimutagênico e antigenotóxico das frações da própolis. No entanto, os dados obtidos permitiram poucas conclusões sobre os mecanismos de ação, evidenciando a grande complexidade para a avaliação e identificação de agentes quimioprotetores. Assim, novos estudos devem ser conduzidos antes que sejam estabelecidas estratégias preventivas ou o uso da própolis como agente terapêutico.

VI – CONCLUSÕES

Com base nos resultados pode-se concluir que:

- ✓ as três frações de própolis (F1, F2 e F3), independente dos protocolos de tratamento, apresentam atividade protetora contra danos primários no DNA induzidos pelo H_2O_2 e 4NQO em células CHO e HepG2 e pelo DEN em células HepG2;

- ✓ as três frações de própolis aumentam os níveis de danos primários no DNA induzidos pelo agente alquilante MMS em células CHO, na dependência da concentração e protocolo de tratamento;

- ✓ as três frações de própolis, independente do protocolo de tratamento e concentração, reduzem a freqüência de micronúcleos induzidos pelo MMS e 4NQO em células CHO;

- ✓ as frações da própolis reduzem ou potencializam o efeito mutagênico (micronúcleo) do H_2O_2, dependendo da concentração e protocolo de tratamento utilizado;

- ✓ não é possível definir qual ou quais compostos presentes nas frações isoladas da própolis apresentam potencial protetor contra danos quimicamente induzidos no DNA;

- ✓ as frações de própolis apresentam efeito antigenotóxico e antimutagênico independente dos protocolos de tratamento, atuando tanto sobre agentes de ação direta como indireta com diferentes mecanismos de lesão ao DNA;

- ✓ os efeitos protetores da própolis ocorrem por meio de diferentes mecanismos;

- ✓ é prematuro o uso dessas frações da própolis em estratégias de quimioprevenção de danos no DNA.

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