Universidade Federal de Pernambuco
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JANAINA KARIN DE LIMA CAMPOS

AVALIAÇÃO DA ATIVIDADE ANTI-INFLAMATÓRIA, ANTINOCICEPTIVA, ANTIPIRÉTICA, E ANTIOXIDANTE DE SUBSTÂNCIAS ISOLADAS DE SEMENTES DE *Indigofera suffruticosa* MILL

Recife
2016

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração Biotecnologia, da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutor em Ciências Biológicas.

Orientadora: Profa. Dra. Vera Lúcia de Menezes Lima
Co-orientador: Prof. Dr. Cesar Augusto da Silva

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A Deus por ser sempre ser o meu caminho e a minha fortaleza.

À minha família, e aos meus amigos pelo incentivo, carinho e apoio.

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“Deves aprender a ser verdadeiro em tudo: pensamentos, palavras e ações. Entre o Bem e o Mal, o ocultismo não admite transigência. Custe o que custar, é preciso fazer o Bem e evitar oMal. Cria esperança e otimismo, onte estiveres, em favor dos outros, sem pedir remuneração. Auxilia muito, e esperes pouco ou nada. Raciocínio sem aspereza; Sentimento sem preguiça, Caridade sem pretensão; Conhecimento sem vaidade; Cooperação sem exigência; Devotamento sem apelo; Dignidade sem orgulho; Firmeza sem petulância; Respeito sem bajulice”.

(Autor desconhecido, 1988)

RESUMO

Conhecida popularmente como “anil” ou “anileira, a *Indigofera suffruticosa*, é empregada na medicina popular para tratamento de processos inflamatórios e infeccioso, porém poucos estudos foram realizados com o objetivo de estabelecer suas possíveis atividades farmacológicas. Com isto, o presente estudo avaliou as possíveis ações antinociceptiva, anti-inflamatória, antipirética, antidiarréica e antioxidante de extratos orgânicos das vagens secas de *I. suffruticosa* e ações antinociceptiva, anti-inflamatória, antipirética e antioxidante de uma fração semipurificada de extrato metanólico de vagens secas de *I. suffruticosa.* Investigações dos metabólitos secundários produzidos por *I. suffruticosa* foram realizados através do método de cromatografia de camada delgada (CCD). A atividade antinociceptiva foi investigada pelos modelos de contorção abdominal e placa quente. A atividade anti-inflamatória foi avaliada pelos modelos de edema de pata e peritonite (20 e 40 mg/kg, fração). A atividade antidiarreica dos extratos metanólicos (200 e 400 mg/kg) foi investigada através de diferentes metodologias utilizando óleo de Rícino com agente indutor de diarréia. A atividade antipirética do extrato metanólico (200 e 400 mg/kg) foi investigada pelo modelo de Yeast Breath. A atividade antioxidante *in vitro* dos extratos orgânicos (50 – 500 µg/ml) foi analisado pelo método de DPPH. No modelo de contorções induzidas por ácido acético todos os extratos (éter, clorofórmio, acetona e metanol – 400 mg/kg) apresentaram excelente inibição das contorções quando comparado com o controle. A fração rica em açúcar de *I. suffruticosa* (20 e 40 mg/kg) também foi capaz de inibir as contorções abdominais. Assim como no modelo de nocicepção de placa quente, apenas os extratos clorofórmio, acetona e metanol (400 mg/kg) se destacaram aumentando o tempo de latência, e também a fração isolada de *I. suffruticosa* na dose mas elevada, apresentando ação duradoura de até 3 horas. No modelo de edema de pata induzida por carragenina observou-se uma diminuição significativa do edema nos animais tratados com os extratos acetônico e metanólico (dose 400 mg/kg - 60,1 e 58,7%, respectivamente), além também da fração nas doses 20 e 40 mg/kg (70,2 e 73,2%, respectivamente) de *I. suffruticosa* num período de 5 horas. Na indução da inflamação no modelo de peritonite induzida por carragenina, ambos os extratos (400 mg/kg) promoveram significativamente uma redução na migração leucocitária e também as doses testada da fração (20 e 40 mg/kg) foram capazes de diminuir o acúmulo de neutrófilos na cavidade peritoneal dos camundongos no tempo de 4 horas. No modelo de diarreia induzida em todas as metodologias aplicadas foi possível observar atividade antidiarréica significativa do extrato. Na indução de febre por yeast breath, ambas as doses do extrato metanólico promoveu significativamente a redução da pirexia no animal. Todos os extratos orgânicos testados no modelo de DPPH apresentaram atividade antioxidante (variação de 59,8 – 73 %). O conjunto de resultados sustenta a hipótese popular que a *Indigofera suffruticosa* possui ações antinociceptivas e anti-inflamatórias, como também atividade antidiarréica, além de contribuir para o acervo científica, destaca também sua potencialidade e utilidade para desenvolvimento de novos fármacos.

Palavras-chave: *Indigofera suffruticosa.* Inflamação. Dor. Diarreia.

ABSTRACT

Popularly known as "indigo" or "anileira the *Indigofera suffruticosa*, is used in folk medicine for treating inflammatory and infectious processes, but few studies have been conducted in order to establish the possible pharmacological activities. With this, the present study evaluated the possible antinociceptive actions, anti-inflammatory, antipyretic, antidiarrheal and antioxidant organic extracts of the dried pods of *I. suffruticosa* and antinociceptive actions, anti-inflammatory, antipyretic and antioxidant of a semipurified fraction of methanol extract of dried pods of *I. suffruticosa*. Investigations of secondary metabolites produced by *I. suffruticosa* were performed using the chromatographic method of thin layer (TLC). The antinociceptive activity was investigated by the models of writhing and hot plate. The anti-inflammatory activity was evaluated by the models of peritonitis and paw swelling (20 and 40 mg/kg fraction). The antidiarrheal activity of methanolic extracts (200 and 400 mg/kg) was investigated through different methodologies using Castor oil with diarrhea-inducing agent. The antipyretic activity of the methanol extract (200 and 400 mg/kg) was investigated by the yeast breath model. The in vitro antioxidant activity of organic extracts (50-500 mg/ml) was analyzed by DPPH method. In the writhing model induced by acetic acid extracts all (ether, chloroform, acetone and methanol - 400 mg/kg) showed excellent inhibition of writhings compared with the control. The sugar rich fraction *I. suffruticosa* (20 and 40 mg / kg) was also able to inhibit writhing. As in the model of nociception hot plate, only the chloroform extracts, acetone and methanol (400 mg/kg) is highlighted by increasing the lag time, and also the isolated fraction *I. suffruticosa* but at a dose high, with long lasting action to 3 hours. In the carrageenan-induced paw was observed a significant reduction of edema model of edema in animals treated with acetone and methanol extracts (dose 400 mg / kg - 60.1 and 58.7%, respectively), and also in the fraction doses 20 and 40 mg / kg (70.2 and 73.2%, respectively) of *I. suffruticosa* a period of 5 hours. In the induction of inflammation in the model of peritonitis induced by carrageenan, both extracts (400 mg / kg) significantly promoted a reduction in leukocyte migration and also the doses tested fraction (20 and 40 mg/kg) were able to decrease the accumulation of neutrophils into the peritoneal cavity of mice the time of 4 hours. In diarrhea model induced in all the applied methodologies it observed significant antidiarrheal activity of the extract. In the induction of fever by yeast breath, both doses of the methanol extract significantly promoted the reduction of pyrexia to the animal. All organic extracts tested in the model presented DPPH antioxidant activity (range 59.8 - 73%). The result set supports the popular hypothesis that *Indigofera suffruticosa* has antinociceptive and anti-inflammatory actions, as well as antidiarrheal activity, and contribute to the scientific collection also highlights its potential and usefulness for development of new drugs.

Keywords: *Indigofera suffruticosa.* Inflammation. Pain. Diarrhea.

LISTA DE ILUSTRAÇÕES DA REVISÃO DA LITERATURA

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LISTA DE ABREVIATURAS E SIGLAS

DNA	Ácido desoxirribonucleico
UV	Ultravioleta
COX 1	Cicloxigenase 1
COX 2	Cicloxigenase 2
PAF	Fator de Ativação de plaquetas
ATP	Trifosfato de adenosina
HETEs	Ácido hidroxieicosatetranóicos
cAMP	Adenosina 3',5'-monofosfato cíclico
OMS	Organização Mundial de Saúde
IL	Interleucina
IASP	International Association for the Study of Pain
TNF	Organização mundial de saúde
LTA4	Leucotrieno A4
LTB_4	Leucotrieno B4
LTC_4	Leucotrieno C4
LTD_4	Leucotrieno D4
PGD_2	Prostaglandina D2
PGE_2	Prostaglandina E2
PGF	Prostaglandina F
PGG_2	Prostaglandina G2
PGH_2	Prostaglandina H2
PGI_2	Prostaciclina
TXA_2	Tromboxano A2
TXB_2	Tromboxano B2
Acetil-CoA	Acetilcoenzima A
EROs	Espécies Reativas de Oxigênio

SUMÁRIO

1 INTRODUÇÃO

Por ser considerado um dos principais sintomas clínicos utilizado na detecção e avaliação de doenças, a dor é uma sensação de suprema relevância para a sobrevivência, pois atua como mecanismo de defesa para manter a integridade do organismo (LE BARS; GOZARIU; CADDEN, 2001). A nocicepção diferente da dor, é um processo fisiológico que utiliza estruturas neurais com campos receptivos específicos para codificar e processar os estímulos. A dor nociceptiva, também chamada de dor fisiológica é transitória e necessita desses estímulos para sua manifestação, enquanto que a dor patológica (dor clínica) é persistente e em geral está associada a processos inflamatórios, por sofrer ações de mediadores químicos comuns na inflamação (MENDELL; SAHENK, 2003). Neste contexto, substâncias com funções de diminuir a condição inflamatória são capazes de serem empregadas no alívio da dor.

A inflamação, resposta imunológica em células e/ou tecidos decorrente de um agente agressor, é caracterizada pelo aumento da permeabilidade vascular, do recrutamento de leucócitos e da liberação de mediadores químicos. A reação inflamatória pode ser de origem endógena, proveniente da degeneração ou necrose tissular, ou de fonte exógena, causada por agentes físicos, químicos ou biológicos. O processo inflamatório também é essencial para a proteção do organismo afetado, pois possui a finalidade de eliminar o agente agressor e manter a homeostase (SCHMID; SCHONBEIN, 2006). Quando a inflamação persiste por dias, semanas ou anos (crônica), as reações celulares em resposta ao estresse provocado causam grandes impactos na formação e progressão de cânceres (DRANOFF, 2004).

Terapias analgésicas e anti-inflamatórias são frequentemente insuficientes pela associação de sua eficácia limitada ou efeitos adversos, tais como: transtornos gastrointestinais e alterações cardiovasculares (CARVALHO; CARVALHO; RIOS-SANTOS, 2004). Por tal razão, novas buscas terapêuticas para tratamentos de dor, inflamação e outras doenças torna-se necessário, principalmente nos casos onde não há respostas farmacológicas. Deste modo, novos agentes bioativos vegetais tem sido investigados com a tentativa de serem mais eficientes, mais benefícos, menos danosos e ascessivel a

população tem sido usados para pesquisas científicas (SULEYMAN et al., 2010).

Desde os tempos primórdios as plantas são utilizadas por várias populações como fonte de tratamento para diversas patologias. O interesse pelo uso de produtos oriundos de vegetais decorre da facilidade de obtenção e baixo custo, como também a crença de que estes são isentos de efeitos tóxicos e colaterais, e que aparentemente são eficazes nos tratamentos em que a medicina tradicional não alcança o resultado esperado (CALIXTO 2000; CARVALHO et al., 2008).

Ao longo do tempo, sucessivas gerações vem acumulando conhecimentos gerais sobre tais produtos e utilizando-os na produção de novos fármacos. As plantas produzem metabólitos essenciais para sua sobrevivência por diferentes propósitos o que incluem regulação do crescimento, interação intra e interespecíficas, proteção contra radiação UV e defesa contra predadores e infecções. Muitos destes compostos apresentam excelentes aplicações biológicas, e são usados com antiinflamatórios, antinociceptivos, antioxidante, andiarréicos e também em tratamentos contra doenças crônicas como diabetes, câncer, entre outras (PUPO et al., 2006), o que desencadeia o aproveitamento frequente na fabricação de novos produtos farmacêuticos.

Estima-se que 25% dos medicamentos prescritos em todo o mundo são provenientes de plantas medicinais (SAHOO; MANCHIKANTI; DEY, 2010), como exemplos relevantes, podemos mencionar a morfina (*Papaver somniferum*), o quinino (casca da *Chinchona* sp), a digoxina (*Digitalis* sp.), o taxol (*Taxus brevifolia*), a vincristina e a vinblastina (*Catharanthus roseus*), dentre outros (RATES, 2001). Assim, as plantas se destacam na terapia moderna por possuir diversos compostos biologicamente ativos ou passíves de modificação e otimização estrutural.

Estes dados evidenciam que o Brasil, que detém a maior biodiversidade no mundo, como também uma rica diversidade cultural e étnica pode se beneficiar deste privilégio para atuar na descoberta de novos potenciais produtos farmacológicos. Além disso, torna-se atrativo o uso de terapias alternativas devido ao difícil acesso aos medicamentos convencionais, tendo em vista o alto custo (RATES, 2001).

As plantas comumentes destacadas como medicinais presentes na flora nativa brasileira são consumidas com pouca ou nenhuma comprovação científica de suas propriedades farmacológicas (VEIGA JUNIOR; PINTO, 2005). A *Indigofera* é o terceiro maior gênero de Leguminosae, composto por aproximadamente 700 espécies. A este gênero pertencente a espécie popularmente conhecida como “anil” ou “anileira”, a *Indigofera suffruticosa*, família Fabaceae, vem se destacando na população por apresentar propriedades antiespasmódica, sedativa e diurética (GARCIA, 2007).

Alguns estudos científicos realizados com esta espécie demonstraram efeitos benéficos nas seguintes atividades biológicas: anticonvulsivante, antiepiléptica e antigenotóxica (ROIG; MESA, 1974; BADELL et al 1998; AJITH et al, 2003; LOPES et al., 2011; ALMEIDA et al., 2013), antimicrobiana, antifúngica (LEITE et al., 2006, CARLI et al., 2010, SANTOS et al., 2015[a], SANTOS et al., 2015[b]) e anti-tumoral de extratos fracionados com Hexano, Acetato de Etila, Metanol e água (LEITE et al., 2006; VIEIRA et al., 2007), gastroprotetora (LUIZ- FERREIRA et al., 2011), e anti-inflamatória (LEITE et al., 2003; CHEN et al, 2013).

No entanto, ainda são escassos os relatos científicos que informem as propriedades presentes nas vagens desta espécie, demonstrando a necessidade ainda de se estudar e avaliar os potenciais efeitos biológicos presentes na *I. suffruticosa*, tal como investigar seus metabólitos secundários para que suas atividades sejam validadas e que seus derivados possam ser utilizados como protótipos de fármacos seguros e eficazes.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Investigar a atividade anti-inflamatória, antinociceptiva, antipirética,antioxidante e antidiareica de extratos orgânicos e fração semipurificada de extrato metanólico de vagens de *Indigofera suffruticosa.*

1.2.1 Objetivos Específicos

a) Avaliar o efeito dos extratos orgânicos (éter, clorofórmio, acetona e metanol) de vagens secas *I. suffruticosa* em modelos experimentais de inflamação aguda e crônico em camundongos;
b) Avaliar o efeito da fração semipurificada de extrato metanólico de vagens secas de *I. suffruticosa* em modelos experimentais de inflamação aguda e crônico em camundongos;
c) Avaliar o efeito dos extratos orgânicos (éter, clorofórmio, acetona e metanol) de vagens secas de *I. suffruticosa* em modelos experimentais de algesia em camundongos;
d) Avaliar o efeito da fração semipurificada de extrato metanólico de vagens secas de *I. suffruticosa* em modelos experimentais de algesia em camundongos;
e) Avaliar o efeito do extrato metanólico de vagens secas de *I. suffruticosa* no modelo experimental febre induzida por *Shaccharomyces cerevisiae* em camundongos;
f) Avaliar o efeito da fração semipurificada de *I. suffruticosa* no modelo experimental febre induzida por *Saccharomyces cerevisiae* em camundongos;
g) Avaliar o efeito antidiarreico de extratos metanólicos de vagens secas de *I. suffruticosa* em diferentes modelos de diarreia induzida em camundongos;
h) Avaliar o efeito antioxidante dos extratos orgânicos (éter, clorofórmio, acetona e metanol) e fração semipurificada de extrato metanólico de vagens de *I. suffruticosa in vitro* e *in vivo*;

2 REVISÃO DE LITERATURA

2.1 DOR

A dor é considerada uma epidemia mundial caracterizada por ser um sintoma clinicamente importante por funcionar como sinalização de alerta de uma ameaça à integridade física do organismo, sendo assim, ela é relevante para a detecção e avaliação de doenças (CHAPMAN; GAVRIN, 1999). A definição da dor pela Associaçao Internacional para o estudo de dor (IASP,

2014) se refere a “uma experiência emocional e sensorial desagradável associada com uma lesão tecidual real ou potencial ou descrita em termos de tal lesão”.

É um fenômeno polivalente, dinâmico e ambíguo, e notoriamente difícil de quantificar, e mesmo com essas limitações, a alta prevalência e incidência existentes em todo o mundo, apesar de terem poucas estimativas não são duvidosas. Mundialmente, estima-se que cerca de 1 em cada 5 adultos sofrem problemas com dor e que mais de 1 em cada 10 adultos tem o diagnostico evoluído para dor crônica a cada ano, ou seja, 60 milhões de pessoas suportam uma dor do tipo crônica (IASP, 2014). A dor afeta independentemente de sexo, etnia, idade, renda ou geografia, em todas as populações, não sendo distribuído igualmente em todo o mundo. Segundo a IASP (2014), as quatro maiores causas de dor são: câncer, artrite e osteoartrite reumatóide, lesões de operações, e problemas de coluna, fazendo com que a etiologia da dor seja um assunto complexo e interdisciplinar e de grande interesse para saúde pública.

Uma sensação dolorosa pode ser transitória, aguda ou crônica. Quando se refere a dor transitória, a ativação de nociceptores não necessita de qualquer dano tecidual. No tipo aguda, geralmente é ocasionada por uma lesão e ativação de nociceptores no local lesionado. Já a dor crônica é proveniente de alguma lesão ou patologia e pode ser perpetuada por fatores que não os causadores iniciais da dor (MENDELL; SAHENK, 2003).

A nocicepção denomina-se o componente sensorial da dor, ou seja, enquanto a dor refere-se a percepção de um estímulo perigoso, a nocicepção envolve manifestações neurofisiológicas geradas por este estímulo. Assim, o alerta de dor reflete na ativação de nociceptores (sensores) que são evocados frente a estímulos potencialmente nocivos (MILLAN, 1999). Os nociceptores podem ser sensibilizados e ativados pela ação de diversas substâncias, denominadas algiogênicas, que inclui a acetilcolina, bradicinina, histamina, leucotrieno, substância P, fator de ativação plaquetário, prostaglandinas, tromboxano, interleucinas, fator de necrose tumoral (TNF-α), entre outras (BEDBROOK, 1976; PIOTROWSKI, 1986).

Os nociceptores estão distribuídos na pele, vasos, articulações, músculos, e visceras. Existem também nociceptores do tipo silencioso (“silent”

ou sleeping"), que são pequenas proporções das fibras aferentes que não responde a estímulos, mas que quando influenciados por mediadores inflamatórios ou administração de agentes flogísticos se tornam ativos espontaneamente ou sensibilizados e então conferem respostas a estímulos sensoriais (JULIUS; BASBAUM, 2001).

São representados pelas terminações livres presentes nas fibras amielínicas C e mielínicas finas Aδ. As fibras do tipo C (diâmetro de 0,4- 1,2 µm), sem a presença da mielina, respondem a estímulos nocivos de origem térmica (mudança de temperatura), mecânica (diferença osmótica) e química (lesão tecidual seguida de inflamação), e por isto são chamados de nociceptores polimodais (LAWSON, 2002; COUTAUX et al., 2005). Esta sensibilização resulta na liberação não somente pelos neurônios sensoriais, mas tambem por fibras simpáticas e por células não neuronais (plaquetas, células endoteliais, fibroblastos, células de Schwann e células inflamatórias) de diversos mediadores químicos, tais como: bradicinina, histamina, serotonina, metabólitos do ácido araquidônico, citocinas, opióides, aminoácidos excitatórios, acetilcolina, entre outros (JULIUS; BASBAUM, 2001).

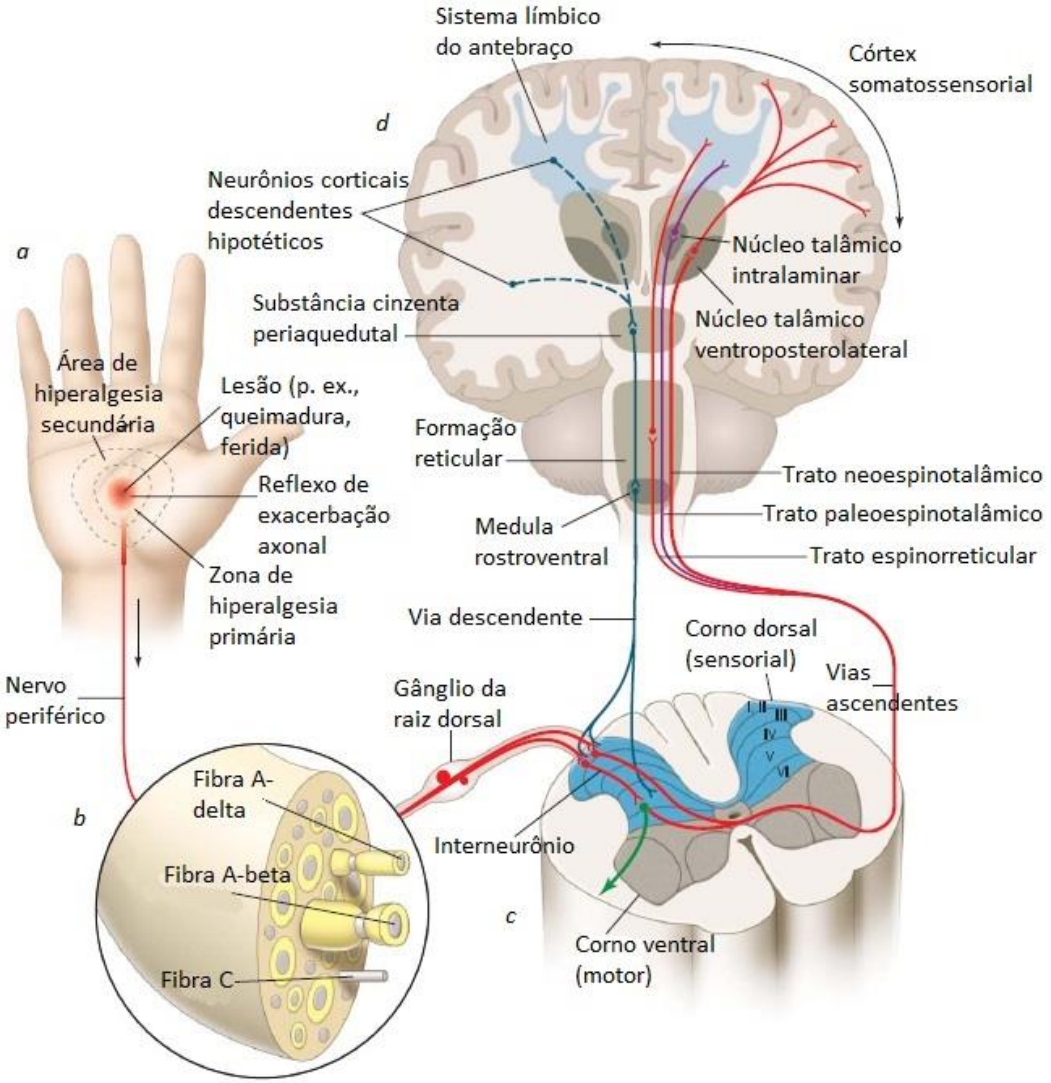
As fibras finas mielinizadas Aβ (diâmetro maior que 10 µm) são originadas de neurônios com corpos celulares de maior diâmetro, sua velocidade de condução é de 30-100 m/s e detectam estímulos inócuos na pele, músculos e articulações, não contribuindo para a nocicepção, porém sua estimulação pode resultar no alívio de dor (JULIUS; BASBAUM, 2001). As fibras Aδ, são pouco mielinizada, variando seu diâmetro (2-6 µm) e propagam o sinal de forma mais rápida que a fibra do tipo C, estando apenas relacionada com estímulos de origem térmica (tipo AδI até 53ºC e tipo AδII menores que 43ºC) e mecânica (MELZACK, 1999).

O corno dorsal na medula espinhal funciona como dispositivo que retransmite o sinal, amplificando-o para a transmissão de dor. Frente a estimulos nocivos o potencial de ação é propagado nos neurônios nociceptivos principalmente pela ação de canais de sódio e cálcio dependentes de voltagem, sendo os canais de cálcio os principais reguladores da liberação de neurotransmissores (MCCLESKEY; GOLD, 1999). Esses neurônios de primeira ordem enviam projeções em diferentes lâminas espinhais, lâmina I (zona marginal) e lâmina II (substância gelatinosa) para região do corno dorsal na

medula espinhal, e através da liberação de neurotransmissores realizam sinapses (MILLAN, 1999).

Após a projeção no corno dorsal, por diferentes tratos aferentes, os axônios dos neurônios de segunda ordem transmitem impulsos nociceptivos para estruturas do tronco cerebral e diencéfalo, incluindo tálamo, substânica cinzenta pariquedutal, região parabraquial, formação reticular da medula, hipotálamo, entre outras (ALMEIDA, 2004). As vias ascendentes projetam os neurônios diretamente para tratos espinotalâmicos, trigemial, espinoparabraquial e espinomesencefálico, sendo os mais importantes para a condução nociceptiva. A via mais utilizada para estudo relacionada com a transmissão de sinal nociceptivo é a via espinotalâmica (MELZACK, 1999). Nesta via, os axônios são direcionados para o tálamo sem sofrer sinapses no decorrer do caminho, porém ao chegar, realizam sinapses em diferentes núcleos e posteriormente liberam neurônios de terceira ordem que são responsáveis por levar informações para várias regiões do córtex cerebral, onde ocorre o processamento que resulta em consciência da dor (CRAIG, 2003) (Figura 1).

Figura 1. Processamento da dor



Fonte:http://www.medicinanet.com.br/conteudos/acp-medicine/5249/dor_cronica_%E2%80%93_anne_louise_oaklander.htm
Acesso em: 10/11/2014

O organismo possui várias regiões e estruturas que são responsáveis em modular mecanismos intrínsecos da sensação dolorosa. Após a sensibilização dos diversos núcleos do tálamo, os sinais são propragados para as diferentes áreas do córtex, substância cinzenta pariaquedutal, hipotálamo, amígdala e cerebelo. A conexão da substância cinzenta pariaquedutal com um modulador endógeno descendente (Núcleo magno da rafe, estruturas adjacentes da medula rostral ventromedial e o corno dorsal da medula) é responsável pela ativação de conexões que podem promover a analgesia

intensa, ocorrendo principalmente pela liberação da serotonina (neurotransmissor) que ativa interneurônios, inibindo assim a transmissão da via espinotalâmica (COFFIELD; BOWEN; MILETIC, 1992; BANDLER; KEAY, 1996).

2.2 INFLAMAÇÃO

A inflamação, do grego *phlogosis* e do latim *flamma*, que significa fogo, é um processo fisiológico que ocorre em decorrência da ativação de alguns mecanismos de defesa contra uma lesão causada por agentes físicos (calor, frio, trauma), químicos (substâncias irritantes) e/ou biológicos (micro-organismos) e que possuema capacidade de provocar alterações nos componentes humorais e celulares após injúria tecidual (SANTOS et al., 2004).

As primeiras descrições clinicas da inflamação foram relatadas pelos egípcios, em aproximadamente 3000 a.C, porém, Celsius um escritor romano do século I, foi o precursor do relato dos quatros sinais cardeais da inflamação: Aumento do fluxo sanguíneo e a dilatação de pequenos vasos, o rubor; O aumento da permeabilidade vascular: tumor; aumento da temperatura local: calor e por fim provocando a dor local (ROCHA; SILVA; GARCIA, 2006).

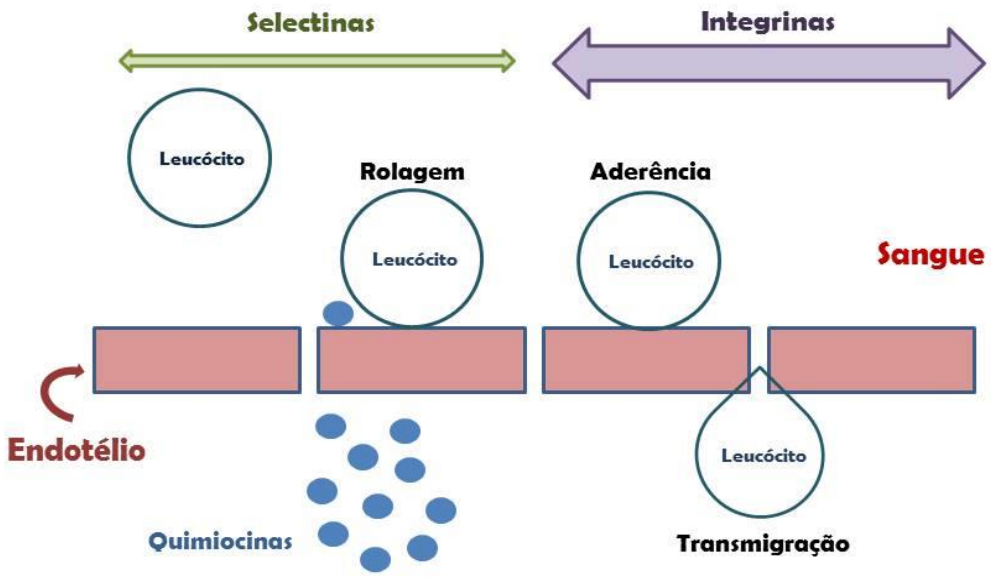
A reação inflamatória é considerada um evento complexo que envolve a ativação de células competentes e a liberação de diversos mediadores responsáveis, capazes de provocar o reconhecimento do agente agressor, sua inativação e posterior destruição e reparação tecidual. Entretanto, se este processo não atuar de maneira eficiente e sincronizada, a resposta imunológica pode evoluir para uma lesão tecidual persistente com o acúmulo de células leucocitárias, colágeno, entre outras substâncias danosas ao organismo (NATHAN, 2002).

Basicamente, a resposta inflamatória tem sido classificada em aguda, sub-aguda ou crônica, dependendo da persistência da lesão e dos seus sintomas clínicos. Inicialmente a reação inflamatória aguda, se caracteriza por ser de curta duração onde ocorre a dilatação arteriolar, aumento da permeabilidade vascular, que são mediados por aminas vasoativas, histamina e serotonina liberados por mastócitos e monócitos. Já na fase sub-aguda, caracterizada pela aderência e migração de leucócitos e células fagocitárias,

favorecidos pela expressão de moléculas no endotélio extravasado pelo exsudato rico em proteínas e água (edema). Este evento celular é nomeado de quimiotaxia e os fatores quimiotáticos são gerados tanto na corrente sangüínea quanto no local da lesão (Ativação do sistema complemento, das cininas e de coagulação e liberação de citocinas pró-inflamatórias (IL-1, TNF-α) (NATHAN, 2002).

Se o endotélio for ativado pela selectinas a adesão dos neutrófilos será fraca, enquanto as integrinas irão promover a adesão forte e as quimiocinas ativam e estimulam a migração dos neutrófilos para o foco inflamatório. Estas interações permitem que os neutrófilos rolem pela parede do vaso e sejam expostos aos fatores quimiotáticos, que irão promover o direcionamento ao local da inflamação (Figura 2) (NATHAN, 2002)..

Figura 2. Mecanismo de migração de leucócitos

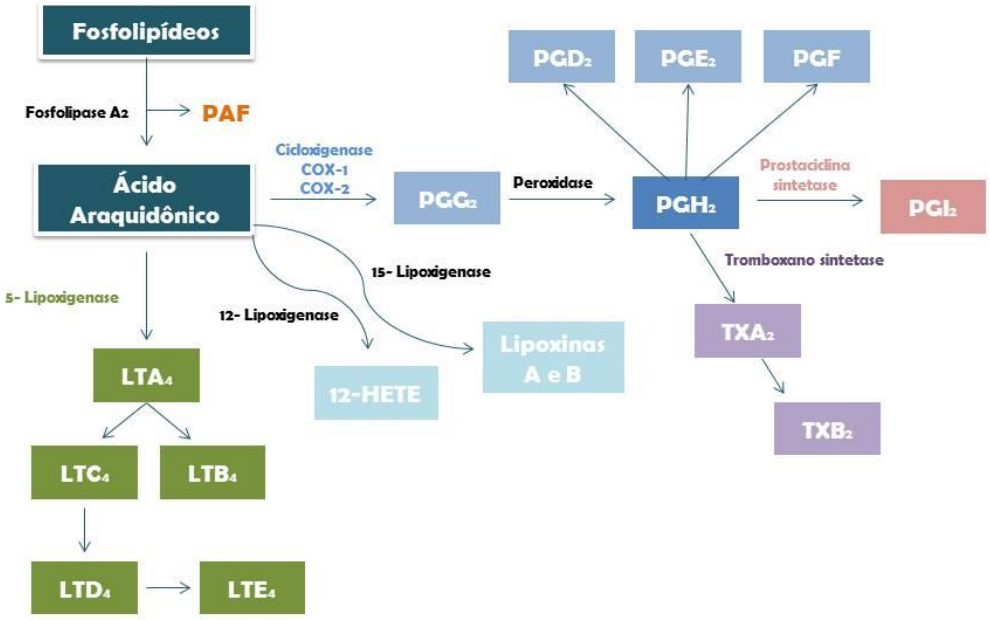


Fonte: CAMPOS, 2015

Simultaneamente a este processo, os mediadores lipídicos, derivados do ácido araquidônico, são produzidos em consequência da ativação de fosfolipases que clivam fosfolipídios constituintes da membrana celular, gerando prostaglandinas, leucotrienos e PAF (fator de ativação plaquetária). Quando a lesão se dá na membrana celular, a enzima fosfolipase A2, presente nos leucócitos e plaquetas, é ativada por citocinas pró-inflamatórias, levando a

degradação dos fosfolipídios, resultando na produção de ácido araquidônico (Figura 3). Este, ao ser metabolizado, dá origem a substâncias com um importante papel na fisiopatologia da inflamação, que podem ser os leucotrienos, pela ação da enzima lipooxigenase, e também forma prostaglandinas, prostaciclinas e tromboxanos, pela ação da enzima ciclooxigenase (HILÁRIO; TERRERI; LEN, 2006). As prostaglandinas produzidas têm funções inflamatórias que induz a febre, a hiperalgesia e a vasodilatação (MESQUITA JR et al, 2008)

Figura 3. Via da síntese Prostaglandinas, Prostaciclinas, Tromboxano A2 e Leucotrienos.



Fonte: CAMPOS, 2015

O processo inflamatório chegará ao fim, quando o agente agressor for eliminado e os mediadores secretados disseminados ou detruídos, dando passagem aos processos de reparo e cicatrização (WALZOG; GAEHTGENS, 2000).

2.3 DIARREIA

A diarreia é um sinal comum de origem gastrointestinal decorrente de vários estímulos caracterizada por um aumento no número de evacuações, que por muitas vezes acompanha também dores abdominais, aumento da fluidez fecal e/ou presença de sangue e muco. Infecções entéricas, alterações na digestão e absorção de alimentos, além de fatores hormonais podem desencadear a presença da diarreia (MATHAN, 1998).

Este sintoma afeta mais de 3 bilhões de pessoas, fazendo com que cerca de 5 milhões de mortes por ano (SHAREEF et al., 2014), por isso é considerado a principal causa de mortalidade nos países desenvolvidos, especialmente quando sua origem é desencadeado por infecções enterotoxina. A Diarreia afeta todas as raças, sexos, idades e regiões geográficas em todo o mundo, estima-se que 3,2% de todas as mortes são devido este processo, desencadeando os 1,5 milhões de mortes em crianças (CHITME et al, 2004; LOPEZ E MATHERS, 2006; YAKUBU E SALIMON, 2015).

O bolo fecal em sua normalidade é excretado até 3 vezes ao dia ou até uma vez a cada três dias que depende basicamente da absorção de água e da intensidade da propulsão intestinal. Os principais mecanismos para alterações fisiológicas nas fezes provocando a diarreia são: secreção excessiva de liquídos e eletrólitos, redução na absorção de liquídos e eletrólitos, aumento na osmolaridade ou distúrbios na motilidade intestinal.

O processo de hipersecreção intestinal é decorrente de ação de enterotoxinas e não ocorre ruptura ou invasão da mucosa intestinal. As enterotoxinas promove a ativação de adenilciclase desencadeando a produção intracelular aumentada de monofosfato de adenosina cíclico (cAMP) resultando na abertura dos canais de cloro nos enterócitos, e no deslocamento do mesmo para a luz intestinal. Com o intuito de preservar a eletroneutralidade ocorre a saída de íons de sódio e de forma passiva se instala fluxo secretório de água por força do gradiente osmótico, consequentemente as fezes se tornam diarréicas aquosas, volumosas, com ausência de sangue ou leucócitos (FIELD E SEMRAD, 1993; CLARKE et al., 1992; MATHAN, 1998).

Na alteração da absorção o mecanismo ocorre devido a invasão e lesão da mucosa intestinal, ocorrendo ruptura da barreira e comprometendo a capacidade de absorção de fluido, eletrólitos e nutrientes no intestino delgado. A presença de nutrientes que foram parcialmente absorvidos no cólon

desencadea a diarreia osmótica. São liberados neste processo ao mesmo tempo células inflamatórias, sangue e soro na luz intestinal além de peptideos que agem sobre a motilidade e secreção entérica (FIELD E SEMRAD, 1993).

Além deste processo, muitos enteropatógenos invasores como: rotavirus, *E coli enteropatogenica, Cryptosporidium parvum, Salmonella sp, Shigella sp e a Entamoeba histolytica*, desencadeam a síntese e liberação de citocinas pela células intestinais epiteliais, como a interleucina 8 (IL-8) que promove a quimioatração de leucócitos polimorfonucleres, intensificando o processo inflamatório e a lesão epitelial através da liberação de espécies reativas de oxigênio e consequentemente evacuações fezes sanguinolentas, em pequenas quantidades, com a presença de cólicas abdominais baixas e urgência intestinal, e eventualmente febre e choque séptico (CRAWFORD, 1996).

Com a presença de substâncias osmoticamente ativas na luz intestinal ocorre a elevação da osmolaridade, incapacitando a reabsorção de água no interior do intestino. Estas substâncias podem ser usadas como fármacos laxantes minerais, e tipicamente podem ser evitadas quando os pacientes se mantem em jejum. Além deste processo, algumas patologias provocam distúrbios na motilidade intestinal, como o hipertiroidismo e alguns tumores neuroendocrinos intestinais (FIELD E SEMRAD, 1993).

A diarreia pode ser classificada de acordo com a duração da mesma, em aguda, persistente ou crônica. Quando de forma aguda apresenta tempo de duração menor que duas semanas. Quando se extende por mais de 14 dias é considerada persistente, e se persistir por mais de um mês de duração, classificada em diarreia crônica (MATHAN, 1998).

Alguns fármacos também são responsáveis por provocar diarreia, tais como: antimicrobianos, antiácidos contendo magnésio, produtos contendo lactose ou sorbitol, análogos de prostaglandinas, colchicina, antineoplásicos, drogas antiarrítimicas e agentes colinérgicos (CHASSANY et al., 2000).

Normalmente dirigido para finalizar um ataque agudo, induzir a remissão, prevenção de recaída e controlar sintomas crônicos o tratamento da diarreia é usado. A abordagem de tratamento é possível para várias opções, como a introdução imediata de terapia de reidratação oral (utilizados com sucesso para gerenciamento deste sintoma em crianças), alimentação

contínua, probióticos e medicamentos como a loperamida ou outros (SHARMA E SHARMA, 2007).

Das diversas abordagens terapêuticas empregadas na redução do quadro diarréico, os medicamentos atualmente usados agem na motilidade intestinal. Dentre eles, os principais aplicados no tratamento são: antibióticos, opiáceos e antagonistas de receptores muscarínicos (RANG et al., 2011). Entretanto, alguns opiáceos, tais como, codeína, difenoxilato e a loperamida podem provocar efeitos adversos como constipação e distensão abdominal, limitano o seu uso (RANG et al., 2011; WANG et al., 2005) .

Deste modo, a fim de superar os sintomas de diarreia, especialmente nos países em desenvolvimento, a Organização Mundial da Saúde (OMS, 2004) incentiva o uso de drogas oriundos de plantas medicinais devido à sua acessibilidade, os conhecimentos adquiridos pelos antepassados e eficácia demonstradas, tais como: *psidium guajava, Jatrpha curcus* L.*, Lantana camara* L., *Xylocarpus granatum, Asparagus pubescens*, dentr outros (GAITEN et al., 2000; MUJUMDAR et al., 2000, NWAFOR et al 2000; SAGAR et al.,2005). Desta forma, ressalva a relevância de pesquisas com abordagens na temática, objetivando o desenvolvimento de novos fármacos mais acessiveis.

2.4 FEBRE

A Termogênese é um processo de produção de energia em forma de calor, e é considerado de extrema relevância para a manutenção da homeostasia do organismo, promovendo a manutenção da estabilidade da temperatura corporal. A baixa da temperatura promove a redução da eficiência das enzimas e da capacidade de difusão, a disponibilidade de energia e o fluxo de íons nas membranas. Enquanto que o aumento da temperatura corporal diminui a viabilidade de instalação de patógenos, despertando uma resposta imunológica (MORRISON, et al., 2008).

A febre é denominada um transtorno da termorregulação normal, ou seja, a elevação controlada da temperatura corporal em resposta a um trauma, lesão ou agente infeccioso, sendo, portanto considerado um dos sintomas de resposta inflamatória aguda. Essa alteração da temperatura interna do organismo é regulada pela ativação de receptores periféricos e centrais que

transmitem a informação para o hipotálamo anterior, que funcionando como um termostato natural ajusta o impulso eferente desencadeando a produção ou a perda de calor (ARNOW E FLAHERTY., 1997).

Na elevação corporal da temperatura decorrente a infecção ou processos inflamatórios, ocorre à produção e liberação de substâncias pirógenas endógenas (ex.: interleucina 1β, IL-6, TNF-α) que atuam na liberação de prostaglandina E2 (principal mediador da febre no encéfalo) aumentando o ponte de ajuste no centro termoregulador no hipotálamo e consequentemente a temperatura se mantém mas elevada (KLUGER, 1991), pois promove ações termoefetoras que desencadeiam inúmeras respostas autônomas e comportamentais, como, piloereção, vasoconstricção cutânea, inibição da sudorese, aumento na produção de calor e a busca por ambientes quentes (BLATTEIS et al., 2004).

Diferente da febre, a hipertermia, aumento passivo da temperatura, é um distúrbio no controle da temperatura, ou seja, ocorre o quando a faixa de temperatura esta acima da normalidade, como exemplos: produção excessiva de calor durante exercício físico intenso, perda da capacidade de dissipação do calor no processo de desidratação (DALE, 1992), e exposições em ambiente com temperaturas elevadas, sem interferir no termostato hipotalâmico (ARNOW E FLAHERTY., 1997). Porém é notado que o processo febril em casos infecciosos é acompanhado de algumas mudanças comportamentais, tais como hiperalgesia, letargia, sonolência, anorexia, diminuição da atividade motora, e outros, o conjunto coordenado destas mudanças também é chamado de *"Sickness behavior"* (BLATEIS, 2003).

Inúmeros estudos relaciona a importância da elevação da temperatura corporal em um processo inflamatório com uma melhor eficácia do sistema imunológico. Neste processo febril sucede um aumento da mobilidade e atividade leucocitária (BLATTEIS, 2003), estimulação da produção e liberação de Interferon – α e ativação dos linfócitos T (KUGLER, 1991). Tais, processos inflamatórios promovem também a liberação de quantidades equilibradas ou desequilibradas de radicais livres, podendo desencadear danos ao organismo. Alguns estudos relatam que o aumento da temperatura corporal a níveis febris podem resultar em convulsões, que acomete comumente crianças (ARONOFF; NEILSON, 2001). A terapia antipirética é necessária em pacientes, pois

promove o alivio do desconforto e a ansiedade promovida pela reação febril, que comumente este efeito está associado ao fato dos fármacos além de serem antipiréticos, são também analgésicos e anti-inflamatórios promovendo a recuperação do individuo após a terapia.

2.5 RADICAIS LIVRES

Os radicais livres são átomos ou moléculas altamente reativos, que apresentam elétrons desemparelhados em sua estrutura (HALLIWELL, 1994), tornando-as muito instáveis, com meia vida reduzida, podendo até promover reação danosa com qualquer molécula que se apresente em contato (HALLIWELL; GUTTERIDGE, 1998). Também conhecidos com Espécies Reativas de Oxigênio (EROs) são encontrados em todos os sistemas biológicos, produzidos do metabolismo celular e liberados durante o processo de redução de oxigênio, sendo relacionados com a produção de energia, fagocitose, regulação do crescimento celular, sinalização intercelular e síntese de substâncias essenciais ao organismo (ANDERSON, 1996; YU, ANDERSON, 1997). Além de sua origem proveniente do metabolismo celular, estas moléculas também podem ser formadas em resposta a diferentes estímulos externos: radiação ionizante, poluição, agentes oxidantes e quimioterápicos (HALLIWELL; GUTTERIDGE, 2007).

Tais moléculas podem atuar de forma benéfica contra agentes infecciosos, formação de ATP, regulação do crescimento celular, e, produção de lipoxigenases e cicloxigenases, quando presente em baixas concentrações. Entretanto, sua produção descontrolada ou o déficit de defesa decorrente de desnutrição podem ser tornar nocivos, e consequentemente promover a oxidação de lipídios de membrana, proteínas, enzimas, carboidratos e DNA, induzindo o desequilíbrio e posteriormente o estresse ou danos oxidativos (HALLIWELL; GUTTERIDGE, 2007). Tal processo, pode contribui para a degeneração de células somáticas e indução de doenças crônico-degenerativas, tais como: câncer, aterosclerose, doenças inflamatórias, Parkison, Alzheimer e catarata (SCALBERT et al., 2005), por isso, o sistema biológico tenta por diferentes mecanismos manter o equilíbrio entre os

promotores da oxidação e os agentes de defesa (Antioxidantes) (SCALBERT et al., 2005).

Os antioxidantes são substâncias de origem natural ou sintética que promovem o retardamento ou inibição da oxidação das moléculas alvo, evitando com isto as reações em cadeias da oxidação. Possuem em sua estrutura química aromática e contém pelo menos uma hidroxila, podendo ser classificados em primários e secundários. Os antioxidantes primários suspendem as reações em cadeia envolvidas na oxidação lipídica, tornando-os mais termodinamicamente estável pela doação de um elétron ou hidrogênio aos radicais livres. Enquanto que os antioxidantes secundários diminuem ou atrasam a taxa de iniciação da oxidação por decompor hidroperóxidos (NACZK; SHAHIDI, 2004).

Algumas enzimas destacam-se com antioxidantes endógenos, tais como: glutationa peroxidase, catalase e superóxido desmutase, prevenindo a formação de radicais (·OH). Além destes também recebem destaque os exógenos, provenientes da dieta como: vitamina C, E, A, carotenóides e flavonóides, como inibidores de ciclooxigenase, alguns cofatores enzimáticos, sequestrantes de espécies reativas de oxigênio, entre outros (NACZK; SHAHIDI, 2004).

Pesquisas toxicológicas com antioxidantes sintéticos (butilhidroxianisol, Butilhidroxitolueno e terq-butilhidroquinona) têm revelado a associação destes com efeitos nocivos no organismo, tais como: doenças pulmonares (HOCMAN, 1988), hiperplasia gastrointestinal, redução de hemoglobinas, hiperplasia de células basais (RAMALHO; JORGE, 2006). Desta forma, diante dos indícios decorrentes do uso elevado de tais compostos, inúmeras pesquisas têm sido propagadas no intuito de se encontrar de forma natural os antioxidantes, para que possam agir de forma menos nociva (DURAN; PADILLA, 1993).

2.6 PRODUTOS NATURAIS COMO FONTES DE NOVOS FÁRMACOS

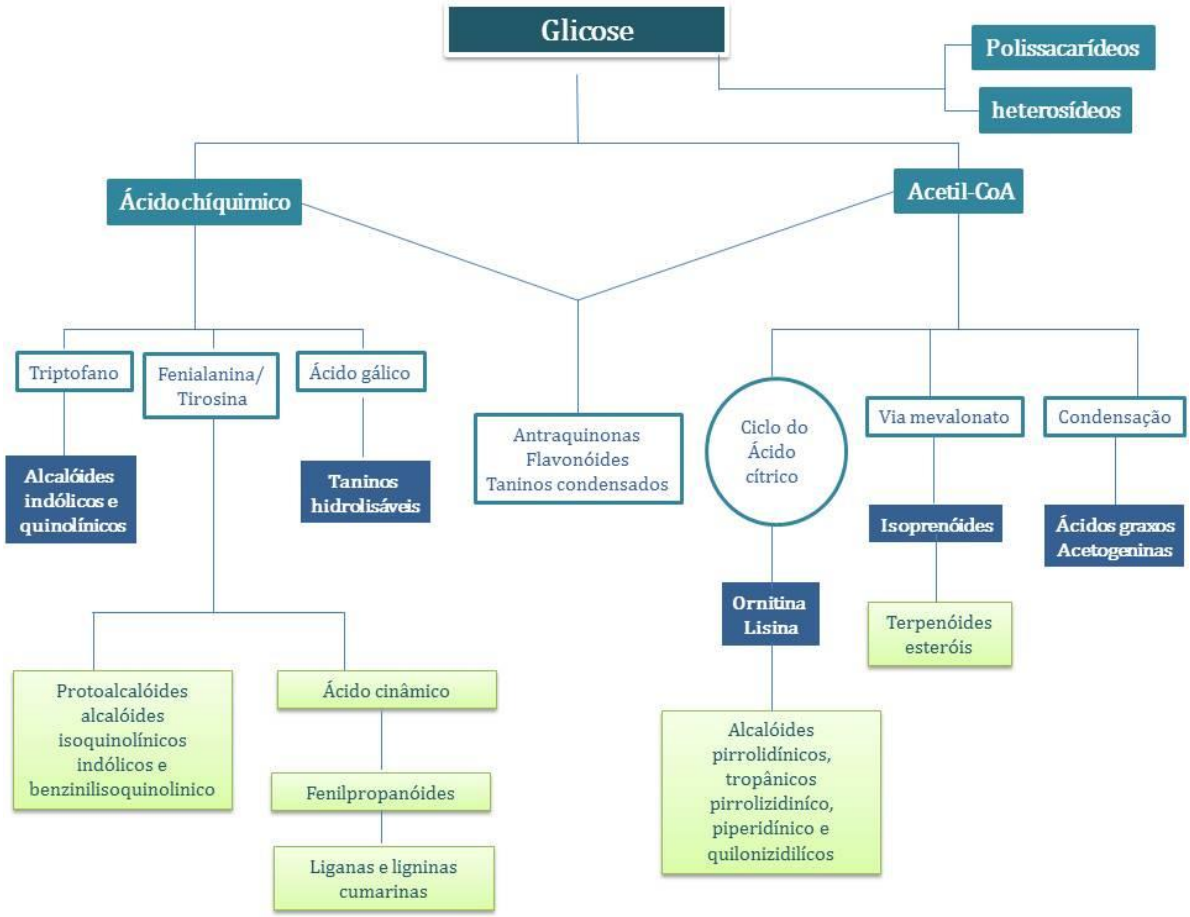
As plantas são usadas como fonte de cura de doenças por diversos povos desde os tempos pré-históricos. Segundo a OMS, 80% da população depende dos fitoterápicos como ferramenta chave para cuidados básicos de saúde (CASTARDO et al., 2008). Cerca de 25% dos medicamentos são oriundos de

plantas e compostos isolados das mesmas (CALIXTO, 2003). No processo cotidiano de sobrevivência da planta, ela responde a diversos estimulos ambientais, de natureza física, química ou biológica, e tal mecanismo é responsável por desencadeiar a biosíntese de tais compostos essenciais para o seu desenvolvimento e sua proteção.

Os metabolitos de origem primária (carboidratos, proteínas, glicerídeos, ácidos nucléicos) estão envolvidos com a formação de proteoplastos e energia, enquanto que, os metabólitos secundários se destacam não só pelas vastas atividades biológicas produzidas em resposta ao meio ambiente, mas também pela sua potencial atividade farmacológica demonstrada (BRAT, 2000). Estes são gerados seletivamente através de diferentes vias biossintéticas (Figura 4), estando relacionados com o mecanismo de evolução das espécies, e portanto define-se que tais compostos sejam ferramentas consideráveis para compreenção das relações filogenéticas entre as diferentes espécies de plantas (BRAT, 2000; WATERMAN, 2007). Tais metabólitos secundários, podem exercer funções de defesa, sinalização e também funções fisiológicas variadas (ex.: regulação do crescimento) (FOLEY; MOORE, 2005; THEIS; LERDAU, 2003).

Muitos destes compostos, tais como, flavanóides, alcalóides, terpenos, entre outros, tem pronunciados efeitos no organismo humano, e são utilizados como agentes medicinais no tratamento de patologias (THEIS; LERDAU, 2003).

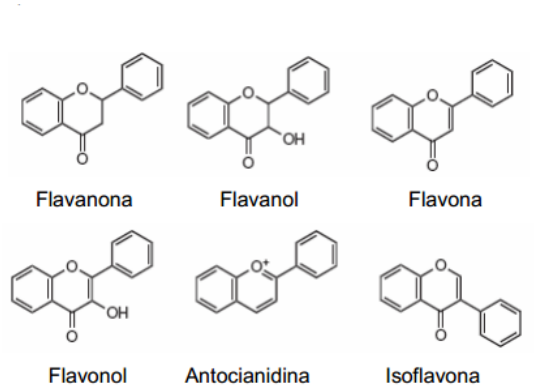
Figura 4. Vias biossintéticas dos metabólitos secundários



Fonte: Campos, 2015

Os flavonóides constituem a maior classe de compostos fenólicos de plantas e são subdivididos em classes de acordo com seu grau de insaturação e oxidação do anel C que inserem os grupos: flavonas, flavonóis, flavanonas, flavonas, isoflavonas (isoflavonóides) e Antocianidinas (RICE-EVANS, 1996; ROBARDS; ANTOLOVIC, 1997; AHERNE; O'BRIEN, 2002).

Figura 5. Estrutura química das principais classes de Flavonóides



Fonte: SHAHIDI; NACZK, 2004

Apresentam um esqueleto básico de dois anéis aromáticos ligados entre si por uma ponte de três carbonos, e podem expor substituições por grupos hidroxil e açúcares (ROBBINS, 2003). Das adições de carboidratos, podemos destacar a glicose, seguido da galactose, ramnose, xilose e arabinose. Este processo permite a solubilização dos flavonódes em água (ROBARDS & ANTOLOVIC, 1997).

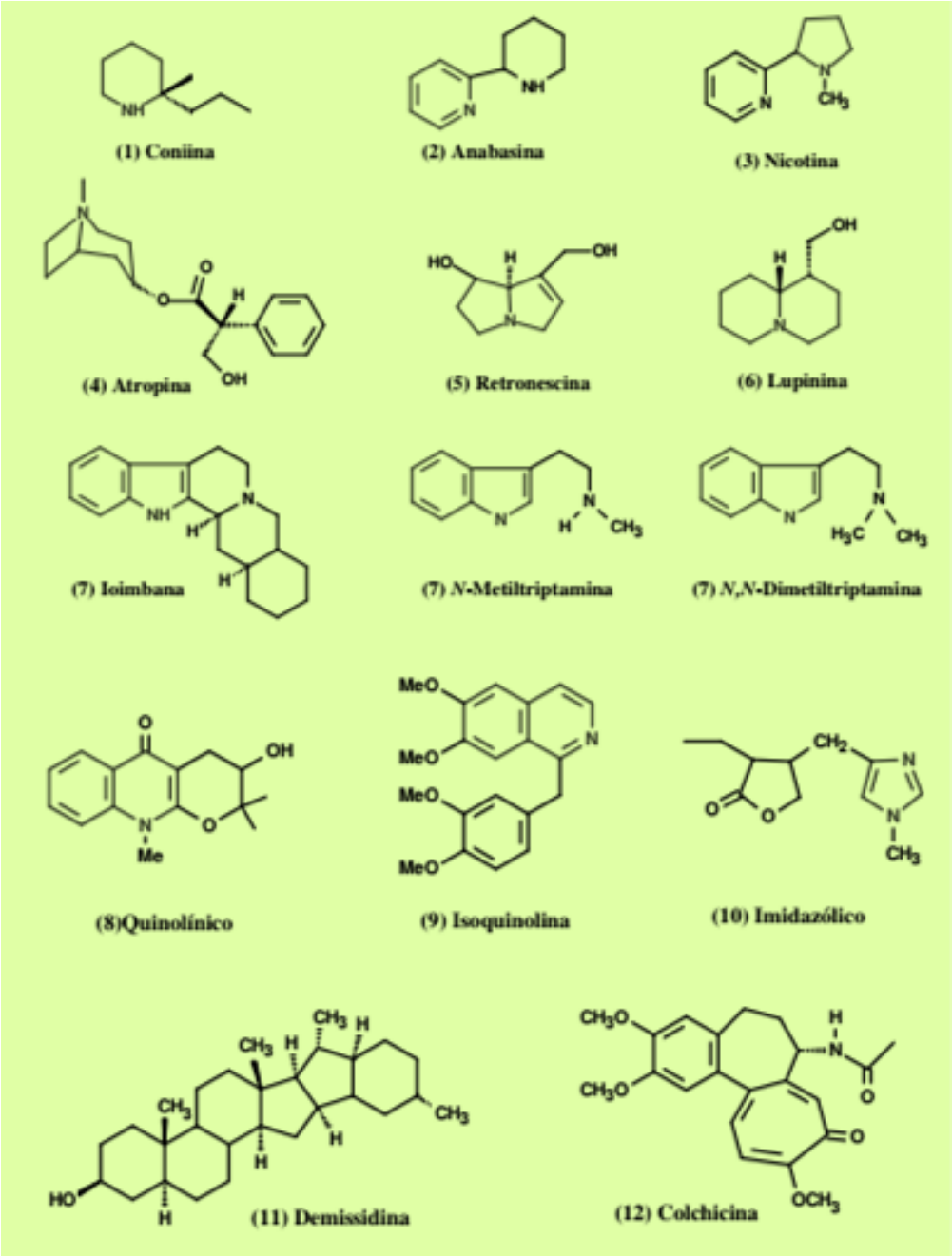
Os flavonóides são considerados antioxidantes naturais potentes, em virtude da capacidade de estabilizar radicais livres e espécies reativas de oxigênio presente, devido aos grupos hidroxila ligados à estrutura do anel aromático. Possuem uma diversidade de importantes funções nos vegetais e diversas atividades biológicas. Nas plantas produtoras de flores e frutos, os flavonóides se destacam por ser fontes de pigmentos para sua coloração. São extremamente relevantes para a proteção da planta contra insetos, fungos, vírus, bactérias, e além de serem também antioxidantes, auxiliam no controle da ação de hormônios vegetais, e são agentes alelopáticos, inibidores de enzimas. São essenciais também para fornecer elementos importantes na proteção dos vegetais contra a incidência de raios ultravioleta e visível (SALISBURY & ROSS, 1991; HARBONE & WILLIAMS, 2002; RIJE et al., 2006).

Esta classe química têm se destacado por apresentar diferentes propriedades benéficas ao metabolismo do nosso organismo, tais como: atividades antialérgicas, antivirais e fungicidas. Outras atividades como

anticâncer, anti-inflamatória e envolvimento na diminuição de risco de doenças cardiovasculares têm sido conferidas às propriedades antioxidantes presentes que se relacionam com a prevenção da peroxidação lipídica da membrana celular e com a proteção de substâncias de importante papel fisiológico, como a vitamina C e a adrenalina (CUSHINE & LAMB, 2005; MAGALHÃES et al., 2007).

Outro grupo químico de grande relevância biológica, são os alcalóides, denominados compostos azotados complexos (nitrogênio amínico) de natureza alcalina, localizados em folhas, sementes, raízes e caules. Estão presentes nos vácuolos das células, porém em forma de sais encontram-se nas paredes celulares e podem apresentar coloração amarela, roxa ou incolor (MARTINS, 1995). Podem ser divididos em vários grupos, segundo a sua composição química e estrutura molecular, como por exemplo, Piperidínicos (1), piridínicos (2), pirrolidínicos (3), tropânico (4), izidínicos (5), quinolizidínicos (6), indólicos (7), quinolínicos (8), isoquinolínicos (9), imidazólicos (10), alcalóides esteroidais (11), alcalóides desprovidos do elemento químico nitrogênio como heteroátomo, contido num ciclo (12) (BREITMAIER; VOELTER, 1990) (Figura 6).

Figura 6. Diferentes tipos de alcalóides nas diversas classes



Fonte: BREITMAIER; VOELTER, 1990

Suas funções internas nas plantas ainda não foram bem esclarecidas, porém acredita-se que eles estajam associados a defesa contra insetos e herbívoros, e reserva para síntese de proteínas. No organismo podem atuar no sistema nervoso central (calmante, sedativo, estimulante, anestésico, analgésicos), podem também ser cancerígenos e outros antitumorais (BREITMAIER; VOELTER, 1990).

Os terpenos também constituem um grande grupo de metabólitos secundários podendo ser chamados também de terpenóides, que são derivados do isopreno (C5). São encontrados em plantas, organismos marinhos, algas, microrganismos e em menor extensão em fungos. As estruturas químicas formadas a partir da união de vários unidades isoprênicas classificam os terpenos em monoterpenos (C10), sesquiterpeno (C15), diterpenos (C20), triterpenos (C30) e tetraterpenos (C40) (DEWICK, 2009). Os terpenos são usados como matéria-pima para diversas indústrias: produtos de resinas para papéis e texteis, aglutinantes usados em inseticidas, antissépticos, produtos farmacêuticos, perfumes e condimentos (DEWICK, 2009).

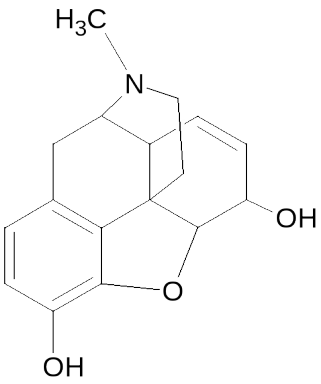
Os mono e sesquiterpenos são os principais componentes dos óleos essenciais e das resinas, as demais classes se apresentam na maioria no estado sólido e podem ser encontrados na forma livre, glicosilados ou como ésteres. Estima-se que tenha cerca de 30.000 terpenos identificados, no qual 4.000 refere-se a triterpenos. Esta classe têm despertado grande interesse devido as suas diversas atividades biológicas, tais como anti-inflamatória, antibacteriana, antifúngica, antiviral, antitumoral, antidiabética, antiulcerogênica, anticariogênica, hepatoprotetora, neuroprotetora, antiparasítica, analgésica e antioxidante (COLOMA et al., 2011).

Outros produtos originados dos metabólitos secundários, são os glucosídeos, compostos por duas partes: um açúcar inativo mas que favorece a sua solubilidade, a sua absorção e o seu transporte para determinado órgão; e um aglícono, que cofere seu efeito terapêutico. Segundo a composição química, pode se diferenciar em diversos grupos: tioglucosídeos, glucosídeos derivados do ácido cianídrico, glucosídeos antraquinônicos, glucosídeos antraquinônicos, cardioglucosídeos e glucosídeos fenólicos (MARTINS, 1995). Vários destes bio-produtos são extraídos de plantas em larga escala para comercialização e muitos deles têm sido utilizados como protótipos para síntese ou semi-síntese de drogas com um perfil farmacológico. Metade dos 25 fármacos de maior utilização mundial são oriundos de metabólitos secundários de vegetais (ALVES, 2005).

Um exemplo marcante de produto natural que impactou a humanidade foi a descoberta do ópio, proveniente dos bulbos de *Papaver somniferum*, conhecida a milhares de anos por apresentar prorpiedades analgésicas e

soporíferas. Utilizada pelos Sumérios desde 4000 A.C., há relatos da utilização da papoula do ópio correlacionando-a com Morfeu o deus do sono (HOSTETTMANN, 2003). Este relato desencadeou em 1803 as primeiras investigações sobre a composição química do ópio, e possibilitou em 1804 o isolamento do composto majoritário, a morfina (Figura 7) (SIMOES et al., 2001). Atualmente, ainda é utilizada na terapêutica segundo recomendação da OMS, para tratamento de dor intensa, especialmente em casos de pacientes com câncer terminal. Sua ação analgésica ocorre por sua interação com receptores específicos μ, δ, e κ, distribuídos no sistema aferente e eferente, que participam da transmissão da sensibilidade dolorosa e modulam a informação nociceptiva.

Figura 7. Estrutura química da morfina

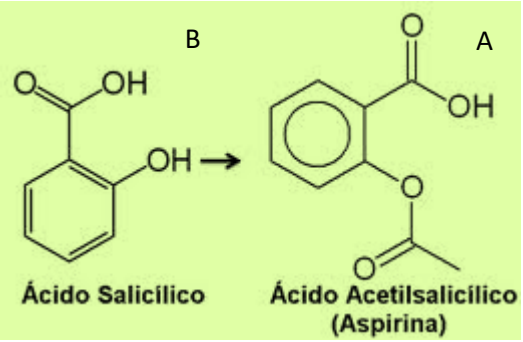


Fonte: http://dc392.4shared.com/doc/qE8uYeGd/preview.html;
Acesso em: 20/11/2014.

Outro marco de grande relevância para o desenvolvimento de fármacos a partir de plantas também pode ter sido o descobrimento dos salicilatos obtidos por *Salix alba*. Edward stone (1763) descobriu as propriedades antipirética e analgésica de extratos desta planta. Uma rivalidade entre Alemanha e França em 1828 foi criada no intuito de descobrir o principio ativo da *Salix alba* responsável por essas tais propriedades, mas só em 1860, Hermann Kolbe e seus alunos síntetizaram o Ácido salicílico e o seu sal sódico a partir do fenol. Porém, só em 1898 que Felix Hofmann em busca da cura da artrite que acometia seu pai e que sofria com os efetos colaterais do salicilato de sódio, descobriu o Ácido Acetil Salicílico (Figura 8 A) o qual era menos ácido que o

Ácido salicílio (Figura 8 B) e apresentava o mesmo efeito analgésico por atuar acetilando irreversivelmente a cicloxigenage (GANGREIRO et al., 2008).

Figura 8. Estrutura química do Ácido salicílico e Ácido Acetil salicílico



Fonte: http://www.brasilescola.com/quimica/Acido-acetilsalicilico-aas.htm
Acesso em: 20/11/2014.

Diversos fármacos poderiam ser ressaltados neste contexto, que utilizaram dos produtos naturais como fonte inicial, como por exemplo: o alcalóide com funções anti-térmicas, anti-maláricas e analgésicas, quinino (casca da *Chinchona* sp); o glicosídeo digoxina (*Digitalis* sp.) utilizado no tratamentos de problemas cardíacos; os alcalóides anti-cancerígenos vincristina e a vinblastina (*Catharanthus roseus*), dentre outros (RATES, 2001). Novas substâncias com fins terapêuticos podem ser desenvolvidas por vários processos, tais como: sínteses de novas moléculas, modificação molecular de substâncias naturais, como também extração, isolamento e purificação de compostos provenientes de plantas, o qual possui quantidades inesgotáveis de compostos ativos a ser usado como fármacos (BRITO et al., 2003).

Estas buscas vem reassumindo um papel importante na ciência, ampliando não só o uso com plantas que já apresentam relatos populacionais terapêuticos, mas também aquelas que possam constituir matéria prima ou fornecer intermediários para a fabricação de novos medicamentos sintéticos (FERREIRA et al., 1998; SIXEL & PECINALLI, 2002). Apesar de existir inúmeras publicações sobre o uso popular e farmacêutico de plantas, ainda é escasso os relatos sobre as propriedades farmacológicas e identificação de substâncias bioativas.

O Brasil é considerado um dos países mais ricos do mundo em biodiversidade, ocupando o primeiro lugar dentre os 17 selecionados (China, Índia, França, Alemanha, entre outros.)(RATES, 2001), o que revela a sua atração pelas indústrias famacêuticas para a produção de fitoterápicos. Dentro dessa biodiversidade, encontra-se a família Fabaceae, considerada a terceira maior família de plantas que possui cerca de 19.500 espécies (OLIVEIRA; PAIVA, 2005), é subdividida em três subfamílias: Mimosoideae, Caesalpinioideae e Papilionoideae e mostra uma característica comum em quase todas, de apresentar frutos semelhantes a legumes, conhecidos como vagens (RIBEIRO et al., 1999; DUTRA et al., 2005).

A família Fabaceae é de grande importância, pois apresenta vasta variedade de espécies alimentícias, além de ser usada como ração para animais, látex, resinas, matéria-prima na fabricação de tintas, inseticidas, fitoterápicos (*Dioclea megacarpa, Vatairea paraensis e Dipteryx puncata*) e árvores ornamentais. Alguns exemplos das espécies empregadas como fontes alimentícias são: o grão-de-bico (*Cicer arietnum*), ervilha (*Pisum sativum*), feijão (*Phaseolus vulgaris*), lentilha (*Lens cultivaris*) e a soja (*Glycine max*) (RIBEIRO et al., 1999).

2. 5 *Indigofera suffruticosa* Mill.

O gênero *Indigofera* pertencente a familia Fabaceae e se destaca por ser usado como forrageira (SHERMAN, 1982), adubo verde e cobertura de solo (FROMAN, 1975). Esta planta é conhecida na população vulgarmente por “anil”, “anileira”, ou “índigo”, cujo nome provém do alemão, devido a produção de um pigmento azul extraído, que é obtido por infusão quente a partir da fermentação de suas folhas, e utilizados comumente para tingimento de fios (PESAVENTO, 2005). Até o início do nosso século as indústrias utilizavam este pigmento como fonte de coloração, até ser substituído pela anilina sintética. Há também relatos que esse pigmento foi bastante utilizado como tintas em rituais, nos templos Maias, Toltecas e Olmecas, e como matéria-prima para desenvolvimento do corante conhecido pelo nome “azul de maia” (MATADAMAS-ORTIZ, 2002).

Este gênero possui aproximadamente 700 espécies distribuídas na Ásia, África Tropical, Austrália, América do Norte e Sul. No Brasil é possível encontrar três espécies: *Indigofera truxillensis*, *I. hirsuta* e *I. suffruticosa*. São consideradas plantas silvestres que crescem em todos os tipos de solos, tolerando secas, inundações e elevadas salinidades (PESAVENTO, 2005).

A *Indigofera suffruticosa* Mill. (Figura 10) é uma espécie originária da Antilha e América Central (ALMEIDA, 1993) mais predominante por toda a América Tropical. No Brasil, encontra-se distribuída em alguns estados: Mato Grosso (FERNANDES, 1987), Alagoas (RIBEIRO, 1984), Paraíba (RIET-CORREA, 2000), Ceará, Rio Grande do Norte, Pará e Pernambuco (NETO et al., 2001). É descrita como uma planta arbustiva, medindo entre 1 - 2 m de altura, possuindo ramos pubescentes, caule anguloso, de cor acizentada, folhas pinadas compostas por 7 a 15 folíolos oblongos ou ovais, glabros na face e no verso, apresentando flores pequenas, numerosas, albo-roseas ou amareladas, em racemos axilares, e seu fruto é uma pequena vagem falciforme com 6 a 10 sementes medindo 25 mm de comprimento (BRAGA, 1976).

Figura 10. Vagens de *Indigofera suffruticosa*

Fonte: http://blog.163.com/gzxmq@126/blog/static/114514038201201585751588
Acesso: 10/11/2014

A *I. suffruticosa* pode ser relacionada a outros nomes populares, tais como, jiquilite, tzitzupu, anil do campos, anileira-da-índia, anileira verdadeira, caá-chica, caá-chira, timbó-mrim, timbozinho, e indigueira. As primeiras investigações dos componentes químicos de *I. suffruticosa* foram realizados

por Miller e Smith, 1973, utilizando extrato de sementes. O isolamento de esteres de glicose de ácido 3-nitropropanóico desta espécie é destacado por possuir efeitos tóxicos, devido a sua conversão para o ácido 3-nitropropanóico, uma toxina respiratória que inibe enzimas mitocôndriais. Além deste isolado, Kamal e Mangla (1993), identificaram, caracterizaram e quantificaram seis rotenóides de diferentes partes de *I. suffruticosa*, com atividade biológica eficaz contra larvas de *Anopheles* e *Callosobruchus chinensis* adultos. Estudos preliminares de folhas, sementes e caules de *I. suffruticosa* demostram a presença de polifenóis e flavonóides, alcalóides, triterpenóides, e carboidratos (LEITE et al, 2003).

Esta espécie apresenta diversas propriedades farmacológicas: antiespasmódicas, sedativas, diuréticas, purgativas, odontálgicas (LORENZI, 1982; BRAGA, 1985). Estudos cientificos realizados com esta espécie mostram também as atividades anticonvulsivante (ALEJO et al, 1996; ALMEIDA et al., 2013), antigenotóxica (BADELL, et al 1998) e antiepiléptica (ROIG & MESA, 1974).

Estudos farmacológicos da *I. suffruticosa*, destacam as aplicações clinicas utilizando partes aéreas e de folhas com as seguintes atividades: atividade citotóxica (LEITE et al., 2004, LOPES et al., 2011) , gastroprotetora (LUIZ-FERREIRA et al., 2011) antimicrobiana (LEITE et al., 2006, CARLI et al., 2010, SANTOS et al., 2015[a], SANTOS et al., 2015[b]), antifúngica (LEITE et al., 2006), anti- tumoral (VIEIRA et al., 2007), e anti-inflamatória (LEITE et al., 2003; CHEN et al, 2013).

Ainda são escassos estudos químicos e biológicos com as vagens de *I. suffruticosa*, o que evidência a importância de se intensificar tais pesquisas para auxiliar na terapia medicamentosa popular, visto que, sua utilização e as suas propriedades farmacológicas em relação a outras partes da planta verificadas são extremamente destacadas. Desta forma, o presente estudo investigou as suas atividades biológicas, usando diversos modelos experimentais *in vivo* e *in vitro*.

4 ARTIGOS

4.1 Artigo**:** Anti-inflammatory, Antinociceptive and Antioxidant Activity of Organic Extracts of *Indigofera suffruticosa* pods.

Artigo a ser submetido ao periódico *Journal of Ethnopharmacology* no formato *Original Research Article* (**FI:** 2.998; **QUALIS CB II:** B1).

Anti-inflammatory, Antinociceptive and Antioxidant Activity of Organic Extracts of *Indigofera suffruticosa* pods.

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ABSTRACT

Ethnopharmacological relevance: Indigofera suffruticosa is a plant commonly used on traditional medicine to treat infections, throes, inflammation, and other diseases. But is scarce scientific studies using the pods of this species for such biological activities.

Aim of the study: The present study observed novel antioxidant, anti-inflammatory and antinociceptive action of the organic extracts obtained from pods of *I. suffruticosa* in different models.

Materials and methods: The anti-inflammatory activity was evaluated by paw edema and peritonitis assays (carrageenan 1%) while the antinociceptive activity was evaluated by the classical writhing (acetic acid 0.8%) and hot plate test (55 ± 1.0ºC). The antioxidant activity of *I. suffruticosa* extracts was evaluated using a 2,2-diphenyl-β-picrylhydrazyl (DPPH) assay. Acute toxicity study was performed according OECD guuidelines (maximum dose of 1000 mg/kg).

Results: Acetone and methanol extracts of *I. suffruticosa* (400 mg/kg, i.p.) have produced significant inhibition of paw edema inflammatory (60 and 59%, respectively). On the other hand, chloroform, acetone and methanol extracts (400mg/kg, i.p.) have produced notable inhibition of inflammation on peritonitis test (59, 46 and 49%, respectively). Interestingly, all organic extracts (ether, chloroform, acetone and methanol, 400mg/kg, i.p.) have shown significant inhibition of nociception in writhing test (85.3, 88.7, 97 and 100 %, respectively). Chloroform, acetone and methanol extracts showed a significant reduction in the latency time (seconds) ($p<0.0001$). In addition, the acetone and methanol extracts presented striking antioxidant activity (65 and 68%, respectively).

Conclusions: All results presented indicate that *I. suffruticosa* have not only remarkable anti-inflammatory properties but also potential antinociceptive and antioxidant properties.

Keywords: *Indigofera suffruticosa, inflammation, pain.*

1. Introduction

One of the main clinical symptoms used in the detection and evaluation of ilness, pain is a feeling of supreme importance for survival because it acts as a defense mechanism to keep the body's integrity (Le Bars, Gozariu & Cadden, 2001). Persistent pain is usually associated with an inflammatory process, as it is a consequence of site-specific inflammatory mediators activity(Mendell e Sahenk, 2003). These molecules are primarily produced by local immune cells in response to a harmful agent in order to enable a proper environment to rapid injury cessation. Such modifications are characterized by increased vascular permeability, recruitment of leukocytes and the release of chemical mediators (Schmid-Schonbein, 2006). Classical Analgesic and anti-inflammatory therapies are often inadequate because of their limited efficacy and the presence of adverse side effects, such as gastrointestinal and cardiovascular disorders (Carvalho, Carvalho & Rios-Santos, 2004). New therapeutic options have emerged from plants and plant-derived compounds with the help of drug therapy aimed at making them more efficient and causing less damage to the body (Olonode et al., 2015).

Indigofera suffruticosa, Fabaceae family, is known as "*indigo*" or "*anileira*" because of the blue pigment commonly extracted for cloth dyeing. It has increasing use among countryside populations of Brazilian Northeast region as an herbal medicine with antispasmodic, sedative and diuretic properties (Santos et al., 2015a; Santos et al., 2015b). *I. suffruticosa* is a species originated from Antilla and Central America described as a shrub plant, measuring 1-2 m tall. It also has other popular names such as *jiquilite, tzitzupu, anil do campos, anileira-da-índia, anileira verdadeira* (Almeida, 1993).

Previous Pharmacological studies with *I. suffruticosa* highlighted the clinical applications with the following activities: cytotoxic to embryonic cells in mice (Leite et al, 2004), antimicrobial (Leite et al, 2006; Carli et al, 2010, Santos et al, 2015a; Santos et al. , 2015b), antifungal (Leite et al, 2006), anticonvulsant (Almeida et al, 2013), gastroprotective (Luiz-Ferreira et al., 2011), cytotoxic activity in tumor cell lines of murine (Lopes et al , 2011), anti-tumor in mice

(Vieira et al., 2007) and inflammation (Leite et al., 2003; Chen et al, 2013).

Few studies have been conducted with the pods of the species *I. suffruticosa*, so popular due their use and their pharmacological properties compared to other parts of the plant verified, the present study investigated their biological activities using inflammation and nociception models *in vivo* and *in vitro* antioxidant activity.

2. Materials and methods

2.1 Plant materials

The pods of *I. suffruticosa* were collected in the municipality of *São Caetano*, within semi-arid region of Pernambuco State, Brazil. Plant samples were identified and authenticated by the Biologist Marlene Barbosa from the Botany Department, Universidade Federal de Pernambuco (UFPE), where a voucher specimen was deposited at the Herbarium at the UFP Geraldo Mariz Herbarium-UFPE (Identification number 45. 217).

2.2 Preparations of plant extracts

The extracts were prepared with the dried pods (with standard greenhouse conditions) finely pulverized (100g) and extracted 3 x 200 mL with increasing polarity solvents continuously (ether, chloroform, acetone and methanol), homogenized for two hours in a mechanical stirrer, kept under refrigeration (4ºC) overnight, and filtered with Whatman filter paper (Nº 1). The solvents were removed by rotary evaporation (BUCHLER INSTRUMENTS, Fort Lee, NJ, USA).

2.3 Drugs administration

All extracts (ether, chloroform, acetone and methanol) were dissolved in isopropyleneglycol (IPG). The vehicle (IPG) alone served as negative control.

Acetyl salicylic acid (ASA - 100 mg/kg) served as positive control and was also dissolved with IPG. ASA, IPG, λ - carrageenan were purchased from Sigma Chemicals Co. (St. Louis, USA) and acetic acid from Merck (Damstadt, Germany). All solvents used for preparation of the extracts were purchased from Vetec (Rio de Janeiro, Brazil).

2.4 Animals

Male Swiss Albino mice (25 - 30g) were divided into groups of six animals each and housed in cages with free access to food (Labina) and water. The animal facility has controlled temperature (20-15ºC) and light/dark cycles of 12 hours each. . Female mice were used exceptionally for the acute toxicity assay. They were kept at the same facility and conditions as the male mice used for the other experiments. All experimental procedures were approved by the animal ethics committee (Case number 0144113/2007-78). Studies of all extracts of *I. suffruticosa* in doses of 100, 200 e 400 mg/kg were tested in animals for paw edema.

2.5 Preliminary Phytochemical Screening

A simple qualitative and semi quantitative phytochemical analysis was performed by screening tests according to Wagner et al. (1984), Markham (1982) and Sharma and Bakhashi (1991). The phytochemical profile of *I. suffruticosa* extracts was evaluated by thin layer chromatography (TLC) opposite the mobile phase solvents containing different proportions and different polarities, and revealing, using as stationary phase, pre-activated silica GF 254 plates GEK (Merck). For identification and differentiation of the disclosed compounds the following parameters were used: staining band and luminescence in UV lamps.

2.6 Anti-inflammatory Activity

2.6.1 Carrageenan-induced paw edema

The paw edema was induced with a subplantar injection of 1% carrageenan (0.1 ml) in saline half hour before the administration of the extracts (n=6, each) (Winter et al., 1962). Dose of 400 mg/kg (i.p) of organic extracts of *I. suffruticosa* (ether, chloroform, acetone and methanol) was chosen because it has best results biologic. The volume of the paw was measured using a caliper ruler (Kanon- Staineless Mardened), before the injection (time 0) and after 1, 2, 3, and 4 hours immediately after the subplantar injection of carrageenan. For the positive control group (n= 6) , animals received ASA in a dose of 100 mg/kg. Animals of the negative control group (n=6) received only the vehicle (IPG). The percentage inhibition was calculated using the formula given below, that represents the time of edema peak (3h): Percentage inhibition (%) = [(Vf-Vi) Control group mean – (Vf-Vi) Test group mean / (Vf-Vi) Control group mean] x 100, where Vf and Vi represent initial and final paw volume.

2.6.2 Carrageenan-induced peritonitis

Peritonitis was conducted as described by Foster et al. (1986). Mice were pre-treated with vehicle (Negative Control – IPG, i.p., n=6), ASA (Positive Control, 100 mg/kg, i.p., n=6), and the different organic extracts of *I. suffruticosa* (400 mg/kg, i.p., n=6, each). After 1 hour, the animals received an injection of 1% carrageenan (i.p.).

Mice were kept for 4 h and then euthanized. Right after that, saline containing EDTA (1mM, i.p.) was injected, a brief massage was performed for further fluid homogenization, and the peritoneal fluid was collected for leukocyte infiltration (mainly neutrophils) quantification in a Cell Counter (ABX MICROS 60). The results were expressed as the number of Leukocytes x $10^3/mm^3$ of peritoneal fluid. The percentage of the leukocyte inhibition (%) = (1 – T/C) x 100, where T represents treated groups and C represents control group leukocyte counts.

2.7 Antinociceptive Activity

2.7.1 Acetic acid-induced abdominal writhing

The abdominal writhing assay was based on the contraction of the abdominal muscle followed by a stretching of the hind limbs, induced by a nociceptive agent (0.8% Acetic acid, i.p.) (Koster et al., 1959). The animals received 400 mg/kg of organic extracts of *I. suffruticosa* (test group, n=6, each), ASA (100 mg/kg, positive control group, n=6) or vehicle (negative control group, n=6) 1 hour before administration of acetic acid. Then, the number of writhing reflexes was recorded during the following 20 min. The percentage inhibition of the writhing response was calculated from the formula: % inhibition = (*D*0 – *D*t) / *D*0×100 where *D*0 was the average writhing response of the control group while *D*t was the average writhing response in the treated mice.

2.7.2 Hot plate test

The central analgesic activity of *I. suffruticosa* against thermal stimuli was studied in male mice using the hot plate test (MacDonald et al., 1946). The control group (n=6) was treated with vehicle, the test group (n = 6 each) with 400 mg/kg (i.p.) of different organic extracts of *I. suffruticosa*, and the positive control group was treated with ASA (100mg/kg, i.p., n=6). All of them 1 hour before performing the experiments. Mice were individually placed in a hot plate heated at a temperature of 55 ± 1.0 ° C, and the reaction time was marked using a timer. Measurements were performed at time 0, 30, 60, 90, and 120 min after the first thermal stimulus. The maximum stay of the animal was 60s to avoid extensive burn.

2.8 Acute Toxicity studies

The acute toxicity assay was performed as described by the Organization for Economic Co-operation and Development Guidelines (OECD, 2004). Swiss

Albino mice of either sex were used. The animals were fasted for 4 h. The maximum dose of *I. suffruticosa* extracts used was 1000 mg/kg through the intraperitoneal route of administration. The mice were observed continuously for behavioral changes for the first 4 h and then observed for mortality, if any, 24 h after drug administration.

2.9 Determination of antioxidant activity by the DPPH radical scavenging method

Different concentrations (50, 100, 200 and 500 µg/mL) of extracts (100 µL) were mixed with a methanol solution (100 µL) of DPPH radicals (150 µM) in duplicate using a 96-well plate. The mixture was kept in dakr for 30 min. Sample mixtures were compared with known concentrations of α-tocopherol (50, 100, 200, 500 µg/mL). DPPH radical reduction was determined by measuring the absorption at 495 nm using a ELX 800 Universal Microplate Reader (Biotek instruments, Inc., Winooski, USA). The radical scavenging activity was calculated as a percentage of DPPH discoloration using the following equation: % AA = [*(Adpph - As*) / *Adpph*] ×100, where *As* was the sample absorbance, and *Adpph* was the DPPH solution absorbance.

3.0 Statistical analysis

The results of activities are presented as the mean ± standard deviation (S.D.). Statistical significance was determined by analysis of variance (ANOVA) followed by Bonferroni's test and Tukey test, with $p<0.05$ considered significant.

3. Results

3.1 Preliminary Phytochemical Screening

The phytochemical analysis of the different extracts of *I. suffruticosa* in TLC showed the presence of flavonoids, alkaloids, phenylpropanoglycosides in all four extracts (Table 1).

3.2 The anti-inflammatory profiles of I. suffruticosa

3.2.1 Oedema paw

The injection of carrageenan in the sub-plantar tissue of the right hind paw of mice in negative control group caused a swelling that ranged from 5.27 to 4.48 mm thick. The positive control group which received a dose of 100 mg/kg of ASA inhibited the paw edema by 60.3% (Table 2). All extracts (400 mg/kg) showed an inhibition of the inflammation 35.2, 27.0, 60.1, 58.7%, for ether, chloroform, acetone and methanol, respectively (Table 2). However, significant differences were observed only in the groups treated with acetone and methanol extracts at all times evaluated (1 to 4 hours) in comparison with the negative control group. The anti-inflammatory effect observed by treatment of acetone and methanol extracts was similar to ASA, but not significantly different ($p > 0.05$) (Table 2).

3.2.2 Carrageenan-induced peritonitis

The results presented in Figure 1 show that the carrageenan in the peritoneal cavity resulted in the migration of leukocytes as shown in the control group after 4 hours. The treatment with ether, chloroform, acetone and methanol extracts from *I. suffruticosa* significantly reduced migration of leukocytes in 23, 59, 46, 49% respectively. It was also possible to verify reduced migration of leukocytes by 60% for the group treated with ASA compared with negative control, $p < 0.0001$. The treatment with chloroform extract present similar effect to ASA.

3.3 The anti-nociceptive profiles of I. suffruticosa

3.3.1 Acetic Acid-induced writing in mice

Figure 2 shows nociceptive effect using the writhing model with intraperitoneal injection of 0.8 % acetic acid. The treatment with all extracts of *I. suffruticosa* reduced significantly the numbers of abdominal contortion in the mice with inhibition of 85.3, 88.7, 97 and 100%, respectively. Thereby, the organic extracts of the *I. suffruticosa* were potent in inhibition of nociception when compared with the control. The treatment with ASA (100mg/kg) presented the significant percentage of inhibition 86.5 %.

3.3.2 Hot-plate test

The anti-nociceptive profile of *I. suffruticosa* assessed using the hot-plate test is shown in Figure 3. Chloroform, acetone and methanol extracts of *I. suffruticosa* showed a significant reduction in the latency time (seconds) in comparison with the control group. Acetonic extract showed the best anti-nociceptive effect in all times evaluated ($p<0.0001$). Chloroform and methanol extracts present antinociceptive effect in 0.5h-1h and 1h-2h, respectively. The ether extract no showed significant difference in all times evaluated. The treatment with ASA (100 mg/kg) presented significant reduction in the latency time in comparison with the control group in all times.

3.4 Acute Toxicity studies

Employed as medicinal plant, *I. suffruticosa* were evaluated *in vivo* models signs of acute toxicity using extracts of *I. suffruticosa* (maximum dose 1000 mg/kg) administered by intraperitoneal injection. It was possible to observe any mortality in the animals tested, however were some symptom observed included minor noticeable pilo-erection during the assessment period.

3.5 Determination of antioxidant activity by the DPPH radical scavenging method

At 50 - 500 ug/ml, *I. suffruticosa* exhibited scavenging activity similar to that α- tocopherol but when the standard was better compared with all extracts. Best

antioxidant activity was presented for the acetone extract (percentage variation from 61.6 to 68 %) followed of methanol extract (percentage variation from 60.8 to 65.4 %), but did not present significant statistic among themselves. In addition noted antioxidant activity reduced in the chloroform extracts (percentage variation from 62.7 to 47.3 %) and ether extract (percentage variation from 59.8 to 55.2 %).

4. **Discussion**

In recent years, there have been ardent interest in plants of traditional society with the consumption of species with therapeutic indications and unknown plants that has scientifically recognized biological actions. Considering that the use of commercially analgesic and anti-inflammatory drugs exert a wide range of side effects (Vane & Botting, 1990), there is currently a strong interest in developing new therapeutic agents from plants (Iwalewa et al., 2007). The present study reported here demonstrated that *I. suffruticosa*, had significant anti-inflammatory and anti-anociceptive effects when assessed in models of chemical inflammation and nociception in mice.

We report here that the acetone and methanol extracts, but not the ether and chloroform extracts obtained from *I. suffruticosa* reduced significantly the paw edema induced by carregeenan. The carrageenam-induced inflammatory response was described in 1969 in the models of mice's paw (Levy, 1969). Since then, for the development new anti-inflammatory drug, this test has been more than used. We note that the acetone extracts and methanol are extremely rich in various types of compounds, the main classes involved in the anti-inflammatory process of these constituents are flavonoids and alkaloids (Barbosa-filho et al., 2006, Serafini et al., 2011).

Flavonoids have actions involving the regulation of inflammatory cells; inhibiting nitric oxide synthesis, metabolism and modulation of arachidonic acid by inhibition of phospholipase A2, modulation of gene expression and production of pro-inflammatory molecules. The alkaloids contribute to the inhibition of inflammation by removal of inflammatory mediators, such as histamine, cytokines (IL-1) and platelet activating factor (PAF) (Seow et al., 1989). Evaluating the effect of both extracts used are present from the onset of

inflammation lasting 4 hours, we suggest that their action mechanisms are involved in the association of such compounds, regulating the release of chemical mediators secreted in the initial phase of inflammation by cell inflammatory. A similar effect was observed in study of methanol extracts of *I. oblongifolia* at a dose of 500mg/kg, which decreased approximately 40% of paw edema in rats (Upwar et al., 2011). Kumal et al. (2013) also observed an approximate result using methanol extracts of *I. cassioides* at concentrations of 200 and 400 mg / kg in rats in the same animal model. These results are suggested due to the involvement of secondary metabolites present in this species to the inhibition of early mediators of inflammation, confirming our contention that the various compounds present in the Fabaceae family, where it is included *I. oblongifolia* have anti-inflammatory action.

Cell recruitment during inflammation depends on the release of local mediators which is responsible for tissue changes as well as for the recruitment of host defense cells. These mediators are able to recruit leukocytes in the inflammation induced with carrageenan, such as neutrophilis. The treatment with chloroform, acetone and methanol extracts of *I. suffruticosa* inhibited leukocyte migration induced by carrageenan (64, 46 and 49%, respectively) ($p<0.0001$). The anti-inflammatory activity should be associated with the presence of different secondary compounds that can act together in the edematous phase, with the release of mediators and influencing leukocyte migration (Nijveldt et al., 2001), or just leukocyte migration as the case of chloroform extract (64% inhibition). This can be triggered by highlighting constituent of action in adhesion and diapedesis of leukocytes by inhibiting the adhesion proteins such as integrin's and selectins. A similar result was presented in another study with a plant, *Piptadenia stipulacea* belonging to the same family of *I. suffruticosa* (Fabaceae), promoting a 36 % reduction in the leukocyte migration (Queiroz et al., 2010).

We mention here that all extracts (ether, chloroform, acetonic and methanol) of *I. suffruticosa* produced an significant antinociceptive effect in models nociception in mice. Moreover, the methanolic extract showed more potent in inhibiting (100%) the acetic acid–induced nociceptive response. The acetic acid-induced has been used to confirm the peripheral anti-nociceptive

activity and is considered non-specific (Chan et al., 1995), because this model test reflexes the direct interaction of the compounds with the various peripheral receptors within the peritoneal cavity (Bentley et al., 1983). The method involves the liberation of mediators, such as histamine, serotonin, cytokines and eicosanoids with an increase in peritoneal fluid that which evokes an inflammation and directly stimulate the nociceptive neurons (Zhang et al., 2005). This analgesic effect of the *I. suffruticosa* could be attributed, at least in part, to its anti-inflammatory effect as, in the pain model, the processor releases arachidonic acid via cyclooxygenase and prostaglandin. Santos et al. (2013) also obtained similar results to ours with inhibition of nociception of 82.3% at the dose of 400 mg/kg of aqueous extract of *Anadenanthera colubrina.* The action submitted by our extracts were better than those presented by Kumal et al. (2013) who used two concentrations (200 and 400 mg/kg) of methanol extract of *I. cassioides* (36.75 and 52.68% inhibition of nociception, respectively). The analgesic effect of the extract *I. cassioides* is also assigned in favor of the anti-inflammatory effect presented, emphasizing our results.

Other test such as the hot-plate test, it's a central pain model, particularly the strong sensitivity to pain and limited tissue damage (Deraedt et al., 1980). The effect exhibited by all *I. suffruticosa* extracts may be due to the synergism of secondary compounds that can acton several mechanisms. This anti-nociceptive effect it has been reported with other study of the *Sutherlandia frutescens*, also Fabaceae family, using aqueous extracts at doses of 5-800 mg/kg in mice (Ojewole, 2004). Nguelefack et al (2010) showed that some plant-derivated extract containing flavonoids and alkaloids may be involved with the opening of potassium channels, therefore hyperpolarization of the cell membrane or inhibition system adenylate cyclase decreasing the production of cyclic adenosine monophosphate (cAMP) which results in the inhibition of voltage gated calcium channels, triggering the reduction of the Ca influx into nerve terminals and inhibition of neurotransmitter and thereby inhibiting the transmission of nerve impulse and the afferent nociceptive. For this reason, the antinociceptive activity presented in our results can be related with the presence of flavonoids and alkaloids in organic extracts of *I. suffruticosa*, at least in part, not excluding the possibility interaction with other constituents present in the

extract.

In addition, we also evaluated the acute oral toxicity of all extracts of *I. suffruticosa*, which did not cause any death of mice at the dose 1000 mg/kg, although showing some clinical signs, it can be considered safe phytotherapic when administered at lower doses, according by the Organization for Economic Co-operation and Development (OECD, 2004). The increase of use of medicinal plants as oppose to the scarcity of scientific evidences on the safety of these have raised concerns regarding toxicity and detrimental effects. *Indigofera suffruticosa* contains several bioactive principles which have the potential to cause beneficial and/or detrimental effects, therefore this results reveals the non-toxic nature of this organic extracts. Other studies (Jayanthi and Lalitha et al., 2011; Lalitha, Sripathi and Jayanthi., 2012). These clinical abnormalities can be attributed to the presence of theses constituents.

The methodology of DPPH based in the reduction of radical DPPH (2,2-diphenyl-1- picrilhidrazil), which change your colour of purple to yellow when receive one electron or one hydrogen radical remained stable (RoginskyLissi, 2005). We noticed that the different extracts of *I. suffruticosa* showed good antioxidant activity, although it is not possible to infer precisely which secondary metabolites are being responsible for this activity, we can partly attribute this action to flavonoids class, as they are significantly present in our extracts and also the numerous studies that described as potent antioxidants (Cioffi et al., 2002; Furusawa et al., 2005; Mohamed et al. 2015; Wu et al., 2015).

Finally, the results point to the significant potential of *Indigofera suffruticosa* extracts for pharmacological use of pain, inflammation and free radicals. Furthermore, results of biological tests show the importance of further scientific knowledge of the species suggest possible because similar effects displayed by drugs commercial standards and also withstand its traditional use of the plant on some inflammatory conditions.

4 Conclusion

In this study, the previous results on the anti-inflammatory and anti-

nociceptive effect of pods *Indigofera suffruticosa*, also justifies in a scientifically way, the use of this plant in the inflammation and pain in popular medicine. These properties showed in this study, make this plant as a potential target for the development of new compounds that can be explored as alternatives to drugs that are already in use.

Appendices

Table 1. Preliminary phytochemical Screening of organics extracts of *I. suffruticosa.*

Tests	Results of the extracts of *I. suffruticosa*			
	Ether	Chloroform	Acetone	Methanol
Alkaloids Dragendorf	++	++	++	++
Flavanoids Neu	+++	+++	+++	+++
Mono, sesqui, diterpene Vanilin sulfuric 2%	++	-	++	++
Steroids and triterpenoids Lieberman Buchard	++	-	+	+
Iridoids Vanilin sulfuric	-	-	-	+++
Coumarins UV	-	-	++	++
Cinnamic derivates Neu	-	-	-	-
Phenylpropanoglycosides UV	+++	+++	+++	+++
Proanthocyanidins Vanilin hydrochloric	-	-	-	+++

+++: Strong intensity reaction; ++: Medium intensity reaction; +: Weak intensity reaction; −: No detected

Table 2. Effect of organics extracts of *I. suffruticosa* (400mg/kg) in the thickness (mm) of the right posterior paw edema of mice.

	Time				
TREATMENT	T0	T1	T2	T3	T4
Control	4.16 ± 0.114	5.27 ± 0.321	5.04 ± 0.281	4.82 ± 0.064	4.71 ± 0064
ASA	4.16 ± 0.182	4.90 ± 0.145*	4.55 ± 0.154*	4.42 ± 0.108*	4.35 ± 0.143*
Éther	4.32 ± 0.07	4.41 ± 0.079	4.51 ± 0.094	4.75 ± 0.161	4.82 ± 0.07
Chloroform	4.26 ± 0.1	4.70 ± 0.188	4.75 ± 0.136	4.74 ± 0.037	4.74 ± 0.037
Acetone	4.13 ± 0.072	4.6 ± 0.121*	4.5 ± 0.121*	4.4 ± 0.108*	4.26 ± 0.075*
Methanol	4.02 ± 0.115	4.43 ± 0.146*	4.39 ± 0.158*	4.29 ± 0.185*	4.2 ± 0.199*

Values are expressed as Mean ± S. D. ANOVA: * $p<0.001$, in relation to control group.

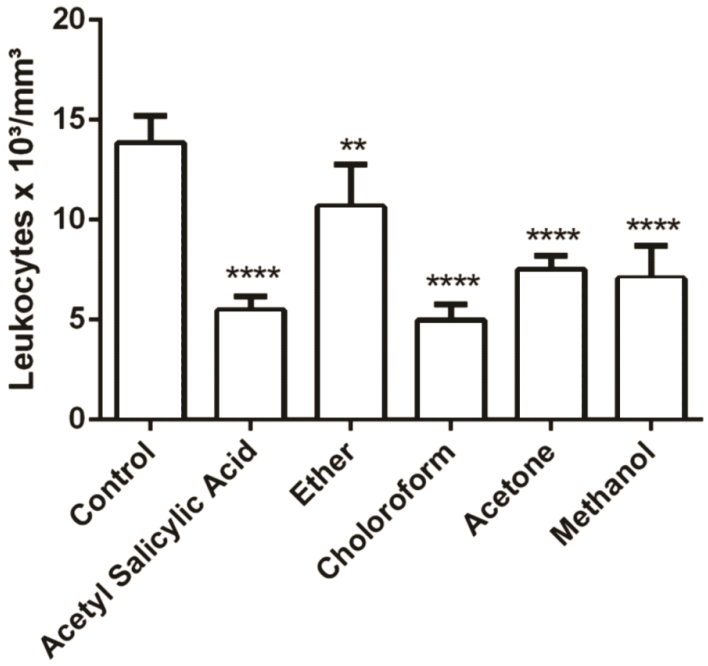

Figure 1. Effect of the different extracts of *I. suffruticosa* in the leucocyte migration in the model of carrageenan-induced peritonitis. Groups of mice were pre-treated with vehicle (Control), Acetyl Salicylic Acid (100 mg/kg), and extracts of *I. suffruticosa* (400mg/kg) (Ether, Chloroform, Acetone and Methanol extracts) 60 min before carrageenan (1%) induced peritonitis. Cell counters were performed at the time 4 h after the injection of carrageenan. Each value represents the mean ± S.D (n=6, group). ** denote statistical significance, $p<0.05$ and ****$p<0.0001$, in relation to control group. ANOVA.

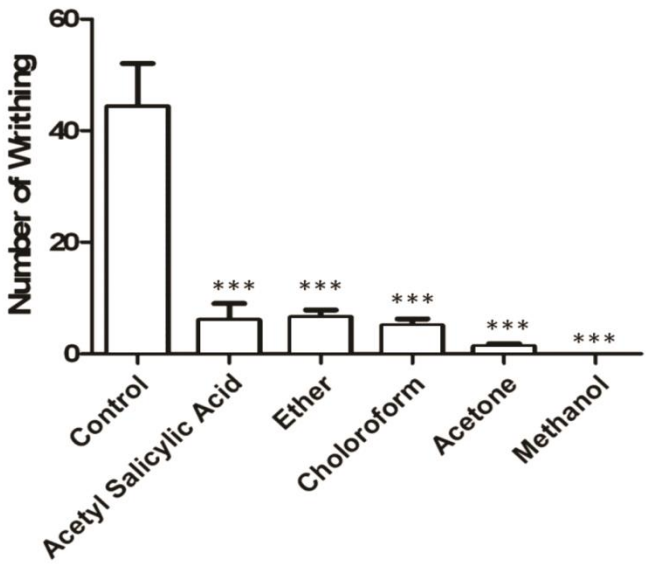

Figure 2. Effect of the different extracts of *I. suffruticosa* in the model of nociception induced by acetic acid. Nociception was registered by the number of writhes, which the animal presented 20 min following i.p. acetic acid injection. Groups of mice were pre-treated with vehicle (Control), Acetyl Salicylic Acid (100 mg/kg), and extracts of *I. suffruticosa* (400mg/kg) Ether, Chloroform, Acetone and Methanol extracts) 60 min before nociception agent. Each column represents the mean ± S.D. *** denote statistical significance, $p<0.001$ in relation to control group. ANOVA.

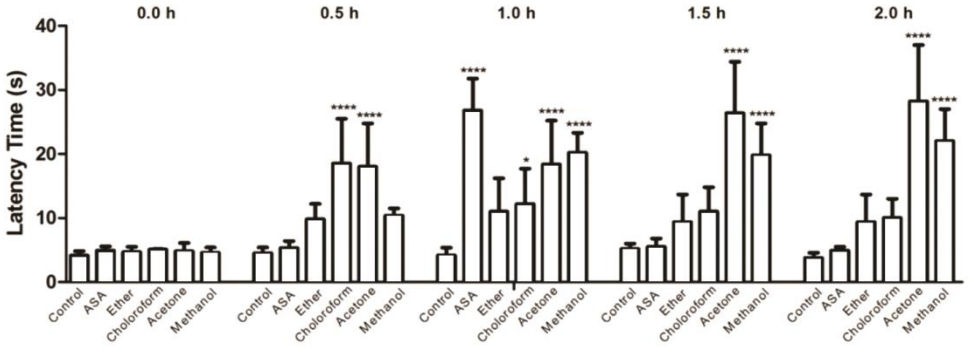

Figure 3. Analgesic effect of the different organics extracts of *I. suffruticosa* and Acetyl Salicylic Acid on hot plate test on mice. Groups of mice were pre-treated with vehicle (Control), Acetyl Salicylic Acid (100 mg/kg), and extracts of *I. suffruticosa* (400mg/kg) Ether, Chloroform, Acetone and Methanol extracts) 60 min before nociception agent. Each column represents the mean ± S.D. * denote statistical significance, $p<0.05$, and **** $p<0.0001$ in relation to control group. Values are expressed as Mean ± S. D. ANOVA.

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4.2 Artigo: *In vivo* antinociceptive, anti-inflammatory and antipyretic activities of sugar-rich fraction isolated from methanol extracts of dried pods of *Indigofera suffruticosa*

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In vivo antinociceptive, anti-inflammatory and antipyretic activities of sugar-rich fraction isolated from methanolic extracts of dried pods of *Indigofera suffruticosa*

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Abstract

Background: *Indigofera suffruticosa* Mill (Fabaceae) or "Anil" is a medicinal plant used in traditional medicine to show proprieties antispasmodic, sedative, diuretic, among others.

Methods: The present study assessed the possible anti-inflammatory, antinociceptive and antipyretic action of the sugar rich fraction from methanol extracts pods of *Indigofera suffruticosa* (Fabaceae) in models of chemical inflammation and nociception in mice.

Materials and methods: Antinociceptive activity was evaluated through writhing and hot plate tests in mice. Anti-inflammatory activity was evaluated through oedema paw and peritonitis test in mice. Antipyretic activity was evaluated through yeast test in mice.

Results: The sugar fraction (SFIs) of *I. suffruticosa* (20 and 40 mg/kg, orally) produced significant reduction of contortion number induced of acetic acid. Only the dose of 40 mg/kg od SFIs led to increased latency time exposed to the hot plate. Both doses too inhibition on inflammation induced by carrageenan for oedema paw (70.2 and 73.2%, respectively). The SFIs produced significant inhibition on inflammation for peritonitis (31 and 49%, respectively) test. Only the dose of 40 mg/kg on day 2 h was able to reduce the pyrexia of each animal.

Conclusions: The findings in this study suggest that the SFIs from methanol extract of *I. suffruticosa* possesses analgesic and anti-inflammatory effects, and discreet effect on fever. These results may be contributing to scientific evidence of its medicinal uses related to the treatment of pain and inflammation.

Keywords*:* anti-inflammatory, antinociceptive, *Indigofera suffruticosa*

Background

In general, pain and inflammation stands out for being considered one of the biggest problems in the population because they affect the quality of life and health [1]. Anti-inflammatory and analgesic therapies are readily available, but are associated with side effects when used long term, as the rise of cardiovascular diseases [2]. So awakened enormous interest in the discovery of new drugs from natural sources with high efficiency and little adverse effect.

Plants are used as a healing source of disease for many people since prehistoric times. They synthesize chemicals essential for its development and protection, such as flavonoids, alkaloids, triterpenoids, tannins, saponins, amongst others, has pronounced effects on the human body and are used as medicinal agents in the treatment of diseases. Several of these bio-products are extracted from plants for large scale marketing and many of them have been used as prototypes for synthetic or semi-synthetic drug with a pharmacokinetic profile [3,4].

The genus *Indigofera* (Syn: APersimmon, ebony), belonging to the Fabaceae family, consists of 19500 species distributed in Ásia, África tropical, Austrália, América do norte e sul [5]. In Brazil it is possible to find three species: *Indigofera truxillensis, I. hirsuta*, and *I. suffruticosa* [6]. Are considered wild plants growing in all types of soils and tolerates drought, flooding and high salinities.

Indigofera suffruticosa Mill (Fabaceae) or “Anil” is a medicinal plant with occurrence in Mato Grosso [7], Alagoas [8], Paraíba [9], Ceará, Rio Grande do Norte, Pará e Pernambuco [10]. It has a long history of popular use by show proprieties antispasmodic, sedative, diuretic, among others [11, 12]. Previous studies have revealed that the leaves of plant presents the activities antimicrobial [13-15], antifungal [13, 16], anti-inflammatory [17-18] and antitumor [19,20]. Phytochemical investigations of this kind show that the extract of leaves has several biologically active chemical constituents including proteins, carbohydrates, steroids, phenols, alkaloids, flavonoids and indigo [21].

Various pharmacological and biological properties of extracts from *I. suffruticosa* have been found in many studies. However, the antinociceptive, antiinflammatory and antioxidative potential of constituents of methanolic extract from *I. suffruticosa* was not so fully characterized. Thus, in the present study,

we evaluated the antinociceptive, antiinflammatory and antioxidative activities of fraction of methanolic extract

Methods

Animals

Experimental protocols and procedures used in this study were approved by the Ethics committee on animal experiments from the center of Biological Sciences, Federal University of Pernambuco (CEEA-UFPE) and in accordance with the guidelines suggested by the "Brazilian College for Animal Experimentation" and with international standards by the "National Institute of Health Guide for Care and Use of Laboratory Animals "(Case No.: 014 413 / 2007-78). Heatlthy male mice (swiss albino), weighing 25-35g were used in the experiments. The mice were obtained from the the Keizo Asami Laboratory of immunopathology (LIKA), Pernambuco, Brasil. The animals were kept under laboratory conditions of 25 ± 2 ° C(constant temperature), 55-65% humidity with 12-hour cycles light/dark, with free access to food and water ad libitum.

Plant material

Pods of *Indigofera suffruticosa* was collected in the region from the São Caetano, the arid region of Pernambuco State, identified and authenticated by the Biologist Marlene Barbosa from the Botany Department, Federal University of Pernambuco (UFPE), Brazil, where a voucher specimen has been deposited at the Herbarium of the Botany department and registered with the number 45 217.

Extraction

The air-dried pods (500 g) were triturated and extracted with ether, chloroform and acetone (200 ml, three times each) and finally dried material was extracted with methanol to whiteness. The methanol extract *I. suffruticosa* (EMeOH) was filtered and evaporated to completely remove the solvent with the aid of a rotary evaporator, to give a solid mass (38g) of EMeOH. This material (30g) was subjected to column chromatography using as stationary phase silica gel 60 0.2-0.5mm / 35-70 mesh ASTM and collect three fractions of different polarities (ethyl acetate-EtOAc to methanol -MEOH). The last fraction (400 mg) was subjected to column chromatography using the same stationary phase and the

same polarity solvents, resulting in 40 fractions. The obtained fractions were analyzed using analytical thin layer chromatography gathering similar fractions and identifying pure specimens. These fractions were subjected to biological tests for the presence of activity. The called active fraction (AF) (200 mg) was subjected to column chromatography using Sephadex LH 20 is a single solvent, in this case methanol to give 15 fractions. The Sephadex was washed with distiled water (Sugar fraction –SFIs) and this liquid was subjected to freeze dry, to extract a fraction permeated (Figure 1).

Phytochemical Screening

A simple qualitative and semiquantitative phytochemical analysis was performed by screening tests according to Wagner et al. [22], Markham [23]. The phytochemical profile of fraction of *I. suffruticosa* were evaluated on thin layer chromatography (TLC) plates in front of the mobile phase solvents containing different proportions and different polarities, and revealing, using as stationary phase, silica G F $_{254+366}$ plates (Merck). For identification and differentiation of the disclosed compounds the following parameters were used: staining band and luminescence in UV lamps.

Carbohydrate quantification

Carbohydrate quantification was determined by the phenol-sulfuric colorimetric method, according to Dubois et al.[24] and quantified by absorption at UV-visible (490 nm), using a calibration curve of 10 points, with a concentration of 10 to 100 µg of glucose and R^2 = 0.99916.

Antioxidant activity

Different concentrations were combined (0.250, 0.500, 1,000 mg / ml) of SFIs in a solution of methanol (100 mL) solution of DPPH (150 uM), in duplicate, using a 96-well plate. The decrease in absorbance was determined at 515 nm at 0min. SFIs were compared with the commercial antioxidant gallic acid. Reduction DPPH radical was evaluated by measuring the absorption at 495 nm using an ELX 800 Universal Microplate Reader (BioTek Instruments, Inc., Winooski, USA). The radical scavenging activity was calculated as a percentage of DPPH discoloration using the following equation:% AA = [(Adpph

- As) / Adpph] × 100, where As is the absorbance of the sample and Adpph was DPPH absorption solution [25].

Acetic acid - induced writhing test

The acetic acid -induced writhing test by method of Koster [26] was conducted for this test. The mice (5 animals per group) were pretreated orally with water (Negative control), Ibuprofen (10 mg/kg) and sephadex SFIs (20 and 40 mg/kg) 1 hour before intraperitoneal injection of 0.8% acetic acid. The induced writhings were observed by the presence of abdominal muscle contraction together with hind limb extension, and were counted for 20 min after the latency period of 5 min. Antinociceptive activity was calculated as follows:

%Inhibition= (Wm0 – Wmt) / Wm0×100 where *Wm0* was the average writhing response of the control group while *Wmt* was the average writhing response in the treated mices.

Hot plate test

The method of MacDonald et al. [27] evaluating the central analgesic was used for this test. Mice were individually placed on a hot plate maintained at 55 ± 1.0 ° C and response time to the stimulus was marked by a timer. Measurements were performed at time 0, 30, 60, 90, and 120 min after the first thermal stimulus. The animals were pretreated with water, morphine (5 mg/kg, sc), and SFIs (20 and 40 mg/kg).

Carrageenan - induced paw oedema

The method of Winter et al. [28] with little modification was used to determine the anti-inflammatory activity of the fraction. The paw edema was induced from a subplantar injection of 0.1 ml carrageenan (1%) in saline half hour before the administration of the SFIs (20 and 40 mg/kg, orally) of *I.suffruticosa.* Acetylsalicylic acid (ASA – 100 mg/kg, orally) was used as a standard drug for the positive control. The negative control animals received just water. The volume of the paw was measured by one caliper rule (Kanon- Staineless Mardened), at the time 0 and intervals of 1, 2, 3 and 4 h immediately after the subplantar injection of carrageenan. The percentage inhibition was calculated using the formula given below, that represents the period of peak edema (3h).

Percentage inhibition (%I) = [(Vf-Vi) Control group mean – (Vf-Vi) Test group mean / (Vf-Vi) Control group mean] x 100, where Vf and Vi represent the volume of the initial and final paw.

Carrageenan - induced Peritonitis

The method was following Foster et al. [29]. The animals were pre-treated with water, Acetylsalicylic acid (Positive Control - ASA, 100 mg/kg, orally), SFIs (20 and 40 mg/kg, orally), and 1h later, the animals received an injection of 1% carrageenan (i.p.). After 4 h, the animals were sacrificed. After, saline containing EDTA (1mM, i.p.) was injected, immediately a brief massage was done for further fluid collection and used for leukocyte (mainly neutrophils) counting in a Cell Counter (ABX MICROS 60). The results were expressed as the number of Leukocytes x $10^3/mm^3$. The percentage of the leukocyte inhibition = (1 – T/C) x 100, where T represents the treated groups leukocyte counts and C represents the control group leukocyte counts.

Yeast induced pyrexia

The method Teotino et al. [30] was used for antipyretic activity. The increase in basal temperature was induced by subcutaneous injection of 20% w/v yeast (10 ml/kg) in 0.5% CMC. The basal temperature was recorded before induction, by rectal insertion (2 cm) of a clinical thermometer for possible exclusion from animals that did not fit within the parameters (basal temperature to or above 37.5 ° C). 24 hours after yeast injection is measured increase in temperature of each animal. The positive control used has received the commercial antipyretic drugs, paracetamol (150 mg / kg). They observed the temperature of each animal at intervals of 1 hour (1 -5) after treatment in each group.

Statistical Analysis

Results are expressed as the mean ± S.D.Data were analysed by one way analysis of variance (ANOVA) and Student t-test followed by a pot hoc Tuckey test, and p values of lesse than 0.05 were considered statistically significant.

Results

Phytochemical Screening

Active fraction showed the presence of alkaloids, carbohydrates and reducing sugars, flavonoids and terpenes. However the SFIs showed just the presence of carbohydrates and reducing sugars (Table 1).

Carbohidrate quantification

SFIs (SFIs - 1mg) showed a total sugar concentration of 5.1891 mg.g-1, which represent the sugars, while the great majority of sugars associated forming polysaccharides.

Acetic acid - induced writhing test

The observed effect by decreasing the number of writhes induced by acetic acid was showed in Figure 2. The doses of 20 and 40 mg/kg ($p < 0.0001$) inhibited the writhing response in a dose dependent Manner with percentage inhibition of 74% and 57.7, respectively. The percentage inhibition of the positive control (ibuprofen) was presented for a higher dose of 10 mg/kg.

Hot plate test

The effect of the SFIs (Figure 3) showed significant ($p < 0.001$) only at the dose of 40 mg/kg, on the mean of Increased latency time to discomfort reaction When Compared with negative control, from the 30 minutes of administration extending throughout the experiment. Morphine showed protective effect at the dose of 10 mg/kg, from time of 60 minutes and remained well until the end of the observation.

Carrageenan - induced paw oedema

Carrageenan injection in the sub-plantar tissue of the right hind paw of mices in the control group caused edema development at 4h post-phologistic agent injection with peaked (4.22 ± 0.1). Compared with control group, SFIs, ASA, 20 and 40 mg/kg produced significant decrease in paw edema. The shown in table 2, which both tested doses of the fraction produced effect on the edema in all times (2-5), but with a few different values, showing an inhibition percentage of 70.2 and 73.2, respectively. ASA also showed a significant reduction of 87% when compared with control group.

Carrageenan - induced Peritonitis

The effects of orally administered SFIs on carrageenan-induced peritonitis are shown in Figure 4. There was intense migration of leukocytes into the peritoneal cavity with caragenina administration (1%) and both doses (20 and 40 mg/kg) of SFIs produced a dose-dependent inhibition of this effect of the order of 31 and 42%, respectivamente. At the higher dose (20 mg/kg) the effect was similar that observed with the ASA (100 mg/kg), with 42% inhibition, the reference used the drug.

Antioxidant activity

At 0.25 - 1000 mg/ml, *I. suffruticosa* exhibited scavenging activity similar to that galic acid but when the standard was better compared with SFIs. Best antioxidant activity was presented in the major concentration (1000 mg) 29% followed of 23.4 and 12% (0.5 and 0.25 mg/mL), was relatively low compared to the gallic acid and did not present significant statistic among themselves.

Yeast induced pyrexia

The effect of the SFIs on Brewer's yeast induced pyrexia in mice is show in table 3. Paracetamol showed decrease significant in pyrexia at all time-points. It was observed only at the 2h time dose of 40 mg/kg SFIs decreased significant in temperature when compared to the negative control. In other time periods, both doses of SFIs (20 and 40 mg / kg) did not cause significant change in pyrexia.

Discussion

Approximately 25% of drugs are derived directly or indirectly from plants form. These assets compounds has received attention in industries due to its potential [31]. Anti-inflammatory and anti-nociceptive drugs have often been associated with severe adverse side effects, such as gastrointestinal bleeding and pepitic ulcers. Many natural medicines derived from plant compounds have been considered safe and effective for the treatment of various diseases, including inflammation and pain.

It was observed that SFIs showed mild antioxidant activity when compared with gallic acid. Yuan et al., 2008. [32] found similar results with polysaccharides Ligusticum chuanxiong fraction (1 mg / ml).

The antinociceptive and anti-inflammatory activity demonstrated of SFIs extracted from methanol extract of *I. suffruticosa* was assessed by various animal protocols. The writhing test evaluates the effect of peripheral and visceral is considered a test model [33], based on the chemoreceptors stimulation, which induces the release of mediators such as histamine, serotonin and cytokines. The release of these mediators, unleash a constriction response in the abdominal wall and trunk twisting followed by extension of the hind legs [34]. These contortions are involved in the development of peripheral inflammation [35, 36], therefore, the fact that the fraction of water significantly decrease the number of writhing in mice in a dose dependent manner (57.7 and 74%, respectively) when compared with the control group, suggests that its effect is involved in the inflammatory process associated with decreased release of chemical mediators or even acting on chemoreceptors lock. However, given the test is not specific, other analgesics models have to be used as confirmatory.

The hot plate test is a useful model for the evaluation of analgesics having central effect, it acts in thermoreceptors causing higher levels of pain, so is not involved in the inflammatory process [37]. It was verified that only the dose of 40mg/kg of the SFIs caused an increase in latency ($P < 0.001$) when compared with the control group. The dose of 20 mg/kg was not active significantly, which was possibly due to their lower dose. With this, it is suggested that the SFIs of the mechanism of action may also be associated with activation of nociceptors involved in the thermal stimulation [38].

To evaluate the anti-inflammatory action potential, the SFIs was tested in models of acute inflammation and paw edema peritonitis. The paw edema test is commonly used to test for drugs with anti-inflammatory activity [39]. The development of edema is characterized by the biphasic response involves the release of various chemical mediators. The first stage is attributed to release of pro-inflammatory agents such as bradykinin, histamine, serotonin, whereas the second phase the release of prostaglandins. Recently, the second phase was

also attributed to the involvement of free radicals, nitric oxid and cyclooxygenase of the mice paw exudate [40].

At both doses (20 and 40 mg/kg) of the SFIs, there was a reduction in paw edema significant 2, 3, 4 and 5 hours (with percentage inhibition 70.2 and 73.2) after the carrageenan injection, suggesting that its action is related from the beginning of first phase, possibly by inhibiting the action of chemical mediators, confirming the date analgesia.

SFIs at 20 and 40 mg/kg significantly reduced cell migration. This result indicates that the fraction of water may be strongly acting in the release of chemical mediators, que thus there avoiding migration of leukocytes to injured area. Some compounds can also be related to the possible inhibition of adhesion proteins, integrins and selectins, which do not favor migration and rolling of leukocytes in the inflammatory focus.

The increase in temperature induced yeast is called pathogenic disease [41]. AINEs can reduce hyperthermia by inhibiting the synthesis of prostaglandins in the hypothalamus [42, 43]. The reduction of fever provided only at time 2 hrs shows that the antipyretic effect of the sugar fraction is light in comparison with the standard. In this study the dose for SFIs must have been relatively small to promote a reduction in rectal temperature, but the light effect can be explained by the inactivation of cyclooxygenase enzyme activity and consequently, inhibition of prostaglandins.

This study evaluated antinociceptive and anti-inflammatory properties of water rich in carbohydrates extracted fraction of methanol extract of *I. suffruticosa* by several experimental protocols on animals, but their actions mechanisms still need confirmation by more specific methods.

Conclusion

The SFIs extracted from methanol extract of *Indigofera suffruticosa* presented with a potent analgesic and anti-inflammatory, which contributes to scientific evidence of its medicinal uses related to the treatment of pain and inflammation. Other investigations are being made to identify and characterize this potentially active fraction.

Competing interests

The authors this work declare does not have competing interests

Authors' contributions

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Appendices

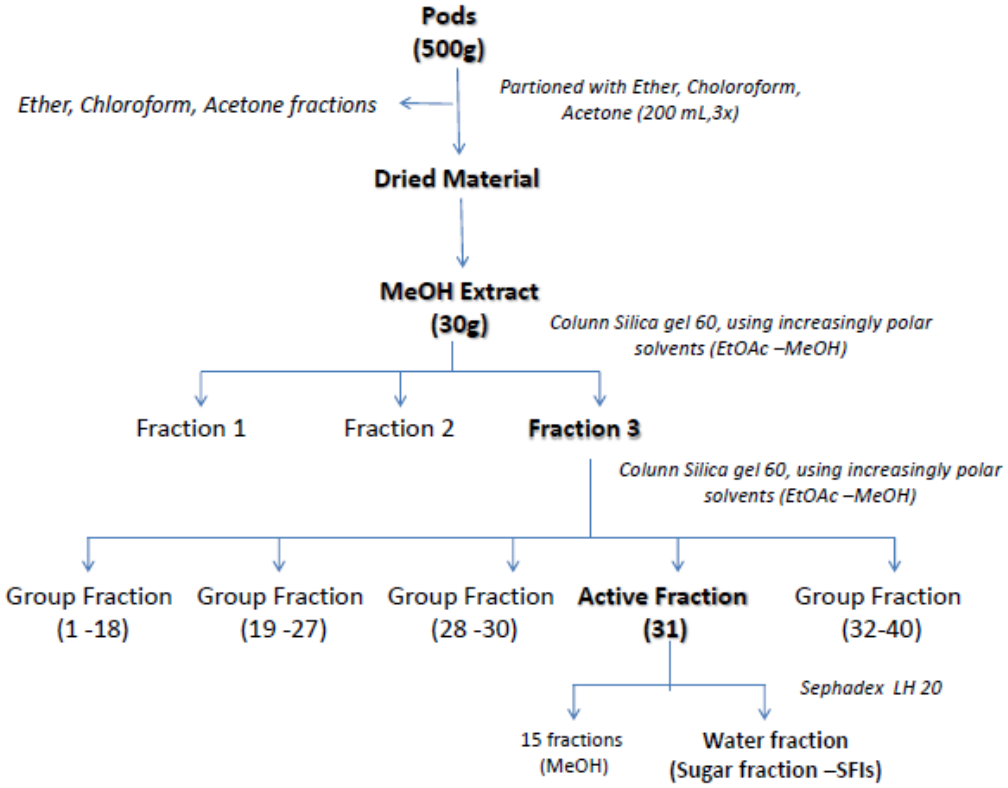

Figure 1. The extraction scheme of SFIs from Methanol extract of *I. suffruticosa*.

Table 1. Preliminary phytochemical Screening of Active fraction and SFIs of *I. suffruticosa.*

Tests	Eluent System	Revelator	Active fraction	Water fraction (SFIs)
Alkaloids	AcOET-N-PrOH-H_2O	Dragendorff	++	-
Proanthocyanidins	AcOET-HCOOH-AcOH- H_2O	Hydrochloric Vanillin	-	-
Saponins			-	-
Tannins	AcOET-HCOOH-AcOH- H_2O	NEU	-	-
Flavonoids	AcOET-HCOOH-AcOH- H_2O	NEU	+++	-
Sugars	AcET-N-PrOH-H_2O	Triphenyltetrazolium	+++	+
Terpenes	Benzen-AcOET	Hydrochloric Vanillin	++	-
Coumarins	Ether-Toluen-AcOH-H_2O	NEU	-	-

+++: Strong intensity reaction; ++: Medium intensity reaction; +: Weak intensity reaction; −: No detected

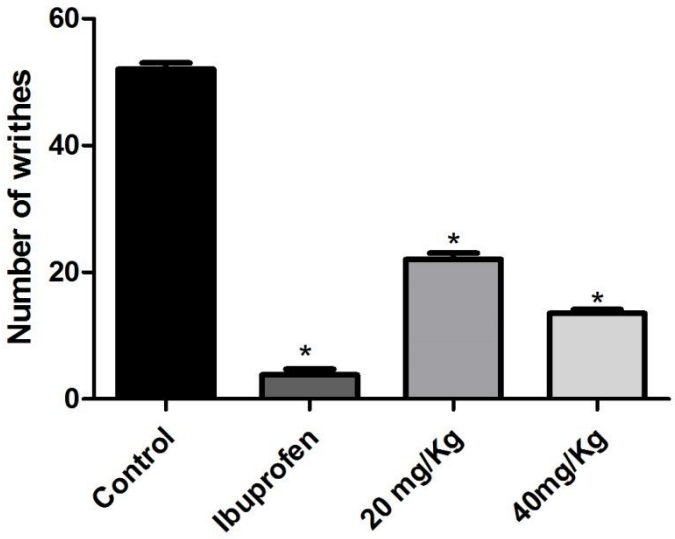

Figure 2. Effect of SFIs and ibuprofen (10mg/kg) on acetic acid –induced writhing in mice. The results are expressed as Mean ± SD (n=5). * Indicates significant difference from the control group ($p<0.001$), One-way ANOVA.

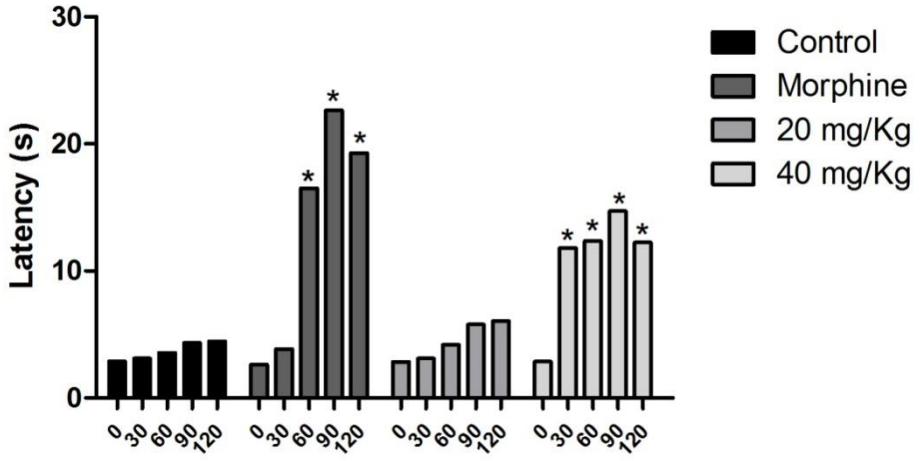

Figure 3. Effect of SFIs and Morphine (5 mg/kg) at different times (0, 30, 60, 90 and 120 minutes) on hot plate test. The results are expressed as Mean ± SD (n=5). * Indicates significant difference from the control group ($p<0.001$), One-way ANOVA.

Table 2. Changes in edema volume (mm) from 1 to 5h after carrageenan injection following oral administration of SFIs (20 and 40 mg/kg), Acetyl salicylic acid (100 mg/kg).

Treatment	**Paw measure (mm)**						
	T0	T1	T2	T3	T4	T5	% I
Control	2.81±0.1	4.42±0.1	4.37±0.1	4.29±0.1	4.22±0.1	4.05±0.1	-
ASA	2.89±0.2	4.37±0.2	4.04±0.1[a]	4.00±0.4[a]	3.66±0.2[a]	3.42±0.2[a]	87.4
20 mg/kg	2.58±0.2	4.28 ±0.1	3.36±0.3[a]	3.2±0.3 [a]	3.01±0.2 [a]	2.85±0.1 [a]	70.2
40 mg/kg	2.82±0.1	4.12±0.2	3.63±0.1 [a]	3.38±0.2[a]	3.21±0.1 [a]	3.03±0.1 [a]	73.2

[a] p<0.001vs. Control**.**

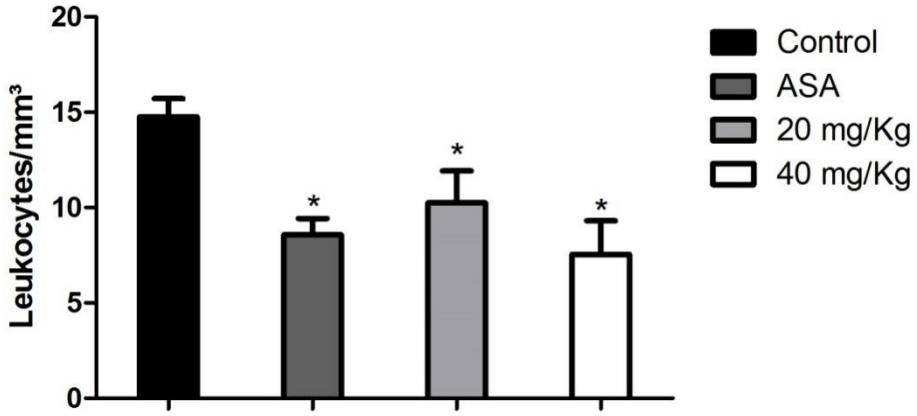

Figure 4. Effect of SFIs and Acetyl salicylic acid (100 mg/kg) on the leukocytes migration. The results are expressed as Mean ± SD (n=5). * Indicates significant difference from the control group ($p<0.01$), One-way ANOVA.

Table 3. Effect of SFIs (20 and 40 mg/kg) on body temperature in yeast induced pyrexia

Groups	Rectal temperature Cº after 24hrs of Yeast injection					
	Baseline	0h	1h	2h	3h	4h
Control	36.4±0.3	37.8±0.2	38.3±0.3	38.4±0.2	38.1±0.3	38±0.4
Paracetamol	36.5±0.8	38.2±0.3	36.4±0.5**	36.2±0.3**	35.8±0.4**	35.8±0.3**
MeOHIs (20 mg/kg)	36.9±0.2	38.4±0.3	38.1±0.2	37.96±0.2	37.8±0.1	37.6±0.1
MeOHIs (40 mg/kg)	37.1±0.22	38.5±0.2	38.3±0.2	37.9±0.1*	37.8±0.2	37.6±0.2

Values are expressed as Mean ± DP, n = 6 in each group, Statistical analysis of data was performed using ANOVA. *indicate $P < 0.05$ ** indicate $p < 0.001$, comparing the same group at different times.

4.3 Artigo: Studies on Antidiarrhoeal and Antipyretic Activities of *Indigofera suffruticosa* (Anil).

Artigo a ser submetido ao periódico *Phytomedicine* no formato *Original Research Article* (**FI:** 3.126; **QUALIS CB I:** B1).

Studies on Antidiarrhoeal and Antipyretic Activities of *Indigofera suffruticosa* (Anil)

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Abbreviations: MeOHIs, Methanol extract of *Indigofera suffruticosa*; UFPE, Universidade Federal de Pernambuco; CMC, Carboximethyl celulose; LIKA, Laboratory of Immunopathology Keizo Asami; LH, Loperamide; DC, Distance travelled; LHI, Length of small intestine; FacePE, Fundação de Apoio à Ciência e Tecnologia do Estado de Pernambuco; SD, Standard deviation; W1, Weight of the fluid- filled; W2, Emptiel intestine; L, Total length.

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Abstract

Background: *Indigofera suffruticosa* generally used in the medicinal popular for its antiespasmodic and antiinflammatory effects. The present study aims to analyze the anti-diarrhoeal and antipyretic activity of methanol extract of *Indigofera suffruticosa* in animal models.

Study design: We investigated the antidiarrheal effects of MeOHIs on diarrheal induced with Castor oil in mice, and antipiretic effect induced with yeast of beer. Mice were randomized into groups termed normal control, loperamide (2 mg/kg), Atropine (0,25 mg/mL), Paracetamol (150 mg/kg), and MeOHIs (200 and 400 mg/kg).

Methods: The methanol extract of *I. suffruticosa* was tested at two doses levels (200 and 400 mg/kg) in mices, against castor oil-induced diarrhoea model, castor oil induced enteropooling, charcoal meal test (intestinal motility test) and antipyretic test. The parameters observed were the onset defecation and consistency of faeces in normal defecation and castor oil induced diarrhoeal model; the weight and size of intestinal content in castor oil induced enteropooling assay; and the distance travelled by charcoal in the intestinal motility test.

Results: Pretreatment with both doses of *Indigofera suffruticosa* extract in dose-response elicited significantly reducing the frequency of bowel movements and the humidity of fecal droppings The administration of MeOHIs to mice produced a significant ($p < 0.0001$) reduction in castor oil-induced fluid accumulation at 200 and 400 mg/kg with respective values of 11.8 ± 2.2 and 15.2 ± 4.4. Treatment with MeOHIs at both doses were able to decrease the distance traveled by the charcoal meal dose-dependent manner ($p <0.0001$) 9.3 ± 2.5 cm when treated with 200 mg/kg, and 5.8 ± 1.3 when treated with dose of 400 mg/kg. There was reduction in rectal temperature in the groups treated with MeOHIs (200 and 400 mg/kg) in all times-points, though not statistically significant.

Conclusion: The methanol extract of pods of *Indigofera suffruticosa* showed anti-diarrhoeal, anti-secretory, anti-motility and antipyretic activity.

Keywords: diarrhoeal, Pyrexia, *Indigofera suffruticosa..*

Introduction

Diarrhea is a common sign of gastrointestinal origin characterized by an increase in the number of bowel movements, it is often accompanied by abdominal cramps, increased secretion of mucous or motility in response to various stimuli. This symptom affects more than 3 billion people, causing about 5 million deaths per year (Shareef et al., 2014), so it is considered the leading cause of mortality in developed countries, especially when their origin is triggered by infections enterotoxin. Diarrhoea affects all races, sexes, ages and geographical areas worldwide it is estimated that 3.2% of all deaths are due this process, triggering the 1.5 million deaths in children (Chitme et al., 2004; Lopez and Mathers, 2006, Yakubu e Salimon, 2015).

Usually directed to finalize an acute attack, induce remission, relapse prevention and control chronic symptoms the treatment of diarrhea is used. The treatment approach is possible for several options such as instant introduction of oral rehydration therapy (often used successfully to manage diarrhea among children), continuous feed, probiotics and medications such as loperamide or others (Sharma and Sharma, 2007)

In order to overcome the symptoms of diarrhea, especially in developing countries, the World Health Organization (WHO, 2004) encourages the use of drugs from medicinal plants due to their affordability, accessibility, knowledge acquired by ancestors and effectiveness demonstrated.

The comumentes highlighted as medicinal plants present in Brazilian native flora are consumed with little or no scientific evidence of its pharmacological properties (VEIGA JUNIOR & PINTO, 2005). The *Indigofera* is the third largest genus of Leguminosae, composed of about 700 species. The species belonging to this genre popularly known as "indigo" or "anileira" the *Indigofera suffruticosa*, Fabaceae family, has been increasing in the population as herbal medicine to present sedative, diuretic and antispasmodic properties (Lorenzi, 1982).

Scientific studies of this species also show the highlighted the clinical applications with the following activities: cytotoxic to embryonic cells in mice (Leite et al, 2004), antimicrobial (Leite et al, 2006; Carli et al, 2010, Santos et al, 2015a; Santos et al. , 2015b), antifungal (Leite et al, 2006), the anticonvulsant

activity (Almeida et al, 2013), gastroprotective activity (Luiz-Ferreira et al., 2011), cytotoxic activity in tumor cell lines of murine (Lopes et al , 2011), anti-tumor in mice (Vieira et al., 2007) and inflammation (Leite et al., 2003; Chen et al, 2013);

Notwithstanding their traditional uses data from the literature revealed that have not yet been investigated its anti-diarrheal pontecial. Thus, the present study was aimed at evaluating the potential anti-diarrheal effect of dried pods *I. suffruticosa* in different experimental models.

Materials and methods

Plants materials

I. suffruticosa were collected in São Caetano, semi-arid region of Pernambuco. Specimens of this species have been identified and authenticated by biologist Marlene Barbosa, Department of Botany, Federal University of Pernambuco (UFPE), Brazil, deposit record with no Herbarium of the Department of Number of Botany 45 217.

Preparations of plants extracts

The dried pods (100g) were finely ground *I. suffruticosa* subjected to a pre fractionation with 200 mL (3x) of increasing polarity solvent (ether, chloroform, acetone and methanol), homogenized for two hours on a mechanical shaker maintained under refrigeration (4 - 10 °) overnight and filtered with Whatman filter paper (nº 1). The methanol extract was subjected to rotary evaporation coupled to a water bath for complete removal of solvent (BUCHLER Instruments, Fort Lee, NJ).

Drugs administration

The Methanol extract of *I. suffruticosa* (MeOHIs) was dissolved in water. The vehicle (water) alone served as negative control. Loperamide hydrochloride (Imosec®, 2 mg/kg), and Atropine sulphate (Pasmodex®, 0.25 mg/mL) served

as positive control in diarrheal models. Paracetamol (Medley, 150 mg/kg) was used as positive control for model pyrexia. Commercial castor oil was used for induction of diarrhea. The charcoal was resuspended in 0.5% Carboximethyl Cellulose (0.5% CMC). The activated charcoal was purchased from the local market. Solvents used for preparation in the extract were purchased from Vetec (Rio de Janeiro, RJ, Brazil).

Animals

The male mice, Swiss Albino (25 - 30 g) were obtained from the Laboratory of Immunopathology Keizo Asami (LIKA). The animals were housed in cages (6 animals each) were kept in light cycle (12 h) and dark (12 hours) with free access to food (Labina) and water ad libitum. The experimental protocols were conducted in accordance with the rules and animal ethics committee regulations of the Federal University of Pernambuco (case Nº 0144113 / 2007-78). All treatments were administered orally. The methanol extract of *I. suffruticosa* were tested in doses 200 and 400 mg/kg in all methodologies.

Castor oil-induced diarrhoea

Castor oil-induced diarrhea was described by Awouters et al. (1975). The animals were fasted for 18 hours to beginning the experiment. Each animal was individually lined glass funnel with filter paper on the floor. The animals were divided into groups: a negative control (Vehicle); a positive control group (LH); and two test groups (MeOHIs – 200 and 400 mg/kg). After one hour of treatment, each animal received 10 mL/kg of castor oil, and the time of appearance of the first diarrheal stool was monitored on for 4 hours. The number of evacuation and consistency of faeces from each animal were noted.

Castor oil-induced enteropooling model

The enteropooling procedure induced by castor oil has been described by Robert et al. (1976). The animals were fasted for 18 hours beginning of the

methodology. The negative and positive controls received the vehicle and atropine sulfate (0.25 mg/kg), Respectively, whereas the test group the administration of MeOHIs (200 and 400 mg/kg). After one hour, all the animals received by oral administration of 10 mL/kg castor oil. After 30 min the oral use of the diarrhea inducing the animals were sacrificed. The small intestine was removed and both its full weight as its empty weight was measured, in addition also for its size. Enteropooling = (W1 - W2)/L where W1 and W2 is the weight of the fluid-filled and emptied intestine, respectively, and “L” refers to the total length of intestin (Valle et al., 2000)

Charcoal meal test

Intestinal motility was assessed by the method described by Yegnanarayan and Shrotri (1982). The animals were fasted for 18 hours. Negative control group received only the vehicle. Positive control group received morphine (10 mL/kg). Test groups received different doses of the methanol extract of *I. suffruticosa* (200 and 400 mg/kg). After 30 minutes every animal received castor oil (0.2 ml). Sequentially after 30 minutes, the animals received orally (0.2 ml), Charcoal meal suspended in CMC (0.5%). The animals were sacrificed 30 minutes after administration of activated charcoal. The small intestine was removed, total size of the pyloric sphincter to the caecum and the distance traveled by the charcoal is measured (cm). Intestinal transit (%): (DC/ LSI) x 100, where DC is distance travelled by charcoal meal, and LSI is length of small intestine.

Yeast induced pyrexia

The antipyretic activity was determined according to Teotino et al. (1963). Pyrexia was induced by subcutaneous injection of 20% w/v brewer’s yeast (10 ml/kg) in 0.5% CMC. The core temperature was measured before induction by rectal insertion depth of 2 cm with a clinical thermometer. After 24 hours the yeast injection was recorded increasing temperature of each animal. Positive control used has received the commercial antipyretic drug, Paracetamol (150 mg/kg). Test groups received different doses of the methanol extract of *I.*

suffruticosa (200 and 400 mg/kg). They observed the temperature of each animal at intervals of 1 hour (1 -5) after treatment in each group.

Statistical evaluation

The results were submitted to analysis of variance (ANOVA) followed by Tukey's multiple comparison test, with $p < 0.05$ considered significant.

Results

Castor oil-induced diarrhea

Castor oil promoted abundant diarrhea in all mice in the control group after 4 hrs. Pretreatment with both doses of *Indigofera suffruticosa* extract in dose-response elicited significantly reducing the frequency of bowel movements and the humidity of fecal droppings (Table 1). The extract also promoted reduction of wet feces abundant diarrhea in all mice in the control group after 4 hrs.

Castor oil-induced enteropooling model

Castor oil administration caused a substantial increase of fluid accumulation in mice with [(W1 - W2) / L] in the enteropooling assay, value of 30.4 ± 4.4 in the control group (Figure 1). The administration of MeOHIs to mice produced a significant ($p < 0.0001$) reduction in castor oil-induced fluid accumulation at 200 and 400 mg/kg with respective values of 11.8 ± 2.2 and 15.2 ± 4.4, when compared with control group. Similar to the effect of Atropine sulphate (13.9 ±3.6) at the dose of 0.25 mg/kg also decreased fluid accumulation.

Charcoal meal test

As shown in Table 2, the intestine lengths of all mice were similar. Treatment with MeOHIs at both doses were able to decrease the distance traveled by the charcoal meal dose-dependent manner ($p < 0.0001$) 9.3 ± 2.5 cm

when treated with 200 mg/kg, and 5.8 ± 1.3 when treated with dose of 400 mg/kg. Morphine also significantly promoted the reduction in the path of charcoal meal (10.2 ± 1.5)

Yeast induced pyrexia

The effect of the extract on Brewer's yeast induced pyrexia in mice is show in table 3. Paracetamol showed decrease in pyrexia at all time-points. There was reduction in rectal temperature in the groups treated with MeOHIs (200 and 400 mg/kg) in all times-points, though not statistically significant. There was statistical significant reduction in the paracetamol and MeOHIs (400 mg/kg) group when compared with control group.

Discussion

National health problem, diarrhea especially affects children and is a major cause of mortality and morbidity. Rehydration therapy in acute diarrhea cases has been very useful, however in chronic cases becomes a problem threatening to cause death. Many plants with medicinal use reported in the population has been investigated for effects against diarrhea.

Both MeOHIs doses studied were used in the model of normal stools, diarrhea induced by castor oil, enteropooling and activated carbon. In diarrhea model induced with castor oil was also noted that both doses (200 and 400 mg/kg) significantly reduced the frequency of stools and stool consistency. For antisecretory research, enteropooling method was also possible to verify significantly inhibitory action of the extract on the two tested doses of the drug with similar effect. The extract also showed significant effect on intestinal motility by activated carbon method, with similar effects to the drug.

Castor Oil is derived from the *Ricinus communis* seed, its main active component is the ricinoleic acid selective agonist of EP3 receptors (Amon et al., 1974). This acid, in the small intestine, it causes local irritation and inflammation of the intestinal mucosa to unleash the release of prostaglandins and motility change in promoting the secretion of water and electrolytes (Pierce et al., 1971).

The standard drug loperamide, commonly used in acute diarrhea acts situations decreasing intestinal motility directly on the circular and longitudinal muscle layers of the intestine inibndo prostaglandin in myenteric plexus and also has anti-secretory action with action on opioid receptors (Craig and Stitizel, 1990). Atropine, extracted from Atropa belladonna plant, is a drug used to block the muscarinic receptors with anti-spasmodic action.

Plants have various constituents of endogenous origin which has beneficial effects on the body. The flavonoids present several biological activity among them anti-diarrheal with inhibitory action of intestinal motility and hydro-electrolyte secretion (Sarin et al., 2013). Recent studies (*in vivo* and *in vitro*) have shown that this group act on inhibition of intestinal secretory response by direct action of prostaglandin E2 by inhibiting electrically induced contraction and induces a variety of agonists, such as 5-HT, acetylcholine, histamine and others (Sarin et al., 2013). It is also known in the scientific literature terpenes, diterpenes and sesquiterpenes act in the release of autocoids and thereby inhibit the motility and intestinal secretion induced by castor oil (Yasmeen et al., 2010). Then on the results presented of phytochemicals present in MeOHIs groups can associate their antidiarrheal owned by the inhibition of prostaglandins, and also by increasing the reabsorption of water and electrolytes, and also inhibit intestinal motility. With this the methanol extract of *Indigofera suffruticosa* contains pharmacologically active compounds with anti-diarrheal properties.

Pyrexia induced by yeast is called pathogenic fever (Ismail et al., 2015). The hyperthermia reduced by AINES, results from the inhibition of prostaglandins synthesis within hypothalamus (Kevin and Gordon, 2007, Pingsusaen et al., 2015). Reduction of fever observed shows that the antipyretic effect of the extract is mild compared to that of the standard. In this study, the mechanism action of methanol extract of *Indigofera suffruticosa* which it causes reduction in rectal temperature may be too blockade of cycloxygenase enzyme activity and consequently the inhibition of prostaglandins.

Conclusion

In conclusion, this study thus proves that the Methanol extract of *Indigofera suffruticosa* pods presents significant antidiarrhoeal property due to its inhibitory effect both on fluid secretion and gastrointestinal propulsion, besides presenting the antipyretic activity expanding the use of this plant in traditional medicine and thus can be developed for use in the treatment of diarrhoea.

Conflict of interest

The authors declared there is not any conflict of interest.

Acknowledgements

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Appendices

Table 1. Effect of MeOHIs (200 and 400 mg/kg) on castor oil induced diarrhea.

Treatment	Dose (mg/kg)	Total nª of stools	Nº of dry stools	Nº of pasty stools	Nº of wet stools
Control	-	12,8 ± 3,81	0 ± 0	0 ± 0	12,8 ± 3,81
Loperamide	2	4,4 ± 4,07	0,8 ± 1,78	0,8 ± 1,78	2,8 ± 4,38*
MeOHIs	200	7,8 ± 7,66	4,2 ± 4,38	1,8 ± 2,48	1,8 ± 1,78*
MeOHIs	400	4,6 ± 2,19	1,6 ± 1,51	1,2 ± 2,16	1,8 ± 1,48*

* Data are presented as mean ± SD. P values are significantly different from control using Tukey post hoc test (n = 6). $p < 0.0001$

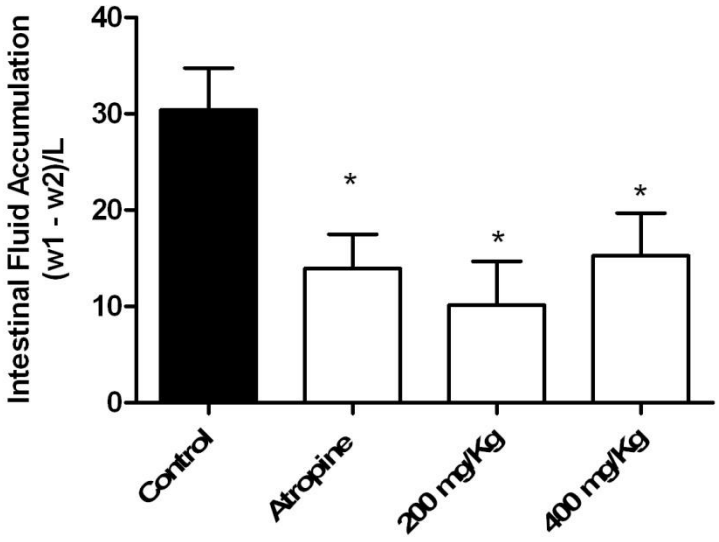


Figure 1. Effect of MeOHIs on enteropooling induced by castor oil in mice. p <0.0001 vs. Control.

Table 2: Effect of MeOHIs (200 and 400 mg/kg) on small intestinal transit in mice

Parameters/ dose	Control	Atropine sulphate	MeOHIs	
			200	400
LSI (cm)	49.8 ± 2.3	51 ± 2*	55.4 ± 3.3*	48.8 ± 2.6*
DC (cm)	39.5 ± 2.7	10.2 ± 1.5*	9.3 ± 2.5*	5.8 ± 1.3*
Intestinal transit (%)	79.3 ± 5.7	19.9 ± 2.8*	16.9 ± 5*	12 ± 3.1*

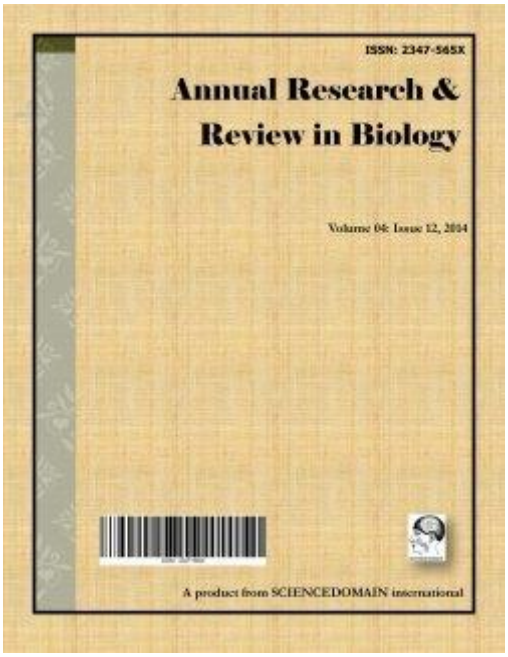
* Data are presented as mean ± SD. P values are significantly different from control using Tukey post hoc test (n = 6). $p < 0.0001$.

Table 3. Effect of MeOHIs (200 and 400 mg/kg) on body temperature in yeast induced pyrexia.

Groups	Rectal temperature C^{o} after 24hrs of Yeast injection					
	Baseline	0h	1h	2h	3h	4h
Control	36.5±0.32	37.8±0.2	38.3±0.4	38.3±0.3	38±0.4	37.6±0.5
Paracetamol	36.6±0.75	38.2±0.3	36.1±0.7**	35.9±0.3**	35.6±0.2**	35.6±0.3**
MeOHIs (200 mg/kg)	36.5±0.57	38.3±0.4	38±0.1†	37.8±0.2†	37.7±0.2†	37.6±0.2†
MeOHIs (400 mg/kg)	36.8±0.54	38.3±0.5	37.7±0.6*†	37.4±0.4**†	37.2±0.6**†	36.9±0.5**†

Values are expressed as Mean ± SD, n = 6 in each group, Statistical analysis of data was performed using ANOVA and Student's "t" test to study the differences amongst the means. *indicate $p < 0.05$ ** indicate $p < 0.001$. $^{\dagger}p < 0.05$, comparing the same group at different times.

4.4 Artigo: *Indigofera suffruticosa* Mill. (Anil)*:* Plant profile, phytochemistry and pharmacology review



Artigo a ser submetido ao periódico *Annual Research & Review in Biology* no formato *Review Article*

Indigofera suffruticosa Mill. (Anil)*:* Plant profile, phytochemistry and pharmacology review

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Authors' contributions

This study was carried out in collaboration between all authors. Authors JKLC and TFSA designed the work and managed the literature searches. Author JKLC, CBS organized figures, conducted the search and prepared the initial draft. Author CAS made corrections. Author VLML managed cross-opinion corrections, reviewed and proof read the manuscript. All authors read and approved the final manuscript

ABSTRACT

Indigofera suffruticosa Mill(Fabaceae), a widely used popular medicine, was known as Anil or Anileira and other names, due to the production of a blue pigment extracted and commonly used for yarn dyeing. It was used with febrifuge, anti-spasmodic, diuretic, abortive, analgesic, against stomach and urinary problems, jaundice, ulcers, purgative, sedative and insecticide. At the same time, *Indigofera suffruticosa* can be used as animal food and pigment. This review aimed to provide widely important date on the botanical, distribution, ethnopharmacology, phytochemical, pharmacological and toxicity of *Indigofera suffruticosa* on scientific literature based. Information on *Indigofera suffruticosa* was gathered via the internet (using Elsevier, NCBI, Sci-hub) and libraries. More than 40 chemical compounds have been identified and a few isolated, and the main origin are the essential oils, organic extracts and aqueous extracts of differents parts of plant. *Indigofera suffruticosa* and its active principles possess wide pharmacological actions in the literature, such as anti-inflammatory, antibacterial, antifungical, antioxidative, antitumor, antimutagenic, anticonvulsivant, gastroprotective and hepatoprotective activities. Therefore, as an important traditional popular medicine, further studies on *Indigofera suffruticosa* can be used to the development of new drugs and therapeutics for various diseases.

Keywords: Popular medicine; Phytochemical; Pharmacological, Indigofera suffruticosa.

1. INTRODUCTION

Present in the Brazilian biodiversity, the Fabaceae family is considered the third largest family of plants that has about 19,500 species [1], is divided into three subfamilies: Mimosoideae, Caesalpinioideae and Papilionoideae and shows a common feature in almost all of presenting similar to fruits vegetables, known as pods [3].

This family is considered of great importance, because among the several varieties of many species are used for food purposes, and is used as animal feed, latex, resins, raw material in the manufacture of paints, pesticides, medicinal drugs in your state gross (*Dioclea megacarpa, Vatairea paraensis* and *Dipteryx puncata*) and ornamental trees. Examples of species used as food sources are: chickpea (*Cicer arietnum*), peas (*Pisum sativum*), beans (*Phaseolus vulgaris*), lentil (*Lens cultivaris*) and soybean (*Glycine max*) [2].

The genus *Indigofera* belonging to Fabaceae family stands out for being used as fodder [4], green manure and ground cover [5]. This genus has about 700 species distributed in Asia, tropical Africa, Australia, North and South America In Brazil it is possible to find three species with same popular name “Anileira”: *Indigofera truxillensis, I. hirsuta, and I. suffruticosa* [6].

A few decades ago, the investigations of *Indigofera suffruticosa* have focused on their biological activities, including their anti-tumor [7], anti-inflammatory [8], antimicrobial [9; 10] and antiepileptic [11]. These studies evaluated the biological potential of different parts of the plant with chemical materials from extractions isolated by various solvents. This review aimed to provide important amplamentes data on the botanical, distribution, ethnopharmacology, phytochemical, pharmacological and toxicity of *Indigofera suffruticosa* based on scientific literature.

2. Botanical characterization and distribuitions

Indigofera suffruticosa is described as a shrub plant, measuring 1m to 2 m tall (A), having branches pubescent, stem angular, of grayish color, pinnate leaves

(B) composed of 7-15 leaflets oblong or oval, hairless on the face and back, with small flowers (D) , numerous albo-pink or yellow, in axillary racemes, and its fruit (C) is a small sickle pod with 6-10 seeds measuring 25 mm in length [12]. Having strong adaptability, they are considered wild plants that grow in all types of soils, tolerating drought, floods and high salinities.

The *Indigofera suffruticosa* Mill. (Figure 1) is a species from the Antilla and Central America [13] more prevalent throughout the tropical America. In Brazil, it is distributed in some states: São Paulo, Sergipe, Bahia, Rio de Janeiro, Minas Gerais, Maranhão [14; 15], Mato Grosso [16], Alagoas [17], Paraíba [18], Ceará, Rio Grande do Norte, Pernambuco and Pará [19].

Figure 1. A) Shrub *Indigofera suffruticosa* measures approximately 1.15 mt. B) leaf and inflorescence; C) branches with leaves and seeds; D) branches with flowers, leaves and inflorescence.

3. Traditional use and ethnopharmacology

This plant is commonly known in the population as "indigo", "anileira" or "indigo", named for German, due to the production of a blue pigment extracted, which is obtained by warm infusion fermentation of its leaves, and commonly used for yarn dyeing. Currently, this extraction is processed by industrial chemical processes and the use of this plant for this economic character was abandoned [6]. The *I. suffruticosa* may also be related to other popular names such as jiquilite, tzitzupu, indigo fields, anileira guinea, real anileira, caá-chica, caá-chira, timbó-mrim, timbozinho and indigueira. The species is widely used in folk medicine in many health problems with uses based on infusions and decoctions of different parts of this plant [20]. They are attributed to this plant febrifuge, anti-spasmodic, diuretic, abortive, analgesic, against stomach and urinary problems, jaundice, ulcers, purgative, sedative and insecticide [21].

4. Chemical constituints

Several studies have identified isolate some chemical constituents of *I. suffruticosa*, including flavonoids, alkaloids, coumarins, and triterpenoids carbohydrates. Early investigations of the chemical components of *I. suffruticosa* were made by Miller and Smith, 1973 using seed extract and with a highlight the rich presence of amino acids and possible toxic effects. According to the Natural Products Alert [22] and Chemical Abstracts the phytochemical profile of this species reveals the presence of alkaloids, polyphenols, terpenoids and/or steroids, reducing sugars, proteins and indigóides.

Paiva et al (1987)[23] conducted quantifying protein and natural fiber of this species making its indication for food for ruminants. Isolation of 3-nitropropanoic acid glucose esters of this type is featured by having toxic effects due to its conversion to the 3-nitropropanoic acid, a respiratory toxin that inhibits mitochondrial enzymes [24; 25] .D-(+)-Pinitol, β -sitosterol and louisfieserone too have isolated from of this plant [25. Apart from this isolated Kamal and Mangla (1993)[26], identified, characterized and quantified six rotenoids from different parts of *I. suffruticosa*. Preliminary studies of leaves, seeds and stems of *I. suffruticosa* demonstrate the presence of polyphenols (coumarin and chlorogenic acid) and flavonoids (quercetin, rutin and gallic acid), alkaloids, triterpenoids, and carbohydrates [27; 28].

The main flavonoid identified and isolated from Methanol extract of *I. suffruticosa* leaves include: quercetin 7-O-β-d-glucopyranoside , quercetin 3-O-[β- d-xylopyranosyl-(1→2)-β-d-galactopyranoside] , quercetin 3-O-[α-l-rhamnopyranosyl-(1 → 6)-β-d-glucopyranoside], quercetin 3-O-[β-d-glucopyranosyl- (1 → 2)-β-d-glucopyranoside. Also were isolated bis-indole derivatives too: indigo and indirubin [28].

Pentadecanoic acid,14-methyl-, methyl Ester, n-hexanedecanoic acid, z-[13, 14-epoxy]tetradec-11-en-1-ol acetate, oleic acid, 9-octadecenoic acid[z]-,2-hydroxy-1-[hydroxyl methyl]ethyl Ester, heptanoic acid, docosyl Ester, octadecanoic acid, 7-hydroxy-, methyl Ester, 6-octadecenoic acid[z]-, 8-octadecenoic acid, methyl ester were indentified of ethanol extract of *I. suffruticosa* leaves [29].

Chen et al. (2013)[30] using aqueous and ethanol extracts of *I. suffruticosa* identified the following different phenolic compounds: Syringic acid, p-Coumaric acid, Vanillin, Syringaldehyde, Salicylic acid, Quercetin, Isoliquiritigenin and Formononetin.

(Z)-3-Hexenyl benzoate, Methyl hexadecanoate, Phytol, Linoleic acid, Methyl linoleate, n-Docosane, n-Tricosane were indentified from leaf oil of *I. suffruticosa* [31].

5. Pharmacological activities

5.1 Embryotoxic and Cytotoxic activity

Leite et al. (2004)[9] investigated the cytotoxic potential of aqueous extracts of leaves of *I. suffruticosa* in mouse embryos and found that at high concentrations of the extract, the growth of the embryos were inhibited, preventing them reached the final stage of embryogenesis, indicating that their use in high doses in humans can be harmful.

Vieira et al., 2007 [7] realized in his studies that aqueous extracts of leaves of *I. suffruticosa* by infusion and maceration in different concentrations (from 6.25 to 50 µg ml^{-1}) tested in cell lines of HEp-2 by MTT method, did not produce any

cytotoxic effect (> 30 µg ml^{-1}) when compared with the control and DMEM (Dulbeccos' Modified Eagle Medium).

In another study, Indigo ethanol extract purifier *I. suffruticosa* showed potent cytotoxic agent showing values of 0.89 for breast tumor cell lines (LM2) and lung (LP07), clarifying that the extract has cellular response inducing apoptosis [32].

Carli et al. (2010)[33] also observed cytotoxic effect on cell viability assays with 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium Bromide (MTT), having inhibitory concentration (IC_{50}) of 200µg / ml.

Vieira et al. (2012)[34] used aqueous *I. suffruticosa* extracts at varying doses (250-1000 mg / ml) in models for embryotoxicity in the development and oviposition of *Aedes aegypti*. In such study found a significant repellent effect on oviposition and also in embryotoxicity, slowing the normal growth of larvae of *Aedes aegypti*.

5.2 *In vivo* activity against Ectoparasite (*Pediculosis capitis*)

Garcia et al. (2011)[35] using ink *I. suffruticosa* 5% reduction in population found *Pediculosis capitis* and removal of lice infestation in cases of persistent in patients, 55 years old, after 2 days of application

5.3 Antimutagenic activity

Calvo et al. (2011)[36] evaluated the ethanol extract of the aerial parts of *I. suffruticosa* in trials with Salmonella showed mutagenic activity, suggesting that such action is due to the presence of indigo and also suggests that the use indiscriminaod and homemade preparations can be harmful to health.

5.4 Antioxidant activity

Ethanolic extracts of leaves of *I. suffruticosa* stands out with potent antioxidant in an experimental model *in vitro* with the free radical 2,2-diphenyl-1-

picrylhydrazyl (DPPH), this action is attributed to high concentrations of gallic acid that has this extract [31].

5.5 Hepatoprotective activities

I. suffruticosa aqueous extract (50 mg / kg, ip) showed protective effect on liver tissue of mice bearing Sarcoma 180 (Silva et al., 2014)[37]. Lima et al. (2014)[38], using the purified compound from the leaves of *I. suffruticosa,* indigo not observed reduction in sarcoma 180 tumor, but found that liver cells remained preserved, emphasizing its hepatoprotective effect.

5.6 Antimicrobial activity

Santos et al. (2015)[39] evaluated the microbial activity ether extract, chloroform and acetone *I. suffruticosa* against nine strains of *Staphylococcus aureus* with minimal inhibitory concentration (MIC) ranging from 0.78 to 6.25 mg / ml. The methanol extract of the aerial parts of *I. suffruticosa* showed significance against *Mycobacterium tuberculosis* with MIC 125 µg/mL, suggesting an important bactericidal source [33].

Fungi isolated endophytic *I. suffruticosa* also showed activity against different bacteria such as *B. subtilis, S. aureus, E. coli, K. pneumoniae, P. aeruginosa*, with a MIC ranging from 0:39 to 6:25 mg / ml, emphasizing that this species has an important potential pharmaceutical [40].

5.7 Antifungal activity

Aqueous extract of leaves of *I. suffruticosa* displays significant results against two strains of *Trichophyton rubrum* and *Microsporum cani*, with concentrations of 5 and 10 mg/ml with MIC ranging between 20 and 15 mm, with similar effect to standard drug ketoconazole (MIC -20 mm)[10].

5.8 Anticonvulsant activity

I. suffruticosa fluid extract (0.06g / kg for 10 days) in experimental models of shock, promotes protective effect in both dose administered orally as intraperitoneally on seizures induced by this model, then highlighting the potential of such antiepilepitic extract [41].

At concentration of 0.06 g/kg also the fluid *I. suffruticosa* extract was tested in models of chronic epilepsy, acting in reducing the concentration of inhibitory amino acids (glycine and tannin) on increasing the excitatory amino acid glutamic acid [42].

In models of seizures induced methanol extract of the leaves of *I. suffruticosa* showed significant activities, their action involving the presence of secondary metabolites such as flavonoids and linolol having action on the GABAergic system [43].

5.9 Gastroprotective activity

Luiz-Ferreira et al. (2011)[44] Exploring the gastroprotective effect Chloroform and Methanol extracts of aerial parts of *I. suffruticosa*, partitioned with ethyl acetate and administered at a dose of 100 mg / kg significantly inhibited the gastric mucosal lesions induced by ethanol and nonsteroidal drugs in rats.

5.10 Anti-tumor activity

Vieira et al., (2007) [7] evaluated the effect of aqueous extracts *I. suffruticosa* processed by maceration and infusion at a dose of 50 mg/kg in sarcoma 180, and found that in both forms used significant effect in reducing the tumor (62.6 and 64.5%), respectively.

5.11 Immunostimulatory activities

Carli et al. (2010)[33], investigating the potential of ethanol extracts of *I. suffurticosa* on immune activity *in vitro* observed that the extract triggered a high nitric oxide production and stimulation of synthesis and TNF-α release, thus

triggering the activation of macrophages, thus promoting production of other molecules that act on the improvement or restoration of responsiveness of the innate immune system reaction against infections.

In another study using a purified compound of *I. suffruticosa*, the Indigo also in experimental models *in vitro* showed an increase in the production and release of nitric oxide and TNF-α [32].

5.12 Anti-inflammatory activity

Aqueous extracts of *I. suffruticosa* (250 mg/kg) in experimental models of mice showed significant anti-inflammatory effect, with similar action to the commercial standard drug, acetyl salicylic acid [8].

In another study, aqueous and ethanolic extracts of leaves of *I. suffruticosa* were used in experimental models of inflammation induced by LPS in macrophages and it was possible to observe a significant anti-inflammatory effect [45].

6. Toxicity

In studies of aqueous extracts of leaves of *I. suffruticosa* by infusion for acute toxicity in mices could check no presence of deaths in the groups tested, however some slight signs of toxicity were observed following i.p. and hours after the lowest concentration to the highest dose tested dose (2400 mg kg^{-1}): Agitation, piloerection, exhaustion, sleepiness, irritability and spasms. Also found that the LD50 of the acute toxicity of aqueous extract of leaves of *I. suffruticosa* made by infusion administered in different doses in mice showed no mortality during 72 h of observation [7].

Methanol extract of leaves of *I. suffruticosa* showed a low toxicity with an LD_{50} of 1600 mg/kg (ip) in mice. The results exhibits a lower significant changes in individual behavioral and parameters slight decrease in spontaneous locomotor activity and an increase inbreathing frequency [43].

7. Conclusions

Plants since ancient times has been used as medicine, and has been daily providing inspiration for new research aiming to highlight the diverse potential and expand the library of biologically active molecules.

I. suffruticosa in recent years has attracted the attention of many researchers, due to their high therapeutic value in the population. Different *I. suffruticosa* extracts of various categories of exhibit pharmacological activities such as antitumor, antioxidant, anti-inflammatory, antimicrobial, antiepileptic, antifungal, anticonvulsant, gastroprotective and hepatoprotective.

These studies were tested *in vivo* in laboratory animals, and the results presented are not sufficient for use in humans. Based on the low toxicity presented by the statement and little research of their phytochemicals, new clinical trials should be conducted in order to fill the gaps in research that need to be overcome in order to establish with certainty the medicinal use of this kind eliminating potential harmful risks and promoting beneficial effects.

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COMPETING INTERESTS

Authors have declared that no interests exist.

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5 CONCLUSÕES

Os extratos orgânicos de *I. suffruticosa* são constituídos predominantemente por fenilpropanóides, alcaloides, flavanóides, protoantocianidina. Todos os extratos apresentaram atividades anti-inflamatória e antinociceptiva em modelos experimentais em camundongos (400 mg/kg), bem como, atividade antioxidante *in vitro* com DPPH.

O extrato metanólico de *I. suffruticosa* apresenta atividade antidiarréica em modelos experimentais induzidos com óleo de Rícino em camundongos (200 e 400 mg/kg), e também atividade antipirética em modelos de febre induzida com *Saccharomyces cerevisiae* em camundongos (200 e 400 mg/kg).

A SFIs apresentou atividade anti-inflamatória e analgésica em ambas as doses testadas (20 e 40mg/kg), enquanto que na atividade antipirética apenas a dose de 40 mg/kg em 2 horas de tratamento apresentou efeito.

Os diversos resultados apresentados corroboram com o uso popular da *Indigofera suffruticosa* que descrevem ações: antinociceptiva, anti-inflamatória, antidiarréica, além da contribuição para o acervo científico, bem como, sua potencial utilização para o desenvolvimento de novos fármacos.

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ANEXOS

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- Conclusions
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BMC Complementary and Alternative Medicine will not edit submitted manuscripts for style or language; reviewers may advise rejection of a manuscript if it is compromised by grammatical errors. Authors are advised to write clearly and simply, and to have their article checked by colleagues before submission. In-house copyediting will be minimal. Non-native speakers of English may choose to make use of a copyediting service.

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Help and advice on scientific writing

The abstract is one of the most important parts of a manuscript. For guidance, please visit our page on Writing titles and abstracts for scientific articles.
Tim Albert has produced for BioMed Central a list of tips for writing a scientific manuscript. American Scientist also provides a list of resources for science writing. For more detailed guidance on preparing a manuscript and writing in English, please visit the BioMed Central author academy.

Abbreviations

Abbreviations should be used as sparingly as possible. They should be defined when first used and a list of abbreviations can be provided following the main manuscript text.

Typography

- Please use double line spacing.
- Type the text unjustified, without hyphenating words at line breaks.
- Use hard returns only to end headings and paragraphs, not to rearrange lines.
- Capitalize only the first word, and proper nouns, in the title.
- All pages should be numbered.
- Use the **BMC Complementary and Alternative Medicine** reference format.
- Footnotes are not allowed, but endnotes are permitted.
- Please do not format the text in multiple columns.
- Greek and other special characters may be included. If you are unable to reproduce a particular special character, please type out the name of the symbol in full. **Please ensure that all special characters used are embedded in the text, otherwise they will be lost during conversion to PDF.**

Units

SI units should be used throughout (liter and molar are permitted, however).

Introduction

PHYTOMEDICINE

International Journal of Phytotherapy and Phytopharmacology

Scope

Phytomedicine is primarily a therapy-oriented Journal, which publishes innovative studies on efficacy, safety, quality and mechanisms of action of specified plant extracts, phytopharmaceuticals and their purified constituents. This includes clinical and preclinical studies of properly standardized herbal medicinal products, herbal preparations and isolated compounds, which have reproducible pharmacological activity.

The journal covers the following sections:

- Clinical pharmacology and toxicology (randomized, placebo controlled, double blind, and observational open label studies),
- Behavioral, mental, affective, and stress-associated disorders,
- Age-associated disorders,
- Neuropharmacology,
- Endocrine pharmacology,
- Metabolic syndrome and obesity,
- Cancer,
- Immunopharmacology, inflammation,
- Infectious diseases,
- Pulmonary diseases,
- Gastrointestinal diseases,
- Cardiovascular diseases,
- Urogenital diseases,

- Systems biology,
- Pre-clinical toxicology of herbal preparations,
- Interaction with drugs,
- Pharmacokinetic of natural compounds,
- Standardization and quality of herbal preparations,
- Legislation of botanicals,
- Invited reviews

Before You Begin

Article requirements

Please note the following requirements for consideration of an article, upon submitting your manuscript:

1. Is your article within the scope of Phytomedicine?
Your article must meet the scope of Phytomedicine (please see above). **Articles that are not in the scope, will be rejected immediately!**

- Articles on the isolation and structure elucidation of novel bioactive compounds or the development of new analytical methods do not fall into the scope of Phytomedicine. However, pharmacological and clinical studies of novel natural products, where new compounds or methods of analysis of active of pharmaceutical ingredients in herbal preparations and biological fluids and tissues are reported (e.g. in pharmacokinetic studies), are welcome.
- Dietary Supplements, "Botanicals" or "Functional Food" are not within the scope of Phytomedicine unless they are specified/standardized and pharmacologically investigated analogues to herbal drugs and if the evidence presented is comparable to therapeutic outcomes with a positive control.
- Studies on pure compounds are not accepted if their origin is not clearly related to the plant kingdom.
- Pharmacological studies of isolated compounds in various forms (salts, ethers, etc.), which do not exist in nature are out of scope of Phytomedicine.
- Screening results of a large number of plant extracts or plant constituents for pharmacological activities will not be considered unless they are focused on those plants or constituents which show superior activities in comparison with generally accepted positive (reference) compounds.

2. Does your article comply with the standard requirements of Phytomedicine?
Your article must meet the criteria assuring reproducible quality and efficacy of herbal preparations.

- Plant name and herbal substance
Latin binomial name and the author, local name and English name and plant part(s) used must by specified for all plants used in the study. It should be stated that the plant name has been checked with http://www.theplantlist.org. The authentication of fresh plants or dried herbal drugs, including those of formulas, must be carried out by means of macroscopic and/or microscopic, molecular biological, chemical, chromatographic and/or other suitable pharmacognostic methods. Voucher specimens of plant materials used for all studies must be deposited and identified with a voucher number, the date and location of collection. The plant material may derive from natural origin, from

cultivated plants, or from an herbal drug market. In case of commercially procured material the source, batch number, and quality control data should be specified. All scientific names of the plants must be written in italics through the whole manuscript!

- Herbal medicinal products and herbal extracts
  Herbal medicinal products or herbal preparations must be declared in accordance to EMA guidelines(http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003272.pdf). In particular, herbal extracts must be clearly and comprehensively described with respect to the plant part used, the drug extract ratio, type and concentration of extraction solvent, extraction conditions etc. They must be sufficiently characterized (e.g. by HPLC fingerprints) and specified for the content of marker compounds to ensure a consistent quality and reproducible pharmacological activity. The choice of marker must be justified. The analytical methods have to be validated for selectivity, accuracy and precision and briefly described, providing the most important information necessary to obtain reproducible results. Traditional and commercial names of herbal preparations should be mentioned in the Introduction of the manuscript, but not in the title. Phytomedicine accepts only international standard terminology – binomial Latin names of the plants and their combinations.
- Herbal combinations
  Studies with herbal drug combinations (e.g. 2-5 plants) will be accepted only if each herbal drug undergo the same authentication and standardization process as described above, each single herbal extracts is HPLC fingerprinted and relevant marker constituents are quantified before and after the extracts are mixed. A 3-D-HPLC-profile of the multiherbal drug combination must be provided, e.g.: Amagaya S. et al. 2001, Phytomedicine 8: 338-347.http://www.sciencedirect.com/science/article/pii/S0944711304700495; For TCM-multi-herbal drug combinations (formula) see: Zeng K.-W. et al. 2012, Phytomedicine 19, 122-129.http://www.sciencedirect.com/science/article/pii/S0944711311002674.Additionally, we encourage the use other relevant and validated physiological, biological, or biochemical methods, which ensure reproducible pharmacological activity of multi-herbal drug combinations.

- Chemicals, phytochemicals and other purified compounds For purified compounds, please provide chemical names using relevant information from the NCBI PubChem which can be found on the websitehttp://www.ncbi.nlm.nih.gov/pccompound. In studies with purified compounds the evidences of their purity (13C NMR or HPLC peak purity test) are required.

- Gene nomenclature Authors should use approved nomenclature for gene symbols. Please consult the appropriate nomenclature data bases for correct gene names and symbols. "Entrez Gene" is a useful resource. Approved

human gene symbols are provided by HUGO Gene Nomenclature committee (HGNC):http://www.gene.ucl.ac.uk/nomenclature Approved Mouse symbols are provided by The Jackson Laboratory:http://www.informatics.jaxorg/mgihome/nomen Approved C. elegans symbols are provided by Caenorhabditis Genetics Center:http://www.cbs.unmn.edu/CGC/Nomenclature/no menguid.htm For approved S. cerevisiae and S. pombe symbols seehttp://yeastgenome.org/help/yeastGeneNomenclature.shtml andhttp://www.sanger.ac.uk/Projects/S_pombe/SP_Name_FAQ.shtml, respectively

- Statistical analysis
  Statistical hypothesis and methods should be described in detail. Actual P values should be used unless less than 0.001. Reporting of 95% confidence intervals is encouraged. The choice of appropriate parametric or nonparametric tools has to be justified. Refer to B.S. Evererett. Statistical Methods for Medica Investigations, Oxford University Press, New York, 1989.

3. Is your article approaching new findings?
Scientific novelty of your study must be clearly demonstrated. The articles limited with a repetition of well-known data or identification of only well-known ubiquitous compounds with little or no relation to activity are not acceptable.

4. Is your article relevant to clinical medicine?
Your article must be based on a thorough study, using proper controls and convincing evidences of therapeutic significance and observations.

Not acceptable are:

- *In vitro* studies with concentrations of active compounds, which could not be implemented in-vivo and that are not appropriate for further pharmaceutical development.
- *In vitro* studies without results on organs, tissues, fluids or cells.
- *In vitro* studies without positive control.
- *In vivo* single dose studies or studies with one set of experiments and few animals.
- Studies on antimicrobial activity with only single dose, very high concentration, measuring only inhibition zones without MIC values, without information on type of activity (or growth inhibition) or microorganisms investigation.
- Pharmacological studies of pure compounds, which are not supported by evidences on pharmacological activity of plant extract where it was identified.

5. Does your article meet the requirements to clinical and pharmacological studies?
Your article must comply with the basic criteria for conducting and reporting clinical and pharmacological studies.

Requirements for clinical studies:

- Clinical studies must meet the current standards for clinical trials (GCP = Good Clinical Practice), which are equivalent to those required for synthetic drugs. http://www.fda.gov/downloads/Drugs/Guidances/ucm073122.pdfhttp://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002832.pdf

- Articles should be in line with Extensions of the Consolidated Standards of Reporting Trials Statement for Herbal Medicinal Interventions (CONSORT), particularly when it comes to description of study medication, which is a strict requirement of acceptance for Phytomedicine. For guidelines and necessary information, please use the following internet addresses: http://www.consort-statement.org http://www.consort-statement.org/extensions?ContentWidgetId=557. http://www.consort-statement.org/Media/Default/Downloads/Extensions/CONSORT Extension for Herbal Interventions.pdfhttp://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003370.pdf. Use of a CONSORT checklist and flow diagram is recommended for illustration of grouping and flow of patients in all clinical studies, randomized clinical trials as well as other trials.
- Clinical studies must be approved by an Institutional Ethics Committee or its equivalent. The Methods section must state that the study followed the guidelines of the Declaration of Helsinki and Tokyo for humans!

Requirements for pharmacological studies (*in vitro*, ex vivo or *in vivo*):

- Investigations with animals must state in the Methods section that the research was conducted in accordance with internationally accepted principles for laboratory animal use and care (e.g. European community guidelines/ EEC Directive of 1986 or the US guidelines/ NIH publication).
- The route of drug administration, different of oral, must be justified.
- Appropriate and justified statistical methods must be used.Positive controls (reference standards must be included in study design).
- Many natural compounds are known for their polyvalent (pleiotropic) activities and are only of interest if one or two pharmacological activities are dominant and somehow superior in comparison with generally accepted reference standards/compounds. Their potential therapeutic application must be justified for specified indication.
- Antimicrobial evaluation of plants are of scientific value only if these plant extracts show superior biological activities in comparison with a synthetic or natural antimicrobial agent standard. It is preferred that *in vitro* activity (MIC) of an extract in not higher than 100 ìg/ml. For the correct determination of MIC values, standardized methodologies such as those of CLSI or EUCAST are preferred.
- All articles that are reporting gene expression profiling data (microarray experiments) should comply with the Minimum Information about Microarray Experiments (MIAME, http://www.mged.org/Workgroups/MIAME/miame.html).
- At least two microarrays should be provided for each experimental condition. Results of selected genes should be validated by a second method (e.g. RT-PCR) or protein data should be provided. In addition functional test (animal experiments/clinical data) undertaken simultaneously are desirable to allow an appraisement of the biological/clinical relevance of the data. Alternatively, results of *in vivo* experiments with comparable dosages can be discussed. The presentation of a sole data collection is not acceptable. Biologically relevant information should be presented.

- We recommend do not overuse specific names, notions and terms from various theories of traditional medical systems (e.g. TCM, Ayurveda, etc.). That makes articles difficult for perceptions and understanding. The essence of these theories should be translated into internationally accepted scientific theories, while traditional names and terms should be converted to English. Final interpretation of the results of the study must adhere to conventional scientific theories.

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Preparation

Types of manuscript

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Articles should not exceed **12-15 typewritten pages** or up to **5,000 words,**including references, tables and figures. Previously reported methods

should be referenced only. The number of references should not exceed 30 (except for review articles or reports on microarray data).

Short communications
Short communications should be condensed to **4-8 typewritten pages** or not more than **2,500 words** including references and a maximum of two illustrations.

Review articles
Review articles will only be by invitation. Review articles can provide concise and critical updates on a subject of current interest. Herbal drug-monographs are only acceptable if they contain the newest pharmacological and toxicological issues and an outlook on future directions.

Prof. Hildebert Wagner Award
The "Prof. Hildebert Wagner Award" was created to honor the outstanding efforts of Prof. Wagner for the journal Phytomedicine. This award will be granted to a graduate student or young post-doctoral researcher who is the first author of a paper reviewed by the Editors of Phytomedicine to be the best one in the Journal during the previous calendar year. The prize will be sponsored by Elsevier with EUR 500 for the awardee and a certificate for every Co-Author. Additionally an official notice will be published on the journal homepage of Phytomedicine(http://www.elsevier.com/locate/phymed), on which the article will be available free of charge for one year. The reviewing editors for the first contribution to be awarded in Phytomedicine will be Prof. Hildebert Wagner himself, Prof. Alexander Panossian, and Prof. Susana Zacchino. To qualify, nominees must be younger than 35 years and an outstanding contribution to the field must be provided. Nominations can be made by first authors (resp. corresponding authors).

Nominations for the first "Prof. Hildebert Wagner Award" in 2016 can be done until June 30, 2016. The announcement of the winner will be by end of October 2016. Please choose Award-Article from the drop-down menu below, if you want your article to be considered for the Award.

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Example:

Anti-stress effects of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol in immobilized mice

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Abstract

A concise and factual abstract is required. Abstracts should summarize the contents of the article in 350 words or less. The abstract should be structured in the following format:
Background: In one or two sentences, summarize the scientific body of knowledge surrounding your study and how this led to your investigation.
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Study Design: Identify the overall design of your study.
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Results: Report the most important results of your study. Only include positive results that are statistically significant, or important negative results that are supported by adequate power. For clinical studies report actual data, not just P values.

Conclusion: State the answer to your original question or hypothesis.Summarize the most important conclusions that can be directly drawn from your study.

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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. Seehttp://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.

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A section of abbreviations should precede the manuscript. Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.
See "Uniform requirements for manuscripts submitted to biomedical journals" (1991) New England Journal of Medicine 324:424–428.

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Introduction

Provide an adequate background, avoiding a detailed literature survey or a summary of the results. State the objectives of the work. No results of the study should be described in this section.

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.

This section should contain some subsections common for almost all studies:

- Plant names and parts used (requirements see above)
- Study medication, herbal extracts (requirements see above)
- Chemical compounds (requirements see above)
- Statistical analysis (requirements see above)
- Assays (requirements see above)
- Animal studies (requirements see above)
- Study design (requirements see above)

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.

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.).

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Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

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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).

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Examples:

Reference to a journal publication:
Wagner., H., Ulrich-Merzenich, G., 2009. Synergy research: Approaching a new generation of phytopharmaceuticals. Phytomedicine 16, 97–110.

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Argyropoulos D, Kudadam J, Müller J, 2009. Color degradation of lemon balm (Melissa officinalis L.) as affected by the drying process. In: 5th International Technical Symposium on Food Processing, Monitoring Technology in Bioprocesses and Food Quality Management, Potsdam, Germany, August 31–September 2, pp. 730–736.

Willcox, M.L., Graz, B., Falquet, J., Diakite, C., Giani, S., Diallo, D., 2011. A "reverse pharmacology" approach for developing an anti-malarial phytomedicine. Malaria J. 10 (Suppl. 1), S8.

Reference to a book:
Cramer, J.A., Spilker, B., 1998. Quality of Life and Pharmacoeconomics. An Introduction. Lippincott-Raven, Philadelphia.

Reference to a chapter in an edited book:
Cragg, G.M., Boyd, M., 1996. Drug discovery and development at the National Cancer Institute: the role of natural products of plant origin. In: Balick, M.J., Elisabetsky, E., Laird, S.A. (Eds.), Medicinal Plan Resources of the Tropical Forest. Columbia University Press, New York, pp. 101–136.

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations: http://www.issn.org/services/online-services/access-to-the-ltwa/.

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4. Review papers:
These papers will not have empirical data acquired by the authors but will include discussion of papers published and data acquired in a specific area. We advise a length of 5000-9000 words, (including 50-150 references plus 3-5 figures and/or tables (if required).

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cannot provide specific guidance for the management of successive patients, they are a record of clinical interactions which help us to frame questions for more rigorously designed clinical studies. Case studies also provide valuable teaching material, demonstrating both classical and unusual presentations which may confront the practitioner. (Ref: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2597880/). Abstract (not more than 250 words) of the Case reports should have the following sections: Aims, Presentation of Case, Discussion and Conclusion. Only Case Reports have word limits: Papers should not exceed 2000 words, 20 references or 5 figures.

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SAMPLE ABSTRACT:

Aims: Here clearly write the aims of this study. Sample: To correlate platelet count, splenic index (SI), platelet count/spleen diameter ratio and portal-systemic venous collaterals with the presence of esophageal varices in advanced liver disease to validate other screening parameters.
Study design: Mention the design of the study here.
Place and Duration of Study: Sample: Department of Medicine (Medical Unit IV) and Department of Radiology, Services Institute of Medical Sciences (SIMS), Services Hospital Lahore, between June 2009 and July 2010.
Methodology: Please write main points of the research methodology applied. Sample: We included 63 patients (40 men, 23 women; age range 18-75 years) with liver cirrhosis and portal hypertension, with or without the medical history of gastrointestinal bleeding. Clinical as well as hematological examination (platelet count) and ultrasonography (gray as well as color Doppler scale including splenic index and splenorenal/ pancreaticoduodenal collaterals) was done besides upper GI endoscopy for esophageal varices. Platelet count/spleen diameter ratio was also calculated.
Results: Kindly make sure to include relevant statistics here, such as sample sizes, response rates, P-values or Confidence Intervals. Do not just say "there were differences between the groups". sample: Out of 63 patients, 36 patients with small varices (F1/F2) and 27 with larger (F3) varices were detected on endoscope. Significant increase in mean splenic index from low (86.7 +/- 27.4) to high (94.7 +/- 27.7) grade varices was documented. Opposite trend was found with platelets (120.2 +/- 63.5 to 69.8 +/- 36.1) and platelets/ splenic diameter ratio (1676.7 to 824.6) declining significantly. Logistic regression showed splenic collaterals and platelets are significantly but negatively associated with esophageal varices grades.
Conclusion: Non-invasive independent predictors for screening esophageal varices may decrease medical as well as financial burden, hence improving the management of cirrhotic patients. These predictors, however, need further work to validate reliability.

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Acknowledgements

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AUTHORS’ CONTRIBUTIONS

Authors may use the following wordings for this section: “ ‘Author A’ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. ‘Author B’ and ‘Author C’ managed the analyses of the study. ‘Author C’ managed the literature searches…… All authors read and approved the final manuscript.”

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1. Hilly M, Adams ML, Nelson SC. A study of digit fusion in the mouse embryo. Clin Exp Allergy. 2002;32(4):489-98.
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For Accepted, unpublished papers.
Same as above, but “In press” appears instead of the page numbers.
1. Saha M, Adams ML, Nelson SC. Review of digit fusion in the mouse embryo. J Embryol Exp Morphol. 2009;49(3): (In press).

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For Articles not in English
Forneau E, Bovet D. Recherches sur l'action sympathicolytique d'un nouveau dérivé du dioxane. Arch Int Pharmacodyn. 1933;46:178-91. French.

Reference to a book:

Personal author(s)
Rang HP, Dale MM, Ritter JM, Moore PK. Pharmacology. 5th ed. Edinburgh: Churchill Livingstone; 2003.

Editor(s) or compiler(s) as authors
Beers MH, Porter RS, Jones TV, Kaplan JL, Berkwits M, editors. The Merck manual of diagnosis and therapy. 18th ed. Whitehouse Station (NJ): Merck Research Laboratories; 2006.

Authored chapter in edited publication
Glennon RA, Dukat M. Serotonin receptors and drugs affecting serotonergic neurotransmission. In: Williams DA, Lemke TL, editors. Foye's principles of medicinal chemistry. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2002.

Reference to Web-resource or Electronic articles.

Hugo JT, Mondal SC. Parallels between tissue repair and embryo morphogenesis: a conceptual framework. Global Health. 2006;16:4. Accessed 29 March 2012. Available: http://www.globalizationandhealth.com/content/1/1/14.
Anonymous. Parallels between tissue repair and embryo morphogenesis: a conceptual framework. Global Health. 2006;16:4. Accessed 29 March 2012. Available: http://www.globalizationandhealth.com/content/1/1/14.

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Diabetes Prevention Program Research Group. A study of digit fusion in the mouse embryo. J Embryol Exp Morphol. 2009;49(2):259–276.

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