RESSALVA

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Resumo

Próp olis é composta por substâncias resinosas, coletadas de várias plantas por abelhas africanizadas, adicionando pólen, cera e secreções glandulares, enquanto para a produção de geoprópolis (Geo) as abelhas sem ferrão utilizam materiais resinosos de plantas, cera, e ainda acrescentam terra ou barro. As atividades biológicas da própolis produzida por *Apis mellifera* têm sido extensivamente estudadas, mas ainda são escassos os trabalhos sobre as propriedades da geoprópolis. Assim, o presente trabalho se propôs a investigar as atividades citotóxica, imunomoduladora, antibacteriana e antifúngica do extrato hidroalcoólico de geoprópolis de *Melipona fasciculata* Smith. Foi realizada a caracterização química da Geo, verificando que o principal grupo de compostos encontrados foram triterpenos. No tocante às células do carcinoma da laringe humana (HEp-2), Geo apresentou ação inibitória em concentrações mais elevadas; entretanto, esta ação foi somente citostática. Geo apresentou ação imunomoduladora sobre monócitos humanos, estimulando a produção das citocinas estudadas (TNF-α e IL-10). Não foi observada ação antibacteriana contra *S. aureus* e *E. coli*, havendo inibição destas linhagens somente com altas concentrações do extrato. Contudo, a associação de Geo com antibióticos favoreceu a ação do cloranfenicol sobre *S. aureus.* A Geo exerceu ação inibitória sobre *Pythium insidiosum*, tanto para o isolado obtido de caso humano como de eqüinos. Os resultados sugerem que a Geo apresentou inúmeras propriedades biológicas; contudo, sua ação é menor em comparação à própolis produzida por abelhas africanizadas, talvez devido ao baixo rendimento do extrato e sua difícil solubilidade.

Palavras-chave: Geoprópolis, *Melipona fasciculata*, Atividades biológicas, Abelhas sem ferrão

Abstract

Propolis is composed of resinous substances collected from various plants by Africanized honeybees, adding polen, wax and bee secretions, while for geopropolis (Geo) production stingless bees use resinous materials from plants, wax, but also adding mud or clay. The biological activities of propolis produced by *Apis mellifera* have been extensively studied, but there are few studies on Geo properties. Thus, the present study investigated the cytotoxic, immunomodulatory, antibacterial and antifungal activities of hydroalcoholic extract of Geo produced by *Melipona fasciculata* Smith. The chemical characterization of Geo was performed, revealing that the main group of compounds was triterpenes. Geo exerted an inhibitory action on human larynx carcinoma cells (HEp-2) using high concentrations, however, it was only a cytostatic action. Geo exhibited immunomodulatory effects on human monocytes, stimulating cytokines production (TNF-α and IL-10). No antibacterial activity was seen against *S. aureus* and *E. coli*. However, the combination of Geo with chloramphenicol favored its effect against *S. aureus*. Geo exerted an inhibitory action against *Pythium insidiosum*, both for human or equine isolates. Data suggested that Geo presented several properties; however, its activity is lower compared to propolis produced by honeybees, perhaps due to the low yield of the extract and its difficult solubility.

Keywords: Geopropolis, *Melipona fasciculata*, Biological activities, Stingless bees

Introdução

1. INTRODUÇÃO

1.1. Própolis e geoprópolis

As abelhas sociais coletam resinas de diferentes partes das plantas e adicionam suas secreções mandibulares produzindo a própolis, um material resinoso de estrutura complexa contendo aproximadamente 50% de resinas e bálsamos vegetais, 30% de cera, 10% de óleos essenciais e aromáticos, 5% de pólen e 5% de outras substâncias, podendo variar de acordo com a flora da região e subespécie de abelha (BURDOCK, 1998).

A palavra própolis tem origem grega, significando: *pro* = em defesa, e *polis* = cidade, pois as abelhas se beneficiam deste produto para vedar frestas, recobrir superfícies irregulares ou insetos e eventuais invasores que morrem no interior da colmeia, com a finalidade de evitar sua decomposição. A própolis também protege a colônia de doenças, por apresentar propriedades antimicrobianas (GHISALBERTI, 1979).

As abelhas indígenas sem ferrão, ou meliponíneos, produzem própolis ou geoprópolis, e a quantidade destes produtos na colmeia varia de acordo com a espécie de abelha (KERR, 1987; NOGUEIRA-NETO, 1997). Diferentemente da própolis, para elaboração da geoprópolis, além do material vegetal, secreção de glândulas, cera e pólen, algumas espécies de abelhas indígenas sem ferrão adicionam terra ou barro à sua composição (Figura 1) (KERR, 1987; NOGUEIRA-NETO, 1997; FREITAS *et al*., 2008). As resinas vegetais compreendem a principal matéria-prima da geoprópolis, mas a presença de terra é a característica marcante deste produto.

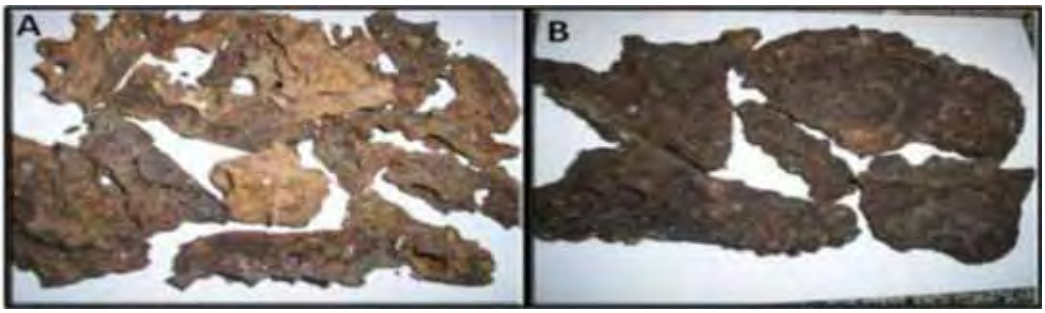

Figura 1. Própolis produzida por *Scaptotrigona* aff. *postica* (A) e geoprópolis produzida por *Melipona fasciculata* (B). Foto: Abigail Araújo.

Em colônias de abelhas sem ferrão, a aplicação da própolis é mais ampla, servindo também para a construção de estruturas externas (tubos de entrada) e internas (favos de cria, lamelas de invólucro e potes de alimento) das colônias. Nos casos em que a própolis é utilizada nas construções, ocorre uma mistura, em proporções variáveis, com outros componentes como cera, fibras vegetais, sementes e barro (MICHENER, 1974; NOGUEIRA-NETO, 1997; MICHENER, 2000; VENTURIERI *et al*., 2003; SANTOS *et al*., 2009).

A quantidade de própolis encontrada nas colônias pode estar associada às estações sazonais, no Rio Grande do Sul. SANTOS *et al*. (2009) mencionaram que, em colônias de *Plebeia emerina,* tal quantidade é maior no período de abril a setembro, havendo acúmulos de própolis na porção anterior das colônias junto à entrada, que é a área de maior risco de invasão por intrusos.

1.2. Origem botânica e composição química da geoprópolis

A geoprópolis possui grande potencial para a geração de renda sustentável no nosso país, no entanto, a falta de certificação e controle de qualidade para a sua produção e comercialização tem se constituído em uma das principais barreiras para a inserção deste produto no mercado. Dessa forma, os parâmetros para a análise da geoprópolis de abelhas sem ferrão baseiam-se na Instrução Normativa 3/2001 do Ministério da Agricultura, a qual se refere aos produtos das abelhas africanizadas.

A composição química da própolis produzida por abelhas africanizadas vem sendo estudada há um bom tempo (BANKOVA *et al*., 1992), e nos últimos anos houve um grande número de publicações sobre sua constituição (BANKOVA, 2005a; BANKOVA, 2005b). Em nossa região (Botucatu, SP), as amostras de própolis são provenientes principalmente de *Baccharis dracunculifolia* DC, conhecida como “alecrim do campo” ou “vassourinha”, *Araucaria angustifolia* (Bertoloni) Otto Kuntze*,* popularmente conhecida como “pinheiro-do-Paraná”, e *Eucalyptus citriodora* Hook (BANKOVA *et al.*, 1999).

Da mesma forma, o conhecimento da composição química e das atividades biológicas da geoprópolis é de extrema relevância, contribuindo para o estabelecimento do controle de qualidade deste produto e de uma legislação específica, além de conscientizar a população sobre a importância da conservação de abelhas nativas, valorizando importantes produtos da nossa biodiversidade.

De modo geral, as amostras de geoprópolis apresentam-se como fragmentos rígidos e com diferentes tamanhos, com grânulos de consistência heterogênea e inodoros, coloração marrom escura e sabor amargo (CUNHA *et al*., 2009). Assim como para a própolis produzida por abelhas africanizadas, o método de preparo dos extratos de geoprópolis pode influenciar sua atividade, uma vez que os diferentes solventes solubilizam e extraem compostos diferentes (CUNHA *et al.*, 2004).

Além da análise da consistência, odor, coloração e principalmente da composição química, BARTH & PINTO DA LUZ (2003) mencionaram que a análise do pólen seria um método útil para caracterização de diferentes amostras de geoprópolis. Ademais, tal análise possibilitaria definir sua origem fitogeográfica. Estas mesmas autoras analisaram 10 amostras de geoprópolis produzidas por três espécies de meliponíneos em São Paulo, Minas Gerais e Espírito Santo, encontrando grãos de pólen, hifas e esporos fúngicos, material orgânico e fragmentos de plantas na maioria das amostras. Terra ou material arenoso foi encontrado em todas as amostras. A análise polínica revelou que o tipo predominante de pólen no Estado de São Paulo foi proveniente de *Eucalyptus* (Myrtaceae); *Schinus* (Anacardiaceae) foi encontrado principalmente nas amostras de Minas Gerais, e o pólen de *Myrcia* (Myrtaceae) foi encontrado em todos os Estados.

Em comparação à própolis produzida por abelhas africanizadas, o pólen encontrado na geoprópolis revela a vegetação regional característica, além de indicar que há uma maior variabilidade de plantas visitadas pelos meliponíneos. Ademais, foi aventado que constituintes do solo bem como a falta de tricomas são características somente da geoprópolis (BARTH & PINTO DA LUZ, 2003).

Diante da importância do conhecimento dos recursos tróficos que as abelhas sem ferrão utilizam para a produção de mel e geoprópolis, MARTINS (2008) identificou as espécies vegetais fornecedoras de pólen e néctar para a espécie *Melipona fasciculata* Smith (Figura 2), na microrregião da baixada ocidental maranhense, com base na análise do pólen presente nas corbículas das operárias. Os grãos de pólen e a sua freqüência mensal nas amostras foram identificados, e o agrupamento foi realizado por famílias botânicas. Os resultados indicaram que as famílias vegetais mais visitadas foram: Fabaceae (26%), Arecaceae (13,30%) e Anacardiaceae (9%). As operárias coletaram o pólen de 64 espécies diferentes, sendo as mais freqüentes: *Senna ala*ta L. (14%), *Orbignya phalerat*a Mart. (13%), *Astronium* P. (6%), *Gustavia augusta* L. (6%), *Pontederia parviflora* Alexander (5%) e *Solanum juripeba* Rich. (5%). A autora concluiu que, embora a espécie *M. fasciculata* possua hábitos generalistas para a captação de recursos alimentares, poucas espécies vegetais apresentaram uma freqüência significativa.

Figura 2. *Melipona fasciculata* Smith em flor de *Euterpe oleracea* (açaí) (Fonte: Giorgio Venturieri).

Em 2011, MARTINS *et al.* analisaram o espectro polínico de 12 amostras de mel produzido por *M. fasciculata* no Estado do Maranhão. Para identificação dos recursos nectaríferos utilizados por essa espécie. Foram encontrados 45 tipos polínicos, sendo *Pontederia parviflora* Alexander (Pontederiaceae) a espécie mais freqüente em todo o período de amostragem

(38,6%), seguida de *Mimosa caesalpiniifolia* Benth (Mimosaceae) com 22,8% de freqüência.

A análise da composição química de amostras de geoprópolis produzidas por *Friesiomellita varia*, *Melipona compressipes*, *Scaptotrigona depilis* e *Paratrigona anduzei* provenientes da Venezuela indicou um perfil fenólico caracterizado pela presença de benzofenonas polipreniladas (TOMÁS-BARBERÁN *et al.,* 1993).

Em 2009, CUNHA *et al*. propuseram a padronização de extrativos de geoprópolis produzida por *M. fasciculata.* As amostras foram coletadas no município de Palmeirândia, localizado na microrregião da baixada ocidental maranhense, e os extratos foram obtidos empregando-se os processos de maceração e extração por aparelho de Soxhlet. As amostras de geoprópolis foram maceradas e extratos foram preparados em diferentes concentrações de etanol (100, 70 ou 50%) ou em água. Foram utilizadas duas relações de hidromódulo (peso/volume = 1:2 e 1:5) e, após 24 horas, as soluções foram filtradas e os resíduos submetidos a uma segunda extração por 24 horas. As soluções extrativas foram concentradas em evaporador rotativo para calcular o peso seco das mesmas. Os dados obtidos permitiram concluir que o extrato de geoprópolis com etanol absoluto e relação de hidromódulo 1:5 apresentou o melhor perfil químico, contendo polifenóis e flavonóides totais em maior quantidade. Os autores concluíram também que tal extrato poderia ser utilizado como parâmetro para a padronização de extratos e controle de qualidade da geoprópolis.

ABREU (2011) analisou a composição química de três amostras de geoprópolis produzida por *M. fasciculata,* oriundas dos municípios de São Bento e São João Batista, localizados no Estado do Maranhão. Os extratos foram macerados com etanol 70% por 48 horas, filtrados e concentrados em rotavapor. Os perfis químicos foram avaliados por cromatografia líquida de alta eficiência com detector espectrofotométrico na região ultra-violeta (CLAE-UV) e os extratos analisados por cromatografia gasosa associada a espectrometria de massa (CG-EM). Foram identificados 30 compostos das classes dos ácidos

fenólicos, flavonóides, triterpenos, açúcares e ácidos graxos, com predomínio de ácido gálico e quercetina em todos os extratos testados.

A análise química do extrato etanólico do ninho de *Trigona spinipes* permitiu a identificação dos seguintes compostos majoritários: triterpenos cicloartanos (trans-3β-hidroxi-24-cicloarteno-26-óico - ácido magniferólico, e ácido 3β-hidroxi-24-metilenocicloartano-26-óico), além dos flavonóides 3'-metil-quercetina, sacuranetina, éter 7-metil canferol, tricetina e éter 7-metil aromadendrina (FREITAS *et al*., 2008).

No caso da amostra de geoprópolis produzida por *Trigona spinipes* no Estado do Ceará, a identificação de sacuranetina, éter 7-metil-canferol e éter 7-metil-aromadendrina no ninho destes meliponíneos e no exsudato de *Eucalyptus citriodora* sugere que esta planta pode ser a principal fonte vegetal visitada por estas abelhas no nordeste brasileiro (FREITAS *et al*., 2008).

O conhecimento sobre as plantas fontes de produtos apícolas ou meliponícolas não é apenas de interesse acadêmico, podendo servir como base para padronização química das amostras (BANKOVA *et al.*, 2000; SALATINO *et al*., 2005). A determinação da origem geográfica e, principalmente, da origem vegetal se faz importante no controle de qualidade e até mesmo na padronização das amostras para uma efetiva aplicação terapêutica (PARK *et al.*, 2002; SFORCIN & BANKOVA, 2011). No entanto, ainda são escassos os trabalhos sobre a composição química e origem botânica da geoprópolis.

1.3. Propriedades biológicas da própolis e geoprópolis

1.3.1. Atividade antitumoral

Tem havido interesse por parte dos pesquisadores em explorar o potencial antitumoral da geoprópolis produzida pelos meliponíneos.

In vitro, CINEGAGLIA (2011) relatou a ação citotóxica da geoprópolis sobre células de osteosarcoma canino ou sarcoma osteogênico, que é uma neoplasia óssea primária mais frequentemente diagnosticada em cães. Os

resultados demonstraram que estas células apresentaram morfologia diferente após exposição à geoprópolis, bem como sensibilidade a este produto de forma tempo e dose-dependente. BORGES *et al*. (2011) evidenciaram a ação da própolis produzida por *Scaptotrigona* sp no Estado do Maranhão sobre glioblastoma humano (U251 e U343) e fibroblastos (MRC-5). Além disso, a associação da própolis à temozolomida apresentou sinergismo na ação antiproliferativa.

ARAÚJO *et al*. (2010) investigaram o efeito do extrato hidroalcoólico de própolis (0,5; 5 ou 50 mg/kg) produzida por *Scaptotrigona* aff. *postica* sobre o desenvolvimento do tumor de Ehrlich em camundongos fêmeas. Os tratamentos foram realizados em dose única, 48 horas antes do inóculo tumoral, verificando que as maiores concentrações de própolis inibiram o desenvolvimento tumoral, o que foi associado a um aumento na celularidade do baço e da medula óssea.

ASSUNÇÃO (2011) avaliou a ação antitumoral da geoprópolis produzida por *Melipona fasciculata in vitro* e *in vivo*. *In vitro*, as células de tumor de Ehrlich apresentaram-se sensíveis à ação direta da geoprópolis, nas concentrações de 62,5 a 500 µg/ml. *In vivo*, camundongos foram tratados com dose única de geoprópolis (0,5; 5 ou 50 mg/kg – via intraperitoneal) e inoculados com tumor ascítico de Ehrlich, também por via intraperitoneal, após 48 horas. Após dez dias do inóculo do tumor, houve inibição do crescimento tumoral nos grupos que receberam a geoprópolis (50 mg/kg) e aumento da sobrevida dos animais. Esta autora também avaliou o efeito de geoprópolis *in vivo* em camundongos, 48 horas antes ou 48 horas após a inoculação do tumor de Ehrlich na forma sólida na pata dos animais. O tratamento com geoprópolis (0,5 e 5 mg/kg) inibiu o desenvolvimento tumoral tanto antes como após o inóculo das células tumorais, sugerindo seu efeito tanto profilático como terapêutico.

Vários pesquisadores têm relatado a propriedade antitumoral da própolis produzida por abelhas africanizadas *in vitro* e *in vivo* (BAZO *et al*., 2002; SFORCIN, 2007). *In vitro*, a própolis apresentou atividade citotóxica de forma tempo-dose dependente sobre as células de tumor venéreo transmissível (TVT)

canino, incluindo o grupo plasmocitóide, considerado o de maior agressividade (BASSANI-SILVA *et al.*, 2007). Também apresentou eficiente ação sobre células de carcinoma de laringe humana (HEp-2) (BÚFALO *et al*., 2009) e sobre células de osteosarcoma canino (CINEGAGLIA, 2011).

In vivo, avaliando o efeito da própolis produzida por abelhas africanizadas sobre a ativação de células *natural killer* (NK) contra células tumorais, foi observado que a administração de própolis a ratos, durante 3 dias, induziu aumento na atividade lítica de células NK contra linfoma murino (SFORCIN *et al.*, 2002). Esta observação reforçou a afirmação prévia de SCHELLER *et al.* (1988), que sugeriram serem os melhores resultados obtidos após tratamento a curto prazo com este apiterápico.

Camundongos tratados com própolis ou alguns de seus componentes (ácido cafeico, CAPE e quercetina) apresentaram menor número de nódulos tumorais no pulmão; entretanto, a própolis exerceu efetiva atividade anti-metastática superior ao tratamento com seus constituintes (ORSOLIC *et al.,* 2004). Camundongos com metástases e tratados com própolis apresentaram recuperação na porcentagem de células T, em favor das células $CD8^{+}$, sugerindo o efeito do produto sobre a geração de células T citotóxicas e imunidade antitumoral específica, seguido de contenção das metástases (ORSOLIC & BASIC, 2003). Assim, este grupo de pesquisa da Croácia sugeriu que a atividade antitumoral da própolis e de alguns de seus componentes pode estar associada à sua ação imunomoduladora em camundongos (ORSOLIC *et al.,* 2006). As últimas revisões têm apontado que os principais mecanismos pelos quais a própolis afeta as células tumorais estão relacionados com inibição do ciclo celular, apoptose e interferência nas vias metabólicas (SFORCIN, 2007; WATANABE *et al*., 2011).

O vasto número de publicações relacionadas à ação antitumoral da própolis e de seus constituintes indica o caráter promissor de sua aplicabilidade, sugerindo a continuidade das investigações para explorar melhor o potencial antitumoral deste produto apícola, o que é de interesse tanto para a Medicina Humana como Veterinária. Do mesmo modo, o potencial da geoprópolis merece ser melhor investigado.

1.3.2. Ação imunomoduladora

As pesquisas sobre a ação imunomoduladora da própolis ganharam maior interesse após os anos 90. Estudos sobre a atividade imunomoduladora de substâncias naturais ou sintéticas podem ser importantes ferramentas na prevenção e tratamento de infecções e doenças neoplásicas (ORSOLIC & BASIC, 2003).

DIMOV *et al.* (1991) relataram que o extrato aquoso da própolis aumentou a sobrevida de camundongos com infecções bacterianas e fúngicas, sugerindo modulação da resposta imunológica inata via ativação de macrófagos. TATEFUJI *et al.* (1996) isolaram seis compostos derivados do ácido cafeoilquínico da própolis brasileira, os quais aumentaram a motilidade de macrófagos murinos. A ação da própolis sobre macrófagos também resulta em aumento da capacidade fagocítica (ORSI *et al.*, 2000), estimulação da secreção de citocinas, tais como o fator de necrose tumoral (TNF)-α, e outros mediadores, como o óxido nítrico (NO) (KHAYAL *et al*., 2003). Em ensaios *in vitro*, ORSI *et al.* (2000) verificaram também que a própolis estimulou a liberação de peróxido de hidrogênio (H_2O_2) por macrófagos peritoneais de camundongos. BACHIEGA *et al*. (2012) investigaram o efeito da própolis e de compostos isolados sobre a produção de citocinas pró- e anti-inflamatórias (IL-1β, IL-6 e IL-10) por macrófagos peritoneais de camundongos BALB/c, em protocolos de desafio com LPS *in vitro*. A própolis e os ácidos cinâmico e *p*-cumárico estimularam a produção de IL-1β, enquanto que, a produção de IL-6 e IL-10 foram inibidas, mesmo em presença do LPS, demonstrando que a própolis pode exercer ações pró- e anti-inflamatória, modulando a resposta imune e inflamatória dependendo da concentração utilizada.

Baseados em estudos pré-clínicos, ORSOLIC *et al.* (2006) sugeriram que a atividade antitumoral da própolis e de alguns de seus compostos está relacionada com a sua ação imunomoduladora, principalmente devido à estimulação da imunidade inata, através da ativação de macrófagos, os quais possuem importante papel na resposta antitumoral, por meio da citotoxicidade celular dependente de anticorpos, secreção de citocinas inibitórias para o

crescimento tumoral e produção de intermediários reativos de oxigênio e de nitrogênio.

Avaliando a ação da própolis sobre monócitos humanos, dados recentes de nosso grupo revelaram que a mesma estimulou a expressão de TLR-4 e de CD80, aumento na produção tanto de TNF-α como de lL-10, e aumento na atividade fungicida contra *Candida albicans* (dados não publicados).

Embora a própolis apresente caráter ativador, também pode exercer papel imunossupressor e anti-inflamatório. Avaliando a influência do extrato de própolis sobre a resposta linfoproliferativa de camundongos e sobre a produção de interferon (IFN)-γ, verificou-se que a própolis apresentou efeito inibitório sobre a proliferação de linfócitos *in vitro* e *in vivo* (SÁ-NUNES *et al*., 2003). ANSORGE *et al.* (2003) relataram que a própolis e alguns de seus compostos isolados são capazes de inibir a síntese de DNA de células mononucleares do sangue periférico (PBMC, na sigla em inglês) e de linfócitos T purificados. Além disso, houve inibição da produção de citocinas derivadas de macrófagos (IL-1β e IL-12) e dos perfis Th1 (IL-2) e Th2 (IL-4), mas não da produção do fator transformador de crescimento β (TGF-β1), sugerindo que a própolis pode inibir tanto as células Th1 como Th2.

Estas observações apontam nitidamente a capacidade deste produto apícola em modular a resposta imunológica, uma vez que, em virtude da concentração, período de incubação ou ingestão, vias de administração, entre outros fatores, os diferentes ensaios realizados *in vitro* ou *in vivo* revelaram ação imunoestimulante ou inibitória.

Considerando que a ação imunomoduladora da geoprópolis é pouco estudada (LIBÉRIO *et al*., 2011), nesta tese a célula escolhida para avaliação da possível ação imunomoduladora da geoprópolis foi o monócito humano. A utilização do sangue periférico como fonte de monócitos é facilitada pelo isolamento dos mesmos através do gradiente de centrifugação pelo Ficoll-Hypaque, devido às diferentes densidades entre as células mononucleares e outros componentes do sangue (RAULF-HEIMSOTH, 2008).

Monócitos possuem uma grande variedade de receptores que reconhecem vários micro-organismos, e quando estimulados podem produzir

grandes quantidades de reativos intermediários de oxigênio, fatores do complemento, prostaglandina, enzimas proteolíticas e citocinas como TNF-α, IL-1β, CXCL-8, IL-6 e IL-10 (AUFFRAY *et al.*, 2009).

As citocinas investigadas neste trabalho foram TNF-α (pró-inflamatória) e IL-10 (anti-inflamatória). O TNF-α, produzido por macrófagos, monócitos, células NK e células T, é o principal mediador da resposta inflamatória aguda contra bactérias Gram-negativas e outros agentes infecciosos, sendo responsável por muitas das complicações sistêmicas de infecções graves. A principal função fisiológica do TNF-α é estimular o recrutamento de neutrófilos e monócitos para locais de infecção e ativá-las na tentativa de erradicação dos micro-organismos (LOCKSLEY *et al*., 2001). IL-10 é secretada por células T, monócitos e macrófagos, sendo um inibidor de macrófagos e células dendríticas ativados, exercendo, portanto, importante papel na imunorregulação (PESTKA *et al*., 2004).

1.3.3. Atividade antimicrobiana

A investigação de produtos naturais com atividade antimicrobiana tem atraído o interesse de muitos pesquisadores, motivados não só pelo aumento da resistência bacteriana às drogas antimicrobianas tradicionais (SHELDON, 2003) como também pelos efeitos colaterais frequentemente observados após o uso de antibióticos (CUNHA, 2001). Dentre os produtos naturais, a própolis tem sido considerada importante adjuvante no tratamento ou na prevenção de muitas doenças infecciosas (LIBÉRIO *et al.*, 2009).

A propriedade antimicrobiana da própolis de abelhas africanizadas tem sido bastante investigada, principalmente contra bactérias e fungos (LILENBAUM & BARBOSA, 1994; BANKOVA *et al*., 1996; BANSKOTA *et al*., 2001). De acordo com BANKOVA (2005a), a própolis não é apenas um material de construção, mas representa a mais importante “arma química” das abelhas contra micro-organismos patogênicos, sendo utilizada pelo homem desde a antiguidade como antibiótico natural.

Nos últimos anos, tem havido maior número de publicações sobre a atividade antimicrobiana da geoprópolis ou da própolis produzida por meliponíneos. LEVY Jr. (1997) avaliou a ação antibacteriana de amostras de própolis produzidas por *Nannotrigona testaceicornis*, *Plebeia droryana*, *Scaptotrigona bipunctata*, *S. postica*, *Tetragonisca angustula* e *T. spinipes* contra *Staphylococcus aureus*, verificando que todas as amostras apresentaram atividade similar. Também foi observado que tais amostras de própolis apresentaram maior atividade antimicrobiana em relação à atividade exercida pelos méis dos meliponíneos.

Analisando diferentes amostras de própolis produzidas por *Melipona compressipes*, proveniente do Piauí, e por *Melipona quadrifasciata anthidioides*, do Paraná, KUJUMGIEV *et al*. (1999) comprovaram sua ação contra *S. aureus*, *Candida albicans* e contra o vírus influenza. Os autores comentaram que estes resultados seriam esperados, uma vez que a própolis faz parte da defesa das abelhas contra infecções, e sugeriram que as atividades biológicas da geoprópolis, assim como da própolis, não são devidas a um só componente ou a uma classe de substâncias, pois as diferentes combinações de constituintes são essenciais para suas propriedades terapêuticas.

Em 2001, nosso grupo investigou a ação antibacteriana da própolis produzida por abelhas africanizadas, comparando-a com aquela produzida pelos seguintes meliponíneos: *Nannotrigona testaceicornis*, *Tetragonisca angustula*, *Trigona spinipes*, *Scaptotrigona* sp, *Partamona* sp, *Melipona scutellaris*, *Melipona* sp e *Melipona quadrifasciata*. Verificou-se que as bactérias Gram positivas (*S. aureus* e *Enterococcus* sp) foram mais suscetíveis que as Gram negativas (*Escherichia coli*), sendo a atividade da própolis produzida por *Partamona* sp e *Melipona* sp semelhante àquela produzida por *Apis mellifera* (FERNANDES JR. *et al.*, 2001).

MIORIN *et al*. (2003) compararam a ação antibacteriana do mel e da própolis produzida por *T. angustula*, verificando que a atividade da própolis contra *S. aureus* foi superior àquela apresentada pelo mel.

A atividade antimicrobiana de amostras de própolis produzidas por *T. angustula* e *A. mellifera* foi investigada contra *C. albicans*, *E. coli*, *Bacillus subtilis* e *S. aureus*, revelando que as duas amostras apresentaram potencial semelhante contra os micro-organismos testados, havendo maior sensibilidade de *S. aureus*. No entanto, nenhuma das amostras foi ativa contra *C. albicans*. *E. coli* e *B. subtilis* foram inibidas somente pela própolis produzida por *A. mellifera*. Segundo os autores, a possível explicação para estes resultados seria a maior concentração de ácidos aromáticos (como os ácidos cafeico e *p*-cumárico) nesta amostra, já tendo inclusive sido relatada a atividade antimicrobiana destes compostos (PEREIRA *et al*., 2003).

RIZZI (2005) avaliou a eficiência da própolis produzida por *Scaptotrigona* sp e da geoprópolis produzida por *M. fasciculata*, comparando suas ações com soluções utilizadas no tratamento endodôntico frente a *Enterococcus faecalis*, visto que a presença de tal micro-organismo é associada a casos de insucesso neste tratamento. Somente o extrato de própolis exerceu atividade antibacteriana, sendo esta atividade maior do que aquela apresentada pelo hipoclorito de sódio 5%, porém menor do que aquela do gel e da solução de clorexidina, os quais são reconhecidamente capazes de eliminar micro-organismos envolvidos na patogênese pulpar e periapical. Resultados semelhantes foram obtidos por MAIA FILHO *et al*. (2008).

Já a própolis produzida por abelhas africanizadas tem sido intensamente investigada no tocante à sua ação antimicrobiana, apresentando eficiente ação contra bactérias Gram positivas, enquanto que bactérias Gram negativas foram mais resistentes (SFORCIN *et al.*, 2000). Estas bactérias possuem uma parede celular quimicamente mais complexa e maior teor lipídico, o que pode explicar a maior resistência aos extratos de própolis (VARGAS *et al*., 2004). É importante ressaltar que, na avaliação da atividade antibacteriana, alguns fatores podem interferir nos resultados, tais como a composição do meio de cultura, os micro-organismos testados, o método de extração da própolis, o pH e a solubilidade das amostras no meio de cultura, entre outros (RIOS *et al.*, 1988; ALVES *et al.*, 2008).

A atividade bactericida da própolis de *A. mellifera* pode ser uma

conseqüência de sua ação sobre a estrutura da parede celular, levando à bacteriólise parcial. A própolis também pode desorganizar o citoplasma, causar alterações na membrana citoplasmática e inibir a síntese protéica de bactérias (MIRZOEVA *et al*., 1997; PINTO *et al.*, 2001).

Diversos trabalhos têm sugerido que os principais compostos responsáveis pela atividade antimicrobiana da própolis de *A. mellifera* são os flavonóides e ésteres de ácidos fenólicos (SFORCIN *et al.*, 2000; CASTALDO & CAPASSO, 2002; PIETTA *et al.*, 2002). UZEL *et al.* (2005) identificaram, como compostos majoritários, os flavonóides pinocembrina e galangina e cafeato de fenil etila (CAPE), cujo mecanismo de ação parece ser baseado na inibição da RNA polimerase bacteriana. Outros flavonóides e os ácidos caféico, benzóico e cinâmico atuam na membrana ou na parede celular bacteriana, causando danos funcionais e estruturais (SCAZZOCCHIO *et al.*, 2005). Com relação à atividade antibacteriana da própolis de abelhas sem ferrão, VELIKOVA *et al.* (2000) correlacionaram esta atividade com ácidos diterpênicos presentes nas amostras de própolis de *M. quadrifasciata*.

A combinação da própolis produzida por abelhas africanizadas com drogas antimicrobianas que atuam na parede bacteriana (amoxilina, ampicilina e cefalexina); na síntese protéica (cloranfenicol, neomicina e tetraciclina); no DNA bacteriano (ciprofloxacina e norfloxacina) e no metabolismo bacteriano (cotrimoxazol) foi analisada, verificando que o sinergismo foi mais eficiente com antimicrobianos que atuam na parede bacteriana e na síntese protéica (ORSI *et al*., 2006; ORSI *et al*., 2012a; ORSI *et al*., 2012b). A ação sinérgica da própolis associada a antibióticos contra cepas resistentes a benzilpenicilina, tetraciclina e eritromicina poderia se constituir como alternativa terapêutica para a resistência microbiana (FERNANDES JR. *et al.*, 2005; LUSTOSA *et al.*, 2008).

Atualmente, há grande necessidade de se descobrir novas drogas antifúngicas. Comparando-se a disponibilidade de fármacos utilizados para o tratamento das infecções bacterianas, são poucas as drogas antifúngicas disponíveis para o tratamento das micoses. A maioria dessas drogas possui a inibição da síntese do ergosterol como mecanismo de ação, ou a ligação da

droga a esta molécula, desestruturando a membrana celular dos fungos. Tais drogas tendem a apresentar toxicidade ao hospedeiro, devido à proximidade filogenética entre fungos e animais (ALEXOPOULOS *et al*., 1996). Além das drogas que atuam no ergosterol fúngico, tem sido avaliada a ação de drogas que atuam na parede celular dos fungos, principalmente inibindo a síntese de β-glucanas, como as equinocandinas, as quais apresentam menor toxicidade ao hospedeiro. No entanto, além do custo do tratamento ser extremamente elevado, há o surgimento de resistência dos fungos patogênicos, o que já é, inclusive, observado clinicamente (KONTOYIANNIS & LEWIS, 2002; MORSCHHAUSER, 2010).

Tem sido observado que a anfotericina-B apresenta ação fungicida contra *Candida* spp, enquanto os azólicos são geralmente agentes fungistáticos para este patógeno e agentes fungicidas para *Aspergillus* spp (MANAVATHU *et al*., 1998). Já as equinocandinas, como caspofungina e micafungina, exibem atividade fungicida para *Candida* spp e atividade fungistática para *Aspergillus* spp. (GULLO, 2009).

CASTRO *et al*. (2011) utilizaram *Sacchromyces cerevisiae* como organismo-modelo para estudos de genética e biologia celular, com o objetivo de compreender o mecanismo de ação da própolis de *A. mellifera* sobre fungos. Inicialmente foi avaliada a sobrevida do mesmo através da análise de determinantes envolvidos na apoptose e/ou necrose deste organismo e, posteriormente, foram investigados os possíveis alvos celulares da própolis. Os autores observaram que o extrato foi capaz de induzir morte celular por apoptose e necrose secundária, e as células fúngicas na fase de desenvolvimento inicial (“fase lag”) e na fase estacionária foram mais resistentes, enquanto células na fase exponencial foram muito mais sensíveis à própolis. Além disso, a própolis induziu acúmulo de espécies reativas de oxigênio durante a apoptose.

S. cerevisiae não possui caspases em seu genoma, mas CASTRO *et al*. (2011) identificaram uma proteína da mesma família, denominada de metacaspase YCA1, possivelmente importante no mecanismo de morte celular induzida por própolis sobre este micro-organismo. Os mesmos autores

investigaram se outros componentes estariam envolvidos no processo de morte celular induzida pela própolis, além de concluir que a molécula citocromo c possui grande importância. Em células de mamíferos, as duas principais vias apoptóticas são a via intrínseca, que precisa do envolvimento da mitocôndria, e a via extrínseca, em que a cascata das caspases é ativada. Umas das principais características da via intrínseca é a liberação de citocromo c no citosol, o qual ativa caspases. Paralelamente, altas concentrações de componentes isolados da própolis (ácido caféico, *p*-cumárico, cinâmico e isosacuranetina) também foram testadas, mas não apresentaram nenhum efeito sobre o fungo. Os autores concluíram que a ação da própolis sobre leveduras envolve mecanismos relacionados com a função mitocondrial, acidificação vacuolar e autofagia.

A pitiose é uma doença de natureza granulomatosa que acomete animais e humanos. Seu agente etiológico é o *Pythium insidiosum*, um micro-organismo semelhante a fungo, pertencente ao Reino Stramenopila, Filo Oomycota. Além da posição taxonômica, há outras diferenças estruturais e bioquímicas que diferem os oomicetos dos fungos verdadeiros, tais como a ausência de ergosterol em sua membrana plasmática, fato este que tem implicações práticas diretas no tratamento, uma vez que as drogas antifúngicas que atuam no ergosterol não têm efeito sobre este patógeno. O tratamento da pitiose é limitado a extensos procedimentos cirúrgicos, o que nem sempre é possível, considerando-se o tamanho e localização das lesões. Torna-se, portanto, de extrema importância a busca por novos compostos e/ou formas de tratamento da pitiose. Neste contexto, a avaliação da geoprópolis sobre o crescimento *in vitro* é uma abordagem interessante e inovadora (JOHNSON *et al*., 2004; BOSCO *et al*., 2005; SANTURIO *et al*., 2006; BROWN *et al*., 2008; CAVALHEIRO *et al*., 2009).

2. Objetivos da tese

Com base nas observações expostas, esta tese teve por objetivos analisar a composição química do extrato hidroalcoólico da geoprópolis (Geo) produzida por *Melipona fasciculata*. Considerando a escassez de dados sobre

as propriedades biológicas, analisamos a possível ação citotóxica do Geo sobre células do carcinoma da laringe humana (HEp-2), avaliando sua morfologia e o número de células viáveis após incubação com Geo em diferentes concentrações e períodos de tempo. Também foi avaliada a possível citotoxicidade da Geo sobre monócitos humanos e sobre a produção de citocinas pró- (TNF-α) e anti-inflamatória (IL-10), por meio de ensaio imunoenzimático. A atividade antibacteriana da Geo foi investigada sobre linhagens de *Staphylococcus aureus* e *Escherichia coli*, determinando sua concentração inibitória mínima e curvas de sobrevivência, em função do tempo, e possíveis interações (sinergismo ou antagonismo) foram avaliadas entre a Geo e drogas antibacterianas convencionais sobre linhagens de *S. aureus* e *E. coli*. Finalmente, analisamos o efeito da Geo sobre a inibição do crescimento *in vitro* de isolados de *Pythium insidiosum* obtidos de humano e equinos.

3. Justificativa e apresentação da tese

Como consequência dos resultados positivos das pesquisas com a própolis produzida por *Apis mellifera*, um grande entusiasmo tem despontado quanto às pesquisas com geoprópolis produzida por *Melipona fasciculata*, porém trabalhos sobre suas atividades citotóxica, imunomoduladora, antibacteriana e antifúngica são escassos.

Devido ao uso crescente deste produto para fins terapêuticos e às suas propriedades biológicas, foi relevante a realização de pesquisas com a finalidade de prover aos produtores e consumidores dados científicos que comprovem os efeitos da geoprópolis de *M. fasciculata*, buscando atingir um perfil de qualidade e posterior certificação. Além disso, os resultados desta tese são pioneiros principalmente com relação à citotoxicidade do extrato hidroalcoólico de geoprópolis de *M. fasciculata* sobre células normais (monócitos) e sobre células tumorais (carcinoma da laringe humana - HEp-2).

A ação imonomoduladora da própolis de *A. mellifera* tem sido observada, tanto na estimulação como na supressão de determinados eventos da resposta imune. Contudo, houve necessidade de ampliação dos estudos

relacionados à sua ação imunomoduladora quando se trata de geoprópolis de abelhas indígenas sem ferrão, devido à escassez de dados na literatura.

É importante ressaltar que a avaliação do efeito antifúngico da geoprópolis em isolados de *Pythium insidiosum* corresponde a uma nova linha de pesquisa que foi implantada no nosso departamento com a realização deste trabalho. Ainda não há droga antifúngica eficiente contra *P. insidiosum*, sendo difícil o tratamento de infecções causadas por este fungo, por apresentar características singulares. Os resultados deste trabalho poderão fornecer subsídios para o surgimento de tratamentos alternativos ou associados àqueles já existentes.

A presente tese é composta por três capítulos, que abordam os temas:

- Capítulo 1: **"*In vitro* cytotoxic activity of geopropolis produced by *Melipona fasciculata* Smith on HEp-2 cells"**, a ser submetido à publicação junto à revista *Journal of Ethnopharmacology*;

- Capítulo 2: **"Chemical analysis and immunomodulatory activity of geopropolis produced by *Melipona fasciculata* Smith on human monocytes"**, a ser submetido ao *Journal of Ethnopharmacology*;

- Capítulo 3: **"Antimicrobial activity of geopropolis produced by *Melipona fasciculata* Smith in northeast Brazil"**, a ser submetido junto à revista *Evidence-Based Complementary and Alternative Medicine.*

Os artigos foram redigidos de acordo com as normas de cada periódico.

Referências bibliográficas*

Abreu BVB. Prospecção química de geoprópolis de *Melipona fasciculata* Smith. São Luis, 2011. 53p. Dissertação (Mestrado em Ciências da Saúde). Centro de Ciências Biológicas e da Saúde, Universidade Federal do Maranhão.

Alves EG, Vinholis AHC, Casemiro LA, Furtado NAJC, Silva MLA, Cunha WR, Martins CHG. Estudo comparativo de técnicas de *screening* para a avaliação da atividade antibacteriana de extratos brutos de espécies vegetais e de substâncias puras. Quim Nova 2008; 31: 1224-1229.

Ansorge S, Reinhold D, Lendeckel U. Propolis and some of its constituents down-regulate DNA synthesis and inflammatory cytokine production but induce TGF-β1 production of human immune cells. Z Naturforsch. 2003; 58c: 580–589.

Araújo MJAM, Dutra RP, Costa GC, Reis AS, Assunção AKM, Libério SA, Maciel MCG, Silva LA, Guerra RNM, Ribeiro MNS, Nascimento FRF. Efeito do tratamento com própolis de *Scaptotrigona* aff. *postica* sobre o desenvolvimento do tumor de Ehrlich em camundongos. Rev Bras Farmacogn. 2010; 20: 580-587.

Assunção AKM. Efeito antitumoral do tratamento com extrato hidroalcoólico de geoprópolis de *Melipona fasciculata* Smith em modelo experimental de tumor de Ehrlich. São Luis, 2011. 68p. Dissertação (Mestrado em Ciências da Saúde). Universidade Federal do Maranhão.

Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol. 2009; 27: 669-692.

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*Referências bibliográficas elaboradas de acordo com o International Committee of Medical Journal Editors. Uniform Requirements for Manuscripts submitted to Biomedical Journal: sample references. [homepage onthe internet]. Bethesda: U.S. National Library of Medicine; 2003 [last upatade 2003 July 09; cited 2005 Jun01]. 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.

Bachiega TF, Orsatti CL, Pagliarone AC, Sforcin JM. The effects of propolis and its isolated compounds on cytokines production by murine macrophages. Phytother Res. 2012; 9: 1308-1313.

Bankova V. Chemical diversity of propolis and the problem of standardization. J Ethnopharmacol. 2005a; 100: 114-117.

Bankova V. Recent trends and important developments in propolis research. Evid-Based Compl Alt Med. 2005b; 2: 29-32.

Bankova V, Boudorova-Krasteva G, Sforcin JM, Frete X, Kujumgiev A, Maimoni-Rodella R, Popov, S. Phytochemical evidence for the plant origin of Brazilian propolis from São Paulo State. Z Naturforsch. 1999; 54: 401-405.

Bankova V, Castro SL, Marcucci MC. Propolis: recent advances in chemistry and plant origin. Apidologie 2000; 31: 3-15.

Bankova V, Dyulgerov A, Popov S, Evstatieva L, Kuleva L, Pureb O, Zamjansan Z. Propolis produced in Bulgaria and Mongolia phenolic compounds and plant origin. Apidologie 1992; 23: 79-85.

Bankova V, Marcucci MC, Simova S, Nikolova N, Kujumgiev A, Popov S. Antibacterial diterpenic acids from Brazilian propolis. Z Naturforsch. 1996; 51c: 277-280.

Banskota AH, Tezuka Y, Kadota S. Recent progress in pharmacological research of propolis. Phytother Res. 2001; 15: 561-571.

Barth OM, Pinto da Luz CF. Palynological analysis of Brazilian geopropolis sediments. Grana 2003; 42: 121-127.

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. 2007; 102: 261-265.

Bazo AP, Rodrigues MAM, Sforcin JM, Camargo JLV, Ribeiro LR, Salvadori DMF. Protective action of propolis on the rat colon carcinogenesis. Teratogen Carcin Mut. 2002; 22: 183-194.

Borges KS, Brassesco MS, Scrideli CA, Soares AEE, Tone LG. Antiproliferative effects of Tubi-bee propolis in glioblastoma cell lines. Genet Mol Biol. 2011; 34: 310-314.

Bosco SMG, Bagagli E, Araújo Jr. JP, Candeias JMG, Franco MF, Marques MEA, Mendoza L, Camargo RP, Marques SA. Human pythiosis, Brazil. Emerg Infect Dis. 2005; 11: 715-717.

Brown TA, Grooters AM, Hosgood GL. *In vitro* susceptibility of *Pythium insidiosum* and a *Lagenidium* sp to itraconazole, posaconazole, voriconazole, terbinafine, caspofungin, and mefenoxam. AJVR 2008; 69: 1463-1468.

Búfalo MC, Candeias JMG, Sforcin JM. *In vitro* cytotoxic effect of brazilian green propolis on human laryngeal epidermoid carcinoma (HEp-2) cells. Evid-Based Compl Alt Med. 2009; 22: 483-487.

Burdock GA. Review of the biological properties and toxicity of bee propolis. Food Chem Toxicol. 1998; 36: 347-63.

Castaldo S, Capasso F. Propolis, an old remedy used in modern medicine. Fitoterapia 2002; 73: 1-6.

Castro PA, Savoldi M, Bonatto D, Barros MH, Goldman MHS, Berretta AA, Goldman GH. Molecular characterization of propolis induced cell death in *Saccharomyces cerevisiae*. Eukaryot Cell. 2011; 10: 398-411.

Cavalheiro AS, Zanette RA, Spader TB, Lovato L, Azevedo MI, Botton S, Alves SH, Santurio JM. *In vitro* activity of terbinafine associated to amphotericin B, fluvastatin, rifampicin, metronidazole and ibuprofen against *Pythium insidiosum*. Vet Microbiol. 2009; 137: 408-411.

Cinegaglia NC. Ação citotóxica da própolis e da geoprópolis sobre as células de osteossarcoma canino. Bandeirantes, 2011. 35p. Monografia. Universidade Estadual do Norte do Paraná.

Cunha BA. Antibiotic side effects. Med Clin North Am. 2001; 85: 149-185.

Cunha IBS, Sawaya ACHF, Caetano FM, Shimizu MT, Marcucci MC, Drezza FT, Povia GS, Carvalho PO. Factors that influence the yield and composition of Brazilian propolis extracts. J Brazil Chem Soc. 2004; 15: 964-970.

Cunha MS, Dutra RP, Batista MCA, Abreu BVB, Santos JR, Neival VA, Amaral FMM, Ribeiro MNS. Padronização de extrativos de geoprópolis de *Melipona fasciculata* Smith (tíuba). Cad Pesq. UFMA 2009; 16: 31-38.

Dimov V, Ivanovska N, Manolova N , Bankova V, Nikolov N, Popov S. Immunomodulatory action of propolis. Influence on anti-infections protection and macrophage function. Apidologie 1991; 22: 155–162.

Fernandes Jr. A, Leomil L, Fernandes AAH, Sforcin JM. The antibacterial activity of propolis produced by *Apis mellifera* L. and Brazilian stingless bees. J Venom Anim Toxins 2001; 7: 173-182.

Fernandes Jr. A, Balestrin EC, Betoni JEC, Orsi RO, Cunha MLRS, Montelli AC. Propolis: anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. Mem. Inst. Oswaldo Cruz 2005; 100: 563-566.

Freitas MO, Ponte FAF, Lima MAS, Silveira ER. Flavonoids and Triterpenes from the nest of the stingless bee *Trigona spinipes*. J Brazil Chem Soc. 2008; 19: 532-535.

Ghisalberti EL. Propolis: a review. Bee World 1979; 60: 59-84.

Gullo A. Invasive fungal infections: the challenge continues. Drugs 2009; 69: 65-73.

Johnson MD, Macdougall C, Ostrosky-Zeichner L, Perfect JR, Rex JH. Combination antifungal therapy. Antimicrob. Agents Chemother. 2004; 48: 693-715.

Kerr WE. Abelhas indígenas brasileiras (meliponíneos) na polinização e na produção de mel, pólen, geoprópolis e cera. Informe Agropecuário 1987; 13: 15-22.

Khayal MT, El-Ghazaly MA, El-Khatib AS, Hatem AM, Vreis PJF, El-Shafei S, Khattab MM. A clinical pharmacological study of the potential beneficial effects of a própolis food product as an adjuvant in asthmatic patients. Fundamental & Clinical Pharmacology 2003; 17: 93-102.

Kontoyiannis DP, Lewis RE. Antifungal drug resistance of pathogenic fungi. Lancet 2002; 359: 1135-1144.

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

Levy Jr NC. Estudo da atividade antimicrobiana de méis e própolis de *Apis mellifera* e Meliponinae brasileiros. Rio Claro, 1997. 116p. Dissertação (Mestrado). Instituto de Biociências, Universidade Estadual Paulista.

Lilenbaum W, Barbosa AV. Avaliação da atividade antimicrobiana da própolis perante *Malassezia pachydermatis* "*in vitro*". Rev Bras Med Vet. 1994; 16: 248-251.

Libério SA, Pereira ALA, Araújo MJAM, Dutra RP, Nascimento FRF, Monteiro-Neto V, Ribeiro MNS, Gonçalves AG, Guerra RNM. The potencial use of propolis as a cariostatic agent and its actions on mutans group streptococci. J Ethnopharmacol. 2009; 125: 1-9.

Libério SA, Pereira ALA, Dutra RP, Reis AS, Araújo MJAM, Mattar, N.S., Silva, L.A., Ribeiro, M.N.S., Nascimento, F.R.F., Guerra, R.N.M., Monteiro-Neto, V., Antimicrobial activity against oral pathogens and immunomodulatory effects and toxicity of geopropolis produced by the stingless bee *Melipona fasciculata* Smith. BMC Complement Altern Med. 2011; 11: 108.

Locksley RM, Killeen N, Leonardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 2001; 104: 487-501.

Lustosa SR, Galindo AB, Nunes LCC, Randau KP, Rolim Neto PJ. Própolis: atualizações sobre a química e a farmacologia. Rev Bras Farmacogn. 2008; 18: 447-454.

Maia-Filho EM, Maia CCR, Bastos ACSC, Novais TMG. Efeito antimicrobiano *in vitro* de diferentes medicações endodônticas e própolis sobre *Enterococcus faecalis*. Rev Gaúcha Odontol. 2008; 56: 21-25.

Manavathu EK, Culright JL, Chandrasekar CHPH. Organism-dependent fungicidal activities of propolis. Antimicrob Agents Ch. 1998; 42: 3018-3021.

Martins ACL. Recursos tróficos de *Melipona fasciculata* Smith, 1854 (Hymenoptera: Apidae: Apini: Meliponina) em uma área da baixada maranhense. São Luís, 2008. Dissertação (Mestrado em Biodiversidade e Conservação) - Centro de Ciências Biológicas e da Saúde, Universidade Federal do Maranhão.

Martins ACL, Rego MMC, Carreira LMM, Albuquerque PMC. Espectro polínico de mel de tiúba (*Melipona fasciculata* Smith, 1854, Hymenoptera, Apidae). Acta Amaz. 2011; 41: 183-190.

Michener CD. The social behaviour of the bees. A comparative study. Cambridge: Harvard University, 1974. 404p.

Michener CD. The bees of the world. Baltimore: The Johns Hopkins University Press, 2000. 913p.

Miorin PL, Levy Junior NC, Custodio AR, Bretz WA, Marcucci MC. Antibacterial activity of honey and propolis from *Apis mellifera* and *Tetragonisca angustula* against *Staphylococcus aureus*. J Appl Microbiol. 2003; 95: 913-920.

Mirzoeva OK, Grishanin RN, Calder PC. Antimicrobial action of propolis and its components, the effects on growth membrane potential, and motility of bacteria. Microbiol Res. 1997; 152: 239-246.

Morschhauser J. Regulation of multidrug resistance in pathogenic fungi. Fungal Genet Biol. 2010; 47: 94-106.

Nogueira-Neto P. A criação de abelhas indígenas sem ferrão. São Paulo: Nogueirapis, 1997. 446p.

Orsi RO, Funari SRC, Soares AMVC, Calvi SA, Oliveira SL, Sforcin JM, Bankova V. Immunomodulatory action of propolis on macrophage activation. J Venom Anim Toxins 2000; 6: 205-219.

Orsi RO, Sforcin JM, Funari SRC, Fernandes Jr. A, Bankova V. Synergistic effect of propolis and antibiotics on the *Salmonella* Typhi. Braz J Microbiol. 2006; 37: 108-112.

Orsi RO, Fernandes Jr. A, Bankova V, Sforcin JM. Antibacterial effects of Brazilian and Bulgarian propolis and synergistic effects with antibiotics acting on the bacterial DNA and folic acid. Nat Prod Res. 2012a; 26: 344-349.

Orsi RO, Fernandes Jr. A, Bankova V, Sforcin JM. The effects of Brazilian and Bulgarian propolis *in vitro* against *Salmonella* Typhi and synergism with antibiotics acting on the ribosome. Nat Prod Res. 2012b; 26: 430-437.

Orsolić N, Basić I. Immunomodulation by water-soluble derivative of propolis: a factor of antitumor reactivity. J Ethnopharmacol. 2003; 84: 265–273.

Orsolić N, Knezević AH, Sver L, Terzić S, Basić I. Immunomodulatory and antimetastatic action of propolis and related polyphenolic compounds. J Ethnopharmacol. 2004; 94: 307–315.

Orsolić N, Saranovic AB, Basić I. Direct and indirect mechanism(s) of antitumour activity of propolis and its polyphenolic compounds. Planta Med. 2006; 72: 20–27.

Park YK, Alencar SM, Scamparine ARP, Aguiar CL. Própolis produzida no sul do Brasil, Argentina e Uruguai: Evidências fitoquímicas de sua origem vegetal. Cienc Rural 2002; 2: 997-1003.

Pereira AS, Bicalho B, Aquino-Neto FR. Comparison of propolis from *Apis mellifera* and *Tetragonisca angustula*. Apidologie 2003; 34: 291-298.

Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y, Fisher PB. Interleukin-10 and related cytokines and receptors. Annu Rev Immunol. 2004; 22: 929-979.

Pietta PG, Gardana C, Pietta AM. Analytical methods for quality control of propolis. Rev Fitoter. 2002; 73: S7-S20.

Pinto MS, Faria JE, Message D, Cassini STA, Pereira CS, Gioso MM. Efeito do extrato de própolis verde sobre bactérias patogênicas isoladas no leite de vacas com mastite. Braz J Vet Res Anim. 2001; 38: 278-283.

Raulf-Heimsoth M. T cell-primary culture from peripheral blood. In: Jones, M.G. & Lympany, P. Allergy Methods and Protocols. Method Mol Med. 2008. Human Press, New Jersey, 17-30.

Rios JL, Recio MC, Villar A. Screening methods for natural products with antimicrobial activity: a review of the literature. J Ethnopharmacol. 1988; 23: 127-149.

Rizzi CC. Estudo *in vitro* da atividade antimicrobiana da própolis, clorexidina, hipoclorito de sódio e hidróxido de cálcio frente a *Enterococcus faecalis*. São Luis, 2005. 52p. Dissertação (Mestrado em Ciências da Saúde) - Centro de Ciências Biológicas e da Saúde. Universidade Federal do Maranhão.

Sá-Nunes A, Faccioli LH, Sforcin JM. Propolis: lymphocyte proliferation and IFN-γ production. J Ethnopharmacol. 2003; 87: 93–7.

Salatino A, Teixeira EW, Negri G, Message D. Origin and chemical variation of Brazilian propolis. Evid-Based Compl Alt Med. 2005; 2: 33-38.

Santos CG, Santos KC, Tirelli FP, Blochtein B. Caracterização sazonal de acúmulos isolados de própolis em colônias de *Plebeia emerina* (Hymenoptera, Apidae) no sul do Brasil. Iheringia, Sér Zool. 2009; 99: 200-203.

Santurio JM, Alves SH, Pereira DB, Argenta JS. Pitiose: uma micose emergente. Acta Sci Vet. 2006; 34: 1-14.

Scazzocchio F, D'auria FD, Alessandrini D, Pantanella F. Multifactorial aspects of antimicrobial activity of propolis. Microbiol Res. 2005; 4: 327-333.

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

Sforcin JM, Bankova V. Propolis: is there a potencial for the development of new drugs? J Ethnopharmacol. 2011; 133: 253-260.

Sforcin JM, Fernandes Jr. A, Lopes CAM, Bankova V, Funari SRC. Seasonal effect on Brazilian propolis antibacterial activity. J Ethnopharmacol. 2000; 73: 243-249.

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.

Scheller S, Gazda G, Pietsz G, Gabrys J, Szumlas J, Eckert L, Shani J. The ability of ethanol extract of propolis to stimulate plaque formation in immunized mouse spleen cells. Pharmacol Res Commun. 1988; 20: 323-328.

Sheldon Jr. AT. Antibiotic resistance: who is winning the war? Introductory remarks. Int J Toxicol. 2003; 22: 129-130.

Tatefuji T, Izumi N, Ohta T, Arai S, Ikeda M, Kurimoto M. Isolation and identification of compounds from Brazilian propolis which enhance macrophage spreading and mobility. Biol Pharm Bull. 1996; 19: 966-970.

Tomás-Barberán FA, Garcia-Viguera C, Vit-Olivier P, Ferreres F, Tomas-Lorente F. Phytochemical evidence for the botanical origin of tropical propolis from Venezuela. Phytochemistry 1993; 34: 191-196.

Uzel A, Sorkun K, Onçag O, Cogulo D, Gençay O, Salih B. Chemical compositions and antimicrobial activities of four different Anatolian propolis samples. Microbiol Res. 2005; 160: 189-195.

Vargas AC, Loguercio AP, Witt NM, Costa MM, Silva MS, Viana LR. Atividade antimicrobiana *in vitro* de extrato alcoólico de própolis. Ciênc Rural 2004; 34: 159-163.

Velikova M, Bankova V, Tsvetkova I, Kujumgiev A, Marcucci MC. Antibacterial ent-kaurene from Brazilian propolis of native stingless bees. Fitoterapia 2000; 71: 693-696.

Venturieri GC, Raiol VFO, Pereira CAB. Avaliação da introdução da criação racional de *Melipona fasciculata* (Apidae, Meliponina), entre os agricultores familiares de Bragança, PA, Brasil. Biotropica 2003; 2: 1-7.

Watanabe MAE, Amarante MK, Conti BJ, Sforcin JM. Cytotoxic constituents of propolis inducing anticancer effects: a review. J Pharm Pharmacol. 2011; 63, 1378-1386.

Capítulo 1

"In vitro cytotoxic activity of geopropolis produced by *Melipona fasciculata* Smith on HEp-2 cells", a ser submetido ao Journal of Ethnopharmacology

***In vitro* cytotoxic activity of geopropolis produced by *Melipona fasciculata* Smith on HEp-2 cells**

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ABSTRACT

Ethnopharmacological relevance: Propolis is produced by Africanized honeybees from resinous materials of plants, adding wax and bee enzymes. Contrarily, geopropolis (Geo) is produced by indigenous stingless bees not only from resinous materials of plants, adding salivary secretions and wax, but also adding mud or clay. Since there are few works regarding Geo cytotoxic action towards tumor cells, the goal of this work was to investigate its possible effect on human laryngeal epidermoid carcinoma (HEp-2) cells.

Materials and methods: Cells were incubated with different concentrations (5, 10, 25, 50 and 100 µg) of Geo for different time periods (6, 24, 48 and 72 h), and the morphology and number of viable HEp-2 cells were analyzed. The same procedure was performed with Geo solvent (70% ethanol) and carboplatin was used as a positive control. Cell viability was assessed using the MTT test.

Results: Data showed that HEp-2 cells exhibited a fast growth, with a large number of fusiform and elongated-shaped cells. A significant decrease in cell viability was observed after 6 h of incubation with 50 and 100 µg. After 24, 48 and 72 h, there was a significant decrease from 25-100 µg. Geo solvent had no effects on morphology and number of viable cells. Carboplatin (400 and 500 mMol/L) exerted only a slight inhibitory effect on HEp-2 cells.

Conclusions: Although almost all tumor cells seem to be sensitive to the action of propolis produced by Africanized honeybees, geopropolis produced by stingless bees showed only a cytostatic action on HEp-2 cells, which were resistant to carboplatin as well. Geo potential should be further investigated in association to chemotherapeutic agents against tumor cells.

Keywords:
Hep-2 cells
Cytotoxic activity
Geopropolis

1. Introduction

The use of bee products for the treatment and relief of various pathological conditions comes from ancient times. Natural products have never been so investigated as currently, mainly for their potential in cancer therapy. In recent decades, several researchers have reported the biological properties of propolis produced by bees of the genus *Apis*. It has also increased the interest of researchers in exploring the potential of geopropolis produced by stingless bees.

Propolis is a material derived from vegetable resins and balsams, wax and enzymes secreted by bees, showing different colors depending on the botanical origin. Unlike propolis, to produce geopropolis (Geo), in addition to plant material, secretions from glands, bee wax and pollen, some species of stingless bees add mud or clay to its composition (Kerr, 1987; Nogueira-Neto, 1997; Castaldo and Capasso, 2002).

In vitro and *in vivo* assays have reported the antitumor effects of propolis produced by *Apis mellifera* (Burdock, 1998; Banskota et al., 2001; Sforcin, 2007), investigating the inhibitory effect of propolis and its chemical compounds on the tumor cell growth in animals and humans (Matsuno et al., 1997a; Matsuno et al., 1997b; Aso et al., 2004; Orsolic and Basic, 2005; Wang et al., 2005; Ahn et al., 2007; Búfalo et al., 2009). Kouidhi et al. (2010) analyzed the cytotoxicity of ethanol extract of propolis produced by *A. mellifera* against neoplastic cell lines (HEp-2, HT-29, A549, RAW 264.7 and Vero), confirming its inhibitory action on all tumor cells.

Our group demonstrated that the administration of hydroalcoholic extract of propolis produced by *A. mellifera* to rats after treatment with the carcinogen dimethyl-hydrazine reduced the number of aberrant crypts in the colon, reflecting suppression of clonal expansion of initiated cells, which characterizes the step of promotion of carcinogenesis (Bazo et al., 2002). Furthermore, propolis cytotoxic effect was assessed on human laryngeal carcinoma (HEp-2) (Búfalo et al., 2009) and on canine transmissible venereal tumor (TVT) (Bassani-Silva et al., 2007), in a dose- and time-dependent way.

With regards to stingless bee products, Araújo et al. (2010) investigated the effect of hydroalcoholic extract of propolis produced by Scaptotrigona aff. postica on Ehrlich tumor development in female mice. Treatments were performed in single dose 48 h before tumor inoculation, verifying that propolis inhibited the tumor growth, which was associated with an increase in bone marrow and splenic cellularity. Borges et al. (2011) also observed the anti-proliferative action of propolis produced by *Scaptotrigona* sp on human glioblastoma (U251 and U343).

There is no data in literature concerning the effects of Geo produced by *Melipona fasciculata* on human laryngeal epidermoid carcinoma (HEp-2) cells, which are derived from laryngeal carcinoma cells of human nasopharyngeal mucosa. Thus, this work was carried out to investigate the cytotoxic activity of Geo compared to carboplatin, which is used clinically to treat several tumors, due to its high efficacy and low toxicity (Caires et al., 1999; Desoize et al., 2002).

2. Materials and Methods

2.1. Hydroalcoholic extract of Geo

Geo produced by *Melipona fasciculata* Smith was collected at Palmeirândia municipality (2º 39' S, 44º 55' W), in the western microregion of Maranhão State, northeast Brazil. The sample was ground and macerated in 70% ethanol at room temperature and moderate shaking. After 24 h, the hydroalcoholic extract of Geo was filtered and the dry weight was calculated (10.5 mg/mL) (Dutra *et al*., 2008).

2.2. Human laryngeal epidermoid carcinoma cells (HEp-2)

HEp-2 cells were grown in minimum essential media (MEM) (Cultilab, Campinas, São Paulo, Brazil) supplemented with 10% fetal bovine serum (FBS) (Cultilab) and gentamycin (40 mg/mL – GIBCO), and cultivated at 37 °C and 5% CO_2. The adherent cells in 25 cm^2 flasks were washed with 5-10 mL of MEM,

and afterwards 1-2 mL of trypsin (0.2% trypsin in 5% EDTA) were added until cell detachment. Cells were counted using 50 µL of Trypan blue 0.2% added to 50 µL of cell suspension in a hemocytometer and adjusted to 2 x 10^5 cells/mL. Cells were cultivated in a 96 wells U-bottom plate (Corning). After preparation of the plates, adherent cells were incubated at 37 °C for 24 h.

2.3. Effects of Geo, 70% ethanol and carboplatin on HEp-2 cells

Geo or its solvent (70% ethanol) were diluted in MEM supplemented with 10% FBS, and specific dilutions were prepared for each assay in order to achieve different concentrations: 5, 10, 25, 50 and 100 µg/well (Búfalo *et al*., 2009). Carboplatin was used as a positive control (Darrow-Vancel® Laboratories A/S), diluted in MEM using the following concentrations: 100, 200, 300, 400 and 500 µMol/L (Konstantinopoulos *et al*., 2008).

After 80% confluent monolayers, HEp-2 cells were incubated separately with Geo, 70% ethanol or carboplatin in a 96 wells plate at 37 °C and 5% CO_2 for 6, 24, 48 and 72 h. Control cells were incubated only with MEM. All experiments were performed in triplicate with 5 repetitions of the assays. After each period, cell morphology was evaluated by optical microscopy.

2.4. Cytotoxicity assay

Cell viability was determined by the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – Sigma®). This method is based on the absorption of MTT salt by the cells, which is reduced within the mitochondria to formazan. After incubation of cell cultures with the stimuli, the supernatant was removed and 100 µL of MTT dissolved in complete MEM was added, followed by subsequent incubation at 37 °C for 3 h. After this period, MTT was removed and 100 µL of dimethyl sulfoxide (DMSO, Dinamica) was added for solubilization of formazan crystals. Plates were read at 492 nm and the percentages of cell viability were calculated.

2.5. Statistical analysis

Analysis of variance (ANOVA) was employed, followed by the Tukey test for multiple comparisons ($P < 0.05$).

3. Results

3.1. Geo and 70% ethanol effects on HEp-2 cells viability

HEp-2 cells are derived from human laryngeal carcinoma, being adherent cells and showing different shapes and sizes, some of them with fusiform aspect, with one or more nucleoli (Lima et al., 2005; Búfalo et al., 2007). Rodrigues et al. (2009) reported that fusiform cells are smaller compared to those with rounded shape, due to functional differences related to cell cycle, once before dividing, cells swell and appear rounded. Control HEp-2 cells exhibited a fast growth and both fusiform and elongated cells (Fig. 1A). After incubation with Geo, HEp-2 cells showed a different morphology than that found in control, with misshapen cells and retracted membranes, and a greater brightness. A significant decrease was found in cell viability after 6 h of incubation with Geo using 50 and 100 µg (Fig. 1C and 2). After 24, 48 and 72 h of incubation, there was a significant decrease in cell viability from 25-100 µg ($P < 0.0001$) (Fig. 1D, 1E, 1F and 2). There was an interaction effect between concentration and incubation time on cell viability ($P < 0.0001$). 70% ethanol, at concentrations equivalent to Geo, had no effect on HEp-2 cells viability (Fig. 1B and 3). There was no interaction between group and concentration on cell viability ($P = 0.98$).

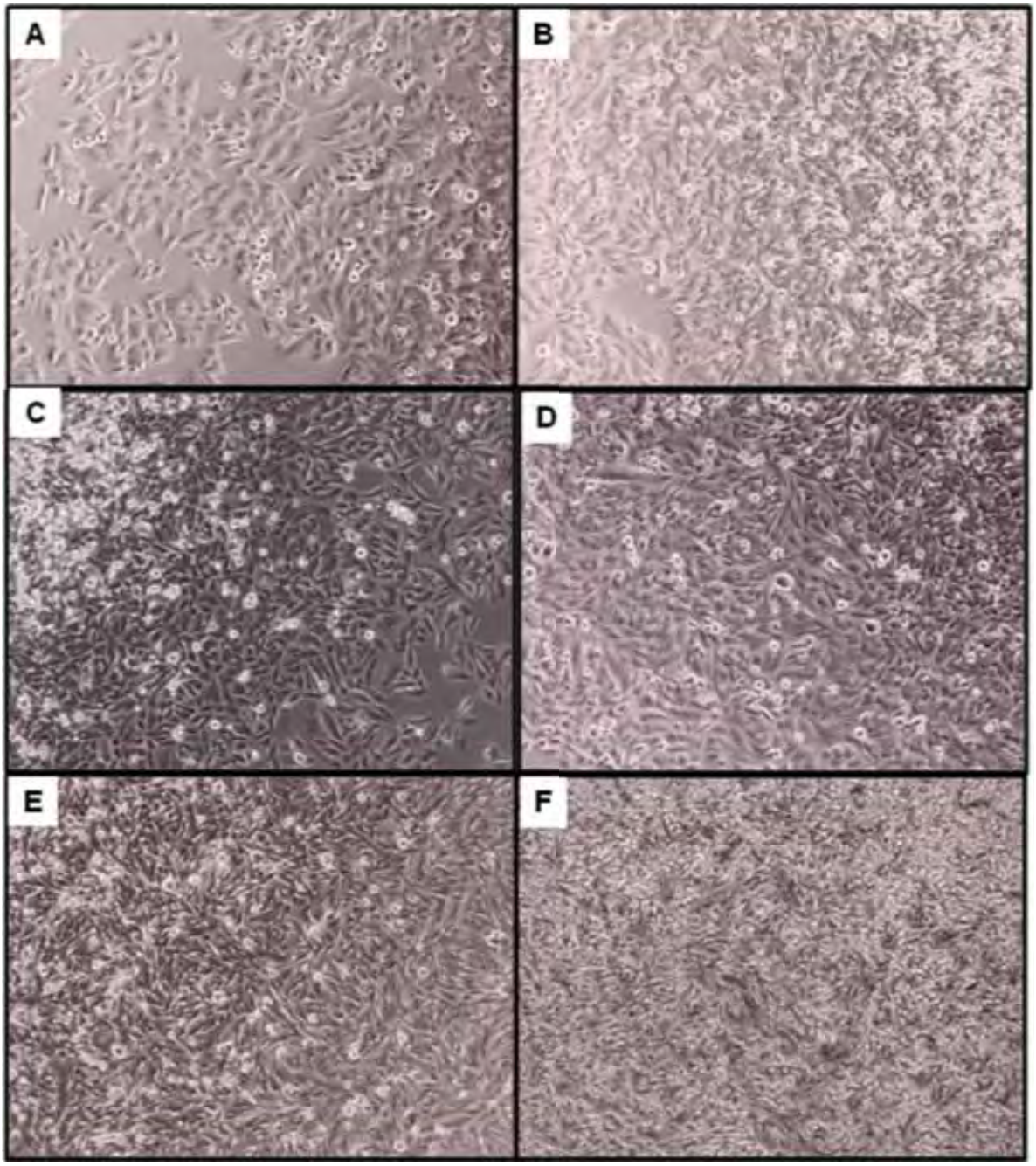

Fig. 1. HEp-2 cells morphology (magnification: 100 x). (A) Control, (B) 70% ethanol, (C) Geo 6 h, 50 µg/well, (D) Geo 24 h, 25 µg/well, (E) Geo 48 h, 25 µg/well, (F) Geo 72 h, 25 µg/well. The figure represents five similar assays performed in triplicate.

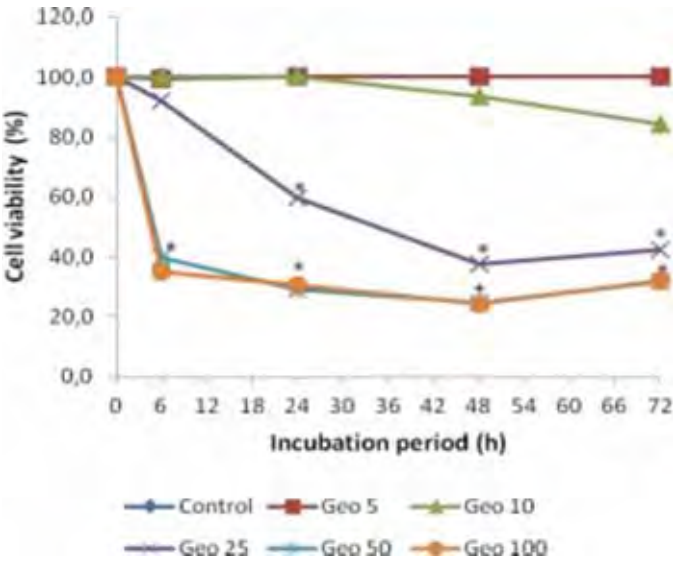

Fig. 2. Geo effects on HEp-2 cells viability. Geo concentrations (5, 10, 25, 50 and 100 µg) were assessed at 6, 24, 48 and 72 h. Data represent the mean of 5 experiments performed in triplicate. * significantly different from control ($P < 0.0001$).

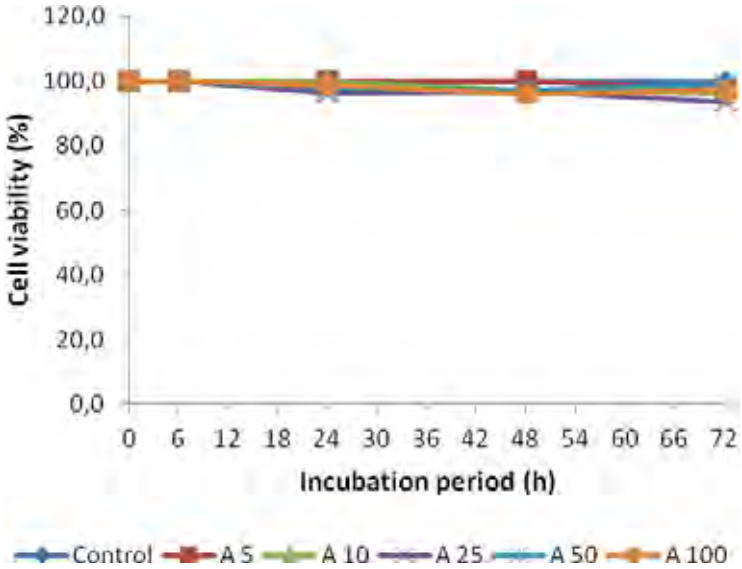

Fig. 3. 70% ethanol effects on HEp-2 cells viability. The concentrations of ethanol were equivalent to those found in 5, 10, 25, 50 and 100 µg of Geo. Data represent the mean of 5 similar experiments performed in triplicate.

3.2. Carboplatin effect on HEp-2 cells viability

The concentrations of 100 and 200 µMol/L recommended in the literature (Konstantinopoulos et al., 2008) exerted no effect on HEp-2 cells. Thus, higher concentrations (300, 400 and 500 µMol/L) of carboplatin were evaluated, verifying that 400 and 500 µMol/L showed only a slight inhibitory effect, what suggest the resistance of tumor cells to conventional drugs (Fig. 4). There was no significant interaction between concentration x group (P = 0.58).

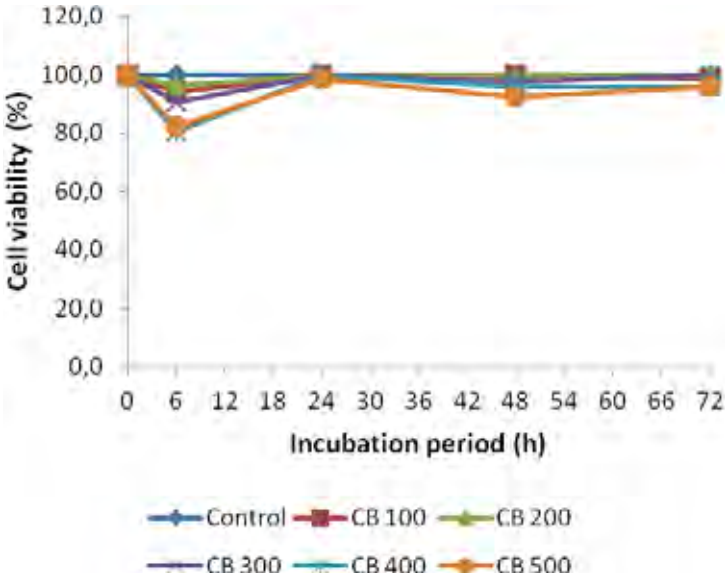

Fig. 4. Carboplatin (CB) effects on HEp-2 cells viability. Concentrations (100, 200, 300, 400 and 500 µMol/L) were assessed in different incubation periods (6, 24, 48 and 72 h). Data represent mean of 5 experiments performed in triplicate.

4. Discussion

Geo produced by *M. fasciculata* exerted an inhibitory action towards HEp-2 cells after 6 h incubation, using 50 and 100 µg. After 24, 48 and 72 h, a significant decrease in cell viability was also seen from 25-100 µg. Geo action was exclusively due to its constituents, with no effects of the solvent. Previous works of our group showed that the cytotoxic concentrations of Geo for canine

osteosarcoma cells were 25, 50 and 100 µg at 72 h incubation. The authors also evaluated the activity of propolis produced by *A. mellifera*, concluding that the concentrations of 50 and 100 µg were more effective after 72 h of incubation. These findings indicated a higher sensitivity of canine osteosarcoma cells to the cytotoxic effect *in vitro* of Geo compared to propolis (Cinegaglia et al., in press). Likewise, Assunção (2011) showed that Geo produced by M. fasciculata displayed an antitumor activity in vitro, inhibiting Ehrlich cells at concentrations ranging from 62.5 to 500 µg/mL.

Umthong et al. (2009) analyzed the antiproliferative activity of aqueous and methanol extracts of propolis produced by *Trigona laeviceps* in Thailand. The aqueous extract of propolis showed a higher antiproliferative activity than methanol extract on human colon cancer cells (SW620), and both extracts induced cell death by necrosis. The same authors evaluated the antiproliferative activity of ethanol, dichloromethane, hexane and methanol extracts of propolis produced by *T. laeviceps* against five neoplastic cell lines derived from lung (Chaco), stomach (KATO-III), colon (SW620), breast (BT474) and liver (Hep-G2) and on two strains of normal cells (fibroblasts-HS-27 and liver-CH). The hexane extract of propolis showed a higher cytotoxic activity against cancer cells, without affecting normal cells.

With respect to propolis produced by Africanized honeybees, Bassani-Silva et al. (2007) analyzed its effect on transmissible venereal tumor canine cells, observing its cytotoxic activity after 6 h of incubation using 100 µg, although the lowest concentrations were also effective (after 24 h from 50 µg, and after 48 h from 25 µg). Búfalo et al. (2009) verified the cytotoxic action of propolis on HEp-2 cells using 25, 50 and 100 µg after 6 h incubation, concluding that the highest concentrations of propolis showed a cytotoxic action in a short-term, while the effect of lower concentrations was observed over time. Búfalo et al. (2010) also examined the *in vitro* cytotoxic activity of *Baccharis dracunculifolia*, the main botanical source of propolis produced in the southeast Brazil, and the activity of caffeic and cinnamic acids, major compounds isolated of propolis. However, propolis action on tumor cells was higher than that presented by *B. dracunculifolia* and the isolated components.

In vitro cytotoxic effects of cinnamic acid derivatives (bacarina and drupanina), flavonoids and phenolic compounds were investigated on HL-60 cells (Akao et al., 2003; Moreno et al., 2005). Flavonoids have been pointed out to affect proliferation, differentiation and apoptosis of cancer cells (Havsteen, 2002). However, they are not the only ones responsible for the cytotoxic and antitumor properties of propolis from *A. mellifera*. Watanabe et al. (2011) reported the antitumor effects of propolis produced in several countries, as well as the action of some constituents, such as flavonoids, terpenes and phenethyl ester of caffeic acid (CAPE), concluding that the main mechanisms by which propolis exerts its action against tumor cells are apoptosis and cell cycle arrest.

These findings suggest the potential of Geo and propolis as anticancer agents; however, most of researches were performed using *in vitro* assays, assessing the direct action on different tumor cell lines. The administration of bee products to animals or humans is followed by its absorption, biotransformation and bioavailability and their antitumoral action *in vivo* should occur due to its immunomodulatory action, exerting therapeutic or chemopreventive effects.

Carboplatin is a chemotherapeutic agent administered in the treatment of various cancers in combination or not with other anticancer agents in different therapies (Caires et al., 1999). This drug has been widely used clinically due to few side effects when compared to cisplatin, the main drug to treat head and neck carcinomas (Hambek et al., 2005; Kuhar et al., 2007). However, in our study the concentrations of 100 and 200 µMol/L recommended in the literature (Konstantinopoulos et al., 2008) exerted no effect on HEp-2 cells. Thus, we investigated higher concentrations (300, 400 and 500 µMol/L) of carboplatin, verifying that 400 and 500 µMol/L showed only a slight inhibitory effect, what suggested the resistance of tumor cells to conventional drugs. Cinegaglia (2011) observed no carboplatin effects (100 and 200 µMol/L) on canine osteosarcoma cells. In fact, some dogs do not respond to cytotoxic chemotherapeutic agents, as observed by Larone and Delprat (2004) and Dernell et al. (2007). The effectiveness of platinum agents against neoplastic cells may be related to the inhibition of DNA synthesis or saturation of the

cellular capacity to repair DNA adducts of platinum (Szymkowski et al., 1992), what may result in changes of the three dimensional structure of DNA induced by adducts metal (Suo et al., 1999). Cells with a higher capacity in repair mechanisms are resistant to platinum compounds (Zamble and Lippard, 1995; Desoize and Madoulet, 2002), and clinically the resistance of cancer cells to platinum agents may occur by different mechanisms such as reduction in tumor blood flow, concentration of these drugs on tumor site, type of tumor and potential metastases. Furthermore, apoptosis induced by platinum is reduced in the presence of extracellular matrix proteins, which can bind to cancer cells (Wernyj and Morin, 2004; Stewart, 2007). The resistance of many cell lines can also be related to the fact that the resistant cells possess a more rigid membrane, with a large amount of sphingomyelin and high cholesterol levels (Popovic et al., 1993; Stewart, 2007). Our data demonstrated that HEp-2 cells were not sensitive to carboplatin treatment, suggesting resistance to chemotherapy, even at very high concentrations.

Herein, Geo inhibitory effects on HEp-2 cells were exclusively due to its constituents, since its solvent showed no cytotoxic effects. Although all tumor cells investigated so far were sensitive to the action of propolis produced by Africanized honeybees, Geo action against HEp-2 cells was less efficient compared to propolis produced by honeybees, perhaps due to the low yield of the extract. However, these results showed a possible continuity of investigation in order to explore the antitumor potential of this bee product associated or not to chemotherapeutic agents, which is of interest to human and veterinary medicine.

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References

Ahn, M., Kumazawa, S., Usui, Y., Nakamura, J., Matsuka, M., Zhu, F., Nakayama, T., 2007. Antioxidant activity and constituents of propolis collected in various areas of China. Food Chemistry 101, 1383-1392.

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

Araújo, M.J.A.M., Dutra, R.P., Costa, G.C., Reis, A.S., Assunção, A.K.M., Libério, S.A., Maciel, M.C.G., Silva, L.A., Guerra, R.N.M., Ribeiro, M.N.S., Nascimento, F.R.F., 2010. Efeito do tratamento com própolis de *Scaptotrigona* aff. *postica* sobre o desenvolvimento do tumor de Ehrlich em camundongos. Revista Brasileira de Farmacognosia 20, 580-587.

Aso, K., Kanno, S., Tadano, T., Satoh, S., Ishikawa, M., 2004. Inhibitory effect of propolis on the growth of human leukemia U937. Biological and Pharmaceutical Bulletin 27, 727-730.

Assunção, A.K.M., 2011. Efeito antitumoral do tratamento com extrato hidroalcoólico de geoprópolis de *Melipona fasciculata* Smith em modelo experimental de tumor de Ehrlich. São Luis, 68p. Dissertação (Mestrado em Ciências da Saúde). Universidade Federal do Maranhão.

Banskota, A.H., Tezuka, Y., Adnyana, I.K., Ishii, E., Midorikawa, K., Matsushige, K., Kadota, S., 2001. Hepatoprotective and anti-*Helicobacter pylori* activities of constituents from Brazilian propolis. Phytomedicine 8, 16-23.

Bassani-Silva, S., Sforcin, J.M., Amaral, A.S., Gaspar, L.F.J.; Rocha, N.S., 2007. Propolis effect *in vitro* on canine transmissible veneral tumor cells. Revista Portuguesa de Ciências Veterinárias 102, 261-265.

Bazo, A.P., Rodrigues, M.A.M., Sforcin, J.M., Camargo, J.L.V., Ribeiro, L.R., Salvadori, DMF, 2002. Protective action of propolis on the rat colon carcinogenesis. Teratogenesis, Carcinogenesis and Mutagenesis 22, 183-194.

Borges, K.S., Brassesco, M.S., Scrideli, C.A., Soares, A.E.E., Tone, L.G., 2011. Antiproliferative effects of Tubi-bee propolis in glioblastoma cell lines. Genetics and Molecular Biology 34, 310-314.

Búfalo, M.C., Candeias, J.M.G., Sforcin, J.M., 2009. *In vitro* cytotoxic effect of Brazilian green propolis on human laryngeal epidermoid carcinoma (HEp-2) cells. Evidence-Based Complementary and Alternative Medicine 22, 483-487.

Búfalo, M.C., Candeias, J.M.G., Sousa, J.P.B., Bastos, J.K., Sforcin, J.M., 2010. *In vitro* cytotoxic effect of *Baccharis dracunculifolia* and propolis against HEp-2 cells. Natural Product Research 24, 1710-1718.

Burdock, G.A., 1998. Review of the biological properties and toxicity of bee propolis (propolis). Food and Chemical Toxicology 36, 347-363.

Caires, A.C.F., Almeida, E.T., Mauro, A.E., Hemerly, J.P., Valentini, S.R., 1999. Síntese e atividade citotóxica de alguns azido-ciclopaladados estabilizados com ligantes bifosfínicos. Química Nova 22, 329-334.

Castaldo, S., Capasso, F., 2002. Propolis, an old remedy used in modern medicine. Fitoterapia 73, 1-6.

Cinegaglia, N.C., 2011. Ação citotóxica da própolis e da geoprópolis sobre as células de osteossarcoma canino. Bandeirantes, 35p. Monografia. Universidade Estadual do Norte do Paraná.

Cinegaglia, N.C., Bersano, P.R.O., Búfalo, M.C., Sforcin, J.M., in press. Cytotoxic action of Brazilian propolis *in vitro* on canine osteosarcoma cells. Phytotherapy Research.

Dernell, W.S., Ehrhart, N.P., Straw, R.C., Vail, D.M., 2007. Tumor of the Sheletal System. In: Withrow, S.J. & Vail, D.M. Small Animal Clinical oncology. 4 ed., 540-582.

Desoize, B., Madoulet, C., 2002. Particular aspects of platinum compounds used at present in cancer treatment. Critical Reviews in Oncology/Hematology 42, 317-325.

Dutra, R.P., Nogueira, A.M.C., Marques, R.R.O., Costa, M.C.P., Ribeiro, M.N.S., 2008. Avaliação farmacognóstica de geoprópolis de *Melipona*

fasciculata Smith da Baixada Maranhense, Brasil. Revista Brasileira de Farmacognosia 18, 557-562.

Hambek, M., Baghi, M., Strebhard, K., Baumann, H., Gstottner, W., Knecht, R., 2005. Reduction of cisplatin dosage by ZD 1839. Anticancer Research 25, 3985-3988.

Havsteen, B.H., 2002. The biochemistry and medical significance of the flavonoids. Pharmacology and Therapeutics 96, 67-202.

Kerr, W.E., 1987. Abelhas indígenas brasileiras (Meliponíneos) na polinização e na produção de mel, pólen, geoprópolis e cera. Informe Agropecuário 13, 15-22.

Konstantinopoulos, P.A., Fountzilas, E., Pillay, K., Zerbini, L.F., Libermann, T.A., Cannistra, S.A., Spentzos, D., 2008. Carboplatin-induced gene expression changes *in vitro* are prognostic of survival in epithelial ovarian cancer. BioMed Central Medical Genomics 1, 1-12.

Kouidhi, B., Zmantar, T., Bakhrouf, A., 2010. Anti-cariogenic and anti-biofilms activity of Tunisian propolis extract and its potential protective effect against cancer cells proliferation. Anaerobe 16, 566-571.

Kuhar, M., Imran, S., Singh, N., 2007. Curcumin and quercetin combined with cisplatin to induce apoptosis in human laryngeal carcinoma HEp-2 cells through the mitochondrial pathway. Journal of Cancer Molecules 4, 121-128.

Larone, D, Delprat, C., 2004. Quimioterapia Anticancerígena. 1ª ed., Roca, p. 79-123.

Lima, M.E., Rissino, J.D., Guimarães, A.C., Overal, D.J., Khayat, A.S., Souza, P.C., Christofolini, D.M., Smith, M.A.C., Burbano, R.R., 2005. Drifter technique: a new method to obtain metaphases in HEp-2 cell line cultures. Brazilian Archives of Biology and Technology 48, 537–540.

Matsuno, T., Chen, C., Basnet, P., 1997a. A tumoricidal and antioxidant compound isolated from an aqueous extract of propolis. Medical Science Research 25, 583-584.

Matsuno, T., Jung, S.K., Matsumoto, Y., Saito, M., Morikawa, J., 1997b. Preferential cytotoxicity to tumor cells of 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C) isolated from propolis. Anticancer Research 17, 3565-3568.

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

Nogueira-Neto, P., 1997. A criação de abelhas indígenas sem ferrão. Nogueirapis, São Paulo, 446p.

Orsolic, N., Basic, I., 2005. Antitumor, hematostimulative and radioprotective action of water-soluble derivative of propolis (WSDP). Biomedicine & Pharmacotherapy 59, 561–570.

Popovic, P., Wong, P., Goel, R., Evans, W.K., Howell, S.B., Stewart, D.J., 1993. Pressure-tuning infrared spectra of cisplatin sensitive and resistant human ovarian cancer cells exposed to cisplatin. Proceedings of the American Association for Cancer Research 34, 404.

Rodrigues, O.G., Lima, E.Q., Sousa, M.R.Q., Medeiros, P.L., Nascimento, S.C., 2009. Caracterização morfológica e cinética de crescimento das células de carcinoma epidermóide de laringe humano – $HEp_{2.}$ Revista de Biologia e Farmácia 3, 43 - 50.

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

Stewart, D.J., 2007. Mechanisms of resistance to cisplatin and carboplatin. Critical Reviews in Oncology/Hematology 63, 12-31.

Suo, Z., Lippard, S.J., Johnson, K.A., 1999. Single d(GpG)/*cis*-diammine-platinum (II) adduct-induced inhibition of DNA polymerization. Biochemistry 38, 715-726.

Szymkowski, D.E., Yarema, K., Essigmann, J.M., Lippard, S.J., Wood, R.D., 1992. An intrastrand d(GpG) platinum crosslink in duplex M13 DNA is refractory to repair by human cell extracts. Proceedings of the National Academy of Sciences of the United States of America 89, 10772-10776.

Umthong, S., Puthong, S., Chanchao, C., 2009. *Trigona laeviceps* propolis from Thailand: antimicrobial, antiproliferative and cytotoxic activities. The American Journal of Chinese Medicine 37, 855-865.

Wang, D., Xiang, D.B., He, Y.J., Li, Z.P., Wu, X.H., Mou, J.H., Xiao, H.L., Zhang, Q.H., 2005. Effect of caffeic acid phenethyl ester on proliferation and apoptosis of colorectal cancer cells *in vitro*. World Journal of Gastroenterology 11, 4008-4012.

Watanabe, M.A.E., Amarante, M.K., Conti, B.J., Sforcin, J.M., 2011. Cytotoxic constituents of propolis inducing anticancer effects: a review. Journal of Pharmacy and Pharmacology 63, 1378-1386.

Wernyj, R.P., Morin, P.J., 2004. Molecular mechanisms of platinum resistance: still searching for the Achiles' heel. Drug Resistance Updates 7, 227-232.

Zamble, D.R., Lippard, S.J., 1995. Cisplatin and DNA repair in cancer chemotherapy. Trends in Biochemical Sciences 20, 435-437.

Capítulo 2

"Chemical analysis and immunomodulatory activity of geopropolis produced by *Melipona fasciculata* Smith on human monocytes", a ser submetido ao Journal of Ethnopharmacology

Chemical analysis and immunomodulatory activity of geopropolis produced by *Melipona fasciculata* Smith on human monocytes

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ABSTRACT

Ethnopharmacological relevance: Geopropolis has been traditionally used in the treatment of digestive diseases and dermatoses. Since there are few studies regarding its pharmacological activities, the aim of this study was to evaluate the chemical composition of geopropolis produced by *Melipona fasciculata* Smith as well as its immunomodulatory action on human monocytes, analysing cytokines (TNF-α and IL-10) production.

Materials and methods: Geopropolis chemical composition was analysed by gas chromatography/mass spectrometry. Monocytes (1 x 10^6 cells/mL) were obtained from peripheral blood of healthy individuals and incubated with geopropolis (5, 10, 25, 50 and 100 µg) or lipopolysaccharide. Supernatants were collected and assayed for cytokines determination by ELISA and cell viability was assessed by the MTT test.

Results: Chemical analysis showed that triterpenes were the major group of compounds found in geopropolis. Data revealed that the highest concentration of geopropolis (100 µg) exerted a cytotoxic action on monocytes, while the concentrations of 10, 25 and 50 µg stimulated significantly both TNF-α and IL-10 production.

Conclusions: Geopropolis stimulated cytokines production, what suggested its activator profile on human monocytes. This activity was due to its chemical constituents, such as hexoses, glucitol, glucuronil acid, inositol, and triterpenes.

Keywords:
Geopropolis
Stingless bees
Chemical composition
Immunomodulatory action

1. Introduction

Social bees collect resins from different parts of plants and add mandibular secretions producing propolis, a gum and balsamic resin of complex structure containing approximately 50% resins and balms plant, 30% wax, 10% of essential oils, 5% pollen, 5% other substances, what may vary according to the flora and bee subspecies (Burdock, 1998). As to geopropolis, some bee species belonging to Meliponini tribe add soil or clay to propolis composition (Freitas et al., 2008). Propolis and geopropolis are used to protect the hive entrance, coat inside walls, disinfect combs and embalm dead insects to keep the internal environment aseptic (Burdock, 1998; Salatino et al., 2005).

Several articles were published in the last decades regarding propolis produced by *Apis mellifera*, due to its biological properties such as immunomodulatory, antitumoral, antimicrobial, antioxidant, hepatoprotective, anti-inflammatory, among others (Orsolic and Basic, 2005; Sforcin, 2007; Bhadauria et al., 2008; Libério et al., 2009). Propolis biological activities are assigned to various groups of chemical compounds, particularly phenolic acids, flavonoids and terpenes. However, its composition may vary according to the seasons, geographical regions and botanical origin. A high chemical variability is expected among tropical propolis samples, due to the flora diversity (Bankova and Popov, 2007).

Recently there has been an increasing interest in geopropolis produced by native bees, however little is known about its chemical composition and immunomodulatory activity (Velikova et al., 2000; Bankova and Popov, 2007; Freitas et al., 2008; Dutra et al., 2008; Libério et al., 2011).

Bankova et al. (1998a) evaluated three samples of Brazilian geopropolis produced by *Melipona compressipes*, *M. quadrifasciata anthidioides* and *Tetragona clavipes,* identifying mainly phenolics (benzophenones, phenolic acids, and pinobanksina) and triterpenes (amyrin). The chemical composition of propolis produced by stingless bees (Meliponinae) of different genera revealed that the main compounds were phenolic acids and terpenes, including

monoterpenes, sesquiterpenes, diterpenes and triterpenes (Bankova and Popov, 2007).

Researches on the immunomodulatory activity of geopropolis are scarce. Libério et al. (2011) assessed the cytokines concentration in the serum of mice that received a gel prepared with geopropolis produced by *Melipona fasciculata* Smith in the oral cavity. Herein, we wish to present for the first time the chemical composition and immunomodulatory activity of geopropolis produced by *Melipona fasciculata* Smith in Maranhão State, Brazil, on human monocytes.

2. Materials and Methods

2.1. Hydroalcoholic extract of geopropolis

Geopropolis produced by *Melipona fasciculata* Smith was collected at the Palmeirândia municipality (2º 39' S, 44º 55' W), in the Western Microregion of Maranhão. The sample was ground and macerated in 70% ethanol at room temperature with moderate shaking. After 24 h, the hydroalcoholic extract of geopropolis was filtered and the dry weight was calculated (10.5 mg/mL) (Dutra et al., 2008). Geopropolis chemical composition was investigated using GC-MS analysis in the Institute of Organic Chemistry with Centre of Phytochemistry, Bulgaria.

2.2. GC-MS analysis

Analysis was performed with a Hewlett Packard Gas Chromatograph 5890 Series II Plus linked to a Hewlett Packard 5972 mass spectrometer system equipped with a 23 m long, 0.25 mm id, 0.5 µm film thickness HP5-MS capillary column. The temperature was programmed from 100 to 310 °C at a rate of 5 °C x min^{-1}. Helium was used as a carrier gas, flow rate 0.7 mL x min^{-1}. Split ratio 1: 80, injector temperature 280 °C. The ionization voltage was 70 eV.

The identification was accomplished using computer searches on a NIST98 MS Data library. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed

on the basis of its mass-spectral fragmentation. If possible, reference compounds were co-chromatographed to confirm GC retention times.

2.3. Human monocytes

Blood samples of 10 healthy donors (40 mL) were collected and put in sterile plastic tubes (Falcon) containing 200 µL of heparin. Tubes were centrifuged at 200 x g for 10 min. Supernatants were discarded and the blood was diluted 1:1 in RPMI 1640 (Cultilab, Brazil). The mononuclear cells were separated by Ficoll-Hypaque sedimentation (400 x g, 30 min) and washed twice in RPMI (200 x g, 10 min) to remove platelets. An aliquot of cells was counted in a hemocytometer, and viability was checked using 0.02% neutral red (1:10). Cells were suspended in RPMI 1640 medium, supplemented with 10% fetal calf serum, cultured in flat-bottomed 24-well plates at a concentration of 1 x 10^6 cells/mL and incubated for 90 min at 37 °C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed by aspiration and the wells washed twice with RPMI medium.

This work was approved by the Ethical Committee of Botucatu Medical School, UNESP (nº 3399-2009).

2.4. Monocytes viability

To assess cell viability, geopropolis was diluted in RPMI medium, supplemented with 10% fetal calf serum. Specific dilutions were prepared for each test, in order to achieve the following concentrations: 5, 10, 25, 50 and 100 µg/mL. The same procedure was performed with geopropolis solvent (70% ethanol) to obtain 0.03, 0.06, 0.15, 0.29 and 0.59%, which are the respective concentrations of alcohol found in geopropolis. Control cells were incubated with culture medium alone.

Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide) assay (Franchi Jr. et al., in press). MTT is captured by cells and reduced intracellularly in a mitochondrion-dependent reaction to yield a formazan product. The ability of cells to reduce MTT indicates the mitochondrial activity and cell viability. After 18 h incubation with different

geopropolis concentrations, the supernatant was removed and 300 µL of MTT (Sigma, USA) dissolved in RPMI medium was added, followed by incubation for 3 h at 37° C and 5% CO_2. After this period, MTT was removed and 200 µL of dimethyl sulfoxide (DMSO – Dinamica) was added for formazan crystals solubilization. The absorbance was read in a Synergy ELISA plate reader (Bio Tek Instruments, Highland Park, Winooski) at 492 nm, and the percentages of cell viability were calculated. Results were expressed compared to control (considered as 100%).

2.5. Cytokine determination

Monocytes cultures (1 x 10^6 cells/mL) were incubated in 24-well plates at 37° C with geopropolis at noncytotoxic concentrations or lipopolysaccharides (LPS – 10 µg/mL). After 18 h, supernatants were collected and stored at -70° C for cytokines quantitation.

TNF-α and IL-10 production were measured by enzyme-linked immunosorbent assay (ELISA), according to manufactured´s instructions (eBiosciences). Briefly, a 96-well flat bottom Maxisorp microtiter plate (Nunc, USA) was coated with capture antibody specific for each cytokine and incubated overnight at 4 °C in a humid chamber. The plate was washed and blocked with 0.1% BSA before 100 µL of the supernatants and serially diluted specific standards were added to the respective wells. Following a series of washing, the cytokine was detected using the specific avidin-peroxidase conjugated detection antibody. The substrate reagent was added into each well and, after color development, the plate was read at 450 nm, using an ELISA plate reader.

2.6. Statistical analysis

Data were analyzed using Prism Graph-Pad statistical software, version 5.0 (Graph-Pad Software, Inc., USA). Significant differences between treatments were determined by analysis of variance (ANOVA), followed by Tukey-Kramer test. Statistic significances were accepted when $P < 0.05$. Data were expressed as mean ± standard deviation.

3. Results

3.1. Geopropolis chemical analysis

Geopropolis sample showed a resinous smell, dark brown color and bitter taste. The chemical analysis of geopropolis produced by *M. fasciculata* was carried out by GC-MS, showing that triterpenes were the main chemical group found in its composition (Table 1), and the major components were hexoses, glucitol, glucuronil acid, inositol, and triterpenes, as shown in Table 2.

Table 1. Major groups of compounds found in geopropolis produced by *Melipona fasciculata* Smith.

Pentoses	1.2
Hexoses	11.9
Sugar alcohols	8.4
Uronic acids	2.6
Disaccharides	4.1
Alkylresorcinols	5.8
Triterpenes	15.9
Others	2.7

Table 2. Geopropolis constituents after silylation by GC-MS. TIC: Total ion chromatogram.

Compound	% TIC	Compound	% TIC
Methylmalonic acid	0.5	Pentadecylresorcinol	1.1
Glycerol	2.2	Disaccharide	1.5
Hexose	4.2	Disaccharide	0.9
Pentose	1.2	Disaccharide	1.4
Hexose	0.4	Heptedecadienylresorcinol	0.8
Xylitol	2.1	Heptadecenylresorcinol	1.2
Hexose	1.0	Heptadecylresorcinol	1.5
Hexose	0.3	Nonadecenylresorcinol	0.9
Inositol	2.4	Nonadecenylresorcinol (isomer)	1.3
Hexose	1.5	Triterpenic ketone	1.0
Hexose	4.5	Triterpenic alcohol	1.8
Glucitol	2.7	Triterpenic ketone	2.0
Sugar alcohol	1.2	Triterpenic ketone	1.4
Glucuronil acid	2.6	Triterpenic alcohol	2.4
Disaccharide	0.3	Triterpene main constituent	7.3

3.2. Geopropolis effects on monocytes viability

Fig. 1 shows that only the highest concentration of geopropolis (100 µg) exerted a cytotoxic effect on monocytes ($p<0.0001$). Thus, the cytokine assays were carried out using only noncytotoxic concentrations.

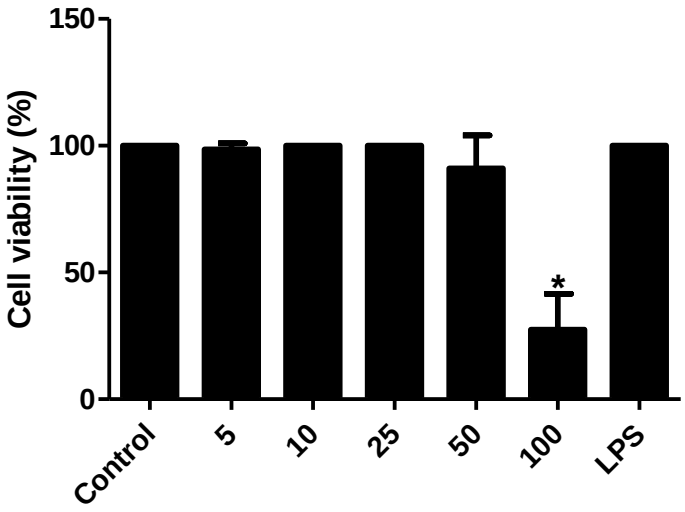

Fig. 1. Monocytes viability after incubation with geopropolis (5, 10, 25, 50 and 100 µg) or LPS (10 µg/mL) for 18 h. Data represent mean and standard deviation of 10 similar assays. * significantly different from control ($p<0.0001$).

3.3. Geopropolis effects on cytokines production

As to TNF-α, geopropolis (10, 25 and 50 µg) increased significantly its production by monocytes ($P < 0.0001$) (**Fig. 2**). An increased IL-10 production was also seen after incubation with geopropolis compared to control ($P < 0.0001$), and the concentrations 10 and 25 µg presented an immunostimulatory profile similar to that of LPS (positive control) (**Fig. 3**). Geopropolis solvent (70% ethanol) did not interfere cytokines production.

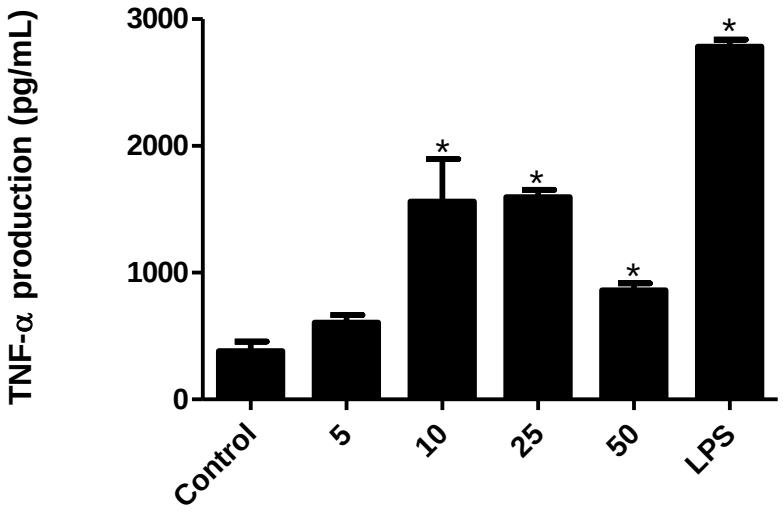

Fig. 2. TNF-α production (pg/mL) by monocytes incubated with geopropolis (5, 10, 25 and 50 µg) or LPS (10 µg/ml) for 18 h. Data represent mean and standard deviation of 10 similar assays. * significantly different from control ($p<0.0001$).

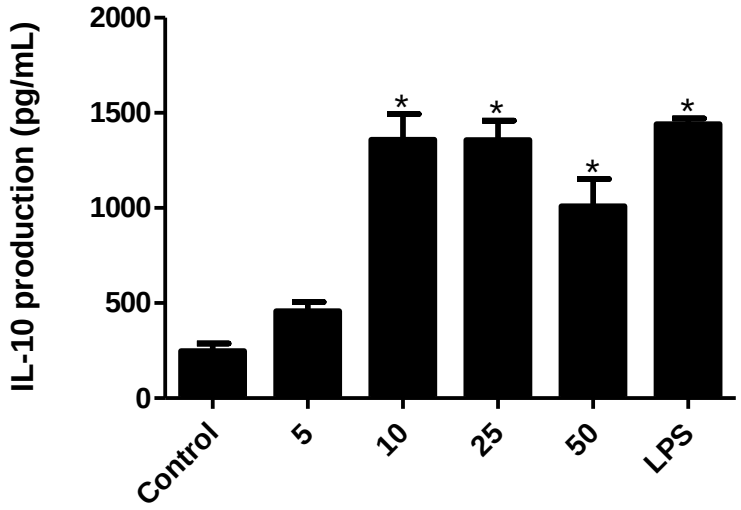

Fig. 3. IL-10 production (pg/mL) by monocytes incubated with geopropolis (5, 10, 25 and 50 µg) or LPS (10 µg/mL) for 18 h. Data represent mean and standard deviation of 10 similar assays. * significantly different from control ($p<0.0001$).

4. Discussion

Geopropolis has been empirically used in the treatment of digestive diseases and dermatoses (Freitas et al., 2008). Nevertheless, there are few studies on the pharmacological activities of geopropolis produced by *Melipona fasciculata*, whose products have been used for centuries in Maranhão State by the indigenous population for honey production. The chemical composition of propolis produced by Africanized honeybees has been widely investigated, unlike geopropolis produced by stingless bees (Boudourova-Krasteva et al., 1997; Bankova et al., 1998b).

Triterpenes were the major chemical group found in our geopropolis sample, and the major components were hexoses, glucitol, glucuronil acid, inositol, and triterpenes. Dutra et al. (2008), using the same geopropolis sample produced *by M. fasciculata* collected in Maranhão State, northeast Brazil, reported that the hydroalcoholic extracts obtained by maceration in 70% ethanol (v/v) showed yields ranging from 1.10 to 5.83%, and this low yield was due to a large amount of clay in the samples (94-98%). Phenolic compounds and triterpenes were found predominantly in the samples.

Bankova et al. (1998a) evaluated the chemical composition of Brazilian geopropolis produced by *Melipona compressipes*, *M. quadrifasciata anthidioides* and *Tetragona clavipes* by GC-MS, revealing that the main compounds were phenolics (benzophenones, phenolic acids, and pinobanksina) and triterpenes (amyrin). Velikova et al. (2000), investigating propolis samples produced by stingless bees and by *A. mellifera* in different regions of Brazil, verified a complex heterogeneity among the samples. The GC-MS analysis revealed that gallic acid was the main constituent in some samples. This compound is the major component found in the resin of *Eucalyptus cytriodora*, indicating that it may be the botanical source of these samples (Bankova et al., 1999). Other samples showed di- and triterpenes as major compounds, but only propolis produced by *A. mellifera* presented prenylated derivatives of *p*-coumaric acid, characteristic of Brazilian propolis.

Miorin et al. (2003) identified high concentrations of derivatives of cinnamic and *p*-coumaric acids in samples produced by *A. mellifera* in Paraná and Minas Gerais States, Brazil. Some of these compounds were present at low concentrations in propolis produced by *Tetragonisca angustula*, although a similar chemical profile was found between samples produced by Africanized honeybees and by stingless bees. Pereira et al. (2003) compared the chemical composition of propolis produced by *A. mellifera* or *T. angustula* in São Paulo State, Brazil. Both samples showed a similar composition, with high concentrations of pentacyclic triterpenes, mainly lupeol and lupeol acetate, with few differences in the concentrations of amino acids, carbohydrates and polyols. Sawaya et al. (2006) compared the composition of propolis produced by *A. mellifera* and by *T. angustula* in different regions of Brazil, observing that the composition of propolis produced by *A. mellifera* depended on collection area, while the composition of samples produced by *T. angustula* showed a similar composition in all regions.

Geopropolis chemical composition depends on the local flora and geographic region, and this aspect is extremely important in order to link its biological properties to its chemical profile, and establish a possible standardization of the assays.

There are few data in literature with regards to the immunomodulatory action of geopropolis produced by stingless bees. Thus, its effect on human monocytes was analyzed, assessing a possible cytotoxic effect on these cells and cytokines production. Only the highest concentration of geopropolis showed cytotoxic effect towards monocytes, and the noncytotoxic concentrations increased TNF-α and IL-10 production by these cells. After stimulation by microorganisms, monocytes may produce reactive oxygen and nitrogen intermediates, complement factors, prostaglandin, proteolytic enzymes and cytokines such as TNF-α, IL-1β, IL-6 and IL-10, among others mediators (Auffray et al., 2009).

TNF-α is a pro-inflammatory cytokine produced by macrophages and monocytes, among other cells, and is the main mediator of acute inflammatory response to Gram-negative bacteria and other infectious agents. One of the

main physiological functions of TNF-α is to stimulate the recruitment of neutrophils and monocytes to infection sites and activate these cells to kill microorganisms (Locksley et al., 2001). IL-10 is secreted by T cells, monocytes and macrophages, and is an inhibitor of activated cells, regulating both innate and adaptive immunity (Pestka et al., 2004). In our study, both TNF-α and IL-10 production was elevated after incubation with geopropolis, suggesting its activator profile although it is necessary to evaluate other mediators, such as hydrogen peroxide (H_2O_2), and cells challenge with microorganisms.

Libério et al. (2011) assessed the cytokine concentration in serum of mice that received a gel with geopropolis produced by *M. fasciculata* in the oral cavity for a minute during four consecutive days. After 7 days, high concentrations of IL-4 and IL-10 were found, with no changes in IFN-γ and TNF-α production. An increased IL-10 production can exert anti-inflammatory activity, what was related to the histopathological changes in the tongue of the animals, probably due to the handling of animals during treatment.

There are few data on the immunomodulatory action of geopropolis, and our work is the first one in assessing human monocytes. Unlike, the immunomodulatory action of propolis produced by Africanized honeybees has been widely investigated (Ivanovska et al., 1995; Sforcin, 2007). It has been reported that propolis can influence the initial events of the immune response, inducing the expression of Toll-like receptors (TLR-2 and TLR-4) and the production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), as well as the generation of oxygen (H_2O_2) and nitrogen (NO) intermediates by peritoneal macrophages of mice (Orsi et al., 2000; Khayal et al., 2003; Orsatti et al., 2010). Propolis also increased the fungicidal and bactericidal activity of murine macrophages (Murad et al., 2002; Orsi et al., 2005).

Propolis increased antibody titers after 15 days of immunization (Sforcin et al., 2005). Fishes that received intraperitoneally inactivated vaccine against *Aeromonas hydrophila* plus propolis presented higher antibody titers and phagocytic activity (Chu, 2006). This author also mentioned that propolis could activate antigen presenting cells, stimulating cytokines production which in turn could activate T and B lymphocytes, suggesting its possible use as vaccine

adjuvant. Mice inoculated with the inactivated vaccine of Suid herpes type-1 (SuHV-1) associated with propolis showed increased levels of antibodies, suggesting their use as vaccine adjuvant (Fischer et al., 2007).

Studies on the immunomodulatory activity of natural or synthetic substances may be important tools for the prevention or treatment of infections and neoplasms (Orsolic and Basic, 2003). Although several mechanisms of action of propolis produced by Africanized honeybees have been proposed (Sforcin, 2007), the investigation of geopropolis immunomodulatory action deserves further investigation, in order to understand its mechanisms of action, as well as to compare its efficacy to propolis produced by honeybees.

In conclusion, geopropolis stimulated cytokines production, suggesting its activator profile on human monocytes. This activity was due to its chemical constituents, including hexoses, glucitol, glucuronil acid, inositol, and triterpenes as major chemical groups.

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References

Auffray, C., Sieweke, M.H., Geissmann, F., 2009. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annual Review of Immunnology 27, 669-692.

Bankova, V., Christov, R., Marcucci, C., Popov, S., 1998a. Constituents of Brazilian geopropolis. Zeitschrift fur Naturforschung 53, 402-406.

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

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

Bankova, V., Popov, M., 2007. Propolis of stingless bees: a promising source of biologically active compounds. Pharmacognosy Reviews 1, 88-92.

Bhadauria, M., Nirala, S.K., Shukla, S., 2008. Multiple treatment of propolis extract ameliorates carbon tetrachloride induced liver injury in rats. Food and Chemical Toxicology 46, 2703–2712.

Boudourova-Krasteva, G., Bankova, V., Sforcin, J.M., Nikolova, N., Popov, S., 1997. Phenolics from Brazilian propolis. Zeitschrift für Naturforschung 52c, 676-679.

Burdock, G.A., 1998. Review of the biological properties and toxicity of bee propolis (propolis). Food and Chemical Toxicology 36, 347-363.

Chu, W.H., 2006. Adjuvant effect of propolis on immunization by inactivated *Aeromonas hydrophila* in carp (*Carassius auratus gibelio*). Fish & Shelfish Immunology 21, 113–117.

Dutra, R.P., Nogueira, A.M.C., Marques, R.R.O., Costa, M.C.P., Ribeiro, M.N.S., 2008. Avaliação farmacognóstica de geoprópolis de *Melipona fasciculata* Smith da Baixada Maranhense, Brasil. Revista Brasileira de Farmacognosia 18, 557-562.

Fischer, G., Conceição, F.R., Leite, F.P.L., Dummer, L.A., Vargas, G.D., Hübner, S.O., Dellagostin, O.A., Paulino, N., Paulino, A.S., Vidor, T., 2007. Immunomodulation produced by a green propolis extract on humoral and cellular responses of mice immunized with SuHV-1. Vaccine 25, 1250–1256.

Franchi Jr., G.C., Moraes, C.S., Toreti, V.C., Daugsch, A., Nowill, A.E., Park, Y.K., in press. Comparison of effects of the ethanolic extracts of Brazilian propolis on human leukemic cells as assessed with the MTT assay. Evidence-Based Complementary and Alternative Medicine.

Freitas, M.O., Ponte, F.A.F., Lima, M.A.S., Silveira, E.R., 2008. Flavonoids and Triterpenes from the nest of the stingless bee *Trigona spinipes*. Journal of the Brazilian Chemical Society 19, 532-535.

Ivanovska, N.D., Dimov, V.B., Pavlova, S., Bankova, V., Popov, S., 1995. Immunomodulatory action of propolis. V. Anticomplementary activity of a water-soluble derivative. Journal of Ethnopharmacology 47, 135–143.

Khayal, M.T., El-Ghazaly, M.A., El-Khatib, A.S., Hatem, A.M., De Vries, P.J.F., El-Shafei, S., Khattab, M.M., 2003. A clinical pharmacological study of the potential beneficial effects of a propolis food product as an adjuvant in asthmatic patients. Fundamental & Clinical Pharmacology 17, 93-102.

Libério, S.A., Pereira, A.L.A., Araújo, M.J.A.M., Dutra, R.P., Nascimento, F.R.F., Monteiro-Neto, V., Ribeiro, M.N.S., Gonçalves, A.G., Guerra, R.N.M., 2009. The potencial use of propolis as a cariostatic agent and its actions on mutans group streptococci. Journal of Ethnopharmacology 125, 1-9.

Libério, S.A., Pereira, A.L.A., Dutra, R.P., Reis, A.S., Araújo, M.J.A.M., Mattar, N.S., Silva, L.A., Ribeiro, M.N.S., Nascimento, F.R.F., Guerra, R.N.M. Monteiro-Neto, V., 2011. Antimicrobial activity against oral pathogens and immunomodulatory effects and toxicity of geopropolis produced by the stingless bee *Melipona fasciculata* Smith. BMC Complementary and Alternative Medicine 11, 108.

Locksley, R.M., Killeen, N., Leonardo, M.J., 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104, 487-501.

Miorin, P.L., Levy Junior, N.C., Custodio, A.R., Bretz, W.A., Marcucci, M.C., 2003. Antibacterial activity of honey and propolis from *Apis mellifera* and *Tetragonisca angustula* against *Staphylococcus aureus*. Journal of Applied Microbiology 95, 913-920.

Murad, J.M., Calvi, S.A., Soares, A.M.V.C., Bankova, V., Sforcin, J.M., 2002. Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against *Paracoccidioides brasiliensis*. Journal of Ethnopharmacology 79, 331–334.

Orsatti, C.L., Missima, F., Pagliarone, A.C., Bachiega, T.F., Búfalo, M.C., Araújo Jr., J.P., Sforcin, J.M., 2010. Propolis immunomodulatory action *in vivo* on

Toll-like receptors 2 and 4 expression and on pro-inflammatory cytokines production in mice. Phytotherapy Research 24, 1141-1146.

Orsi, R.O., Funari, S.R.C., Soares, A.M.V.C., Calvi, S.A., Oliveira, S.L., Sforcin, J.M., Bankova, V., 2000. Immunomodulatory action of propolis on macrophage activation. Journal of Venomous Animals and Toxins 6, 205-219.

Orsi, R.O., Sforcin, J.M., Funari, S.R.C., Bankova, V., 2005. Effects of Brazilian and Bulgarian propolis on bactericidal activity of macrophages against *Salmonella typhimurium*. International Immunopharmacology 5, 359–368.

Orsolic, N., Basic, I., 2003. Immunomodulation by water-soluble derivative of propolis: a factor of antitumor reactivity. Journal of Ethnopharmacology 84, 265–273.

Orsolic, N., Basic, I., 2005. Antitumor, hematostimulative and radioprotective action of water-soluble derivative of propolis (WSDP). Biomedicine & Pharmacotherapy 59, 561–570.

Pereira, A.S., Bicalho, B., Aquino-neto, F.R., 2003. Comparison of propolis from *Apis mellifera* and *Tetragonisca angustula*. Apidologie 34, 291-298.

Pestka, S., Krause, C.D., Sarkar, D., Walter, M.R., Shi, Y., Fisher, P.B., 2004. Interleukin-10 and related cytokines and receptors. Annual Review of Immunnology 22, 929–979.

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.

Sawaya, A.C.H.F., Cunha, I.B.S., Marcucci, M.C., Rodrigues, R.F.O., Eberlin, M.N., 2006. Brazilian propolis of *Tetragonisca angustula* and *Apis mellifera*. Apidologie 37, 398-407.

Sforcin, J.M., Orsi, R.O., Bankova, V., 2005. Effects of propolis, some isolated compounds and its source plant on antibody production. Journal of Ethnopharmacology 98, 301–305.

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

Velikova, M., Bankova, V., Tsvetkova, I., Kujumgiev, A., Marcucci, M.C., 2000. Antibacterial ent-kaurene from Brazilian propolis of native stingless bees. Fitoterapia 71, 693-696.

Capítulo 3

“Antimicrobial activity of geopropolis produced by *Melipona fasciculata* Smith in northeast Brazil”, a ser submetido à Evidence-based Complementary and Alternative Medicine

Antimicrobial activity of geopropolis produced by *Melipona fasciculata* Smith in northeast Brazil

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Propolis is a resinous substance collected by Africanized honeybees from plants, adding bee enzymes, pollen and wax. Contrarily, geopropolis (Geo) is produced by indigenous stingless bees from resinous materials of plants, adding salivary secretions, wax, but also adding mud or clay. The antimicrobial activity of propolis produced by *Apis mellifera* have been extensively studied, whereas there are few studies concerning Geo properties. This study evaluated the antimicrobial activity of Geo hydroalcoholic extract on *Staphylococcus aureus* and *Escherichia coli*, determining the minimal inhibitory concentration, the survival curves, and a possible interaction (synergism or antagonism) with conventional antibiotics. Geo effect was also assessed on *Pythium insidiosum* isolates obtained from human and equines. Geo showed no antibacterial activity against *S. aureus* and *E. coli*, and an inhibitory activity was seen only at high concentrations, with a bacteriostatic action towards *S. aureus*. Moreover, the association of Geo with chloramphenicol showed a synergistic action against *S. aureus* but not on *E. coli*. Geo exerted a fungistatic action against *Pythium insidiosum* isolates. Although Geo exerted an antimicrobial action, it was lower than that exerted by propolis produced by Africanized honeybees, perhaps due to the low yield of the extract and its difficult solubility.

Keywords: Antimicrobial activity, Geopropolis, *Melipona fasciculata*, Stingless bees

1. Introduction

There has been an increased interest by the pharmaceutical industry in the search of natural products to maintain a healthy lifestyle, especially those with antimicrobial activity, due to bacterial and fungal resistance to antimicrobial drugs and side effects [1-3].

Bee products have been widely investigated concerning their biological properties. Social native bees, known as stingless bees, may produce propolis as well as geopropolis (Geo) [4, 5]. Propolis is a balsamic and resinous product made by bees from different parts of plants, adding mandibular secretions, pollen and wax. As to Geo, besides plant material, gland secretions, wax and pollen, some species of stingless bees add mud or clay to its composition [4-7].

The antimicrobial activity of propolis produced by Africanized honeybees has been extensively investigated, [8, 9], and in recent years there has been a great interest in the antibacterial properties of propolis and Geo produced by stingless bees. Geo produced by *Melipona compressipes fasciculata* exerted an antibacterial effect *in vitro* against *Streptococcus mutans* isolated from the oral cavity of young individuals of both gender, suggesting that its use as an alternative for preventing dental caries [10]. The antimicrobial action of Geo produced by *M. fasciculata* from Maranhão State, northeast Brazil, was analyzed against *S. mutans*, *Lactobacillus acidophilus* and *Candida albicans* by the agar diffusion method, confirming its potential to control or prevent infections in the oral cavity [11]. Although there are some data with respect to its antibacterial and antifungal activities, there are no data concerning the effects of geopropolis produced by *M. fasciculata* on *Pythium insidiosum* – a fungus-like organism, which is the causative agent of pythiosis – a pyogranulomatous disease of the subcutaneous tissue that affects mostly horses, dogs and humans as well.

Epidemiologically, pythiosis is closely related to human and animal contact with contaminated water, and zoospores constitute its infective form [12]. This disease is life-threatening and diagnosis is time-consuming. Besides, treatment with conventional antifungal drugs is difficult in most of the cases, requiring extensive surgical debridements. The unsuccessful response to

antifungal drugs is the absence of ergosterol in the plasmatic membrane, which is the main target of azoles, alilamines and polienes. Since propolis and Geo have been used in folk medicine for different purposes, the present work evaluated the antibacterial activity of Geo produced by *Melipona fasciculata* against *Staphylococcus aureus* and *Escherichia coli* strains by determining the minimum inhibitory concentration and survival curves. A possible interaction (synergism or antagonism) between Geo and antibiotics was also investigated. Geo effect on *Pythium insidiosum* isolates was also assessed.

2. Material and methods

2.1. Geopropolis sample

Geo was produced by *Melipona fasciculata* Smith and collected at Palmeirândia municipality (2º 39' S, 44º 55' O), Maranhão State, northeast Brazil. Municipalities of this region comprise different ecosystems such as mangroves, flooding fields, lagoons, forests and babassu fields [13]. Samples were kept under refrigeration at 4 °C before extraction.

Samples (40 g) were ground and macerated in 70% ethanol at room temperature, under moderate shaking. After 24 h, the extract was filtered and the dry weight of Geo hydroalcoholic extract (Geo-1) was calculated (13 mg/mL) [14-16]. Another extract was prepared following the same procedures (Geo-2) but, after filtration, Geo-2 was subjected to solvent evaporation in order to analyze the effect of geopropolis without the influence of the solvent (dry weight: 8 mg/mL). Geo-2 was solubilized using 1% DMSO, which was previously tested in *Pythium insidiosum* isolates, without affecting the pathogen growth.

2.2. Bacterial strains

Staphylococcus aureus strains (n = 31) and 15 strains of *Escherichia coli* were isolated from biological material of patients of the Botucatu Medical School, UNESP. American Type Culture Collection (ATCC) strains (*S. aureus* ATCC 25923 and *E. coli* ATCC 25922) were used. Strains were stored at -70 °C, in the Department of Microbiology and Immunology, IB, UNESP.

This work was approved by the Ethics Committee of Botucatu Medical School, UNESP (3399-2009).

2.3. Susceptibility tests

Susceptibility tests were performed by dilution in agar method, as recommended by the Clinical and Laboratory Standards Institute and determining the $MIC_{90\%}$ values [17, 18].

Bacterial strains were inoculated in Brain Heart Infusion (BHI - Difco, USA) at 37° C for 24 h and standardized at 0.5 on the McFarland scale in sterile saline. Dilutions were performed of each sample to obtain bacterial suspensions with a final cell density of 1×10^6 colony-forming units (CFU)/mL.

Geo-1 was added to Petri dishes containing Mueller Hinton Agar (MHA) (Difco, USA) at final concentrations: 3, 6, 9, 12, 14 and 16% v/v to *S. aureus*, and 9, 12, 14, 16, 18 and 20% v/v to *E. coli*. Control plates contained only 70% ethanol at the same concentrations found in Geo-1. Bacterial strains were inoculated in Petri plates containing different concentrations of Geo-1 or 70% ethanol, using a Steer multiple inoculators, and incubated at 37 °C for 24 h. $MIC_{90\%}$ was considered as the lowest concentration of Geo-1 able to inhibit 90% of microorganisms, showing no visible growth or haze on the surface of the culture medium.

2.4. Survival curve

The survival curve of *S. aureus* and *E. coli* was carried out to observe the bactericidal or bacteriostatic action of Geo-1 according to the period of time, using the $MIC_{90\%}$ values. Bacterial suspensions (1 x 10^6 CFU/mL) were inoculated in BHI plus Tween 80% (0.5% v/v) containing the $MIC_{90\%}$ of Geo-1 or 70% ethanol. After 3, 6, 9 and 24 h of incubation at 37 °C, aliquots of each culture were taken and plated on Plate Count Agar (PCA) (Difco, USA) by the pour plate method. After 24 h at 37 °C, CFU values were counted and the survival percentage was calculated [8].

2.5. Synergistic effects of geopropolis and antimicrobial drugs

Ten strains of *S. aureus* and one ATCC strain were tested. Besides, ten *E. coli* strains and one ATCC were used. Strains were grown in BHI at 35 °C for 18 h, and after this period, microorganisms were standardized in 0.5 McFarland in sterile physiological solution. The synergistic effect of Geo-1 with antibiotics was assayed by the disc diffusion method on MHA, and the following antibiotic disks were used: chloramphenicol, gentamicin, tetracycline, ciprofloxacin and oxacillin (Sigma, USA). The procedures comprised three types of antibiogram: control plates containing only MHA, plates with ½ or ¼ of Geo-1 ($MIC_{90\%}$) or ½ or ¼ of 70% ethanol ($MIC_{90\%}$). Plates were incubated at 37° C for 24 h, and the effect of Geo-1 or 70% ethanol was measured as growth inhibition zones around the discs.

2.6. Geo-2 effect on Pythium insidiosum isolates

Fifteen *P. insidiosum* isolates were used: one human isolate (obtained from the first human pythiosis in Brazil - B 01) and 14 obtained from equine pythiosis of middle west region of São Paulo State.

Isolates were kept in tubes containing Sabouraud (SAB) Agar at 25 °C to promote colonies growth. Each isolate was inoculated into plates containing

SAB Agar at 35 °C for 7 days. Standardized fragments of 5 mm were removed and put into microtubes containing SAB broth and Geo-2. The sensitivity of *P. insidiosum* isolates to Geo-2 was determined according to the radial growth, determining the growth inhibition by measuring the colony diameter. Different concentrations of Geo-2 were diluted in SAB broth: 3.4, 5.0, 7.0, 12.5, 17.7 mg/mL, to obtain a final volume of 1 mL. Control group contained only 1 mL of Sabouraud broth. For each Geo-2 concentration, five standardized fragments of pathogen were added and microtubes were placed under moderate shaking at 35 °C for 24 h, to prevent the extract and the inoculum to precipitate. After this period, fragments were cultured in SAB agar plate at 35 °C for up to 7 days. All experiments were performed in quintuplicate.

Assessment of pathogen growth was carried out by measuring the colonies diameter after 1, 2 and 7 days. All cultures were photographed and colonies diameter was achieved using the software Image J (image processing and analysis in JAVA; http://rsbweb.nih.gov/ij/). Diameters were recorded in cm, in the angles 0, 45, 90 and 135º of each plate.

2.7. Statistical analysis

Data analysis of bacterial growth and interactions between geopropolis and antimicrobial drugs were performed using nonparametric Kruskal-Wallis test to compare independent treatments. Dunn test was used for multiple comparisons. For antifungal activity, Kruskal-Wallis test was employed, followed by the Friedman test ($P < 0.05$).

3. Results and discussion

Geo-1 showed an inhibitory activity for *S. aureus* and *E. coli* only at high concentrations, and $MIC_{90\%}$ values (% v/v) for the strains as well as the range are shown in Table 1.

Table 1. Median, 1st and 3rd quartiles (in brackets) relative to the values of minimum inhibitory concentration ($MIC_{90\%}$, % v/v) for Geo-1 and 70% ethanol against microorganisms by dilution method on Mueller Hinton Agar.

microorganism	**median (Geo-1)**	**median (70% ethanol)**
S. aureus (n=31) $MIC_{90\%}$	14.0 a [12, 16] 15.36%	16.0 a [16, 16] 15.70%
E. coli (n=15) $MIC_{90\%}$	14.0 a [14, 14] 13.75%	16.0 a [16, 16] 15.77%

Data with the same lowercase letters in rows and columns do not differ ($P > 0.05$).

Propolis samples produced by *A. mellifera* and by *Scaptotrigona* sp. were effective against *E. coli*, while the antibacterial activity of propolis produced by *A. mellifera* for *S. aureus* was higher than propolis produced by *Melipona quadrifasciata* and *Scaptotrigona* sp [19]. Although our extract was not effective for the strains, some factors may have affected the results, such as the yield of the extract, pH and samples solubility in the culture medium [18, 20].

The antibacterial activity of Geo produced by *M. fasciculata* against *Streptococcus mutans* was evaluated in the oral cavity, decreasing by 49% the number of *S. mutans* colonies in patients saliva after oral rinses performed with the extract, suggesting its use as an alternative for carie prevention [10]. Furthermore, the antimicrobial activity of three samples of Geo produced by *M. fasciculata* in different regions of Maranhão State, Brazil was analyzed on *S. mutans*, *Lactobacillus acidophilus* and *Candida albicans* by the agar diffusion method and by minimal bactericidal concentration and only one extract showed no activity against *S. mutans* and *C. albicans*. Moreover, the extract of Geo

collected in Palmeirândia municipality showed a higher antimicrobial activity and the highest flavonoids content [11].

Our Geo sample was not effective for bacterial strains, although it was collected in the same region of others authors [11] who showed an inhibitory activity for microorganisms in the oral cavity, reporting its potential for prophylactic or therapeutic use in dental diseases, especially caries and candidiasis, and for the treatment of inflammatory processes.

Propolis antibacterial activity has been investigated by several authors using samples from different geographic regions [21, 22], although there are only few reports on Geo antimicrobial action. Biological activities of propolis or Geo samples depend on their chemical composition, which is related to the plant material that bees collect. In general, Africanized honeybees visit predominantly the same plants to produce propolis, and its chemical composition is qualitatively the same in the geographic region where it was produced [23]. On the other hand, propolis or Geo produced by stingless bees show a great variation even among samples from the same region, since they collect material from plants near their hives, what may explain the differences in the pharmacological activities of such samples [24].

After determining $MIC_{90\%}$ values for *S. aureus* and *E. coli,* the survival curves were carried out to verify a possible bactericidal or bacteriostatic activity of Geo. Bactericidal effects comprised a reduction in three or more log of CFU/mL compared to the initial inoculums [25]. As shown in Figure 1A, a decreased *S. aureus* CFU was seen 24 h after incubation with Geo-1 or 70% ethanol, with an inhibitory effect on this strain after 9 h incubation (Figure 1B). The antibacterial activity of propolis produced by *Apis mellifera* was evaluated against Gram-positive and Gram-negative bacteria. However, the authors observed an inhibitory effect on *S. aureus* after 6 h incubation with propolis using a low concentration (0.5% v/v) [8], while in our study this effect was observed after 9 h incubation using 15.36% v/v, showing that Geo was not as efficient as propolis.

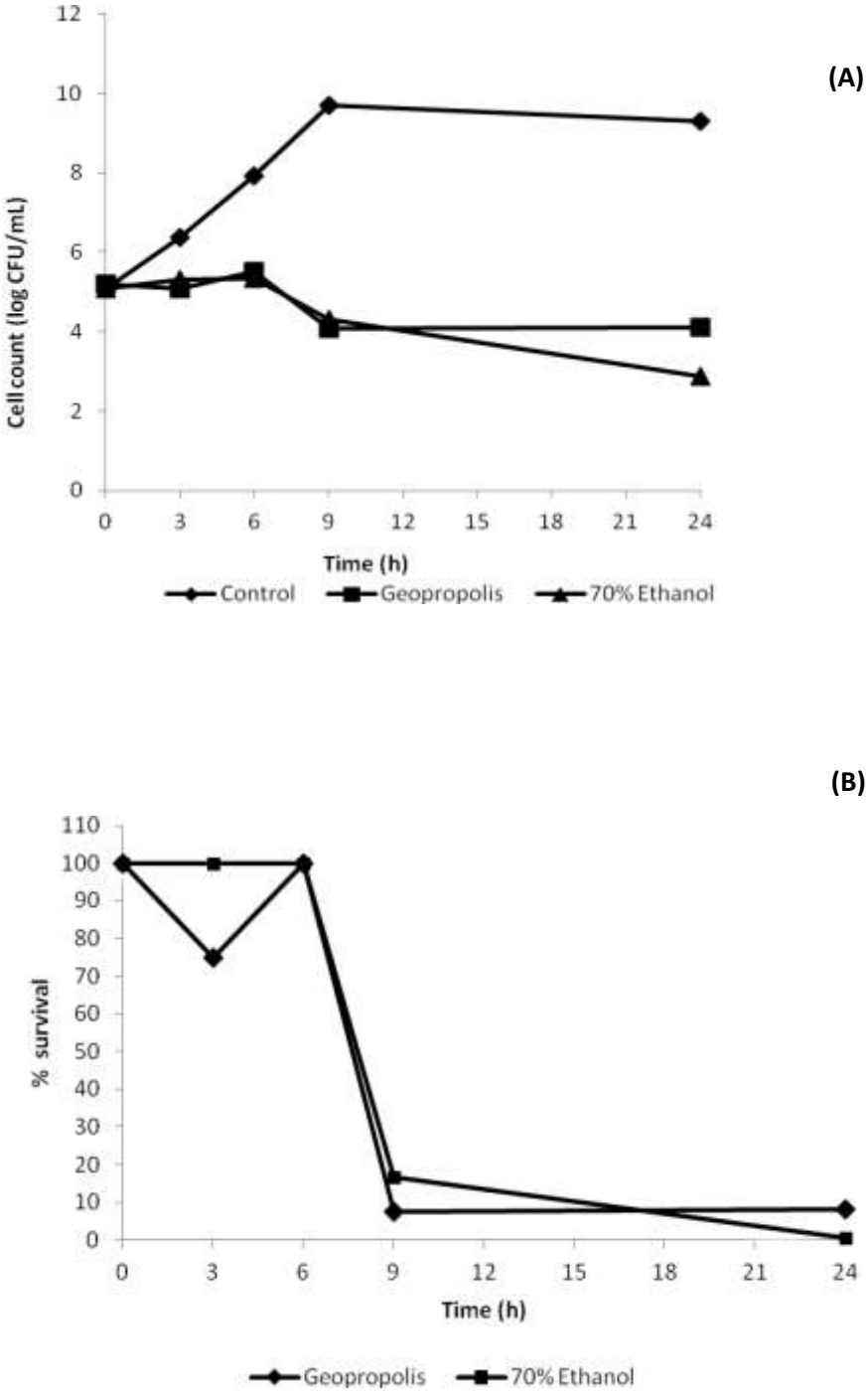

Figure 1. (A) Survival curve (% CFU/mL) and (B) survival percentage of *S. aureus* along the incubation period with Geo-1 (15.36 % v/v).

E. coli was also susceptible to Geo-1 (Figure 2A), but a reduction of CFU was seen only after 24 h incubation. The effect of 70% ethanol was observed after 9 h incubation (Figure 2B).

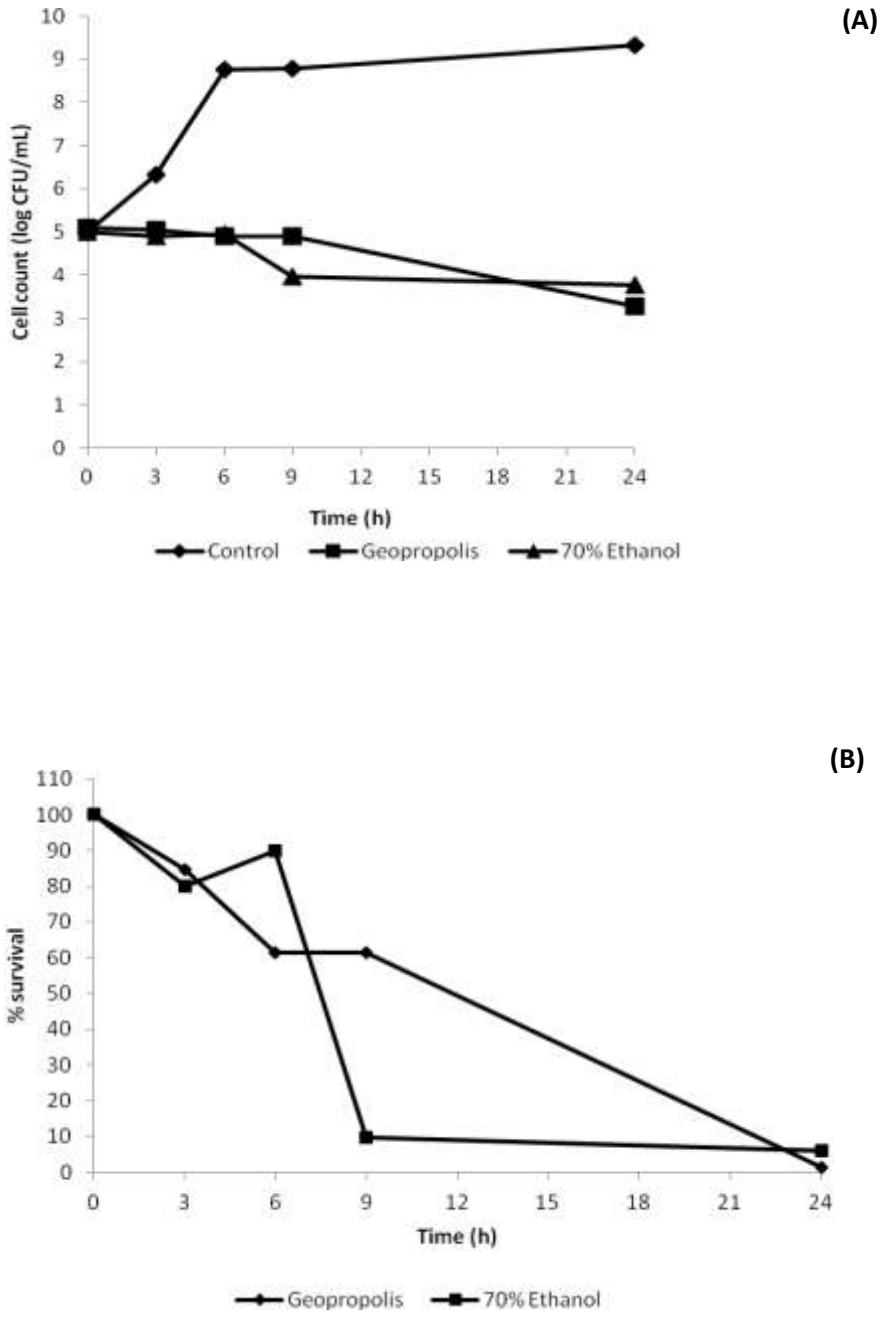

Figure 2. (A) Survival curve (% CFU/mL) and (B) survival percentage of *E. coli* as a function of the incubation period with Geo-1 (13.75% v/v).

Similar results were obtained for *E. coli* using propolis produced by *A. mellifera* ($MIC_{90\%}$ = 8% v/v), with a decreased CFU only after 24 h incubation, with no inhibitory effect of 70% ethanol [8]. These authors also reported that propolis was less efficient against Gram-negative bacteria than Gram-positive ones, since the former have a more chemically complex cell wall and a higher lipid content, what may explain their resistance to propolis extracts [9].

Taken together, our data demonstrated that Geo-1 exhibited an inhibitory activity for both *S. aureus* and *E. coli*; however, this effect was not exclusively due to Geo-1, since similar results were obtained with its solvent.

The interaction between natural products and antibiotics is very important taking into consideration the increasing number of bacterial resistance to antibiotics, due to some mechanisms: 1- modifying the active site of the target which results in decreased efficiency of drugs binding; 2- direct destruction or modification of the antibiotic by enzymes produced by the body or 3- efflux of antibiotics from the cell [26, 27]. Therefore, one strategy employed to overcome these resistance mechanisms is combination of drugs [27].

The association of propolis with antimicrobial drugs has suggested that propolis may potentiate the effects of some antibiotics, mainly those acting in bacterial wall and ribosome [28, 29]. However, there is no data in literature on the synergism of Geo with antibiotics. Our results demonstrated a synergistic action of Geo-1 only with chloramphenicol on *S. aureus* strains, but not on *E. coli* (Table 2). This is an important finding, since one may use a lower concentration of antibiotic combined with Geo in order to reduce side effects and bacterial resistance. Similar effects were found using both ½ and ¼ of Geo-1, suggesting that its lower concentration may lead to better results. Since chloramphenicol acts by inhibiting bacterial protein synthesis, one may speculate that Geo favored its action on bacteria ribosome.

Table 2. Median, 1st and 3rd quartiles, in brackets, relative to the values of the association of ¼ or ½ of the $MIC_{90\%}$ values for Geo-1 or 70% ethanol with antimicrobial drugs against *S. aureus* and *E. coli* strains.

S. aureus

Groups	Chloramphenicol	Gentamicin	Tetracycline	Ciprofloxacin	Oxacillin
control	25.0 [24, 29]	22.0 [12, 22]	14.5 [12, 30]	25.0 [23, 27]	18.5 [0, 19]
¼ Geo-1	30.0 * [26, 32]	15.0 [15, 16]	15.5 [14, 30]	24.0 [19, 25]	17.5 [0, 19]
½ Geo-1	31.5 * [30, 38]	13.0 [13, 14]	19.0 [14, 32]	21.0 [20, 22]	19.5 [0, 21]
¼ 70% ethanol	28.5 [25, 30]	22.0 [12, 23]	14.0 [13, 31]	26.5 [22, 27]	18.5 [0, 20]
½ 70% ethanol	23.0 [19, 26]	23.0 [19, 26]	12.5 [10, 23]	9.0 [0, 12]	27.5 [22, 34]

E. coli

Groups	Chloramphenicol	Gentamicin	Tetracycline	Ciprofloxacin	Oxacillin
control	22.0 [16, 23]	20.5 [19, 21]	22.0 [7, 25]	31.5 [28, 32]	0
¼ Geo-1	19.0 [14, 22]	14.5 [14, 15]	19.0 [0, 22]	24.5 [20, 30]	0
½ Geo-1	19.0 [15, 22]	12.0 [12, 12]	18.5 [0, 21]	21.5 [19, 24]	0
¼ 70% ethanol	20.5 [16, 22]	20.5 [20, 22]	23.0 [8, 26]	31.5 [26, 35]	0
½ 70% ethanol	20.0 [18, 21]	23.0 [22, 24]	26.0 [8, 29]	31.5 [28, 32]	0

* significantly different from control ($P < 0.05$).

Regaring the *Pythium insidiosum* evaluation, 15 isolates were used to determine the inhibition range of their hyphae growth. *Pythium insidiosum* differs from the true fungi in the production of mobile zoospores and in the chemical composition of its cell wall and membrane. Chytridiomycetes (phylum of true fungi belonging to Kingdom Fungi) also produce zoospores in water environment; however, they are uniflagellate, while Oomycetes are biflagellate. As to the chemical composition of the cell wall, true fungi have chitin and α- and β-glucans, whereas *P. insidiosum* has chitin, β-glucans, cellulose and hydroxyproline. The plasma membrane of *P. insidiosum* is deficient in ergosterol – a target molecule of most of antifungal drugs [12]. Thus, there are no effective antifungal drugs against *P. insidiosum* and pythiosis is characterized by a difficult treatment.

There is a great interest of researchers to discover new antifungal drugs. There different groups of antifungal drugs affect the cell membrane or cell wall integrity by inhibiting ergosterol or β-glucan/quitin synthesis, respectively. The problem of drug resistance is the appearance of pathogenic fungi already seen clinically [30, 31].

Our data indicated that human B 01 isolate was inhibited by Geo-2 (5.0 mg/mL) in all incubation periods (1, 2 and 7 days). The concentration 3.4 mg/mL showed only an inhibitory effect on this isolate, with a fungal growth significantly lower than control in all time periods ($P < 0.05$) (Table 3). The growth of B 01 isolate after incubation with Geo-2 is found in Figure 3.

Equine isolates were inhibited by Geo-2 using 7.0 mg/mL after 1 and 2 days of incubation ($P < 0.05$). Table 3 shows the effect of different concentrations of Geo-2 on *P. insidiosum* growth and Figure 4 shows *P. insidiosum* behavior over time after incubation with Geo-2 (5.0 and 7.0 mg/mL).

Table 3. Effect of Geo-2 (3.4, 5.0, 7.0, 12.5, 17.7 mg/mL) on the growth of *Pythium insidiosum* obtained from human or equine isolate after 1, 2 and 7 days of incubation at 37 ºC on SAB agar.

Human isolate

Time (days)	control	3.4	5.0	7.0	12.5	17.7
1	5.82 ± 1,38	0*	0*	0*	0*	0*
2	21.2 ± 0,62	2.39 ± 3,32*	0*	0*	0*	0*
7	90.0 ± 0	30.60 ± 32,97*	0*	0*	0*	0*

Equine isolates

Time (days)	control	3.4	5.0	7.0	12.5	17.7
1	18.25 ± 2.26	14.31 ± 7.84	5.81 ± 7.41*	0*	0*	0*
2	35.96 ± 4.28	30.92 ± 11.22	16.65 ± 13.03*	0*	0*	0*
7	90.00 ± 0	80.55 ± 16.03	53.74 ± 33.99	0*	0*	0*

Data represent mean ± standard deviation of colony diameter (cm) in quintuplicate (*$P < 0.05$ vs control).

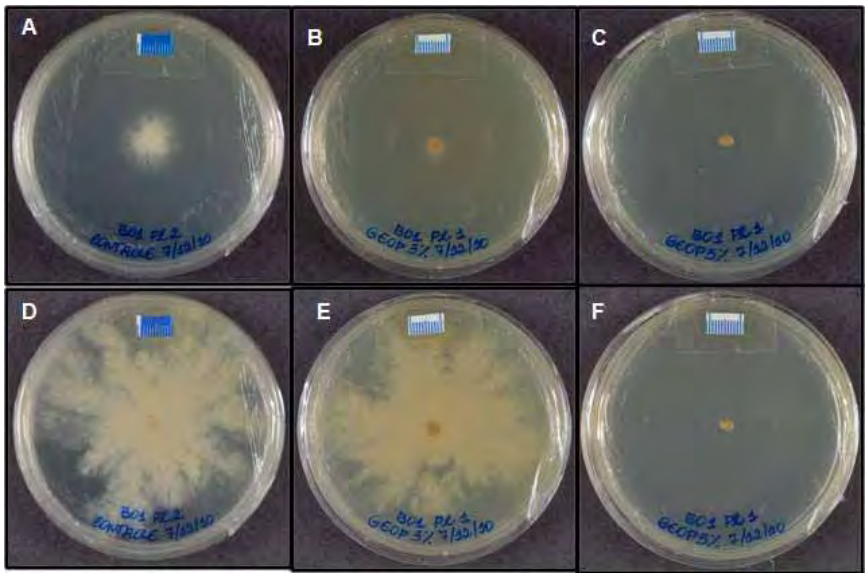

Figure 3. Plates inoculated with human B 01 isolate containing only SAB medium - control (A and D), 3.4 mg/mL of Geo-2 (B and E) and 5 mg/mL of Geo-2 (C and F) after 2 and 7 days of incubation, respectively. The diameter of colonies growth was performed using the software Image J (image processing and analysis in JAVA; http://rsbweb.nih.gov/ij/).

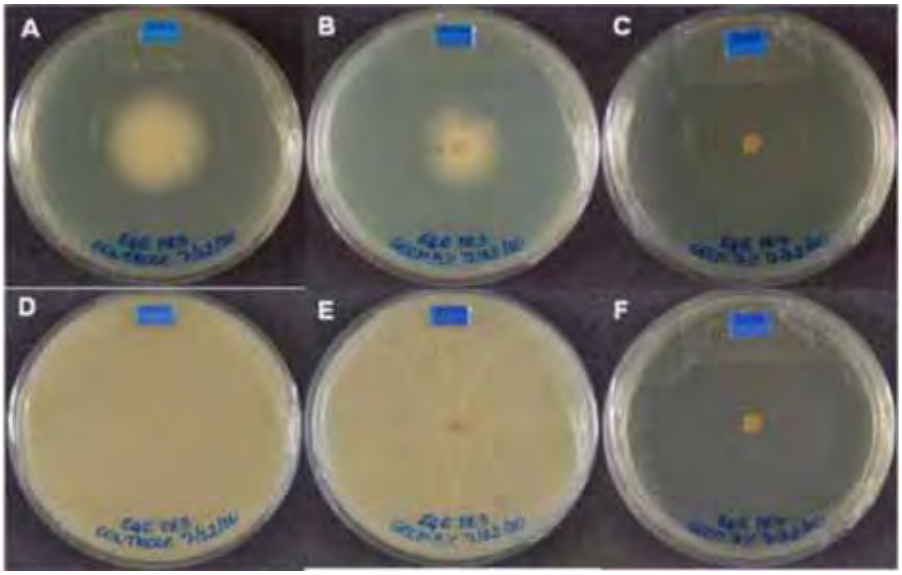

Figure 4. Plates inoculated with equine isolate containing only Sabouraud medium - control (A and D), 5 mg/mL of Geo-2 (B and E) and 7 mg/mL of Geo-2 (C and F) after 2 and 7 days incubation, respectively. The diameter of colonies growth was performed using the software Image J (image processing and analysis in JAVA; http://rsbweb.nih.gov/ij/).

One may verify that Geo-2 exerted a fungistatic activity on *P. insidiosum* isolates. This fact is new and relevant, since there are no data concerning Geo effects on *P. insidiosum*. On the other hand, several works have been published using propolis produced by Africanized honeybees, and its fungicidal action on true fungi such as *Candida tropicalis* and *C. albicans* as well as on dermatophyte fungi of the genus *Trichophyton*, and yeasts that cause onychomycosis were reported [24, 32- 34]. The antifungal activity of propolis from Tucumán (Argentina) against dermatophytes and yeasts was analyzed, and the most susceptible species were *Microsporum gypseum*, *Trichophyton mentagrophytes* and *T. rubrum* [35].

Allicin found in garlic has antimicrobial activities against certain of bacteria and fungi. Its antifungal activity was evaluated *in vitro* against 17 strains of *P. insidiosum* isolated from horses, and all isolates were inhibited by garlic extract, with MIC values ranging from 0.39 to 6.25 mg/mL [36].

Up to now, it is difficult to establish a more accurate comparison between our results and those found in the literature using propolis produced by

Africanized honeybees, since geopropolis extract has a low yield and is difficult to solubilize. Further investigation would assess its association with traditional antifungal agents, reducing their concentration, analyzing a possible synergistic effect with no side effects.

In conclusion, Geo produced by *M. fasciculata* showed no antibacterial action for *S. aureus* and *E. coli*, with inhibitory activities only at high concentrations and similar to the solvent effects. However, the association of Geo with antibiotics favored the effect of chloramphenicol on *S. aureus*, probably affecting protein synthesis. Geo exerted an inhibitory action on *Pythium insidiosum*, for both human and equine isolates. The human isolate exhibited a higher sensitivity to Geo and was inhibited using a lower concentration than that observed for horses. Geo exerted a lower antimicrobial activity compared to propolis produced by Africanized honeybees, perhaps due to the low yield of the extract and its difficult solubility.

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Conflict of Interests

No potential conflict of interests were disclosed.

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REFERENCES

[1] B. A. Cunha, “Antibiotic side effects,” *Medical Clinics of North America*, vol. 85, no. 1, pp. 149-185, 2001.

[2] A. T. Sheldon Jr., “Antibiotic resistance: who is winning the war? Introductory remarks,” *International Journal of Toxicology*, vol. 22, no. 2, pp. 129-130, 2003.

[3] S. A. Libério, A. L. A. Pereira, M. J. A. M. Araújo, R. P. Dutra, F. R. F. Nascimento, V. Monteiro-Neto, M. N. S. Ribeiro, A.G. Gonçalves, and R. N. M. Guerra, “The potencial use of propolis as a cariostatic agent and its actions on mutans group streptococci,” *Journal of Ethnopharmacology*, vol. 125, no. 1, pp. 1-9, 2009.

[4] W. E. Kerr, “Abelhas indígenas brasileiras (Meliponíneos) na polinização e na produção de mel, pólen, geoprópolis e cera,” *Informe Agropecuário*, vol. 13, no. 149, pp. 15-22, 1987.

[5] P. Nogueira-Neto, *A criação de abelhas indígenas sem ferrão*, São Paulo: Nogueirapis, 1997.

[6] S. Castaldo and F. Capasso, “Propolis, an old remedy used in modern medicine,” *Fitoterapia*, vol. 73, no. 1, pp. 1-6, 2002.

[7] M. O. Freitas, F. A. F. Ponte, M. A. S. Lima, and E. R. Silveira, “Flavonoids and triterpenes from the nest of the stingless bee *Trigona spinipes*,” *Journal of the Brazilian Chemical Society*, vol. 19, no. 3, pp. 532-535, 2008.

[8] J. M. Sforcin, A. Fernandes Jr., C. A. M. Lopes, V. Bankova, and S. R. C. Funari, “Seasonal effect on Brazilian propolis antibacterial activity,” *Journal of Ethnopharmacology*, vol. 73, no. 1-2, pp. 243-249, 2000.

[9] A. C. Vargas, A. P. Loguercio, N. M. Witt, M. M. Costa, M. S. Silva, and L. R. Viana, “Atividade antimicrobiana *in vitro* de extrato alcoólico de própolis,” *Ciência Rural*, vol. 34, no. 1, pp. 159-163, 2004.

[10] S. A. C. Duailibe, A. G. Gonçalves, and F. J. M. Ahid, “Effect of a propolis extract on *Streptococcus mutans* counts *in vivo,” Journal* of *Applied Oral Science,* vol. 15, no. 5, pp. 420-423, 2007.

[11] S. A. Libério, A. L. A. Pereira, R. P. Dutra, A. S. Reis, M. J. A. M. Araújo, N.S. Mattar, L.A. Silva, M.N.S. Ribeiro, F.R.F. Nascimento, R.N.M. Guerra, V. Monteiro-Neto, "Antimicrobial activity against oral pathogens and immunomodulatory effects and toxicity of geopropolis produced by the stingless bee *Melipona fasciculata* Smith," *BMC Complementary and Alternative Medicine*, vol. 11, pp. 108.

[12] J. M. Santurio, S. H. Alves, D. B. Pereira, and J. S. Argenta, "Pitiose: uma micose emergente," *Acta Scientiae Veterinariae*, vol. 34, no. 1, pp. 1-14, 2006.

[13] E. G. Moura, *Agroambientes de transição: entre o trópico úmido e o semi-árido do Brasil*, UEMA, São Luís, 2004.

[14] B. V. B. Abreu, M. C. A. Batista, C. C. Azevedo, R. P. Dutra, A. M. C. Nogueira, M. C. P. Costa, and M. N. S. Ribeiro, "Quantificação de polifenóis de geoprópolis de *Melipona fasciculata* Smith (tiúba) coletado no cerrado maranhense," *Revista de Ciências da Saúde***,** vol. 8, no. 1, pp. 18-24, 2006.

[15] R. P. Dutra, A. M. C. Nogueira, R. R. O. Marques, M. C. P. Costa, and M. N. S. Ribeiro, "Avaliação farmacognóstica de geoprópolis de *Melipona fasciculata* Smith da Baixada Maranhense, Brasil," *Revista Brasileira de Farmacognosia*, vol. 18, no. 4, pp. 557-562, 2008.

[16] M. S. Cunha, R. P. Dutra, M. C. A. Batista, B. V. B. Abreu, J. R. Santos, V. A. neival, F. M. M. Amaral, and M. N. S. Ribeiro, "Padronização de extrativos de geoprópolis de *Melipona fasciculata* Smith (Tiúba)," *Cadernos de Pesquisas, São Luís,"* vol. 16, no. 3, pp. 31-38, 2009.

[17] Clinical and Laboratory Standards Institute - CLSI/ National Committee for Clinical Laboratory Standards – NCCLS, *Performance Standards for Antimicrobial Suscetibility Testing*, CLSI document M100-S15, Wayne, PA, USA, 2005.

[18] E. G. Alves, A. H. C. Vinholis, L. A. Casemiro, N. A. J. C. Furtado, M. L. A. Silva, W. R. Cunha, and C. H. G. Martins, "Estudo comparativo de técnicas de *screening* para a avaliação da atividade antibacteriana de extratos brutos de espécies vegetais e de substâncias puras," *Química Nova*, vol. 31, no. 5, pp. 1224-1229, 2008.

[19] A. P. Farnesi, R. Aquino-Ferreira, D. de Jong, J. K. Bastos, and A. E. E. Soares, “Effects of stingless bee and honey bee própolis on four species of bacteria,” *Genetics and Molecular Research*, vol. 8, no. 2, pp. 635-640, 2009.

[20] J. L. Rios, M. C. Recio, and A. Villar, “Screening methods for natural products with antimicrobial activity: a review of the literature,” *Journal of Ethnopharmacology*, vol. 23, no. 2-3, pp. 127-149, 1988.

[21] A. Fernandes Jr., L. Leomil, A. A. H. Fernandes, and J. M. Sforcin, “The antibacterial activity of propolis produced by *Apis mellifera* L. and Brazilian stingless bees,” *Journal of Venomous Animals and Toxins*, vol. 7, no. 2, pp. 173-182, 2001.

[22] S. Silicia and S. Kutluka, “Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region,” *Journal of Ethnopharmacology*, vol. 99, no. 1, pp. 69-73, 2005.

[23] V. Bankova, “Chemical diversity of propolis and the problem of standardization,” *Journal of Ethnopharmacology*, vol. 100, no. 1-2, pp. 114–117, 2005.

[24] M. Velikova, V. Bankova, M. C. Marcucci, I. Tsvetkova, and A. Kujumgiev, “Chemical composition and biological activity of propolis from Brazilian meliponinae,” *Zeitschrift fur Naturforschung*, vol. 55, no. 9-10, pp. 785-789, 2000.

[25] M. Wootton, A. P. Macgowan, and T. R. Walsh, “Comparative bactericidal activities of Daptomycin and Vancomycin against glycopeptide-intermediate *Staphylococcus aureus* (GISA) and heterogeneous GISA isolates,” *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 12, pp. 4195- 4197, 2006.

[26] A. T. Sheldon Jr., “Antibiotic resistance: a survival strategy,” *Clinical Laboratory Science*, vol. 18, no. 3, pp. 170-180, 2005.

[27] S. Hemaiswarya, A. K. Kruthiventi, and M. Doble, “Synergism between natural products and antibiotics against infectious diseases,” *Phytomedicine*, vol. 15, no. 8, pp. 639-652, 2008.

[28] R. O. Orsi, A. Fernandes Jr., V. Bankova, and J. M. Sforcin, “Antibacterial effects of Brazilian and Bulgarian propolis and synergistic effects with antibiotics acting on the bacterial DNA and folic acid,” *Natural Product Research*, vol. 26, no. 4, pp. 344-349, 2012.

[29] R. O. Orsi, A. Fernandes Jr., V. Bankova, and J. M. Sforcin, “The effects of Brazilian and Bulgarian propolis *in vitro* against *Salmonella* Typhi and synergism with antibiotics acting on the ribosome,” *Natural Product Research*, vol. 26, no. 5, pp. 430-437, 2012.

[30] D. P. Kontoyiannis and R.E. Lewis, “Antifungal drug resistance of pathogenic fungi,” *Lancet*, vol. 359, no. 9312, pp. 1135-1144, 2002.

[31] J. Morschhauser, “Regulation of multidrug resistance in pathogenic fungi,” *Fungal Genetics and Biology*, vol. 47, no. 2, pp. 94-106, 2010.

[32] J. M. Sforcin, A. Fernandes Jr., C. A. M. Lopes, S. R. C. Funari, and V. Bankova, “Seasonal effect on Brazilian propolis on *Candida albicans* and *Candida tropicalis*,” *Journal of Venomous Animals and Toxins*, vol. 7, no. 1, pp. 139-144, 2001.

[33] A. B. Siqueira, B. S. Gomes, I. Cambuim, R. Maia, S. Abreu, C. M. Souza-Motta, L. A. Queiroz, and A. L. Porto, “Trichophyton species susceptibility to green and red propolis from Brazil,” *Letters Applied Microbiology***,** vol. 48, no. 1, pp. 90-96, 2009.

[34] S. R. Lustosa, A. B. Galindo, L. C. C. Nunes, K. P. Randau, and P. J. Rolim Neto, “Própolis: atualizações sobre a química e a farmacologia,” *Revista Brasileira de Farmacognosia*, vol. 18, no. 3, pp. 447-454, 2008.

[35] M. B. Aguero, M. Gonzalez, B. Lima, L. Svetaz, M. Sánchez, S. Zacchino, G. E. Feresin, G. Schmeda-Hirschmann, J. Palermo, D. Wunderlin, A. Tapia, “Argentinean propolis from *Zuccagnia punctata* Cav. (Caesalpinieae) exudates: phytochemical characterization and antifungal activity,” *Journal of Agricultural and Food Chemistry*, vol. 58, no. 1, pp. 194-201, 2010.

[36] R. A. Zanette, P. E. R. Bitencourt, C. Weiblen, M. B. Pilotto, A. S. Pigatto, R. B. Ceolin, M. B. Moretto, S. H. Alves and J. M. Santurio, “*In vitro*

susceptibility of *Pythium insidiosum* to garlic extract," *African Journal of Microbiology Research*, vol. 5, no. 29, pp. 5316-5318, 2011.

Conclusões

Após a realização deste projeto de pesquisa, os dados obtidos permitiram obter as seguintes conclusões:

- A caracterização química da Geo revelou que o principal grupo de compostos encontrados foram triterpenos, possibilitando associar as ações observadas ao perfil químico desta amostra e abrindo perspectivas para novos estudos, avaliando compostos identificados em nossa amostra, tais como triterpenos, hexoses, glucitol, ácido glicurônico e inositol;

- Embora praticamente todas as linhagens tumorais sejam sensíveis à ação da própolis produzida por abelhas africanizadas, a geoprópolis (Geo) apresentou ação inibitória sobre as células HEp-2; entretanto, esta ação foi somente citostática;

- A Geo apresentou ação imunomoduladora, estimulando a produção das citocinas estudadas (TNF-α e IL-10), sugerindo seu caráter ativador sobre monócitos humanos;

- A Geo não apresentou atividade antibacteriana sobre *S. aureus* e *E. coli*, havendo inibição destas linhagens somente com concentrações elevadas do extrato, e com influência do solvente. Além disso, a associação da Geo com antibióticos favoreceu o efeito do cloranfenicol sobre *S. aureus*, o qual atua interferindo a síntese proteica. Estes resultados apontam a necessidade de investigação futura quanto à avaliação do sinergismo da geoprópolis com antibióticos;

- A Geo exerceu ação inibitória sobre *Pythium insidiosum*, tanto para o isolado obtido de caso humano como de eqüinos. O isolado humano demonstrou maior sensibilidade à geoprópolis, sendo inibido com concentração inferior àquela observada para equinos;

- A Geo exerceu menor atividade em comparação à própolis produzida por abelhas africanizadas, talvez devido ao baixo rendimento do extrato e à sua difícil solubilidade. Entretanto, nossos achados abrem perspectivas para a investigação de novas formas de utilização da Geo, concentrando melhor seus extratos. Também seria plausível avaliar a ação de seus componentes majoritários, evidenciando o potencial farmacológico ou terapêutico dos mesmos;

- Os dados obtidos junto a esta tese de doutoramento revelam o ineditismo dos trabalhos, ao avaliar pioneiramente os efeitos da Geo nos modelos experimentais adotados.