UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE – UFCSPA
PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA

Jeferson Gustavo Henn

Desenvolvimento de um extrato hidroetanólico das folhas de *Plantago australis* (Kunth) Rahn padronizado em verbascosídeo e determinação de sua segurança toxicológica

Porto Alegre

2016

Jeferson Gustavo Henn

Desenvolvimento de um extrato hidroetanólico das folhas de *Plantago australis* (Kunth) Rahn padronizado em verbascosídeo e determinação de sua segurança toxicológica

Dissertação submetida ao Programa de Pós-Graduação em Patologia da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre

Orientador: Dra. Dinara Jaqueline Moura
Co-orientadora: Dra. Jenifer Saffi

Porto Alegre
2016

Catalogação na Publicação

Henn, Jeferson Gustavo
Desenvolvimento de um extrato hidroetanólico das folhas de Plantago australis (Kunth) Rahn padronizado em verbascosídeo e determinação de sua segurança toxicológica / Jeferson Gustavo Henn. -- 2016.
172 p. : 30 cm.

Dissertação (mestrado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Patologia, 2016.

Orientador(a): Dinara Jaqueline Moura ; coorientador(a): Jenifer Saffi.

1. Plantago australis. 2. Verbascosídeo. 3. Extrato hidroetanólico. 4. Validação analítica. 5. Segurança toxicológica. I. Título.

Sistema de Geração de Ficha Catalográfica da UFCSPA com os dados fornecidos pelo(a) autor(a).

“Daher ist die Aufgabe nicht sowohl zu sehen was noch Keiner gesehen hat, als, bei Dem, was Jeder sieht, zu denken, was noch Keiner gedacht hat.”

(Arthur Schopenhauer)

Agradecimentos

À minha orientadora, Profª Dra. Dinara Jaqueline Moura, a quem tive a oportunidade de conhecer durante a graduação, na UNISC, e desde então pude admirar sua capacidade de incitar o conhecimento científico em seus alunos. Querida “Dinja”, obrigado por me proporcionar a concretização deste trabalho e por ter confiado em mim e participado do meu crescimento científico e pessoal, que levarei por toda a vida.

À minha coorientadora e “big boss”, Profª Dra. Jenifer Saffi, por ter me proporcionado a vivência no mundo da genética toxicológica, pelas conversas em francês e por instigar em mim a vontade de ser um pesquisador.

À Dra. Valéria Flores Peres, ou simplesmente “Val”, pelo auxílio no desenvolvimento analítico deste trabalho, por todo o conhecimento repassado e principalmente pela amizade cultivada.

Às Dras. Izabel Vilella, Miriana Machado e Jaqueline Picada, e ao M.Sc. Gustavo Hassemer, pela colaboração na execução deste projeto.

À Helen e Nathalia, por terem feito essa caminhada comigo desde o início e compartilhado as alegrias e angústias diárias da vida de um pós-graduando. Tudo valeu a pena! Contem sempre comigo, onde quer que estejamos! Helen, obrigado pelos cafés, bolos e pelas risadas; por ser como uma irmã e ter encarado comigo as maratonas da seleção e as incansáveis viagens da zona norte até a UFCSPA. “Nathi”, obrigado pela amizade e cumplicidade; pela paciência, pelas “fofuras” e pelos ranços.

À Betânia e ao Rodrigo, meus “filhotes” e alunos de iniciação científica. Serei eternamente grato por terem me dado a oportunidade de repassar meu

conhecimento. Vocês são especiais na minha vida! Aos demais integrantes da “Família Dinja”: Aline, Ana, Bruna I., Giovana, Luiza, Paula T. e Rick. Vocês são a essência e o sentido de uma verdadeira amizade! Obrigado pelas festas, discussões de artigos e pela companhia nos cafés de todos os dias.

Aos colegas e amigos do Laboratório de Genética Toxicológica: Bruna C., Bruna L., Bruna N., Cassiana, Débora, Ducival, Elisa, Fabiana, Franciele, Grasiela, Helena, Isabel Cristina, Jaqueline, Jéssica, Lisiane, Leopoldo, Marcele, Margarida, Marina, Natália B., Natalia M. Natascha, Paula M., Renata, Roberto, Vanessa, Verônica e Victória. Obrigado pela eterna amizade e por terem crescido comigo, dia após dia!

À minha família de Porto Alegre: Aline, Clarissa, Gustavo, Julia, Lucas, “Pina” e Vanessa. Vocês me acolheram e nós nos acolhemos, e nessa mistura de sentimentos pude perceber que família vai além dos laços de sangue... É rir, conversar, discutir, beber, dar adeus, e mesmo assim jamais esquecer a importância que cada um teve!

Ao Thiago, parceiro nos estudos sobre cromatografia, nas “indiadas” com o skate e nas pizzas do Cavanhas. Obrigado por ser como um irmão para mim! Ao Guilherme e à Thamires, amigos desde os tempos de graduação, e que assim como eu encararam o mestrado com muita determinação. Espero que possamos vencer mais obstáculos juntos!

Aos meus amigos e colegas de mestrado: Alessandra, Alexandre, Ana Paula, Daniélle, Elias, Francine, Graziele, Maiquidiéli e Melissa, pela troca de experiências, amizade, descontração e pelas junções no Mariu’s.

À secretária do PPG, Maristela, por sempre estar à disposição para resolver qualquer situação em favor dos alunos. Aos funcionários dos

laboratórios de Farmacotécnica, Bioquímica, Farmacologia e da Central Analítica da UFCSPA, pelo auxílio e cedência de espaço para a realização dos experimentos.

A CAPES, FAPERGS e ao CNPq, pelo auxílio financeiro.

Aos familiares e amigos de Sinimbu e Santa Cruz do Sul, que mesmo de longe sempre me apoiaram e acreditaram em mim, possibilitando a concretização deste trabalho.

Por fim, e de maneira imensurável, agradeço aos meus pais, Maria e Nestor, pelo apoio incondicional nos meus estudos, acreditando no meu esforço, sendo para mim um exemplo de honestidade, simplicidade, coragem e perseverança. Amo vocês!

Apresentação

Este trabalho foi desenvolvido no Laboratório de Genética Toxicológica da Universidade Federal de Ciências da Saúde de Porto Alegre. O projeto foi subsidiado pela Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) e pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

A dissertação está dividida em quatro partes. A primeira parte contém o referencial teórico sobre o tema deste trabalho; na segunda parte é apresentado, no Capítulo I, um artigo de revisão a ser submetido à revista *Phytotherapy Research*; na terceira parte é apresentado no Capítulo II um artigo de dados, a ser submetido à revista *Journal of Ethnopharmacology,* e na quarta parte apresentam-se as considerações finais do trabalho, conclusões, perspectivas e anexos com resultados adicionais e informações sobre as revistas.

Sumário

Lista de abreviaturas utilizadas

AAS: ácido acetilsalicílico

ALT: alanina transaminase

AST: aspartato transaminase

CLAE: cromatografia líquida de alta eficiência

DNA: *deoxyribonucleic acid* (ácido desoxirribonucleico)

DPPH•: radical 2,2-difenil-1-picrilhidrazila

His-: auxotrofia para histidina

His+: prototrofia para histidina

MN: micronúcleo

MTT: (4,5-dimetiltiazol-2-il)2,5-difenil brometo de tetrazolium

NRU: ensaio de captação do vermelho neutro (*neutral red uptake*)

OECD: *Organisation for Economic Co-operation and Development*

(Organização para a Cooperação e Desenvolvimento Econômico)

OMS: Organização Mundial da Saúde

UVA: radiação ultravioleta do tipo A

UVB: radiação ultravioleta do tipo B

XTT: 2,3-bis-(2-metoxi-4-nitro-5-sulfofenil)-5-fenilalanina carbonil-2H-tetrazolium hidróxido

Resumo

Introdução: *Plantago australis* Kunth (Rahn) (Plantaginaceae) é uma planta perene da América Latina, muito encontrada no sul do Brasil e popularmente conhecida como “tansagem”. Suas folhas e sementes são usadas no tratamento de diversas doenças e sintomas, e sua composição fitoquímica inclui inúmeros metabólitos, e dentre eles, o verbascosídeo tem sido amplamente estudado. Pouco se sabe ainda sobre a segurança toxicológica de ambos.

Objetivos: O presente estudo teve o objetivo de desenvolver um extrato hidroetanólico das folhas de *P. australis*, através de planejamentos experimentais, utilizando extração por ultrassom e o verbascosídeo como marcador. Em seguida, foi determinada a segurança toxicológica do extrato e do verbascosídeo em modelos *in vitro*.

Material e métodos: A preparação dos extratos etanólicos (70%) das folhas de *P. australis* foi feita pelos métodos de extração por percolação e ultrassom. A extração pelo ultrassom foi otimizada através de planejamentos experimentais (fatorial completo 2^2 e metodologia de superfície de resposta). As análises por CLAE/DAD visaram à pesquisa dos constituintes fitoquímicos aucubina, baicaleína, ácidos ursólico e oleanólico e verbascosídeo. O método de quantificação do constituinte fitoquímico presente nos extratos foi validado. A segurança toxicológica do extrato otimizado e do constituinte fitoquímico (verbascosídeo) foi avaliada por ensaios de mutagenicidade e genotoxicidade (teste de *Salmonella*/microssoma e ensaio cometa), citotoxicidade (MTT, NRU), além de fototoxicidade para o constituinte fitoquímico presente.

Resultados: Análises preliminares por CLAE/DAD demonstraram a presença do constituinte verbascosídeo nos extratos produzidos. A otimização da extração de verbascosídeo pelo método de extração por ultrassom apresentou maior rendimento com o tempo de extração de 40 min e temperatura do banho-maria de 25 °C. A avaliação *in vitro* da segurança toxicológica revelou que tanto o extrato quanto o verbascosídeo não induzem mutagenicidade e genotoxicidade nas concentrações testadas, enquanto que a citotoxicidade só foi observada nas maiores concentrações de ambos. Além disso, o verbascosídeo não demonstrou fototoxicidade nas concentrações utilizadas no teste.

Conclusão: O conjunto de resultados permitiu elaborar um extrato hidroetanólico das folhas de *P. australis* padronizado em verbascosídeo a 6%. Quanto à segurança toxicológica *in vitro* de ambos, não foram demonstradas atividades genotóxicas e mutagênicas, e a citotoxicidade foi vista apenas em concentrações elevadas, bem como o verbascosídeo não se mostrou fototóxico. Estes resultados embasam a segurança de uso do extrato hidroetanólico e do verbascosídeo.

Abstract

Introduction: *Plantago australis* Kunth (Rahn) (Plantaginaceae) is a perennial plant in Latin America, much found in Southern Brazil and popularly known as "tansagem". The leaves and seeds are used in the treatment of various diseases and symptoms and their phytochemical composition includes numerous metabolites, and among them, verbascoside has been widely studied. Little is yet known about the toxicological safety of both of them.

Objectives: This study aimed to develop a hydroethanolic extract of the leaves of *P. australis*, by experimental design with ultrasound-assisted extraction, using verbascoside as an marker. Then it was determined the toxicological safety of the extract and verbascoside in *in vitro* models.

Material and methods: Preparation of hydroethanolic extract (70%) of *P. australis* leaves was taken by percolation and ultrasound methods. Ultrasound-assisted extraction was optimized by complete factorial design (2^2) and response surface methodology. The analysis by HPLC/DAD aimed to research the phytochemical constituents aucubin, baicalein, oleanolic and ursolic acids and verbascoside. The quantification method of the phytochemical constituent was validated. The toxicological safety of the optimized extract and the phytochemical constituent was evaluated by mutagenicity and genotoxicity tests (Ames test and comet assay), cytotoxicity (MTT and NRU), and a phototoxicity assay was carried out with the phytochemical constituent.

Results: Preliminary analysis by HPLC/DAD showed the presence of the constituent verbascoside in produced extracts. The optimization of verbascoside extraction by ultrasound showed higher performance at the

extraction time of 40 min and water-bath temperature at 25 °C. The *in vitro* evaluation of toxicological safety revealed that both the extract and verbascoside did not induce mutagenicity and genotoxicity in all concentrations tested, while the cytotoxicity was only observed at the highest concentrations. Furthermore, verbascoside showed no phototoxicity in all concentrations used in the test.

Conclusion: The results allowed the development of a hydroethanolic extraction method of the leaves of *P. australis*, standardized in verbascoside (6%). As for *in vitro* toxicological safety of both of them, genotoxic and mutagenic activities were not demonstrated and cytotoxicity was seen only at high concentrations, as well as verbascoside did not show phototoxic effects. These results support the safety use of the hydroethanolic extract and verbascoside.

1. Introdução

A Organização Mundial da Saúde (OMS) considera plantas medicinais aquelas empregadas com a finalidade de prevenir, aliviar, curar ou modificar um estado fisiológico ou patológico, ou como fonte e matéria-prima para fármacos e medicamentos (Rates, 2001). Apesar de apresentarem diferentes alvos e mecanismos de ação, esse grupo tem sido pouco investigado, uma vez que o estudo fitoquímico e biológico de plantas envolve apenas uma pequena parcela do reino vegetal, se comparada ao amplo conhecimento etnofarmacológico e às inúmeras espécies espalhadas pelo mundo (Filho e Yunes, 1998; Simões *et al.*, 2004).

Os produtos naturais fazem parte do cotidiano da humanidade há milhares de anos, e vêm acompanhando desde então o desenvolvimento das civilizações. Como prova disso, podemos citar a medicina tradicional chinesa, com seus diversos preparados vegetais atualmente difundidos pelo mundo, as técnicas químicas egípcias e greco-romanas para a preservação de corpos e o desenvolvimento de venenos utilizados para fins de defesa (Viegas Jr. *et al.*, 2006).

O valor dessa sabedoria popular tem o reconhecimento da medicina moderna, ainda mais quando observada a dificuldade de acesso às políticas públicas de saúde enfrentada em diversos países. Neste contexto, e em razão de fatores como sua notória biodiversidade e o conhecimento empírico indígena trazido desde os tempos da colonização, o Brasil destaca-se como um importante cenário na exploração de plantas medicinais e investigação de novas moléculas com potencial terapêutico (Simões *et al.*, 2004; Calixto, 2005).

Atualmente, a indústria farmacêutica vem resgatando o segmento fitoterápico, aplicando cada vez mais investimentos nessa área. Por esse motivo, nota-se também a intensificação dos esforços para que se tenha uma legislação mais rigorosa e clara referente aos fitoterápicos, garantindo o máximo de segurança no seu uso, e devido à complexidade química apresentada pelas plantas, faz-se necessário um rigoroso controle de qualidade desde o cultivo, passando pela coleta e extração, até a eventual elaboração do medicamento final. Para tal, o aporte financeiro e tecnológico ao estudo botânico, químico e farmacológico dessas espécies é evidente, proporcionando a pesquisa de novas drogas e o aprimoramento da utilização e do registro de fitoterápicos, evitando assim o uso indiscriminado e a possível extinção de muitas espécies vegetais (Turolla e Nascimento, 2006).

1.1. Família Plantaginaceae

A família Plantaginaceae, comumente incluída na ordem Plantaginales, subclasse Asteridae (Cronquist, 1988), foi reclassificada na ordem Lamiales devido a diversos estudos filogenéticos, morfológicos, embriológicos e químicos, que deslocaram os dois demais gêneros para o nível de subgêneros (Rahn, 1996).

Atualmente é aceita a inclusão de 90 gêneros e 2000 espécies em Plantaginaceae (Albach *et al.*, 2005; APG, 2016), que em razão da diversificação evolutiva resultaram nas mais variadas morfologias, sendo que no Brasil são descritos 25 gêneros e 130 espécies. Especificamente sobre o gênero *Plantago*, em torno de 250 espécies são conhecidas mundialmente,

com distribuição principalmente nas regiões temperadas, incluindo o sul e sudeste do Brasil. No Brasil existem 16 espécies do gênero *Plantago*, popularmente conhecidas como tansagem ou tanchagem, sendo seis dessas espécies endêmicas (Souza e Hassemer, 2016).

Em sua grande maioria, as espécies de *Plantago* são ervas perenes ou anuais, de caráter invasor, distribuição restrita a cosmopolita e difícil taxonomia, tendo nos tricomas e sementes as maiores diferenças morfológicas (Rahn, 1996; Rønsted *et al.*, 2002; Ishikawa *et al.*, 2009; Meudt, 2012). As tansagens são muito utilizadas na medicina popular, tendo as atividades anti-inflamatória e cicatrizante como as mais descritas (Lorenzi, 1982; Chiang *et al.*, 2003), e as espécies *P. australis* L., *P. lanceolata* L., *P. major* L. e *P. ovata* Forssk. as mais estudadas (Helfer *et al.*, 2011).

1.1.1. Farmacologia do gênero *Plantago* e de seus constituintes fitoquímicos

A família Plantaginaceae tem sido alvo de diversos estudos norteados pela relação entre a composição fitoquímica de suas espécies e atividades farmacológicas, sejam elas *in vitro* ou *in vivo*. No gênero *Plantago*, mais especificamente, os extratos *n*-hexano e etil-acetato de *P. asiatica* L. foram testados quanto à sua atividade antioxidante pelo método de capacidade de absorbância de radicais oxigenados. Os resultados demonstraram que a forma etil-acetato do extrato exerceu notável capacidade sequestrante de radicais livres (Amakura *et al.*, 2012).

Muito utilizadas na medicina chinesa, *P. depressa* Willd. e *P. lanceolata* foram descritas em diferentes estudos como portadoras de atividade gastroprotetora. A primeira espécie foi eficaz em casos de constipação e a segunda, em um estudo mais aprofundado com seu extrato etanólico, teve seus efeitos antiespasmódicos reproduzidos em modelos de contração do íleo e da traqueia em porquinhos-da-índia (Fleer e Verspohl, 2007; Shen *et al.*, 2010). A *P. lanceolata* também possui descrita atividade antioxidante pela redução do radical 2,2-difenil-1-picrilhidrazila (DPPH•) em ensaios *in vitro* (Adam *et al.*, 2009).

O extrato etanólico das folhas de *P. major*, por sua vez, foi testado em ratos submetidos ao modelo de hepatotoxicidade induzida por paracetamol. Os resultados demonstraram marcante redução de citocinas pró-inflamatórias, bem como dos níveis séricos de alanina transaminase (ALT) e aspartato transaminase (AST), importantes marcadores de disfução hepática (Hussan *et al.*, 2015). Em outro estudo, o mesmo tipo de extrato apresentou atividade sequestradora de radicais livres em ensaios com mitocôndrias de fígado de rato e na linhagem de carcinoma hepatocelular humano (Mello *et al.*, 2015). Por fim, o extrato hidroalcoólico da mesma espécie foi submetido a testes *in vivo* para o tratamento da asma e comparado ao medicamento padrão, a teofilina. Os achados revelaram que ratos asmáticos tratados com o extrato de *P. major* apresentaram normalização das características histopatológicas do pulmão, evidenciando sua atividade protetora contra a doença (Farokhi e Khaneshi, 2013).

Amplamente empregada na medicina popular, diversas preparações de *P. ovata* foram relatadas na literatura devido a uma gama de atividades

farmacológicas. Suas fibras, por exemplo, demonstraram efeito protetor na mucosa duodenal de coelhos submetidos a lesões por ácido acetilsalicílico (AAS), provavelmente mediante a limitação da penetração do AAS nas células do epitélio local. De maneira semelhante, suas folhas e sementes possuem relatos de eficácia no tratamento de úlceras pépticas e da constipação (Fernández-Bañares *et al.*, 1999; Sahagún *et al.*, 2015), bem como na redução do desconforto gastrointestinal causado pela doença de Parkinson, estabilizando os níveis séricos do medicamento padrão do tratamento, a levodopa, aumentando sua eficácia terapêutica (Fernandez-Martinez *et al.*, 2014).

Um estudo ecológico realizado na Espanha também correlacionou o consumo de *P. ovata* e a mortalidade por câncer colorretal, e os achados revelaram que, entre 1995 e 2000, o consumo da planta foi inversamente proporcional à mortalidade pela doença, com valor significativo (López *et al.*, 2009). Quanto à atividade hipolipemiante, a casca de *P. ovata* é conhecida por sua propriedade redutora dos níveis séricos de colesterol, e este efeito foi comprovado também pelas suas sementes, que em um modelo *in vivo* diminuiram os níveis da lipoproteína de baixa densidade (LDL) mediante alteração do metabolismo hepático e ação direta na regulação enzimática da síntese e catabolismo do colesterol (Romero *et al.*, 2002).

O gênero *Plantago* contêm quatro grupos principais de compostos biologicamente ativos: compostos fenólicos, flavonoides, glicosídeos iridoides e terpenoides (Rønsted *et al.*, 2000; Samuelsen, 2000). Essas classes compreendem dezenas de metabólitos e têm sido amplamente estudadas em razão da etnofarmacologia associada às tansagens.

Os compostos fenólicos têm papel essencial na reprodução, crescimento e defesa das espécies vegetais, bem como são responsáveis pela coloração de plantas e pelo sabor dos frutos (Balasundram *et al.*, 2006; Huang *et al.*, 2009). Dentre esses, o ácido clorogênico tem descritas as atividades antioxidante em modelos *in vitro* e de isquemia intestinal, antidiabética e como potencializador dos efeitos do 5-fluorouracil em modelos *in vitro* utilizando linhagens celulares de carcinoma hepatocelular humano (Sato *et al.*, 2010; Chowdhury *et al.*, 2014; Yan *et al.*, 2015).

Dois compostos dessa classe foram inicialmente isolados em plantagináceas e possuem atividades farmacológicas descritas na literatura. A plantaginina foi estudada em razão de seu efeito protetor na membrana celular renal por meio de ensaios *in vitro* em células do túbulo proximal, demonstrando efetiva prevenção da perda de lactato desidrogenase local, comprovando sua elevada proteção da membrana renal (Yokozawa *et al.*, 1999), enquanto que o plantamajosídeo teve a atividade antiespasmódica testada e comprovada em modelos *in vivo* utilizando porquinhos-da-índia (Fleer e Verspohl, 2007).

Os flavonoides compõem um dos maiores grupos de produtos naturais encontrados na natureza, caracterizando-se como importante fonte de novos compostos com potencial terapêutico (Cazarolli *et al.*, 2008). A apigenina, por exemplo, teve atividade inibitória comprovada em modelos celulares de melanoma, leucemia e glioblastoma (Wang *et al.*, 1999; Caltagirone *et al.*, 2000; Santos *et al.*, 2015;). A baicaleina exibiu atividade sinérgica com penicilâmicos contra *Staphylococcus aureus* produtores de penicilinases. Da mesma forma, induziu citotoxicidade e supressão de metaloproteinases em células de câncer mamário, e atividades anti-inflamatória mediante inibição de

marcadores pró-inflamatórios em modelos *in vivo* de pancreatite e mastite, e antioxidante *in vivo* na proteção renal contra danos causados pela cisplatina e *in vitro* na inibição da produção de espécies reativas de oxigênio por neutrófilos (Reina *et al.*, 2013; Chang *et al.*, 2015; He *et al.*, 2015; Li *et al.*, 2015; Qian *et al.*, 2015; Sahu *et al.*, 2015; Santos *et al.*, 2015).

A luteolina, um flavonoide amplamente encontrado em diversas plantas, teve evidenciada em ensaios *in vitro e in vivo* as propriedades antiespasmódica, anti-inflamatória e neuroprotetora, antimicrobiana contra *Candida glabrata*, antitumoral por inibição da angiogênese local e protetora da membrana celular renal. A rutina, ou vitamina P, tem mostrado efeitos similares sobre a integridade renal, além de efeitos inibitórios contra linhagens celulares de glioblastoma (Yokozawa *et al.*, 1999; Fleer e Verspohl, 2007; Talib *et al.*, 2012; Ambasta *et al.*, 2015; Nabavi *et al.*, 2015).

Não tão estudados, o crisoeriol apresentou efeito broncodilatador em testes com porquinhos-da-índia; a hispidulina exibiu inibição da peroxidação lipídica *in vitro* e demonstrou-se antiproliferativa em células de câncer mamário, e a homoplantaginina foi capaz de modular a sensibilidade à insulina mediante inibição da inflamação em modelo celular de células de endotélio umbilical humano. Por fim, os compostos mangiferina e escutelareina suprimiram a expressão de marcadores inflamatórios e a geração de radicais livres em modelos de ratos (Sanz *et al.*, 1994; Talib *et al.*, 2012; Wu *et al.*, 2012; Khan e Gilani, 2015; Tsubaki *et al.*, 2015).

Os iridoides constituem uma importante classe de metabólitos no reino vegetal, atuando principalmente na proteção das espécies contra predadores, sendo por isso, encontrados em um largo grupo de plantas, geralmente na

forma de glicosídeos (Dinda *et al.*, 2007; Tundis *et al.*, 2008). Neste grupo, a aucubina tem sido amplamente estudada, tendo comprovada sua eficácia *in vitro* na redução da lipotoxicidade e do estresse do retículo endoplasmático na doença hepática gordurosa não alcoólica, no tratamento de alergias inflamatórias, na inibição de marcadores pró-inflamatórios e radicais livres e na proteção de fibroblastos de pele humana contra a radiação ultravioleta do tipo B (UVB). Da mesma forma, em um modelo *in vivo*, o composto apresentou atividade cicatrizante em feridas orais, acelerando a recuperação da matriz epitelial em ratos (Ho *et al.*, 2005a; Ho *et al.*, 2005b; Park *et al.*, 2007; Shim *et al.*, 2007; Oku *et al.*, 2011; Lee *et al.*, 2013; Zhang *et al.*, 2013; Lee *et al.*, 2014).

Outro iridoide largamente analisado é o catalpol, que em modelo celular apresentou capacidade de inibir a proliferação celular e induzir apoptose em células de câncer ovariano, reduzir a apoptose neuronal e a secreção de marcadores pró-inflamatórios. Em experimentos envolvendo linhagens celulares e ratos, o composto atenuou o déficit cognitivo e estresse oxidativo, os danos neuronais e aumentou a angiogênese pós-oclusão arterial; produziu efeito antidepressivo similar ao cloridrato de fluoxetina; preveniu o envelhecimento ovariano e a falência renal; reduziu a alergia inflamatória e a pancreatite aguda. Finalmente, um estudo conduzido com testes *in vitro* em células de linfócitos humanos e *in vivo* em ratos comprovaram a atividade radioprotetora do catalpol mediante redução da produção de radicais livres (Park *et al.*, 2007; Oku *et al.*, 2011; Chen *et al.*, 2012; Chen *et al.*, 2013; Gao *et al.*, 2014; Zhu *et al.*, 2010; Haicheng *et al.*, 2014; Wang *et al.*, 2014; Xiao *et al.*, 2014; Zhu *et al.*, 2015).

O asperulosídeo desempenhou importante atividade hipolipemiante em ratos, reduzindo o peso corporal, a ingesta de alimentos e os níveis plasmáticos de glicose e lipídeos, bem como a alergia inflamatória e os sintomas de artrite reumatoide em modelos *in vivo* com ratos e *in vitro* com lisozimas de ovos de galinha, respectivamente (Li *et al.*, 2006; Oku *et al.*, 2011; Fujikawa *et al.*, 2012). O ácido geniposídico e o mussaenosídeo também apresentaram características anti-inflamatórias, sendo o primeiro detentor de propriedades antioxidantes, todos em ensaios *in vitro* (Park *et al.*, 2007; Vogl *et al.*, 2013; Zhang *et al.*, 2013).

Outra classe, os terpenoides são os constituintes básicos dos óleos essenciais em plantas, atuando principalmente como repelentes, hormônios e na interação entre diferentes espécies vegetais e com seus predadores e patógenos (Grassmann, 2005). Dentre os representantes desse grupo, o linalol demonstrou efeitos analgésicos ao reduzir a dor aguda em ratos, induziu citotoxicidade via estimulação da imunidade antitumoral em linhagens celulares de câncer de cólon, fígado, mama e pulmão, e apresentou atividade antioxidante por meio da inibição da peroxidação lipídica e do sequestro de radicais livres em ensaios *in vitro* com pâncreas de ratos (Oboh *et al.*, 2015; Yang, *et al.*, 2015).

O β-sitosterol, em estudos *in vitro* e *in vivo*, revelou importante atividade quimiopreventiva contra modelos de câncer de cólon, e sua propriedade anti-inflamatória foi evidenciada em testes *in vitro* por indução de edema de pata em ratos (Baskar *et al.*, 2010; Chittur *et al.*, 2011; Bhalke e Pal, 2012). O ácido 18β-glicirretínico, por sua vez, mostrou resultados promissores no tratamento da malária, inicialmente em triagem *in vitro* utilizando *Plasmodium falciparum*,

posteriormente confirmados em ensaios *in silico* seguidos de um modelo de infecção em ratos. Ainda, o mesmo composto foi empregado em ensaios *in vivo* com ratos, como estratégia terapêutica na esclerose múltipla, sendo capaz de modular a ativação da micróglia, promover a remielinização e inibir a inflamação do sistema nervoso (Kalani *et al.*, 2013; Zhou *et al.*, 2015).

Por último, um conjunto de dois ácidos triterpênicos é alvo de diferentes estudos envolvendo inúmeros alvos terapêuticos. Os ácidos oleanólico e ursólico vêm sendo amplamente testados e ambos demonstraram possuir as atividades antidiabética por inibição da enzima α-glicosidade e regulação da sinalização da insulina, antimicrobiana contra *Streptococcus mutans*, e hepatoprotetora e hipolipemiante via inibição da peroxidação lipídica em ensaios *in vivo* com ratos (Ho *et al.*, 2005b; Lee *et al.*, 2013; Lee *et al.*, 2014; Oku *et al.*, 2011; Reina *et al.*, 2013; Shim *et al.*, 2007; Zhang, Su, e Zhang, 2013; Park *et al.*, 2015; Rezaei-Golmisheh *et al.*, 2015).

A atividade antioxidante desses dois compostos também é conhecida, sendo o ácido oleanólico, em modelos experimentais com ratos, capaz de recuperar a atividade de enzimas antioxidantes e melhorar as funções renal e hepática, além de agir indiretamente na proteção contra a citotoxicidade induzida por radicais livres, uma vez que aumenta a expressão de fatores de transcrição sensíveis ao estresse oxidativo (Gao *et al.*, 2009; Wang *et al.*, 2010a). De maneira semelhante, o ácido ursólico suprimiu a geração de peróxido de hidrogênio em mitocôndrias de ratos, conferindo-o possível caráter cardioprotetor, e também mediou efeito protetor no dano ao DNA gerado por radicais livres induzidos por radiação UVB (Ramachandran e Prasad, 2008; Liobikas *et al.*, 2011).

No que diz respeito à atividade antitumoral, tanto o ácido oleanólico como o ursólico possuem diversos mecanismos descritos. O ácido oleanólico apresentou considerável inibição tumoral em camundongos transplantados com células de câncer pancreático humano e inibição da proliferação e aumento do estresse oxidativo em linhagens celulares de adenocarcinoma mamário (Hsu *et al.*, 2005; Jutooru *et al.*, 2010; Sánchez-Quesada *et al.*, 2015). O ácido ursólico, por sua vez, exerceu bloqueio do crescimento tumoral em linhagem celular de sarcoma, inibiu a proliferação de linhagens tumorais de leucemia e câncer de língua, e induziu apoptose em células dos cânceres de mama, fígado e estômago, fragilizou os mecanismos de sobrevivência de células de carcinoma pulmonar e aumentou a citotoxicidade em células de glioma (Manu e Kuttan, 2008; Wang *et al.*, 2010b; Wang *et al.*, 2012; Mazumder *et al.*, 2013; Li *et al.*, 2014a; Kim e Moon, 2015; Kim *et al.*, 2015; Zhang *et al.*, 2015).

Em estudos distintos, o ácido oleanólico reduziu o estresse oxidativo em comparações realizadas entre células de câncer de mama e de epitélio normal, inibiu potencialmente a enzima acetilcolinesterase, reduzindo a morte neuronal e o déficit de memória induzida pelo acúmulo de placas β-amiloides em ensaios *in vivo* de doença de Alzheimer em ratos, regulou a sinalização pró-inflamatória por supressão gênica, e apresentou propriedade antiparasitária contra *Leishmania donovani* (Yoo e Park, 2012; Hwang *et al.*, 2014; López *et al.*, 2015; Sánchez-Quesada *et al.*, 2015). Por outro lado, o ácido ursólico melhorou a atuação de enzimas-chave no controle do metabolismo lipídico e regulou a intolerância à glicose, reduzindo a esteatose hepática e mostrando-se como um potente candidato no tratamento da doença hepática gordurosa não alcoólica e da obesidade, bem como teve papel neuroprotetor contra a doença de

Alzheimer, uma vez que foi capaz de inibir a acetilcolinesterase, reduzir os danos oxidativo causados pelo acúmulo de placas β-amiloides e bloquear suas citocinas pró-inflamatórias (Kunkel *et al.*, 2012; Yoo e Park, 2012; Li *et al.*, 2014b).

1.1.2. *Plantago australis* (Kunth) Rahn

A espécie *P. australis* (Figura 1) é uma planta perene e largamente distribuída em toda a América Latina. No Brasil, sua ocorrência é mais acentuada nas regiões Sudeste e Sul, sendo expressivamente encontrada nos três estados do Sul. Seu habitat é adaptado a ambientes fortemente modificados pela ação humana, formando densas populações nos mais variados tipos de solo. A floração ocorre geralmente de setembro a fevereiro, podendo acontecer também nos outros meses do ano (Helfer *et al.*, 2011).

Figura 1: Habitat de *P. australis*. Fonte: Arquivo fotográfico do autor (2015).

Suas raízes são adventícias, escondidas entre numerosas raízes secundárias fibrosas, caule com comprimento de 0,4 a 0,7 cm e folhas com 7,0 a 34,3 cm x 2,5 a 5,5 cm, margem inteira ou levemente denteada e ápice agudo. Seu pecíolo não é distinto da lâmina, a inflorescência é laxa, com 13 a 23 cm de comprimento, e a bráctea tem 1,6 a 3,9 x 1,0 a 1,5 mm. O ovário possui três lóculos, com um óvulo por lóculo, originando três sementes, cada uma com 1,2 a 2,8 x 1,0 a 1,4 mm (Figura 2) (Rocha *et al.*, 2002; Helfer *et al.*, 2011).

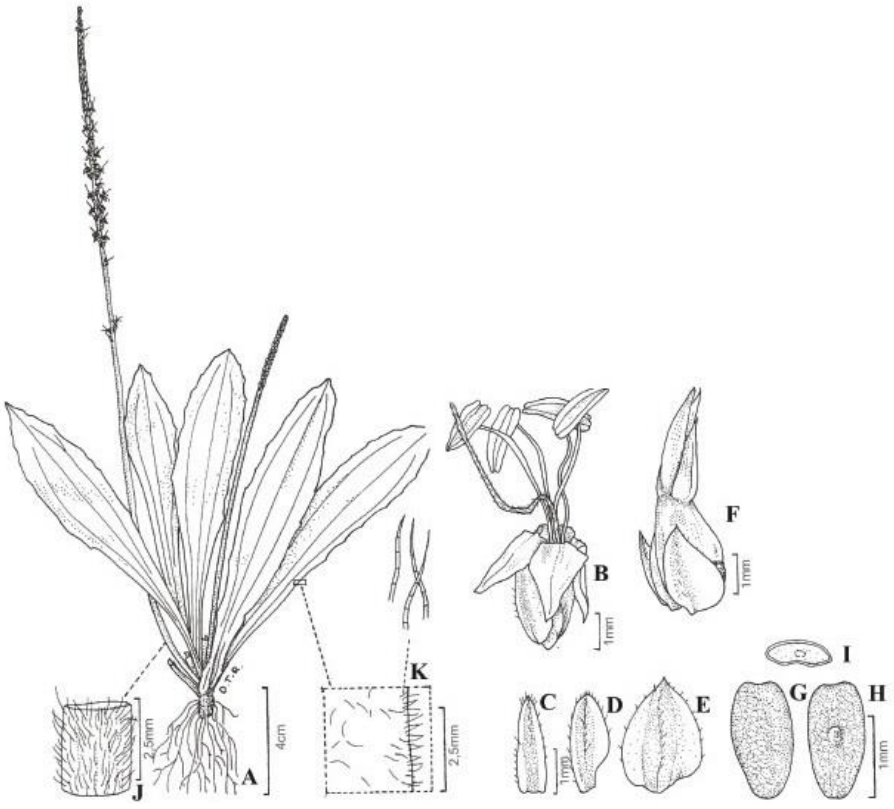


Figura 2: Morfologia de *P. australis* (A: hábito; B: flor; C: bráctea; D: sépala anterior; E: sépala posterior; F: fruto; G: semente, face externa; H: semente, face interna; I: semente, corte transversal; J: tricomas e escapo; K: tricomas, detalhe). Fonte: Adaptado de Helfer *et al.*, 2011.

1.1.2.1. Composição química de *P. australis* e atividades farmacológicas relacionadas

Estudos primários com *P. australis* identificaram na planta o composto verbascosídeo (Figura 3) (Andary *et al.*, 1988), e posteriormente outro trabalho utilizando um extrato hidroetanólico da planta identificou o glicosídeo irioide aucubina (Figura 4) e os derivados do ácido cafeico, isoverbascosídeo (Figura 5) e salidrosídeo (Figura 6) (Rønsted *et al.*, 2000).

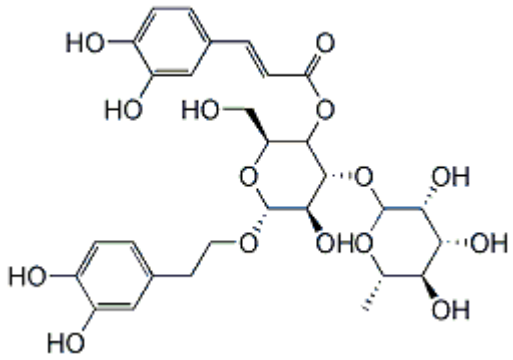


Figura 3: Estrutura química do verbascosídeo. Fonte: Biosynth Chemistry & Biology (2016).

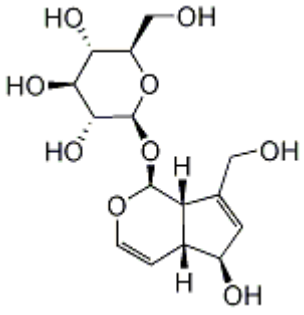


Figura 4: Estrutura química da aucubina. Fonte: Biosynth Chemistry & Biology (2016).

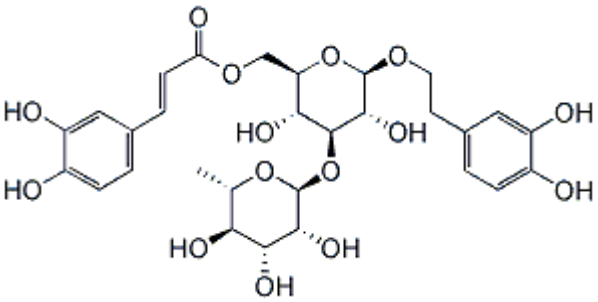


Figura 5: Estrutura química do isoverbascosídeo. Fonte: Biosynth Chemistry & Biology (2016).

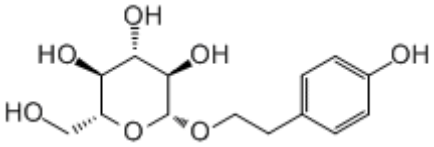


Figura 6: Estrutura química do salidrosídeo. Fonte: Biosynth Chemistry & Biology (2016).

O conhecimento etnofarmacológico de *P. australis* é difundido, e seu uso está relacionado ao tratamento de doenças renais e da bexiga, às atividades antiviral, antimicrobiana e anti-inflamatória para a garganta e ovários, e como cicatrizante, antiulceroso e antidiarreico (Souza *et al.*, 2004; Andrade-Cetto, 2009).

Embora ainda pouco estudada se comparada a outras espécies da família, *P. australis* já tem algumas atividades farmacológicas comprovadas. Seu extrato aquoso foi capaz de inibir a replicação do vírus da estomatite vesicular em ensaios *in vitro*, sem apresentar efeitos citotóxicos, enquanto que o extrato hidroalcoólico das folhas, sementes e dos frutos demonstrou propriedades analgésica e anti-inflamatória em ratos. A atividade gastroprotetora do extrato etanólico das folhas da espécie foi relatada em diferentes modelos de indução de úlceras em ratos, tendo sido capaz de reduzir o índice de úlceras e inclusive aumentar a secreção da camada

mucoprotetora estomacal (Abad *et al.*, 1999; Bürger *et al.*, 2002; Palmeiro *et al.*, 2002).

Da mesma forma, pouco se sabe sobre a toxicidade de *P. australis* e seus extratos. O único estudo realizado verificou a toxicidade subcrônica do extrato aquoso das folhas da planta em ratos. Os animais foram submetidos a 60 dias de administração diária do extrato nas concentrações de 850 e 1700 mg/kg, e foram monitorados quanto ao peso e parâmetros bioquímicos e histopatológicos. Os resultados demonstraram elevação dos níveis séricos de alanina transaminase na menor concentração testada do extrato, indicando possível hepatotoxicidade do mesmo, embora avaliações toxicológicas adicionais devam ser realizadas para fins de confirmação dos resultados (Palmeiro *et al.*, 2003).

1.1.2.2. Verbascosídeo: componente majoritário do extrato hidroetanólico de *P. australis*

O verbascosídeo, também conhecido como acteosídeo, pertence ao grupo dos glicosídeos fenilpropanoides, ou glicosídeos feniletanoides, e é amplamente distribuído em plantas dicotiledônias pertencentes a famílias como Gesneriaceae, Lamiaceae, Oleaceae, Orobanchaceae, Scrophulariaceae, Verbenaceae e Plantaginaceae, sendo por isso um dos compostos mais estudados em *Plantago* ssp. (Li *et al.*, 2014b; Zhang *et al.*, 2015). Estruturalmente, esse composto é formado por quatro grupamentos químicos: o ácido cafeico, uma aglicona feniletanoide, uma molécula de glicose e um grupamento ramnose (Figura 7) (Wen *et al.*, 2016).

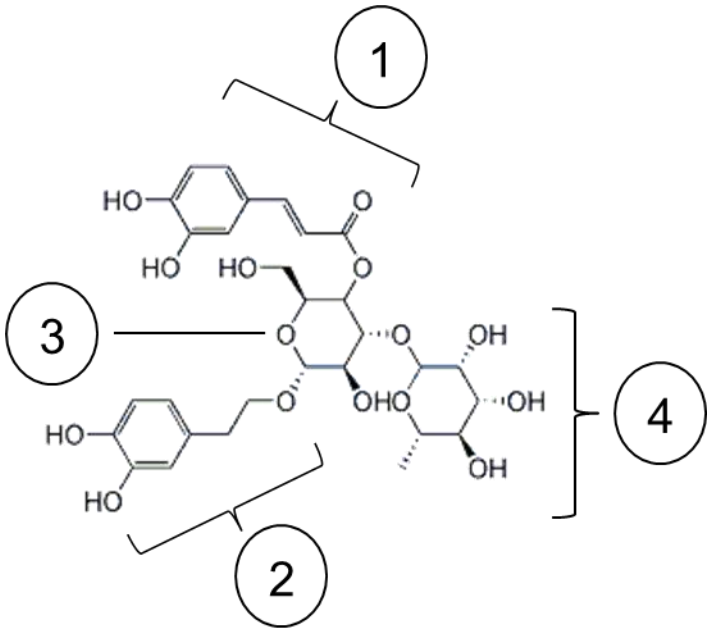


Figura 7: Estrutura química do verbascosídeo e seus grupamentos. 1: ácido cafeico; 2: aglicona feniletanoide; 3: glicose; 4: ramnose. Fonte: Adaptado de Biosynth Chemistry & Biology (2016).

Sua farmacologia envolve a atividade antigenotóxica via proteção de células sanguíneas periféricas e leucêmicas humanas contra danos ao DNA induzidos pelo peróxido de hidrogênio; gastroproteção pela redução das contrações do íleo e da traqueia induzidas por diversos agonistas em modelos *in vivo*, e atividades anti-inflamatória e cicatrizante em modelos *in vitro* utilizando células leucêmicas, macrófagos murinos e células de queratinócitos tratados com marcadores pró-inflamatórios, e *in vivo* mediante modelo de lesão ocular em leporídeos (Fleer e Verspohl, 2007; Korkina *et al.*, 2007; Fabiani *et al.*, 2008; Speranza *et al.*, 2010; Gyurkovska *et al.*, 2011; Ambrosone *et al.*, 2014).

Quanto à farmacocinética, ensaios *in vivo* revelaram que sua absorção por via oral é imediata e sua concentração plasmática é proporcional à dose administrada. Sua biodisponibilidade em mamíferos de pequeno porte foi de

apenas 1%, enquanto que nos de grande porte essa taxa atingiu 4%, com tempo de meia-vida de 90 minutos e eliminação rápida (Wen *et al.*, 2016).

1.2. Análise fitoquímica no desenvolvimento de medicamentos fitoterápicos

A pesquisa fitoquímica tem um papel essencial nos estudos químicos de espécies de interesse popular, principalmente quando não se tem ainda um conhecimento completo sobre a planta em questão. Essa etapa permite elucidar os diferentes compostos presentes no vegetal, identificando seus grupos de metabólitos, que posteriormente possam vir a ser utilizados como marcadores analíticos e ativos no desenvolvimento de extratos e fitoterápicos (Simões *et al.*, 2004; Leite, 2009).

Avanços no entendimento da fitoquímica e o desenvolvimento de novas técnicas são importantes e estão diretamente relacionados ao progresso nessa área. Com base nesses requisitos, nota-se que a qualidade da matéria-prima vegetal é primordial para o bom andamento da análise fitoquímica. De um modo geral, amostras frescas da planta são ideais para o processo de extração, embora em alguns casos o material triturado e seco também seja útil (Harborne, 1998).

A extração e a purificação amostral são passos cruciais na análise de plantas medicinais, uma vez que constituem as etapas iniciais da preparação das amostras. A escolha do método e do solvente de extração influencia no tipo de substância a ser extraída, sendo que para a extração de compostos hidrofílicos usam-se solventes polares como metanol, etanol e acetato de etila,

enquanto que os constituintes lipofílicos são melhor obtidos com os solventes diclorometano puro ou misturado com metanol. Costumeiramente, o etanol é um dos solventes mais utilizados em razão de sua propriedade preliminar de extrair diversos componentes, e para os processos seguintes, o extrato é filtrado e concentrado por rotaevaporação, tendo o processo de liofilização como etapa opcional (Harborne, 1998; Sasidharan *et al.*, 2011).

Em seguida, os métodos cromatográficos garantem uma análise qualitativa e/ou quantitativa da amostra em questão. Essa etapa envolve fatores determinantes no processo, como a escolha das fases móvel e estacionária, a relação de polaridade entre elas e a variação do método a ser empregado, onde podemos citar as cromatografias em camada delgada, gasosa e líquida de alta eficiência (Nyiredi, 2004).

Devido à sua versatilidade e robustez, a cromatografia líquida de alta eficiência (CLAE) é umas das técnicas mais utilizadas na análise de produtos naturais, sendo atualmente considerada a impressão digital do controle de qualidade dessas matérias-primas. Esse sistema é capaz de oferecer características como a alta resolução, rápida separação, monitoramento contínuo da eluição, e automação dos processos com a possibilidade de melhoramento dos dados obtidos (Sarker *et al.*, 2005; Fan *et al.*, 2006; Swami *et al.*, 2008).

Brevemente, o sistema é composto por uma bomba de eluição da fase móvel, um dispositivo de injeção da amostra, uma coluna cromatográfica contendo a fase estacionária e por onde a fase móvel é eluída juntamente com a amostra, um detector responsável por emitir sinais elétricos correspondentes aos analitos encontrados, que são interpretados e registrados na forma de

picos cromatográficos por um programa computacional acoplado ao sistema. A detecção dos picos amostrais dependerá da afinidade da amostra pelas fases móvel e estacionária, sendo que quanto maior esse fator pela fase estacionária, mais lentamente a amostra eluirá pela coluna e mais tardiamente o pico será detectado, ocorrendo o contrário caso a afinidade for maior pela fase móvel (Sasidharan *et al.*, 2011).

Dessa forma, a análise de extratos vegetais por CLAE é um importante passo para a análise de extratos vegetais, contribuindo para o posterior desenvolvimento de testes biológicos. Tais resultados servem de base para a condução e otimização dos ensaios pré-clínicos subsequentes, exigidos para o registro de fitoterápicos.

1.3. Ensaios pré-clínicos no desenvolvimento de medicamentos fitoterápicos

Devido à relevância socioeconômica que o uso de plantas medicinais exerce na qualidade de vida de grande parte da população, principalmente àquelas de baixa renda, o processo de levantamento, resgate de informações e identificação de espécies é de fundamental importância para garantir a segurança de seus usuários (Rodrigues e Carvalho, 2001; Silva *et al.*, 2010).

A classificação e consequente normatização dos fitoterápicos variam conforme o país ou região. Enquanto que nos Estados Unidos as preparações à base de plantas são consideradas suplementos nutricionais, na Europa e no Brasil esses produtos são categorizados como medicamentos. Em razão dessas diferenças, observam-se condutas distintas em relação aos ensaios

para o registro de fitoterápicos, a fim de se garantir a máxima eficácia e segurança no uso desses medicamentos (Figura 8) (Turolla e Nascimento, 2006).

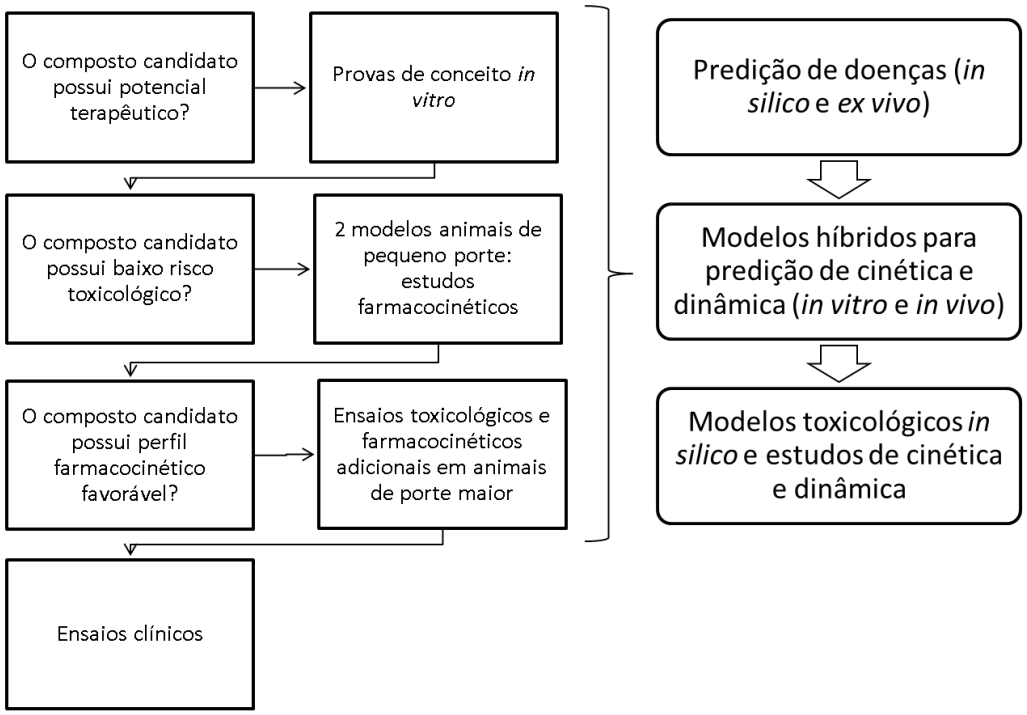


Figura 8: Fluxo tradicional para a avaliação de compostos e/ou fitoterápicos com potencial terapêutico. Fonte: Adaptado de Turolla e Nascimento, 2006.

Atualmente, várias diretrizes são utilizadas na condução de ensaios pré-clínicos para os diferentes tipos de medicamentos, sendo o *Guideline for Testing of Chemicals*, da *Organisation for Economic Co-operation and Development* (OECD), o mais aceito. O guia prevê uma série de testes de citotoxicidade, genotoxicidade e mutagenicidade, que objetivam melhorar a validade e a aceitação internacional dos dados obtidos; otimizar os recursos disponíveis; racionalizar o uso de animais de laboratório e minimizar as questões comerciais dos produtos (OECD, 2016). No Brasil, o registro de medicamentos e o registro e a notificação de produtos tradicionais fitoterápicos

é regido pela RDC n° 26/2014, da Agência Nacional de Vigilância Sanitária (Anvisa, 2014).

1.3.1. Ensaios de citotoxicidade

A citotoxicidade pode ser entendida como a capacidade intrínseca de qualquer material ou composto de promover modificações metabólicas em células de cultura, acarretando ou não em morte celular. Essa propriedade pode ser mensurada por ensaios de viabilidade celular que comparam as taxas de células sobreviventes e mortas (Freshney, 2011).

Os ensaios de citotoxicidade *in vitro* são os primeiros testes realizados, e por isso são extremamente úteis para a definição da toxicidade basal do composto estudado nos estágios iniciais do desenvolvimento farmacológico. Dessa forma, podem-se definir os limites de concentração das substâncias para os ensaios posteriores, além de prever informações e parâmetros adicionais para testes de genotoxicidade e mutagenicidade (Eisenbrand *et al.*, 2002; Putnam *et al.*, 2002).

Dentre os ensaios mais empregados, grande parte baseia-se na avaliação da alteração da permeabilidade celular (uso de corantes, como o azul de Trypan); da função mitocondrial, como o (4,5-dimetiltiazol-2-il)2,5-difenil brometo de tetrazolium (MTT) e o 2,3-bis-(2-metoxi-4-nitro-5-sulfofenil)-5-fenilalanina carbonil-2H-tetrazolium hidróxido (XTT); da função lisossomal, como a captação do corante vermelho neutro (NRU), e nas alterações da morfologia e proliferação celular (ensaio clonogênico e de proliferação celular) (Eisenbrand *et al.*, 2002; Riss *et al.*, 2011).

O NRU, em especial, é recomendado pela OECD para a avaliação da citotoxicidade de compostos, servindo como teste preliminar na definição de doses e na condução dos ensaios subsequentes, principalmente os relacionados à toxicidade oral aguda sistêmica. Brevemente, esse modelo baseia-se na capacidade das células viáveis incorporarem o corante vermelho neutro, que é fracamente catiônico, em seus lisossomos, que possuem matriz aniônica. Agentes tóxicos podem modificar a superfície celular ou a membrana lisossomal, fragilizando seu sistema e causando alterações que podem vir a ser irreversíveis. Esses eventos acarretam na inibição do crescimento ou morte celular, ocasionando a redução da captação do corante e consequente queda da viabilidade celular (OECD, 2010).

Além disso, o NRU consiste na etapa final da avaliação de outro importante ensaio, a fototoxicidade de compostos. O conceito de fototoxicidade é atribuído a uma substância quando essa apresenta uma resposta tóxica se aplicada ao corpo seguida de exposição à luz, ou que é induzida por irradiação da pele após a administração sistêmica de uma substância (OECD, 2004). A predição *in vitro* da fototoxicidade de compostos é extremamente acessível e validada por diversos órgãos regulatórios. O teste baseia-se na comparação da citotoxicidade de uma substância em contato com a linhagem celular de fibroblastos de ratos (3T3), na ausência e presença de radiação ultravioleta do tipo A (UVA), seguida do ensaio de NRU, 24 h após a exposição ao agente testado (Peters e Holzhütter, 2004).

1.3.2. Ensaios de genotoxicidade e mutagenicidade

O objetivo principal dos estudos de genotoxicidade e mutagenicidade é detectar compostos capazes de induzir danos ao DNA de maneira direta ou indireta, discriminando mutações em células germinativas (devido ao seu possível envolvimento na etiologia de defeitos genéticos humanos herdáveis) e somáticas (envolvidas em transformações neoplásicas). Devido aos diferentes tipos de danos existentes, a abordagem usual prevê uma bateria de testes que, ao serem compilados, fornecem informações sobre mutações em genes, aberrações cromossômicas e a potencial indução de carcinogenicidade (Eastmond *et al.*, 2009).

Como testes preconizados para essa fase de estudos de segurança toxicológica, um dos ensaios mais utilizados é a eletroforese de célula única em gel de agarose, também conhecido como ensaio cometa. Capaz de detectar quebras simples e duplas na molécula de DNA, assim como oxidação de purinas e pirimidinas, o ensaio cometa consiste em fixar células tratadas com o agente de interesse em lâminas pré-cobertas com gel de agarose, submetê-las a uma solução de lise para que o material genético fique exposto. Após lisadas, essas lâminas são submetidas a uma corrente elétrica em uma cuba contendo uma solução tampão, e o material genético, que possui carga negativa, tenderá a migrar para o polo positivo da cuba, sendo que quanto mais degradado esse estiver, maior será sua taxa de migração. Em seguida, as lâminas são fixadas e coradas para avaliação dos danos ao DNA, sob microscopia ótica convencional (Collins, 2004).

O teste de micronúcleos (MN), por sua vez, pode indicar possíveis alterações citogenéticas com consequentes modificações cromossômicas. Os MN são pequenas massas nucleares, delimitadas por membrana e separadas do núcleo principal, formadas a partir de quebras nas fitas de DNA ou devido à ruptura de fibras do fuso acromático, e, portanto passíveis de ocorrerem somente em células que estão em divisão celular. Dessa forma, a detecção de MN representa perda de cromatina, sendo por isso considerada uma mutação, uma vez que pode ser transmitida às células filhas no ciclo de divisão seguinte (Fenech, 2000).

O princípio do teste *in vitro* baseia-se no bloqueio da citocinese celular com citocalasina-B em culturas tratadas com o agente em estudo, a fim de se sincronizar as células e excluir danos causados por alterações na cinética da divisão celular. Como a citocalasina-B bloqueia a citocinese, mas não impede a divisão celular, as células tratadas por esse composto acabam adquirindo o caráter binucleado, e na sequência são submetidas a um tratamento hipotônico, fixadas em lâminas de microscopia e coradas para posterior contagem de células binucleadas com e sem a presença de MN (Bonacker *et al.*, 2004).

Na avaliação da carcinogênese, apesar do uso dos ensaios já citados, outro modelo, o teste de *Salmonella*/microssoma, possui acurácia mais elevada, podendo detectar agentes carcinogênicos causadores de substituição de pares de base e de mutações do tipo *frameshift* (alteração do quadro de leitura dos códons) (Kim *et al.*, 2013). Diferentemente dos demais, esse teste faz uso de cepas específicas da bactéria *Salmonella typhimurium*, que carregam mutações em genes envolvidos na síntese do aminoácido histidina.

Uma vez constituindo-se de cepas mutantes auxotróficas (*His*-), elas necessitam da histidina para seu crescimento, porém não a produzem. Por esse motivo, o método testa a capacidade da substância de interesse criar mutações que resultem na reversão da bactéria ao seu estado prototrófico (*His*+) quando inserida em um meio livre de histidina. A contagem é feita levando-se em conta o número de colônias formadas, comparando-as com o controle do teste. Adicionalmente, enzimas metabolizadoras podem ser inseridas, a fim de se certificar de que os achados do teste possam vir a ser causados não pelo agente em questão, mas sim por seus metabólitos (Ames *et al.*, 1973; Hakura *et al.*, 1999).

1.4. Referências Bibliográficas

Abad M, Bermejo P, Gonzales E, Iglesias I, Irurzun A, Carrasco L. Antiviral activity of Bolivian plant extracts. Gen Pharmacol. 1999;32(4):499-503.

Adam M, Dobiáš P, Eisner A, Ventura K. Extraction of antioxidants from plants using ultrasonic methods and their antioxidant capacity. J Sep Sci. 2009;32:288-94.

Albach DC, Meudt HM, Exelman B. Piecing together the “new” Plantaginaceae. Am J Bot. ;92(2):297-315.

Amakura Y, Yoshimura A, Yoshimura M, Yoshida, T. Isolation and characterization of phenolic antioxidants from *Plantago* herb. Molecules. 2012;17(5):5459-66.

Ambasta R, Jha S, Kumar D, Sharma R, Jha N, Kumar P. Comparative study of anti-angiogenic activities of luteolin, lectin and lupeol biomolecules. J Transl Med. 2015;13(1):307.

Ambrosone L, Guerra G, Cinelli M, Filippelli M, Mosca M, Vizzarri F, *et al.* Corneal Epithelial Wound Healing Promoted by Verbascoside-Based Liposomal Eyedrops. Biomed Res Int. 2014;2014:1-8.

Ames BN, Lee FD, Durston WE. An Improved Bacterial Test System for the Detection and Classification of Mutagens and Carcinogens. Proc Nat Acad Sci. 1973;70(3):782-6.

Andary C, Motte-Florac ME, Gargadennec A, Wylde R, Heitz A. Les esters caféiques du genre *Plantago*. Identification et valeur chimiotaxinomique. Plant Med Phytother. 1988;22:17-22.

Anvisa. Resolução RDC n° 26, de 13 de maio de 2014. Ministério da Saúde – MS. Agência Nacional de Vigilância Sanitária – Anvisa.Disponível em: http://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2014/rdc0026_13_05_2014.pdf. Acesso em 30 jun. 2016.

Andrade-Cetto A. Ethnobotanical study of the medicinal plants from Tlanchinol, Hidalgo, México. J Ethnopharmacol. 2009 Feb 3;122(1):163-71.

APG (The Angiosperm Phylogeny Group). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Bot J Linn Soc. 2016:1-20.

Balasundram N, Sundram K, Samman S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. Food Chem. 2006;99(1):191-203.

Baskar A, Ignacimuthu S, Paulraj G, Numair K. Chemopreventive potential of β-Sitosterol in experimental colon cancer model - an *In vitro* and *In vivo* study. Bmc Complem Altern M. 2010;10(1):24.

Bhalke RD, Pal SC. Anti-infflamation and Antinociceptive Activity of *Pterospermum acerifolium* Leaves. Asian J Pharm Clin Res. 2012;5(2):23-26.

Biosynth. Biosynth Chemistry & Biology [Internet]. [acesso em: 2016 fev 29]. Disponível em: https://www.biosynth.com/.

Bonacker D, Stoiber T, Wang M, Böhn KJ, Prots I, Unger E, *et al.* Genotoxicity of inorganic mercury salts based on disturbed microtubule function. Arch Toxicol. 2004;78:575-83.

Bürger ME, Ghedini PC, Baldisserotto B, Palmeiro NMS, Almeida CE, Lenzi CF, *et al.* Effect of *Plantago australis* leaves on different gastric ulcer models. Rev Bras Farmacogn. 2002;12:113-4.

Calixto J. Twenty-five years of research on medicinal plants in Latin America: a personal view. J Ethnopharmacol. 2005;100(1-2):131-4.

Caltagirone S, Rossi C, Poggi A, Ranelletti F, Natali P, Brunetti M, *et al.* Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. Int J Cancer J Int Du Cancer. 2000;87(4):595-600.

Cazarolli L, Zanatta L, Alberton E, Figueiredo M, Folador P, Damazio R, *et al.* Flavonoids: prospective drug candidates. Mini Rev Medicinal Chem. 2008;8(13):1429-40.

Chang H-T, Chou C-T, Kuo D-H, Shieh P, Jan C-R, Liang W-Z. The Mechanism of Ca2+ Movement in the Involvement of Baicalein-Induced Cytotoxicity in ZR-75-1 Human Breast Cancer Cells. J Nat Prod. 2015;78(7):1624-34.

Chen C, Chen Z, Xu F, Zhu C, Fang F, Shu S, *et al.* Radio-protective effect of catalpol in cultured cells and mice. J Radiat Res. 2012;54(1):76-82.

Chen W, Li X, Jia L-Q, Wang J, Zhang L, Hou D, *et al.* Neuroprotective activities of catalpol against CaMKII-dependent apoptosis induced by LPS in PC12 cells. Brit J Pharmacol. 2013;169(5):1140-52.

Chiang L-C, Ng LT, Chiang W, Chang M-Y, Lin C-C. Immunomodulatory Activities of Flavonoids, Monoterpenoids, Triterpenoids, Iridoid Glycosides and Phenolic Compounds of *Plantago* Species. Planta Med. 2003;69:600-604.

Chittur S, Parr B, Marcovici G. Inhibition of inflammatory gene expression in keratinocytes using a composition containing carnitine, thioctic Acid and saw palmetto extract. Evid Based Complement Alternat Med. 2011;2011:985345.

Chowdhury SS, Islam MN, Jung HA, Choi JS. In vitro antidiabetic potential of the fruits of *Crataegus pinnatifida*. Res Pharm Sci. 2014;9(1):11-22.

Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol. 2004;26(3):249-61.

Cronquist A. The Evolution and Classification of Flowering Plants. 2.ed. New York: The New York Botanical Garden;1988.

Dinda B, Debnath S, Harigaya Y. Naturally Occurring Iridoids. A Review, Part 1. Chem Pharm Bull. 2007;55(2):159-222.

Eastmond DA, Hartwig A, Anderson D, Anwar WA, Cimino MC, Dobrev I, *et al.* Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme. Mutagenesis. 2009;24(4):341-349.

Fabiani R, Rosignoli P, Bartolomeo A, Fuccelli R, Servili M, Montedoro G, *et al.* Oxidative DNA damage is prevented by extracts of olive oil, hydroxytyrosol, and other olive phenolic compounds in human blood mononuclear cells and HL60 cells. J Nutrition. 2008;138(8):1411-6.

Fan X-H, Cheng Y-Y, Ye Z-L, Lin R-C, Qian Z-Z. Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. Anal Chim Acta. 2006;555:217-224.

Farokhi F, Khaneshi F. Histophatologic changes of lung in asthmatic male rats treated with hydro-alcoholic extract of *Plantago major* and theophylline. Avicenna J Phytomed. 2013;3(2):143-51.

Fenech M. The in vitro micronucleus technique. Mutat Res. 2000;455(1-2):81-95.

Fernández-Bañares F, Hinojosa J, Sánchez-Lombraña JL, Navarro E, Martínez-Salmerón JF, García-Pugés A, *et al.* Randomized clinical trial of *Plantago ovata* seeds (dietary fiber) as compared with mesalamine in maintaining remission in ulcerative colitis. Spanish Group for the Study of Crohn's Disease and Ulcerative Colitis (GETECCU). Am J Gastroenterol. 1999;94(2):427-33.

Fernandez-Martinez MN, Hernandez-Echevarria L, Sierra-Vega M, Diez-Liebana MJ, Calle-Pardo A, Carriedo-Ule D, *et al.* A randomised clinical trial to evaluate the effects of *Plantago ovata* husk in Parkinson patients: changes in levodopa pharmacokinetics and biochemical parameters. BMC Complement Altern Med. 2014;14(296):1-10.

Filho V, Yunes R. Estratégias para a obtenção de compostos farmacologicamente ativos a partir de plantas medicinais: conceitos sobre modificação estrutural para otimização da atividade. Quim Nova. 1998;21(1):99-105.

Fleer H, Verspohl EJ. Antispasmodic activity of an extract from *Plantago lanceolata* L. and some isolated compounds. Phytomedicine. 2007;14(6):409-15.

Freshney RI. Culture for animal cells: a manual of basic technique and specialized applications. 6.ed. Indianapolis: Wiley-Blackwell;2011.

Fujikawa T, Hirata T, Hosoo S, Nakajima K, Wada A, Yurugi Y, *et al.* Asperuloside stimulates metabolic function in rats across several organs under high-fat diet conditions, acting like the major ingredient of Eucommia leaves with anti-obesity activity. J Nutritional Sci. 2012;1.

Gao D, Li Q, Li Y, Liu Z, Fan Y, Liu Z, *et al.* Antidiabetic and Antioxidant Effects of Oleanolic Acid from *Ligustrum lucidum* Ait in Alloxan-induced Diabetic Rats. Phytoter Res. 2009;23:1257-1262.

Gao N, Tian J-X, Shang Y-H, Zhao D-Y, Wu T. Catalpol Suppresses Proliferation and Facilitates Apoptosis of OVCAR-3 Ovarian Cancer Cells through Upregulating MicroRNA-200 and Downregulating MMP-2 Expression. Int J Mol Sci. 2014;15(11):19394-405.

Grassmann J. Terpenoids as Plant Antioxidants. Vitamins Hormones. 2005;505-35.

Gyurkovska V, Alipieva K, Maciuk A, Dimitrova P, Ivanovska N, Haas C, *et al.* Anti-inflammatory activity of Devil's claw in vitro systems and their active constituents. Food Chem. 2011;125(1):171-8.

Haicheng Z, Jing Liu, Liyuan R, Wei L, Qian X, Lolo M, *et al.* Relationship between spatial memory in diabetic rats and protein kinase Cγ, caveolin-1 in the hippocampus and neuroprotective effect of catalpol. Chin Med J. 2014;127(5):916-923.

Hakura A, Suzuki S, Satoh T. Advantage of the use of human liver S9 in the Ames test. Mutat Res. 1999;438(1):29-36.

Harborne JB. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 3.ed. London: Chapman & Hall;1998.

He X, Wei Z, Zhou E, Chen L, Kou J, Wang J, *et al.* Baicalein attenuates inflammatory responses by suppressing TLR4 mediated NF-κB and MAPK signaling pathways in LPS-induced mastitis in mice. Int Immunopharmacol. 2015;28(1):470-6.

Helfer SM, Rodrigues WA, Cervi AC. O gênero *Plantago* L. (Plantaginaceae) na região Sul do Brasil. Rev Bras Biocienc. 2011 Jul/Sep;9(3)297-321.

Ho JN, Lee YH, Park JS, Jun WJ, Kim HK, Hong BS, *et al.* Protective Effects of Aucubin Isolated from *Eucommia ulmoides* against UVB-Induced Oxidative Stress in Human Skin Fibroblasts. Biol Pharm Bull. 2005a;28(7):1244-1248.

Ho J-N, Lee Y-H, Lee Y-D, Jun W-J, Kim H-K, Hong B-S, *et al.* Inhibitory effect of Aucubin isolated from Eucommia ulmoides against UVB-induced matrix metalloproteinase-1 production in human skin fibroblasts. Biosci Biotechnology Biochem. 2005b;69(11):2227-31.

Huang W-Y, Cai Y-Z, Zhang Y. Natural Phenolic Compounds From Medicinal Herbs and Dietary Plants: Potential Use for Cancer Prevention. Nutr Cancer. 2009;61(1);1-20.

Hussan F, Mansor AS, Hassan SN, Kamaruddin TNE, Budin SB, Othman F. Anti-Inflammatory Property of *Plantago major* Leaf Extract Reduces the Inflammatory Reaction in Experimental Acetaminophen-Induced Liver Injury. Evid Based Complement Alternat Med. 2015;347861:1-7.

Hsu H-F, Houng J-Y, Chang C-L, Wu C-C, Chang F-R, Wu Y-C. Antioxidant Activity, Cytotoxicity, and DNA Information of *Glossogyne tenuifolia*. J Agric Food Chem. 2005;53(15):6117-6125.

Hwang Y-J, Song J, Kim H-R, Hwang K-A. Oleanolic acid regulates NF-κB signaling by suppressing MafK expression in RAW 264.7 cells. Bmb Rep. 2014;47(9):524-9.

Ishikawa N, Yokoyama J, Tsukaya H. Molecular evidence of reticulate evolution in the subgenus *Plantago* (Plantaginaceae). Am J Bot. 2009;96(9):1627-35.

Jutooru I, Chadalapaka G, Abdelrahim M, Basha M, Samudio I, Konopleva M, *et al.* Methyl 2-Cyano-3,12-dioxooleana-1,9-dien-28-oate Decreases Specificity Protein Transcription Factors and Inhibits Pancreatic Tumor Growth: Role of MicroRNA-27a. Mol Pharmacol. 2010;78(2):226-36.

Kalani K, Agarwal J, Alam S, Khan F, Pal A, Srivastava S. *In Silico* and *In Vivo* Anti-Malarial Studies of 18β Glycyrrhetinic Acid from *Glycyrrhiza glabra*. Plos One. 2013;8(9):e74761.

Khan A-U, Gilani A-H. Natural Products Useful in Respiratory Disorders: Focus on Side-Effect Neutralizing Combinations. Phytotherapy Res Ptr. 2015;29(9):1265-85.

Kim HR, Park YJ, Shin DY, Oh SM, Chung KH. Appropriate *In Vitro* Methods for Genotoxicity Testing of Silver Nanoparticles. Environ Health Toxicol. 2013;28:1-8.

Kim E-SS, Moon A. Ursolic acid inhibits the invasive phenotype of SNU-484 human gastric cancer cells. Oncol Lett. 2015 Feb;9(2):897-902.

Korkina LG, Mikhal'Chik EV, Suprun MV, Pastore S, Dal Toso R. Molecular Mechanisms Underlying Wound Healing and Anti-infflamatory Properties of Naturally Occurring Biotechnologically Produced Phenylpropanoid Glycosides. Cell Mol Biol. 2007;53(5):84-91.

Kunkel SD, Elmore CJ, Bongers KS, Ebert SM, Fox DK, Dyle MC, *et al.* Ursolic Acid Increases Skeletal Muscle and Brown Fat and Decreases Diet-Induced Obesity, Glucose Intolerance and Fatty Liver Disease. Plos One. 2012 Jun;7(6):e39332.

Lee H-Y, Lee G-H, Lee M-R, Kim H-K, Kim N, Kim S-H, *et al. Eucommia ulmoides* Oliver Extract, Aucubin, and Geniposide Enhance Lysosomal Activity to Regulate ER Stress and Hepatic Lipid Accumulation. Plos One. 2013;8(12):e81349.

Lee G-H, Lee M-R, Lee H-Y, Kim S, Kim H-K, Kim H-R, *et al. Eucommia ulmoides* Cortex, Geniposide and Aucubin Regulate Lipotoxicity through the Inhibition of Lysosomal BAX. Plos One. plos; 2014;9(2):e88017.

Leite JPV. Fitoterapia: bases científicas e tecnológicas. 1.ed. São Paulo: Editora Atheneu;2009.

Li B, Zhang D-M, Luo Y-M, Chen X-G. Three New and Antitumor Anthraquinone Glycosides from *Lasianthus acuminatissimus* MERR. Chem Pharm Bull. 2006;54(3):297-300.

Li S, Meng F, Liao X, Wang Y, Sun Z, Guo F, *et al.* Therapeutic Role of Ursolic Acid on Ameliorating Hepatic Steatosis and Improving Metabolic Disorders in High-Fat Diet-Induced Non-Alcoholic Fatty Liver Disease Rats. Plos One. 2014a;9(1):e86724.

Li Y, Gan L, Li GQ, Deng L, Zhang X, Deng Y. Pharmacokinetics of plantamajoside and acteoside from *Plantago asiatica* in rats by liquid chromatography-mass spectrometry. J Pharm Biomed Anal. 2014b Feb 6;89:251-6.

Li J, Wu Y, Zhang S, Zhang J, Ji F, Bo W, *et al.* Baicalein protect pancreatic injury in rats with severe acute pancreatitis by inhibiting pro-inflammatory cytokines expression. Biochem Bioph Res Co. 2015;466(4):664-9.

Liobikas J, Majiene D, Trumbeckaite S, Kursvietiene L, Masteikova R, Kopustinskiene D, *et al.* Uncoupling and antioxidant effects of ursolic acid in isolated rat heart mitochondria. J Nat Prod. 2011;74(7):1640-4.

López JCC, Villanueva R, Martínez-Hernández D, Albaladejo R, Regidor E, Calle MEE. *Plantago ovata* consumption and colorectal mortality in Spain, 1995-2000. J Epidemiol. 2009;19(4):206-11.

López D, Cherigo L, Spadafora C, Loza-Mejía M, Martínez-Luis S. Phytochemical composition, antiparasitic and α–glucosidase inhibition activities from *Pelliciera rhizophorae*. Chem Cent J. 2015;9(1):53.

Lorenzi H. Plantas daninhas do Brasil: terrestres, aquáticas, parasitas, tóxicas e medicinais. Nova Odessa: Instituto Plantarum;1982.

Manu KA, Kuttan G. Ursolic acid induces apoptosis by activating p53 and caspase-3 gene expressions and suppressing NF-κB mediated activation of bcl-2 in B16F-10 melanoma cells. Int Immunopharmacol. 2008;8(7):974-81.

Mazumder K, Tanaka K, Fukase K. Cytotoxic Activity of Ursolic Acid Derivatives Obtained by Isolation and Oxidative Derivatization. Molecules. 2013;18(8):8929-44.

Mello JC, Gonzalez MV, Moraes VW, Prieto T, Nascimento OR, Rodrigues T. Protective Effect of *Plantago major* Extract against t-BOOH-Induced Mitochondrial Oxidative Damage and Cytotoxicity. Molecules. 2015;20(10):17747-59.

Meudt HM. A taxonomic revision of native New Zealand *Plantago* (Plantaginaceae). New Zeal J Bot. 2012;50(2):101-178.

Nabavi S, Braidy N, Gortzi O, Sobarzo-Sanchez E, Daglia M, Skalicka-Woźniak K, *et al.* Luteolin as an anti-inflammatory and neuroprotective agent: A brief review. Brain Res Bull. 2015;119(Pt A):1-11.

Nyiredy S. Separation strategies of plant constituents–current status. J Chromatogr B. 2004;812:35-51.

Oboh G, Akinbola I, Ademosun A, Sanni D, Odubanjo O, Olasehinde T, *et al.* Essential Oil from Clove Bud (*Eugenia aromatica* Kuntze) Inhibit Key Enzymes Relevant to the Management of Type-2 Diabetes and Some Pro-oxidant Induced Lipid Peroxidation in Rats Pancreas *in vitro*. J Oleo Sci. 2015;64(7):775-82.

OECD – Organisation for Economic Co-operation and Development. OECD guideline for testing of chemicals: *In vitro* 3T3 NRU phototoxicity test. 2004;432:1-15.

OECD – Organisation for Economic Co-operation and Development. Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxycity tests. 2010;129:1-54.

OECD. Organisation for Economic Co-operation and Development. [Internet]. [acesso em: 2016 mar 03]. Disponível em: http://www.oecd.org/.

Oku H, Ogawa Y, Iwaoka E, Ishiguro K. Allergy-Preventive Effects of Chlorogenic Acid and Iridoid Derivatives from Flower Buds of *Lonicera japonica*. Biological Pharm Bulletin. 2011;34(8):1330-3.

Palmeiro NS, Almeida CE, Ghedini PC, Goulart LS, Baldisserotto B. Analgesic and Anti-inflammatory Properties of *Plantago australis* Hydroalcoholic Extract. Acta Farm Bonaerense. 2002;21(2):89-92.

Palmeiro NM, Almeida CE, Ghedini PC, Goulart LS, Pereira MC, Huber S, *et al.* Oral subchronic toxicity of aqueous crude extract of *Plantago australis* leaves. J Ethnopharmacol. 2003 Sep 1;88(1):15-8.

Park K, Kim B, Chang I-M. Inhibitory Potencies of Several Iridoids on Cyclooxygenase-1, Cyclooxygnase-2 Enzymes Activities, Tumor Necrosis Factor-α and Nitric Oxide Production In Vitro. Evid-based Compl Alt. 2007;7(1):41-5.

Park S-N, Ahn S-J, Kook J-K. Oleanolic acid and ursolic acid inhibit peptidoglycan biosynthesis in *Streptococcus mutans* UA159. Braz J Microbiol. 2015;46(2):613-7.

Peters B, Holzhütter H-G. *In Vitro* Phototoxicity Testing: Development and Validation of a New Concentration Response Analysis Software and Biostatistical Analyses Related to the Use of Various Prediction Models. Altern Lab Anim. 2002;30:415-432.

Qian M, Tang S, Wu C, Wang Y, He T, Chen T, *et al.* Synergy between baicalein and penicillins against penicillinase-producing *Staphylococcus aureus*. Int J Med Microbiol Ijmm. 2015;305(6):501-4.

Rahn K. A phylogenetic study of the Plantaginaceae. Bot J Linn Soc. 1996;120:145-198.

Ramachandran S, Prasad N. Effect of ursolic acid, a triterpenoid antioxidant, on ultraviolet-B radiation-induced cytotoxicity, lipid peroxidation and DNA damage in human lymphocytes. Chem-biol Interact. 2008;176(2-3):99-107.

Rates SMK. Promoção do uso racional de fitoterápicos: uma abordagem no ensino de Farmacognosia. Rev Bras Farmacogn. 2001;11(2):57-69.

Reina E, Al-Shibani N, Allam E, Gregson K, Kowolik M, Windsor L. The Effects of *Plantago major* on the Activation of the Neutrophil Respiratory Burst. J Traditional Complementary Medicine. 2013;3(4):268-72.

Rezaei-Golmisheh A, Malekinejad H, Asri-Rezaei S, Farshid AA, Akbari P. Hawthorn ethanolic extracts with triterpenoids and flavonoids exert hepatoprotective effects and suppress the hypercholesterolemia-induced oxidative stress in rats. Iran J Basic Med Sci. 2015 Jul;18(7):691-699.

Riss TL, Moravec RA, Niles AL. Cytotoxicity Testing: Measuring Viable Cells, Dead Cells, and Detecting Mechanism of Cell Death. Methods Mol Biol. 2011;740:103-114.

Rocha JF, Rosa MMT, Frade CCM, Diersmann EM. Estudo anatômico e histoquímico em folhas de *Plantago major* L. e *Plantago australis* Lam. (Plantaginaceae). Revista Universidade Rural: Série Ciências da Vida. 2002;22(1):33-41.

Rodrigues VEG, Carvalho DA. Levantamento Etnobotânico de Plantas Medicinais no Condomínio Cerrado na Região do Alto Rio Grande –Minas Gerais. Ciênc agrotec. 2001 Jan/Fev;25(1):102-123.

Romero AL, West KL, Zern T, Fernandez ML. The seeds from *Plantago ovata* lower plasma lipids by altering hepatic and bile acid metabolism in guinea pigs. J Nutr. 2002;132(6):1194-8.

Rønsted N, Göbel E, Franzyk H, Jensen SR, Olsen CE. Chemotaxonomy of *Plantago*. Iridoid glucosides and caffeoyl phenylethanoid glycosides. Phytochemistry. 2000 55:337-348.

Rønsted N, Chase M, Alch D, Bello M. Phylogenetic relationships within *Plantago* (Plantaginaceae): evidence from nuclear ribosomal ITS and plastid trnL-F sequence data. Bot J Linn Soc. 2002;139(4):323-38.

Sahagún AM, Vaquera J, García JJ, Calle AP, Diez M-J, Fernández N, *et al.* Study of the protective effect on intestinal mucosa of the hydrosoluble fiber *Plantago ovata* husk. BMC Complement Altern Med. 2015;15(298):2-8.

Sahu BD, Kumar JM, Sistia R. Baicalein, a Bioflavonoid, Prevents Cisplatin-Induced Acute Kidney Injury by Up-Regulating Antioxidant Defenses and Down-Regulating the MAPKs and NF-κB Pathways. Plos One. 2015;10(7) e0134139.

Samuelsen A. The traditional uses, chemical constituents and biological activities of *Plantago major* L. A review. J Ethnopharmacol. 2000;71(1-2):1-21.

Sánchez-Quesada C, López-Biedma A, Gaforio J. Oleanolic Acid, a Compound Present in Grapes and Olives, Protects against Genotoxicity in Human Mammary Epithelial Cells. Mol Basel Switz. 2015;20(8):13670-88.

Santos B, Oliveira M, Coelho PC, Pitanga B, Silva A, Adelita T, *et al.* Flavonoids suppress human glioblastoma cell growth by inhibiting cell metabolism, migration, and by regulating extracellular matrix proteins and metalloproteinases expression. Chem-biol Interact. 2015;242:123-38.

Santos-Cruz LF, Ávila-Acevedo JG, Ortega-Capitaine D, Ojeda-Duplancher JC, Perdigón-Moya JL, Hernández-Portilla LB, *et al.* Verbascoside is not genotoxic in the ST and HB crosses of the Drosophila wing spot test, and its constituent, caffeic acid, decreases the spontaneous mutation rate in the ST cross. Food Chem Toxicol. 2012;50(3-4):1082-90.

Sanz M, Ferrandiz M, Cejudo M, Terencio M, Gil B, Bustos G, *et al.* Influence of a series of natural flavonoids on free radical generating systems and oxidative stress. Xenobiotica Fate Foreign Compd Biological Syst. 1994;24(7):689-99.

Sarker SD, Latif Z, Gray AI. Natural Products Isolation. 2.ed. Totowa: Humana Press;2005.

Sasidharan S, Chen Y, Saravanan D, Sundram KM, Latha LY. Extraction, Isolation and Characterization of Bioactive Compounds From Plants' Extracts. Afr J Trad Complement Altern Med. 2011;8(1):1-10.

Sato Y, Itagaki S, Kurokawa T, Ogura J, Kobayashi M, Hirano T, *et al.* In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid. Int J Pharm. 2010;403(1-2):136-8.

Shen, S., Qian, J., & Ren, J. Ethnoveterinary plant remedies used by Nu people in NW Yunnan of China. J Ethnobiol Ethnomed. 2010;6(24): 1-10.

Shim K, Choi S, Jeong M-J, Kang S. Effects of aucubin on the healing of oral wounds. Vivo Athens Greece. 2007;21(6):1037-41.

Silva NLA, Miranda FAA, Conceição GM. Triagem Fitoquímica de Plantas de Cerrado, da Área de Proteção Ambiental Municipal do Inhamum, Caxias, Maranhão. Sci Plena. 2010;6(2):1-17.

Simões MO, Schenkel EP, Gossmann G, Mello JCP, Mentz LA, Petrovick PR. Farmacognosia: da planta ao medicamento. 5.ed. Porto Alegre / Florianópolis: Editora da UFRGS / Editora da UFSC;2004.

Souza G, Haas APS, Poser GL, Schapoval EES, Elisabetsky E. Ethnopharmacological studies of antimicrobial remedies in the south of Brazil. J Ethnopharmacol. 2004;90(1):135-43.

Souza VC, Hassemer G. Lista de Espécies da Flora do Brasil [Internet]. Rio de Janeiro. [acesso em: 2016 jan 28]. Disponível em: http://floradobrasil.jbrj.gov.br/reflora/listaBrasil/PrincipalUC/PrincipalUC.do#CondicaoTaxonCP.

Speranza L, Franceschelli S, Pesce M, Reale M, Menghini L, Vinciguerra I, *et al.* Antiinflammatory effects in THP-1 cells treated with verbascoside. Phytother Res. 2010;24(9):1398-404.

Swami HS, Singh KSP, Gennaro L, Dutt RD. Extraction Technologies for Medicinal and Aromatic Plants. 1.ed. Trieste: International Centre for Science and High Tecnology;2008.

Talib W, Zarga M, Mahasneh A. Antiproliferative, antimicrobial and apoptosis inducing effects of compounds isolated from *Inula viscosa*. Mol Basel Switz. 2012;17(3):3291-303.

Tsubaki M, Takeda T, Kino T, Itoh T, Imano M, Tanabe G, *et al.* Mangiferin suppresses CIA by suppressing the expression of TNF-α, IL-6, IL-1β, and RANKL through inhibiting the activation of NF-κB and ERK1/2. Am J Transl Res. 2015;7(8):1371-81.

Tundis, R, Loizzo MR, Menichini F, Statti GA, Menichini F. Biological and Pharmacological Activities of Iridoids: Recent Developments. Mini Rev Med Chem. 2008;8:399-420.

Turolla M, Nascimento E. Informações toxicológicas de alguns fitoterápicos utilizados no Brasil. Rev Bras Cienc Farm. 2006;42(2):289-306.

Viegas Jr C, Bolzani VS, Barreiro EJ. Os produtos naturais e a química medicinal moderna. Quim Nova. 2006;29(2):326-337.

Vogl S, Atanasov A, Binder M, Bulusu M, Zehl M, Fakhrudin N, *et al.* The Herbal Drug *Melampyrum pratense* L. (Koch): Isolation and Identification of Its Bioactive Compounds Targeting Mediators of Inflammation. Evid-based Compl Alt. 2013;2013:1-10.

Wang I, Lin-Shiau S, Lin J. Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. European J Cancer Oxf Engl 1990. 1999;35(10):1517-25.

Wang X, Ye X-L, Liu R, Chen H-L, Bai H, Liang X, *et al.* Antioxidant activities of oleanolic acid *in vitro*: Possible role of Nrf2 and MAP kinases. Chem Biol Interact. 2010a;184:328-337.

Wang J, Ren T, Xi T. Ursolic acid induces apoptosis by suppressing the expression of FoxM1 in MCF-7 human breast cancer cells. Med Oncol. 2010b;29(1):10-5.

Wang J, Li Y, Wang X, Jiang C. Ursolic Acid Inhibits Proliferation and Induces Apoptosis in Human Glioblastoma Cell Lines U251 by Suppressing TGF-β1/miR-21/PDCD4 Pathway. Basic Clin Pharmacol Toxicol. 2012;111:106-112.

Wang J, Cui Y, Feng W, Zhang Y, Wang G, Wang X, *et al.* Involvement of the central monoaminergic system in the antidepressant-like effect of catalpol in mice. BioScience Trends. 2014;8(5):248-252.

Wen Y, Huo S, Zhang W, Xing H, Qi L, Zhao D, *et al.* Pharmacokinetics, Biodistribution, Excretion and Plasma Protein Binding Studies of Acteoside in Rats. Drug Res (Stuttg). 2016;66(3):148-53.

Wu F, Wang H, Li J, Liang J, Ma S. Homoplantaginin Modulates Insulin Sensitivity in Endothelial Cells by Inhibiting Inflammation. Biological Pharm Bulletin. 2012;35(7):1171-7.

Xiao W, Yin G, Fan Y, Qiu L, Cang X, Yu G, *et al.* Catalpol Ameliorates Sodium Taurocholate-Induced Acute Pancreatitis in Rats via Inhibiting Activation of Nuclear Factor Kappa B. Int J Mol Sci. 2014;15(7):11957-72.

Yan Y, Li J, Han J, Hou N, Song Y, Dong L. Chlorogenic acid enhances the effects of 5-fluorouracil in human hepatocellular carcinoma cells through the inhibition of extracellular signal-regulated kinases. Anti-cancer Drug. 2015;26(5):540.

Yang X-N, Khan I, Kang S. Chemical composition, mechanism of antibacterial action and antioxidant activity of leaf essential oil of *Forsythia koreana* deciduous shrub. Asian Pac J Tropical Medicine. 2015;8(9):694-700.

Yokozawa T, Dong E, Kawai Y, Gemba M, Shimizu M. Protective effects of some flavonoids on the renal cellular membrane. Exp Toxicol Pathol. 1999;51(1):9-14.

Yoo K-Y, Park S-Y. Terpenoids as Potential Anti-Alzheimer's Disease Therapeutics. Molecules. mdpi; 2012;17(3):3524-38.

Zhang Q, Su Y, Zhang J. Seasonal Difference in Antioxidant Capacity and Active Compounds Contents of *Eucommia ulmoides* Oliver Leaf. Molecules. 2013;18(2):1857-68.

Zhang W, Huo S-XX, Wen Y-LL, Xing H, Zhang Q, Li N, *et al.* Pharmacokinetics of acteoside following single dose intragastric and intravenous administrations in dogs. Chin J Nat Med. 2015 Aug 6;13(8):634-40.

Zhou J, Cai W, Jin M, Xu J, Wang Y, Xiao Y, *et al.* 18β-glycyrrhetinic acid suppresses experimental autoimmune encephalomyelitis through inhibition of microglia activation and promotion of remyelination. Sci Reports. 2015;5:13713.

Zhu H-F, Wan D, Luo Y, Zhou J-L, Chen L, Xu X-Y. Catalpol increases brain angiogenesis and up-regulates VEGF and EPO in the rat after permanent middle cerebral artery occlusion. Int J Biol Sci. 2010;6(5):443-53.

Zhu J, Chen X, Wang H, Yan Q. Catalpol protects mice against renal ischemia/reperfusion injury via suppressing PI3K/Akt-eNOS signaling and inflammation. Int J Clin Exp Med. 2015;8(2):2038-2044.

2. Objetivos

2.1. Objetivo geral

Desenvolver um extrato etanólico das folhas de *Plantago australis*, padronizado com o composto verbascosídeo, e avaliar a segurança toxicológica deste extrato e do verbascosídeo em modelos *in vitro*.

2.2. Objetivos específicos

a) Realizar uma revisão bibliográfica sobre a família Plantaginaceae, seus constituintes fitoquímicos e atividades farmacológicas relacionadas.
b) Elaborar extratos etanólicos a 70% das folhas de *P. australis*, empregando diferentes métodos de extração (ultrassom e percolação).
c) Realizar análises qualitativas, por cromatografia líquida de alta eficiência (HPLC/DAD), para verificar a presença dos principais constituintes fitoquímicos citados na literatura para a espécie.
d) Desenvolver um método por HPLC/DAD para a quantificação do seu marcador analítico, o verbascosídeo.
e) Otimizar o processo extrativo, utilizando a técnica de ultrassom, para obtenção de um extrato hidroetanólico a 70%, padronizado em verbascosídeo.
f) Realizar uma validação de metodologia analítica por HPLC/DAD para a quantificação do verbascosídeo.

g) Realizar o teste de *Salmonella*/microssoma em cepas de S*almonella* tratadas com o extrato hidroetanólico de *P. australis* e com o verbascosídeo.

h) Realizar testes de viabilidade celular pelos métodos de redução do (4,5-dimetiltiazol-2-il)2,5-difenil brometo de tetrazolium (MTT) e captação do vermelho neutro em células de fibroblastos pulmonares de hamster chinês (V79) tratadas com o extrato hidroetanólico de *P. australis* e com o verbascosídeo.

i) Realizar o ensaio cometa (versão alcalina) em células V79 tratadas com o extrato hidroetanólico de *P. australis* e com o verbascosídeo.

j) Realizar o ensaio de fototoxicidade em células de fibroblastos embrionários de *Mus musculus* (3T3) tratadas com o verbascosídeo.

3. Capítulo I – artigo de revisão

A review of the therapeutic potential of the major constituents from species of *Plantago* (Plantaginaceae)

Artigo a ser submetido à revista *Phytotherapy Research*

(Para facilitar a leitura do manuscrito as tabelas foram inseridas no texto)

A review of the therapeutic potential of the major constituents from species of *Plantago* (Plantaginaceae)

Jeferson Gustavo Henn[a], Nathalia Denise de Moura Sperotto[a], Gustavo Hassemer[b], Valéria Flores Péres[a], Jenifer Saffi[a], Dinara Jaqueline Moura[a*]

[a] Laboratório de Genética Toxicológica, Universidade Federal de Ciências da Saúde de Porto Alegre (UFSCPA), Porto Alegre, RS, Brazil

[b] Statens Naturhistoriske Museum, Københavns Universitet, Sølvgade 83S, 1307 Copenhagen, Denmark

[*]Corresponding author: dinaram@ufcspa.edu.br

Abstract

Plantago is a largely used genus in folk medicine. Belonging to the Plantaginaceae family, this genus has a cosmopolite distribution and includes about 250 species. Some of these species have been used for millennia for the treatment of several diseases, and different extract forms and isolated compounds, such as iridoid glucosides, phenolic compounds, flavonoids and terpenoids, have already been related to important biological activities. Some extracts from *Plantago* species showed analgesic, anti-asthmatic, anti-inflammatory, antioxidant, antitumoral, gastroprotective, hypolipemiant and neuroprotective properties, while isolated compounds are related to antidiabetic, anti-inflammatory, antioxidant, antitumoral, hypolipemiant and neuroprotective properties. On the other hand, isolated compounds are also related to angiogenic, antidepressant, antidiabetic, antigenotoxic, antimicrobial, photoprotective, radioprotective, renal membrane protection and wound healing activities. This information, once collected, may be helpful to guide researches and to support further investigation about the common properties of *Plantago* species and evaluation as a source of active compounds.

Introduction

The Plantaginaceae as currently accepted (APG, 2016) comprise a morphologically very diverse family which includes approximately 90 genera and 2000 species (Albach et al., 2005). One of the largest among these genera, *Plantago* L. is a cosmopolitan genus which includes about 250 species of herbs or rarely subshrubs concentrated in temperate regions and at high elevations in the tropics (Pilger, 1937; Rahn, 1996; Hassemer et al., 2015, 2016). Here we follow the subgeneric classification of *Plantago* as in Rønsted et al. (2002), with the updates of Hoggard et al. (2003), i.e., accepting *Littorella* P.J.Bergius as a genus separate from *Plantago*.

The classification and identification of *Plantago* is known to be notoriously difficult, because of a very simplified morphology and complicated taxonomy (Rahn, 1996; Ishikawa et al., 2009; Tay et al., 2010a, 2010b; Meudt, 2011; Shipunov, 2015). Therefore, it is very frequent that herbarium specimens of *Plantago* are found to be misidentified. Furthermore, sometimes studies focusing on aspects of *Plantago* other than taxonomy are not careful regarding species identification and nomenclature, which results in unreliable, unverifiable and ultimately non-repeatable results. Therefore, maximum care should be taken to correctly identify and present the species included in any research, preferably with the help of specialist taxonomists.

Some species of *Plantago* have been shown to have biologically active compounds with several pharmacological activities (Marlett et al., 2000; Samuelsen, 2000; Fischer et al., 2004; Singh, 2007; Barua et al., 2011; Beara et al., 2012a, 2012b; Weryszko-Chmielewska et al., 2012; Zhou et al., 2013). Also, species of *Plantago* have been shown to contain at least four classes of biologically active compounds: iridoid glucosides (i.e., aucubine and catalpol), phenolic compounds (i.e., verbascoside, chlorogenic acid and vannilic acid), flavonoids (i.e., baicalein and baicalin) and terpenoids (i.e, oleanolic acid and ursolic acid) (Rønsted et al., 2000; Samuelsen, 2000).

These compounds have been largely studied due their potential pharmacological activity, and combined with the traditional folk use of some species of *Plantago*, comprise an important source of research. In this review, our aim was to analyze the major isolated constituents of species of *Plantago* and their biological activities – described or not in this genus – however the traditional uses will be considered.

Ethnopharmacology

Some species of *Plantago* have been used in traditional medicine around the world, in the treatment of inflammation, cancer, disorders of reproduction, respiratory, digestive and skin systems (Chiang et al., 2003). The leaves and seeds are the most commonly used parts of the plant, as infusion or as fresh leaves forms (Vogl et al., 2013a).

As example, seeds of *P. ovata* Forssk. are used in medicine as a mild laxative in southern Europe and India. In Japan, *P. asiatica* L. is used as anti-inflammatory, against diarrhea, atitussive and as antiasthmatic (Samuelsen et al., 1999). *Plantago major* L. has been used for hundreds of years in Taiwan for treating colds, conjunctivitis and hepatitis (Chiang et al., 2003). In Brazil, leaves and seeds of *P. major* are used as antiseptic, anti-inflammatory and antibacterial, and *P. australis* Lam. has been used in southern Brazil as a laxative, diuretic, anti-inflammatory, anti-bacterial and wound healing (Palmeiro et al., 2002). *Plantago lanceolata* L. can be used as syrup or tea, as well as fresh leaves and was indicated to insects bites, viral infects, skin and respiratory tract (Vogl et al., 2013a) . In Norway, the leaves of *P. major* are well known in traditional medicine for its wound healing properties (Samuelsen et al., 1999).

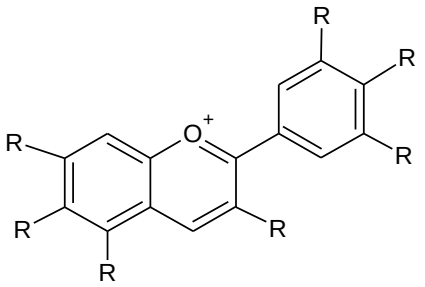
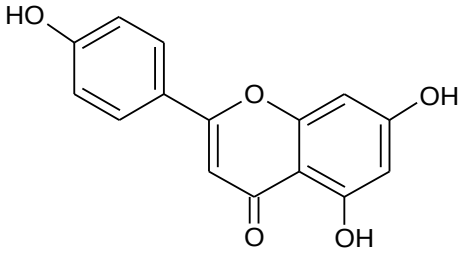
Phytochemistry and chemotaxonomy

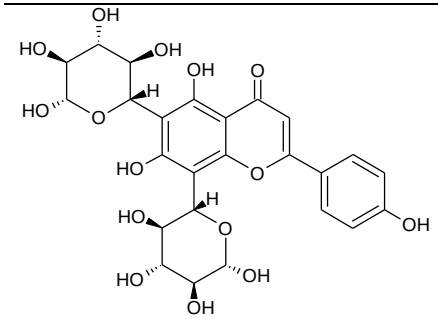
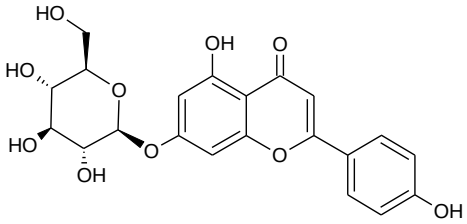
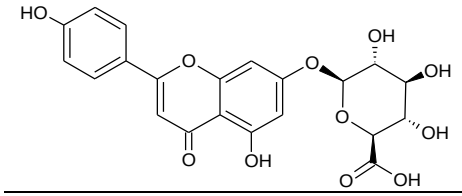
Flavonoids

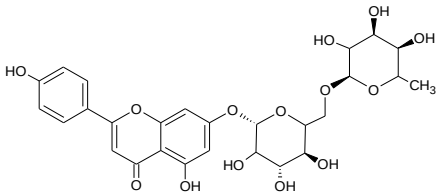
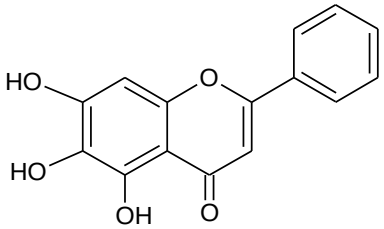
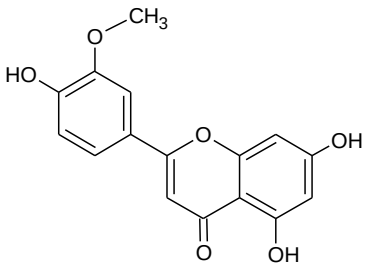
Flavonoids are polyphenolic compounds biosynthesized from acetic acid and shikimic acid pathways, and they are predominantly present in nature as glycosides. Its diffuse presence in nature makes flavonoids the major group of natural products known (Cazarolli et al., 2008).

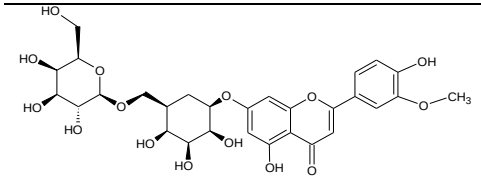
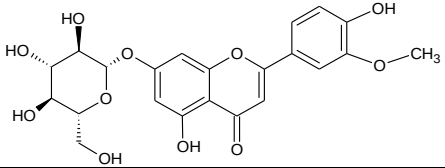
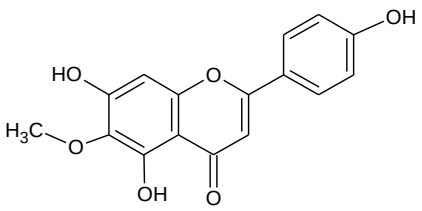
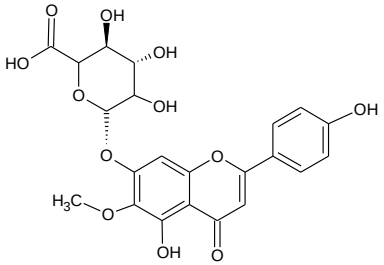
The flavonoids from *Plantago* and their chemical structures are listed in Table 1.

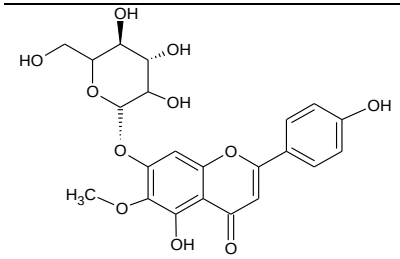
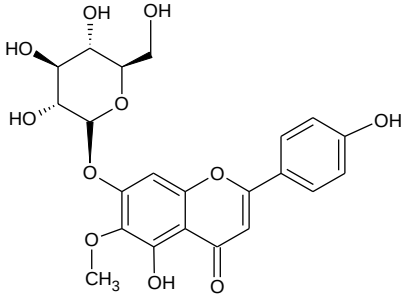
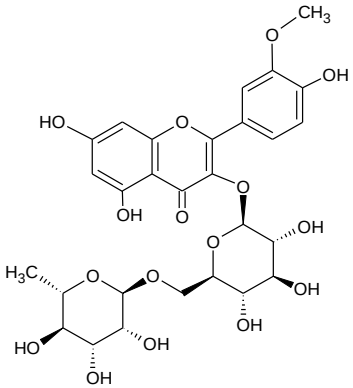
Table 1
Flavonoids from *Plantago*.

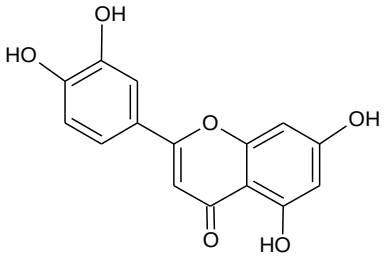
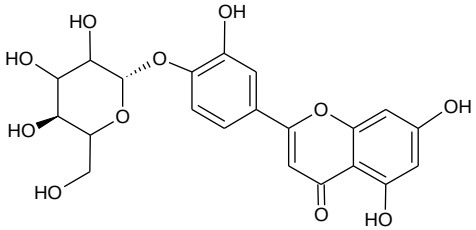
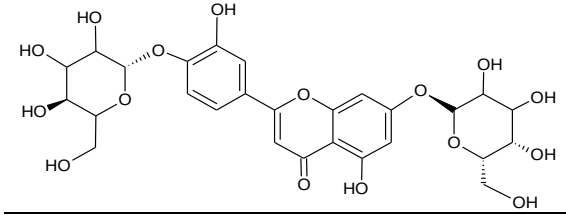
Flavonoids	***Plantago* species**	**Part of plant**	**References**
Anthocyanins 	*P. lanceolata* L.	Whole plant	Dalar et al., 2012
Apigenin 	*P. crypsoides* Boiss. *P. lanceolata* L. *P. albicans* L. *P. asiatica* L. *P. atrata* Hoppe *P. coronopus* L. *P. reniformis* Beck *P. schwarzenbergiana* Schur	Leaves and stems Leaves and stems Leaves and stems Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Nishibe et al., 1995 Janković et al., 2012 Janković et al., 2012 Janković et al., 2012 Janković et al., 2012
Apigenin-6,8-di-C-glucoside	*P. commutata* Guss. *P. crypsoides* Boiss. *P. lanceolata* L. *P. afra* L. *P. arabica* Boiss. *P. arenaria* Waldst. & Kit.	Leaves and stems Leaves and stems Leaves and stems Leaves and stems Leaves and stems Leaves and stems	Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994

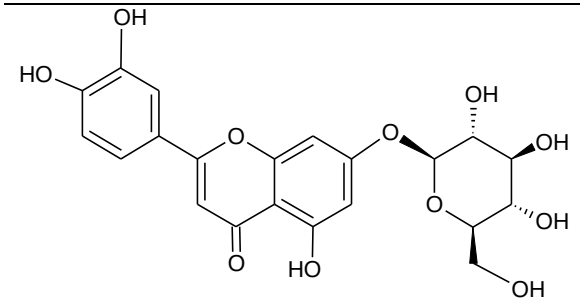
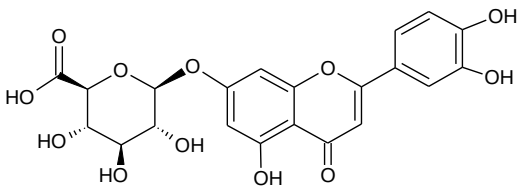
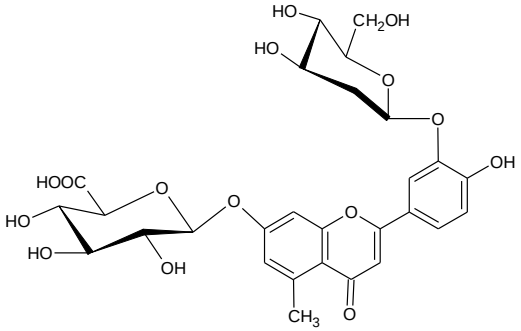
			
Apigenin-7-glucoside 	*P. major* L. *P. lanceolata* L. *P. albicans* L. *P. ovata* Forssk. *P. notata* Lag. *P. ciliata* Desf. *P. afra* L. *P. phaeostoma* Boiss. & Heldr. *P. coronopus* L. *P. holosteum* Scop. *P. schwarzenbergiana* Schur *P. argentea* Chaix *P. maritima* L. *P. media* L.	Leaves and stems Leaves and stems Leaves and stems Leaves and stems Leaves and stems Leaves and stems Leaves and stems Leaves and stems Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Janković et al., 2012 Janković et al., 2012 Janković et al., 2012 Beara et al., 2009 Beara et al., 2009 Beara et al., 2009
Apigenin-7-glucuronide 	*P. lanceolata* L. *P. phaeostoma* Boiss. & Heldr.	Leaves and stems Leaves and stems	Kawashty et al., 1994 Kawashty et al., 1994

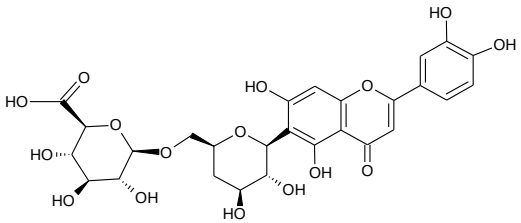
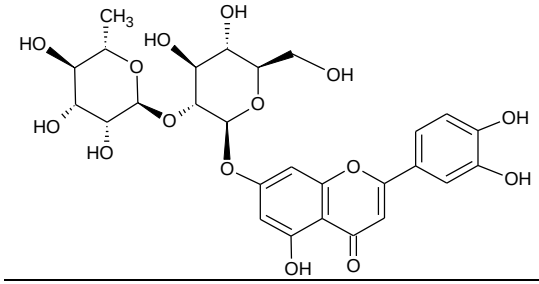
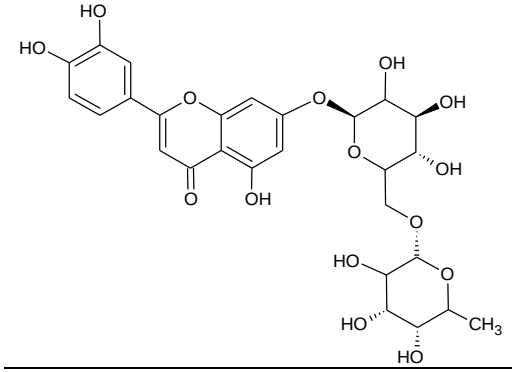
Apigenin-7-rutinoside 	*P. amplexicaulis* Cav.	Leaves and stems	Kawashty et al., 1994
Baicalein 	*P. major* L.	Leaves	Maksyutina et al., 1971
Chrysoeriol 	*P. albicans* L. *P. afra* L. *P. phaeostoma* Boiss. & Heldr.	Leaves and stems Leaves and stems Leaves and stems	Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994
Chrysoeriol-7-gentiobioside	*P. albicans* L. *P. cylindrica* Forssk. *P. afra* L.	Leaves and stems Leaves and stems Leaves and stems	Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994

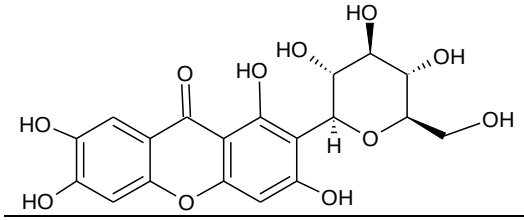
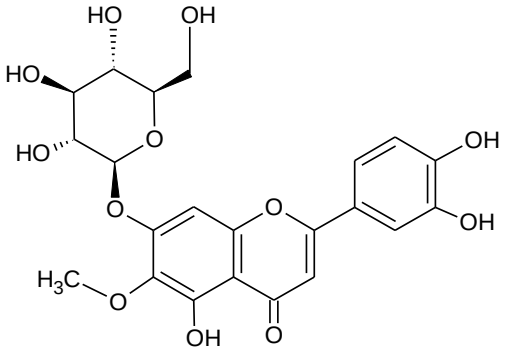
			
Chrysoeriol-7-glucoside 	*P. afra* L. *P. squarrosa* Murray *P. phaeostoma* Boiss. & Heldr.	Leaves and stems Leaves and stems Leaves and stems	Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994
Hispidulin 	*P. major* L.	Leaves and stems	Kawashty et al., 1994
Hispidulin-7-glucoronide 	*P. major* L.	Leaves and stems	Kawashty et al., 1994
Hispidulin-7-glucoside	*P. major* L. *P. asiatica* L.	Leaves and stems Leaves	Kawashty et al., 1994 Kawashty et al., 1994

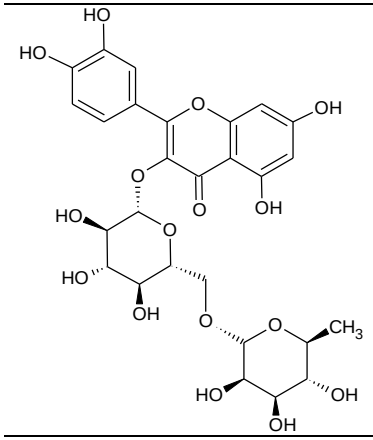
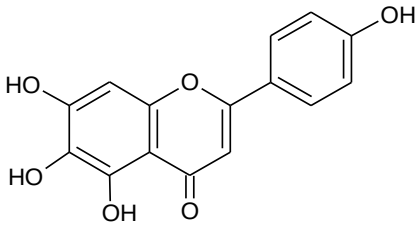
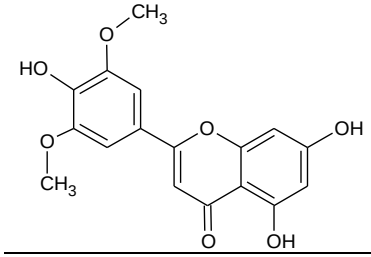
			
Homoplantaginin 	*P. major* L. *P. asiatica* L.	Aerial parts Aerial parts	Skari et al., 1999 Aritomi, 1967
Isorhamnetin-3-rutinoside 	*P. albicans* L. *P. cylindrica* Forssk. *P. notata* Lag.	Leaves and stems Leaves and stems Leaves and stems	Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994

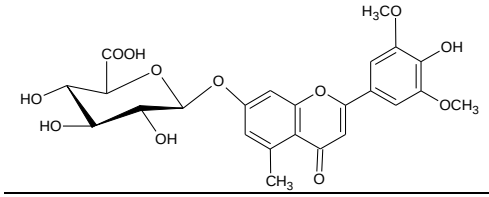
Luteolin 	*P. commutata* Guss. *P. lanceolata* L. *P. atrata* Hoppe *P. coronopus* L. *P. holosteum* Scop. *P. schwarzenbergiana* Schur *P. reniformis* Beck	Leaves and stems Leaves and stems Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Kawashty et al., 1994 Fleer & Verspohl, 2007 Janković et al., 2012 Janković et al., 2012 Janković et al., 2012 Janković et al., 2012 Janković et al., 2012
Luteolin-4'-glucoside 	*P. ovata* Forssk.	Leaves and stems	Kawashty et al., 1994
Luteolin-7-diglucoside 	*P. major* L.	Leaves and stems	Kawashty et al., 1994
Luteolin-7-glucoside	*P. major* L. *P. commutata* Guss. *P. crypsoides* Boiss. *P. lanceolata* L. *P. lagopus* L.	Leaves and stems Leaves and stems Leaves and stems Leaves and stems Leaves and stems	Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994

	*P. albicans* L.	Leaves and stems	Kawashty et al., 1994
	P. cylindrica Forssk.	Leaves and stems	Kawashty et al., 1994
	P. ovata Forssk.	Leaves and stems	Kawashty et al., 1994
	P. notata Lag.	Leaves and stems	Kawashty et al., 1994
	P. ciliata Desf.	Leaves and stems	Kawashty et al., 1994
	P. afra L.	Leaves and stems	Kawashty et al., 1994
	P. squarrosa Murray	Leaves and stems	Kawashty et al., 1994
	P. atrata Hoppe	Aerial parts	Janković et al., 2012
	P. coronopus L.	Aerial parts	Janković et al., 2012
	P. holosteum Scop.	Aerial parts	Janković et al., 2012
	P. argentea Chaix	Aerial parts	Beara et al., 2009
	P. maritima L.	Aerial parts	Beara et al., 2009
Luteolin-7-glucuronide 	*P. commutata* Guss.	Leaves and stems	Kawashty et al., 1994
	P. crypsoides Boiss.	Leaves and stems	Kawashty et al., 1994
	P. lanceolata L.	Aerial parts	Fleer & Verspohl, 2007
	P. arenaria Waldst. & Kit.	Leaves and stems	Kawashty et al., 1994
Luteolin-7-glucuronide-3'-glucoside 	*P. crypsoides* Boiss.	Leaves and stems	Kawashty et al., 1994
	P. commutata Guss.	Leaves and stems	Kawashty et al., 1994
	P. amplexicaulis Cav.	Leaves and stems	Kawashty et al., 1994
	P. lanceolata L.	Leaves and stems	Kawashty et al., 1994
	P. arabica Boiss.	Leaves and stems	Kawashty et al., 1994

Luteolin-7-glucuronylglucoside 	*P. amplexicaulis* Cav.	Leaves and stems	Kawashty et al., 1994
Luteolin-7-neohesperidoside 	*P. lagopus* L.	Leaves and stems	Kawashty et al., 1994
Luteolin-7-rutinoside 	*P. lagopus* L.	Leaves and stems	Kawashty et al., 1994
Mangiferin	*P. atrata* Hoppe	Aerial parts	Janković et al., 2012

			
Nepetin-7-glucoside 	*P. major* L.	Leaves and stems	Kawashty et al., 1994
Rutin	*P. atrata* Hoppe	Aerial parts	Janković et al., 2012

			
Scutellarein 	*P. major* L.	Leaves	Maksyutina et al., 1971
Tricin 	*P. afra* L. *P. squarrosa* Murray *P. phaeostoma* Boiss. & Heldr. *P. arabica* Boiss. *P. arenaria* Waldst. & Kit. *P. pumila* L.f.	Leaves and stems Leaves and stems Leaves and stems Leaves and stems Leaves and stems Leaves and stems	Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994
Tricin-7-glucuronide	*P. afra* L.	Leaves and stems	Kawashty et al., 1994

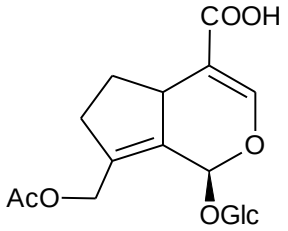
	*P. squarrosa* Murray *P. arabica* Boiss. *P. arenaria* Waldst. & Kit. *P. pumila* L.f.	Leaves and stems Leaves and stems Leaves and stems Leaves and stems	Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994 Kawashty et al., 1994

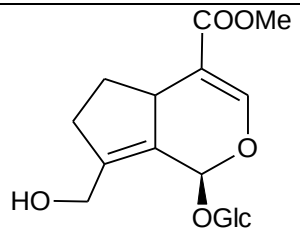
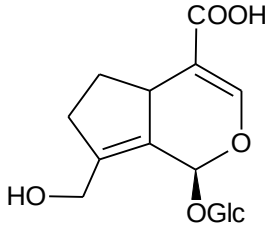
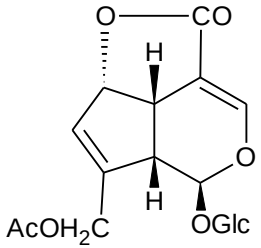
Iridoid glucosides

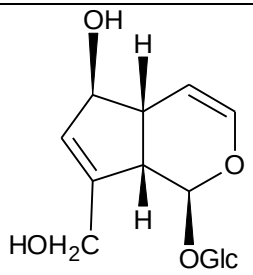
Iridoids are secondary metabolites commonly related with plant protection from predators. They are basically formed by a cyclopenta*[c]*pyran and are found in a large group of plants, mainly as glucosides (Tundis et al., 2008) .

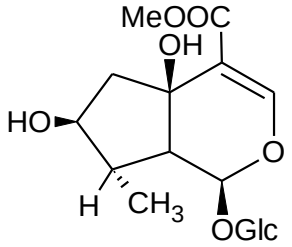
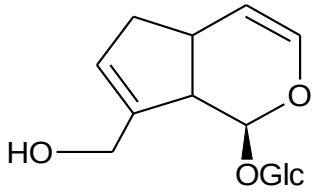
The iridoid glucosides from *Plantago* and their chemical structures are listed in Table 2.

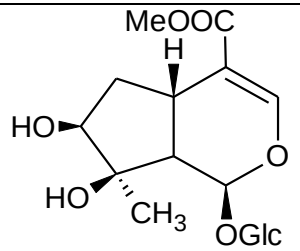
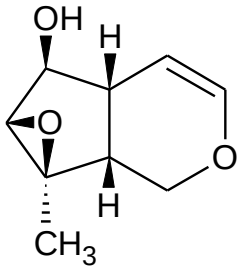
Table 2
Iridoid glucosides from *Plantago*.

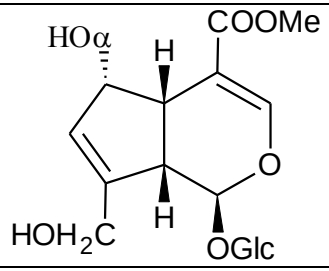
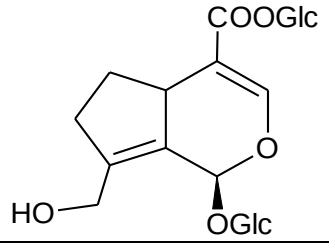
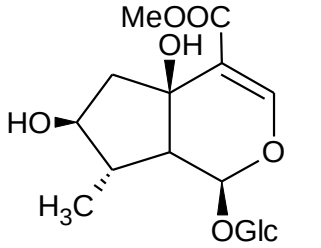
Iridoid glucoside	***Plantago* species**	**Part of plant**	**References**
Alpinoside 	*P. lundborgii* Sparre *P. alpina* L. *P. debilis* R.Br.	Aerial parts Aerial parts Aerial parts	Rønsted et al., 2000 Jensen et al., 1996 Rønsted et al., 2003
Arborescoside	*P. arborescens* Poir. *P. ovata* Forssk. *P. webbii* Barnéoud *P. palmata* Hook.f. *P. spathulata* Hook.f.	Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2003 Rønsted et al., 2003

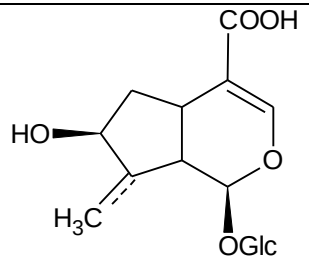
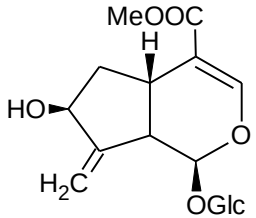
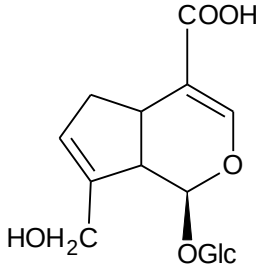
			
Arborescosidic acid 	*P. atrata* Hoppe *P. maritima* L. *P. stauntonii* Reichardt *P. subulata* L. *P. tenuiflora* Waldst. & Kit.	Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2003
Asperuloside 	*P. major* L. *P. lanceolata* L. *P. altissima* L. *P. argentea* Chaix *P. lagopus* L. *P. bellardii* All. *P. cretica* L. *P. ovata* Forssk.	Flowers Flowers Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Bianco et al., 1984 Taskova et al., 2002 Taskova et al., 2002 Taskova et al., 2002 Taskova et al., 2002 Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2000
Aucubin	*P. major* L. *P. tenuiflora* Waldst. & Kit. *P. cornuti* Gouan *P. coronopus* L. *P. subulata* L. *P. media* L. *P. atrata* Hoppe	Leaves Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Taskova et al., 2002 Taskova et al., 2002 Taskova et al., 2002 Taskova et al., 2002 Taskova et al., 2002 Taskova et al., 2002 Taskova et al., 2002

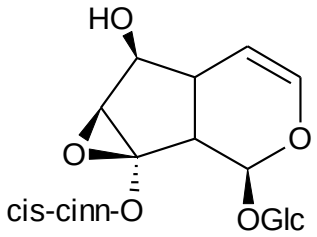
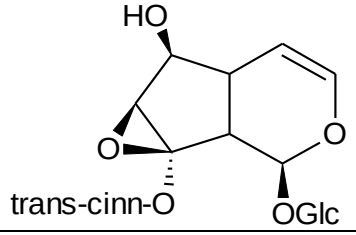
	*P. lanceolata* L.	Aerial parts	Taskova et al., 2002
	P. altissima L.	Aerial parts	Taskova et al., 2002
	P. argentea Chaix	Aerial parts	Taskova et al., 2002
	P. lagopus L.	Aerial parts	Taskova et al., 2002
	P. afra L.	Aerial parts	Taskova et al., 2002
	P. amplexicaulis Cav.	Aerial parts	Rønsted et al., 2000
	P. australis Lam.	Aerial parts	Rønsted et al., 2000
	P. cretica L.	Aerial parts	Rønsted et al., 2000
	P. stauntonii Reichardt	Aerial parts	Rønsted et al., 2000
	P. lundborgii Sparre	Aerial parts	Rønsted et al., 2000
	Littorella uniflora (L.) Asch.	Aerial parts	Rønsted et al., 2000
	P. maritima L.	Aerial parts	Rønsted et al., 2000
	P. webbii Barnéoud	Aerial parts	Rønsted et al., 2000
	P. ovata Forssk.	Aerial parts	Rønsted et al., 2000
	P. raoulii Decne.	Aerial parts	Rønsted et al., 2000
	P. arborescens Poir.	Aerial parts	Rønsted et al., 2000
	P. reniformis Beck	Aerial parts	Rønsted et al., 2000
	P. bellardii All.	Aerial parts	Rønsted et al., 2000
	P. patagonica Jacq.	Whole plant	Bowers, 1996
	P. myosuros Lam.	Whole plant	Franzyc et al., 1998
	P. hookeriana Fisch. & C.A.Mey.	Aerial parts	Damtoft et al., 1994
	P. sempervirens Crantz	Aerial parts	Damtoft et al., 1994
	P. alpina L.	Aerial parts	Andrzejewska-Golec et al., 1984
	P. asiatica L.	Whole plant	Jensen et al., 1996
	P. holosteum Scop.	Aerial parts	Oshio & Inouye, 1982
	P. reniformis Beck	Aerial parts	Janković et al., 2012
	P. schwarzenbergiana Schur	Aerial parts	Janković et al., 2012
	P. tandilensis (Pilg.) Rahn	Aerial parts	Janković et al., 2012
	P. palmata Hook.f.	Aerial parts	Rønsted et al., 2003
	P. rugelii Decne.	Aerial parts	Rønsted et al., 2003

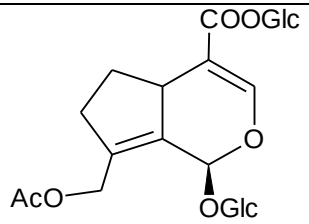
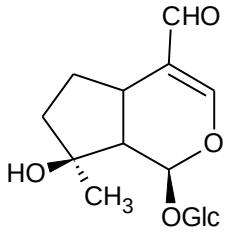
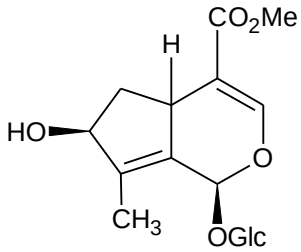
	P. maxima Juss. ex Jacq.	Aerial parts	Rønsted et al., 2003
	P. camtschatica Link	Aerial parts	Rønsted et al., 2003
	P. debilis R.Br.	Aerial parts	Rønsted et al., 2003
	P. spathulata Hook.f.	Aerial parts	Rønsted et al., 2003
	P. tomentosa Lam.	Aerial parts	Rønsted et al., 2003
	P. serraria L.	Aerial parts	Rønsted et al., 2003
	P. arenaria Waldst. & Kit.	Aerial parts	Rønsted et al., 2003
	P. aristata Michx.	Aerial parts	Rønsted et al., 2003
Auroside (5-hydroxy-8-epiloganin) 	*P. sempervirens* Crantz	Aerial parts	Rønsted et al., 2003
	P. maxima Juss. ex Jacq.	Aerial parts	Rønsted et al., 2003
Bartsioside 	*P. afra* L.	Aerial parts	Andrzejewska-Golec et al., 1993
	P. arborescens Poir.	Aerial parts	Andrzejewska-Golec et al., 1984
	P. sempervirens Crantz	Aerial parts	Andrzejewska-Golec et al., 1984
	P. webbii Barnéoud	Aerial parts	Andrzejewska-Golec et al., 1984
	P. arenaria Waldst. & Kit.	Aerial parts	Andrzejewska-Golec et al., 1984
	P. subulata L.	Aerial parts	Rønsted et al., 2000
	P. arenaria Waldst. & Kit.	Aerial parts	Rønsted et al., 2003
	P. sarcophylla Boiss. & Zohary	Aerial parts	Rønsted et al., 2003
Caryoptoside	*P. maxima* Juss. ex Jacq.	Aerial parts	Rønsted et al., 2003

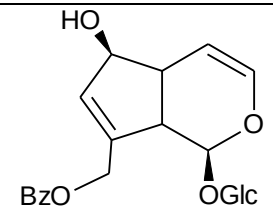
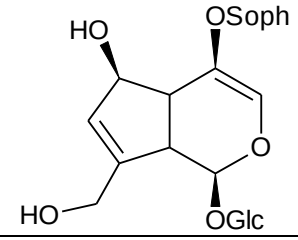
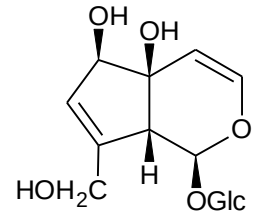
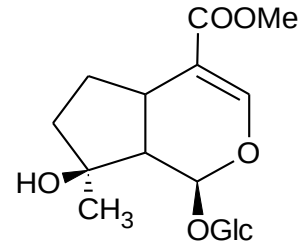
			
Catalpol 	*P. major* L.	Aerial parts	Murai et al., 1996
	P. atrata Hoppe	Aerial parts	Taskova et al., 2002
	P. lagopus L.	Aerial parts	Taskova et al., 2002
	P. lanceolata L.	Aerial parts	Taskova et al., 2002
	P. altissima L.	Aerial parts	Taskova et al., 2002
	P. argentea Chaix	Aerial parts	Taskova et al., 2002
	P. amplexicaulis Cav.	Aerial parts	Rønsted et al., 2000
	P. lundborgii Sparre	Aerial parts	Rønsted et al., 2000
	P. nivalis Boiss.	Aerial parts	Rønsted et al., 2000
	P. ovata Forssk.	Aerial parts	Rønsted et al., 2000
	P. patagonica Jacq.	Aerial parts	Rønsted et al., 2000
	Littorella uniflora (L.) Asch.	Aerial parts	Rønsted et al., 2000
	P. cornuti Gouan	Ground parts	Handjieva et al., 1992
	P. hookeriana Fisch. & C.A.Mey.	Aerial parts	Damtoft et al., 1994
	P. tandilensis (Pilg.) Rahn	Aerial parts	Rønsted et al., 2003
	P. aristata Michx.	Aerial parts	Rønsted et al., 2003
Deacetylasperulosidic acid methyl ester	*P. lagopus* L.	Aerial parts	Taskova et al., 2002
	P. altissima L.	Aerial parts	Handjieva et al., 1991
	P. lanceolata L.	Aerial parts	Handjieva et al., 1991

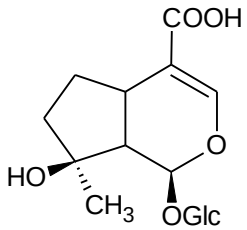
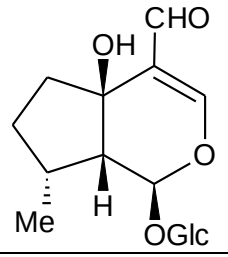
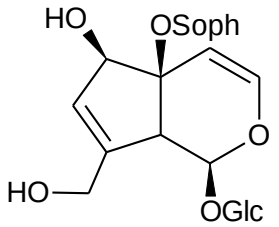
			
Desacetylhookerioside 	*P. subulata* L. *P. altissima* L. *P. tenuiflora* Waldst. & Kit. *P. serraria* L.	Aerial parts Aerial parts Aerial parts Aerial parts	Rønsted et al., 2000 Jensen et al., 1996 Rønsted et al., 2003 Rønsted et al., 2003
Epiloganin 	*P. maxima* Juss. ex Jacq.	Aerial parts	Rønsted et al., 2003
Gardoside	*P. major* L. *P. arborescens* Poir. *P. atrata* Hoppe *P. lundborgii* Sparre *P. nivalis* Boiss. *P. ovata* Forssk.	Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Murai et al., 1996 Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2000

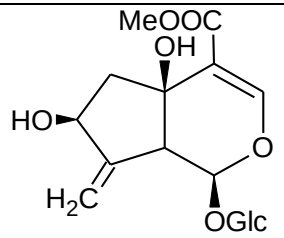
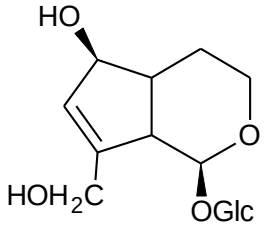
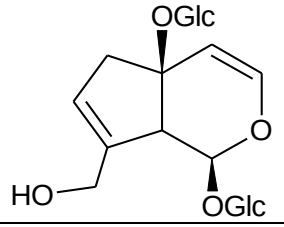
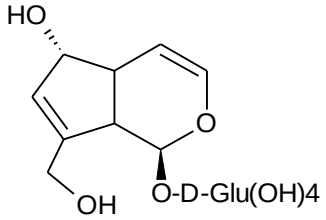
	*P. reniformis* Beck	Aerial parts	Rønsted et al., 2000
	P. stauntonii Reichardt	Aerial parts	Rønsted et al., 2000
	P. webbii Barnéoud	Aerial parts	Rønsted et al., 2000
	P. alpina L.	Aerial parts	Jensen et al., 1996
	P. altissima L.	Aerial parts	Jensen et al., 1996
	P. palmata Hook.f.	Aerial parts	Rønsted et al., 2003
	P. maxima Juss. ex Jacq.	Aerial parts	Rønsted et al., 2003
	P. tenuiflora Waldst. & Kit.	Aerial parts	Rønsted et al., 2003
	P. camtschatica Link	Aerial parts	Rønsted et al., 2003
	P. spathulata Hook.f.	Aerial parts	Rønsted et al., 2003
	P. serraria L.	Aerial parts	Rønsted et al., 2003
	P. tandilensis (Pilg.) Rahn	Aerial parts	Rønsted et al., 2003
Gardoside methyl ester 	*P. maxima* Juss. ex Jacq.	Aerial parts	Rønsted et al., 2003
	P. tomentosa Lam.	Aerial parts	Rønsted et al., 2003
Geniposidic acid 	*P. major* L.	Aerial parts	Murai et al., 1996
	P. tenuiflora Waldst. & Kit.	Aerial parts	Taskova et al., 2002
	P. coronopus L.	Aerial parts	Taskova et al., 2002
	P. lanceolata L.	Aerial parts	Taskova et al., 2002
	P. bellardii All.	Aerial parts	Rønsted et al., 2000
	P. lundborgii Sparre	Aerial parts	Rønsted et al., 2000
	P. reniformis Beck	Aerial parts	Rønsted et al., 2000
	P. alpina L.	Aerial parts	Jensen et al., 1996
	P. asiatica L.	Seeds	Toda et al., 1985
	P. serraria L.	Aerial parts	Rønsted et al., 2003

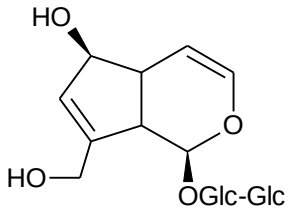
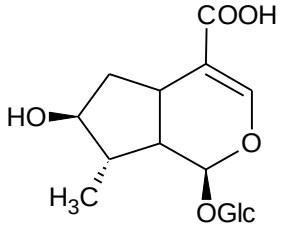
	P. sarcophylla Boiss. & Zohary	Aerial parts	Rønsted et al., 2003
	P. altissima L.	Aerial parts	Rønsted et al., 2003
	P. ovata Forssk.	Aerial parts	Rønsted et al., 2003
	P. aristata Michx.	Aerial parts	Rønsted et al., 2003
	P. palmata Hook.f.	Aerial parts	Rønsted et al., 2003
	P. rugelii Decne.	Aerial parts	Rønsted et al., 2003
	P. camtschatica Link	Aerial parts	Rønsted et al., 2003
	P. debilis R.Br.	Aerial parts	Rønsted et al., 2003
	P. spathulata Hook.f.	Aerial parts	Rønsted et al., 2003
	P. tomentosa Lam.	Aerial parts	Rønsted et al., 2003
Globularicisin 	*P. tandilensis* (Pilg.) Rahn	Aerial parts	Rønsted et al., 2003
Globularin 	*P. lanceolata* L.	Aerial parts	Rønsted et al., 2003
	P. altissima L.	Aerial parts	Rønsted et al., 2003
	P. argentea Chaix	Aerial parts	Rønsted et al., 2003
	P. lagopus L.	Aerial parts	Rønsted et al., 2003
	P. tandilensis (Pilg.) Rahn	Aerial parts	Rønsted et al., 2003
Hookeroside	*P. altissima* L.	Aerial parts	Jensen et al., 1996
	P. hookeriana Fisch. & C.A.Mey.	Aerial parts	Damtoft et al., 1994
	P. tomentosa Lam.	Aerial parts	Rønsted et al., 2003

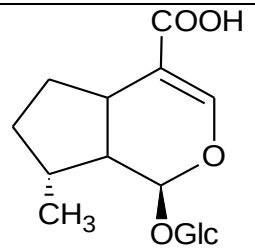
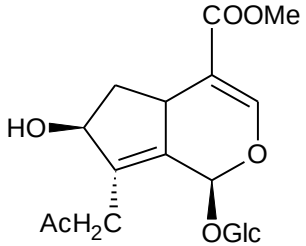
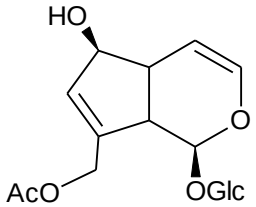
	*P. tandilensis* (Pilg.) Rahn	Aerial parts	Rønsted et al., 2003
Ixoroside 	*P. major* L.	Aerial parts	Afifi et al., 1990
Majoroside 	*P. major* L. *P. cornuti* Gouan *P. camtschatica* Link	Aerial parts Aerial parts Aerial parts	Handjieva et al., 1991 Taskova et al., 2002 Rønsted et al., 2003
Melampyroside (10-benzoylaucubin)	*P. major* L.	Aerial parts	Rønsted et al., 2003

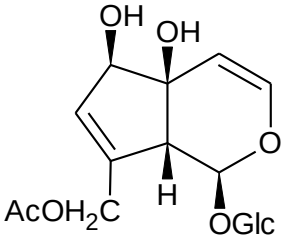
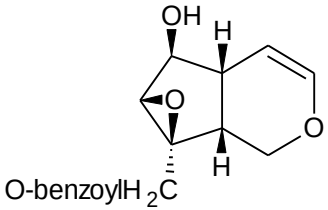
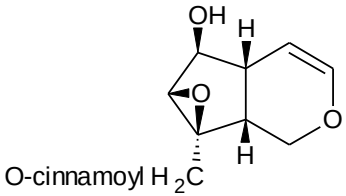
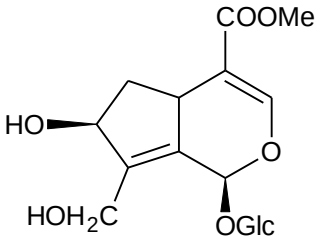
			
Mellitoside 	*P. major* L. *P. subulata* L. *P. media* L. *P. maritima* L. *P. cornuti* Gouan *P. alpina* L.	Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Murai et al., 1996 Taskova et al., 2002 Taskova et al., 2002 Rønsted et al., 2000 Rønsted et al., 2003 Rønsted et al., 2003
Monomellitoside 	*P. subulata* L. *P. media* L. *P. alpina* L.	Aerial parts Aerial parts Aerial parts	Taskova et al., 2002 Taskova et al., 2002 Jensen et al., 1996
Mussaenoside 	*P. maxima* Juss. ex Jacq.	Aerial parts	Rønsted et al., 2003

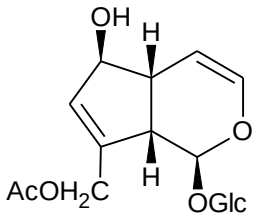
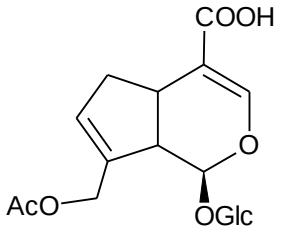
Mussaenosidic acid 	*P. arborescens* Poir. *P. nivalis* Boiss. *P. sempervirens* Crantz *P. webbii* Barnéoud *P. alpina* L. *P. maxima* Juss. ex Jacq. *P. sarcophylla* Boiss. & Zohary	Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2000 Jensen et al., 1996 Rønsted et al., 2003 Rønsted et al., 2003
Plantarenaloside 	*P. major* L. *P. afra* L. *P. coronopus* L. *P. sempervirens* Crantz *P. webbii* Barnéoud *P. arenaria* Waldst. & Kit. *P. lagopus* L.	Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Afifi et al., 1990 Taskova et al., 2002 Rønsted et al., 2000 Rønsted et al., 2000 Rønsted et al., 2000 Andrzejewska-Golec et al., 1984 Afifi et al., 1990
Rehmannioside D 	*P. maritima* L.	Aerial parts	Rønsted et al., 2000
Strictoloside	*P. maxima* Juss. ex Jacq.	Aerial parts	Rønsted et al., 2003

			
3,4-dihydroaucubin 	*P. atrata* Hoppe *P. subulata* L. *P. asiatica* L.	Aerial parts Aerial parts Whole plant	Handjieva et al., 1991 Saadi et al., 1991 Oshio et al., 1982
6-deoxymellitoside 	*P. subulata* L.	Aerial parts	Rønsted et al., 2000
6-epiaucubin 	*P. subulata* L.	Aerial parts	Andrzejewska-Golec et al., 1984

6’-o-beta-glucosylaucubin  	*P. asiatica* L.	Whole plant	Oshio et al., 1982
8-epiloganic acid  	*P. arborescens* Poir.	Aerial parts	Rønsted et al., 2000
	P. atrata Hoppe	Aerial parts	Rønsted et al., 2000
	P. lundborgii Sparre	Aerial parts	Rønsted et al., 2000
	P. ovata Forssk.	Aerial parts	Rønsted et al., 2000
	P. reniformis Beck	Aerial parts	Rønsted et al., 2000
	P. stauntonii Reichardt	Aerial parts	Rønsted et al., 2000
	P. alpina L.	Aerial parts	Jensen et al., 1996
	P. altissima L.	Aerial parts	Jensen et al., 1996
	P. hookeriana Fisch. & C.A.Mey.	Aerial parts	Damtoft et al., 1994
	P. palmata Hook.f.	Aerial parts	Rønsted et al., 2003
	P. rugelii Decne.	Aerial parts	Rønsted et al., 2003
	P. tenuiflora Waldst. & Kit.	Aerial parts	Rønsted et al., 2003
	P. camtschatica Link	Aerial parts	Rønsted et al., 2003
	P. debilis R.Br.	Aerial parts	Rønsted et al., 2003
	P. spathulata Hook.f.	Aerial parts	Rønsted et al., 2003
	P. tomentosa Lam.	Aerial parts	Rønsted et al., 2003
	P. webbii Barnéoud	Aerial parts	Rønsted et al., 2003
	P. tandilensis (Pilg.) Rahn	Aerial parts	Rønsted et al., 2003
8-epideoxyloganic acid	*P. spathulata* Hook.f.	Aerial parts	Rønsted et al., 2003
	P. webbii Barnéoud	Aerial parts	Rønsted et al., 2003

			
10-acetoxymajoroside 	*P. major* L. *P. tenuiflora* Waldst. & Kit. *P. cornuti* Gouan	Aerial parts Aerial parts Aerial parts	Taskova et al., 2002 Taskova et al., 2002 Rønsted et al., 2003
10-acetylaucubin 	*P. media* L. *P. subulata* L.	Aerial parts Aerial parts	Rønsted et al., 2003 Rønsted et al., 2003
10-acetylmonomellitoside	*P. subulata* L. *P. media* L.	Aerial parts Aerial parts	Taskova et al., 2002 Taskova et al., 2002

			
10-benzoylcatalpol 	*P. lagopus* L. *P. hookeriana* Fisch. & C.A.Mey. *P. patagonica* Jacq. *P. aristata* Michx. *P. tandilensis* (Pilg.) Rahn	Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Taskova et al., 2002 Damtoft et al., 1994 Rønsted et al., 2000 Rønsted et al., 2003 Rønsted et al., 2003
10-cinnamoylcatalpol 	*P. lanceolata* L. *P. altissima* L. *P. argentea* Chaix *P. lagopus* L.	Aerial parts Aerial parts Aerial parts Aerial parts	Taskova et al., 2002 Taskova et al., 2002 Taskova et al., 2002 Taskova et al., 2002
10-hydroxymajoroside 	*P. major* L. *P. tenuiflora* Waldst. & Kit. *P. cornuti* Gouan *P. rugelii* Decne.	Aerial parts Aerial parts Ground parts Aerial parts	Taskova et al., 2002 Taskova et al., 2002 Handjieva et al., 1992 Rønsted et al., 2003

10-o-acetylaucubin 	*P. subulata* L. *P. media* L.	Aerial parts Aerial parts	Taskova et al., 2002 Taskova et al., 2002
10-o-acetylgeniposidic acid 	*P. alpina* L. *P. tomentosa* Lam.	Aerial parts Aerial parts	Jensen et al., 1996 Rønsted et al., 2003

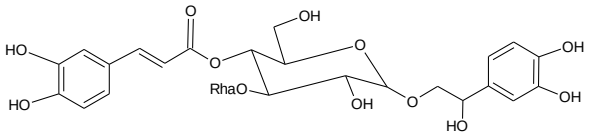
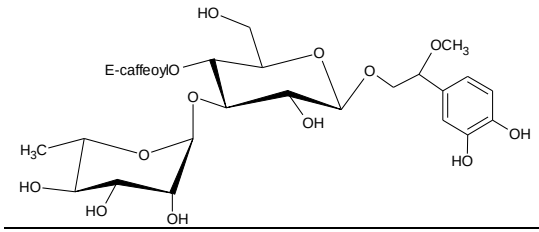
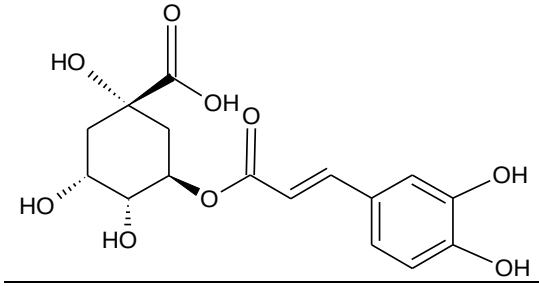
Other phenolic compounds

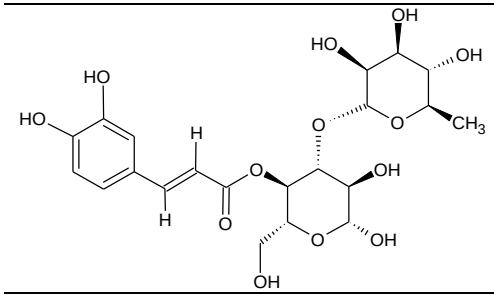
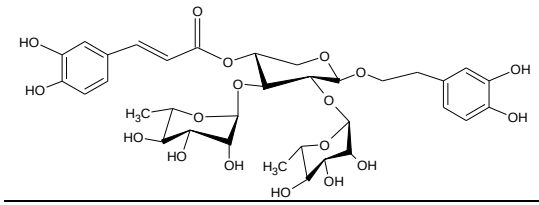
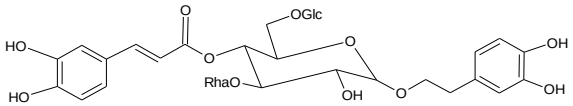
In addition to the flavonoids, they are other phenolic compounds in *Plantago*. These compounds are structurally formed by an aromatic ring, bearing one or more hydroxyl group. They have an essential role in the reproduction, growth and defense of plants, and are responsible for the color of plants and flavor of fruits (Balasundram et al., 2006; Huang et al., 2010).

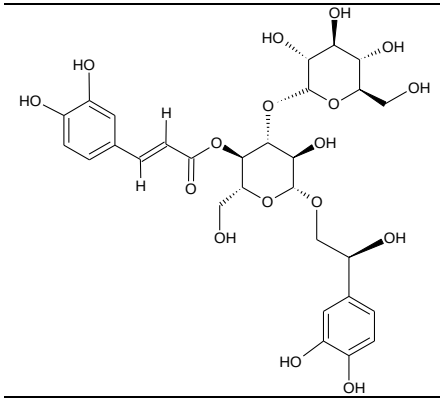
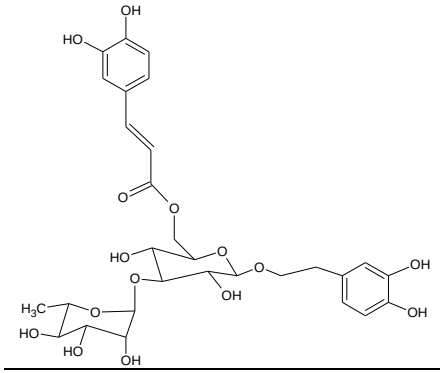
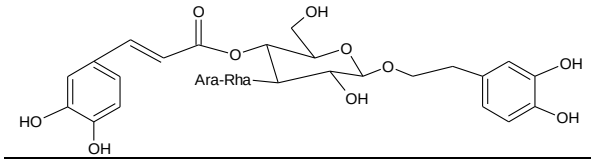
The phenolic compounds from *Plantago* and their chemical structures are listed in Table 3.

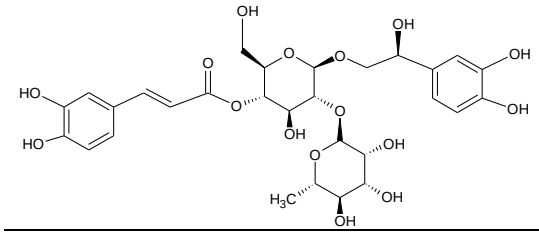
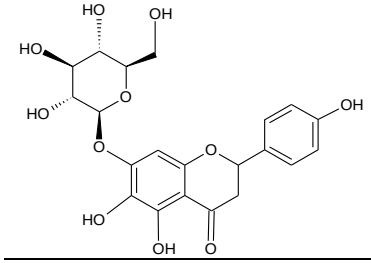
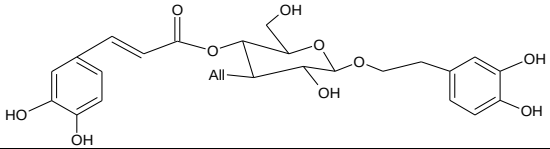
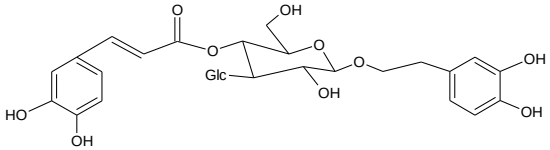
Table 3
Phenolic compounds from *Plantago*.

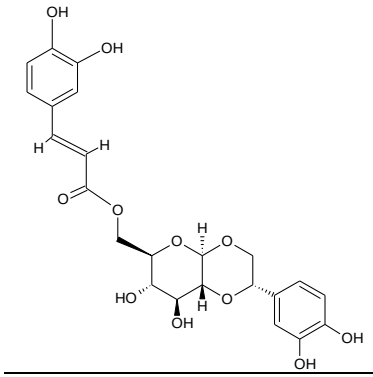
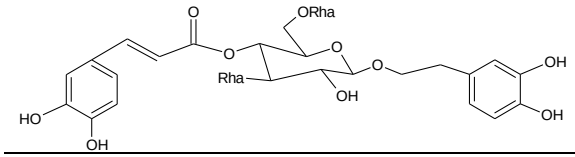
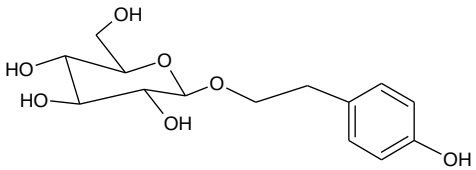
Phenolic coumponds	***Plantago* species**	**Part of plant**	**References**
β-Hydroxyacteoside	*P. tenuiflora* Waldst. & Kit.	Aerial parts	Rønsted et al., 2003

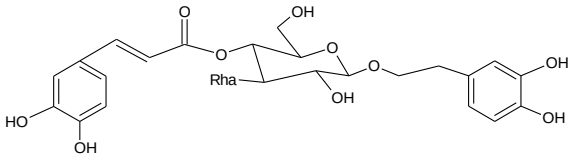
	*P. camtschatica* Link *P. tomentosa* Lam. *P. crassifolia* Forssk. *P. macrorhiza* Poir. *P. depressa* Willd. *P. serraria* L. *P. macrorhiza* Poir. *P. lanceolata* L.	Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Rønsted et al., 2003 Rønsted et al., 2003 Rønsted et al., 2003 Rønsted et al., 2003 Nishibe et al., 1993 Rønsted et al., 2003 Rønsted et al., 2003 Fleer & Verspohl, 2007
Campenoside I 	*P. depressa* Willd.	Aerial parts	Nishibe et al., 1993
Chlorogenic acid 	*P. major* L. *P. cretica* L. *P. bellardii* All.	Aerial parts Aerial parts Aerial parts	Rønsted et al., 2003 Rønsted et al., 2000 Rønsted et al., 2000
Cistanoside F	*P. depressa* Willd.	Aerial parts	Nishibe et al., 1993

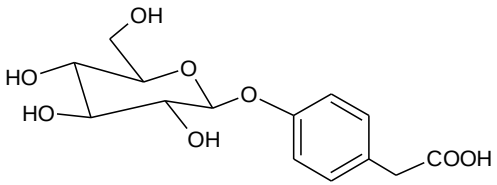
			
Crassifolioside 	*P. crassifolia* Forssk.	Leaves	Andary et al., 1988
Echinacoside 	*P. maritima* L.	Aerial parts	Rønsted et al., 2000
Hellicoside	*P. asiatica* L.	Aerial parts	Ravn et al., 1990

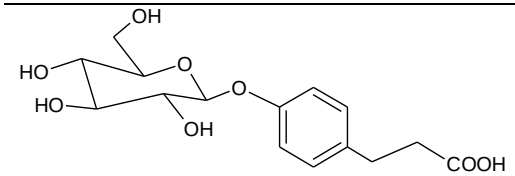
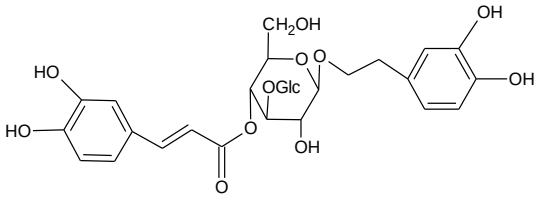
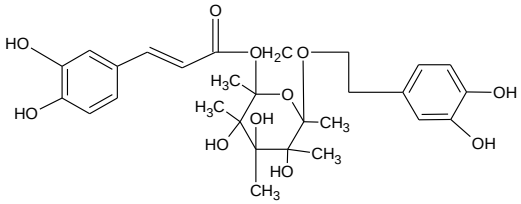
			
Isoverbascoside 	*P. australis* Lam. *P. lanceolata* L.	Aerial parts Aerial parts	Rønsted et al., 2000 Murai et al., 1995
Lavandufolioside 	*P. lanceolata* L.	Aerial parts	Murai et al., 1995
Orobanchoside	*P. depressa* Willd.	Aerial parts	Nishibe et al., 1993

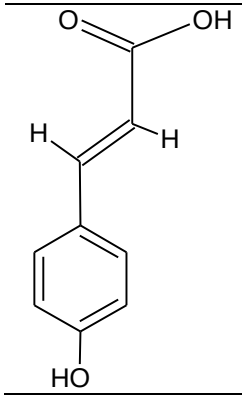
			
Plantaginin 	*P. asiatica* L.	Aerial parts	Ravn et al., 1990
Plantalloside 	*P. crassifolia* Forssk. *P. myosuros* Lam.	Aerial parts Aerial parts	Rønsted et al., 2003 Rønsted et al., 2003
Plantamajoside 	*P. major* L. *P. asiatica* L. *P. palmata* Hook.f. *P. tenuiflora* Waldst. & Kit. *P. debilis* R.Br. *P. sarcophylla* Boiss. & Zohary *P. media* L. *P. arborescens* Poir.	Aerial parts Leaves Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts Aerial parts	Zubair et al., 2012 Ravn et al., 1990 Biringanine et al., 2007 Rønsted et al., 2003 Rønsted et al., 2003 Rønsted et al., 2003 Andary et al., 1988 Rønsted et al., 2000

	P. webbii Barnéoud	Aerial parts	Rønsted et al., 2000
	P. lagopus L.	Aerial parts	Rønsted et al., 2003
	P. bellardii All.	Aerial parts	Rønsted et al., 2003
	P. ovata Forssk.	Aerial parts	Rønsted et al., 2000
	P. lanceolata L.	Aerial parts	Murai et al., 1995
Plantasioside 	*P. asiatica* L.		
Poliumoside 	*P. tandilensis* (Pilg.) Rahn	Aerial parts	Rønsted et al., 2003
Salidroside 	*P. maxima* Juss. ex Jacq.	Aerial parts	Rønsted et al., 2003
	P. australis Lam.	Aerial parts	Rønsted et al., 2000
	P. coronopus L.	Aerial parts	Rønsted et al., 2000
	P. tandilensis (Pilg.) Rahn	Aerial parts	Rønsted et al., 2003

Verbascoside (acteoside)  	*P. major* L.	Leaves	Ravn & Brimer, 1988
	P. asiatica L.	Aerial parts	Ravn et al., 1990
	P. palmata Hook.f.	Leaves	Biringanine et al., 2007
	P. rugelii Decne.	Aerial parts	Rønsted et al., 2003
	P. maxima Juss. ex Jacq.	Aerial parts	Rønsted et al., 2003
	P. tenuiflora Waldst. & Kit.	Aerial parts	Rønsted et al., 2003
	P. camtschatica Link	Aerial parts	Rønsted et al., 2003
	P. spathulata Hook.f.	Aerial parts	Rønsted et al., 2003
	P. tomentosa Lam.	Aerial parts	Rønsted et al., 2003
	P. crassifolia Forssk.	Aerial parts	Rønsted et al., 2003
	P. sarcophylla Boiss. & Zohary	Aerial parts	Rønsted et al., 2003
	P. debilis R.Br.	Aerial parts	Rønsted et al., 2003
	P. tandilensis (Pilg.) Rahn	Aerial parts	Rønsted et al., 2003
	P. aristata Michx.	Aerial parts	Rønsted et al., 2003
	P. reniformis Beck	Aerial parts	Rønsted et al., 2003
	P. depressa Willd.	Aerial parts	Nishibe et al., 1993
	P. raoulii Decne.	Aerial parts	Rønsted et al., 2000
	P. stauntonii Reichardt	Aerial parts	Rønsted et al., 2000
	P. myosuros Lam.	Whole plant	Franzyk et al., 1998
	P. australis Lam.	Aerial parts	Rønsted et al., 2003
	P. subulata L.	Aerial parts	Rønsted et al., 2000
	P. serraria L.	Aerial parts	Rønsted et al., 2003
	P. coronopus L.	Aerial parts	Rønsted et al., 2003
	P. macrorhiza Poir.	Aerial parts	Rønsted et al., 2003
	Littorella uniflora (L.) Asch.	Aerial parts	Rønsted et al., 2000
	P. arborescens Poir.	Aerial parts	Rønsted et al., 2000
	P. webbii Barnéoud	Aerial parts	Rønsted et al., 2000
	P. sempervirens Crantz	Aerial parts	Rønsted et al., 2000
	P. arenaria Waldst. & Kit.	Aerial parts	Rønsted et al., 2000
	P. afra L.	Aerial parts	Rønsted et al., 2003

	P. atrata Hoppe	Aerial parts	Rønsted et al., 2003
	P. nivalis Boiss.	Aerial parts	Rønsted et al., 2003
	P. lagopus L.	Aerial parts	Rønsted et al., 2003
	P. lanceolata L.	Aerial parts	Fleer & Verspohl, 2007
	P. cretica L.	Aerial parts	Rønsted et al., 2003
	P. bellardii All.	Aerial parts	Rønsted et al., 2003
	P. ovata Forssk.	Aerial parts	Rønsted et al., 2000
	P. lundborgii Sparre	Aerial parts	Rønsted et al., 2003
	P. patagonica Jacq.	Aerial parts	Rønsted et al., 2000
	P. hookeriana Fisch. & C.A.Mey.	Aerial parts	Damtoft et al., 1994
	P. altissima L.	Aerial parts	Jensen et al., 1996
	P. alpina L.	Aerial parts	Jensen et al., 1996
	P. holosteum Scop.	Aerial parts	Janković et al., 2012
	P. reniformis Beck	Aerial parts	Janković et al., 2012
	P. schwarzenbergiana Schur	Aerial parts	Janković et al., 2012
2-[4-(β-D-glucopyranosyloxy) phenyl] acetic acid 	*P. tandilensis* (Pilg.) Rahn	Aerial parts	Rønsted et al., 2003
3-[4-(β-D-glucopyranosyloxy) phenyl] propionic acid	*P. aristata* Michx.	Aerial parts	Rønsted et al., 2003

			
3,4-dihydroxy-β-phenethyl-O- β-D-glucopyranosyl (1 → 3)-4- O-caffeoyl-β-D-glucopyranoside 	*P. major* L.	Leaves	Ravn et al., 1988
3,4-dihydroxyphenethyl alcohol-6-O-caffeoyl-β-D-glucoside 	*P. asiatica* L.	Aerial parts	Ravn et al., 1990
4-Hydroxycinnamic acid	*P. lanceolata* L.	Whole plant	Dalar et al., 2012

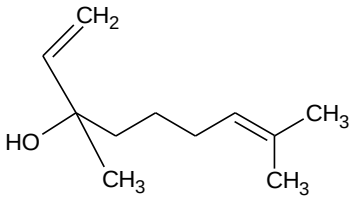
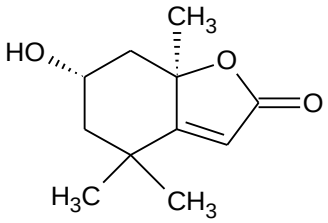
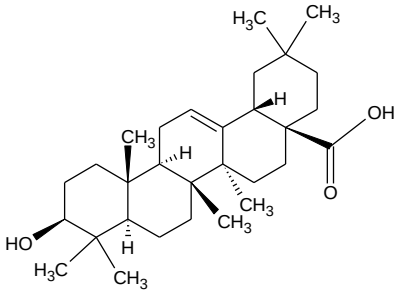
			

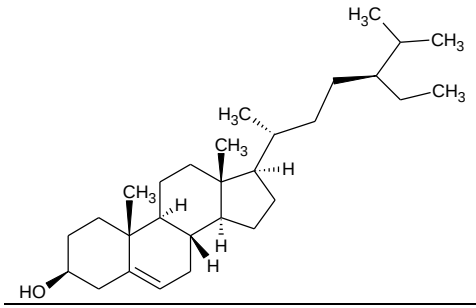
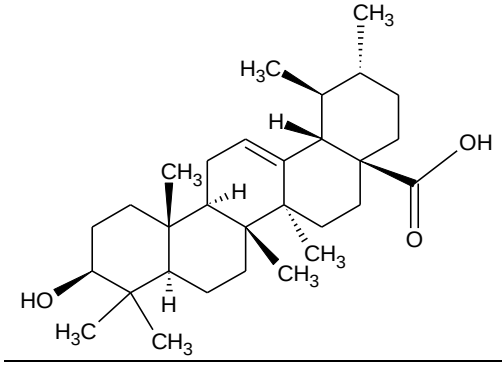
Terpenoids

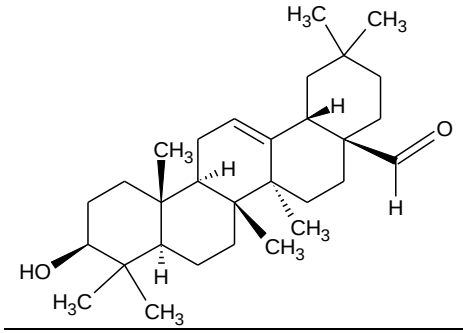
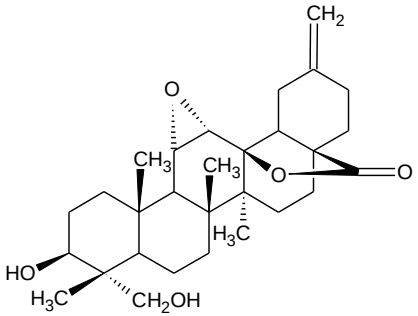
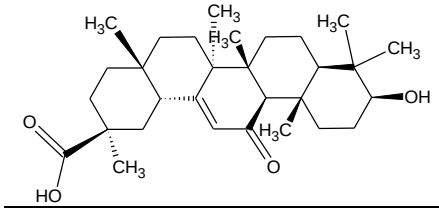
Terpenoids are derived from five-carbone isoprene units, synthetized either by the mevalonate or the methylerythritol-4-phosphate (MEP) pathway. They are the basic constituents of essential oils in plants, and play roles as repellants, plant-plant and plant-pathogen/herbivore interaction, and hormones (Graßmann, 2005).

The terpenoids from *Plantago* and their chemical structures are listed in Table 4.

Table 4
Terpenoids from *Plantago*.

Terpenoid	***Plantago* species**	**Part of plant**	**References**
Linalool 	*P. major* L. *P. asiatica* L.	Whole plant Leaves	Chiang et al., 2003 Chung et al., 2007
Loliolid 	*P. major* L. *P. lanceolata* L.	Leaves Leaves	Pailer & Haschke-Hofmeister, 1969 Tóth et al., 1976
Oleanolic acid 	*P. major* L.	Leaves	Abud et al., 2012

Sitosterol 	*P. major* L. *P. lanceolata* L. *P. ovata* Forssk.	Aerial parts Leaves Seeds	Kolak et al., 2011 Pankoke & Müller, 2013 Nakamura et al., 2005
Ursolic acid 	*P. major* L. *P. lanceolata* L.	Whole plant Leaves	Ringbom et al., 1998 Sosa et al., 2011

3β-hydroxyolean-12-en-28-al 	*P. amplexicaulis* Cav.	Aerial parts	Salama & Saffan., 2003
11α, 12α-Epoxy-3β, 23-dihydroxy-30-norolean-20(29)-en-28, 13-β-olide 	*P. amplexicaulis* Cav.	Aerial parts	Salama & Saffan, 2003
18β-glycyrrhetinic acid 	*P. major* L.	Leaves	Ringbom et al., 1998

Biological activities of *Plantago* constituents

Analgesic activity

The analgesic activity of *P. australis* hydroalcoholic extract on mice was evaluated by acetic acid induced writhing, and the analgesic effect was probably reported by the inhibition of prostaglandin synthesis (Palmeiro et al., 2002). Linalool also was performed to reduce the acute pain in mice treated with paclitaxel. The results offer evidence for the involvement of peripheral opioids in this mechanism, promoting an anti-hyperalgesia (Katsuyama et al., 2012).

Antidiabetic activity

Recent studies revealed that triterpenes, as oleanolic and ursolic acids, have antidiabetic activity. Both of them exhibited higher inhibition of α-glucosidase enzyme, with more satisfactory results than the positive control, acarbose, in *in vitro* experiments. Oleanolic acid also regulates the intracellular redox status and directly modulates the carbohydrate metabolism and insulin signaling, and ursolic acid may exhibits immunomodulatory effects by growing insulin levels and modulating blood glucose levels in *in vivo* models (Jang et al., 2009; Castellano et al., 2013; López et al., 2015).

Antigenotoxic activity

Additionally results obtained from a study who submitted human breast cancer cells and noncancerous human mammary epithelial MCF10A cells, revealed that oleanolic acid can reduce oxidative stress and oxidative damage to the DNA in the epithelial cells (Sánchez-Quesada et al., 2015). Other studies, this time involving the verbascoside, indicated a protection of human peripheral blood cells and human leukemia HL-60 cells against H_2O_2-induced DNA damage, and no genotoxic effects in the *Drosophila* wing spot test. However, when tested in human lymphocytes, verbascoside induced genotoxicity reported by the increase chromosome aberrations and chromatid exchanges (Fabiani et al., 2008; Santoro et al., 2008; Santos-Cruz et al., 2012).

Anti-inflammatory activity

Hydrosoluble fiber of *P. ovata* showed protective effect in duodenal mucosa of rabbits submitted to acetylsalicylic acid (AAS)-induced lesions, probably by limiting AAS penetration in epithelial cells (Sahagún et al., 2015). Similarly, *P. ovata* leaves has been used in the treatment of peptic ulcer, and its seeds are largely used in constipation cases (Fernández-Bañares et al., 1999). In the other hand, *P. major* leaf extract was tested in rats with acetaminophen-induced hepatotoxicity. As results, the plant extract revealed significantly anti-inflammatory property, reducing the proinflammatory cytokine and hepatic enzymes (ALT and AST) levels (Hussan et al., 2015). The anti-inflammatory activity of *P. australis* hydroalcoholic extract on mice was evaluated by the rat paw edema induced by carrageenan, and the favorable results were probably reported by the inhibition of prostaglandin synthesis (Palmeiro et al., 2002)

In vitro and *in vivo* assays showed that mussaenoside and geniposidic acid have potent anti-inflammatory activity by targeting mediators of inflammation and reducing the induced edema in rats (Carrillo-Ocampo et al., 2013; Vogl et al., 2013b). Another study revealed that geniposidic acid also inhibits cyclooxygenase enzymes activities, together with the other iridoids, aucubin, catalpol and verbascoside. In addition, catalpol ameliorates acute pancreatitis in rats by inhibiting activation of nuclear factor kappa B (NF-κB) (Park et al., 2007; Gyurkovska et al., 2011; Xiao et al., 2014). The NF-κB inhibition by baicalein, mangiferin and oleanolic acid was also verified in LPS-induced mastitis, collagen-induced arthritis – both in mice, and in an *in vitro* assay with RAW 264.7 macrophage cell line, respectively, suggesting its use in clinical treatment of rheumatoid arthritis, oxidative stress induced diseases related to inflammatory consequences and mastitis (Hwang et al., 2014; He et al., 2015; Tsubaki et al., 2015).

Due the role of inflammation in the development of insulin resistance, an *in vitro* study using homoplantaginin showed its participation in the modulation of insulin sensitivity in endothelial cells. This compound usefully decreased nitric oxide production, inhibiting inflammation and ameliorating the endothelial disfunction (Wu et al., 2012). Other anti-inflammatory properties were verified in several studies with sitosterol, who inhibited inflammatory genes expression in keratinocytes – which may be useful in the treatment of alopecia, and significantly reduced carrageenan rat paw edema, as well as with verbascoside, who reduced pro-inflammatory cytokines expression and reactive oxygen species (ROS) production in cultured cells with pro-inflammatory stimuli (Speranza et al., 2010; Chittur et al., 2011; Bhalke & Pal, 2012).

Antimicrobial activity

Compound baicalein exhibited synergistic activity with penicillins against penicillinase-producing *Staphylococcus aureus*. The triterpenes oleanolic and ursolic acids significantly inhibited the peptidoglycan biosynthesis in *Streptococcus mutans*, and oleanolic acid displayed antiparasitic activity against *Leishmania donovani* (Talib et al., 2012; López et al., 2015; Park et al., 2015; Qian et al., 2015).

In the search for new anti-malarial drugs, *in vitro* studies involving 18β-glycyrrhetinic acid revealed its significant anti-malarial potential against *Plasmodium falciparum*. These results allowed an *in silico* trial which confirmed that this compound holds drug-like properties, and to support these results, an *in vivo* evaluation indicated a dose dependent activity of 18β-glycyrrhetinic acid in the Swiss mice infection test (Kalani et al., 2013).

Antioxidant activity

N-hexane, ethyl acetate (EtOAc) and water extracts of the whole part of *P. asiatica* were tested for their antioxidant activity by the oxygen radical absorbance capacity (ORAC) method. As results, EtOAc extract showed the mostly marked antioxidant activity, and was chromatographed by HPLC, resulting in seven isolated compounds, all of them with markedly antioxidant activity (Amakura et al., 2012). Another research, this time involving *P. major* ethanolic extract, exhibited significant free radical scavenger activity against DPPH radicals in isolated rat liver mitochondria and in human hepatocellular carcinoma HepG2 cells, with no cytotoxic effects. *P. lanceolata* extract also performed similar activity on the reduction of DPPH radical (Adam et al., 2009; Mello et al., 2015).

According two studies, baicalein and aucubin played important role in antioxidant defenses. First, both of them inhibited ROS production by human neutrophils, and baicalein also exerted up-regulation antioxidant defense in cisplatin-induced renal damage (Reina et al., 2013; Sahu et al., 2015). Similarly, chlorogenic acid showed antioxidant properties in an intestinal ischemia-reperfusion model, sitosterol contributed to the antioxidant activity of compounds produced by carbonyl-amine reactions and linalool inhibited some pro-oxidant lipid peroxidation in rats' pancreas (Hidalgo et al., 2009; Sato et al., 2011; Oboh et al., 2015).

Several studies with oleanolic and ursolic acids have been shown their antioxidant activity. An *in vitro* study involving oleanolic acid resulted in a higher

protection of cells against cytotoxicity induced by ROS generation induced by tert-Butyl hydroperoxide (tBHP) and alloxan, and also protected rats' liver against tetrachloride-induced injury (Bai et al., 2007; Gao et al., 2009; Wang et al., 2010). On the other hand, ursolic acid showed significant radical scavenger activity using the DPPH assay, as well as modulated the UVB-induced ROS in human lymphocytes (Ramachandran & Prasad, 2008; do Nascimento et al., 2013).

Antitumoral activity

According López et al. (2009), the consumption of *P. ovata* appears to be inversely associated with colorectal cancer mortality. Similarly, but not described in *Plantago*, apigenin have shown inhibitory effects against melanoma and its metastatic potential, leukaemia and glioblastoma (as rutin) in cell lines (Wang et al., 1999; Caltagirone et al., 2000; Santos et al., 2015).

Other studies conducted in cell lines have shown that baicalein and isoharmnetin are involved in the induced cytotoxicity and by downregulating the matrix metalloproteinase activity in human breast cancers. Catalpol suppresses proliferation and induces apoptosis in ovarian cancer; linalool shows cytotoxic effects by stimulating antitumor immunity and β-sitosterol has chemopreventive activity against colon cancer models, including *in vivo* models (Baskar et al., 2010; Chang & Shen, 2014; Gao et al., 2014; Chang et al., 2015; Li et al., 2015a).

The triterpene, mainly oleanolic and ursolic acids, are broadly described as antitumor compounds. Oleanolic acid showed considerable tumor inhibition in nude mice transplanted with human pancreatic cancer L3.6PL cells (Jutooru et al., 2010). Also, it reported cytotoxicity, although it was weak, and inhibition of proliferation and increase of oxidative stress in human breast adenocarcinoma MCF-7 and MDA-MB-231 cells (Hsu et al., 2005; Sánchez-Quesada et al., 2015). Meanwhile, ursolic acid exercised blockade in tumor growth in mouse sarcoma S180 cells, and also inhibited proliferations of HL-60 and human tongue cancer TSCC-α cells (Manu & Kuttan, 2008; Wang et al., 2012a; Wang et al., 2012b), and induced apoptosis of MCF-7 cells. Recent studies presented more mechanistic effects of ursolic acid in several cancer types, as by weakening surveillance mechanisms in mice lung carcinoma cells, by causing cytotoxicity in mouse glioma C6 cells, and by inhibiting the invasive phenotype or promoting apoptosis of human gastric cancer SNU-484 and SGC-7901 cells, respectively (Mazumder et al., 2013; Li et al., 2014a; Kim & Moon, 2015; Kim et al.,

2015). Due its poor solubility, experiments involving nanosized delivery systems loading ursolic acid indicated that these nanoparticles could inhibit the growth of mouse hepatocellular carcinoma H22 cells and induce more efficiently apoptosis (Zhang et al., 2015).

Gastroprotective activity

A Chinese study with medicinal plants described that *P. depressa* Willd. is commonly used in stomach gas and constipation cases (Shen et al., 2010). Similarly, ethanolic spissum extract of *P. lanceolata* and its compounds luteolin, acteoside (verbascoside) and plantamajoside were investigated for antispasmodic activity on isolated ileum and trachea of the guinea-pig. The results showed that the extract decreased the contractions of the trachea and the ileum induced by various agonists, and these results were also demonstrated by the compounds luteolin, acteoside and plantamajoside (Fleer & Verspohl, 2007).

Two studies tested aucubin as possible therapy against non-alcoholic fatty liver disease (NAFLD). The results demonstrated that both compounds regulate lipotoxicity by enhancing lysosomal activity and inhibiting the endoplasmic reticulum stress in HepG2 cells (Lee et al., 2013; Lee et al., 2014). Rats with severe acute pancreatitis were also submitted to a treatment with baicalein, and the compound protected the animals against many pro-inflammatory cytokines effects (Li et al., 2015b).

Another research, this time involving ursolic acid, the compound improved key enzymes in the controlling lipids metabolism, decreasing the hepatic steatosis and constituting this terpenoid as a new candidate for NAFLD therapy (Li et al., 2014b). Oleanolic and ursolic acids also showed similar effects *in vivo*, revealing that these compounds can exert hepatoprotective and hypolipidemic properties by lowering the lipid peroxidation in the liver (Rezaei-Golmisheh et al., 2015).

Hypolipemiant activity

The *P. ovata* husks are largely used to decrease plasma cholesterol, and similarly effect was noted when the seeds of *P. ovata* were tested in guinea pigs. As results, the administration of seeds reduced plasma triglycerides and LDL cholesterol by altering hepatic and bile acid metabolism, acting directly in regulatory enzymes of cholesterol synthesis and its catabolism (Romero et al., 2002).

The anti-obesity activity of asperuloside was evaluated in rats, and the effect of this compound suggested its important role by suppressing the body weight, food intake and plasma levels of glucose, insulin and lipids, and increasing many enzymes related to the metabolic function in several organs under high-fat diet conditions (Fujikawa et al., 2012). As well as in NAFLD, ursolic acid has potential capacity to increase skeletal muscle and brown fat, regulating the glucose intolerance and reducing the obesity (Kunkel et al., 2012).

Neuroprotective effects

A recent clinical trial evaluated the effects of *P. ovata* in Parkinson patients treated with levodopa, since this plant is commonly used to reduce the symptoms of gastrointestinal disorders caused by this disease. As results, *P. ovata* stabilized levodopa concentrations by causing a homogenization of its absorption, improving the pharmacotherapy (Fernandez-Martinez et al., 2014).

Oleanolic and ursolic acids have shown potential inhibition of acetylcholinesterase (AChE). The cleavage of acetylcholine by this enzyme has a strong correlation with the degree of cognitive loss in patients with Alzheimer's disease (AD). *In vitro* assays demonstrated that the ursolic acid inhibits AChE activity and also reduces beta-amyloid(Aβ)-induced oxidative damage, as well acts blocking the proinflammatory cytokines production stimulated by Aβ, playing a neuroprotective role against AD. Oleanolic acid has similar functions, reducing the neuronal death and the Aβ-induced memory deficit in *in vivo* assays (Yoo & Park, 2012).

Another constituent, the 18β-glycyrrhetinic acid, has been used in *in vivo* assays as a therapeutic strategy for multiple sclerosis (MS). This terpenoid could modulate the microglia activation, inhibiting the central nervous system inflammation, promoting the remyelination and suppressing the disease severity of autoimmune encephalomyelitis (Zhou et al., 2015). The iridoid catalpol also acts as an important neuroprotective compound, by attenuating cognitive deficit, oxidative stress and neuronal damage in *in vivo* tests, and decreasing the apoptosis in LPS-induced neurodegeneration in *in vitro* models (Chen et al., 2013a; Haicheng et al., 2014). Finally, luteolin has many studies due its neuroprotective effects, suggesting its use in cases of autism spectrum disorders, AD, Parkinson's disease, diabetes-associated cognitive decline, traumatic brain injury and multiple sclerosis (Kempuraj et al., 2008; López-Lázaro, 2009; Choi et al., 2014; Hu et al., 2014; Xu et al., 2014).

Respiratory tract activity

The *P. major* is widely used for treatment of respiratory diseases. Thus, an *in vivo* study compared the hydro-alcoholic extract of *P. major* and the standard therapy with theophylline, and the results showed that the administration of *P. major* extract in asthmatic rats reestablished the lung histopathologic characteristics, evidencing its protective effect in asthma (Farokhi & Khaneshi, 2013).

About the compounds described in *Plantago*, chrysoeriol has been related as a selective bronchodilator, according a study which tested its properties in guinea pigs. The results demonstrated a relaxant effect in airways through K^+ channel activation (Khan & Gilani, 2015).

Wound healing activity

An *in vivo* study examined the effects off 0.1% aucubin on oral wound healing. As results, both re-epithelization and matrix formation of mice buccal mucosa treated with aucubin occurred earlier when compared with the control group. The number of inflammatory cells was also fewer, making aucubin a useful compound to topical wound healing formulations (Shim, et al., 2007).

According two different studies, verbascoside also showed wound healing activity. First, a study involving adult hares performed the corneal epithelial wound healing activity of liposomal eyedrops containing verbascoside. The effect of this administration was positive, reducing the latency time to three hours and the healing process to 48 hours (Ambrosone et al., 2014). Another study, with *in vitro* and *in vivo* experiments, verified that verbascoside significantly inhibited chemokines secretion and exhibited anti-inflammatory action in the excision wound model (Korkina et al., 2007).

Other activities

Experiments using mice reported the involvement of the iridoid catalpol with the central monoaminergic system. As results, catalpol may produce an antidepressant-like effect, by increasing serotonin levels, and not affecting levels or norepinephrine or dopamine, equating this compound to the clinical antidepressant fluoxetine (Wang et al., 2014).

Catalpol also plays an important role in brain angiogenesis by upregulating erythropoietin and vascular endothelial growth factor and decrease neurological deficits in a rat model of stroke (Zhu et al., 2010). In addition, it was demonstrated ovarian anti-

aging properties in female rats' models by nourishing ovarian tissues and improving the quality of follicles (Wei et al., 2014) . At last, this compound proved allergy-preventive effects in *in vivo* experiments (Oku et al., 2011).

There are some studies involving radioprotective and photoprotective effects of the natural compounds in cultured cells and mice submitted to a treatment with catalpol before irradiation. Probably by its effects on reducing ROS generation, catalpol inhibited ionizing radiation-induced cell apoptosis *in vitro* and increased plasma endogenous antioxidants *in vivo* (Chen et al., 2013b). Similarly, aucubin significantly reduced UVB-induced oxidative stress in human skin fibroblasts by inhibiting matrix metalloproteinase-1 production, reducing skin photoaging (Ho et al., 2005).

Finally, some compounds displayed relation with renal membrane protection. The first study, performed in cultured renal cells, concluded that the treatment with flavonoids luteolin, plantaginin and rutin inhibited the release of lactate dehydrogenase and decreased the lipid peroxidation, making them as effective protector compounds on the renal cellular membrane (Yokozawa et al., 1999). In the other hand, an *in vivo* study submitted mice with renal ischemia/reperfusion-injury (IRI) to a treatment with catalpol, and as results, this compound significantly reduced some inflammatory markers, attenuating renal IRI and contributing to the renal protection (Zhu et al., 2015).

Concluding remarks

Plantago has been largely studied in the medical field. Its traditional folk use in treating several diseases makes its species a significant source of studies of new molecules with therapeutic properties. In this context, different compounds such as iridoid glucosides, phenolic compounds, flavonoids and terpenoids, have shown some biological activities, involving *Plantago* extracts or isolated compounds, with significantly results, mainly related to the antidiabetic, anti-inflammatory, antioxidant, antitumoral, hypolipemiant and neuroprotective activities.

Due the requirement for new researching lines to provide new drugs development, and due the long traditional use of species of *Plantago*, it seems to be worth the effort of exploring this plant genus further.

References

Abud, M. A., Molina, A., Wendel, G. H., Hikawczuk, V. E. J., Pelzer, L. E., María, A. O., & Saad, J. R. (2012). Gastroprotective Effects of *Plantago major* and Metabolites in Rats. *Latin Americann Journal of Pharmacy, 31(8),* 1203-6.

Adam, M., Dobiáš, P., Eisner, A., & Ventura, K. (2009). Extraction of antioxidants from plants using ultrasonic methods and their antioxidant capacity. *Journal of Separation Science, 32,* 288-94.

Afifi, M. S., Salama, O. M., & Maatooq, G. T. (1990). Phytochemical study of two *Plantago* species. Part II: Iridoid glucosides. *Mansouri Journal of Pharmaceutical Sciences, 6,* 16-25.

Albach, D. C., Meudt, H. M., Oxelman, B. (2005) Piecing together the "new" Plantaginaceae. *American Journal of Botany,* 92(2), 297-315.

Amakura, Y., Yoshimura, A., Yoshimura, M., & Yoshida, T. (2012). Isolation and characterization of phenolic antioxidants from *Plantago* herb. *Molecules (Basel, Switzerland), 17*(5), 5459–66.

Ambrosone, L., Guerra, G., Cinelli, M., Filippelli, M., Mosca, M., Vizzarri, F., … Costagliola, C. (2014). Corneal epithelial wound healing promoted by verbascoside-based liposomal eyedrops. *BioMed research international, 2014,* 471642.

Andary, C., Ravn, H. W., Wylde, R., Heitz, A., & Motte-Florac, E. (1989). Crassifolioside, a caffeic acid glycoside ester from *Plantago crassifolia*. *Phytochemistry, 28(1),* 288-90.

Andrzejewska-Golec, E., & Swiatek, L. (1984). Chemotaxonomic studies of the genus Plantago. I. Analysis of iridoid fraction. *Herba Polonica, 0(1),* 9-16.

Andrzejewska-Golec, E., Ofterdinger-Daegel, S., Calis, I., Swiatek, L. (1993). E, Ofterdinger-Daegel S, Calis I, & Swiatek L. 1993. Chemotaxonomic aspects of iridoids occurring in *Plantago* subgen. *Psyllium* (Plantaginaceae). *Plant Systematics and Evolution, 185,* 85-9.

APG (The Angiosperm Phylogeny Group). (2016). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. *Botanical Journal of the Linnean Society,* 1-20.

Aritomi, M. (1967). Homoplantaginin, a New Flavonoid Glycoside in Leaves of *Plantago asiatica* LINNAEUS. *Chemical and Pharmaceutical Bulletin, 15(4),* 432-4.

Bai, X., Qiu, A., Guan, J., & Shi, Z. (2007). Antioxidant and protective effect of an oleanolic acid-enriched extract of A. deliciosa root on carbon tetrachloride induced rat liver injury. *Asia Pacific journal of clinical nutrition, 16 Suppl 1,* 169–73.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, *99*(1), 191–203.

Barua, C. C., Pal, S. K., Roy, J. D., Buragohain, B., Talikdar, A., Barua, A. G., Borah, P. (2011). Studies on the anti-inflammatory properties of *Plantago erosa* leaf extract in rodents. *Journal of Ethnopharmacology*, 134, 62-6.

Baskar, A. A., Ignacimuthu, S., Paulraj, G. M., & Al Numair, K. S. (2010). RCesheaercmh aroticple reventive potential of β-Sitosterol in experimental colon cancer model - an *In vitro* and *In vivo* study. *BMC Complementary and Alternative Medicine, 10(24)*, 2-10.

Beara, I. N., Lesjak, M. M., Jovin, E. D., Balog, K. J., Anackov, G. T., Orcić, D. Z., & Mimica-Dukić, N. M. (2009). Plantain (*Plantago* L.) species as novel sources of flavonoid antioxidants. *Journal of agricultural and food chemistry*, *57*(19), 9268–73.

Beara, I. N., Lesjak, M. M., Orčić, D. Z., Simin, N. Đ., Četojević-Simin, D. D., Božin, B. N., Mimica-Dukić, N. M. (2012a). Comparative analysis of phenolic profile, antioxidant, anti-inflammatory and cytotoxic activity of two closely-related Plantain species: *Plantago altissima* L. and *Plantago lanceolata* L. *LWT - Food Science and Technology*, 47, 64-70.

Beara, I. N., Lesjak, M. M., Četojević-Simin, D. D., Orčić, D. Z., Janković, T., Anačkov, G. T., Mimica-Dukić, N. M. (2012b). Phenolic profile, antioxidant, anti-inflammatory and cytotoxic activities of endemic *Plantago reniformis* G. Beck. *Food Research International*, 49, 501-7.

Bhalke, R. D., & Pal, S. C. (2012). Anti-inflammatory and antinociceptive activity of *Pterospermum acerifolium* leaves. *Asian Journal of Pharmaceutical and Clinical Research, 5(2)*, 23-6.

Bianco, A., Guiso, M., & Passacantilli, P. Iridoid and phenylpropanoid glycosides from new sources. (1984). *Journal of Natural Products, 47(5)*, 901-2.

Biringanine, G., Rønsted, N., Vanhaelen-Fastré, T., Vanhalens, M., & Duez, P. Iridoids and phenylethanoid glycosides from *Plantago palmata* Hook. F. S. (2007). *Journal de la Société Ouest-Africaine de Chimie, 23*, 35-40.

Bowers, M. D. (1996). Variation in iridoid glycosides in a population of *Plantago patagonica* Jacq. (Plantaginaceae) in Colorado. *Biochemical Systematics and Ecology*, *24*(3), 207–210.

Caltagirone, S., Rossi, C., Poggi, A., Ranelletti, F. O., Natali, P. G., Brunetti, M., … Piantelli, M. (2000). Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. *International journal of cancer*, *87*(4), 595–600.

Carrillo-Ocampo, D., Bazaldúa-Gómez, S., Bonilla-Barbosa, J. R., Aburto-Amar, R., Rodríguez-López, V. (2013). Anti-Inflammatory Activity of Iridoids and Verbascoside Isolated from *Castilleja tenuiflora*. *Molecules*, 18, 12109-18.

Castellano, J. M., Guinda, A., Delgado, T., Rada, M., & Cayuela, J. A. (2013). Biochemical basis of the antidiabetic activity of oleanolic acid and related pentacyclic triterpenes. *Diabetes*, *62*(6), 1791–9.

Cazarolli, L. H., Zanatta, L., Alberton, E. H., Figueiredo, M. S., Folador, P., Damazio, R. G., ... Silva, F. R. (2008). Flavonoids: prospective drug candidates. *Mini Reviews in Medicinal Chemestry, 8(13)*, 1429-40.

Chang, M-Y., & Shen, Y-L. (2014). Linalool Exhibits Cytotoxic Effects by Activating Antitumor Immunity. *Molecules, 19*, 6694-706.

Chang, H-T., Chou, C-T., Kuo, D-H., Shieh, P., Jan, C-R., & Liang, W-Z. (2015). The Mechanism of Ca^{2+} Movement in the Involvement of Baicalein-Induced Cytotoxicity in ZR-75-1 Human Breast Cancer Cells. *Journal of Natural Products, 78*, 1624-34.

Chen, W., Li, X., Jia, L-Q., Wang, J., Zhang, L., Hou, D., … (2013a). Ren, L. Neuroprotective activities of catalpol against CaMKII-dependent apoptosis induced by LPS in PC12 cells. *Brithis Journal of Pharmacology, 169*, 1140-52.

Chen, C., Chen, Z., Xu, F., Zhu, C., Fang, F., Shu, S., … Ling, C. (2013b). Radio-protective effect of catalpol in cultured cells and mice. *Journal of radiation research*, *54*(1), 76–82.

Chiang, L.-C., Chiang, W., Chang, M.-Y., & Lin, C.-C. (2003). In vitro cytotoxic, antiviral and immunomodulatory effects of *Plantago major* and *Plantago asiatica*. *The American journal of Chinese medicine, 31*(2), 225–34.

Chittur, S., Parr, B., & Marcovici, G. (2011). Inhibition of inflammatory gene expression in keratinocytes using a composition containing carnitine, thioctic Acid and saw palmetto extract. *Evidence-based complementary and alternative medicine : eCAM*, *2011*, 985345.

Choi, S.-M. M., Kim, B. C., Cho, Y.-H. H., Choi, K.-H. H., Chang, J., Park, M.-S. S., … Kim, J.-K. K. (2014). Effects of Flavonoid Compounds on β-amyloid-peptide-induced Neuronal Death in Cultured Mouse Cortical Neurons. *Chonnam medical journal*, *50*(2), 45–51.

Chung, M., Park, K., Kim, K., Kim, C.-T., Baek, J., Bang, K.-H., … Lee, S.-J. (2007). Asian plantain (*Plantago asiatica*) essential oils suppress 3-hydroxy-3-methyl-glutaryl-co-enzyme A reductase expression in vitro and in vivo and show hypocholesterolaemic properties in mice. *British Journal of Nutrition*, *99*(01).

Dalar, A., Türker, M., & Konczak, I. (2002). Antioxidant capacity and phenolic constituents of *Malva neglecta*Wallr. and *Plantago lanceolata* L. from Eastern Anatolia Region of Turkey. *Journal of Herbal Medicine, 2*, 42-51.

Damtoft, S., Falkesgaard, E., & Jensen, S. (1994). Iridoid glucosides from *Plantago hookeriana*. *Phytochemistry*, *35*(5), 1367–1368.

Do Nascimento, P. G. G. G., Lemos, T. L., Bizerra, A. M., Arriaga, Â. M. M., Ferreira, D. A., Santiago, G. M., … Costa, J. G. G. (2013). Antibacterial and antioxidant activities of ursolic acid and derivatives. *Molecules (Basel, Switzerland)*, *19*(1), 1317–27.

Fabiani, R., Rosignoli, P., De Bartolomeo, A., Fuccelli, R., Servili, M., Montedoro, G. F., & Morozzi, G. (2008). Oxidative DNA damage is prevented by extracts of olive oil, hydroxytyrosol, and other olive phenolic compounds in human blood mononuclear cells and HL60 cells. *The Journal of nutrition*, *138*(8), 1411–6.

Farokhi, F., & Khaneshi, F. (2013). Histophatologic changes of lung in asthmatic male rats treated with hydro-alcoholic extract of *Plantago major* and theophylline. *Avicenna journal of phytomedicine*, *3*(2), 143–51.

Fernández-Bañares, F., Hinojosa, J., Sánchez-Lombraña, J. L., Navarro, E., Martínez-Salmerón, J. F., García-Pugés, A., … Gassull, M. A. (1999). Randomized clinical trial of *Plantago ovata* seeds (dietary fiber) as compared with mesalamine in maintaining remission in ulcerative colitis. Spanish Group for the Study of Crohn's Disease and Ulcerative Colitis (GETECCU). *The American journal of gastroenterology*, *94*(2), 427–33.

Fernandez-Martinez, M. N., Hernandez-Echevarria, L., Sierra-Vega, M., Diez-Liebana, M. J., Calle-Pardo, A., Carriedo-Ule, D., … Garcia-Vieitez, J. J. (2014). A randomised clinical trial to evaluate the effects of *Plantago ovata* husk in Parkinson patients: changes in levodopa pharmacokinetics and biochemical parameters. *BMC complementary and alternative medicine*, *14*, 296.

Fischer, M. H., Yu, N., Gray, G. R., Ralph, J., Anderson, L., Marlett, J. A. (2004). The gel-forming polysaccharide of psyllium husk (*Plantago ovata* Forsk). *Carbohydrate Research*, 339, 2009-17.

Fleer, H., & Verspohl, E. J. (2007). Antispasmodic activity of an extract from *Plantago lanceolata* L. and some isolated compounds. *Phytomedicine : international journal of phytotherapy and phytopharmacology*, *14*(6), 409–15.

Franzyk, H., Husum, T. L., & Jensen, S. R. (1998). A caffeoyl phenylethanoid glycoside from *Plantago myosuros*. *Phytochemistry, 47(6)*, 1161-2.

Fujikawa, T., Hirata, T., Hosoo, S., Nakajima, K., Wada, A., Yurugi, Y., … Nishibe, S. (2012). Asperuloside stimulates metabolic function in rats across several organs under high-fat diet conditions, acting like the major ingredient of Eucommia leaves with anti-obesity activity. *Journal of nutritional science*, *1*, e10.

Gao, D., Li, Q., Li, Y., Liu, Z., Fan, Y., Liu, Z., … Han, Z. (2009). Antidiabetic and antioxidant effects of oleanolic acid from *Ligustrum lucidum* Ait in alloxan-induced diabetic rats. *Phytotherapy research : PTR*, *23*(9), 1257–62.

Gao, N., Tian, J-X., Shang, Y-H., Zhao, D-Y., & Wu, T. (2014). Catalpol Suppresses Proliferation and Facilitates Apoptosis of OVCAR-3 Ovarian Cancer Cells through

Upregulating MicroRNA-200 and Downregulating MMP-2 Expression. *International Journal of Molecular Sciences, 15*, 19394-405.

Graßmann, J. (2005). Terpenoids as Plant Antioxidants. *Vitamins & Hormones* (Vol. 72, pp. 505–535).

Gyurkovska, V., Alipieva, K., Maciuk, A., Dimitrova, P., Ivanovska, N., Haas, C., … Georgiev, M. (2011). Anti-inflammatory activity of Devil's claw in vitro systems and their active constituents. *Food Chemistry*, *125*(1), 171–178.

Haicheng, Z., Jing, L., Liyuan, R., Wei, L., Qian, X., Lili, M., … Jianling, D. (2014). Relationship between spatial memory in diabetic rats and protein kinase Cγ, caveolin-1 in the hippocampus and neuroprotective effect of catalpol. *Chinese Medical Journal, 127(5)*, 916-23.

Handjieva, N., Spassov, S., Bodurova, G., Saadi, H., Popov, S., Pureb, O., & Zamjansan, J. (1991). Majoroside, an iridoid glucoside from *Plantago major*. *Phytochemistry, 30*(4), 1317–1318.

Handjieva, N., Taskova, R., & Popov, S. (1992). 10-Hydroxymajoroside, an Iridoid Glucoside from *Plantago cornuti* Gouan L. *Zeitschrift für Naturforschung, 48c*, 827-8.

Hassemer, G., De Giovanni, R., Trevisan, R. (2016a). The use of potential distribution models in the study of the distribution and conservation status of plants: The case of *Plantago* L. (Plantaginaceae) in Brazil. *Journal of the Torrey Botanical Society*, 143(1), 38-49.

Hassemer, G., Trevisan, R., Meudt, H. M., Rønsted, N. (2016b). Taxonomic novelties in *Plantago* section *Virginica* (Plantaginaceae) and an updated identification key. *Phytotaxa*, 221(3), 226-46.

He, X., Wei, Z., Zhou, E., Chen, L., Kou, J., Wang, J., Yang, Z. (2015). Baicalein attenuates inflammatory responses by suppressing TLR4 mediated NF-κB and MAPK signaling pathways in LPS-induced mastitis in mice. *International Immunopharmacology, 28*, 470-6.

Hidalgo, F., León, M., & Zamora, R. (2009). Effect of β-sitosterol in the antioxidative activity of oxidized lipid–amine reaction products. *Food Research International*, *42*(8), 1215–1222.

Ho, J. N., Lee, Y. H., Park, J. S., Jun, W. J., Kim, H. K., Hong, B. S., … Cho, H. Y. (2005). Protective effects of aucubin isolated from *Eucommia ulmoides* against UVB-induced oxidative stress in human skin fibroblasts. *Biological & pharmaceutical bulletin*, *28*(7), 1244–8.

Hoggard, R. K., Kores, P. J., Molvray, M., Hoggard, G. D., Broughton, D. A. (2003). Molecular systematics and biogeography of the amphibious genus *Litorella* (Plantaginaceae). *American Journal of Botany*, 90(3), 429-35.

Hsu, H.-F. F., Houng, J.-Y. Y., Chang, C.-L. L., Wu, C.-C. C., Chang, F.-R. R., & Wu, Y.-C. C. (2005). Antioxidant activity, cytotoxicity, and DNA information of *Glossogyne tenuifolia*. *Journal of agricultural and food chemistry*, *53*(15), 6117–25.

Hu, L.-W. W., Yen, J.-H. H., Shen, Y.-T. T., Wu, K.-Y. Y., & Wu, M.-J. J. (2014). Luteolin modulates 6-hydroxydopamine-induced transcriptional changes of stress response pathways in PC12 cells. *PloS one*, *9*(5), e97880.

Huang, W.-Y. Y., Cai, Y.-Z. Z., & Zhang, Y. (2010). Natural phenolic compounds from medicinal herbs and dietary plants: potential use for cancer prevention. *Nutrition and cancer*, *62*(1), 1–20.

Hussan, F., Mansor, A. S., Hassan, S. N., Tengku Nor Effendy Kamaruddin, T. N., Budin, S. B., & Othman, F. (2015). Anti-Inflammatory Property of *Plantago major* Leaf Extract Reduces the Inflammatory Reaction in Experimental Acetaminophen-Induced Liver Injury. *Evidence-based complementary and alternative medicine : eCAM*, *2015*, 347861.

Hwang, Y.-J. J., Song, J., Kim, H.-R. R., & Hwang, K.-A. A. (2014). Oleanolic acid regulates NF-κB signaling by suppressing MafK expression in RAW 264.7 cells. *BMB reports*, *47*(9), 524–9.

Ishikawa, N., Yokohama, J., Tsukaya, H. Molecular evidence of reticulate evolution in the subgenus *Plantago* (Plantaginaceae). *American Journal of Botany*, 96(9), 1627-35.

Jang, S.-M. M., Yee, S.-T. T., Choi, J., Choi, M.-S. S., Do, G.-M. M., Jeon, S.-M. M., … Lee, M.-K. K. (2009). Ursolic acid enhances the cellular immune system and pancreatic beta-cell function in streptozotocin-induced diabetic mice fed a high-fat diet. *International immunopharmacology*, *9*(1), 113–9.

Janković, T., Zdunić, G., Beara, I., Balog, K., Pljevljakušić, D., Stešević, D., & Šavikin, K. (2012). Comparative study of some polyphenols in *Plantago* species. *Biochemical Systematics and Ecology*, *42*, 69–74.

Jensen, S. R., Olsen, C. E., Rahn, K., & Rasmussen, J. H. (1996). Iridoid glucosides in *Plantago alpina* and *P. altissima*. *Phytochemistry, 42(6)*, 1633-6.

Jutooru, I., Chadalapaka, G., Abdelrahim, M., Basha, M. R., Samudio, I., Konopleva, M., … Safe, S. (2010). Methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate decreases specificity protein transcription factors and inhibits pancreatic tumor growth: role of microRNA-27a. *Molecular pharmacology*, *78*(2), 226–36.

Kalani, K., Agarwal, J., Alam, S., Khan, F., Pal, A., & Srivastava, S. K. (2013). *In silico* and *in vivo* anti-malarial studies of 18β glycyrrhetinic acid from *Glycyrrhiza glabra*. *PloS one*, *8*(9), e74761.

Katsuyama, S., Kuwahata, H., Yagi, T., Kishikawa, Y., Komatsu, T., Sakurada, T., & Nakamura, H. (2012). Intraplantar injection of linalool reduces paclitaxel-induced acute pain in mice. *Biomedical research (Tokyo, Japan)*, *33*(3), 175–81.

Kawashty, S. A., Gamal-el-din, E., Abdalla, M. F., & Saleh, N. A. M. (1994). Flavonoids of *Plantago* species in Egypt. *Biochemical Systematics and Ecology, 22*(7), 729–733.

Kempuraj, D., Tagen, M., Iliopoulou, B. P., Clemons, A., Vasiadi, M., Boucher, W., … Theoharides, T. C. (2008). Luteolin inhibits myelin basic protein-induced human mast cell activation and mast cell-dependent stimulation of Jurkat T cells. *British journal of pharmacology, 155*(7), 1076–84.

Khan, A.-U. U., & Gilani, A.-H. H. (2015). Natural Products Useful in Respiratory Disorders: Focus on Side-Effect Neutralizing Combinations. *Phytotherapy research*, 29(9), 1265-85.

Kim, E.-S. S., & Moon, A. (2015). Ursolic acid inhibits the invasive phenotype of SNU-484 human gastric cancer cells. *Oncology letters*, *9*(2), 897–902.

Kim, S.-H. H., Ryu, H. G., Lee, J., Shin, J., Harikishore, A., Jung, H.-Y. Y., … Kim, K.-T. T. (2015). Corrigendum: Ursolic acid exerts anti-cancer activity by suppressing vaccinia-related kinase 1-mediated damage repair in lung cancer cells. *Scientific reports*, *5*, 15864.

Kolak, U., Boğa, M., Uruşak, E. A., Ulubelen, A. (2011). Constituents of *Plantago majo*r subsp. *intermedia* with antioxidant and anticholinesterase capacities. *Turkish Journal of Chemistry, 35*, 637-45.

Korkina, L. G., Mikhal'chik, E., Suprun, M. V., Pastore, S., & Dal Toso, R. (2007). Molecular mechanisms underlying wound healing and anti-inflammatory properties of naturally occurring biotechnologically produced phenylpropanoid glycosides. *Cellular and molecular biology (Noisy-le-Grand, France), 53*(5), 84–91.

Kunkel, S. D., Elmore, C. J., Bongers, K. S., Ebert, S. M., Fox, D. K., Dyle, M. C., … Adams, C. M. (2012). Ursolic acid increases skeletal muscle and brown fat and decreases diet-induced obesity, glucose intolerance and fatty liver disease. *PloS one*, *7*(6), e39332.

Lee, G.-H. H., Lee, M.-R. R., Lee, H.-Y. Y., Kim, S. H., Kim, H.-K. K., Kim, H.-R. R., & Chae, H.-J. J. (2014). *Eucommia ulmoides* cortex, geniposide and aucubin regulate lipotoxicity through the inhibition of lysosomal BAX. *PloS one*, *9*(2), e88017.

Lee, H.-Y. Y., Lee, G.-H. H., Lee, M.-R. R., Kim, H.-K. K., Kim, N. Y., Kim, S.-H. H., … Chae, H.-J. J. (2013). *Eucommia ulmoides* Oliver extract, aucubin, and geniposide enhance lysosomal activity to regulate ER stress and hepatic lipid accumulation. *PloS one*, *8*(12), e81349.

Li, R., Wang, X., Zhang, X.-H. H., Chen, H.-H. H., & Liu, Y.-D. D. (2014a). Ursolic acid promotes apoptosis of SGC-7901 gastric cancer cells through ROCK/PTEN mediated mitochondrial translocation of cofilin-1. *Asian Pacific journal of cancer prevention : APJCP*, *15*(22), 9593–7.

Li, S., Liao, X., Meng, F., Wang, Y., Sun, Z., Guo, F., … Sun, C. (2014b). Therapeutic role of ursolic acid on ameliorating hepatic steatosis and improving metabolic disorders in high-fat diet-induced non-alcoholic fatty liver disease rats. *PloS one*, *9*(1), e86724.

Li, C., Yang, D., Zhao, Y., Qiu, Y., Cao, X., Yu, Y., ... Yin, X. (2015a). Inhibitory Effects of Isorhamnetin on the Invasion of Human Breast Carcinoma Cells by Downregulating the Expression and Activity of Matrix Metalloproteinase-2/9. *Nutrition and cancer, 67(7)*, 1191-200.

Li, J., Wu, Y., Zhang, S., Zhang, J., Ji, F., Bo, W., … Li, Z. (2015b). Baicalein protect pancreatic injury in rats with severe acute pancreatitis by inhibiting pro-inflammatory cytokines expression. *Biochemical and Biophysical Research Communications, 466(4)*, 664-9.

López, D., Cherigo, L., Spadafora, C., Loza-Mejía, M. A., & Martínez-Luis, S. (2015). Phytochemical composition, antiparasitic and α-glucosidase inhibition activities from *Pelliciera rhizophorae*. *Chemistry Central journal*, *9*(1), 53.

López, J. C. C., Villanueva, R., Martínez-Hernández, D., Albaladejo, R., Regidor, E., & Calle, M. E. E. (2009). *Plantago ovata* consumption and colorectal mortality in Spain, 1995-2000. *Journal of epidemiology / Japan Epidemiological Association*, *19*(4), 206–11.

López-Lázaro, M. (2009). Distribution and biological activities of the flavonoid luteolin. *Mini reviews in medicinal chemistry*, *9*(1), 31–59.

Maksyutina, N. P. (1971). Baicalein and scutellarein derivatives in the leaves of *Plantago major*. *Chemistry of Natural Compounds, 7(3)*, 352.

Manu, K. A., & Kuttan, G. (2008). Ursolic acid induces apoptosis by activating p53 and caspase-3 gene expressions and suppressing NF-kappaB mediated activation of bcl-2 in B16F-10 melanoma cells. *International immunopharmacology*, *8*(7), 974–81.

Marlett, J. A., Kajs, T. M., Fischer, M. H. (2000). An unfermented gel component of psyllium seed husk promotes laxation as a lubricant in humans. *The American Journal of Clinical Nutrition*, 72, 784-9.

Mazumder, K., Tanaka, K., & Fukase, K. (2013). Cytotoxic activity of ursolic acid derivatives obtained by isolation and oxidative derivatization. *Molecules (Basel, Switzerland)*, *18*(8), 8929–44.

Mello, J. C., Gonzalez, M. V., Moraes, V. W., Prieto, T., Nascimento, O. R., & Rodrigues, T. (2015). Protective Effect of *Plantago major* Extract against t-BOOH-Induced Mitochondrial Oxidative Damage and Cytotoxicity. *Molecules (Basel, Switzerland)*, *20*(10), 17747–59.

Meudt, H. M. (2011). Amplified fragment length polymorphism data reveal a history of auto- and allopolyploidy in New Zealand endemic species of *Plantago* (Plantaginaceae): new perspectives on a taxonomically challenging group. *International Journal oof Plant Sciences*, 172(2), 220-37.

Murai, M., Tamayama, Y., & Nishibe, S. (1995). Phenylethanoids in the herb of *Plantago lanceolata* and inhibitory effect on arachidonic acid-induced mouse ear edema. *Planta medica*, *61*(5), 479–80.

Murai, M., Takenaka, T., & Nishibe, S. (1996). Iridoids from *Plantago major*. *Natural Medicines, 50*, 306.

Nakamura, Y., Yoshikawa, N., Hiroki, I., Sato, K., Ohtsuki, K., Chang, C.-C. C., … Trosko, J. E. (2005). Beta-sitosterol from psyllium seed husk (*Plantago ovata* Forsk) restores gap junctional intercellular communication in Ha-ras transfected rat liver cells. *Nutrition and cancer*, *51*(2), 218–25.

Nishibe, S., Sasahara, M., Jiao, Y., Yuan, C., & Tanaka, T. (1993). Phenylethanoid glycosides from *Plantago depressa*. *Phytochemistry*, *32*(4), 975–7.

Nishibe, S., Murai, M., & Tamayama, Y. Studies on Constituents of Plantaginis Herba 7: Flavonoids from *Plantago asiatica* and *P. hostifblia*. (1995). *Natural Medicines, 49(3)*, 340-2.

Oboh, G., Akinbola, I. A., Ademosun, A. O., Sanni, D. M., Odubanjo, O. V., Olasehinde, T. A., & Oyeleye, S. I. (2015). Essential Oil from Clove Bud (*Eugenia aromatica* Kuntze) Inhibit Key Enzymes Relevant to the Management of Type-2 Diabetes and Some Pro-oxidant Induced Lipid Peroxidation in Rats Pancreas *in vitro*. *Journal of Oleo Science, 64(7)*, 775-82.

Oku, H., Ogawa, Y., Iwaoka, E., & Ishiguro, K. (2011). Allergy-preventive effects of chlorogenic acid and iridoid derivatives from flower buds of *Lonicera japonica*. *Biological & pharmaceutical bulletin*, *34*(8), 1330–3.

Oshio, H., & Inouye, H. (1982). Two new iridoid glucosides of *Plantago asiatica*. *Planta medica*, *44*(4), 204–6.

Pailer, M., & Haschke-Hofmeister, E., Inhaltsstoffe aus *Plantago major*. (1969). *Planta Medica, 17(2)*, 139-45.

Palmeiro, N. S., Almeida, C. E., Ghedini, P. C., Goulart, L. S., & Baldisserotto, B. (2002). Analgesic and Anti-inflammatory Properties of *Plantago australis* Hydroalcoholic Extract. *Acta Farmacéutica Bonaerense, 21(2)*, 89-92.

Pankoke, H., & Müller, C. (2013). Impact of defoliation on the regrowth capacity and the shoot metabolite profile of *Plantago lanceolata* L. *Plant physiology and biochemistry : PPB / Société française de physiologie végétale*, *71*, 325–33.

Park, K., Kim, B., & Chang, I.-M. (2007). Inhibitory Potencies of Several Iridoids on Cyclooxygenase-1, Cyclooxygnase-2 Enzymes Activities, Tumor Necrosis factor-α and Nitric Oxide Production In Vitro. *Evidence-based complementary and alternative medicine : eCAM*, *7*(1), 41–5.

Park, S.-N. N., Ahn, S.-J. J., & Kook, J.-K. K. (2015). Oleanolic acid and ursolic acid inhibit peptidoglycan biosynthesis in *Streptococcus mutans* UA159. *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]*, *46*(2), 613–7.

Pilger, R. K. F. (1937). Plantaginaceae. *In:* Das Pflanzenreich. Wilhelm Engelmann, Leipzig, Germany, 102, 1-466.

Qian, M., Tang, S., Wu, C., Wang, Y., He, T., Chen, T., & Xiao, X. (2015). Synergy between baicalein and penicillins against penicillinase-producing *Staphylococcus aureus*. *International journal of medical microbiology : IJMM*, *305*(6), 501–4.

Rahn, K. (1996) A phylogenetic study of the Plantaginaceae. *Botanical Journal of the Linnean Society, 120*, 145-98.

Ravn, H., & Brimer, L. Structure and antibascterial activity of plantamajoside, a caffeic acid sugar ester from *Plantago major* subsp. *major*. (1988). *Phytochemistry, 27(11)*, 3433-7.

Ravn, H., Nishibe, S., Sasahara, M., & Xuebo, L. Phenolic compounds from *Plantago asiatica*. (1990). *Phytochemistry, 29(11)*, 3627-31.

Ramachandran, S., & Prasad, N. R. (2008). Effect of ursolic acid, a triterpenoid antioxidant, on ultraviolet-B radiation-induced cytotoxicity, lipid peroxidation and DNA damage in human lymphocytes. *Chemico-biological interactions*, *176*(2-3), 99–107.

Reina, E., Al-Shibani, N., Allam, E., Gregson, K. S., Kowolik, M., & Windsor, L. J. (2013). The Effects of *Plantago major* on the Activation of the Neutrophil Respiratory Burst. *Journal of traditional and complementary medicine*, *3*(4), 268–72.

Rezaei-Golmisheh, A., Malekinejad, H., Asri-Rezaei, S., Farshid, A. A., & Akbari, P. (2015). Hawthorn ethanolic extracts with triterpenoids and flavonoids exert hepatoprotective effects and suppress the hypercholesterolemia-induced oxidative stress in rats. *Iranian journal of basic medical sciences*, *18*(7), 691–9.

Ringbom, T., Segura, L., Noreen, Y., Perera, P., & Bohlin, L. (1998). Ursolic acid from *Plantago major*, a selective inhibitor of cyclooxygenase-2 catalyzed prostaglandin biosynthesis. *Journal of natural products*, *61*(10), 1212–5.

Romero, A. L., West, K. L., Zern, T., & Fernandez, M. L. (2002). The seeds from *Plantago ovata* lower plasma lipids by altering hepatic and bile acid metabolism in guinea pigs. *The Journal of nutrition*, *132*(6), 1194–8.

Rønsted, N., Gøbel, E., Franzyk, H., Jensen, S. R., & Olsen, C. E. (2000). Chemotaxonomy of *Plantago*. Iridoid glucosides and caffeoyl phenylethanoid glycosides. *Phytochemistry*, *55*(4), 337–48.

Rønsted, N., Chase, M. W., Albach, D. C., Bello, M. A. (2002). Phylogenetic relationships within *Plantago* (Plantaginaceae): evidence from nuclear ribosomal ITS

and plastid *trnL-F* sequence data. *Botanical Journal of the Linnean Society*, 139, 323-338.

Rønsted, N., Franzyk, H., Mølgaard, P., Jaroszewski, J., & Jensen, S. (2003). Chemotaxonomy and evolution of *Plantago* L. *Plant Systematics and Evolution*, *242*(1-4), 63–82.

Saadi, H., Handjieva, N. Ivanova, A., & Popov, S. Iridoids from *Plantago carinata* Schrad. (1991). *Zeitschrift für Naturforschung, 46(11-12)*, 1001-2.

Sahagún, A. M., Vaquera, J., García, J. J., Calle, A. P., Diez, M-J., Fernández, N., ... Sierra, M. (2015). Study of the protective effect on intestinal mucosa of the hydrosoluble fiber *Plantago ovata* husk. *BMC Complementary & Alternative Medicine, 15(298)*, 2-8.

Sahu, B. D., Kumar, J. M., & Sistia, R. (2015). Baicalein, a Bioflavonoid, Prevents Cisplatin-Induced Acute Kidney Injury by Up-Regulating Antioxidant Defenses and Down-Regulating the MAPKs and NF-κB Pathways. *PloS One, 10(7)*, e0134139.

Salama, H. M. H., & Saffan, E-S. (2003). Triterpenoids and flavonoids from the air-dried aerial parts of *Plantago amplexicaulis*. *Kuwait Journal of Science and Engineering, 30(2)*, 109-18.

Samuelsen, A. B., Lund, I., Djahromi, J. M., Paulsen, B. S., Wold, J. K., & Knutsen, S. H. (1999). Structural features and anti-complementary activity of some heteroxylan polysaccharide fractions from the seeds of *Plantago major* L. *Carbohydrate Polymers, 38*, 133-43.

Samuelsen, A. B. (2000). The traditional uses, chemical constituents and biological activities of *Plantago major* L. A review. *Journal of Ethnopharmacology*, *71*(1-2), 1–21.

Sánchez-Quesada, C., López-Biedma, A., & Gaforio, J. J. J. (2015). Oleanolic Acid, a Compound Present in Grapes and Olives, Protects against Genotoxicity in Human Mammary Epithelial Cells. *Molecules (Basel, Switzerland)*, *20*(8), 13670–88.

Santoro, A., Bianco, G., Picerno, P., Aquino, R., Autore, G., Marzocco, S., … Bifulco, M. (2008). Verminoside- and verbascoside-induced genotoxicity on human lymphocytes: Involvement of PARP-1 and p53 proteins. *Toxicology Letters*, *178*(2), 71–76.

Santos, B. L., Oliveira, M. N., Coelho, P. L., Pitanga, B. P., da Silva, A. B., Adelita, T., … Costa, S. L. (2015). Flavonoids suppress human glioblastoma cell growth by inhibiting cell metabolism, migration, and by regulating extracellular matrix proteins and metalloproteinases expression. *Chemico-biological interactions*, *242*, 123–38.

Santos-Cruz, L., Ávila-Acevedo, J., Ortega-Capitaine, D., Ojeda-Duplancher, J., Perdigón-Moya, J., Hernández-Portilla, L., … Heres-Pulido, M. (2012). Verbascoside is not genotoxic in the ST and HB crosses of the *Drosophila* wing spot test, and its

constituent, caffeic acid, decreases the spontaneous mutation rate in the ST cross. *Food and Chemical Toxicology*, *50*(3-4), 1082–1090.

Sato, Y., Itagaki, S., Kurokawa, T., Ogura, J., Kobayashi, M., Hirano, T., … Iseki, K. (2011). *In vitro* and *in vivo* antioxidant properties of chlorogenic acid and caffeic acid. *International journal of pharmaceutics*, *403*(1-2), 136–8.

Shen, S., Qian, J., & Ren, J. (2010). Ethnoveterinary plant remedies used by Nu people in NW Yunnan of China. *Journal of ethnobiology and ethnomedicine*, *6*, 24.

Shim, K. M., Choi, S. H., Jeong, M.-J. J., & Kang, S. S. (2007). Effects of aucubin on the healing of oral wounds. *In vivo (Athens, Greece)*, *21*(6), 1037–41.

Shipunov, A. (2015). *Plantago schrenkii* is *P. maritima*: morphological and molecular evidence. *Annales Botanici Fennici*, 52, 33-37.

Singh, B. (2007). Psyllium as therapeutic and drug delivery agent. *International Journal of Pharmaceutics*, 334, 1-14.

Skari, K. P., Malterud, K. E., & Nishioka, I. (1999). Radical scavengers and inhibitors of enzymatic lipid peroxidation from *Plantago major*, a medicinal plant. Poster 495 at 2000 Years of Natural Products Research — Past, Present and Future, Amsterdam, The Netherlands.

Sosa, S., Faudale, M., Zacchigna, M., Cateni, F., Del Favero, G., Tubaro, A., & Della Loggia, R. (2011). Topical anti-inflammatory activity of *Plantago lanceolata* L. leaves: the relevance of triterpenic acids. *Planta medica, 77(12)*.

Speranza, L., Franceschelli, S., Pesce, M., Reale, M., Menghini, L., Vinciguerra, I., … Grilli, A. (2010). Antiinflammatory effects in THP-1 cells treated with verbascoside. *Phytotherapy research : PTR*, *24*(9), 1398–404.

Talib, W. H., Zarga, M. H., & Mahasneh, A. M. (2012). Antiproliferative, antimicrobial and apoptosis inducing effects of compounds isolated from *Inula viscosa*. *Molecules (Basel, Switzerland)*, *17*(3), 3291–303.

Taskova, R., Evstatieva, L., Handjieva, N., & Popov, S. (2002). Iridoid patterns of genus *Plantago* L. and their systematic significance. *Zeitschrift für Naturforschung. C, Journal of biosciences*, *57*(1-2), 42–50.

Tay, M. L., Meudt, H. M., Garnock-Jones, P. J., Ritchie, P. A. (2010a). DNA sequences from three genomes reveal multiple long-distance dispersals and non-monophyly of sections in Australasian *Plantago* (Plantaginaceae). *Australian Systematic Botany*, 23, 47-68.

Tay, M. L., Meudt, H. M., Garnock-Jones, P. J., Ritchie, P. A. (2010b). Testing species limits of New Zealand *Plantago* (Plantaginaceae) using internal transcribed spacer (ITS) DNA sequences. *New Zealand Journal of Botany*, 48(3-4), 205-224.

Toda, S., Miyase, T., Arichi, H., Tanizawa, H., & Takino, Y. (1985). Natural Antioxidants. II. Antioxidative Components Isolated from Seeds of *Plantago asiatica* LINNE. *Chemical and Pharmaceuticals Bulletin, 3,* 1270-3.

Tsubaki, M., Takeda, T., Kino, T., Itoh, T., Imano, M., Tanabe, G., … Nishida, S. (2015). Mangiferin suppresses CIA by suppressing the expression of TNF-α, IL-6, IL-1β, and RANKL through inhibiting the activation of NF-κB and ERK1/2. *American journal of translational research, 7*(8), 1371–81.

Tundis, R., Loizzo, M. R., Menichini, F., Statti, G. A., & Menichini, F. (2008). Biological and pharmacological activities of iridoids: recent developments. *Mini reviews in medicinal chemistry, 8*(4), 399–420.

Tóth, G., Háznagy, A., & Bula, E. (1976). Isolation and NMR-study of loliolid from *Plantago lanceoata* using a displacement reagent. *Die Pharmazie, 31*(1), 51–2.

Vogl, S., Atanasov, A. G., Binder, M., Bulusu, M., Zehl, M., Fakhrudin, N., … Kopp, B. (2013a). The Herbal Drug *Melampyrum pratense* L. (Koch): Isolation and Identification of Its Bioactive Compounds Targeting Mediators of Inflammation. *Evidence-based complementary and alternative medicine : eCAM*, *2013*, 395316.

Vogl, S., Picker, P., Mihaly-Bison, J., Fakhrudin, N., Atanasov, A. G., Heiss, E. H., … Kopp, B. (2013b). Ethnopharmacological in vitro studies on Austria's folk medicine - an unexplored lore in vitro anti-inflammatory activities of 71 Austrian traditional herbal drugs. *Journal of ethnopharmacology*, *149*(3), 750–71.

Wang, I. K., Lin-Shiau, S. Y., & Lin, J. K. (1999). Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. *European journal of cancer (Oxford, England : 1990)*, *35*(10), 1517–25.

Wang, X., Ye, X-l., Liu, R., Chen, H-L., Bai, H., Liang, X., ... Hai, C-X. (2010). Antioxidant activities of oleanolic acid in vitro: Possible role of Nrf2 and MAP kinases. *Chemico-Biological Interactions, 184*, 328-37.

Wang, J., Li, Y., Wang, X., & Jiang, C. (2012a). Ursolic acid inhibits proliferation and induces apoptosis in human glioblastoma cell lines U251 by suppressing TGF-β1/miR-21/PDCD4 pathway. *Basic & clinical pharmacology & toxicology*, *111*(2), 106–12.

Wang, J. S., Ren, T. N., & Xi, T. (2012b). Ursolic acid induces apoptosis by suppressing the expression of FoxM1 in MCF-7 human breast cancer cells. *Medical oncology (Northwood, London, England)*, *29*(1), 10–5.

Wang, J., Cui, Y., Feng, W., Zhang, Y., Wang, G., Wang, X., & Zhou, G. (2014). Involvement of the central monoaminergic system in the antidepressant-like effect of catalpol in mice. *Bioscience trends*, *8*(5), 248–52.

Wei, M., Lu, Y., Liu, D., & Ru, W. (2014). Ovarian failure-resistant effects of catalpol in aged female rats. *Biological & pharmaceutical bulletin*, *37*(9), 1444–9.

Weryszko-Chmielewska, E., Matysik-Woźniak, A., Sulborska, A., Rejdak, R. (2012). Commercially important properties of plants of the genus *Plantago*. *Acta Agrobotanica*, 65(1), 11-20.

Wu, F., Wang, H., Li, J., Liang, J., & Ma, S. (2012). Homoplantaginin Modulates Insulin Sensitivity in Endothelial Cells by Inhibiting Inflammation. *Biological and Pharmaceutical Bulletin*, *35*(7), 1171–1177.

Xiao, W. Q., Yin, G. J., Fan, Y. T., Qiu, L., Cang, X. F., Yu, G., … Wan, R. (2014). Catalpol Ameliorates Sodium Taurocholate-Induced Acute Pancreatitis in Rats via Inhibiting Activation of Nuclear Factor Kappa B. *Internationa Journal of Molecular Sciences, 15*, 11957-72.

Xu, J., Wang, H., Ding, K., Zhang, L., Wang, C., Li, T., … Lu, X. (2014). Luteolin provides neuroprotection in models of traumatic brain injury via the Nrf2-ARE pathway. *Free radical biology & medicine, 71*, 186–95.

Yokozawa, T., Dong, E., Kawai, Y., Gemba, M., & Shimizu, M. (1999). Protective effects of some flavonoids on the renal cellular membrane. *Experimental and toxicologic pathology : official journal of the Gesellschaft für Toxikologische Pathologie*, *51*(1), 9–14.

Yoo, K.-Y. Y., & Park, S.-Y. Y. (2012). Terpenoids as potential anti-Alzheimer's disease therapeutics. *Molecules (Basel, Switzerland), 17*(3), 3524–38.

Zhang, H., Zheng, D., Ding, J., Xu, H., Li, X., & Sun, W. (2015). Efficient delivery of ursolic acid by poly(N-vinylpyrrolidone)-block-poly (ε-caprolactone) nanoparticles for inhibiting the growth of hepatocellular carcinoma *in vitro* and *in vivo*. *International journal of nanomedicine, 10*, 1909–20.

Zhou, Q., Weiying, L., Niu, Y., Liu, J., Zhang, X., Gao, B., … Yu, L. (2013). Identification and quantification of phytochemical composition and anti-inflammatory, cellular antioxidant, and radical scavenging activities of 12 *Plantago* species. *Journal of Agricultural and Food Chemistry*, 61, 6693-702.

Zhou, J., Cai, W., Jin, M., Xu, J., Wang, Y., Xiao, Y., … Huang, R. (2015). 18β-glycyrrhetinic acid suppresses experimental autoimmune encephalomyelitis through inhibition of microglia activation and promotion of remyelination. *Scientific reports*, *5*, 13713.

Zhu, H.-F. F., Wan, D., Luo, Y., Zhou, J.-L. L., Chen, L., & Xu, X.-Y. Y. (2010). Catalpol increases brain angiogenesis and up-regulates VEGF and EPO in the rat after permanent middle cerebral artery occlusion. *International journal of biological sciences*, *6*(5), 443–53.

Zhu, J., Chen, X., Wang, H., & Yan, Q. (2015). Catalpol protects mice against renal ischemia/reperfusion injury via suppressing PI3K/Akt-eNOS signaling and inflammation. *International journal of clinical and experimental medicine*, *8*(2), 2038–44.

Zubair, M., Nybom, H., Ahnlund, M., & Rumpunen, K. (2012). Detection of genetic and phytochemical differences between and within populations of *Plantago major* L. (plantain). *Scientia Horticulturae*, *136*, 9–16.

4. Capítulo II – artigo de dados

Toxicological safety evaluation of an hydroethanolic extract from leaves of *Plantago australis* and its major compound, verbascoside

Artigo a ser submetido à revista *Journal of Ethnopharmacology*

(Para facilitar a leitura do manuscrito as tabelas e figuras foram inseridas no texto)

Toxicological safety evaluation of an hydroethanolic extract from leaves of Plantago australis and its major compound, verbascoside

Jeferson Gustavo Henn[a], Betânia de Souza Ponce[a], Nathalia Denise de Moura Sperotto[a], Rodrigo Moisés Veríssimo[a], Fernanda Brião Menezes Boaretto[b], Gustavo Hassemer[c], Valéria Flores Péres[a], Jaqueline Nascimento Picada[b], Jenifer Saffi[a], Dinara Jaqueline Moura[a*]

[a]Laboratório de Genética Toxicológica, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil

[b]Laboratório de Genética Toxicológica, Universidade Luterana do Brasil (ULBRA), Canoas, RS, Brazil

[c]Statens Naturhistoriske Museum, Københavns Universitet, Sølvgade 83S, 1307 Copenhagen, Denmark

[*] Corresponding author: dinaram@ufcspa.edu.br

Abstract

Ethnopharmacological relevance: Plantago australis (Kunth) Rahn is a perennial plant widely distributed in Latin America, and its seeds and leaves are used in folk medicine to treat many diseases and conditions. Among its various chemical compounds, verbascoside is one of the most present, and has several pharmacological activities described, but there is no information about the toxicity of both of them.

Aims of the study: The aims of the present study were to optimize the extraction of verbascoside from *P. australis* leaves through ultrasound methods. Also, a validated HPLC method to quantify verbascoside was developed, and we evaluated the toxicological safety of extraction and verbascoside in *in vitro* assays.

Materials and methods: Dried leaves of *P. australis* were submitted to different extraction methods (percolation and ultrasound). The optimization of the ultrasound extraction was carried out by complete factorial design (2^2) and response surface methodology (RSM). Furthermore, HPLC analysis was performed for the estimation of aucubin, baicalein, oleanolic acid, uroslic acid and verbascoside as marker compounds. Mutagenicity of PAHE and verbascoside was performed by *Salmonella*/microsome mutagenicity assay. Cytotoxicity and genotoxicity were carried out in Chinese hamster lung fibroblast V79 cells, by reduction of tetrazolium salt (MTT) and neutral red uptake (NRU) assay, and alkaline comet assay, respectively. Due the potential use of verbascoside in skin formulations, its phototoxicity was tested in *Mus musculus* embryo fibroblast 3T3 cells by the NRU phototoxicity assay.

Results: Among the marker compounds, only verbascoside was founded in PAHE, and its highest concentration was obtained in the ultrasound-assisted extraction (UAE) method, optimized in 40 min and 25 °C, and the method validation was successfully applied. Both PAHE and verbascoside did not show mutagenic or genotoxic activities. Cytotoxicity assays demonstrated that both of them reduced cell viability only in the highest concentrations, and verbascoside has no phototoxic properties.

Conclusion: The extraction of verbascoside from *P. australis* leaves through ultrasound methods was optimizing. The HPLC method to quantify verbascoside was validated. The results suggest the toxicological safety of PAHE and verbascoside, corroborating the use of *P. australis* in folk medicine, and also propose verbascoside as a potential ingredient in skin formulations.

Keywords: *Plantago australis*, hydroethanolic extract, verbascoside, response surface methodology, mutagenicity, cytotoxicity, genotoxicity, phototoxicity.

1. Introduction

Medicinal plants are part of the humanity's daily lifes for thousands of years. As examples, the traditional Chinese medicine, and the Egyptian and Greco-Roman chemical techniques are widely used until today (Viegas Jr. et al., 2006). Despite their different therapeutic targets and pharmacological mechanisms, plants have been poorly investigated, since the phytochemical and biological study of plants involves only a small portion of the plant kingdom, when compared to the ethnopharmacological knowledge of different species around the world (Filho and Yunes, 1998; Simões et al., 2004).

In addition, due the plants' chemical complexity, it is necessary a strict quality control from cultivation, through the harvest and extraction, till the eventual development of a phytotherapic product, supporting the financial and technological funding to botanical, chemical and pharmacological studies of plants, providing the research for new drugs and thus avoiding the indiscriminate use and the possible extinction of some plants (Turolla and Nascimento, 2006).

Plantago australis (Kunth) Rahn (Plantaginaceae) is a perennial plant, popularly known as *tansagem* or *transagem*, and is outspread in all Latin America, with a widely distribution in Southern Brazil (Helfer et al., 2011). In folk medicine, *P. australis* is largely used because its anti-inflammatory, antimicrobial, antiviral, gastric antiulcer, antidiarrheal and healing properties, as well as in the treatment of kidney, bladder and ovary diseases, but there is a lach of results on its safety (Palmeiro et al., 2003; Souza et al., 2004; Andrade-Cetto, 2009).

Phytochemical studies revealed several compounds in *P. australis*, some of them already having pharmacological activities described. As examples, we have the iridoid glucoside aucubine and the phenolic compounds, salidroside, isoverbascoside and verbascoside, which were described in hydroethanolic extracts (Andary et al., 1988; Rønsted et al., 2000).

Verbascoside, also known as acteoside, is an ester structurally formed by the phenylpropanoid caffeic acid, the phenylethanoid hydroxytyrosol and the sugar alpha-*L*-rhamnopyranosyl-(1->3)-β-*D*-glucopyranose. Belonging to the caffeoyl phenylethanoid glycoside group, it is widely founded in numerous plant families, including

Plantaginaceae, and therefore is one of the most studied compounds in *Plantago* (Li et al., 2014b; Zhang et al., 2015; Wen et al., 2016). Pharmacologically, verbascoside has antigenotoxic, gastric antiulcer, anti-inflammatory and healing activities (Fleer and Verspohl, 2007; Korkina et al., 2007; Fabiani et al., 2008; Speranza et al., 2010; Gyurkovska et al., 2011; Ambrosone et al., 2014).

Due to the limited studies about *P. australis*, especially in relation to its toxicity, and also because of the need to improve the plant phytochemical analysis, this study aims to develop a hydroethanolic extract of the leaves of *P. australis* standardized in its analytical marker, verbascoside, and determinate the toxicological safety of the extract and the marker in *in vitro* models.

2. Material and methods

2.1. Chemicals

DMEM (Dulbeco's Modified Eagle Medium), FBS (fetal bovine serum), newborn calf serum, PBS (phosphate-buffered saline), trypsin-EDTA, L-glutamine, penicillin/streptomycin and Trypan blue were obtained from Gibco-BRL (Grand Island, NY, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), neutral red, MMS (methyl methanesulfonate), aucubin, baicalein, oleanolic acid, ursolic acid, verbascoside and Cytochalasin-B were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid, acetonitrile, ethanol, formic acid, methanol and phosphoric acid were purchased from EMD Millipore (Darmstadt, Germany).

2.2. Plant material

The leaves of *P. australis* (Kunth) Rahn were collected in the city of Santa Cruz do Sul, RS, Brazil, in October of 2014, in a delimited area under coordinates 29°41'46.8"S 52°26'27.4"W. The plant was identified by the specialist botanic M.Sc. Gustavo Hassemer (Statens Naturhistoriske Museum, Københavns Universitet, Denmark), and the voucher specimen (ICN: 179648) was deposited in the herbarium of Federal University of Rio Grande do Sul (UFRGS), Brazil. The leaves were dried in a greenhouse (renewal of air) at 40 °C, manually triturated and submitted to a sieve analysis (Bertel, Brazil). Triplicates of PAHE were prepared by two different techniques, percolation and ultrasound.

2.3. Ultrasound-assisted extraction and optimization of process parameters

The ultrasound-assisted extraction (UAE) was performed in an ultrasonic water bath (Unique Group USC 1800, São Paulo, Brazil). The bath consisted of a rectangular container with 37 kHz transducers annealed to the bottom. In experimental runs, 2.5 g of grounded *P. australis* leaves were mixed in 25 mL of a hydroethanolic solution (30% water and 70% ethanol) and sonicated in an ultrasonic bath under different temperature and time parameters, according optimization of process parameters. The extracts were paper-filtered and concentrated by rotary evaporator at low temperature (<40 °C) under vacuum, until dried evaporated the solvent. The mass yields (*y*%) were also calculated, and the extracts were stored at -20 °C until further use.

The extraction of the verbascoside content by ultrasound was performed by employing various extraction conditions. In this study, a complete full factorial design (2^2) with two levels and two factors was applied to determine the best combination of extraction variables. The factors selected include extraction time from 30 and 60 min. and temperature from 25 and 40 °C. All of the extrations variantions were performed in triplicate.

A central composite design of response surface methodology (RSM) was used to optimize the verbascoside concentration. The RSM is a collection of statistically based experimental designs that have been established as a convenient method for optimizing several processes (Paz et al., 2015). In this study, RSM was used to optimize the extraction of verbascoside from *P. australis*. Table 1 shows the experimental condition of RSM. All extracts were paper-filtered and concentrated by rotary evaporator at low temperature (<40 °C) under vacuum, until dried evaporated the solvent. The mass yields (*y*%) were also calculated, and the extracts were stored at -20 °C until further use.

Table 1
RSM employed for the extraction of *P. australis* leaves.

			Levels		
Variables		Code units	-1	0	1
1	Temperature (°C)	X_1	25	30	35
2	Extraction time (min)	X_2	45	60	75

2.4. Percolation

Onde hundred grams of *P. australis* leaves were mixed in 130 mL of a hydroethanolic solution, followed by 2 h of stirring and rest. The material was passed to a percolation system, covered with the hydroethanolic solution and rested for 24 h. After this, the percolation system was opened with a flow of 1.0 mL/min until complete 1 L of percolated solution. The extract was paper-filtered and concentrated by rotary evaporator at low temperature (<40 °C) under vacuum, until dried evaporated the solvent. The mass yiels (*y*%) was also calculated, and the extracts were stored at -20 °C until further use.

2.5. High performance liquid chromatography (HPLC) analysis

The HPLC method was developed in a Shimadzu Prominence (Japan) chromatograph equipped with a quaternary, low-pressure mixing pump and inline vacuum degassing, controlled by a CBM-20A interface module, an automatic injector (SIL-20A) and Diode Array Detector (SPD-M20A). The separation was carried out by a gradient system, using a reverse-phase Phenomenex Luna 5 mm C18(2) (250 x 4.0 mm^2) column maintained at 30 °C. All compounds where identified, when present, by comparing the retention times of samples and its authentic standard. The compounds were analyzed qualitatively and whenever possible the quantification was performed and expressed in percentage, correlating the area of the analyte in sample with the area of the authentic standard.

The HPLC conditions to analyze verbascoside were made according Li et al. (2005), with some modifications. The mobile phase consisted of 2% (v/v) acetic acid solution (solvent A) and acetonitrile (solvent B). The composition gradient used was: 2% (B) to 25% (B) in 40 min, 25% (B) to 100% (B) in 5 min, 100% (B) to 2% (B) in 5 min. The injection volume was 10 µL with a flow rate of 1.0 mL/min, and the samples were monitored at 320 nm. Aucubin was analyzed according Kartini et al. (2012). The mobile phase comprised 93% Milli-Q® water and 7% acetonitrile, the injection volume was 20 µL with a flow rate of 0.6 mL/min, and the samples were monitored at 204 nm. Baicalein was analyzed allowing a method described by Gao et al. (2008). The mobile phase consisted of methanol (solvent A) and a 0.1% (v/v) formic acid solution (solvent B). The composition gradient was: 55% (B) to 40% (B) in 20 min, 40% (B) to 30% (B) in 20 min, 30% (B) to 55% (B) in 5 min. The injection volume was 10 µL with a flow rate of 0.6 mL/min, and the samples were monitored at 270 nm. Oleanolic and ursolic

acids were analyzed based in Tarvainen et al. (2010). The mobile phase comprised 90% acetonitrile and 10% of a 1% (v/v) phosphoric acid solution, the injection volume was 10 µL with a flow rate of 0.5 mL/min, and the samples were monitored at 206 nm.

2.6. Verbascoside method validation by HPLC

The current HPLC-DAD assay was validated for selectivity, linearity, intraday and inter-day precisions, accuracy and robustness according RE 899/2003 (Brazil, 2003). The selectivity of the HPLC method was examined by peak identification; no interferences appeared at the retention time of verbascoside in the chromatogram from sample solution and standard solution. The linearity was studied in a range of concentrations to 49.6 to 74.4 µg/mL (49.6, 55.8, 62, 68.2 and 74.4 µg/mL) from the verbascoside standard solution (1 mg/mL). Each sample was analyzed in triplicate. The calibration curves were obtained by the analysis of least squares linear regression of the verbascoside peak area vs. the verbascoside concentration. The mean, standard deviation (SD) and coefficient of variation (CV) were calculated for each concentration. Besides, we estimated the values of the slope and intercept, correlation and determination coefficients. The precision of the method was evaluated by repeatability and intermediate precision. Repeatability was examined by six evaluations of the same concentration sample, on the same day (intraday), under the same experimental conditions. The intermediate precision was assessed by carrying out the analysis on different day (inter-day) and also by other analyst performing the analysis in the same laboratory (between-analysts). Accuracy was evaluated by the addition (fortification) of verbascoside in three levels of concentration: low (49,6 µg/ml), medium (62 µg/ml) and high (74,4 µg/ml), in PAHE. Recovery (R%) was calculated according to the following equation: R% = (measured concentration/nominal concentration) × 100.

2.7. *Salmonella*/microsome mutagenicity assay

Salmonella typhimurium strains TA98, TA97a, TA100, TA1535, and TA102 were provided by MOLTOX® (Molecular Toxicology Inc., USA). Mutagenicity was assayed according to the preincubation procedure. The S9 metabolic activation mixture (S9 mix) was prepared according to Maron and Ames (1983). Briefly, 100 µL of test bacterial cultures (1-2x10^9 cells/mL) were incubated at 37 °C with different amounts of PAHE and verbascoside in the presence or absence of S9 mix for 20 min, without shaking. Subsequently, 2 mL of soft agar (0.6% agar, 0.5% NaCl, 50 µM histidine, 50

µM biotin, pH 7.4, 42 °C) were added to the test tube and poured immediately onto a plate of minimal agar (1.5% agar, Vogel-Bonner E medium, containing 2% glucose). Aflatoxin B1 (1.0 µg/plate) was used as positive control for both strains in the presence of metabolic activation (with S9 mix). In the absence of metabolic activation, 4-nitroquinoline-oxide (4-NQO, 0.5 µg/plate) was used for TA98, TA97a, and TA102 strains and sodium azide (1 µg/plate) was employed for TA100 and TA1535 strains. Plates were incubated in the dark at 37 °C for 48 h before counting the revertant colonies. Assays were repeated three fold and the plating for each dose was in triplicate.

2.8. Cells culture and treatments

Chinese hamster lung fibroblast V79 cells and *Mus musculus* embryo fibroblast 3T3 cells were obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil). V79 cells were cultured under standard conditions in DMEM supplemented with 10% heat-inactivated FBS, 0.2 mg/mL L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin, and 3T3 cells were cultured in the same conditions, but using newborn calf serum. Cells were kept in tissue-culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2 in air and were harvested by treatment with 0.15% trypsin-0.08% EDTA in PBS. For all treatments, water stock solutions of *P. australis* and verbascoside were prepared immediately prior to. The appropriate concentrations were obtained by dilution of stock solutions in distilled water. The solvent control (distilled water) included in the tests were found to be negative.

2.9. MTT assay

The V79 cells were seeded in a 96-well tissue culture microtiter plate ($1x10^5$/mL) in complete media and grown for 24h prior to treatment with PAHE and verbascoside before evaluation by MTT assay, allowing Huang et al. (2010). Briefly, after treatments, cells were washed with PBS before the addition of 100 µL serum-free media containing yellow tetrazolium salt (1 mg/mL) dye and incubated for 3 h at 37 °C. After incubation, the supernatant was removed, the residual purple formazan product solubilized in 200 µL DMSO and its absorbance measured at 570 nm. The absorbance of negative control cells was set as 100% viability and the values of treated cells were calculated as percentage of control.

2.10. Neutral red uptake assay

To the neutral red uptake (NRU) assay, V79 cells were seeded in a 96-well tissue culture microtiter plate ($1,3x10^5$/mL) in complete media and grown for 24 h prior to treatment with PAHE and verbascoside before evaluation, according Borenfreund and Puerner (1985), with some modifications. Briefly, after treatment, cells were washed with 250 µl of PBS before the addition of 250 µl of neutral red dye (25 µg/ml) dissolved in serum-free media and incubated for 3 h at 37 °C in a humidified 5% CO_2 incubator. Cells were washed with PBS, and 125 µl of a desorb solution (ethanol:acetic acid:water, 50:1:49) was added followed by gentle shaking for 30 min for complete dissolution. Absorbance was recorded at 540 nm using a microtiter plate reader. Cell viability was expressed as a percentage of the control.

2.11. Alkaline comet assay

The alkaline comet assay was performed as previously described by Singh et al. (1988), with minor modifications. Briefly, 60 µL of V79 cell suspension (1×10^4) treated with PAHE or verbascoside were mixed with 180µL low-melting point agarose, spread on two normal agarose precoated microscope slide, and placed at 4°C for 10 min to allow for solidification. Cells were lysed in high concentration of salt and detergent solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris with 1% Triton X-100 and 10% DMSO freshly added) for 24 h. Slides were removed from lysing solution and washed three times with PBS. Subsequently, cells were exposed to alkali conditions (300 mM NaOH/1 mM Na_2EDTA, pH>13, 15 min, 4 °C) to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 20 min at 25 V and 300 mA, and after this, the slides were neutralized and silver stained. MMS, at 50 µg/mL concentration, was used as positive control, and the vehicle as negative control. One hundred cells were scored visually according to the tail length and the amount of DNA present in the tail. Each comet was given an arbitrary value of 0 to 4 (0, undamaged; 4, maximally damaged), as described by Collins et al. (1995). Damage score was thus assigned to each sample and can range from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4).

2.12. Phototoxicity assay

The *in vitro* 3T3 NRU phototoxicity test was carried out as described by the OECD guideline 432 (2004). Briefly, 96-well plates were seeded with $1x10^5$ cells/mL 3T3 cells, and subsequently incubated at 37 °C in a humidified 5% CO_2 incubator for 48 h. After this, the media was removed and the cells were washed with PBS and exposed to various concentrations of verbascoside for 1 h. Following incubation for 24 h in a CO_2 incubator at 37°C, duplicate plates were either exposed to UVA/visible light at 5 J/cm^2 in a UV instrument (Vilber-Lourmat, Eberhardzell, Germany) or kept in the dark for 50 min. Following irradiation, the media were discarded from all the plates and the cells were washed with culture medium. The cells were then reincubated in culture medium overnight. On day 3, the medium was removed and the cells were washed with PBS and added to 150 µL of neutral red medium (100 µg/ml, serum-free). Samples were then incubated for 3 h in a CO_2 incubator at 37°C, and subsequently 100 µL of desorb solution (ethanol:acetic acid:water, 50:1:49) was added to the plates. Absorbance was recorded at 540 nm using a microtiter plate reader. Cell viability was expressed as a percentage of the control. Norfloxacin, at 120 µg/mL concentration, was used as positive control, and the own media as negative control.

2.13. Statistical analysis

Complete factorial design and RSM were analyzed by ANOVA, according Teófilo and Ferreira (2006). All *in vitro* experiments were independently repeated at least threefold, with triplicate samples for each treatment, and results are expressed as mean ± SD and statistical significance was determined by One-Way Analysis of Variance (ANOVA) followed by Dunnett's test or Tukey's test using statistical software GraphPad Prism®, version 5 (La Jolla, CA, USA). In all comparisons, $p<0.05$ was considered as indicating statistical significance. A test substance was considered mutagenic from the *Salmonella*/microsome assay when significant ANOVA variance was observed, and the mean number of revertants on test plates was at least twice as high as that observed in the negative control plates (or at least three times higher, for the TA1535 strain).

3. Results and discussion

3.1. Plant extraction, optimization and validation of the analytical method

Two different methods of plant extraction were used in this study, and their yields and verbascoside concentration are presented in Table 2. As results, the UAE

method provided the highest verbascoside concentration and the percolation the highest *y*%.

Table 2
Yields of the different techniques of *P. australis* leaves extraction.

Extraction technique	*y*%	Verbascoside concentration (%)
Percolation	10,43	5,5411 ± 0,1409
UAE – PAHE*	9,92	6,2604 ± 0,0185**

*PAHE - ultrasonic bath at 37 kHz for 40 min at 25 °C. **Results repeatability.

In this study, we investigated the presence of aucubin, baicalein, oleanolic acid, ursolic acid and verbascoside in percolation extract and ultrasound-assisted extract. Only verbascoside was present in extracts. The chromatograms of *P. australis* extracts and verbascoside standard are presented in Fig. 1.

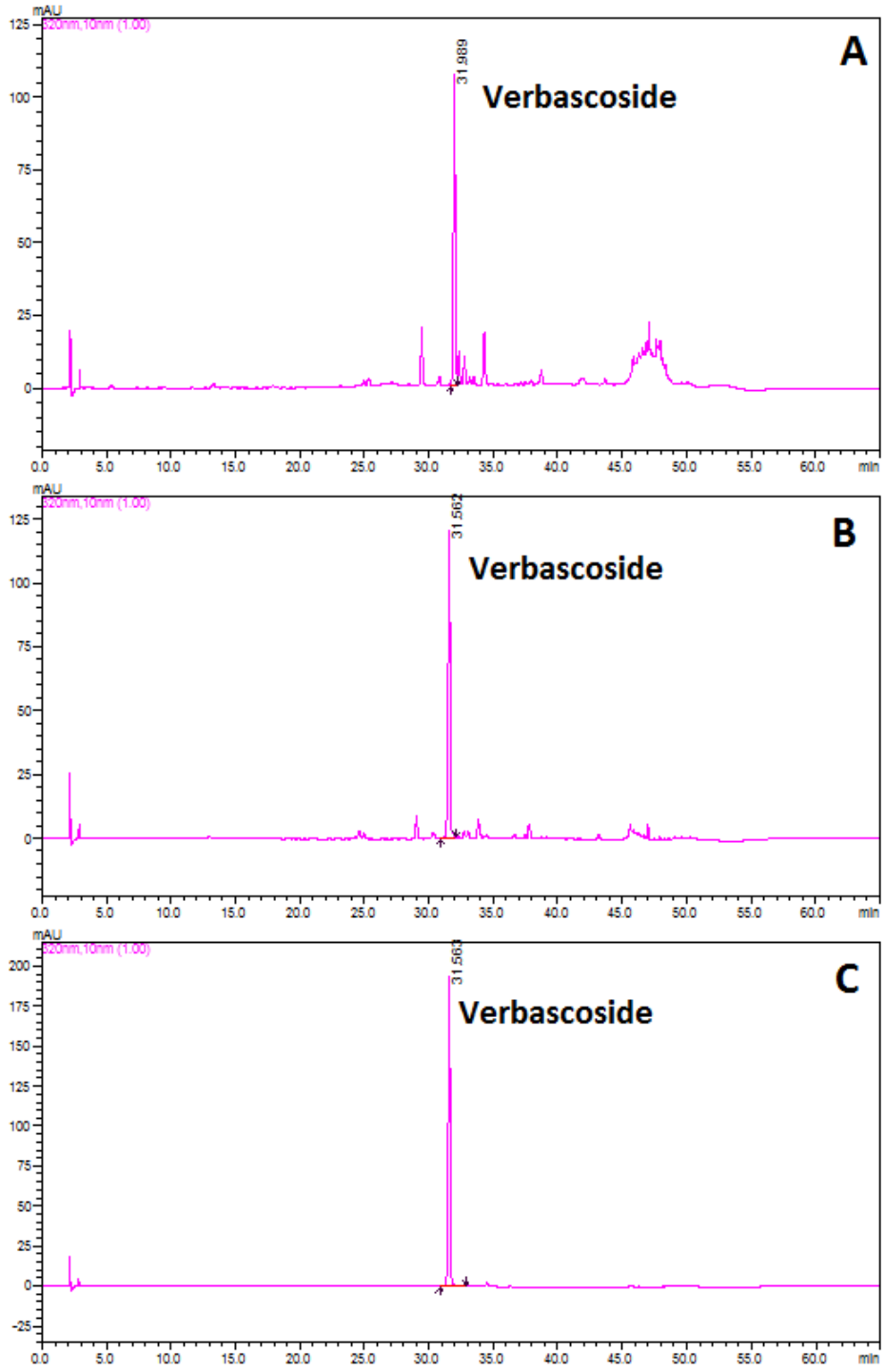


Fig. 1. Chromatograms of percolation extract (A), ultrasound-assisted extraction (B) and standard verbascoside (C).

In 2^2 full factorial design, attention was specifically focused on the two selected factors, according to the list presented above in Table 3, with two values for each factor. This experimental program provides the opportunity to analyze the influence of a pertinent selection of experimental parameters on a verbascoside concentration. In this

modeling procedure, it was chosen for simplicity to represent the influence of each factor through a linear variation. By analyzing the results quantitatively, it results from this assumption, the correlation between the verbascoside concentration and the two selected factors (X_1,X_2) can be represented by the following polynomial model:

$Y = 3,522 + 0,1312\ X_1 – 0,776\ X_2 + 0,331\ X_1X_2 + 0,054197.$

Table 3
Experiment matrix of 2^2 full factorial design and respective responses.

Experiment	Independent variables		Symbol code		Verbascoside concentration (%) (mean ± SD)
	Extraction Time (min)	Temperature (°C)	X_1	X_2	
1	30	40	-1	1	2,903 ± 0,063
2	60	40	1	1	3,365 ± 0,030
3	30	25	-1	-1	4,010 ± 0,165
4	60	25	1	-1	3,810 ± 0,055

The sign of each coefficient shows how the related factor influences the response. If the coefficient is positive, the response is increased (synergistic effect) as the factor moves from low level to high level; the contrary is obtained (inverse relationship/antagonist effect) if the coefficient is negative (Deshmukh, 2013). Thereafter, the optimum variable values were identified as 60 min and 25 °C and these conditions were applied to the RSM process.

The RSM was used to optimize the effect of process variables (time and temperature) on extraction procedure. The influence of selected parameters was evaluated by a central composite design, where time was modulated from 60 to 45 and 75 min, and temperature was modified from 25 to 30 and 35 °C. Results from this modulation are described in Table 4.

Table 4
Experiment matrix of RSM design – modulation step and respective responses.

Experiments	Variables		Symbol code		Verbascoside concentration (%) (mean ± SD)
	Time (min)	Temperature (°C)	Time	Temperature	
1	45	25	-1	-1	4,714 ± 0,148
2	75	25	1	-1	4,176 ± 0,160
3	45	35	-1	1	3,944 ± 0,147
4	75	35	1	1	3,107 ± 0,068
5	60	30	0	0	4,323 ± 0,106
6	60	30	0	0	4,400 ± 0,162
7	60	30	0	0	4,516 ± 0,048

The first step of RSM design revealed that the response variable, which presented the highest verbascoside concentration (4.714 %), was observed in 45 min and 25 °C. Therefore, in the displacement step of RSM design, temperature parameter was fixed at 25 °C and time parameter was moved to 40 and 50 min. The results are outlined in Table 5.

Table 5
Experiment matrix of RSM design – displacement step and respective responses.

Experiments	Variables		
	Time (min)	Temperature (°C)	Verbascoside concentration (%) (mean ± SD)
1	40	25	6,079 ± 0,09
2	40	25	6,169 ± 0,209
3	40	25	6,040 ± 0,021
4	50	25	4,491 ± 0,09
5	50	25	4,822 ± 0,160
6	50	25	5,697 ± 0,255

Based on these results, the optimal extraction point of verbascoside was estimated in 40 min and at 25 °C. After this, a *P. australis* hydroethanolic extract (PAHE) was prepared using 395.0 g of leaves and 3950 mL of hydroethanolic solution (30% water and 70% ethanol) and sonicated in an ultrasonic bath at 37 kHz for 40 min at 25 °C. The extract was paper-filtered and concentrated by rotary evaporator at low temperature (<40 °C) under vacuum, until dried evaporated the solvent. The mass yield (*y*%) was calculated and the extract was stored at -20 °C until further use. The PAHE was used in the validation of method.

To evaluate the selectivity, the retention times of the verbascoside in the chromatograms of the standard and samples solutions and the respective UV–PDA spectra were compared. Similarities in retention times and the same UV absorption profile were observed in the chromatograms of the standard and sample solutions. The purity of verbascoside peak constituent was checked by a diode array detector coupled to the HPLC system, comparing the UV spectra of verbascoside peak with those of the authentic sample. So, the proposed chromatographic method has adequate selectivity for verbascoside analysis in PAHE.

The precision of the method was obtained at two levels: in the first, repeatability assay was carried to evaluate the concordance between the results within a short period

of time with the same analyst and the same instrumentation. The second level, which comprises the intermediate precision, analyzes the correlation between the results obtained in different days with different analysts. The results of these tests are shown in Table 6.

Table 6
Results of the precision tests for determination of verbascoside in PAHE.

Test	Verbascoside concentration (%)			Mean ± SD (%CV)
Repeatability	6,2600	6,2328	6,2569	
	6,2880	6,2782	6,2463	6,2604 ± 0,0185 (0,2952)
Intermediate precision (analyst 2)	6,2593	6,2512	6,2659	6,2426 ± 0,0231 (0,3702)
	6,2425	6,2353	6,2011	

Linearity was evaluated on a calibration curve constructed with methanol standard solutions of verbascoside in the range of 50.0–80.0 µg/mL. A linear model was adjusted to the experimental data of the calibration (peak area and concentration) by applying the linear regression technique and a lack of fit test at 95% confidence (Table 7). Results demonstrated that the linear model is appropriate to establish the relationship between the area and the concentration. The recuperation (R) values for the three levels of fortification of verbascoside were in the extract of *P. australis* were 92.15% (low), 96.78% (medium) and 97.04% (high).

Table 7
Linear regression analysis of calibration curves (n=2).

Analyte	Linearity range (µg/mL)	Regression equation	Correlation coefficient (r^2)
Verbascoside	50 - 80	y = 24713x + 175618	0,9952

3.2 *In vitro* assays

To determine the safety of medicines, systematic toxicological studies must be performed using experimental models to predict toxicity and to set criteria for selecting a safe dose in humans. Despite the popular use of *P. australis* in traditional medicine as anti-inflammatory and wound healing, little information on *P. australis* toxicity is available. In this sense in this work, the hydroethanolic extract of *P. australis*

standardized in its analytical marker verbascoside, were tested to determine the toxicological safety *in vitro* models.

The PAHE was tested for TA100 strain toxicity at concentrations of 100–5,000 µg/plate in Salmonella/microsome assay. The test-substance was considered toxic if the mutagenic index (colony counts on the test plate/average counts on the negative control plates; MI) value was lower than 0.60 in at least two of the tested concentrations. The results of the range-finder experiment were used to define the dose range to be applied in the mutagenicity test, which concentration range should be the highest allowed by the toxicity or the solubility of the test substance. The range finding results indicate no cytotoxicity in tested concentrations. No PAHE mutagenicity effect was detected, even at the highest concentration, on TA97a (detects frameshift mutation in DNA target –C–C–C–C–C–; +1 cytosine), TA98 (detects frameshift mutation in DNA target –C–G–C–G–C–G–C–G–), TA100 and TA1535 (base-pair substitution mutation results from the substitution of a leucine [GAG] by a proline [GGG]) or TA102 (detect oxidative and alkylating mutagens by an ochre mutation TAA in the hisG gene), in the absence or presence of metabolic activation (Table 6). The verbascoside also be tested using TA100 at concentration of 10-500 µg/plate in microssoma assay and in the same way no verbascoside mutagenicity was observed in TA98 and TA100 strains (Table 7). The results on the mutagenic potential of verbascoside are conflicting. Santos-Cruz et al. (2012) showed absence of mutagenic effect of verbascoside using the *Drosophila* wing spot test. On the other hand, Santoro et al. (2008) indicates that verbascoside induces a significant increase of structural chromosome aberrations (CAs) and sister chromatid exchanges (SCEs) on normal human lymphocytes associated with a reduction of the MI, besides, these authors observed enhanced protein expression levels of PARP-1 and p53 that are key regulatory proteins involved in cell proliferation and DNA repair.

Table 8.
Induction of *his*+ revertants in *S. typhimurium* strains by *Plantago australis* hydroethanolic extract (PAHE) with and without metabolic activation (S9 mix).

		S. typhimurium strains									
Substance	Concentration (µg/plate)	TA98		TA97a		TA100		TA1535		TA102	
		Rev/plate[a]	MI[b]	Rev/plate[a]	MI[b]	Rev/plate[a]	MI[b]	Rev/plate[a]	MI[b]	Rev/plate[a]	MI[b]
Without metabolic activation (-S9)											
NC[c]	-	22.7±0.6	-	95.0±11.8	-	114.3±12.1	-	8.3±2.5	-	445.3±52.5	-
PAHE	100	24.3±5.5	1.07	91.3±14.2	0.96	89.7±15.1	0.78	12.3±1.5	1.48	429.7±28.4	0.96
	500	24.0±2.6	1.06	85.7±16.0	0.90	112.0±9.6	0.98	11.7±4.0	1.40	486.3±45.0	1.09
	1000	32.7±7.1	1.44	69.7±18.2	0.73	116.0±11.8	1.01	14.0±3.6	1.68	470.0±48.0	1.06
	2000	28.7±5.5	1.26	73.3±9.8	0.77	110.7±10.2	0.97	12.0±3.0	1.44	569.0±26.2*	1.28
	5000	50.3±11.5***	2.22	106.3±19.2	1.12	142.0±3.5*	1.24	11.3±0.6	1.36	727.3±55.7***	1.63
PC[d]	0.5 (4NQO) 1 (NaN_3)	$370.0_{\pm 28.3}$***	**16.32**	$521.0_{\pm 36.8}$***	**5.48**	$2658.0_{\pm 681.7}$***	**23.25**	$540.0_{\pm 46.6}$***	**64.83**	$4832.0_{\pm 616.6}$***	**10.85**
With metabolic activation (+S9)											
NC[c]	-	31.7±2.9	-	125.3±25.2	-	143.3±17.8	-	11.7±2.3		429.7±28.5	-
PAHE	100	40.7±5.5	1.28	125.0±13.0	1.00	158.0±13.1	1.10	7.7±3.5	0.66	473.3±77.5	1.10
	500	31.3±5.7	0.99	114.0±4.4	0.91	166.0±2.6	1.16	8.7±3.8	0.74	433.0±21.0	1.01
	1000	32.0±2.6	1.01	109.0±13.8	0.87	159.3±9.0	1.11	13.7±3.1	1.17	459.7±9.1	1.07
	2000	33.7±7.6	1.06	115.7±2.1	0.92	169.7±5.9*	1.18	12.0±4.4	1.03	423.0±37.7	0.98
	5000	35.7±3.2	1.13	103.0±10.8	0.82	174.0±6.2*	1.21	12.0±4.6	1.03	414.3±50.8	0.96
PC[d]	1 (AFB_1)	$715.0_{\pm 63.6}$***	**22.58**	$706.0_{\pm 99.0}$***	**5.63**	$400.5_{\pm 27.6}$***	**2.79**	$119.0_{\pm 18.5}$***	**10.20**	$1598.0_{\pm 19.8}$***	**3.72**

[a]Number of revertants/plate: mean of three independent experiments ± SD; [b]MI: mutagenic index (nº. of *his*+ induced in the sample/nº. of spontaneous *his*+ in the negative control); [c]NC: negative control (phosphate buffer pH 7.4 used as a solvent for the extract). [d]PC: positive control (-S9) sodium azide to TA100 and TA1535; 4-NQO to TA97a, TA98 and TA102; (+S9) aflatoxin B1; Significantly different in relation to the negative control. * $p<0.05$; *** $p<0.001$ (ANOVA, Dunnett's test).

Table 9.
Induction of *his*$^+$ revertants in *S. typhimurium* TA98 and TA100 strains by verbascoside with and without metabolic activation.

Substance	Concentration (µg/plate)	TA98		TA100	
Without metabolic activation (S9)		Rev/plate[a]	MI[b]	Rev/plate[a]	MI[b]
NC[c]	-	24.3±1.2	-	128.3±17.0	-
Verbascoside	10	28.0±7.9	1.15	127.0±7.0	0.99
	25	27.7±6.0	1.14	120.3±7.1	0.94
	50	26.0±3.0	1.10	141.0±12.1	1.10
	100	35.7±6.5	1.47	128.0±14.0	1.00
	500	38.3±7.1*	1.58	154.3±6.5	1.20
4NQO[d]	0.5	165.0±27.7***	6.78	-	-
NaN_3[e]	1	-		1460.0±50.9***	11.38
With metabolic activation (S9)		Rev/plate	MI	Rev/plate	MI
NC	-	41.0±10.4	-	140.3±17.0	-
Verbascoside	10	38.3±6.4	0.93	131.0±13.9	0.93
	25	38.3±7.5	0.93	144.0±23.1	1.03
	50	43.0±2.0	1.05	158.3±10.3	1.13
	100	41.3±7.1	1.01	152.0±32.4	1.08
	500	34.7±3.2	0.85	155.0±14.7	1.10
AFB-1[f]	1	296.0±40.5***	7.22	910.0±183.8***	6.49

[a]Number of revertants/plate: mean ± SD; [b]MI: mutagenic index (nº. of *his*+ induced in the sample/nº. of spontaneous *his*+ in the negative control); [c] Negative control (phosphate buffer pH 7.4 used as solvent of the verbascoside); [d] 4-nitroquinoline oxide used as positive control (without S9mix) to TA98; [e]sodium azide used as positive control (without S9mix) to TA100; [f]aflatoxin B_1 used as positive control (with S9mix) to TA98 and TA100. Significantly different in relation to the NC. ** $p<0.01$; *** $p<0.001$ (ANOVA, Dunnett's test).

The MTT and NRU assays were used in order to obtain information on PAHE and verbascoside in V79 cells. The results are showed in Fig. 3. The PAHE induces a significant cytotoxicity in concentrations up to 1000 µg/mL in both assays. After verbascoside treatment there is a cytotoxic effect at 50µg/mL and higher by NRU assay. MTT reduction assesses the functional integrity of mitochondria based on the enzymatic reduction of a tetrazolium salt by the mitochondrial dehydrogenase of viable cells. NRU is based on accumulation of the dye in the lysosome of viable cells. The use of different tests confirms the PAHE and verbascoside induces cytotoxic effects only at higher tested concentrations. Previously, was determined the effect of oral administration of crude aqueous extract of the leaves from *P. australis* on some biochemical, hematological and histopathological parameters in rats and the authors did not show significant changes in relation to control animals (Palmeiro et al., 2003).

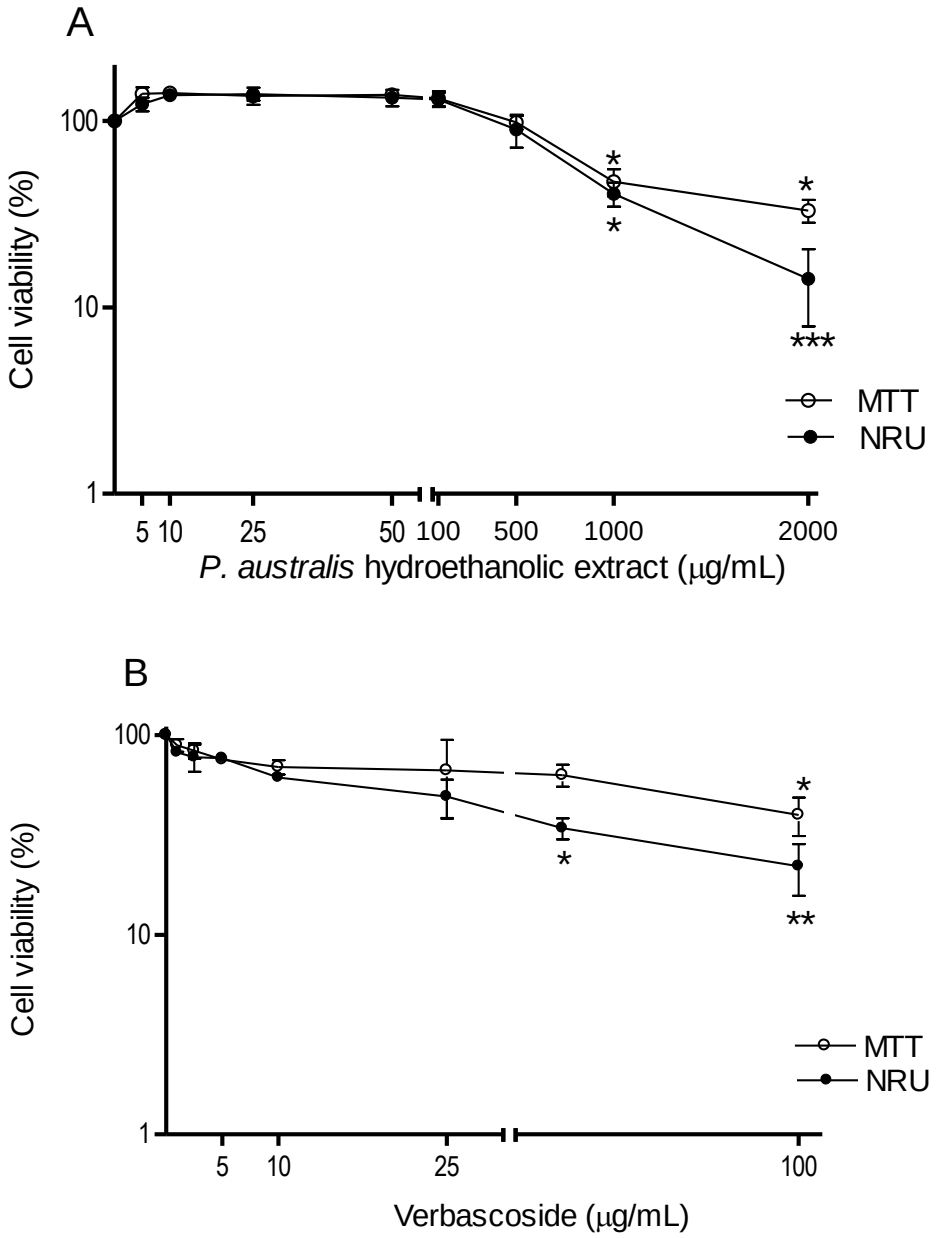


Fig. 3. Sensitivities of V79 cells to PAHE (**A**) and verbascoside (**B**). Cells were exposed to treatments for 24 h and cell viability was assessed by MTT and NRU assays right after the treatment. Results are expressed as percentage of control group growth for each data point and represent the mean (± SE) of three independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$. *** $p \leq 0.001$ (ANOVA Tukey's test).

To increase the knowledge about genotoxicity safe we also evaluated the induction of DNA single- and double-strand breaks and alkali-labile sites in the V79 mammalian cells using alkaline comet assay. Our results showed that PAHE and verbascoside does not generate DNA-strand breaks at tested concentrations (Fig. 4). The comet assay has achieved the status of a standard test in the battery of tests used to

assess the genotoxic safety of novel drugs (Hartmann et al., 2003; Collins, 2004). The results presented here indicate that the PAHE and verbascoside are not genotoxic by comet assay. These results are in accordance with Ames test presented before.

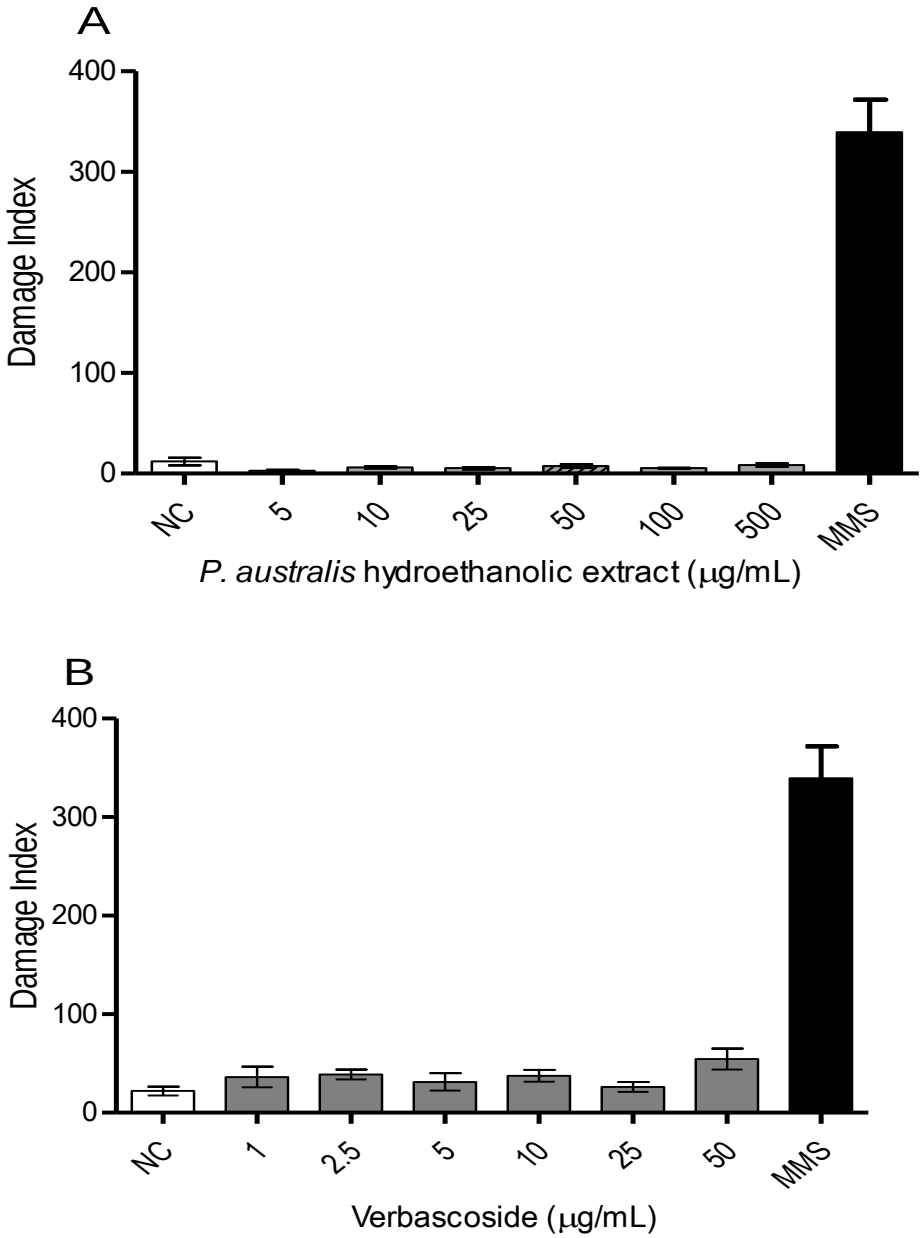


Fig. 4. Induction of DNA damage (relative tail intensity) measured with the alkaline comet assay in V79 cells treated for 24 h with PAHE (**A**) and verbascoside (**B**). **NC** = control group; **MMS** = positive control group. Results are expressed as damage index of control group damage for each data point and represent the mean (± SE) of three independent experiments (ANOVA Tukey's test).

In view of verbascoside potential use in cosmetics as active ingredient in solution or in dermocosmetic preparations, by presenting properties such as skin repair

promotes and ameliorates skin inflammation due to its ROS scavenging, antioxidant, iron chelating and anti-inflammatory activity (Kostyuka et al., 2011; Speranza et al., 2010; Espozito et al., 2010), we decided evaluated its phototoxicity properties. 3T3 NRU phototoxicity was tested *in vitro* according to the OECD 432 guideline. For the assay, norfloxacin was selected as a positive control, as the OECD guideline suggests that this drug exhibits phototoxicity by UV irradiation in 3T3 cells. The phototoxicity factor (PIF) of norfloxacin was 19.1, confirming the phototoxicity rate (data not shown). Various concentrations of verbascoside were tested and as shown in Fig. 5, no difference in cell cytotoxicity profile was observed with and without UV, suggesting that verbascoside did not induce phototoxicity in this *in vitro* 3T3 NRU phototoxicity test.

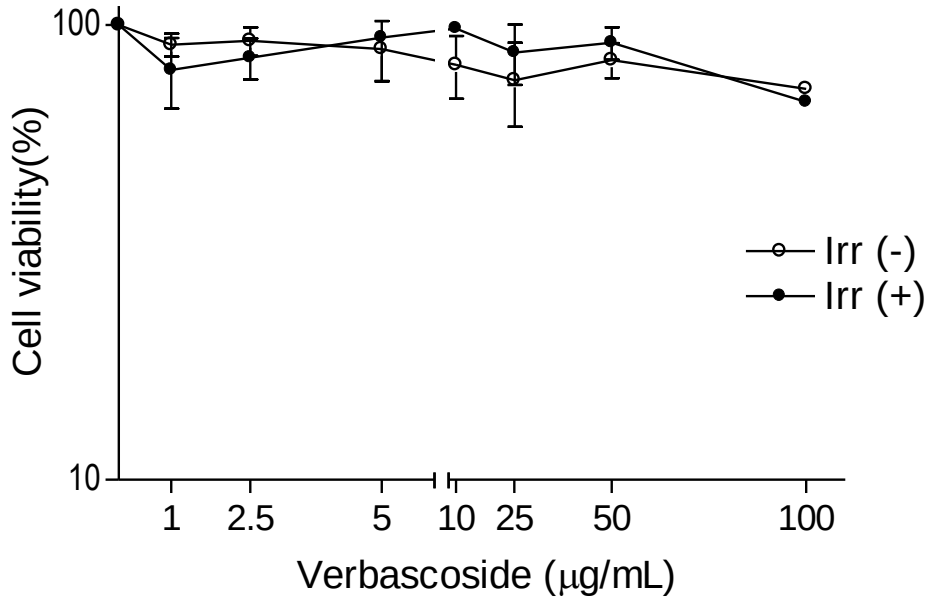


Fig. 5. Phototoxicity data for verbascoside in the 3T3 NRU phototoxicity assay. 3T3 cells were treated for 1 h with different concentrations of verbascoside and irradiated with UVA (5 J/cm²). **Irr (-)** = response in the 3T3-NRU assay in the absence of UVA irradiation; **Irr (+)** = response in the 3T3-NRU assay in the presence of UVA irradiation. Results are expressed as percentage of control group growth for each data point and represent the mean (± SE) of three independent experiments (ANOVA Tukey's test).

4. Conclusion

The extraction of verbascoside from *P. australis* leaves through ultrasound methods was optimized. The HPLC method to quantify verbascoside was validated. Our *in vitro* assays demonstrated that PAHE does not exert mutagenic and genotoxic effects

in all tested concentrations, and its cytotoxicity was observed only in higher concentrations, suggesting *P. australis* as a safe plant to be used as an herbal remedy. Our analytical marker, verbascoside, showed similar effects in the mutagenic, genotoxic and cytotoxic assays. Additionally, we demonstrated that verbascoside has no phototoxic effects, making this compound a potential candidate to active ingredient in skin formulations, corroborating its anti-inflammatory and healing activity.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Research was supported by grants from FAPERGS (Fundação de Amparo à Pesquisa do Rio Grande do Sul, Grant n° 13/1124-6). J.G. Henn, N.D.M. Sperotto and V.F. Péres received fellowships from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil). J. Saffi received fellowships from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil).

References

Ambrosone L., Guerra G., Cinelli M., Filippelli M., Mosca M., Vizzarri F., Giorgio D., Costagiola C., 2014. Corneal Epithelial Wound Healing Promoted by Verbascoside-Based Liposomal Eyedrops. Biomed Research International 2014:1-8.

Andary C., Motte-Florac M.E., Gargadennec A., Wylde R., Heitz A., 1988. Les esters caféiques du genre *Plantago*. Identification et valeur chimiotaxinomique. Plant Med Phytother 22:17-22.

Andrade-Cetto A., 2009. Ethnobotanical study of the medicinal plants from Tlanchinol, Hidalgo, Mexico. Journal of Ethnopharmacology 3;122(1):163-71.

Borenfreund E., Puerner J.A., 1985. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. Toxicology Letters 24:119-24.

Brazil – Agência Nacional de Vigilância Sanitária (ANVISA), 2003. Resolução RE n° 899, de 29 de maio de 2003. Brazil: 1-15.

Collins A.R., Ai-guo M., Duthie S.J., 1995. The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. Mutation Research 336:69-77.

Collins A.R., 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Molecular Biotechnology 26(3):249-61.

Deshmukh R.K., Naik J.B., 2013. Diclofenac sodium-loaded Eudragit® microspheres: optimization usin statistical experimental design. Journal of Pharmaceutical Innovation 8(4):276-87.

Esposito E., Mazzon E., Paterniti I., Dal Toso R., Pressi G., Caminiti R., Cuzzocrea S., 2010. PPAR-α contributes to the anti-Inflammatory activity of verbascoside in a model of inflammatory bowel disease in mice. PPAR Research 917312:1-10.

Fabiani R., Rosignoli P., Bartolomeo A., Fuccelli R., Servili M., Montedoro G., Morozzi G., 2008. Oxidative DNA damage is prevented by extracts of olive oil, hydroxytyrosol, and other olive phenolic compounds in human blood mononuclear cells and HL60 cells. The Journal of nutrition 138(8):1411-6.

Filho V., Yunes R., 1998. Estratégias para a obtenção de compostos farmacologicamente ativos a partir de plantas medicinais: conceitos sobre modificação estrutural para otimização da atividade. Química Nova 21(1):99-105.

Fleer H., Verspohl E.J., 2007. Antispasmodic activity of an extract from *Plantago lanceolata* L. and some isolated compounds. Phytomedicine 14(6):409-15.

Gao J., Sanchez-Medina A., Pendry B.A., Hughes M.J., Webb G.P., Corcoran O., 2008. Validation of a HPLC method for flavonoid biomarkers in skullcap (Scutellaria) and its use to illustrate wide variability in the quality of commercial tinctures. Journal of Pharmacy and Pharmaceutical Sciences 11(1):77-87.

Gyurkovska V., Alipieva K., Maciuk A., Dimitrova P., Ivanovska N., Haas C., Bley T., Georgiev M., 2011. Anti-inflammatory activity of Devil's claw in vitro systems and their active constituents. Food Chemistry 125(1):171-8.

Hartmann A., Aqurell E., Beevers C., Brendler-Schwaab S., Burlinson B., Collins A., Smith A., Speit G., Thybaud V., Tice R.R, 4th International Comet Assay Workshop, 2003. Recommendations for conducting the in vivo alkaline Comet assay. 4th International Comet Assay Workshop. Mutagenesis 18(1):45-51.

Helfer S.M., Rodrigues W.A., Cervi A.C., 2011. O gênero *Plantago* L. (Plantaginaceae) na região Sul do Brasil. Revista Brasileira de Biociências 9(3)297-321.

Huang F.-M., Yang S.-F., Zhao J.-H., Chang Y.-C., 2010. Platelet-rich fibrin increases proliferation and differentiation of human dental pulps cells. Basic Research – Biology 36(10):1628-32.

Kartini, Azminah, 2012. Chromatographic fingerprinting and clustering of *Plantago major* L. from different areas in Indonesia. Asian Journal of Pharmaceutical and Clinical Research 5(4):191-5.

Korkina L.G., Mikhal'Chik E.V., Suprun M.V., Pastore S., Dal Toso R., 2007. Molecular Mechanisms Underlying Wound Healing and Anti-infflamatory Properties of

Naturally Occurring Biotechnologically Produced Phenylpropanoid Glycosides. Cellular and Molecular Biology 53(5):84-91.

Kostyuka V.A., Potapovich A.I., Suhan T.O., de Luca C., Korkina L.G., 2011. Antioxidant and signal modulation properties of plant polyphenols in controlling vascular inflammation. European Journal of Pharmacology 658:248-256.

Li L., Tsao R., Liu Z., Liu S., Yang R., Young C., Zhu H., Deng Z., Xie M., Fu Z., 2005. Isolation and purification of acteoside and isoacteoside from *Plantago psyllium* L. by high-speed conter-current chromatography. Journal of Chromatography A 1063(1-2):161-9.

Li Y., Gan L., Li G.Q., Deng L., Zhang X., Deng Y., 2014. Pharmacokinetics of plantamajoside and acteoside from *Plantago asiatica* in rats by liquid chromatography-mass spectrometry. Journal of Pharmaceutical Biomedical Analysis 6;89:251-6.

Maron D.M., Ames B.N., 1983. Revised methods for the Salmonella mutagenicity test. Mutation Research 113(3-4):173-215.

OECD – Organisation for Economic Co-operation and Development., 2004. OECD guideline for testing of chemicals: *In vitro* 3T3 NRU phototoxicity test. OECD 432:1-15.

Palmeiro N.M., Almeida C.E., Ghedini P.C., Goulart L.S., Pereira M.C., Huber S., da Silva J.E., Lopes S., 2003. Oral subchronic toxicity of aqueous crude extract of *Plantago australis* leaves. Journal of Ethnopharmacology 88(1):15-8.

Paz J.E.W., Márquez D.B.M., Ávila G.C.G.M., Cerda R.E.B., Aguilar C.N., 2015. Ultrasound-assisted extraction of polyphenols from native plants in the Mexican desert. Ultrasonics Sonochemistry 22:474-81.

Rønsted N., Göbel E., Franzyk H., Jensen S.R., Olsen C.E., 2000. Chemotaxonomy of *Plantago*. Iridoid glucosides and caffeoyl phenylethanoid glycosides. Phytochemistry 55:337-348.

Simões M.O., Schenkel E.P., Gossmann G., Mello J.C.P., Mentz L.A., Petrovick P.R., 2004. Farmacognosia: da planta ao medicamento. 5.ed. Porto Alegre / Florianópolis: Editora da UFRGS / Editora da UFSC.

Santoro A., Bianco G., Picerno P., Aquino R.P., Autore G., Marzocco S., Gazzerro P., Lioi M.B., Bifulco M., 2008. Verminoside- and verbascoside-induced genotoxicity on human lymphocytes: involvement of PARP-1 and p53 proteins. Toxicology Letters 178(2):71-6.

Santos-Cruz L.F., Ávila-Acevedo J.G., Ortega-Capitaine D., Ojeda-Duplancher J.C., Perdigón-Moya J.L., Hernández-Portilla L.B., López-Dionicio H., Durán-Díaz A., Dueñas-García I.E., Castañeda-Partida L., García-Bores A.M., Heres-Pulido M.E., 2012. Verbascoside is not genotoxic in the ST and HB crosses of the *Drosophila* wing spot test, and its constituent, caffeic acid, decreases the spontaneous mutation rate in the ST cross. Food and Chemical Toxicology 50(3-4):1082-90.

Singh N.P., McCoy M.T., Tice R.R., Schneider E.L., 1988. A simple technique for quantification of low levels of DNA damage in individual cells. Experimental Cell Research 175:184-91.

Souza G., Haas A.P.S., Poser G.L., Schapoval E.E.S., Elisabetsky E., 2004. Ethnopharmacological studies of antimicrobial remedies in the south of Brazil. Journal of Ethnopharmacology 90(1):135-43.

Speranza L., Franceschelli S., Pesce M., Reale M., Menghini L., Vinciguerra I., De Lutiis M.A., Felaco M., Grilli A., 2010. Antiinflammatory effects in THP-1 cells treated with verbascoside. Phytotherapy Research 24(9):1398-404.

Tarvainen M., Suornela J.-P., Kallio H., Yang B., 2010. Triterpene acids in *Plantago major*: identification, quantification and comparison of different extraction methods. Chromatographia 71(3):279-84.

Teófilo, R.F., Ferreira M.M.C., 2006. Quimiometria II: planilhas eletrônicas para cálculos de planejamentos experimentais, um tutorial. Química Nova 29(2):338-50.

Turolla M., Nascimento E., 2006. Informações toxicológicas de alguns fitoterápicos utilizados no Brasil. Revista Brasileira de Ciências Farmacêuticas 42(2):289-306.

Viegas Jr C., Bolzani V.S., Barreiro E.J., 2006. Os produtos naturais e a química medicinal moderna. Química Nova 29(2):326-337.

Wen Y., Huo S., Zhang W., Xing H., Qi L., Zhao D., Li N., Xu J., Yan M., Chen X., 2016. Pharmacokinetics, Biodistribution, Excretion and Plasma Protein Binding Studies of Acteoside in Rats. Drug research 66(3):148-53.

Zhang W., Huo S.-X.X., Wen Y.-L.L., Xing H., Zhang Q., Li N., Zhao D., Sun X.L., Xu J., Yan M., Chen X.J., 2015. Pharmacokinetics of acteoside following single dose intragastric and intravenous administrations in dogs. Chinese journal of natural medicines 6;13(8):634-40.

5. Considerações finais

Tendo em vista a ampla utilização de plantas medicinais pela população, muitas vezes sem levar em consideração os potenciais riscos toxicológicos que norteiam este conhecimento empírico, faz-se plausível a padronização e investigação das características fitoquímicas, biológicas e toxicológicas destes preparados vegetais.

A presente dissertação, intitulada “Desenvolvimento de um extrato hidroetanólico das folhas de *Plantago australis* (Kunth) Rahn padronizado em verbascosídeo e determinação de sua segurança toxicológica”, teve por objetivo desenvolver, otimizar e padronizar um extrato hidroetanólico das folhas de *P. australis* mediante um marcador, o verbascosídeo, utilizando-se de modelos matemáticos atrelados a análises fitoquímicas por HPLC. Uma vez elaborado o extrato, ele e seu marcador verbascosídeo foram submetidos a uma bateria de ensaios *in vitro* para determinação da segurança toxicológica.

Ao final do trabalho, considerou-se que os objetivos foram adequadamente atingidos, constituindo um importante passo para a realização de teste adicionais *in vitro* e *in vivo* com o intuito de corroborar o uso de ambos, seja na forma empírica ou como possível matéria-prima para a indústria farmacêutica.

6. Conclusão

Esse trabalho confirma a difundida utilização de espécies da família Plantaginaceae na medicina caseira, embora os relatos sobre *P. australis* serem mais escassos, bem como descreve inúmeros compostos encontrados nessas plantas, muitos deles com atividade farmacológica documentada. Dessa forma, os resultados obtidos no presente estudo permitiram demonstrar que:

- O método de extração que apresentou o maior rendimento em massa foi a percolação e o método que apresentou maior rendimento em verbascosídeo foi o ultrassom.
- Análises preliminares por CLAE dos extratos produzidos por percolação e ultrassom permitiram identificar o verbascosídeo em ambos os extratos.
- A otimização do processo de extração de verbascosídeo por ultrassom revelou que o ponto ótimo de extraçãofoi em 40 min e a temperatura de 25 °C.
- Um extrato etanólico, a 70%, foi desenvolvido, utilizando os parâmetros do processo de otimização da extração, sendo ao final padronizado em 6% de verbascosídeo.
- A validação do método de quantificação do verbascosídeo por CLAE foi bem sucedida, com resultados de seletividade, precisão, linearidade e exatidão significativos, garantindo a correta quantificação do verbascosídeo no extrato.

Além disso, a segurança toxicológica *in vitro* do extrato hidroetanólico das folhas de *P. australis* e do seu marcador verbascosídeo, foi verificada e demonstrou resultados satisfatórios:

- Ambos não apresentaram atividade mutagênica no teste de *Salmonella*/microssoma.
- Tanto o extrato quanto o verbascosídeo não apresentaram citotoxicidade em baixas concentrações quando testados em células V79 mediantes dois diferentes métodos colorimétricos, o MTT e o NRU, cujos resultados foram similares. Concentrações maiores de ambos demonstraram-se citotóxicas.
- Não foi verificada indução significativa de dano ao DNA pelo extrato e pelo verbascosídeo na versão alcalina do ensaio cometa em células V79.
- O verbascosídeo não apresentou fototoxicidade quando testado em células 3T3, indicando seu potencial uso em formulações dérmicas com ação cicatrizante e anti-inflamatória.

7. Perspectivas

O conjunto de dados obtdos nesse trabalho possibilitou padronizar um extrato etanólico das folhas de *P. australis*, bem como validar a metodologia para a quantificação do verbascosídeo. Da mesma forma, foi possível realizar e determinar a segurança toxicológica do extrato e do seu marcador mediantes ensaios *in vitro* de mutagenicidade, citotoxicidade, genotoxicidade e fototoxicidade. Desta forma, as perspectivas de continuidade do presente estudo incluem:

a) Avaliar a genotoxicidade do verbascosídeo por meio do ensaio *in vitro* de formação de micronúcleos.
b) Realizar ensaio *in vitro* para a avaliação da fototoxicidade do extrato hidroetanólico de *P. australis*.
c) Avaliar a mutagenicidade do extrato hidroetanólico de *P. australis* e do verbascosídeo por meio do teste *in vitro* de mutação gênica em células de linfoma de camundongos.
d) Realizar ensaios de toxicidade aguda, subcrônica e de segurança toxicológica em ratos *Wistar*.
e) Elaborar uma formulação fitoterápica utilizando o extrato hidroetanólico de *P. australis.*

8. Anexos

8.1. Ensaio de genotoxicidade do extrato hidroetanólico de *P. australis* pelo teste de formação de MN em células V79

Dando continuidade aos ensaios de segurança toxicológica do extrato hidroetanólico de *P. australis* e de seu marcador analítico, verbascosídeo, foi verificado o potencial mutagênico do extrato pelo ensaio de formação de MN em células V79. Brevemente, as células V79 foram tratadas com diferentes concentrações do extrato, mantidas em meio completo e incubadas por 24 h sob condições normais. Após, as mesmas foram lavadas com o próprio meio e foi adicionada a citocalasina-B, mantendo-as em cultivo por mais 21 h. em seguida, as células foram tripsinizadas e a suspensão celular foi centrifugada, ressuspendida em tratamento hipotônico com 0,075 M KCl e mantida a 4 °C por 3 min. As células foram então centrifugadas fixadas em lâminas de microscopia com uma solução de metanol:ácido acético (3:1). As lâminas foram coradas com uma solução de Giemsa 10% por 3 a 4 min. O controle positivo do teste foi o metilmetanosulfonato (MMS), e os micronúcleos foram contados em 1000 células binucleadas por tratamento, conforme Fenech (2000).

A frequência de MN encontrada nas concentrações mais altas do extrato foi em torno de duas vezes maior que o controle negativo do teste, com significância estatística (Figura 9), sugerindo uma possível mutagenicidade do extrato em concentrações elevadas, embora o mesmo não tenha sido visualizado no teste *Salmonella*/microssoma e no ensaio cometa.

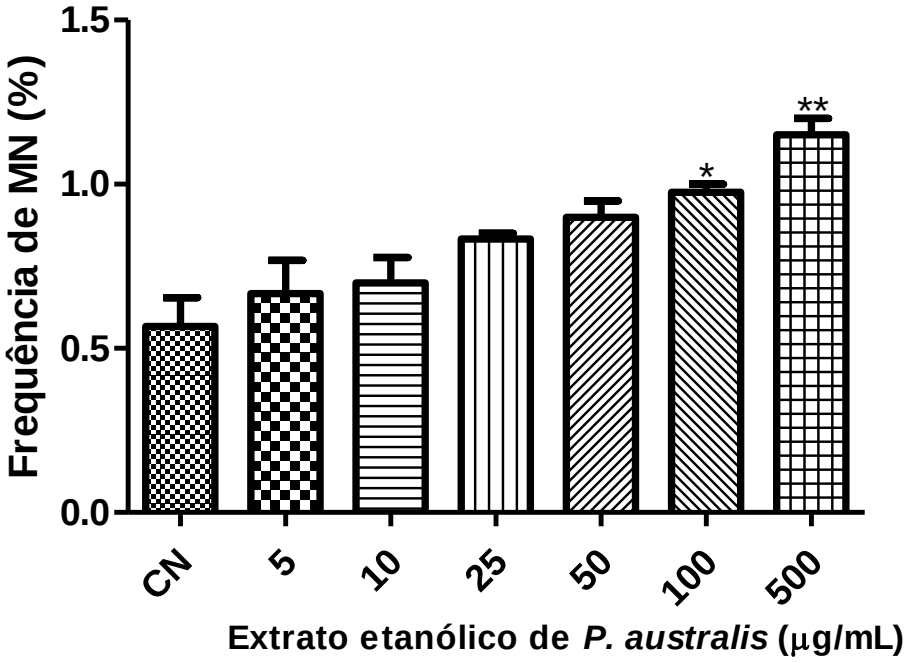


Figura 9. Frequência de micronúcleo (MN) em células V79 tratadas por 24 h com diferentes concentrações do extrato hidroetanólico de *P. autralis*. **CN =** grupo controle negativo. Os resultados estão expresso em frequência de MN (%) em comparação ao CN, e cada barra representa a média (± DP) de três experimento independentes realizados em triplicata. (* p≥0,05; ** p≥0,01).

8.2. Normas para publicação de artigos na revista *Phytotherapy Research*

Author Guidelines

NIH Public Access Mandate

For those interested in the Wiley Blackwell policy on the NIH Public Access Mandate, please visit our policy statement

For additional tools visit Author Resources - an enhanced suite of online tools for Wiley Online Library journal authors, featuring Article Tracking, E-mail Publication Alerts and Customized Research Tools.

- Permission Request Form

Author Guidelines

Phytotherapy Research is a monthly, international journal for the publication of original research papers, short communications, reviews and letters on medicinal plant research. Key areas of interest are pharmacology, toxicology, and the clinical applications of herbs and natural products in medicine, from case histories to full clinical trials, including studies of herb-drug interactions and other aspects of the safety of herbal medicines. Papers concerned with the effects of common food ingredients and standardised plant extracts, including commercial products, are particularly welcome, as are mechanistic studies on isolated natural products.

Short communications dealing with the pharmacology and screening of crude or uncharacterised extracts will be considered for publication only if they are clearly of interest to our international readership and are not deemed more suitable for a regional audience.

Phytotherapy Research does not publish agricultural, phytochemical, structure elucidation, quality control or botanical identification papers unless directly pertinent to the pharmacological effects or overall safety of plant based medicines currently in use.

Manuscript Submission. *Phytotherapy Research* operates an online submission and peer review system that allows authors to submit articles online and track their progress via a web interface. Please read the remainder of these instructions to authors and then clickhttp://mc.manuscriptcentral.com/ptr to navigate to the *Phytotherapy Research* online submission site.

All papers must be submitted via the online system.

Authors are welcome to submit the names and contact details of up to three suggested reviewers, using the online system. Submission of a manuscript will be held to imply that it contains original unpublished work and is not being submitted for publication elsewhere at the same time.

File types. Preferred formats for the text and tables of your manuscript are *.doc, *.docx and *.rtf. Figures must be provided in *.tiff or *.eps format.

Upon acceptance, authors **must** supply by e-mail to the Production Editor: permission grants, quoting the manuscript code. If the manuscript contains extracts, including illustrations, from other copyright works (including material from on-line or intranet sources) it is the author's responsibility to obtain written

permission from the owners of the publishing rights to reproduce such extracts using the Wiley Permission Request Form.

Manuscript Style. The language of the journal is English. Please ensure that your manuscript has been checked by an English language expert if there is concern for grammatical or other errors. All submissions including book reviews must have a title, be double-line spaced with type no smaller than 12 point, and have a margin of 3cm all round. Tables must be on separate pages after the reference list, and not be incorporated into the main text. Figures should be uploaded as separate Image files.

- The **title page** must list the full title, short title of up to 60 characters and names and affiliations of all authors. Give the full address, including email, telephone and fax, of the author who is to check the proofs.
- Include the name(s) of any **sponsor(s)** of the research contained in the paper, along with**grant number(s)**.
- Supply an **abstract** of up to 200 words for all articles. An abstract is a concise summary of the whole paper, not just the conclusions, and is understandable without reference to the rest of the paper. It should contain no citation to other published work.
- Include up to six **keywords** that describe your paper for indexing purposes.
- Authors may suggest up to 3 potential reviewers
- A concise **introduction** is required of the background to the subject, its significance and its relationship to earlier works, with references.
- **Materials and methods** should be presented with clarity and detail. State the original and important findings in the **results**. Illustrate these with figures or tables where necessary but keep these to a minimum.
- **Results and discussion** may be combined as one section. Discuss the principal conclusions drawn from the results and their important implications.
- **Convention on biodiversity.** Authors must indicate that they have obtained authority to access plant samples (other than freely available commercial crops or herbal products) used for research and that this has been authorised by the appropriate agent of the government of the source country as required under the framework of the United Nations Convention on Biodiversity.
- **Botanical aspects.** Plant materials used must be adequately described using the Latin binomial for the plant, author of the name, plant family, source (e.g. country and region of collection, name of the collector and collection number) means of unambiguous identification (e.g. name and affiliation of expert botanist or the results of comparison with a published monograph and/or authenticated reference specimen). The reference number and place of deposition of a voucher specimen of the plant material must be given. For papers relating to crude plant extracts, the method of extraction and the yield of dried extract as a percentage weight of the starting fresh or dried plant material must also be stated. These should be submitted as short communications (see below).
- **Experimental procedures.** Bioassay data for plant extracts or isolated compounds must be accompanied by data for positive and negative controls. All animal experiments should be conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, the EEC Directive of 1986 (86/609/EEC) or the NIH guide for the care and use of laboratory animals (NIH Publication No. 80-23; revised 1978). The Editors will reject papers if there is any doubt about the suitability of the animal procedures used.
- Use Chemical Abstracts nomenclature for chemical names and structures. Use proper or proprietary names with caution. Common acronyms for biomedical names are acceptable but define all others when first mentioned. Define abbreviations when first mentioned and do not use in the title or abstract. Define non-standard units.
- Keep acknowledgements brief and place them at the end of the paper.

Original Papers. These should not exceed five printed pages including a maximum of four figures and/or four tables and 30 references, (where one page comprises 800 words or the equivalent in illustrative and tabular material).

Short Communications. These must be complete, self-contained papers, and not preliminary reports. These should not exceed two printed pages including a maximum of two figures and/or two tables and 10 references. To exceed the limit may delay acceptance or publication of the paper.

Reviews and Keynote Lecture Transcripts. These will usually be written at the invitation of the Editors. Unsolicited reviews and manuscripts based on Conference Keynote Lectures will be welcome but authors wishing to submit these are requested to consult the Editor beforehand, ideally prior to commencement of

writing. Reviews should include a Table of Contents and will normally be limited to 10,000 words including references and should be submitted via the online system.

Correspondence. Items submitted for the **correspondence columns**, which need have no fixed format are intended for constructive comments on published work or for putting forward new ideas and are published at the discretion of the Editors.

Pre-Submission English Language Editing Authors for whom English is a second language may choose to have their manuscript professionally edited before submission to improve the English. A list of independent suppliers of editing services can be found athttp://www.blackwellpublishing.com/bauthor/english_language.asp. Japanese authors can also find a list of local English improvement services athttp://www.wiley.co.jp/journals.editcontribute.html. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

Reference style. References should be quoted in the text as name and year, and listed at the end of the paper alphabetically. All references must be complete and accurate. *Phytotherapy Research* uses *Index Medicus Style* abbreviations for journals cited. For correct abbreviations visithttp://www.ncbi.nlm.nih.gov/books/NBK7251/#IX-E. If necessary, cite unpublished or personal work in the text but do not include it in the reference list. References should be listed in the following style:

Journals: Wright CW, Phillipson JD. 1990. Natural products and the development of selective antiprotozoal drugs. *Phytother Res* **4:** 127-139.

Books: Wagner H, Bladt S. 1996. *Plant Drug Analysis: A Thin Layer Chromatography Atlas* (2nd edn). Springer-Verlag: Berlin Heidelberg.

Chapters in Books: Kips RH. 1985. Environmental aspects. In *Pesticide Application: Principles and Practice* , Haskel PT (ed). Oxford University Press: Oxford; 1-34.

Illustrations. Upload each figure as a separate file in either .tiff or .eps format, with the lead author's name, the figure number and the top of the figure indicated. Compound figures e.g. 1a, b, c should be uploaded as one figure. Tints are not acceptable. Lettering must be of a reasonable size that would still be clearly legible upon reduction, and consistent within each figure and set of figures. Please supply artwork at the intended size for printing, sized to the text width of 84mm/single column, 176mm/double column.Where a key to symbols is required, please include this in the artwork itself, not in the figure legend. All illustrations must be supplied at the correct resolution:

- Black and white and colour photos - 300 dpi
- Graphs, drawings, etc. - 800 dpi preferred; 600 dpi minimum
- Combinations of photos and drawings (black and white and colour) - 500 dpi

The cost of printing **colour** illustrations in the journal will be charged to the author. If colour illustrations are supplied electronically in either TIFF or EPS format, they **may** be used in the PDF of the article at no cost to the author, even if this illustration was printed in black and white in the journal. The PDF will appear on the *Wiley Online Library* site.

OnlineOpen. OnlineOpen is available to authors of articles who wish to make their article open access. With OnlineOpen the author, their funding agency, or institution pays a fee to ensure that the article is made available to non-subscribers upon publication via Wiley Online Library, as well as deposited in PubMed Central and PMC mirror sites. In addition to publication online via Wiley Online Library, authors of OnlineOpen articles are permitted to post the final, published PDF of their article on a website, institutional repository, or other free public server, immediately on publication.

Copyright Transfer Agreement. If your paper is accepted, the author identified as the formal corresponding author for the paper will receive an email prompting them to login into Author Services;

where via the Wiley Author Licensing Service (WALS) they will be able to complete the license agreement on behalf of all authors on the paper.

For authors signing the copyright transfer agreement. If the OnlineOpen option is not selected the corresponding author will be presented with the copyright transfer agreement (CTA) to sign. The terms and conditions of the CTA can be previewed in the samples associated with the Copyright FAQs below:

CTA Terms and Conditions

For authors choosing OnlineOpen. If the OnlineOpen option is selected the corresponding author will have a choice of the following Creative Commons License Open Access Agreements (OAA):

Creative Commons Attribution License OAA

Creative Commons Attribution Non-Commercial License OAA

Creative Commons Attribution Non-Commercial -NoDerivs License OAA

To preview the terms and conditions of these open access agreements please visit the Copyright FAQs hosted on Wiley Author Services and visithttp://www.wileyopenaccess.com/details/content/12f25db4c87/Copyright--License.html.

If you select the OnlineOpen option and your research is funded by The Wellcome Trust and members of the Research Councils UK (RCUK) you will be given the opportunity to publish your article under a CC-BY license supporting you in complying with Wellcome Trust and Research Councils UK requirements. For more information on this policy and the Journal's compliant self-archiving policy please visit: http://www.wiley.com/go/funderstatement.

Further Information. Proofs will be sent to the author for checking. This stage is to be used only to correct errors that may have been introduced during the production process. Prompt return of the corrected proofs, preferably within two days of receipt, will minimise the risk of the paper being held over to a later issue. Free access to the final PDF offprint of your article will be available via Author Services only (unless otherwise stated). Please therefore sign up for Author Services if you would like to access your article PDF offprint and enjoy the many other benefits the service offers. Reprints of your article and copies of the journal may be ordered. There is no page charge to authors.

Authors Resources: Manuscript now accepted for publication?

If so, check out our suite of tools and services for authors and sign up for:

- Article Tracking
- E-mail Publication Alerts
- Personalization Tool

PEER Project

This journal is participating in the PEER Project, an EU-funded initiative to examine the impact on scholarly journals of depositing accepted manuscripts in web-based repositories. If the corresponding author is based in an EU country, we will deposit the accepted manuscript into a central project repository (DRIVER). The article's availability will be subject to a post-publication embargo. This project has no effect on your rights or obligations under the terms of the copyright agreement pertaining to this journal.

Conflict of Interest. All authors must declare financial/commercial conflicts of interest. Even if there is none, this should be stated in a separate paragraph following on from the Acknowledgements section. This is a mandatory requirement for **all** articles.

8.3. Normas para publicação de artigos na revista *Journal of Ethnopharmacology*

Guide for Authors

Author information pack

Introduction

The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their biological and pharmacological effects based on the principles established through international conventions. Early people, confronted with illness and disease, discovered a wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of indigenous remedies. Chemists continue to use plant-derived drugs (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

Please note that figures and tables should be embedded in the text as close as possible to where they are initially cited. It is also mandatory to upload separate graphic and table files as these will be required if your manuscript is accepted for publication.

Classification of your paper

Please note that upon submitting your article you will have to select **at least one classification** and **at least three of the given keywords**. You can preview the list of classifications and keywords (here). This information is needed by the Editors to more quickly process your article. In addition to this, you can submit free keywords as described below under "Keywords".

The "rules of 5"

The Editors and Editorial Board have developed the "Rules of 5" for publishing in JEP. We have produced five clear criteria that each author needs to think about before submitting a manuscript and setting the whole process of editing and reviewing at work. Click here.

For more details on how to write a world class paper, please visit our Pharmacology Author Resources page.

Authors are encouraged to submit video material or animation sequences to support and enhance your scientific research. For more information please see the paragraph on video data below.

Types of paper

The *Journal of Ethnopharmacology* will accept the following contributions:

1. Original research articles - whose length is not limited and should include Title, Abstract, Methods and Materials, Results, Discussion, Conclusions, Acknowledgements and References. As a guideline, a full length paper normally occupies no more than 10 printed pages of the journal, including tables and illustrations.
2. Short Communications - whose average length is not more than 4 pages in print (approx. 2000-2300 words, including abstract and references). A maximum of 2 illustrations (figures or tables) is allowed. See paragraph below for description and format.
3. Letters to the Editors.
4. Reviews - Authors intending to write review articles should consult and send an outline to the Reviews Editor (see inside front cover for contact information) before preparing their manuscripts. The organization and subdivision of review articles can be arranged at the author's discretion. Authors should keep in mind that a good review sets the trend and direction of future research on the subject matter being reviewed. Tables, figures and references are to be arranged in the same way as research articles in the journal. Reviews on topics that address cutting-edge problems are particularly welcome.
Outlines for potential reviews need to include:

- A detailed abstract using the structure provided in the guidelines
- An annotated table of contents
- A short CV of the lead author

5. Book reviews - Books for review should be sent to the Reviews Editor.
6. Commentaries - *invited*, peer-reviewed, critical discussion about crucial aspects of the field but most importantly methodological and conceptual-theoretical developments in the field and should also provide a standard, for example, for pharmacological methods to be used in papers in the *Journal of Ethnopharmacology*. The scientific dialogue differs greatly in the social / cultural and natural sciences, the discussions about the common foundations of the field are ongoing and the papers published should contribute to a transdisciplinary and multidisciplinary discussion. The length should be a maximum of 2-3 printed pages or 2500 words. Please contact the Reviews Editor j.ethnopharmacol@pharmacy.ac.uk with an outline.
7. Conference announcements and news.

Before You Begin

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication
see https://www.elsevier.com/publishingethics andhttps://www.elsevier.com/journal-authors/ethics.

Policy and ethics

In the covering letter, the author must also declare that the study was performed according to the international, national and institutional rules considering animal experiments, clinical studies and biodiversity rights. See below for further information. The ethnopharmacological importance of the study must also be explained in the cover letter.

Animal and clinical studies - Investigations using experimental animals must state in the Methods section that the research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in for example the European Community guidelines (EEC Directive of 1986; 86/609/EEC) or the US guidelines (NIH publication #85-23, revised in 1985). Investigations with human subjects must state in the Methods section that the research followed guidelines of the Declaration of Helsinki and Tokyo for humans, and was approved by the institutional human experimentation committee or equivalent, and that informed consent was obtained. The Editors will reject papers if there is any doubt about the suitability of the animal or human procedures used.

Biodiversity rights - Each country has its own rights on its biodiversity. Consequently for studying plants one needs to follow the international, national and institutional rules concerning the biodiversity rights.

Author contributions

For each author the contribution to the publication should be mentioned.

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See alsohttps://www.elsevier.com/conflictsofinterest. Further information and an example of a Conflict of Interest form can be found at:http://service.elsevier.com/app/answers/detail/a_id/286/supporthub/publishing.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, seehttps://www.elsevier.com/sharingpolicy), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheckhttps://www.elsevier.com/editors/plagdetect.

Changes to authorship

Authors are expected to consider carefully the list and order of authors **before**submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Article transfer service

This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. Please note that your article will be reviewed again by the new

journal. More information about this can be found here:https://www.elsevier.com/authors/article-transfer-service.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright, seehttps://www.elsevier.com/copyright). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consulthttps://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult https://www.elsevier.com/permissions.

For open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (for more information seehttps://www.elsevier.com/OAauthoragreement). Permitted third party reuse of open access articles is determined by the author's choice of user license (seehttps://www.elsevier.com/openaccesslicenses).

Author rights

As an author you (or your employer or institution) have certain rights to reuse your work. For more information see https://www.elsevier.com/copyright.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies

Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some authors may also be reimbursed for associated publication fees. To learn more about existing agreements please visit https://www.elsevier.com/fundingbodies.

Open access

This journal offers authors a choice in publishing their research:

Open access
• Articles are freely available to both subscribers and the wider public with permitted reuse.
• An open access publication fee is payable by authors or on their behalf (e.g. by their research funder or institution).
Subscription
• Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs (https://www.elsevier.com/access).
• No open access publication fee payable by authors.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

Creative Commons Attribution (CC BY)
Lets others distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), include in a collective work (such as an anthology), text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)
For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

The open access publication fee for this journal is **USD 3300**, excluding taxes. Learn more about Elsevier's pricing policy:https://www.elsevier.com/openaccesspricing.

Green open access

Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our green open access page for further information (http://elsevier.com/greenopenaccess). Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form.

This journal has an embargo period of 12 months.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Additional information

Authors who want to submit a manuscript should consult and peruse carefully recent issues of the journal for format and style. Authors must include the following contact details on the title page of their submitted manuscript: full postal address; fax; e-mail. All manuscripts submitted are subject to peer review. The minimum requirements for a manuscript to qualify for peer review are that it has been prepared by strictly following the format and style of the journal as mentioned, that it is written in good English, and that it is complete. Manuscripts that have not fulfilled these requirements will be returned to the author(s).

In addition, you are recommended to adhere to the research standards described in the following articles:

Cos P., Vlietinck A.J., Berghe D.V., et al. (2006) Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. Journal of Ethnopharmacology, 106: 290-302.

Matteucci, E., Giampietro, O. (2008) Proposal open for discussion: defining agreed diagnostic procedures in experimental diabetes research. Journal of Ethnopharmacology,115: 163-172.

Froede, T.SA. and Y.S. Medeiros, Y.S. (2008) Animal models to test drugs with potential antidiabetic activity. Journal of Ethnopharmacology 115: 173-183.
Gertsch J. (2009) How scientific is the science in ethnopharmacology? Historical perspectives and epistemological problems. Journal of Ethnopharmacology, 122: 177-183.

Chan K., et al. (2012) Good practice in reviewing and publishing studies on herbal medicine, with special emphasis on traditional Chinese medicine and Chinese Materia Medica. Journal of Ethnopharmacology 140: 469-475.

Heinrich, M., Edwards. S., Moerman. D.E.. and Leonti. M. (2009), Ethnopharmacological field studies: a critical assessment of their conceptual basis and methods. J. Ethnopharmacol, 124: 1-17.

Preparation

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: https://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work.

In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Glossary
Please supply, as a separate list, the definitions of field-specific terms used in your article.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the

purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

The author should divide the abstract with the **headings *Ethnopharmacological relevance*, *Aim of the study* , *Materials and Methods*, *Results*, and *Conclusions***. Click here to see an example.

Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. Seehttps://www.elsevier.com/graphicalabstracts for examples.
Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

Keywords

After having selected a classification in the submission system, authors must in the same step select 5 keywords. These keywords will help the Editors to categorize your article accurately and process it more quickly. A list of the classifications and set keywords can be found here.

In addition, you can provide a maximum of 6 specific keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Chemical compounds

You can enrich your article by providing a list of chemical compounds studied in the article. The list of compounds will be used to extract relevant information from the NCBI PubChem Compound database and display it next to the online version of the article on ScienceDirect. You can include up to 10 names of chemical compounds in the article. For each compound, please provide the PubChem CID of the most relevant record as in the following example: Glutamic acid (PubChem CID:611). The PubChem CIDs can be found viahttp://www.ncbi.nlm.nih.gov/pccompound. Please position the list of compounds immediately below the 'Keywords' section. It is strongly recommended to follow the exact text formatting as in the example below:

Chemical compounds studied in this article
Ethylene glycol (PubChem CID: 174); Plitidepsin (PubChem CID: 44152164); Benzalkonium chloride (PubChem CID: 15865)
More information is available at: https://www.elsevier.com/PubChem.

Plant names

In the Materials and Methods section there must be a separate heading for describing the material used. That includes official name, local name, English name (if known), GPS position in case of collection in the wild or cultivation, a voucher specimen must be deposited in an official herbarium for possible future comparison. In the text it should be stated that the plant name has been checked with www.theplantlist.org mentioning the data of accessing that website.
In case of commercially procured material should mention the source, batch number, quality control data. Data on chemical characterization (metabolomics, chromatographic methods) should also be presented, in case of known active compounds their quantitative analysis should be presented.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Math formulae

Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
A detailed guide on electronic artwork is available on our website:
https://www.elsevier.com/artworkinstructions.
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Please note that figures and tables should be embedded in the text as close as possible to where they are initially cited. It is also mandatory to upload separate graphic and table files as these will be required if your manuscript is accepted for publication.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. For further information on the preparation of electronic artwork, please seehttps://www.elsevier.com/artworkinstructions.

Figure captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure

itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with "Unpublished results". "*Personal communication*" will not be accepted as a reference. Citation of a reference as "in press" implies that the item has been accepted for publication.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

Reference management software

Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles (http://citationstyles.org), such as Mendeley (http://www.mendeley.com/features/reference-manager) and Zotero (https://www.zotero.org/), as well as EndNote (http://endnote.com/downloads/styles). Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link:
http://open.mendeley.com/use-citation-style/journal-of-ethnopharmacology

When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Reference style

Text: All citations in the text should refer to:
1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.
Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.
Reference to a website:
Cancer Research UK, 1975. Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ (accessed 13.03.03).

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 150 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect:http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video

instruction pages athttps://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Supplementary material

Supplementary material can support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Please note that such items are published online exactly as they are submitted; there is no typesetting involved (supplementary data supplied as an Excel file or as a PowerPoint slide will appear as such online). Please submit the material together with the article and supply a concise and descriptive caption for each file. If you wish to make any changes to supplementary data during any stage of the process, then please make sure to provide an updated file, and do not annotate any corrections on a previous version. Please also make sure to switch off the 'Track Changes' option in any Microsoft Office files as these will appear in the published supplementary file(s). For more detailed instructions please visit our artwork instruction pages at https://www.elsevier.com/artworkinstructions.

Data in Brief

Authors have the option of converting any or all parts of their supplementary or additional raw data into one or multiple Data in Brief articles, a new kind of article that houses and describes their data. Data in Brief articles ensure that your data, which is normally buried in supplementary material, is actively reviewed, curated, formatted, indexed, given a DOI and publicly available to all upon publication. Authors are encouraged to submit their Data in Brief article as an additional item directly alongside the revised version of their manuscript. If your research article is accepted, your Data in Brief article will automatically be transferred over to *Data in Brief* where it will be editorially reviewed and published in the new, open access journal, *Data in Brief*. Please note an open access fee is payable for publication in*Data in Brief*. Full details can be found at http://www.journals.elsevier.com/data-in-brief. Please use the following template to write your Data in Brief:https://www.elsevier.com/dib-template.

Database linking

Elsevier encourages authors to connect articles with external databases, giving readers access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See https://www.elsevier.com/databaselinking for more information and a full list of supported databases.

AudioSlides

The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are

shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available athttps://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.
Ensure that the following items are present:
One author has been designated as the corresponding author with contact details:
• E-mail address
• Full postal address
All necessary files have been uploaded, and contain:
• Keywords
• All figure captions
• All tables (including title, description, footnotes)
Further considerations
• Manuscript has been 'spell-checked' and 'grammar-checked'
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Internet)
Printed version of figures (if applicable) in color or black-and-white
• Indicate clearly whether or not color or black-and-white in print is required.
For any further information please visit our customer support site athttp://support.elsevier.com.

After Acceptance

Use of the Digital Object Identifier

The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. Example of a correctly given DOI (in URL format; here an article in the journal *Physics Letters B*):
http://dx.doi.org/10.1016/j.physletb.2010.09.059
When you use a DOI to create links to documents on the web, the DOIs are guaranteed never to change.

Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.
If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.
We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints

The corresponding author, at no cost, will be provided with a personalized link providing 50 days free access to the final published version of the article onScienceDirect. This link can also be used for sharing via email and social networks. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's WebShop (http://webshop.elsevier.com/myarticleservices/offprints). Authors requiring printed copies of multiple articles may use Elsevier WebShop's 'Create Your Own Book' service to collate multiple articles within a single cover (http://webshop.elsevier.com/myarticleservices/booklets).

Author Inquiries

You can track your submitted article at https://www.elsevier.com/track-submission. You can track your accepted article at https://www.elsevier.com/trackarticle. You are also welcome to contact Customer Support via http://support.elsevier.com.