UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
FACULDADE DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Avaliação da segurança do extrato aquoso obtido da casca de *Plinia cauliflora* (Mart.) Kausel: Da toxicidade aguda a disrupção endócrina.

Rhanany Alan Calloi Palozi

Dourados - MS
2021

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ATA DA DEFESA DE TESE DE DOUTORADO APRESENTADA POR **RHANANY ALAN CALLOI PALOZI** ALUNO DO PROGRAMA DE PÓS-GRADUAÇÃO *STRICTO SENSU* EM CIÊNCIAS DA SAÚDE, ÁREA DE CONCENTRAÇÃO "FARMACOLOGIA".

Aos vinte e oito dias do mês de setembro de dois mil e vinte e um, às 14 horas, em sessão pública, realizou-se por videoconferência a Defesa de Tese de Doutorado intitulada **"Avaliação da segurança do extrato aquoso obtido da casca de *Plinia cauliflora* (Mart.) Kausel: da toxicidade aguda a disrupção endócrina"** apresentada pelo aluno **Rhanany Alan Calloi Palozi**, do Programa de Pós-Graduação em Ciências da Saúde, à Banca Examinadora constituída pelos membros: Prof. Dr. Arquimedes Gasparotto Junior - PPGCS/UFGD (presidente), Dr. Emerson Luiz Botelho Lourenço/UNIPAR, Dr. Marcio Eduardo de Barros - PPGCS/UFGD, Dr.ª Raquel dos Santos Donatini/UFGD e Dr.ª Virgínia Demarchi Kappel Trichez/UFGD. Iniciados os trabalhos, a presidência deu a conhecer ao candidato e aos integrantes da Banca as normas a serem observadas na apresentação da Tese. Após o candidato ter apresentado a sua explanação, os componentes da Banca Examinadora fizeram suas arguições. Terminada a Defesa, a Banca Examinadora, em sessão secreta, passou aos trabalhos de julgamento, tendo sido o candidato considerado APROVADO, fazendo *jus* ao título de **DOUTOR EM CIÊNCIAS DA SAÚDE**. **O presidente da banca abaixo-assinado atesta que todos os membros participaram de forma remota[1] desta defesa de tese, considerando o candidato APROVADO, conforme declarações anexas**. Nada mais havendo a tratar, lavrou-se a presente ata, que vai assinada pela presidente da Comissão Examinadora.

Dourados, 28 de setembro de 2021.

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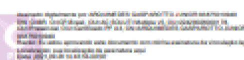

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1 Participação remota dos membros da banca conforme § 3º do Art. 1º da Portaria RTR/UFGD n. 200, de 16/03/2020 e Art. 2º e 5º da Instrução Normativa PROPP/UFGD Nº 1, de 17/03/2020

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DEDICATÓRIA

Dedico este trabalho às pessoas mais especiais de minha vida: Minha mãe Cláudia e meu pai Clóvis, pelos exemplos de vida que são.

Meu irmão, Gabriel, pelo incentivo direto ou indireto.

Meu grande amor, Zara, por estar ao meu lado nos melhores momentos de minha vida e me ajudar a remover as pedras que surgiram em nossa caminhada.

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MUITO OBRIGADO!

EPÍGRAFE

Agir, eis a inteligência verdadeira. Serei o que quiser. Mas tenho que querer o que for. O êxito está em ter êxito, e não em ter condições de êxito. Condições de palácio tem qualquer terra larga, mas onde estará o palácio se não o fizerem ali?

(FERNANDO PESSOA)

LISTA DE ABREVIATURAS E SÍMBOLOS

DL_{50}	Dose necessária de uma dada substância capaz matar 50% de uma população teste
EHHG	Eixo Hipotálamo-Hipófise-Gonadal
ER	Receptor estradiol
LC-DAD-MS	Cromatografia Líquida Acoplada ao Espectrômetro de Massas
MN	Micronúcleo
PA	Pressão Arterial
SNC	Sistema Nervoso Central
SR	Sistema Respiratório
SCV	Sistema Cardiovascular
SEIPC	Sobrenadante etanólico do infuso de *Plinia cauliflora*
TdP	Torsades de pointes

Avaliação da segurança do extrato aquoso obtido da casca de *Plinia cauliflora* (Mart.) Kausel: Da toxicidade aguda a disrupção endócrina.

RESUMO

Popularmente conhecida como “jaboticaba” ou “jabuticaba”, a *Plinia cauliflora* (Mart.) Kausel (Myrtaceae) e uma fruta apreciada tanto no consumo *in natura* quanto na produção de geleias, sucos e licores. Seu uso tradicional mais difundido envolve o tratamento da diarreia, que aproveita todas as partes da planta, inclusive as cascas das frutas. O estudo objetivou elucidar os possíveis riscos da administração de uma fração solúvel em etanol obtida a partir da infusão de cascas de frutas de *P. cauliflora* (SEIPC). Para isso, realizou-se uma série de experimentos para avaliar a possível toxicidade do SEIPC, nos quais o mesmo foi administrado por via oral, aguda e repetidamente por 28 dias. Também avaliamos os possíveis efeitos desreguladores endócrinos e genotóxicos em células eucarióticas através dos ensaios de cometa e micronúcleo. O SEIPC foi produzido e caracterizado quimicamente por LC-DAD-MS. A toxicidade aguda e as alterações comportamentais e fisiológicas foram avaliadas no teste de Irwin modificado. Após 28 dias de tratamento oral, foram avaliados parâmetros como frequência respiratória, gasometria arterial, eletrocardiografia, frequências respiratória e cardíaca, pressão arterial, análises hematológicas, bioquímicas e histopatológicas. O ensaio de cometa, o teste do micronúcleo, o teste uterotrófico, o bioensaio de Hershberger e o teste AMES foram realizados usando protocolos apropriados. Do sobrenadante etanólico do infuso de *Plinia cauliflora* foram identificados por LC-DAD-MS ácidos fenólicos, como ácido gálico e ácido elágico e derivados, flavonóis e antocianidinas, bem como ácido cítrico. Após os tratamentos agudo e prolongado, o presente estudo constatou que o SEIPC não causou alterações significativas em vários sistemas corporais, incluindo atividade elétrica cardíaca, temperatura corporal, frequência respiratória e pressão arterial. Ademais, não foram observadas alterações dos parâmetros bioquímicos, hematológicos ou gasométricos. Foi possível constatar ainda que o SEIPC não causou quaisquer perturbações do sistema endócrino ou efeitos mutagênicos, citotóxicos ou genotóxicos. Esses achados comprovam o uso seguro de *P. cauliflora*.

Palavras-chave: Myrtacea. Jaboticaba. Toxicidade. Fitoterapia

Safety evaluation of the aqueous extract obtained from the peels of *Plinia cauliflora* (Mart.) Kausel: From acute toxicity to endocrine disruption.

ABSTRACT

Popularly known as “jaboticaba” or “jabuticaba”, a *Plinia cauliflora* (Mart.) Kausel (Myrtaceae) and a fruit appreciated both for fresh consumption and for the production of jellies, juices and liqueurs. Its most widespread traditional use involves the treatment of diarrhea, which takes advantage of all parts of the plant, including the fruit peels. The study aimed to elucidate the possible risks of administering an ethanol-soluble fraction obtained from the infusion of *P. cauliflora* fruit peels (SEIPC). For this, a series of experiments were carried out to evaluate a possible toxicity of SEIPC, in which it was administered orally, acutely and repeatedly for 28 days. Also evaluated the possible endocrine disrupting and genotoxic effects on eukaryotic cells through comet and micronucleus assays. SEIPC was produced and chemically prepared by LC-DAD-MS. Acute toxicity and how behavioral and physiological changes were evaluated in the modified Irwin test. After 28 days of oral treatment, parameters such as respiratory rate, arterial blood gases, electrocardiography, respiratory and heart rates, blood pressure, hematological, biochemical and histopathological analyses were obtained. The comet assay, the micronucleus test, the uterotrophic test, the Hershberger bioassay, and the AMES test were performed using external links. Phenolic acids such as gallic acid and ellagic acid and derivatives, flavonols and anthocyanidins, as well as citric acid were identified from the ethanolic supernatant of the infusion of *Plinia cauliflora* by LC-DAD-MS. After both acute and prolonged treatments, the present study found that SEIPC did not cause significant changes in several body systems, including electrical activity, body temperature, respiratory rate and blood pressure. Furthermore, no changes in biochemical, hematological or blood gas parameters were observed. It was also possible to verify that SEIPC did not cause any disturbances in the endocrine system or mutagenic, cytotoxic or genotoxic effects. These findings support the safe use of *P. cauliflora.*

Keywords: *Myrtacea. Jaboticaba. Toxicity. Phytotherapy*

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1 INTRODUÇÃO

Estudos de segurança são essenciais para o desenvolvimento de novos medicamentos (TORNATORE et al., 2019). Este tipo de estudo tem por objetivo investigar os potenciais efeitos indesejáveis de novas drogas utilizando doses presentes na janela terapêutica e acima da mesma (BASS; KINTER; WILLIAMS, 2004). Um dos motivos que levam a estudos de segurança farmacológica são os graves efeitos adversos que podem ser observados após a administração, principalmente nos sistemas nervoso central, respiratório e cardiovascular (BRIGGS et al., 2015; HAMDAM et al., 2013).

A padronização de protocolos de estudo da segurança farmacológica trouxe normativas importantes no que diz respeito à condução dos experimentos para esse fim. Afinal, todos os ensaios realizados são baseados em documentos publicados que regem as minúcias da metodologia científica envolvida no processo. Vale salientar que, embora existam protocolos bem definidos para a avaliação de segurança farmacológica e toxicidade, essas publicações ainda não resolveram todos os desafios apresentados frente ao estudo de novas drogas, especialmente no que diz respeito à detecção de riscos de efeitos adversos raros e letais. Um dos maiores desafios é o de como conduzir ensaios que detectem precocemente, com precisão e exatidão, efeitos adversos. Este é um problema específico para efeitos adversos raros mas potencialmente letais. (PUGSLEY; AUTHIER; CURTIS, 2008).

Da mesma forma que os os medicamentos alopáticos, os fitoterápicos precisam ter comprovação de sua segurança, eficácia e qualidade antes de serem colocados à venda para a população em geral. (CARVALHO et al., 2008). À medida que o uso global de produtos fitoterápicos continua a crescer e mais produtos são introduzidos no mercado, as questões de saúde pública e as preocupações em torno de sua segurança também são cada vez mais reconhecidas. Embora alguns medicamentos fitoterápicos tenham potencial promissor e sejam amplamente utilizados, muitos deles permanecem não testados e seu uso também não monitorado. Isso torna o conhecimento de seus potenciais efeitos adversos muito limitado e a identificação das terapias mais seguras e eficazes, bem como a promoção de seu uso racional, mais difícil (EKOR, 2014).

Semelhante a outras populações mundiais, a população brasileira utiliza diferentes espécies vegetais como importantes fontes de alimentos e medicamentos. Apesar do amplo uso desses agentes, os estudos de segurança relativos aos fitocomplexos são relativamente restritos e limitados principalmente a compostos com amplo uso industrial. Um exemplo de uma espécie vegetal utilizada no Brasil, é *Plinia cauliflora* (Mart.) Kausel (Myrtaceae). Esta espécie é

endêmica na América do Sul e seus frutos são encontrados nos mais diversos biomas brasileiros, como Cerrado, Caatinga, Mata Atlântica, Floresta Amazônica e Pampa (BORGES; CONCEIÇÃO; SILVEIRA, 2014).

Conhecida popularmente como "jabuticaba", a fruta é consumida *in natura* ou utilizada para a produção de licor, vinagre, vinho, suco e geleia (INADA et al., 2015a). Vários compostos fenólicos, incluindo flavonoides e antocianinas, estão presentes na casca da fruta de *P. cauliflora* que exercem inúmeros efeitos biológicos (PEREIRA et al., 2017). Diversos estudos têm sido realizados utilizando diferentes extratos obtidos de cascas de frutas desta espécie, destacando seu efeito antioxidante (DE SOUZA et al., 2017), vasorrelaxante e hipotensor (LOBO DE ANDRADE et al., 2015), antiobesidade (MOURA et al., 2018), antiinflamatório (HSU et al., 2016), hipolipemiante (ARAÚJO et al., 2014) e antidiarreico (SOUZA-MOREIRA et al., 2011).

Apesar do uso difundido de casca de fruta de *P. cauliflora* na produção de diferentes materiais bioativos, existem poucos dados disponíveis na literatura sobre a segurança desta preparação.

2 REVISÃO DE LITERATURA

2.1 Ensaios de toxicidade e segurança

Nos Estados Unidos estima-se que 50 a 60% dos adultos ingerem suplementos fitoterápicos ou dietéticos regularmente. Um estudo relatou que pelo menos 23.000 visitas ao departamento de emergência nos Estados Unidos a cada ano são atribuídas a eventos adversos ocorridos com suplementos dietéticos, herbais ou substâncias homeopáticas. O relatório anual de 2019 da American Association of Poison Control Centers (AAPCC) documentou 69.618 eventos relacionados a suplementos dietéticos, herbais ou substâncias homeopáticas. A AAPCC também relatou que a exposição aos suplementos acima relatados está aumentando na última década, com uma média de 95 exposições por ano (GELLER et al., 2015).

A maioria das exposições relatadas ao Sistema Nacional de Dados sobre Envenenamento (National Poison Data System – NPDS, Estados Unidos) são em crianças com idade inferior a 5 anos. Em 2019, foram relatadas 48.537 exposições ocorridas com crianças nessa faixa etária. Das 7.053 exposições tratadas em unidades de saúde, 51 foram relatados como tendo efeitos adversos importantes e 2 mortes foram observadas (GUMMIN et al., 2020).

No Brasil, o número de intoxicações ocasionados por plantas em 2012 foi de 1.026 casos. Este número correspondente a 1,2% dos casos de intoxicação ocorrida em humanos. As espécies vegetais ocuparam o 13° lugar, em número de casos de intoxicação apresentando 1.185 casos registrados (CAMPOS et al., 2016).

Produtos naturais podem produzir vários efeitos e podem afetar vários sistemas orgânicos, incluindo os sistemas nervoso, cardiovascular, gastrointestinal, hepático, renal e hematológico.

Um estudo, publicado em 2014, que investigou as intoxicações em humanos por alcaloides reportou que estas exposições ocorrem como ingestões não intencionais ou ingestões intencionais. Como ingestões não intencionais, o artigo apresenta contaminação e/ou rotulagem incorreta com treze notificações e identificação errada: onze notificações. Já as ingestões intencionais, foram nove notificações de overdose (ADAMSE et al., 2014).

Outro ponto que merece ser comentado é a contaminação dos alimentos que ocorre quando plantas tóxicas são acidentalmente misturadas com plantas comestíveis durante a colheita ou processamento. Por outro lado as ingestões intencionais podem ser o resultado do consumo para fins recreativos (efeitos alucinógenos) ou para propriedades médicas ou homicídios e suicídios. Um exemplo de exposição internacional, mas indesejada, foi o incidente ocorrido na Holanda em 2013, onde a ingestão de chá de ervas contendo raiz de *Althaea officinalis* estavam contaminadas por *Atropa belladonna,* o que levou quatro pessoas a serem internadas(OERLEMANS; DE VRIES; VAN RIEL, 2017).

Os protocolos experimentais que estudam a segurança e toxicidade de futuros fármacos, são definidos como os estudos que investigam os potenciais efeitos farmacodinâmicos indesejáveis de uma substância nas funções fisiológicas em relação à exposição. Consequentemente, esses estudos são parte integrante da avaliação pré-clínica de novas substâncias. Os testes realizados antes da primeira administração de novos medicamentos em pacientes humanos devem ser constituídos de uma ampla bateria de testes toxicológicos, bem como quaisquer estudos complementares que os justifiquem (CLAUDE; CLAUDE, 2004).

Outro acontecido que ratifica a importância dos protocolos de segurança, foi o ocorrido com a terfenadina nos Estados Unidos, onde em meados de 1990 o anti-histamínico foi retirado das prateleiras das farmácias após crescente discussão e conscientização de que o fármaco poderia evocar, mesmo em pacientes saudáveis, uma síndrome cardíaca potencialmente fatal. Conhecida como torsades de pointes (TdP), esta síndrome é caracterizada por distúrbios da atividade elétrica cardíaca, também denominada de síndrome do QT longo (JUNE; NASR, 1997; MONAHAN et al., 1990).

Antes disso, a percepção geral era de que apenas os compostos utilizados para efeitos cardíacos/cardiovasculares eram considerados como potenciais possuidores de tal efeito. O problema era que a terfenadina, um fármaco não cardiovascular, tinha pequena probabilidade de desencadear a TdP, tornando-a um evento tão raro que exigia vários milhões de prescrições antes que sua administração se tornasse suspeita. A outra consideração importante é que a indicação para a qual a terfenadina foi prescrita, está longe de representar uma ameaça à vida. Portanto, é melhor ficar com o nariz congestionado a correr o risco de desenvolver uma síndrome do QT longo (ROSEN, 1996).

2.2 Toxicidade aguda e teste de Irwin modificado

A toxicidade aguda compreende os efeitos adversos de uma substância que resultam de uma única exposição ou de múltiplas exposições em um curto período de tempo (geralmente menos de 24 horas). Os testes de toxicidade aguda em animais usam a mortalidade como o principal ponto final de observação a fim de determina a DL50 (WALUM, 1998).

O Teste de Irwin é usado para avaliar os efeitos qualitativos do composto teste sobre o comportamento e a função fisiológica, desde a primeira dose que tem efeitos observáveis, até doses que induzem a toxicidade comportamental evidente ou a morte. Esse teste também fornece uma estimativa inicial da duração da ação da substância em diferentes momentos do experimento. Como essas medidas envolvem avaliação subjetiva de diferentes aspectos do comportamento animal, para garantir a reprodutibilidade, o teste é realizado de maneira altamente padronizada por observadores experientes e sempre com um grupo controle como parâmetro de comparação. Modificações comportamentais, sinais de neurotoxicidade, temperatura retal e diâmetro da pupila são registrados. Outros sinais específicos a serem observados incluem morte, convulsões, tremor, sedação, excitação, deambulação, incoordenação motora, tônus muscular alterado, catalepsia, perda de tração, perda de equilíbrio, piloereção, comportamentos estereotípicos, frequência respiratória, agressão, medo, reatividade alterada ao toque, ptose, exoftalmia, perda do reflexo de endireitamento, perda do reflexo corneal, analgesia, defecação/diarreia, dentre outros (CASTAGNÉ; HERNIER; PORSOLT, 2014). Embora padronizado inicialmente por Irwin para camundongos (IRWIN, 1968), o teste foi adaptado posteriormente para ratos (ESTEVE; FARRÉ; ROSER, 1988).

2.3 Toxicidade após doses repetidas

Os estudos de toxicidade após doses repetidas são a espinha dorsal do programa de desenvolvimento de medicamentos pré-clínicos em termos de elucidação de efeitos danosos ou indesejáveis à órgãos-alvo, estimativa da dose humana e possíveis marcadores prodrômicos que podem ser usados para monitorar o início das respostas adversas no homem. Eles também definem a relação dose-resposta para quaisquer toxicidades observadas e a dose na qual elas aparecem (KILLE, 2013)

Diversos são os parâmetros que podem ser analisados nos protocolos de toxicidade após doses repetidas. Independente da aplicação final dos compostos testados, todos os novos candidatos a agentes terapêuticos devem se mostrar seguros, ou seja, não causar nenhum efeito deletério ao sistema nervoso central (SNC), sistema respiratório (SR) e sistema cardiovascular (SCV). Esses três sistemas compreendem o núcleo da segurança farmacológica (FDA, 2020).

Com relação ao SNC, a atividade motora, mudanças comportamentais e as respostas reflexas sensoriais/motoras e a temperatura corporal devem ser avaliadas. Da mesma forma, os efeitos da substância sobre o sistema cardiovascular devem ser avaliados de forma adequada. Desta forma, a pressão arterial, a frequência cardíaca e o eletrocardiograma também devem ser registrados e devidamente avaliados. Além disso, os efeitos da substância frente ao sistema respiratório também deve ser investigado. Para isso, a frequência respiratória, o volume corrente ou a saturação de oxigênio também devem ser avaliados (FDA, 2001).

2.4 Parâmetros hemodinâmicos

A avaliação dos efeitos de uma droga no SCV mais especificamente, na pressão arterial (PA) é importante na segurança farmacológica. Se por um lado detectar grandes variações na pressão arterial é uma tarefa desafiadora, por outro lado, detectar pequenas alterações é um grande desafio, e a interpretação dos resultados requer uma avaliação cuidadosa. A detecção de mudanças sutis na pressão arterial é importante, em particular no que diz respeito às elevações, uma vez que a pressão arterial acima da faixa normal está associada a um risco aumentado de acidente vascular cerebral e morte cardíaca súbita. Os protocolos de segurança são necessários para fornecer melhor garantia de que o agente terapêutico é seguro e distinto de qualquer evento indesejável ou adverso na pressão arterial. A diretriz ICH S7A, documento gerado pelo FDA que fornece definição, princípios gerais e recomendações para estudos de farmacologia de segurança, foi desenvolvida para incluir a avaliação da hemodinâmica antes dos estudos clínicos em humanos. Vale salientar que embora existam normativas para a avaliação dos níveis pressóricos, a extensão do conteúdo ainda é pequena em comparação com o conteúdo relacionado à eletrofisiologia cardíaca (PUGSLEY; AUTHIER; CURTIS, 2008; FDA, 2020)

Por outro lado, a segurança farmacológica cardiovascular também tem se preocupado com as mudanças induzidas por fármacos sobre a função elétrica cardíaca (CHAMPEROUX et al., 2013; LACROIX; PROVOST, 2000). Desde meados da década de 1990, o foco na segurança cardiovascular tem sido as alterações induzidas por substâncias no eletrocardiograma, ou seja, como elas se relacionam, principalmente, aos efeitos sobre o intervalo QT. Eventos cardíacos e arritmias fatais podem ocorrer quando o intervalo QT é prolongado (AL-AKCHAR; SIDDIQUE, 2021).

2.5 Estudos de mutagenicidade e genotoxicidade

A mutagenicidade refere-se à indução de alterações na quantidade ou estrutura do material genético das células ou organismos. Essas mudanças podem envolver um único gene, vários genes ou cromossomos. Uma mutação é uma mudança permanente e hereditária no material genético, que pode resultar em função proteica alterada e mudanças fenotípicas. A mudança genética é conhecida como mutação e o agente causador da mudança como um mutagênico (DURLAND; AHMADIAN-MOGHADAM, 2021).

A mutagênese é a força motriz da evolução; no entanto, também pode causar câncer e doenças hereditárias. Os mutagênicos conhecidos de células germinativas também são mutagênicos em células somáticas *in vivo*. Substâncias que são mutagênicas em células somáticas podem produzir efeitos hereditários se elas, ou seus metabólitos ativos, tiverem a capacidade de interagir com o material genético das células germinativas. Não se espera que as substâncias que não induzem mutações em células somáticas in vivo sejam mutagênicos de células germinativas (ZHANG; VIJG, 2018).

Os estudos de genotoxicidade podem ser definidos como testes *in vitro* e *in vivo* concebidos para identificar substância ou compostos que possam induzir danos ao material genético direta ou indiretamente por vários mecanismos. Esses testes devem permitir a identificação do perigo com relação ao dano e fixação do DNA (CIMINO, 2006).

Como parte do processo de avaliação da segurança de uma determinada substância, autoridades regulatórias em todo o mundo exigem informações sobre o potencial genotóxico dos novos compostos. A genotoxicidade é geralmente avaliada junto com outros pontos finais toxicológicos durante a avaliação de segurança (GEORGE, 2011).

2.5.1 Ensaio do cometa

O ensaio do cometa *in vivo* também pode ser chamado de ensaio do cometa alcalino, referindo-se ao uso de tampão de eletroforese com pH > 13. Embora, outras versões

usando tampões de eletroforese com um pH diferente também podem ser usados (SILVA et al., 2000).

Em condições alcalinas, algumas formas de danos ao DNA podem ser expressas por fragmentação do DNA nuclear, aumentando a migração do DNA durante a eletroforese. Outras formas de dano (*crosslinks*) agregam o DNA nuclear, diminuindo a migração do mesmo. As quebras da fita de DNA pode ser induzidas diretamente por radiação ionizante, produtos químicos formadores de radicais livres ou por substâncias que interagem fisicamente com o DNA. Os locais suscetíveis a lise alcalina podem ser induzidos por agentes que fazem a alquilação do DNA. Alguns produtos químicos se ligam a apenas uma fita de DNA, enquanto outros podem se ligar de forma covalente a ambas as fitas de DNA, formando ligações cruzadas, os chamados *crosslinks* (PFUHLER; WOLF, 1996).

Ao contrário dos outros ensaios, o ensaio do cometa mede o dano genético transitório que não é uma mudança fixa no DNA. As quebras de DNA podem ser reparadas, daí a necessidade de intervalos de tempo muito curtos entre o final do tratamento e a avaliação do dano. O ensaio do cometa pode ser conduzido *in vitro* usando células únicas de linhagens celulares específicas para esse fim, ou *ex vivo* (KRISHNA et al., 2019).

No nível molecular, a formação de cometas no DNA após o desafio genotóxico pode ser visualizada através do método de eletroforese em gel e indica quebras de fita de DNA. Isso acontece devido o DNA danificado migrar durante a eletroforese a uma taxa diferente do DNA não danificado. No ensaio do cometa, quando uma suspensão de células contendo DNA danificado é incorporada em agarose de baixo ponto de fusão e submetida à eletroforese, o DNA danificado migra para longe do corpo nucleoide contendo DNA não danificado, parecendo a estrutura de um cometa, daí o nome do ensaio. Na estrutura do cometa, a parte nucleoide do DNA não danificado é referida como a "cabeça" e a faixa de DNA danificada é referida como a "cauda" (KUMARAVEL et al., 2009).

A porcentagem de fragmentos de DNA na cauda é diretamente proporcional à porcentagem de danos ao DNA que ocorreram em uma determinada célula. Assim, ao contar uma amostra representativa de células, é possível chegar à porcentagem média de dano ao DNA acumulado em um determinado tecido (AZQUETA; COLLINS, 2013).

2.5.2 Teste do micronúcleo

O teste do micronúcleo (MN) é um teste usado na triagem toxicológica de compostos genotóxicos potenciais. O ensaio é reconhecido como um dos ensaios mais bem sucedidos e

confiáveis para carcinógenos genotóxicos. Este teste é baseado na formação de MN em células do organismo tratado com a droga teste (HAYASHI, 2016; LUZHNA; KATHIRIA; KOVALCHUK, 2013).

Os MN são formados durante a anáfase de fragmentos cromossômicos ou cromossomos inteiros que são deixados para trás quando o núcleo se divide. Com o tempo, o ensaio evoluiu para a inclusão de um pré-tratamento com uma droga controle. Isso permite uma pontuação mais precisa e a capacidade de filtrar as células em divisão das que não se dividem, reduzindo assim a incidência de falsos positivos (HAYASHI, 2016).

O aumento da incidência de formação de MN`s serve como um biomarcador para danos genotóxicos. Nos eritrócitos, os MN`s foram identificados há mais de 100 anos, como os corpos de Howell-Jolly. A primeira sugestão do uso da frequência do aparecimento destas inclusões eritrocitárias como uma medida quantitativa de dano cromossômico foi apresentada há mais de seis décadas (EVANS; NEARY; WILLIAMSON, 1959).

Micronúcleos em amostras de sangue periférico humano têm sido usados para acompanhar a terapêutica com agentes quimioterápicos. Um MN é um fragmento de cromossomo quebrado ou raramente um cromossomo inteiro que permanece fora do núcleo após a divisão celular. Um MN pode ser formado após dano direto ao DNA (mecanismo clastogênico) ou após interação secundária com o aparelho de replicação do DNA (mecanismo aneugênico indireto). Durante o estágio de divisão celular denominado anáfase, os fragmentos de DNA acêntricos formados devido a danos no DNA, podem não ser incorporados ao núcleo. Assim, os fragmentos cromossômicos resultam na formação de MN após os estágios de telófase. A formação de MN pode ser medida tanto *in vitro* como *in vivo* (CARDINALE; BRUZZI; BOLOGNESI, 2012).

O ensaio *in vitro* é normalmente realizado com e sem ativação metabólica exógena. Para o ensaio de *in vivo*, a medula óssea ou amostras de sangue periférico de roedores tratados com doses múltiplas de composto teste, veículo e controle positivo apropriado são analisadas para a formação de MN (HAYASHI, 2016).

Como apresentado anteriormente, a formação de MN pode ser devido a um evento de dano ao DNA clastogênico e/ou aneugênico. No entanto, os MN formados por clastogênese carecem de centrômeros, enquanto os micronúcleos resultantes da perda de uma cromátide ou cromossomo inteiro, ou seja, oriundos de evento aneugênico, contêm um centrômero. Assim, por meio da coloração de centrômero usando vários métodos, como hibridização fluorescente *in situ* (FISH) ou outros métodos de imunofluorescência, pode-se determinar não só a presença

destas inclusões nos eritrócitos mas também se os micronúcleos são resultado de eventos clastogênicos ou aneugênicos (NADERI, 2020).

Ainda se discute se o tamanho do MN também pode servir como um indicador de um evento aneugênico ou clastogênico. Isso acontece porque o tamanho do MN formado devido a eventos aneugênicos é geralmente maior do que aquele resultante de um evento clastogênico (HASHIMOTO et al., 2010). De qualquer forma, as tecnologias de detecção de MN, tornam essa técnica apropriada para medir danos genotóxicos.

2.6 Ensaio de mutação bacteriana reversa - Teste de Ames

O teste de Ames é um ensaio que foi nomeado para homenagear Bruce Ames, que primeiro identificou e relatou a utilidade deste ensaio na detecção de mutações em 1974 (AMES, 1974).

Ames criou uma série de cepas geneticamente modificadas de *Salmonella typhimurium,* com mutações no gene da histidina, de tal forma que essas cepas requerem histidina para o seu crescimento. No ensaio de Ames, as bactérias auxotróficas (auxotrofia é a incapacidade de um organismo de sintetizar um composto orgânico específico necessário para seu crescimento) que requerem o aminoácido histidina para crescimento, são tratadas com os compostos teste em uma placa de ágar sem histidina. Portanto, apenas as bactérias que voltaram ao *locus* gênico são capazes de crescer e formar colônias (FÖLLMANN et al., 2013).

O aumento do número de colônias revertentes (aquelas que voltaram ao *locus* gênico) em placas tratadas com a substância em estudo, em comparação com placas não tratadas ou de controle, é uma indicação do potencial mutagênico do produto em estudo. O ensaio de Ames é um dos ensaios mais comumente usados para prever o potencial mutagênico de um composto teste, e é amplamente utilizado porque, historicamente, os produtos químicos que se mostraram positivos no ensaio de Ames também se mostraram positivos quando testados em roedores para medir o potencial carcinogênico (KIRKLAND et al., 2005).

O ensaio de Ames é realizado usando cepas bacterianas de *S. typhimurium* que detectam substituição de pares de bases e mutações chamadas frameshift (tipo de mutação que ocorre quando há adição ou remoção de bases de DNA e a estrutura de leitura de um gene se altera) em pares de bases G e C (guanina e citosina). Essas cepas são geneticamente manipuladas para aumentar sua sensibilidade para detectar a frequência de mutação (BEEDANAGARI et al., 2014)

As bactérias, como várias outras linhagens de células de roedores ou humanos, podem ter ou têm o potencial de ativação metabólica limitado, dito isto, o ensaio de Ames é quase

sempre realizado com e sem ativação metabólica exógena, geralmente na presença de fração S9 (homogenato de fígado de ratos), com a finalidade de simular o efeito de primeira passagem metabólica (KISHINO et al., 2019).

2.7 Teste de Hershberger

O teste de Hershberger é um ensaio *in vivo* de curta duração utilizado para avaliar o potencial androgênico ou antiandrogênico de uma substância teste. Este teste se baseia nas mudanças de peso de tecidos andrógeno-dependentes em ratos machos púberes ou pré-puberes castrados. Os tecidos analisados são as vesículas seminais, músculo levantador do ânus e glande do pênis. Um aumento ou diminuição significativos nos pesos dos órgãos indica um resultado positivo para ação androgênica e antiandrogênica, respectivamente (OECD, 2009).

O princípio do teste de Hershberger é calcado na necessidade de testosterona para o crescimento e desenvolvimento de órgãos sexuais durante o período da puberdade em machos saudáveis não castrados. A castração leva à abrupta regressão e involução dos órgãos sexuais e consequentemente a diminuição de seus pesos relativos, que são os parâmetros centrais deste ensaio. Quando um andrógeno é administrado (propionato de testosterona), ocorre o retorno às condições prévias à castração (DESSÌ-FULGHERI et al., 1983)

Este ensaio pode ser realizado por dois métodos, o primeiro é o ensaio que impõe a castração de animais adultos jovens; já o segundo, utiliza animais recém-desmamados não castrados. Ambos protocolos apresentam sensibilidade adequada para a realização dos testes, pois os animais estão com mínima produção endógena de andrógenos. Como os níveis de andrógenos séricos são baixos, o eixo hipotálamo-hipófise-gonadal não apresenta capacidade de exercer feedback sobre os órgãos alvo específicos (OECD, 2009)

2.8 Teste uterotrófico

O teste uterotrófico é um protocolo *in vivo* que avalia a capacidade de uma substância teste induzir atividades estrogênicas ou antiestrogênicas em modelos animais (KANNO et al., 2001). Por definição, as substâncias com potencial de interagir com os sistemas hormonais são denominados disruptores endócrinos. Os possíveis mecanismos para esses efeitos incluem a ligação ao receptor dos hormônios, inibição enzimática, interferência na esteroidogênese, bem como os efeitos sobre o desenvolvimento dos órgãos sexuais reprodutivos (DIAMANTI-KANDARAKIS et al., 2009).

O estradiol (17β-estradiol) é um hormônio endógeno que estimula a divisão celular e o crescimento de vários tecidos em fêmeas de mamíferos. Em particular, os tecidos do trato

reprodutivo (útero, colo do útero e vagina) passam por um ciclo estral de crescimento e regressão (STILLWELL, 2016). A duração de todo o ciclo é relativamente curta em ratos e camundongos, compreendendo apenas 4 a 5 dias, e é controlado pelo eixo hipotálamo-hipófise-gonadal (EHHG) (CALIGIONI, 2009; MARCONDES; BIANCHI; TANNO, 2002).

O resultado final da modulação da transcrição gênica após a ligação do 17β-estradiol ao seu receptor é uma resposta biológica do tecido alvo, como a divisão e o crescimento das células uterinas. Os processos mediados pelo receptor (ER) também são suscetíveis à inibição por antagonistas. Estes antagonistas exibem alta afinidade para o ER e inibem competitivamente a ligação do ligante 17β-estradiol nativo e sua atividade biológica subsequente (MARINO; GALLUZZO; ASCENZI, 2006).

Durante o ciclo estral normal, os níveis aumentados de 17β-estradiol normalmente resultam na divisão celular e no crescimento do tecido uterino e da vagina (MESIANO, 2019). Portanto, o útero se apresenta como um tecido ideal para detectar efeitos estrogênicos pois o tempo de resposta natural ao 17β-estradiol é relativamente curto, o aumento de seu peso é facilmente medido e a sua sensibilidade pode ser aumentada na ausência de estrogênios endógenos. O ensaio uterotrófico utiliza um sistema no qual o EHHG não é funcional, o que leva a baixos níveis de estrogênio endógeno circulante, isso maximiza a resposta ao 17β-estradiol exógeno administrado no protocolo. Vale salientar que o eixo EHHG não funcional é obtido usando uma das duas versões do ensaio. A primeira utiliza fêmeas adultas jovens após ovariectomia, com tempo adequado para a regressão dos tecidos uterinos. Já a segunda versão utiliza fêmeas imaturas, após o desmame e antes da puberdade (KANNO et al., 2001).

2.9 Produtos naturais

Ao longo do tempo os humanos tiveram a natureza como fonte primária para atender às suas necessidades básicas. Isso se aplica tanto à alimentação como para fontes de agentes terapêuticos. As plantas, em particular, formaram a base de sistemas sofisticados da medicina tradicional, com os primeiros registros, datando de cerca de 2600 a.C., documentando o uso de aproximadamente 1.000 substâncias derivadas de plantas na Mesopotâmia. Estes incluíam, entre outras, o *Cedrus spp* (cedro), *Cupressus sempevirens* (cipreste), *Glycyrrhiza glabra* (alcaçuz), espécies do gênero *Commiphora* (mirra) e *Papaver somniferum* (papoula); todos utilizados até os dias atuais para o tratamento de tosses e resfriados, infecções parasitárias e inflamações (BORCHARDT, 2002; KAPOOR, 2017).

Se voltarmos nossos olhos para a medicina egípcia, veremos que ela data de cerca de 2900 a.C., mas o registro mais conhecido daquele país, é o "Papiro Ebers", datado de 1500 a.C.,

documentando mais de 700 medicamentos, a maioria de origem vegetal (BORCHARDT, 2002). O mesmo ocorre na ásia, a Matéria Médica chinesa, um documento que documenta e organiza a fitoterapia daquele país, foi amplamente documentada ao longo dos séculos (HUANG, 1998), com o primeiro registro datando de cerca de 1100 a.C. (Wu Shi Er Bing Fang, contendo 52 prescrições), seguido por obras como o Shennong Herbal (~ 100 a.C.; 365 prescrições) e Tang Herbal (659 a.C.; 850 prescrições) (DEV, 1999; KAPOOR, 2017).

Como constituintes das espécies vegetais, figuram os metabólitos primários, que tem como objetivo a síntese de compostos essenciais para a sobrevivência das espécies. Ao metabolismo primário, estão associados os processos de fotossíntese que originam a formação de ácidos carboxílicos, aminoácidos, carboidratos, ácido graxos, proteínas e ácidos nucleicos, ou seja, todos envolvidos nos processos vitais das plantas (MAEDA, 2019).

Já os compostos de interesse humano, são denominados metabólitos secundários. Nas plantas, esses compostos apresentam função de defesa contra pragas, atração de agentes polinizadores, proteção contra grandes variações de temperatura, etc. Nos seres humanos, estas substâncias apresentam a capacidade de interagir e explorar nossos sistemas biológicos. Vale salientar que muitas vezes os efeitos observados podem ser indesejáveis, fato que chamamos de intoxicação. As principais classes de metabólitos secundários são os flavonoides, alcaloides, cumarinas, antraquinonas, terpenos, esteroides, saponinas e taninos (GUERRIERO et al., 2018)

Além de trazer os benefícios esperados para o tratamento de problemas de saúde, a utilização de plantas medicinais também apresenta um componente cultural e por vezes ritualístico ou sagrado. Em um país como o Brasil onde as populações ribeirinhas, aldeias indígenas além das figuras das benzedeiras se mantém vivas e ativas nos dias de hoje, é natural que estes tenham a sua importância destacada neste trabalho (MACIEL; GUARIM NETO, 2006).

Um estudo realizado com populações indígenas das etnias Kaiowá e Guarani na Reserva de Caarapó, Mato Grosso do Sul, mostrou que 34 espécies de plantas medicinais eram usadas por 22 famílias para o tratamento de diversas doenças. Na ocasião foi constatado que o modo de consumo dessas essas espécies vegetais era através da infusão ou sucos e que as aplicações eram as mais diversas possíveis, indo desde o tratamento para dores de cabeça até acidentes ofídicos (BUENO et al., 2005). Na região amazônica, índios Yanomami utilizam quase uma centena de plantas medicinais para o tratamento de malária (MILLIKEN, 1998).

Nas cidades de Cascavel e Foz do Iguaçu no estado do Paraná o conhecimento etnomedicinal já é colocado à favor da saúde da população atendida em unidades básicas de

saúde. Este fato demonstra a real importância de tal conhecimento e seu impacto na sociedade em que vivemos (BRUNING; MOSEGUI; VIANNA, 2012).

2.10 *Plinia cauliflora*

A *Plinia cauliflora* [Mart.] Kausel [Myrtaceae] é uma espécie nativa do Brasil popularmente conhecida como "jabuticaba" ou "jaboticaba". O nome jabuticaba tem origem na língua Tupi e significa "alimento de jabuti", pois quando as jabuticabeiras estão produzindo, alguns frutos caem no chão e os jabutis aproveitam para se alimentar (NAVARRO; SUASSUNA, 2015).

Os nomes populares e a classificação botânica ainda são bem variados, havendo divergências entre os autores. Embora o nome científico da espécie seja *Plinia cauliflora*, diversos sinônimos são encontrados em bases de dados científicos, tais como *Eugenia cauliflora, Eugenia jaboticaba, Myrcia jaboticaba, Myrciaria cauliflora, Myrciaria jaboticaba, Myrtus cauliflora* e *Myrtus jaboticaba.*

Se apresenta como uma árvore perene rústica e longeva, podendo atingir 20 metros de altura e viver até 100 anos (BORGES; MELO, 2003). Sua popularidade foi comparada à das uvas em outros países (REYNERTSON et al., 2006). Via de regra é obtida por plantio de semente, e demora de 8 a 12 anos para produzir os primeiros frutos que se formam diretamente no tronco. Esta espécie apresenta flores brancas e folhas lanceoladas e opostas. Sua madeira apresenta elevada dureza, e por isso pode ser utilizada na marcenaria (BORGES; MELO, 2003), porém o interesse pelos benefícios dos frutos inviabilizou a derrubada das árvores para este fim.

Seus frutos se apresentam como baga globosa de até 4 cm de diâmetro, com cascas que variam do preto-avermelhado ao verde-brônzeo com faixas vermelhas e roxas. A polpa é branca de aspecto mucilaginoso e apresenta de uma a quatro sementes (WANG et al., 2014). A *P. cauliflora* pode ser consumida *in natura* ou como geleias e compotas. Com a fermentação de sua polpa se produz vinho, vinagre e licor. (INADA et al., 2015b). A polpa de *P. cauliflora* apresenta vários nutrientes como ácido ascórbico, fósforo, ferro e niacina. A casca é rica em antocianinas (PITZ et al., 2016) e nela estão presentes fibras, pectina e peonidina (BORGES; CONCEIÇÃO; SILVEIRA, 2014). Quando analisadas separados dos frutos, as cascas apresentam teor de antocianinas de 4 a 7 vezes maior do que a fruta inteira, sugerindo que a casca é a principal fonte deste metabólito secundário (LEITE-LEGATTI et al., 2012).

As cascas dos frutos de *P. cauliflora* contêm fibras insolúveis e solúveis (pectina), β-caroteno, ácidos fenólicos (ácido gálico, ácido elágico e ácido transcinâmico) e flavonoides (rutina, miricetina e quercitrina). As cascas também apresentam antocianinas,

predominantemente cianidina-3-glicosídeo (curomanina) e delfinidina-3-glicosídeo (mirtilina) e diversos elagitaninos (casuarinina, casuarictina, pedunculagina e casuarina) e galotaninos (PLAZA et al., 2016).

Utilizando um extrato etanólico 70% preparado a partir de cascas de frutas de *P. cauliflora*, Romão et al., (2019) identificaram 37 compostos, incluindo ácidos orgânicos, derivados do ácido fenólico, flavonoides, antocianinas e taninos hidrolisáveis, cujos principais constituintes eram ácido gálico, ácido síngico, ácido elágico, O-hexosil cianidina, O-hexosil quercetina, O-hexosil ácido elágico, O-hexosil delfinidina, O-galoil desidrohexosídeo, di-O-galoil hexosídeo, tri-O-galoil hexosídeo, O-desoxihexosil miricetina, O-desoxihexosil ácido elágico, O-desoxihexosil quercetina, O-cinamoil, O-galoil hexosídeo, O-pentosil ácido elágico, quercetina, hexahidroxidifenil (HHDP)-galloyl O-hexosídeo, HHDP galoil O-hexosídeo (isômero de corilagina), di-HHDP galoil Ohexoside, di-HHDP galoil O-hexosídeo (isômero de casuarinina), di-HHDP galoil O-hexosídeo (isômero de castalagina), di-HHDP galoil O-hexosídeo (isômero de vescalagina), di-HHDP O-hexosídeo e HHDP tri-galoil O-hexosídeo. Ácido gálico, ácido elágico, O-hexosil cianidina, ácido O-hexosil elágico, ácido O-pentosil elágico, ácido O-desoxihexosil elágico e O-desoxihexosil quercetina também foram identificados neste extrato etanólico de 70% de cascas de frutas (PALOZI et al., 2019).

Praticamente todas as partes da espécie são utilizados na medicina popular. Sua casca é adstringente, o que justifica seu uso contra diarreia e disenterias; além disso, apresenta indicações na medicina popular contra asma e enterite (INADA et al., 2015b), dor de garganta e corrimento vaginal (PAIVA et al., 2017). As folhas são usadas na terapêutica da gripe, labirintite; feridas e bronquite (BIESKI et al., 2015; MACIEL; GUARIM NETO, 2006; ZENI; BOSIO, 2011).

Na literatura científica, diversos são os estudos já publicados com preparações obtidas à partir de *P. cauliflora*. Podem ser encontrados resultados publicados em artigos que avaliaram o potencial antinflamatório, hipoglicêmico, hipolipidêmico e analgésico (BRITO et al., 2021), antioxidante e antiproliferativo em células de carcinoma oral (WANG et al., 2014), atividade antinflamatória para tratamento de doença pulmonar obstrutiva crônica (ZHAO et al., 2019) e anti cândida (SOUZA-MOREIRA et al., 2013).

Além disso, estudos realizados *in vitro* demonstraram propriedades antimicrobiana para bactérias gram-positivas, gram-negativas e antifúngica (SOUZA-MOREIRA et al., 2018), antioxidante e de diminuir a produção de Interleucina-8 em células epiteliais de vias aéreas expostas à fumaça de cigarro (WU; LONG; KENNELLY, 2013). Já os estudos *in vivo*

apresentam efeitos antimicrobiano para *Staphylococcus aureus* resistente à meticilina (MRSA) (DOS SANTOS et al., 2019), antioxidante e cardioprotetor (ROMÃO et al., 2019).

3 OBJETIVOS

3.1 GERAL

Avaliar a toxicidade e segurança do sobrenadante etanólico do infuso das cascas de *Plinia cauliflora.*

3.2 ESPECÍFICOS

- Produzir o sobrenadante etanólico do infuso das cascas de *P. cauliflora* (SEIPC);
- Identificar os constituintes do SEIPC por cromatografia líquida de alta eficiência acoplada ao espectrômetro de massas (LC-DAD-MS);
- Investigar a toxicidade oral aguda e realizar o teste de Irwin modificado;
- Investigar a toxicidade prolongada do SEIPC após a administração de doses repetidas por 28 dias;
- Avaliar se o tratamento prolongado com o SEIPC interfere no traçado eletrocardiográfico, frequência respiratória, medida da pressão arterial, gasometria arterial, além de parâmetros bioquímicos e hematológicos;
- Investigar o potencial genotóxico através do ensaio do cometa e do teste de micronúcleo;
- Investigar o potencial mutagênico do SEIPC através do teste de AMES.
- Avaliar possíveis efeitos disruptores endócrinos do SEIPC através dos testes uterotrófico e Hershberger.

4. ARTIGOS PUBLICADOS DURANTE O PERÍODO DO DOUTORADO

Ao longo do período de doutorado, tive a oportunidade de contribuir em diversos outros projetos de pesquisa, tanto na própria Universidade Federal da Grande Dourados – UFGD como

em outras Universidades. O resultado de tais projetos pode ser conferido nos artigos abaixo listados.

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5. MARQUES, ALINE APARECIDA MACEDO; LORENÇONE, BETHÂNIA ROSA; ROMÃO, PAULO VITOR MOREIRA; GUARNIER, LUCAS PIRES; **PALOZI, RHANANY ALAN CALLOI**; MORENO, KARYNE GARCIA TAFARELO; TIRLONI, CLEIDE ADRIANE SIGNOR; DOS SANTOS, ARIANY CARVALHO; SOUZA, ROOSEVELT ISAÍAS CARVALHO; KLIDER, LISLAINE MARIA; LOURENÇO, EMERSON LUIZ BOTELHO; TOLOUEI, SARA EMILIA LIMA; BUDEL, JANE MANFRON; KHAN, SHABANA I.; SILVA, DENISE BRENTAN; GASPAROTTO JUNIOR, ARQUIMEDES. Ethnopharmacological investigation of the cardiovascular effects of the ethanol-soluble fraction of *Aloysia polystachya* (Griseb.) Moldenke leaves in spontaneously hypertensive rats. JOURNAL OF ETHNOPHARMACOLOGY, v. 274, p. 114077-14, 2021.

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6 APÊNDICES

Artigo 1: Revista Journal of Ethnopharmacology (Qualis A2, FI: 3.690)

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From general toxicology to DNA disruption: A safety assessment of *Plinia cauliflora* (Mart.) Kausel

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ABSTRACT

Ethnopharmacological relevance: *Plinia cauliflora* (Mart.) Kausel (Myrtaceae) is popularly known as "jaboticaba" or "jaboticaba". The fruit is appreciated for both fresh consumption and the manufacture of jelly, juice, ice cream, fermented beverages, and liqueurs. The more widespread traditional use of the plant involves the treatment of diarrhea, which utilizes all parts of the plant, including the fruit peels.

Aim of the study: We sought to elucidate possible risks of the administration of an ethanol-soluble fraction that was obtained from an infusion of *P. cauliflora* fruit peels (SEIPC). We performed a series of experiments to evaluate possible toxicity, in which we administered SEIPC orally both acutely and repeatedly for 28 days. We also evaluated possible endocrine-disruptive and genotoxic effects in eukaryotic cells. The possible mutagenic activity of SEIPC was evaluated using reverse mutation (Ames) assays.

Materials and methods: SEIPC was produced and chemically characterized by LC-DAD-MS. Acute toxicity and behavioral and physiological alterations were evaluated in the modified Irwin test. Respiratory rate, arterial blood gas, electrocardiography, respiratory rate, heart rate, and blood pressure were evaluated, and hematological, biochemical, and histopathological analyses were performed after 28 days of oral treatment. The comet assay, mammalian erythrocyte micronucleus test, uterotrophic test, Hershberger bioassay, and AMES test were performed using appropriate protocols.

Results: From SEIPC, ellagic acid and derivatives, flavonols and anthocyanidins, as well as citric acid and gallic acid, were annotated by LC-DAD-MS. We did not observed any significant toxic effects after acute or prolonged SEIPC treatment. No endocrine-disruptive or mutagenic effects were observed.

Conclusions: The present study found that SEIPC did not cause any significant alterations of various corporeal systems, including cardiac electrical activity, body temperature, respiratory rate, and arterial pressure. No alterations of biochemical, hematological, or blood gas parameters were observed. SEIPC did not cause any perturbations of the endocrine system or mutagenic, cytotoxic, or genotoxic effects. These findings substantiate the safe clinical use of *P. cauliflora*.

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Abbreviations

ABG Arterial blood gas
ANOVA One-way analysis of variance
BB Buffer Base
BE Base Excess
BE_{ecf} Base excess in the extracellular fluid compartment
Ca^{++} Calcium
$_{c}HCO3$ Bicarbonate concentration
Cl^{-} Chloride
CNS Central nervous system
$_{ct}CO_{2\ (B)}$ Concentration of total carbon dioxide of whole blood
$_{ct}CO_{2\ (P)}$ Concentration of total carbon dioxide in plasma
$_{ct}O_2$ Concentration of total oxygen
DBP Diastolic blood pressure
ECG Electrocardiography
SEIPC Soluble ethanolic infuse of Plinia cauliflora
H^+ Hydrogen ion dissociated
Hct Hematocrit
HH_b Deoxyhemoglobin
K^+ Potassium
LA Left arm
LC-DAD-MS Liquid chromatography coupled to a diode array detector and mass spectrometer
LL Left leg
MAP Mean arterial pressure
MHC Mean Corpuscular Hemoglobin
MCHC Mean corpuscular hemoglobin concentration
MCV Mean corpuscular volume
MPV Mean platelet volume
RDW Red blood cell distribution width
Na^+ Sodium Na_2CO_3
Sodium carbonate O_2Hb
Oxyhemoglobin P_{50} Half of the maximum hemoglobin saturation
PCO_2 Partial pressure of carbon dioxide pH
Potential of hydrogen PO_2
Partial pressure of oxygen RA Right arm
RBC red blood cells
RL Right leg
S.E.M. Standard error of the meanz
SBP Systolic blood pressure
SO_2 Level of hemoglobin-saturation by oxygen
$_{t}Hb$ Hemoglobin
UFLC Ultra fast liquid chromatograph
WBC White blood cells

1. Introduction

In the 16th century, Paracelsus said, "What is there that is not poison? All things are poison, and nothing is without poison. Solely the dose determines that a thing is not a poison." Most substances that are used by mankind have potential risks. The concept of safety is relative because all medicines, both natural and synthetic, can be both harmful and beneficial (Grandjean, 2016). Safety and efficacy must always be considered in new drug development. Pharmacological safety studies are important because they ensure the safety of these compounds before testing and use in humans. These protocols seek to map and identify possible harmful or undesirable effects in vitally important systems, including the cardiovascular, respiratory, endocrine, and central nervous systems (Pugsley et al., 2008).

Most products or compounds that are used by the world's population have passed safety tests in the pharmaceutical industry. Preparations from natural sources, however, commonly follow a "popular use flow." It is not uncommon to find people who say, "If it's natural, it's not harmful," but we know this is not necessarily true (Kaitin et al., 1993; Latten et al., 2018; Schuhmacher et al., 2018). Without safety and quality tests, events like the New England Compounding Center disaster can happen, in which a nationwide fungal meningitis outbreak in 2012 caused infections in 793 patients, 64 of whom died (Akers, 2015).

A drug should only be used when it can benefit patients. Risk-benefit assessments consider both the ability of a drug to produce desirable effects and the kind and likelihood of adverse effects (Juhaeri, 2019). Similar classes of secondary metabolites can present different and even antagonistic effects. One example is *Veratrum californicum*, one component of which is the alkaloid cyclopamine, which is known to be teratogenic (Turner et al., 2019). Alkaloids that are present in *Catharanthus roseus* (e.g., vinblastine, vincristine, vindesine, and vinorelbine) are used to treat some types of cancer, such as breast cancer, testicular cancer, and lymphoma (Abouzeid et al., 2019; Le Roux and Guéritte, 2017).

Plinia cauliflora (Mart.) Kausel (Myrtaceae) is popularly known as "jaboticaba" or "jabuticaba. The commercial potential of *P. cauliflora* is high because of its organoleptic characteristics. The fruit is appreciated for both fresh consumption and the manufacture of jelly, juice, ice cream, fermented beverages, and liqueurs (Magalhães et al., 1996; Balerdi et al., 2006). The more widespread traditional use of the plant involves the treatment of diarrhea, which utilizes all parts of the plant. Different parts of the species are also used for antidiarrhea in several states of Brazil, including bark in Bahia (Agra et al., 2008), leaves and fruits in Santa Catarina (Zeni and Bosio, 2011), and fruit peels in Bahia state (Silva et al., 2012).

Several studies have reported cardioprotective (Romão et al., 2019), antiobesity (Moura et al., 2018), antioxidant (Inada et al., 2015; de Souza et al., 2017), antiinflammatory (Hsu et al., 2016), hypotensive (Lobo de Andrade et al., 2015), antibacterial (Souza-Moreira et al., 2011), and hypolipidemic (Araújo et al., 2014) effects of *P. cauliflora*. However, these studies did not perform batteries of safety tests to demonstrate its safe use and consumption.

To elucidate possible risks that are associated with *P. cauliflora* consumption, we administered an ethanol-soluble fraction that was obtained from an infusion of *P. cauliflora* fruit peels (SEIPC) in rats. The rats received SEIPC orally both acutely and repeatedly for 28 days to evaluate possible toxicity. We evaluated possible endocrine-disruptive and genotoxic effects in eukaryotic cells. The possible mutagenic activity of SEIPC was evaluated by reverse mutation (Ames) assays.

2. Materials and methods

2.1. Animals

Healthy male and female Wistar rats, 3 months old, were obtained from the Central Vivarium of the Federal University of Grande Dourados (UFGD, Brazil). The animals were housed in the vivarium at a constant temperature (22 °C ± 2 °C) under 12 h/12 h light/dark cycle. The animals received food and water *ad libitum*. The Institutional Ethics Committee of UFGD (protocol no. 18/2018) approved all of the experimental procedures.

2.2. Plant material

Plinia cauliflora fruits were collected in Esperança Nova, Paraná, Brazil (-23.719864, -53.802104). A voucher specimen (no. 6337) was authenticated by Dr. Zefa Valdivina Pereira and deposited in the herbarium of UFGD. The fruit peels were manually removed and dried by forced air circulation for 5 days. The peels were pulverized in a knife mill and stored in plastic bags at 2–8 °C until use.

2.2.1. Production of ethanol-soluble fraction of the infusion of P. cauliflora fruit peels

Extract production began by infusing 1 L of boiling water for each 100 g of dried and pulverized fruit peels. After filtration to remove plant material, the infusion was treated with 3 vol of 95% ethanol, which gave rise to a precipitate and an ethanol-soluble fraction (SEIPC). SEIPC was filtered to remove the precipitate. Ethanol was totally removed by evaporation, and the resulting fraction was lyophilized to produce a yield of 11.03%.

2.2.2. Identification of the constituents from the infusion of the P. cauliflora fruit peels (SEIPC) by LC-DAD-MS

A Shimadzu Prominence UFLC Shimadzu device coupled to a diode array detector (DAD) and mass spectrometer MicrOTOF-Q III (Bruker Daltonics, Billerica, MA, USA) was applied to determine the constituents from SEIPC. The column was a Kinetex C18 column (2.6 µm, 150 × 2.1 mm, Phenomenex). SEIPC was prepared at 2 mg/mL, filtered on syringe filter (Millex 0.22 µm, PTFE, Millipore®) and injected (6 µL) in the chromatographic system. The chromatographic and mass spectrometric parameters were the same reported by Romão et al. (2019). The compounds were identified based on UV spectra, accurate mass and compared to data from *P. cauliflora* extracts previously published by our research group (Romão et al., 2019). The molecular formulas were determined by accurate mass considering errors up 8 ppm and mSigma below than 25.

2.3. Acute oral toxicity and modified Irwin test

This protocol was performed according to Acute Oral Toxicity–Acute Toxic Class Method guidelines (Organisation for Economic Cooperation and Development [OECD] guideline 423, 2002). Ten female Wistar rats were used in this experiment. After a 7-day acclimatization period, they were randomly assigned to two groups: naive and SEIPC (n = 5/group). SEIPC (2000 mg/kg) was administered orally by gavage. The naive group received only drinking water (5 ml/kg). Possible effects on the central nervous system were evaluated using the modified Irwin test. Food was given to the animals only 1 h after the treatments. The effects of the treatments were evaluated 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h after treatment and then daily for 2–14 days after treatment.

We evaluated excitation (i.e., convulsions, tremor, straub tail, hyperactivity, jumping, increases in fear/startle, increases in reactivity to touch, increases in abdominal muscle tone, and aggression), stereotypy (i.e., head-twitches, head movements, chewing, sniffing, and scratching), motor activity (catalepsy, akinesia, rolling, tip-toe, motor incoordination, loss of traction, and loss of grasping ability), sedation (i.e., hypoactivity, decreases in fear/startle, decreases in reactivity to touch, and decreases in abdominal muscle tone), pain (i.e., writhing and analgesia), autonomic effects (i.e., ptosis, exophthalmia, myosis, mydriasis, piloerection, defecation/diarrhea, salivation, and lacrimation), and other effects (i.e., respiration). Food and water consumption and body weight were monitored for 14 days.

After 14 days, the animals were euthanized by deep anesthesia in a saturation chamber (isoflurane saturation > 20%). Before euthanasia, after a 6-h fast, the final body weight was determined. The liver, heart, kidneys, lungs, and spleen were removed, cleaned, and weighed to determine absolute and relative organ weights (weight × 100/body weight).

2.4. Repeated treatment with SEIPC for 28 days and oral toxicity and genotoxicity studies

The doses of SEIPC were based on the popular use of *P. cauliflora*. On average, an adult eats 12 jabuticaba fruits with the peel (i.e., ~100 g), indicating ~6 g of dried peels. In the present study the SEIPC yield was 11.03%. The low dose was ~30 mg/kg, and the high dose was 10-times the low dose (300 mg/kg). The intermediate dose of 100 mg/kg was then 3-times higher than the low dose and 3-times lower than the high dose. The highest dose of 1000 mg/kg was based on "Repeated Dose 28-Day Oral Toxicity Study in Rodents" (OECD guideline 407, 2008).

Sixty female and 60 male Wistar rats were used. The animals were randomly divided into six groups for both males and females (n = 10/group): naive (vehicle), SEIPC (30, 100, 300, and 1000 mg/kg), and satellite. The satellite group was composed of animals that were treated with 1000 mg/kg SEIPC for 28 days. Unlike the other experimental groups, which were euthanized on day 29 after treatment, the animals in the satellite group were euthanized 2 weeks after completing the 28-day treatment regimen, with the goal of evaluating possible delayed toxicity. The volume that was administered daily did not exceed 1 ml/100 g body weight. Throughout the study, body weight and food and water consumption were monitored. On day 29, body temperature was measured using an anal thermometer.

2.4.1. Electrocardiography

To evaluate electrocardiographic (ECG) waves and intervals, the animals were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (20 mg/kg) intramuscularly, which was then administered every 40 min as necessary. To record signals from the cardiac cycle, electrodes were connected to a Wincardio–Micromed electrocardiograph and positioned on the animals (Guarnier et al., 2019) to record three bipolar leads (DI, DII, and DIII) and three amplified leads (aVR, aVL, and aVF). The following data were collected: P segment (ms), PR segment (ms), QRS segment (ms), QT segment (ms), QTc segment (ms), P amplitude (mV), Q amplitude (mV), R amplitude (mV), S amplitude (mV), and T amplitude (mV).

2.4.2. Respiratory rate

Respiratory rate was recorded by whole-body plethysmography according to Kabir et al. (2010).

2.4.3. Arterial pressure measurement

Immediately after ECG and respiratory rate recordings, all of the animals subcutaneously received a bolus injection of heparin (20 IU). Tracheotomy was performed to allow the animals to breathe spontaneously. Systolic blood pressure, diastolic blood pressure, mean arterial pressure, and heart rate were measured in all of the groups. The left carotid artery was isolated, cannulated, and connected to the PowerLab system and Chart 8.1 software (both from ADInstruments, Castle Hill, Australia). The different hemodynamic parameters were recorded for 20 min (15 min for stabilization and 5 min for data analysis; Gasparotto Junior et al., 2011).

2.4.4. Arterial blood gas

To determine arterial blood gas, arterial blood was collected from the left carotid artery, which was previously cannulated, into heparinized syringes (lithium heparin, 33.6 USP Units, Becton Dickinson, Franklin Lakes, NJ, USA) and tested using a Cobas b121 arterial blood gas analyzer. The following parameters were analyzed: pH, pCO_2 (mmHg), pO_2 (mmHg), SO_2 (%), Hct (%), tHb (g/dL), Na^+ (mmol/L), K^+ (mmol/L), Ca^{++} (mmol/L), Cl^- (mmol/L), AnGap (mmol/L), mOsm (mOsm/kg), glucose (mg/dl), lactate (mmol/L), CO oximetry, fO_2Hb (%), fHHb (%), fCOHb (%), fMetHb (%), base excess (B) (mmol/L), base

excess-extracellular fluid (mmol/L), HCO_3^- actual (mmol/L), and $ctCO_2$ (mmol/L).

2.4.5. Serum biochemical analyses

While the animals were still anesthetized, blood was collected from the catheter into Clot Activator/Polymer tubes (BD Hemogard) and centrifuged at 3500 rotations per minute for 15 min. γ-Glutamyl transferase (U/L), urea (mg/dl), magnesium (mg/dl), creatinine (mg/dl), triglycerides (mg/dl), total cholesterol (mg/dl), high-density lipoprotein (mg/dl), total protein (mg/dl), albumin (mg/dl), thyroid-stimulating hormone (TSH; uIU/ml), lactate dehydrogenase (LDH; U/l), sodium (mmol/L), calcium (mg/dl), potassium (mmol/L), chloride (mmol/L), alanine aminotransferase (U/L), aspartate aminotransferase (U/L), total bilirubin (mg/dl), and direct bilirubin (mg/dl) were measured using a Cobas 8000 modular analyzer (Roche Diagnostics).

2.4.6. Hematological parameters

To analyze different hematological parameters, blood was collected from the cannulated left carotid artery into BD Hemogard K_2EDTA 5.4 mg tubes. White blood cells (10^4/μl), red blood cells (10^4/μl), hemoglobin (g/dl), hematocrit (%), MCV (fl), MCH (pg), MCHC (g/dl), platelets (10^3/μl), RDW (%), MPV (fl), neutrophils (10^3/μl), lymphocytes (10^3/μl), monocytes (10^3/μl), eosinophils (10^3/μl), and basophils (10^3/μl) were measured using a Sysmex XN-3100 automated hematology system.

2.4.7. Organ collection and histopathological analysis

At the end of the experiments, the animals were euthanized by deep anesthesia in a saturation chamber (isoflurane saturation > 20%). After euthanasia, median xiphoid laparotomy was performed to remove the organs of interest. The liver, heart, kidneys, lungs, spleen, encephalon, testicles, adrenal gland, epididymis, prostate, seminal vesicle, ovaries, uterus, and cervix were removed, cleaned, and weighed to determine absolute and relative organ weights (weight × 100/body weight). Tissue samples from the pituitary glands, encephalon, lungs, heart, liver, kidneys, spleen, adrenal gland, thyroid, testicles, epididymis, seminal vesicle, prostate, uterine body, cervix, ovaries, and vaginal canal were dehydrated in different concentrations of alcohol, embedded in paraffin, and sectioned at the 5 μm thickness. The sections were dyed with hematoxylin and eosin (H&E) according to an accepted procedure for light microscopy.

2.4.8. Comet assay

The Comet assay was performed according to OECD guideline 489 for chronic exposure. According to Villas Boas et al. (2018), whole blood was used to perform the test in animals after 28 days of treatment. Blood samples were collected through a caudal puncture and transferred to a container with low-melting-point agarose (1.5%) at 37 °C. The blood and agarose were then homogenized and transferred to plates that were precoated with normal agarose (5%). After the solidification of agarose, the slides were transferred to a final lysis solution. After lysis, the slides were placed in an electrophoresis pool that was filled with alkaline buffer and left at rest for 20 min to denature the DNA. After the denaturation step, the electrophoretic run began.

Afterward, the slides were removed from the tank and covered by neutralization solution in three cycles of 5 min each. The slides were fixed in ethanol for 10 min and transferred to a refrigerator at 2-8 °C until the analysis. The slides were stained with ethidium bromide solution and analyzed using a fluorescence microscope that was equipped with an excitation filter (420–490 nm) sand barrier filter (520 nm). Each slide was examined in a blinded fashion, in which 100 cells were analyzed per animal. The Comet classifications were the following: class 0 (no damage), class 1 (comet tail shorter than the diameter of the nucleoid), class 2 (comet tail once or twice the diameter of the nucleoid), and class 3 (comet tail greater than twice the size of the nucleoid). Two parameters were calculated for each animal: damage frequency and damage index. Damage frequency refers to the number of cells, out of 100 analyzed cells, that have some kind of damage. The damage index refers to the extent and severity of damage to the cells.

2.4.9. Micronucleus test

The micronucleus test was performed according to OECD guideline 474 (Mammalian Erythrocyte Micronucleus Test, 2016) and Villas Boas et al. (2018). The tests were performed with polychromatic erythrocytes (PCEs) from bone marrow after the animals received SEIPC for 28 days. Immediately after euthanasia, the right femur was removed and dissected from adjacent muscles and tissues. The epiphyses were removed to expose the bone marrow. The bone marrow cavity was washed with 1 ml of fetal bovine serum to place the bone marrow into a centrifuge microtube. The suspension that contained fetal bovine serum and bone marrow was centrifuged at $1000 \times g$ for 5 min. The supernatant was discarded, and bone marrow pellets that were formed after centrifugation were used to perform smears on slides. The slides were fixed with methanol for 10 min and stained with Giemsa for 15 min. Each slide was analyzed in a blinded fashion. A total of 2000 PCEs were analyzed per animal. Each erythrocyte was identified by the presence or absence of micronuclei, also known as Howell-Jolly bodies. To evaluate possible cytotoxic effects, the ratio of polychromatic and normochromatic erythrocytes (PCEs/NCEs) was calculated by analyzing 200 random erythrocytes per sample.

2.5. Endocrine disruption assays

2.5.1. Uterotrophic test

The uterotrophic bioassay was performed according to OECD guideline 440 (2007). Thirty-two immature female rats (postnatal day [PND] 21) were randomly divided into four experimental groups (n = 8/group) to investigate possible estrogenic effects of SEIPC. Two groups received different doses of SEIPC (30 and 300 mg/kg). The negative control group received vehicle only (filtered water, 2 ml/kg). The positive control group received ethynyl estradiol (3 μg/kg). The doses were calculated daily according to the animals' body weight.

All of the animals were treated orally by gavage for three consecutive days from PND 22 to PND 24. Twenty-four hours after the last administration (PND 25), the animals were individually weighed and euthanized by an overdose of isoflurane anesthesia (inhalation) followed by decapitation. During necropsy, the uterine horns were cut just above the junction with the cervix and at the junction of the uterine horns with the ovaries. Each uterus was then removed from the body wall and trimmed free of excess fat. The uterus with and without luminal fluid was weighed. Absolute and relative organ weights (weight × 100/body weight) were recorded.

2.5.2. Hershberger bioassay

This test was performed according to OECD guideline 441 (2009). Fifty-six male pubertal rats (PND 43) were castrated via a midline scrotal incision under intraperitoneal anesthesia with ketamine (75 mg/kg) and xylazine (1.5 mg/kg), followed by ibuprofen (20 mg/ml) by oral gavage. After castration, the animals immediately received gentamicin (0.1 ml/100 g) intramuscularly. Ibuprofen (20 mg/ml) was administered for two consecutive days. The surgical site was carefully cleaned with iodine-soaked cotton for two consecutive days. All of the castrated rats were placed in cages (4 animals/cage) that contained 5 cm of wood shavings as bedding and left in the bioterium for 2 weeks to recover from surgery. After this period, the animals were randomly divided into seven experimental groups (n = 8/group) to investigate the possible (anti)androgenic activity of SEIPC. To test antiandrogenicity, two doses of SEIPC (30 and 300 mg/kg) were administered orally by gavage followed by subcutaneous testosterone administration (0.25 mg/kg). Flutamide (5 mg/kg) was used as the positive control of antiandrogenicity, which was dissolved in distilled water and administered orally by gavage (2 mg/kg), followed by subcutaneous

testosterone administration (0.25 mg/kg). To test androgenicity, two doses of SEIPC (30 and 300 mg/kg) were administered orally by gavage, followed by subcutaneous canola oil administration (1 ml/kg). A positive control for androgenicity was established, in which vehicle (filtered water) was administered orally by gavage, followed by subcutaneous testosterone administration (0.25 mg/kg). A negative control group that was treated with vehicle (filtered water, 2 ml/kg) orally by gavage, followed by subcutaneous canola oil administration (1 ml/kg), was also established and used for both tests. All of the animals were treated individually for 10 days. Doses were calculated daily based on the animals' body weight. Twenty-four hours after the last treatment, the animals were weighed and euthanized by an overdose of isoflurane anesthesia (inhalation) followed by decapitation.

The seminal vesicle (without fluid), levator ani muscle/bulbocavernosus muscle (LABC), and glans penis were removed and dissected. Absolute and relative organ weights (weight × 100/body weight) were calculated and recorded.

2.6. Reverse mutation (Ames) assays

The mutagenic potential of SEIPC was evaluated using the Ames test, with preincubation according to Mortelmans and Zeiger (2000)) and OECD guideline 471 (1997). *Salmonella typhimurium* strains were provided by the Toxicology and Genotoxicity Division of the Environmental Company of the State of São Paulo, Brazil (CETESB). First, 100 µl of the *S. Typhimurium* TA98 and TA100 strains were reactivated in 20 ml of Nutrient Broth (Oxoid no. 2) and incubated at 37 °C for 16 h in an orbital shaker incubator. After this period, the lineages were standardized in a spectrophotometer at 650 nm to obtain a concentration of 1 × 108 bacteria/ml. The strains were then concentrated by centrifugation at 1500 × *g* at 4 °C for 10 min and resuspended in 4 ml of 0.2 M phosphate buffer. The assays were performed in the presence and absence of exogenous metabolization (S9 fraction [S9F]) to test for the presence of mutagens both directly and indirectly. In test tubes without S9F, we added 50 µl of 0.2 M phosphate buffer. In test tubes with S9F, 50 µl of S9F was added. Samples (5 µl) at concentrations of 5000, 1500, 500, 150, and 50 µg/plate were added to 50 µl of the inoculum. The test tubes were preincubated at 37 °C for 90 min. After the incubation period, 2 ml of top agar (0.6% agar, 0.6% NaCl, 0.05 mM L-histidine, and 0.05 mM biotin, pH 7.4, 45 °C) was poured into the plates with minimal agar (1.5% agar, 50% Voguel-Boner solution, and 10% glucose solution). The plates were incubated at 37 °C for 48–66 h. Afterward, revertant colonies were counted. 2-Anthramine (2.5 µg/plate) was used as a positive control in the metabolic activation assays for both strains. In the tests without metabolic activation, the positive control for TA98 was 4-nitro-phenylenediamine (10 µg/plate). The positive control for TA100 was sodium azide (2.5 µg/plate). Distilled water was used as a negative control. The assays were performed in triplicate.

2.7. Statistical analysis

For the *in vivo* assays, the data are expressed as mean ± standard error of the mean (SEM). Differences between groups were assessed using analysis of variance (ANOVA), followed by Dunnett's *post hoc* test. Values of $p < 0.05$ were considered statistically significant. GraphPad Prism 7.0 software was used to draw the graphs and for the statistical analysis.

The Ames assay results were analyzed using the Salanal statistical program (U.S. Environmental Protection Agency, Monitoring Systems Laboratory, NC, USA; version 1.0). The mutagenicity index was calculated as the following: *mutagenicity index = number of induced revertants/number of spontaneous revertants*. A compound is considered to have mutagenic potential when the mutagenicity index is ≥ 2 for at least one of the concentrations tested.

3. Results

3.1. Identification of the constituents from SEIPC by LC-DAD-MS

The SEIPC was analyzed by LC-DAD-MS and 19 compounds were annotated (Table 1, Fig. 1). The compound 1, 3 and 4 showed intense ions at *m/z* 191.0178, 205.0354 and 219.0506 [M-H]⁻, which were putatively identified as citric acid, methyl citrate, and dimethyl citrate, respectively. The compound 2 revealed an absorption band at the wavelength 270 nm in the UV spectrum and an intense ion at *m/z* 169.0121 [M-H]⁻, which was identified and confirmed as gallic acid by injection of authentic standard. The metabolites 1 and 2 have been

Table 1
Identification of the constituents from infusion of the *P. cauliflora* fruit peels *Plinia cauliflora* (SEIPC) by LC-DAD-MS.

Peak	RT (min)	Compound	UV (nm)	MF	Negative mode (*m/z*)		Positive mode (*m/z*)	
					MS $[M-H]^-$	MS/MS	MS $[M+H]^+$	MS/MS
1	2.2	Citric acid	–	$C_6H_8O_7$	191.0178	–	–	
2	2.5	Gallic acid[st]	270	$C_7H_6O_5$	169.0146	–	–	
3	3.2	Methyl citrate	–	$C_7H_{10}O_7$	205.0354	–	229.0301[Na]	
4	6.1	Dimethyl citrate	–	$C_8H_{12}O_7$	219.0506	–	221.0605	203, 157
5	13.4	*O*-hexosyl cyanidin	280, 512	$C_{21}H_{21}O_{11}^+$	447.0909	285, 255, 162, 147	449.1072	287
6	14.4	NI	–	$C_{16}H_{20}O_{10}$	371.0985	–	373.1162	–
7	15.7	*O*-hexosyl ellagic acid	255, 360	$C_{20}H_{16}O_{13}$	463.0503	301	465.0689	303
8	15.9	NI	270	$C_{15}H_{12}O_8$	319.0456	–	321.0602	–
9	16.7	NI	270	$C_{10}H_{16}O_7$	–	–	249.0950	–
10	16.8	di-HHDP-galloyl O-hexoside (castalagin/vescalagin isomer)	275	$C_{41}H_{24}O_{25}$	–	–	917.0660*	–
11	18.1	*O*-pentosyl ellagic acid	265, 360	$C_{19}H_{14}O_{12}$	433.0447	301	435.0570	303
12	18.5	Ellagic acid[st]	255, 362	$C_{14}H_6O_8$	301.0002	283, 257, 229, 185	303.0120	275, 257, 229, 201
13	18.7	*O*-deoxyhexosyl myricetin	260, 355	$C_{21}H_{20}O_{12}$	463.0890	–	465.1024	319
14	19.2	*O*-hexosyl quercetin	260, 355	$C_{21}H_{20}O_{12}$	463.0899	301	465.1002	303
15	19.5	*O*-hexosyl quercetin	265, 355	$C_{21}H_{20}O_{12}$	463.0897	301	465.1020	303
16	19.7	NI	280	$C_{27}H_{34}O_{15}$	597.1815	395	599.1973	581, 397
17	20.9	*O*-pentosyl quercetin	270, 350	$C_{20}H_{18}O_{11}$	433.0797	–	435.0956	303
18	21.8	*O*-deoxyhexosyl quercetin	261, 350	$C_{21}H_{20}O_{11}$	447.0924	300, 271, 255, 243, 179, 151	449.1081	303
19	35.7	NI	–	$C_{16}H_{33}NO_3$	–	–	288.2527	–

St: confirmed by authentic standard; * $[M + H-H_2O]^+$, Na: $[M+Na]^+$; MF: molecular formula.

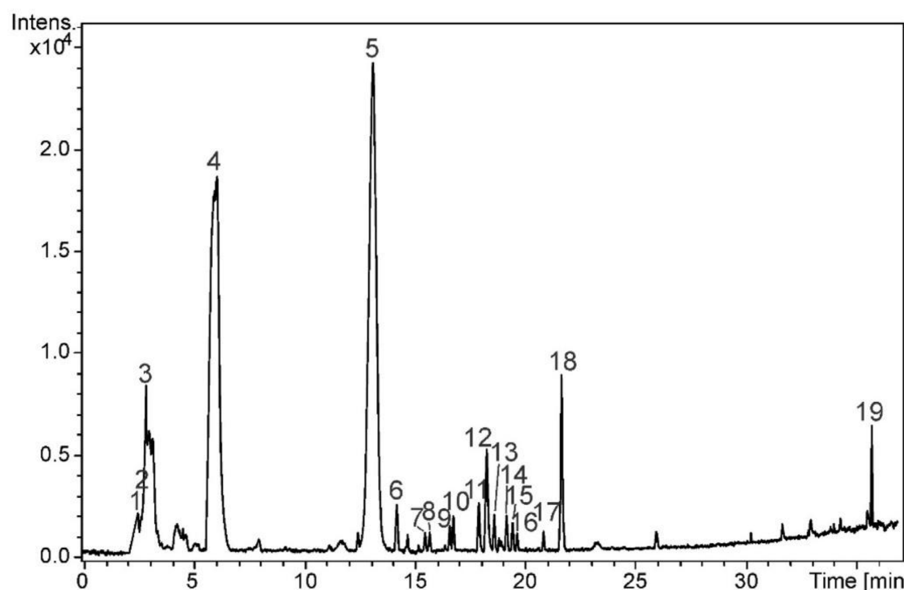

Fig. 1. Base peak chromatogram (positive ion mode) from infusion of the *P. cauliflora* fruit peels *Plinia cauliflora* (SEIPC).

previousy reported in *P. cauliflora* fruits (Romão et al., 2019).

The substances 7, 11 and 12 showed bands in the UV spectra at absorptions at ≈ 260 and 360 nm that suggested the chromophore group relative to ellagic acid. The compound 12 exhibited the molecular formula $C_{14}H_6O_8$ (from ions *m/z* 301.0002 [M-H]$^-$ and 303.0120 $[M+H]^+$), as well as the fragment ions at *m/z* 283, 257 and 229, which are yielded from losses of a water, CO_2 and CO molecules. This fragmentation pathway is similar to ellagic acid profile described in the literature (Neves et al., 2018; Ambigaipalan et al., 2016), and it was confirmed by injection of authentic standard. The substances **7** (*m/z* 463.0503 [M-H]$^-$ and 465.0689 $[M+H]^+$, $C_{20}H_{16}O_{13}$) and 11 (*m/z* 433.0447 [M-H]$^-$ and 435.0570 $[M+H]^+$, $C_{19}H_{14}O_{12}$) yielded the fragment ions at *m/z* 301 and 303 at negative and positive ion modes, respectively, from losses of a hexosyl (162 *u*) and pentosyl (132 *u*) groups. Thus, the compounds 7 and 11 were annotated as *O*-hexosyl ellagic acid and *O*-pentosyl ellagic acid. The compounds 7, 11 and 12 were recently reported in *P. cauliflora* fruits (Romão et al., 2019).

The compound 5 showed characteristic UV spectrum of anthocyanin (λ_{max} = 280 and 512 nm), while the compounds 13, 14, 15 and 17–18 showed characteristic UV spectra of flavonols (λ_{max} ≈ 265 and 355 nm) (Markham, 1982). The ions at *m/z* 447.0909 [M-H]$^-$ and 449.1072 $[M+H]^+$ from **5** confirmed the molecular formula $C_{21}H_{21}O_{11}{}^+$. The fragment ions at *m/z* 285 and 287, yielded from the loss of 162 *u*, indicate a hexosyl substituent. Thus, the compound **5** was identified as *O*-hexosyl cyanidin.

The substances **13**, **14** and **15** exihibited intense ions at *m/z* 463.0890/463.0899/463.0897 compatible to molecular formula $C_{21}H_{20}O_{12}$. The fragment ions at *m/z* 301, relative to quercetin aglycone, were observed from **14** and **15** and they were yielded from loss of 162 *u* and indicated the hexosyl substituent. While, the loss of deoxyhexosyl (146 *u*) from **13** yielded the fragment ion *m/z* 319 compatible to myricetin aglycone. Thus, **14/15** and **13** were identified as *O*-hexosyl quercetin and *O*-deoxyhexosyl myricetin. The compounds **17** (*m/z* 433.0797 [M-H]$^-$ and 435.0956 $[M+H]^+$, $C_{20}H_{18}O_{11}$) and **18** (*m/z* 447.0924 [M-H]$^-$, $C_{21}H_{20}O_{11}{}^-$ and 449.1081 $[M+H]^+$, $C_{21}H_{20}O_{11}$) yielded the fragment ions at *m/z* 303 [quercetin + H]$^+$ and 300 [quercetin-H]$^{-\cdot}$. These fragment ions suggested losses relative to pentosyl (132 *u*) and deoxyhexosyl (146 *u*) groups. Thus, the compounds **17** and **18** were identified as *O*-pentosyl quercetin and *O*-deoxyhexosyl quercetin.

3.2. Acute oral toxicity

No signs of toxicity or deaths were observed in animals that were treated with SEIPC or vehicle. Acute treatment did not cause any behavioral abnormalities, including excitation (i.e., convulsions, tremor, straub tail, hyperactivity, jumping, increases in fear/startle, increases in reactivity to touch, increases in abdominal muscle tone, or aggression), stereotypy (i.e., head-twitches, head movements, chewing, sniffing, or scratching), motor activity (i.e., catalepsy, akinesia, rolling, tip-toe, motor incoordination, loss of traction, or loss of grasping ability), sedation (i.e., hypoactivity, decreases in fear/startle, decreases in reactivity to touch, or decreases in abdominal muscle tone), pain (i.e., writhing or analgesia), autonomic activity (i.e., ptosis, exophtalmia, myosis, mydriasis, piloerection, defecation/diarrhea, salivation, or lacrimation), or other parameters (i.e., respiration; Table 2). No alterations of vital organ weight (Table 3) were observed in any of the animals that received SEIPC or vehicle.

3.3. Toxicity and genotoxicity studies after repeated oral treatment for 28 days

3.3.1. Food and water consumption and body temperature

Treatment with SEIPC for 28 days did not cause perturbations in thermoregulation homeostasis at any of the doses tested, either in male or female rats (Fig. 2). Food and water consumption were not different between the SEIPC and vehicle groups (data not shown).

3.3.2. Electrocardiography

As shown in Table 4, we did not observe any differences in ECG data

Table 2
Effects of acute treatment with SEIPC in female Wistar rats on behaviors and clinical signals observed in Irwin modified test.

Treatment		Saline 5 ml/kg														SEIPC 2000 mg/kg													
Observation Time (Days)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Death		Non observed														Non observed													
Excitation	Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Straub tail	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Increased activity	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Jumping	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Increased fear/startle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Increased reactivity to touch	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Increased abdominal muscle tone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Aggression	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stereotypy	Head-twitches	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Head movements	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Chewing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sniffing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Scratching	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Motor	Catalepsy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Akinesia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Rolling	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Tiptoe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Motor incoordination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Loss of traction	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	Loss of grasping	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sedation	Decreased activity	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	Decreased fear/startle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Decreased reactivity to touch	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Decreased abdominal muscle tone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pain	Writhing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Analgesia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Autonomic	Ptosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Exophthalmia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Myosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Mydriasis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Piloerection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Defecation/diarrhea	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Salivation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Lacrimation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Others	Increased respiration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Decreased respiration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

between groups. The P, PR, QRS, QT, and QTc segments and P, Q, R, S, and T waves were unaltered by the treatments.

3.3.3. Respiratory rate and arterial blood gas

In this assay, we evaluated several acid-base and respiratory homeostasis elements: respiratory rate, pH, pCO_2 (mmHg), pO_2 (mmHg), SO_2 (%), Hct (%), tHb (g/dl), Na^+ (mmol/L), K^+ (mmol/L), Ca^{++} (mmol/L), Cl^- (mmol/L), AnGap (mmol/L), mOsm (mOsm/kg), glucose (mg/dl), lactate (mmol/L), $_fO_2Hb$ (%), $_fHHb$ (%), $_fCOHb$ (%), $_fMetHb$ (%), base excess (B) (mmol/L), base excess-extracellular fluid (mmol/L), HCO_3^- actual (mmol/L), and $ctCO_2$ (mmol/L). We did not observe any differences among these arterial blood gas parameters in male or female Wistar rats (Tables 5 and 6).

3.3.4. Arterial pressure measurement

Basal systolic blood pressure, diastolic blood pressure, and mean arterial pressure that were recorded for 5 min after the 15-min stabilization period were 123.60 ± 10.77 mmHg, 73.39 ± 8.86 mmHg, and 97.67 ± 10.37 mmHg, respectively, in naive animals. Oral SEIPC administration did not significantly alter systolic blood pressure, diastolic blood pressure, or mean arterial pressure compared with the control group. Heart rate was not significantly different between groups (Table 7).

3.3.5. Serum biochemical analyses

The serum biochemical results from animals that were treated with SEIPC or vehicle for 28 days are shown in Table 8. The rats that were treated with SEIPC exhibited no significant changes compared with naive animals.

3.3.6. Hematological parameters

As shown in Table 9, none of the hematological parameters were significantly different between groups.

3.3.7. Relative organ weight and histopathological analysis

Vital and reproductive organs that were analyzed after treatment for

Table 3
Body weight (g) and relative weight (%) of vital organs of female Wistar rats orally treated with SEIPC 2000 mg/kg and vehicle (naive group) for acute oral toxicity protocol.

Groups	Body weight	Liver	Heart	Kidney	Lung	Spleen
Naive	217.40 ± 3.969	3.83 ± 0.309	0.275 ± 0,009	0.285 ± 0.008	0.473 ± 0.023	0.241 ± 0.008
SEIPC 2000 mg/kg	219.4 ± 3.171	3.48 ± 0.06	0.279 ± 0,009	0.274 ± 0.009	0.445 ± 0.010	0.224 ± 0.008

Values are expressed as mean ± SEM (standard error of the mean). n = 10. Statistical analyses were performed using unpaired *t-test*.

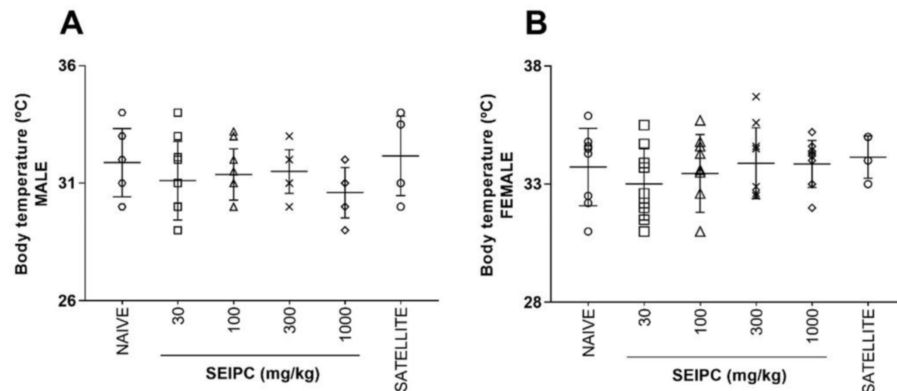

Fig. 2. Effect of 28-day oral administration of SEIPC in different doses on body temperature of male (A) and female (B) Wistar rats. Statistical analyses were performed using one-way ANOVA followed by Dunnett post hoc test. The results are expressed as mean ± standard error of the mean (S.E.M.) and p-value of less than 0.05 was considered statistically significant. SEIPC: Soluble ethanolic infuse of *Plinia* cauliflora; SATELLITE: animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity.

28 days did not exhibit alterations of absolute or relative weight (Table 14) or significant histological changes. Representative photomicrographs of each evaluated tissue from the experimental groups are presented in suplemmentary material (Fig. S1 a-f and Fig. S2 a-f).

3.3.8. Comet assay

A significant increase in the DNA damage index in male and female rats in the cyclophosphamide group was observed compared with the naive and SEIPC groups (Table 10). The DNA damage index and damage frequency in the SEIPC group were similar to the naive group, indicating that SEIPC did not cause genotoxic effects.

3.3.9. Micronucleus test

The mean values of micronucleated polychromatic erythrocytes are presented in Table 11. Only rats in the positive control (cyclophosphamide) group exhibited a significant increase in the number of micronucleated polychromatic erythrocytes compared with the naive group and SEIPC group at all doses tested.

Table 4
Effects on electrocardiogram (ECG) of male and female Wistar rats treated with SEIPC for 28 days.

	MALE					
	NAIVE	SEIPC 30 mg/kg	SEIPC 100 mg/kg	SEIPC 300 mg/kg	SEIPC 1000 mg/kg	SATELLITE
SEGMENTS (ms)						
P	33.80 ± 1.42	33.67 ± 1.64	33.43 ± 1.41	31.50 ± 0.78	35.71 ± 3.13	34.10 ± 2.84
PR	46.50 ± 3.00	48.30 ± 1.85	46.89 ± 1.06	44.86 ± 2.18	45.00 ± 1.16	49.71 ± 2.98
QRS	36.30 ± 1.05	36.90 ± 0.95	35.44 ± 1.37	38.29 ± 1.82	34.00 ± 0.80	37.43 ± 1.13
QT	74.50 ± 3.04	84.50 ± 1.88	78.89 ± 2.89	78.43 ± 1.60	69.00 ± 4.24	85.86 ± 4.78
QTc	144.60 ± 8.09	157.90 ± 6.72	148.90 ± 5.75	156.30 ± 5.15	138.50 ± 10.22	174.60 ± 13.02
WAVES-Amp (mV)						
P	0.041 ± 0.011	0.085 ± 0.0040	0.062 ± 0.015	0.076 ± 0.003	0.070 ± 0.004	0.073 ± 0.010
Q	-0.016 ± 0.003	-0.022 ± 0.003	-0.019 ± 0.002	-0.020 ± 0.003	-0.015 ± 0.002	-0.018 ± 0.003
R	0.280 ± 0.024	0.300 ± 0.034	0.278 ± 0.020	0.360 ± 0.023	0.255 ± 0.018	0.298 ± 0.013
S	0.077 ± 0.022	0.086 ± 0.011	0.063 ± 0.008	0.076 ± 0.020	0.064 ± 0.006	0.060 ± 0.012
T	0.122 ± 0.018	0.178 ± 0.021	0.174 ± 0.023	0.167 ± 0.016	0.117 ± 0.008	0.104 ± 0.011
FEMALE						
SEGMENTS (ms)						
P	35.44 ± 2.74	36.89 ± 4.67	37.13 ± 4.18	35.50 ± 1.45	34.88 ± 2.77	36.86 ± 3.96
PR	48.89 ± 3.82	50.22 ± 6.39	56.63 ± 7.04	47.88 ± 3.27	45.88 ± 2.86	55.00 ± 4.00
QRS	41.00 ± 1.23	42.00 ± 1.95	39.88 ± 1.12	39.50 ± 1.77	41.13 ± 2.15	38.29 ± 0.83
QT	93.00 ± 3.12	84.11 ± 3.14	86.13 ± 1.85	86.63 ± 3.17	83.75 ± 3.83	84.57 ± 3.48
QTc	173.40 ± 6.43	149.2 ± 8.23	153.1 ± 8.24	142.00 ± 21.03	150.30 ± 7.19	143.00 ± 9.42
WAVES-Amp (mV)						
P	0.091 ± 0.012	0.059 ± 0.012	0.058 ± 0.008	0.065 ± 0.007	0.061 ± 0.007	0.069 ± 0.009
Q	-0.026 ± 0.004	-0.023 ± 0.003	-0.015 ± 0.003	-0.028 ± 0.004	-0.020 ± 0.004	-0.015 ± 0.003
R	0.394 ± 0.040	0.353 ± 0.036	0.380 ± 0.040	0.345 ± 0.029	0.331 ± 0.014	0.387 ± 0.027
S	0.046 ± 0.008	0.073 ± 0.019	0.042 ± 0.012	0.056 ± 0.010	0.051 ± 0.012	0.063 ± 0.005
T	0.159 ± 0.033	0.155 ± 0.036	0.212 ± 0.015	0.193 ± 0.016	0.181 ± 0.014	0.171 ± 0.009

Statistical analyses were performed using one-way ANOVA followed by Dunnett post hoc test. The results are expressed as mean ± standard error of the mean (S.E.M.). A p-value of less than 0.05 was considered statistically significant. SEIPC: Soluble ethanolic infuse of *Plinia* cauliflora; SATELLITE: animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity. WAVE-Amp (wave amplitude).

Table 5
Effects of the prolonged (28-day) treatment with SEIPC on respiratory rate, blood gases, electrolytes, and metabolites parameters of male Wistar rats.

	NAIVE	SEIPC 30 mg/kg	SEIPC 100 mg/kg	SEIPC 300 mg/kg	SEIPC 1000 mg/kg	SATELLITE
Respiratory rate	91.10 ± 4.89	88.00 ± 3.14	92.50 ± 4.02	90.90 ± 4.22	88.20 ± 3.17	93.00 ± 4.08
Blood gases						
pH	7.32 ± 0.02	7.32 ± 0.02	7.36 ± 0.01	7.37 ± 0.02	7.32 ± 0.04	7.22 ± 0.01
pCO_2 (mmHg)	50.03 ± 3.48	44.87 ± 2.52	44.85 ± 2.21	41.05 ± 3.90	49.82 ± 7.05	51.83 ± 5.30
pO_2 (mmHg)	119.20 ± 4.21	119.50 ± 4.52	132.80 ± 2.62	129.10 ± 3.44	117.60 ± 7.00	135.10 ± 4.45
SO_2 (%)	94.54 ± 0.52	94.38 ± 0.49	96.51 ± 0.72	94.18 ± 1.10	93.74 ± 1.08	93.66 ± 1.11
Hct (%)	46.50 ± 1.66	47.50 ± 1.46	48.44 ± 0.76	49.13 ± 1.17	43.60 ± 2.94	48.67 ± 0.76
tHb (g/dL)	15.80 ± 0.57	16.56 ± 0.31	16.54 ± 0.26	16.70 ± 0.38	14.84 ± 1.02	16.80 ± 0.32
Electrolytes						
Na^+ (mmol/L)	134.10 ± 0.51	135.10 ± 1.14	133.30 ± 0.75	133.40 ± 0.33	134.20 ± 1.23	135.60 ± 1.28
K^+ (mmol/L)	5.89 ± 0.19	5.64 ± 0.17	5.92 ± 0.26	5.90 ± 0.24	5.49 ± 0.10	5.83 ± 0.26
Ca^{++} (mmol/L)	1.29 ± 0.01	1.31 ± 0.01	1.34 ± 0.02	1.30 ± 0.01	1.35 ± 0.02	1.27 ± 0.01
Cl^- (mmol/L)	99.75 ± 0.79	101.90 ± 1.10	100.30 ± 0.64	100.00 ± 0.62	101.20 ± 1.35	102.60 ± 0.68
AnGap (mmol/L)	14.42 ± 0.79	16.02 ± 1.04	14.47 ± 0.62	15.66 ± 0.50	14.34 ± 0.62	17.08 ± 0.58
mOsm (mOsm/kg)	286.00 ± 1.28	286.60 ± 1.50	283.40 ± 1.91	284.10 ± 2.04	286.30 ± 1.03	279.70 ± 2.67
Metabolites						
Glucose (mg/dL)	320.80 ± 16.69	295.70 ± 29.73	292.30 ± 28.43	352.60 ± 25.02	323.20 ± 41.91	159.20 ± 9.95
Lactate (mmol/L)	2.76 ± 0.24	2.42 ± 0.12	2.48 ± 0.05	2.43 ± 0.10	2.34 ± 0.13	2.03 ± 0.20
CO-Oximetry						
fO_2Hb (%)	94.00 ± 0.46	93.97 ± 0.51	93.35 ± 0.25	93.79 ± 1.11	93.32 ± 1.09	93.23 ± 1.10
*f*HHb (%)	5.42 ± 0.44	5.62 ± 0.49	4.26 ± 0.23	5.80 ± 1.08	6.26 ± 1.08	6.75 ± 1.51
*f*COHb (%)	0.16 ± 0.01	0.15 ± 0.01	0.14 ± 0.02	0.15 ± 0.02	0.16 ± 0.02	0.14 ± 0.02
*f*MetHb (%)	0.28 ± 0.01	0.26 ± 0.01	0.25 ± 0.01	0.26 ± 0.01	0.26 ± 0.02	0.30 ± 0.00
Calculated values						
BE (B) (mmol/L)	-1.60 ± 0.90	-2.38 ± 1.39	-0.97 ± 0.60	-2.34 ± 0.43	-2.20 ± 0.35	-2.97 ± 0.89
BE_{ecf} (mmol/L)	-0.96 ± 0.94	-2.19 ± 1.40	-0.67 ± 0.53	-2.80 ± 0.60	-1.94 ± 0.80	-1.57 ± 0.76
$HCO_3^-{}_{act}$ (mmol/L)	25.02 ± 0.77	22.79 ± 0.84	24.52 ± 0.36	22.69 ± 0.77	24.18 ± 1.38	23.28 ± 0.43
$_{ct}CO_2$ (mmol/L)	26.55 ± 0.81	24.14 ± 0.86	25.89 ± 0.39	23.91 ± 0.39	25.70 ± 1.60	23.98 ± 0.40

Statistical analyses were performed using one-way ANOVA followed by Dunnett post hoc test. The results are expressed as mean ± standard error of the mean (S.E.M.). A p-value of less than 0.05 was considered statistically significant. SEIPC: Soluble ethanolic infuse of *Plinia cauliflora*; SATELLITE: animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity. pH: potential of Hydrogen; pCO_2: partial pressure of carbon dioxide; SO_2: level of hemoglobin-saturation by oxygen; tHb: hemoglobin; Na^+: sodium; K^+: potassium; Ca^{++}: calcium; Cl^-: chloride; AnGap: anionic interval; mOsm: Osmolar gap; $_fO_2Hb$: fraction of oxygenated hemoglobin in relation to the total of all hemoglobin's present; $_f$HHb: fraction of unoxygenated hemoglobin to total hemoglobin; $_f$COHb: carboxyhemoglobin; $_f$MetHb: methemoglobin; BE (B): base excess; BE_{ecf}: base excess in the extracellular fluid compartment; HCO_3^-: bicarbonate concentration; $_{ct}CO_2$ (P): concentration of total carbon dioxide in plasma.

3.4. Endocrine disruption assays

3.4.1. Uterotrophic test

No significant differences in the relative empty uterus weight were observed in animals that were treated with 30 and 300 mg/kg SEIPC compared with the negative control (vehicle) group. Female rats that were treated with ethynyl estradiol exhibited a significant increase in the relative empty uterus weight compared with the negative control group (Fig. 3). All of the female rats were healthy, with no signs of toxicity during the experimental period (data not shown).

3.4.2. Hershberger bioassay

The animals that received testosterone as a positive control for androgenicity exhibited an increase in relative weights of the seminal vesicles, glans penis, and LABC compared with the control (vehicle + canola oil) group. Similarly, animals in the positive control (flutamide + testosterone) group for antiandrogenicity exhibited a significant decrease in the relative weights of these organs compared with the positive control (water + testosterone) group. The weight of androgenic-dependent organs in the groups that were treated with 30 and 300 mg/kg SEIPC combined with testosterone was not significantly different from the positive control (water + testosterone) group. Thus, no significant changes in relative organ weights were observed in animals that were treated with 30 and 300 mg/kg SEIPC (combined with canola oil) compared with the negative control (water + canola oil) group (Table 12).

3.5. Ames assay

None of the tested doses of SEIPC (50–5000 μg/plate) in the presence and absence of exogenous metabolization significantly increased the number of revertant colonies of the *S. typhimurium* TA98 and TA100 strains. All of the doses presented a mutagenicity index > 2, indicating that the samples did not have the potential to cause frameshift and base-pair substitution mutations (Table 13).

4. Discussion

Although experiments that evaluate the effectiveness of possible therapeutic agents are conducted appropriately and carefully, experimental protocols that are used to evaluate possible risks that are associated with the use of these agents do not always follow the same rigor because of either an inability to perform such studies or conflicts of interest (Lüde et al., 2016). Toxicological studies seek to provide data to support or refute the use of drugs and determine whether they are damaging or safe to various organs and bodily systems (Valentin and Hammond, 2008). Among the protocols that are performed in preclinical studies, we highlight those that evaluate acute and prolonged toxicity, genotoxicity, cytotoxicity, and pharmacological safety with prolonged use of the different compounds (Pugsley et al., 2008).

Natural products are often wrongly assumed to be safe for indiscriminate use (Lüde et al., 2016). Since time immemorial, humans have known about the existence of compounds that are harmful to health as herbal preparations. In classic Greek antiquity, *Conium maculatum* L. (a plant species that contains cicutin, a neurotoxic alkaloid with similar effects as curare) was used to execute those who were condemned to the death penalty. The most notable of such deaths was the execution of Socrates in 399 B.C. (Hotti and Rischer, 2017).

Acute toxicity tests are performed to determine the LD_{50} of a given compound. The LD_{50} is the main acute toxicity test that is performed.

Table 6
Effects of the prolonged (28-day) treatment with SEIPC on respiratory rate, blood gases, electrolytes, and metabolites parameters of female Wistar rats.

	NAIVE	SEIPC 30 mg/kg	SEIPC 100 mg/kg	SEIPC 300 mg/kg	SEIPC 1000 mg/kg	SATELLITE
Respiratory rate	83.17 ± 3.56	85.00 ± 1.91	81.83 ± 2.67	84.67 ± 1.73	85.17 ± 1.50	81.67 ± 2.16
Blood gases						
pH	7.27 ± 0.02	7.280 ± 0.030	7.28 ± 0.03	7.29 ± 0.030	7.29 ± 0.02	7.27 ± 0.03
pCO_2 (mmHg)	55.41 ± 3.26	53.450 ± 4.110	53.34 ± 3.75	52.56 ± 4.98	50.61 ± 3.60	53.04 ± 5.18
pO_2 (mmHg)	121.80 ± 3.18	126.100 ± 9.010	127.70 ± 9.91	125.90 ± 8.68	137.70 ± 1.35	119.30 ± 4.24
SO_2 (%)	94.09 ± 0.53	94.280 ± 0.720	93.92 ± 1.15	94.39 ± 0.72	94.00 ± 0.92	93.60 ± 0.87
Hct (%)	45.11 ± 1.50	44.200 ± 0.820	45.44 ± 0.70	44.20 ± 1.04	45.11 ± 0.97	46.14 ± 1.24
tHb (g/dL)	15.36 ± 0.50	15.060 ± 0.270	15.34 ± 0.26	14.86 ± 0.43	15.31 ± 0.34	15.70 ± 0.40
Electrolytes						
Na^+ (mmol/L)	132.00 ± 1.04	133.900 ± 0.970	132.10 ± 1.50	134.00 ± 1.10	133.70 ± 1.53	130.60 ± 1.70
K^+ (mmol/L)	4.60 ± 0.24	4.800 ± 0.350	5.14 ± 0.16	4.64 ± 0.21	4.70 ± 0.15	5.54 ± 0.40
Ca^{++} (mmol/L)	1.32 ± 0.01	1.330 ± 0.010	1.33 ± 0.01	1.31 ± 0.02	1.32 ± 0.02	1.33 ± 0.02
Cl^- (mmol/L)	100.00 ± 0.90	101.400 ± 0.900	100.60 ± 1.12	101.20 ± 0.80	101.30 ± 1.20	99.860 ± 1.03
AnGap (mmol/L)	12.03 ± 0.82	12.920 ± 0.580	12.13 ± 1.01	13.54 ± 0.68	13.50 ± 1.30	14.000 ± 1.21
mOsm (mOsm/kg)	278.00 ± 2.46	281.700 ± 2.180	280.50 ± 3.78	284.20 ± 2.44	280.30 ± 2.42	276.00 ± 3.13
Metabolites						
Glucose (mg/dL)	253.40 ± 25.30	252.000 ± 22.720	240.20 ± 20.12	252.50 ± 22.50	212.60 ± 35.40	265.60 ± 25.44
Lactate (mmol/L)	2.380 ± 0.30	2.310 ± 0.260	2.25 ± 0.16	2.40 ± 0.35	2.44 ± 0.340	1.900 ± 0.09
CO-Oximetry						
fO_2Hb (%)	93.68 ± 0.54	93.880 ± 0.720	93.53 ± 1.17	93.97 ± 0.73	93.56 ± 0.92	93.20 ± 0.88
$fHHb$ (%)	5.91 ± 0.53	5.710 ± 0.710	6.06 ± 1.14	5.62 ± 0.72	5.99 ± 0.93	6.340 ± 0.86
$fCOHb$ (%)	0.13 ± 0.01	0.160 ± 0.020	0.12 ± 0.03	0.17 ± 0.01	0.16 ± 0.02	0.140 ± 0.02
$fMetHb$ (%)	0.27 ± 0.01	0.250 ± 0.010	0.27 ± 0.01	0.24 ± 0.01	0.30 ± 0.01	0.270 ± 0.02
Calculated values						
BE (B) (mmol/L)	-3.22 ± 0.74	-2.87 ± 0.67	-2.83 ± 0.75	-3.250 ± 0.470	-3.26 ± 1.01	-4.13 ± 0.66
BE_{ecf} (mmol/L)	-2.36 ± 0.67	-2.05 ± 0.80	-2.10 ± 0.72	-2.670 ± 0.460	-2.76 ± 1.40	-3.53 ± 0.66
$HCO_3^-{}_{act}$ (mmol/L)	24.53 ± 0.44	24.46 ± 0.88	24.53 ± 0.68	23.900 ± 0.740	23.63 ± 1.38	23.31 ± 0.84
${}_{ct}CO_2$ (mmol/L)	26.24 ± 0.46	26.08 ± 0.98	26.19 ± 0.75	25.490 ± 0.870	25.13 ± 1.48	24.96 ± 0.98

Statistical analyses were performed using one-way ANOVA followed by Dunnett post hoc test. The results are expressed as mean ± standard error of the mean (S.E.M.). A p-value of less than 0.05 was considered statistically significant. SEIPC: Soluble ethanolic infuse of *Plinia cauliflora*; SATELLITE: animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity. pH: potential of Hydrogen; pCO_2: partial pressure of carbon dioxide; SO_2: level of hemoglobin-saturation by oxygen; tHb: hemoglobin; Na^+: sodium; K^+: potassium; Ca^{++}: calcium; Cl^-: chloride; AnGap: anionic interval; mOsm: Osmolar gap; ${}_fO_2Hb$: fraction of oxygenated hemoglobin in relation to the total of all hemoglobin's present; ${}_fHHb$: fraction of unoxygenated hemoglobin to total hemoglobin; ${}_fCOHb$: carboxyhemoglobin; ${}_fMetHb$: methemoglobin; BE (B): base excess; BE_{ecf}: base excess in the extracellular fluid compartment; HCO_3^-: bicarbonate concentration; ${}_{ct}CO_2$ (P): concentration of total carbon dioxide in plasma.

Table 7
Hemodynamics parameters of male and female Wistar rats treated with SEIPC 30, 100, 300 and 1000 mg/kg and vehicle (drinking water).

PARAMETERS	NAIVE	SEIPC 30 mg/kg	SEIPC 100 mg/kg	SEIPC 300 mg/kg	SEIPC 1000 mg/kg	SATELLITE
MALE						
SBP (mm Hg)	123.60 ± 10.77	140.80 ± 5.63	130.90 ± 5.08	136.4 ± 6.53	136.40 ± 17.33	123.10 ± 5.42
DBP (mm Hg)	73.39 ± 8.86	85.21 ± 4.63	73.61 ± 6.94	78.92 ± 6.57	73.31 ± 9.64	80.07 ± 3.03
MAP (mm Hg)	97.67 ± 10.37	112.20 ± 5.04	102.90 ± 6.32	107.70 ± 6.23	104.90 ± 13.55	100.30 ± 4.01
HR (BPM)	322.60 ± 22.38	326.40 ± 21.12	361.70 ± 10.54	320.50 ± 29.00	340.00 ± 31.35	287.00 ± 22.03
FEMALE						
SBP (mm Hg)	114.60 ± 8.02	119.10 ± 5.38	110.20 ± 6.82	111.6 ± 6.292	103.50 ± 4.63	119.10 ± 5.38
DBP (mm Hg)	68.71 ± 5.25	64.98 ± 2.55	61.47 ± 4.36	65.35 ± 4.469	59.34 ± 3.54	64.98 ± 2.55
MAP (mm Hg)	90.92 ± 6.42	91.88 ± 3.37	87.51 ± 5.76	88.06 ± 5.468	81.35 ± 4.28	91.88 ± 3.37
HR (BPM)	248.10 ± 19.21	273.20 ± 20.06	257.10 ± 22.66	274 ± 21.03	257.30 ± 32.07	273.20 ± 20.06

Statistical analyses were performed using one-way ANOVA followed by Dunnett post hoc test. The results are expressed as mean ± standard error of the mean (S.E.M.). A p-value of less than 0.05 was considered statistically significant. SEIPC: Soluble ethanolic infuse of *Plinia cauliflora*; SATELLITE: animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity. SBP: Systolic blood pressure, DBP: Diastolic blood pressure, MAP: Mean arterial pressure, HR: Heart rate.

Acute toxicity studies seek to characterize dose/response relationships that lead to calculation of the LD_{50}. This parameter represents the statistical probability that a specific dose causes a lethal effect in 50% of the animals in a population. Such a calculation is useful for identifying the relative toxicity of various substances (Erhirhie et al., 2018).

Acute toxicity tests are intended to simulate a single administration or possible accidental ingestion of a compound or drug. Prolonged toxicity protocols simulate the use of a drug by patients who are afflicted with chronic diseases, such as diabetes, arterial hypertension, and atherosclerosis, among others. Prolonged toxicity tests provide data on possible changes in target organs and changes in indicators of corporeal homeostasis, including biochemical and hematological profiles, body temperature, ECG, respiratory rate, arterial blood gas, and arterial blood pressure (Pugsley et al., 2008; Roberts et al., 2015).

The hemodynamic profile is one of the least explored areas in safety evaluations of natural products. Although effects on blood pressure have been systematically evaluated, cardioelectric activity patterns that are observed after treatment with different natural products are not commonly determined (Calapai et al., 1999; Joukar, 2012; Joukar and Mahdavi, 2014). Some studies have demonstrated the ability of some drugs to affect the duration of cardiac action potential. Some natural products are classic blockers of sodium channels (e.g., quinidine) in cardiac muscles (Bozic et al., 2018; Yang et al., 2009). Several drugs can induce prolongation of the Q-T interval and may initiate ventricular arrhythmias (Fermini and Fossa, 2003). Any preparation that is intended for prolonged use should be investigated with regard to

Table 8
Effects of prolonged (28-day) treatment with SEIPC on serum biochemical parameters of male and female Wistar rats.

	NAIVE	SEIPC 30 mg/kg	SEIPC 100 mg/kg	SEIPC 300 mg/kg	SEIPC 1000 mg/kg	SATELLITE
MALE						
Sodium (mmol/L)	149.44 ± 2.29	148.14 ± 1.96	149.36 ± 1.51	151.06 ± 4.19	149.95 ± 3.39	138.00 ± 0.43
Potassium (mmol/L)	6.40 ± 0.31	6.47 ± 0.22	6.58 ± 0.28	6.08 ± 0.28	6.43 ± 0.32	5.29 ± 0.30
Calcium (mg/dL)	11.41 ± 0.31	11.16 ± 0.29	11.18 ± 0.49	11.88 ± 0.40	11.075 ± 0.43	10.87 ± 0.07
Chloride (mmol/L)	109.64 ± 1.90	109.3 ± 1.78	109.84 ± 0.95	110.17 ± 3.18	110.45 ± 2.93	101.74 ± 0.92
Total cholesterol (mg/dL)	62.4 ± 2.926	66.53 ± 5.52	68.45 ± 5.95	65.48 ± 5.55	81.55 ± 15.93	66.84 ± 10.48
Triglycerides (mg/dL)	45.52 ± 4.71	44.14 ± 4.29	57.80 ± 9.56	45.56 ± 5.86	59.85 ± 13.00	66.96 ± 6.04
Total protein (g/dL)	6.48 ± 0.12	6.48 ± 0.14	6.55 ± 0.17	6.24 ± 0.11	6.27 ± 0.09	6.76 ± 0.17
Albumin (g/dL)	3.91 ± 0.24	4.16 ± 0.15	4.19 ± 0.10	4.16 ± 0.16	3.74 ± 0.26	4.18 ± 0.13
AST (U/L)	196.18 ± 46.29	172.86 ± 26.64	153.76 ± 10.40	180.86 ± 23.94	180.86 ± 29.12	127.85 ± 22.71
ALT (U/L)	74.54 ± 7.79	61.02 ± 2.30	80.52 ± 9.58	68.27 ± 4.94	74.25 ± 17.23	50.61 ± 5.00
LDH (U/L)	488.82 ± 188.71	285.78 ± 51.72	490.61 ± 81.37	367.44 ± 60.60	316.63 ± 23.01	404.42 ± 101.28
BUN (mg/dL)	60.22 ± 2.90	71.56 ± 2.50	62.31 ± 3.29	63.20 ± 2.67	61.90 ± 5.91	54.35 ± 1.29
Creatinine (mg/dL)	0.39 ± 0.01	0.40 ± 0.02	0.42 ± 0.02	0.43 ± 0.02	0.40 ± 0.02	0.38 ± 0.01
Magnesium (mg/dL)	2.89 ± 0.10	3.03 ± 0.16	3.18 ± 0.09	3.15 ± 0.14	3.15 ± 0.14	2.63 ± 0.07
FEMALE						
Sodium (mmol/L)	142.30 ± 1.86	148.30 ± 2.18	144.81 ± 5.65	147.94 ± 2.27	146.26 ± 2.16	148.09 ± 1.68
Potassium (mmol/L)	6.69 ± 0.31	6.90 ± 0.27	5.82 ± 0.34	7.04 ± 0.35	7.24 ± 0.24	7.28 ± 0.35
Calcium (mg/dL)	12.61 ± 0.34	12.32 ± 0.37	12.24 ± 0.60	13.12 ± 0.45	12.44 ± 0.34	10.34 ± 1.76
Chloride (mmol/L)	106.30 ± 2.28	106.73 ± 1.91	107.82 ± 1.0594	108.05 ± 3.12	108.54 ± 1.89	101.02 ± 1.34
Total cholesterol (mg/dL)	73.64 ± 4.28	74.51 ± 4.55	73.00 ± 4.89	65.48 ± 5.55	88.87 ± 7.30	82.12 ± 8.43
HDL (mg/dL)	47.60 ± 4.55	42.31 ± 2.13	45.01 ± 5.12	47.72 ± 6.29	53.31 ± 8.10	54.93 ± 8.27
Triglycerides (mg/dL)	7.13 ± 0.13	7.07 ± 0.17	7.23 ± 0.21	6.87 ± 0.12	7.03 ± 0.13	7.44 ± 0.19
Total protein (g/dL)	4.30 ± 0.27	4.58 ± 0.16	4.61 ± 0.11	4.58 ± 0.18	4.11 ± 0.28	4.59 ± 0.14
Albumin (g/dL)	155.31 ± 25.34	163.63 ± 28.29	153.91 ± 12.02	181.07 ± 23.97	189.20 ± 22.42	128.01 ± 22.74
AST (U/L)	67.07 ± 11.29	49.64 ± 4.90	73.11 ± 7.64	60.30 ± 2.51	81.98 ± 10.10	66.96 ± 4.85
ALT (U/L)	332.84 ± 56.10	357.08 ± 45.80	474.16 ± 183.05	255.17 ± 33.69	489.85 ± 88.08	392.29 ± 98.24
LDH (U/L)	59.68 ± 1.67	58.81 ± 2.39	56.83 ± 3.36	62.83 ± 2.84	62.46 ± 8.17	58.20 ± 2.56
BUN (mg/dL)	0.34 ± 0.01	0.36 ± 0.01	0.37 ± 0.01	0.39 ± 0.01	0.38 ± 0.02	0.38 ± 0.01
Creatinine (mg/dL)	2.84 ± 0.06	3.09 ± 0.15	3.05 ± 0.10	3.22 ± 0.11	3.29 ± 0.19	3.53 ± 0.91
Magnesium (mg/dL)	142.30 ± 1.86	148.30 ± 2.18	144.81 ± 5.65	147.94 ± 2.27	146.26 ± 2.16	148.09 ± 1.68

Statistical analyses were performed using one-way ANOVA followed by Dunnett post hoc test. The results are expressed as mean ± standard error of the mean (S.E.M.). A p-value of less than 0.05 was considered statistically significant. SEIPC: Soluble ethanolic infuse of *Plinia* cauliflora; SATELLITE: animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity; HDL: high density lipoprotein, GGT: gamma-glutamyl transpeptidase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, BUN: blood urea nitrogen.

cardiovascular safety. In the present study, ECG components in SEIPC-treated animals did not show variations, including parameters that integrate the cardiac cycle, such as atrial depolarization, ventricular depolarization, and ventricular repolarization. The conduction time of electrical impulses (from its origin in the sinus node until reaching the sinoatrial node and then its migration to the cardiac apex) was also unaffected. These findings indicate that no pathological events occurred that acted on electrical conduction or cardiac function.

Another factor that is crucial for the dynamic balance of organisms is body temperature. In humans, a normal body temperature is considered 37 °C (98.6 °F). The body's ability to maintain this stable temperature is called thermoregulation, which is one of many physiological reactions that comprise an organism's homeostasis. Abnormal body temperature can be classified into three categories. Hypothermia is characterized by rectal temperature below 35 °C (95 °F). Pyrexia is characterized by oral temperature above 37.5 °C (99.5 °F) or rectal temperature above 38 °C (100.5 °F). Hyperpyrexia is characterized by body temperature above 41.1 °C (106 °F; Geneva et al., 2019). Endocrine abnormalities, such as low levels of thyroid hormones (Gustafson, 2015), pancreatic disorders (e.g., diabetes), pituitary gland problems (Doberentz and Madea, 2017), and adrenal insufficiency (Francisco and Minson, 2018), in addition to antipsychotic medicines (Zonnenberg et al., 2017), can lower body temperature. Some substances, called pyrogens, can significantly raise body temperature. Antibiotics (Labbus et al., 2018), opioids (Rawls and Benamar, 2011), and antihistamines (Lundius et al., 2010) are examples of pyrogens. If this increase in body temperature is sustained for a prolonged period of time, then enzymatic-protein denaturation can occur, which can be life-threatening (Walter et al., 2016). In the present study, the prolonged administration of SEIPC did not alter body temperature or influence thermoregulation.

In addition to thermoregulation, the acid-base balance is important for ideal biological function. Arterial blood gas analysis provides valuable information about homeostasis and the acid-base balance. Arterial blood gas is composed of several parameters that are sensitive to the smallest changes that can occur in the organism (Hamm et al., 2015). Arterial blood gas parameters include serum levels of blood gases, electrolytes, metabolites, and CO oximetry. Low oxygen saturation or respiratory acidosis is considered an important indicator of systemic toxicity (Burns, 2014; Pompey and Abraham-Settles, 2019). SEIPC treatment for 28 days did not affect the acid-base balance or its components.

Biological organisms must maintain an accurate and delicate balance among biochemical reactions. This balance is called homeostasis (Modell et al., 2015). Any variations or breakdown of homeostasis generates a biological trail that can be traced to determine its origin and relative importance (Bas et al., 2015; Christmann et al., 2016; Lin et al., 2011). In the present study, we found no evidence of deleterious effects of 28 days of oral treatment with SEIPC at different doses.

Other biological markers that assist in monitoring mammalian homeostasis are hematological parameters (Cluitmans et al., 2016; Zhang and Enns, 2009). In addition to measurements of different cells in blood (e.g., leukocytes, erythrocytes, and platelets), hematological assessments also include the presence of hematimetric indices, specific counts for each type of leukocyte, and derived platelet data. Changes in red blood cell count or hematimetric indices can reveal issues with bone marrow function (Fliedner et al., 2002), such as anemia (Ishii and Young, 2015). Likewise, erythrocytes, leukocytes and platelets may reflect deficits in bone marrow activity or problems with cellular defense systems and even coagulopathies. Such failures can be caused by drugs or compounds that interfere with hematopoiesis (Risitano et al.,

Table 9
Effects of prolonged (28-day) treatment with SEIPC on hematological parameters of male and female Wistar rats.

PARAMETERS	NAIVE	SEIPC 30 mg/kg	SEIPC 100 mg/kg	SEIPC 300 mg/kg	SEIPC 1000 mg/kg	SATELLITE
MALE						
WBC (10^3/μL)	8.800 ± 1.060	8.600 ± 0.970	6.970 ± 0.600	11.000 ± 1.570	11.630 ± 2.970	7.120 ± 0.930
RBC (10^4/μL)	8.720 ± 0.260	8.840 ± 0.220	8.940 ± 0.180	8.800 ± 0.150	8.100 ± 0.570	9.01 ± 0.300
Hemoglobin (g/dL)	14.340 ± 0.400	15.430 ± 0.260	15.210 ± 0.300	14.900 ± 0.250	13.700 ± 0.930	15.320 ± 0.360
Hematocrit (%)	44.240 ± 1.520	46.110 ± 1.130	46.510 ± 0.960	45.750 ± 0.750	41.780 ± 2.850	47.560 ± 1.720
MCV (fL)	50.660 ± 0.370	51.730 ± 0.250	51.710 ± 0.270	51.830 ± 0.220	51.620 ± 0.680	52.220 ± 0.340
MCH (pg)	16.470 ± 0.160	16.880 ± 0.070	16.850 ± 0.140	16.870 ± 0.070	16.920 ± 0.180	16.810 ± 0.050
MCHC (g/dL)	32.500 ± 0.310	32.750 ± 0.150	32.710 ± 0.130	32.550 ± 0.190	32.800 ± 0.110	32.20 ± 0.210
Platelets (10^3/μL)	769.600 ± 26.920	740.400 ± 19.390	714.100 ± 18.860	743.900 ± 19.120	739.800 ± 31.580	801.000 ± 34.410
RDW (%)	16.680 ± 0.470	16.430 ± 0.480	16.890 ± 0.380	16.780 ± 0.250	16.380 ± 0.710	18.370 ± 0.460
MPV (fL)	7.560 ± 0.110	7.600 ± 0.080	7.530 ± 0.110	7.670 ± 0.140	7.740 ± 0.240	7.430 ± 0.070
Neutrophil (10^3/μL)	0.870 ± 0.150	1.000 ± 0.120	0.810 ± 0.090	0.850 ± 0.130	1.130 ± 0.320	0.740 ± 0.190
Lymphocyte (10^3/μL)	8.260 ± 1.160	7.120 ± 0.720	7.060 ± 0.830	8.890 ± 1.580	9.500 ± 2.120	6.270 ± 0.840
Monocyte (10^3/μL)	0.070 ± 0.040	0.020 ± 0.010	0.070 ± 0.030	0.020 ± 0.004	0.010 ± 0.005	0.010 ± 0.004
Eosinophil (10^3/μL)	0.070 ± 0.010	0.080 ± 0.010	0.070 ± 0.010	0.100 ± 0.030	0.100 ± 0.010	0.070 ± 0.010
Basophil (10^3/μL)	0.010 ± 0.005	0.020 ± 0.003	0.010 ± 0.004	0.030 ± 0.010	0.020 ± 0.010	0.020 ± 0.005
FEMALE						
WBC (10^3/μL)	11.120 ± 1.570	10.020 ± 1.100	10.460 ± 1.260	8.360 ± 1.120	7.060 ± 0.680	11.120 ± 1.570
RBC (10^4/μL)	8.240 ± 0.160	8.490 ± 0.160	8.210 ± 0.220	7.950 ± 0.220	8.010 ± 0.150	8.240 ± 0.160
Hemoglobin (g/dL)	14.230 ± 0.250	15.100 ± 0.260	14.200 ± 0.310	14.420 ± 0.320	14.230 ± 0.280	14.230 ± 0.250
Hematocrit (%)	44.250 ± 0.900	46.050 ± 0.490	43.880 ± 1.180	44.130 ± 0.940	43.630 ± 0.870	44.250 ± 0.900
MCV (fL)	53.700 ± 0.540	55.200 ± 0.700	53.960 ± 0.370	55.250 ± 0.460	54.400 ± 0.470	53.700 ± 0.540
MCH (pg)	53.700 ± 0.540	55.200 ± 0.690	53.960 ± 0.370	55.250 ± 0.460	54.400 ± 0.470	53.700 ± 0.540
MCHC (g/dL)	32.160 ± 0.160	32.230 ± 0.280	32.090 ± 0.150	32.540 ± 0.250	32.630 ± 0.150	32.160 ± 0.160
Platelets (10^3/μL)	882.600 ± 27.870	854.600 ± 27.420	870.500 ± 20.360	820.700 ± 31.980	888.400 ± 24.070	882.600 ± 27.870
RDW (%)	13.840 ± 0.190	15.260 ± 0.870	13.590 ± 0.330	14.140 ± 0.700	14.440 ± 0.340	13.840 ± 0.190
MPV (fL)	7.390 ± 0.090	7.270 ± 0.030	7.440 ± 0.090	7.630 ± 0.150	7.600 ± 0.070	7.390 ± 0.090
Neutrophil (10^3/μL)	0.500 ± 0.080	0.640 ± 0.050	0.600 ± 0.080	0.460 ± 0.070	0.360 ± 0.050	0.500 ± 0.080
Lymphocyte (10^3/μL)	10.030 ± 1.430	9.140 ± 1.000	9.140 ± 1.000	7.740 ± 1.10	6.600 ± 0.600	10.030 ± 1.430
Monocyte (10^3/μL)	0.290 ± 0.200	0.020 ± 0.003	0.070 ± 0.040	0.080 ± 0.030	0.040 ± 0.030	0.290 ± 0.200
Eosinophil (10^3/μL)	0.060 ± 0.010	0.080 ± 0.010	0.070 ± 0.010	0.050 ± 0.010	0.030 ± 0.010	0.060 ± 0.010
Basophil (10^3/μL)	0.030 ± 0.004	0.010 ± 0.002	0.020 ± 0.010	0.020 ± 0.006	0.020 ± 0.010	0.030 ± 0.004

Statistical analyses were performed using one-way ANOVA followed by Dunnett post hoc test. The results are expressed as mean ± standard error of the mean (S.E.M.) and p-value of less than 0.05 was considered statistically significant. SEIPC: Soluble ethanolic infuse of *Plinia* cauliflora; SATELLITE: animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity; WBC: White blood cells; RBC: red blood cells; MCV: Mean corpuscular volume; MHC: Mean Corpuscular Hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red blood cell distribution width; MPV: Mean platelet volume.

2007). Antiinflammatory drugs (e.g., ibuprofen and naproxen), antibiotics (e.g., sulfa antibiotics and β-lactams), anticonvulsants (e.g., carbamazepine and phenytoin), and antipsychotics (e.g., haloperidol) can cause thrombocytopenia (Bakchoul and Marini, 2018). Chloramphenicol, an antibiotic that is widely used in clinical practice, can suppress bone marrow activity, causing not only leukopenia but also pancytopenia (Cho et al., 2019). In the present study, 28 days of oral SEIPC treatment did not exert any harmful hematological effects in either male or female Wistar rats.

Some substances that are considered safe may not have overt toxic

Table 10
Frequency of damage by the comet assay in rats treated orally for 28 days with SEIPC.

Groups	Injured Cells	Classes of damage				Score
		0	1	2	3	
Male						
Naive	4.78 ± 0.88[a]	94.62 ± 0.65	4.12 ± 0.95	0.87 ± 0.40	0.37 ± 0.26	6.22 ± 0.97[a]
Cyclophosphamide	72.80 ± 1.46[b]	27.20 ± 1.46	37.60 ± 1.63	22.00 ± 2.55	13.20 ± 1.80	121.20 ± 2.45[b]
SEIPC 30 mg/kg	5.50 ± 1.17 [a]	94.50 ± 1.17	3.50 ± 0.76	1.50 ± 0.72	0.50 ± 0.34	8.00 ± 1.96 [a]
SEIPC 100 mg/kg	4.87 ± 0.72[a]	95.00 ± 0.70	2.25 ± 0.70	1.25 ± 0.84	1.57 ± 0.65	8.87 ± 1.75[a]
SEIPC 300 mg/kg	5.75 ± 0.86[a]	94.37 ± 0.77	3.00 ± 0.38	1.25 ± 0.56	1.50 ± 0.50	10.00 ± 2.23[a]
SEIPC 1000 mg/kg	6.00 ± 1.22[a]	94.00 ± 1.22	2.20 ± 1.02	2.20 ± 0.80	1.60 ± 0.81	11.40 ± 3.12[a]
SATELLITE	6.28 ± 0.89[a]	93.71 ± 0.89	4.28 ± 0.81	1.57 ± 0.29	0.43 ± 0.43	8.71 ± 1.66[a]
Female						
Naive	3.89 ± 0.63[a]	96.11 ± 0.63	3.22 ± 0.44	0.66 ± 0.23	0.00 ± 0.00	4.55 ± 0.85[a]
Cyclophosphamide	71.20 ± 1.68[b]	28.80 ± 1.68	35.40 ± 2.96	28.40 ± 1.88	7.40 ± 2.13	114.40 ± 3.95[b]
SEIPC 30 mg/kg	4.60 ± 0.91 [a]	95.40 ± 0.91	3.70 ± 0.74	0.90 ± 0.27	0.00 ± 0.00	5.50 ± 1.12 [a]
SEIPC 100 mg/kg	4.66 ± 0.64[a]	95.33 ± 0.64	3.33 ± 0.68	0.77 ± 0.27	0.55 ± 0.37	6.55 ± 0.88[a]
SEIPC 300 mg/kg	5.30 ± 0.65[a]	94.70 ± 0.65	3.10 ± 0.43	1.20 ± 0.39	1.00 ± 0.33	8.50 ± 1.28[a]
SEIPC 1000 mg/kg	7.25 ± 0.62[a]	92.75 ± 0.62	4.75 ± 0.75	2.00 ± 0.82	0.50 ± 0.26	9.75 ± 1.53[a]
SATELLITE	4.43 ± 1.17[a]	95.57 ± 1.17	3.28 ± 1.08	0.57 ± 0.29	0.57 ± 0.37	6.14 ± 1.42[a]

Values expressed as mean ± SEM. n = 10 animals/group. Different letters indicate statistically significant differences ($p < 0.05$; ANOVA/Dunnett). [b] Significantly different from naive and treatments groups. Class of damage 0: no damage, Class of damage 1: tail of comet shorter than the diameter of nucleoid, Class of damage 2: tail of comet once or twice the diameter of nucleoid; Class of damage 3: tail of comet more than twice the diameter of nucleoid. SEIPC: Soluble ethanolic infuse of *Plinia* cauliflora; SATELLITE: animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity.

Table 11
Micronucleated polychromatic erythrocytes and the ratio between polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) in rats treated orally for 28 days with different doses of SEIPC.

Experimental groups	Micronucleus		PCE/NCE	
	Female	Male	Female	Male
Naive	8.55 ± 1.53[a]	9.22 ± 0.62[a]	0.48 ± 0.06[b]	0.48 ± 0.06[b]
Cyclophosphamide	27.60 ± 2.42[b]	28.20 ± 3.21[b]	0.14 ± 0.03[a]	0.14 ± 0.03[a]
SEIPC 30 mg/kg	7.90 ± 0.58[a]	10.00 ± 1.63[a]	0.51 ± 0.06 [b]	0.51 ± 0.06[b]
SEIPC 100 mg/kg	8.89 ± 1.29 [a]	11.25 ± 0.75[a]	0.47 ± 0.07 [b]	0.47 ± 0.07[b]
SEIPC 300 mg/kg	7.90 ± 1.22 [a]	11.28 ± 1.19[a]	0.51 ± 0.04 [b]	0.51 ± 0.04[b]
SEIPC 1000 mg/kg	9.00 ± 1.31[a]	12.80 ± 0.58[a]	0.53 ± 0.09[b]	0.53 ± 0.09[b]
SATELLITE	8.85 ± 1.59 [a]	8.50 ± 1.31[a]	0.56 ± 0.05 [b]	0.56 ± 0.05[b]

Values expressed as mean ± SEM. Different letters indicate statistically significant differences ($p < 0.05$; ANOVA/Dunnett). [a] Naive; [b] Cyclophosphamide. SEIPC: Soluble ethanolic infuse of *Plinia* cauliflora; SATELLITE: animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity.

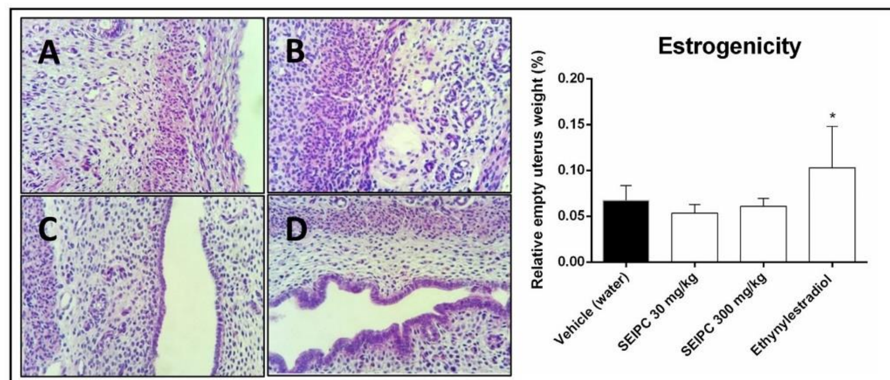

Fig. 3. Photomicrograph showing the uterine cornua of the animals tested in the uterotrophic protocol. The pictures show no alterations between all the experimental groups. Positive Control (A), Negative Control (B), SEIPC-30 mg/kg (C) and SEIPC-300 mg/kg (D). Relative empty uterus weight (%) from female rats treated orally from PND 22 to PND 24 with SEIPC 30 and 300 mg/kg for 3 days. The negative control group received the vehicle (filtered water) alone and the positive control group received ethynylestradiol (3 μg/kg). Bars represent the mean ± S.E.M (n = 8). * Represents significantly different from controls ($p < 0.05$) by ANOVA followed by Dunnett's test.

Table 12
Body weight (g) and relative organ weight (%) of pubertal castrated male rats after 10 days of treatment in the Hershberger assay.

	Experimental groups						
Parameters	Vehicle + Oil	Water + Test	Flut + Test	SEIPC 30 + Test	SEIPC 300 + Test	SEIPC 30 + Oil	SEIPC 300 + Oil
Body weight	327 ± 14.20	337 ± 20.40	342 ± 23.03	348 ± 35.39	336 ± 26.25	333 ± 10.70	318 ± 31.36
Seminal vesicle	0.011 ± 0.00	0.081 ± 0.01*	0.012 ± 0.00[#]	0.075 ± 0.00*	0.087 ± 0.00*	0.011 ± 0.00	0.012 ± 0.00
Glans penis	0.006 ± 0.00	0.018 ± 0.00*	0.007 ± 0.00[#]	0.041 ± 0.06*	0.019 ± 0.00*	0.005 ± 0.00	0.004 ± 0.00
LABC	0.068 ± 0.00	0.156 ± 0.01*	0.088 ± 0.01[#]	0.150 ± 0.01*	0.155 ± 0.01*	0.076 ± 0.01	0.074 ± 0.01

Data are expressed as mean ± SD of body weight before euthanasia (g) and relative (%) organ weights (n = 8 animals per group). *Significantly different from vehicle + oil ($p < 0.05$ ANOVA/Dunnett test); [#]Significantly different from water + test ($p < 0.05$ ANOVA/Dunnett test). Test: Testosterone (0.25 mg/kg/day); Flut: Flutamide (5 mg/kg/day); SEIPC: ethanol-soluble fraction of *Plinia cauliflora* (30 or 300 mg/kg/day); LABC: Levator ani/bulbocavernosus muscle.

or harmful effects macroscopically but instead act directly on cells that undergo rapid cell division and cause serious damage to biological systems. One example is doxorubicin, which is a natural product that is isolated from *Streptomyces peucetius* that causes myelosuppression and eliminates bone marrow stem cells by catalyzing ceramide glycosylation (Pugazhendhi et al., 2018). In the present study, the results of the Comet assay and micronucleus test indicated that 28 days of oral treatment with SEIPC did not cause any DNA damage in eukaryotic cells or genotoxic effects. Complementing these data, we also performed the Ames test to investigate the possible mutagenic activity of SEIPC. Compared with the negative control, with and without exogenous metabolic activation, the mutagenic index for all SEIPC concentrations tested (50, 150, 500, 1500, and 5000 μg/plate) indicated that SEIPC was inert and did not induce mutagenicity in *Salmonella typhimurium* strains. The histopathological data corroborated these findings, in which no significant morphofunctional changes were found between experimental groups.

Possible estrogenic and (anti)androgenic effects of SEIPC were evaluated in male and female Wistar rats using the uterotrophic and Hershberger assays, respectively. These tests reliably identify substances that can interact with the endocrine system (OECD, 2007; OECD, 2009). Our uterotrophic bioassay results demonstrated that SEIPC caused no estrogenic effects in immature female rats, in which the relative empty uterus weight in each group did not differ

Table 13
Mutagenic activity expressed by the mean of revertants/plate ± standard deviation and mutagenicity index **(MI)** of the sample against TA98 and TA100 strains of *Salmonella typhimurium* with metabolic activation (S +) and without metabolic (S-) activation.

	TA98		TA100	
Concentrations (μg/plaque)	S+	S-	S+	S-
0[a]	20.33 ± 1.24	23.33 ± 1.24	122.30 ± 1.88	111.33 ± 1.69
50	14.66 ± 0.88 **(0.72)**	17.77 ± 0.44 **(0.76)**	113.33 ± 1.11 **(0.92)**	104.66 ± 4.88 **(0.94)**
150	18.66 ± 0.88 **(0.91)**	17.00 ± 1.33 **(0.72)**	109.00 ± 6.00 **(0.89)**	105.00 ± 2.00 **(0.94)**
500	15.33 ± 0.44 **(0.75)**	18.20 ± 1.33 **(0.78)**	113.66 ± 13.77 **(0.92)**	104.00 ± 3.66 **(0.93)**
1500	17.00 ± 2.00 **(0.83)**	20.33 ± 2.22 **(0.87)**	105.00 ± 4.00 **(0.85)**	102.00 ± 5.33 **(0.91)**
5000	18.00 ± 1.33 **(0.88)**	20.66 ± 0.88 **(0.88)**	112.00 ± 13.33 **(0.91)**	106.50 ± 6.50 **(0.95)**
C+	321.00 ± 11.00[d]	1315.00 ± 8.00[b]	1338.00 ± 11.00[d]	1428.00 ± 6.00[c]

Values expressed as mean ± SEM. Different letters indicate statistically significant differences ($p < 0.05$; ANOVA/Tukey) from: [a]Distilled water; Positive control (C +): [b]4-nitro-o-phenylenediamine (10 μg/plaque); [c]Sodium azide (2.5 μg/plaque); [d]2AA-aminoanthracene (2.5 μg/plaque).

Table 14
Body weight (g) and relative weight (%) of vital organs of male and female Wistar rats orally treated with SEIPC (30. 100. 300 or 1000 mg/kg) or vehicle (naive group) for 28 days.

MALE	NAIVE	SEIPC 30 mg/kg	SEIPC 100 mg/kg	SEIPC 300 mg/kg	SEIPC 1000 mg/kg	SATELLITE
Body weight	350.000 ± 14.270	326.700 ± 11.480	360.400 ± 11.520	380.500 ± 6.173	404.600 ± 18.740	336.00 ± 13.980
Liver	3.020 ± 0.100	3.100 ± 0.090	2.750 ± 0.400	3.320 ± 0.120	3.080 ± 0.120	2.200 ± 0.040
Heart	0.270 ± 0.010	0.280 ± 0.010	0.270 ± 0.010	0.260 ± 0.010	0.260 ± 0.010	0.250 ± 0.010
Kidney	0.320 ± 0.010	0.350 ± 0.010	0.330 ± 0.010	0.340 ± 0.010	0.350 ± 0.020	0.280 ± 0.020
Lung	0.470 ± 0.030	0.450 ± 0.030	0.430 ± 0.020	0.420 ± 0.030	0.400 ± 0.010	0.420 ± 0.020
Spleen	0.190 ± 0.010	0.170 ± 0.010	0.190 ± 0.010	0.170 ± 0.005	0.180 ± 0.010	0.170 ± 0.010
Encephalon	0.570 ± 0.010	0.550 ± 0.020	0.570 ± 0.010	0.540 ± 0.010	0.500 ± 0.010	0.600 ± 0.020
Testicle	0.460 ± 0.020	0.420 ± 0.010	0.420 ± 0.010	0.400 ± 0.010	0.420 ± 0.020	0.420 ± 0.020
Adrenal gland	0.010 ± 0.001	0.020 ± 0.002	0.010 ± 0.002	0.020 ± 0.003	0.010 ± 0.000	0.010 ± 0.004
Epididymis	0.240 ± 0.010	0.210 ± 0.010	0.210 ± 0.010	0.200 ± 0.010	0.210 ± 0.010	0.190 ± 0.010
Prostate and Seminal vesicle	0.930 ± 0.040	0.950 ± 0.050	0.880 ± 0.040	0.890 ± 0.030	0.800 ± 0.030	0.780 ± 0.040

FEMALE	NAIVE	SEIPC 30 mg/kg	SEIPC 100 mg/kg	SEIPC 300 mg/kg	SEIPC 1000 mg/kg	SATELLITE
Body weigh	253.300 ± 8.070	220.050 ± 15.230	226.400 ± 8.550	221.300 ± 5.310	241.40 ± 3.206	259.400 ± 4.508
Liver	3.350 ± 0.120	3.570 ± 0.140	3.130 ± 0.050	3.380 ± 0.100	3.050 ± 0.180	3.120 ± 0.140
Heart	0.300 ± 0.010	0.310 ± 0.010	0.290 ± 0.010	0.300 ± 0.010	0.290 ± 0.010	0.300 ± 0.010
Kidney	0.350 ± 0.010	0.330 ± 0.010	0.310 ± 0.010	0.330 ± 0.010	0.320 ± 0.010	0.300 ± 0.006
Lung	0.470 ± 0.010	0.510 ± 0.010	0.500 ± 0.010	0.510 ± 0.020	0.480 ± 0.010	0.460 ± 0.020
Spleen	0.210 ± 0.010	0.230 ± 0.010	0.200 ± 0.010	0.200 ± 0.010	0.210 ± 0.010	0.200 ± 0.010
Encephalon	0.800 ± 0.020	0.850 ± 0.040	0.800 ± 0.010	0.820 ± 0.010	0.770 ± 0.010	0.710 ± 0.010
Adrenal gland	0.020 ± 0.003	0.020 ± 0.002	0.010 ± 0.001	0.070 ± 0.040	0.020 ± 0.001	0.020 ± 0.003
Right ovary	0.030 ± 0.003	0.030 ± 0.005	0.070 ± 0.030	0.030 ± 0.001	0.030 ± 0.003	0.030 ± 0.003
Left ovary	0.030 ± 0.002	0.030 ± 0.010	0.030 ± 0.003	0.030 ± 0.002	0.030 ± 0.001	0.030 ± 0.003
Uterus and cervix	0.270 ± 0.030	0.230 ± 0.030	0.250 ± 0.040	0.200 ± 0.010	0.250 ± 0.020	0.260 ± 0.030

Values are expressed as mean ± SEM (standard error of the mean). n = 10. Statistical analyses were performed using one-way ANOVA followed by Dunnett *post hoc* test.

significantly from the negative control group. We also found that SEIPC did not induce androgenic or antiandrogenic effects in male rats, in which it did not significantly alter the weight of androgen-dependent tissues compared with controls. We did not visualize or evaluate the ventral prostate or bulbourethral glands because some androgen-dependent organs tend to be atrophied after castration (Gu et al., 2016; Peters et al., 2010). Additionally, SEIPC treatment began 2 weeks after the castration procedure, and this length of time may have resulted in even more atrophy of these organs post-castration. Nonetheless, our treatment period was in accordance with OECD guideline 441 (2009), which states that treatment can start on PND 49 but not later than PND 60.

5. Conclusion

The present study found that an infusion of *Plinia cauliflora* fruit peels (SEIPC) did not cause any significant alterations in various corporeal systems, including cardiac electrical activity, body temperature, respiratory rate, and arterial pressure, and did not influence various biochemical, hematological, or blood gas parameters. Moreover, SEIPC did not cause any endocrine-disruptive, mutagenic, cytotoxic, or genotoxic effects. These findings support the safe clinical use of *P. cauliflora*.

Author's contributions

All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; RACP, BRL, LPG, PVMR, AAMM, APCRH, ELBL, SELT, GNS, TZC, MTP, PRD, FHSA and SAO conducted the experiments; DBS and SRN were involved with the preparation and chemical analysis of extract. RICS and ACS were responsible for histopathological analizes. RACP, SELT, KMPO, PFC and AGJ were responsible for data discussion and manuscript correction. AGJ was the senior researcher responsible for this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.112916.

Supplementary materials

Supplementary data associated with this article can be found in the online version.

0: absence of any changes in clinical signs; 1–2: slight changes in clinical signs; 3–4: moderate changes in clinical signs; 5–6: marked changes in clinical signs. SEIPC: Soluble ethanolic infuse of *Plinia cauliflora*; SATELLITE: animals treated for 28 days with SEIPC 1000 mg/kg with the purpose of evaluating possible late toxicity.

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7 CONCLUSÕES

Diante dos dados expostos, fica evidenciado que sobrenadante etanólico do infuso obtida das cascas de *Plinia cauliflora* (SEIPC) não causou alterações significativas nos sistemas biológicos avaliados, incluindo atividade cardioelétrica, temperatura corporal, frequência respiratória e pressão arterial.

Além disso, não houve alteração da homeostasia bioquímica e hematológica, ou de parâmetros de gasometria. Ademais, o SEIPC não causou qualquer desregulação endócrina, mutagênica, citotóxica ou genotóxica.

Com esses dados é possível afirmar que a avaliação da toxicidade e segurança do sobrenadante etanólico do infuso das cascas de *Plinia cauliflora* se mostrou seguro frente ao modelo de exposição utilizado.

8 ANEXOS

8.1 PARECER DE APROVAÇÃO DO COMITÊ DE ÉTICA

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 14 de novembro de 2018.

CERTIFICADO

Certificamos que a proposta intitulada ***"Avaliação dos efeitos antihipertensivo e antiaterosclerótico de Plinia cauliflora em ratas ovariectomizadas submetidas à um modelo experimental de aterosclerose associado à hipertensão renovascular."***, registrada sob o protocolo de nº 33/2018, sob a responsabilidade de *Arquimedes Gasparotto Junior* – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata, subfilo Vertebrata* (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 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 da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 09/11/2018.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*19/11/2018 a 10/01/2021*
Espécie/linhagem/raça	*Rattus norvegicus*
Nº de animais	144 *Wistar*
Peso/idade	90 dias
Sexo	Fêmeas
Origem	Biotério Central UFGD

Melissa Negrão Sepulvida

Melissa Negrão Sepulvida
Coordenadora CEUA

Comissão de Ética no Uso de Animais – CEUA/UFGD – Rua João Rosa Góes, 1761 – Vila Progresso. Dourados/MS. E-mail: ceua@ufgd.edu.br

Materiais suplementares

SUPPLEMENTAL INFORMATION
Appendix A

Figure S1. Photomicrographs showing the histology of different vital organs and reproductive structures of **male** Wistar rats that received SEIPC or vehicle (NAIVE) for 28 days. Letters a, b, c, d, e and f refers to NAIVE, SEIPC-30 mg/kg, SEIPC-100 mg/kg, SEIPC-300 mg/kg, SEIPC-1000 mg/kg and Satellite respectively. **SEIPC:** Soluble ethanolic infuse of *Plinia cauliflora*; **SATELLITE:** animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity. **HE:** hematoxylin & eosin.

Figure S2. Photomicrographs showing the histology of different vital organs and reproductive structures of **female** Wistar rats that received SEIPC or vehicle (NAIVE) for 28 days. Letters a, b, c, d, e and f refers to NAIVE, SEIPC-30 mg/kg, SEIPC-100 mg/kg, SEIPC-300 mg/kg, SEIPC-1000 mg/kg and Satellite respectively. **SEIPC:** Soluble ethanolic infuse of *Plinia cauliflora*; **SATELLITE:** animals treated for 28 days with SEIPC 1000 mg/kg and euthanized two weeks after completing the 28-day treatment with the purpose of evaluating possible late toxicity. **HE:** hematoxylin & eosin.

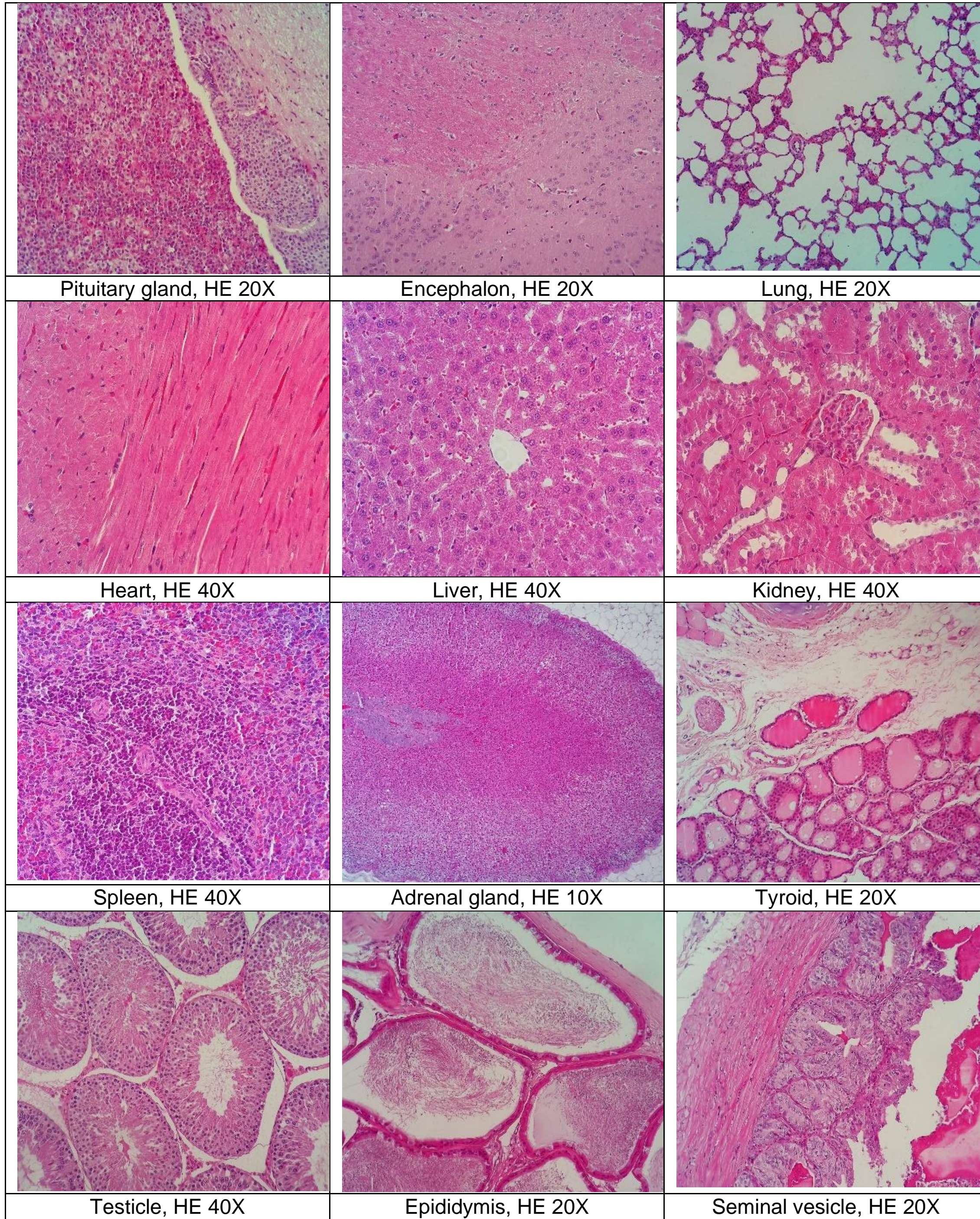

Figure 1 (a) NAIVE

Palozi et al.

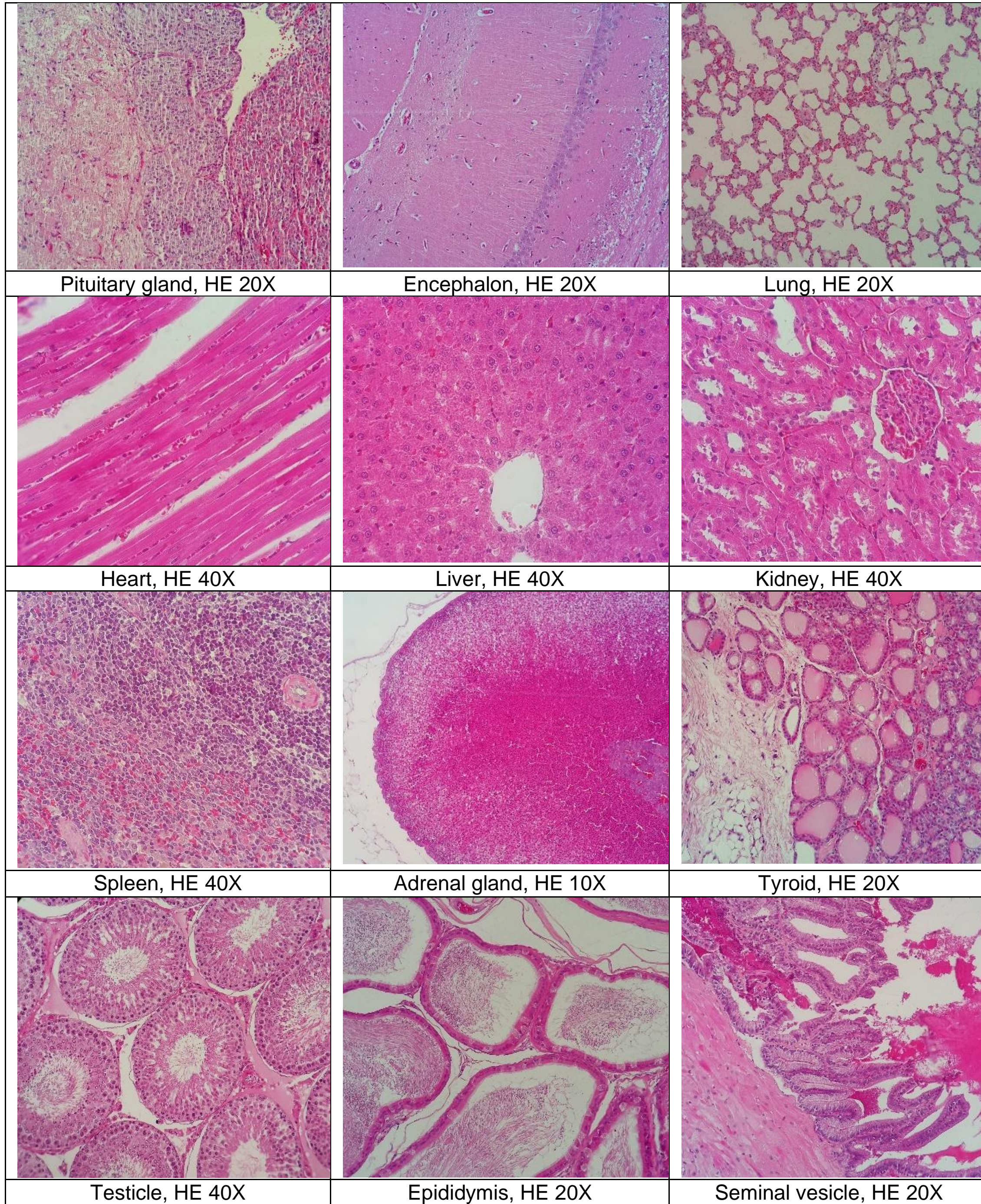

Figure 1 (b) SEIPC-30 mg/kg

Palozi et al.

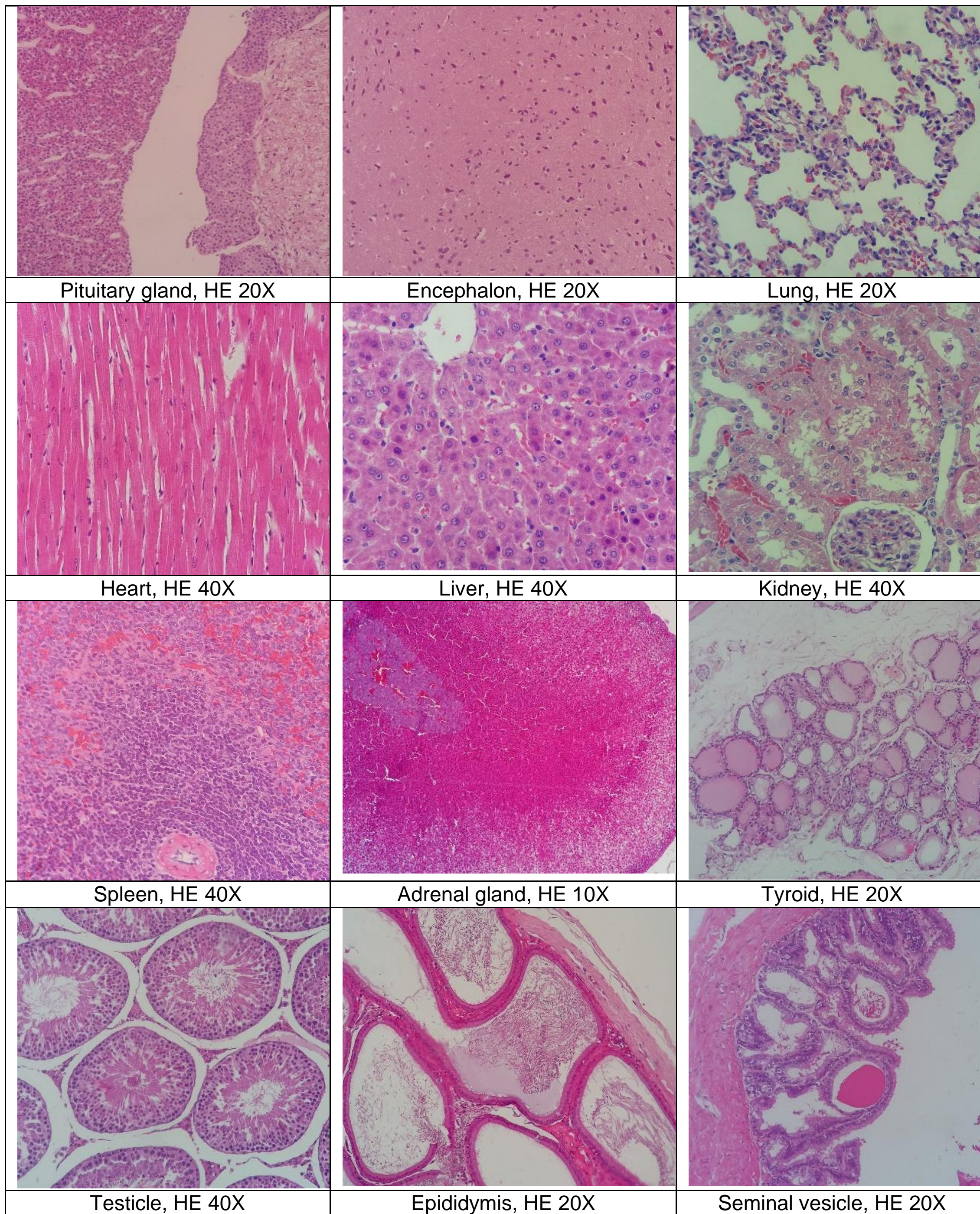

Figure 1 (c) SEIPC-100 mg/kg

Palozi et al.

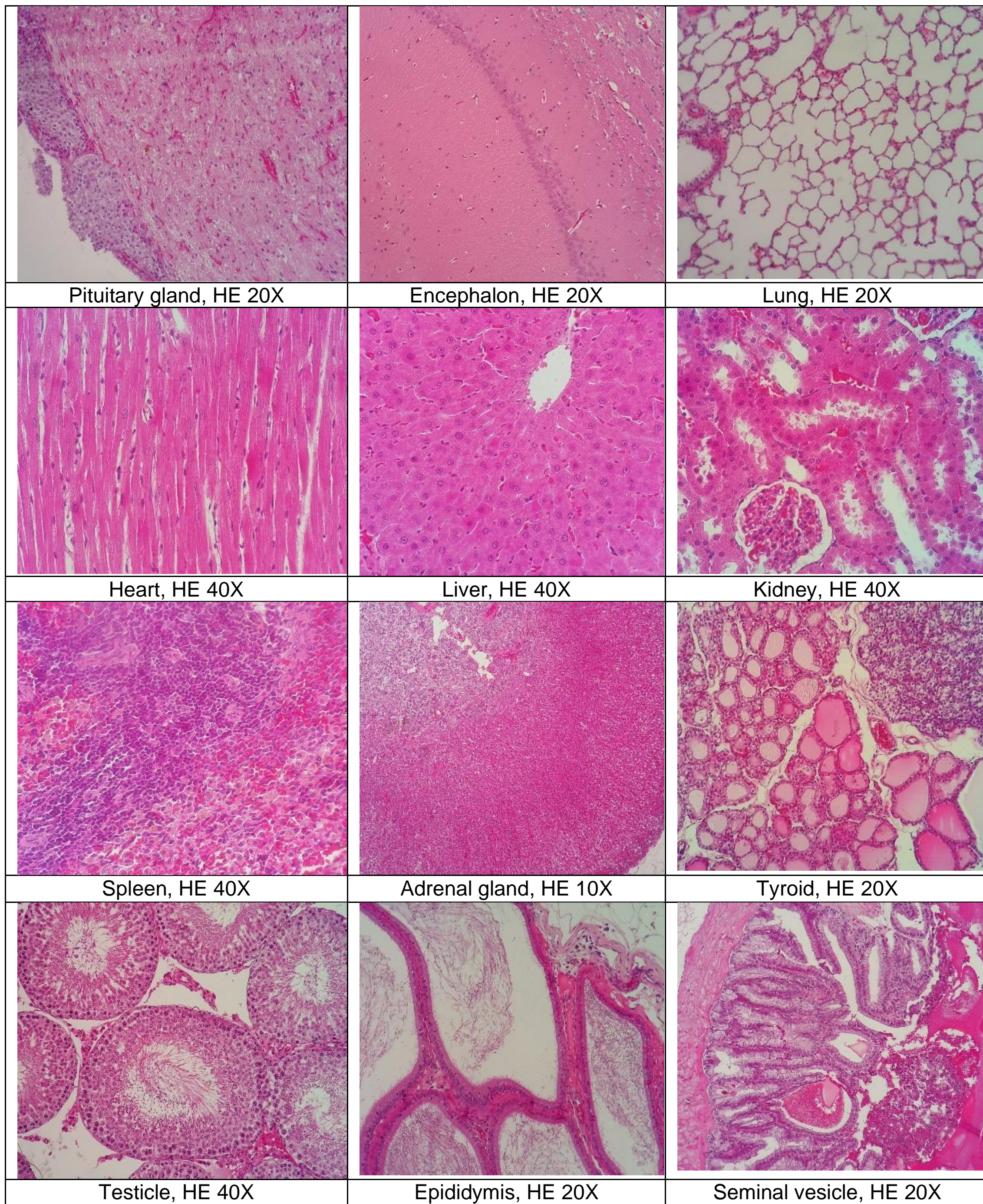

Figure 1 (d) SEIPC-300 mg/kg

Palozi et al.

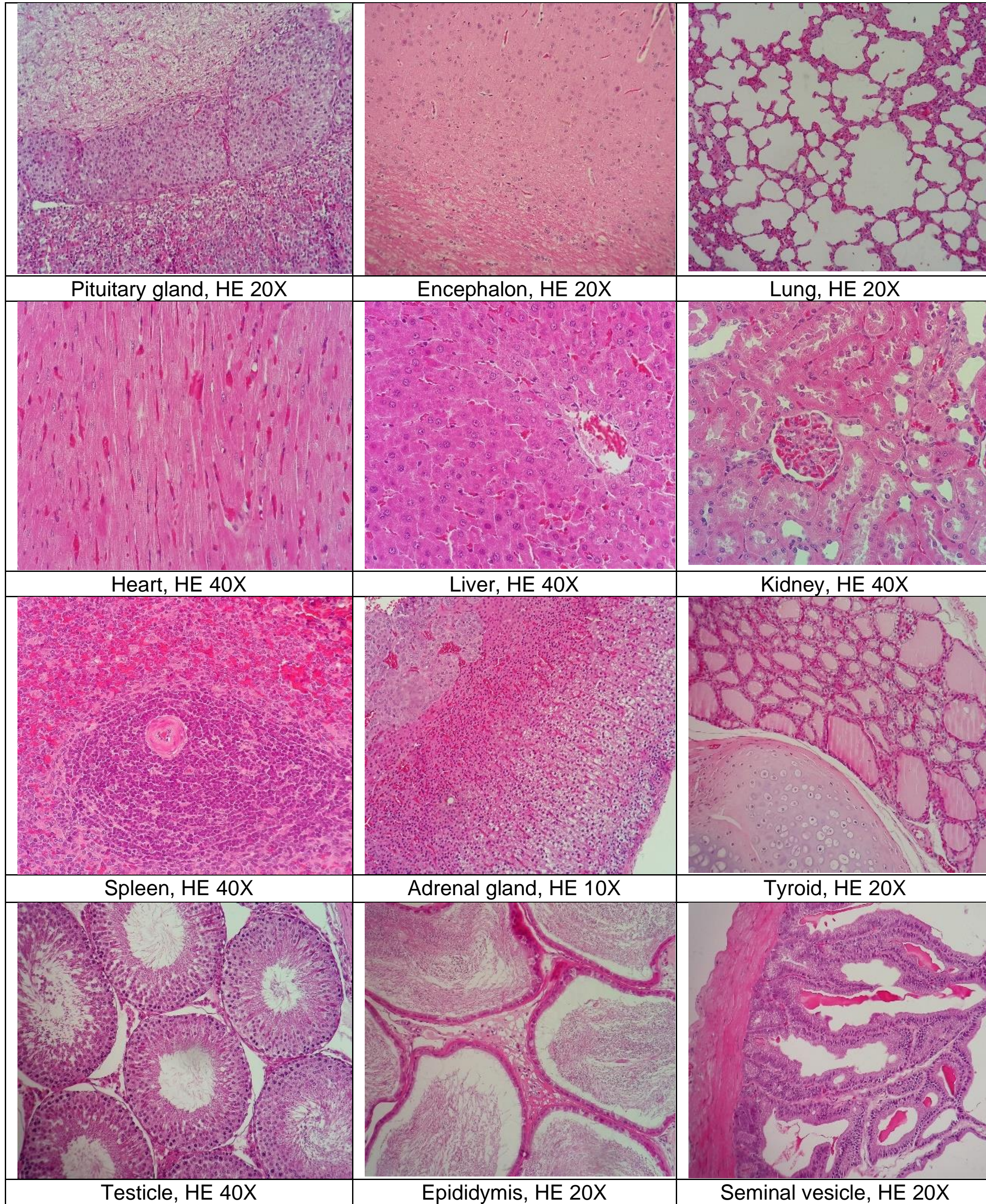

Figure 1 (e) SEIPC-1000 mg/kg

Palozi et al.

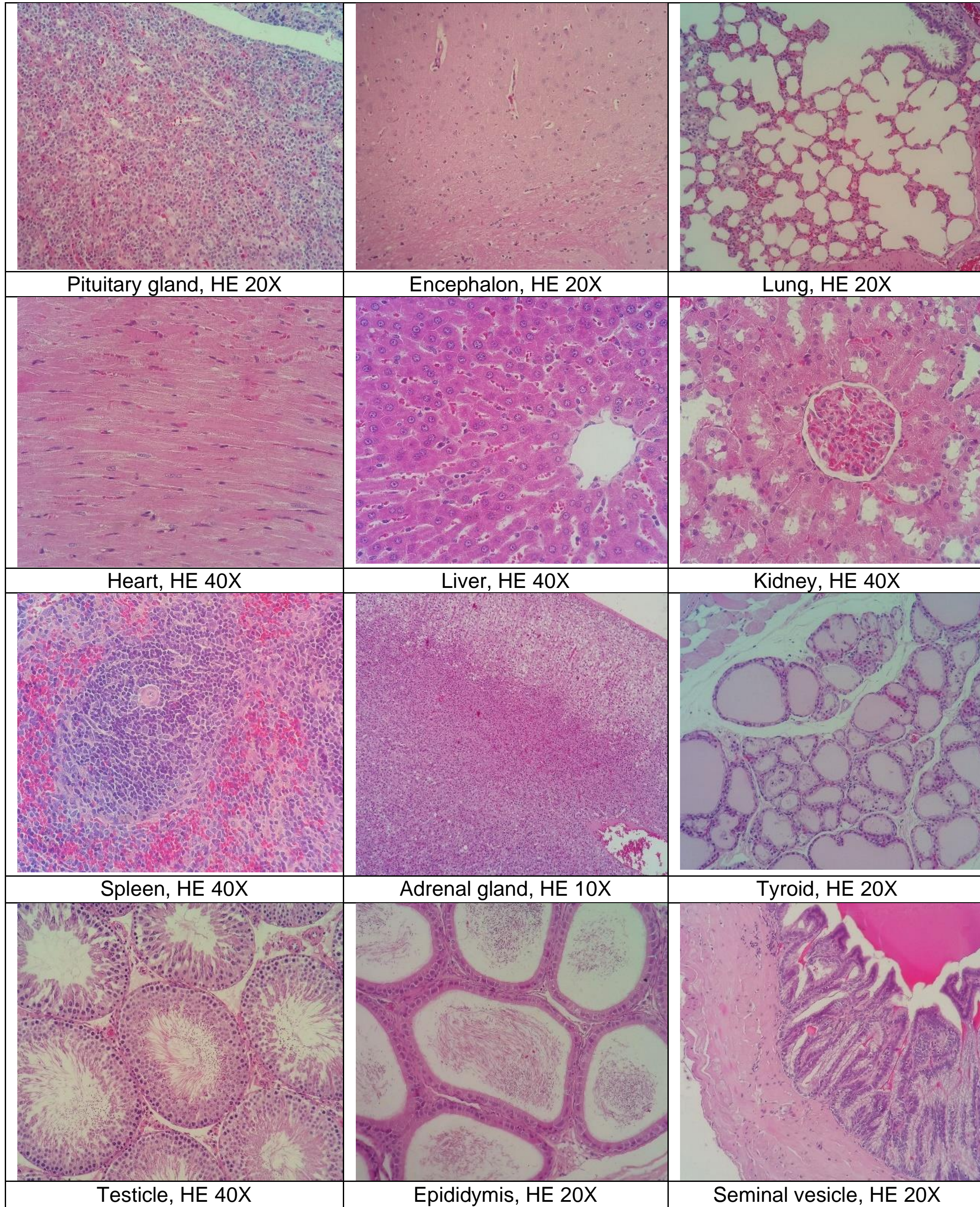

Figure 1 (f) Satellite

Palozi et al.

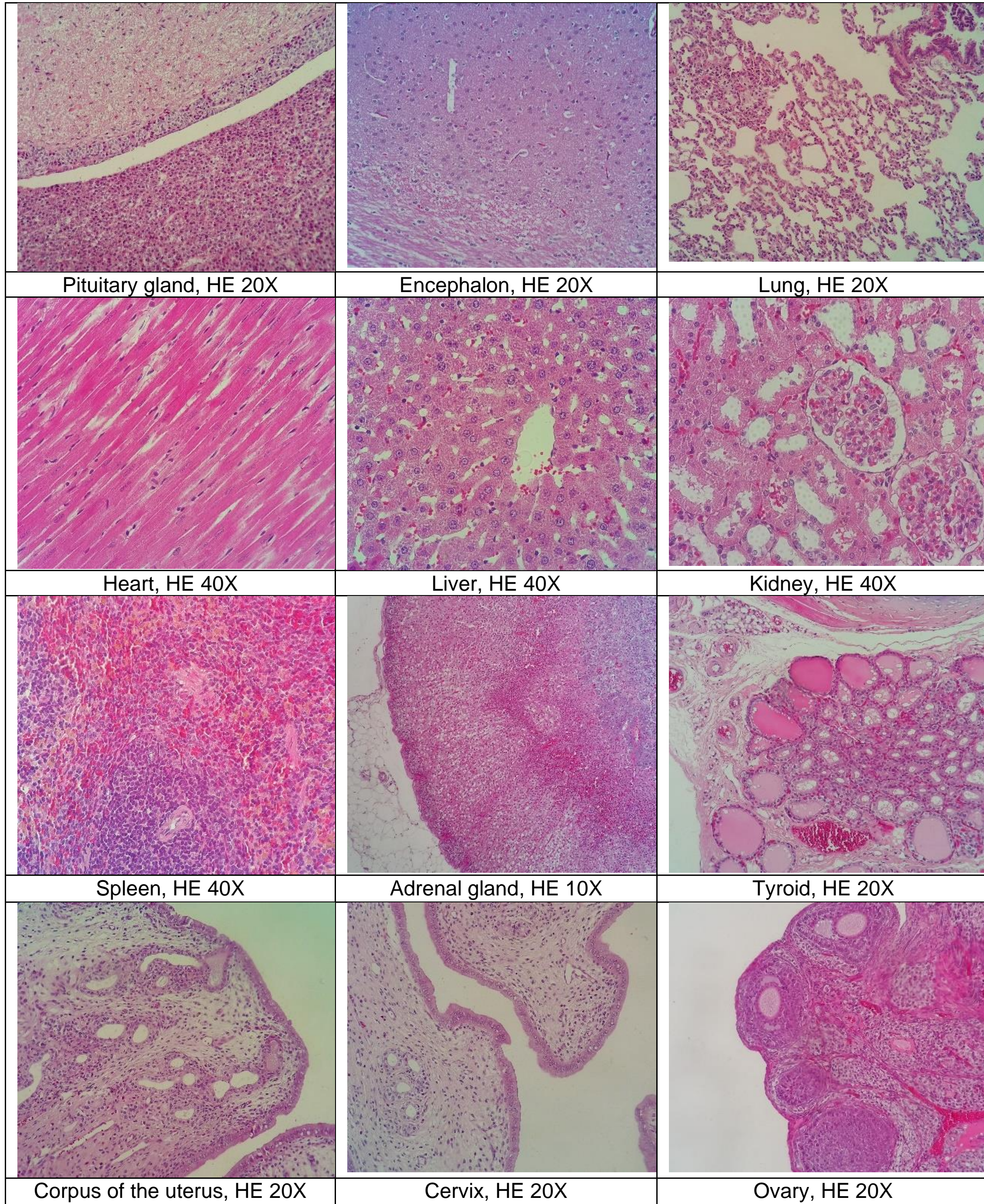

Figure 2 (a) NAIVE
Palozi et al.

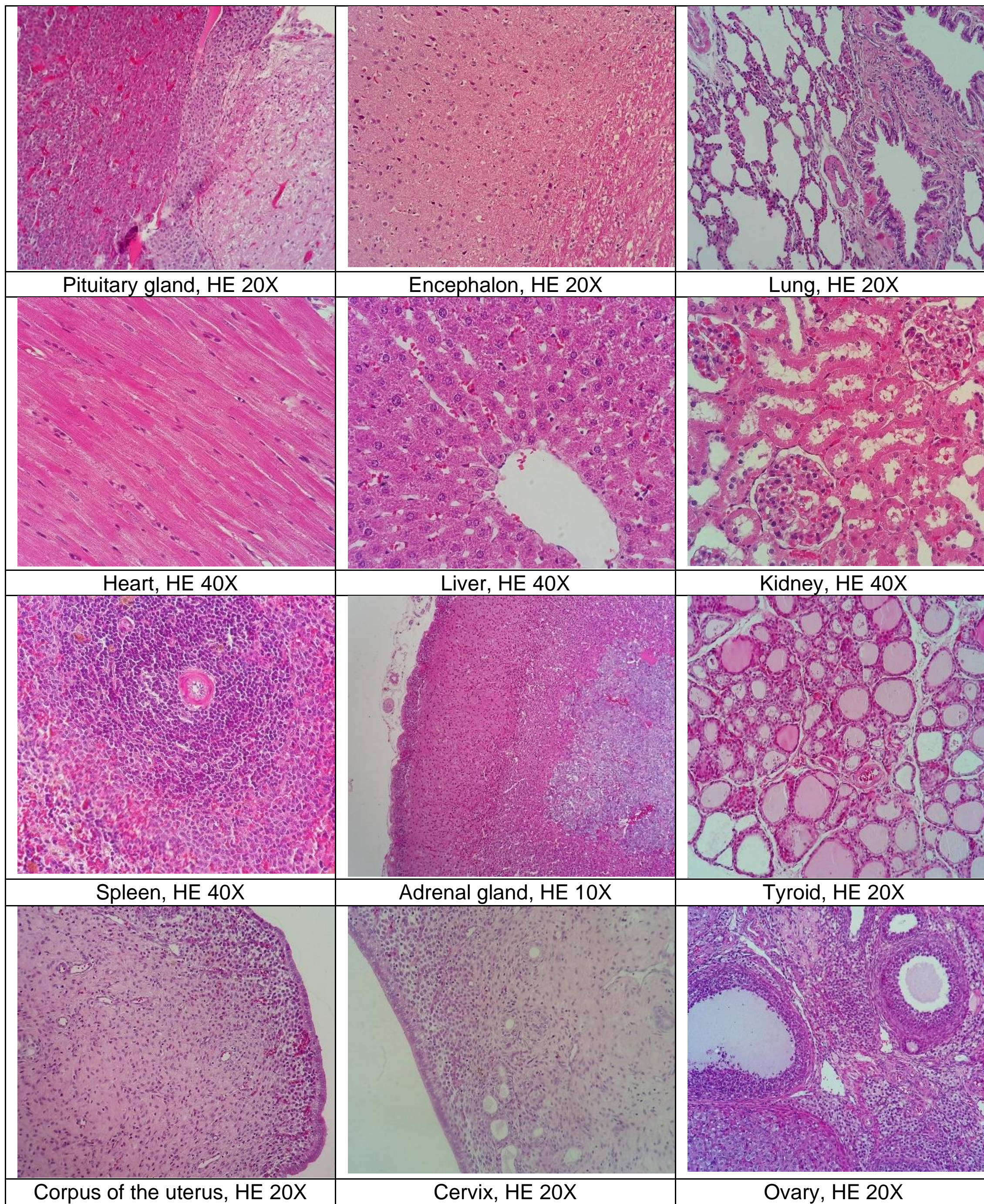

Figure 2 (b) SEIPC-30 mg/kg

Palozi et al.

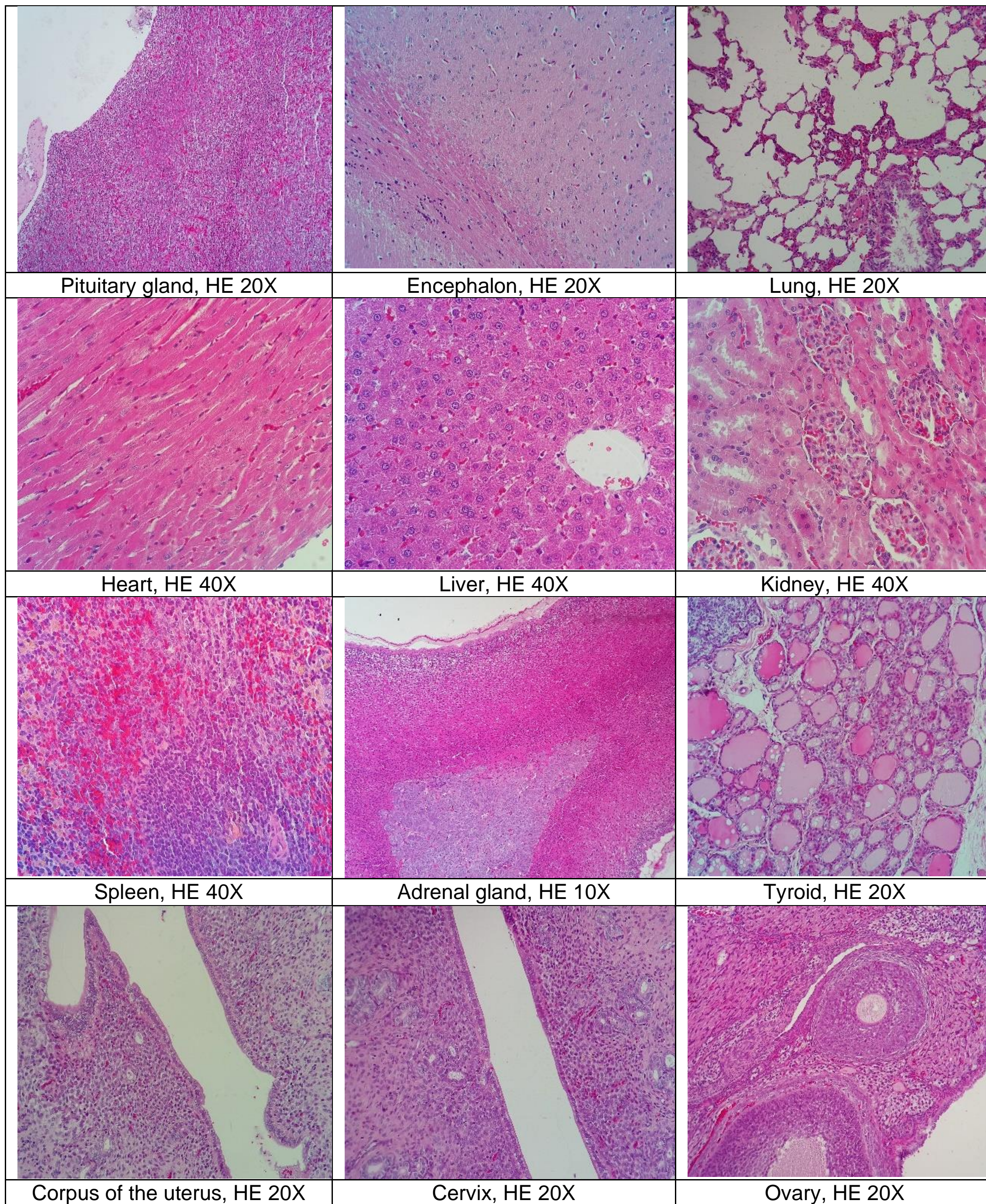

Figure 2 (c) SEIPC-100 mg/kg

Palozi et al.

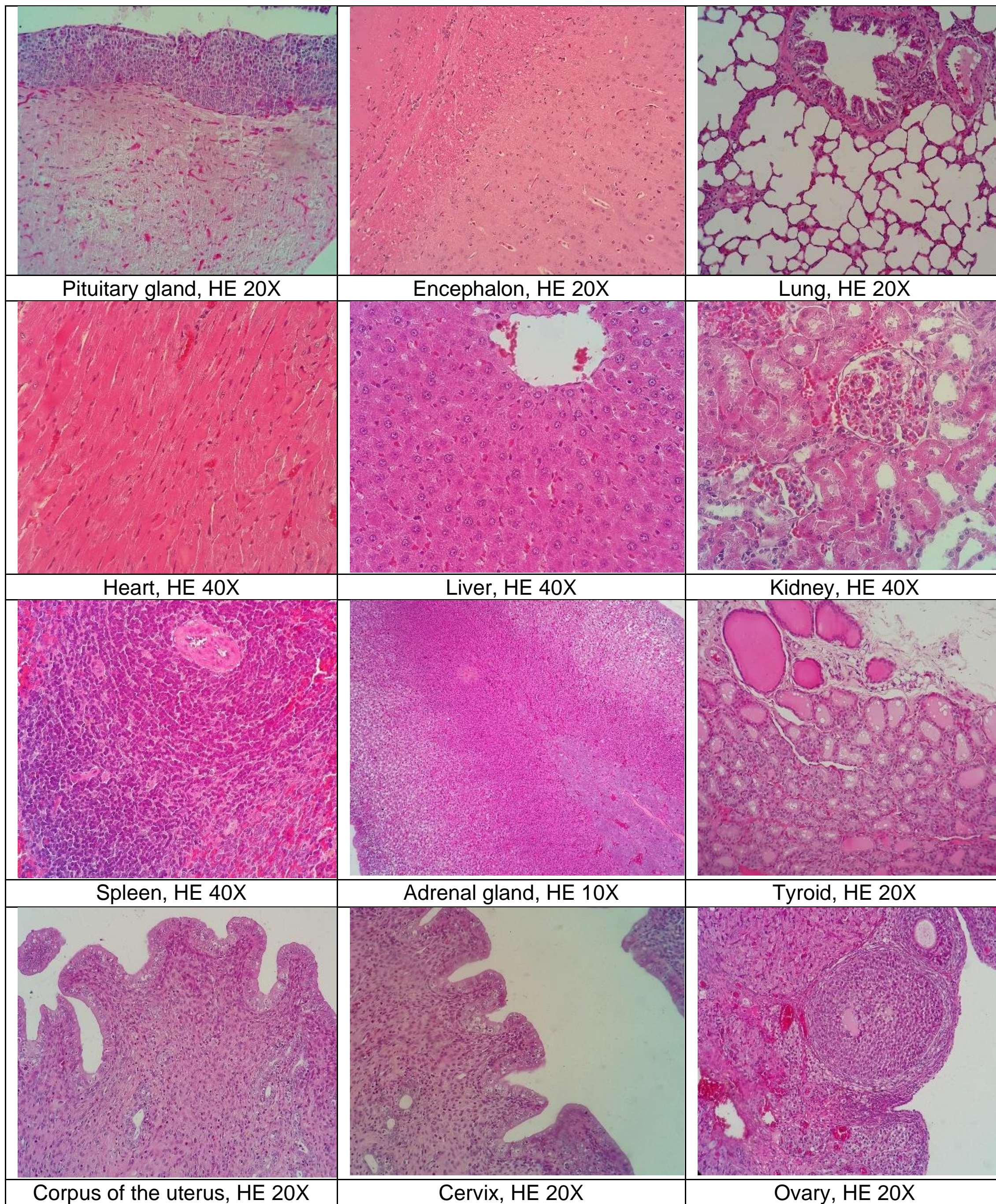

Figure 2 d) SEIPC-300 mg/kg

Palozi et al.

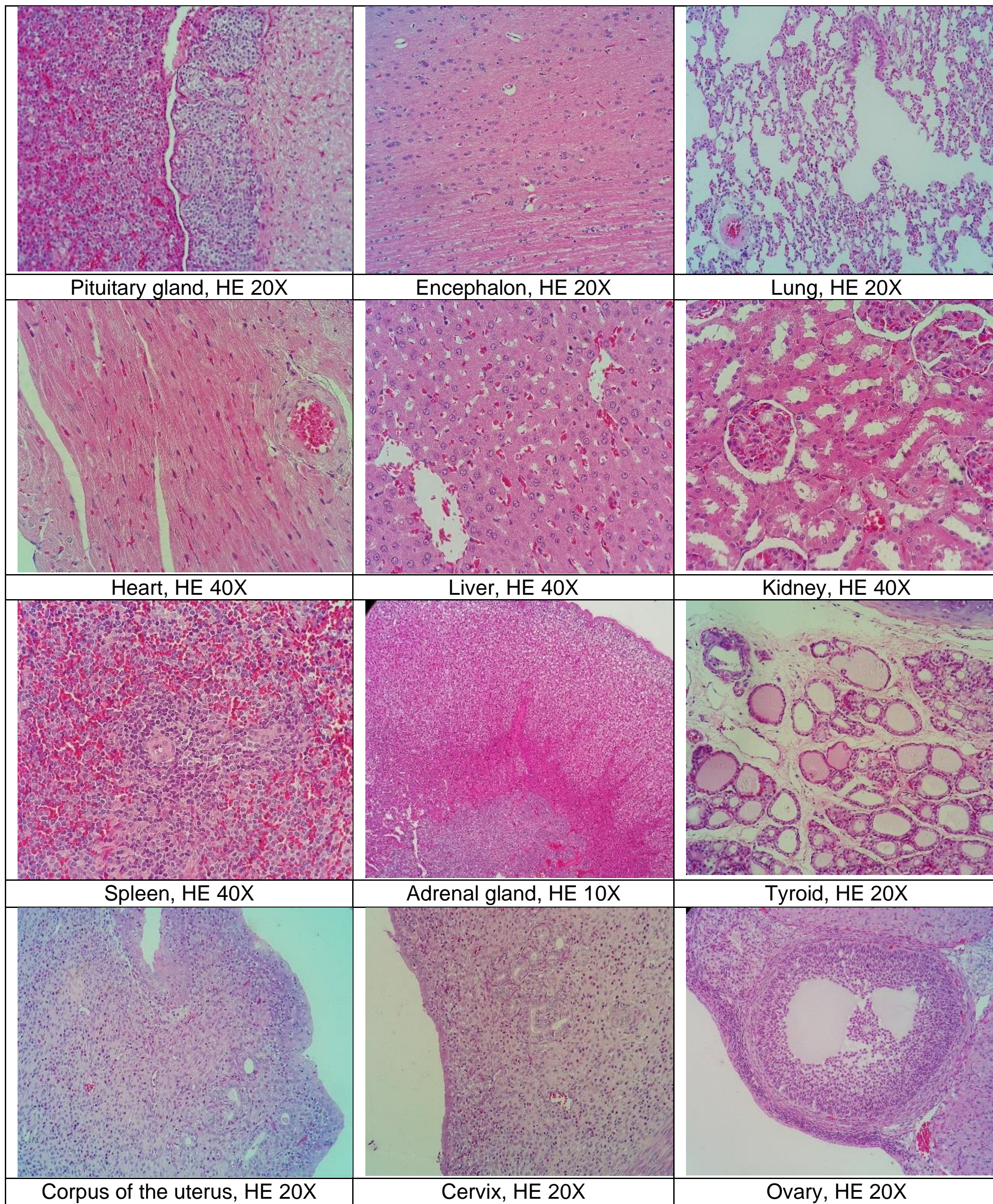

Figure 2 (e) SEIPC-1000 mg/kg

Palozi et al.

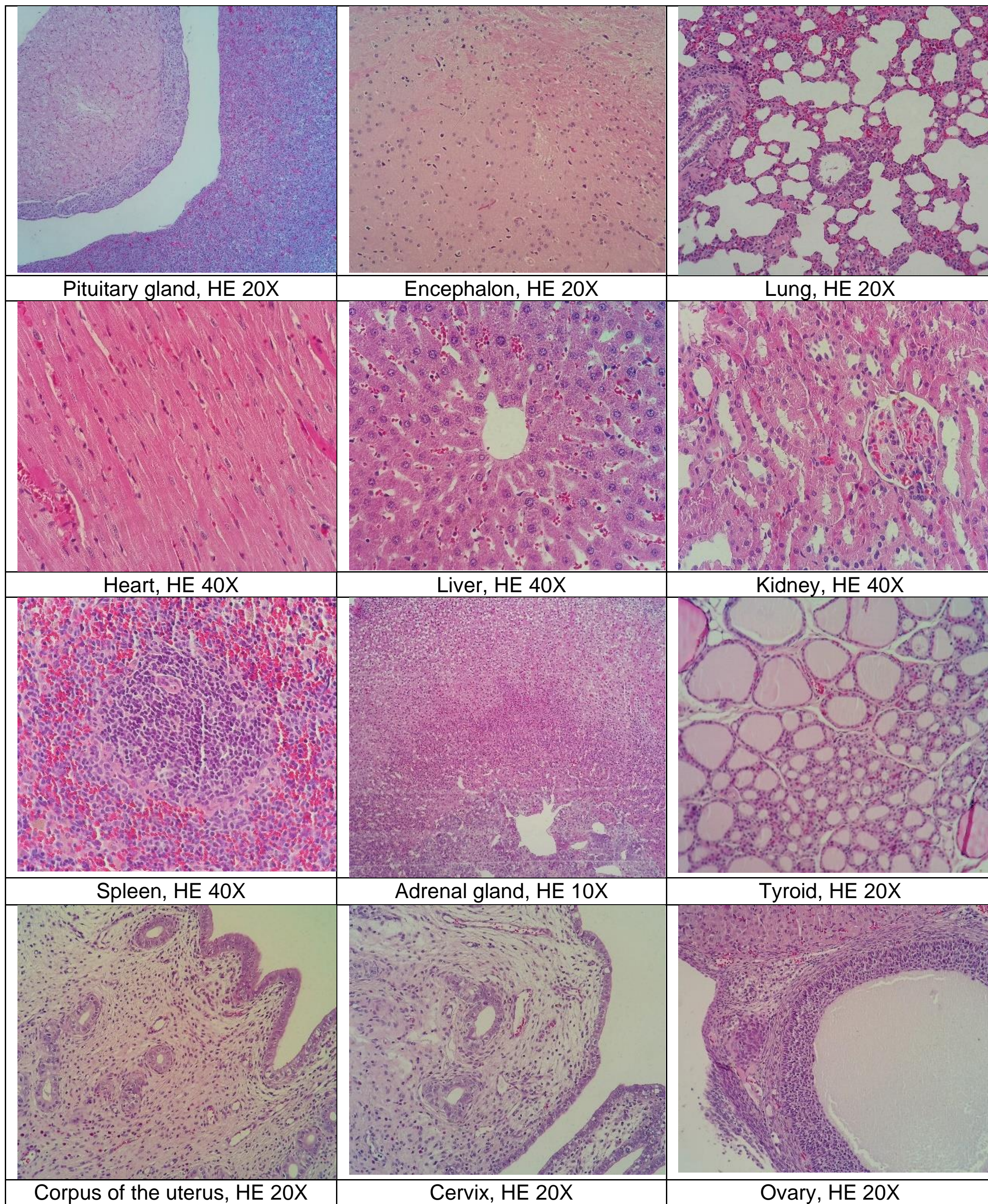

Figure 2 (f) Satellite

Palozi et al.