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**ESTUDO DE ESPÉCIES DE SENNA DA CAATINGA E DO CERRADO:  
DOCUMENTAÇÃO QUÍMICA E FARMACOLÓGICA NA BUSCA DE NOVOS  
FÁRMACOS**

**FORTALEZA  
2021**

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Tese de Doutorado submetida à avaliação da banca examinadora do Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia da Universidade Federal do Ceará como parte dos requisitos para obtenção do título de Doutora em Biotecnologia. Área de concentração: Biotecnologia de Produtos Naturais.

Orientadora: Profa. Dra. Maria Goretti de Vasconcelos Silva.

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## RESUMO

A busca por compostos naturais com propriedades farmacológicas tem sido um desafio para ciência, notadamente devido à complexidade das matrizes vegetais. Espécies do gênero *Senna* apresentam em sua composição uma ampla variedade de compostos, incluindo metabólitos das classes dos alcaloides, flavonoides e antraquinonas, que se destacam por seu potencial farmacológico, econômico e biotecnológico. Este trabalho objetivou realizar um estudo químico e farmacológico de sete espécies do gênero *Senna*, nativas da Caatinga e do Cerrado. Nesse sentido foi desenvolvido um método analítico para a análise comparativa, sistemática e abrangente, do perfil químico de folhas, flores e frutos das espécies selecionadas. O método desenvolvido foi validado, e sua abrangência foi avaliada através da aplicação das técnicas analíticas HPLC-DAD e UPLC-QTOF-MS, sendo possível a identificação de 46 compostos de várias classes de metabólitos secundários, nas formas livres e glicosiladas. Os resultados obtidos indicam que o método desenvolvido também é aplicável ao estudo de outras matrizes vegetais complexas. Alternativamente, foi empregada a técnica de Extração Online – OLE acoplado a UPLC-MS, para gerar o perfil químico das espécies de *Senna* de forma rápida, eficiente e verde. Observou-se que o perfil cromatográfico obtido pela técnica OLE, é muito semelhante ao obtido por meio da técnica de extração e análise convencional, porém com importantes ganhos em eficiência de extração, tempo e quantidade de amostras e solventes envolvidos. A técnica OLE foi eficiente na obtenção do perfil químico completo das amostras de *Senna* spp. A documentação farmacológica das espécies foi realizada através da avaliação das atividades antioxidante, citotóxica e anticolinesterásica de extratos das flores de espécies de *Senna macranthera* e *Senna spectabilis* var. *excelsa*. Na avaliação da atividade citotóxica e anticolinesterásica as espécies estudadas apresentaram potencial significativo. Para o extrato das flores de *Senna trachypus* além das atividades antioxidante e citotóxica, foi avaliado ainda a toxicidade e atividade locomotora em peixe-zebra adulto. Os resultados demonstraram que o extrato tem significativo potencial biológico e não é tóxico, ausência de toxicidade aponta para um possível uso fitoterápico.

**Palavras-chave:** *Senna*; UPLC-QTOF-MS; OLE; citotoxicidade; acetilcolinesterase; zebrafish.

## ABSTRACT

The search for natural compounds with pharmacological properties has been a challenge for science, mainly due to the complexity of plant matrices. Species of the *Senna* genus have in their composition a wide variety of compounds, including metabolites from the classes of alkaloids, flavonoids and anthraquinones, which stand out for their pharmacological, economic and biotechnological potential. This work aimed to carry out a chemical and pharmacological study of seven species of the genus *Senna*, native to Caatinga and Cerrado. In this sense, an analytical method was developed for the comparative, systematic and comprehensive analysis of the chemical profile of leaves, flowers and fruits of the selected species. The developed method was validated, and its scope was evaluated through the application of analytical techniques HPLC-DAD and UPLC-QTOF-MS, making it possible to identify 46 compounds from various classes of secondary metabolites, in free and glycosylated forms. The results obtained indicate that the developed method is also applicable to the study of other complex plant matrices. Alternatively, the technique of Online Extraction - OLE coupled to UPLC-MS was used to generate the chemical profile of *Senna* species in a fast, efficient and green way. It was observed that the chromatographic profile obtained by the OLE technique is very similar to that obtained through the conventional extraction and analysis technique, but with important gains in extraction efficiency, time and amount of samples and solvents involved. The OLE technique was efficient in obtaining the complete chemical profile of the *Senna* spp. The pharmacological documentation of the species was performed by evaluating the antioxidant, cytotoxic and anticholinesterase activities of extracts from flowers of *Senna macranthera* and *Senna spectabilis* var. excellent. In the evaluation of cytotoxic and anticholinesterase activity, the studied species showed significant potential. For the extract of the flowers of *Senna trachypus*, in addition to the antioxidant and cytotoxic activities, the toxicity and locomotor activity in adult zebrafish was also evaluated. The results showed that the extract has significant biological potential and is non-toxic, absence of toxicity points to a possible herbal use.

**Keywords:** *Senna*; UPLC-QTOF-MS; OLE-LC; cytotoxicity; acetylcholinesterase; zebrafish.

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## LISTA DE ABREVIATURAS E SIGLAS

ACN	Acetonitrila
BHT	<i>Butylated hydroxytoluene</i>
CCD	Cromatografia em camada delgada
CLAE/HPLC	Cromatografia líquida de alta eficiência ( <i>High performance liquid chromatography</i> )
DAD	Detector de arranjo de diodos ( <i>Diode array detector</i> )
DMSO	Dimetilsulfóxido
HRMS	Espectrometria de massas de alta resolução ( <i>High resolution mass spectometry</i> )
IC <sub>50</sub>	Concentração efetiva que mata 50% das cepas
<i>m/z</i>	Massa/carga
MeOH	Metanol
MS/MS	Espectrometria de massas sequencial
OLE	Extração em linha ( <i>On-line Extraction</i> )
QTOF	Analisador híbrido quadrupolo-tempo de voo
UHPLC	Ultra high performance liquid chromatography
UV/Vis	Ultravioleta/Visível
var.	Variedade

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## 1 INTRODUÇÃO

A química de produtos naturais é uma das subáreas mais antigas e mais importantes da química orgânica. Visa à investigação da composição química e o isolamento de compostos bioativos procedentes de organismos como plantas, bactérias, fungos e organismos marinhos entre outras. Os produtos naturais são fontes inestimáveis para o desenvolvimento de fármacos, e nas ultimas décadas mais de 50% de todos os medicamentos aprovados contém como ativos pequenas moléculas derivados de produtos naturais ou de estruturas semelhantes (GUO *et al.*, 2018).

O Brasil apresenta posição privilegiada na ciência de produtos naturais visto que possui uma mega-biodiversidade com biomas riquíssimos e ainda pouco explorados (BERLINCK *et al.*, 2017). As plantas, em especial, produzem uma grande variedade de compostos com potencial bioativo denominados de metabólitos secundários, que são necessários a sua sobrevivência, mas que podem apresentar atividades terapêuticas importantes (WOLFENDER *et al.*, 2019).

A família Fabaceae/Leguminosae abrange cerca de 730 gêneros e mais de 19.000 espécies. O gênero *Senna* se destaca nessa família por possuir quase 350 espécies amplamente distribuídas em zonas tropicais e subtropicais (KHALAF *et al.*, 2018). Das espécies que ocorrem no Brasil, 33 são endêmicas. Apresentam-se como árvores, arbustos ou subarbustos e se destacam por suas belas flores amarelas. Este gênero é conhecido por ser rico em diferentes metabólitos secundários, especialmente antraquinonas (AZEVEDO; CONCEIÇÃO, 2017).

Na medicina popular as plantas deste gênero são utilizadas na forma de extratos brutos ou infusões para tratar infecções fúngicas, dores de cabeça, feridas, picadas de cobra, febre, queimaduras, hemorroidas, insônia, ansiedade, gripe e dor de estômago (ALONSO-CASTRO *et al.*, 2019; NKAMGUIE NKANTCHOUA *et al.*, 2018; ANDRADE *et al.*, 2015). Porém são conhecidas principalmente devido ao efeito purgativo que várias espécies apresentam, notadamente *Senna alexandrina* e *Senna fistula*, que compõem fitoterápicos disponíveis comercialmente como por exemplo Tamarine®. Esse efeito foi justificado pela presença de glicosídeos antraquinônicos denominados sennosídeo A e B, que hoje são os fármacos mais ativos com esta propriedade e que são relacionadas ao gênero (BRADLEY MORRIS; TONNIS; WANG, 2019).

O potencial farmacológico de espécies do gênero *Senna* também foi relatado para diferentes tipos de enfermidades, incluindo doenças dermatológicas, hiperglicemia, e como cicatrizante (AHMED; SHOHAEL, 2019), como também efeitos antineoplásicos, anti-inflamatórios, antiartríticos, antioxidante, antifúngico, antibacterianos, antivirais, antiplaquetários e neuroprotetores (VILANOVA-SANCHEZ *et al.*, 2018). A efetividade dessas espécies nas mais variadas atividades farmacológicas pode ser justificada pela presença de diversas classes de metabólitos secundários como alcaloides, compostos fenólicos, flavonoides, triterpenos, antraquinonas e lipídios (NKAMGUIE NKANTCHOUA *et al.*, 2018).

No entanto a maioria dos registros disponíveis sobre a composição química e atividades biológicas das espécies de *Senna* spp. são limitados as folhas, sendo o estudo das outras partes como flores e frutos, desconhecidos ou preliminares. Para avaliar diferenças reais entre os respectivos materiais vegetais, é necessária a aplicação de técnicas analíticas e métodos confiáveis para um estudo comparativo direto com informações abrangentes. Para isso o uso de instrumentos analíticos avançados e análises multivariadas vêm se mostrando como técnicas eficientes que proporcionam grande número de informações sobre as amostras de plantas (FARAG *et al.*, 2019). A aplicação de técnicas espectroscópicas (UV, RMN e MS) e o desenvolvimento métodos em sistemas cromatográficos como cromatografia líquida de alta eficiência (HPLC), são as formas mais comuns de se produzir *fingerprintings* químicos representativos (DHANANI *et al.*, 2017).

Mesmo diante de ferramentas analíticas avançadas a extração dos metabólitos secundários ainda é o passo mais importante na purificação de compostos bioativos de fontes de produtos naturais (GUO *et al.*, 2018). Podendo destacar ainda a crescente necessidade por métodos de extração verde que se baseiam em processos que reduzem ou eliminem o consumo de água, energia, solventes de petróleo e tempo de trabalho. Estes desafios tendem a tornar centros acadêmicos e indústrias mais econômicas, inovadoras e, principalmente, com maior responsabilidade ambiental (CHEMAT *et al.*, 2019).

Esse trabalho se dispôs a realizar o estudo químico e farmacológico de sete espécies do gênero *Senna* da Caatinga e do Cerrado, utilizando ferramentas analíticas avançadas e respeitando os princípios da química verde, sempre que possível.

A elaboração dessa tese obedece aos padrões estabelecidos pela coordenação do curso de Pós-Graduação em Biotecnologia da Universidade Federal do Ceará, bem como as regras e normas básicas da ABNT.

## 2 CONSIDERAÇÕES BOTÂNICAS

### 2.1 A Família Leguminosae e o Gênero *Senna*

A família Leguminosae é considerada a terceira maior família de angiospermas, possui uma ampla distribuição geográfica com cerca de 750 gêneros e mais de 19.000 espécies (KHALAF *et al.*, 2018). Espalhadas em todo o mundo, especialmente nas regiões tropicais e subtropicais, têm estruturas variadas indo de ervas perenes até árvores de alto porte. No Brasil podem ser encontradas 2.100 espécies nativas pertencentes a 188 gêneros distribuídas na maioria das formações vegetacionais (LIMA *et al.*, 2007). Também são de grande importância econômica pela produção de alimentos como: soja (*Glycine max*); ervilha (*Pisum sativum*); feijão (*Phaseolus vulgaris*) e alfafa (*Medicago sativa*). Além disso, as leguminosas são conhecidas por proverem minerais essenciais e metabólitos secundários que poderiam ser efetivos contra variadas doenças humanas como o câncer (MADESIS *et al.*, 2012).

O gênero *Senna* Mill. pertence à tribo Cassieae Bronn, subtribo Cassinae Irwin & Barneby (Irwin & Barneby 1981), juntamente com os gêneros *Chamaecrista* e *Cassia*, dos quais se diferencia pelas flores sem bractéolas no pedicelo, folhas usualmente com nectários interfoliolares e frutos indeiscentes ou tardivamente deiscentes. Tratamento taxonômico de Irwin & Barneby (1981), separaram estes três gêneros (RODRIGUES *et al.*, 2005; SILVA; SANTOS; SOUZA, 2018), embora ainda se encontrem atribuições botânicas confusas. O gênero *Senna* tem distribuição em todo o Brasil e em outros países da América do Sul. No Brasil é representado por 80 espécies sendo 33 endêmicas, sendo que mais de 40 destas espécies foram coletadas e identificadas por pesquisadores da Universidade Federal do Ceará e suas exsicatas estão depositadas no Herbário Prisco Bezerra-UFC. Na tabela 01 estão listadas as localidades onde foram coletadas as espécies cujos estudos químicos estão descritos nesta tese.

Estas espécies podem se apresentar como pequenos arbustos, como *Senna occidentalis* (mata-pasto) ou árvores com 4-9 metros de altura, como *Senna fistula* (chuva de ouro). Suas folhas podem ser pequenas com 10 a 20 pares de folhetos ou grandes com 2 pares de folhetos.

Suas flores amarelas são vistosas, o que explica seu uso como plantas ornamentais, e seus frutos do tipo vagem são encontrados das mais variadas formas: alongados, chatos, grossos e com sementes marrons (SELEGATO et al., 2016; SOBEH *et al.*, 2017). A diferença entre as espécies deste gênero também se dá em seus perfis químicos, sendo algumas já conhecidas por apresentarem classes compostos específicos como é o caso da *Senna spectabilis* que é rica em alcaloides, enquanto outras espécies como a *Senna macranthera* é mais ricas em flavonoides e ainda outra como *Senna martiana*, rica em antraquinonas. Isso mostra que não se tem uma relação de constituição química estabelecida entre as espécies deste gênero. Todas as plantas foram registradas no Sistema Nacional de Gerenciamento de Patrimônio Genético (SISGEN; #AB06D11).

Tabela 01: Locais de coletas das espécies estudadas neste trabalho

<b>Espécie</b>	<b>Cidade</b>	<b>Coordenadas</b>	
<i>S. cearensis</i>	Crateús	05.14714 (S)	40.54686 (W)
<i>S. macranthera</i>	Quixeramobim	05.22175 (S)	39.57692 (W)
<i>S. obtusifolia</i>	Crateús	05.13109 (S)	40.52286 (W)
<i>S. reticulata</i>	Caucaia	02.58347 (S)	39.53439 (W)
<i>S. spectabilis</i>	Quixeramobim	05.22175 (S)	39.57692 (W)
<i>S. splendida</i>	Crateús	05.13974 (S)	40.53621 (W)
<i>S. trachypus</i>	Senador Sá	03.13760 (S)	40.55911 (W)

S= sul; W= oeste

## 2.1 *Senna cearensis* Afr.Fern

*Senna cearensis* é comumente encontrada no estado do Ceará e norte de Pernambuco, especialmente nas Chapadas do Araripe e do Planalto da Ibiapaba. Foi classificada botanicamente somente em 2000 e é conhecida popularmente como “besouro”. Trata-se de um arbusto de 1-2 m de altura, com ramos jovens vilosos, suas folhas têm de 3-4 pares de folhíolos e prefere solos arenosos (QUEIROZ, 2009).

## 2.2 *Senna macranthera* (DC. ex Collad.) H.S. Irwin & Barneby

*Senna macranthera* (DC. ex Collad.) H.S. Irwin & Barneby é conhecida popularmente como pau-fava e fedegoso é uma espécie arbórea de ambiente tropical nativa do Brasil, mas também nasce na Colômbia, Equador, Peru e Venezuela (ANDRADE *et al.*, 2015). Usada como árvore ornamental tem de 6-8 m de altura, tronco de até 30 cm de diâmetro e pode ser encontrada de norte a sul do Brasil (GUARIZE *et al.*, 2012). As folhas são compostas por dois pares de folhetos opostos, durante a floração exibem simultaneamente um grande número de inflorescências juntamente com frutos em diferentes estágios de desenvolvimento (AQUILA; BRAGA; DIETRICH, 2012).

### **2.3 *Senna obtusifolia* (L.) H.S. Irwin & Barneby**

*Senna obtusifolia* (L.) H.S. Irwin & Barneby comumente conhecida como vagem-foice, mata-pasto-liso ou simplesmente mata-pasto, é uma erva daninha problemática devido ao seu alto caráter infestante. No Brasil causa impacto na agricultura principalmente na produção de soja, além de relatos de intoxicação em bovinos no estado de Mato Grosso do Sul (CARVALHO *et al.*, 2014). Pode ser encontrada desde o México até a Argentina e também na África e Ásia tropicais. Ocorre em quase todos os estados do Brasil. Presente na Caatinga, Cerrado e Pantanal, prefere áreas antropizadas, margens de estradas, em solos arenoso-argilosos. É um subarbusto com cerca de 70 cm de altura, de flores amarelas e folhas compostas de formato oval (MATOS *et al.*, 2019; PANG *et al.*, 2019).

### **2.4 *Senna reticulata* (Willd.) H.S. Irwin & Barneby**

*Senna reticulata* conhecida popularmente como mangerioba ou maria mole é uma espécie com distribuição geográfica em quase toda América do Sul, Central e parte da América do Norte - Brasil, Bolívia, Peru, Equador, Colômbia, Venezuela, Guianas, Panamá e México. Apresenta-se como arbusto ou como árvore com uma coroa arredondada, pode chegar a alcançar de 2 a 15 metros de altura. O tronco é geralmente curto de 3 a 10 cm de diâmetro e geralmente é sustentado por raízes pequenas. É considerada uma planta pioneira das áreas abertas das planícies da Amazônia, cresce muito rápido e adapta-se facilmente em planícies inundadas. Prefere locais de baixas elevações, úmidos ou muito úmidos. É frequente seu aparecimento em margens de rios, córregos e áreas pantanosas (LIMA *et al.*, 2015).

### **2.5 *Senna spectabilis* var. *excelsa* (Schrad.) H.S.Irwin & Barneby**

*Senna spectabilis* var. *excelsa* (Schrad.) H.S.Irwin & Barneby é conhecida popularmente como canafístula, cássia ou cássia do nordeste. São encontradas em áreas com

climas tropicais e subtropicais. Ocorrem em países da África, Ásia, América do Sul e Latina. Possui pequeno porte com até 6 m altura. Espécimes de *Senna espectralis* são empregados no Brasil, principalmente em paisagismo, devido à grande beleza de suas flores amarelas, sendo uma espécie com grande potencial para recomposição de áreas degradadas (NKAMGUIE NKANTCHOUA *et al.*, 2018; SILVA; OLIVEIRA; BRAZ-FILHO, 2010).

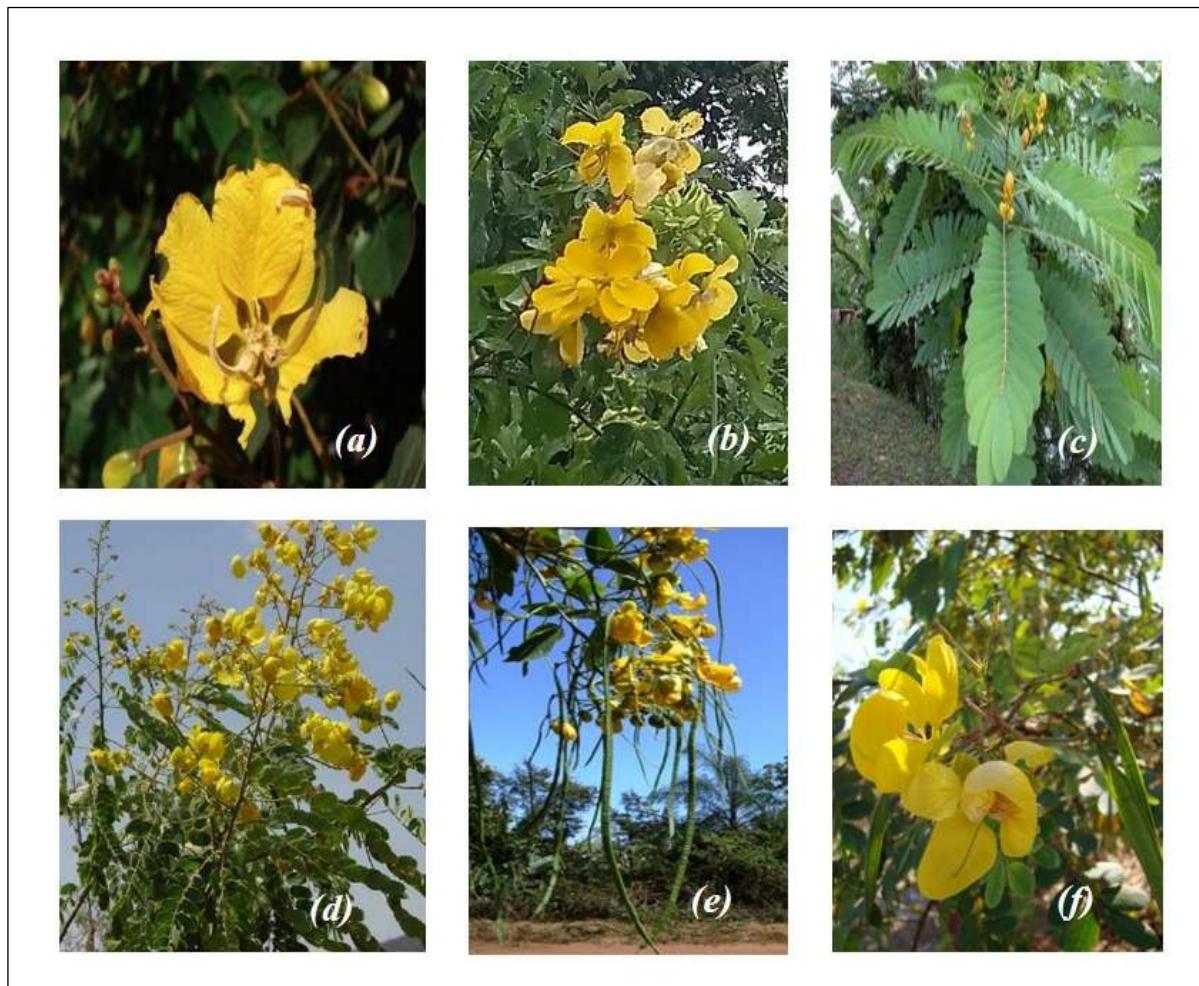
## **2.5 *Senna splendida* (Vogel) H.S. Irwin & Barneby**

*Senna splendida* (Vogel) H.S. Irwin & Barneby é conhecida popularmente como amendoim-bravo, ocorre em países como Paraguai, Uruguai e Brasil. Neste último ocorre nas regiões Nordeste, Centro-Oeste e no Sul, nos domínios Caatinga, Cerrado, floresta ciliar, floresta ombrófila e restinga. Pode ser encontrados em locais preservados com solo argilo-arenosos ou sobre afloramentos rochosos em altitudes que variam 500 a 800 m. Trata-se de um arbusto 2–4 m altura com ramos verdes, cilíndricos e com 4 folíolos, tem frutificação e floração por volta do mês de setembro (MATOS *et al.*, 2019; SILVA, 2017).

## **2.6 *Senna trachypus* (Mart. ex Benth.) H.S. Irwin & Barneby**

*Senna trachypus* (Mart. ex Benth.) H.S. Irwin & Barneby também conhecida como quebra machado e canafistula única é uma espécie encontrada em ambientes com solo raso e pedregoso. Ocorre no bioma do cerrado e da caatinga percorrendo os estados do Maranhão, Piauí e noroeste da Bahia e no Ceará. Trata-se de um arbusto com cerca de 2,5 m de altura com ramos densamente cobertos por estruturas secretoras. Suas folhas têm menos de 14 pares de folíolos e inflorescência vistosa e fruto plano (CÓRDULA, 2008; CÓRDULA; MORIM; ALVES, 2014). O registro fotográfico das espécies estudadas em seu habitat natural encontra-se na Figura 02.

Figura 02. Registro fotográfico de *Senna cearensis* (a) *Senna macranthera* (b) *Senna reticulata* (c) *Senna spectabilis* var. *excelsa* (d) *Senna splendida* (e) *Senna trachypus* (f).



Fonte: MGVSilva.

### **3 EVOLUÇÃO NAS TÉCNICAS DE EXTRAÇÃO E IDENTIFICAÇÃO DE METABÓLITOS SECUNDÁRIOS: UMA REVISÃO.**

#### **3.1 Extração de metabólitos secundários**

A preparação das amostras vegetais para a análise cromatográfica é sem dúvida um processo muito complexo. Tem-se como objetivo atenuar a complexidade da amostra e eliminar as interferências da matriz antes de serem inseridas nos equipamentos de separação e detecção, para que não causem defeitos ou diminuição da vida útil dos componentes da análise. Entretanto, mesmo com cautela a preparação da amostra é a etapa com maior chance de causar problemas, como o alto consumo de tempo, alto custo, baixos rendimentos de extração e fonte de erros analíticos. Além disso, os métodos convencionais não são consistentes com os objetivos da química verde, pois consomem grande quantidade de solvente orgânico e produzem resíduos que são descartados na natureza, causando grande impacto ambiental (GIACOMETTI *et al.*, 2018).

Os métodos convencionais de purificação de amostras geralmente incluem as seguintes etapas: (1) secagem e extração por solvente; (2) redução do solvente por aquecimento prolongado; (3) dissolução do extrato em solvente apropriado; (4) filtração para remover impurezas; (5) métodos de análises para isolamento e/ou identificação (GUO *et al.*, 2018). Os processos de extração básicos são normalmente utilizados por usuários de plantas medicinais, eles incluem processos como maceração, decocção, infusão, ebulação sob-refluxo e percolação. O processo de maceração é o mais simples, envolve apenas a imersão do material em um solvente adequado para a extração. A percolação é um processo dinâmico que envolve a substituição constante dos solventes extractores saturados por solventes frescos. Já a extração por refluxo é a que requer menos solvente tornando o processo mais eficaz, porém não é aplicável a compostos sensíveis a altas temperaturas. Já as técnicas mais atuais de extração incluem extração por solvente orgânico auxiliada por ultrassom (EAU), entre outras (PATRA *et al.*, 2018)

O processo de extração consiste na transferência do componente alvo da matriz sólida da planta para a fase líquida do extrator, a eficiência do processo depende da natureza da matriz da amostra, do composto a ser extraído e da localização do composto dentro da matriz (MUSTAFA; TURNER, 2011). O método de extração por solvente orgânico envolve o uso de um solvente caracterizado por um conjunto de propriedades que proporcionam a difusão do analito no extrato. É comum o uso, por exemplo, de solventes como etanol e água para a

extração de ácidos fenólicos e flavonoides, enquanto o hexano e o cloreto de metileno são usados para extrair compostos fracamente polares. O rendimento da extração pode ser maximizado por meio do ajuste da temperatura e/ou pressão, os métodos de extração subcrítica e supercrítica são baseados nesses princípios. Para acelerar o processo, impactos ultrassônicos são aplicados adicionalmente (MILEVSKAYA; PRASAD; TEMERDASHEV, 2019)

A extração assistida por ultrassom (EAU) tem sido destaque nas metodologias de extração, por intensificar o processo e elevar as taxas de rendimento em menores tempos (PANDEY *et al.*, 2018). O sistema ultrassônico gera o processo de cavitação (crescimento e colapso de bolhas), capaz de produzir efeitos nas matrizes vegetais, tais como: a circulação do líquido no sistema e a formação de turbulências que podem auxiliar no aumento da transferência de massa. Ou seja, a cavitação facilita a penetração do solvente nas paredes celulares da matriz, permitindo que o conteúdo intracelular seja liberado mais eficazmente. Por esses motivos a EAU apresenta um baixo consumo de solventes e pode evitar danos térmicos a compostos bioativos causados por altas temperaturas, já que opera em temperaturas inferiores, destacando-se ainda por ser uma técnica simples fácil e barata com um escopo amplo de aplicação nas indústrias química (CHAKRABORTY; UPPALURI; DAS, 2020).

A etapa de filtração ou limpeza dos extratos também é de suma importância nos processos de preparação de amostras, visto que esta é a responsável por remover interferentes ou composto indesejável que podem atrapalhar a análise, causando aumento no tempo de análise, contaminação e até entupimento de colunas cromatográfica ate a inutilização do equipamento. Dentre as técnicas utilizadas para a limpeza do extrato, também chamada de *clean up*, está à extração em fase sólida (EFS). Esta técnica consiste na retirada de analitos de uma amostra por meio da passagem desta por adsorvente (pode ser um polímero de Si-C18), que geralmente está empacotado em cartuchos. Um solvente orgânico escolhido de acordo com a polaridade da fase estacionária e da amostra é usado para passar o extrato contendo os compostos pela fase estacionária (CARDOSO *et al.*, 2011). Apesar de muita utilizada, a etapa de *clean up* possui algumas desvantagens, como por exemplo, a incerteza sobre a intensidade da modificação que procedimento provoca na amostra.

### **3.2 Desreplicação de Metabólitos Secundários**

Extratos vegetais costumam ser extremamente complexos contendo centenas a milhares de metabólitos secundários. Assim, a abordagem clássica geralmente requer muitas

etapas cansativas e complexas de purificação, isolamento e elucidação estrutural. Para agilizar estes procedimentos, é de crucial importância o uso de estratégias modernas como a desreplicação, que objetiva acelerar o processo de identificação de compostos já conhecidos presentes em extratos brutos ou frações parcialmente purificadas, evitando o re-isolamento ou elucidação estrutural de substâncias não inéditas, minimizando tempo, esforço e custo (SILVA *et al.*, 2019).

O termo “desreplicação” (do inglês, dereplication) foi introduzido na área acadêmica a partir dos anos 90 como uma importante estratégia no processo de triagem de extratos brutos. Essa metodologia faz uso de instrumentação analítica hifena, principalmente de LC-DAD-MS e GC-MS, combinada com rotinas computacionais complexas associadas a banco de dados globais. Essa hifenação instrumental e o uso de bancos de dados têm obtido grande sucesso no entendimento dos sistemas fitoquímicos. Esses fatos causaram um aumento considerável no número de bancos de dados de metabólitos secundários, com dados gratuitos e confiáveis, compreendendo variadas informações espectrais e químicas dos compostos (SILVA *et al.*, 2019; GALLON; JAIYESIMI; GOBBO-NETO, 2018). As técnicas de desreplicação estão continuamente evoluindo quando se fala de produtos naturais, isso se dá, pela sua alta versatilidade, rapidez e reproduzibilidade na análise de extratos brutos. Esta técnica ajuda na escolha de substância a serem isoladas e quais extratos são mais interessantes em relação ao ineditismo de seus compostos. Assim, a perspectiva é que nos próximos anos a desreplicação torne-se cada vez mais aprimorada, favorecendo o desenvolvimento das pesquisas na área de produtos naturais (SOMENSI, 2012).

### **3.3 Técnicas cromatográficas**

#### **3.3.1 Cromatografia Líquida de Alta Eficiência - HPLC**

A cromatografia líquida de alta eficiência (CLAE; sigla em inglês: HPLC) é uma das técnicas mais populares, modernas e versáteis usadas atualmente na separação, identificação e quantificação de compostos em amostras complexas, como por exemplo, a obtenção de perfis químicos de extratos vegetais (NAHAR; ONDER; SARKER, 2019). Em comparação com a técnica de cromatografia gasosa (GC) a cromatografia líquida se destaca por sua versatilidade e eficiência sendo possível analisar compostos com uma extensa gama de massas moleculares, sem limitação de volatilidade ou estabilidade térmica e sem a necessidade de modificação química prévia da amostra. A técnica também é utilizada para a análise de macromoléculas e

espécies iônicas, podendo ser usadas nas áreas farmacêutica, clínica, alimentícia, ambiental, etc. (RIGANO *et al.*, 2019).

O detector mais comumente utilizado na técnica de separação por cromatografia líquida de alta eficiência para a determinação de compostos em extratos de produtos naturais é o UV/vis, esse fato é justificado, devido ao detector ser mais barato e mais facilmente disponível. O detector UV/visível mede a absorbância da luz monocromática em comparação com o feixe de referência. Existem três tipos de detetores UV/Vis: 1. comprimento de onda fixo; 2. Comprimento de onda variável, mas que monitora um comprimento de onda por vez; 3. UV/Vis com arranjo de diodos (DAD ou PDA), que monitora todos os comprimentos de onda ao mesmo tempo (MANSOURI *et al.*, 2020).

A separação eficiente dos analíticos de acordo com a sua polaridade também é outro destaque da técnica de cromatografia líquida. Normalmente usa-se uma coluna compacta com 2,0 a 4,6 mm de diâmetro e 20 a 250 mm de comprimento empacotada com uma fase estacionaria, por exemplo, fase reversa de sílica C18 com tamanho de partículas de 2-5 µm. A sílica é sem dúvida a fase estacionária mais utilizada nesta técnica, devido a sua versatilidade e características físicas. Possui ampla gama de seletividade, porém uma desvantagem que vale a pena salientar é a forte interação que é estabelecida entre compostos básicos e os grupos silanóis residuais livres na superfície da fase estacionária. Nas ultimas décadas houve um aumento significativo no estudo e no desenvolvimento de fases estacionárias a fim de melhorar eficiência, permeabilidade e estabilidade da coluna (PINO; AFONSO, 2012). Combinado com o tipo de fase móvel, o tamanho da partícula também é de grande importância na eficiência da separação, a redução do tamanho da partícula foi crucial para o sucesso dessa técnica, pois a utilização de partículas pequenas permite a aplicação de altas taxas de fluxo, resultando em uma aceleração significativa da separação melhorando significativamente os limites de detecção e resolução (SYKORA; VOZKA; TESAROVA, 2016).

### **3.3.2 Cromatografia Líquida de Alta Eficiência (HPLC) - Espectroscopia de massa- (MS)**

Apesar da excelência já confirmada da técnica de separação por cromatografia líquida de alta eficiência, ainda é necessária uma técnica confirmatória para análises qualitativas e quantitativas. A espectrometria de massas (MS) é uma técnica poderosa que fornece informações estruturais, sendo que o acoplamento entre estas duas técnicas dá origem a uma ferramenta analítica versátil e de grande potencial. O resultado da hifenização entre

espectrômetro de massas MS e cromatografia líquida de alta eficiência é comumente conhecida como HPLC-MS. A combinação dos dois métodos analíticos reduz o erro experimental e melhora a precisão na identificação dos compostos. Essa técnica é muito útil em aplicações que envolvem um grande número de compostos em matrizes complexas, os componentes da mistura são separados de acordo com suas propriedades físicas e químicas, sendo possível identificar os compostos de acordo com a comparação de dados como o tempo de retenção, espectros no UV/Vis e espectros de massas. A amostra pode ser identificada também com o auxílio da base de dados de espectros de massas presentes no *software* do próprio equipamento (MICHEL *et al.*, 2015).

O vazão do solvente na técnica hifena deve ser menor que o usado em análises de LC isoladas, isso é necessário para garantir uma ionização completa e manter a sensibilidade de detecção do MS, que começa a diminuir a partir de 200 µL/min. Por isso as colunas utilizadas são muito menores para melhor acomodar as menores taxas de vazão do solvente e da amostra. Os tipos de analisadores mais comuns são quadrupolo, íon trap e tempo de voo (TOF), estes oferecem vários graus de precisão em massa e possibilidades MS/MS. A fonte de ionização mais comumente empregada é a electrospray (ESI), ela é preferível para a análise de compostos muito polares, iônicos, termolábeis, ou com massa molecular elevada (superior a 1.000 Da). É uma técnica de ionização bastante robusta, sendo a mais utilizada nos trabalhos atuais. Pode ser aplicada para análise de proteínas, aminoácidos, metabólitos secundários e várias substâncias de interesse em bioanalítica, alimentos e área farmacêutica (LANÇAS, 2009).

Aplicando a técnica de LC-MS Fan *et al.* (2020), analisou a composição fenólica de extratos de *Malus toringoides* (Rehd.) Hughes e de acordo com as características estruturais, foram identificados 14 compostos que incluíram 5 flavonas, 3 flavanóis, 3 di-hidrochalcones, 1 isoflavona, 1 ácido fenólico e 3 outros compostos. Já Moreno-Rojas et al 2018, caracterizou os metabólitos presentes no extrato da cebola preta, determinando com sucesso 53 compostos entre flavonoides, aminoácidos e compostos organossulfurados.

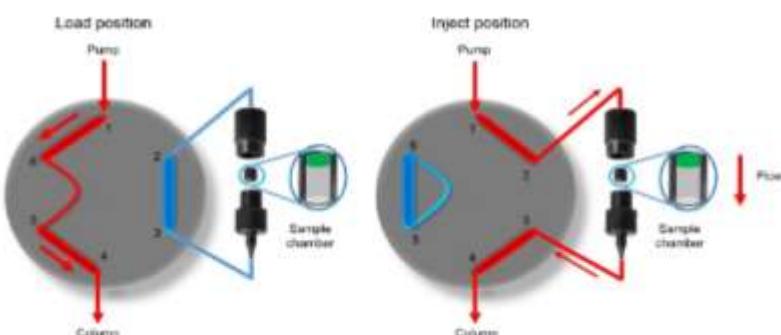
### **3.3.2 Extração on-line - Cromatografia Líquida de Alta Eficiência - Espectroscopia de massas- (OLE-HPLC-MS)**

O estudo do perfil químico de produtos naturais sempre compreendeu etapas de pré-tratamento da amostra, seguidas das etapas de análise baseadas em cromatografia líquida de alta eficiência. Como vimos anteriormente as etapas de análise em HPLC são sempre

concluídas em alguns minutos enquanto, as etapas de pré-tratamento da amostra, incluindo extração e eliminação de solvente, levam horas ou até dias. Dessa forma, a principal etapa limitante no estudo do perfil químico de produtos naturais são as etapas de pré-tratamento da amostra. Atualmente, esforços conjuntos vêm sendo feitos para desenvolver métodos de pré-tratamento de amostras com eficiência, baixo consumo de solvente e menor tempo (TONG *et al.*, 2018b).

A técnica de Extração Online - OLE, recentemente desenvolvida propõe uma nova configuração analítica otimizada e simples, na qual a extração de uma amostra sólida é acoplada diretamente a análise por cromatografia líquida de alta eficiência. Especificamente, a técnica substitui o loop de amostra de uma válvula de injeção do cromatógrafo líquido (LC) convencional por câmara de amostra, que é preenchida com a amostra sólida. Assim a fase móvel flui através da câmara de amostra e realiza a extração dos analitos, assistida pela alta pressão desenvolvida no sistema de LC. Os analitos extraídos são então transferidos diretamente para a coluna LC para separação e análise. Além disso, a extração também é feita no modo gradiente, de forma que analitos de todas as polaridades podem ser sequencialmente extraídos e transferidos para a coluna cromatográfica, tornando o procedimento totalmente abrangente. O acoplamento direto da extração online à análise é realizado sem a necessidade de aparelhos adicionais para integrar a extração com ao sistema de detecção, somado a isso o fato da eliminação de todas as etapas de pré-tratamento da amostra, esta técnica se destaca pela simplificação do processo de preparação e análise de amostras sólidas (FERREIRA *et al.*, 2016). A Figura 03 mostra a configuração da válvula de seis portas e duas posições usada para a extração on-line (“load” and “inject” positions) e a Figura 04 apresenta um exemplo de coluna de proteção (pré-coluna) utilizada em análises.

Figura 03. Representação da configuração da válvula de seis portas e duas posições usada para a extração on-line (“load” and “inject” positions).



Fonte: (FERREIRA *et al.*, 2016)

Figura 04. Imagem da câmara de amostra.



Fonte: Próprio autor

Comparando-se as etapas de pré-tratamento das amostras nos métodos off-line (convencional) e on-line, podemos observar que teremos uma série de vantagens como: (a) métodos eficientes, os procedimentos de pré-tratamento de amostra foram eliminados; (b) processo verde, sem a utilização de solventes extras, exceto o da fase móvel; (c) economia de energia, pois, evita processos de extração seguidos e concentração do extrato. Todos esses fatores tornam a análise mais rápida, diminui a possibilidade de degradação da amostra e os erros aleatórios, além de excluir a perda de compostos termicamente sensíveis (TONG *et al.*, 2018a; VOTANI; CHISVERT; GIOKAS, 2020).

Essa nova abordagem já foi efetivamente realizada para purificação de compostos fenólicos de *Manganolia officinalis* e alcaloides em *Piper nigrum* (GUO *et al.*, 2018), para a caracterização de polifenóis em *Citrus paradisi* (TONG *et al.*, 2018a), para identificação flavonoides de *Fructus aurantii immaturus* (TONG *et al.*, 2018b), e na determinação da cafeína no café, chá e cacau (RUSSO *et al.*, 2018).

## 4 OBJETIVOS

### 4.1 Objetivo Geral

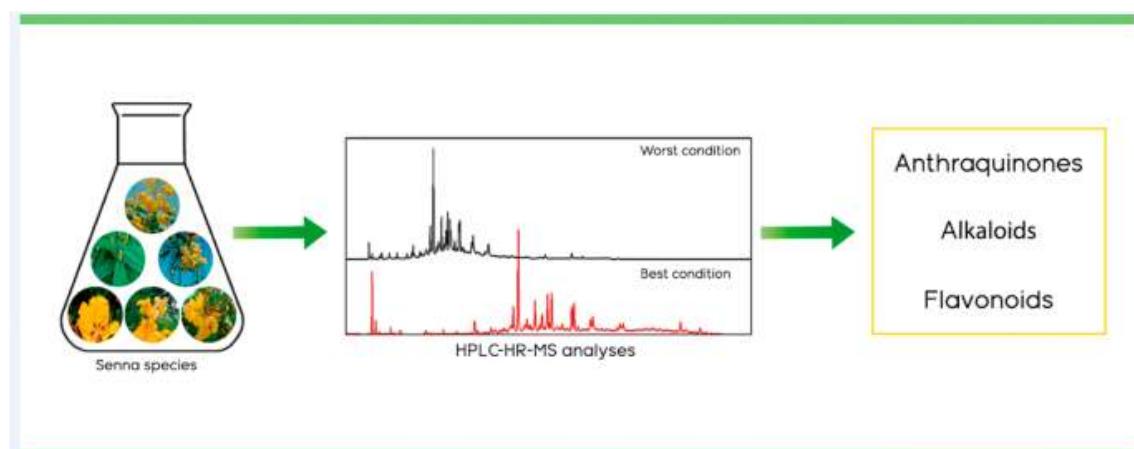
Realizar estudo químico e farmacológico de sete espécies do gênero *Senna* que ocorrem na Caatinga e no Cerrado.

### 4.2 Objetivos Específicos

- Caracterizar e documentar o perfil cromatográfico das espécies *Senna cearensis* Afr.Fern, *Senna macranthera* (DC. ex Collad.) H.S. Irwin & Barneby, *Senna obtusifolia* (L.) H.S. Irwin & Barneby, *Senna reticulata* (Willd.) H.S. Irwin & Barneby, *Senna spectabilis* var. *excelsa* (Schrad.) H.S.Irwin & Barneby, *Senna splendida* (Vogel) H.S. Irwin & Barneby e *Senna trachypus* (Mart. ex Benth.) H.S. Irwin & Barneby;
- Aplicar a técnica de Extração Online – OLE acoplado com UPLC-MS para gerar de forma rápida, eficientemente e verde, o perfil químico das folhas das espécies de *Senna* spp.;
- Investigar atividades antiacetilcolinesterásica e citotóxica dos extratos das flores de *Senna* spp.

## 5 A COMPREHENSIVE LC-DAD-QTOF-MS METHOD FOR DEREPLICATION OF BIOACTIVE COMPOUNDS IN SENNA EXTRACTS

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## A Comprehensive LC-DAD-QTOF-MS Method for Dereplication of Bioactive Compounds in Senna Extracts

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### Abstract

A reversed-phase analytical method was developed and validated by using a combination of high-performance liquid chromatography with diode array detection and ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry systems to dereplicate compounds in samples prepared by mixing extracts from various organs of different *Senna* species, Fabaceae. The pre-treatment of the sample by solid phase extraction was also investigated. The results obtained showed that the clean-up of the plant extract produces relevant changes in its chemical profile, resulting in false fingerprints. The efficiency of the method was applying to compare the chemical profile of the extracts of seven different *Senna* species: *S. cearensis* Afr. Fern, *S. macranthera* (Collad.) H.S.Irwin & Barneby, *S. obtusifolia* (L.) H.S.Irwin & Barneby, *S. reticulata* (Willd.) H.S.Irwin & Barneby, *S. spectabilis* var. *excelsa* (DC.) H.S.Irwin & Barneby, *S. splendida* (Vogel) H.S.Irwin & Barneby, and *S. trachypus* (Benth.) H.S.Irwin & Barneby. These results suggest that the comprehensive developed method can be applied to the study and dereplication of the chemical composition of other *Senna* species, preferentially without clean-up of the samples.

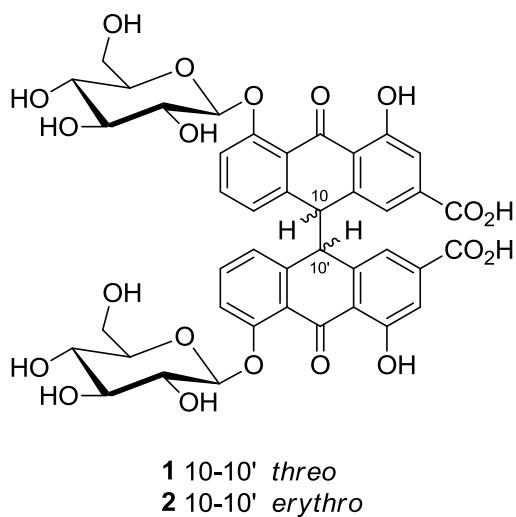
**KEYWORDS:** Dereplication, Extract pre-treatment, Liquid chromatography, Method optimization, Sennosides

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## 1. Introduction

*Senna* species in the legume family Fabaceae can be found in tropical and subtropical regions of the world. Around 82 species of this plant genus are widely distributed in the Northeast, South, and Southeastern regions of Brazil, 26 of which are considered endemic (Silva et al. 2016; Azevedo and Conceição 2017). *Senna* plant species appear as small shrubs or trees with 4-9 meters in height. The leaves are pinnate with opposite paired leaflets. The plant species have flamboyant yellow flowers, and this explains their large use as ornamental plants. The inflorescences are racemes at the ends of branches or emerging from the leaf axils. The species are known to bear legume pod-like fruits, which are mostly long and black in color with several brown seeds (Selegato et al. 2017).

Previous studies have described the chemical composition of *Senna* species. One that deserves mention was conducted by Silva et al. (2016) in which the authors evaluated 12 leaf extracts of 4 *Senna* species, namely *S. gardneri*, *S. macranthera*, *S. splendida* and *S. trachypus*, and identified the presence of 34 compounds, including carboxylic acids, fatty acids, alcohols, long chain alkanes, diterpenes, triterpenes and sterols, as well as flavonoids (such as chrysin and quercetin). Pivatto et al. (2005) elucidated the structures of 4 new alkaloids isolated from *S. spectabilis* ethanolic extracts. Bradley et al. (2019) have also demonstrated the importance of the anthraquinones sennosides A (**1**) and B (**2**), originally identified in *S. alexandriana*, which are widely used as purgative agents and are among the most important pharmaceutical products of plant origin.



Considering the wide diversity of *Senna* species, many of them have poorly been chemically and pharmacologically studied, the development of an efficient analytical chemical approach is essentially relevant for comparing the metabolite profiles as well as

identifying potentially active compounds present in these plant species (Patriarca et al. 2018). The present work reports the development, optimization and validation of a method in reverse mode (C-18) HPLC-DAD potentially useful for the analysis of extracts from *Senna* species. The analytical method was developed using a composite sample prepared with extracts from different organs of seven *Senna* spp. The chromatographic parameters evaluated included flow rate and composition mobile phase (solvent B and formic acid contents), gradient time, column temperature and selection of an internal standard. The optimal conditions for sample preparation were also investigated. In order to evaluate the method efficacy, the chemical profile of extracts (leaves, flowers and fruits) of seven *Senna* species was obtained; these included *S. cearensis* Afr. Fern, *S. macranthera* (Collad.) H.S.Irwin & Barneby, *S. obtusifolia* (L.) H.S.Irwin & Barneby, *S. reticulata* (Willd.) H.S.Irwin & Barneby, *S. spectabilis* var. *excelsa* (DC.) H.S.Irwin & Barneby, *S. splendida* (Vogel) H.S.Irwin & Barneby, and *S. trachypus* (Benth.) H.S.Irwin & Barneby. The chemical composition of the extracts was dereplicated through a combination of UPLC-QTOF-MS technique and automated data processing software with *in-house* database containing 180 compounds, which have already been identified in *Senna* species.

## 2. Materials and Methods

### 2.1 Plant Material

The leaves, flowers and fruits of *Senna cearensis* Afr. Fern, *S. macranthera* (Collad.) H.S.Irwin & Barneby, *S. obtusifolia* (L.) H.S.Irwin & Barneby, *S. reticulata* (Willd.) H.S.Irwin & Barneby, *S. spectabilis* var. *excelsa* (DC.) H.S.Irwin & Barneby, *S. splendida* (Vogel) H.S.Irwin & Barneby, and *S. trachypus* (Benth.) H.S.Irwin & Barneby, Fabaceae, were collected in Crateús, Senador Sá, Quixeramobim and Caucaia, cities of the Ceará State, Brazil, between May and June, 2017. The species were authenticated by the Prisco Bezerra Herbarium staff at the Universidade Federal do Ceará, Fortaleza, Ceará, Brazil, and the voucher deposited under the accession numbers 60424, 60415, 60416, 60419, 60421, 60420, 60432, respectively. The access to the plant material was granted by the Sistema Nacional de Gestão do Patrimônio Genético (SisGen AB06D11). After collection, the plant parts were dried in an oven at 40 °C and subsequently grounded. The dried and ground plant material (1 g) was subjected to ultrasound-assisted extraction with MeOH (30 ml) three times and then dried on a rotatory evaporator. For the method development, a *Senna* composite sample was prepared by blending the extracts from different plant parts. Approximately 0.5 mg of each

extract was dissolved in 500 µl of MeOH/H<sub>2</sub>O 1:1 (v/v), mixed in the same vessel, subsequently dried and weighed. From this mixture, 15 mg were separated, dissolved in MeOH (1 ml) and subjected to C-18 solid-phase extraction using reversed phase silica (Merck C-18 cartridge, 2 1 cm 40-63 µm) and pure MeOH as eluent. The eluate was dried, dissolved in MeOH (10 mg/ml), filtered through a 0.22 µm nylon membrane and finally used for the HPLC method development.

## 2.2 HPLC-DAD Analysis

Mixtures of *Senna* extracts as a composite sample were analyzed to evaluate the performance of HCO<sub>2</sub>H 0.1% as solvent A in CH<sub>3</sub>CN, MeOH and EtOH (solvent B). Then, three gradient programs were tested in order to increase de number of bands in the chromatograms: tG1 (3/45/100 %B in 0/30/40 min), tG2 (5/45/100 %B in 0/45/60 min) and tG3 (3/15/35/100 %B in 0/8/45/60 min). After that, factorial planning with central compound 3<sup>3</sup> (significance level of 0.05) was applied to optimized temperature (x<sub>1</sub>= 35, 45 and 55 °C), HCO<sub>2</sub>H concentration (x<sub>2</sub>= 0.10, 0.25 and 0.50%) and flow rate (x<sub>3</sub>= 0.8, 1.0 and 1.2 ml/min). The number of chromatographic bands at 254 nm was used as dependent variable in order to find the best chromatographic conditions.

## 2.3 Precision

Instrumental accuracy was evaluated based on the peak area and retention time of the internal standard (IS) solution (butylated hydroxytoluene, 0.7 mg/ml) with detection at 254 nm, after six injections times using the assay concentration. Four replicates of *S. splendida* leaf extract containing internal standard (IS) solution at 0.7 mg/ml concentration were analyzed in two different days. Three representative chromatographic peaks were selected (*t<sub>R</sub>* = 17.9, 18.9 and 19.5 min) and monitored at 254 nm, to measure the relative standard deviations of the retention times and peak areas. The stability of sample solution was performed using of the *S. splendida* leaf extract, stored in the autosampler for three days and analyzed every 24 h. Three peaks were selected (*t<sub>R</sub>* = 17.9, 18.9 and 19.5 min) from the resulting chromatograms and the relative standard deviation (RSD) was calculated for each analyst and in both days.

## 2.4 Solid Phase Extraction for Sample Clean-up

The fruit extracts of *S. macranthera* were prepared based on three procedures: i) UAE with MeOH/H<sub>2</sub>O (1:1) and IS (0.7 mg/ml); ii) UAE using MeOH/H<sub>2</sub>O (1:1) without IS; and

iii) UAE with MeOH/H<sub>2</sub>O (1:1) and IS of the plant residue used in first procedure. All extracts were divided into two aliquots: one was subjected to clean-up by C-18 solid phase extraction (SPE); the second was not subjected to clean-up and was directly analyzed by HPLC. The clean-up with C-18 SPE consisted of the application of 1 ml of MeOH/H<sub>2</sub>O extracts on the SPE column, which has been previously activated and conditioned with MeOH and MeOH/H<sub>2</sub>O (1:1). Elution was carried out with an additional 1 ml of MeOH/H<sub>2</sub>O (1:1) and 2 ml of pure MeOH, all collected in the same vial. The eluate was dried under airstream and weighed.

## 2.5 Sample Preparation

Approximately 100 mg of the plant material, accurately weighed, were subjected to UAE using acetonitrile/water 1:1 (v/v) and 0.7 mg/ml of butylated hydroxytoluene as internal standard. The extracts were dried, weighed, and stored at -18 °C until analysis. The extract solutions were prepared at concentrations of 10 mg/ml to HPLC analysis and 1 mg/ml for UPLC-QTOF-MS analysis. Both solutions were filtered using Chromafil®Xtra RC-20/25 membranes with 0.20 µm pores before analysis.

## 2.6 HPLC-DAD Analysis

HPLC-DAD analyses were carried out in a Shimadzu UFC system (Kyoto, Japan), which consisted of a quaternary pump system, degasser (DGU-20A3), auto-injector (SIL-20A), with a UV-Vis diode array detector (Shimadzu SPD-M20), a control system (CBM-20A), and LC solution software (Shimadzu Version 1.22sp). The separation process was performed using a Phenomenex Luna C-18 column (250 4.60 mm, 5 µm), at a flow rate of 0.8 ml/min, oven temperature at 30 °C and injection volume of 10 µl. The UV-Vis spectral data were obtained in the range of 200–800 nm and the chromatograms were recorded at 254 nm.

## 2.7 UPLC-QTOF Analysis

The analysis was performed on an ACQUITY Ultra Performance LC system equipped with Q-Tof Premier LC-MS-MS spectrometer (Waters). The analysis of chromatographic profile was performed using an ACQUITY UPLC® HSS T3 column (Waters, 100 2.1 mm, 1.8 µm), temperature of 40 °C and injection volume of 1 µl. Elution was performed using a binary gradient system, which consisted of [A] acetonitrile (Synth, Brazil) and [B] water, both

containing 0.1% formic acid (Synth, Brazil), applied at flow rate of 0.58 µl/min. The gradient elution was set as follows: 3/45/100/100 % B in 0/4.34/5.78/6.50 min. The data were collected in centroid mode, using a lock spray frequency of 10 s, with an average of 10 scans, in both positive and negative ionization modes.

## 2.8 Statistical analysis

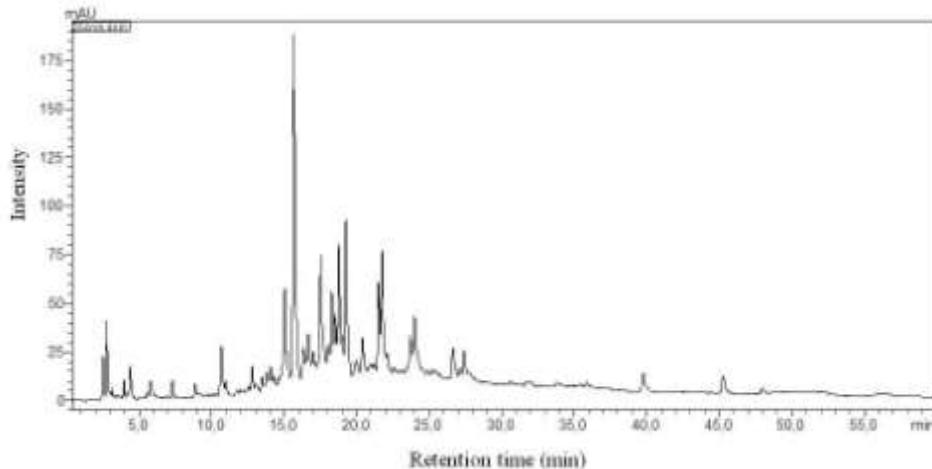
All tests were performed on Microsoft Office Excel 2007. Statistical significance was set at  $p < 0.05$ .

## 3. Results and discussion

### 3.1 Optimization of the Chromatographic Method

The characterization of secondary metabolites in plants is regarded essentially important because it helps one to have a better understanding of plants and their medicinal properties for the efficient and safe development of plant-based medicines (Navarro et al. 2017). However, the chemical investigation of plants is extremely difficult due to a high degree of chemodiversity, such as the species belonging to the *Senna* genus. However, the use of HPLC or UHPLC coupled to hybrid state-of-the-art mass spectrometers is becoming a key tool for the rapid and accurate analysis and dereplication of bioactive substances in complex plant matrices to rapidly estimate their pharmacological potential. The present work describes the development of an efficient method for the analysis and dereplication of the chemical profile of *Senna* species through the use of the UPLC-QTOF-MS technique and automated data processing software with in-house database. To conduct the experiments, a *Senna* composite sample (SCS) prepared with extracts of different organs from seven species of *Senna* was used as a representative sample of the molecular diversity of these plants (Fig. 1). This analytical approach was adopted to reduce the time required for the development of a comprehensive chromatographic method, suitable for conducting comparative analyses of all the samples.

Fig. 1 Chromatographic profile of the *Senna* composite sample (SCS). Gradient: 5/100 %B in 0/60 min, at 254 nm; móible phase: water (A) and acetonitrile (B)

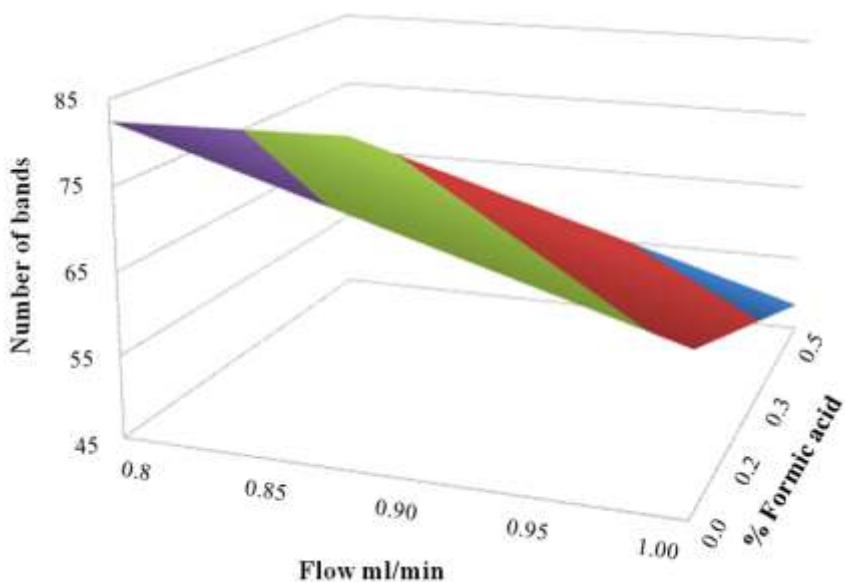


Initially, the performance of some solvent B ( $\text{CH}_3\text{CN}$ ,  $\text{MeOH}$ , or  $\text{EtOH}$ ) and gradient programs were evaluated. Acetonitrile was selected by exhibiting a higher number of total chromatographic bands and more bands with  $R_s \geq 1$  in comparison with the alcohols, apart from presenting better UV performance (cutoff: 190 nm) and lower operating pressure. Then, adjustments were made to the elution gradient program with the best results obtained with tG3 (3/15/35/100 %B in 0/8/45/60 min), which provided a greater number of bands in a relatively shorter time and bands better distributed throughout the analysis time. A central compound factorial design of  $3^3$  was used to optimize the additional chromatographic factors: temperature ( $x_1$ ), formic acid ( $x_2$ ), and flow rate ( $x_3$ ). The quadratic model obtained from Eq. 1 provided the best fit at a confidence level of 95%. The relationship between the factor levels, the coded values, and the results of the fractional factorial experimental planning are shown in supplementary file Table S1.

$$N=68.4+2.4x_1-9.4x_2-7.7x_3-5.1x_{11}-3.8x_{22}+4.1x_{33}-1.8x_{12}+0.5x_{13}+4.2x_{23} \quad (\text{Equation 1})$$

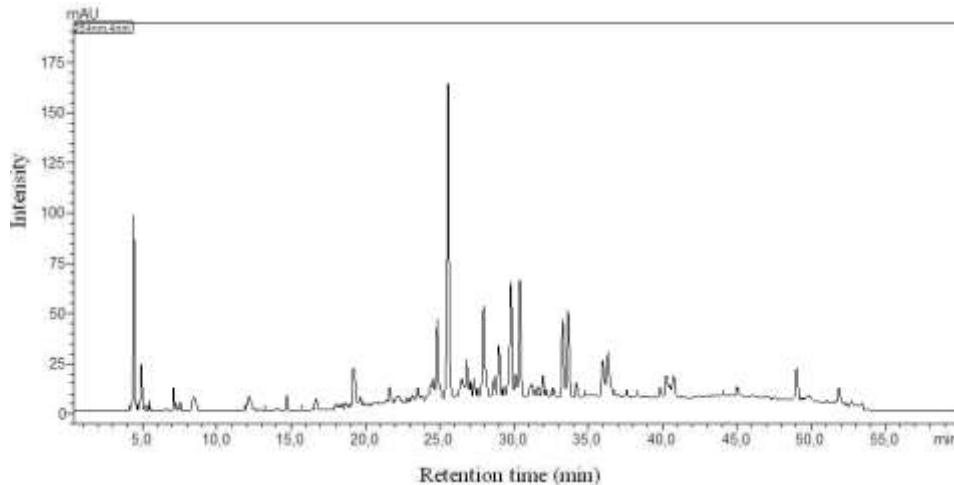
At a significance level of 0.05, the variables found to be statistically significant were as follows: the flowrate of mobile phase ( $X_2$ ) and the percentage of formic acid ( $X_3$ ). This result shows that the aforementioned variables exert an influence over the number of chromatographic bands. One can better observe this through the graphs shown in Fig. 2, which demonstrate the relationship between the flow rate, the percentage of formic acid, and the number of bands. Based on the result obtained, the flow rate of 0.8 ml/min and 0.1% of formic acid in the mobile phase were chosen for conducting the analysis. Considering that the factorial design showed that the temperature level had no influence over the variation of the number of bands, the temperature of 35 °C was chosen for conducting the experimental analyses.

Fig. 2 Response surface related to the influence of flow rate and formic acid content on the total number of bands observed in the chromatograms



The reliability of the results obtained in the factorial design can be evaluated through the ANOVA table, which allows one to study the relationship between different parameters of the model. The sum of the square (SS) should ideally be as high as possible, while the sum residuals, error, and lack of adjustment should be preferably small. One will note that each SS has an associated number of degrees of freedom (DF), and dividing the SS by the corresponding DF leads you to the square mean (SM) of the model parameters. In order to verify the relevance of the parameters, one needs to compare the SM values through the application of two F tests. All variables are within their expected ranges as can be observed in Table S2; this implies that the model has no lack of adjustment and produces reliable results. The chromatographic profile of the *Senna* composite sample (SCS) under optimized conditions is shown in Fig. 3.

Fig. 3 Chromatographic profile of the *Senna* composite sample (SCS) in optimized conditions. Gradient: 3/45/100/100 %B in 0/30/40/45 min, flow rate of 0.8 ml/min, and temperature of 35 °C. Solvent A: W; solvent B: ACN; both solvents contained 0.1% of formic acid



### 3.2 Method Validation

The relative standard deviations obtained in the repeatability test (Table S3) were lower than 1%, as recommended (Lanças 2004). The intermediate precision, evaluated by the calculated relative standard deviations (RSDs) of the ratios between the areas of the peaks, presented values below 6%; this implies good intermediate precision in the preparation and injection of the samples (Table S3). It is worth noting that, when it comes to complex matrices, RSD values below 10% are considered satisfactorily good (Bueno et al. 2015). The stability test evaluates how long the solution containing the sample remains stable after preparation. In this test, it was possible to observe that the results obtained in the three days remained without significant variation with values of standard deviation less than 10% (Table S3). The analytical procedure developed was validated in accordance with ICH Q2(R1) in terms of precision and stability of sample solution (Guideline 2005). The optimized chromatographic conditions were as follows: Phenomenex Luna C-18 column ( $250 \times 4.6$  mm,  $5 \mu\text{m}$ ), gradient elution conditions: 3/45/100/100% B in 0/30/40/45 min, flow rate of 0.8 ml/min, and temperature of  $35^\circ\text{C}$ . Solvent A: H<sub>2</sub>O; solvent B: ACN; both solvents contained 0.1% of formic acid.

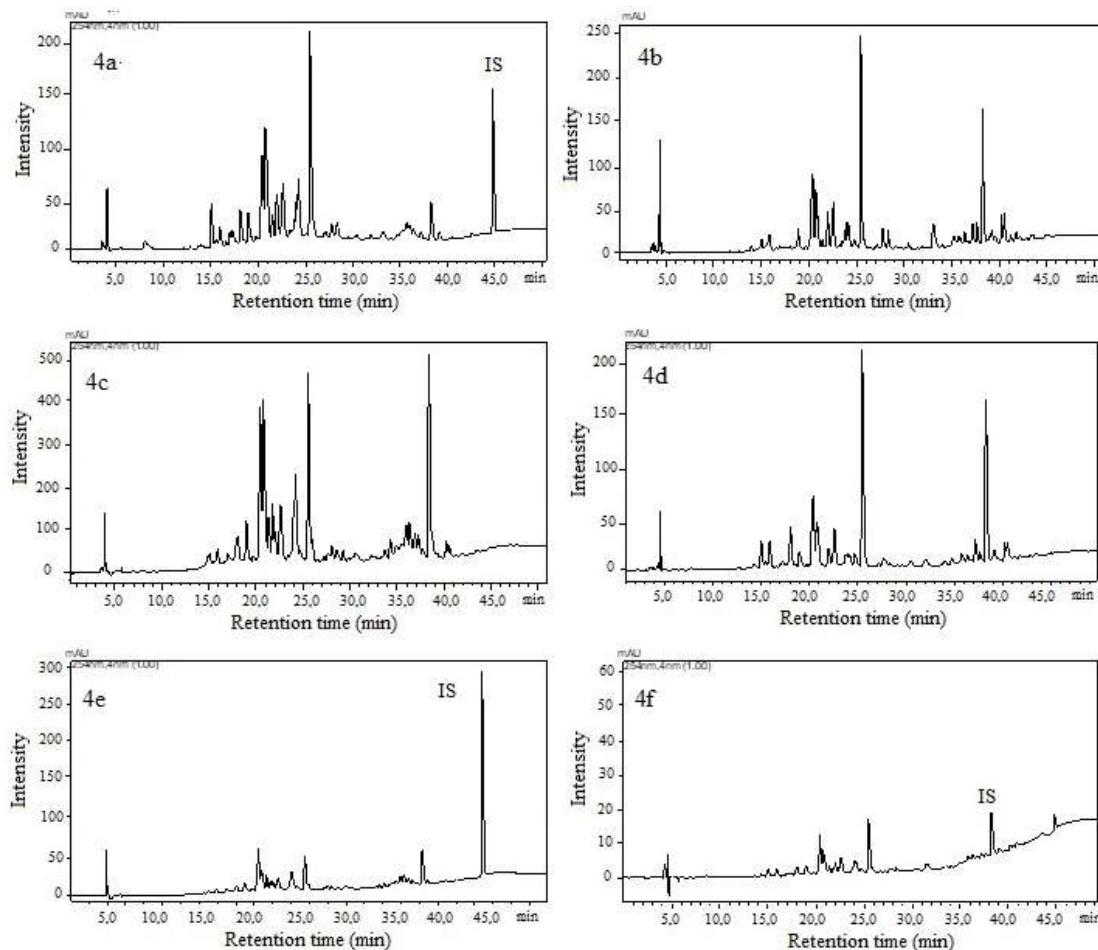
### 3.3 Pre-treatment of the Extracts

Solid phase extraction (SPE) is a relevant, validated and powerful clean-up procedure recommended to be carried out prior to subjecting plant extracts to HPLC analysis (Epifano et al. 2020). This procedure is useful for the removal of interferents or undesirable compounds, apart from contributing toward the matrix simplification of complex samples and protecting the columns. However, the modifications that this procedure causes in the chemical profile of

plant extracts are usually unclear, particularly when a comprehensive analysis is required (Cardoso et al. 2011).

In order to investigate the interference of SPE C-18 in the chemical profile of the analyzed plant extracts, three experiments were performed using dried and ground fruits of *S. macranthera*. In the first experiment, the extract was prepared by UAE with MeOH/H<sub>2</sub>O (1:1, v/v) and IS without SPE C-18 clean-up (Fig. 4A) and with SPE C-18 clean-up (Fig. 4B). The comparison of these two sample preparation procedures showed that SPE led to a reduction in the IS band, which implied a partial adsorption in the reversed-phase. In the second experiment that was conducted in the absence of IS, significant differences were observed in the chromatographic profiles of the samples analyzed in the absence of SPE C-18 clean-up and in the presence of SPE C-18 clean-up (Fig. 4C and D). In the third experiment, an eventual matrix effect on the adsorption of the IS was evaluated by extracting the plant residue from the second experiment with MeOH/H<sub>2</sub>O in the presence of IS. However, a comparison of the chromatograms obtained before and after the SPE C-18 clean-up showed that the internal standard was retained in the adsorbent, even after elution with pure MeOH (Fig. 4E and F), as observed in the first experiment. It is necessary to reinforce that besides IS retention in the SPE C18, significant differences were observed in intensity and occurrence of bands in the middle of the chromatograms, what could be explained by partial or total adsorption of phenolics and basic compounds by residual silanols and/or metal impurities of the packing material of SPE cartridges. Based on these results, it is possible to conclude that performing clean-up via SPE results in false fingerprinting, which compromises the comprehensiveness of the method. Therefore, the SPE clean-up procedure was not applied for qualitative analysis described in the present study

Fig. 4 Comparison of sample preparation procedures for HPLC analysis. Chromatograms of *Senna macranthera* fruit extract at 254 nm; a and b: (i) extraction with MeOH/W + BHT, without or with SPE C-18 clean-up; c and d: (ii) MeOH/W extraction, without or with SPE C-18 clean-up; e and f: second extraction of the plant residue used in (ii), with MeOH/W + BHT, without or with SPE C-18 clean-up. \*BHT (IS).



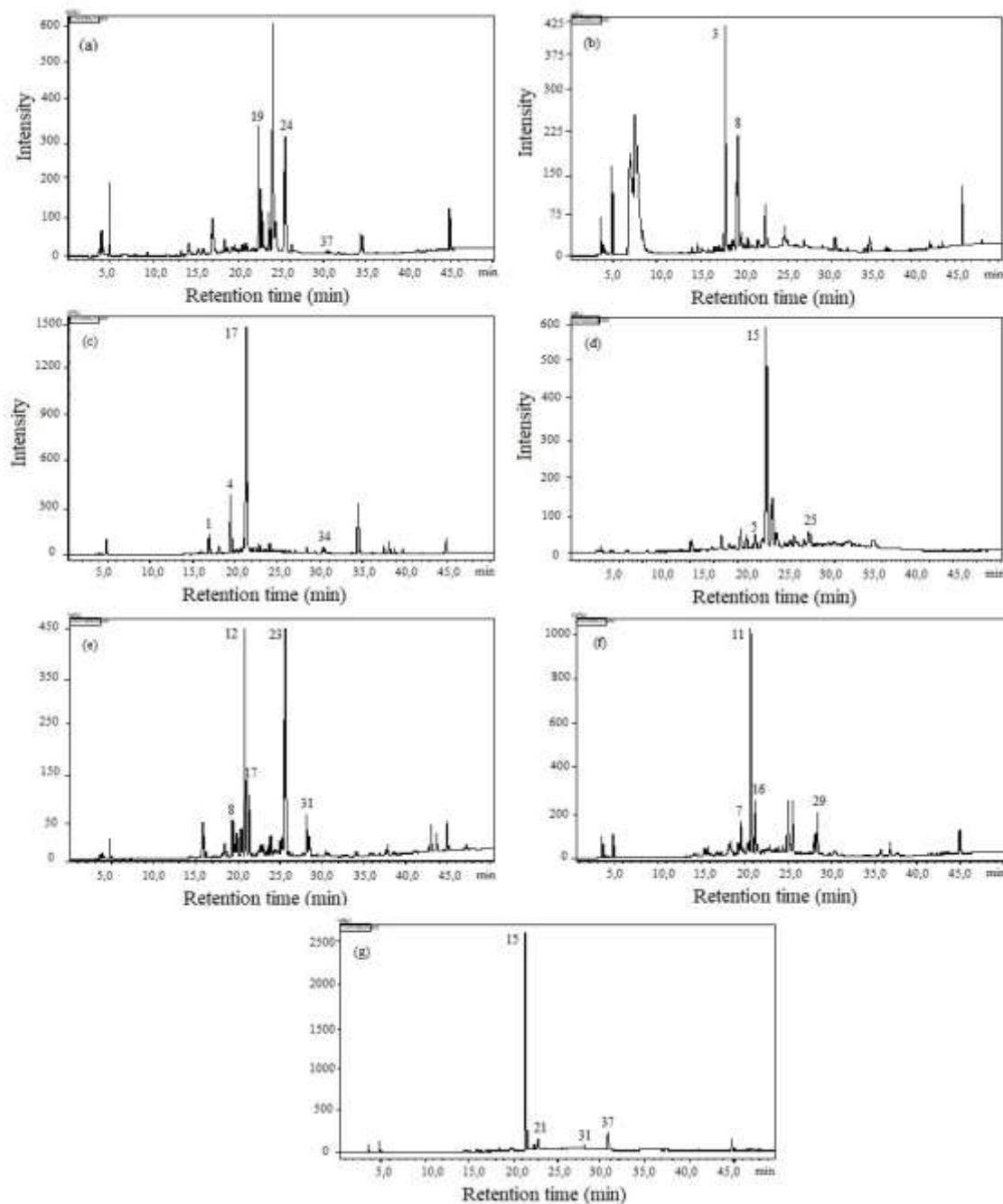
### 3.4 Qualitative Analysis

Extracts obtained from leaves, fruits, and flowers of seven *Senna* species (*S. macranthera*, *S. spectabilis* var. *excelsa*, *S. splendida*, *S. trachypus*, *S. reticulata*, *S. obtusifolia*, and *S. cearensis*) were individually analyzed by HPLC-DAD and UPLC-QTOF-MS in order to compare their chemical profiles and to derePLICATE the main known metabolites present in the samples. The qualitative analyses were performed based on the following procedures: (1) comparison of *t* (min), UV/Vis spectra, and HRMS of secondary metabolite standards available in the laboratory; (2) implementation of in-house database with 180 compounds previously reported in the literature for *Senna* species; (3) recording of the chemical constituents of the extracts through the comparison of the data obtained from UPLC-QTOF-MS analyses with those of the UNIFI information platform (Waters Corporation) containing the Traditional Medicine Library database with more than 6000 compounds.

The compounds identified are shown in Table S4. All the fragments are provided in supplementary data (Table S4), as well as the MS spectra of some compounds (Fig. S4). The chromatograms related to the leaves of the seven *Senna* species are shown in Fig. 5 and the chromatograms related to the flowers and fruits of the plant species can be found in the

supplementary material (Figs. S1 and S2). The application of this experimental approach helped to identify 46 compounds, mostly belonging to the class of anthraquinones, flavonoids, and alkaloids. Clearly, while some compounds were expected to be found in the different species of the *Senna* genus analyzed in this work (as was the case of alkaloids found in the *S. spectabilis* species), the identification of sennosides A (1) and B (1) for the first time in *S. reticulata* was a remarkable finding considering that these compounds are of significant medicinal and economic importance.

Fig. 5 Representative HPLC-DAD chromatograms of extracts from leaves of seven *Senna* spp.: (a) *S. obtusifolia*, (b) *S. spectabilis* var. *excelsa*, (c) *S. reticulata*, (d) *S. cearensis*, (e) *S. macranthera*, (f) *S. splendida*, (g) *S. trachypus*. Chromatographic conditions: Phenomenex Luna C-18 column ( $250 \times 4.6$  mm,  $5 \mu\text{m}$ ), gradient elution conditions: 3/45/100/100 %B in 0/30/40/45 min, flow rate of 0.8 ml/min, and temperature of 35 °C. Solvent A: W; solvent B: ACN; both solvents contained 0.1% of formic acid. Samples: extracts without SPE C-18 clean-up



#### **4. Conclusion**

The experimental approach applied in this work, which involved the study of preparation and pre-treatment stages of the samples, showed that the clean-up of the plant extracts by SPE C-18 produces relevant modifications in the chemical profile of the samples; these modifications lead to false fingerprinting when one aims to obtain a comprehensive chemical profile. The optimization of the parameters (mobile phase, gradient, temperature, flow rate, and percentage of formic acid), using a mixture of extracts as a representative sample of various organs and species, enabled us to develop and validate a comprehensive analytical method based on HPLC-DAD. The application of this method allowed the efficient representation of the chemical profile of leaves, fruits, and flowers of seven species of *Senna*. The method applied, based on the HPLC-DAD and UPLC-QTOF-MS techniques, helped to identify 46 compounds of various classes of metabolites, including anthraquinones, flavonoids, and alkaloids. This study provided relevant information regarding the method of treatment and composition of the samples of *Senna* species and can serve as a basis for future chemical studies of other herbal crude drugs.

#### **Author Contribution**

All authors contributed to the study design. The collection and preparation of plant material were done by MGAF, MGVS, and AJC. Funds were raised by MGVS and AJC. Supervision was carried out by AJC. The first draft of the manuscript was written by MGAF and all authors read and approved the final manuscript.

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#### **Declarations Conflict of Interest**

The authors declare no competing interests

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## Material suplementar

### A Comprehensive LC-DAD-QTOF-MS Method for Dereplication of Bioactive Compounds in *Senna* Extracts

**Table S1.** Factor levels, coded values and results of the fractional factorial experimental planning ( $3^3$ ) for variables selection. N\* = number of chromatographic bands

Test	Factor levels				Coefficients	Error	Interval
	X1	X2	X3	N*			
1	-1	-1	-1	83	b0	68.4	3.7
2	0	-1	-1	85	b1	2.4	2.1
3	1	-1	-1	92	b2	-9.4	2.1
74	-1	0	-1	77	b3	-7.7	2.1
5	0	0	-1	81	b11	-5.1	3.5
6	1	0	-1	89	b22	-3.8	3.5
7	-1	1	-1	43	b33	4.1	3.5
8	0	1	-1	79	b12	-1.8	2.6
9	1	1	-1	39	b13	0.5	2.6
10	-1	-1	0	64	b23	4.2	2.6
11	0	-1	0	68			
12	1	-1	0	70			
13	-1	0	0	65			
14	0	0	0	64			
15	0	0	0	66			
16	0	0	0	60			
17	1	0	0	61			
18	-1	1	0	57			
19	0	1	0	66			
20	1	1	0	58			
21	-1	-1	1	57			
22	0	-1	1	70			
23	1	-1	1	71			
24	-1	0	1	61			
25	0	0	1	62			
26	1	0	1	60			
27	-1	1	1	43			
28	0	1	1	53			
29	1	1	1	53			

**Table S2.** ANOVA table of the proposed model for the optimization of the analysis conditions

Table ANOVA					
Source of variation	SS	DF	SM	F	Table F
Regression	3415	(p-1) 9	379.49	4.62	2.42
Waste	1562	(n-p) 19	82.19	-	-
Lack of Adjustment	47.5	(m-p) 2	23.75	3.75	19.44
Pure error	1514	(n-m) 17	89.07	-	-
Total	4977	(n-1) 28	-	-	-

Legend: p = 10 (number of coefficients); n = 29 (number of experiments); m = 12 (number of independent experiments)

**Table S3.** Repeatability, intermediate precision and stability. Relative standard deviation (RSD) values for internal standard (IS) BHT and replicates of *Senna splendida* leaf extracts

RSD%	Intermediate Precision		Stability		Repeatability BHT (IS)	
	R <sub>t</sub>	Area	R <sub>t</sub>	Area	R <sub>t</sub>	Area
Peak 1	0.25	5.37	0,10	8,43	0.02	0.59
Peak 2	0.11	4.97	0,16	5,01		
Peak 3	0.18	4.92	0,08	5,32		

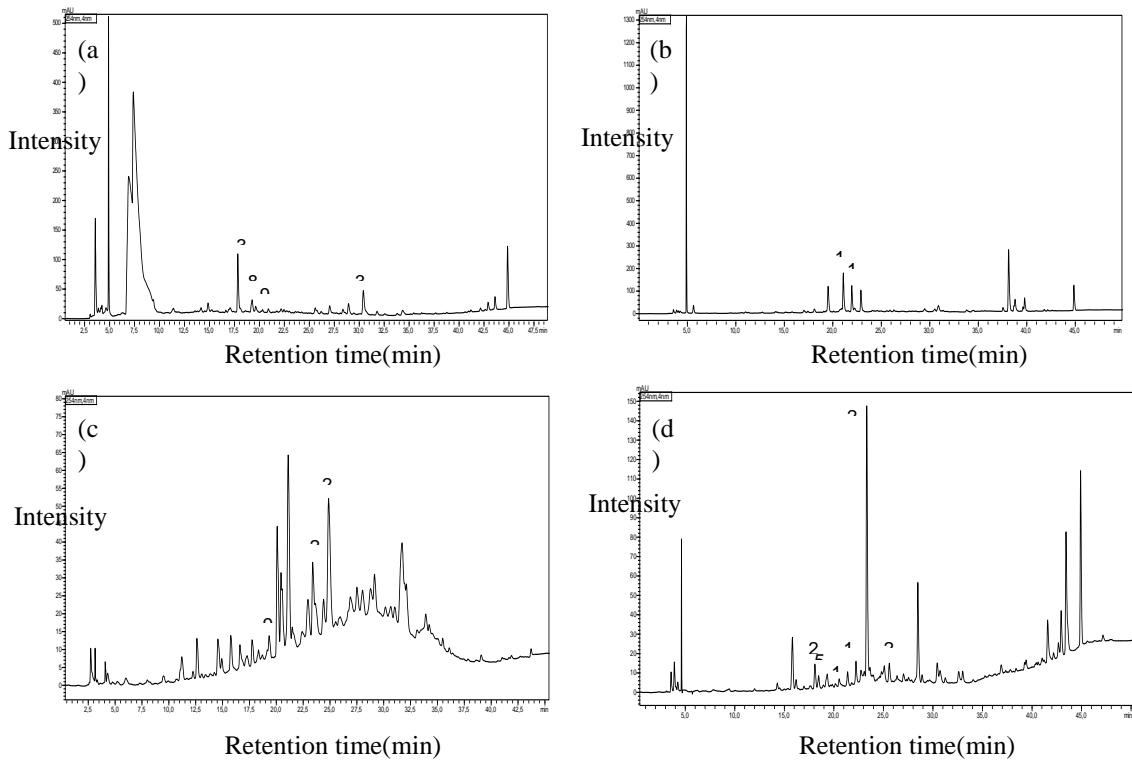
**Table S4.** Compounds annotated in *Senna* spp extracts by LC-DAD-MS

Nº	Component name	Observed RT MS (min)	Observed RT DAD (min)	Observed m/z	Mass Calcd	Mass Obsd	Error (ppm)	Molecular formula	<i>Senna</i> spp.
1	4- <i>O</i> -Caffeoylquinic acid <sup>(3)</sup>	2.77	16.94	377.0843 [M-Na] <sup>-</sup>	354.0951	354.0950	0.3	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	SrLe
2	Procyanidin B1 or B2 or B3	2.95	17.60	577.1344[M-H] <sup>-</sup>	578.1424	578.1417	1.3	C <sub>30</sub> H <sub>26</sub> O <sub>12</sub>	SmFl
3	Genistein-7,4'-di- <i>O</i> -β-D-glucoside <sup>(3)</sup>	3.00	17.78	593.1495 [M-H] <sup>-</sup>	594.1590	594.1584	1.0	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>	SseLe, SseFl, SSeFr
4	Myricetin-3- <i>O</i> -galactoside <sup>(2)(3)</sup>	3.11	19.49	479.0871 [M-H] <sup>-</sup>	480.0895	480.0903	1.7	C <sub>21</sub> H <sub>20</sub> O <sub>13</sub>	SrLe, SrFr
5	Procyanidin B1 or B2 or B3 <sup>(3)</sup>	3.13	19.33	577.1338 [M-H] <sup>-</sup>	578.1424	578.1417	1.2	C <sub>30</sub> H <sub>26</sub> O <sub>12</sub>	ScLe, ScFr
6	Procyanidin B1 or B2 or B3	3.14	19.50	577.1343[M-H] <sup>-</sup>	578.1424	578.1416	1.4	C <sub>30</sub> H <sub>26</sub> O <sub>12</sub>	SmFl
7	Malvidin-3- <i>O</i> -(6- <i>O</i> -acetyl-β-D-glucoside)-5- <i>O</i> -β-D- glucoside <sup>(3)</sup>	3.18	19.58	755.2045 [M-CH <sub>3</sub> COO] <sup>+</sup>	696.1901	696.1906	0.7	C <sub>31</sub> H <sub>37</sub> O <sub>18</sub>	SsLe
8	Apigenin-7- <i>O</i> -apioglucoside <sup>(3)</sup>	3.22	19.22	563.1391 [M-H] <sup>-</sup>	564.1479	564.1484	0.9	C <sub>26</sub> H <sub>28</sub> O <sub>14</sub>	SseLe, SseFr, SmLe,
9	Quercetin 3,4'-diglucoside	3.22	19.22	626.1483[M-H] <sup>-</sup>	624.1480	626.1480	0.5	C <sub>27</sub> H <sub>30</sub> O <sub>17</sub>	ScFl, SseFl
10	Kaempferol 3-glucoside-(1→6)-glucoside-7-alpha-L-rhamnoside <sup>(3)</sup>	3.40	20.51	757.2185[M+H] <sup>-</sup>	756.2110	756.2112	0.3	C <sub>33</sub> H <sub>40</sub> O <sub>20</sub>	SmFl
11	Isorhamnetin-3- <i>O</i> -(2G-α-L-rhamnosyl)-rutinoside <sup>(3)</sup>	3.42	20.73	769.2179 [M-H] <sup>-</sup>	770.2269	770.2282	1.7	C <sub>34</sub> H <sub>42</sub> O <sub>20</sub>	SsLe, StFr
12	Kaempferide-3- <i>O</i> -α-L-(4-O-acetyl)-rhamnosyl-7-O-α-L-rhamnoside <sup>(3)</sup>	3.42	20.79	635.1970 [M+H] <sup>+</sup>	634.1897	634.1923	4.1	C <sub>30</sub> H <sub>34</sub> O <sub>15</sub>	SmLe, SmFr
13	Quercetin-3- <i>O</i> -Arabinoglucoside	3.44	21.02	595.1317[M-H] <sup>-</sup>	596.1377	596.1389	2.0	C <sub>26</sub> H <sub>28</sub> O <sub>16</sub>	SmFl
14	Sennoside A or B <sup>(2)(3)</sup>	3.48	21.07	861.1873 [M-H] <sup>-</sup>	862.1955	862.1956	0.1	C <sub>42</sub> H <sub>38</sub> O <sub>20</sub>	SrFl, SrFr

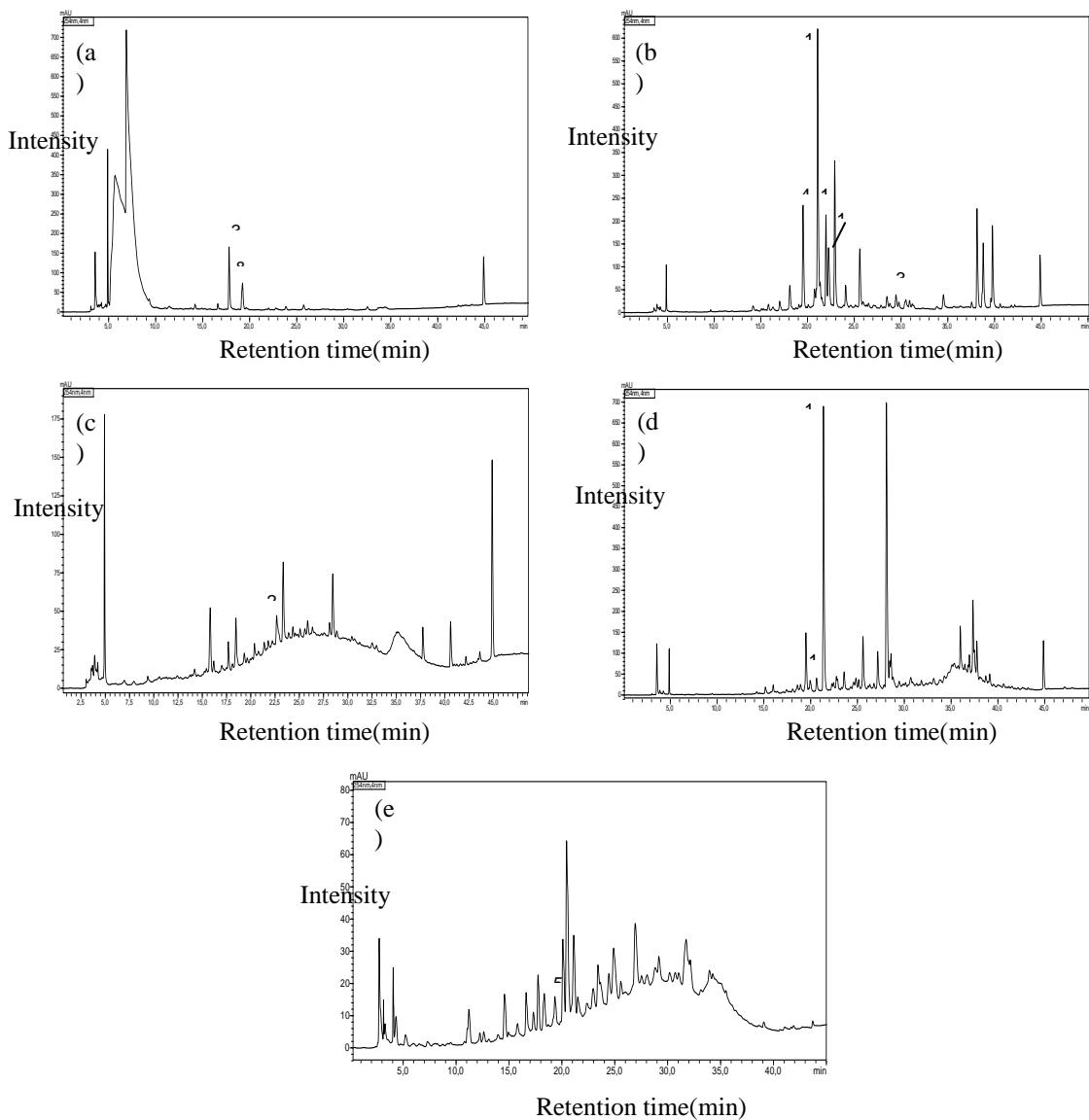
15	Rutin <sup>(1)(2)(3)</sup>	3.50	21.34	609.1454 [M-H] <sup>-</sup>	610.1533	610.1527	1.0	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	ScLe, StLe, StFr, SrFr	
16	6-Methoxyluteolin <sup>(3)</sup>	3.50	21.26	317.0670 [M+H] <sup>+</sup>	316.0588	316.0586	0.6	C <sub>16</sub> H <sub>12</sub> O <sub>17</sub>	SsLe	
17	Kaempferol-3,7-di-O- β-D-glucoside <sup>(2)(3)</sup>	3.52	21.32	609.1456 [M-H] <sup>-</sup>	610.1533	610.1543	1.6	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	SrLe, SmLe, SmFr	
18	Sennoside A or B <sup>(2)(3)</sup>	3.63	22.00	861.1873 [M-H] <sup>-</sup>	862.1956	862.1971	1.7	C <sub>42</sub> H <sub>38</sub> O <sub>20</sub>	SrFl, SrFr	
19	Quercetin-3-O-β-D- glucuronide <sup>(2)(3)</sup>	3.64	22.76	479.0220 [M+H] <sup>+</sup>	478.0747	478.0745	0.4	C <sub>21</sub> H <sub>18</sub> O <sub>13</sub>	SoLe	
20	3,8-Di-C- glucosylapigenin <sup>(3)</sup>	3.72	22.90	617.1479 [M+Na] <sup>+</sup>	594.1584	594.1587	0.5	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>	SsFr	
21	Kaempferol-3-O- glucorhamnoside	3.75	23.40	593.1504[M-H] <sup>-</sup>	594.1584	594.1577	1.4	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>	StLe	
22	Quercetin 3-O-β-D- glucopyranoside	3.76	23.50	464.0950[M-H] <sup>-</sup>	464.0954	464.0955	0.9	C <sub>21</sub> H <sub>20</sub> O <sub>12</sub>	SmFl, ScFl	
23	Icariside-I <sup>(3)</sup>	3.89	25.59	529.1479 [M-H] <sup>-</sup>	530.1785	530.1781	0.8	C <sub>27</sub> H <sub>30</sub> O <sub>11</sub>	SmLe	
24	Kaempferol 3-O-β-D- glucuronopyranoside (2)(3)	3.91	25.60	461.0717 [M-H] <sup>-</sup>	462.0798	462.0790	1.5	C <sub>21</sub> H <sub>18</sub> O <sub>12</sub>	SoLe	
25	Mirificin <sup>(3)</sup>	3.93	25.43	547.1479 [M-H] <sup>-</sup>	548.1529	548.1532	0.5	C <sub>26</sub> H <sub>28</sub> O <sub>13</sub>	ScLe, ScFl	
26	Gallocatequichin	3.93	25.43	305.0670[M-H] <sup>-</sup>	306.0739	306.0744	1.1	C <sub>15</sub> H <sub>14</sub> O <sub>7</sub>	SmFl	
27	Acacetin 7-O-[6-O- acetyl-glucopyranosyl (1→2)] α- rhamnopyranosyl(1→ 6)- glucopyranoside <sup>(3)</sup>	4.13	ND	819.228 [M+Na] <sup>+</sup>	796.2425	796.2388	4.6	C <sub>36</sub> H <sub>44</sub> O <sub>20</sub>	SmLe	
28	Leptophyllin A <sup>(2)(3)</sup>	4.14	ND	316.2842 [M+H] <sup>+</sup>	315.2773	315.2775	0.6	C <sub>12</sub> H <sub>25</sub> NO <sub>3</sub>	SseLe, SseFr, SmLe	
29	Rubrofusarin <sup>(2)(3)</sup>	4.15	28.10	271.0610 [M-H] <sup>-</sup>	272.0684	272.0686	0.7	C <sub>15</sub> H <sub>12</sub> O <sub>5</sub>	SsLe	
30	Naringenin-6-C- glucoside <sup>(2)</sup>	4.30	28.50	433.1133 [M-H] <sup>-</sup>	434.1213	434.1214	0.2	C <sub>21</sub> H <sub>22</sub> O <sub>10</sub>	SmLe	
31	Narirutin <sup>(3)</sup>	4.63	28.01	579.1718 [M-H] <sup>-</sup>	580.1792	580.1791	0.4	C <sub>27</sub> H <sub>32</sub> O <sub>14</sub>	StLe	
32	Physcion <sup>(2)(3)</sup>	4.63	ND	283.0610 [M-H] <sup>-</sup>	284.0684	284.0688	1.4	C <sub>16</sub> H <sub>12</sub> O <sub>5</sub>	SseLe	

33	9-Methyl-rubrofusarin <sup>(2)</sup>	4.71	ND	287.0913 [M+H] <sup>+</sup>	286.0841	286.0840	0.3	C <sub>16</sub> H <sub>14</sub> O <sub>5</sub>	SsLe, SSeFr, SmLe, SmFl
34	Kaempferol-7-O- $\alpha$ -L-rhamnoside <sup>(3)</sup>	4.82	30.38	431.0983 [M-H] <sup>-</sup>	432.1057	432.1056	0.2	C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>	SrLe
35	1,3,6-Trihydroxy-2-methylantraquinone-3-O- $\beta$ -D-glucoside <sup>(2)</sup>	4.83	30.50	431.0979 [M-H] <sup>-</sup>	432.1056	432.1053	0.7	C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>	SrFr
36	Kaempferol	4.83	30.50	285.0409[M-H] <sup>-</sup>	286.0477	286.0482	0.6	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>	SseFl
37	Quercetin <sup>(1)(2)(3)</sup>	4.84	30.63	303.0430 [M+H] <sup>+</sup>	302.0425	302.0427	0.7	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	StLe, StFr, SoLe
38	7-Hydroxycassine <sup>(2)</sup>	4.82	ND	314.269 [M+H] <sup>+</sup>	313.2620	313.2623	1.0	C <sub>18</sub> H <sub>35</sub> NO <sub>3</sub>	SseLe, SseFr
39	Carnavaline <sup>(2)</sup>	4.98	ND	300.2904 [M+H] <sup>+</sup>	299.2830	299.2833	1.0	C <sub>18</sub> H <sub>37</sub> NO <sub>2</sub>	SseFl
40	Cassine <sup>(2)</sup>	5.11	ND	298.2746 [M+H] <sup>+</sup>	297.2667	297.2674	2.4	<u>C<sub>18</sub>H<sub>35</sub>NO<sub>2</sub></u>	SseLe, SseFl, SseFr, SrLe, SrFr
41	Spectaline <sup>(2)(3)</sup>	5.77	ND	326.3058 [M+H] <sup>+</sup>	325.2980	325.2977	0.9	C <sub>20</sub> H <sub>39</sub> NO <sub>2</sub>	SseLe, SseFl, SseFr, SrLe
42	3-O-Feruloylcassine <sup>(2)<sub>(3)</sub></sup>	5.90	ND	474.3214 [M+H] <sup>+</sup>	473.3141	473.3140	0.2	C <sub>28</sub> H <sub>43</sub> NO <sub>5</sub>	SseFl, SseFr
43	1,3,8-Trihydroxyanthraquinone <sup>(2)</sup>	6.03	ND	255.0298[M-H] <sup>-</sup>	256.0371	256.0373	0.8	C <sub>14</sub> H <sub>8</sub> O <sub>5</sub>	SrFl
44	Cassiamin B <sup>(2)(3)</sup>	6.36	ND	537.0836 [M-H] <sup>-</sup>	538.0900	538.0897	0.6	C <sub>30</sub> H <sub>18</sub> O <sub>10</sub>	SrFl
45	Alatonal <sup>(2)</sup>	6.40	ND	283.0249 [M-H] <sup>-</sup>	284.0320	284.0323	1.1	C <sub>15</sub> H <sub>8</sub> O <sub>6</sub>	SrFl
46	Tenacigenin B <sup>(3)</sup>	6.89	ND	365.1360 [M+H] <sup>+</sup>	364.2249	36412259	2.7	C <sub>21</sub> H <sub>32</sub> O <sub>5</sub>	SrLe

**Fig. S1.** Representative HPLC-DAD chromatograms extracts from flowers species of *Senna*  
 (a) *S. spectabilis* var. *excelsa* (b) *S. reticulata* (c) *S. cearensis* (d) *S. macranthera*



**Fig. S2.** Representative HPLC-DAD chromatograms extracts from fruits species of *Senna* (a) *S. spectabilis* var. *excelsa* (b) *S. reticulata* (c) *S. splendida* (d) *S. macranthera* (e) *S. cearensis*



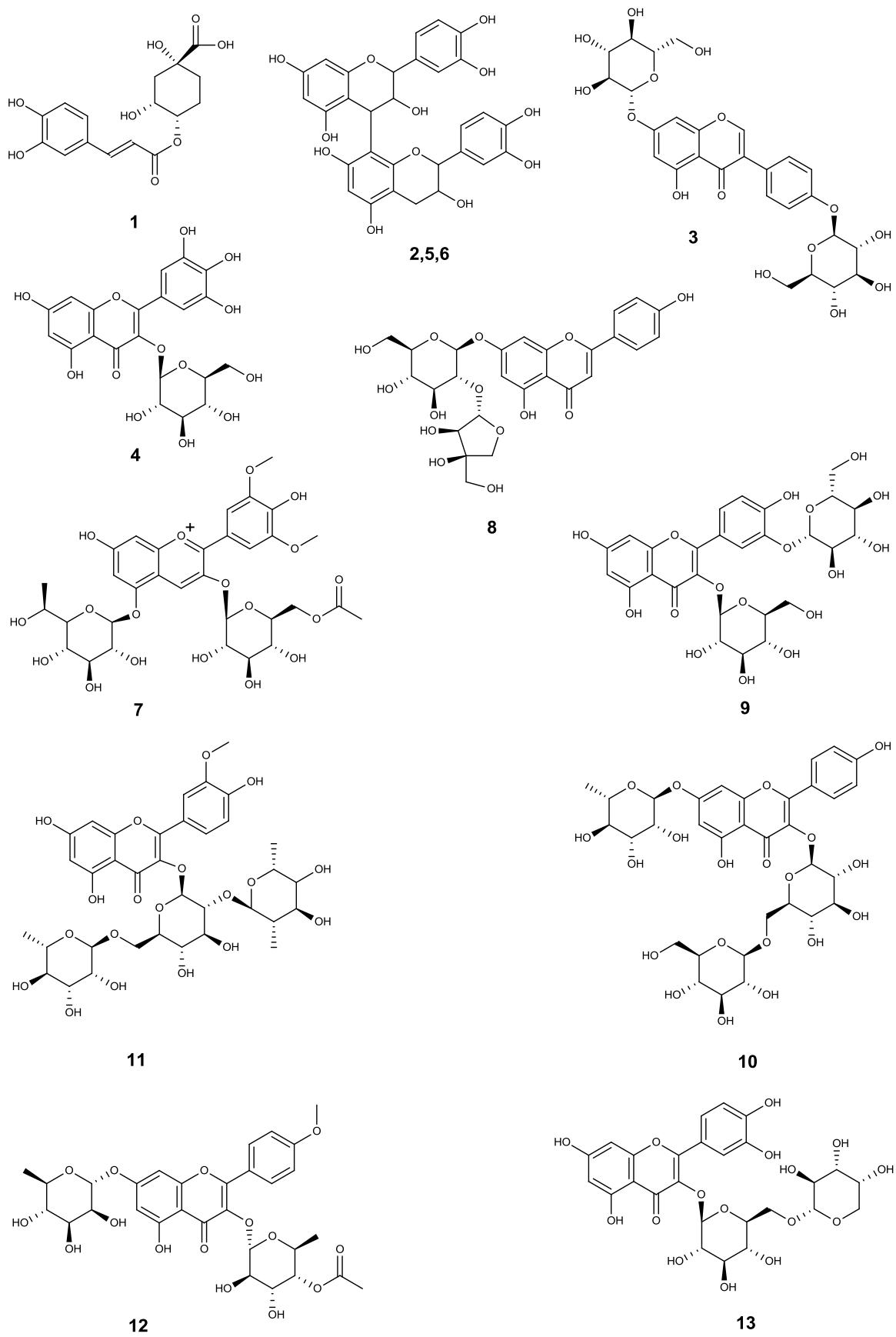
**Table S5.** UPLC-QTOF-MS fragmentation of compounds detected in extracts of various botanical parts of *Senna spp.*

N°	Component name	Observed <i>m/z</i>	Major Fragments	References
1	4- <i>O</i> -Caffeoylquinic acid	377.0843 [M-Na] <sup>+</sup>	<b>163.0386</b>	Granica et al. 2013
2	Procyanidin B1 or B2 or B3	577.1344[M-H] <sup>+</sup>	<b>289.0706</b> , 407.0749, 425.0861	Granica et al. 2013 and Rue et al. 2018
3	Genistein-7,4'-di- <i>O</i> -β-D-glucoside	<b>593.1495</b> [M-H] <sup>+</sup>	353.0660, 297.0762, 473.1075	March et al. 2004
4	Myricetin-3- <i>O</i> -galactopyranoside	<b>479.0871</b> [M-H] <sup>+</sup>	<b>299.0192</b> , 317.0276	Wang et al. 2014
5	Procyanidin B1 or B2 or B3	577.1338 [M-H] <sup>+</sup>	<b>289.0712</b> , 305.066, 407.0760	Granica et al. 2013 and Rue et al. 2018
6	Procyanidin B1 or B2 or B3	577.1343[M-H] <sup>+</sup>	<b>289.0706</b> , 407.0749, 425.0861	Granica et al. 2013 and Rue et al. 2018
7	Malvidin-3- <i>O</i> -(6- <i>O</i> - acetyl-β-D-g glucoside)-5- <i>O</i> -β-D-glucoside	<b>755.2045</b> [M-CH <sub>3</sub> COO] <sup>+</sup>	625.13992, 535.2827, 491.1793	Srivastava and Vankar, 2010
8	Apigenin-7-apioglucoside	<b>563.1391</b> [M+H] <sup>+</sup>	353.0661, 297.0761, 383.0764	Dueñas et al., 2009
9	Quercetin 3,4'-diglucoside	625.1385 [M-H] <sup>+</sup>	<b>301.0343</b> , 463.0856	Sobral et al. 2019
10	Kaempferol-3-glucoside-(1→6)-glucoside-7-alpha-L-rhamnoside	757.2185[M-H] <sup>+</sup>	<b>209.0809</b> , <b>287.0552</b> , 449.1082	Kachlicki et al. 2008
11	Isorhamnetin-3- <i>O</i> -(2G-α-L-rhamnosyl)-rutinoside	<b>769.2179</b> [M-H] <sup>+</sup>	314.0429, 725.1912	Chen et al. 2015
12	Kaempferol-3- <i>O</i> -α-L-(4- <i>O</i> -acetyl)-rhamnosyl-7- <i>O</i> -α-L-rhamnoside	635.1970[M+H] <sup>+</sup>	<b>279.0933</b> , 317.0650	Jang et al. 2016
13	Quercetin-3- <i>O</i> -Arabinoglucoside	595.1317[M-H] <sup>+</sup>	300.0334, <b>301.0348</b> , 447.0918	Cuyckens et al. 2002 and Sobral

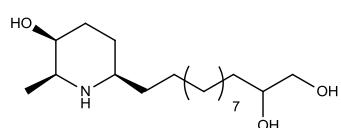
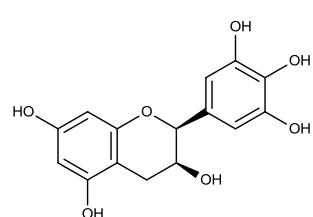
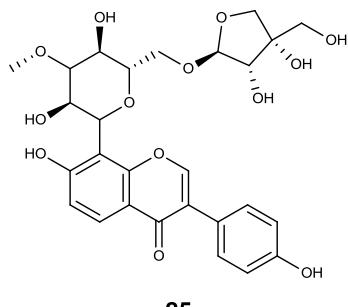
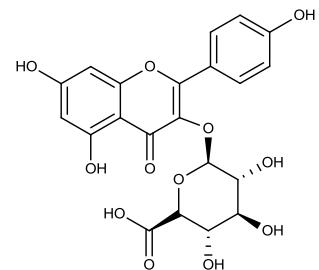
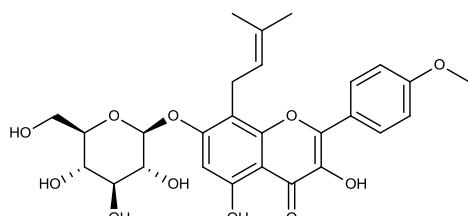
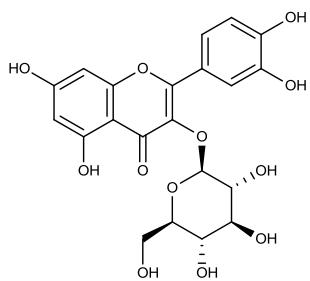
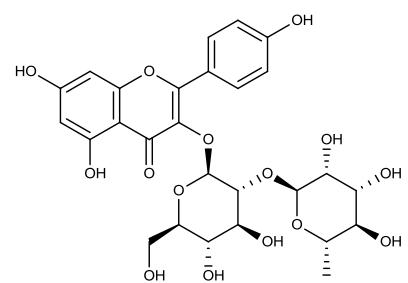
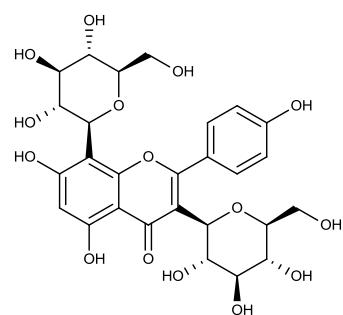
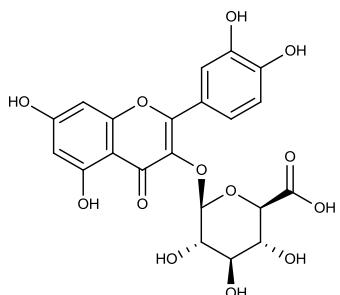
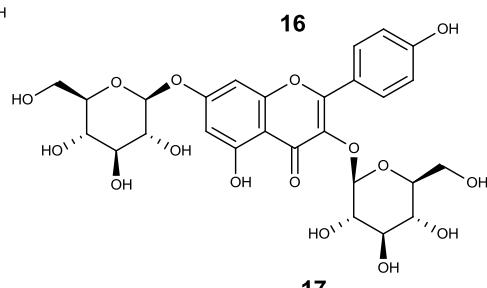
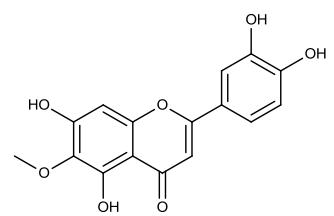
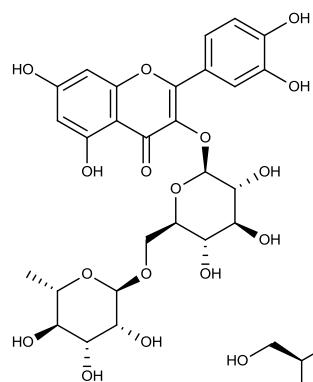
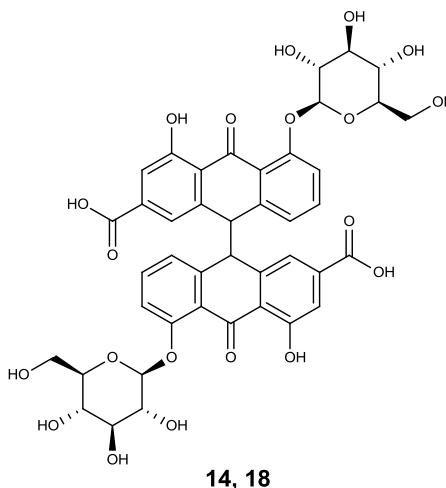
				et al. 2019
14	Sennoside A or B	<b>861.1873</b> [M-H] <sup>-</sup>	699.133, 847.2084	Lin et al. 2006
15	Rutin	<b>609.1454</b> [M-H] <sup>-</sup>	271.02441, 300.0273	Makita et al. 2016 and Sobral et al. 2019
16	6-Methoxyluteolin	<b>317.0670</b> [M+H] <sup>+</sup>	303.0496	Lee et al. 2018
17	Kaempferol-3,7-di-O- $\beta$ -D-glucoside	<b>609.1456</b> [M-H] <sup>-</sup>	284.0322, 285.0400	Sobral et al. 2019
18	Sennoside A or B	<b>861.1873</b> [M-H] <sup>-</sup>	701.1506, 845.1929	Lin et al., 2006
19	Quercetin-3-O- $\beta$ -D-glucuronide	479.0220 [M+H] <sup>+</sup>	163.0704, <b>303.0501</b> , 304.0553	Sobral et al. 2019
20	3,8-Di-C-glucosylapigenin	<b>617.1479</b> [M+Na] <sup>+</sup>	271.0600	Sato et al. 2006
21	Kaempferol-3-O-glucorhamnoside	593.1504[M-H] <sup>-</sup>	<b>285.0391</b> , 284.0315	Chen et al. 2015
22	Quercetin 3-O- $\beta$ -D-glucopyranoside	<b>464.0950</b> [M-H] <sup>-</sup>	301.0347, 300.0269	Dong et al. 2017
23	Icariside-I	529.1479 [M-H] <sup>-</sup>	<b>243.0651</b> , 269.0808, 409.0917	Shen et al. 2009
24	Kaempferol 3-O- $\beta$ -D-glucuronopyranoside	461.0717 [M-H] <sup>-</sup>	227.0341, <b>447.0915</b>	Makita et al. 2016 and Jang et al. 2016
25	Mirificin	548.154 [M+H] <sup>-</sup>	179.0340, <b>305.0668</b> , 545.1441-	Du et al. 2010
26	Gallocatequin	305.0670[M-H] <sup>-</sup>	<b>179.0346</b>	Dong et al. 2017 and Jang et al. 2016
27	Acacetin 7-O-[6-O-acetyl-glucopyranosyl (1 $\rightarrow$ 2)] $\alpha$ -rhamnopyranosyl(1 $\rightarrow$ 6)-glucopyranoside	819.228 [M+Na] <sup>+</sup>	<b>287.0551</b> , 285.0585, 651.1797	Marin et al. 2001
28	Leptophyllin A	<b>316.2842</b> [M+H] <sup>+</sup>	262.2528, 280.2632	Bolzani et al. 1995
29	Rubrofusarin	<b>271.0610</b> [M-H] <sup>-</sup>	230.0570	Maia et al. 2018

30	Naringenin-6-C-glucoside	433.1133 [M-H] <sup>-</sup>	271.0605, <b>305.0678</b>	Xie et al. 2017
31	Narirutin	<b>579.1718</b> [M-H] <sup>-</sup>	296.0682, 459.1278	Su et al. 2010
32	Physcion	<b>283.0610</b> [M-H] <sup>-</sup>	240.0425	Jin et al. 2019
33	9-Methyl-rubrofusarin	<b>287.0913</b> [M+H] <sup>+</sup>	272.0637	Li et al. 2001
34	Kaempferol-7-O- $\alpha$ -L-rhamnoside	431.0983 [M-H] <sup>-</sup>	268.0370, <b>287.0398</b>	Santos et al. 2020
35	1,3,6-Trihydroxy-2-methylantraquinone-3-O- $\beta$ -D- glucoside	431.0979 [M-H] <sup>-</sup>	<b>269.0443</b> , 285.0398	Itokawa et al. 1989
36	Kaempferol	<b>285.0409</b> [M-H] <sup>-</sup>	116.9290, 164.8341	Jang et al. 2016
37	Quercetin	<b>303.0430</b> [M+H] <sup>+</sup>	224.1268	Dong et al. 2017 and Jang et al. 2016
38	7-Hydroxycassine	<b>314.269</b> [M+H] <sup>+</sup>	252.2318	Viegas Jr. et al. 2013
39	Carnavaline	<b>300.2904</b> [M+H] <sup>+</sup>	82.2790, 264.2689	Bolzani et al. 1995
40	Cassine	<b>298.2746</b> [M+H] <sup>+</sup>	198.1855, 280.2641	Bolzani et al. 1995
41	Spectaline	<b>326.3058</b> [M+H] <sup>+</sup>	292.3001, 308.2952	Bolzani et al. 1995
42	3-O-Feruloylcassine	474.3214 [M+H] <sup>+</sup>	<b>280.2638</b> , 298.2744	Viegas Jr. et al. 2007
43	1,3,8-Trihydroxyanthraquinone	255.0298[M-H] <sup>-</sup>	<b>239.0347</b>	Huang et al. 2016
44	Cassiamin B	537.0836 [M-H] <sup>-</sup>	<b>523.1026</b>	Cheng et al. 2004
45	Alatonal	283.0249 [M-H] <sup>-</sup>	<b>239.0346</b>	Yadav, 2013
46	Tenacigenin B	<b>365.1360</b> [M+H] <sup>+</sup>	269.1530, 163.0746	Zhao et al. 2015

Structures of compounds identified in extracts of *Senna* spp.



Structures of compounds identified in extracts of *Senna* spp.



Structures of compounds identified in extracts of *Senna* spp.

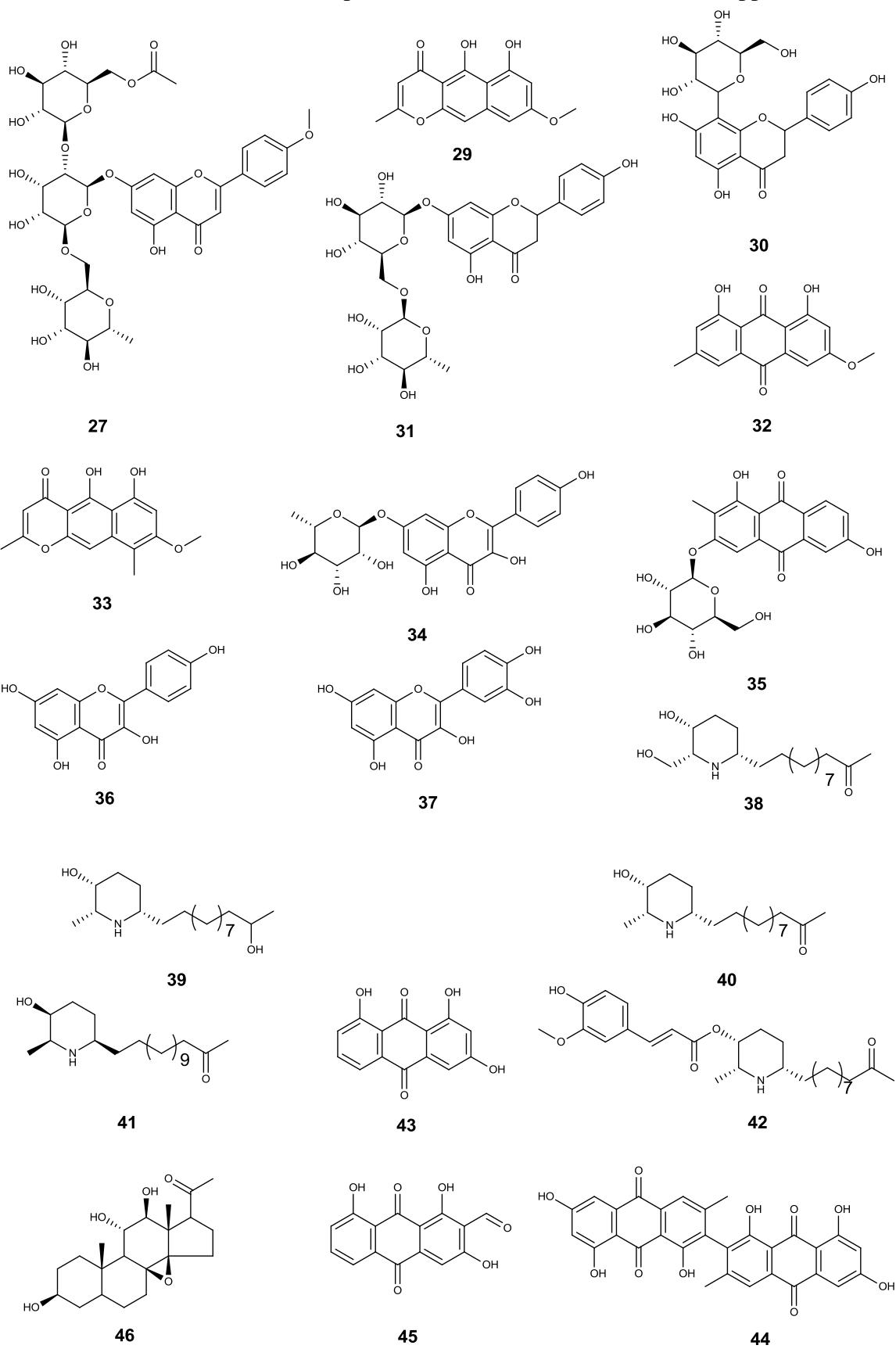
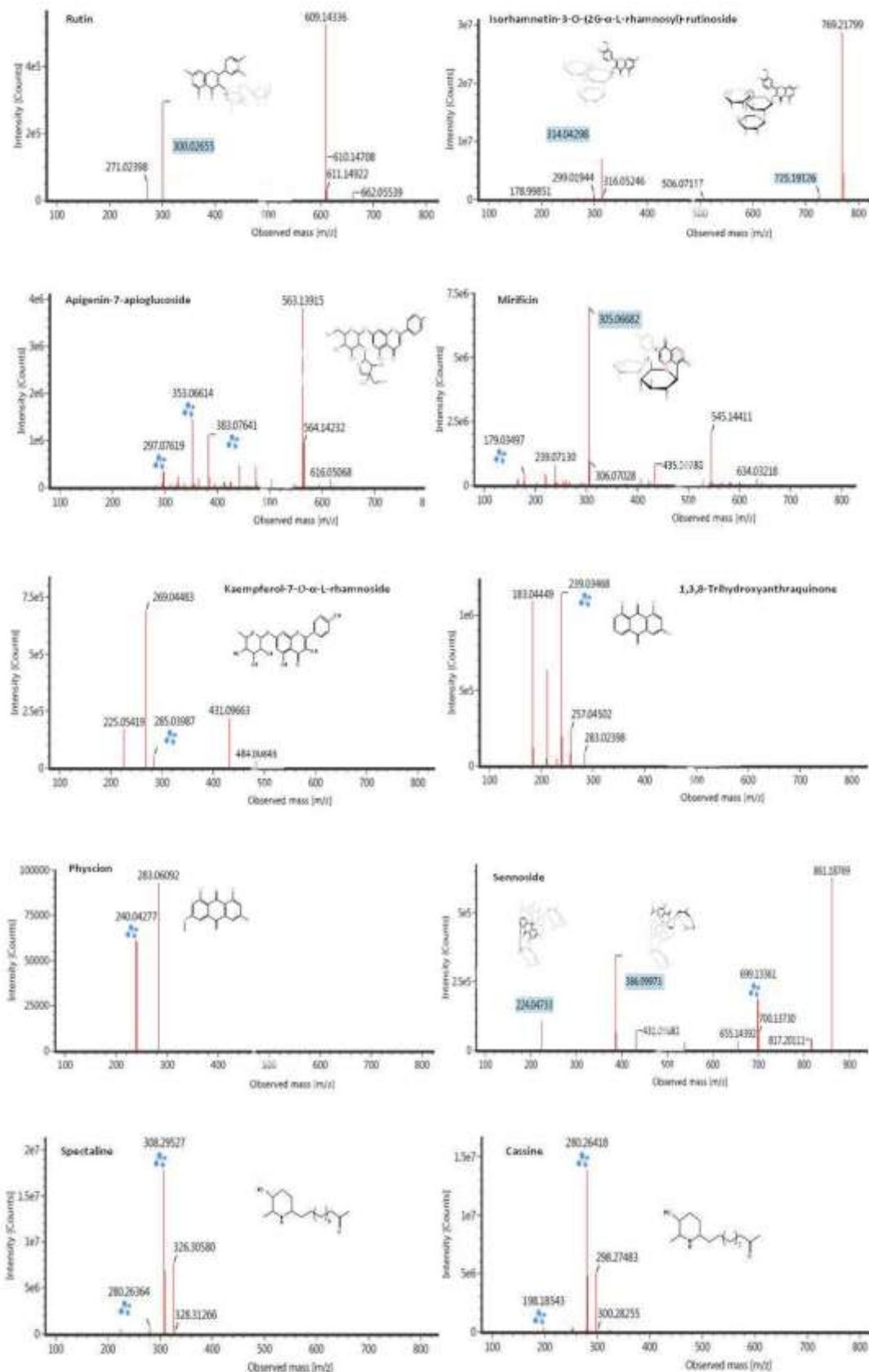


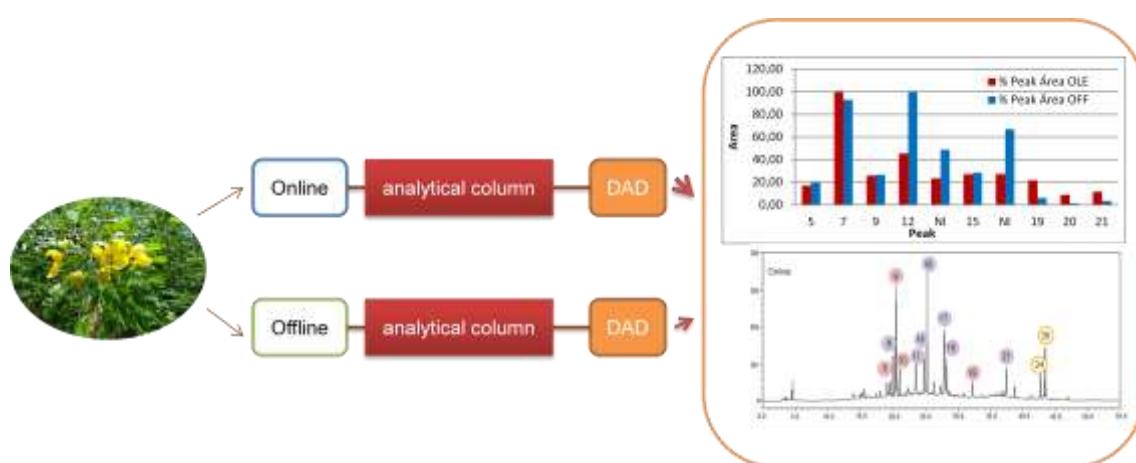
Fig.

**S3.** The MS spectra and fragmentation pathway of some significant compounds in negative ion mode



**CAPÍTULO 6 - *ONLINE EXTRACTION COUPLED TO LIQUID CHROMATOGRAPHY ANALYSIS OF LEAVES EXTRACTS FROM SENNA SPP***

A ser submetido no Journal of Separation Science



## ***Online Extraction Coupled to Liquid Chromatography Analysis of Leaves Extracts from Senna spp.***

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### **Abstract**

Sample preparation processes for application in chromatographic techniques are often criticized due to the high number of steps employed. The Online Extraction (OLE) technique proposes an optimized analytical configuration in which the extraction of a solid sample is directly coupled with HPLC analysis (OLE-LC). Making analysis faster and reducing random errors. In this work, the OLE-LC technique was applied to quickly, efficiently and green generate the chemical profile of the leaves extracts of *Senna* species (*S. macranthera*, *S. obtusifolia* and *S. splendida*). As a result, it was observed that the profile obtained by the OLE technique was very similar to that found by the conventional extraction technique. But some differences were noted in favor of OLE-LC, which includes more efficient extraction with chromatographic bands at least 2 or 4 times larger than offline extraction. In addition, significant changes were observed in the peak intensity of compounds from the classes of phenolic acids, benzochromenones and anthraquinones, this result is mainly related to extraction in gradient mode. This study is important since *Senna* species are rich in compounds of these classes; it also shows that the OLE-LC technique provides more complete chemical profiles of *Senna* spp.

**Keywords:** *Senna*, green chemistry, OLE-LC, phenolic acids, benzochromenones, anthraquinones.

## 1. Introduction

The method of online extraction with liquid chromatography analysis (OLE-LC) is a technique that allows the direct use of small amounts of plant material without the need for equipment modifications, such as adding pumps, valves, extra devices or solvents in addition to those that make up the HPLC device. Thus, it is an interesting alternative to optimize the obtaining of the chemical profile of solid samples [1] and to turn around the problems pointed above, related to sample preparation.

Moreover there is a current effort to carry out extraction replication procedures as a fundamental step in the research for new active molecules. This procedure involves extraction steps, solvent removal, sample redissolution, clean-up (usually by SPE) and finally analysis by LC-DAD-MS and qualitative analysis using retention and spectral information, available in databases. However, these pre-chromatographic steps are still relatively laborious, they are potential sources of error and they consume time, solvent and energy. In addition they do not necessarily provide a chemical profile (fingerprinting) which faithfully represents the chemical constitution of the plant tissue under study. In this sense the risk of degradation of thermally sensitive compounds and the chance of sample contamination are possibilities that are taken into consideration [2-3]. Thus the simplification and integration of extractive and chromatographic-spectrometric methods would have indisputable advantages in terms of viability, cost reduction and environmental resources [4-5].

The species which are currently included in the genera *Senna* have already been the subject of several taxonomic reviews, although there is controversy regarding the species positioning in some cases [6]. Studies aiming at a comprehensive characterization of the chemical profile regarding some of these species are limited by the sample preparation procedures (extraction and clean-up) and even by the chromatographic conditions employed . Recently, we demonstrated a simple clean-up of the extracts of a *Senna* spp. using SPE-C18 resulted in a fake chromatographic fingerprinting [7]. In addition, the great chemical diversity of these species makes chemical analysis a challenge, with no efficient method reported in the literature to portray the general chemical profile.

In this study, the OLE-LC method is applied to obtain metabolic fingerprinting in the selected samples of *Senna* (*S. macranthera*, *S. obtusifolia*, and *S. splendida*) and its performance to the ultrasound assisted extraction (UAE), which is an efficient offline extraction method.

## 2. Materials and Methods

### 2.1. Chemicals and reagents

The acetonitrile solvent (J. T. Baker, USA) used was of chromatographic grade. Formic acid (Synth, Brazil) was of analytical grade (ACS). Ultrapure water was obtained using a Millipore water purification system (Millipore, USA).

### 2.2. Plant material

The leaves of *Senna macranthera*, *S. obtusifolia* and *S. splendida* were collected between May and June 2017, in various locations in the state of Ceará, Brazil. All plant specimens were authenticated by Herbarium Prisco Bezerra Federal University of Ceará (Ceará, Brazil) under the numbers 60424, 60421, 60420, 60419, 60415 and registered in the National System of Genetic Heritage Management (SISGEN; #AB06D11). After collection, the leaves were dried in an oven at 40 °C, grinding in analytical mill (IKA A11 basic) and sieved in 350 mesh steel sieves.

### 2.3. Preparation of samples for offline extraction

Plant material (particle size 350 µm). The extraction was assisted by ultrasound (UAE) in the proportion of 100 mg of plant material to 3 mL of acetonitrile/water 1:1 (v/v). After filtration the extracts were dried, weighed and kept in freeze (-18 °C). Before analysis, the extracts were dissolved in W/CAN 1:1 (v/v) at concentration of 10 mg/mL, filtered on Chromafil®Xtra RC-20/25 membranes with 0.20 µm pores and 10 µL was injected on the HPLC. The extraction of each sample was made in duplicate.

### 2.4. Preparation of samples for online extraction

The dried, crushed and sieved leaves of *Senna* spp. were weighed (1 mg) and placed in the sample chamber of the on line extractor. Finally, this sample chamber is inserted into the holder, which is positioned on the HPLC injection valve, in the "loop" position [1]. The

extraction was performed by the mobile phase, in gradient mode, as described below. The extraction of each sample was also made in duplicate.

## 2.5. Chromatographic instrumentation and conditions

The chromatographic analysis was carried out using Shimadzu HPLC system (Kyoto, Japan) instrument that consisting in a quaternary pump system (LC-20AD), degasser (DGU-20A3), auto-injector (SIL-20A), and an UV-Vis diode array detector (Shimadzu SPD-M20), a control system (CBM-20A) and LC solution software (Shimadzu Version 1.22sp). Separation was performed using a Phenomenex Luna® C18 column (250 x 4.60 mm, 5 µm). The chromatographic conditions were as previously described [13] and includes flow rate at 0.8 mL/min, oven temperature at 30 °C, and an elution gradient program 3/45/100/100% B in 0/30/40/45 min, being [A] acetonitrile and [B] ultrapure water, both with 0,1% of formic acid. The UV-Vis spectral data were collected in the range of 200–800 nm and the chromatograms were recorded at 280 nm.

The extracts from UAE were also analyzed by LC-DAD-MS Shimadzu system (Kyoto, Japan) coupled to a diode array detector (DAD) and an ion trap mass spectrometer (amaZon SL, Bruker, Billerica, MA, USA) with electrospray ionisation (ESI) in negative ion mode. The spectrometer analysis parameters were 7.0 kV capillary, ESI in negative ion mode, a 500 V end plate offset, a 50.0 psi nebulizer, a dry gas (N2) flow rate of 10.0 L/h, and a temperature of 300 °C. Spectra (m/z 50–1000) were recorded every 2.0 s.

## 3. Results and Discussion

### 3.1 Online extraction coupled to high efficiency chromatography (OLE-LC)

In order to generate the leaves of *Senna* spp. in a quicker, greener, and more efficient way, the conventional sample preparation process that preceded the HPLC analysis was substituted by a new strategy of analysis called online extraction (OLE) that provides a comprehensive chemical profile of solid samples without sample clean-up procedures. The annotation of main peaks of each extract (Table 01) was based on UV-Vis and MS data and on our previous paper [7].

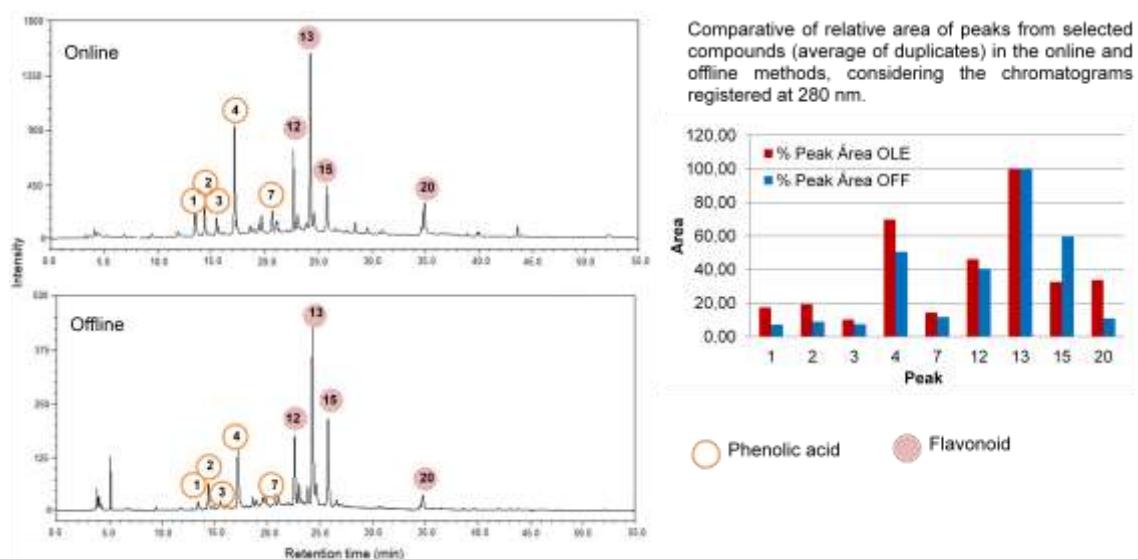
The analysis using the OLE-LC showed a chromatographic profile of *Senna* leaves that was very similar to that found using the offline extraction assisted by ultrasound (UAE). However, some important differences should be highlighted in favor of the OLE-LC, such as

it being the most efficient extraction, as can be seen in Figures 1-3, where the chromatographic bands are at least 2 or 4 times higher than in the offline extraction using equivalent amounts of solid samples.

Regarding the extraction efficiency, the UAE procedure resulted in a 10 % yield, so 10  $\mu$ L of the extract solution (10 mg/mL) injected into the HPLC system corresponds to the extract from 1.0 mg of the dried and ground leaves of *Senna* species. However, 100 mg of dry material were used to prepare these offline extracts, while just 1.0 mg of the dried and ground leaves was enough to carry out the OLE-LC analyses while still providing much more intense chromatographic bands. This means that the OLE-LC approach was more efficient per mass unity of dried leaves than the reference procedure (UAE).

Another marked difference between the chromatographic profiles obtained is observed in the relative proportion between the peaks, as shown in the graphs on Figures 1-3. In this analysis, the peak areas were normalized in each chromatogram, considering the largest peak as 100%. For the chemical profile of leaves from *S. obtusifolia* (Figure 1), flavonoid 13 was taken as reference (100%) and the relative intensity of cinnamoylquinic acids (compounds 1, 2, 3, 4 and 7) is higher in OLE-LC than UAE. However, the relative proportion between flavonoids (compounds 12, 13, 15 and 20) is variable, depending on the type of extraction and the specific flavonoid.

Figure 1. Fingerprinting comparison obtained by online extraction (OLE-LC) and offline extraction (UAE) of leaves from *Senna obtusifolia*.



In the fingerprints of leaves from *S. splendida* (Figure 2), a higher intensity was observed for benzocromenones 14, 16 and 18 in the offline extract, while for benzocromenones 22 and 23 and for anthraquinones 24, 25 and 26 the inverse occurred. Finally, in the case of *S. macranthera*, the relative proportions have changed in last peaks, with increase of relative amount on benzocromenones 17 and 21, to flavonoid 19 and to anthraquinones 24 and 26 (Figure 3). The region where the smallest variations in the relative proportions occurred was between 20-25 min, this is close to acn/w 1:1. The before and after variations are more significant, demonstrating the importance of gradient extraction to increase the extraction efficiency of compounds with a wide polarity range. The UV-Vis spectra characteristic of the classes identified in this work are shown in Figure 4.

Figure 2. Fingerprinting comparison obtained by online extraction (OLE-LC) and offline extraction (UAE) of leaves from *Senna splendida*.

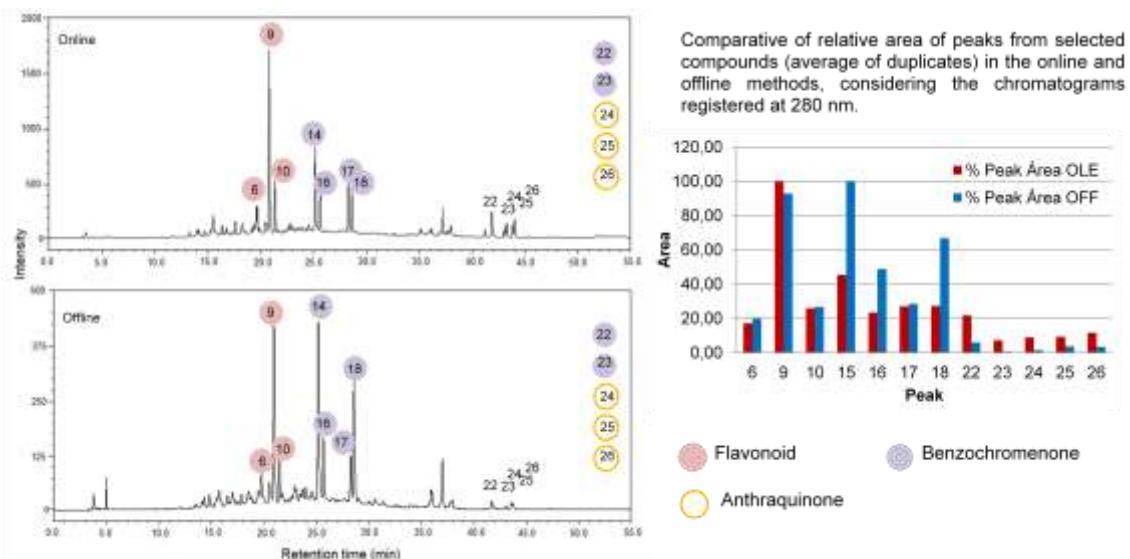
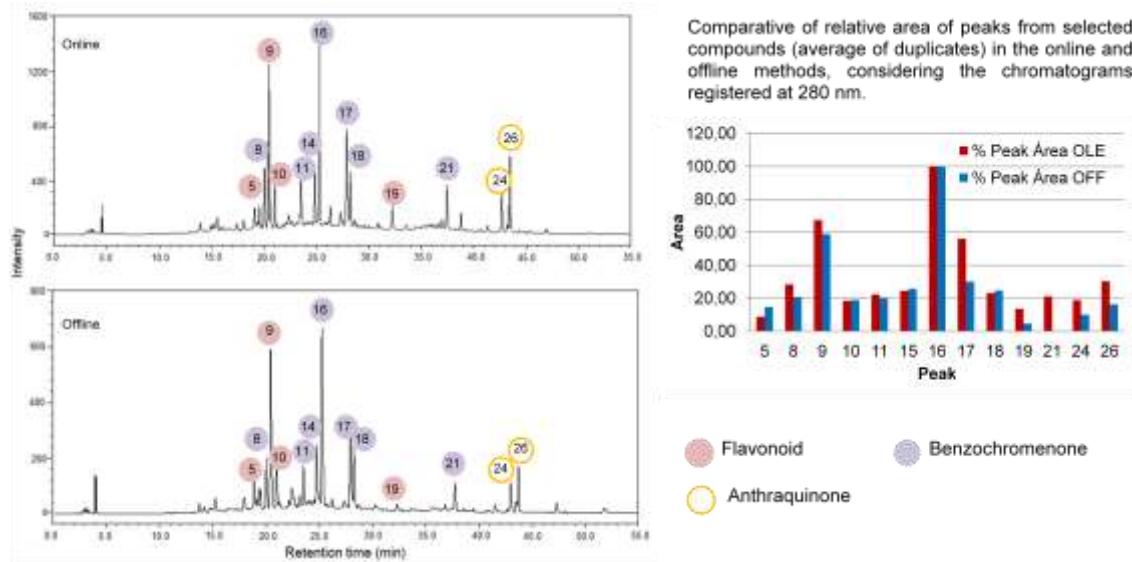


Figure 3. Fingerprinting comparison obtained by online extraction (OLE-LC) and offline extraction (UAE) of leaves from *Senna macranthera*.



We can observe that the change in peak intensity when extraction is done online (OLE) happens in compounds of the anthraquinone and phenolic acid classes. The study of this behavior is important since a great part of the compounds present in species of the genus *Senna* belong to these classes. The most efficient extraction of these compounds in the online method (OLE) may be associated with a combination of pressure increase and gradient mode extraction, that is, the proportion of solvents changes with the time of analysis. Factors that do not occur in offline extraction, where pressure is atmospheric and solvent proportion are constant.

According to Milevskaya et al. [8], it is possible to intensify the process of extracting plant materials and increase the efficiency of the process by increasing pressure. The more efficient extraction of certain compounds when exposed to higher pressures may be associated with their location in the plant matrix [9]. Compounds that are linked to cell wall substances are more difficult to extract.

Regarding the more efficient extraction of some compounds in the online technique (OLE) associated with the proportion of solvent used in the extraction, we see that the proportion of solvent is equivalent to the gradient, and its composition can vary from 5 to 100% of acetonitrile. That is, as the analysis time elapses, the water/acetonitrile ratio changes. For example, for initial retention times, the mobile phase composition favors the extraction of more polar compounds such as phenolic acids, for a retention time of 40 min, the extraction is taking place with 100% acetonitrile, which may favor a more efficient extraction of nonpolar compounds such as anthraquinones. In offline extraction the extraction solvent ratio is always

the same 1:1 water/acetonitrile. This composition favors the extraction of compounds from the flavonoid classes that did not show important changes when we compare the two extraction techniques.

This type of information is very relevant to untargeted metabolomic analyses, since significant alterations in the content of some metabolite are needed when proposing an initial hypothesis about its role in the ecophysiological event under study. In this type of approach, any sample preparation prior to chromatographic and/or spectrometric analysis, such as the classic cleanup by SPE-C18, can remove potentially important compounds, making the analysis not as comprehensive as desired. This fact was demonstrated by Franca et al. [7] when they compared chromatographic profiles of extracts from the *Senna* species, obtained by UAE and analyzed by HPLC-DAD, with and without cleanup by SPE-C18.

Figure 4. UV-VIS absorption spectra of the classes: phenolic acid (a), flavonoid (b), benzochromenone (c) and anthraquinone (d), in acetonitrile:water (1:1).

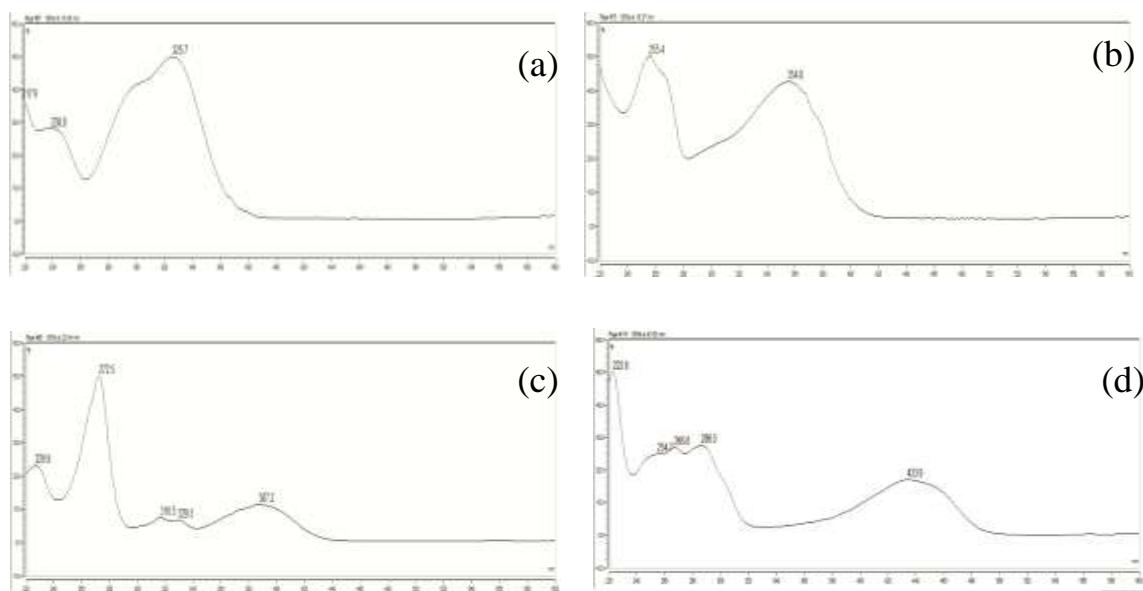


Table 1. Compounds and respective peak areas found in extracts from leaves of *Senna* spp.

Peak	Rt (min)	Observed m/z	Compound or class	MS2	$\lambda_{\text{max}}$ (nm)	Reference
1	13.08	353.20 [M-H]-	3- Caffeoylquinic acid	191.07, 179.00	325.7	[10], [11]

2	13.94	371.27 [M-H]-	Unidentified acid	191.00, 209.02	328	
3	15.09	353.23 [M-H]-	5- Caffeoylquinic acid	191.00, 219.02	328.2	[12], [13]
4	16.74	353.30 [M-H]-	4- Caffeoylquinic acid	191.00	325.7	[12], [13]
5	19.37	563.1391 [M-H]-	Apigenin-7- apioglucoside	301.0343, 463.0856	255.4, 354.8	[7]
6	19.49	625.50 [M-H]-	Unidentified flavonoid	179.03, 300.20, 445.29	255, 354	[7]
7	20.21	367.27 [M-H]-	Feruloylquinic acid	191.07, 173.00	311.9	[12], [13]
8	20.12	595.40 [M-H]-	Unidentified benzochromenone	254.13, 269.12	272.3, 316.8, 384.3	
9	20.50	769.2179 [M-H]-	Isorhamnetin-3- <i>O</i> - (2G- $\alpha$ -L-rhamnosyl)- rutinoside	314.0429, 725.1912	252.5, 355.1	[7]
10	21.05	609,39 [M-H]-	Unidentified flavonoid	301.17, 343.21	255.2, 354.4	
11	25.64	593.29 [M+H]+	Unidentified benzochromenone	299.17, 461.29	272.5, 316.5, 387.2	
12	22.76	479.0220 [M-H]-	Quercetin-3- <i>O</i> - $\beta$ -D- glucuronide	163.0704, 303.0501, 304.0553	264.2, 351.7	[7]
13	23.78	447.27 [M-H]-	Unidentified flavonoid	285.15, 323.23	265.5, 346.7	

14	24.84	595.31 [M-H]-	Unidentified benzochromenone	271.15, 256.25	278, 324.3, 398.7	[7]
15	25.13	461.0717 [M+H]+	Kaempferol 3- <i>O</i> - $\beta$ -D-glucuronopyranoside	227.0341, 447.0915	264, 295.8, 343.7	[7]
16	25.32	461.47 [M-H]-	Unidentified benzochromenone	415.41, 341.23, 299.20	260.9, 410.8	
17	27.90	271.0610 [M-H]-	Rubrofusarin	230.0570	270.6, 346.8, 421.7	[7]
18	28.26	433.113 [M-H]-	Unidentified benzochromenone	271.0605, 305.0678	280, 332.5, 413	
19	32.24	491.29 [M-H]-	Not identified	283.15, 445.29	222.7, 271.8, 420.6	[14]
20	34.25	461.35 [M-H]-	Unidentified flavonoid	179.06, 285.11	265.6, 365	
21	37.49	287.21 [M-H]-	Unidentified benzochromenone	269.21, 283.36	227.4, 272.2, 317.6, 394.1	
22	41.56	565.61 [M-H]-	Unidentified benzochromenone	256.13, 271.18	224.6, 276.8, 403.2	
23	42.77	455.44 [M-H]-	Unidentified	177.03, 277.34	231.5, 257.8,	

			benzochromenone		411.4	
24	42.76	253.25 [M-H] <sup>-</sup>	Chrysophanol	253.25	257.7, 278, 287.5, 428.5	[14], [15]
25	43.41	503.32 [M-H] <sup>-</sup>	Unidentified anthraquinone	245.08, 461.39	226.5, 254, 281.6, 424.7	
26	43.63	517.39 [M-H] <sup>-</sup>	Unidentified anthraquinone	471.25	222.7, 266.9, 286.1	[15], [16]

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#### 4. Conclusion

These results suggest that the online extraction technique is efficient, providing a complete chemical profile of the *Senna* species, which was very similar to that obtained when the extraction was done by the UAE, an offline extraction procedure. Thus, it can facilitate the process by eliminating sample preparation steps, in addition to making the process greener. The OLE-LC analysis also provided some gains in the quality of the analyses, such as a better yield and an increase in the relative intensities of compounds belonging to the classes of cinnamoyl-quinic acids, benzocromenones, and anthraquinones, a fact mainly related to gradient extraction. This result is of remarkable relevance, since the species of the genus *Senna* studied in this work are rich in compounds of the referred classes and, additionally, shows that the OLE-LC technique could be more useful in obtaining metabolic fingerprinting in target metabolomic analysis of plant tissues.

#### Acknowledgments

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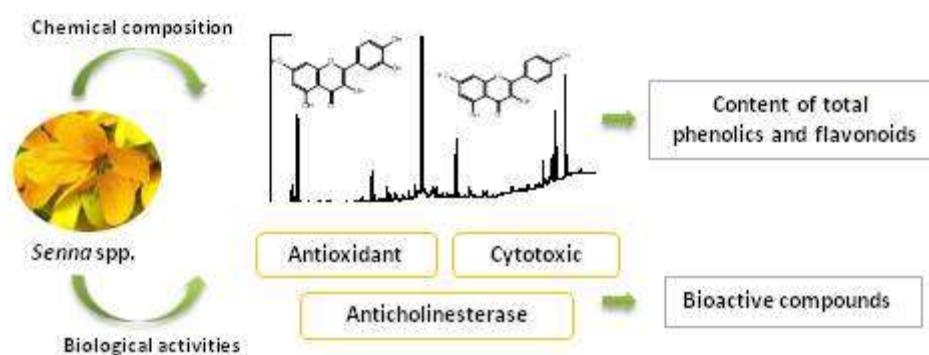
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## 7 CHEMICAL COMPOSITION, ANTIOXIDANT CONTENT, ENZYME INHIBITION, AND CYTOTOXIC POTENTIAL OF FLOWER EXTRACTS OF *SENNA SPECTABILIS* VAR *EXCELSA* AND *SENNA MACRANTHERA*

Submetido a Natural Product Research em 07 de julho de 2021



## **Chemical composition, antioxidant content, enzyme inhibition, and cytotoxic potential of flower extracts of *Senna spectabilis* var *excelsa* and *Senna macranthera***

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### **Abstract**

In this study, we investigated the phenolic and antioxidant content, cytotoxic, and anticholinesterase activities of flower extracts of *Senna spectabilis* and *Senna macranthera*. The antioxidant activities performed by the DPPH and ABTS methods showed that the extracts showed good antioxidant activity, with emphasis on the *S. macranthera* extract, which obtained results very similar to the rutin pattern. In the evaluation of the cytotoxic activity, the species *S. spectabilis* presented expressive cytotoxicity against the cellular lines PC3 and HL60 with IC<sub>50</sub> values of 21.08 and 31.37 µg/mL respectively. The values of anticholinesterase activity showed that both plants induced enzyme inhibition, reaching 14 mm of inhibition in the case of *S. spectabilis*. These results suggest that the extracts studied are promising sources of bioactive compounds.

**Keywords:** *Senna*, antioxidant, cytotoxicity, acetylcholinesterase.

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## 1. Introduction

Species of the genus *Senna* have been popularly used for several medicinal purposes such as in the treatment of fungal infections, wounds, burns, hemorrhoids, insomnia, flu, stomach pain, among others. However, they are known mainly for the purgative activity of their leaves (Alonso-Castro et al. 2019; Nkantchoua et al. 2018; Andrade et al. 2015). There are scientific reports on its potential for anti-inflammatory, antioxidant, antifungal, antibacterial and antiviral activities (Nkantchoua et al. 2018). The good performance of these species in biological tests may be associated with the presence of secondary metabolites with relevant antioxidant activity. Despite these perspectives, only the leaves of these species have been studied. Recent studies in other species have shown that flowers have a biological potential and chemical variety equal to or greater than that of leaves, in addition to being renewable and abundant in most species (Saleem et al. 2018). Thus, in the present study we report the antioxidant activity, enzyme inhibition, cytotoxic potential and the relationship with the chemical composition of flower extracts of the species *S. macranthera* I & B, *S. spectabilis* var. *excelsa* I & B.

## 2. Results and discussion

### 2.1. Previous work

In a previous work, we described the presence of compounds from the classes of flavonoids and alkaloids in the extract of the flowers of *S. splendida*, among them the compounds Apigenin-7-apioglucoside, Quercetin 3,4'-diglucoside, Cassine and Spectaline. For *S. macranthera* extracts, several compounds of the flavonoid classes have been described, among them are Procyanidin, Kaempferol, and Quercetin (Franca et al. 2021).

### 2.2. Antioxidant activity

The results obtained for the antioxidant activity, using the DPPH and ABTS methods and the total content of phenolic compounds, are shown in Table S1. The *S. macranthera* extract showed good antioxidant values in both methods, with IC<sub>50</sub> values close to the standard, especially in the ABTS method, with an IC<sub>50</sub> of  $5.653 \pm 0.20 \mu\text{g/mL}$ ; it also showed a high percentage of phenolic compounds, which is consistent with the LC-MS test that identified six phenolic compounds. The extract *S. spectabilis* also obtained considerable members, standing out in the ABTS method where it presented an IC<sub>50</sub> of  $15,000 \pm 0.59$

$\mu\text{g}/\text{mL}$ . These results suggest that the phenolic compounds are the main responsible factors for the strong antioxidant potential of these plants. These findings are consistent with those of de Silva et al. (2014), which identified good antioxidant activity numbers in root and leaf extracts of *S. macranthera*.

### 2.3. Cytotoxic Activity

The cell lines SNB 19, HCT-116, PC3, and HL60 were used to determine the cytotoxic potential of flower extracts. The results were promising and are presented in the Table S2 and Table S3. The *S. spectabilis* extract showed an IC<sub>50</sub> value that is considered very strong against the prostate cancer lineage (PC3) and a moderate value for the leukemic cell lines (HL60), with an IC<sub>50</sub> ranging from 21.5 to 37.08  $\mu\text{g}/\text{mL}$ . The *S. macranthera* did not show significant results. The good performance of the *S. spectabilis* extract may be related to the presence of compounds from alkaloid classes, such as carnavaline, cassine, spectaline, and 3-O-feruloylcassine (Kamo et al. 2003). According to Selegato et al. (2017), cyclic nitrogen compounds have a strong antitumor action.

### 2.4. Acetylcholinesterase activity

The results were evaluated from the formation of white halos at the point where the samples were applied. Both extracts inhibited the AChE enzyme (Table S4) and the best result was obtained by the *S. spectabilis* extract, with 14 mm of inhibition. This excellent outcome of the *S. spectabilis* extract may be related to the antioxidant power of the combination of compounds from the flavonoid and alkaloid classes, since Alzheimer's disease is characterized by a great oxidative stress in the brain region, caused by radicals that are reduced by compounds of these classes of substances (Morais et al. 2017).

## 3. Conclusion

To our knowledge, this is the first work that relates the study of the chemical composition and the biological activities of flower extracts from *Senna spectabilis* and *Senna macranthera*. The samples presented expressive biological actions with emphasis on the extract of *S. spectabilis* var. excelsa that showed strong cytotoxic and anticholinesterase action. These results suggest that *Senna* spp. flower extracts are an important source of bioactive compounds with remarkable antioxidant, cytotoxic and anticholinesterase actions.

## Conflict of Interest

The authors declare that they have no conflict of interest.

### Acknowledgements

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## Material suplementar

Chemical composition, antioxidant content, enzyme inhibition, and cytotoxic potential of flower extracts of *Senna spectabilis* var *excelsa* and *Senna macranthera*

### **1. Materials and Methods**

#### **1.1. Chemicals and reagents**

The solvents acetonitrile and methanol (J. T. Baker, USA) are of chromatographic grade. Formic acid (Synth, Brazil) is of analytical grade (ACS). Ultrapure water was obtained using a Millipore water purification system (Millipore, USA).

#### **1.2. Plant material**

The flowers of the *S. macranthera* and *S. spectabilis* var. *excelsa*, species were collected between the months of May and June 2017 from the cities of Quixeramobim and Senador Sá in the state of Ceará, Brazil. All these plant species have been authenticated by the Prisco Bezerra Herbarium of the Federal University of Ceará under the numbers 60424 and 60419. All are registered in the National System for the Management of Genetic Heritage (SISGEN; #AB06D11).

#### **1.3. Sample preparation**

After the collection, the flowers were dried in an oven at 40°C and ground. The dried and ground samples were extracted with the aid of an ultrasound (UAE) in the proportion of 1 g of plant material to 30 mL of methanol for 45 minutes. The extracts were filtered and then concentrated under vacuum on a rotary evaporator. The extracts were dried, weighed, and stored at -18°C until analysis.

#### **1.4. Total phenolic content**

The determination of the total phenolic content was carried out using the Folin-Ciocalteu spectrometric method according to Maia et al. (2017) with modifications. Initially, 9.0 mg of each sample was dissolved in 9 mL of methanol, 7.5 mL of this mixture was transferred to a flask and its volume was adjusted to 50 mL with methanol. A 50 µL aliquot of this solution was stirred with 250 µL of the Folin-Ciocalteu reagent and 3.0 mL of distilled water for 1 minute. After that, 1 mL of 15% sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) was added to the mixture and stirred for 30 seconds. Finally, the solution had its volume adjusted to 5 mL with

distilled water. After 30 min, the absorbance of the samples was read at 750 nm using a 700 plus UV-VIS FEMTO spectrophotometer. The tests were performed in triplicate.

### ***1.5. Determination of DPPH radical scavenging activity***

The evaluation of the free radical scavenging capacity of plant extracts was carried out using 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) as described by Yepez et al. (2002). The extract solutions (0.1 mL) in the concentrations of 1, 5, 10, 50, 100, 500, 1000, and 50000 ppm were added with 3.9 mL of methanol solution containing DPPH radical. After 60 minutes, the absorbances were measured using a UV-Vis spectrophotometer at 515 nm. The inhibition of free radical (DPPH) in percentage (I%) was calculated according to the formula:

$$\text{IV\%} = (\text{Acontrol} - \text{Asample}) / \text{Acontrol} \times 100;$$

in which Acontrol is the absorbance of the control reaction (containing all reagents with the exception of the mushroom extracts) and Asample is the absorbance of the test compound. The values of IV% and their respective concentrations were used to calculate the efficient concentration that inhibits 50% of the free radicals in the test system ( $\text{IC}_{50}$ ). The tests were carried out in triplicate. Rutin was used as a positive control.

### ***1.6. Determination of ABTS radical scavenging activity***

The samples' potential to inactivate the diammonium salt of the radical 2,2'-azinobis-(ethylbenzo-thiazoline-6-sulfonic) of the radical cation (ABTS) was estimated according to Martins et al. (2018). The ABTS solution was prepared by mixing 5 mL of the standard ABTS radical (7 mmol.L-1) with 88  $\mu$ L of potassium persulfate (140 mmol.L-1). The mixture was then stirred and kept in the dark for 16 h at room temperature. After that, the mixture was diluted with ethanol to give an absorbance of  $0.70 \pm 0.02$  units at 734 nm using a spectrophotometer. The extract solutions were prepared at a concentration of 5000, 1000, 500, 100, 10, and 5  $\mu$ g/mL with PA ethanol. Then, 30  $\mu$ L of these solutions were removed, added to 3.0 mL of the ABTS radical solution, and allowed to react for 6 min. The reading was performed at 734 nm and the capacity for scavenging free radicals was expressed by  $\text{IC}_{50}$  values ( $\mu$ g/mL), which were determined by using the same equation described previously for the DPPH method. All measurements were performed in triplicate by using rutin as standard.

### ***1.7. Cell culture and cytotoxicity assay***

The cytotoxic activity of the flower extracts was evaluated in the following tumor lines: HCT-116 (colon - human), SNB-19 (glioblastoma), PC3 (prostate), and HL60 (leukemia). The first three were provided by the National Cancer Institute (USA) and the HL60 line was acquired by the Rio de Janeiro Cell Bank (BCRJ). The tumor lines were grown in RPMI 1640 medium and they were supplemented with 10% fetal bovine serum and 1% antibiotics. All cells were kept in an oven at 37 °C and an atmosphere containing 5% of CO<sub>2</sub>. The cells were plated at concentrations of 0.7 x 10<sup>5</sup>, 0.1 x 10<sup>6</sup>, and 0.3 x 10<sup>6</sup> cells/ml for the HCT-116, SNB-19/PC3, and HL60 lines, respectively. Subsequently, the samples were solubilized in dimethyl sulfoxide (DMSO) and diluted in cell culture medium (100 µg/mL).

The plates were incubated with the substance for 72 hours in an oven at 5% CO<sub>2</sub> at 37 °C. At the end of this process, they were centrifuged and the supernatant was removed. Then, 100 µL of the MTT solution (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (tetrazolium salt) was added and the plates were incubated for 3 h (Mosmann 1983). After incubation, the plates were again centrifuged to remove the MTT solution. The absorbance was read after dissolving the formazan precipitate with 100 µL of pure DMSO in a plate spectrophotometer at 595 nm. The samples that showed a percentage of cell growth inhibition above 75% in at least two of the cell lines tested were then diluted in (1.56, 3.13, 6.25, 12.5, 25, 50, and 100 µg/mL) DMSO and the process was repeated. The absorbance values of these samples were converted to a half of the maximum inhibitory concentration (IC<sub>50</sub>). All experiments were carried out in triplicate.

### **1.8. Anticholinesterase activity assay**

This activity was performed according to the method described by Elmann et al. (1961), with adaptations; 5 µl aliquots of the extracts were prepared at a concentration of 10 mg/mL and applied to a chromatographic plate (DC-Alufolien, Silicagel 60 F254, 0.2 mm Merck). After the complete evaporation of the solvent, a 1:1 mixture of acetylcholine:iodide (ATCI) 1 mmol.L<sup>-1</sup> was sprayed with Ellman's reagent (5.5'-Dithiobis-(2-nitrobenzoic acid, DTNB, 1 mmol.L<sup>-1</sup>) and left standing for 3 minutes for drying, then the enzyme acetylcholinesterase (18 U/ml) was sprayed. After 10 minutes, a yellow color appeared. There was the formation of a white halo around the points where the samples were applied, which indicates enzyme inhibition. As a positive control, the eserina salt standard solution (1 mg/mL) was used.

### **1.9. Statistical analysis**

The single concentration experiments were analyzed according to the mean  $\pm$  standard deviation (SD) of the percentage of cell growth inhibition using the GraphPad Prism 5.0 program.

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Table S1. Antioxidant activity of the extract of *Senna* spp.

Extract	DPPH	ABTS	TPC
	(IC <sub>50</sub> µg/mL)	(IC <sub>50</sub> µg/mL)	(GAE. g <sup>-1</sup> dry extract)
Sm	6.295 $\pm$ 0,20	5.653 $\pm$ 0.20	651.275 $\pm$ 60.47
Sse	45.05 $\pm$ 1,76	15.00 $\pm$ 0.59	102.306 $\pm$ 14.52
Rut*	4.645 $\pm$ 0.01	2.176 $\pm$ 0.02	-

Sm = *S. macranthera*; Sse = *S. spectabilis* var. *excelsa*. Rut= rutin. IC<sub>50</sub>: content of the extract able to inhibit 50% of DPPH radicals. TPC (total phenolic compound) is expressed in mg gallic acid equivalent per gram of the extract.

Table S2: Average percentage of cell growth inhibition (GI%) in single concentration 100 µg/mL.

<b>Sample</b>	<b>SNB</b>	<b>HCT116</b>	<b>PC3</b>	<b>HL60</b>
Sm	2.67 ± 3.73	71.18 ± 2.25	28.53 ± 3.81	86.56 ± 2.76
Sse	97.44 ± 1.11	99.51 ± 0.22	100 ± 0.08	87.91 ± 1.97

Sm = *S. macranthera*; Sse = *S. spectabilis* var. *excelsa*. HCT-116 (Colon - human), SNB-19 (Glioblastoma), PC3 (Prostate) and HL60 (Leukemica)

Table S3: Cytotoxic activity of flower extracts from *Senna* ssp.

<b>Sample</b>	<b>MTT</b>			
	<b>HL60</b>	<b>HCT-116</b>	<b>SNB-19</b>	<b>PC3</b>
Sse	31.37 (26.7-37.0)	37.08 (29.3-47.0)	32.9 (24.0-45.2)	21.5 (17.9-25.9)

$IC_{50}$  was defined as the satisfactory concentration to obtain 50% of the maximum inhibitory effect on cell viability. Sse = *S. spectabilis* var. *excelsa*. HCT-116 (Colon - human), SNB-19 (Glioblastoma), PC3 (Prostate) and HL60 (Leukemica).

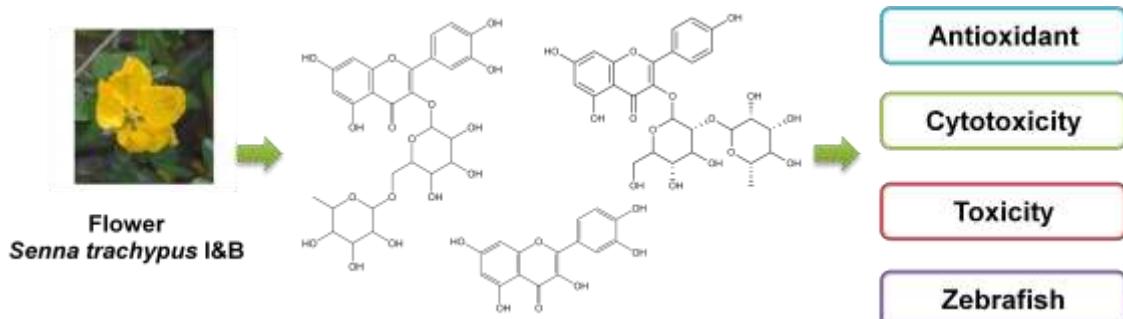
Table S4: Halo diameter of the acetylcolinesterase AChE inhibition of extract from *Senna* ssp.

<b>AChE inhibitor</b>	<b>Result</b>	<b>Halo diameter (mm)</b>
Sm	Positive	7
Sse	Positive	14
Eserin	Positive	10

Sm = *S. macranthera*; Sse = *S. spectabilis* var. *excelsa*.

**8 CHEMICAL COMPOSITION, ANTIOXIDANT CONTENT, *IN VITRO* CYTOTOXIC POTENTIAL AND ASSESSMENT OF TOXICITY EFFECT OF *SENNA TRACHYPOUS* FLOWER IN ADULT ZEBRAFISH (*DANIO RERIO*)**

A ser submetido na Fitoterapia



**Chemical composition, antioxidant content, *in vitro* cytotoxic potential and assessment of toxicity effect of *Senna trachypus* flower in adult zebrafish (*Danio rerio*)**

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**Abstract**

The aim of this study was to evaluate the content of phenolic compounds, antioxidant content, cytotoxic activitie, toxicity and locomotor activity in adult zebrafish (aZF) of flower extract from *Senna trachypus*. The UPLC-QTOF-MS technique was used to identify the compounds. The antioxidant activities were performed by DPPH and ABTS methods.. Cytotoxicity was tested against human tumor cell lines HCT-116 (colon - human), SNB-19 (glioblastoma), PC3 (prostate), and HL60 (leukemia) using the MTT method. aZF were used to assess the acute toxicity of the extract and the animals' locomotor activity. The investigation of the chemical composition of the extract by UPLC-QTOF-MS led to the identification of compounds belonging to the flavonoid class, with rutin being the major compound. The extract showed good antioxidant activity with IC<sub>50</sub> value 3.396±0.17 µg/mL in the DPPH method. The evaluation of cytotoxic activity demonstrated significant results against the tested cell lines, with more expressive cytotoxicity against the HL60 cell with IC<sub>50</sub> of 50.8 µg/ml. The results showed that the extract was not toxic against aZF within 96h of analysis and did not alter of locomotor activity of the animals. These results suggest that the studied extract is promising source of natural antioxidant, showing a positive relationship between chemical composition, cytotoxic and toxicity activities.

**Keywords:** *Senna trachypus*, flower, cytotoxicity, flavonoids, adult zebrafish

## 1. Introduction

Cancer is still the leading cause of death in the world, totaling about 8.7 million deaths per year. Cancer is part of a group of diseases with a common characteristic of uncontrolled cell proliferation, determined by several genetic, epigenetic and biochemical causes. An estimated 18 million new cases of cancer arose and 9.6 million deaths occurred worldwide in the year 2018 (Bray et al., 2018). Even with improvements in treatments over time, there are still many remaining shortcomings such as the high toxicity cost of current anticancer drugs. Chemotherapy, the best-known treatment for cancer, often causes adverse side effects and does not control its progression. Thus, the search for natural products for cancer therapy represents an area of great interest (Percia et al., 2020).

Antioxidants are substances capable of reducing or preventing the oxidation of other chemical substances (Wei et al., 2021). Antioxidant substances can work by inhibiting reactive oxygen species (ROS). ROS are metabolic by-products that can trigger various pathologies such as cancer (Hadi et al., 2021). Antioxidant compounds occur naturally in plants, are safer and more abundant than synthetic ones (Ersoy et al., 2020). Compounds of the flavonoid class are excellent antioxidants that act by different mechanisms, for example, scavenging radicals or binding to metal ions and inhibiting the enzyme systems responsible for generating free radicals. Flavonoids have already been shown to be effective as cytotoxic agents against cancer cells in different mechanisms, which generates great interest in the development of flavonoid-based cytostatics for anticancer therapy (Oliveira et al., 2020, Sghaier et al., 2011).

Research has shown that species of the genus *Senna* are rich sources of compounds from the flavonoid class such as rutin, quercetin, apigenin, kaempferol, among others (Nascimento et al., 2020). These metabolites may be related to important biological activities, such as antioxidant, anti-inflammatory, antimicrobial, antifungal, antimalarial, hepatoprotective and anticancer (Oladeji et al., 2021). Most studies with species of this genus are carried out with leaves, branches or roots, but recent work has shown that flowers are also invaluable sources of secondary metabolites (Franca et al., 2021). Liquid chromatography coupled with mass spectrometry (LC-MS) represents a quick method and a useful tool to identify biologically active substances in plant extracts, contributing to the improvement of cancer treatment in the next decade (Rabêlo et al., 2021).

Animal models are widely used in pharmacological tests in drug screening as well as in the assessment of toxicity. The zebrafish stands out in this scenario because it is easy to handle, has high genetic similarity with humans (70–80%) and is low cost (Gonçalves et al., 2020). Works involving the use of zebrafish in the analysis of the effect of extracts from *Senna alexandrina* Mill leaves and its cytokine suppression fractions have already been reported by Yuniarto et al., 2019. Researches also report that the adult zebrafish model can be used to determine the toxicity of plant extracts (Batista et al., 2018).

In this context, the present study aimed to evaluate the content of phenolic compounds, antioxidant content, cytotoxic activity, toxicity and locomotor activity in adult zebrafish (aZF) of flower extract from *Senna trachypus*.

## **2. Materials and Methods**

### **2.1. Chemicals and reagents**

The solvents acetonitrile and methanol (J. T. Baker, USA) are of chromatographic grade. Formic acid (Synth, Brazil) is of analytical grade (ACS). Ultrapure water was obtained using a Millipore water purification system (Millipore, USA). Diazepam (Teuto), dimethylsulfoxide (DMSO; Dynamic®; 3%), Folin-Ciocalteu, 2,2'-azinobis-(ethylbenzothiazoline-6-sulfonic) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethyl alcohol PA (99.5%; Dynamic®), Ferric chloride, gallic acid.

### **2.2. Plant material**

The flowers of the *S. trachypus* was collected between the months of May and June 2017 in Caucaia, state of Ceará, Brazil. All plant species have been authenticated by Herbário Prisco Bezerra (EAC) of the Federal University of Ceará under the number 60432. All are registered in the National System for the Management of Genetic Heritage (SISGEN) under the number AB06D11.

### **2.3. Sample preparation**

After collection, the flowers were dried in an oven at 40°C and ground. The dried and ground samples were extracted with the aid of an ultrasound (UAE) in the proportion of 1 g of plant material to 30 mL of methanol for 45 minutes. The extracts were filtered and then concentrated under vacuum on a rotary evaporator. The extracts were dried, weighed, and

stored at -18°C until analysis. 26.6% of extraction yield of methanol extract of *S. trachypus* flowers was obtained.

#### **2.4. *Chemical Analysis***

The analysis was performed on an ACQUITY Ultra Performance LC system equipped with Q-Tof Premier LC- MS-MS spectrometer (Waters). The analysis of chromatographic profile was performed using an ACQUITY UPLC® HSS T3 column (Waters, 100 2.1 mm, 1.8 µm), temperature of 40 °C, and injection volume of 1 µl. Elution was performed using a binary gradient system, which consisted of [A] acetonitrile (Synth, Brazil) and [B] water, both containing 0.1% formic acid (Synth, Brazil), applied at flow rate of 0.58 µl/min. The gradient elution was set as follows: 3/45/100/100 %B in 0/4.34/5.78/6.50 min. The data were collected in centroid mode, using a lock spray frequency of 10 s, with an average of 10 scans, in both positive and negative ionization modes (Franca et al., 2021)

#### **2.5 *Total phenolic content***

The determination of the total phenolic content was carried out using the Folin-Ciocalteu spectrometric method according to Maia et al. (2017) with modifications. Initially, 9.0 mg of extract sample was dissolved in 9 mL of methanol, 7.5 mL of this was transferred to a flask and its volume was adjusted to 50 mL with methanol. A 50 µL aliquot of this solution was stirred with 250 µL of the Folin-Ciocalteu reagent and 3.0 mL of distilled water for 1 minute. After that time, 1 mL of 15% sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) was added to the mixture and stirred for 30 seconds. Finally, the solution had its volume adjusted to 5 mL with distilled water. After 30 min, the absorbance of the samples was read at 750 nm using a 700 plus UV-VIS FEMTO spectrophotometer. The tests were performed in triplicate.

#### **2.6. *Determination of DPPH radical scavenging activity***

The evaluation of the free radical scavenging capacity of plant extracts was carried out using 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) as described by Yepez et al. (2002). The extract solutions (0.1 mL) in the concentrations of 1, 5, 10, 50, 100, 500, 1000, and 50000 ppm were added with 3.9 mL of methanol solution containing DPPH radical. After 60 minutes, the absorbances were measured using a UV-Vis spectrophotometer at 515 nm. The inhibition of free radical (DPPH) in percentage (I%) was calculated according to the formula:

$$\text{IV\%} = (\text{Acontrol} - \text{Asample}) / \text{Acontrol} \times 100;$$

where Acontrol is the absorbance of the control reaction (containing all reagents with the exception of the mushroom extracts) and Asample is the absorbance of the test compound. The values of IV% and their respective concentrations were used to calculate the efficiency concentration that inhibits 50% of the free radicals in the test system ( $\text{IC}_{50}$ ). The tests were carried out in triplicate. Rutin was used as a positive control.

### **2.7. Determination of ABTS radical-scavenging activity**

The potential for the samples to inactivate the diamonium salt of the radical 2,2'-azinobis- (ethylbenzo-thiazoline-6-sulfonic) of the radical cation (ABTS) was realized according to Martins et al. (2018). The ABTS solution was prepared by mixing 5 mL of the standard ABTS radical ( $7 \text{ mmol.L}^{-1}$ ) with 88  $\mu\text{L}$  of potassium persulfate ( $140 \text{ mmol.L}^{-1}$ ). The mixture was then stirred and kept in the dark for 16 h at room temperature. After that time, the mixture was diluted with ethanol to give an absorbance of  $0.70 \pm 0.02$  units at 734 nm using a spectrophotometer. The extract solutions were prepared at a concentration of 5000, 1000, 500, 100, 10, and 5  $\mu\text{g/mL}$  with PA ethanol. Then, 30  $\mu\text{L}$  of these solutions were removed, added to 3.0 mL of the ABTS radical solution, and allowed to react with the ABTS solution for 6 min. The reading was performed at 734 nm and the capacity for scavenging free radicals was expressed by  $\text{IC}_{50}$  values ( $\mu\text{g/mL}$ ) determined by using the same equation described previously for the DPPH method. All measurements were performed in triplicate using rutin as standard.

### **2.8. Cell culture and cytotoxicity assay**

The cytotoxic activity of the flower extracts was evaluated in the tumor lines, HCT-116 (colon - human), SNB-19 (glioblastoma), PC3 (prostate), and HL60 (leukemia). The first three were provided by the National Cancer Institute (USA) and the HL60 was acquired by the Rio de Janeiro Cell Bank (BCRJ), having been grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 1% antibiotics. All cells are kept in an oven at 37 °C and an atmosphere containing 5% of  $\text{CO}_2$ . The cells were plated at concentrations of  $0.7 \times 10^5$ ,  $0.1 \times 10^6$ , and  $0.3 \times 10^6$  cells/ml for the HCT-116, SNB-19/PC3, and HL60 lines, respectively. Subsequently, the samples were solubilized in dimethyl sulfoxide (DMSO) and diluted in cell culture medium (100  $\mu\text{g/mL}$ ).

The plates were incubated with the substance for 72 hours in an oven at 5% CO<sub>2</sub> at 37 °C. At the end of this process, they were centrifuged and the supernatant removed. Then, 100 µL of the MTT solution (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (tetrazolium salt) was added and the plates were incubated for 3 h (Mosman 1983). After incubation, the plates were again centrifuged to remove the MTT solution. The absorbance was read after dissolving the formazan precipitate with 100 µL of pure DMSO in a plate spectrophotometer at 595 nm. For samples that showed a percentage of cell growth inhibition above 75% in at least two of the cell lines tested, they were now diluted in (1.56, 3.13, 6.25, 12.5, 25, 50, and 100 µg/mL) DMSO and the process was repeated. The absorbance values of these samples were converted to a half of the maximum inhibitory concentration (IC<sub>50</sub>). All experiments were carried out in triplicate.

## **2.9 Zebrafish**

Wild zebrafish (*Danio rerio*) (ZFa), both sexes, aged 60-90 days, sizes of  $3.5 \pm 0.5$  cm and weight  $0.4 \pm 0.1$  g, obtained from Comércio de Produtos Veterinário LTDA, a supplier located in Fortaleza (Ceará, Brazil). Groups of 50 fish were acclimatized for 24 h in glass aquaria (40 x 20 x 25 cm) containing dechlorinated water (ProtecPlus® anti-chlorine) and air pumps with submerged filters, at 25 °C and pH 7.0, with a circadian cycle of 14: 10 h of light/dark. The fish were fed (Spirulina®) ad libitum 24 h before the experiments. After the experiments, the animals were sacrificed by immersion in cold water (2-4 °C), for 10 minutes, until the loss of opercular movements (Concea, 2018). All experimental procedures were approved by the animal ethics committee of the State University of Ceará (CEUA-UECE), under protocol No. 3344801/2017.

### **2.9.1. General protocol**

The zebrafish tests were performed based on methodologies proposed by (Magalhães et al., 2017). On the day of the experiments, the fish were selected randomly, transferred to a wet sponge and treated with the test or control samples via intraperitoneal (*i.p.*). Then, the animals were individually placed to rest in glass cups (250 mL) containing 150 mL of aquarium water. For treatments via *i.p.*, an insulin syringe (0.5 mL; Ultra FinaTM BD) with a 30G needle was used.

### **2.9.2. Acute toxicity 96 h**

The study of acute toxicity was performed against adult zebrafish (*Danio rerio*) according to OECD guidelines (OECD, 1992) and Batista et al. (2019). The animals (n = 6/each) were treated with 20 µL (*i.p.*) of flower extracts (4.0 or 2.0 or 40 mg/kg) or Control (3% DMSO, vehicle) and left at rest for the mortality rate to be analyzed. After 96 hours of treatments, the number of dead fish in each group was recorded and the lethal dose capable of killing 50% of the animals (LD<sub>50</sub>) was determined using the mathematical method Trimmed Spearman-Karber with a 95% confidence interval (Arellano-Aguilar et al., 2015).

#### **2.9.3. Locomotor activity (Open Field Test)**

The open field test was carried out (Magalhães et al., 2017) to assess whether changes occurred or not in the animals' motor coordination, either by sedation and/or muscle relaxation. Initially, the animals (n= 6/group) were treated with 20 µL (*i.p.*) of flower extracts (4.0 or 2.0 or 40 mg/kg) or vehicle (3% DMSO). A group of animals without treatments was included (Naive). After 30 min of the treatments, the animals were added in glass Petri dishes (10 x 15 cm), containing the same water from the aquarium, marked with four quadrants, and the locomotor activity was analyzed by counting the number of crossing lines (CL). Using the CL value of the Naive group as a baseline (100%), the percentage of locomotor activity (AL%) was calculated individually for 0-5 minutes.

#### **2.10. Statistical analysis**

The results were expressed as values of the mean ± standard error of the mean for each group of 6 animals. After confirming the normal distribution and homogeneity of the data, the differences between the groups were subjected to analysis of variance (one-way ANOVA), followed by the Tukey test. All analyzes were performed using the GraphPad Prism software v.5.0.1. The level of statistical significance was set at 5% (p <0.05).

### **3. Results and discussion**

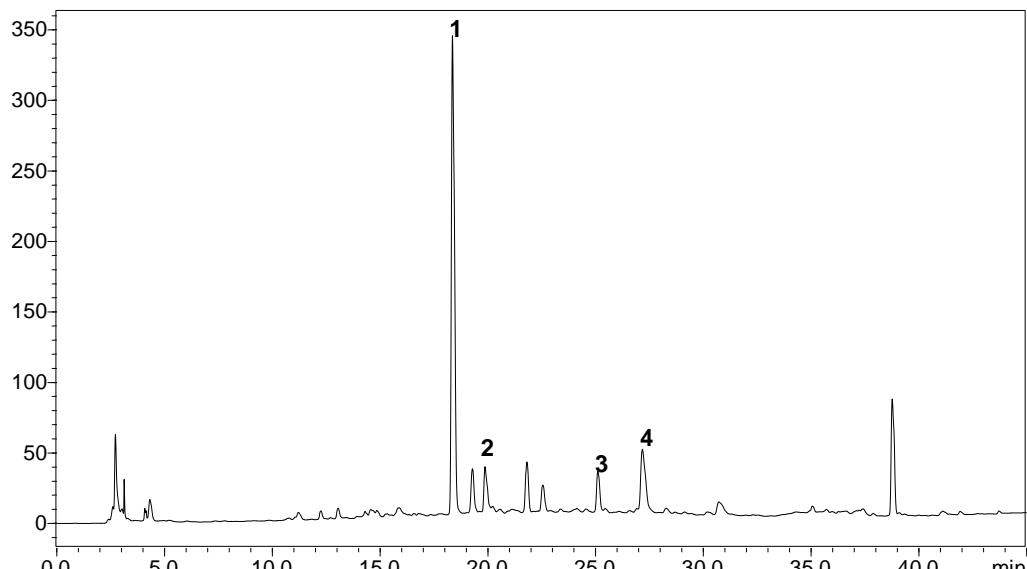
#### **3.1. Chemical analysis**

The extract obtained from the flowers of *S.trachypus* was analyzed by HPLC-DAD and by UPLC-QTOF-MS, in order to obtain more robust data. The identification of compounds was made by comparing tR (min) and UV/Vis with standards of secondary metabolites available in the laboratory and by comparing the obtained fragments with literature data. This analysis led to the identification of four compounds, all belonging to the

phenolic compounds class. The identified compounds are shown in Table 01. The chemical profile obtained is shown in Figure 1.

Compound 1 has been identified as rutin (quercetin rutinoside) with a precursor ion at m/z 609.1454 [M-H]<sup>-</sup>. The standard fragmentation of molecule 1 is consistent with that previously published by Makita et al. 2016 and Sobral et al. (2019). Compound 2 which has m/z 593.1504 as precursor ion was identified as Kaempferol-3-O-glucorhamnoside. The characteristic fragmentation of 284,0315 is justified by the cleavage of the glycosidic bond (Chen et al., 2015). Compound 3 identified as Narirutin has precursor ion m/z 579.1718 [M-H]<sup>-</sup>. The formation of fragment ions 151,0682, 459,1278 follows the same explanation given for the first two compounds (Zengin et al., 2018; Su et al., 2010). Finally, compound 4 was identified as quercetin, a very common compound in *Senna* species that has precursor ion m/z 303.0430 [M+H]<sup>+</sup> (Jang et al. 2016; Dong et al., 2017).

Figure 1: Representative HPLC-DAD chromatogram extract from *S. trachypus* flower



Compound: 1= rutin; 2=Kaempferol-3-O-glucorhamnoside; 3= Narirutin; 4= quercetin

### 3.2 Determination of antioxidant activity and total phenol contents

The extract of *S. trachypus* flowers showed expressive antioxidant activity both by the DPPH method ( $IC_{50} = 3,396 \pm 0.17 \mu\text{g/mL}$ ) and by the ABTS method ( $IC_{50} = 5.8 \pm 0.08 \mu\text{g/mL}$ ). With values very close to the standard rutin  $IC_{50} = 4.645 \pm 0.01 \mu\text{g/mL}$  in DPPH and  $IC_{50} = 2.176 \pm 0.02 \mu\text{g/mL}$  in ABTS (Table 01).

Table 01. Antioxidant activity of *S. trachypus* flower extract.

Sample	DPPH (IC <sub>50</sub> : $\mu$ g/mL)	ABTS (IC <sub>50</sub> : $\mu$ g/mL)	TPC (GAE. g <sup>-1</sup> dry extract)
Extract	3.396 ± 0.17	5.8 ± 0.08	469.881 ± 26.95
Rut*	4.645 ± 0.01	2.176 ± 0.02	-

St = *S. trachypus*. Rut= rutin. IC<sub>50</sub>: content of the extract able to inhibit 50% of DPPH radicals. TPC (total phenolic compound) is expressed in mg gallic acid equivalent per gram of the extract.

The results of antioxidant activity are consistent with the high content of phenolic compounds found in this extract 469.881 ± 26.95 GAE. g<sup>-1</sup> dry extract. A high phenolic content automatically has a high content of antioxidants, since phenolic compounds are excellent natural antioxidants (Monteiro et al., 2018). Phenolic compounds are the main class of secondary metabolites responsible for the antioxidant action against free radicals (Colantuano et al., 2017). These results are considered positive and are also in agreement with the metabolites identified, mainly flavonoids such as rutin, quercetin and kaempferol-3-O-glucorhamnoside, the compound rutin has already been widely reported for its antioxidant potential and as we can see in Figure 1 it is the marjoritarian compound (Oliveira et al., 2020).

### 3.3 Cytotoxic Activity

Cytotoxicity assays are commonly the first methods performed to assess a compound's biological potential (Custer & Sweder, 2008). The MTT assay is a commonly used screening method to measure cell viability (Mosmann, 1983). In this work, samples were initially tested at a single concentration of 100  $\mu$ g/mL in cell lines SNB19, HCT116, PC3 and HL60 and EC<sub>50</sub> were calculated from the dose-response curve. It was observed that the ethanol extract of the flowers of *S. trachypus*, presented a percentage of cell growth inhibition (GI) above 70% in the cell lines SNB-19, HCT-116 and HL60 being then indicated to evaluate the concentration sufficient to obtain 50% of the effect maximum inhibitory IC<sub>50</sub> (Table 02).

Table 02: Cytotoxic activity of *S. trachypus* flower extract

Test	MTT			
	HL60	HCT-116	SNB-19	PC3
GI% (SD)	77.72 ± 2.12	72.65 ± 1.63	72.51 ± 3.76	52.93 ± 3.46
CI <sub>50</sub> $\mu$ g/mL	50.8	91.6	79.2	>100
(SD)	(44.4-58.1)	(78.6-106.8)	(58.3-107.6)	-

GI% percentage of cell growth inhibition; IC<sub>50</sub> was defined as the concentration sufficient to obtain 50% of the maximum inhibitory effect on cell viability; SD – standart desviation; HL60 (Leukemica); HCT-116 (Colon - human); SNB-19 (Glioblastoma); PC3 (Prostate).

For IC<sub>50</sub> tests, samples were tested in serial dilution on HL60, HCT-116, SNB-19 and PC3 lines starting with a maximum concentration of 100 µg/mL. Data investigation showed that the tested extract showed expressive cytotoxic potential against the HL60 cell line (Leukemica). In this cell line, the IC<sub>50</sub> found ranged from 44.4-58.1 µg/mL. This result may also be associated with the presence of flavonoids in this extract. It is widely described in the literature that flavonoids are excellent cytotoxic agents that also act in the chemoprevention of some types of cancer (Sharmila et al., 2014; Takemura et al., 2013). These results are in agreement with those carried out by Sánchez-Gutiérrez et al (2020) who identified the *Asclepias linaria* Cav. the flavonoids rutin and quercetin, this extract showed good cytotoxic activity against HL60 tumor cells which may also be related to its chemical composition.

### **3.4     Toxicity and Locomotor activity evaluation (Open Field Test)**

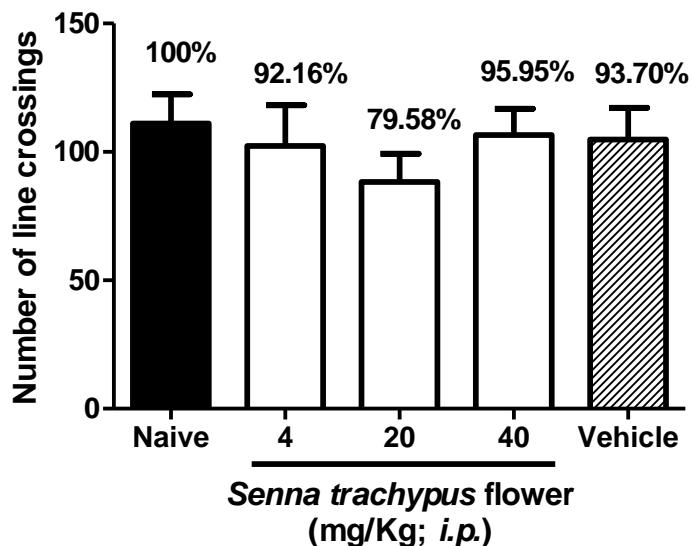
Plant species have a wide variety of secondary metabolites that can be potentially toxic to humanity. To ensure safety, studies are needed to show the safety profiles of certain studied plant species (Sharif et al., 2015; Kale et al., 2019).

Animal toxicity tests provide toxicity information relevant to the studied environment, serving as a transition between a cellular and human assessment (Muthulakshmi et al., 2018). In recent years, zebrafish have been used as the preferred vertebrate animal model in assessing the toxicity of compounds or drugs (Haque and Ward, 2018; Xia et al., 2018).

The toxicity test of plant extracts has already been reported in the literature by Batista et al., 2018, the researchers analyzed the toxicity of extracts from the fruit of neem (*Azadirachta indica* A. Juss.) using the adult zebrafish model. The acute toxicity of samples of the ethanol extract of *S. trachypus* flowers is being reported for the first time in the literature through the animal model of adult zebrafish. The extract was not toxic against adult zebrafish in 96 h of analysis, since there was no mortality of animals treated with the extract (4.0 or 2.0 or 40 mg/kg; i.p.). The results of this work are similar to those developed by Ferreira et al (2019) and Lira et al (2020) who also used zebrafish for the toxicity test. These results are important since the absence of toxicity points to a possible herbal use.

The open field test was adapted by Magalhães et al. (2017) as an alternative method to the use of rodents to investigate the possible effects that the test drugs may have on the

central nervous system of adult zebrafish, causing or not locomotor impairment. Here, we evaluated the same test and extract of *S. trachypus* flower no interference in the locomotor activity of animals (Figure 2). These results suggest that all concentration tested no significant alterations in fish motor coordination, sedation, or muscle relaxation. Batista et al. (2018), Silva et al (2020) and Bezerra et al (2020) also used the open field test to assess locomotor activity in the adult zebrafish model.



**Fig. 2.** Effect of methanolic extract of *Senna trachypus* flower on the locomotor activity of adult zebrafish in the open-field test (0-5 min). The numbers above the columns indicate percentage of locomotor activity. Naïve-control 1 (untreated group). Vehicle (3% DMSO; 20 µL; *i.p.*). One-way ANOVA with Tukey post hoc test. The values represent the mean ± standard error of the mean for 6 animals/group. The numbers above each column indicate the percentage of locomotor activity (% LA).

#### 4. Conclusion

The methanolic extract of *S. trachypus* flower is rich in flavonoids (rutin, Kaempferol-3-*O*-glucorhamnoside, Narirutin e quercetin), justifying the expressive antioxidant activity against DPPH, potential cytotoxicity against the tested cell lines, with more expressive cytotoxicity against the HL60 (Leukemic), and did not exert acute toxicity against zebrafish and no interference in the locomotor activity of animals. These results encourage the continuation of the study aiming to isolate and characterize the active agent. Therefore, this

study adds new scientific evidence and highlights the potential of the the *S. trachypus* flower in the development of phytomedicines with citotoxicity properties.

### **Conflict of Interest**

The authors declare that they have no conflict of interest.

### **Acknowledgements**

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## 9 CONSIDERAÇÕES FINAIS

Este trabalho relatou a documentação química e ensaios farmacológicos com extratos de plantas do gênero *Senna* spp. Um método abrangente, aplicável a diversas espécies foi desenvolvido e possibilitou a identificação de compostos das principais classes de metabólitos, já relatados para o gênero, como alcaloides, flavonoides e antraquinonas nos extratos de sete espécies analisadas (*Senna cearensis*, *Senna macranthera*, *Senna obtusifolia*, *Senna reticulata*, *Senna spectabilis* var. *excelsa*, *Senna splendida* e *Senna trachypus*), por meio da técnica UPLC-MS mostrando sua eficiência. O artigo resultante desse trabalho foi publicado na *Revista Brasileira de Farmacognosia* (<https://doi.org/10.1007/s43450-021-00137-6>). Um segundo artigo produzido apresenta a busca por minimizar as etapas envolvidas na preparação de amostras e tornar o processo mais verde. Para isso foi aplicado à técnica de extração online (OLE) para a obtenção de perfis químicos das espécies de *Senna* spp. Os resultados obtidos sugerem que a técnica de extração online é eficiente, fornecendo um perfil químico completo das espécies de *Senna*. Foi observado ganhos de qualidade expressivos no rendimento e no aumento nas intensidades relativas de compostos pertencentes às principais classes já relatados para esse gênero. Esses resultados serão submetidos no *Journal of Separation Science*. A análise das atividades antioxidante, citotóxica e anticolinesterásica das flores das espécies de *Senna macranthera* e *Senna spectabilis* var. *excelsa* e a sua relação com a composição química também foi realizada, mostrando resultados interessantes. Esses resultados possibilitaram a elaboração de um terceiro artigo submetido a *Natural Product Research*. O extrato das flores de *Senna trachypus* também foi avaliado quanto a sua composição química e a relação com o conteúdo antioxidante, potencial citotóxico e avaliação do efeito da toxicidade em peixes-zebra adultos (*Danio rerio*), os resultados promissores foram escritos dando origem ao quarto artigo apresentado nessa tese que será submetido na revista *Fitoterapia*. Assim, que esta pesquisa contribuiu para o conhecimento e a valorização das espécies de *Senna*, e relata seu potencial como fonte de compostos bioativos.

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