AVALIAÇÃO DAS ATIVIDADES ANTI-HIPERTENSIVA, ANTIMICOBACTERIANA, ANTI-INFLAMATÓRIA E CITOTÓXICA DE ALCALOIDES INDÓLICOS ISOLADOS DE ESPÉCIES DO GÊNERO *Aspidosperma* (Apocynaceae)

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CAMPOS DOS GOYTACAZES – RJ
FEVEREIRO – 2023

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M775 Monteiro, Noemi Oliveira.

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87 f. : il.
Inclui bibliografia.

Tese (Doutorado em Produção Vegetal) - Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Ciências e Tecnologias Agropecuárias, 2023.
Orientador: Ivo Jose Curcino Vieira.

1. *Aspidosperma*. 2. Alcaloides. 3. Hipertensão. 4. Tuberculose. 5. Câncer. I. Universidade Estadual do Norte Fluminense Darcy Ribeiro. II. Título.

CDD - 630

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Aprovada em 27 de fevereiro de 2023

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AGRADECIMENTOS

Primeiramente a Jesus Cristo, por ter renovado as minhas forças para prosseguir nos momentos mais difíceis;

Ao meu esposo Hebert por sempre acreditar em mim e ser um grande incentivador;

À minha filha Alice, que ressignificou tudo em minha vida, me fazendo uma pessoa mais forte e persistente;

Aos meus pais, Oziel Carlos Monteiro e Edmea de Jesus O. Monteiro, por serem minha base e estarem sempre por perto para me ajudar no que for preciso. Agradeço em especial a minha mãe por ter me ajudado a descascar, limpar e secar o material vegetal, além de cuidar de Alice para eu poder terminar a confecção da tese;

À minha irmã Priscila Oliveira M. Mendes por todo apoio e incentivo;

Ao professor Ivo José Curcino Vieira pela orientação, paciência, incentivo e todo ensinamento;

Ao professor Raimundo Braz pela coorientação e pela sua grande dedicação ao realizar as elucidações estruturais;

Ao professor Milton Masahiko Kanashiro, Marina Silva, Leide Laura Maciel e os demais alunos e técnicos do laboratório de Biologia do Reconhecer (LBR), pela colaboração na atividade anticancerígena;

À professora Elena Lassounskaia, Sanderson Dias Calixto, Thatiana Lopes e Michelle F. Muzitano pela colaboração nas atividades antimicobacteriana, anti-inflamatória e citotóxica;

À professora Fernanda Antunes, Theresa Moura e aos demais alunos e técnicos da Unidade de Experimentação Animal (UEA), pela colaboração na atividade anti-hipertensiva;

A todos os amigos do Laboratório de Química de Produtos Naturais (Michel Passos, Samyra Boeno, Thalya Nogueira, Lara Nascimento, Mayara Arantes, Renata Robaina, Jacqueline César e Mariah Correa) pelo companheirismo, apoio e momentos de descontração;

À FAPERJ pela bolsa concedida;

Ao programa de Pós-graduação em Produção Vegetal pela vaga concedida e por todo suporte prestado.

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RESUMO

MONTEIRO, Noemi Oliveira. D.Sc.; Universidade Estadual do Norte Fluminense Darcy Ribeiro, fevereiro 2023. AVALIAÇÃO DAS ATIVIDADES ANTI-HIPERTENSIVA, ANTIMICOBACTERIANA, ANTI-INFLAMATÓRIA E CITOTÓXICA DE ALCALOIDES INDÓLICOS ISOLADOS DE ESPÉCIES DO GÊNERO *Aspidosperma* (Apocynaceae). Orientador: D.Sc. Ivo José Curcino Vieira. Co-orientador: D.Sc. Raimundo Braz Filho.

Aspidosperma é o mais importante gênero da família Apocynaceae no Brasil, composto por cerca de 57 espécies, todas restritas às regiões tropicais e subtropicais da América. As espécies desse gênero são quimicamente caracterizadas pela produção de alcaloides indólicos monoterpênicos, aos quais são atribuídas diversas atividades biológicas, como atividade antimalárica, antileishmania, anti-inflamatória e anti-hipertensiva. Com o objetivo de investigar novas atividades biológicas neste gênero, foi realizado o estudo fitoquímico das cascas de *A. desmanthum* levando a identificação do alcaloide aspidocarpina (**1**) e a mistura entre um novo alcaloide N_a-hidroxi-11-desmetoxi-10-metoxitubotaiwina (**2**) e 11-metoxitubotaiwina (**3**). Além disso, das sementes de *A. pyricollum* foram identificados os alcaloides estemadenina (**4**) e aparicina (**5**). O isolamento das substâncias foi realizado utilizando as técnicas de cromatografia clássica em coluna e cromatografia em camada delgada preparativa. As determinações

estruturais das substâncias foram realizadas com base em dados espectroscópicos de Ressonância Magnética Nuclear (RMN), Espectrometria de Massas (EM) e comparação com dados descritos na literatura. Essa tese foi dividida em dois trabalhos. No primeiro trabalho, a atividade cardiovascular do alcaloide **1** foi determinada por infusão intravenosa em ratos Wistar e o mecanismo de ação sugerido pelo teste do Rotarod. Esse alcaloide reduziu significativamente ($p < 0,05$) as pressões arteriais sistólica, mediana e diastólica de roedores, sem causar incoordenação motora e desequilíbrio no teste do Rotarod. No segundo trabalho, as atividades antimicobacteriana, anti-inflamatória e citotóxica contra células de macrófago RAW 264.7 e a linhagem celular de leucemia humana Molt-4 dos alcaloides **1-5** foram avaliadas através do micro ensaio colorimétrico utilizando MTT (3-(4,5- dimetiltiazol-2-il)-2,5-difenil brometo de tetrazólio). Os alcaloides apresentaram atividade antimicobacteriana com concentrações inibitórias mínimas (CIM_{50}) variando de 49,9 a ≥500 µg/mL e atividade anti-inflamatória, através da inibição da produção de óxido nítrico (ON), com CI_{50} variando de 18,3 a 138,7 µg/mL. Os compostos não apresentaram citotoxicidade contra as células de macrófagos RAW264.7 e apresentaram valores de CI_{50} variando de 59,8 a ≥500 µg/mL contra as células Molt-4.

ABSTRACT

MONTEIRO, Noemi Oliveira. D.Sc.; State University of North Fluminense Darcy Ribeiro, february 2023; EVALUATION OF ANTI-HYPERTENSIVE, ANTIMYCOBACTERIAL, ANTI-INFLAMMATORY AND CYTOTOXIC ACTIVITIES OF ISOLATED INDOLE ALKALOIDS FROM SPECIES OF THE GENUS *Aspidosperma* (Apocynaceae). Advisor: D.Sc. Ivo José Curcino Vieira. Co-supervisor: D.Sc. Raimundo Braz Filho.

Aspidosperma is the most important genus of the Apocynaceae family in Brazil, comprising about 57 species, all restricted to tropical and subtropical regions of America. Species of this genus are chemically characterized by the production of monoterpene indole alkaloids, to which several biological activities are attributed, such as antimalarial, antileishmania, anti-inflammatory and antihypertensive activity. With the objective of investigating new biological activities in this genus, a phytochemical study of the bark of *A. desmanthum* was carried out, leading to the identification of the alkaloid aspidocarpine **(1)** and the mixture between a new alkaloid N_a-hydroxy-11-demethoxy-10-methoxytubotaiwine **(2)** and 11-methoxytubotaiwine **(3)**. Furthermore, from the seeds of *A. pyricollum*, the alkaloids stemmadenine **(4)** and apparicine **(5)** were identified. The isolation of substances was carried out using column chromatography (CC) and preparative thin layer chromatography (PTLC) techniques. The chemical profiles were then

analysed by NMR analysis, mass spectrometry, and comparison with data described in the literature. This thesis was divided into two works. In the first work, the cardiovascular activity of alkaloid **1** was determined by intravenous infusion in Wistar rats and the mechanism of action suggested by the rotarod test. This alkaloid significantly reduced ($p < 0.05$) systolic, median and diastolic blood pressure in rodents, without causing motor incoordination and imbalance in the rotarod test. In the second work, the antimycobacterial, anti-inflammatory and cytotoxic activities against RAW 264.7 macrophage cells and the human leukemia cell line Molt-4 of alkaloids **1-5** were evaluated through the colorimetric micro assay using MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The alkaloids showed antimycobacterial activity with MIC_{50} ranging from 49.9 to ≥500 µg/mL and anti-inflammatory activity, through inhibition of nitric oxide production (NO), with IC_{50} ranging from 18.3 to 138.7 µg/mL. The compounds did not show cytotoxicity against RAW264.7 macrophage cells and showed IC_{50} values ranging from 59.8 to ≥500 µg/mL against Molt-4 cells.

1. INTRODUÇÃO

Ao longo da história, as plantas têm sido utilizadas como fonte de substâncias naturais para o tratamento de várias doenças e até os dias atuais continuam desempenhando um importante papel na descoberta de novos medicamentos (Atanasov et al.; Dey; Mukherjee, 2018).

Boa parte das drogas prescritas no mundo são de origem vegetal. Estima-se que pelo menos 25% de todos os medicamentos modernos e até 60% dos medicamentos antitumorais e antimicrobianos sejam derivados de produtos naturais (Newman & Cragg, 2016; WHO, 2011).

As propriedades terapêuticas de espécies vegetais têm sido associadas, pelo menos em parte, com a ocorrência de metabólitos secundários, que têm demonstrado exercer uma série de atividades biológicas (Vo & Kim, 2013).

Neste sentido, a família Apocynaceae é uma das mais importantes fontes vegetais de metabólitos secundários, destacando-se pela produção de alcaloides com grande diversidade estrutural. Dentro desta família, as espécies do gênero *Aspidosperma*, caracterizam-se pela produção de alcaloides indólicos monoterpênicos, frequentemente associados à atividade antimalárica, antileishmaniose e doenças inflamatórias (Brandão et al., 1992; Brandão et al., 2006).

Desta forma, mediante a importância química e biológica das substâncias bioativas do gênero *Aspidosperma*, este trabalho tem como objetivo avaliar as atividades anti-hipertensiva, antimicobacteriana, anti-inflamatória e citotóxica dos alcaloides isolados das espécies *A. desmanthum e A. pyricollum*.

2. REVISÃO BIBLIOGRÁFICA

2.1 Família Apocynaceae

A família Apocynaceae pertence ao reino Plantae, classe Magnoliopsida, subclasse Asteridae, ordem Gentianiales e divisão Angiospermae, possui 4600 espécies distribuídas em 424 gêneros, agrupados em 17 tribos pertencentes a cinco subfamílias: Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae e Asclepiadoideae (Endress et al., 2014).

Os membros desta família podem ser encontrados em regiões tropicais, subtropicais e zonas temperadas; apresentam-se como árvores, arbustos, subarbustos e ervas rasteiras que contêm um látex leitoso (Endress; Bruyns, 2000; Endress; Liede-Schumann; Meve, 2014; Bhadane et al., 2018). Este látex pode conter em sua composição hidrocarbonetos poliisoprênicos (borracha), triterpenos, ácidos graxos, fitoesteróis e alcaloides, substâncias que conferem à planta propriedades cicatrizantes e proteção contra herbívoria (Demarco et al., 2006).

Várias espécies são amplamente cultivadas ornamentalmente, como a *Nerium oleander*, *Allamanda cathartica, Acanthoscurria violacea, Wrightia tinctoria,* entre outras (Joselin et al., 2012); já algumas espécies, conhecidas

popularmente como “peroba” e “guatambu”, são comercialmente importantes para a fabricação de móveis (Guimarães et al., 2012). Além disso, alguns membros de Apocynaceae são consumidos por pessoas na área rural como alimento e outros são usados como veneno (Aiyambo, 2010).

Grande parte das plantas desta família são quimicamente caracterizadas pela produção de alcaloides, que apresentam grande diversidade estrutural e importância medicinal (Dey et al., 2017).

Entre os alcaloides isolados em Apocynaceae, a vincristina **(1)** e vimblastina **(2)**, isolados de *Catharanthus roseus*, destacam-se na medicina por apresentarem uma marcante atividade antitumoral (Dewick, 2002; Alam et al., 2017).

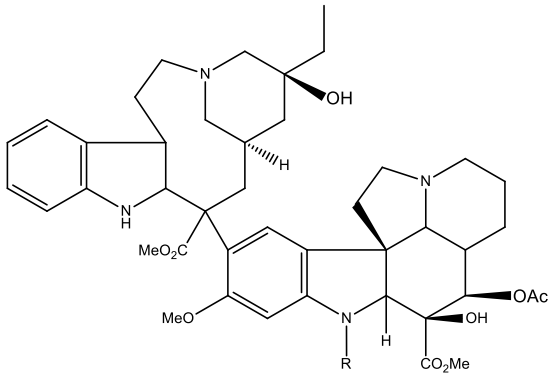


Figura 1: Estrutura das substâncias **1**-**2**

Além disso, outros alcaloides isolados de plantas dessa família são conhecidos por desempenharem as mais diversas atividades biológicas, como por exemplo a ajmalicina **(3)** que funciona como agente antiarrítmico, alstonina **(4)** que possui propriedades antipsicótica, reserpina **(5)** atividade anti-hipertensiva (Deshmukh et al., 2012), aspidospermina **(6)** atividade antiprotozoárias (Mitaine-Offer et al., 2002), entre outras.

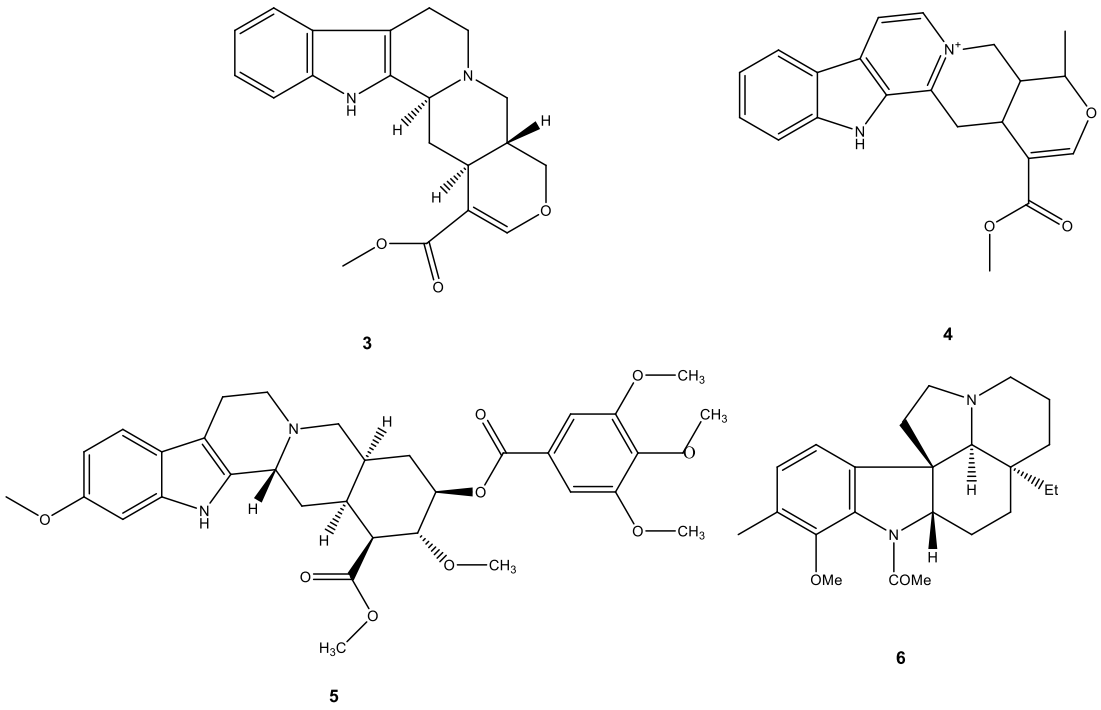


Figura 2: Estruturas das substâncias **3**-**6**

2.2 Gênero *Aspidosperma*

O gênero *Aspidosperma* está incluído na subfamília Rauwolfioideae, da tribo Alstoniae (Endress & Bruyns, 2000). O gênero inclui 52 espécies divididas nas séries: Rigida, Nitida, Quebranquinas, Polyneura, Pyricolla, Nobile, Macrocarpa e Tomentosa (**Tabela 1**), todas restritas às regiões tropicais e subtropicais da América (Pereira et al., 2016). No Brasil, as espécies de *Aspidosperma* ocorrem nas regiões da floresta amazônica, mata atlântica, cerrado e caatinga (Almeida et al., 2019).

Na Amazônia brasileira, as espécies de *Aspidosperma* são popularmente conhecidas como carapanaúba ou araracanga, enquanto em outras regiões do país elas são conhecidas principalmente como peroba, guatambu ou pereiro (Koch et al., 2015).

Tabela 1. Classificação das espécies de *Aspidosperma*

Série	Espécies
Rigida	*A. rigidum*
Nitida	*A. auriculatum, A. carapanauba, A. compactinervium, A. discolor, A. eburneum, A. excelsum, A. marcgravianum, A. nitidum, A. oblongum e A. spegazzinii*
Quebrachines	*A. chakensis e A. quebracho-blanco*
Polyneura	*A. cuspa, A. cylindrocarpon, A. dispermum, A. peroba, A. polyneuron e A. sessiflorum.*
Pyricolla	*A. australe, A. campus-belus, A. gomesianum, A. multiflorum, A. nigricans, A. olivaceum. A. parvifolium, A. populifolium, A. pyricollum, A. pyrifolium, A. quirandy, A. refractum, A. rhombeosignatum, A. subincanum, A. tomentosum, A. ulei e A. vargasii.*
Nobile	*A. album, A. desmanthum, A. exalatum, A. fendleri, A. limae, A. megalocarpon, A. melanocalyx, A. neblinae, A. obscurinervium, A. sandwithianum e A. spruceanum*
Macrocarpa	*A. duckei, A. macrocarpon e A. verbascifolium*
Tomentosa	*A. formosanum e A. dasycarpon*

Aspidosperma está entre os gêneros de Apocynaceae de alto valor comercial devido à boa qualidade da sua madeira. Além disso, as cascas de algumas espécies são usadas na forma de infusões pela medicina popular para tratar várias doenças (De Oliveira et al., 2009; De Almeida et al., 2019).

Em relação à composição química do gênero, destaca-se a presença de alcaloides indólicos monoterpênicos, com considerável diversidade estrutural, podendo ser encontrados em diferentes partes do vegetal (Oliveira et al., 2009; De Almeida et al., 2019).

Entre as espécies de *Aspidosperma*, foram isolados aproximadamente 250 alcaloides indólicos. Estas substâncias, além de apresentarem diversas atividades biológicas, também são consideradas marcadores quimiotaxonômicos para classificação botânica das espécies deste gênero (Pereira et al., 2007; Henrique; Nunomura; Pohlit, 2010).

2.2.1 Atividades biológicas de espécies do gênero *Aspidosperma*

As espécies de *Aspidosperma* são conhecidas popularmente por apresentarem várias aplicações terapêuticas, o que tem despertado o interesse de pesquisadores. Estudos realizados com extratos destas plantas têm relatado uma grande diversidade de atividades biológicas, como mostra a **tabela 2**.

Tabela 2. Atividades biológicas de extratos de espécies de *Aspidosperma*

Espécie	Extrato	Parte da Planta	Atividade Biológica	Referência
A. Rigidum	Diclorometano e butanol	Casca	Antiplasmodial	(Oliveira et al., 2015)
A. excelsum	Diclorometano	Casca	Antiplasmodial	(De Nascimento et al., 2019)
	Metanólico	Casca	Antimicrobiana	(Roumy et al., 2020)
A. nitidum	Etanólico	Casca	Antiplasmodial	(Coutinho et al., 2013)
A. ramiflorum	Etanólico	Casca	Antibacteriana	(De Oliveira et al., 2009)
A. olivaceum	Metanólico	Casca e folhas	Antiplasmodial	(Chierrito et al., 2014)
A. pyrifolium	Etanólico	Casca	Antiplasmodial	(Ceravolo et al., 2018)
A. subincanum	Diclorometano e Etanólico	Folhas e galhos	Inibidora da AChE Antioxidante Antibacteriano	(Rocha et al., 2018)
	Etanólico	Casca	Diurética	(Ribeiro et al., 2015)
	Etanólico	Casca	Hipotensiva	(Bernardes et al., 2013)
A. tomentosum	Etanólico	Casca	Antinociceptiva e anti-inflamatória	(De Aquino et al., 2013)
	Etanólico	Caule	Antileishmanial	(De Paula et al., 2019)
	Etanólico	Folhas e madeira	Antiplasmodial	(Dolabela, 2012)
	Etanólico	Casca	Anti-hipertensiva e Vasorelaxante	(Furtado et al., 2017)
A. ulei	Etanólico	Casca da Raiz	Relaxante	(Campos et al., 2008)

Tabela 2, Cont.

	Etanólico	Casca da Raiz	Pró-eréctil	(Campos et al., 2006)
A. fendleri	Etanólico	Sementes	Hipotensiva e bradicárdia	(Estrada et al., 2015)
A. macrocarpon	Etanólico	Casca da Raiz	Antiplasmodial	(De Mesquita et al., 2007)
A. cuspa	Aquoso	Casca	Antinociceptiva	(Pérez et al., 2012)

Os alcaloides indólicos isolados neste gênero apresentam principalmente atividade antiprotozoária (Brandão et al., 2006). Como exemplo, pode-se citar os alcaloides aspidocarpina **(7)** e aparicina **(8)**, isolados das cascas do caule de *A. olivaceum,* que demonstraram potente atividade contra o *Plasmodium falciparum*, apresentando valores de CI_{50} de 5,4 ± 2,5 µg/mL e 3,0 ± 1,4 µg/mL, respectivamente (Chierrito et al., 2014).

Já os alcaloides ramiflorina A **(9)** e ramiflorina B **(10)** obtidos da casca do caule de *Aspidosperma ramiflorum* mostraram atividade *in vitro* contra as formas promastigotas de *Leishmania amazonensis*, com valores de DL_{50} de 18,5 ± 6,5 µg/mL e 12,6 ± 5,5 µg/mL, respectivamente (Tanaka et al., 2007; Cunha et al., 2012). Além disso, também apresentaram atividade significativa contra *S. aureus* (CIM = 25 µg / mL) e *E. faecalis* (CIM = 50 µg / mL) (Tanaka et al., 2006).

A atividade anticancerígena também tem sido relatada, especialmente para o alcaloide ellipticina **(11)**, comumente encontrado em espécies de *Aspidosperma* (Woodward et al., 1959). Esta substância apresentou citotoxicidade contra adenocarcinoma de mama MCF (IC_{50} 1,25 ± 0,13µM), células leucêmicas HL-60 (CI_{50} 0,67 ± 0,06 µM) e CCRF-CEM (CI_{50} 4,70 ± 0,48 µM), neuroblastoma IMR-32 (CI_{50} 0,27 ± 0,02 µM) e UKF-NB (CI_{50} 0,44 ± 0,03 µM) e glioblastoma U87MG (CI_{50} 1,48 ± 0,62 µM) (Stiborová et al., 2011). Os mecanismos propostos para a atividade antitumoral envolvem intercalação com o DNA, inibição da atividade da topoisomerase II e ligação covalente ao DNA (Miller & McCarthy, 2012).

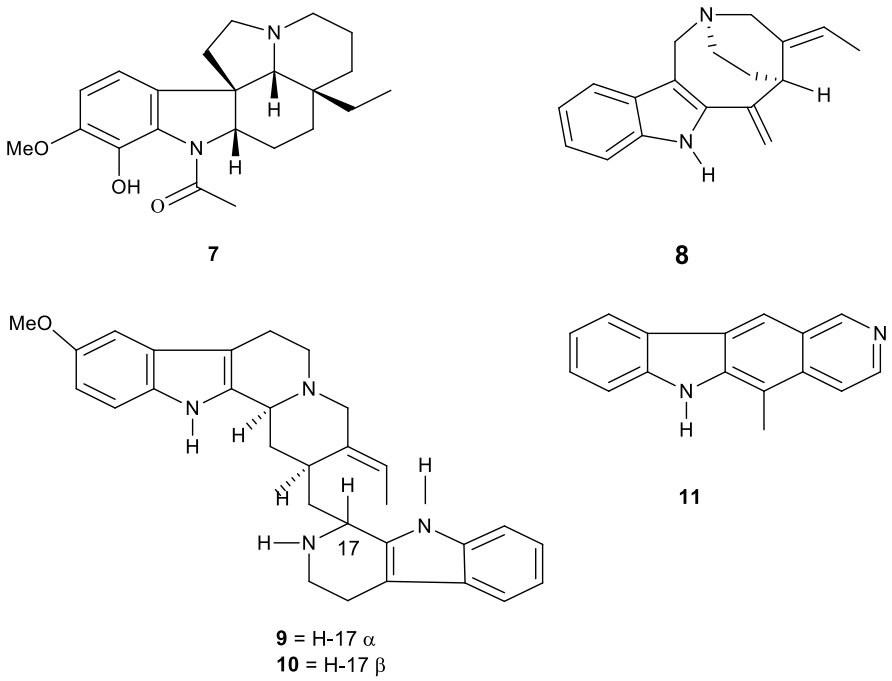


Figura 3: Estrutura das substâncias **7-11**

2.3 *Aspidosperma desmanthum*

Conhecida popularmente como araracanga, araraúba, araraíba, pau-marfim, para-tudo-branco, jacamim, peroba, pequiá, pequiá-marfim-do-roxo, amargoso, pau-de-arara, araracanga-preta, marfim, peroba-mica, araracanga-vermelha, pequiá-marfim, araracanga-marfim, quina, piquiá-marfim-do-roxo e quina-da-mata (Cruz, 2018).

É caracterizada por árvores que medem de 10 a 35 m de altura e troncos com diâmetro de 30 a 70 cm, revestido por uma espessa camada de cortiça, apresentando galhos pequenos, porém resistentes. As folhas são alternas ou aproximadas, oval-alongadas ou arredondadas, medindo de 7-15 cm de comprimento e 3-7 cm de largura, apresentando coloração verde claro e látex avermelhado. As flores são amareladas e florescem no período de junho a setembro. O fruto é deiscente, orbicular, oblíquo, ligeiramente acuminado, contendo de 8 a 10 sementes aladas (**Figura 4**) (Woodson, 1951; Pereira et al., 2016).

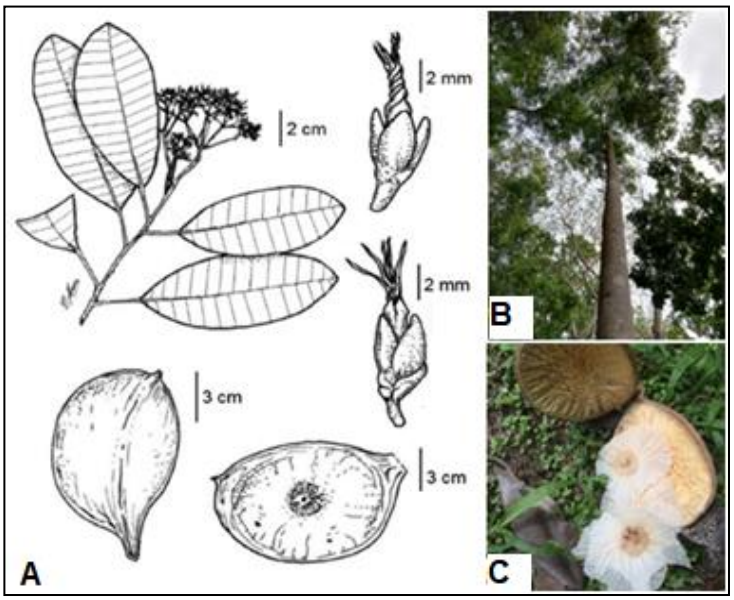
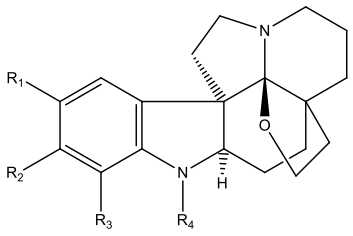
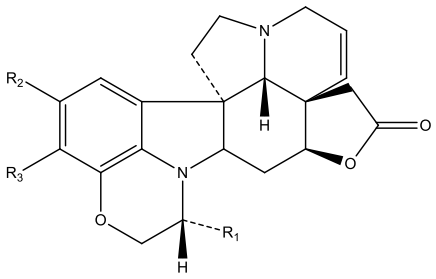


Figura 4: (**A**) Esquema botânico da espécie *A. desmanthum* (Pereira, Simões, Santos, 2016). (B) árvore de *A. desmanthum e* (C) Fruto e sementes. Fonte: https://www.inaturalist.org/observations/21651145.

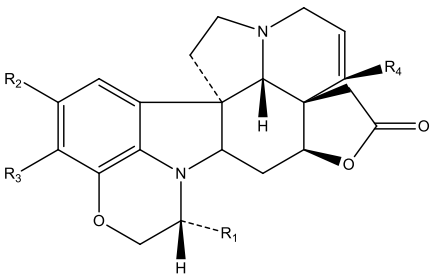
Na revisão realizada por Pereira e colaboradores (2007) foi relatada a presença dos alcaloides aspidoalbina **(12)**, N-acetilaspidoalbina **(13)** e Des-O-metilaspidolimidina **(14)** em *A. desmanthum***.** Posteriormente, um estudo fitoquímico desta espécie relatou o isolamento das substâncias: obscurinervina **(15)**, obscurinervidina **(16)**, dihidroobscurinervina **(17)**, 6β-hidroxi-dihidroobscurinervina **(18)**, E-akuammidina **(19)**, pagicerina **(20)**, aspidodasycarpina **(21)**, spruceanumina A **(22)**, desacetilakuammilina **(23)**, aspidolimina **(24)**, aspidolimidina **(25)**, aspidocarpina **(7)**, rhazinilam **(26)**, 19,20-dihidro-11-metoxi-(+)-limatinina **(27)**, 19,20-dihidro-11-metoxi-(+)-limatina **(28).** Estes alcaloides foram isolados de diferentes partes da planta (Reina et al., 2014).



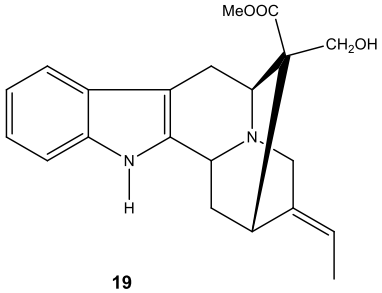
12: R_1= R_2= OMe; R_3= OH; R_4=COEt
13: R_1= R_2= R_3= OMe; R_4= COEt
14: R_1= R_2= OMe; R_3= OH; R_4= COMe



15: R_1= CH_2CH_3; R_2= R_3= OMe
16: R_1= CH_3; R_2= R_3= OMe



17: R_1= CH_2CH_3; R_2= R_3= OMe; R_4= H
18: R_1= CH_3; R_2= R_3= OMe; R_4= OH



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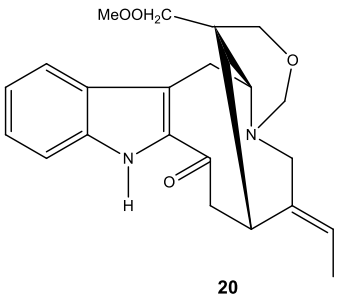

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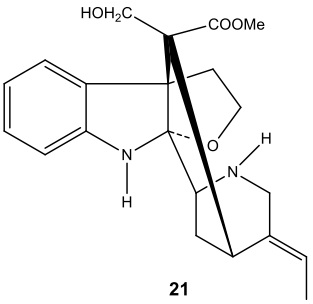

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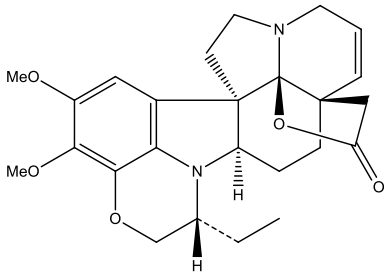

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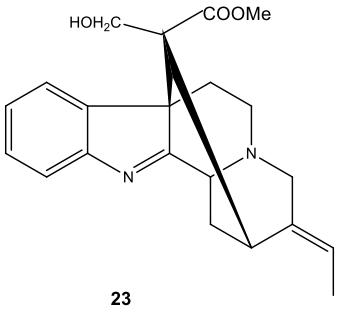

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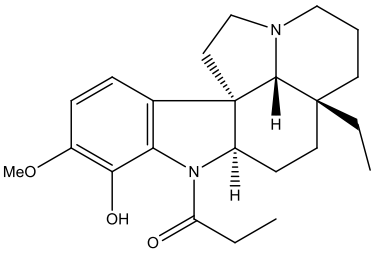

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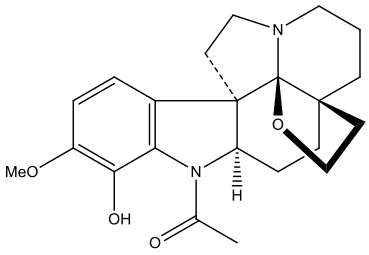

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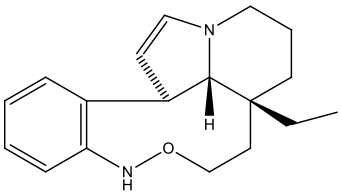

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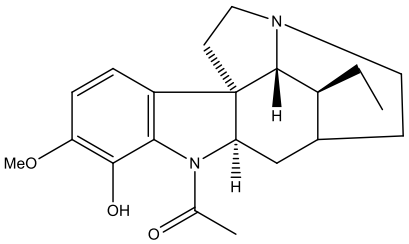

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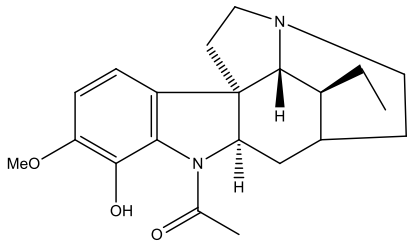

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Figura 5: Estrutura das substâncias **12**-**28**.

2.4 *Aspidosperma pyricollum*

Conhecida popularmente como guatambu, pequiá, pequiá da restinga e amarelão. Esta espécie é caracterizada por árvores que medem de 3 a 10 m de altura, os galhos são relativamente delgados. As folhas possuem látex branco, formato oval com extremidades agudas a arredondadas, disposição alterna e medem 3-10 cm. O fruto é lenhoso, marrom e com lenticelas brancas. A floração ocorre de outubro a dezembro (**Figura 6**) (Woodson, 1951).

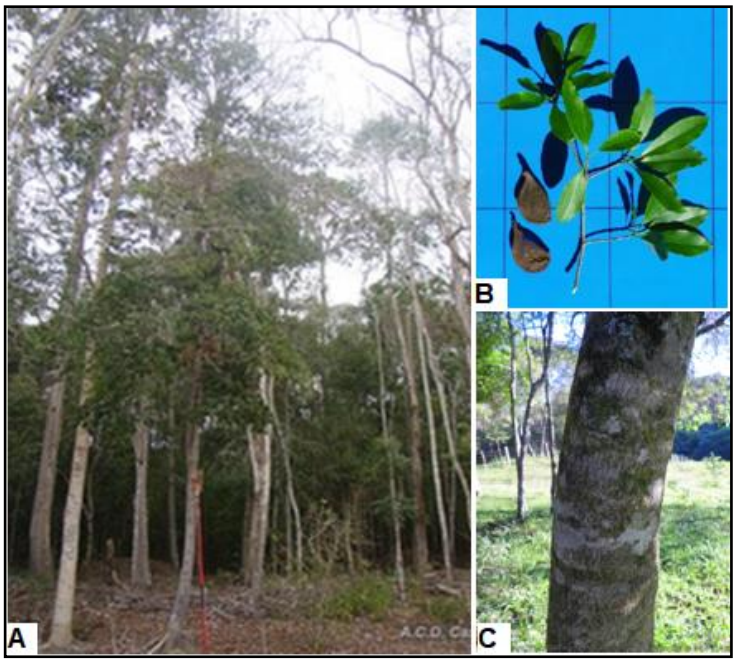

Figura 6: (**A**) Árvores da espécie *A. pyricollum.* **(B)** Folhas e Frutos de *A. pyricollum* e **(C)** Tronco. Fonte: https://www.inaturalist.org/observations/21651145.

A revisão realizada por Pereira e colaboradores (2007), relatou a presença dos alcaloide (+)-aspidospermina **(6)**, (+)-Des-*O*-metilaspidospermina **(29)**, (–)-β-ioimbina **(30)**, heteroioimbina **(31)** 19,20-Desidro-β-ioimbina **(32)**, estemadenina **(33),** uleína **(34),** 3-epi-uleína **(35)**, dasicarpidona **(36) e** (–)-aparicina **(8)**. Posteriormente, as substâncias pyricolluminol **(37)** e sitsirikina **(38)** foram isoladas das cascas dessa planta (do Carmo, Braz-Filho, Vieira, 2015).

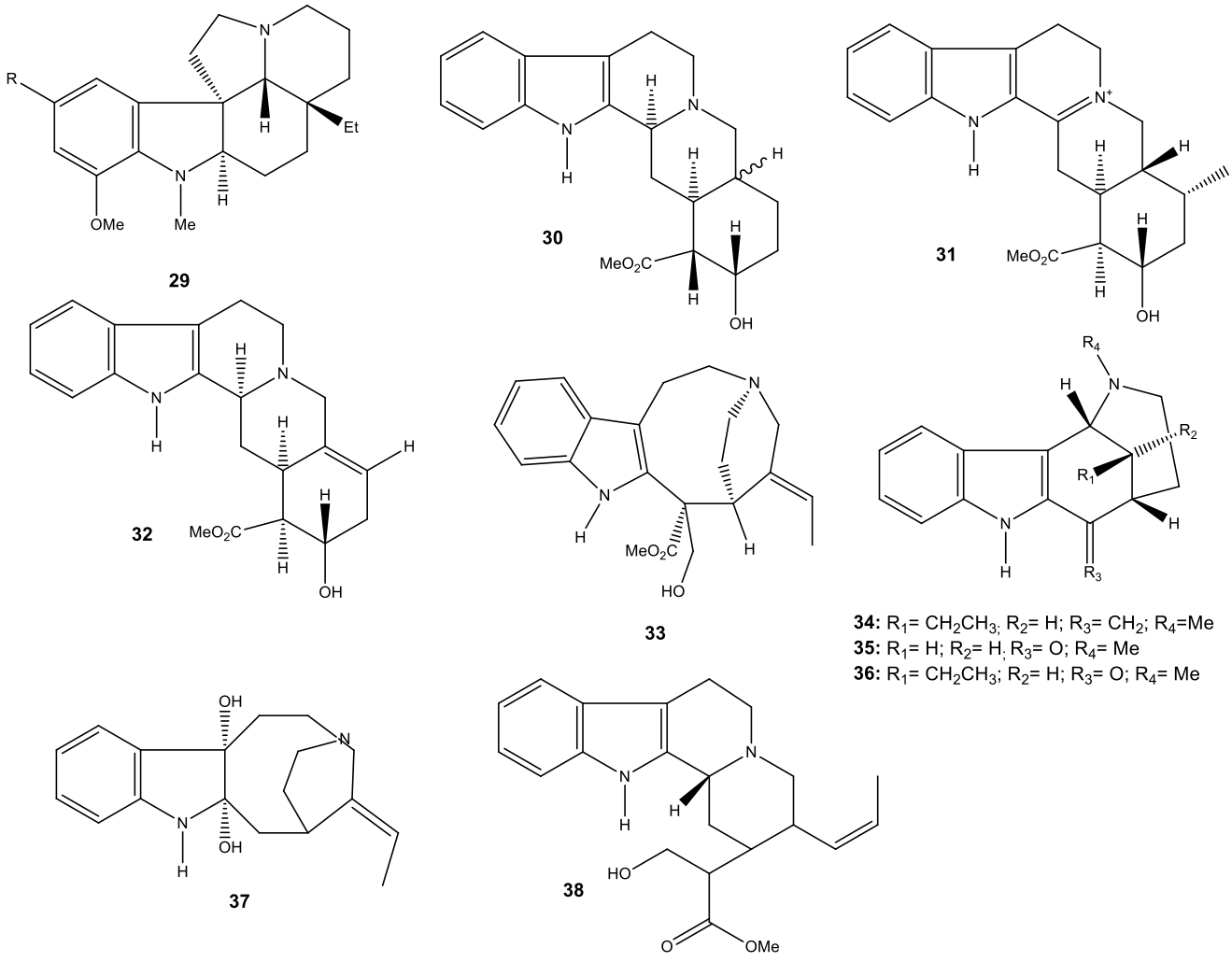

Figura 7: Estrutura das substâncias **29**-**38.**

2.5 Alcaloides

A palavra alcaloide foi relatada por Meisner e colaboradores em 1819 e refere-se a alcalino. Os alcaloides são compostos heterocíclicos de origem biológica, que contém um ou mais átomos de nitrogênio ligados a pelo menos dois átomos de carbono (Jayakumar & Murugan, 2016). Em sua estrutura o átomo de nitrogênio é derivado de aminoácidos, enquanto o esqueleto carbônico pode ser derivado de acetato, terpenoides, esteroides, entre outros. A classificação dos alcaloides ocorre de acordo com o aminoácido precursor (Dewick, 2008).

No caso dos alcaloides indólicos, o aminoácido precursor é triptofano, e podem ser classificados em dois grupos:

1. Simples: formados por reações simples de descarboxilação, metilação ou hidroxilação do L-triptofano, tendo como resultado os alcaloides triptamina e serotonina (**Figura 8**).

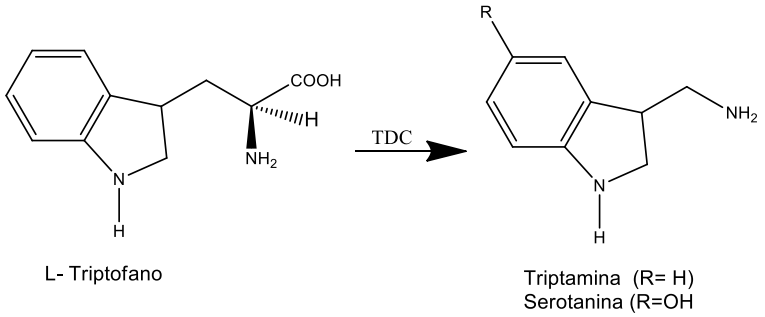

Figura 8: Reação promovida pela enzima triptofano-descarboxilase (TDC).

2. Monoterpênicos: A estrictosidina forma o esqueleto básico dos alcaloides indólicos monoterpênicos.

A estrictosidina é um glicosídeo formado pela condensação de triptamina com a secologanina, constituindo as suas unidades indólicas e terpênica, respectivamente (**Figura 9A**). Na maioria dos casos, o esqueleto da triptamina é conservado e a secolonanina pode ser reorganizada (**Figura 9B**) (Dewick, 2002).

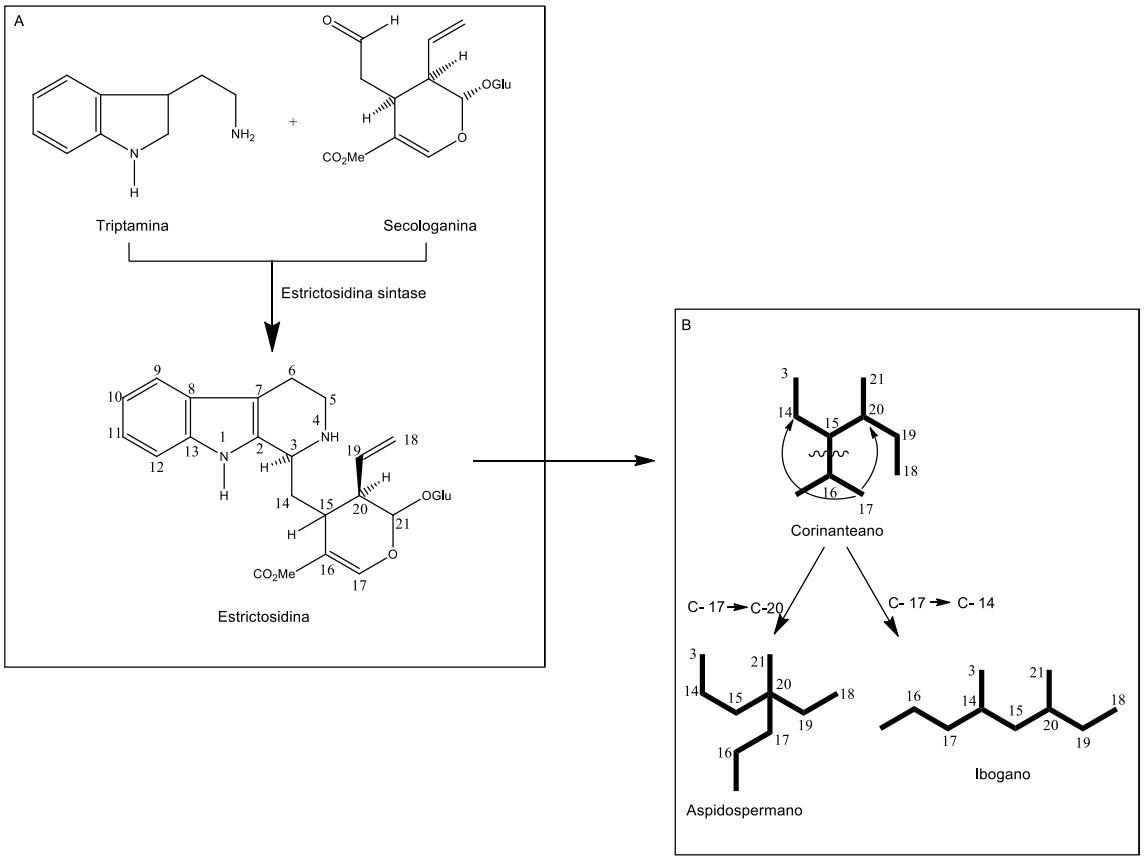

Figura 9: Via Biosintética dos alcaloides indólicos monoterpênicos. A) Reação de formação de estrictosidina e B) Rearranjos na cadeia terpênica da estrictosidina.

Rearranjos na parte terpênica da estrictosidina levam à formação de três classes diferentes de alcaloides indólicos: corinanteano (unidade monoterpênica não sofre rearranjo), aspidospermano (rearranjo de carbono C-17→ C-20) e Ibogano (rearranjo de carbono C-17 → C-14). Cada uma dessas classes pode ser subdividida em três, formando nove subclasses principais, apresentadas na **figura 10** (Danieli & Palmisano, 1986).

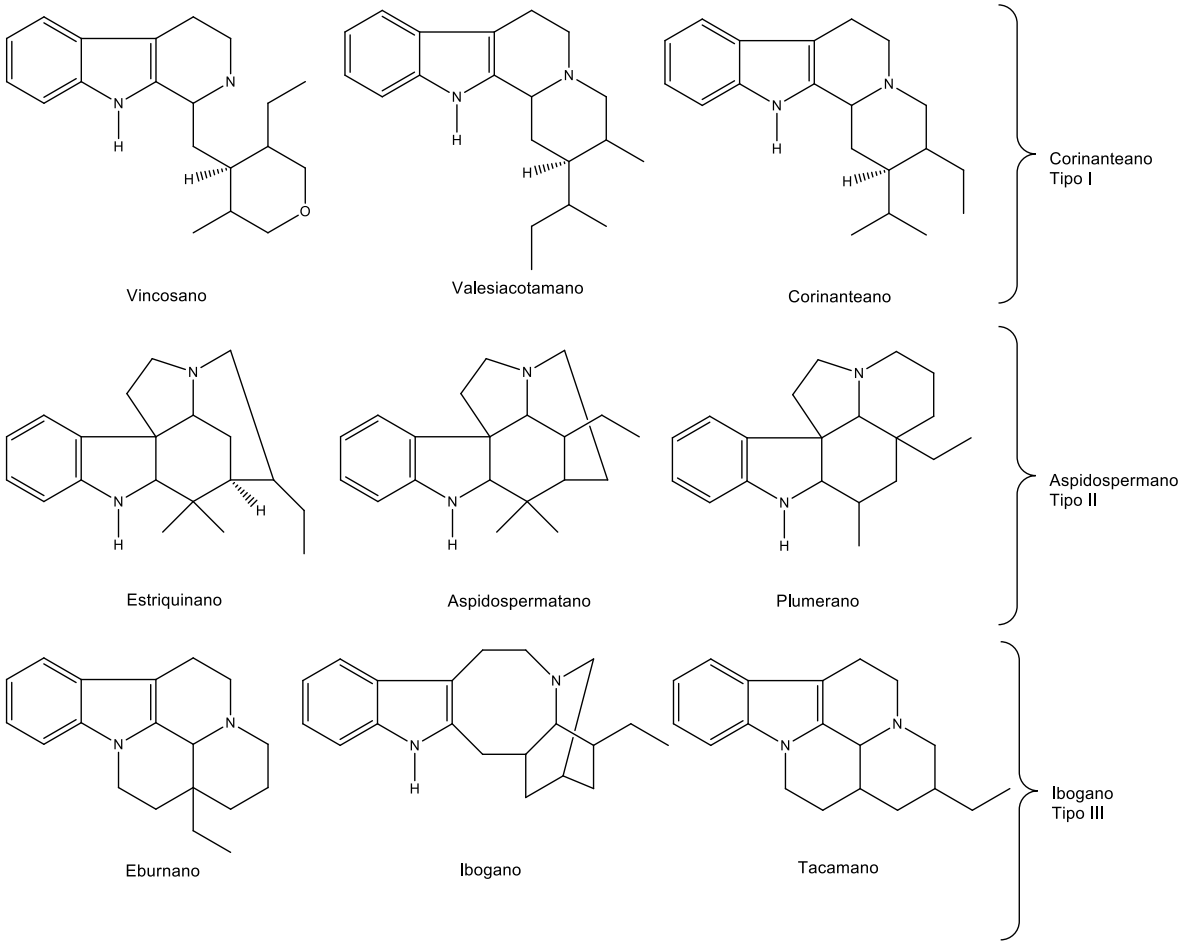

Figura 10: Esqueletos das nove subclasses de alcaloides indólicos monoterpênicos.

3. TRABALHOS

3.1 Antihypertensive Activity of the Alkaloid Aspidocarpine in Normotensive Wistar Rats

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Molecules 2022, 27(20), 6895; https://doi.org/10.3390/molecules27206895
Received: 10 September 2022 / **Revised:** 2 October 2022 / **Accepted:** 10 October 2022 / **Published:** 14 October 2022

ABSTRACT

The alkaloid Aspidocarpine was isolated from the bark of *Aspidosperma desmanthum*. Its structure was elucidated by spectral data of ^{1}H and ^{13}C-NMR (1D and 2D) and high resolution spectrometry mass (HRESIMS). The antihypertensive activity was investigated by intravenous infusion in Wistar rats. This alkaloid significantly reduced ($p < 0.05$) the systolic, median, and diastolic blood pressures of rodents, without causing motor incoordination and imbalance in the rotarod test. The results indicate that the alkaloid Aspidocarpine exerts its antihypertensive activity without causing sedation and impairment of motor functions.

Keywords: *Aspidosperma desmanthum*; alkaloid; Aspidocarpine; blood pressure; rotarod

1. INTRODUCTION

Hypertension is a major public health problem and the leading cause of cardiovascular mortality worldwide, mainly affecting people living in low- and

middle-income countries (Chockalingam, 2008). Although this disease can be controlled through pharmacological interventions, the drugs commonly used can present several side effects and high cost. (Bachheti et al., 2022). In this sense, medicinal plants represent a good alternative, as many have demonstrated hypotensive capacity, causing few side effects and low cost. (Chan et al., 2000; Greenway et al., 2011).

The genus *Aspidosperma* (Apocynaceae) is composed of approximately 50 species, all restricted to tropical and subtropical regions of America, where they are popularly known as "peroba" and "guatambu" (Bolzani et al., 1987; Garcia & Brown, 1976; A. S. de S. Pereira et al., 2016). Many of this species are used in folk medicine for the treatment of malaria (TOMCHINSKY et al., 2017), fever (D. R. Oliveira et al., 2015), inflammation, diabetes, high blood pressure (Vásquez et al., 2014), and cardiovascular diseases (Ribeiro et al., 2015). In addition, studies carried out with extracts from several species of *Aspidosperma* showed important antihypertensive effects (Bernardes et al., 2013; Estrada et al., 2015; Herculano et al., 2012; M. P. Oliveira et al., 2012).

The biological activities presented by plant species have been associated with the occurrence of secondary metabolites (Vo & Kim, 2013). In *Aspidosperma*, the most abundant secondary metabolites are indole alkaloids, to which several biological activities are attributed (Chierrito et al., 2014), such as antiplasmodial (Mitaine-Offer et al., 2002), antiprotozoal (Reina et al., 2014), cytotoxic (Bannwart et al., 2013), anti-inflammatory (Shang et al., 2010), and antihypertensive (Gao & Li, 2021).

Considering the biological importance of alkaloids, the antihypertensive activity of the alkaloid Aspidocarpine, isolated from the bark of *A. desmanthum*, was determined. This work presents the first report of the antihypertensive activity of the alkaloid Aspidocarpine.

2. RESULTS AND DISCUSSION

2.1. Identification and Structure of the Compound

The chromatographic fractionation of the dichloromethane partition, obtained through the methanolic extract of the bark of *Aspidosperma desmathum*, led to the isolation of the alkaloid Aspidocarpine (Figure 1). The chemical profile was then analyzed by mass spectrometry, NMR analysis (Supplementary material), and by comparing its spectral data from ^{1}H-, ^{13}C - NMR with values from the literature.

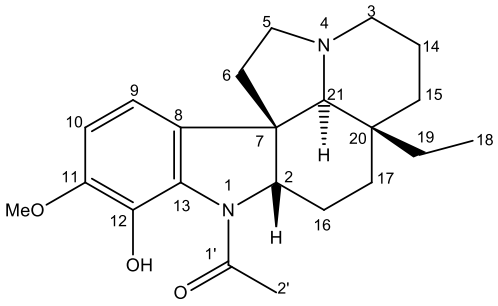

Figure 1. Chemical structure of Aspidocarpine.

Aspidocarpine was obtained as a yellow crystal. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 10.91 (s, OH-12), 6.60 (d, *J* = 8.1, H-10), 6.52 (d, *J* = 8.1, H-9), 4.03 (dd, *J* = 11.3, 6.2, H-2), 3.77 (s, OMe-11), 3.06 (td, *J* = 9.0, 2.1, H-5a), 2.97 (br d, *J* = 10.9, H-3a), 2.23 (s, 3H-2'), 2.20 (s, H-21), 2.20 (m, H-14a), 2.15 (m, H-5b), 1.98 (m, H-6a), 1.98 (m, H-17a), 1.92 (m, H-14b), 1.90 (m, H-3b), 1.75 (m, H-16a), 1.60 (m, H-15a), 1.45 (m, H-6b), 1.40 (m, H-16b), 1.25 (m, H-19a), 1.02 (m, H-15b), 1.02 (m, H-17b), 0.80 (m, H-19b) and 0.64 (t, J = 7.4, 3H-18). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ_C 169.2 (C-1'), 149.2 (C-11), 137.3 (C-12), 132.9 (C-8), 127.4 (C-13), 112.3 (CH-9), 109.9 (CH-10), 70.6 (CH-21), 69.3 (CH-2), 56.4 (OMe-11), 53.5 (CH_2-3), 52.2 (CH_2-5), 51.9 (C-7), 39.1 (CH_2-6), 35.7 (C-20), 33.8 (CH_2-15), 30.0 (CH_2-19), 24.9 (CH_2-16), 22.7 (CH_2-17), 22.6 (CH_3-2'), 21.2 (CH_2-14), 7.0 (CH_3-18). The molecular formula was confirmed to be $C_{22}H_{30}N_2O_3$ by its HRESIMS (*m/z* = 371.2324 $[M+H]^+$, $C_{22}H_{31}N_2O_3$). The data are in accordance with those previously published (Andrade-Neto et al., 2007; Guimarães et al., 2012; Henrique et al., 2010; McLean et al., 1960, 1987).

2.2. In Vivo Blood Pressure Assessment

Indole alkaloids are the main secondary metabolites produced in *Aspidosperma* and are responsible for most of the biological activities reported in the genus, including antihypertensive activity. An intravenous infusion of the alkaloid Aspidocarpine (1 and 3 mg/kg) was administered in rats to investigate whether this compound can cause changes in blood pressure. After administration of a 1mg/kg infusion of Aspidocarpine, systolic and diastolic pressures were significantly reduced in relation to negative controls and a positive control (DMSO), and after administration of a 3mg/kg infusion, all parameters analyzed were significantly reduced in relation to negative controls and a positive control (DMSO) in Wistar rats at 5% probability (Figure 2).

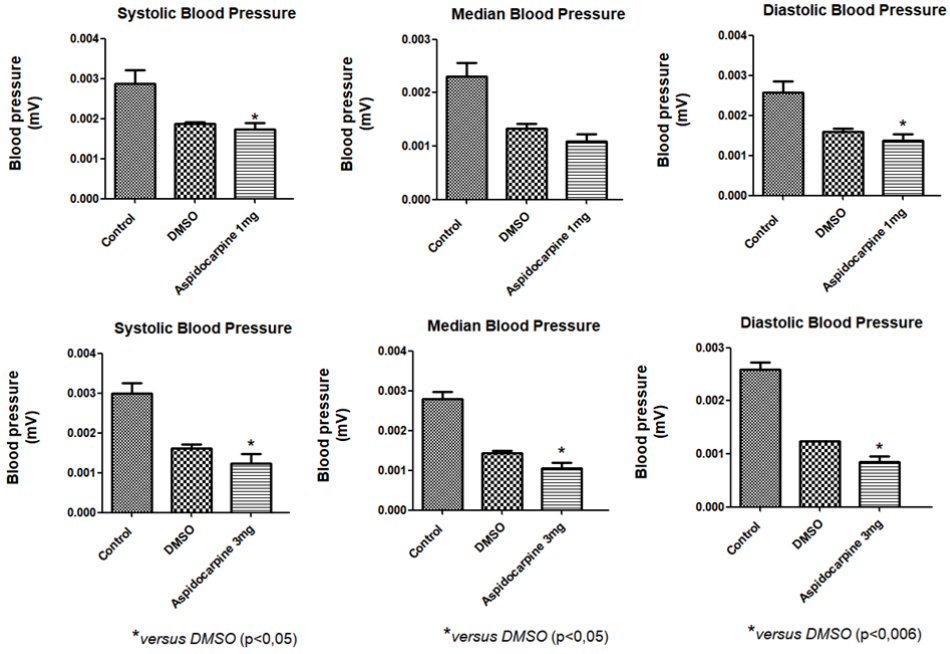

Figure 2. Effects of Aspidocarpine (1 and 3 mg/kg) on blood pressure in Wistar rats. The values are expressed as mean ± SEM (n=4). One-way ANOVA followed by Newman-Keuls test (* $p < 0.05$ compared to control).

The Tubotaiwine alkaloid, commonly isolated from *Aspidosperma* (M. de M. Pereira et al., 2007), significantly reduced the cadmium-induced increase in systolic and diastolic blood pressure in rats at doses of 2.5, 5, and 10 mg/kg (Gao & Li, 2021). In the present study, Aspidocarpine showed a similar antihypertensive response, but in lower concentrations. In addition, extracts of *A. subincanum* (Bernardes et al., 2013), *A. pyrifolium* (Herculano et al., 2012), *A. fendleri* (Estrada

et al., 2015), and *A. macrocarpum* (M. P. Oliveira et al., 2012) showed significant hypotensive effects, consistent with the results presented by Aspidocarpine. These reports confirm that the genus *Aspidosperma* is an important source of alkaloids with cardiovascular activity.

2.3. Rotarod Test

To assess the possible effects of Aspidocarpine on motor performance, the rotarod test was used. Intraperitoneal administration of 1 mg/kg and 3 mg/kg Aspidocarpine did not affect the time the mice remained on the rotating bar. In contrast, diazepam (positive control) significantly reduced locomotor activity and performance on the rotarod (Table 1).

Table 1. Effects of the Aspidocarpine on Swiss mice (*Mus musculus*) in the Rotarod test.

	5 min	15 min	30 min	60 min
Control	180.00 s ± 0.00	180.00 s ± 0.00	180.00 s ± 0.00	180.00 s ± 0.00
DZP (5mg/Kg)	178.14 s ± 1.29*	179.45 s ± 0.94*	179.47 s ± 0.83*	179.51 s ± 0.81*
ASP (1mg/Kg)	179.82 s ± 0.36	180.00 s ± 0.00	179.78 s ± 0.42	179.70 s ± 0.59
ASP (3mg/Kg)	179.62 s ± 0.59	179.26 s ± 0.83	179.95 s ± 0.13	180.00 s ± 0.00

Values are expressed as means ± SEM (n=6). One-way ANOVA followed by Newman-Keuls test (* $p < 0.05$ compared to control). DZP= Diazepam, ASP= Aspidocarpine. Permanence in seconds (s).

The treatment with Aspidocarpine did not produce motor incoordination, hypolocomotion, immobility, clinical signs of sedation or stimulant effect in mice, and did not cause lethality at the doses studied. This result is in agreement with previous studies that reported that the ethanolic extract of *A. nitidum* did not reduce the time spent by the animals on the rotating bar, indicating that the antinociceptive action presented was not related to neurological or motor alterations (M. M. Pereira et al., 2006). In addition, the alkaloid-rich extract of *A. ulei* induced an increase in motor locomotion, but did not cause motor impairment in the rotarod test in contrast to diazepam, indicating a central nervous system stimulant effect (Adriana R. Campos et al., 2006). Furthermore, the extract of *A.*

pyrifolium seeds demonstrated a neuroprotective effect by reducing motor incoordination in a rat model of Parkinson's disease (De Araújo et al., 2018).

The performance of aspidocarpine in the rotarod test was similar to that of other alkaloids that showed neuroprotective activity associated with anti-inflammatory activity (Dey & Mukherjee, 2018; Jing et al., 2021). In addition, the ability of some alkaloids to inhibit the release of inflammatory factors was also related to cardioprotective activities (Bachheti et al., 2022; Vijayalakshmi et al., 2011). Thus, future studies that evaluate the anti-inflammatory activity of Aspidocarpine are necessary to determine this possible mechanism of action of the antihypertensive activity.

The present work is the first to report the influence of an alkaloid isolated from the genus *Aspidosperma* on motor coordination.

3. MATERIALS AND METHODS

3.1. General Experimental Procedures

^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR data were obtained on a Bruker Advance II 9.4 T instrument (Centro de Ciências e Tecnologia, UENF) using deuterated chloroform as a solvent. HR-ESI-MS mass spectra was obtained on a micrOTOF-Q II Bruker Daltonics mass spectrometer (Billerica, MA, USA) using the positive ion mode of analysis. Chromatographic purifications were performed using silica gel 60 (0.063–0.200 mm, MERCK). Aluminum-backed Sorbent silica gel plates, w/UV 254, were used for analytical thin-layer chromatography (Merck) with visualization under UV (254 and 366 nm), vanillin, and dragendorff. The solvents used were methanol (MetOH), ethyl acetate (AcOEt), dichloromethane (CH_2Cl_2), and n-butanol, purchased from Synth (São Paulo, Brazil). Hemodynamic parameters were measured with the Bioamp equipment (Adinstrumentes, Australia) and the Graph Lab software (version 7.0; AD Instruments). The

automated Rota Rod instrument (EFF 411, Insight®) and the Hot plate (EFF-361, Insight®) were used.

3.2. Plant Material

The *A. desmanthum* bark was collected in Linhares, Espírito Santo State, Brazil. A voucher specimen was identified and deposited in the herbarium of the Vale Natural Reserve under code CVRD-9470.

3.3. Extraction and Isolation

A. desmanthum bark (2.5 kg) was extracted with methanol, three times, at room temperature, providing 530 g of crude extract. The was then suspended in H_2O:MeOH (3:1) and partitioned using dichloromethane, ethyl acetate, and n-butanol. The dichloromethane fraction (10.1284 g) was subjected to repeated silica gel column chromatography (CC) using CH_2Cl_2:MeOH, leading to the identification of Aspidocarpine (263.9 mg).

3.4. Animals

The tests were carried out with male and female Wistar rats (*Rattus norvegicus*) weighing between 250 and 300 g and male Swiss mice (*Mus musculus*) weighing between 25 and 30 g from the Animal Experimentation Unit of the Universidade Estadual do Norte Fluminense (UEA—UENF), that were kept in an environment with a controlled temperature of 19°C, humidity of 50 to 60%, 12h and light/dark cycle. Water and food were provided ad libitum. The present study was approved by the Ethics Committee for the Use of Animals of UENF, registered as an additive under protocol number 353, approved on June 2th 2022.

3.5. *In Vivo* Blood Pressure Assessment

Wistar rats were anesthetized by intraperitoneal administration of ketamine (50 mg/k) and xylazine (5 mg/kg) and restrained for insertion of a catheter into the left carotid artery, to measure the following parameters: systolic, median, and diastolic blood pressure. The cannula was heparinized with sodium heparin and 0.9% sodium chloride solution to prevent blood clotting. Another catheter was inserted into the jugular vein for intravenous infusion of the alkaloid Aspidocarpine at a dose of 1 mg/kg (n=4) and 3 mg/kg (n=4), diluted in DMSO, with a volume of

0.1 mL per animal. Prior to the tests, DMSO alone was infused at the same dose to serve as a control in order to eliminate the hypotensive effects of DMSO on the results.

3.6. Rotarod Test

The swiss mice (*Mus muscullus*) were previously tested on the rotating bar. Those that fell two or more times in the three-minute period were discarded. After selection of the animals, the alkaloid Aspidocarpine (1 and 3 mg/kg) and the positive control of diazepam (5 mg/kg) were administered intraperitoneally, with a volume of 0.03 mL. Six animals were used per group.

Each individual was placed with all four legs on a rotating bar 8 cm in diameter, 20 cm from the bottom of the equipment, already in motion (20 rpm). The time the mice were able to balance before falling was measured. The mice were observed at the times of 5, 15, 30, and 60 min after sample administration, and remained on the rotating bar for three minutes. At the moment of falling, the chronometer used to verify the time of equilibrium stopped automatically, the animals were returned to their respective bars, and the chronometer was reactivated, so that the total falls after the three minutes could be counted, while a general timer measured the total time of the test (120 min).

3.7. Statistical Analysis

All experiments were performed in triplicate, and the results are expressed as mean ± standard error of the mean (SEM). The results obtained were tabulated by the LabChart 7 program, and statistically analyzed through GraphPad Prisma 5. The analysis of variance (ANOVA) was defined, followed by the Newman-Keuls and Bonferroni mean test, with a reliability index of 95%. Significant difference was taken as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

4. CONCLUSIONS

This work presents the first report on the antihypertensive potential of the alkaloid Aspidocarpine, isolated from the bark of *A. desmanthum*, analyzed by induction of a decrease in blood pressure evaluated *in vivo*. The alkaloid Aspidocarpine showed effective hypotensive activity without causing significant changes in motor coordination. Therefore, this alkaloid may be a promising alternative for the treatment of pathological conditions related to hypertension.

Supplementary Materials: ^{1}H-, ^{13}C-DEPTQ NMR spectra, COSY, HSQC, and HMBC spectra correlations; HRESIMS and Low-resolution mass spectrum and proposal of mass spectra fragmentation of Aspidocarpine; NMR spectral data for Aspidocarpine, including results obtained by heteronuclear 2D shift-correlated HSQC and HMBC and comparison with values described in the literature are available as Supplementary Materials available online.

Author Contributions: N.O.M. and I.J.C.V. conceived and designed the experiments; A.R.d.C.J., R.B.-F., and T.S.R.N. performed the experiments on NMR analysis; R.B.-F., I.J.C.V. and T.S.R.N. performed the high resolution spectrometry analysis experiments; N.O.M., T.M.M., and F.A. performed the antihypertensive analysis; N.O.M., J.R.C., L.P.S.N. and K.A.C wrote the article; D.B.O. and G.R.S. performed a writing-review of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This article received no external funding.

Acknowledgments: The authors are grateful to the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials

Antihypertensive Activity of the Alkaloid Aspidocarpine in Normotensive Wistar Rats

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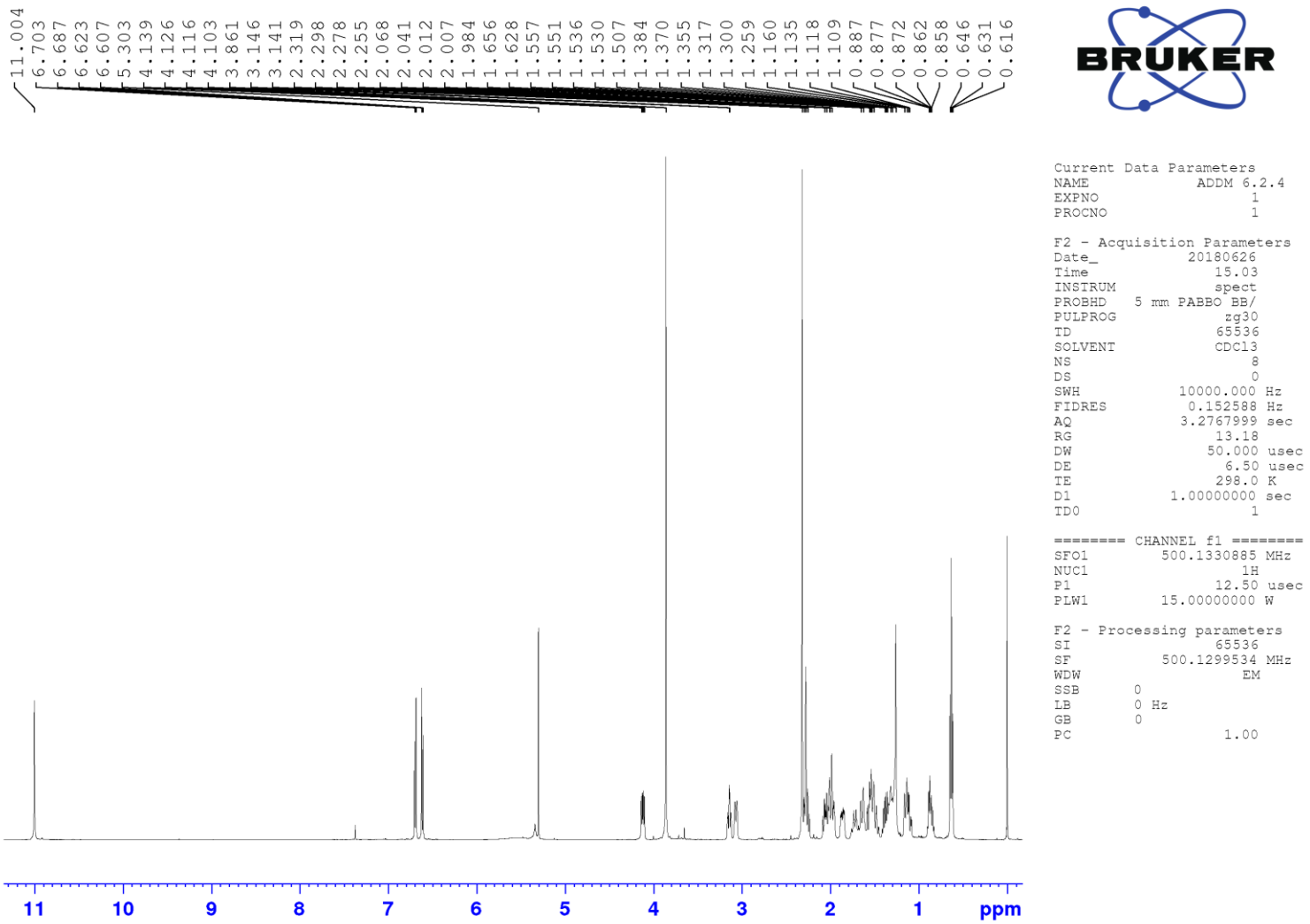
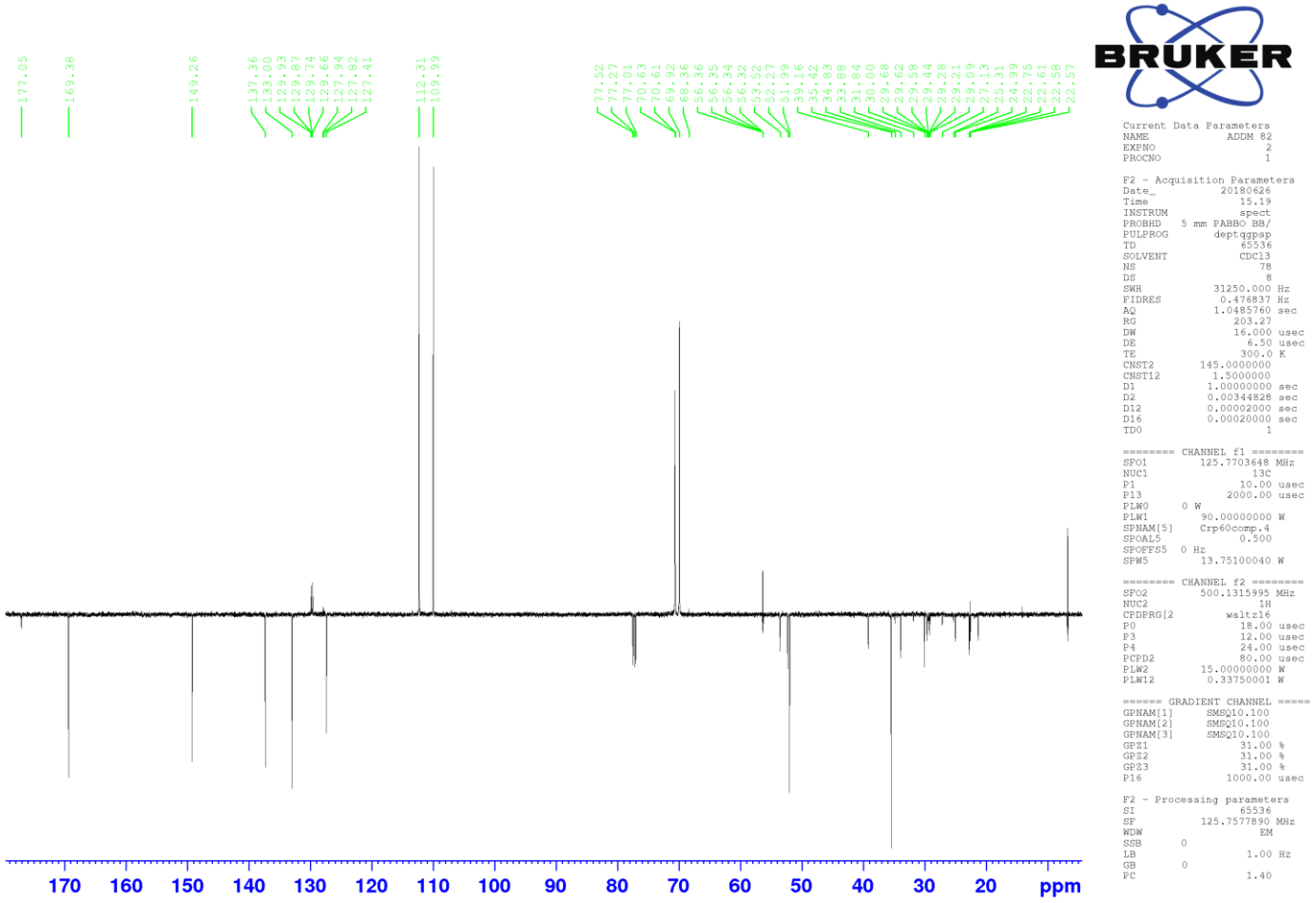


Figure S1: ^{1}H NMR spectrum of Aspidocarpine ($CDCl_3$, 500 MHz).



BRUKER

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PROCNO                      1

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D12                 0.00002000 sec
D16                 0.00020000 sec
TD0                         1

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P1                       10.00 usec
P13                    2000.00 usec
PLW0        0 W
PLW1               90.00000000 W
SPNAM[5]      Crp60comp.4
SPOAL5                  0.500
SPOFFS5   0 Hz
SPW5               13.75100040 W

======== CHANNEL f2 ========
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NUC2                        1H
CPDPRG[2           waltz16
P0                       18.00 usec
P3                       12.00 usec
P4                       24.00 usec
PCPD2                    80.00 usec
PLW2               15.00000000 W
PLW12               0.33750001 W

====== GRADIENT CHANNEL =====
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GPNAM[2]       SMSQ10.100
GPNAM[3]       SMSQ10.100
GPZ1                    31.00 %
GPZ2                    31.00 %
GPZ3                    31.00 %
P16                   1000.00 usec

F2 - Processing parameters
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WDW                           EM
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LB                       1.00 Hz
GB          0
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Figure S2: ^{13}C DEPTQ NMR spectrum of Aspidocarpine ($CDCl_3$, 125 MHz).

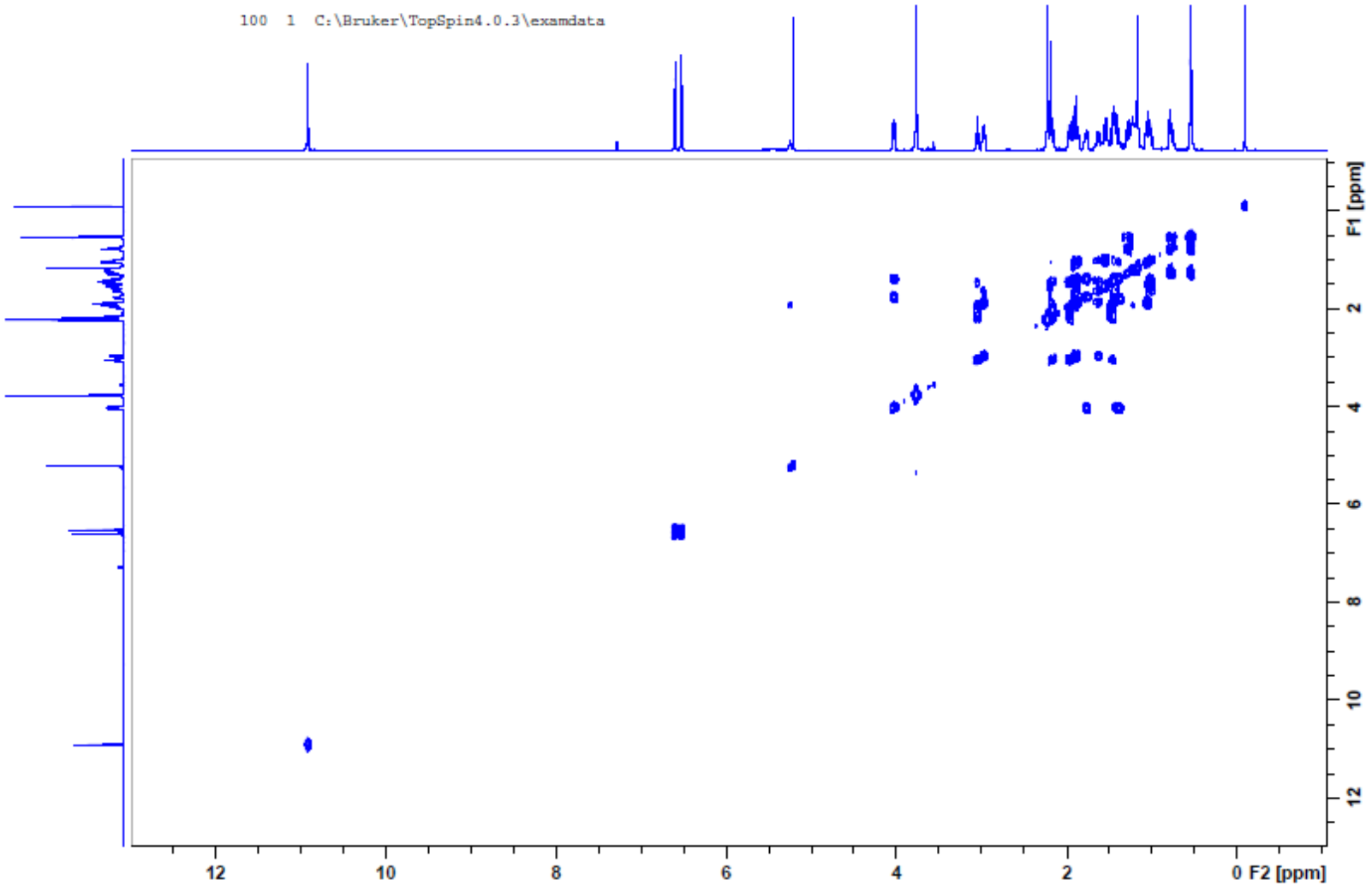

Figure S3: ^{1}H-^{1}1H-COSY spectrum of Aspidocarpine ($CDCl_3$, 500 MHz).

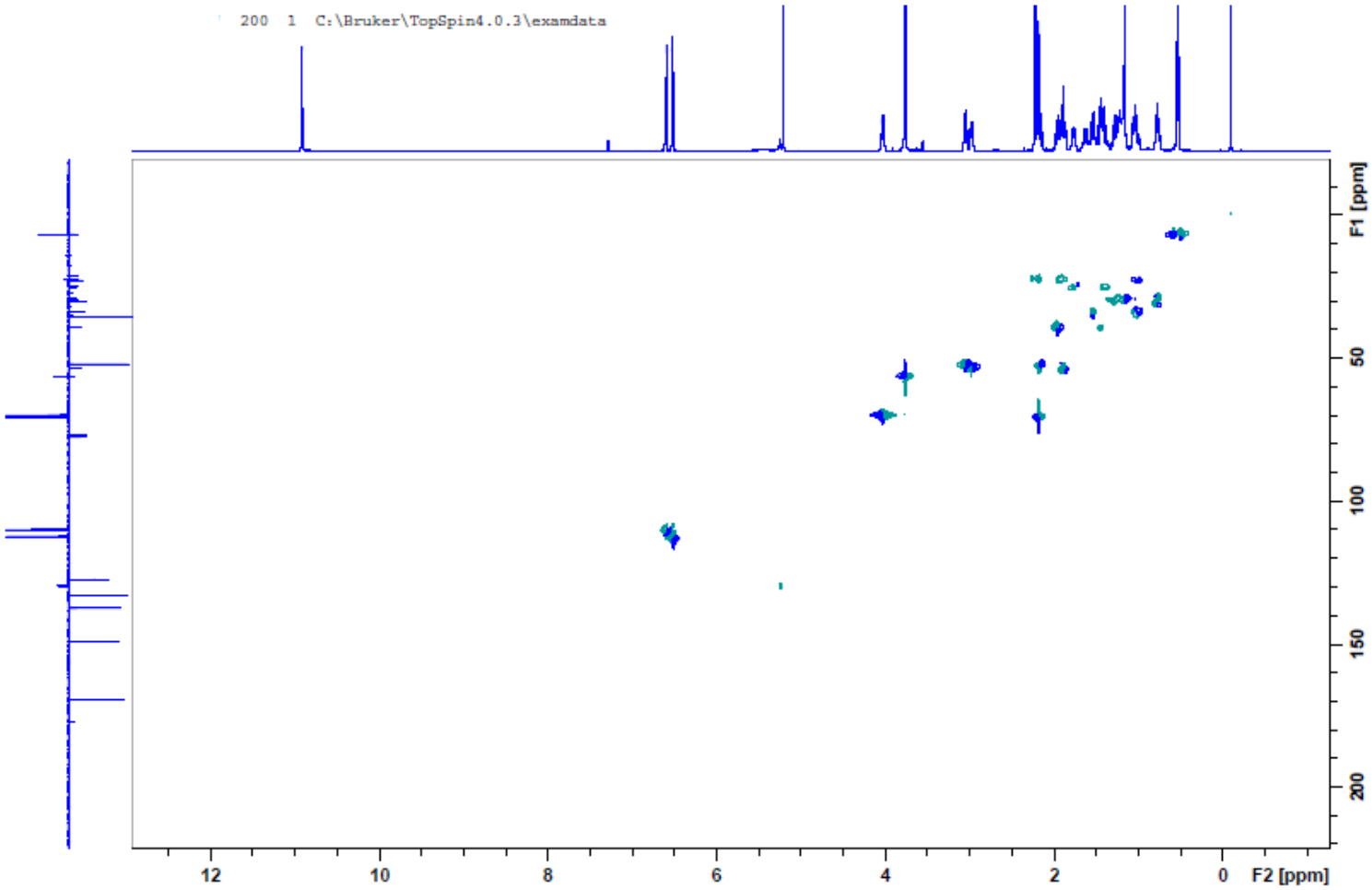

Figure S.4: HSQC spectrum of Aspidocarpine ($CDCl_3$, 500 MHz).

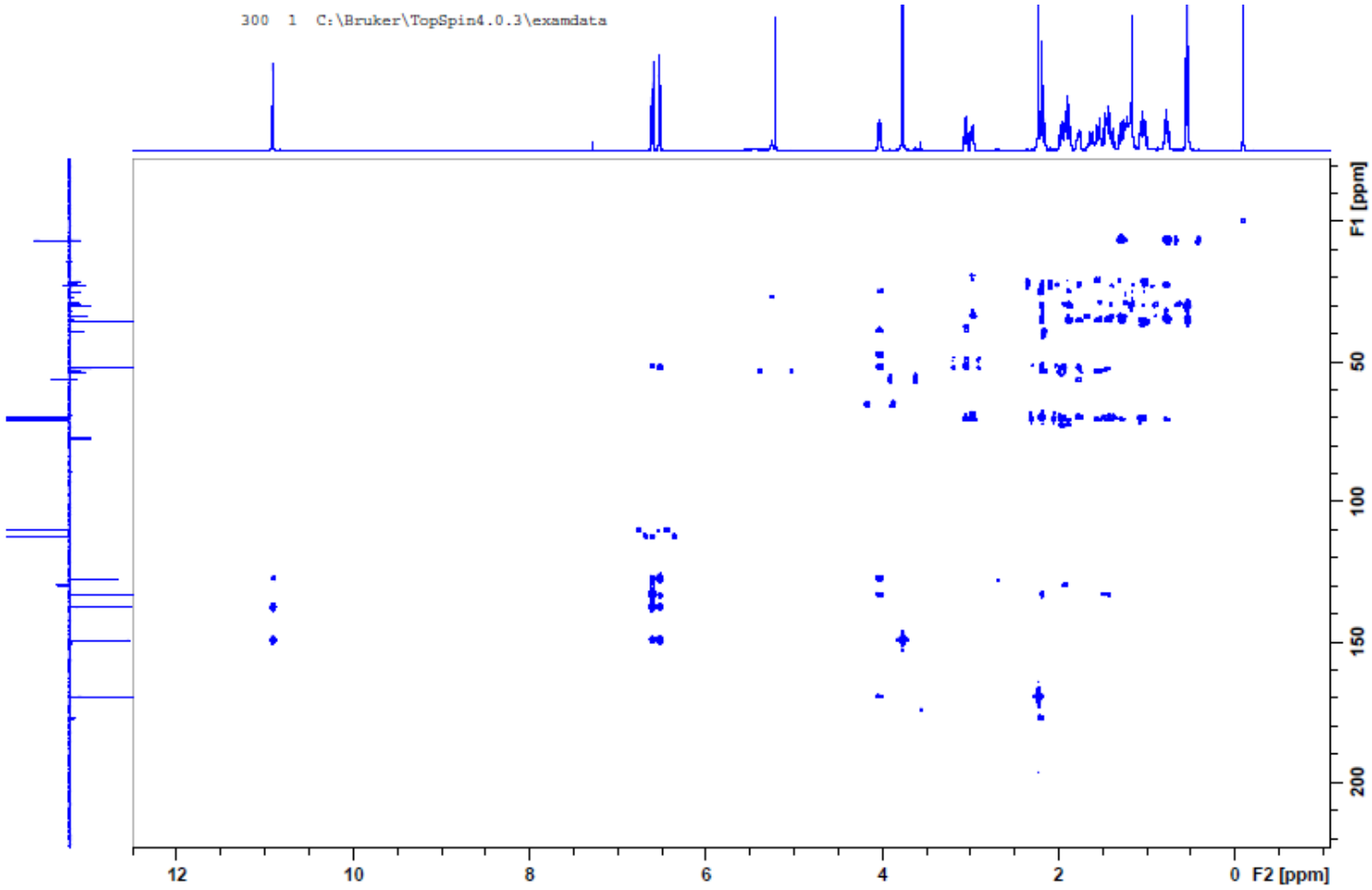

Figure S.5: HMBC spectrum of Aspidocarpine ($CDCl_3$, 500 MHz).

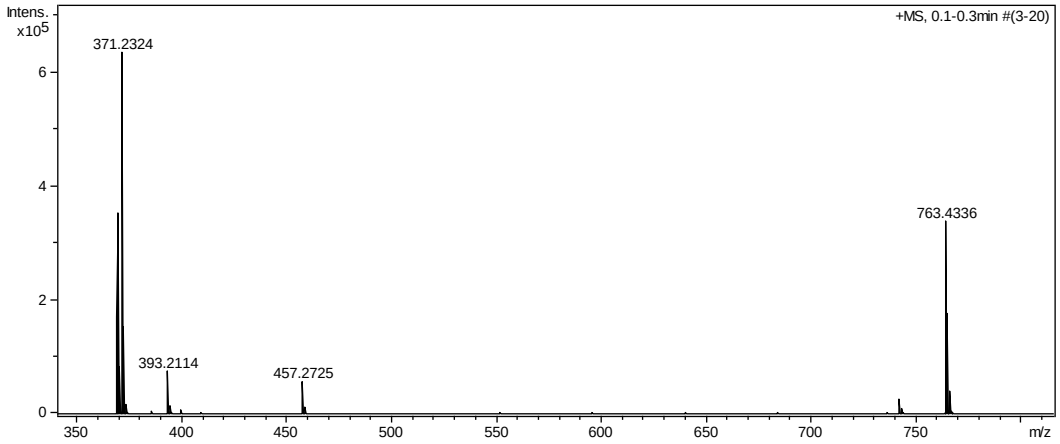

Figure S6: High-resolution mass spectrum (HRESIMS/positive mode) of Aspidocarpine.

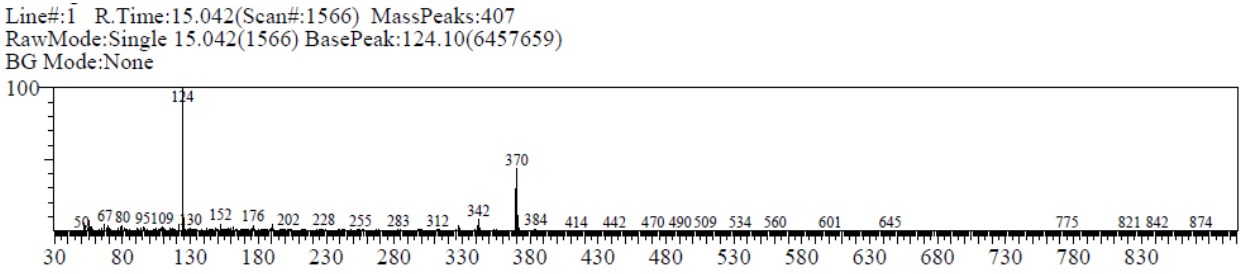

Figure S7: Low-resolution mass spectrum of Aspidocarpine.

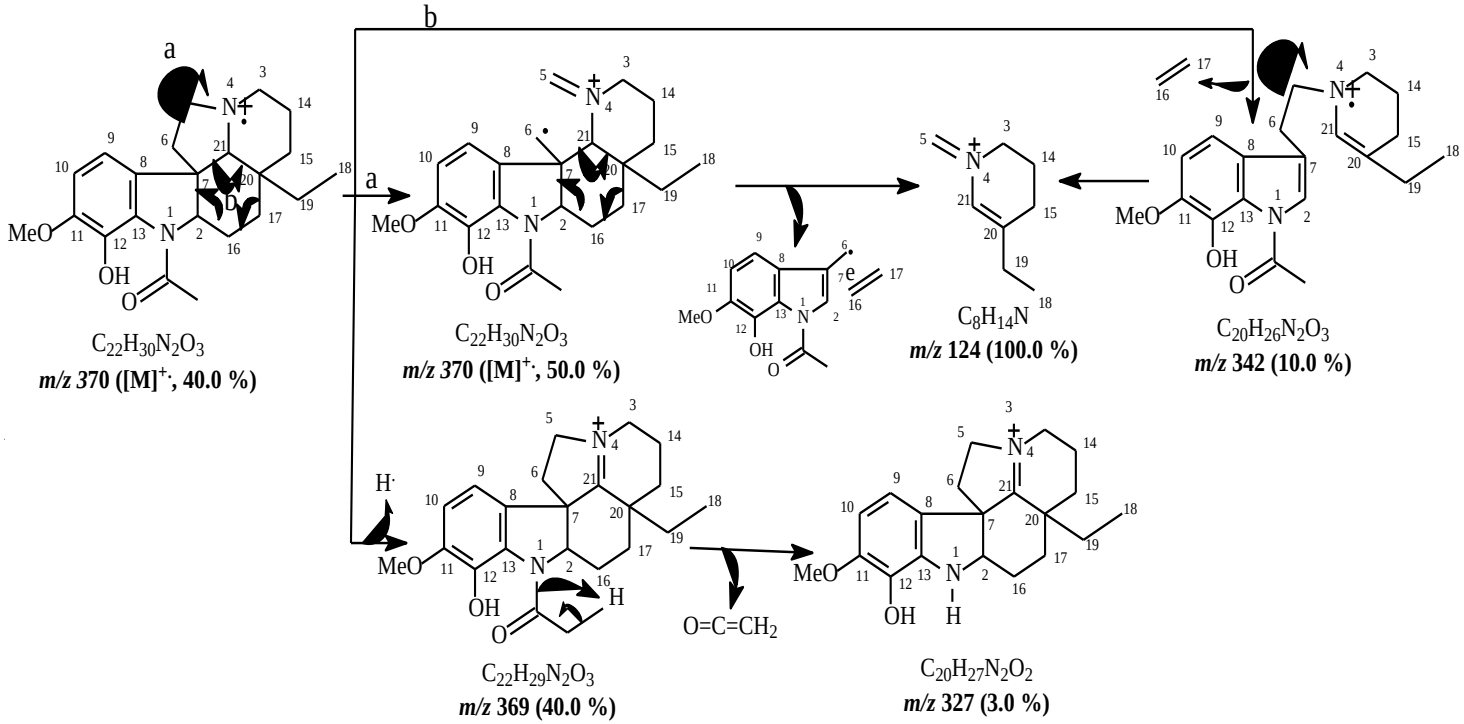

Figure S8: Proposal of mass spectra fragmentation of Aspidocarpine.

Table S1: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data for Aspidocarpine, including results obtained by heteronuclear 2D shift-correlated HSQC ($^{1}J_{CH}$) and HMBC ($^{n}J_{CH}$, n=2 and 3) and comparison with values described in the literature [16, 17]), in $CDCl_3$ as solvent. Chemical shifts (*d*, ppm) and coupling constants (*J*, Hz, in parenthesis).

	HMQC		HMBC		[17]		[16]	
	δ_C	δ_H	$^{2}J_{CH}$	$^{3}J_{CH}$	δ_C	δ_H	δ_C	δ_H
C								
7	51.99	-	H-2	2H-5; H-9	52.2	-	52.20	-
8	132.99	-	H-9	H-2; H-10; H-21	133.1	-	133.13	-
11	149.26	-	H-10	H-9; MeO-11; HO-12	149.4		149.35	-
12	137.36	-	HO-12	H-10	137.5		137.47	-
13	127.40	-		H-2; H-9; HO-12	127.5	-	127.51	-
20	35.42	-	2H-19	H-16a; 3H-18	35.5	-	35.46	-
1'	169.28	-	3H-2'	H-2	169.3	-	169.31	-
CH								
2	69.31	4.03 (dd, 11.3, 6.2)			70.3		70.25	4.07 (dd, 11.0, 6.0))
9	112.31	6.52 (d, 8.1)	H-10		112.4	6.61 (d, 8.0)	112.36	6.61 (d, 8.0)
10	109.99	6.60 (d, 8.1)	H-9		110.0	6.69 (d, 8.0)	110.04	6.69 (d, 8.0)
21	70.63	2.20 (s)		H-3a; H-5a; 2H-19	70.6	2.25 (s)	70.62	2.25 (s)
CH_2								
3	53.52	2.97 (dl, 10.9(1.90			53.7	3.04 (dm, 12 1.98 (td, 12.0, 4.0)	53.67	3.04 (dm, 12.0) 1.98 (td, 12.0, 4.0)
5	52.27	3.06 (td, 9.0, 2.1) 2.15			52.4	3.12 (m), 2.27 (m)	52.43	3.12 (m), 2.27 (m)
6	39.16	1.98, 1.45	H-5b	H-2	39.4	2.04 (m), 1.57(m)	39.37	2.04 (m), 1.57 (m)
14	21.27	2.20, 1.92	H-15b		21.5	1.72(tm, 12.0) 1.53 (dm, 12.0, 4.0)	21.50	1.72 (tm, 12.0), 1.53 (dm, 12.0)
15	33.88	1.60, 1.02		H-3[a]	34.0	1.65 (dt, 12.0, 4.0) 1.11 (td, 12.0, 4.0)	34.02	1.65 (dt, 12.0, 4.0), 1.11 (dt, 12.0, 4.0)
16	24.99	1.88, 1.52			25.1	1.86 (m), 1.52 (m)	25.10	1.86 (m), 1.52 (m)
17	22.75	1.98, 1.20		H-19b	22.9	2.00 (td, 14.0, 12.0) 1.15 (dm, 12.0)	22.92	2.00 (td, 12.0, 14.0), 1.15 (dm, 12.0)
19	30.00	1.25, 0.80	3H-18		30.0	1.44 (m), 0.93 (m)	30.03	1.44 (m), 0.93 (m)
CH_3								
18	6.71	0.64 (t, 7.4)	2H-19		6.8	0.63 (t, 7.5)	6.76	0.63 (d, 7.5)
MeO	56.36	3.77 (s)			56.4	3.88 (s)	56.43	3.88 (s)
2'	22.60'	2,23 (s)			22.7	2.33 (s)	22,68	2.33 (s)
HO	-	10.91 (s)			-	10.98 (s)	-	10.98 (s)

3.2 ANTIMYCOBACTERIAL, ANTI-INFLAMMATORY AND CYTOTOXIC ACTIVITIES OF ALKALOIDS FROM *ASPIDOSPERMA desmanthum* AND *A. pyricollum* (APOCYNACEAE)

ABSTRACT

The alkaloid aspidocarpine (**1**) and the mixture between a new alkaloid N_a-hydroxy-10-demethoxytubotaiwine (**2**) and 11-methoxytubotaiwine (**3**) were isolated from the *A. desmanthum*. Stemmadenine (**4**) and apparicine (**5**) were isolated from the *A. pyricollum* seeds. The structures were elucidated based on by the spectral data of 1H and 13C NMR (1D and 2D), high-resolution mass spectrometry (HRESIMS), and data comparison with values described in the literature. The compounds **1, 2, 3, 4** e **5** were evaluated for their antimycobacterial, anti-inflammatory and cytotoxic activity against RAW 264.7 macrophage cells and the human leukemia cell line Molt-4 (acute lymphoblastic). These compounds showed antimycobacterial activity with MIC_{50} ranging from 49.9 to ≥500 µg/mL and inhibitory activity of NO production with IC_{50} ranging from 18.3 to 138.7 µg/mL. The compounds did not show cytotoxicity against macrophages RAW264.7 and showed values of IC_{50} ranging from 59.8 to ≥500 µg/mL against the cells Molt-4.

1. INTRODUCTION

The genus *Aspidosperma* belongs to the Apocynaceae family, has about 50 species distributed in tropical and subtropical areas of America [1]. Infusions of parts of these plants are used in folk medicine to treat various diseases such as: malaria, fever [2], erectile dysfunction [3], diabetes, headache, fighting free radicals [4], high blood pressure, inflammation [5], among others. Studies carried out with extracts highlight the activities antiprotozoal [6–8], anti-inflammatory [9,10], action on the central nervous system [3,11,12] and antimicrobial [13].

Monoterpenic indole alkaloids are the most frequently isolated secondary metabolites among species of *Aspidosperma* [14] and most biological activities reported in this genus are attributed to them [15].

The present study aimed to isolate monoterpenic indole alkaloids from the bark of *A. desmanthum* and from the seeds of *A. pyricollum* and to evaluate their antimycobacterial, anti-inflammatory and cytotoxic activities against macrophage RAW 264.7 and human leukemia cell line Molt-4 (acute lymphoblastic).

2. RESULTS AND DISCUSSION

2.1 Chemical Study

The chromatographic fractionation of the dichloromethane partition, obtained through the methanolic extract of the bark of *A. desmanthum*, led to the isolation of the alkaloid aspidocarpine (**1**) [16,17] and the mixture of an unknown compound Na-hydroxy-11-desmetoxy-10-methoxytubotaiwine (**2**) and 11-methoxytubotaiwine (**3**), isolated for the first time in this species [18]. Furthermore,

the chromatographic purification of the methanolic extract of the *A. pyricollum* seeds led to the identification of the alkaloids stemmadenine (**4**) [19,20] and apparicine (**5**) [21].

The identification of the compounds was based on the analysis of the spectral data of ^{1}H and ^{13}C NMR (1D e 2D) and high-resolution mass spectrometry (HRESIMS), and comparison with data described in the literature. The structures are shown in **Figure 1**.

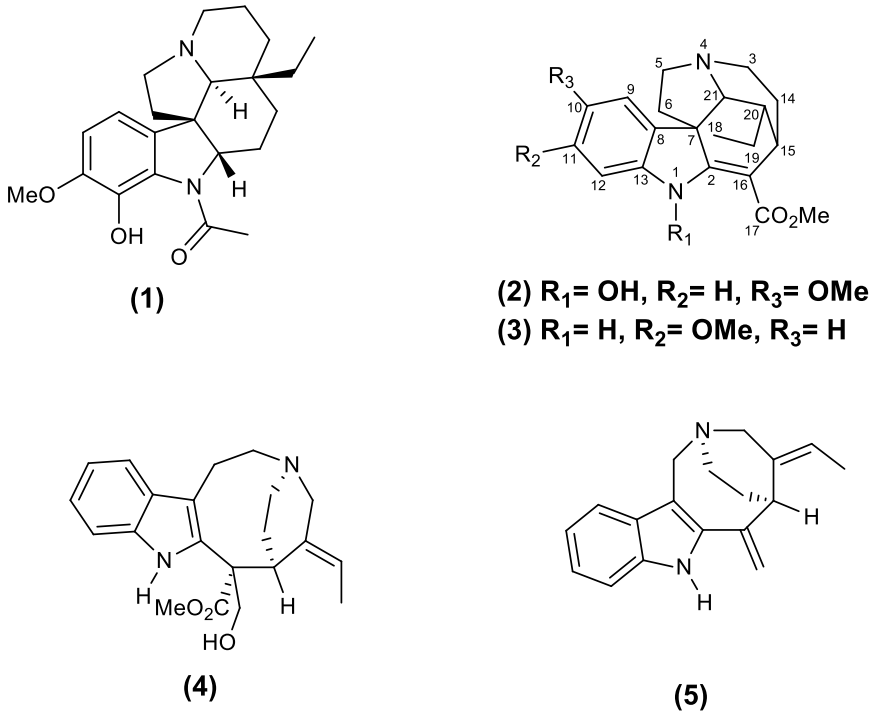

Figure 1: Compounds isolated from *A. desmanthum* and *A. pyricollum*.

Compound **2** was obtained as brownish amorphous solid. ^{1}H NMR ($CDCl_3$, 500 *MHz*): δ_H 7.33 (d=8.3 Hz; H-12), 7.22 (d=2.3 Hz; H-9), 6.92 (dd=2.3, 8.3 Hz, H-11), 4.39 (brl; H-21) 4.01 (s; MeO-17), 3.62 (m; 2H-5b), 3.42 (m; 2H-5a), 3.84 (s; MeO-10), 3.38 (m; H-15), 3.35 (m; 2H-6b), 2.63 (m; 2H-14b), 2.32 (m; 2H-14a), 2.30 (m; H-20), 2.20 (m; 2H-6a), 1.07 (m; 2H-19b), 0.59 (m; 2H-19a) and 0.74 (t=7.2 Hz, 3H-18). ^{13}C NMR ($CDCl_3$, 125 *MHz*): δ_C 177.8 (C-2), 169.2 (C-17),

161.0 (C-10), 152.6 (C-8), 135.9 (C-13), 120.5 (CH-12), 114.6 (CH-11), 109.0 (CH-9), 96.2 (C-16), 68.8 (CH-21), 57.2 (C-7), 55.7 (MeOH-10), 54.8 (CH_2-5), 54.3 (MeOH-17), 44.8 (CH_2-3), 39.7 (CH-20), 36.3 (CH-15), 34.9 (CH_2-6), 29.9 (CH_2-14), 22.1 (CH_2-19) and 11.6 (CH_3-18). The HRESIMS revealed a molecular formula of [$C_{21}H_{26}N_2O_4$] of *m/z* 371.1910 ([M+H]+, calc. *m/z* 371.1971).

Compound **3** was obtained as brownish amorphous solid. ^{1}H-NMR ($CDCl_3$, 500 *MHz*): *δ*H 7.12 (d= 8.0 Hz; H-9), 6.47 (d= 2.2 Hz; H-12), 6.49 (dd= 8.0, 2.2 Hz; H-10), 4.43 (brl; H-21), 3.70 (m; 2H-3b), 3.62 (m; 2H-5b), 3.14 (m; 2H-5a), 2.92 (m; 2H-3), 2.70 (m; 2H-6b), 2.25 (m; 2H-14b), 2.20 (m; 2H-6a), 2.05 (m; 2H-14a), 0.98 (m; 2H-19b), 0.89 (m; 2H-19a) and, 0.76 (t=7.3 Hz; 3H-18). ^{13}C-NMR ($CDCl_3$, 125 *MHz*): *δ*C 167.93 (C-17), 167.16 (C-2), 160.79 (C-11), 144.45 (C-13), 125.02 (C-8), 120.79 (CH-9), 106.18 (CH-10), 106.18 (CH-10), 98.24 (CH-12), 96.15 (C-16), 64.47 (CH-21), 55.57 (MeO-11), 52.01 (C-7), 51.65 (MeO-17), 51.55 (CH_2-5), 44.94 (CH_2-3), 40.59 (CH_2-6), 36.62 (CH-20), 28.26 (CH-15), 29.90 (CH_2-14), 22.67 (CH_2-19), 11.04 (CH_3-18). The HRESIMS revealed a molecular formula of [$C_{21}H_{26}N_2O_3$] of *m/z* 355.1993 ([M+H]+, calc. *m/z* 355.2022).

2.2 Antimycobacterial, anti-inflammatory and cytotoxic activity

All compounds were evaluated for antimycobacterial, anti-inflammatory and cytotoxic activities (**Table 1**). Antimycobacterial activity was tested against *Mycobacterium tuberculosis* strains H37Rv and M299 (highly virulent clinical isolate).

Table 1. Inhibitory effects of the compounds isolated from the bark of *A. desmanthum* and *A. pyricollum* seeds on growth of *M. tuberculosis* H37Rv and M299 strains in culture, NO production by LPS-stimulated RAW 264.7 macrophages, and assessment of cytotoxicity Compounds.

Compounds	MIC_{50} (µg/mL)		IC_{50} (µg/mL)	
	H37Rv	M299	NO	MTT
1	133 ± 1.0	120.3 ± 0.1	84.3 ± 0.8	≥500
2 e 3	49.92 ± 0.7	72.68 ± 0.2	18.3 ± 0.8	≥500
4	≥500	≥500	137.9 ± 0.5	≥500
5	468.2 ± 0.1	≥500	138.7 ± 0.8	≥500
Rifampicina[1]	0.8 ± 0.1	0.9 ± 0.2	XX	XX
L-NMMA[2]	XX	XX	13.2 ± 0.6	XX

[1] Standard antimycobacterial drug; [2] Standard nitric oxide inhibitor; Mean value ± SD; XX - not defined.

All alkaloids, except compound **4**, were able to inhibit *M. tuberculosis* H37Rv in vitro. Compounds **1** and **5** showed low activity and the mixture between compounds **2** and **3** showed moderate antimycobacterial activity against both strains. Compounds **4** and **5** were inactive against the highly virulent strain M299.

There are no reports in the literature on antimycobacterial activity in *Aspidosperma* species. However, the antimycobacterial activity of alkaloids has been reported by several authors. The alkaloids strictosidine and 5-α-carboxy-strictosidine showed promising activity against *M. tuberculosis* H37Rv, with MIC_{50} values ranging from 7.1 µg/mL to 19.2 µg/mL, respectively [22]. Another alkaloid, globospiramine, isolated from *Voacanga globosa*, showed a high potential for inhibiting *M. tuberculosis* H37Rv (MIC_{50} 4.0 µg/mL), along with three other alkaloids of similar structure that showed MIC_{50} over 50 µg/mL [23].

Tubotaiwine, a strychnine indole alkaloid, obtained from a bioactive fraction of *Alstonia scholaris*, was tested against *M. tuberculosis H37Rv* and presented a MIC_{50}=100 µg/mL, demonstrating better activity in relation to the other identified alkaloids of the subtype vallesamine – apparicine, suggesting that the monoterpenoid skeleton of indole alkaloids has the greatest influence on their activity against *M. tuberculosis* H37Rv [24]. In the present study, the mixture between the alkaloids N_a-hydroxy-11-demetoxy-10-methoxytubotaiwine (**2**) and 11-methoxytubotaiwine (**3**) showed the best inhibitory activity against *M. tuberculosis* H37Rv and M299 (MIC_{50} 49.92 and 72.68 µg/mL) among the other alkaloids isolated from different subtypes. However, the structural differences of the indole portion of alkaloids **2** and **3** in relation to tubotaiwine seem to have favoured the antimycobacterial activity.

The anti-inflammatory activity of the isolated alkaloids was evaluated by inhibiting NO expression in RAW 264.7 macrophages induced by LPS. All compounds showed some activity, highlighting the mixture between compounds **2** and **3** (18.3 ± 0.8), which showed good activity in relation to the L-NMMA control (13.2 ± 0.6 µg/mL). Furthermore, none of the compounds tested showed significant cytotoxicity against RAW 264.7 macrophage cells.

Aspidosperma species are known for their anti-inflammatory properties. Several studies carried out with extracts of *A. pyrifolium* and *A. tomentosum* showed in vivo anti-inflammatory activity [9,10,25]. In this work, the isolated alkaloids showed anti-inflammatory activity, through the inhibition of NO

production, with IC_{50} values ranging from 18.3 to 138.7 µg/mL without showing cytotloxicity against RAW 264.7 macrophage cells. In this way, we can suggest that the indole alkaloids described in this study may be contributing to the anti-inflammatory activity reported for *Aspidosperma* extracts.

Furthermore, the cytotoxicity of compounds **1**-**5** was evaluated against the leukemia cell line Molt-04, using cisplatin as a positive control. IC_{50} values are shown in **Table 2**.

Table 2. Inhibitory effect of compounds **1, 2, 3, 4, 5** and cisplatin against the MOLT-4 cell line.

Compounds	**IC_{50} (µg/mL)**
1	112.7± 1.1
2 and **3**	59.83 ± 1.1
4	≥500
5	129.6 ± 1.3
Cisplatin	22.00 ± 1.0

Compounds **1** and **5** showed low activity, the mixture between compounds **2** and **3** showed moderate activity and compound **4** was inactive compared to cisplatin (positive control).

In the genus *Aspidosperma*, the anticancer activity is mainly related to the ellipticine alkaloid. This alkaloid showed high efficiency against several types of cancer such as breast adenocarcinoma, leukemia, neuroblastoma and glioblastoma, with IC_{50} values ranging from 0.27 to 1.48 µM [26]. Another alkaloid kopsanone, isolated from *A. macrocarpon*, showed cytotoxicity against U251 glioma cancer cells and K-562 leukemic cells, with GI_{50} values of 20.6 µg/mL and 8.7 µg/mL, respectively [27]. The alkaloids described in this work showed low to moderate cytotoxic activity against MOlt-4 leukemic cells, with IC_{50} values ranging from 59.83 to ≥500 µg/mL. These results expand the amount of indole alkaloids that have anticancer activity in the genus *Aspidosperma*.

3. Experimental

3.1. General procedures

NMR spectra were acquired by using Bruker Ascend 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) in $CDCl_3$. Chemical shifts (δ) in ppm and coupling constants (*J*) in Hz. HRESI data were acquired on a micrOTOF-Q II Bruker Daltonics, with the use of the positive ion mode of analysis. Column chromatography (CC) was performed on silica 60 (0.063–0.200 mm, MERCK) and for the preparative thin layer chromatography (PTLC) was used silica gel 60 PF_{254} (MERCK) on glass plates. TLC plates contained with silica gel 60 F_{254} (MERCK) were used for analytical analysis; detection was under UV (254 and 365 nm) and chromogenic reagent (Vanillin/sulfuric acid). The solvents used were methanol (MetOH), ethyl acetate (AcOEt), dichloromethane (CH_2Cl_2) and *n*-butanol purchased from Synth (São Paulo, Brazil).

3.2. Plant material

The bark of *A. desmanthum* and the seeds of *A. pyricollum*, were collected in Reserva Natural Vale, Linhares City, Espírito Santo state, Brazil. A voucher specimen (CVRD-9470 and 3573, respectively) was deposited in the herbarium of the Reserva Natural Vale.

3.3. Extraction and isolation

A. desmanthum bark (2.5 kg) was extracted with methanol, three times, at room temperature, providing 530 g of crude extract. The was then suspended in H_2O:MeOH (3:1) and partitioned using dichloromethane, ethyl acetate, and *n*-butanol. The dichloromethane fraction (10.1284 g) was subjected to silica gel column chromatography (CC) using CH_2Cl_2:MeOH, affording 9 fractions. Fraction 6 (139 g) was rechromatographed affording 10 fractions. From fraction 2 (1.17 g) compound **1** was (263.9 mg). Fraction 8 (578,3 mg) was rechromatographed affording 3 fractions. Fraction 2 (130 mg) was purified by preparative TLC leading to the identification of a mixture of compounds **2** and **3** (28.3 mg).

A. pyricollum seeds (2.0 kg) were extracted with methanol three times at room temperature, resulting in 380 g of crude extract that was subjected to chromatography on a silica gel column (CC) using CH_2Cl_2:MeOH, resulting in 9 fractions. From fraction 6 (1.145 g) that was subjected to chromatography on a silica gel column (CC) using CH_2Cl_2:MeOH leading to the compound **4** (946 mg).

Fraction 5 (497.8 mg) was rechromatographed affording 5 fractions. Fraction 5 (376.7 mg) was rechromatographed affording 6 fractions. Fraction 3 (74 mg) was purified by preparative TLC, leading to the compound **5** (35 mg).

3.4 Culture of mycobacteria and evaluation of bacterial growth

M. tuberculosis H37Rv ATCC 27294 and highly virulent Mtb strain Beijing M299, were cultivated in suspension of Middlebrook 7H9 broth, containing 10% dextrose albumin complex (ADC), 0.5% glycerol and 0.05% Tween-80. The compounds **1**-**5** were evaluated for their antimycobacterial activity using an MTT assay (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazole) in a 96-well plate. During the logarithmic growth phase, 50 µL of bacterial suspensions were plated at 1×10^6 UFC/well and incubated with 50 µL of samples at concentrations of 4, 20, 100 and 500µg/mL. The sealed plate was incubated at 37°C and 5% CO_2 for 5 days. After this period, the bacterial culture was then incubated for 3 h with 10 µL of tetrazolium salt (5 mg/mL in sterile phosphate-buffered saline [PBS]) and then 100 µL of lyses buffer (20% w/v SDS/50% DMF - dimethylformamide in distilled water pH 4.7).

3.5 Quantification of NO production

The macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD, USA) was cultivated in DMEM F-12 supplemented with 10% FCS and gentamicin (50 µg/mL), and incubated in the presence of 5% CO_2 at 37°C. The cells were seeded in 96-well plates (1×10^5 cells/well) in the presence or absence of samples (4, 20, 100 and 500µg/mL) and/or lipopolysaccharide (LPS-*Escherichia coli* 055: B5; Sigma Aldrich). After a 24 h incubation, supernatants were collected and the concentration of nitrite was determined according to the Griess test. A NO inhibitor (NG-methyl-Larginine acetate salt - L-NMMA) was used as a positive control of NO inhibition at 20 µg/mL (32.7 ± 0.9 µM/reducing NO production by 52.5 ± 1.3%) in the experiments.

3.6 Cytotoxicity assay

Cytotoxic effects of samples on RAW 264.7 cell viability in cultures stimulated with LPS were determined using the MTT assay. The optical density was measured at 570 nm employing a microplate reader after incubation for 2 h at

37°C with MTT solution (5 mg/ml in sterile PBS). The negative control was used untreated macrophages and the positive control (stimulated macrophages) culture treated with 1% (v/v) Triton X-100.

3.8. Antineoplastic activity

3.8.1. Cell culture and Analysis of cell viability

Human leukemia cell line Molt-4 (acute lymphoblastic) was cultured in DMEM-F12 medium (Gibco, BRL) supplemented with 10% fetal bovine serum and gentamicin (20 µg/mL, Gibco, BRL) and maintained at 37°C incubator (Forma, Thermo Scientific, USA) in a humidified atmosphere containing 5% CO_2.

Cells at 1×10^5 cells/mL final concentration were plated in 96 well plate and immediately treated with different concentrations (0–100 µM) of the compounds for 48 h in DMEM-F12 medium. DMSO (0.02%) was used as the solvent control and cisplatin (Sigma-Aldrich) was used as positive control. Cell viability was measured by the colorimetric micro assay (MTT assay). Twenty microliters of MTT [3-(4,5-dimethyl- 2-thihazyl)-2,5-diphenyl-2Htetrazolium bromide] stock solution (5 mg/mL) were added to each well and incubated at 37°C for a further 4 h. Then, the MTT-formazan produced by viable cells was dissolved in an isopropanol-HCl solution. Using a Microplate Reader (Epoch™, BioTek® Instruments, Inc.) the optical density (OD) values were measured at 570 nm. The values of IC_{50} were obtained from dose -response curves using GraphPad Prism 5.0. The IC_{50} was defined as the concentration of the compound required to reduce the viability of treated cells to 50% in comparison to untreated control cells. All experiment s were carried out in triplicate.

3.9 Statistical analysis

The results were tabulated by the LabChart 7 program and analysed statistically through the software GraphPad Prisma 4. The tests were performed in three replicates, and values were expressed as mean ± SD.

4. CONCLUSIONS

An investigation of the barks of *A. desmanthum* resulted into isolation of aspidoscarpine (**1**) and the mix between alkaloid N_a-hydroxy-11-demetoxy-10-methoxytubotaiwine (**2**) and 11-methoxytubotaiwine (**3**). Compound **2** is new in the literature and compound **3** is being reported for the first time in this species. Stemmadenine (**4**) and apparicine (**5**) alkaloids were isolated from *A. pyricollum* seeds. The mixture between compound **2** and **3** showed the best antimycobacterial, anti-inflammatory and cytotoxic activity against molt-4 human leukemia cells. None of the compounds showed cytotoxicity against RAW 264.7 macrophage. These results contributed with new biological activities of the genus *Aspidosperma*.

5. Acknowledgements

The authors are grateful to Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Finance Code 001.

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Supplementary Materials

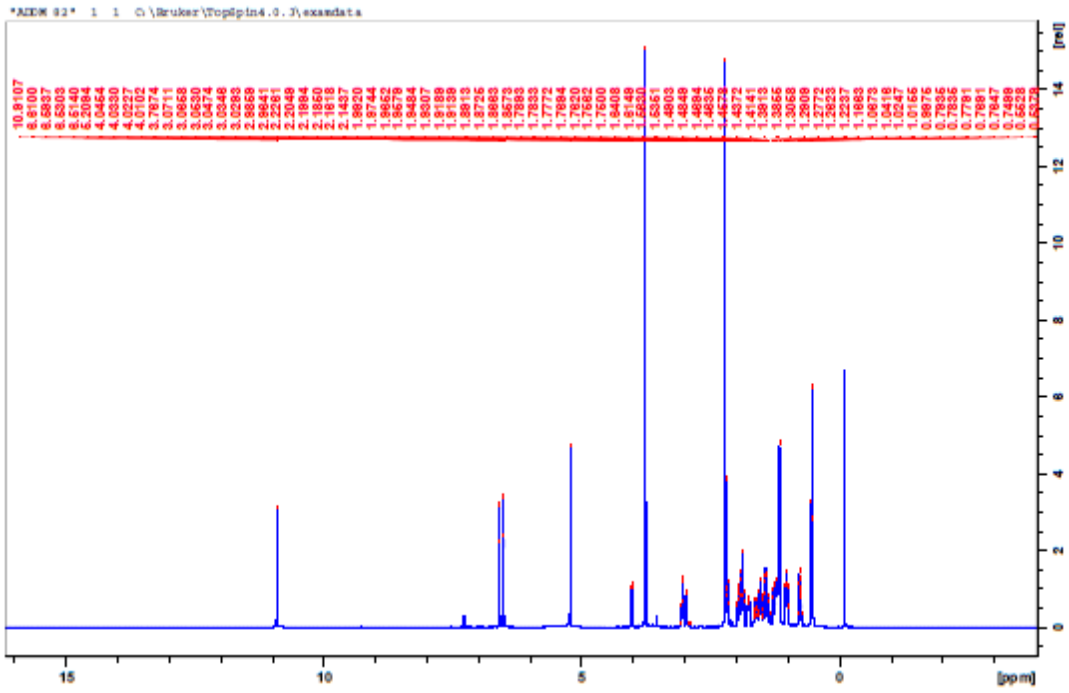

Figure S1: ^{13}C DEPTQ NMR spectrum of compound **1** ($CDCl_3$, 125 MHz).

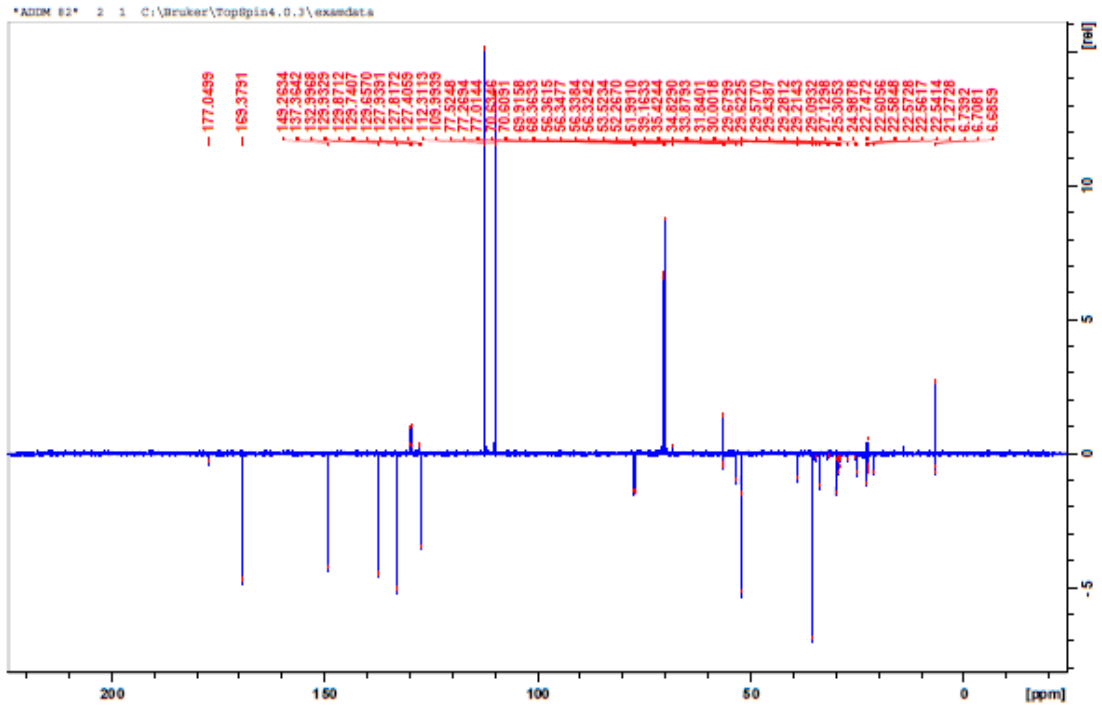

Figure S2: ^{1}H NMR spectrum of compound **1** ($CDCl_3$, 500 MHz).

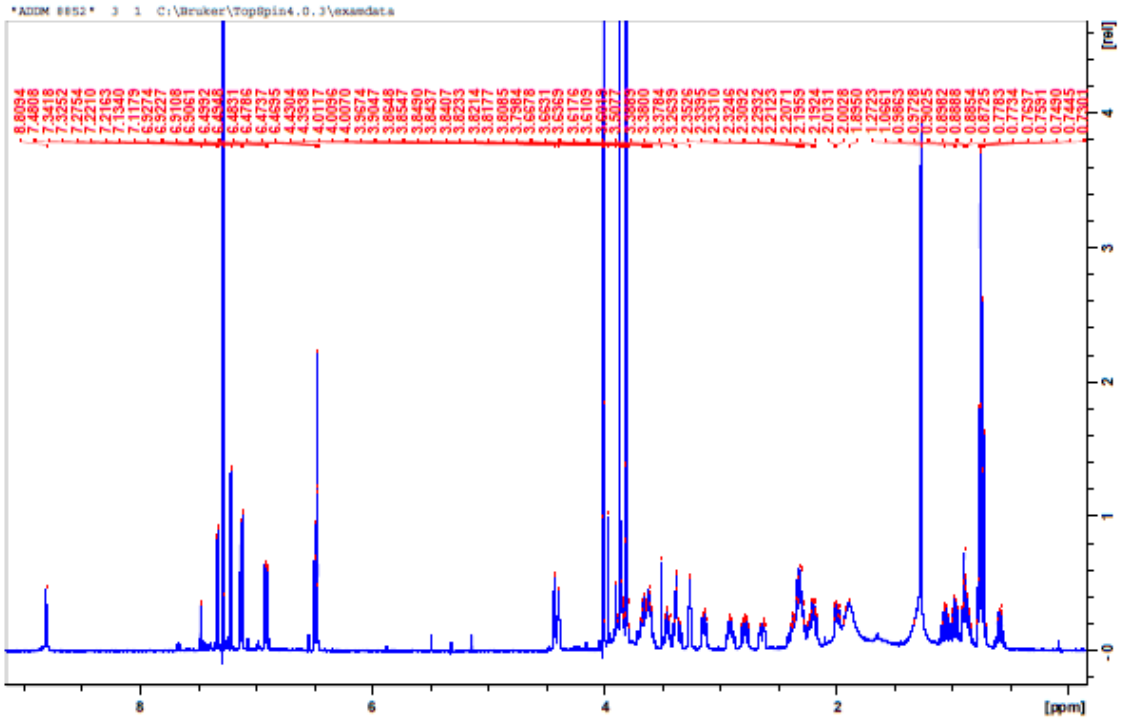

Figure S3: ^{1}H NMR spectrum of mixture compounds **2** and **3** ($CDCl_3$, 500 MHz).

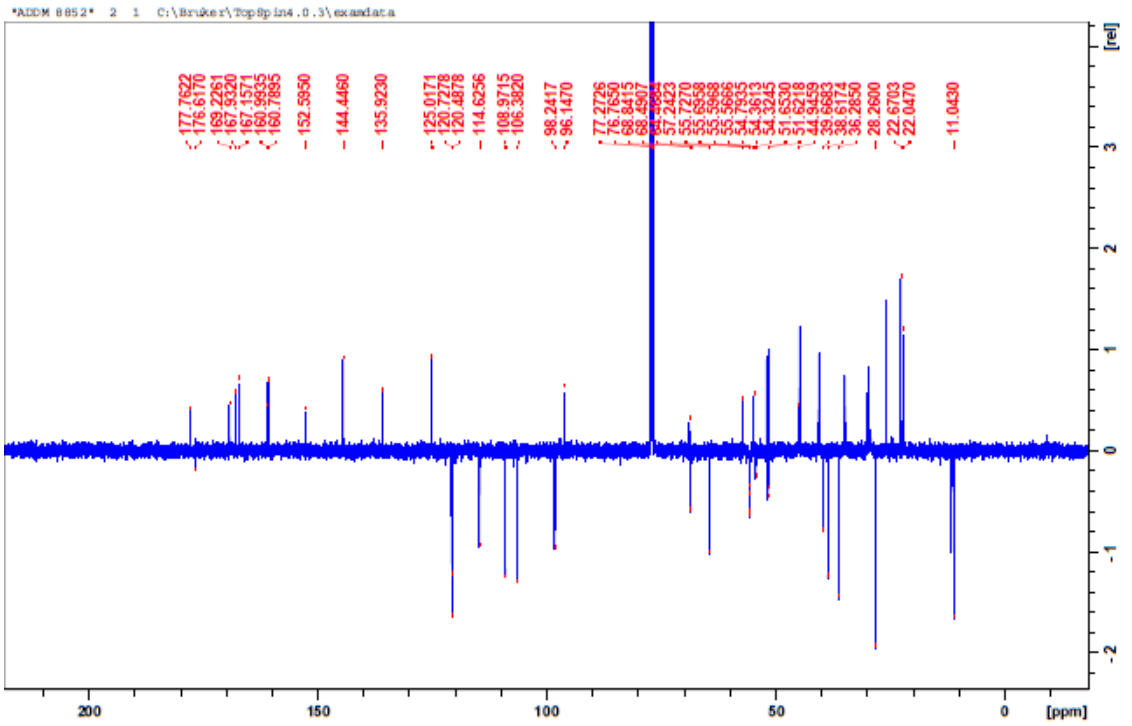

Figure S4: ^{13}C DEPTQ NMR spectrum of mixture of compounds **2** and **3** ($CDCl_3$, 125 MHz).

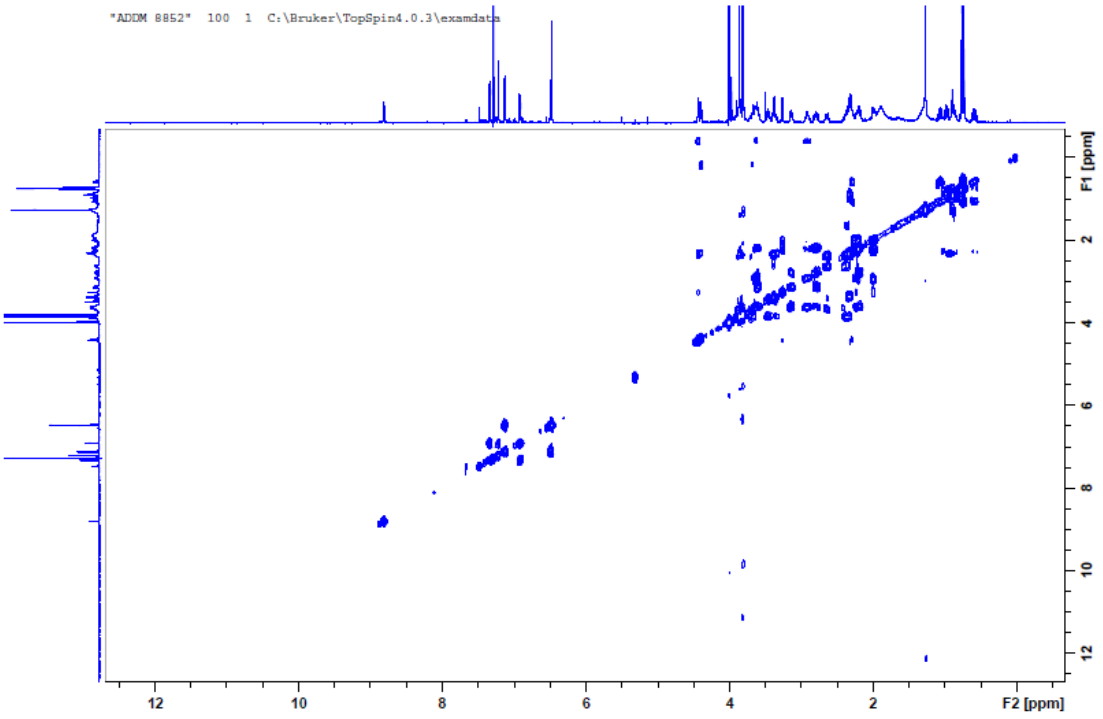

Figure S5: ^{1}H-^{1}1H-COSY spectrum of mixture of compounds **2** and **3** ($CDCl_3$, 500 MHz).

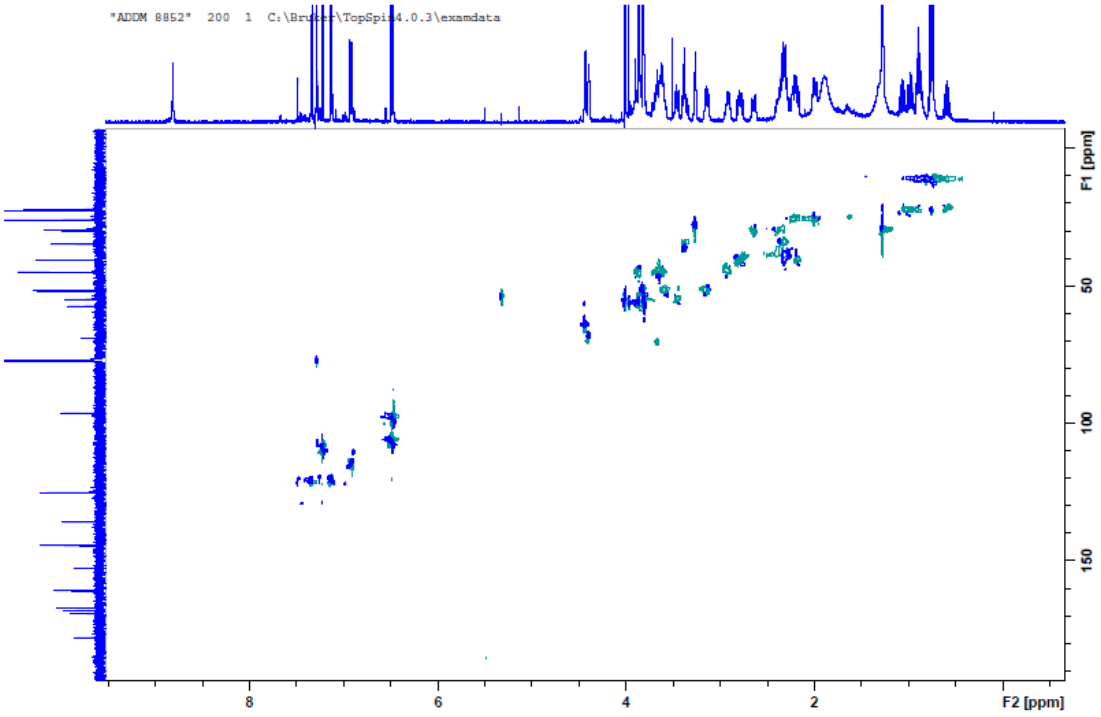

Figure S.6: HSQC spectrum of mixture of compounds **2** and **3** ($CDCl_3$, 500 MHz).

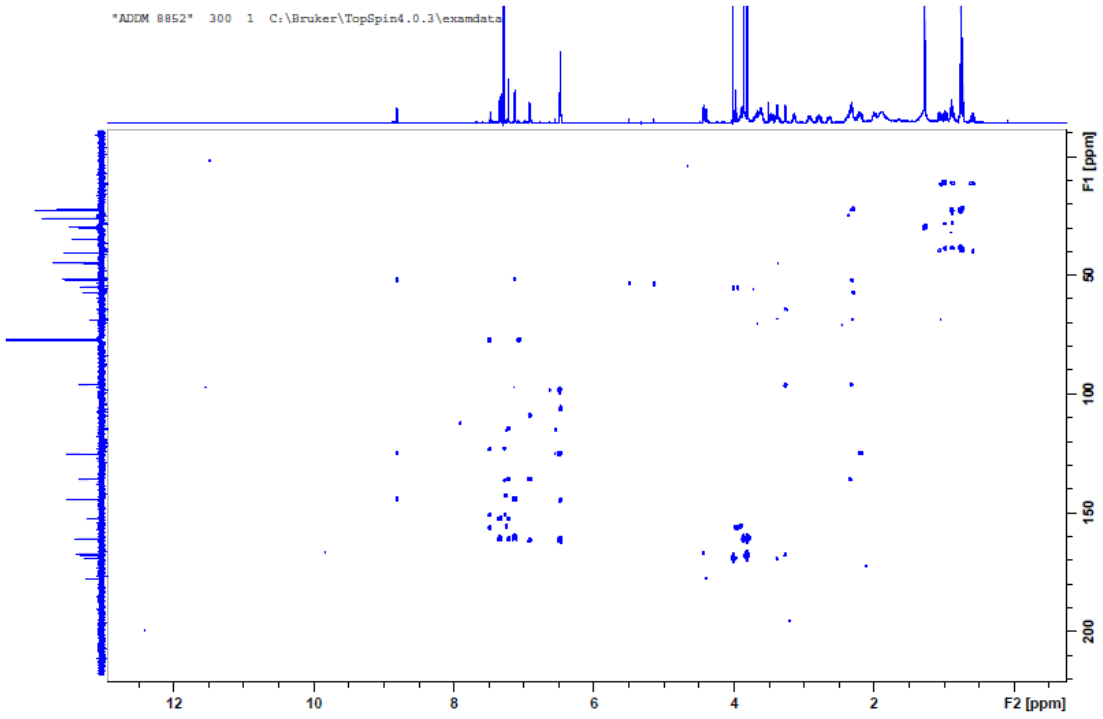

Figure S.7: HMBC spectrum of mixture of compounds **2** and **3** ($CDCl_3$, 500 MHz).

Compounds 2 and 3 HRESIMS - positive mode

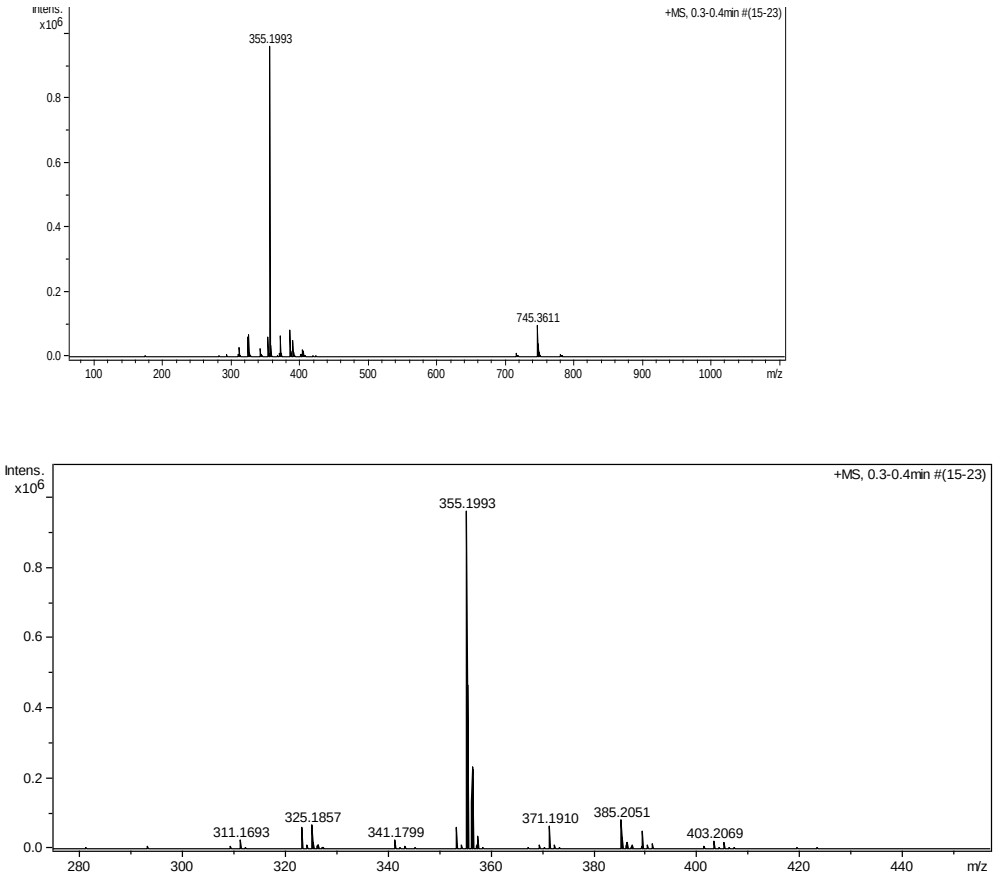

Figure S8: high-resolution mass spectrum of compounds **2** and **3**.

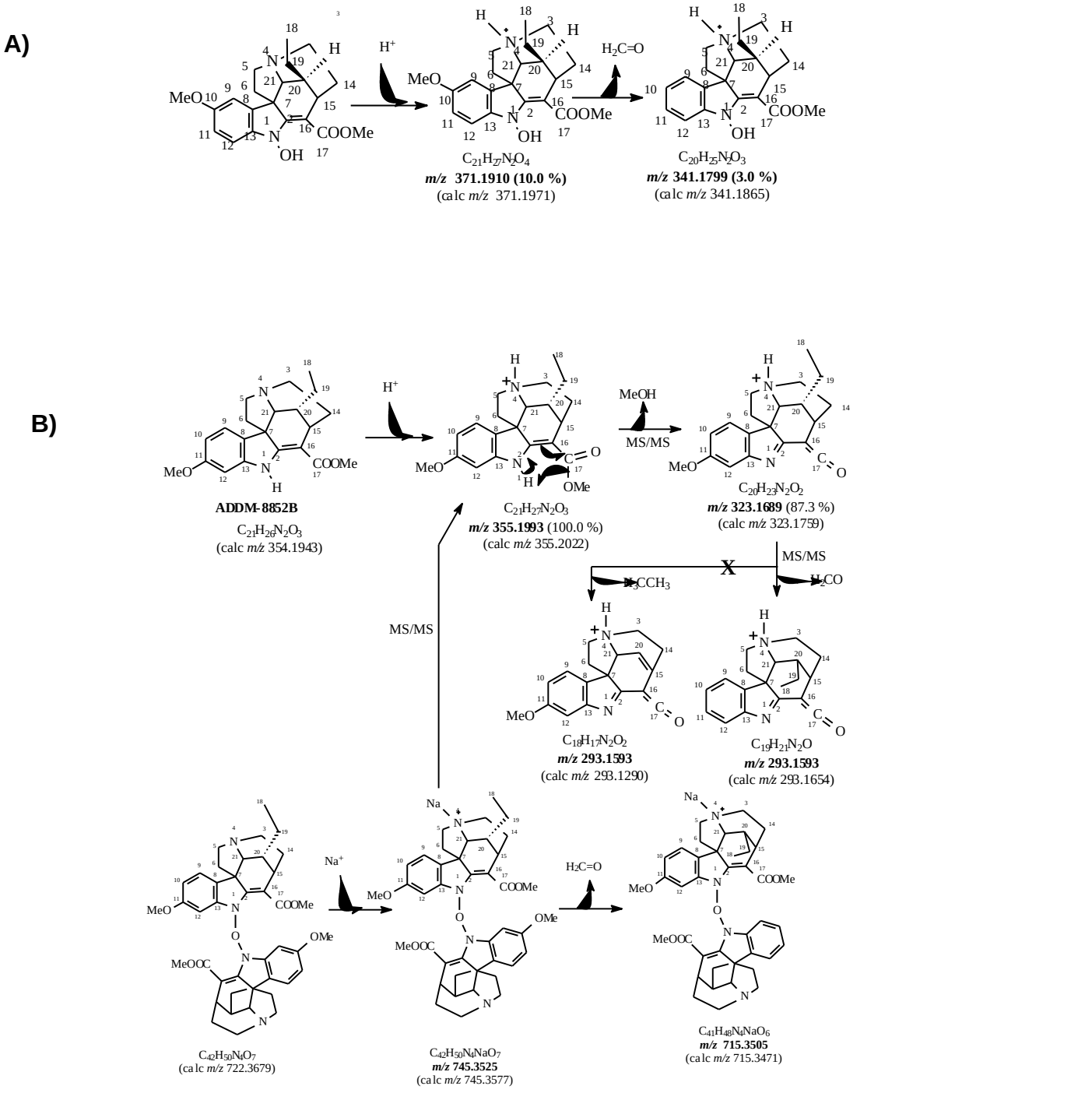

Figure S9: Proposal of mass spectra fragmentation of compounds **2** and **3**.

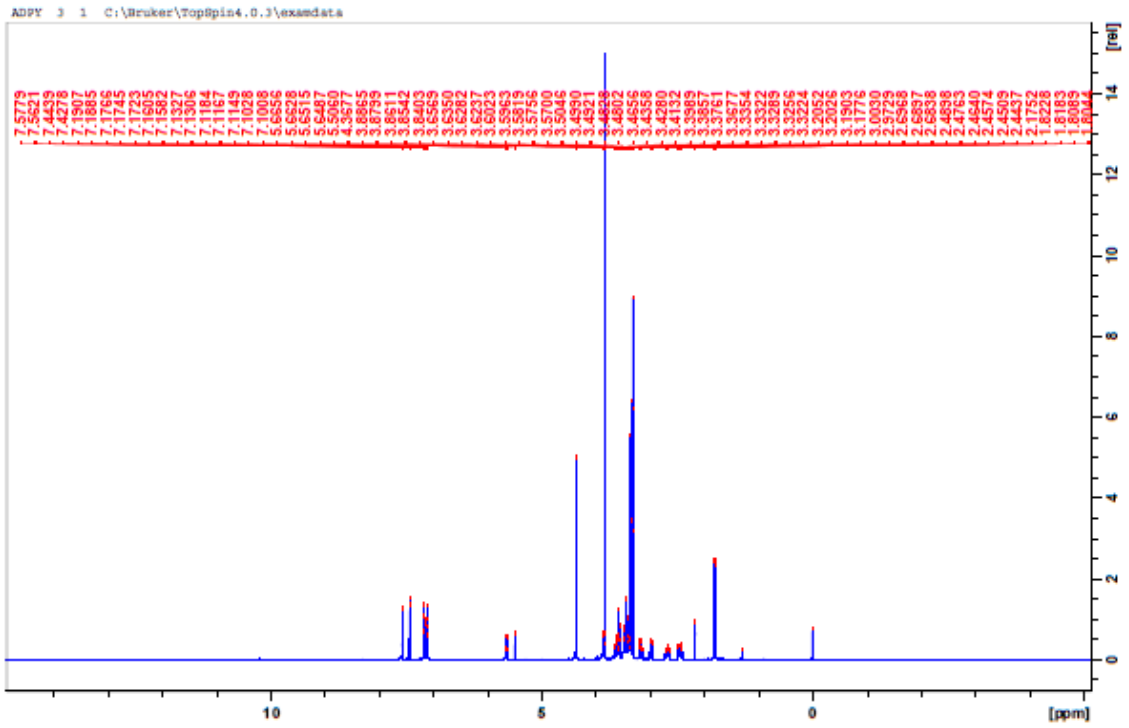

Figure S10: ^{1}H NMR spectrum of compound **4** (CD_3OD, 500 *MHz*).

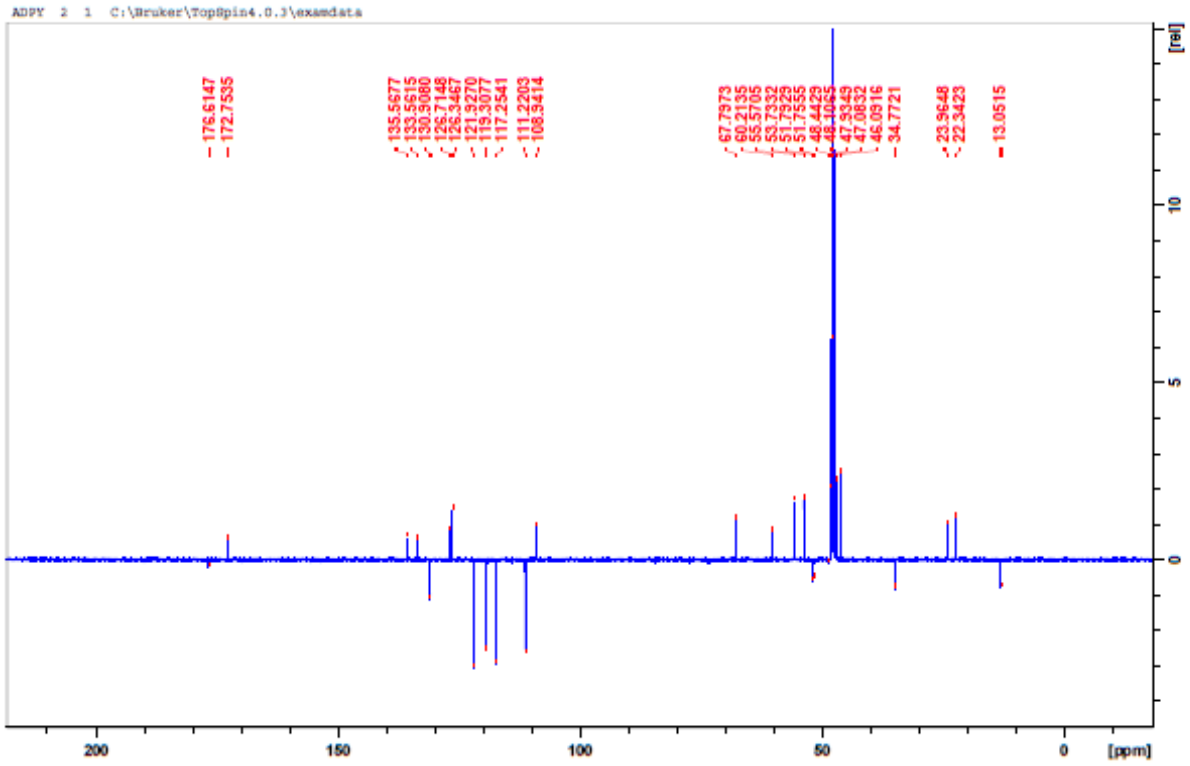

Figure S11: ^{13}C DEPTQ NMR spectrum of compound **4** (CD_3OD, 125 MHz).

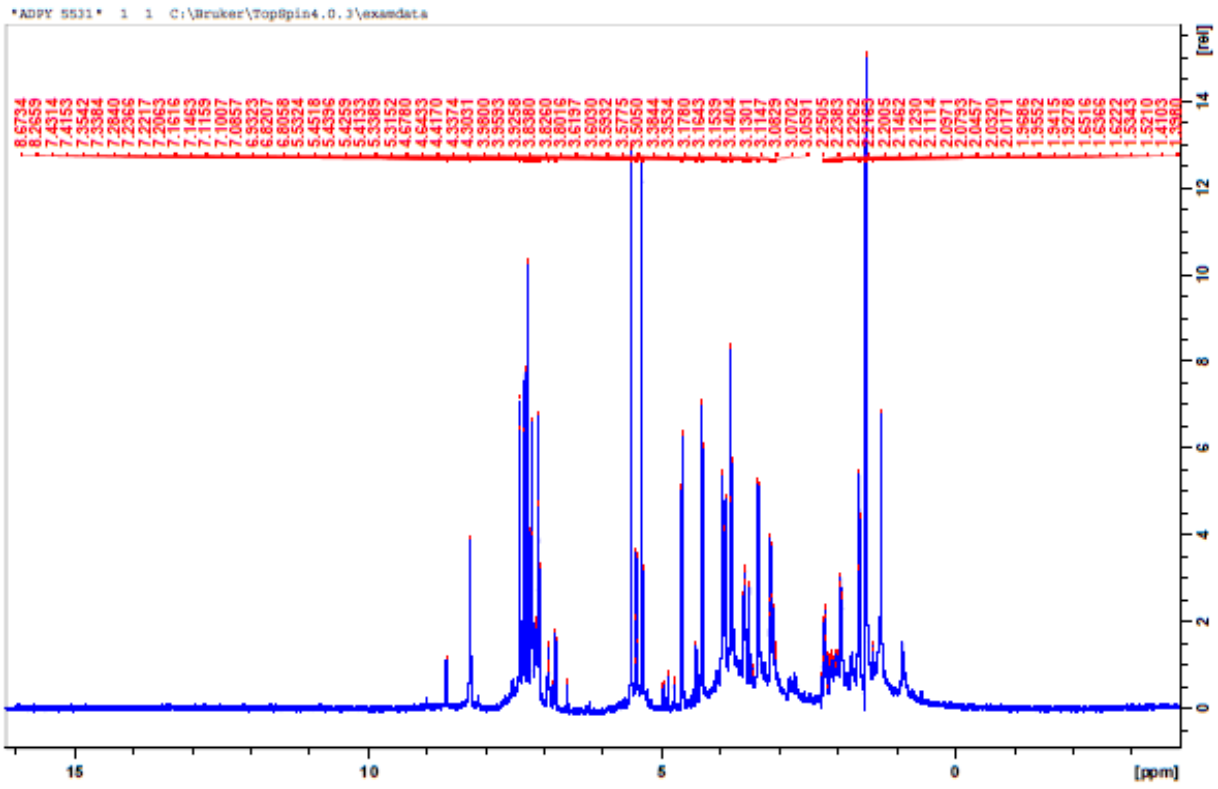

Figure S12: ^{1}H NMR spectrum of compound **5** (CD_3OD, 500 *MHz*).

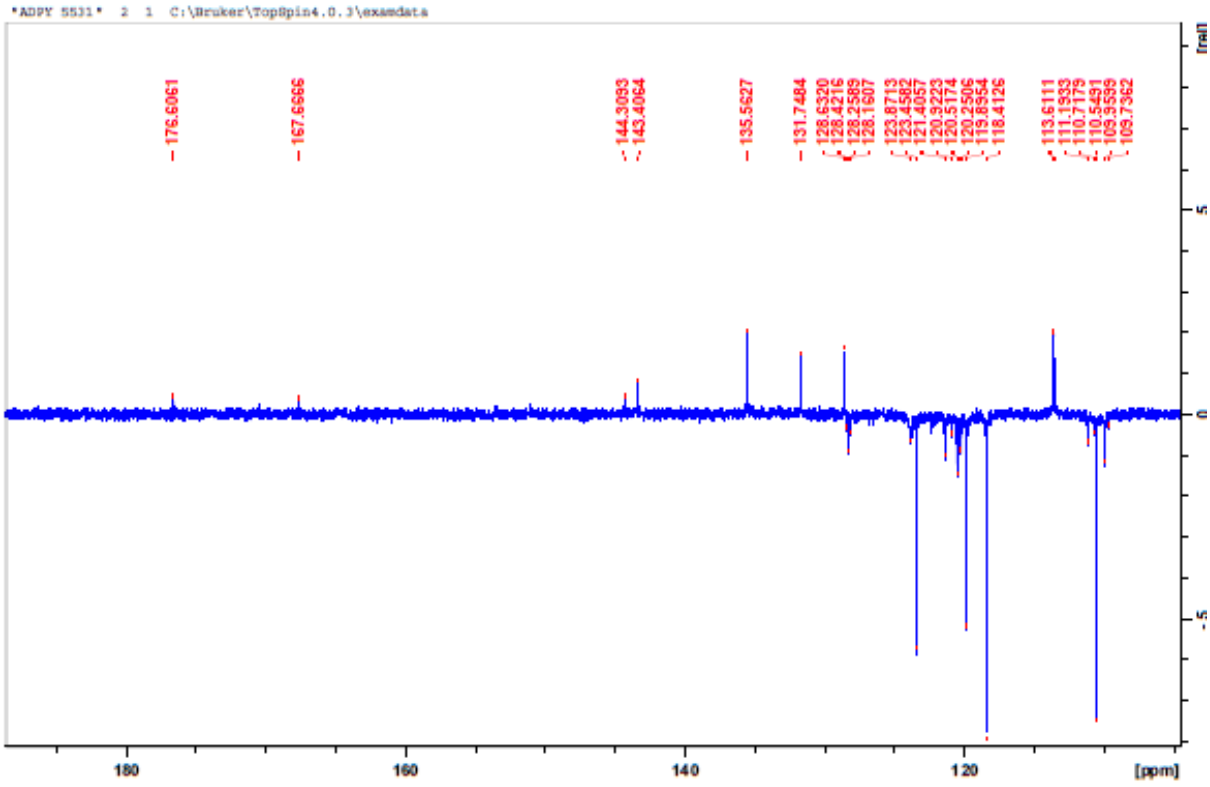

Figure S13: ^{13}C DEPTQ NMR spectrum of compound **5** (CD_3OD, 500 MHz).

Table S1: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data for Na-hydroxy-10-demethoxytubotaiwine (**2**) and 11-methoxytubotaiwine (**3**), including results obtained by heteronuclear 2D shift-correlated HSQC ($^{1}J_{CH}$) and HMBC ($^{n}J_{CH}$, n=2 and 3) and comparison with values described in the literature [18]), in $CDCl_3$ as solvent. Chemical shifts (*d*, ppm) and coupling constants (*J*, Hz, in parenthesis).

	(2)				(**3**)					
	HMQC		HMBC		HMQC		HMBC		**[18]**	
	δ_C	δ_H	$^{2}J_{CH}$	$^{3}J_{CH}$	δ_C	δ_H	$^{2}J_{CH}$	$^{3}J_{CH}$	δ_C	δ_H
C										
2	167.16	-		H-21; H-15	177.76	-		H-21	170.9	-
7	52.01	-		HN-1; H-9	57.24	-	H-6b		54.5	-
8	125.02	-		H-6b; HN-1; H-10; H-12	152.59	-		H-12	129.4	-
10	-	-	-		160.99	-	H-11	H-12; MeO-10	-	-
11	160.79	-	H-10; H-11	H-9; MeO-11	-	-	-	-	159.6	-
13	144.45	-	HN-1; H-12	H-9	135.92	-		H-9; H-11	144.8	-
16	96.15	-	H-15	H-20	96.15	-			95.9	-
17	167.93	-		H-15; MeO-17	169.23	-		H-15; MeO-17	169.0	-
CH										
9	120.79	7.12 (d, 8.0)			108.97	7.22 (d, 2.3)		H-11	120.0	6.97 (d, 9.0)
10	106.18	6.49 (dd, 8.0, 2.2)		H-12	-	-	-	-	105.5	6.35 (d. 9.0)
11	-	-	-	-	114.62	6.92 (dd, 2.3; 8.3)		H-9	-	-
12	98.24	6.47 (d, 2.2)		H-10	120.47	7.33 (d, 8.3)			97.2	6.3 (s)
15	28.26	3.26 (m)		2H-19	36.28	3.38 (m)			30.9*	3.01 (m)
20	36.62	2.32	2H-19	3H-18	39.67	2.30	2H-19	3H-18	41.3	1.94 (m)
21	64.47	4.43 (sl)			68.79	4.39 (sl)		H-6b	65.4	3.79 (m)
CH_2										
3	44.94	3.70 2.92 (m)			44.81				45.3	2.91 (m) 2.41 (m)
5	51.55	3..62 3.14			54.79	3.62 3.42 (dd, 11.9, 6.7)			53.9	2.95 (m) 2.82 (m)
6	40.59	2.70 2.20			34.85	3.35 2.20			44.0	
14	25.90	2.25 2.05			29.93	2.63 2.32			28.5*	1.78 (m)
19	22.67	0.98 (m) 0.89 (m)	H-20; 3H-18		22.05	1.07 (m) 0.59 (m)	H-20; 3H-18		23.9	0.81 (m)
CH_3										
18	11.04	0.76 (t, 7.3)	2H-19		11.57	0.74 (t, 7.2)	2H-19		11.6	0.71 (t, 6.4)
MeO-10	-	-	-	-	55.69	3.84 (s)			55.5	3.76 (s)
MeO-11	55.57	3.81 (s)			-	-	-	-	-	-
MeO-17	51.65	3.82 (s)			54.32	4.01 (s)			51.1	3.74 (s)

4. RESUMO E CONCLUSÕES

O estudo fitoquímico das cascas de *A. desmanthum* levou a identificação dos alcaloides aspidocarpina e a mistura entre um novo alcaloide N_a-hidroxi-11-desmetoxi-10-metoxitubotaiwina e 11-metoxitubotaiwina, isolado pela primeira vez nessa espécie.

Através do estudo fitoquímico das sementes de *A. pyricollum* foram identificados os alcaloides estemadenina (**4**) e aparicina (**5**).

O alcaloide aspidocarpina demonstrou potencial anti-hipertensivo, através da infusão intravenosa em ratos Wistar, sem causar incoordenação motora e desequilíbrio no teste do Rotarod.

Os alcaloides identificados demonstraram de moderada a baixa atividade antimicobacteriana (CIM_{50} 49,9 a ≥500 µg/mL) e atividade inibitória da produção de óxido nítrico de boa a moderada (CI_{50} 18,3 a 138,7 µg/mL). Os compostos não apresentaram citotoxicidade contra as células de macrófagos RAW264.7 e apresentaram valores de CI_{50} variando de 59,8 a ≥500 µg/mL contra as células de leucemia humana Molt-4.

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