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VIVIANE ULBRICH FERREIRA

CARACTERIZAÇÃO QUÍMICA, ATIVIDADES ANTIOXIDANTE, ANTILEUCÊMICA E ANTIMICROBIANA DA PRÓPOLIS ÂMBAR SUL BRASILEIRA

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RESUMO

A própolis é um composto utilizado pelas abelhas, com a finalidade de vedar a colmeia e evitar contaminações, que se destaca por suas atividades biológicas as quais têm sido muito estudadas para fins terapêuticos. Quando produzida por abelhas da espécie *Apis mellifera* a substância é composta por cerca de 50 % de resina vegetal misturada a enzimas presentes em sua saliva e cera. Este produto natural pode variar dependendo da origem botânica/geográfica e mais de 300 compostos já foram descritos. No caso do Brasil, existe grande variabilidade da composição química que é facilmente explicada pela sua grande biodiversidade. O objetivo deste trabalho foi analisar o perfil químico e as atividades antioxidante, antileucêmica e antimicrobiana da própolis de São Gabriel/Rio Grande do Sul, a qual denominamos própolis Âmbar, e comparar suas propriedades com as própolis brasileiras Vermelha e Verde. As análises do perfil químico foram realizadas pelas técnicas de GC-MS, HPLC e quantificação dos flavonoides e fenóis totais. A atividade antioxidante foi aferida pelas técnicas DPPH°, $ABTS^{\circ+}$ e FRAP. A atividade antileucêmica foi analisada nas linhagens celulares K562, Jurkat e U937 pelos parâmetros de IC50, viabilidade, apoptose e ciclo celular. E a atividade antimicrobiana foi analisada aferindo-se o crescimento das espécies *E. coli* e *S. aureus*. Os resultados obtidos da análise por GC-MS das própolis Âmbar, dos dois anos coletados, identificam um total de 99 compostos dentre os quais apenas 16 foram identificados para as própolis Verde e Vermelha. Também foi possível notar que grande parte dos compostos encontrados são descritos para o gênero *Eucalyptus* que parece ser uma fonte vegetal importante para a produção da própolis Âmbar. Quanto aos fenóis totais, flavonóides totais e atividade antioxidante, as própolis apresentaram resultados diferentes entre si, sendo os valores obtidos para a própolis Âmbar sempre menores que os encontrados para as própolis Verde e Vermelha. Porém quanto à atividade antileucêmica a própolis Âmbar apresentou resultados similares a própolis Vermelha nas análises de IC50 e viabilidade. E na análise do efeito antimicrobiano todas as própolis igualaram seu efeito em concentrações acima de 500 µg/mL, apresentando também atividades semelhantes no tratamento com *E.coli* na concentração de 100 µg/mL. Tipificação, identificação

da origem geográfica/botânica e quantidade de estudos sobre suas atividades biológicas agregam valor à própolis. Esperamos com este trabalho expandir o conhecimento técnico-científico da própolis Âmbar contribuindo com o desenvolvimento regional e ampliando o conhecimento sobre própolis.

Palavras-chave: Própolis brasileira; Atividade antimicrobiana; Atividade antileucêmica; Atividade antioxidante.

ABSTRACT

Propolis is a compound used by bees to seal the hive and prevent contamination that stands out because of its biological activities which have been studied for therapeutic purposes. When produced by *Apis mellifera* bees specie the substance is composed by about 50% vegetable resin mixed with enzymes present in its saliva and wax. This natural product may vary depending on the botanical/geographical origin and more than 300 compounds have already been described. In Brazil there is a great variability of chemical composition that is easily explained by the Brazilian biodiversity. The goal of this work was to analyze the chemical profile and antioxidant, antileukemic and antimicrobial activities of São Gabriel/Rio Grande do Sul propolis, which we call Amber propolis, and to compare its properties with the Brazilian propolis Red and Green. The chemical analyzes were performed by total flavonoids and total phenols quantification, GC-MS and HPLC. The antioxidant activity was measured by DPPH°, $ABTS^{\circ+}$ and FRAP techniques. The antileukemic activity was analyzed in the K562, Jurkat and U937 cell lines taken into account IC50, viability, apoptosis and cell cycle parameters. The antimicrobial activity was analyzed by *E. coli* and *S. aureus* growth. The results obtained from the GC-MS of Amber propolis, collected in two years, identified a total of 99 compounds among, from these only 16 were identified for the Green and Red propolis. It was also possible verify that most of the compounds found are described for the genus *Eucalyptus* which seems to be an important source of compounds for the Amber propolis production. Values for total phenolics, total flavonoids and antioxidant activity, were different among the propolis, being the values obtained for the Amber propolis always smaller than those found for the Green and Red propolis. However, regarding the antileukemic activity, the propolis Amber presented similar results to the Red propolis for IC50 analyzes and viability. In the analysis of the antimicrobial effect, all the propolis presented similar effects above 500 µg/mL and also presenting some levels of activity at 100 µg / mL in *E. coli*. Typification, identification of the geographical/botanical origin and quantity of studies on its biological activities add value to propolis. We hope with this work to expand the technical-scientific knowledge of propolis Amber contributing to regional development and expanding knowledge about propolis.

Keywords: Brazilian propolis; Anti-microbial activity; Anti-leukemic activity; Anti-oxidant activity.

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1. INTRODUÇÃO

Própolis, “cola de abelha” e/ou “cera negra”, é uma substância resinosa semelhante à cera natural, encontrada em colmeias. A composição da própolis bruta é dividida basicamente em 50% de resina de vegetais, 30% de cera de abelha, 10% de óleos essenciais, 5% de pólen e 5% de detritos (Ghisalberti et al, 1978).

A história da relação entre homens e as abelhas é muito antiga, havendo registros de representações de abelhas e da apicultura em trabalhos arqueológicos datados do ano 13.000 a.C (Kuropatnicki et al., 2013). A própolis também tem sido utilizada pelo homem há séculos, com registros que sugerem o seu uso pelos antigos egípcios, persas e romanos (Chan et al., 2013). Na cultura egípcia, onde os rituais fúnebres tinham grande importância, a própolis era utilizada como substância de “embalsamento”, inspirando-se na utilização pelas abelhas da própolis e cera para cobrir animais que foram mortos dentro das colmeias, com a finalidade de proteger a colmeia, daí o significado da palavra própolis, que é derivado do grego onde (pro = em defesa da; polis = população) (Bankova et al., 2000; Castaldo e Capasso, 2002; Kuropatnicki et al., 2013; Salatino et al., 2005).

Na Idade Média a própolis perdeu sua popularidade e seu uso na medicina tradicional logo desapareceu, mas algumas fontes do século XII descrevem preparações medicinais contendo cola de abelha, que foram utilizadas no tratamento de infecções de boca e faringe, como cárie dentaria (Kuropatnicki et al., 2013).

O interesse pela própolis retornou no inicio do século XIX, mas no Brasil a primeira publicação sobre a própolis ocorreu apenas no ano de 1984, em um estudo comparativo do efeito antibiótico (Pereira et al., 2002). Apesar do início tardio, na década de 90 o país aumentou bastante o número de trabalhos ficando entre os principais países em quantidade de publicações. Contudo deve-se destacar que no mesmo período o número de patentes brasileiras depositadas sobre a própolis foi reduzido (3 patentes/37 trabalhos publicados), ao contrário de países como o Japão que teve 43 trabalhos publicados e depositou 98 patentes, incluindo patentes sobre a publicação de compostos isolados inicialmente de amostras de própolis brasileira (Pereira et al., 2002). Este interesse industrial se deve às inúmeras atividades biológicas associadas à própolis.

Entre as atividades biológicas mais estudadas da própolis encontram-se as atividades antimicrobiana (Dodrowolski et al.,1991; Grange e Davey, 1990; Kujumgiev et al., 1999), antioxidante (Frozza et al., 2013; Kumazawa et al., 2004) e antitumoral (Chan et al., 2013; Scheller et al.,1989); porem a lista se estende para efeitos antivirais (Amoros et al., 1992; Kujumgiev et al., 1999), anti e proinflamatório (Conti et al., 2015; Dodrowolski et al., 1991), anti-hipertensivo (Kubota et al., 2004; Mishima et al., 2005) redutor dos níveis de colesterol (Yu et al., 2011), ansiolítico e antidepressivo (Reis et al., 2014) entre outras.

Em geral, as abelhas coletam resinas de plantas em seu ambiente, e as depositam como "própolis" (Simone-Finstrom e Spivak, 2010), devido às suas propriedades físicas. Por outro lado, este material é também a sua defesa contra micro-organismos, com base na sua composição química (Chan et al., 2013). Embora, a própolis seja considerada um produto animal, uma porção considerável de seus componentes, principalmente aqueles que possuem atividade biológica, são derivados de plantas (Salatino et al., 2005). Estes compostos vegetais, conhecidos como metabólitos secundários, defendem as plantas contra herbívoros e microrganismos patogênicos; as três principais classes de metabólitos secundários são os terpenos, compostos fenólicos e compostos nitrogenados (Tai e Zeiger, 2004).

O desenvolvimento de pesquisas sobre a composição da própolis está inteiramente relacionado com o desenvolvimento da química, começando na década de 1970 com os avanços em métodos analíticos cromatográficos como, por exemplo, a cromatografia em camada fina, que permitiu a separação e extração de vários compostos da própolis. Em 1970 Vanhaelen e Vanhaelen-Fastré utilizaram cromatografia gasosa (GC) e cromatografia líquida de alto desempenho (HPLC) para analisar a própolis. Mais tarde a aplicação de espectrometria de massas acoplada a cromatografia gasosa (GC-MS) levou a identificação de açúcares em própolis (Kuropatnicki et al., 2013).

A composição química complexa é um grande problema para o uso da própolis brasileira na “fitoterapia ou apiterapia” devido à alta variabilidade que é influenciada pela localização geográfica, época de colheita e genética da abelha (Bankova et al., 2000; Kumazawa et al., 2004; Park et al., 2002; Pereira et al., 2002). No caso do Brasil essa variação das propriedades biológicas e composição química

são facilmente explicadas pela grande biodiversidade brasileira que tornam a análise da própolis uma tarefa complexa, onde há mais exceções do que regras (Kuropatnicki et al., 2013; Pereira et al., 2002).

Sabe-se que as resinas de algumas plantas como o eucalipto, *Corymbia citriodora*, *Araucaria angustifolia*, *Baccharis dracunculifolia e Dalbergia ecastophyllum* são preferidas no Brasil pelas abelhas, quando disponíveis (Park et al., 2002 and 2004; Silva et al., 2008), mas há muitas espécies co-ocorrendo com essas plantas nas regiões em que estão localizadas, e não está claro como ou porque as abelhas, entre diferentes plantas, escolhem uma e não outra para recolher resina. Também não está claro se certas plantas resinosas são mais benéficas para as abelhas do que outras (Wilson et al., 2013).

1.1. Interesse regional

A história da Apicultura no Brasil tem forte relação com o sul do país desde sua implementação, que corresponde ao período entre 1839 a 1955, quando ocorreu o início da exploração da apicultura pelos colonizadores europeus que foi alicerçada com tecnologias importadas da Europa, em especial alemã, destacando-se sua influência no Município de Rio Pardo (RS), berço da apicultura brasileira e que aos poucos se expandiu para o Sudoeste brasileiro e demais regiões, antes da chegada das abelhas africanas (*Apis mellifera scutellata*) ao Brasil em 1956 (Oliveira e Cunha, 2005).

Até o último censo em 2015 a Região Sul se manteve como a maior produtora de mel e foi responsável por 37,3% do total nacional, seguida pelas Regiões Nordeste (32,6%), Sudeste (23,4%), Centro-Oeste (4,2%) e Norte (2,5%), mas apresentou redução de 14,2% na sua produção em relação ao ano anterior (IBGE, 2015). O Rio Grande do Sul que teve uma queda de 17,8% na produção de 2014, embora fosse o maior produtor, sofreu uma nova queda em 2015 (-17,2%) e perdeu a posição para o Paraná que cresceu 10,5% (IBGE, 2014 e 2015).

Cabe salientar que o Rio Grande do sul, embora seja um ator de destaque na produção de mel, não se destaca nacionalmente na produção de outros produtos apícolas.

O Brasil apresenta características especiais de flora e clima que, aliados à presença da abelha africanizada, lhe conferem um potencial fabuloso para a atividade apícola, ainda pouco explorada. A produtividade brasileira ainda se encontra reduzida quando comparada com a produção internacional. A baixa produtividade dos apiários brasileiros se explica pela pouca utilização de recursos tecnológicos na produção (SEBRAE, 2006).

O atual interesse comercial na própolis pode ser mais um atrativo na retomada da sua produção, uma vez que, além do seu uso fitoterápico, ela também é um recurso importante para a sanidade apícola, mantendo a colmeia saudável e reduzindo consideravelmente o crescimento de microrganismos (Finstrom e Spivak, 2010).

O último Censo Agropecuário realizado pelo IBGE, no ano de 2015, não apresentou dados unitários para a produção de própolis, mas em Junho de 2014 o SEBRAE apresentou um boletim apenas do mercado da própolis (SEBRAE, 2014), uma vez que a crescente produção de artigos científicos relacionados à aplicação e composição química da própolis brasileira ocasionou um aumento na produção da própolis, sendo então o Brasil o terceiro maior produtor mundial, chegando a 150 toneladas anuais (Brighenti et al., 2014).

O Japão é o principal importador de própolis, com uma preferência manifestada pela própolis do Brasil (Kuropatnicki et al., 2013; Salatino et al., 2005; SEBRAE, 2014). Brasil é responsável por apenas 15% de toda a produção mundial de própolis e 67% do que é produzido no Brasil é exportado para Japão, Estados Unidos, Alemanha e China (Brighenti et al., 2014). O comércio Brasil/Japão movimenta cerca de 300 milhões por ano, 92% da própolis consumida no Japão é de origem brasileira (Toledo, 2007 *apud* Brighenti et al., 2014; SEBRAE, 2014). Outro aspecto de grande importância nesta área tem sido a estabilização dos preços do produto no mercado, custando 500 reais o Kg de material bruto, e de acordo com dados da Japan Trade Organization o extrato alcoólico da substância é vendido no Japão a US$ 110 o frasco (SEBRAE, 2014). Consulta recente no site Amazon permite observar que 30 mL de extrato de própolis Brasileira (não especifica o tipo de própolis) custa entre 15 e 40 dolares (acessado em 20 de outubro de 2016, usando o termo “brazilian propolis” no site www.amazon.com).

O valor do produto está agregado a sua tipificação, identificação de origem geográfica/botânica e teor total de fenóis e flavonoides. Ausência de contaminantes químicos e biológicos têm sido alguns itens fundamentais na valorização e melhor comercialização do produto.

Apesar disso, o mercado ainda valoriza o aspecto visual da própolis, supervalorizando alguns tipos como a própolis verde produzida na Região Sudeste, a resina provem de botões florais de *Baccharis dracunculifolia* e a vermelha produzida nos mangues de Alagoas e a resina provem de *Dalbergia ecastophyllum*, o boletim do SEBRAE de 2014 fala apenas do mercado desses dois tipos de própolis que são denominadas verde e vermelha devida a sua coloração (Park et al., 2002 and 2004; Silva et al., 2008). Existem regiões que não produzem própolis verde nem vermelha, sendo discriminados no mercado, desmotivando sua produção.

1.2. Atividade antileucêmica da Própolis

A leucemia é a enfermidade em que a medula óssea produz glóbulos brancos anormais, as células leucêmicas, que podem se proliferar rapidamente, se dividem de forma descontrolada e possuem resistência à morte programada. O baixo nível de células sanguíneas normais pode tornar mais difícil para o corpo para obter oxigênio para os tecidos, controle de sangramento, ou combater infecções. Além disso, as células leucêmicas podem se espalhar para outros órgãos, como os linfonódos, baço e cérebro (NCI, 2013). O INCA estima que em 2016 ocorreram no Brasil 10.070 novos casos de leucemia, sendo 5.540 homens e 4.530 mulheres (INCA, 2016).

Os quatro principais tipos de leucemia são: leucemia linfoide crônica (LLC), que afeta células linfoblásticas e se desenvolvem lentamente; leucemia mieloide crônica (LMC), afeta células mieloblásticas e se desenvolvem lentamente, é extremamente diferenciadas das células mieloides (medula), sugerindo a diferenciação de células-tronco mieloide em diversos tipos celulares distintos; leucemia linfoide aguda (LLA), surgem tanto em células B (80%) como em linhagens de células T (20%) de linfócitos e agrava-se rapidamente; e leucemia mieloide aguda

(LMA), na qual as células possuem um núcleo grande com uma pequena camada ao seu redor de citoplasma é uma doença que avança rapidamente (NIH, 2017).

As células K562 foram isoladas de uma paciente diagnosticada com Leucemia Mielóide Crônica (LMC) em crise de explosão (Lozzio e Lozzio,1975). São células que possuem uma translocação cromossômica 9:22, envolvendo o gene ontogênico c-abl, que é uma característica comum dos pacientes com LMC. Além disso, o gene c-abl é amplificado de 4 a 8 vezes em células K562 (Collins e Groudine, 1983).

A linhagem celular Jurkat, originalmente nomeada de JM, é utilizada para o estudo da Leucemia Linficítica Aguda. Estabelecida a partir do sangue periférico de um paciente, a linhagem expressa características de células T e receptores do complemento (Schneider et al., 1077). E também possui vários receptores de quimiocinas susceptível a entrada viral, particularmente HIV e são capazes de produzir interleucina 2 (Takeuchi et al., 2008).

A linhagem celular hematopoiética humana (U937) foi obtida de um paciente com linfoma histiocítico verdadeiro generalizado por Sundstrom e Nilsson (1976), é proveniente de precursor mieloide e possui muitas características de células monociticas servindo como modelo *in vitro* para diferenciação monócitos/macrófagos (ATCC, 2017; ABCAM, 2017).

As pesquisas para o tratamento do câncer têm como principal alvo fármacos que bloqueiam o ciclo celular e induzem a apoptose sem induzir inflamação ou danos em células normais (Abubakar et al., 2014; Gautam et al., 2014). O ciclo celular regula a transição da quiescência (G0), para a proliferação e as fases associadas com a síntese de DNA (fase S) e mitose (M) que são separadas por intervalos G1(*Gap* 1) e G2 (*Gap* 2)/M. Células normais assim que completam o ciclo celular recebem sinais para seguir crescendo e dividindo ou para entrar em estado não proliferativo (fase G0), no entanto as células cancerosas tem sua sinalização do controle celular normal rompido (Weinberg, 2008), ou seja, não finaliza o ciclo de replicação celular (não retorna a fase G0), assim passa da fase M para nova fase G1 (Almeida et al., 2005).

A apoptose, ou morte celular programada, apresenta alterações morfológicas e bioquímicas como encolhimento celular, fragmentação do DNA, formação de prolongamentos da membrana celular (*blebs*), condensação da cromatina, perda de

adesão e arredondamento. Sendo regulada por várias proteínas, exemplos as proteínas p53, IAPs (*Inhibitor of Apoptosis Protein*), caspases e Bcl. O aumento no número de cópias, mutação ou a delação dessas proteinas faz com que as células crescam de forma independente, dessa forma as células neoplásicas param de checar os erros e falhas que provocariam a morte por apoptose ou parada no ciclo celular (Abubakar et al., 2012; Belizário, 2002).

Sendo a própolis um produto natural que tem sido utilizada na medicina popular desde tempos antigos, recentemente, tornou-se um assunto de especial interesse na área de pesquisa oncológica, como uma fonte de compostos com atividades biológicas valiosas para a prevenção e tratamento do câncer. A própolis não pode ser usada diretamente como matéria-prima e deve ser purificada por extração para remover o material inerte e preservar a fração com atividade biológica (Szliszka e Krol, 2013).

Franchi Jr. et al., (2012) mostraram por teste de MTT (3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina) que a própolis vermelha e a verde tem compostos químicos capaz de inibir o crescimento de diferentes células de linhagem leucêmica. Efeitos citotóxicos da própolis vermelha (extrato hidroalcoólico) em linhagens celulares de câncer de Hep-2 e HeLa e em células de linhagem não tumoral (HEK-283), também foram mostrados por (Frozza et al., 2013), onde extrato de própolis foi capaz de inibir a proliferação das linhagens de células de câncer de forma significativa quando comparado a células de linhagem não tumoral.

O efeito inibidor contra o crescimento de células de câncer por diferentes amostras de própolis pode estar relacionado com um efeito geral de compostos químicos presentes em cada extrato, na região e ano em que as amostras foram recolhidas. Os resultados *in vitro* confirmaram os efeitos citotóxicos da própolis em diferentes linhagens de células de câncer, indicando uma atividade antitumoral, tendo como principal efeito inibir a proliferação do crescimento celular (Sawicka et al., 2012).

Parece haver barreiras aos estudos clínicos humanos das atividades da própolis contra a doença, provavelmente porque a própolis é uma mistura complexa de compostos ativos diferentes que podem ser difíceis de padronizar, principalmente no Brasil onde há grande biodiversidade que tornam a análise da própolis uma tarefa ainda mais complexa (Bankova et al. 200o; Sforcin e Bankova, 2011).

1.3. Atividade antimicrobiana da Própolis

Além de sua utilização puramente mecânica como espécie de cola e cimentação, a própolis e sua base química podem servir para conter putrefação e propagação de infecções e doenças, proporcionando um ambiente hostil para o crescimento de bactérias e outros microrganismos. O estudo mais antigo da atividade antibacteriana da própolis foi realizada por Kivalkina na década de 1940 demonstrando que a própolis utilizada possuia actividade bacteriostática contra *Streptococcus*, contra o bacilo da febre tifóide, e algumas outras bactérias (Ghisalberti,1979).

Lindenfelser (1967) realizou uma análise abrangente da atividade antimicrobiana com 15 diferentes amostras de própolis contra 25 espécies diferentes de bactérias (incluindo: *Mycobacterium spp*., *Pseudomonas spp*., *Xanthomonas spp*. e *Bacillus spp*.) e 20 espécies diferentes de fungos (incluindo: *Aspergillus spp.*, *Trichophyton spp.*, e *Claviceps purpurea*). Esse estudo descobriu que pelo menos uma das 15 amostras de própolis testadas na dosagem de 100 µg/mL inibia cada patógenos individualmente. Dos 45 patógenos testados, *Paenibacillus larvae* foi inibida por todas as 15 amostras de própolis (Wilson, 2014).

Em outro exemplo o uso da própolis inibiu completamente o crescimento de *Staphylococcus aureus*, incluindo a estirpe MRSA (*S. aureus* resistente à meticilina). Também inibindo o crescimento de *Escherichia coli* parcialmente, indicando assim um efeito preferencial em cocos e bacilos Gram-positivos (Grange e Davey, 1990).

Parece que a própolis tem uma atividade antimicrobiana geral, particularmente contra bactérias gram-positivas (Burdock, 1998; Grange e Davey 1990; Kujumgiev et al., 1999; Marcucci, 1995). De fato, uma das doenças mais agressiva para colmeia é causada por uma bactéria Gram-positiva, *Paenibacillus larvae*, formadora de endosporos que causa a Loque Americana em abelhas. Contudo resultados com bactérias Gram-negativas sugerem que a ação da própolis depende da espécie, possivelmente pode estar relacionada com a proteína porina ou a molécula lipopolissacarídeo que compõem a membrana. (Mirzoeva et al., 1997) ou pode variar dependendo da região em que a resina para produção da própolis foi coletada (Burdock, 1998; Kujumgiev et al., 1999).

Este remédio natural também parece poder inibir a replicação do DNA e, indirectamente, a divisão celular, como demonstrado por estudos de microscopia eletrônica e microcalorimetria de *Streptococcus agalactiae* tratadas com própolis. Além disso, a análise de proteínas celulares e segregadas de células tratadas com própolis indicou que esta inibe a síntese e secreção de proteínas das células bacterianas. Demonstrando um mecanismo complexo e que não pode ser comparado com qualquer antibiótico clássico (Takaisi-Kikuni e Schilcher, 1994).

A própolis parece conter também constituintes que aumentam a permeabilidade da membrana e inibem a motilidade bacteriana demonstrando que a própolis contém componentes que atuam como ionóforos (Mirzoeva et al., 1997). Outro aspecto é que a actividade da própolis em bactérias cultivadas em ágar sólido foi mais fraca do que a das bactérias incubadas em meio líquido (Mirzoeva et al., 1997). Esta situação também foi observada em dois estudos que compararam diferentes métodos para testar o efeito do extrato de própolis contra espécies de *Candida*, *Staphylococcus* e *Streptococcus*; neste estudos, os resultados mais claros foram obtidos por diluição em série em tubo ou placas do que pelo ensaio de difusão (Sawaya et al., 2002 e 2004).

Há um grande potencial para descobrir novos compostos biologicamente ativos na própolis. Além da capacidade de inibir diretamente o crescimento microbiano, a própolis tem sido relatada como aumentando a susceptibilidade de bactérias gram-positivas (*Bacillus subtilus*) e gram-negativas (*Escherichia coli*) aos antibióticos tradicionais 1,2 a 1,75 vezes, mesmo quando o tratamento com própolis não apresenta nenhum efeito aparente sobre o crescimento bacteriano (Mirzoeva et al., 1997).

1.4. Atividades antioxidante da Própolis

O uso clínico da própolis como mistura ainda é tímido, mas muitos compostos com atividades biológicas já foram isolados de amostras de própolis, como ácido 3,5-diprenil-4-hidroxicinâmico (Artepillin C) a partir da própolis verde brasileira, o qual tem se mostrado um dos principais componentes com efeitos imunomoduladores (Cheung et al., 2011). E o éster fenílico do ácido caféico (CAPE), considerado como um importante composto ativo da própolis vermelha, o qual acredita-se ser o

principal responsável pelas atividades terapêuticas antitumorais da própolis (Sawicka et al., 2012).

Apesar da complexidade da composição química da própolis, muitos autores atribuem sua atividade biológica à concentração de compostos fenólicos, particularmente os flavonóides (Burdock, 1998; Castaldo e Capasso, 2002; Ghisalberti, 1979, Grange e Davey, 1990; Marcucci, 1995).

Os flavonóides são um grupo diverso de fitoquímicos que são produzidos por diversas plantas em quantidades elevadas (Kuropatnicki et al., 2013; Tais e Zeiger, 2004). Possuem atividade antioxidante potente eliminando radicais livres, que podem interferir amplamente com o metabolismo da célula normal. Também possuem um amplo espectro de atividades biológicas no corpo humano, grande parte resultado de seus efeitos antioxidantes. Eles protegem os lipídeos e outros compostos, tais como a vitamina C de ser oxidada ou destruída (Kurek-Górecka et al., 2014; Kuropatnicki et al., 2013).

Neste trabalho o foco foi análisar as atividades antioxidantes, antimicrobiana e antileucemica da própolis produzida em florestas de eucalipto associadas ao bioma Pampa no estado do Rio Grande do Sul cujo extrato etanólico apresenta coloração âmbar. Essas atividades biologicas já foram relatadas para as própolis vermelha e verde que utilizamos como referência neste estudo. Também estudamos a composição química desta a qual denominamos própolis Âmbar, devido a sua coloração e caracterítica física, a fim de conhecer melhor sua principal fonte botânica. Estes conhecimentos podem tornar a produção dessa própolis mais atrativa para os apicultores e agregar valor ao que é considerado um produto secundário da colméia.

2. OBJETIVOS

2.1 Objetivo Geral

Estudar a própolis do Município de São Gabriel (RS) (aqui denominada como própolis âmbar) quanto aos efeitos antileucêmico, antimicrobiano e antioxidante, avaliando também sua composição química.

2.2 Objetivos específicos

Analisar as propriedades antioxidantes e perfil de polifenóis dos extratos etanólicos das própolis Âmbar (2014/2015), comparando-as com as própolis Vermelha e Verde;

Estudar o efeito antileucêmico dos extratos etanólicos das própolis Âmbar, coletadas nos anos 2014 e 2015, através de análise da viabilidade celular, ciclo celular e apoptose nas linhagens leucêmicas K562, U937 e Jurkat, comparando-as com as própolis Vermelha e Verde;

Definir o IC50 dos extratos etanólicos das própolis Âmbar (2014/2015), Vermelha e Verde nas linhagens celulares K562, U937 e Jurkat;

Estudar o efeito antimicrobiano dos extratos etanólicos das própolis Âmbar (2014/2015), comparando-as com as própolis Vermelha e Verde no enfrentamento contra culturas líquidas de *Escherichia coli* e *Staphylococcus aureus;*

Caracterizar sua composição química por cromatografia gasosa associada a espectrometria de massas, a fim de definir sua provável origem botânica.

3. MANUSCRITO

South Brazilian amber propolis chemical profile and its antimicrobial, antioxidant, and antileukemic activities

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Title: **South Brazilian amber propolis chemical profile and its antimicrobial, antioxidant, and antileukemic activities**

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ABSTRACT

Propolis is composed mainly of resin collected by bees from plants, mixed with saliva and wax; it is used by bees to waterproof the hive and to preserve hive health. Considering it well known biological activities propolis is widely consumed due to its benefits to human health. In this work, we analyzed the chemical profile and evaluated the antioxidant, antileukemic, and antimicrobial activity of a new Brazilian propolis we named “amber”, collected in 2014 and 2015 at São Gabriel city (Rio Grande do Sul state). Also, we compared its activities with Red and Green propolis samples. Despite the substantial difference in the chemical composition among the three propolis. Amber propolis presented high antileukemic and antimicrobial activities, similar to red propolis and higher than green propolis, although it presented low phenolic compound concentration and lower antioxidant activity than red and green propolis. GC-MS analysis revealed that amber propolis is rich in essential oil compounds and that most of the compounds found have already been described for *Eucalyptus*, indicating this genus as an important and stable source of compounds for amber propolis.

Keywords: Brazilian propolis; Anti-microbial activity; Anti-leukemic activity; Anti-oxidant activity.

1. Introduction

Propolis or "bee glue" is a resinous substance produced by several eusocial Hymenoptera such as the bees in order to protect the hive. The name derives from the Greek (pro = in defense; polis = population) and is comprised of approximately 50 % resins and vegetable balsams which bees collect from leaves and shoots, 30 % wax, 10 % essential oils, 5 % pollen and 5 % of other components and debris (Burdock, 1998; Cirasino et al., 1987; Ghisalberti, 1979; Marcucci, 1995; Monti et al., 1983).

Bees use propolis to seal unwanted openings in the hive, to create a smooth surface for the comb, to embalm parasites and predators and to protect the hive against microbial pathogens (Ghisalberti, 1979) in a self-medication mechanism (Finstrom and Spivak, 2012).

Humans have been using bee propolis for its benefits to health since ancient times, dating from the year 300 BC (Ghisalberti, 1979), and its use continues nowadays (Burdock, 1998; Castaldo and Capasso, 2002; Kuropatnicki et al., 2013; Sforcin, 2016).

The most investigated activities of propolis, described so far, are the antimicrobial (Dodrowolski et al., 1991; Grange and Davey, 1990; Kujumgiev et al., 1999), antioxidant (Frozza et al., 2013; Zhao et al., 2016) and antineoplastic (Chan et al., 2013; Scheller et al., 1989) activities; however, the list extends to the following activities: antiviral (Amoros et al., 1992;. Kujumgiev et al., 1999; Vynograndet al., 2000), anti or proinflammatory (Dodrowolski et al., 1991; Conti et al., 2015; Mirzoeva and Calder, 1996;), antihypertensive (Kubota et al., 2004; Mishima et al., 2005b) cholesterol levels reduction (Yu et al., 2011), anxiolytic and antidepressant (Reis et al., 2014), among others.

Chemical analyses of different type of propolis described more than 300 compounds. Among these compounds, phenolic acids, flavonoids, terpenoids, fatty acids, beeswax, bioelements and other components such as vitamins, proteins, amino acids and sugars were detected; for example, in the Polish propolis the amount of biologically active compounds can reach 70 % and 58 % of this amount are part of the polyphenols group and 20 % are flavonoids (reviewed in Kurek-Górecka et al., 2014).

Polyphenols are suggested to be the potentially active compounds in antioxidant and antineoplastic activity (Abubakar et al., 2014 and Kurek-Górecka et al., 2014). However, the chemical composition of propolis varies both geographically and seasonally; thus, each propolis has particular therapeutic potentials and it would not be correct to attribute its potential to a single or a cluster of substances, neither attribute identical properties for distinct propolis (Kujumgiev et al., 1999). For example, regarding the effect on the immune system, some propolis can develop pro-inflammatory effects and other anti-inflammatory effects (Conti et al., 2015).

Brazil has a gargantuan area and has a wide range of ecosystems and, for these reasons, it would be very difficult to estimate the number of propolis varieties that could be found. In order to catalog these propolis Park et al. (2000) analyzed 12 types of propolis, collected from 7 Brazilian states, based on their physico-chemical characteristics, cataloging them by their color and absorption spectra; at the same time, each propolis were tested for antimicrobial, antioxidant and anti-inflammatory activity, among them the propolis G12 (called “green propolis”) showed the better results. The same research group identified the botanical origin of the green propolis as coming from the resin buds of *Baccharis dracunculifolia* (Park et al., 2002 and 2004). Later, a thirteenth type of propolis was collected in Alagoas state mangrove hives and was called red propolis; the analysis of the chemical compounds of red propolis showed its base derived from the *Dalbergia ecastophyllum* resin (Silva et al., 2008). Another typified Brazilian propolis is the brown propolis, produced at Paraná State, derived from *Araucaria heterophylla* (Sawaya et al., 2011).

Brazilian green and red propolis are extensively studied and both present several biological activities such as anti-microbial and anti-neoplastic, among other, being more pronounced in the Red propolis (Franchi Jr. et al., 2012; Machado et al., 2016). However, taking into account the extension of the Brazilian territory, it is possible to find other varieties of propolis that possess similar biological activities to those presented by the red or green propolis.

In this work, we characterized a new type of propolis collected in Southern Brazil, which we called “amber propolis”, based in its color appearance, by evaluating its antioxidant, antileukemic and antibacterial properties, as well as profiling its chemical composition by HPLC and GC-MS.

2. Materials and methods

2.1. Reagents

The medium for cell lines growth, RPMI 1640 medium (Applichem, Germany), Fetal Bovine Serum, penicillin and streptomycin (Gibco, Brazil) were used. The bacterial culture medium was composed by tryptone (Neogen USA) and HiMedia yeast extract (Acumedia Mumbai, India). YOPRO®-1 iodide was purchased from Invitrogen (USA), Propidium Iodide (≥ 94.0 % purity), Folin-Ciocalteu radical 2,2-diphenyl-1-picrylhydrazyl (DPPH°), diammonium salt 2 2-azinobis- [3-ethyl-benzotiazolin-6-sulfonic acid] sodium acetate, 2,4,6-tris (2-pyridyl) -s-triazine (TPTZ), quercetin, rutin and luteolin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gallic acid, aluminum chloride, potassium persulfate, ammonium sulfate, iron (II) hexahydrate and dimethyl sulfoxide (DMSO) were purchased from Vetec Fine Chemicals LTD (Rio de Janeiro, RJ, Brazil). Acetonitrile, phosphoric acid, chlorogenic acid, caffeic acid, p-coumaric acid and ellagic acid were purchased from Merck (Darmstadt, Germany). All other chemicals used in this work have analytical grade.

2.2. Origin of Propolis

We tested Brazilian propolis samples (here named amber propolis) produced by *Apis mellifera*, collected in 2014 (March) and 2015 (September) from São Gabriel city, located in Rio Grande do Sul state (the Southernmost state of Brazil). As reference for comparisons, we used commercial raw propolis from Alagoas state (red propolis) and Minas Gerais state (green propolis). In all state cited, bees are considered Africanized.

Figure 1. Image of the propolis *in natura*. Propolis Amber (A), Red Propolis (B) and Green Propolis (C).

2.3. Propolis extract production

All ethanolic propolis extracts (EEP) tested in this work were initially prepared by dilution of raw propolis in ethanol (10 % w/v), with regular stirring, during 7 days at room temperature. On the 7th day, the extracts were centrifuged at 1.600 × g for 10 min and the supernatant was removed and filtered with a filter paper. Ethanol was evaporated in a vacuum concentrator (Eppendorf Concentrator Plus) at 60 °C until complete evaporation. The extracts were solubilized in dimethyl sulfoxide (DMSO) (EEP/DMSO) for the treatment of leukemic cells or in absolute ethanol (EEP/EtOH) to observe antimicrobial activity (final concentration of 10 % w/v). Prior to testing, all the extracts were filtered through 0.45 μM pore-sized membrane. Propolis extracts used to analyze anti-leukemic and anti-microbial activity were also used to analyze the antioxidant properties. For HPLC and GC-MS analysis, the ethanolic extracts were solubilized at 10 % (w/v) in methanol (EEP/MeOH).

2.4. Analyses of propolis through GC-MS

GC-MS analysis was performed using a gas chromatograph coupled to a mass spectrometer (GC/MS), Shimadzu model GC/MS QP-2010*Plus* (Shimadzu Corporation, Kyoto, Japan). GC was equipped with RTX-5MS capillary column (30 m x 0.25 mm i.d x 0.25 μm film thickness) consisting of a stationary phase 5 % diphenyl and 95 % dimethyl polysiloxane. The injection was carried out in CT splitless mode at an injector temperature of 250 ºC. Helium gas was used as a carrier gas with a flow rate of 0.95 mL/min. The oven temperature programming was as follows: the initial oven temperature was held 50 ºC for 5 min, and then increased to 300 ºC at a rate of 10 ºC/min held for 30 min. The ion source and transfer line temperature were at 280 ºC. Identification of the compounds was performed by comparing their mass spectra with NIST library available in the instrument.

2.5. Quantification of compounds by HPLC-DAD

High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector (DAD) and LC solution 1.22 SP1 software.

For the analysis of the propolis extracts, 50 μL was injected at concentration of 10 % (m/v) into a Phenomenex C_{18} column (4.6 mm x 250 mm) packed with 5 μm diameter particles and eluted at 0.6 mL/min. The mobile phase was consisted of solvent A (methanol: water; 9:1, v/v) adjusted to pH 3.5 with phosphoric acid and solvent B (acetonitrile: water: methanol; 60:20:20, v/v/v). At a flow rate of 0.6 mL/min, the following linear gradient was used: 0 min, 100 % A; 10 min 30 % A; 20 min, 40 % A; 60 min, 0 % A; held at 0 % A for 15 min. Five min of equilibration at 100 % A was conducted before and after each injection (Bitencourt et al., 2016). All solvents and samples were filtered through a 0.45 μm Millipore filter and then degassed by ultrasonic bath prior to use. The wavelengths used were 327 nm for *p*-coumaric acid, chlorogenic acid, caffeic acid and ellagic acid; and 366 nm for rutin, quercetin and luteolin. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030 – 0.500 mg/mL. Chromatography peaks were confirmed by comparing the retention time with those of reference standards and by DAD spectra (200 to 500 nm). All chromatography operations were carried out at room temperature in triplicate.

2.6. Analysis of the in vitro Antioxidant Properties

The analysis of antioxidant properties *in vitro* were performed spectrophotometrically in 96 well plates using the EnSpire multimode plate reader (PerkinElmer, USA).

2.6.1. DPPH° Radical Scavenging Assay

The activity was determined by evaluating the scavenging capacity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH°) radical according to the method of Baltrušaitytė et al. (2007) with some modifications. Briefly, 100 μL of DPPH° (300 μM) diluted in ethanol were mixed with 50 μL of propolis (0.1 g/mL) in a 96-well plate adjusting the final volume of each well to 300 μL with ethanol. As positive control, ascorbic acid was used. After incubation for 45 min, absorbance was determined at 517 nm. The results were expressed as μmol ascorbic acid equivalents (AAEs) per 100 g of propolis.

2.6.2. ABTS$^{\circ+}$ radical scavenging assay

The antioxidant activity of the propolis samples in the reaction with ABTS$^{\circ+}$ radical was determined according to the method of Baltrušaitytė et al. (2007) with some modifications. The ABTS$^{\circ+}$ radical solution was generated by oxidation of the stock solution (7 mM) of 2,2-azino-bis(3-ethyl-benzotiazolin-6-sulphonic acid) diammonium salt with 2.5 mM potassium persulphate ($K_2S_2O_8$). 200 μL of ABTS$^{\circ+}$ solution were mixed with 10 μL of propolis solution (0.1 g/mL) in a microtitre plate and the decrease in absorbance was measured after 10 min at 734 nm. Ascorbic acid (1 mM) was used as positive control and the results were expressed as μmol ascorbic acid equivalents (AAEs) per g of propolis.

2.6.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric ion reducing capacity of propolis samples was analyzed with the method of Benzie and Strain (1996), adjusted to the analysis of propolis samples. Propolis samples (0.1 g/mL) were mixed with 270 μL of FRAP reagent containing 2.5 mL 0.3 M acetate buffer pH 3.6, 250 μL of 10 mM 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) solution and 250 μL of $FeCl_3 \cdot 6H_2O$. The mixture was shaken and incubated at 37 °C for 30 min. Absorbance was determined at 595 nm. To calculate the standard curve was used Ammonium iron (II) sulfate hexahydrate (100 – 2000 μM). The reducing capacity of propolis was expressed as μmol of Fe(II) equivalent/g of propolis.

2.7. Determination of the total phenolics compounds

Phenolic compounds derived from propolis samples were detected by the Folin-Ciocalteu method described by Singleton et al. (1998) with minor modifications. Briefly, a propolis solution (0.5 mg/mL) was mixed with 35 μL of 1N Folin-Ciocalteu´s reagent, followed by addition of 70 μL 15 % Na_2CO_3 solution. The final volume was adjusted to 284 μL with distilled water. The mixture was incubated in the dark for 2 hours and read by spectrophotometry, measuring the absorbance at 760 nm. Gallic acid was used as standard (10-300 μg/mL). The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of propolis.

2.8. Determination of flavonoids content

The total flavonoid content was determined using the method adapted by Dowd Arvouet-Grande et al. (1994). Briefly, 150 μL of 2 % aluminum chloride were mixed with the same volume of the propolis solution (0.5 g/mL). The values correction was performed using 150 μL of blank sample, composed of distilled water and propolis, but without $AlCl_3$. After 10 min, the absorbance were read in a spectrophotometer at 415 nm. Quercetin was used as standard (0.625 to 25 mg/mL), and the results were expressed in mg of quercetin equivalents (QE) per 100 g of propolis.

2.9. Cell culture and treatments

K562, Jurkat and U937 cell lines were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were maintained at 37 °C in humidified atmosphere containing 5 % CO_2, and medium was completely changed every 2-3 days. After reaching approximately 80 % confluence, cells were transferred to plastic culture dishes (24 or 96 well plates). The initial cell concentration for analysis of viability, IC50, apoptosis, and cell cycle was 0.5×10^5 cells/mL and the cells were maintained for 24 hours in fresh culture medium before treatment. We tested red, green, amber

2014 and amber 2015 propolis extract diluted in DMSO [10 % (w/v)]. The extract were used at different concentrations according to the treatment. Cells treated with DMSO were used as negative control. All analyzes were performed in triplicate.

2.10. Analysis of cell viability and determination of IC50 dose

Cell viability was measured using the Propidium Iodide (PI) exclusion assay. Briefly, cells were seeded in 24-well plates at 0.5 x 10^5 cells/well, and after 24 hours the cells were treated with propolis extract (100 μg/mL) for 72 hours. At analysis time, cells were centrifuged at 750 × g for 5 min and resuspended in 200 μL of complete medium containing PI (1.25 μg/mL). Cell viability was analyzed by flow cytometry, acquiring 10.000 gated cells in FSC-H vs. SSC-H density plot and discriminating live and dead cells in FL2-H vs. SSC-H density plot. To analyze IC50 dose (50 % maximal inhibitory concentration) cell viability was performed as described above by treating the cells with different extract concentrations (10-100 μg/mL) for 24 hours.

2.11. Apoptosis assay

To distinguish between apoptotic and necrotic cells, membrane permeability assay was performed using the YOPRO/PI system according to the manufacturer's instructions (Invitrogen, USA), with slight modifications. Cells were seeded in 24-well plates at 0.5 × 10^5 cells/well and after 24 hours they were treated with propolis extract at IC50 dose. For analysis, the cells were collected by centrifugation at 750 × g for 5 min and resuspended in 500 μL of complete medium containing 100 nM YO-PRO-1, and 150 nM PI and incubated for 5 min. The assay was performed without the washing procedure with PBS. After incubation, 30.000 gated events were analyzed by flow cytometry in FL1-H (YOPRO) vs. FL3-H (PI) density plot.

2.12. Cell cycle analysis

We used the method described by Overton and McCoy (1994), slightly modified for cell cycle analysis. Cells were seeded in 24-well plates at 0.5 × 10^5 cells/well for 24 hours. Cells were treated with propolis extract at IC50 dose. At analysis time, cells were collected by centrifugation at 750 × g for 5 min and resuspended in lysis buffer containing 10 µg/mL propidium iodide, 50 mM Trizma base, 50 mM NaCl, EDTA 1 mM and 0.5 % NP-40. After 5 min, cells were analyzed by flow cytometry by collecting 5.000 gated events, in slow mode, FL2-H vs. FL2-A density plot. Later, cell cycle was analyzed in FL2-A histograms by FlowJo X v.0.7 software.

2.13. Antimicrobial activity

Escherichia coli and *Staphylococcus aureus* strains were grown in Luria-Bertani medium containing 1 % Tryptone, 0.5 % NaCl and 0.5 % yeast extract to examine the effect of propolis extracts in their growth. Initially, bacteria culture were incubated at 37 °C under constant agitation (180 RPM) until the optical density of approximately 0.4 was reached for *E. coli* and 0.2 for *S. aureus* (600 nm in a SP-22 spectrophotometer Biospectro, Brazil). A total of 100 µL of bacterial culture were transferred to 96-well plates containing 100 µL of LB medium. The plates were incubated at 37 °C with agitation (50 RPM) and the absorbance measured at 600 nm during 12 hours, at 1, 3, 6, 12 hours intervals, in a spectrophotometer EnSpire® multimode (PerkinElmer, USA). For negative controls and blanks we replicated the treatment conditions, but without bacteria. As positive controls we used ampicillin and streptomycin.

2.14. Statistical analysis

Results are expressed as mean ± standard deviation (SD) of at least three samples. Statistical analyzes were performed by two-way ANOVA followed by Tukey post-hoc test, to analyze the differences among treatments, or by post-hoc Dunnett, to compare each of a number of treatments with a single control. The statistical

differences were determined using GraphPad Prism version 6.0, and values were considered significant at $p \leq 0.05$.

The IC50 dose was determined in GraphPad Prism version 6.0 by using non-linear regression fit with a sigmoid dose-response equation, representing the correlation between the inhibition percentage and propolis concentration.

Differences between groups of HPLC were assessed by an analysis of variance model and Tukey's test. The level of significance for the analyses was set to $p \leq 0.05$. These analyses were performed by using the free software R version 3.1.1. (R Core Team, 2014).

3. Results

3.1. Analysis of propolis compounds by gas chromatography with mass spectrometric detection (GC-MS)

GC-MS analysis identified 69 compounds in amber propolis 2014 (72 % are terpenes) and 62 compounds in amber propolis 2015 (77 % are terpenes). Approximately 50 % of the compounds are shared between propolis collected in 2014 and 2015. A total of 99 different compounds were identified in both amber propolis combined. The GC-MS chromatograms of both amber propolis share the same pattern, taking into account the predominant peaks (Figure 2). The higher peak (approximately 42 min) could not be discriminated by the used library. Probably the peak possesses several compounds. The second predominant peak (approximately 50 min) represented a tetra-cyclic compound, but with approximately 75 % of similarity and it was not included in the compound list.

As the beehives for propolis collection were located in an eucalyptus, forest we searched for eucalyptus compounds and 70 amber propolis compounds have already been described in *Eucalyptus sp.* (Table 1). We also analyzed red and green propolis by GC-MS. Amber propolis 2014 shared 11 compounds with red propolis and 6 with green propolis. On the other hand, amber propolis 2015 shared 6 compounds with red propolis and 5 compounds green propolis. Some of these compounds are shared between all propolis analyzed in this study. Table 1 presents

the compounds found in amber propolis, highlighting compounds shared with red and green propolis.

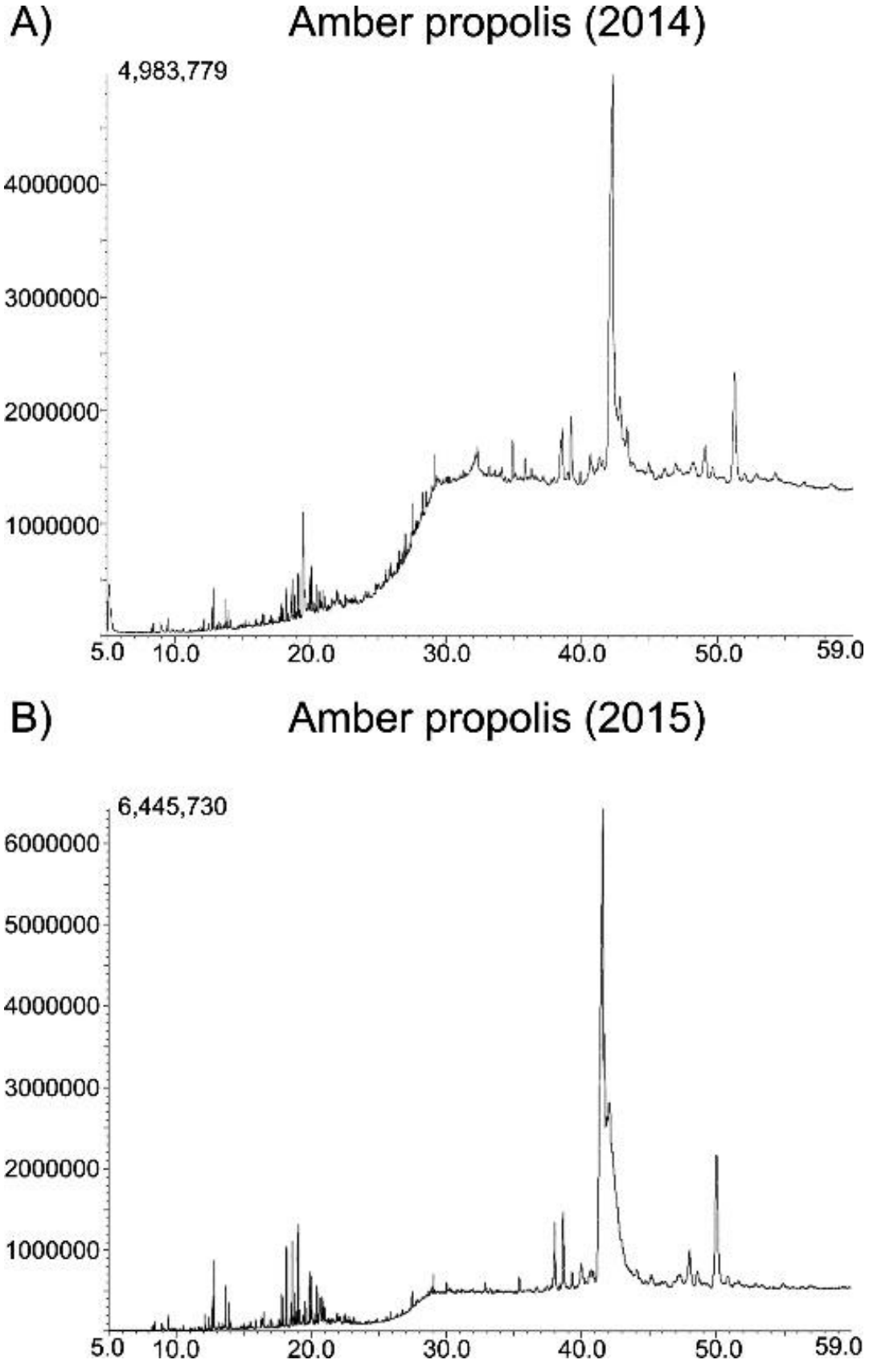

Figure 2. GC-MS profile of the ethanolic extract of amber propolis samples collected in 2014 (A) and 2015 (B).

Table 1: Chemical composition of amber propolis ethanolic extracts

Compounds	Identified in *Eucalyptus sp.*	Year 2014 - %	Year 2015 - %
Terpenes			
(E,E,E)-3,7,11,15-Tetramethylhexadeca-1,3,6,10,14-pentaene	-	+	
(S)-cis-Verbenol	Kumari et al., 2014	+	+
(z,e)-farnesol	-	+	
1,6,10,14,18,22-tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-e)-	-	+	
4,8,13-Duvatriene-1,3-diol	-	+	
4-Thujanol	Gupta et al., 2015		+
6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	-	+	
7-tetracyclo[6.2.1.0(3.8)0(3.9)]undecanol, 4,4,11,11-tetramethyl	-	+	
9-methoxycalamenene	-	+	
Alloaromadendrene	Zini et al., 2003		+
Alpha-Cadinol	von Mühlen et al., 2008	$+_R$	$+_R$
Alpha-Calacorene	Kumari et al., 2014	+	+
Alpha-Cubebene	Zini et al., 2003	$+_R$	$+_R$
Alpha-Guaiene	Kumari et al., 2014	+	+
Alpha-Gurjunene	von Mühlen et al., 2008	+	+
Alpha-Phellandrene	von Mühlen et al., 2008		+
Alpha-Pinene	von Mühlen et al., 2008	+	+
Alpha-Thujene (3-Thujene)	Kumari et al., 2014	+	
Aromadendrene	Zini et al., 2003		+
Aromadendrene oxide	-	+	
Beta-elemene	Kumari et al., 2014	+	
Beta-pinene	von Mühlen et al., 2008	+	+
Beta-Pinene epoxide (2,10-Epoxypinane)			+
Beta-Selinene	von Mühlen et al., 2008	$+_G$	
Beta-Thujene (2-thujene)	-	+	
Betulin	-	$+_{R,G}$	
Bicyclo[3.2.0]heptan-3-ol, 2-methylene-6,6-	-	+	

dimethyl-			
Bicyclo[5.3.0]decane, 2-methylene-5-(1-methylvinyl)-8-methyl-	Mathur et al., 2014		+
Bornyl acetate	von Mühlen et al., 2008	+	+
Cadala-1(10),3,8-triene	-	+	+
Cadinol	Kumari et al., 2014	$+_R$	$+_R$
Camphenol, 6-	von Mühlen et al., 2008		+
Carvone	Kumari et al., 2014		+
Cis-Beta-Guainene	El-Ghorab et al., 2009	+	
Cis-Carveol	von Mühlen et al., 2008	+	+
Cis-Sabinol	von Mühlen et al., 2008		+
Copaene (Alpha-Copaene)	von Mühlen et al., 2008	$+_R$	$+_R$
Cosmene	-		+
Cubenol (10.beta.H-Cadin-4-en-1-ol)	Kumari et al., 2014	+	
Cyclo Sativene	El-Ghorab et al., 2009	+	
D-carvone	Ashraf et al., 2010		+
Delta-cadiene (Cadina-1(10),4-diene)	Kumari et al., 2014	$+_{R,G}$	$+_{R,G}$
Delta-Guaiene	Kumari et al., 2014		+
Eucalyptol (Cineole)	Kumari et al., 2014		+
Farnesol	Kumari et al., 2014	+	
Gamma-Gurjunene	von Mühlen et al., 2008	+	
Gamma-Muurolene	Kumari et al., 2014		$+_G$
Gamma-Terpinene	Kumari et al., 2014		+
Geranylgeraniol	-	+	
Globulol	Kumari et al., 2014	$+_G$	$+_G$
Isoaromadendrene epoxide	Goldbeck et al., 2014		+
Juniper camphor (Eudesm-7(11)-en-4-ol)	Mejdoub et al., 1998	+	
Lanosterol		+	+
Ledene	Joshi et al., 2016	+	
Ledol	Luís et al., 2015	+	+
Limonene	von Mühlen et al., 2008		+
Methyl palustrate	-		+
Myrcene	von Mühlen et		+

	al., 2008		
Myrtenol	von Mühlen et al., 2008	+	+
Neral (2,6-Octadienal, 3,7-dimethyl-)	von Mühlen et al., 2008	+	+
Nerolidol	Kumari et al., 2014	$+_G$	$+_G$
O-cymene	Luís et al., 2015		+
Oplopanone	-	+	
Perilla Alcohol (Para-mentha-1,8-dien-7-ol)	Tsiri et al., 2003	+	+
Perillene	Pino et al., 2001	+	
Pinanediol	Joshi et al., 2016		+
Pinocarveol	Kumari et al., 2014	+	+
Pinocarvone (Alpha-Pinocarvone)	Kumari et al., 2014	+	+
Sabinene (Sabinene, (1R)-isomer)	Kumari et al., 2014	+	+
Spathulenol	Kumari et al., 2014	$+_G$	$+_G$
Squalene	Ge et al., 2015	$+_R$	
Thuja-2,4(10)-diene	Luís et al., 2015		+
Verbenone (D-Verbenone)	von Mühlen et al., 2008	+	+
Aldehydes			
2-isopropenyl-5-methylhex-4-enal	-	+	
8-hexadecenal, 14-methyl-, (z)-	-		+
Alpha-Campholenal (Campholenic aldehyde)	Kumari et al., 2014	+	+
Germacrene D	El-Ghorab et al., 2009	+	+
Phellandral	Pino et al., 2001		+
Urs-12-en-28-al	-	+	
Ketones			
Guaiacylacetone	Nunes et al., 2010	+	
Megastigmatrienone	-	+	+
Ethers			
Verbenyl ethyl ether	-		+
Esters and Fatty acids			
Benzyl benzoate	Skariyachan et al., 2011	+	
Dodecanoic acid	Domingues et al., 2011	$+_R$	
Heneicosane	Domingues et al., 2011		+
Hexadecanoic acid	Domingues et al., 2011	+	
Nonanoic acid	Domingues et	+	

	al., 2011		
Ethyl oleate	Zhang et al., 2009	$+_R$	$+_R$
Oleic acid	Domingues et al., 2011	$+_R$	
Phthalic acid (1,2-Benzenedicarboxylic acid)	Ge et al., 2015		+
Alcohols			
3,7-nonadien-2-ol, 4,8-dimethyl-	-	+	+
9,19-cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	-	+	
Humulane-1,6-dien-3-ol	-	+	
Selina-6-en-4-ol	-	+	
Trans-3(10)-caren-2-ol	Qi et al., 2010	+	
Others			
2-octene, 2-methyl-6-methylene-	-	+	+
Biphenylene, 1,2,3,6,7,8,8a,8b-octahydro-4,5-dimethyl-	-		+
Decane	Krock et al., 1994		+
Oxirane, [(dodecycloxy)methyl]-		$+_R$	
N-butylpyrrole	-		+

R indicates compounds shared with red propolis; G compounds shared with green propolis.

3.2. Quantification of propolis flavonoids and phenolics by HPLC-DAD

The analysis of propolis extracts by HPLC-DAD revealed the presence of phenolic compounds: chlorogenic acid (retention time - tR = 21.65 min, peak 1), caffeic acid (tR = 25 min; peak 2), p-coumaric acid (tR = 28.13 min, peak 3), ellagic acid (tR = 36.04 min, peak 4), rutin (tR = 49.11 min; peak 5), quercetin (tR = 53.87 min, peak 6), and luteolin (tR = 56.75 min; peak 7). It was not possible to detect caffeic acid nor rutin in amber propolis. In reference to these amber propolis they had lower flavonoid amounts, except ellagic acid whose concentration was higher than in red and green propolis. The chromatograms are showed in Figure 3 and Table 2.

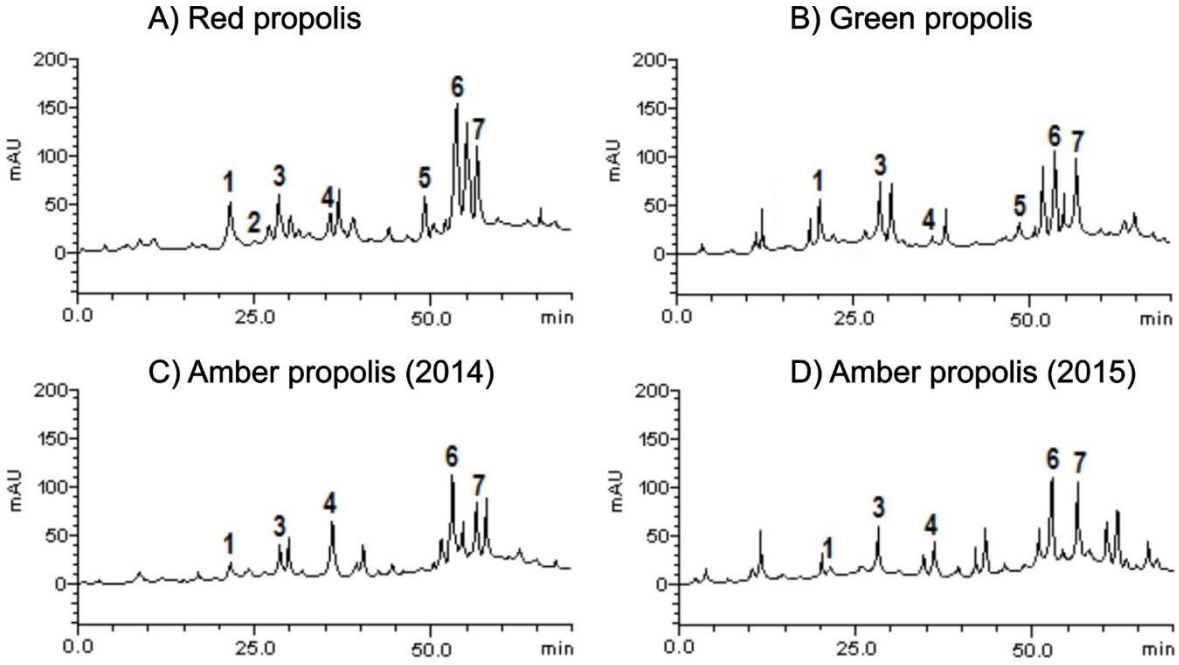

Figure 3. Representative high performance liquid chromatography profile of propolis extracts Red (A), Green (B), Amber 2014 (C) and Amber 2015 (D). Chlorogenic acid (peak 1), caffeic acid (peak 2), *p*-coumaric acid (peak 3), ellagic acid (peak 4), rutin (peak 5), quercetin (peak 6) and luteolin (peak 7).

Table 2 – Phenolic profile of propolis ethanolic extracts evaluated by HPLC.

Compounds	**Red**	**Green**	**Amber 2014**	**Amber 2015**
	mg/g	**mg/g**	**mg/g**	**mg/g**
Chlorogenic acid	2.03 ± 0.04	1.86 ± 0.03	0.73 ± 0.01	0.28 ± 0.02
Caffeic acid	0.23 ± 0.01	-	-	-
p-Coumaric acid	1.97 ± 0.02	2.13 ± 0.01	1.04 ± 0.03	1.95 ± 0.04
Ellagic acid	1.16 ± 0.03	0.29 ± 0.01	2.35 ± 0.02	1.64 ± 0.01
Rutin	2.07 ± 0.01	0.76 ± 0.03	-	-
Quercetin	7.84 ± 0.01	4.15 ± 0.05	4.18 ± 0.01	2.11 ± 0.05
Luteolin	4.31 ± 0.05	4.09 ± 0.01	2.06 ± 0.04	2.09 ± 0.03

Results are expressed as mean ± standard deviations (SD) of three determinations.

3.3. Analysis of the in vitro antioxidant properties

The analysis of antioxidant properties of different propolis extracts showed lower contents of phenols and flavonoids in amber propolis than red and green propolis (approximately four and two times, respectively) (Figure 4 A and B). The activity of DPPH radical scavenging showed no statistical difference between the red propolis, green and amber collected in 2015 when diluted in DMSO and they showed superior activity than amber propolis collected in 2014. When diluted in ethanol, amber propolis 2014 and 2015 had similar activities to green propolis, but approximately 30 % lower than red propolis (Figure 4 C). Analysis of $ABTS^{\circ+}$ radical scavenging and Ferric Reducing Antioxidant Power (FRAP) showed similar effects between the green and red propolis, that presented activity two times greater than the amber propolis (Figure 4 D and E).

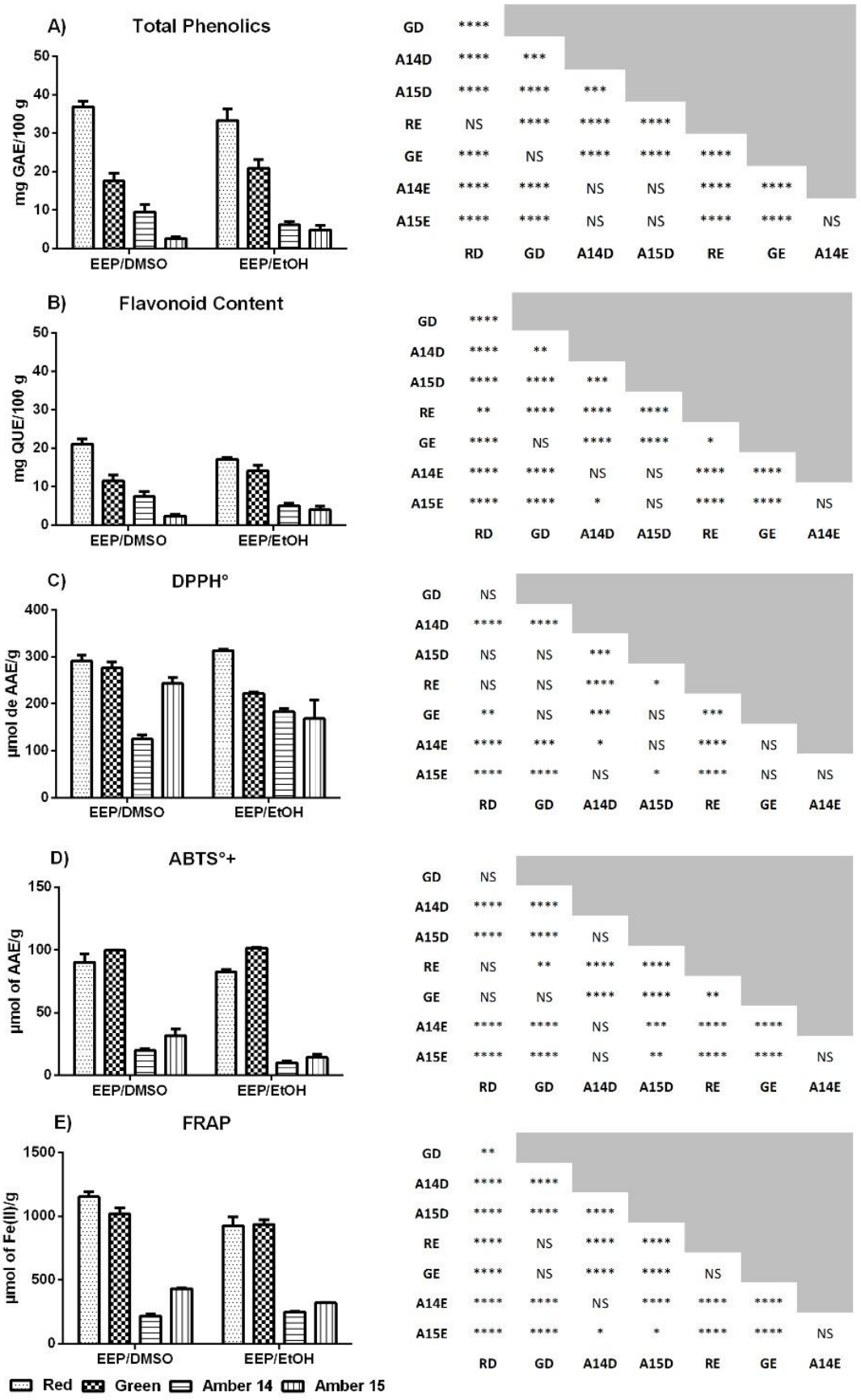
A) Total Phenolics
mg GAE/100 g
EEP/DMSO
EEP/EtOH
B) Flavonoid Content
mg QUE/100 g
C) DPPH°
µmol de AAE/g
D) ABTS°+
µmol of AAE/g
E) FRAP
µmol of Fe(II)/g
Red
Green
Amber 14
Amber 15
GD **** | A14D **** *** | A15D **** **** *** | RE NS **** **** **** | GE **** NS **** **** **** | A14E **** **** NS NS **** **** | A15E **** **** NS NS **** **** NS
RD GD A14D A15D RE GE A14E
GD **** | A14D **** ** | A15D **** **** *** | RE ** **** **** **** | GE **** NS **** **** * | A14E **** **** NS NS **** **** | A15E **** **** * NS **** **** NS
RD GD A14D A15D RE GE A14E
GD NS | A14D **** **** | A15D NS NS *** | RE NS NS **** * | GE ** NS *** NS *** | A14E **** *** * NS **** NS | A15E **** **** NS * **** NS NS
RD GD A14D A15D RE GE A14E
GD NS | A14D **** **** | A15D **** **** NS | RE NS ** **** **** | GE NS NS **** **** ** | A14E **** **** NS *** **** **** | A15E **** **** NS ** **** **** NS
RD GD A14D A15D RE GE A14E
GD ** | A14D **** **** | A15D **** **** **** | RE **** NS **** **** | GE **** NS **** **** NS | A14E **** **** NS **** **** **** | A15E **** **** * * **** **** NS
RD GD A14D A15D RE GE A14E

Figura 4. Antioxidant content of ethanolic extracts solubilized in 99.9% dimethyl sulfoxide (DMSO) (EEP/DMSO) or absolute ethanol (EEP/EtOH). Data are expressed as Mean ± SEM. Asterisks represent statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. The abbreviations correspond to: Red DMSO (RD), Green DMSO (GD), Amber 2014 DMSO (A14D), Amber 2015 DMSO (A15D) as well as for ethanol (RE, GE, A14E, A15E).

3.4. Analysis of cell viability and IC50

Treatment of K562, Jurkat and U937 cells with different type of propolis at 100 μg/mL caused a decrease in cell viability after 24 hours treatment. Both amber propolis and red propolis had similar cytotoxic effect (no statistical differences), killing more than 90 % of cells after 24 hours in the three cell lines; green propolis had minor effect ($p \leq 0.0001$ related to amber and red propolis), but also significant when compared to control ($p \leq 0.0001$) (Figures 5 A, B and C).

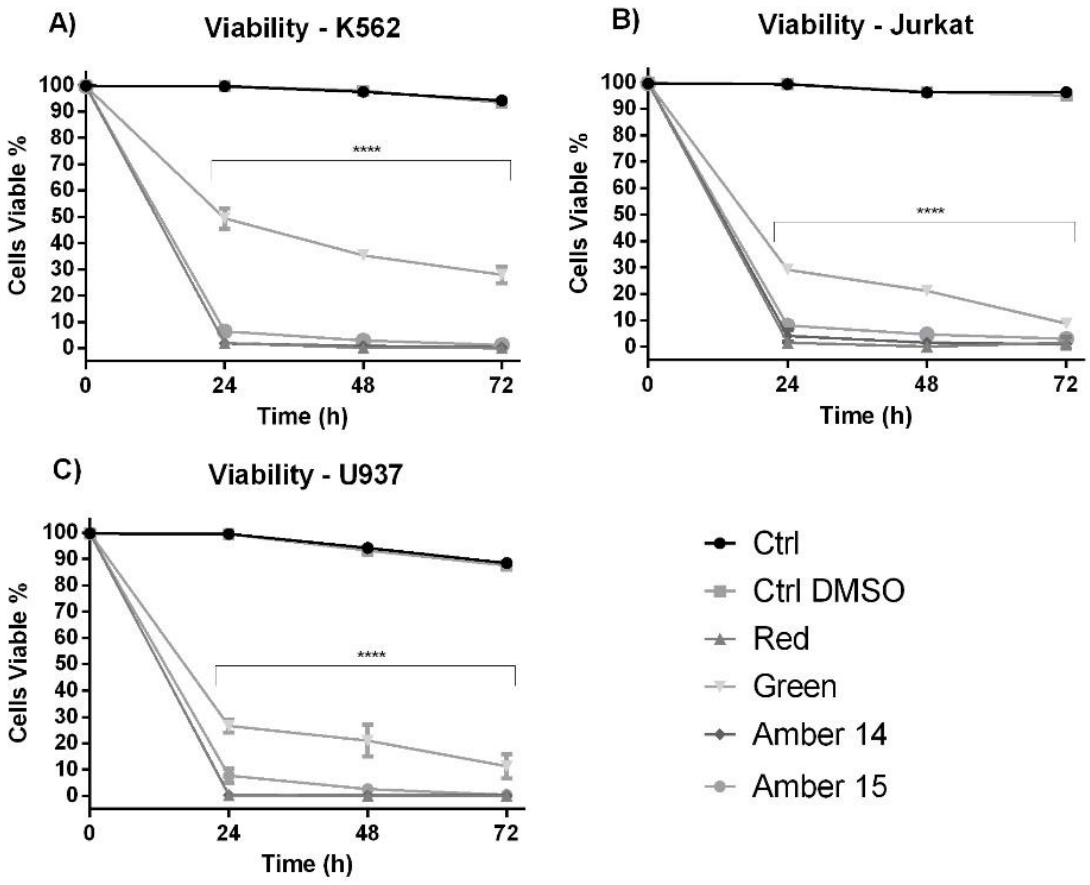

Figure 5. Citotoxic effects of amber, green and red propolis extract on K562 (A), Jurkat (B) and U937 (C) cell lines. Cells were treated with the different propolis at 100 μg/mL concentration for 24, 48 and 72 hours. Data are expressed as Mean ± SEM. The asterisks represent statistical significance: **** $p \leq 0.0001$.

Also, we determined the mean inhibitory concentration (IC50) of different propolis in different cell lines. K562 cells did no showed statistical differences when compared treated with either red propolis or amber propolis with IC50 values of about 30 μg/mL, green propolis presented IC50 values approximately 3 times higher ($p \leq 0.0001$). Jurkat and U937 cell lines did not showed statistical differences regarding IC50 dose when treated with each propolis; however, there were differences between propolis treatments. Red propolis showed the lower IC50 dose (approximately 10 μg/mL), amber propolis showed dose of approximately 25 μg/mL; however, only amber propolis collected in 2015 showed statistical difference when compare with red propolis ($p \leq 0.05$ in both cell lines). Green propolis dose was approximately 65 μg/mL, showing statistically significant differences when compared to red and amber propolis ($p \leq 0.0001$ in both of the cell lines). Regarding the effect between cell lines, both red and green propolis had significantly lower IC50 values for U937 and Jurkat lines than for K562 cells ($p \leq 0.01$ and $p \leq 0.0001$, respectively); nevertheless, amber propolis showed no statistical difference in their effect on different strains. The values of the median inhibitory concentration range (IC50) of each propolis in the different cell lines are shown in Figure 6.

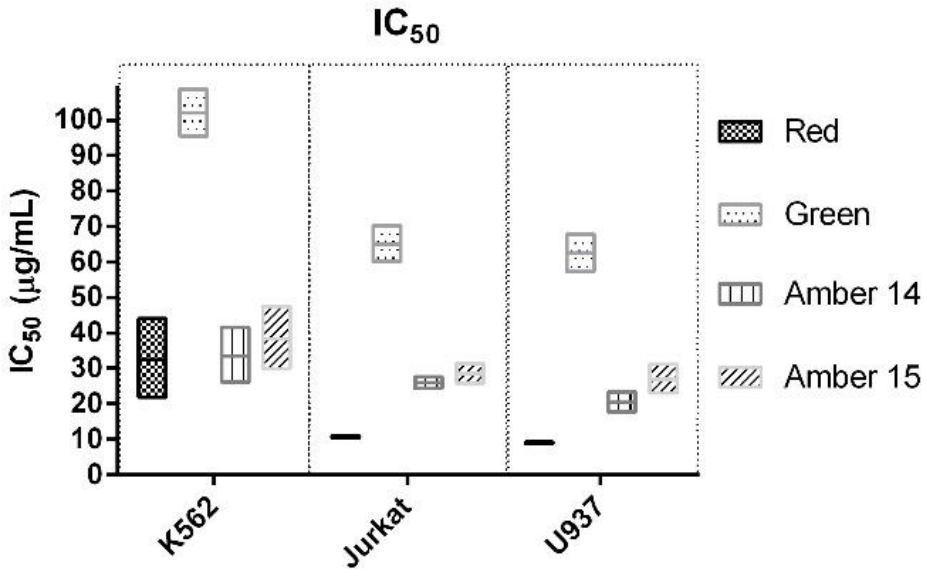

Figure 6. Mean of inhibitory concentration (IC50) values of amber, green and red propolis extract were determined by treating the K562, Jurkat and U937 cell lines with different extract concentrations (10-100 µg/mL) for 24 hours. Red propolis: 31.12 µg/mL (K562), 10.68 µg/mL (Jurkat), 9.08 µg/mL (U937). Green propolis: 101.90 µg/mL (K562), 64.94 µg/mL (Jurkat), 62.29 µg/mL (U937). Amber propolis 2014: 32.97 µg/mL (K562), 26.01 µg/mL (Jurkat), 20.28 µg/mL (U937). Amber propolis 2015: 37.73 µg/mL (K562), 28.52 µg/mL (Jurkat), 26.85 µg/mL (U937).

3.5. Induction of apoptosis by propolis

The treatment of leukemic cell lines with different types of propolis in the median inhibitory concentration (IC50) showed significant cell death compared to control, from 12 hours treatment in all the cell lines tested (Figure 7 A, C and E). In K562 cells, apoptosis was only observed after 12 hours treatment with red propolis, whereas green and amber propolis showed apoptosis events after 24 hours treatment (Figure 7 A and B). Treatment with green propolis did not presented statistical significant apoptosis in leukemic cell lines Jurkat and U937 neither in 12 hours nor in 24 hours (Figure 7 C - F). Treatment of Jurkat cells with red, amber 2014 and 2015 propolis induced significant apoptosis at 12 and 24 hours (Figure 7 C and D). In U937 cells only amber propolis 2014 and 2015 induced significant death by

apoptosis after the 12 hours treatment (Figure 7 E and F) and after 24 hours treatment red propolis showed significantly apoptosis induction (Figure 7 F). Supplementary Figure 1 (S1) shows representative cytometry graphs emphasizing the gates created for the discrimination of different cell groups.

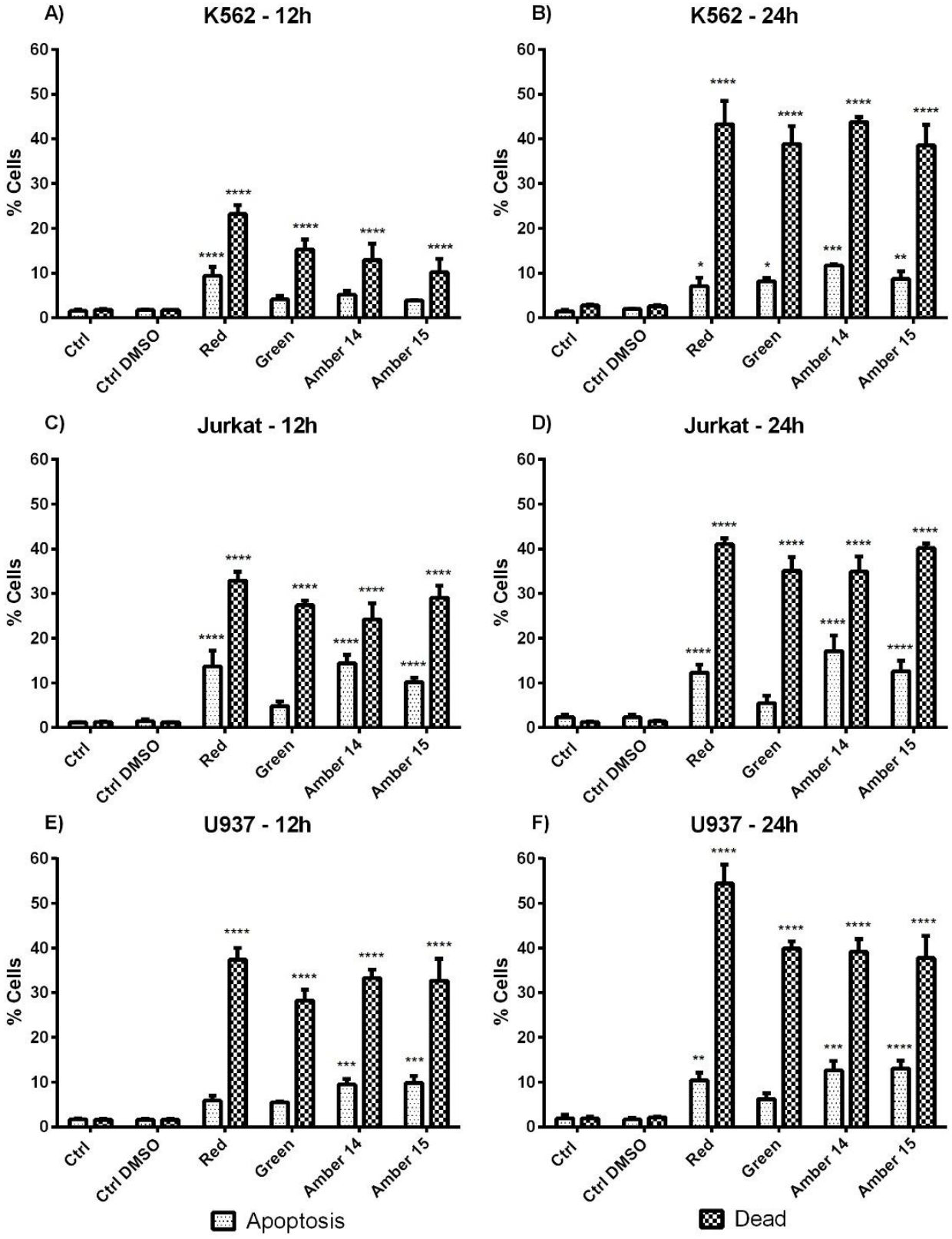

Figure 7. Effect of propolis on apoptotic cell death in K562 (A and B) Jurkat (C and D) and U937 (E and F) cell lines. The cells were treated for 24 hours at the concentration values defined by IC50. Data are expressed as Mean ± SEM. The

asterisks represent statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.6. Effect of propolis treatment on cell cycle

The cell cycle analysis by flow cytometry revealed that all propolis (used at IC50 dose) caused significant cell cycle arrest in G2/M phase at 24 hours of treatment in comparison with the controls (Figure 8 B, D and F). G2/M arrest was observed in K562 cells since 12 hours of treatment for red, amber 2014 and amber 2015 propolis but not for green propolis (Figure 8 A). In Jurkat cells, no propolis caused G2/M arrest at 12 hours treatment (Figure 8 C). In U937 cells all the propolis presented G2/M arrest from 12 hours of treatment (Figures 8 E). Supplementary Figure 2 (S2) shows representative histograms of all propolis treatments.

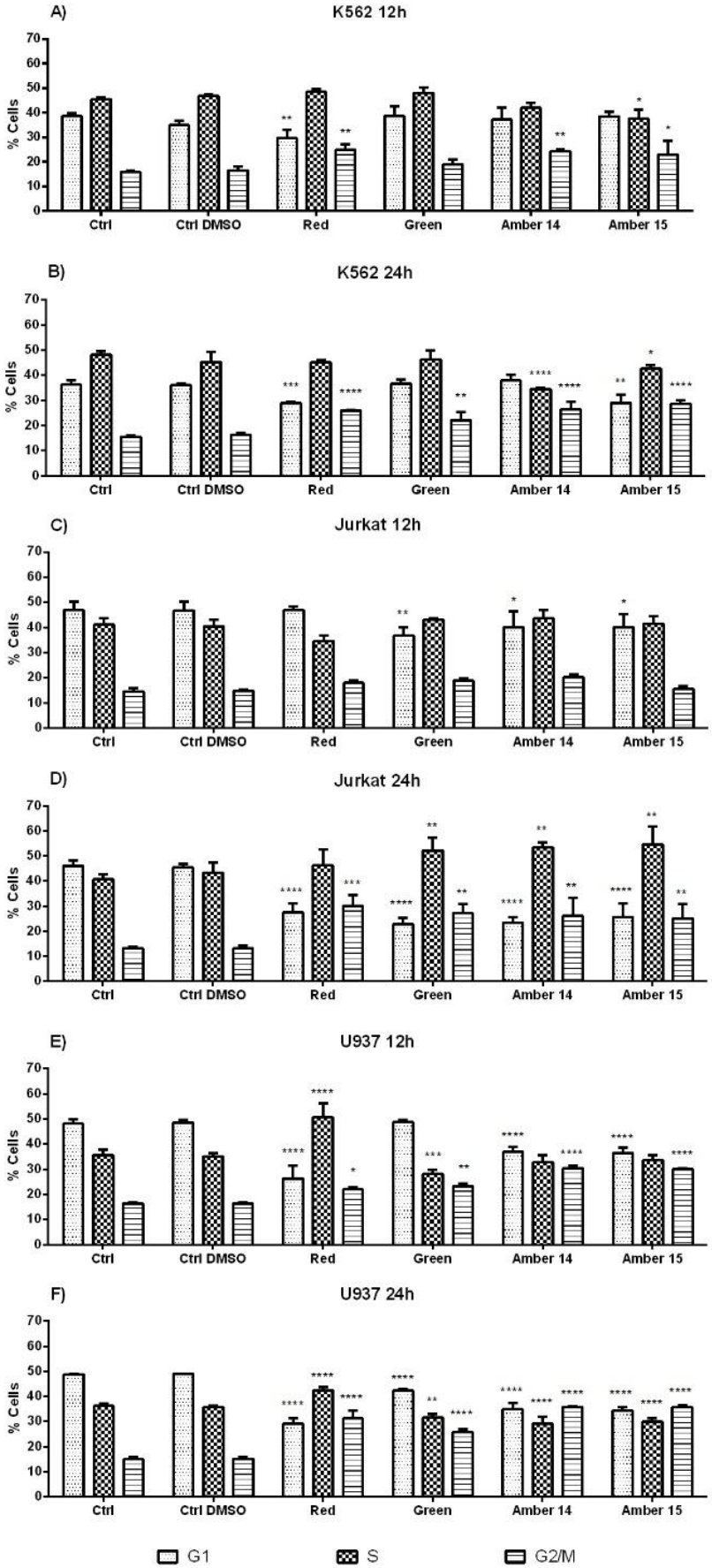
A)
K562 12h
B)
K562 24h
C)
Jurkat 12h
D)
Jurkat 24h
E)
U937 12h
F)
U937 24h
% Cells
Ctrl
Ctrl DMSO
Red
Green
Amber 14
Amber 15
G1
S
G2/M

Figure 8. Effect of propolis on the cell cycle of cell lines K562 (A, B), Jurkat (C, D) and U937 (E, F). The cells were treated for 24 hours at the concentration values defined by IC50 and analyzed at 12 and 24 hours. Data are expressed as Mean ± SEM. The asterisks represent statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.7. Antibacterial effect of propolis

The effect of propolis on *Escherichia coli* and *Staphylococcus aureus* growth was studied during 12 hours treatment at concentrations of 100 μg/mL and 500 μg/mL. In *E. coli,* 100 μg/mL propolis caused decreasing in the grouth from 3 hours of treatment without statistical differences between propolis types (Figure 9 A). In this species, the positive control (antibiotic solution 100 U/mL penicillin and 100 μg/mL streptomycin) had better effect than propolis ($p \leq 0.01$); although, at 500 μg/mL all propolis inhibited the bacterial growth (Figure 9 B). In *S. aureus* the three types of propolis showed better effect than antibiotic from the lowest concentration tested from 3 hours of treatment ($p \leq 0.0001$). Red propolis was capable of inhibiting bacterial growth from the lowest concentration tested (100 μg/mL). Amber propolis collected in 2014 and 2015 did not shown significant difference between them, but they showed higher effect than green propolis ($p \leq 0.01$), but neither of them showed totally growth inhibition as red propolis ($p \leq 0.0001$) (Figure 9 C). All 500 μg/mL propolis tested inhibited bacterial growth (Figure 9 D) in relation to control.

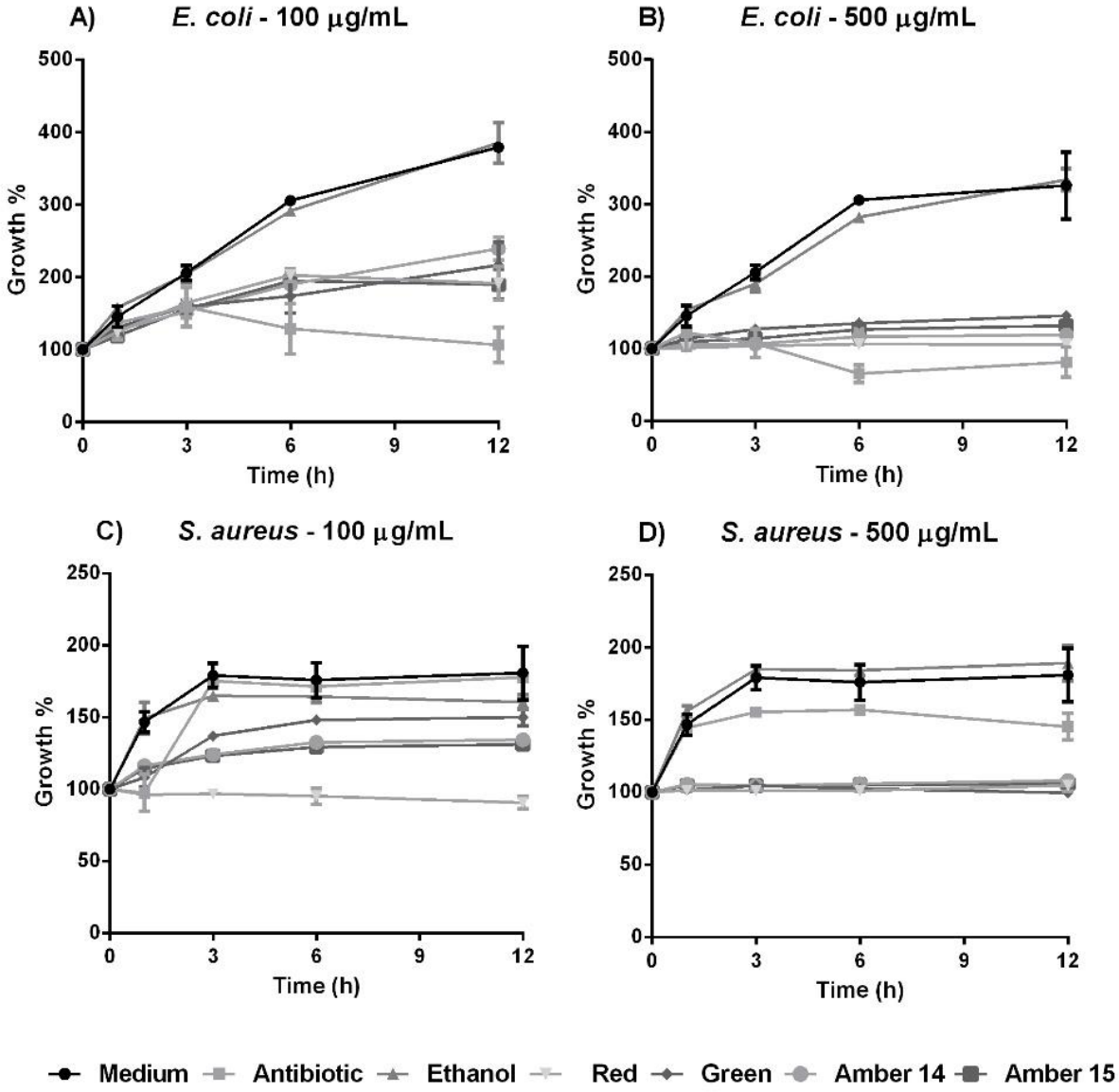

Figure 9. Propolis extracts effect in *Escherichia coli* growth at 100 µg/mL (A) and 500 µg/mL (B) concentrations and in *Staphylococcus aureus* growth at 100 µg/mL (C) and 500 µg/mL (D) concentrations. Data are expressed as Mean ± SEM.

4. Discussion

The first published work with different types of Brazilian propolis has cataloged them in 12 types (Park et al., 2000). From Rio Grande do Sul state (the southernmost state of Brazil) 2 types were cataloged (without specified location) showing negative or low antimicrobial activity against *S. aureus*; concomitantly, Rio Grande do Sul

propolis showed high antioxidant activity but low anti-inflammatory effect. This fact rendered its production unattractive due to the low market value, when compared with the green propolis produced at São Paulo and Minas Gerais States.

The expansion of beekeeping in the Rio Grande do Sul state, from the increase in *Eucalyptus* forest for commercial purposes, is rising up the demand for beekeeping diversification. Therefore, in this work we characterized the propolis produced in the southern region of Rio Grande do Sul State - Brazil, by studying its chemical profile and the antileukemic, antioxidant, and antimicrobial activities. As references we use the green and red propolis that are already well characterized.

The propolis samples collected in 2014 and 2015 produced an ethanolic extract with light amber color that we decided to name “amber propolis” to differentiate it from propolis called yellow by Park et al. (2000), whose analyzes have not shown interesting biological activities. The characterization of amber propolis by GC-MS showed large differences in their chemical profile when compared to the green and red propolis. This variation was expected by the great biodiversity and climate differences between the local where the different propolis were collected, as already suggested by other authors (Bankova et al., 2000; Conti et al., 2015; Park et al., 2002; Pereira et al., 2002).

Amber propolis was collected from apiaries located in eucalyptus forests associated with the Brazilian Pampa Biome and were rich in essential oils. Most of the detected compounds have already been described for the *Eucalyptus* genus, including one of the characteristic substances of this genus, eucalyptol (1,8-Cineole) (Elaissi et al., 2012), indicating that the eucalyptus could be an important and stable source of substances for the production of amber propolis.

The predominance of monoterpenes, *α* and *β*-pinene and limonene, were also detected in the volatile oil extracted from propolis samples collected in three cities of Rio Grande do Sul State, about 200 Km north of the city where the propolis were collected for our work; however, this work does not discriminate the type of propolis or the characteristics of the sampling sites (Simionatto et al., 2012). Similar chemical composition of volatile compounds was also found in three samples of propolis from Uruguay, one of them characterized by high amounts of limonene (Kaškonienė et al., 2014). However, these analyzes did not detect eucalyptol which, to the best of our knowledge, only has been reported in low-quality green propolis samples (Nunes and

Guerreiro, 2012) and in propolis samples from Estonia (Kaškonienė et al., 2014) and from China (Yang et al., 2010).

Comparing the total phenols and flavonoids content, our data showed that red propolis have the highest content of phenols and amber propolis the lowest, and these data are in agreement with the literature where Brazilian propolis collected in sub-tropical climate are poor for this type of substances (Bankova et al., 1995; Bankova et al., 1996; Sawaya et al., 2011). Several authors attribute the potent antioxidant activity of propolis to their phenols and flavonoids content (Alencar et al., 2007; Banskota et al., 2001; Kurek-Górecka et al., 2014; Kuropatnicki et al., 2013; Marcucci et al., 2001). Our results showed a direct relation between the total concentration of phenols and flavonoid and the effect on DPPH°, $ABTS^{\circ+}$ and FRAP; however, the fourfold difference between red propolis and amber propolis is only observable for $ABTS^{\circ+}$ and FRAP. For DPPH° this difference is not observed, suggesting that other compounds present in the amber propolis may be contributing in this activity. The solution of DPPH° is composed of ethanol or methanol having favorable characteristics to measure the antioxidant value of less polar compounds like terpenes. And this technique has already been used to determine the antioxidant value of essential oils in cintrus, resinous exudate of *Heliotropium spp.* and mixtures of beeswax (Sánchez-Moreno, 2002).

We also analyzed the phenols and flavonoid content and profile by HPLC-DAD as used previously for eucalyptus honey by our group (Cruz et al., 2015). Among these substances, amber propolis were characterized by the absence of caffeic acid (present in eucalyptus honey) and rutin; on the other hand, they presented high content of ellagic acid compared to the green and red propolis.

To evaluate their antileukemic activity, initially we tested the effect of propolis in a concentration of 100 μg/mL, frequently reported in the literature, and there were no differences between amber and red propolis, which presented greater cytotoxic effect than green propolis. Subsequently, we determine the IC50 values of each propolis in K562, Jurkat and U937 cell lines. Our data for the green and red propolis were similar to those obtained by Franchi Jr. et al. (2012) when treated the same cells lines. Amber propolis showed no difference in cytotoxicity between the samples obtained in 2014 and 2015 and showed IC50 values similar to red propolis for K562 cells and slightly higher than the red propolis in U937 and Jurkat cells for 2015

propolis, but not significant when compare to 2014 propolis; at the same time, in all the cell lines the amber propolis IC50 was much lower than that for green propolis. Interestingly, unlike the green and red propolis, that had a greater effect on U937 and Jurkat cells lines than in K562, amber propolis showed no statistical differences in IC50 between three cell lines suggesting that alternative signaling pathways could be involved in the cytotoxic effect of this propolis when compared with the green and red propolis.

For treatment of cancer, researches aim drugs that arrest cell cycle and induce apoptosis without inducing inflammation or damage in normal cells (Abubakar et al., 2014; Gautam et al., 2014). As the induction of apoptosis has already been documented in the cell lines used in this study treated with green or red propolis (Aso et al., 2004; Franchi Jr. et al., 2012; Mishima et al., 2005a), we tested the induction of apoptosis by amber propolis in its IC50 dose and compare this activity with the green and red propolis. In this analysis, it was observed that amber propolis causes apoptosis in cells treated with an IC50 dose, detected from 12 hours in Jurkat and U937 cells and from 24 in K562 cells. At the same time, the analysis of IC50 dose of each propolis on the cell cycle showed the amber propolis, as well as green and red propolis, blocks the cell cycle at G2 /M phase in K562, Jurkat and U937 cell lines. The cell cycle arrest was already showed for prostate cell lines treated with Brazilian green and brown propolis (Li et al., 2007), for colon cell lines treated with Chinese propolis (Ishihara el at., 2009) and U937 leukemic cell treated with Japanese propolis (Motomura et al., 2008) but, to the best of our knowledge, this is the first time that the effect of Brazilian propolis over the cell cycle in leukemic cell lines is demonstrated.

The propolis is a complex mix of substances; thus, the determination of the compounds with antileukemic activity in amber propolis would be an arduous task; however, some of the compounds identified by GC-MS in amber propolis samples have already been studied individually, killing or avoiding proliferation of neoplastic cells. For example, α-pinene isolated from essential oils of *Schinus terebinthifolius* demonstrated anti-metastatic activity on a metastatic melanoma model, avoiding the spread of cancer to other body parts and inhibiting tumor proliferation (Matsuo et al., 2011); leukemic cells treated with eucalyptol (1,8-cineole) underwent apoptosis (Moteki et al., 2002); β-elemene induces cell cycle arrest in the G2/M phase in H460 and A549 lung cancer cells (Wang et al., 2005) and induces cell cycle arrest in G2/M

phase leading to apoptosis in HepG2 liver cancer cells (Dai et al., 2013) and causes apoptosis in K562 (Zou et al., 2001). Limonene induces apoptosis in leukemic cells (Ji et al., 2006), and inactivation of Akt in colon cancer cells (LS174T) (Jia et al., 2013), among other results. Thus, the high effect of amber propolis could be due to de high content of active anti-leukemic compounds. In addition, much of these active molecules differ from those present in green or red propolis and an alternative pathway could be targeted by them.

The antimicrobial effect of propolis is naturally necessary for the bees, since it is produced to protect the hive against microbial pathogens (Ghisalberti, 1979), in a self-medication mechanism (Finstrom and Spivak, 2012). Also, the propolis mechanism towards bacterial cells is complex and cannot be compared with any classical antibiotic (Takaisi-Kikuni and Schilcher, 1994) and can be used to treat infections caused by super-resistant bacteria. For example, almost three decades ago it has been shown that the use of French propolis completely inhibited the growth of *S. aureus*, including MRSA strain (a methicillin-resistant *S. aureus*) (Grange and Davey, 1990). Actually, the red and green Brazilian propolis showed great antimicrobial activity (Alencar et al., 2007; Daugsch et al., 2008; Machado et al., 2016; Righi et al., 2011; Sawaya et al., 2004). However, several antimicrobial analyzes were performed by the inhibition zone method, which may not be the best method for comparing the antimicrobial effect of propolis, because the more water soluble compounds tend to disperse more effectively into the agar, resulting in a higher inhibition zone, than those without soluble active compound, as may be the case of amber propolis. This situation was clearly observed in two studies that compared different methods to test the effect of propolis extract against *Candida* species, *Staphylococcus* and *Streptococcus*; in those studies, the clearest results were obtained by serial dilution in tube or plates than by the diffusion assay (Sawaya et al., 2002 and 2004).

At the same time, despite the complexity of the chemical composition of propolis, many authors attribute the biological activity to the concentration of phenolic compounds, particularly the flavonoids, and green and red propolis stand out again in this regard (Burdock, 1998; Castaldo and Capasso, 2002; Choi et al., 2006; Ghisalberti, 1979; Grange and Davey, 1990; Marcucci, 1995; Righi et al., 2011; Savickas et al., 2005). Nevertheless, amber propolis demonstrates antimicrobial

activity similar to that showed by red propolis and, in some cases, slightly higher than green propolis, even with low phenolic and flavonoids content compared to the red and green propolis. Thus, our data support the data published by Kujumgiev et al., (1999), which evaluated the antimicrobial, antifungal, and antiviral effect of propolis obtained from different countries and bee species and observed similar biologic activity in samples with chemical compositions completely different, observing that propolis with low phenolic content had no decrease on their antimicrobial activity.

Our results for red and green propolis were similar to those obtained by other studies previously performed using *E. coli* or *S. aureus*. (Alencar et al., 2007, Daugsch et al., 2008; Machado et al., 2016; Righi et al., 2011). Therefore, taking together the IC50 values obtained on the antileukemic activity, our data for antibacterial activity validate the quality of the red and green propolis used in this study as references and, at the same time, demonstrate the high antibiotic potential of amber propolis against gram-positive and gram-negative bacteria, as well as their potential for use as antineoplastic agent.

5. Conclusion

This study describes propolis samples collected in southern Brazil that was named “amber propolis” from the color of its ethanolic extract. This propolis is chemically characterized by the presence of eucaliptol, together with α and β-pinene, limonene, globulol, myrtenol and high content of ellagic acid, among other substances, which suggests the genus *Eucalyptus* as a major source of amber propolis base. At the same time, regarding the flavonoid profile, this propolis does not have rutin or caffeic acid that differs it from the green and red propolis. The content of total phenols, flavonoids and antioxidant potential of amber propolis is lower than that of green and red propolis; however, this does not affect its antineoplastic or antibacterial potential, having similar activity than red propolis. Finally, the present results stimulate further studies aiming at elucidation of potential therapeutic applications of amber propolis and new studies should be conducted to identify the area where amber propolis may be produced in South America.

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Competing interests

The authors declare no competing or financial interests.

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Supplemental material

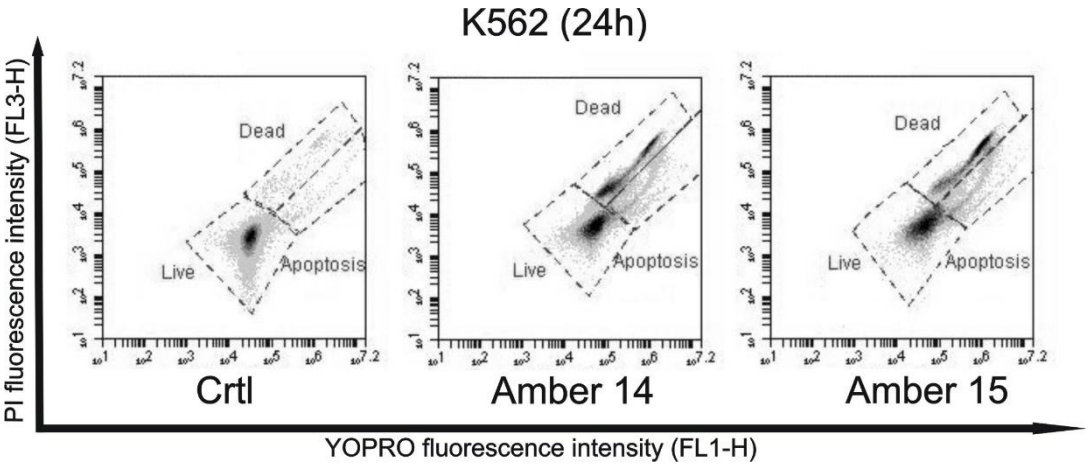

Figure S1. Representative graphics of flow cytometry apoptosis assay, showing K562 treated during 24 hours with amber propolis (2014 and 2015) and untreated cells. Three gates were created to discriminate apoptotic cells from live and dead cells.

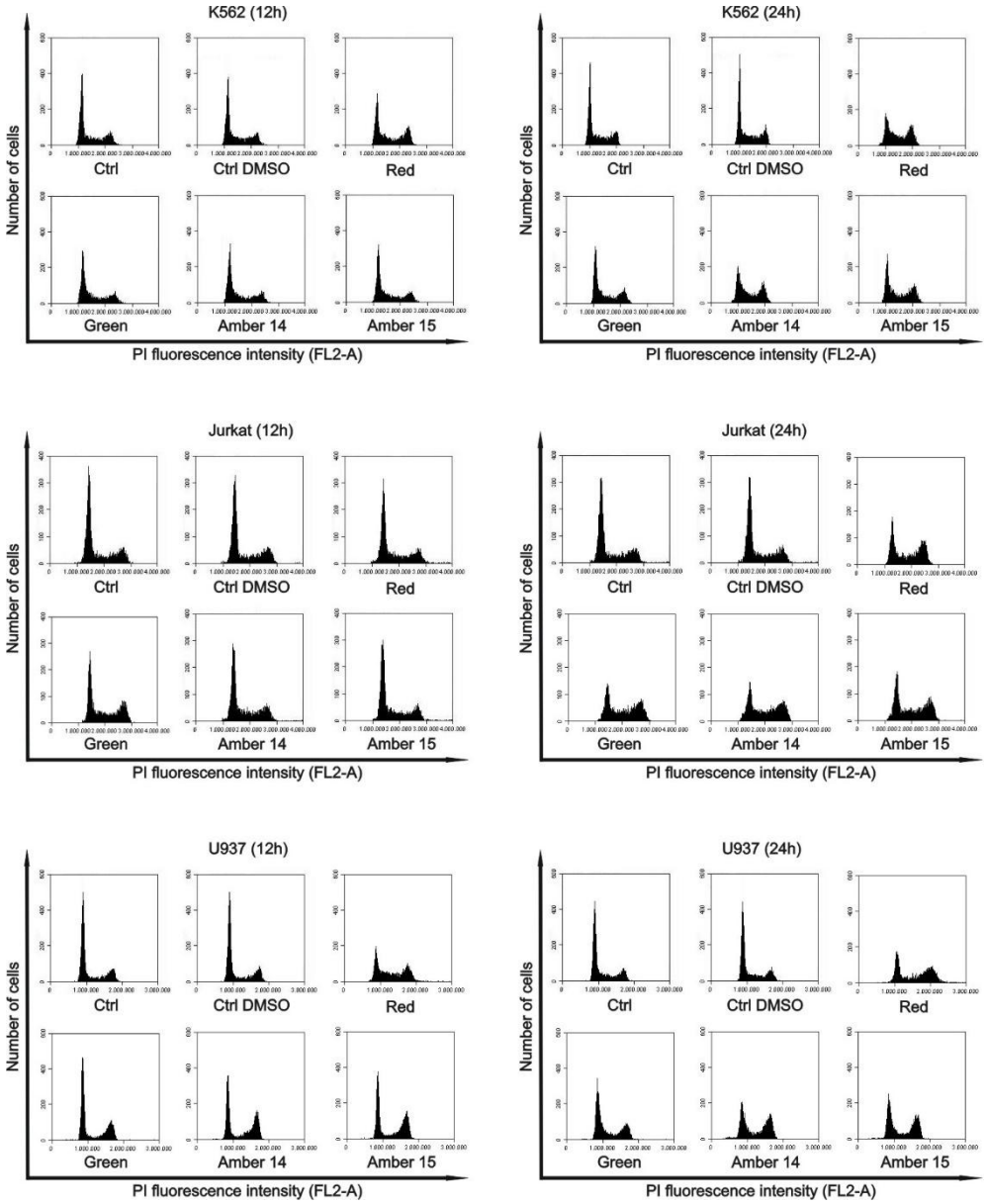

Figure S2. Representative histograms of flow cytometry cell cycle assay, showing K562, Jurkat and U937 cells untreated or treated during 12 and 24 hours with amber, green and red propolis.

4. CONSIDERAÇÕES FINAIS

Própolis de diferentes origens têm demonstrado aplicabilidade na melhoria da saúde humana. A Própolis Âmbar, produzida em florestas de eucalipto associadas ao bioma Pampa e rica em óleos essenciais, também segue este padrão e apresenta atividade antileucêmica e antimicrobiana semelhante aos resultados obtidos pela própolis vermelha e melhor que os da própolis verde, apesar do baixo teor de propriedades antioxidantes. A documentação destas propriedades deve tornar a produção da própolis mais atrativa aos apicultores, colaborando na sanidade apícola e no desenvolvimento regional do setor apícola de São Gabriel.

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