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Avaliação de parâmetros imunológicos, inflamatórios e bioquímicos em pacientes HIV-positivos sob uso da terapia antirretroviral em associação com a própolis

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutora em Ciências, área de Patologia.

Orientador: Prof. Dr. José Maurício Sforcin
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Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar.

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RESUMO

A infecção pelo HIV representa um grave problema de saúde pública no mundo, contando com milhões de pessoas infectadas e novos casos registrados anualmente. A terapia antirretroviral (TARV) mudou completamente o cenário da infecção, propiciando marcante redução da mortalidade das pessoas que vivem com HIV/aids (PVHA). Apesar dos benefícios da TARV, seu uso crônico pode resultar em efeitos adversos, como citotoxicidade e distúrbios metabólicos, processos que contribuem com a persistente ativação imune e estado inflamatório intenso que as PVHA apresentam. Assim, objetivamos investigar se a própolis, produto apícola que possui inúmeras propriedades terapêuticas, seria capaz de promover benefícios à saúde de PVHA assintomáticas em uso de TARV. Para isso, após randomização de 40 pacientes em caráter duplo-cego, os dois grupos (500 mg/dia própolis versus placebo) foram analisados em dois momentos: antes (M0) e 3 meses após (M1) a intervenção. Resultados da carga viral plasmática do HIV, contagem de células T $CD4^+$/$CD8^+$, hemograma e o perfil bioquímico/metabólico foram obtidos via prontuário eletrônico. A concentração de citocinas plasmáticas (TNF-α, IL-6, IL-10, IL-17, IL-2 e IL-4) e a proliferação de linfócitos foram determinadas por citometria de fluxo. A produção de citocinas (IFN-γ, IL-5, IL-17, IL-10, IL-18, IL-1β e IL-33) por células mononucleares do sangue periférico (PBMC) foi determinada por método imunoenzimático (ELISA). A expressão dos genes T-bet, GATA-3, RORγt e Foxp3 foi avaliada pela reação em cadeia da polimerase quantitativa em tempo real (RT-qPCR). Houve aumento na atividade de creatinofosfoquinase no grupo tratado com própolis, embora sem significância clínica. Observou-se, no mesmo grupo, aumento na concentração de magnésio, correlação positiva entre IL-10 (citocina anti-inflamatória) e contagem de células T $CD4^+$, correlação negativa entre IL-10 e IFN-γ (citocina pró-inflamatória), além do aumento da expressão de Foxp3 e da proliferação de linfócitos T $CD4^+$. Assim, os resultados indicam que o uso da própolis é seguro e pode ser uma alternativa para melhora da resposta imune e redução da inflamação nos pacientes assintomáticos, através da indução de células T reguladoras. Resultados adicionais obtidos com linhagem celular de monócitos humanos, em projeto paralelo, indicaram que a própolis exerce atividade antioxidante, potencialmente envolvida em sua ação anti-inflamatória e antialérgica, o que também pode ser uma contribuição para as PVHA. No entanto, mais estudos

são necessários para confirmar esses achados e poder considerar a própolis uma intervenção efetiva para as PVHA, em especial àquelas com comorbidades ou falha terapêutica, não contempladas neste estudo.

ABSTRACT

HIV infection represents a serious public health problem in the world, with millions of people infected and new cases registered annually. Antiretroviral therapy (ART) has completely changed the infection scenario, providing a marked reduction in the mortality of people living with HIV/AIDS (PLWHA). Despite the benefits of the ART, its chronic use can lead to side effects such as cytotoxicity and metabolic disorders, what contributes to persistent immune activation and inflammatory status in PLWHA. Thus, we aimed to investigate whether propolis, a bee product presenting several therapeutic properties, could be able to improve the health of asymptomatic PLWHA under ART. After a double-blind randomization of 40 patients, two groups (500 mg/day propolis versus placebo) were analyzed in two moments: before (M0) and 3 months after (M1) the intervention. Plasma HIV viral load, $CD4^+$/$CD8^+$ T cell count, complete blood count and biochemical/metabolic profile were obtained via electronic medical record. The concentration of plasma cytokines (TNF-α, IL-6, IL-10, IL-17, IL-2 and IL-4) and lymphocyte proliferation were determined by flow cytometry. The production of cytokines (IFN-γ, IL-5, IL-17, IL-10, IL-18, IL-1β and IL-33) by peripheral blood mononuclear cells (PBMC) was determined by Enzyme-Linked Immunosorbent Assay (ELISA). The expression of the T-bet, GATA-3, RORγt and Foxp3 genes was evaluated by real time reverse transcription polymerase chain reaction (RT-qPCR). There was an increase in creatinophosphokinase activity after propolis intake, although without clinical significance. The same group also showed increased magnesium levels, a positive correlation between IL-10 (anti-inflammatory cytokine) and $CD4^+$ T cell count, a negative correlation between IL-10 and IFN-γ (proinflammatory cytokine), an increased Foxp3 expression and $CD4^+$ T lymphocytes proliferation. Our findings indicate that the use of propolis is safe and may be an alternative for improving the immune response and reducing inflammation in asymptomatic patients, inducing regulatory T cells. Additional findings were obtained using a human monocytic cell line in a parallel project, indicating that propolis exerted an antioxidant activity, potentially involved in its anti-inflammatory and antiallergic action, what can also be a contribution to PLWHA. However, more studies are needed to confirm these findings and consider propolis an effective intervention for PLWHA, especially those with comorbidities or therapeutic failures, who was not included in this study.

SUMÁRIO

Introdução

1. Vírus da Imunodeficiência Humana (HIV) e aids

1.1. Aspectos gerais e história natural da doença

A infecção pelo HIV representa um dos maiores problemas de saúde pública no mundo, com 38 milhões de pessoas infectadas, sendo 1,7 milhão somente em 2019 (UNAIDS, 2020). Apesar dos intensos avanços tecnológicos e das pesquisas relacionadas ao seu diagnóstico precoce e tratamento eficaz, a infecção pelo HIV ainda é emergente, em função do elevado número de novos casos a cada ano e de sua gravidade (Brasil, 2020).

O HIV começou a propagar-se, de forma silenciosa, na década de 70 (Gallo, 2006; Montagnier, 2010). Em 1980, ocorreu o primeiro caso da doença no Brasil (Brasil, 2008) e, em 1981, foi identificada e denominada “síndrome da imunodeficiência adquirida”, conhecida pela sigla AIDS (Gallo, 2006; Montagnier, 2010). Em 1982, foram identificados os modos de transmissão do vírus: via sangue, relação sexual e da mãe para o filho (Galvão, 2002). No mesmo ano, foram identificados os então chamados grupos de risco, denominados de "5 H's": homossexuais, usuários de heroína, haitianos, hemofílicos e *hookers* (do inglês, “prostitutas”) (Gallo, 2006). Em 1983, o HIV foi finalmente isolado e, no ano de 1984, foi confirmado como agente causal da aids (Montagnier, 2010).

O entendimento inicial da patogênese do HIV e o rápido avanço na pesquisa levaram à expectativa de que a aids pudesse ser prontamente resolvida, porém, com experiência em retrovírus, os cientistas sabiam que esse seria um longo caminho e, até mesmo, uma infecção permanente (Gallo, 2006).

Os anos seguintes caracterizaram um período de mobilização global, com campanhas de educação quanto à prevenção da infecção, um grande avanço prático que continua até os dias atuais, mas com resultados que variam a nível local e temporal (Gallo, 2006).

O HIV pertence ao gênero *Lentivirus* e à família *Retroviridae.* É um vírus de RNA, de estrutura esférica, com tamanho médio de 100 a 120 nm, encapsulado por um nucleocapsídeo, um capsídeo e um envelope externo com bicamada fosfolipídica (ICTV, 2017; Brasil, 2018a). Seu genoma inclui, principalmente, os genes *gag, pol e env,* que codificam proteínas estruturais e enzimas virais, além de outros genes regulatórios e acessórios (Miller, 2010).

A entrada do vírus na célula do hospedeiro é mediada, inicialmente, pela glicoproteína (gp) 120, presente no envelope viral, que se liga ao receptor CD4 presente nas células do hospedeiro, como linfócitos T $CD4^+$, células dendríticas (DCs) e macrófagos. Essa ligação desestabiliza a gp120 e expõe sua alça V3, que interage com o co-receptor CCR5 ou CXCR4. Em seguida, ocorre a ativação da gp41 e a fusão do envelope viral com a membrana celular, permitindo a entrada do vírus na célula do hospedeiro (Kwong *et al.*, 1998; Goodsell, 2015). Após liberação do material genético viral na célula do hospedeiro, O RNA é convertido em DNA pela ação da enzima viral transcriptase reversa, e, posteriormente, o DNA viral é inserido no DNA do hospedeiro com auxílio da enzima viral integrase. A partir desse momento e pela ação da protease viral, a célula hospedeira produz RNA e proteínas virais para formação de novos vírus, que são então liberados da célula por brotamento e, após sofrer maturação, são capazes de infectar outras células (Goodsell, 2015).

Após o contato inicial com o HIV, macrófagos, DCs e células T residentes no local de entrada são as primeiras células a serem infectadas (Espíndola *et al.*, 2016). A resposta imune inata é desencadeada no foco da infecção, com posterior atração de células T, levando ao aumento da replicação e disseminação do vírus (McMichael *et al.*, 2010). No decorrer da infecção, o HIV é disseminado para os reservatórios virais, principalmente nos tecidos linfoides (Trigo & Costa, 2016).

Desde a entrada do vírus no organismo até à viremia inicial, decorrem cerca de 4 a 11 dias (Trigo & Costa, 2016), com pico em torno de 21 a 28 dias após a exposição (McMichael *et al.*, 2010).

Essa primeira fase da infecção, denominada de fase aguda, geralmente é sintomática, com duração de algumas semanas. As queixas e sinais clínicos mais observados nesse momento são febre, adenopatia, astenia, faringite, exantema,

mialgia e cefaleia. Nessa fase, são observados valores elevados de carga viral plasmática do HIV, maior número de células infectadas e queda no número de células T $CD4^+$ (McMichael *et al.*, 2010; Espíndola *et al.*, 2016; Brasil, 2018a).

Durante a disseminação sistêmica viral, a resposta imune específica é desencadeada para controlar a infecção (Langford *et al.*, 2007). Essa resposta conta com a ação de linfócitos T citotóxicos específicos contra o HIV e produção de anticorpos neutralizantes, levando à diminuição da viremia até o nível de *set point* viral e o estabelecimento de uma infecção latente (Ascher & Sheppard, 1988; Trigo & Costa, 2016). Assim, é estabelecida uma fase crônica que perdura por muitos anos. O exame clínico nessa etapa costuma ser normal, com exceção da linfadenopatia que pode ser persistente, e alguns achados laboratoriais como anemia e leucopenia leves (Brasil, 2018b).

No entanto, a resposta imune desencadeada é tardia e insuficiente para evitar a progressão e erradicação da infecção (McMichael *et al.*, 2010). Com a progressão da infecção, ocorre a evolução para aids, caracterizada pela contagem de linfócitos T $CD4^+$ inferior a 200 células/mm^3, presença de infeções oportunistas e neoplasias (Trigo & Costa, 2016). Assim, o principal dano causado pelo HIV é a deterioração progressiva do sistema imunológico, especialmente das células T $CD4^+$, acarretando grave imunodepressão, o que torna o indivíduo mais suscetível às doenças oportunistas e determinadas neoplasias (Gallo, 2006; Brasil, 2014).

O curso da infecção depende dos fatores virológicos e também do hospedeiro (Okulicz *et al.*, 2009). Estima-se que o tempo entre o contato inicial com o vírus e surgimento da aids, em indivíduos não tratados, seja de aproximadamente 10 anos (Brasil, 2013). Esse quadro acontece com os chamados “progressores típicos”, no entanto, cerca de 5% dos infectados permanecem sem sintomas ou evidência clínicas da infecção por um período superior, sendo denominados de “progressores lentos” (Kumar, 2013). Há, ainda, um grupo mais raro (≤ 1% dos infectados), os chamados “controladores de elite”, que mesmo sem tratamento apresentam carga viral indetectável (Poropatich & Sullivan, 2011). No entanto, desde a recomendação do tratamento antirretroviral imediato para todas as pessoas que vivem com HIV/aids (PVHA), tornou-se mais escassa a identificação desses grupos (Brasil, 2018b).

Como as células T $CD4^+$ são essenciais para o desenvolvimento da resposta imune adaptativa, sua depleção resulta em significativa redução na capacidade de elaboração da resposta imune específica do hospedeiro (Langford *et al.*, 2007; Said *et al.*, 2010). Melhores respostas linfoproliferativas a antígenos específicos do HIV são observadas em indivíduos que apresentam progressão mais lenta da doença, em comparação aos indivíduos que demonstram rápida progressão. Dessa forma, a contagem de células T $CD4^+$ consiste em um importante marcador para acompanhar o desenvolvimento da doença, sendo que baixas contagens estão relacionadas com maiores chance de progressão (Langford *et al.*, 2007). Além de reduzir a quantidade dessas células, a infecção viral também ocasiona menor função efetora das mesmas (Said *et al.*, 2010).

1.2. Ativação imune e inflamação no HIV/aids

A infecção pelo HIV induz intensa desregulação do sistema imunológico, gerando perda da função celular e inflamação crônica. A exaustão imunológica observada nas PVHA é caracterizada pelo aumento do fenótipo ativado das células T e B, porém diminuição da capacidade proliferativa e perda da capacidade efetora das células do sistema imune (Gutiérrez *et al.*, 2019).

Dentre as respostas imunes adaptativas existentes, as subpopulações celulares mais estudadas são os linfócitos T *helper* (Th) 1, Th2, Th17 e T regulador (Treg). Nunnari *et al.* (2016) descreveram que a progressão para a aids deve-se provavelmente ao grande desbalanço que ocorre entre esses diferentes perfis de resposta.

Clereci & Shearer (1993) revelaram que o desequilíbrio das respostas Th1 e Th2 está associado com resistência à infecção pelo HIV ou progressão para aids. Os autores descreveram que a exposição ao HIV desencadeia forte resposta Th1 contra o vírus, enquanto a progressão para aids está relacionada com a perda da produção de IL-2 e IFN-γ e aumento de produção de IL-4 e IL-10 (Clereci & Shearer, 1993). Orsilles *et al.* (2006) observaram que pacientes HIV^+ sem uso da terapia antirretroviral (TARV) e com contagem de CD4 ≥ 200 células/mm^3 apresentavam maior concentração de IL-2, ao passo que aqueles com contagem de CD4 < 200

céls/mm^3 apresentavam maior produção de IL-10, comparados ao controle. No entanto, não foram observadas diferenças significativas desses valores em pacientes sob uso de TARV, demonstrando que a desregulação das citocinas pode ser revertida pela terapia (Orsilles *et al.*, 2006).

De fato, o HIV é capaz de modular a secreção de diversas citocinas durante a progressão da infecção, mas a influência dessas moléculas na patogênese do vírus é diversa e não completamente entendida (Chinen & Shearer, 2002). Roberts *et al*. (2010) observaram elevação de diversas citocinas inflamatórias na infecção aguda pelo HIV, como IL-1α, IL-1β, IL-6, IL-8 e TNF-α. Os autores também descreveram que as citocinas IL-12 e IFN-γ estão associadas com baixa carga viral, enquanto IL-7 e IL-15 estão associadas com carga viral elevada (Roberts *et al.*, 2010). Nunnari *et al.* (2016) identificaram que indivíduos HIV$^+$ sem tratamento, com alta viremia ou controladores de elite, apresentaram maior expressão de citocinas pró-inflamatórias (IL-1β, INF-γ, IL-18) e quimiocinas (CCL3, CCR6, CCL5, CCR7, CXCL16 e CXCR4) do que indivíduos saudáveis. Também as moléculas de HLA I e II, CD3, CD4, CD28, CD69, CTLA4 e LAG3 estavam positivamente reguladas nas PVHA (Nunnari *et al*., 2016), demonstrando o estado de ativação imune na infecção pelo HIV.

A principal característica da infecção pelo HIV é a secreção de citocinas pró-inflamatórias, as quais podem favorecer a replicação do vírus, a apoptose das células T (Decrion *et al.*, 2005) e contribuir com o desenvolvimento de comorbidades não-associadas à aids (Tasca *et al.*, 2012). Além das citocinas, outros biomarcadores associados a processos inflamatórios encontram-se elevados nas PVHA, como a alfa-1-glicoproteína ácida (Cordialli *et al.*, 1992), proteína C reativa (PCR) e creatinofosfoquinase (CPK) (Monteiro *et al.*, 2013; Neuhaus *et al.*, 2010). Além disso, PCR e CPK são fatores também associados ao risco de desenvolvimento de doenças cardiovasculares, corroborando o fato de que as PVHA frequentemente apresentam maior estado inflamatório e danos cardiovasculares em relação à população não infectada (Deeks, 2011). Tien e colaboradores (2010) sugeriram que, mesmo em PVHA com contagens de CD4 > 500 células/mm^3 relativamente preservadas, a inflamação ainda é um importante fator de risco para mortalidade dessa população.

Além dos marcadores inflamatórios, também a proteína de morte celular programada 1 (PD-1) apresenta-se superregulada em pacientes HIV^+ sem uso de TARV (Said *et al.*, 2010). Outros marcadores associados à coagulação (D-dímero) e função renal (cistatina C), encontram-se elevados na infecção pelo HIV, mesmo após adesão à TARV e supressão da carga viral (Neuhaus *et al.*, 2010).

O HIV também induz ativação do inflamassoma, um complexo citoplasmático multiproteico que contribui com o processo inflamatório (Hernandez *et al.*, 2014). A ativação do inflamassoma induz a maturação das citocinas IL-1β, IL-18 e IL-33 em suas formas biologicamente ativas, as quais são então liberadas das células e desempenham papel crucial na inflamação. A ativação do inflamassoma parece ter participação em uma variedade de doenças inflamatórias, como aterosclerose, gota e diabetes do tipo 2 (Yang *et al.*, 2012). Na infecção pelo HIV, observa-se alta expressão de IL-1β, IL-18 e caspase-1 tanto no tecido linfoide associado ao intestino (GALT) quanto nas células mononucleares do sangue periférico (PBMC) (Feria *et al.*, 2018).

Para o controle de resposta imunológica exacerbada, diversos mecanismos são acionados, como a diferenciação celular para o perfil de células Tregs (Raphael *et al.*, 2015). Os linfócitos Treg secretam citocinas anti-inflamatórias e suprimem respostas inflamatórias em diversos contextos biológicos, sendo considerados um fator vital de regulação negativa da inflamação, inclusive em processos infecciosos crônicos (Plitas & Rudensky, 2016). No entanto, na infecção pelo HIV é observado um estado de hiperativação imune, com redução no número e função das Tregs (Eggena *et al.*, 2005; Prendergast *et al.*, 2010), sendo a contagem dessas células ainda menor na ausência da TARV (Gutiérrez *et al.*, 2019).

As PVHA também apresentam diminuição do perfil Th17 - resposta importante para manutenção da homeostase no GALT. Essa resposta previne a inflamação e evita alterações na estrutura da mucosa intestinal, diminuindo o risco de ruptura da barreira epitelial, dificultando a translocação microbiana do lúmen intestinal para a corrente sanguínea (Wacleche *et al.*, 2017). A presença de produtos microbianos circulantes nas PVHA, principalmente o lipopolissacarídeo (LPS), está diretamente correlacionado com aumento da ativação da resposta imune inata e adaptativa, e com aumento da produção de citocinas pró-inflamatórias (Klatt *et al.*,

2013). Assim, na falha de uma resposta protetora no GALT, todos estes processos são intensificados, comprometendo ainda mais o *status* clínico das PVHA (Brenchley *et al.*, 2006; Hsu *et al.*, 2013).

A translocação microbiana, bem como os danos genômicos e estresse oxidativo podem contribuir para senescência celular acelerada na infecção pelo HIV. Muitas das anormalidades do sistema imune, geralmente relacionadas com aumento da idade, são observadas mais cedo e mais frequentemente nas PVHA, como a redução do número e função das células-tronco hematopoiéticas, disfunção tímica, repertório de células T reduzido, potencial proliferativo limitado, capacidade de resposta reduzida às vacinas, concentrações aumentadas de citocinas pró-inflamatórias, e proporções CD4/CD8 diminuídas, conforme revisado por Deeks (2011).

A ativação das células T $CD8^+$ específicas para o controle da infecção, apesar de suprimir a replicação viral, contribui também para o aumento da ativação imune crônica e seus marcadores, tais como a expressão de CD38 e HLA-DR. Durante a progressão da infecção, essas células adquirem um fenótipo característico de imunosenescência, com capacidade proliferativa e funcional limitadas, o que pode ser observado mesmo nos indivíduos sob uso de TARV (Warren *et al.*, 2019).

Assim, a ativação do sistema imune e o processo inflamatório são persistentes durante todo o curso da infeção, sendo decorrentes, provavelmente, de vários fatores, incluindo efeitos diretos das proteínas virais, a própria resposta imune ao vírus, translocação microbiana, produção desregulada de citocinas e quimiocinas, replicação residual do HIV, estímulo provocado por outros patógenos, perda de células regulatórias, fibrose do timo e linfonodo (Hunt, 2012; Kamat *et al.*, 2012; Brasil, 2013; Hsu *et al.*, 2013; Zicari *et al.*, 2019), além de efeitos adversos da própria TARV, como citotoxicidade, promoção de estresse oxidativo e distúrbios metabólicos.

As persistentes ativação imune e inflamação observadas nas PVHA merecem atenção, pois são fatores potencialmente determinantes de morbidade e mortalidade não associadas à aids, mesmo nos indivíduos sob TARV e que

apresentam supressão viral adequada (French *et al.*, 2009; Deeks, 2011; Aberg, 2012; Hunt *et al.*, 2013; Zicari *et al.*, 2019).

1.3. Terapia antirretroviral (TARV)

A TARV foi considerada o maior avanço da pesquisa na área HIV/aids (Gallo, 2006), diminuindo consideravelmente a morbidade e mortalidade das PVHA (Brasil, 2013), além de impactar na economia do sistema público de saúde, evitando gastos com internações e tratamento de infecções oportunistas (Galvão, 2002).

O tratamento antirretroviral no Brasil teve início no ano de 1989, com a zidovudina (AZT), a qual passou a ser distribuída em 1991 de forma gratuita pelo Sistema Único de Saúde (SUS). No ano de 1996, foi instituída a lei brasileira que garantia às PVHA o acesso universal e gratuito aos medicamentos antirretrovirais. Desde então, terapias mais eficazes e refinadas foram surgindo no país (Brasil, 2008). Estima-se que, após um ano de tratamento, pelo menos 80% das PVHA apresentam carga viral inferior a 50 cópias/mL, supressão viral que se mantém no decorrer do tempo (Brasil, 2018b).

De acordo com o Programa Conjunto das Nações Unidas sobre HIV/AIDS (UNAIDS), até o fim de 2019, aproximadamente 25,4 milhões de PVHA tinham acesso à TARV, o que é considerado um grande aumento comparado aos 7,7 milhões em 2010. Além disso, desde 2010, a mortalidade relacionada à aids diminuiu consideravelmente devido à esta terapia (UNAIDS, 2020). De acordo com o Boletim Epidemiológico HIV/Aids do Ministério da Saúde (2020), a taxa de mortalidade sofreu decréscimo de 28,1% entre 2014 e 2019, possivelmente, em consequência da recomendação do “tratamento para todos” e da ampliação do diagnóstico precoce da infecção pelo HIV (Brasil, 2020).

A TARV engloba vários tipos de fármacos, que atuam em diferentes fases do ciclo viral. As classes utilizadas para o tratamento da infecção pelo HIV incluem os inibidores de transcriptase reversa análogos de nucleosídeos (ITRN), inibidores de transcriptase reversa não análogos de nucleosídeos (ITRNN), inibidores de protease (IP), de fusão, de integrase e antagonistas do correceptor CCR5. Tais classes são utilizadas em associação, compondo diferentes esquemas terapêuticos,

cuja prescrição depende de diversos fatores, como presença de comorbidades, intolerância medicamentosa, uso de outros medicamentos, presença de co-infecções, risco cardiovascular elevado, ocorrência de falha virológica, entre outros (Brasil, 2018b).

Considerando os benefícios da TARV, como supressão viral, melhora da função imunológica e redução da transmissão do HIV, a Organização Mundial da Saúde passou a recomendar o início imediato da terapia a todos pessoas diagnosticadas com a infecção, independente do estágio clínico ou imunológico (Ford *et al*., 2018).

Assim, a adesão à TARV tem promovido maior sobrevida e mais benefícios às PVHA, levando à redução do reservatório viral e proteção da imunidade inata e adquirida (Sáez-Cirión *et al.*, 2013). Valdez *et al.* (2002) descreveram que a maioria das mudanças na restauração imunológica das PVHA ocorre já durante o primeiro ano do uso da TARV.

Apesar dos benefícios promovidos pela TARV, o início terapêutico tem ocorrido cada vez mais cedo, ocasionando surgimento precoce de eventos adversos causados pelos antirretrovirais, como distúrbios metabólicos, doenças cardiovasculares, toxicidade óssea, desenvolvimento de hepatopatias, alterações renais e pancreáticas (Rose *et al.*, 2006; Aberg, 2012; Hsu *et al.*, 2013; Pinto Neto *et al.*, 2013; Deeks *et al.*, 2015; Ngala *et al.*, 2015; Vos *et al.*, 2018). Além disso, mesmo sob terapia, a inflamação e ativação imune ainda estão presentes nas PVHA, quando comparado aos indivíduos não infectados (Deeks, 2011; Kamat *et al.*, 2012).

Entre as alterações metabólicas oriundas da TARV, a dislipidemia, caracterizada pelo aumento dos níveis séricos de triglicérides, colesterol total, LDL-colesterol, e redução do HDL-colesterol, é mais prevalente em PVHA do que na população de não-infectados (Rose *et al.*, 2006). Uma vez iniciada a TARV, observa-se que 33% a 82% dos pacientes desenvolvem hipercolesterolemia e 43% a 66% hipertrigliceridemia (Yu *et al.*, 2005), alterações estas que contribuem para o aparecimento de doenças cardiovasculares nesses pacientes (Venkataramana, 2013).

As alterações do perfil metabólico estão associadas também com o desenvolvimento da resistência à insulina, que, por sua vez, pode contribuir com o desenvolvimento de hipertensão arterial sistêmica. Essas alterações provocadas pela TARV, somadas às alterações no perfil lipídico e inflamatório, resultam em aumento no risco de doenças cardiovasculares em PVHA (Brasil 2013, 2018b).

Outro evento adverso provavelmente associado à TARV é o desenvolvimento da lipodistrofia - desordem do tecido adiposo caracterizada por níveis alterados de gordura em diferentes partes do corpo (Araújo-Vilar & Santini, 2019). Além dos fatores intrínsecos do indivíduo, da própria infecção e da TARV, a concentração elevada de proteínas inflamatórias também está associada ao seu desenvolvimento (Caron-Debarle *et al.*, 2010). Como exemplo, a citocina TNF-α, cuja expressão está aumentada no tecido lipoatrófico das PVHA, é responsável pela indução da apoptose das células de gordura. A lipodistrofia é um fator impactante na vida das PVHA, causando problemas psicológicos, sociais, diminuindo adesão à terapia e aumento da morbimortalidade nesses indivíduos (Brasil, 2013).

Alterações renais podem ser decorrentes da nefrotoxicidade causada pelos antirretrovirais ou das alterações metabólicas relacionadas a estas medicações (Brasil, 2013). A doença renal crônica apresenta alta prevalência em PVHA sob TARV, com índices que só são observados na população geral com mais de 60 anos de idade (Menezes *et al*., 2011). PVHA também apresentam maior risco de desenvolver injúria renal aguda do que indivíduos não infectados (Wyatt, 2014).

A hepatotoxicidade grave acomete até 10% das PVHA sob TARV (Brasil, 2013), pois tanto os antirretrovirais quanto o vírus desencadeiam aumento da atividade de aspartato aminotransferase (AST) e alanina aminotransferase (ALT) (Mgogwe *et al.*, 2012; Venkataramana *et al.*, 2013). Além disso, o aumento desses marcadores está associado a maior risco de mortalidade em PVHA (Viana *et al.*, 2011).

As alterações pancreáticas também são frequentes nas PVHA (Chehter *et al.*, 2000), sendo consideradas umas das principais causas de morbidade nessa população (Raza *et al.*, 2013) e também podem estar associadas ao uso dos antirretrovirais (Oliveira *et al.*, 2014).

A prevalência de osteopenia e osteoporose é pelo menos três vezes maior em pessoas infectadas pelo HIV do que em não infectadas e, da mesma forma, as fraturas também são mais comuns nessa população (Deeks, 2011). A própria infecção pelo HIV pode causar distúrbios de mineralização óssea, através da ação direta do vírus nas células osteogênicas, e também pela presença persistente de citocinas pró-inflamatórias e alterações no metabolismo da vitamina D (Brasil, 2013). Além disso, a TARV também contribui nesse cenário, em decorrência da toxicidade dos medicamentos e da persistente inflamação sistêmica (Deeks, 2011). Os antirretrovirais podem interferir no metabolismo do fósforo e da vitamina D (Brasil, 2018b), inibir a formação óssea ou influenciar na diferenciação e função dos osteoclastos (Brasil, 2013). Estima-se que, nos primeiros dois anos da terapia, a densidade óssea sofre uma redução de 2 a 6%, com posterior estabilização (Brasil, 2018b).

Também as neoplasias passaram a ser responsáveis por um número maior de óbitos nas PVHA após a implementação da TARV, associada ao aumento da média de idade das PVHA, presença de co-infecções, exposição prolongada a fatores ambientais de risco e estilo de vida. Entre os tipos de cânceres mais comuns nessa população estão os de pele, pulmão, colorretal/anal, carcinoma hepatocelular, e linfoma de Hodgkin's, conforme revisado por Brugnaro *et al.* (2015).

Há que se considerar, também, outros fatores que podem contribuir com o surgimento ou agravamento dessas comorbidades, como tabagismo, etilismo, sedentarismo, alimentação desbalanceada, estresse, co-infecções com vírus da hepatite B e C, doenças pré-existentes, uso de outros medicamentos, fatores genéticos, entre outros (Deeks, 2011; Brasil, 2018b).

Assim, atualmente as principais causas de óbito em PVHA estão associadas às comorbidades não-aids, diferentemente da era pré-TARV, em que as causas de óbito eram associadas principalmente com a profunda imunossupressão e desencadeamento de infecções oportunistas. As principais comorbidades atualmente presentes nas PVHA são doenças típicas do envelhecimento, como doença cardíaca isquêmica, acidente vascular encefálico e outras alterações do sistema nervoso central, dislipidemias, hepatopatias, doença renal crônica, diabetes mellitus, alterações ósseas, cânceres não relacionados à aids, entre outras (Crum-

Cianflone *et al.*, 2010; Deeks, 2011; Guaraldi *et al.*, 2011; Venkataramana, 2013; Ramana, 2014; Ngala *et al.*, 2015). Estas comorbidades são frequentes em indivíduos infectados pelo HIV (Hsu *et al.*, 2013; Maciel *et al.*, 2018), e aparecem cerca de 15 anos mais cedo nas PVHA do que na população em geral, caracterizando o “envelhecimento precoce” (Guaraldi *et al.*, 2011). Além disso, o processo inflamatório crônico apresentado pelas PVHA também é um importante mecanismo desencadeador do envelhecimento mais acelerado (Wing, 2016).

Portanto, muitas das comorbidades encontradas nas PVHA podem ser consequência do dano direto do vírus bem como da inflamação persistente e da toxicidade da terapia antirretroviral (Anthony *et al.*, 2006; Brasil, 2013; Zicari *et al.*, 2019).

Diante deste cenário, pode-se observar que ainda há necessidade de encontrar novas alternativas quanto à melhoria da saúde das PVHA, visando atenuar o impacto da própria infecção e dos efeitos adversos da TARV nestes indivíduos. Assim, tivemos a iniciativa de investigar um produto apícola – a própolis, a qual apresenta inúmeras propriedades terapêuticas, na promoção de benefícios à saúde das PVHA sob TARV.

2. Própolis

2.1. Aspectos gerais

De acordo com a Agência Nacional de Vigilância Sanitária (ANVISA), a própolis é classificada como um produto opoterápico, coletada a partir de espécies vegetais e que sofre adição de secreções da abelha (Brasil, 2011). A própolis é elaborada pelas abelhas a partir de material coletado de diferentes partes das plantas, como brotos, ramos, cascas de árvores, exsudatos resinosos e botões florais, ao qual as abelhas adicionam enzimas salivares, cera e pólen (Bankova, 2005). Dessa forma, a própolis caracteriza-se como um material lipofílico, resinoso, balsâmico (Fokt *et al.*, 2010) e de matriz complexa (Braakhuis, 2019).

Em relação às suas características organolépticas, a própolis apresenta aspecto duro e quebradiço em baixas temperaturas, e apresenta-se como material

flexível e pegajoso em temperaturas mais elevadas. A coloração é variável, podendo ser encontrada nas cores verde, vermelha e marrom, em função de sua origem botânica. Seu odor é característico e intenso, sendo considerado aromático e agradável (Silva-Carvalho *et al.*, 2015).

A própolis apresenta, de modo geral, resinas, bálsamos, cera de abelha, óleos voláteis, pólen e outras substâncias, além de impurezas que podem ser removidas durante o processamento da amostra (Burdock, 1998). Sua composição química é dependente da localização geográfica e da flora local onde foi produzida, apresentando, assim, caracterização química extremamente complexa e variável (Bankova *et al.*, 2000). Já foram identificados centenas de constituintes na própolis, incluindo ácidos fenólicos, flavonoides, ésteres, diterpenos, sesquiterpenos, lignanas, aldeídos aromáticos, álcoois, aminoácidos, ácidos graxos, vitaminas e minerais (Braakhuis, 2019). Em geral, amostras de própolis verde brasileira são caracterizadas pela presença do ácido 3,5-diprenil-4-hidroxicinâmico, artepelin C, ácidos cinâmicos prenilados e derivados do ácido cafeico (Cornara *et al.*, 2017).

Na zona temperada do Hemisfério Norte, a produção de própolis ocorre apenas no período compreendido entre o final da primavera e o início do outono, ao contrário de outros países, como o Brasil, onde a própolis é produzida durante todo o ano. No entanto, não são observadas variações sazonais na composição da própolis brasileira, que apesar de apresentar algumas variações quantitativas, possui concentração significativa de compostos biologicamente ativos em todas as estações do ano (Bankova *et al.*, 1998). Vale ressaltar que diferentes compostos podem estar relacionados a diferentes atividades biológicas, sendo, por isso, essencial que os estudos realizados com própolis estejam sempre associados à composição química da amostra utilizada (Sforcin, 2016).

Etimologicamente, a palavra própolis, originada do grego, significa “pro” = defesa, e “polis” = cidade (Ghisalberti, 1979), sendo utilizada pelas abelhas com o objetivo de defender a colmeia. Este produto apícola é empregado pelas abelhas para: 1) selar aberturas, afim de controlar a entrada de invasores na colmeia e também manter a temperatura interna; 2) bloquear buracos e rachaduras; 3) cobrir invasores mortos, evitando sua decomposição e a propagação de doenças, entre outras finalidades (Salatino *et al.*, 2005). Desde a domesticação das abelhas, o

homem também tem utilizado a própolis para seu benefício próprio (Silva-Carvalho *et al.*, 2015).

O Brasil lidera o ranking de pesquisas sobre produtos apícolas, incluindo a própolis (Przybyłek & Karpinski, 2019). A própolis é extensivamente produzida e comercializada para diferentes finalidades, como formulação de produtos farmacêuticos, suplementação alimentar e também na indústria de cosméticos, devido às suas diversas atividades biológicas (Silva-Carvalho *et al.,* 2015). Ademais, as pesquisas sobre própolis intensificaram nas últimas décadas.

2.2. Propriedades biológicas da própolis

Estima-se que a própolis tenha sido utilizada na medicina popular desde 300 a.C. (Silva-Carvalho *et al.,* 2015). O primeiro relato científico sobre suas atividades e composição química foi anunciado em 1908 (Helfenberg, 1908; Anjum *et al.*, 2019). Mas foi em 1985 que a própolis começou a ser considerada promissora na área da farmacologia, sendo um dos poucos produtos naturais que manteve sua popularidade ao longo do tempo (Silva-Carvalho *et al.,* 2015).

Na antiguidade, a própolis foi utilizada por diversas populações para diferentes finalidades. Devido à sua propriedade antiputrefativa, os egípcios utilizavam-na para mumificação de cadáveres, impedindo a propagação de infecções. Gregos e romanos, bem como os combatentes da segunda guerra mundial, utilizavam a própolis para o tratamento de feridas, graças à sua ação antimicrobiana e cicatrizante. Já o povo persa empregava a própolis para tratamento de eczemas, mialgia e reumatismo, enquanto os incas utilizavam-na como agente antipirético (Silva-Carvalho *et al.,* 2015; Anjum *et al.*, 2019).

A própolis tem sido utilizada até os dias atuais e tem se destacado pela possibilidade de aplicação na indústria farmacêutica (Sforcin, 2016). Ademais, a própolis é geralmente considerada segura e não tóxica para seres humanos ou animais de experimentação a curto, médio ou longo prazo (Sforcin *et al.*, 2002; Mani *et al.*, 2006; Rocha *et al.*, 2013; Silveira *et al.*, 2019). No entanto, alguns casos isolados de alergia e dermatite de contato à própolis foram descritos, principalmente em alguns apicultores (Oryan *et al*., 2018).

Dentre as diversas atividades biológicas, a própolis destaca-se por sua ação antimicrobiana, atuando contra bactérias (Sforcin *et al.*, 2000; Chen *et al.*, 2018; Przybyłek & Karpinski, 2019), fungos (Sforcin *et al*., 2001; Ota *et al.,* 2001; Siqueira *et al.*, 2009), parasitas (Dantas *et al.*, 2006; Freitas *et al*., 2006; Salomão *et al.*, 2011; Da Silva *et al.*, 2013), e também contra vírus (Búfalo *et al.*, 2009; Yildirim *et al.*, 2016; Labská *et al.*, 2018; Ripari *et al.*, 2021), incluindo o HIV (Harish *et al.*, 1997; Gekker *et al.*, 2005).

A própolis é reconhecida também por sua atividade antitumoral (Sforcin *et al.*, 2002; Ahn *et al.*, 2007), antioxidante (Kalogeropoulos *et al.*, 2009; Cole *et al.*, 2010), anti-inflamatória (Cole *et al.*, 2010; Orsatti *et al.*, 2010), imunomoduladora (Orsatti *et al.*, 2010; Búfalo *et al.*, 2014; Conti *et al.*, 2016), entre outras.

O uso da própolis como agente imunomodulador tem sido considerado uma alternativa para a prevenção e cura de diversas patologias (Orsolic & Basic, 2003). Vários estudos descreveram sua atividade imunoestimulante, com capacidade de ativar macrófagos (Orsi *et al.*, 2000) e células *natural killer* (Takeda *et al.*, 2018), aumentar a proliferação de linfócitos (Ivanovska *et al.*, 2005) e modular as respostas imunes humoral e celular (Sforcin, 2016; Al-Hariri, 2019). Trabalhos do nosso grupo já documentaram sua ação imunomoduladora em modelos animais, como ratos e camundongos (Orsi *et al.*, 2000; Orsatti *et al.*, 2010), e em células imunes humanas, tais como monócitos e células dendríticas (Búfalo *et al.*, 2014; Conti *et al.*, 2015; Conti *et al.*, 2016; Cardoso *et al.*, 2017; Conte *et al.*, 2021). A própolis também é capaz de modular diversos eventos da resposta imunológica, promovendo indução da expressão de receptores semelhante a *Toll* (TLR)-2, TLR-4 (Orsatti *et al.*, 2010), da molécula co-estimulatória CD80 (Búfalo *et al.*, 2014), modulação de vias do sistema complemento (Ivanovska *et al.*, 1995), do fator de transcrição NF-κB (Conti *et al.*, 2016) e aumento da produção de anticorpos (Sforcin *et al*., 2005).

Diversos trabalhos têm demonstrado também a potente ação anti-inflamatória da própolis, tanto na inflamação aguda quanto crônica (Araújo *et al.*, 2011) por induzir a produção do mediador anti-inflamatório IL-10 (Khayyal *et al.*, 2003; Conti *et al.,* 2015) e diminuir a concentração de marcadores inflamatórios como as citocinas IFN-γ, IL-1 β (Bueno-Silva *et al.*, 2015), TNF-α, IL-6 (Shang *et al.*,

2020), a molécula de adesão intercelular-1 (ICAM-1), as prostaglandinas E_2 e $F_{2\alpha}$ e leucotrienos D4 (Khayyal *et al.*, 2003). A própolis também é capaz de inibir a expressão da óxido nítrico-sintase induzida (Song *et al.*, 2002), a ativação do inflamassoma (Hori *et al.,* 2013) e produção de quimiocinas como CXCL1/KC e CXCL2/MIP-2 (Bueno-Silva *et al.*, 2016). Assim, sugere-se que a própolis possa ter papel importante no controle de doenças com caráter inflamatório, sendo considerada uma promissora estratégia terapêutica (Hori *et al.,* 2013).

A própolis também é capaz de modular a produção de diferentes marcadores bioquímicos e metabólicos, promovendo ação antineurodegenerativa (Chen *et al.*, 2008), cardioprotetora (Chopra *et al.,* 1995; Daleprane & Abdalla, 2013), melhora do perfil glicêmico e lipídico (Al Ghamdi *et al.*, 2015; Samadi *et al.* 2017; Nna *et al.,* 2018), conferindo proteção hepática (Banskota *et al.*, 2001, Ahmed *et al.*, 2012; Bhadauria, 2012), pancreática (Büyükberber *et al.*, 2009) e renal (El Rabey *et al.*, 2017; El Menyiy *et al.*, 2018).

Além dos ensaios *in vitro* e com animais de experimentação *in viv*o, ensaios clínicos utilizando a própolis como intervenção têm demonstrado efeitos benéficos à saúde de indivíduos saudáveis (Mujica *et al.,* 2017), fumantes (Koo *et al.*, 2019), portadores de doença renal crônica (Silveira *et al.,* 2019) e portadores de diabetes (Fukuda *et al.*, 2015, Samadi *et al.* 2017; Afsharpour *et al.,* 2017; Zakerkish *et al.*, 2019; Hesami *et al.*, 2019).

Dessa forma, considerando as atividades biológicas da própolis supracitadas, a importância de seu uso por PVHA merece ser investigada, especialmente considerando a abordagem do *status* inflamatório crônico e o desenvolvimento de comorbidades não associadas à aids muito precoce nesta população.

Vale ressaltar que os extratos de própolis têm sido preparados e utilizados deliberadamente com diferentes recomendações, sendo que, nem sempre são mencionados os métodos de preparo e composição química. Neste sentido, estudos clínicos poderiam preencher estas lacunas e contribuir com o estabelecimento de novas aplicações terapêuticas da própolis, desde que utilizando amostras devidamente caracterizadas e com propriedades biológicas conhecidas (Sforcin, 2016).

2.3. Extrato Padronizado de Própolis (EPP-AF®)

Os comprimidos contendo própolis utilizados em nosso trabalho foram gentilmente fornecidos pela Apis Flora Indl. Coml. Ltda., empresa sediada na cidade de Ribeirão Preto, SP, e que atende todo o território nacional e também internacional. A Apis Flora Indl. Coml. Ltda. comercializa diferentes tipos de produtos para saúde, desenvolvidos a partir de bioativos da biodiversidade, com ênfase nos segmentos de própolis, mel e extratos de plantas medicinais.

Nesse trabalho, optamos por trabalhar com o EPP-AF® em virtude do mesmo possuir padronização e rigoroso controle de qualidade. Nesse sentido, o EPP-AF® já foi documentado na literatura quanto ao seu processo de obtenção e padronização, garantindo a reprodutibilidade lote-a-lote. A caracterização química revelou presença de artepelin C, isosacuranetina, ácido *p*-cumárico, aromadendrina, ácido cafeico, e ácido cinâmico no extrato (Berretta *et al.*, 2012; Marquiafável *et al.*, 2015). Os comprimidos foram produzidos e avaliados pelos laboratórios de controle de qualidade, visando a aprovação dos mesmos. Ademais, um estudo conduzido *in vivo* com adultos saudáveis demonstrou que a ingestão dos comprimidos de EPP-AF® (375 mg/dia; 15 dias) não ocasiona interação com medicamentos de uso comum pela população, tais como cafeína, losartana, omeprazol, metoprolol, midazolam e fexofenadina (Cusinato *et al.*, 2019).

Os comprimidos também foram avaliados quanto à sua segurança, utilizando modelos experimentais. *In vitro,* foram realizados o Teste de Ames e micronúcleos, e *in vivo* foram avaliados modelos de exposição aguda e de longa duração. O estudo pré-clínico foi realizado com ratos Wistar (machos e fêmeas, n=10/grupo) e consistiu de dose oral única de 2500 mg/kg para avaliação de toxicidade oral aguda e doses repetidas de 1000 mg/kg por 28 dias, para avaliar o efeito da exposição prolongada. Foram avaliados parâmetros toxicológicos como sinais clínicos, peso corpóreo, análises bioquímicas e histopatológicas. Não foram observados sinais de toxicidade e tampouco morte de animais em ambos os protocolos, comparados ao grupo sem a intervenção (dados ainda não publicados).

Posteriormente, a Agência Nacional de Vigilância Sanitária (Anvisa) concedeu aprovação do EPP-AF® para estudos de fase 1 e 2 em seres humanos.

Um estudo clínico de fase 1 foi realizado na Unidade de Pesquisa Clínica do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (UPC/HC/FMRP/USP), contando com 14 voluntários sadios (6 homens e 8 mulheres) que fizeram uso de dose diária de 375 mg de EPP-AF® (3 comprimidos de 125 mg ao dia) pelo período de 5 dias. O estudo conduzido foi do tipo aberto, não aleatorizado. Os participantes da pesquisa foram submetidos à avaliação clínica (histórico médico e exame físico), exames clínicos, eletrocardiograma e exames laboratoriais (hemograma, urina tipo I, ureia, creatinina, glicemia, bilirrubina total, aspartato aminotransferase, ácido úrico, CPK, colesterol total e frações, triglicérides, beta-HCG para mulheres, sorologias para vírus da hepatite B, hepatite C e HIV). Os resultados demonstraram ausência de toxicidade aguda nos indivíduos. Houve alteração de CPK em um paciente, sem presença de mioglobinúria e de sinais clínicos de rabdomiólise, recomendando-se a monitorização sistemática de CPK nos protocolos clínicos futuros. Também foi observada variação positiva no HDL-colesterol, e estudos com um número maior de pacientes estão sendo realizados para confirmar se há efeito benéfico ao sistema cardiovascular (dados ainda não publicados).

Após a devida análise e aprovação do protocolo pela Anvisa, foi realizado também um estudo clínico de fase 2 na mesma instituição (UPC/HC/FMRP/USP), demonstrando os efeitos anti-inflamatórios dos comprimidos (dados ainda não publicados).

Desde então, a eficácia pré-clínica e clínica do EPP-AF® vem sendo documentada por diversos autores, quanto às atividades antimicrobiana e cicatrizante (Berretta *et al.*, 2012; Barud *et al.*, 2013; Marquele-Oliveira *et al.*, 2019), anti-inflamatória (Hori *et al.*, 2013), antioxidante (Diniz *et al.*, 2020) e imunomoduladora (Piñeros *at al.*, 2020), bem como seu potencial no tratamento de estomatite dentária (Pina *et al.*, 2017), doença renal crônica (Silveira *et al.*, 2019) e como tratamento adjuvante para pacientes hospitalizados com a doença do coronavírus 2019 (Silveira *et al.*, 2021).

Para o presente trabalho, foi utilizada a dosagem de EPP-AF® 500mg/dia, considerando os modelos de conversão de dose de animais para humanos, seguindo o *Guideline* do *Food and Drug Administration* (FDA), demonstrando ser

uma dosagem segura. Também, a mesma dosagem é corroborada pelos compêndios canadenses, que adotam 500 mg/dia de própolis.

3. Justificativa do trabalho

Um dos atuais desafios na área de HIV/aids é o melhor entendimento sobre a ativação imune persistente e a inflamação crônica que as PVHA apresentam, bem como os mecanismos que refletem no envelhecimento precoce observado nesta população. Tais indivíduos se deparam com um grande desafio atualmente: o de postergar o aparecimento de comorbidades não associadas à aids, como distúrbios metabólicos, desenvolvimento de doenças cardiovasculares, comprometimento renal ou hepático, alterações ósseas, neoplasias, entre outras.

Apesar dos incontestáveis benefícios da TARV, há diminuição apenas parcial dos níveis de ativação imune e inflamação em PVHA, mesmo naquelas que apresentam supressão viral adequada, quando comparados aos indivíduos não infectados pelo HIV. Há que se considerar, também, os efeitos deletérios do uso prolongado da TARV, como sua contribuição no desenvolvimento das doenças não associadas à aids.

Assim, estudos de intervenção com componentes anti-inflamatórios e imunomoduladores são necessários, principalmente após a indicação de início terapêutico cada vez mais precoce. Nesse sentido, a própolis, além de ser um produto natural isento de efeitos colaterais, apresenta diversas propriedades biológicas que podem promover a saúde humana.

Por estas razões, o intuito do presente estudo foi analisar os efeitos da própolis em alguns parâmetros imunológicos, metabólicos e inflamatórios em PVHA que fizeram seu uso diário concomitantemente à TARV. Esse projeto foi delineado visando atenuar os efeitos tóxicos da terapia, contribuir para a melhoria da resposta imune e postergar/evitar o desenvolvimento de comorbidades, a fim de melhorar a qualidade de vida e sobrevida das PVHA.

Objetivos

Objetivo geral

Verificar o efeito da suplementação com própolis por três meses em PVHA, sob terapia e supressão viral adequada, em relação ao perfil bioquímico/metabólico, inflamatório e imunológico.

Objetivos específicos

Analisar antes e após a intervenção:

- O perfil bioquímico/metabólico (triglicérides, colesterol total, HDL-colesterol, LDL-colesterol, PCR, CPK, glicose em jejum, ureia, creatinina, ácido úrico, AST, ALT, gamaglutamiltransferase, bilirrubina, ácido úrico, fosfatase alcalina, fósforo, cálcio, magnésio, sódio, potássio, amilase, desidrogenase láctica, alfa1-glicoproteína ácida), hemograma e velocidade de hemossedimentação;
- O perfil imunovirológico (contagem de T $CD4^+$/$CD8^+$ e carga viral plasmática do HIV);
- A concentração de citocinas plasmáticas (TNF-α, IL-6, IL-10, IL-17, IL-2 e IL-4);
- A produção de citocinas relacionadas aos diferentes perfis de respostas (IFN-γ [Th1], IL-5 [Th2], IL-17 [Th17] e IL-10 [Treg]) e à ativação do inflamassoma (IL-18, IL-1β, IL-33) produzidas por PBMC;
- A fenotipagem dos linfócitos T por meio da expressão dos fatores de transcrição T-bet (Th1), GATA-3 (Th2), RORγt (Th17) e Foxp3 (Treg);
- A proliferação de linfócitos.

O presente trabalho foi aprovado pelo Comitê de Ética em Pesquisa com Seres Humanos (CAAE nº 58694816.6.0000.5411 – Anexo 1) e todos os pacientes assinaram o Termo de Consentimento Livre e Esclarecido (Anexo 2). O projeto também foi aprovado pela "Comissão Interna de Biossegurança" (CQB-164/02-16) e encontra-se registrado no "Registro Brasileiro de Ensaios Clínicos" (nº RBR-33mjbg).

Os pacientes incluídos no estudo receberam um kit contendo os comprimidos de própolis ou placebo e uma cartilha explicativa (Anexo 3). A cartilha continha informações básicas e acessíveis a respeito da infecção pelo HIV, da TARV e da importância do nosso estudo. Ademais, informava aos pacientes o modo correto de ingerir os comprimidos e continha espaços para anotações do registro alimentar e de possíveis esquecimentos ou sintomas, ao longo do tratamento.

O delineamento experimental, assim como os resultados do estudo e a discussão dos dados obtidos estão apresentados a seguir na forma de manuscritos.

Os dados sociodemográficos/clínicos, esquema de tratamento, hemograma, perfil imunovirológico, bioquímico e metabólico estão apresentados no manuscrito intitulado "**Propolis intake is safe and increases magnesium levels in HIV patients: a randomized, double-blinded, placebo-controlled clinical study**" submetido à *Evidence-Based Complementary and Alternative Medicine.*

Os resultados das análises do perfil imunológico e inflamatório estão apresentados no manuscrito intitulado "**Propolis increases Foxp3 expression and lymphocyte proliferation in HIV-infected people: a randomized, double blind, parallel-group and placebo-controlled study**", que será submetido à publicação junto à *Phytomedicine*.

Experimentos adicionais foram realizados durante o doutorado sanduíche na Universidade de Coimbra (Portugal), com o objetivo de compreender melhor alguns mecanismos modulados pela própolis em monócitos. Os resultados obtidos durante o doutorado sanduíche estão apresentados no artigo intitulado "**Exploring the antioxidant, anti-inflammatory and antiallergic potential of Brazilian propolis in monocytes**", submetido ao *Journal of Pharmacy and Pharmacology*.

Referências Bibliográficas

Aberg JA. Aging, inflammation, and HIV. Top Antiviral Med. 2012, 20(3):101-105.

Afsharpour F, Hashemipour S, Khadem-Haghighian H, Koushan Y. Effects of Iranian propolis on glycemic status, inflammatory factors, and liver enzyme levels in type 2 diabetic patients: a randomized, double-blind, placebo-controlled, clinical trial. JNSD. 2017, 3(2):9-14.

Ahmed KM, Saleh EM, Sayed EM, Shalaby KAF. Anti-Inflammatory effect of different propolis extracts in thioacetamide-induced hepatotoxicity in male rat. AJBAS. 2012, 6(6):29-40.

Ahn MR, Kunimasa K, Ohta T, Kumazawa S, Kamihira M, Kaji K, Uto Y, Hori H, Nagasawa H, Nakayama T. Suppression of tumor-induced angiogenesis by Brazilian propolis: major component artepillin C inhibits in vitro tube formation and endothelial cell proliferation. Cancer Lett. 2007, 252: 235-243.

Al Ghamdi AA, Badr G, Hozzein WN, Allam A, Al-Waili NS, Al-Wadaan MA, Garraud O. Oral supplementation of diabetic mice with propolis restores the proliferation capacity and chemotaxis of B and T lymphocytes towards CCL21 and CXCL12 by modulating the lipid profile, the pro-inflammatory cytokine levels and oxidative stress. BMC Immunol. 2015, 16:1-14.

Al-Hariri M. Immune's-boosting agent: immunomodulation potentials of propolis. J Family Community Med. 2019, 26(1):57-60.

Anjum SI, Ullah A, Khan KA, Attaullah M, Khan H, Ali H, Bashir MA, Tahir M, Ansari MJ, Ghramh HA, Adgaba N, Dash CK. Composition and functional properties of propolis (bee glue): A review. Saudi J Biol Sci. 2019, 26(7):1695-1703.

Anthony IC, Ramage SN, Carnie FW, Simmonds P, Bell JE. Accelerated Tau deposition in the brains of individuals infected with human immunodeficiency virus-1 before and after the advent of highly active anti-retroviral therapy. Acta Neuropathol. 2006, 111:529-538.

Araújo MJAM, Mattar NS, Reis AS, Serra ICPB, Fialho EMS, Assunção AKM, Dutra RP, Nogueira AMC, Libério SA, Guerra RNM, Lopes AS, Ribeiro MNS, Nascimento FRF. Pharmacognostic and acute toxicological evaluation of *Scaptotrigona* aff. *postica* propolis extract in pre-clinical assays. Nat Prod Res. 2011, 25:1037-1046.

Araújo-Vilar D, Santini F. Diagnosis and treatment of lipodystrophy: a step-by-step approach. J Endocrinol Invest. 2019, 42(1):61-73.

Ascher MS, Sheppard HW. AIDS as immune system activation: a model for pathogenesis. Clin Exp Immunol. 1988, 73(2):165-167.

Bankova V, Boudourova-Krasteva G, Popov S, Sforcin JM, Funari SRC. Seasonal variations of the chemical composition of Brazilian propolis. Apidologie. 1998, 29:361-367.

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

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

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

Barud HS, De Araújo Jr AM, Saska S, Mestieri LB, Campos JA, De Freitas RM, Ferreira NU, Nascimento AP, Miguel FG, Vaz MM, Barizon EA, Marquele-Oliveira F, Gaspar AM, Ribeiro SJ, Berretta AA. Antimicrobial Brazilian propolis (EPP-AF) containing biocellulose membranes as promising biomaterial for skin wound healing. Evid Based Complement Alternat Med. 2013, 2013:1–10.

Berretta AA, Nascimento AP, Bueno PCP, Vaz MMOLL, Marchetti JM. Propolis standardized extract (EPP-AF®), an innovative chemically and biologically reproducible pharmaceutical compound for treating wounds. Int J BiolSci. 2012, 8(4):512-521.

Bhadauria M. Propolis prevents hepatorenal injury induced by chronic exposure to carbon tetrachloride. Evid Based Complement Alternat Med. 2012, 2012:1-12.

Braakhuis A. Evidence on the health benefits of supplemental propolis. Nutrients. 2019, 11:1-15.

Brasil, ANVISA. Agência Nacional de Vigilância Sanitária. Resolução da Diretoria Colegiada - RDC Nº 24, de 14 de Junho de 2011. Dispõe sobre o registro de medicamentos específicos. 2011.

Brasil, Ministério da Saúde. Departamento de DST, Aids e Hepatites Virais. Boletim Epidemiologia DST AIDS. 2014.

Brasil, Ministério da Saúde. Departamento de DST, Aids e Hepatites Virais. Protocolo Clínico e Diretrizes Terapêuticas para Manejo da Infecção pelo HIV/aids em Adultos. 2013.

Brasil, Ministério da Saúde. Secretaria de Vigilância em Saúde. Boletim Epidemiológico HIV/Aids. 2020.

Brasil, Ministério da Saúde. Secretaria de Vigilância em Saúde. Manual de Prevenção das DST/HIV/Aids em Comunidades Populares. 2008.

Brasil, Ministério da Saúde. Secretaria de Vigilância em Saúde. Manual técnico para o diagnóstico da infecção pelo HIV em adultos e crianças. 2018a.

Brasil, Ministério da Saúde. Secretaria de Vigilância em Saúde. Protocolo clínico e diretrizes terapêuticas para manejo da infecção pelo HIV em adultos. 2018b.

Brenchley JM, Price DA. Schacker TW, Asher TE, Silvestri G, Rao S, Kazzaz Z, Bornstein E, Lambotte O, Altmann D, Blazar BR, Rodriguez B, Teixeira-Johnson L, Landay A, Martin JN, Hecht FM, Picker LJ, Lederman MM, Deeks SG, Douek DC. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med. 2006, 12:1365-1371.

Brugnaro P, Morelli E, Cattelan F, Petrucci A, Panese S, Eseme F, Cavinato F, Barelli A, Raise E. Non-acquired immunodeficiency syndrome defining malignancies among human immunodeficiency virus-positive subjects: epidemiology and outcome after two decades of HAART era. World J Virol. 2015, 4(3):209-218.

Bueno-Silva B, Kawamoto D, Ando-suguimoto ES, Alencar SM, Rosalen PL, Mayer MPA. Brazilian red propolis attenuates inflammatory signaling cascade in LPS-activated macrophages. PLoS One. 2015, 10(12):1-14.

Bueno-Silva B, Franchin M, Alves CF, Denny C, Colón DF, Cunha TM, Alencar SM, Napimoga MH, Rosalen PL. Main pathways of action of Brazilian red propolis on the modulation of neutrophils migration in the inflammatory process. Phytomedicine. 2016, 23(13):1583-1590.

Búfalo MC, Figueiredo AS, Sousa JPB, Candeias JMG, Bastos JK, Sforcin, JM. Anti-poliovirus activity of *Baccharis dracunculifolia* and propolis by cell viability determination and real-time PCR. J App Microbiol. 2009, 107(5):1669-1680.

Búfalo MC, Bordon-Graciani AP, Conti BJ, Golim MA, Sforcin JM. The immunomodulatory effect of propolis on receptors expression, cytokine production and fungicidal activity of human monocytes. J Pharm Pharmacol. 2014, 66:1497-1504.

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

Büyükberber M, Savaş MC, Bağci C, Koruk M, Gülşen MT, Tutar E, Bilgiç T, Deveci R, Küçük C. The beneficial effect of propolis on cerulein-induced experimental acute pancreatitis in rats. Turk J Gastroenterol. 2009, 20(2):122-128.

Cardoso EO, Conti BJ, Santiago KB, Conte FL, Oliveira LPG, Hernandes RT, Golim MA, Sforcin JM. Phenolic compounds alone or in combination may be involved in propolis effects on human monocytes. J Pharm Pharmacol. 2017, 69:99-108.

Caron-Debarle M, Lagathu C, Boccara F, Vigouroux C, Capeau J. HIV-associated lipodystrophy: from fat injury to premature aging. Trends Mol. Med. 2010, 16:218-229.

Chehter EZ, Longo MA, Laudanna AA, Duarte MI. Involvement of the pancreas in AIDS: a prospective study of 109 post-mortems. AIDS. 2000, 14(13):1879-1886.

Chen YW, Ye SR, Ting C, Yu YH. Antibacterial activity of propolins from Taiwanese green propolis. J Food Drug Anal. 2018, 26(2):761-768.

Chen J, Long Y, Han M, Wang T, Chen Q, Wang R. Water-soluble derivative of propolis mitigates scopolamine-induced learning and memory impairment in mice. Pharmacol Biochem Behav. 2008, 90:441-446.

Chinen J, Shearer WT. Molecular virology and immunology of HIV infection. J Allergy Clin Immunol. 2002, 110 (2): 189-198.

Chopra S, Pillai KK, Husain SZ, Giri DK. Propolis protects against doxorubicin-induced myocardiopathy in rats. Exp Mol Pathol. 1995, 62(3):190-198.

Clerici M, Shearer GM. A ThI→Th2 switch is a critical step in the etiology of HIV infection. Immunol Today. 1993, 14(3): 1-5

Cole N, Sou PW, Ngo A, Tsang KH, Severino JA, Arun SJ, Duke CC, Reeve VE. Topical 'Sydney' propolis protects against UV-radiation-induced inflammation, lipid peroxidation and imune suppression in mouse skin. Int Arch Allergy Immunol. 2010, 152:87-97.

Conte FL, Santiago KB, Conti BJ, Cardoso EO, Oliveira LPG, Feltran GS, Zambuzzi WF, Golim MA, Cruz MT, Sforcin JM. Propolis from southeastern Brazil produced by *Apis mellifera* affects innate immunity by modulating cell marker expression, cytokine production and intracellular pathways in human monocytes. J Pharm Pharmacol. 2021, 73:135-144.

Conti BJ, Santiago KB, Cardoso EO, Freire PP, Carvalho RF, Golim MA, Sforcin, JM. Propolis modulates miRNAs involved in TLR-4 pathway, NF-κB activation, cytokine production and in the bactericidal activity of human dendritic cells. J Pharm Pharmacol. 2016, 68(12):1604-1612.

Conti BJ, Santiago KB, Búfalo MC, Frión-Herrera Y, Alday E, Velazquez C, Hernandez J, Sforcin JM. Modulatory effects of propolis samples from Latin America (Brazil,Cuba and Mexico) on cytokine production by human monocytes. J Pharm Pharmacol. 2015, 67(10):1431-1438.

Cornara L, Biagi M, Xiao J, Burlando B. Therapeutic properties of bioactive compounds from different honeybee products. Front Pharmacol. 2017, 8:1-20.

Crum-Cianflone N, Ganesan A, Teneza-Mora N, Riddle M, Medina S, Barahona I, Brodine S. Prevalence and factors associated with renal dysfunction among HIV infected patients. AIDS Patient Care STDS. 2010, 24(6):353-360.

Cusinato DAC, Martinez EZ, Cintra MTC, Filgueira GCO, Berretta AA, Lanchote VL, Coelho EB. Evaluation of potential herbal-drug interactions of a standardized propolis extract (EPP-AF®) using an in vivo cocktail approach. J Ethnopharmacol. 2019, 245:112174. doi: 10.1016/j.jep.2019.112174.

Da Silva SS, Thomé GS, Cataneo AHD, Miranda MM, Felipe I, Andrade CGTJ, Watanabe MAE, Piana GM, Sforcin, JM, Pavanelli WR, Conchon-costa I. Brazilian propolis antileishmanial and immunomodulatory effects. Evid Based Complement Alternat Med. 2013, 2013:1-7.

Daleprane JB, Abdalla DS. Emerging roles of propolis: antioxidant, cardioprotective, and antiangiogenic actions. Evid Based Complement Alternat Med. 2013, 2013:1-8.

Dantas AP, Olivieri BP, Gomes FH, De Castro SL. Treatment of *Trypanosoma cruzi*-infected mice with propolis promotes changes in the immune response. J Ethnopharmacol. 2006, 103(2):187-193.

Decrion AZ, Dichamp I, Varin A, Herbein G. HIV and Inflammation. Curr HIV Res. 2005, 3(3):243-259.

Deeks, S. HIV Infection, inflammation, immunosenescence, and aging. Ann Rev Med. 2011, 62:141-155.

Deeks SG, Overbaugh J, Phillips A, Buchbinder S. HIV infection. Nat Rev Dis Primers. 2015, 1:15035.

Diniz DP, Lorencini DA, Berretta AA, Cintra MACT, Lia EN, Jordão AA Jr, Coelho EB. Antioxidant Effect of Standardized Extract of Propolis (EPP-AF) in Healthy Volunteers: A "Before and After" Clinical Study. Evid Based Complement Alternat Med. 2020, 2020:7538232.

Eggena MP, Barugahare B, Jones N, Okello M, Mutalya S, Kityo C, Mugyenyi P, Cao H. Depletion of regulatory T cells in HIV infection is associated with immune activation. J Immunol. 2005, 174(7):4407-4414.

El Menyiy N, Al-Waili N, El Ghouizi A, Al-Waili W, Lyoussi B. Evaluation of antiproteinuric and hepato-renal protective activities of propolis in paracetamol toxicity in rats. Nutr Res Pract. 2018, 12(6):535-540.

El Rabey HA, Al-Seeni MN, Bakhashwain AS. The antidiabetic activity of *Nigella sativa* and propolis on streptozotocin-induced diabetes and diabetic nephropathy in male rats. Evid Based Complement Alternat Med. 2017, 2017:1-14.

Espíndola MS, Soares LS, Galvão-Lima LJ, Zambuzi FA, Cacemiro MC, Brauer VS, Frantz FG. HIV infection: focus on the innate immune cells. Immunol Res. 2016, 64:1118-1132.

Feria MG, Taborda NA, Hernandez JC, Rugeles MT. HIV replication is associated to inflammasomes activation, IL-1β, IL-18 and caspase-1 expression in GALT and peripheral blood. PLoS One. 2018,13(4):1-14.

Fokt H, Pereira A, Ferreira A, Cunha A, Aguiar C. How do bees prevent hive infections? The antimicrobial properties of propolis. Curr Res Technol Educ Top Appl Microbiol Microbial Biotechnol. 2010, 1:481-493.

Freitas SF, Shinohara L, Sforcin JM, Guimarães S. In vitro effects of propolis on *Giardia duodenalis* trophozoites. Phytomedicine. 2006, 13:170-175.

French MA, King MS, Tschampa JM, da Silva BA, Landay AL. Serum immune activation markers are persistently increased in patients with HIV infection after 6 years of antiretroviral therapy despite suppression of viral replication and reconstitution of CD4+ T cells. J Infect Dis. 2009, 200:1212-1215.

Ford N, Migone C, Calmy A, Kerschberger B, Kanters S, Nsanzimana S, Mills EJ, Meintjes G, Vitoria M, Doherty M, Shubber Z. Benefits and risks of rapid initiation of antiretroviral therapy. AIDS. 2018, 32(1):17-23.

Fukuda T, Fukui M, Tanaka M, Senmaru T, Iwase H, Yamazaki M, Aoi W, Inui T, Nakamura N, Marunaka Y. Effect of Brazilian green propolis in patients with type 2 diabetes: a double-blind randomized placebo-controlled study. Biomed Rep. 2015, 3 (3): 355-360.

Gallo RC. A reflection on HIV/AIDS research after 25 years. Retrovirology. 2006, 3:1-7.

Galvão, J. 1980-2001: Uma cronologia da epidemia de HIV-AIDS no Brasil e no mundo. Rio de Janeiro; Coleçäo ABIA: políticas públicas, 2, 2002. 30 p.

Gekker G, Hu S, Spivak M, Lokensgard JR, Peterson PK. Anti-HIV-1 activity of propolis in $CD4^+$ lymphocyte and microglial cell cultures. J Ethnopharmacol. 2005, 102 (2): 158–163.

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

Goodsell DS. Illustrations of the HIV life cycle. Curr Top Microbiol Immunol. 2015, 389:243-252.

Guaraldi G, Orlando G, Zona S, Menozzi M, Carli F, Garlassi E, Berti A, Rossi E, Roverato A, Palella F. Premature age-related comorbidities among HIV-infected persons compared with the general population. Clin Infect Dis. 2011, 53(11):1120–1126.

Gutiérrez C, Lopez-Abente J, Pérez-Fernández V, Prieto-Sánchez A, Correa-Rocha R, Moreno-Guillen S, Muñoz-Fernández MÁ, Pion M. Analysis of the dysregulation between regulatory B and T cells (Breg and Treg) in human immunodeficiency virus (HIV)-infected patients. PLoS One. 2019,14(3):1-18.

Harish Z, Rubinstein A, Golodner M, Elmaliah M, Mizrachi Y. Suppression of HIV-1 replication by propolis and its immunoregulatory effect. Drugs Exp Clin Res. 1997, 23(2):89-96.

Helfenberg K. The analysis of beeswax and propolis. Chemiker Zeitungm. 1908, 31:987–998.

Hernandez JC, Latz E, Urcuqui-Inchima S. HIV-1 induces the first signal to activate the NLRP3 inflammasome in monocyte-derived macrophages. Intervirology. 2014, 57(1):36-42.

Hesami S, Hashemipour S, Shiri-Shahsavar MR, Koushan Y, Khadem Haghighian H. Administration of Iranian Propolis attenuates oxidative stress and blood glucose in ty pe II diabetic patients: a randomized, double-blind, placebo-controlled, clinical trial. Caspian J Intern Med. 2019,10(1):48-54.

Hori JI, Zamboni DS, Carrão DB, Goldman GH, Berretta AA. The inhibition of inflammasome by Brazilian propolis (EPP-AF). Evid Based Complem Alternat Med. 2013; 2013:1-11.

Hsu DC, Sereti I, Ananworanich J. Serious non-AIDS events: immunopathogenesis and interventional strategies. AIDS Res Ther. 2013, 10: 1-15.

Hunt PW. HIV and Inflammation: mechanisms and consequences. Curr HIV/AIDS Rep. 2012, 9:139-147.

Hunt PW, Martin JN, Sinclair E, Bredt B, Hagos E, Lampiris H, Deeks SG. T Cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with sustained viral suppression during antiretroviral therapy. J Infect Dis. 2013, 187(10):1534-1543.

ICTV (International Committee on Taxonomy of Viruses). Virus Taxonomy: The Classification and Nomenclature of Viruses. The Online (10th) Report of the ICTV, 2017. Disponível em: <https://talk.ictvonline.org/ictv-reports/ictv_online_report/>. Acesso em: 15 janeiro 2021.

Ivanovska ND, Dimov VB, Bankova VS, Popov SS. Immunomodulatory action of propolis. VI. Influence of a water soluble derivative on complement activity *in vivo*. J Ethnopharmacol. 1995, 47:145–147.

Ivanovska N, Neychev H, Stefanova Z, Bankova V, Popov S. Influence of cinnamic acid on lymphocyte proliferation, cytokine release and Klebsiella infection in mice. Apidologie. 2005, 26:73-81.

Joint United Nations Programme on HIV/AIDS (UNAIDS). Global report: UNAIDS report on the global AIDS epidemic. s.l.: Disponível em: https://unaids.org.br/estatisticas/?gclid=EAIaIQobChMItszK-d3z7QIVhQeRCh1q1wGuEAAYASABEgLocvD_BwE. 2020.

Kalogeropoulos N, Konteles SJ, Troullidou E, Mourtzinos J, Karathanos VT. Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. Food Chem. 2009, 116:452–461.

Khayyal MT, El-Ghazaly MA, el-Khatib AS, Hatem AM, de Vries PJ, el-Shafei S, Khattab MM. A clinical pharmacological study of the potential beneficial effects of a propolis food product as an adjuvant in asthmatic patients. Fundam Clin Pharmacol. 2003, 17(1):93-102.

Kamat A, Misra V, Cassol E, Ancuta P, Yan Z, Li C, Morgello S, Gabuzda D. A plasma biomarker signature of immune activation in HIV patients on antiretroviral therapy. PLoS One. 2012, 7(2):1-11.

Klatt NR, Funderburg NT, Brenchley JM. Microbial translocation, immune activation, and HIV disease. Trends Microbiol. 2013, 21:6-13.

Koo HJ, Lee KR, Kim HS, Lee BM. Detoxification effects of aloe polysaccharide and propolis on the urinary excretion of metabolites in smokers. Food Chem Toxicol. 2019, 130:99-108.

Kumar P. Long term non-progressor (LTNP) HIV infection. Indian J Med Res. 2013, 138(3):291-293.

Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature. 1998, 393:648-659.

Labská K, Plodková H, Pumannová M, Sensch KH. Antiviral activity of propolis special extract GH 2002 against *Varicella zoster* virus *in vitro*. Pharmazie. 2018, 73(12):733-736.

Langford SE, Ananworanich J, Cooper AD. Predictors of disease progression in HIV infection: a review. Aids Res Ther. 2007, 4:1-14.

Maciel RA, Klück HM, Durand M, Sprinz E. Comorbidity is more common and occurs earlier in persons living with HIV than in HIV-uninfected matched controls, aged 50 years and older: a cross-sectional study. Int J Infect Dis. 2018, 70:30-35.

Mani F, Damasceno HCR, Novelli ELB, Martins EAM, Sforcin JM. Propolis: effect of different concentrations, extracts and intake period on seric biochemical variables. J Ethnopharmacol. 2006, 105:95-98.

Marquele-Oliveira F, da Silva Barud H, Torres EC, Machado RTA, Caetano GF, Leite MN, Frade MAC, Ribeiro SJL, Berretta AA. Development, characterization and pre-clinical trials of an innovative wound healing dressing based on propolis (EPP-AF®)-containing self-microemulsifying formulation incorporated in biocellulose membranes. Int J Biol Macromol. 2019, 136:570-578.

Marquiafável FS, Nascimento AP, Barud HS, Marquele-Oliveira F, Freitas LAP, Bastos JK, Berretta AA. Development and characterization of a novel standardized propolis dry extract obtained by factorial design with high artepillin C content. J Pharm Technol Drug Res. 2015, 4:1-13.

McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF. The immune response during acute HIV-1 infection: clues for vaccine development. Nat Rev Immunol. 2010, 10(1):11-23.

Menezes AM, Torelly J Jr, Real L, Bay M, Poeta J, Sprinz E. Prevalence and risk factors associated to chronic kidney disease in HIV-Infected patients on HAART and undetectable viral load in Brazil. PLoS One. 2011, 6(10):1-5.

Mgogwe J, Semvua H, Msangi R, Mataro C, Kajeguka D, Chilongola J. The evolution of haematological and biochemical indices in HIV patients during a six-month treatment period. Afri Health Sci. 2012, 12(1):2-7.

Miller LE. Laboratory Diagnosis of HIV Infection. In: STEVENS, C. D. (Ed.). Clinical immunology and serology: a laboratory perspective. 3. ed. Philadelphia: F.A. Davis Company, 2010, cap. 23.

Montagnier L. 25 years after HIV discovery: prospects for cure and vaccine. Virology. 2010, 397(2):248-54.

Monteiro P, Perez I, Pich J, Gatell JM, Martínez E. Creatine kinase elevation in HIV-1-infected patients receiving raltegravir-containing antiretroviral therapy: a cohort study. J Antimicrob Chemother. 2013, 68(2):404-408.

Mujica V, Orrego R, Pérez J, Romero P, Ovalle P, Zúñiga-Hernández J, Arredondo M, Leiva E. The role of Propolis in oxidative stress and lipid metabolism: A randomized controlled trial. Evid Based Complement Alternat Med. 2017, 2017: 1-11.

Neuhaus J, Jacobs DR Jr, Baker JV, Calmy A, Duprez D, La Rosa A, Kuller LH, Pett SL, Ristola M, Ross MJ, Shlipak MG, Tracy R, Neaton JD. Markers of inflammation, coagulation, and renal function are elevated in adults with HIV infection. J Infect Dis. 2010, 201(12):1788-1795.

Ngala RA, Opoku D, Asare G. Effects of HIV infection and Highly Active Antiretroviral Therapy (HAART) on the liver of HIV patients. Trends Med Res. 2015, 10(1):1-11.

Nna VU, Bakar ABA, Lazin RL, Mohamed M. Antioxidant, anti-inflammatory and synergistic anti-hyperglycemic effects of Malaysian propolis and metformin in streptozotocin–induced diabetic rats. Food Chem Toxicol. 2018, 120:305-320.

Nunnari G, Fagone P, Condorelli F, Nicolett F, Malaguarnera L, Di Rosa M. CD4+ T-cell gene expression of healthy donors, HIV-1 and elite controllers: immunological chaos. Cytokine. 2016, 83:127-135.

Okulicz JF, Marconi VC, Landrum ML, Wegner S, Weintrob A, Ganesan A, Hale B, Crum-Cianflone N, Delmar J, Barthel V, Quinnan G, Agan BK, Dolan MJ, Infectious Disease Clinical Research Program (IDCRP) HIV Working Group. Clinical outcomes of elite controllers, viremic controllers, and long-term nonprogressors in the US Department of Defense HIV natural history study. J Infect Dis. 2009, 200(11):1714-1723.

Oliveira NM, Ferreira FA, Yonamine RY, Chehter EZ. Antiretroviral drugs and acute pancreatitis in HIV/AIDS patients: is there any association? A literature review. Einstein (Sao Paulo). 2014, 12(1):112-119.

Orsatti CL, Missima F, Pagliarone AC, Sforcin JM. Th1/Th2 cytokines' expression and production by propolis-treated mice. J Ethnopharmacol. 2010, 129(3):314-318.

Orsi RO, Funari SRC, Soares AMVC, Calvi SA, Oliveira SL, Sforcin JM, Bankova V. Immunomodulatory action of propolis on macrophage activation. J Venom Anim Toxins. 2000, 6(2):205-219.

Orsilles MA, Pieri E, Cooke P, Caula C. IL-2 and IL-10 serum levels in HIV-1-infected patients with or without active antiretroviral therapy. APMIS. 2006, 114:55-60.

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

Oryan A, Alemzadeh E, Moshiri A. Potential role of propolis in wound healing: Biological properties and therapeutic activities. Biomed Pharmacother. 2018, 98:469-483.

Ota C, Unterkircher C, Fantinato V, Shimizu MT. Antifungal activity of propolis on different species of Candida. Mycoses. 2001, 44:375-378.

Pina GMS, Lia EN, Berretta AA, Nascimento AP, Torres EC, Buszinski AFM, de Campos TA, Coelho EB, Martins VP. Efficacy of propolis on the denture stomatitis treatment in older adults: a multicentric randomized trial. Evid Based Complement Alternat Med. 2017, 2017:1-9.

Piñeros AR, de Lima MHF, Rodrigues T, Gembre AF, Bertolini TB, Fonseca MD, Berretta AA, Ramalho LNZ, Cunha FQ, Hori JI, Bonato VLD. Green propolis increases myeloid suppressor cells and $CD4^{+}Foxp3^{+}$ cells and reduces Th2 inflammation in the lungs after allergen exposure. J Ethnopharmacol. 2020, 252:1-9.

Pinto Neto LFDS, Neves MB, Ribeiro-Rodrigues R, Page K, Miranda AE. Dyslipidemia and fasting glucose impairment among HIV patients three years after the first antiretroviral regimen in a Brazilian AIDS outpatient clinic. Braz J Infect Dis. 2013, 17(4):438-443.

Plitas G, Rudensky AY. Regulatory T Cells: Differentiation and Function. Cancer Immunol Res. 2016, 4(9): 721–725.

Poropatich K, Sullivan DJ Jr. Human immunodeficiency virus type 1 long-term non-progressors: the viral, genetic and immunological basis for disease non-progression. J Gen Virol. 2011, 92(2):247-268.

Prendergast A, Prado JG, Kang YH, Chen F, Riddell LA, Luzzi G, Goulder P, Klenerman P. HIV-1 infection is characterized by profound depletion of CD161+ Th17 cells and gradual decline in regulatory T cells. AIDS. 2010, 24(4):491-502.

Przybyłek I, Karpiński TM. Antibacterial properties of propolis. Molecules. 2019, 24(11):1-17.

Ramana KV. Effect of highly active antiretroviral therapy (HAART) on human immunodeficiency virus disease pathogenesis and progression. Am J Public Health Res. 2014, 2(3):68-74.

Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. Cytokine. 2015, 74(1):5-17.

Raza S, Chaudhry NA, Brown JD, Aghaie S, Rezai D, Khan A, Tan Pde L, Berger BJ. To study the clinical, biochemical and radiological features of acute pancreatitis in HIV and AIDS. J Clin Med Res. 2013, 5(1):12-17.

Ripari N, Sartori AA, Honorio MS, Conte FL, Tasca KI, Santiago KB, Sforcin JM. Propolis antiviral and immunomodulatory activity: a review and perspectives for COVID-19 treatment. J Pharm Pharmacol. 2021, 73: 281-299.

Roberts L, Passmore JA, Williamson C, Little F, Bebell LM, Mlisana K, Burgers WA, van Loggerenberg F, Walzl G, Djoba Siawaya JF, Karim QA, Karim SS. Plasma cytokine levels during acute HIV-1 infection predict HIV disease progression. AIDS. 2010, 24(6):819-831.

Rocha BA, Bueno PC, Vaz MM, Nascimento AP, Ferreira NU, Moreno Gde P, Rodrigues MR, Costa-Machado AR, Barizon EA, Campos JC, de Oliveira

PF, Acésio Nde O, Martins Sde P, Tavares DC, Berretta AA. Evaluation of a propolis water extract using a reliable RP-HPLC methodology and in vitro and in vivo efficacy and safety characterisation. Evid Based Complement Alternat Med. 2013, 2013:1-11.

Rose H, Woolley I, Hoy J, Dart A, Bryant B, Mijch A, Sviridov D. HIV infection and high-density lipoprotein: the effect of the disease vs the effect of treatment. Metabolism. 2006, 55(1):90-95.

Sáez-Cirión A, Bacchus C, Hocqueloux L, Avettand-Fenoel V, Girault I, Lecuroux C, Potard V, Versmisse P, Melard A, Prazuck T, Descours B, Guergnon J, Viard JP, Boufassa F, Lambotte O, Goujard C, Meyer L, Costagliola D, Venet A, Pancino G, Autran B, Rouzioux C, ANRS VISCONTI Study Group. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. PLoSPathog. 2013, 9(3):1-12.

Said EA, Dupuy FP, Trautmann L, et al. Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. Nat Med. 2010, 16(4):452-459.

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

Salomão K, De Souza EM, Henriques-Pons A, Barbosa HS, De Castro SL. Brazilian green propolis: effects *in vitro* and *in vivo* on *Trypanossoma cruzi*. Evid Based Complement Alternat Med. 2011, 2011:1-11.

Samadi N, Mozaffari-Khosravi H, Rahmanian M, Askarishahi M. Effects of bee propolis supplementation on glycemic control, lipid profile and insulin resistance indices in patients with type 2 diabetes: a randomized, double-blind clinical trial. J Integr Med. 2017, 15(2):124-134.

Sforcin JM. Biological properties and therapeutic applications of propolis. Phytother Res. 2016, 30(6):894-905.

Sforcin JM, Orsi RO, Bankova V. Effects of propolis, some isolated compounds and its source plant on antibody production. J Ethnopharmacol. 2005, 98:301-305.

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

Sforcin JM, Fernandes Jr A, Lopes CAM, Funari SRC, Bankova V. Seasonal effect of Brazilian propolis on *Candida albicans* and *Candida tropicalis*. J Venom Anim Toxins. 2001, 7:139-144.

Sforcin JM, Novelli ELB, Funari SRC. Seasonal effect of Brazilian propolis on seric biochemical variables. J Venom Anim Toxins. 2002, 8:244-254.

Shang H, Bhagavathula AS, Aldhaleei WA, Rahmani J, Karam G, Rinaldi G, Clark C, Salehisahlabadi A, Yuan Q. Effect of propolis supplementation on C-reactive protein levels and other inflammatory factors: A systematic review and meta-analysis of randomized controlled trials. J King Saud Univ Sci. 2020, 32(2):1694-1701.

Silva-Carvalho R, Baltazar F, Almeida-Aguiar C. Propolis: a complex natural product with a plethora of biological activities that can be explored for drug development. Evid Based Complement Alternat Med. 2015, 2015:1-29.

Silveira MAD, De Jong D, Berretta AA, Galvão EBS, Ribeiro JC, Cerqueira-Silva T, Amorim TC, Conceição LFMR, Gomes MMD, Teixeira MB, Souza SP, Santos MHCA, San Martin RLA, Silva MO, Lírio M, Moreno L, Sampaio JCM, Mendonça R, Ultchak SS, Amorim FS, Ramos JGR, Batista PBP, Guarda SNF, Mendes AVA, Passos RH, for the BeeCovid Team. Effcacy of Brazilian green propolis (EPP-AF®) as an adjunct treatment for hospitalized COVID-19 patients: A randomized, controlled clinical trial. Biomed Pharmacother. 2021, 138:111526.

Silveira MAD, Teles F, Berretta AA, Sanches TR, Rodrigues CE, Seguro AC, Andrade L. Effects of Brazilian green propolis on proteinuria and renal function in patients with chronic kidney disease: a randomized, double-blind, placebo-controlled trial. BMC Nephrol. 2019, 20(1):1-12.

Siqueira ABS, Gomes BS, Cambuim I, Maia R, Abreu S, Souza-Motta CM, De Queiroz LA, Porto AL. *Trichophyton* species susceptibility to green and red propolis from Brazil. Lett Appl Microbiol. 2009, 48:90-96.

Song YS, Park E, Hur GM, Ryu YS, Kim YM, Jin C. Ethanol extract of propolis inhibits nitric oxide synthase gene expression and enzyme activity. J Ethnopharmacol. 2002, 80:155-161.

Takeda K, Nagamatsu K, Okumura K. A water-soluble derivative of propolis augments the cytotoxic activity of natural killer cells. J Ethnopharmacol. 2018, 218:51-58.

Tasca KI, Calvi SA, Souza LR. Immunovirological parameters and cytokines in HIV infection. Rev Soc Bras Med Trop. 2012, 45(6):663-669.

Tien PC, Choi AI, Zolopa AR, Benson C, Tracy R, Scherzer R, Bacchetti P, Shlipak M, Grunfeld C. Inflammation and mortality in HIV-infected adults: analysis of the FRAM study cohort. J Acquir Immune Defic Syndr. 2010, 55(3):316-322.

Trigo D, Costa JB. HIV: epidemiology, natural course and diagnosis. Revista SPDV. 2016, 743(4):371-374

Valdez H, Connick E, Smith KY, Lederman MM, Bosch RJ, Kim RS, St Clair M, Kuritzkes DR, Kessler H, Fox L, Blanchard-Vargas M, Landay A. Limited immune restoration after 3 years' suppression of HIV-1 replication in patients with moderately advanced disease. AIDS. 2002, 16(14):1859-1866.

Venkataramana K. A study of biological markers in HIV disease progression and management in the highly active antiretroviral therapy (HAART) era. Am J Biosc Bioeng. 2013, 1(2):24-37.

Viana GMC, Brandão MDS, Ferreira AM, Rabelo EMF, Diniz Neto JA, Galvão CS, Santos AC, Santos Júnior OM, Oliveira RA, Binda Júnior JR. Evaluation of laboratory markers of progression of HIV disease to death. Rev Soc Bras Med Trop. 2011, 44(6):657-660.

Vos AG, Chersich MF, Klipstein-Grobusch K, Zuithoff P, Moorhouse MA, Lalla-Edward ST, Kambugu A, Kumarasamy N, Grobbee DE, Barth RE, Venter WD. Lipid levels, insulin resistance and cardiovascular risk over 96 weeks of antiretroviral therapy: a randomised controlled trial comparing low-dose stavudine and tenofovir. Retrovirology. 2018, 15(1):1-8.

Wacleche VS, Landay A, Routy JP, Ancuta P. The Th17 lineage: from barrier surfaces homeostasis to autoimmunity, cancer, and HIV-1 pathogenesis. Viruses. 2017, 9(10):303. doi:10.3390/v9100303.

Warren JA, Clutton G, Goonetilleke N. Harnessing CD8+ T cells under HIV antiretroviral therapy. Front Immunol. 2019, 10:291. doi:10.3389/fimmu.2019.00291.

Wing EJ. HIV and aging. Int J Infect Dis. 2016, 53:61-68.

Wyatt CM. Antiretroviral therapy and the kidney. Top Antivir Med. 2014, 22(3):655-658.

Yang CS, Shin DM, Jo EK. The role of NLR-related protein3 inflammasome in host defense and inflammatory diseases. Int Neurourol J. 2012, 16:2-12.

Yildirim A, Duran GG, Duran N, Jenedi K, Bolgul BS, Miraloglu M, Muz M. Antiviral activity of Hatay propolis against replication of herpes simplex virus type 1 and type 2. Med Sci Monit. 2016, 22:422-430.

Yu PC, Calderaro D, Lima EMO, Caramelli B. Hypolipidemic therapy under special conditions: acquired immune deficiency syndrome. Arq. Bras. Cardiol. 2005, 85:58-61.

Zakerkish M, Jenabi M, Zaeemzadeh N, Hemmati AA, Neisi N. The effect of Iranian propolis on glucose metabolism, lipid profile, insulin resistance, renal function and inflammatory biomarkers in patients with type 2 diabetes mellitus: A randomized double-blind clinical trial. Sci Rep. 2019, 9(1):1-11.

Zevin AS, McKinnon L, Burgener A, Klatt NR. Microbial translocation and microbiome dysbiosis in HIV-associated immune activation. Curr Opin HIV AIDS. 2016, 11(2):182-190.

Zicari S, Sessa L, Cotugno N, Ruggiero A, Morrocchi E, Concato C, Rocca S, Zangari P, Manno EC, Palma P. Immune activation, inflammation, and non-AIDS co-morbidities in HIV-infected patients under long-term ART. Viruses. 2019, 11(3):200.

Manuscrito 1

Propolis intake is safe and increases magnesium levels in HIV patients: a randomized, double-blinded, placebo-controlled clinical study

Running head: "HIV: Blood and body changes after propolis use"

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Conflict of interest
The authors declare that they have no conflicts of interest.

Abstract

Objectives: The antiretroviral therapy (ART) promotes alterations in the metabolic and nutritional profile contributing to the early development of non-aids comorbidities. In order to include new candidates to mitigate ART effects, this study aimed to investigate the action of propolis in people living with HIV/aids (PLWHA) with sustained viral suppression.

Methods: Forty PLWHA (20 propolis and 20 placebo) were enrolled in this randomized double-blind, placebo-controlled clinical trial. The influence of propolis (EPP-AF®, 500mg/day) daily intake on their biochemical and nutritional status was assessed at two moments (M0: before and M1: after 3 months). The participant's dietary pattern was also verified.

Key findings: Both groups were homogeneous regarding clinical and sociodemographic characteristics. An increased creatinophosphokinase (CPK) average ($p=0.011$) was seen in the propolis group in M1, not exceeding the standard reference range. The magnesium concentration was higher in propolis group ($p=0.003$), which can contribute to maintaining the body's homeostasis. No other parameters were affected by propolis use.

Conclusions: The participants'diet did not change during the intervention period and, except for the increased CPK and magnesium, propolis did not affect their nutritional, biochemical or metabolic profiles after 3 months of its consumption, indicating that the daily intake of propolis was safe for asymptomatic PLWHA.

Keywords: HIV, Propolis, Nutritional status, Dietary profile, Biochemical exams, Randomized double-blind trial; Safety.

1. INTRODUCTION

Globally, there were 38.0 millions (31.6–44.5 million) of people living with HIV at the end of 2019 and about 33 million people have died since the beginning of the epidemic [1]. Although the antiretroviral therapy (ART) has improved the prognosis and life expectancy of people living with HIV/aids (PLWHA), its use may lead to side effects and continuous immune/inflammatory activation. Consequently, illnesses such as cardiovascular, neurocognitive, kidney and liver impairment, bone disorders, and insulin resistance have increased [2-5]. In addition, ART can intensify the metabolic syndrome development due to changes in body composition and lipid/glycaemic profiles [2], triggering many of the aforementioned diseases, specially cardiovascular ones [4,6,7].

The nutritional profile of PLWHA, previously characterized by intense weight loss and malnutrition, is currently changing to an excessive weight gain and obesity [8,9]. However, factors other than infection and therapy, such as heredity, life style and dietary habit may be involved in metabolic abnormalities [8,10].

It has been demonstrated that PLWHA have similar age-related comorbidities to non-HIV controls, but 10 years earlier [5]. In a Brazilian cohort, the prevalence of multimorbidity was significantly higher in HIV-positive subjects than negative ones, which was associated both with duration of HIV infection and time on treatment in years [5]. The increased number of comorbidities among PLWHA is associated with higher resource utilization and direct medical costs for the health care system (ie, $300-5000 more for PLWHA with comorbidities monthly) [11].

Therefore, there is an urgent need to investigate new products that can reverse this situation or delay at least in part the onset of these comorbidities. In this scenario, propolis could be a candidate to promote health benefits for such patients. Propolis is a bee product that displays numerous pharmacological properties, such as antiinflammatory, antioxidant, antitumor, antimicrobial and immunomodulatory [12-15]. In addition, propolis is nontoxic and no adverse effects were reported after its administration to humans or experimental animals in the short, medium or long-term [16,17]. In addition to the nontoxic behavior, propolis also demonstrated a very low potential interaction with other drugs [18], being safe to be used concomitantly with

other medications without any risk. Thus, propolis has widely attracted the attention of researchers and of the pharmaceutical industry due to its potential for the development of new drugs. Experimental findings have also demonstrated the cardioprotective, vasoprotective, anti-atherosclerotic, anti-inflammatory and antiangiogenic action of some propolis constituents [19].

Clinical studies have also shown the beneficial effects of propolis, decreasing proteinuria levels in diabetic patients with chronic kidney disease [20], reducing glucose, insulin [21,22], inflammatory cytokines and increasing HDL-cholesterol (HDL-c) in diabetic patients [23]. An increased HDL-c level was also observed in healthy people ingesting 15 drops of propolis/day [24]. Moreover, other biochemical parameters improved after its administration, such as reduction in creatinine, glucose and total bilirubin levels in smokers [25] and the inflammatory status (C-reactive protein and tumor necrosis factor α) [26]. Recently, intervention with propolis has shown to be effective even in patients with COVID-19, showing a decrease in hospitalization time in those who used it as an adjuvant [27]. However, there are no clinical trials in the literature dealing with propolis treatment in HIV-infected patients and its possible clinical benefits.

Thus, the aim of this study was to perform, for the first time, routine laboratory tests (metabolic and biochemical variables, HIV viral load, CD4+/CD8+ T lymphocyte count, complete blood count) on PLWHA, as well as body composition and anthropometric measurements and safety assessment before and after 3 months of intervention with standard propolis pills (500mg/day). Besides, we verified if possible alterations were related to changes in the participants' dietary pattern during this period.

2. METHODS

2.1. Study design

This work was a randomized double blind placebo-controlled clinical trial conducted between September 2016 and December 2019, approved by the Research Ethics Committees of the Botucatu Medical School (FMB), UNESP (CAAE nº 58694816.6.0000.5411) and by Brazilian Clinical Trials Registry **(**ReBec; nº RBR-

33mjbg). This research followed the principles of the Helsinki Declaration and all individuals included in this assay signed an informed consent. Participants were recruited at the Specialized Outpatient Service for Infectious Diseases “Domingos Alves Meira” (SAEI-DAM) – Botucatu Medical School Complex (FMB)-UNESP, São Paulo State, Brazil.

In order to determine the sample size of the study, we adopted the variables “glucose, cholesterol, interleukins IL1-β and IL-10, and reactive oxygen species” reported by Al Ghamdi *et al.* [28] in diabetic rats with and without propolis supplementation. A sample of 20 individuals/intervention per group was determined to be necessary, assuming errors of type I and II (5% and 20%, respectively) and a loss of follow-up of 30%. This calculation was performed by professionals from the institution’s Research Support Office (EAP - FMB/UNESP).

Forty eligible subjects (Fig 1) were randomized into two groups: propolis (n=20) and placebo (n=20). The method used to generate a random allocation sequence was a random-numbers table (blocking randomization). Participants of propolis group ingested daily oral pills containing Brazilian green propolis – EPP-AF® 500mg/day for 90 consecutive days [29]. Propolis-containing pills and placebo were kindly provided by the *Apis Flora Company*, Ribeirão Preto, SP, Brazil (Patent Letter no. 0405483-0, approved by Industrial Property Magazine on July 23th, 2019). The chemical characterization and some biological activities of this EPP-AF® standardized extract were previously reported by Berretta *et al.* [30] and Marquiafável *et al.* [31]. Placebo group received the pills without propolis.

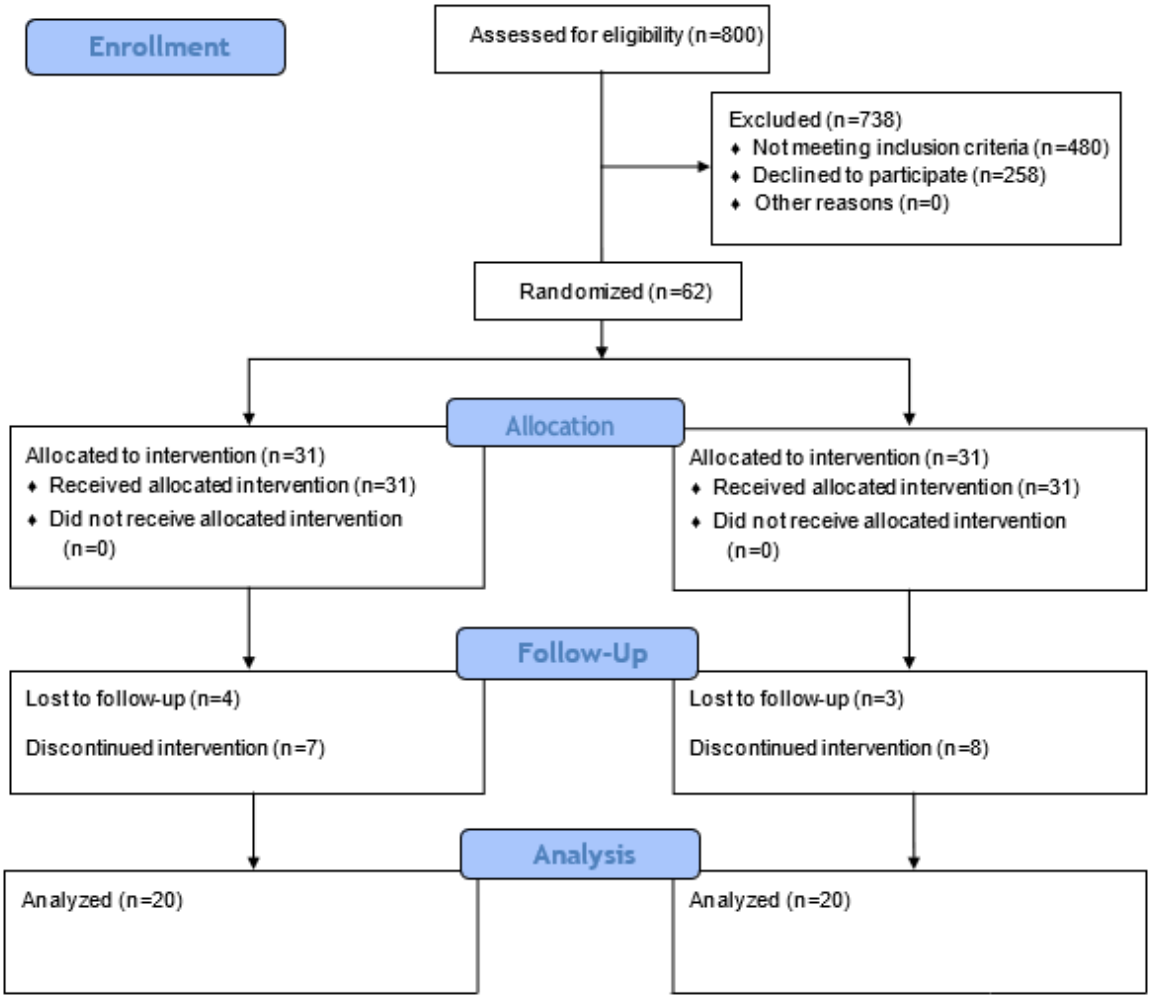

Fig 1: Flow chart showing the sequence of patient inclusion for the clinical trial - CONSORT 2010 Flow Diagram. Inclusion criteria: Age between 20 and 55 years, confirmed HIV infection, and regular ART use + undetectable viral load + $CD4^{+}$T cells count >500, for more than 2 years. Exclusion criteria: pregnancy, cancer history, organ transplant, underlying diseases (autoimmune, genetic, thyroid, cardiovascular diseases, diabetes mellitus, cancer, etc.), co-infections (tuberculosis, chronic viral hepatitis, syphilis, human T-lymphotropic virus, papillomavirus and other sexually transmitted diseases), taking any antibiotics, anxiolytics, antidepressants, vitamin, minerals, or other nutritional supplements; and use of illicit drugs or excessive alcohol consumption.

2.2. Inclusion and exclusion criteria

Inclusion criteria were: HIV-seropositive asymptomatic patients; 20-55 years old; regular ART use for at least two years and good immunological response in the same period, verified by medication history system (for adherence – *SICLOM*

System) and the clinical exam history, i.e., undetectable plasma HIV-1 viremia levels (<40 copies of RNA/mL) and $CD4^+$ T lymphocyte count stabilized above 500 cells/ml for more than five years (*SISCEL System*).

Exclusion criteria were: participation in a potentially conflicting research protocol, lack of desire to continue participating in the study, pregnancy, cancer history, organ transplantation, underlying diseases (autoimmune, genetic, thyroid, cardiovascular diseases, diabetes mellitus, cancer, etc.), co-infections (tuberculosis, chronic viral hepatitis, syphilis, human T-lymphotropic virus, papillomavirus and other sexually transmitted diseases), intake of antibiotics, anxiolytics, antidepressants, vitamins, minerals, or other nutritional supplements; and use of illicit drugs or excessive alcohol consumption.

2.3. Biochemical and nutritional profiles of the subjects

The socio-demographic, clinical and therapeutic characteristics of the subjects were obtained from electronic medical records and by interviews and the following variables were analysed: gender, age, skin colour, time of HIV infection (considering the date of diagnosis), time of ART use, current ART regimen and current $CD4^+$ and $CD8^+$ T lymphocyte count.

For biochemical/metabolic analyses, the variables were collected from electronic medical records prior and at the end of intervention (M0 and M1): fasting blood glucose, triglycerides, total cholesterol, low and high density lipoprotein (LDL-c and HDL-c), alpha-1-acid glycoprotein (AGP), C-reactive protein (CRP), creatinophosphokinase (CPK), erythrocyte sedimentation rate (ESR), red and white blood cells, urea, creatinine, aspartate and alanine aminotransferases (AST, ALT), uric acid, gamma-glutamyl transpeptidase (GGT), total protein, albumin, amylase, lactic dehydrogenase (LDH), alkaline phosphatase (ALP), phosphorus, calcium, sodium, magnesium and potassium. Complete blood count was also evaluated.

For nutritional diagnosis, the following tools were used:

- Anthropometric and body composition assessment: weight, height and circumference of the neck (NC), waist (WC), hip (HC) and mid-upper arm circumference (MUAC) were measured at moments M0 and M1, as recommended by Lohman *et al.* [32], in addition to the body mass index (BMI) according to the World

Health Organization for adults reference [33]. The Multifrequency Electrical Bioimpedance Equipment (EB) (Inbody®, 570 model, USA) with straight segmental system was used to measure lean mass (LM) and total body fat percentage (TBFP).

- Dietary profile: An interview-based 24-hour dietary recall (24hR) was accomplished twice at M0 and M1. Besides, to assess the dietary pattern, participants were asked to answer monthly a self-reported food records (considering 3-day food intake records, reporting three non-consecutive days including one weekend day, totalling nine records per participant). For comparison purposes, the first and the third months were analysed.

Aftewards, the professional *Dietbox*® website/app was used to determine energy intake (total caloric value [TCV]), saturated fat (SFA), macronutrients (protein [PTN], carbohydrates [CHO], lipids [LPD]) and micronutrients (vitamins and minerals). Nutrient values were expressed as nutrient content of the edible portion of the food per 100g [34].

2.4. Statistical analyses

All analyses were performed using SAS for Windows (version 9.2) with the assistance of professionals from the institution's Research Support Office (EAP - FMB/UNESP). The Chi-square (x^2) test was used to compare the differences in proportions between arms for categorical variables.

The symmetry of the data was tested by Shapiro-Wilk test. In order to study the changes in both groups (Propolis and Placebo) over time (M0 and M1) and their interactions, a repeated measure design fitting a generalized linear models was adopted for continuous data: Poisson Distribution for count variables; Gamma Distribution for asymmetric data; and Analysis of Variance (ANOVA) followed by Tukey's post-hoc test adjusted for variables under normal distribution. All differences were considered statistically significant when p values were <.05.

3. RESULTS

The 40 individuals enrolled in this work were mainly male (65.5%), mean age 40.2 (±7.6) years, white skin colour (77.5%) and heterosexuals (55%). The average time since diagnosis and under ART were 9.4 years (±5.5) and 7.9 years (±4.8), respectively. ART regimen containing Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) were used by the majority of patients (57.5%), followed by those on Protease Inhibitor (IP, 32.5%) and Integrase Inhibitor (INI, 10%). The groups were homogeneous for all the epidemiological, clinical and therapeutic variables shown in Table 1.

Table 1. Epidemiological, clinical and therapeutic characteristics of 40 PLWHA.

	PLACEBO		**PROPOLIS**			
	Mean ±SD		Mean ±SD		*p*	Test
Age (years)	38.75	±7.93	41.6	±7.24	0.223	
Time since HIV diagnosis (years)	8.65	±5.25	10.2	±5.91	0.368	*Anova*
Time on ART (years)	7.6	±4.37	8.32	±5.27	0.635	
	N	%	N	%	*p*	Test
Skin colour						
White	17	85.0%	14	70.0%		
Brown	0	0.0%	4	20.0%	0.106	x^2
Black	3	15.0%	2	10.0%		
Gender						
Male	13	65.0%	13	65.0%	1.000	x^2
Female	7	35.0%	7	35.0%		
Sexual orientation						
Heterosexual	11	57.9%	11	61.1%		
Homosexual	8	42.1%	5	27.8%	0.263	x^2
Bissexual	0	0.0%	2	11.1%		
Current ART scheme						
PI/r	10	50.0%	3	15.0%		
NNRTI	8	40.0%	15	75.0%	0.065	x^2
INI	2	10.0%	2	10.0%		

PLWHA: people living with HIV/aids; SD: standard deviation; N: sample number; %: percentage; ART: antiretroviral therapy; PI/r: protease inhibitors reinforced with ritonavir; NNRTI: non-nucleoside reverse transcriptase inhibitor; INI: integrase inhibitor x^2: chi-square.

No changes were observed regarding the clinical variables previously controlled (adequate viral suppression [<40 copies/mL] and in the sustained immune response [$CD4^+$ T cells >500cell/mm^3]) after propolis intervention (Table 2). No patients reported adverse events or complaints during the intervention period.

Table 2. Viral load, $CD4^+$/$CD8^+$ T cell count, WBC, RBC, and biochemical variables of 40 PLWHA before (M0) and after (M1) intervention, represented by mean values and SD.

	PLACEBO			PROPOLIS			
	M0	M1	*p*	M0	M1	*p*	Test
HIV viral load*	undetectable	undetectable	-	undetectable	undetectable	-	-
$CD4^+$T cells (mm^3)	937.6 ± 470.3	953.6 ± 467.7	NS	918.6 ± 369.6	912.0 ± 409.4	NS	*Poisson*
$CD8^+$T cells (mm^3)	956.5 ± 353.7	917.4 ± 201.2	NS	844.7 ± 279.6	841.4 ± 248.4	NS	*Poisson*
Leukocytes (%)	6.8 ± 1.9	7.6 ± 2.6	NS	6.0 ± 2.0	5.9 ± 1.8	NS	*Poisson*
Neutrophils (%)	53.0 ± 8.8	55.9 ± 12.1	NS	53.8 ± 9.2	54.5 ± 9.0	NS	*Poisson*
Lymphocytes (%)	35.6 ± 7.8	32.5 ± 8.7	NS	36.2 ± 7.8	35.4 ± 8.3	NS	*Poisson*
Monocytes (%)	6.9 ± 2.4	6.9 ± 3.5	NS	6.5 ± 1.9	6.4 ± 2.1	NS	*Poisson*
Eosinophils (%)	3.7 ± 2.7	3.1 ± 2.1	NS	2.8 ± 1.4	2.8 ± 1.9	NS	*Poisson*
Basophils (%)	0.9 ± 0.3	0.6 ± 0.3	**0.015**	0.7 ± 0.2	0.9 ± 0.5	NS	*Poisson*
RBC (million/mm^3)	4.8 ± 0.3	4.8 ± 0.4	NS	4.6 ± 0.5	4.6 ± 0.5	NS	*Poisson*
Platelet ($x10^3/mm^3$)	261.1 ± 66.5	259.4 ± 65.7	NS	255.0 ± 69.6	241.6 ± 69.3	0.061	*Poisson*
ESR (mm/h)	15.1 ± 9.9	15.3 ± 11.7	NS	11.6 ± 12.7	12.0 ± 11.4	NS	*Gamma*
T. Chol (mg/dL)	178.7 ± 42.6	179.2 ± 43.9	NS	185.5 ± 44.3	188.8 ± 37.9	NS	*Anova*
LDL-c (mg/dL)	100.3 ± 41.4	108.1 ± 45.6	NS	100.8 ± 37.4	107.9 ± 31.1	NS	*Anova*
HDL-c (mg/dL)	45.6 ± 17.0	46.7 ± 15.0	NS	50.0 ± 14.9	48.7 ± 14.6	NS	*Anova*
Glucose (mg/dL)	99.5 ± 43.7	97.0 ± 31.8	NS	88.3 ± 9.7	87.4 ± 7.0	NS	*Gamma*
GGT (U/L)	50.3 ± 40.7	42.6 ± 19.6	NS	57.9 ± 41.2	52.2 ± 33.9	NS	*Gamma*
ALT (U/L)	41.4 ± 25.5	42.0 ± 24.3	NS	40.4 ± 25.7	41.2 ± 18.9	NS	*Gamma*
AST (U/L)	32.5 ± 14.6	32.3 ± 12.2	NS	28.9 ± 14.2	31.3 ± 9.8	NS	*Gamma*
AGP (mg/dL)	84.0 ± 30.3	88.6 ± 29.9	NS	81.6 ± 31.5	87.7 ± 34.2	NS	*Anova*
CRP (mg/dL)	0.8 ± 0.5	0.8 ± 0.4	NS	0.8 ± 0.6	0.8 ± 0.6	NS	*Gamma*
LDH (U/L)	357.6 ± 116.6	332.9 ± 143.2	NS	365.5 ± 148.2	363.4 ± 146.0	NS	*Anova*
Urea (mg/dL)	26.8 ± 8.1	31.7 ± 8.1	**0.006**	31.0 ± 7.3	30.5 ± 7.1	NS	*Anova*
Creatinine(mg/dL)	0.8 ± 0.2	0.9 ± 0.2	**0.029**	0.8 ± 0.1	0.8 ± 0.2	NS	*Anova*
Uric Acid (mg/dL)	4.8 ± 1.3	5.3 ± 1.3	**0.001**	4.6 ± 1.3	4.8 ± 1.7	NS	*Anova*

T. protein (g/dL	7.8 ± 0.5	7.6 ± 0.6	NS	7.6 ± 0.5	7.5 ± 0.6	NS	*Anova*
Albumin (g/dL)	4.4 ± 0.3	4.3 ± 0.4	NS	4.4 ± 0.3	4.4 ± 0.4	NS	*Anova*
Sodium (mmol/L)	140.6 ± 4.2	141.1 ± 3.6	NS	139.3 ± 3.6	140.4 ± 3.4	NS	*Anova*
Potass (mmol/L)	4.61 ± 0.5	4.7 ± 0.4	NS	4.5 ± 0.5	4.6 ± 0.4	NS	*Anova*
Phospho (mmol/L)	3.5 ± 0.6	3.7 ± 0.6	NS	3.5 ± 0.6	3.5 ± 0.4	NS	*Anova*
Calcium (mg/dL)	9.6 ± 0.5	9.3 ± 0.5	0.053	9.4 ± 0.4	9.4 ± 0.5	NS	*Anova*
ALP (U/L)	81.8 ± 16.3	80.6 ± 15.7	NS	77.0 ± 26.3	75.1 ± 24.9	NS	*Anova*
Amylase (U/L)	78.7 ± 25.6	75.9 ± 22.7	NS	91.5 ± 48.2	91.8 ± 30.9	NS	*Anova*

PLWHA: people living with HIV/aids; SD: standard deviation; %: percentage from the total leukocytes; WBC: white blood cell; RBC: red blood cell; ESR: erythrocyte sedimentation rate; T. chol: total cholesterol; LDL-c and HDL-c: low and high density lipoprotein; GGT: gamma-glutamyl transpeptidase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; AGP: alpha-1-acid glycoprotein; CRP: C-reactive protein; LDH: lactic dehydrogenase; Potass: potassium; Phospho: phosphorus; ALP: alkaline phosphatase; NS: non-significant value. No difference between groups, both fixing M0 and M1. *undetectable HIV viral load: <40 copies/ml.

There was an increased magnesium concentration and CPK activity in the propolis group (Fig 2). However, categorizing the data according to the reference values (normal range) commonly used in clinical practice in relation to CPK, no differences were observed in the frequency of altered results (Fig 2B). No changes were seen in other parameters in propolis-receiving patients (Table 2). Unexpectedly, the placebo group showed a decreased triglycerides level after intervention (Figure 2C) and increased levels of urea, creatinine and uric acid (Table 2). Except for basophils, the other variables did not change in the placebo group (Table 2).

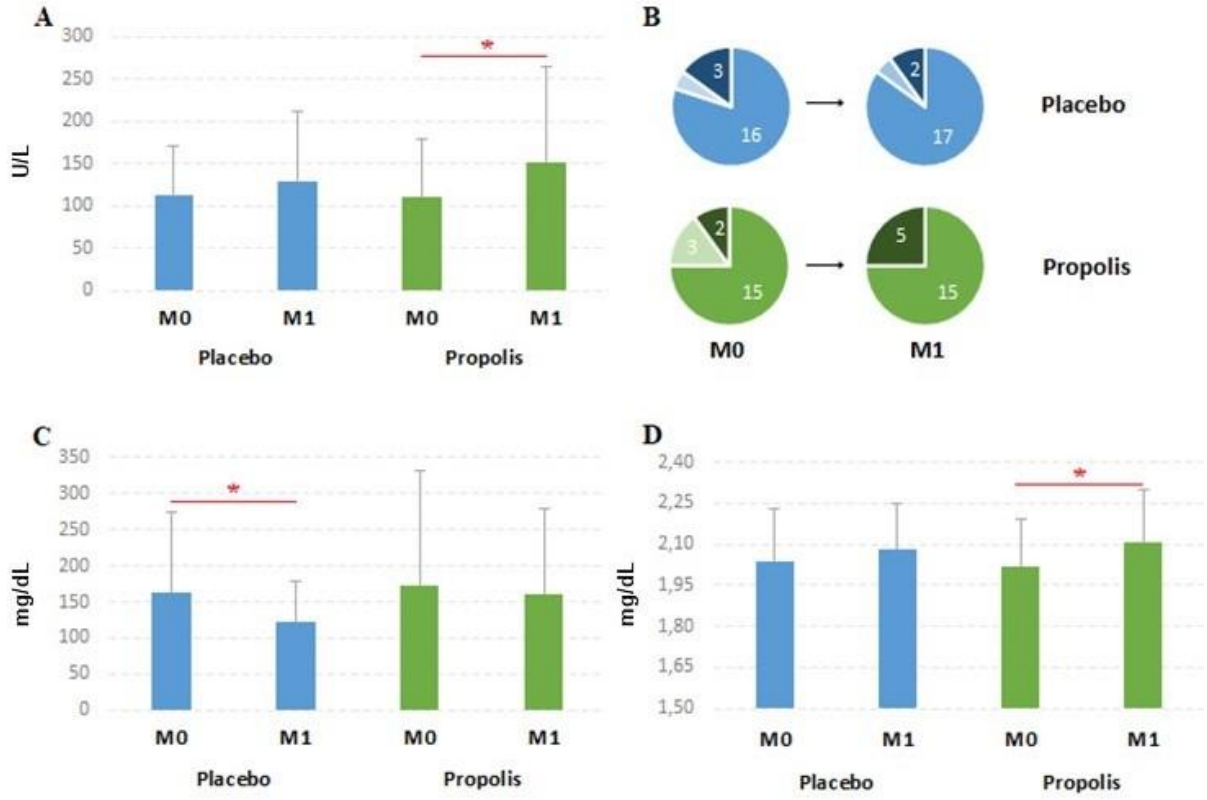

Fig 2. Significant differences presented by propolis and placebo groups, considering the 40 PLWHA before (M0) and after (M1) intervention, represented by mean values and standard deviation. PLWHA: people living with HIV/aids; **(A)** Creatinophosphokinase (CPK) concentration (**p=0.001, Anova*); **(B)** Frequency (%) of normal and abnormal CPK results, according to reference range 55 - 170 U/L – dark colour: values above the range; slight colour: values below the range. There was no statistical difference between the groups, fixing each moment separately regarding the distribution of these frequencies (association table). **(C)** Triglycerides levels (**p=0.017, Gamma*); (**D)** Magnesium levels (**p=0.003, Anova*).

The nutritional status, anthropometric measurements and body composition of 40 PLWHA are shown in Table 3, showing no changes in the parameters. In order to categorize patients' BMI into normal (<25), overweight (25-30) and grade I obesity (30-35), the frequency of participants were: 47.5%, 45.0%, 7.5% respectively.

Table 3. Nutritional status, anthropometric measurements and body composition of 40 PLWHA, at the moments (M0) and after (M1) the intervention with propolis and placebo, represented by mean values and SD.

	PLACEBO			PROPOLIS			
	M0	M1	*p*	M0	M1	*p*	Test
NC (cm)	37.47 ± 2.98	36.53 ± 3.53	NS	37.76 ± 3.71	37.85 ± 3.82	NS	*Anova*
WC (cm)	89.15 ± 10.53	86.42 ± 16.18	NS	88.32 ± 9.79	88.51 ± 9.40	NS	*Anova*
HC (cm)	102.16 ± 6.20	102.00 ± 6.50	NS	101.71 ± 6.98	102.3 ± 6.78	NS	*Anova*
MUAC (cm)	31.21 ± 3.85	31.36 ± 4.38	NS	32.21 ± 3.45	32.05 ± 4.47	NS	*Anova*
LM (%)	31.79 ± 5.64	31.84 5.89	NS	30.62 ± 7.06	30.48 ± 6.65	NS	*Poisson*
TBFP (%)	25.92 ± 7.65	26.44 ± 9.56	NS	26.34 ± 10.58	25.41 ± 8.20	NS	*Poisson*
Weight (kg)	76.00 ± 12.84	77.23 ± 13.61	NS	74.66 ± 14.91	74.63 ± 13.68	NS	*Anova*
Height (m)	1.72 ± 0.09	1.72 ± 0.09	NS	1.70 ± 0.11	1.70 ± 0.11	NS	*Anova*
BMI (kg/m^2)	23.1 ± 4.4	23.3 ± 4.8	NS	22.5 ± 5.2	22.5 ± 5.1	NS	*Anova*

PLWHA: people living with HIV/aids; SD: standard deviation; NC: neck circumference; WC: waist circumference, HC: hip circumference; MUAC: mid-upper arm circumference; LM: lean mass (percentage); TBFP: total body fat percentage. BMI: body mass index; NS: non-significant value.

The analysis of food profile calculated through the 24hR indicated that propolis group exhibited a higher fiber intake (M0: 10.00±4.89; M1: 13.05±5.14 g; p=0.011) and lower TCV (M0: 1643.66±481.67; M1: 1357.05±303.03 kcal; p=0.011) after intervention, while the placebo group showed no differences and only a tendency to lower % carbohydrate intake was seen in M1 (M0: 48.92±14.52; M1: 43.48±12.26 %; p=0.066) (Suppl 1).

With regard to food records, which is an accuracy tool to estimate/trace a food pattern, no significant changes that could attribute bias were seen here, since the two nutrients that showed different consumption values were the highest vitamin B intake and the lowest carbohydrate intake, both presented only by the placebo group (Table 4).

Table 4. Food profile of 40 PLWHA, calculated through the analysis of three food records made monthly, during the period of intervention, represented by mean values and SD.

	PLACEBO				PROPOLIS			
	1st Record	2nd Record	3th Record	*P*	1st Record	2nd Record	3th Record	*p*
EI (kcal)	1374 ± 461	1439 ± 645	1429 ± 434	NS	1214 ± 421	1229 ± 384	1248 ± 359	NS
CHO (g)	179 ± 79	167 ± 65	162 ± 47	NS	146 ± 76	154 ± 73	147 ± 50	NS
CHO (%)	**50.0 ± 11.6***	**47.9 ± 13.1**	**44.7 ± 9.3***	**0.004**	46.5 ± 12.6	48.8 ± 12.1	47.48 ± 9.9	NS
PTN (g)	63.5 ± 27.1	68.9 ± 33.1	73.7 ± 27.8	NS	63.8 ± 28.5	62.2 ± 26.5	64.22 ± 23.23	NS
PTN (%)	18.6 ± 6.9	19.5 ± 6.5	20.2 ± 6.3	NS	21.9 ± 9.0	21.5 ± 11.5	21.41 ± 7.47	NS
LPD (g)	49.9 ± 26.9	57.7 ± 49.8	58.0 ± 22.2	NS	56.6 ± 95.7	42.5 ± 19.3	43.95 ± 19.02	NS
LPD (%)	31.4 ± 9.9	32.6 ± 10.5	35.1 ± 7.9	NS	32.1 ± 8.7	30.8 ± 8.3	30.64 ± 7.99	NS
SFA (g)	16.6 ±9.9	19.2 ± 15.0	20.1 ± 8.8	NS	15.6 ± 7.7	14.7 ± 7.7	15.16 ± 7.57	NS
Fibra (g)	13.6 ±5.8	11.7 ± 5.3	12.0 ± 5.8	NS	10.8 ± 5.1	10.4 ± 4.5	10.23 ± 4.74	NS
VitC (mg)	239 ± 288	279 ± 323	268 ± 231	NS	281 ± 365	256 ± 325	249 ± 216	NS
Selenium (ug)	65.5 ± 34.8	62.6 ± 34.2*****	81.9 ± 41.1*****	0.006	73.5 ± 37.1	79.2 ± 35.1	72.59 ± 30.22	NS
Zinc (mg)	7.2 ± 3.7	8.6 ± 4.1	9.1 ± 5.8	NS	8.6 ± 4.5	8.5 ± 5.0	8.26 ± 3.94	NS
VitB (mg)	**2.6 ± 1.9**	**2.7 ± 2.1**	**3.8 ± 3.5****	**<0.05**	3.5 ± 2.8	4.4 ± 6.6	3.58 ± 2.63	NS
VitE (mg)	9.3 ± 7.3	8.8 ± 9.0	9.1 ± 6.6	NS	5.7 ± 5.0	5.8 ± 3.8*	7.2 ± 5.78*	0.044

PLWHA: people living with HIV/aids; SD: standard deviation; %: percentage from the kcal; EI: energy intake; CHO: carbohydrate; PTN: protein; LPD: lipids; SFA: satured fat; VIT: vitamin; NS: non-significant value. For final comparison purposes, we used the differences between first and the third month (highlighted in bold); * difference between the groups specified with the symbol; ** difference in relation to the others.

In order to establish a general analysis of the composition of the participants' diet, most of individuals reached the recommendations of the Recommended Daily Intake (IDR) for macronutrients: carbohydrates, 79.4% of them; proteins, 100%; and total fats 73.5%, but none of them reached the recommended

fiber intake. Regarding micronutrients, a high rate of patients with inadequate consumption was identified, such as iron, zinc, vitamin C and vitamin E (44.1%, 76.4%, 82.4 and 97% of the participants, respectively). No individual reached the recommendations for calcium and vitamin A. As for selenium and vitamin B12, 11.8% and 35% reached the recommendations, respectively.

4. DISCUSSION

Since PLWHA under effective ART usually exhibit changes in the metabolic, biochemical and nutritional profile [2,8], this study aimed at investigating the influence of propolis intake in their routine medical examination.

The viral load remained undetectable in M1, demonstrating the absence of propolis interference in the therapeutic response. Accordingly, Cusinato *et al.* [18] verified the absence of a potential interaction between Brazilian propolis and some commonly prescribed drugs (sub therapeutic doses of caffeine, losartan, omeprazole, metoprolol, midazolam and fexofenadine). However, no evidence of drug interactions between this bee product and ART has been reported so far. Thus, we proposed only the inclusion of participants presenting a sustained viral suppression in our experimental design in order to avoid the influence of the virus in the investigated parameters. Although our protocol did not allow an assessment of propolis effectiveness in decreasing HIV load, propolis and some of its isolated components (such as moronic acid and flavonoids) seemed to inhibit HIV replication *in vitro* [35-38]. Moreover, propolis activity against other viruses such as Varicella zoster virus and Herpes simplex virus type 1 and type 2 was also reported [39,40].

Valdez *et al*. [41] described that the main changes in the immune restoration of PLWHA occurred during the first year of ART use. Thus, we assume our patients were in a phase of clinical stability (CD4+/CD8+) considering the high T cell count, which was a requirement for the participants inclusion. The role of propolis and its isolated compounds in modulating these immunological markers has been well documented. Park *et al.* [42] observed a significant CD4+ T cells increase in caffeic acid phenethyl ester (CAPE)-treated mice, whereas the proportion of CD8+ T cells was not affected. In HIV infected cultures, propolis may inhibit cell lysis

mediated by the HIV nef protein and increase the proliferation of CD4+ T cells [43]. Thus, the potential immunomodulatory action of propolis should be taken into account and maybe this effect could be more evident in “non-immune responders” PLWHA, i.e., those under treatment, with a persistent history of CD4+ T count ≤500 cells/mm3 despite undetectable viral load for several years.

Blood biochemical determinations revealed a significantly higher CPK activity after propolis intake for 3 months (p=0.001); however, the averages were within the normal range in both moments and groups (55 to 170 U/L) and patients had no signs of rhabdomyolysis, suggesting no clinical significance. Elevations in CPK activity and myopathies in PLWHA have been associated with the use of the integrase inhibitor (INI) raltegravir [44]; however, none of the regimens used by the participants included this drug (data not shown). Regarding INI, only four patients were under dolutegravir-based ART, showing no association between its use and CPK changes [45]. Ishikawa *et al.* [46] suggested that Brazilian propolis may lead to side effects in muscle tissue, since an increased CPK was observed in patients with recent colon removal and receiving capsules containing propolis components (165 µmol/day of artepillin C and 150 µmol/day of other polyphenols for three months), compared to the levels from the placebo group.

However, experimental studies have revealed different outcomes. Chopra *et al.* [47] reported that the pre-treatment of rats with propolis, followed by the administration of a cardiomyopathy-inducing agent, significantly reduced CPK levels compared to the untreated group, suggesting propolis’ cardioprotective effect. Similarly, decreased CPK levels were observed in rats with ischemia-reperfusion after treatment with the isolated compound CAPE [48]. Although CPK averages increased in the propolis group, one may speculate that our finding is not relevant from a clinical point of view, since there was no significant difference in the frequency of altered values between M0 and M1. Although CPK is a non-specific marker, it is still important that it be checked in the standard examinations in this population.

In the present study, the intervention with propolis was able to increase magnesium concentration (p=0.003). This ion is an essential cofactor and critical stabilizer in hundreds of enzymatic reactions, including those responsible for muscle contraction and glucose utilization, and is indispensable for the synthesis of nucleic

acids, fats and proteins; however, the significance of this ion is underestimated in the literature and clinical practice [49].

Gröber *et al.* [50] reported that low levels of magnesium may lead to neuromuscular, cardiac or nervous disorders and were associated with Alzheimer's disease, insulin resistance and type 2 diabetes mellitus, hypertension, among other chronic diseases. It is worth mentioning that the role of homeostatic regulation of magnesium also occurs in kidney and bone diseases and its deficiency is related to worsening prognosis, as observed by Biagioni et al. [51] investigating PLWHA with acute kidney injury and by Castiglioni *et al.* [52] showing magnesium contribution for bone health in experimental and clinical studies. Moreover, ART-treated patients may exhibit decreased micronutrients levels, including magnesium [53], and its supplementation and higher plasma levels were inversely associated to depressive symptoms in virally controlled PLWHA [54,55].

Similarly, several studies using animal models found that propolis may improve magnesium levels. Propolis (0.3 g/kg and 0.6 g/kg) exerted a notable effect on femur bone mineralization in diabetic rats [56]. Haro *et al.* [57] demonstrated that the addition of propolis (1g/kg) to the diet of rats with nutritional ferropenic anemia displayed a positive effect on phosphorus and calcium metabolism, maintaining an adequate level of magnesium. Magnesium supplementation of HIV-1-transgenic rats attenuated hyperlipidemia and oxidative/nitrosative/inflammatory stress caused by ART [58]. Thus, elevation in plasma magnesium is encouraging since higher levels may contribute to reducing some ART toxic effects.

Although there is conflicting evidence about COVID-19 risks for PLWHA [59,60], Tan *et al.* (2020) [61] reported that magnesium / vitamin D / vitamin B12 in combination reduced the proportion of patients with clinical deterioration requiring oxygen support or intensive care support in a cohort study enrolling elderly COVID-19 patients. However, this hypothesis needs to be better investigated.

The positive effects of propolis intake have been observed in several chronic human conditions including hypertension, heart and Alzheimer's disease [62] and principally diabetes. Fukuda *et al.* [63] observed an improvement in uric acid and glomerular filtration rate in diabetic people receiving Brazilian propolis (226.8 mg/day, two months). Silveira *et al.* [20] verified that the administration of Brazilian green

propolis (500 mg/day, 12 months) was safe and well tolerated in both diabetic and non-diabetic patients and reduced proteinuria. An improvement of glycaemic and lipid levels was seen in diabetic patients treated with propolis (900mg/day, two months) [22]. Afsharpour *et al.* [21] also showed an improvement in the glycaemic profile and in the inflammatory status of diabetics after Iranian propolis intervention (1500 mg/day, two months), with no changes in AST and ALT determination. On the other hand, a decreased AST, ALT, urea nitrogen, post-prandial blood glucose, serum insulin, insulin resistance, and inflammatory cytokines was seen after Iranian propolis use (100mg/day, three months) by diabetic individuals, and increased HDL levels [23].

The analysis of the dietary pattern and its influence in intervention studies has become essential in order to exclude possible confounding effects in the final analysis. Therefore, the assessment of nutritional status is crucial in defining the nutritional diagnosis and subsequent intervention, and must be composed of anthropometric indicators, body composition measurement and biochemical markers [64]. Here, there was no difference between groups or moments in relation to anthropometric and bioimpedance measurements and more than 50% of individuals exhibited overweight and grade I obesity. Our findings are in agreement with Soares *et al.* [65], showing a 45.7% prevalence of overweight PLWHA under ART. Although propolis has not been able to change this prevalence, Oršolić *et al.* [66] showed a regulation in serum and liver lipid profiles and decreased body weight gain after propolis administration to mice fed with a high-fat diet, as well as a significantly lower atherogenic indices, suggesting the use of propolis to treat obese patients with hyperlipidemia.

The 24hR and food records revealed similar nutrients values in M0 and M1, indicating no changes in dietary pattern of patients during the investigation. The highest fiber consumption ($p=0.011$) and total caloric value ($p=0.011$) in M1 in the propolis group did not seem to affect the variables analysed in the present study, since this method evaluates the consumption for just one day, with no certain influence in the blood exams performed the next day and therefore may not indicate change in the dietary pattern. For dietary records, vitamin B12 consumption was higher in the placebo group in the last month of the intervention ($p<0.05$), in addition

to a lower percentage of carbohydrate intake (p=0.004), which possibly may be associated with reduced triglyceride levels in M1 that occurred only in placebo. To date, only one study has considered the dietary pattern and its influence on the markers chosen after propolis intake: the double-blind trial conducted by Afsharpour *et al.* [21] evaluating diabetic adults. The authors analysed the patients' diet using three-day food diaries spanning all 8 weeks of the intervention, showing no differences in the averages of total caloric value, protein, fat, saturated fatty acids, unsaturated fatty acids and fibers.

The fact that the participants had an inadequate intake of microfibers and since there is a micronutrient deficiency frequently observed in PLWHA under ART [67], further investigation should be done to prevent and treat nutritional disorders in this population. The nutritional orientation of an adequate and balanced diet may promote weight maintenance, prevent infections, improve response to treatment, improve immune function and, thus, positively impact the life quality of these individuals.

The adopted concentration of propolis was recommended by Apis Flora Company for our study and other clinical trials, standardizing the comparison of data using the same pills. However, a longer intervention period may be assessed in future studies potentially increasing propolis concentration. Our findings are reliable since participants did not modify their diet during the intervention period as noticed in the analysis of the food surveys, and the groups were homogeneous in relation to the main clinical, sociodemographic and therapeutic variables. Since HIV infection (viruses and treatment) and dietary patterns may affect metabolic abnormalities and body composition, further study is recommended to assess the effects of propolis intake on this population in order to obtain a healthy lifestyle that can guarantee higher expectations and life quality for PLWHA.

5. CONCLUSION

Despite the slight increase in CPK averages, propolis intake promoted an increase in magnesium levels, which can contribute to the maintenance of body

homeostasis, since this ion is involved in several metabolic reactions and it is an important physiological and hormonal regulator.

Additionally, propolis-treated PLWHA reported no adverse events or complaints during the intervention period, and they showed no changes in their biochemical, metabolic, nutritional and dietary profiles during the intervention period. Our original clinical trial indicated that the daily use of propolis (500mg/day for 3 months) is safe for asymptomatic PLWHA under ART, benefiting from its several biological properties.

AUTHORS' CONTRIBUTIONS

Participated in the design of the study: KIT and JMS. Randomized patients: KBS, EOC, BJC, JEC. Included and followed patients: KIT and FLC. Accomplished nutritional monitoring: KIT, FLC, ACMMA and LBS. Fed the database and analysis of medical records: KIT, FLC and ACMMA. Calculated and interpreted the results and statistical analysis: KIT, FLC, ACMMA, JMS, KBS, EOC, BJC, AAB and LRS. Contributed to the reagents/materials/analysis tools: LBS, JEC, AAB, and LRS. Wrote the paper: KIT, FLC and JMS. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. WHO: World Health Organization. Estimated antiretroviral therapy coverage among people living with HIV (%), 2021. https://www.who.int/data/gho/data/indicators/indicator-details/GHO/estimated-antiretroviral-therapy-coverage-among-people-living-with-hiv-(-). [accessed 11 March 2021].
2. Collaboration ATC. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. The Lancet. 2008; 372(9635):293-9. doi: 10.1016/S0140-6736(08)61113-7.
3. Tasca KI, Manfio VM, Vidal VVMF, Barbosa AN, Souza LR. Contributory role of ART in the development of non-Aids comorbidities in asymptomatic PLWHA. J Appl Biomed; 2021. doi: 10.32725/jab.2021.002.
4. Nsagha DS, Assob JCN, Njunda AL, Tanue EA, Kibu OD, Ayima CW, et al. Risk factors of cardiovascular diseases in HIV/AIDS patients on HAART. Open AIDS J. 2015; 9:51-9. doi: 10.2174/1874613601509010051.
5. Maciel RA, Klück HM, Durandc M, Sprinz E. Comorbidity is more common and occurs earlier in persons living with HIV than in HIV-uninfected matched controls, aged 50 years and older: A cross-sectional study. Int J Infec Dis. 2018; 70:30-5. doi: 10.1016/j.ijid.2018.02.009.

6. Kaur J. A comprehensive review on metabolic syndrome. Cardiol Res Pract. 2014; 2014:943162. doi: 10.1155/2014/943162.
7. Saboya PP, Bodanese LC, Zimmermann PR, Gustavo AD, Assumpção CM, Londero F. Metabolic syndrome and quality of life: a systematic review. Rev Lat Am Enfermagem. 2016; 24:e2848. doi: 10.1590/1518-8345.1573.2848.
8. Silva EFR, Lewi DS, Vedovato GM, Garcia VRS, Tenore SB, Bassichetto KC. Estado nutricional, clínico e padrão alimentar de pessoas vivendo com HIV/Aids em assistência ambulatorial no município de São Paulo. Rev Bras Epidemiol. 2010; 13(4):677-88.
9. Mankal PK, Kotler DP. From wasting to obesity, changes in nutritional concerns in HIV/AIDS. Endocrinol Metab Clin North Am. 2014; 43(3):64763. doi: 10.1016/j.ecl.2014.05.004.
10. de Paula EP, Neres S, Santini E, Reis ADF. Considerações nutricionais para adultos com HIV/Aids. Rev Mato-gros Enf. 2011; 148-65.
11. Lerner AM, Eisinger RW, Fauci AS. Comorbidities in persons with HIV: the lingering challenge. JAMA. 2020; 323(1):19-20. doi:10.1001/jama.2019.19775.
12. Cole N, Sou PW, Ngo A, Tsang KH, Severino JAJ, Arun SJ, et al. Topical 'Sydney' propolis protects against UV-radiation-induced inflammation, lipid peroxidation and immune suppression in mouse skin. Int Arch Allergy Immunol. 2010; 52:87-97. doi: 10.1159/000265530.
13. Ahn MR, Kunimasa K, Ohta T, Kumazawa S, Kamihira M, Kaji K, et al. Suppression of tumor induced angiogenesis by Brazilian propolis: major component artepillin C inhibits in vitro tube formation and endothelial cell proliferation. Cancer Lett. 2007; 252:235-43. doi: 10.1016/j.canlet.2006.12.039.
14. Park YK, Koo MH, Abreu JA, Ikegaki M, Cury JA, Rosalen PL. Antimicrobial activity of propolis on oral microorganisms. Curr Microbiol. 1998; 36(1):24-8. doi: 10.1007/s002849900274.
15. Sforcin JM, Kaneno R, Funari SRC. Absence of seasonal effect on the immunomodulatory action of Brazilian propolis on natural killer activity. J Venom Anim Toxins. 2002; 8:19-29.
16. Sforcin JM. Biological properties and therapeutic applications of propolis. Phytother Res. 2016; 30:894-905. doi: 10.1002/ptr.5605.

17. Mani F, Damasceno HCR, Novelli ELB, Martins EAM, Sforcin JM. Propolis: effect of different concentrations, extracts and intake period on seric biochemical variables. J Ethnopharmacol. 2006; 105(1-2):95-8. doi: 10.1016/j.jep.2005.10.011.
18. Cusinato DC, Martinez EZ, Cintra MOT, Filgueira GCO, Berretta AA, Lanchote VL, et al. Evaluation of potential herbal-drug interactions of a standardized propolis extract (EPP-AF®) using an in vivo cocktail approach. J Ethnopharmacol. 2019; 245:112174. doi: 10.1016/j.jep.2019.112174.
19. Daleprane JB, Abdalla DS. Emerging roles of propolis: antioxidant, cardioprotective, and antiangiogenic actions. Evid-Based Complement Altern Med. 2013; 2013:175135. doi: 10.1155/2013/175135.
20. Silveira MAD, Teles F, Berretta AA, Sanches TR, Rodrigues CE, Seguro AC, et al. Effects of Brazilian green propolis on proteinuria and renal function in patients with chronic kidney disease: a randomized, double-blind, placebo-controlled trial. BMC Nephrol. 2019; 20:140. doi: 10.1186/s12882-019-1337-7.
21. Afsharpour F, Hashemipour S, Khadem-Haghighian H, Koushan Y. Effects of Iranian propolis on glycaemic status, inflammatory factors, and liver enzyme levels in type 2 diabetic patients: a randomized, double-blind, placebo-controlled, clinical trial. JNSD. 2017; 3(2):9-14.
22. Samadi N, Mozaffari-Khosravi H, Rahmanian M, Askarishahi M. Effects of bee propolis supplementation on glycemic control, lipid profile and insulin resistance indices in patients with type 2 diabetes: a randomized, double-blind clinical trial. J Integr Med. 2017; 15(2):124-34. doi: 10.1016/S2095-4964(17)60315-7.
23. Zakerkish M, Jenabi M, Zaeemzadeh N, Hemmati AA, Neisi N. The Effect of Iranian propolis on glucose metabolism, lipid profile, insulin resistance, renal function and inflammatory biomarkers in patients with type 2 diabetes mellitus: A Randomized Double-Blind Clinical Trial. Sci Rep. 2019; 9:7289. doi: 10.1038/s41598-019-43838-8.
24. Mujica V, Orrego R, Pérez J, Romero P, Ovalle P, Zúñiga-Hernández J, et al. The Role of propolis in oxidative stress and lipid metabolism: a randomized controlled trial. Evid-Based Complementary Altern Med. 2014; 2017:11. doi: 10.1155/2017/4272940.

25. Koo HJ, Lee KR, Kim HS, Lee B. Detoxification effects of aloe polysaccharide and propolis on the urinaryexcretion of metabolites in smokers. Food Chem Toxicol. 2019; 130:99-108. doi: 10.1016/j.fct.2019.05.029.
26. Jalali M, Ranjbar T, Mosallanezhad Z, Mahmoodi M, Moosavian SP, Ferns GA, et al. Effect of propolis intake on serum C-reactive protein (CRP) and tumor necrosis factor-alpha (TNF-α) levels in adults: a systematic review and meta-analysis of clinical trials. Complement Ther Med. 2020; 50:102380. doi: 10.1016/j.ctim.2020.102380.
27. Silveira MAD, Jong DD, Galvão EBS, Ribeiro JC, Silva TC, Berretta AA, et al. Efficacy of propolis as an adjunct treatment for hospitalized COVID-19 patients: a randomized, controlled clinical trial. MedRxiv 2021. doi: 10.1101/2021.01.08.20248932.
28. Al Ghamdi AA, Badr G, Hozzein WN, Allam A, Al-Waili NS, Al-Wadaan MA, et al. Oral supplementation of diabetic mice with propolis restores the proliferation capacity and chemotaxis of B and T lymphocytes towards CCL21 and CXCL12 by modulating the lipid profile, the pro-inflammatory cytokine levels and oxidative stress. BMC Immunol. 2015; 16:54. doi: 10.1186/s12865-015-0117-9.
29. Berretta AA, Arruda C, Miguel FG, Baptista N, Nascimento AP, Marquele-Oliveira F, et al. Functional Properties of Brazilian Propolis: From Chemical Composition Until the Market. In: Superfood and Functional Food - AnOverview of Their Processing and Utilization. Waisundara VY, Shiomi N (editors). IntechOpen; 2017. p. 55-98.
30. Berretta AA, Nascimento AP, Bueno PCP, Vaz MMOLL, Marchetti JM. Propolis Standardized Extract (EPP-AF®), an Innovative Chemically and Biologically Reproducible Pharmaceutical Compound for Treating Wounds. Int J Biol Sci. 2012; 8(4):512-21. doi: 10.7150/ijbs.3641.
31. Marquiafável FS, Nascimento AP, Barud HS, Marquele-Oliveira F, Freitas LAP, Bastoset JK, et al. Development and characterization of a novel standardized propolis dry extract obtained by factorial design with high artepillin C content. J Pharm Technol Drug Res. 2015; 4:1. doi: 10.7243/2050-120X-4-1.
32. Lohman TG, Roche AF, Martorell R. Anthropometric standardization reference manual. Human kinetics books; 1988.

33. WHO: World Health Organization. BMI classification WHO 2013, http://www.euro.who.int/en/health-topics/disease-prevention/nutrition/a-healthy-lifestyle/body-mass-index-bmi [accessed 20 January 2020].
34. FAO: Food and Agriculture Organization. Dietary Assessment: A resource guide to method selection and application in low resource settings. Rome, 2018, http://www.fao.org/3/i9940en/I9940EN.pdf [accessed 12 April 2020].
35. Harish Z, Rubinstein A, Golodner M, Elmaliah M, Mizrachi Y. Suppression of HIV-1 replication by propolis and its immunoregulatory effect. Drugs Exp Clin Res. 1997; 23:89-96
36. Ito J, Chang FR, Wang HK, Park YK, Ikegaki M, Kilgore N, et al. Anti-AIDS agents. Anti-HIV activity of moronic acid derivatives and the new melliferone-related triterpenoid isolated from Brazilian propolis. J Nat Prod. 2001; 64(10):1278-81. doi: 10.1021/np010211x.
37. Gekker G, Hu S, Spivak M, Lokensgard JR, Peterson PK. Anti-HIV-1 activity of propolis in CD4+ lymphocyte and microglial cell cultures. J Ethnopharmacol. 2005; 102(2):158-63. doi: 10.1016/j.jep.2005.05.045.
38. Silva CCF, Salatino A, Motta LB, Negri G, Salatino MLF. Chemical characterization, antioxidant and anti-HIV activities of a Brazilian propolis from Ceará state. Rev Bras Farmacogn. 2019; 29(3):309-18. doi: 10.1016/j.bjp.2019.04.001.
39. Yildirim A, Duran GG, Duran N, Janedi C, Bolgul BS, Miraloglu M, et al. Antiviral activity of Hatay propolis against replication of herpes simplex virus type 1 and type 2. Med Sci Monit. 2016; 22:422-30. doi: 10.12659/msm.897282.
40. Labská K, Plodková H, Pumannová M, Sensch KH. Antiviral activity of propolis special extract GH 2002 against Varicella zoster virus in vitro. Pharmazie. 2018; 73(12):733-6. doi: 10.1691/ph.2018.8672.
41. Valdez H, Connick E, Smith KY, Lederman MM, Bosch RJ, Kim RS, et al. Limited immune restoration after 3 years' suppression of HIV-1 replication in patients with moderately advanced disease. AIDS. 2002; 16(14):1859-66. doi: 10.1097/00002030-200209270-00002.

42. Park JH, Lee JK, Kim HS, Chung ST, Eom JH, Kim KA, et al. Immunomodulatory effect of caffeic acid phenethyl ester in Balb/c mice. Int Immunopharmacol. 2004; 4:429-36. doi: 10.1016/j.intimp.2004.01.013.
43. Azad AA. Novel drugs and vaccines based on the structure and function of HIV pathogenic proteins including nef. Ann NY Acad Sci. 2005; 1056:279-92. doi: 10.1196/annals.1352.013.
44. Madeddu G, De Socio GV, Ricci E, Quirino T, Orofino G, Carenzi L, et al. Muscle symptoms and creatine phosphokinase elevations in patients receiving raltegravir in clinical practice: Results from the SCOLTA project long-term surveillance. Int J Antimicrob Agents. 2015; 45(3):289-94. doi: 10.1016/j.ijantimicag.2014.10.013.
45. Chen GJ, Sun HY, Cheng A, Chuang YC, Huang YS, Lin KY, et al. Risk of elevation of serum creatine kinase among HIV-positive individuals receiving dolutegravir-based combination antiretroviral therapy. Medicine (Baltimore). 2019; 98(26):e16235. doi: 10.1097/MD.0000000000016235.
46. Ishikawa H, Goto M, Matsuura N, Murakami Y, Goto C, Sakai T, et al. A pilot, randomized, placebo-controlled, double-blind phase 0/biomarker study on effect of artepillin C-rich extract of Brazilian propolis in frequent colorectal adenoma polyp patients. J Am Coll Nutr. 2012; 31(5):327-37. doi: 10.1080/07315724.2012.10720434
47. Chopra S, Pillai KK, Husain SZ, Giri DK. Propolis protects against doxorubicin-induced myocardiopathy in rats. Exp Mol Pathol. 1995; 62(3):190-8. doi: 10.1006/exmp.1995.1021.
48. Ozyurt B, Iraz M, Koca K, Ozyurt H, Sahin S. Protective effects of caffeic acid phenethyl ester on skeletal muscle ischemia-reperfusion injury in rats. Mol Cell Biochem. 2006; 292(1-2):197-203. doi: 10.1007/s11010-006-9232-5.
49. Jahnen-Dechent W, Ketteler M. Magnesium basics. Clin Kidney J. 2012; 5(Suppl 1):i3-i14. doi: 10.1093/ndtplus/sfr163.
50. Gröber U, Schmidt J, Kisters K. Magnesium in prevention and therapy. Nutrients. 2015; 7(9):8199-226. doi: 10.3390/nu7095388.
51. Biagioni SMS, Seguro AC, Andrade L. Hypomagnesemia is a risk factor for nonrecovery of renal function and mortality in AIDS patients with acute kidney

injury. Braz J Med Biol Res. 2010; 43(3):316-23. doi: 10.1590/s0100-879x2010007500002.

52. Castiglioni S, Cazzaniga A, Albisetti W, Maier JAM. Magnesium and osteoporosis: current state of knowledge and future research directions. Nutrients. 2013; 5(8):3022-33. doi: 10.3390/nu5083022.
53. Mudzinge D, Nyazika TK, Chisango TJ, Zhou DT. Differences in serum levels of magnesium, phosphate, and albumin for HAART-experienced and HAART-naïve female patients attending Parirenyatwa Opportunistic Infections Clinic in Harare, Zimbabwe. ISRN AIDS. 2013; 2013:383214. doi: 10.1155/2013/383214.
54. Derom ML, Sayon-Orea C, Martinez-Ortega JM, Martinez-Gonzalez MA. Magnesium and depression: a systematic review. Nutr Neurosci. 2013; 16(5):191-206. doi: 10.1179/1476830512Y.0000000044.
55. Currie J, Perazzo J, Schreiner N, Webel A. R. Association between magnesium intake and depressive symptoms in people living With HIV Infection. J Assoc Nurses AIDS Care. 2020; 31(2):255-60. doi: 10.1097/JNC.0000000000000132.
56. Al-Hariri M, Eldin TG, Abu-Hozaifa B, Elnour A. Glycemic control and anti-osteopathic effect of propolis in diabetic rats. Diabetes Metab Syndr Obes. 2011; 4:377-84. doi: 10.2147/DMSO.S24159.
57. Haro A, Lopez-Aliaga I, Lisbona F, Barrionuevo M, Alferez MJM, Campos MS. Beneficial effect of pollen and/or propolis on the metabolism of iron, calcium, phosphorus, and magnesium in rats with nutritional ferropenic anemia. J. Agric. Food Chem. 2000; 48:5715-22. doi: 10.1021/jf000635h.
58. ElZohary L, Weglicki WB, Chmielinska JJ, Kramer JH, Mak IT. Mg-supplementation attenuated lipogenic and oxidative/nitrosative gene expression caused by Combination Antiretroviral Therapy (cART) in HIV-1-transgenic rats. PLoS One. 2019; 14(1):e0210107. doi: 10.1371/journal.pone.0210107.
59. Tesoriero JM, Swain CE, Pierce JL, Zamboni L, Wu M, Holtgrave DR, et al. Elevated COVID-19 outcomes among persons living with diagnosed HIV infection in New York state: results from a population-level match of HIV, COVID-19, and hospitalization databases. MedRxiv, 2020. doi: 10.1101/2020.11.04.20226118.

60. Lee MJ et al. Comparative outcomes in hospital admissions with COVID-19 in people living with HIV and people living without HIV: A retrospective study. 23rd International AIDS Conference, abstract LBPEB09, 2020.
61. Tan CW, Ho LP, Kalimuddin S, Cherng BPZ, Teh YE, et al. Cohort study to evaluate the effect of vitamin D, magnesium, and vitamin B12 in combination on progression to severe outcomes in older patients with coronavirus (COVID-19). Nutrition. 2020; 79-80:111017. doi: 10.1016/j.nut.2020.111017.
62. Braakhuis A. Evidence on the health benefits of supplemental propolis. Nutrients. 2019; 11:2705. doi: 10.3390/nu11112705.
63. Fukuda T, Fukui M, Tanaka M, Senmaru T, Iwase H, Yamazaki M, et al. Effect of Brazilian green propolis in patients with type 2 diabetes: a double-blind randomized placebo-controlled study. Biomed Rep. 2015; 3(3):355-60. doi: 10.3892/br.2015.436.
64. Sacilotto LB, Pereira PCM, Manechini JPV, Papini SJ. Body composition and metabolic syndrome components on lipodystrophy different subtypes associated with HIV. J Nutr Metab. 2017; 2017:8260867. doi: 10.1155/2017/8260867.
65. Soares LR, Silva DC, Gonsalez CR, Batista FG, Fonseca LAM, Duarte AJS, et al. Discordance between body mass index and anthropometric measurements among HIV-1-infected patients on antiretroviral therapy and with lipoatrophy/ lipohypertrophy syndrome. Rev Inst Med Trop S Paulo. 2015; 57(2):105-10. doi: 10.1590/S0036-46652015000200002.
66. Oršolić N, Jurčević IL, Đikić D, Rogić D, Odeh D, Balta V, et al. Effect of propolis on diet-induced hyperlipidemia and atherogenic indices in mice. Antioxidants (Basel). 2019; 8(6):156. doi: 10.3390/antiox8060156.
67. Drain PK, Kupka R, Mugusi F, Fawzi WW. Micronutrients in HIV-positive persons receiving highly active antiretroviral therapy. Am J Clin Nutr. 2007; 85:333-45. doi: 10.1093/ajcn/85.2.333.

Supplementary 1: Analysis of the 24-hour dietary recall (24hR) of 40 PLWHA in the moments before (M0) and after (M1) t intervention with propolis and placebo. Values represent mean and SD.

	PLACEBO			PROPOLIS			
	M0	M1	*p*	M0	M1	*p*	Test
EI (kcal)	1513.85 ± 615.44	1644.39 ± 698.68	NS	1643.66 ±481.67	1357.05 ± 303.03	**0.011**	*Anova*
CHO (g)	177.87 ± 8.39	167.44 ± 62.31	NS	210.01 ± 84.40	172.90 ± 64.58	0.055	*Anova*
CHO (%)	48.92 ± 14.52	43.48 ± 12.26	0.066	53.35 ± 12.42	50.03 ± 10.61	NS	*Poisson*
PTN (g)	72.06 ± 30.84	75.94 ± 33.14	NS	63.21 ± 20.02	64.40 ± 33.69	NS	*Anova*
PTN (%)	20.39 ± 6.08	19.62 ± 6.73	NS	16.70 ± 5.37	19.51 ± 11.30	NS	*Poisson*
LPD (g)	49.33 ± 28.77	70.72 ± 52.46	NS	49.60 ± 20.31	45.26 ± 9.92	NS	*Gamma*
LPD (%)	30.69 ± 12.34	36.33 ± 11.05	NS	29.75 ± 9.28	30.46 ± 5.11	NS	*Anova*
SFA (g)	17.20 ± 9.05	24.58 ± 16.61	NS	18.56 ± 9.01	15.48 ± 5.43	NS	*Anova*
Fiber (g)	10.08 ± 5.40	10.05 ± 7.00	NS	10.00 ± 4.89	13.05 ± 5.14	**0.011**	*Anova*
VitC (mg)	288.57 ± 262.27	303.18 ± 203.25	NS	369.71 ± 646.88	292.75 ± 312.45	NS	*Anova*
Selenium(ug)	71.59 ± 44.79	76.25 ± 40.89	NS	73.26 ± 43.88	75.23 ± 77.40	NS	*Anova*
Zinc (mg)	8.53 ± 4.46	8.79 ± 6.25	NS	8.28 ± 4.23	7.11 ± 3.22	NS	*Anova*
VitB12(mg)	2.69 ± 2.42	3.22 ± 2.94	NS	2.66 ± 1.88	3.01 ± 4.00	NS	*Gamma*
VitE (mg)	10.01 ± 10.74	10.26 ± 15.02	NS	4.88 ± 3.89	5.68 ± 4.34	NS	*Gamma*

PLWHA: people living with HIV/aids; SD: standard deviation; %: percentage from kcal; EI: energy intake; CHO: carbohydrate; PT protein; LPD: lipids; SFA: satured fat; VIT: vitamin; NS: non-significant value.

Manuscrito 2

PROPOLIS INCREASES FOXP3 EXPRESSION AND LYMPHOCYTE PROLIFERATION IN HIV-INFECTED PEOPLE: A RANDOMIZED, DOUBLE BLIND, PARALLEL-GROUP AND PLACEBO-CONTROLLED STUDY

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Abstract

HIV infection and the prolonged use of antiretroviral therapy (ART) contribute to persistent inflammation and immune deregulation in people living with HIV/aids (PLWHA). Propolis is a bee product with plenty of biological properties, including immunomodulatory and anti-inflammatory action. This work aimed to evaluate possible changes in the immune/inflammatory response in PLWHA under ART after propolis intake. Asymptomatic PLWHA were double-blindly randomized into parallel groups receiving propolis (500 mg/day, n=20) for 3 months or placebo (n=20). Plasma cytokines (TNF-α, IL-2, IL-4, IL-6, IL-10 and IL17) were evaluated by cytometric bead array; cytokine production by PBMC (IFN-γ, IL-5, IL-17, IL-10, IL-1β, IL-18, and IL-33) was assessed by ELISA; gene expression (T-bet, GATA-3, RORγt and Foxp3) was determined by RT-qPCR, and cell proliferation was analysed by flow cytometry using CFSE staining. The average of gender, age, $CD4^+/CD8^+$ T cell count, time of diagnosis and treatment were similar in both groups. No differences were observed in cytokine levels nor in inflammasome activation. However, Pearson's correlation showed that IL-10 was directly correlated to $CD4^+$ T cell count and inversely correlated to IFN-γ after treatment with propolis. Foxp3 expression and lymphocyte proliferation increased in the propolis group. Data suggested that daily

propolis consumption may improve the immune response and decrease the inflammatory status in asymptomatic PLWHA under ART.

Keywords: HIV/aids, antiretroviral therapy, propolis, immunomodulation, inflammation, Treg cells

1. INTRODUCTION

HIV infection represents one of the biggest public health problems in the world, with a large number of cases and new infections every year (UNAIDS, 2020). Despite the numerous benefits of the antiretroviral therapy (ART), people living with HIV/AIDS (PLWHA) present a persistent immune activation and chronic inflammation (Gutiérrez et al., 2019) due to many factors, including direct pathogenic virus effects, microbial translocation imbalance, cytotoxic effects of ART, deregulated cytokine/chemokine production and loss of regulatory T cells (Eggena et al., 2005; Deeks, 2011; Hsu et al., 2013).

These factors contribute to the development of non-AIDS comorbidities (Hsu et al., 2013) and the main causes of death in PLWHA are associated with cardiovascular disease, diabetes mellitus, dyslipidemia, neurologic disorders, liver and kidney disease, bone disorders and cancers not related to AIDS (Deeks, 2011; Guaraldi et al., 2011; Venkataramana, 2013; Ramana, 2014). Thus, it is important to find interventions to improve the quality of life and health of PLWHA, to mitigate the impact of the infection and the adverse effects of ART for these individuals.

Propolis is a resinous and balsamic product made by bees from different parts of plants (Bankova, 2005), exhibiting several biological properties, especially immunomodulatory and anti-inflammatory action (Sforcin, 2016). Propolis is an immunomodulatory agent, acting on different cells in innate and adaptive immune response (Al-Hariri, 2019). This natural product is able to increase the concentration of anti-inflammatory mediators like IL-10 (Khayyal et al., 2003; Conti et al., 2015) and to decrease the concentration of many inflammatory markers, such as IFN-γ, IL-1β (Bueno-Silva et al., 2015), TNF-α, IL-6 (Shang et al., 2020), the intercellular adhesion molecule-1 (ICAM-1), leukotrienes D4, prostaglandins E2 and F2α (Khayyal et al.,

2003). Propolis also inhibits the expression of induced nitric oxide synthase (Song et al., 2002), the inflammasome activation (Hori et al., 2013) and the production of the chemokines CXCL1/KC and CXCL2/MIP-2 (Bueno-Silva et al., 2016). Data from literature suggested that propolis may have an important role in controlling immunological disorders (Al-Hariri, 2019) and inflammatory diseases (Hori et al., 2013). Therefore, the development of clinical trials is essential in considering the routine use of propolis as a therapeutic agent (Chan et al., 2013).

This work aimed to investigate whether propolis intake (500 mg/day for 3 months) could modulate the immunological response and decrease the inflammatory status of PLWHA under ART, in order to postpone the development of comorbidities and improve the quality of life and survival of these individuals.

2. MATERIAL AND METHODS

2.1. Study design

A randomized, double blind, parallel-group, placebo-controlled trial was conducted in the Specialized Outpatient Service for Infectious Diseases “Domingos Alves Meira” (SAEI-DAM) - Botucatu Medical School Complex (FMB)-UNESP, in São Paulo State, Brazil.

Inclusion criteria were HIV^+ patients, 20-55 years old, regular ART use for at least two years, with verified adherence of pharmacy discharge history, and sustained immunovirological response during the same period ($CD4^+$ T lymphocyte count ≥ 500 cells/mm^3 and plasma HIV-1 viremia <40 copies of RNA/ml).

Exclusion criteria for participants included registration in potentially conflicting research protocols, cancer history, diabetes mellitus, cardiovascular or autoimmune diseases, pregnancy, co-infections, transplants, patients who perform intense physical exercises regularly, genetic diseases, treatment with anxiolytics or antidepressants, vitamin or nutritional supplementation, use of illicit drugs or excessive alcohol consumption.

After determining the sample size and eligibility assessment according to Tasca et al. (submitted), 40 PLWHA were randomized by random-numbers table

(blocking randomization) in a group receiving propolis 500 mg/day (n=20) or placebo (n=20) for 3 months. Blood samples were collected twice: before (moment 0 = M0) and after (moment 1 = M1) propolis intake.

Tablets containing a standardized propolis extract (EPP-AF®) were kindly provided by Apis Flora Company, Ribeirão Preto, SP, Brazil (Patent Letter nº 0405483–0, approved by Industrial Property Magazine on July 23th, 2019). The chemical composition of propolis was previously analyzed by Berretta *et al.* (2012) and contained artepillin C, isosakuranetin, *p*-coumaric acid, aromadendrin, caffeic acid, and *trans*-cinnamic acid.

This work was approved by the Research Ethics Committees of the Botucatu Medical School (FMB), UNESP (CAAE nº 58694816.6.0000.5411) and by the Brazilian Clinical Trials Registry (ReBec; nº RBR-33mjbg). The research was carried out according to the ethical principles of the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference on Harmonization. An informed consent was obtained from all participants.

2.2. Socio-demographic, clinical and therapeutic data

Socio-demographic, clinical and therapeutic characteristics of PLWHA were obtained from electronic medical records and by interviews. The following variables were analysed: gender, age, skin color, time of HIV infection (considering the diagnosis), time of ART, current ART type and $CD4^+$ and $CD8^+$ T lymphocyte count.

2.3. Determination of plasma cytokines

Blood samples were centrifuged for 10 minutes at 200 x *g* to obtain the plasma. TNF-α, IL-6, IL-10, IL-17, IL-2, IL-4 levels were determined using the Human Th1/Th2/Th17 cytokine kit BD™ Cytometric Bead Array (CBA), according to the manufacturer's guidelines. Cytokine levels were assessed using a FACS Calibur TM cytometer (Becton Dickinson, USA).

2.4. Peripheral blood mononuclear cells (PBMC)

After plasma collection, cells were centrifuged at 400 x *g* for 30 min at room temperature in the presence of Ficoll-Paque (GE Healthcare Bio-Sciences, Sweden – density = 1.077) to obtain the peripheral blood mononuclear cells (PBMC). The interface layer of PBMC was aspirated and washed twice with RPMI at 200 x *g* for 10 min.

2.5. Cytokine production by PBMC

PBMC ($1x10^6$ cells/ml) were incubated in the presence of LPS (10 µg/ml; Sigma-Aldrich, USA), for 18 h at 37ºC and 5% CO_2. The supernatant was collected and cytokine production was measured by enzyme-linked immunosorbent assay (ELISA).

Cytokines involved in inflammasome activation (IL-1β, IL-18 and IL-33) were measured using DuoSet ELISA kit (R&D Systems, USA); IFN-γ, IL-17, IL-10 were measured by ELISA Max Deluxe kit (BioLegend, USA) and IL-5 by BD OptEIA™ Set Kit (BD Biosciences, USA), according to manufacturer's guidelines.

The plates were read at 450 nm using an ELISA plate reader (BioTek, UK).

2.6. Gene expression of T cell transcription factors

T-bet, GATA-3, RORγt and Foxp3 expression was carried out using the real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR).

After PBMC separation, total RNA was extracted using the "*Total RNA Purification*" kit (Norgen Biotek, Canada), according to the manufacturer's instruction. RNA concentration was evaluated spectrophotometrically using a NanoDrop 2000c (Thermo Scientific, USA). RNA integrity was verified using the "*Agilent RNA 6000 Nano Kit*" (Agilent Technologies, Germany). Total RNA was incubated with RNase-free DNase (Promega, USA) to remove the genomic DNA. Subsequently, the synthesis of complementary DNA (cDNA) was performed, using *ImProm-IITM Reverse Transcription System* (Promega, USA). Gene expression was evaluated by RT-qPCR, using SYBR™ Green Master Mix (Promega, USA) and the primers listed

in table 1. The reaction was carried out on 7300 fast real-time PCR Systems (Applied Biosystems, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize gene expression. The differential expression was performed using a standard curve (Larionov *et al.,* 2005).

Tabela 1. Genes and primer sequences.

Gene	Primer Sequence (5'–3')	GeneBank
T-bet	Forward: (906) GGATGCGCCAGGAAGTTTCA (925) Reverse: (993) TGGAGCACAATCATCTGGGT (974)	NM_013351
GATA-3	Forward: (174) CTCTTCGCTACCCAGGTGAC (193) Reverse: (269) ACGACTCTGCAATTCTGCGA (250)	NM_001002295.1
RORγt	Forward: (363) CATGTCCCGAGATGCTGTCA (382) Reverse: (473) GGTTCCTGTTGCTGCTGTTG (454)	NM_005060.3
Foxp3	Forward: (614) AGGAAGGACAGCACCCTTT (633) Reverse: (726) GGAAGTCCTCTGGCTCTTCG (707)	NM_014009
GAPDH	Forward: (684)CGTGGAAGGACTCATGACCA (703) Reverse: (801)GGCAGGGATGATGTTCTGGA (782)	NM_002046.4

2.7. Lymphocyte proliferation

Lymphocyte proliferation assay was performed using the CellTrace™ Cell Proliferation kit (Life Technologies, USA). PBMC ($1x10^6$ cells/ml) were labeled with 0.2 µl/ml CFSE and incubated with the mythogen phytohemagglutinin (PHA) (3 µg/ml) for 120h. Proliferating cells were phenotyped using the monoclonal anti-CD4-PerCP/Cy5.5 and anti-CD3-APC (BioLegend, USA) antibodies. In each sample, 10.000 events were analyzed by flow cytometry, using FACSCanto II™ (BD Biosciences, USA). Data was analysed using *Flowjo software (Tree Star)*.

2.7. Statistical analysis

The analysis study population comprised only patients who completed the treatment originally allocated (Per-protocol analysis). Analyses was performed using SAS for Windows (version 9.2) software, with the assistance of the institution's Research Support Office (EAP - FMB/UNESP). Data was analysed before and after 3

months of intervention using Poisson, Gamma distribution and Analysis of Variance (ANOVA) followed by the Tukey-Kramer test. Significant differences were considered when $p \leq 0.05$.

3. RESULTS

3.1. Data from medical records and interviews

The groups were homogeneous regarding the epidemiological, therapeutic and clinical variables. PLWHA enrolled in the study were predominantly male (65.5%), with mean age 40.2 (±7.6) years, white skin color (77.5%) and heterosexuals (55%). The average diagnosis time was 9.4 years (±5.5) and the use of ART was 7.9 years (±4.8). ART containing Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) was used by the majority of patients (57.5%), followed by a Protease Inhibitor (IP, 32.5%) and Integrase Inhibitor (INI, 10%). Viral load remained undetectable (<40 copies/ml) and no changes were seen in $CD4^+$ and $CD8^+$ T cells count after the intervention. Additionally, no adverse events or complaints were reported during propolis intervention, and the patients showed no significant changes in their biochemical, metabolic, nutritional and dietary profiles during the intervention period, as previously reported by Tasca et al. (submitted).

3.2. Plasma cytokines

Plasma cytokine levels in PLWHA before and after the intervention are seen in table 2.

Significant differences were observed only in IL-2 and IL-4 in the placebo group after treatment. High frequencies of undetectable cytokines were obtained pre and post-treatment: TNF-α was undetected for 90% of PLWHA (both M0 and M1); 50% (M0) and 40% (M1) for IL-6; 90% (M0) and 77.5% (M1) for IL-10; 97.5% (M0 and M1) for IL-17; 92.5% (M0 and M1) for IL-2; 97.5 (M0) and 100% (M1) for IL-4.

Tabela 2. Concentration of plasma cytokines (fg/ml) in the placebo or propolis group, before (M0) and after (M1) the intervention. Data represent mean ± standard deviation of 40 PLWHA.

	PLACEBO			PROPOLIS		
Cytokines (fg/ml)	M0	M1	*p*	M0	M1	*p*
TNF-α	38.90 ± 152.93	26.26 ± 117.42	NS	894.37 ± 3798,57	861.63 ± 3444.56	NS
IL-6	797.70 ± 1374.10	626.99 ± 843.75	NS	1474.95 ± 4283.18	2053.36 ± 4638.10	NS
IL-10	126.05 ± 548.78	44.28 ± 107.53	NS	101.16 ± 342.58	4.56 ± 13.86	NS
IL-17	0.00 ± 0.00	0.00 ± 0.00	NS	372.55 ± 1666.08	382.00 ± 1708.35	NS
IL-2	1.42 ± 6.37	0.00 ± 0.00	<.0001*	682.41 ± 2950.60	631.35 ± 2570.68	NS
IL-4	8.79 ± 39.30	0.00 ± 0.00	<.0001*	0.00 ± 0.00	0.00 ± 0.00	NS

PLWHA: people living with HIV/aids; SD: standard deviation; TNF-α: *Tumor necrosis fator alpha*; IL-6: *Interleukin 6*; IL-10: *Interleukin 10*; IL-17: *Interleukin 17*; IL-2: *Interleukin 2*; IL-4: *Interleukin 4*. NS: non-significant value. * Significantly different from M0 ($p < 0.05$)

3.2. Cytokine production by PBMC

There was no significant difference in cytokine production by PBMC (Table 3). No detectable IL-33 levels were registered (data not shown). However, Pearson's correlation analysis demonstrated that IL-10 was directly associated with $CD4^+$ T cell count ($p = 0.0459$) and inversely correlated with IFN-γ ($p = 0.0046$) in propolis-treated group (table 4).

Tabela 3. Cytokine production (pg/ml) by PBMC ($1x10^6$ cells/ml) incubated with LPS (10 µg/ml) for 18h, before (M0) and after (M1) treatment with placebo or propolis. Data represent mean ± standard deviation of 40 PLWHA.

	PLACEBO			PROPOLIS		
Cytokines (pg/ml)	M0	M1	*p*	M0	M1	*p*
IFN-γ	116.48 ± 153.83	144.16 ± 228.78	NS	198.35 ± 227.44	233.32 ± 268.99	NS
IL-5	17.18 ± 21.75	10.83 ± 14.39	NS	9.18 ± 8.58	8.25 ± 9.28	NS
IL-17	1.70 ± 0.87	1.66 ± 1.10	NS	2.05 ± 1.00	2.05 ± 2.02	NS
IL-10	5411.76 ± 2447.56	4985.99 ± 2853.01	NS	5595.18 ± 2893.33	5605.81 ± 3212.26	NS
IL-18	99.96 ± 36.74	139.12 ± 144.82	NS	92.63 ± 70.52	111.87 ± 93.33	NS
IL-1β	8334.16 ± 3341.41	9600.20 ± 5215.10	NS	7664.57 ± 5258.88	9545.82 ± 7755.81	NS

PLWHA: people living with HIV/aids; SD: standard deviation; IFN-γ: *Interferon-gamma*; IL-5: *Interleukin-5*; IL-17: *Interleukin-17*; IL-10: *Interleukin-10*; IL-18: *Interleukin-18*; IL-1β: *Interleukin-1 beta*. NS: non-significant value.

Table 4. Pearson's correlation analysis in the propolis group.

	IL-10	***p***
T CD4⁺ cells	**0,45107**	**0,0459***
IFN-γ	**-0,60589**	**0,0046***

IFN-γ: *Interferon-gamma*; IL-10: *Interleukin-10. *p* < 0.05*

3.3. Gene expression of transcription factors

Regarding gene expression, an increased Foxp3 expression was observed in the propolis group (p = 0.0406), as shown in figure 1. No differences were seen with respect to T-bet, GATA-3 and RORγt relative expression.

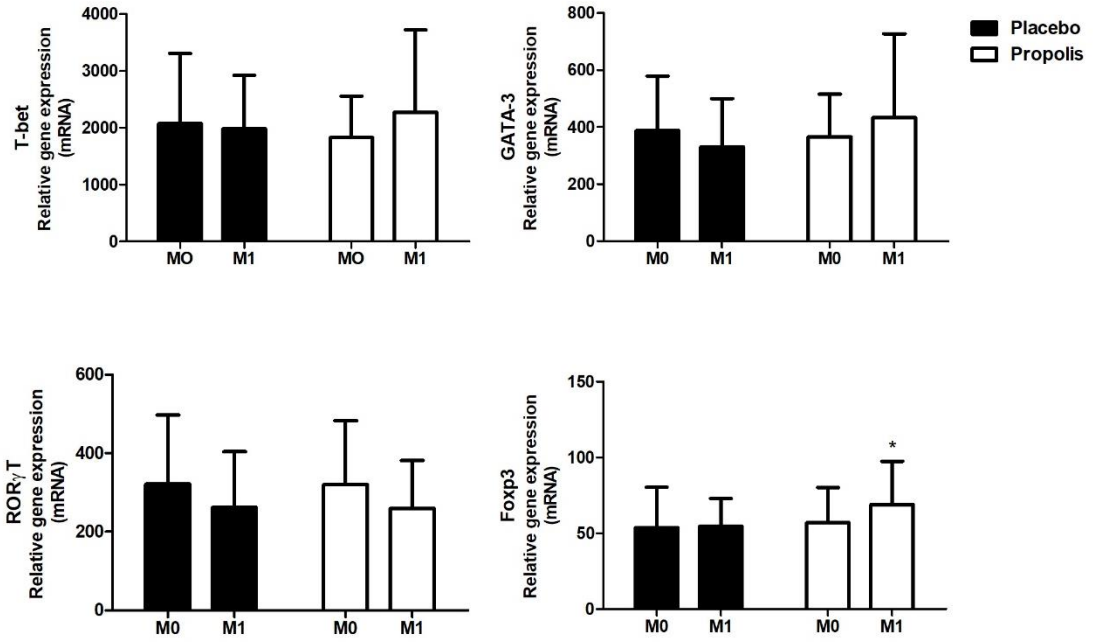
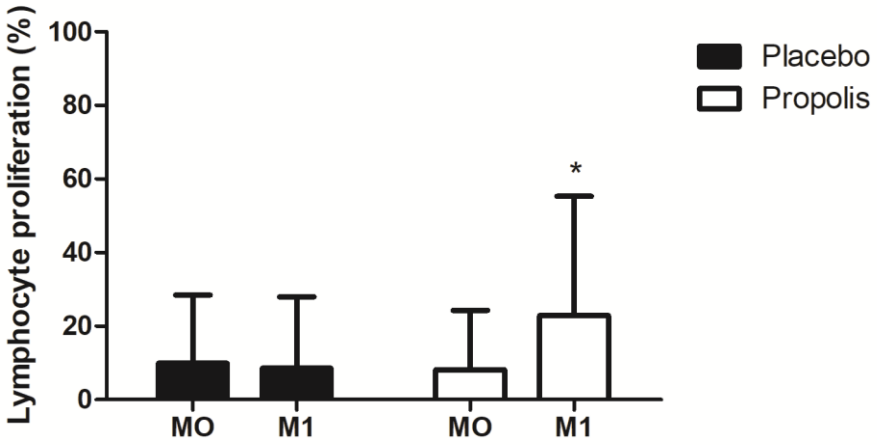


Figure 1. Relative gene expression of T-bet, GATA-3, RORγt and Foxp3 by PBMC ($1x10^6$ cells/mL), before (M0) and after (M1) treatment with placebo or propolis. Data represent mean ± standard deviation of 40 PLWHA. * Significantly different from propolis M0 ($p < 0.05$)

3.4. Proliferation assay

Data from cell proliferation (figure 2) revealed that the propolis group presented a higher lymphocyte proliferation after treatment (p = 0.0058). Gate strategies for samples analysis are shown in the supplementary file - figure 1.

A)



B)

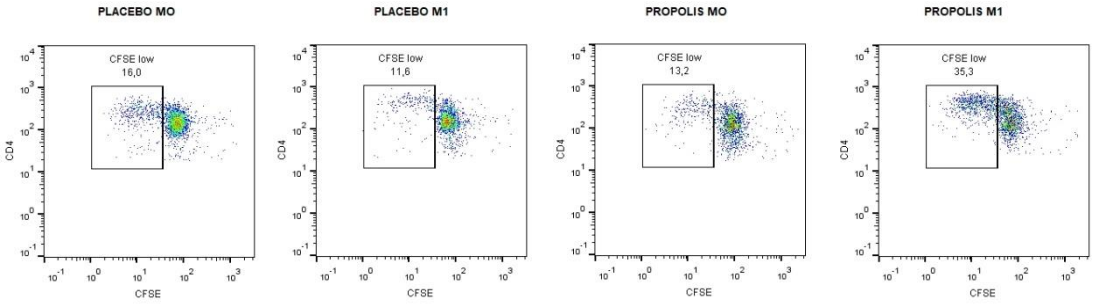


Figure 2. A) Proliferation (%) of T $CD3^{+}CD4^{+}$ lymphocytes ($1x10^{6}$ cells/ml) after 120 h of culture with PHA (3 µg/ml), before (M0) and after (M1) treatment with placebo or propolis. Data represent mean ± standard deviation of 40 PLWHA. *Significantly different from propolis M0 ($p < 0.05$). **B)** Representative dot plots of lymphocytes proliferation in placebo and propolis groups, before (M0) and after (M1) the treatments.

4. DISCUSSION

The control of the persistent immune activation and chronic inflammation is a current challenge for PLWHA, reflecting on the development of non-AIDS comorbidities. Thus, interventions with anti-inflammatory and immunomodulatory drugs are necessary, making propolis an important candidate to improve the quality of life and survival of PLWHA.

In this study, PLWHA in both groups were homogeneous regarding the epidemiological, clinical and therapeutic variables, minimizing the influences of

possible distorting effects in our findings. HIV viral load remained undetectable (<40 copies of RNA/ml) and there was no change in the $CD4^+/CD8^+$ T lymphocyte count, demonstrating the absence of propolis interference in antiretroviral response. Additionally, no adverse events or changes in the biochemical and metabolic profile were seen, signalling propolis as a safe intervention for asymptomatic PLWHA (Tasca et al., submitted).

PLWHA present high levels of inflammatory cytokines that favors the development of non-AIDS comorbidities (Tasca et al., 2012). HIV also leads to inflammasome activation, a multiprotein cytoplasmic complex that contributes to the inflammatory process (Hernandez et al., 2014). Propolis is able to modulate cytokine production (Al-Hariri, 2019), thus we evaluated whether propolis treatment could affect cytokine levels in PLWHA under ART.

A low frequency of plasma cytokines was observed. In contrast, evaluating the cytokines in PBMC supernatant by ELISA, detectable values were seen in almost all cell cultures, only without producing IL-33. Furthermore, low levels of IL-17 were observed - a cytokine that seemed to be less prominent in HIV-infected individuals (Wacleche et al., 2017). Regarding IL-4, Pina et al. (2018) found no detectable concentration of this cytokine in PLWHA and Gorenec et al. (2016) reported that IL-4 levels were increased in the acute HIV infection. This may be a possible explanation for the difficulty in detecting IL-4 in the samples of our patients, who were HIV-positive for at least 2 years.

It is worth mentioning that no change was seen in the concentration of cytokines related to the inflammasome activation (IL-1β, IL-18 and IL-33), whereas the same propolis sample exerted an inhibitory effect on IL-1β secretion by murine macrophages (Hori et al., 2013). Since IL-1β exerts a pro-inflammatory action, the fact that propolis decreases or maintains its levels reinforces the anti-inflammatory effect of this bee product.

Although propolis consumption did not change the cytokine levels, the propolis-treated group presented an important correlation related to IL-10, a cytokine that exerts an anti-inflammatory activity and is able to regulate the immune response, suppressing the synthesis of pro-inflammatory cytokines (Raphael et al., 2015). PLWHA exhibit a persistent inflammatory status and high levels of biomarkers

associated with inflammation (Deeks, 2011; Hsu et al., 2013). Even in PLWHA with a regular $CD4^+$ T cell count, inflammation is still an important risk factor for mortality (Tien et al., 2010). In the propolis group, $CD4^+$ T cell count was positively correlated with IL-10 production, and higher levels of IL-10 were associated with lower production of the inflammatory cytokine IFN-γ. Evaluating subcutaneous injection of IL-10 in HIV-1 infected individuals for 4 weeks, Pott et al. (2007) observed a lower production of IFN-γ and IL-1β compared to the placebo, demonstrating its anti-inflammatory and immunomodulatory features.

Our findings indicated that propolis use may be associated with suitable immunity and decreased inflammatory status, as is in agreement with other studies. Conti et al. (2015) observed that Brazilian green propolis stimulated IL-10 production by human monocytes. Increased IL-10 levels were seen in asthmatic patients treated with propolis, as well as a significant reduction in the pro-inflammatory cytokines TNF-α, IL-8 and IL-6 (Khayyal et al., 2003). Propolis treatment of murine macrophages pretreated with LPS attenuated IFN-γ and IL-1β production, among other inflammatory mediators (Bueno-Silva et al., 2015). Hori et al. (2013) observed that propolis reduced IL-1β production by murine macrophages stimulated with LPS. Propolis treatment of diabetic rats reduced IL-1β and increased IL-10 levels, compared to the control group (Nna et al., 2018). Isolated compounds, such as caffeic acid phenethyl ester (CAPE), administered to LPS-treated rats decreased pro-inflammatory cytokines production and increased the anti-inflammatory ones (Korish & Arafa, 2011). Thus, data from literature demonstrated the immunomodulatory action of propolis, generally favoring anti-inflammatory responses.

HIV infection also promotes a profound imbalance in the immune response of patients (Cleirici & Shearer, 1993; Orsilles et al., 2006; Nunnari et al., 2016), leading to a reduction of T regulatory (Treg) cells (Prendergast et al., 2010). We observed in an unprecedented way that propolis increased Foxp3 expression, a marker of Treg lymphocytes. Furthermore, propolis was also able to increase the number of Treg cells in asthmatic mice, reducing lung inflammation (Piñeros et al., 2020). Since Treg cells are responsible for regulating immune responses, secreting anti-inflammatory cytokines, controlling exacerbated inflammatory responses and maintaining immune homeostasis (Raphael et al., 2015; Wan et al., 2020), propolis may be a beneficial

alternative in controlling the immune activation and inflammation in PLWHA. Data from literature indicated that propolis can also prevent Th17 (Okamoto et al., 2012) and Th1 differentiation (Okamoto et al., 2013), what might be useful for controlling inflammatory diseases.

Our data demonstrated that propolis influenced the gene expression of transcription factors without interfering in cytokine production, increasing speculation for a longer-term intervention or a higher propolis dosage. Additionally, it is important to note that our study included only young PLWHA, with good immune status and without comorbidities, what could make it difficult to observe other effects of propolis.

HIV infection induces an intense immune deregulation with loss of cell function. The observed immune exhaustion in PLWHA is characterized by an increase in a given activated T cell phenotype, with a decreased proliferative and effector capacity of immune cells (Gutiérrez et al., 2019). Thus, immune cells become increasingly dysfunctional during chronic HIV infection (Korencak et al., 2019).

Korencak et al. (2019) reported that the use of integrase inhibitors (elvitegravir and dolutegravir) is associated with slow proliferation, impaired cell respiration and mitochondrial dysfunction, resulting in decreased $CD4^+$ T cell functions. This data demonstrates that the immune function impaired by HIV still remains even after ART.

Our results indicated for the first time that daily propolis intake is able to restore cell activity in PLWHA under ART, inducing the proliferation of $CD4^+$ T cells and favouring an anti-inflammatory profile. Thus, propolis can be useful in complementary medicine to restore the effector cell ability, the immunity of PLWHA and to prevent inflammatory response.

Other nonclinical studies have already demonstrated the ability of propolis and its constituents to modulate $CD4^+$ T cell activities. Kimoto et al. (1998) described that artepillin C increased the ratio of $CD4^+/CD8^+$ T cells and the total number of T helper cells. Park et al. (2004) observed an increased $CD4^+$ T cell count in mice treated with CAPE. Assessing the effects of irradiation after propolis administration in mice, Takagi et al. (2005) verified the protective action of the bee product on $CD4^+$ T cells. Al Ghamd et al. (2015) observed an increased T and B lymphocyte proliferation in diabetic mice treated with propolis, demonstrating its potential to improve the function of immune cells in this disease, which is characterized by impaired immunity.

Regarding HIV infection, propolis inhibited cell lysis mediated by the HIV nef protein and increased $CD4^+$ T cell proliferation (Azad, 2005). Investigating the action of propolis as an adjuvant in vaccine formulations containing HIV proteins (tat/env/pol/gag), Mojarab et al. (2020) verified that aqueous and ethanolic extracts of propolis induced lymphocyte proliferation in mice.

Several experimental studies conducted by our group have documented the immunomodulatory action of Brazilian propolis in animal models, such as rats and mice (Sforcin, 2007; Orsi et al., 2000; Orsatti et al., 2010), and in human immune cells, like monocytes and dendritic cells (Búfalo et al., 2014; Conti et al., 2015; Conti et al., 2016; Sforcin, 2016; Cardoso et al., 2017).

In addition, propolis is able to improve humoral and cellular immunity, being considered a promising candidate to strengthen the immune system in various pathological conditions (Al-Hariri, 2019).

5. CONCLUSION

The daily intake of propolis (500 mg) can be an alternative therapy to improve the immune response of PLWHA due to its ability to induce T cell proliferation, mitigating the inflammatory status in PLWHA through the activation of Treg cells. However, further investigation is still needed to indicate propolis as an effective intervention for these patients in the long-term, especially for immunological non-responders, symptomatic patients and those with concomitant non-aids illnesses.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests to disclose.

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REFERENCES

Al Ghamdi AA, Badr G, Hozzein WN, Allam A, Al-Waili NS, Al-Wadaan MA, Garraud O. Oral supplementation of diabetic mice with propolis restores the proliferation capacity and chemotaxis of B and T lymphocytes towards CCL21 and CXCL12 by modulating the lipid profile, the pro-inflammatory cytokine levels and oxidative stress. BMC Immunol. 2015, 16:1-14.

Al-Hariri M. Immune's-boosting agent: Immunomodulation potentials of propolis. J Family Community Med. 2019; 26(1):57-60.

Azad AA. Novel drugs and vaccines based on the structure and function of HIV pathogenic proteins including nef. Ann NY Acad Sci. 2005; 1056:279-292.

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

Berretta AA, Nascimento AP, Bueno PCP, Vaz MMOLL, Marchetti JM. Propolis Standardized Extract (EPP-AF®), an Innovative Chemically and Biologically Reproducible Pharmaceutical Compound for Treating Wounds. Int J BiolSci. 2012, 8(4):512-521.

Bueno-Silva B, Kawamoto D, Ando-suguimoto ES, Alencar SM, Rosalen PL, Mayer MPA. Brazilian red propolis attenuates inflammatory signaling cascade in LPS-activated macrophages. PLoS One. 2015, 10 (12):1-14.

Bueno-Silva B, Franchin M, Alves CF, Denny C, Colón DF, Cunha TM, Alencar SM, Napimoga MH, Rosalen PL. Main pathways of action of Brazilian red propolis on the modulation of neutrophils migration in the inflammatory process. Phytomedicine. 2016, 23(13):1583-1590.

Búfalo, M.C., Bordon-Graciani, A.P., Conti, B.J., Golim, M.A., Sforcin, J.M., 2014. The immunomodulatory effect of propolis on receptors expression, cytokine production and fungicidal activity of human monocytes. J. Pharm. Pharmacol. 66, 1497-1504.

Búfalo MC, Figueiredo AS, Sousa JPB, Candeias JMG, Bastos JK, Sforcin, JM. Anti-poliovirus activity of Baccharis dracunculifolia and propolis by cell viability determination and real-time PCR. J App Microbiol. 2009, 107(5):1669-1680.

Cardoso EO, Conti BJ, Santiago KB, Conte FL, Oliveira LPG, Hernandes RT, Golim MA, Sforcin JM. Phenolic compounds alone or in combination may be involved in propolis effects on human monocytes. J Pharm Pharmacol. 2017, 69:99-108.

Chan GC, Cheung K, Sze DM. The immunomodulatory and anticancer properties of propolis. Clin Rev Allergy Immunol. 2013, 44(3):262-273.

Clerici M, Shearer GM. A ThI→ Th2 switch is a critical step in the etiology of HIV infection. Immunology Today. 1993, 14(3):1-5.

Conti BJ, Santiago KB, Búfalo MC, Frión-Herrera Y, Alday E, Velazquez C, Hernandez J, Sforcin JM. Modulatory effects of propolis samples from Latin America (Brazil,Cuba and Mexico) on cytokine production by human monocytes. J Pharm Pharmacol. 2015, 67(10):1431-1438.

Conti BJ, Santiago KB, Cardoso EO, Freire PP, Carvalho RF, Golim MA, Sforcin, JM. Propolis modulates miRNAs involved in TLR-4 pathway, NF-κB activation, cytokine production and in the bactericidal activity of human dendritic cells. Journal of Pharmacy and Pharmacology. 2016, 68(12):1604-1612.

Deeks, S. HIV Infection, inflammation, immunosenescence, and aging. Ann Rev Med. 2011, 62:141-155.

Eggena MP, Barugahare B, Jones N, Okello M, Mutalya S, Kityo C, Mugyenyi P, Cao H. Depletion of regulatory T cells in HIV infection is associated with immune activation. J Immunol. 2005, 174(7):4407-4414.

Gekker G, Hu S, Spivak M, Lokensgard JR, Peterson PK. Anti-HIV-1 activity of propolis in $CD4^+$ lymphocyte and microglial cell cultures. J Ethnopharmacol. 2005, 102 (2):158-163.

Gorenec L, Lepej SZ, Grgic I, Planinic A, Bes JI, Vince A, Begovac J. The comparison of Th1, Th2, Th9, Th17 and Th22 cytokine profiles in acute and chronic HIV-1 infection. Microb Pathog. 2016, 97:125-130.

Guaraldi G, Orlando G, Zona S, Menozzi M, Carli F, Garlassi E, Berti A, Rossi E, Roverato A, Palella F. Premature age-related comorbidities among HIV-infected persons compared with the general population. Clin Infect Dis. 2011, 53(11):1120-1126.

Gutiérrez C, Lopez-Abente J, Pérez-Fernández V, Prieto-Sánchez A, Correa-Rocha R, Moreno-Guillen S, Muñoz-Fernández MÁ, Pion M. Analysis of the dysregulation

between regulatory B and T cells (Breg and Treg) in human immunodeficiency virus (HIV)-infected patients. PLoS One. 2019,14(3):1-18.

Harish Z, Rubinstein A, Golodner M, Elmaliah M, Mizrachi Y. Suppression of HIV-1 replication by propolis and its immunoregulatory effect. Drugs Exp Clin Res. 1997, 23(2):89-96.

Hernandez JC, Latz E, Urcuqui-Inchima S. HIV-1 induces the first signal to activate the NLRP3 inflammasome in monocyte-derived macrophages. Intervirology. 2014, 57(1):36-42.

Hori JI, Zamboni DS, Carrão DB, Goldman GH, Berretta AA. The inhibition of inflammasome by brazilian propolis (EPP-AF). Evid-Based Complem and Altern Med. 2013; 2013:1-11.

Hsu DC, Sereti I, Ananworanich J. Serious Non-AIDS events: Immunopathogenesis and interventional strategies. AIDS Res Ther. 2013, 10:1-15.

Ito J, Chang FR, Wang HK, Park YK, Ikegaki M, Kilgore N, Lee KH. Anti-AIDS agents. 48.(1) Anti-HIV activity of moronic acid derivatives and the new melliferone-related triterpenoid isolated from Brazilian propolis. J Nat Prod. 2001, 64(10):1278-1281.

Khayyal MT, El-Ghazaly MA, el-Khatib AS, Hatem AM, de Vries PJ, el-Shafei S, Khattab MM. A clinical pharmacological study of the potential beneficial effects of a propolis food product as an adjuvant in asthmatic patients. Fundam Clin Pharmacol. 2003, 17(1):93-102.

Kimoto T, Arai S, Kohguchi M, Aga M, Nomura Y, Micallef MJ, Kurimoto M, Mito K. Apoptosis and suppression of tumor growth by artepillin C extracted from Brazilian propolis. Cancer Detect Prev. 1998; 22(6):506-515.

Korencak M, Byrne M, Richter E, Schultz BT, Juszczak P, Ake JA, Ganesan A, Okulicz JF, Robb ML, de Los Reyes B, Winning S, Fandrey J, Burgess TH, Esser S, Michael NL, Agan BK, Streeck H.
Effect of HIV infection and antiretroviral therapy on immune cellular functions.
JCI Insight. 2019; 4(12):e126675.

Korish AA, Arafa MM. Propolis derivatives inhibit the systemic inflammatory response and protect hepatic and neuronal cells in acute septic shock. Braz J Infect Dis. 2011, 15(4):332-338.

Labská K, Plodková H, Pumannová M, Sensch KH. Antiviral activity of propolis special extract GH 2002 against Varicella zoster virus in vitro. Pharmazie. 2018, 73(12):733-736.

Larionov A, Krause A, Miller W. A standard curve based method for relative real time PCR data processing. BMC Bioinformatics. 2005, 6:62.

Mojarab S, Shahbazzadeh D, Moghbeli M, Eshraghi Y, Bagheri KP, Rahimi R, Savoji MA, Mahdavi M. Immune responses to HIV-1 polytope vaccine candidate formulated in aqueous and alcoholic extracts of Propolis: Comparable immune responses to Alum and Freund adjuvants. Microb Pathog. 2020, 140:103932.

Nna VU, Bakar ABA, Lazin RL, Mohamed M. Antioxidant, anti-inflammatory and synergistic anti-hyperglycemic effects of Malaysian propolis and metformin in streptozotocin–induced diabetic rats. Food Chem Toxicol. 2018, 120:305-320.

Nunnari G, Fagone P, Condorelli F, Nicolett F, Malaguarnera L, Di Rosa M. CD4+ T-cell gene expression of healthy donors, HIV-1 and elite controllers: Immunological chaos. Cytokine. 2016, 83:127-135.

Okamoto Y, Hara T, Ebato T, Fukui T, Masuzawa T. Brazilian propolis ameliorates trinitrobenzene sulfonic acid-induced colitis in mice by inhibiting Th1 differentiation. International Immunopharmacology. 2013, 16:178-183.

Okamoto Y, Tanaka M, Fukui T, Masuzawa T. Brazilian propolis inhibits the differentiation of Th17 cells by inhibition of interleukin-6-induced phosphorylation of signal transducer and activator of transcription 3. Immunopharmacology and Immunotoxicology. 2012, 34(5):803-809.

Orsatti CL, Missima F, Pagliarone AC, Sforcin JM. Th1/Th2 cytokines' expression and production by propolis-treated mice. J Ethnopharmacol. 2010, 129(3):314-318.

Orsi RO, Funari SRC, Soares AMVC, Calvi SA, Oliveira SL, Sforcin JM, Bankova V. Immunomodulatory action of propolis on macrophage activation. J Venom Anim Toxins. 2000, 6(2):205-219.

Orsilles MA, Pieri E, Cooke P, Caula C. IL-2 and IL-10 serum levels in HIV-1-infected patients with or without active antiretroviral therapy. APMIS. 2006, 114:55-60.

Park JH, Lee JK, Kim HS, Chung ST, Eom JH, Kim KA, et al. Immunomodulatory effect of caffeic acid phenethyl ester in Balb/c mice. Int Immunopharmacol. 2004, 4:429-436.

Pina GM, Lia EN, Berretta AA, Nascimento AP, Torres EC, Buszinski AF, de Campos TA, Coelho EB, Martins VP. Efficacy of Propolis on the Denture Stomatitis Treatment in Older Adults:A Multicentric Randomized Trial. Evid Based Complement Alternat Med. 2017, 2017:1-9.

Piñeros AR, de Lima MHF, Rodrigues T, Gembre AF, Bertolini TB, Fonseca MD, Berretta AA, Ramalho LNZ, Cunha FQ, Hori JI, Bonato VLD. Green propolis increases myeloid suppressor cells and $CD4^{+}Foxp3^{+}$ cells and reduces Th2 inflammation in the lungs after allergen exposure. J Ethnopharmacol. 2020, 252:1-9.

Pott GB, Sailer CA, Porat R, Peskind RL, Fuchs AC, Angel JB, Skolnik PR, Jacobson MA, Giordano MF, Lebeaut A, Grint PC, Dinarello CA, Shapiro L. Effect of a four-week course of interleukin-10 on cytokine production in a placebo-controlled study of HIV-1-infected subjects. Eur Cytokine Netw. 2007, 18(2):49-58.

Prendergast A, Prado JG, Kang YH, Chen F, Riddell LA, Luzzi G, Goulder P, Klenerman P. HIV-1 infection is characterized by profound depletion of CD161+ Th17 cells and gradual decline in regulatory T cells. AIDS. 2010, 24(4):491-502.

Ramana KV. Effect of Highly Active Antiretroviral Therapy (HAART) on Human Immunodeficiency Virus Disease Pathogenesis and Progression. Am J Public Health Res. 2014, 2(3):68-74.

Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. Cytokine. 2015, 74(1):5-17.

Sforcin JM. Biological Properties and Therapeutic Applications of Propolis. Phytotherapy research. 2016, 30(6): 894-905.

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

Shang H, Bhagavathula AS, Aldhaleei WA, Rahmani J, Karam G, Rinaldi G, Clark C, Salehisahlabadi A, Yuan Q. Effect of propolis supplementation on C-reactive protein levels and other inflammatory factors: A systematic review and meta-analysis of randomized controlled trials. Journal of King Saud University - Science. 2020, 32(2):1694-1701.

Silva CCF, Salatino A, Motta LB, Negri G, Salatino MLF. Chemical characterization, antioxidant and anti-HIV activities of a Brazilian propolis from Ceará state. Revista Brasileira de Farmacognosia. 2019, 29(3):309-318.

Song YS, Park E, Hur GM, Ryu YS, Kim YM, Jin C. Ethanol extract of propolis inhibits nitric oxide synthase gene expression and enzyme activity. J Ethnopharmacol.2002, 80:155-161.

Takagi Y, Choi IS, Yamashita T, Nakamura T, Suzuki I, Hasegawa T, Oshima M, Gu YH. Immune activation and radioprotection by propolis. Am J Chin Med. 2005, 33(2):231-240.

Tasca KI, Calvi SA, Souza LR. Immunovirological parameters and cytokines in HIV infection. Revista da Sociedade Brasileira de Medicina Tropical. 2012, 45(6):663-669

Tien PC, Choi AI, Zolopa AR, Benson C, Tracy R, Scherzer R, Bacchetti P, Shlipak M, Grunfeld C. Inflammation and mortality in HIV-infected adults: analysis of the FRAM study cohort. J Acquir Immune Defic Syndr. 2010, 55(3):316-322.

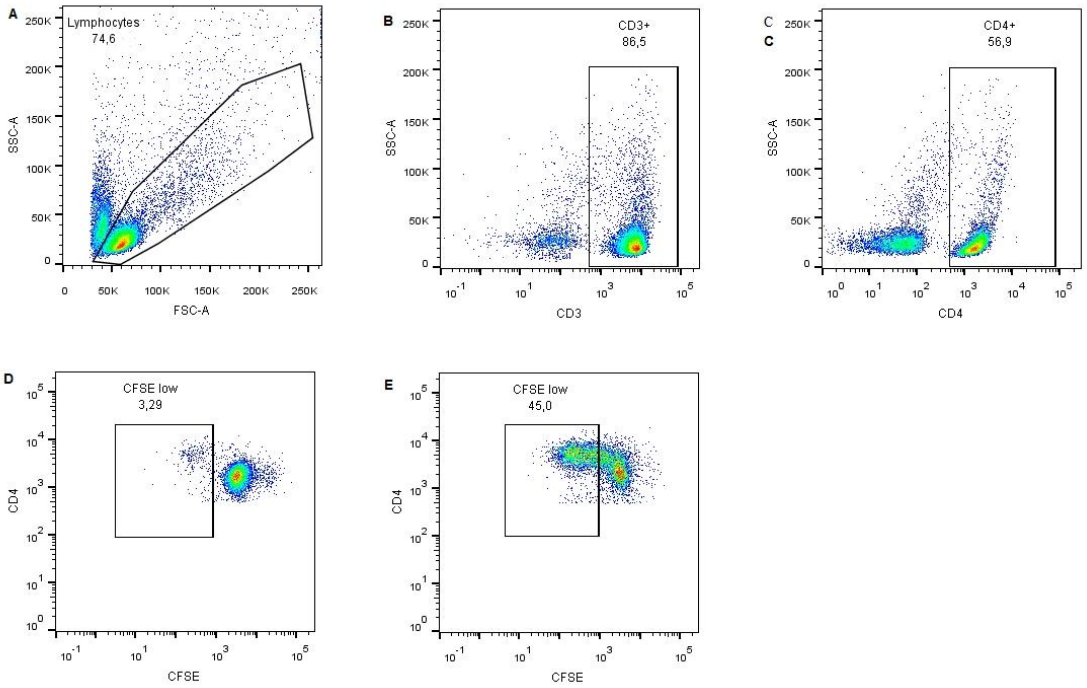
Valdez H, Connick E, Smith KY, Lederman MM, Bosch RJ, Kim RS, St Clair M, Kuritzkes DR, Kessler H, Fox L, Blanchard-Vargas M, Landay A. Limited immune restoration after 3 years' suppression of HIV-1 replication in patients with moderately advanced disease. AIDS. 2002, 16(14):1859-1866.

Venkataramana K. A study of biological markers in HIV disease progression and management in the highly active antiretroviral therapy (HAART) era. Am J Biosc Bioeng. 2013, 1(2):24-37.

Wacleche VS, Landay A, Routy JP, Ancuta P. The Th17 lineage: from barrier surfaces homeostasis to autoimmunity, cancer, and HIV-1 pathogenesis. Viruses. 2017, 9(10):303.

Wan Z, Zhou Z, Liu Y, Lai Y, Luo Y, Peng X, Zou W. Regulatory T cells and T helper 17 cells in viral infection. Scand J Immunol. 2020, 91(5):1-14.

Yildirim A, Duran GG, Duran N, Jenedi K, Bolgul BS, Miraloglu M, Muz M. Antiviral activity of Hatay propolis against replication of herpes simplex virus type 1 and type 2. Med Sci Monit. 2016, 22:422-430.



Supplementary Figure 1. Representative dot plots of lymphocyte proliferation after 120h of culture. A) Gate of lymphocyte size x granularity. B) $CD3^+$ T lymphocyte gate. C) $CD4^+$ T lymphocyte gate within the $CD3^+$ T population. D) Proliferation of $CD3^+CD4^+$ T lymphocytes without PHA. E) Proliferation of $CD3^+CD4^+$ T lymphocytes stimulated with PHA (3 µg/ml).

Manuscrito 3

Exploring the antioxidant, anti-inflammatory and antiallergic potential of Brazilian propolis in monocytes

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ABSTRACT

Propolis is a product made by bees, used since ancient times due to its diverse applications, potentially able to prevent or treat inflammatory and oxidative injury. Since oxidative stress and inflammation are present in many diseases, we aimed to investigate the antioxidant and anti-inflammatory action of Brazilian propolis, envisaging its potential application in inflammatory-related pathologies. Propolis

biological acitivities were evaluated in the human monocytic cell line (THP-1) stimulated with two different pro-inflammatory stimuli, the skin allergen 1-fluoro-2,4-dinitrobenzene (DNFB), responsible for allergic contact dermatitis, a skin inflammatory condition, or the Toll-like receptor (TLR)-4 agonist lipopolysaccharide (LPS) evoking a systemic pro-inflammatory response. Cell viability was assessed by resazurin assay, antioxidant activity was evaluated by DPPH assay, and superoxide dismutase (SOD) 1 and 2 activity was evaluated by colorimetric assay. CD86 expression was determined by flow cytometry. IL-1β and HMOX-1 expression was analysed by Western blot. Propolis did not affect THP-1 cell viability and exhibited a potent antioxidant activity. Propolis alone induced cytoplasmatic SOD 1 activity. Propolis did not affect SOD 2 activity, while LPS stimulated it, concomitantly or not with propolis. In the presence of LPS, propolis induced HMOX-1 expression. Propolis inhibited CD86 expression stimulated by DNFB. LPS induced pro-IL-1β expression and propolis did not affect its action. Propolis exerted an antioxidant activity, potentially involved in its anti-inflammatory/antiallergic action.

Keywords:
Propolis
Monocytes
Antioxidant activity
Antiallergic action

1. Introduction

Propolis is a mixture of components made by bees from different plant sources, including the bark and buds of trees, and contains flavonoids, phenylpropanoids, terpenes, stilbenes, lignans, coumarins, and their prenylated derivatives. Its chemical composition varies according to the geographical location and botanical origin (Burdock, 1998; Huang et al., 2014).

Propolis popular use comes from around 300 BC, used by ancient civilizations for different purposes, such as wound treatment, as a mouth disinfectant, for mummification of corpses, anti-eczema, anti-myalgia, and anti-rheumatism agent, among other applications (Silva-Carvalho et al., 2015). Research on propolis action has intensified over the last decades, demonstrating remarkable immunomodulatory, antimicrobial and antitumor properties (Sforcin, 2016). Due to its diverse biological activities, propolis may be used in food supplementation, cosmetics and in the pharmaceutical industry (Silva-Carvalho et al., 2015).

Oxidative stress is characterized by the excessive production of reactive oxygen species (ROS) and the inability of the natural antioxidants to control their generation. When an imbalance occurs between the oxidant and antioxidant systems, cellular damage and tissue injury can be observed, which contributes to the development of the inflammatory process (Pisoschi & Pop, 2015). Since oxidative stress and inflammation are present in several diseases, it is important to search for alternative treatments to minimize these effects and to promote human health. In recent years, research in natural products has grown due to their beneficial properties (Hussain et al., 2016) and less side effects compared to synthetic medication (Toreti et al., 2013).

Data from literature has shown the beneficial action of propolis in inflammatory diseases such as neuronal degenerative disease, diabetes, chronic kidney disease, skin disorders, among others (Silva-Carvalho et al., 2015). However, the mechanisms by which propolis performs these activities are not yet fully understood and are somewhat controversial.

Our group has been investigating the action of propolis on human monocytes, which are phagocytic cells quickly recruited to sites of injury or infection, mediating the initial defense against pathogens (Geissmann et al., 2008) and playing an important role in many inflammatory diseases. THP-1 cell is a human monocytic cell lineage with relatively similar response patterns to primary monocytes. This cell is widely used in *in vitro* assays, avoiding genetic variability between individuals (Chanput et al., 2014) and providing a better comprehension of propolis mechanisms of action.

In this work, we aimed at investigating the antioxidant potential of propolis and its ability to prevent the activation of inflammatory/oxidative pathways in THP-1 monocytes stimulated with the skin allergen 1-fluoro-2,4-dinitrobenzene (DNFB), responsible for a skin inflammatory condition, or the Toll-like receptor (TLR)-4 agonist lipopolysaccharide (LPS) responsible for a systemic pro-inflammatory response.

2. Material and Methods

2.1. Propolis sample

Propolis was produced in the Beekeeping Sector, UNESP, Botucatu. The plants visited by bees to produce propolis were identified and their vouchers specimens were stored at the Herbarium BOTU of the Institute of Biosciences, UNESP, obtaining the following registration numbers: *Araucaria angustifolia* (Bert.) O. Kuntze - BOTU 09866 - 18.03.98, *Baccharis dracunculifolia* DC - BOTU 09867 - 18.03.98 and *Eucalyptus citriodora* Hook - BOTU 04502 - 22.09.98. Plant names were checked at http://www.theplantlist.org.

Its chemical composition was previously analyzed by gas chromatography–mass spectrometry (GC-MS) and high performance liquid chromatography coupled to photodiode-array detector and interfaced with a electrospray ionization mass spectrometer (HPLC-PDA-ESI/MS^n). The analysis revealed that the main constituents or chemical groups are benzoic acid, dihydrocinnamic acid, 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C), *p*-coumaric acid, prenyl-*p*-coumaric acid,

caffeic acid, 1,3- and 4,5-dicaffeoylquinic acids, 3,4,5-tricaffeoylquinic acid, flavones (6,8 di-*C*-hexosyl apigenin, 6-*C*-pentosyl-8-*C*-hexosyl apigenin and 6-*C*-hexosyl-8-*C*-pentosyl apigenin), trihydroxymethoxy flavanone, tetrahydroxy flavanone and triterpenes (Búfalo et al., 2013; Conti et al., 2015).

Propolis was ground and ethanolic extracts were perfomed (30g of propolis/100 mL ethanol 70%), at room temperature, in the absence of light and moderate shaking. After a week, extracts were filtered and the dry weight was determined. Specific dilutions were prepared for each assay in RPMI 1640 culture medium (Sigma-Aldrich Chemical, USA) containing 18 mM sodium bicarbonate, 25 mM glucose, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich Chemical, USA). The extract was filtered using a Millipore membrane filter 0.22 µm pore size, 30 mm diameter (Kasvi, Brazil).

2.2. THP-1 cell culture

THP-1 human monocytic cell line (American Type Culture Collection TIB-202; InvivoGen, France) was cultured in RPMI 1640 medium supplemented with 10% SFB and 1% penicillin-streptomycin and incubated at 37 °C and 5% CO_2. Cells were maintained at $0.5x10^6$ to $1x10^6$ cells/mL and were sub-cultured every 2-3 days. All experiments were performed in duplicate with three independent assays.

2.3. Cell viability

THP-1 cells were cultured at 0.2 x 10^6 cell/well in a final volume of 200 µL. Cells were treated with propolis (1, 5, 10, 20 and 50 µg/mL) or 70% ethanol with the same proportion found in 50 µg/mL. After 20 h of incubation at 37 °C and 5% CO_2, resazurin (20 µL – final concentrarion 50 µM) was added and incubated in the same conditions for 4 h. Absorbance was determined at 570 and 600 nm using the spectrophotometer Synergy HT Multi Detection Microplate Reader (Biotek Instruments, USA).

2.4. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant capacity of propolis was determined by its ability to capture the free radical DPPH. Thus, 10 µL of propolis samples (5 to 50 µg/mL) were assessed by their reactivity with 50 µL of DPPH methanolic solution 500 µM (Sigma-Aldrich Chemical, USA), with 100 µL of acetate buffer 0.2 M (pH=6) and 140 µL of methanol (Merck, USA). The plate was incubated for 30 min at room temperature in dark conditions. The absorbances were read at 517 nm using the Multiskan FC spectrophotometer (Thermo Scientific, USA). Propolis effective concentration decreasing the absorbance of DPPH by 50% (EC50) was calculated from the calibration curve determined by linear regression.

2.5. Superoxide dismutase (SOD) 1 and 2 activity

THP-1 cells ($2x10^6$ cells/well, final volume 2 mL) were incubated with propolis (50 µg/mL) or its solvent for 30 min before LPS (1 µg/mL – Sigma-Aldrich Chemical, USA) stimulation. Cells were incubated at 37 °C and 5% CO_2 for 24h.

After, cells were centrifuged at 300g for 5 min at 4 °C and washed with cold PBS. Cells were lysed using 100 µL of RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 2 mM ethylenediaminetetraacetic acid) for 30 min on ice. After centrifugation at 12000g for 10 min at 4 °C, the supernatant containing the extracts was collected and protein concentration was determined by the bicinchoninic acid (BCA) method.

SOD total activity was determined by the colorimetric assay, using the SOD Determination Kit (Sigma-Aldrich Chemical, USA), according to the manufacturer's instructions. Briefly, 20 µL of each sample was mixed with 200 µL of the working solution containing WST-1 [2-(4-iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium, monosodium salt] and 20 µL of the working solution containing the

enzyme. To evaluate SOD 2 activity, 2 µL of the SOD 1 inhibitor – potassium cyanide (final concentration 2 mM) were added. The reaction was incubated for 20 min at 37 °C and the absorbance was performed at 470 nm using the Synergy HT Multi Detection Microplate Reader (Biotek Instruments, USA). SOD 1 was determined by the formula: SOD 1 = total SOD – SOD 2.

2.6. CD86 expression by flow cytometry

THP-1 cells ($0.8x10^6$ cells/well, final volume 1.5 mL) were incubated in the presence of propolis (50 µg/mL) or its solvent (ethanol 70%) for 30 min at 37 °C and 5% CO_2. Then, 1.5 µL of the allergen DNFB (8 mM – Sigma-Aldrich Chemical, USA) was added to the culture and the cells were incubated for 24 h.

The cells were washed twice at 300g/5 min and resuspended in 100 µL of phosphate buffered saline (PBS) supplemented with 1% FBS. Cells were incubated with 3 µL of monoclonal antibody anti-CD86-Alexa488 (Clone IT2.2, BioLegend) for 30 min at 4 °C. For each sample, 10.000 events were analyzed using a BD AccuriTM C6 flow cytometer (BD, USA). The data represents a percentage (%) of cells expressing the surface markers.

2.7. Western blot analysis

THP-1 cells ($2.4x10^6$ cells/well, final volume 3 mL) were incubated in the presence of propolis (50 µg/mL) or 70% ethanol for 30 min before LPS (1 µg/mL) stimulation. Cells were incubated at 37 °C and 5% CO_2 for 24h.

Cell lysates were obtained with 100 µL of RIPA lysis buffer supplemented with 1 mM dithiothreitol, protease and phosphatase inhibitor cocktails (Roche, Germany) for 30 min on ice. Cells were centrifuged at 12000 g for 10 min at 4 °C to remove cell debris. Protein concentration was determined by the BCA method. Subsequently, the lysates were denatured at 95 ºC for 5 min in a buffer containing 0.125 mM Tris pH 6.8, 2% (w/v) sodium dodecyl sulphate, 100 mM dithiothreitol, 10% glycerol and bromophenol blue.

Proteins were separated by 4-10% (v/v) SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Sweden). The membranes were incubated with the primary antibody for IL-1β (1:200; Santa Cruz Biotechnology, USA), heme oxygenase-1 (HMOX-1) (1:1000; Thermo Scientific, USA) and the control β-tubulin (1:20000; Sigma-Aldrich Chemical, USA) overnight at 4 ºC. The following day, the membranes were washed and incubated with secondary antibodies (1:20000; GE Healthcare, UK) for 1h at room temperature. The immunoreactive bands were visualized using the ECF substrate and the imaging system ThyphoonTM FLA 9000 (GE Healthcare, UK). The bands densitometry were quantified using the TotalLab TL120 software (Molecular Dynamics, Amersham Biosciences).

2.8. Statistical analysis

Data was analyzed by Graph Pad statistical software (Graph Pad Software, USA) using one-way ANOVA with Dunnett's post-hoc test. Results are presented as means ± standard deviation (SD) of 3 different assays, in duplicate. The differences were considered significant at $p < 0.05$.

3. Results

3.1. Cell Viability

In order to disclose the safe concentrations of propolis to be further exploitated in the subsequent biological assays, a dose-response curve was performed on THP-1 cells treated with propolis for 24 h. THP-1 cell viability was not affected after incubation with propolis concentrations (1 to 50 µg/mL) or its solvent 70% ethanol (Fig. 1), highlighting the safety profile of propolis to human cells.

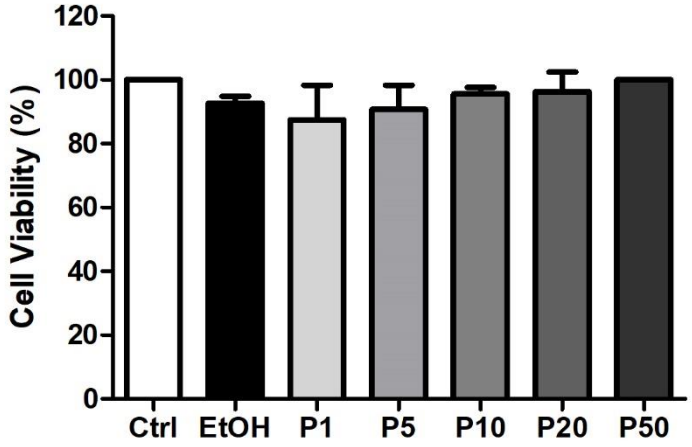


Fig. 1. Percentage (%) of THP-1 cells viability after incubation with culture medium (Ctrl), 70% ethanol (EtOH) or propolis (P – 1, 5, 10, 20 and 50 µg/mL) for 24 h. Data represent mean ± SD of 3 indepent assays in duplicate. $p > 0.05$.

3.2. Propolis antioxidant activity

Since many inflammatory pathologies are fuelled by oxidative stress, we further investigated whether propolis displays an antioxidant activity, using a cell free approach. As shown in Fig. 2, propolis presented a dose-dependent antioxidant activity and the effective concentration for reducing 50% of DPPH was 18.51978 µg/mL.

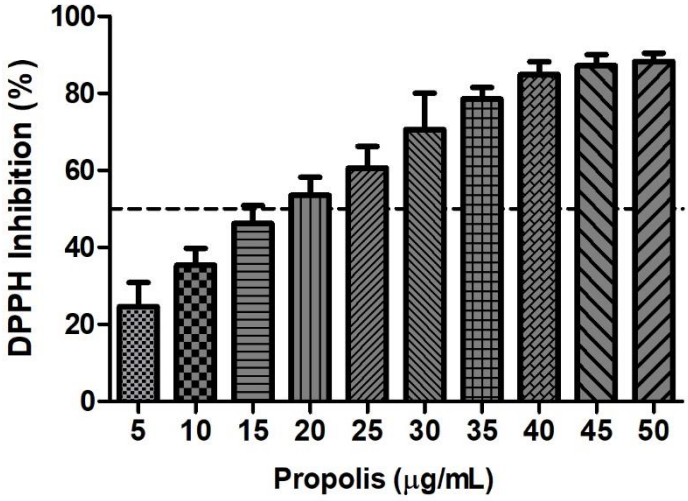


Fig. 2. DPPH inhibition (%) after incubation with different propolis concentrations (5-50 μg/mL) for 30 min. Data represent mean ± SD of 3 indepent assays in duplicate. The dashed line indicates the concentration of propolis that inhibited 50% of DPPH.

3.3. SOD 1 and SOD 2 activity and HMOX expression

Superoxide dismutases are present in eukaryotic cells as the main antioxidant defense systems against oxidative stress in the organism. Therefore, since propolis previously demonstrated an antioxidant activity in a cell-free system, we thought to investigate its action on a cell based-model, focusing on SOD activity in THP-1 cells. Interestingly, propolis alone induced cytoplasmatic SOD 1 activity. Regarding mitochondrial SOD 2, propolis did not affect its activity, while LPS stimulated it, concomitantly or not with propolis (Fig. 3).

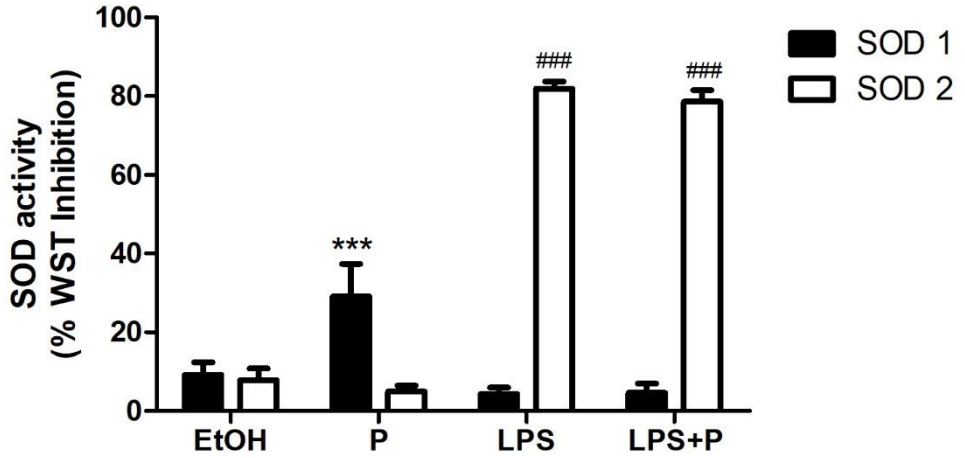


Fig. 3. SOD 1 and 2 activity (%WST inhibition) after THP-1 cells incubation with 70% ethanol (EtOH), propolis (P - 50 µg/mL), LPS (1 µg/mL) and LPS 1 µg/mL + propolis 50 µg/mL (LPS+P) for 24 h. Data represent mean ± SD of 3 indepent assays in duplicate. *** p<0.0001 different from EtOH (SOD 1); ### p<0.0001 different from EtOH (SOD 2).

Since HMOX-1 is upregulated during oxidative stress and it is critical in the response against oxidant-induced injury in many pathological conditions, we also investigated the effects of propolis on HMOX-1 expression in an attempt to furtherconfirm its potential in the management of oxidative stress-related pathologies. Although a slight expression of HMOX-1 was observed in propolis or LPS-treated cells, the levels were not statistically different from the control. Interestingly, in the presence of LPS, propolis significantly induced the expression of the antioxidant enzyme HMOX-1 (Fig. 4).

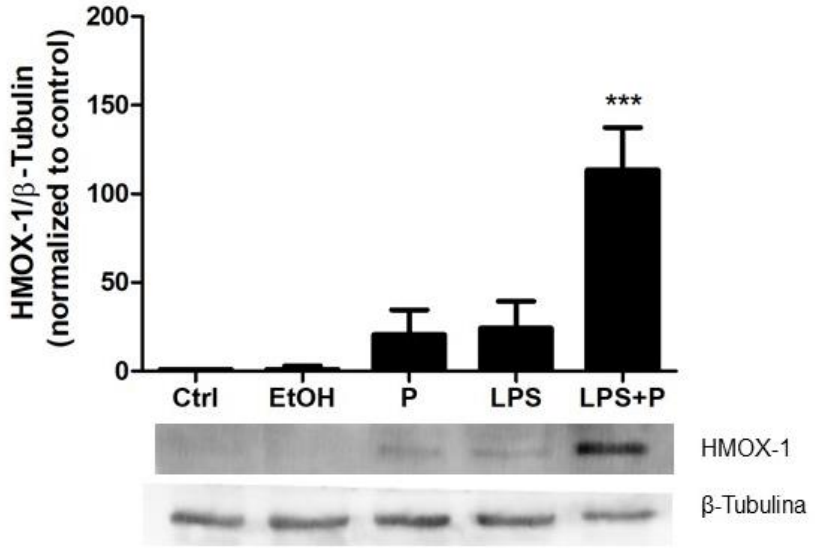


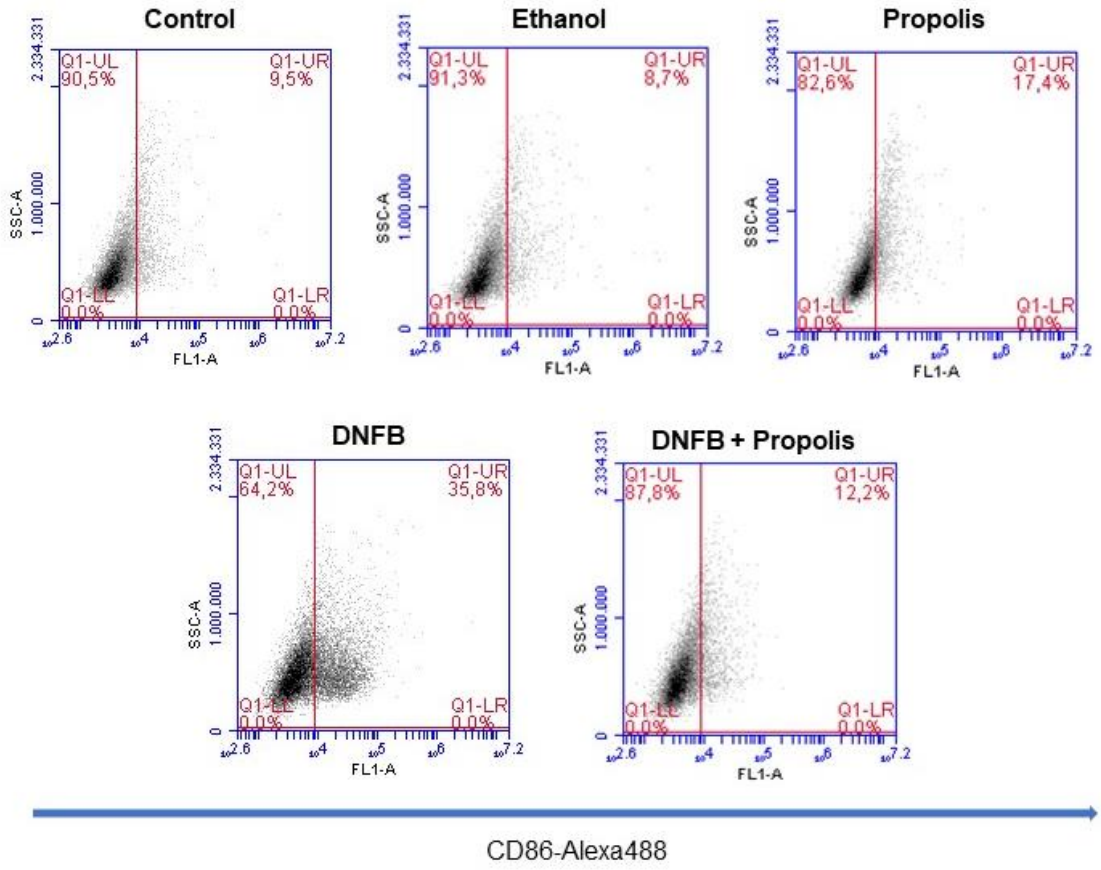
Fig. 4. HMOX production by THP-1 cells after incubation with culture medium (Ctrl), ethanol (EtOH), propolis (P - 50 µg/mL), LPS (1 µg/mL) and LPS 1 µg/mL + propolis 50 µg/mL (LPS+P) for 24 h. Data represent mean ± SD of 3 indepent assays and a representative blot. *** p<0.0001 different from control. HMOX-1 = 32 kDa; β-tubulin = 55 kDa.

3.4. Pro-inflammatory molecules: CD86 and pro-IL-1β expression

Since several pro-inflammatory events are evoked by oxidative stress we hypothesize that, besides its antioxidant properties, propolis could also mitigate the production of pro-inflammatory molecules in monocytes. Therefore, we evaluated the effect of propolis on THP-1 cells stimulated with two different pro-inflammatory stimuli, the skin allergen DNFB and the TLR-4 agonist LPS. DNFB causes allergic contact dermatitis, a Type IV [delayed-type] hypersensitivity response, characterized by excessive ROS production and inflammation and manifests as a local skin rash, itchiness, redness, swelling, and lesions. THP-1 cells are frequently used to detect skin allergens since they evoke the upregulation of cell surface co-stimulatory molecules expression, for instance CD86. Since this assay could be used to screen molecules with anti-allergic potential, we investigated whether propolis could mitigate the increase on CD86 expression triggered by the strong skin allergen DNFB. As expected, DNFB induced the expression of the co-stimulatory molecule CD86 and,

interestingly, propolis inhibited CD86 expression stimulated by the allergen (Fig. 5), highlighting its antiallergic potential.

A)



B)

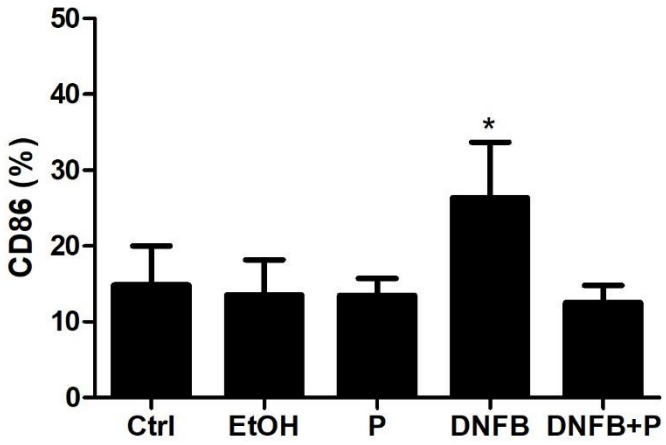


Fig. 5. CD86 expression (%) by THP-1 cells after incubation with culture medium (Ctrl), 70% ethanol (EtOH), propolis (P - 50 µg/mL), DNFB (8 µM) and DNFB 8 µM + propolis 50 µg/mL (DNFB + P) for 24h. **A)** Representative Dotplots. **B)** Data represent mean ± SD of 3 indepent assays carried out in duplicate. * p <0.05 compared to the control.

We further addressed the effect of propolis on THP-1 cells stimulated with LPS, specifically on the expression of the precursor form of IL-1β. Indeed, release of proinflammatory cytokines such as IL-1β by monocytes may be associated to an inflammatory response in the host. The results demonstrated that LPS induced the expression of IL-1β precursor form (pro-IL-1β) and propolis did not affect its action (Fig. 6).

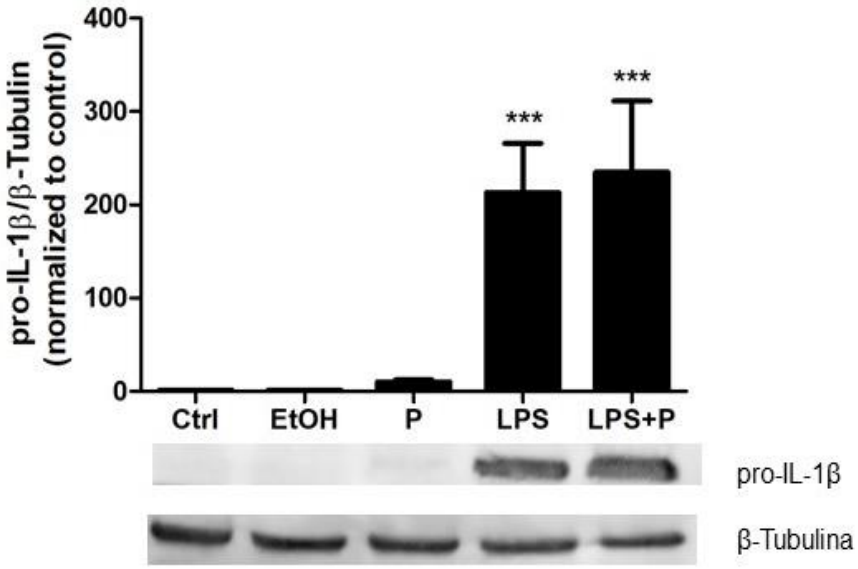


Fig. 6. Pro-IL-1β production by THP-1 cells after incubation with culture medium (Ctrl), ethanol (EtOH), propolis (P - 50 µg/mL), LPS (1 µg/mL) and LPS 1 µg/mL + propolis 50 µg/mL (LPS+P) for 24 h. Data represent mean ± SD of 3 indepent assays and a representative blot. *** p<0.0001 different from control. Pro-IL-1β = 31 kDa; β-tubulin = 55 kDa.

4. Discussion

Although human has used propolis for centuries, in the last years, propolis has gained attention due to its potential application in the pharmaceutical industry, motivating a better understanding of its antioxidant and anti-inflammatory activity.

As expected, our propolis sample and its solvent did not affect cell viability, what was also observed in human monocytes using concentrations up to 100 µg/mL (Búfalo et al., 2014). As none of the concentrations used in our assay affected cell

viability, the highest concentration of propolis (50 µg/mL) was used in the subsequent assays.

Propolis exerted a potent antioxidant action evidenced by the ability to reduce DPPH in low concentrations (EC50 = 18.51978 µg/mL); moreover, our findings were exactly the same as reported by Búfalo et al. (2013), who used the same propolis sample and obtained an EC50 = 18.51 µg/mL, demonstrating that the same propolis sample maintained its antioxidant action after being frozen for years. Data from literature revealed that other propolis samples may have significantly higher EC50, varying from 0.0700 to 0.9320 mg/mL (Duca et al., 2019). These findings confirm the importance of working with chemically characterized propolis samples, since different chemical compositions may be responsible for divergence in results (Sforcin, 2016).

Because of the remarkable antioxidant activity observed in this work using the DPPH test, a cell-free assay, we aimed to confirm the anti-oxidant effect of propolis in THP-1 cells, investigating different cell pathways. Pro-inflammatory stimuli, like LPS, can induce SOD 2 activity in an attempt to contain the generated oxidative stress (Ishihara et al., 2015), as observed in our results. Although there are several works correlating propolis to total SOD activity, little is known about its action in SOD 1 and 2 pathways. Interestingly, our reslts demonstrated that propolis increase SOD 1 activity relatively to the vehicle. Rats treated with propolis (100 mg/kg), followed by doxorubicin (DOX) treatment, presented higher SOD 1 and SOD 2 levels than the group treated only with DOX and similar levels to the control (Wided et al., 2014). An increased SOD 1 activity was observed in mice treated with propolis (250 mg/kg), which was not observed using the concentrations 100 and 500 mg/kg (Curti et al., 2019). Similarly, an increased SOD 1 activity was seen in glioma cells incubated with propolis at 250 and 500 µg/mL, but not with 100 µg/mL (Coskun et al., 2020). Thus, the potential of propolis to modulate the activity of SOD 1 and 2 enzymes may differ according to the models adopted in vitro and in vivo and to propolis concentrations.

We also analyzed the expression of HMOX-1 – a cytoprotective enzyme with a powerful antioxidant and anti-inflammatory action (Exner et al., 2004). Under normal conditions, HMOX-1 is expressed at low levels in most tissues; however, its activity is highly induced in response to oxidative stress, reducing ROS generation and controling the inflammatory response (Wu et al., 2011). Since propolis significantly

increased HMOX-1 expression in the presence of the inflammatory stimulus LPS, this may be one of the strategies by which propolis can exert its antioxidant activity. Our data agrees with those in the literature, supporting propolis modulatory activity in the HMOX-1 pathway. An extract of Brazilian propolis induced the expression of HMOX-1 in human skin fibroblast cell line submitted to oxidative stress by ultraviolet A irradiation, which was associated with accelerated nuclear factor erythroid 2-related factor 2 (Nrf2) translocation (Saito et al., 2015). Yuan et al. (2019) also observed that Brazilian green propolis induced the expression of HMOX-1 and Nrf2 in human umbilical vein endothelial cells exposed to vascular endothelial injury. Thus, under oxidative stress, treatment with propolis can be beneficial by overexpressing the HMOX-1 pathway.

Subsequently, we investigated whether the antioxidant action exerted by propolis could be beneficial in the presence of the allergen DNFB. Indeed, skin allergens induce ROS production, which accounts for the overexpression of co-stimulatory proteins, for instance CD86, involved in the development of allergic contact dermatitis, a skin inflammatory condition. Propolis was able to protect THP-1 cells from DNFB action by inhibiting CD86. As far as we know, this is the first work to report propolis action in DNFB-induced cell activation. Phenolic compounds have been recognized for their ability to prevent or reduce the progression of various skin disorders (Dzialo et al., 2016). The extract from the leaves of *Sapium sebiferum* (L.) Roxb., rich in phenolic compounds, reduced symptoms of DNFB dermatitis, which was related to the antioxidant capacity of this extract (Fu et al., 2015). The propolis sample used in our work contains several hydroxycinnamic acids and derivatives, widely believed to have an important antioxidant and anti-inflammatory potential, predominantly on the skin (Taofiq et al., 2017). Isolated compounds found in our propolis sample have been described for their ability to moderate DNFB-induced inflammation, such as caffeic acid (Jeon et al., 2015) and *p*-coumaric acid (Moon et al., in press). Since natural compounds have been considered promising in reducing skin disorders and our propolis inhibited one of the cell activation mechanisms induced by DNFB, our results open perspectives for research on its potential antiallergic action.

As expected, LPS induced pro-IL-1β expression in our assay, while the pretreatment with propolis had no effect. After pro-IL-1β processing, mature IL-1β is rapidly secreted by immune cells and plays a crucial role in the inflammatory response (Lopez-Castejon & Brough, 2011). Although its production is harmful in some circumstances, this cytokine is essential for the control and resolution of infections (Gabay et al., 2010).

Propolis was able to modulate IL-1β production under different conditions. Propolis inhibited IL-1β, TNF-α and IL-6 production by MG6 microglia submitted to hypoxia (Wu et al., 2013), inhibited IL-1β production by J774A.1 macrophages stimulated with LPS + IFN-γ (Szliszka et al., 2013), and reduced IL-1β secretion by murine bone marrow-derived macrophages stimulated with LPS + nigericin or *Legionella pneumophila* (Hori et al., 2013). Using the same propolis sample of the current work, Bachiega et al. (2012) reported an increased IL-1β production by murine peritoneal macrophages. Although in general propolis may exhibit an inhibitory effect on IL-1β production, this was not reported in our assay conditions.

On these bases, our data indicated that propolis has a potent antioxidant activity and may be beneficial in preventing skin allergy via CD86 inhibition, probably by inducing the activation of antioxidant pathways, such as SOD and HMOX-1. These findings open perspectives for the inclusion of propolis in the treatment of diseases and conditions that generate oxidative stress.

5. Conclusion

Propolis exerted an antioxidant activity, potentially involved in its anti-inflammatory/antiallergic action.

Author Contributions

FC, CP, MB, JS and MC designed the study. FC, AP, GB, IF, ACS, AIS and PM developed the protocols and performed the experiments. FC, MB, JS and MC structured and wrote the manuscript. All authors reviewed the manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

References

Bachiega, T.F., Orsatti, C.L., Pagliarone, A.C., Sforcin, J.M., 2012. The effects of propolis and its isolated compounds on cytokine production by murine macrophages. Phytother. Res. 26, 1308-1313.

Búfalo, M.C., Bordon-Graciani, A.P., Conti, B.J., Golim, M.A., Sforcin, J.M., 2014. The immunomodulatory effect of propolis on receptors expression, cytokine production and fungicidal activity of human monocytes. J. Pharm. Pharmacol. 66, 1497-1504.

Búfalo, M.C., Ferreira, I., Costa, G., Francisco, V., Liberal, J., Cruz, M.T., Lopes, M.C., Batista, M.T., Sforcin, J.M., 2013. Propolis and its constituent caffeic acid suppress LPS-stimulated proinflammatory response by blocking NF-κB and MAPK activation in macrophages. J. Ethnopharmacol. 149, 84-92.

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

Chanput, W., Mes, J.J., Wichers, H.J., 2014. THP-1 cell line: na in vitro cell model for immune modulation approach. Int. Immunopharmacol. 23, 37-45.

Conti, B.J., Bankova, V., Sforcin, J.M., 2015. Chemical composition of the same Brazilian propolis sample analysed in 1997 and in 2012: no freezing effect. Nat. Prod. Commun. 10, 1279-1280.

Coskun, Z.M., Ersoz, M., Gecili, M., Ozden, A., Acar, A., 2020. Cytotoxic and apoptotic effects of ethanolic propolis extract on C6 glioma cells. Environ. Toxicol. 35, 768-773.

Curti, V., Zaccaria, V., Tsetegho Sokeng, A.J., Dacrema, M., Masiello, I., Mascaro, A., D'Antona, G., Daglia, M., 2019. Bioavailability and in vivo antioxidant activity of a standardized polyphenol mixture extracted from brown propolis. Int. J. Mol. Sci. 20, 1250.

Duca, A., Sturza, A., Moacă, E.A., Negrea, M., Lalescu, V.D., Lungeanu, D., Dehelean, C.A., Muntean, D.M., Alexa, E., 2019. Identification of resveratrol as bioactive compound of propolis from western Romania and characterization of phenolic profile and antioxidant activity of ethanolic extracts. Molecules 24, 3368.

Dzialo, M., Mierziak, J., Korzun, U., Preisner, M., Szopa, J., Kulma, A., 2016. The potential of plant phenolics in prevention and therapy of skin disorders. Int. J. Mol. Sci. 17, 160.

Exner, M., Minar, E., Wagner, O., Schillinger, M., 2004. The role of heme oxygenase-1 promoter polymorphisms in human disease. Free Radic. Biol. Med. 37, 1097-1104.

Fu, R., Zhang, Y., Peng, T., Guo, Y., Chen, F., 2015. Phenolic composition and effects on allergic contact dermatitis of phenolic extracts *Sapium sebiferum* (L.) Roxb. Leaves. J. Ethnopharmacol. 162, 176-180.

Gabay, C., Lamacchia, C., Palmer, G., 2010. IL-1 pathways in inflammation and human diseases. Nat. Rev. Rheumatol. 6, 232-241.

Geissmann, F., Auffray, C., Palframan, R., Wirrig, C., Ciocca, A., Campisi, L., Narni-Mancinelli, E., Lauvau, G., 2008. Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. Immunol. Cell Biol. 86, 398-408.

Hori, J.I., Zamboni, D.S., Carrão, D.B., Goldman, G.H., Berretta, A.A., 2013. The inhibition of inflammasome by Brazilian propolis (EPP-AF). Evid. Based Complement. Alternat. Med. 2013, 418508.

Huang, S., Zhang, C.P., Wang, K., Li, G. Q., Hu, F.L., 2014. Recent advances in the chemical composition of propolis. Molecules 19, 19610-19632.

Hussain, T., Tan, B., Yin, Y., Blachier, F., Tossou, M.C., Rahu, N., 2016. Oxidative stress and inflammation: what polyphenols can do for us? Oxid. Med. Cell. Longev. 2016, 7432797.

Ishihara, Y., Takemoto, T., Itoh, K., Ishida, A., Yamazaki, T., 2015. Dual role of superoxide dismutase 2 induced in activated microglia: oxidative stress tolerance and convergence of inflammatory responses. J. Biol. Chem. 290, 22805-22817.

Jeon, Y.D., Kee, J.Y., Kim, D.S., Han, Y.H., Kim, S.H., Kim, S.J., Um, J.Y., Hong, S.H., 2015. Effects of *Ixeris dentata* water extract and caffeic acid on allergic inflammation in vivo and in vitro. BMC Complement. Altern. Med. 15, 196.

Lopez-Castejon, G., Brough, D., 2011. Understanding the mechanism of IL-1β secretion. Cytokine Growth Factor Rev. 22, 189-195.

Moon, P.D., Han, N.R., Lee, J.S., Kim, H.M., Jeong, H.J. *p*-coumaric acid, an active ingredient of *Panax ginseng*, ameliolates atopic dermatitis-like skin lesions through inhibition of thymic stromal lymphopoietin in mice. J. Ginseng Res., in press.

Pisoschi, A.M., Pop, A., 2015. The role of antioxidants in the chemistry of oxidative stress: A review. Eur. J. Med. Chem. 97, 55-74.

Saito, Y., Tsuruma, K., Ichihara, K., Shimazawa, M., Hara, H., 2015. Brazilian green propolis water extract up-regulates the early expression level of HO-1 and accelerates Nrf2 after UVA irradiation. BMC Complement. Altern. Med. 15, 421.

Sforcin, J.M., 2016. Biological Properties and Therapeutic Applications of Propolis. Phytother. Res. 30, 894-905.

Silva-Carvalho, R., Baltazar, F., Almeida-Aguiar, C., 2015. Propolis: a complex natural product with a plethora of biological activities that can be explored for drug development. Evid. Based Complement. Alternat. Med. 2015, 1-29.

Szliszka, E., Kucharska, A.Z., Sokół-Łętowska, A., Mertas, A., Czuba, Z.P., Król, W., 2013. Chemical composition and anti-inflammatory effect of ethanolic extract of Brazilian green propolis on activated J774A.1 macrophages. Evid. Based Complement. Alternat. Med. 2013, 976415.

Taofiq, O., González-Paramás, A.M., Barreiro, M.F., Ferreira, I.C.F.R., 2017. Hydroxycinnamic acids and their derivatives: cosmeceutical significance, challenges and future perspectives, a review. Molecules 22, 281.

Toreti, V.C., Sato, H.H., Pastore, G.M., Park, Y.K., 2013. Recent progress of propolis for its biological and chemical compositions and its botanical origin. Evid. Based Complement. Alternat. Med. 2013, 1-13.

Wided, K., Hassiba, R., Mesbah, L., 2014. Polyphenolic fraction of Algerian propolis reverses doxorubicin induced oxidative stress in liver cells and mitochondria. Pak. J. Pharm. Sci. 27, 1891-1897.

Wu, M.L., Ho, Y.C., Lin, C.Y., Yet, S.F., 2011. Heme oxygenase-1 in inflammation and cardiovascular disease. Am. J. Cardiovasc. Dis. 1, 150-158.

Wu, Z., Zhu, A., Takayama, F., Okada, R., Liu, Y., Harada, Y., Wu, S., Nakanishi, H., 2013. Brazilian green propolis suppresses the hypoxia-induced neuroinflammatory responses by inhibiting NF-κB activation in microglia. Oxid. Med. Cell. Longev. 2013, 906726.

Yuan, W., Chang, H., Liu, X., Wang, S., Liu, H., Xuan, H., 2019. Brazilian green propolis inhibits Ox-LDL-stimulated oxidative stress in human umbilical vein endothelial cells partly through PI3K/Akt/mTOR-mediated Nrf2/HO-1 pathway. Evid. Based Complement. Alternat. Med. 2019, 5789574.

Glossary:

BCA, bicinchoninic acid; CD, cluster of differentiation; DNFB, 1-fluoro-2,4-dinitrobenzene; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; EC, effective concentration; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate conjugate; GC-MS, gas chromatography–mass spectrometry; HCl, *hydrochloric acid; HEPES,* 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; HMOX-1, heme oxygenase-1; HPLC–PDA–ESI/MS^n, high performance liquid chromatography coupled to photodiode-array detector and interfaced with a electrospray ionization mass spectrometer; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; IFN-γ, *interferon gamma*; LPS, lipopolysaccharide; NaCL, *sodium chloride*; Nrf2, *nuclear factor-erythroid-2–related factor 2;* PBS, phosphate buffered saline; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; SDS-PAGE, *sodium dodecyl sulfate–polyacrylamide gel electrophoresis;* SOD, superoxide dismutase; TNF-α, tumor necrosis factor alpha; WST-1, [2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium.

Conclusões

O consumo de própolis durante 3 meses (500 mg/dia) por PVHA assintomáticas sob TARV promoveu:

▪ Aumento na atividade de CPK, considerado uma alteração sem importância clínica relevante. Visto que os pacientes não apresentaram mioglobinúria e sinais clínicos de rabdomiólise, a administração da própolis pode ser considerada segura e sem efeitos adversos;

▪ Aumento na concentração de magnésio, indicando que a própolis pode contribuir para a manutenção da homeostase do organismo, uma vez que esse íon está envolvido em várias reações metabólicas e é um importante regulador fisiológico e hormonal;

▪ Associação entre imunidade adequada (alta contagem de células T $CD4^+$) e menor estado inflamatório (altos níveis de IL-10), o que foi reforçado pela correlação inversa entre as citocinas anti-inflamatória IL-10 e a pró-inflamatória IFN-γ;

▪ Aumento na expressão de Foxp3, fator de transcrição característico de células T reguladoras, indicando que o consumo de própolis modula a resposta imune das PVHA, favorecendo um perfil anti-inflamatório;

▪ Aumento na proliferação de linfócitos T $CD4^+$, indicando que o consumo de própolis pode melhorar a resposta imunológica das PVHA sob TARV.

Considerações finais

Considerando a importância da utilização da TARV e o início da terapia cada vez mais precoce, procuramos nesse trabalho uma alternativa que minimizasse seus efeitos adversos e promovesse melhor qualidade de vida às PVHA. Nesse sentido, a própolis apresentou resultados interessantes e promissores, não acarretando efeitos colaterais e conferindo, consequentemente, segurança após sua ingestão.

A própolis aumentou a concentração de magnésio, a linfoproliferação e promoveu perfil anti-inflamatório em PVHA assintomáticas sob TARV. Esses achados não foram associados às características sociodemográficas e terapêuticas, tampouco às mudanças nos hábitos alimentares destes indivíduos.

Ademais, resultados adicionais obtidos com linhagem celular de monócitos humanos, em projeto paralelo, indicaram que a própolis exerce atividade antioxidante, potencialmente envolvida em sua ação anti-inflamatória e antialérgica, o que também pode ser uma contribuição para as PVHA.

Novos estudos são necessários para a adoção da própolis como intervenção efetiva para as PVHA, em especial àquelas com comorbidades ou falha terapêutica, não contempladas neste estudo.

ANEXO 1

UNESP -FACULDADE DE MEDICINA DE BOTUCATU

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Associação da própolis à terapia antirretroviral para indivíduos infectados pelo HIV: inovação no tratamento e possíveis benefícios em parâmetros imunovirológicos, inflamatórios e de estresse oxidativo.

Subprojeto 1: Avaliação de parâmetros imunológicos, inflamatórios e bioquímicos em pacientes HIV-positivos sob uso da terapia antirretroviral em associação com a própolis.
Conduzido por Fernanda Lopes Conte, orientado por José Maurício Sforcin, com obtenção de título de Doutora

Pesquisador: Karen Ingrid Tasca

Área Temática:

Versão: 4

CAAE: 58694816.6.0000.5411

Instituição Proponente: Departamento de Microbiologia e Imunologia

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.110.071

ANEXO 2

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Registrado no Ministério da Saúde em 30 de abril de 1997

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

RESOLUÇÃO 466/2012

CONVIDO o Senhor (a) para participar do Projeto de Pesquisa intitulado **"Avaliação de parâmetros imunológicos, inflamatórios e bioquímicos em pacientes HIV-positivos sob uso da terapia antirretroviral em associação com a própolis"**, que será desenvolvido por mim, Fernanda L. Conte, com a orientação do Prof. Dr. José M. Sforcin do Instituto de Biociências de Botucatu (IBB) e colaboração da Dra. Karen I. Tasca e das Profas. Drs. Lenice R. Souza e Camila R. Correa, da Faculdade de Medicina de Botucatu (FMB), Unesp.

Informo que o objetivo deste Projeto de Pesquisa é permitir que, no futuro, pessoas com a infecção pelo HIV e que estão sob terapia antirretroviral, possam ter melhorias em sua saúde/bem-estar utilizando a própolis, um produto natural conhecido por ter vários efeitos positivos para nossa saúde. Assim, nosso intuito é verificar se o uso da própolis seria capaz de diminuir alguns efeitos prejudiciais provocados pelos antirretrovirais, além de reduzir a inflamação e melhorar o perfil imunológico.

O estudo é composto por dois grupos que tomarão quatro comprimidos por dia, sendo que, um destes grupos receberá comprimidos contendo a própolis e, o outro grupo, receberá comprimidos sem esta substância, ou seja, que não apresentará qualquer efeito. A princípio, nem o (a) Senhor (a), nem a entrevistadora saberão qual tipo de comprimido estará sendo recebido. Durante o período do estudo, nós doaremos ao (a) Senhor (a) **os comprimidos para uso via oral (pela boca, com ajuda de água ou outro líquido), durante três meses** e, por isso, **contamos com seu compromisso / seriedade / responsabilidade na participação deste estudo, fazendo o uso correto das medicações**.

Para isso, utilizarei os dados coletados pela Dra. Karen I. Tasca, como peso, altura e medida do abdômen, os dados do prontuário médico, como colesterol, carga viral, entre outros, além dos dados da entrevista e do inquérito alimentar. Precisarei de **20 mL do seu sangue**, **em dois momentos** (hoje e após três meses do uso dos comprimidos), para realização dos testes.

Dessa forma, **serão aproveitados os 12 mL coletados** para a Dra. Karen I. Tasca, pois ela utilizará somente uma parte do sangue, chamada de plasma, e eu utilizarei as células, além disso

será coletado 8 mL adicionais, totalizando, assim, os 20 mL necessários. O risco com a coleta de sangue será apenas o possível aparecimento de uma mancha roxa após a picada da agulha, mas ela desaparecerá bem rapidamente. Após o processamento do sangue coletado, o material obtido será armazenado no laboratório em que trabalho, na Unesp, e utilizado para os testes somente desse trabalho.

Fique ciente de que sua participação neste estudo é voluntária e que mesmo após ter dado seu consentimento (aceite) para participar da pesquisa, o (a) Senhor (a) poderá retirá-lo a qualquer momento, sem qualquer prejuízo no seguimento do seu tratamento.

Este TCLE é elaborado em duas vias idênticas, o qual uma via será entregue ao (a) Senhor (a) devidamente assinada, e a outra via será arquivada e mantida pelos pesquisadores por um período de cinco anos após o término da pesquisa.

Qualquer dúvida adicional sobre os termos éticos da pesquisa, o (a) Senhor (a) poderá entrar em contrato com o Comitê de Ética em Pesquisa através dos telefones (14) 3880-1608 ou 3880-1609 que funciona de 2ª a 6ª feira das 8:00 as 11:30 e das 14:00 às 17:00 horas, na Chácara Butignolli s/nº em Rubião Júnior – Botucatu - São Paulo.

Após terem sido esclarecidas todas minhas dúvidas a respeito deste estudo, **CONCORDO EM PARTICIPAR de forma voluntária,** estando ciente que todos os meus dados estarão seguros através do **sigilo** que os pesquisadores se comprometeram. Estou ciente que os resultados desse estudo poderão ser publicados em revistas científicas, no entanto**, sem que minha identidade seja revelada**.

_____	_____	___/___/___
Nome do Sujeito do Estudo	Assinatura	Data

_____
Pesquisador

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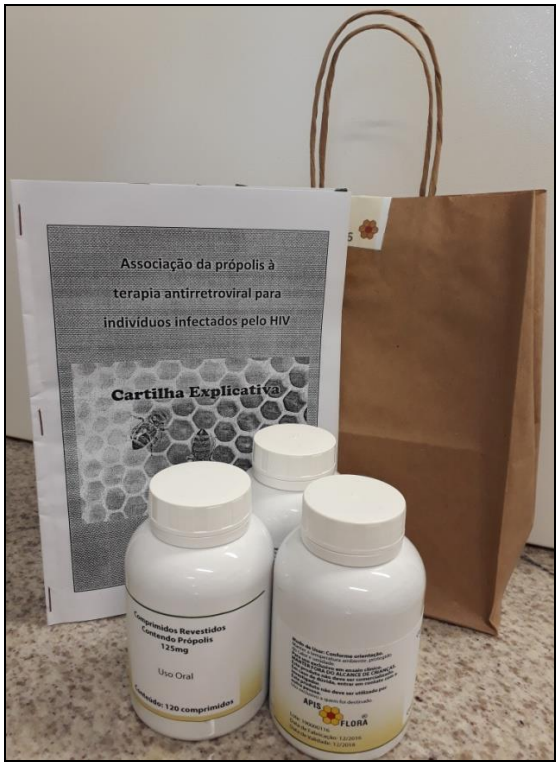
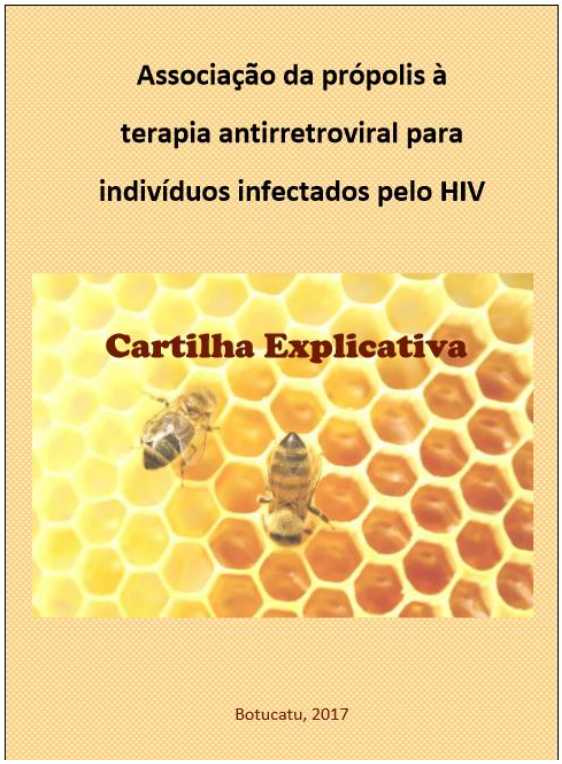
** favor rubricar a página anterior*

ANEXO 3

Kit contendo os frascos de comprimidos de própolis ou placebo e a cartilha explicativa.





Informações gerais

A terapia antirretroviral utilizada para o tratamento de pessoas que vivem com HIV/AIDS é responsável pela maior sobrevida desses indivíduos e reduz a transmissão do vírus.

No entanto, apesar de muitas vantagens dessa terapia, existem também alguns efeitos adversos, principalmente as alterações metabólicas e a toxicidade das células. Esses fatores podem levar ao desenvolvimento mais rápido de algumas doenças como diabetes, alterações do sistema nervoso central, rins e fígado, doenças cardíacas, câncer, entre outros.

Além disso, pessoas que vivem com HIV/AIDS apresentam o sistema imunológico sempre ativado e com maior inflamação, do que os indivíduos que não têm a infecção. Esses fatores também levam ao desenvolvimento dessas doenças citadas acima.

Importância do estudo

Assim, esse estudo foi desenvolvido com o objetivo de **diminuir o aparecimento de efeitos colaterais e melhorar a qualidade de vida** das pessoas que vivem com o HIV/AIDS e que fazem uso da terapia antirretroviral.

Para isso, serão utilizados comprimidos contendo extrato de **própolis**, desenvolvidos pela empresa *Apis Flora Indl. Coml. Ltda*. A própolis é um produto natural produzido pelas abelhas e tem vários benefícios comprovados. Provavelmente, a própolis também pode retardar a evolução da infecção pelo HIV e diminuir efeitos adversos da terapia antirretroviral. Além disso, os comprimidos contendo própolis foram bastante estudados, são eficazes e seguros, podendo ser utilizados sem causar efeitos colaterais.

Como tomar os comprimidos

▪ Tome 2 comprimidos a cada 12 horas (aproximadamente), totalizando **4 comprimidos por dia**. Tome todos os dias no mesmo horário, pra não esquecer. Por exemplo, 2 depois do café da manhã e 2 depois da janta, todos os dias.

▪ Os comprimidos podem ser tomados com qualquer tipo de líquido, **exceto bebidas alcoólicas**.

▪ Os comprimidos devem ser utilizados pelo período de 3 meses, sem interrupções (então, os 3 frascos que você levará embora serão utilizados, ou seja, 1 frasco durará 1 mês).

IMPORTANTE: a terapia antirretroviral não deve ser interrompida.

Sintomas:

Caso você sinta algum mal estar durante o período de tratamento, anote aqui a data, qual o sintoma, intensidade (fraco, moderado, forte, muito forte) e duração (em horas).

Dia: ____ Sintoma:_____ Intensidade:_____ Duração:_____

Dia: ____ Sintoma:_____ Intensidade:_____ Duração:_____

Dia: ____ Sintoma:_____ Intensidade:_____ Duração:_____

Dia: ____ Sintoma:_____ Intensidade:_____ Duração:_____

Em caso de esquecimento

▪ Caso você esqueça de tomar:

- os 2 primeiros comprimidos do dia, tome 4 de uma vez a noite.
- os 2 últimos comprimidos do dia, pule eles e anote na sua ficha. E, no dia seguinte, volte a tomar normalmente (4 por dia, de preferencia, de 2 em 2).
- os 4 comprimidos do dia, pule este dia e anote na sua ficha. No dia seguinte, volte a tomar normalmente (4 por dia, de preferencia, de 2 em 2).

Anote aqui os dias e horários de esquecimento:

Dia: _____ Horário: _____

Dia: _____ Horário: _____

Dia: _____ Horário: _____

Dia: _____ Horário: _____

Dia: _____ Horário: _____

Dia: _____ Horário: _____

Dia: _____ Horário: _____

Dia: _____ Horário: _____

Registro alimentar 3

Preencha aqui como foi a sua alimentação (horários, quais alimentos e o quanto comeu) durante o segundo mês de tratamento.

Dia da semana:	Domingo (___/___)	Terça (___/___)	Quinta (___/___)
Café da manhã Horário:	Alimentos/quantidade:	Alimentos/quantidade:	Alimentos/quantidade:
Lanche da manhã Horário:	Alimentos/quantidade:	Alimentos/quantidade:	Alimentos/quantidade:
Almoço Horário:	Alimentos/quantidade:	Alimentos/quantidade:	Alimentos/quantidade:
Lanche da tarde Horário:	Alimentos/quantidade:	Alimentos/quantidade:	Alimentos/quantidade:
Jantar Horário:	Alimentos/quantidade:	Alimentos/quantidade:	Alimentos/quantidade:
Ceia Horário:	Alimentos/quantidade:	Alimentos/quantidade:	Alimentos/quantidade:

Dados do voluntário da pesquisa

Número de identificação: _____

Data da primeira coleta de sangue: _____

Data de início dos comprimidos: _____

Data do primeiro registro alimentar: _____

Data do segundo registro alimentar: _____

Data do terceiro registro alimentar: _____

Data de finalização dos comprimidos: _____

Data da segunda coleta de sangue: _____

Outras anotações: