UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA - RENORBIO

Uso de técnicas hifenadas (CLAE-DAD/CL-EM) na identificação de metabólitos secundários da atemoia (*Annona cherimola* x *Annona squamosa*) e avaliação da atividade citotóxica

SUZANA VIEIRA RABÊLO

Recife-PE
2019

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Dedico este trabalho aos que verdadeiramente me conhecem e amam:
Minha família!

AGRADECIMENTOS

À Deus;

À minha mãe, meu pai (*in memorian*), minhas irmãs, meu sobrinho, meu companheiro, meus parentes e amigos... Vocês são fonte de forças para eu seguir em frente.

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“O que dizem sobre nós só deverá ser considerado se estiver comprometido com nossa verdade ou favorecer o nosso crescimento. Caso contrário, deverá ser interpretado como mais um ruído que dispersamos com o esquecimento.”

Pe Fábio de Melo.

RESUMO

Atemoieira é um híbrido resultante do cruzamento de duas espécies do gênero *Annona* (*A. cherimola* e *A. squamosa*) que são conhecidas pela riqueza de compostos biologicamente ativos. Devido ao número crescente de novos casos de câncer a cada ano e aos tratamentos agressivos, e muitas vezes ineficientes, faz-se necessário a busca por novos compostos citotóxicos eficazes. O uso de técnicas hifenadas de separação e identificação de moléculas de matrizes vegetais tem sido bastante valorizadas, pois conseguem acelerar as buscas por esses compostos potencialmente ativos. Por isso, o objetivo deste trabalho foi analisar o potencial citotóxico de extratos e fases de diferentes partes da planta atemoia e correlacionar os resultados com sua composição química definida por meio de diferentes técnicas de isolamento e identificação. Para tal, folhas da planta foram coletadas em Petrolina-PE e extraídas com hexano seguido por metanol para obtenção dos extratos brutos hexânico (EHB-F) e metanólico (EMB-F), respectivamente. O EMB-F foi submetido à uma extração ácido base para obtenção da fração alcaloídica. O uso de técnicas cromatográficas comuns levou ao isolamento de um alcaloide inédito (aqui chamado de dehidroanomuricina *N*-óxido) e dos alcaloides anonaina, asimilobina, lanuginosina, liriodenina, lisicamina, pronuciferina, estefarina e anomuricina, cujas identificações foram feitas por análise de espectros de massas, de Ressonância Magnética Nuclear uni e bidimensionais e por comparação com dados da literatura. Anomuricina e dehidroanomuricina *N*-óxido tiveram suas fragmentações propostas. Além disso, partes aéreas da planta (talos e folhas), passaram pelo mesmo procedimento que as folhas para obtenção dos extratos brutos hexânico (EHB-PA), metanólico (EMB-PA) e fração alcaloídica (FAT) que foi submetida ainda à extração sólido-líquido para obtenção das suas subfrações - hexânica (FAT-Hex), clorofórmica (FAT-$CHCl_3$), acetato de etila (FAT-AcOEt) e metanólica (FAT-MeOH). Todas as fases, frações e subfrações obtidas foram testadas quanto à sua citotoxicidade. Os resultados foram comparados com estudos de cromatografia líquida e cromatografia gasosa, ambas acopladas a espetrômetro de massas (CL-EM e CG-EM, respectivamente), para correlação química com potencial biológico. Todos os procedimentos de acesso ao patrimônio genético e conhecimentos tradicionais associados foram realizados e o projeto foi registrado no SisGen (Registro #ABD9AA7). Como resultado das análises por CL-EM foi possível identificar a

presença de anomuricina, dehidroanomuricina *N*-óxido, esculerina, reticulina, isocoridina, norisocoridina, asimilobina, nornuciferina, anonaina e liriodenina no EMB-PA. A análise do EHB-PA por CG-EM levou a identificação de 29 compostos, sendo o espatulenol o majoritário. Este extrato (EHB-PA) inibiu mais de 90% do crescimento das células de glioblastoma testadas (SF-295). Foi possível observar ainda, que o EMB-PA teve sua atividade potencializada quando fracionada, com diferença de até 41%, aproximadamente. Anomuricina foi testada diante de nove linhagens celulares e apresentou potencial citotóxico diante de células leucêmicas (HL-60) com índice de seletividade de 1,66. As diferentes partes do híbrido apresentaram alto potencial citotóxico e por isso esta planta pode ser considerada uma fonte promissora de compostos citotoxicamente ativos.

Palavras-chave: Citotoxicidade, Espectrometria de Massas, Ressonância Magnética Nuclear, Cromatografia Líquida e Gasosa.

ABSTRACT

Atemoieira is a hybrid resulting from the crossing of two species of the *Annona* genus (*A. cherimola* and *A. squamosa*) that are known for the richness of biologically active compounds. Due to the increasing number of new cases of cancer each year and aggressive and often inefficient treatments, it is necessary to search for new effective cytotoxic compounds. The use of hyphenated techniques of separation and identification of plant matrix molecules has been highly valued, since they can accelerate searches for these potentially active compounds. Therefore, the objective of this work was to analyze the cytotoxic potential of extracts and phases of different parts of the atemoia plant and to correlate the results with their chemical composition defined by means of different isolation and identification techniques. For this, plant leaves were collected in Petrolina-PE and extracted with hexane followed by methanol to obtain crude extracts hexane (EHB-F) and methanolic (EMB-F), respectively. The EMB-F was subjected to an acid-base extraction to obtain the alkaloidal fraction. The common chromatographic techniques used led to the isolation of a novel alkaloid (here called dehidro-*N*-oxide anomuricine) and the anonaine, asimilobine, lanuginosine, liriodenine, lisicamine, pronuciferine, stefarine and anomuricine alkaloids whose identifications were made by analysis of spectra masses, uni and bidimensional Nuclear Magnetic Resonance and by comparison with literature data. Anomuricine and dehydro-*N*-oxide-anomuricine had their fragmentations proposed. In addition, the aerial parts of the plant (mixture of stems and leaves), went through the same procedure that leaves to obtain the hexane crude extracts (EHB-PA), methanol (EMB-PA) and alkaloidal fraction (FAT-PA) which was further subjected to extraction solid-liquid to obtain subfractions - hexane (FAT-Hex), chloroform (FAT-$CHCl_3$), ethyl acetate (FAT-AcOEt) and methanol (FAT-MeOH). All the extracts, fractions and subfractions obtained were tested for their cytotoxicity. The results were compared to liquid chromatography and gas chromatography, both coupled to mass spectrometer (LC-MS and GC-MS, respectively), for chemical correlation with biological potential. All procedures for access to genetic heritage and associated traditional knowledge were carried out and the project was registered with SisGen (Record # ABD9AA7). As result of analysis by LC-MS was possible to identify the presence of anomuricine, dehydro-*N*-oxide-anomuricine, scoulerine, reticuline, isocorydine, norisocorydine, asimilobine, nornuciferine, liriodenine, anonaine in the

EMB-PA. The analysis of EHB-PA by GC-MS led to the identification of 29 compounds, with spatulenol being the major. This extract (EHB-PA) inhibited more than 90% of the growth of glioblastoma cells tested (SF-295). It was also possible to observe that the EMB-PA had its activity potentiated when fractioned, with a difference of up to 41%, approximately. Anomuricin was tested against nine cell lines and presented a cytotoxic potential against leukemic cells (HL-60) with selectivity index of 1.66. The different parts of the hybrid showed high cytotoxic potential and therefore this plant can be considered a promising source of cytotoxically active compounds.

Key-Words: Cytotoxicity, Mass Spectrometry, Nuclear Magnetic Resonance, Liquid and Gas Chromatography.

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1. INTRODUÇÃO

Atualmente, os produtos naturais desempenham um papel fundamental como fonte de novos medicamentos ou como fonte de substâncias que levam ao desenvolvimento de novos medicamentos. Entre a gama de potencialidades farmacêuticas, estes produtos aparecem como uma alternativa ao combate a microrganismos patogênicos e múltiplas drogas, devido ao uso indiscriminado de antimicrobianos, assim como potenciais agentes anticâncer (GUIMARÃES et al., 2010; KHAN, 2018).

O câncer, que é caracterizado por diferenciação e proliferação celular anormal (DOLL e PETO, 1981), especialmente tumores malignos, é responsável por um número significativo e crescente de pacientes em todo o mundo e representa a segunda principal causa de morte na população mundial (COSTA-LOTUFO, 2010). Essa crescente incidência e as várias limitações da terapia convencional, incluindo alto custo e alta toxicidade das atuais drogas antineoplásicas, têm enfrentado um sério desafio para todos os pesquisadores. É o desafio de projetar e desenvolver uma alternativa estratégica, econômica e eficaz no processo de identificação e aquisição de novos medicamentos.

Nesse sentido, as técnicas hifenadas como cromatografia gasosa acoplada à espectrometria de massas (CG-EM) e cromatografia líquida acoplada a espectrometria de massas (CL-EM), por exemplo, são apresentadas como ferramentas mais rápidas e úteis na tentativa de identificar substâncias de extratos vegetais e biologicamente ativos mais rapidamente. E é nesse cenário que acredita-se que as fitomoléculas devem revolucionar o tratamento do câncer na próxima década (IQBAL, 2017).

Assim, a busca por moléculas bioativas inicia-se com a escolha do material vegetal e neste âmbito, as espécies da família Annonaceae são consideradas boas fontes de substâncias bioativas provenientes do seu metabolismo secundário. Esta família compreende cerca de 2500 espécies distribuídas em aproximadamente 135 gêneros (CHATROU et al., 2009), nos quais o gênero *Annona* se destaca por produzir frutos muito saborosos e nutritivos, que agregam grande valor comercial (LEMOS, 2014).

Dentre as anonáceas, a atemóia é um híbrido resultante do cruzamento entre duas espécies do gênero *Annona:* a pinha e a cherimoia (*Annona squamosa* x *Annona cherimola*, respectivamente) (WONGS-AREE; NOICHINDA, 2011). Estas espécies paternais da atemoia já foram bastante estudadas fitoquimicamente e entre seus

componentes estão inclusos, pincipalmente, alcaloides, diterpenos, acetogeninas e óleos essenciais que apresentam atividades significativas, como citotóxicos, antimicrobianos, antioxidantes e antiparasitários, por exemplo (RABÊLO et al., 2016). Dentre os alcaloides comuns, anonaína, asimilobina, liriodenina e reticulina são considerados marcadores taxonômicos das espécies deste gênero (CRUZ et al., 2011).

Ainda sobre a atemoia, investigações fitoquímicas realizadas em espécies coletadas em ChiaYi, Taiwan, os alcaloides atemoina e cleistofolina foram apresentados como produto do metabolismo secundário (WU, 2005). Os monoterpenos linalol, α-pineno, β-pineno, *trans*-ocimeno e o sesquiterpeno biciclogermacreno, entre outros, foram identificados como constituintes do óleo essencial extraído dos frutos de atemoia coletados em Havana e na Austrália (WYLLIE et al., 1987; PINO; ROSADO, 1999).

Dentre as diferentes regiões do mundo, o Brasil é um país que possui um grande potencial para a exploração da biodiversidade e tem um amplo conhecimento tradicional acumulado por populações locais, que têm acesso à natureza e aos produtos da biodiversidade. Dentre as regiões brasileiras, a região Nordeste é caracterizada por altos valores de radiação solar, elevadas temperaturas, e pela irregularidade no regime pluviométrico. Características estas que proporcionam à vegetação aqui existente, períodos abundantes de stresse hídrico que afetam a síntese de metabólitos secundários (GOBBO-NETO; LOPES, 2007). Assim, o isolamento e a determinação estrutural de substâncias orgânicas produzidas pelo metabolismo secundário das plantas provenientes desta região representam importância fundamental para o desenvolvimento científico da própria química de produtos naturais e contribuem para avanço de outras atividades científicas e tecnológicas no país (BRAZ-FILHO, 2010).

Diante do exposto, foi objetivo deste trabalho analisar o potencial citotóxico de extratos brutos, fases alcaloídicas e subfrações das fases alcaloídicas provenientes de folhas e partes aéreas (mistura de folhas e talos) de atemoia, e identificar e/ou isolar os constituintes químicos nelas presentes.

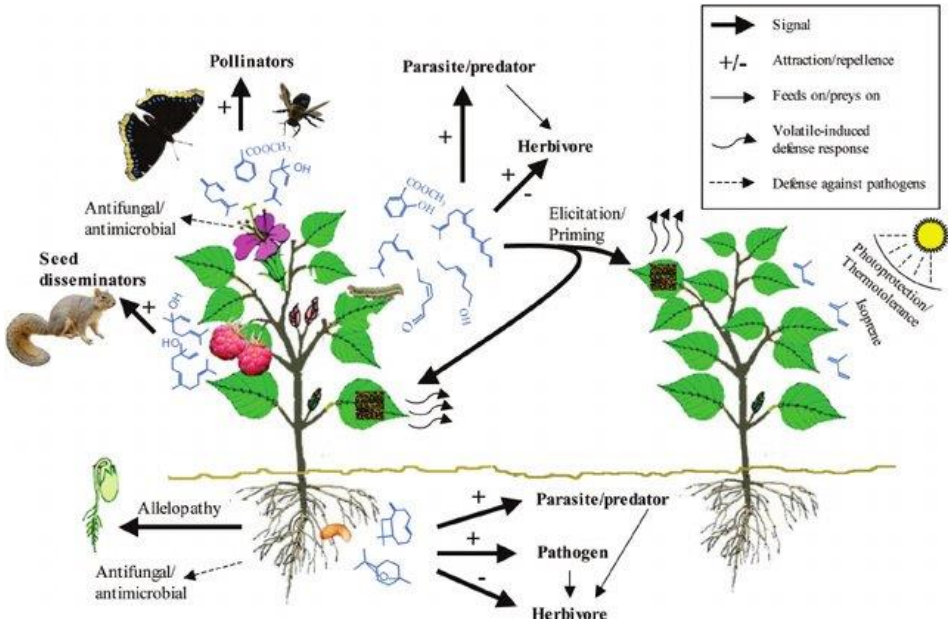
2. REVISÃO DE LITERATURA

2.1. Metabolismo secundário de plantas

As plantas produzem uma enorme variedade de metabólitos que tem funções diversas em todas as etapas da sua vida que vão desde o nascimento até a e morte. Estes metabólitos são moléculas orgânicas que distribuem-se entre os metaboslimos primário e secundário, e são mais viriáveis nas plantas do que os metabólitos produzidos pela maioria dos outros organismos (FANG, 2018; BRAZ FILHO, 2010).

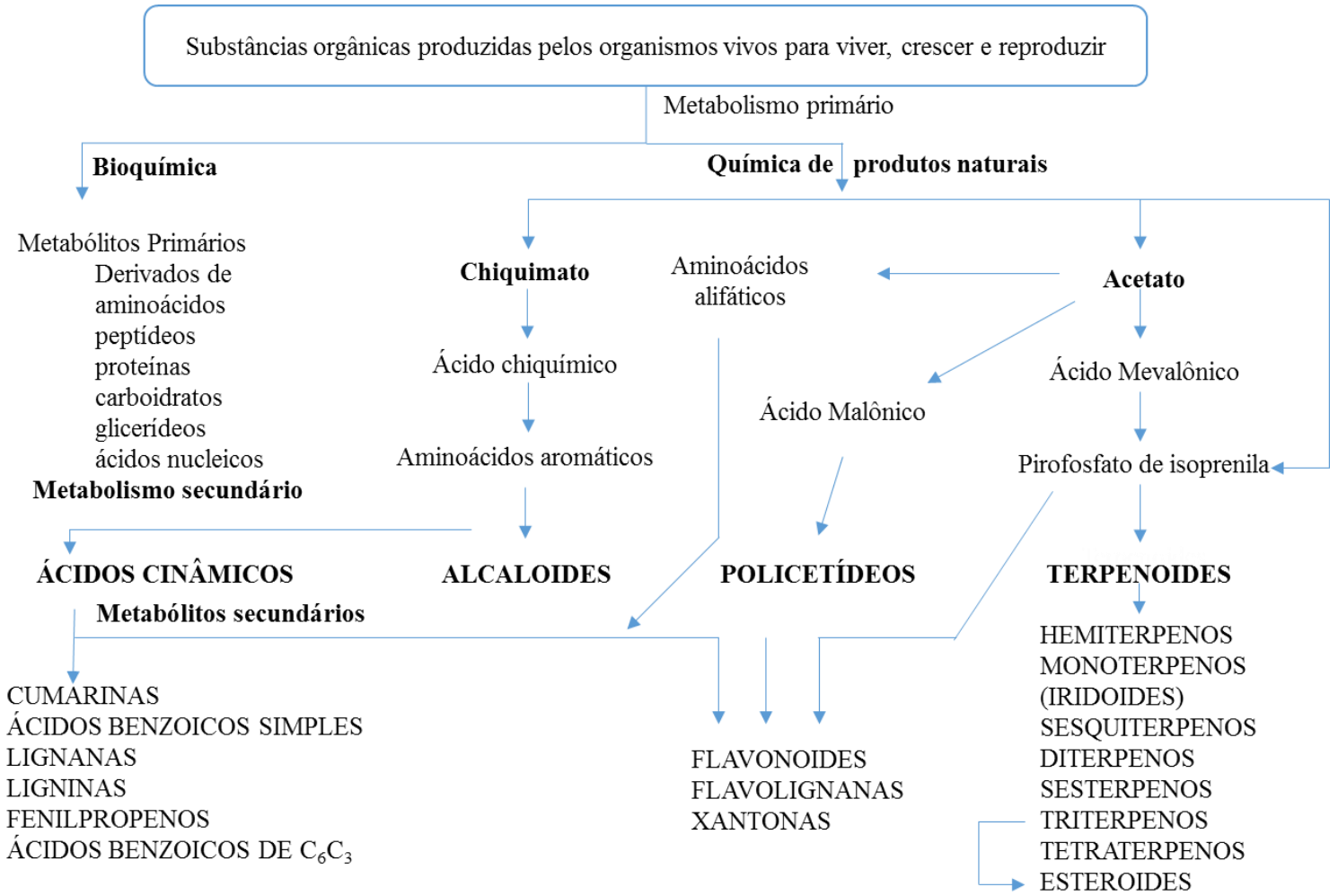
Apesar de não ter papel principal na manutenção de processos vitais fundamentais nas plantas que os sintetizam, os metabólitos secundários podem servir para deter potenciais herbívoros ou patógenos, para atrair polinizadores ou simbiontes, ou para promover os interesses da planta de outras maneiras, como é o caso do isopreno que confere fotoproteção e termotolerância às plantas (DUDAREVA, 2006). Eles são produzidos em partes específicas de plantas em estágios definidos de desenvolvimento e as quantidades são frequentemente baixas (menos de 1% do peso seco) e altamente variáveis (DAVEY, 2017). A Figura 1 e o Esquema 1, a seguir, são representações das interações entre constituintes voláteis do metabolismo secundário e substâncias orgânicas produzidas pelos metabolismos primário e secundário de plantas, respectivamente.

Figura 1. Imagem ilustrativa de uma visão geral das interações de plantas mediadas por compostos voláteis com o ambiente circundante.



(Fonte: DUDAREVA, 2006)

Esquema 1. Substâncias orgânicas produzidas pelos organismos vivos para viver, crescer e reproduzir.



(Adaptado de BRAZ FILHO, 2010).

As moléculas do metabolismo secundário desempenham funções biológicas importantes para os seres humanos e por isso tem sido cada dia mais investigadas pelos grupos de pesquisa com interesse em química medicinal. Isso deve-se a descobertas importantes que teve como marco o isolamento de quinina (alcaloide) das cascas de *Cinchona* pelos cientistas franceses Caventou e Pelletier em 1920 (PHILLIPSON, 2001; OLIVEIRA E SZCZERBOWSKI, 2009).

Após o isolamento de quinina, o princípio ativo do "pó dos Jesuítas" (pó feito com as raízes das árvores de *Cinchona*) que é uma mistura utilizada pelos índios - por eles chamada "quina-quina" - para tratar malária, muitos outros estudos com outras espécies de plantas e de síntese molecular em laboratórios de fitoquímica foram alavancados (OLIVEIRA E SZCZERBOWSKI, 2009).

Com o passar dos anos, muitas classes de metabólitos secundários de plantas são conhecidos e testados para diversos fins farmacêuticos, medicinais ou agrícolas como repelentes de insetos, herbicidas, pesticidas naturais, larvicidas, auxinas, fitoalexinas, anti-helmínticos, anti-filariais, neuro-ativos, cardiovasculares, anti-hiperlipidêmicos, protetores do fígado, anti-inflamatórios, antimicrobianos, anti-cânceres, anti-oxidantes , anti-diabéticos, analgésicos, anti-espasmódicos, digestivos, purgativos, anti-úlceras, afrodisíacos, tônicos, anti-alérgenos, etc (KHAN, 2018).

Como agentes anticancerígenos de origem vegetal, as moléculas vincristina e vimblastina, isoladas de *Catharantus roseus*, pertencem a classe dos alcaloides (assim como a quinina), se destacam como dois dos mais importantes agentes quimioterápicos de uso clínico corrente contra o câncer e são de grande utilidade no tratamento de linfoma de Hodkin, sarcoma de Kaposi, câncer do ovário e testículos e leucemia linfoblástica aguda infantil (BRAZ FILHO, 2010; BRANDÃO et al., 2010).

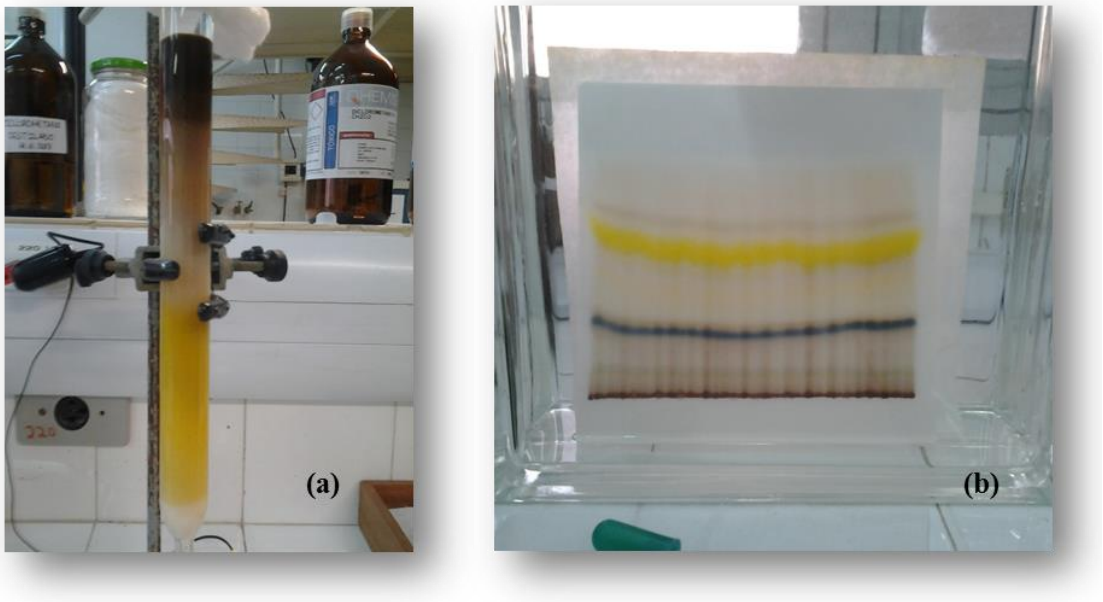
Outras classes de metabólitos secundários de interesse farmacológico são os Flavonoides, terpenoides, cumarinas, antraquinonas etc, que apresentam atividades biológicas variadas (SIMÕES et al., 2010).

2.2. Uso de técnicas hifenadas

Á princípio as técnicas de separação dos constituintes de matrizes eram os chamados métodos clássicos, que ainda são comumente utilizados nos laboratórios de

produtos naturais (PN's). No entanto, estes métodos, que envolvem a cromatografia em coluna (CC), cromatografia em camada delgada analítica (CCDA) e preparativa (CCDP) – Figura 2 –, por exemplo, são metodologias que geralmente permitem o isolamento de substâncias majoritárias, sem que haja seletividade para o isolamento de novas moléculas ou de moléculas que ainda não foram estudadas quanto ao seu potencial biológico. Já os métodos de identificação como espectroscopia de infravermelho (IV), ultravioleta (UV), ressonância magnética nuclear (RMN) e espectrometria de massas (EM) são técnicas importantes e comumente utilizadas, porém, a velocidade e confiabilidade dos resultados de elucidação estão diretamente relacionados aos conhecimentos técnicos do pesquisador.

Figura 2. Imagem ilustrativa de sistema de cromatografia em coluna (a) e em camada delgada preparativa (b).

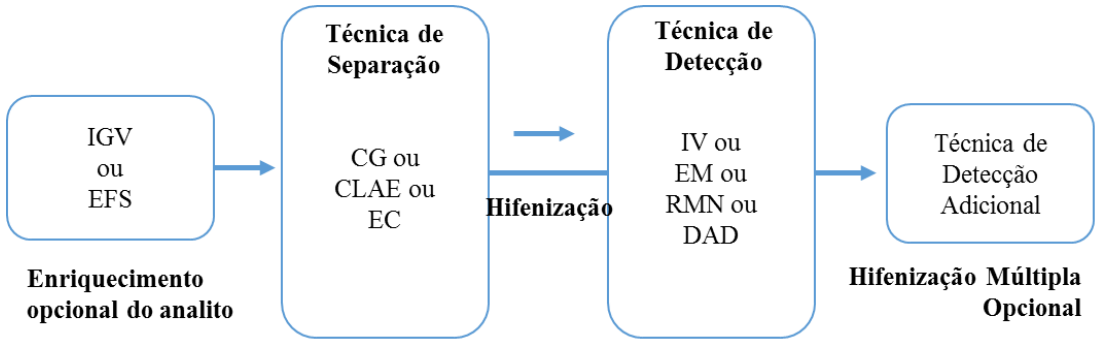


(Fonte: Próprio autor)

Por isso, na prospecção por moléculas bioativas os avanços tecnológicos que proporcionaram a hifenação das técnicas de separação às técnicas de identificação permitem a elucidação estrutural completa (ou parcial), de misturas complexas, com ajuda ainda de plataformas que permitem identificação *on-line*. Com isso, evita-se, portanto, a perda de tempo com isolamentos de compostos de baixo interesse estrutural e/ou de atividade biológica, além de se permitir a caracterização de compostos lábeis e/ ou voláteis de difícil isolamento (CASS E BARREIRO, 2011).

Nas técnicas hifenadas os aparelhos são, em sua maioria, compostos por um elemento de separação de alta eficiência como cromatografia gasosa (CG), cromatografia líquida (CL), cromatografia líquida de alta eficiência (CLAE) ou eletroforese capilar (EC), por exemplo, acoplado a um detector com capacidade de varredura espectrofotométrica como IV, EM, RMN ou DAD (detector de arranjo de diodos), e por isso são uma importante ferramenta na prospecção por novos fármacos (Esquema 2). No entanto, a hifenação não precisa acontecer apenas entre duas técnicas; o acomplamento pode envolver mais de uma técnica de separação ou detecção como CL-DAD-EM, CL-EM-EM, CL-RMN-EM, CL-DAD-RMN-EM e ainda ser submetido previamente a um enriquecimento utilizando a injeção de grandes volumes (IGV) ou extração em fase sólida (EFS), por exemplo (PATEL et al., 2010).

Esquema 2. Possibilidade de acoplamento de técnicas de separação e detecção de constituintes de matrizes complexas.



(Fonte: Adaptado de Patel et al., 2010)

No Brasil, técnicas como CG-EM, CLAE-EM, CLAE-UV-EM, CLAE-EM/EM, CLAE-EM_n, CLAE-RMN, estão sendo cada vez mais utilizadas por inúmeros grupos de pesquisa, em decorrência de uma necessidade da atualização das abordagens para investigação do metabolismo secundário de fontes naturais (BERLINCK et al., 2017). O *screening* químico utilizando estas técnicas fornece rapidamente ampla informação estrutural, possibilitando em alguns casos a identificação inequívoca de compostos sem a necessidade do isolamento clássico dos mesmos, auxiliando assim, na racionalização dos estudos fitoquímicos (FUNARI et al., 2013).

A IES (ionização por *electrospray*) é a técnica de ionização mais utilizada para acoplamento com CLAE, já que permite a análise de moléculas polares, passíveis de sofrer ionização. Esse tipo de ionização ocorre à pressão atmosférica, onde um campo

elétrico intenso dispersa uma amostra líquida em um gás, na forma de um fino *spray* de gotas carregadas que, por evaporação, ejetam íons na fase gasosa (PETTA, 2008; CROTTI et al., 2006).

Na IES-EM (ionização por *electrospray* acoplada à espectrometria de massas) as substâncias que apresentam grupamentos básicos, principalmente aminas, amidas e ésteres, normalmente são analisadas em modo positivo, dadas à relativa facilidade de protonação desses grupos funcionais. Por outro lado, substâncias contendo funções ácidas, tais como ácidos carboxílicos e fenóis, são mais facilmente desprotonadas e, consequentemente, são analisadas em modo negativo (CROTTI et al., 2006).

Assim, esta técnica permite identificar uma gama de moléculas do metabolismo secundário das plantas, permitindo selecionar grupos de maior interesse. Como exemplo, Silva e colaboradores (2012), identificaram alcaloides aporfínicos e oxoaporfínicos de extratos de *Unonopsis guattrioides*, utilizando espectrômetro *ion trap* equipado com uma fonte de *electrospray*, operando no modo positivo e programado para monitorar a faixa de *m/z* 200-400, adequada aos alcaloides aporfínicos.

O acoplamento de um espectrômetro de massas a algum tipo de instrumento cromatográfico, como um cromatógrafo a gás (CG-EM) ou um cromatógrafo a líquido (CL-EM), é comum. Os espectrômetros de massas são muito uteis na analise de compostos cujo espectro de massas é conhecido e na análise de compostos de estrutura completamente desconhecida. No caso de compostos conhecidos, uma busca computadorizada compara o espectro de massas do composto em questão com uma biblioteca de espectros de massas. A coincidência dos espectros de massas é uma evidencia convincente da identificação que é, muitas vezes, aceita em procedimentos legais. No caso de compostos desconhecidos, o íon molecular, a sequencia de fragmentações e evidencias de outros tipos de espectrometria (por exemplo, IV e RMN) podem levar àidentificação de novos compostos (SILVERSTEIN et al., 2005).

É importante salientar que a introdução de abordagens assistidas por computador tem sido cada vez mais utilizada e é considerada por alguns como o futuro da fitoquímica pois economiza tempo e dinheiro associados à pesquisa, variando desde a descoberta de compostos bioativos até a identificação dos metabólitos (SARKER e NAHAR, 2018).

A Plataforma *online* GNPS (Global Natural Products Social Molecular Networking) é uma base de conhecimento de acesso aberto para organização e compartilhamento de dados de massa (WANG et al., 2016) e tem sido cada dia mais utilizada pelos pesquisadores da área de produtos naturais pois permite que substâncias

conhecidas e comuns aos gêneros de plantas tenham seus espectros compartilhados para comparação e identificação mais rápida de resultados e pode ser alimentada frequentemente, tornando-se cada vez maior.

2.3. Considerações sobre a família Annonaceae

Levando-se em consideração os tipos de metabólitos secundários identificados em espécies de diferentes famílias, as espécies da família Annonaceae se tornam uma boa fonte de busca por moléculas bioativas. Esta família possui aproximadamente 2.500 espécies distribuídas em aproximadamente 135 gêneros (CHATROU et al., 2012) e, embora os estudos sobre fitoquímica e atividade biológica tenham se iniciado há muito tempo, recentemente tais estudos têm sido intensificados devido à presença das acetogeninas, que possuem ampla atividade biológica tais como citotóxica, imunossupressora, pesticida, antiparasitária e antimicrobiana (LIMA et al., 2010).

Dados quimiotaxonômicos caracterizam a família Annonaceae pela presença de outros compostos boativos importantes tais como alcaloides, flavonoides e terpenoides, principalmente diterpenos (SILVA et al., 2009).

No que se diz respeito às atividades biológicas dos metabólitos secundários, os compostos fenólicos tem sido responsabilizados por algumas atividades, tais como antioxidante, antibacteriana, antiviral, expectorante, colerética e analgésica (SIMÕES et al., 2010).

2.4. Considerações sobre o gênero *Annona* e o híbrido atemoia:

O gênero *Annona* L. pertence à família Annonaceae e compreende cerca de 162 espécies de árvores e arbustos (CHATROU et al., 2012). No Brasil, existem cerca de 60 espécies, com maior ocorrência em florestas e com poucos representantes em áreas abertas (COSTA et al., 2011).

Derivado da palavra em Latim “colheita anual”, o gênero *Annona* é a fonte mais importante de frutos comestíveis da família Annonaceae tendo, consequentemente, grande destaque econômico. Normalmente suas frutas são consumidas "in natura" ou utilizadas em sucos, sobremesas e preparações de sorvetes (LORENZI; MATOS, 2002).

Muitas espécies do gênero *Annona* são reconhecidas por suas propriedades medicinais, como é o caso dos frutos de *Annona spinescens*, popularmente conhecida como araticum-do-rio ou araticum-do-alagadiço, que é usado no tratamento de úlceras. Outro exemplo é a *Annona muricata*, cujo decoto das folhas é muito utilizado contra diarreia e espasmos, e seu chá é empregado como agente emagrecedor e como medicação contra alguns tipos de câncer (BARATA et al., 2009).

Investigações prévias sobre química e atividade biológica de algumas espécies deste gênero têm indicado a presença de compostos bioativos importantes, exibindo diversas atividades. Entre elas, a citotoxicidade contra várias linhagens de células tumorais, atividades antimicrobiana, antioxidante e anti-plaquetária e propriedades antiparasitárias, em particular contra a *Leishmania* sp. e *Trypanosoma cruzi*. Essas atividades são geralmente atribuídas à presença de metabólitos secundários como alcaloides, acetogeninas e terpenos (COSTA et al., 2011).

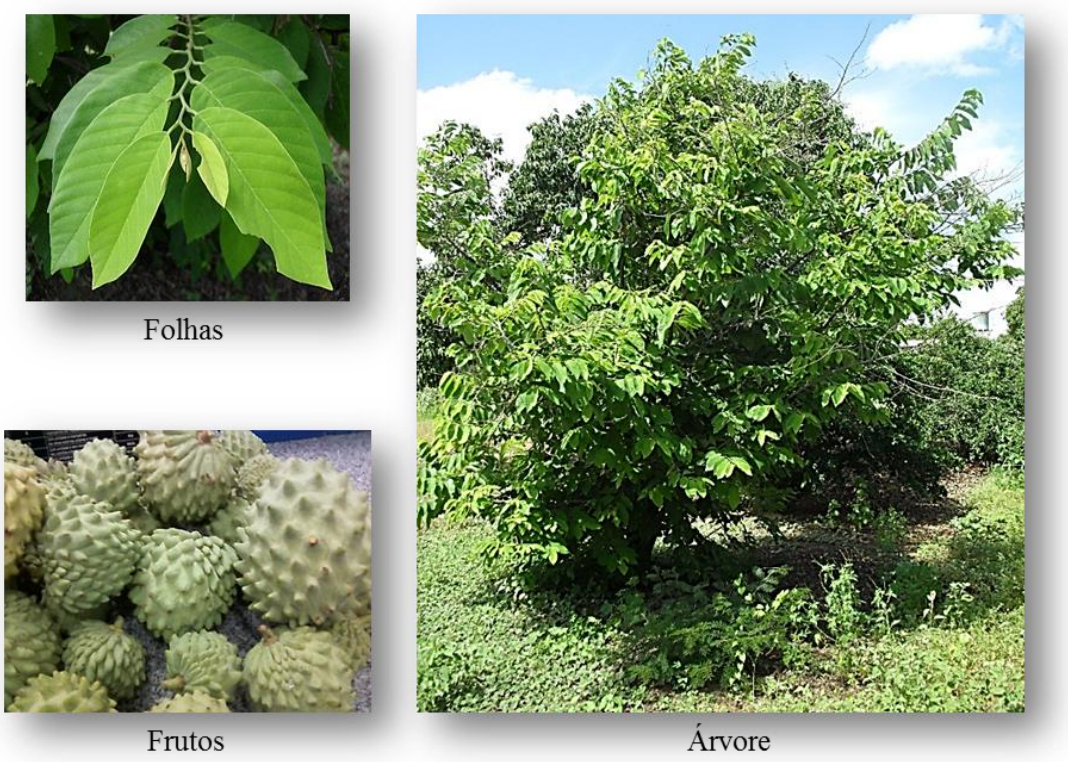
A atemoia é um híbrido interespecífico entre a cherimoia (*A. cherimola* Mill.) e a pinha ou fruta-do-conde (*A. squamosa* L.) (Figura 3) e seu interesse comercial tem aumentado no decorrer dos anos. Ao considerar os resultados de estudos realizados com suas espécies geradoras, este híbrido se torna uma boa fonte de moléculas bioativas (OLIVEIRA et al., 2010).

Figura 3. Espécies paternais da atemoia.

(Fonte: Google imagens – imagem adaptada)

Deste híbrido (Figura 4) já foram identificados os alcaloides atemoina e cleistofolina das sementes de uma especime coletada em Taiwan (WU et al., 2005), assim como algumas acetogeninas (DURET et al., 1997). Mais recentemente ela foi considerada uma planta promissora no controle de lagartas de *Anticarsia gemmatalis*, apresentando assim, potencial fitoinseticida (GORRI et al., 2018).

Figura 4. Folhas, frutos e árvore de atemoia.



(Fonte: Próprio autor)

2.5. Considerações sobre atividade citotóxica

Câncer é o nome dado a um conjunto de doenças cuja principal característica é o crescimento descontrolado das células. Este termo deriva da palavra grega "*carcinos"* que quer dizer caranguejo. Comumente, esta palavra é usada para designar tumores malignos (BOMFIM, 2013).

A busca por substâncias com atividade citotóxica e potencialmente anticancerígena sempre foi uma das prioridades da química medicinal e um grande número de abordagens diferentes vem sendo utilizado nessa busca. No entanto, a

descoberta de substâncias antitumorais seletivas permanece como um objetivo na pesquisa contra câncer (PISCO et al., 2006).

Apesar dos recentes estudos na área de química medicinal, atualmente os produtos naturais desempenham papel fundamental como fonte de novas drogas ou protótipos para o desenvolvimento de fármacos. Recentemente, muitos estudos estão sendo realizados com o objetivo de isolar acetogeninas que, como citado anteriormente, são uma classe de compostos encontrados exclusivamente em espécies da família Annonaceae e que possuem ampla atividade biológica.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Testar o potencial citotóxico, Isolar e identificar diferentes metabólitos secundários de folhas e partes aéreas (mistura de folhas e talos) de atemoia (*Annona cherimola* x *Annona squamosa*) utilizando técnicas hifenadas de CLAE-DAD-EM, CL-EM e CG-EM e, assim, contribuir para o estudo químico e biológico da família Annonaceae.

3.2. OBJETIVOS ESPECÍFICOS

- Obter extratos e frações alcaloídicas das folhas e partes aéreas (mistura de folhas e talos) do híbrido atemoia para realização do estudo fitoquímico e análise do potencial citotóxico *in vitro*;
- Submeter extratos e frações alcaloídicas das folhas e partes aéreas (mistura de folhas e talos) do híbrido atemoia a diferentes técnicas de separação e identificação (clássicas e avançadas) de seus constituintes;
- Analisar o perfil químico dos extratos citotoxicamente ativos por meio de métodos cromatográficos como CLAE-DAD, CLAE-DAD-EM e CG-EM;
- Identificar e/ou elucidar a estrutura dos constituintes químicos de atemoia por meio de técnicas de UV, EM e RMN (uni e bidimensionais);
- Isolar moléculas provenientes do metabolismo secundário de atemoia para posterior avaliação da sua atividade citotóxica.

CAPÍTULO 1

ALCALOIDES ISOLADOS DE FOLHAS DE ATEMOIA

(*Annona cherimola x Annona squamosa*)

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Alkaloids isolated from the leaves of atemoya (*Annona cherimola* Mill. x *Annona squamosa* L.)

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Abstract: Atemoya is an interspecific annonaceous hybrid between *Annona cherimola* Mill. and *Annona squamosa* L. Its phytochemical investigation led to seven alkaloids, including two aporphine (anonaine and asimilobine), three oxoaporphine (lanuginosine, liriodenine and lysicamine) and two proaporphine (pronuciferine and stepharine). These alkaloids were identified by a series of spectrometric methods, mainly MS and NMR (1D

and 2D), as well as by comparison with literature data. Our findings showed that this species is an important source of aporphine alkaloids and have high relationship with other *Annona* species.

Keywords: Annonaceae, *Annona*, alkaloids, atemoya, Caatinga.

Introduction

Annonaceae is a large family comprising about 135 genera and more than 2500 species distributed mainly in tropical and subtropical regions (Chatrou et al., 2004). Chemically, this family is characterized by the presence of isoquinoline alkaloids, mainly aporphines.

Regarding the species of Annonaceae, those from the genus *Annona* L. comprises approximately 175 species of trees and shrubs. In Brazil the genus *Annona* contains around 60 species, with the largest part occurring in forests and few representatives in open areas (Costa et al., 2011). Moreover, economically, this genus is the most important of the family Annonaceae due to its edible fruits and medicinal properties (Dutra et al., 2012).

Atemoya is a fruitful plant of the Annonaceae family, being a hybrid resulting from a cross between the "cherimoia" (*Annona cherimola* Mill.) and the "pinha" or "fruta do conde" (*Annona squamosa* L.). It was achieved at the beginning of the century in the Florida (USA), the crosses being repeated in other countries in order to obtain hybrids adapted to tropical climate, as *A. squamosa*, and subtropical as *A. cherimola* (Silva and Muniz, 2011).

The introduction of atemoya in the Northeast region of Brazil is recent, with a predominance of the cultivar Gefner from Israel, originally grown in the irrigation projects of the Vale do São Francisco. A recent study carried out by our research group quantified the levels of total phenols and flavonoids as well as evaluated the antioxidant and antimicrobial activities of extracts obtained from stems and leaves of atemoya (Rabêlo et al., 2014a).

Due the expansion of atemoya cultivation and its increasing consumption in the main Brazilian markets, it is necessary to investigate the chemical composition of this plant. In the present paper, we report results of the first phytochemical study of the atemoya collected in the Vale do São Francisco, and the isolation and chemical characterization of seven alkaloids by spectrometric methods.

Materials and Methods

NMR experiments were acquired in $CDCl_3$, at 303 K on a Bruker AVANCE III 600 NMR spectrometer operating at 14.1 Tesla, observing ^{1}H and ^{13}C at 600 and 150 MHz, respectively. The spectrometer was equipped with a 5-mm multinuclear inverse detection probe with *z*-gradient. One-bond and long range ^{1}H-^{13}C correlation from HSQC and HMBC NMR experiments were optimized for average coupling constants $^{1}J_{(H,C)}$ and $^{LR}J_{(H,C)}$ of 140 and 8 Hz, respectively. All ^{1}H and ^{13}C NMR chemical shifts are given in ppm related to the TMS signal at 0.00 ppm as internal reference, and the coupling constants (*J*) in Hz. High-resolution ESI-MS data were taken in the positive ion mode, on a Bruker micrOTOF II - ESI-TOF Mass Spectrometer. Silica gel 60 (F_{254}) was used for analytical thin layer chromatography (TLC), while silica gel 60 (230 to 240 mesh) was used for column chromatography (CC). Spots on chromatograms were detected under

exposure to UV light (254 and 365 nm). When necessary, Dragendorff's reagent was used to visualize the spots on the TLC plates.

Leaves of atemoya (*Annona cherimola* Mill. x *Annona squamosa* L.) were collected in Petrolina (Coordinates: 9°20'30" S and 40°40'42" W), state of Pernambuco, Brazil, in July of 2013. The species was identified by Prof. José Alves de Siqueira Filho, and a voucher specimen (16310) was deposited in the Herbário Vale do São Francisco (HVASF) of the Universidade Federal do Vale do São Francisco (UNIVASF).

Dried and powdered leaves of atemoya (1163.0 g) were extracted with hexane (3.0 L, three times), followed by MeOH (3.0 L, three times), yielding of hexane (63.0 g) and MeOH (120.0 g) extracts, after each solvent removal under reduced pressure.

TLC analysis also indicated a high concentration of alkaloids in the MeOH extract, which was initially subjected to an acid-base extraction (Costa et al., 2006) to give alkaloid (1.50 g) and neutral (7.5 g) fractions. The alkaloidal fraction was subjected to silica gel CC previously treated with a 10% $NaHCO_3$ solution, eluted with increasing concentrations of hexane, CH_2Cl_2, EtOAc and MeOH, giving 297 fractions (40 mL each). These fractions were evaluated and pooled according to TLC analysis yielding eighteen groups. Group 5 (81.8 mg) was subjected to a preparative TLC eluted with CH_2Cl_2:MeOH (95:05, *v/v*, three times), giving asimilobine (**1**) (2.3 mg), a mixture of alkaloids asimilobine and pronuciferine (**2**) (6.5 mg), and a mixture of three oxoaporphine alkaloids lanuginosine (**3**), liriodenine (**4**) and lysicamine (**5**) (5.9 mg). Group 7 (66.9 mg) was subjected to a preparative TLC eluted with CH_2Cl_2:MeOH (95:05, *v/v*, three times), affording a mixture of alkaloids asimilobine and anonaine (**6**) (3.3 mg) and the proaporphine alkaloid stepharine (**7**) (2.5 mg) (Fig. 1).

All isolated compounds (Fig. 1) were identified by a series of spectrometric methods, mainly MS and NMR (1D and 2D) data, as well as comparison with those

reported in the literature. Alkaloids in mixtures were established through totally independents datasets from the 1D and 2D NMR experiments including signal areas. Each dataset was consistent with only one structure. Moreover, it was observed the same results in several samples investigated.

Asimilobine (**1**): yellow amorphous solid. Molecular formula: $C_{17}H_{17}NO_2$. Positive ESI-MS *m/z*: 268.1354 $[M + H]^+$. ^1H NMR (600 MHz, $CDCl_3$): δ 8.27 (1H, *d*, *J*= 7.8 Hz, H-11), 7.22–7.33 (3H, *m*, H-8, 9, and 10), 6.72 (1H, *s*, H-3), 3.59 (3H, *s*, C1–OCH_3). The MS and ^1H-NMR data are in agreement with the literature (Guo et al., 2011; Costa et al., 2015).

Pronuciferine (**2**): brown amorphous solid. Molecular formula: $C_{19}H_{21}NO_3$. Positive ESI-MS *m/z*: 312.1284 $[M + H]^+$. ^1H NMR (600 MHz, $CDCl_3$): δ 6.41 (1H, *dd*, *J*= 8.1 and 2.8 Hz, H-12), 6.89 (1H, *dd*, *J*= 7.1 and 2.8 Hz, H-8), 7.00 (1H, *dd*, *J*= 8.1 and 2.8 Hz, H-9), 6.29 (1H, *dd*, *J*= 8.10 and 2.00 Hz, H-11), 6.64 (1H, *s*, H-3), 3.60 (3H, *s*, C1–OCH_3), 3.80 (3H, *s*, C2–OCH_3), 3.05 (3H, *s*, N–CH_3). ^{13}C NMR (150 MHz, $CDCl_3$): δ 184.42 (C-10), 153.34 (C-8), 128.49 (C-9), 127.10 (C-11), 149.80 (C-12), 61.15 (1-OMe), 56.48 (2-OMe), 44.83 (N-Me). The MS, ^1H-NMR and ^{13}C-NMR data are in agreement with the literature (Thuy et al., 2005).

Lanuginosine (**3**): dark brown solid. Molecular formula: $C_{18}H_{11}NO_4$. Positive ESI-MS *m/z*: 306.0762 $[M + H]^+$. The MS data are in agreement with the literature (Wijeratne et al., 1996).

Liriodenine (**4**): yellow amorphous solid. Molecular formula: $C_{17}H_9NO_3$. Positive ESI-MS *m/z*: 276.0659 $[M + H]^+$. The MS data are in agreement with the literature (Guo et al., 2011; Costa et al., 2011).

Lysicamine (**5**): yellow amorphous solid. Molecular formula: $C_{18}H_{13}NO_3$. Positive ESI-MS *m/z*: 292.0971 $[M + H]^+$. The MS data are in agreement with the literature (Harrigan et al., 1994).

Anonaine (**6**): yellow powder. Molecular formula: $C_{17}H_{15}NO_2$. Positive ESI-MS *m/z*: 266 $[M + H]^+$. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.15 (1H, *d*, *J*= 7.74 Hz, H-11), 7.22–7.32 (3H, *m*, H-8, 9, 10), 6.58 (1H, *s*, H-3), 6.09 and 5.94 (each 1H, *s*, –OCH_2O–). The MS and ^{1}H-NMR data are in agreement with the literature (Guo et al., 2011; Costa et al., 2015).

Stepharine (**7**): amorphous brown solid. Molecular formula: $C_{18}H_{19}NO_3$. Positive ESI-MS *m/z*: 298.1438 $[M + H]^+$. ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.39 (1H, *dd*, *J*= 8.4 and 2.0 Hz, H-12), 6.87 (1H, *dd*, *J*= 7.4 and 2.0 Hz, H-8), 6.98 (1H, *dd*, *J*= 7.4 and 2.00 Hz, H-9), 6.28 (1H, *dd*, *J*= 8.4 and 2.0 Hz, H-11), 6.64 (1H, *s*, H-3), 3.60 (3H, *s*, C1–OCH_3), 3.80 (3H, *s*, C2–OCH_3). The MS and ^{1}H-NMR data are in agreement with the literature (Thuy et al., 2005; Costa et al., 2015).

Results and Discussion

The phytochemical investigation from the leaves of the hybrid atemoya, an edible fruit of the Annonaceae family, resulted in seven alkaloids, including two aporphine (anonaine and asimilobine), three oxoaporphine (lanuginosine, liriodenine and lysicamine) and two proaporphine (pronuciferine and stepharine). All of them are being described for the first time in the hybrid atemoya.

This family is considered the centre of distribution of isoquinoline alkaloids. Recent chapter published by our research group presented an overview of the chemistry and pharmacology of the alkaloids found in species of the Annonaceae family. Within the

Annonaceae family, the genera *Annona*, *Duguetia*, and *Guatteria* have led to many important publications. The alkaloids of the aporphine type represent the predominant group in this family. Many of the isolated alkaloids exhibit unique structures. The chapter could be considered as a contribution for the scientific community, mainly to enable the search for alkaloids in species belonging to the Annonaceae family (Lúcio et al., 2015). Another work reviewed articles published in the literature regarding alkaloids isolated from plants of the genus *Annona*. This review covers a period from 1930 to 2013 and shows the identification of 147 alkaloids in *Annona* species (Rabêlo et al., 2014b).

The aporphine asimilobine and anonaine, and the oxoaporphine liriodenine have been described in several species of *Annona*. Recent studies describe the isolation of asimilobine and liriodenine in some species of *Annona*, such as *Annona foetida* Mart., *Annona pickelii* (Diels) H. Rainer, *Annona salzmannii* A. DC., and *Annona sericea* Dunal, all native to Brazil (Campos et al., 2008; Costa et al., 2011; Cruz et al., 2011; Dutra et al., 2012). These alkaloids were found in leaves and barks of *A. crassiflora* from the Guianas (Hocquemiller et al., 1982). Anonaine, asimilobine and liriodenine have been described in several species of *Annona*, and could be considered as chemotaxonomic markers of this genus (Cruz et al., 2011).

Other studies described the isolation of the alkaloid pronuciferine in *Annona cherimola* (Chen et al., 1999); lanuginosine in *A. cherimola*, *A. rugulosa* and *A. squamosa;* lysicamine in *A. acuminata*, *A. cherimola*, *A. glabra*, *A. hayesii*, *A. pickelii*, *A. purpurea* and *A. sericea*; and stepharine in *Annona cacans*, *A. glabra*, *A. hayesii* and *A. spinescens* (Rabêlo et al., 2014b). All these compounds are being described for the first time in the hybrid atemoya collected in the Vale do São Francisco.

Conflict of interest

All authors declare that there are no conflicts of interests and they affirm that this paper consists of original and unpublished work.

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Authors' contributions

SVR, XPN and MFCS were responsible for the collection, preparation of the extracts and phytochemical studies. EVC, AB and LMD conducted the experiments of nuclear magnetic resonance. JCT, GGO and NPL conducted the experiments of mass spectrometry. EVC and JRGSA analyzed and interpreted the data, and drafted the manuscript. All the authors have read the final manuscript and approved the submission.

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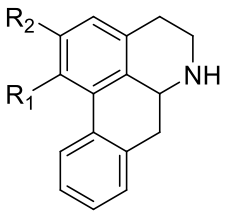
FIGURE CAPTIONS:

Figure 1. Alkaloids isolated from the leaves of atemoya.



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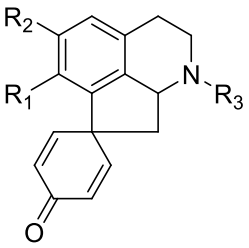
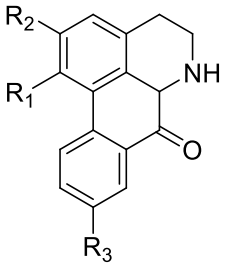
6 R_1-R_2=O-CH_2-O



2 R_1=R_2=OCH_3; R_3=CH_3
7 R_1=R_2=OCH_3; R_3=H



3 R_1-R_2=O-CH_2-O; R_3=OCH_3
4 R_1-R_2=O-CH_2-O; R_3=H
5 R_1=R_2=OCH_3; R_3=H

CAPÍTULO 2

UM NOVO ALCALOIDE BENZILISOQUINOLÍNICO *N*-OXIDO DE ATEMOIA (*Annona cherimola* x *Annona squamosa*)

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A new *N*-oxide benzylisoquinoline alkaloid isolated from the leaves of atemoya (*Annona cherimola* x *Annona squamosa*)

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Abbreviations

NMR: Nuclear Magnetic Resonance; DEPT 135º: Distortionless Enhancement by Polarization Transfer; NOE: Nuclear Overhalser Efect; COSY: Correlated Spectroscopy; HSQC: Heteronuclear Single Quantum Coherence; HMBC: Heteronuclear Multiple Bond Coherence; *J*: Coupling constant; δ: Chemical shift; ESI-MS: Electrospray Ionization Mass Spectrometry; TOF: Time of Flight; TLC: Thin-Layer Chromatography; CC: Column Chromatography; UV: Ultraviolet; MeOH: Methanol; EtOAc: Etil Acetate; HRESIMS: High-Resolution Electrospray Ionization Mass Spectrometry; MTT: Thiazolyl Blue Tetrazolium Bromide; DMSO: Dimethylsulfoxide; PC3: prostate carcinoma; PC-3M: metastasis-derived variant of PC3; PC9: lung adenocarcinoma; COLO 205: colorectal adenocarcinoma; SW620: colorectal adenocarcinoma; B16F10: skin carcinoma; SF-295: gliobastoma; HL60: promyelocytic leukemia; HCT-116: human colon carcinoma; IC_{50}: Half-maximal inhibitory concentration; SI: Selectivity Index.

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Abstract Phytochemical investigation of the atemoya aerial parts by LC-MS-IT led to the identification of a new *N*-oxide alkaloid (dehydro-*N*-oxide-anomuricine) and other eleven alkaloids: scoulerine, reticuline, isocorydine, norisocorydine, asimilobine, nornuciferine, anonaine, and liriodenine. The new alkaloid dehydro-*N*-oxide-anomuricine and anomuricine were also isolated. The structures of compounds were determined based on spectroscopic and spectrometric data interpretation, and the fragmentation was proposed. The investigation of the cytotoxic activity of crude methanolic extract and the alkaloidal fraction were analyzed and presented high cytotoxicity, showing that atemoya can be considered a promising source of substances useful for the cancer treatment.

KEYWORDS

Anomuricine; Cytotoxicity; Mass spectrometry; LC-MS; NMR; *N*-oxide.

1. Introduction

Atemoya is a hybrid resulting from mixing of two species of the genus *Annona*: sugar apple (*Annona cherimola* Mill) and cherimoya (*Annona squamosa* L.) [1]. The genus *Annona* is represented by 175 species and is the main genus of the Annonaceae family [2], which is well known to produce alkaloids as main secondary metabolites and considered as chemotaxonomic markers of the genus. For example, the alkaloids anonaine, asimilobine, liriodenine and reticuline are very common [3].

Previous phytochemical investigations carried out with atemoya, the alkaloids atemoine and cleistofoline were found in the seeds of a specimen collected in ChiaYi City, Taiwan [4]. Besides, the aporphines anonaine and asimilobine, the oxoaporphine lanuginosine, liriodenine and lysicamine, and the proaporphine pronuciferine and stepharine alkaloids were found in the leaves of an atemoya specimen collected in Petrolina, Brazil [5].

Currently hyphenated techniques have been widely used in phytochemical studies since it allows a quick and sensitive identification of the chemical constituents. These analyzes, coupled with *in vitro* biological activity investigations, allow us to obtain fast and targeted results in the search for biologically active compounds.

Therefore, this work reports the results of a phytochemical investigation and cytotoxic potential of aerial parts of atemoya, with a detailed description of the identification of a new *N*-oxide benzylisoquinoline alkaloid (6,7-dimethoxy-1-[(4-methoxyphenyl)methyl]-2-oxo-3,4-dihydro-2λ^5-isoquinolin-5-ol, named as dehydro-*N*-oxide-anomuricine) and 1D and 2D NMR data attributed unambiguously for the benzyltetrahydroisoquinoline alkaloid, anomuricine.

2. Experimental

2.1. Plant material

Aerial parts of atemoya (*Annona cherimola* Mill. × *Annona squamosa* L.) were collected in July 2013 and September 2014, in Petrolina (Coordinates: 9°20′30″ S and 40°40′42″ W), state of Pernambuco, Brazil. The plant was identified by Prof. José Alves de Siqueira Filho (a plant taxonomist of the Federal University of San Francisco Valley, Petrolina, PE, Brazil), and a voucher specimen (#16310) was deposited in the Herbário Vale do São Francisco (HVASF) of the Federal University of San Francisco Valley (UNIVASF). All procedures for access to genetic patrimony and associated traditional knowledge were carried out and the project was registered in SisGen (Register #ABD9AA7).

2.2. Extraction and isolation

The dried and powdered aerial parts of atemoya (2,200 g) were extracted with hexane (3 L, three times), followed by MeOH (3 L, three times), yielding 84.1 and 8.5 g of hexane and MeOH extracts, respectively, after each solvent removal under reduced pressure. The MeOH extract was submitted to an acid-base extraction to give an alkaloid and a neutral fraction. The alkaloidal fraction (FAT) was submitted to solid-liquid partition with Hex, $CHCl_3$, AcOEt and MeOH, using silica gel previously treated with an aqueous solution of $NaHCO_3$ (10¢ *v*/*v*) as adsorbent, giving the phases FAT-Hex, FAT-$CHCl_3$, FAT-AcOEt and FAT-MeOH. These samples were submitted to LC-MS-IT analysis and led to identification of compounds **1** to **11** (Figure 1 and 2).

The dried and powdered leaves of atemoya (1163 g) were extracted with hexane and MeOH at the same conditions described previously, yielding 63 and 120 g of hexane and MeOH extracts, respectively. The MeOH extract was submitted to an acid-base

extraction give to 1.5 g of alkaloidal fraction, which was submitted to silica gel CC previously treated with a 10% $NaHCO_3$ solution, eluted with increasing concentrations of hexane, CH_2Cl_2, EtOAc and MeOH, giving 297 fractions (40 mL each). These fractions were evaluated and pooled according to TLC analysis, yielding eighteen groups. Group 7 (66.9 mg) was submitted to a preparative TLC eluted with CH_2Cl_2:MeOH (95:05, *v*/*v*, three times), giving compounds **7** (1,3 mg) and **9** (9,3 mg). These were identified by analyzing the 1D and 2D NMR spectra and by mass spectrometry data.

Anomuricine (**7**): brown amorphous solid; molecular formula $C_{19}H_{23}NO_4$; HRESIMS *m/z* 330.1764 $[M+H]^+$; ^1H and ^{13}C NMR data, see Table 1.

Dehydro-*N*-oxide-anomuricine (**9**): yellowish powder; molecular formula $C_{19}H_{21}NO_5^+$. HRESIMS *m/z* 344.1498 $[M+H]^+$; ^1H and ^{13}C NMR data, see Table 1.

INSERT TABLE 1

2.3. LC-MS procedures

The FAT, FAT-Hex, FAT-$CHCl_3$, FAT-AcOEt and FAT-MeOH fractions were solubilized in methanol (HPLC grade) at 1 mg/mL concentration and individually analyzed by LC-MS using an octadecylsilane column (250 x 4,6 mm, 5 µm, Luna® C18, Phenomenex®) as stationary phase and mobile phase consisting of 2 solvents: solvent A – 0.1% formic acid in ultrapure water and solvent B 0.1% formic acid in methanol (HPLC grade) with flow rate of 1.0 mL/min, in gradient according to Table 2.

INSERT TABLE 2

The stationary phase was maintained at 30 °C and the injected volume was 20 µL for the samples in the HPLC-DAD-MS for monitored analyzes of 190 to 400 nm and 50 to 1000 *m/z*.

The analyzes developed used a Shimadzu® LC-20 equipped with a quaternary pump system LC-20ADVP model, DGU-20A degasser, PDA detector model SPD-20AVP, CTO-20ASVP model oven, SIL-20ADVP model automatic injector and SCL-20AVP model controller coupled to an ESI-IT mass spectrometer from Bruker Daltonics, equipped with an electrospray ionization source operating in the analyzer mode, and by trapping positive ions to divide the HPLC eluent, a flow rate of 0.2 mL/min was introduced in the source. The parameters of the mass spectrometer used were: capillary voltage, 3.5 kV; desolvation temperature 330 °C; gas flow of 10 L/min; pressure of 60 PSI using nitrogen as drying gas and misting [6].

The **7** and **9** molecules fragmentation were taken in the positive ion mode, on a Bruker Daltonics TOF-Q-II Mass Spectrometer and the parameters were: capillary voltage 3.5 kV, nitrogen as the mist gas at a flow of 4 L/min, pressure 0.4 Bar, temperature 180 °C. The equipment was calibrated with NaTFA at 200 µg/mL. The sample was solubilized in methanol at concentration of 100 µg/mL, acidified with formic acid when necessary and directly injected on mass spectrometer.

2.4 NMR Procedures

The NMR data were acquired on a Bruker AVANCE III 600 NMR spectrometer operating at 14.1 Tesla, observing ^{1}H and ^{13}C at 600 and 150 MHz, respectively. The spectrometer was equipped with a 5-mm multinuclear inverse detection probe with *z*-gradient. One-bond and long range ^{1}H–^{13}C correlation from HSQC and HMBC NMR experiments were optimized for average coupling constants $^{1}J_{(H,C)}$ and $^{LR}J_{(H,C)}$ of 140 and 8 Hz, respectively.

All ^{1}H and ^{13}C NMR chemical shifts were given in ppm related to the TMS signal at 0.00 ppm as internal reference, and the coupling constants (*J*) in Hz. Silica gel 60 (F_{254}) was used for analytical thin-layer chromatography (TLC), while silica gel 60 (230-240 mesh) was used for column chromatography (CC). Spots on chromatograms were detected under exposure to UV light (254 and 365 nm). When necessary, Dragendorff's reagent was used to visualize the spots on the TLC plates.

2.5. Cytotoxic activity of extracts and fractions

The cytotoxic study was conducted by the MTT method [7], which has the capacity to analyze the viability and the metabolic state of the cell. For this, the cells were plated at 0.7 x 10^5, 0.1 x 10^6 and 0.3 x 10^6 cells/mL concentrations for the HCT-116, SF295 e HL60 cell lines, respectively. The plates were incubated with the samples by 72 hours in greenhouse at 5% of CO_2 and 37 ºC. At the end, the cells were centrifuged and the supernatant removed. Then, 150 μL of the MTT (tetrazolium salt) solution were added and the plates were incubated for 3 hours. After incubation, the plates were centrifuged again for the MTT solvent removal. The absorbance was read in spectrophotometer at 595 nm after dissolution of the formazan precipitate with 150 μL of pure DMSO. The single concentration experiments were analyzed according to the mean ± standard deviation (SD) of the percentage of cell growth inhibition using the GraphPad Prism 5 program.

2.6. Cytotoxic activity of Anomuricine

Anomuricine was diluted in sterile pure dimethylsulfoxide (DMSO) at 50 μg/mL. The tumor cell lines were provided by the National Cancer Institute (USA), and were maintained in RPMI 1640, supplemented with 10% fetal bovine serum and 1%

antibiotics, which were incubated at 37 °C under 5% CO_2. The cell lines used were seeded at concentrations of 0.1 x 10^6 cels/mL for the cell lines PC3 (prostate carcinoma), PC-3M (metastasis-derived variant of PC3), PC9 (lung adenocarcinoma), COLO 205 (colorectal adenocarcinoma), SW620 (colorectal adenocarcinoma), B16F10 (skin carcinoma) and SF-295 (glioblastoma), HL60 (promyelocytic leukemia) 0.3 x 10^6 cels/mL and 0.7 x 10^5 cels/mL for the HCT-116 (human colon carcinoma) cell line. The plates were incubated for 72 hours in an oven at 5% CO_2 at 37 °C. At the end of treatment the MTT (tetrazolium salt) was added, and incubated for 3 h. The absorbance was measured at 595 nm. The negative control received the same amount of DMSO and doxorubicin was used as a positive control.

3. Results and discussion

3.1. Chemical analysis

The analysis of the chromatogram of the alkaloidal fraction from atemoya (FAT) allowed observing the presence of 11 compounds (Figure 1 and 2) and the confirmation was made based on the identification of the precursor ion, the fragmentation profile of the chemical constituents and comparison with literature data. Table 3 shows which alkaloids were identified in FAT and in its fractions.

INSERT FIGURE 1

INSERT FIGURE 2

INSERT TABLE 3

The benzylisoquinolines scoulerine (**1**), reticuline (**2**), anomuricine (***7***) and the new *N*-oxide benzylisoquinoline, here call of dehydro-*N*-oxide anomuricine (**9**); the aporphines isocorydine (**3**), norisocorydine (**4**), asimilobine (**5**), nornuciferine (**6**) and anonaine (**8**); and the the oxoaporphines liriodenine (**10**) and lanuginosine (**11**) alkaloids were identified.

Asimilobine, lanuginosine, liriodenine and anonaine had already been identified in the leaves of atemoya in a study conducted by our research group previously [5]. The other compounds were identified at the first time in this hybrid. Anomuricine (**7**) is being chemically characterized in this work with uni and bidimensional NMR data and proposed fragmentation presented for the first time, unequivocally. The new *N*-oxide alkaloid, dehydro-*N*-oxide anomuricine (**9**), is an unprecedented compound and its chemical characterization is described below.

3.2. Dehydro-N-oxide-anomuricine

The new alkaloid (Figure 2) was obtained as a yellowish powder with the molecular formula, $C_{19}H_{21}NO_5$, as determined by HRESIMS (*m/z* 344.1498 $[M+H]^+$) and NMR data. The ^{1}H-NMR spectrum of the compound displayed three signals characteristic of an aromatic hydrogen nuclei, one consisting of the signal at δ 6.51 (*s*, 1H, H-8), shielded by oxygenation in the *ortho* and *para* positions, indicating a 1,2,3,4,5-pentasubstituted benzene ring [8] (see Table 1 and supplementary material). On the other hand, two doublets signals at δ 6.81 (*d*, 2H, H-3'/H-5') and δ 7.23 (*d*, 2H, H-2'/H-6') showed *spin-spin* interaction in *ortho* (*J* = 8.6 Hz), which indicates a *para*-disubstituted benzene ring, typically of an AA'BB' spin system [8]. The proposed fragmentation is shown in Figure 3. In addition, one spin system atδ 3.07 (*t*, *J*= 7.7 Hz, 2H, H-4) and δ 4.16 (*t*, *J*= 7.7 Hz,

2H, H-3) attributed to B ring of benzylisoquinoline alkaloid. Furthermore, two singlets corresponding to methoxyl groups at δ 3.76 (*s*, 6H, OCH_3-7 and OCH_3-4') and δ 3.90 (*s*, 3H, OCH_3-6).

INSERT FIGURE 3

The complete assignments of the ^{1}H and $^{13}C\{^{1}H\}$ NMR chemical shifts were established based on one-bond and long-range ^{1}H-^{13}C correlation map from HSQC and HMBC experiments. The singlet at δ 4.26 (2H, H-α) showed long-range ^{1}H-^{13}C correlation with the carbons at δ 126.0 (C-8a), 129.7 (C-2), 130.1 (C-2'and C-6') and 144.5 (C-1). In contrast, the signal at δ 6.51 (1H, H-8) displayed correlation with the carbons at δ 111.4 (C-4a), 136.6 (C-6), 144.5 (C-1) and 151.6 (C-7). The presence of a hydroxyl group in the molecule located in the A ring at C-5 was established based on long-range ^{1}H-^{13}C correlation of the hydrogen H-4 with the carbon δ 146.4, which showed no correlation with the methoxyl groups. However, the singlets at δ 3.76 and 3.90 showed long-range correlation with the carbons at δ 151.6 (C-7) and 136.6 (C-6), respectively, indicating the presence of the two methoxyl groups in the pentasubstituted benzene ring (Table 1).

The *para*-disubstituted benzyl group was established in C ring based on direct ^{1}H-^{13}C correlation of hydrogens at δ 7.23 (2H, H-2' and H-6') and 6.81 (2H, H-3' and H-5') with the carbons at δ 114.8 and 130.1, respectively, and long-range ^{1}H-^{13}C correlation with the carbon δ 158.9 supporting the presence of methoxyl group in C-4'.

The correlation map of HSQC NMR experiment allowed establishing the direct correlations ($^{1}J_{CH}$) (Table 1), while the long-range ^{1}H-^{13}C correlation HMBC NMR

experiment permitted to establish the location of substituents in the molecule, as well as the methylene carbons C-α, C-4 and the *N*-linked C-3 (Figure 4).

INSERT FIGURE 4

The *N*-oxide group was supported by mass spectrum that revealed a molecular ion at 344.1559 (Figure S1). The overall analysis of 1D and 2D NMR experiments enabled the complete and unambiguous assignments of ^{1}H and ^{13}C NMR chemical shifts (Table 1, Figure S1-S5 and Figure S12-S16). These data allowed identifying the compound as 6,7-dimethoxy-1-[(4-methoxyphenyl)methyl]-2-oxo-3,4-dihydro-2λ^{5}-isoquinolin-5-ol alkaloid, named as dehydro-*N*-oxide-anomuricine. 1D NOE NMR experiments supported the proposed structure (Figure S6 –S11).

3.3. Anomuricine

The compound **7** was obtained as a brown amorphous solid with the molecular formula, $C_{19}H_{23}NO_4$, as determined by HRESIMS (*m/z* 330.1665 $[M+H]^+$). The mass spectrum (Figure S17) showed ionic fragments (Figure 5) similar to those described in literature [9]. The NMR data for this compound was very similar to those observed to dehydro-*N*-oxide-anomuricine, except by the signal at δ 144.5 on the $^{13}C\{^{1}H\}$ NMR spectrum which was replaced by the signal at δ 56.9 indicating that the quaternary carbon was replaced by a methine group (Table 1). This information was supported by an emerging double doublet signal at δ 4.11 (Table 1).

The carbons at δ 23.0 (C-4), 39.7 (C-3), 41.2 (C-α), and 56.9 (C-1) in combination with the other signals revealed the presence of a benzyltetrahydroisoquinoline alkaloid. The AA'BB' system was also observed with the signals in δ 114.0 (C-3' and C-5') and

130.3 (C-2’ and C-6’), which suggested a *para*-disusbstituted benzyl group. In addition, three signals corresponding to methoxyl carbons were observed at δ 60.9 (H_3CO-6), 55.8 (H_3CO-7) and 55.2 (H_3CO-4’). The overall analysis of 1D and 2D NMR experiments enabled the complete and unambiguous attribution of ^1H and ^{13}C NMR chemical shifts (Figure 6 and Table 1). Furthermore, it was observed a singlet at δ 6.24 (1H, H-8), indicative of an 1,2,3,4,5-pentasubstituted benzene ring, as well as a spin system at δ 6.86 (*d*, 2H, *J* = 8.5 Hz, H-3’/H-5’) and 7.16 (*d*, 2H, *J* = 8.5 Hz, H-2’/H-6’), compatible with the AA'BB' system described above.

The ^1H NMR spectrum revealed the presence of two spin systems, one consisting of the signals at δ 2.94 (*dt*, 1H, H-3, *J* = 12.1 and 6.0 Hz) and 3.21 (*dt*, 1H, H-3, *J* = 12.1 and 6.0 Hz), as well as 2.67 (*t*, 2H, H-4, *J* = 6.0 Hz) and other comprising the signals at δ 2.92 (*dd*, 1H, H-α, *J* = 13.8 and 9.2 Hz), 3.13 (*dd*, 1H, H-α, *J* = 13.8 and 4.6 Hz) and 4.11 (*dd*, 1H, H-1, *J* = 9.2 and 4.6 Hz) (Figure 6). Similar to dehydro-*N*-oxide-anomuricine, singlets corresponding to methoxyl groups at δ 3.87 (*s*, 3H, OCH_3-6), 3.78 (*s*, 3H, OCH_3-7) and 3.79 (*s*, 3H, OCH_3-4') were observed. The respective location of the methoxyls at the C-6, C-7 and C-4’ carbons was defined based on the NMR data and HMBC correlation map (Figure S-18-S23). The HSQC correlation map (Figure S22) allowed establishing the correlations at one-bond ($^1J_{CH}$) (Table 1). The long-range ^1H-^{13}C correlation experiment allowed observing the correlation of the aromatic hydrogen at δ 6.24 with the carbons at δ 56.9 (C-1), 114.9 (C-4a), 133.7 (C-6) at three bonds ($^3J_{HC}$) and two bonds ($^2J_{HC}$) with the carbon at δ 150.0 (C-7), supporting the assignments the hydrogen in C-8. Likewise, the signal at δ 4.11 showed long-range ^1H-^{13}C correlation with the carbons at δ 41.2 (C-α), 101.5 (C-8), 114.9 (C-4a) and 133.8 (C-8a), as well as, the methylene hydrogen H-α at δ 2.92 and 3.13 with the carbons in δ 56.9 (C-1) and 130.3 (C-2’/C-6’) were observed. By analyzing the correlation map from COSY NMR experiment, it was

possible to confirm the assignments of the aliphatic hydrogen nuclei. Comparison with the literature data allowed us to identify compound **7** as the alkaloid 6,7-dimethoxy-1-(4'-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline-5-ol, known as anomuricine ($C_{19}H_{23}NO_4$), isolated for the first time from the *Annona muricata* stem in 1981 [10]. It is the first time that this molecule is isolated from atemoya leaves and the data of carbon and hydrogen 1D and 2D are pointed out unequivocally.

3.4. Cytotoxic activity

In this study, cytotoxicity analyzes were done by the MTT method which is commonly used in these assays. Large reference centers use this method, such as the Screening Program of the National Cancer Institute of the United States (NCI), which tests more than 10,000 substances each year [11]. It is a fast, sensible and inexpensive method.

It was first described by Mosman (1983) and allows analyze the viability and the metabolic state of the cell. It is a colorimetric analysis based on the conversion of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide salt (MTT) in formazam blue, from mitochondrial enzymes present only in metabolically active cells. The cytotoxic study by the MTT method allows to easily define cytotoxicity, but not the mechanism of action [12].

In this work, the crude extracts from aerial parts of atemoya were evaluated *in vitro* against three cancer cell lines: HL-60 (leukemia), HCT-116 (human colon) and SF295 (central nervous system). The most significant activity was observed toward SF295 and the EHB and EMB were highly active with percentual values of 91.77 and 86.21, respectively (Table 4).

INSERT TABLE 4

This is not the first time that *Annona* extracts are tested against cancer cells. Species of this genus are known for presenting cytotoxicity, as observed in the study with extract from roots of *Annona crassiflora* against SF95 and HL60 cells [13]. In a recent study, Agu and contributors [14] presented some of the possible *in vitro* anticancer mechanisms of *Annona muricata*. The focus of their work was on the group of important secondary metabolites of the Annonaceae family, the acetogenins. However, another class of molecules of this family are important in the search for new anticancer medicines, are the alkaloids. Some works realized with alkaloids found in Annonaceae species, has showed that this compounds presented high cytotoxic activity [15-18].

A brief search on the *Science Direct* platform, in July of 2018, revealed a total of 21,685 results in the search for publications of studies on alkaloid cytotoxicity. Of these, 411 are related to *Annona* alkaloids; 82 of them only between 2017 and 2018. This result shows the growing interest in this topic. For this reason, the alkaloidal fraction (FAT) from the methanolic crude extract (EMB) of atemoya was tested, and its hexane, chloroform, ethyl acetate and methanolic subfractions were also tested.

The results presented in Table 4 show that the cytotoxic potential of FAT was greater than 95% for all tested cells, being slightly increased in the hexane and chloroform phases, and reduced in the ethyl acetate and methanol phases.

Many of the alkaloids identified in the active fraction from atemoya have already been studied for cytotoxicity, such as anonaine and asimilobine [19], isocorydine [20] and reticuline [21]. Nornuciferine still showed moderate activity against KB cells [22] and was considered important chemical constituent of the cytotoxic active alkaloid fraction from *Annona hypoglauca* [23].

In this study, anomuricine (**7**) was tested against nine cancer cell and the results are showed in Table 5. Anomuricine showed moderate cytotoxic activity against all evaluated cell lines. The IC_{50} presented a large cytotoxic activity against the leukemic cell (HL60) 41.03 µM, while for lines of human colon the IC_{50} was 78.75 µM for SW620, 87.90 µM for COLO 205, and 110.73 µM for HCT-116.

The selectivity index (SI) indicate the selectivity of a compound between a neoplastic lineage and a normal lineage, suggesting the potential of its use for future clinical trials. The calculation of this index corresponds to the division between the IC_{50} value of each test compound in the L929 non-tumor cell line and the IC_{50} value of each compound in the neoplastic cell line (SI= IC_{50} L929 / IC_{50} neoplastic cells) (Table 5).

INSERT TABLE 5

According to Suffness and Pezzuto (1991) [23], SI values ≤ 2.0 indicates that the activity is twice as high in neoplastic cell line than in non-tumor cells. The selectivity index found for anomuricine ranged from 0.59 -1.66. These results suggested that atemoya can be considered a promising source of substances with cytotoxic potential.

4. Conclusion

This work described the isolation and characterization of a new alkaloid, dehydro-*N*-oxide-anomuricine, the alkaloids scoulerine, reticuline, anomuricine, isocorydine, norisocorydine and nornuciferine for the first time in atemoya. These findings contribute to the chemotaxonomy of the Annonaceae family, especially to the genus *Annona* and show how the hiphenated techniques can help in the rapid discovery of plant compounds.

Moreover, atemoya can be considered a promising source of substances useful for the cancer treatment.

Conflict of interest

The authors declare that there are no conflicts of interests and they affirm that this paper consists of original and unpublished work.

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Author's contributions

SVR and MFCS were responsible for botanical material collection and phytochemical investigations. EVC, AB, RBF performed the NMR experiments. SVR, LAR, JCT and NPL performed MS experiments. COP, MOMF and MFSS performed cytotoxic assays. SVR, ECCA, XPN, EVC and JRGSA analyzed and interpreted the data, and drafted the manuscript. All the authors have read the final manuscript and approved the submission.

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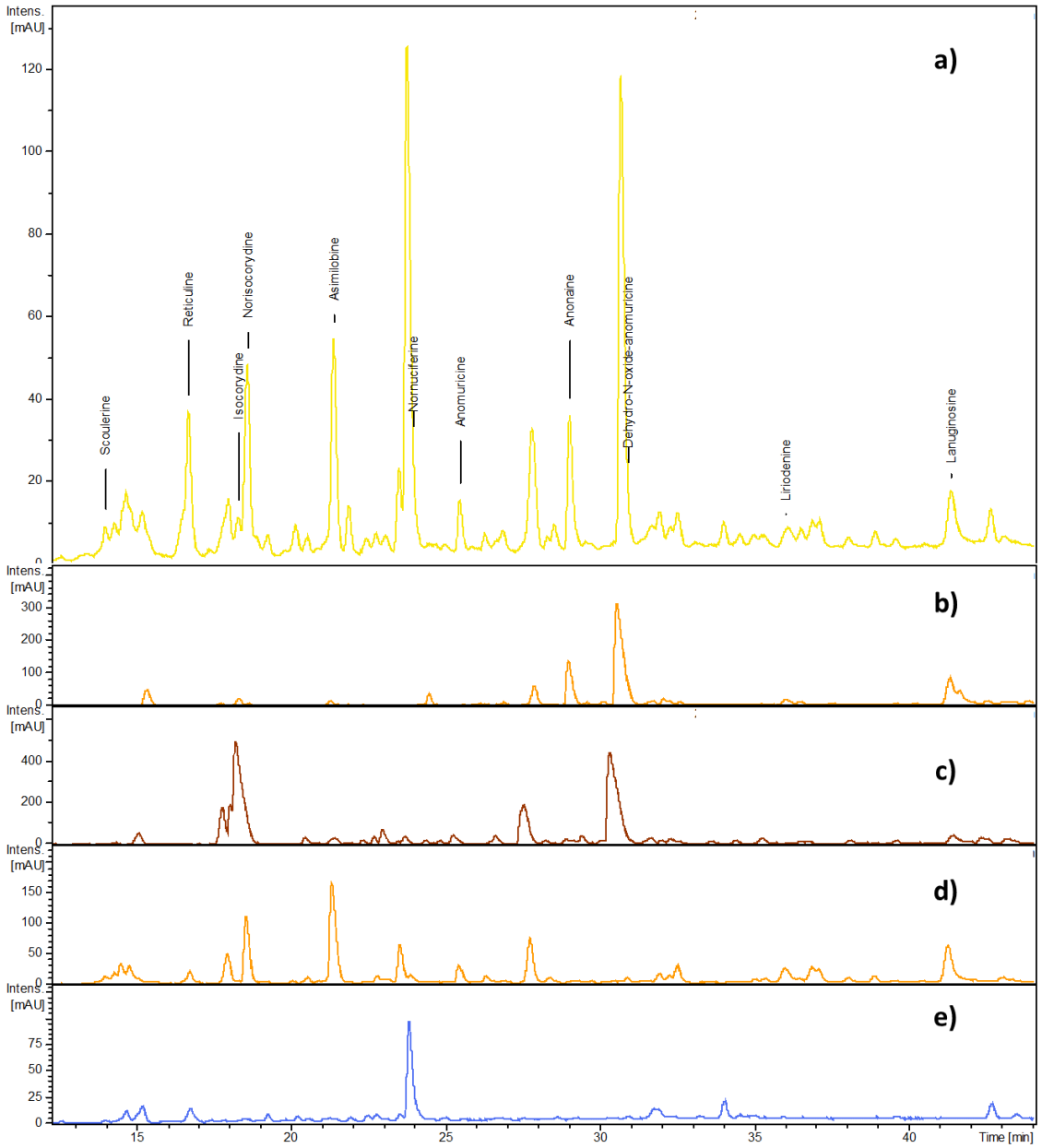

Figure 1. Chromatogram of total alkaloid fraction and fractions obtained by partition of atemoya: (a) FAT, (b) FAT-Hex, (c) FAT-$CHCl_3$, (d) FAT-AcOEt and (e) FAT-MeOH.

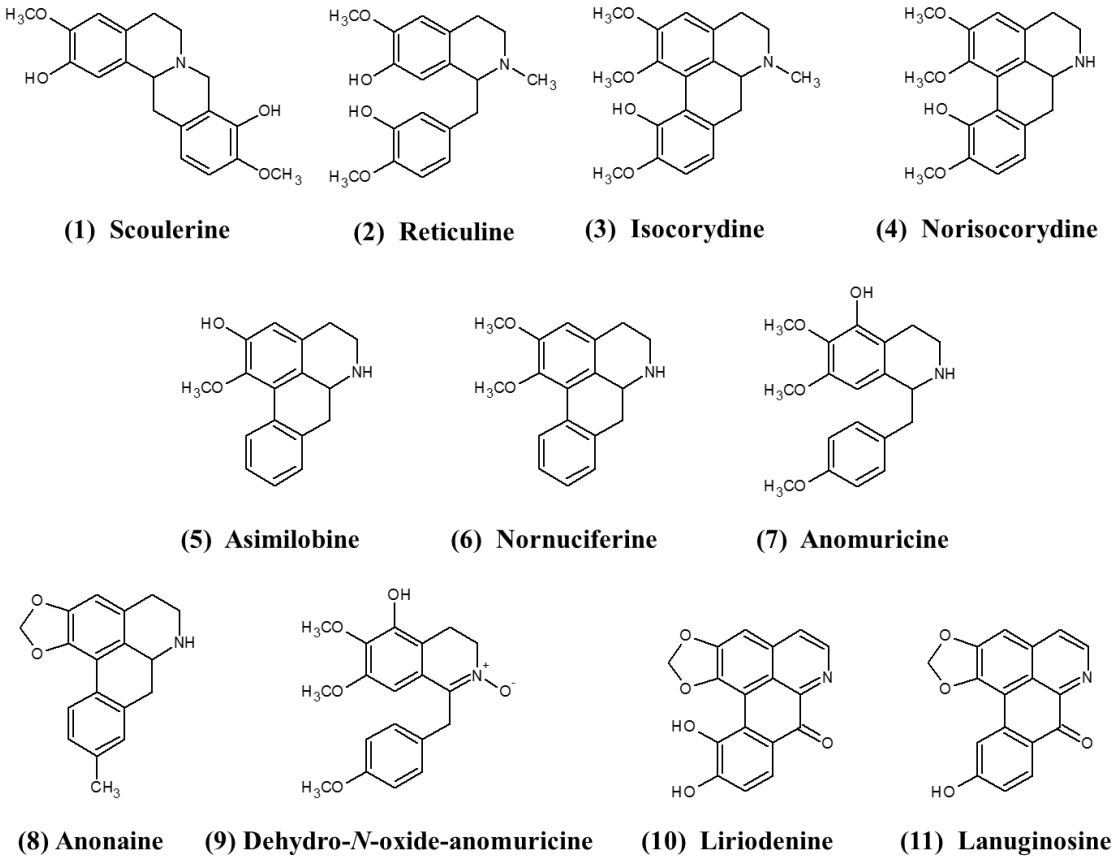

Figure 2. Alkaloids identified in the alkaloidal fraction of atemoya.

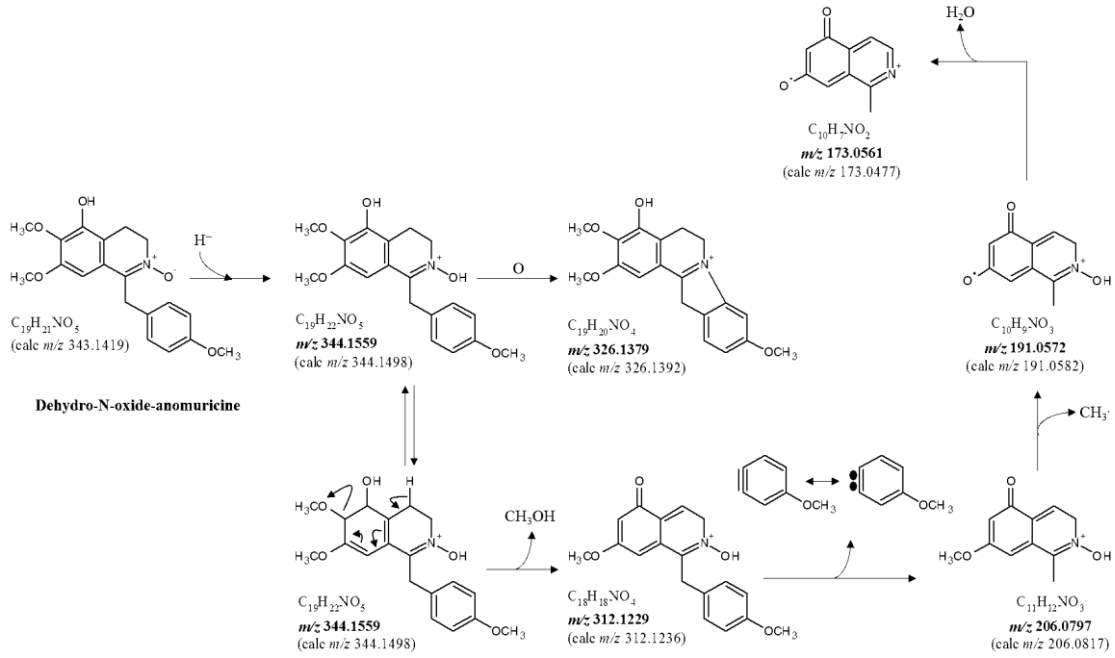

Figure 3. Proposed fragmentation of dehydro-*N*-oxide-anomuricine.

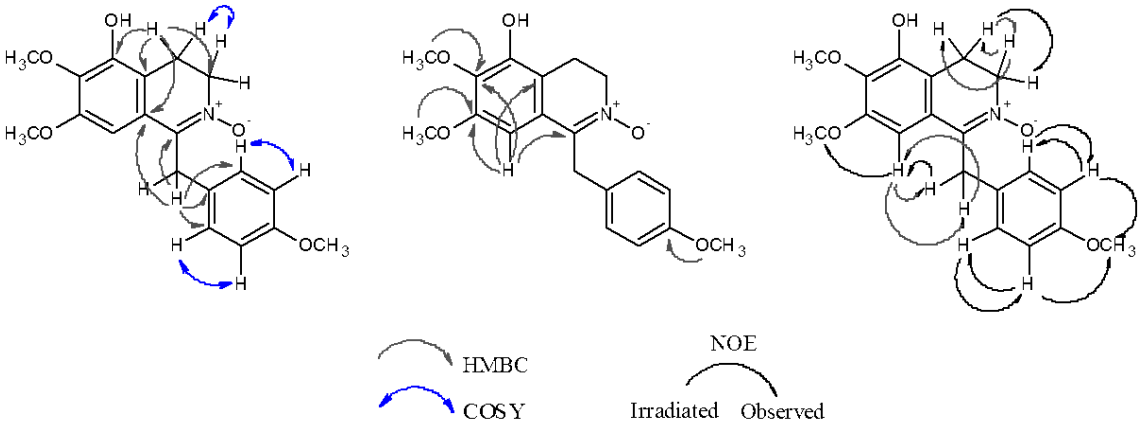

Figure 4. Correlations observed in HMBC, COSY and NOE NMR experiments for dehydro-*N*-oxide-anomuricine.

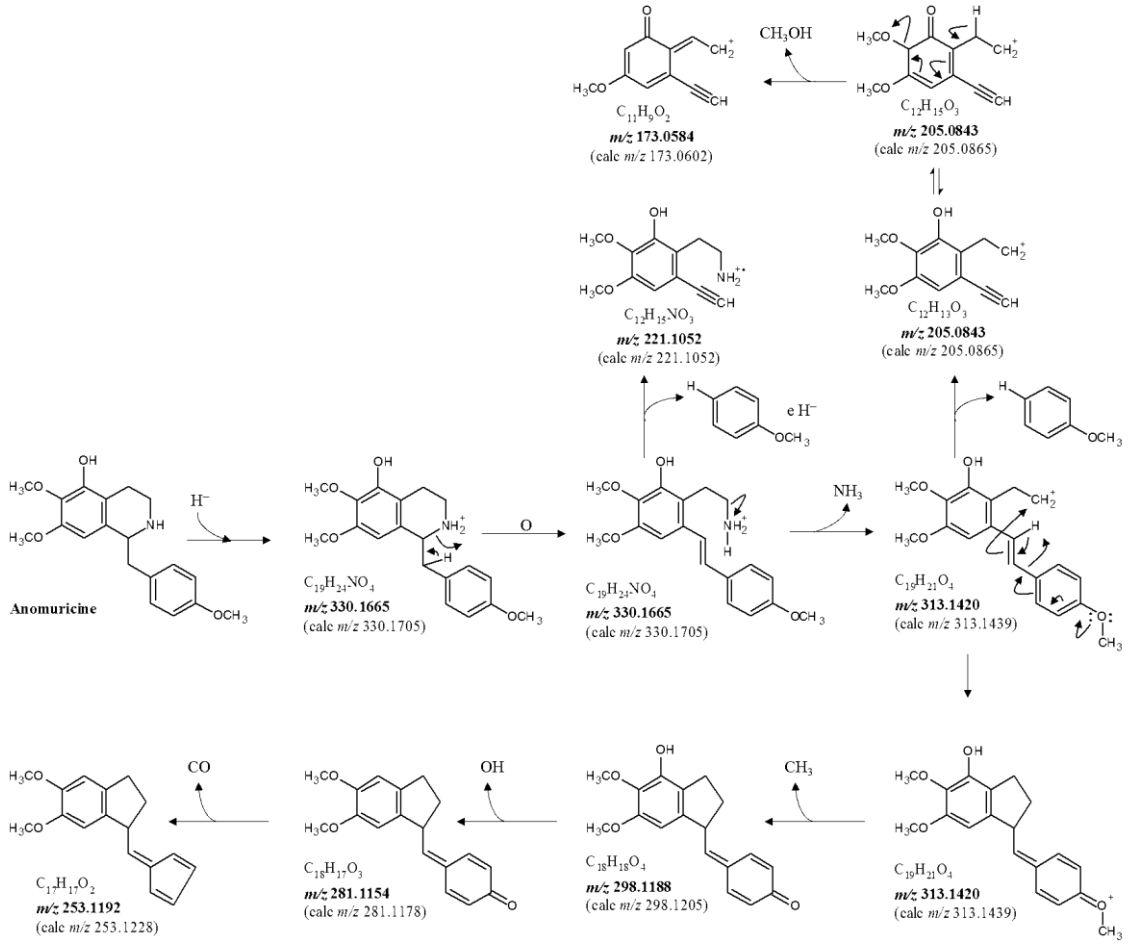

Figure 5. Proposed fragmentation of anomuricine.

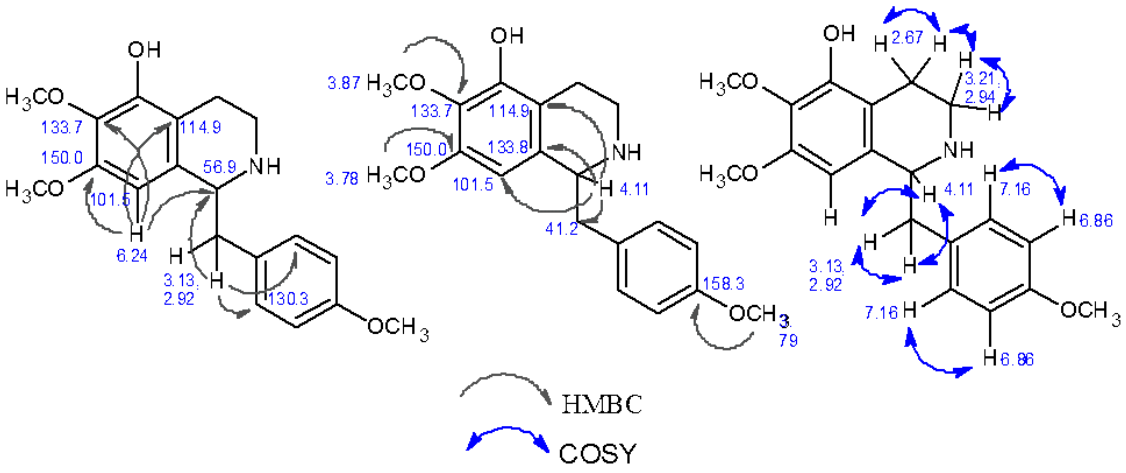

Figure 6. Main NMR correlations by HMBC and COSY for anomuricine.

Table 1. NMR spectroscopic data for dehydro-*N*-oxide-anomuricine and anomuricine (600 MHz for ^{1}H and 150 MHz for ^{13}C, $CDCl_3$)**.**

	Dehydro-*N*-oxide-anomuricine		**Anomuricine**	
Position	δ_C, Type	δ_H, (mult., *J* in Hz)	δ_C, Type	δ_H, (mult., *J* in Hz)
1	144.5, C	-	56.9, CH	4.11 (*dd*, 9.2 and 4.6)
3	58.8, CH_2	4.16 (*t*, 7.7)	39.7, CH_2	2.94 (*dt*, 12.1; 6.0) 3.21 (*dt*, 12.1; 6.0)
4	21.2, CH_2	3.07 (*t*, 7.7)	23.0, CH_2	2.67 (*t*, 6.0)
4a	111.4, C	-	114.9, C	-
5	146.4, C	-	146.8, C	-
6	136.6, C	-	133.7, C	-
7	151.6, C	-	150.0, C	-
8	101.4, CH	6.51 (*s*)	101.5, CH	6.24 (*s*)
8a	126.0, C	-	133.8, C	-
1’	129.7, C	-	130.6, C	-
4’	158.9, C	-	158.3, C	-
2’/6’	130.1, CH	7.23 (*d*,8.6)	130.3, CH	7.16 (*d*, 8.5)
3’/5’	114.8, CH	6.81 (*d*,8.6)	114.0, CH	6.86 (*d*, 8.5)
α	32.1, CH_2	4.26 (*s*)	41.2, CH_2	2.92 (*dd*, 13.8; 9.2) 3.13 (*dd*, 13.8; 4.6)
H_3CO-4’	55.9	3.76	55.2	3.83 (*s*)
H_3CO-6	61.3	3.90 (*s*)	60.9	3.91 (*s*)
H_3CO-7	56.0	3.76 (*s*)	55.8	3.78 (*s*)

The experiments were obtained at 293 K with TMS as internal reference (0.00 ppm).

Table 2. Table of the mobile phase gradient used for qualitative determination of alkaloid fractions of atemoya.

Time (min)	Solvent A (%)	Solvent B (%)
0.00	90	10
60.00	10	90
70.00	0	100
80.00	0	100
85.00	90	10
90.00	90	10

Table 3. LC-MS data of alkaloids of alkaloidic fraction and phases from atemoya.

Compound identified in FAT (Nº)	**Molecular Formula**	**RT**	**$^{+}$MS**	**$^{+}$MS^{2}**	**$^{-}$MS**	**$^{-}$MS^{2}**	**Literature**	**Sample**			
								FAT-Hex	FAT-$CHCl_3$	FAT-AcOEt	FAT-MeOH
Scoulerine (**1**)	$C_{19}H_{21}NO_4$	14.0	328.15	300.13 178.02	326.20	312.15 301.20 248.96 209.86	[25 and 26]	-	-	+	+
Reticuline (**2**)	$C_{19}H_{23}NO_4$	16.7	330.16	299.08 192.03	328.22	313.05 175.92 135.94 121.94	[25 - 27]	-	-	+	+
Isocorydine (**3**)	$C_{20}H_{23}NO_4$	18.3	342.17	311.13 279.13 165.02	-	-	[25 and 28]	+	+	-	-

Norisocorydine (**4**)	$C_{19}H_{21}NO_4$	18.6	328.13	311.13 279.11	324.19	-		-	+	+	-
Asimilobine (**5**)	$C_{17}H_{17}NO_2$	21.4	268.04	251.07 219.01	-	-	[27 and 29]	+	+	+	+
Nornuciferine (**6**)	$C_{18}H_{19}NO_2$	23.8	282.09	265.08	280.07	252.04	[27 and 29]	-	+	+	+
Anomuricine (**7**)	$C_{19}H_{23}NO_4$	25.5	330.18	313.14	-	-		-	+	+	-
Anonaine (**8**)	$C_{17}H_{15}NO_2$	29.0	266.12	249.06	-	-	[27 and 29]	+	+	-	-
Dehydro-*N*-oxide anomuricine (**9**)	$C_{19}H_{21}NO_5^+$	30.7	344.15	327.22 311.16 279.06	-	-	-	+	+	-	-
Liriodenine (**10**)	$C_{17}H_9NO_3$	36.1	276.04	-	-	-	[5 and 28]	+	-	+	+
Lanuginosine (**11**)	$C_{18}H_{11}NO_4$	41.4	306.11	-	-	-	[5]	+	+	+	-

Table 4. Mean percentage of cell growth inhibition (CI) of the samples at the single concentration of 50 μg.mL^{-1}.

Sample	HCT116		SF 295		HL60	
	CI% (mean)	SD	CI% (mean)	SD	CI% (mean)	SD
EMB	65.77	3.01	86.21	2.62	54.23	8.72
FAT	95.68	0.25	95.65	2.05	95.54	0.35
FAT-Hex	96.98	0.59	97.97	1.07	97.24	0.92
FAT-$CHCl_3$	95.32	0.25	87.49	1.15	96.64	0.50
FAT-AcOEt	79.81	1.42	64.08	1.64	90.07	2.69
FAT-MeOH	54.58	4.94	74.74	7.54	44.30	4.18

SD – Standard Deviation.

Table 5. IC_{50} and selectivity index values for anomuricine.

Cell line	IC_{50} µM (Interval)	Selectivity index
SF-295 (Glioblastoma)	**96.26** (71,0 – 130.49)	**0.71**
PC3 (Prostate)	**114.62** (75.94 – 172.98)	**0.59**
HL60 (Leukemia)	**41.03** (29.42 – 57.2)	**1.66**
HCT-116 (Colon)	**110.73** (76,82 – 160.64)	**0.61**
PC9 (Lung)	**109.91** (98.33 – 123.25)	**0.62**
B16F10 (Skin)	**105.07** (98.27 – 116.81)	**0.64**
COLO 205 (Colorectal)	**87.90** (72.46 – 106.63)	**0.77**
SW620 (Colorectal)	**78.75** (68.51 – 90.55)	**0.86**
PC3M (Prostate)	**>152**	**ND**
L929 (Fibroblast)	**67.99** (59.33 – 78.21)	**-**

*Not defined. Selectivity index values IC_{50} (non-tumor cells)/IC_{50} (tumor cell)

Supplementary Information

A new *N*-oxide benzylisoquinoline alkaloid isolated from the leaves of atemoya (*Annona cherimola* x *Annona squamosa*)

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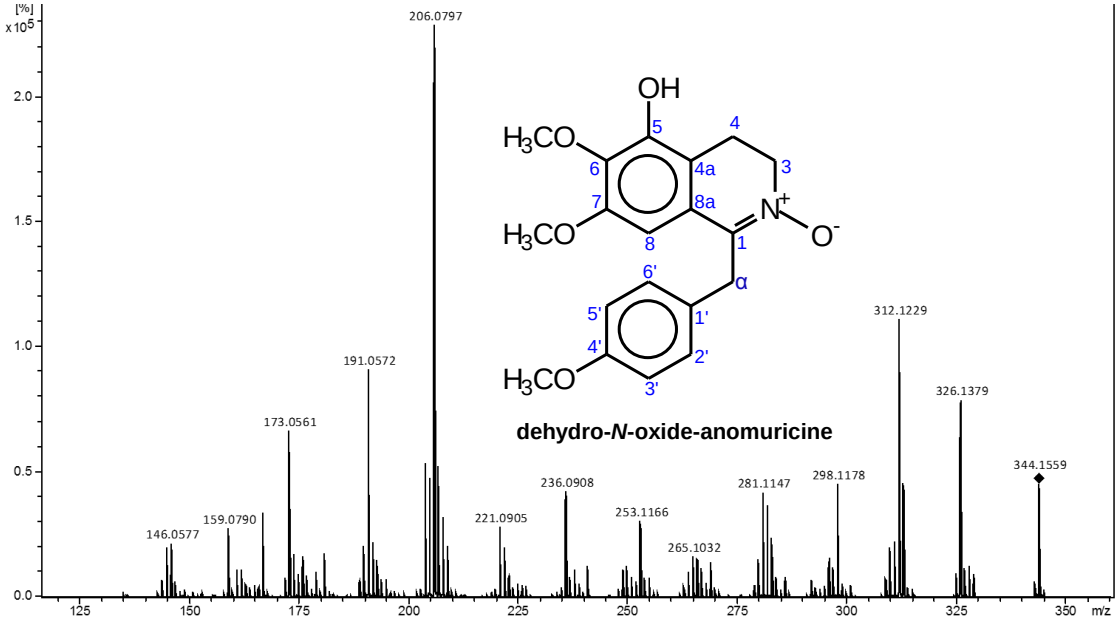

Figure S1. HRESIMS mass spectra of the $[M+H]^+$ ion of dehydro-*N*-oxide-anomuricine.

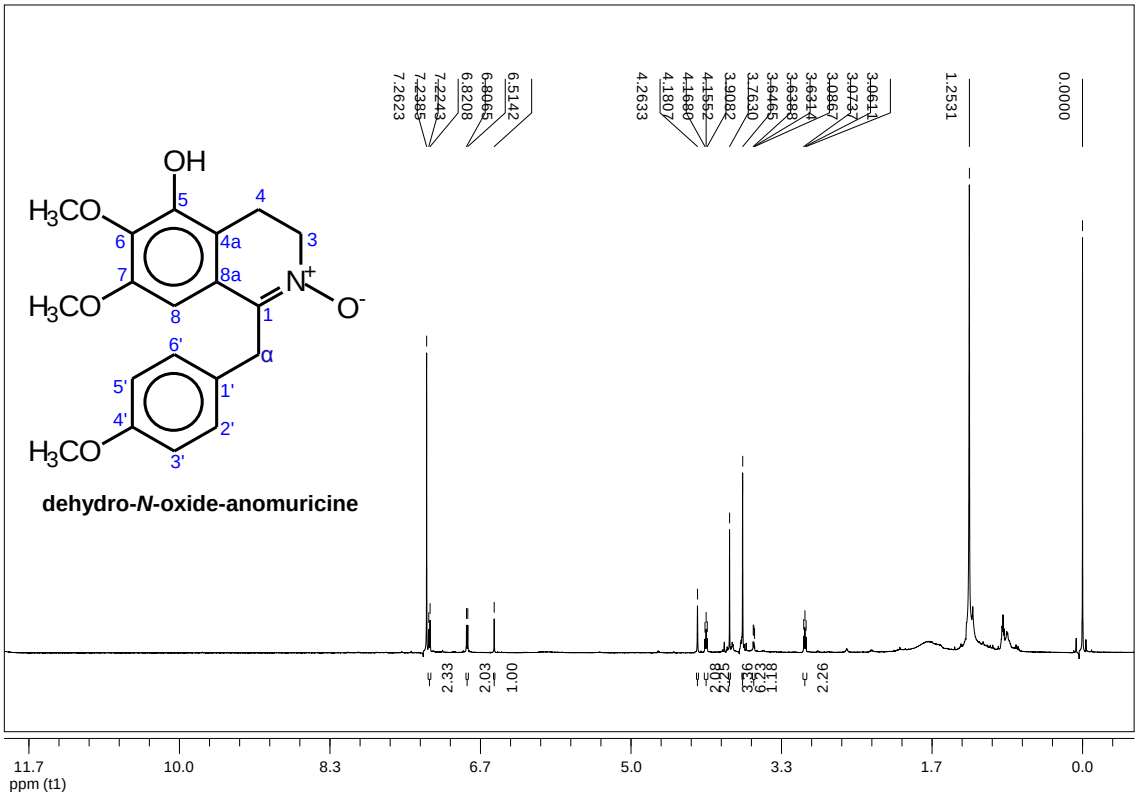

Figure S2. ^{1}H NMR spectrum of dehydro-*N*-oxide-anomuricine (600 MHz, $CDCl_3$).

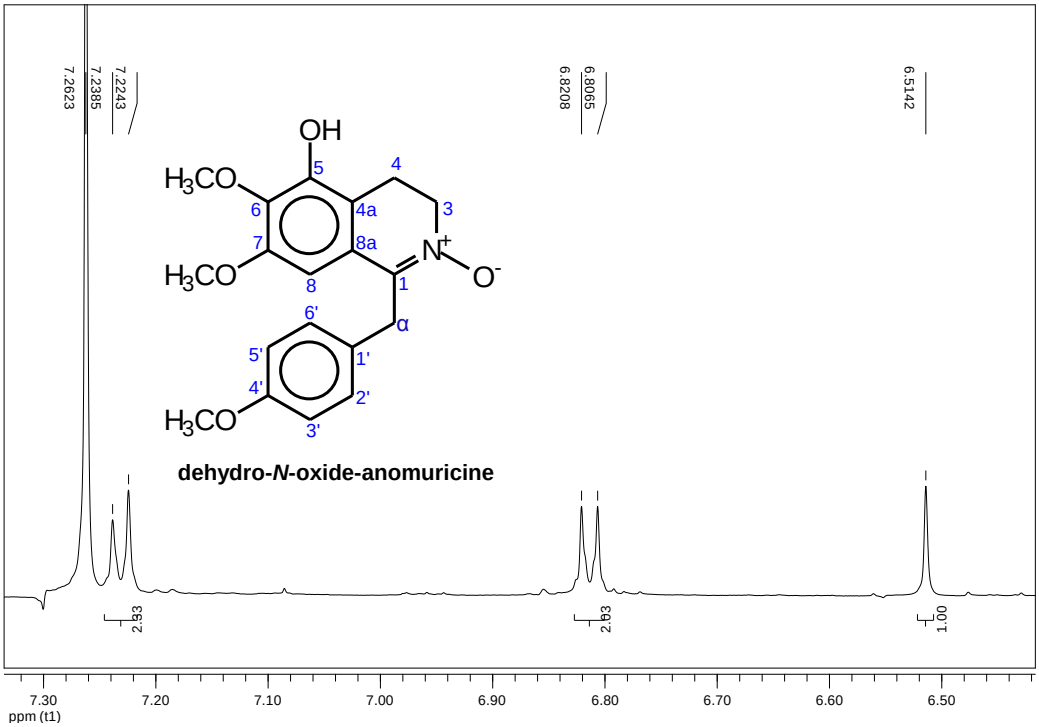

Figure S3. Expansion of the ^{1}H NMR spectrum of dehydro-*N*-oxide-anomuricine (600 MHz, $CDCl_3$), showing the region at δ 7.30-6.50 ppm.

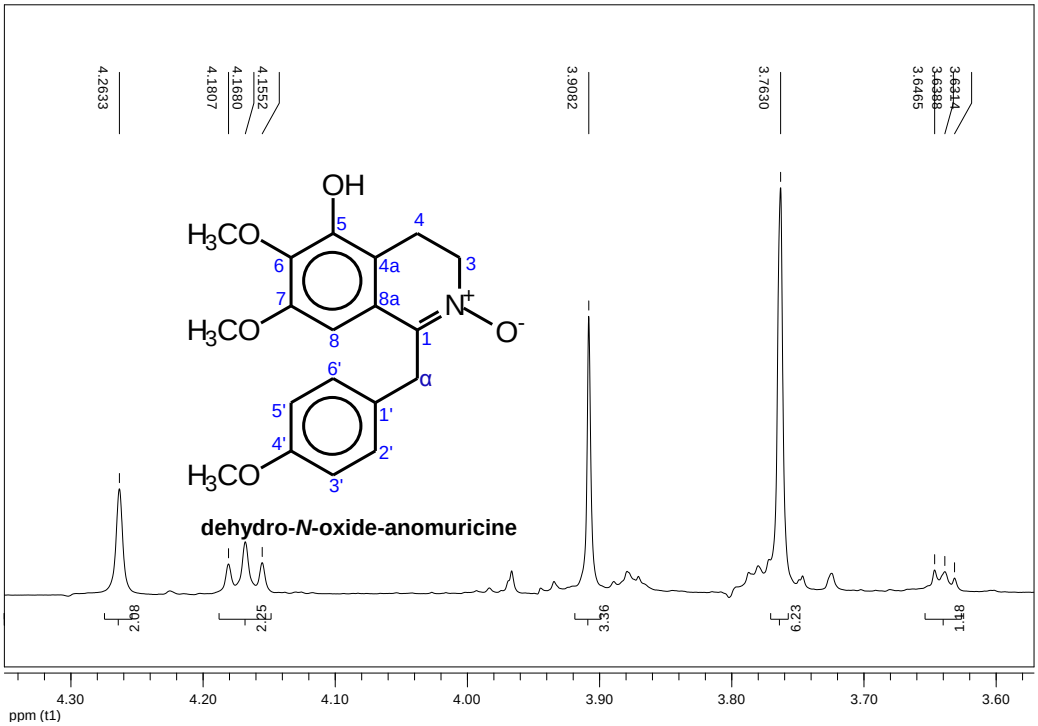

Figure S4. Expansion of the ^{1}H NMR spectrum of dehydro-*N*-oxide-anomuricine (600 MHz, $CDCl_3$), showing the region at δ 4.30-3.60 ppm.

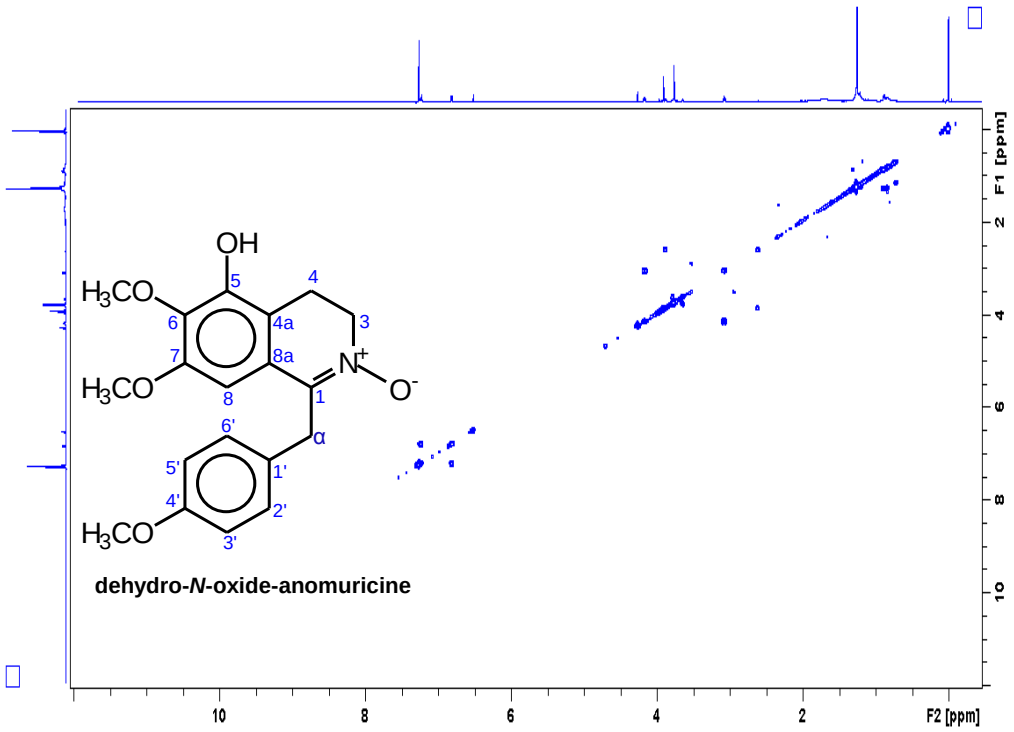

Figure S5. ^{1}H-^{1}H correlation map from COSY NMR experiment of dehydro-*N*-oxide-anomuricine (600 MHz, $CDCl_3$).

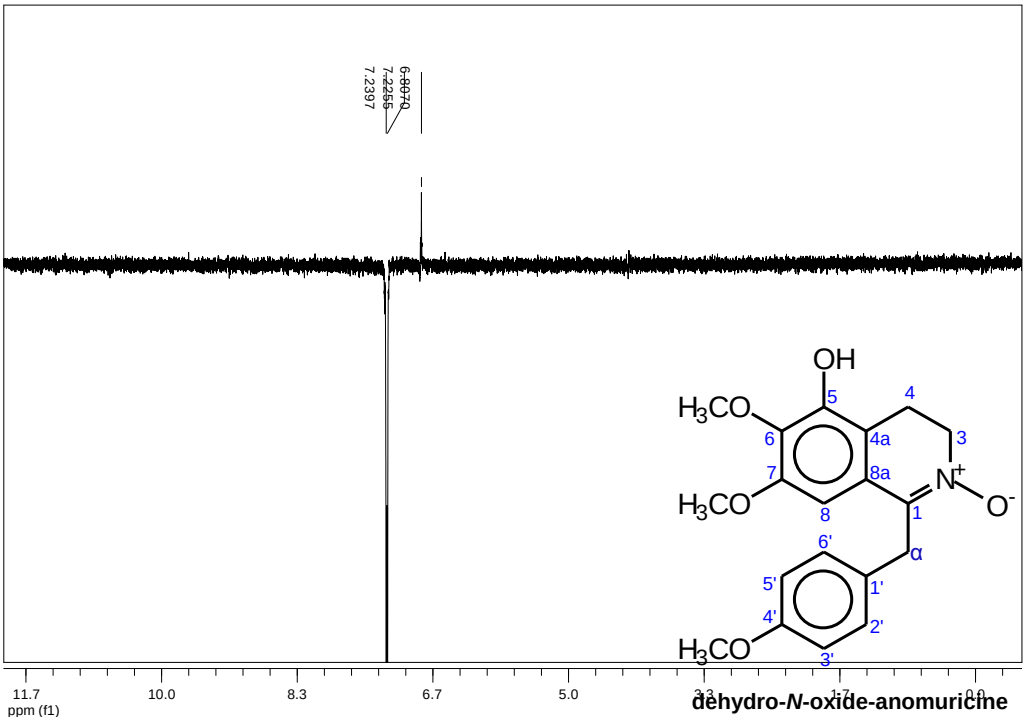

Figure S6. 1D NOE NMR experiment for dehydro-*N*-oxide-anomuricine, showing the selective irradiation of the resonance frequency of the hydrogen H-2'/H-6' at δ 7.23 (600 MHz, $CDCl_3$) .

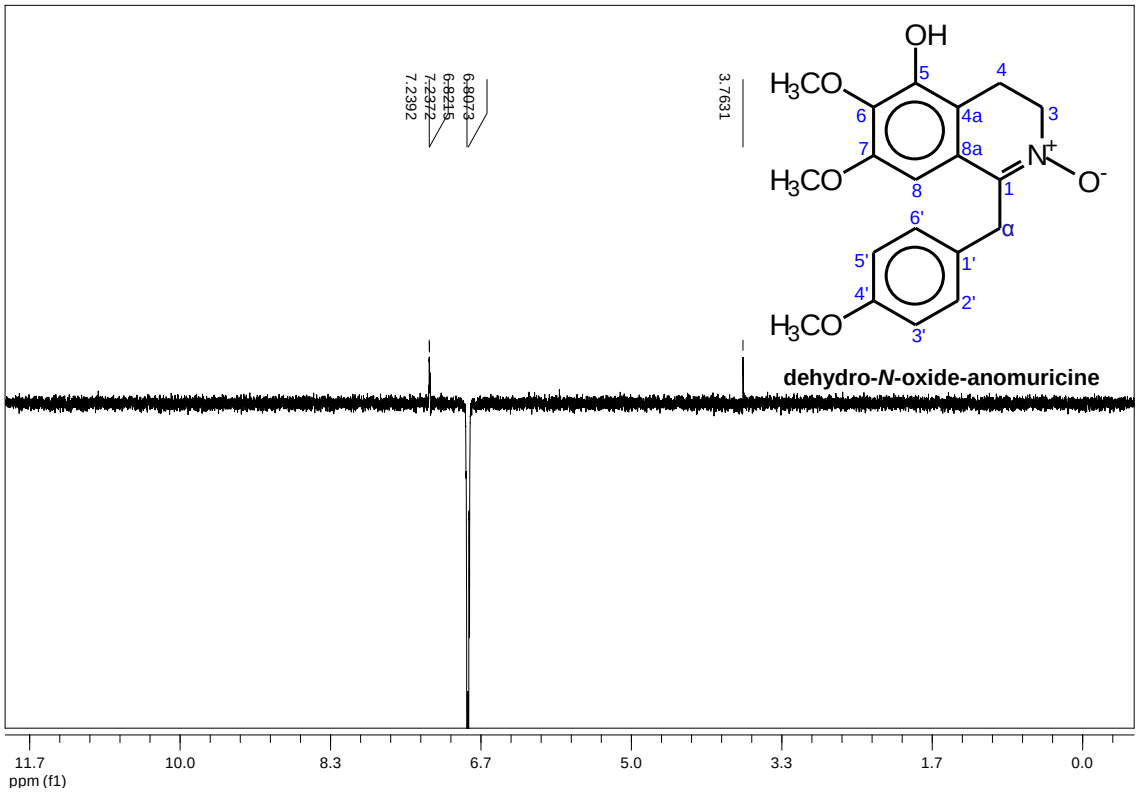

Figure S7. 1D NOE NMR experiment for dehydro-*N*-oxide-anomuricine, showing the selective irradiation of the resonance frequency of the hydrogen H-3'/H-5' in δ 6.81 (600 MHz, $CDCl_3$).

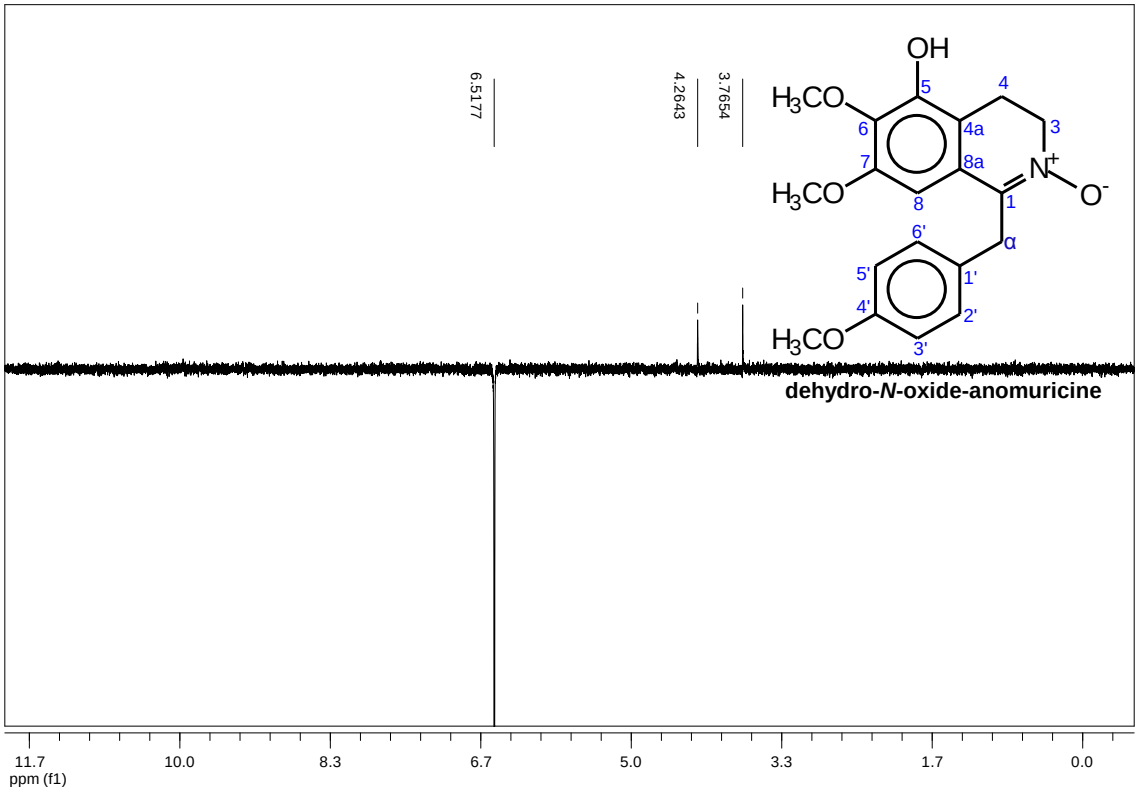

Figure S8. 1D NOE NMR experiment for dehydro-*N*-oxide-anomuricine, showing the selective irradiation of the resonance frequency of the hydrogen H-8 at δ 6.51 (600 MHz, $CDCl_3$).

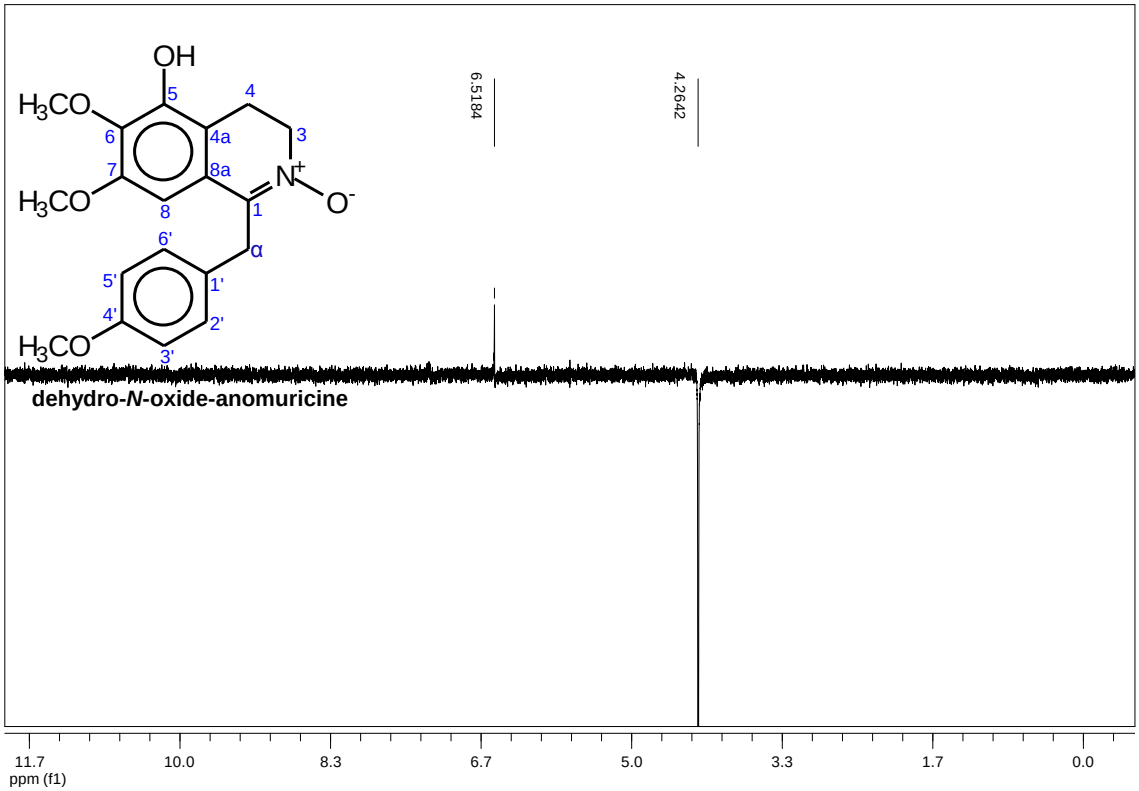

Figure S9. 1D NOE NMR experiment for dehydro-*N*-oxide-anomuricine, showing the selective irradiation of the resonance frequency of the hydrogen H-α at δ 4.26 (600 MHz, $CDCl_3$)

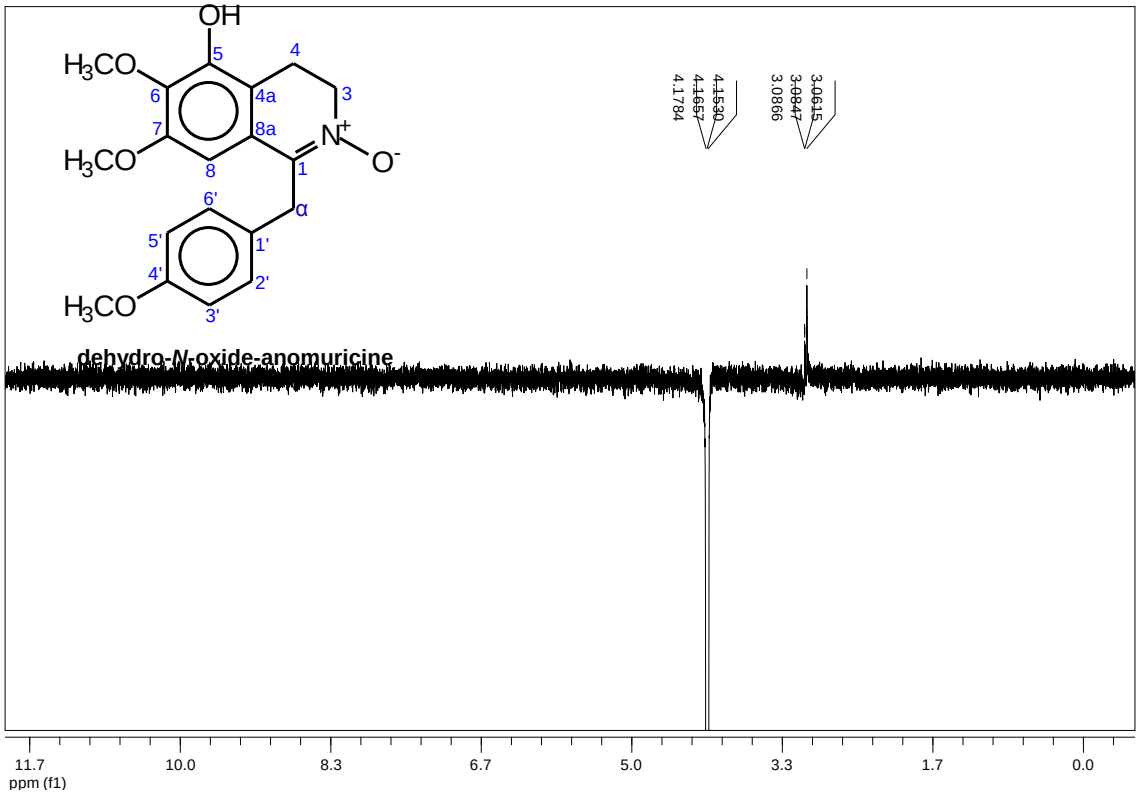

Figure S10. 1D NOE NMR experiment for dehydro-*N*-oxide-anomuricine, showing the selective irradiation of the resonance frequency of the hydrogen H-3 at δ 4.16 (600 MHz, $CDCl_3$).

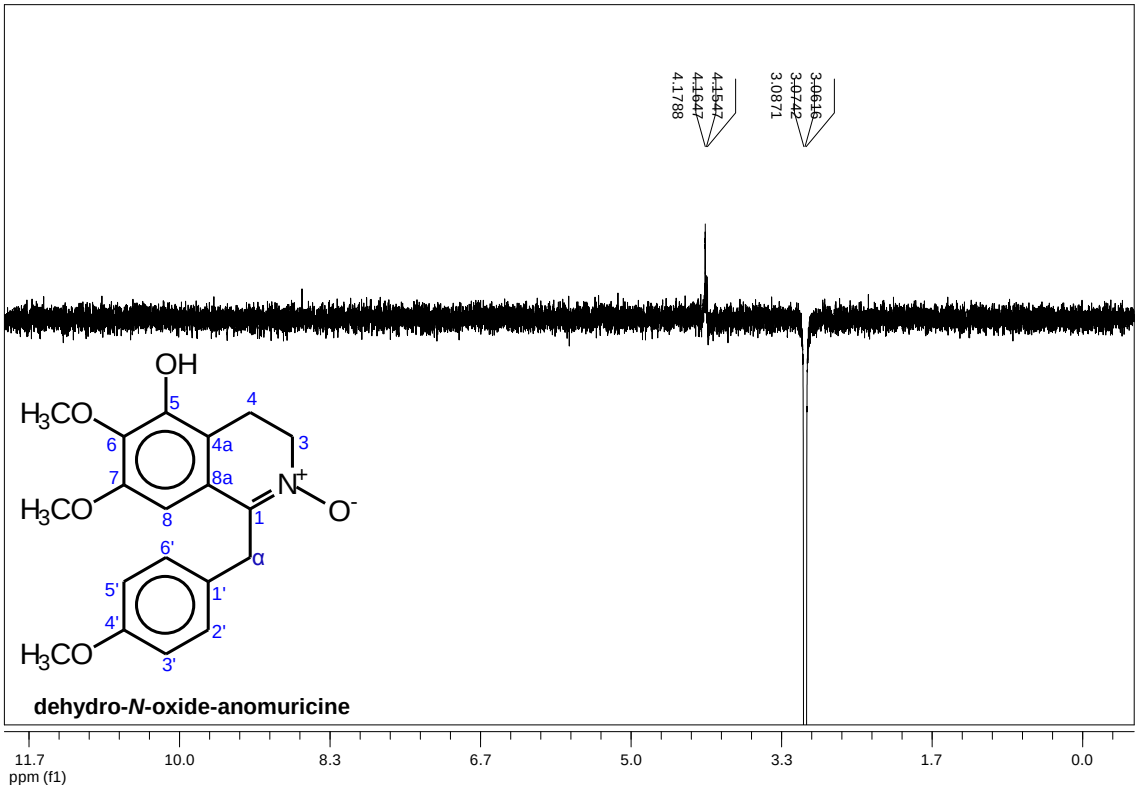

Figure S11. 1D NOE NMR experiment for dehydro-*N*-oxide-anomuricine, showing the selective irradiation of the resonance frequency of the hydrogen H-4 at δ 3.07 (600 MHz, $CDCl_3$)

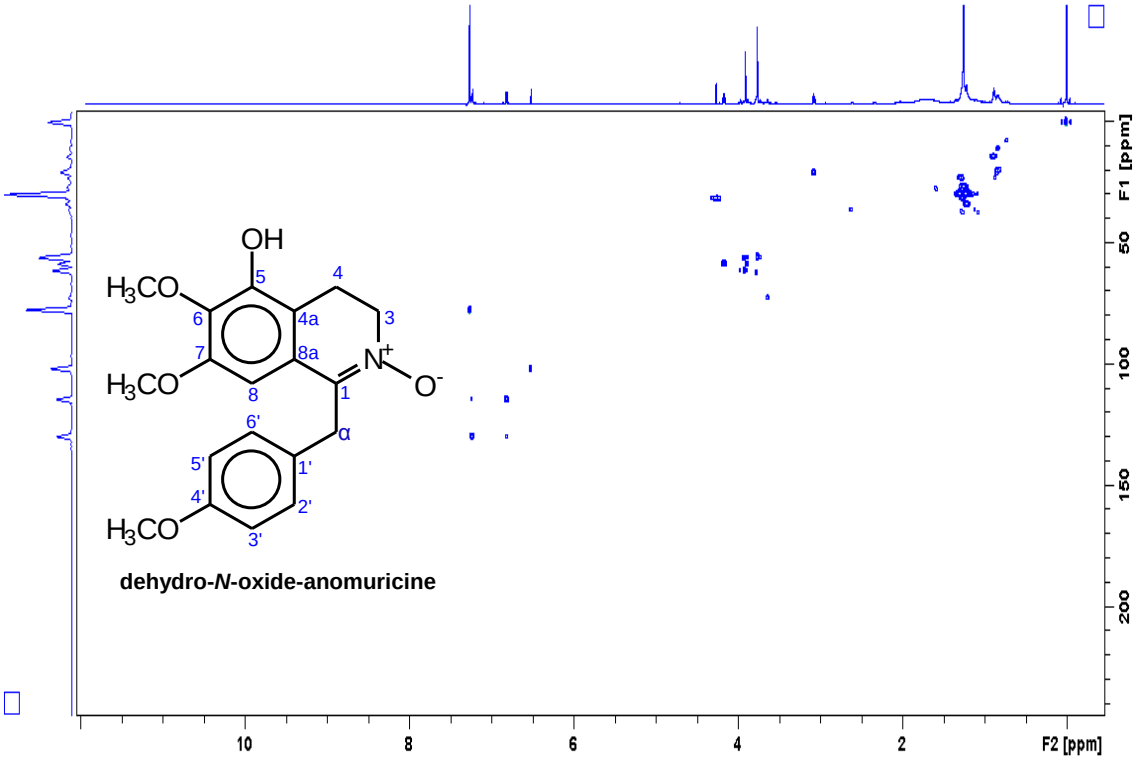

Figure S12. ^{1}H-^{13}C one-bond correlation map from HSQC NMR experiment of dehydro-*N*-oxide-anomuricine (600 and 150 MHz, $CDCl_3$).

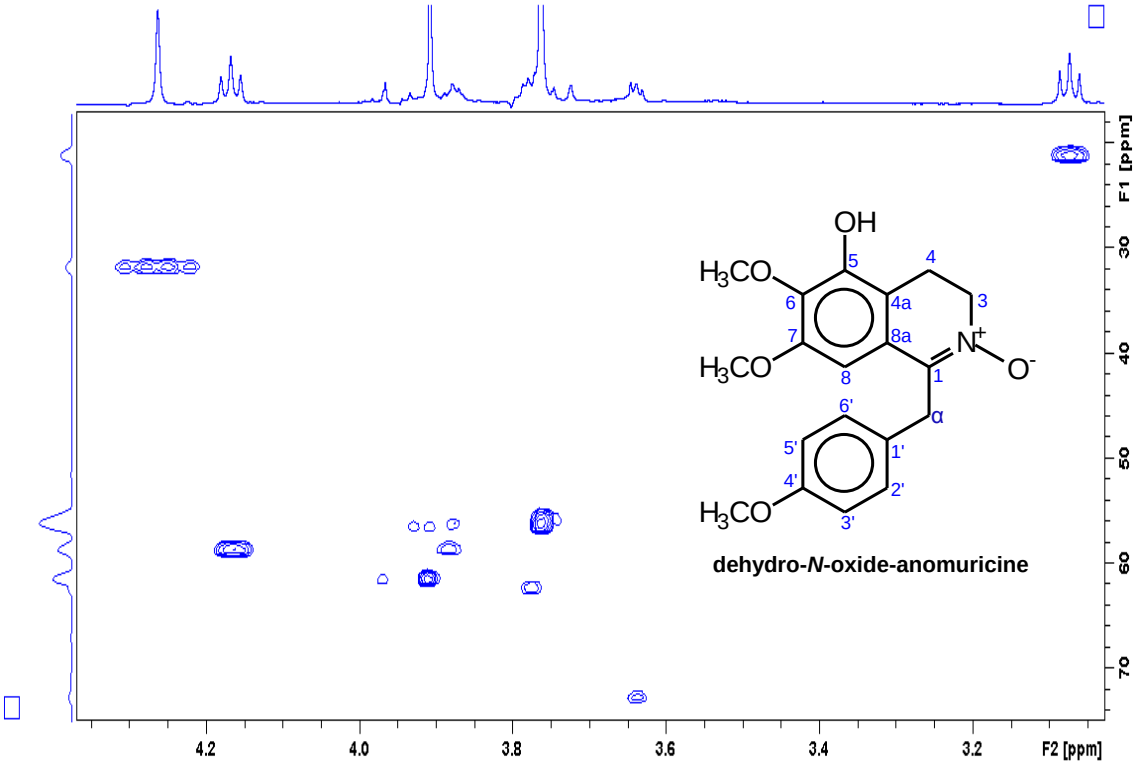

Figure S13. Expansion of the ^{1}H-^{13}C one-bond correlation map from HSQC NMR experiment of dehydro-*N*-oxide-anomuricine (600 and 150 MHz, $CDCl_3$), showing the correlations for signals at δ 3.07 (H-4), 3.76 (H_3CO-7,4'), 3.91 (H_3CO-6), 4.16 (H-3), and 4.26 (H-α), respectively.

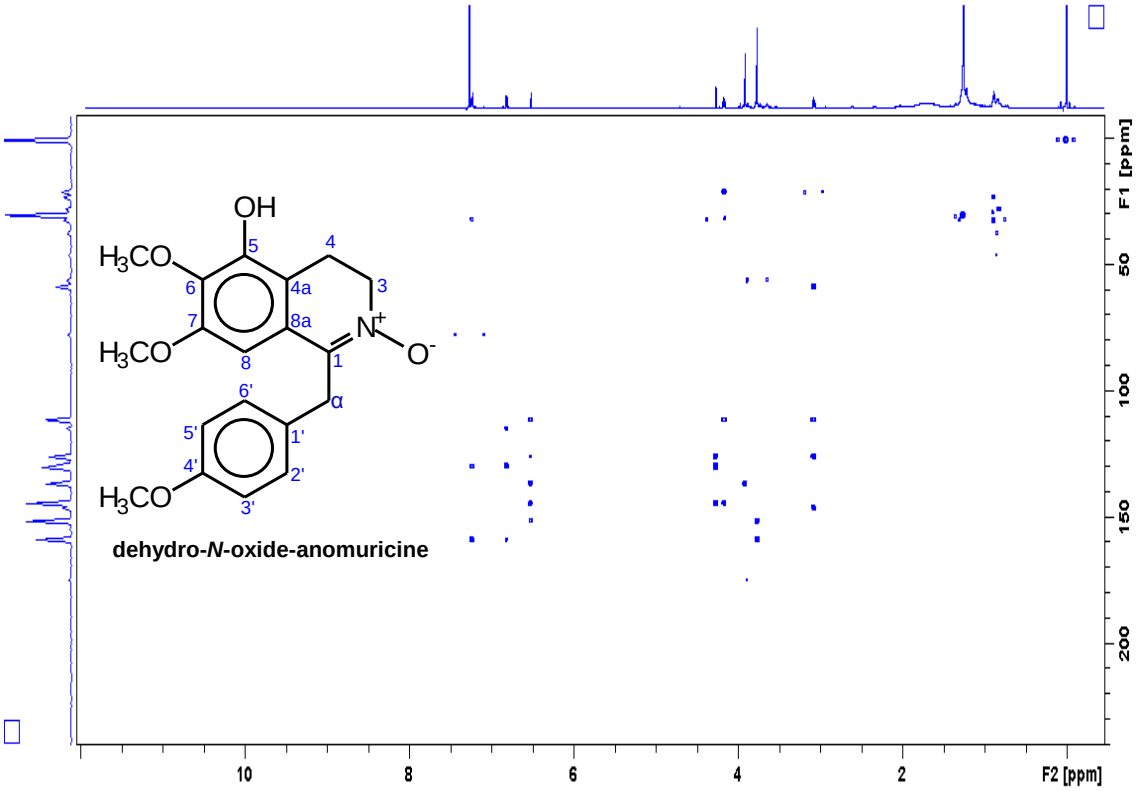

Figure S14. ^{1}H-^{13}C long-range correlation map from HMBC NMR experiment of dehydro-*N*-oxide-anomuricine (600 and 150 MHz, $CDCl_3$).

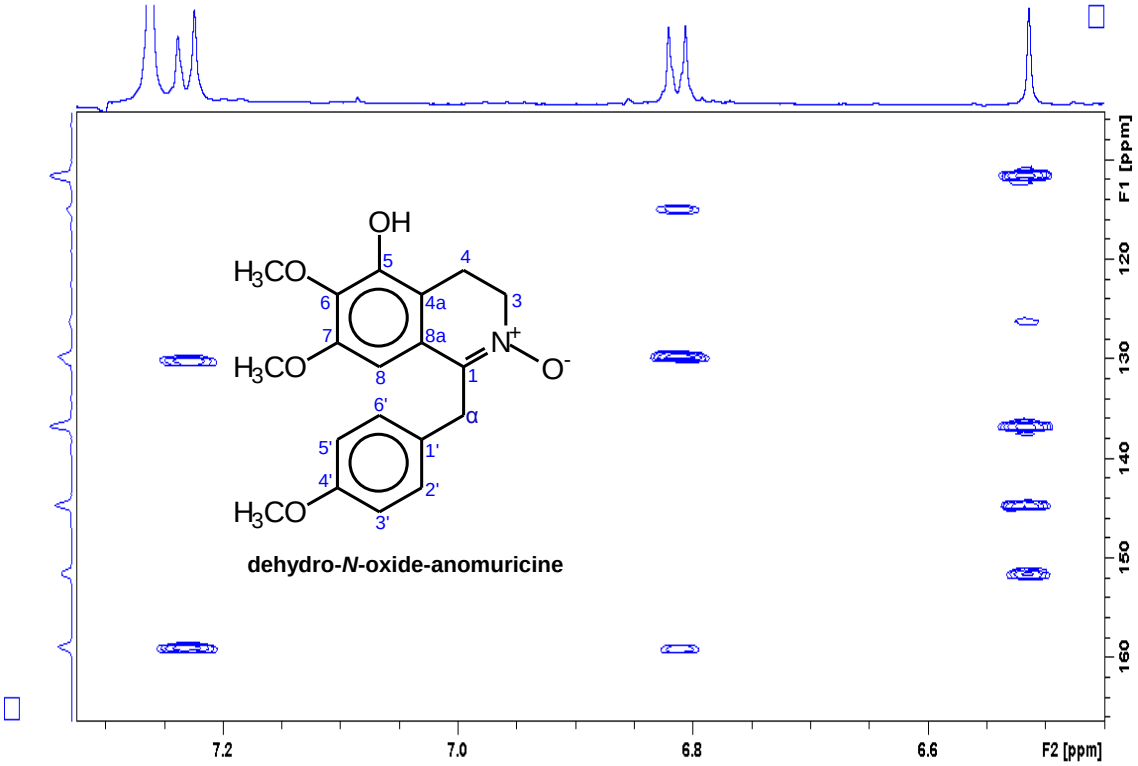

Figure S15. Expansion of the ^{1}H-^{13}C long-range correlation map from HMBC NMR experiment of dehydro-*N*-oxide-anomuricine (600 and 150 MHz, $CDCl_3$), showing the correlations for signals at δ 6.51 (H-8), 6.81 (H-3'/5') and 7.23 (H-2'/6'), respectively.

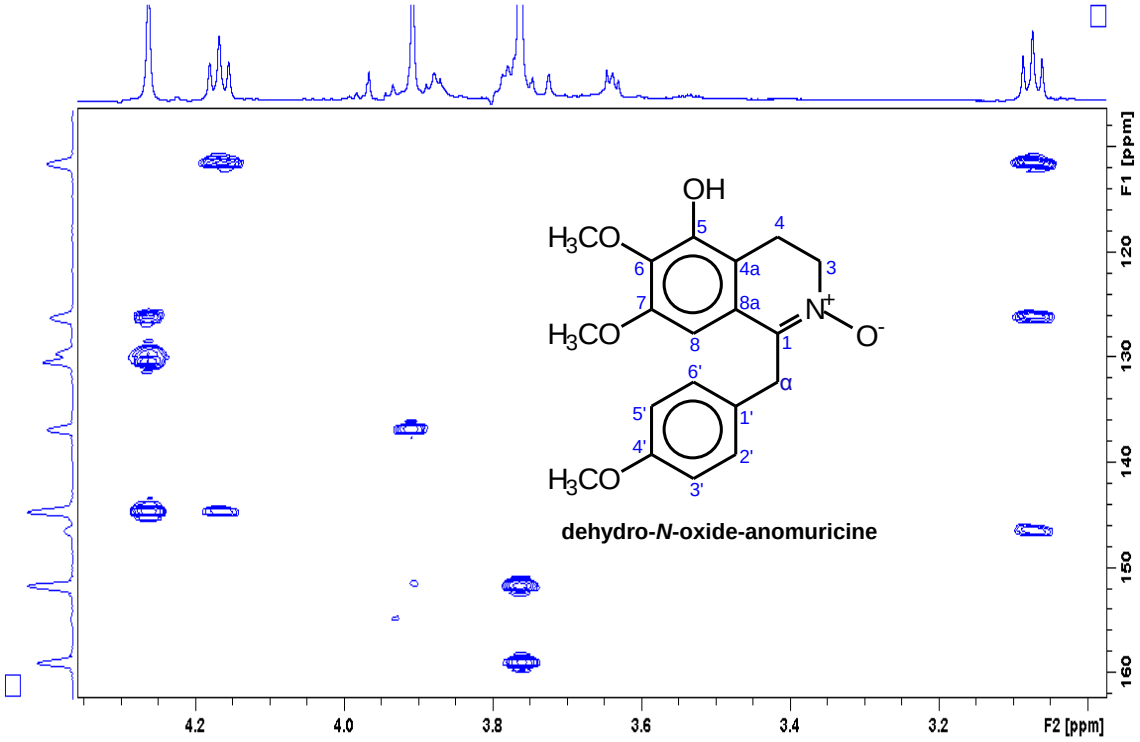

Figure S16. Expansion of the ^{1}H-^{13}C long-range correlation map from HMBC NMR experiment of dehydro-N-oxide-anomuricine (600 and 150 MHz, $CDCl_3$), showing the correlations for signals at for signals at δ 3.07 (H-4), 3.76 (H_3CO-7,4'), 3.91 (H_3CO-6), 4.16 (H-3), and 4.26 (H-α), respectively.

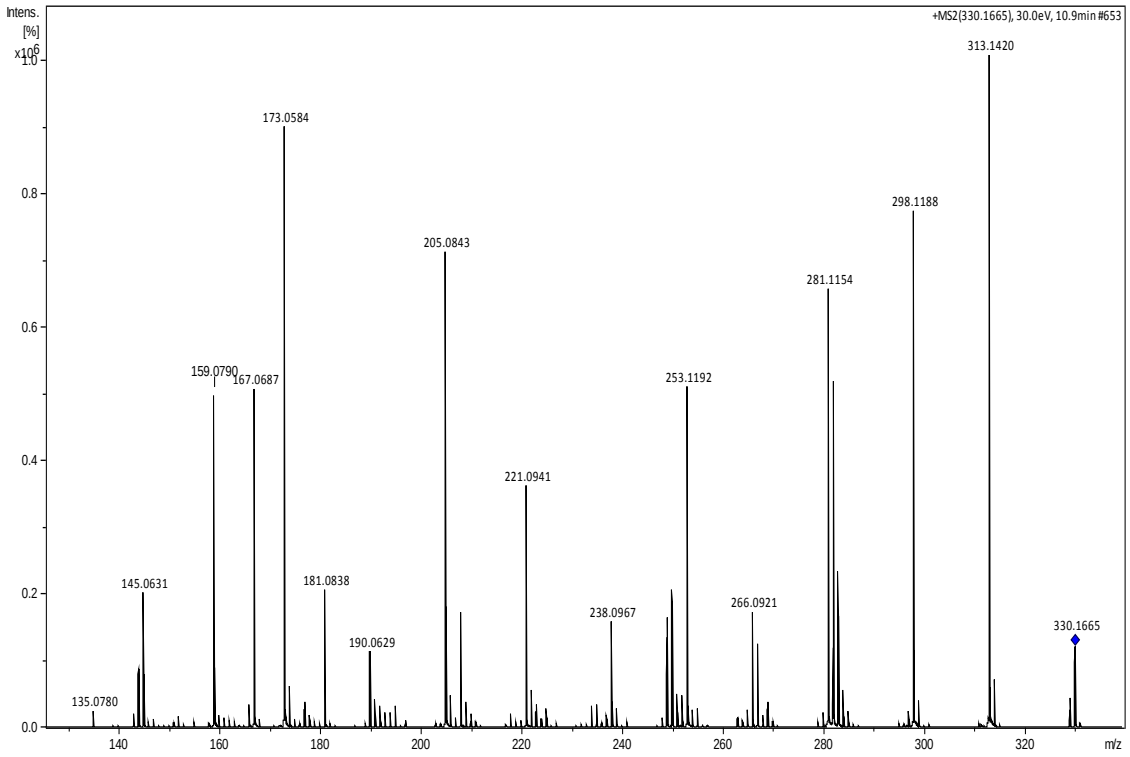

Figure S17. HRESIMS mass spectra of the $[M+H]^+$ ion (*m/z* 330) of anomuricine.

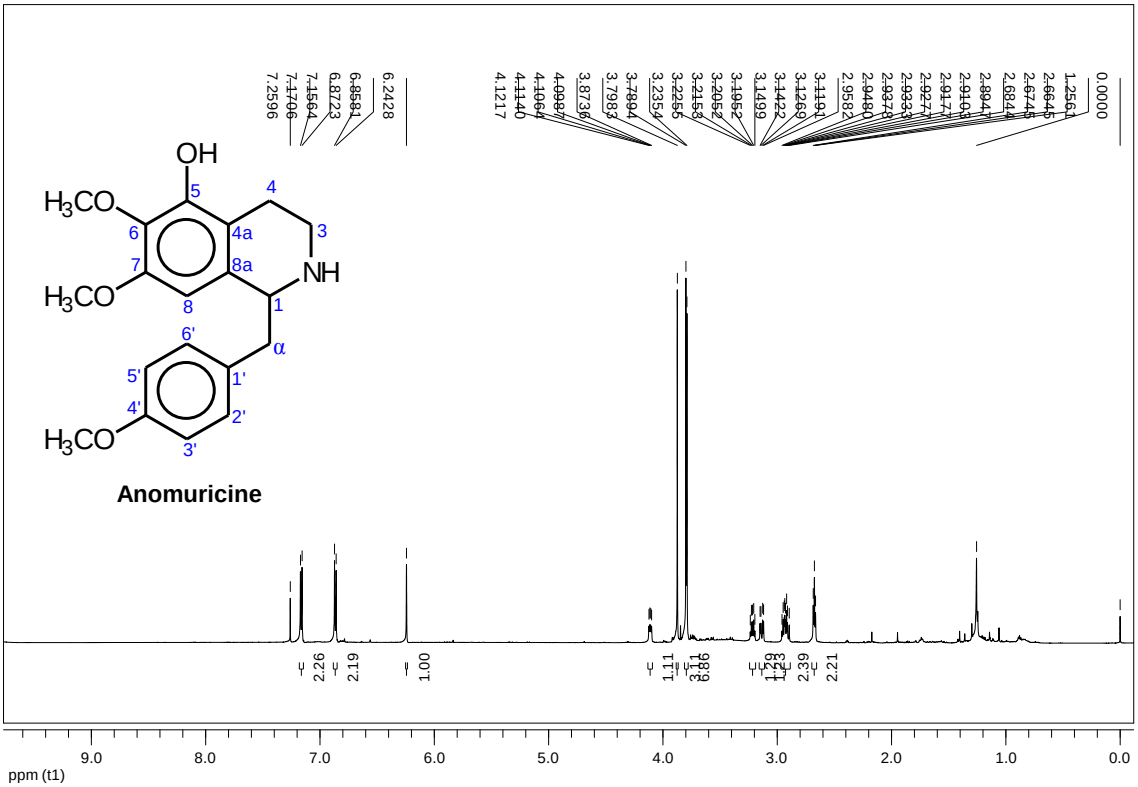

Figure S18.[1]H NMR spectrum of anomuricine (600 MHz, $CDCl_3$).

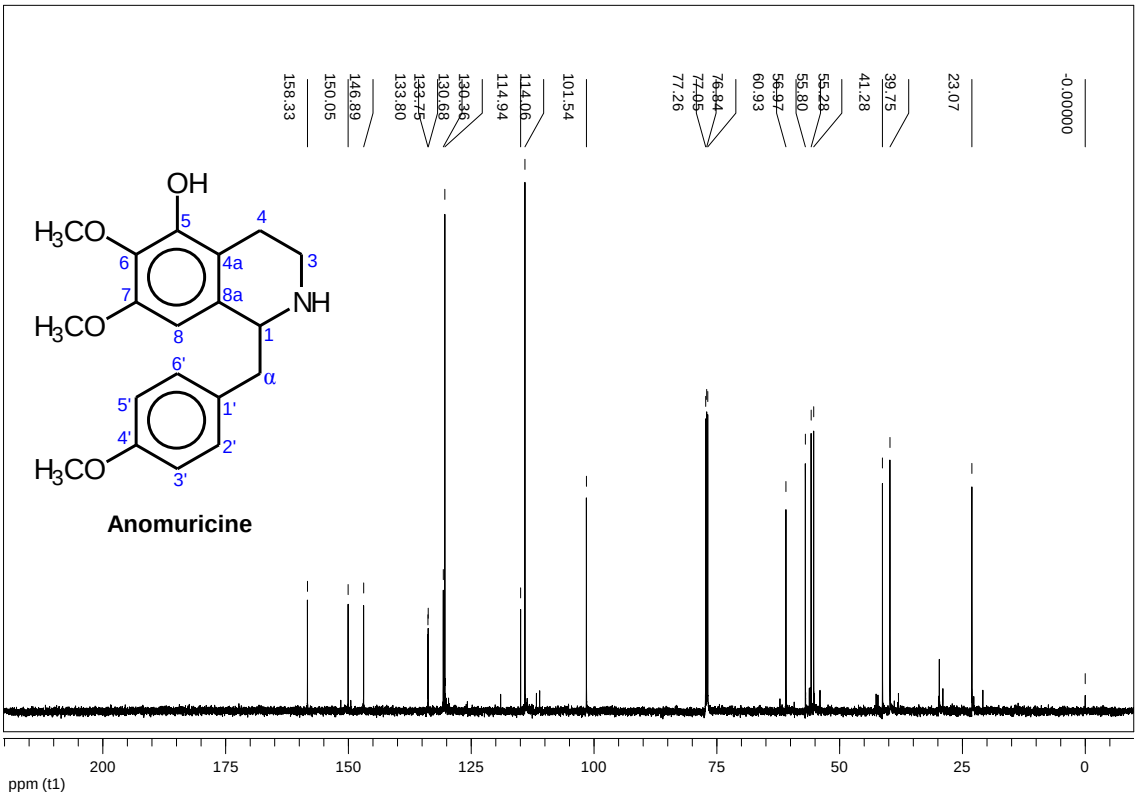

Figure S19. $^{13}C\{^{1}H\}$ NMR spectrum of anomuricine (150 MHz, $CDCl_3$).

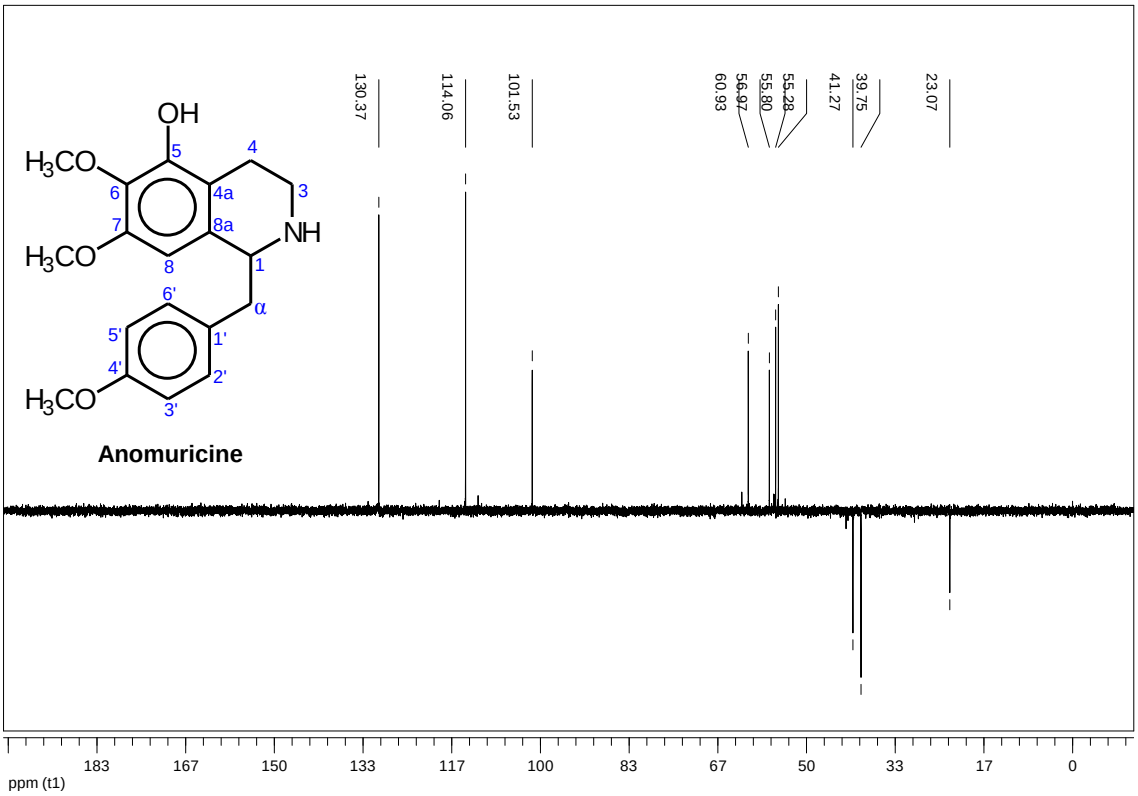

Figure S20. DEPT 135 NMR spectrum of anomuricine (150 MHz, $CDCl_3$).

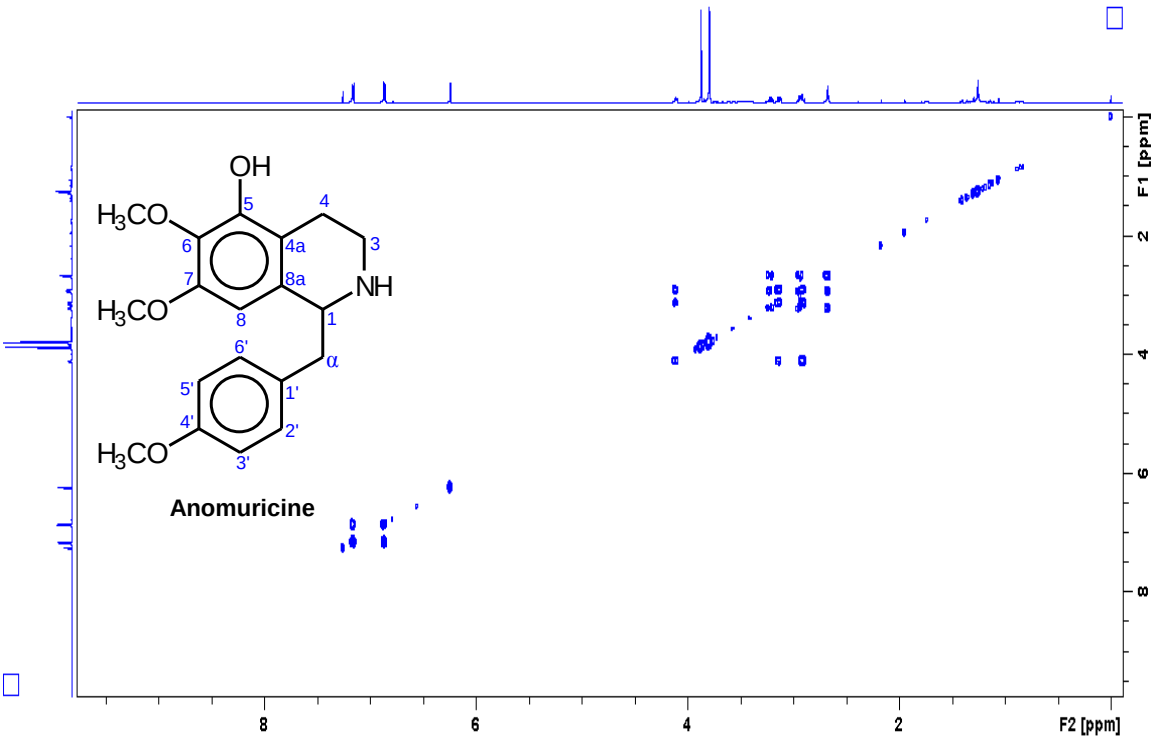

Figure S21. ^{1}H - ^{1}H correlation map from COSY NMR experiment of anomuricine (600 MHz, $CDCl_3$).

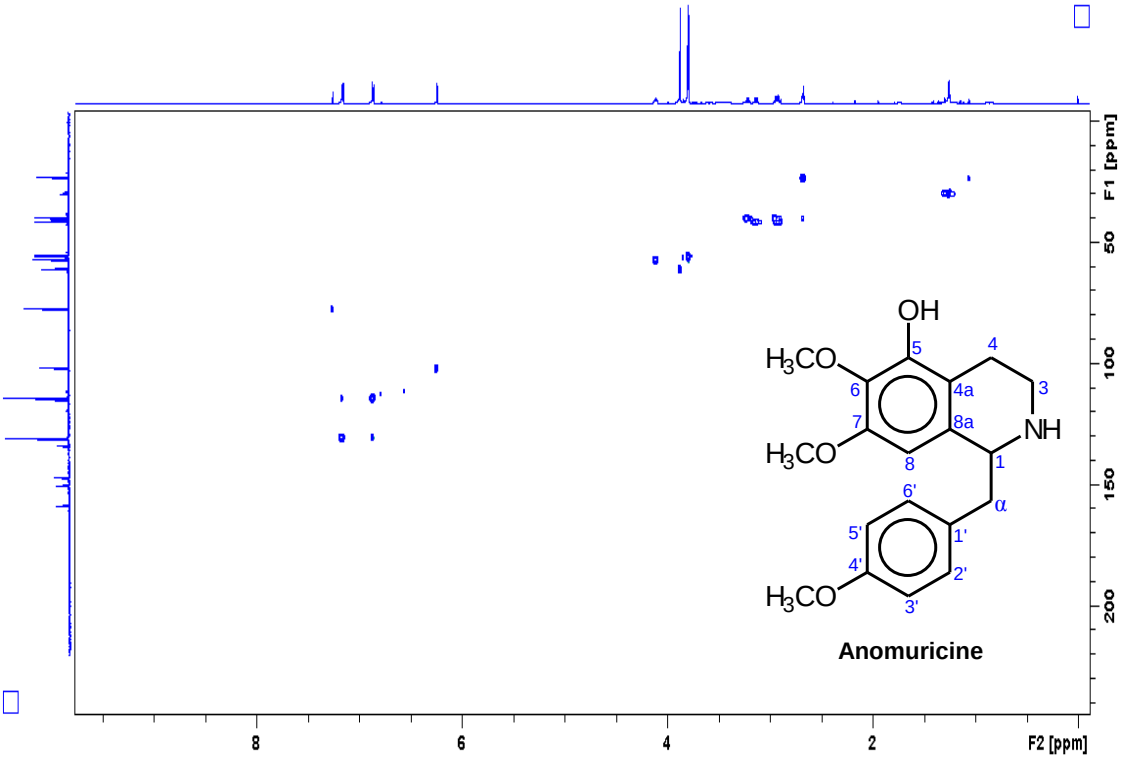

Figure S22. ^{1}H-^{13}C one-bond correlation map from HSQC NMR experiment of anomuricine (600 and 150 MHz, $CDCl_3$)

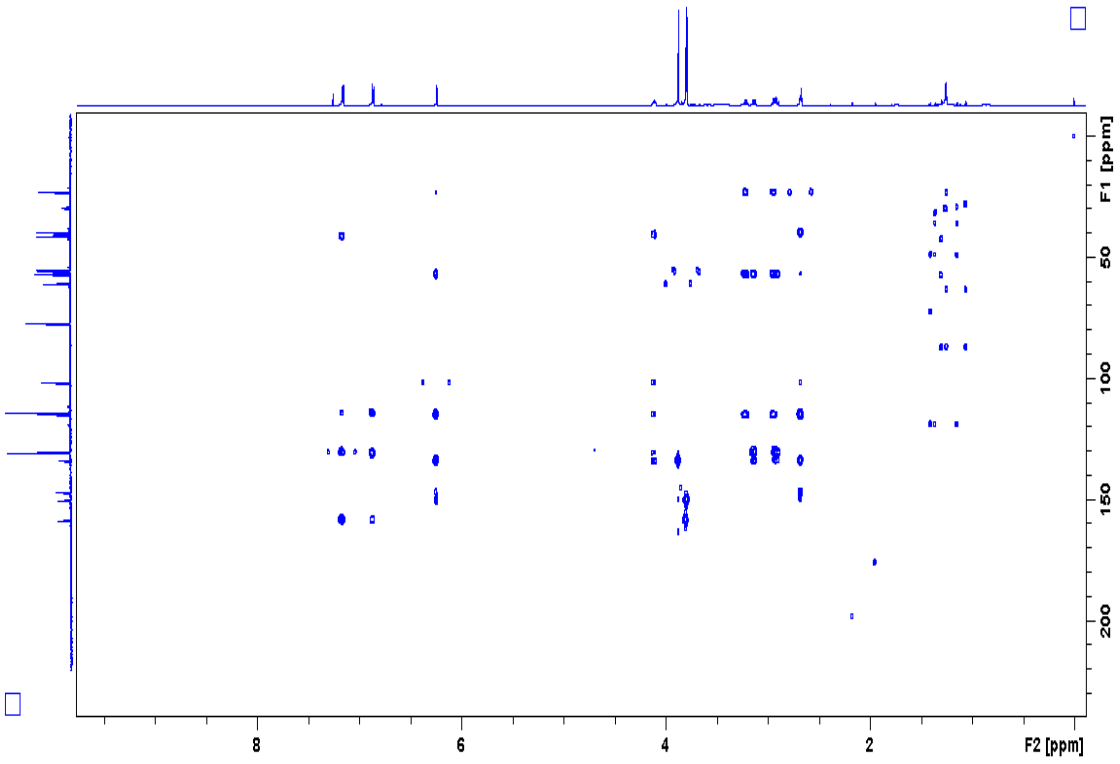

Figure S23. ^{1}H-^{13}C long-range correlation map from HMBC NMR experiment of anomuricine (600 and 150 MHz, $CDCl_3$).

CAPÍTULO 3

CHEMICAL COMPOSITION OF THE ESSENTIAL OIL FROM FRUITS OF ATEMOYA AND EVALUATION OF THE CYTOTOXIC AND ANTIMICROBIAL ACTIVITY

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Chemical Composition of the Essential Oil from Fruits of Atemoya and Evaluation of the Cytotoxic and Antimicrobial Activity

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Key words

Annona, GC-MS, volatile compounds, essential oil, cytotoxicity, antimicrobial activity.

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ABSTRACT

This study aimed to identify the chemical composition of the essential oil from fruits as well as from active hexanic crude extract from aerial parts (At-Hex) of atemoya (*Annona cherimola* x *Annona squamosa*), a hybrid belonging to the Annonaceae family. Cytotoxic and antimicrobial activity was also evaluated. The essential oil (EOAF) was obtained by hydrodistillation in a Clevenger-type apparatus. Gas chromatograph coupled to mass spectrometry (GC-MS) analyses was performed to identify the chemical composition of the oil. Cytotoxicity was tested against human tumor cell lines - HCT-116 (colon carcinoma), SF-295 (glioblastoma), OVCAR-8 (ovarian carcinoma) and HL60 (leukemia) - using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, and the antimicrobial activity was conducted by bioauthography method against eleven microorganisms strains. A total of twenty four compounds were identified in the EOAF. The monoterpenes linalool (25.70%), α-pinene (10.38%), β-pinene (9.12%) and *trans*-ocimene (7.43%), and the sesquiterpene bicyclogermacrene (12.58%) were the major constituents. Twenty nine compounds were identified in At-Hex, and the sesquiterpene spathulenol was the major compound. At-Hex showed a high cytotoxicity against SF-295. These findings are an important chemotaxonomic contribution for the Annonaceae family and the *Annona* genus. Atemoia proved to be a good source of research in the search for cytotoxic substances.

Introduction

Atemoya is a hybrid resultant from the crossing between two species of the *Annona* genus, belonging to the Annonaceae family [1]. This family comprises about 2500 species in about 135 genera [2], in which the *Annona* genus stands to produce very tasty and nutritious fruit, which add great commercial value [3].

From the phytochemical point of view, both the family and genus, are considered good sources of bioactive substances from their secondary metabolism. These components mainly include alkaloids, diterpenes, acetogenins and essential oils that exhibit significant activities, such as cytotoxic, antimicrobial, antioxidant and anti-parasitic, for example [4].

Cancer is characterized by abnormal cell proliferation and differentiation [5], especially malignant tumors, is responsible for a significant and increasing number of patients around the world, and represents the second leading cause of death in the world population [6]. That increasing incidence and the several limitations in conventional therapy, including high cost and high toxicity of current anticancer drugs, have faced a serious challenge for all researchers to design and develop a strategic, economical and effective alternative in the identification and procurement process of new drugs. In this sense, gas chromatography coupled to mass spectrometry (GC-MS) is shown as a faster and useful tool in the attempt to identify substances from biologically active plant oil and extracts faster and, under this scenario, phytomolecules are expected to revolutionize cancer treatment in the next decade [7].

Natural products currently play a fundamental role as a source of new drugs or leads for drug development. These focus not only in the treatment of cancer but also - to exemplify - as an alternative to the fight against pathogenic microorganisms and multiple drugs due to the indiscriminate use of antimicrobials [8]. In this scenary, Brazil holds a great potential for biodiversity exploitation and, among its regions, the Northeast has areas characterized by high solar radiation values, elevated temperatures, and the irregularity in rainfall regime. These characteristics provide the existing vegetation, abundant periods of water stress affecting the synthesis of secondary metabolites [9].

Thus, this work presents for the first time the chemical composition of essential oil of atemoya fruits (EOAF) and the volatile compounds from aerial parts hexanic crude extract (At-Hex) grown in Northeast Brazil, and the results of their cytotoxic activities. The antimicrobial activity of the essential oil was also tested.

Results and Discussion

The analysis of EOAF by GC-MS identified a total of 24 compounds, representing 96.23% of the constituents of the sample. The oil was characterized by the abundance of monoterpenes (60.84%), and sesquiterpenes (34.54%). Only one diterpene was identified in the sample, corresponding to 0.85% of the chemical composition. More than 60% of the oil consists of only five components: the monoterpenes linalool (25.70%), α-pinene (10.38%), β-pinene (9.12%) and *trans*-ocimene (7.43%), and the sesquiterpene bicyclogermacrene (12.58%) (**Table 1**).

These same components were also identified in essential oils of leaves, flowers and fruits of *Annona cherimola* [10] and oils from atemoya fruits from Australia and Havana [11-12].

The larger proportion of α-pinene and bicyclogermacrene has been observed in many essential oils of *Annona* species, indicating that they may be considered as chemotaxonomic markers of the genus [13].

Linalool, the major constituent of EOAF, is an open-chain tertiary monoterpene alcohol widely used in perfumery and cosmetics industry [14]. It presents depressant effect on the central nervous system, resulting in hypnotic properties, hypothermic and anticonvulsants [15-16], and also has antihelmintic efficacy [17]. The enantiomers of this substance when inhaled separately have opposite effects on the human cardiovascular system. While the (+)-linalool stimulates the cardiovascular system, (-)-linalool has depressive effect [18]. This oil is common in essential oils extracted from the *Annona* genus, it has also been identified in *A. muricata* [12] and *A. senegalensis* [19].

The presence of monoterpenes such as α-pinene, β-pinene, (*E*)-caryophyllene or sesquiterpenes as germacrene D and bicyclogermacrene are quite common in the oils from species of the genus *Annona*, with the occurrence of at least two of these constituents in *A. muricata*, *A. senegalensis*, *A. ermaginata*, *A. squamosa*, *A. pickeli* and *A. salzmanii* [20-22].

The analysis of the chemical composition of At-Hex was also done using the hyphenated technique of GC-MS. This is often used in the identification of compounds that are suitably volatile, small and stable at high temperatures, by interpreting the fragmentations and comparing with data from a library [23].

By means of this analysis it was possible to identify 29 compounds representing 54% of the volatile compounds of the At-Hex. Among them, the majority spathulenol (13.91%), octadecanal (10.05%) and kauran-18-al-17-acetoxy (4.01%). This and the other compounds are shown in Table 1 and are frequently found in species of the *Annona* genus as can be observed in species *A. vepretorum* [24]*, A. reticulata* [4], *A. squamosa* [25] and *A. leptopetala* [26].

Spathulenol, present in most quantity in At-Hex, is a sesquiterpene alcohol which was considered to be the main responsible for antioxidant, anti-inflammatory and antimycobacterial activities, as well as antiproliferative potential against ovarian (OVCAR-3) cell line shown by essential oil from *Psidium guineense* Sw., Myrtaceae [27]. This compound was also identified in EOAF, but in different quantity, just 4.44%. This compound was the major constituent of essential oil extracted from immature atemoia fruits collected in Taiwan so as the α-cubebene, α-copaene, germacrene D, δ-cadinene and caryophyllene oxide [28]. The compounds β-Elemene, Germacrene D and δ-Cadinene were identified in both sample analyzed here, EOAF and At-Hex.

In EOAF, the cytotoxicity tests showed that the cell growth inhibition percentage of the tumor cells varied from 2.47% to OVCAR-8 to 49.67% for HCT-116, and for SF-295, the inhibition percentage was 10.30% (**Table 2**).

Studies with other Annonaceae species showed higher cytotoxic potential than the obtained in this study, such as fruit essential oil of *Annona vepretorum*, that demonstrate moderate activity against the HCT-116 cells, with inhibition percentage of 65.57%. Against OVCAR-8 cells and NS-295, the cell growth inhibition was 13.15% and 30.32%, respectively [29].

Another study on cytotoxicity of essential oil and extracts from the leaves of *Annona senegalensis* found that the moderate cytotoxicity results would be related to the presence of

caryophyllene oxide, main constituent identified in the fraction considered moderately cytotoxic (64.5% of the composition) [30]. The essential oil extracted from leaves of *Annona muricata* showed noticeable cytotoxic activity in vitro on MCF-7 cells (breast) with 99.2% cell death (100 µg/mL). These cytotoxicity data were assigned to the major constituents of the essential oil that was (*E*)-caryophyllene (38.9%), and eugenol (30.2%) [31].

Taking into consideration that the percentage of (*E*)-caryophyllene in the essential oil of fruits atemoya was 1.06%, and eugenol and caryophyllene oxide were not identified, the low cytotoxic activity may be related in part to the absence of a greater amount of these constituents, and the way of interaction of the chemical constituents present.

At-Hex was tested against HCT-116 (colon carcinoma), SF-295 (glioblastoma) and HL60 (leukemia) cells. The results, presented in table 2, show the antiproliferative effect of the extract against the lineage of SF-295, with a mean of 91.77% inhibition (**Table 2**). The At-Hex was not considered cytotoxic against the other tumor lines tested, since the percentages of growth inhibition were below 75%.

The SF-295 cells correlate with human glioblastoma cell lines, which is the most common, aggressive and lethal type of brain tumor [32] with increasing estimates of mortality and new cases each year[7]. Recent studies have shown that less than 1% of patients with this type of cancer can survive 10 years or more [33] and that, despite advances in surgery and chemotherapy, patients have an average survival rate of only year [34]. Therefore, the search for less aggressive and more effective drugs against this disease is an important scientific contribution.

The high cytotoxic potential presented by At-Hex against SF-295 demonstrates that the atemoya hybrid is a promising source of active molecules important in the treatment of cancer diseases. Among the constituents identified spathulenol (the major in At-Hex) is frequently associated with cytotoxic activity and is common in species of *Annona* [11-13]. As some of these constituents here showed have not yet been tested, it is important that these molecules be isolated for further evaluation of the potential.

In the analysis of antimicrobial activity (**Table 3**), the oil showed minimum inhibitory concentration values (MIC) greater than 2 mg/mL against all the tested strains, indicating low activity.

The antimicrobial activity of essential oils of other species and its individual constituents has been widely studied. There are studies indicating that oxygenated compounds, phenolic or alcoholic or ester groups tend to be more potentially active [35]. A study with the species *Annona cherimola* indicates that different parts exhibit significant and moderate antimicrobial activity. These activities are justified by the presence of linalool and *trans*-caryophyllene [15].

The EOAF presented 36.06% of oxygenated compounds of which 25.70% just of linalool. By that it was expected a good antimicrobial activity, but the low observed activity from EOAF must be related to the way of interaction of its various constituents.

Materials and Methods

Collection of plant material

For essential oil extraction, atemoya immature fruits were collected in Petrolina, Pernambuco, Brazil (Coordinates: 9°23'19" S, 40°29'3" W), in May 2012. The botanical material was identified by Prof. José Alves de Siqueira Filho and the voucher specimen (#16310) was deposited in the Herbário Vale do São Francisco (HVASF) at Universidade Federal do Vale do São Francisco (UNIVASF). The aerial parts for the prepare of the hexanic crude extract were collected in September of 2014 in Petrolina, state of Pernambuco (Coordinates: 9°23'19" S and 40°29'3" W). The plant was identified by Professor José Alves de Siqueira Filho by comparation with the voucher specimen #16310. All procedures for access to genetic patrimony and associated traditional knowledge were carried out and the project was registered in SisGen (Register #ABD9AA7).

Preparation and characterization of essential oil

The essential oil from atemoya fruits was obtained by hydrodestillation using a Clevenger-type apparatus for 2 h, from 1503.5 g of fruits. The initial time was recorded from the steam condensing in the Clevenger apparatus. Residual water of the extraction process was removed by decreasing the temperature to the freezing point of water so that, once solidified, the residue was separated from this the essential oil that remains in the liquid phase.

The characterization was carried out by gas chromatography coupled to mass spectrometry (GC-MS). The analysis was conducted on a Hewlett-Packard chromatograph 5890 series II, equipped with a Hewlett-Packard 5971 mass selective detector, split/splitless injector, using a capillary column HP-5 (25 m x 0.20 mm x 0.33 diameter). Temperatures: injector = 220 °C, detector = 280 °C, column = 60 °C, 3 °C/min, 240 °C (7 min). Carrier gas flow rate (super dry He) = 1.0 mL/min. The GC-MS electron ionization system was 70 eV. The retention indices were determined by co-injection of hydrocarbons standards. The oil components were identified by comparison with published data from the profiles 138 and Wiley Nist 98 libraries and by co-injection of a series of *n*-alkanes [36]. The sample was diluted with ethyl acetate (10 mg/mL).

Preparation and characterization of hexanic crude extract of atemoya aerial parts

The aerial parts (2.200 g) were dried at 45 ºC by seven days in stove with air circulation, and then powdered in a mechanical mill obtain 707 g of powder. The powder was extracted with hexane (3 L, three times) yielding 84.1 g of hexane extract after solvent removal under reduced pressure in rotavapor.

The compounds in the hexanic crude extract were investigated on a Shimadzu QP-2010 (Shimadzu Corporation, Kyoto, Japan) gas chromatograph coupled to a mass spectrometer (GC-MS). The following conditions were used: DB-5MS column Agilent Technologies (30 m × 0.25 mm × 0.25 µm); helium (99.999%) carrier gas at a constant flow of 1.1 mL/min; injection volume of 1.0 µL; injector split ratio of 1:10; injector temperature of 250 ºC; electron impact mode at 70 eV; ion-source temperature of 280 ºC and transfer line temperature of 260 ºC. The oven temperature was programmed from 60 ºC, with an increase of 3 ºC/min to 240 ºC. A mixture of

linear hydrocarbons (C_8H_{18}–$C_{20}H_{42}$) was injected under the same experimental conditions. The identification of the constituents in the At-Hex was performed by comparing the spectra obtained with those of the equipment database (Wiley 7 lib and Nist 08 lib) and by using the Retention Index (RI), calculated for each constituent as previously described [36]. The data were acquired and processed on a PC with Shimadzu GC-MS Solution software (Shimadzu Corporation, Kyoto, Japan).

Evaluation of cytotoxic activity of essential oil and At-Hex

The cytotoxic study was conducted by the MTT method [37], which has the capacity to analyze the viability and the metabolic state of the cell. The oil was diluted in sterile DMSO and the substances were tested in the concentration of 5 µg/mL. The tumor cell lines used, HCT-116 (colon carcinoma), HL-60 and SF-295 (glioblastoma) were provided by the National Cancer Institute (USA), and were maintained in RPMI 1640, supplemented with 10% fetal bovine serum and 1% antibiotics, which were incubated at 37 °C under 5% CO_2. The cell lines used were seeded at concentrations of 0.7 x 10^6 cels/mL (HCT-116), 0.1 x 10^6 cels/mL (OVCAR-8) e 0.1 x 10^5 cels/mL (SF-295).

The plates were incubated for 72 hours in an oven at 5% CO_2 at 37 °C. Then, they were centrifuged and the supernatant was removed. Then, 150 µl of the solution of MTT (tetrazolium salt) were added, and the plates were incubated for 3 h. The absorbance was read after dissolution of the precipitate with 150 µl of DMSO pure in a plate spectrophotometer at 595 nm. The experiments were analyzed according to the mean of the percentage of inhibition of cell growth using GraphPad Prism. The sample was tested in triplicate in two independent experiments. An intensity scale was used to assess the cytotoxic potential of the tested sample: No activity sample (NA), with low activity (LA, inhibiting cell growth varying from 1 to 50%), with moderate activity (MO, inhibition of cell growth ranging from 50 to 75%) and much activity (MA inhibition growth ranging 75 to 100%). The EHB was tested against the HCT-116, SF-295 e HL60 cells in the same conditions describe previouslly. HL60 cells were prepared at 0.3 x 10^6 cells/mL concentration.

Evaluation of antimicrobial activity of essential oil

Antimicrobial activity tests were conducted against 11 strains of bacteria and fungi: *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella choleraesuis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus hirae*, *Candida albicans*, *Candida dubliniensis*, *Candida krusei*, *Candida glabrata* e *Candida tropicalis*. The bacteria were preserved on nutrient agar and *C. albicans* yeast on sabouraud dextrose agar.

Preliminary tests of antimicrobial activity were conducted by bioauthography method [38]. A suspension of microorganisms was inoculated by *pour plate* technique on culture media, using 1:1000 (v/v) rate. A 0.5 ml aliquot of 1 mg/mL of triphenyl tetrazolium chloride (TTC) was added to the inoculated media and then the media was poured into Petri plates, on thin layer chromatography (TLC) plates where oils were applied. After homogenization, the material was incubated at 37 °C for 24 hours for the bacterial stains. For *C. albicans* yeast was directly determined the minimum inhibitory concentration (MIC) of oil.

The MIC was determined in a solid medium in a Petri plate through the addition of the revelator 2,3,5-triphenyl tetrazolium chloride. The concentration was determined in a solid medium in a Petri dish through the addition of the developer 2,3,5-triphenyl tetrazolium chloride and different oil concentrations up to 2.0 mg/mL, to the media. The oil dilution was made in sterile water containing Tween 80. As positive standards Chloramphenicol was used for bacteria, and Nystatin, for yeast. The MIC was defined as the lowest concentration of the oil able to prevent the appearance of red color, conferred on the cells by TTC when they have respiratory activity.

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Conflict of Interest

The authors declare no conflict of interest.

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Table 1 Chemical composition of the essential oil extracted from atemoya fruits (EOAF) and of hexanic crude extract from aerial parts (At-Hex).

Nº	Name	EOAF		At-Hex	
		Rt (min)	% rel.	Rt (min)	% rel.
01	4,8,12-tetradecatrienal,5,9,13-trimethyl	-	-	27.95	0.74
02	Aromadendrene	25.17	0.81	-	-
03	Bicyclogermacrene	27.51	12.58	-	-
04	Camphene	6.20	0.33	-	-
05	Caryophyllene oxide	-	-	19.34	2,00
06	Dehydro-Aromadendrene	26.00	0.58	-	-
07	*epi*-α-Cadinol	33.05	2.63	-	-
08	Farnesyl acetone	-	-	34.21	0,27
09	Germacrene D*	26.88	4.56	16.86	2.14
10	Globulol	30.91	1.87	-	-
11	Isobornyl acetate	18.84	1.15	-	-
12	Kaur-16-en-18-yl acetate	-	-	35.39	1.59
13	Kaur-16-ene	-	-	30.32	3.16
14	Kauran-16-ol	52.06	0.85	-	-
15	Kauran-18-al,17-acetoxy	-	-	37.38	4.01
16	Kaurenoic acid	-	-	35.18	0.34
17	Limonene	8.60	3.70	-	-
18	Linalool	11.27	25.70	-	-
19	Naphtalene	-	-	17.00	0.31
20	Neophytadiene	-	-	24.74	1.28
21	Octadecanal	-	-	45.56	10.05
22	Palmitic acid	-	-	27.33	0.82
23	Palmitone	-	-	47.14	0.54
24	Phytol	-	-	48.08	1.46
25	Salvial-4(14)-em-1-one	-	-	19.59	0.28
26	Spathulenol*	30.68	4.44	19.24	13.91

27	Squalene	-	-	40.40	0.34
28	Terpin-4-ol	14.32	0.47	-	-
29	*trans*-caryophylene*	24.37	1.06	15.32	2.92
30	*trans*-Ocimene	9.26	7.43	-	-
31	Viridiflorol	31.20	1.00	-	-
32	α-Bisabolene	27.83	0.97	-	-
33	α-Cadinol	33.56	2.13	-	-
34	α-Copaene	-	-	14.18	0.49
35	α-Cubebene	-	-	13.48	0.22
36	α-Gurjunene	-	-	16.27	0.29
37	α-Humulene	-	-	16.18	0.51
38	α-Muurolene	-	-	17.31	0.26
39	α-Pinene	5.82	10.38	-	-
40	α-Selinene	-	-	17.21	0.49
41	α-Terpineol	14.90	0.58	-	-
42	α-Tocopherol	-	-	44.79	2.78
43	β-Elemene*	23.28	0.73	14.58	1.82
44	β-Eudesmol	-	-	20.91	0.16
45	β-Myrcene	7.34	1.98	-	-
46	β-Pinene	6.99	9.12	-	-
47	γ-Muurolene	-	-	16.73	0,39
48	δ-Cadinene*	28.55	1.18	17.86	0,39

Rt: retention time, NI: not identified, % rel.: relative percentage. *found in both sample.

Table 2 Cytotoxic activity of the essential oil of atemoya fruit (EOAF) and hexanic crude extract from aerial parts (At-Hex).

Sample	SF-295	HCT-116	OVCAR-8	HL60
EOAF	10.39%	49.67%	2.47%	NA
APHCE	91.77%	66.25%	NA	49.72%

Percentages inhibition of growth with 95% confidence intervals obtained by non-linear regression performed in triplicate on three tumor cell lines tested at the maximum dose of 5 µg/mL. NA – Not Analyzed.

Table 3 Antimicrobial activity of the essential oil of atemoya fruit (*Annona cherimola* Mill x *Annona squamosa* L.)

Microorganisms	MIC
Escherichia coli	*
Pseudomonas aeruginosa	*
Salmonella choleraesuis	*
Staphylococcus aureus	*
Streptococcus pneumoniae	*
Enterococcus hirae	*
Candida albicans	*
Candida dubliniensis	*
Candida krusei	*
Candida glabrata	*
Candida tropicalis	*

MIC - minimum inhibitory concentration, *MIC > 2 mg/mL.

CONSIDERAÇÕES FINAIS

- Este trabalho descreve a presença dos alcaloides esculerina, reticulina, isocoridina, norisocoridina, asimilobina, nornuciferina, anonaina, liriodenina, anomuricina e dehidroanomuricina *N*-óxido em partes aéreas (mistura de folhas e talos) de atemoia, cuja identificação foi feita por meio do uso de técnica de CL-DAD-EM.
- Dehidroanomuricina *N*-óxido foi apresentado como alcaloide inédito e teve seus dados de RMN e Massas apresentados pela primeira vez na literatura.
- Propostas de fragmentação para os alcaloides benzilisoquinolínicos anomuricina e dehidroanomuricina *N*-óxido foram apresentadas.
- Os alcaloides asimilobina, lanuginosina, liriodenina, lisicamina, pronuciferina, estefarina, anomuricina e o novo alcaloide benzilisoquinolíno N-óxido (dehidroanomuricina *N*-óxido) foram isolados.
- Extratos brutos, frações alcaloídicas e subfrações da fração alcaloídica de folhas e partes aéreas (mistura de folhas e talos) de atemoia foram testados quanto a citotoxicidade *in vitro* diante de linhagens HCT 116, SF-295 e HL-60, e apresentaram alto potencial, com valores de inibição do crescimento celular variando entre 44 e 97 %.
- O subfracionamento da fração alcaloídica em subfrações hexânica, clorofórmica, acetato de etila e metanólica, mostrou que o potencial citotóxico diante das linhagens testadas foi diminuído de acordo com a ordem crescente de polaridade: FAT-Hex > FAT-CHCl3 > FAT-AcOET > FAT-MeOH.
- Anomuricina foi submetida a testes de citotoxicidade diante de nove linhagens celulares diferentes e apresentou melhor potencial citótóxico diante de células leucêmicas HL-60 com índice de seletividade de 1,66.
- Vinte e nove compostos voláteis foram identificados no extrato hexânico bruto das partes aéreas de atemoia por meio do uso da técnica de cromatografia gasosa acoplada a espectrometria de massas, estando o espatulenol presente em maior quantidade (14 %, aproximadamente).
- Nossos resultados mostraram que atemoia é uma importante fonte de alcalóides e tem alta semelhança com outras espécies do gênero *Annona*.
- Todos estes compostos estão sendo descritos pela primeira vez como resultado do metabolismo secundário da planta atemóia coletada no Vale do São Francisco.

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ANEXOS

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Short communication

Alkaloids isolated from the leaves of atemoya (*Annona cherimola* × *Annona squamosa*)

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ABSTRACT

Atemoya is an interspecific annonaceous hybrid between *Annona cherimola* Mill. and *Annona squamosa* L. Its phytochemical investigation led to seven alkaloids, including two aporphine (anonaine and asimilobine), three oxoaporphine (lanuginosine, liriodenine and lysicamine) and two proaporphine (pronuciferine and stepharine). These alkaloids were identified by a series of spectrometric methods, mainly MS and NMR (1D and 2D), as well as by comparison with literature data. Our findings showed that this species is an important source of aporphine alkaloids and have high relationship with other *Annona* species.

Introduction

Annonaceae is a large family comprising about 135 genera and more than 2500 species distributed mainly in tropical and subtropical regions (Chatrou et al., 2004). Chemically, this family is characterized by the presence of isoquinoline alkaloids, mainly aporphines.

Regarding the species of Annonaceae, those from the genus *Annona* L. comprises approximately 175 species of trees and shrubs. In Brazil the genus *Annona* contains around 60 species, with the largest part occurring in forests and few representatives in open areas (Costa et al., 2011). Moreover, economically, this genus is the most important of the family Annonaceae due to its edible fruits and medicinal properties (Dutra et al., 2012).

Atemoya is a fruitful plant of the Annonaceae family, being a hybrid resulting from a cross between the "cherimoia" (*Annona cherimola* Mill.) and the "pinha" or "fruta-do-conde" (*Annona squamosa* L.). It was achieved at the beginning of the century in the Florida (USA), the crosses being repeated in other countries in order to obtain hybrids adapted to tropical climate, as *A. squamosa*, and subtropical as *A. cherimola* (Silva and Muniz, 2011).

The introduction of atemoya in the Northeast region of Brazil is recent, with a predominance of the cultivar Gefner from Israel, originally grown in the irrigation projects of the Vale do São Francisco. A recent study carried out by our research group quantified the levels of total phenols and flavonoids as well as evaluated the antioxidant and antimicrobial activities of extracts obtained from stems and leaves of atemoya (Rabêlo et al., 2014a).

Due to expansion of atemoya cultivation and its increasing consumption in the main Brazilian markets, it is necessary to investigate the chemical composition of this plant. In the present paper, we report results of the first phytochemical study of the atemoya collected in the Vale do São Francisco, and the isolation and chemical characterization of seven alkaloids by spectrometric methods.

Materials and methods

NMR experiments were acquired in $CDCl_3$, at 303 K on a Bruker AVANCE III 600 NMR spectrometer operating at 14.1 Tesla, observing ^1H and ^{13}C at 600 and 150 MHz, respectively. The spectrometer

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