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ALISSON MACÁRIO DE OLIVEIRA

AVALIAÇÃO DA TOXICIDADE AGUDA E SUBAGUDA E EFEITOS ANTINOCICEPTIVO, ANTIPIRÉTICO E ANTI-OBESIDADE DE EXTRATO DO CAULE DE *Pilosocereus gounellei*

Recife
2019

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de Doutor em Bioquímica e Fisiologia.

Área de concentração: Bioquímica e Fisiologia

Orientador: Prof. Dr. Thiago Henrique Napoleão

Coorientador: Prof. Dr. Bruno de Melo Carvalho

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“Não seja o de hoje. Não suspires por ontem. Não queiras ser o de amanhã. Faze-te sem limites no tempo”.

(MEIRELES, 1982)

RESUMO

Pilosocereus gounellei, conhecida como xique-xique, é usada popularmente para o tratamento de inflamações. A presente tese avaliou a toxicidade aguda e subaguda e os efeitos antinociceptivo, antipirético e anti-obesidade do extrato salino do caule de *P. gounellei* (ES) em camundongos. A composição química foi investigada por cromatografia de camada delgada e cromatografia líquida de alta eficiência. No ensaio de toxicidade aguda, foram utilizadas doses únicas de 2000 e 5000 mg/kg (v.o.) e análise 7 dias após a administração. Os animais também foram submetidos aos testes de campo aberto e *rotarod*. No teste de toxicidade subaguda, ES (250, 500 e 1000 mg/kg, v.o.) foi administrado diariamente durante 28 dias. Para avaliação das atividades biológicas foram utilizadas as doses 125, 250 e 500 mg/kg (v.o.). Atividade antinociceptiva foi avaliada através dos testes de contorções abdominais induzidas por ácido acético, de imersão da cauda e da formalina. A atividade antipirética foi investigada utilizando modelo de febre induzida por levedura. O efeito anti-obesidade foi testado sobre camundongos submetidos à dieta hiperlipídica. Foram avaliados tolerância à glicose, resistência à insulina, perfil lipídico e índices aterogênicos, bem como marcadores de estresse oxidativo e organização histológica de fígado, músculo e tecido adiposo epididimal. Os animais obesos foram tratados com ES ou solução salina diariamente por 21 dias; um grupo não obeso foi utilizado como controle. A análise fitoquímica revelou presença de flavonoides e açúcares redutores. No teste de toxicidade aguda, o ganho de peso e consumo de ração e água não foram afetados no tratamento a 2000 mg/kg. Não foram detectadas alterações histológicas no fígado, coração, pulmão, rim e baço. Os testes de campo aberto e *rotarod* mostraram que ES não afetou a função motora e a atividade exploratória. No ensaio de toxicidade subaguda, os tratamentos não afetaram ganho de peso, consumo de água e ração e parâmetros hematológicos. Proteinúria e alterações histológicas no fígado e nos pulmões foram detectadas em camundongos tratados com 1000 mg/kg. Atividade antinociceptiva foi detectada nos três modelos utilizados, indicando interferência tanto na dor não-inflamatória quanto inflamatória. ES (500 mg/kg) apresentou efeito antipirético a partir de 60 minutos após ser administrado. Animais obesos tratados com ES apresentaram peso menor e redução nos níveis de colesterol total, LDL-colesterol, triglicerídeos, marcadores da função hepática e renal, bem como aumento da tolerância à glicose e redução da resistência à insulina, em comparação com animais obesos tratados com salina. Os índices aterogênicos também foram menores nos animais tratados com ES, atingindo valores semelhantes ao controle não-obeso no tratamento a 500 mg/kg. De forma geral, ES evitou o aumento da peroxidação lipídica nos órgãos avaliados e aumentou os níveis

de superóxido dismutase. Os animais tratados com ES apresentaram redução da esteatose, da deposição de colágeno e da inflamação no fígado, em comparação ao tratamento com solução salina. A gordura epididimal também foi reduzida nos grupos tratados com extrato. Em conclusão, ES apresentou baixa toxicidade e tem potencial para avaliações futuras visando sua aplicação como agente analgésico e antitérmico e para o controle de obesidade.

Palavras-chave: xique-xique. Análise toxicológica. Atividade antinociceptiva. Obesidade. Atividade antitérmica.

ABSTRACT

Pilosocereus gounellei, known as xique-xique, is popularly used for the treatment of inflammation. The present thesis evaluated the acute and subacute toxicity and the antinociceptive, antipyretic and antiobesity effects of the saline extract (SE) of *P. gounellei* stem in mice. The chemical composition was investigated by thin layer chromatography and high-performance liquid chromatography. In the acute toxicity test, single doses of 2000 and 5000 mg/kg (*per os*) were used and analysis made 7 days after administration. The animals were also submitted to the open field and rotarod tests. In the subacute toxicity test, SE (250, 500 and 1000 mg/kg, *per os*) was administered daily for 28 days. For the evaluation of biological activities, the doses 125, 250 and 500 mg/kg (*per os*) were used. Antinociceptive activity was assessed through the abdominal writhings induced by acetic acid, tail immersion, and formalin tests. Antipyretic activity was investigated using a yeast-induced fever model. The anti-obesity effect was tested on mice submitted to the hyperlipidic diet. Glucose tolerance, insulin resistance, lipid profile and atherogenic indexes, as well as markers of oxidative stress and histological organization of liver, muscle and epididymal adipose tissue were evaluated. Obese animals were treated with SE or saline daily for 21 days; a non-obese group was used as control. Phytochemical analysis revealed the presence of flavonoids and reducing sugars. In the acute toxicity test, weight gain and food and water intake were not affected in the treatment at 2000 mg/kg. No histological changes were detected in the liver, heart, lung, kidney and spleen. Open field and rotarod tests showed that SE did not affect motor function and exploratory activity. In the subacute toxicity test, treatments did not affect weight gain, water and food intake, and hematological parameters. Proteinuria and histological changes in the liver and lungs were detected in mice treated with 1000 mg/kg. Antinociceptive activity was detected in the three models used, indicating interference in both non-inflammatory and inflammatory pain. SE (500 mg/kg) showed antipyretic effect from 60 minutes after being given. Obese animals treated with SE presented lower weight and reduced levels of total cholesterol, LDL-cholesterol, triglycerides, markers of liver and renal function, as well as increased glucose tolerance and reduced insulin resistance compared to obese animals treated with saline. Atherogenic indexes were also lower in SE-treated animals, reaching similar values to the non-obese control in the treatment at 500 mg/kg. In general, SE prevented the increase of lipid peroxidation in the evaluated organs and increased levels of superoxide dismutase. SE-treated animals had a reduction in steatosis, collagen deposition and inflammation in the liver compared to treatment with saline solution. Epididymal fat was also reduced in the extract-treated groups.

In conclusion, SE presented low toxicity and has potential for future evaluations aiming its application as an analgesic and antipyretic agent and for the control of obesity.

Key words: Xique-xique. Toxicological survey. Antinociceptive activity. Obesity. Antithermal activity.

LISTA DE FIGURAS

Artigo 1

(2000 and 5000 mg/ kg oral). Livers: the centrilobular vein (v) is seen in all images. In control, the hepatocyte bundles are preserved and ordered. A few, small greasy micro- vesicles of diffuse form (arrows) in control mice and those treated with the extract at 2000 and 5000mg/kg are visible. Heart: Normal structure, with cardiac muscle (m) and pericardium. No signs of degeneration and necrosis/ inflammation or other changes. Lungs: bronchiole (Br), alveolar duct (Da), al- veolar sac (Sa), and interalveolar septa (short arrows) can be visualized, with preserved architecture in both control and extract-treated mice. Hematoxylin and eosin staining was used. Magnification: 400x. 46

Figure 3 - Representative photo- micrographs of the kidneys and spleens from control mice (oral saline) and those treated with Pilosocereus gounellei stem extract (2000 and 5000 mg/kg oral). Kidneys: Renal glomeruli (Gr) and contorted tubules without altera- tions are visible in control and extract- treated groups. Spleens: The lymphatic nodes (Nd) are well-defined in control and treated groups. Hematoxylin and eosin staining was used. Kidney images: 400x. Spleen images: 100x(upper pa- nels) and 400x(lower panels). 47

Figure 4 - Antinociceptive effect of saline extract from *Pilosocereus gounellei* stem (125, 250, and 500 mg/kg oral) and the reference drugs indomethacin (20 mg/ kg intraperitoneal) and morphine (10 mg/kg intraperitoneal) in the acetic acid- induced writhing assay. The bars represent the mean numbers of writhing ± SD. (***) indicates significant difference ($p < 0.01$) in the number of writhings versus the control. 47

Figure 5 - Antinociceptive effect of saline extract from *Pilosocereus gounellei* stem (125, 250, and 500 mg/kg, oral) and the reference drugs, indomethacin (20 mg/kg, intraperitoneal) and morphine (10 mg/kg, intraperitoneal) on both phases of the formalin assay. The bars represent the mean time spent by the mice licking their paws ± SD. The involvement of opioid receptors in the antinociceptive effect was also evaluated by administering naloxone (20 mg/kg, intraperitoneal) to the mice 30 min before the administration of the extract or morphine. (*) indicates significant difference ($p < 0.01$) in the licking time versus the control. 48

Artigo 2

signs of degeneration, necrosis/inflammation or other changes. Hematoxylin-eosin staining was used. Magnification: 400 ×.

Figure 6 - Representative photomicrographs of the spleens of male and female mice from control group and treated *per os* with the *P. gounellei* stem extract at the doses of 250, 500 and 1000 mg/kg. The lymph nodes (Nd) are well-defined in control and treated groups. Spleens of females that received the dose of 1000 mg/kg showed a considerable activation of lymph nodes. Hematoxylin-eosin staining was used. Magnification: 400 ×. 59

Figure 7 - Monitoring of rectal temperature of yeast-infected and hyperthermic mice after administration of the *P. gounellei* stem extract at the doses of 125, 250 and 500 mg/kg *per os* or 10 mg/kg diclofenac (positive control). 60

Artigo 3

Figure 1 - Intraperitoneal challenge tests of glucose (ipGTT) (A) and insulin (ipITT) tolerance in non-obese (control) and diet-induced obese mice treated with saline (DIO group), metformin (MET group) or with the saline extract of *Pilosocereus gounellei*. Data represent the means ± SEM (n=6). Statistical analysis was performed: (*) indicates $p < 0.05$ vs. control. (a) indicates $p < 0.05$ vs. DIO group. 78

Figure 2 - Representative photomicrographs of the adipose, liver and muscle of male mice from the control group, metformin (MET) and the groups treated daily with the saline extract from *P. gounellei* stem at the doses of 125, 250 and 500 over 21 days. **Adipose:** Significantly reduced in the extract-treated groups, especially at the highest dose, in comparison with the DIO group treated with saline, differently of the metformin group. Additionally, the size of epididymal adipose tissue cells were larger in the DIO and MET groups than in the extract treatment at 500 mg/kg **Liver:** Not detected neutral intracellular lipids in the liver slices of the control group. In the DIO group, there was an increase in the amount of detectable collagen deposition in the perivenular spaces. In the MET group, it was observed a reduction in intracellular fat. In the extract treatments, the livers showed collagen deposition in the perivenular spaces but in a less extent than DIO group. **Muscle:** no alterations were observed in the experimental groups when compared to the control group. The muscle fibers 79

remained in their usual morphology. Hematoxylin-eosin staining was used. Magnification: 400×.

LISTA DE TABELAS

Artigo 1

Artigo 2

Artigo 3

LISTA DE ABREVIATURAS E SIGLAS

AC	Atherogenic coefficient
AIP	Atherogenic index of plasma
ALP	Alkaline phosphatase
ALT	Alanina aminotransferase
ANVISA	Agência Nacional de Vigilância Sanitária
AST	Aspartato aminotransferase
CAT	Catalase
CK	Creatine kinase
CMHC	Concentração média de hemoglobina corpuscular (em inglês MCHC – *mean corpuscular hemoglobin concentration*)
CRI-II	Castelli's Risk Index II
CRR	Cardiac risk ratio
GGT	Gama glutamil transferase
HCM	Hemoglobina corpuscular média (em inglês MCH – *mean corpuscular hemoglobin*)
HDL-c	High-density lipoprotein cholesterol
HFD	High - fat diet
IL-1β	Interleucina 1β
ipGTT	Intraperitoneal glucose tolerance test
ipITT	Intraperitoneal insulin tolerance test
MDA	Malondialdehyde
OECD	*The Organization for Economic Co-operation and Development*
OMS	Organização Mundial de Saúde
PI3K	Fosfatidilinositil-3-quinase

SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive substances
TC	Total cholesterol
TG	Triglycerides
TLC	*Thin layer chromatography*
TNF-α	Fator de Necrose Tumoral alfa
VCM	Volume corpuscular médio (em inglês MCV – *mean corpuscular volume*)
VLDL-c	Very low-density lipoprotein cholesterol

SUMÁRIO

1 INTRODUÇÃO

A procura pela utilização de terapias alternativas e complementares para o tratamento ou cura de enfermidades tem sido cada vez maior devido ao alto custo e aos efeitos adversos dos medicamentos alopáticos. Dentre essas alternativas, estão incluídas as plantas medicinais e os produtos derivados delas (LIMA et al., 2015; NOVELLO et al., 2012). Tendo em vista o uso crescente dessa modalidade de terapia, há uma preocupação quanto ao discurso de que tais produtos são isentos de efeitos adversos e/ou toxicidade simplesmente por terem origem natural (VIEIRA DE SOUZA et al., 2016).

Estudos de plantas medicinais comprovaram que várias espécies vegetais possuem substâncias potencialmente perigosas e, por esta razão, devem ser utilizadas respeitando-se os riscos toxicológicos (VEIGA JR; PINTO; MACIEL, 2005). Além das interações com medicamentos e/ou alimentos, possíveis reações adversas inerentes de preparações vegetais podem depender de características do paciente como gênero, idade e estado de nutricional, entre outros (BALBINO; DIAS, 2010; PAUMGARTTEN, 2016).

O Brasil apresenta a biodiversidade como uma característica marcante de seus ecossistemas, com habitats específicos para plantas não encontradas em outras regiões do planeta (FANG et al, 2015). Dentre eles, merece destaque a Caatinga, cuja diversidade química e farmacológica é pouco explorada, considerando a diversidade de suas fontes vegetais. Ainda, a riqueza cultural do semiárido nordestino representa uma importante fonte de informações para o desenvolvimento de estudos etnobotânicos, os quais constituem uma importante ferramenta para o início de estudos científicos e aproveitamento adequado dos recursos naturais da região (CREPALDI et al, 2016).

A espécie *Pilosocereus gounellei* (F.A.C. Weber ex K. Schum.) Byles & G.D. Rowley, conhecida popularmente como xique-xique, é uma planta cactácea que se desenvolve inclusive nas áreas mais secas da Caatinga. Estudo etnobotânico listou usos trófico (alimentação humana e animal), decorativo e medicinal do xique-xique (ALBUQUERQUE, 2002). Do ponto de vista medicinal, é descrita a utilização de uma pomada produzida a partir do miolo do xique-xique macerado para tratamento de processos inflamatórios decorrentes de perfurações nos membros superiores e inferiores (OLIVEIRA, 2011). Preparações das raízes do xique-xique são utilizadas no tratamento de inflamações prostáticas e na uretra (AGRA et al., 2008; ROQUE et al., 2010).

Atualmente, diferentes drogas analgésicas, incluindo opioides e não-opioides, são usadas para o alívio da dor. No entanto, esses agentes estão associados a vários efeitos adversos, tais como comprometimento psicomotor e dependência (VELLA-BRINCAT e MACLEOD,

2007), o que tem estimulado a busca por alternativas. Vários compostos à base de plantas têm sido relatados como tendo propriedades analgésicas significativas, com poucos efeitos adversos, e estão sendo considerados substitutos terapêuticos para modular a nocicepção (FRANZOTTI et al., 2000; PINHEIRO et al., 2010).

Segundo a Organização Mundial de Saúde, estima-se em cerca de 2,3 milhões o número de pessoas com sobrepeso em 2025, sendo que mais de 700 milhões estarão obesas. A obesidade é descrita como uma condição inflamatória subclínica de curso crônico, caracterizada pela ocorrência de resistência à insulina, fenômeno pelo qual esse hormônio não consegue exercer suas funções anabólicas. A atividade hipoglicemiante de plantas medicinais pode ocorrer através da sua habilidade em restaurar a função pancreática resultando em aumento da liberação de insulina, da inibição da absorção intestinal de glicose, diminuindo a glicemia pós-alimentar, ou ainda por facilitar os processos metabólicos dependentes de insulina, sendo que em alguns casos seus efeitos são similares ao dos fármacos convencionais (MALAVIYA et al., 2010).

Diante do contexto apresentado acima, este trabalho investigou a toxicidade aguda e subaguda de extrato salino do caule de *P. gounellei*, bem como avaliou seu potencial farmacológico como agente antinociceptivo, antipirético e anti-obesidade em camundongos.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Avaliar extrato salino do caule de *P. gounellei* quanto à toxicidade e aos efeitos antinociceptivo, antipirético e anti-obesidade em camundongos.

1.1.2 Objetivos Específicos

- Investigar os metabólitos secundários presentes no extrato salino do caule de *P. gounellei*.
- Avaliar a toxicidade aguda e subaguda do extrato em camundongos utilizando parâmetros comportamentais, bioquímicos, hematológicos e histológicos.
- Investigar o potencial antinociceptivo do extrato em modelos de dor inflamatória e não-inflamatória.
- Avaliar a atividade antipirética do extrato em modelo de febre induzida por levedura.
- Investigar a influência do tratamento com o extrato na sensibilidade à insulina, tolerância à glicose e em parâmetros bioquímicos em camundongos obesos (submetidos à dieta hiperlipídica).
- Avaliar os níveis de marcadores de estresse oxidativo no fígado, baço e tecido adiposo de animais obesos tratados ou não com o extrato.
- Investigar alterações histológicas em fígado e tecido adiposo dos camundongos obesos tratados ou não tratados com o extrato.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 PLANTAS MEDICINAIS E FITOTERÁPICOS

O Brasil é considerado um dos países de maior diversidade biológica por abrigar 25% da flora mundial. Atualmente, existe uma crescente demanda para o uso de produtos naturais, o que impulsiona investigações científicas e a busca por drogas naturais, como ponto de partida para o desenvolvimento de novos medicamentos (JESUS et al., 2010; KANTATI et al. 2016; BERLINCK, et. al., 2017).

Os produtos naturais possuem diversas denominações, que dependem da técnica farmacêutica utilizada para o processamento. A princípio temos a planta medicinal, que como definição, é um vegetal que é utilizado com fins terapêuticos. Seu uso compreende as formas frescas, em geral *in natura*, ou secas. Já o fitoterápico é aquele obtido exclusivamente de matérias-primas ativas vegetais utilizando técnicas elaboradas com finalidade profilática, paliativa ou curativa (BRASIL, 2010).

As plantas medicinais e os fitoterápicos se mostram como uma alternativa terapêutica interessante, pois os medicamentos industrializados desencadeiam diversas reações adversas que podem não existir ou ocorrer em menor grau quando são usadas plantas medicinais. Ainda, os fitoterápicos podem ser uma alternativa aos fármacos sintéticos, que são geralmente mais onerosos e para os quais há relatos de resistência pelos pacientes (GUPTA, DUBEY, KUMAR, 2016).

Nas últimas décadas, o número de pessoas que usam produtos fitoterápicos no Brasil cresceu notavelmente (ABIFISA, 2007; SANTOS et al., 2011, SILVA, 2015). Com isso a ANVISA exige a avaliação da eficácia, segurança e de qualidade destes produtos para fins medicinais, pois estes apresentam uma composição complexa de substâncias químicas (FOLASHADE *et al*., 2012; SAHOO *et al*., 2010). A Resolução nº 48, de 16 de março de 2004, da Agência Nacional de Vigilância Sanitária (ANVISA), normatizou o registro de medicamentos fitoterápicos. Em 2006, foi criada a Política Nacional de Práticas Integrativas e Complementares no Sistema Único de Saúde (SUS), visando ampliar as opções terapêuticas oferecidas aos usuários, com a proposta de garantir à população o acesso a plantas medicinais, fitoterápicos e outros serviços relacionados, com segurança, eficácia e qualidade (BALBINO; DIAS, 2010).

2.2 ESTUDOS TOXICOLÓGICOS

A toxicidade e a segurança de uma determinada substância química estão relacionadas com sua concentração e tempo de permanência e/ou exposição, devendo os testes de toxicidade ser realizados de forma padronizada e em condições replicáveis (SILVERIA, 2007). Além das interações com medicamentos e/ou alimentos, possíveis reações adversas inerentes às preparações vegetais podem depender de características do paciente como gênero, idade e estado de nutricional, entre outros (BALBINO; DIAS, 2010).

Os estudos toxicológicos de xenobióticos visam garantir a segurança do homem frente a possíveis efeitos negativos, estabelecendo limites seguros para a aplicabilidade de uma nova substância e contribuindo, assim, para o desenvolvimento de novos fármacos (HODGSON, 2003). Os testes toxicológicos permitem a avaliação de parâmetros como alterações de massa corporal, consumo de água e ração (JAHN; GÜNZEL, 1997), condições anatomopatológicas dos órgãos, parâmetros bioquímicos e hematológicos e influência na reprodução (REBOREDO et al., 2007).

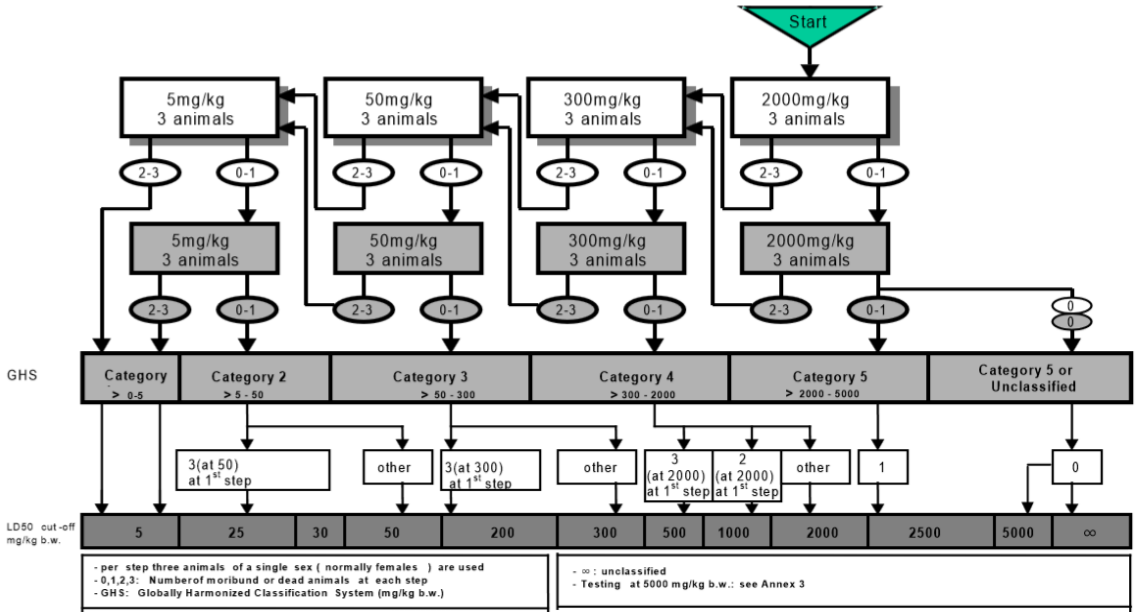
Dentre vários métodos ou testes empregados para avaliar efeitos toxicológicos *in vivo*, o teste de toxicidade aguda permite definir o grau de toxicidade intrínseca do composto, identificar órgãos que podem ser alvos de efeitos indesejados e selecionar doses para estudos de longa duração (ALMEIDA, 2011). A Figura 1 apresenta o protocolo estabelecido pela Organização para Cooperação e Desenvolvimento Econômico (OECD; www.oecd.org) com orientações para avaliação da toxicidade aguda de drogas. O teste é iniciado a partir da administração dos produtos na dose de 2000 mg/kg, e estabelece classificações do nível de toxicidade de acordo com a mortalidade em diferentes doses.

Além da avaliação da mortalidade, a análise toxicológica pode ser complementada pela avaliação de alterações em órgãos e em parâmetros bioquímicos e fisiológicos. Adicionalmente, a triagem comportamental permite avaliar se uma determinada droga modifica a atividade cerebral, através do registro de alguns sinais ou alterações de condutas apresentados pelos animais (ALMEIDA, 2011).

O estudo de toxicidade oral com exposição contínua às substâncias (doses repetidas) é utilizado para mimetizar a forma mais comum de exposição humana (CAMPOS; AMARAL, 2009). O estudo de toxicidade em doses repetidas é conduzido de acordo com protocolo nº 407 da OECD, sendo realizado com grupos de dez animais, para roedores, utilizando machos e fêmeas. A duração do tratamento com a substância-teste depende do tempo de uso proposto, variando até quatro semanas (BRASIL, 2014; OECD, 2008). Durante o período do teste são

avaliados sinais clínicos gerais, alterações comportamentais, de peso corpóreo e consumo de água e ração. Ao final devem ser avaliados parâmetros hematológicos, bioquímicos, análises macroscópicas, histopatológicas dos órgãos e tecidos selecionados e o peso dos órgãos (OECD, 2001b; OECD, 2008; BRASIL, 2014).

Figura 1 - Avaliação de toxicidade aguda de acordo com o guia da Organização para Cooperação e Desenvolvimento Econômico (OECD, 423).



Fonte: OECD (2001).

Dentro dos estudos de toxicidade, é válida a realização do *screening* hipocrático (triagem farmacológica), ou seja, testes que possam avaliar de forma mais específica sinais comportamentais que sejam apresentados pelos animais quando estão sob ação de substâncias-teste. Estes possibilitam um direcionamento dos demais estudos e testes que possam ser realizados. São exemplos os testes de campo aberto e de *rotarod*.

O teste do campo aberto é utilizado para verificar as alterações comportamentais e motoras, baseado no comportamento espontâneo dos animais. Trata-se de uma placa de acrílico pintada de branco e com 12 campos marcados de preto e delimitada com dois círculos, o mais externo apresentando 56 x 56 cm, sendo circundado por um cilindro transparente com 30 cm de altura (Figura 2). Na realização deste teste, animais tratados e do grupo controle são avaliados quanto a: movimentação espontânea (ambulação) registrada pelo número de cruzamentos com as quatro patas entre as divisões de campo; comportamento de autolimpeza; ação de levantar; e número de bolos fecais, como índice de sua emocionalidade (CARLINI et al., 1986). Substâncias estimulantes do SNC tendem a aumentar os parâmetros

comportamentais registrados neste teste, enquanto que substâncias depressoras tendem a diminuí-los (COELHO, 2010). Verifica-se que a tendência do animal em um ambiente novo é a de explorá-lo, apesar do conflito com o medo provocado pelo ambiente novo (LAPA et al., 2003).

Figura 2 - Instrumento utilizado no teste do Campo aberto.

Fonte: Arquivo pessoal

O teste do *rotarod* é utilizado para avaliar a coordenação motora, a aprendizagem de competências e o equilíbrio em roedores, que são os indicativos básicos da função motora normal (BROOKS et al., 2012). Essa função motora normal é dependente da interação de vários neurotransmissores em diversas regiões cerebrais, tais como o córtex motor, o estriado, o tronco cerebral e o cerebelo (WICHMANN e DELONG, 2002). O aparelho utilizado (Figura 3) consiste de uma barra giratória não escorregadia, medindo 45 cm de altura, 54 cm de largura e 35 cm de comprimento, dividida em 4 compartimentos com velocidade regulável em rotações por minuto (r.p.m). Ainda, neste aparelho é possível contabilizar o tempo de permanência do animal na barra giratória. Na realização do teste, o animal é posto em uma barra giratória em velocidade constante e o tempo de permanência dos mesmos é registrado em no máximo três reconduções (DUNHAM; MIYA, 1957; MORAIS et al., 1998).

Figura 3 - Aparelho utilizado no teste do *Rotarod*.

Fonte: Arquivo pessoal

2.3 ATIVIDADE ANTINOCICEPTIVA

A dor é um dos sinais cardinais da inflamação e, segundo a Associação Internacional para Estudo da Dor (IASP), pode ser definida como uma experiência sensorial e emocional desagradável, associada a dano tecidual real ou potencial. Além de envolver um estímulo potencialmente nocivo, a dor tem uma conotação individual e é representada por uma experiência subjetiva, incluindo componentes afetivos e emocionais, que amplificam ou diminuem a sensação dolorosa (KLAUMANN; WOUK; SILLAS, 2008).

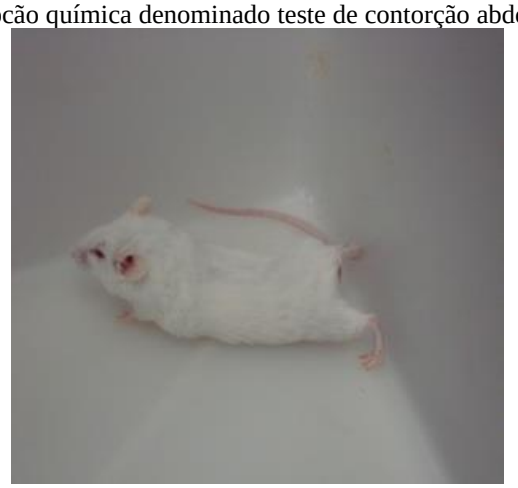
Do ponto de vista fisiológico, a dor pode ser classificada em cinco tipos de mecanismos distintos, de acordo com o tipo de lesão e/ou os mediadores envolvidos: nociceptiva, quando ocorre por estimulação excessiva dos nociceptores; neurogênica, quando ocorre lesão do tecido neuronal; neuropática, quando ocorre a disfunção de um nervo; psicogênica, que ocorre por fatores psicológicos; ou inflamatória, resultante de processos inflamatórios (MILLAN, 1999).

A busca por novas moléculas que atuem no sistema nervoso central (SNC) e que possam ter fins terapêuticos começou no século XIX, e muito do que a ciência moderna sabe hoje acerca da neuroquímica do encéfalo e das funções do SNC pode ser relacionado ao estudo de produtos naturais psicoativos. Como exemplo, os estudos sobre o alcaloide morfina da *Papaver somniferum* L. (Papaveraceae) conduziram à identificação do sistema opioide; os estudos com os canabinoides da marijuana (*Cannabis sativa* L. Cannabaceae) possibilitaram a identificação dos receptores e do sistema endocanabinoide; e os estudos dos alcaloides da *Rauwolfia serpentina* Benth. (Apocynaceae), como a reserpina, forneceram a base da compreensão sobre a transmissão medida por serotonina, norepinefrina e dopamina, bem como sobre a etiologia da depressão, além da hipertensão (GOMES et al., 2009; PRISINZANO, 2009). Efeitos adversos, tais como comprometimento psicomotor e dependência, têm sido associados aos fármacos analgésico atualmente disponíveis (VELLA-BRINCAT e MACLEOD, 2007). Nesse contexto a busca por novas opções farmacológicas a partir de plantas medicinais torna-se uma alternativa interessante (FRANZOTTI et al., 2000; PINHEIRO et al., 2010).

O teste de contorções abdominais é um modelo utilizado como triagem, uma vez que avalia a ação nociceptiva de forma não específica, sendo sensível a substâncias analgésicas de ação central ou periférica dotadas dos mais variados mecanismos de ação (SHAFIEE et al., 2003). O principio do teste se baseia na administração da substância a ser avaliada salina 1 hora antes do ácido acético. Cada animal recebe uma injeção intraperitoneal de 0,85% (v/v) de ácido acético em solução salina e é colocado em uma caixa de polietileno para registrar o

período de latência (tempo até a primeira contorção) e o número de contorções (Figura 4) no intervalo correspondente 5 a 15 minutos após a injeção de ácido acético.

Figura 4 - Fotografia de camundongo realizando movimento de extensão dos membros posteriores em modelo de nocicepção química denominado teste de contorção abdominal.

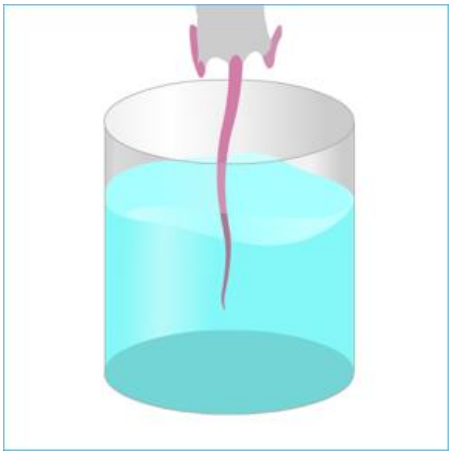


Fonte: Arquivo pessoal

O limiar nociceptivo térmico é comumente avaliado pelo teste de retirada da cauda descrito por D'Amour (1941), método este amplamente utilizado para avaliar analgésicos de ação central (ZAKARIA et al., 2006; FISCHER et al., 2008). É útil para identificar drogas de ação central cujos mecanismos se assemelhem aos promovidos pelos analgésicos opioides (LE BARS; GOZARIU; CADDEN, 2001), uma vez que a morfina administrada sistemicamente é capaz de suprimir respostas de neurônios espinhais ao estímulo térmico nocivo na cauda (DOUGLASS; CARSTENS, 1997).

A Figura 5 mostra a imersão da cauda de camundongo em água morna (55 ± 1 °C) e animais que removem suas caudas em menos de 5 s são selecionados para o ensaio. Para a realização do teste os camundongos são divididos em grupos que receberão veículo (controle), uma droga de referência ou a substância teste. O período de latência (isto é, o tempo requerido para cada camundongo remover a cauda) é determinado 0, 15, 30, 45 e 60 min após a administração dos tratamentos. Um período de latência de 20 s é considerado analgesia completa e a cauda é retirada para evitar lesões (KHATUN et al., 2015).

Figura 5 - Representação da nocicepção térmica pelo teste de retirada de cauda.



Fonte: http://www.neurofit.com/tech-anim-tail-flick.html

O teste da formalina é um modelo válido e confiável de nocicepção, sensível para várias classes de fármacos analgésicos, sendo utilizado no esclarecimento dos possíveis mecanismos do efeito antinociceptivo. Este teste tem por objetivo avaliar a ação das substâncias testadas tanto sobre a dor neurogênica quanto sobre a dor inflamatória (HUNSKAAR; HOLLE, 1987). Consiste na injeção intraplantar de solução de formaldeído (formalina) na pata posterior do animal, o que induz dor intensa pela estimulação direta dos nociceptores, caracterizada por vigorosas lambidas, mordidas e batidas na pata injetada (Figura 6) (HESS, 2006).

Neste teste é possível avaliar dois tipos diferentes de dor ao longo de um período de tempo (RANDOLPH, 1997). As respostas comportamentais à formalina possuem duas fases: a primeira é a fase inicial aguda, que se inicia após a injeção de formalina e se estende pelos primeiros 5 min (dor neurogênica ou aguda). A segunda fase, que é a mais prolongada com atividade comportamental aumentada, pode durar até cerca de uma hora. A segunda fase tem início entre 15 e 30 min após a injeção de formalina e está relacionada com a liberação de vários mediadores pró-inflamatórios, como bradicinina, prostaglandinas, serotonina, entre outros (HUNSKAAR; HOLE, 1987). Esse teste, considerado de longa duração, permite a verificação de sinais presentes na fase de modulação dos impulsos nervosos, e também, observar a participação de sistemas endógenos como o dos opioides (MUNGUIA, 2007).

Figura 6 - Fotografia de camundongo realizando o movimento de lambida de pata após injeção de formalina (A) e do instrumento utilizado para avaliar o tempo de lambida de pata pelo camundongo (B).

Fonte: Arquivo pessoal

Zétola *et al.* (2002) demonstraram o efeito antinociceptivo da fração hidroalcoólica da *Lippia alba*, planta conhecida como falsa melissa, e correlacionaram o seu efeito às quantidades de flavonoides presentes nessa fração. O látex de *Calotropis procera*, rico em enzimas proteolíticas, demonstrou atividade anti-inflamatória em diferentes modelos experimentais (ALENCAR et al., 2004), bem como analgésica (SOARES et al., 2005). O látex de *C. procera* inibe também as contorções abdominais induzidas por ácido acético em ratos (DEWAN, SANGRAULA e KUMAR, 2000). A atividade antinociceptiva das proteínas do látex de *C. procera*, quando administradas intraperitonealmente, foi detectada usando-se três modelos experimentais diferentes de nocicepção em camundongos: contorções abdominais induzidas por ácido acético, teste da formalina e teste da placa quente (SOARES et al., 2005).

O extrato etanólico das folhas de *Luehea divaricata*, possui atividade antinociceptiva, aumentando o limiar da dor induzida quimicamente por injeções intraperitoneal de ácido acético e intraplantar de formalina (BATISTA et al., 2016) Rosa et al. (2014), avaliando a atividade antinociceptiva do extrato bruto de *L. divaricata*, observou que esse extrato foi eficaz na redução de contorção abdominal na dose de 30 mg/kg.

Pereira et al. (2006), tomando por base que *Aspidosperma nitidum* era utilizada no tratamento de inflamações, avaliaram a atividade antinociceptiva do extrato etanólico do cerne, através dos testes de nocicepção induzida por formalina, da latência para o reflexo de retirada da cauda (“tail-flick”), de performance motora (“rota-rod”), e edema induzido pela injeção intraplantar da carragenina. O efeito antinociceptivo foi detectado na 2ª fase, indicando que não houve a participação do sistema opioidérgico (baseado na liberação de substâncias como as endorfinas e metaencefalinas, como parte do controle antinociceptivo).

2.4 ATIVIDADE ANTIPIRÉTICA

A temperatura tem papel crítico na homeostase, já que as reações bioquímicas envolvidas no metabolismo são exponencialmente influenciadas pela temperatura corporal (GILLOOLY et al., 2001). A febre é definida como o aumento controlado da temperatura corporal decorrente de uma alteração no controle térmico exercido pelo hipotálamo, particularmente na área pré-óptica do hipotálamo anterior, e é um dos sinais clínicos da resposta inflamatória sistêmica (DINARELLO, 1996). Quando a febre se inicia, a temperatura corporal está inferior à definida como referência. Cerca de 24 mecanismos de geração e conservação de calor são ativados, como tremor, mudanças comportamentais (procura por um ambiente mais quente) e vasoconstrição (CANNON, 2013).

A febre induzida por levedura, que representa a febre patogênica, apresenta um método econômico e confiável para avaliar novos agentes antipiréticos (Tomazetti, et al 2005). A febre se deve à presença de determinadas proteínas na levedura, gerando uma resposta inflamatória (PASIN, 2010). Além disso, a produção de citocinas pró-inflamatórias, como interleucina-1β (IL-1β) e IL-6, interferon-α (IFN-α), fator de necrose tumoral α (TNF-α) e prostaglandinas como PGE2 e PGI2 são responsáveis para elevar a temperatura corporal, agindo no cérebro (LUHESHI et al 1998; SAPER, 1994). Os antipiréticos, como o paracetamol, atuam de várias maneiras, reduzindo os níveis de prostaglandinas como também aumentando a mensagem antitérmica no cérebro e estimulando sinais anti-inflamatórios no local da lesão (ARONOFF, NEILSON, 2001).

Estudos realizados revelaram que as plantas medicinais que apresentam propriedade analgésica também demonstraram atividades antipiréticas e anti-inflamatórias (AFSAR et al, 2015; RAUF et al, 2014), já que o mecanismo para a supressão da dor, febre e inflamação pode ser correlacionado via inibição dos mediadores da inflamação. A presença de flavonoide em plantas medicinais tem sido associada a propriedades analgésicas e antipiréticas (KUMAR et al., 2015). Tesema et al. (2015) avaliaram o efeito antipirético das folhas de *Ocimum suave* e concluíram que há uma correlação com o efeito analgésico, semelhante ao fármaco de referência (ácido acetilsalicílico).

2.5 OBESIDADE

A obesidade é descrita como uma condição inflamatória subclínica de curso crônico, caracterizada pela ocorrência de resistência à insulina, fenômeno pelo qual o esse hormônio não

consegue exercer suas funções anabólicas. Esse é reconhecidamente o elo entre a obesidade e doenças correlatas, como diabetes tipo 2, esteato-hepatite não alcoólica e disfunções cardiovasculares (WELLEN et al., 2005). A base da doença consiste em um balanço energético positivo, resultando em ganho de peso (PINHEIRO et al. 2004). O excesso de peso está claramente associado com o aumento da morbidade e mortalidade e este risco aumenta progressivamente de acordo com o ganho de peso.

A via de sinalização da insulina é desencadeada a partir da ligação do hormônio ao seu receptor específico (IR), o qual possui a habilidade de se autofosforilar em resíduos de tirosina, iniciando a propagação do sinal (PESSIN; SALTIEL, 2000). Devido à sua atividade tirosina quinase, o receptor de insulina fosforila, também em resíduos de tirosina, uma família de proteínas denominadas substratos do receptor de insulina (IRS 1-6), as quais por sua vez ativam a fosfatidilinositil-3-quinase (PI3K) (SALTIEL; KAHN, 2001). A ativação da PI3K leva à fosforilação da proteína Akt (também conhecida como proteína quinase B) que, então, promove as ações inerentes à função da insulina, dentre elas a translocação de GLUT4 para a membrana plasmática de células musculares e do tecido adiposo e inibição da produção hepática de glicose (SALTIEL, 2000).

Foi demonstrado que a resistência à insulina está vinculada à modulação da atividade de proteínas da via de sinalização deste hormônio, como o receptor e seus substratos, em diversos modelos de resistência à insulina. Estudos demonstraram que o TNF-α (uma citocina pró-inflamatória) tem sua expressão aumentada no tecido adiposo de camundongos obesos, introduzindo a inflamação como componente importante da obesidade e diabetes (HOTAMISLIGIL et al., 1993). Estudo conduzido por Pedersen et al. (2015) demonstrou que a redução dos níveis de insulina em camundongos obesos, causada pelo tratamento com estreptozotocina, diminuiu o conteúdo de macrófagos no tecido adiposo e aumentou o nível de fosforilação de Akt estimulada pela insulina e a lipogênese *de novo*. Os autores ainda observaram que a administração de insulina em condições de constante euglicemia estimulou a expressão de citocinas no tecido adiposo. Dessa forma, eles concluíram que a hiperinsulinemia associada à obesidade leva à inflamação do tecido adiposo, a qual contribui para a supressão da síntese *de novo* de lipídeos pelos adipócitos.

Kumar et al. (2012) testaram o efeito do extrato etanólico das folhas de *Allangium lamarkii* durante 14 dias nas doses de 250 e 500 mg/kg, em ratos diabéticos induzidos por estreptozotocina. Foi constatada uma redução significativa da glicemia com a dose mais elevada, além da reversão do quadro hiperlipidêmico, diminuição dos níveis de triglicerídeos, colesterol total e LDL e aumento dos níveis de HDL. A administração do extrato também

resultou no aumento dos níveis das enzimas superóxido dismutase (SOD) e catalase (CAT), importantes enzimas antioxidantes.

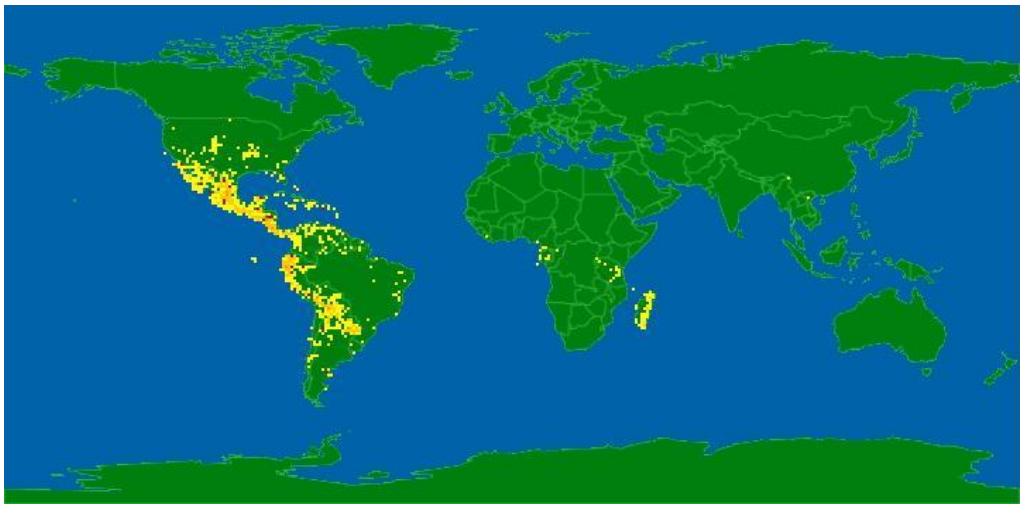
Estudo de Sharma et al. (2008) demonstrou que o extrato rico em flavonoides das sementes de *Eugenia jambolona* (Myrtaceae) apresentou atividade hipoglicemiante e hipolipidêmica em animais diabéticos. A administração do extrato aumentou os níveis de glicogênio hepático e muscular, além de aumentar os níveis de insulina em estudos *in vivo* e *in vitro*. Bansal et al. (2012) avaliaram a atividade hipoglicemiante da fração rica em flavonoides do extrato de *Pilea microphylla* e, após tratamento de 28 dias, o extrato administrado na dose de 100 mg/kg foi capaz de diminuir significativamente os níveis glicêmicos de jejum em animais diabéticos, bem como diminuir os níveis de triglicerídeos, colesterol total e aumentar os níveis das enzimas antioxidantes como GSH, CAT e SOD.

Araujo et al. (2015) demonstraram que o tratamento com as partes áreas de *Parkinsonia aculeata* L., aumentou significativamente a sensibilidade à insulina e normalizou os níveis séricos de glicose, insulina e leptina em jejum, sem afetar o peso de animais obesos por indução de dieta hiperlipídica.

2.6 *Pilosocereus gounellei*

A família Cactaceae compreende 127 gêneros e 1.450 espécies (HERNÁNDEZ-HERNÁNDEZ et al., 2011), distribuídas em regiões de zonas temperadas e tropicais das Américas (Figura 7), ocupando uma ampla variedade de hábitats, desde regiões áridas até florestas úmidas (REBMAN; PINKAVA, 2001). As plantas dessa família crescem até mesmo em solos pedregosos (GOMES, 1977) e apresentam formas variadas, sendo principalmente suculentas e sem folhas, com aréolas conspícuas, tendo espinhos e cerdas ou pelos (XU e DENG, 2017).

Figura 7 - Distribuição geográfica da família Cactaceae no mundo.



FONTE: http://www.tropicos.org/Name/42000071?tab=maps

Pilosocereus gounellei (F.A.C. Weber ex K. Schum.) Byles & G.D. Rowley, conhecida popularmente como xique-xique, alastrado ou sodoro, é uma planta cactácea que pode ser encontrada nas diversas áreas da Caatinga, até mesmo crescendo em cima de rochas, solos rasos e pedregosos (ARRUDA et al., 2005; BARBOSA, 1997; AGRA et al., 2008). Na Figura 8, podemos observar características como: caule ereto com galhos ramificados (8A), sendo cobertos por espinhos (8B); flores de coloração branca (8C) e protegidas por um tipo de algodão característico; e frutos do tipo baga de tamanho mediano, verdes no exterior e vermelhos no interior (8D), contendo as sementes, que servem de alimento para aves e outros animais da Caatinga (GOMES; 1977; CAVALCANTI & RESENDE, 2007).

Do ponto de vista medicinal, é descrita a utilização de uma pomada produzida a partir do miolo do xique-xique macerado para tratamento de processos inflamatórios decorrentes de perfurações nos membros superiores e inferiores (OLIVEIRA, 2011). Preparações das raízes do xique-xique são utilizadas no tratamento de inflamações prostáticas e na uretra (AGRA et al., 2008; ROQUE et al., 2010). Glaubert e colaboradores (2018) descreveram que o extrato etanólico de *P. gounellei* tem atividade gastroprotetora em diferentes modelos de indução de lesões gástrica, sugerindo como um candidato para o tratamento de patologias gástricas.

Figura 8 - *Pilosocereus gounellei*. (A) Hábito da planta. (B) Espinhos. (C) Flor. (D) Fruto.

Fonte: www.cactiguide.com

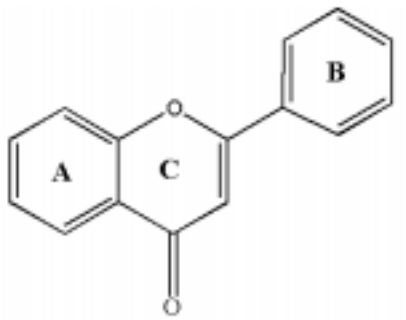
2.7 FLAVONOIDES

Os compostos fenólicos formam diversas classes de metabólitos secundários, como flavonoides, taninos, cumarinas, entre outros, sendo substâncias em que pelo menos um hidrogênio do anel aromático está substituído por hidroxila (SIMÕES et al., 2017). Esses compostos estão relacionados com a atividade antioxidante de alguns alimentos de origem vegetal, o que contribui para a saúde humana devido à capacidade de reduzir substratos, evitando assim a geração excessiva de radicais livres e seus subsequentes danos (VIEIRA et al., 2011).

Os flavonoides constituem uma família de compostos fenólicos que consistem de dois anéis aromáticos, que estão conectados por um anel pirano (Figura 9). Estes compostos podem ser agrupados em seis subgrupos principais: flavonas, flavonóis, catequinas ou flavanóis, flavanonas, antocianinas e isoflavonas (HOFFMANN-RIBANI; RODRIGUEZ-AMAYA, 2008). Há um grande interesse econômico pelos flavonoides, por alguns apresentarem cor, podendo ser utilizados como pigmentos, e por possuírem importância farmacológica, atribuídas a propriedades como: antitumoral, anti-inflamatória, antioxidante, antiviral, entre outros.

Alguns medicamentos também são elaborados a partir de flavonoides, para o tratamento de doenças circulatórias e hipertensão (SIMÕES et al., 2017). Os flavonoides têm sido associados a resultados benéficos à saúde humana, por isso sua quantificação em espécies vegetais é tão importante. Alguns estudos epidemiológicos revelaram que seu consumo elevado ajuda a proteger contra doenças crônicas, como as cardiovasculares, e que têm ação antioxidantes, anti-inflamatória e antitrombogênica (VALADARES, 2017).

Figura 9 - Núcleo fundamental dos flavonoides



A oxidação é um processo essencial aos organismos aeróbios, sendo os radicais livres (RL) produzidos naturalmente, como consequência desse processo de oxidação, ou por alguma disfunção biológica (BARREIROS et al., 2006). Como substâncias antioxidantes mais estudadas e sintetizados pelas plantas estão os compostos fenólicos dentre os quais se destacam os flavonoides. Nesta classe há grandes variações estruturais, dependendo do nível de hidrogenação, hidroxilação, metilação e sulfonação das moléculas. Além disso, flavonoides formam complexos com açúcar, lipídios, aminas e ácidos carboxílicos (DUTHIE; DUTHIE; KYLE, 2000; SUN; SIMONYI; SUN, 2002).

Estudos *in vitro* indicam que os polifenóis de plantas podem efetivamente participar de processos que possam ter implicações anti-carcinogênicas e anti-aterogênicas. Entre estes processos, o mais evidente é a capacidade antioxidante atribuída ao poder redutor do grupo hidroxila aromático, que podem doar elétrons e reduzir os radicais livres reativos. Essa atividade dos polifenóis é influenciada pelo número e posição dos grupos OH, assim como pelas posições de glicosilação. Ao contrário do ácido ascórbico e α-tocoferol, que agem em meio aquoso e na camada fosfolipídica, respectivamente, os flavonoides podem se localizar nas duas fases (MCKAY; BLUMBERG, 2002).

3 RESULTADOS

Os resultados desse trabalho são apresentados na forma de artigos.

3.1. ARTIGO 1 - SALINE EXTRACT OF *Pilosocereus gounellei* STEM HAS ANTINOCICEPTIVE EFFECT IN MICE WITHOUT SHOWING ACUTE TOXICITY AND ALTERING MOTOR COORDINATION

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Saline extract of *Pilosocereus gounellei* stem has antinociceptive effect in mice without showing acute toxicity and altering motor coordination

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ABSTRACT

People broadly use medicinal plants although their safety and efficacy are often neglected. Several Cactaceae plants are used for medicinal purposes, but *Pilosocereus gounellei* (xique-xique) has been little studied for their pharmacological properties. In this study, a saline extract from *Pilosocereus gounellei* stem was evaluated for acute toxicity, effects on motor function, and antinociceptive activity in mice. Thin-layer chromatography and high-performance liquid chromatography revealed the presence of flavonoids and reducing sugars. No death of mice was noted with oral treatments at 2000 and 5000 mg/kg. Body weight gain, food intake, and water consumption were not affected with treatment at 2000 mg/kg. The number of segmented leukocytes was higher in the treated mice than in control, and reduced serum levels of cholesterol and triglycerides were detected after both extract treatments. No histological alterations in the liver, heart, lung, kidney, and spleen were detected. Open field and rotarod tests showed that the extract did not affect motor function and exploratory activity. Antinociceptive activity was detected in acetic acid-induced writhing, tail-immersion, and formalin tests, indicating interference with both non-inflammatory and inflammatory pain. In conclusion, the *P. gounellei* extract has antinociceptive activity in mice without showing toxicity and altering motor coordination.

1. Introduction

The Cactaceae family is composed of species known as cacti, which are used for human consumption, animal fodder, and for medicinal and ornamental purposes (Novoa et al., 2015). The pharmacological potential of this family has been evaluated, but the studies have been performed mainly with species from the *Opuntia* and *Pereskia* genera, which showed cytotoxic, antimicrobial, hypotensive, diuretic, and anti-inflammatory properties (Abdul-Wahab et al., 2012; Kazama et al., 2012; Pinto and Scio, 2014; Siddiqui et al., 2016). *Opuntia ficus-indica* has been reported as a source of compounds with several beneficial effects on human health (El-Mostafa et al., 2014). The compounds vitexin and sitosterol, isolated from *Pereskia bleo*, showed antinociceptive effects (Guilhon et al., 2015), and polysaccharides from *Opuntia dillenii* were demonstrated to be useful for the treatment of ischemia and neurodegenerative diseases induced by oxidative stress (Huang et al., 2008).

Pilosocereus gounellei (F.A.C. Weber ex K. Schum.) Byles & G.D. Rowley is a cactus exclusively found in Brazilian Caatinga, where it is popularly known as “xique-xique.” It develops very well in the drier areas of the Brazilian semi-arid region, growing in shallow soils as well as sandy and rocky outcrops (Gomes, 1977; Monteiro et al., 2015). In traditional medicine, several parts of *P. gounellei* (stem, root, and flowers) have been used to treat prostate and urethra inflammation, jaundice, hyperglycemia, and wounds (Agra et al., 2008; Roque et al., 2010). Recent studies have demonstrated the antioxidant potential of *Pilosocereus* cacti compounds, such as flavonoids (Gonçalves et al., 2015; Maciel et al., 2016).

Opioids and non-opioids are the major classes of analgesic drugs used for the relief of pain. However, these agents are associated with various adverse effects, including those involving the central nervous system, such as psychomotor impairment (Vella-Brincat and Macleod,

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2007). This necessitates the search for alternative approaches. Several plant-based compounds have been reported to have significant analgesic properties with few adverse effects, and these are being considered as substitute therapeutics to modulate nociception (Franzotti et al., 2000; Pinheiro et al., 2010).

Despite the use of *P. gounellei* in folk medicine, studies on the toxicity of this plant as well as pharmacological trials are scarce in the literature. Accordingly, this work aimed to evaluate the acute toxicity in mice of a saline extract from *P. gounellei* stem and to evaluate its effect on motor function and antinociceptive activity.

2. Materials and methods

2.1. Extract preparation

P. gounellei stem was collected from Limoeiro City, Pernambuco, northeast of Brazil, with authorization (36301) of the *Instituto Chico Mendes de Conservação da Biodiversidade* from the Brazilian Ministry of Environment. The material was immediately transferred into plastic boxes to the laboratory (in a period of about 2 h), all the thorns were removed, and the stem was cut into pieces and dried for 3 days at 56 °C. The material was pulverized using a blender and stored at −20 °C and submitted to extraction in a maximum of 1 week. The stem powder was suspended in 0.15 M NaCl (at 5%, w/v) and homogenized for 16 h at 28 °C using a magnetic stirrer. Next, the suspension was centrifuged (3000 g, 15 min, 25 °C), and the supernatant was collected as the extract.

2.2. Phytochemical analysis

Thin-layer chromatography (TLC) was used in the screening of the extract for the presence of alkaloids, coumarins, cinnamic acid derivatives, flavonoids, tannins, terpenes/steroids, and sugars. The plates were developed in a high-performance thin-layer chromatography (HPTLC) system (CAMAG, Switzerland), consisting of a Linomat V sample applicator equipped with a 100 µL syringe (Hamilton, Switzerland) connected to compressed air, and the winCATS® software (CAMAG, Switzerland). Silica gel plates (60-F_{254}; Merck, Germany) were placed in a twin trough vertical glass chamber (10 × 10 cm, CAMAG, Switzerland), and the plates were developed using the mobile phase required for each compound class (Markhan, 1982; Abreu, 2000; Wagner and Bladt, 2001). Saturation time for the mobile phase was 30 min at 25 ± 2 °C. After development, the plates were derivatized by spraying with the reagents specific to each class compound, and visualized under 254 or 365 nm ultraviolet light. The images were acquired using the MultiDoc-It™ Imaging System (Model 125, USA) with UVP software and a Canon camera (Rebel T3, EOS 1100 D).

2.3. High-performance liquid chromatography (HPLC) analysis

The extract (100 mg) was dissolved in 5 mL of HPLC grade methanol (Tedia, USA) and then diluted in ultrapure water (Purelab® Classic UV, ELGA LabWater, USA) to obtain a 5 mg/mL solution. The analysis was conducted on an HPLC UltiMate™ 3000 system (Thermo Fisher Scientific, USA) coupled to a Diode Array Detector (DAD; Thermo Fisher Scientific) and equipped with a binary pump (HPG-3x00RS, Thermo Fisher Scientific), a degasser, and an autosampler with a loop of 20 µL (ACC-3000, Thermo Fisher Scientific). The wavelength was set at 350 nm for detection of flavonoids. The chromatographic separations were achieved with a C_{18} column (250 mm × 4.6 mm i.d., particle size 5 µm; Dionex, USA) equipped with a guard column (C_{18}, 4 mm × 3.9 µm; Phenomenex, USA). The separations were performed at a temperature of 25 ± 2 °C. The mobile phase consisted of purified water (A) and methanol (B), both acidified with 0.05% trifluoroacetic acid, at a flow rate of 0.8 mL/min. A gradient program was applied as follows: 0–10 min, 5–20% B; 10–13.5 min, 20–25% B; 13.5–18 min, 25–40% B; 18–25 min, 40–80% B; 25–30 min, 80% B; 30–34 min, 80-5% B. For data analysis and processing, the software Chromeleon™ version 6 (Dionex/Thermo Fisher Scientific, USA) was used.

The standards (vitexin, rutin, quercetin, and kaempferol; Sigma-Aldrich, USA), were dissolved in HPLC-grade methanol and diluted in purified water to obtain 10 µg/mL solutions. These were submitted for analysis under the same conditions as described for the sample.

2.4. Artemia salina lethality assay

A. salina cysts (San Francisco Bay Brand, Inc., USA) were incubated in natural sea water at 25–30 °C, with the pH adjusted to 9.0 with sodium bicarbonate. After nauplii hatching, the assay was performed as described by Meyer et al. (1982). The *P. gounellei* extract was diluted in sea water in Falcon tubes to achieve 5-mL solutions at 1, 3, 10, 30, 100, 300, 500, 750 and 1000 µg/mL. Each assay contained 10–15 nauplii. In the negative control, the nauplii were incubated in sea water. After 24 h, the survival rates were determined and the concentration required to kill 50% of the nauplii was calculated. Three independent experiments were performed in triplicate.

2.5. Animals

Male and female Swiss mice (*Mus musculus*), 60–90 day-old and weighing 30–40 g, were obtained from the bioterium of the *Laboratório de Imunopatologia Keizo Asami* from the *Universidade Federal de Pernambuco* (Recife, Brazil). The animals were used in the assays after an adaptation week in the experimental laboratories located at the *Núcleo de Cirurgia Experimental* of the *Universidade de Pernambuco* (Recife, Brazil) or at the *Centro de Biotecnologia* of the *Universidade Federal da Paraíba* (João Pessoa, Brazil).The mice were housed at a temperature of 22 °C, with a 12-h:12-h light:dark cycle and *ad libitum* access to food (Purina, Nestlé Brasil Ltda., Brazil) and water. The Ethics Committee on Animal Experimentation of the *Universidade Federal de Pernambuco* approved all the experiments (processes numbers 23076.048175/2015–12 and 23076.039336/2016–50).

2.6. Acute toxicity evaluation

The acute toxicity of *P. gounellei* stem extract was evaluated according to the instructions of the Organization for Economic Cooperation and Development (2001). Female mice were separated into three groups (n = 3 for each group) that received a single dose of the treatment by gavage (in a final volume of 0.1 mL). The first group of mice received only saline solution (control). The extract was evaluated at doses of 2000 and 5000 mg/kg. Behavioral alterations were evaluated during the first 60 min after treatment administration, divided into four periods (0–15, 15–30, 30–45, and 45–60 min). The following parameters were evaluated: piloerection, stool appearance, sensitivity to sound and touch, mobility, and aggressive behavior (Malone, 1983; Almeida et al., 1999). On the 15th day after the start of treatment, body weight variations, as well as water and food consumption, were determined. In addition, peripheral blood was collected. Next, the mice were euthanized, and the livers, kidneys, lungs, spleens, and hearts were removed, weighed, macroscopically analyzed, and processed for histological evaluation.

The collected blood was used to evaluate hematological and biochemical alterations. The following hematologic parameters were analyzed using an automatic analyzer (Animal Blood Counter – ABC Vet, Montpellier, France) and optical microscopy: erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and total and differentiated analysis of leukocytes. For biochemical analysis, the blood was evaluated for albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, gamma glutamyl transferase (GGT), total cholesterol,

triglycerides, urea, and creatinine, using specific kits (Labtest Diagnóstica, Lagoa Santa, Brazil).

Pieces of the organs were fixed in buffered formalin (10%, v/v) and then dehydrated in ethanol (70–100%), diaphonized in xylene, and embedded in paraffin. Sections of 5 μm were stained with hematoxylin and eosin and mounted using coverslips with Entellan® resin (Merck, Germany) (Kiernan, 2008). The slides were observed with a Motic BA200 microscope coupled to a Moticam 1000 1.3 MP digital camera (Motic Incorporation Ltd., Hong Kong).

2.7. Evaluation of extract effects on motor function and exploratory activity

2.7.1. Open-field test

The open field test is a model of anxiety-like behavior used to evaluate locomotor and exploratory activity (Prut and Belzung, 2003). Female and male Swiss mice (six per group, of the same sex) received saline (control) or *P. gounellei* extract (250, 500, or 1000 mg/kg) orally, and after 30 and 60 min, each animal was subjected individually to the open-field test in a square arena (56 × 56 cm) for a period of 5 min. The parameters observed were: ambulation (number of segments crossed by the animal with all four legs), grooming frequency, number of raising occurrences, and number of fecal masses (Mota et al., 2011).

2.7.2. Rotarod test

The rotarod test evaluates muscle relaxation and motor incoordination caused by a substance (Dunham and Miya, 1957). Twenty hours before the assay, male mice were prescreened according to their ability to remain on the rotating bar (7 rpm) for at least 3 min in 3 trials. The preselected mice were then divided into 4 groups (n = 6 per group), orally treated with saline (control) or with the *P. gounellei* extract (250, 500, or 1000 mg/kg), and then evaluated 30, 60, and 120 min after treatment. The time spent on the rotating rod for a period of up to 3 min (from 5 to 40 rpm) was recorded for each mouse. The animals that fell were repositioned on the bar for up to three attempts. Diazepam (10 mg/kg, intraperitoneal) was used as a reference drug.

2.8. Evaluation of antinociceptive activity

2.8.1. Tail-immersion test

Male mice were prescreened for sensitivity by immersing their tails in warm water (55 ± 1 °C), and the animals that removed their tails in less than 5 s were selected for the assay. The test was performed as described by Khatun et al. (2015). The mice were divided into 5 groups (n = 4 per group) that received oral saline (control), diclofenac sodium (positive control, 100 mg/kg, intraperitoneal), or oral *P. gounellei* extract (125, 250, or 500 mg/kg). The latency period (i.e., the time required for each mouse to remove its tail) was determined 0, 15, 30, 45, and 60 min after administration of the treatments. A latency period of 20 s was considered complete analgesia, and the tail was withdrawn to avoid injury (Khatun et al., 2015).

2.8.2. Acetic acid-induced writhing test

Male mice were separated into six groups (n = 8 per group) and treated with oral saline (control), oral *P. gounellei* extract (125, 250, or 500 mg/kg), intraperitoneal morphine (10 mg/kg), or intraperitoneal indomethacin (20 mg/kg). Saline solution and the plant extract were administered 1 h before the acetic acid, while morphine or indomethacin was administered 30 min before. Each animal received an intraperitoneal injection of 0.85% (v/v) acetic acid in saline, and was then placed in a polyethylene box to record the latency period (time until the first writhing) and the number of writhes in the interval corresponding to 5–15 min after the injection of acetic acid.

2.8.3. Formalin test

Male mice were divided into 6 groups (n = 6 per group) and the following pre-treatments were administered: oral saline (control), oral *P. gounellei* extract (125, 250, or 500 mg/kg), intraperitoneal morphine (10 mg/kg), or intraperitoneal indomethacin (20 mg/kg). After 60 min, 20 μL of 2.5% (v/v) formalin in saline was injected into the subplantar region of the right hind paw of each animal. The time spent by the mouse licking its paw was recorded during the first 5 min after formalin injection (first phase: neurogenic pain), as well as 15–30 min after the injection (second phase: inflammatory pain) (Hunskaar and Hole, 1987). For evaluation of the involvement of opioid receptors in the effect of the extract, naloxone (20 mg/kg, intraperitoneal) was administered 30 min before the extract (500 mg/kg) or morphine (10 mg/kg).

2.9. Statistical analysis

The results are expressed as the means of replicates ± standard deviation (SD). Analysis of variance (ANOVA) was performed followed by Bonferroni's test for multiple comparisons. A p value < 0.05 was adopted as the significance level.

3. Results and discussion

The use of medicinal plants in the treatment of several types of diseases is an ancient practice, and the World Health Organization has recognized its relevance to healthcare once quality, safety, and efficacy are demonstrated (World Health Organization, 2013). However, this caution is frequently neglected, and people often wrongly consider a medicinal plant safe simply because it was obtained from a natural source (Adewale et al., 2016). Indeed, several plants may be harmful to humans depending on the dose and mode of use (Lewis and Elvin-Lewis, 2003). In the present study, we evaluated the toxicity to mice of a saline extract from *P. gounellei* stem before determining its pharmacological properties.

TLC analysis revealed the presence of flavonoids and reducing sugars in the *P. gounellei* saline extract. Alkaloids, coumarins, cinnamic acid derivatives, tannins, terpenes, and steroids were not detected. An HPLC-DAD analysis was then performed at an absorbance detection wavelength of 350 nm for flavonoids. The chromatogram profile showed four main peaks (Fig. 1A), with an absorption spectrum characteristic of flavonoids with maximums at approximately 265.0 and 360.0 nm (Fig. 1B). The compounds did not correspond exactly to the standards available (vitexin, rutin, quercetin, and kaempferol), but the profiles of the UV absorption spectrum of these peaks were similar to that of kaempferol. So, we assumed that the unknown compounds in the extract were derivatives of kaempferol. Thus, the flavonoid content in the sample was calculated as 0.21% (w/w) and was expressed as kaempferol equivalents.

The stems (cladodes) of *P. gounellei* were mainly composed of carbohydrates, proteins, and lipids, but also contained a small amount of flavonoids and carotenoids (Nascimento et al., 2012). Previous experiments performed in our laboratory showed that the saline solution was effective in extracting both proteins and carbohydrates from *P. gounellei* stem (unpublished data). Thus, in the present study, we decided to work with this extract, in order to use a preparation containing both primary and secondary metabolites. Indeed, cacti polysaccharides have been reported to exert different biological activities, such as neuroprotective effects (Huang et al., 2009), antioxidant activity (Yang et al., 2013), improvement in the functioning of erythrocyte membranes (Chenfeng et al., 2007), and antitumor effects (Liang et al., 2008). Bioactive proteins have not been extensively studied in cacti, but reports of the presence of lectins in *O. ficus-indica* and *Machaerocereus eruca* were found (Zenteno et al., 1988; Santana et al., 2009). Lectins were not detected in the saline extract of the *P. gounellei* stem (data not shown), and our research group is investigating the presence of other classes of bioactive proteins.

Flavonoids are known for their antioxidant activity, which can protect cells against oxidative damage associated with the oxidative

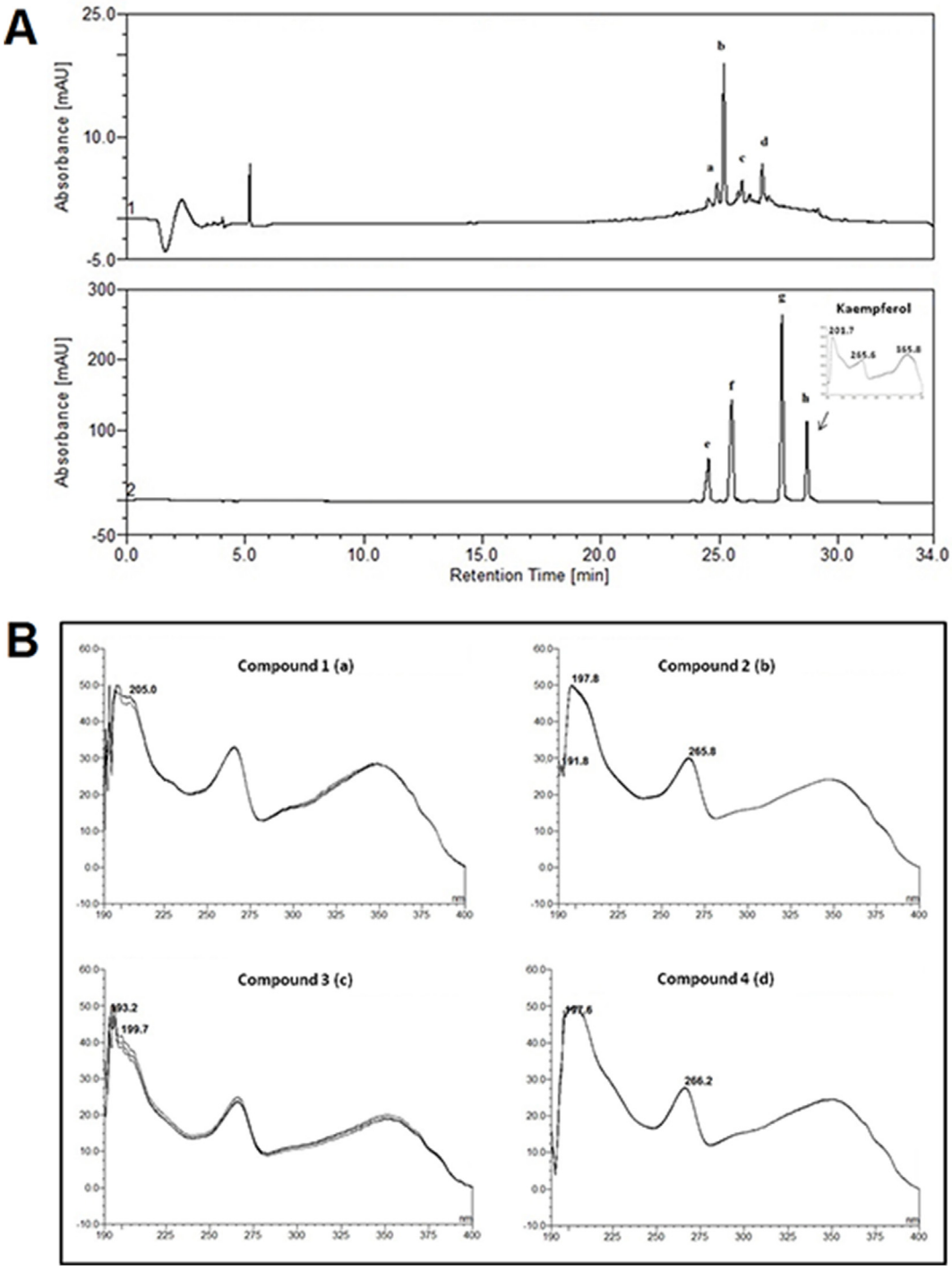

Fig. 1. High-performance liquid chromatography (HPLC) profile of saline extract from *Pilosocereus gounellei* stem. (A) Chromatogram of the extract (a to d = compounds 1 to 4, respectively) and reference substances (vitexin-e; rutin-f; quercetin-g, and kaempferol-h). The inset shows the UV spectrum of kaempferol. (B) UV spectrum of the four main peaks detected in the extract (a–d).

stress caused by free radicals. In addition, flavonoids exhibit a wide range of therapeutic effects, such as cardiotonic, anti-inflammatory, antimicrobial, antinociceptive, hepatoprotective, and antineoplastic activities (Martinez-Florez et al., 2002; Rauf et al., 2016). Flavonoid content has been correlated with the antioxidant activity of the fruits of *Pilosocereus arrabidae* (Gonçalves et al., 2015). A study conducted by Maciel et al. (2016) revealed the presence of pinostrobin, β-sitosterol, hydroxyphaeophytin, phaeophytin, mariannein, N-trans-feruloyltyramine, and the flavonoids kaempferol and quercetin in the *P. gounellei* cladodes.

One way to assess the safety of medicinal plants is to determine their toxicity. The *Artemia salina* lethality assay is used as a preliminary indicator of general toxicity of plant compounds since this microcrustacean is usually very sensitive to toxic substances. The *P. gounellei* extract promoted mortality of *A. salina* nauplii, with an LC_{50} of 767.62 μg/mL. This result suggests some toxic potential of the extract, which prompted us to evaluate its acute toxicity in mice, as well as its possible effects on hematological and biochemical parameters and organ histology.

The acute toxicity assay is usually conducted to determine the safety

of a substance at determined dose ranges, but it can also provide initial information on toxicity mechanisms. No animal deaths after treatment with 2000 mg/kg of the extract were recorded. Since the folk use of *P. gounellei* includes the ingestion of boiled stem medulla or soaked stems (Costa, 2011), resulting in doses that may be greater than 2000 mg/kg, a higher dose was administered to mice. Again, no mortality was observed, giving a LD_{50} > 5000 mg/kg, which classifies this extract as safe according to the guidelines of the OECD (2001). Therefore, the extract can be included in Category 5 (low toxic or atoxic) according to the classification of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

The evaluation of behavior parameters revealed that the animals that received the extract at both doses (2000 and 5000 mg/kg) showed irritability (0–15 min period), followed by prostration and decreased touch response (15–30 min), suggesting a depressant action of the extract. However, these responses to the extract were temporary and were not observed after 30 min. This can be related to a short half-life of the compound(s) due to the activity of metabolic/excretion detoxification mechanisms in the mice (Eaton, 1996). However, there may be agents of pharmacological interest among these possible short half-life compounds, which would not be interesting. Thus, the antinociceptive activities whose results will be shown below were evaluated after a period longer than 30 min after administration of the extract, to observe the action of compounds that were able to remain for more time in the animal body.

There were no significant differences ($p > 0.05$) in the body weight gain as well as food and water consumption between the control mice and those treated with 2000 mg/kg extract (Table 1). In contrast, the mice treated with the extract at 5000 mg/kg showed a reduction in food consumption and gained 23% less weight than the control mice did ($p < 0.05$). This may be directly linked to the decrease in food intake and not necessarily caused by metabolic changes. In addition, a sample is generally considered toxic only when there is a weight loss greater than 10% (Vaghasiya et al., 2011). The determination of such parameters is important in the study of the safety of a product with therapeutic purpose, as proper intake of nutrients and water are essential to the physiological status of the animals (Iversen and Nicolaysen, 2003).

The hematopoietic system is very sensitive to toxic compounds and serves as an important indicator for physiological and pathological changes in humans and animals (Adeneye et al., 2006). Hematological analysis (Table 2) showed that there were no significant ($p > 0.05$) differences in erythrocyte numbers, hematocrit, hemoglobin, MCV, MCH, and MCHC among the groups. In the group that received 5000 mg/kg, there was a significant ($p < 0.05$) increase in the total number of leukocytes, but a reduction in the proportion of lymphocytes. However, the number of segmented cells in both extract treatments was higher ($p < 0.05$) than in control mice. All these results may be related to a response of the immune system to compounds present in the *P. gounellei* extract. An increase in the number of segmented leukocytes has been found in other studies evaluating the acute toxicity of plant extracts (Oliveira et al., 2015, 2016). Carneiro et al. (2015), evaluating the acute and subchronic toxicity of rhodium (II) citrate in Balb/c mice, detected an increase in segmented neutrophils and correlated this with a transitory reaction that usually occurs in response to drugs.

Table 1
Water and food consumption and weight gain of control mice and those treated orally with saline extract from *Pilosocereus gounellei* stem for 14 days.

Parameter	Control	Extract	
		2000 mg/kg	5000 mg/kg
Water consumed (mL)	30.00 ± 1.14	28.92 ± 0.86	28.61 ± 0,97
Food consumed (g)	13.58 ± 0.25	13.13 ± 0.85	12.42 ± 0.40*
Weight gain (g)	2.45 ± 0.10	2.78 ± 0.17	1.87 ± 0.28*$^{\Delta}$

*Significantly different ($p < 0.05$) from control. $^{\Delta}$Significantly different ($p < 0.05$) from the 2000 mg/kg treatment. Data are the means ± standard deviations. Statistical analysis was performed by analysis of variance (ANOVA), followed by Bonferroni's test.

Table 2
Hematological parameters of blood of control mice and those treated orally with saline extract from *Pilosocereus gounellei* stem for 14 days.

Parameter	Control	Treatments	
		Extract (2000 mg/kg)	Extract (5000 mg/kg)
Erythrocytes (10^6/mm^3)	9.31 ± 0.15	8.90 ± 0.41	9.85 ± 0.17
Hematocrit (%)	44.63 ± 0.88	42.33 ± 1.56	45.40 ± 0.61
Hemoglobin (g/dL)	15.49 ± 0.34	15.31 ± 0.41	16.00 ± 0.62
MCV (fL)	47.66 ± 0.30	47.81 ± 0.55	48.10 ± 0.44
MCH (pg)	16.55 ± 0.06	16.14 ± 0.09	16.48 ± 0.14
MCHC (%)	34.44 ± 0.03	34.39 ± 0.12	34.31 ± 0.11
Leukocytes (10^3/mm^3)	8.87 ± 0.91	8.94 ± 0.80	11.1 ± 0.78*
Segmented (%)	51.44 ± 0.22	61.66 ± 0.41*	71.12 ± 1.12*$^{\Delta}$
Lymphocytes (%)	37.00 ± 0.18	27.36 ± 0.31*	17.66 ± 0.61*$^{\Delta}$
Monocytes (%)	11.56 ± 0.49	10.98 ± 0.30	11.22 ± 0.12

*Significantly different ($p < 0.05$) from control. $^{\Delta}$Significantly different ($p < 0.05$) from the 2000 mg/kg treatment. Data are the means ± standard deviations. Statistical analysis was performed by analysis of variance (ANOVA), followed by Bonferroni's test. MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

The results from the biochemical analysis (Table 3) revealed decreased serum levels of cholesterol and triglycerides in treatments with the extract at both doses, in comparison with those of the control group. The other biochemical parameters did not vary significantly ($p > 0.05$) among the groups. These data suggest a decrease in lipid profiles promoted by the *P. gounellei* extract, which warrants future in-depth studies on this issue. The levels of ALT, AST, alkaline phosphatase, and GGT were not altered, showing that, at the doses tested, the extract of *P. gounellei* did not cause hepatotoxicity. Our results also showed that the *P. gounellei* extract did not affect renal function and did not interfere with the ability of the kidney to excrete metabolites, since blood levels of urea and creatinine were not significantly different among the groups (Table 3).

Organ histology revealed a few, small greasy microvesicles of diffuse form in the hepatocytes of the mice treated with the extract at 2000 and 5000 mg/kg (Fig. 2). However, these alterations are considered reversible. The hearts of control and treated mice showed preserved myocardial structure, with no signs of degeneration, necrosis, inflammation, or other changes (Fig. 2). The lungs of mice treated with the extract also showed a normal morphology as in the control group (Fig. 2). The kidneys showed no alterations (Fig. 3), and the spleens showed well-defined lymphatic nodes in the control and treated groups (Fig. 3).

Acute toxicity evaluation has been performed with other Cactaceae species, and low or no toxicity has been described. Methanolic extracts of *Pereskia bleo* and *Pereskia grandifolia* leaves, orally administered to mice (2500 mg/kg), did not cause death or behavioral changes, and showed no effect on weight gain (Sim et al., 2010). Similarly, an ethanolic extract from *Pereskia aculeata* leaves orally administered to rats (1250, 2500, and 5000 mg/kg) did not cause clinical or histopathological alterations (Silva et al., 2017). *O. ficus-indica* seed oil was reported to have low toxicity to mice, although it was able to induce death with LD_{50} values of 2.72 and 43.0 mL/kg, when administered orally and intraperitoneally, respectively (Boukeloua et al., 2012).

Based on the results from the toxicological evaluation, the *P. gounellei* stem extract was tested in doses up to 1000 mg/kg in the next assays. In the open field test, effects of the extract on motor function

Table 3
Biochemical parameters of blood of control mice and those treated orally with saline extract from *Pilosocereus gounellei* stem for 14 days.

Parameter	Control	Treatments	
		Extract (2000 mg/kg)	Extract (5000 mg/kg)
Albumin (g/dL)	1.99 ± 0.09	2.01 ± 0.13	2.05 ± 0.15
ALT (U/L)	88.40 ± 1.12	86.3 ± 1.02	89.33 ± 0.93
AST (U/L)	122.09 ± 1.23	121.97 ± 1.10	120.44 ± 1.11
Total protein (g/dL)	7.12 ± 0.20	7.09 ± 0.23	7.13 ± 0.19
Alkaline phosphatase (IU/L)	12.12 ± 0.10	11.99 ± 0.22	12.04 ± 2.65
GGT (U/L)	10.70 ± 0.50	10.27 ± 0.26	10.19 ± 0.51
Urea (mg/dL)	44.02 ± 0.11	44.39 ± 0.20	44.20 ± 0.14
Creatinine (mg/dL)	0.41 ± 0.09	0.40 ± 0.11	0.39 ± 0.16
Total cholesterol (mg/dL)	137.8 ± 9.69	113.46 ± 9.42*	110.13 ± 9.88*
Triglycerides (mg/dL)	149.21 ± 10.21	128.88 ± 10.30*	130.34 ± 10.42*$^{\Delta}$

*Significantly different ($p < 0.05$) from control. $^{\Delta}$Significantly different ($p < 0.05$) from the 2000 mg/kg treatment. Data are the means ± standard deviations. Statistical analysis was performed by analysis of variance (ANOVA), followed by Bonferroni's test. ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma glutamyl transferase.

and exploratory activity of the mice were not observed. The open field test is a classic model used to investigate the exploratory activity and emotional behavior of rodents (Prut and Belzung, 2003; Pellow et al., 1985). The suppression of exploratory behavior suggests a sedative and central nervous system depressant activity (File and Wardill, 1975; Mujumdar et al., 2000). In the rotarod test, there were no significant differences ($p > 0.05$) in the mean times spent on the rotating rod among the mice from the control and extract-treated groups (Table 4). In contrast, treatment with the reference drug, diazepam (250 mg/kg intraperitoneal), significantly decreased the time that the animals remained on the rod.

The antinociceptive activity of the *P. gounellei* extract was evaluated by three methods: tail-immersion, acetic acid-induced writhing, and formalin tests. Treatment with the extract at all doses significantly inhibited ($p < 0.01$) the number of abdominal writhings induced by the intraperitoneal administration of acetic acid, compared with that of control mice (Fig. 4). The groups treated with extract doses of 125, 250, and 500 mg/kg showed a reduction of 52, 58, and 73%, respectively, in the number of writhings. The reference drugs, morphine and indomethacin, also showed antinociceptive effects, as expected. The acetic acid-induced writhing is caused by the irritation of the intraperitoneally injected acid and involves the stimulation of nociceptors and consequent behavioral reactions. The injection of acetic acid triggers the release of a variety of mediators, such as substance P, bradykinins, prostaglandins, and pro-inflammatory cytokines, stimulating the peripheral nociceptors and neurons that are sensitive to inflammatory mediators (Le Bars et al., 2001). The results show that the extract was effective in reducing local and inflammatory pain.

The results from the tail-immersion test (Table 5) showed that the response time was significantly increased ($p < 0.05$) in mice that received 250 mg/kg of the extract at least 30 min beforehand compared to that of control mice. With the extract treatment at 500 mg/kg, the antinociceptive effect was detected from time zero after extract administration. The diclofenac sodium also showed an antinociceptive

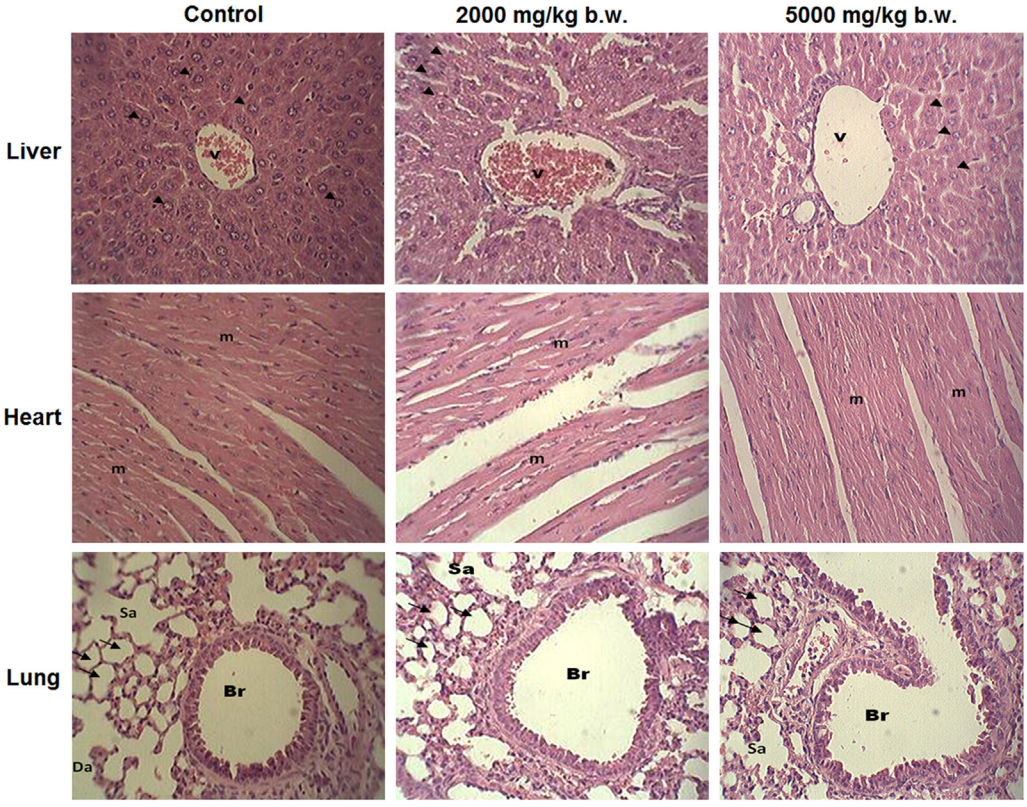

Fig. 2. Representative photomicrographs of the liver, heart, and lung of mice from control (oral saline) and treatments with *Pilosocereus gounellei* stem extract (2000 and 5000 mg/kg oral). Livers: the centrilobular vein (v) is seen in all images. In control, the hepatocyte bundles are preserved and ordered. A few, small greasy microvesicles of diffuse form (arrows) in control mice and those treated with the extract at 2000 and 5000 mg/kg are visible. Heart: Normal structure, with cardiac muscle (m) and pericardium. No signs of degeneration and necrosis/inflammation or other changes. Lungs: bronchiole (Br), alveolar duct (Da), alveolar sac (Sa), and interalveolar septa (short arrows) can be visualized, with preserved architecture in both control and extract-treated mice. Hematoxylin and eosin staining was used. Magnification: 400x.

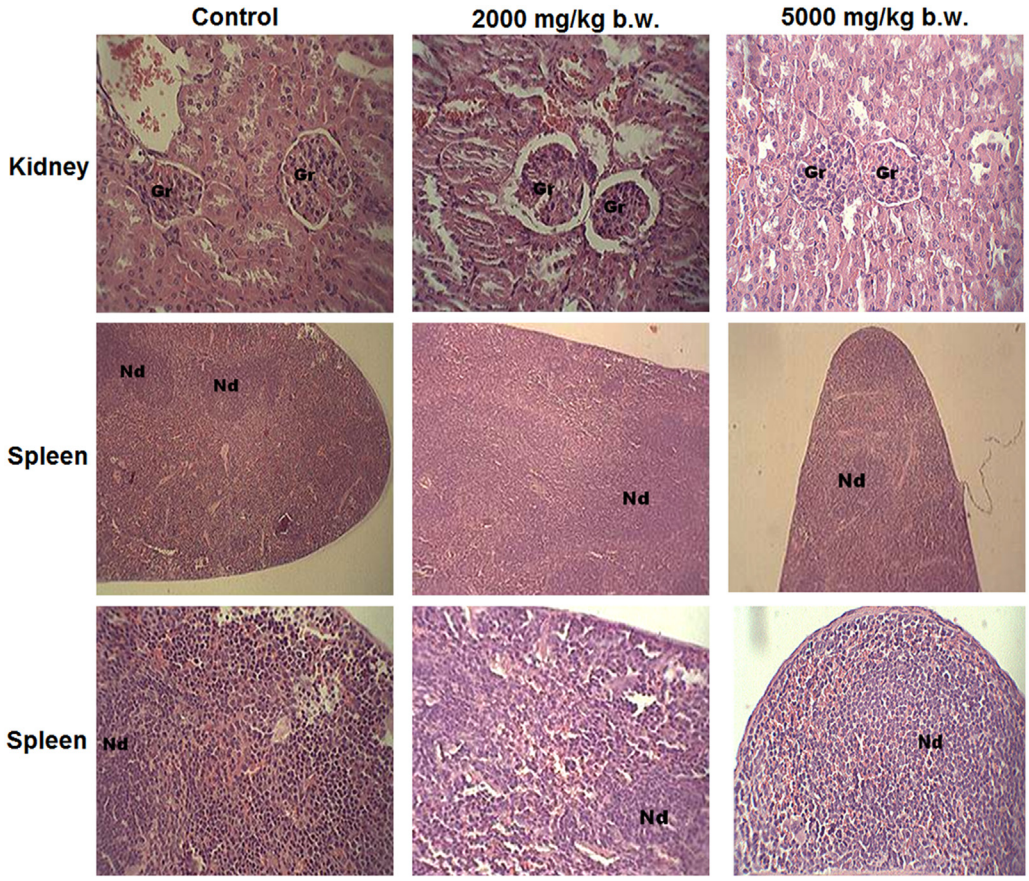

Fig. 3. Representative photomicrographs of the kidneys and spleens from control mice (oral saline) and those treated with *Pilosocereus gounellei* stem extract (2000 and 5000 mg/kg oral). Kidneys: Renal glomeruli (Gr) and contorted tubules without alterations are visible in control and extract-treated groups. Spleens: The lymphatic nodes (Nd) are well-defined in control and treated groups. Hematoxylin and eosin staining was used. Kidney images: 400x. Spleen images: 100x(upper panels) and 400x(lower panels).

Table 4
Time spent on the rotating rod by mice treated with saline (control), *Pilosocereus gounellei* stem extract, or the reference drug, diazepam, determined 30, 60, and 120 min after treatment.

Treatment	Dose (mg/kg)	Period after administration		
		30 min	60 min	120 min
		Time spent on rod (s)		
Control (*per os*)	–	178.4 ± 0.58	177.8 ± 0.51	176.9 ± 0.56
Diazepam (i.p.)	10	176.9 ± 1.41	175.2 ± 0.95	176.1 ± 1.11
Diazepam (i.p.)	250	60.2 ± 8.3*	71.2 ± 10.3*	63.2 ± 11.7*
Extract (*per os*)	500	177.6 ± 1.05	175.0 ± 1.41	177.0 ± 0.89
Extract (*per os*)	1000	178.8 ± 1.03	176.1 ± 1.00	175.8 ± 1.13

*Significantly different ($p < 0.05$) from control. Data are the means ± standard deviations. i.p.: intraperitoneal.

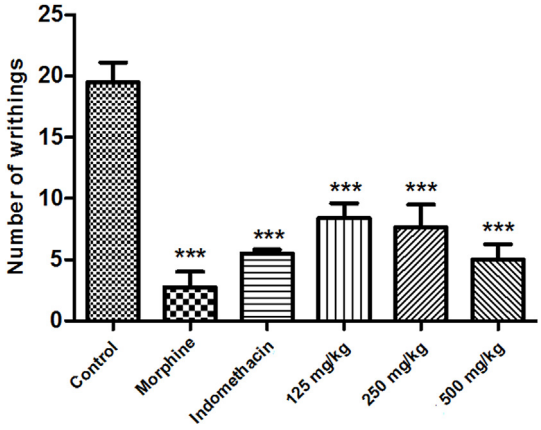

Fig. 4. Antinociceptive effect of saline extract from *Pilosocereus gounellei* stem (125, 250, and 500 mg/kg oral) and the reference drugs indomethacin (20 mg/kg intraperitoneal) and morphine (10 mg/kg intraperitoneal) in the acetic acid-induced writhing assay. The bars represent the mean numbers of writhing ± SD. (***) indicates significant difference ($p < 0.01$) in the number of writhings versus the control.

effect in all periods evaluated. The tail removal response is mediated by a spinal reflex, and several drugs produce peripheral analgesia by spinal sensory fibers (Chapman et al., 1985; Scholz and Wolf, 2002). The data obtained in this assay indicate that the saline extract of *P. gounellei* may interfere with the central nociceptive pathway, and may also be effective against noninflammatory pain.

In the formalin test, antinociceptive activity of the *P. gounellei* extract was detected for all doses and in both neurogenic and inflammatory phases (Fig. 5). The extract administration at all doses decreased the time spent licking the paw by 36–50% in the first phase (neurogenic pain). In the second phase, the extract showed an antinociceptive effect only when administered at 500 mg/kg, reducing the licking time by 75%. The reference drug, indomethacin, suppressed the response in the second phase (inflammatory pain), while morphine was active in both phases. The administration of naloxone 30 min before the extract reversed the antinociceptive effect in the first phase and partially inhibited the extract effect in the second phase. Since naloxone is a non-selective opioid receptor antagonist (Lewanowitsch et al., 2006), this suggests that the antinociceptive effect of the extract is mediated, at least in part, by activation of opioid receptors.

The mechanism of nociceptive action in the neurogenic phase is characterized by direct activation of sensory C fibers through transient receptor potential cation channel, subfamily A, member 1 (TRPA1) (McNamara et al., 2007). Drugs that act at the central level (e.g., opioids) are the main agents that act in the neurogenic phase of nociception (Ferreira et al., 2006). In the second phase of the assay,

Table 5
Mean latency to remove the tail from warm water for mice treated with saline (control), *Pilosocereus gounellei* stem extract, or the reference drug, diclofenac sodium, determined at different periods after treatment.

Treatment	Dose (mg/kg)	Period after administration				
		0	15 min	30 min	45 min	60 min
		Mean latency (min)				
Control	–	2.55 ± 0.45	2.32 ± 0.21	2.57 ± 0.51	2.75 ± 0.31	2.55 ± 0.29
Diclofenac sodium (i.p.)	100	4.62 ± 0.21*	5.98 ± 0.11*	7.11 ± 0.23*	6.89 ± 0.19*	8.29 ± 0.22*
Extract (*per os*)	250	2.71 ± 0.35	2.98 ± 0.41	3.45 ± 0.89*	4.01 ± 0.17*	4.22 ± 0.26*
Extract (*per os*)	500	3.08 ± 0.43*	4.01 ± 0.22*	6.65 ± 1.13*	7.02 ± 0.31*	7.24 ± 0.22*

*Significantly different ($p < 0.05$) from control treatment. Data are the means ± standard deviations. i.p.: intraperitoneal.

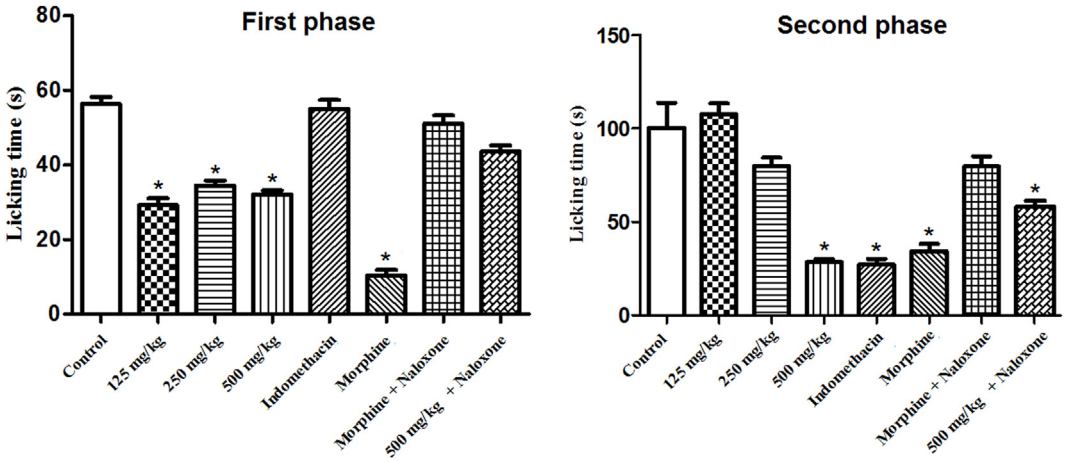

Fig. 5. Antinociceptive effect of saline extract from *Pilosocereus gounellei* stem (125, 250, and 500 mg/kg, oral) and the reference drugs, indomethacin (20 mg/kg, intraperitoneal) and morphine (10 mg/kg, intraperitoneal) on both phases of the formalin assay. The bars represent the mean time spent by the mice licking their paws ± SD. The involvement of opioid receptors in the antinociceptive effect was also evaluated by administering naloxone (20 mg/kg, intraperitoneal) to the mice 30 min before the administration of the extract or morphine. (*) indicates significant difference ($p < 0.01$) in the licking time versus the control.

inflammatory mediators are released in response to formalin administration, including prostaglandins, serotonin, bradykinins, histamine, tumor necrosis factor-α (TNF-α), and interleukins (Hunskaar and Hole, 1987; Tornos et al., 1999; Milano et al., 2008). The extract of *P. gounellei* was active in both phases, which corroborates its effectiveness in both acetic acid-induced abdominal writhings and tail-immersion tests. Abdul-Wahab et al. (2012) also showed that fractions obtained from leaves of the cactus *P. bleo* presented peripheral antinociceptive effects in tests of acetic acid-induced abdominal writhing and of formalin sensitivity.

Flavonoids have been reported as antinociceptive agents and may be responsible for the effects found with *P. gounellei* extract. The flavonoid glycosides, kaempferol-3,7-*O*-α-dirhamnoside and quercetin-3,7-*O*-α-dirhamnoside, isolated from the leaves of *Tilia argentea*, showed antinociceptive effects at 50 mg/kg *per os* in the *p*-benzoquinone-induced writhing test, without evidence of acute toxicity or gastric damage (Toker et al., 2004). Flavonoids isolated from a chloroform fraction of *Pistacia integerrima* galls also showed antinociceptive activity in acetic acid-induced writhing tests and in both phases of formalin tests (Rauf et al., 2016). However, the authors reported that opioid receptors are not involved in the effect of *P. integerrima* flavonoids, unlike the results obtained by us with *P. gounellei* extract.

4. Conclusion

The saline extract of *P. gounellei* stem has antinociceptive activity in mice without toxic effects, as demonstrated by hematological, biochemical, and histological analyses, and with no effects on motor coordination. The extract was active against noninflammatory and inflammatory pain. The results also suggest that antinociceptive activity is associated with interference of the opioid receptor pathway. This work contributes to the knowledge of the bioactive composition of Cactaceae plants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2018.04.004.

Conflicts of interest

The authors declare no conflict of interest.

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3.2. ARTIGO 2 - ASSESSMENT OF TWENTY-EIGHT-DAY ORAL TOXICITY AND ANTIPYRETIC ACTIVITY OF SALINE EXTRACT FROM *Pilosocereus gounellei* (CACTACEAE) STEM IN MICE

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Assessment of 28-day oral toxicity and antipyretic activity of the saline extract from *Pilosocereus gounellei* (Cactaceae) stem in mice

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ABSTRACT

Ethnopharmacological relevance: *Pilosocereus gounellei* is a plant found in the Brazilian Caatinga and is popular due to its traditional uses in the treatment of inflammation. The present study was conducted to investigate the sub-acute toxicity of the saline extract from the stem of *P. gounellei.*

Aim of the study: To evaluate the 28-day oral toxicity (through behavioral, biochemical, hematological, and morphological analysis) and the antipyretic activity of the extract in mice.

Materials and methods: A single oral dose (250, 500, and 1000 mg/kg) was administered daily over 28 consecutive days to male and female mice. Body weight, food and water intake, blood biochemical and hematological parameters, and urine composition were recorded. Histopathological examinations of the liver, kidney, spleen, lungs, and heart were performed and oxidative stress in the organs was evaluated by lipid peroxidation, superoxide dismutase (SOD), catalase (CAT), and nitrite analysis. The antipyretic effect of the 500 mg/kg dose was assessed using a yeast-induced pyrexia model.

Results: Oral administration of the extract over 28 days did not affect body weight gain, food and water consumption, body temperature, and hematological parameters in male and female mice. Blood glucose, total cholesterol, and triglyceride levels in male and female mice were reduced. Protein in the urine and histological alterations in both the liver and lungs were detected in male and female mice treated with the highest dose of the extract. SOD levels in the liver and the spleen increased significantly in both sexes, whereas lipid peroxidation decreased in the spleen of male mice. The extract also exerted an antipyretic effect after the first 60 min of the evaluation until the end of the observation duration (180 min).

Conclusion: The saline extract from the stem of *P. gounellei* did not present significant toxic effects over 28 consecutive days and demonstrated antipyretic activity when administered orally. Moreover, the results suggest that the extract has potential hypoglycemic and hypolipidemic effects. Future studies are needed to investigate its pharmacological potential.

1. Introduction

The use of extracts from different parts of plants is common in folk medicine. Traditionally, these extracts are used for therapeutic purposes and are prepared using various techniques (Jordan et al., 2010). The natural origin of these extracts do not preclude them from adverse reactions and toxic potential. Indeed, substances present in plants can be highly toxic and cause both acute and chronic effects (Kharchoufa et al., 2018). According to Mezzasalma et al. (2017), plants are among the most frequent causes of poisonings worldwide. These are usually associated with plant misidentification, plant-based supplements or medicines with no toxicity testing, and the lack of regulation of herbal products.

The Cactaceae family comprises various plants that grow in arid regions; these plants are mostly succulent and leafless with conspicuous areoles and bear spines, hairs, or bristles. *Pilosocereus gounellei* (F.A.C.

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Weber ex K.Schum.) Byles & G.D. Rowley, popularly known as xique-xique, is a cactus used in the treatment of various inflammatory processes including those resulting from perforations on the hands and feet and prostate and urethral inflammations (Agra et al., 2008; Roque et al., 2010). Sousa et al. (2018) demonstrated the gastroprotective effect of ethanol extracts from the stem and roots of *P. gounellei* on experimental ulcer models induced by oral administration of absolute ethanol.

Scientific information on the safety of alternative medicines is needed before they can be used as an herbal therapy. Despite the wide range of traditional uses of *P. gounellei*, there are few reports of in vivo toxicity testing of this plant. Acute toxicity in mice was evaluated via oral administration (2000 mg/kg b.w.) of the ethanol extract from the stem and roots of *P. gounellei*. No signs of toxicity were observed and no macroscopic changes or lesions in the internal organs (Sousa et al., 2018). Oliveira et al. (2018) reported that a saline extract from the stem of *P. gounellei* containing flavonoids and reducing sugars demonstrated antinociceptive activity in mice. An acute toxicity assay revealed that it did not have adverse effect on survival, hematological, biochemical, and histological parameters as well as on motor coordination.

In the present study, we evaluated the toxicity of the saline extract from the stem of *P. gounellei* administered orally to mice over 28 days. Behavioral, hematological, biochemical, and morphological parameters were analyzed. Considering the popular use of this plant in the treatment of inflammation, its antipyretic properties were also examined.

2. Materials and methods

2.1. Plant material collection and preparation of extract

Stems of *P. gounellei* were collected in 10 May 2017 from Afogados da Ingazeira City (7 ° 44' 10.9" S 37° 35' 29" W), Pernambuco, Brazil, under authorization (number 36301) from the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) within the Brazilian Ministry of the Environment. Access was recorded (ADF1667) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). A voucher specimen (number 82,853) was deposited at the herbarium "UFP – Geraldo Mariz" in the *Universidade Federal de Pernambuco*. After removal of the thorns, the stem was cut into small pieces and dried at 28 °C for 3 days. The dry material was powdered and stored at − 20 °C. The powder was mixed with 0.15 M NaCl (in a proportion of 10%, w/v) and stirred for 16 h at 28 °C. The mixture was centrifuged (3000 × *g*, 15 min) and the supernatant corresponded to the extract.

2.2. Total flavonoid content (TFC)

Standard solutions of rutin (0.1 g/mL) and the *P. gounellei* extract (0.1 g/mL) were prepared in 50% (v/v) ethanol. Each solution was transferred to a 25 mL flask, 5% (w/v) aluminum chloride (2 mL) was added and brought to volume (25 mL) with 50% (v/v) ethanol. The samples were scanned in an ultraviolet/visible spectrophotometer (Evolution 60 S, Thermo Scientific, Waltham, MA, USA) for absorbance at 410 nm. Solutions of the extract and standard without the addition of the aluminum chloride reagent were used as controls. All analyses were carried out in triplicate. The results are expressed as the percentage of TFC, calculated as rutin equivalents using the following formula:

$$TFC = \frac{A \times DF}{w \times E_{1\,cm}^{1\%}}$$

where A = absorbance; DF = dilution factor; w = mass of plant material (g); $E_{1cm}^{1\%}$ = specific absorption for the rutin-$AlCl_3$ complex.

2.3. Carbohydrate and protein contents

Carbohydrate content was determined as described by Galvão et al. (2014). The extract (0.5 g) was transferred to a 250 mL round bottom flask and 50 mL of distilled water was added. The solution was heated at 85 °C under reflux in a water bath for 30 min, cooled and left to decant. The supernatant was filtered through a filter paper into a 50-mL volumetric flask and volume was adjusted to 50 mL with distilled water yielding the stock solution (SS). A 3-mL aliquot of SS was transferred to a 250 mL round bottom flask and 1 mL of 5% (w/v) phenol solution and 5 mL of sulfuric acid were added. The mixture was heated at 85 °C in a water bath under reflux for 30 min. After cooling at 28 °C, 1.5 mL of this solution was transferred to a 10 mL volumetric flask, and brought to volume with distilled water yielding the probe solution (PS). The absorbance at 490 nm of the PS was determined with a spectrophotometer after 5 min using water as a blank. Maltose was used as a standard. The carbohydrate content was calculated using the following equation:

$$TPC = \frac{A_1 \times m_2}{m_1 \times A_2} \times DF \times 100$$

where TPC = total polysaccharide content (g %); A_1 = absorbance of PS; m_1 = weight of extract; m_2 = weight of standard; A_2 = absorbance of standard solution; DF = dilution factor.

2.4. Animals

All the experiments were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee on Animal Experimentation of the *Universidade Federal de Pernambuco* (23076.048175/2015-12). Male and female Swiss mice (35–45 g) were used in the experiments after a one week adaptation period in the *Núcleo de Cirurgia Experimental* (NUCEX) at *Universidade de Pernambuco*. The animals were housed under a light/dark cycle of 12 h at 22 °C, and food (Presence Nutrição Animal, Paulínia, Brazil) and water were available ad libitum.

2.5. Experimental procedures

Healthy male and female mice were randomly divided into five groups for each sex (n = 10 per group). The *P. gounellei* extract was dissolved in distilled water before administration to the animals. Mice in the control group received distilled water. The mice in the treated groups received daily doses of 250, 500, and 1000 mg/kg b.w. (in a final volume of 0.1 mL) via oral administration for 28 consecutive days following guideline 407/2008 of the Organization for Economic Cooperation and Development (OECD, 2008). During the experiment, body weight, food consumption, and water intake were recorded weekly. Mice were observed twice daily for signs of toxicity (piloerection, diarrhea, and changes in locomotor activity) and mortality. Body temperature and blood glucose (after 6 h fasting) were assessed weekly. At the end of the 28-day treatment period, the mice were fasted overnight, although water was made available ad libitum. They were then anesthetized with thiopental (35 mg/kg, i.p.), and blood samples were obtained by retro-orbital puncture using capillary tubes. Blood was collected into two tubes: one containing the anticoagulant ethylenediamine tetra acetic acid (EDTA) and one tube without anticoagulant. The urinary bladder was perforated using a sterile syringe to collect urine. The liver, kidneys, spleen, lungs, heart, brain, intestine, testicles and ovaries were removed, weighed and their relative weights (g/10 g of animal body weight) were calculated.

2.6. Blood glucose assay and body temperature measurement

Blood glucose levels were measured using a glucometer (Accu-Chek Active, Roche Diagnóstica Brasil Ltda., São Paulo, Brazil). The rectal temperatures were measured without subjecting the animals to anesthesia by placing the thermometer (Animed, Brazil) approximately 2 mm from the anus. The measurement was performed at room temperature.

2.7. Hematological and biochemical analyses

Hematological analyses were carried out immediately after blood collection using an automatic hematology analyzer (Coulter STKS, Beckman Coulter, Miami, FL, USA) and optical microscopy. Parameters included counting of red blood cells (RBC) and white blood cells (WBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), and differential leukocyte counts (segmented lymphocytes and monocytes).

For biochemical analyses, the blood was centrifuged at 1480 × *g* for 10 min to obtain serum that was stored at − 20 °C. The following parameters were determined: albumin, blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglycerides, total protein, alkaline phosphatase, gamma-glutamyl transferase (GGT) and bilirubin. The dosages of these parameters were performed using specific kits (Labtest Diagnóstica, Lagoa Santa, Brazil) and a COBAS Mira Plus analyzer (Roche Diagnostics Systems, Basel, Switzerland).

2.8. Urine analysis

Urine density, glucose, bilirubin, ketones, pH, protein, urobilinogen, and nitrite concentrations were determined using urine strips (10 Multistix® SG, Siemens Healthineers Brazil).

2.9. Histological analysis

Histological analyses of the liver, kidney, spleen, lung, and heart of mice from the control group and the treatment groups were performed by optical microscopy. Sections of the organs were fixed in buffered formalin (10%, v/v), dehydrated through a graded ethanol series (70–100%), diaphanized in xylol, and embedded in paraffin. Histological slices (5 μm) were stained with hematoxylin-eosin and mounted using cover slips with Entellan resin (Merck, Germany) (Kiernan, 2008). The samples were observed using a Motic BA200 microscope coupled to a Moticam 1000 1.3 MP digital camera (Motic Incorporation Ltd., Causeway Bay, Hong Kong).

2.10. Oxidative stress evaluation

2.10.1. Liver, kidney, and spleen homogenates

Homogenates of the liver, kidney, and spleen (500 μg) were prepared in 50 mM Tris-HCl pH 7.4 containing 1 mM EDTA, 1 mM sodium orthovanadate, and 2 mM phenylmethanesulfonyl fluoride and centrifuged at 2500 × *g* for 10 min at 4 °C. The supernatant was collected and used in the experiments described below. Aliquots of tissue homogenates were used to determine the total protein content as described by Bradford (1976).

2.10.2. Lipid peroxidation assay

Lipid peroxidation was evaluated by estimating the level of thiobarbituric acid-reactive substances (TBARS) (Ohkawa et al., 1979). Briefly, the reaction was developed by the addition of homogenate (100 μL), 30% (w/v) trichloroacetic acid (10 μL), and 10 mM Tris-HCl pH 7.4 (390 μL). The mixture was centrifuged at 2500 × *g* for 10 min. After centrifugation, the supernatant was transferred to another tube and 0.8% (v/v) thiobarbituric acid (10 μL) was added. The mixture was boiled in a water bath for 30 min and after cooling, the absorbance of the organic phase was read at 535 nm using a spectrophotometer. Results are expressed as nmol of malondialdehyde (MDA) per mg of protein.

2.10.3. Superoxide dismutase (SOD) activity

The determination of total tissue SOD activity was performed using liver, kidney, and spleen homogenates (80 μL) incubated with 880 μL of 0.05% (w/v) sodium carbonate (pH 10.2) containing 0.1 mM EDTA in a water bath at 37 °C. The reaction was developed by the addition of 20 μL of 30 mM epinephrine in 0.05% (v/v) acetic acid. The SOD activity was measured by evaluating the kinetics of inhibition of epinephrine autoxidation according to the absorbance at 480 nm. One unit of SOD activity was defined as the amount of protein required to inhibit the autoxidation of 1 μmol of epinephrine per minute. Tissue SOD enzymatic activity is expressed as U/mg protein.

2.10.4. Catalase (CAT) activity

Liver, kidney, and spleen homogenates (80 μL) were used to measure CAT activity. The homogenates were mixed in a buffer solution of potassium phosphate (pH 7.4) and centrifuged at 3000 rpm for 15 min. A solution of hydrogen peroxide (0.059 M) in this buffer was used as substrate. In a quartz cuvette, 0.1 mL of the supernatant was mixed with 1 mL of hydrogen peroxide solution and 1.9 mL of distilled water. The enzyme activity was measured at 240 nm to monitor the change in absorbance between the first and sixth minutes (Beers and Sizer, 1952). One unit of CAT activity was defined as the amount of protein required to convert 1 μmol of H_2O_2 per minute to H_2O. Tissue CAT enzymatic activity is expressed as mU/mg protein.

2.10.5. Nitrite analysis

Nitrite levels were estimated using Griess reagent as an indicator of nitric oxide production (Green et al., 1982). Equal volumes (100 μL) of homogenate and reagent [1%, w/v, sulfanilamide in 2.5% (v/v) phosphoric acid and 0.1% (w/v) 1-naphthylethylene diamine dihydrochloride in water] were placed in a 96-well microplate and allowed to react for 10 min at 20 °C. The absorbance at 540 nm was read. The results are expressed as pmol of nitrite/mg protein using a standard curve prepared from sodium nitrite as a reference.

2.11. Antipyretic studies

For the analysis of the antipyretic activity of the *P. gounellei* extract, male mice with rectal temperatures between 35.0 and 38.0 °C were selected. The model of yeast-induced hyperthermia was performed following the method previously described by Aoueya et al. (2016) with some modifications. Five groups of mice (n = 6) were formed and hyperthermia was induced by subcutaneous injection of *Saccharomyces cerevisiae* (1 mL/100 g b.w., 15%, w/v, in saline). Rectal temperatures were recorded again 18 h later. Mice that had a body temperature increase greater than 1 °C were treated with distilled water (control), *P. gounellei* extract (125, 250, and 500 mg/kg, *per os*), 10 mg/kg diclofenac (positive control, *per os*; Malhotra et al., 2013). Rectal temperatures were recorded using a digital thermometer at 30, 60, 90, 120, and 180 min post-treatment.

2.12. Statistical analyses

The results are expressed as mean ± S.D. or mean ± S.E.M. One-way analyses of variance (ANOVA) followed by Bonferroni's posttest was used to calculate statistical significance between groups with GraphPad Prism Software 5.0 (GraphPad Software, La Jolla, CA, USA). A p value < 0.05 was considered significant.

3. Results and discussion

The *P. gounellei* extract evaluated here was the same as that studied by Oliveira et al. (2018), who showed that it contains derivatives of kaempferol (0.21%, w/w) and reducing sugars. The flavonoid content determined was 0.054 ± 0.0025 g% and the polysaccharide content was 17.17 ± 0.0045 g%. As stated in this previous work, saline solution was effective in extracting both proteins and carbohydrates (the major components of *P. gounellei* stem) together with flavonoids, yielding a preparation containing both primary and secondary

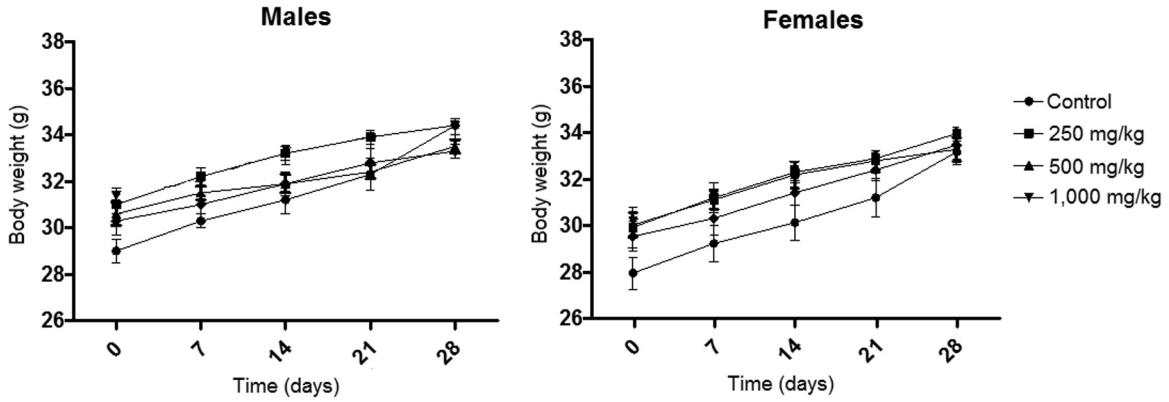

Fig. 1. Body weight gain (g) in male and female mice in the control group and the groups treated daily with the saline extract from *P. gounellei* stem at 250, 500, and 1000 mg/kg over 28 days. Values are expressed as mean ± S.E.M.

metabolites. Here, we evaluated long-term toxicity of this extract in mice via daily oral administration at various doses over 28 days.

The mice that received the *P. gounellei* extract at 250 and 500 mg/kg doses did not show behavioral alterations throughout the study, similar to the control group. Mice of both sexes treated with a dose of 1000 mg/kg showed irritability and increased motility within 15–30 min after administration. However, these signs were not observed after this period. Hippocratic screening of the effects of a hydroethanolic extract of *Dilodendron bipinnatum* bark in mice showed that the highest dose of 5000 mg/kg (administered orally) caused reduction in motility, increased respiratory rate, and tail erection (Mahon et al., 2014). Similar to the results presented here, the effects reversed after a short period and were considered toxicologically irrelevant.

Changes in body weight are indicators of adverse effects of drugs and chemicals (Tofovic and Jackson, 1999). Over the period of treatment, the increase in body weight was similar among both sexes in treated and control mice (Fig. 1). In addition, there were non-significant or no alterations in food and water consumption, among the groups (Fig. 2). These findings indicate that oral administration of repeated doses of the *P. gounellei* extract did not have effects on the growth, feeding activity, and maintenance of body fluid volume.

Glycemic evaluation showed that all groups treated with *P. gounellei*

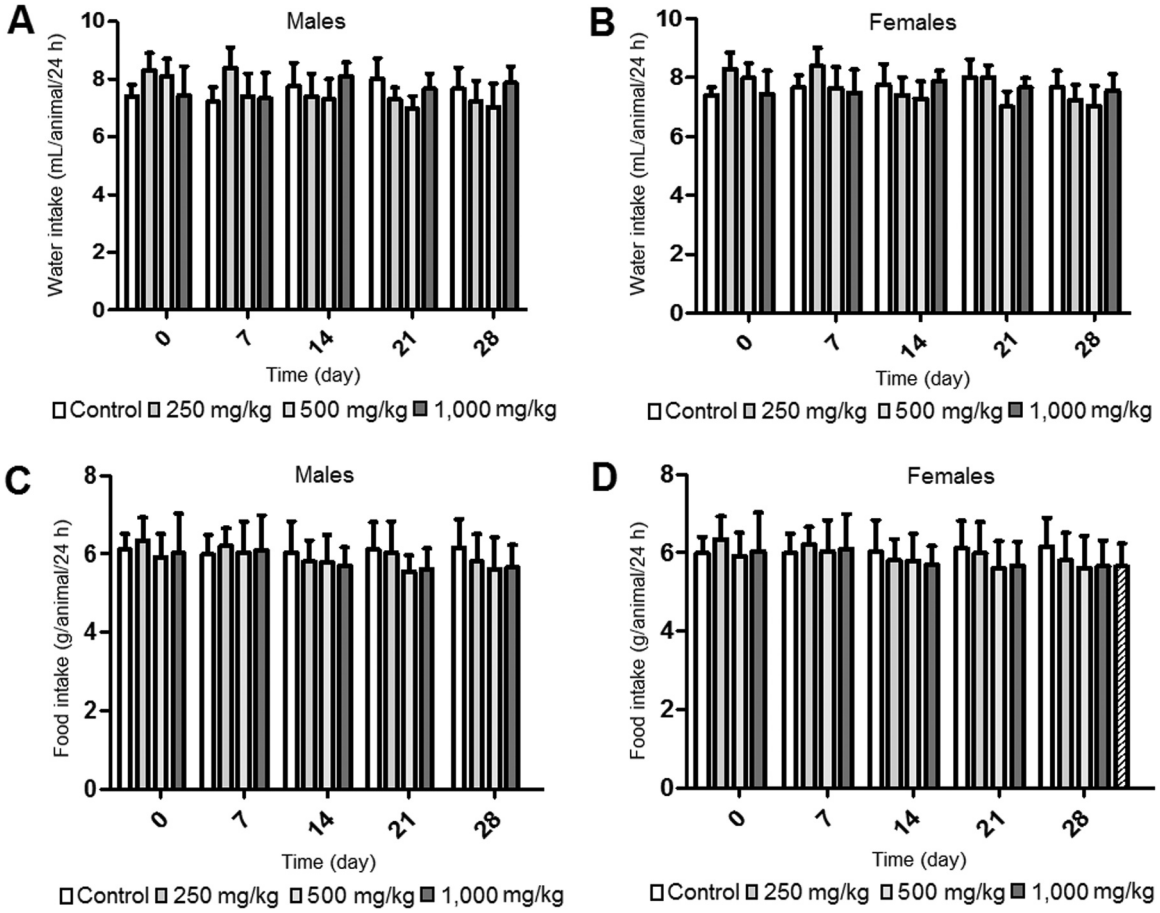

Fig. 2. Consumption of water (A and B) and food (C and D) by male and female mice from the control group and the groups treated daily with the saline extract from *P. gounellei* stem at 250, 500, and 1000 mg/kg over 28 days. No significant differences ($p > 0.05$) were observed between the groups.

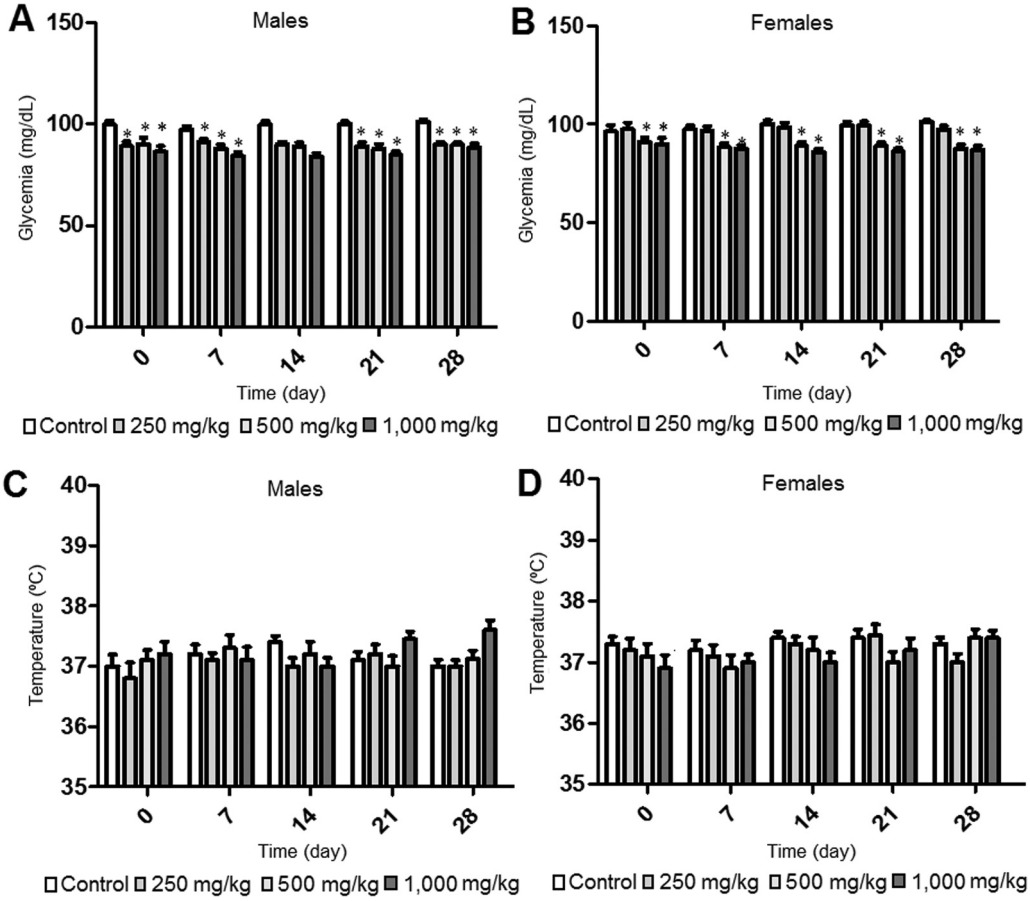

Fig. 3. Evaluation of glycemia (A and B) and temperature (C and D) in male and female mice from the control group and the groups treated daily with the saline extract from *P. gounellei* stem at 250, 500, and 1000 mg/kg over 28 days. (*) indicates significant difference ($p < 0.05$) in comparison with control.

extract demonstrated a significant decrease in blood glucose when compared with controls (Fig. 3A and B), although the levels remained within the normal range. The recorded body temperatures of the treated groups were not significantly different from the respective controls (Fig. 3C and D).

Evaluation of hematological parameters has a high predictive value for toxicity in mammals and the data from animal studies may be translated to humans (Olson et al., 2000). In addition, hematological analyses provide information about the hematopoietic system and immunological responses (Igwebuike and Obidike, 2007). For example, increased production of leukocytes is usually considered as a marker of stress and indicates an immune response. In the present study, none of the hematological parameters (erythrocytes, HCT, Hb, MCH, MCHC, RDW, and leukocytes) were significantly different among treated and control groups (Table 1).

The biochemical analyses showed no significant differences in the blood levels of albumin, alkaline phosphatase, total proteins, BUN, creatinine, and bilirubin among the groups (Table 2). Total cholesterol levels significantly decreased in male and female mice that received doses of 500 and 1000 mg/kg. Triglycerides levels were also lower in

Table 1
Hematological parameters of mice treated daily with the saline extract from *P. gounellei* stem *per os* for 28 days.

Parameter	Females				Males			
	Control	Extract			Control	Extract		
		250 mg/kg	500 mg/kg	1000 mg/kg		250 mg/kg	500 mg/kg	1000 mg/kg
Erythrocytes (10^6/mm^3)	9.54 ± 0.19	9.21 ± 0.32	9.66 ± 0.20	9.19 ± 0.38	9.94 ± 0.77	9.62 ± 0.51	9.75 ± 0.93	9.61 ± 0.72
Hematocrit (%)	46.22 ± 0.65	45.54 ± 0.15	46.09 ± 0.17	45.87 ± 0.19	47.26 ± 0.41	46.25 ± 0.61	45.87 ± 0.22	46.45 ± 0.76
Hemoglobin (%)	16.41 ± 0.55	16.52 ± 0.22	16.39 ± 0.32	15.89 ± 0.26	17.19 ± 0.34	17.22 ± 0.21	16.49 ± 0.88	17.01 ± 0.86
MCV (%)	45.23 ± 0.16	46.01 ± 0.28	45.86 ± 0.62	46.12 ± 0.17	47.12 ± 0.27	47.35 ± 0.46	48.01 ± 0.77	47.68 ± 0.52
MCH (%)	17.88 ± 0.27	17.71 ± 0.31	17.33 ± 0.15	17.44 ± 0.63	18.03 ± 0.34	17.89 ± 0.52	17.90 ± 0.24	17.72 ± 0.13
MCHC (%)	37.31 ± 0.18	37.89 ± 1.09	38.01 ± 0.66	37.87 ± 0.27	38.03 ± 0.51	38.14 ± 0.32	37.93 ± 0.47	37.79 ± 0.48
Leukocytes (10^3/mm^3)	11.29 ± 1.02	10.90 ± 1.17	11.02 ± 1.12	10.99 ± 1.19	10.07 ± 0.74	9.94 ± 1.02	10.12 ± 0.89	10.11 ± 1.03
Segmented (%)	50.34 ± 0.18	49.34 ± 0.33	50.22 ± 0.67	51.01 ± 0.38	52.31 ± 0.41	52.04 ± 0.11	51.84 ± 0.62	51.22 ± 0.80
Lymphocytes (%)	38.38 ± 0.07	39.10 ± 0.12	38.82 ± 0.21	37.23 ± 0.18	35.13 ± 0.71	35.62 ± 0.45	36.17 ± 0.68	36.58 ± 0.31
Monocytes (%)	11.28 ± 0.65	11.56 ± 0.09	10.96 ± 0.78	11.76 ± 0.33	12.56 ± 0.55	12.34 ± 0.32	11.99 ± 0.29	12.20 ± 0.31

MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration. Values represent the mean ± SEM (n = 10/group). No significant differences ($p > 0.05$) were found in comparison with control.

Table 2
Biochemical parameters of blood of mice treated daily with the saline extract from *P. gounellei* stem *per os* for 28 days.

Parameter	Females				Males			
	Control	Extract			Control	Extract		
		250 mg/kg	500 mg/kg	1000 mg/kg		250 mg/kg	500 mg/kg	1000 mg/kg
Albumin (g/dL)	3.89 ± 0.12	3.82 ± 0.16	3.84 ± 0.19	3.76 ± 0.21	3.41 ± 0.18	3.52 ± 0.21	3.44 ± 0.16	3.58 ± 0.15
ALT (U/L)	67.6 ± 0.30	67.1 ± 0.44	66.9 ± 0.39	67.4 ± 0.28	69.4 ± 0.42	69.1 ± 0.28	68.9 ± 0.34	68.0 ± 0.33
AST (U/L)	110.2 ± 0.23	111.0 ± 0.51	110.7 ± 0.35	109.9 ± 0.55	102.4 ± 0.33	102.5 ± 0.12	102.9 ± 0.51	101.8 ± 0.69
Alkaline phosphatase (IU/L)	12.3 ± 0.22	13.5 ± 0.22	12.3 ± 0.24	12.5 ± 0.31	11.9 ± 0.34	11.5 ± 0.27	12.2 ± 0.22	11.7 ± 0.41
GGT (U/L)	10.21 ± 0.23	11.0 ± 0.44	10.5 ± 0.31	11.3 ± 0.21	9.83 ± 0.10	9.68 ± 0.24	10.1 ± 0.11	9.97 ± 0.18
Total protein (g/dL)	5.79 ± 0.12	5.29 ± 0.21	5.42 ± 0.33	5.61 ± 0.27	4.98 ± 0.19	4.96 ± 0.13	5.02 ± 0.15	5.01 ± 0.17
BUN (mg/ dL)	38.5 ± 0.12	36.3 ± 0.16	35.9 ± 0.18	37.7 ± 0.20	35.7 ± 0.22	36.1 ± 0.26	35.6 ± 0.31	35.3 ± 0.23
Creatinine (mg/ dL)	0.31 ± 0.06	0.34 ± 0.10	0.29 ± 0.12	0.33 ± 0.04	0.27 ± 0.04	0.28 ± 0.03	0.30 ± 0.01	0.31 ± 0.04
Bilirubin	0.22 ± 0.01	0.20 ± 0.01	0.23 ± 0.02	0.23 ± 0.04	0.19 ± 0.03	0.21 ± 0.01	0.22 ± 0.02	0.20 ± 0.02
Total Cholesterol (mg/dL)	149.4 ± 10.32	145.8 ± 11.46	119.4 ± 12.52 *	104.1 ± 11.3 *	110.3 ± 9.11	106.3 ± 10.89	96.3 ± 9.07 *	97.3 ± 11.09 *
Triglycerides (mg/dL)	135.3 ± 12.05	133.2 ± 12.09	126.8 ± 8.26 *	117.1 ± 9.21 *	129.3 ± 9.45	125.7 ± 10.11	118.5 ± 9.87 *	120.3 ± 9.43 *

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: gamma-glutamyl transferase; BUN: blood urea nitrogen. Values represent the mean ± SEM (n = 10/group). (*) indicates significant difference ($p < 0.05$) in comparison with control.

Table 3
Analysis of urine collected from mice treated daily with the saline extract from *P. gounellei* stem *per os* for 28 days.

Parameter	Females				Males			
	Control	Extract			Control	Extract		
		250 mg/kg	500 mg/kg	1000 mg/kg		250 mg/kg	500 mg/kg	1000 mg/kg
Density	1.21	1.24	1.19	1.21	1.22	1.21	1.22	1.20
Glucose	–	–	–	–	–	–	–	–
Bilirubin	–	–	–	–	–	–	–	–
Ketones	–	–	–	–	–	–	–	–
pH								
Protein	–	–	–	+	–	–	–	+
Urobilinogen	–	–	–	–	–	–	–	–
Nitrite	–	–	–	–	–	–	–	–
Blood	–	–	–	–	–	–	–	–

(-): absent. (+): present.

male and female mice that received doses of 500 and 1000 mg/kg than controls (Table 2).

A study of *Opuntia ficus indica* (Cactaceae) revealed that a diet containing its seeds resulted in a pronounced hypolipidemic effect with significant decreases in the levels of triglycerides in the serum and total lipids in the liver in rats (Ennouri et al., 2007). Zanchet et al. (2018) demonstrated that the hydroalcoholic extract of *Celtis iguanaea* was effective in the prevention of hypercholesterolemia and provided protection against atherosclerosis and hyperglycemia.

Urine was also evaluated; the treated groups did not show any differences when compared with controls, except for the presence of proteins in the treated groups (male and female; 1000 mg/kg) (Table 3). Urinalysis is commonly used to evaluate kidney function and can aid in the diagnosis of various renal diseases. The presence of protein in the urine of mice treated with the highest dose may or may not be indicative of renal lesions. For example, it can be linked to hemodynamic alterations in glomerular blood flow due to exercise, fever, seizures, and stress, or due to overflow of elevated normal or abnormal plasma proteins, in order that the tubular cells cannot reabsorb all that is filtered (Narchi, 2008). Then, the possibility of kidney lesion was investigated by histopathological analysis.

The macroscopic analysis of the organs of the mice treated with the *P. gounellei* extract did not show significant changes in color or texture when compared with the control groups. There were no significant differences in the relative weights of livers in male and female mice treated with the extract compared with controls (Table 4).

Fig. 4 shows photomicrographs of the livers and kidneys of mice (both sexes) in the control and treated groups. The liver of male mice treated with 250 and 500 mg/kg of the extract showed well delimited hepatocytes, nuclei with visible chromatin, bile ducts, central lobular veins with preserved characteristics, and the absence of fibrosis. However lymphocytic infiltrates were observed in the hepatic parenchyma around the bile duct in male mice (1000 mg/kg), compared with the control group. Similarly, the presence of lymphocytic infiltrates near the centrilobular vein was noted in the liver of mice treated with 1000 mg/kg of the extract. No alterations were observed in the kidneys, suggesting that the presence of protein in the urine of the mice treated with the highest dose is not the result of a pathological process.

Photomicrographs of the lungs and hearts of male and female mice, both treated or non-treated with the extract, are presented in Fig. 5. Infiltration of the parenchyma with thickening of the interalveolar septa was observed in the lungs of male mice treated with 1000 mg/kg, while the other groups did not show alterations in lungs. No changes were observed in the heart tissue of male mice in any group. Female mice treated with the extract at 250 and 500 mg/kg doses did not show histological changes in the liver and heart when compared with the control group. However, thickening of the interalveolar septa in the lungs of females treated with a dose of 1000 mg/kg was observed. The spleen of treated male and female mice did not show histological alterations in comparison with controls, except in the case of females that received a dose of 1000 mg/kg, which showed a considerable activation of lymph nodes (Fig. 6).

The *P. gounellei* extract administered over 28 days at different doses did not induce any significant changes in the MDA, SOD, CAT, and

Table 4
Evaluation of the relative weight (g/10 g animal body weight) of mice treated daily with the saline extract from *P. gounellei* stem *per os* for 28 days.

Organ	Females				Males			
	Control	Extract			Control			
		250 mg/kg	500 mg/kg	1000 mg/kg		250 mg/kg	500 mg/kg	1000 mg/kg
Brain (g/10 g)	0.36 ± 0.01	0.38 ± 0.02	0.39 ± 0.00	0.38 ± 0.02	0.39 ± 0.02	0.38 ± 0.01	0.39 ± 0.02	0.38 ± 0.03
Liver (g/10 g)	2.18 ± 0.11	2.19 ± 0.19	2.18 ± 0.18	2.19 ± 0.17	2.25 ± 0.22	2.24 ± 0.36	2.24 ± 0.41	2.24 ± 0.32
Kidney (g/10 g)	0.31 ± 0.01	0.29 ± 0.04	0.31 ± 0.06	0.31 ± 0.10	0.26 ± 0.09	0.28 ± 0.03	0.32 ± 0.10	0.30 ± 0.08
Lung (g/10 g)	0.24 ± 0.04	0.24 ± 0.2	0.24 ± 0.04	0.24 ± 0.00	0.26 ± 0.01	0.25 ± 0.02	0.26 ± 0.00	0.28 ± 0.04
Heart (g/10 g)	0.19 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.17 ± 0.02	0.21 ± 0.02	0.22 ± 0.02	0.21 ± 0.00	0.23 ± 0.03
Spleen (g/10 g)	0.29 ± 0.01	0.29 ± 0.00	0.29 ± 0.02	0.29 ± 0.04	0.29 ± 0.01	0.29 ± 0.02	0.30 ± 0.01	0.29 ± 0.03
Intestine (g/10 g)	0.26 ± 0.01	0.28 ± 0.02	0.29 ± 0.03	0.27 ± 0.02	0.30 ± 0.00	0.28 ± 0.04	0.27 ± 0.03	0.28 ± 0.02
Testicle (g/10 g)	–	–	–	–	0.08 ± 0.02	0.10 ± 0.02	0.07 ± 0.02	0.08 ± 0.01
Ovary (g/10 g)	0.11 ± 0.01	0.10 ± 0.02	0.07 ± 0.03	0.09 ± 0.02	–	–	–	–

Values represent the mean ± SEM (n = 10/group). No significant differences ($p > 0.05$) were found in comparison with control.

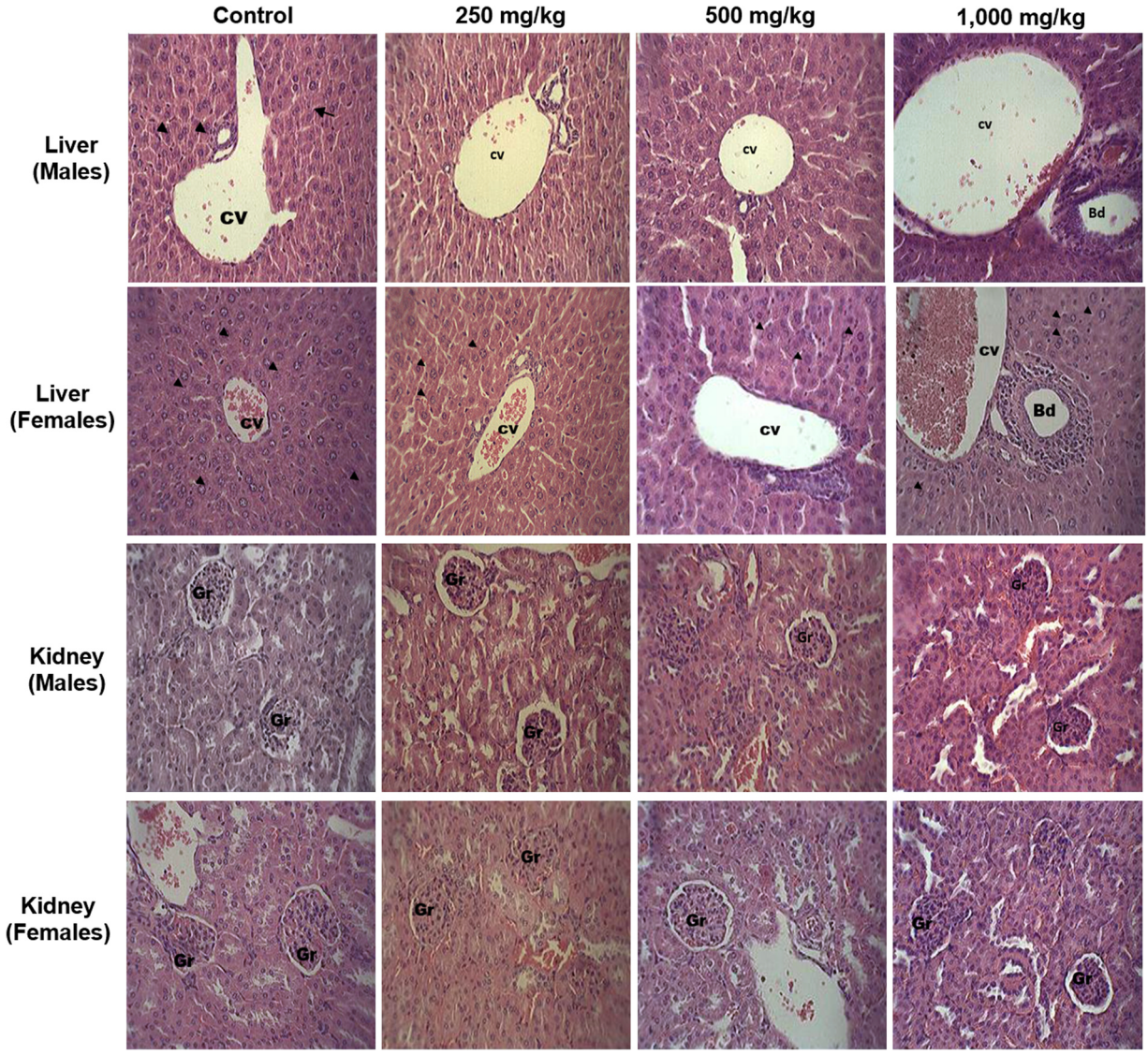

Fig. 4. Representative photomicrographs of the livers and kidneys of male and female mice from the control group and the groups treated daily with the saline extract from *P. gounellei* stem at the doses of 250, 500, and 1000 mg/kg over 28 days. **Livers**: the centrilobular vein (cv) is seen in all images. In control, the hepatocyte bundles (arrow heads) are preserved and ordered. Lymphocytic infiltrates are visible around the bile duct (Bd) in male and female mice treated with the extract at 1000 mg/kg. **Kidneys:** Renal glomeruli (Gr) and contorted tubules without alterations are visible in control and extract-treated groups. Hematoxylin-eosin staining was used. Magnification: 400×.

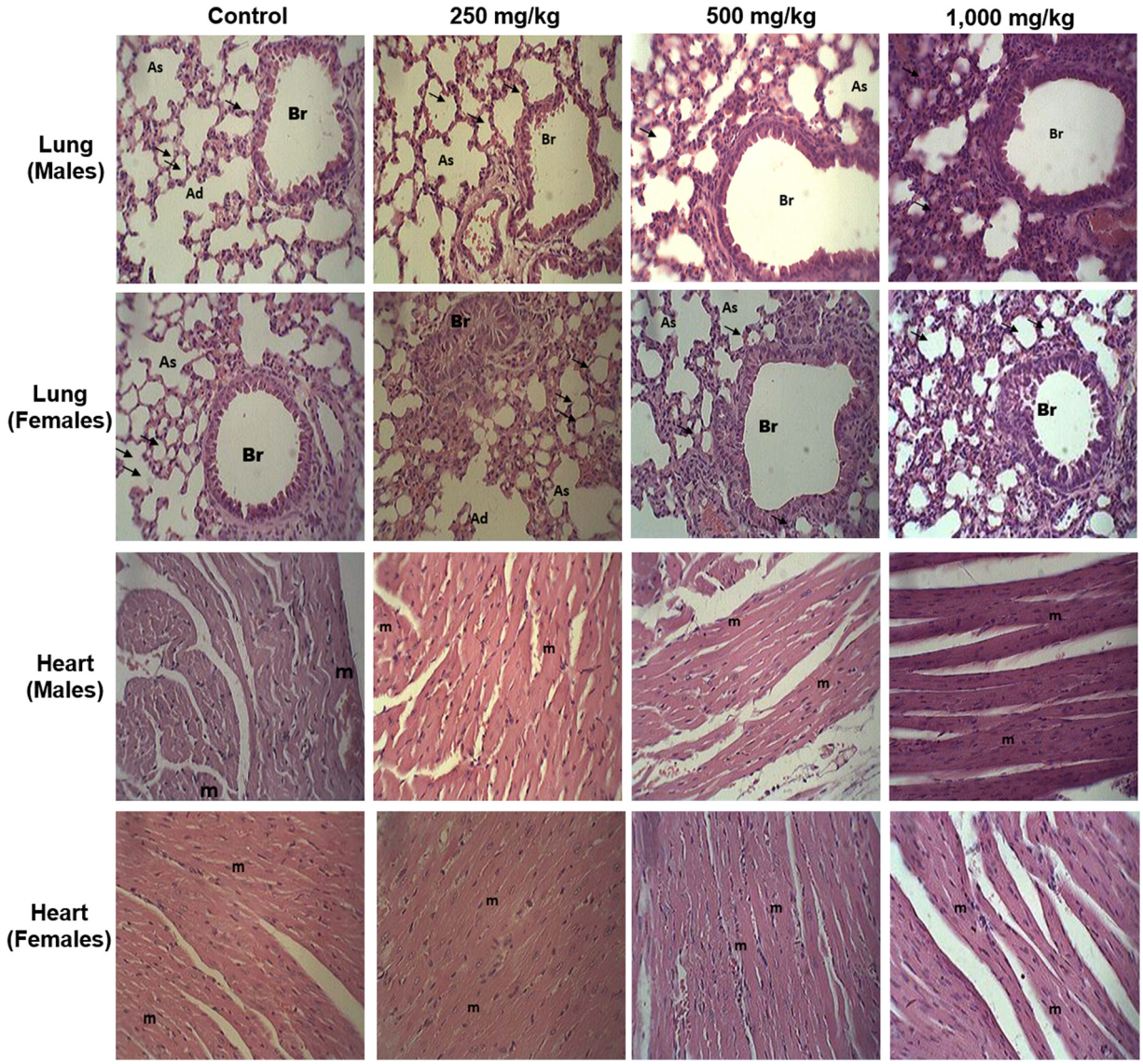

Fig. 5. Representative photomicrographs of the lungs and hearts of male and female mice from the control group and the groups treated daily with the saline extract from *P. gounellei* stem at the doses of 250, 500, and 1000 mg/kg over 28 days. **Lungs:** bronchiole (Br), alveolar sac (As), and interalveolar septa (short arrows) can be visualized, with preserved architecture in control and male and female mice treated with the extract at 250 and 500 mg/kg. The alveolar duct can also be seen in one image. The groups of male and female mice that received the dose of 1000 mg/kg showed thickening of the interalveolar septa. **Heart:** Cardiac muscle (m) and pericardium with normal structures in all groups. No signs of degeneration, necrosis/inflammation, or other changes. Hematoxylin-eosin staining was used. Magnification: 400×.

nitrite levels in the kidneys of both sexes of mice (Table 5). The liver is the primary source of xenobiotic metabolism whereby the process of degradation and elimination of drugs tend to cause oxidative stress in liver (Chen et al., 2014), accompanied by inhibition of antioxidant enzymes and elevation of lipid peroxidation (Morsy et al., 2016). In the livers of both male and female rats that were treated with 500 and 1000 mg/kg doses, there was a significant increase in SOD activity. In the spleen, there was no significant change in MDA levels in comparison with the control group in females; there was a significant decrease in male mice treated at all doses. The SOD levels in the spleen increased in male and female mice. These results may suggest that the aqueous extract of *P. gounellei* at a high dose (1000 mg/kg) stimulates the protection against free radicals in the female and male liver and spleen.

There is evidence of gender differences in response to a wide variety of drugs (Czerniak, 2001; Sin et al., 2007; Kadekar et al., 2012). The gender differences observed in mice may be due to one or more factors such as hepatic metabolism (which may include differential expression of cytochrome P450 enzymes), pharmacokinetic parameters (plasma clearance and volume), and differences in both phase I and phase II metabolism (Czerniak, 2001). In our study, we did not find remarkable sex-related differences between treated and control groups.

Based on the toxicity results, the antipyretic activity of the extract was evaluated using doses up to 500 mg/kg. Subcutaneous injection of a yeast suspension markedly elevated the rectal temperature after 18 h of administration. Treatment with 500 mg/kg of the extract significantly decreased ($p < 0.05$) the rectal temperature of the mice (Fig. 7). The antipyretic effect began after 60 min and continued until the end of the evaluation (180 min). The standard drug group (sodium

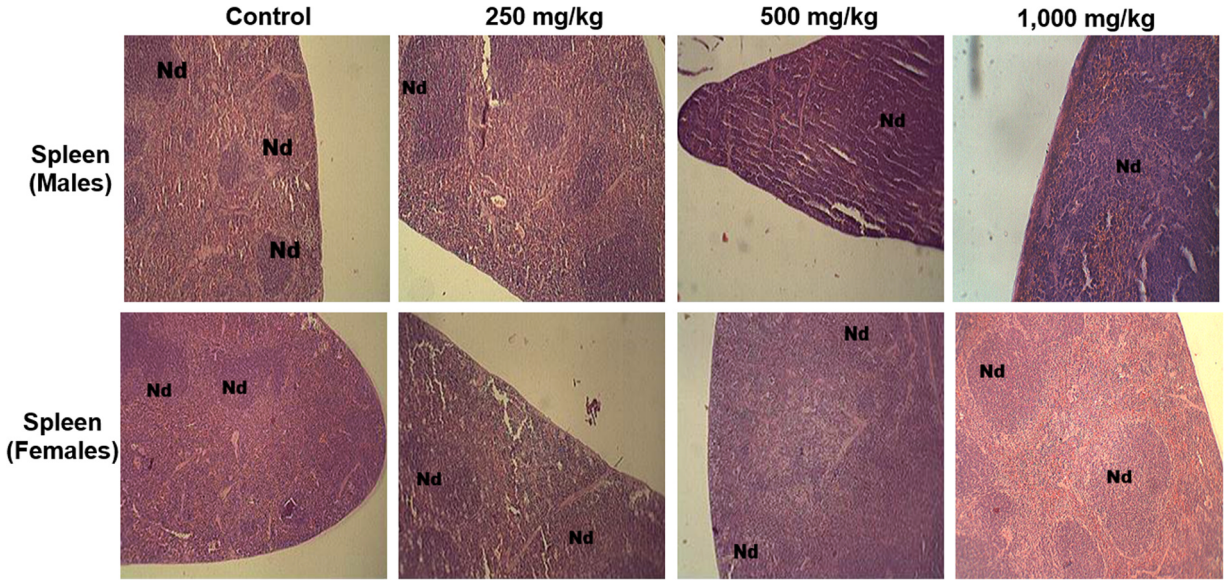

Fig. 6. Representative photomicrographs of the spleens of male and female mice from the control group and the groups treated daily with the saline extract from *P. gounellei* stem at the doses of 250, 500, and 1000 mg/kg over 28 days. The lymph nodes (Nd) are well-defined in the control and treated groups. Spleens of females that received a dose of 1000 mg/kg showed a considerable activation of lymph nodes. Hematoxylin-eosin staining was used. Magnification: 400×.

Table 5
Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and nitrite levels in organs of mice treated daily with the saline extract from *P. gounellei* stem *per os* for 28 days.

Parameter	Females				Males			
	Control	Extract			Control	Extract		
		250 mg/kg	500 mg/kg	1000 mg/kg		250 mg/kg	500 mg/kg	1000 mg/kg
Liver								
MDA (nM/mg of protein)	4.94 ± 0.88	4.42 ± 0.37	4.56 ± 0.55	4.37 ± 0.41	5.01 ± 0.23	5.12 ± 0.60	4.98 ± 0.29	4.72 ± 0.90
SOD (U/mg of protein)	10.96 ± 0.06	11.89 ± 0.62	12.81 ± 0.34*	13.88 ± 0.34*	9.65 ± 0.06	9.44 ± 0.09	10.22 ± 0.11*	12.57 ± 0.08*
CAT (nM/mg of protein)	0.33 ± 0.03	0.36 ± 0.02	0.35 ± 0.02	0.36 ± 0.01	0.31 ± 0.12	0.29 ± 0.11	0.30 ± 0.08	0.30 ± 0.02
Nitrite (pmol/ng of protein)	0.90 ± 0.02	0.91 ± 0.01	0.90 ± 0.02	0.91 ± 0.01	0.89 ± 0.03	0.89 ± 0.01	0.88 ± 0.05	0.88 ± 0.03
Spleen								
MDA (nM/mg of protein)	1.04 ± 0.01	1.05 ± 0.03	1.03 ± 0.02	1.03 ± 0.02	1.45 ± 0.11	1.22 ± 0.09*	1.27 ± 0.06*	1.24 ± 0.05*
SOD (U/mg of protein)	0.48 ± 0.01	0.50 ± 0.04	0.61 ± 0.02*	0.73 ± 0.02*	0.49 ± 0.01	0.50 ± 0.04	0.61 ± 0.03*	0.63 ± 0.05*
CAT (nM/mg of protein)	3.44 ± 0.08	3.43 ± 0.11	3.47 ± 0.09	3.49 ± 0.12	3.31 ± 0.03	3.29 ± 0.11	3.30 ± 0.07	3.32 ± 0.06
Nitrite (pmol/ng of protein)	0.92 ± 0.03	0.90 ± 0.02	0.88 ± 0.03	0.87 ± 0.04	0.91 ± 0.06	0.93 ± 0.08	0.90 ± 0.05	0.92 ± 0.07
Kidney								
MDA (nM/mg of protein)	5.93 ± 0.59	5.88 ± 0.33	5.98 ± 0.12	5.92 ± 0.59	5.91 ± 0.14	5.96 ± 0.34	5.87 ± 0.09	5.88 ± 0.23
SOD (U/mg of protein)	0.99 ± 0.07	0.96 ± 0.02	0.97 ± 0.05	0.96 ± 0.03	0.82 ± 0.03	0.85 ± 0.05	0.81 ± 0.08	0.83 ± 0.05
CAT (nM/mg of protein)	0.011 ± 0.002	0.013 ± 0.001	0.010 ± 0.001	0.011 ± 0.002	0.008 ± 0.001	0.010 ± 0.002	0.013 ± 0.001	0.012 ± 0.002
Nitrite (pmol/ng of protein)	0.99 ± 0.07	0.97 ± 0.02	0.94 ± 0.08	0.98 ± 0.06	0.88 ± 0.02	0.89 ± 0.04	0.90 ± 0.04	0.87 ± 0.07

Values represent the mean ± SEM (n = 10/group). (*) indicates significant difference ($p < 0.05$) in comparison with control.

diclofenac 10 mg/kg) showed a significant decrease in temperature when compared with that in the control group (Fig. 7). The treatment at 500 mg/kg did not show significant difference ($p > 0.05$) in comparison with diclofenac group.

Injection of yeast promotes the release of proinflammatory cytokines that stimulate the synthesis of prostaglandin E2 in the surroundings of the thermoregulatory centers of the hypothalamus, provoking the temperature rise (Morrison, 2016). The results found here suggest that the extract of *P. gounellei* may have anti-inflammatory effects. The presence of flavonoids in medicinal plants has been associated with analgesic and antipyretic properties (Kumar et al., 2015). The methanol and petroleum ether fractions of *Schoenoplectus grossus* significantly lowered the temperature in yeast-induced pyrexia, which is assumed to be mediated through interference of prostaglandin synthesis and inhibition of cytokine release (Subedi et al., 2016).

4. Conclusion

When administered orally on a daily basis, the saline extract from the stem of *P. gounellei* was not associated with significant toxic effects. Moreover, it showed antipyretic activity along with hypoglycemic and hypolipidemic effects. Further studies are needed to investigate the pharmacological potential of *P. gounellei.*

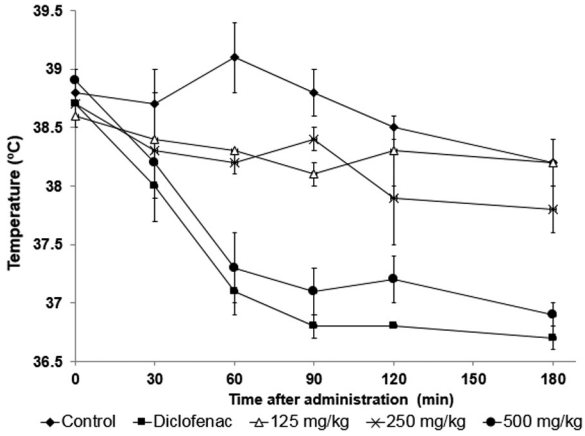

Fig. 7. Monitoring of rectal temperature of yeast-infected and hyperthermic mice after daily administration of the saline extract from *P. gounellei* stem at the doses of 125, 250, and 500 mg/kg or 10 mg/kg diclofenac (positive control) over 28 days.

CRediT authorship contribution statement

Alisson Macário de Oliveira: Investigation, Methodology, Data curation, Formal analysis, Conceptualization, Writing - original draft. **Wliana Alves Viturino da Silva:** Investigation, Methodology. **Magda Rhayanny Assunção Ferreira:** Investigation, Methodology, Data curation, Formal analysis. **Patrícia Maria Guedes Paiva:** Funding acquisition, Resources, Supervision, Data curation, Formal analysis, Writing - review & editing. **Paloma Lys de Medeiros:** Investigation, Methodology. **Luiz Alberto Lira Soares:** Funding acquisition, Resources, Supervision, Data curation, Formal analysis. **Bruno Melo Carvalho:** Funding acquisition, Resources, Supervision, Data curation, Formal analysis, Writing - review & editing. **Thiago Henrique Napoleão:** Funding acquisition, Resources, Supervision, Data curation, Formal analysis, Conceptualization, Writing - original draft, Writing - review & editing.

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Conflicts of interest

The authors declare no conflict of interest.

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3.3. ARTIGO 3 – *Pilosocereus gounellei* STEM EXTRACT DECREASES INSULIN RESISTANCE, HYPERLIPIDEMIA, CARDIAC RISK AND OXIDATIVE STRESS IN OBESE MICE

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Abstract

Pilosocereus gounellei is a popular Caatinga plant used for treatment of inflammatory processes. Previous studies revealed the low acute and sub-acute toxicity of a saline extract from *P. gounellei* stem in mice and evidences of a hypolipidemic effect. The present work evaluated the anti-obesity activity of this extract in mice fed on a high-fat diet (HFD). It was evaluated glucose intolerance, insulin resistance, serum lipid profile, atherogenic indices as well as the levels of oxidative stress markers and histological organization in liver, muscle and epididymal adipose tissue. Diet-induced obese mice were treated daily with saline (DIO group), the extract (125, 250 or 500 mg/kg) or 400 mg/kg metformin (MET group) *per os* for 21 days. The study included a non-obese group as control. Animals treated with the extract showed mean weight lower than DIO and MET groups. Reduction in the levels of total cholesterol, LDL-cholesterol, and triglycerides was observed in the extract-treated groups. The treatment also restored glucose tolerance to levels similar to the control and decreased insulin resistance. The atherogenic indices were also lower in animals-treated with the extract than DIO group; in the treatment at 500 mg/kg, these values were similar to those from control. The treatment with the

extract prevented lipid peroxidation and increased SOD levels in the three organs evaluated. The extract treatment reduced steatosis, collagen deposition, and inflammation in liver, when compared to DIO group. Epididymal fat was also reduced in the extract-treated groups. In conclusion, the *P. gounellei* saline extract was able to improve the physiological parameters from obese mice, suggesting its therapeutic application against the deleterious effects of the obesity state.

Keywords: *Pilosocereus gounellei*, Caatinga, saline extract, obesity.

1. Introduction

The obesity is the result of the increase in the size (hypertrophy) and number (hyperplasia) of adipocytes and is usually associated with comorbidities such as cardiovascular and respiratory complications, type-II diabetes, dyslipidemia, certain types of cancers, neuropsychiatric disorders and osteoarthritis; in addition, obesity is prevalent in all age and sex groups being considered a major health concern worldwide (Kopelman, 2000; Garaulet et al., 2010). The International Diabetes Federation (IDF) reported that diabetes afflicted 425 million people in 2017 and the number is expected to rise to 552 million by 2030.

Insulin resistance is defined as a state of reduced metabolic response to the circulating levels of this hormone (Harcourt et al., 2013) and is directly related to abnormalities in peripheral tissues (muscle, liver and adipose tissue), in the central nervous system (hypothalamic neurons involved in the control of food intake) and pancreatic beta-cells (Kahn et al., 2014; McArdle et al., 2013). The insulin resistance decreases energy expenditure, which contributes to the metabolic dysfunctions. Among the causative mechanisms involved in the insulin signaling impairment is the excess reactive oxygen species (Canto and Auwerx, 2009).

The most commonly used drugs to treat diabetes mellitus linked to insulin resistance have many side effects (e.g. gastrointestinal discomfort, flatulence, diarrhea, insomnia, and headache) and high doses are required in order to obtain any improvement (Kahn et al., 2014; Lee et al., 2015). For this reason, there is a continuous need to develop new, safer and more effective pharmaceuticals for the management and treatment of this dysfunction. Furthermore, many people believe that plants correspond to less toxic treatment option and their employment is more accessible and less expensive than the synthetic drugs. Their huge advantage is that they can be ingested in the daily diet. Several medicinal plants are used to improve diabetes mellitus in many countries (ALMUAIGEL, et al, 2017).

The Cactaceae family comprises variously shaped plants that grow in dry regions, being mostly succulent and leafless, with conspicuous areoles, bearing spines and hairs or bristles (Xu and Deng, 2017). *Pilosocereus gounellei*, popularly known as xique-xique, is a cactus used, for example, to treat inflammatory processes resulting from perforations in the hands and foots (Oliveira, 2011) as well as in the treatment of prostate and urethral inflammations (Agra et al., 2008; Roque et al., 2010). Oliveira et al. (2018) showed that saline extract from *P. gounellei* reduced serum levels of cholesterol and triglycerides without acute toxicity to mice. In another study, Oliveira et al. (2019) reported that this extract did not present toxic effects over 28 consecutive days and evidences of hypoglycemic and hypolipidemic effects were also observed. In regard to the chemical composition, it was verified the presence of reducing sugars and flavonoids in the extract.

In the present work, it was investigated the effects of the oral administration of the saline extract from *P. gounellei* stem in diet-induced obese (DIO) mice, using a model that mimics a metabolic syndrome and diabetes mellitus disorder associated to an unhealthy lifestyle.

2. Material and methods

2.1 Plant material and preparation of the extract

Stem of *P. gounellei* was collected in Afogados da Ingazeira City, Pernambuco, Brazil, under authorization (number 36301) of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from Brazilian Ministry of the Environment. Access was recorded (ADF1667) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). A voucher specimen (number 82,853) is deposited at the herbarium "UFP – Geraldo Mariz" from the *Universidade Federal de Pernambuco* (UFPE). After removal of the thorns, the stem was cut into small pieces and dried at 28°C for 3 days. Next, the material was powdered and stored at -20ºC. The stem powder was added to 0.15 M NaCl (in proportion of 10%, w/v) and the mixture was stirred for 16 h at 28°C. Next, the material was centrifuged (3000 *g*, 15 min) and the supernatant corresponded to the extract.

2.2 Animals

All the experiments were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and were approved (process no.

23076.048175/2015-12) by the Ethics Committee on Animal Use of UFPE. Male Swiss mice (35–45 g), obtained from the *Laboratório de Imunopatologia Keizo Asami* (LIKA) of UFPE, were used in experiments after a one-week adaptation period in the *Núcleo de Cirurgia Experimental* (NUCEX) of the *Universidade de Pernambuco* (UPE) where they were kept under light/dark 12-h cycle and 22 °C. Water and food (Labina, Purina, Brazil - 8% fat, 26% protein, 54% carbohydrate) were available *ad libitum*.

2.3 Experimental procedure

The animals were assigned to six groups of six animals each. Group 1 (control) was formed by non-obese animals that received saline solution (0.9% NaCl); group 2 (DIO) corresponded to DIO mice that received saline solution; groups 3, 4 and 5 (EXT) were DIO animals treated with 125, 250, or 500 mg/kg b.w. of saline extract from *P. gounellei* stem; group 6 (MET) comprised DIO animals treated with metformin (Sigma-Aldrich, USA) at 400 mg/kg as reference drug. The treatments were administered orally by stomach tube, once daily, for 21 days. The obesity in DIO, EXT and MET groups was induced by feeding on a high-fat diet (HFD) for 10 weeks before the start of the experiment. The HFD consisted in 55% of energy derived from fat, 29% from carbohydrates and 16% from protein. The control animals received standard food described in the previous section. The HFD remained being offered to the animals, except from control group, during all the treatment period.

Mice were weighed weekly and observed for behavioral changes, feeding and drinking habits, and general morphological changes. At the end of the 21-days treatment period, the mice were fasted for 12 h and then anesthetized by administration (i.p) of ketamine (500 mg/kg b.w.) (Ringer, 1979). Blood samples were collected from post vena cava of mice into plain sample tubes for serum generation for biochemical analysis. Serum was obtained after allowing blood to coagulate for 30 min and centrifuged at 4000 *g* for 10 min using bench top centrifuge (MSE Minor, England). The supernatant serum samples were collected using dry Pasteur pipette and stored in the refrigerator for further analysis. All analyses were completed within 24 hours of sample collection (Wolford, 1986).

2.4 Biochemical analysis

Serum samples were analyzed for determination of the levels of urea, albumin, total protein, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), triglycerides

(TG), creatine kinase (CK), uric acid, alkaline phosphatase (ALP), aspartate transaminase (AST) and alanine transaminase (ALT) using commercial kits and the semi-automatic photometer Humalyzer 3500. Very low-density lipoprotein cholesterol (VLDL-c) and low-density lipoprotein cholesterol (LDL-c) were calculated according to Friedewald (1972): (1): LDL-c (mg/dL) = [TC – HDL-c – TG]/5; (2) VLDL-c (mg/dL) = TG/5.

Atherogenic indices were calculated as follows: cardiac risk ratio (CRR) = TC/HDL-c (Martirosyan et al., 2007); atherogenic coefficient (AC) = (TC – HDL-c)/HDL-c (Brehm et al., 2004); atherogenic index of plasma (AIP) = log (TG/HDL-c) (Dobiasova et al., 2004); Castelli's Risk Index II (CRI-II) = LDL-c/HDL-c (Castelli et al., 1983).

2.5 Intraperitoneal glucose tolerance test (ipGTT)

The ipGTT test was performed after a 6-h fast. After collection of an unchallenged sample (time 0), a bolus of 1.0 g/kg body weight of glucose was administered into the peritoneal cavity, and the blood samples were collected from the tail vein at different time points up to 120 min to determine blood glucose levels (Araujo et al., 2012; Caricilli et al., 2011).

2.6. Intraperitoneal insulin tolerance test (ipITT)

After fasting for 6 h, systemic insulin sensitivity was analyzed using the ipITT. Briefly, tail blood samples were withdrawn before (0 min) and at 5, 10, 15, 20, 25 and 30 min after an intraperitoneal injection of 1.5 IU/kg of regular insulin (Humulin-R, Eli-Lilly, Brazil). Glucose concentrations were measured using a glucometer and these values were used to calculate the constant rate of blood glucose disappearance (Kitt), which is based on the linear regression of the naperian logarithm of glucose concentrations obtained from 0 to 30 min of the test. Kitt was calculated using the formula $0.693/t_{1/2}$ (the plasma glucose $t_{1/2}$, was calculated from the slope of the least-square analysis of the plasma glucose concentrations during the linear decay phase) (Bonora et al., 1989).

2.7. Oxidative stress evaluation

2.7.1. Liver, adipose tissue, and muscle homogenates

Homogenates of liver, adipose tissue, and spleen were prepared in 50 mM Tris-HCl pH 7.4 containing 1 mM EDTA, 1 mM sodium orthovanadate, and 2 mM phenylmethanesulfonyl fluoride (PMSF) and centrifuged at 2500 × *g* for 10 min at 4 °C. The supernatant was collected and used in the experiments described below. Aliquots of tissue homogenate were used for measuring the total protein content as described by Bradford (1976).

2.7.2. Lipid peroxidation assay

Lipid peroxidation was evaluated by estimating the level of thiobarbituric acid-reactive substances (TBARS) (Ohkawa and Ohishi, 1979). Briefly, the reaction was developed by the sequential addition of homogenate (100 μL), 30% (w/v) trichloroacetic acid (10 μL) and 10 mM Tris-HCl pH 7.4 (390 μL). The mixture was centrifuged at 2500 × *g* for 10 min. After centrifugation, the supernatant was transferred to another tube and it was added 0.8% (v/v) thiobarbituric acid (10 μL). The assay was boiled in water-bath for 30 min and, after cooling, the absorbance of the organic phase was read at 535 nm in a spectrophotometer. Results were expressed as nmol of malondialdehyde (MDA) per mg of protein.

2.7.3. Superoxide dismutase (SOD) activity

SOD enzyme activity was determined in homogenates as proposed by Kostyuk and Potapovich (1989). Briefly, homogenates aliquots (25μL) were added to a medium containing 0.016 M phosphate buffer, 0.8 mM *N,N,N',N'*-Tetramethylethylenediamine, and 0.08 mM EDTA (final pH of the medium was 10). The kinetic analysis of SOD was measured spectrophotometrically at 406 nm after quercetin addition (1.5 mg of quercetin in 10mL of *N,N*-Dimethylformamide).The results were corrected by protein content and expressed as unit per milligram of protein. One unit of SOD activity is defined as the amount of enzyme that inhibited the quercetin oxidation reaction by 50% of maximal inhibition.

2.7.4. Catalase (CAT) activity

CAT activity was determined according to Aebi (1984). Diluted tissue homogenate tissue (1:7 v/v) was treated with chloroform-ethanol (0.6:1 v/v). Assay buffer (50 mM potassium phosphate buffer, pH 7.0; 8 μL), sample (80 μL), and 10 mM H_2O_2 were used for

determination. Detection was performed at 360 nm. The amount of CAT was expressed as U/g tissue.

2.8. Histological analysis

Histological analyses of liver, gastrocnemius muscle and epididymal adipose of animals were performed by optical microscopy. Fragments of the organs were fixed in buffered formalin (10%, v/v) and then dehydrated through a graded ethanol series (70–100%), diaphonized in xylol, and embedded in paraffin. Histological sections (5 μm) were stained with hematoxylin-eosin and mounted using cover slips with Entellan resin (Merck, Germany) (Kiernan, 2008). The materials were observed under a Motic BA200 microscopy coupled to a Moticam 1000 1.3 MP digital camera (Motic Incorporation Ltd., Causeway Bay, Hong Kong).

2.9. Statistical analysis

Data were expressed as mean SEM (Standard Error of Mean). Repeated measures ANOVA and One-way ANOVA followed by Dunnett's multiple comparison were performed to analyze data sets. $P < 0.05$ was considered significant. Statistical program used was Graphpad Prism (version 6.02; GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

Recent studies have reported that Cactaceae extracts can protect against dysfunctions associated with unbalance in lipid metabolism. For instance, *Opuntia ficus-indica* seed extract attenuated hepatic steatosis and promoted M2 macrophage polarization in high-fat diet–fed mice (Kang, 2015). Also, Oliveira et al. (2018, 2019) reported that the ingestion of saline extract from *P. gounellei* stem resulted in decrease of lipid levels in serum of mice. In the present work, we investigated the effect of this *P. gounellei* extract on insulin sensitivity, serum parameters linked to obesity, cardiac markers and oxidative stress in obesity model induced by diet with high lipid content.

Table 1 summarizes the data on the evaluation of the weight of the animals. At the end of the treatments, all the groups whose animals fed on HFD showed significantly higher ($p < 0.001$) weight than control group. However, the animals that received the *P. gounellei* extract showed mean weight significantly ($p < 0.001$) lower than the DIO and MET groups.

The analysis of the serum parameters in DIO group mice showed that the HFD led to significant ($p < 0.05$) increase of albumin, total protein, alkaline phosphatase, GGT, AST, ALT, CK, total cholesterol, LDL-c and TG levels, as well as to a decrease of HDL-c, in comparison with control group (Table 2). MET animals showed the same alterations, but the increase of total cholesterol and TG levels was lower than in the obese mice treated with saline. Reduction in the levels of total cholesterol, LDL-c and TG was observed with the increase of the dose of *P. gounellei* extract administered to obese mice. In the treatment with the highest dose (500 mg/kg), the animals showed a lipidic profile similar ($p > 0.05$) to that of non-obese animals (control). Interestingly, the HDL-c levels did not decrease in the animals that received the extract at all doses. The treatments with *P. gounellei* extract also have benefits on the AST, ALT, GGT and creatinine levels (Table 2), reducing the negative impact of the obesity on liver and kidney. In the treatment with the highest dose (500 mg/kg), the values were similar or near to those found in control.

Excessive triglyceride levels not only cause lipids to accumulate but also interfere with the physiological function of adipocytes (Sun et al., 2011). Excessive lipid deposits in adipocytes impair insulin sensitivity and lead to metabolic syndrome abnormalities. The insulin-resistant state is characterized by a reduction in the ability of insulin to activate its signaling pathway in the main target tissues such as muscle, fat and liver (Pessin and Saltiel, 2000, Saad et al., 1992). Insulin plays a crucial role in metabolism, mainly by increasing glucose uptake in both muscle and adipose tissue, and inhibiting glucose output in the liver. In this context, an ipGTT test was conducted to estimate glucose tolerance. During the tolerance tese, the DIO group showed higher glucose blood levels when compared to the control. However, treatment with *P. gounellei* for 21 days improved glucose tolerance in obese mice to the same extent as the group treated with metformin (Figure 1A). We then performed an insulin challenge, which showed that the treatments with both doses of the extract, as well as with metformin, were able to completely restore insulin tolerance in obese mice, as demonstrated by the glucose disappearance rate (Kitt) (Figure 1B).

The hyperlipidemia contributes significantly in the manifestation and development of atherosclerosis and coronary heart diseases. Coronary artery disease, which often results in acute myocardial infarction is a debilitating/fatal disease among the leading causes of death worldwide (Hausenloy and Yellon, 2013). Acute myocardial ischemia and infarction can develop when coronary arteries are partially or completely occluded, reducing oxygen supply and nutrients, which may lead to cell death (Buia, 2005; Carden and Granger, 2000). Thus, we evaluated the vascular risk of the animals treated or not with the *P. gounellei* extract. The

atherogenic indices were lower in animals treated with the extract than in DIO group (Table 3). In the group treated with the extract at 500 mg/kg, the AC and AIP indices were similar ($p > 0.05$) to those found in non-obese control and the CRR and CRI-II were significantly lower than in MET group.

Oxidative stress in liver, spleen and adipose tissue of the animals was investigated and the data can be seen in Table 4. Generally, the treatments with the extract prevented lipid peroxidation in the three organs, with MDA levels similar ($p > 0.05$) to those found in control animals. On the other hand, the tissues from DIO and MET groups showed increased level of lipid peroxidation. The CAT levels were not statistically different among all the groups while SOD levels were increased in the organs of animals that received the extract as well as in the liver of metformin-treated animals. The SOD is the primary enzyme catalyzing ROS by dismutating superoxide and producing H_2O_2 and oxygen. The mitochondrial SOD is inducible form and acts protecting from oxidative stress, being by far the most protective SOD isoform for eliminating ROS (NELSON et al, 2005). Our results suggest that the treatment with the extract led to the activation of SOD and consequent enhancement of antioxidant and anti-inflammatory mechanisms in liver, spleen and adipose tissue.

In the histopathological analysis of the liver, it was not detected neutral intracellular lipids in the liver slices of the control group. In the DIO group, there was an increase in the amount of detectable collagen deposition in the perivenular spaces. In the MET group, it was observed a reduction in intracellular fat, when compared to the livers of the DIO group. In addition, in the extract treatments, the livers showed collagen deposition in the perivenular spaces but in a less extent than DIO group (Figure 2).

The epididymal fat significantly reduced in the extract-treated groups, especially at the highest dose, in comparison with the DIO group treated with saline, differently of the metformin group. Additionally, the size of epididymal adipose tissue cells were larger in the DIO and MET groups than in the extract treatment at 500 mg/kg (Figure 2). These results suggested that extract reverse the obesity. In the muscle, no alterations were observed in the experimental groups when compared to the control group. The muscle fibers remained in their usual morphology.

In summary, our study demonstrated that the *P. gounellei* saline extract was able to improve the physiological parameters from obese mice, suggesting its therapeutic application against the deleterious effects of the obesity state.

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Table 1. Body weight of non-obese mice (control) and diet-induced obese mice treated with saline (DIO group), metformin (MET group) or with the saline extract of *Pilosocereus gounellei*.

Groups	Obesity induction step			End of treatments
	Initial weight (g)[a]	Final weight (g)[b]	Weight gain (g)	Weight (g)[c]
Control	36.87 ± 1.05	46.03 ± 0.45	10.02 ± 0.15	41.75 ± 0.15
DIO	36.87 ± 1.05	69.92 ± 0.85	34.61 ±0.43	73.04 ±0.43*
MET	35.07 ± 2.78	70.8 ± 2.01	35.66 ± 0.42	71.88 ± 0.32*
Extract (125 mg/kg)	36.57 ± 2.05	70.33 ± 2.05	34.39 ± 0.41	58.7 ± 0.41*#
Extract (250 mg/kg)	35.23 ± 2.15	71.08 ± 1.84	36.04 ± 0.72	57.6 ± 0.72*#
Extract (500 mg/kg)	36.5 ± 1.76	69.95 ± 1.47	34.79 ± 0.59	58.44 ± 0.59*#

Obesity was induced by high-fat diet offer to all groups, except the control animals, which received normal diet along all the experiment. [a]Weight of the animals before the start of the high-fat diet offer. [b]Weight of animals 10 weeks after the start of the high-fat diet offer. [c]Weight after 21 days of treatments. (*) $p < 0.001$ in comparison with control. (#) $p<0.001$ in comparison with MET group.

Table 2. Biochemical parameters in blood serum of non-obese mice (control) and diet-induced obese mice treated with saline (DIO group), metformin (MET group) or with the saline extract of *Pilosocereus gounellei*.

Parameter	Groups					
	Control	DIO	MET	Extract (125 mg/kg)	Extract (250 mg/kg)	Extract (500 mg/kg)
Albumin (g/dL)	1.99±0.09	4.02±0.42	1.99±0.09*	2.01±0.13*	2.05±0.15*	1.99±0.09*#
Total protein (g/dL)	7.12±0.20	9.21±0.31	7.12±0.20*	7.09±0.23*#	7.13±0.19*#	7.12±0.20*#
Alkaline phosphatase (IU/L)	12.12±0.10	18.07±0.14	12.12±0.10*	11.99±0.22*	12.04±2.65*	12.12±0.10*
Urea (mg/dL)	44.02±0.11	44.18±0.15	44.02±0.11	44.39±0.20	44.20±0.14	44.02±0.11
Creatinine (mg/dL)	0.41±0.09	0.41±0.07	0.41±0.09	0.40±0.11	0.39±0.16	0.41±0.09
Total cholesterol (mg/dL)	137.8±2.69	205.5±2.98	192.8±5.22*	200.46±7.42	189.13±3.88*#	148.8±2.39*#
LDL-cholesterol (mg/dL)	8.41±0.12	14.12±0.90	12.09±0.78*	10.01±0.55*	9.01±0.09*	8.72±0.38#
HDL-cholesterol (mg/dL)	85.12±1.66	69.96±0.98	76.91±0.45*	84.96±0.98*#	85.07±0.51*#	85.86±0.33*#
VLDL-cholesterol (mg/dL)	44.12±0.22	68.23±0.85	65.45±1.02*	60.32±1.22*#	53.21±1.08*#	49.54±1.13*#
Triglycerides (mg/dL)	99.21±10.21	189.21±9.28	157.21±9.20	168.88±10.30*	130.34±10.42*#	105.22±9.28*
ALT (U/L)	67.6 ± 0.30	177.12 ± 1.31	156.12 ± 1.31*	154.1 ± 2.05*	120.12 ± 1.03*#	87.22 ± 0.77*#
AST (U/L)	110.2 ± 0.23	188.2 ± 1.55	171.2 ± 1.43*	169.2 ± 0.88*	140.2 ± 0.91*#	120.2 ± 2.01*#
GGT (U/L)	10.70±0.50	14.12±0.21	13.70±0.50*	12.27±0.26*	10.19±0.51*	10.70±0.50*
CK (U/L)	399.83± 1.26	411.09± 4.11	422.91± 2.47	450.83± 2.50	398.66± 1.20	396.05± 4.82

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatine kinase; GGT: gamma-glutamyl transferase; Values represent the mean ± SEM (n = 6). (*) indicates significant difference ($p < 0.05$) in comparison with DIO group treated with saline. (#) indicates significant difference ($p < 0.05$) in comparison with metformin-treated group.

Table 3. Atherogenic indices determined for non-obese mice (control) and diet-induced obese mice treated with saline (DIO group), metformin (MET group) or with the saline extract of *Pilosocereus gounellei*.

Index	Groups					
	Control	DIO	MET	Extract (125 mg/kg)	Extract (250 mg/kg)	Extract (500 mg/kg)
CRR	79.4±0.81	155.8±1.05	142.5±1.12*	143.2±1.33*#	121±1.24*#	92.5±1.13*#
AC	129.2±1.44	269.8±2.11	204.2±2.06*	190.5±2.02*#	167.5±1.86*#	135.9±1.06*#
AIP	80.2±1.08	81.9±0.09	80.2±0.98	81.5±0.85	80.8±0.72	80.5±0.81
CRI-II	40.1±0.72	71.1±0.72	69.1±0.72*	65.5±0.88*#	58.2±0.91*#	45.1±1.01*#

CRR: cardiac risk ratio. AC: atherogenic coefficient. AIP: atherogenic index of plasma. CRI-II: Castelli's Risk Index II. (*) indicates significant difference ($p < 0.05$) in comparison with DIO group treated with saline. (#) indicates significant difference ($p < 0.05$) in comparison with metformin-treated group.

Table 4. Malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) levels in organs from non-obese mice (control) and diet-induced obese mice treated with saline (DIO group), metformin (MET group) or with the saline extract of *Pilosocereus gounellei*.

Parameter	Groups					
	Control	DIO	MET	Extract (125 mg/kg)	Extract (250 mg/kg)	Extract (500 mg/kg)
Liver						
MDA	5.01±0.23	7.12±0.60	6.98±0.29	5.12±0.90*#	5.56±0.55*#	5.37±0.41*#
SOD	9.65±0.06	7.44±0.09	10.22±0.11*	12.57±0.08*#	12.81± 0.34*#	13.88±0.34*#
CAT	0.31±0.12	0.29±0.11	0.30±0.08	0.30±0.02	0.35±0.02	0.46±0.01*#
Muscle						
MDA	1.45±0.11	4.22±0.09	1.57±0.06*	1.74±0.05*#	1.03±0.02*#	1.03±0.02*#
SOD	0.49±0.01	0.50±0.04	0.61±0.03*	0.63±0.05*#	0.61±0.02*#	0.73±0.02*#
CAT	3.31±0.03	3.29±0.11	3.30±0.07	3.32±0.06	3.47±0.09	3.49±0.12
Adipose tissue						
MDA	5.91±0.14	8.96±0.34*	5.87±0.09*#	5.88±0.23*#	5.98±0.12*#	5.92±0.59*#
SOD	0.69±0.03	0.44±0.05*	0.67±0.08	0.80±0.05*#	0.77±0.05*#	1.16±0.03*#
CAT	0.008±0.001	0.010±0.002	0.013±0.001	0.012±0.002	0.010±0.001	0.011±0.002

Values represent the mean ± SEM (n = 6). (*) indicates significant difference ($p < 0.05$) in comparison with DIO group.

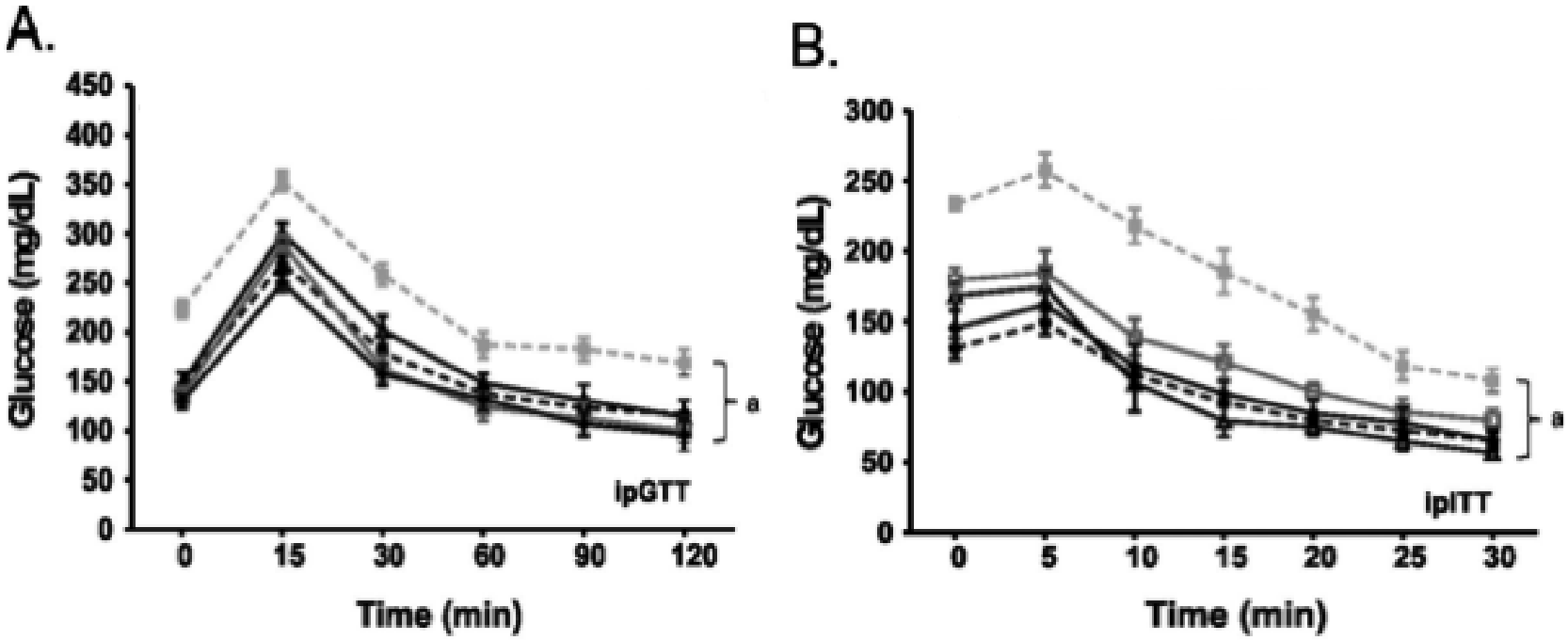

Figure 1. Intraperitoneal challenge tests of glucose (ipGTT) (A) and insulin (ipITT) tolerance in non-obese (control) and diet-induced obese mice treated with saline (DIO group), metformin (MET group) or with the saline extract of *Pilosocereus gounellei*. Data represent the means ± SEM (n=6). Statistical analysis was performed: (*) indicates $p < 0.05$ vs. control. (a) indicates $p < 0.05$ vs. DIO group.

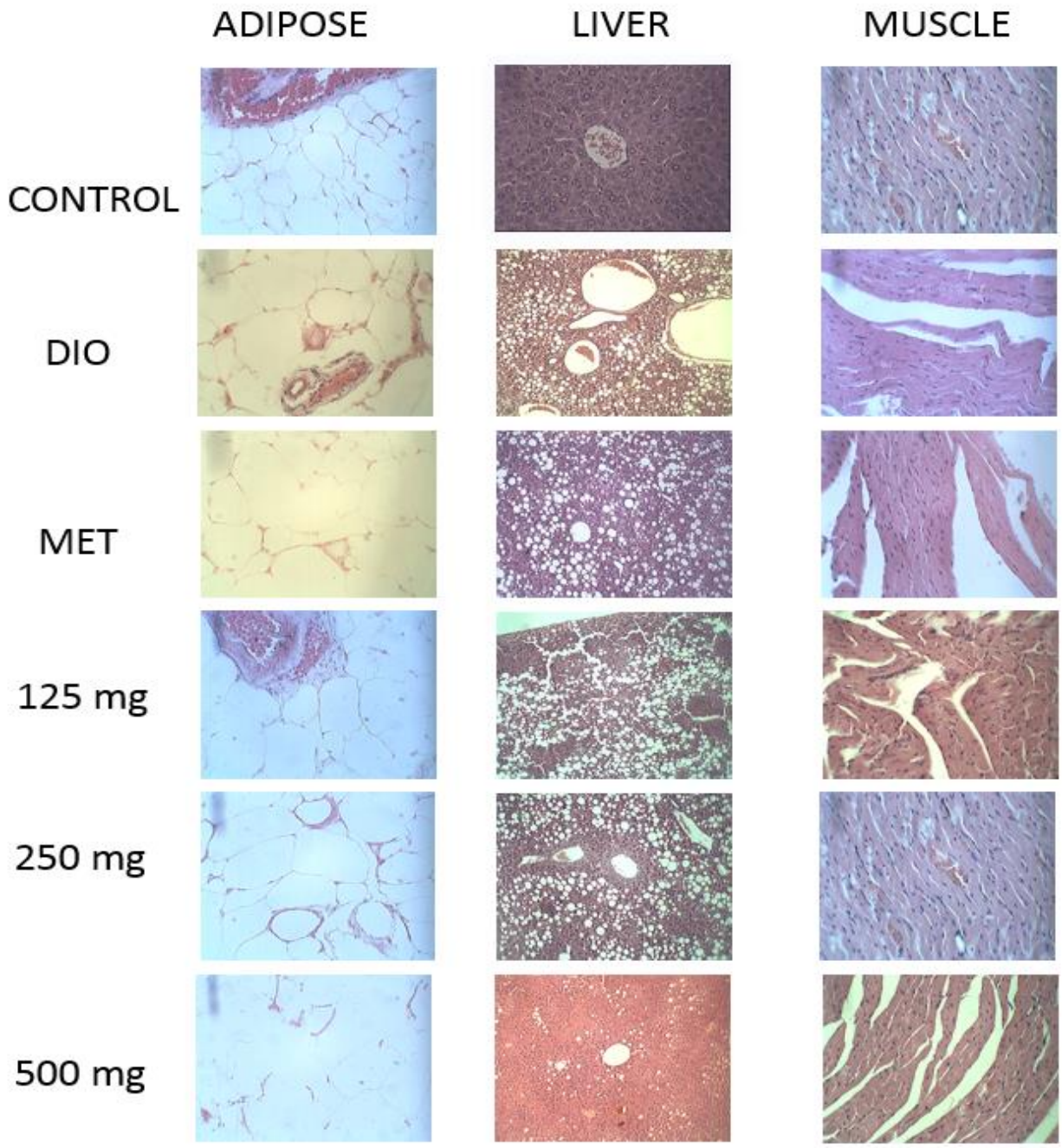

Figure 2: Representative photomicrographs of the adipose, liver and muscle of male mice from the control group, metformin (MET) and the groups treated daily with the saline extract from *P. gounellei* stem at the doses of 125, 250 and 500 over 21 days. **Adipose:** Significantly reduced in the extract-treated groups, especially at the highest dose, in comparison with the DIO group treated with saline, differently of the metformin group. Additionally, the size of epididymal adipose tissue cells were larger in the DIO and MET groups than in the extract treatment at 500 mg/kg **Liver:** Not detected neutral intracellular lipids in the liver slices of the control group. In the DIO group, there was an increase in the amount of detectable collagen deposition in the perivenular spaces. In the MET group, it was observed a reduction in intracellular fat. In the extract treatments, the livers showed collagen deposition in the perivenular spaces but in a less extent than DIO group. **Muscle:** no alterations were observed in the experimental groups when

compared to the control group. The muscle fibers remained in their usual morphology. Hematoxylin-eosin staining was used. Magnification: 400×.

4 CONCLUSÕES

- O extrato salino do caule de *P. gounellei*, nas doses de 2000 e 5000 mg/kg, apresentou baixa toxicidade aguda, como demonstrado por análises hematológicas, bioquímicas e histológicas, bem como não afetou a coordenação motora dos animais.
- Também de acordo com análises hematológicas, bioquímicas e histológicas, o extrato apresentou baixa toxicidade subaguda, quando administrado diariamente por 28 dias consecutivos nas doses de 250, 500 e 1000 mg/kg.
- Em doses consideradas seguras, o extrato apresentou:
  - ação antinociceptiva contra dor inflamatória e não-inflamatória, atuando através vias ligadas a receptores opioides.
  - ação antipirética em modelo de febre causada por levedura.
  - efeito anti-obesidade em animais submetidos à dieta hiperlipídica, melhorando o perfil lipídico, aumentando a tolerância à glicose, reduzindo a resistência à insulina e índices aterogênicos, diminuindo o estresse oxidativo em fígado, músculo e tecido adiposo, bem como reduzindo o quadro de esteatose e inflamação no fígado.

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ANEXO A – AUTORIZAÇÃO DO COMITÊ DE ÉTICA

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Recife, 14 de março de 2016.

Ofício nº 14/16

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Thiago Henrique Napoleão**
Departamento de Bioquímica
Centro de Biociências
Universidade Federal de Pernambuco
Processo nº 23076.048175/2015-12

Os membros da Comissão de Ética no Uso de Animais do Centro de Bioências da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Avaliação de extrato inibidor de protease do caule de *Pilosocereus gounellei* quanto aos efeitos sobre o perfil de resistência à insulina em camundongos obesos e quanto às atividades citotóxica e anti-inflamatória."**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem do animal: Biotério do LIKA/UFPE; Animal: camundongos *Swiss*; Idade: 4 semanas; Sexo: machos e fêmeas; Peso: 35g; Nº total de animais: 334.

Atenciosamente,

Pedro V. Lodi

ANEXO B – AUTORIZAÇÃO DO COMITÊ DE ÉTICA

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Recife, 09 de agosto de 2017.

Ofício nº 70/17

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Thiago Henrique Napoleão**
Departamento de Bioquímica
Centro de Biociências
Universidade Federal de Pernambuco
Processo nº **23076.039336/2016-50**

Certificamos que a proposta intitulada "**Avaliação toxicológica e atividades biológicas do extrato do caule de *Pilosocereus gounellei***", registrada com o **nº 23076.039336/2016-50** sob a responsabilidade de **Prof. Thiago Henrique Napoleão** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 02/08/2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	11 meses
Espécie/ linhagem/raça	Camundongos heterogênicos/Swiss
Nº de animais	194
Peso/Idade	30-40g / 60 dias
Sexo	Machos
Origem	Bioterio do LIKA/UFPE

Atenciosamente,