UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO"
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EFEITO PROTETOR DA PRÓPOLIS CONTRA DANOS QUIMICAMENTE INDUZIDOS NO DNA

DANIELLE CRISTINA DE ALMEIDA

DRA. DAISY MARIA FÁVERO SALVADORI

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ABSTRACT

Since it is not always possible to reduce human exposure to mutagens, attempts have been directed to identify potential antimutagens and anticarcinogens for protecting human population against environmental diseases. Propolis has been used in folk medicine since ancient times, and its antimicrobial, antiparasitic, antiviral, anti-inflammatory, antitumoral and antioxidant properties have been described. In view of the great therapeutic interest on this bee product, this study was designed to evaluate the antigenotoxic and antimutagenic effect of an aqueous extract of propolis (AEP) and two of its fractions (F4 and F5) on Chinese Hamster Ovary (CHO) and Human Hepatoblastoma (HepG2) cell lines by using comet assay and cytokinesis-block micronucleus assay (CBMN). Chemicals with different mechanisms of mutagenicity (hydrogen peroxide, 4-nitroquinoline-1-oxide, methyl methanesulphonate (MMS), and or n-nitrosodiethylamine) were used in order to better understand propolis chemopreventive action. Data showed that both AEP and the fractions reduced genotoxicity as depicted by the comet assay. However, lower protective effect was detected in the micronucleus assay. In conclusion, the results confirmed propolis as an effective chemopreventive agent, mainly on primary-induced DNA damage. This effect might be attributed to its antioxidant activity and also to its capability in modulate drug metabolizing enzymes.

RESUMO

Considerando que as estratégias para a prevenção de doenças relacionadas a danos no DNA, ou que envolvam a eliminação da exposição humana a fatores de risco nem sempre são possíveis, a identificação de agentes quimioprotetores torna-se uma alternativa relevante. Nesse contexto, a própolis, por seu amplo espectro de ação biológica e devido a sua complexa composição química, fácil obtenção e importância econômica para o país, é um composto que vem sendo bastante estudado, especialmente com relação a sua atividade terapêutica na saúde humana. Assim sendo, o presente estudo objetivou avaliar a ação protetora do extrato aquoso de própolis verde (12,5, 25, 50, 100 e 200 μg/ml) e duas de suas frações (F4 e F5; 25, 50 e 100 μg/ml) sobre danos quimicamente induzidos no DNA de células das linhagens CHO (células de ovário de hamster chinês) e HepG2 (células de hepatoblastoma humano), sob três protocolos de tratamento. Como agentes indutores de danos genéticos foram utilizados o peróxido de hidrogênio (H_2O_2), a 4-n-óxido-nitroquinolina (4NQO), o metil metano sulfonato (MMS) e a n-dietilnitrosamina. Os dados mostraram que tanto o extrato aquoso da própolis quanto suas frações foram eficientes na proteção contra danos genotóxicos, nas duas linhagens celulares e nos diferentes protocolos de tratamento, quando avaliados pelo teste do cometa. No entanto, quando a própolis foi testada pelo teste do micronúcleo, sua atividade antimutagênica foi menos evidente, sendo detectada apenas em situações específicas. Concluindo, os resultados demonstraram que tanto o extrato aquoso da própolis como suas frações foram capazes de reduzir os danos quimicamente induzidos no DNA, por mecanismos antioxidantes e de modulação de sistema enzimático de metabolismo de drogas.

I. INTRODUÇÃO

I.1 Considerações inicias

A quimioprevenção de danos no DNA tem se mostrado como alternativa importante para a prevenção de doenças relacionadas a mutações, uma vez que o uso de estratégias que envolvem a redução ou a eliminação da exposição humana a alguns fatores de risco nem sempre é possível (Ribeiro et al., 2006). Já foram identificados vários compostos com ação antimutagênica e anticarcinogênica, muitos dos quais com atividade antioxidante e encontrados em verduras, frutas e em extratos de plantas (Reddy et al., 2003). Knasmüller et al. (2002) estimaram que na última década cerca de 25.000 artigos foram publicados na área de antimutagênese e anticarcinogênnese, sendo que mais de 80% dos estudos foram realizados com agentes de origem vegetal e utilizados na dieta ou com propósitos medicinais. Embora o mecanismo de proteção desses compostos não seja ainda completamente conhecido, já é bem estabelecido que o consumo de frutas e verduras diminui a incidência de câncer (Reddy et al., 2003). Nesse contexto, o presente estudo aborda o potencial protetor da própolis, resina elaborada por abelhas a partir de componentes presentes no reino vegetal, contra danos quimicamente induzidos no DNA.

I.2 Própolis

A palavra própolis tem origem grega, em que *pro* significa em/para defesa de, e *polis* cidade (defesa da colméia). As abelhas utilizam a própolis para vedar aberturas e revestir as paredes internas da colméia, cobrir a carcaça de invasores e para proteger a colônia de doenças, graças a sua ação antiséptica e propriedades antimicrobianas (Salantino et al., 2005). A própolis é uma resina que é mastigada pelas abelhas, misturada a enzimas salivares, parcialmente digerida e, por fim, misturada à cera

(Ghisalberti, 1979; Marcucci, 1995). Sua coloração pode variar do amarelo claro ao marrom escuro, sendo que, em geral, a própolis é composta por 50% de resina, 30% de cera, 10% de extratos vegetais, 5% de pólen e 5% de outras substâncias (Cerasino et al., 1987; Monti et al., 1983). O fracionamento da própolis não é um procedimento simples, sendo que no início do século XX, apenas a sua composição bruta havia sido determinada (resina, cera, componentes voláteis e substâncias insolúveis). Os primeiros componentes identificados foram o ácido cinâmico, vanilina e crisina. A partir da década de 60 é que foram identificados os flavonóides e outros compostos fenólicos (Sawaya et al., 2004).

A composição química da própolis varia de acordo com a vegetação da região da colméia, sendo os compostos das espécies de *Populus* os principais componentes encontrados na própolis européia e norte americana (Greenaway et al., 1990). Devido à grande diversidade da flora brasileira, a composição da própolis encontrada no país é única e muito variável, (Park et al., 2004). A principal fonte botânica da própolis brasileira é a *Baccharis dracunculifolia.* Estudos realizados recentemente, comparando a composição química da própolis verde e do extrato de *B. dracunculifolia*, mostraram que os dois extratos são quimicamente similares, apresentando, inclusive, a mesma quantidade de flavonóides, compostos conhecidos por sua capacidade de seqüestrarem radicais livres (Park et al., 2004).

Atualmente, vários estudos vêm sendo realizados com o objetivo de avaliar as atividades da própolis e de seus componentes, principalmente no que se refere à sua atividade antitumoral, antioxidante, antiinflamatória, antibiótica (Akao et al., 2003), antimutagênica (Alves de Lima et al., 2005) e anticarcinogênica (Bazo et al., 2002). Kismet et al.(2008) observaram que a própolis apresentou efeito hepatoprotetor em um modelo experimental de icterícia obstrutiva. Os autores sugerem que esse efeito possa

estar relacionado à sua atividade antioxidante, antiinflamatória e imunomoduladora. Benkovic et al. (2008), utilizando modelo *in vitro*, demonstraram que o extrato etanólico da própolis e a quercetina (componente da própolis), são capazes de proteger as células sanguíneas contra danos induzidos DNA pela radiação ionizante.

Em 2007, Chen et al, isolaram dois compostos (propolin A e propolin B) da própolis tailandesa e observaram que ambos eram potentes agentes antioxiantes e também capazes de induzir apoptose em células A2058. Anteriormente, Chen et al. (2004), utilizando células de melanoma humano já haviam observado que a própolis induzia apoptose por ativação de caspases, Bid (proteína pró-apoptótica) e citocromo C. Por outro lado, Russo et al. (2006) também demonstraram 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, aumentando a fertilidade. Além disso, estudos em animais evidenciaram que o extrato etanólico da própolis reduz em 50% as aberrações cromossômicas induzidas por radiação gama (Montoro et al., 2005).

Estudos realizados anteriormente em nosso laboratório mostraram que a própolis apresenta atividade protetora no processo de carcingogênese de cólon, diminuindo o número de focos de criptas aberrantes induzidos pela dimetil-hidrazina em ratos Wistar (Bazo et al., 2002). Mais recentemente, Alves de Lima et al. (2005) observaram que o extrato aquoso da própolis reduz significativamente os danos no DNA induzidos pelo DMH nas células de cólon de ratos. Efeitos antigenotóxico e antimutagênico de frações do extrato aquoso da própolis foram também detectados em células de ovário de hamster chinês (CHO) e de hepatoblastoma humano (HepG2), *in vitro,* em diferentes protocolos de tratamento com mutágenos de ação direta e indireta (Alves de Lima, 2007).

Os inúmeros estudos realizados com a própolis, embora demonstrem o potencial quimioprotetor dessa resina, ressaltam a importância dos delineamentos experimentais e sistemas-teste para a avaliação dos mecanismos de ação e identificação de seus componentes ativos.

I.3 - Delineamentos Experimentais para Estudos de Quimioproteção

Culturas Celulares

Os ensaios *in vitro*, com células de mamíferos, são realizados com bastante eficácia e sucesso para explorar os mecanismos pelos quais os compostos mutagênicos e antimutagênicos exercem suas atividades (Waters et al., 1996). As culturas celulares possibilitam a análise de compostos em menor período de tempo, variações nos protocolos de tratamento, além de permitir o uso de linhagens com diferentes características.

A escolha do protocolo experimental, embora dependa do tipo de agente em estudo e das características das células empregadas, é uma ferramenta útil na identificação dos mecanismos envolvidos no processo de antimutagênese. No caso do composto quimioprotetor ser administrado anteriormente ao mutágeno (pré-tratamento), e, portanto, supostamente já estar no interior da célula quando da entrada deste, poderia induzir a produção de enzimas metabólicas que atuariam na detoxificação do mutágeno. Nos tratamentos em que os dois agentes são utilizados simultaneamente, o composto quimioprotetor pode interagir diretamente com o mutágeno inativando-o ou impedindo sua interação com o DNA. Quando o agente antimutagênico é administrado posteriormente, estar-se-a avaliando seu potencial na reversão de danos no DNA, isto é, sua capacidade de atuar sobre o sistema de reparo do DNA (Kuroda et al., 1992).

A escolha da linhagem celular também é um importante instrumento para a definição dos mecanismos de ação dos compostos quimioprotetores. Nesse contexto, as

células HepG2, isoladas de hepatoblastoma humano, têm sido bastante utilizadas por preservarem os sistemas enzimáticos de metabolização, podendo, portanto, detectar com maior fidelidade os efeitos de substâncias que necessitam ser metabolizadas para exercer suas atividades genotóxicas ou mutagênicas (Uhl et al., 2000). Por outro lado, as células de ovário de hamster chinês (CHO), que não têm a capacidade de metabolização, mas são de fácil crescimento e manipulação, são úteis para a detecção do potencial quimioprotetor sobre compostos que agem diretamente sobre o DNA sem necessidade de serem previamente ativados ou metabolizados (Aardema et al., 2006).

Agentes Mutagênicos

Outro aspecto relevante na elaboração do delineamento experimental para estudos de antimutagênese é a escolha do agente mutagênico Para a avaliação do efeito protetor da própolis contra danos no DNA, foram utilizados compostos com diferentes mecanismos de genotoxicidade e mutagenicidade. O peróxido de hidrogênio (H_2O_2) é um composto que reage com o DNA induzindo quebras de fita simples e dupla, e que pode causar alterações em bases púricas e pirimidínicas por meio da formação de espécies reativas de oxigênio (Slupphaug et al., 2003). Devido a sua alta solubilidade e baixa reatividade, o H_2O_2 pode se difundir livremente através das membranas celulares antes de reagir com alvos moleculares específicos (Go et al., 2004). Outro mutágeno utilizado, a 4 n-óxido nitroquinolina (4NQO), além de promover forte estresse oxidativo intracelular por gerar espécies reativas de oxigênio, é um composto eletrofílico que mimetiza a ação da radiação UV. A 4 NQO promove a formação de aductos de DNA levando à formação de diversos tipos de lesões como quebras de fitas simples, formação de dímeros de pirimidina e sítios abásicos (Mambo et al., 2003; Kanojia et al., 2006).O metil metano sulfonato (MMS), por sua vez, é um típico agente alquilante

monofuncional com capacidade de transformar as bases guanina e adenina e induzir pareamentos errôneos e bloqueio da replicação do DNA, e que vem sendo bastante utilizado em modelos experimentais para a elucidação dos mecanismos de toxicidade genética dos agentes alquilantes (Franke et al., 2005; Ludin et al., 2005). A dimetilnitrosamina (DEN) é um potente carcinógeno ambiental que está presente no tabaco, em alimentos processados derivados do leite e em bebidas alcoólicas (Shahjahan et al., 2005). É utilizado como agente iniciador em protocolos de hepatocarcinogênese em roedores e, após sua bioativação por isoenzimas do citocromo P450, torna-se altamente reativo com o DNA, formando, de maneira proporcional à dose, aductos pró-mutagênicos (Verna et al., 1996).

I.4 Testes para a identificação de agentes antigenotóxicos e antimutagênicos

Técnicas citogenéticas vêm sendo amplamente utilizadas para a detecção de danos no DNA e para a identificação de compostos mutagênicos e antimutagênicos. Dentre essas, as que detectam aberrações cromossômicas numéricas e estruturais, troca entre cromátides irmãs e micronúcleo, têm sido as mais utilizadas, embora apresentem limitações, pois requerem proliferação celular e detectam apenas lesões visíveis em nível cromossômico. Assim, o emprego de técnicas moleculares amplia e complementa o espectro de danos genéticos que podem ser avaliados nos estudos de toxicogenética

Teste do Cometa

Östling & Johanson (1984) foram os primeiros pesquisadores a desenvolver a técnica de eletroforese em microgel para a detecção de danos no DNA. Nessa técnica, células embebidas em agarose eram colocadas em lâminas, lisadas por detergentes e o

DNA liberado era exposto a eletroforese sob condições neutras. As células com frequência aumentada de quebras de fitas duplas apresentavam maior migração que era quantificada corando-se as lâminas com brometo de etídio e medindo a intensidade da fluorescência entre dois pontos fixos dentro de um padrão de migração. Mais tarde, Singh et al.(1988) reproduziram essa técnica sob condição alcalina de eletroforese (pH >13), que propiciou maior migração do DNA, a qual foi atribuída a quebras de fitas simples na molécula e que não apareciam em condições neutras. Como a imagem obtida ao microscópio era semelhante a de um cometa a técnica de *Single Cell Gel Electrophoresis Assay* (SCGE) ficou mais conhecida por *Comet Assay* (teste do cometa) Olive (1989) (Figura 1).

Desde a introdução da versão alcalina em 1988, a utilização do teste do cometa para avaliação de agentes genotóxicos cresce exponencialmente. Quando comparada a outras técnicas, esta apresenta vantagens como: 1) ser sensível, mesmo para a detecção de baixos níveis de danos no DNA; 2) requerer pequeno número de células; 3) ser mais flexível, já que pode, teoricamente, em qualquer tipo celular; 4) ter custo relativamente baixo; 5) ser de fácil aplicação (Tice et al., 2000).

Teste do Micronúcleo

Nas técnicas citogenéticas clássicas, os cromossomos são avaliados pela observação e contagem de alterações visualizadas em células metafásicas (Natarajan et al., 1982). Essas técnicas proporcionam a possibilidade de detecção de alterações cromossômicas numéricas e estruturais, porém são complexas e laboriosas. Dessa forma, a técnica do micronúcleo foi padronizada e vem sendo amplamente utilizada, pois, além de fornecer informações citogenéticas relevantes é de análise muita mais simples e rápida.

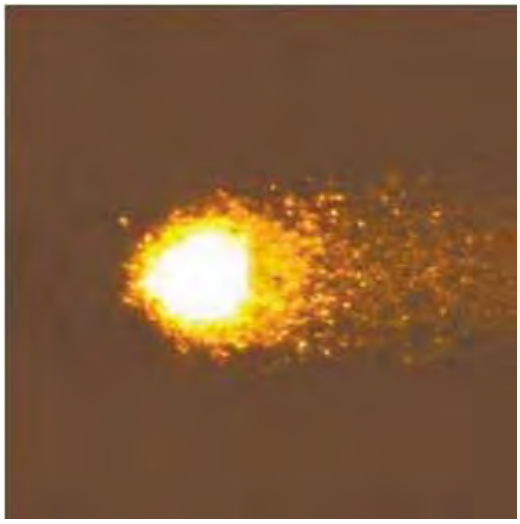

Figura 1. Fotomicrografia mostrando a imagem de célula CHO (nucleóide) com danos no DNA, obtida pela técnica do cometa. Microscopia de fluorescência com coloração brometo de etídio (aumento 400X).

Micronúcleos são fragmentos cromossômicos acêntricos ou cromossomos inteiros que não são incluídos ao núcleo principal durante a telófase da mitose, quando o envelope nuclear é reconstituído ao redor dos cromossomos das células filhas (Fenech, 1997). O teste do micronúcleo foi inicialmente desenvolvido para ser aplicado em células de medula óssea de roedores, *in vivo*, sendo sua aplicação *in vitro* dificultada pela impossibilidade do reconhecimento das células que haviam passado por um ciclo de divisão, etapa sem a qual não há a formação de micronúcleo. Em 1985, Fenech & Morley demonstraram que utlização da citocalasina B (CtB), composto que bloqueia a citocinese sem bloquear a divisão nuclear e a mitose, permitia identificar as células que passaram por um ciclo de divisão, reduzindo, assim, a limitação do teste do micronúcleo para estudos *in vitro* (Figura 2).

A princípio, o teste do micronúcleo com citocalasina B foi proposto para ser aplicado em cultura de linfócitos humanos. No entanto, após adaptações, a técnica vem sendo utilizada com sucesso em vários tipos celulares, não apenas para a detecção de quebras cromossômicas e aneuploidias, mas, também, de outros parâmetros que refletem instabilidade genômica (Fenech & Morley, 1985).

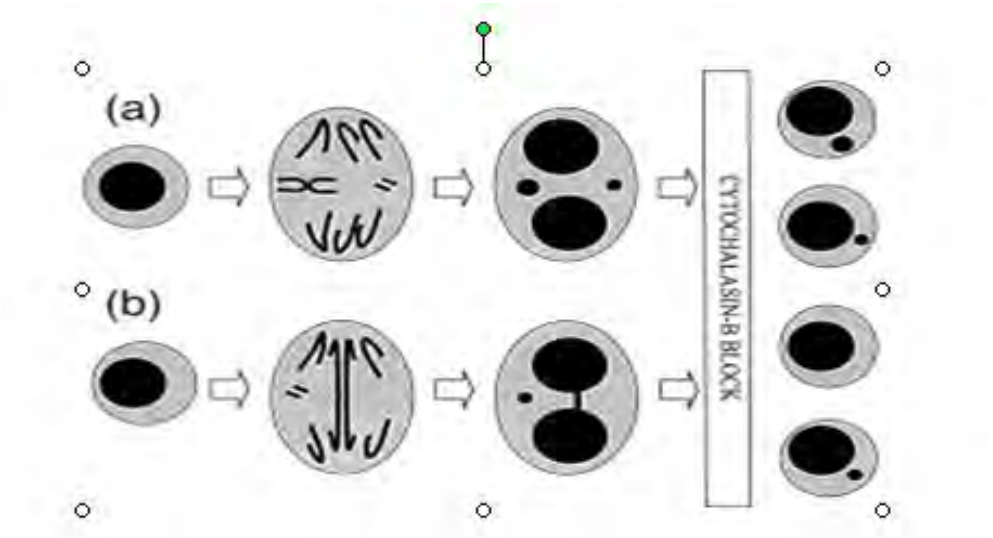

Figura 2. Esquema da formação do micronúcleo em ensaio utilizando a citocalasina B (tirado do livro Mutagênese Ambiental, 1ª ed., 2003).

Com base nas considerações apresentadas, pode-se concluir que a identificação de compostos naturais que atuem na prevenção de danos genotóxicos é de grande relevância para saúde humana, especialmente se forem de fácil obtenção e de baixo custo. Além disso, a utilização de delineamentos experimentais que contribuam para o entendimento do mecanismo de ação de tais compostos torna-se fundamental para que se possam estabelecer estratégias para seu uso em populações humanas.

II. OBJETIVOS

Com base na literatura e em estudos prévios que demonstraram o potencial antimiutagênico e anticarcinogênico da própolis, os objetivos atuais foram:

- avaliar o efeito antigenotóxico e antimutagênico do extrato aquoso bruto da própolis e de suas frações contra danos induzidos no DNA das células CHO pelos mutágenos H_2O_2, 4NQO ou MMS através do teste do cometa e do teste do micronúcleo;
- avaliar o efeito antigenotóxico e antimutagênico das frações do extrato bruto da própolis contra danos induzidos no DNA das células HepG2 pelos mutágenos H_2O_2, 4NQO ou DEN através dos testes do cometa e micronúcleo;
- avaliar diferentes protocolos de tratamento (pré-, simultâneo e pós-), tanto com o extrato bruto, quanto com as frações, visando o entendimento do modo de ação da própolis na prevenção de danos no DNA.

PROTECTIVE EFFECT OF BRAZILIAN PROPOLIS ON CHEMICALY-INDUCED DNA DAMAGE

DANIELLE CRISTINA DE ALMEIDA[1]; RODRIGO OTÁVIO ALVES DE LIMA[1]; MARIA CRISTINA MARCUCCI[2]; DAISY MARIA FÁVERO SALVADORI[1]

[1] – Dept. of Pathology, Botucatu Medical School, UNESP – São Paulo State University – Brazil.

[2] – Dept. of Pharmacology, Bandeirantes University of São Paulo, UNIBAN – Brazil.

Key words: Antimutagenesis, Chemoprevention; DNA damage; Micronucleus; Propolis

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Abstract

Propolis is a complex mixture of plant resins, bee wax, essential oils and pollen, with a highly complex and variable chemical composition, which is intimately related to the region visited by the honeybees. This resin has been used in folk medicine because of its antimicrobial, antiparasitic, antiviral, antiinflamatory, and antioxidant properties. In the present study, we investigated the protective effect of propolis on chemically-induced DNA damage in Chinese hamster ovary cells (CHO), treated with the mutagens hydrogen peroxide (H_2O_2), 4-nitroquinoline 1-oxide (4NQO) and methyl methanesulphonate (MMS). An aqueous extract of crude propolis (AEP: 12.5, 25, 50, 100 or 200 μg/ml), and two of its fractions (F4 and F5: 25, 50 and 100 μg/ml) were tested under three different treatment protocols: pre-, simultaneous and post-treatment with the mutagens. The comet and cytokinesis-block micronucleus (MN) assays were used to evaluate primary damage and chromossome aberrations, respectively. Data showed that both the whole extract (AEP) and its two fractions (F4 and F5) were capable of reducing primary DNA damage, as depicted by the comet assay, but not the frequency of micronucleated cell. In conclusion, our findings demonstrated the effective protective activity of propolis on easily repaired DNA damage induced by chemicals with different mechanisms of genotoxicity.

Introduction

Epidemiological studies have indicate that vegetables, fruits and other natural products can prevent a variety of human genetic-related disease, mainly cancers, protecting cells against the gentoxic action of some free radicals. In particular, propolis has gained popularity because of its several biological and pharmacological activities, such as immunomodulatory, antitumor, antimicrobial, and antiinflamatory (Bankova et al., 2000), as well as the potent antioxidant properties (Matsushige et al. (1995). Propolis is a complex mixture of plant resins, bee wax, essential oils and pollen, with a highly complex and variable chemical composition, which is intimately related to the region visited by the honeybees (Ghisalberti, 1979). At present, about 300 components, phenolic compounds, have been identified, most of them, (Simões et al., 2004).

Antitumor activities of propolis have been documented by various authors throughout the years. Matsuno (1997) has reported the citotoxicity of a clerodane-type diterpene isolated from Brazilian propolis on human hepatocellular carcinoma HuH13 cell line; Bazo et al. (2002) have detected the inhibition of 1,2 dimethylhydrazine (DMH)-induced colon aberrant crypt foci development in rats treated with a hydroalcoholic extract of propolis; Akao et al. (2003) have showed that two cinnamic acids derivatives from propolis exhibited antitumor activity on HL60 cell line. Recently, Chen et al. (2007) have demonstrated that the prenylflavones propolin A and propolin B, two strong antioxidants isolated and characterized from Taiwanese propolis, may trigger apoptosis of A2058 cells through mitochondria-dependent pathways. Additionally, Alves de Lima et al. (2005) have demonstrated that an aqueous extract of crude Brazilian propolis was effective in protecting against DNA damage induced by DMH in colon cells of Wistar rats. On the other hand, recently, Resende et al. (2007) have reported the antimutagenic activity of *Baccharis dracunculifolia* on doxorubicin-induced micronucleus *in vivo*. *Baccharis dracunculifolia* is the main botanical source of Brazilian propolis and its chemical composition is very similar to those of green propolis.

In the present study, we evaluated the ability of an aqueous extract of crude propolis and two of its fractions (F4 and F5) for preventing chemically induced primary DNA damage as depicted by the single cell gel electrophoresis (comet) assay, and

chromosome aberrations by the cytokinesis-block micronucleus assay in Chinese Hamster Ovary (CHO) cells.

Materials and Methods

Aqueous Extract of Propolis

Propolis, produced by *Apis mellifera L.*, was collected from Chaves Farm (Itapecerica, State of Minas Gerais, Brazil). An aqueous extract of propolis (AEP) was prepared as previously described by Alves de Lima et al. (2005), and used in the experiments at concentrations of 12.5, 25, 50, 100 and 200 µg/ml. Part of this extract was lyophilized, the powder ressuspended in 10% methanol (V/V) and introduced into a Sephadex LH-20 (G&E) column (40x200mm), to be chemically fractioned. The two fractions obtained (F4 and F5), 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 distillated 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 (Tables 1 and 2). Each fraction was used at concentrations of 25, 50 and 100 µg/ml.

Table 1. Chemical profile of fraction 4 (F4)

Compound	**Concentration (µg/g)**	**Concentration (%)**
3-[4-hydroxy-3-(oxobutenyl)-phenylacrilic acid	78.84	0.008
3-prenyl-4-hydroxycinnamic acid	186.43	0.019
caffeic acid	68.17	0.007
p-coumaric acid	153.74	0.015
(E)-3-{4-hydroxy-3-[(E)-4-(2.3)-dihydrocinamoiloxy-3-methyl-2-butenyl]-5-prenylphenyl-2-propenoic acid	40.79	0.004
3,4-dihydroxy-5-prenylcinnamic acid	81.26	0.008
Total	**609.25**	**0.061**

Table 2. Chemical profile of fraction 5 (F5)

Compound	Concentration (µg/g)	Concentration (%)
2,2-dimethyl-6-carboxyetenil-2H-1-benzopirane	362.19	0.036
(E)-3-{4-hydroxy-3-[(E)-4-(2.3)-dihydrocinamoiloxy-3-methyl-2-butenyl]-5-prenylphenyl-2-propenoic acid	42.33	0.042
3,4-dihydroxy-5-prenylcinnamic acid	1783.54	0.178
3,5-diprenyl-4-hydroxycinnamic acid (Artepillin C)	301.18	0.030
3-prenyl-4-(2-methylpropionyloxy)cinamic acid	291.58	0.029
3-prenyl-4-hydroxycinnamic acid	301.18	0.030
6-propenoic-2.2-dimethyl-8-prenyl-2H-1-benzopirane acid	363.99	0.036
caffeic acid	705.14	0.070
p-coumaric acid	620.52	0.062
Total	**7289.40**	**0.729**

Chemicals

The mutagens methyl methanesulphonate (MMS – 80 µg/ mL; Sigma – USA), 4-nitroquinoline 1-oxide (4NQO – 0.01 µM; 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 medium just before use. The selected concentrations were obtained in preliminary experiments. or were based on literature.

Cell lines and culture conditions

Chinese hamster ovary cells (CHO K-1) were grown until confluence in 75 cm^2 tissue culture flasks. at 37° C and 5% CO_2 atmosphere, using Ham´s F10 medium supplemented with 10% fetal calf serum (Cultilab – Brazil) and antibiotics (penicillin 100 U/mL and streptomycin 0.1 mg/ mL; Cultilab - Brazil). Confluent cells were detached with 0.15% trypsin (Cultilab – Brazil) for 5 min and. 0.2 x 10^6 cells/well in 24-well plates. or 1 x 10^6 cells/plastic Petri dish (94 mm). were seeded for the comet assay or micronucleus assay, respectively.

Cell viability, using Trypan Blue, was checked after all treatments and it was always higher than 75% (data not showed).

Comet (Single Cell Gel Electrophoresis) Assay – Experimental Design

Three treatment protocols were used, and always performed in duplicate to ensure the reproducibility.

Pre-treatment: twenty-four hours after seeding, medium was removed and cells incubated with AEP at concentrations of 12.5. 25. 50, 100 and 200 µg/mL, or with F4 or F5 (25. 50 or 100 µg/mL) in fresh medium, for 24 hours. Then, cells were washed with PBS and treated with 4NQO, H_2O_2, or MMS for 5 minutes. Further, cells were washed twice with PBS. Trypsinized, centrifuged at 180 *g* for 3 min and ressuspended into 100 µL fresh medium for the comet assay.

Simultaneous treatment: twenty-four hours after seeding, medium was removed and cells were simultaneously treated with AEP or with one of the fractions. and with one of the mutagens (4NQO, H_2O_2 or MMS) for 5 minutes. Then, cells were washed twice with PBS. Trypsinized, centrifuged at 180 *g* for 3 min, and ressuspended into 100 µL fresh medium for comet assay.

Post-treatment: twenty-four hours after seeding, medium was removed and cells treated with the mutagen 4NQO, H_2O_2 or MMS for 5 minutes. Then, cells were washed with PBS and incubated with AEP or with fractions in fresh medium for 24 hours. After this period, cells were washed twice with PBS. Trypsinized, centrifuged at 180 *g* for 3 min and ressuspended into 100 µL fresh medium for the comet assay.

The comet assay was performed according to Singh et al. (1988) and Tice et al. (1991). In brief, a volume of 10 µL of the cells 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. 10mM 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 into a horizontal electrophoresis chamber and left immersed in alkaline solution (300mM NaOH. 1mM EDTA. pH 13) for 20 min. Electrophoresis was carried out for 20 minutes. at 25V and 300 mA. After electrophoresis, slides were neutralized with 0.4< Tris-HCL (pH 7.5) solution, fixed with 100% ethanol, and stored until analysis. After stained with Sybr Gold (1:10.000 - Invitrogen), 50 randomly selected “nucleoids” per culture were

examined at 200X 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 intensity (% of migrated DNA in the tail).

Citokinesis-block micronucleus (CBMN) assay – Experimental Design

The concentrations of AEP fractions and mutagens were the same used for the comet assay.

. *Pre-treatment:* twenty-four hours after seeding, medium was removed and cells incubated with AEP or with one of the fractions in fresh medium, for 24 hours. Then, cells were washed with PBS. and treated with MMS, 4NQO or H_2O_2 for 5minutes. Further, cells were washed twice with PBS and incubated into a fresh medium containing cytochalasin B (final concentration 1.2 µg/mL) for 24 hours.

Simultaneous treatment: twenty-four hours after seeding, medium was removed and cells simultaneously treated with the AEP or its fractions, and with one of the mutagens (4NQO, H_2O_2 or MMS) for 5 minutes. 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, medium was removed and cells treated with one of the mutagens for 5 minutes. After washing with PBS, cells were incubated with AEP or fractions in fresh medium for 24 hours. Then, cells were washed twice with PBS, and incubated for 24 hours into a fresh medium containing cytochalasin B.

Each protocol was performed in duplicate to ensure reproducibility (Fenech, 2000). After harvesting, cells were treated with cold hypotonic solution (KCl 0.075 M), centrifuged (800 rpm. 8 min), and fixed with acetic acid/methanol (1:3) solution. Air-dried slides were prepared and stained with 5% Giemsa solution for 7 min. A total of 1000 binucleated cells (BNC) were analyzed from each culture.

Statistical Analysis

It was adjusted a model following a completely randomized design, using a general linear model with gamma distribution. The multiple comparisons were done by DIFF option from PROC GENMOD from the SAS program for Windows. v- 9.1.3.

Results

Tables 3, 4, and 5 show the level of DNA damage (*tail intensity*) in CHO cells treated with H_2O_2, 4NQO or MMS, and with AEP at five concentrations (12.5, 25, 50, 100, and 200 µg/ml). Data showed that in pre- and pos-treatment protocols all the concentrations of AEP reduced the genotoxicity of the three mutagens. When AEP and mutagens were used simultaneously, different effects were observed: 1) clear chemopreventive activity was detected against 4NQO genotoxicity, with all the concentrations decreasing the level of DNA damage (Table 4); 2) both the highest and lowest. or the highest concentrations of AEP decreased H_2O_2 and MMS genotoxicity, respectively (Tables 3 and 5); 3) three of the AEP concentrations increased MMS-induced primary DNA damage (Table 5).

The levels of DNA damage (tail intensity) in cells treated with one of the AEP fractions (F4 or F5) and with mutagens (H2O2, 4NQO or MMS) are presented in Tables 4. 5. and 6. Data showed that in pre- and post-treatment protocols both fractions reduced H_2O_2-induced primary DNA damage, although at 100 µg/ml. in the pre-treatment protocol. F4 was more effective than F5 (Table 6). Similar results were observed for treatments with F4 and F5, and 4NQO and MMS, where the three protocols fractions were capable of reducing DNA damage, but in different ways (Tables 7 and 8)

The frequency of micronucleated cells (MNC) after exposure to the mutagens (H_2O_2, 4NQO or MMS), and to the AEP (12.5, 25, 50, 100, and 200 µg/ml) or its fractions (25. 50 and 100 µg/ml) are presented in Tables 9-14. Data showed no antimutagenic activity, both for AEP and its fractions, except when AEP at concentration of 50 µg/ml was used before or after H_2O_2 (Table 9).

Table 3. DNA damage (*tail intensity)* in CHO cells treated with hydrogen peroxide and with aqueous extract of propolis (AEP)

AEP (μg/ml)	Treatment	Tail Intensity Mean ± SD
12.5	C-	9.3 ± 10.9
	C+	33.9 ± 16.1#
	Pre-	17.5 ± 14.9*
	Simultaneous	7.7 ± 10.5*
	Post-	17.9 ± 15.7*
25	C-	6.8 ± 8.6
	C+	39.6 ± 19.0#
	Pre-	18.1 ± 18.3*
	Simultaneous	16.6 ± 18.3
	Post-	6.8 ± 8.2*
50	C-	11.2 ± 11.6
	C+	28.7 ± 16.1#
	Pre-	15.4 ± 13.8*
	Simultaneous	16.3 ± 15.1
	Post-	17.6 ± 14.1*
100	C-	7.6 ± 9.8
	C+	34.4 ± 17.1#
	Pre-	11.3 ± 13.0*
	Simultaneous	10.3 ± 12.7
	Post-	8.5 ± 9.4*
200	C-	9.1 ± 10.7
	C+	27.8 ± 15.0#
	Pre-	10.0 ± 10.4*
	Simultaneous	9.8 ± 11.0*
	Post-	7.0 ± 9.8*

C-: negative control; C+: positive control (H_2O_2 – 0.6 mM); pre-treatment: AEP before H_2O_2; post-treatment: AEP after H_2O_2

# $p<0.05$, compared to C-

* $p< 0.05$, compared to C+

Table 4. DNA damage (*tail intensity*) in CHO cells treated with 4-nitroquinoline 1-oxide and with aqueous extract of proplis (AEP)

AEP (μg/ml)	Treatment	Tail Intensity Mean ± SD
12.5	C-	2.1 ± 1.7
	C+	36.1 ± 16.0#
	Pre-	12.5 ± 11.4*
	Simultaneous	8.4 ± 9.5*
	Post-	8.7 ± 10.6*
25	C-	2.1 ± 1.7
	C+	31.5 ± 13.4#
	Pre-	14.1 ± 13.1*
	Simultaneous	10.2 ± 11.1*
	Post-	9.1 ± 9.4*
50	C-	7.6 ± 10.8
	C+	19.6 ± 16.6#
	Pre-	12.1 ± 13.7*
	Simultaneous	13.7 ± 14.5*
	Post-	10.3 ± 11.7*
100	C-	10.2 ± 12.8
	C+	25.1 ± 17.1#
	Pre-	16.1 ± 14.9*
	Simultaneous	17.6 ± 15.1*
	Post-	13.3 ± 13.5*
200	C-	6.5 ± 9.8
	C+	32.2 ± 19.0#
	Pre-	10.9 ± 13.1*
	Simultaneous	10.1 ± 9.7*
	Post-	17.1 ± 16.2*

C-: negative control; C+: positive control (4NQO – 0.01μM); pre-treatment: AEP before 4NQO; post-treatment: AEP after 4NQO

# $p<0.05$, compared to C-

* $p<0.05$, compared to C+

Table 5. DNA damage (*tail intensity*) in CHO cells treated with methyl methanesulfonate and with aqueous extract of propolis (AEP)

AEP (μg/ml)	Treatment	Tail Intensity Mean ± SD
12.5	C-	8.6 ± 11.6
	C+	51.0 ± 14.9#
	Pre-	12.1 ± 10.1*
	Simultaneous	76.2 ± 10.2*
	Post-	15.8 ± 12.1 *
25	C-	9.6 ± 11.0
	C+	63.2 ± 12.6#
	Pre-	8.7 ± 10.7*
	Simultaneous	71.6 ± 10.9*
	Post-	14.6 ± 12.4*
50	C-	6.7 ± 7.4
	C+	53.8 ± 13.7#
	Pre-	24.1 ± 13.9*
	Simultaneous	54.1 ± 13.4
	Post-	22.7 ± 16.9*
100	C-	5.8 ± 7.4
	C+	68.1 ± 16.4#
	Pre-	53.6 ± 26.8*
	Simultaneous	69.7 ± 12.6
	Post-	20.1 ± 14.8 *
200	C-	9.3 ± 10.6
	C+	73.2 ± 16.6#
	Pre-	21.7 ± 12.8*
	Simultaneous	59.2 ± 15.3*
	Post-	20.2 ±13.2*

C-: negative control; C+: positive control (MMS – 80 μg/ml); pre-treatment: AEP before MMS; post-treatment: AEP after MMS

# $p<0.05$, compared to C-

* $p< 0.05$, compared to C+

Table 6. DNA damage (*tail intensity*) in CHO cells treated with hydrogen peroxide and with fractions (F4 or F5) of the aqueous extract of propolis

Treatment	Fraction/concentration (μg/ml)	*Tail Intensity* Mean ± SD
PRE-	C-	2.3 ± 2.3
	C+	15.8 ± 12.0#
	F4/25	14.5 ± 14.9
	F4/50	12.1 ± 12.3
	F4/100	7.2 ± 10.4* Ψ
	F5/25	12.4 ± 13.0
	F5/50	7.8 ± 8.9*
	F5/100	10.0 ± 10.5*
SIMULTANEOUS	C-	7.2 ± 6.9
	C+	20.8 ± 18.5#
	F4/25	16.5 ± 13.2
	F4/50	22.5 ± 16.6
	F4/100	16.0 ± 13.5
	F5/25	19.7 ± 17.1
	F5/50	17.3 ± 16.9
	F5/100	26.3 ± 21.0
POST-	C-	1.5 ± 1.4
	C+	21.1 ± 8.7#
	F4/25	9.2 ± 9.0*
	F4/50	8.2 ± 8.5*
	F4/100	8.3 ± 8.1*
	F5/25	9.3 ± 9.9*
	F5/50	7.4 ± 8.2*
	F5/100	6.9 ± 9.0*

C-: negative control; C+: positive control (H_2O_2 - 0.6 mM); pre- treatament: F4 or F5 before H_2O_2; post-treatament: F4 ou F5 after H_2O_2;

# $p<0.05$, compared to C-

* $p< 0.05$, compared to C+

Ψ F4 ≠ F5 at the same concentration ($p<0.05$).

Table 7. DNA damage (*tail intensity*) in CHO cells treated with 4-nitroquinoline 1-oxide and with fractions (F4 or F5) of the aqueous extract of propolis

Treatment	Fraction/concentration (μg/ml)	*Tail Intensity* Mean ± SD
PRE-	C-	1.7 ± 1.1
	C+	28.3 ± 11.7#
	F4/25	12.1 ± 12.3* Ψ
	F4/50	8.6 ± 10.2*
	F4/100	9.9 ± 11.6*
	F5/25	7.6 ± 9.0*
	F5/50	8.6 ± 9.3*
	F5/100	12.7 ± 14.4*
SIMULTANEOUS	C-	1.7 ± 1.2
	C+	34.6 ± 15.8#
	F4/25	12.1 ± 13.7*
	F4/50	14.0 ± 13.1* Ψ
	F4/100	20.5 ± 17.4*
	F5/25	14.9 ± 15.2*
	F5/50	10.0 ± 10.5*
	F5/100	15.8 ± 15.7*
POST-	C-	1.6 ± 4.3
	C+	24.6 ± 7.8#
	F4/25	10.4 ± 10.6*
	F4/50	8.7 ± 9.9*
	F4/100	6.9 ± 8.5*
	F5/25	7.8 ± 9.7*
	F5/50	8.1 ± 11.1*
	F5/100	6.1 ± 7.9*

C-: negative control; C+: positive control (4NQO – 0.01 μM); pre-treatament: F4 or F5 before 4 NQO; post-treatment: F4 or F5 after 4 NQO

# $p<0.05$, compared to C-

* $p< 0.05$, compared to C+

Ψ F4 ≠ F5 at the same concentration ($p<0.05$).

Table 8. DNA damage (*tail intensity*) in CHO cells treated with methyl methanesulphonate and with fractions (F4 or F5) of the aqueous extract of propolis

Treatment	Fraction/concentration (μg/ml)	Tail Intensity Mean ± SD
PRE-	C-	1.8 ± 1.5
	C+	58.1 ± 22.6#
	F4/25	63.9 ± 23.5
	F4/50	59.7 ± 26.3
	F4/100	58.1 ± 25.3
	F5/25	37.2 ± 27.7*
	F5/50	52.4 ± 27.3
	F5/100	65.2 ± 15.3
SIMULTANEOUS	C-	1.9 ± 2.0
	C+	77.5 ± 9.7#
	F4/25	63.4 ± 15.7*
	F4/50	59.2 ± 19.9* Ψ
	F4/100	62.5 ± 14.5* Ψ
	F5/25	64.6 ± 16.0*
	F5/50	70.8 ± 16.1*
	F5/100	68.8 ± 19.8*
POST-	C-	2.2 ± 2.7
	C+	33.0 ± 12.1#
	F4/25	21.4 ± 17.8* Ψ
	F4/50	15.6 ± 14.8* Ψ
	F4/100	17.7 ± 18.7*
	F5/25	9.1 ± 8.5*
	F5/50	9.2 ± 10.7*
	F5/100	13.7 ± 12.7*

C-: negative control; C+: positive control (MMS - 80 μg/ml); pre-treatament: F4 or F5 before MMS; post-treatment: F4 or F5 after MMS

# $p<0.05$, compared to C-

* $p< 0.05$, compared to C+

Ψ F4 ≠ F5 at the same concentration ($p<0.05$).

Table 9. Frequency of micronucleated cells (MNC) after three treatment protocols with hydrogen peroxide and aqueous extract of propolis (AEP)

AEP (μg/ml)	Treatment	Number of MNC	MNC/1000 BNC Mean ± SD
12.5	C-	6	3.0 ± 2.8
	C+	22	11.0 ± 1.4#
	Pre-	16	8.0 ± 2.8
	Simultaneous	17	8.5 ± 0.7
	Post-	18	9.0 ± 1.7
25	C-	5	2.5 ± 0.7
	C+	19	9.5 ± 0.7#
	Pre-	17	8.5 ± 0.7
	Simultaneous	15	7.5 ± 2.1
	Post-	17	8.5 ± 3.5
50	C-	9	4.5 ± 0.7
	C+	25	12.5 ± 3.5#
	Pre-	9	4.5 ± 2.1*
	Simultaneous	16	8.0 ± 1.4
	Post-	12	6.0 ± 1.4*
100	C-	4	2.0 ± 0
	C+	20	10.0 ± 2.8#
	Pre-	18	9.0 ± 1.4
	Simultaneous	19	9.5 ± 2.1
	Post-	18	9.0 ± 0
200	C-	5	2.5 ± 0.7
	C+	22	11.0 ± 0#
	Pre-	14	7.0 ± 1.4
	Simultaneous	16	8.0 ± 1.4
	Post-	17	8.5 ± 2.0

C-: negative control; C+: positive control (H_2O_2 - 0.6 mM); pre-treatment: AEP before H_2O_2; post-treatment: AEP after H_2O_2; BNC: binucleated cell.
# $p<0.05$, compared to C-
* $p< 0.05$, compared to C+

Table 10. Frequency of micronucleated cells (MNC) after three treatment protocols with 4-nitroquinoline 1-oxide and aqueous extract of propolis (AEP)

AEP (μg/ml)	Treatment	Number of MNC	MNC/1000 BNC Mean ± SD
12.5	C-	3	1.5 ± 0.7
	C+	18	9.0 ± 1.4#
	Pre-	14	7.0 ± 2.8
	Simultaneous	15	7.5 ± 0.7
	Post-	16	8.0 ± 0
25	C-	4	2.0 ± 1.4
	C+	21	10.5 ± 2.1#
	Pre-	19	9.5 ± 0.7
	Simultaneous	18	9.0 ± 1.4
	Post-	16	8.0 ± 0
50	C-	5	2.5 ± 0.7
	C+	21	10.5 ± 2.1#
	Pre-	15	7.5 ± 0.7
	Simultaneous	18	9.0 ± 0
	Post-	17	8.5 ± 2.1
100	C-	4	2.0 ± 0
	C+	19	9.5 ± 2.1#
	Pre-	15	7.5 ± 0.7
	Simultaneous	16	8.0 ± 1.4
	Post-	17	8.5 ± 0.7
200	C-	5	2.5 ± 0.7
	C+	20	10.0 ± 1.4#
	Pre-	18	9.0 ± 1.4
	Simultaneous	16	8.0 ± 0
	Post-	16	8.0 ± 1.4

C-: negative control; C+: positive control (4NQO – 0.01μM); pré-treatment: AEP before 4NQO; post-treatment: AEP after 4NQO; BNC: binucleated cell.
# $p<0.05$, compared to C-

Table 11. Frequency of micronucleated cells (MNC) after three treatment protocols with methyl methanesulphonate and aqueous extract of propolis (AEP)

AEP (μg/ml)	Treatment	Number of MNC	MNC/1000 BNC Mean ± SD
12.5	C-	4	2.0 ± 0
	C+	15	7.5 ± 0.7#
	Pre-	06	3.0 ± 1.4
	Simultaneous	10	5.0 ± 0
	Post-	09	4.5 ± 0.7
25	C-	3	1.5 ± 0.7
	C+	11	5.5 ± 0.7#
	Pre-	06	3.0 ± 0
	Simultaneous	06	3.0 ± 0
	Post-	07	3.5 ± 0.7
50	C-	3	1.5 ± 0.7
	C+	11	5.5 ± 0.7#
	Pre-	7	3.5 ± 0.7
	Simultaneous	6	3.0 ± 0
	Post-	7	3.5 ± 0.7
100	C-	4	2.0 ± 0
	C+	13	6.5 ± 0.7#
	Pre-	8	4.0 ± 1.4
	Simultaneous	7	3.5 ± 0.7
	Post-	8	4.0 ± 0
200	C-	4	2.0 ± 0
	C+	13	6.5 ± 0.7#
	Pre-	7	3.5 ± 0.7
	Simultaneous	10	5.0 ± 0
	Post-	6	3.0 ± 0

C-: negative control; C+: positive control (MMS – 80 μg/ml); pré-treatment: AEP before MMS; post-treatment: AEP after MMS; BNC: binucleated cell.
# $p<0.05$, compared to C-

Table 12. Frequency of micronucleated cells (MNC) after three treatment protocols with hydrogen peroxide and the two fractions (F4 and F5) of aqueous extract of propolis (AEP)

Treatment	Fractions/concentration µg/ml	Number of MNC	MNC/1000 BNC Mean ± SD
PRE-	C-	4	2.0 ± 0
	C+	18	9.0 ± 0#
	F4/25	10	5.0 ± 0
	F4/50	8	4.0 ± 0
	F4/100	9	4.5 ± 0.7
	F5/25	11	5.5 ± 2.1
	F5/50	11	5.5 ± 0.7
	F5/100	13	6.5 ± 0.7
SIMULTANEOUS	C-	5	2.5 ± 0.7
	C+	16	8.0 ± 1.4#
	F4/25	11	5.5 ± 0.7
	F4/50	9	4.5 ± 0.7
	F4/100	9	4.5 ± 2.1
	F5/25	10	5.0 ± 1.4
	F5/50	11	5.5 ± 2.1
	F5/100	12	6.0 ± 1.4
POST-	C-	4	2.0 ± 0
	C+	17	8.5 ± 2.1#
	F4/25	12	6.0 ± 0
	F4/50	12	6.0 ± 1.4
	F4/100	12	6.0 ± 1.4
	F5/25	11	5.5 ± 0.7
	F5/50	15	7.5 ± 0.7
	F5/100	12	6.0 ± 1.4

C-: negative control; C+: positive control (H_2O_2 - 0.6 mM); pre-treatament: F4 or F5 before H_2O_2; post-treatament: F4 or F5 after H_2O_2; BNC: binucleated cell.
# $p<0.05$, compared to C-

Table 13. Frequency of micronucleated cells (MNC) after three treatment protocols with 4-nitroquinoline 1-oxide and the two fractions (F4 and F5) of aqueous extract of propolis (AEP)

Treatment	Fractions/Concentration (µg/ml)	Number of MNC	MNC/1000 BNC Mean ± DP
PRE-	C-	6	3.0 ± 0
	C+	21	10.5 ± 0.7#
	F4/25	16	8.0 ± 1.4
	F4/50	13	6.5 ± 0.7
	F4/100	15	7.5 ± 0.7
	F5/25	13	6.5 ± 0.7
	F5/50	15	7.5 ± 2.1
	F5/100	15	7.5 ± 0.7
SIMULTANEOUS	C-	6	3.0 ± 1.4
	C+	18	9.0 ± 1.4#
	F4/25	12	6.0 ± 2.8
	F4/50	8	4.0 ± 1.4
	F4/100	16	8.0 ± 1.4
	F5/25	14	7.0 ± 1.4
	F5/50	14	7.0 ± 1.4
	F5/100	15	7.5 ± 0.7
POST-	C-	7	3.5 ± 2.1
	C+	25	12.5 ± 0.7#
	F4/25	19	9.5 ± 2.1
	F4/50	19	9.5 ± 2.1
	F4/100	21	10.5 ± 0.7
	F5/25	21	10.5 ± 2.1
	F5/50	22	11.0 ± 2.8
	F5/100	21	10.5 ± 0.7

C-: negative control; C+: positive control (4 NQO – 0.01 µM); pre-treatament: F4 or F5 before 4 NQO; post-treatament: F4 or F5 after 4 NQO; BNC: binucleated cell.
# $p<0.05$, compared to C-

Table 14. Frequency of micronucleated cells (MNC) after three treatment protocols with methyl methanesulphonate and the two fractions (F4 and F5) of aqueous extract of propolis (AEP)

Treatment	Fraction/concentration (µg/ml)	Number of MNC	MNC/1000 BNC Mean ± SD
PRE-	C-	7	3.5 ± 2.1
	C+	25	12.5 ± 0.7#
	F4/25	19	9.5 ± 2.1
	F4/50	19	9.5 ± 2.1
	F4/100	21	10.5 ± 0.7
	F5/25	21	10.5 ± 2.1
	F5/50	22	11.0 ± 2.8
	F5/100	21	10.5 ± 0.7
SIMULTANEOUS	C-	5	2.5 ± 0.7
	C+	22	11.0 ± 2.8#
	F4/25	20	10.0 ± 0
	F4/50	19	9.5 ± 2.1
	F4/100	17	8.5 ± 2.1
	F5/25	21	10.5 ± 2.1
	F5/50	19	9.5 ± 0.7
	F5/100	18	9.0 ± 2.8
POST-	C-	4	2.0 ± 0
	C+	19	9.5 ± 0.7#
	F4/25	16	8.0 ± 1.4
	F4/50	17	8.5 ± 0.7
	F4/100	15	7.5 ± 0.7
	F5/25	16	8.0 ± 0
	F5/50	18	9.0 ± 0
	F5/100	17	8.5 ± 2.1

C-: negative control; C+: positive control (MMS - 80 µg/ml); pre-treatament: F4 or F5 before MMS; post-treatament: F4 or F5 after MMS; BNC: binucleated cell.
# $p<0.05$, compared to C-

Discussion

In 2003, Aruoma suggested that a range of chronic degenerative diseases, including not only cancer, but also cardiovascular and other genetic-related diseases. may. at least in part, result from cellular damage, through exposure to reactive oxygen species (ROS). Based on this information, we investigated the effect of propolis on chemically-induced primary DNA damage and chromosome aberrations (micronucleus), since mutations are closely associated with the initial steps of those pathologies.

It is well known that propolis and its constituents have a high antioxidant activity. Matsushige et al. (1995) have studied 5 different samples of Brazilian propolis and have observed strong antioxidant effects of all of them. The antioxidant activities of propolis, and its ability to scavenger reactive oxygen species, have also been investigated by Simões et al. (2004), who have demonstrated the biological effects of different extracts and fractions of green propolis. Kismet et al. (2007) have showed that the antioxidant properties of propolis play an important role in their potent hepatoprotective activity in chemically-induced liver injury models. Ahn et al. (2009) have studied a correlation between antiangiogenic and antioxidant activities of various components from propolis and have observed that artepillin C, caffeic acid, galangin, kaempferol, and quercetin might represent a new class of dietary-derived antioxidative compounds with antiangiogenic activities. However, there is little information available in order to confirm which components are responsible for the proposed effects of this resin (Shimizu et al., 2004). Kumazawa et al. (2004) have analyzed the chemical composition of a Brazilian green propolis and they have observed a high concentration of triterpenoids and prenylated derivatives of p-coumaric acids. Propolis *in natura* has 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen and other substances (Burdock, 1998). Herein, we firstly tested an aqueous extract of crude propolis, because this is one of the main solution commonly used as medicine. Then, we isolated two of its fractions (F4 and F5), trying to identify and differentiate the mechanisms by which the main components of propolis would be responsible for its chemopreventive activity. The chemical profile of the two fractions showed that both have basically the same compounds, and their major constituents are cinnamic acids and caffeic acids, although in different concentrations. Therefore, once they both showed protective effects, we cannot attribute this activity to specific identified compounds.

Despite of some more constituents were identified in F5, and others were in higher concentrations, in some situations F4 showed to be more effective than F5.

In the present study, xenobiotics with different mechanisms of mutagenicity were used in order to better understand the chemopreventive potencial of propolis. Hydrogen peroxide is one of the most known mutagens with ability to interact with DNA through highly reactive oxygen and radical species, causing extensive oxidative damage (Ratnam et al., 2006). Similarly, 4NQO, an alkylating compound and potent mutagen, is able to induce DNA oxidative lesions, but also DNA adducts, leading to single strand breaks, incomplete repair and alkali-labile sites (Arima et al., 2006; Kim et al., 2006). On the other hand, methyl methanesulphonate (MMS), is a DNA alkylating agent that has been used for many years as a DNA damaging agent to induce mutagenesis and in recombination experiments. MMS mainly modifies guanine (to 7-methylguanine) and adenine (to 3-methyladenine), causing base mispairing and replication blocks, respectively (Beranek, 1990).

Our data showed that both AEP and its fractions clearly reduced primary DNA damage caused by H_2O_2 and 4NQO. Similarly, using an *in vivo* experimental model, Kanbur et al. (2008) have reported that propolis exhibits antiradical and antioxidant effects against damage caused by propetamphos. a potent pesticide. However, we did not detected protective effect on the frequency of micronucleated cells, except when AEP. at concentration of 50µg/ml. was used simultaneously to H_2O_2. The lack of propolis effective activity on chemically induced-chromosome damage (micronucleus) suggests that this bee product mainly prevent those DNA damage easily repaired, and which are not fixed as DNA mutation. Similar result was observed when the effect of AEP and of its fractions were checked against the alkylating mutagen MMS. It was noticed that AEP reduced primary DNA damage in pre- and post-treatments, while F4 and F5 fractions were more effective at simultaneous and post-treatment protocols. Nevertheless, it must be emphasized that, when used simultaneously to MMS, AEP, at the concentrations of 12.5, 25, and 100 µg/ml, instead of protecting DNA, it increased the level of damage induced by MMS. Moreover, no chemopreventive effect was detected when the end-point of genetic damage was the frequency of micronucleated cell.

According to Tavares et al. (2006), propolis is able to show characteristics of a "Janus" compound (a term used to designate substances that show both genotoxic and antigenotoxic effects depending on the experimental conditions used; von Borstel and

Higgins, 1998). In their study, these authors have observed that propolis was genotoxic at higher concentrations, while at lower concentrations it displayed chemopreventive effect on doxorubicin-induced mutagenicity in CHO cells (Tavares et al., 2006). Furthermore, it has also been reported that flavonoids, which are present in propolis, act mainly by its capacity of scavenging free radicals (Pietta, 2000), although their pro-oxidant abilities leading to DNA damage have also been described (Halliwell et al., 2005).

In conclusion, the present study showed that both aqueous extract of propolis and its fractions (F4 and F5) presented protective activity against chemically-induced genetic damage arisen from oxidative stress and base alkylation. Although this chemopreventive effect was not dependent on the treatment protocol or concentrations used, propolis showed to be effective only on primary damage, as depicted by the comet assay. Therefore, before establishing chemopreventive strategies using this bee product, more studies are necessary to better understand under which conditions propolis may prevent genetic instability and promotes health.

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PROPOLIS ACTIVITY AGAINST CHEMICALLY INDUCED DNA DAMAGE IN A DRUG-METABOLIZING (HEPG2) CELL LINE

DANIELLE CRISTINA DE ALMEIDA[1]; RODRIGO OTÁVIO ALVES DE LIMA[1]; MARIA CRISTINA MARCUCCI[2]; DAISY MARIA FÁVERO SALVADORI[1]

1 – Dept. of Pathology, Botucatu Medical School, UNESP – São Paulo State University – Brazil
2 – Dept. of Pharmacology, Bandeirantes University of São Paulo, UNIBAN – Brazil

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Abstract

Propolis is a resinous hive product collected by honeybees from various plant sources. It is a popular folk medicine with a broad spectrum of biological and pharmacological activities. Several studies have demonstrated the capability of propolis to prevent chemically-induced DNA damage. In the present study, we investigated the antigenotoxic/antimutagenic effects of propolis in a drug-metabolizing cell line (HepG2) treated with hydrogen peroxide (H_2O_2), 4-nitroquinoline-1-oxide (4NQO) or n-nitrosodiethylamine (DEN). Two chemical fractions (F4 and F5) of an aqueous extract of propolis (at 25, 50 and 100 μg/ml) were tested under three different protocols: pre-, simultaneously, and post-treatment with the mutagens. The comet and cytokinesis-block micronucleus (MN) assays were used to evaluate primary DNA damage and chromosome aberrations, respectively. Data showed that both fractions were capable for reducing primary DNA damage induced by H_2O_2, 4NQO, and DEN, although this chemopreventive activity was dependent on the treatment protocol used. On the other hand, significant decrease of micronucleated cells was detected only when propolis was used simultaneously to DEN. In conclusion, our findings confirmed the effective activity of propolis components against chemically-induced DNA damage. Further studies should be conducted to better understand the mechanisms by which this bee product exerts its chemopreventive activity.

Introduction

Propolis is a complex resinous bee product with a physical appearance that varies widely, depending on various factors. Its color may be cream, yellow, green, light or dark brown. Some samples have a hard friable texture, while other samples may be elastic and gummy (Ghisalberti, 1979). Propolis is a complex mixture of plant resins, bee wax, essential oils and pollen, and it is considered a natural remedy, which has been used since ancient times (Nakajima et al., 2007). This honeybee resin has been widely recommended by phytotherapists due to its anti-inflammatory (Marcucci, 1995), hepatoprotective (Liu et al., 2004), antimicrobial (Orsi et al., 2005), and antioxidant properties (Shimazawa et al., 2005). A hepatoprotective effect of Cuban propolis was described by González et al. (1994) using a mouse model of acute hepatotoxicity induced by a high oral dose of paracetamol. Additionally, Suzuki et al. (1996) have also demonstrated an anti-tumor effect of immunologically active fractions obtained from Brazilian propolis on mice Ehrlich carcinoma.

Several studies have confirmed that the pharmacological properties of propolis are mainly related to the presence of flavonoids, and with their action against free radicals (Arvouet-Grand et al., 1994; Mirzoeva & Calder, 1996; Vynograd et al., 2000). Flavonoids are metabolized into various types of phenolic acids, many of which are also able to inhibit the action of free radicals (Pietta, 2000). Kimoto et al. (2000) have shown that artepillin C, a compound found in large amounts in green propolis, significantly reduced the formation of lipid peroxidases, reducing renal toxicity and consequently the incidence of renal adenocarcinoma. These activities have been intrinsically related to the propolis concentration of flavonoids, since the blockage of lipid peroxidases is due to these compounds (Shimizu et al., 2004). Sun et al. (2000) have also described a high concentration of vitamin C in kidney, stomach, and small and large intestines, of vitamin E-deficient rats treated with propolis. These authors suggest that some components of propolis are absorbed into the blood, behave as hydrophilic antioxidants and conserve vitamin C. Ozen et al. (2004), have reported that CAPE (caffeic acid phenethyl ester), an active compound of honeybee extract, confers protection against oxidative damage caused by cisplatin in renal tissues, and attribute this effect to its free-oxygen radical scavenging activity.

In the present study, we used a drug metabolizing cell line (HepG2) to evaluate the capability of two fractions (F4 and F5) of an aqueous extract of propolis to protect against chemically-induced primary DNA damage and chromosome aberrations.

Materials and Methods

Propolis fractions

Propolis, produced by *Apis mellifera L.*, was collected from Chaves Farm (Itapecerica, State of Minas Gerais, Brazil). An aqueous extract of propolis (AEP) was prepared as previously described by Alves de Lima et al. (2005). After lyophilization, the powder was ressuspended in 10% methanol (V/V), and introduced into a Sephadex LH-20 (G&E) column (40x200mm) previously washed with distillated water. The two obtained fractions (denominated F4 and F5) 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 distillated 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 (Table 1 and 2). Each fraction was used at concentrations of 25, 50 and 100 µg/ml.

Table 1. Chemical profile of fraction 4 (F4)

Compound	**Concentration (µg/g)**	**Concentration (%)**
3-[4-hydroxy-3-(oxobutenyl)-phenylacrilic acid	78.84	0.008
3-prenyl-4-hydroxycinnamic acid	186.43	0.019
caffeic acid	68.17	0.007
p-coumaric acid	153.74	0.015
(E)-3-{4-hydroxy-3-[(E)-4-(2.3)-dihydrocinamoiloxy-3-methyl-2-butenyl]-5-prenylphenyl-2-propenoic acid	40.79	0.004
3,4-dihydroxy-5-prenylcinnamic acid	81.26	0.008
Total	**609.25**	**0.061**

Table 2. Chemical profile of fraction 5 (F5)

Compound	Concentration (µg/g)	Concentration (%)
2,2-dimethyl-6-carboxyetenil-2H-1-benzopirane	362.19	0.036
(E)-3-{4-hydroxy-3-[(E)-4-(2.3)-dihydrocinamoiloxy-3-methyl-2-butenyl]-5-prenylphenyl-2-propenoic acid	42.33	0.042
3,4-dihydroxy-5-prenylcinnamic acid	1783.54	0.178
3,5-diprenyl-4-hydroxycinnamic acid (Artepillin C)	301.18	0.030
3-prenyl-4-(2-methylpropionyloxy)cinnamic acid	291.58	0.029
3-prenyl-4-hydroxycinnamic acid	301.18	0.030
6-propenoic-2.2-dimethyl-8-prenyl-2H-1-benzopirane acid	363.99	0.036
caffeic acid	705.14	0.070
p-coumaric acid	620.52	0.062
Total	**7289.40**	**0.729**

Chemicals

The mutagens 4-nitroquinoline 1-oxide (4NQO – 0.01 µM; 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 DMEM medium, just before use. The selected concentrations were obtained in preliminary experiments, or were based on literature.

Cell lines and culture conditions

Human hepatoblastoma cells (HepG2) were grown to confluence into 75 cm^2 tissue culture flasks, at 37° C and 5% CO_2 atmosphere, using DMEM medium supplemented with 15% fetal calf serum (Cultilab – Brazil), and antibiotics (penicillin 100 U/mL and streptomycin 0.1 mg/ mL; Cultilab - Brazil). Confluent cells were detached with 0.15% trypsin (Cultilab – Brazil) for 5 min, and 0.2 x 10^6 cells/well in 24-well plates, or 1 x 10^6 cells/plastic Petri dish (94 mm), were seeded for the comet assay or micronucleus assay, respectively. Cell viability, using Trypan Blue, was checked after all treatments and it was always higher than 75% (data not showed).

Comet (Single Cell Gel Electrophoresis) Assay – Experimental Design

Three treatment protocols were used, and always performed in duplicate to ensure the reproducibility.

Pre-treatment: twenty-four hours after seeding, medium was removed and cells incubated with F4 or F5 (25, 50 and 100 µg/mL) in fresh medium, for 24 hours. Then, cells were washed with PBS, and treated with 4NQO or H_2O_2 for 5 minutes or with DEN for 1 hour. Then, cells were washed twice with PBS, trypsinized, centrifuged at 180 *g* for 3 min and ressuspended into 100 µL fresh medium for the comet assay.

Simultaneous treatment: twenty-four hours after seeding, medium was removed and cells were simultaneously treated with one of the fractions (F4 or F5) and with one of the mutagens (4NQO, H_2O_2 or DEN). Then, cells were washed twice with PBS, tripsinized, centrifuged at 180 *g* for 3 min and ressuspended into 100 µL fresh medium for the comet assay.

Post-treatment: twenty-four hours after seeding, medium was removed and cells treated with 4NQO or H_2O_2 for 5 minutes or with DEN for 1 hour. Then, cells were washed with PBS and incubated with F4 or F5, in fresh medium, for 24 hours. After this period, cells were washed twice with PBS, tripsinized, centrifuged at 180 *g* for 3 min and ressuspended into 100 µL fresh medium for the comt assay.

The comet assay was performed according to Singh et al. (1988) and Tice et al. (1991). In brief, a volume of 10 µL of the cells 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, 10mM 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 (300mM NaOH, 1mM EDTA, pH 13) for 20 min. Electrophoresis was carried out for 20 minutes, at 25V and 300 mA. After electrophoresis, slides were neutralized with 0.4< Tris-HCL (pH 7.5) solution, fixed with 100% ethanol, and stored until analysis. After stained with Sybr Gold (1:10.000 - Invitrogen), 50 randomly selected “nucleoids” per culture were examined at 200X 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 intensity (% of migrated DNA in the tail).

Citokinesis-block micronucleus (CBMN) assay – Experimental Design

The concentrations of the fractions and mutagens were the same used for the comet assay.

. *Pre-treatment:* twenty-four hours after seeding, medium was removed and cells incubated with the fractions of propolis (F4 or F5) in fresh medium, for 24 hours. Then, cells were washed with PBS, and treated with H_2O_2 or 4NQO for 5 minutes or with DEN for 1 hour. Then, cells were washed twice with PBS and incubated into a fresh medium containing cytochalasin B (final concentration 2.4 µg/mL) for 48 hours.

Simultaneous treatment: twenty-four hours after seeding, medium was removed and cells were simultaneously treated with F4 or F5, and with one of the mutagens. 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, medium was removed and cells treated with one of the mutagens (H_2O_2 or 4NQO for 5 minutes, or with DEN for 1 hour). After washing with PBS, cells were incubated with F4 or F5, in fresh medium, for 24 hours. Then, cells were washed twice with PBS, and incubated for 24 hours into a fresh medium containing cytochalasin B.

Each protocol was performed in duplicate to ensure reproducibility (Fenech, 2000). After harvesting, cells were treated with cold hypotonic solution (KCl 0.075 M), centrifuged (800 rpm, 8 min.), and fixed with acetic acid/methanol (1:3) solution. Air-dried slides were prepared and stained with 5% Giemsa solution for 7 min. A total of 1000 binucleated cells (BNC) were analyzed from each culture.

Statistical Analysis

It was adjusted a model following a completely randomized design, using a general linear model with gamma distribution. The multiple comparisons were done by DIFF option from PROC GENMOD from the SAS program for Windows, v- 9.1.3.

Results

Tables 3, 4 and 5 show the level of DNA damage (*tail intensity*) in HepG2 cells treated with the two propolis fractions (F4 or F5) and with H_2O_2, 4NQO and DEN. Data showed that at simultaneous treatment protocol both F4 and F5 were capable of reducing DNA damage caused by H_2O_2 and 4NQO, as depicted by the comet assay (Tables 3 and 4). For H_2O_2, F4 significantly decreased DNA lesions at the three concentrations used (25, 50, and 100 µg/ml), while F5 only at the intermediate concentration (50 µg/ml) (Table 3). A larger protective effect of the two fractions occurred when they were used with DEN (Table 5). Both F4 and F5 were capable of reducing damage at the three protocols, but F4 was more effective when used simultaneously to DEN, and F5 when used before the mutagen.

Frequencies of micronucleated cells (MNC) after exposure to propolis fractions and to H_2O_2, 4NQO, and DEN are presented in Tables 6, 7, and 8, respectively. Significant decreases in the number of MNC induced by DEN were detected when F4 was incubated before (25 µg/ml) and simultaneously (50 and 100 µg/ml), and F5 simultaneously (25 µg/ml) to the mutagen (Table 8). A protective effect was also detected for F5 (100 µg/ml) and H_2O_2 at post-treatment protocol (Table 6). None change was observed on the 4NQO mutagenicity (Table 7).

Table 3. DNA damage (*tail intensity*) in HepG2 cells treated with hydrogen peroxide and with two fractions of propolis (F4 or F5)

Treatment Protocol	Fraction/concentration (μg/ml)	Tail Intensity Mean ± SD
PRE-	C-	8.2 ± 10.5
	C+	12.0 ± 14.8#
	F4/25	13.9 ± 15.2
	F4/50	10.2 ± 12.9
	F4/100	13.5 ± 14.4
	F5/25	11.0 ± 13.7
	F5/50	9.9 ± 12.4
	F5/100	11.6 ± 13.6
SIMULTANEOUS	C-	7.5 ± 11.1
	C+	20.5 ± 17.5#
	F4/25	11.6 ± 13.1*
	F4/50	14.5 ± 17.9*
	F4/100	11.7 ± 14.3*
	F5/25	18.6 ± 16.5
	F5/50	15.2 ± 16.5*
	F5/100	21.8 ± 21.5
POST-	C-	7.2 ± 9.8
	C+	9.7 ± 11.5#
	F4/25	9.4 ± 10.3
	F4/50	8.4 ± 10.2
	F4/100	10.6 ± 12.9
	F5/25	8.8 ± 10.5
	F5/50	7.4 ± 10.0
	F5/100	8.4 ± 10.8

C-: negative control; C+: positive control (H_2O_2 – 0.6 mM); pre-treatment: F4 or F5 before H_2O_2; post-treatment: F4 or F5 after H_2O_2

# $p<0.05$, compared to C-

* $p< 0.05$, compared to C+

Table 4. DNA damage (*tail intensity*) in HepG2 cells treated with 4-nitroquinoline 1-oxide and with two fractions of propolis (F4 or F5)

Treatment Protocol	Fraction/concentration (μg/ml)	Tail Intensity Mean ± SD
PRE-	C-	5.5 ± 8.2
	C+	10.7 ± 12.6#
	F4/25	10.1 ± 11.5
	F4/50	11.3 ± 12.7
	F4/100	9.2 ± 9.7
	F5/25	13.6 ± 16.2
	F5/50	13.4 ± 16.2
	F5/100	9.6 ± 12.0
SIMULTANEOUS	C-	8.1 ± 11.6
	C+	28.2 ± 17.3#
	F4/25	26.7 ± 17.8
	F4/50	21.7 ± 17.8*
	F4/100	23.2 ± 16.9
	F5/25	18.4 ± 13.8*
	F5/50	24.9 ± 18.2
	F5/100	23.0 ± 17.9
POST-	C-	4.6 ± 6.9
	C+	10.1 ± 11.4#
	F4/25	9.5 ± 11.3
	F4/50	8.9 ± 10.8
	F4/100	9.1 ± 12.0
	F5/25	9.4 ± 10.0
	F5/50	8.8 ± 9.9
	F5/100	10.2 ± 11.1

C-: negative control; C+: positive control (4 NQO – 0.01 μM); pre-treatment: F4 or F5 before 4NQO; post-treatment: F4 or F5 after 4NQO

# $p<0.05$, compared to C-

* $p< 0.05$, compared to C+

Table 5. DNA damage (*tail intensity*) in HepG2 cells treated with n-nitrosodiethylamine and with two fractions of propolis (F4 or F5)

Treatment Protocol	Fraction/concentration (μg/ml)	Tail Intensity Mean ± SD
PRE-	C-	8.0 ± 9.1
	C+	18.9 ± 17.5#
	F4/25	8.7 ± 11.6*
	F4/50	16.3 ± 16.8
	F4/100	9.4 ± 11.2*
	F5/25	12.1 ± 13.1*
	F5/50	10.9 ± 11.7*
	F5/100	13.7 ± 13.5*
SIMULTANEOUS	C-	7.9 ± 10.5
	C+	16.6 ± 16.4#
	F4/25	10.3 ± 12.1*
	F4/50	10.8 ± 12.6*
	F4/100	10.9 ± 11.5*
	F5/25	14.4 ± 15.1
	F5/50	18.7 ± 17.5
	F5/100	11.1 ± 12.3*
POST-	C-	7.0 ± 9.8
	C+	12.5 ± 14.8#
	F4/25	10.7 ± 15.0
	F4/50	10.3 ± 12.0
	F4/100	8.7 ± 11.6*
	F5/25	10.8 ± 11.5
	F5/50	8.4 ± 11.4*
	F5/100	7.7 ± 10.6*

C-: negative control; C+: positive control (DEN – 50 mM); pre-treatment: F4 or F5 before DEN; post-treatment: F4 or F5 after DEN

# $p<0.05$, compared to C-

* $p< 0.05$, compared to C+

Table 6. Frequency of micronucleated cells (MNC) after different treatment protocols with hydrogen peroxide and propolis fractions (F4 and F5)

Treatment Protocol	Fraction/concentration (μg/ml)	Number of MNC	MNC/1000 BNC Mean ± SD
PRE-	C-	4	2.0 ± 0
	C+	18	9.0 ± 1.4#
	F4/25	12	6.0 ± 0
	F4/50	10	5.0 ± 0
	F4/100	8	4.0 ± 0
	F5/25	15	7.5 ± 0.7
	F5/50	11	5.5 ± 0.7
	F5/100	11	5.5 ± 0.7
SIMULTANEOUS	C-	5	2.5 ± 0.7
	C+	18	9.0 ± 2.8#
	F4/25	9	4.5 ± 0.7
	F4/50	8	4.0 ± 0
	F4/100	13	6.5 ± 0.7
	F5/25	8	4.0 ± 1.4
	F5/50	13	6.5 ± 2.1
	F5/100	8	4.0 ± 1.4
POST-	C-	5	2.5 ± 0.7
	C+	18	9.0 ± 1.4#
	F4/25	13	6.5 ± 0.7
	F4/50	14	7.0 ± 0
	F4/100	10	5.0 ± 0
	F5/25	11	5.5 ± 0.7
	F5/50	9	4.5 ± 0.7
	F5/100	7	3.5 ± 0.7*

C-: negative control; C+: positive control (H_2O_2 – 0.6 mM); pre-treatment: F4 or F5 before H_2O_2; post-treatment: F4 or F5 after H_2O_2; BNC: binucleated cells

# $p<0.05$, compared to C-

* $p< 0.05$, compared to C+

Table 7. Frequency of micronucleated cells (MNC) after different treatment protocols with 4-nitroquinoline 1-oxide and propolis fractions (F4 and F5)

Treatment Protocols	Fraction/Concentration (μg/ml)	Number of MNC	MNC/1000 BNC Mean ± SD
PRE-	C-	6	3.0 ± 0
	C+	17	8.5 ± 0.7#
	F4/25	10	5.0 ± 0
	F4/50	9	4.5 ± 0.7
	F4/100	15	7.5 ± 0.7
	F5/25	9	4.5 ± 0.7
	F5/50	10	5.0 ± 0
	F5/100	11	5.5 ± 0.7
SIMULTANEOUS	C-	7	3.5 ± 0.7
	C+	21	10.5 ± 0.7#
	F4/25	17	8.5 ± 2.1
	F4/50	17	8.5 ± 0.7
	F4/100	14	7.0 ± 0
	F5/25	18	9.0 ± 1.4
	F5/50	16	8.0 ± 1.4
	F5/100	17	8.5 ± 0.7
POST-	C-	4	2.0 ± 0
	C+	16	8.0 ± 1.4#
	F4/25	11	5.5 ± 0.7
	F4/50	9	4.5 ± 0.7
	F4/100	10	5.0 ± 1.4
	F5/25	7	3.5 ± 0.7
	F5/50	8	4.0 ± 1.4
	F5/100	10	5.0 ± 2.8

C-: negative control; C+: positive control (4NQO – 0.01μM); pre-treatment: F4 or F5 before 4NQO. post-treatment: F4 or F5 after 4NQO; BNC: binucleated cells.
# $p<0.05$, compared to C-

Table 8. Frequency of micronucleated cells (MNC) after different treatment protocols with n-nitrosodiethylamine and propolis fractions (F4 and F5)

Treatment Protocol	Fraction/Concentration (µg/ml)	Number of MNC	MNC/1000 BNC Mean ± SD
PRE-	C-	5	2.5 ± 0.7
	C+	17	8.5 ± 0.7#
	F4/25	7	3.5 ± 0.7*
	F4/50	8	4.0 ± 1.4
	F4/100	8	4.0 ± 0
	F5/25	9	4.5 ± 0.7
	F5/50	8	4.0 ± 0
	F5/100	10	5.0 ± 1.4
SIMULTANEOUS	C-	6	3.0 ± 0
	C+	24	12.0 ± 0#
	F4/25	14	7.0 ± 0
	F4/50	12	6.0 ± 0*
	F4/100	8	4.0 ± 0*
	F5/25	12	6.0 ± 0*
	F5/50	13	6.5 ± 0.7
	F5/100	15	7.5 ± 0.7
POST-	C-	5	2.5 ± 0.7
	C+	18	9.0 ± 1.4#
	F4/25	10	5.0 ± 1.4
	F4/50	8	4.0 ± 0
	F4/100	13	6.5 ± 0.7
	F5/25	8	4.0 ± 1.4
	F5/50	8	4.0 ± 1.4
	F5/100	10	5.0 ± 0

C-: negative control; C+: positive control (DEN – 50 mM); pre-treatment: F4 or F5 before DEN; post-treatment: F4 or F5 after DEN; BNC: binucleated cell

# $p<0.05$, compared to C-

* $p< 0.05$, compared to C+

Discussion

In the present study, we evaluated the chemopreventive activity of two propolis fractions (F4 and F5) on genetic damage induced by chemicals with different mechanisms of mutagenicity. The investigation was done using the HepG2 cell line, a useful tool to study regulation of drug-metabolizing enzymes (Wilkening et al., 2003). This cell line retains many characteristics of hepatocytes, such as the activity of phase I and phase II enzymes, which play key roles in the activation and detoxification of DNA-reactive mutagens (Mersch-Sundermann et al., 2004). Furthermore, two end-points of genetic damage - primary DNA lesions and micronucleus - were analyzed in order to better understand the antimutagenic potential of propolis.

The continuous generation and removal of reactive oxygen species (ROS) seems to be a common phenomenon in aerobic life. However, conditions of increased production or decreased removal of these very reactive radicals may lead to enhanced steady-state levels in cells and tissues, a situation generally called “oxidative stress” (McCord, 2000). Oxidative stress is considered to be actively involved in a variety of pathological conditions, including cancer, cardiovascular and neurodegenerative diseases and aging (Surh & Ferguson, 2003). Therefore, the use of therapeutic strategies using several antioxidants to prevent or attenuate these diseases has increased in the last two decades. In this context, the antioxidant properties of propolis and many of its components have been widely studied and well documented. Matsushige et al. (1995) have studied 5 different samples of Brazilian propolis and have observed strong antioxidant effects in all of them. Additionally, antioxidant activities of green propolis, and also its ability to scavenger reactive oxygen species, have also been reported (Simões et al. 2004). Thus, in this present study we used H_2O_2 and 4NQO, mutagens with ability to interact with DNA through highly reactive oxygen and radical species (Arima et al., 2006; Ratnam et al., 2006), to evaluate the capability of propolis components to protect DNA against oxidative stress. Hydrogen peroxide represents a particularly important molecule because it is generated under nearly all oxidative stress conditions and it can participate in several fundamental intracellular processes (Rhee, 2006). It 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). On the other hand, 4NQO is a model chemical carcinogen which has been extensively studied with respect to carcinogenicity,

mutagenicity, DNA adduct formation, and DNA repair (Bailleul et al., 1989; Ribeiro et al., 2004; Ribeiro & Salvadori, 2007). 4NQO has been shown to induce a potent intracellular oxidative stress because it can undergo redox cycling and produce ROS (Ninoshiba & Demple, 1993). Upon metabolic activation 4NQO can also be converted to an active carcinogen metabolite which reacts with DNA forming adducts primarily at guanine bases and to a lesser extent at adenine base (Galiegue-Zouitina et al., 1985), and also causing genetic damage through formation of alkali labile sites and SSBs (Vondracek et al., 2001). Regarding to DNA repair, 4NQO-induced damage can behave very similarly to those from UV light (Ikenega et al., 1975). Both bulky 4NQO adducts and UV light-induced pyramidine dimers are repaired by nucleotide excision repair pathways (Sancar & Sancar, 1988).Because of that 4NQO is often been referred as a UV mimetic agent

Herein, clear antigenotoxic effect of F4 on H_2O_2-induced DNA lesions at simultaneous treatment protocol was detected by the comet assay. F5 acted only at the intermediate concentration, although it was also capable of reducing chromosome aberrations as depicted by the micronucleus assay. Conversely, no evident protection was observed on 4NQO-induced genetic damage, although, Heo et al. (2001) have previously described that polyphenolic compounds from propolis are capable to modulate some enzyme activities and to suppress alkylating and radiomimetic chemical genotoxicity. Ahn et al. (2008) studying the antioxidant activities of various components from propolis (acacetin, apigenin, artepillin C, CAPE, chrysin, p-coumaric acids, galangin, kaempferol, pinocembrin and quercetin) showed that all of them have antioxidant properties. Kanbur et al. (2008) have also observed that propolis exhibits antiradical and antioxidant effects *in vivo* against damage caused by propetamphos, which is an organic phosphorus pesticide known to exhibit toxic effects through the generation of free radicals (Milatovic et al., 2006). Therefore, it is reasonable to think that propolis components could have protected HepG2 cell against H_2O_2-induced DNA lesions by their antioxidant activity. On the other hand, the lower effectiveness of this bee resin on 4NQO could be due to other mechanisms evolved on its genotoxicity.

Since HepG2 cells have the ability for metabolizing drugs, we also checked the capacity of F4 and F5 to reduce damage induced by DEN. This, is a potent procarcinogen that needs metabolic biotransformation to produce the promutagenes O_6-ethydeoxyguanosine and O_4- and O_6-ethyldeoxythymidine, which may initiate liver carcinogenesis (Dragan et al, 1994; Verna et al., 1996). Our data showed that both

fractions of propolis inhibited DNA damage in all protocols of treatment evaluated by the comet. Simultaneous and pre-treatment with F4 and simultaneous treatment with F5 also reduced the number of MNC caused by DEN. It is known that propolis has phenolic acids in its composition, therefore we might suppose that these compounds could have acted on phase II enzymes and increased DEN detoxification. Yeh et al. (2005) have demonstrated that phenolic acids increase the levels of phase II metabolizing enzymes in HepG2, suggesting that these compounds could be important in detoxification pathways. However, we might not discard the possibility that propolis has acted as a free radical scavenging, blocking DEN metabolites to interact with DNA.

The chemical profile of the two fractions (F4 and F5) showed that they have basically the same compounds, and their major constituents are cinnamic acids and caffeic acids, although in different concentrations. Since both showed protective effects, we could not attribute this activity to specific identified compounds. Therefore, based on our findings, we might suggest that propolis constituents can prevent DNA damage by different mechanism, mainly by their direct action on reactive oxygen species and by the modulation of detoxifying enzymes.

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V. DISCUSSÃO FINAL

Nas últimas décadas, as atividades biológicas e farmacológicas da própolis têm levado a um aumento considerável de sua utilização na medicina popular, e também atraído o interesse de um número crescente de pesquisadores. Tem sido relatado, que tais atividades da própolis estão relacionadas à sua complexa e variável composição química, a qual está intimamente relacionada à flora de cada região onde é coletada. (Bankova et al., 2000, Banskota et al., 2001; Simões et al., 2004). Portanto, com base em tais informações, o presente estudo foi delineado como o objetivo de avaliar o efeito protetor de um extrato aquoso de própolis bruta e duas de suas frações químicas (F4 e F5) sobre danos quimicamente induzidos no DNA de duas linhagens celulares *in vitro*. Mais especificamente, o estudo visou contribuir para o entendimento do(s) mecanismo(s) pelo(s) qual(ais) a própolis exerce atividade quimioprotetora contra os efeito de agentes reconhecidamente mutagênicos.

Inicialmente, foi obtido um extrato aquoso a partir de amostras de própolis do Estado de Minas Gerais. A seguir, esse extrato foi fracionado com o objetivo de isolar alguns compostos na tentativa de identificar aqueles com maior potencial quimioprotetor. No entanto, as análises cromatográficas posteriormente realizadas mostraram que as duas frações obtidas apresentavam basicamente compostos derivados do ácido cinâmico, embora em concentrações distintas. Alguns desses compostos (artepilina C, drupanina, ácido p-cumárico e ácido cafeico) são os que se apresentam em maiores concentrações na própolis da região sudeste do Brasil (Kumazawa et al., 2003). Na fração 4, dos 6 compostos identificados, 4 eram ácidos cinâmicos e, do mesmo modo, na fração 5, dos 9 compostos, 6 eram ácidos cinâmicos.

Na expectativa de identificar o tipo específico de dano genético, sobre o qual a própolis poderia exercer sua atividade protetora, dois parâmetros foram investigados:

danos primários no DNA (ou lesões genotóxicas) e alterações cromossômicas (efeito mutagênico), detectados, respectivamente, pelo teste do cometa e pelo teste do micronúcleo. Além disso, foram utilizados compostos químicos com diferentes mecanismos de indução de danos genéticos e com necessidade, ou não, de serem metabolicamente ativados para exercerem tal efeito. Para melhor avaliar se a própolis poderia atuar sobre os sistemas de ativação e detoxificação drogas, foram utilizadas duas linhagens celulares, uma metabolicamente ativa (HepG2) e outra sem capacidade para metabolizar agentes químicos (CHO).

Com o teste do cometa em células CHO, pode-se observar o efeito protetor do extrato aquoso da própolis e de suas frações (F4 e F5), principalmente sobre os compostos que induzem danos oxidativos no DNA (H_2O_2 e 4 NQO), sem a necessidade de serem metabolizados pelo sistema de citocromo P-450. Tal efeito foi visualizado nos três protocolos de tratamentos, isto é, quando a própolis foi utilizada antes, simultaneamente ou após o tratamento com o mutágenos. Nas células da linhagem HepG2, contudo, a ação dos componentes da própolis sobre o H_2O_2 e 4 NQO não foi tão evidente, já que somente quando utilizada simultaneamente com os xenobióticos é que protegeu o DNA contra os radicais oxidantes.

O efeito protetor da própolis e de seus constituintes tem sido atribuído às suas atividades antioxidante e sequestradora de radicais livres (Shimazawa et al., 2005). Sabe-se que a principal fonte dos componentes da própolis brasileira é a planta *Baccharis dracunculifolia* (Park et al., 2004). Em geral, tanto a própolis quanto o extrato da *Baccharis dracunculifolia* contem grande quantidade de flavonóides em sua composição, os quais podem ser metabolizados em vários tipos de ácidos fenólicos e muitos desses são capazes inibir a ação de radicais livres (Russo et al., 2002; Pietta, 2000). Os flavonóides e outros compostos fenólicos têm se mostrado excelentes agentes

antioxidantes em estudos *in vitro* e, portanto, muitos pesquisadores têm sugerido que suas inclusões na dieta poderia trazer efeitos positivos sobre o estresse oxidativo e doenças relacionadas (Urquiaga et al., 2000). Nakajima et al, (2009), comparando a própolis com outros produtos fabricados pelas abelhas, observaram que a própolis tem ação antioxidante não apenas mais forte esses produtos, mas, também, que outros conhecidos compostos antioxidantes, como por exemplo, o ácido ascórbico. Um composto comumente encontrado na própolis brasileira e que possui alta atividante antioxidante é a artepelina C (Nakanishi et al., 2003). De acordo com Shimizu et al. (2006), quando a artepelina C chega ao fígado, é capaz de estimular atividades antioxidantes e induzir a expressão de enzimas de fase II, que auxiliam na detoxificação de carcinógenos e, consequentemente, reprimem a formação de lesões pré-neoplásicas.

Quando o efeito da própolis foi avaliado sobre o agente alquilante MMS, foi também observada diminuição dos danos no DNA em células CHO. Além do possibilidade de inibição da formação ou da ligação de radicais alquilantes no DNA, a própolis poderia ter favorecido a atuação do sistema de reparo, já que seu efeito protetor pode ser visualizado quando utilizada após o MMS. Há informações na literatura que as bases metiladas por agentes como o MMS, podem ser eficientemente reparadas em células CHO pelo sistema de excisão de bases (Ludin et al., 2005).

Como dito anteriormente, em células HepG2 foi também detectado o efeito protetor das frações do extrato da própolis sobre compostos que induzem danos oxidativos (H_2O_2 e 4 NQO), sendo este melhor visualizado no protocolo de tratamento simultâneo. Com isso, poder-se-ia sugerir que a própolis teria atuado como um desmutágeno, isto é, inativando química ou enzimaticamente os mutágenos. Sabe-se que as células da linhagem HepG2 possuem enzimas de ativação e detoxificação de xenobióticos, como por exemplo, as superóxido desmutases (Murakimi et al., 2002).

Por outro lado, ao se observar a atividade de compostos da própolis sobre danos no DNA causados pela DEN, poder-se-ia também sugerir a ação sobre o sistema de enzimas da família P450, já que a DEN é um pré-mutágeno que necessita de biotransformação e formação de metabólitos ativos para reagir e lesar o DNA (Verna et al., 1996).

Dado que causou surpresa foi a capacidade da própolis em aumentar o nível de danos no DNA causados pelo MMS no teste do cometa, muito embora isso não tenha sido detectado com nenhum dos mutágenos em células HepG2. Esse resultado sugere que compostos presentes na própolis poderiam potencializar efeitos genotóxicos. Tavares et al. (2006), já haviam descrito que o extrato etanólico da própolis apresenta característica de compostos com efeito "Janus", isto é, aqueles que podem atuar tanto protegendo como induzindo danos genotóxicos, dependendo das condições experimentais. De maneira similar, flavonóides, os quais são encontrados em grande quantidade na própolis brasileira, podem também apresentar atividades antioxidante ou pro-oxidante (Halliwell et al., 2005).

Concluindo, nossos resultados confirmam a capacidade do extrato aquoso de própolis e de suas frações em prevenir contra danos genotóxicos quimicamente induzidos em células CHO e HepG2.

VI. CONCLUSÕES

Com base nos resultados do presente estudo pode-se concluir que:

1) os principais compostos presentes nas duas frações do extrato da própolis pertencem à família dos ácidos cinâmicos;

2) o extrato aquoso da própolis e suas duas frações, quando administrados antes e após o mutágeno H_2O_2 e em todos os protocolos com 4NQO, foram capazes de reduzir danos oxidativos no DNA das células CHO;

3) as frações do extrato aquoso da própolis foram capazes de reduzir os danos genotóxicos induzidos pela DEN nas células HepG2, em todos os protocolos utilizados sugerindo que a própolis pode atuar modulando enzimas de metabolização;

4) o extrato aquoso da própolis quanto e suas duas frações foram capazes de reduzir a freqüência de micronúcleos em células CHO e HepG2 tratadas com H_2O_2 e DEN, respectivamente.

VII. REFERÊNCIAS

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