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Própolis: teor em fenóis totais e actividades antimicrobiana e inibitória da enzima hialorunidase

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Resumo

Hoje em dia na literatura está disponível uma grande quantidade de informação sobre os aspetos químicos e biológicos dos produtos apícolas, no entanto a informação científica fundamentada sobre a sua utilização terapêutica é limitada. O objetivo deste estudo foi avaliar o perfil fenólico, a atividade antimicrobiana *in vitro* e o efeito sobre a enzima hialuronidase (amplamente relacionado com o processo de inflamação) do própolis Português. Foi também comparada a eficácia da extração do própolis em três extractos (hidro-alcoólico, metanólico e aquoso). Foi escolhido o extrato hidro-alcoólico, porque foi o mais eficaz na extração de fenóis totais.

Foi analisada a atividade antimicrobiana do própolis contem bactérias Gram-positivas e Gram-negativas e leveduras, isoladas de diferentes fluídos biológicos. Os resultados foram comparados com os obtidos para microrganismos de referência. O própolis de Bragança foi o que possuiu o mais alto teor de fenóis totais. A amostra de Beja evidenciou a inibição menos significativa da enzima hialuronidase.

Em relação à atividade antimicrobiana, *Candida albicans* foi a mais resistente e *Staphylococcus aureus* a mais sensível. Os micorganismos de coleção foram mais sensíveis do que os isolados a partir de fluídos biológicos.

Palavras-chave: atividade antimicrobiana, hialuronidase, inflamação, compostos fenólicos, própolis.

Abstract

Nowadays a great amount of information regarding chemical and biological aspects of bee products is available in the literature, but few data on their therapeutic uses are found. The aim of this study was to evaluate the phenolic profile, the in vitro antimicrobial activity and effect in the hyaluronidase enzyme (widely related with the inflammation process) of propolis harvested in Portugal. The efficacy of three extracts (hydro-alcoholic, methanolic and aqueous) was also compared. It was chosen the hydro-alcoholic extract, because this was the most effective for extracting phenolic compounds. The antimicrobial activity was accessed in Gram-positive and Gram-negative bacteria and yeasts, isolated from different biological fluids and the results were then compared with the obtained for reference microorganisms. The propolis from Bragança was the one that possessed the highest polyphenols' content. The sample from Beja showed less significant inhibition of the hyaluronidase enzyme. Concerning the antimicrobial activity, *Candida albicans* was the most resistant and *Staphylococcus aureus* the most sensitive microganism. The reference microorganisms were more sensitive than the ones isolated from biological fluids.

Keywords: antimicrobial activity, hyaluronidase, inflammation, phenolic compounds, propolis.

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CAPÍTULO I
Introdução

1. QUE É O PRÓPOLIS

Entende-se por própolis o produto oriundo de substâncias resinosas, viscosas e balsâmicas, colhidas pelas abelhas (*Apis mellifera*), dos brotos, flores e exsudados de algumas árvores e plantas, nas quais as abelhas acrescentam secreções salivares, cera e pólen para elaboração final do produto (Regulamento Técnico para fixação de identidade e qualidade de própolis).

A abelha recolhe os exsudados com as suas mandíbulas, durante este processo, enzimas presentes na saliva das abelhas são misturadas com as resinas recolhidas produzindo-se a hidrólise de alguns compostos. A abelha armazena estas substâncias nas patas traseiras, até chegar à colmeia (Sforcin, 2007). Posteriormente, estas substâncias são modificadas com a adição de ceras.

Figura 1- Abelha levando própolis à colmeia (Bioapis, Lda.)

Figura 2- Abelha a cubrir uma grelha com própolis (Bioapis, Lda.)

O termo própolis, do Grego *pro* = em defesa e *polis* = cidade ou comunidade, que significa “em defesa da comunidade”, reflete a importância deste composto para as abelhas. As abelhas utilizam o própolis na colmeia para selar as paredes e fissuras, fortificar os alvéolos, proteger a colmeia das doenças através da sua eficácia antisséptica e das propriedades antimicrobianas (Salatino et al., 2005), bem como cobrir os corpos de animais que morrem dentro da colmeia, evitando a sua decomposição (Bankova et al., 2000).

Em geral, o própolis é composto por 50% de resinas e balsamos vegetais, 30% de cera, 10% de óleos essenciais e aromáticos, 5% pólen e 5% de outras substâncias, incluindo restos orgânicos (Burdock, 1998). A cera e os restos orgânicos são removidos no processamento, normalmente a extração etanólica, e a tintura do própolis resultante, contém grande parte dos constituintes bioativos do própolis.

O própolis tem sido utilizado como remédio pelos humanos desde os tempos mais antigos. É usado na medicina popular em muitas partes do mundo. Egípcios, Gregos e Romanos reportam o uso deste produto apícola geralmente pelas suas qualidades curativas. O própolis tem sido reconhecido como um agente anti-inflamatório e curativo de chagas e úlceras. No antigo Egipto era usado para mumificar, e mais recentemente utilizado na *Guerra Bôeres* como curativo de feridas e regeneração de tecidos (Ghisalberti et al, 1978). Atualmente ainda é utilizado em remédios, produtos higiénicos e outras preparações. É um dos medicamentos mais usado nos Países Balcãs (Wollenweber et al., 1990; Bankova, 2005a). Convém no entanto salientar que o conhecimento sobre estas propriedades é empírico pois só nestas últimas décadas está a ser estudado para conhecer os seus constituintes e suas propriedades.

Vários autores demostraram que a administração do própolis, tanto em humanos como em ratos, não evidenciou efeitos secundários (Kaneeda and Nishina, 1994; Sforcin et al., 1995, 2002b; Jasprica et al., 2007). Ramadan et al. (2012) demostraram que o extrato etanólico de própolis não foi tóxico e não causou mortalidade nem sinais de toxicidade em ratos, quando foi administrado oralmente a doses superiores a 5 g/kg de massa vivo ao nascimento, tendo estabelecido este valor como a sua LD_{50}.

Mesmo que em pequeno número, verificaram-se alguns casos de alergia ao própolis e dermatites de contacto (Hausen et al., 1987; Hegyi et al., 1990; Silvani et al., 1997; Callejo et al., 2001). Estas alergias foram diferentes da alergia provocada pelo mel, que é derivada de alguns alérgenos presentes nas flores. Normalmente são os apicultores os que apresentam esta sensibilidade ao própolis (Rudeschko et al., 2004; Gulbahar et al.,2005; Sforcin, 2007).

2. CARACTERÍSTICAS E COMPOSIÇÃO QUÍMICA

O própolis é uma substância resinosa e muito adesiva, dai a baixa solubilidade em água. Na sua extração devem utilizar-se solventes orgânicos como éter etilico, acetona, tolueno e tricloroetileno ou álcoois como metanol e etanol que permitem a dissolução da maior parte dos compostos do própolis. Para além disso tem sido desenvolvidas patentes com novos métodos ou solventes para extrair os compostos presentes no própolis, que utilizam óleos vegetais comestíveis, triglicéridos e ácidos gordos (Kasuma and Kenichi, 2001a, 2001b; Namiki et al., 2005).

À temperatura ambiente é um produto de consistência sólida, a partir de 30ºC torna-se maleável e funde entre os 60-70ºC podendo chegar a seu ponto de fusão até aos 100ºC dependendo da sua composição.

A sua coloração pode variar de verde até amarelo, vermelho, castanho, castanho-escuro ou preto (Ghisalberti et al., 1978) dependendo da origem botânica da resina.

O seu odor é característico e extremamente forte devido maioritariamente aos compostos voláteis nele existentes (Anon, 1927)

A composição química do própolis é muito complexa. O própolis bruto contém mais de 300 compostos entre os quais se encontram flavonoides, ácidos fenólicos e seus ésteres, sesquiterpenos, quininas, esterois, vitaminas, aminoácidos, açúcares e proteínas (Kumar et al., 2009; Lotfy, 2006; Markham et al., 1996; Trusheva et al., 2007). Vários destes compostos encontram-se de forma natural nos alimentos ou são utilizados como aditivos alimentares dentro do grupo de sustâncias geralmente reconhecidas como seguras ou sustâncias Generally Recognised as Safe (GRAS) (Burdock et al., 1998). Este facto sugere que o própolis é uma sustância apropriada para ser utilizada como conservante natural em alimentos, satisfazendo a atual procura por parte dos consumidores, de antioxidantes e antimicrobianos naturais. Deste modo é possível obter alimentos minimamente processados, com conservantes naturais ou que utilizam conservantes sintéticos em concentrações muito baixas (Chaillou and Nazareno, 2009; Han and Park, 1995; Oldoni et al., 2011; Tosi et al., 2007).

Os estudos da composição química do própolis têm sido centrados principalmente nos compostos que apresentam propriedades bioativas, principalmente os polifenóis. Dentro destes compostos vários autores destacaram a presença de ácidos fenólicos (ácido caféico, cumárico, ferúlico e elágico) e seus ésteres e de flavonoides (hespertina, crisina, naringenina, kaempferol, vainillina, galanina) (Chunying et al., 2011; De Vecchi and Drago, 2007; Falcão et al., 2010; Ghassan et al., 2011; Gregoris and Stevanato, 2010; Ilhami et al., 2010; Medana et al., 2008) que são considerados os principais responsáveis pelas suas propriedades biológicas e farmacológicas (Carvalho et al., 2011; De Castro, 2001; Kumazawa et al., 2004; Sforcin, 2007; Nolkemper et al., 2010).

No entanto, o teor nestes compostos e, consequentemente, as suas propriedades bioativas variam segundo a origem geográfica, dependendo da flora local e da fenologia das plantas de origem (Ahn et al., 2007; Kujumgiev et al., 1999; Sforcin, 2007), bem como de outros fatores como a estação do ano (Isla et al., 2009; Teixeira et al., 2010; Valencia et al., 2012). Alguns autores descrevem a presença de sustâncias específicas, nomeadamente os trepenos, em função da origem geográfica do mesmo (Oldoni et al., 2011; Popova et al., 2009; Popova et al., 2010; Trusheva et al., 2003). Esta grande variabilidade constitui um impedimento à aprovação oficial do própolis para uso em preparações farmacológicas ou produtos nutracêuticos, sendo necessária uma estandardização química que garantir-se a sua qualidade, segurança e eficácia (Bankova, 2005a; Marcucci, 1995; De Castro, 2001).

3. PROPRIEDADES BIOACTIVAS DO PRÓPOLIS

O própolis tem sido utilizado extensivamente desde os primórdios da humanidade. Egípcios beneficiaram das propriedades anti putrefação do própolis usando-o para embalsamar os seus mortos. Médicos Gregos e Romanos utilizaram o própolis como agente antisséptico e cicatrizante. Os Incas empregaram o própolis como agente antipirético, e as farmacopeias Londrinas do século 17 listaram o própolis como uma droga oficial. Hoje em dia este produto tem diversas aplicações. De facto possui um amplo espectro de atividades biológicas, incluindo anti- cancerígeno (Bufalo et al., 2009; Hernandez et al., 2007; Li et al., 2009), antiviral (Amoros et al., 1994), cardioprotector (Daleprane et al., 2012), antioxidante (Ahn et al., 2004; Lima et al., 2009; Velazquez et al., 2007), antimicrobiana (Jorge et al., 2008; Majiene et al., 2007; Popova et al., 2005; Sforcin et al., 2001; Sforcin et al., 2000; Velazquez et al., 2007) e anti- inflamatória (Paulino et al., 2003) entre outras propriedades. Por estas razões, o própolis tem sido usado como um remédio popular na medicina tradicional, em apiterapia, alimentos saudáveis e em outros fins (Bankova et al., 2000; Banskota et al., 2001; Ghisalberti, 1978).

3.1 Atividade anti tumoral

O própolis foi reportado como um agente anti tumoral, pela sua capacidade anti proliferativa das células tumorais, quer *in vitro* quer *in vivo* (Banskota et al., 2001; Buriol et al., 2009; Chen et al., 1996; Kim et al., 2008; Rao et al.,1992).O seu principal mecanismo da ação anti tumoral esta relacionado com a inibição do crecimento celular e indutor do processo de apoptose (Sforcin 2007).

Vários estudos demostraram que compostos isolados do própolis como o ácido cafeico e seus esteres são os responsáveis por esta atividade. Lee et al., 2005 observaram que o ácido cafeico interfere com o ciclo celular, a análise do fluxo citométrico demonstrou a interrupção da célula na fase G2/M. Orsolic et al., 2004, verificaram que, compostos polifenólicos como o acido caféico e seus ésteres assim como a quercetina, diminuíram o numero de nódulos tumorais do pulmão. Contudo, o efeito anti metastático observado no própolis inteiro, foi superior aos compostos isolados. Esta ação do própolis pode ser resultado de ações sinergéticas dos compostos polifenólicos (Orsolic et al., 2005).

Carvalho et al., (2011) demonstraram o efeito citotóxico de diferentes extratos de própolis *in vivo*, através do modelo Sarcoma 180 (tumor frequentemente induzido em ratos para estudos anti tumorais *in vivo*) transplantado em ratos, em que todos os extratos de própolis demonstraram significativas reduções do crescimento do tumor.

3.2 Atividade antiviral

Atividade farmacológica do própolis, foi observada contra várias infeções virais, tais como, o vírus da gripe (Serkedjieva et al., 1992), vírus imunodeficiência humana (Ito et al., 2001) , adenovírus (Amoros et al., 1992a) e herpes simplex vírus(Debiaggi et al., 1990; Amoros et al., 1992b; Amoros et al., 1994; Huleihel e Isanu, 2002).

Este ultimo, o vírus herpes simplex, são agentes patogénicos generalizados para os seres humanos, especialmente recém-nascidos e para pacientes imunosuprimidos

(Chakrabarti et al., 2000). Existem dois tipos, o tipo 1 é transmitido através de gotículas e recorrentemente provoca herpes labiais, ao passo que o tipo 2 é transmitido principalmente por via sexual e é o agente causador do herpes genital (Smith e Robinson, 2002; Stranska et al., 2005).

Galangina e crisina, constituintes do própolis, reduziram a formação de placas de vírus herpes simplex tipo 1 livre em 68,0% e 56,4%, respetivamente, quando comparados com os controlos não tratados.

A sinergia dos compostos do própolis pode ser demonstrada quando combinações binárias flavo-flavonóis foram testadas contra o vírus herpes simplex, isto explica o facto do própolis ser mais ativo do que seus compostos individuais (Scheller et al., 1999).

3.3 Atividade cariostática

Vários produtos de higiene oral têm na sua base ativa propriedades cariostáticas. No própolis também foram encontrados compostos que por diferentes mecanismos de ação são capazes de controlar a aparição de cáries.

Analises antimicrobianas realizadas com alguns compostos do própolis revelaram que o sesquiterpeno tt-farnesol é eficaz conta o *Streptococcus mutans*, microrganismo responsável pela formação caries, com concentrações mínimas inibitórias (MIC) de 14 –28 g/ml e concentrações mínimas bactericidas (MBC) de 56–112 g/ml (Koo et al., 2002).

Outro composto, o flavonoide apigenina demonstrou ser um potencial inibidor da atividade glucosiltransferase tanto em solução como num ensaio feito com grânulos de hidroxiapatite revestido com saliva (Koo et al., 2000). Esta enzima é responsável pela formação de biofilmes que dão resistência ao *Streptococcus mutans* tornando-o menos sensível às substâncias antibacterianas.

A apigenina evidencia capacidade para inibir a atividade enzimática, mas não possui capacidade anti bacteriana, enquanto tt-farnesol apresenta capacidade anti bacterianas moderada contra o bio filme, mas o seu efeito em glucosiltransferase não foi significativo. Os dois compostos interferem na acumulação e na composição polissacarídica do biofilme do *Streptococcus mutans* sem causar um grande impacto na viabilidade bacteriana (Koo et al., 2003).

Juntamente com o flureto de sodio, a apigenina e o tt-farnesol são capazes de reduzir a virulência do *Streptococcus mutans*, e promover o efeito cariostatico do floreto. Neste estudo foi evidente que a combinação destes compostos diminui a quantidade de glucanos insolúveis extracelulares no bio filme do *Streptococcus mutans* e que esta combinação pode ser uma alternativa aos agentes químicos normalmente utilizados para a prevenção do processo cariostático (Koo et al., 2005).

3.4 Atividade hepatoprotetora

As doenças hepáticas são consideradas um dos maiores problemas de saúde, sendo o fígado um órgão importante tanto na detoxicação como deposição de substâncias endógenas e exógenas (Sehrawat et al., 2006)

As lesões provocadas no fígado pelo consumo excessivo de remédios halopáticos nos últimos anos, tem levado a muitas patologias crónicas deste órgão. O tratamento das disfunções hepáticas, sejam funcionais ou lesionais, envolvem substâncias que têm ação hepatoprotetora (que atuam nos hepatócitos protegendo-os dos danos diversos: químicos, microbiológico).

Vários extratos de própolis apresentaram forte atividade hepatoprotetora. Os extratos metanólicos do própolis do Brasil, China, Peru e da Holanda apresentaram grande atividade hepatoprotetora da D-galactosamine (D-GalN)/ fator de necrose tumoral -a (TNF-a) – morte celular induzida em culturas primarias de hepatócitos de ratos (Banskota et al., 2000).

Sugimoto et al. (1999), verificaram que o extrato etanólico (95%) do própolis Brasileiro possui forte atividade hepatoprotetora da D-GalN – lesão hepática induzida em ratos, González et al. (1994, 1995) observaram que o extrato etanólico (95%) do própolis Cubano exibiu forte atividade hepatoprotetora do paracetamol – induzindo danos no fígado em ratos e do CCl_4 i - lesão hepática induzida em ratos.

Também no extrato aquoso Brasileiro foram observados constituintes hepatoportetores e antioxidantes (Basnet et al., 1996; Matsushige et al., 1996).

A atividade hepatoprotetora do Própolis Brasileiro deve-se principalmente aos compostos fenólicos, tais como os flavonoides.

3.5 Atividade antioxidante

Os radicais livres de oxigénio são produtos normais do metabolismo celular provocando o *stress oxidativo* (Valko et al., 2007). Estes são responsáveis por várias anomalias celulares e o consumo regular de antioxidantes parece limitar ou prevenir os efeitos provocados por estes radicais livres (Kaur and Geetha, 2006).

Embora nosso organismo possua as suas próprias defesas contra estes radicais estas podem ser insuficientes devido às agressões sofridas pelo nosso organismo no dia-a-dia. Assim, o consumo de produtos com capacidade antioxidante como o própolis torna-se um importante fator na defesa do nosso organismo (Mohammadzadeh et al., 2007).

As propriedades antioxidantes do própolis são devidas principalmente aos compostos fenólicos. Estes componentes apresentam um efeito notável de proteção contra as reações de oxidação, devido às suas propriedades redox, que lhes permitem atuar como agentes redutores, doadores de hidrogénio, quelantes de metais e supressores de singletos de oxigénio (Parr and Bolwell, 2000). Própolis também tem sido relatado como um inibidor da formação do anião superóxido. Além disso, o própolis pode reverter o consumo de glutationoxidase, uma das principais enzimas antioxidantes, que é sintetizada no fígado, e apresenta capacidade captadora de radicais libres (Castaldo and Capasso, 2002).

As propriedades antioxidantes do própolis além das suas propriedades antibacteriana e anti fungicida, juntamente com o facto de vários dos seus constituintes estarem presentes nos alimentos ou em aditivos alimentares, e ser reconhecido como GRAS (Burdock, 1998), torna-o bastante atrativo como conservante alimentar natural. De facto, tem-se verificado um aumento da procura de antioxidantes e antimicrobianos naturais, por parte dos consumidores em geral e particularmente por parte dos que preferem alimentos minimamente processados (Han & Park, 1995; Tosi et al., 2007).

Os compostos antioxidantes presentes no própolis podem aumentar a vida de prateleira dos produtos alimentares, retardando o processo de peroxidação lipídica, o que é uma das principais razões para a deterioração dos produtos alimentares durante o processamento e armazenamento (Halliwell, 1997; Halliwell and Gutteridge, 1999).

3.6 Atividade antimicrobiana

Um dos maiores problemas de difícil resolução ao nível da saúde pública, desde o início do seculo, é o aumento de resistência dos vários microrganismos aos agentes antimicrobianos. A evolução incessante de resistências aliada à diminuição do desenvolvimento de novos agentes antimicrobianos ativos contra patogénicos resistentes conduziu a um aumento do número de casos de microrganismos resistentes à maioria ou a praticamente todos os fármacos disponíveis para uso clinico. Perante esta situação são necessárias alternativas terapêuticas eficazes e se possível que não induzam resistências.

O própolis pode constituir uma destas alternativas ao mostrar efeitos sinergeticos com antibióticos. Oksuz et al. (2005) verificou o efeito sinergetico do propolis com ciprofloxacin no tratamento de *Staphylococcus aureus keratitis*. Segundo Orsi et al. (2006), o própolis tem a capacidade de diminuir a resistência das paredes da bactéria à entrada dos antibióticos (amoxicilina, ampicilina e cefaloxina) e demonstrou efeitos sinérgicos com antibióticos que atuam no ribossoma (cloranfenicol, tetraciclina e neomicina).

Esta atividade não é útil só na terapia antimicrobiana humana se não também no tratamento de doenças das próprias abelhas. *Paenibacillus larvae*, o agente responsável pelo Loque Americano, tem vindo a ganhar resistências aos antibióticos convencionais, e os extratos de própolis de vários estados do Brasil inibiram significativamente estes microrganismos (Bastos et al., 2008).

De todas as propriedades que o própolis possui, a atividade antimicrobiana é a mais extensivamente estudada (Chaillou e Nazareno, 2009; Kalogeropoulos et al., 2009; Libério et al., 2009; Petrova et al., 2010; Popova et al., 2009) e tem sido documentada contra diferentes tipos de bactérias, fungos e parasitas (Freitas et al., 2006; Guven et al., 2011; Popova et al. 2009; Sforcin et al., 2000; Sforcin et al., 2001).

In vitro, o própolis pode agir diretamente no microrganismo e *in vivo* pode agir estimulando o sistema imunitário, envolvendo-se em mecanismo que resultam na morte dos microrganismos. A atividade antimicrobiana do própolis está relacionada principalmente com o seu conteúdo em flavonoides (Cushnie and Lamb, 2005; Salomao et al., 2004) como assim os compostos fenólicos, terpenos e ácidos aromáticos e esteres mostraram também atividade antimicrobiana (Bankova, 2005b; Burdock et al., 1998; Popova et al., 2005).

3.7 Atividade anti-inflamatória

O processo inflamatório é desencadeado por diversos produtos químicos e/ou biológicos, incluindo enzimas pró-inflamatórias e citocinas, compostos de baixo peso molecular, tais como eicosanóides ou a degradação enzimática dos tecidos (Dao et al., 2004).

Diversos estudos acordam que a ciclooxigenase-2 (COX-2), uma isoforma da ciclooxigenase (COX), que catalisa a transformação de ácido araquidónico a prostaglandina, é a enzima mais relacionada com o processo inflamatório (Griswold and Adams, 1996; Cho et al., 2004). A outra isoforma é a ciclooxigenase-1 (COX-1), que regula os processos de homeostase (Dao et al., 2004).

Nestes últimos 30 anos, diversos estudos demonstram as propriedades anti-inflamatórias do própolis, essas propriedades devem-se basicamente à presença de flavonoides que inibem o desenvolvimento de inflamações provocadas por diversos agentes (Teixeira et al., 2005; Mani et al., 2006).

Galangina, um composto flavonoide, é capaz de inibir a atividade da COX e lipo-oxigenase, limitar a ação da poligalacturonase e reduzir a expressão da isoforma induzível da COX-2 (Raso et al 2001; Rossi et al 2002a, 2002b).

Crisina, outro composto flavonoide de grande interesse presente no própolis, também mostra atividade anti-inflamatória (Kim et al 2002; Ko et al., 2003).O seu mecanismo de ação esta relacionado com a supressão das atividades pró-inflamatórias da COX-2 e induzível sintase do óxido nítrico (Cho et al., 2004).

Outro composto, o ácido cafeico fenetil éster (CAPE), também presente no própolis, mostra atividade anti-inflamatória através da inibição da libertação de ácido araquidónico a partir da membrana celular, o que conduz á supressão da atividade de COX-1 e COX-2 e inibe a ativação da expressão génica de COX-2 (Mirzoeva and Calder 1996; Lee et al., 2004).

4. REFERÊNCIAS

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CAPÍTULO II
Antimicrobial activity, phenolic profile and role in the inflammation of propolis

Antimicrobial activity, phenolic profile and role in the inflammation of propolis

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1. Introduction

Propolis is a beehive product prepared by bees of the *Apis mellifera* species, using resinous substances collected from various plants. These substances are mixed with β-glycosidase enzyme of their saliva, partially digested and added to bee wax to form the final product (Umthong et al., 2011). Propolis is used, by bees, as a sealing wax for filling cracks in beehives and as a protective barrier against the pathogenic microorganisms. It is considered the most important "chemical weapon" (Falcão et al., 2010).

The composition of this sticky resin and its physico-chemical properties, biological activities and therapeutic uses depend on the vegetation where the hives are placed, the climate and the variety of the queen (Quiroga et al., 2006). According to Kumazawa et al. (2004), in samples from Brazil terpenoids and prenylated derivatives of *p*-coumaric acids predominated whilst the samples from China and Europe mostly contained phenolic acid esters and flavonoids. In spite of the possible differences in composition, most propolis samples share considerable similarity in their overall chemical nature: 50% resin, 30% wax, 10% essential oils, 5% pollen and 5% of other organic compounds (Gómez-Caravaca et al., 2006). In fact, propolis has more than 300 different compounds identified, such as: aliphatic acids, esters, aromatic acids, fatty acids, carbohydrates, aldehydes, amino acids, ketones, chalcones, dihydrochalcones, terpenoids, vitamins and inorganic substances (Bankova et al., 2000).

This product has been used as remedy and as a food preservative by humans since ancient times (Umthong et al., 2011). In the last years, this product has been the subject of intensive studies, highlighting its biological and pharmacological properties, such as antibacterial (Velazquez et al., 2007) antiviral (Schnitzler et al. 2010), antioxidant (Moreira et al., 2008), hepatoprotective (Banskota et al., 2001), cariostatic (Libério et al., 2009) and anticancer (Valente et al., 2011). For these reasons, propolis awakened interest in the pharmaceutical industries, being introduced in products for human consumption, such as drinks, food and cosmetics (Moreira et al., 2008).

In addition, the emergence of antibiotic-resistant microorganisms, that are known by their dangerous action in wounds, decrease the treatment options. This led to an increase research of antimicrobial activity of natural products as possible alternatives (Morais et al., 2011). In fact, this was the most studied biological property of propolis. However, European propolis studies are scarce, and particularly in Portugal are non-existent.

This product is also used is medicine as an anti-inflammatory. Inflammation is a process by which the body's white blood cells and chemicals protect us from infection and foreign substances such as bacteria and viruses (Park et al., 2002). This process is associated with the liberation of inflammatory mediators, like prostaglandins, through enzimatic reactions, in which are involved: lipoxygenases, cyclooxygenases, phospholipase A2 and hyaluronidase (Braga et al., 2006).

The hyaluronic acid is an important component of articular cartilage and plays an important role in tissues' renovation. Its degradation, by the hyaluronidase enzyme, may cause bone loss, inflammation and pain (Libby et al., 2002). As consequence, the determination of the hyaluronidase enzyme is an indirect way to assess the anti-inflammatory activity.

Both the anti-inflammatory and antimicrobial activities of Portuguese propolis have never been studied, even though beekeeping has great importance in the economy of this country.

In this context it is necessary to ensure the consistency of pharmacological and clinical research, to understand the biological activity of propolis as well as to achieve a reliable standardization on propolis types and to enhance product quality control.

In the present work and for the first time, it is evaluated the antimicrobial activity against multi-resistant microorganisms and the anti-inflammatory activity, assessed by the effect on the hyaluronidase enzyme, of propolis samples from Portugal. Simultaneously, it was also studied the effect of extraction solvents on these biological activities.

2. Material and Methods

2.1. Chemicals and Reagents

All the reagents were of analytical grade purity. Methanol (CH_3OH) and ethanol (CH_3 CH_2OH) were supplied by Pronolab (Lisbon, Portugal). The Folin–Ciocalteu reagent, chloroform ($CHCl_3$), sodium carbonate (Na_2CO_3), gentaminice and fluconazol were obtained from Merck (Darmstadt, Germany). Gallic acid and (+)-catechin were purchased from Sigma (St. Louis, MO, USA). The bovine testicular hyaluronidase (350 units) and the potassium salt of human umbilical cord hyaluronic acid were obtained from Sigma (St. Louis, MO, USA). The culture mediums were purchased from Himedia (Mumbai, India). The TTC solution (2,3,5-Triphenyl-2H-tetrazolium chloride) was supplied by Fluka (Buchs, Switzerland). The other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). High purity water (18 MΩ cm), used in all experiments, was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Propolis Samples

Propolis samples were collected by beekeepers in the fall of 2010 from *Apis mellifera* hives located in different zones of Portugal: Bragança (42º 48' N; 6º 45' W); Coimbra (40º 15' N; 8º 27' W) and Beja (38º 1' N, 7º 52' W). Three samples (n=3) were collected from each place and all the analysises were performed in triplicate. They were obtained after the honey extraction by scratching the hive walls and frames. Upon receipt, each sample was inspected in order to find rests of bees, wood, plant, pupa of moth, among others. The major visible impurities were removed from the samples. Each sample was weighed and frozen at -20°C until analysis.

2.3. Palynological identification

Palynological processing of the samples followed the standard methodology, described in detail previously by Moreira et al. (2008). In briefly, 0.5 g of scraped propolis was extracted overnight with ethanol. Next, the sediment was treated with KOH (10%), sonicated for 15 min. and sieved through a 20 mesh stainless steel screen to eliminate large fragments. In this stage, three propolis microscope slides were mounted with sediment obtained after centrifugation (10000 g for 1 min) for observation of plant trichomes and other organic residues that may be destroyed in sequence. Then acetolysis was applied, and two additional microscope slides were prepared using glycerin jelly, one stained with basic fuchsine and the other without stain. Approximately 300 pollen grains in each sample were counted. Pollen grain identification was performed by optical microscope with total magnification (x400 and x1000). A reference collection of CIMO – Mountain Research Centre (Agricultural College of Bragança) and different pollen morphology guides (CUPOD, Cambridge University Palynological Online Database) were used for the recognition of the pollen types.

2.4. Extraction procedure

2.4.1. Aqueous extract

Propolis (5g) was chopped into small pieces and extracted with 50 mL of water (80ºC) for 3 h (Midorikawa et al., 2001). Afterwards, the resulting mixture was filtered and the residue was re-extracted in the same conditions. The next step was the mixture of both filtrated solutions, which were then frozen at -20ºC.

2.4.2. Methanolic extract

The propolis samples were broken into small pieces and homogenized. The samples were extracted with 80% of methanol/water (1/10, v/v) at 45 °C for 1 h. The mixtures were filtered, and the residue was re-extracted following the same procedure. After, the filtrated solutions were combined, concentrated and frozen at −20 °C.

2.4.3. Hydro-alcoholic extract

Prior to the extraction, the propolis was grounded and homogenized. The samples were extracted with 80% of ethanol/water (1/10, v/v) at 45 °C for 1 h, the resulting mixtures were filtered, and the residues were re-extracted in the same conditions. After the second extraction, the filtrated solutions were combined, concentrated and frozen at −20 °C.

2.5. Total Phenolics and Flavonoids

The total phenolic content in the extracts were recorded using the Folin–Ciocalteu method as described by Moreira et al. (2008). Briefly, a dilute solution of each propolis in MeOH (MeOH-propolis; 500 µL of 1:10 g/mL) was mixed with 500 µL of Folin-Ciocalteu reagent and 500 µL of Na_2CO_3 (10% w/v). After incubation in dark at room temperature for 1 h, the absorbance of the reaction mixture at 700 nm was determined against the blank (the same mixture without the MeOH-propolis) using a Unicam Helios Alpha UV–visible spectrometer (Thermo Spectronic, Cambridge, UK). Galic Acid standard solutions ($0.01x10^{-3}$ - $0.08x10^{-3}$ M) were used for constructing the calibration curve (y=2.3727x+0.0022; R^2=0.9998). Total phenols content were expressed as mg of Galic Acid equivalents per g of propolis (GAEs).

For flavonoids' contents the aluminium chloride method was used. In briefly, MeOH-propolis (250 µL) was mixed with 1.25 mL of distilled H_2O and 75 µL of a 5% $NaNO_2$ solution. After 5 min, 150 µL of a 10% $AlCl_3 \cdot H_2O$ solution was added. After 6 min, 500 µL of 1M NaOH and 275 µL of distilled H_2O were added to the mixture and vortexed. The solution was well mixed and the intensity of pink colour was measured at 510 nm. Catechin standard solutions ($0.022x10^{-3}$ - $0.34x10^{-3}$ M) were used for constructing the calibration curve (y = 0.9689x-0.0092; R^2 = 0.9987). Total flavonoids content were expressed as mg of catechin equivalents per g of propolis (CAEs).

2.6. UV-Visible Absorption Spectroscopy

The determination of the UV-Visible spectra of the extracts (aqueous, methanolic and hydro-alcoholic) was performed according to Koo et al. (2002). 25 µL of each propolis extract were diluted into 30 mL of ethanol. The absorption spectra were determined in the wavelength range from 200 to 500 nm.

2.7. Anti-inflammatory activity – Hyaluronidase assay

The inhibition of hyaluronidase activity was determined using de method described by Park et al. (1998). The reaction mixture is constituted by 50 µL of propolis' extract and 50 µL (350 units) of hyaluronidase enzyme (Type IV-S: bovine testes), was incubated at 37ºC for 20 min. Then, calcium chloride was added (1.2 µL, $2.5x10^{-3}$ M/L) to activate the enzyme and the mixture was incubated at 37ºC for 20 min. To start the reaction 0.5 mL of hyaluronic acid sodium salt (0.1 M/L) were added. The mixture was incubated at 37ºC for 40 minutes. After this, 0.1 mL of potassium tetraborate 0.8M was added and it was incubated in water-bath at ebullition for 3 minutes. The mixture was placed at 10ºC and 3 mL of p-dimethylaminebenzaldehyde were added. Afterwards, it was incubated at 37ºC for 20 minutes. Finally, the absorbance was measured at 585 nm using water as control.

2.8. Antimicrobial activity

The microorganisms used as test organisms are presented in Table 1. The microorganisms were isolated from biological fluids (in the Hospital Centre) and identified in the Microbiology Laboratory of Escola Superior Agrária de Braganca. It were also used reference strains, obtained from the authorized distributor of ATCC (LGC Standards S.L.U., Barcelona.) The isolates were stored in Muller–Hinton medium plus 20% glycerol at -70 ºC, before experimental use. The inoculum for the assays were prepared by diluting cell mass in 0.85% NaCl solution, adjusted to 0.5 MacFarland scale, confirmed by spectrophotometrical reading at 580 nm for bacteria and 640 nm for yeasts. Cell suspensions were finally diluted to 10^4 CFU/mL in order to use them in the activity assays. Antimicrobial tests were carried out according to Morais et al. (2011), using Nutrient Broth (NB) or Yeasts Peptone Dextrose (YPD) on microplate (96 wells). Propolis extracts were diluted in dimethylsulfoxide (DMSO) and transferred into the first well, and serial dilutions were performed. The inoculum was added to all wells and the plates were incubated at 37 ºC for 24 h (bacteria) and 25 ºC for 48 h (yeast). Fluconazol and gentamicine were used as controls. In each experiment a positive control (inoculated medium) and a negative control (medium) and DMSO control

(DMSO with inoculated medium) was introduced. Antimicrobial activity was detected by adding 20 µL of 0.5% TTC solution.

The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of propolis extract that inhibited visible growth, as indicated by the TCC staining (dead cells are not stained by TTC). All the tests were performed in triplicate (n=3). The results are expressed as mg/mL.

Table 1 – Microorganisms used in the present study to test antimicrobial activity of propolis extracts.

Microorganism	Reference	Origin
Escherichia coli	**ATCC 29998™**	**Reference culture**
Cephalosporins resistant Escherichia coli	ESA 37	Urine
	ESA 54	Hemoculture
Candida albicans	**ATCC 10231™**	**Reference culture**
Fluconazol resistant Candida albicans	ESA 500	Faeces
	ESA 502	Urine
Pseudomonas aeruginosa	**ATCC 15442™**	**Reference culture**
Imipenem resistant Pseudomonas aeruginosa	ESA 22	Expectoration
	ESA 23	Gingival exudates
Staphylococcus aureus	**ATTC 6538™**	**Reference culture**
Methicillin resistant Staphylococcus aureus	ESA 175	Pus
	ESA 159	Expectoration

ESA (Escola Superior Agrária de Bragança); ATCC (American Type Culture Colection)

2.9. Statistical analysis

Each propolis sample was analysed in triplicate. Results are shown as arithmetic mean values ± standard deviation. In each parameter, the differences between propolis were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD. P values less than or equal to 0.05 were evaluated as statistically significant. This treatment was carried out using SAS v. 9.1.3 program (SAS Inc, New York City, USA).

3. Results and Discussion

3.1. Palynological identification

Results of bee pollens' profile analysis allow scientists to infer the vegetation present in the area and to date and ascertain any biodiversity change, as for example, the presence and distribution of invasive or exotic plants (Morais et al., 2011). The quantification of the pollens' present in propolis aims to determinate its floral origin. In fact, this origin is one of the factors that influence the bioactive properties of this product. In accordance with melissopalynological criteria (Louveaux et al. 1970), the following designations of pollen frequency were used: PD for dominant (>45%), PA for accessory (15-45%), and PI for isolated pollen loads but important to characterize the phytogeographical origin of the sample (3-15%).

The pollen profiles obtained for the three samples are presented in Figure 3. Marked differences were found among the samples. In the propolis from Bragança, with brown colour, the botanical specie with higher percentage was *Erica* sp. (47.29% ± 5.89), followed by *Castanea sativa* (21.08% ± 2.27). Concerning the sample from Coimbra (dark yellow propolis), *Populus tremula* was the dominant specie (55.10% ± 5.87), however the species *Salix* sp. (35.82% ± 3.78) and Rubus sp. (9.08% ± 0.99) were also found. In propolis from Beja (green-brown colour), *Eucalyptus* sp. was the predominant pollen, with a percentage of 60.02 and a standard deviation of 5.89. Pollens from *Rubus sp*. and *Populus tremula* were found in all the analysed samples. The pollens' profile was significantly different for all the samples under study ($p<0.05$).

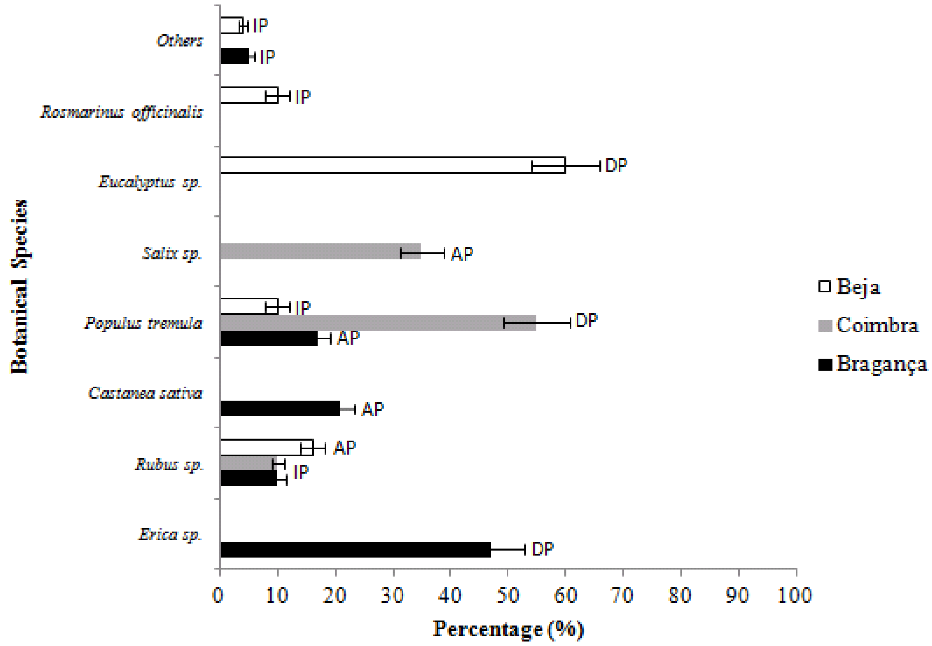

Figure 3 - Palynological spectrum of bee pollen samples. DP – Dominant Pollen (>45%); AP - Acessory Pollen (15%-45%); IP – Isolated Pollen (<15%)

3.2. Total Phenolics and Flavonoids

According to the literature (Bankova et al., 2000; Gómez-Caravaca et al., 2006), the majority of compounds already identified in propolis are polyphenols. These compounds have been extracted using different solvents: water, methanol and ethanol. In this context, the efficiency of these substances was assessed.

Table 2 shows the contents of phenolic and flavonoids compounds present in the different propolis extracts (hydro-alcoholic, methanolic and aqueous), for each place (Bragança, Coimbra and Beja). For all the propolis studied (n=3), water was the less effective solvent. In fact, the concentration of phenolics and flavonoids of the aqueous extract was lower than the concentration found in the other extracts, which showed significantly higher amounts of those compounds. The hydro-alcoholic extract, that is not as toxic as methanolic, was found to be the best solvent for the compounds under study. It extracted ≈4.34 times more total phenolics than the aqueous extract and ≈1.52 times more than the methanolic extract. The flavonoids' concentration obtained for the hydro-alcoholic extract was significantly higher than the amounts extracted by methanol and by water.

Concerning the different places, propolis from Bragança was the one that possessed higher concentration of total phenolics (277.17 mg GAE/g ± 7.50) and flavonoids (142.32 mg GAE/g ± 4.52), followed by Coimbra's propolis. For a 95% confidence interval ($p=0.05$), significant differences were found among the samples with different origins (Table 2).

Table 2 – Concentration (mg GAE/g) of phenolics and flavonoids in propolis extracts from different locations (n=9)

	Extract	Phenolics (mg/g)	Flavonoids (mg/g)
Bragança [x]	Hydro-alcoholic	277.17 ± 7.50[a]	142.32 ± 5.75[a]
	Methanolic	181.31 ± 4.71[b]	135.51 ± 6.22[b]
	Aqueous	72.15 ± 1.20[c]	42.30 ± 2.10[c]
Coimbra [y]	Hydro-alcoholic	157.31 ± 1.52[a]	98.30 ± 6.54[a]
	Methanolic	102.32 ± 0.59[b]	55.25 ± 0.33[b]
	Aqueous	35.15 ± 0.88[c]	9.0 ± 1.00[c]
Beja [z]	Hydro-alcoholic	87.15 ± 4.80[a]	25.15 ± 2.53[a]
	Methanolic	58.61 ± 3.10[b]	13.62 ± 2.49[b]
	Aqueous	18.52 ± 1.35[c]	6.34 ± 0.55[c]

a, b, c - Means with different letters are significantly different for microorganisms.
x, y, z - Means with different letters are significantly different for locations.

Globally, our results are in agreement with the data obtained by Moreira et al. (2008), who studied propolis from the northeast of Portugal. However, Miguel et al. (2010) obtained inferior values when analyzing propolis from the south of the same country. This discrepancy may be due to the great distances between the local of origin and the different apicultural practices. In fact, our data suggest that propolis from different places have different concentrations of polyphenols.

The values obtained for catechin and gallic acid, which were used as standards, were bellow the concentration obtained in this study for flavonoids. This is in agreement with the reported by Falcão et al. (2010) and Popova et al. (2004) that refer the minor importance of gallic acid in propolis from temperate zones. This phenolic acid is mostly found in tropical samples. In propolis from the Mediterranean region prevailed flavonoids and esters of caffeic and ferulic acids.

Considering that the hydro-alcoholic extract was the most effective, it was used in all the assays performed after.

3.3. UV-Visible Absorption Spectroscopy

The absorption spectrum of the hydro-alcoholic extracts is shown in Figure 4. The spectrums of the analysed propolis were similar, with the maximum absorption between 290 nm and 370 nm. In agreement with Castro et al. (2007) the absorption profile between the 270 - 330 nm (wavelength) are attributed to flavonoids and phenolics. This suggests that the polyphenols are the biggest constituents of propolis. The small differences (p=0.103) in absorbance values reflect the concentrations of phenolic and flavonoids present in each propolis: propolis from Bragança possesses the highest amount of polyphenols and also has the highest value of absorbance.

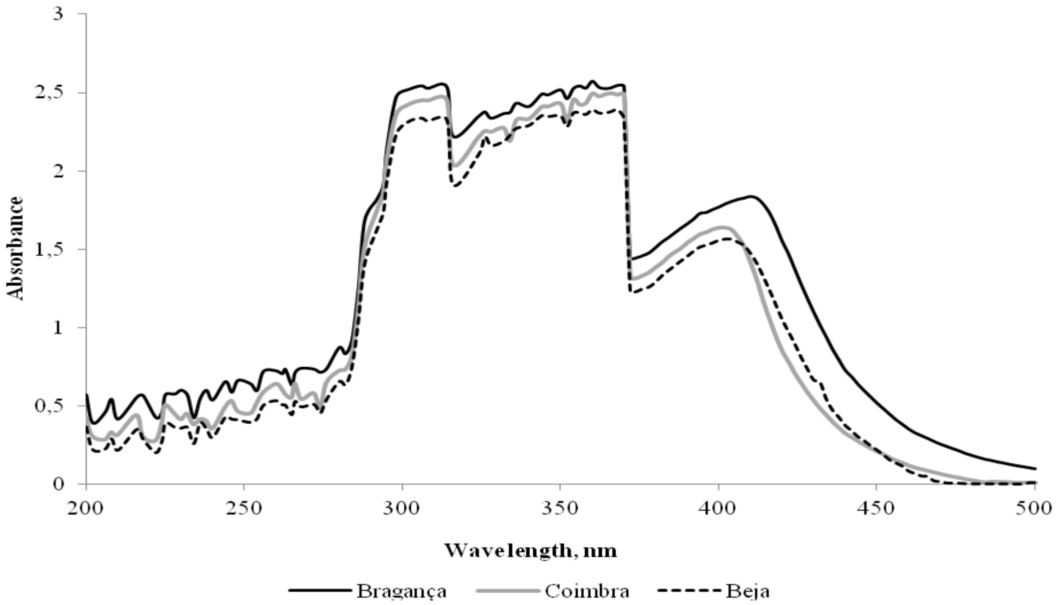

Figure 4 - Absorption spectra of the ethanolic propolis extracts from different locations.

3.4. Anti-inflammatory activity

The inflammation process involves production and/or release of mediators from neurons or damaged tissues, which are responsible for different responses including pain. Scavenging of free radicals, generated by neutrophils in inflammatory processes, is the principal mechanism of conventional anti-inflammatory drugs, and is also a known property of propolis (Paulino et al., 2003). In this study, we verified that all the extracts inhibited the hyaluronidase enzyme in a dose-dependent manner (Figure 5). The propolis that showed higher inhibitory activity was the one from Bragança and the product from Beja was the less effective. When the concentration of propolis was 25mg/mL, the percentage of inhibition was 75.79±2.17% (Bragança), 70.48±3.12% (Coimbra) and 53.76±2.87% (Beja).

Concerning the inhibition, it weren't found significant differences between the samples from Bragança and Coimbra, despite the differences amongst the polyphenols' concentrations. This suggests that these compounds are not the only factor responsible for the bioactive properties of this beehive product. In fact, other constituents like vitamins and proteins are also involved in this activity (Almeida-Muradian et al., 2005).

The action mechanisms of this product haven't yet been figured out. However, Hu et al. (2005) claimed that propolis inhibited the increase of prostaglandin E_2 and nitric oxid production, suggesting that both effects could decrease the inflammatory process.

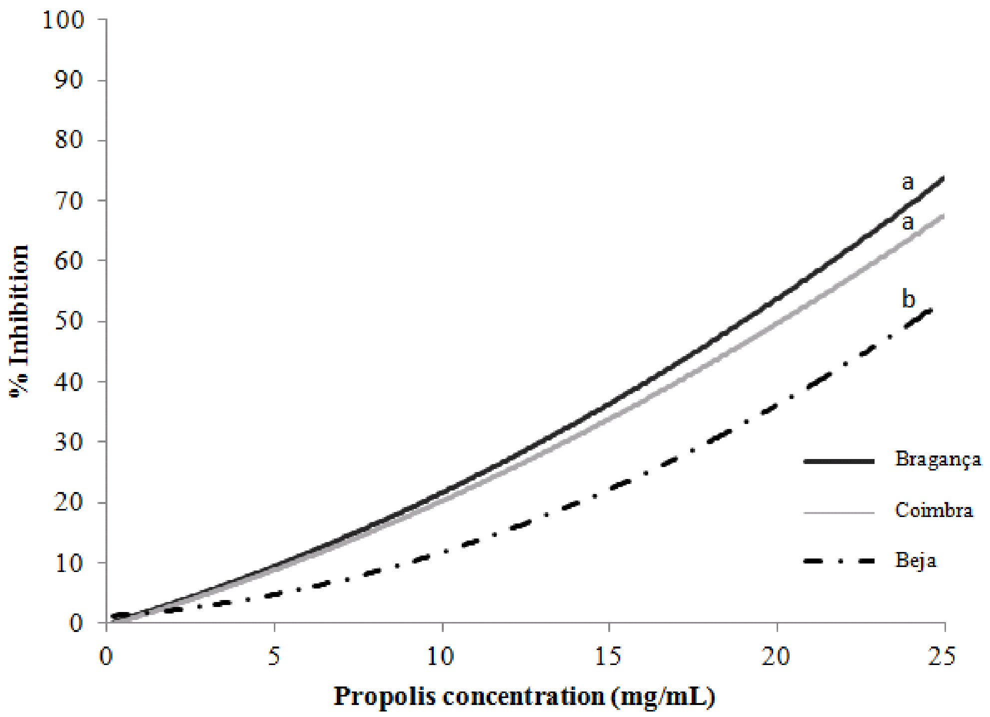

Figure 5 - Inhibition of the activity of Hyaluronidase by the propolis extracts for each concentration. The letters (a,b) represent which samples are different by Tukey test with significance of p = 0.05

3.5. Antimicrobial activity

Some of the common nosocomial infections are urinary tract infections, respiratory pneumonia, surgical site wound infections, bacteremia, gastrointestinal and skin infections. According to the Center for Disease Control and Prevention (Atlanta, USA), the most common pathogens that cause these infections are *Staphylococcus aureus* (Gram-positive), *Pseudomonas aeruginosa* (Gram-negative), *Escherichia coli* (Gram-negative) and *Candida albicans* (yeast). As it is very difficult to eliminate these microorganisms, due to their resistance to most antimicrobial agents, we decided to test the effect of propolis against them. Table 3 and Figure 6 depict the obtained results. All the propolis studied presented antimicrobial activity, but this effect depended on the origin of the product and the microorganism under study. Significant differences were found between the sample from Beja and the samples from Coimbra and Bragança ($p<0.001$). The activity of the last two samples didn't differ significantly ($p=0.142$). For all the microorganisms, the propolis from Beja was the least effective. Concerning the microorganisms, the post-hoc test indicates that *Candida albicans* (all the strains) was significantly different from the others ($p<0.001$). Once its MIC value was the highest (13.19 ± 7.21; 13.44 ± 8.23; 13.90 ± 7.512 mg/mL), it was the most resistant to the propolis' effect. The *Staphylococcus aureus* was the most sensitive to the propolis' effect (MIC: 0.59 ± 0.30; 1.36 ± 0.74; 1.72 ± 0.87 mg/mL). As it can be seen in Table 3, the propolis showed greater activity against Gram-positive bacteria than against Gram-negative. These results are in agreement with those of Vardar-Unlu et al. (2008) and Kim and Chung (2011). This may be explained by the structural differences of the bacterial cell wall of Gram-positive and Gram-negative bacteria. Gram-negative bacteria, apart from the cell membrane, possess an additional outer layer membrane, which consists of phospholipids, proteins and lipopolysaccharides, and this membrane is impermeable to most molecules (Silici and Kutluca, 2005).

Even though the action mechanisms aren't fully understood, the antimicrobial activity is potentially due to rutin, quercetin, naringenin. These compounds increase the permeability of the inner bacterial membrane, nullifying its potential, decreasing the ATP production, the membrane transport and its mobility (Tsuchiya and Iinuma, 2000). In addition, they inhibit the DNA gyrase which involves in the mechanism of DNA and RNA synthesis of bacteria (Mirzoeva et al., 1997).

Globally, the drug-resistance strains were more resistant to the hydro-alcoholic extract action than the reference strains (Table 3). Apart from *Candida*, it were found significant differences between the reference stains and the ones isolated from biological fluids.

These results, which are corroborated by Onlen et al. (2007), suggest that the simultaneous use of propolis and antibiotics may reduce the acquisition of resistances and consequently avoid the use of more powerful antibiotics.

Table 3 – Minimum Inhibitory Concentration (mg/mL) for the studied microorganisms and relation between the same specie (reference culture and isolated microorganisms), independently of propolis origin.

Microorganism	Mean ± standard deviation	Microorganism's effect (pvalue)
S. aureus ATCC	0.59 ± 0.30[b]	
S. aureus ESA 175	1.36 ± 0.74[a]	(<0.001)***
S. aureus ESA 159	1.72 ± 0.87[a]	
P. aeruginosa ATCC	1.56 ± 0.67[b]	
P. aeruginosa ESA 22	2.56 ± 1.07[a]	(0.035)*
P. aeruginosa ESA 23	2.81 ± 1.18[a]	
E. coli ATCC	3.19 ± 0.93[b]	
E. coli ESA 37	4.94 ± 1.42[a]	(0.043)*
E. coli ESA 54	4.86 ± 1.90[a]	
C. albicans ATCC	13.19 ± 7.21[a]	
C. albicans ESA 500	13.44 ± 8.23[a]	NS (0.968)
C. albicans ESA 502	13.90 ± 7.512[a]	

The letters a and b symbolize means that are significantly different.
NS- non significant; * p-value < 0,05; *** p-value < 0,001

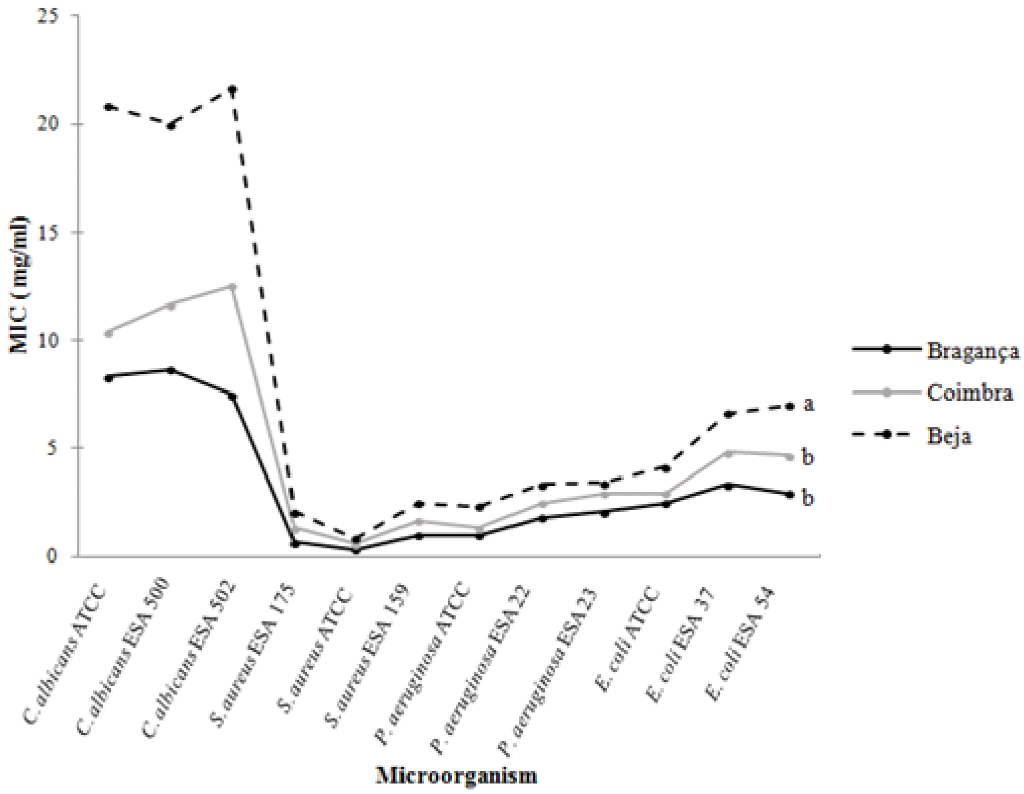

Figure 6 - Minimum Inhibitory Concentration (mg/mL) for each place and microorganism. The letters (a,b) represent which samples are different by Tukey test with significance of $p = 0.05$.

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CAPÍTULO III
Considerações finais

Considerações finais

Este estudo aborda as atividades antimicrobiana e anti-inflamatória do própolis Português. Os resultados obtidos permitem-nos tecer as seguintes considerações finais:

- A solução hidra alcoólica foi a mais eficaz na extração dos fenóis totais.

- A concentração em polifenois dependeu da origem botânica e geográfica do propolis.

- O extrato hidra alcoólico de própolis, em concentrações muito baixas inibiu a enzima hialuronidase.

- O própolis inibiu o crescimento de todos os microrganismos (leveduras, bactérias Gram-negativas e Gram-positivas) dependendo o efeito do tipo e da concentração de propolis bem como do microrganismo em estudo.

- As bactérias gram positivas foram os microrganismos mais sensíveis aos efeitos negativos induzidos pelo propolis no crescimento, sendo as leveduras as mais resistentes.

É necessário efetuar estudos adicionais, *in vivo* e *in vitro*, para confirmar a atividade anti-inflamatória do própolis Português.

Estes resultados sugerem que este produto apícola poderá vir a ser utilizado como alternativa terapêutica o completar no tratamento de doenças causadas por microrganismos resistentes a drogas e na prevenção das inflamações.

Em estudos posteriores, pretende - se elucidar os mecanismos de ação do própolis na célula e identificar os compostos responsáveis pelas suas propriedades biológicas, a fim de tirar vantagem deste produto natural, que, aparentemente, não apresenta efeitos secundários indesejáveis.