UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA
CAMPUS DE BOTUCATU

EFEITO DA PRÓPOLIS E DE EXTRATOS OBTIDOS DE *Baccharis dracunculifolia* SOBRE A REPLICAÇÃO DO POLIOVÍRUS TIPO 1 EM CÉLULAS HEp-2

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Dissertação apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista - UNESP, para obtenção do título de Mestre em Patologia.

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Sumário

SUMÁRIO

Capítulo I – Revisão da Literatura

1. Própolis: considerações gerais

As abelhas utilizam a própolis para a construção e manutenção de suas colméias, sendo empregada para selar aberturas na colméia, visando controlar variações de temperatura principalmente no inverno. A própolis também é utilizada para embalsamar insetos invasores que morrem no interior da colméia, evitando sua decomposição, além de contribuir para manter o ambiente interno asséptico, protegendo-o contra bactérias e vírus (Salatino et al., 2005).

A matéria-prima da própolis é composta por 50% de resina, 30% de cera de abelha, 10% de óleos aromáticos e essenciais, 5% de pólen e 5% de outras substâncias variadas, além de impurezas (Burdock, 1998). A coloração da própolis pode variar entre amarelo claro e vermelho escuro, passando pelo verde e marrom, dependendo da sua origem botânica e época do ano. Neste sentido, a cor verde característica de algumas amostras de própolis brasileira é uma conseqüência de sua origem botânica, pois as abelhas coletam matéria-prima de tecidos jovens contendo clorofila, como brotos vegetativos de *Baccharis dracunculifolia*. As folhas jovens contêm pêlos secretórios, com óleos voláteis e aromáticos, conferindo o aroma típico da própolis (Salatino et al., 2005).

À temperatura de 15° C a própolis é uma substância dura, tornando-se maleável a partir de 30° C. Os constituintes da própolis, como materiais cerosos, bálsamos, óleos essenciais e derivados fenólicos, podem ser extraídos em solventes como etanol, éter, acetona, tolueno e tricloroetileno (Cunha et al., 2004). Quimicamente, sua composição é considerada extremamente complexa, variando conforme a localização geográfica e a ecologia vegetal da região. A literatura revela ainda que foram identificados mais de 300 componentes presentes na própolis; destes, os flavonóides são, em geral, considerados os principais compostos responsáveis pelas atividades antimicrobianas e demais propriedades farmacológicas da própolis européia, tendo como origem botânica principalmente as plantas

do gênero *Populus*, enquanto que os principais componentes biologicamente ativos da própolis brasileira são os ácidos diterpênicos e ácidos *p*-cumáricos prenilados. Encontram-se também, chalconas, ácido benzóico, benzoaldeído, álcoois, acetona, compostos fenólicos, ácido cinâmico e derivados, ácido cafeico e derivados, di- e triterpenos (De Castro, 2001).

Nas zonas temperadas do hemisfério norte, as abelhas produzem a própolis somente no verão, incluindo o final da primavera e o começo do outono. No Brasil, a coleta da própolis procede o ano todo e variações sazonais são possíveis. Neste sentido, nosso grupo de pesquisa coletou amostras de própolis ao longo do ano para posterior análise de sua constituição, bem como para avaliar seus efeitos em diferentes ensaios biológicos. Nossos resultados evidenciaram que variações sazonais na composição da própolis não são significativas, havendo compostos biologicamente ativos em todas as estações sazonais. Este fato indica que as abelhas coletam a própolis do mesmo grupo de plantas, na mesma região geográfica (Boudourova-Krasteva et al., 1997).

A própolis possui inúmeras propriedades biológicas e farmacológicas, tendo sido utilizada desde a antiguidade. Os sacerdotes do antigo Egito utilizavam-na para embalsamar os mortos. Posteriormente, os gregos beneficiaram-se de suas propriedades anti-séptica e cicatrizante. Países do leste europeu utilizavam este produto apícola no tratamento de doenças infecciosas. No Brasil, seu uso é amplamente difundido, sendo empregada na medicação popular para o tratamento da tuberculose, distúrbios gástricos, redução da febre, doenças inflamatórias, como agente anestésico, anticancerígeno, bem como por sua propriedade antimicrobiana (Salatino et al., 2005).

1.1. Interação entre *Apis mellifera* e *Baccharis dracunculifolia* na elaboração da própolis

A própolis pode ser produzida a partir de uma variedade de plantas. O gênero *Baccharis* pertence à família Asteraceae e possui cerca de 500 espécies distribuídas

principalmente no Brasil, Argentina, Paraguai e Uruguai. No Brasil, estão descritas cerca de 120 espécies de *Baccharis* (Verdi et al., 2005). Devido à enorme importância medicinal, comercial e biológica, inúmeras espécies de *Baccharis* têm atraído a atenção de muitos pesquisadores das mais diversas áreas (Teixeira et al., 2005).

Baccharis dracunculifolia DC (Asteraceae), conhecida popularmente como "alecrim do campo" e "vassourinha", é um arbusto lenhoso, podendo atingir até 4 metros de altura, nativo do Brasil, onde ocorre nas regiões sul, sudeste e centro-oeste, principalmente nas áreas de cerrado, pastagens abandonadas e áreas em processo de sucessão (Park et al., 2004). Esta planta é uma espécie perene, dióica, reproduzida por sementes, possuindo galhos bastante ramificados. Suas folhas apresentam tricomas tectores e glandulares (Hellwing, 1992), os quais além de atuarem como barreira ao ataque de predadores que tentam alimentar-se dos tecidos vegetais (Spring, 2000), auxiliam na interação desta espécie com as abelhas para a coleta do material resinoso (Teixeira et al., 2005). De acordo com Bankova et al. (1999) e Bastos (2001), *Baccharis dracunculifolia* é a principal fonte botânica da própolis produzida nos estados de Minas Gerais e São Paulo.

Para elaboração da própolis, as abelhas *Apis mellifera* fragmentam ápices vegetativos de *B. dracunculifolia*, tais como brotos, primórdios foliares e folhas jovens (Park et al., 2004; Teixeira et al., 2005). Utilizando o primeiro par de patas, movem o material para as patas medianas e, então, a colocam na corbícula. Muito raramente, o material é transferido diretamente do primeiro par de patas para a corbícula. O tempo gasto para a coleta da resina e seu depósito na corbícula é, em média, 7 minutos (Teixeira et al., 2005). As abelhas visitam os ápices foliares, por alguns segundos, com o auxílio de suas antenas, e então, movem para outras plantas, pois suas antenas possuem alta capacidade olfatória (Loayza et al., 1995). Após a coleta, este material é transportado até a colméia, onde as abelhas modificam sua composição, adicionando cera e secreções glandulares (Burdock, 1998).

Os óleos voláteis ou essenciais, produtos do metabolismo secundário, são definidos como misturas complexas de mono-, sesqui- e diterpenos, os quais possuem grande valor devido às suas aplicações em medicamentos, cosméticos, alimentos e agro- químicos (Pinto et al., 2002). O rendimento do óleo e a concentração de cada constituinte podem variar durante as fases de crescimento do vegetal, bem como por influência de fatores ambientais tais como: pluviosidade, radiação solar, temperatura, exposição solar, entre outros (Palomino et al., 2002; Agostini et al., 2005). No óleo volátil extraído das folhas de *B. dracunculifolia*, os componentes majoritários encontrados *β*-selineno (10%), *β*-cariofileno (10%), germacreno-*D* (10%), espatulenol (10%) e nerolidol (20%) (Ferracini, 1995). Assim, considera-se de grande importância a avaliação das atividades biológicas desses compostos, visando aproveitar o grande potencial desta planta para o desenvolvimento de novos fármacos.

1.2. Propriedades biológicas da própolis e de extratos obtidos de *Baccharis dracunculifolia*

Muitos artigos científicos ilustram a diversidade de atividades biológicas da própolis, e nosso grupo tem investigado principalmente suas ações imunomoduladora, antitumoral e antimicrobiana (Sforcin, 2007).

Com relação ao sistema imunológico, trabalhos experimentais de nosso laboratório indicaram aumento na atividade natural *killer* em ratos tratados com própolis (Sforcin et al., 2002). Este produto apícola também aumentou a geração de espécies reativas do oxigênio (H_2O_2) e inibiu a produção de óxido nítrico (NO) por macrófagos peritoneais de camundongos (Orsi et al., 2000). Camundongos estressados e tratados com própolis apresentaram potencialização na geração de H_2O_2 e inibição da produção de NO por macrófagos peritoneais (Missima & Sforcin, 2008). O aumento da produção de H_2O_2 por

macrófagos peritoneais também foi evidenciado quando estes foram submetidos ao tratamento com extratos de *Baccharis* e alguns de seus compostos isolados como óxido de *Baccharis* e friedelanol, sugerindo a ação ativadora de extratos e compostos isolados desta planta sobre macrófagos (Missima et al., 2007).

Ivanovska et al. (1995) verificaram que a concentração de interleucina (IL)-1β no soro de animais tratados com ácido cinâmico (constituinte da própolis) apresentou-se elevada, possivelmente devido à ativação dos macrófagos por este composto.

Dados de nosso laboratório revelaram que a própolis também aumentou a atividade fungicida de macrófagos contra *Paracoccidioides brasiliensis* (Murad et al., 2002) e a atividade bactericida contra *Samonella* Typhimurium, envolvendo a participação de metabólitos do oxigênio e do nitrogênio (Orsi et al., 2005). Ratos tratados com própolis apresentaram maior produção de anticorpos, não havendo efeito de compostos isolados ou de *B. dracunculifolia* neste ensaio (Sforcin et al., 2005). Camundongos inoculados com vacina inativada de herpes tipo 1 Suid (SuHV-1) associada à própolis apresentaram aumento dos níveis de anticorpos (Fischer et al., 2007a). O uso do extrato etanólico da própolis, associado à uma emulsão oleosa e à vacina inativada de herpes vírus bovino tipo 5 (BoHV-5), aumentou a resposta imune humoral a este vírus (Fischer et al., 2007b), e tais resultados sugerem o uso da própolis em vacinas como adjuvante.

No tocante à ação antitumoral da própolis, nossos estudos confirmaram a atividade antitumoral da própolis, *in vitro,* sobre células de tumor venéreo transmissível canino (TVT) (Bassani-Silva et al., no prelo) e células de carcinoma de laringe humano (HEp-2) de maneira tempo-dose-dependente (Búfalo et al., no prelo). A administração do extrato hidroalcoólico da própolis a ratos, após o tratamento com o carcinógeno di-metil-hidrazina (DMH), reduziu o número de criptas aberrantes no cólon, refletindo supressão da expansão clonal das células iniciadas, o que caracteriza o passo da promoção da carcinogênese (Bazo

et al., 2002). Alves De Lima et al. (2005) realizaram investigações com o extrato aquoso da própolis neste mesmo modelo de carcinogênese, verificando o efeito protetor da própolis na genotoxicidade induzida pelo DMH.

Nosso grupo também avaliou a ação antimicrobiana da própolis, destacando-se sua eficiente ação inibidora, *in vitro*, sobre várias linhagens de bactérias Gram positivas, como *Staphylococcus aureus,* e limitada ação contra bactérias Gram negativas, como *Escherichia coli* e *Pseudomonas areuginosa* (Sforcin et al., 2000). De acordo com Ferronato et al. (2007), os óleos essenciais obtidos de *Baccharis dracunculifolia* também apresentaram efeito bactericida sobre estas mesmas bactérias.

Nossos estudos confirmaram a atividade antiprotozoária da própolis sobre *Giardia duodenalis* (Freitas et al., 2006) e atividade antifúngica sobre *Candida albicans* e *Candida tropicalis* (Sforcin et al., 2001). O potencial biológico da própolis parece ser devido a um sinergismo que ocorre entre os muitos constituintes presentes em sua composição química.

2. O vírus

2.1. Poliovírus tipo 1

O poliovírus (PV), agente etiológico da poliomielite, foi identificado pela primeira vez em 1909, quando Karl Landsteiner e Erwin Popper inocularam o vírus de um paciente com doença paralítica em macacos (Blondel et al., 1998; De Jesus, 2007). Desde então, o PV tem sido classificado como um membro da família *Picornaviridae*, a qual inclui muitos importantes vírus humanos e animais (Calvez et al., 1993; Blondel et al., 1998; De Jesus, 2007). O PV pertence ao gênero *Enterovírus* e é classificado em três sorotipos (PV-1, PV-2 e PV-3) (Minor, 1996; De Jesus, 2007).

O poliovírion possui morfologia esférica e diâmetro variável entre 27 a 30 nm. É constituído de RNA de fita simples, polaridade positiva e envolto por capsídeo protéico de

simetria icosaédrica não envelopado. O capsídeo consiste de 60 cópias de cada uma das quatro proteínas estruturais virais (VP1 – VP4), sendo que VP1 – VP3 são proteínas superficiais e responsáveis pela diversidade antigênica entre os poliovírus (Blondel et al., 1998; Simonet & Gantzer, 2006; Brandenburg et al., 2007), ao passo que VP4 localiza-se no interior do capsídeo, associado ao ácido nucléico (Blondel et al., 1998).

O genoma do PV é constituído de um único filamento linear de RNA com 7500 nucleotídios, o qual é poliadenilado na região terminal 3' e covalentemente ligado a uma pequena proteína viral VPg na região terminal 5'. O genoma do PV é dividido em três regiões: uma longa estrutura de leitura (ORF – *open reading frame*) flanqueada por duas regiões não codificadas (NCR) de 742 nucleotídios na terminação 5' e 72 na 3', envolvidas no processo de replicação do genoma viral (Blondel et al., 1998; Mueller et al., 2005; Simonet & Gantzer, 2006; De Jesus, 2007) (Figura 1).

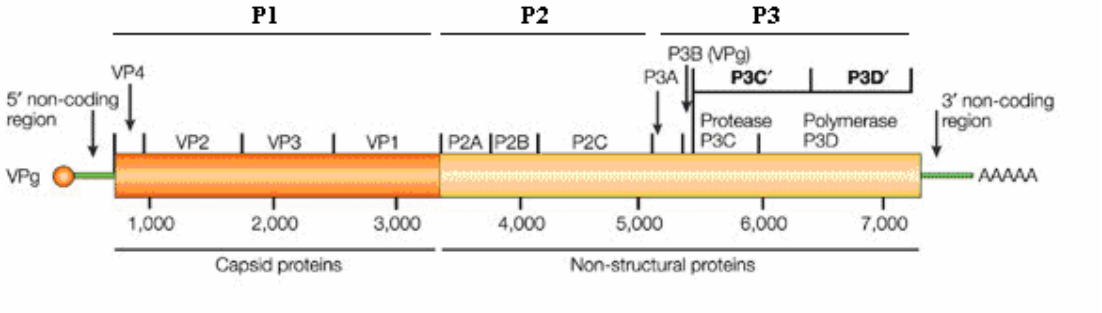

Figura 1. Organização do genoma do poliovírus (Minor, 2004).

O ciclo replicativo do PV ocorre inteiramente no citoplasma da célula hospedeira. A infecção é iniciada através da ligação do vírion ao receptor de superfície celular do PV humano (hPVR) ou CD155, o qual é membro da superfamília das imunoglobulinas (Blondel et al., 1998; Mueller et al., 2005; Brandenburg et al., 2007; De Jesus, 2007). Além de ser responsável pela interação do vírus à superfície da célula, o hPVR induz desestabilização do vírion, levando a mudanças conformacionais no capsídeo viral para que

ocorra a liberação do RNA viral no citoplasma da célula infectada (BLONDEL et al., 1998; BRANDENBURG et al., 2007). Uma vez que o RNA viral encontra-se no citoplasma da célula, a proteína VPg é liberada da região terminal 5' do genoma viral e a tradução é processada pelos ribossomos da célula hospedeira. O RNA viral pode funcionar como mRNA (Mueller et al., 2005; De Jesus, 2007). O RNA viral é traduzido em uma proteína autocatalítica, a qual é clivada por proteases virais em proteínas menores. A região P1 codifica as proteínas do capsídeo – VP0 (precursor de VP4 e VP2), VP3 e VP1; enquanto que as regiões P2 e P3 codificam proteínas não estruturais como as proteases virais, a proteína VPg e a polimerase viral (Blondel et al., 1998; Mueller et al., 2005; De Jesus, 2007).

Na montagem final, o VP0 é clivado em VP2 e VP4, tranformando o provírion em partícula viral madura (Blondel et al., 1998; Mueller et al., 2005; De Jesus, 2007). Uma vez estruturados, os vírions acumulam-se no citoplasma das células infectadas, sendo liberados através da lise da célula infectada (Calvez et al., 1993; Blondel et al., 1998; De Jesus, 2007).

2.2. Patogênese do PV

O PV tem o homem como hospedeiro natural, afetando principalmente crianças menores de 5 anos de idade (Minor, 1996). Multiplica-se nas mucosas laríngea, linfóide e intestinal, e sua progênie é eliminada pelas fezes - veículo de transmissão pela via fecal-oral, através da contaminação da água e de alimentos. O tempo de incubação do vírus é de 7 a 14 dias, podendo variar entre 2 a 35 dias (Blondel et al., 1998; Mueller et al., 2005). Após a replicação inicial, o vírus é encontrado no sangue, podendo disseminar para sítios distantes, incluindo o sistema nervoso central (SNC) (Minor, 1996). A patogenia clássica do

PV é a poliomielite, doença infecciosa aguda resultante do acometimento do SNC, caracterizada pelo quadro clássico de paralisia flácida (Blondel et al., 1998).

Em 1988, países membros da Organização Mundial da Saúde (OMS) adotaram uma campanha global para erradicar o PV até o ano de 2000. Desde então, progressos consideráveis têm sido obtidos na luta contra a doença (De Jesus, 2007) e, programas epidemiológicos e campanhas de vacinação têm sido cada vez mais reforçados, utilizando a vacina constituída de vírus atenuado (Sabin), pela maior facilidade de administração, baixo custo da aplicação e indução do aparecimento de imunidade duradoura (Gonçalvez et al., 2002). A vacinação proporcionou resultados eficazes, reduzindo a incidência da poliomielite de 350.000 casos em 1998 para 1.874 em 2006 (De Jesus, 2007). Atualmente, a doença é endêmica em países dos continentes africano e asiático, sendo notificados 229 casos na Nigéria, 392 na Índia, 17 no Paquistão e 12 no Afeganistão (De Palma et al., 2008).

Ao considerarmos mais especificamente as propriedades antivirais da própolis, os estudos têm-se concentrado, principalmente, sobre o vírus da herpes (HSV), vírus influenza, vírus da imunodeficiência humana (HIV), adenovírus tipo-2 e vírus da estomatite vesicular (VSV) (Ito et al., 2001; Gekker et al., 2005; Moreno et al., 2005). Para o tratamento da herpes genital, a própolis mostrou-se eficaz na cicatrização das lesões genitais e na redução de sintomas. Burdock (1998) também verificou o efeito do extrato da própolis, *in vitro*, em vários vírus de DNA e RNA, incluindo o vírus da herpes dos tipos 1 e 2, revelando ação inibidora da própolis sobre a replicação destes vírus. Cushnie & Lamb (2005) relataram que constituintes da própolis, em especial os flavonóides, têm ação inibitória sobre o vírus da herpes e, o extrato alcoólico da própolis apresentou efeito sobre a expressão viral do HIV, *in vitro*. Quanto ao vírus influenza, os resultados revelaram que sua replicação é inibida em presença da própolis (Burdock, 1998; Gekker et al., 2005).

Estudos com o gênero *Baccharis trinervis* revelam potente atividade antiviral contra o HSV, havendo inibição de 100% da sua atividade viral, e moderada atividade sobre o HIV e VSV (Palomino et al., 2002).

A escassez de dados na literatura a respeito da utilização da própolis e de extratos de *Baccharis dracunculifolia* em processos antivirais, de modo geral e mais especificamente com o poliovírus tipo 1, motivaram-nos a pesquisar sua ação frente a esse vírus de importância patológica. Uma forma de obter tais respostas seria averiguar a ação do extrato etanólico de própolis e de *B. dracunculifolia* sobre a replicação deste vírus.

Esta dissertação encontra-se apresentada sob a forma de dois manuscritos a serem submetidos a publicação. Anteriormente aos ensaios antivirais, avaliamos a citotoxicidade das variáveis sobre a célula utilizada, resultando no manuscrito I ("***In vitro* cytotoxic activity of** *Baccharis dracunculifolia* **and propolis against HEp-2 cells"**). Em seguida, avaliamos o potencial antiviral das variáveis, apresentado no manuscrito II ("**Antiviral activity of** *Baccharis dracunculifolia* **and propolis and poliovirus quantification by real-time PCR"**).

3. Referências Bibliográficas*

Agostini F, Santos ACA, Rossato M, Pansera MR, Zattera F, Wasum R, et al. Estudo do óleo essencial de algumas espécies do gênero *Baccharis* (Asteraceae) do sul do Brasil. Rev Bras Farmacog. 2005; 15: 215-20.

Alves De Lima RO, Bazo AP, Said RA, Sforcin JM, Bankova V, Darros BR, et al. Modifying effect of propolis on dimethylhydrazine-induced DNA damage but not colonic aberrant crypt foci in rats. Environ Mol Mutagen. 2005; 45: 8-16.

Bankova VS, Popov SS, Marekov N. On the chemical composition of some propolis fractions with antiviral action. Acta Microbiol Bulg. 1998; 23: 52-7.

Bankova VS, Brasteva GB, Sforcin JM, Frete X, Kujumgiev A, Rodella RM, Popov J. Phytochemical evidence for the plant origin of Brazilian propolis from São Paulo state. Z Naturforsch. 1999; 54: 401-5.

Bankova VS. Recent trends and important developments in propolis research. Evid Based Complement Alternat Med. 2005; 2: 29-32.

Bassani-Silva S, Sforcin JM, Amaral AS, Gaspar LFJ, Rocha NS. Propolis effect *in vitro* on canine transmissible venereal tumor cells. Rev Port Ciênc Vet., no prelo.

Bastos EM. Origem botânica e indicadores de qualidade da própolis verde produzida no estado de Minas Gerais, Brasil, [Dissertação]. Ribeirão Preto: Universidade de São Paulo; 2001.

---

*Referências bibliográficas elaboradas de acordo com o International Committee of Medical Journal Editors. Uniform Requirements for Manuscripts submitted to Biomedical Journal: sample references. [homepage on the internet]. Bethesda: U.S. National Library of Medicine; 2003 [last upatade 2003 July 09; cited 2005 Jun 01]. Avaliable from: http://www.nlm.nih.gov.bsd/uniform_requirements.html. National Library of Medicine. List of journals indexed in Index Medicus. Washington, 2003. 240p.

Bazo AP, Rodrigues MAM, Sforcin JM, Camargo JLV, Ribeiro LR, Salvadori DM.F. Protective action of propolis on the rat colon carcinonogenesis. Teratog Carcinog Mutagen. 2002; 22: 183-94.

Blondel B, Duncan G, Couderc T, Delpeyroux F, Pavio N, Colbère-Garapin F. Molecular aspects of poliovirus biology with a special focus on the interactions with nerve cells. J Neurovirol. 1998; 4: 1-26.

Boudourova-Krasteva G, Bankova V, Sforcin JM, Nikolova N, Popov S. Phenolics from Brazilian propolis. Z Naturforsch. 1997; 52c: 676-79.

Brandenburg B, Lee LY., Lakadamyali M, Rust MJ., Zhuang X, Hogle JM. Imaging poliovirus entry in live cells. PLoS Biol. 2007; 5: 1543-55.

Búfalo MC, Candeias JMG, Sforcin JM. In vitro cytotoxic effect of brazilian green propolis on human laryngeal epidermoid carcinoma (HEp-2) cells. Evid Based Complement Alternat Med.**,** no prelo.

Burdock G. A review of the biological properties and toxicity of bee propolis (propolis). Food Chem Toxicol. 1998; 36: 347-63.

Calvez V, Pelletier I, Borzakian S, Colbère-Garapin F. Identification of a region of the poliovirus genome involved in persistent infection of HEp-2 cells. J Virol. 1993; 67: 4432-35.

Cunha IBS, Sawaya ACHF, Caetano FM, Shimizu MT, Marcucci, MC, Drezza FT, et al. Factors that influence the yield and composition of Brazilian propolis extracts. J Braz Chem Soc. 2004; 15: 964-70.

Cushnie TPT & Lamb AJ. Antimicrobial activity of flavonoids. Intern J Antimicrob Agents. 2005; 26: 343-56.

De Castro SL. Propolis: biological and pharmacological activities. Therapeutic uses of this bee-product. Annu Rev Biomed Sci. 2001; 3: 49-83.

De Jesus NH. Epidemics to eradication: the modern history of poliomyelitis. Virol J. 2007; 4: 1-18.

De Palma AM, Purstinger G, Wimmer E, Patick AK, Andries K, Rombut B, et al. Potential use of antiviral agents in polio irradication. Emerg Infect Dis. 2008; 14: 545-51.

Ferracini VL. Óleos essenciais de Baccharis e sua interação com insetos polinizadores. [Dissertação] Campinas: Universidade de Campinas; 1995.

Fischer G, Conceição FR, Leite FPL, Dummer LA, Vargas GA, Hubner SOT, et al. Immunomodulation produced by a green propolis extract on humoral and cellular responses of mice immunized with SuHV-1. Vaccine. 2007a; 25: 1250-56.

Fischer G, Cleff MB, Dummer LA, Paulino N, Paulino AS, Vilela FSC, et al. Adjuvant effect of green propolis on humoral immune response bovines immunized with bovine herpesvirus type 5. Vet Immunol Immunopathol. 2007b; 116: 79-84.

Freitas SF, Shinohara L, Sforcin JM, Guimarães S. *In vitro* effects of propolis on *Giardia duodenalis* trophozoites. Phytomedicine. 2006; 13: 170-75.

Gekker G, Hu S, Spivak M, Lokensgard JR, Peterson PK. Anti-HIV- 1 activity of propolis in $CD4^+$ lymphocyte and microglial cell cultures. J Ethnopharmacol. 2005; 102: 158-63.

Gonçalvez JLS, Hubinger MGV, Wermelinger MCMW. Viroses do Sistema Nervoso Central. In: Santos NSO, Romanos MT, Wigg MD, editors. Introdução a Virologia Humana. Rio de Janeiro: Guanabara Koogan; 2002. p. 164-75.

Hellwing FH. Studies on the trichomes of some Asteraceae (Compositae). Flora. 1992; 186: 425-44.

Ito J, Chang FR, Wang HK, Park YK, IkegakI M, Kilgore N, et al. Anti-HIV activity of moronic acid derivates and the new melliferone-related triterpenoid isolated from Brazilian propolis. J Nat Prod. 2001; 64: 1278-81.

Ivanovska N, Stefanova Z, Valeva V, Neychev H. Immunomodulatory action of propolis: A comparative study on cinnamic and caffeic lysine derivatives. Compt Rend Acad Bulg Sci. 1995; 46: 115-7.

Loayza I, Abujder D, Aranda R, Jakupovic J, Collin G, Deslauriers H, et al. Essential oils of *Baccharis salicifolia*, *B. latifolia* and *B. dracunculifolia*. Phytochemistry 1995; 38: 381-89.

Minor P D. Poliovirus biology. Structure. 1996; 4: 775-78.

Minor P D. Polio erradication, cessation of vaccination and re-emergence of disease. Nat Rev Microbiol [serial online]. 2004; 2: 473-482. Avaliable from: http://www.nature.com/nrmicro/journal/v2/n6/box/nrmicro906_Bx1.html

Missima F & Sforcin JM. Green Brazilian propolis action on macrophages and lymphoid organs of chronically stressed mice. Evid Based Complement Alternat Méd. 2008; 5: 71-5.

Missima F, Da Silva Filho AA, Nunes GA, Bueno PCP, De Sousa JPB, Bastos JK, et al. Effects of *Baccharis dracunculifolia* D.C. (Asteraceae) extracts and its isolated compounds on macrophage activation. J Pharm Pharmacol. 2007; 59: 463-68.

Moreno MIN, Zampini IC, Ordonez RM, Jaime GS, Vattuone MA, Isla MI. Evaluation of the cytotoxicity, genotoxicity, mutagenicity and antimutagenicity of propolis from Tucuman, Argentina. J Agricult Food Chem. 2005; 53: 8957-62.

Mueller S, Wimmer E, Cello J. Poliovirus and poliomyelitis: a tale of guts, brains and an accidental event. Virus Res. 2005; 111: 175-93.

Murad JM, Calvi SA, Soares AMVC, Bankova V, Sforcin JM. Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against *Paracoccidioides brasiliensis*. J Ethnopharmacol. 2002; 79: 331-34.

Orsi RO, Funari SRC, Soares AMVC, Calvi SA, Oliveira SL, Sforcin JM. Immunomodulatory action of propolis on macrophage activation. J Venom Anim Toxins. 2000; 6: 205-19.

Orsi RO, Sforcin JM, Funari SRC, Bankova V. Effects of Brazilian and Bulgarian propolis on bactericidal activity of macrophages against *Salmonella* Typhimurium. Int Immunopharmacol. 2005; 5: 359-68.

Palomino SS, Abad MJ, Bedoya LM, Garcia J, Gonzales E, Chiriboga X, et al. Screening of south american plants against human immunodeficiency vírus: preliminary fractionation of aqueous extract from *Baccharis trinervis*. Biol Pharm Bull. 2002; 25: 1147-50.

Park YK, Peredes-Guzman JF, Aguiar CL, Alencar SM, Fujiwara FY. Chemical constituents in *Baccharis dracunculifolia* as the main botanical origin of southeastern Brazilian propolis. J Agricult Food Chem. 2004; 52: 1100-03.

Pinto CA, Bolzani VS, Silva DHS, Lopes NP, Epifanio RA. Produtos naturais: Atualidades, desafios e perspectivas. Quím Nova. 2002; 25: 45-61.

Salatino A, Teixiera EW, Negri G, Message D. Origin and chemical variation of Brazilian propolis. Evid Based Complement Alternat Med. 2005; 2: 33-8.

Sforcin JM, Fernandes JR A, Lopes CAM, Bankova V, Funari SRC. Seasonal effect on Brazilian propolis antibacterial activity. J Ethnopharmacol. 2000; 73: 243-49.

Sforcin JM, Fernandes JR A, Lopes CAM, Bankova V, Funari SRC. Seasonal effect of Brazilian propolis on *Candida albicans* and *Candida tropicalis*. J Venom Anim Toxins. 2001; 7: 39-44.

Sforcin JM, Kaneno R, Funari SRC. Absence of seasonal effect on the immunomodulatory action of Brazilian propolis on natural *killer* activity. J Venom Anim Toxins. 2002; 8: 19-29.

Sforcin JM, Orsi RO, Bankova V. Effect of propolis, some isolated compounds and its source plant on antibody production. J Ethnopharmacol. 2005; 98: 301-05.

Sforcin JM. Propolis and the immune system: a review. J Ethnopharmacol. 2007; 113: 1-14.

Simonet J, Gantzer C. Degradation of the poliovirus 1 genome by chlorine dioxide. J Appl Microbiol. 2006; 100: 862-70.

Spring O. Chemotaxonomy based on metabolites from glandular trichomes. Advances in Botanical Research. 2000; 31: 153-74.

Teixeira EW, Negri G, Meira RMSA, Message D, Salatino A. Plant origin of green propolis: bee behavior, plant anatomy and chemistry. Evid Based Complement Alternat Med. 2005; 2: 85-92.

Verdi LG, Brighente IMC, Pizzolatti MG. Gênero Baccharis (Asteraceae): aspectos químicos, econômicos e biológicos. Quim Nova. 2005; 28: 84-9.

*Capítulo II – Manuscrito I**

*De acordo com as normas da revista *Natural Product Research*

In vitro **cytotoxic activity of** *Baccharis dracunculifolia* **and propolis against HEp-2 cells**

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Abstract

Baccharis dracunculifolia is the most important vegetal source of propolis in southeast Brazil, and researchers have been investigating its biological properties. Propolis is a complex resinous hive product collected by bees from several plants, showing a very complex chemical composition. It has been employed since ancient times, due to its therapeutic properties, such as antimicrobial, antiinflammatory, antioxidant, immunomodulatory and antitumoral activities, among others. The goal of this work was to compare the cytotoxic action of *B. dracunculifolia*, propolis and 2 isolated compounds (caffeic and cinnamic acids) on HEp-2 cells *in vitro*. These cells were incubated with different concentrations of each variable, and cell viability was assessed by crystal violet method. Lower concentrations of *B. dracunculifolia* (extract and essential oil), propolis as well as caffeic and cinnamic acids, showed no cytotoxic activity against HEp-2 cells. On the other hand, elevated concentrations (50 and 100 μg/100μL) exerted a cytotoxic action, and propolis showed a more efficient action than its vegetal source and isolated compounds. Further investigation is still needed in order to explore the potential of these variables as antitumor agents and to understand their mechanisms of action.

Keywords: propolis; *Baccharis dracunculifolia*; HEp-2 cells; antitumor action

1. Introduction

Baccharis dracunculifolia D. C. (Asteraceae), a native plant from Brazil commonly known as “alecrim-do-campo” and “vassoura”, has been indicated as the most important botanical source of a Brazilian propolis, called green propolis because of its color (Bankova et al., 1999; Sousa et al., 2007a). It was observed that many phenolic compounds present in

B. dracunculifolia are also found in green propolis (Park et al., 2004; Sousa, et al., 2007b).

An extensive variety of chemical compounds isolated from green propolis and *B. dracunculifolia* samples have displayed plenty of biology activities. Da Silva Filho et al. (2004) have showed the presence of flavonoids (isosakuranetin, aromadendrin-4'-methyl ether) and cinnamic acid derivatives (caffeic acid, *p*-coumaric acid, ferulic acid) with trypanocidal activity, and Missima et al. (2007) identified diterpenes and triterpenes with immunomodulatory activity. Resende et al. (2007) have reported the antimutagenic activity of the hydroalcoholic extract of the leaves of this plant. Akao et al. (2003) have verified that prenylated *p*-coumaric acid derivatives (artepellin C, drupanin and baccharin), which are presented in both green propolis and this plant, exhibited antitumoral properties. In addition, Klopell et al. (2007) have found that (*E*)-nerolidol, the major constituent of the volatile fraction from *B. dracunculifolia* leaves, exhibited anti-ulcer activity.

Propolis is a natural resinous substance used to seal holes in the hive, smooth out the internal walls and to protect the entrance against intruders (Bankova, 2005). This product has attracted researcher's interest in the last decades because of its several biological and pharmacological properties, such as immunomodulatory, antitumoral, antimicrobial, antiinflammatory, antioxidant, among others (Salatino et al., 2005). Our group has been investigating mainly its immunomodulatory, antimicrobial and antitumoral activities (Sforcin, 2007). Previous works of on laboratory confirmed the cytotoxic action of propolis on canine transmissible venereal tumor cells in a concentration- and time- dependent manner *in vitro* (Bassani-Silva et al., in press).

Human laryngeal epidermoid carcinoma (HEp-2) cell line is derived from laryngeal carcinoma cells of human nasopharyngeal mucosa. Slow-growing tumors, these cells develop in animals hosts as wells as in tissue culture (Lima et al., 2005). Propolis effects on HEp-2 cells were previously investigated (Búfalo et al., in press); however, the effects of *B.*

dracunculifolia on these cells have not been investigated yet. In the present work, we compared propolis effects with *B. dracunculifolia* (extracts and essential oil), in order to verify its possible cytotoxic action of on HEp-2 cells *in vitro*. The effects of caffeic and cinnamic acids were also evaluated, and cell viability was assessed after incubation with different concentrations of each variable.

2. Materials and Methods

2.1. Plant material, oil and extract isolation and major compounds

B. dracunculifolia leaves were collected in ten different regions of Brazil (São Paulo, Minas Gerais and Paraná State) in their natural habitat, between May 2004 and April 2005. Plants were identified by Dr. Nelson Ivo Matzenbacher (Department of Bioscience - PUC/RS - Brazil), and voucher specimens (n^o 1298) were deposited in the Herbarium of the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA), University of Campinas, São Paulo, Brazil.

Extracts of *B. dracunculifolia* were obtained from dried and powdered leaves samples (500 mg), dissolved in 20 mL of 90% ethanol in 125 mL erlenmeyer flasks, which were stirred at 40 ºC and 170 rpm on a shaker (Innova 4300). After two hours, flasks were cooled down to room temperature and filtered using analytical filter papers. Aliquots (5.0 mL) of the hydroalcoholic extracts were transferred to an appropriate vial and dried under air circulation (40 ºC). The yielding of crude extracts from *B. dracunculifolia* (90 mg) was of 18%.

The essential oil of the dried leaves (500 g) was extracted by hydro-distillation using a Clevenger type apparatus. After extraction, the volume of essential oil obtained was measured and the essential oil was stored in hermetically sealed glass containers with rubber lids, covered with aluminum foil to protect the contents from light and kept under

refrigeration at 8 ºC. The oil yield was 0.6% based on the dry weight of the plant. Caffeic and cinnamic acids were purchased from Acros Organics (Morris Plains, NJ, USA).

Extracts, caffeic and cinnamic acids were diluted in 100µL of 70% ethanol. Essential oil (1 mL) was diluted in culture medium containing 0.2% dimethylsulfoxide (DMSO – Sigma-Aldrich, USA).

Specific dilutions of each variable (*Baccharis* extract and essential oil, propolis, caffeic and cinnamic acids) were prepared for each assay in order to achieve: 5, 10, 25, 50 and 100 µg/100µL. The same procedure was carried out with 0.2% DMSO (essential oil solvent) and 70% ethanol (extract and isolated compounds solvent).

2.2. Propolis sample

Propolis was collected in the Beekeeping Section, UNESP, Campus of Botucatu, Brazil. Propolis was ground and 30% ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 mL with 70% ethanol), in the absence of bright light, at room temperature, with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated (120 mg/mL). Propolis chemical composition was investigated using thin-layer chromatography (TLC), gas-chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) analysis (Bankova et al., 1998).

Propolis was diluted in minimum essential medium (MEM – GIBCO, USA) supplemented with 0.1 g/L of L-glutamine, 2.2 g/L sodium bicarbonate, 10 mL/L non-essential amino acids and 10% fetal calf serum (LGC, Biotecnologia LTDA., Brazil), and afterwards specific dilutions were prepared for each assay in order to achieve different propolis concentrations: 5, 10, 25, 50 and 100 µg/well. The same procedure was carried out

with 70% ethanol (propolis solvent), in order to obtain 0.03, 0.06, 0.15, 0.29 and 0.59% ethanol, which are the respective concentrations of alcohol in propolis concentrations.

2.3. HEp-2 cells

HEp-2 cells were grown in MEM supplemented with 10% fetal calf serum. Cells were cultured in 25 cm^2 flasks and further washed with 10 mL of MEM. Afterwards, 2 mL of trypsin (0.2% trypsin in 5% EDTA) were added to each flask until cells detachment. Cells were counted using a hemocytometer and cultured in a 96 well U-bottom plate (Corning, USA) at a final concentration of 2 x 10^5 cells/well.

2.4. Cytotoxicity assay

HEp-2 cells cultures were incubated at 37 ºC and 5% CO_2 for 48h in the presence of different concentrations of *B. dracunculifolia* (extracts and essential oil), propolis, caffeic and cinnamic acids (5, 10, 25, 50 and 100 µg/100µL) and with their solvents (70% ethanol and 0.2% DMSO). Control cells were incubated with medium alone. After incubation, the medium was removed and cell viability was evaluated by crystal violet method (Ait-Mbarek et al., 2007), adding 100 µL 0.5% crystal violet solution. After 10 minutes of incubation at room temperature, plates were washed and viable crystal violet-stained cells were lysed with 1% sodium dodecyl sulfate (SDS). Optical densities (O.D.) were read at 492 nm and the percentage of cell viability was calculated, using the formula: [O.D. test / O.D. control] x 100. Assays were carried out in triplicate.

2.5. Statistical analysis

Significant differences between treatments were assessed by Friedman non-parametric test and Kruskal-Wallis test, with the level of significance at $p < 0.05$ (Zar, 1999).

3. Results and Discussion

Phenolic compounds are secondary metabolites, which occur in abundance in all plant material, belonging to a large and heterogeneous group of biologically active non-nutrients. Their synthesis depends on several enzymes involved in different metabolic pathways and their metabolism is completely integrated into morphological and biochemical regulatory patterns of plants. Stress caused by pathogens, adverse environmental conditions or wounding affects the biosynthesis of phenolic compounds, and one of the well-known and important function of phenols is their action on plant defense mechanisms (Solar et al., 2006).

Detailed analysis carried out by Sousa et al. (in press) reported the chemical composition of essential oil and crude extract from *B. dracunculifolia* used in present work. Ten samples of extracts and essential oil of *B. dracunculifolia* were used in the beginning of this project, and their biological behavior was similar. Thus, one extract and one essential oil were chosen, and their chemical composition is showed in Figures 1 and 2, respectively. Analysis of essential oil of this plant identified 14 compounds, accounting for 70% of the volatile constituents. The main groups of constituents in all samples were composed by oxygenated sesquiterpenes (49.61%), followed by sesquiterpene hydrocarbons (11.83%), minoritary oxygenated sesquiterpenes (7.59%) and monoterpenes hydrocarbons (0.97%). The major oxygenated sesquiterpenes were (*E*)-nerolidol (32%) and spathulenol (17%). With respect to phenolic compounds, caffeic, *p*-coumaric, ferulic, cinnamic, and 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran (DCBEN) acids, along with isosakuranetin,

aromadendrin-4′-methyl ether – AME, baccharin, drupanin and artepillin C were the major phenolic compounds in hydroalcoholic extracts of both *B. dracunculifolia* and green propolis (Sousa et al., 2007b), which can guarantee the authenticity of plant material, propolis resin and their products. These studies corroborate the use of this plant for the production of green propolis by honeybees.

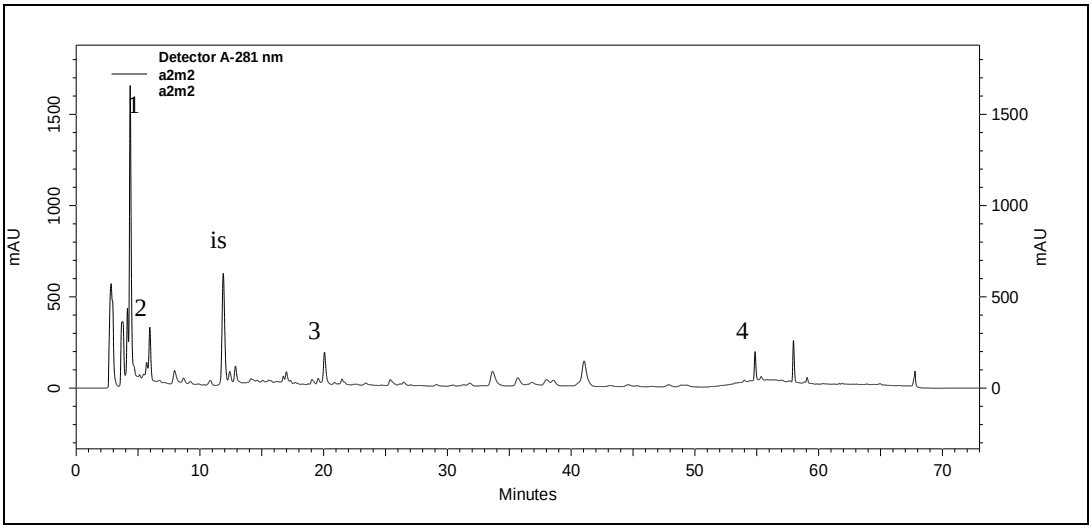

Figure 1. HPLC profile of *B. dracunculifolia* extract: caffeic acid (1); ferulic acid (2); aromedendrin-4'-methyl ether (3) and artepellin C (4), internal standard (is) (veratraldehyde).

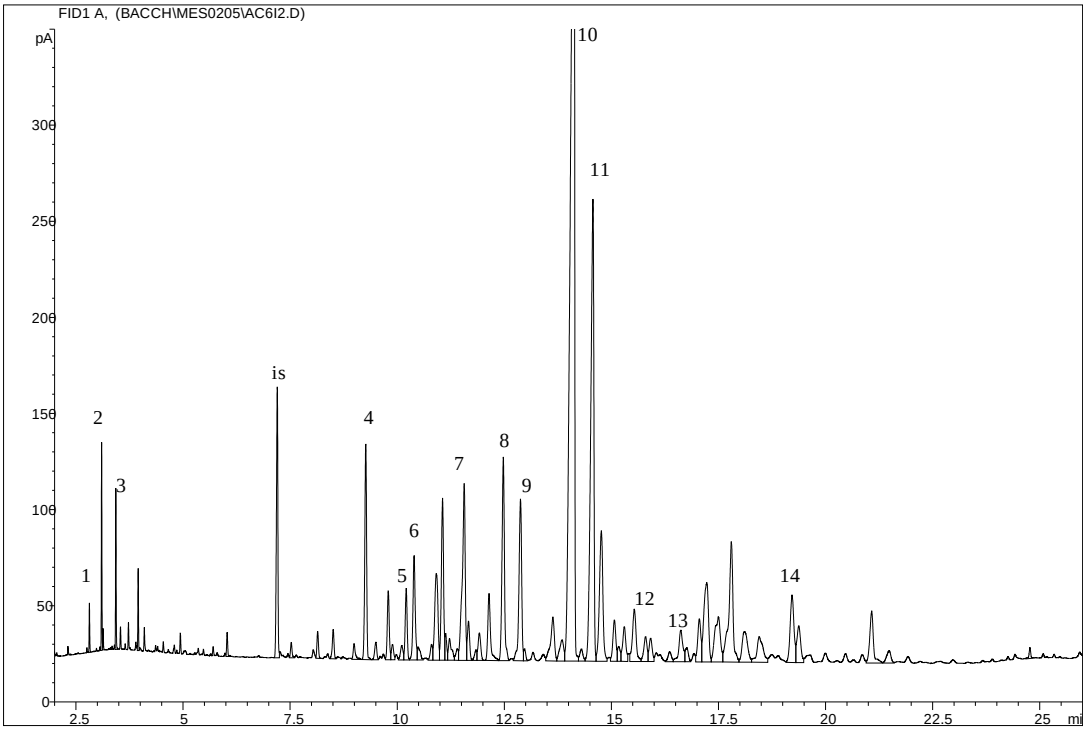

Figure 2. Chromatographic profile of *B. dracunculifolia* essential oil: α-pinene (1), β-pinene (2), limonene (3), trans-caryophyllene, (4), aromadendrene (5), α-humulene (6), germacrene D (7), bicyclogermacrene (8), δ-cadinene (9), nerolidol (10), spathulenol (11), viridiflorol (12), guaiol (13), α-muurolol (14) and internal standard (is) (piperonal).

In order to investigate the biological properties of *B. dracunculifolia*, this work aimed to analyze its cytotoxic action against tumor (HEp-2) cells. Propolis and some isolated compounds (caffeic and cinnamic acids) were also investigated in these assays. Table 1 and Figure 3 show the percentage of cell viability after incubation with each variable in different concentrations.

Table 1. Median, 1st and 3rd quartiles between brackets of cell viability percentage of each variable according to concentration.

Variable	5 µg	10 µg	25 µg	50 µg	100 µg	*p* *
Propolis	64.6 aB [62.6-88.3]	66.2 aB [64.78-72.8]	28.0 abB [19.5-32.2]	21.0 abC [8,70-24,5]	20.1 bA [8.4-21.8]	0.02
Extract	94.8 aB [92.9-96.9]	89.1 aAB [87.5-98.3]	86.1 aA [84.2-93.9]	76.5 abB [74.6-84.2]	13.0 bA [11.73-57.79]	0.02
Essential oil	102.2 aA [99.9-115.2]	101.3 aA [100.3-101.3]	96.7 aA [92.4-98.2]	85.7 abB [77.1-88.6]	16.9 bA [16.53-18.8]	0.02
Cafeic acid	75.6 aB [73.95-76.43]	68.9 abB [67.48-69.7]	65.2 abAB [62.2-62.6]	60.0 abB [59.55-66.08]	19.4 bA [17.6-19.85]	0.03
Cinnamic acid	65.3 aB [63.80-75.28]	71.6 aB [65.75-75.8]	63.1 aAB [60.7-63.8]	55.4 abB [54.1-56.3]	49.4 bA [49.0-50.2]	0.03
Ethanol	80.9 aB [74.45-85.25]	78.5 aB [77.3-82.7]	94.6 aA [90.5-95.5]	83.8 aB [77.3-88.5]	94.6 aA [91.75-96.78]	0.06
DMSO	92.5 aB [89.88-97.30]	94.6 aB [93.7-97.08]	92.8 aA [90.7-97.4]	95.7 aA [94.2-97.7]	99.3 aA [33.3-99.6]	0.08
p #	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p > 0.05$	

Different small letters indicate significant differences between each concentration of same variable by Friedman test (*). Different capital letters indicate significant differences between each variable in the same concentration by Kruskal-Wallis test (#).

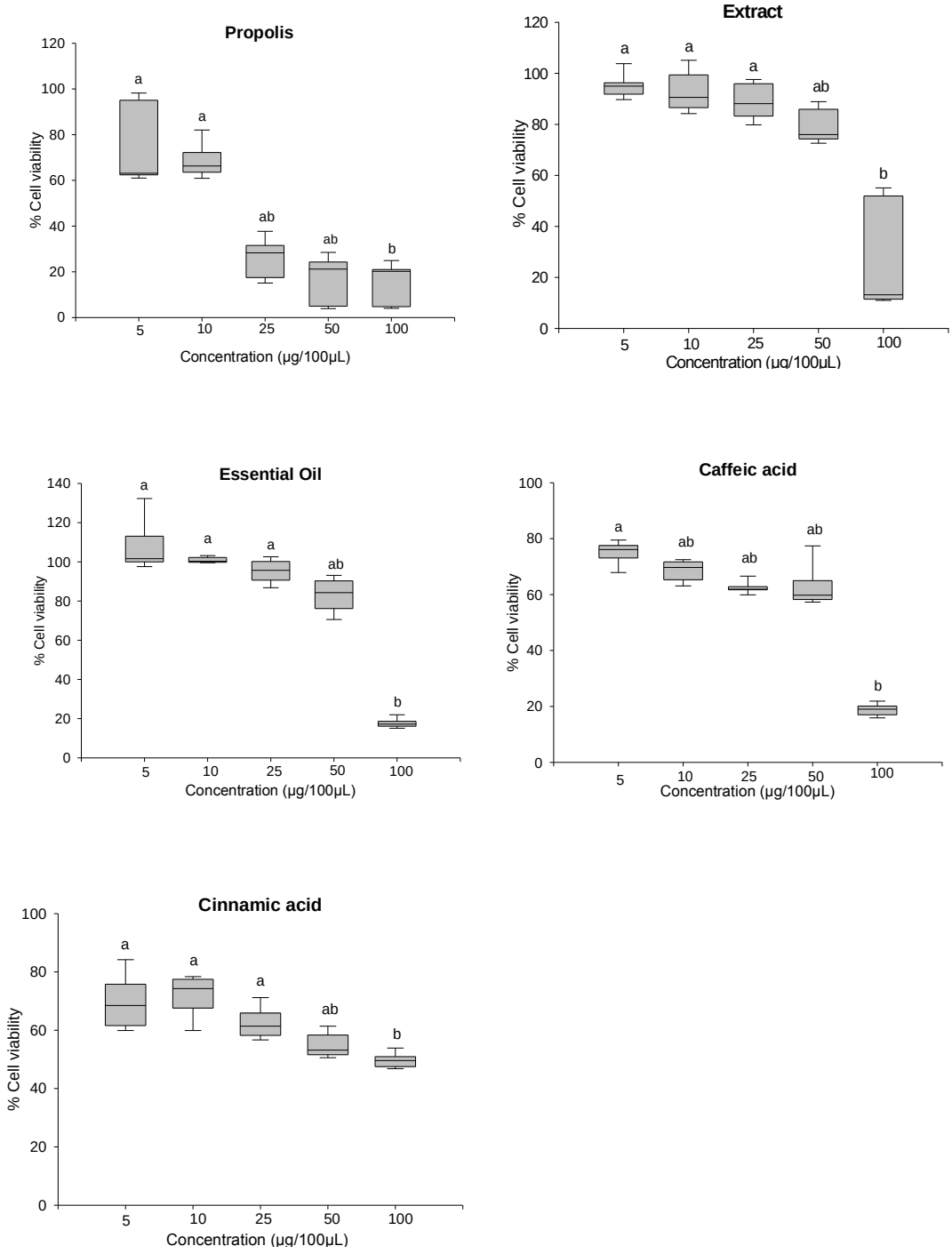

Figure 3. *Box-Plot* representing the median of cell viability percentage after 48 h incubation with different concentrations of *B. dracunculifolia* (extract and essential oil), propolis, caffeic and cinnamic acids. Data represent 3 similar assays. Lower and upper quartiles represent 25% and 75% of values, respectively; the horizontal lines represent the median, and error bars correspond to 5th and 95th percentiles.

B. dracunculifolia (extract and essential oil – 5, 10 and 25 μg/100μL) had no effect in cell viability ($p > 0.05$). However, higher concentrations exhibited a cytotoxic action against HEp-2 cells ($p < 0.05$) in a concentration-dependent way. Fukuda et al. (2006) have reported that the leaf extracts from *B. dracunculifolia* and isolated compounds (sesquiterpene and terpene phenols) exhibited a potent cytotoxic activity against leukemia cells (L1210).

As to propolis, the main constituents of our propolis sample were investigated by TLC, GC and GC-MS: flavonoids were present in small quantities (kaempferid, 5,6,7-trihydroxy-3,4'-dimethoxyflavone, aromadendrine-4'-methyl ether); a prenylated *p*-coumaric acid and two benzopyranes: *E* and *Z* 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes); essential oils (spathulenol, (2Z,6E)-farnesol, benzyl benzoate and prenylated acetophenones); aromatic acids (dihydrocinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, 3,5-diprenyl-*p*-coumaric acid, 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzo-pyran); di- and triterpenes were also found, among others (Bankova et al., 1998).

Our data showed that 5 and 10 μg/100μL of propolis had no effect in cell viability ($p > 0.05$), whereas higher concentrations (25, 50 and 100 μg/100μL) had an efficient activity against HEp-2 cells, in a concentration-dependent manner ($p < 0.05$). These data are in agreement with those previously reported by our group, using the same concentrations of propolis: with 5 and 10 μg/100μL, HEp-2 cells showed a typical morphology and epithelioid-shaped cells were observed, with few rounded cells. On the other hand, changes in cell morphology such as lysis and disorganization of the monolayer were seen after incubation with 25, 50 and 100 μg/100μL (Búfalo et al., in press). Propolis possesses a significant cytotoxicity towards various tumor cells. It was observed its antiproliferative

activity against human HT-1080 fibrosarcoma, and human lung A549 adenocarcinoma (Banskota et al., 2002).

It has been reported that the antiproliferative action of propolis on tumor cells may be the result of the synergistic effect of its constituents. Kujumgiev et al. (1999) suggested that the biological properties of propolis are due to a natural mixture of its components, and a single propolis constituents does not have an activity greater than that of the total extract. In order to investigate the antitumoral action of isolated compounds, found both in propolis and in *B. dracunculifolia*, as well as to understand their mechanisms of action, caffeic and cinnamic acids were evaluated in our assays. Cinnamic acid (5, 10 and 25 μg/100μL) and caffeic acid (5 μg/100μL) had no effect in cell viability in vitro ($p > 0.05$), and cytotoxic effects were seen using higher concentrations ($p < 0.05$).

Caffeic acid phenethyl ester (CAPE) is one of propolis constituents most investigated with regards to the antitumor action (Castaldo and Capasso, 2002; Kartal et al., 2003), although other phenolic compounds and diterpenoids isolated from propolis also have antitumor properties (Banskota et al., 2001). CAPE had a dose-dependent effect on the cytotoxicity of the C6 glioma cells, increasing the proportion of hypodiploid DNA, as an indication of apoptosis (Lee et al., 2003). CAPE also inhibited the enzymatic activity of matrix metalloproteinases, which is often found to be elevated in tumor tissues and malignant cells (Jin et al., 2005). CAPE derivatives were investigated on oral cancer cell line and normal human oral fibroblast, showing cytotoxic effects on tumor cells but not on normal ones (Lee et al., 2005). According to Jin et al. (2008), the treatment of human myeloid leukemia U937 cell line with CAPE decreased cell viability in a dose- and time-dependent manner, probably due to apoptosis.

Cinnamic acid derivatives from propolis (baccharin and drupanin) exhibited antitumor activity *in vitro* and this effect was due to apoptosis in human myelocytic

leukemia cell line (HL60) (Akao et al., 2003). The cytotoxic properties of propolis and *B. dracunculifolia* may also be explained due to flavonoids or other phenolic compounds (Moreno et al., 2005).

Ethanol and DMSO had no effects on cell viability in our assays, evidencing that the cytotoxic effects were exclusively due to the variables.

On the basis of these results, our data reinforce propolis' antitumoral activity *in vitro*, previously related by our group and other researchers (Sforcin, 2007). Moreover, *B. dracunculifolia* showed a cytotoxic activity against tumor cells, but not as efficient as propolis. Since caffeic and cinnamic acids also showed a cytotoxic activity, one may speculate that these acids may be involved in the mechanisms of action of propolis and *B. dracunculifolia* against HEp-2 cells. Further investigation is still needed in order to explore the potential of these variables as antitumor agents and to understand their mechanisms of action.

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References

Ait-Mbarek, L.A., Mouse, H.A., Elabbadi, N., Bensalah, M., Gamouh, A., Aboufatima, R., Benharref, A., Chait, A., Kamal, M., Dalal, A., Zyad, A. (2007). Anti-tumor properties of blackseed (*Nigella sativa* L,) extracts. Brazilian Journal of Medical and Biological Research 40, 839-847.

Akao, Y., Maruyama, H., Matsumoto, H., Ohguchi, K., Nishigawa, K., Sakamoto, T., Araki, Y., Mishima, S., Nozawa, Y. (2003). Cell growth inhibitory effect of cinnamic acid derivates from propolis on human tumor cell lines. Biological and Pharmaceutical Bulletin 26, 1057-1059.

Bankova, V.S., Boudourova-Krasteva, G., Popov, S., Sforcin, J.M., Funari, S.R.C. (1998). Seasonal variations of the chemical composition of Brazilian propolis. Apidologie 29, 361-367.

Bankova, V.S., Boudourova-Krasteva, G., Sforcin, J.M., Frete, X., Kujumgiev, A., Rodella, R.M., Popov, S. (1999). Phytochemical evidence for the plant origin of Brazilian propolis from São Paulo State. Zeitschrift für Naturforschung 54, 401-405.

Bankova, V.S. (2005). Recent trends and important developments in propolis research. Evidence-based Complementary and Alternative Medicine 2, 29-32.

Banskota, A.H., Tezuka, Y., Kadota, S. (2001). Recent progress in pharmacological research of propolis. Phytotherapy Research 15, 561-571.

Banskota, A.H., Nagaoka, T., Sumioka, L.Y., Tezuka, Y., Awale, S., Midorikawa, K., Matsushige, K., Kadota, S. (2002). Antiproliferative activity of the Netherlands propolis and its active principles in cancer cell lines. Journal of Ethnopharmacology 80, 67-73.

Bassani-Silva, S., Sforcin, J.M., Amaral, A.S., Gaspar, L.F.J., Rocha, N.S. (in press). Propolis effect *in vitro* on canine transmissible venereal tumor cells. Revista Portuguesa de Ciências Veterinárias.

Búfalo, M.C., Candeias, J.M.G., Sforcin, J.M. (in press). In vitro cytotoxic effect of Brazilian green propolis on human laryngeal epidermoid carcinoma (HEp-2) cells. Evidence-based Complementary and Alternative Medicine.

Castaldo, S., Capasso, F. (2002). Propolis, an old remedy used in modern medicine. Fitoterapia 73, S1-S6.

Da Silva Filho, A.A., Bueno, P.C.P., Gregório, L.E., Andrade e Silva, M.L., Albuquerque, S., Bastos, J.K. (2004). In-vitro trypanocidal activity evaluation of crude extract and isolated compounds from *Baccharis dracunculifolia* D. C. (Asteraceae). Journal of Pharmacy and Pharmacology 56, 1195-1199.

Fukuda, M., Ohzoshi, E., Makino, M., Fujimoto, Y. (2006). Studies on the constituents of the leaves of *Baccharis dracunculifolia* (Asteraceae) and their cytotoxic activity. Chemical & Pharmaceutical Bulletin 54, 1465-1468.

Jin, U.H., Chung, T.W., Kang, S.K., Suh, S.J., Kim, J.K., Chung, K.H., Gu, Y.H., Suzuki, I., Kim, C.H. (2005). Caffeic acid phenyl ester in propolis is a strong inhibitor of matrix metalloproteinase-9 and invasion inhibitor: isolation and identification. Clinica Chimica Acta 362, 57-64.

Jin, U.H., Song, K.H., Motomura, M., Suzuki, Y., Gu, Y.H., Kang Y.J., Moon, T.C., Kim, C.H. (2008). Caffeic acid phenethyl induces mitochondria-mediated apoptosis in human myeloid leukemia U937 cells. Molecular and Cellular Biochemistry 310, 43-48.

Kartal, M., Yildiz, S., Kaya, S., Kurucu, S., Topçu, G. (2003). Antimicrobial activity of propolis samples from two different regions of Anatolia. Journal of Ethnopharmacology 86, 69-73.

Klopell, F.C., Lemos, M., Sousa, J.P.B., Comunello, E., Maistro, E.L., Bastos, J.K., Andrade, S.F. (2007). Nerolidol, an antiulcer constituent from essential oil of *Baccharis dracunculifolia* DC (Asteraceae). Zeitschrift für Naturforschung 62c, 537-542.

Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Christov, R., Popov, S. (1999). Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. Journal of Ethnopharmacology 64, 235-240.

Lee, Y.J., Kuo, H.C., Chu, C.H., Wang, C.J., Lin, W.C., Tseng, T.H. (2003). Involvement of tumor suppressor protein p53 and p38 MAPK in caffeic acid phenethyl ester-induced apoptosis of C6 glioma cells. Biochemical Pharmacology 66, 2281-2289.

Lee, Y.T., Don, M.J., Hung, P.S., Shen, Y.C., Lo, Y.S., Chang, K.W., Chen, C.F., Ho, L.K. (2005). Cytotoxic of phenolic acid phenethyl esters on oral cancer cells. Cancer Letters 223, 19-25.

Lima, M.E., Rissino, J.D., Guimarães, A.C., Overal, D.J., Khayat, A.S., Souza, P.C. (2005). Drifter technique: a new method to obtain metaphases in HEp-2 cell line cultures. Brazilian Archives of Biology and Technology 48, 537-340.

Missima, F., Silva, Filho, A.A., Nunes, G.A., Bueno, P.C.P., De Sousa, J.P.B., Bastos, J.K., Sforcin, J.M. (2007). Effects of *Baccharis dracunculifolia* D.C. (Asteraceae) extracts and its isolated compounds on macrophage activation. Journal of Pharmacy and Pharmacology 59, 463-468.

Moreno, M.I.N., Zampini, I.C., Ordonez, R.M., Jaime, G.S., Vattuone, M.A., Isla, M.I. (2005). Evaluation of the cytotoxicity, genotoxicity, mutagenicity and antimutagenicity of propolis from Tucuman, Argentina. Journal of Agricultural and Food Chemistry 53, 8957-8962.

Park, Y.K., Paredes-Guzman, J.F., Aguiar, C.L., Alencar, S.M., Fujiwara, F.Y. (2004). Chemical constituents in *Baccharis dracunculifolia* as the main botanical origin of southeastern Brazilian propolis. Journal of Agricultural and Food Chemistry 52, 1100-1103.

Resende, F.A., Alves, J.M., Munari, C.C., Senedese, J.M., Sousa, J.P.B., Bastos, J.K., Tavares, D.C. (2007). Inhibition of doxorubicin-induced mutagenicity by *Baccharis dracunculifolia*. Mutation Research 634, 112-118.

Salatino, A., Teixeira, E.W., Negri, G., Message, D. (2005). Origin and chemical variation of Brazilian propolis. Evidence-based Complementary and Alternative Medicine 2, 33-38.

Sforcin, J.M. (2007) Propolis and the immune system: a review. Journal of Ethnopharmacology 113, 1-14.

Solar, A., Colaric, M., Usenik, V., Stampar, F. (2006). Seasonal variations of selected flavonoids, phenolic acids and quinones in annual shoots of common walnut (*Juglans regia* L.). Plant Science 170, 453–461.

Sousa J.P.B., Bueno, P.C.P., Gregório, L.E., Da Silva Filho, A.A., Furtado, N.A.J.C., De Sousa, M.L., Bastos, J.K. (2007b). A reliable quantitative method for the analysis of phenolic compounds in Brazilian propolis by reverse phase high performance liquid chromatography. Journal of Separation Science 30, 2656-2665.

Sousa, J.P.B., Furtado, N.A.J.C., Jorge, R., Soares, A.E.E., Bastos, J.K. (2007a). Perfis físico-químico e cromatográfico de amostras de própolis produzidas nas microrregiões de Franca (SP) e Passos (MG), Brasil. Revista Brasileira de Farmacognosia 17, 85-93.

Sousa, J.P.B., Jorge, R.F., Da Silva Filho, A.A., Furtado, N.A.J.C., Leite, M.F., Queiroga, C.L., Magalhães, P.M., Soares, A.E.E., Bastos, J.K. Seasonal variation of the (*E*)-nerolidol and other volatile compounds within ten different cultivated populations of *Baccharis dracunculifolia* D.C. (Asteraceae). Journal of Essential Oil Research, in press.

Zar, J.H. (1999). Biostatistical analysis. 4th ed. New Jersey: Pretince Hall

*Capítulo III– Manuscrito II**

*De acordo com as normas da revista *Antiviral Research*

Antiviral activity of *Baccharis dracunculifolia* **and propolis and poliovirus quantification by real-time PCR**

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ABSTRACT

Baccharis dracunculifolia is the most important botanical source of the southeastern Brazilian propolis, and its potential for the development of new phytotherapeutic medicines has been investigated. Propolis is commonly used for its antimicrobial and immunomodulatory activities. Nevertheless, *B. dracunculifolia* and propolis effects on poliovirus type 1 (PV1) were not investigated yet. Thus, the aim of this work was to evaluate the antiviral activities of *B. dracunculifolia* (extract and essential oil), propolis and some isolated compounds (caffeic and cinnamic acids) against PV1 replication in HEp-2 cells in three different protocols (pre-, simultaneous and post-treatment), to verify the effect of addition time of the variables on PV1 replication by crystal violet method and relative viral RNA quantification by real-time PCR, and to analyze in which step of virus replication the variables could interfere. Data revealed that the *B. dracunculifolia* (25 μg/100 μL) and propolis (10 μg/100 μL) showed the best antiviral activity percentages in the simultaneous treatment, and lower relative viral quantification in real-time PCR. Some explanations are proposed, however, new investigation is still needed in order to explore the potential of these variables as antiviral agents and to understand their mechanisms of action against PV1 cycle replication.

Keywords: Poliovirus; Antiviral action; *Baccharis dracunculifolia*; Propolis; Real-time PCR

1. Introduction

A variety of natural products and their derivatives have been considered as potential candidates for the treatment of human viral diseases (Huleihel and Isanu, 2002; Gekker et al., 2005). Recently, the inhibitory effects of medicinal plants extracts on the replication of

several viruses have been reported. Herpes simplex virus (HSV), human immunodeficiency virus (HIV), hepatitis B virus (HBV) and severe acute respiratory syndrome (SARS) virus were strongly inhibited by various plants extracts (Mukhtar et al., 2008).

Poliovirus (PV) is a single-stranded RNA, non-enveloped virus, belonging to the family Picornaviridae and genus *Enterovirus*. It is the etiologic agent of poliomyelitis, and once it reaches the central nervous system, one may develop paralytic poliomyelitis – a disease characterized by a classic manifestation of flaccid paralysis. The incidence of paralytic poliomyelitis has been reduced over the last decades, especially by the systematic use of vaccines; however, this disease is still endemic in Asia and Africa (Felipe et al., 2006). Because of its replication in several types of cultured cells, PV is one of the most studied and understood viral models (Faccin et al., 2007).

As to PV, extracts of *Tridax procumbens, Carissa carandas, Mallotus philippensis, Agaricus brasiliensis, Euphorbia grantii,* among others were found to be efficient against the replication of poliovirus type 1 (PV1) (Semple et al., 2001; Felipe et al., 2006). A potent activity against HIV and HSV in vitro was observed using extracts of *Baccharis trinervis* (Palomino et al., 2002). In the present study, we evaluated the effect of *Baccharis dracunculifolia* against PV1 replication.

B. dracunculifolia is used in folk medicine as an anti-inflammatory agent and for the treatment of gastrointestinal diseases. This plant it is the most important botanical origin of Brazilian propolis, called green propolis because of its color (Bankova et al., 1999; Da Silva Filho et al., 2004).

Propolis is the generic name for the resinous substance produced by honeybees and commonly used to improve health and to prevent several diseases. It has been used for medicinal purposes since ancient times, and its antimicrobial, antitumoral, and immunomodulatory activities have been reported (Sforcin, 2007). With regards to propolis

antiviral properties, its inhibitory effect on several virus including influenza, HIV, HSV, adenovirus and vesicular stomatitis virus has been well documented (Ito et al., 2001; Gekker et al., 2005; Moreno et al., 2005). These findings have indicated the potential of propolis as a possible antiviral drug. However, *B. dracunculifolia* antiviral effects have not been investigated yet.

In the present study, we used PV1 as a model for the investigation of the antiviral activity of *B. dracunculifolia* (extract and essential oil), propolis and isolated compounds (caffeic and cinnamic acids). These variables were incubated with HEp-2 cells prior, simultaneously or subsequently to PV1 addition to the cell cultures in order to understand their mechanism of action.

2. Materials and methods

2.1.Plant material, oil and extract isolation and major compounds

B. dracunculifolia leaves were collected in ten different regions of Brazil (São Paulo, Minas Gerais and Paraná State) in their natural habitat, between May 2004 and April 2005. Plants were identified by Dr. Nelson Ivo Matzenbacher (Department of Bioscience - PUC/RS - Brazil), and voucher specimens (n^{o} 1298) were deposited in the Herbarium of the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA), University of Campinas, São Paulo, Brazil.

Extracts of *B. dracunculifolia* were obtained from dried and powdered leaves samples (500 mg), dissolved in 20 mL of 90% ethanol in 125 mL erlenmeyer flasks, which were stirred at 40 ºC and 170 rpm on a shaker (Innova 4300). After two hours, flasks were cooled down to room temperature and filtered using analytical filter papers. Aliquots (5.0 mL) of the hydroalcoholic extracts were transferred to an appropriate vial and dried under

air circulation (40 ºC). The yielding of crude extracts from *B. dracunculifolia* (90 mg) was of 18%.

The essential oil of the dried leaves (500 g) was extracted by hydro-distillation using a Clevenger type apparatus. After extraction, the volume of essential oil obtained was measured and the essential oil was stored in hermetically sealed glass containers with rubber lids, covered with aluminum foil to protect the contents from light and kept under refrigeration at 8 ºC. The oil yield was 0.6% based on the dry weight of the plant. High performance liquid chromatography (HPLC) profile of *B. dracunculifolia* extract and flame ionization detector (FID) capillary gas chromatogram of the essential oil were carried out (Sousa et al., 2007).

Caffeic and cinnamic acids were purchased from Acros Organics (Morris Plains, NJ, USA).

Extract, caffeic and cinnamic acids were diluted in 100 µL of 70% ethanol, and subsequently diluted in minimum essential medium (MEM – GIBCO, USA) supplemented with 0.1 g/L of L-glutamine, 2.2 g/L sodium bicarbonate, 10 mL/L non-essential amino acids and 10% fetal calf serum (LGC, Biotecnologia LTDA., Brazil). Essential oil (1 mL) was diluted in culture medium containing 0.2% dimethylsulfoxide (DMSO – Sigma-Aldrich, USA).

Specific dilutions of each variable (*Baccharis* extract and essential oil, caffeic and cinnamic acids) were prepared for each assay in order to achieve 5, 10, 25, 50 and 100 µg/100 µL. The same procedure was carried out with 0.2% DMSO (essential oil solvent) and 70% ethanol (extract and isolated compounds solvent), to obtain the respective concentrations of DMSO in the essential oil, and of alcohol in the extract, caffeic and cinnamic acids.

2.2. Propolis sample

Propolis was collected in the Beekeeping Section, UNESP, Campus of Botucatu, Brazil. Propolis was ground and 30% ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 mL with 70% ethanol), in the absence of bright light, at room temperature, with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated (120 mg/mL). Propolis chemical composition was investigated using thin-layer chromatography (TLC), gas-chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) analysis (Bankova et al., 1998). Propolis was diluted in MEM, and specific dilutions were prepared to achieve 5, 10, 25, 50 and 100 µg/100 µL. The same procedure was carried out with 70% ethanol (propolis solvent).

2.3. Cells and virus

HEp-2 cells (human laryngeal epidermoid carcinoma cells) were stored in liquid nitrogen. Afterwards, cells were cultured in flasks with MEM supplemented with 10% fetal calf serum and, before use, 2 mL of trypsin (0.2% trypsin in 5% EDTA) were added to each flask until cells detachment. Cells were counted using a haemocytometer and cultured in a 96 well flat-bottomed plate (Corning, USA), at a final concentration of 2×10^5 cells/well.

Poliovirus type 1 (vaccinal strain Sabin I, stored at –80 ºC in our laboratory) was propagated in HEp-2 cells cultures and maintained at –80 ºC. The virus titer was determined according to Reed and Muench method (Lennette, 1995) and expressed in 50% tissue culture infectious dose ($TCID_{50}$).

2.4. Cytotoxic assay

Prior to antiviral assays, cell viability after incubation with each variable in different concentrations was assessed, in order to carry out the assays only with non-cytotoxic concentrations. The evaluation of citotoxicity was carried out by crystal violet method (Ait-Mbarek et al., 2007).

HEp-2 cells grown in flat-bottomed 96-well microplates were incubated with different concentrations (5, 10, 25, 50 and 100 μg/100 μL) of *B. dracunculifolia* (extract and essential oil), propolis, caffeic and cinnamic acids, as well as with 70% ethanol and 0.2% DMSO. Control cells were incubated with medium alone. The final volume in each well was 100 μL.

Cell viability was determined after 48 h incubation at 37 ºC and 5% CO_2. The medium was removed and 100 μL of 0.5% crystal violet solution were added to the cells. After 10 min of incubation at room temperature, plates were washed and viable crystal violet-stained cells were lysed with 1% sodium dodecyl sulphate (SDS). Optical densities (O. D.) were read at 492 nm in an ELISA reader and the percentage of cell viability was calculated using the formula: [O. D. test / O. D. control] x 100. Assays were carried out in triplicate.

2.5. Antiviral assays

The antiviral effect of the variables on PV1 replication was determined according to Faccin et al. (2007) with minor modifications, in 3 different protocols, as follows:

Pre-treatment: HEp-2 cells were resuspended at a concentration of 2 x 10^6 cells/mL in MEM, cultured in flat-bottomed 96-well plates and incubated with the variables at non-cytotoxic concentrations: *B. dracunculifolia* (extract and essential oil), caffeic and cinnamic acids = 5, 10 and 25 μg/100 μL; propolis = 5 and 10 μg/100 μL. After 2 h, the medium was

removed and 100 $TCID_{50}$/100 µL ($10^{2,5}$) of virus suspension were added and incubated for 48h at 37 ºC and 5% CO_2.

Simultaneous treatment: HEp-2 cells (2 x 10^6 cells/mL) were incubated simultaneously with non-cytotoxic concentrations of each variable and 100 $TCID_{50}$/100 µL ($10^{2,5}$) of virus suspension for 48 h.

Post-treatment: HEp-2 cells (2 x 10^6 cells/mL) were incubated with 100 $TCID_{50}$/100 µL ($10^{2,5}$) of virus suspension for 2 h for virus adsorption. After incubation, the medium was removed and variables at non-cytotoxic concentrations were added and incubated for 48 h.

Cells with virus but without variables were considered as a positive control. Each variable was evaluated in triplicate and assays were repeated three times. The percentage of antiviral activity, corresponding to viable HEp-2 cells, was assessed by crystal violet method as described above.

In these same conditions, other plates were prepared for relative viral RNA quantification by real-time polymerase chain reaction (real-time PCR) in order to verify in which step of virus replication the variables could interfere.

2.6. Quantification of the viral genome

After incubation with *B. dracunculifolia* (extract and essential oil), propolis, caffeic and cinnamic acids, 100 µL of supernatant were collected and centrifuged at 400 x g for 15 min to remove cellular debris. Afterwards, the cell monolayer was treated with 100 µL of lysis buffer of the RNAspin Mini RNA Isolation Kit (GE – Healthcare, USA) and the lysate

was collected. Samples of supernatant and cell lysates were submitted to total RNA extraction.

2.6.1. Extraction of total RNA and *cDNA synthesis*

Total RNA was extracted using the RNAspin Mini RNA Isolation Kit (GE – Healthcare, USA), according to the manufacturer's instructions. Extracted RNA preparations were stored at –80 ºC.

Total RNA extracted (4 µL) was reverse-transcribed with 1 µL of random primer (250 ng/µL) and the mixture was incubated for 5 min at 70 ºC. For each sample, the master mix was prepared with 4 µL of reaction buffer (Improm II 5x), 2.4 µL of $MgCl_2$ (25 mM), 1 µL of RNase out (10 units), 0.5 µL of dNTP (20 mM), 0.5 µL Improm RT II (Promega, USA), 6.6 µL of free nuclease water. Afterwards, samples were cooled down to 4 ºC and 15 µL of master mix were added, incubating for 5 min at 25 ºC for denaturation, 60 min at 42 ºC for annealing of primers and for 15 min at 70 ºC for polymerization and extension. Finally, tubes were incubated at 22 ºC. Each cDNA was stored at –20 ºC.

2.6.2. Real-time PCR

Each tube of reaction contained 4 µL of PV cDNA template, 10 µL of the master mix SYBR Green I (Invitrogen), 0.4 µL of each primer (final concentration: 200 mM), 0.4 µL ROX reference dye and 5.2 µL of PCR-grade water. Specific primers for PV capsid gene (VP1-VP4) were 5'-AGT TTC ACC GAA GGC GGA-3' (F) and 5'-CGC TGA CAC AAA ACC AAG GA-3' (R) (GeneBank accession no. **AY184219**), resulting in a 102 bp amplified product. The PCR program consisted of the following steps: 95 ºC for 10 min for initial denaturation, amplification for 40 cycles (95 °C for 15 s for denaturation, 60 °C for 1 min for annealing and extension), and to confirm the PCR product one cycle of melting

curve analysis at 95 ºC for 15 s, 60 ºC for 30 s and 95 ºC for 15 s. Fluorescence data were collected during each annealing/extension step and analysed using ABI PRISM® 7300 Sequence Detector (Applied Biosystems, USA) and software SDS version 1.2.3 ("Sequence Detection Systems" 1.2.3 – 7300 Real Time PCR System - Applied Biosystems, USA).

In every PCR run, negative (no template) and positive PV controls were processed as a routine quality control of the assay. Assays were carried out in duplicate.

2.6.3. Standard-curve

The standard-curve was generated by performing serial dilutions of the PV1 RNA extracted of cell culture. To the smallest dilution of RNA standard it was given the relative value 100 and, following the same reason of dilution, the other 3 points were 50, 25 and 12.5. The virus quantity in the samples was expressed in relation to the standard-curve.

2.7. Statistics

Wilcoxon test was used to detect differences between propolis concentrations, and Friedman test to the other variables, with the significant level at $P<0.05$ (Zar, 1999).

3. Results

3.1. B. dracunculifolia *(extract and essential oil) and propolis chemical composition*

The chromatographic profiles of *B. dracunculifolia* leaves (extract and essential oil) are showed in Figs. 1 and 2, respectively. From HPLC analysis and comparatives studies, involving authentic standard and the samples investigated here, it was possible to carry out the characterization of the extract this plant. According to Fig. 1, caffeic acid, ferulic acid,

aromadendrin-4'-methyl ether and artepillin C were the main phenolics detected in the plant hydroalcoholic extract (Sousa et al., 2007). GC-MS and FID capillary gas chromatography analysis of the essential oil of *B. dracunculifolia* revealed that the main chemical components were: α-pinene, β-pinene, limonene, trans-caryophyllene, aromadendrene, α-humulene, germacrene D, bicyclogermacrene, α-cadinene, nerolidol, spathulenol, viridiflorol, guaiol, α-muurolol (Fig. 2).

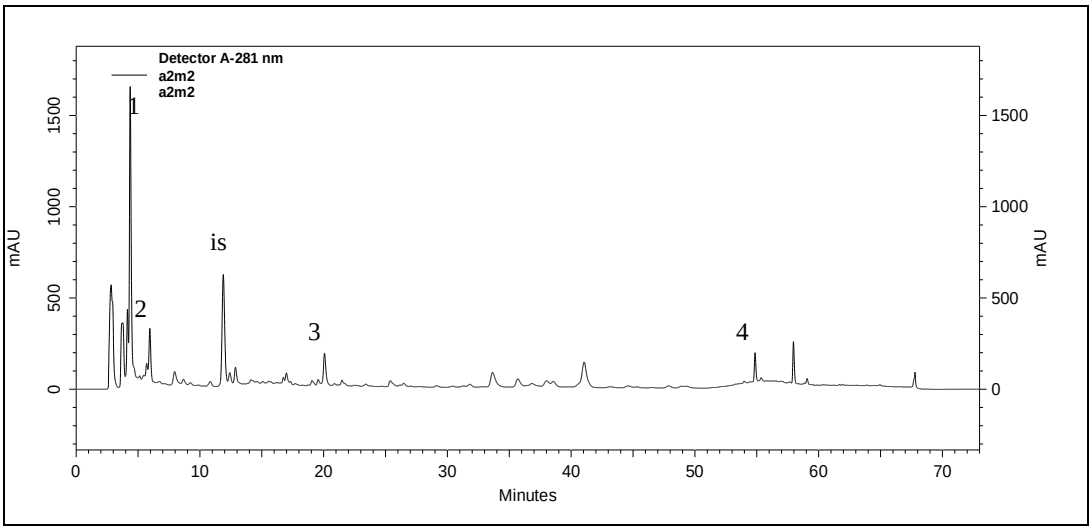

Fig. 1. HPLC profile of *B. dracunculifolia* extract: caffeic acid (1); ferulic acid (2); aromadendrin-4'-methyl ether (3) and artepellin C (4), internal standard (is) (veratraldehyde).

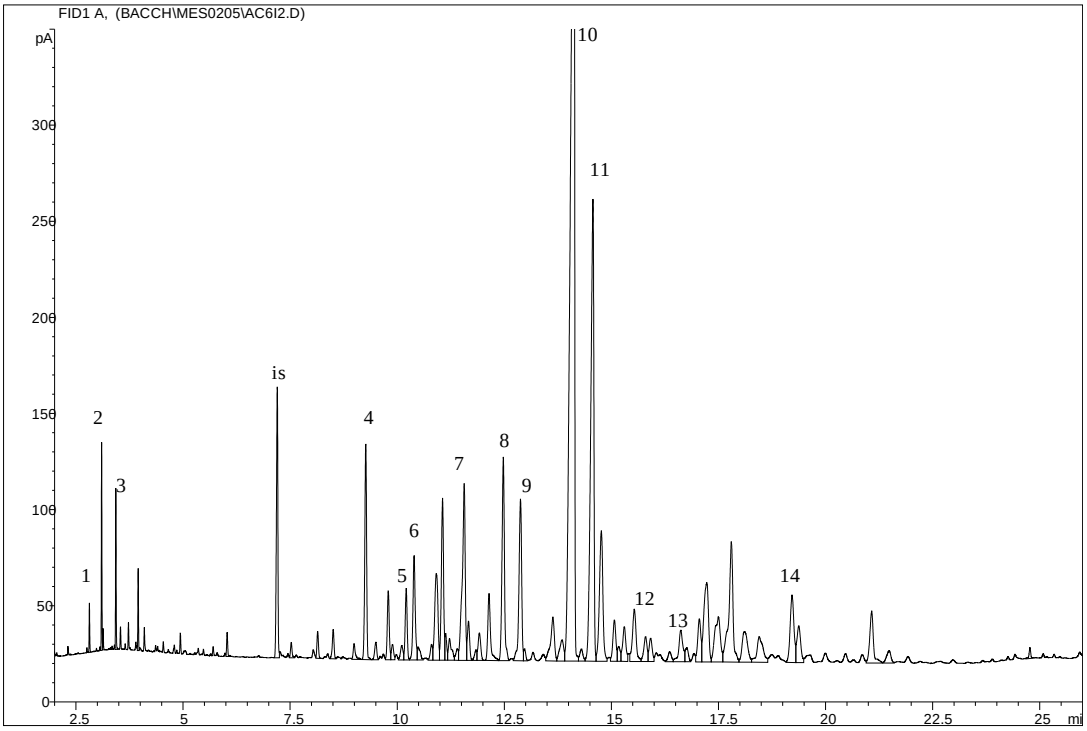

Fig. 2. FID capillary gas chromatogram of essential oil of *B. dracunculifolia*: α-pinene (1), β-pinene (2), limonene (3), trans-caryophyllene, (4), aromadendrene (5), α-humulene (6), germacrene D (7), bicyclogermacrene (8), δ-cadinene (9), nerolidol (10), spathulenol (11), viridiflorol (12), guaiol (13), α-muurolol (14) and internal standard (is) (piperonal).

The main constituents of our propolis sample, investigated by TLC, GC and GC-MS analysis, were: flavonoids (kaempferid, 5,6,7-trihydroxy-3,4'-dimethoxyflavone, aromadendrine-4'-methyl ether); a prenylated *p*-coumaric acid and two benzopyranes: *E* and *Z* 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes); essential oils (spathulenol, (2Z,6E)-farnesol, benzyl benzoate and prenylated acetophenones); aromatic acids (dihydrocinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, 3,5-diprenyl-*p*-coumaric acid, 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzo-pyran); di- and triterpenes, among others (Bankova et al., 1998).

3.2. Cytotoxic assay

Data showed that the non-cytotoxic concentrations for each variable were: *B. dracunculifolia* (extract and essential oil), caffeic and cinnamic acids = 5, 10 and 25 μg/100 μL, and propolis = 5 and 10 μg/100 μL. Ethanol and DMSO had no effects in cell viability (Búfalo et al., submitted).

3.3. Antiviral assay

For the antiviral assays, non-cytotoxic concentrations of each variable described in 3.2. were used.

In the pre-treatment, *B. dracunculifolia* (extract and essential oil – 25 μg/100 μL) showed the most efficient antiviral action in comparison to the other concentrations ($P<0.05$) (Figs. 3A e 4A). No significant differences were seen between the different concentrations of propolis, caffeic and cinnamic acids ($P>0.05$) (Figs. 5A, 6A and 7A, respectively).

In the simultaneous treatment, propolis (10 μg/100 μL) and *Baccharis* extract, caffeic and cinnamic acids (25 μg/100 μL) inhibited significantly the virus replication ($P<0.05$). There were no significant differences between the different concentrations of *Baccharis* essential oil ($P>0.05$) (Figs. 3B-7B).

Data from the post-treatment showed that propolis (10 μg/100 μL) and of *Baccharis* (extract and essential oil) and caffeic acid (25 μg/100 μL) inhibited significantly the PV1 replication ($P<0.05$). No significant differences were seen between the concentrations of the cinnamic acid ($P>0.05$) (Figs. 3C-7C).

A comparison between the variables regarding their concentrations and antiviral action in all protocols (pre-, simultaneous and post-treatment) revealed that *Baccharis* extract (25 μg/100 μL) showed the best antiviral activity (74.0%) in the simultaneous

treatment, followed by 10 µg/100 µL of propolis (52.2%), in the same treatment (p<0.05) (Table 1).

Table 1. Comparison between the protocols (pre-, simultaneous and post-treatment) with regards to the antiviral activity of each variable.

Variables	5 µg / 100 µL			10 µg / 100 µL			25 µg / 100 µL		
	Pre-	Simultaneous	Post-	Pre-	Simultaneous	Post-	Pre-	Simultaneous	Post-
Propolis	b	ab	a	b	**a (52.2%)**	ab	-	-	-
Extract	b	a	ab	b	a	ab	b	**a (74.0%)**	ab
Essential oil	a	a	a	b	ab	a	b	ab	a
Caffeic acid	a	a	a	b	a	ab	b	a	ab
Cinnamic acid	a	a	a	b	a	b	ab	a	b

Different small letters indicate significant differences between the treatments in the same concentration by Friedman test (p< 0.05). The best antiviral activities are showed in bold, and the percentages are between parentheses.

3.4. Relative quantification of viral RNA

PCR efficiency (78.1%) was determined using the slope of the standard-curves, and the real time PCR reproducibility was represented by the correlation coefficient (R = 0.99). Amplicons specificity was confirmed by melting curve analysis. A single melting peak at 80 °C to specific amplicon of 102 bp was obtained, indicating that the primers used in this study were specific for PV1 (data not shown).

In the pre-treatment, the lowest relative viral RNA quantification was seen in the supernatant and cell lysate samples after incubation with *B. dracunculifolia* extract (25 µg/100 µL) and propolis (10 µg/100 µL), followed by the essential oil, caffeic and cinnamic acids (25 µg/100 µL) (Figs. 3A-7A).

In the simultaneous treatment, the lowest relative viral RNA concentrations were found in the supernatant after incubation with *B. dracunculifolia* extract (25 µg/100 µL) and

propolis (10 µg/100 µL). In cell lysate, these same concentrations of propolis and *Baccharis* extract led to decreased RNA quantification. *Baccharis* essential oil, caffeic and cinnamic acids were also efficient, but not as the extract and propolis (Figs. 3B-7B).

In the post-treatment, propolis (10 µg/100 µL) led to the lowest relative viral RNA in the supernatant and cell lysate, followed by the other variables (Figs. 3C-7C).

The best results were obtained in the simultaneous treatment, followed by the pre- and post-treatment for *B. dracunculifolia* (extract and essential oil), propolis and caffeic acid, either in the supernatants or in cells lysates. Cinnamic acid showed the same pattern in the supernatant, but the evaluation of cell lysate showed that the simultaneous treatment was better than post- and pre-treatment. Higher relative viral RNA quantification was observed in the cell lysate in comparison to the supernatants, in all protocols and variables (Figs. 3-7).

One may verify that real-time PCR data are in agreement with the Table 1, and an association between the highest antiviral activities of the variables in the crystal violet method could be established with the lowest relative viral quantification in the real-time PCR.

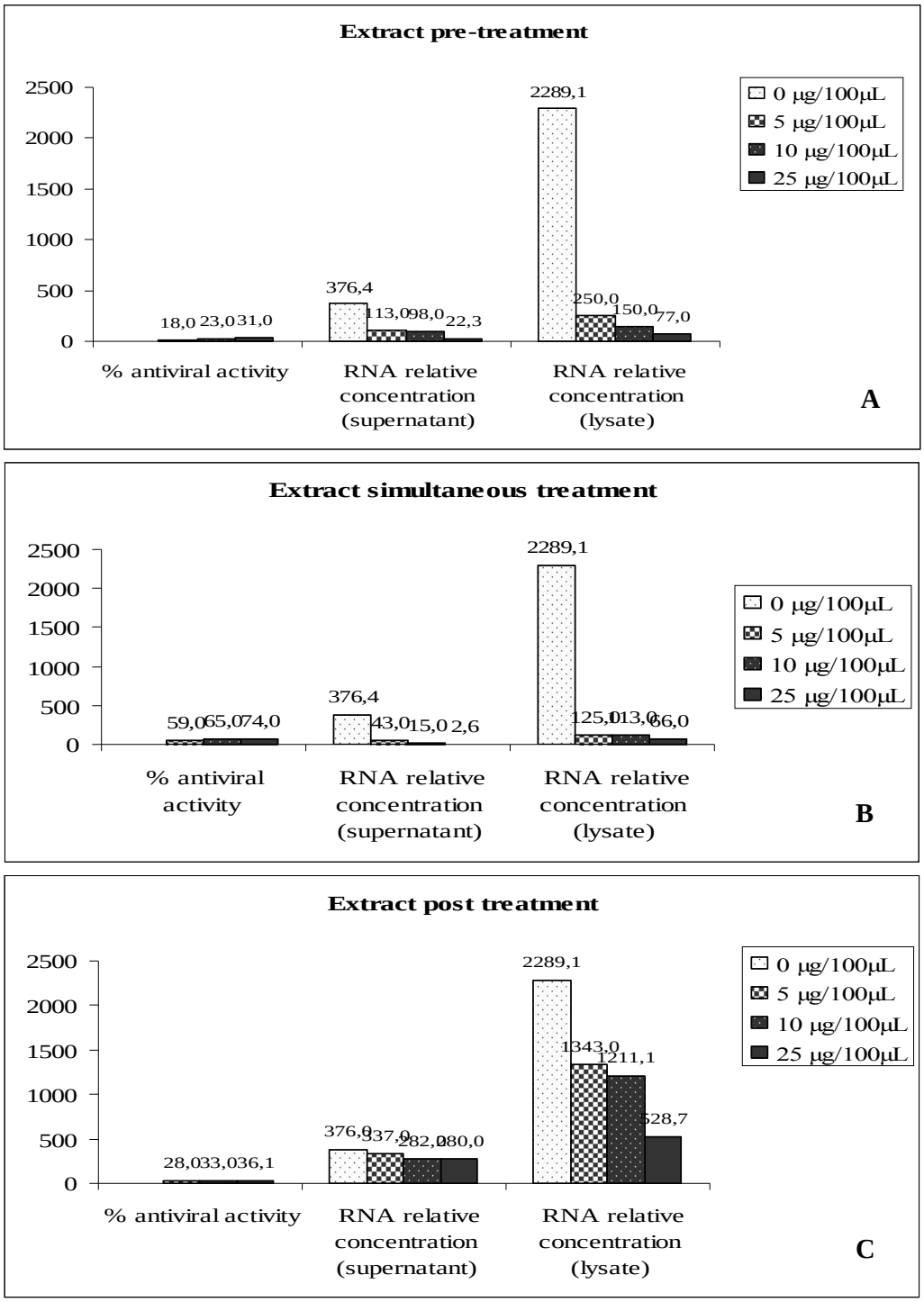

Fig. 3. Antiviral activity percentage and relative RNA viral quantification (supernatant and cell lysate) of the extract. A: pre-treatment, B: simultaneous treatment, C: post-treatment. Friedman test ($P<0.05$).

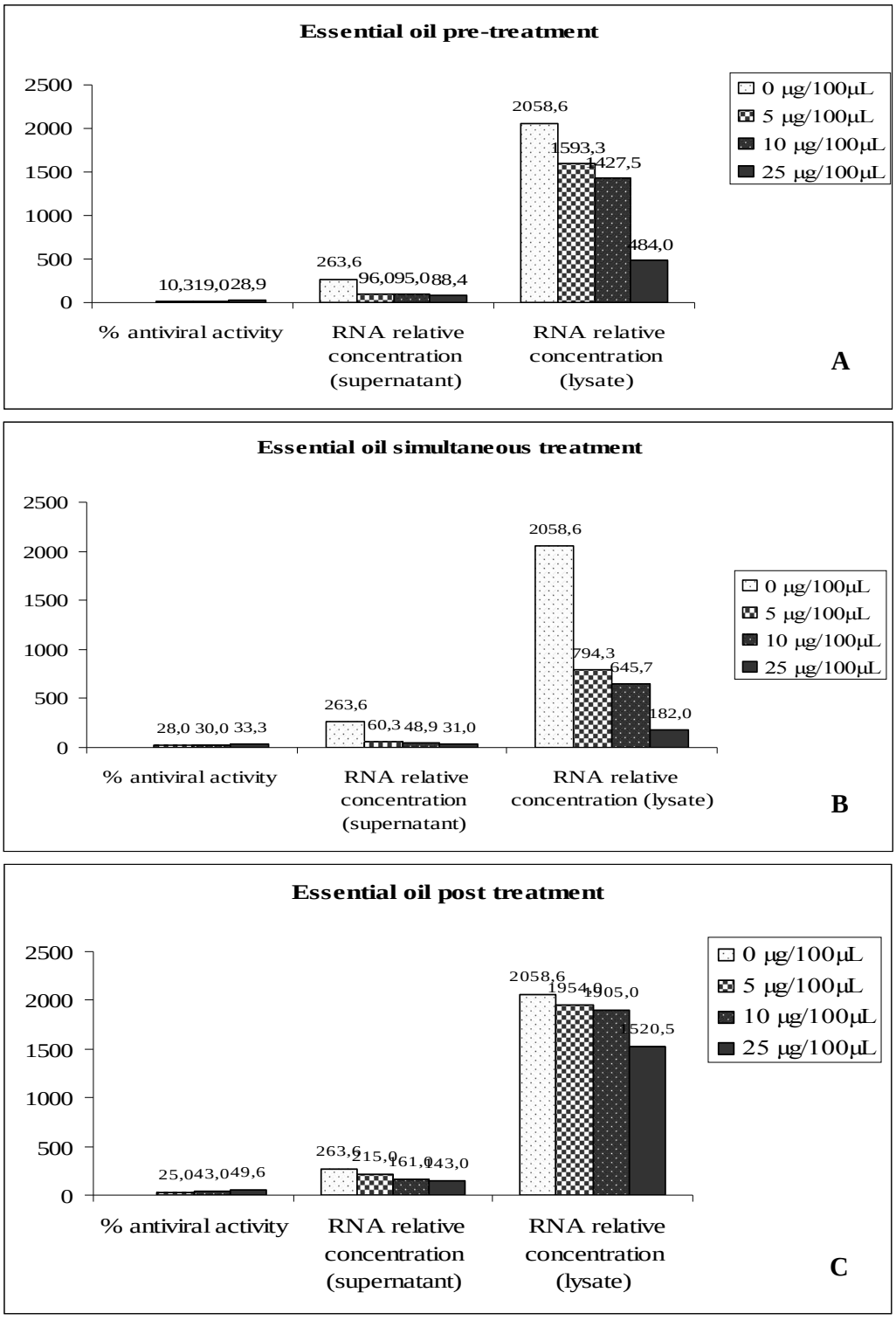

Fig. 4. Antiviral activity percentage and relative RNA viral quantification (supernatant and cell lysate) of the essential oil. A: pre-treatment, B: simultaneous treatment, C: post-treatment. Friedman test (P<0.05).

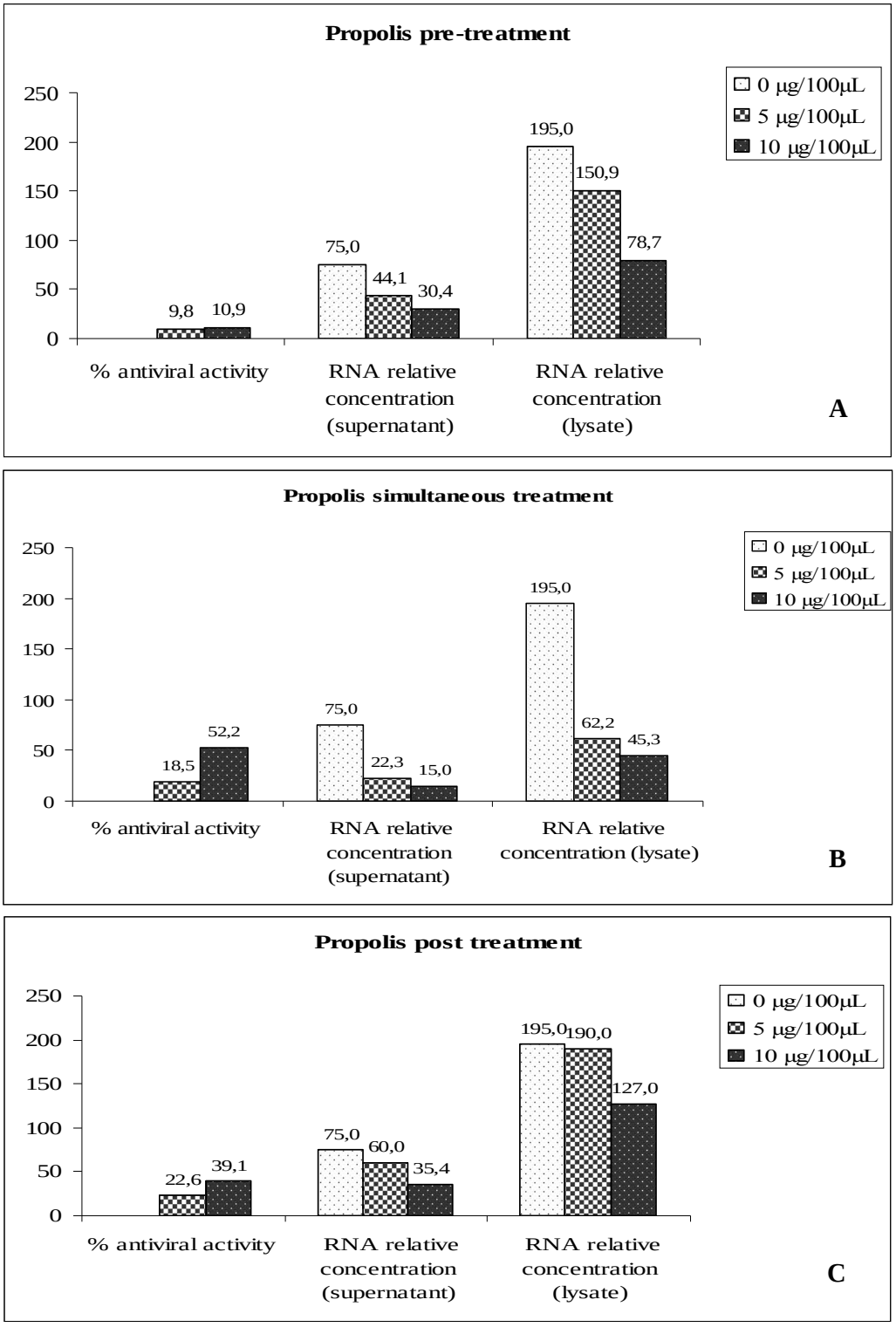

Fig. 5. Antiviral activity percentage and relative RNA viral quantification (supernatant and cell lysate) of the propolis. A: pre-treatment, B: simultaneous treatment, C: post-treatment. Wilcoxon test (P<0.05).

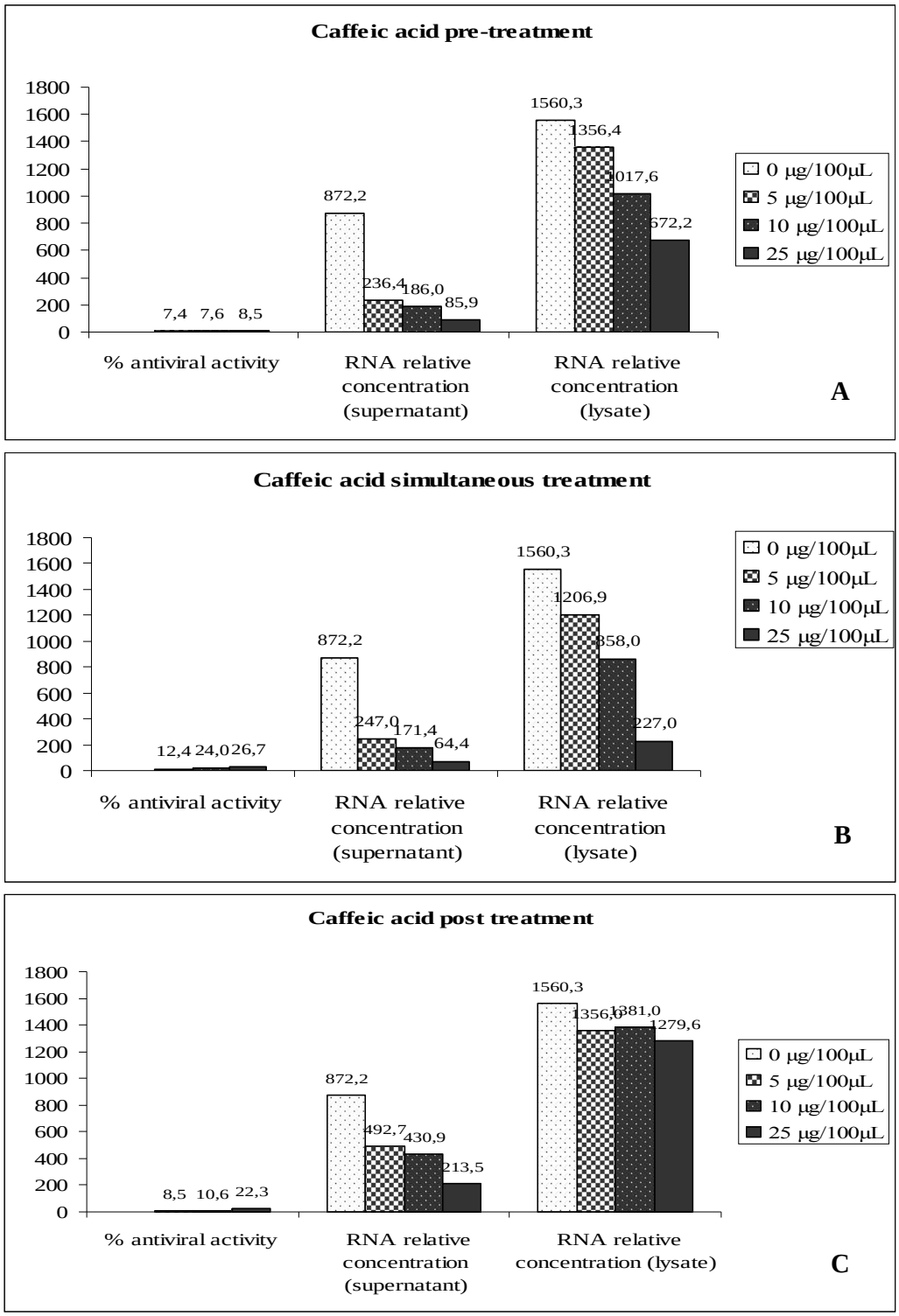

Fig. 6. Antiviral activity percentage and relative RNA viral quantification (supernatant and cell lysate) of the caffeic acid A: pre-treatment, B: simultaneous treatment, C: post-treatment. Friedman test (P<0.05).

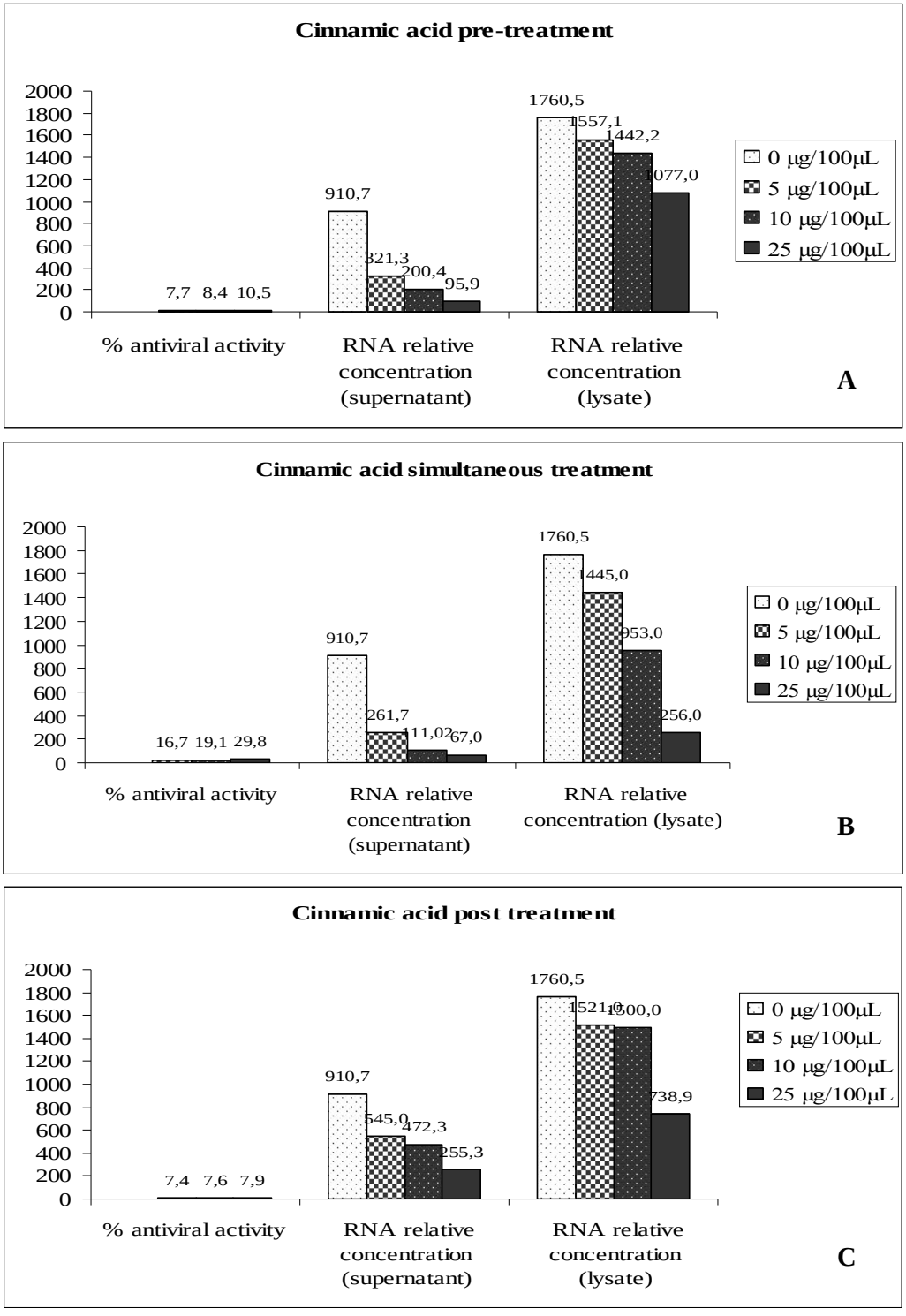

Fig. 7. Antiviral activity percentage and relative RNA viral quantification (supernatant and cell lysate) of the cinnamic acid A: pre-treatment, B: simultaneous treatment, C: post-treatment. Friedman test ($P<0.05$).

4. Discussion

Medicinal plants have been widely used to treat a variety of infectious and non-infectious diseases and 25% of the commonly used medicines contain compounds isolated from plants (Mukhtar et al., 2008). The investigation of natural products with antiviral action has attracted researchers interest; nevertheless, no articles are found in the literature dealing with *B. dracunculifolia* antiviral activity. In the present study, we wish to report for the first time the antiviral activity of this plant on PV1 replication.

With regards to the antiviral assays, the highest percentage of viral inhibition and consequently smallest relative RNA viral quantification were obtained with 25 μg/100 μL of *B. dracunculifolia* (extract) followed by 10 μg/100 μL of propolis, in the simultaneous treatment. *B. dracunculifolia* essential oil showed a lower antiviral activity compared to the extract.

When PV1 was added to the cells simultaneously with the extract or propolis, there was a decreased RNA quantification in cell lysate as well as in the supernatant samples, however, higher amounts of RNA were found in the lysate in comparison to the supernatant. Although the viral entry into cells could have been inhibited, *B. dracunculifolia* and propolis exerted their antiviral activity probably when PV1 was within cells, affecting the cycle replication.

In the pre-treatment, variables were removed before adding the virus, what could lead cells more resistant to virus attack. However, there was a higher viral entry into cells, in comparison to the simultaneous treatment, discarding our hypothesis. On the other hand, in the post-treatment, variables were added after the virus, and the RNA viral quantification was higher than that the simultaneous treatment. One may speculate that variables interfered on virus output by infected cells, or led to RNA degradation in the supernatant after virus output.

Extracts of *Baccharis trinervis* showed inhibitory effects on HSV and HIV replication when added simultaneously to the virus, suggesting that the extracts inhibited the virus-cell attachment, virus-cell fusion and cell-to-cell fusion (Palomino et al., 2002).

As to propolis, Amoros et al. (1992) verified the effect in vitro of alcoholic extract of propolis against several viruses of DNA and RNA, including HSV, adenovirus, and vesicular stomach virus, showing that propolis reduced HSV titers, but the other viruses were less susceptible to its action.

Serkedjieva et al. (1992) reported that the pre-treatment of canine kidney epithelial (MDCK) cells with propolis had no effect on influenza virus replication. However, a reduced viral infectivity was seen adding propolis simultaneously with the virus or immediately after virus adsorption, suggesting that adsorption and the penetration of virus were inhibited in the initial stage of replication cycle.

Ito et al. (2001) verified the efficient activity of propolis anti-HIV in vitro. Gekker et al. (2005), using microglial cell cultures, showed propolis activity against HIV in a concentration-dependent manner suggesting that this effect may have occurred due to caffeic acid phenethyl ester (CAPE) action, involving, in part, the inhibition of viral entry into cells.

According to Huleihel and Isanu (2002), propolis caused 50% inhibition of HSV infection, and indirect evidence pointed out to a strong interaction between propolis and the surface of Vero cells, but not with HSV particles. Administration of propolis before or simultaneously to infection yielded the most significant inhibitory effect, suggesting that this effect were due to propolis blockage of the cell membrane receptors for HSV or to changes inside the cells, which could in turn affect the virus replication cycle.

Huleihel and Ishano (2001) and Matsuo et al. (2005) suggested that the antiviral activity of propolis might be attributed to flavonoids action, which play a significant role in

the antiviral process. Tait et al. (2006) related that natural and synthetic flavonoids might interfere with picornavirus replication preventing the decapsidation of viral particles and RNA release within cells or blocking viral RNA synthesis.

Isolated compounds are important to understand the possible mechanism of action of propolis and its vegetal source. In our work, caffeic and cinnamic acids showed a lower antiviral activity as compared to *B. dracunculifolia* and propolis, suggesting that these acids may be involved in *B. dracunculifolia* and propolis' antiviral effects. It has been reported that the action of propolis and *Baccharis* are due to a natural mixture of its components, and a single constituents would not have an activity greater than that the total extract (Kujumgiev et al., 1999).

The real time PCR has been described as a quantitative detection method for nucleic acids (Min et al., 2006). This method provides higher sensitivity and specificity to quantify viral nucleic acids, and an association between the concentrations of viral nucleic acid and cell culture infectivity by PV1 could be established herein.

In our work, *B. dracunculifolia* (25 μg/100 μL) and propolis (10 μg/100 μL) showed an efficient percentage of antiviral activity evidenced by the crystal violet method (74.0% and 52.2% respectively) in the simultaneous treatment, which was associated to the lowest relative viral quantification in the real-time PCR. Some explanations to these effects may be arised: a) variables might block partially the viral entry within cells; b) variables could have affected the steps of viral cycle replication into cells; c) RNA degradation before the virus entry into cells or after virus release to the supernatant. However, new investigation is still needed in order to explore the potential of these variables as antiviral agents and to understand their mechanisms of action against PV1 cycle replication.

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References

Ait-Mbarek, L., Ait-Mouse, H., Elabbadi, N., Bensalah, M., Gamouh, A., Aboufatima, R., Benharref, A., Chait, A., Kamal, M., Dalal, A., Zyad, A., 2007. Anti-tumor properties of blackseed (*Nigella sativa* L.) extracts. Braz. J. Med. Biol. Res. 40, 839-847.

Amoros, M., Sauvager, F., Girrel, C., Cormier, M., 1992. In vitro antiviral activity of propolis. Apidologie. 23, 231-240.

Bankova, V.S., Popov, S.S., Marekov, N.L., 1998. On the chemical composition of some propolis fractions with antiviral action. Acta Microbiol. Bulg. 23, 52-57.

Bankova, V.S., Boudourova-Krasteva, G., Sforcin, J.M., Frete, X., Kujumgiev, A., Maimoni-Rodella, R., Popov, J., 1999. Phytochemical evidence for the plant origin of Brazilian propolis from São Paulo state. Z. Naturforsch. 54, 401-405.

Búfalo, M.C., Candeias, J.M.G., Sousa, J.P.B., Bastos, J.K., Sforcin, J.M., *In vitro* cytotoxic activity of *Baccharis dracunculifolia* and propolis against HEp-2 cells. J. Nat. Prod., submitted.

Da Silva Filho, A.A., Bueno, P.C.P., Gregório, L.E., Andrade e Silva, M.L., Albuquerque, S., Bastos, J.K., 2004. In-vitro trypanocidal activity evaluation of crude extract and isolated compounds from *Baccharis dracunculifolia* D. C. (Asteraceae). J Pharm Pharmacol. 56, 1195-1199.

Faccin, L.C., Benati, F., Rincão, V.P., Mantovani, M.S., Soares, S.A., Gonzaga, M.L., Nozawa, C., Carvalho Linhares, R.E., 2007. Antiviral activity of aqueous and ethanol

extracts and of an isolated polysaccharide from *Agaricus brasiliensis* against poliovirus type 1. Lett. Appl. Microbiol. 45, 24-28.

Felipe, A.M.M., Rincão, V.P., Benati, F.J., Linhares, R.E.C., Galina, K.J., De Toledo, C.E.M., Lopes, G.C., De Mello, J.C.P., Nozawa, C., 2006. Antiviral effect of *Guazuma ulmifolia* and *Stryphnodendron adstringens* on Poliovirus and Bovine Herpesvirus. Biol. Pharm. Bull. 29, 1092-1095.

Gekker, G., Hu, S., Spivak, M., Lokensgard, J.R., Peterson, P.K., 2005. Anti-HIV- 1 activity of propolis in $CD4^+$ lymphocyte and microglial cell cultures. J. Ethnopharmacol. 102, 158-163.

Huleihel, M, Isanu, V., 2002. Anti-herpes simplex virus effect of an aqueous extract of propolis. Isr. Med. Assoc. J. 4, 923-927.

Huleihel, M., Ishano, V., 2001. Effect of propolis extract on malignant cell transformation by moloney murine sarcoma virus. Arch. Virol. 146, 1517-1526.

Ito, J., Chang, F.R., Wang, H.K., Park, Y. K., Ikegaki, M., Kilgore, N., Lee, K.H., 2001. Anti-HIV activity of moronic acid derivates and the new melliferone-related triterpenoid isolated from Brazilian propolis. J. Nat. Prod. 64, 1278-1281.

Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Christov, R., Popov, S., 1999. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J. Ethnopharmacol. 64, 235-240.

Lennette, D.A., 1995. General principles for laboratory diagnosis of viral, rickettsial, and chlamydial infections, in: Lennette, E.H., Lennette D.A., Lennette, E.T. (Eds.), Diagnostic procedures for viral, rickettsial, and chlamydial infections**,** Washington, pp. 3-25.

Matsuo, M., Sasaki, N., Saga, k., Kaneko, T., 2005. Citotoxicity of flanonoids toward cultured normal human cells. Biol. Pharm. Bull. 28, 253-259.

Min, B.S., Noh, Y.J., Shin, J.H., Balk, S.Y., Min, K.I., Ryu, S.R., Kim, B.G., Park, M.K., Choi, S.E., Yang, E.H., Park, S.N., Hur, S.J., Ahn, B.Y., 2006. Assessment of the quantitative real-time polymerase chain reaction using cDNA standard for human group A rotavirus. J. Virol. Methods. 137, 280-286.

Moreno, M.I.N., Zampini, I.C., Ordonez, R.M., Jaime, G.S., Vattuone, M.A., Isla, M.I., 2005. Evaluation of the cytotoxicity, genotoxicity, mutagenicity and antimutagenicity of propolis from Tucuman, Argentina. J. Agricult. Food. Chem. 53, 8957-8962.

Mukhtar, M., Arshad, M., Ahmad, M., Pomerantz, R.J., Wigdahl, B., Parveen, Z., 2008. Antiviral potentials of medicinal plants. Virus Res. 131, 111-120.

Palomino, S.S., Abad, M.J., Bedoya, L.M., Garcia, J., Gonzales, E., Chiriboga, X., Bermejo, P., Alcami, J., 2002. Screening of South American plants against human immunodeficiency vírus: preliminary fractionation of aqueous extract from *Baccharis trinervis*. Biol. Pharm. Bull. 25, 1147-1150.

Semple, S.J., Pyke, S.M., Reynolds, G.D., Flower, R.L.P., 2001. In vitro antiviral activity of the anthraquinone chrysophanic acid against poliovirus. Antiviral Res. 49, 169-178.

Serkedjieva, J., Manolova, N., Bankova, V., 1992. Anti-influenza virus effect of some propolis constituents and their analogues (esters of substituted cinnamic acids). J. Nat. Prod. 55, 294-297.

Sforcin, J.M., 2007. Propolis and the immune system: a review. J. Ethnopharmacol. 113, 1-14.

Sousa J.P.B., Bueno, P.C.P., Gregório, L.E., Da Silva Filho, A.A., Furtado, N.A.J.C., De Sousa, M.L., Bastos, J.K., 2007. A reliable quantitative method for the analysis of phenolic compounds in Brazilian propolis by reverse phase high performance liquid chromatography. J. Sep. Sci. 30, 2656-2665.

Tait, S., Salvati, A.L., Desideri, N., Fiore, L., 2006. Antiviral activity of substituted homoisoflavonoids on enterovirus. Antiviral Res. 72, 252-255.

Zar, J.H., 1999. Biostatistical analysis, fourth ed. New Jersey: Pretince Hall.

Conclusões

Conclusões

- Concentrações elevadas das variáveis analisadas apresentaram citotoxicidade para as células HEp-2, de forma concentração-dependente;
- Concentrações não tóxicas de todas as variáveis investigadas apresentaram atividade antiviral nos 3 tratamentos (pré-, simultâneo e pós-tratamento), porém as melhores atividades foram obtidas utilizando-se 10 µg/100µL de própolis e 25 µg/100µL de *B. dracunculifolia*, no tratamento simultâneo;
- Houve associação entre as técnicas utilizadas: quanto maior a porcentagem de atividade antiviral evidenciada pelo método colorimétrico do cristal violeta, menor a quantidade relativa de RNA viral detectada pela técnica de PCR em tempo real;
- Prováveis mecanismos relacionados à ação antiviral da própolis e de sua principal fonte vegetal incluiriam o bloqueio parcial, pelas variáveis analisadas, da entrada do vírus nas células; a interferência em etapas da replicação viral no citoplasma celular, ou degradação do RNA viral no sobrenadante da cultura celular.

Anexo

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Ética

Registrado no Ministério da Saúde em 30 de abril de 1997

Botucatu, 05 de novembro de 2.007

OF. 459/2007-CEP

Ilustríssimo Senhor
Prof. Dr. José Maurício Sforcin
Departamento de Microbiologia e Imunologia
Instituto de Biociências de Botucatu.

Prezado Prof. Sforcin,

De ordem da Senhora Coordenadora de CEP, estou enviando cópia do Parecer expedido por este CEP com respeito à consulta formulada por Vossa Senhoria sobre a necessidade ou não de se obter parecer ético referente ao Projeto de Pesquisa **"Efeitos da própolis e de extratos obtidos de Baccharis dracunculifolia sobre a replicação do poliovírus tipo1 em células HEp-2"**, que será conduzido por Michelle Cristiane Búfalo, orientada por Vossa Senhoria.

PARECER DO CEP: APROVADO COM RECOMENDAÇÃO (CÓPIA EM ANEXO) , A QUAL DEVERÁ SER ATENDIDA ANTES DO INÍCIO DESTE PROJETO.

Atenciosamente,

Alberto Santos Capelluppi
Secretário do CEP.

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