ERIKA IZUMI

“EFEITOS DO ÓLEO DE COPAÍBA E CONSTITUINTES EM *TRYPANOSOMA CRUZI*”

Londrina
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Tese apresentada ao Programa de Pós Graduação em Microbiologia, da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor em Microbiologia.

Orientador: Prof. Dr. Celso Vataru Nakamura
Co-Orientador: Prof. Dr. Valdir Florêncio da Veiga Júnior

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Dedico este trabalho a Deus, por tudo, e a minha família, que sempre esteve ao meu lado.

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“Não sei o que posso parecer para o mundo, para mim mesmo, porém, pareço ter sido somente como um menino que brinca à beira do mar, tendo me distraído em encontrar vez por outra um seixo mais liso ou mais bonito que o comum, enquanto o imenso oceano da verdade se estende à minha frente, inteiramente desconhecido.”

Isaac Newton

IZUMI, E**. Efeitos do óleo de copaíba e constituintes em *Trypanosoma cruzi***. 2010. 95 f. Tese (Doutorado em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2010.

RESUMO

A doença de Chagas é causada pelo protozoário *Trypanosoma cruzi* e afeta 15 milhões de pessoas na América Latina. Atualmente, o único fármaco disponível para tratamento é muito tóxico, com diversos efeitos colaterais, e apresenta baixa eficácia na fase crônica. Novas alternativas para o tratamento dessa doença podem surgir da associação de fármacos, supostamente mais eficaz e menos tóxica, e também dos produtos naturais. Substâncias de origem natural têm sido usadas em outras doenças com resultados promissores, tanto isoladas como em associação. O gênero *Copaifera* pertence à família Fabaceae, uma das maiores famílias em importância etnofarmacológica. A planta produz um óleo que exsuda do tronco, chamado de óleo de copaíba, muito utilizado nas regiões Norte e Centro-Oeste para fins medicinais. No presente trabalho foi avaliado a atividade tripanocida do óleo de copaíba e constituintes contra as formas epimastigota, tripomastigota e amastigota de *T. cruzi*, bem como seus efeitos citotóxicos em eritrócitos e células LLC-MK_2, na perda da integridade de membrana celular e mitocondrial do parasita, na capacidade de causar estresse oxidativo, alterações ultraestruturais e sinergismo. As espécies estudadas foram *C. reticulata*, *C. langsdorffii*, *C. paupera*, *C. martii*, *C. multijuga*, *C. officinalis*, *C. lucens* e *C. cearensis*. As substâncias estudadas foram o ácido copálico (1), ácido hidroxi-copálico (2), β-cariofileno (3), ácido agático (4), ácido caurenóico (5), ácido pinifólico (6), ácido poliáltico (7) e copalato de metila (8). Todos os óleos de copaíba e constituintes avaliados mostraram atividade contra *T. cruzi* em todos os estágios de vida. Para os óleos de copaíba, o IC_{50} em epimastigotas variou de aproximadamente 17 a 50 μg/ml, em tripomastigotas o EC_{50} foi de 90 a 280 μg/ml e nas formas amastigotas, houve redução de 50% na replicação intracelular em concentrações abaixo de 5 μg/ml para a maioria das espécies. Para as substâncias isoladas, a atividade de 50% sobre formas epimastigotas variou entre 13 e 53 μg/ml, de 120 a 525 μg/ml em tripomastigotas e em amastigotas intracelulares os valores de IC_{50} obtidos foram de 0,4 a 13 μg/ml. O óleo de *C. reticulata*, *C. martii*, *C. officinalis* e as substâncias 1 e 3, causaram peroxidação lipídica em *T. cruzi*. Alterações de permeabilidade da membrana celular e mitocondrial foram detectadas após tratamento com óleo de *C. martii*, *C. paupera*, *C. officinalis* e com a maioria das substâncias isoladas. A citotoxicidade foi maior para células de linhagem do que para eritrócitos humanos, tanto no tratamento com o óleo de copaíba como seus constituintes. A associação das substâncias 1 e 3 foi sinérgica em tripomastigotas, enquanto que 1 e 2, 2 e 3, e 1, 2 e 3 com benzonidazol foram aditivas. A sazonalidade influenciou na atividade tripanocida do óleo de copaíba de *C. multijuga* em formas epimastigotas. Alterações ultraestruturais no protozoário tratado com o óleo de copaíba e alguns constituintes mostram desorganização nuclear, "swelling" mitocondrial, formação de vacúolos membranosos, destacamento do flagelo e diminuição do volume celular.

Palavras-chave: *Trypanosoma cruzi*. *Copaifera*. Diterpeno. Sesquiterpeno. Sazonalidade. Sinergismo. Ultraestrutura.

IZUMI, E. **Effects of copaiba oil and its compounds against *Trypanosoma cruzi***. 2010. 95 f. Thesis (Doctarade in Microbiology) – Universidade Estadual de Londrina, Londrina, 2010.

ABSTRACT

Chagas' disease is caused by the protozoa parasite *Trypanosoma cruzi* and affects 15 million people in Latin America. The available drug is toxic, presents several side effects and presents low efficacy in the chronic phase. New alternatives for the treatment of this disease can be directioned to the combinatory use of drugs and to natural products. Compounds from natural origins have been evaluated against other diseases with promissory results, both isolated or in combination. *Copaifera* genus belongs to Fabaceae family and it is one of the most important families of plants on ethnopharmacology. *Copaifera* produces an oilresin that exsudates from the trunk, named copaiba oil, and it is very used by natives from North and West parts of the country for its medicinal properties. In the present work it was evaluated the trypanocidal activity of copaíba oils and their isolated compounds against all stages of *T. cruzi*, also their citotoxic effects against erythrocytes and $LLCMK_2$ cells, loss of mitochondrion and cell membranes integrity, ability to cause oxidative stress, ultrastructural alterations and synergism. Species studied were *C. reticulata*, *C. langsdorffii*, *C. paupera*, *C. martii*, *C. multijuga*, *C. officinalis*, *C. luces* and *C. cearensis*. Compounds tested were copalic acid (1), hidroxy-copalic acid (2), β-caryophylene (3), agathic acid (4), kaurenoic acid (5), pinifolic acid (6), polyaltic acid (7) e methyl copalate (8). All copaiba oils and compounds tested showed activity against all life forms of *T. cruzi*. For oleoresins, the IC_{50} in epimastigotes varied from 17 to 50 μg/ml, in trypomastigotes the EC_{50} was between 90 and 280 μg/ml, and against amastigotes, there was 50% of reduction on proliferation in concentrations below 5 μg/ml for the majority of species. For compounds, the activity of 50% on epimastigotes varied from 13 to 53 μg/ml, from 120 to 525 μg/ml in trypomastigotes, and against intracellular amastigotes the IC50 obtained varied from 0.4 to 13 μg/ml. Copaiba oils collected from *C. reticulata*, *C. martii*, *C. officinalis* and compounds 1 and 3, caused lipid peroxidation in *T. cruzi.* Alterations on potential of cell and mitochondrion membranes were detected after treatment with oils from *C. martii*, *C. paupera*, *C. officinalis* and with the majority of the isolated compounds. Citotoxicity was higher to line cells than to human erythrocytes, for both oils and compounds. Compounds 1 and 3 were synergic against trypomastigotes, while 1 and 2, 2 and 3, 1, 2 and 3 with benznidazole were additives. Sazonality influenciated the trypanocidal activity of copaíba oils against epimastigotes. Ultrastructural alterations of protozoan treated with some oils and compounds showed nuclear disorganization, mitochondrion swelling, membraneous vacuoles, flagellar detachment and reduction of cell volume.

Keywords: *Trypanosoma cruzi*. *Copaifera*. Diterpene. Sesquiterpene. Sazonality. Synergism. Ultrastruture.

LISTA DE FIGURAS

ANEXO A

ANEXO B

RESULTADOS COMPLEMENTARES

LISTA DE ABREVIATURAS E SIGLAS

PBS	Tampão Salina-fostato
pH	Potencial de Hidrogênio
ml	Mililitro
µl	Microlitro
µm	Micrômetro
mg	Miligrama
µg	Micrograma
M	Molar
mM	Milimolar
Rh123	Rodamina 123
IP	Iodeto de Propídeo
ºC	Graus Celsius
DMSO	Dimetilsulfóxido
MDA	Malondialdeído
TBA	Ácido Tiobarbitúrico
IC_{50}	Inibição de crescimento de 50%

SUMÁRIO

1 REVISÃO BIBLIOGRÁFICA

Doença de Chagas e *Trypanosoma cruzi*

A doença de Chagas, descoberta e descrita inicialmente por Carlos Chagas em 1909, é endêmica das Américas, principalmente América Latina, onde se estima que aproximadamente 15 milhões de pessoas estejam infectadas e quase 30 milhões vivam em áreas de risco (WHO, 2007). Embora a descoberta da doença tenha ocorrido há cerca de cem anos, estudos em paleoparasitologia revelam que a doença de Chagas já estava presente em populações sul-americanas há mais de nove mil anos (Araújo e cols., 2009).

As populações dos países em desenvolvimento sofrem com diversas doenças, negligenciadas pelas indústrias farmacêuticas, como malária, leishmaniose, tuberculose, doença de Chagas, esquistossomose, entre outras. Fatores sócio-econômicos desses países influenciam as indústrias farmacêuticas a determinarem o valor do investimento em pesquisa e desenvolvimento de novos fármacos, visto que a maior parte dos infectados vive com menos de US$ 2.00 por dia e não pode arcar com os custos do tratamento (Liese, Schubert, 2009).

A população afetada pela doença de Chagas é de um modo geral, de baixa renda, habitando regiões rurais ou comunidades isoladas, com pouco ou nenhum acesso a serviços de saúde e saneamento básico, de baixa escolaridade e uma parte significativa desta população desconhece a doença. Devido a esses fatores, a doença de Chagas é considerada negligenciada porque recebe pouco fianciamento para pesquisa, quando comparada a outras doenças, e o conhecimento produzido não é revertido em avanços terapêuticos, em grande parte por falta de interesse das indústrias farmacêuticas (Liese, Schubert, 2009).

A distribuição geográfica da doença se correlaciona com a distribuição de seus vetores e reservatórios, que se estendem desde o sul dos Estados Unidos até o sul da Argentina. Os vetores são insetos da ordem Hemiptera, família Reduviidae, subfamília Triatominae. Mais de 130 espécies, pertencentes a 3 gêneros – *Rhodnius*, *Panstrongylus* e *Triatoma*, já foram identificadas como transmissores da doença. Os insetos transmissores são popularmente conhecidos como bicho barbeiro, possuem hábito noturno, são hematófagos em todos os estágios de vida, tanto o macho quanto a fêmea, e podem viver até dois anos dependendo das condições ambientais (Lent e cols., 1979).

Os animais reservatórios da doença são mamíferos domésticos ou silvestres, dependendo do ciclo de transmissão da doença. No ciclo silvestre, os reservatórios incluem,

entre outros, tatus, tamanduás, preguiças, coelhos, roedores, marsupiais, morcegos, carnívoros e outros primatas. No ciclo doméstico da doença, os reservatórios são, em geral, cães, gatos e roedores presentes nas habitações humanas (Coura, Dias, 2009).

Além da transmissão vetorial da doença, também pode ocorrer a transmissão congênita, por transfusão sanguínea, via oral e também por acidentes médico-laboratoriais. Embora a transmissão vetorial ainda seja importante em várias regiões onde as condições de habitações não são adequadas, nos últimos anos tem aumentado os casos de transmissão por via oral, principalmente na região amazônica, em comunidades pouco instruídas sobre hábitos de higiene alimentar (Coura, 2006).

A doença de Chagas apresenta fase aguda e crônica. Na fase aguda observa-se o chagoma de inoculação, edema que surge no local de entrada do parasita. Outros sintomas desta fase também são comuns como febre, mal estar geral, dores abdominais, entre outros, que podem durar algumas semanas. Os sintomas iniciais são decorrentes da multiplicação e disseminação do parasita, fase caracterizada também pela alta parasitemia sanguínea.

A fase crônica, que pode persistir assintomaticamente na maioria dos indivíduos por um longo período, se manifesta através de alterações cardíacas, digestivas ou em menor proporção, envolvimento do sistema nervoso. Os sintomas dessa fase provêm da presença de ninhos de amastigotas, que levam a disfunção da célula infectada e causam reações inflamatórias locais, nos tecidos, resultando em inchaço e diminuição da função do órgão comprometido. Na fase crônica, em geral, não se observa a presença do protozoário na corrente sanguínea (Moncayo, Silveira, 2009).

O agente etiológico da doença de Chagas é o protozoário parasita *Trypanosoma cruzi*, uniflagelado, pertencente à ordem Kinetoplastida e família *Trypanosomatidae*. Apesar de ser eucarioto, o parasita apresenta algumas características peculiares, como a presença de uma única mitocôndria que se estende por toda a célula, e cujo material genético se concentra em uma porção específica da organela, chamada de cinetoplasto, característica marcante dos membros da ordem Kinetoplastida.

O parasita apresenta também outras características, entre elas a bolsa flagelar, de onde emerge o flagelo e é um local de intensa endocitose; as sete primeiras enzimas da via glicolítica estão compartimentalizadas em glicossomos e não ocorre livremente no citoplasma; reservas de origem proteica e lipídica podem ser encontradas em reservossomos, assim como algumas proteases; nos acidocalcissomos, quantidades significativas de íons, em especial cálcio e polifosfatos, são estocadas para auxiliar processos

celulares; o vacúolo contrátil, descoberto recentemente, exerce sua função na osmoregulação celular (Souza, 2009).

A reprodução, descrita para *T. cruzi* até o momento, é assexuada por fissão binária. Durante o processo de divisão celular a membrana nuclear não se desintegra e não ocorre condensação da cromatina. Este fator dificultou, por muito tempo, a identificação exata do número de cromossomos da espécie, atualmente reconhecido como sendo dez (Stuart e cols., 2008).

T. cruzi apresenta diferentes formas ao longo de seu ciclo de vida, destacando-se a forma epimastigota - replicativa extracelular, não infectiva - presente no tubo digestivo do inseto vetor; a forma tripomastigota - não replicativa, infectiva - presente nas porções finais do intestino do inseto e na corrente sanguínea de mamíferos infectados; e a forma amastigota - replicativa intracelular, infectiva - presente no interior das células infectadas.

O ciclo de vida de *T. cruzi* se passa em dois hospedeiros, invertebrado (triatomíneos) e vertebrado (mamífero), caracterizando-se como heteroxênico. O barbeiro ingere sangue de uma pessoa infectada e juntamente com ele as formas tripomastigotas. No intestino do inseto ocorre a diferenciação para as formas epimastigotas, que se multiplicam e migram para a porção final do intestino, onde se diferenciam novamente em formas tripomastigotas. O inseto, ao realizar o repasto sanguíneo, defeca imediatamente, depositando na pele do mamífero as excretas contendo as formas tripomastigotas. Ao coçar a região, o mamífero leva os tripomastigotas em direção ao próprio ferimento de repasto ou então até uma superfície mucosa, onde o parasita consegue penetrar. No hospedeiro ocorre a infecção de diversas células, tais como fibroblastos, macrófagos residentes, células musculares lisas e estriadas e neurônios. No interior destas células ocorre a diferenciação para amastigotas, que se multiplicam no citoplasma e novamente se diferenciam para tripomastigotas, ocasionando rompimento da célula hospedeira e liberação dos parasitas na corrente sanguínea. Estes podem infectar novas células ou serem ingeridos por outro inseto.

A população de *T. cruzi* não é homogênea. Ao longo do tempo, a espécie foi circulando por uma imensa variedade de espécies de vetores e mamíferos, formando ciclos domésticos e silvestres, o que levou a seleção natural de diversas cepas com virulência, resistência a fármacos e tropismo tissulares distintos. Tal heterogeneidade foi caracterizada molecularmente em dois grupos, TcI e TcII, esta última com 5 subdivisões, TcII a, b, c, d, e. TcI estaria mais relacionado ao ciclo silvestre e geograficamente mais presente na região amazônica, enquanto TcII está relacionado ao ciclo doméstico e circulante nos demais

territórios abaixo da amazônia legal (Zingales, 1999; Yeo e cols., 2005). Recentemente, a divisão em DTU (Discrete Typing Unity) 1 e 2, sendo este último também com 5 subdivisões, tem tentado relacionar os genótipos por marcadores moleculares e imunológicos comuns e contribuir para o esclarecimento da influência genética do parasita na patogênese da doença de Chagas (Brisse e cols., 2000, 2003).

Quimioterapia da doença de Chagas

No presente, existem apenas dois fármacos eficazes: benzonidazol e nifurtimox, ambos desenvolvidos nos anos 70 pelas indústrias farmacêuticas Roche e Bayer, respectivamente. O Laboratório Farmacêutico do Estado de Pernambuco (LAFEPE) é o atual responsável por produzir o benzonidazol, o único fármaco utilizado para o tratamento da doença de Chagas no Brasil, e cabe ao governo brasileiro sua distribuição para os outros países da América Latina.

O benzonidazol, embora não atenda os preceitos para um fármaco ideal de acordo com a Organização Mundial de Saúde, é mais eficaz na fase aguda da doença, curando cerca de 80% dos infectados. Entretanto, após o estabelecimento da fase crônica, sua eficácia é reduzida para cerca de 20%. Além da taxa variável de cura, o fármaco causa diversos efeitos colaterais que fazem muitos pacientes abandonarem o tratamento. Esta alta toxicidade com severos efeitos colaterais não é visualizado apenas no tratamento da doença de Chagas, mas está presente em geral, no tratamento de outras doenças causadas por protozoários parasitas, como *Plasmodium*, *Entamoeba*, entre outros..

Na busca de alternativas terapêuticas para uma série de doenças estão os produtos naturais, ou seja, a procura por substâncias de origem natural que podem oferecer novos mecanismos de ação e menor toxicidade. Os estudos bioprospectivos, utilizando substâncias da forma como existem na natureza, fornecem subsídios valiosos para identificação de tratamentos para utilização futura.

Em geral, na pesquisa de fármacos derivados de produtos naturais ou síntese, o esqueleto principal é mantido e é feito alterações químicas para melhorar a atividade. Um exemplo de sucesso foi a quinina, isolada das cascas de *Cinchona* sp., uma planta nativa da América do Sul. A quinina foi o primeiro medicamento com eficácia contra *Plasmodium*, causador da malária. Este produto natural sofreu, e ainda sofre, alterações químicas que permitem a molécula aumentar sua atividade e diminuir sua toxicidade, gerando derivados utilizados no tratamento da doença atualmente. Para cepas resistentes aos derivados

da quinina estão sendo testadas as artemisinas, substâncias também de origem natural, identificadas na planta chinesa *Artemisia annua*, com ótimos resultados (Schwikkard, Heerden, 2002).

Diversos estudos em produtos naturais aplicados a doença de Chagas tem sido realizados na última década, na expectativa de também detectar alguma substância de origem natural que seja efetiva. O alvo na prospecção de novos fármacos não são somente plantas, mas animais e micro-organismos, cujas substâncias produzidas podem gerar novas perspectivas no tratamento da doença. Prytzyk e cols. (2003) avaliaram o efeito de extratos de própolis em formas tripomastigotas e verificou efeito semelhante ao cristal violeta.

Extratos da espécie de coral *Macrorhynchia philippina*, assim como um esterol isolado de *Carijoa riisei*, foram testados para atividade em formas tripomastigotas de *T. cruzi*, por Reimão e cols. (2008), e apresentaram efeitos similares ao benzonidazol. Veiga-Santos e cols. (2010) relataram estudo em que outra substância de origem marinha, o sesquiterpeno elatol, isolado da alga *Laurencia dendroidea*, foi eficaz contra as formas tripomastigotas e amastigotas, com efeito tóxico de 50% abaixo de 2 μM para ambas as formas.

Luize e cols. (2006a) mostraram a atividade de lignanas isoladas de *Piper regnellii*, para as quais o IC_{50} variou de 7 a 8 μg/ml em epimastigotas, e a molécula mais ativa, o eupomatenoide-5, também mostrou-se efetivo para amastigotas sendo 5 μg/ml a concentração capaz de reduzir em 50% a multiplicação intracelular do parasita, após 48 h (Luize e cols., 2006b). A substância partenolídeo, isolado de *Tanacetum parthenium*, foi avaliado sobre formas replicativas do *T. cruzi*, mostrando alta toxicidade contra o parasita, com IC_{50} para ambas as formas abaixo de 1 μg/ml (Izumi e cols. 2008)

Ancistrotanzazina A e B, duas substâncias isoladas de *Ancistrocladus tanzaniensis* por Bringmann e cols. (2003), foram ativas contra amastigotas nas concentrações de 1,7 e 1,5 μg/ml. Senn e cols. (2007) conseguiram isolar a substância 8-hidroxiheptadeca-1-ene-4,6-diin-3-il etanoato da planta *Cussonia zimmermannii*, cujo IC_{50} em amastigotas intracelulares foi de 0,2 μg/ml após 96 h.

Outra forma possível de quimioterapia para doença de Chagas seria a associação de fármacos. Estudos de efeito sinérgico em *T. cruzi* têm sido realizados nos últimos anos, sendo uma abordagem mais recente para doenças parasitárias. Em geral, as substâncias mais estudadas combinadamente são sintéticas, na maioria das vezes em conjunto com o próprio benzonidazol, entre elas fármacos que afetam a via do ergosterol, como os

derivados azólicos utilizadas para infecções fúngicas, fármacos para arritimia, entre outras (Benaim e cols., 2006; Faundez e cols., 2008; Lopez-Muñoz e cols., 2000).

A utilização de substâncias de origem natural, para testes sinérgicos em protozoários parasitas, está ganhando espaço aos poucos. Na literatura encontram-se ainda poucos estudos de produtos naturais associados com fármacos sintéticos já conhecidos para *Plasmodium* e *Leishmania*. Em *T. cruzi*, um estudo realizado em 2006, por Hall e cols., avaliaram a associação de duas substâncias isoladas da planta *Solanum* sp., α-solasonine e α-solamargine, porém o efeito sinérgico não foi o mesmo encontrado para células tumorais e fúngicas, as quais já haviam sido relatadas.

Produtos naturais

Os produtos naturais compreendem uma fonte crescente de novos fármacos que, utilizados de maneira natural ou modificados sinteticamente, contribuem para o tratamento de muitas doenças. Estima-se que mais de 20% das substâncias químicas atuais disponíveis comercialmente têm origem natural ou são derivados de produtos naturais (Harvey, 2007).

Os produtos naturais são utilizados pelos seres humanos desde tempos remotos para seu benefício, seja como óleos perfumados para atrativos, temperos para alimentos ou chás para doenças. O uso de extratos de plantas para a cura de males da saúde foi o primeiro recurso médico disponível em tempos remotos e, ainda hoje, em comunidades isoladas em diversos continentes, é o principal meio de tratamento de doenças.

A sobrevivência é o fator essencial para as espécies persistirem no ambiente natural. Para conseguirem sobreviver, plantas, animais e micro-organismos tiveram que desenvolver mecanismos de competição e proteção, que permitiram aos melhor adaptados tanto se estabelecer no ambiente e se alimentar, quanto se proteger de predação e doenças. Esses mecanismos de competição e proteção, em muitos casos, estão relacionados às substâncias químicas que a espécie produz e que são responsáveis pelo efeito desejado.

Tais substâncias químicas presentes nas diferentes espécies são originárias do metabolismo secundário, ou seja, substâncias produzidas de modo a conferir características extras para melhor adaptação às condições ambientais, porém não são essenciais para o organismo. Atualmente existem mais de cem mil moléculas, derivadas do metabolismo secundário, conhecidas e já isoladas de plantas superiores. Além da função exercida na planta, também podem ser aplicadas industrialmente na área farmacêutica, agronômica, cosmética e

alimentícia. Estes metabólitos podem sofrer variações quanto a diversidade e quantidade, dependendo da espécie, disponibilidade de nutrientes e pressão ambiental (Goossens e cols., 2003).

As plantas apresentam variações de composição química, de suas partes estruturais e reprodutivas, ao longo de seu tempo de vida. Entretanto, essa variação química ocorre também devido a variações sazonais e ambientais que podem influenciar diretamente na atividade medicinal das plantas. Em geral, os relatos populares sobre a utilização de uma determinada planta medicinal já incluem algumas características sobre a melhor época de coleta e como preparar a planta para o uso.

Entre os produtos naturais, os de origem vegetal são os mais conhecidos e utilizados. As florestas, sempre presentes ao redor das comunidades, eram uma fonte imensa de remédios, cujos conhecimentos de utilização serviram para moldar profissões específicas de saúde, como curandeiros, que eram passadas de geração a geração. Apesar do costume de se tratar com ervas tenha diminuído devido ao avanço da medicina moderna e a redução das áreas florestais, nos países em desenvolvimento, grande parte da população ainda usa este recurso.

Os estudos em produtos naturais visam exatamente entender as razões biológicas para a produção de substâncias bioativas pelas diferentes espécies e consequentemente aplicá-las em benefício da humanidade, sendo assim, a importância não é somente a possibilidade de encontrar novos fármacos para curar doenças, mas também entender os mecanismos celulares que ocorrem na presença de tais substâncias (Pucheault, 2008).

Família Fabaceae e o gênero *Copaifera*

Fabaceae (Leguminosae) é a terceira maior família de angiospermas, com mais de 700 gêneros e quase 20 mil espécies descritas, desde pequenas ervas até árvores de grande porte (Lewis e cols., 2005). A família é dividida nas subfamílias Caesalpinoideae, Mimosoideae e Faboideae (Papilionoideae), sendo esta última a maior, com quase 500 gêneros e aproximadamente 14 mil espécies. A família possui representantes distribuídos ao redor do mundo em diferentes ecossistemas, altitudes e latitudes, porém, as subfamílias Caesalpinoideae e Mimosoideae ocorrem principalmente em regiões tropicais e subtropicais (Raven e cols., 2001).

Uma característica marcante da família Fabaceae é a simbiose em suas raízes com *Rhizobium*, o que contribui para a fixação do nitrogênio atmosférico. Por esta razão, as plantas desta família são frequentemente colonizadoras primárias em solos relativamente inférteis e são muito utilizadas em rotatividade na agricultura para manter a fertilidade do solo e alta produtividade das demais culturas (Raven e cols., 2001).

A importância desta família não se deve somente a fatores ecológicos pela grande quantidade e distribuição de suas espécies ou fixação de nitrogênio, mas também fatores econômicos. Diversas espécies são exploradas, pelo potencial madeireiro, na construção civil e fabricação de móveis. O legume, o fruto típico de Fabaceae, é um dos itens mais comumente encontrados nas refeições da maioria dos povos, como exemplo temos o feijão, ervilhas, lentilhas, soja, entre outros. Algumas plantas são usadas como ornamentais ou tem sua exploração voltada para área medicinal ou cosmética, principalmente pelos óleos produzidos. Devido a todos esses fatores, a família Fabaceae é a segunda em importância econômica, ficando atrás de Poaceae (Wojciechowski, 2004 ; Sprent, 2001).

A subfamília Caesalpinoideae compõe um dos grupos mais importantes em produção de resinas e um dos mais conhecidos componentes dos ecossistemas equatoriais da África e América do Sul (Lee, Langenheim, 1975). O gênero *Copaifera* pertence à família Fabaceae e subfamília Caesalpinoideae, presente nas regiões tropicais dos continentes Americano e Africano. Nas Américas, pode ser encontrado desde o México até o norte da Argentina, sendo que 16 espécies, das mais de 70 descritas, são encontradas somente no Brasil. As primeiras descrições referentes ao gênero foram realizadas ainda no século XVI, já que a planta era muito conhecida pelos indígenas.

No Brasil, embora o gênero possa ser encontrado em todo o território nacional, é encontrado mais frequentemente nas regiões Norte e Centro-Oeste. As espécies mais abundantes são *Copaifera officinalis*, *C. guianensis*, *C. reticulata*, *C. multijuga*, *C. confertiflora*, *C. langsdorffii*, *C. coriacea* e *C. cearensis*, encontradas na região amazônica e em alguns estados do nordeste, com exceção da espécie *C. langsdorffii* que se distribui por todo o país (Veiga Júnior, Pinto, 2002).

As árvores de copaíba, copaibeiras ou pau-d'óleo, como são conhecidas, podem atingir até quarenta metros de altura e quatro metros de largura, possuem crescimento lento e chegam a viver centenas de anos. O tronco tem coloração escura e a copa ampla e densa. A floração ocorre, em geral, entre outubro e julho, mas pode variar de acordo com a região e clima. As flores são pequenas e importantes na identificação das diferentes espécies, o que causa uma dificuldade na identificação botânica correta, já que algumas copaibeiras, em

determinadas condições ambientais, podem não florescer anualmente (Veiga Júnior, Pinto, 2002).

O óleo de copaíba, composição química e atividade biológica

A copaibeira produz um óleo-resina de odor forte e característico, com propriedades medicinais, chamado de óleo de copaíba. O óleo, que exsuda do tronco ferido, tem coloração que vai do amarelo-claro ao marrom-avermelhado, viscosidade e composição química variável e é muito utilizado pelos nativos em feridas, por ter características cicatrizantes e antiinflamatórias (Veiga Júnior, Pinto, 2002).

Algumas características fazem com que a produtividade de óleo das copaíbas varie. A quantidade produzida depende da espécie, das características genéticas da planta, do tipo de solo, da disponibilidade de água e da época do ano. Todos esses fatores influenciam diretamente na densidade, viscosidade, coloração e composição química (Leite, 2004).

O óleo de copaíba é muito comercializado em todo o país, em especial na região amazônica, tanto em feiras livres como em lojas especializadas em produtos naturais. Embora o óleo seja muito difundido na cultura popular, a identificação da espécie da qual ele foi coletado, na grande maioria das vezes, não é realizada. A maioria dos óleos disponíveis no mercado vem de espécies diferentes, árvores diferentes e muitos ainda sofrem adulteração com outros óleos comerciais.

A composição química do óleo é uma mistura variável de terpenos. Os terpenos, ou terpenóides, estão presentes em todas as plantas e compõem a maior classe de metabólitos secundários. Existem mais de 20.000 compostos terpenóides descritos de plantas, os quais exercem funções fisiológicas, metabólicas e estruturais importantes (Connolly, Hill, 1991). Uma única planta pode sintetizar muitos terpenos diferentes, em partes distintas do organismo e em épocas diferentes, para uma grande diversidade de propósitos ao longo de seu desenvolvimento. Dentre esses se encontram pigmentos fotossintéticos (fitol, carotenóides), hormônios (giberelinas, ácido abscísico), carreadores de elétrons (ubiquinona, plastoquinona), componentes de membrana (fitosteróis), além de antimicrobianos e toxinas contra outras plantas e herbívoros (McGarvey, Croteau, 1995).

O terpenóide mais simples é o hidrocarboneto isopreno (C_5H_8). Todos os terpenóides podem ser classificados de acordo com o seu número de unidades de isopreno. Eles são formados a partir de reações que envolvem ligações de várias subunidades, oxidação,

redução, isomerização, e outras transformações específicas de cada composto. As categorias mais bem conhecidas de terpenóides são os monoterpenóides (duas unidades de isopreno), os sesquiterpenóides (3 unidades) e os diterpenóides (quatro unidades) (Raven e cols., 2001).

No óleo de copaíba estão presentes sesquiterpenos e diterpenos, sendo o primeiro mais abundante e responsável pelo aroma típico do óleo de copaíba. Entre os sesquiterpenos mais relatados na literatura sobre copaíba estão o β-cariofileno, α-copaeno, β-humuleno, α-cubebeno, entre outros. Dos diterpenos, a maioria encontrada são ácidos diterpênicos, e entre os mais relatados podemos citar os ácidos copálico, hardwíckiico, covalênico e clorequínico. O ácido copálico é o diterpeno mais encontrado nos óleos estudados, podendo ser considerado uma marca química de copaíba (Veiga Júnior & Pinto, 2002).

Além das propriedades cicatrizantes e antinflamatórias, o óleo de copaíba tem sido estudado para outros fins medicinais, não somente o óleo como é extraído da árvore, mas também algumas substâncias químicas isoladas. Como bioinseticida, Silva e cols. (2003) demonstraram que o óleo de *C. reticulata* foi capaz de eliminar todos os estádios larvais do inseto *Culex quinquefasciatus*, transmissor da filariose, em concentrações entre 15-180 ppm por 48 h. Em outro estudo, Geris e cols. (2008) avaliaram quatro diterpenos isolados do óleo de *C. reticulata* contra estádios larvais de *Aedes aegypti*, transmissor da dengue e febre amarela urbana, e verificaram que o ácido 3-β-acetoxilabdano-8(17)-13-dien-15-óico teve efetividade de 50% com apenas 0,8 ppm, após 48 h.

Em um estudo de Paiva e cols. (1998), o óleo de *C. langsdorffii* mostrou-se protetor da mucosa gástrica de ratos, prevenindo a ulceração induzida quimicamente, em doses diárias variáveis de 200-400 mg/kg. Isolado do óleo desta mesma espécie de copaíba, por Cavalcanti e cols. (2006), o ácido caurenóico foi testado quanto o seu potencial genotóxico e os pesquisadores mostraram que a concentração de 60 μg/ml, por 2 h, foi suficiente para levar a fragmentação de DNA em fibroblastos de hamsters, visualizada através da técnica do cometa e formação de micronúcleos.

Alguns trabalhos comprovam *in vivo* o efeito antinflamatório já creditado ao óleo de copaíba. Veiga Júnior e cols. (2007) estudaram os óleos de três espécies de copaíbas – *C. multijuga, C. cearensis, C. reticulata* – quanto ao potencial antinflamatório e observaram que 5 μg/ml dos óleos de *C. multijuga* e *C. cearensis* foram capazes de inibir parcialmente a produção de óxido nítrico por macrófagos murinos e todos os óleos reduziram a pleurisia induzida por zimosano, sendo o óleo de *C. multijuga* o mais eficaz. Em outra pesquisa mais

recente, Gomes e cols. (2010) confirmaram o efeito analgésico e antinflamatório de frações do óleo de *C. multijuga* em camundongos.

Santos e cols. (2008a) estudaram o efeito antimicrobiano do óleo de várias espécies de copaíba – *C. reticulata, C. multijuga, C. martii, C. langsdorffii, C. cearensis, C. officinalis, C. lucens, C. paupera*. Neste estudo, somente os óleos de *C. reticulata*, *C. martii* e *C. officinalis* foram efetivos contra bactérias Gram-positivas e nenhuma espécie inibiu o crescimento de Gram-negativas. No teste antifúngico, *C. paupera* e *C. lucens* mostraram atividade moderada contra *Tricophyton rubrum* e *Microsporum canis*, enquanto que *C. multijuga*, *C. cearensis* e *C. langsdorffii* inibiram parcialmente somente *T. rubrum*. Nenhum óleo mostrou atividade contra fungos leveduriformes.

A atividade antiparasitária do óleo de diferentes espécies de copaíba foi avaliada por Santos e cols. (2008b), em que promastigotas de *L. amazonensis* tiveram o crescimento inibido em 50% por concentrações abaixo de 22 μg/ml, sendo os mais efetivos os óleos coletados de *C. reticulata*, *C. paupera* e *C. multijuga*, cujos IC_{50} foram de 5, 11 e 10 μg/ml, respectivamente, após 72 h.

Considerando as variações do próprio óleo de copaíba e também sua coleta indiscriminada, um estudo mais abrangente sobre suas propriedades medicinais faz-se necessário. Ainda não há na literatura estudos medicinais a respeito do óleo de copaíba na doença de Chagas, muito menos estudos comparativos entre várias espécies, suas substâncias e avaliação sazonal.

No presente trabalho, investigamos a atividade do óleo de copaíba de diferentes espécies em *T. cruzi*, bem como a alteração de atividade devido à sazonalidade. A atividade de algumas substâncias presentes no óleo e seu possível efeito sinérgico também foi avaliada. Uma revisão sobre produtos naturais na doença de Chagas também foi feita para auxiliar na compreensão de como outras substâncias de origem natural podem ser melhor utilizadas para buscar tratamento para esta enfermidade.

2 OBJETIVOS

- Mostrar o efeito dos óleos de copaíba coletado de diferentes espécies vegetais e substâncias isoladas nas formas epimastigotas, tripomastigotas e amastigotas de *T. cruzi*;
- Quantificar a toxicidade dos óleos de diferentes espécies de copaiberias e substâncias isoladas em células de linhagem e hemáceas de humanos;
- Avaliar o efeito do óleo de algumas espécies de copaiba e substâncias isoladas na permeabilidade da membrana celular e mitocondrial de *T. cruzi*;
- Observar o efeito do óleo de algumas espécies de copaíba e substâncias isoladas na peroxidação lipídica em *T. cruzi*;
- Isolar substâncias majoritárias de *C. multijuga* e *C. officinalis*;
- Avaliar o efeito do óleo de *C. multijuga*, coletado em diferentes épocas sazonais, em formas epimastigotas;
- Visualizar as alterações morfológicas e ultraestruturais de *T. cruzi* causadas por amostras de óleo de copaíba e substâncias isoladas, nas formas epimastigotas, tripomastigotas e amastigotas;
- Estudar o efeito combinatório de algumas substâncias isoladas em *T. cruzi*, entre si e com benzonidazol, em formas tripomastigotas.

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ANEXOS

ANEXO A – ARTIGO

***TRYPANOSOMA CRUZI*:** A COMPARATIVE STUDY OF ACTIVITY OF DIFFERENT *COPAIFERA* SP. OLEORESINS

Erika Izumi; Tânia Ueda-Nakamura; Valdir F. Veiga-Júnior; Benedito P. Dias-Filho; Celso V. Nakamura.

***TRYPANOSOMA CRUZI*: A COMPARATIVE STUDY OF ACTIVITY OF DIFFERENT *COPAIFERA* SP. OLEORESINS**

Erika Izumi[1]; Tânia Ueda-Nakamura[2]; Valdir F. Veiga-Júnior[3]; Benedito P. Dias-Filho[1,2]; Celso V. Nakamura[1,2].

Abstract

The genus *Copaifera* is present in the Amazon region and it produces an oleoresin, which is used by natives due to medicinal properties. Its use is done with no discrimination of species, and until today no comparative study of parasitic activity of copaiba oils from different species has been published against *Trypanosoma cruzi*. Copaiba oils from *C. reticulata*, *C. multijuga*, *C. martii*, *C. cearensis*, *C. langsdorffii*, *C. paupera*, *C. officinalis* and *C. lucens* were evaluated for activity against all lifestages of *T. cruzi*, citotoxic effect on mammalian cells, alterations in mithocondrion and cell membranes permeability, lipid peroxidation and ultrastructural damages. All copaiba oils had effect on all parasite life stages, in special against replicative forms. *C. martii* and *C. officinalis* showed the best activity promoting the total elimination of intracellular amastigotes with less than 10 μg/ml. Copaiba oils present moderate citotoxicity on cell line monolayers but low citotoxicity over erythrocytes. *C. martii* and *C. officinalis* oleoresins were able to cause lipid peroxidation. Oleoresins from *C. martii*, *C. paupera* and *C. officinalis* increased cell membrane permeability and caused mitochondrion potential alteration. Ultrastructural changes could be visualized after treatment of the epimastigote and intracellular amastigote forms of the parasite, where detachment of cell membrane, swelling of the kinetoplast, disruption of organelles membrane and condensation of the chromatin were observed. Medicinal potential differs among oleoresins from different copaiba species, what can influence on efficiency of the treatment, mainly in populations that are far from health assistance.

Keywords: Fabaceae. *Copaifera*. Oleoresin. *Trypanosoma cruzi*. Copaiba oil. Antiprotozoal activity.

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1 INTRODUCTION

Natural products are a growing source of new drugs that, used in its natural or synthetically modified form, contribute to the treatment of many diseases. It is estimated that more than 20% of the new chemicals available recently have natural origins or they are derived from natural products (Harvey, 2007). The importance of such natural products is not only to cure diseases but it to help the understanding of cellular mechanisms that occur in their presence (Pucheault, 2008).

American trypanosomiasis, caused by the protozoan *Trypanosoma cruzi*, is a neglected disease since its coverage is the developing countries of Latin America, where many social problems persist. International Amazon region, which includes part of the territory of nine countries, has low demographical density and many rural communities are far from health assistance (Aguilar et al., 2007). Besides the pre-existent Chagas disease in the local population, it has occurred non-vectorial transmissions like oral infections due to poor food hygiene (Coura, 2006).

Chagas disease presents symptoms in the beginning of infection in only 10% of the infected people, and its symptoms can be mistaken with other diseases. This fact makes it difficult to identify plants used to treat it since infected people do not know about their condition. Thus many plants studied against *T. cruzi* are the ones that had effect against other protozoa in laboratory assays or they are used by natives to treat others protozoa infections such as leishmaniasis, sleeping sickness and malaria (Gachet et al., 2010; Abdel-Sattar et al., 2010).

The genus *Copaifera* belongs to *Fabaceae* (Leguminosae) family and it is represented by many species in the American and African continent. Copaiba trees can get forty meters tall, live hundreds of years and their oleoresin exsudated from the trunk are known for having a remarkable scent. For these reasons, the wood is used for construction and copaiba oils for industrial purposes, as cosmetic.

Copaifera sp. botanical identification is difficult due to the similarity among the species, which can be differentiated mainly by characteristics of the flowers and fruits (Veiga Júnior & Pinto, 2002; Martins-Silva et al., 2008). The oleoresin has medicinal properties and it is largely commercialized by natives of the Amazon region who use it to treat several infectious and inflammatory disorders which include blenorragy, cystitis, bronquitis, pneumonia, psoriasis, tetanus, herpes and as antiseptic and cicatrizing. The collect of the oleoresin is made by native population with no discrimination of species and most of

times the flasks of the commercialized copaiba oils contain oleoresins collected from different individuals (Veiga Júnior & Pinto, 2002).

In the last decades many studies have been done with different *Copaifera* species to investigate the medicinal activity of its oleoresins. Gomes et al. (2006) described the antinociceptive activity of the oleoresins obtained from *C. multijuga* Hayne and *C. reticulata* Ducke. For *C. langsdorffii*, Paiva et al. (1998) demonstrated that it was able to protect gastric mucosa from damage and prevent ulceration. A comparative antimicrobial activity of oleoresins from *Copaifera* species was investigated by Santos et al. (2008a) in which it was demonstrated the susceptibility of some Gram-positive bacteria and dermatophyte fungi to some copaiba oils. Antiprotozoal activity against *Leishmania amazonensis* was also investigated by Santos et al. (2008b), indicating an antileishmanial property of oleoresins collected from different species.

The aim of this work was to evaluate the activity of copaiba oils collected from different species of *Copaifera* against all life stages of *T. cruzi* and determine the toxicity over mammalian cells. This is the first comparative study of biological activity of species from the genus *Copaifera*, a medicinal genus of plants, against *T. cruzi*.

2 MATERIALS AND METHODS

2.1 OLEORESINS

It was evaluated the activity of oleoresins from eight different *Copaifera* species. *C. reticulata* and *C. martii* (colected in Pará State), *C. langsdorfii* (São Paulo), *C. paupera* (Acre), *C. cearensis* (Minas Gerais), *C. multijuga* (Amazonas), *C. officinalis* (Rondônia), *C. lucens* (Rio de Janeiro), all collected, identified and cataloged as described by Santos et al. (2008a). All copaiba oils were solubilized in dimethylsulfoxide and then diluted with appropriate medium to the concentrations used on the experiments, since the final concentration of DMSO did not exceed 1%.

2.2 PARASITES AND HOST CELLS

Trypanosoma cruzi (Y strain - TcII) was cultivated at 28 °C in log-phase culture every 96 h (epimastigotes) in Liver Infusion Tryptose medium - LIT (Camargo, 1964) supplemented with 10% of fetal bovine serum - FBS (GIBCO Invitrogen). Cell line $LLCMK_2$

was used as host cells to obtain trypomastigotes, from harvesting the supernatant, every 120 h in Dulbecco's Modified Eagle Medium - DMEM (GIBCO Invitrogen) at 37 °C and 5% CO_2 atmosphere.

2.3 Epimastigotes Growth Inhibition Assay

Epimastigotes from log-phase culture were inoculated at $1x10^6$ parasites/ml, in 24-well plates, in LIT medium supplemented with 10% of FBS. Different concentrations of copaiba oils (10 to 100 μg/ml) were added to the wells and the plates were incubated for 96 h at 28 °C. Parasite population was estimated by counting using a Neubauer hemocytometer chamber and the inhibitory concentration of 50% (IC_{50}) was determined.

2.4 Trypomastigotes Toxicity Assay

Trypomastigotes were harvested from the supernatant of infected monolayers of $LLCMK_2$ cell line as previously described. In 96-well plates, trypomastigotes were inoculated at $1x10^7$ parasites/ml in DMEM medium containing copaiba oils (50 to 500 μg/ml) together with 20% of FBS and 10% of mice blood (male Balb/C strain). The plate was then incubated for 24 h at 37 °C and 5% CO_2. The effective concentration of 50% (EC_{50}) was determined by counting the living parasites by Brener method (Brener, 1962).

2.5 Amastigotes Survival Assay

The activity of the oils on intracellular amastigotes was determined by treating infected cells. In 24-well plates, $3x10^5$ cells/ml ($LLCMK_2$) were inoculated on round glass coverslips in a manner to form a confluent monolayer after 24 h of incubation at 37 °C and 5% CO_2, in DMEM medium supplemented with 10% of FBS. After obtaining the monolayer, it was added $1x10^7$ trypomastigotes/ml to the wells and it was incubated in the same conditions before. The wells were then washed to remove the parasites remaining in the supernatant and 5 and 10 μg/ml of the oils were added, followed by the incubation of the plate for 96 h. The infected monolayers were submitted to fixation with methanol and Giemsa staining. Amastigotes survival was calculated by counting 200 cells and following the equation: survival index = n° infected cells x n° amastigotes, and then the percentage of survival was determined.

2.6 CYTOTOXICITY

LLCMK$_2$ Cells were inoculated to form confluent monolayers in 96-well plates. Different concentrations of the oils (1 to 1000 μg/ml) were added to the wells in DMEM medium and the plate was incubated for 96 h at 37 °C and 5% CO_2. After the incubation time, the cells were fixed with 10% trichloroacetic acid (Synth) for 1 h and then the wells were washed. 0.4% sulforhodamine-B (Sigma-Aldrich, Chemical Co., St. Louis, Mo, USA) was added to the wells and incubated for 30 min. The wells were washed again with 1% acetic acid to remove the dye excess and an aliquot of 150 μl of 10 mM Tris buffer (Invitrogen) were added to each well, homogenized for 15 min, and the reading of the absorbance was performed at 530 nm. The cytotoxic concentration of 50% (CC_{50}) was determined comparing the treated monolayers to control.

2.7 HEMOLYSIS

Human blood (type A^+) was collected, from a voluntary healthy donor, defibrinated and washed in isotonic dextrose saline solution. A suspension of 3% of erythrocytes was prepared in 96-well plates with concentrations ranging from 5 to 500 μg/ml of copaiba oils. The plates were incubated for 3 h at 37 °C. Triton X-100 1% was used as positive control (PC) and isotonic dextrose saline as negative control (NC). After incubation the plates were centrifuged at low rotation and the supernatant had its absorbance determined at 550 nm, as described by Reimão et al. (2008) with modifications. The hemolysis percentage was obtained by the equation:

% hemolysis = (sample absorbance – NC) x 100 / PC.

2.8 LIPID PEROXIDATION

Epimastigote cultures, maintained in LIT medium in exponential phase, were treated with 100 μg/ml of copaiba oils for 6 h, at 28 °C. After the treatment, cells were washed with phosphate buffer, homogenized and added to a solution of 0.37% thiobarbituric acid in 15% trichloroacetic acid and 0.25 N HCl. Samples were submitted to heat at 95 °C for 45 min, followed by cooling and centrifugation for 5 min at 2000 *g*. Supernatant was

homogenized with butanol (1:1), centrifuged for separation, and it was read at 532 nm. Lipoperoxidation was determined as the amount of thiobarbituric acid reactive substances (TBARS) in terms of malondialdehyde (MDA), expressed in MDA nmol x protein mg^{-1}.

2.9 Flow Cytometry

Epimastigotes were treated with 100 μg/ml of copaiba oils from *C. martii*, *C. paupera* and *C. officinalis*, for 3 h at 28 °C. After incubation, parasites were analyzed to verify a possible alteration in cell membrane integrity and mitochondrion membrane potential. Treated parasites were washed in PBS and incubated with 0.2 μg/ml of propidium iodide (PI – Sigma Aldrich) for 10 min and 5 μg/ml of rhodamine 123 (Rh123 – Sigma Aldrich)) for 15 min, followed by another cell wash. Samples had 10,000 events analysed each, using a FACSCalibur flow cytometer (Becton-Dickinson).

2.10 Transmission Electron Microscopy

Replicative forms were prepared for transmission electron microscopy to investigate the ultrastructural alterations caused by copaiba oils. For epimastigotes, a log-phase culture was treated during 96 h with IC_{50} of *C. reticulata* and for intracellular amastigotes, infected cell monolayers were treated with 5 μg/ml of the oils from *C. martii*, *C. paupera* and *C. officinalis* for 96 h. Samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Pos fixation was done in 1% osmium tetroxide and 0.8% potassium ferrocyanide in cacodylate buffer. In the same buffer the parasites were washed and then dehydrated in growing concentrations of acetone (50, 80, 90, 95 and 100%) and gradually embedded in Epon resin and polymerized. Samples were submitted to ultramicrotome for obtaining thin sections, which were stained with uranyl acetate and lead citrate. A Zeiss 900 transmission electron microscope was used to examine the ultrastructure of the parasites.

2.11 Statistical Analysis

Experiments were performed in three different occasions and the results were submitted to statistical analysis with GraphPad Prism 5.0 software, in which Tukey, Dunnet and T tests were done, depending on the analysis, and values of $p < 0.05$ were considered significant.

3 RESULTS

3.1 ACTIVITY AGAINST EPIMASTIGOTE AND TRYPOMASTIGOTE FORMS

For epimastigotes, the oleoresins obtained from *C. reticulata*, *C. martii*, *C. paupera*, *C. multijuga* and *C. officinalis* presented the best activity, inhibiting 50% of parasite growth with about 17-21 µg/ml after 96 h. Moderate activity was observed for *C. cearensis* and *C. langsdorffii*, with IC_{50} about 30-35 µg/ml. Copaiba oil from *C. lucens* presented low activity compared to others which its IC_{50} was about 51 µg/ml (Figure 1).

For trypomastigotes it was needed a higher concentration of copaiba oils to obtain some activity. *C. martii* and *C. officinalis* had the best activity, about 100 µg/ml the effective concentration of 50%, after 24 h. *C. reticulata*, *C. cearensis*, *C. paupera*, *C. langsdorffii*, *C. multijuga* and *C. lucens* presented similar moderate activity, with EC_{50} against the non proliferative forms of the parasite with about 175-285 µg/ml (Figure 1).

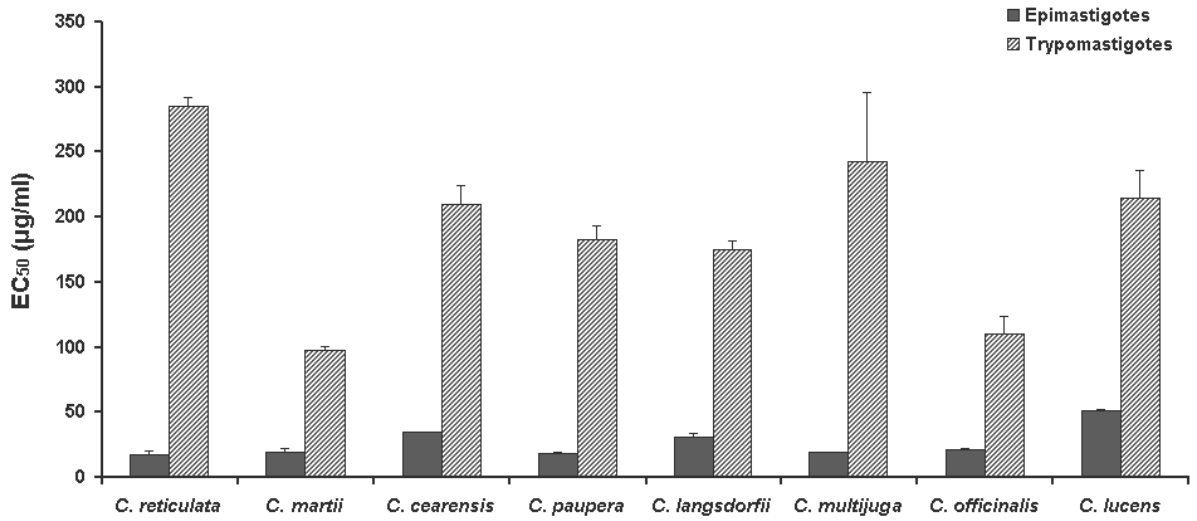

Figure 1 – Effective concentration that cause 50% of growth inhibition in epimastigotes and 50% of lysis in trypomastigotes, treated with different *Copaifera* oleoresins. Significant difference among species ($p < 0.001$).

3.2 ACTIVITY AGAINST INTRACELLULAR AMASTIGOTES

For intracellular forms the activity of copaiba oils was the best among all life stages of the parasite, after 96 h of treatment. At the concentration of 5 µg/ml, *C. martii* and *C. officinalis* presented great activity, inhibiting *T. cruzi* growth about 99%. *C. paupera*

and *C. multijuga* had also high activity inhibiting more than 80% of proliferation. *C. reticulata* and *C. langsdorffii* presented amastigote survival of 35% and 75%, respectively. *C. cearensis* and *C. lucens* had no effect at this concentration. At the concentration of 10 μg/ml, *C. martii*, *C. paupera* and *C. officinalis* treatment lead to absence of intracellular forms. *C. reticulata*, *C. cearensis* and *C. multijuga* inhibited amastigotes replication in more than 90%, while *C. langsdorffii* and *C. lucens* had inhibitory effect of 66% and 47%, respectively. IC_{50} values were determinate as 7.3, 7.7 and 10 μg/ml, for *C. cearensis*, *C. langsdorffii* and *C. lucens*, respectively, while for other species IC_{50} were below 5 μg/ml (Table 1).

3.3 Citotoxicity to Mammalian Cells

All copaiba oils presented similar toxicity to nucleated $LLCMK_2$ host cells, with toxic concentration of 50% varying from 20-32 μg/ml, after 96 h of treatment. Toxicity to erythrocytes was lower than to nucleated cells. *C. reticulata*, C. cearensis, *C. multijuga* and *C. lucens* presented hemolytic activity of 50% above 500 μg/ml. *C. martii* promoted 50% of hemolysis with 450 μg/ml, and for *C. paupera* and *C. langsdorffii* the toxic concentrations were 340 and 260 μg/ml, respectively. *C. officinalis* oleoresin was the most lytic one with 75 μg/ml. Amphotericin B and benznidazole, used as control drugs, showed CC_{50} of 13 and 160 μg/ml against $LLCMK_2$, respectively, while against erythrocytes CC_{50} was about 35 and above 500 μg/ml, respectively (Table 1).

Table 1 – Toxic concentrations of copaiba oils from different species on intracellular amastigotes and mammalian cells.

Copaifera species	IC_{50} (μg/ml)	CC_{50} (μg/ml)	
	Intracellular amastigotes	$LLCMK_2$	Erythrocytes
C. reticulata	< 5	27 ± 4.2	> 500
C. martii	< 5	17.5 ± 7.7	450 ± 3
C. cearensis	7.3 ± 0.1	30 ± 4.2	> 500
C. paupera	< 5	21.5 ± 2.1	340 ± 6
C. langsdorfii	7.7 ± 0.2	32.5 ± 3.5	260 ± 9
C. multijuga	< 5	31 ± 11.3	> 500
C. officinalis	< 5	21.5 ± 13.4	75 ± 2
C. lucens	10 ± 2.1	18 ± 1.4	> 500

IC_{50}, inhibitory concentration of 50%. CC_{50}, citotoxic concentration of 50%.

3.4 LIPOPEROXIDATION

Two copaiba oils promoted lipid peroxidation of epimastigotes of *T. cruzi*. *C. martii* and *C. officinalis* oleoresins caused a two-fold increase on the peroxidation of parasite cells, after six hours of treatment, when compared to control. Other copaiba oils showed the same result, without significant difference among them (Figure 2).

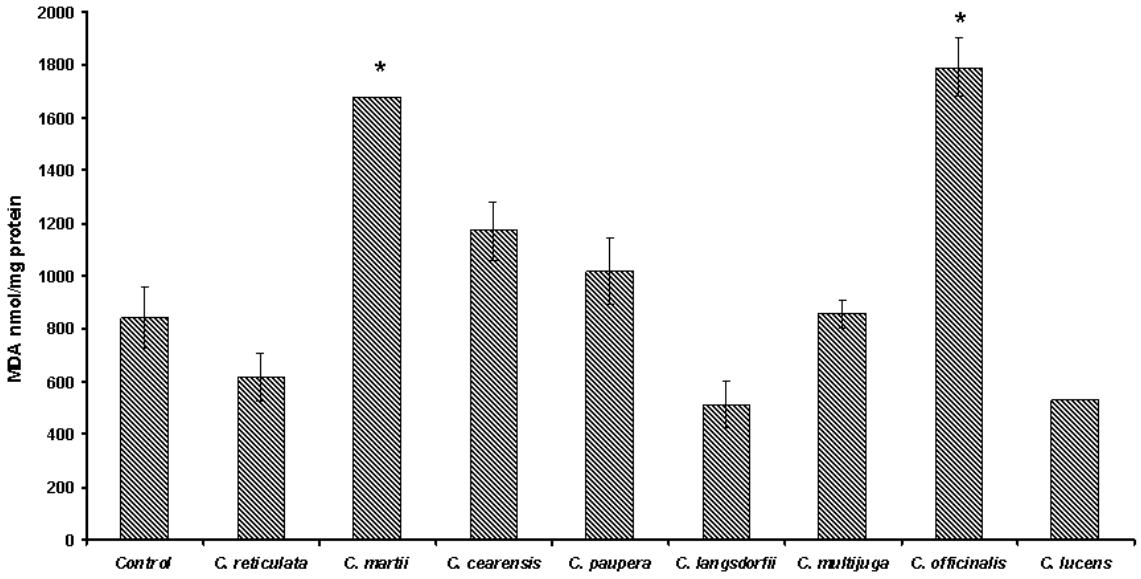

Figure 2 – Lipid peroxidation of epimastigotes of *T. cruzi* after 6 h of treatment with *Copaifera* oleoresins. Asterisk ($p < 0.001$).

3.5 FLOW CYTOMETRY

After 3 h of incubation, all oleoresins tested were able to cause damage to cell membrane, allowing propidium iodide entrance in almost 100% of the parasite population. Mitochondrion potential was altered similarly among copaiba oils, causing alterations in 40-50% of the parasites, after the incubation time (Figure 3).

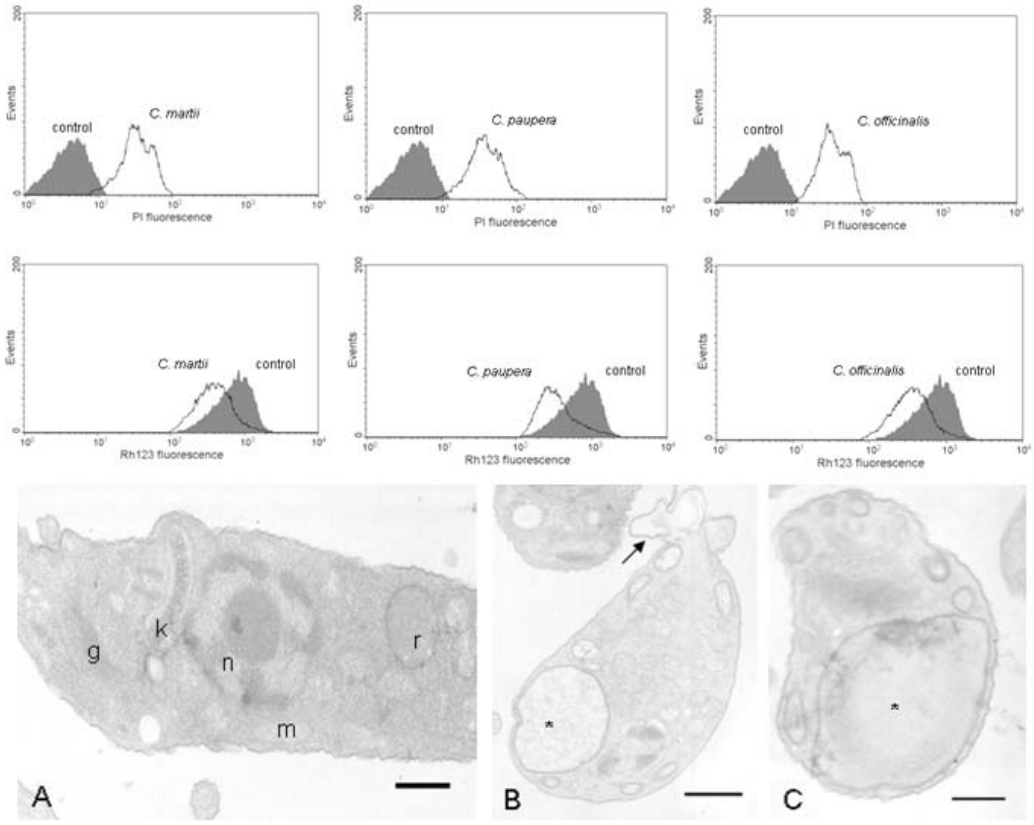

Figure 3 – Flow cytometry and transmission electron microscopy in epimastigotes of *T. cruzi* treated with copaiba oils from *C. martii*, *C. paupera*, *C. officinalis* and *C. reticulata*. Alterations in cell membrane permeability and mitochondrial potential loss can be seen in the population analysis, agreeing with ultrastructural damages visualized after treatment with *C. reticulata*. A, control cell; B and C, treated with IC_{50}; n, nucleous; m, mitochondrion; k, kinetoplast; g, Golgi apparatus; r, reservossomes; asterisks, mitochondrial swelling; arrow, cell membrane disarrangements. Bars = 10 μm.

3.6 TRANSMISSION ELECTRON MICROSCOPY

Epimastigotes treated with oleoresin obtained of *C. reticulata* IC_{50} showed fragmentation of organelles, a great swelling of the mitochondrion and a disarrangement of the cell membrane (Figure 3). Treatment with 5 μg/ml of oleoresins against intracellular amastigotes showed some alterations that can be responsible for the inhibition of parasite replication and death inside host cells (Figure 4). *C. martii* and *C. paupera* promoted disruption of cell membranes. *C. officinalis* caused swelling of kinetoplast together with chromatin condensation in both nucleus and mitochondrion. All treatments caused reduction of parasite dimensions and disorganization of membranes, which became not possible in some cases, like in *C. officinalis*, the clear distinction of organelles.

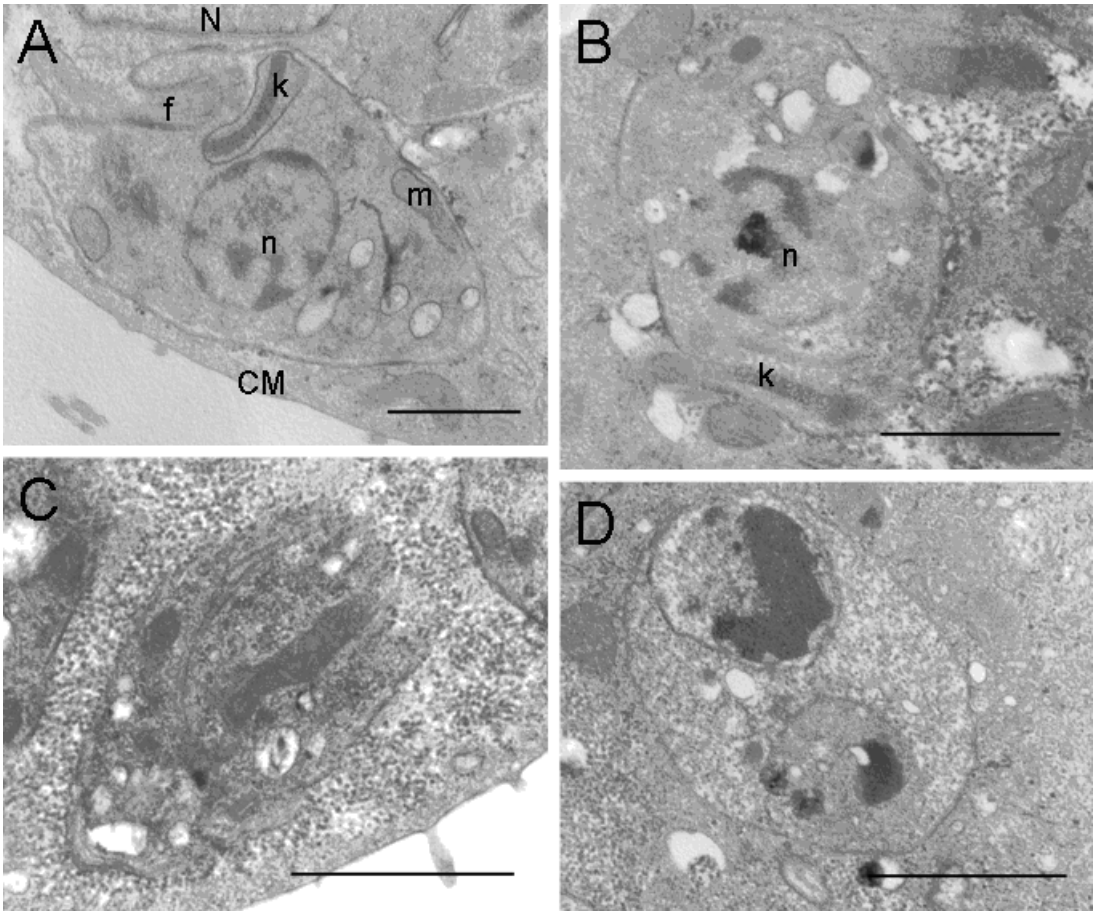

Figure 4 – Transmission electron microscopy of $LLCMK_2$ cells infected with *T. cruzi* and treated with *Copaifera* oleoresins for 96 h. **A**, control; **B**, *C. martii*; **C**, *C. paupera*; **D**, *C. officinalis*. N, cell nucleus; CM, cell membrane; n, parasite nucleus; k, kinetoplast; m, mitochondrion; f, flagellum. Bars = 1 μm

4 DISCUSSION

Amazon rainforest is a great source of natural compounds, which the majority has not been submitted to scientific validation yet. Native communities are spread all over the forest territory and many are far from medical care. Their ancient knowledge about the utilization of plants and animal secretions for healing wounds or treating diseases can be subjected to scientific proof so as in the case of *Copaifera* species, used the same way by population, can be discriminated which one has the best medicinal activity. Until the present moment, this is the first comparative study of species of the genus *Copaifera* against a neglected disease present in the Amazon region, where the genus is abundant.

In a general way, all copaiba oils showed activity against *T. cruzi* life stages, in special against replicative forms. For epimastigotes, the extracellular replicative stage, *C. lucens* was less active than other species. Oleoresins from *C. reticulata* showed moderate

activity as *C. cearensis* and *C. langsdorffii*. *C. martii*, *C. paupera*, *C. multijuga* and *C. officinalis* presented the best growth inhibition. There are not many studies with *Copaifera* species and even less against protozoal diseases. The same species were studied for activity against bacterial and fungal models, and the same way that in the present work, the best activity was described for *C. martii* and *C. officinalis* (Santos et al., 2008a). Against *Leishmania*, copaiba oil from *C. reticulata* demonstrated high activity, but in the present work we observed this effect only for epimastigotes (Santos et al., 2008b).

For amastigotes, the intracellular replicative stage, all copaiba oils were very effective, with low concentrations causing total elimination of the parasite as in *C. martii* and *C. officinalis*. Also *C. paupera* decreased almost 99% of parasite's survival. *C. multijuga* had the fifth best inhibition, while *C. cearensis*, *C. langsdorffii* and *C. lucens* had the lowest effect. *C. reticulata* was evaluated against amastigotes of *L. amazonensis* (Santos et al., 2008b) and it was needed concentrations 3-folds higher, to inhibit amastigotes survival in 50%, than the necessary to obtain the same result against *T. cruzi* amastigotes.

C. martii and *C. officinalis* had the best activity against trypomastigotes, the non-replicative form. Similar activities among other species were observed. Trypomastigotes are reached easily by drugs since it is free in the blood stream, what does not happen with amastigotes, because the drug must transposes the cell membrane to reach the parasite. *Copaifera* oleoresins were able to affect more amastigotes than trypomastigotes, and in some cases, it was needed a concentration more than twenty-folds higher, to get the same effect against the non-replicative form. *T. cruzi* organelles differ among lifestages due to differential gene expression that lead to alterations in surface molecules, metabolic pathway enzymes or structural organization. Such modifications can increase or decrease the susceptibility of *T. cruzi* to drugs, exposing or hiding possible drug targets.

Comparing the citotoxicity of the oleoresins, they were more citotoxic to nucleated cells than to erythrocytes. Amphotericin B, a common drug used for fungal infections and also leishmaniasis, presents more citotoxicity than copaiba oils. The citotoxic effect over cell line monolayers is questionable since they do not represent a real situation in a living being, otherwise, citotoxicity evaluation on red blood cells shows better a real toxic concentration, and in case of *Copaifera* oleoresins, erythrocytes that use to be more sensible to drugs, did not presented lysis in low concentrations.

In other studies, toxic effects of oleoresins and diterpenes isolated from *C. reticulata* and *C. langsdorfii* were evaluated against *Culex quinquefasciatus* (Silva et al., 2003), *Aedes aegypti* (Geris et al., 2008), embryonic cells of sea urchin, tumor cells and

erythrocytes (Costa-Lotufo et al., 2002), showing that this toxicity can control the development of bancroftian filariasis and Dengue's vector, inhibits cleavage in the beginning of embryogenic stages, reduce tumor development and also cause lysis of erythrocytes, the same way described here for copaiba oils.

Alterations on the mitochondrion function can generate reactive oxygen species, leading to oxidative stress, which can cause cell damage because of their reactivity with cellular components (Hernandez et al., 2006). *C. martii* and *C. officinalis* oleoresins treatments are possibly causing an increase of oxidative stress on parasite cells, which can be observed by the lipid peroxidation. *C. cearensis* and *C. paupera* oils tended an augmentation on the MDA generation, not significantly after 6 h, but it could be more distinct in a longer treatment. Oleoresins from *C. reticulata*, *C. langsdorffii* and *C. lucens*, on the contrary, initiated a reduction on the amount of MDA, which could suggest their action as possibly scavenger of reactive species, preventing partially the lipoperoxidation that occurs normally on the cells. *C. multijuga* oil showed lipoperoxidation similar to control cells. The oxidative stress caused by treatment with drugs can be originated not only by alterations on mitochondrion structure, but also inhibiting or blocking the activity of enzymes responsible for detoxifying the cell. Reactive oxygen species generation can also be a cause or a consequence of autophagy and apoptosis (Scher-Shouval & Elazar, 2007)

The simultaneous alterations on the cell membrane and mitochondrion permeability could be responsible for the death of the parasite, not only promoting cell lysis by the cell membrane damage, but also permitting a greater entrance of the oil compounds in the cell, leading to other lethal alterations by affecting other cellular components.

Transmission electron microscopy of epimastigotes showed a great detachment of the cell membrane and swelling of the kinetoplast. Alterations of the amastigotes inside host cells shows that the membrane of the parasite suffered disorganization and no organelles can be distinguished. Also a great condensation of the chromatin can be visualized after treatment with copaiba oils, in both nucleus and kinetoplast. A study about genotoxicity of a diterpene isolated from *C. langsdorffii* (Cavalcanti et al., 2006) against hamster lung fibroblast resulted in cell DNA damage above concentrations of 30 μg/ml. In our study, the presence of 5 μg/ml was not enough to cause any alterations on the host cell, but a detection of chromatin condensation with kinetoplast membrane swelling was possible.

5 CONCLUSIONS

The comparative study with species commonly used by natives is important to determinate the real biological activities of the genus and the best specie to be used by population. *C. martii* and *C. officinalis* presented a great inhibition on the development of all *T. cruzi* life stages, specially for intracellular forms. It means that *Copaifera* oleoresins can be considered as a potential source of compounds that have effect over *Trypanosoma cruzi*.

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ANEXO B – ARTIGO

DITERPENES AND SESQUITERPENES FROM *COPAIFERA* OLEORESINS: TRYPANOCIDAL AND SYNERGIC ACTIVITIES AGAINST *TRYPANOSOMA CRUZI*

Erika Izumi; Tânia Ueda-Nakamura; Valdir F. Veiga-Júnior; Ângelo C. Pinto; Benedito P. Dias-Filho; Celso V. Nakamura*

DITERPENES AND SESQUITERPENES FROM *COPAIFERA* OLEORESINS: TRYPANOCIDAL AND SYNERGIC ACTIVITIES AGAINST *TRYPANOSOMA CRUZI*

Erika Izumi[1]; Tânia Ueda-Nakamura[2]; Valdir F. Veiga-Júnior[3]; Ângelo C. Pinto[4]; Benedito P. Dias-Filho[1,2]; Celso V. Nakamura[1,2]*

Running Title: "Antitrypanosomal synergism of copaiba oil terpenoids"

Keywords: *Copaifera*. Diterpenes. Sesquiterpene. Synergism. *Trypanosoma cruzi*.

Synopsis

Objectives: To evaluate the trypanocidal and synergistic activity of terpenes isolated from *Copaifera* oleoresins on *Trypanosoma cruzi*, and investigate their possible mechanism of action.

Methods: Seven diterpenes (methyl copalate and copalic, hydroxy-copalic, agathic, pinifolic, polyaltic and kaurenoic acids) and one sesquiterpene (β-caryophylene) were obtained and assayed for activity against all life forms of *T. cruzi*, cytotoxicity on mammalian red blood cells, effect on parasite lipid peroxidation and permeability changes of cell and mitochondrion membranes. Ultrastructural alterations were investigated.

Results: Synergistic effect occurred between β-caryophylene and copalic acid. Cytotoxicity was moderate to erythrocytes. The majority of compounds were able to

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cause changes in mitochondrion and cell membrane permeability, while β-caryophylene and copalic acid had effect on lipid peroxidation. Hydroxy-copalic acid, copalic acid and β-caryophylene caused ultrastructural alterations, such as the formation of membranous vacuoles and mitochondrial swelling.

Conclusions: This is the first study showing synergic activity between two terpenes against *T. cruzi*. Natural compounds combinations can show great activity and can lead to new alternative treatments in the future.

Introduction

Chagas' disease is a major endemic disease in Latin America that affects over 15 million people[1]. This disease is caused by *Trypanosoma cruzi*, a kinetoplastid protozoa that is traditionally transmitted by bugs. Especially in the Amazon region, Chagas' disease has occurred in oral infections due to food contamination with vector excretes containing the parasite[2].

The *Fabaceae* family is one of the most important families of plants in ethnopharmacology. The *Copaifera* genus belongs to *Fabaceae* and is distributed throughout many countries on the American and African continents. In Brazil, copaiba trees are present in many territories, but they are found most often in the Amazon region and in western parts of the country. *Copaifera* sp. trees produce an oleoresin known as copaiba oil that is popularly used for its medicinal properties[3].

Copaiba oil exudes from the trunk of these trees and is essentially comprised of sesquiterpenes and acidic diterpenes, the former being more abundant and responsible for the oil's aroma. The chemical composition of the oils differs between species and also within the same species depending on the environmental conditions. Generally, the most cited sesquiterpenes in literature are β-caryophylene, α-copaene and β-humulene, and the most cited diterpenes are copalic acid, hardwickiic acid and clorequinic acid[4,3].

There have been several studies that investigated copaiba oils and their medicinal properties, including the antinociceptive, anti-inflammatory, antimicrobial, antileishmanial and bioinsecticidal activities[5,6,7,8,9]. For isolated compounds that have been isolated from these oils, kaurenoic acid is described as genotoxic, causing DNA fragmentation and micronucleus formation[10]. Two diterpenoids from *Copaifera* were recently found to have bioinsecticidal properties, 3-β-acetoxylabdan-8(17)-13-dien-15-oic acid and alepterolic acid[11].

In the present study, we describe the antiprotozoal and synergistic activities of diterpenes and one sesquiterpene found in *Copaifera* oleoresins and their cytotoxicity against mammalian cells. Ultrastructural alterations as well as the integrity of mitochondria and cell membranes were also evaluated together with parasite oxidative stress.

Materials and methods

Compounds

Compounds utilised in this work include (sesquiterpene) β-caryophylene purchased from Sigma (Sigma-Aldrich, Chemical Co., St. Louis, Mo, USA) as well as (diterpenes) copalic acid, hydroxy-copalic acid, kaurenoic acid, polyaltic acid, pinifolic acid, agathic acid and methyl copalate (Figure 1), all isolated from *Copaifera* oleoresins.

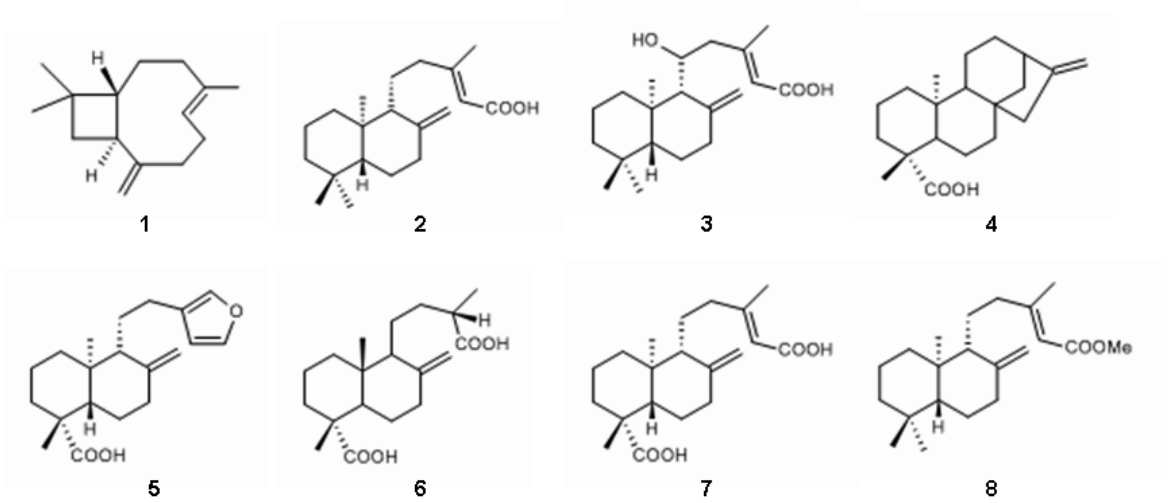

Figure 1 – Compounds isolated from Copaifera oleoresins. 1, β-caryophylene; 2, copalic acid; 3, hydroxy-copalic acid; 4, kaurenoic acid; 5, polyaltic acid; 6, pinifolic acid; 7, agathic acid; 8, methyl copalate.

Activity in epimastigotes

Epimastigotes from cultures kept in log-phase were inoculated at $1x10^6$ parasites/mL in LIT medium supplemented with 10% foetal bovine serum. These cells were kept in 24-well plates with concentrations of the compounds varying from 1 to 100 μg/mL. The plates were incubated at 28 °C for 96 h, and the parasites were then counted in a Neubauer Chamber. The 50% growth inhibitory concentrations (IC_{50}) were determined.

Activity in trypomastigotes

The mammalian $LLCMK_2$ cell line was maintained in DMEM (Gibco Invitrogen Co., Grand Island, NY) medium with 10% foetal bovine serum (FBS – Gibco Invitrogen Co., Grand Island, NY). The cells were kept in flasks for 72 h at 37 °C and with 5% CO_2. To obtain the trypomastigotes, $LLCMK_2$ cell monolayers were infected with trypomastigotes of *T. cruzi,* and the supernatant was harvested after 120 h. The trypomastigotes were inoculated at $1x10^7$ parasites/mL in DMEM medium with 10% of Balb/C mice blood and 20% of FBS. The experiment was prepared in 96-well plates that were incubated for 24 h at 37 °C with different concentrations of the compounds. Parasites were counted by the Brenner method to determine the activity (EC_{50}) of the drugs on 50% of the trypomastigotes.

Synergic activity

Hydroxy-copalic acid, copalic acid and β-caryophylene were combined with benznidazole in different concentrations: 10-250 μg/mL for the isolated compounds and 0.5-10 μg/mL for benznidazole. The experiment was done in 96-well plates in DMEM medium containing $1x10^7$ trypomastigotes/mL, 10% of mice blood and 20% of FBS, followed by incubation for 24 h at 37 °C. The parasites were counted as cited above. Fractional inhibitory concentration index (F.I.C.I.) were calculated and interpreted as in Odds[12].

Activity in amastigotes

Infected $LLCMK_2$ monolayers were prepared in round coverslips in 24-well plates and treated with concentrations of the compounds ranging from 0.5-15 μg/mL. The plates were incubated at 37 °C with 5% CO_2 for 96 h. Coverslips containing the monolayers were washed, fixed with methanol, stained with Giemsa and permanently prepared in Permount resin. The number of infected cells and amastigotes were counted in a total of 200 cells, and the percentage of inhibition of the amastigotes was calculated.

Haemolytic assay

The cytotoxicity of the compounds was evaluated against human red blood cells. Erythrocytes were obtained voluntarily from a healthy human donor, A^+ blood type. Blood was collected, defibrinated and washed in glycosylated saline to avoid free haemoglobin from the defibrinisation process. Red blood cells were inoculated at 3% in glycosylated saline, with different concentrations of the compounds in 96-well plates. The plates were incubated for 3 h at 37 °C, and the supernatant was read at 550 nm. Triton x-100 at 1% was the positive control.

Lipid peroxidation

Epimastigotes were treated with 100 μg/mL of the compounds for 6 h at 28 °C. After treatment, cells were washed with phosphate buffer and added to tubes containing 0.37% thiobarbituric acid solution, prepared in 15% trichloroacetic acid and 0.25 N HCl. Tubes were submitted to heating at 95 °C for 45 min, followed by cooling and centrifugation. The supernatant was collected, homogenised with butanol (1:1) and centrifuged for phase-separation; the upper phase was read at 532 nm. Lipoperoxidation was determined as the amount of thiobarbituric acid reactive substances (TBARS) in terms of malondialdehyde (MDA), expressed in MDA nmol x protein mg^{-1}.

Flow cytometry

The cell and mitochondria membrane integrity of epimastigotes and trypomastigotes was evaluated after treatment with the isolated compounds. The epimastigotes were treated with 100 μg/mL of the sounds and the trypomastigotes with concentrations from 100 to 300 μg/mL for 3 h. After incubation, parasites were washed in PBS and incubated with 0.2 μg/mL of propidium iodide (PI – Sigma-Aldrich Chemical Co., St. Louis, Mo, USA) for 10 min and 5 μg/mL of rhodamine 123 (Rh123 – Sigma-Aldrich Chemical Co., St. Louis, Mo, USA) for 15 min. Cells were then washed again and submitted for counting of 10,000 events each using a FACSCalibur flow cytometer (Becton-Dickinson).

Transmission electron microscopy

Epimastigotes were treated with IC_{50} concentrations of hydroxy-copalic acid, copalic acid and β-caryophylene for 96 h at 28 °C. Cells were fixed with 2.5% glutaraldehyde prepared in 0.1 M cacodylate buffer and then pos-fixed in 1% osmium tetroxide and 0.8% potassium ferrocyanide, prepared in a cacodylate buffer. Parasites were washed and then dehydrated in growing concentrations of acetone (50, 70, 80, 90, 95 and 100%), embedded in Epon resin gradually and polymerised at 60 °C. Ultra-thin sections of the samples were obtained by a ultramicrotome cut and stained with uranyl acetate and lead citrate. A Zeiss 900 transmission electron microscope was used to examine the ultrastructure of the parasites.

Statistical analysis

Experiments were performed on different occasions, and the statistical analysis was performed with GraphPad Prism 5.0 software, one-way ANOVA and Dunnett's test. Values of $p < 0.05$ were considered significant.

Results

Epimastigotes

All compounds showed activity against the epimastigotes until the maximum concentration tested. Copalic and hydroxy-copalic acids together with β-caryophylene were the most active, with IC_{50} ranges between 12-16 μg/mL. Agathic acid and methyl copalate had moderate activity, approximately 30 μg/mL, and kaurenoic and polyaltic acids needed higher concentrations to inhibit parasite proliferation, approximately 50 μg/mL. The IC_{50} of pinifolic acid after 96 h was above 100 μg/mL. (Table 1).

Trypomastigotes

Trypanosoma cruzi in their non-proliferative form were more resistant to the toxic effects of the compounds. Copalic and hydroxyl-copalic acids were more effective, but kaurenoic acid and methyl copalate showed similar activities, with EC_{50} values varying from

130 to 190 µg/mL. Caryophylene, agatic and polyaltic acids had activities around 300 µg/mL. Pinifolic acid was the least active compound against trypomastigotes, with an EC_{50} of about 500 µg/mL (Table 1).

Synergy

Combinations of copalic and hydroxy-copalic acids, β-caryophylene and benznidazole were tested for trypomastigotes. β-caryophylene associated with copalic acid showed a strong synergy, resulting in a F.I.C.I. value of 0.12. The EC_{50} of both drugs in association were twelve and forty times reduced, from 120 to 9.5 µg/mL and 300 to 7 µg/mL for copalic acid and β-caryophylene, respectively. Indifferent index were obtained by combinating β-caryophylene and hydroxy-copalic acid (F.I.C.I. = 0.86). When associated to hydroxy-copalic acid, the copalic acid IC_{50} had a sixty-fold reduction, from 120 to 2 µg/mL against trypomastigotes, but the index obtained from the combination was above synergy (F.I.C.I. = 0.6). Associations of the compounds with benznidazole showed indifferent index, all showing F.I.C.I. around 1.0. (Figure 2).

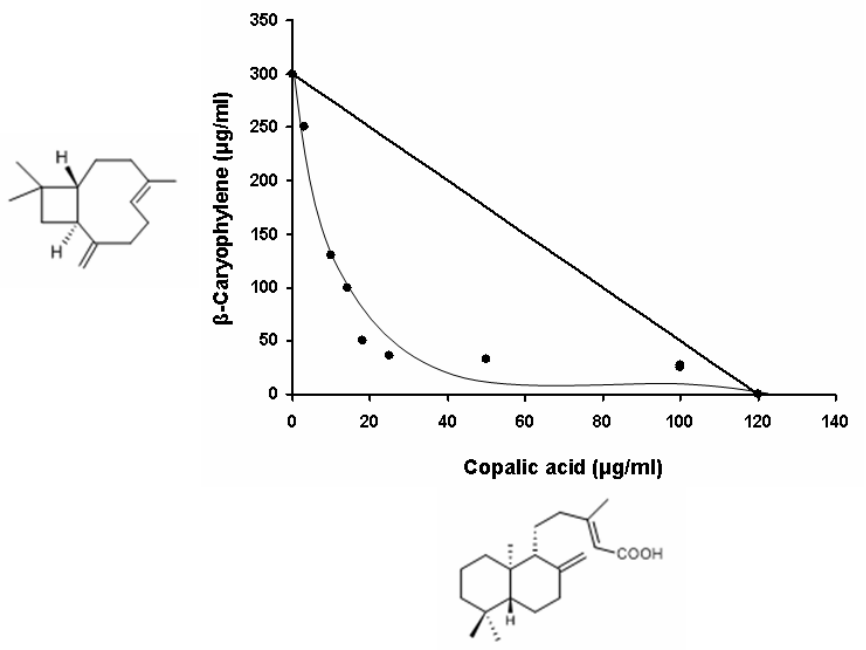

Figure 2 – Isobologram representing the synergy activity between β-caryophylene and copalic acid (F.I.C.I. = 0.12).

Amastigotes were more sensitive to the presence of different compounds from *Copaifera*. After 96 h, copalic and hydroxy-copalic acids as well as methyl copalate inhibited intracellular multiplication of the parasite with concentrations below 1 μg/mL. Agathic, kaurenoic and pinifolic acids had similar effects between 5-6 μg/mL. Polyaltic acid and β-caryophylene presented IC_{50} values of 9 and 13 μg/mL, respectively (Table 1).

Table 1 – Effective concentrations of compounds isolated from *Copaifera* for inhibition of *Trypanosoma cruzi*.

Compounds	μg/mL		
	Epimastigotes	Trypomastigotes	Amastigotes
	IC_{50}/96 h	EC_{50}/24 h	IC_{50}/96 h
β-caryophylene	16 ± 1	325 ± 35	13 ± 2
Copalic acid	13 ± 2	135 ± 49	0.4 ± 0.02
Hydroxy-copalic acid	13.2 ± 0.3	145 ± 35	0.6 ± 0.02
Agathic acid	29 ± 5	275 ± 35	5 ± 1
Kaurenoic acid	50.5 ± 0.7	180 ± 28	5 ± 1
Polyaltic acid	53 ± 2	305 ± 21	9 ± 2
Methyl copalate	26.5 ± 0.7	120 ± 28	0.8 ± 0.02
Pinifolic acid	275 ± 35	525 ± 35	6 ± 1

IC_{50}, inhibitory concentration of 50%; EC_{50}, effective concentration of 50%. Significant differences between compound's activity and control cell growth, ANOVA ($p < 0.0001$).

Hemolysis

The hemolytic assay showed different toxicities of the compounds on human red blood cells. The majority of compounds showed low toxic effects, resulting in 50% hemolysis in concentrations above 200 μg/mL. Pinifolic acid was the least toxic drug, causing 8% hemolysis at 500 μg/mL. Copalic and hydroxy-copalic acids were hemolytic for 50% of the erythrocytes in concentrations below 50 μg/mL (Figure 3).

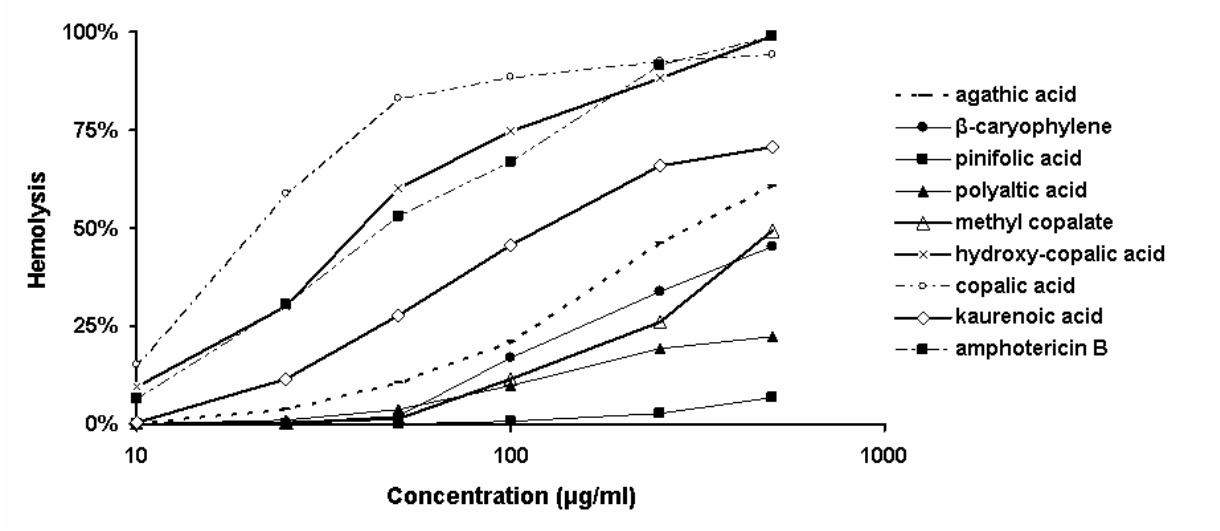

Figure 3 – Hemolytic activity of compounds isolated from *Copaifera* oleoresins. Standard deviation bars were excluded to facilitate visualisation.

Lipoperoxidation

Quantification of lipid peroxidation showed that only β-caryophylene and copalic acid were able to cause alterations. Both compounds were responsible for an increase of oxidative stress in the parasite of approximately 30-40%. Hydroxy-copalic, agathic, polyaltic, kaurenoic and pinifolic acids and methyl copalate caused no lipoperoxidation after 6 h of treatment (Figure 4).

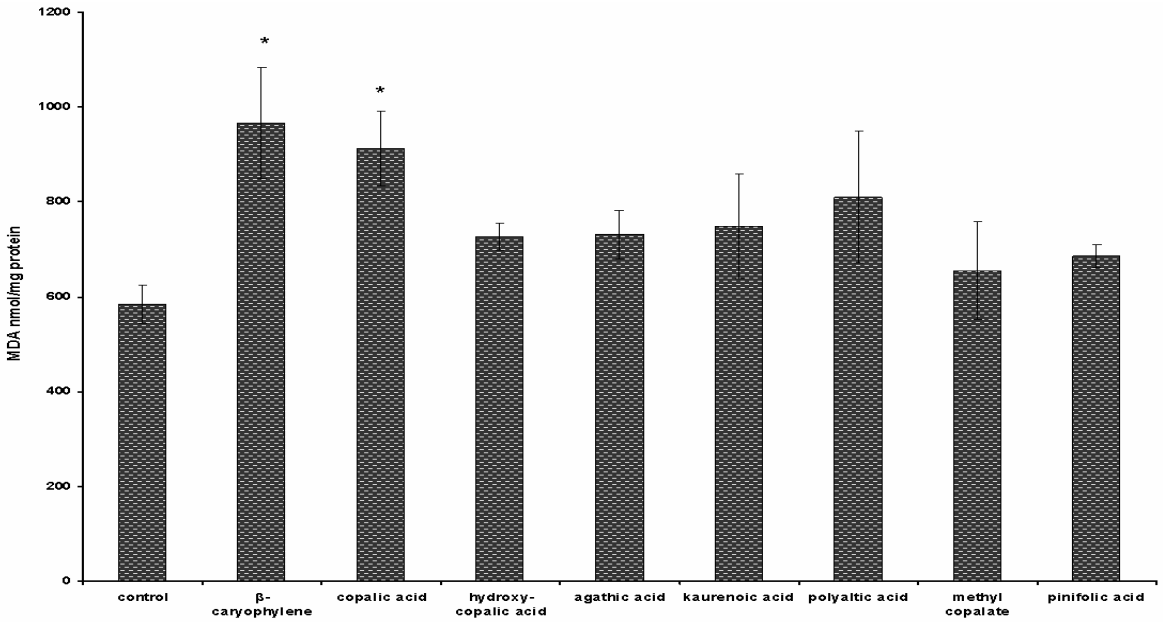

Figure 4 – Lipid peroxidation of epimastigotes of *T. cruzi* treated with different compounds from copaiba oil. ANOVA-Dunnett (* p = 0.02).

Flow cytometry

Only epimastigotes were susceptible to drug interference. Cell membrane integrity was affected by six compounds, and β-caryophylene, copalic, hydroxy-copalic and agathic acids promoted such permeability in almost 100% of the parasite population. Kaurenoic and polyaltic acids affected about 50-60% of the total population after 3 h of treatment. Pinifolic acid and methyl copalate did not interfere with cell permeability (Figure 5).

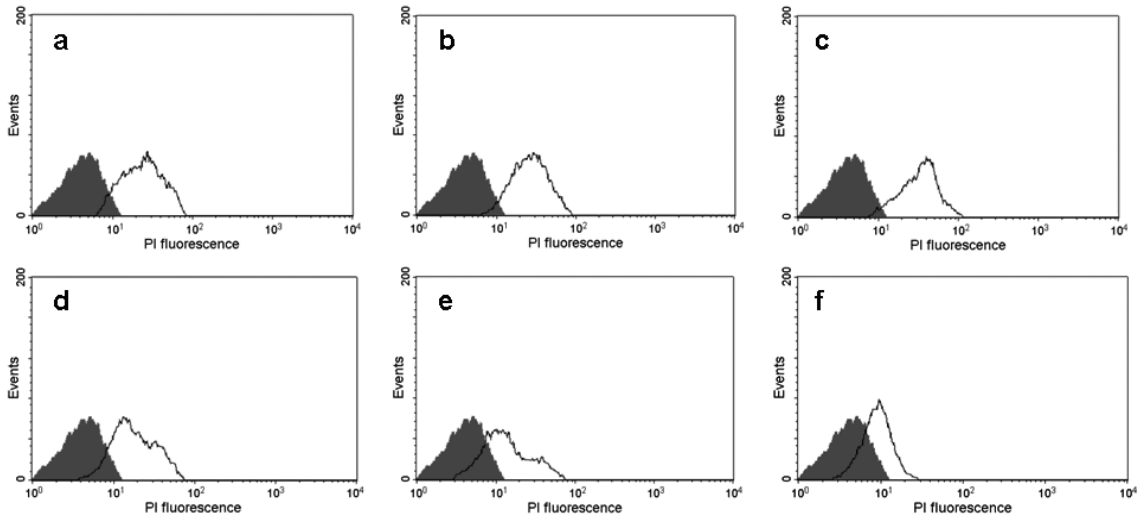

Figure 5 – Flow cytometry analyses of cell membrane integrity after treatment for 3 h with (a) β-caryophylene, (b) copalic acid, (c) hydroxy-copalic acid, (d) agatic acid, (e) kaurenoic acid and (f) polyaltic acid.

As seen in cell membranes, alteration of mitochondrion permeability was also detected for β-caryophylene, copalic, hydroxy-copalic and agathic acids, but kaurenoic and polyaltic acids did not affect mitochondrion. The same results were seen with other compounds (Figure 6).

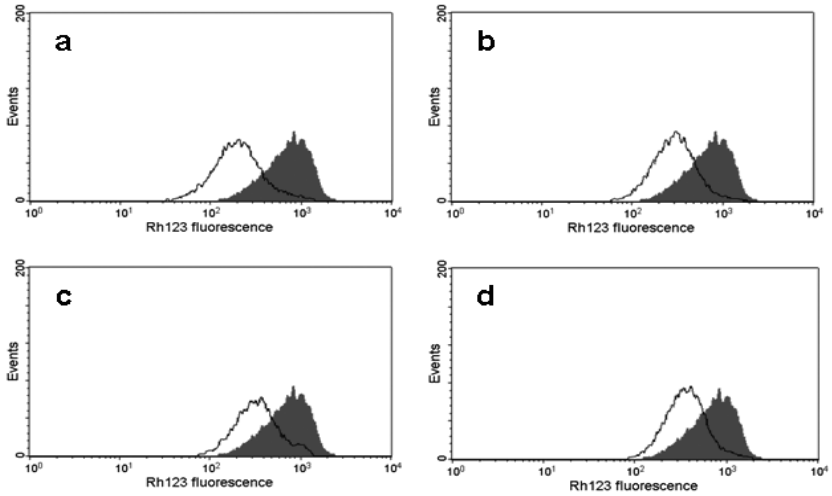

Figure 6 – Flow cytometry analyses of mitochondrial membrane potential after 3 h treatment with (a) β-caryophylene, (b) copalic acid, (c) hydroxy-copalic acid and (d) agatic acid.

Ultrastructural alterations

Epimastigotes treated with the sesquiterpene β-caryophylene showed total disorganisation of the kinetoplast and an initial formation of concentric membranous vacuoles. Diterpenes also caused changes in the parasite ultrastructure, though in different ways. Copalic acid caused a great swelling of the mitochondrion on almost all treated cells, while hydroxy-copalic acid caused organelle disorganisation and membranous vacuole formation present in all parasite body parts (Figure 7).

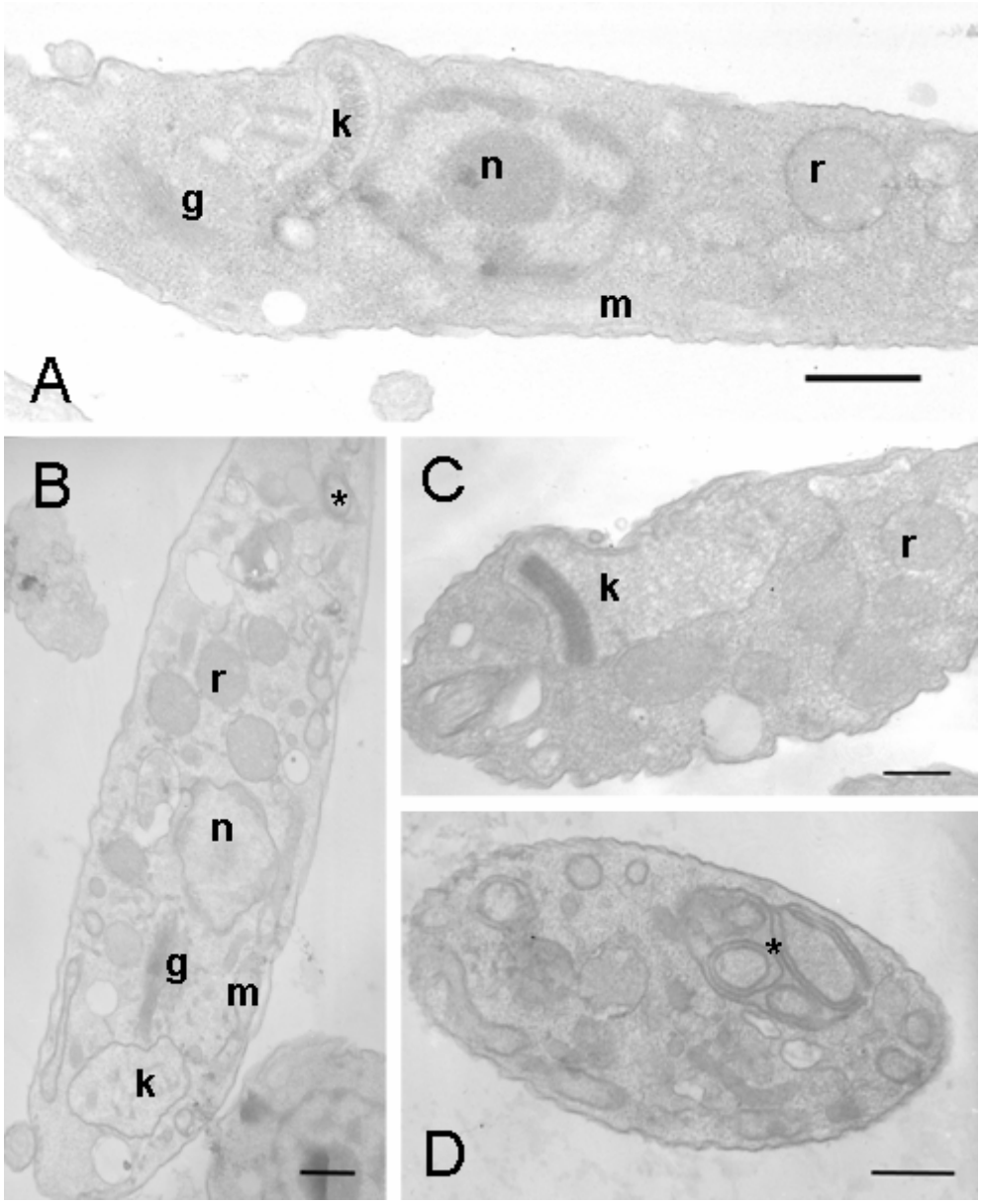

Figure 7 – Transmission electron microscopy of epimastigotes treated for 96 h with IC_{50} concentrations. (A) Control, (B) β-caryophylene, (C) copalic acid, (D) hydroxy-copalic acid, (n) nucleus, (k) kinetoplast, (r) reservossomes, (g) Golgi apparatus, (m) mitochondrion, (asterisk) membranous vacuoles formation. Bars = 1 μm.

Discussion

Natural product research is a growing field, partly due to its potential as a source for new medicines and treatments for diseases, such as Chagas' disease, that do not have a cure or for which the available treatment has severe side effects or low effectiveness. Studies with natural products usually involve an isolation process followed by evaluation of the biological activity of the compound obtained. It is difficult to find studies in the scientific

literature that compare compounds obtained from the same plant. Usually, all compounds are evaluated separately or with a synthetic drug.

In this work, we tried to show that combinations of compounds obtained from the same plant could present better results than the standard drug method. Copalic acid and hydroxy-copalic acid were the most active compounds of those we tested alone. In the case of copalic acid, the absence of a hydroxyl changed the "indifferent activity" (with hydroxy-copalic acid), to "synergic activity" when associated with β-caryophylene. The sesquiterpene β-caryophylene showed good activity against replicative forms, but it was after combination with copalic acid that the synergic activity was revealed, increasing forty times the activity against trypomastigotes. In a recent study, Pelizzaro-Rocha *et al*.[13] evaluated the activity of parthenolide, a sesquiterpene lactone isolated from *Tanacetum parthenium*, and it showed a strong synergistic effect with benznidazole against epimastigote forms.

Against intracellular amastigotes, methyl copalate showed good activity in addition to copalic and hydroxy-copalic acids. The toxicity over erythrocytes showed that methyl copalate is one of the less haemolytic compounds. Its good activity on intracellular forms and low toxicity to host cells results in higher selectivity, which permits the drug to affect the parasite where it is more difficult to be reached.

Lipid peroxidation is a reaction that occurs in the presence of reactive oxygen species (ROS) and can be associated with mitochondrial damage or detoxification system inhibition. Almost all compounds did not promoted lipid peroxidation, with the exception of β-caryophylene and copalic acid, which caused oxidative stress during the treatment. These compounds also interfered in the mitochondrial membrane potential. Hydroxy-copalic and agathic acids altered mitochondrion potential but did not lead to oxidative stress. A recent study showed that the trypanocidal action of the naphthofuranquinones is associated with mitochondrial dysfunction, leading to increased ROS generation and parasite death[14].

Changes in mitochondrial membrane potential can be associated with many cellular processes, including oxidative stress, apoptosis and autophagy[15]. β-caryophylene, copalic and hydroxy-copalic acids showed activities on cell membrane permeability, lipid peroxidation and mitochondrial potential changes. Part of these results can be correlated to ultrastructural alterations visualised by electron transmission microscopy. Mitochondrial and kinetoplast swelling, visualised after treatment with β-caryophylene and copalic acid, can

justify the lipoperoxidation and the loss of labelling with Rh123. For the hydroxy-copalic acid treatment, the presence of all membranous vacuoles could imply an autophagy processes.

In this study, treatment with the obtained compounds resulted in a decrease in cell volume. The fact that almost all of these compounds interfered with cell membrane permeability due to DNA labelling with IP can be a possible cause of this effect. The activity of a drug on cell membranes can be by insertion into the lipid bilayer, ion or amino acid transporter blocking, which is an important mechanism of osmotic regulation in *T. cruzi*, or several other reasons[16]. This increase in permeability can promote the loss of intracellular content, and these uncontrolled osmotic changes can also initiate a programmed cell death process.

Menna-Barreto *et al*.[17] studied another diterpene, geranylgeranyol, isolated from *Pterodon pubescens* (Fabaceae) that was also more effective against amastigotes than against other life stages of the parasite. Geranylgeranyol caused alterations in mitochondrial potential, and, by the visualisations of concentric membranous arrangements, it was suggested that this compound causes an autophagic pathway that leads to cell death.

Trypomastigotes did not suffer any changes detected by flow cytometry. No compound was able to cause alterations in cell or mitochondrion membrane, even in concentrations 3-fold higher than those used for epimastigotes. This suggests that the mechanism of action for the majority of the compounds can be associated with a specific component in the parasite cell that is present or more expressed in epimastigotes.

Conclusion

Some terpenes found in copaiba oils can cause parasite death by different mechanisms, including oxidative stress and autophagy. Copalic acid and β-caryophylene present synergistic activity against *T. cruzi*, and considering that both compounds are found in several species, this synergism could occur in almost all copaiba oils and may be one of the reasons for its medicinal properties. This investigative work studying natural compounds isolated from *Copaifera* alone and in combination is the first of its kind for *T. cruzi*.

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ANEXO C – ARTIGO

NATURAL PRODUCTS AND CHAGAS' DISEASE: A REVIEW OF PLANT COMPOUNDS STUDIED FOR ACTIVITY AGAINST *TRYPANOSOMA CRUZI*

Erika Izumi; Valdir Florêncio Veiga Júnior; Tânia Ueda-Nakamura; Benedito Prado Dias Filho; Celso Vataru Nakamura

NATURAL PRODUCTS AND CHAGAS' DISEASE: A REVIEW OF PLANT COMPOUNDS STUDIED FOR ACTIVITY AGAINST *TRYPANOSOMA CRUZI*

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Covering: 1995-2010

Many plant derivatives have been studied for different purposes. The diversity of families and species of plants in nature is very large compared to the number of studies performed, most of which were carried out with respect to applications to infectious diseases. Here, we review a significant number of studies that investigated the activity of plant-derived compounds against *Trypanosoma cruzi*, the etiologic agent of Chagas' disease. In the last decade, more than 300 species belonging to almost 100 families were evaluated for activity, and from some of them, isolated compounds were obtained as described in the 85 references cited.

Natural products and Chagas' disease

Natural products are an increasing source of new drugs that may, in the near future, replace current medications that have severe collateral effects. Natural products with antibacterial, antiprotozoal, antimycobacterial, antileishmanial, antitumor, and anti-HIV[1,2,3,4,5] properties have been identified in recent decades. Ancient customs led to modern plant research because, for centuries, plant extracts were the only known medicines available. However, with advances in technology, large numbers of natural compounds from animals, marine organisms, and free-living or symbiotic microorganisms can also be investigated for new drugs[6,7].

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American trypanosomiasis, also known as Chagas' disease, is endemic in Latin America, where the World Health Organization estimates that about 15 million people are infected and that almost 30 million live in risk areas[8]. The disease was first described in 1909 by the Brazilian physician Carlos Chagas, who identified the symptoms and described both the vectors and the life cycle of the parasite, *Trypanosoma cruzi*[9]. Although Chagas' disease has been recognised for more than a century, paleobiology experiments detected the presence of the protozoan in human populations from 9,000 years ago, specifically in tissues collected from mummies in the Andean region[10,11].

T. cruzi is a parasitic flagellate protozoan that belongs to the order Kinetoplastida and the family Trypanosomatidae, along with *Leishmania* and *T. brucei*, which are also human parasites of large impact on human health.. In its natural cycle, *T. cruzi* is transmitted to wild mammals, its vertebrate hosts, by hematophagous Triatominae insects. The proximity of human populations to natural habitats originated another parasite cycle, the domestic one, in which the vertebrate hosts are humans and their domestic animals[12]. *T. cruzi* is known to have the following three main morphological forms during its life cycle: the epimastigote (replicative, noninfective), which is found in the midgut of the invertebrate vector; the trypomastigote (nonreplicative, infective), which is the form of the parasite found in both hosts; and the amastigote (replicative, infective), which is the intracellular form found only in mammals and the most difficult to reach by drugs[13,14].

The few symptoms apparent at the beginning of an infection with *T. cruzi* include fever, malaise, and pain, which can be diagnosed as many other infectious diseases such as dengue or influenza. Constipation, as well as chest pain and fatigue, when manifested in people of advanced age, may be associated with aging problems and not with the symptoms of the late stage of Chagas' disease. The asymptomatic phase of the disease can persist for decades before the first manifestations begin, or it can last for the host's entire lifetime, which occurs in the majority of cases[15]. This asymptomatic situation may be one explanation for the difficulty in finding medicinal plants used popularly to treat Chagas' disease. In such cases, plants used for other protozoal diseases, especially those with taxonomic similarities, may show better results[16].

Socioeconomic factors in countries where the disease is endemic influence the level of funding for research and development that pharmaceutical companies will dedicate to finding new drugs for neglected diseases because most infected people live on less than US $ 2 per day and cannot pay for expensive treatments. Moreover, the long chronic-

asymptomatic phase makes it difficult to accurately estimate the number of sufferers, which is higher than official data show[17].

In the 1970s, nifurtimox and benznidazole were synthesised and belong to the nitrofurans and nitroimidazols, respectively. These drugs are highly toxic to mammalian cells. In Brazil, benznidazole is the only available drug, and its action results in a cure rate of approximately 70-80% in the acute phase but only 10-20% for chronic infection[18,19]. The toxicity manifests in severe side effects, and many patients abandon the treatment. Even after decades of research there still are no compounds able to cure all chagasic patients, and no substitute for the two drugs has been developed[20].

In attempts to find new therapies, synthetic drugs have been designed and evaluated, either alone or in combination with other drugs, against this parasite[21,22,23]. The advantage of the synthetic drugs is that, because of the known chemical structures of benznidazole and nifurtimox, alterations of their basic structural frameworks or the construction of new molecules with similar functional groups can guarantee high activity[24,25]. Discovery of new molecules from natural sources, however, is much more difficult. In the case of malaria, for example, quinine, an active compound against *Plasmodium* sp., was isolated from *Cinchona* sp. bark in the 17th century. Until recently, quinine was the only natural product with adequate activity to prevent and treat the disease and from which synthesised derivatives became commercially successful. The isolation of artemisinin from *Artemisia annua*, however, has improved the prospects for treatment of quinine-derivative-resistant malarial parasites[26,27,28].

The search for natural compounds extracted from plants has intensified in previous years, and the results are promising. The possibility of finding an active molecule with low toxicity is increasing as more plant species are screened. However, the question remains as to whether plants are being screened in the right way to reveal their optimum activity.

The efficiency of the available drugs and of the new drugs tested also depends on the strain of the parasite[29]. Some strains are more resistant to the commonly used drugs, and this difference should be carefully analysed because it may result from different mechanisms of action and defense. A few studies have used more than a single strain, and this comparison could be important to improve the evaluation of the effects of new extracts and compounds[30]. The similarities and differences in the effects of new drugs on different strains can provide new and useful information.

How natural products have been studied against *Trypanosoma cruzi*

There are many incentives for studying the medicinal benefits of natural products. For Chagas' disease, the prospect of developing new drugs has led over the last 15 years to the screening of almost 400 species belonging to more than 100 plant families for activity against *T. cruzi*. Many extraction processes used only one type of solvent and a single part of the plant; however, others have extracted compounds from various plant parts and used different solvents. Usually, the plant part subjected to the extraction process is the same that is used traditionally, but other parts may be assessed to determine which part contains the highest concentration of the active compounds. In screening for activity, solvents of different polarity should be used for the extractions because it is not certain that only the most polar compounds will show the highest activity.

Among the various studies, the following 18 plant species (listed by family, genus, and species) were evaluated by different investigators against different life stages of *T. cruzi*: Anacardiaceae, *Schinus molle*[31,32]; Annonaceae, *Annona muricata*[33,34], *A. reticulata*[33,35,36]; Asteraceae, *Mikania cordifolia*[37,36], *Neurolaena lobata*[38,35,36], *Tagetes lucida*[35,36], *Tanacetum parthenium*[33,39], *Tridax procumbens*[38,35,36,40]; Euphorbiaceae, *Croton guatemalensis*[35,36]; Fabaceae, *Gliricidia sepium*[38,35,36]; Lamiaceae, *Marrubium vulgare*[33,32]; Malpighiaceae, *Byrsonima crassifolia*[38,35,36]; Myrtaceae, *Psidium guajava*[33,36]; Phytolaccaceae, *Petiveria alliacea*[38,35]; Poaceae, *Cymbopogon citrates*[40,41]; Polypodiaceae, *Phlebodium aureum*[33,36]; Smilacaceae, *Smilax lundelli*[35,36]; and Sterculiaceae, *Chiranthodendron pentadactylon* [33,36]. The plant part used and the solvent chosen often differ. Even with the same extraction process, the results obtained were not the same, principally because of the different methodologies employed.

Promising results were obtained upon exposing the parasite to some plant extracts. Hexanic extracts of *Polygala sabulosa* and aqueous extracts of *P. cyparissias* (Polygalaceae) showed 50% inhibition of epimastigote growth after 72 h of treatment at concentrations of 1 and 2 μg mL^{-1}, respectively[31]. For trypomastigotes, *Piptadenia africana* (Mimosaceae) methanolic extract caused lysis in 50% of the parasites at 4 μg mL^{-1} after 96 h[40], and a methanolic extract from *Gardenia lutea* (Rubiaceae) also promoted the same effect at approximately 22 μg mL^{-1} after 72 h[42]. Against amastigotes, a surprising inhibition of 50% at less than 0.25 μg mL^{-1} after 7 days of treatment was detected using methanolic extracts from the following 8 species: *Hypoestes forsskalii* (family Acanthaceae), *Kleinia odora* and *Psiadia punctulata* (Asteraceae), *Capparis spinosa* (Capparidaceae), *Euphorbia schimperiana*

and *Ricinus communis* (Euphorbiaceae), *Marrubium vulgare* (Lamiaceae), and *Solanum villosum* (Solanaceae)[32].

All of these interesting results could possibly lead to the discovery of new active compounds, especially against intracellular forms of the parasite. Despite the screening of hundreds of species, the major, possibly active compound was only isolated, identified, and evaluated for antiparasitic activity in approximately 10% of the cases. A total of 136 compounds tested against *T. cruzi* in the last decade are described in Table 1. They are organised by family and species, and the methodology utilised to identify them are indicated to compare all reported activities.

TABLE 1

Antiparasitic studies have been carried out in a variety of differnt ways. Due to the great variation of methods, it is not a simple matter to capture data and determine which method yields the best results and should be continued or used as the standard for evaluation. All life stages of the parasite have their own proper culture media (e.g., LIT or BHI). The host cell type chosen for the amastigote and trypomastigote forms also can be different, as can the media used to culture them, for example, DMEM or RPMI 1640. Some data are described precisely, whereas others are given as a range of concentrations, which makes it impossible to discern the true value. These "hidden" values prevent merging information with other reported values and prevent comparisons and, hence, discussion and analysis of the activity.

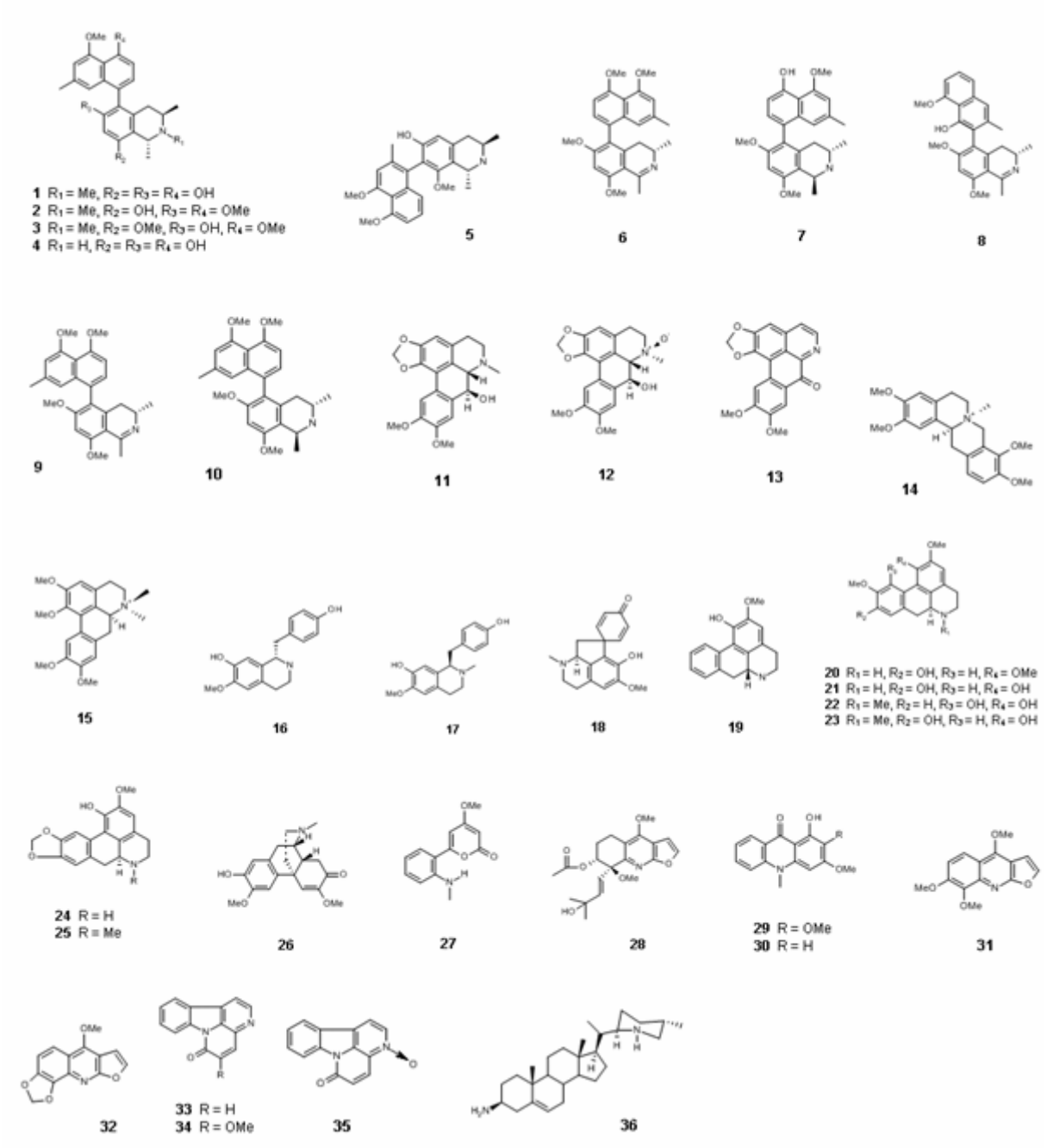
The majority of the processes used isolated alkaloids (**1-36**), which compose almost 29% of the compounds listed. All compounds isolated from members of the families Ancistrocladacea, Annonacea, Rutaceae, and Solanaceae, and some from Lauraceae, are alkaloids.

Against epimastigotes, only a steroidal alkaloid **36** was tested, resulting in growth inhibition of 50% at 10 μg mL^{-1} after 48 h. For investigation of amastigote survival, isoquinoline alkaloids from the family Ancistrocladaceae were identified. The most active were compounds **6**, **8,** and **9**, which all reduced intracellular replication at concentrations varying from 1.5 to 2.35 μg mL^{-1}. Similar activities were seen for **1** and **4**, with IC_{50} values of approximately 39 and 30 μg mL^{-1}, respectively, demonstrating that a methyl group bound to nitrogen causes a reduction of almost 10 μg mL^{-1} in IC_{50}. Compounds **2**, **7,** and **10** also had similar activities at approximately 17 μg mL^{-1}. Comparing **7** and **10**, it is clear that changes in the position of the methyl and the substitution of a methoxyl for a hydroxyl did not interfere

with activity, as was also observed for the presence of a methyl bound to nitrogen in compound **2**.

Aporphine alkaloids evaluated against trypomastigotes did not show high antiparasitic activity, except for **11**, which caused the death of half the parasite population at 9.32 μM after 24 h. An oxide bound to positive nitrogen could be responsible for the loss of activity of **12**, while the absence of a dioxol ring and a hydroxyl on **15** could explain its activity loss. Other alkaloids from Lauraceae showed moderate activity against trypomastigotes. Compounds **19** and **24** caused lysis of 70% and 76% of parasites, respectively; the only difference between these two compounds is the presence of a dioxol ring on **24**. Between compounds **24** and **25**, the presence of a methyl bound to nitrogen on **25** caused inactivation.

The *in vivo* evaluation of β-carboline alkaloids indicated decreased parasitemia. Compound **32**, a quinoline alkaloid, had moderate activity compared to **28** and **31**, which have hydrogenated carbons and methoxyls in place of a dioxol ring. Protoberberine, quinoline, acridine, and other alkaloids showed poor activity against trypomastigotes. Compound **36**, a steroidal alkaloid, was tested against epimastigotes and demonstrated an IC_{50} of 10 μg mL^{-1} after 48 h.

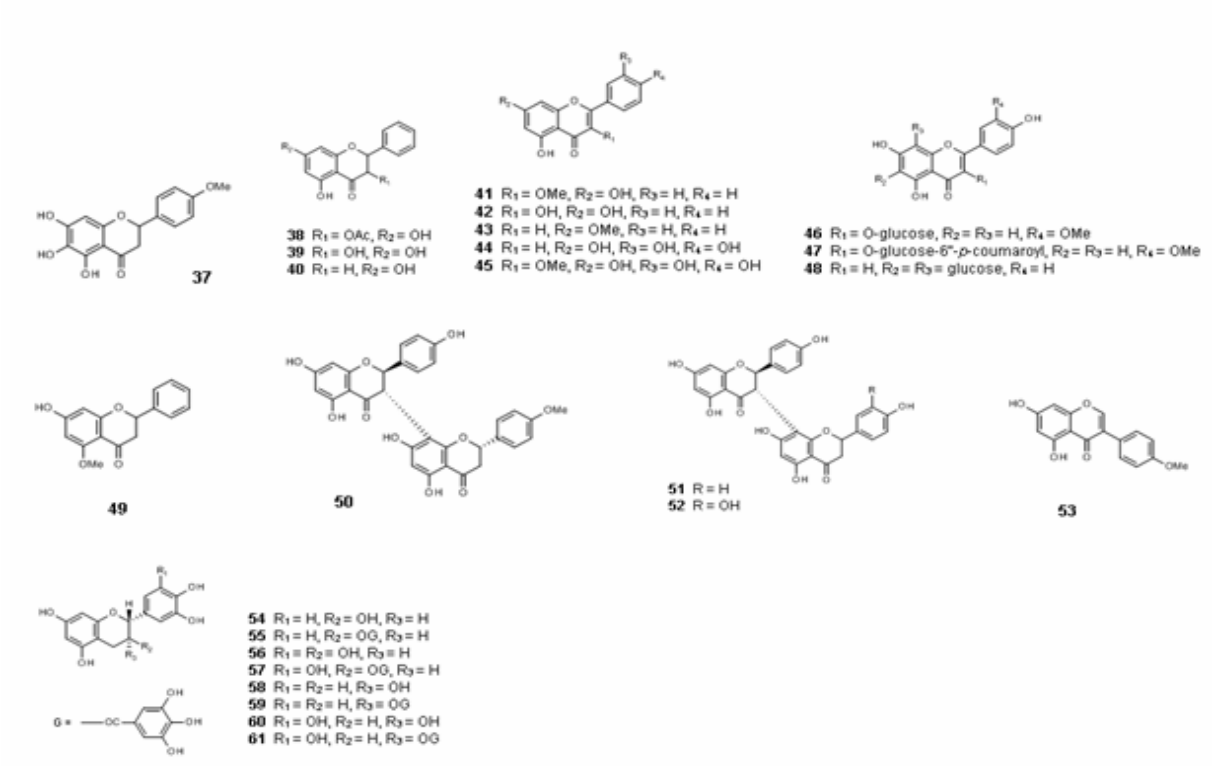


Flavonoids (**37**-**61**) are the second most abundant group of isolated compounds, represented here by the families Asteraceae, Bignoniaceae, Clusiaceae, Fabaceae, and Theaceae. Among flavonoids evaluated against trypomastigotes, structures **37** and **53** presented the best activity, with approximately 20 μg mL^{-1} needed to cause 50% parasite lysis. Both compounds have the same functional groups bound to the rings, but **53** is an isoflavonoid in which the aromatic ring is bound to the carbon closest to the carbonyl. Comparing glucosylated flavonoids, compound **48**, glucosylated at the A ring and not

presenting methoxyl groups, was more active than both **46** and **47**, which are both glucosylated in the C ring and have a methoxyl in the B ring.

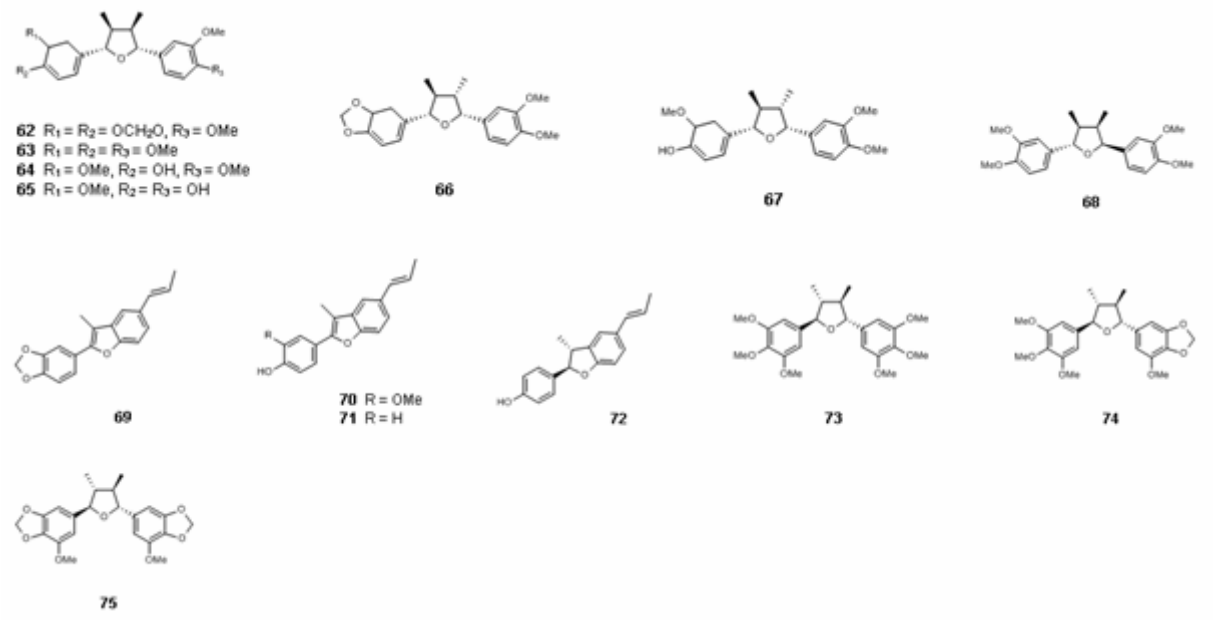
Compound **49** was inactive at the concentration tested and differs from **40**, which is active, in that it has a methoxyl in the A ring instead of a hydroxyl. The presence of methoxyl groups in the B or C rings reduces significantly the trypanocidal activity of these flavonoids. In contrast, **43**, with a methoxyl in the A ring and a double bond in the C ring, had a considerable lytic effect on the parasite. Compounds **44** and **45**, with unsaturated C rings and dihydroxylated B rings, had similar activity; however, **45** lacks a methoxyl in the C ring and was less effective than **44**.

Several catechins, such as compound **60**, for which the IC_{50} was 0.0000012 □M after 24 h, have shown good activity against epimastigotes. Among catechins, compounds trihydroxylated in the A ring and also trihydroxylated in the B ring were more active against *T. cruzi* than other catechins. Biflavonoids were evaluated for activity only against amastigotes. Compound **52** is dihydroxylated in the B ring and was the only inactive compound of this type. Against the intracellular form, compound **50** inhibited 50% of parasite replication at 34.7 μM after 168 h. Compounds **50** and **51** were effective in different concentrations, with the former being more toxic to the parasites and presenting in its structure a methoxyl in the B ring of the inferior flavonoid.



Lignans (**62-75**) were isolated from the families Lauraceae and Piperaceae, with all compounds obtained from the latter family being lignans. In general, lignans showed good activity against all *T. cruzi* forms. Among tetrahydrofuran lignans from the family Lauraceae, compounds **62** and **63** eliminated 50% of trypomastigotes within 24 h at concentrations of 2.2 and 4.4 μM, respectively. Other good activities against the nonreplicative form of the parasite were shown by **66** and **68**, with the two being toxic at almost the same concentration of approximately 12 μM. Comparing **63** and **68,** we find that a change in stereoconfiguration is responsible for the 3-fold higher concentration of drug necessary to exhibit the same activity. Also, the only difference between **64**, which showed no effect, and **67** is the position (up or down) of a methyl group.

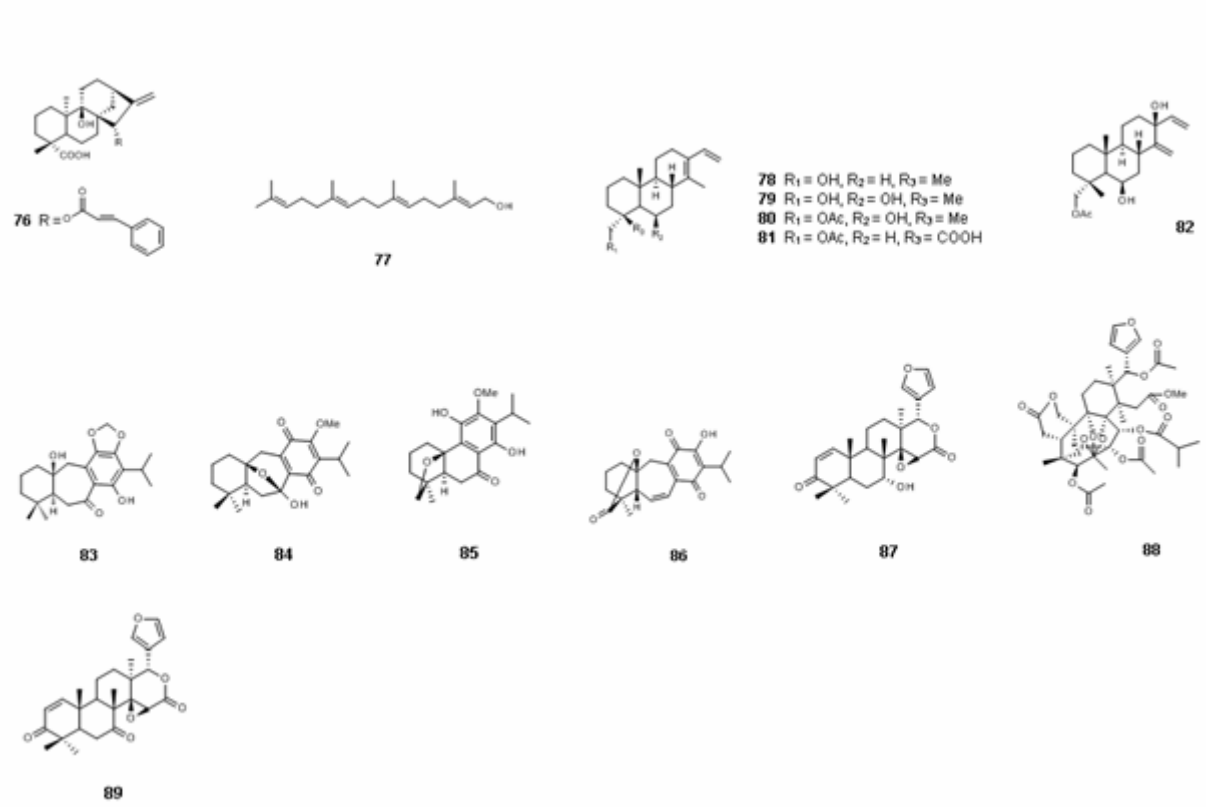
From the family Piperaceae, compounds were active at low concentrations, and **75** presented the best result, eliminating 50% of trypomastigotes at 3.47 μg mL^{-1}. The presence of two dioxol rings seems to increase the antiparasitic effect for these tetrahydrofuran lignans. Neolignans were toxic to epimastigotes, of which **70**, **71**, and **72** were active at similar concentrations ranging from 7 to 8 μg mL^{-1} after 96 h. In this case, the dioxol ring of **69** in the place of methoxyl and hydroxyl groups may be the cause for the reduction of activity. The only lignan assayed against amastigotes was **70**, and this compound inhibited intracellular replication at 5 μg mL^{-1} after 48 h.



Diterpenes (**76-89**) have been isolated from members of the families Meliaceae, Asteraceae, Fabaceae, and Lamiaceae. Only compound **77**, of all 136 listed in

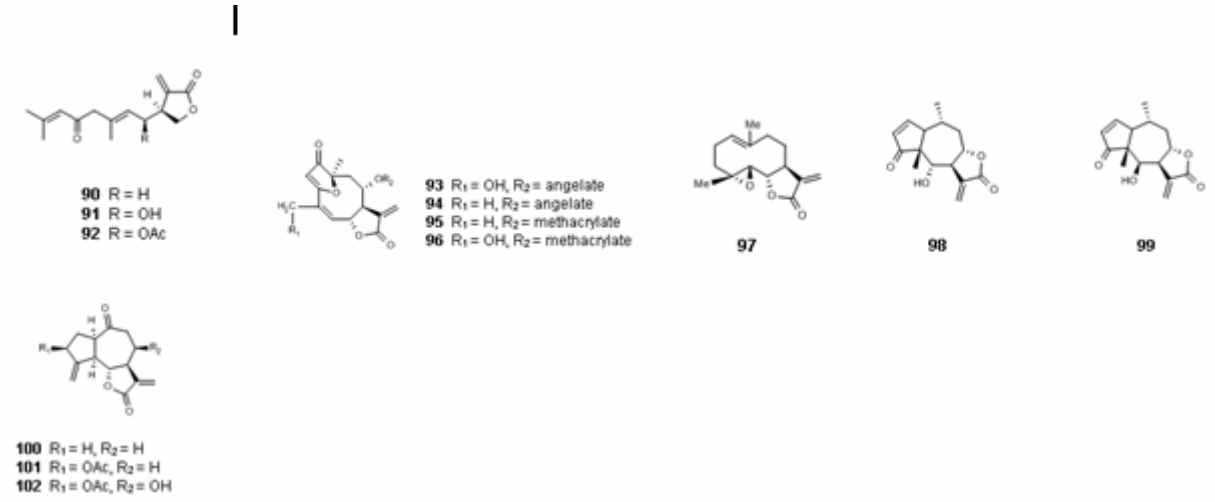
Table 2, has been evaluated against all forms of the parasite. It showed 50% inhibition of epimastigotes and amastigotes at 12.5 and 2 μg mL^{-1}, respectively, after 96 h. Against trypomastigotes, compound **77** had a 50% effect at 15.3 μg mL^{-1} after 24 h. Among other diterpenes from the family Fabaceae, **80** presented good activity together with **82** in epimastigotes, but compound **81** had an IC_{50} above 100 μM. Independent of the existence of a double bond or a hydroxyl, the carboxyl group could be reducing the activity of the latter.

Compound **84** from the family Lamiaceae also showed strong activity against epimastigotes, with 0.4 μM causing total lysis of the parasite after 24 h. Diterpenes from the family Meliaceae were screened against amastigote forms of *T. cruzi*. Compound **87**, another diterpene with good activity against intracellular forms, had an IC_{50} of 5.2 μg mL^{-1} after 96 h. Compounds **88** and **89** showed no activity except at the maximum concentration tested. Comparing **87** and **89**, a hydroxyl that has replaced the carbonyl group is responsible for the activity detected.

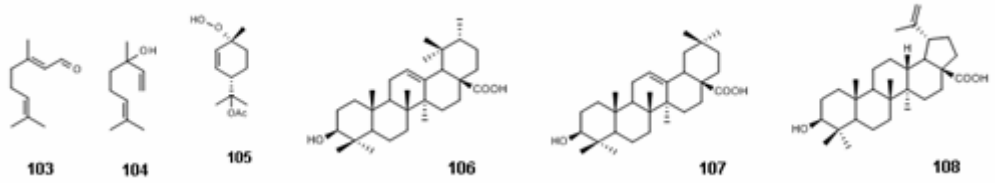


Sesquiterpenes (**90**-**102**) were obtained from members of the families Asteraceae and Lauraceae, and all isolated compounds are sesquiterpene lactones. The activity against *T. cruzi* was better for both replicative forms than for the nonreplicative form. From Asteraceae, sesquiterpenes were assayed against amastigotes, and **91** was the best,

inhibiting the parasite inside host cells at 5.72 μg mL^{-1}. Compounds **90** and **92** presented moderate and low activity, respectively. For those sesquiterpenes, an acetoxyl group in the place of a hydroxyl inactivated the compound. Against epimastigotes, compound **97** exhibited an IC_{50} of 0.5 μg mL^{-1} after 96 h, while for amastigotes, **91** showed 50% inhibition at 5.3 μg mL^{-1} for the same incubation time. Compound **96** was evaluated at 250 μg mL^{-1} against trypomastigotes, and after 24 h, 98.9% of the parasites were lysed.

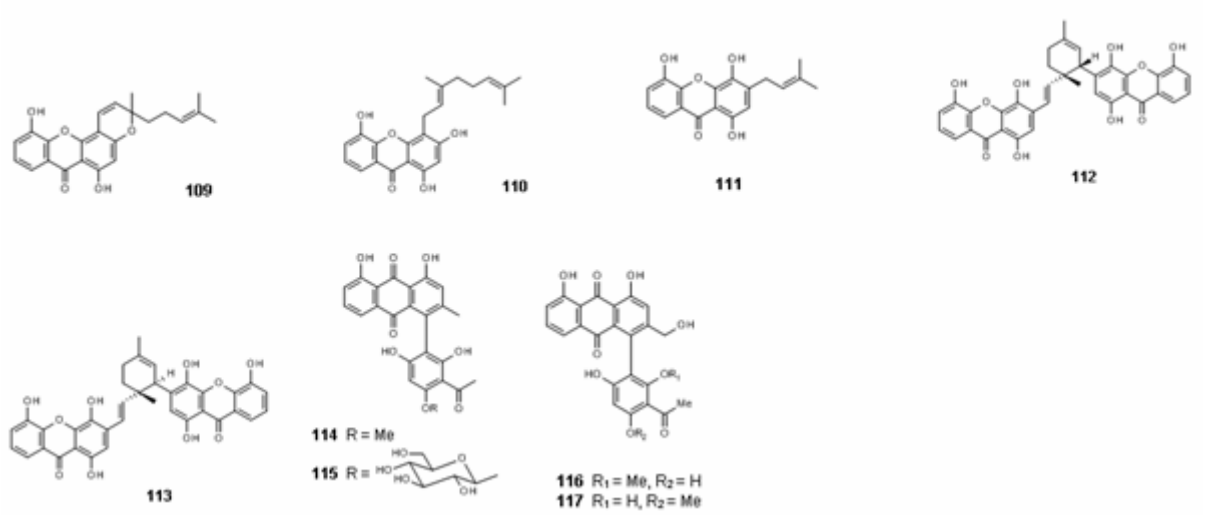


Monoterpenes (**103-105**) were isolated from members of families Gramineae, Lamiaceae, and Lauraceae, while triterpenes (**106-108**) were obtained from species of the Lecythidaceae and Bignoniaceae families. For epimastigotes, **103** showed 50% parasite-inhibiting activity at 42 μg mL^{-1} after 24 h; it had better activity against trypomastigotes, with 14.2 μg mL^{-1} causing 50% lysis. Compound **106** had low activity against trypomastigotes after 24 h, with 400 μg mL^{-1} being necessary to achieve 100% lysis. In comparison, the concentration of **107** to achieve the same results was approximately 4-fold higher.



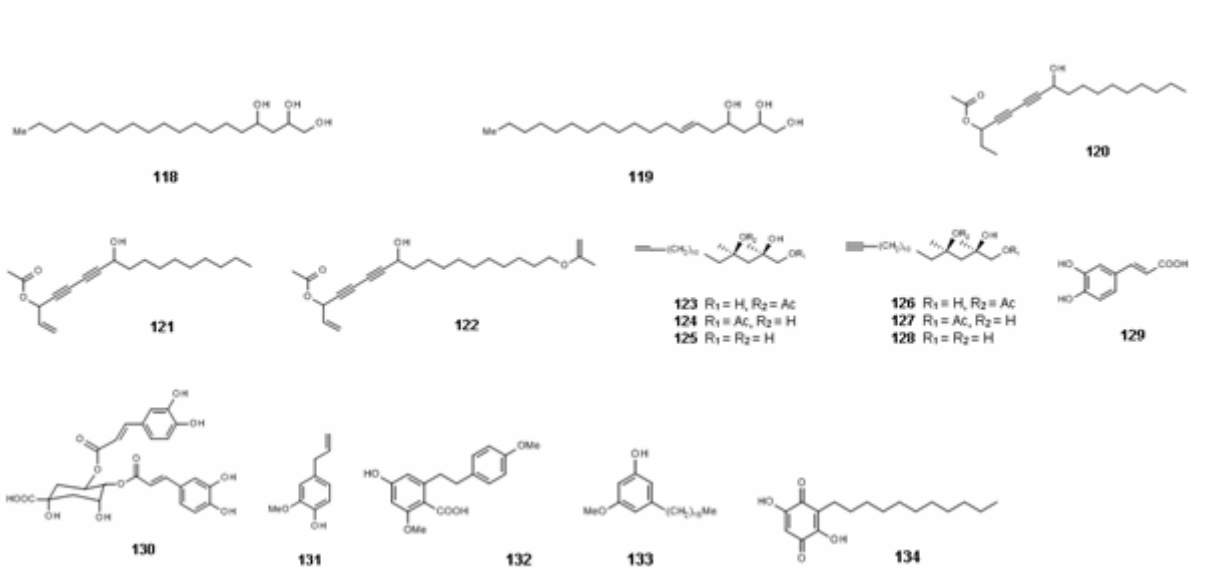
Xanthones (**109-113**) were obtained from the family Clusiaceae, and anthraquinones (**114-117**) from the family Asphodelaceae. The compounds from both groups

have been evaluated only against the intracellular form of the parasite. Compounds **110** and **112** inhibited 50% of amastigote growth after 168 h at 5.7 and 4 μM, respectively, while compounds **114** and **115** showed IC_{50} values of 7.6 and 6.8 μg mL^{-1} after 96 h, respectively. Comparing **112** with **113**, the latter was almost 10 times less active due to a hydrogen stereoconfigured differently.. Compounds **114** and **115** had similar activity, and despite they contain different radicals, these radicals may not affect the activity of these compounds.. However, when comparing compounds **116** and **117**, the former was three times more active than the latter, probably because of the switched positions of the methoxyl and hydroxyl groups.



Oxygenated hydrocarbons (**118-128**) were isolated from members of the families Araliaceae and Lauraceae, and other aromatic compounds (**129-134**), including phenylpropanoids, were isolated from members of the families Asteraceae, Myrtaceae, Oxalidaceae, and Polypodiaceae. Compounds **118** and **119**, which differ from each other in saturation at position six, showed similar antiparasitic activities. For epimastigotes, **123** exhibited an IC_{50} of 82 μM after 24 h, whereas against trypomastigotes, its activity was moderate, with 198 μg mL^{-1} causing 100% lysis after 24 h. For amastigotes, **121** and **122** showed IC_{50} values of 0.2 and 0.15 μg mL^{-1}, respectively, after 96 h, which is the best result obtained for oxygenated hydrocarbons. Compounds **120** and **121** are differentiated from each other by the presence of a double bond at position one in compound **121**, the activity of which is increased nearly 40-fold. For compound **123**, an acetoxyl group in position four and a double bond at position sixteen promoted high activity. Of other aromatic compounds, none has been tested against amastigotes, but compounds **131** and **132** showed moderate activity

against epimastigotes and trypomastigotes, with 50% lysis detected at doses above 50 μg mL^{-1} in both cases.



In research on Chagas' disease, natural products are most often active against the epimastigote form of the parasite. This form is more sensitive to drugs and is an easy model for the screening of new plant derivatives. The cell structures present in this form differ from the nonreplicative trypomastigote and the replicative intracellular amastigote. In particular, epimastigotes have reservosomes; these contain cruzipain, a cysteine protease that is important for the process of differentiation into an infective form and for invasion of host cells[75]. An evaluation of reservosomes and their contents, as well as of other proteases, using specific inhibitors in order to determine their effects on the differentiation process may show if an enzymatic alteration is occurring.

Organelles, including acidocalcisomes, glycosomes, kinetoplast, and the contractile vacuole, may be affected by the presence of some plant compounds. The fact that these structures are not present in the host cells allows them to serve as targets for the development of new drugs[76]. However, the actual functions of many parasitic cell structures are still unexplained, which makes the goal of finding new compounds able to alter or regulate the parasite life cycle by affecting these structures a difficult proposition at best.

Many tests are not carried out with trypomastigote and amastigote forms of the parasite because many laboratories do not have the financial resources required to maintain infected animal models or cell cultures. As a result, many compounds are screened

only against noninfective forms. Even those that show good results are not studied thoroughly, and often no active compound is isolated.

Trypomastigotes can easily evade immune mechanisms and persist in the blood. Even fewer compounds are capable of killing the parasite inside the host, and their effects on the immune response should also be evaluated. Natural products influence the immune system, and their effects can be manipulated to produce a greater or lesser immune response[77,78]. An evaluation of immune responses upon exposure to plant compounds could provide answers about how isolated plant compounds function inside the host. Because the parasite is capable of overcoming the host's defenses, it is undesirable to discover or employ compounds that interfere with immune responses and, thus, increase host susceptibility to the disease.

The amastigote form is the most difficult to affect because its natural habitat is inside host cells, which have many mechanisms for protecting the cell against cytotoxic agents. Compounds must be able to pass through the plasma membrane of the host, and therefore, substances that increase its permeability or act on the efflux pumps must be considered. Compounds active against amastigote parasites in cell monolayers have been obtained from plants and were able to reduce the proliferation of the parasite[62]. The activity of natural compounds in multilayer tissues should be investigated further by using animal models that would enable an evaluation of the activity of various compounds in conditions more closely reflecting actual Chagas' disease.

Just as some compounds affect metabolic pathways by inactivating enzymes or altering the structure of other proteins, it is also possible for a compound to cause an overexpression of specific genes. Several alterations in gene expression could be detected in the cell after exposure to various extracts or compounds, thus opening a new avenue for attacking the parasite in a specific manner. Observing nuclear alterations, membrane disorganisation, organelle deformation, or simple changes in the external structures of the parasite would provide more information about the target of the drug.

Walking together and helping each other

Studying natural substances has the potential to provide beneficial results, especially against diseases for which a cure does not yet exist or for which the available treatment is difficult for the patient. Determining the efficacy of a tested drug or its optimum

concentration is very important, but providing information about the target of the drug, the biology of the parasite, or its interaction with the host cell is also a valuable additional step[79].

Fixed parameters such as the percent efficacy of the drug and time of treatment should be utilised more frequently, as should standardisation, to possibly make better comparisons among the activities of extracts and compounds. An index of 50% activity in parasite lysis, for example, is more comprehensible as a measure of compound efficacy than merely evaluating the activity of a given compound at the same concentration as the standard drug. In some cases, the best activity of an extract or compound can be ten times below the standard drug value, but no activity was observed because only one or two concentrations were analysed. It is necessary to generate activity curves for screening tests with which the percentage of inhibition/lysis can be compared to several concentrations of the drug and used to determine the concentration for 50% activity as a reference datum, and perhaps the concentrations for 90% or 100% activity as well.

It is also important to use a standard treatment period, i.e., to expose the parasite and host cells to the drug for the same time period and then to transform the data obtained into a more accurate selectivity index. To expose the cell and the parasite to different incubation periods may lead to an erroneous interpretation of the compound's toxicity because each cell type will require different time periods for detoxification processes and the replacement of damaged structures.

In vivo studies always correlate the weight of the drug with the weight of the animal, usually given as mg kg^{-1}, and arrive at an agreement as to how the drugs are to be dosed in humans, thus facilitating the determination of the toxic dose. In vitro studies may show a similar dosage but correlate the weight of the drug with the volume of the solvent and not simply the molecular weight of the compound. When a drug is reported in mg mL^{-1} or µg mL^{-1}, for example, it is easier to comprehend an increase or decrease in the activity of the drug, and it would help many researchers who study the same family or genus to know the prospects for each one. The molecular weights of these compounds must be available as reference values, but many publications do not properly provide this information. To provide only the name of the compound or its molecular structure and then show the results only in terms of weight makes it difficult to compare the evaluated compounds because this obligates the reader to personally calculate the molecular weight by analysing the molecular structure to determine which compound shows the best activity compared to the control drug. This process therefore necessitates a large amount of time to reach a conclusion that could easily e given by the writer. More information given for the activity of natural products by the authors

of the study will facilitate additional comparative studies and lead to more rapid advances in research on protozoal diseases.

Differences among parasitic protozoa such as *Trypanosoma* sp., *Leishmania* sp., *Plasmodium* sp., and *Trichomonas vaginalis* are well known. Some investigators have demonstrated the effects of several plant extracts against these protozoa[80,42,67,81,82,64,34]. The results are interesting not only because of the high activity of some extracts, but also because they have similar effects on protozoa from different orders and families. Might this similar activity be related to similar targets? What do these parasites have in common that makes them susceptible to the same drug? What experiments can we design to explore these ideas?

There still is a black hole at the center of our knowledge of protozoal diseases. Part of this is due to the problem of lack of easy coordination and comparison of information, which could be resolved if many authors analysed their results better and cross-referenced them with others. This "globalisation" of scientific data must continue to enable research to progress and to avoid repeating the same steps.

Isolation and purification of natural compounds is neither an easy nor a simple process. Extractions have been carried out with hundreds of plant species, and only 136 compounds have been obtained, as described above. The fact that so few compounds have been studied is itself the source of another problem, namely that only a few of these have been evaluated against different strains or different protozoa[61]. At minimum, the determination of the major compounds in the extracts can help to answer questions about the biological activity of the species.

Toxicity is the major impediment in the development of new drugs for parasitic diseases. The treatments for Chagas' disease, leishmaniasis, sleeping sickness, malaria, and toxoplasmosis, for example, all have serious side effects on the hosts. In the case of Chagas' disease, a low index of cure is achieved after the establishment of the chronic phase, and the treatment itself causes the patient to suffer as a consequence of the toxicity. Only 37% of the articles published in the last decade reported results for the toxicity of the extracts or compounds they studied. The differences between our cells and those of parasites different and the discovery of natural products able to act specifically on one type of cell are topics to be studied in the near future and when screening of plants can be done in a more optimal manner.

The determinations of lethal doses in animals and investigations of toxicity to mammalian cell lines can help us to understand and address the toxic effects of natural products, thus determining whether a possible drug can be used in the same form in which it

was isolated or whether it requires structural modifications. Experiments with active compounds isolated from plants have shown that some modifications in the molecule increase activity significantly; however, such alterations of the original compound are rarely performed, and opportunities to find new drugs are perhaps being missed[83].

Synergetic effects on *T. cruzi* exposed to multiple compounds have mostly been studied using synthetic drugs, and almost no results for natural products used together have been published. Recently, a synergistic effect on *T. cruzi* epimastigotes was demonstrated between parthenolide, a sesquiterpene lactone isolated from *Tanacetum vulgare* (family Asteraceae), and benznidazole; the IC_{50} of benznidazole was reduced 23-fold when combined with the natural compound[84]. Against *Plasmodium*, this association of natural compounds has been studied, resulting in the detection of moderate synergism[85]. Associations of compounds are already used to treat some diseases such as malaria and sleeping sickness, especially when treatment with the standard drug fails. The possibility of finding natural compounds or derivatives able to replace the usual drug or that can be used in combination with other drugs should be taken more seriously.

One of the most popular means of treatment with medicinal plants is the preparation and ingestion of teas. This ancient tradition includes secret recipes in which different plant parts are mixed in appropriate concentrations and sometimes taken in peculiar ways to obtain the desired result. This means that, from the beginning, humankind has known of the advantages of mixing products from nature to cure its ills. Current scientific practice is to subdivide or simplify the object of study to discover its minimally relevant or functional part, and this is also occurring with studies of natural products. However, we must not neglect to put all these minimal parts together again so as to understand the complexity of nature on the macroscale, in which things work together and not separately.

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Table 1 – Isolated compounds from plants evaluated against *Trypanosoma cruzi*.

FAMILIES Species	Compounds	Activity in *T. cruzi*			Ref.
		E	T	A	
ANCISTROCLADACEA					
Ancistrocladus congolensis	ancistrocongoline A (**1**)	NT	NT	39.9 [(a)****]	43
	ancistrocongoline B (**2**)	NT	NT	17.4 [(a)****]	43
	ancistrocongoline C (**3**)	NT	NT	> 90 [(a)****]	43
	ancistrocongoline D (**4**)	NT	NT	30.1 [(a)****]	43
	korupensamine A (**5**)	NT	NT	14.5 [(a)****]	43
Ancistrocladus ealaensis	ancistroealaine A (**6**)	NT	NT	2.35 [(a)****]	44
	ancistroealaine B (**7**)	NT	NT	17.6 [(a)****]	44
Ancistrocladus tanzaniensis	ancistrotanzanine A (**8**)	NT	NT	1.7 [(a)****]	45
	ancistrotanzanine B (**9**)	NT	NT	1.5 [(a)****]	45
	ancistrotectoriline A (**10**)	NT	NT	17.8 [(a)****]	45
ANNONACEAE					
Duguetia furfuracea	duguetine (**11**)	NT	9.32 [(b)*]	NT	46
	duguetine-β-N-oxide (**12**)	NT	30.79 [(b)*]	NT	46
	dicentrinone (**13**)	NT	18.83 [(b)*]	NT	46
	N-methyltetrahydropalmatine (**14**)	NT	9072 [(b)*]	NT	46
	N-methylglaucine (**15**)	NT	4957 [(b)*]	NT	46
ASPHODELACEAE					
Bulbine frutescens	knipholone (**114**)	NT	NT	7.6 [(a)****]	47
	4'-*o*-demethylknipholone-4'-*o*-β-D-glucoside (**115**)	NT	NT	6.8 [(a)****]	47
	gaboroquinone A (**116**)	NT	NT	33.1 [(a)****]	47
	gaboroquinone B (**117**)	NT	NT	> 90 [(a)****]	47
ASTERACEAE					
Anthemis auriculata	anthecotulide (**90**)	NT	NT	18.05 [(a)****]	48
	4-hydroxyanthecotulide (**91**)	NT	NT	5.72 [(a)****]	48
	4-acetoxyanthecotulide (**92**)	NT	NT	> 30 [(a)****]	48
Baccharis retusa	5,6,7-trihydroxy-4′-methoxyflavanone (**37**)	NT	20.39 [(a)*]	NA [(a)***]	49
Lychnophora pohlii	centratherin (**93**)	NT	52.60 [(c)*]	NT	50
	galangin 3-methyl ether (**41**)	NT	10.17 [(c)*]	NT	50
	lychnopholide (**94**)	NT	66.67 [(c)*]	NT	50
	pinobanksin 3-acetate (**38**)	NT	16.67 [(c)*]	NT	50
	15-deoxygoyazensolide (**95**)	NT	65.21 [(c)*]	NT	50
	pinobanksin (**39**)	NT	35.09 [(c)*]	NT	50
	galangin (**42**)	NT	13.43 [(c)*]	NT	50
	goyazensolide (**96**)	NT	98.88 [(c)*]	NT	50
	tectochrysin (**43**)	NT	42.30 [(c)*]	NT	50
	luteolin (**44**)	NT	38.46 [(c)*]	NT	50
	vicenin-2 (**48**)	NT	41.82 [(c)*]	NT	50
	caffeic acid (**129**)	NT	15.94 [(c)*]	NT	50
	pinocembrin (**40**)	NT	40.13 [(c)*]	NT	50
	isorhamnetin 3-*o*-glucoside (**46**)	NT	18.44 [(c)*]	NT	50
	4,5-di-*o*-*E*-caffeyolquinic acid (**130**)	NT	74.77 [(c)*]	NT	50
	quercetin-3-methyl ether (**45**)	NT	32.10 [(c)*]	NT	50
	isorhamnetin 3-*o*-(6'-*p*-coumaroyl) glucoside (**47**)	NT	17.15 [(c)*]	NT	50
Tanacetum parthenium	parthenolide (**97**)	0.5 [(a)****]	NT	93 [(i)**]	39
Gaillardia megapotamica	helenalin (**98**)	1.9 [(b)****]	NT	NT	51
	mexicanin (**99**)	3.8 [(b)****]	NT	NT	51
Mikania stipulacea	ent-9α-hydroxy-15β-*E*-cinnamoyloxy-16-kauren-19-oic acid (**76**)	NT	62.75 [(c)*]	NT	52
Mikania hoehnei	8β-hydroxyzaluzanin D (**102**)	NT	56.64 [(c)*]	NT	52
ARALIACEAE					
Cussonia zimmermannii	8-hydroxyheptadeca-4,6-diyn-3-yl ethanoate (**120**)	NT	NT	7.9 [(a)****]	53
	8-hydroxyheptadeca-1-ene-4,6-diyn-3-yl ethanoate (**121**)	NT	NT	0.2 [(a)****]	53
	16-Acetoxy-11-hydroxyoctadeca-17-ene-12,14-diynyl ethanoate (**122**)	NT	NT	0.15 [(a)****]	53
BIGNONIACEAE					
Arrabidaea triplinervia	ursolic acid (**106**)	NT	400 [(e)*]	NT	54
	oleanolic acid (**107**)	NT	1,600 [(e)*]	NT	54
	alpinetine (**49**)	NT	NA [(e)*]	NT	54
CLUSIACEAE					
Garcinia livingstonei	6,11-dihydroxy-3-methyl-3-(4-methylpent-3- enyl) pyrano[2,3-*c*]-xanthen-7(3*H*)-one (**109**)	NT	NT	8 [(b)#]	55
	4[(*E*)-3,7-dimethylocta-2,6-dienyl]-1,3,5-trihydroxy-9H-xanthen-9-one (**110**)	NT	NT	5.7 [(b)#]	55
	1,4,5-trihydroxy-3-(3-methylbut-2-enyl)-9H-xanthen-9-one (**111**)	NT	NT	7 [(b)#]	55
	garcilivin A (**112**)	NT	NT	4 [(b)#]	55
	garcilivin C (**113**)	NT	NT	39.2 [(b)#]	55
	ent-naringeninyl-(I-3*R*,II-8)-4-*o*-methylnaringenin (**50**)	NT	NT	34.7 [(b)#]	55
	(+)-volkensiflavone (**51**)	NT	NT	56 [(b)#]	55
	(+)-morelloflavone (**52**)	NT	NT	NA [(b)#]	55

FAMILIES (cont.) Species	Compounds	Activity in *T. cruzi*			Ref.
		E	T	A	
FABACEAE					
Cassia fistula	biochanin A (**53**)	NT	18.32 [(a)*]	NT	56
Pterodon pubescens	geranylgeraniol (**77**)	12.5 [(a)****]	15.3 [(a)*]	2 [(a)****]	57
Myrospermum frutescens	18-hydroxycassan-13,15-diene (**78**)	48.6 [(b)***]	NT	17.4 [(b)#]	58
	6β,18-dihydroxycassan-13,15-diene (**79**)	56 [(b)***]	NT	16.6 [(b)#]	58
	6β-hydroxy-18-acetoxycassan-13,15-diene (**80**)	11.5 [(b)***]	NT	25.9 [(b)#]	58
	18-acetoxy-13,15-diene-19-cassanoic acid (**81**)	104 [(b)***]	NT	ND	58
	6β,13β-dihydroxy-18-acetoxycassan-14(17),15-diene (**82**)	16.5 [(b)***]	NT	35.8 [(b)#]	58
GRAMINEAE					
Cymbopogon citratus	citral (**103**)	42 [(a)*]	14.2 [(a)*]	NT	59
LAMIACEAE					
Dracocephalum komarovi	cyclocoulterone (**83**)	20 [(j)*]	NT	NT	60
	komaroviquinone (**84**)	0.4 [(j)*]	NT	NT	60
	dracocephalone A (**85**)	200 [(j)*]	NT	NT	60
Salvia gilliessi	5-epi-icetexone (**86**)	32-41 [(g)**]	NT	NT	61
Ocimum basilicum	linalool (**104**)	162.5 [(a)*]	264 [(a)*]	NT	59
LAURACEAE					
Laurus nobilis	dehydrocostus lactone (**100**)	NT	NT	75 [(h)****]	62
	zaluzanin D (**101**)	NT	NT	38 [(h)****]	62
	(1*R*,4*S*)-1-hydroperoxy-p-menth-2-en-8-ol acetate (**105**)	NT	NT	83 [(h)****]	62
Nectandra megapotamica	machilin G (**62**)	NT	2.2 [(b)*]	NT	63
	galgravin (**63**)	NT	4.4 [(b)*]	NT	63
	nectandrin A (**64**)	NT	17407[(b)*]	NT	63
	nectandrin B (**65**)	NT	47.3 [(b)*]	NT	63
	calopiptin (**66**)	NT	12.6 [(b)*]	NT	63
	aristolignin (**67**)	NT	34.8[(b)*]	NT	63
	ganschisandrine (**68**)	NT	12.2 [(b)*]	NT	63
Persea americana	4-acetoxy-1,2-dihydroxyheptadec-16-ene (**123**)	183 [(j)*]	244 [(j)*]	NT	33
	1-acetoxy-2,4-dihydroxyheptadec-16-ene (**124**)	213 [(j)*]	228 [(j)*]	NT	33
	1,2,4-trihydroxyheptadec-16-ene (**125**)	245 [(j)*]	198 [(j)*]	NT	33
	4-acetoxy-1,2-dihydroxyheptadec-16-yne (**126**)	276 [(j)*]	337 [(j)*]	NT	33
	1-acetoxy-2,4-dihydroxyheptadec-16-yne (**127**)	307 [(j)*]	307 [(j)*]	NT	33
	1,2,4-trihydroxyheptadec-16-yne (**128**)	327 [(j)*]	294 [(j)*]	NT	33
	1,2,4-trihydroxynonadecane (**118**)	189 [(j)*]	153 [(j)*]	NT	33
	(*E*)-1,2,4-trihydroxynonadec-6-ene (**119**)	223 [(j)*]	159 [(j)*]	NT	33
Ocotea lancifolia	coclaurine (**16**)	NT	10 [(c)*]	NT	64
	N-methycoclaurine (**17**)	NT	22 [(c)*]	NT	64
	crostparine (**NME**)	NT	0 [(c)*]	NT	64
	glaziovine (**18**)	NT	42 [(c)*]	NT	64
	caaverine (**19**)	NT	70 [(c)*]	NT	64
	laurotetanine (**20**)	NT	7 [(c)*]	NT	64
	nordomesticine (**24**)	NT	76 [(c)*]	NT	64
	norisoboldine (**21**)	NT	0 [(c)*]	NT	64
	norantenine (**NME**)	NT	12 [(c)*]	NT	64
	corytuberine (**22**)	NT	0 [(c)*]	NT	64
	domesticine (**25**)	NT	0 [(c)*]	NT	64
	isoboldine (**23**)	NT	7 [(c)*]	NT	64
	pallidine (**26**)	NT	8 [(c)*]	NT	64
LECYTHIDACEAE					
Bertholletia excelsa	betulinic acid (**108**)	NT	42.41 [(c)*]	NT	65
MELIACEAE					
Pseudocedrela kotschyi	7-deacetylgedunin (**87**)	NT	NT	5.2 [(a)****]	66
	kotschyin A (**88**)	NT	NT	> 30 [(a)****]	66
	7-deacetyl-7-oxogedunin (**89**)	NT	NT	> 30 [(a)****]	66
MYRTACEAE					
Syzygium aromaticum	eugenol (**131**)	246 [(a)*]	76 [(a)*]	NT	59
OXALIDACEAE					
Oxalis erythrorhiza	3-heptadecyl-5-methoxy-phenol (**133**)	NT	50 [(d)*]	NT	67
	embelin (**134**)	NT	100 [(d)*]	NT	67
PIPERACEAE					
Piper regnellii	eupomatenoid-3 (**69**)	26.3 [(a)****]	NT	NT	68
	eupomatenoid-5 (**70**)	7 [(a)****]	NT	5 [(a)**]	68,69
	eupomatenoid-6 (**71**)	7.5 [(a)****]	NT	NT	68
	conocarpan (**72**)	8 [(a)****]	NT	NT	68
Piper solmsianum	grandisin (**73**)	NT	8.74 [(a)*]	NT	70
	rel-(7*R*,8*R*,7'*R*,8'*R*)-3',4'-methylenedioxy-3,4,5,5'-tetramethoxy-7,7'-epoxylignan (**74**)	NT	17.6 [(a)*]	NT	70
	rel-(7*R*,8*R*,7'*R*,8'*R*)-3,4,3',4'-dimethylenedioxy-5,5'-dimethoxy-7,7'-epoxylignan (**75**)	NT	3.47 [(a)*]	NT	70

FAMILIES (cont.) Species	Compounds	Activity in *T. cruzi*			Ref.
		E	T	A	
POLYPODIACEAE					
Notholaena nivea	isonotholaenic acid (**132**)	50 [(a)***]	> 100 [(a)*]	NT	30
RUTACEAE					
Almeidea rubra	4-methoxy-6-[2-(methylamino)phenyl]-2H-pyran-2-one (**27**)	NT	1271 [(b)*]	NT	71
	rel-(7*R*,8*R*)-8-[(*E*)-3-hydroxy-3-methyl-1-butenyl]-4,8-dimethoxy-5,6,7,8-tetrahydrofuro[2,3-b]quinoline-7-yl acetate (**28**)	NT	977 [(b)*]	NT	71
	arborinine (**29**)	NT	1231 [(b)*]	NT	71
	N-methyl-1-hydroxy-3-methoxyacridone (**30**)	NT	2600 [(b)*]	NT	71
	skimmianine (**31**)	NT	1455 [(b)*]	NT	71
	kokusagine (**32**)	NT	559 [(b)*]	NT	71
Zanthoxylum chiloperone	canthin-6-one (**33**)	NT	D [(f)]	NT	72
	5-methoxycanthin-6-one (**34**)	NT	D [(f)]	NT	72
	canthin-6-one-*N*-oxide (**35**)	NT	D [(f)]	NT	72
SOLANACEAE					
Saracha punctata	sarachine (**36**)	10 [(a)**]	NT	NT	73
THEACEAE					
Camellia sinensis	catechin (**54**)	0.000067 [(b)*]	NT	NT	74
	epicatechin (**55**)	0.000085 [(b)*]	NT	NT	74
	gallocatechin (**56**)	0.0000105 [(b)*]	NT	NT	74
	epigallocatechin (**57**)	0.000013 [(b)*]	NT	NT	74
	catechin gallate (**58**)	0.000048 [(b)*]	NT	NT	74
	epicatechin gallate (**59**)	0.000056 [(b)*]	NT	NT	74
	gallocatechin gallate (**60**)	0.0000012 [(b)*]	NT	NT	74
	epigallocatechin gallate (**61**)	0.0000053 [(b)*]	NT	NT	74

E:epimastigotes; T: trypomastigotes; A:intracellular amastigotes. D: decreased parasitemia, NA: no activity detected, NT: not tested, NME: no molecular structure. Ref.: references.

(a) concentration in μg mL^{-1} able to cause lysis or inhibit growth of 50% of the cells

(b) concentration in μM able to cause lysis or inhibit growth of 50% of the cells

(c) percentage of lysis or inhibition of growth in the presence of 250 μg mL^{-1} of the compound

(d) percentage of lysis or inhibition of growth in the presence of 100 μg mL^{-1} of the compound

(e) concentration in μg mL^{-1} able to cause death/lysis of 100% of the cells

(f) parasitemia in animal model

(g) percentage of death/lysis in 4.5 μM of the compound

(h) percentage of inhibition of growth in 1 μg mL^{-1} of the compound

(i) percentage of inhibition of growth in 4 μg mL^{-1} of the compound

(j) Concentration in μM able to cause death/lysis of 100% of the cells

Time of incubation: * 24 h, ** 48 h, *** 72 h, **** 96 h, # 168 h.

RESULTADOS COMPLEMENTARES

Isolamento de substâncias do óleo de copaíba.

Colunas cromatográficas (35 mm x 600 mm), contendo 100 g de sílica-gel (70-230 mesh) impregnada com KOH, foram utilizadas para a extração de ácidos diterpênicos de interesse, de amostras de 5 g de óleo de copaíba. Foram utilizandos os solventes diclorometano (600 mL) para filtração e metanol (800 mL) para coleta da fração ácida. A fração metanólica (aproximadamente 2 g) foi concentrada em rotaevaporador, sob pressão reduzida, e submetida à partição ácido-base com diclorometano (100 mL) e água (50 mL), em pH 10, 8, 6 e 4. As subfrações pH 10 e pH 8 (aproximadamente 0,1-0,5 g) foram rotaevaporadas, seguindo as condições anteriores, e submetidas a cromatografia em coluna de sílica-gel (15 g de sílica, 15 mm x 300 mm), em hexano e concentrações crescentes de acetato de etila, para obtenção de substâncias isoladas. O processo de fracionamento e isolamento foi acompanhado de cromatografia em camada fina com diversos solventes, como hexano, acetato de etila, diclorometano, clorofórmio, isopropanol, acetona e metanol, em diferentes proporções a fim de se obter uma melhor separação das substâncias. O óleo utilizado para obtenção das substâncias foi coletado de *Copaifera multijuga* e *C. officinalis*.

A cromatografia em coluna de sílica-gel impregnada com KOH se mostrou eficiente em separar a fração contendo ácidos diterpênicos das demais substâncias, que são filtradas na fração diclorometano. A partir da fração metanólica, a partição ácida de 4 pHs contribuiu para melhorar a separação das substâncias em grupos menores (Figura 1).

Figura 1 – Cromatografia em camada fina do fracionamento do óleo de *C. officinalis*. D, fração dicloro. 10,8,6 e 4, pHs da partição ácido-base da fração metanólica. Revelação em sulfato cérico 10% seguido de aquecimento.

Quando as frações de diferentes pHs foram submetidas à cromatografia em coluna, foi possível observar a separação das substâncias ao longo do subfracionamento, das substâncias menos polares eluindo primeiramente em hexano puro, e outras seguindo o aumento da polaridade com a adição de diferentes porcentagens de acetato de etila, até finalmente acetato de etila puro e metanol (Figura 2).

Seguindo essa metodologia de fracionamento, foi possível obter 3 substâncias isoladas (ácido copálico, ácido hidroxi-copálico e ácido agático) provenientes do óleo de *Copaifera officinalis* e *Copaifera multijuga*.

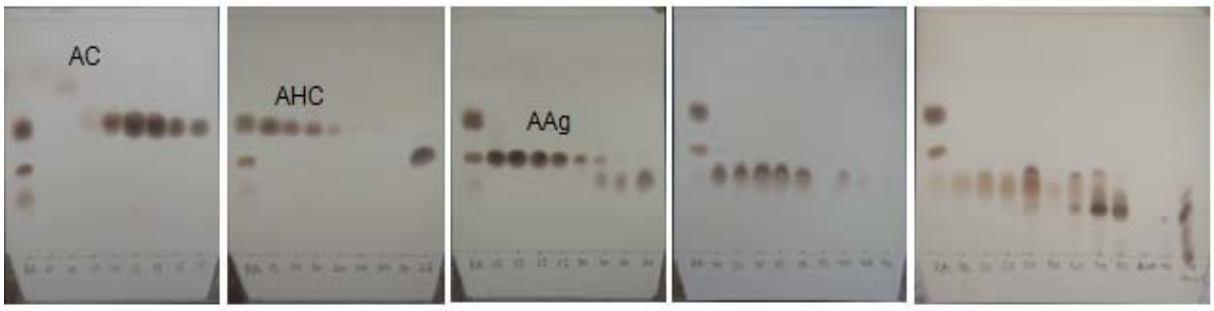

Figura 2 – Cromatografia em camada fina do subfracionamento da fração obtida em pH 10. Revelação com sulfato cérico 10% seguido de aquecimento. AC, ácido copálico; AHC, ácido hidroxi-copálico; AAg, ácido agático. Subfracionamento em Hexano com concentrações crescentes de acetato de etila.

Avaliação da sazonalidade do óleo de copaíba em *T. cruzi.*

Amostras do óleo de copaíba de *C. multijuga* foram coletadas, de diferentes indivíduos, em épocas sazonais distintas. Na região amazônica a sazonalidade diz respeito ao

período de chuvas e seca, que ocorrem de maneira cíclica. A quantidade de água disponível para as plantas pode influenciar na produção de substâncias provenientes do metabolismo secundário, entre elas, as substâncias presentes no óleo de copaíba. Para avaliar o fator sazonal na atividade parasitária do óleo, foram analisadas 53 amostras de 35 indivíduos, das quais algumas são sazonais e outras são coletas únicas. Também a característica do solo onde estão os indivíduos foi considerada nas coletas.

A avaliação de atividade tripanocida das amostras de óleo de copaíba, em formas epimastigotas, mostrou diferença significativa, tanto para épocas diferentes do ano, como para diferentes indivíduos (Figura 3). A diferença sazonal pode estar relacionada a quantidade de água disponível para a planta, bem como outras funções vegetais que também variam com a época do ano, como o florescimento e frutificação, por exemplo. No ano de coleta das amostras, houve uma progressiva estiagem que atingiu novembro do ano seguinte, nota-se pela atividade mostrando um valor mais baixo para IC_{50}, comparando com a primeira coleta na estação chuvosa do ano anterior. Já para as diferenças individuais, considerando que a região amazônica está sujeita, de uma maneira geral, a mesma variação climática em quase toda sua extensão, essa diferença de atividade pode estar relacionada com o tipo de solo em que se encontra a planta, que pode conter diferentes nutrientes. Como visto nos resultados sobre a atividade tripanocida de algumas substâncias encontradas no óleo de copaíba, a maior ou menor quantidade de cada substância pode levar a diferenças individuais de atividade, assim como o potencial sinérgico de algumas dessas substâncias.

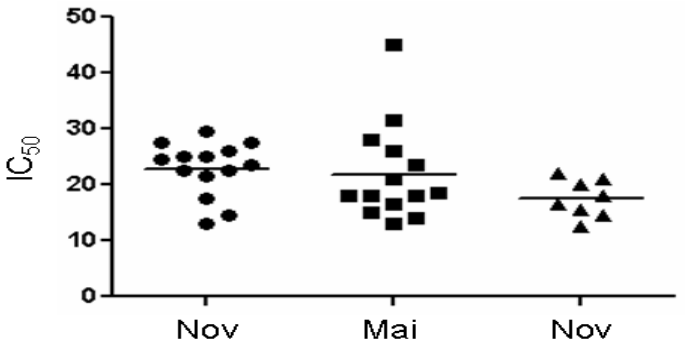

Figura 3 – Concentração inibitória de 50% em epimastigotas tratados por 96 h com o óleo coletado de diferentes estações. Nov, novembro (final da estação seca); Mai, maio (final da estação chuvosa).