INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior Agrária

PROPRIEDADES NUTRACÊUTICAS DO PRÓPOLIS PORTUGUÊS

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Dissertação apresentada à Escola Superior Agrária de Bragança para obtenção do Grau de Mestre em Qualidade e Segurança Alimentar

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Bragança
2009

Agradecimentos

Após todo o esforço que tornou possível a realização deste trabalho, queria agradecer às pessoas que tornaram possível a sua concretização.

Em primeiro lugar, à Professora Doutora Letícia Estevinho, por todos os conhecimentos transmitidos, também pela dedicação, disponibilidade, amizade e grande ajuda prestada em todos os momentos.

Ao Professor Doutor Luís Dias, por todos os conhecimentos transmitidos, pela disponibilidade e acompanhamento ao longo de todo este trabalho.

À Professora Doutora Teresa Dias, pelos conhecimentos e disponibilidade em todos os momentos e colaboração no trabalho escrito.

Ao Professor Doutor José Alberto, pela amizade demonstrada e pela ajuda prestada.

À Professora Doutora Ermelinda Pereira, pelos materiais disponibilizados, indispensáveis à realização deste trabalho.

Às funcionárias do Laboratório de Microbiologia e Biologia, Dª Arminda, Dª Fátima, Dª Isabel e Dª Amélia, pela amizade, pelo apoio e colaboração prestados. À Elsa, ao Rui e a todos os outros estagiários que por lá têm passado, pelos bons momentos proporcionados.

Às minhas colegas e amigas de laboratório, Ana Paula e Margarida, por todos os bons momentos proporcionados e por toda a ajuda na realização deste trabalho.

À minha família, em especial aos meus pais e irmã, por todo o apoio e incentivo nos bons e maus momentos.

À Mónica, pelo companheirismo, afecto e ajuda no trabalho laboratorial, e à sua família, por todo o carinho e bons momentos passados, em especial ao Sr. Francisco e ao Rúben por todo o esforço e colaboração neste trabalho.

Ao João e à Margarida, pela colaboração e disponibilidade sempre que necessário.

Aos meus amigos de licenciatura e companheiros de toda a vida que sempre estiveram presentes e me apoiaram nos momentos mais importantes, Alexandra, Ana Cláudia, Bruno, Goreti, Isabel, Joana, Natália, Sílvia e Susana, a eles devo e recordarei para sempre os melhores momentos académicos, momentos felizes primando muitos deles pelo inédito do imprevisto.

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Resumo

O propolis é uma substância resinosa colhida das plantas pelas abelhas. A composição deste produto depende da flora envolvente, da estação do ano, e da localização geográfica de proveniência. Este produto apícola contém normalmente uma alta variedade de compostos químicos como, polifenóis, esteróides e aminoácidos, e é usado desde a Civilização Egípcia.

Pela primeira vez, a origem polínica, os fenóis totais e a actividade antioxidante, foram estudadas em Portugal. Os fenóis totais foram determinados pelo método colorimétrico. Os resultados obtidos foram 329 mg/g de EAG para o própolis de Bornes, e 151 mg/g de EAG para o própolis do Fundão. A actividade antioxidante foi avaliada pelo efeito bloqueador de radicais livres de DPPH (2,2-difenil-1-picrilhidrazil) e pelo método do poder redutor. Os resultados evidenciaram valores de EC_{50} muito baixos no método de DPPH, 6,22 µg/mL (Bornes) e 52,00 µg/mL (Fundão). Para o método do poder redutor os valores obtidos foram 9,00 µg/mL (Bornes) e 55,00 µg/mL (Fundão). Estes valores indicam que o própolis Português é uma importante fonte de polifenóis e compostos antioxidantes, o que prova o alto benefício para a saúde Humana deste produto.

A esferocitose hereditária (EH) é um tipo de anemia hereditária, que origina eritrócitos microcíticos e hipercrómicos. Clinicamente, o paciente apresenta desde uma condição assintomática a uma anemia hemolítica severa. O objectivo deste estudo foi avaliar o efeito de dois própolis, de diferentes regiões, na fragilidade osmótica da membrana dos eritrócitos de um paciente com EH. Constatou-se que o própolis diminui a fragilidade da membrana dos eritrócitos, sendo o propolis de Bornes mais efectivo comparativamente com o de Fundão. Os resultados obtidos sugerem que o stress oxidativo aumenta a fragilidade da membrana de pacientes com EH, e que este efeito possivelmente deve-se à capacidade antioxidante do própolis. Estes resultados abrem portas a futuras investigações com o objectivo de elucidar os mecanismos, e identificar os compostos envolvidos na protecção da membrana dos eritrócitos.

Palavras-chave: propolis Português, propriedades nutracêuticas, análise polinica, fenóis totais, actividade antioxidante, esferocitose hereditária, fragilidade osmótica, membrana eritrocitária.

Abstract

Propolis is a resinous substance collected from plants by bees. The propolis composition depends on the surrounding vegetation, the season, and the area from which it derives. This hive product usually contains a high variety of chemical compounds such as polyphenols (flavonoids, phenolic acids and esters), steroids and amino acids, and it's used since Egyptian Civilization.

Polinic analysis, total phenols content and antioxidant activity were studied for the first time in Portuguese samples of propolis. Total phenols content was determined by colorimetric assay and its amount was of 329 mg/g of GAE, in Bornes sample, and 151 mg/g of GAE in Fundão propolis. The antioxidant capacity of propolis extracts was assessed through the scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl) and reducing power assay. It was verified a very low EC_{50} on DPPH scavenging assay, of 6.22 µg/mL (Bornes propolis) and 52.00 µg/mL (Fundão propolis) and for reducing power the values were 9.00 µg/mL, for Bornes propolis, and 55.00 µg/mL, for Fundão propolis. The results obtained indicate that Portuguese propolis is an important source of phenols and antioxidative compounds that prove the high beneficial effects of propolis for human health.

The hereditary spherocytosis (HS) is a type of transmission of hereditary anaemia that results from the presence of microcytic and hyperchromic red cells. Clinically, subjects usually present from asymptomatic conditions to severe haemolytic anaemia. The aim of this study was to evaluate the effect of two propolis extracts, from different regions, in the osmotic fragility of patient red blood cell (RBC) membrane with HS. It was found that propolis decrease the erythrocytes membrane fragility, being the effect of Bornes propolis more pronounced comparatively to Fundão propolis. The obtained results suggest that *in vitro*, the membrane fragility may be increased under oxidative stress conditions in patient RBC's, and the protection effect of propolis is possibly due to its antioxidant properties. These results open doors for future investigations in order to elucidate the mechanisms, and identify the most relevant compounds involved in the fragility of the erythrocyte membrane.

Keywords: Portuguese propolis, nutraceutical properties, polinic analysis, total phenols, antioxidant activity, hereditary spherocytosis, osmotic fragility, erythrocyte membrane.

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CAPÍTULO I

Introdução

1. Breve introdução ao tema

O própolis também denominado por cola das abelhas, é um material resinoso recolhido pelas abelhas *Apis mellifera*. Desde os primórdios da humanidade que o própolis tem sido usado com diversos fins terapêuticos na medicina tradicional. As principais propriedades nutracêuticas associadas ao própolis são: antimicrobiana, antiviral, antiprotozoárica, antioxidante, anti-inflamatória, imunomodulatória e anti-tumoral (Lustosa et al., 2008; Sforcin, 2007; Simoes et al., 2008a).

Os produtos apícolas Portugueses encontram-se subvalorizados, facto que pode ser verificado por comparação do custo dos produtos apícolas praticados a nível internacional. Esta conjuntura poderá dever-se à concorrência de países Asiáticos e da América do Sul, que obriga a que o mel de qualidade seja comercializado abaixo dos custos de produção. Os apicultores nacionais ainda preferem desperdiçar o própolis, a procederem à sua exploração, talvez pelos "mitos" sobre este procedimento diminuir a produção de mel, ou pela falta de informação fundamentada sobre a importância deste produto no panorama económico apícola. Este produto pode constituir uma fonte secundária de rendimento, que poderá contribuir de forma importante para que os apicultores consigam superar as dificuldades financeiras por que estão a passar. Actualmente, o própolis nacional está a ser comercializado abaixo de cinquenta euros por kilograma, enquanto os hómologos de outros países estão a ser comercializados acima dos cento e cinquenta euros por kilograma. A subvalorização deste produto nacional resulta da falta de estudos científicos sobre as suas propriedades bioactivas. Torna-se imperiosa a realização de estudos a nível nacional com o objectivo de caracterizar e avaliar as propriedades farmacêuticas deste produto apícola nacional, com vista à sua valorização económica no mercado nacional e internacional.

Neste trabalho pretendeu-se, como objectivo global, estudar o própolis proveniente de duas regiões de Portugal: Trás-os-Montes e Beira Interior.

Ao nível dos objectivos específicos, o estudo do própolis incidiu na:

- Avaliação da origem floral através da análise melissopalinológica (Capitulo II)
- Quantificação dos fenóis totais (Capitulo II)
- Avaliação da actividade antioxidante (Capitulo II)
- Avaliação do efeito quelante (Capitulo III)
- Avaliação do efeito protector na membrana dos eritrócitos (Capitulo III)

A seguir, efectua-se a introdução dos subcapítulos:

2. Própolis na História da Humanidade
3. Caracterização e Composição Química
4. Propriedades Nutracêuticas
    4.1. Antimicrobiana
    4.2. Antiviral
    4.3. Antiprotozoárica
    4.4. Antioxidante
    4.5. Anti-inflamatória e Imunomodulatória
    4.6. Antitumoral

2. Própolis na História da Humanidade

O própolis é uma mistura complexa de substâncias recolhida pelas abelhas da espécie *Apis mellifera*, dos brotos de algumas árvores e plantas, que contêm substâncias como materiais lipofílicos, látex, resinas, bem como de exsudados de feridas nas plantas (Bankova, 2005; Castaldo and Capasso, 2002; Lustosa et al., 2008). A abelha recolhe os exsudados com as suas mandíbulas, mistura com a cera por si produzida, e com as patas dianteiras empurra para os sacos das patas traseiras, onde armazena o material recolhido (Fig. 1) (Sforcin, 2007).

Figura 1 – Abelha a recolher exsudados de uma planta, com própolis armazenado nas patas (fonte: www.flirck.com).

Durante este processo as enzimas fermentativas da saliva são misturadas com o material bruto recolhido, o que provoca a hidrólise de compostos, nomeadamente dos glicosil-flavonóides, que na presença da enzima β-glicosidase originam flavonóides agliconas (Lustosa et al., 2008; Park and Ikegaki, 1998; Ramos and Miranda, 2007). Quando a abelha completa a recolha, voa para a colmeia onde deposita o própolis que será utilizado na construção e conservação da colmeia (Fig. 2) (Bankova et al., 2006).

Figura 2 – Abelha transportando própolis dentro da colmeia (fonte: www.pastorronilto.blogspot.com).

Nomeadamente, na protecção contra microrganismos e insectos, na reparação de fendas ou partes danificadas da colmeia, na garantia séptica de locais para a postura de ovos pela abelha rainha e na mumificação de insectos e outros invasores (Lustosa et al., 2008; Marcucci, 1996).

O termo própolis remonta ao léxico grego e resulta da confluência de *pro*- (defesa) e *polis*- (cidade ou comunidade), o que significa em "defesa da cidade", ou seja, da colmeia (dos Santos Pereira et al., 2002b; Simoes et al., 2008b). O uso deste produto natural remonta aos primórdios da Humanidade, sendo que as primeiras referências a este produto e às suas propriedades surgem com a Civilização Egípcia em 300 a.C. (da Silva et al., 2006; Ghisalberti, 1979; Lustosa et al., 2008), onde era usado pelos sacerdotes devido às suas qualidades terapêuticas e era um importante material no processo de mumificação dos Faraós para preservar as vísceras dos cadáveres (Bankova et al., 2000; Castaldo and Capasso, 2002; Marcucci, 1996), existindo referências ao própolis nos túmulos Egípcios de Luxor e nos papiros Ebers e Beck Badog (Figura 3).

Figura 3 – Hieróglifo que simboliza a importância dos produtos da colmeia no antigo Egipto (fonte: www.wikipedia.org)

Na civilização Persa, nomeadamente Avicena, um brilhante médico e filósofo do seu tempo, afirmou que o própolis tem a capacidade de "eliminar pontas de flechas e espinhas, vivifica, limpa facilmente e amacia fortemente" (Marcucci, 1996).

Mais tarde na Grécia antiga, este produto adquiriu uma grande popularidade entre as mentes mais brilhantes desse tempo, como Aristóteles (brilhante pensador do seu tempo, referenciou este produto como sendo útil nas infecções de pele, chagas e suturas), Hipócrates (por muitos considerado o "pai da medicina", prescrevia a utilização deste produto natural para auxiliar na cicatrização de feridas e úlceras, tanto a

nível interno como externo) e Galeno (cita este produto nos seus estudos, como benéfico para a saúde Humana) (Castaldo and Capasso, 2002; Lustosa et al., 2008). Nesta época, também as caravanas islâmicas transportavam este produto, devido à sua elevada importância e valor comercial, tão relevantes quanto a do petróleo ou mesmo do trigo.

Também na Bíblia este produto é mencionado com o nome de Bálsamo Galaad ou da Judéia. O Corão por sua vez aconselha o seu uso para efeitos medicinais, juntamente com o mel, denominando-se Kitharpikse.

A civilização Inca por sua vez usava este produto no tratamento de doenças infecciosas e estados febris (Castaldo and Capasso, 2002).

Na Europa, no século XIX os franceses utilizaram-no no tratamento de chagas, e na Guerra de Boers (1899-1902 África do Sul) era usado no tratamento de feridas infectadas e como cicatrizante (Lustosa et al., 2008; Pereira et al., 2002).

Durante a Segunda Guerra Mundial, a União Soviética financiou estudos aprofundados sobre este produto, e começou a ser utilizado no tratamento de inúmeras doenças (Lustosa et al., 2008; Pereira et al., 2002).

Desde essa época, o interesse por este produto tem vindo a aumentar, verificando-se actualmente um acréscimo na investigação científica acerca deste produto, paralelamente ao aumento do seu uso na indústria cosmética e farmacêutica.

3. Características e Composição Química

As propriedades bioactivas que tornaram o própolis um eminente produto natural, estão ligadas à sua composição química. No entanto, esta composição química é multivariável (Bankova, 2005; de Sousa et al., 2007), dependendo do clima e da ecologia da região de proveniência (Park et al., 2002), da estação do ano, bem como da variabilidade genética da rainha (Moreira et al., 2008; Park and Ikegaki, 1998), existindo mais de 300 compostos descritos (Castaldo and Capasso, 2002; dos Santos Pereira et al., 2002b). Este facto constitui um dos maiores problemas na aplicação do própolis, como agente terapêutico (dos Santos Pereira et al., 2002a). Contudo, diversos estudos têm vindo a ser desenvolvidos com vista à sua padronização, controlo de

qualidade e posterior aplicação terapêutica. A padronização e controlo da qualidade, segundo estudos recentes, devem incluir a comparação dos compostos químicos do própolis com os encontrados nas plantas que lhe deram origem (Bankova, 2005; Park et al., 2002), uma vez que, alguns compostos encontram-se em todos os tipos de própolis, enquanto outros ocorrem somente em própolis que derivam de determinadas plantas (Lustosa et al., 2008).

Entre os imensos compostos existentes no própolis destacam-se acacetina, ácido cinámico, cumárico, galangina, campferol, pinocembrina, preniletina, crisina, viscidona, vanilina, 5,7-dihidroxiflavona, 3,5,7-trihidroxiflavona e 5,7-dihidroxiflavona (Astudillo et al., 2000; Munoz et al., 2001a; Munoz et al., 2001b; Pena, 2008), pelos efeitos na saúde Humana.

A coloração do própolis pode variar entre castanho, amarelo, verde e vermelho (Ghisalberti et al., 1978). Possui um odor extremamente forte e característico, maioritariamente originado pelos compostos voláteis que nele existem ([Anon], 1927).

À temperatura ambiente é um produto de consistência sólida, sendo que a partir de 30ºC torna-se maneável. O seu ponto de fusão varia entre 60-70ºC, podendo ir até aos 100ºC, dependendo da sua composição.

Trata-se de um produto de baixa solubilidade em água, aconselhando-se por isso a utilização de solventes como éter, etanol, acetona, metanol, tolueno e tricloroetileno, que permitem a dissolução de grande parte dos compostos do própolis. Geralmente, obtêm-se duas fases: uma em que os materiais como ceras (cerca de 30%), bálsamos, derivados fenólicos (aproximadamente 60%) e óleos essenciais, estão dissolvidos de forma homogénea (fase solúvel); outra em que existe o depósito de materiais insolúveis como materiais orgânicos de origem vegetal e animal, grãos de pólen, entre outros (fase insolúvel) (Vanhaelen and Vanhaelen-Fastre, 1979).

Na actualidade o própolis, à semelhança de outros produtos naturais, é amplamente utilizado na medicina alternativa, na indústria cosmética e, embora ainda em baixa escala, na indústria farmacêutica (Kaneya et al., 2009; Myung et al., 2009).

4. Propriedades Nutracêuticas

O própolis na actualidade é considerado um dos produtos naturais de maior destaque nos estudos farmacológicos. Como já foi referido anteriormente, é um produto conhecido pelas diversas propriedades biológicas que possui: antimicrobiana, antiviral, antiprotozoárica, antioxidante, anti-inflamatória, imunomodulatória e anti-tumoral (Lustosa et al., 2008; Sforcin, 2007; Simoes et al., 2008a).

4.1. Antimicrobiana

De todas as propriedades que o própolis possui, a actividade antimicrobiana é a mais extensivamente estudada (Kujumgiev et al., 1999), tendo sido a primeira a despertar o interesse na comunidade científica, pois o própolis era usado desde o Antigo Egipto como conservante.

A actividade antimicrobiana que o própolis apresenta, segundo alguns autores, deve-se aos flavonóides, em especial à pinocembrina, à galangina e ao éster de ácido caféico, com um mecanismo de acção baseado na inibição da RNA polimerase (Cushnie and Lamb, 2005; Uzel et al., 2005). Outros estudos revelam que o ácido caféico, ácido benzóico e ácido cinâmico causam danos tanto a nível funcional, como estrutural na membrana e parede celular (Scazzocchio et al., 2006). O mecanismo de acção envolve a destruição da membrana citoplasmática, com perda dos iões potássio e consequente autólise (Viuda-Martos et al., 2008). A quercetina, outro dos compostos do própolis, é conhecida por aumentar a permeabilidade da membrana e anular o seu potencial. Deste modo, a célula perde a capacidade de produção de ATP, o transporte membranar e a sua mobilidade (Mirzoeva et al., 1997).

O campo de acção do própolis ao nível antimicrobiano é amplo, conseguindo inibir o crescimento de bactérias, bolores e leveduras, mas a sua eficácia depende do microrganismo em causa, uma vez que estudos recentes evidenciam uma maior eficácia contra bactérias Gram-positivas do que contra bactérias Gram-negativas (Lu et al., 2005; Lustosa et al., 2008; Marcucci et al., 2001). Tal facto pode ocorrer

devido à maior complexidade da parede celular das bactérias Gram negativas (Campos et al., 2004; Cushnie and Lamb, 2005).

A actividade antimicrobiana do própolis, ao contrário do que muitos investigadores consideravam, resulta do sinergismo dos compostos que compõem o própolis. Estudos efectuados com fracções de própolis revelam que estas não possuem qualquer efeito antimicrobiano, mas quando se voltam a juntar as fracções o efeito antimicrobiano verifica-se novamente (Marcucci, 1996).

Durante as últimas décadas, tem-se verificado um aumento no número de microrganismos resistentes aos antibióticos habitualmente utilizados em clínica. Tal facto provocou um aumento na investigação de novos produtos microbicidas ou que exerçam sinergismo com antibióticos, nomeadamente o própolis. Estudos realizados com biomicina, tetraciclina, neomicina, polimixina, penicilina e estreptomicina contra *Staphilococcus aureus* e *Escherichia coli* evidenciaram que o efeito dos antibióticos na presença de própolis foi potenciado entre 10 a 100 vezes (Marcucci, 1996). Deste modo, a aplicação simultânea de própolis em associação com um antibiótico, pode diminuir a aquisição de resistências nos microrganismos, evitando o uso de antibióticos cada vez mais potentes (Fernandes et al., 2005; Onlen et al., 2007a; Stepanovic et al., 2003).

No que respeita ao sinergismo com antimicóticos o própolis mostrou-se ser igualmente potenciador, mas em menor escala. Adicionalmente, Marcucci (1996) verificou que o extracto aquoso não possui qualquer efeito sobre *Candida albicans* enquanto o extracto etanólico possui um efeito moderado. O própolis evidenciou igualmente actividade fungicida *in vitro* contra leveduras causadoras das oncomicoses (Lustosa et al., 2008; Oliveira et al., 2006a). Estudos realizados com o própolis Mexicano mostraram que o própolis em concentrações muito baixas tem capacidade fungistática enquanto para concentrações mais altas o efeito é fungicida (Quintero-Mora et al., 2008).

Na tabela 1 resume-se a pesquisa efectuada dos efeitos do própolis nos microrganismos.

Tabela 1 – Microrganismos cujo crescimento é afectado pelo própolis, com as referências que avaliam a concentração mínima inibitória (CMI) e as que citam esta actividade.

Microrganismo	***CMI (mg/mL)***	***Referências***
Actinomyces sp.	0,002 – 0,390	(Castro et al., 2009; Ferreira et al., 2007; Koru et al., 2007)
	---	(Koo et al., 2000a; Marcucci, 1996; Park et al., 1998)
Aerobacter aerogenes	---	(Marcucci, 1996)
Alcaligenes sp.	---	(Marcucci, 1996)
Aspergilus niger	0,500	(Mohammadzadeh et al., 2007a)
Bacillus brevis	0,005 – 0,060	(Garedew et al., 2004)
	---	(Marcucci, 1996)
Bacillus cereus	0,020 – 0,250	(Erkmen and Ozcan, 2008; Kalogeropoulos et al., 2009; Silici et al., 2007b; Stepanovic et al., 2003)
	---	(Aga et al., 1994; Kivman et al., 1978; Marcucci, 1996; Menezes et al., 1997; Pavilonis et al., 2008)
Bacillus megatherium	0,005 – 0,060	(Garedew et al., 2004)
	---	(Marcucci, 1996)
Bacillus polymyxa	---	(Marcucci, 1996)
Bacillus pumilus	---	(Marcucci, 1996; Tolba et al.)
Bacillus sphaericus	---	(Marcucci, 1996)
Bacillus subtilis	0,020 – 0,310	(Erkmen and Ozcan, 2008; Garedew et al., 2004; Mohammadzadeh et al., 2007a; Stepanovic et al., 2003)
	---	(Choi et al., 2006; Kartal et al., 2003; Kosalec et al., 2005; Kumar et al., 2008; Marcucci, 1996; Menezes et al., 1997; Muli and Maingi, 2007; Myung et al., 2009; Pavilonis et al., 2008)
Bordetella bronchiseptica	---	(Marcucci, 1996)
Branhella catarrhalis	---	(Kartal et al., 2003; Marcucci, 1996)
Candida albicans	0,004 – 8,000	(Gebara et al., 2002; Jorge et al., 2008; Kalogeropoulos et al., 2009; Melliou et al., 2007; Mohammadzadeh et al., 2007a; Ota et al., 2001; Quintero-Mora et al., 2008; Silici et al., 2007b; Stepanovic et al., 2003; Uzel et al., 2005)
	---	(Abd El Hady and Hegazi, 2002; Azevedo et al., 1999; Bruschi et al., 2006; Chen and Shen, 2008; Choi et al., 2006; D'Auria et al., 2003; Hegazi and El Hady, 2002; Kaneya et al., 2009; Kartal et al., 2003; Koo et al., 2000a; Kosalec et al., 2005; Kumar et al., 2008; Marcucci, 1996; Oliveira et al., 2006b; Popova et al., 2009; Salomao et al., 2004; Salomao et al., 2008; Silici et al., 2005; Ugur and Arslan, 2004)

Candida glabrata	0,500 – 2,900	(Melliou et al., 2007)
	---	(Azevedo et al., 1999; Koc et al., 2007; Popova et al., 2009; Silici et al., 2005)
Candida krusei	0,004 – 9,000	(Ota et al., 2001; Silici et al., 2007b; Uzel et al., 2005)
Candida tropicalis	0,016 – 10,000	(Kalogeropoulos et al., 2009; Melliou et al., 2007; Ota et al., 2001; Uzel et al., 2005)
	---	(Azevedo et al., 1999; Oliveira et al., 2006b; Popova et al., 2009)
Cellulomonas fimi	---	(Marcucci, 1996)
Clostridium sp.	0,781	(Ferreira et al., 2007)
	---	(Boyanova et al., 2006; Marcucci, 1996)
Corynebacterium	---	(Kartal et al., 2003; Marcucci, 1996)
Enterococcus sp.	0,002 – 6,425	(Ferreira et al., 2007; Kalogeropoulos et al., 2009; Silici et al., 2007b; Stepanovic et al., 2003; Uzel et al., 2005)
	---	(Awawdeh et al., 2009; Drago et al., 2007; Erkmen and Ozcan, 2008; Kartal et al., 2003; Koo et al., 2000a; Marcucci, 1996; Melliou et al., 2007; Myung et al., 2009; Oncag et al., 2006; Pavilonis et al., 2008; Popova et al., 2009)
Escherichia coli	0,016 – 5,000	(Garedew et al., 2004; Gebara et al., 2002; Jorge et al., 2008; Kalogeropoulos et al., 2009; Melliou et al., 2007; Mohammadzadeh et al., 2007a; Stepanovic et al., 2003; Uzel et al., 2005)
	---	(Dabiza, 2007; Drago et al., 2007; Erkmen and Ozcan, 2008; Farnesi et al., 2009; Garcia Bernal et al., 2007; Hegazi and Abd El Hady, 2002; Hegazi et al., 2000a; Kumar et al., 2008; Muli and Maingi, 2007; Myung et al., 2009; Pavilonis et al., 2008; Popova et al., 2001)
Fusobacterium nucleatum	0,001 – 0,781	(Ferreira et al., 2007; Gebara et al., 2002; Koru et al., 2007; Santos et al., 2003)
Klebsiella pneunomiae	1,250 – 8,500	(Melliou et al., 2007; Stepanovic et al., 2003)
	---	(Kartal et al., 2003; Popova et al., 2009)
Lactobacillus acidophilus	0,004 – 0,128	(Koru et al., 2007)
Lactobacillus bulgaricus	0,600	(Kalogeropoulos et al., 2009)
Lactobacillus casei	0,300 – 0,600	(Kalogeropoulos et al., 2009)
	---	(Bruschi et al., 2006)
Lactobacillus fermentum	0,600 – 2,500	(Kalogeropoulos et al., 2009)
Lactobacillus helveticus	0,150 – 0,600	(Kalogeropoulos et al., 2009)
Leuconostoc mesenteroides	---	(Marcucci, 1996)

Lysteria monocytogenes	0,040 – 1,000	(Erkmen and Ozcan, 2008; Kalogeropoulos et al., 2009; Silici et al., 2007b; Stepanovic et al., 2003)
Micrococcus luteus	0,004 – 5,000	(Garedew et al., 2004; Uzel et al., 2005)
	---	(Assegid and Lamprecht, 1997; Farnesi et al., 2009)
Micrococcus lysodeikticus	---	(Marcucci, 1996)
Microsporum sp.	---	(Leite et al.; Marcucci, 1996)
Nocardia globerula	---	(Marcucci, 1996)
Paenibacillus larvae	---	(Antunez et al., 2008; Bastos et al., 2008; Fuselli et al.)
Peptococcus sp.	---	(Marcucci, 1996)
Peptostreptococcus sp.	---	(Koru et al., 2007; Marcucci, 1996)
Porphyromonas gingivalis	0,001 – 0,032	(Ferreira et al., 2007; Gebara et al., 2002; Koru et al., 2007)
	---	(Koo et al., 2000a; Santos et al., 2002)
Prevotella nigrescens	0,001 – 0,049	(Ferreira et al., 2007; Gebara et al., 2002)
	---	(Santos et al., 2002)
Propionibacterium sp.	---	(Boyanova et al., 2006; Marcucci, 1996)
Proteus vulgaris	---	(Dimov et al., 1992a; Marcucci, 1996)
Pseudomonas aeruginosa	0,032 – 7,100	(Jorge et al., 2008; Melliou et al., 2007; Mohammadzadeh et al., 2007a; Stepanovic et al., 2003; Uzel et al., 2005)
	---	(Drago et al., 2007; Garcia Bernal et al., 2007; Kumar et al., 2008; Marcucci et al., 2001; Muli and Maingi, 2007; Myung et al., 2009; Pavilonis et al., 2008; Pepeljnjak and Kosalec, 2004; Silici and Kutluca, 2005; Ugur and Arslan, 2004)
Salmonela typhimurium	0,032 – 5,000	(Kalogeropoulos et al., 2009; Stepanovic et al., 2003; Uzel et al., 2005)
	---	(Choi et al., 2006)
Sarcina lutea	---	(Marcucci, 1996)
Serratia marcescens	1,250 – 5,000	(Stepanovic et al., 2003)
	---	(Marcucci, 1996; Zampini et al.)
Shigella dysenteriae	2,500	(Kalogeropoulos et al., 2009)
Staphylococcus aureus	0,001 – 6,800	(Alencar et al., 2007; Castro et al., 2009; Fernandes et al., 2005; Gebara et al., 2002; Jorge et al., 2008; Kilic et al., 2005; Lu et al., 2005; Melliou et al., 2007; Miorin et al., 2003; Mohammadzadeh et al., 2007a; Salomao et al., 2008; Silici et al., 2007b; Stepanovic et al., 2003; Uzel et al., 2005)

	---	(Abd El Hady and Hegazi, 2002; Chaillou and Nazareno, 2009; Drago et al., 2007; Erkmen and Ozcan, 2008; Farnesi et al., 2009; Garcia Bernal et al., 2007; Gonsales et al., 2006; Hegazi and Abd El Hady, 2002; Hegazi et al., 2000b; Hegazi and Abd El Hardy, 2001; Kaneya et al., 2009; Kim et al., 2005; Koo et al., 2000a; Kumar et al., 2008; Lu et al., 2003; Marcucci et al., 2001; Muli and Maingi, 2007; Oksuz et al., 2005; Onlen et al., 2007a; Onlen et al., 2007b; Pavilonis et al., 2008; Pepeljnjak and Kosalec, 2004; Popova et al., 2001; Salomao et al., 2004; Sayed et al., 2009; Sforcin et al., 2000; Silici and Kutluca, 2005; Velazquez et al., 2007; Velikova et al., 2000; Yang et al., 2007)
Staphylococcus capitis	2,500	(Scazzocchio et al., 2006)
Staphylococcus epidermidis	0,008 – 7,200	(Kalogeropoulos et al., 2009; Melliou et al., 2007; Mohammadzadeh et al., 2007a; Scazzocchio et al., 2006; Stepanovic et al., 2003; Uzel et al., 2005)
	---	(Garcia Bernal et al., 2007; Popova et al., 2009; Yildirim et al.)
Staphylococcus haemolyticus	2,500	(Scazzocchio et al., 2006)
Staphylococcus hominis	2,500	(Scazzocchio et al., 2006)
Staphylococcus warnerii	2,500	(Scazzocchio et al., 2006)
Streprococcus viridans	2,500	(Scazzocchio et al., 2006)
	---	(Popova et al., 2009)
Streptococcus beta-haemolyticus	0,620	(Scazzocchio et al., 2006)
Streptococcus critecus	---	(Koo et al., 2000a)
Streptococcus faecalis	2,500	(Scazzocchio et al., 2006)
	---	(Bruschi et al., 2006; Koo et al., 2000a; Kosalec et al., 2005; Marcucci et al., 2001; Uzel et al., 2005)
Streptococcus mutans	0,008 – 0,064	(Castro et al., 2009; Hayacibara et al., 2005; Uzel et al., 2005)
	---	(Bruschi et al., 2006; Duarte et al., 2003; Duarte et al., 2006; Duran et al., 2007; Koo et al., 2000a; Koo et al., 2000b; Leitao et al., 2004; Park et al., 1998; Ugur and Arslan, 2004; Yang et al., 2007)
Streptococcus pneumoniae	0,002 – 1,250	(Salomao et al., 2008; Scazzocchio et al., 2006)
	---	(Kartal et al., 2003; Salomao et al., 2004; Speciale et al., 2006)
Streptococcus pyogenes	0,500 – 1,000	(Silici et al., 2007b)
	---	(Bosio et al., 2000; Drago et al., 2007; Kartal et al.,

		2003; Kosalec et al., 2005; Nieva Moreno et al., 1999; Speciale et al., 2006)
Streptococcus sanguis	---	(Bhattacharya et al.; Koo et al., 2000a; Nostro et al.)
Streptococcus sobrinus	0,002 – 0,050	(Castro et al., 2009; Hayacibara et al., 2005; Uzel et al., 2005)
	---	(Bruschi et al., 2006; Duarte et al., 2003; Duarte et al., 2006; Koo et al., 2000a; Koo et al., 2000b; Koo et al., 1999)
Trichophyton sp.	0,256 – 1,024	(Siqueira et al., 2009)
	---	(Heinze et al., 1998; Kim, 2008; Koc et al., 2005; Koc and Silic, 2008; Silici et al., 2007a)
Yersinia enterocolitica	1,250 – 2,500	(Kalogeropoulos et al., 2009; Stepanovic et al., 2003)
	---	(Erkmen and Ozcan, 2008)

4.2. Antiviral

Os estudos sobre a actividade antiviral são escassos. Marcucci (1996) cita que o vírus do herpes, na presença dos flavonóides crisina e campferol (isolados do própolis), sofre uma redução na replicação intracelular, directamente proporcional à concentração de própolis (Lustosa et al., 2008). Adicionalmente, verificou-se que a quercetina possui um efeito reduzido e que a acacetina e galangina não possuem qualquer efeito sobre o processo de replicação. Ainda no mesmo estudo, constatou-se que o própolis apresentava também efeito virucida contra poliovírus, reduzindo a taxa de replicação mil vezes (Marcucci, 1996), e actividade contra o vírus da estomatite vesicular e o adenovírus, embora de menor efeito (Lustosa et al., 2008). Estudos desenvolvidos na Ucrânia referem que o própolis possui um efeito superior na eliminação do vírus do herpes genital tipo 2 e na cicatrização das lesões, quando comparado com dois fármacos (Lustosa et al., 2008).

Ensaios *in vitro* e *in vivo* sugerem um alto potencial do própolis nas variantes X4 e R5 do HIV-1, actuando possivelmente na replicação do vírus e na sua entrada na célula (Gekker et al., 2005b; Ito et al., 2001; Lustosa et al., 2008). Estima-se que os componentes antiretrovirais do propolis sejam mais de 180 compostos, não se sabendo o número exacto (Burdock, 1998), sendo o grupo dos flavonóides o mais efectivo nesta actividade (Harish et al., 1997; Ito et al., 2001). Desta forma, o própolis constitui um produto natural não tóxico com efeito imunorregulador e anti-

HIV-1, que poderá ser usado em clínica após estudos mais aprofundados (Gekker et al., 2005a; Lustosa et al., 2008).

4.3. Antiprotozoárica

A actividade antiprotozoárica do própolis, até à actualidade, não foi alvo de muitos estudos. No entanto, segundo estudos realizados, o própolis tem efeito sobre a viabilidade de *Trichomonas vaginalis e Giardia lamblia* (Marcucci, 1996).

O própolis foi testado em parasitas do género *Leishmania*, que provoca uma patologia denominada Leishmaniose, caracterizada por úlceras cutâneas. Os testes laboratoriais realizados provaram um efeito superior do própolis, quando comparado com os resultados obtidos com o antibiótico Anfotericina B (Duran et al., 2008; Pontin et al., 2008). Estas observações podem resultar da activação do sistema imunitário pelo própolis (Orsi et al., 2005). Adicionalmente, o própolis evidenciou acelerar o processo de cura das lesões mais eficientemente que o fármaco Glucantime (Duran et al., 2008; Pontin et al., 2008). Pois a expansão destas lesões resulta geralmente do desenvolvimento bacteriano na lesão, as propriedades antimicrobianas do própolis podem constituir o mecanismo pelo qual a cura é mais rápida (Bankova et al., 2000).

Em amebas verificou-se que este produto natural é capaz de eliminar trofozoítos, mas não produz qualquer efeito nos cistos (Topalkara et al., 2007; Vural et al., 2007).

4.4. Antioxidante

Os radicais livres são compostos responsáveis pelo dano e envelhecimento celular e por diversas patologias como as doenças cardiovasculares, cancro, diabetes, artrite, doença de Parkinson e Alzeimer (Lustosa et al., 2008; Marquele et al., 2006; Mohammadzadeh et al., 2007b; Viuda-Martos et al., 2008). A sua ligação à etiologia destas patologias resulta da junção dos radicais livres com outros agentes oxidativos, o que possibilita a acção de toxinas no organismo humano

(Nagai et al., 2001). Embora o nosso organismo possua as suas próprias defesas contra estes radicais – enzimáticas (superóxido dismutase, catalase e glutationa oxidase) e não enzimáticas (vitaminas) – estas podem ser por vezes insuficientes devido às agressões sofridas pelo nosso organismo no quotidiano (Mohammadzadeh et al., 2007b).

Desta forma, o consumo de produtos com capacidade antioxidante, como o própolis, torna-se um importante factor na defesa do nosso organismo (Mohammadzadeh et al., 2007b). Durante os últimos anos, a procura de alimentos funcionais benéficos para a saúde Humana tem vindo a aumentar pelo consumidor (Viuda-Martos et al., 2008). Esta procura originou o aparecimento no mercado de bebidas e alimentos que previnem contra patologias do foro cardiovascular e imunitário (Bankova et al., 2000; Burdock, 1998; Mohammadzadeh et al., 2007b).

As diversas investigações realizadas tanto a nível Europeu como Americano, revelaram que o própolis é um poderoso antioxidante, com capacidades similares às da vitamina C (Bankova et al., 2000; Basnet et al., 1997; Kumar et al., 2008; Lustosa et al., 2008; Mohammadzadeh et al., 2007b). Esta eficácia está relacionada com o alto teor de polifenóis existentes no própolis, que bloqueiam os radicais livres (Choi et al., 2006; da Silva et al., 2006; Lustosa et al., 2008).

Outro campo de aplicação dos antioxidantes é na conservação de alimentos (Viuda-Martos et al., 2008). As reacções de oxidação em alimentos diminuem a sua vida útil, originando odores, sabores e alterações de cor, para além de alterar o seu valor nutricional (Viuda-Martos et al., 2008). Logo, o própolis poderá ser usado na indústria alimentar para a conservação e preservação das qualidades organolépticas e nutricionais dos alimentos.

4.5. Anti-inflamatória e Imunomodulatória

A inflamação é um processo complexo de resposta a estímulos prejudiciais (Sforcin, 2007). Entende-se como estímulo prejudicial qualquer agressão capaz de causar lesão celular ou tecidual, podendo ser de origem bacteriana, viral ou outra.

O dano celular causado pelo agente patogénico é responsável pela libertação de mediadores pro-inflamatórios (proteases do plasma, citocinas, mediadores lipídicos, entre outros) (Ramos and Miranda, 2007; Sforcin, 2007). Ocorre libertação de citocinas celulares (IL-1 e IL-6) que participam no processo de sinalização do local e de interacção celular. Quando os macrófagos são activados pelos antigénios, estes produzem novas citocinas (IL-1, IL-2, IL-4, TNF-α, IFN-γ, etc.), o que promove a vasodilatação, o aumento da permeabilidade vascular, o aumento da aderência das células e a migração de leucócitos do sangue para os tecidos por diapedese. Numa fase seguinte, os fosfolípidos membranares activam as fosfolipases intra e extra-celulares, que activam outras enzimas, como as ciclooxigenases (COX) e lipoxigenases (LOX) (Ramos and Miranda, 2007; Sud'ina et al., 2008; Venerito et al., 2008). Estas, por sua vez, actuam no processo metabólico de ácido araquidónico e de eicosanóides, originando prostaglandinas e leucotrienos, que mantêm o processo inflamatório (Girgin et al., 2009; Ramos and Miranda, 2007; Sforcin, 2007). Também as cininas, o complemento, as aminas vasoactivas e o óxido nítrico (NO) podem levar à inflamação (Mani et al., 2006; Ramos and Miranda, 2007; Sforcin et al., 2008).

Estas inflamações em alguns casos podem ser tratadas com medicamentos de actuação específica, mas estes podem produzir efeitos tóxicos indesejados no organismo (Ramos and Miranda, 2007), causados pela produção de radicais livres e NO (quando sintetizado em grandes quantidades). Estas moléculas degradam os lípidos das membranas, quebram as proteínas de membrana e podem induzir mutações no ADN (Ramos and Miranda, 2007; Sforcin, 2007).

O própolis é um conhecido anti-inflamatório de origem natural (Bankova et al., 2000), que não possui efeitos colaterais indesejáveis, contrariamente ao que se verifica com a generalidade dos anti-inflamatórios usados em clínica (Sforcin, 2007), podendo tornar-se uma mais-valia a sua utilização.

Até 1990, a informação na comunidade científica sobre a acção imunomodulatória do própolis era escassa, mas os estudos realizados na última década têm contribuído para a sua melhor compreensão (Sforcin, 2007).

Alguns autores sugerem que o propolis modula a imunidade inespecífica através da activação dos macrófagos (Dimov et al., 1991). Estudos realizados revelam que

o própolis estimula a produção de citocinas, como a IL-1 e TNF-α dos macrófagos na cavidade peritoneal de ratos (Sforcin, 2007). Este produto apícola também demonstrou ser capaz de modular *in vivo* e *in vitro* a produção de C1q pelos macrófagos, de alterar o funcionamento do receptor do complemento (Dimov et al., 1992b; Sforcin, 2007) e de inibir a via clássica e a via alternativa do sistema complemento (Ivanovska et al., 1995; Sforcin, 2007). Os flavonóides são referenciados como os principais agentes anti-complemento, sendo a molécula C3 um dos principais alvos de acção (Georgieva et al., 1997). Outros estudos revelam que os derivados do ácido cafeoilquinico são responsáveis pelas alterações nos macrófagos ao nível da mobilidade e propagação (Sforcin, 2007; Tatefuji et al., 1996). A exposição dos macrófagos a patogénios, como referido anteriormente, leva à produção de moléculas que produzem alterações no metabolismo celular, nomeadamente na produção de espécies reactivas de oxigénio (EROs) (O_2^-, $OH^\bullet$, OCl^- e H_2O_2), que representa um dos mecanismos pelo qual os macrófagos são microbicidas (Sforcin, 2007). As EROs para além de terem a função de destruir os patogénios fagocitados, podem destruir biomoléculas importantes, e consequentemente provocar lesão tecidual (Missima et al., 2007; Sforcin, 2007). Estudos realizados *in vitro* com o objectivo de estudar o efeito do própolis na produção de H_2O_2 evidenciaram que para concentrações entre 5 a 20 µg/mL de própolis existe um aumento na produção de H_2O_2 (Sforcin, 2007). Contudo, quando este efeito foi avaliado com dois compostos isolados do própolis (ácido cinâmico e ácido caféico) comprovou-se que os compostos em separado possuem um efeito diferente do própolis, assim sendo o ácido cinâmico inibe a produção de H_2O_2 e o ácido caféico estimula a sua produção (Krol et al., 1996; Sforcin, 2007), o que evidencia que as funcionalidades nutracêuticas do própolis variam com a origem do própolis, e consequentemente com a sua composição química. Em relação à produção de aniões superóxido, segundo um estudo realizado por Simoes (2004), concentrações que variaram entre 2 a 25 µg/mL o própolis inibiu a produção deste tipo de radicais livres. Contudo, embora estejam a ser desenvolvidos vastos trabalhos nesta área, ainda não se conseguiu perceber os mecanismos pelos quais o própolis inibe a produção de radicais livres pelos macrófagos (Cuesta et al., 2005).

O NO é outro dos indicadores da activação dos macrófagos, de extrema importância na função microbicida dos macrófagos, através da inibição da síntese

de ADN, da respiração mitocondrial e do transporte activo da membrana dos patogénios (Chan et al., 1992; MacMicking et al., 1997; Sforcin, 2007). Todavia, o NO possui outras funções no organismo humano, tais como vasodilatador, neurotransmissor e co-factor na reparação tecidular (Paulino et al., 2008). O própolis, a concentrações compreendidas entre 50 e 1000 µg/mL, tem a capacidade de inibir a produção de NO (Krol et al., 1996; Moriyama et al.; Paulino et al., 2008), sendo os flavonóides os principais responsáveis por esta actividade (Hu et al., 2005).

Com o objectivo de implementar a sua aplicação clínica, diversos estudos iniciaram a pesquisa sobre as plantas a partir das quais o própolis é produzido, no sentido de verificar se a eficácia dos extractos dessas plantas se assemelhava à do própolis, mas após a realização dos ensaios laboratoriais, verificou-se que o efeito é diferente, o que comprova que a acção do própolis deve-se ao sinergismo entre os compostos nele existentes e possivelmente também à fermentação dos compostos pelas enzimas salivares da abelha (Lopes et al., 2003).

As investigações sobre a actividade imunomodulatória do própolis sempre se limitaram à pesquisa da sua acção sobre os macrófagos, pensando-se que não interferia com os linfócitos (Dimov et al., 1991). No entanto, estudos realizados com flavonóides demonstraram que estes possuem efeito imunossupressor na linfoproliferação (You et al., 1998), desencadeando a investigação direccionada para a linfoproliferação com o própolis, uma vez que este é rico em flavonóides (Bankova et al., 1998). Estudos recentes revelam que o própolis possui efeito inibitório *in vitro* na proliferação de linfócitos (Sa-Nunes et al., 2003). Como já referido, o própolis tem a capacidade de pré-activar os macrófagos *in vivo* para produzirem NO. O NO segundo alguns autores pode ser o composto responsável pela inibição da linfoproliferação (Sforcin, 2007), uma vez que tanto a activação dos linfócitos como a produção de NO está dependente do IFN-γ e, este aumenta na presença de própolis e Con A (Sa-Nunes et al., 2003). O própolis é capaz de inibir a produção de IL-2, IL-4, IL-10 e IL-12, e estimular a produção de TGF-β1 (Sforcin, 2007). TGF-β1 é uma citocina com alta influência na divisão celular e efeito inibitório de outras citocinas, o que poderá explicar o decréscimo de algumas interleucinas (Sforcin, 2007). A IL-2 está envolvida na diferenciação dos linfócitos

T em linfócitos T auxiliares, e como o própolis inibe este tipo de interleucina, a diferenciação é impedida (Ansorge et al., 2003). Outros estudos sugerem que a administração de própolis a uma concentração de 200 mg/kg por 14 dias em ratos tem a capacidade de inibir a IL-1β, IL-6, IFN-γ, IL-2 e IL-10 em células do baço, o que pode ser uma mais-valia na utilização do própolis como anti-inflamatório, pois a maioria destas citocinas estão envolvidas nas doenças inflamatórias crónicas (Sforcin et al., 2008).

O própolis possui em grande efeito sobre as células envolvidas na resposta inata (Orsi et al., 2005), sobretudo na produção de anticorpos, em que a administração de própolis a 10% mostrou ao final de 15 dias ser suficiente para aumentar a produção de anticorpos em ratos (Sforcin et al., 2005). Segundo este investigador, a quercetina e o ácido caféico isolados do própolis não possuem efeito sobre a produção de anticorpos, o que corrobora a hipótese do efeito do própolis resultar do efeito sinergístico dos seus constituintes.

4.6. Antitumoral

O própolis é um agente antitumoral, pela sua capacidade antiproliferativa das células tumorais, tanto *in vitro* como *in vivo* (Banskota et al., 2001; Chen et al., 1996; Kim et al., 2008; Rao et al., 1992).

As investigações que têm vindo a ser desenvolvidas nesta área possibilitaram a identificação de alguns compostos envolvidos nesta actividade, como: PMS-1 (Matsuno, 1995); PRF-1 (Matsuno et al., 1997a); Artepillin C (ácido 3,5-difenil-4-hidroxicinámico) (Kimoto et al., 1998; Matsuno et al., 1997b); ésteres de ácido caféico (Lee et al., 2003; Liao et al.); Plukenetione A (Diaz-Carballo et al., 2008a; Diaz-Carballo et al., 2008b).

O composto PMS-1 foi isolado por Matsuno (1995) no própolis Brasileiro e identificado como sendo um diterpeno clerodano. O PMS-1 inibe o crescimento das células tumorais do fígado, por estagnação das células tumorais na fase S do crescimento (Matsuno, 1995; Sforcin, 2007).

Em 1997, a mesma equipa de investigação descobriu um novo composto denominado PRF-1, isolado a partir do extracto aquoso de própolis, demonstrando ser antioxidante e citotóxico para as células do carcinoma hepatocelular (Matsuno et al., 1997a).

O Artepillin C isolado do própolis Brasileiro evidenciou actividade antitumoral, que está relacionada com a fragmentação do ADN e consequente o desencadeamento da apoptose (Matsuno et al., 1997b), diminuindo o crescimento tumoral. Testes realizados *in vivo* demonstraram que este composto também estimula o sistema imune, por aumento da razão das células T CD4/CD8 (Kimoto et al., 1998).

Os ésteres de ácido caféico, genericamente denominados CAPE, aumentam a fosforilação e expressão de p53 e Bax, que podem acelerar o processo de apoptose (Lee et al., 2003). Outros estudos relatam que a acção deste composto resulta da indução pela via das caspases da apoptose (Aso et al., 2004). O tratamento *in vivo* com este composto mostrou reduzir significativamente o número de células mitóticas e de células proliferativas (Kuo et al., 2006).

A plukenetiona A (PA) foi referenciada pela primeira vez em 1996 (Henry et al., 1996), como contendo propriedades antitumorais. Estudos mais recentes realizados com o própolis Cubano revelaram que a actividade anti-metastática desse produto deve-se em grande parte à presença de PA na sua constituição (Diaz-Carballo et al., 2008b). Este composto provou ser eficaz contra uma grande linha de células de várias origens tumorais, além disso, não se verificou qualquer resistência dessas linhas celulares a este composto (Diaz-Carballo et al., 2008b). Os alvos celulares segundo este estudo são a topoisomerase I e a ADN polimerase, alterando desta forma o ciclo celular da célula e consequentemente a multiplicação celular. Os resultados deste estudo mostram serem promissores no campo da aplicação clínica destes conhecimentos ao nível da quimioterapia.

No capítulo II apresenta-se o trabalho sobre a actividade antioxidante e quantificação dos compostos fenólicos no própolis Português. No capítulo III, o efeito do própolis na membrana dos eritrócitos de pacientes com esferocitose hereditária.

Finalmente, no último capítulo fazem-se as considerações finais sobre os trabalhos efectuados bem como os resultados obtidos mais relevantes.

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CAPÍTULO II

Antioxidant properties, total phenolics and polinic analysis of propolis samples from Portugal

Food and Chemical Toxicology, **46** (2008) 3482–3485

Antioxidant properties, total phenols and polinic analysis of propolis samples from Portugal

ABSTRACT

Polinic analysis, total phenols content and antioxidant activity were studied for the first time in Portuguese samples of propolis from Bornes and Fundão region. Total phenols content were determined by colorimetric assay and their amount was of 329 mg/g of GAE, in Bornes sample, and 151 mg/g of GAE in Fundão propolis. The antioxidant capacity of propolis extracts was assessed through the scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl) and reducing power assay. A concentration-dependent antioxidative capacity was verified in DPPH and reducing power assays, with very low EC_{50} on DPPH scavenging assay, of 6.22 µg/mL (Bornes propolis) and 52.00 µg/mL (Fundão propolis) and for reducing power the values were 9.00 µg/mL, for Bornes propolis, and 55.00 µg/mL, for Fundão propolis. The high activity of propolis from Bornes could be related with their different polinic composition. The results obtained indicate that Portuguese propolis is an important source of phenols and antioxidative compounds that prove the high beneficial effects of propolis for human health.

Keywords: Portugal, propolis, polinic analysis, total phenols, antioxidant activity.

1. INTRODUCTION

Propolis is a product based on resins collected from resinous sprouts and exudates of some plants by bees of *Apis mellifera* specie. When the bees reap the propolis, they mix the resinous substance collected from plants with the 13-glicosidase enzyme of their saliva, causing the hydrolysis of the glucosyl flavonoids, originating flavonoids agliconas (Pereira *et al*., 2002). In the beehive, the propolis is used by the bees to defend them from the invaders (causing the death of those from asphyxia) and promotes the conservation of their bodies, protecting the beehive from the resultant plagues of putrefaction. Another propolis function is the thermal isolation of the beehive, serving to fill eventual cracks or apertures that can appear (Bankova *et al*., 2002).

In the past few years, the suspected toxicity of some synthetic compounds used in food has raised the interest in natural products (Stone *et al.*, 2003). Some industries, such as those related to food additive production, cosmetics, and pharmaceuticals, have increased their efforts in obtaining bioactive compounds from natural products by extraction and purification. Antioxidant compounds can increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products during processing and storage (Halliwell, 1997; Halliwell and Gutteridge, 1999).

Propolis has been used in the traditional medicine since the primordial times of humanity, having acquired popularity between Egyptians Arabs, Greeks, and many other civilizations (Abd El Hady and Hegazi, 2002). In the present moment, from all the apicultural products, the most increase is verified in propolis. The main reasons of the increasing interest are associated to their therapeutic properties. In fact, different works attribute important properties to propolis, namely antibacterial action against different pathogenic bacteria (Kujumgiev *et al*., 1999), antifungal and anti-inflammatory (Wang *et al*., 1993), anti-viral (Amoros *et al*., 1994), curative, anesthetical and anti-tumoural properties (Kimoto *et al*., 2001; Matsuno, 1995). Recently, Kim *et al.* (2005) showed that propolis is able to inhibit the action the enzyme hyaluronidase, allowing slow the aging of cells. For all these reasons, this natural product awakened interest in the pharmaceutical industry, mainly in Asian countries, being propolis introduced in different products for human consumption like drinks, foods and cosmetics (Pereira *et*

al., 2002). Different authors have been attributed the biological properties to the phenolic composition (Lahouel *et al.*, 2004).

Some studies were developed about propolis characterization, their biological properties and the action of their constitution. No previous works were reported in Portugal about this hive product. In this work, and for the first time, the Portuguese propolis, from two different regions, were studied regarding their total phenols content, polinic characterization and antioxidant activities. Antioxidant potential was accessed by the reducing power assay, and the scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals.

2. MATERIALS AND METHODS

2.1. Samples

Two different samples of propolis were analysed. Bornes sample proceed from Serra de Bornes in the Northeast of Portugal and Fundão sample proceed from the Beira Interior Region in the Centre of Portugal (Figure 1). Between regions, marked differences were registered in terms of climatic conditions and vegetation.

Figure 1. Localization of the different region of sample origin (Bornes and Fundão).

2.2. Reagents

Absolute alcohol and 1.1-diphenyl-2-picryl-hydrazyl (DPPH) were obtained from Sigma-Aldrich (Germany). Methanol HPLC grade was obtained from Pronolab (Lisboa, Portugal). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Sample preparation

Samples were prepared by mixing propolis with methanol (1:1 v/v) and were leave in agitation *over-night*. After this step, the obtained solution was filtered through Whatman nº 4 paper for separate the solid trashes. The residue was then extracted with methanol two times, as described earlier. The combined methanolic extracts were frozen. After 12 h the extract was filtered to eliminate waxes. The methanol was evaporated with a rotary evaporator. The extracts were redissolved in the corresponding solvent at a concentration of 50 mg/mL, and analysed for their content in phenols.

2.4. Polinic analyse

The polinic analyse was executed by the methodology described by Barth *et al.* (1999). The pollen attainment was initiated by the extraction of 0.5 grams of propolis with 15mL of absolute alcohol during at least 24 h. The sediment gotten after the centrifugalization was boiled during 2 minutes in KOH 10% in bath-water, washed in distilled water, filtered, remaining in acid ascetic glacial during a night. After that, it was submitted to the mixture of acetolise (oxidation sediment in a mixture 9:1 of ascetic and sulphuric acid anhydride in bath-water until reaching the temperature of 80°C for about three minutes). After careful laundering in water and glycerinate-water, prepared the sediment on two buckets of microscopy in gelatine-glycerinate, with or without courante (fuchsine basic), forbidding itself with paraffin.

2.5. Determination of total phenol content

Total phenolic content in the methanolic extract of the different propolis were estimated by a colorimetric assay based on procedures described by Kumazawa *et al*.,

2002 and Singleton *et al*., 1999 with some modifications. Methanolic extract solution (0.5 mL) was mixed with 0.5 mL of the Folin-Ciocalteau reagent and 0.5 mL of 10% Na_2CO_3, and the absorbance was measured at 700nm (Unicam UV-Visible Spectrometry Heλios, United Kingdom). The reaction was kept in the dark at room temperature for 1 h, after which the absorbance was read at 700 nm. Methanolic extract samples were evaluated at the final concentration of 20 mg/mL. Gallic acid was used for constructing the standard curve. Total phenols content were expressed as mg of gallic acid equivalents/g of extract (GAEs).

2.6. Scavenging of DPPH radicals

The scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was assayed following the method of Hatano *et al.* (1989). One millilitre of the extract solution was dissolved in MeOH with 1:1 v/v of DPPH solution (0.1 mM). The mixture was shaken vigorously and left to stand for 50 min in the dark at room temperature (until stable absorbance values were obtained).

The reduction of the DPPH-radical was measured by continuous monitoring of the decrease of absorption at 517 nm (Unicam UV-Visible Spectrometry Heλios, United Kingdom). DPPH scavenging effect was calculated as a percentage of DPPH discolouration using the equation: % scavenging effect = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration in the solution.

2.7. Scavenging of Reducing Power

The reducing power was determined according to the method described by Shi and Dalal (1991). The propolis extract (2.5 mL) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 10 mg/mL potassium ferricyanide.

The mixture was incubated at 50 ºC for 20 min. After 2.5 mL of 100 mg/mL trichloroacetic acid (v/v) were added, the mixture was centrifuged at 650 g for 10 min

(Eppendorf centrifuge 5810R, Germany). The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 1.0 mg/mL of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm (higher absorbance indicates higher reducing power) in an spectrophotomer (Unicam UV-Visible Spectrometry Heλios, United Kingdom). Extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration in the solution.

3. RESULTS AND DISCUSSION

3.1. Polinic analysis

The polinic analysis of the different propolis samples were presented in Table 1. Marked differences were observed between samples. *Castanea sativa* was the dominant pollen in Bornes propolis, that represents 45% of the total, and it is absent in Fundão propolis. On the other side, *Populus tremula* was the main constitute of Fundão (50%) and represents 30% in Bornes propolis. *Pinus sp.* only was observed in Fundão sample (Table 1).

Table 1. Polinic composition (%) of different propolis samples.

	Polinic analysis (%)	
Species	Bornes	Fundão
Populus tremula	30	50
Castanea sativa	45	0
Pinus sp.	0	15
Others	25	35

3.2. Total phenolic contents

The total phenols content in propolis extracts was different according to the provenience region (Table 2). Bornes propolis showed the high amount of these compounds, with 329.00 mg/g of GAE, being that was approximately two fold higher than Fundão propolis (151.00 mg/g of GAE).

Table 2. Scavenging effect and reducing power EC_{50} values (mg/mL), and total phenol content (mg/g) of propolis extracts from different regions.

Propolis	DPPH antioxidant activity (mg/mL)	Reducing power (mg/mL)	Total phenolics (GAE) (mg/g)
Bornes	0.006 ± 0.003	0.009 ± 0.001	329.00 ± 0.0005
Fundão	0.052 ± 0.003	0.055 ± 0.001	151.00 ± 0.0005

Propolis is commercialized in different parts of the world and it is recognized as an important source of compounds with pharmacological properties (Fujimoto, 1992; Miyataka *et al.*, 1997). Bornes samples showed the highest values of total phenols in comparison to the propolis samples from other countries. The amounts of total phenols in other regions were also high in Chinese samples from Hebei, 302±4.3mg/g of GAE (Ahn *et al.*, 2007), and Hubei, 299±0.5mg/g of GAE (Kumazawa *et al.*, 2004); and Korean propolis from Yeosu, with 212.7±7.4mg/g of GAE (Choi *et al.*, 2006). In our work Fundão samples, from the centre land of Portugal, showed approximately an half part (151 mg/g of GAE) of Bornes samples, but their amount were also higher than the propolis from Brazil, with 120±3.5mg/g of GAE (Choi *et al.*, 2006), and Thailand, with 31.2±0.7mg/g of GAE (Kumazawa *et al.*, 2004).

Some studies were developed concerning the phenolic composition of propolis samples (Marcucci and Bankova, 1999; Tazawa *et al.*, 2000). The compounds reported by different authors are caffeic acid; p-coumaric acid; 3,4-dimethoxycinnamic acid; quercetin; pinobanksin 5-methyl ether; apigenin; kaempferol; pinobanksin; cinnamylideneacetic acid; chrysin; pinocembrin; galangin; pinobanksin 3-acetate; phenethyl caffeate; cinnamyl caffeate; tectochrysin; artepillin C (Marcucci and Bankova, 1999; Medic-Saric *et al.*, 2004; Kumazawa *et al.*, 2004).

3.3. Scavenging of DPPH radicals

The scavenging activity on DPPH radicals has been widely used to determine the free radical-scavenging activity of different matrices by our research group (Pereira *et al.*, 2006; Sousa *et al.*, 2008; Oliveira *et al.*, 2007, 2008). DPPH is a stable free radical which dissolves in methanol, and its purple colour shows a characteristic absorption at

517 nm. Antioxidant molecules scavenge the free radical by hydrogen donation, the colour from the DPPH assay solution becomes light yellow resulting in a decrease in absorbance at 517 nm. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation (Hatano *et al.*, 1989). In this assay, results are expressed as the ratio percentage of the absorbance decrease of DPPH radical solution in the presence of extract at 517 nm to the absorbance of DPPH radical solution at the same wave length.

In this work the pattern of DPPH radicals inhibition showed a concentration-dependent pattern for both samples propolis (Figure 2). Propolis from Bornes presents a very high scavenging activity at very low extract concentration, at 0.001 mg/mL of propolis extract the scavenging activity was 33% and at 0.020 mg/mL reaching 94% of scavenging activity. However from Fundão propolis the activity was low. These propolis only showed a few scavenging at 0.007 mg/mL, with 1%, when Bornes propolis scavenge 57%. The same situation was observed at 0.020 mg/mL when Fundão extract propolis scavenge 18% (Figura 2).

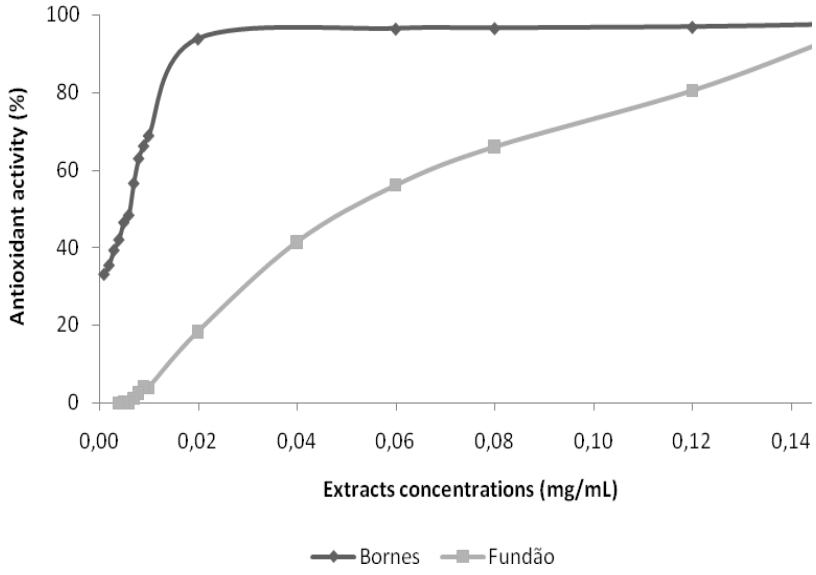

Figure 2. Scavenging effect of propolis from Bornes and Fundão extracts.

The EC_{50} value was very low that revelled the high antioxidant activity of the propolis extract. The EC_{50} value for Bornes extract was 0.006 mg/mL while for Fundão extract was 0.052 mg/mL, approximately 10-fold more (Table 2). In our work the activity of the extracts was in accordance with the total phenols contents. Bornes

samples presented a very high concentration of phenols and also a strong scavenging activity against DPPH radicals. The total phenols content was considered the main responsible from the antioxidant activity of different extracts (Kumazawa *et al.*, 2004). In fact, Pereira *et al*. (2008) and Sousa *et al.* (2008) proved that the EC_{50} values were statistically correlated with total phenols content in the analysed samples.

3.4. Scavenging of Reducing Power

Propolis extracts revealed a strong reducing power. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) monitored at 700 nm (Sousa *et al*., 2008). In this assay, the yellow colour of the test solution changes to green depending on the reducing power of the test specimen. In both analysed samples, a concentration-dependent activity was observed (Figure 3).

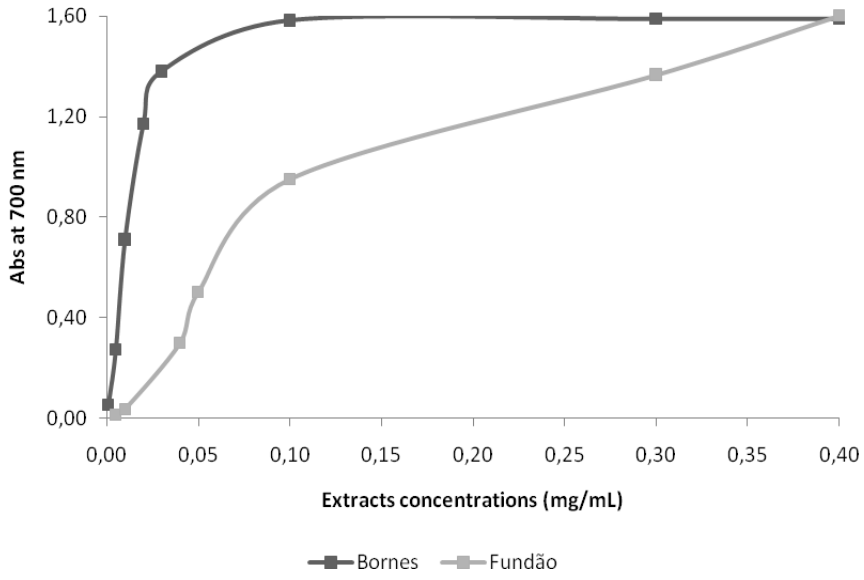

Figure 3. Reducing power values of of propolis from Bornes and Fundão extracts.

When extract concentration increase the absorbance at 700 nm also increase. At very low extract concentrations, for example 0.1 mg/mL high values of absorvence were observed, namely 0.950 for Fundão propolis and 1.582 for Bornes sample. The results obtained in our work were much better than the results obtained in Brazilian propolis by

(Wang *et al.*, 2004). These authors for the same extract concentration obtained approximately an half of activity, when we compare their results with Fundão propolis, and approximately 30% of the activity of Bornes propolis.

In the present work EC_{50} values obtained for methanol extracts were of 0.009 mg/mL, for Bornes propolis, and 0.055 mg/mL to the Fundão propolis. In general, extracts with high total phenols content presented lower EC_{50} values in reducing power assay and in the order (Table 2).

The results obtained in our work permit to precede a one preliminary polinic characterization of Portuguese propolis. We demonstrate also the high concentration in total phenols and a strong antioxidant activity of this natural product.

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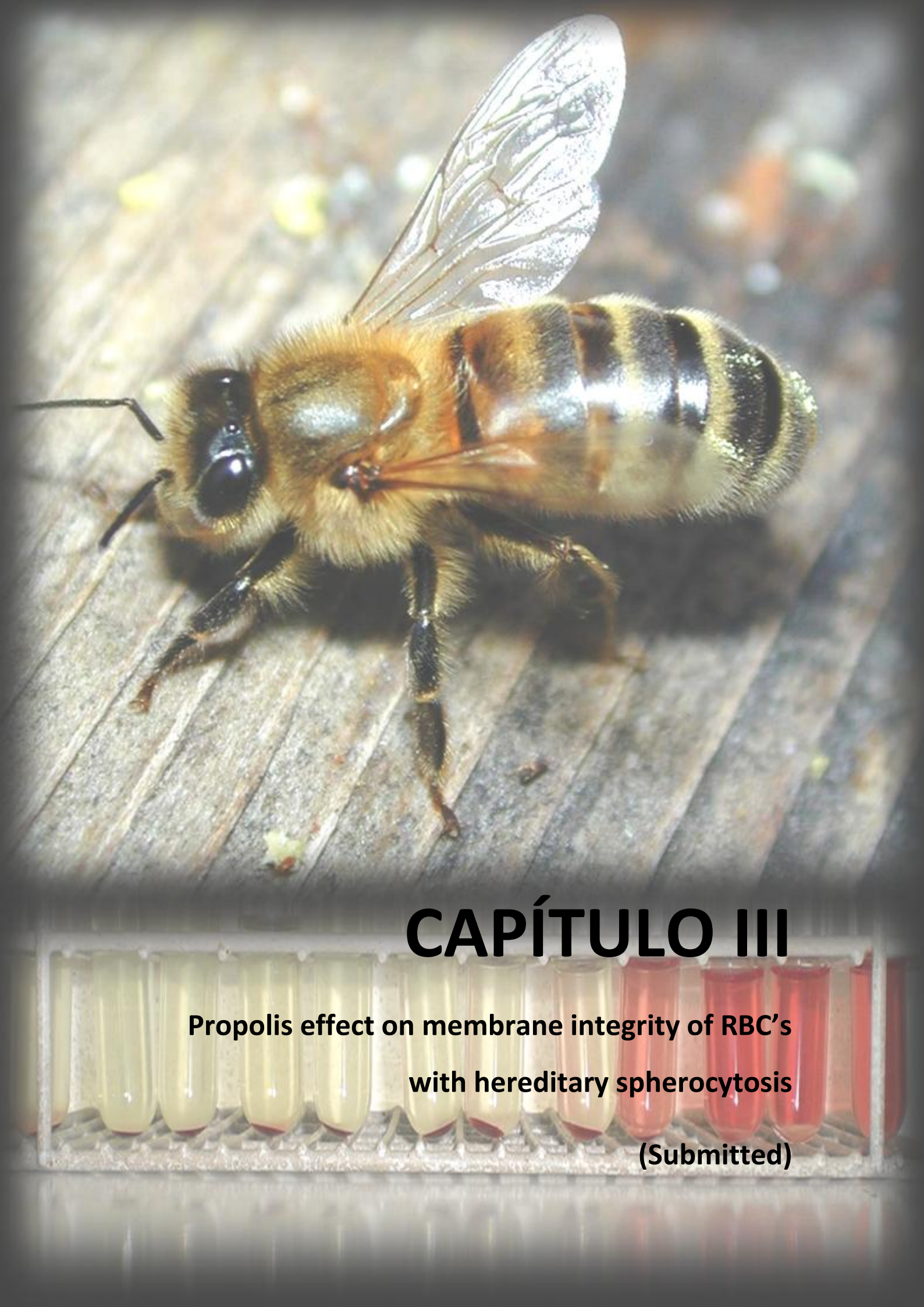

CAPÍTULO III

Propolis effect on membrane integrity of RBC's with hereditary spherocytosis

(Submitted)

Propolis effect on membrane integrity of RBC's with hereditary spherocytosis

ABSTRACT

Propolis is a resinous substance collected from plants by bees. The propolis composition depends on the surrounding vegetation, the season, and the area from which derived. This hive product usually contains a high variety of chemical compounds such as polyphenols (flavonoids, phenolic acids and esters), steroids and amino acids, and it's used since Egyptian Civilization.

The hereditary spherocytosis (HS) is a type of transmission of hereditary anaemia that results from the presence of microcytic and hyperchromic red cells, spherical and without central pallor. Clinically, subjects usually present from asymptomatic conditions to severe haemolytic anaemia.

The objective of this study was to evaluate the effect of two propolis extracts, from different regions, in the osmotic fragility of patient red blood cell (RBC) membrane with HS. It was found that propolis decrease the erythrocytes membrane fragility, being the effect of Bornes propolis more pronounced comparatively to Fundão propolis. The obtained results suggest that *in vitro*, the membrane fragility may be increased under oxidative stress conditions in patient RBC's, and the protection effect of propolis is due to its antioxidant properties. These results open doors for future investigations in order to elucidate the mechanisms, and identify the most relevant compounds involved in the fragility of the erythrocyte membrane.

Keywords: propolis, hereditary spherocytosis, osmotic fragility, erythrocyte membrane.

1. INTRODUCTION

Propolis is a beehive product, produced by bees of *Appis mellifera* specie from the treatment of resinous substances collected from various plants and mixed with β-glicosidase enzyme of their saliva (Moreira et al., 2008), being partially digested and added to bee wax before being used. Bees use it to seal holes on the beehive walls, and to protect themselves from invaders by involving them on propolis. The invaders die from asphyxia and their bodies are preserved, preventing the putrefaction of the honeycomb (Bankova et al., 2002).

As a natural substance, it's been used since the primordial times by ancient civilizations as Egyptians, Arabs, Greeks, and many others, particularly on traditional medicine due to its therapeutic properties (Abd-El Hady et al., 2002). Nowadays, there is an increasing interest on beehive products, particularly on propolis, mostly because of its application on health care, cosmetics and food addictive production that has been demonstrated by different studies (Krell, 1996). The health care applications of propolis are a particularly important area because it's been shown in various studies, different activities, as anti-inflammatory (Wang et al., 1993), antibacterial (Kujumgiev et al., 1999), antifungal (Wang et al., 1993), anti-viral (Amoros et al., 1994), curative, anesthetical, anti-tumour (Kimoto et al., 2001; Matsuno, 1995) and antioxidant properties (Moreira et al., 2008). Researchers claim efficiency on wound healing, tissue regeneration, treatment of burns, psoriasis, herpes simplex and genitalis, rheumatism, sprains, dental medicine and immune system support and improvement (Burdock, 1998).

The hereditary spherocytosis (HS) is a congenital haemolytic anaemia, with origin in the modification of membrane proteins of erythrocytes, which leads to increased susceptibility to hemolysis and a decrease of the cell over-life (Favero et al., 2003). The HS is the most common red blood cell (RBC) membrane disorder in European Caucasians, with a prevalence of roughly 200-300 per 10^6, and to Japanese population 5.7-20.3 per 10^6 (Satchwell et al., 2009; Orcutt et al., 1995; Granjo et al., 2003).

The HS is caused by a defect in the erythrocyte membrane, resulting in instability of the cell cytoskeleton. On a smear from peripheral blood, it's possible to confirm the erythrocyte morphology – microspherocytosis – when the surface loses its integrity, the erythrocyte acquire a spherical form, and smaller size. Consequently, these cells are

quickly kidnapped by the movement of the spleen, the production of erythrocytes in bone marrow is increased, and there is an augmentation in circulating reticulocytes level. There are four types of abnormalities in the erythrocyte membrane protein that can cause the HS: deficiency of spectrin, spectrin deficiency associated with anquirin, the band 3 deficiency or defects in protein 4.2. A deficiency of spectrin is the most common cause (Granjo et al., 2003).

The identification of the presence of microspherocytes is definitively diagnostic to HS, because it is not difficult to identify the characteristic cells on blood film, and it's a quick procedure. However, the osmotic fragility test is the most critical one. This test measure the *in vitro* lysis of RBCs suspended in solutions of decreasing osmolarity (Tracy et al., 2008).

The work aims were: to assess the effect of propolis extract in the osmotic fragility of patients with HS, with and without oxidation stress conditions; to compare two different propolis effects.

This is a very important work, since it's the first time that it was studied the use of Portuguese propolis in hematologic diseases. These results and the information provided can be used for future investigations in order to elucidate the mechanisms by which propolis affect the fragility of the erythrocyte membrane.

2. MATERIALS AND METHODS

2.1. Propolis samples preparation

The origin of two propolis samples was Bornes - Trás-os-Montes and Fundão - Beira Interior (Portugal).

Samples were prepared by mixing propolis with methanol (1:1 v/v) and were left in agitation *over-night*. After this step, the obtained solution was filtered through Whatman nº 4 paper for separate the solid trashes. The residue was then extracted with methanol twice, as described earlier. The combined methanolic extracts were frozen, during 12h, followed by a filtration to eliminate waxes. The methanol was evaporated with a rotary evaporator, and the extracts were re-dissolved in DMSO (50%).

2.2. Patient

The patient is a 22-years-old male, from Bragança, Portugal, was born healthy, with non-consanguineous parents and no known family history of anaemia. His clinical history includes: an intravascular hemolysis at 9-months-old which required hospitalization; at 12-months-old was diagnosed a hereditary spherocytosis; at 18-months-old he had a severe anaemia (4.2 g/dL) that required two erythrocytes transfusion; at 2-years-old the patient was splenectomized and its gallbladder was also removed, and since then, the patient take oral penicillin twice a day. Except for HS, he had a normal health and development.

2.3. Peripheral blood smear and Wright's stain

Peripheral blood smear (PBS) is made by placing a drop of blood on one end of a slide, and using another slide to disperse the blood over the slide's length. The aim is to get a region where the cells are spaced far enough apart to be analyzed the erythrocytes morphology.

When the PBS is dry, we proceeded to Wright's staining. For this purpose was placed 1mL of Wright's stain solution in the PBS. After 3 min, 2mL of distilled water was added for the same time of the first step. PBS was washed with distilled water until the edges show faintly pinkish-red. Finely, the PBS was carefully dried, and analyzed by microscopy.

2.4. Saline solutions preparation

In order to prepare the saline solutions it was made a stock solution with 100g of NaCl, 13.65g of Na2HPO4, 2.34g of NaH2PO4.2H2O, and 1L of deionized water. Later, from the stock solution were prepared the following concentrations (g/L): 0.0; 1.0; 2.0; 3.0; 3.5; 4.0; 4.5; 5.0; 5.5; 6.0; 6.5; 7.0; 8.0; 9.0; 10.0.

2.5. RBC membrane integrity evaluation – Osmotic fragility test (OFT)

2.5.1. Propolis influence on OFT

With the aim to evaluate the influence of propolis on OFT, RBCs was exposed to decreasing concentrations of hypotonic saline solutions, in the propolis presence.

In order to evaluate the OFT it was used heparinised blood of patient and control subjects. The test was done immediately, inside the recommended interval of two hours, when the sample was at room temperature.

Heparinised blood was exposed for 10 min to propolis samples solution, and was washed twice with a physiological saline solution. After this step, 50µL of RBCs was placed in series of tubes of decreasing concentrations (g/L) of sodium chloride solutions (0.0; 1.0; 2.0; 3.0; 3.5; 4.0; 4.5; 5.0; 5.5; 6.0; 6.5; 7.0; 8.0; 9.0; 10.0) and incubated at room temperature for 30 min. The tubes were carefully shacked and the haemolysis was quantificated at 540nm, using the tube with 10.0g/L NaCl as blank. Haemolysis percentage was calculated considering the tube with 0.0g/L NaCl, 100% of hemolysis, and was represented through a graph that related the concentration of saline solution with the degree of hemolysis.

2.5.2. Oxidation effect on OFT

Oxidation effect on osmotic fragility was made using hydrogen peroxide as oxidant agent. In this assay was evaluated the osmotic fragility in oxidative stress conditions, of erythrocytes treated/untreated with propolis, with periferical blood of HS patient. The procedure was similar to the osmotic fragility test but modified, in order to include 1mM hydrogen peroxide.

2.6. Chelating activity

The chelating activity of propolis extracts was measured as reported by Topçu et al. (2007).

Each sample (1mL) was mixed with 3.7 mL of deionized water, and then was reacted with 0.1 mL of $FeCl_2$ 2 mM and 0.2 mL of ferrozine 5 mM. The absorvance was determined spectrophotometrically at 562 nm.

Chelating activity of propolis samples on Fe^{2+} was calculated using the following formula:

Chelating activity (%) = [1 – (absorbance of sample) / (absorbance of control)] x 100

2.7. Statistical analysis

It was performed a statistical analysis (Statistical Package for Social Sciences v17) by one-way analysis of variance (ANOVA) followed by Tukey's Test, to verify which variables are significantly different, with α=0.05.

3. RESULTS AND DISCUSSION

3.1. Confirmation of hereditary spherocytosis

In this work two adults were studied, one with the syndrome HS splenectomized and one healthy used as control. Diagnosis of HS was made by clinical features, identification of spherocytes on peripheral blood smears and abnormal osmotic fragility.

In microscopic observation of peripheral blood smears of the subject with HS was observed the presence of microspherocytes, that appears smaller and spherical, more intensely haemoglobinised without a central pallor (Figure 1).

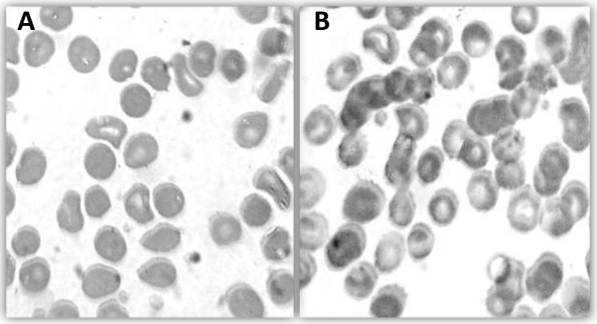

Figure 1. Microscopic observation of blood film with Wright coloration (1000x). A - Subject with HS spherocytes (arrows), characterized by a lack of central pallor, occur in hereditary spherocytosis; B - Control subject.

Thus, the microscopic observation confirmed the clinical diagnostic. Amongst the various laboratory procedures for diagnosing HS, the osmotic fragility test is the most critical one (Rocha et al., 2009). The erythrocyte is characterized by a biconcave shape giving it an excess of surface area in relationship to its volume. The osmotic fragility test evaluates the relationship of the erythrocyte's surface area to its volume. When

there is a decrease in the surface area to the cell volume (as in the spherocyte), the osmotic fragility is increased. As a result, these spherocytes are more sensitive to hemolysis than normal RBCs when they are suspended in hypotonic NaCl solutions. The amount of haemoglobin, which escapes into supernatant, is determined spectrophotometrically to estimate the osmotic fragility of the red cell membrane.

Regarding the results obtained in osmotic fragility test, the HS patients under investigation was classified as presenting typical HS, since, that the hemolysis of RBCs started at the concentration of 5.5g/L of NaCl.

3.2. Propolis effect in erythrocyte membrane integrity

The aim of the present work was to determine if propolis extracts could affect the red cell membrane integrity and comparing the effect of two propolis extracts from different regions (Bornes - Trás-os-Montes; Fundão - Beira Interior). In order to evaluate the effect of propolis extract in membrane integrity, we performed in vitro assays in which propolis extract was added before RBCs are submitted to osmotic fragility test, as described in material and methods. The results show, the erythrocyte membrane fragility in both subjects were affected by two propolis extracts, as we can verify in the curve a dislocation when the test was performed in the propolis presence (Figure 2, 3 and 4).

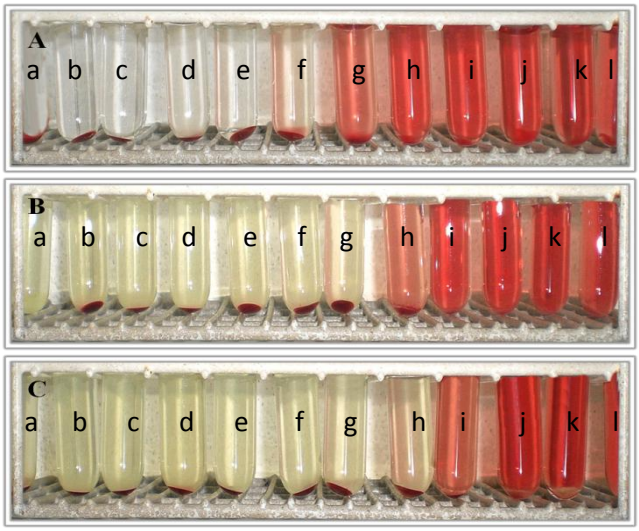

Figure 2. Osmotic fragility test results: A – control; B – with Fundão propolis; C – with Bornes propolis. NaCl concentrations in buffer: a-10.0g/L; b-9.0g/L; c-7.5g/L; d-6.5g/L; e-6.0g/L; f-5.5g/L; g-5.0g/L; h-4.0g/L; i-3.5g/L; j-3.0g/L; k-2.0g/L; l-1.0g/L.

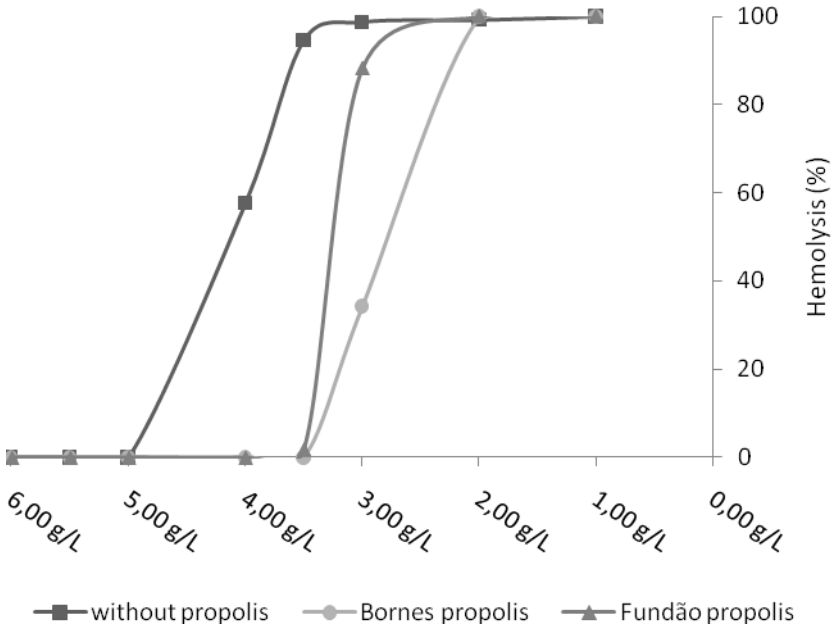

Figure 3. Osmotic fragility curves of control subject, with and without exposure to propolis.

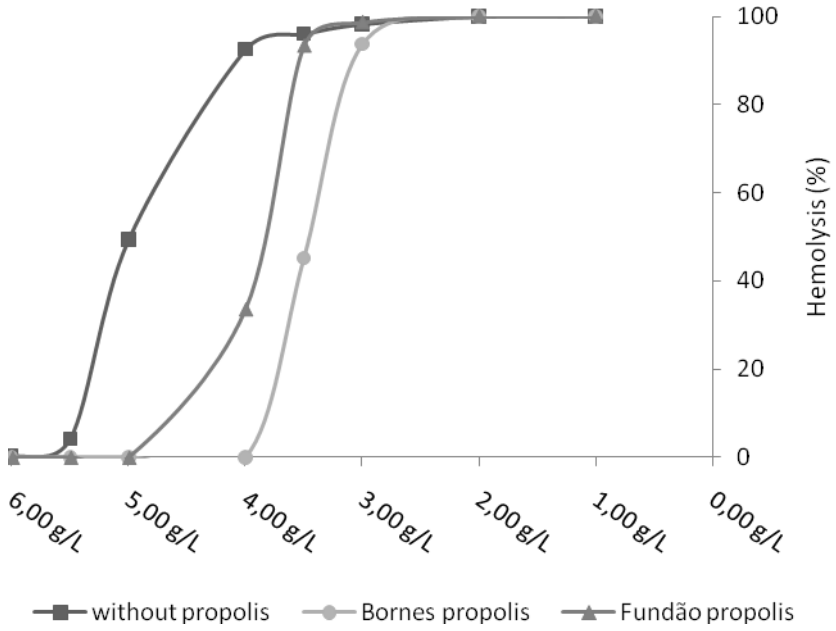

Figure 4. Osmotic fragility curves of subject with HS, with and without exposure to propolis.

In control subject, the haemolysis without propolis addition started at concentration of 5g/L NaCl buffer solution and with the addition of both propolis extracts started at 3,5g/L. However, differences were observed between the propolis samples: 80% of hemolysis (Fundão propolis), and 30% (Bornes propolis), at 3g/L of NaCl. Similar behaviour was observed in patient with HS, however the hemolysis without propolis started at concentration of 5.5g/L, as expected, while in the propolis presence was 5.0g/L and 4.0g/L, for Fundão and Bornes propolis respectively. The obtained results establish that in vitro treatment of RBCs with propolis from Bornes protect more efficiently the cells membrane integrity.

In literature was referred that the bioactive properties of propolis can be due to the phenolic compounds, but propolis is a complex mix of compounds. The propolis composition depend the flora, the region, the climatic changes and the season. As this study wasn't employed the same concentration of phenolic compounds, the differences observed in the effectiveness of two propolis, can be due to this different concentration, 300 mg/g GAE (Gallic acid equivalent) and 151 mg/g, to Bornes and Fundão, respectively (Moreira et al., 2008).

In clinical diagnosis, osmotic fragility test is used to determine the integrity of erythrocyte membrane. In normal subject the hemolysis starts approximately at 5.0g/L. The assay obtained results can prove that in vitro treatment of cells with propolis changed the erythrocyte osmotic fragility, how we can observe in Figure 2 (A - control; B - Bornes propolis; C - Fundão propolis). In Figure 2A, the hemolysis start in the concentration of 6.0g/L, however results analysis (Figure 3 and 4) has shown that propolis has affected the fragility of erythrocyte membrane in the control and in the subject with HS, and was verified a curve dislocation to the right when the tested was performed in the presence of propolis. In subject with HS, the hemolysis without propolis started for the concentration of 5.5g/L of NaCl, while in the presence of propolis was 5.0g/L and 4.0g/L, for and Bornes propolis and Fundão propolis, respectively.

It is believed that the responsible compounds for this activity are phenolic compounds, however, no conclusive studies were verified in actuality.

Given the importance of these results, since there aren't any invasive treatments for this disease, our team will work in future studies to identify the compounds involved in

this activity, as well as the identification of molecular targets in the erythrocyte membrane.

3.3. Chelating effect on ferrous ions

The transition metal ions, as Fe^{2+}, possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals (Aboul-Enein et al. 2003). Chelating activity was performed in order to verify the propolis effect on availability of transition metal ions involved on ROS production. The chelating effect of propolis was evaluated by the method of Topçu et al. (2007). Ferrozine can form complexes with Fe^{2+}, originating a violet colour. However, in the presence of propolis, the complex formation is disrupted, and it's possible to verify a decrease on colour formation, proportionally with propolis concentration.

The results suggested a high effectiveness of Bornes propolis in ferrous ions complexation in relation to Fundão propolis (Bornes IC_{50} 10.3 mg/mL; Fundão IC_{50} 17.8 mg/mL) (Figure 6). Possible explanations for these results are that Bornes propolis has twice more total phenolic compounds that Fundão propolis, and some phenolic compounds can contribute more to this result. However, the synergistic effect between phenolic compounds should also be considered (Figure 5).

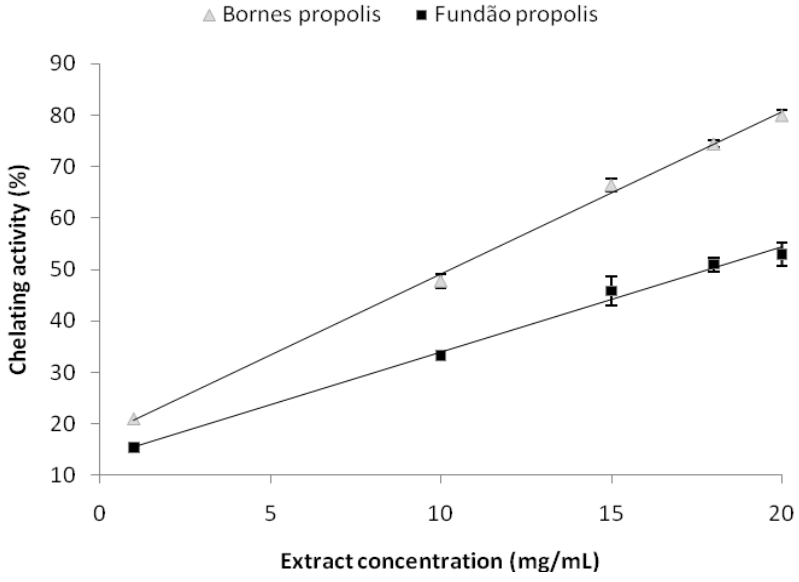

Figure 5. Chelating activity of propolis extracts: Bornes and Fundão.

Margetis et al. (2007) reported increased amounts of hemoglobin in HS patient RBC's (Rocha et al., 2008). Hemoglobin increasing implies an augmentation of iron ions, so HS RBC's seem to be more prone to develop oxidative reactions. As expected, Bornes propolis showed twice more capacity in iron complexation. This result suggest that phenol compounds are involved on stability of RBC membrane, and that this action leads to decrease the damages caused by ROS on membrane, through the sequestration of iron ions and/or by its antioxidant activity.

3.4. RBCs under H_2O_2-induced oxidative stress and propolis inhibition

It is generally accepted that the HS is characterized by clinical and laboratory heterogeneity and according to molecular studies, by genetic heterogeneity (Granjo et al., 2003; Favero et al., 2003). Recent studies support the notion that the type and amount of secondary protein deficiencies are involved in the haematological and clinical outcome of the disease (Rocha et al., 2009). There is also a possibility for an oxidative damage of red blood cell membrane in HS, similar to the one recorded in other haemolytic anaemia (Margetis et al., 2009). Indeed, the spherocytes were found to be more sensitive than normal erythrocytes to the action of oxidation inducing drugs (Margetis et al., 2007). Phenolic compounds of propolis have a large spectrum of pharmaceutical properties, however, the most studied was the antioxidant activity. The aim of the present work was to determine if hemolysis of RBC cells could be induced by oxidative stress conditions, and to verify if propolis can inhibit the hemolysis due to its antioxidant properties. We performed *in vitro* assays in which RBCs of both subjects were exposed to oxidative stress conditions with 1 mM H_2O_2 during 30 min and hemolysis level was measured at 540 nm. To evaluate the effect of propolis in RBCs membrane integrity under oxidative stress conditions, was added 10µg/mL of propolis during 10 min and after was added 1 mM of H_2O_2 during 30 min. In these tests Bornes propolis was used because it gave better results in previous tasks. A preliminary study was made using 10 µg/mL of propolis and 2.65 M of hydrogen peroxide, but in the presence of hydrogen peroxide without propolis it was verified a haemoglobin oxidation with formation of methaemoglobin (green colour solution). In the presence of propolis, the oxidation wasn't verified consequently the solution stays red, since the propolis inhibited the oxidation process (data not showed). No differences were observed in

RBCs hemolysis of control subject incubated with 1 mM of H_2O_2 (data not showed). This result is in accordance with the results obtained by El-Missiry and Abou-Seif (2000), since they only observed oxidative hemolysis with *m*-CPBA after RBCs photosensibilization, increasing for this way the oxidative stress. However, when we have studied the oxidation effect on RBCs of patient it was verified that propolis doesn't affect the hemolysis start concentration (Table 1).

Table 1. Osmotic fragility results of two subjects (with and without HS) and with/without propolis (Bornes and Fundão).

	Without propolis				**Bornes propolis**				**Fundão propolis**			
	Control		*With HS*		*Control*		*With HS*		*Control*		*With HS*	
NaCl in buffer (g/L)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
10.0	-	-	-	-	-	-	-	-	-	-	-	-
9.0	-	-	-	-	-	-	-	-	-	-	-	-
7.5	-	-	-	-	-	-	-	-	-	-	-	-
6.5	-	-	-	-	-	-	-	-	-	-	-	-
6.0	-	-	0.3	±0.2	-	-	-	-	-	-	-	-
5.5	-	-	4.2	±0.2	-	-	-	-	-	-	-	-
5.0	-	-	49.4	±0.1	-	-	-	-	-	-	-	-
4.0	57.7	±0.4	92.4	±0.1	-	-	-	-	-	-	33.8	±0.3
3.5	94.7	±0.3	96.0	±0.1	-	-	45.2	±0.1	1.9	±0.2	93.7	±0.1
3.0	98.7	±0.2	98.2	±0.1	34.3	±0.2	93.8	±0.2	87.8	±0.4	99.1	±0.1
2.0	99.2	±0.2	99.9	±0.1	99.9	±0.1	100.0	±0.3	99.5	±0.2	100.0	±0.4
1.0	100.0	±0.2	100.0	±0.0	100.0	±0.1	100.0	±0.3	100.0	±0.8	100.0	±0.6

Regarding the oxidation effect on patient RBC's, it was observed the same hemolysis start concentration (6.0 g/L). However, the OFT from RBC's under oxidative stress had a curve dislocation to the left which means that oxidant increased the percentage of patient RBC's hemolysis. This effect was blunted when the RBCs were incubated with propolis (Figure 6).

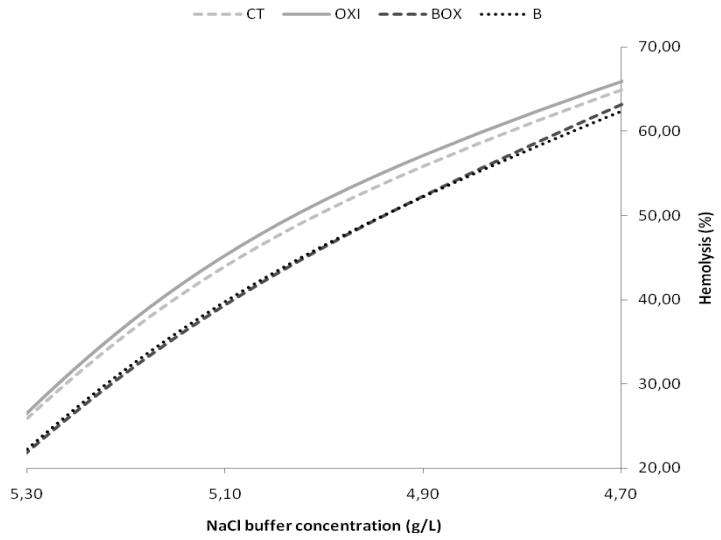

Figure 6. Oxidation effect on osmotic fragility with and withpout propolis on hereditary spherocytosis subject (CT – control; OXI – with hydrogen peroxide; BOX – with hydrogen peroxide and propolis; and B – with propolis).

The obtained results from statistical analysis showed that oxidation effect on RBC's membrane integrity was significant. That means that oxidant occurrence increase the percentage of RBC's hemolysis, in the patient (Table 2).

Table 2. Statistical significance of osmotic fragility curves (Tukey test p=0.05).

Curves	**Significance**
Control - Oxidant	<0.001
Control - Propolis/Oxidant	<0.001
Control - Propolis	<0.001
Oxidant - Propolis/Oxidant	<0.001
Oxidant - Propolis	<0.001
Propolis - Propolis/Oxidant	0.112

This effect was blunted when the RBCs were incubated with propolis extract, which might indicate that propolis act as free radical scavenger protecting the membrane integrity against oxidative effect.

This study suggests that *in vitro*, the osmotic fragility may be increased by oxidative stress conditions in RBCs of HS patient, supporting the concept that the protection of membrane integrity by propolis is due to its antioxidant properties. Furthers studies will be required, like the measurement *in vitro* of the effect of propolis in the oxidation level of membrane proteins and lipids under oxidative stress conditions.

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CAPÍTULO IV

Considerações Finais

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O própolis português é um produto natural com diversas propriedades bioactivas, pelo que a sua inserção na dieta Humana poderá representar uma mais-valia. Estas propriedades estão associadas à presença de compostos fenólicos, como os flavonóides e os ácidos orgânicos.

Os compostos fenólicos são compostos químicos que se encontram nas plantas, e têm a função de protecção celular. Além disso, possuem uma alta importância na indústria farmacêutica, devido às evidências científicas de que estão envolvidos na prevenção de muitas doenças, bem como no seu possível tratamento. Os ensaios realizados revelaram que o própolis Português apresenta um alto teor em compostos fenólicos, que varia com a origem geográfica. A região de Trás-os-Montes apresenta um dos valores mais altos verificados tanto a nível nacional como internacional, segundo a pesquisa realizada. Este facto pode resultar da alta diversidade e singularidade da flora envolvente, bem como do clima característico desta região.

Através das análises melissopalinologicas verificamos que os pólens predominantes na região de Trás-os-Montes são *Castanea sativa* (castanheiro) e *Populus tremula* (choupo), enquanto na região Beira Interior são *Populus tremula* (choupo) e *Pinus sp.* (pinheiro).

Os antioxidantes são compostos promotores da saúde Humana, uma vez que previnem o dano celular causado por radicais livres. O própolis nacional apresentou na actividade antioxidante baixos valores de IC_{50} (Bornes 6 µg/mL; Fundão 52 µg/mL), quando submetido aos testes de bloqueio de radicais livres e poder redutor. A utilização deste produto nacional como suplemento alimentar antioxidante é uma hipótese plausível, dada a baixa concentração necessária para se obter cinquenta por cento de inibição dos radicais livres nos testes *in vitro*. No entanto, o própolis de Trás-os-Montes demonstrou uma maior eficácia em todos os ensaios efectuados, o que pode estar relacionado com a concentração de compostos fenólicos.

Adicionalmente, este trabalho teve por objectivo o estudo do efeito do própolis nacional na membrana do eritrócito de indivíduos saudáveis e com esferocitose hereditária. Os resultados dos testes de fragilidade osmótica evidenciam uma alta

eficácia do própolis na protecção da integridade da membrana eritrocitária, tanto no indivíduo saudável como no paciente com esferocitose hereditária. Na tentativa de elucidar os factores responsáveis por esta protecção, foi estudado o efeito do própolis na oxidação dos eritrócitos e o efeito quelante de iões. Os resultados sugerem que o própolis possui capacidade quelante de iões metálicos (valores de IC_{50}: Bornes 10,3 mg/mL e Fundão 17,8 mg/mL), e desta forma poderá interferir na disponibilidade de iões de transição envolvidos na produção de espécies reactivas de oxigénio. A indução do stress oxidativo com peróxido de hidrogénio aumentou a fragilidade dos eritrócitos do paciente.

Na presença de própolis, o efeito negativo do oxidante foi anulado, verificando-se uma descida na hemólise. Estes dados suportam a hipótese que o própolis protege a membrana eritrocitária, através do bloqueio das espécies reactivas de oxigénio e da sua produção.

Este estudo foi importante, na medida que permitiu uma primeira abordagem à possibilidade de utilização do própolis Português, como alimento funcional. A valorização deste produto ainda muito pouco conhecido a nível nacional é muito importante para aumentar o rendimento das explorações apícolas nacionais, que se deparam actualmente com graves problemas económicos.