UNIVERSIDADE FEDERAL DO AMAPÁ
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

LETÍCIA ELIZANDRA MEHL BOETTGER

EFEITO QUIMIOPREVENTIVO DO EXTRATO HIDROETANÓLICO PADRONIZADO DE *Euterpe oleracea* Mart. NO CARCINOSSARCOMA DE WALKER 256 IMPLANTADO EM CÉRVICE UTERINA DE RATAS WISTAR

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Amapá para obtenção do Título de Mestre em Ciências Farmacêuticas.

Orientadora: Profª. Drª. Clarissa Silva Lima

Macapá
2019

Dados Internacionais de Catalogação na Publicação (CIP)
Biblioteca Central da Universidade Federal do Amapá
Elaborado por Cristina Fernandes – CRB-2/1569

Boettger, Letícia Elizandra Mehl
Efeito quimiopreventivo do extrato hidroetanólico padronizado de *Euterpe oleracea* Mart. no carcinossarcoma de walker 256 implantado em cérvice uterina de ratas Wistar / Letícia Elizandra Mehl Boettger; Orientadora, Clarissa Silva Lima. – Macapá, 2019.
78 f.

Dissertação (Mestrado) – Fundação Universidade Federal do Amapá, Programa de Pós-Graduação em Ciências Farmacêuticas.

1. Câncer – Tratamento. 2. Câncer em animais. 3. Aparelho genital feminino – Câncer. I. Lima, Clarissa Silva, orientadora. II. Fundação Universidade Federal do Amapá. IV.Título.

616.99 B732e
CDD. 22 ed.

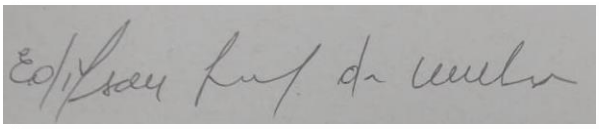
Programa de Pós-Graduação em Ciências Farmacêuticas
da Universidade Federal do Amapá

BANCA EXAMINADORA

Aluna: Letícia Elizandra Mehl Boettger

Orientadora: Profª. Drª. Clarissa Silva Lima

Profª. Drª. Clarissa Silva Lima
Presidente
Programa de Pós-Graduação em Ciências Farmacêuticas - PPGCF



Prof°. Dr°. Edilson Leal da Cunha
Membro Titular
Faculdade de Tecnologia do Amapá – META

Profª. Drª. Raphaelle Sousa Borges
Membro Titular
Programa de Pós-Graduação em Ciências Farmacêuticas – PPGCF

Data: 12/08/2019

Dedico este trabalho a minha família, especialmente ao meu Pai, José Maria Mehl (in memoriam), que sempre me apoiou e partiu durante o desenvolvimento desta pesquisa.

AGRADECIMENTOS

Agradeço primeiramente a Deus, o principal orientador da minha vida;

Aos meus familiares, por suportarem a minha ausência e me incentivarem em cada decisão;

A minha orientadora, Prof.ª Dr.ª Clarissa Silva Lima, pelos ensinamentos, paciência, parceria e amizade;

As equipes do Laboratório de Bioquímica e Citologia, do Laboratório de Toxicologia Reprodutiva e, do Laboratório de Pesquisa em Fármacos, da Universidade Federal do Amapá, especialmente ao Ivanilson, Fernanda, Clarice, Helison, Heitor, Brenda e, ao Prof. Dr. José Carlos Tavares, pelo apoio durante o desenvolvimento desta pesquisa;

Pelo suporte da equipe do Laboratório de Oncologia Experimental da Universidade Federal do Ceará, em nome do Prof. Dr. Manoel Odorico de Moraes e, do Laboratório Multiusuário de Caracterização e Análises da Universidade Federal da Paraíba, em nome do Prof. Dr. Josean Fechine Tavares;

Aos colegas e professores do Programa de Pós-Graduação em Ciências Farmacêuticas, especialmente ao Robson, Edmilson e Ana Paula pela amizade e companheirismo durante toda esta jornada;

A todas as pessoas que contribuíram para a realização deste trabalho.

SUMÁRIO

LISTA DE FIGURAS

%	Porcentagem
C3G	Cianidina-3-glicosídeo
CAT	Catalase
CCU	Câncer do colo do útero
DNA	Ácido desoxirrubonucléico
EHPEo	Extrato Hidroetanólico Padronizado de *Euterpe oleracea* Martius
ERO	Espécies reativas do oxigênio
Gpx	Glutationa peroxidase
HBV	Vírus da hepatite B
HPV	*Papilomavírus humano*
HSIL	Lesão intraepitelial escamosa de alto grau
Kg	Quilograma
L	Litro
LSIL	Lesão intraepitelial escamosa de baixo grau
mg	Miligrama
mm	Milímetro
NF-kB	Fator nuclear κB
NO	Óxido nítrico
Nrf2	Fator nuclear eritróide 2 relacionado ao fator 2
p53	Proteína do gene supressor de tumor
pRb	Proteína do gene supressor de tumor retinoblastoma
SOD	Superóxido dismutase
TNF-α	Fator de necrose tumoral α

W256	Tumor de Walker 256

RESUMO

EFEITO QUIMIOPREVENTIVO DO EXTRATO HIDROETANÓLICO PADRONIZADO DE *Euterpe oleracea* Mart. NO CARCINOSSARCOMA DE WALKER 256 IMPLANTADO EM CÉRVICE UTERINA DE RATAS WISTAR

Introdução: O câncer é caracterizado pelo crescimento descontrolado de células que podem invadir diversos tecidos, sendo uma das principais causas de morte no mundo. O dano oxidativo é uns dos principais promotores da carcinogênese. Diversos problemas na terapia do câncer, como baixa efetividade, toxicidade e desenvolvimento de resistência ao tratamento impulsiona a busca por novos fármacos. Nesse contexto, destacam-se os agentes quimiopreventivos que são fitoquímicos com alta capacidade antioxidante, que podem suprimir, impedir ou reverter a carcinogênese. *Euterpe oleracea* Martius (açaí) é uma palmeira tropical, com frutos amplamente investigados por sua composição química, na qual as antocianinas são os polifenóis mais abundantes e biologicamente ativos. **Objetivo:** Investigar o efeito quimiopreventivo do Extrato Hidroetanólico Padronizado de *E. oleracea* Mart. (EHPEo) no crescimento tumoral de Walker 256 (W256) em cérvice uterina de ratas Wistar. **Metodologia:** O extrato foi obtido a partir de um planejamento multifatorial visando à obtenção de maior concentração de antocianinas. A caracterização química foi realizada utilizando FTIR, CLAE-UV e CLAE-ESI-MS^n. O tratamento quimiopreventivo foi administrado, via oral, nos 5 grupos experimentais: Controle Negativo (0,5 mL de água destilada/70 dias), Controle Positivo (N-Acetilcisteína 300mg/Kg/70 dias), Tratado I (EHPEo 150mg/Kg/70 dias), Tratado II (EHPEo 300mg/Kg/70 dias) e Tratado III (EHPEo 300mg/Kg/82 dias). As células W256 foram inoculadas no canal vaginal das ratas e, avaliadas por citologia e histopatologia. **Resultados:** A análise fitoquímica revelou a presença de cianidina-3-O-glicosídeo e cianidina-3-O-rutinosídeo, que são as antocianinas mais abundantemente encontradas em *E. oleracea*. O índice de pega tumoral foi de 100%, com menor crescimento tumoral na concentração de 300 mg/Kg do EHPEo. Os critérios citomorfológicos de malignidade, pleomorfismo celular e hipercromasia, foram mais evidentes no Controle Negativo. A análise histopatológica revelou menor celularidade tumoral na concentração de 300 mg/Kg. **Conclusão:** Portanto, estes resultados sugerem que o EHPEo teve capacidade quimiopreventiva na dose de 300 mg/Kg.

Palavras-Chave: câncer; cérvice uterina; quimioprevenção; *Euterpe oleracea*; antocianina; antioxidante; tumor de Walker 256; citologia vaginal.

Agradecimentos: PPP/FAPEAP; UFC; UFPB.

ABSTRACT

THE CHEMOPREVENTIVE EFFECT OF THE STANDARDIZED HYDROETHANOLIC EXTRACT OF *Euterpe oleracea* Mart. IN THE WALKER 256 CARCINOSARCOMA IMPLANTED ON UTERINE CERVIX OF FEMALE WISTAR RATS

Introduction: Cancer is characterized by the uncontrolled growth of cells that might invade several tissues, being one of the most common causes of death in the world. The oxidative damage is considered one of the main promoters of carcinogenesis. Several problems in cancer therapy, such as low effectiveness, toxicity and development of resistance to the treatment have been driving the search for new drugs. In this context, the chemopreventive agents stand out, which are phytochemicals with has high antioxidant capacity, which may suppress, prevent or reverse carcinogenesis. *Euterpe oleracea* Martius (açai palm) is a tropical palm, with fruits widely investigated by the chemical composition, in which the anthocyanins are the most abundant and biologically active polyphenols. **Objective:** To investigate the chemopreventive effect of the Standardized Hydroethanolic Extract of *E. oleracea* Mart. (SHEEo) in the tumor growth of Walker 256 (W256) in the uterine cervix of female Wistar rats. **Methodology:** The extract was obtained by using multifactorial planning with the purpose of obtaining a higher concentration of the anthocyanins. Using FTIR, HELC-UV-S-UV and HELC-UV-S-MSn made the chemical characterization. The chemopreventive treatment was carried out in the animals, orally, in the 5 experimental groups: Negative Control Group (0.5mL of distilled water/70 days), Positive Control Group (N-Acetyl Cysteine 300mg/Kg/70 days), Treated Group I (SHEEo 150mg/Kg/70 days), Treated Group II (SHEEo 300mg/Kg/70 days) and Treated Group III (SHEEo 300mg/Kg/82 days). The W256 cells were inoculated in the vaginal canal of the rats and were assessed by cytology and histopathology. **Results:** The phytochemical analysis revealed the presence of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside, which are the most abundantly found anthocyanins in *E. oleracea*. The tumor engraftment index was of 100%, with a lower tumor growth at the concentration of 300mg/Kg of the SHEEo. The cytomorphological criteria of malignancy, cell pleomorphism and hyperchromasia were more evident in the Negative Control Group. The histopathological analysis revealed less tumor cellularity at the concentration of 300mg/Kg. **Conclusions:** Therefore, these results suggest that the SHEEo presented chemopreventive capacity at the dosage of 300mg/Kg.

Keywords: cancer; uterine cervix; chemoprevention; *Euterpe oleracea*; anthocyanin; antioxidant; Walker 256 tumor; vaginal cytology.

Acknowledgements: PPP/FAPEAP; UFC; UFPB.

1 INTRODUÇÃO

1.1 CÂNCER

Apesar dos modernos avanços na terapêutica médica, o câncer ainda representa a principal causa de morte e a barreira mais importante para aumentar a expectativa de vida em todos os países do mundo (BRAY et al., 2018), sendo responsável por 9 milhões de mortes no ano de 2016 (WHO, 2018). Para o Brasil, estima-se a ocorrência de 600 mil novos casos de câncer para cada ano do biênio 2018-2019 (INCA, 2017b).

O câncer, também denominado neoplasia maligna, é uma condição anormal na qual um grupo de células desconsidera as regras fisiológicas de divisão celular e não responde aos sinais de ativação do ciclo celular normal, devido à uma auto-suficiência, a qual às leva ao crescimento descontrolado e à proliferação de células alteradas (ABBAS; REHMAN, 2018). As células tumorais apresentam características que favorecem o seu crescimento, como uma resposta alterada ao estresse celular, que permite à sua sobrevida, além da modulação imune, do microambiente favorável, vascularização, invasão e metástase (FOUAD; AANEI, 2017).

Por apresentar atividade celular descontrolada, o câncer desenvolve uma síndrome chamada de caquexia, que é a principal causa de morbidade e mortalidade em pacientes com câncer em estágio avançado, caracterizada por um intenso consumo generalizado dos tecidos corporais, muscular e adiposo, com uma perda progressiva e involuntária de peso. A caquexia também é configurada pela presença de anemia, astenia, balanço nitrogenado negativo, disfunção imune e alterações metabólicas, geralmente associadas à anorexia, a qual é intensificada pelas alterações no metabolismo dos nutrientes (carboidratos, proteínas e lipídios), alterações hormonais e aumento de citocinas circulantes (SILVA, 2006).

Uma proporção substancial de cânceres pode ser evitada, incluindo aqueles causados pelo uso do tabaco e por outros comportamentos não saudáveis, como a inatividade física, o excesso de peso corporal, o consumo excessivo de álcool, a má nutrição e a exposição excessiva ao sol, além daqueles causados por agentes infecciosos, como P*apilomavírus humano* (HPV), vírus da hepatite B (HBV), vírus da hepatite C e *Helicobacter pylori*, os quais podem ser prevenidos através de mudanças comportamentais ou vacinação para evitar a infecção (ACS, 2019).

Os tumores são nomeados dependendo do tipo de célula na qual se originam, podendo ser carcinomas (células epiteliais, constituindo a maior proporção todos os tipos de câncer), sarcomas (ossos, músculos, gorduras e tecido conjuntivo), leucemia (glóbulos brancos), linfoma (sistema linfático ou células derivadas da medula óssea) e mielomas (glóbulos brancos específicos para síntese de anticorpos) (ABBAS; REHMAN, 2018). Carcinossarcomas são caracterizados pela sua morfologia bifásica única, como um tumor composto de ambos os elementos, epiteliais e mesenquimais, os quais estão microscopicamente intermitentemente misturados ou distintos, sendo o componente epitelial, frequentemente, um adenocarcinoma endometrial, associado a um componente estromal maligno (BROWN, 2008).

Foi estimado, para o ano de 2018, 18,1 milhões de novos casos de câncer no mundo todo e, 9,6 milhões de mortes, destacando o câncer de pulmão como o mais comum (11,6% do total de casos) e a principal causa de morte por câncer (18,4% do total de mortes por câncer), seguido pelo câncer de mama feminino (11,6%), de próstata (7,1%) e colorretal (6,1%) para incidência e, câncer colorretal (9,2%), de estômago (8,2%) e de fígado (8,2%) para mortalidade (BRAY et al., 2018).

A origem e o avanço do câncer dependem de muitos fatores dentro da célula (mutações, condições imunes e hormônios), bem como fatores externos ao ambiente (tabagismo, químicos, organismos infecciosos e radiações), os quais agem juntos para causar comportamento celular anormal e proliferação descontrolada. De acordo com o modelo mais aceito para a causa do câncer, mutações no gene supressor tumoral e oncogenes são os principais fatores para o desenvolvimento do câncer (ABBAS; REHMAN, 2018). Segundo Jopkiewicz (2019), 70-90% dos cânceres humanos estão associados a fatores comportamentais, ambientais e dietéticos, de forma que o efeito acumulativo dos carcinógenos, em diferentes intervalos de tempo, é responsável pela maior incidência de câncer em idosos.

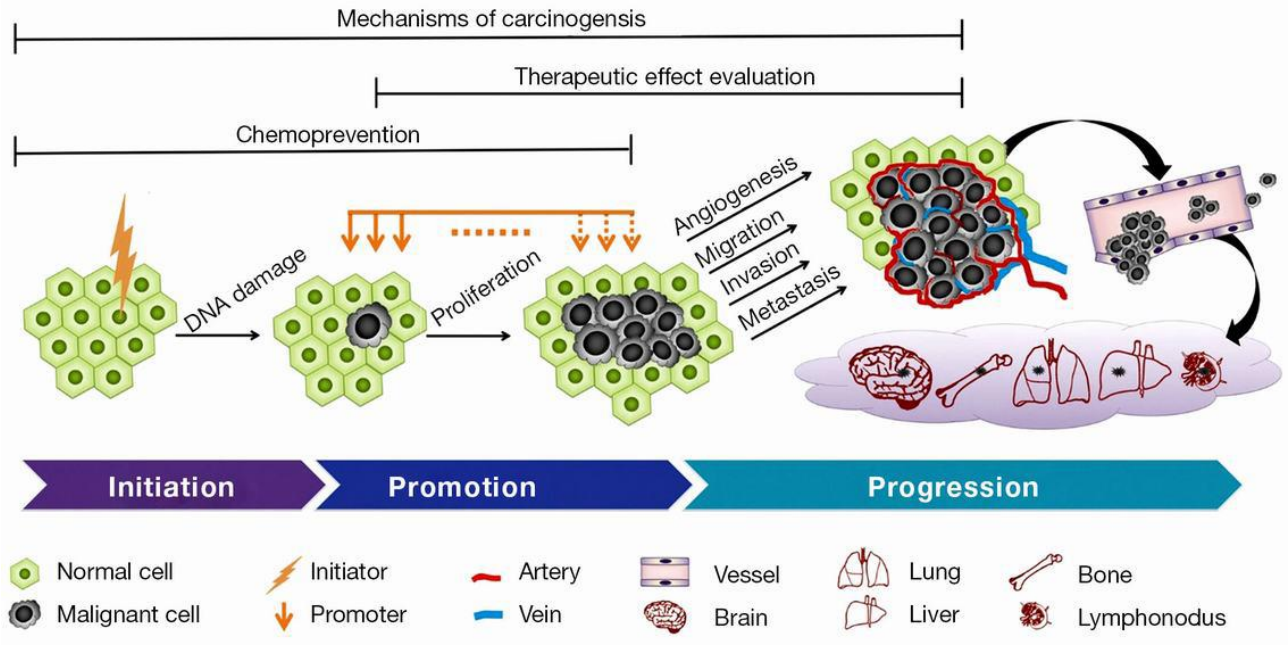
A carcinogênese é um processo lento composto por múltiplas etapas, que resulta em alterações na arquitetura do tecido, em conjunto com a formação de nódulos neoplásicos, que por sua vez, irão preceder o aparecimento do câncer (FEITELSON et al., 2015). Embora o processo inclua múltiplos eventos moleculares e celulares que levam à transformação de células normais em células neoplásicas malignas, são definidos três principais estágios no processo de carcinogênese: a iniciação, a promoção e a progressão (TANAKA et al., 2013; LIU et al., 2015).

Durante o primeiro estágio, a iniciação, as células entram em contato com agentes cancerígenos, os quais, por sua vez, irão provocar mutações irreversíveis no ácido

desoxirribunocléico (DNA) da célula, fazendo com que esta fique "iniciada" para os próximos estágios da carcinogênese (MALARKEY et al., 2013). O segundo estágio, promoção, ocupa a maior parte do período de latência envolvendo mecanismos epigenéticos, com mudanças na expressão gênica e seleção clonal e, ativação dos oncogenes, que levam ao crescimento e proliferação das células iniciadas (JOPKIEWICZ, 2019).

Por sua vez, a progressão caracteriza-se pela irreversibilidade, instabilidade genética, produção de fatores de crescimento, invasão, metástases e alterações na bioquímica, metabolismo e morfologia das células afetadas, sendo a neoangiogênese fundamental para esta etapa (TANAKA et al., 2013) **(Figura 1)**.

Figura 1 – As três etapas da carcinogênese e seus mecanismos que levam à formação de metástases.



Fonte: LIU et al. (2015).

No câncer invasivo, as células migram para outras camadas celulares do órgão, ganham a corrente sanguínea ou linfática e têm a capacidade de se disseminar para outras partes do corpo onde produzem outros tumores, sendo esses novos focos da doença conhecidos como metástases (INCA, 2017a). Segundo Abbas e Rehman (2018), 90% das mortes por câncer são devido à propagação de células cancerígenas para outros tecidos.

No decorrer do intenso processo de proliferação e formação de um tumor, as células malignas, assim como as células normais, necessitam de um suprimento sanguíneo para receber nutrientes e oxigênio e para remover produtos de excreção, ocorrendo uma expressiva indução da formação de novos vasos sanguíneos, processo conhecido como

angiogênese. As células tumorais promovem um aumento na atividade angiogênica por meio da liberação de fatores como o fator de crescimento vascular endotelial. Desta forma, a angiogênese apresenta uma correlação com a malignidade tumoral, já que é requerida não somente para o crescimento celular, mas também para o acesso à vasculatura e posterior formação de mestástases. Assim, quanto maior a atividade angiogênica, maior é a potência de metastatização do câncer e mais rápida é a sua progressão (HANAHAN; WEINBERG, 2011).

1.1.1 Câncer do colo do útero

Entre as mulheres de todo o mundo, com uma estimativa de 570.000 casos e 311.000 mortes em 2018, o câncer do colo do útero (CCU), mesmo sendo prevenível, classifica-se como o quarto mais frequente e a quarta principal causa de morte por câncer (BRAY et al., 2018). Em termos globais, a maioria dos casos de CCU (70%) ocorre em áreas com menores níveis de desenvolvimento humano, de forma que quase nove de cada dez óbitos ocorrem em regiões menos desenvolvidas, onde o risco de morrer por este câncer antes dos 75 anos é três vezes maior (FERLAY et al., 2015).

Para o Brasil, estimam-se 16.370 casos novos de CCU para cada ano do biênio 2018-2019, com um risco estimado de 15,43 casos a cada 100 mil mulheres, ocupando a terceira posição. A Região Norte destaca-se pelo CCU ser o primeiro mais incidente, se desconsiderar os tumores de pele não melanoma (25,62/100 mil) (INCA, 2017b).

Configura-se como uma doença de desenvolvimento lento, frequentemente assintomática em estágio inicial, com manifestações de sangramento vaginal, dispareunia e corrimento vaginal anormal, que pode ser aquoso, mucóide ou purulento e, fétido. Dor pélvica e/ou lombar, com irradiação para a região posterior dos membros são geralmente sintomas de doença avançada. Os casos mais extremos podem evoluir com sintomas decorrentes da invasão das estruturas adjacentes, como o trato geniturinário e o reto, levando à hematúria e suboclusão intestinal, entre outros (DIZ; MEDEIROS, 2009).

Os vírus têm sido reconhecidos como causa de alguns tipos de câncer e, a infecção crônica persistente pelos subtipos oncogênicos do HPV é a principal causa do CCU, também conhecido como câncer cervical (COHEN et al., 2019). A infecção pelo HPV é muito comum entre populações humanas, com estimativas de prevalência variando de 2% a 44% entre as mulheres de todo o mundo (MEHTA et al., 2017).

O HPV pode ser classificado de acordo com o seu potencial cancerígeno em HPV de baixo risco (principalmente os subtipos HPV6 e HPV11), associando-se às verrugas

anogenitais benignas e, HPV de alto risco (principalmente HPV16, HPV18, HPV31, HPV33, HPV35, HPV45) os quais estão relacionados ao câncer e lesões precursoras, de forma que sequências de DNA de HPVs de alto risco são encontradas em praticamente todos os tumores cervicais (NUNES et al., 2018).

Aproximadamente 200 subtipos de HPV já foram identificados, porém, o HPV16 e o HPV18 são responsáveis por aproximadamente 60-80% de todos os casos de câncer cervical e, embora a infecção e colonização do epitélio do colo uterino pelo HPV de alto risco seja pré-requisito para o desenvolvimento do câncer, a resposta imune local é considerada uma importante determinante da progressão e desfecho desta doença (MEHTA et al., 2017). Desta forma, torna-se compreensível o fato de que as infecções por HPV em mulheres saudáveis sejam comuns, porém raramente causem câncer (ACS, 2019).

De acordo com Cohen et al. (2019), outros fatores de risco associados corroboram, severamente, para o desenvolvimento do CCU, por prejudicarem a resposta imune à infecção pelo HPV, a qual é imprescindível para a eliminação viral. Os cofatores de risco relacionados à oncogênese cervical podem ser imunológicos (pacientes transplantadas ou coinfecção com o vírus da imunodeficiência humana - HIV) e genéticos (como o polimorfismo da p53 – proteína do gene supressor de tumor), além do tabagismo, do uso prolongado de contraceptivos orais, do início precoce da atividade sexual, da multiplicidade de parceiros, da baixa escolaridade e renda, da multiparidade, da baixa ingestão de vitaminas e da história de infecções sexualmente transmissíveis (coinfecção com *Chlamydia trachomatis*) (ANJOS et al., 2010; RODRIGUES et al., 2012; STEWART; WILD, 2014, SILVA et al., 2018b).

Todos os cofatores de risco do CCU aumentam os níveis de óxido nítrico (NO) no microambiente cervical, o qual é observado em níveis significativamente elevados em pacientes com câncer cervical quando em comparação com pacientes saudáveis, sugerindo que o NO tem potencial atividade mutagênica e carcinogênica neste tipo de câncer, pois atua como cofator molecular da infecção pelo HPV na carcinogênese cervical, antecipando a expressão do mRNA, diminuindo os níveis de pRb (proteína do gene supressor de tumor retinoblastoma) e p53 (CHOUDHARI et al., 2013).

Embora as mulheres que iniciam a atividade sexual em idade precoce ou que tiveram muitos parceiros sexuais tenham um risco aumentado de infecção pelo HPV e progressão para o CCU, uma mulher pode estar infectada pelo HPV mesmo que tenha tido apenas um parceiro sexual (ACS, 2019). O hábito de fumar associado à infecção pelo HPV

dobra o risco de ocorrências de lesão pré-cancerosa e carcinoma do colo uterino, sendo que a cessação do tabagismo reduz este risco em duas vezes (COHEN et al, 2019).

Nutrientes antioxidantes, como as vitaminas A, E e C, podem inibir a formação de espécies reativas do oxigênio (ERO), impedir a amplificação do DNA viral e a evolução das lesões malignas no epitélio do colo uterino, atuando como moduladores da resposta imune frente à presença e/ou à persistência da infecção por HPV, impedindo a progressão das lesões e o desenvolvimento do CCU (KONG; LILLEHEI, 1998).

A cérvice uterina é constituída pelo epitélio escamoso (composto pelas camadas de células basais, parabasais, intermediárias e superficiais), pelo epitélio endocervical (composto por uma camada única de células cilíndricas ciliadas) e, pelo epitélio metaplásico (CONSOLARO; MARIA-ENGLER, 2016). O CCU caracteriza-se pela replicação desordenada de algum destes epitélios, comprometendo o tecido subjacente (estroma) e, podendo invadir estruturas e órgãos contíguos ou à distância (INCA, 2011).

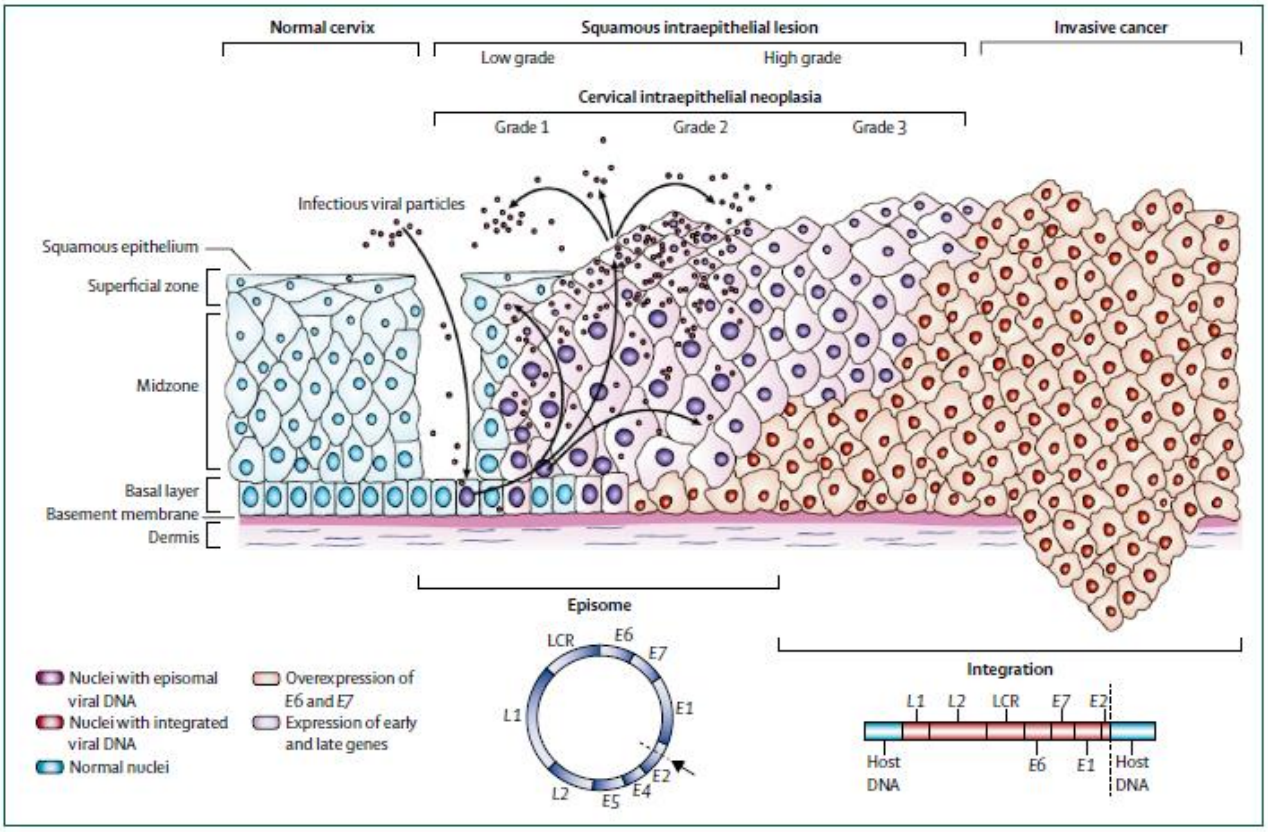
A complexidade das alterações morfológicas que ocorrem no epitélio cervical para levar ao processo de carcinogênese depende da exposição ao HPV, do subtipo de HPV envolvido, da persistência do HPV e das condições preexistentes do hospedeiro (SAMPAIO; ALMEIDA, 2009). Na maioria dos indivíduos afetados por esse vírus, as infecções são espontaneamente resolvidas, mas nos casos em que as infecções se apresentam persistentes, pode haver progressão para o CCU em 10 a 20 anos após a infecção (WHO, 2014).

A infecção inicial pelo HPV ocorre nas células da camada basal do epitélio escamoso através de microlesões. Estas células servem de reservatório de DNA viral, e não são lisadas pela produção de novos vírus, continuando a proliferação (SAMPAIO; ALMEIDA, 2009). Após a entrada do HPV no núcleo da célula hospedeira, o genoma viral se estabiliza e se replica. As células infectadas, ao se dividirem, distribuem equitativamente o DNA viral entre as células filhas, que migram a partir da camada basal em direção as camadas mais superficiais do epitélio e iniciam o programa de diferenciação celular (MARTIN; LEARY, 2011) **(Figura 2)**.

A carcinogênese pelo HPV resulta da expressão de dois genes virais, o E6 e o E7, que interferem com a função das proteínas pRb e p53 da célula hospedeira. A proteína p53 está relacionada ao controle do ciclo celular, principalmente pela função de manutenção da integridade do genoma, induzindo a apoptose (morte celular) quando ocorre alguma alteração nuclear. A pRb está envolvida no ciclo celular diretamente na transição das fases G1 para S (SAMPAIO; ALMEIDA, 2009; SILVA et al., 2018b). Através da inibição do p53 e pRb, há uma perda no controle de proliferação celular e da morte celular

programada, o que permite que células infectadas pelo HPV continuem a se dividir, mesmo na presença de DNA danificado, aumentando o risco de células infectadas evoluírem para a malignidade (SOUTO et al., 2005).

Figura 2 - Patogênese do câncer do colo do útero.



Fonte: Cohen et al. (2019).

O CCU demora muitos anos para se desenvolver e as alterações das células (lesões pré-cancerosas ou precursoras) que podem desencadear o câncer são descobertas facilmente no exame preventivo (Papanicolaou), portanto, a realização periódica deste exame a cada três anos após dois exames anuais consecutivos negativos é extremamente importante (INCA, 2017a). As vacinas contra o HPV não podem proteger contra infecções estabelecidas nem contra todos os tipos de HPV, e é por isso que as mulheres vacinadas ainda devem ser rastreadas pelo teste do Papanicolaou, um procedimento simples em que uma pequena amostra de células é coletada do colo do útero e examinada sob um microscópio (ACS, 2019).

A nomenclatura mais utilizada para classificar as lesões no epitélio cervical é a do Sistema Bethesda, que as denomina em lesão intraepitelial escamosa de baixo grau (LSIL) e lesão intraepitelial escamosa de alto grau (HSIL), sendo, esta última, a lesão precursora do CCU (NAYAR; WILBUR, 2017). As lesões celulares são identificadas pelos critérios

citomorfológicos de malignidade, que se relacionam à fisiologia alterada das células, podendo ser observados tanto no núcleo quanto no citoplasma, dentre os quais destacam-se o pleomorfismo celular, que define o achado de diversas formas celulares diferentes, refletindo uma população geneticamente instável propensa à progressão tumoral e, a hipercromasia, que reflete maior conteúdo de DNA no núcleo, revelando a instabilidade cromossômica que é comum em muitos tumores (FISCHER et al., 2010).

Na LSIL, as alterações ocorrem nas células maduras do epitélio escamoso (intermediárias e superficiais), as quais apresentam núcleos aumentados de 2 a 3 vezes quando comparados ao tamanho do núcleo de uma célula intermediária normal e, são geralmente hipercromáticos, podendo ocorrer multinucleação (KIETPEERAKOOL et al., 2014). Por sua vez, a HSIL ocorre nas células imaturas do epitélio escamoso (basais e parabasais) e, nas células do epitélio metaplásico, as quais apresentam um aumento do tamanho nuclear (cariomegalia), com alterações no formato e tamanho dos núcleos, que se apresentam hipercromáticos (CONSOLARO; MARIA-ENGLER, 2016).

O CCU pode originar-se do epitélio escamoso da ectocérvice (carcinoma de células escamosas) ou do epitélio cilíndrico do canal endocervical (adenocarcinoma endocervical), os quais representam, respectivamente, 90% e 10% dos casos de CCU (TSUCHIYAL et al., 2017).

No carcinoma de células escamosas observa-se um intenso pleomorfismo celular, com células em formato de fibra ou girino, isoladas ou agrupadas, com hipercromasia acentuada (CONSOLARO; MARIA-ENGLER, 2016). O adenocarcinoma endocervical é caracterizado por grupamentos celulares com formato papilar ou glandular, os quais podem estar arranjados em forma de tira ou roseta, podendo apresentar macronucléolos (KOSS; GOMPEL, 2016).

O prognóstico no CCU depende da extensão da doença no momento do diagnóstico, estando sua mortalidade fortemente associada ao diagnóstico em fases avançadas (CARVALHO et al., 2018). Os tipos de terapia disponíveis incluem cirurgia e radioterapia para casos diagnosticados precocemente, ou radioterapia/quimioterapia para casos mais avançados, entretanto, os efeitos secundários e morbidade causados por essas terapias afetam profundamente a qualidade de vida dessas mulheres (CORREIA et al., 2018).

Uma associação inversa foi observada entre os fatores antioxidantes presentes na dieta e a tumorigênese do HPV, sugerindo que antioxidantes naturais podem proteger contra a persistência do HPV e o desenvolvimento tumoral. Além disso, estudos utilizando compostos naturais presentes em extratos de plantas, como a curcumina e resveratrol, têm

sido conduzidos para sensibilizar as células tumorais para a radioterapia/quimioterapia, sugerindo que moléculas com estas propriedades podem ser utilizadas como um complemento para o tratamento do câncer (SILVA et al., 2018b).

1.2 TUMORES EXPERIMENTAIS

As pesquisas sobre câncer têm sido uma das mais fascinantes e interessantes áreas da pesquisa pela sua diversidade, buscando a compreensão da origem e a localização da doença, os tipos de células alvos de drogas, o diagnóstico e as diversas formas de tratamento. Qualquer que seja a área de estudo o objetivo final permanece o mesmo, tornar a doença curável (NAVALE, 2013). Até tempos recentes, o diagnóstico de uma forma progressiva de câncer significava, invariavelmente, que haveria pouca chance de sobrevivência em longo prazo. Entretanto, esta noção mudou recentemente graças aos avanços na pesquisa e desenvolvimento na área do câncer, apoiados por um enorme financiamento público e privado (BREITENBACH; HOFFMANN, 2018).

Inúmeros esforços foram empregados nas últimas décadas para a compreensão do processo de tumorigênese, nos quais a maior parte provém da análise *in vitro* de tecidos tumorais em estágios avançados de desenvolvimento, removidos a partir de pacientes, que elucidou muitas questões referentes a mudanças genômicas apresentadas pelas células tumorais, mas pouco contribuiu na obtenção de informações sobre os fatores que influenciam os estágios iniciais do seu desenvolvimento *in vivo* (PANTALEÃO; LUCHS, 2010).

A descoberta de novas drogas contra o câncer continua a evoluir em um ritmo fenomenal e elevada quantidade de recursos financeiros estão empenhados na descoberta de novos medicamentos (NAVALE, 2013). Os estudos envolvendo o câncer dependem de modelos representativos e confiáveis. Em mais de 100 anos de pesquisa, os modelos experimentais vêm mudando constantemente, de forma que, hoje, a seleção do modelo mais apropriado para melhor refletir a uma dada entidade tumoral é um dos maiores desafios para os pesquisadores que tem como objetivo de estudo o câncer (BREITENBACH; HOFFMANN, 2018).

Grande parte da pesquisa genética do câncer em humanos depende de modelos experimentais em animais, nos quais os modelos tumorais em ratos revolucionaram nossa capacidade de estudar a função de genes e proteínas *in vivo* e entender melhor suas vias e mecanismos moleculares (CEKANOVA; RATHORE, 2014). A riqueza dos dados gerados a partir de modelos sobre as funções biológicas dos genes e das vias de sinalização

envolvidas no processo tumoral permitiu a construção do conceito atual de metástase (PANTALEÃO; LUCHS, 2010).

Genomicamente, ratos e humanos são razoavelmente próximos, podendo relacionar cerca de 80% de todos os genes codificadores de proteínas de seres humanos com os dos ratos, incluindo a maioria dos genes reconhecidamente envolvidos no câncer. Além disso, o tempo médio de vida em laboratório (2,5 anos), a possibilidade de introduzir quase todas as alterações genéticas desejadas para estudar a biologia tumoral e, a ocorrência freqüente de câncer, mesmo na ausência de agentes cancerígenos, com aumento exponencial na velhice, como nos seres humanos, torna os modelos tumorais em ratos muito atraentes (BREITENBACH; HOFFMANN, 2018).

Entre os modelos experimentais tumorais envolvendo animais destacam-se os tumores espontâneos, os induzidos por vírus, radiação ou quimicamente induzidos e, os modelos de tumores transplantáveis (NAVALE, 2013).

1.2.1 Tumor de Walker 256

O tumor Walker 256 (W256) é um carcinossarcoma de crescimento rápido, descoberto nos Estados Unidos, no ano 1928, pelo Dr. George Walker, que o descreveu como um tumor progenitor do tamanho de um pequeno ovo e, aparentemente, originado do tecido mamário. Microscopicamente, foi descrito como uma típica estrutura de um adenocarcinoma: algumas áreas de estrutura glandular inalteradas, epitélio proliferativo, lóbulos distorcidos e epitélio de massas irregulares (EARLE, 1935). Este modelo tumoral tem sido amplamente descrito na literatura, revelando alto índice de pega e fácil manuseio (MORANO et al., 2011).

As células são mantidas em laboratório por meio de passagens semanais na cavidade intraperitoneal de ratos e, quando necessário o tumor sólido, este é induzido por via subcutânea ou intramuscular, se tornando palpável cerca de quatro dias pós-implante, podendo crescer até um diâmetro médio de 20-30 mm dentro de oito dias. Este modelo permite observar as três etapas da carcinogênese em um breve período de 12 a 16 dias (AMARAL et al., 2019).

As características do tumor são alteradas de acordo com o local de inoculação, de forma que os tumores inoculados no tecido subcutâneo apresentavam uma característica encapsulada de modo geral e, a inoculação intramuscular que apresenta infiltrações mais numerosas, demonstra um comportamento tumoral mais agressivo. As tentativas de inoculação interespécies não foram bem sucedidas, afirmando desta forma,

que o tumor W256 é um modelo de tumor espécie-específico para ratos (SCHREK, 1935; GOLDACRE; SYLVEN, 1962). Segundo Schrek e Avery (1937), o crescimento e o tempo decorrido entre a inoculação e o aparecimento do tumor são diretamente proporcionais ao número de células injetadas, por via intramuscular.

O implante do tumor sólido é realizado após o teste de exclusão com Azul de Tripan na câmara de Neubauer, seguido da ressuspenção das células e da adição de uma solução antibiótica para evitar a contaminação microbiana (AMARAL et al., 2019).

O tumor W256 é um carcinossarcoma de origem espontânea com comportamento biológico agressivo, sendo localmente invasivo e com alto poder de metástase por via linfática e hematogênica (ALVES et al., 2004), as quais tem sido observadas, após inoculação por via intramuscular, nos rins, baço, pulmões, fígado, suprarrenais, medula-óssea, coração e língua (SILVA et al., 2006).

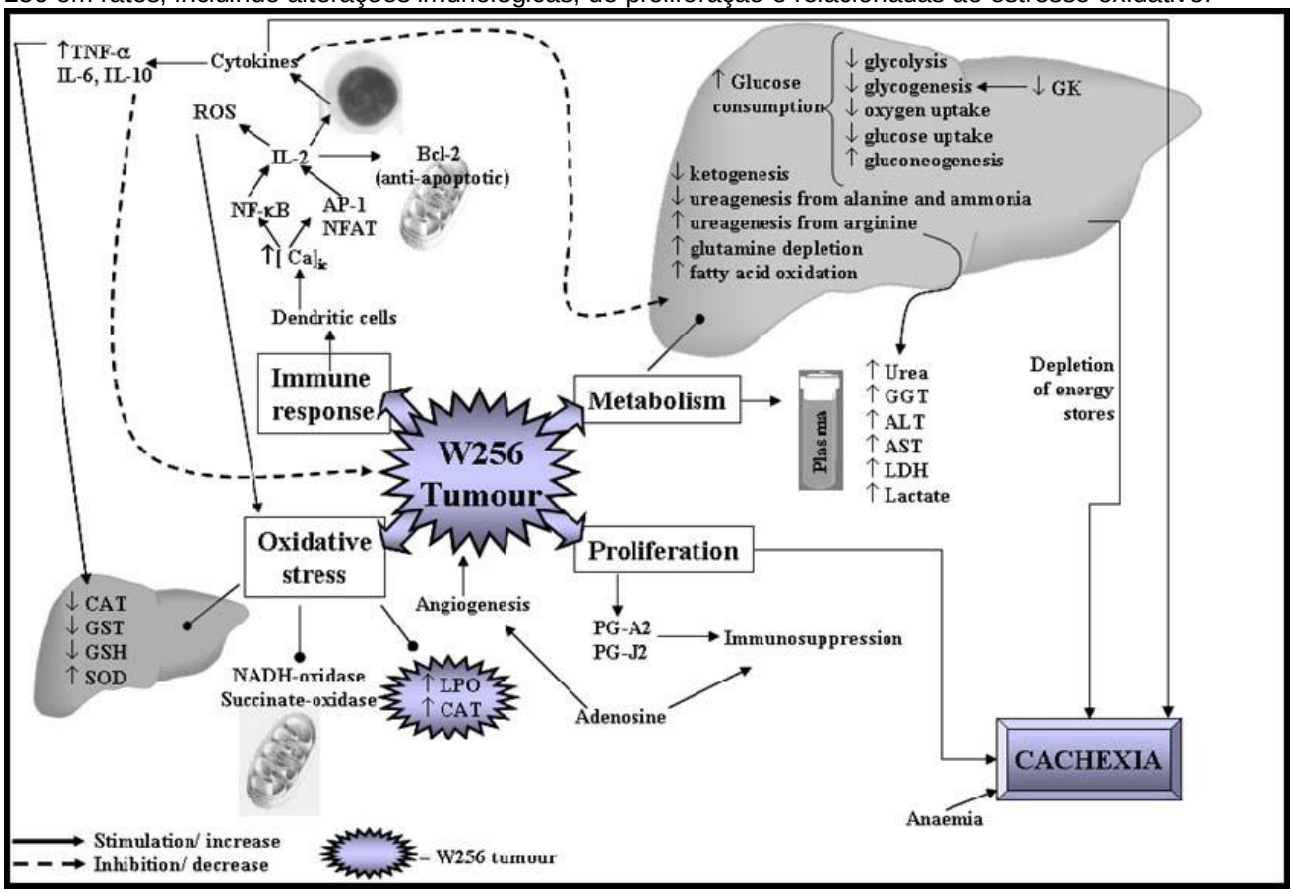
Animais portadores do tumor W256 apresentam significativas alterações hepáticas que influenciam no metabolismo da glicose, da uréia e dos ácidos graxos, principalmente porque os tumores sólidos têm uma tendência de ter fornecimento deficiente de sangue, o que pode levar à hipóxia. Após sua implantação, ocorre redução no peso do animal, dificuldade de ingestão adequada de alimento (anorexia), catabolismo de proteínas, lipídeos e carboidratos. Aos 14 dias após o implante, a massa tumoral pode representar uma fração considerável do peso do animal e a morte pode ocorrer após este período (VICENTINO et al., 2002).

Esta linhagem tumoral tem sido amplamente utilizada em estudos envolvendo dor, atividade enzimática, metabolismo, farmacocinética e farmacodinâmica, drogas antineoplásicas e, caquexia induzida pelo tumor, sendo facilmente transplantado e possuindo uma estabilidade *in vitro* e *in vivo* através do tempo. Alterações decorrentes da formação tumoral estão relacionadas à indução de estresse oxidativo e modificações na resposta inflamatória e imunológica, principalmente pela diminuição da atividade de enzimas antioxidantes, pela estimulação de citocinas pró-inflamatórias e por modificações no metabolismo da glicose e ácidos graxos (ACCO et al., 2012).

Os mecanismos celulares envolvidos no desenvolvimento do tumor W256 estão relacionados com o aumento na expressão de fator de necrose tumoral alfa (TNF-α), interleucina 6, Interleucina 1 e fator de transformação do crescimento beta 1 (TGF-β1) (FOLADOR et al., 2009).

As principais alterações provocadas pela inoculação do tumor W256 no metabolismo hepático, no estresse oxidativo, na resposta imune, na proliferação tumoral e, na caquexia, estão esquematizadas na **figura 3**.

Figura 3 - Principais alterações metabólicas e inflamatórias causadas pela inoculação do tumor de Walker 256 em ratos, incluindo alterações imunológicas, de proliferação e relacionadas ao estresse oxidativo.



Fonte: Acco et al. (2012).

Dentre as inúmeras pesquisas utilizando este modelo tumoral, destacam-se as que envolveram a boca (ALVES et al., 2004), os rins (SILVA et al., 2002), a bexiga (DORNELAS et al., 2006), a vagina e o colo do útero (BRITO et al., 2010), a atividade física (KRYCZYK et al., 2014; MOREIRA et al., 2018), a anorexia-caquexia (REBECA et al., 2008; NASCIMENTO et al., 2016), a angiogênese (BRIGATTE et al., 2016), a dor (SHENOY et al., 2017; KOPRUSZINSKI et al., 2019), a hipertermia (MORANO et al., 2011), a morte celular (BORGHETTI et al., 2015; STIPP et al., 2017), o metabolismo de lipídeos (CHEKHUN et al., 2018), as drogas antineoplásicas (TOSCA et al., 2019), entre outras.

1.3 TRATAMENTO DO CÂNCER

A solução definitiva do câncer é eliminar as células tumorais em sua totalidade (LAI, 2018). Para isto, as três principais formas de tratamento incluem a cirurgia, a radioterapia e a quimioterapia, as quais podem ser usadas em conjunto. Atualmente, poucas neoplasias malignas são tratadas com apenas uma modalidade terapêutica (INCA, 2017a). Pela baixa taxa de sobrevida dos pacientes submetidos à radioterapia e

quimioterapia e, pelo desenvolvimento de resistência das células tumorais, novos agentes terapêuticos mais eficazes e menos tóxicos vem sendo pesquisado ao longo dos anos (CHEN et al., 2014; ABBAS; REHMAN, 2018).

Pesquisas apontam que a quimioterapia tem sido um pilar do tratamento sistêmico do câncer, porém, este tipo de tratamento está fortemente relacionado à ocorrência de efeitos adversos aos medicamentos, entre os quais ocorrem comumente a náusea, o vômito, a fadiga, a alopecia, a neurogenia, a diarréia, a constipação intestinal, as alterações do sistema tegumentar e as neurotoxidades, que trazem vários transtornos aos pacientes, interferindo negativamente na qualidade de vida (COSTA et al., 2015). Além disso, a resistência aos fármacos quimioterápicos é um grande obstáculo ao tratamento bem sucedido, resultando de uma variedade de alterações farmacocinéticas e moleculares, que podem invalidar os tratamentos mais bem planejados, como a absorção e a liberação deficientes do fármaco, a variabilidade geneticamente determinada no transporte, a ativação e depuração do fármaco e, as mutações, amplificações ou supressões nos alvos dos fármacos (CHABNER, 2012).

Outras categorias modernas de tratamento incluem a terapia baseada em hormônios, as modalidades anti-angiogênicas, as terapias com células-tronco, a imunoterapia e a imunoterapia baseada nas células dendríticas (ABBAS; REHMAN, 2018). Todos os tratamentos disponíveis são onerosos e nem sempre resultam na cura, então, a implementação de medidas de prevenção e de detecção precoce vêm sendo apontadas como estratégias baratas e eficientes para a redução nas taxas de incidência e mortalidade (THUN et al., 2010).

No Brasil, os gastos relacionados ao câncer são mais altos do que os gastos com o benefício da aposentadoria, de forma que o custo dos benefícios por incapacidade do câncer foram, aproximadamente, 73,4 milhões de dólares em 2010 e 131,5 milhões em 2015. O custo econômico total do câncer foi estimado em 49,5 bilhões de dólares em 2010 e 59,8 bilhões em 2015 e, está previsto um aumento exponencial, podendo chegar a quase 81 bilhões em 2020 (SIQUEIRA et al., 2017).

1.4 QUIMIOPREVENÇÃO

A quimioprevenção pode ser definida como uso de substâncias naturais ou sintéticas capazes de suprimir, impedir e reverter a carcinogênese nos seus estágios iniciais (DAS et al., 2012; LANDIS-PIWOWAR; IYER, 2014; RATHER; BHAGAT, 2018), podendo ocorrer por meio da utilização de fitoquímicos dietéticos naturais ou drogas terapêuticas

com toxicidade relativamente baixa (FUENTES et al., 2015; CHIKARA et al., 2018; KAPINOVA et al., 2019).

Como a carcinogênese é um processo de múltiplas etapas, substâncias com potencial quimiopreventivo bloqueador e supressor podem agir nas etapas de iniciação e após a promoção/progressão, respectivamente (JOHNSON, 2007), as quais devem ter pouca ou nenhuma toxicidade, alta eficácia em múltiplos locais, possibilidade de ser consumido oralmente, mecanismos de ação conhecidos, baixo custo e aceitação humana (RAJAMANICKAM; AGARWAL, 2008).

Os agentes quimiopreventivos do câncer podem ser hormônios (antiestrogênios e antiandrogênios), medicamentos (estatina, metformina, aspirina e outros anti-inflamatórios), vacinas (HBV e HPV) e, micronutrientes relacionados à dieta (antioxidantes) (BENETOU et al., 2015).

O estresse oxidativo resulta de um desequilíbrio na formação e eliminação de espécies oxidantes, que podem levar à disfunção celular, como uma conseqüência de modificações oxidativas acumuladas, anteriormente, em várias biomoléculas (SILVA et al., 2018b). O dano oxidativo é considerado uns dos principais promotores da carcinogênese e da evolução do câncer, de forma que a atividade antioxidante é importante desde o início da carcinogênese, evitando os danos no DNA pelo estresse oxidativo e a progressão das alterações até o desenvolvimento do câncer (DAI; MUMPER, 2010).

Antioxidantes são conhecidos como depuradores de ERO, pois são capazes de interagir e neutralizar estas espécies, podendo ser produzidos naturalmente pelo organismo (endógenos) e, adquiridos de fontes externas (frutas, legumes e grãos) pela dieta. Estudos comprovam que os antioxidantes são capazes de prevenir danos celulares induzidos por ERO, sugerindo que o desenvolvimento do câncer pode ser retardado no cenário de aumento dos níveis endógenos e exógenos destas substâncias (KOTECHA et al., 2016).

Os sistemas antioxidantes são atualmente divididos em enzimas e grupos não enzimáticos. O grupo enzimático compreende a catalase (CAT), a superóxido dismutase (SOD), a glutationa peroxidase (Gpx) e a glutationa-S-transferase. O não enzimático é composto de moléculas como vitaminas C e E, ácido lipóico, carotenóides, flavonóides e outros (SILVA et al., 2018b).

As ERO são consideradas uma significante classe de carcinogênicos, participando da iniciação, progressão e metástase das neoplasias. São espécies químicas que possuem um ou mais elétrons não pareados na última camada eletrônica, o que os torna muito instáveis, sendo os principais encontrados nas células o superóxido, o hidroxil, o alcoxil, o peroxil e o hidroperoxil (ROSA; COIMBRA, 2009). As fontes exógenas de ERO

são os alimentos, o tabaco, o fumo, as drogas, os xenobióticos, a radiação e outros mediadores, porém elas também são produzidas endogenamente, através de múltiplos mecanismos, incluindo mitocôndrias, peroxissomos, retículo endoplasmático e complexo NADPH oxidase nas membranas celulares (KOCYIGIT et al., 2017).

Em células normais, baixas concentrações destes compostos são necessárias para a transdução de sinais, no entanto, níveis excessivos de ERO podem induzir danos a todos os componentes celulares, incluindo proteínas, lipídios, carboidratos e ácidos nucléicos, oxidando as bases pirimidinas, purinas, e desoxirriboses, levando à mutagênese (KOTECHA et al., 2016). A presença de altas concentrações de biomoléculas oxidadas é associada à alterações no metabolismo aeróbico, na resposta inflamatória, à exposição a radiação UV, hipóxia, anomalias na proliferação celular e infecções virais, estando diretamente associado à várias condições patológicas, incluindo tumores associados ao HPV (SILVA et al., 2018b).

As ERO estão associadas com os três estágios de carcinogênese. Na formação do câncer elas contribuem para o início de mutações no DNA nuclear ou mitocondrial, incluindo mutações pontuais, deleções, translocações cromossômicas e outras. Elas transformam as células normais em cancerígenas, promovendo a proliferação celular, a inibição da apoptose, a regulação de genes e modificações no estado redox para preservar fenótipos malignos (KOCYIGIT et al., 2017).

Compreendendo que aproximadamente 35% dos cânceres estão relacionados com o padrão alimentar (GIBSON et al., 2010) e, que um maior consumo de frutas e verduras está associado com a redução do risco de vários tipos de cânceres (VELMURUGAN et al., 2010), renova o interesse na pesquisa fitoquímica dietética, principalmente nos compostos fitoquímicos que têm sido revelados pelo seu potencial efeito quimiopreventivo ou quimioterápico, confirmando a redução significativa do risco de câncer através de seu consumo regular (ALBULESCU, 2015; KRISTO et al., 2016; MITSIOGIANNI et al., 2019). Uma dieta rica em vegetais e frutas parece fornecer mais proteção do que os micronutrientes individuais (BENETOU et al., 2015).

Os fitoquímicos constituem um conjunto heterogêneo de compostos bioativos que incluem polifenóis, alcalóides, carotenóides e os compostos nitrogenados, os quais são encontrados naturalmente em frutas, legumes, grãos e outros produtos vegetais e são muitas vezes responsáveis por características distintas da planta, como pigmentação de cor e cheiro (KOTECHA et al., 2016).

Os compostos fenólicos são os maiores responsáveis pela atividade antioxidante em frutos, destacando-se pela capacidade de eliminar e evitar a produção de ROS (HEIM

et al., 2002; FOLMER et al., 2014). Atualmente, muita ênfase tem sido dada aos polifenóis, os quais apresentam diversos grupos hidroxilas ligados aos seus anéis aromáticos (CEDRIM et al., 2018), apresentando diversas propriedades, dentre as quais se destacam a antioxidante, a antiinflamatória e a anticarcinogênica, sendo considerados agentes quimiopreventivos primários (KANG et al., 2011).

Os efeitos quimiopreventivos dos compostos fenólicos na carcinogênese se devem às modificações no estado redox e regulações de funções celulares comumente alteradas na doença, como o ciclo celular, a apoptose, a inflamação, a angiogênese e a metástase (DAI; MUMPER, 2010). Estudos iniciais revelaram que estes compostos são capazes de afetar a proliferação celular pela regulação do ciclo celular, e geralmente participam de múltiplas vias de sinalização que são frequentemente interrompidas na iniciação, na proliferação e na propagação tumoral (KOTECHA et al., 2016). Do ponto de vista molecular, estes compostos bioativos apresentam potencial ação modulatória de mecanismos epigenéticos e/ou de vias de sinalização da carcinogênese (PAN et al., 2015).

Os polifenóis promovem as vias celulares anticâncer através de vários mecanismos, incluindo o metabolismo de xenobióticos, o suporte na produção inata de antioxidantes e a estimulação das fases I e II de desintoxicação de carcinógenos, as quais são ativadas através de suas vias pró-oxidantes, nas quais células normais estressadas ativam o sistema de resposta antioxidante humana e o fator nuclear eritróide 2 relacionado ao fator 2 (Nrf2), o qual desencadeia uma infinidade de genes aumentando a produção de antioxidantes, reduzindo o estresse e os danos oxidativos e ativando a desintoxicação de químicos pontencialmente prejudiciais (CHAMP; KUNDU-CHAMP, 2019).

A angiogênese é a chave para o desenvolvimento do câncer, sendo considerada um passo importante na transição dos tumores de um estado benigno para maligno. A antiangiogênese é o processo que impede a formação de novos vasos sanguíneos que fornecem oxigênio para as células tumorais e, neste sentido, fitoquímicos com potencial atividade antiangiogênica, como os flavonóides e antocianinas, contribuem para a quimioprevenção do câncer (KHOO et al., 2017).

A identificação de substâncias que previnam o desenvolvimento do câncer é uma importante medida de saúde pública e, entre as pesquisas descritas na literatura, destacam-se aquelas abrangendo a curcumina (WANG et al., 2016; SANTOS FILHO et al., 2018), o licopeno (SAHIN et al., 2018; BI et al., 2019), o resveratrol (SINHA et al., 2016; ESPINOZA; INAOKA, 2017) e outros polifenóis. Todavia, apesar do potencial benefício para a saúde pública e importância científica, a quimioprevenção do câncer ainda não foi amplamente adotado na prática clínica (KOTECHA et al., 2016).

O conceito de quimioprevenção é intuitivamente atraente, pois implica em evitar o sofrimento causado pelo diagnóstico do câncer, a doença em si e seu tratamento, porém, a latência do câncer está na faixa de anos, sendo mais longa do que a duração dos ensaios clínicos para abordar a eficácia dos agentes quimiopreventivos, que deve considerar a preservação da bioatividade dos compostos na digestão, a determinação da dose adequada e, a segurança em longo prazo, a fim de de identificar até mesmo efeitos colaterais raros (BENETOU et al., 2015).

1.4.1 Antocianinas

A palavra antocianina é derivada do grego 'anthos' que significa flor e 'kyanos' azul escuro. São compostos solúveis em água que fornecem cores para as plantas (folhas, caules, raízes, flores e frutos) que variam de vermelho, roxo a azul, de acordo com o pH ambiental e sua composição estrutural, sendo o mais importante grupo de flavonóides em plantas, com mais de 600 compostos identificados na natureza (RAMOS et al., 2014).

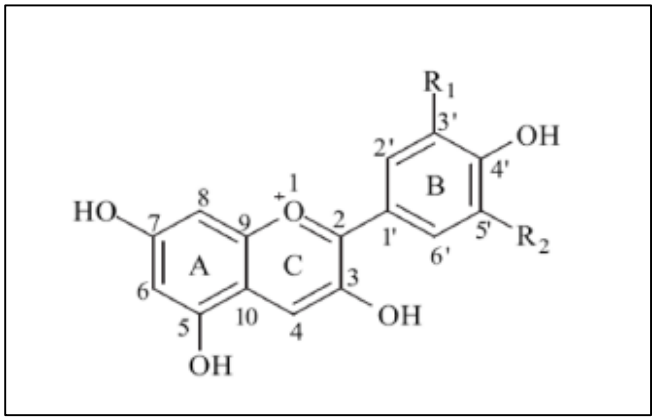
São utilizadas, tradicionalmente, como corantes alimentícios e como remédio para tratar várias doenças devido aos seus efeitos antidiabético (SIVAMARUTHI et al., 2018), anti-inflamatório (LI et al., 2019), antimicrobiano (CHEN et al., 2018), anti-obesidade (AZZINI et al., 2017) e cardioprotetores (LIOBIKAS et al., 2016). Também tem sido extensivamente estudadas por sua propriedades anticancerígenas (LIN et al., 2017), bem como antiangiogênicas (JOSHUA et al., 2017), baseadas em estudos *in vitro*, de culturas celulares e modelos tumorais em animais. As formas desglicosiladas ou agliconas das antocianinas são conhecidas como antocianidinas, das quais, as mais comumente encontradas nas plantas são a cianidina, a delfinidina, a pelargonidina, a peonidina, a malvidina e a petunidina (KHOO et al., 2017) **(Figura 4)**.

A estrutura dos polifenóis tem vários anéis de benzeno com um ou mais grupos hidroxila, sendo dividido como flavonóides e não flavonóides. Entre os flavonóides, as antocianinas possuem dois anéis de benzeno que estão ligados por um anel C de pirona heterocíclico (MARTINS et al., 2018). Esta estrutura fenólica é responsável pela atividade antioxidante, conferida pelos grupos hidroxilo na posição 3 do anel C e também nas posições 3′, 4′ e 5′ do anel B. Em geral, a atividade antioxidante das antocianidinas é superior às suas respectivas antocianinas (glicosídeos), e diminui conforme o número de frações de açúcar aumenta (WANG; STONER, 2008).

As antocianinas são capazes de doar átomos de hidrogênio, quebrar cadeias de oxidação e íons de metais quelantes de transição, inibindo a formação de ERO, através da

indução expressiva de enzimas antioxidantes como a SOD, a CAT, e a Gpx (BARBOSA et al., 2016), evitando a peroxidação lipídica das membranas celulares e os danos ao DNA (PERVAIZ et al., 2017).

Figura 4 – Estrutura química das antocianidinas.



Nome	R1	R2
Delfinidina	OH	OH
Petunidina	OCH3	H
Cianidina	OH	H
Pelargonidina	H	H
Peonidina	OCH3	H
Malvidina	OCH3	OCH3

Fonte: Wang e Stoner (2008).

A cianidina-3-O-glicosídeo (C3G) é um das antocianinas mais disponíveis e, parece impedir a ativação do TNF-α e fator nuclear κB (NF-kB), impedindo a formação de ERO, melhorando o status redox e aumento da ativação do Nrf2. A C3G e a delfinidina-3-glicosídeo inibem a apoptose através da regulação das enzimas caspase 3 e linfoma de células B2 (Bcl-2), proteínas que regulam a morte celular (MARTINS et al., 2018).

1.4.2 *Euterpe oleracea* Martius

O açaizeiro pertence à família Arecaceae que possui, aproximadamente, 200 gêneros e cerca de 2.600 espécies, com distribuição tropical e subtropical em sua maior parte, destacando a *Euterpe oleracea* Martius como uma das mais importantes espécies encontradas no Brasil (SILVA, 2017). *E. oleracea* é uma palmeira tropical, perene e ribeirinha, encontrada tipicamente na região amazônica brasileira (FERREIRA et al., 2011) assim como nos países que fazem fronteira com o norte do Brasil, como o Suriname e as Guianas. Pode chegar a medir cerca de 30 a 40 metros de altura e sua inflorescência pode produzir até dois quilos de fruto, o qual é amplamente conhecido como açaí e, quando maduro, apresenta cor violácea, é redondo, pesa em média 1,5 gramas e tem grande importância econômica, em especial ao estado do Pará (SCHAUSS et al., 2010) **(Figura 5)**.

O açaí tem um consumo identificado como um hábito comum nas regiões Norte e Nordeste do Brasil, sendo considerado um dos principais antioxidantes da dieta brasileira

(TORRES; FARAH, 2017; MARTINEZ et al., 2018). Além do Brasil, o consumo do açaí aumentou significativamente nos últimos anos na Europa e nos Estados Unidos, passando a ser reconhecido como uma “super fruta” (SANTOS et al., 2014; ALESSANDRA-PERINI et al., 2018; LEE, 2019).

Figura 5 - *Euterpe oleracea* Mart., família: Arecaceae, frutos maduros.

Fonte: Silva (2017).

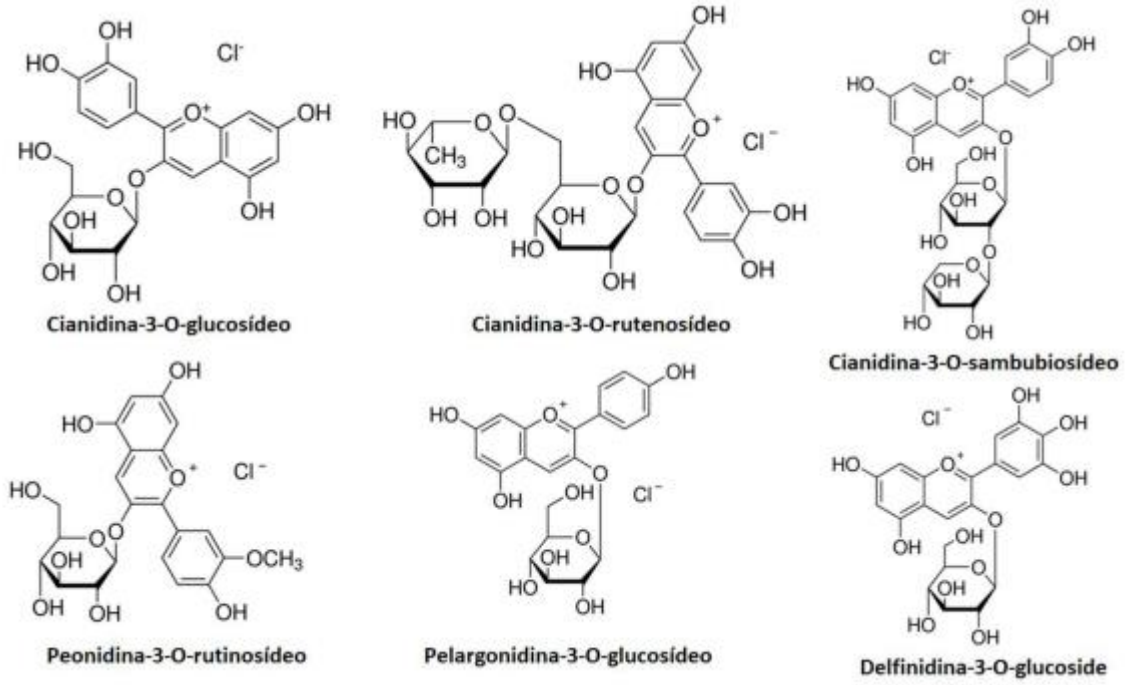
Seu uso como planta medicinal é identificado em diversos países. Na Guiana Francesa, o açaí é usado em combinação com outras plantas medicinais como antimalárico (VIGNERON et al., 2005) e no tratamento da Leishmaniose (SILVA et al., 2018a).

É reconhecido pelo seu valor energético, por conter alto teor de lipídios, como os ácidos graxos essenciais Ômega 6 e Ômega 9, além da abundância de carboidratos, fibras, vitamina E, proteínas e minerais, como o manganês, o ferro, o zinco, o cobre e o crômio (PORTINHO et al., 2012). Além disso, a composição química dos frutos de *E. oleracea* tem uma alta concentração de polifenóis, dos quais o flavonóide antocianina destaca-se como o mais abundante e mais biologicamente ativo (HOGAN et al., 2010; CEDRIM et al., 2018).

Os níveis de antocianinas contidos na polpa de açaí preparada com frutos coletados a partir das mesmas árvores, em diferentes anos, estão entre 88-211 mg/L, de forma que a C3G e a cianidina-3-O-rutinosídeo são as mais prevalentes, seguidas da peonidina-3-rutinosídeo e a peonidina-3-glicosídeo, enquanto a cianidina-3-arabinosil-

arabinosídeo, a cianidina-3-arabinosídeo, a cianidina-3-sambubiosídeo, a cianidina-3-acetil-hexose e a delfinidina-3-glicosídeo são as menos comuns **(Figura 6)**. Outros flavonóides encontrados são a quercetina, a orientina, a epicatequina, a catequina, a rutina, a homoorientina, a isovitexina, a escoparina, a luteolina, entre outras (SILVA, 2017). A presença das antocianinas promove à este fruto intensa capacidade antioxidante e antiinflamatória *in vitro* e *in vivo* quando comparada a outros frutos, sendo considerado, portanto, um alimento funcional (POULOSE et al., 2012).

Figura 6 – Antocianinas encontradas em *Euterpe oleracea* Mart.



Fonte: Cedrim et al. (2018).

Após a ingestão, a absorção das antocianinas inicia-se, na sua forma intacta, no estômago, porém, pelo menos 75% alcançam o cólon, onde são biotransformadas pela ação de bactérias entéricas, sendo degradas em ácidos fenólicos, que são absorvidos. Acredita-se que estes produtos da biotransformação pelas bactérias entéricas estão relacionados aos efeitos benéficos à saúde e, que o perfil da microbiota intestinal de cada indivíduo influencia nos metabólitos finais produzidos e impacta na sua biodisponibilidade, além de que estes estes metabolitos fenólicos podem modular a microbiota intestinal, refletindo no efeito anti-obesidade deste fitoquímico (MARTINS et al., 2018).

Entre os diversos estudos que envolvem a *E. oleracea*, sua fração mais utilizada é a polpa, seguida do suco, do óleo e das sementes, sendo administrada principalmente pela

via oral, com dose variando de 30 mg/kg a 40.000 mg/kg, administradas durante 10 a 35 semanas (ALESSANDRA-PERINI et al., 2018).

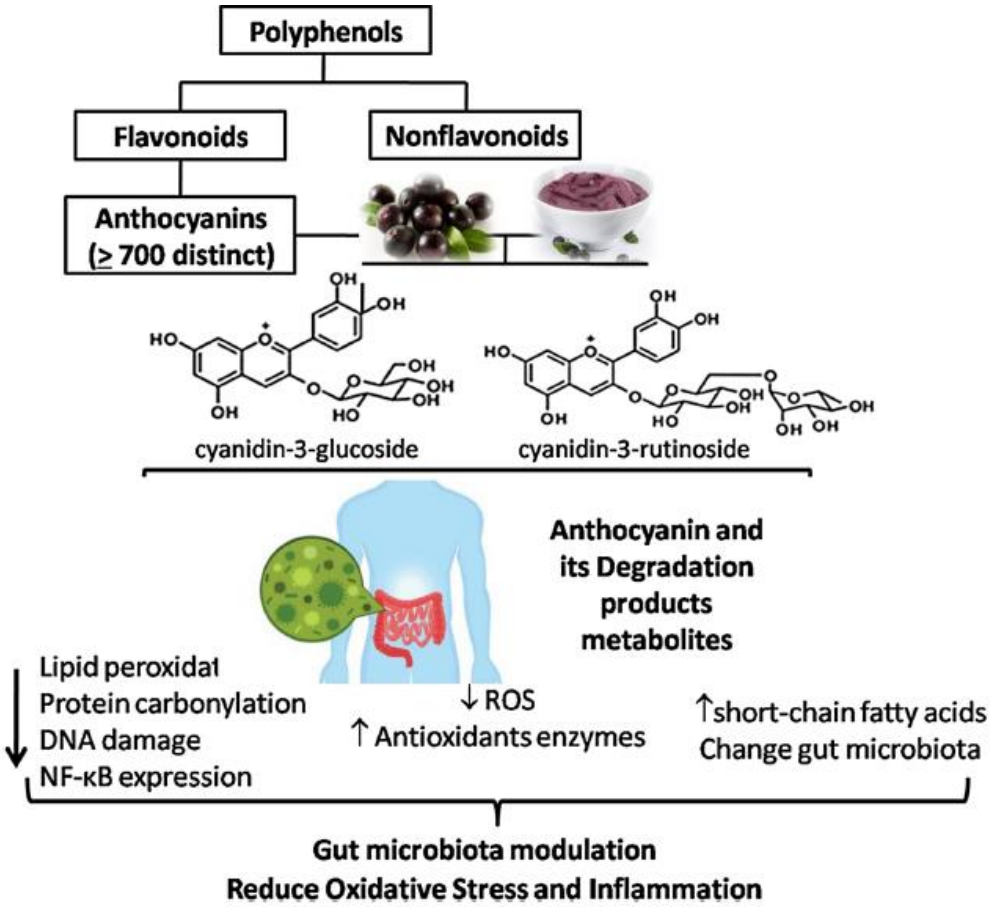
E. oleracea é amplamente investigada em pesquisas científicas, pela gama de fitoquímicos em sua composição, principalmente pela presença das antocianinas, as quais lhe conferem significativas atividades antioxidante (BARBOSA et al., 2016; NASCIMENTO et al., 2016; GARZÓN et al., 2017), anti-inflamatória (POULOSE et al., 2012; MARTINS et al., 2018), antimicrobiana (DIAS-SOUZA et al., 2018; PINA-PÉREZ et al., 2018), cardioprotetiva (ZAPATA-SUDO et al., 2014; MOURA; RESENDE, 2016), anticarcinogênica (CHOI et al., 2017; FREITAS et al., 2017; MONGE-FUENTES et al., 2017; MARTINEZ et al., 2018) e quimiopreventiva (WANG; STONER, 2008; STONER et al., 2010; GOUVÊA et al., 2012; FRAGOSO et al., 2013; DIAS et al., 2014; FRAGOSO et al., 2018).

As atividades anticarcinogênica e quimiopreventiva de *E. oleraceae* são evidenciadas em diversos modelos experimentais de câncer, pela redução da incidência, proliferação, multiplicidade e tamanho de tumores (ALESSANDRA-PERINI et al., 2018). Diferentes pesquisas envolvendo esta espécie corroboram entre si na hipótese de que a *E. oleracea* modula o estresse oxidativo, diminui a produção de ERO e a inflamação nas células endoteliais, previne a peroxidação lipídica, aumenta as enzimas antioxidantes e, influencia na expressão de Nrf2 e NF-kB, além de aumentar os níveis dos ácidos graxos de cadeia curta e regular a microbiota intestinal (MARTINS et al., 2018) **(Figura 7).**

No entanto, existem poucos estudos com um ponto de vista metodológico preciso que poderia ajudar a definir um efeito dose-dependente e seu mecanismo de ação, o qual, dado seu desempenho em vários sistemas biológicos, parece ser complexo. A maioria dos estudos propõe o impacto do consumo de *E. oleracea*, mas não especificam o possível composto ativo, dado a diversidade de fitoquímicos presentes. Em conjunto a isso, todas as características favoráveis ao consumo de *E. oleraceae*, acrescidas da sua exuberante disponibilidade na região Norte, foram os fatores determinantes na escolha desta espécie para esta pesquisa e, embora ela represente um dos principais antioxidantes da dieta brasileira, com consumo aumentando consideravelmente no Brasil e em todo mundo, ainda existem poucos estudos acerca de sua atividade antitumoral e quimiopreventiva em modelos que avaliem o crescimento tumoral, especialmente em cérvice uterina.

Desta forma, este trabalho objetiva testar o efeito quimiopreventivo de um extrato hidroetanólico padronizado de *E. oleracea* (SILVA, 2017) em modelo tumoral de W256, baseando-se na hipótese de que o estresse oxidativo, estimulado pela carcinogênese do

Figura 7 – Modelo hipotético do mecanismo de ação de *E. oleracea* no estresse oxidativo, inflamação e microbiota intestinal.



Fonte: MARTINS et al. (2018).

do tumor W256, propicia a carcinogenêse colo uterina pelo HPV e, a intensa quantidade de antocianinas estáveis no extrato atuam como potentes antioxidantes promovendo a quimioprevenção do tumor.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o efeito quimiopreventivo do extrato hidroetanólico padronizado de *Euterpe oleracea* Mart. (EHPEo) sobre o crescimento tumoral de Walker 256, induzido na cérvice uterina de ratas Wistar.

2.2 OBJETIVOS ESPECÍFICOS

a) Caracterizar quimicamente o EHPEo;
b) Avaliar o efeito quimiopreventivo do EHPEo sobre crescimento tumoral;
c) Avaliar as alterações citológicas nas células cervicais;
d) Caracterizar macroscopicamente e microscopicamente o tumor.

Submetido para publicação no periódico Frontiers in Pharmacology.

THE CHEMOPREVENTIVE EFFECT OF THE STANDARDIZED HYDROETHANOLIC EXTRACT OF *Euterpe oleracea* Mart. ON THE TUMOR GROWTH OF WALKER 256 IN THE UTERINE CERVIX OF FEMALE WISTAR RATS

Letícia Elizandra Mehl Boettger[1,3], Clarice Flexa da Rocha[2], Helison de Oliveira Carvalho[2], Heitor Ribeiro da Silva[2], Fernanda Monteiro Rocha[1], Ivanilson Lobato da Costa[1], Abrahão Victor Tavares de Lima Teixeira dos Santos[2], Brenda Lorena Sánchez Ortíz[2], Lucas Silva Abreu[4], Josean Fechine Tavares[4], José Carlos Tavares Carvalho,[2,3], Clarissa Silva Lima,[1,3,4*]

[1]Laboratory of Research on Reproductive Toxicology, Department of Biological Sciences and Health of the Federal University of Amapá, Macapá, AP, Brazil, [2]Laboratry of Research on Drugs, Department of Biological Sciences and Health, Federal University of Amapá, Macapá, AP, Brazil,, [3]Postgraduate Program in Pharmaceutical Sciences, Department of Biological Sciences and Health, Federal University of Amapá, Macapá, AP, Brazil, [4]Postgraduate studies in Natural and Synthetic Bioactive Products, Multi-user Laboratory for Characterization and Analysis; Department of Pharmaceutical Sciences, Institute of Research on Drugs and Medications, Federal University of Paraíba, João Pessoa, PB, Brazil.

**Correspondence:*
Dr. Clarissa Silva Lima
clarissalima@unifap.br

Cancer is characterized by the uncontrolled growth of cells that might invade several tissues, being one of the most common causes of death in the world. The oxidative damage is considered one of the main promoters of carcinogenesis and cancer evolution. Several problems in cancer therapy, such as low effectiveness, toxicity and development of resistance to the treatment have been driving the search for new drugs. In this context, the chemopreventive agents stand out, which are phytochemicals obtained from fruits and vegetables, and that has high antioxidant capacity. *Euterpe*

oleracea Martius (açai palm) is a tropical palm tree typically found in the Amazon rainforest region, widely investigated by the chemical composition of its fruits, in which the anthocyanins are the most abundant and more biologically active polyphenols. The purpose of this work is that of investigating the chemopreventive effect of the standardized hydroethanolic extract of *E. oleracea* Mart. (SHEEo) in the tumor growth of Walker 256 (W256) in the uterine cervix of female Wistar rats. The extract was obtained by using multifactorial planning with the purpose of obtaining a higher concentration of e anthocyanins. Using FTIR, HELC-UV-S-UV and HELC-UV-S-MSn made the chemical characterization. The chemopreventive treatment was carried out in the animals, which were divided into 5 experimental groups: Negative Control Group (0.5mL of distilled water/70 days), Positive Control Group (N-Acetyl Cysteine 300mg/Kg/70 days), Treated Group I (SHEEo 150mg/Kg/70 days), Treated Group II (SHEEo 300mg/Kg/70 days) and Treated Group III (SHEEo 300mg/Kg/82 days). The W256 carcinosarcoma tumor cells were inoculated in the vaginal canal of the rats and were assessed by cytology and histopathology. The phytochemical analysis revealed the presence of cyanidin-3-glucoside and cyanidin-3-rutinoside, which are the most abundantly found anthocyanins in *E. oleracea*. The tumor engraftment index was of 100%, with a lower tumor growth at the concentration of 300mg/Kg of the SHEEo. The cytomorphological criteria of malignancy, cell pleomorphism and hyperchromasia were more evident in the Negative Control Group. The histopathological analysis revealed less tumor cellularity at the concentration of 300mg/Kg. Therefore, the conclusion is that the SHEEo presented chemopreventive capacity at the dosage of 300mg/Kg.

Keywords: cancer, uterine cervix, chemoprevention, *Euterpe oleracea*, anthocyanin, antioxidant, Walker 256 tumor, vaginal cytology.

1 INTRODUCTION

Cancer is characterized by the uncontrolled growth of cells that might invade several tissues, being classified as the main cause of death and the most important barrier to be crossed in order to increase life expectancy of the world (Bray et al., 2018). According to the World Health Organization, cancer was responsible for 9 million deaths in 2016 (WHO, 2018).

Globally speaking, the majority of the cases of uterine colon cancer (UCC) occur in areas where there are lower levels of human development (Ferlay et al., 2015). With regards to Brazil, the estimation is of 16,370 new cases of UCC for each year of the biennium 2018-2019, with an estimated risk of 15.43 cases every other 100 thousand women, being the third the most frequent one. The North

Region of the country stands out because the UCC is the most incidental, if the non-melanoma skin tumors (25.62/100 thousand) are taken from the equation (INCA, 2017).

Even being preventable, the UCC is classified as the forth most frequent type of cancer and the fourth cause of death by cancer amongst women in the world, with an estimation of 570,000 cases and 311,000 deaths in 2018 (Bray et al., 2018).

The viruses have been recognized as causing some types of cancer, and the persistent chronic infection by the oncogenic subtypes of the *human Papillomavirus* (HPV) is the main cause of the UCC. However, other associated risk factors strongly corroborate for the development of this type of cancer, since they impair the immune response to the infection by the HPV (Cohen et al., 2019). The effective triggering of the immune response is indispensable for the viral elimination (Song et al., 2015); therefore, an improper release of proinflammatory mediators, such as the reactive oxygen species (ROS), might contribute for the development of the UCC in the chronification process of the infection (Boda et al., 2018; Nunes et al., 2018).

The oxidative damage is considered one of the main promoters of carcinogenesis and cancer evolution (Dai and Mumper, 2010). When the ROS concentration increases so that it is higher than the endogenous antioxidant defense capacity, the oxidative stress occurs and, in this context, the phenolic compounds stand out due to their capacity of eliminating and avoiding the production of ROS (Folmer et al., 2014).

Even though chemotherapy and radiotherapy are the main cancer treatments, on account of their low survival rate, new therapeutic agents that are more efficient and less toxic have been researched along the years (Chen et al., 2014; Abbas and Rehman, 2018). The cancer chemoprevention is an important strategy for inhibiting the malignant transformation of the cells during the carcinogenesis promotion stage or progression stage (Landis-Piwowar and Iyer, 2014; Rather and Bhagat, 2018), which might happen by the use of natural dietary phytochemicals or therapeutic drugs with relatively low toxicity (Fuentes et al., 2015; Chikara et al., 2018; Kapinova et al., 2019).

Different classes of phytochemicals compounds isolated from fruits and vegetables have been revealed because of their potential chemopreventive or chemotherapeutic effects, confirming the significant decrease in the risk of developing cancer by regularly consuming them (Albulescu, 2015; Kristo et al., 2016; Kocyigit et al., 2017; Mitsiogianni et al., 2019). In this sense, the research with turmeric (Wang et al., 2016; Santos Filho et al., 2018), lycopene (Sahin et al., 2018; Bi et al., 2019), resveratrol (Sinha et al., 2016; Espinoza and Inaoka, 2017) and other polyphenols stand out; however, despite their potential benefits for public health and scientific importance, cancer chemoprevention has not yet been widely adopted in the clinical practice (Benetou et al., 2015; Kotecha et al., 2016).

Euterpe oleracea Martius is a tropical palm tree typically found in the Amazon rainforest region (Martins et al., 2018), the fruit of which is widely known as açai, and its consumption has been

identified as a common habit in the North and Northeast regions of Brazil (Torres and Farah, 2017; Martinez et al., 2018). Besides Brazil, the consumption of açai has significantly increased in recent years in Europe and in the United States, acquiring the reputation of "superfruit" (Santos et al., 2014; Alessandra-Perini et al., 2018; Lee, 2019).

The chemical composition of the *E. oleracea* fruits have been widely investigated, in which the anthocyanins have stood out as being the most abundant polyphenols (Cedrim et al., 2018), providing significant antioxidant activities (Barbosa et al., 2016; Nascimento et al., 2016; Garzón et al., 2017), as well as anti-inflammatory (Poulose et al., 2012; Martins et al., 2018), antimicrobial (Dias-Souza et al., 2018; Pina-Pérez et al., 2018), cardioprotective (Zapata-Sudo et al., 2014; Moura and Resende, 2016), anticarcinogenic (Choi et al., 2017; Freitas et al., 2017; Monge-Fuentes et al., 2017; Martinez et al., 2018) and chemopreventive (Stoner et al., 2010; Fragoso et al., 2013; Dias et al., 2014; Fragoso et al., 2018).

The chemopreventive effects of the phenolic compounds on carcinogenesis are due to the modifications in the redox state and regulations of cell functions commonly changed in the disease, such as the cell cycle, apoptosis, inflammation, angiogenesis and metastasis (Dai and Mumper, 2010). From the molecular point of view, these bioactive compounds present a potential modulatory action of epigenetic mechanisms and/or carcinogenesis signaling pathways (Pan et al., 2015).

Even though *E. oleracea* represents one of the main antioxidants of the Brazilian diet (Torres and Farah, 2017), and its consumption has considerably increased in Brazil and all over the world, there are still only a few studies regarding its antitumor and chemopreventive activities in models that assess the tumor growth, especially in the uterine cervix. Therefore, this word has as purpose to test the chemopreventive effects of a standardized extract of *E. oleracea* (Silva et al., 2018) in a Walker 256 (W256) tumor model (Earle, 1935).

2 MATERIALS AND METHODS

2.1 Material Vegetal

Ripe fruits of *Euterpe oleracea* Mart. were collected in the morning, in the municipality of Santana – Amapá, Brazil (S 0° 00'14''/W 51° 13'56'') for the preparation of the hydroethanolic extract. Samples of leaves, inflorescences, flowers and fruits of *E. oleracea* were filed, for the purposes of identification, with the Herbarium of Amapá (HAMAB – Herbarium of Amapá/Institute of Scientific and Technological Research of the State of Amapá – IEPA), according to registration: Brazil, Amapá, Santana, Matapi River, Bela Vista Farm, 17.IV.2019, *L.E.M. Boettger*, 1 (HAMAB).

2.2 Standardized Hydroethanolic Extract of *Euterpe oleracea* Mart. (SHEEo)

The extraction process was developed based on a multifactorial planning, with the purpose of obtaining a higher concentration of anthocyanins in the fruits, as per the methodology described by Silva et al. (2018). In order to obtain the extract, 100g of the ripe fruit were macerated into 400mL of 92% alcohol (Start®) and 75mL of P.A. glacial acetic acid (Qhemis®), for 4 hours, being constantly agitated in an automatic agitator (Nova Ética®). After that, the extract was filtered in #1 Whatman paper filter and preserved from the incidence of light in an amber vessel. All of the processes were carried out at ambient temperature (25 ± 2 °C).

The SHEEo was concentrated in a vacuum rotary evaporator (Ika-Werke®) at a temperature lower than 35 °C until all of the residual solvents had evaporated. After that, the extract was frozen at -20 °C and later, lyophilized for 72 hours (LS 3000, Terroni®). The lyophilized extract was kept in a desiccator for 24 hours.

2.3 Spectroscopic profile in the infrared region through the Fourier (FTIR) transformation of the SHEEo

In order to obtain the spectroscopic profile in region IV of the SHEEo, the readings were made in an IRAffinity-1 (Shimadzu®) spectrometer, recording the readings in the wavelength within the rage of 4000 cm^{-1} – 450 cm^{-1}, with a 1 cm^{-1} resolution and the accumulation of 100 scans (scan rate of 0.5 cm^{-1}/s). Appropriate amounts of Potassium Bromide (KBr) were used as blank (Silva et al., 2018).

2.4 Profile through highly efficient liquid chromatography associated with the ultraviolet spectroscopy (HELC-UV-S) of the SHEEo

The HELC-UV-S profile of the SHEEo was obtained by using a highly efficient liquid chromatograph associated with a spectroscope (Shimadzu® Class-VP) equipped with a high-pressure pump (LC-20 AT), an automatic sampler (SIL-20AC) and a UV SPD 20A detector. A C-18 Agilent column was used, at 30 °C, with a flow of 0.5ml/min and a gradient programming with two mobile phases: phase A, methanol: water: acetic acid (14: 85: 1), and phase B, acetonitrile: acetic acid (99: 1). The process started with a ratio of 95: 5 (A: B) and was completed with a ratio of 80:20 (A: B). Its execution time was of 15 minutes. The wavelength used was of 520 nm. The standard used was cyanidin-3-O-glucosíde (Sigma-Aldrich®) (Silva et al., in press).

2.5 Liquid chromatography–high-resolution and iontrap (MS^n) mass spectrometry instrumentation and conditions

A Shimadzu® (Kyoto, Japan) High Performance Liquid Chromatography System, coupled with a Amazon X or micrOTOF II (Bruker Daltonics, Billerica, MA, USA) with an electrospray ion (ESI)

source, was used to perform the ESI-IT-MS/MS and ESI-TOF-MS analysis, respectively. The LC System consisted of a LC-20AD solvent pump unit (flow rate of 600 µL.min^{-1}); a DGU-20A$_5$ online degasser; a CBM-20A system controller and a SPD-M20A (190 – 800 nm) diode array detector. The LC separation was performed on a Kromasil C-18 5mm 100Å, 250 x 4.6 mm (Kromasil, Bohus, Sweden) analytical column. Injections (20 µL) were performed using an autosampler (SIL-20A). The mobile phase consisted of 0.1% formic acid in water (solvent A) and methanol (solvent B). Exploratory gradient was performed to eluition in 60 minutes. The analysis parameters are as follows: capillary 4.5 kV, ESI in positive mode, final plate offset 500 V, 40 psi nebulizer, dry gas (N2) with flow rate of 8 mL/min and a temperature of 300 ºC. CID fragmentation was achieved in auto MS/MS mode using advanced resolution mode for MS and MS/MS mode. The spectra (m/z 50–1000) were recorded every two seconds (Abreu et al., 2017).

2.6 Animals

Female rats of the Wistar lineage were used (Rattus novergicus albinus), being 90 days old and weighing approximately 250g, kept in ventilated racks (Alesco®), under controlled temperature (22 ± 2 ° C), humidity (80%) and light-dark cycle (12/12 hours). The rats were fed with ration (Primor®) and filtered water ad libitum.

The Committee on Ethics in the Use of Animals approved the experimental protocol of this study (Federal University of Amapá) under registration n^{o}. 021/2017.

2.7 Experimental design

After the adaptation period, the animals were randomly distributed into five groups (n = 5/group). The chemopreventive treatment, given orally, was started in the 90th day after their birth, previously inoculation of tumor cells. The groups were set forth and treated as follows:

Negative Control Group (0.5ml of distilled water/70 days);
Positive Control Group (N-Acetyl cysteine 300mg/Kg/70 days);
Treated Group I (SHEEo 150mg/Kg/70 days);
Treated Group II (SHEEo 300mg/Kg/70 days);
Treated Group III (SHEEo 300mg/Kg/82 days).

2.8 Assessment of the ponderal evolution and the consumption of water and feed

These analyses occurred during the entire period of chemoprevention and tumor growth, until the day when the animals were submitted to euthanasia (172th day after their birth). Scales (BG400, Gehaka®) with a capacity of 4200g and precision of 0.1g were used for the daily assessment of the body mass. 200ml of water were daily made available, and the water consumption was measured in a 500ml

beaker. For the feed consumption, 50g of ration were daily made available for each animal and said consumption was measured in scales (BG400, Gehaka®).

2.9 *In vivo* aliquot of W256 carcinosarcoma cells

The maintenance of the W256 tumor cells occurred through successive transplants carried out each 7 days. The tumor removed from the donating animal was incubated for 5 minutes in a Petri dish containing 5.0ml of ringer's lactate solution (Eurofarma®) and a Gentamicin Sulphate solution (10mg/ml) (Novafarma®). Afterwards, the tumor tissue without necrotic focuses was macerated into small fragments of approximately 2 millimeters, which were then transferred to another dish containing a ringer's lactate solution and a Gentamicin Sulphate solution at the same concentrations as before, remaining incubated for 3 minutes. After that, the fragments were filtered in a sterile gaze and 1.0ml of the suspension of 2,0 x 10^6 cells/ml was intramuscularly inoculated into the inner right thigh of female Wistar rats, with ages between 6 to 8 weeks (Oliveira et al., 1998; Morano et al., 2011).

2.10 Inoculation of the tumor cells

One day before the tumor cells were inoculated (70^{th} day of treatment with the SHEEo), all of the animals were anesthetized with Xylazine (10mg/kg) and Ketamine (70mg/kg). In order to induce an inflammatory process in the vaginal mucosa, 0.3ml of 10% acetic acid (Qhemis®) was introduced in the vaginal canal of the rats. After 24 hours, under the anesthetic effect in the same conditions previously described, scarification was performed with an endocervical brush (Kolplast®) in the vagina and uterine cervix of the rats, followed by the inoculation of 0.3ml of 2.0 x 10^6 tumor cells/ml (Brito et al., 2010).

2.11 Vaginal cytology

The collection of the vaginal smears was carried out during the four days before the inoculation of the tumor cells (67^{th} to 70^{th} days of treatment with the SHEEo) and, after that, on the four days before submitting the animals to euthanasia (78^{th} to 81^{st} days of treatment with the SHEEo). In order to do so, 0.2ml of physiologic serum was introduced into the vaginal canal of the rats, with the aid of a micropipette (Digipet®), in a depth of approximately 5 through 10mm. After that, the vaginal washes obtained were deposited in previously identified sheets. In order to fix the cells, the spray cytological fixative (Adlin Diagnóstico®) was used, and these cells were stained with the use of the Papanicolaou staining kit (Newprov®) (Consolaro and Maria-Engler, 2016). In order to assess the presence of cell changes compatible with malignant tumors, the sheets were analyzed in an optical microscope (Nikkon® E200), with an amplification of 10x and 40x (Cora et al., 2015).

2.12 Macroscopic and microscopic analyses of the organs

On the 12th day after tumor cells were implanted, the animals were euthanizes and necropsied for the macroscopic ad microscopic analyses of the organs (heart, lung, uterus, ovary, kidneys, liver, spleen and pancreas) and the tumor, which were weighed and fixed in 10% formalin. For the histopathological analysis, they were included in transversal sections in automatic microtome (Slee medical® CUT 6062) with a thickness of 5 µm. All of the sheets were stained with the use of the Harris hematoxylin technique (Laborclin®) and yellowish eosin (Inlab®), and mounted with Canadian balm. Five sheets per animal were prepared, with each of them containing 1-3 cuts, and they were analyzed in an optical microscope (Olympus® CX21LED) with magnification of 10x and 40x, photographed with a MDCE-5C USB 2.0 (digital) camera, and blindly examined by two observers, at the Laboratory of Reproductive Toxicology of the Postgraduate Department/UNIFAP. The analyzed parameters were: infiltration of undifferentiated neoplastic small cells, tissue necrosis, infiltration of granulocytic cells and hemorrhage areas (Silva et al., 2006).

2.13 Statistical analysis

The results obtained in the several analyses are expressed in average ± standard error of the average (average ± S.E.A.) of each experimental group. In order to compare the data from the assessed groups, the variance analysis (ANOVA) was applied, followed by the Tukey test. The significance level considered was of 5% ($p < 0.05$). The software used was GraphPad Prism® (version 5.03).

3 RESULTS

3.1 Spectroscopic profile of the infrared per Fourier transformation (FTIR) of the SHEEo

The infrared spectrum of the SHEEo can be seen in Figure 1. The spectrum of the infrared region showed a band associated with the vibration of the stretch of the O-H couplings (3362.07cm-1), a characteristic band of the stretches in the C–H couplings (2924.21cm-1) and another of the ester group carbonyl (1753.67 cm-1). A band corresponding to the C–C stretch of the aromatic rings (1608.70cm-1) and another relating to the axial deformation of the C=C coupling of the aromatic rings (1512.25cm-1) were verified. The spectrum also revealed a band referring to the deformation of the C–H couplings (1440.88cm-1) and another corresponding to the stretch of the C–O couplings of phenols (1026.17cm-1). Other bands were observed between 1456-1419cm-1; 1377-1340cm-1 and 1155-889cm-1.

3.2 Profile through highly efficient liquid chromatography associated with the ultraviolet spectroscopy (HELC-UV-S) of the SHEEo

The HELC-UV-S digital imprint of anthocyanin is represented in **Figure 2**. The chromatographic profile per HELC-UV-S revealed four peaks with retention times: 5,175; 6,348; 6,786 and 7,712. The fourth peak was identified as cyanidin-3-O-glucoside (by the retention time as compared to the standard and coelution). The UV spectrum demonstrated the characteristic peaks of the anthocyanins in 281nm and 523nm.

3.3 Profile through highly efficient liquid chromatography coupled with the ion trap mass spectrometry

The liquid chromatogram obtained through HELC-MS^n of the SHEEo is presented in **Figure 3**. In the positive ionization mode **(Figure 3A),** a peak at 25,4 minutes and another one at 26.0 minutes (majority peak) are observed, the mass spectrum of which showed fragments that are characteristic of cyanidin-3-glucoside and cyanidin-3-rutinoside, which are the anthocyanins that are more abundantly found in the pulp of *E. oleracea*. In the negative ionization mode **(Figure 3B),** the majority peak occurred in the retention time of 32.2 minutes, with fragments that are characteristic of the orientin flavonoid. The characterization of the compounds of the SHEEo identified via HELC-MS^n is described in **Table 1.**

3.4 Assessment of the SHEEo chemoprevention on the tumor growth

The implantation and W256 tumor growth rates were of 100%, both in the vagina and in the uterine cervix. In the macroscopic examination of the organs, no visceral impairment was observed on near structures possibly affected by the proximity, except for the urethra and rectum, which were compressed by the expansion of the tumor growth, causing urine and feces retention in the animals of the Negative Control Group and Treated Group I (SHEEo 150mg/Kg/70 days). The tumor grew, in all of the animals of all of the groups, in the form of an abnormal mass of bulky, solid, consistent and reddish tissue.

The tumor growth was different amongst the assessed groups. A statistical difference was observed when comparing the Treated Group II (SHEEo 300mg/Kg/70 days) with the Negative Control Group, showing that the SHEEo had a chemopreventive effect at the concentration of 300mg/Kg. However, when continuing with the treatment with the SHEEo at the dosage of 300mg/Kg for more than 12 days after the tumor cells had been inoculated which growth inhibition process was not observed **(Figure 4).**

3.5 Assessment of the ponderal evolution and the consumption of water and ration

The ponderal evolution of the animals showed no statistic differences between the groups, that is, the body mass gain was similar in all animals **(Figure 5).** With regards to the weight of the organs

of the animals (liver, heart, lungs, kidneys and spleen), there was no statistic difference amongst the groups **(Table 2).**

The consumption of water did not differ statistically between groups, however, there is an abrupt decrease in the consumption of water by all animals groups after tumor cells inoculation **(Figure 6).** Animal feed intake also did not differ statistically between the groups, however, a mild decrease in the consumption of ration by the animals was observed after the tumor cells were inoculated **(Figure 7).**

3.6 Vaginal cytology

The analysis of the cell morphology of the vaginal epithelium of all of the rats, after the Walker 256 carcinosarcoma was inoculated, revealed the presence of roundish, basophilic and nucleated small cells, which are characteristics compatible with those of the W256 tumor cells **(Figure 8).** The tumor cellularity varied amongst the groups, being more abundant in the Negative Control Group. Changes were observed in the pattern of the cell types present, when compared to the cytology before and after the tumor inoculation. These cells were identified by cytomorphological´s criteria of malignancy which had been found in post-inoculation smear cells.

The presence of cell alteration´s which as pleomorphism and hyperchromasia was primarily verified in the cervical cells after the W256 had been inoculated, which attended malignancy´s criteria and it is cellular alterations compatible with malignant cells that had been more evident in the Negative Control **(Figures 9 and 10)**.

3.7 Histopathological analysis

Histopathological analysis of the solid tumor mass **(Figure 11)** revealed that the SHEEo, at the dosage of 300mg/kg/70days, was able to promote a chemopreventive effect in the uterine cervix after the implantation of the W256 carcinosarcoma **(Figure 11D)**. Analysis of the other groups revealed neoplastic findings by the presence of pleomorphic cells and invasion of other tissues. However, in the treated group III, which received doses of 300 mg/kg and the treatment lasted 82 days (that continued after the tumor cells were inoculated), the tumoral cellularity found, in your totality, was lower, when compared to the other groups **(Figure 11E).**

Regarding the histopathological analysis of organs such as heart, uterus, ovary, kidneys, and liver, metastasis was observed in all groups analyzed. However, in some organs (spleen, pancreas, and lung), no changes or metastases were found during the analysis that met criteria compatible with malignancy.

4 DISCUSSION

In the extractive process, the use of a weak acid and a more concentrated (92%) ethanol solution, at ambient temperature, increase the solubility and the stability of the anthocyanins, and the lyophilization is necessary in order to preserve the chemical and pharmacological properties, as well as the physiochemical and microbiological stability (Silva et al., in press). In this study, these procedures were indispensable in order to improve the quality of the SHEEo.

All of the bands observed in the infrared spectrum obtained from the SHEEo corresponded to the bands revealed by other authors for different extracts of anthocyanins and by standard of cyanidin-3-O-glucoside (Teixeira-Neto et al., 2009; Ahmed et al., 2015). The bands observed between 1456-1419 cm^{-1}, 1377-1340 cm^{-1} and 1150-889 cm^{-1} are associated with the presence of monosaccharides, such as glucose and galactose, which are commonly present in these metabolites (Silva et al., in press).

The majority peak (7.71 min) found in the digital imprint HELC-UV-S of the SHEEo refers to an anthocyanin cyanidin-3-O-glucoside. Gouvêa et al. (2012) also isolated the cyanidin-3-O-glucoside using HELC-UV-S of a lyophilized extract of *E. oleracea*, which showed a high level of purity (98.9%). The UV spectrum revealed the characteristic peaks of the anthocyanins at 281nm and 523nm. Silva et al. (in press) showed a maximum absorption band for anthocyanins in the UV interval between 270 and 282nm. According to Lopes et al. (2007), the anthocyanins have an intense absorption within the range of 520 through 560 nm (visible region).

The chromatogram obtained through HELC-MS^n of the SHEEo, in the positive ionization mode, presented peaks characteristic of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside, which are the anthocyanins more abundantly found in the pulp of açai (Silva et al., 2016). In the negative ionization mode, the majority peak represented the orientin flavonoid. These results corroborate other studies related to the identification of the chemical composition of *E. oleracea* (Rosso et al., 2008; Mena et al., 2012; Dias et al., 2013; Garzón et al., 2017).

Polyphenols promote the anticancer cell pathways through several mechanisms, including the metabolism of xenobiotics, the support in the natural production of antioxidants and stimulation of phases I and II of carcinogens detoxing, which are activated through their pro-oxidant pathways, in which normal stressed cells activate the human antioxidant response system and the Erythroid Nuclear Factor 2 (Nrf2), which initiates an infinity of genes, thus increasing the production of antioxidants, reducing both stress and the oxidative damages, activating the detoxing of potentially harmful chemicals (Champ and Kundu-Champ, 2019).

The antioxidant activity is important from the beginning of the carcinogenesis, avoiding damages to the DNA caused by the oxidative stress, the progression of the changes until cancer is developed (Dai and Mumper, 2010). Lee et al. (2014) suggested that the anthocyanins reduce the ROS levels

through the activation of Nrf2 in activated lipopolysaccharide macrophages (LPS), however, the anti-inflammatory effect was independent of Nrf2.

The anticarcinogenic and chemopreventive activities of the *E. oleracea* are evidenced in several experimental models by the decrease in the incidence of tumors, in the proliferation of tumor cells, as well as in the multiplicity and size of the tumors (Alessandra-Perini et al., 2018). Martinez et al. (2018) suggested that the *E. oleracea* has a high antioxidant capacity and might have a protective effect against the lung cancer. Monge-Fuentes et al. (2017) reported that the *E. oleracea* is an efficient photosensitizing agent in the decrease in the carcinogenesis of the melanoma.

Nascimento et al. (2016) also described the anticarcinogenic effect of the *E. oleracea*, in the anorexia-cachexia syndrome induced by W256 tumors. *E. oleracea* proved efficient in the carcinogenesis of the colon induced by 1,2-dimethylhydrazine (DMH) (Fragoso et al., 2013) and induced by azoxymethane (AOM) associated with the sodium sulphate dextran (SSD) (Choi et al., 2017). Freitas et al. (2017) demonstrated the cytotoxic potential of the *E. oleracea* in the cell lineage of breast cancer (MCF-7).

Amongst the several factors involved with the etiology of the UCC, the persistent infections caused by the HPV oncogenic subtypes (mainly the HPV16 and the HPV18) stand out; however, the precocious beginning of the sexual activity, the multiparity, the immunosuppression, the tabagism and the sustained use of oral contraceptives (estrogen) are considered cofactors associated with the development of cervical cancer (INCA, 2017).

According to Choudhari et al. (2013), all of the UCC risk cofactors increase the levels of nitric acid (NO) in the cervical microenvironment, which is observed at levels significantly high in patients with cervical cancer when compared to healthy patients, suggesting that the NO has a potential mutagenic activity in this kind of cancer, acting as a molecular cofactor of the infection by the HPV in the cervical carcinogenesis.

Sampaio and Almeida (2009) point out the low ingestion of antioxidant vitamins as another risk cofactor for developing UCC. According to these authors, antioxidant nutrients such as the vitamins A, E and C, might inhibit the ROS formation and the evolution of malignant lesions in the epithelium of the uterine colon, acting as modulators of the immune response in face of the presence and/or persistence of the infection by HPV, preventing the progression of the lesions and, as a consequence of that, the development of the UCC. Guo et al. (2015) also suggest that the antioxidant vitamins (mainly α-carotene, β-carotene and the vitamins E and C) might be beneficial in the reduction of the risk of the invasive cervical cancer.

In vivo studies demonstrated that the dietary anthocyanins inhibit the gastrointestinal tract cancer and, when topically applied, they inhibit the skin cancer (Wang and Stoner, 2008). This research was based on the hypothesis that the oxidative stress, which improves the carcinogenesis of the W256

tumor, also promotes the uterine colon carcinogenesis by the HPV. Therefore, it is suggested that a high concentration of stable anthocyanins, revealed in the chemical characterization of the SHEEo, acted as a strong antioxidant, promoting the chemopreventive effect verified by the lower tumor growth, the decrease in the malignancy cytological criteria and lower histopathological tumor cellularity.

According to Acco et al. (2012), after W256 tumor cells are implanted, there is a decrease in the animal's weight, difficulty in ingesting food properly (anorexia), protein, lipids and carbohydrates catabolism and, 14 days after the implantation, the tumor mass might represent a considerable fraction of the animal's weight, and death might occur. The several metabolic alterations arising out of the W256 tumor make this model recognized for developing the cachexia syndrome (anorexia, muscular atrophy, weight loss, weakness and, frequently, death) essentially due to an increase in NO, PGE2 (prostaglandin E2) and decrease in proinflammatory cytokines, such as IL-6 (Interleukin 6), with the development of inflammatory processes of the tumor tissue (Rebeca et al., 2008). Our research revealed a decrease in the consumption of water and ration by the animals of all of the experimental groups after the tumor was inoculated; however, there was no decrease in the animals' weight, suggesting that the SHEEo protected these animals from cachexia.

An inflammation state is established in association with the solid tumor growth, so that the monocytes are recruited from the systemic circulation to the tumor tissue, in response to chemokines secreted by the tumor cells and, macrophages are differentiated, which, associated with the tumor, modulate the migration of tumor cells, overflow and also the angiogenesis (Mantovani et al., 2004; Siveen and Kuttan, 2009). Hao et al. (2011) affirm that the activated macrophages play a tumoricidal effect, secreting molecules such as hydrogen peroxide (H_2O_2), NO and lipoxins (LXs).

Moreira et al. (2018) verified that the aerobic training started during adolescence might attenuate the W256 tumor growth, due to the improvement in the insulin sensitivity, the lower levels of glucose and insulin and/or reduction in the secretion of insulin stimulated by glucose. On the other hand, Kryczyk et al. (2014) investigated the effects of the physical exercise associated with the supplementation with shark liver oil on the W256 tumor growth and found out that the supplementation and the exercises alone help avoid the installation of the cachexia state and also reduce the tumor growth, but the association of both causes a pronounced effect only on the tumor growth, since they involve the induction of the apoptotic process through the increase in the lipid peroxidation in the tumor tissue and modification in the expression pattern of the proteins connected with the induction of cell death.

The effects of crotoxin, which is the main venom of the South American rattlesnake (*Crotalus durissus terrificus),* on the W256 tumor growth were analyses by Brigatte et al. (2016), who observed that the treatment reduced the tumor growth through the inhibition of the formation of new blood

vessels and decrease in the diameter of the blood vessels, suggesting that the crotoxin compromised the angiogenesis. The active proliferation of tumor cells, which generally accompanies the initial phase of the tumor growth, is balanced by the cell death caused by the removal of blood supply from the tumor, being angiogenesis parallel to the metastasis (Ribatti et al., 2004).

The microscopic analysis of the types of cells present in vaginal smears have been used for assessing the stages of the estrous cycle of the rats, which is the main measure in determining the reproductive cyclicity and an auxiliary test in reproductive toxicological studies (Cora et al., 2015). The estrous cycle in rats and mice last for an average of 4 to 5 days, being a repetitive but dynamic process, in which different types of cells appear and regress, reflecting the changes at the levels of estradiol and progesterone secreted by the ovarian follicles, being divided into four stages — proestrus, estrus, metestrus and diestrus (Goldman et al., 2007). For this reason, in this research, the cytology was performed in the 4 days before the tumor cells were inoculated and on the 4 days before euthanasia, in order to assess the morphology of all of the cell types and their possible alterations.

The cellular types found in the different stages of the estrous cycle comprise neutrophils, small and big nucleated epithelial cells and enucleated keratinized epithelial cells (Cora et al., 2015). In this research, alterations in the pattern of the cellular types present were observed, when compared to the cytology carried out before and after the tumor was inoculated. According to Fischer et al. (2010), the malignancy cytological criteria are related to the altered physiology of the cancer cells, and they can be observed both in the nucleus and in the cytoplasm. In this research, the main criteria observed were the cellular pleomorphism and hyperchromasia. These same authors affirm that the pleomorphism defines the finding of several different cellular forms, reflecting a population that is genetically unstable and susceptible to the tumor progression, while hyperchromasia reflects a higher content of deoxyribonucleic acid (DNA) in the nucleus, revealing the chromosomal instability that is common in many tumors.

The W256 tumor is a carcinosarcoma the origin of which is spontaneous from the mammal glands of rats and that has an aggressive biological behavior, being locally invasive and with a high power of metastasis by lymphatic and hematogenous routes (Alves et al., 2004). Metastasis after intramuscular inoculation have been observed in the kidneys, spleen, liver, suprarenal glands, bone marrow, heart and tongue (Silva et al., 2006). Due to the high engraftment index, the subcutaneous route is preferable, thus avoiding the emergence of metastases or invasions of the other cavities (Moraes et al., 2000). In this work, the cervicovaginal route favored the formation of a more widely spread tumor; however, no alterations or metastases were verified in the animals' spleens. The resistance of the spleen to the implantation of metastases have been attributed to its anatomical functional and immunological characteristics (Schon et al., 2006).

Carcinosarcomas are characterized by their unique biphasic morphology, being a tumor composed of both elements, epithelial and mesenchymal, which are microscopically and intermittently mixed or distinct, with the epithelial component frequently an endometrial adenocarcinoma, associated to a malignant stromal component (Brown, 2008). The uterine carcinosarcoma has the higher rate of lung metastases amongst the uterine malignancies, which are due to the positivity of retroperitoneal lymph nodes, the deep myometrial invasion, the extension of the cervical tumor, the vascular invasion and the low degree of differentiation (Bharwani et al., 2010). According to Ho et al. (2008), the most common locations for the metastatic deposit include lungs (49%), peritoneum (44%), pelvic lymph nodes or periaortic lymph nodes (35%), adrenal gland or bone (19%), heart or pericardium (9%) and/or brain (7%).

5 CONCLUSION

In this study, the results demonstrated that the SHEEo had chemopreventive capacity at the dosage of 300mg/Kg. Other *in vivo* investigations using other tumor experimental models, as well as *in vitro* trials are necessary for better understanding the chemopreventive mechanism involved.

CONTRIBUTIONS FROM THE AUTHORS

LB, CR, FR and IC performed *in vivo* tests under the supervision of CL. HS, AS, LA, JT and JC carried out the phytochemical study. LB and CR performed the cytological and histological analysis. HC and BO assisted in the analysis of statistical data. LB and CL prepared and revised the manuscript.

FUNDING

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES) - PROCAD (Proc. 88887.313607/2019-00), the Research Support Foundation of Amapá - FAPEAP (Proc. 250.203.060/2017) and the Federal University of Paraíba (UFPB) - PROPESQ/UFPB n. 02/2019 for the financial support.

Abbreviations: AOM, azoxymethane; AUC, area under the curve; UCC, uterine colon cancer; HELC-MS ^{n}, highly efficient liquid chromatography coupled with the ion trap mass spectrometry; HELC-UV-S, highly efficient liquid chromatography associated with the ultraviolet spectroscopy; S.D., standard deviation; DMH, 1,2-dimetilhidrazina; DNA, deoxyribonucleic acid; SSD, sodium sulphate dextran; *E. oleracea, Euterpe oleracea* Martius; SHEEo, standardized hydroethanolic extract of *E. oleracea* Mart.; FTIR, spectroscopy in the infrared region through the Fourier (FTIR) transformation; H_2O_2, hydrogen peroxide; HPV, *Papillomavirus humano*; HPV16, *Papillomavirus humano* subtype 16;

HPV18, *Papillomavirus humano* subtype 18; KBr, Potassium bromide; LPS, lipopolysaccharides; LXs, lipoxins; MCF-7, breast cancer cell lineage; NAC, N Acetylcysteine; NO, nitric oxide; Nrf2, Erythroid nuclear factor 2; PGE2, prostaglandin E2; W256, Walker 256 tumor.

Statement of Conflict of Interest: The authors state that the research was carried out in the absence of any commercial or financial relationships that might be construed as a potential conflict of interests.

ACKNOWLEDGEMENTS

To Professor Doctor Manoel Odorico de Moraes Filho, from the Laboratory of Experimental Oncology of the School of Medicine of the Federal University of Ceará, for his kind supply of the Walker 256 carcinosarcoma cells.

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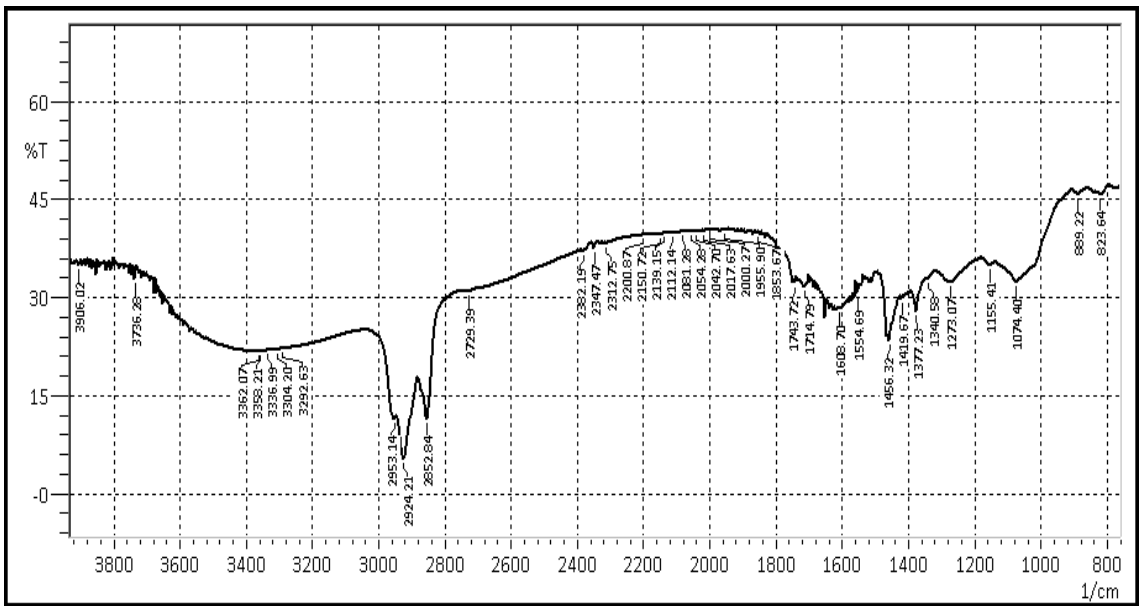

Figure 1. Spectrum in the infrared region through the Fourier (FTIR) transformation of the Standardized Hydroethanolic Extract of the *Euterpe oleracea* Mart. (SHEEo).

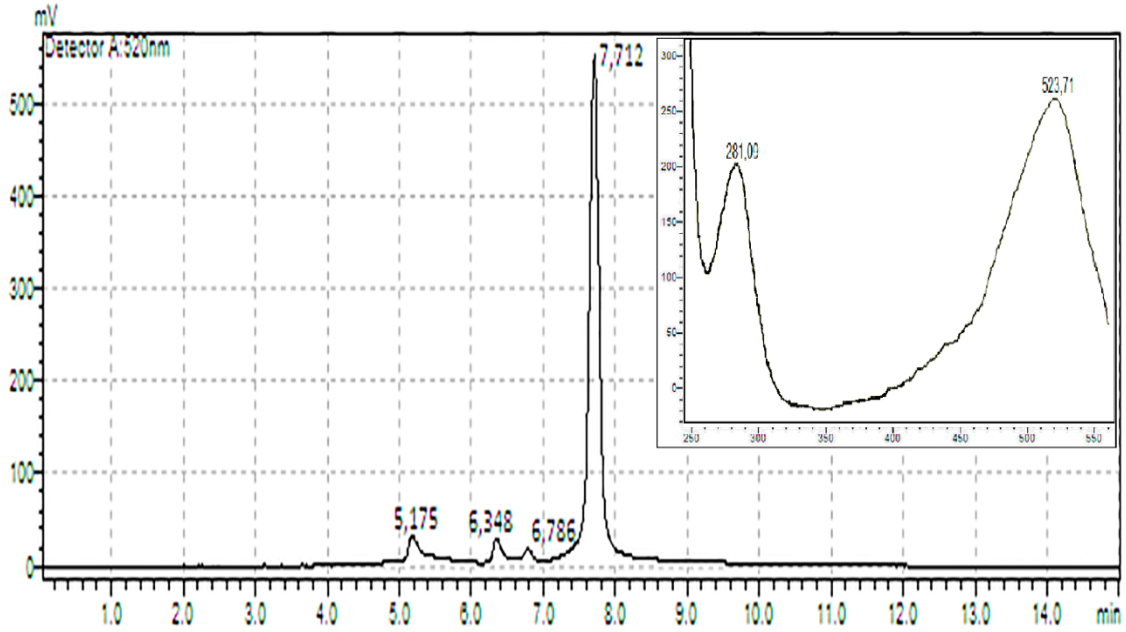

Figure 2. Profile through highly efficient liquid chromatography associated with the ultraviolet spectroscopy (HELC-UV-S) of the Standardized Hydroethanolic Extract of the *Euterpe oleracea* Mart. (SHEEo)**.**

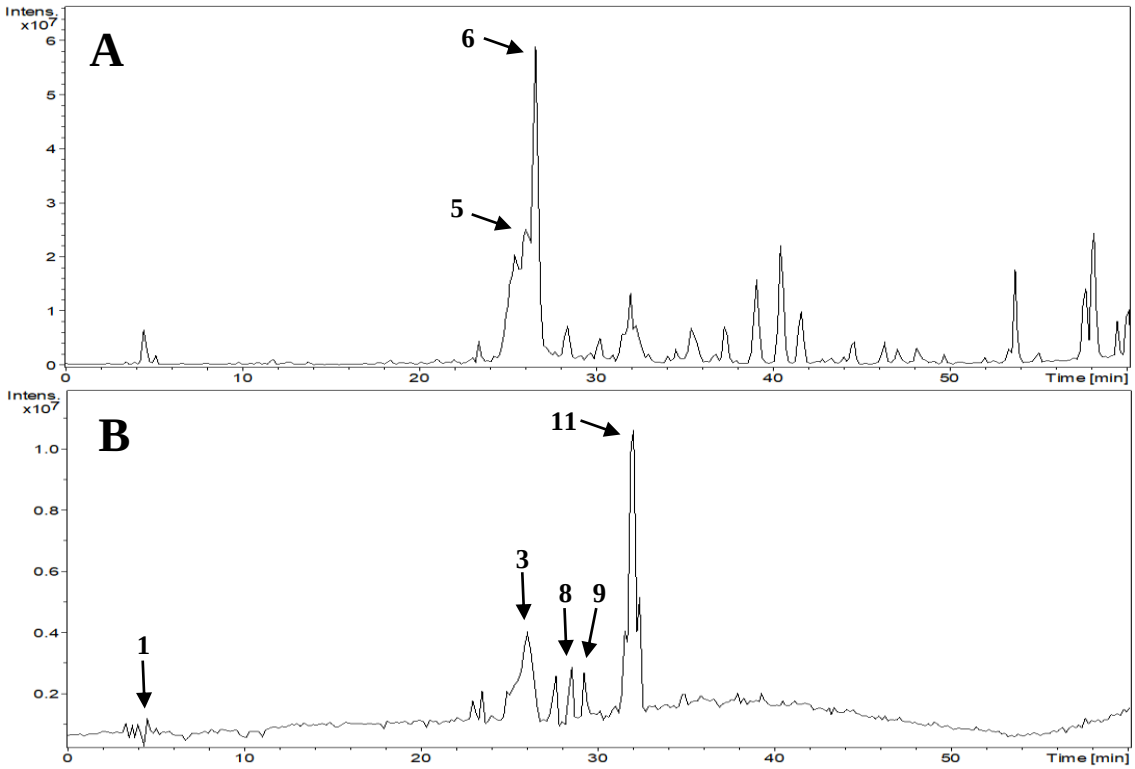

Figure 3. Base peak chromatograms (BPC) in positive ion mode **(A)** and negative ion mode **(B)** of the Standardized Hydroethanolic Extract of the *Euterpe oleracea* Mart. (SHEEo) through HELC–DAD–MS/MS.

Table 1. Characterization of the compounds identified through highly efficient liquid chromatography coupled with the ion trap mass spectrometry HELC-MS^n of the Standardized Hydroethanolic Extract of the *Euterpe oleracea* Mart. (SHEEo).

Pico N°.	t_R (min.)	$[M+H]^+$/ $[M-H]^-$	MS/MS	Identification Attempt	Reference
1	5.2	-/325	MS^2 [325]: 179; 163	*p*- Coumaroyl hexoside	Garzón et al., 2017
2	17.9	-/329	MS^2 [329]: 167; 152	Vanillic-hexosíde acid	Mena et al., 2012
3	24.5	-/353	MS^2 [353]: 191	5- caffeoylquinic acid	Garzón et al., 2017
4	24.6	581/-	MS^2 [581]: 449; 287	Cyanidin 3,5-hexose pentose	Garzón et al., 2017; Rosso et al., 2008
5	25.4	449/-	MS^2 [449]: 287	Cyanidin 3-glicoside	Garzón et al., 2017; Rosso et al., 2008
6	26.0	595/-	MS^2 [595]: 449; 287	Cyanidin 3-rutinoside	Garzón et al., 2017; Rosso et al., 2008
7	27.7	609/-	MS^2 [609]: 463; 301	Peonidin 3-rutinoside	Garzón et al., 2017; Rosso et al., 2008
8	28.0	-/335	MS^2 [335]: 291; 179; 161; 155; 135	Caffeoylquinic acid isomer I	Garzón et al., 2017
9	29.2	-/335	MS^2 [335]: 291; 179; 161; 155; 135	Caffeoylquinic acid isomer II	Garzón et al., 2017
10	31.7	-/447	MS^2 [447]: 357; 327; 285	Isoorientin	Garzón et al., 2017; Dias et al., 2013
11	32.2	-/447	MS^2 [447]: 357; 327; 285	Orientin	Garzón et al., 2017; Dias et al., 2013
12	33.2	-/431	MS^2 [431]: 341; 311; 283	Vitexin	Garzón et al., 2017; Dias et al., 2013
13	34.3	-/449	MS^2 [449]: 269	Deoxyhexose Taxifolin	Garzón et al., 2017; Dias et al., 2013
14	34.6	-/431	MS^2 [431]: 413; 341; 311; 283	Isovitexin	Garzón et al., 2017; Dias et al., 2013
15	36.2	-/609	MS^2 [609]: 301; 300; 271; 255	Rutin	Garzón et al., 2017; Dias et al., 2013

t_R = retention time; $[M+H]^+$ = positive ion mode; $[M-H]^-$ = negative ion mode; MS/MS or MS^2= mass spectrometry/mass spectrometry.

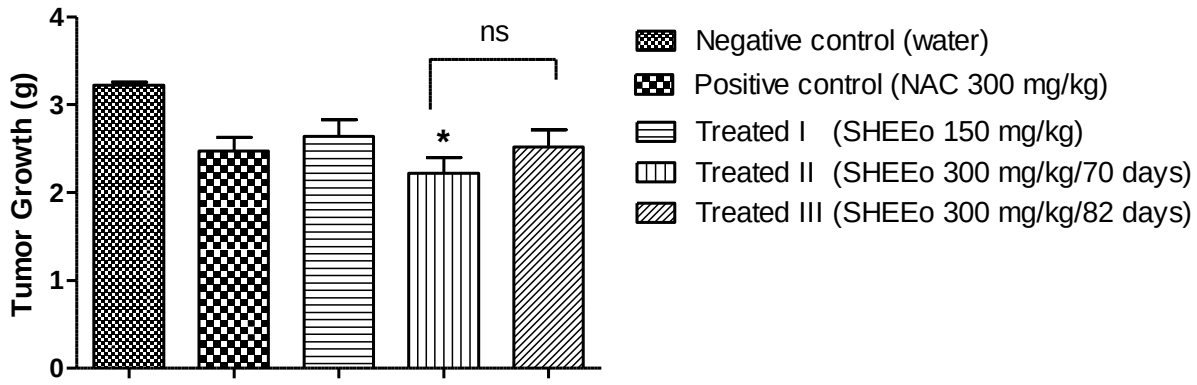

Figure 4. Effect of the Standardized Hydroethanolic Extract of *Euterpe oleracea* Mart. (SHEEo) on the tumor growth (Walker 256) in the vaginal canal and uterine cervix of the Wistar rats after. The differences between the groups (n=5 animals) were calculated through the One-way (ANOVA) variance analysis, followed by the Tukey test. *($p<0.05$). Statistically significant result versus Negative Control Group (Water); NAC = N Acetylcysteine.

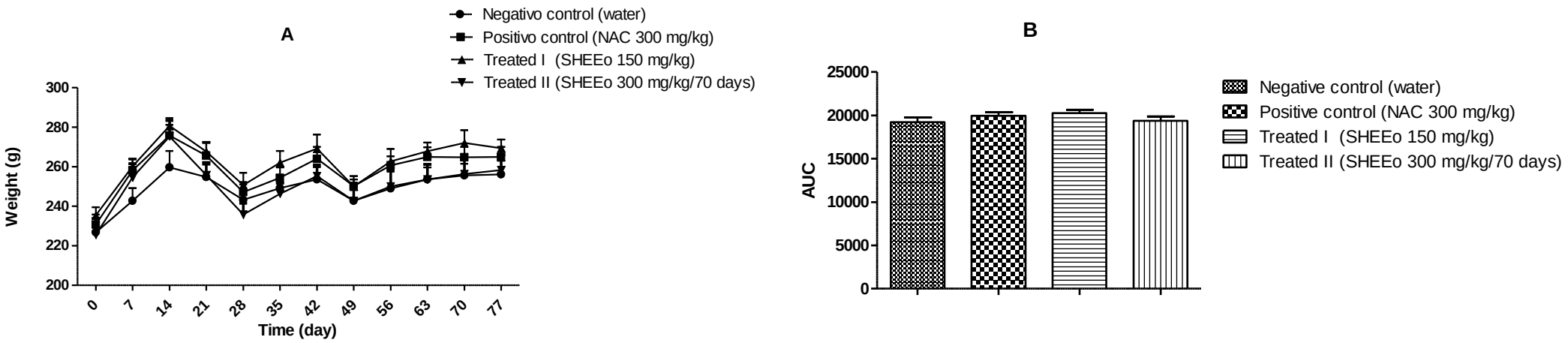

Figure 5. Evolution of the body mass gain during the treatment with the Standardized Hydroethanolic Extract of *Euterpe oleracea* Mart. (SHEEo) **(**150 and 300mg/kg) and inoculation of the Walker 256 tumor cells. Data obtained on a weekly basis **(A)** and area under the curve of the result **(B)**. The differences between the groups (n=5 animals) were calculated through the One-way (ANOVA) variance analysis, followed by the Tukey test. NAC = N Acetylcysteine; AUC = Area under the curve.

Table 2. Chemopreventive effect, on the 12th day after the Walker 256 carcinosarcoma tumor cells were inoculated, on the weight of the organs of the animals treated with the Standardized Hydroethanolic Extract of *Euterpe oleracea* Mart. (SHEEo).

	Negative Control (Water)	**Positive Control (NAC 300mg/kg)**	**Treated I SHEEo (150mg/Kg)**	**Treated II SHEEo (300mg/Kg)**
Liver	10.40±1.51	10.89±1.23	11.36±1.18	11.46±1.37
Heart	1.02±0.07	1.06±0.12	1.05±0.07	0.97±0.05
Lungs	1.81±0.23	1.87±0.18	1.82±0.19	1.96±0.19
Kidneys	2.54±0.39	2.72±0.26	2.88±0.21	2.74±0.33
Spleen	0.82±0.07	0.88±0.07	1.00±0.07	0.93±0.04

The results are presented as the average ± S.D. (n=5 animals). The differences amongst the groups were calculated through One-way (ANOVA) variance analysis, followed by the Tukey test. NAC = N Acetylcysteine.

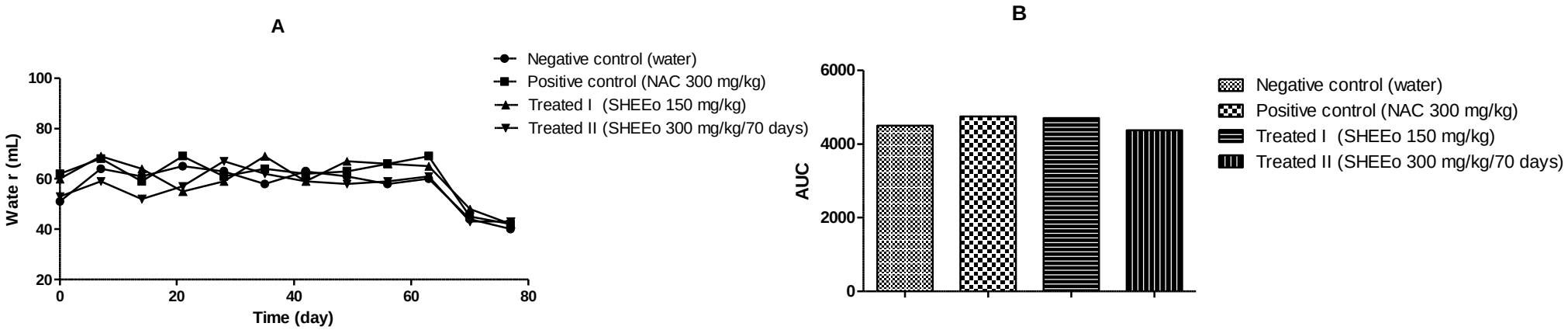

Figure 6. Variance in the consumption of water during the treatment period with the Standardized Hydroethanolic Extract of *Euterpe oleracea* Mart. (SHEEo) (150 and 300mg/kg) and inoculation of the Walker 256 tumor cells. The data were obtained on a weekly basis **(A)**, and area under the curve of the result **(B)**. The differences amongst the groups (n=5 animals) were calculated through One-way (ANOVA) variance analysis, followed by the Tukey test. NAC = N Acetylcysteine; AUC = Area under the curve.

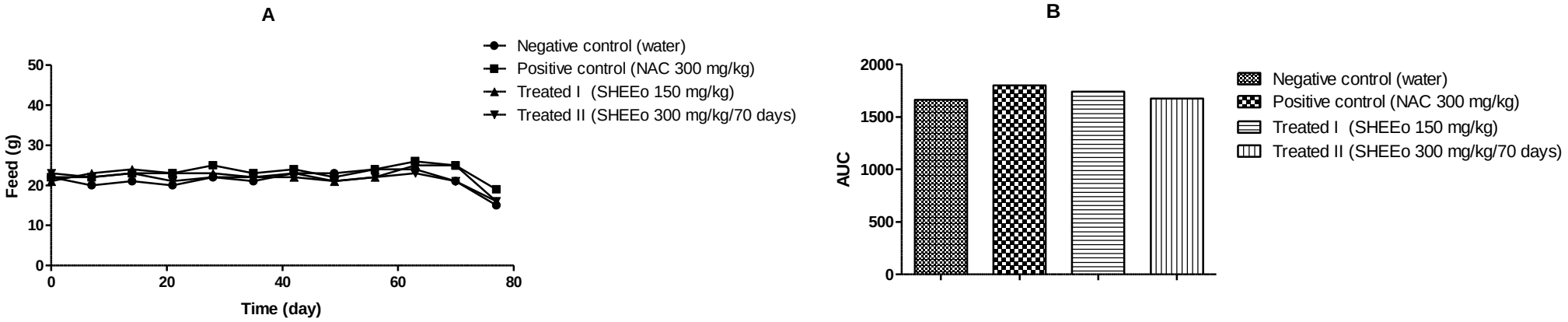

Figure 7. Assessment of the consumption of ration during the treatment period with the Standardized Hydroethanolic Extract of *Euterpe oleracea* Mart. (SHEEo) (150 and 300mg/kg) and inoculation of the Walker 256 tumor cells. The data were obtained on a weekly basis **(A)**, and area under the curve of the result **(B)**. The differences amongst the groups (n=5 animals) were calculated through One-way (ANOVA) variance analysis, followed by the Tukey test. NAC = N Acetylcysteine; AUC = Area under the curve.

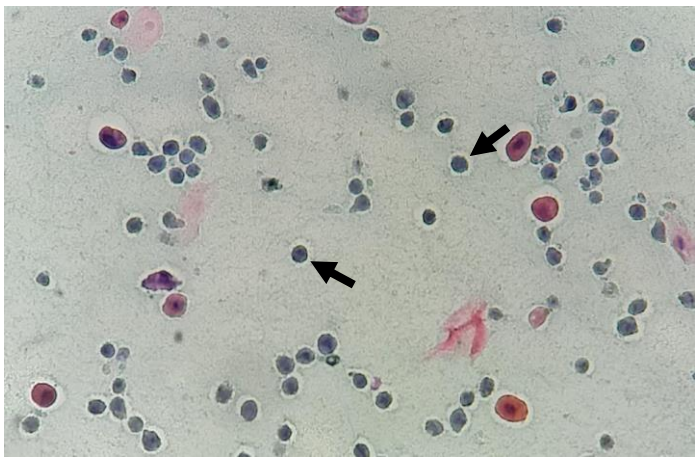

Figure 8. Vaginal smear after the inoculation of Walker 256 tumor cells of an animal from the Treated II Group that received the Standardized Hydroethanolic Extract of *Euterpe oleracea* Mart. (SHEEo), at the concentration of 300mg/kg, during 70 days. Observe the presence of small round, basophilic and nucleated cells, characteristic of Walker 256 tumors (arrows). Papanicolaou. 40x.

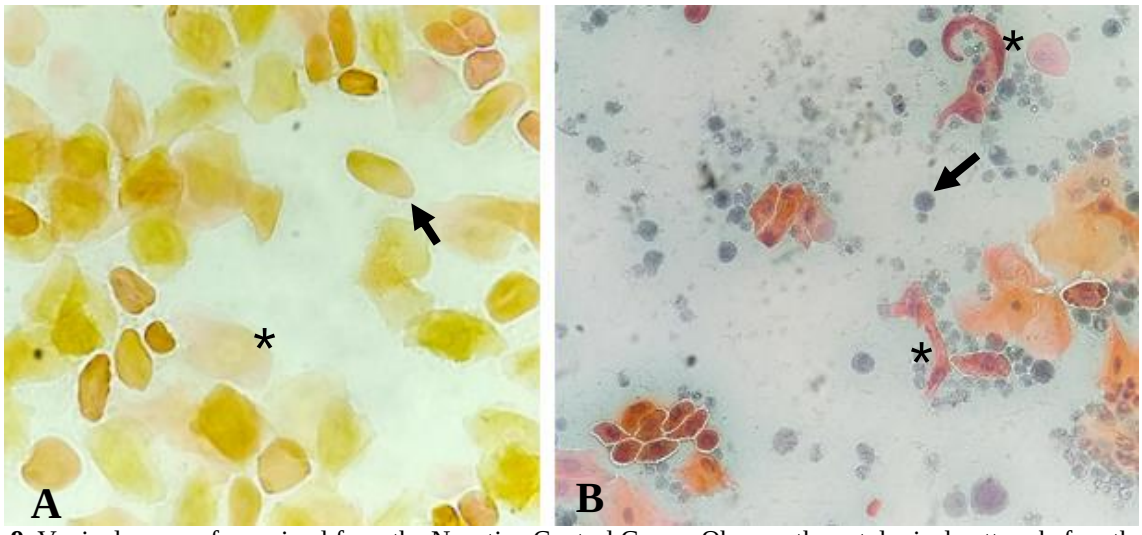

Figure 9. Vaginal smear of an animal from the Negative Control Group. Observe the cytological pattern before the tumor inoculation **(A)**, with oval enucleated cells (arrow) and polygonal enucleated cells (*), characteristic of the phase of the estrous cycle of the rat. In the cytology of the collected vaginal smear after the Walker 256 tumor was inoculated **(B)**, observe the presence of small round, basophilic and nucleated cells (Walker 256 tumor – arrow) and cervical cells altered, with keratinization and pleomorphism (bizarre shapes) (*). Papanicolaou. 40x.

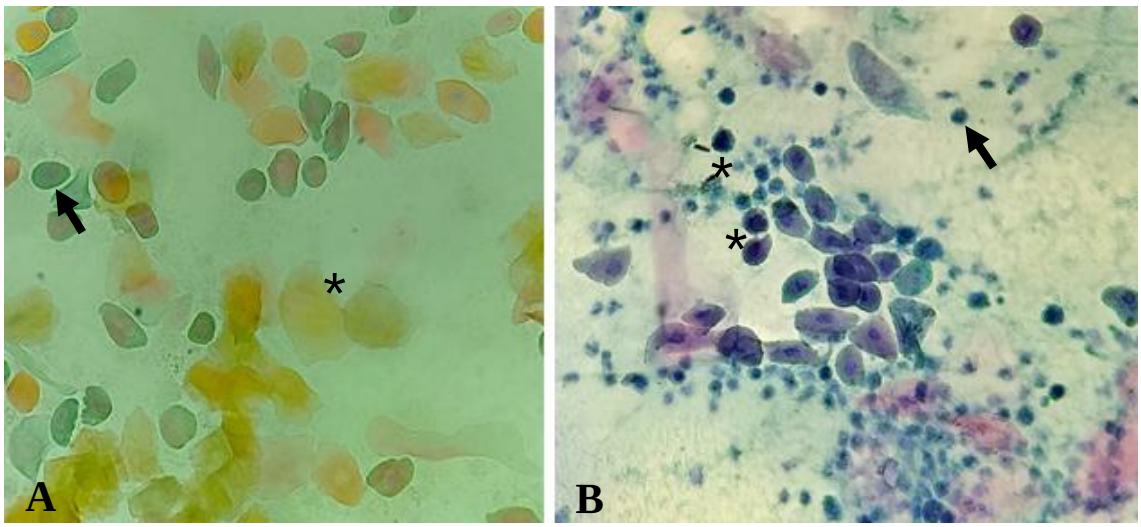

Figure 10. Vaginal smear of an animal from the Treated Group I that received 150mg/Kg d that received the Standardized Hydroethanolic Extract of *Euterpe oleracea* Mart. (SHEEo), on a daily basis, during 70 days. Observe the cellular pattern before the tumor was inoculated **(A)**, with basophilic, oval and nucleated cells (arrow) and eosinophilic, polygonal enucleated cells (*), characteristic of the phase of the estrous cycle of the rat. In the cytology dafter the Walker 256 tumor was inoculated **(B)**, observe the presence of small round, basophilic and nucleated cells (Walker 256 tumor – arrow), and basophilic cervical cells, with an intense hyperchromasia (*). Papanicolaou. 40x.

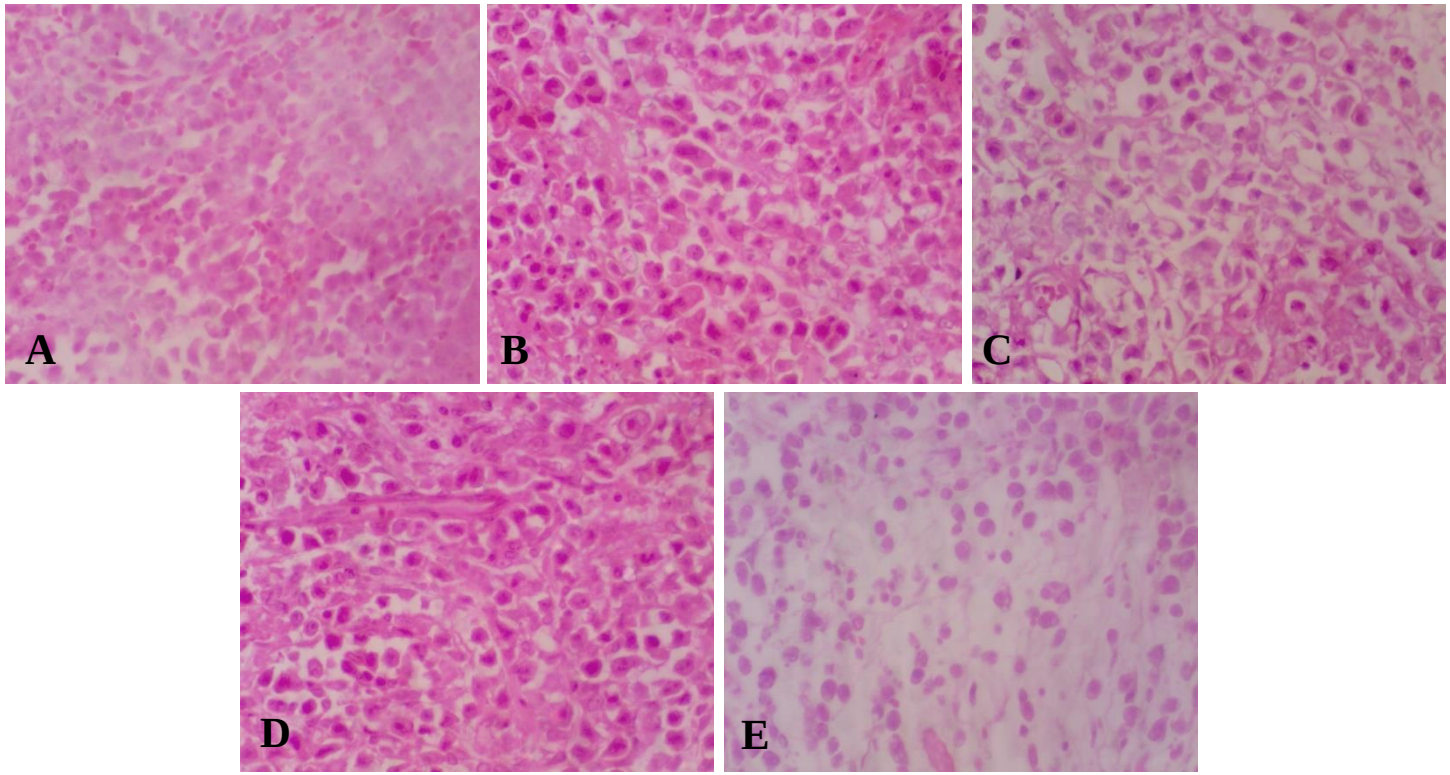

Figure 11. Photomicrographies, in a transversal section, of histologic cuts of the Walker 256 tumor inoculated in the uterine cervix of Wistar rats. Observe that the total of the tumor cells are round, characterized by scarce cytoplasm, bulky nucleus and clear delimitation of the cell membrane, with lower tumor cellularity in E. **(A)** = Negative Control Group = Water/70 days; **(B)** = Positive Control Group = N-Acetylcysteine 300mg/kg/70 days; **(C)** = Treated Group I (SHEEo 150mg/kg/70 days); **(D)** = Treated Group II (SHEEo 300mg/kg/70 days); **(E)** = Treated Group III (SHEEo 300mg/kg/82 days). SHEEo = Standardized Hydroethanolic Extract of *Euterpe oleracea* Mart. HE. 40x.

4 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Considerando os inúmeros benefícios, a ingestão de *E. oleracea* deve fazer parte da dieta como uma estratégia nutricional. Seus efeitos poderiam ser potencializados em reduzir o estresse oxidativo, a inflamação e, consequentemente, a carcinogênese, utilizando-se extratos padronizados, com elevado teor de antocianinas. Todavia, ainda há necessidade de mais estudos, principalmente a longo prazo, que estabeleçam uma relação de causa e efeito quimiopreventivo. Outro fator que pode ser considerado é o isolamento de compostos presentes no extrato de *E. oleracea*, para identificar quais mecanismos farmacológicos estão envolvidos no processo quimiopreventivo. No entanto, é necessário manter e comparar os resultados entre compostos isolados, frações e o extrato padronizado e avaliar a ocorrência de sinergismo farmacológico. Novas pesquisas tornam-se viáveis pelo grandioso consumo de *E. oleracea* no Brasil, principalmente na região Norte e, pelo considerável aumento de exportação e consumo em diversas regiões do mundo.

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Anexo 1 – Parecer do Comitê de Ética

UNIVERSIDADE FEDERAL DO AMAPÁ
PRÓ-REITORIA DE PESQUISA E PÓS- GRADUAÇÃO
COMITE DE ÉTICA NO USO DE ANIMAIS – CEUA – UNIFAP

CERTIFICADO

A Comissão de Ética no Uso de Animais da Universidade Federal do Amapá **APROVOU,** na data de 05 de janeiro de 2018, o parecer referente ao protocolo no. **021/2017** e certifica que o Projeto de Pesquisa intitulado "**EFEITO PROTETOR DO EXTRATO HIDROETANÓLICO PADRONIZADO DO AÇAÍ (*Euterpe oleracea* Mart.) NA INDUÇÃO DO TUMOR DE WALKER 256 EM CÉRVICE UTERINA DE RATAS WISTAR**" coordenado por **Letícia Elizandra Mehl Boettger**, está de acordo com os princípios de ética e bem estar animal.

CERTIFICATE

The Ethics Committee on Animal Use of the Amapá Federal University **APPROVED** at the meeting of 05 January 2018, the final decision about the Protocol **021/2017** and certify that the research project entitled "**EFEITO PROTETOR DO EXTRATO HIDROETANÓLICO PADRONIZADO DO AÇAÍ (*Euterpe oleracea* Mart.) NA INDUÇÃO DO TUMOR DE WALKER 256 EM CÉRVICE UTERINA DE RATAS WISTAR**" coordinated by **Letícia Elizandra Mehl Boettger**, is in accordance with the principles of ethics and animal welfare.

Macapá, 05 de janeiro de 2018

Prof. Tit. José Carlos Tavares Carvalho
Presidente CEUA-UNIFAP
Port. No. 1733/2014

Universidade Federal do Amapá
Pró-Reitoria de Pesquisa e Pós- Graduação
Comitê de Ética no Uso de Animais – CEUA – UNIFAP
Rod. Juscelino Juscelino Kubitschek, km 02 – Campus Marco Zero,

Anexo 2 – Comprovante de submissão do artigo científico

---- Mensagem encaminhada ----
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Para: clarissalima@unifap.br
Enviadas: Quinta-feira, 25 de julho de 2019 23:50:09
Assunto: Frontiers: Your manuscript submission - 487357

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Frontiers Pharmacology Editorial Office has sent you a message. Please click 'Reply' to send a direct response

We are pleased to inform you that we have received the manuscript "THE CHEMOPREVENTIVE EFFECT OF THE STANDARDIZED HYDROETHANOLIC EXTRACT OF Euterpe oleracea Mart. ON THE TUMOR GROWTH OF WALKER 256 IN THE UTERINE CERVIX OF FEMALE WISTAR RATS " to be considered for publication in Frontiers in Pharmacology, section Ethnopharmacology.

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Your Frontiers in Pharmacology team

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Manuscript ID: 487357
Authors: Letícia Elizandra Mehl Boettger, Clarice Flexa Da Rocha, Helison Oliveira Carvalho, Heitor Ribeiro Silva, FERNANDA MONTEIRO ROCHA, Ivanilson Lobato Da Costa, Abrahão Victor Tavares Dos Santos, Brenda Lorena Sánchez -Ortiz, LUCAS SILVA ABREU, Josean Fechine Tavares, José Carlos Tavares, Clarissa Silva Lima
Journal: Frontiers in Pharmacology, section Ethnopharmacology
Article type: Original Research
Submitted on: 26 Jul 2019

Research Topic: Ethnopharmacological Perspectives on Cancer Prevention

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Anexo 3 – Normas de publicação no periódico Frontiers in Pharmacology

1. Summary Table

Please view the table below for a summary on currently accepted article types and general manuscript style guidelines. Article types may vary depending on journal.

	Abstract (max. length)	Running title (5 words)	Figures and/or tables (combined)	Manuscript (max. length)	Peer review	Author fees
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2.3.1. General standards

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2.3.1.12. Text

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Your manuscript is organized by headings and subheadings. The section headings should be those appropriate for your field and the research itself. For Original Research Articles, it is recommended to organize your manuscript in the following sections or their equivalents for your field:

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Succinct, with no subheadings.

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This section may be divided by subheadings. This section should contain sufficient detail so that when read in conjunction with cited references, all procedures can be repeated. For experiments reporting results on animal or human subject research, an ethics approval statement should be included in this section (for further information, see section Materials and Data Policies).

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AB, CDE and FG contributed conception and design of the study; AB organized the database; CDE performed the statistical analysis; FG wrote the first draft of the manuscript; HIJ, KL, AB, CDE and FG wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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This study was carried out in accordance with the principles of the Basel Declaration and recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee].

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