Universidade Federal do Amazonas – UFAM

Instituto de Ciências Biológicas – ICB

Programa de Pós-graduação em Imunologia Básica e Aplicada – PPGIBA

BIOPROSPECÇÃO DE COMPOSTOS NATURAIS, SINTÉTICOS E DERIVADOS BIOATIVOS COM POTENCIAL ANTICANCERÍGENO E IMUNOMODULADOR

CARLOS EDUARDO DE CASTRO ALVES

MANAUS-AMAZONAS

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À minha amada família que sempre me apoia, incentiva e, acima de tudo, ama-me. Eu os amo incondicionalmente.

Dedico

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito.
Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes”.

Marthin Luther King

Resumo

Introdução: a toxicidade resultante das terapias disponíveis contra neoplasias continua sendo um fator altamente debilitante para os pacientes. Plantas medicinais e derivados sintéticos têm sido fontes de descoberta de diversos compostos com atividades antineoplásicas, antinflamatórias e analgésicas. **Objetivo:** este estudo teve como objetivo avaliar a atividade anticancerígena e imunomoduladora *in vitro* de derivados bioativos e sintéticos contra linhagens de células lêucemicas. **Material e métodos:** foram utilizadas linhagens celulares cancerígenas HL60 (leucemia mielóide aguda) e K562 (leucemia mielóide crônica), assim como células mononucleadas do sangue periférico humanas (PBMC) e Vero (epitelial do rins de *Cercopithecus aethiops*). A avaliação da citotoxicidade dos composto foi realizadas por meio do teste de viabilidade celular MTT. Ensaios do ciclo celular foram conduzidos para determinar o efeito dos os compostos nos diferentes estágios do ciclo celular. A ação dos compostos sobre o crescimento clonal de linhagens cancerígenas foi avaliada por meio do teste de formação de colônia. A atividade imunomoduladora foi realizada por meio da dosagem de citocinas pró e anti-inflamatórias e da capacidade de inibição da ciclooxigenase 1 e 2 (COX-1 e COX-2) em PBMC humano ou células HL60 tratadas previamente com diferentes concentrações dos compostos. **Resultados e conclusão:** Ao total foram avaliados os efeitos citotóxicos de 11 compostos, no qual 3 apresentaram efeito citotóxicos seletivos contra as linhagens cancerígenas. Destes, dois compostos sintéticos, um nova síntese de pirimidina e um novo derivado de espiroindolonas, e um natural, uma fração enriquecida com polifenol extraída de *Myrtus communis* (PEMC). A nova síntese de pirimidina apresentou efeito citotóxico tanto para células HL60 (IC_{50}:25,93µg/mL) quanto para K562 (IC_{50}:10,42 µg/mL), enquanto que o derivado de espiroindolonas possui efeito citotóxico contra K562 (IC_{50}:25,27µg/mL). Ambos os compostos apresentaram ainda efeito imunomodulador sobre a estimulação de citocinas em PBMCs humanas. Já a fração PEMC apresentou efeito antiproliferativo contra linhagens HL60 (IC_{50}:19.87µM) e K562 (IC_{50}:29.64µM) demonstrando ainda um significativo potencial anti-inflamatório e cicatrizante. Em conclusão, os compostos identificados neste estudo exibiram potenciais farmacológicos promissores como agentes antineoplásicos no tratamento de leucemias mieloides.

Palavras-chave: citotoxicidade, imunomodulação, biocompostos, sintéticos, leucemias.

Abstract

Introduction: the resulting toxicity of available therapies against neoplasms continues to be a highly debilitating factor for patients. Medicinal plants and synthetic derivatives have been sources of discovery of several compounds with antineoplastic, anti-inflammatory and analgesic activities. **Objective:** this study aimed to evaluate the in vitro anticancer and immunomodulatory activity of bioactive and synthetic derivatives against leukemia cell lines. **Material and methods:** HL60 (acute myeloid leukemia) and K562 (chronic myeloid leukemia) cancer cell lines were used, as well as human peripheral blood mononuclear cells (PBMC) and Vero (kidney epithelium from Cercopithecus aethiops). The evaluation of the cytotoxicity of the compounds was performed using the MTT cell viability test. Cell cycle assays were conducted to determine the effect of the compounds on different stages of the cell cycle. The action of the compounds on the clonal growth of cancerous strains was evaluated using the colony formation test. Immunomodulatory activity was determined by measuring pro and anti-inflammatory cytokines and the ability to inhibit cyclooxygenase 1 and 2 (COX-1 and COX-2) in human PBMC or HL60 cells previously treated with different concentrations of compounds. **Results and conclusion:** In total, the cytotoxic effects of 11 compounds were evaluated, in which 3 showed selective cytotoxic effects against cancerous strains. Of these, two synthetic compounds, a new synthesis of pyrimidine and a new derivative of spiroindolones, and a natural one, a fraction enriched with polyphenol extracted from Myrtus communis (PEMC). The new pyrimidine synthesis showed a cytotoxic effect both for HL60 cells (IC_{50}:25.93µg/mL) and for K562 (IC_{50}:10.42 µg/mL), while the spiroindolone derivative has a cytotoxic effect against K562 (IC_{50}:25.27µg/mL). Both compounds also showed an immunomodulatory effect on cytokine stimulation in human PBMCs. The PEMC fraction, on the other hand, showed an antiproliferative effect against HL60 (IC_{50}:19.87µM) and K562 (IC_{50}:29.64µM) strains, also demonstrating a significant anti-inflammatory and healing potential. In conclusion, the compounds identified in this study exhibited promising pharmacological potential as antineoplastic agents in the treatment of myeloid leukemias.

Keywords: cytotoxicity, immunomodulation, biocomposed, synthetic, leukemias.

LISTA DE FIGURAS

Figura 1. Representação espacial de taxas ajustadas de incidência por 100 mil homens (A) e 100 mil mulheres (B), estimadas para o ano de 2020, de acordo com Unidade da Federação (todas as neoplasias malignas, com exceção as de pele e melanoma). 18
Figura 2. Distribuição proporcional dos dez tipos de neoplasias mais incidentes estimados para 2020-22 por sexo (exceto pele não melanoma). *Números arredondados para múltiplos de 10. 19
Figura 3. Processo de imunoedição do câncer. Este processo consiste em três fases: (A) eliminação, (B) equilíbrio, (C) escape. 20
Figura 4. Pertubação hematopoiéticas de citocinas inflamatórias durante a leucemia. As citocinas inflamatórias associadas a leucemia medeiam a diferenciação, abundância e funções celulares de fatores da imunidade inata e adaptativa. 21
Figura 5. Esquema de patogenia das leucemias. 24
Figura 6. Fluxograma de atividades realizadas no decorrer do estudo.**Erro! Indicador não definido.**
CAPÍTULO 1:
Figura 1. Cytotoxicity of the polyphenol-enriched fraction extracted from Myrtus communis for K562 and HL60 cells……53
Figura 2. Cytotoxicity of the polyphenol-enriched fraction extracted from Myrtus communis for the normal Vero cell line……...54
Figura 3. Evolution of the hemolysis level……..54
Figura 4. Inhibition percent of the edema volume after the treatment with PEMC…….55
Figura 5. Wound healing assessment after treatment with PEMC containing ointments.55
Figura 6. Antioxidant activity of the PEMC, the results are expressed as mean ± standard deviation……..56
Figura 7. Bodyweight development of mice treated with a single oral administration of the PEMC…….57
CAPÍTULO 2:
Figura 1. Synthesis of 2-(4-(2,5-dimethyl-1H-pyrrol-1-yl)-1H-pyrazol-3-yl)pyridine …….....66
Figura 2. Cytotoxic effect of compound 3 on cancerous and non-cancerous cells…….68
Figura 3. Effect of compound 3 on HL60 and K562 leukemic cell colony formation…69
Figura 4. Cell cycle analysis of cells treated with compound 3……..70
Figura 5. Effect of compound 3 on total cyclooxygenase activity (%)……70
Figura 6. Immunomodulatory effect of the compound 3…….71
Capítulo 3:
Figura 1. Synthesis of spiroindolones…….78
Figura 2. Cytotoxic activity of compound 3a in cancerous and non-cancerous cells…..81
Figura 3. Effect of the compound 3a on the formation of colonies of K562 cells……..81
Figura 4. Cell cycle analysis of K562 cells after treatment with 3a……82
Figura 5. Immunomodulatory effect of compound 3a…….83

LISTA DE TABELAS E QUADROS

Tabela 1. Mecanismos de ação de alguns dos principais quimioterápicos. 29
Tabela 2. Compostos bioativos derivados de plantas com atividade anticâncer 31
Tabela 3. Compostos avaliados quanto atividade citotóxica em linhagens HL60, K562, Vero e PBMCs humanas e suas respectivas IC_{50} 43
CAPÍTULO 1:
Tabela 1. Constituents identified in the polyphenol-enriched fraction extracted from Myrtus communis and their relative abundances……..52
Tabela 2. Biochemical analysis on hepatic and renal biomarkers in the serum of mice treated with different doses of the polyphenol-enriched fraction extracted from *Myrtus communis*…….57
Tabela 3. The PEMC effect on relative weight (g) of several vital organs……..57

LISTA DE ABREVIATURAS E SIGLAS

µg	Micrograma
µl	Microlitro
CD	Cluster de diferenciação
CO_2	Gás carbônico
CTLA-4	Antígeno-4 do linfócito T citotóxico
DNA	Ácido desoxirribonucleico
DMEM	*Dulbecco's modified Eagle's médium*
ELISA	Ensaio imunoabsorvente enzimático
FBS	Soro Fetal Bovino
GAPDH	Gliceraldeído-3-fosfato desidrogenase
GLOBOCAN	Observatório Global do Câncer
HEMOAM	Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas
HER-2	Receptor de fator de crescimento epidérmico humano tipo 2
HL60	Linhagem de leucemia mielóide aguda
IC_{50}	Concentração inibitória de 50%
IL	Interleucinas
INCA	Instituto Nacional de Câncer
IFNγ	Interferon gama
INPA	Instituto Nacional de Pesquisas da Amazônia
K562	Linhagem de leucemia mielóide crônica
LLA	Leucemia linfoide aguda
LLC	Leucemia linfoide crônica
LMA	Leucemia mieloide aguda
LMC	Leucemia mieloide crônica
LPS	Lipopolissacarídeo
MTT	Metiltiazoletrazolium
NK	Do inglês *Natural Killer*
nm	Nanômetros
OD	Densidade óptica
PBMC	Células mononucleares de sangue periférico
PBS	Tampão fosfato-salino
PD-1	Ligante 1 de morte celular programa
PEMC	Polifenol extraída de Myrtus communis
RNA	Ácido ribonucleico
RNase A	Ribonuclease A
RNS	Espécie reativa de nitrogênio
ROS	Espécie reativa de oxigênio
RPMI	Meio do Instituto Roswell Park Memorial
Th1	Células T *helper* 1
Th2	Células T *helper* 2
TNFα	Fator de Necrose Tumoral Alfa
Treg	Células T reguladoras

SUMÁRIO

1. INTRODUÇÃO 15

2. REFERENCIAL TEÓRICO 17

2.1. Câncer: aspectos gerais 17

2.2. Imunopatogênese do câncer 19

2.3. Leucemias 23

2.3.1. Leucemia mieloide aguda (LMA) 25

2.3.2. Leucemia mieloide crônica (LMC) 26

2.4. Terapias convencionais no tratamento do câncer 28

2.5. Compostos naturais como estratégia terapêutica contra o câncer 31

2.6. Compostos sintéticos e atividade anticancerígena 34

3. OBJETIVOS 37

3.1. Objetivo geral 37

3.2. Objetivos específicos 37

4. MATERIAL E MÉTODOS 38

4.1. Fluxograma de atividades 38

4.2. Aspectos éticos 39

4.3. Obtenção de substâncias naturais 39

4.4. Cultura de células 39

4.5. Isolamento e preparação de células mononucleares do sangue periférico humano (PBMC) 39

4.6. Avaliação da viabilidade celular 40

4.7. Ensaio de formação de colônia 40

4.8. Análise do ciclo celular 41

4.9. Ensaio de Ciclooxigenase-1 e -2 (COX-1 e COX-2) 41

4.10. Dosagem de citocinas 41

4.11. Análise estatística 42

5. RESULTADOS 43

5.1. Análise citotóxica dos compostos 43

CAPÍTULO 1: 45

Antileukemic, Antioxidant, Anti-Inflammatory and Healing Activities Induced by a Polyphenol-Enriched Fraction Extracted from Leaves of *Myrtus communis* L. 45

CAPÍTULO 2: 64

Cytotoxic and Immunomodulatory Potential of a Novel Pyrimidine Ensemble [2-(4-(2,5-dimethyl-1H-pyrrol-1-yl)-1H-pyrazol-3-yl)pyridine] in Myeloid Leukemia..... 64

CAPÍTULO 3: 74

Pharmacological assessment of the Antineoplastic and Immunomodulatory Properties of a new spiroindolone derivative (7',8'-Dimethoxy-1',3'-dimethyl-1,2,3',4'-tetrahydrospiro[indole-3,5'-pyrazolo[3,4-c]isoquinolin]-2-one) in Chronic Myeloid Leukemia 74

6. CONCLUSÃO..... 89

7. REFERÊNCIAS 90

1. INTRODUÇÃO

O termo “câncer” é utilizado para designar um conjunto de doenças que são caracterizadas pela expansão autônoma e disseminação de um clone celular somático. Este clone pode fazer uso da ativação/estimulação de várias vias de sinalização celular, impedindo a vigilância imunológica, o que favorece sua proliferação, invasão de barreiras epiteliais e disseminação para órgãos adjacentes (INCA, 2019). De acordo com a Organização Mundial da Saúde (OMS), o câncer é uma das principais causas de morte no mundo e supõe-se que sua incidência aumente em mais de 50% nas próximas décadas (WHO, 2019). Somente no ano de 2020, estimou-se cerca de 18,1 milhões de novos casos e 9,9 milhões de óbitos em todo mundo (SUNG et al., 2021).

As células cancerígenas sofrem constantes mutações e frequentemente evadem da resposta imunológica, o que as tornam resistentes a intervenções terapêuticas, gerando dificuldades para uma cura (VINAY et al., 2015). Como forma de tratamentos principais, a radioterapia e a quimioterapia são as mais empregadas no tratamento de várias neoplasias (TERWILLIGER; ABDUL-HAY, 2017).

No entanto, essas terapias podem gerar intensos efeitos colaterais aos pacientes. Como exemplo, a quimioterapia possui problemas com efeitos de direcionamento e toxicidade e resistencia, enquanto que a radioterapia pode produzir efeitos colaterais tóxicos locais e sistêmicos e levar à supressão imunológica sistêmica (CAO et al., 2018). Além disso, a interação entre abordagens quimioterápicas e o sistema imunológico podem gerar uma influência decisiva no resultado dos pacientes (SERRANO-DEL VALLE et al., 2020). Assim, a busca por estratégias terapêuticas de baixa toxicidade e com base em substâncias naturais ou sintéticas que auxiliem nas terapias convencionais, têm sido crescente entre a comunidade científica (BUENZ; VERPOORTE; BAUER, 2018).

Segundo a OMS, cerca de 60% da população global faz uso de ervas medicinais e 80% da população de países em desenvolvimento utiliza essas ervas como principal forma de tratamento primário (WHO, 2019). As biomoléculas ativas naturais têm sido uma fonte para muitas formulações terapêuticas direcionadas a diferentes processos patológicos (PIRES et al., 2012). Por possuírem atividades anticancerígenas, muitas destas moléculas são farmacologicamente exploradas como alternativas de tratamento

para diversos tipos de neoplasias (SUBRAMANIAM; SELVADURAY; RADHAKRISHNAN, 2019).

Muitos medicamentos utilizados atualmente no tratamento de neoplasias são provenientes de espécies de plantas ou análogos sintéticos derivados de plantas, como vimblastina, vincristina, irinotecan, dentre outros (CRAGG; NEWMAN, 2005; NEWMAN; CRAGG, 2020). Estas substâncias podem agir pode diversos mecanismos celular, como nas tubulinas, impedindo a formação correta de microtúbulos, o que promove a interrupção ciclo celular e desencadeia a apoptose (NEWMAN; CRAGG, 2020). Por exemplo, a vincristina foi isolada da planta *Catharanthus roseus* e possui a capacidade de causar depressão da medula óssea causando diminuição da contagem de leucócitos, sendo utilizada no tratamento da leucemia linfoide aguda (LLA) (MADSEN et al., 2019).

O câncer tornou-se um dos maiores desafios para saúde pública mundial e exige a realização de ações profiláticas e terapêuticas com graus diversificados de complexidade. Diante disto, estudos que visam a utilização de produtos naturais para o desenvolvimento de estratégias terapêuticas de melhor custo-benefício e baixa toxicidade para o paciente com câncer têm se tornado cada vez mais necessários. Dessa forma, este estudo se propôs a avaliar o potencial antineoplásico e imunomodulador de compostos naturais, derivados bioativos e sintéticos.

2. REFERENCIAL TEÓRICO

2.1. Câncer: aspectos gerais

O câncer é resultado de uma proliferação contínua de células geneticamente alteradas e que apresentam, em diferentes graus, características que as distiguem das células normais, como proliferação descontrolada, perda de função, invasividade e metástase (HANAHAN; WEINBERG, 2011a). Este grupo de doenças apresenta uma etiologia multifatorial decorrente de causas internas, como alterações genéticas e imunológicas, e causas externas, como infecções virais e exposição à radiação ou substâncias químicas (PARSA, 2012).

Naturalmente, as células apresentam mutações espontâneas, mas que não afetam seu crescimento normal (CAMPBELL; MARTINCORENA, 2015). Por outro lado, quando essas mutações ocorrem em genes específicos, chamados de proto-oncogenes, estes se transformam em oncogenes que passam a atuar na transformação de células normais em células cancerosas (SAITO; KOYA; KATAOKA, 2021). Dentre os oncogenes frequentemente ligados a neoplasias estão o fator de transcrição MYC, genes da família RAS e tirosina-quinase RET. Todos esses genes expressam proteínas com funções regulatórias no ciclo celular, apoptose e diferenciação celular. Porém, quando são expressos de forma aberrante, desencadeiam o crescimento e proliferação celular anormal (PRIOR; HOOD; HARTLEY, 2020; SAITO; KOYA; KATAOKA, 2021).

Para casos moderados e avançados são indicadas três principais abordagens terapêuticas: cirurgia, quimioterapia e radioterapia. A cirurgia tende a ser voltada a remoção de tumores sólidos comuns, enquanto que a quimioterapia (administração de agentes citotóxicos) tem a função de impedir a proliferação de células neoplásicas (TOHME; SIMMONS; TSUNG, 2018). A radioterapia trabalha com o tratamento local por meio de radiações ionizantes, afetando células cancerosas e o microambiente tumoral (JAROSZ-BIEJ et al., 2019).

A GLOBOCAN (Observatório Global do Câncer) estima que até o ano de 2040 haja um aumento de 61,7% na incidência de câncer mundialmente, ou seja, cerca de 27,5 milhões de novos casos a cada ano (BRAY et al., 2018). Os tipos de câncer de maior incidência no ano de 2020 foram o câncer o câncer de mama (2.261.419 casos), pulmão (2.206.771 casos), próstata (1.414.259 casos) e cólon (1.148.515 casos) (SUNG et al.,

2021). Entre os principais fatores atrelados a este aumento estão o crescimento e envelhecimento populacional, assim como o status socioeconômico da população, os quais tem relação direta com hábitos e situações que favorecem o desenvolvimento do câncer, como sedentarismo, alimentação inadequada, precariedade sanitária, não acesso a planos de saúde, dentre outros (BRAY et al., 2018).

No Brasil, segundo levantamento da distribuição de incidência por região geográfica do Instituto Nacional de Câncer (INCA), a região Sudeste apresenta 60% da incidência de casos de câncer, acompanhada da região Nordeste (27,8%) e Região Sul (23,4%) (INCA, 2019). Quando estratificados por sexo, a incidência de neoplasias entre homens e mulheres difere de acordo com as regiões do país (**Figura 1**) (INCA, 2019). Na região Norte para o ano de 2020, estimou-se para o estado do Amazonas um total de 2.350 novos casos de câncer em homens e 2.900 em mulheres. Em Manaus, a incidência estimada para o mesmo ano foi de 1.910 casos em mulheres e 1.610 em homens.

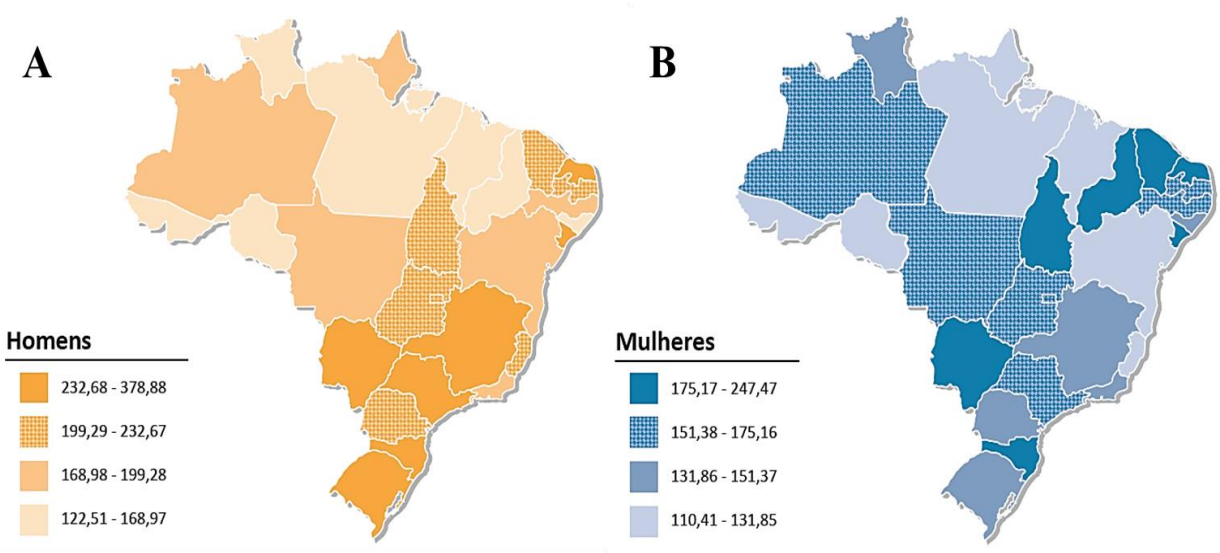


Figura 1. Representação espacial de taxas ajustadas de incidência por 100 mil homens (A) e 100 mil mulheres (B), estimadas para o ano de 2020, de acordo com Unidade da Federação (todas as neoplasias malignas, com exceção as de pele e melanoma).

Fonte: MS, INCA, 2019.

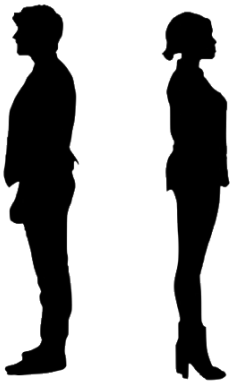
Localização primária	Casos	%	Homens	Mulheres	Localização primária	Casos	%
Próstata	65.840	29,2			Mama feminina	66.280	29,7
Cólon e reto	20.520	9,1			Cólon e reto	20.470	9,2
Traqueia, brônquio e pulmão	17.760	7,9			Colo do útero	16.590	7,4
Estômago	13.360	5,9			Traqueia, brônquio e pulmão	12.440	5,6
Cavidade oral	11.180	5,0			Glândula tireoide	11.950	5,4
Esôfago	8.690	3,9			Estômago	7.870	3,5
Bexiga	7.590	3,4			Ovário	6.650	3,0
Linfoma não Hodgkin	6.580	2,9			Corpo do útero	6.540	2,9
Laringe	6.470	2,9			Linfoma não Hodgkin	5.450	2,4
Leucemias	5.920	2,6			Sistema Nervoso Central	5.220	2,3

Figura 2. Distribuição proporcional dos dez tipos de neoplasias mais incidentes estimados para 2020-22 por sexo (exceto pele não melanoma). *Números arredondados para múltiplos de 10.

Fonte: adaptado de MS, INCA, 2019.

Segundo estimativas para 2020-22, dentre os 10 tipos de câncer mais incidentes no Brasil, o câncer de próstata e o de mama serão os de maior incidência entre homens (29,2%) e mulheres (29,7%), respectivamente (**Figura 2**). As leucemias apresentam uma maior incidência entre homens com 2,6% de casos. Além disso, estima-se que no Brasil, para cada ano do triênio 2020-22, ocorrerão cerca de 625 mil novos casos de câncer, sendo 10 mil casos de leucemias (INCA, 2019).

2.2. Imunopatogênese do câncer

Diversos fatores imunes como células da imunidade inata e adquirida, expressão de receptores celulares, citocinas, quimiocinas e enzimas desempenham importante papel no reconhecimento, controle e inibição de células malignas ou anormais. Entretanto, o câncer pode ter seu desenvolvimento atrelado à deficiência da vigilância imunológica, especialmente devido a mecanismos de escape imunológico de células cancerígenas (KIM; EMI; TANABE, 2007). Contudo, o papel de fatores imunes no processo de oncogênese ou em esquema terapêuticos ainda não está completamente esclarecido.

O sistema imunológico pode exercer papel duplo no câncer ao restringir ou promover o desenvolvimento/progressão de células cancerígenas por meio de um processo denominado imunoedição do câncer, que consiste em três fases: eliminação,

equilíbrio e escape **(Figura 3)** (SCHREIBER; OLD; SMYTH, 2011). O processo de imunoedição do câncer ocorre normalmente durante a progressão natural da doença. No entanto, pode vir a ocorrer novamente, devido à quimiorresistência a tratamentos convencionais (O'DONNELL; TENG; SMYTH, 2019).

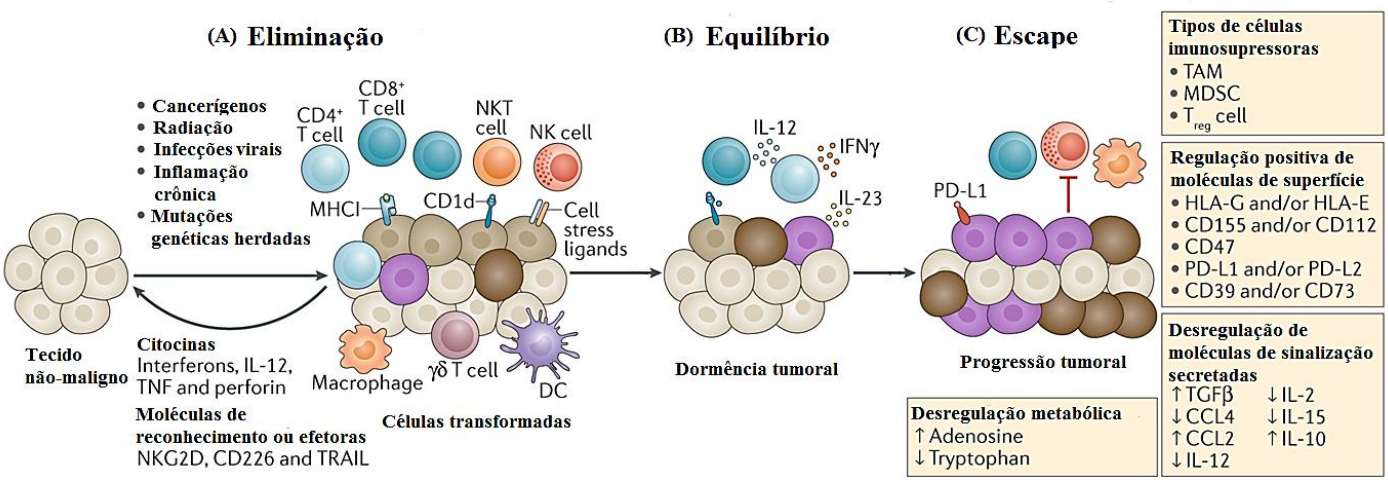


Figura 3. Processo de imunoedição do câncer. Este processo consiste em três fases: (A) eliminação, (B) equilíbrio, (C) escape.

Fonte: adaptado de O'DONNELL; TENG; SMYTH, 2019

A fase de eliminação (**Figura 3.A**) é um componente da teoria da vigilância imunológica caracterizada pelo reconhecimento e ação conjunta da imunidade inata e adaptativa no combate e destruição de células cancerígenas (SCHREIBER; OLD; SMYTH, 2011; SUBRAMANIAM; SELVADURAY; RADHAKRISHNAN, 2019). Exemplo dessas ações é a secreção de perforina por células imunes citolíticas, como células *Natural Killer* (NK), células NKT, células T $CD8^+$ e células dendríticas atuando na lise das células cancerosas (VESELY et al., 2011).

A fase de equilíbrio (**Figura 3.B**) consiste em subclones cancerígenos que não foram erradicados durante a fase de eliminação, porém as respostas imunes ainda se encontram ativas contra o câncer e a imunogenicidade celular é editada pelo sistema imune adaptativo (SCHREIBER; OLD; SMYTH, 2011; SUBRAMANIAM; SELVADURAY; RADHAKRISHNAN, 2019). Nessa fase, células T helper 1 (Th1) participam mantendo as células cancerosas em um estado de dormência por meio da liberação de citocinas, dentre elas a IL-12, IL-23 e IFNγ, que neutralizam e reduzem o potencial maligno de células cancerígenas (O'DONNELL; TENG; SMYTH, 2019).

A fase de escape (**Figura 3.C**) representa a terceira e última fase do processo de imunoedição, caracterizada pelo crescimento progressivo de células cancerígenas, estabelecimento do ambiente imunossupressor, e na qual o câncer passa a ser diagnosticado clinicamente (O'DONNELL; TENG; SMYTH, 2019; SCHREIBER; OLD; SMYTH, 2011). Os fatores que levam ao escape de células cancerígenas do sistema imunológico são diversos, relacionados principalmente ao desenvolvimento de um microambiente cancerígeno imunossupressor (SAUSSEZ et al., 2010).

No microambiente de neoplasias hematológicas, algumas citocinas também têm sido relacionadas a promoção de células malignas. Mudanças qualitativas e quantitativas no perfil dessas proteínas promovem a desdiferenciação de células neoplásicas, gerando um fenótipo maligno (RAJA et al., 2017). Esse fato é observado principalmente em citocinas pró-inflamatórias, como TNFα, IL1β e IL6, mas também em citocinas anti-inflamatórias, como a IL-10, que juntas estão associadas a progressão de células leucêmicas (**Figura 4**) (CAMACHO; KUZNETSOVA; WELNER, 2021).

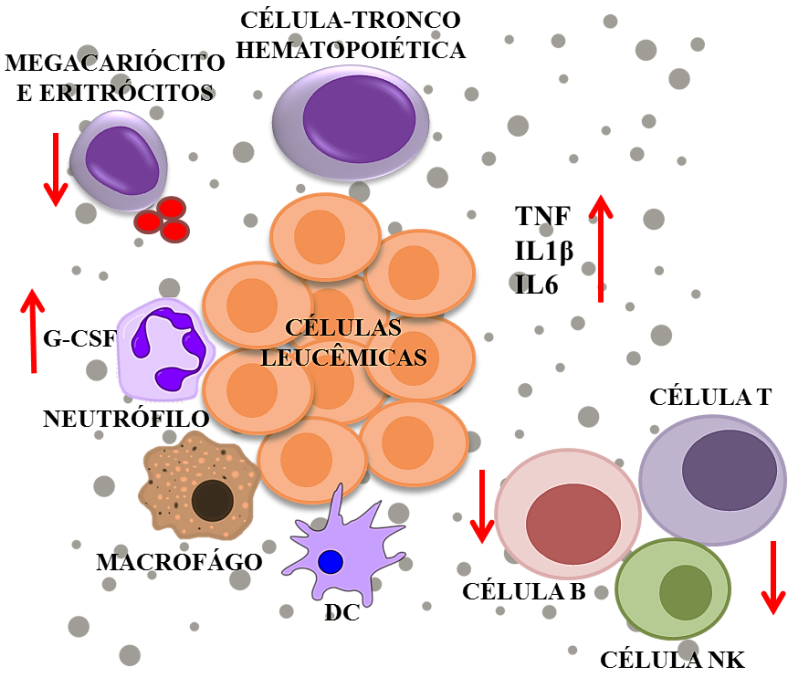


Figura 4. Pertubação hematopoiéticas de citocinas inflamatórias durante a leucemia. As citocinas inflamatórias associadas a leucemia medeiam a diferenciação, abundância e funções celulares de fatores da imunidade inata e adaptativa.

Fonte: adapatado de CAMACHO; KUZNETSOVA; WELNER, 2021.

O fator de necrose tumoral alfa (TNF-α) é uma citocina que tem papel importante no processo de apoptose e homeostase celular, sendo secretado principalmente por macrófagos, células NK e linfócitos (BALKWILL, 2009). Entretanto, TNF-α também é capaz de induzir a iniciação e progressão de células cancerígenas (BALKWILL, 2009). Está citocina é encontrada em altos níveis em microambiente leucêmico atuando na evasão imunológica, sobrevivência e resistência à quimioterapia (JACOBSON et al., 2019; ZHOU; LI; ZHOU, 2017). Essa indução de células cancerígenas é mediada pela capacidade de TNF-α na ativação de vias dependentes de fator nuclear k-B (NF-kB), proteína quinase Cα (PKCα-) e proteína adaptadora 1 (AP-1) (WU; ZHOU, 2010).

A IL-6 é uma citocina pleiotrópica pró-inflamatória que possui importante papel na defesa do organismo contra patógenos e estresse agudo (YAO et al., 2014). Porém sua expressão desregulada está envolvida na patogênese de neoplasias (YAO et al., 2014). No processo de oncogênese, a IL-6 possui funções antiapoptóticas e promotora do crescimento de células cancerígenas ao estimular a liberação de ROS e RNS, além de estimular a produção de proteínas Bcl, reguladoras chave da apoptose (PU et al., 2004; WEI et al., 2001). Por outro lado, a IL-6 pode promover efeito antineoplásico ao mobilizar o tráfego de células T para o microambiente tumoral e modular a resposta dessas células de um estado supressivo para um estado responsivo, assim agindo contra células cancerígenas (FISHER; APPENHEIMER; EVANS, 2014; MIKUCKI et al., 2013)

A IL1β é uma potente citocina pró-inflamatória produzida por monócitos e células mieloides (CAMACHO; KUZNETSOVA; WELNER, 2021). Altos níveis de IL1β são observados em neoplasias hematológicas, fazendo desta um importante alvo terapêutico (ARRANZ; ARRIERO; VILLATORO, 2017). Na LMC este aumento está atrelado a um mau-prognóstico, além de contribuir para resistência a inibidores de tirosina quinase (TKIs) aumentando a viabilidade de células cancerígenas (LEE et al., 2016; ZHAO et al., 2014). Na LMA o aumento da IL1β está associado à riscos da doença através de estresses crônicos, também gerando infiltrações de células leucêmicas (LIU et al., 2021).

A IL10 apresenta perfil anti-inflamatório tendo papel essencial na regulação da resposta imune ao suprimir a atividade de macrófagos e citocinas pró-inflamatórias (OFT, 2014). Sua participação na patogênese do câncer ainda é controversa, no entanto alguns estudos indicam que ela pode está atrelada ao escape e desenvolvimento de células

cancerígenas ao diminuir a resposta imune antineoplásica (MANNINO et al., 2015). Além disso, polimorfismos no nucleotídeo do gene promotor da IL10 e níveis séricos alterados estão associados à susceptibilidade a câncer de cabeça e pescoço (MAKNI et al., 2019). Por outro lado, estudos apontam que deficiências na sinalização de IL10 podem levar ao desenvolvimento preoce do câncer (NEVEN et al., 2013).

As células T CD4+ também podem apresentar duplo papel durante a leucemogênese. Em um papel protetor, células CD4+ Th1 auxiliam nas funções de células T CD8+, facilitando a eliminação de células cancerígenas, ao passo que células CD4+ Th2, promovem a produção de anticorpos por células B, contribuindo para destruição de células cancerígenas (SAUSSEZ et al., 2010). Em contrapartida, células T CD4+ Th17, por meio da produção de IL-17, estimulam positivamente a secreção de citocinas e quimiocinas possibilitando a inflamação do ambiente leucêmico, o que possibilita, quando em frequência, a proliferação de células cancerígenas e metástase (BI; LIU; YANG, 2007). Também, células Treg, através da secreção de IL-10, IL-35 e PGE2, podem inibir ação de células T CD8+ e células NK, possibilitando o escape e promoção de células cancerígenas (CURIEL et al., 2004)

O sistema imunológico como resposta ao câncer age de forma complexa, contando com a participação de diversos fatores pró- e anti-inflamatórios (CICCHESE et al., 2018). Contudo, é essencial que este sistema, ao tentar controlar a progressão neoplásica, evite uma resposta inflamatória exacerbada que cause danos ao organismo e favoreça a oncogênese (CICCHESE et al., 2018). Desta forma, compreender a dinâmica da modulação imune no contexto do câncer é essencial para a formulação de estratégias terapêuticas que promovam o controle ou inibição de células cancerígenas.

2.3. Leucemias

As leucemias são um grupo heterogêneo de neoplasias caracterizadas pelo comprometimento de células-tronco hematopoiéticas (BROWN, 2022). As leucemias são categorizadas de acordo com o tipo de linhagem celular acometida, aspectos clínicos, prognóstico e resposta à terapia (BROWN, 2022). Dessa forma, podem ser classificadas em: leucemia mielóide aguda (LMA), leucemia mielóide crônica (LMC), leucemia linfoide aguda (LLA) e leucemia linfoide crônica (LLC) (**Figura 5**) (ARBER et al., 2016a; BAIN; ESTCOURT, 2013).

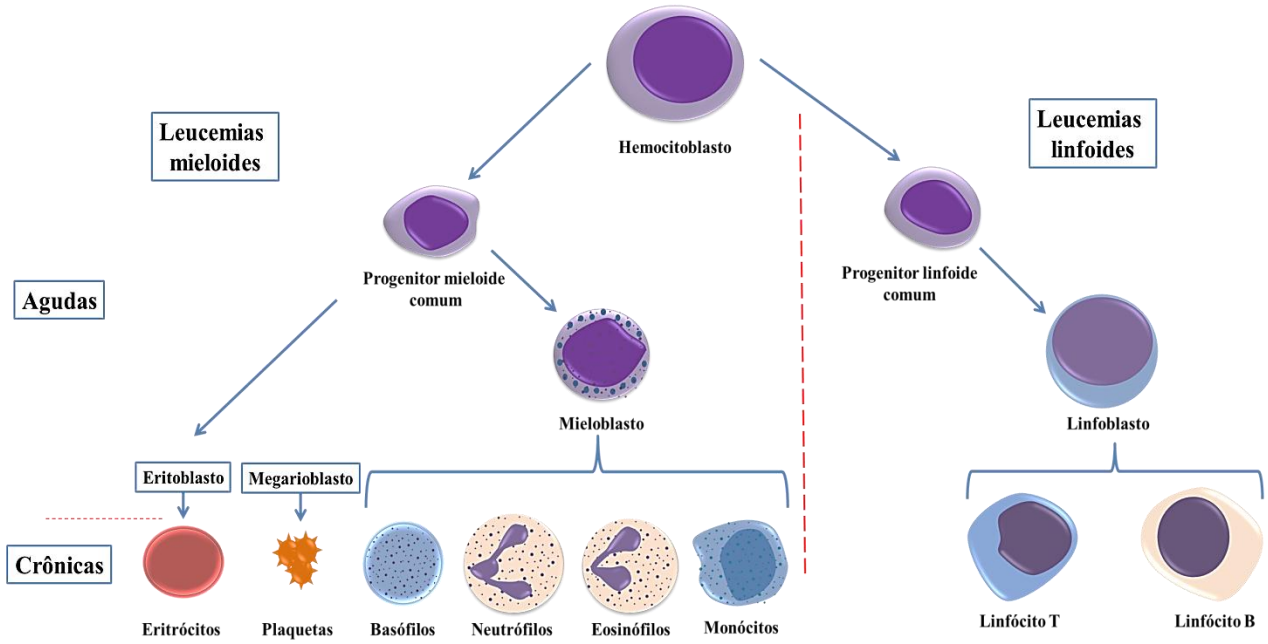


Figura 5. Esquema de patogenia das leucemias.
Fonte: confecção própria.

Segundo dados da GLOBOCAN, foram estimados 474.519 novos casos de leucemias e 311.594 óbitos para o ano de 2020 (SUNG et al., 2021). No Brasil, para cada ano do triênio 2020-2022, são estimados cerca de 5.920 casos de leucemias em homens e 4.890 casos em mulheres. Na Região Norte, a leucemia em homens é a quinta neoplasia mais frequente, representando risco estimado de 4,45 para cada 100 mil habitantes (INCA, 2019).

As leucemias compõe um grupo de doenças multifatoriais desencadeadas pela interação de fatores genéticos e ambientais, com menos de 10% dos casos atribuídos à hereditariedade (CRUMP et al., 2015; WIEMELS, 2012). No geral, estima-se que fatores ambientais, dietéticos, infecções e alergias também exerçam influência na etiologia da doença (LARIOU et al., 2013; WANG et al., 2019).

O diagnóstico é baseado em características morfológicas, imunofenotípicas, cariótipo e/ou análise molecular, o que leva à classificação da leucemia como aguda ou crônica, dependendo do estágio de proliferação e maturação (ARBER et al., 2016b; GRIMWADE, 2017).

2.3.1. Leucemia mieloide aguda (LMA)

A LMA é resultado de alterações genéticas e baixa produção de células hematopoiéticas saudáveis que culminam na inibição da diferenciação celular e induzem a proliferação do acúmulo de mieloblastos anormais na médula óssea, sangue e outros tecidos (PRADA-ARISMENDY; ARROYAVE; RÖTHLISBERGER, 2017). É a leucemia aguda de maior incidência entre adultos acima de 65 anos de idade (80% dos casos), sendo ligeiramente mais comum entre homens do que mulheres (DE KOUCHKOVSKY; ABDUL-HAY, 2016).

A LMA possui elevada incidência em países desenvolvidos, como os Estados Unidos da América (EUA), no qual é frequentemente diagnosticada em homens de ancestralidade caucasiana e é responsável pela maior porcentagem (62%) de óbitos dentre os subtipos de leucemia no país (SHALLIS et al., 2019). Segundo dados do programa *The Surveillance, Epidemiology and End Results* (SEER), para o ano de 2021, estima-se 20.240 novos casos de LMA e 11.400 óbitos nos EUA. Semelhante aos dados encontrados nos EUA, países como Suécia e Dinamarca apresentam alta taxa de incidência para LMA (JULIUSSON et al., 2012; OCIAS et al., 2016).

No Brasil, de acordo com estudo do Observatório de Oncologia realizado entre os anos de 2008 a 2017, dos 63 mil óbitos por leucemia, 36% foram ocasionados por LMA, sendo o estado de São Paulo detendor da maior taxa de mortalidade absoluta. Na população infanto-juvenil, segundo estudos epidemiológicos, a LMA foi observada principalmente no sexo masculino e em pacientes acima de 10 anos de idade (DE LIMA et al., 2016; MORAIS et al., 2021).

Um estudo analisou o perfil epidemiológico da LMA em 266 pacientes entre o ano de 2005 a 2015 no estado do Amazonas (SILVA-JUNIOR et al., 2019). Observou-se que a maioria dos pacientes eram homens (56,77%), acima de 60 anos de idade (26,05%), residentes da cidade de Manaus (65,22%) e apresentavam alguma comorbidade (68,05%), tendo maior número de casos registrado em 2012 (34 casos). Dos pacientes estudados, 59,77% vieram a óbito.

O desenvolvimento da LMA está associado a frequentes mutações que ocorrem durante o processo de hematopoiese. De acordo com modelo de leucemogênese, as mutações associadas à LMA podem ser classificadas em classe I e classe II, as quais

ocorrem em genes envolvidos na ativação de vias pro-proliferativas e inibição da diferenciação hematopoiética normal, respectivamente (TAKAHASHI, 2011). Para classe I, mutações em *FLT3* (duplicações internas em tandem, ITD e mutações no domínio TK) ocorrem em 28% dos casos (KENNEDY; SMITH, 2020). Quanto à classe II, o exemplo comum inclui mutações em *NPM1* (nucleofosmina) e *CEBPA* (CCAAT/proteína alfa de ligação do realçador), que ocorrem em 27 e 6% dos casos, respectivamente (GREEN et al., 2010).

Com base em informações genéticas, imunofenótipo e apresentação clínica, a OMS classificou a LMA em 6 tipos: LMA com anormalidades genéticas recorrentes, LMA relacionada à mielodisplasia; LMA relacionada à terapia; LMA sem outra especificação; sarcoma mieloide; e proliferação mieloide relacionadas à Síndrome de Down (ARBER et al., 2016c). A LMA também pode ser classificada em três categorias de risco, sendo elas: favorável, intermediário e desfavorável (ESTEY et al., 2017). Estes grupos se distinguem pelas características citogenéticas e no reconhecimento de subconjuntos moleculares que respondem de diferentes formas à terapia padrão (ESTEY et al., 2017). Quando dividida por perfil de risco, a taxa de sobrevida global de pacientes abaixo de 55 anos de idade é de 64% pra risco favorável, 42% para intermediário e 12% para desfavorável. Essas taxas diminuem em idosos acima de 65 anos de idade (PATEL et al., 2013).

O principal tratamento para LMA consiste num regime intensivo de citarabina e antaciclina, junto ao transplante de células-tronco alogênico para indivíduos elegíveis (DE KOUCHKOVSKY; ABDUL-HAY, 2016). Os pacientes jovens respondem bem a estas intervenções obtendo um bom prognóstico. Por outro lado, pacientes idosos geralmente são mais vulneráveis e não suportam o tratamento, resultando em um prognótico ruim (ALMEIDA; RAMOS, 2016; MEYERS et al., 2013). Apenas 5% dos pacientes idosos sobrevivem 5 anos após o diagnóstico de LMA e cerca de 70% vêm a óbito 1 ano após o diagnóstico (MEYERS et al., 2013).

2.3.2. Leucemia mieloide crônica (LMC)

A LMC é uma doença maligna mieloproliferativa clonal, constituída por dois ou três cursos clínicos: fase crônica indolente (FC) seguida por uma fase acelerada (FA) e posterior fase blástica agressiva (FB) (APPERLEY, 2015). A FC tem duração de 5 a 6 anos com sintomas leves ou indolentes. A FA pode durar de semanas a anos e consiste no

surgimento de blastos no sangue ou médula óssea, além de agravamentos, como a trombocitose. Já a FB representa o estágio final, no qual blastos predominam (>20%) fora da médula óssea e a sobrevida é medida em meses (APPERLEY, 2015; SOVERINI et al., 2018).

A LMC representa de 15 a 20% dos casos de leucemias em adultos com incidência de 10-12 casos por 100.000 indivíduos, ocorrendo com mais frequênia em homens de 40 a 60 anos de idade (HÖGLUND; SANDIN; SIMONSSON, 2015; SOSSELA; ZOPPAS; WEBER, 2017). Em crianças, a LMC se mostra rara tendo uma incidência de 0,6-1,2 milhões crianças/ano (DE LA FUENTE et al., 2014). Para os EUA, segundo banco de dados do programa SEER, são estimados 9.110 novos casos e 1.220 óbitos por LMC para o ano de 2021.

No Brasil, existem poucos dados epidemiológicos atualizados acerca da LMC. Segundo o Observatório de Oncologia, a LMC representou 7% dos 63 mil casos de óbitos por leucemia registrados entre 2008 a 2017, com elevada taxa em pacientes acima de 50 anos no estado do Rio Grande do Sul (2,2% ao ano). No Estado de Pernambuco, um estudo observou o perfil epidemiológico da LMC entre os anos de 2004 a 2015 e observou uma incidência de 3-10 casos por 100.000 habitantes, com predomínio em homens com idade média de 47 anos (NEVES et al., 2019).

Na maioria dos casos (90 a 95%) o processo de leucemogênese da LMC está associado a uma anormalidade persistente no cromossomo Filadélfia (Ph), produto da translocação recíproca entre os cromossomos 9 e 22, t (9:22) (ZHOU; MEDEIROS; HU, 2018). Esta translocação cromossômica gera o oncogene de fusão *BCR-ABL*$_1$, a partir da associação do gene *ABL*$_1$ (Abelson tirosina quinase) no cromossomo 9 com o gene *BCR* (região de cluster de ponto de interrupção) presente no cromossomo 22 (QUINTÁS-CARDAMA; CORTES, 2009).

O oncogene *BCR-ABL*$_1$ expressa a proteína BCR-BL$_1$, uma TK constitutivamente ativa que confere aumento de proliferação, perda de adesão estromal e resistência a apoptose às células da LMC (GOLDMAN; MELO, 2001; QUINTÁS-CARDAMA; CORTES, 2009). Esse fenótipo lêucemico gerado por BCR-BL$_1$ se dá através de vias a jusante, especialmente RAF, RAS, Jun quinase, MYC e STAT (ALBAJAR et al., 2011; JABBOUR; KANTARJIAN, 2014).

O tratamento da LMC consiste em terapia celular através de transplante de células-tronco hematopoieticas, ou na administração de inibidores de TK (TKIs), organizados em primeira geração (imatinibe), segunda geração (desatinibe, nilotinibe e bosutinibe) e terceira geração (ponatinibe) (SOVERINI et al., 2019).

Antes da introdução de TKIs, a sobrevida média de pacientes com LMC era de apenas 5 a 7 anos (APPERLEY, 2015). Entretanto, cerca de 10% dos pacientes apresentam resistência ao tratamento por estes medicamentos (BACCARANI; ROSTI; SOVERINI, 2019). Isso se deve por mutações pontuais presentes no domínio de ABL_1 que impedem a ligação de TKIs. Exemplo frequente é a mutação que leva a troca de treonina por isoleucina 315 (T315l) que dificulta a ligação de TKIs de primeira e segunda geração (SOVERINI et al., 2014). Já como efeito adverso, o uso do imatinibe pode estar associado à hipogamaglobulinemia em crianças e adolescentes portadoras de LMC (TOTADRI et al., 2020).

2.4. Terapias convencionais no tratamento do câncer

Atualmente, as principais terapias convencionais que compreendem o tratamento do câncer envolvem a cirurgia, quimioterapia, radioterapia e transplante de médula óssea (BRASIL, 2014). Geralmente são adotadas em conjunto, variando de importância e ordem de administração. Embora essas terapias sejam indicações curativas indispensáveis, algumas destas podem ocasionar intensos efeitos nocivos ao paciente, como dores, náuseas, cardiotoxicidade, perda capilar, ressecamento ou escurecimento da pele, úlceras, assim como alterações hormonais e imunológicas (METRI et al., 2013; SERENO et al., 2008).

Há anos a radioterapia tem se tornado uma especialidade reconhecida no tratamento médico de cânceres, sendo uma abordagem eficaz e não invasiva, reduzindo o risco de morte e recorrência (BASKAR et al., 2012). Esta terapia consiste no emprego de um agente físico usado para eliminar células cancerosas através de danos diretos ao DNA, impedindo a divisão e proliferação celular. A radioterapia utiliza a radiação ionizante (γ ou raios X) aplicada diretamente na área de localização do tumor (JAROSZ-BIEJ et al., 2019).

Aproximadamente 50% dos pacientes com câncer receberão radioterapia durante o curso da doença, estimando-se um tratamento curativo contribuinte em cerca de 40%

dos casos (BARNETT et al., 2009). Por outro lado, estudos apontam que a radiação apresenta efeitos prejudiciais à células não cancerígenas, podendo resultar em necrose de tecido ou até mesmo o desenvolvimento de outros tipos de cânceres, devido à utilização de equipamentos de baixa precisão (BARNETT et al., 2009; HALL, 2006). Para que a radioterapia tenha uma taxa maior de eficácia, é necessário fazer seu uso combinado com a quimioterapia, ou outras terapias alternativas (HALL, 2006).

A quimioterapia tem sido utilizada como tratamento mais comum contra o câncer desde sua aprovação, há mais de 60 anos (BAUDINO, 2015). Este tratamento é principalmente indicado em concomitância com a radioterapia como terapia neoadjuvante (pré-cirurgia) para dimunir o número de cirurgias remocivas necessárias. Contudo, a quimioterapia também pode ser empregada como terapia adjuvante (pós-cirurgia), devido à possibilidade de não remoção total de tumores (BAUDINO, 2015; BRASIL, 2014).

A quimioterapia consiste no uso de substâncias químicas, chamadas de quimioterápicos, que agem em células cancerigenas via diferentes mecanismos, tendo como função principal inibir proliferação celular ou induzir apoptose (**Tabela 1**) (LARIONOVA et al., 2019). No entanto, são comuns os casos de resistência às substâncias utilizadas nesta abordagem, o que resulta na eliminação incompleta das células cancerígenas, ocasionando a ocorrência de recidivas ou o surgimento de câncer mais agressivo (BAUDINO, 2015). O efeito citotóxico em células não cancerígenas também preocupa, como o caso de redução de glóbulos brancos e vermelhos, o que pode aumentar a susceptibilidade a infecções (CRAWFORD; DALE; LYMAN, 2004; ROHATGI et al., 2014). Outra desvantagem conhecida são efeitos colaterais comuns da terapia, como a neutropenia, anemia, estomatite, mucosite, diarreia e vômitos (HAUNER; MAISCH; RETZ, 2017).

Tabela 1. Mecanismos de ação de alguns dos principais quimioterápicos.

Quimioterapico	Mecanismo de Ação	Câncer alvo
Paclitaxel e docetaxel	Inibem a polimerização/despolimerização da tubulina	Câncer de mama, câncer de ovário, câncer de pulmão, etc.
Doxorrubicina (Adriamicina)	Interropem o reparo do DNA mediado pela topoisomerase-II	Câncer de mama, LFA, câncer de bexiga, etc.

Cisplatina e carboplatina	Formação de reticulações inter- e intra-fita de DNA	Câncer de testículo, câncer de ovário, câncer de mama, etc.
5-fluorouracil	Inibe a enzima timidilato sintetase	Câncer de cólon, câncer de esôfago, câncer de estômago, etc.
Gencitabina	Atua como antimetabólito de nucleosídeo de pirimidina	Câncer de pâncreas, câncer de pulmão, câncer de mama metastásico, etc.
Trabectedina	Ligam-se ao sulco menor do DNA; inibe a transcrição e reparo do DNA	Sarcomas avançados de tecidos moles, câncer de ovário.

Fonte: adaptado de (LARIONOVA et al., 2019).

Além das terapias citadas, a imunoterapia tem se tornado uma abordagem promissora no tratamento de neoplasias. Os agentes imunoterapêuticos melhoram a citotoxicidade e a capacidade proliferativa de linfócitos T infiltrantes por meio do bloqueio de receptores imunossupressores, como PD-1 e o antígeno-4 do linfócito T citotóxico (CTLA-4) (SEIDEL; OTSUKA; KABASHIMA, 2018). Outro método imunoterapêutico é a utilização de células T de receptor de antígeno quimérico específico (CAR-T). Essas células são desenvolvidas de acordo com especificidades alvo da célula tumoral, como CART-T voltados para mesotelina, glicoproteína altamente expressa em CMTN (KEENAN; TOLANEY, 2020). Entretanto, essa estratégia além do custo elevado, também pode ocasionar efeitos colaterais inflamatórios específicos no órgão tratado e síndrome de neurotoxicidade associada a células efetoras imunológicas (NEELAPU et al., 2018).

Uma forma de amenizar os efeitos colaterais gerados por essas terapias é a utilização de medidas profiláticas direcionadas, como monitoramento e avaliação, e gerenciamento de efeitos colaterais, além de um esclarecimento adequado junto ao paciente feito pelos profissionais da saúde (BRASIL, 2014). Além disso, como estratégia emergente, a pesquisa voltada para a utilização de produtos naturais ou novos sintéticos

como potenciais terapias alternativas para o tratamento único ou complementar de neoplasias (ou como quimiopreventivos) tem sido cada vez mais frequentes. Isso se deve ao fato destes produtos poderem apresentar baixa citotoxicidade às células normais e modularem a resposta imunológica contra o câncer (PIRES et al., 2012).

2.5. Compostos naturais como estratégia terapêutica contra o câncer

A quimioterapia e a radioterapia são as principais intervenções terapêuticas contra o câncer. Contudo, os medicamentos e abordagens utilizados nestes processos podem resultar em toxicidade para células normais, resistência à substâncias e outros efeitos nocivos ao paciente, o que dificulta o sucesso terapêutico, como já mencionado (CRAWFORD; DALE; LYMAN, 2004; ROHATGI et al., 2014). Dessa forma, na busca pelo equilíbrio entre eliminar células cancerígenas de forma eficaz e não gerar prejuízos ao paciente, estudos nacionais e internacionais têm investido na pesquisa de compostos naturais para uso na terapia oncológica (DA SILVA MESQUITA et al., 2020; OLIVEIRA et al., 2020; SHIRODE et al., 2015).

Uma grande porcentagem dos agentes terapêuticos contra o câncer utilizados atualmente são oriundos de fontes naturais como plantas, microrganismos e organismos marinhos (CRAGG; NEWMAN, 2013). Dos medicamentos anticancerígenos, 75% foram concebidos e desenvolvidos a partir de compostos naturais derivados de plantas (NEWMAN; CRAGG, 2016).

Atualmente, diversos estudos têm avaliado a atividade antineoplásica e/ou imunomoduladora de compostos naturais e derivados bioativos de plantas (**Tabela 2**). Em alguns casos, muitos destes compostos podem atuar como atenuantes de efeitos colaterais gerados pelo uso dos agentes quimioterápicos convencionais (LEE et al., 2004; LU et al., 2017; SHIRODE et al., 2015). Esses compostos podem agir na interrupção do ciclo celular, ativar a via de apoptos e inibir a angiogênese e metástase de células cancerígenas (SUBRAMANIAM; SELVADURAY; RADHAKRISHNAN, 2019).

Tabela 2. Compostos bioativos derivados de plantas com atividade anticâncer

Câncer alvo	Biocomposto	Atividade biológica	Dosagem/ Concentração	Referência

Mama	Fucoxantina	Anticâncer	10 µM	(RWIGEMERA ; MAMELONA; MARTIN, 2015)
	Punicalagin	Anticâncer	10 mg/ml	(SHIRODE et al., 2015)
	Curcumina	Apoptose	5-50 µg/ml	(NADAF; KILLEDAR, 2018)
	Galato de epigalocatequina	Apoptose	3-25 µg/ml	(LEE et al., 2004)
Leucemias	Rosavin	Anticâncer	8 µg/ml	(SKOPIŃSKA-RÓŻEWSKA et al., 2008)
	Br-Ell-SO_3Na	Anticâncer/ imunomodulador	35 µM	(OLIVEIRA et al., 2020)
	Stepharine & 5-N-metilmaiteina	Anticâncer/ imunomodulador	19,55 µM	(DA SILVA MESQUITA et al., 2020)
Pulmão	Antocianina	Antiproliferativo	400 µg/ml	(LU et al., 2017)
	Saponinas	Anticâncer, apoptose	50 µg/ml	(LI et al., 2015a)
Pancreático	Genistein	Anticâncer	60 µM	(XIA et al., 2014)
	Garcinol	Antiproliferativo	7 µM	
	Limonoides	Antiproliferativo	18-42 µM	(PARASRAMKA; GUPTA, 2012)
Colorretal	Crocin	Apoptose	10 g/L	(BAKSHI et al., 2010)
	Carotenoides	Antiproliferativa	250 µg/ml	(CASTRO-PUYANA et al., 2017)
	B-sitosterol	Anticâncer, apoptose	266,2 µM	(BASKAR et al., 2010)

	Saponinas	Anticâncer	5, 10 ou 20 mg/kg	(LI et al., 2015b)
	Genistein	Antiproliferativo	50 µM	(QI et al., 2011)
	Ácido gálico	Anticâncer	100 µg/ml	(CHEN et al., 2009)
Próstata	Neobavaisoflavonas, psoralidina	Apoptose	50 µM	(SZLISZKA et al., 2011)
	Rodioflavonosídeo	Apoptose	80 µg/ml	(MING et al., 2005)
	Corilagin	Apoptose	20-40 µM	(JIA et al., 2013)
Ovariano	Ácido gálico	Antiproliferativo	40 µM	(HE et al., 2015)
	Ácido elágico	Anti-metástase	50 mg/kg	(LIU et al., 2017)

Fonte: adaptado de (SUBRAMANIAM; SELVADURAY; RADHAKRISHNAN, 2019),

A molécula flavoneglaziovianin A, isolada de folhas da árvore brasileira *Ateleia glazioviana* demonstrou eficácia contra leucemia, linfomas e tumores sólidos (PRAKASH et al., 2013). Esta molécula possui atividade citotóxica contra células leucêmicas promielocíticas, atuando em mecanismos envolvidos na inibição da polimerização da tubulina (YOKOSUKA et al., 2007).

A curcumina (ou diferuloilmetano) é um composto polifenólico bioativo isolado da espécie vegetal *Curcuma longa*, planta nativa da região do Sudeste Asiático e pertencente à família Zingiberacae (CHATTOPADHYAY et al., 2004). Este composto pode exercer atividade anticancerígena por meio de diversos mecanismos, como inibição da angiogênese, inibição da proliferação e metástase, diminuição da inflamação crônica e indução da apoptose de células cancerosas mutantes (SUBRAMANIAM; SELVADURAY; RADHAKRISHNAN, 2019). A curcumina possui ação anticancerígena e imunomoduladora identificada em diversos tipos de neoplasias, dentre elas a LMC, LMA e câncer de mama (LIU et al., 2009; RAO et al., 2011; WU et al., 2003).

O resveratrol (ou 3,5,4'-Triidroxi-trans-estilbeno) é uma fitoalexina encontrada na uva (*Vitis vinífera*), mas também na amora, amendoim e no vinho tinto, possuindo efeito antioxidante e anticancerígeno (VARONI et al., 2016). Na leucemia, foi

demonstrado que resveratrol ocasiona apoptose em células HL60 dependente da sinalização de mTOR reguladas por LKB1-AMOK e PI3K/AKT (FAN et al., 2018).

Nosso grupo de pesquisa avaliou o potencial anticâncer e imunomodulador de um derivado da Elipticina, Br-Ell-SO_3Na (OLIVEIRA et al., 2020). O composto apresentou significativa atividade antiproliferativa com IC_{50} de 35µM frente à linhagem leucêmica K562, além de aumentar a expressão de IL-6 e diminuir a IL-8 em PBMCs. Em outro estudo, avaliamos a atividade anticâncer e imunomoduladora dos alcaloides stepharine e 5-N-metilmaiteina, extraídos de *Abutapanurensis* Eichler (DA SILVA MESQUITA et al., 2020). Os compostos apresentaram atividade citotóxica contra duas linhagens leucêmicas, K562 e U937, nas concentrações de IC_{50} 11,77µM e 28,48µM, respectivamente. Quanta à atividade imunomoduladora, os alcaloides foram capaz de inibir a produção de IL-6, assim como IL-8.

Mesmo com os avanços da medicina, o tratamento do câncer ainda se mantem bastante desafiador (SUBRAMANIAM; SELVADURAY; RADHAKRISHNAN, 2019). Como observado, o uso de compostos naturais e derivados têm se mostrado promissores no combate e prevenção de diversas neoplasias. Diversos compostos naturais com potencial anticancerígeno foram descobertos nos últimos anos, por outro lado, a exploração da biodiversidade disponível como fonte de novos fármacos anticancerígenos ainda é incipiente.

2.6. Compostos sintéticos e atividade anticancerígena

Como citado, a busca por compostos naturais como medida terapêutica oncológica vem sendo realizada a anos por pesquisadores ou povos tradicionais. Entretanto, derivados de metodologia sintética também se apresentam como potentes agentes anticancerígenos direcionados a diversos tipos de neoplasias. Certas vantagens de compostos sintéticos se encontram na possibilidade em modificar estruturas químicas a fim de gerar diferentes relações estrutura-atividade (Liew et al., 2020). Essas sínteses podem auxiliar na diminuição de efeitos colaterais ao aumentar a seletividade e especificidade do composto (LIEW et al., 2020).

Atualmente existem diversos análogos sintéticos derivados de plantas, avaliados cientificamente e indicados no tratamento de diversos tipos de cânceres. Segundo

levantamento de medicamentos aprovadas pela *Food and Drug Administration* (FDA) entre os anos de 1981 e 2019, cerca de 41 a 75% dos compostos são considerados sintéticos ou semissintéticos (NEWMAN; CRAGG, 2020). São exemplos de drogas antitumorais placitaxel (Taxols) e os análogos docetaxel (Taxoteres) e cabazitaxel (Jevtanas), vimblastina (Velbans), vincristina (Oncovins) e seus análogos vindesina (Eldisines) e vinorelbina (Navelbines), e camptotecina e análogos belotecano (Camptobells), topotecano (Hycamtins) e irinotecano (Camptosars), dentre outros (Sharifi-Rad et al., 2019).

As porções heterocíclicas são os principais constituintes de compostos naturais e também de derivados sintéticos, apresentando grande versatilidade, características físico-químicas distintas e sendo encontrados em vários medicamentos (Farhat et al., 2022). Os compostos heterocíclicos apresentam ampla atividade biológica, como ação anticancerígena, antimicrobiana, anti-inflamatória, antimalárica, dentre outras (LIU et al., 2018). São exemplos de drogas antitumorais que contêm núcleo heterocíclico: vinblastina, metotrexato, vincristina, daunorrubicina, 5-fluorouracil e doxorrubicina (KUMAR; GOEL, 2022). Eles atuam contra células cancerígenas através de diferentes mecanismos celular, como estimulação de apoptose, interferência da polimerização de tubulina, inativação de gene *Bcl2*, inibição de p53/MDM2, dentre outros (KUMAR; GOEL, 2022).

Outro exemplo são as neolignanas que são produtos naturais formados pela união de duas unidades C_6C_3, normalmente encontradas em espécies da família *Myristicaceae* e com uma ampla atividade biológica (ZÁLEŠÁK; BON; POSPÍŠIL, 2019). São de imenso interesse sintético e biológico, principalmente na busca de novos agentes anticancerígenos. Por exemplo, um estudo avaliou a atividade citotóxica da síntese de uma nova subclasse de 8,4’-oxyneoligan em diversas linhagens cancerígenas, como leucemias e carcinomas (SOUZA et al., 2017). Observou-se que as sínteses foram capazes de inibir a proliferação da maioria das linhagens cancerígenas, tendo o composto 1-oxo-1-fenilpropan atividade significativa contra 12 linhagens de leucemias.

Dessa forma, o uso de compostos sintéticos no tratamento do câncer, assim como produtos naturais, têm sido uma parte importante na medicina oncológica. Os compostos sintéticos ou semissintéticos oferecem uma ampla gama de opções para o tratamento de diversos tipos de câncer, incluindo as terapias-alvo. Embora esses compostos possam

oferecer uma solução efetiva para o tratamento de neoplasias, é fundamental analisar seus possíveis efeitos citotóxicos aos pacientes. Para isso, a pesquisa vem evoluindo no desenvolvimento de drogas que não tragam intensos efeito colaterais ao indivíduo.

3. OBJETIVOS

3.1. Objetivo geral

- Avaliar a atividade antineoplásica e imunomoduladora *in vitro* de compostos naturais, sintéticos e derivados bioativos.

3.2. Objetivos específicos

- Analisar a atividade citotóxica *in vitro* dos compostos contra linhagens células lêucemicas e contra células não-cancerígenas;
- Observar a influência dos compostos sobre o crescimento clonal de linhagens cancerígenas;
- Avaliar o potencial imunomodulador dos compostos;
- Avaliar o efeito dos compostos sobre cada fase do ciclo celular de células cancerígenas.

4. MATERIAL E MÉTODOS

4.1. Fluxograma de atividades

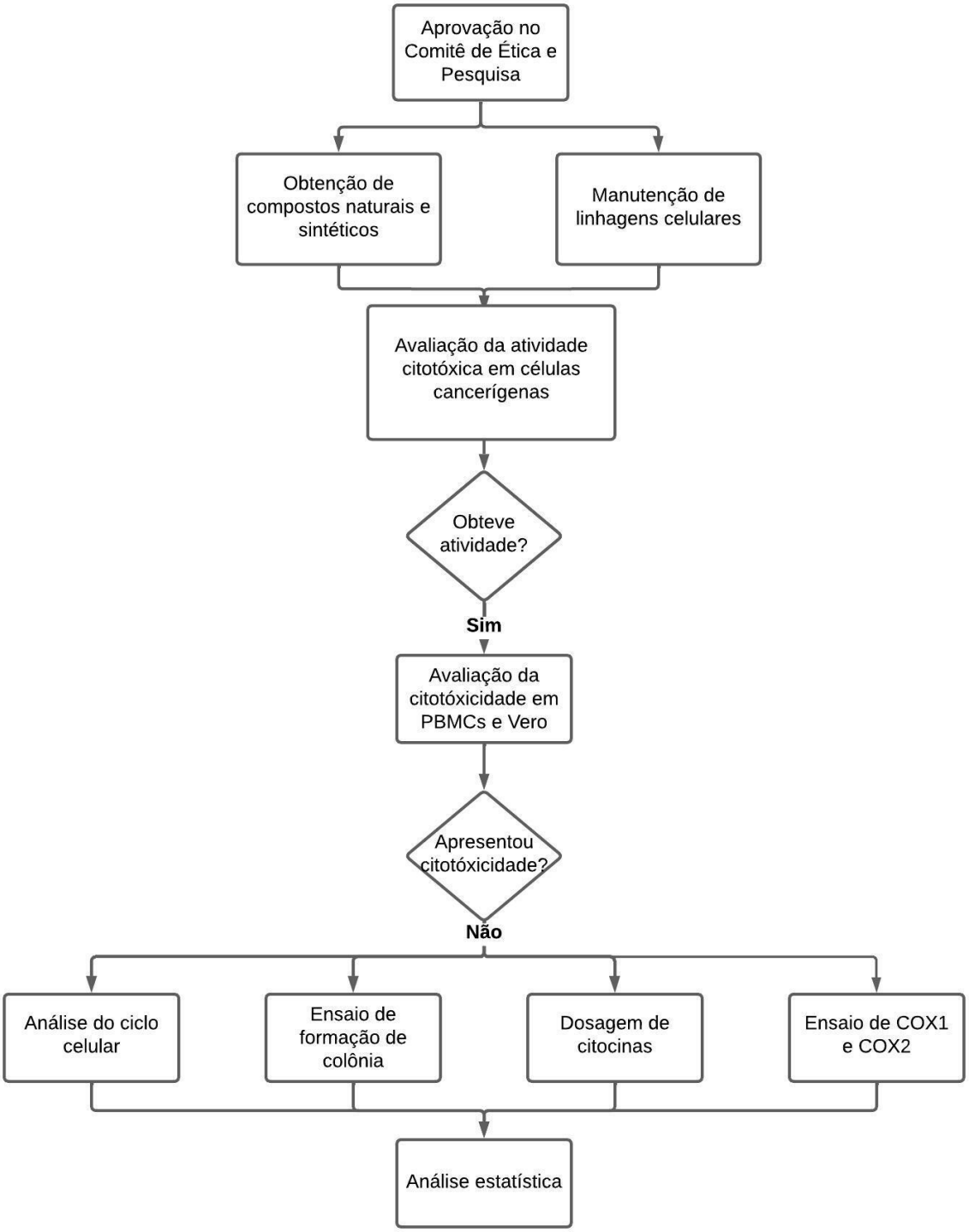


Figura 6. Fluxograma de atividades realizadas no decorrer do estudo.

4.2. Aspectos éticos

O presente estudo foi aprovado pelo Comitê de Ética e Pesquisa da Fundação HEMOAM (Número do Parecer: 3.138.343) aprovado dia 08 de fevereiro de 2019, em acordância com a resolução 466/2012 do Conselho Nacional de Saúde, a qual preconiza as diretrizes e Normas Regulamentares da Pesquisa Envolvendo Seres Humanos.

4.3. Obtenção de substâncias naturais

Os compostos utilizados neste estudo foram isoladas e purificadas pelo Instituto de Química Orgânica da Academia Nacional de Ciência da Ucrânia, Universidade de Helsinki da Finlândia e Universidade de Mohamed Ben Abdellah de Marrocos. Devido ao acordo firmado entre as instituições colaboradoras, alguns compostos estão sob sigilo devido à possibilidade de patente. São utilizados códigos para nomear estes compostos. Este estudo faz parte do projeto Horizon2020-MSCA-RISE-2016-734759, acrônimo VAHVISTUS, realizado em parceria com o Instituto Nacional de Pesquisa da Amazônia – INPA via acordo firmado com a União Europeia.

4.4. Cultura de células

Linhagens de leucemia mieloide aguda (HL60), leucemia mieloide crônica (K562) e células mononucleares do sangue periférico (PBMCs) foram cultivadas em meio RPMI (RPMI 1640/*Gibco, Rockville*, MD) suplementado com 10% de soro bovino fetal ativado (*FBS; Gibco*), 100 µg/ml de penicilina e 100 µg/ml de estreptomicina. A linhagem não-cancerígena Vero (*Cercopithecus aethiops*) foi cultivadas em meio Dulbecco's modified Eagle's médium (DMEM) suplementado com 10% de soro fetal bovino (FBS; *Gibco*) e 100 µg/mL de streptomicina. Todas as células foram mantidas a 5% de CO2 e temperatura de 37°C na incubadora de CO_2. As culturas de linhas celulares leucêmicas foram obtidas do Laboratório de Biologia Molecular – UNICAMP. A linhagem Vero foi gentilmente cedida pelo Instituto Leônidas e Maria Deane, Fiocruz-AM.

4.5. Isolamento e preparação de células mononucleares do sangue periférico humano (PBMC)

Foram coletadas amostras de sangue periférico de aproximadamente 10 pacientes portadores de leucemia e 10 doadores saudáveis rotineiramente atendidos na Fundação HEMOAM. As células mononucleares de sangue periférico (PBMC) foram isoladas por meio de centrifugação gradiente de densidade utilizando *Ficoll-Hypaque* (*GE Healthcare*). Em seguida, as células mononucleares foram coletadas e lavadas três vezes em RPMI antes de serem cultivadas sob as mesmas condições mencionadas anteriormente.

4.6. Avaliação da viabilidade celular

A proliferação celular foi medida por ensaio de Metiltiazoletrazolium (MTT), conforme descrito anteriormente (OLIVEIRA et al., 2020). As linhagens celulares permanentes ($1x10^5$ células/poço) e as primárias (PBMCs, $5x10^5$ células/poço) foram cultivadas em placas de cultura de 96 poços e tratadas com diferentes concentrações dos compostos (3-100µg/mL). As placas foram incubadas por 24, 48 e 72h a 37ºC. Após o período de incubação, foi adicionado 10µl nos poços de uma solução de MTT (5 mg/mL) e incubado a 37°C durante 4 h. A reação foi interrompida utilizando 100µL de 0,1 NHCl em isopropanol anidro. O crescimento celular foi avaliado por meio da absorbância através de espectrofotometria utilizando um filtro de comprimento de onda de 570 nm.

A viabilidade celular relativa das células foi estimada usando a seguinte equação: (expressa como absorbância óptica A_{570} da amostra tratada) / (A_{570} da amostra não tratada) × 100. Todos os testes foram realizados em triplicata.

4.7. Ensaio de formação de colônia

Para avaliar a capacidade dos compostos em inibir e/ou reduzir a formação de colônias, o crescimento clonal das linhagens HL60 e K562 foi avaliado por meio de ensaio de formação de colônias. Em resumo, cerca de 0,5 x 10^3 das linhagens celulares foram cultivadas nas concentrações de IC_{50} e $2xIC_{50}$ em placa de 12 poços com metilcelulosesemi-sólida (*MethoCult* 4230, *StemCell Technologies Inc., Vancouver*, BC, Canadá). As placas foram incubadas a 37ºC e 5% por 24horas em estufa. Após este período, as células foram lavadas com PBS (1X) e foi adicionado meio de cultura contendo SFB (10%). As células foram novamente incubadas nas condições descritas. As colônias foram avaliadas microscopicamente após 8 a 10 dias de cultura. Foi adicionado junto aos poços o reagente MTT (1mg/ml) e os resultados foram avaliados pelo *software*

de quantificação *Image J* (*US National Institute of Health, Bethesda, MD, EUA*). Todos os experimentos foram realizados em triplicata.

4.8. Análise do ciclo celular

Para determinar a ação dos compostos de acordo com a fase do ciclo celular, as linhagens celulares HL60 e K562 foram cultivadas em uma placa de cultura de 6 poços e incubadas com as concentrações de IC_{50} e $2xIC_{50}$ das substâncias por 24 horas. Após a incubação, as células foram coletadas, lavadas e fixadas com etanol 70% a 4°C por 24h. Após lavagem com PBS, as células foram incubadas com 100 µl de inibidor de ribonuclease (RNase A; 1mg/ml) e 100 µl de iodeto de propídio (PI, 400 µg/ml) a 37°C durante 30 min. As células foram caracterizadas por meio de citometria de fluxo (*FACS Calibur, BD Biosciences, San Jose,CA*) usando o módulo de discriminação de dupleto e os dados foram adquiridos usando o *software Cell Quest* (*BD Biosciences*). O ciclo celular foi modelado usando o *software FlowJoTM (Cytek® Bioscience Inc., EUA)* que permite calcular as porcentagens de células nas fases S, G1, G2 e M.

4.9. Ensaio de Ciclooxigenase-1 e -2 (COX-1 e COX-2)

Os compostos foram avaliadas quanto à atividade inibitória de COX-1 e -2 *in vitro* através do Kit Cayman's COX (Ovine/Human) Inhibitor Screening Assay (Cayman Chemical Company, Ann Arbor, MI, EUA). Em resumo, células HL60 foram cultivadas em placa de 24 poços numa densidade de $>1x10^8$ e estimuladas com LPS (10µg/mL) por 6h. Após este período, as células foram tratadas nas concentrações de IC_{50} do composto por 24h a 37ºC a 5%. Em seguida, as células foram coletadas, lisadas em tampão frio (0,1 M Tris-HCl, pH 7,8, contendo 1 mM de EDTA) e seu sobrenadante colhido para ensaio de COX. Para o ensaio, as amostras foram adicionadas em poços junto a tampão de ensaio e hemina e incubadas a temperatura ambiente por 5 minutos. A reação foi iniciada com a adição do substrato colorimétrico e solução de ácido araquidônico e deixada agir por 5 min. Por fim, a placa foi lida num comprimento de onda de 590 nm usando leitor de microplacas

4.10. Dosagem de citocinas

As PBMCs isoladas foram cultivadas a $5x10^5$ células/poço na placa de 96 poços e incubadas a 37°C e 5% de CO_2 por um período de 24h na presença das concentrações

de IC_{50} e $2xIC_{50}$ das substâncias. Após a incubação, os sobrenadantes foram coletados para dosagem de citocinas pró- e anti-inflamatórias.

Os níveis das interleucina-4 (IL4), IL6, IL10, IL12, interferon-γ (IFNγ) e fator de necrose tumoral-α (TNFα) foram avaliados por meio de kit de ensaio imunoenzimático (ELISA) comercialmente disponível (ImunoTools, Friesoythe, Germany). O ensaio foi realizado de acordo com instruções do fabricante. Foram utilizados poços em duplicata para cada amostra individual. Os resultados das concentrações de cada citocina são expressos em pg/mL.

4.11. Análise estatística

Os dados obtidos foram analisados por meio do *software GraphPad Prism* (v.8.0). Todas as variáveis numéricas estão expressas por média ± desvio padrão. Variáveis categóricas foram expressas por valor absoluto (n) e frequência relativa (%). Foi utilizado o teste Qui-quadrado ou exato de Fisher para variáveis categóricas. Teste 't' de Student, Mann-Whitney ou ANOVA foram utilizados para avaliar citotoxicidade relativa das substâncias testadas. A IC_{50} dos compostos foi calculada por meio do teste de regressão não linear.

5. RESULTADOS

5.1. Análise citotóxica dos compostos

Conforme mencionado anteriormente, para avaliar a atividade citotóxica dos compostos as linhagens celulares HL60 e K562 foram tratadas em diferentes concentrações dos compostos (3-100 µg/ml) numa cinética de 24h, 48h e 72h. Os resultados obtidos são descritos na tabela 3.

Tabela 3. Atividades citotóxicas dos compotos avaliados em linhagens HL60, K562, Vero e PBMCs humanas e suas respectivas IC_{50}

COMPOSTOS	**IC_{50} (µg/mL)**				
	Linhagens celulares				
	HL60	**K562**	**Vero**	**PBMCs**	**Instituição de procedência**
IOCh-01*,[B]	>100	>100	-	-	1
IOCh-022*,[B]	>100	>100	-	-	1
IOCh-037*,[S]	>100	>100	-	-	1
2-(4-(2,5-dimethyl-1H-pyrrol-1-yl)-1H-pyrazol-3-yl)pyridine [S]	25.93	10.42	>100	>100	1
7',8'-demethoxy-1',3'-dimethyl-1,2,3',4'-tetrahydrospiro[índole-3,5'-pyrazolo[3,4-c]isoquinolin]-2-one [S]	-	25.27	>100	>100	1
Composto 023[S]	>100	>100	-	-	2
SAP CE*,[N]	>100	>100	12.47	-	3
FLAV CE*,[N]	>100	>100	17.07	-	3
BUH*,[N]	17.90	24.08	14.19	-	3
T. A.*,[N]	6.36	11.25	5.52	-	3
Polifenol extraída de *Myrtus communis* (PEMC)[N]	19.87	29,64	>100	>100	3

*****Identidade protegida por acordo de confidencialidade firmado com a instituições colaboradoras; **(-)** atividade citotóxica não avaliada; **N:** composto natural, **B:** derivado bioativo, **S:** composto sintético; **1:** Instituto de Química Orgânica da Academia Nacional de Ciência, Ucrânia, **2:** Universidade de Helsinki, Finlândia, **3:** Universidade de Mohamed Ben Abdellah,Marrocos.

Ao todo foram avaliados 11 compostos, sendo 5 de origem natural, 2 derivados bioativos e 4 sintéticos. Quanto a procedência destes, 5 compostos são provenientes do Instituto de Química Orgânica da Academia Nacional de Ciências da Ucrânia, 1 composto da Universidade de Helsinki e 5 da Universidade de Mohamed Ben Abdellah, Marrocos.

Dos 11 compostos avaliados, apenas 3 (2-(4-(2,5-dimethyl-1H-pyrrol-1-yl)-1H-pyrazol-3-yl)pyridine, 7',8'-demethoxy-1',3'-dimethyl-1,2,3',4'-tetrahydrospiro[índole-3,5'-pyrazolo[3,4-c]isoquinolin]-2-one e PEMC apresentaram atividades anticancerígenas seletivas, pois a citotoxicidade observada foi direcionada apenas contra as linhagens cancerígenas HL60 e/ou K562, não apresentando, portanto, efeito citotóxico contra as células Vero e PBMCs humanas. Desta forma, os testes subsequentes para avaliar as atividades anticancerígenas e imunomodulodoras foram realizados apenas com esses três compostos.

Os compostos BUH e T.A. apresentaram significativo efeito antiproliferativo contra células HL60 e K562, entretanto diminuíram a viabilidade de células não-cancerígenas Vero. Já os compostos IOCh-01, -022, -037, 023, SAP CE e FLAV CE não apresentaram qualquer efeito citotóxico nas linhagens cancerígenas.

Os resultados detalhados relacionados as atividades anticancerígenas e imunomoduladoras apresentadas pelos compostos 2-(4-(2,5-dimethyl-1H-pyrrol-1-yl)-1H-pyrazol-3-yl)pyridine, 7',8'-demethoxy-1',3'-dimethyl-1,2,3',4'-tetrahydrospiro[índole-3,5'-pyrazolo[3,4-c]isoquinolin]-2-one e PEMC são descritos a seguir em forma de artigo, divididos em capítulos.

CAPÍTULO 1:

Artigo científico publicado no *Nutrients Journal:*

Antileukemic, Antioxidant, Anti-Inflammatory and Healing Activities Induced by a Polyphenol-Enriched Fraction Extracted from Leaves of *Myrtus communis* L.

nutrients

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Article

Antileukemic, Antioxidant, Anti-Inflammatory and Healing Activities Induced by a Polyphenol-Enriched Fraction Extracted from Leaves of *Myrtus communis* L.

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Abstract: Natural products have offered a number of exciting approaches in cancer treatment over the years. In this study, we investigated the prophylactic and therapeutic effects of the polyphenol-enriched fraction extracted from *Myrtus communis* (PEMC) on acute and chronic leukemia. According to the UHPLC-MS^n, the fraction is rich in flavonoids. Protective activity of the PEMC was assessed by evaluating the antioxidant, anti-inflammatory, wound healing, and hemolysis potential in a series of in vivo and in vitro assays, while the therapeutic approach consisted of the evaluation of cytotoxic activity of the PEMC against HL60 and K562 leukemia cell lines. Safety of the fraction was also evaluated on a non-cancerous Vero cell line and by an acute toxicity test performed in mice. The PEMC demonstrated a significant anti-inflammatory and healing potential. The activities found at the dose of 100 mg/kg were better than those observed using a reference drug. The PEMC demonstrated a significant antioxidant effect and a specific cytotoxicity towards HL60 (IC_{50} = 19.87 µM) and K562 (IC_{50} = 29.64 µM) cell lines being non-toxic to the Vero cell line. No hemolytic activity was observed in vitro and no toxicity effect was found in mice. Thus, the PEMC has a pharmacological potential as both preventive and therapeutic agent. However, further research is necessary to propose its mechanism of action.

Keywords: anticancer; HL60; K562; wound healing activity; carrageenan-induced edema test; hemolysis test; beta carotene bleaching test; ferric reducing antioxidant power; total antioxidant capacity; acute toxicity study

1. Introduction

Bioactive products prepared from natural sources have a number of biological and pharmacological properties, they have been extensively used as a source of medicine since the ancient times [1,2]. Natural products from plant sources have been attracting a constantly growing interest owing to many different pharmacological activities, such as antioxidant, anticancer, immunomodulatory, anti-inflammatory, antidiabetic, diuretic, osteogenic, wound healing, and so on [3,4]. Furthermore, in recent years, evaluation of

naturally produced bioactive compounds or extracts has resulted in identification of many active ingredients with high therapeutic potential [2,5–11].

Cancer is one of the leading causes of mortality worldwide. The incidence of cancer varies significantly and depends on different variables, such as age, gender, race, environment, social status, and geographic origin [12]. There is a number of medicines for cancer treatment; nevertheless, the higher-than-expected toxicity and resistance underscore the need for novel compounds that may either limit the resistance to current therapies or possess a reduced toxicity [13].

Multiple cellular activities in terms of oxidative stress and inflammation may be involved in the genesis and progression of cancer, including cell proliferation, angiogenesis, migration, metabolic reprogramming, and evasion of controlled cell death [14,15]. NF-B, AP-1, p53, HIF-1, PPAR-, catenin/Wnt, and Nrf2 are transcription factors that may be activated by an oxidative stress [16]. Over 500 genes, including growth factors, inflammatory cytokines, chemokines, cell cycle regulatory molecules, and anti-inflammatory molecules, may be expressed upon activation of those transcription factors [17]. Both the oxidative stress and inflammation are cellular outcomes of a biological defense mechanism that may feed cancer and other pathophysiological manifestations [18].

Leukemia, often known as blood cancer, is a hematological disease that starts in the bone marrow, where hematopoietic stem cells are improperly cloned [19]. Acute myeloid leukemia (AML) is a different kind of leukemia that accounts for a majority of occurrences. Fms-like tyrosine kinase 3 (FLT3) has long been recognized to be expressed in the malignant cells of most AML patients [20]. The FLT3 is a transmembrane receptor that controls hematopoietic progenitor cell development, proliferation, and differentiation through the RAS/RAF/MEK/ERK and PI3K/Akt/mTOR pathways [21]. Sunitinib and midostaurin have been authorized as targeted anticancer medicines. However, owing to frequent FLT3 mutations and an emergence of the drug resistance, targeted AML therapies have not progressed much in recent decades [22].

Philadelphia chromosome and associated chimeric oncoprotein BCR-ABL distinguish a chronic myeloid leukemia (CML). The BCR-ABL protein, which has constitutive tyrosine kinase activity, causes persistent activation of downstream signaling cascades (e.g., PI3K/Akt/mTOR) that lead to uncontrolled clonal growth of leukemia cell [23]. In recent years, tyrosine kinase inhibitors (TKI) that target the BCR-ABL have attracted growing attention. However, responses of some CML patients to the TKI treatment were unsatisfactory, since the developing drug resistance began to pose significant therapeutic difficulties [24]. Therefore, the development of less harmful and more effective medicines for leukemia patients remains a critical challenge.

Myrtus communis L. is an aromatic shrub of a Myrtaceae family, widespread around the Mediterranean basin and well-known for its medicinal properties since ancient times [25]. A plethora of the plant's pharmacological properties have been reported, those include antimicrobial, anti-inflammatory, antidiarrheal, antispasmodic, antidiabetic, vasodilator, antiulcer, antioxidant, anticancer, and anxiolytic activities [26]. Mimica-Dukić et al. [27] described that essential oil of the *M. communis* revealed an antimutagenic effect by reducing the percentage of spontaneous and *tert*-butylhydroperoxide-induced mutagenesis in *Escherichia coli*. Several constituents of the oil like myricetin 3-O-galactoside and myricetin 3-O-rhamnoside demonstrated a similar effect, they reduced the mutagenicity caused by nifuroxazide, aflatoxin-B1, and hydrogen peroxide by modulating the expression of several genes involved in the apoptosis, DNA repair, and oxidative stress [28]. A myrtucommulone isolated from myrtle leaves induced apoptotic death of cancer cells by activating caspases, which resulted in a cleavage of poly-(ADP-ribose)polymerase, a release of nucleosomes, a fragmentation DNA, and a potential loss of mitochondrial membrane [29]. The plant extracts are rich in polyphenols, especially in flavonoids, which could be behind the observed activities.

Flavonoids are the most prevalent kind of plant phenol compounds, which may be found in abundance in many edible plants and their products [30]. Over 15,000 distinct

flavonoids have been discovered across the plant kingdom, with at least several hundreds of them found in the edible components [31]. Flavonoids are plant secondary metabolites that are not required for the plant's development or survival. Instead, flavonoids help plants to defend themselves against pests and herbivores, as well as plant diseases, season and climate, environment, and other local constraints, boosting the plant's overall survival capacity [32–34]. Flavonoids have anti-inflammatory properties through different mechanisms such as inhibition of regulatory enzymes and transcription factors that have an important role in the control of mediators involved in the inflammation [35]. They can modulate gene expression via transcription factors, such as NF-κB, GATA-3, STAT-6, and hence the transcription of proinflammatory genes [35]. Flavonoids may also be used as chemotherapeutic and chemopreventive drugs [36,37]. It is a well-known fact that diets rich in fresh fruits and vegetables, which are abundant in A, C, and E vitamins, carotene, flavonoids, and other components, protect against malignancies such as lung, breast, prostate, and colon cancers [38].

Therapeutic properties of flavonoids are also associated with acute and chronic wound healing [39,40]. Flavonoids act on fibroblasts, endothelial cells, and macrophages by playing a regulatory role in a dynamic balance between pro- and anti-inflammatory responses [41]. Skin lesions are a serious public health problem worldwide. Only in the United States, 6.5 million people suffer from chronic or complex wounds, which cost approximately USD 10 billion annually [42,43]. Thus, a continuous search for therapeutic alternatives to the available treatment is necessary to improve both the health assistance and the treatment cost-effectiveness.

In the present study, we investigated the antileukemic, anti-inflammatory, antioxidant, and healing potential of the polyphenol-enriched fraction extracted from leaves of *M. communis* looking for an effective and safe new potential alternative to conventional drugs.

2. Materials and Methods

2.1. Materials and Reagents

The following commercial cell lines and reagents were used in the study: HL60 (ATCC® CCL-240TM-Human acute promyelocytic leukemia), K562 (ATCC® CCL-243TM—chronic myelogenous leukemia), and Vero cells (kidney epithetical cell line derived from African green monkey, *Chlorocebus* sp.). RPM 1640 and DMEM—Dulbecco's Modified Eagle Medium, both from Gibco (Rockville, MD, USA) were used as culture media. Heat inactivated fetal bovine serum with Penicillin, Amphotericin B, and Streptomycin (FBS, Gibco) was used to supplement the media. 3-(4,5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Merck, Darmstadt, Germany) was used in the cell viability assays.

2.2. Plant Material

The leaves of *Myrtus communis* L. were harvested in August 2018 in the province of Taounate (34.4913° N, 5.1263° W), Morocco. Their botanical identification was carried out by a specialist botanist, and a voucher specimen (BPRN60) was deposited at the Laboratory of Biotechnology, Environment, Agrifood and Health, Faculty of Sciences Dhar el Mahraz, University of Sidi Mohamed Ben Abdellah, Fez, Morocco.

2.3. Polyphenol Extraction

Polyphenols are usually obtained from plant materials by liquid phase extraction. The extraction was performed with 100 mL of aqueous methanol 70% in an ultrasonic apparatus at 35 kHz frequency at 40 °C. The powder of dried leaves of *M. communis* (10 g) was sonicated for 45 min, and the extract was filtered at 40 °C through a filter paper and then concentrated on a rotary evaporator. The product was washed twice with dichloromethane and chloroform to eliminate pigments and to obtain the fraction enriched in polyphenols, which was concentrated until dryness on a rotary evaporator. A solid

fraction (0.27 g, yield 2.7%) was stored at 4 °C. It was dissolved in a distilled water before further experiments [44].

2.4. Polyphenol Composition Analysis

2.4.1. Sample Preparation

Two different extraction procedures were employed.

For a hydrophilic extraction, a portion of the PEMC sample (80 mg) was treated with 1 mL of ethanol. The Eppendorf tube was vortexed and incubated in a sonication bath at 45 °C for 60 min. The liquid phase was filtered and lyophilized.

For a lipophilic extraction, the portion of 80 mg was treated with 1 mL of acetonitrile and 500 µL of benzene. The Eppendorf tube was vortexed and incubated in a sonication bath at 45 °C for 60–105 min. The liquid phase was filtered and lyophilized.

10 mg of dry products from both hydrophilic and lipophilic extractions were dissolved in the LCMS-grade acetonitrile to produce the sample concentration of 0.5 mg/mL used for UHPLC/MS analyses.

2.4.2. Qualitative Analysis

Qualitative analysis was performed using a Shimadzu Ultra-High-Performance Liquid Chromatograph (Nexera XR LC 40) coupled to an MS/MS detector (LCMS 8060, Shimadzu Italy, Milan, Italy). The MS/MS was operated with electrospray ionization (ESI) and controlled by Lab Solution software, which simultaneously provided quick switching from a low-energy scan at 4 V (full scan MS) to a high-energy scan (10–60 V ramping) during a single LC run. The source parameters were set as follows: nebulizing gas flow 2.9 L/min, heating gas flow 10 L/min, interface temperature 300 °C, DL temperature 250 °C, heat block temperature 400 °C, and drying gas flow 10 L/min. The analysis was performed by flow injection with the mobile phase consisting of acetonitrile: water + 0.01% formic acid (5:95, *v*/*v*). The instrument was set for a SIM experiment in negative mode (only syringic acid in positive ESI). Sample peaks were considered "positive" if the area under them was higher in magnitude than that of the blank (See Supplementary Files S1–S4).

2.5. Animals

In this study, we used both sexes of Wistar rats weighing between 150–200 g and Swiss albino mice weighing between 23–29 g. The animals were acquired from the animal facility at the Department of Biology, at the Faculty of Sciences Dhar el Mahraz, Fez, Morocco.

Before each test, the selected animals were subjected to an acclimatization period of 14 days with controlled parameters of temperature (22 °C ± 2 °C), humidity (45–50%), and 12:12 h light–dark cycle. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board at the Faculty of Sciences, Dhar el Mahraz, Fez, Morocco (02/17-LBEAS-04 and 03/01/2020).

2.6. Antileukemic Activity

2.6.1. Cell Culture

Human cancer and Vero cell lines were obtained from the National Institute of Amazonian Research, Manaus (AM), Brazil. HL60 (ATCC® CCL-240™ Human acute promyelocytic leukemia), K562 (ATCC® CCL-243™—human chronic myelogenous leukemia) and Vero cell lines (2×10^4 cells per well) were cultured into a 96-well plate containing 0.2 mL per well of the RPMI medium supplemented with 10% FBS, penicillin-streptomycin, and fungizone, in an atmosphere of 5% CO_2 at 37 °C for 24 h. After the formation of a sub-confluent monolayer, the cells were treated with different concentrations of the PEMC and incubated again at the same conditions for 24, 48, and 72 h.

2.6.2. Cytotoxicity Assay

We assessed the cytotoxicity of the PEMC by the MTT assay. The HL60, K562, and Vero cell lines (2×10^4 per well) were cultured into a 96-well plate containing 0.2 mL per well of

the RPMI medium supplemented with 10% FBS, penicillin-streptomycin, and fungizone, in an atmosphere of 5% CO_2 at 37 °C for 24 h.

Once the sub-confluent monolayer had been formed, the cells were treated with different concentrations of the PEMC (diluted in PBS with 0.5% of DMSO) and incubated again at the same conditions for 24, 48, and 72 h. Sterile PBS and DMSO 0.5% were used as a negative control and DMSO 100% as a positive one. Subsequently, the medium was removed from all wells, and 10 µL of the MTT (5 mg/mL in sterile PBS) diluted in 100 µL of the DMEM medium (without a phenol red to avoid misinterpretation) was added into the wells and incubated for 4 h at the same conditions as stated above. After that, the MTT was removed, and 50 µL of MTT lysis buffer was added to each well. The content was gently homogenized to dissolve formazan crystals and incubated for 10 min at 37 °C. All assays were performed in triplicate. Optical densities of the samples were measured on a microplate reader at a wavelength of 570 nm. The relative viability of the cells was estimated using the following equation:

$$Cell\ viability = \frac{\text{A570 of the treated sample}}{\text{A570 of the untreated sample}} \times 100$$

2.7. In Vitro Hemolysis, Hemolytic Effect of the PEMC

The hemolytic effect of the polyphenol-enriched fraction under investigation was evaluated according to a slightly modified method of Li and Liu [45]. Blood samples from rats were collected in heparinized tubes. After centrifugation at 1500 rpm for 5 min, the supernatant was removed, the pellet was washed three times with the PBS (125 mM NaCl, 10 mM sodium diphosphate, pH 7.4) and centrifugated. The last centrifugation lasted 10 min.

The cell pellet was diluted with the PBS solution to obtain a hematocrit of 2%. The PEMC was diluted in PBS to obtain different concentrations of 20, 10, 5, and 1 mg/mL. In each tube, 1 mL of the PEMC at different concentrations, 2.5 mL of the PBS, and 3.5 mL of the prepared erythrocyte suspension were added. The tubes were mixed gently and left in a shaker incubator at 37 °C for 60 min.

After centrifugation at 1500 rpm for 5 min, the absorbance of each tube was read at 630 nm with a UV-Vis spectrophotometer against a blank containing PBS. A negative control tube was prepared in the same experimental steps, composed of erythrocyte suspension and the PBS buffer solution in the absence of the PEMC. The experiment was repeated three times, and the rate of hemolysis at different PEMC concentrations was calculated as a percentage of total hemolysis according to the following formula:

$$\textbf{Hemolysis rate}\ (\%) = \frac{\mathbf{Abs_{extract}} - \mathbf{Abs_{negative\ control}}}{\mathbf{Abs_{total\ hemolysis}}} \times 100\%$$

2.8. Anti-Inflammatory Activity

2.8.1. In Vivo Carrageenan-Induced Edema Test

This test was performed following the method of Winter and Porter [46]. Injection of carrageenan under the plantar fascia of the hind paw of a rat resulted in an edema in the metatarsal region. Intensity of the edema was assessed by the increase in the volume of the paw as a percentage of the initial volume. Preventive administration of an anti-inflammatory product significantly reduces the edema development.

The rats were divided into four groups (5 rats in each group) homogeneous in weight:

Group 1: animals receiving the PEMC at 50 mg/kg b.w.

Group 2: animals receiving the PEMC at 100 mg/kg b.w.

Group 3: animals receiving placebo only (distilled water).

Group 4: reference group receiving diclofenac at 10 mg/kg b.w.

1% solution of carrageenan in distilled water was used in the experiment. The circumference of the left hind leg was measured for each rat before the carrageenan injection. The

doses of the PEMC treatment, diclofenac, and placebo were administered by oral gavage. One hour after the gavage, each rat received an injection of 50 µL of 1% carrageenan solution under the plantar pad of the left hind paw to induce edema. The edema evolution was monitored by measurement of its volume 1, 3, 4, 5, and 6 h after the carrageenan injection. The edema inhibition percentage was calculated according to the formula:

$$\textbf{nhibition}\ (\%) = \frac{\textbf{Average volume}_{\textbf{control group}} - \textbf{Average volume}_{\textbf{test group}}}{\textbf{Average volume}_{\textbf{control group}}} \times 100\%$$

2.8.2. In Vivo Wound Healing Activity

A. Formulation of Ointments

The ointments containing the polyphenol-enriched fraction extracted from leaves of the *M. communis* L. were prepared by mixing together accurately weighed amounts of the PEMC and a petrolatum (vaseline) used as an excipient. The latter was added in several steps by portions. The mixture was thoroughly homogenized after the addition of each portion of the excipient [47].

Two ointments were prepared containing 0.05% and 0.1% of the PEMC, respectively. For the PEMC-0.05% ointment, 25 mg of the PEMC were mixed with 49.975 g of vaseline. For the PEMC-0.1% ointment, 50 mg of the PEMC were mixed with 49.95 g of vaseline.

B. Wound Healing Assessment

The animals were divided into 4 groups of 5 rats each, and all treatments were applied topically. A control group received a placebo (Vaseline only) treatment, the other groups were treated with madecassol (1%), PEMC-0.05%, and PEMC-0.1% ointments, respectively.

On the first day (D 0) of the experiment, the animals were anesthetized and the down on the animal's back was shaved with a sterilized clipper. Subsequently, two circular wounds of 10 mm diameter were created on each side of the spine. During the entire experiment (Day 0 to Day 18), the wounds were cleaned and measured daily.

To evaluate the wound healing potential of the PEMC, the evolution of the wound surfaces was observed every day. The wound size reduction was calculated as follows:

$$\textbf{Reduction}\ (\%) = \frac{\textbf{Average wound area}_{\textbf{initial}} - \textbf{Average wound area}_{\textbf{test}}}{\textbf{Average wound area}_{\textbf{control}}} \times 100\%$$

2.9. Antioxidant Activity

2.9.1. β-Carotene Bleaching Test

The antioxidant activity of the PEMC was evaluated by a coupled oxidation of β-carotene and linoleic acid in an aqueous emulsion as described in [48]. The antioxidant capacity was determined by measuring the inhibition of β-carotene oxidative degradation (decolorization) by linoleic acid oxidation products [49].

Kinetics of the emulsion decolorization was monitored at 490 nm at regular time intervals for 48 h both in the presence and in the absence of the antioxidant. Butylated hydroxytoluene (BHT) was used as a reference sample. The test tubes were prepared in parallel with the negative control where the sample was replaced by 350 µL of methanol.

The relative antioxidant activity of the extracts (RAA) was calculated according to the following equation:

$$\textbf{RAA}\ (\%) = \frac{\textbf{Absorbance}\ 48\ \textbf{h}_{\textbf{sample}}}{\textbf{Absorbance}\ 48\ \textbf{h}_{\textbf{BHT}}} \times 100\%$$

2.9.2. Assessment of the Ferric Reducing Antioxidant Power (FRAP)

The reducing power of the extracts was determined according to the method of Hong et al. [50]. To a test tube containing 0.1 mL of sample, 2 mL of phosphate buffer (0.2 M, pH 6.6) solution was added followed by 2 mL of potassium hexacyanoferrate

$[K_3Fe(CN)_6]$ (10 g/L). The preparation was heated in a water bath at 50 °C for 20 min. After that, 2 mL of trichloroacetic acid (100 g/L) was added and the mixture was centrifuged at 3000 rpm for 10 min. Finally, 2 mL of the supernatant was mixed with 2 mL of distilled water and 0.4 mL of ferric chloride $FeCl_3$ (1 g/L). A blank without a sample was prepared under the same conditions. The readings were measured at 700 nm. Ascorbic acid was used as a positive control

2.9.3. Total Antioxidant Capacity (TAC)

A volume of 0.3 mL of the PEMC was mixed with 3 mL of a reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tube was screwed down and incubated at 95 °C for 90 min. After cooling, the absorbance of the solution was measured at 695 nm against the blank containing 3 mL of the reagent solution and 0.3 mL of methanol and incubated under the same conditions. The total antioxidant capacity was expressed in milligram equivalents of ascorbic acid per gram of dry matter (mg-eqv AA/g) [51].

2.10. Acute Toxicity Study

The acute toxicity of the polyphenol-enriched fraction extracted from leaves of *Myrtus communis* L. was evaluated according to the OECD Guideline 423 in groups of 5 Swiss Albino mice. The PEMC was administered by gavage in a single oral dose of 50, 100, and 2000 mg/kg body weight. The animals were observed for 14 days after the treatment, they had free access to food and water during the experiment.

After 14 days, the animals were weighed and blood samples were collected for biochemical analysis. Subsequently, the animals were euthanized, and their liver, spleen, and kidneys were collected.

2.11. Statistical Analysis

Statistical analyses were performed using a Graph Pad Prism software version 8.0. Data were expressed as means ± standard deviation. The results were analyzed using analysis of variance and Tukey as a post hoc test. The differences of cell viability observed for treatments with different PEMC concentrations were evaluated using *t*-test and ANOVA. The IC_{50} was estimated using non-linear regression. The results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Polyphenol Analysis

The UHPLC/MS-MS analysis revealed the presence of seventeen components in the prepared polyphenol-enriched fraction, namely: gallic acid, quercetin, *p*-coumaric acid, hesperidin, amentoflavone, luteolin, quercetin-3-O-glucoside, quercetin-3-O-glucuronic acid, isorhamnetin-7-O-pentose, luteolin 7-O-glucoside, kaempferol-3-O-glucuronic acid, kaempferol-3-O-pentose, kaempferol-3-O-hexose deoxyhexose, catechin gallate, procyanidin, kaempferol, and naringin (Table 1). Relative abundance of the constituents in the fraction was estimated on the peak area basis by comparison of those of the sample with the blank (see Supplementary Files S1–S4). Quercetin-3-O-glucoside, isorhamnetin-7-O-pentose, and luteolin 7-O-glucoside were the most abundant molecules in the PEMC. It was also noted that most of the constituents (viz., 15 of 17) belong to flavonoids, therefore, the fraction may be considered as a flavonoid-rich one.

Table 1. Constituents identified in the polyphenol-enriched fraction extracted from *Myrtus communis* and their relative abundances.

Constituent	$[M - H]^-$	Relative Abundance
gallic acid	168.9	++
quercetin	301	+

Table 1. *Cont.*

Constituent	$[M - H]^-$	Relative Abundance
p-coumaric acid	162.9	+
hesperidin	301.3	+
amentoflavone	537.1	++
luteolin	284.9	+
quercetin-3-O-glucoside	463.1	+++
quercetin-3-O-glucuronic acid	477	+
isorhamnetin-7-O-Pentose	447.1	+++
luteolin 7-O-glucoside	447	+++
kaempferol-3-O-glucuronic acid	461.1	+
kaempferol-3-O-pentose	417.1	+
kaempferol-3-O-hexose deoxyhexose	593.1	+
catechin gallate	441	+
procyanidin	577	+
kaempferol	285	+
naringin	579	+

Relative peak intensities: + medium, ++ strong, +++ very strong.

3.2. Antileukemic Activity

The cytotoxic activity of the PEMC was evaluated using three cell lines: human acute promyelocytic leukemia (HL60 cell line), human chronic myelogenous leukemia (K562 cell line), and normal Vero cells. The cytotoxicity indices were estimated as cell viability percentage measured by the MTT assay in a dose-dependent manner after 24, 48, and 72 h of treatment with increasing doses (0 to 100 µg/mL) of the PEMC.

The polyphenol-enriched fraction was able to inhibit the proliferation of HL60 (IC_{50} = 19.87 µM) and K562 (IC_{50} = 29.64 µM) cancerous cell lines. The 50% reduction in the cell viability was found after 48 h at the dose of 100 µg/mL for both cancerous cells (Figure 1). For the HL60, the maximum cytotoxicity of 80% was reached at 50 µg/mL concentration after 72 h, while for the K562 cells the maximum cytotoxicity of 60% was noted for 100 µg/mL concentration after 48 h treatment. No cytotoxicity effect was observed for Vero cells at all concentrations tested (IC_{50} > 100 µM) (Figure 2).

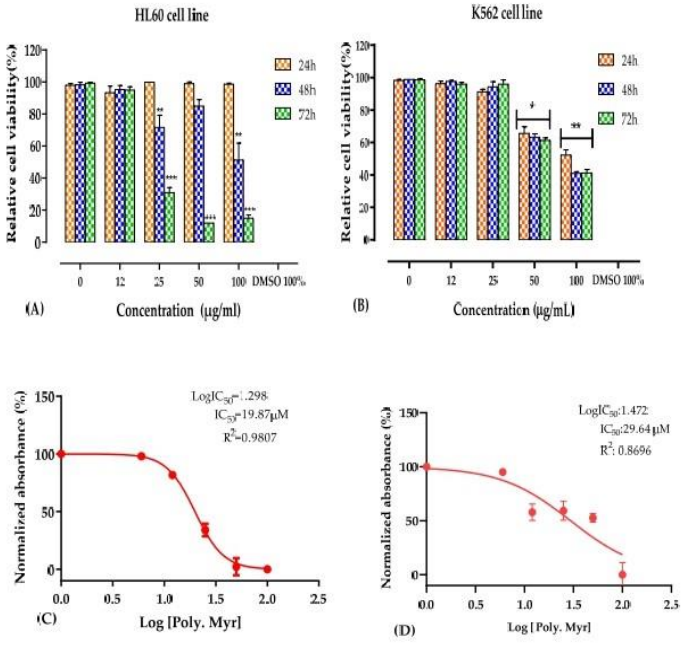

Figure 1. Cytotoxicity of the polyphenol-enriched fraction extracted from *Myrtus communis* for K562 and HL60 cells. (**A**) K562 and (**B**) HL60 cell viability after 24–72 h of treatment with different

concentrations of the PEMC (12–100 µg/mL). The IC_{50} values for K562 (**C**) and HL-60 (**D**) were estimated using nonlinear regression. The absorbance values were measured at the wavelength of 570 nm, and the mean values ± SD of three experiments are displayed along with a representative IC_{50} curve. The cell viability was estimated by the MTT assay. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

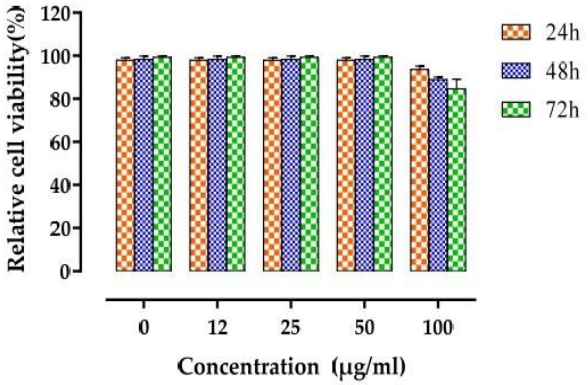

Figure 2. Cytotoxicity of the polyphenol-enriched fraction extracted from *Myrtus communis* for the normal Vero cell line. Relative viability of Vero cells after 24–72 h of treatment with different concentrations of the PEMC (12–100 µg/mL). The absorbance values were measured at the wavelength of 570 nm, and the mean values ± SD of three experiments, the cell viability was estimated by the MTT assay.

3.3. In Vitro Hemolysis

To study the hemolytic effect of the polyphenol-enriched fraction extracted from *Myrtus communis* L., the effect was tested on erythrocytes isolated from blood samples collected from rats. The results shown in Figure 3 illustrate the evolution of the hemolysis rate of the red blood cells as a function of the PEMC concentration. The hemolysis ratio less than 5% indicates that the fraction was non-hemolytic, and therefore may be safe for use.

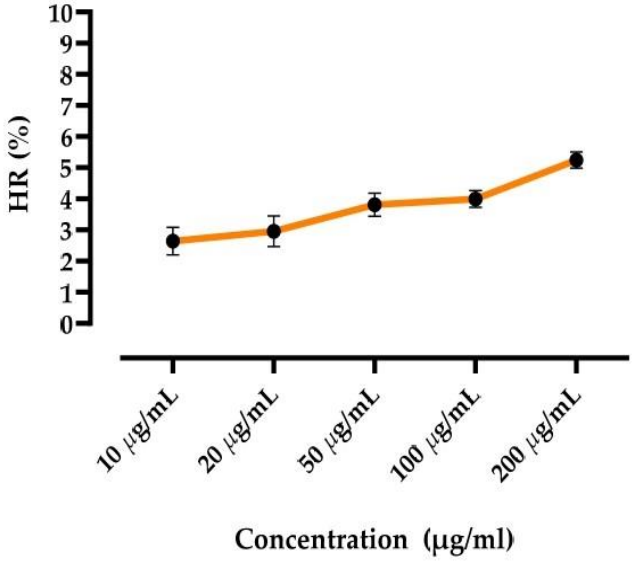

Figure 3. Evolution of the hemolysis level. The results are expressed as mean ± standard deviation. The assays were performed in triplicate. HR = Hemolysis ratio.

3.4. Anti-Inflammatory Activity

3.4.1. In Vivo Carrageenan-Induced Edema Test

Figure 4 shows the PEMC effect on carrageenan-induced inflammatory edema on the left paw of the rats. Oral administration of diclofenac (10 mg/kg) prevented significantly

the increase in the left paw volume with 42.61, 57.22, 68.75, 80.94, and 94.7 inhibition percentages after 1, 3, 4, 5, and 6 h of the carrageenan injection, respectively. Oral administration of the PEMC in doses of 50 and 100 mg/kg b.w. resulted in a decrease in the edema at a rate of 23.49, 44.44, 62.5, 78.57, and 89.41% (for 50 mg/kg b.w.) and 48.08, 68.25, 81.25, 95.23 and 100% (for 100 mg/kg b.w.) after 1, 3, 4, 5, and 6 h, respectively. The data obtained confirm that PEMC at the dose of 100 mg/kg b.w. exhibits a remarkable anti-inflammatory activity exceeding that of diclofenac. The anti-inflammatory activity maximum was observed 5 h after the edema induction.

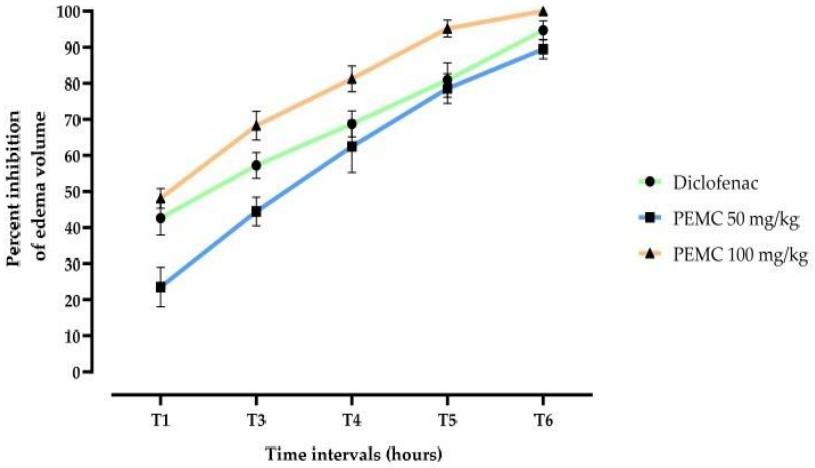

Figure 4. Inhibition percent of the edema volume after the treatment with PEMC. Results are expressed as mean ± standard deviation. The experiments were performed in quintuplicate.

3.4.2. In Vivo Wound Healing Activity

According to the results obtained, the ointments containing 0.05% and 0.1% of PEMC reduced significantly the wound surface area (Figure 5) as a function of time, when compared to the control treatment. The wound healing acceleration was observed, with the maximum (≈100%) reached on the twelfth day of the treatment. Remarkably, the positive control group receiving the madecassol (1%) ointment arrived to the maximum value (≈100%) only on the 18th day, while the placebo group reached the maximum of the wound healing process only after the 18th day.

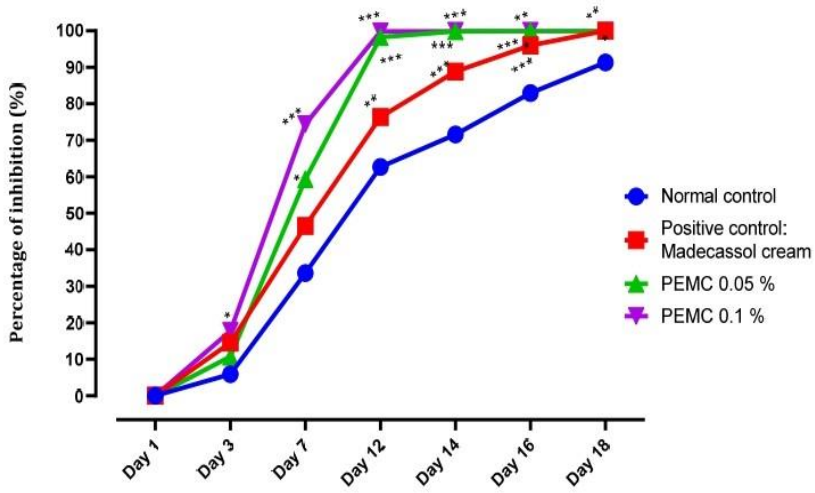

Figure 5. Wound healing assessment after treatment with PEMC containing ointments. The results are expressed as mean ± standard deviation. The tests were done in quintuplicates; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to the normal control.

3.5. Antioxidant Activity

3.5.1. β-Carotene Bleaching Test

The PEMC inhibit the coupled oxidation of linoleic acid and β-carotene compared to the negative control as shown in Figure 6. However, it was less effective than the BHT (butylated hydroxytoluene).

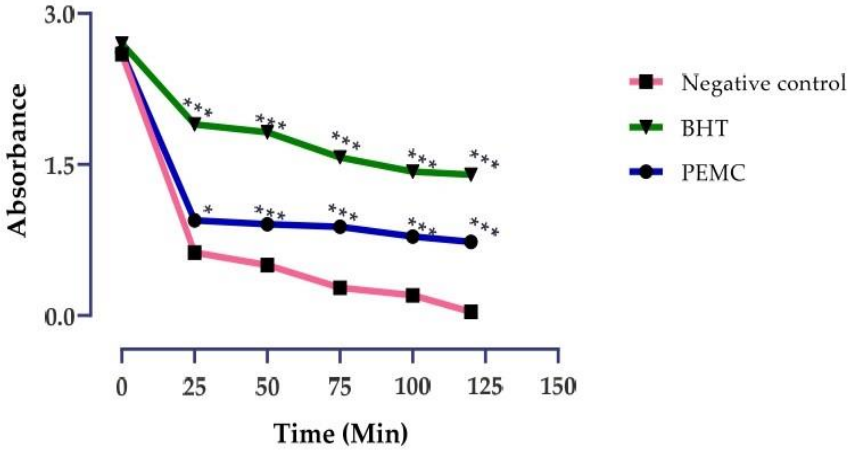

Figure 6. Antioxidant activity of the PEMC, the results are expressed as mean ± standard deviation. The tests were realized in triplicate. * $p < 0.05$; *** $p < 0.001$ compared to the normal control.

A bleaching delay observed in the test indicates that the PEMC may perform as a free radical scavenger [16] protecting the β-carotene against free radicals generated by linoleic acid peroxidation. The antioxidant effect could be explained by the presence of polyphenols in the fraction.

3.5.2. Ferric Reducing Antioxidant Power (FRAP)

The antioxidant activity of polyphenolic extracts of *Myrtus Communis* L. (PEMC) was also evaluated using the FRAP method. It is a universal, simple, rapid, and reproducible assay that can be applied either in organic or aqueous extracts [52]. The presence of reductants in plant extracts causes the reduction in Fe^{3+} in a ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be assessed by measuring and monitoring the increase in blue color density in the reaction medium at 700 nm.

The results obtained show that the reducing power of the PEMC is dose-dependent and it is much lower (EC_{50}: 3.033 ± 0.378 EAA/g) than that of quercetin (EC_{50}: 0.032 ± 0.002 EAA/g).

3.5.3. Total Antioxidant Capacity (TAC)

The total antioxidant capacity of the PEMC is 0.171 ± 0.003 mg EAA/g (mg of ascorbic acid equivalent per g of the dry fraction) indicate a good antioxidant activity.

3.6. In Vivo Acute Toxicity Study

3.6.1. Body Weight and General Aspect

A single oral administration of the PEMC at the dose of 50 or 100 mg/kg did not disturb the mice growth (Figure 7). It is interesting to note that even a single oral administration of the PEMC at the dose of 2000 mg/kg did not disturb the growth of the animals and showed no toxic effect. The results suggest that the PEMC is non-toxic, since no mortality or changes in general condition were observed in mice that received the dose of 2000 mg/kg.

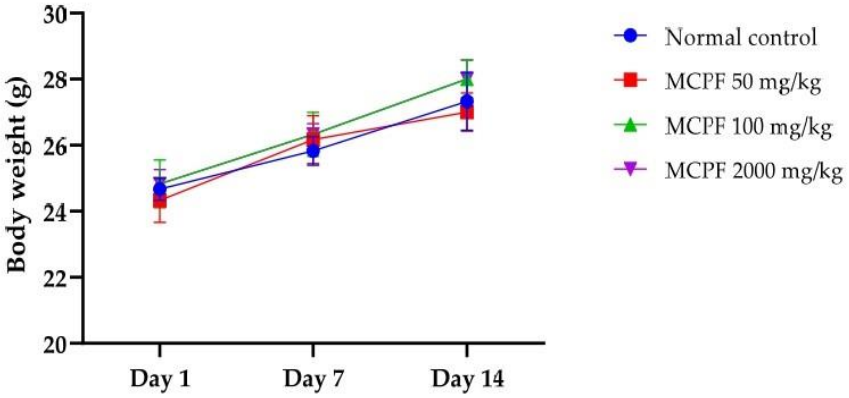

Figure 7. Bodyweight development of mice treated with a single oral administration of the PEMC.

3.6.2. Biochemical Analysis

The results of biochemical analysis of blood serum taken from the mice groups treated orally with the doses of 50, 100, and 2000 mg/kg showed no significant change in the levels of ASAT and ALAT transaminases suggesting that the PEMC is not hepatotoxic. Additionally, the urea and creatinine values relating to the health of kidneys did not altered within the study, even for the highest dose of 2000 mg/kg (Table 2).

Table 2. Biochemical analysis on hepatic and renal biomarkers in the serum of mice treated with different doses of the polyphenol-enriched fraction extracted from *Myrtus communis*.

	Normal Control	PEMC 50 mg/kg	PEMC 100 mg/kg	PEMC 2000 mg/kg
ALAT	36.80 ± 1.11	37.67 ± 1.76	41.33 ± 2.6	33.67 ± 2.48 *
ASAT	307.7 ± 20.37	284.33 ± 17.12	318.67 ± 26.76	304 ± 31.33 **
Urea	0.28 ± 0.02	0.26 ± 0.02	0.3 ± 0.01	0.28 ± 0.02 *
Creatinine	3.40 ± 0.31	3.93 ± 0.6	3.3 ± 0.24	3.6 ± 0.34 *

The results are expressed as mean ± standard deviation. The assays were performed in triplicates; * $p < 0.05$, ** $p < 0.01$ compared to normal control.

3.6.3. Effect of Acute Administration of the PEMC on the Relative Weight of Organs

The acute oral toxicity of the PEMC effect on different vital organ weights was evaluated in mice. At the end of the experiment, kidneys, livers, and spleens were collected from different mice groups and weighed. The average weights of the internal organs in question had no significant deviations when compared to the normal control (Table 3). Those data are in good agreement with the biochemical analysis described above. Moreover, no gastric toxicity was found by a macroscopic stomach observation.

Table 3. The PEMC effect on relative weight (g) of several vital organs.

	Normal Control	PEMC 50 mg/kg	PEMC 100 mg/kg	PEMC 2000 mg/kg
Liver (g)	7.15 ± 0.73	6.34 ± 0.41	6.41 ± 0.44	6.92 ± 0.51
Kidneys (g)	1.19 ± 0.24	1.12 ± 0.12	1.17 ± 0.14	1.21 ± 0.13
Spleen (g)	0.44 ± 0.63	0.46 ± 0.04	0.47 ± 0.05	0.42 ± 0.09

Results are expressed as mean ± standard deviation. Experiments were performed in triplicates.

4. Discussion

Our findings demonstrated a significant specific cytotoxicity effect of the PEMC towards the cancerous cells (HL60 and K652) without affecting the non-cancerous ones (Vero).

When studying anticancer properties of natural compounds, it is very important to consider antioxidant activity as well. Oxidative stress is responsible for causing many different diseases, including cancer, especially because of an imbalance between the formation and neutralization of prooxidant moieties. Free radicals initiate the oxidative stress that ultimately cause protein and DNA damage and may provoke a peroxidation of lipids as well [1,2]. Those physiological changes contribute to carcinogenesis. The antioxidant activity of phenolic compounds is related to their redox characteristics, which enable them to function as reducing agents, hydrogen donors, free radical scavengers, singlet oxygen quenchers, and metal chelating agents, among other functions [53]. It was shown that a total phenolic content in a variety of seeds, fruits, and vegetables directly correlates with the antioxidant activity [54]. The present study demonstrated the antioxidant role of the PEMC through three different methods (TAC, FRAP and β-carotene bleaching), confirming its action as either a free radical scavenger or reducing agent. Those findings support the pharmaceutical activity of the PEMC as a prophylactic anticancer agent.

In this study, we identified seventeen compounds in the PEMC of *Myrtuus communis*, fifteen of them were flavonoids. Quercetin is found in a variety of edible plants. It possesses antihypertensive, anticarcinogenic, anti-inflammatory, antiulcer, and antiviral properties [55]. It is one of the most studied flavonoids in epigenetic research, with numerous studies attempting to uncover its anticancer potential on various cancer cell lines, such as MCF-7 and MDA-MB-231 [56], colon cancer [57], osteosarcoma [58] and many other. Tseng et al. investigated DNA fragmentation, PARP, and procaspases activities to discover that quercetin might induce apoptosis in HL-60 human leukemia cells in a dose-dependent manner [59]. Luteolin is a flavone found in many fruits and vegetables. It demonstrated a capability to decrease the viability of lung (LNM35), colon (HT29), liver (HepG2), and breast (MCF7/6 and MDA-MB231-1833) cancer cells mainly through an inhibition of histone deacetylases (HDAC) [60]. Gallic acid is an phenolic acid widely distributed in plants. It has protective effects against human cancers by targeting DNA methyltransferases (DNMTs) [61,62]. Amentoflavone is a biflavonoid found in many plants, such as *Ginkgo biloba*, *Chamaecyparis obtusa*, *Xerophyta plicata*, etc. [63]. It was reported that amentoflavone can reduce cell viability and induce apoptosis in glioma cell lines in a dose-dependent manner [64]. Isorhamnetin-7-O-pentose, quercetin-3-O-glucoside, and luteolin 7-O-glucoside were the most abundant components of the PEMC fraction. Although only a few studies of the pharmacological potential of the isorhamnetin-7-O-pentose have been reported, the flavonoid shows a great therapeutic potential for type 2 diabetes by down-regulating the protein tyrosine phosphatase-1B (PTP1B) expression [65]. The quercetin-3-O-glucoside demonstrated anti-inflammatory and antileukemic activities by inhibiting cyclooxygenase-1/2 pathway and inducing apoptosis, respectively [66,67]. The luteolin 7-O-glucoside is a glycosyloxyflavone that acts as an antioxidant and an anti-inflammatory agent through a regulation of inflammatory mediators and oxidative stress [68–70]. Additionally, the luteolin 7-O-glucoside may induce apoptosis of nasopharyngeal carcinoma cells via the AKT signaling pathway [71].

Anticancer drugs that demonstrate anti-inflammatory activities tend to be more reliable for tumor treatment, since inflammation plays an important role in the formation of carcinogenic state [3]. We assessed the anti-inflammatory activity of the PEMC using both in vitro (hemolysis) and in vivo (carrageenan-induced edema and wound healing) assays. The carrageenan-induced paw edema is a very sensitive and reproducible test for nonsteroidal anti-inflammatory medications, which has been used for a long time to look for novel anti-inflammatory treatments [72]. The assay helps to identify active compounds through oral administration and has a high predictive value for anti-inflammatory medicines operating via acute inflammation mediators [73], since the carrageenan injection results in an immediate and localized inflammatory response. Histamine, serotonin, and bradykinin are the first mediators to be implicated in the early phase (0–1 h), while prostaglandins and other cytokines such as IL-1, IL-6, IL-10, and TNF are implicated in the second phase [72,73]. Increased vascular permeability, increased blood flow, and infil-

tration of neutrophils and macrophages are hallmarks of an acute inflammatory response. Furthermore, exudation of fluid and plasma proteins together with a buildup of leukocytes near the inflammatory site often result in edema, which was treated efficiently by the polyphenol-enriched fraction. It is interesting to note that the PEMC at the dose of 100 mg/kg demonstrated the best anti-inflammatory effect throughout the study. It was even superior than the diclofenac used as the positive control.

As the PEMC showed a pronounced anti-inflammatory activity, we checked its wound healing potential, bearing in mind that those two biological activities are intrinsically correlated. Wound healing is a dynamic process involving numerous biochemical reactions that aim to restore an injured cellular structure to its original condition. Inflammation, proliferation, and remodeling are three consecutive and overlapping stages in the traditional wound healing cascade [74]. Topical administration of the prepared ointments (0.1 and 0.05%) of the PEMC demonstrated a powerful wound healing effect in compassion with both positive (madecassol) and negative controls.

Hemolysis studies demonstrated stability of red blood cells when contacting with a foreign substance (i.e., the PEMC), and thus the fraction was considered non-hemolytic (hemolysis rate was below 5%) according to the biological safety guidelines [75].

Safety and tolerability of chemotherapy have a significant influence on patients' quality of life and may sometimes dissuade them from continuing the treatment [76]. Prescribed tyrosine kinase inhibitors, such as imatinib and others, have been linked to liver damage [77], chronic tiredness [78], nausea, rash, superficial edema, muscle cramps, and myelosuppression [79] similar to chronic myeloid leukemia therapy. The findings of both in vivo and in vitro experiments show that the PEMC has no toxicity, which enables to focus more attention on its antileukemic activity.

5. Conclusions

Our findings demonstrated that the PEMC has a selective cytotoxicity effect against leukemia cell lines, since no toxicity towards the non-cancerous Vero cell line was observed. Furthermore, the toxicological experiments in vivo and in vitro clearly demonstrated the absence of toxicity, which makes the polyphenol-enriched fraction a good candidate for prospective studies on pharmaceutical approaches. Besides the antileukemic activity, this study also revealed a high anti-inflammatory, antioxidant, and wound healing potential of the PEMC; thus, the polyphenol-enriched fraction could be a new effective and safe alternative to current treatments. However, additional research related to the profile of chemokines, growth factors, and cytokines elicited by the PEMC is necessary to better characterize the rich pharmaceutical potential of the polyphenol-enriched fraction extracted from leaves of *M. communis*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14235055/s1, Files S1–S4.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board at the Faculty of Sciences, Dhar el Mahraz, Fez, Morocco (02/17-LBEAS-04 and 03/01/2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available upon request.

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CAPÍTULO 2:

Artigo científico publicado no *Biomedicine & Pharmacotherapy Journal:*

Cytotoxic and Immunomodulatory Potential of a Novel Pyrimidine Ensemble [2-(4-(2,5-dimethyl-1H-pyrrol-1-yl)-1H-pyrazol-3-yl)pyridine] in Myeloid Leukemia

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Cytotoxic and immunomodulatory potential of a novel [2-(4-(2,5-dimethyl-1H-pyrrol-1-yl)−1H-pyrazol-3-yl)pyridine] in myeloid leukemia

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ABSTRACT

Cancer ranks among the leading causes of mortality worldwide. However, the efficacy of commercially available anticancer drugs is compromised by the emerging challenge of drug resistance. This study aimed to investigate the anticancer and immunomodulatory potential of a recently developed a novel [2-(4-(2,5-dimethyl-1 H-pyrrol-1-yl)− 1 H-pyrazol-3-yl) pyridine]. The cytotoxic potential of the compound was assessed using the MTT assay on both cancerous HL60 (acute myeloid leukemia) and K562 (chronic myeloid leukemia) cell lines, as well as non-cancerous Vero cells and human peripheral blood mononuclear cells (PBMCs). A clonogenic assay was employed to evaluate the anticancer efficacy of the compound, while flow cytometry was utilized to investigate its effect on cell cycle arrest. Furthermore, the immunomodulatory potential of the compound was assessed by quantifying inflammatory and anti-inflammatory biomarkers in the supernatant of PBMCs previously treated with the compound. Our study revealed that the novel pyridine ensemble exhibits selective cytotoxicity against HL60 (IC_{50} = 25.93 μg/mL) and K562 (IC_{50} = 10.42 μg/mL) cell lines, while displaying no significant cytotoxic effect on non-cancerous cells. In addition, the compound induced a decrease of 18% and 19% in the overall activity of COX-1 and COX-2, respectively. Concurrently, it upregulated the expression of cytokines including IL4, IL6, IL10, and IL12/23p40, while downregulating INFγ expression. These findings suggest that the compound has the potential to serve as a promising candidate for the treatment of acute and chronic myeloid leukemias due to its effective antiproliferative and immunomodulatory activities, without causing cytotoxicity in non-cancerous cells.

1. Introduction

Cancer comprises a cluster of diseases characterized by abnormal cell growth and the potential to invade and destroy normal body tissues, resulting in a high annual mortality rate worldwide [1,2]. In 2020, the number of new cases of cancer was estimated to be 18.1 million, with 9.9 million cancer-related deaths reported globally [3]. The World Health Organization (WHO) has projected that the incidence of cancer will rise by more than 50% in the upcoming decades [4].

Leukemias are a heterogeneous group of neoplasms characterized by clonal impairment of hematopoietic stem cells [5]. In 2020, GLOBOCAN estimated 474,519 new cases of leukemia, which resulted in 311,594 deaths [3]. Leukemia is broadly categorized into two types: myeloid and lymphoid. Myeloid leukemia is further subcategorized into acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). AML is typified by specific genetic mutations in the *FLT3*, *NPM1*, and *CEBPA* genes [6,7]. On the other hand, the majority (90–95%) of CML cases are attributed to the persistent abnormality of the Philadelphia chromosome

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(Ph), which arises from the reciprocal translocation between chromosomes 9 and 22, t (9:22)[8].

Currently, the main treatments for leukemia therapy are radiotherapy or chemotherapy [9–11]. Despite being indispensable curative measures, they can present certain side effects to the patient, such as toxicity to healthy tissues, hormonal or immunological alterations [12, 13]. The escalation of resistance to conventional treatments represents a prevalent challenge in the field of leukemia management [14,15]. This trend has resulted in a decrease in treatment efficacy across diverse geographical regions [16]. Consequently, it is imperative to continually advance research efforts and treatment options in order to address this urgent and pressing issue.

The compound 2-(4-(2,5-dimethyl-1 H-pyrrol-1-yl)− 1 H-pyrazol-3-yl)pyridine is a novel pyridine ensemble synthesized in three steps from 2-(4-nitro-1 H-pyrazol-5-yl)pyridine [17]. This compound is a heterocyclic ensemble consisting of pyridine, pyrazole and pyrrole ring consecutively connected. Since this synthesis is unprecedented, the biological activity of this compound remains unknown. Nevertheless, several heterocyclic compounds have been identified and shown to possess clinical applications, including anticancer, antifungal, antibacterial, antiviral, and anti-inflammatory properties [18–20]. Moreover, nitrogen heterocyclics have been demonstrated to be safe and effective and serve as important structural components in approximately 59% of unique drugs approved by the U.S. Food and Drug Administration (FDA) [21].

The purpose of this study was to examine the anticancer and immunomodulatory potential of a recently developed nitrogen heterocycle, known as 2-(4-(2,5-dimethyl-1 H-pyrrol-1-yl)− 1 H-pyrazol-3-yl) pyridine, in the context of both acute and chronic myeloid leukemia.

2. Materials and methods

2.1. Preparation of 2-(4-nitro-1H-pyrazol-5-yl)pyridine

The synthesis of the target compound **3** (as illustrated in Fig. 1) was achieved in a two-step process, utilizing 2-(4-nitro-1H-pyrazol-5-yl) pyridine **1** as described previously [17]. Notably, compound **1** can be easily obtained through a three-step procedure started from 2-acetylpyridine, and the reduction of compound **1** is documented in a patent [22]. The nitro group was reduced under mild conditions, via hydrogenation over 5% Pd/C at 5 bar pressure at room temperature. Following reduction, amine **2** was employed in the condensation reaction without additional purification, while analytical samples were prepared by recrystallizing the compound from acetonitrile. It is worth noting that the compound has low solubility in organic solvents. While a high-quality ^{1}H NMR spectrum was obtained in DMSO, the ^{13}C NMR spectrum had poor resolution, but was better resolved in CF_3COOD. MS, HRMS, and elemental analyses confirmed the assigned structure. Additional details on the synthesis can be found in the supporting information.

2.2. Experimental procedures

2.2.1. 3-(pyridin-2-yl)−1H-pyrazol-4-amine (2)

To a solution of nitropyrazole **1** (19 g, 0.1 mol) dissolved in methanol (400 mL) was added 5% Pd/C (5 g). The solution was transferred into a 1 L autoclave. The hydrogenation was carried out under 5 bar pressure at room temperature. The reaction was monitored by TLC. The catalyst was filtered off. All volatiles were evaporated till dryness. The residue was recrystallized from acetonitrile to give amine **2** as dark beige solid mp 155–156 °C. 13.6 g, 85%. ^{1}H NMR (400 MHz, DMSO-d6): δ 4.96 (bs, 2 H, NH_2), 7.1–7.2 (m, 2 H, Py), 7.7–7.9 (m, 2 H, Py, Pyr), 8.53 (s, 1 H, Py), 12.37 (bs, 0.5 H, NH), 12.67 (bs, 0.5 H, NH) ppm. ^{13}C (125.6 MHz, CF_3COOD): δ 159.8 (bs, C, Py), 146.8 (s, CH, 2-Py), 140.8 (s, CH, 4-Py), 140.6 (s, C, Pyr), 134.4 (s, C, Pyr), 127.7 (s, CH, Py), 125.9 (s, CH, Pyr), 125.2 (s, CH, Pyr) ppm. MS (GC/MS): $m/z = 104.95$ (100%), 159.95 (88.3%). HRMS (ESI) m/z [M + H]+ calcd for $C_8H_9N_4$ 161.0824, found 161.0819.

2.2.2. 2-(4-(2,5-dimethyl-1H-pyrrol-1-yl)−1H-pyrazol-3-yl)pyridine (3)

A mixture of amine **2** (10 g, 62 mmol), hexane-2,5-dione (7.2 g, 63 mmol), and p-toluenesulfonic acid (30 mg) (PTSA) in isopropanol (10 mL) were refluxed for 2 days. After cooling to rt, the precipitated solid was collected by filtration, washed with hexane and dried. It was dissolved in methylene chloride (100 mL), and washed with diluted hydrochloric acid (pH 4–5). The both layers were collected. The organic layer was dried over Na_2SO_4 and the solvent was evaporated to give the target compound as light-brown solid 7.8 g. The aqueous layer was neutralized with aqueous NaOH until pH = 8–9. The precipitated solid was filtered and dried. It is a slightly darker target compound 5.9 g. Total weight 13.7 g, 92%. The compound can be recrystallized from isopropanol, mp 139–140 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.99 (s, 6 H, Me), 6.02 (s, 2 H, Pyrrole), 6.37 (d, $J = 8$ Hz, 1 H), 7.26 (m, 1 H), 7.61 (t, $J = 8$ Hz, 1 H), 7.73 (s, 1 H), 8.77 (d, $J = 4$ Hz, 1 H) ppm. ^{13}C NMR (125.7 MHz, $CDCl_3$): δ 12.2 (s, CH, Me), 106.2 (s, CH), 118.3 (s, C), 119.3 (s, CH), 122.9 (s, CH), 128.4 (s, C), 136.8 (s, C), 137.3 (s, CH), 139.0 (s, CH), 146.1 (s, C, Py), 149.0 (s, CH) ppm. MS (GC/MS): $m/z = 238$ [M^+] 100%. HRMS (ESI) m/z [M + H]+ calcd for (**3**) 239.1296, found 239.1285.

2.3. Cell culture

The anticancer potential of the novel pyridine ensemble was assessed in acute promyelocytic (HL60 - ATCC® CCL-240™) and chronic myeloid leukemia (K562 - ATCC® CCL-243™) cell lines. Furthermore, the compound's cytotoxicity was evaluated for safety using both the Vero cell line and human peripheral blood mononuclear cells (PBMCs) obtained from human blood donors who provided informed consent to participate in the experimentation involving human subjects. Approval for the utilization of human PBMCs was granted by the Research Ethics Committee (REC) of the Amazonas State Hematology and Hemotherapy Foundation (HEMOAM), Brazil (approval number: 3.138.343; approved on February 8, 2019). The HL60, K562 cells, and PBMCs were cultured in RPMI medium supplemented with 10% activated fetal bovine serum,

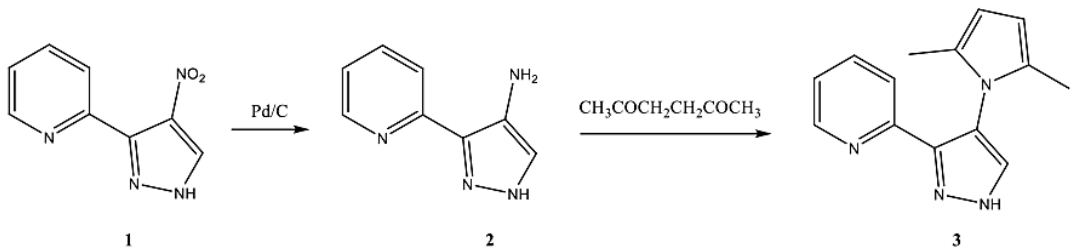

Fig. 1. Synthesis of 2-(4-(2,5-dimethyl-1H-pyrrol-1-yl)−1H-pyrazol-3-yl)pyridine **3**.

100 μg/mL penicillin, and 100 μg/mL streptomycin. On the other hand, the Vero cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 100 μg/mL streptomycin. All cells were maintained at a temperature of 37 °C and 5% CO_2 throughout the tests.

2.4. MTT cytotoxicity assay

The cytotoxicity of the new pyridine ensemble was assessed using the 3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [23]. Briefly, a 96-well plate was seeded with HL60 and K562 cancerous cell lines, or non-cancerous Vero cell (1×10^5 cells/well) or PBMCs (5×10^5 cells/well) and treated with different concentrations of the compound (3–100 μg/mL). The cells were then incubated at 37 °C in a CO_2 incubator for 24, 48, and 72 h. The assay included both untreated cells and cells treated with 100% DMSO as negative and positive controls, respectively. After each incubation period, 10 μl of an MTT solution (5 mg/mL) was added to each well and the cells were incubated for an additional 4 h under the same conditions described above. The reaction was stopped using 100 μl of 0.1 N HCl in anhydrous isopropanol. Cell viability was assessed by measuring the absorbance using spectrophotometry with a 570 nm wavelength filter. Cell viability was estimated by calculating the relative cell viability of the treated cells, which was determined using the following equation: (optical absorbance at 570 nm of the treated sample) / (optical absorbance at 570 nm of the untreated sample) × 100.

2.5. Forming-colony assay

The effectiveness of the compound in inhibiting or reducing the clonogenic potential of the cells was assessed through a forming-colony assay, performed as described previously with a few modifications [24]. Briefly, approximately 0.5×10^3 HL60 or K562 cells lines were seeded in a 12-well plate and treated with IC_{50} concentrations of the compound and methylcellulose semisolid (MethoCult 4230, StemCell Technologies Inc., Vancouver, BC, Canada). The plate was then incubated at 37°C in an atmosphere of 5% CO_2. After 8–10 days of culture, the colonies were detected by adding MTT reagent (1 mg/mL) and evaluated using ImageJ quantification software (US National Institute of Health, Bethesda, MD, USA).

2.6. Cell cycle assay

To investigate the effect of compound **3** on cell cycle progression, HL60 and K562 cell lines were cultured in a 6-well plate and treated with the IC_{50} concentrations of the compound for 24 h at 37° C in an atmosphere of 5% CO_2. After the incubation period, cells were collected, washed, and fixed in 70% ethanol at 4 °C for 24 h. Subsequently, the fixed cells were washed with PBS (1x) and incubated with 100 μl of ribonuclease inhibitor (RNase A; 1 mg/mL) and 100 μl of propidium iodide (PI, 400 μg/mL) at 37 °C for 30 min. The cells were then subjected to flow cytometry analysis (FACS Calibur, BD Biosciences, San Jose, CA) with doublet discrimination module, and data were acquired using Cell Quest software (BD Biosciences). To determine the percentage of cells in G_1, S, G_2, and M phases, FlowJo™ software (Cytek® Bioscience Inc., EUA) was utilized to model the cell cycle distribution.

2.7. Cyclooxygenase-1 and − 2 assay (COX-1 and COX-2)

The inhibitory activity of compound **3** on COX-1 and − 2 was assessed using a commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA). To accomplish this, HL60 cells were cultured in a 24-well plate at a density of 1×10^6 cells and stimulated with LPS (10 μg/mL) for 6 h at 37 °C in an atmosphere of 5% CO_2. Following incubation, the cells were treated with the IC_{50} concentration of the compound for 24 h under the same conditions described earlier. After treatment, the cells were collected, lysed in a cold buffer (0.1 M Tris-HCl, pH 7.8, containing 1 mM EDTA), and the supernatant was collected for COX assay. For the assay, the samples were added into wells along with assay buffer and hemin and then incubated at room temperature for 5 min. The reaction was initiated with the addition of colorimetric substrate and arachidonic acid solution and incubated for 5 min. Finally, the plate was read at a wavelength of 590 nm using a microplate reader to measure the level of COX inhibition.

2.8. Dosage of cytokines

To evaluate the immunomodulatory activity of the compound **3**, peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors and seeded at a density of 5×10^5 cells/well in a 96-well plate. The cells were treated with the IC_{50} concentration of the compound and maintained for 18 h at 37 °C in a 5% CO_2 atmosphere. After the incubation period, the supernatants were collected and the levels of interleukin-2 (IL2), IL4, IL6, IL8, IL10, IL12/23p40, interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) were evaluated using a commercially enzyme-linked immunosorbent assay (ELISA) kit (ImunoTools, Friesoythe, Germany) in accordance with the manufacturer's instructions. The concentrations of each cytokine were measured in pg/mL.

2.9. Statistical analysis

Numerical variables were reported as mean ± standard deviation, while categorical variables were presented as absolute values (n) and relative frequencies (%). To evaluate the statistical significance of the results, Student's t-test, Mann-Whitney or ANOVA were employed as appropriate. Non-linear regression test was performed to estimate the IC_{50} of the compound. A p-value of less than 0.05 was considered statistically significant. All experiments were conducted in triplicate to ensure the reliability of the findings.

3. Results

3.1. Cytotoxic activities

Our findings demonstrated that compound **3** has significant cytotoxic activity against both HL60 and K562 cell lines in a concentration-dependent manner (Fig. 2). In the HL60 cell line, the compound exhibited significant cytotoxic activity at concentrations of 25 μg/mL ($p < 0.001$) and 50 μg/mL ($p < 0.001$) after 48 h and 72 h of treatment, as well as at a concentration of 100 μg/mL in all treatment periods ($p < 0.05$), as compared to control (Fig. 2A). Similarly, in the K562 cell line, the compound reduced cell viability significantly at concentrations of 12.5 μg/mL ($p < 0.001$), 25 μg/mL ($p < 0.001$), 50 μg/mL ($p < 0.001$), and 100 μg/mL ($p < 0.001$) in all three treatment periods compared to control (Fig. 2B). The IC_{50} values of compound **3** for HL60 and K562 cells were 25.93 μg/mL and 10.42 μg/mL, respectively (Fig. 2. C and 2. D). Moreover, the results showed no significant cytotoxic activity of compound **3** against Vero cells (Fig. 2. E) and human PBMCs (Fig. 2. F), suggesting that the compound exhibits selectivity towards cancerous cells without affecting normal cells.

3.2. Colony formation analysis

The results indicate that treatment with compound **3** significantly decreased the colony formation of HL60 and K562 cells by 76% and 70%, respectively, compared to untreated cells ($p < 0.001$) (Fig. 3A). Moreover, the size of the colonies was significantly reduced upon treatment with compound **3**, as evident by the decrease in colony size compared to controls (Fig. 3B). These findings suggest that compound **3** is an effective inhibitor of clonal proliferation of leukemic cells, and therefore, holds potential as a therapeutic agent for leukemia.

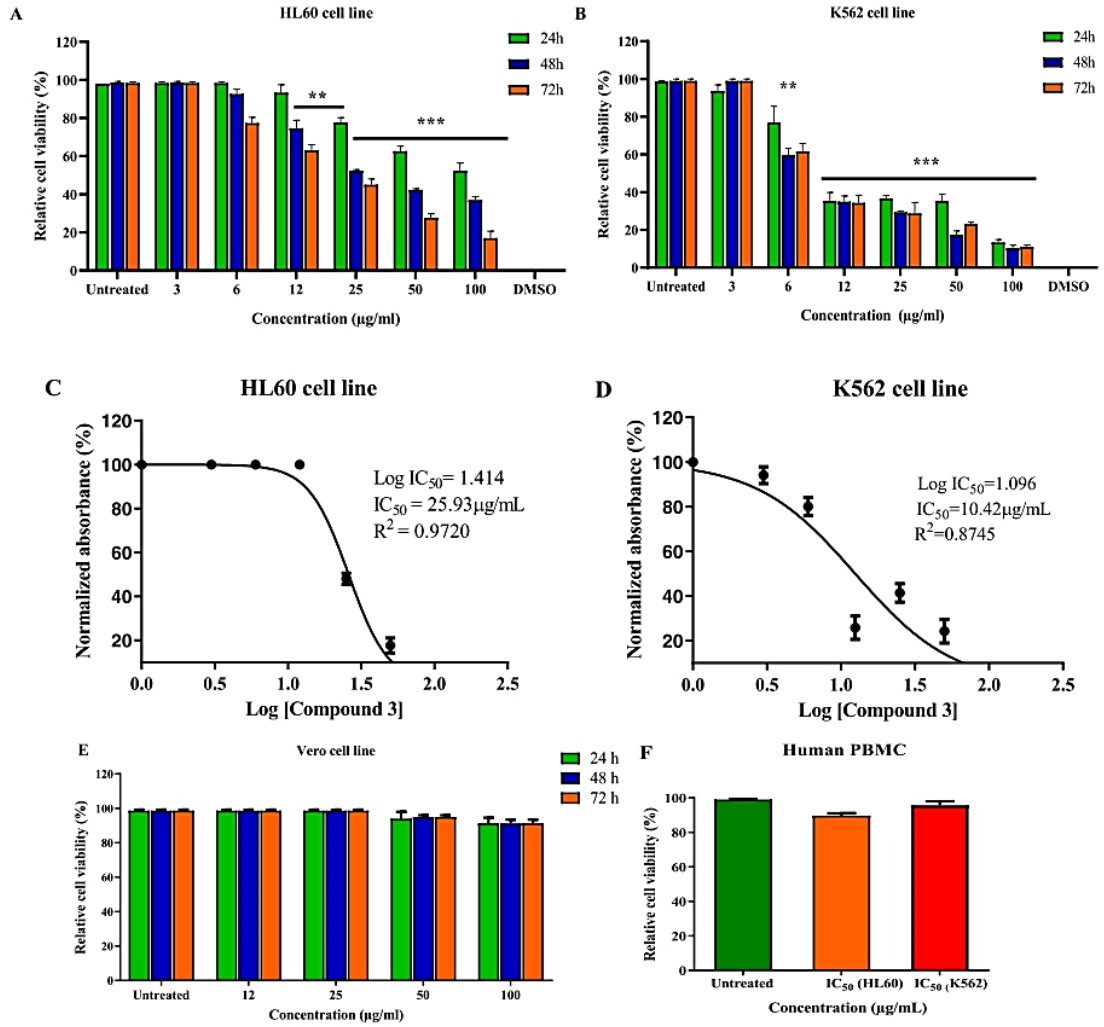

Fig. 2. Cytotoxic effect of compound **3** on cancerous and non-cancerous cells. Viability of HL60 (A) and K562 (B) cells treated with varying concentrations (3–100 µg/mL) of compound **3** for 24, 48, and 72-hour time points. IC_{50} values for HL60 (C) and K562 (D) were estimated through non-linear regression. Cell viability of non-cancerous cells, Vero (E), and human PBMCs (F) after treatment with compound **3**. Statistical significance was represented by asterisks; $^{**}p < 0.01$ and $^{***}p < 0.001$.

3.3. Cell cycle analysis

Our findings indicate that when HL60 cells were treated with the IC_{50} (25.93 µg/mL) concentration of compound **3**, there was a significant increase of 58% ($p < 0.0001$) in the cellular content of the S phase compared to the control group (Fig. 4A and C). Also, it is possible to observe a considerable increase of 12% ($p < 0.0001$) in the distribution of the G2 phase in relation to the control. Treatment with $2xIC_{50}$ (51.86 µg/mL) yielded comparable results to those of IC_{50}, albeit to a lesser extent. The distribution of the S phase showed a significant increase ($p < 0.0001$), as did the G_2 phase ($p < 0.0001$), compared to the control and the previous distribution at IC_{50}. These findings suggest that the compound is primarily effective in arresting the cell cycle in the S phase, but also has an impact on the G_2 phase (Fig. 4A and C).

Similar results were observed in K562 cells. Treatment with the IC_{50} concentration (10.42 µg/mL) increased in 34% the cellular content during the S phase ($p < 0.001$), in comparison to the control group (Fig. 4B and D). However, treatment with a $2xIC_{50}$ (20.84 µg/mL) concentration resulted in a significant increase in the number of cells arrested in the G_2 phase (68%) followed by a decrease in the S phase, as compared to the IC_{50} treatment (Fig. 4B and D). These findings indicate that compound **3** can induce cell cycle arrest in a dose-dependent manner both in the S and the G_2 phases of K562 and HL60 cells.

3.4. Analysis of the total activity of COX-1 and COX-2

Our findings indicate that compound **3** inhibits both COX-1 and COX-2 enzymes similarly, as compared to the untreated control cells. The inhibitory activity of compound **3** at the concentration of 25.93 µg/mL (IC_{50}) inhibits 18% and 19% COX-1 and COX-2, respectively, compared to the untreated cells (Fig. 5A and B). These results suggest that compound **3** may not selectively target either COX-1 or COX-2.

3.5. Compound 3 immunomodulatory activity

The results revealed that the treatment of PBMCS with a concentration of 25.93 µg/mL of compound **3** resulted in a significant upregulation of IL4 ($p < 0.05$), IL6 ($p < 0.001$), IL10 ($p < 0.01$), and IL12/23p40 ($p < 0.01$) compared to untreated cells (Fig. 6). Conversely, the levels of IFNγ were significantly decreased by the compound ($p < 0.05$) compared to the control.

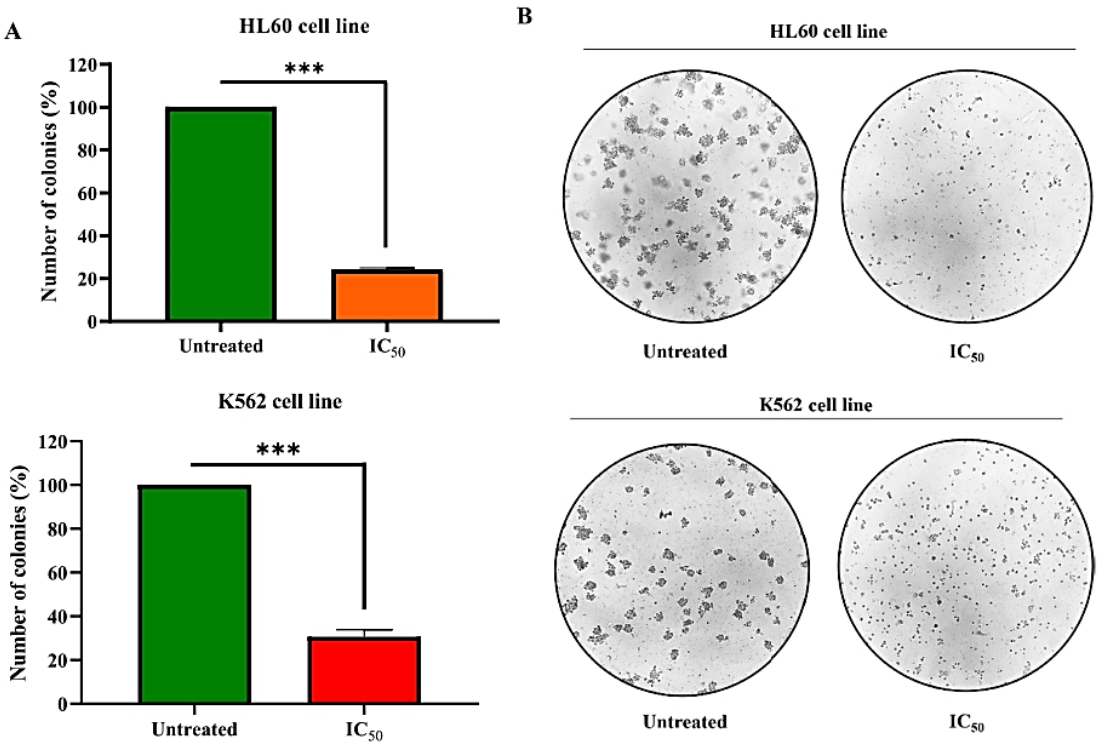

Fig. 3. Effect of compound **3** on HL60 and K562 leukemic cell colony formation. Quantification of colonies formed by HL60 and K562 cells subsequent to treatment with compound **3**, at their respective IC_{50} concentrations of 25.93 μg/mL and 10.42 μg/mL (A). Additionally, the representative optical microscopy image (10x) portrays the colony formation for HL60 and K562 cells (B). Statistical significance was represented by asterisks; ***$p < 0.001$.

4. Discussion

The present study demonstrates the potential of a newly developed pyridine compound, namely 2-(4-(2,5-dimethyl-1 H-pyrrol-1-yl)− 1 H-pyrazol-3-yl)pyridine **3**, to selectively inhibit cancerous cells without harming non-cancerous cells. This compound exhibited IC_{50} values of 10.42 μg/mL and 25 μg/mL against K562 and HL60 cell lines, respectively, indicating its promising pharmaceutical potential as an anti-cancer agent. Furthermore, the compound was able to inhibit at a great rate the clonal proliferation of both HL60 and K562 cells.

The efficacy of nitrogenous heterocyclic compounds against leukemia cell lines is well-established, and one such compound is 4-CP.P, which has shown encouraging activity against leukemia cells [25]. Furthermore, a recent investigation has reported the antiproliferative activity of 2,6-di-(4-carbamoyl-2-quinolyl)pyridine derivatives against HL60 and K562 strains, with an IC_{50} range of 3–18 μM [26].

Our investigation, using flow cytometry to analyze the cell cycle, revealed that compound **3** induces mainly S-phase cell cycle arrest in both HL60 and K562 cells, but can generate arrest in G_2, especially in K562. The objective of chemotherapeutic agents is to target dividing cells, primarily by interfering with their proliferation (cell cycle), to trigger apoptosis in cancer cells [13]. The regulation of cell proliferation is a highly coordinated process, controlled by multiple regulatory checkpoints in different stages of the cell cycle [27]. The S phase is characterized by cellular DNA synthesis, while G_2 signifies the process of cell division through mitosis [28]. The ability of compound **3** to induce cell cycle arrest in both phases indicates a disturbance in maintaining genomic integrity and cell division, ultimately leading to apoptosis in both HL60 and K562 cells. The specific arrest of the S phase by compound **3** suggests that this compound may act through direct damage to the DNA of leukemic cells or through inhibition of cyclin-dependent kinases (CDKs), such as A-CDK, which is responsible for maintaining the S phase [27,29]. Further comprehensive analyses are necessary to fully elucidate the molecular mechanism of compound **3**.

The cellular mechanisms underlying the anticancer activity of compound **3** are likely to be multifaceted, and are influenced by its chemical structure. Specifically, compound **3** is composed of three nitrogenous heterocyclic groups, including pyrrole, pyrazole and pyridine. As previously described, the compound **3** consists of three nitrogenous heterocyclic groups, namely pyrrole, pyrazole and pyridine. Pyrrole-containing compounds and their analogues have been shown to exhibit a range of biological activities, including anti-cancer and anti-inflammatory effects [30–32].

Studies have demonstrated that pyrrole-based agents can exert diverse anticancer effects, such as inhibition of tubulin polymerization, activation of pro-apoptotic proteins of the Bcl-2 family, and inhibition of histone deacetylase, among others [32–34]. Pyrazole and its derivatives are also promising due to their ability to be synthesized with various moieties, allowing for the development of a range of analogues [35]. Pyrazole has been shown to induce DNA fragmentation, which can lead to apoptosis of cancer cells, and this may explain the observed S phase arrest induced by compound **3** [36,37]. On the other hand, pyridine is the second most common nitrogenous heterocyclic group, and is present in the chemical structure of various drugs [21]. Anticancer compounds containing pyridine-based functional groups primarily induce apoptosis by causing mitochondrial dysfunction or oxidative stress through the accumulation of free radicals [25,38,39]. However, in addition to the primary anti-cancer properties, therapeutic compounds should also possess other properties, such as anti-inflammatory effects.

The COX-2 isoenzyme expression is implicated in neoplasm pathogenesis due to its ability to induce cancer stem cell activity, promote an inflammatory microenvironment, and generate apoptotic resistance [40]. It is crucial to identify a compound that can inhibit cancer cell proliferation while also reducing or not inducing inflammation during treatment. In this study, we evaluated the impact of compound **3** on the total activity of COX-1 and COX-2 and found that it mildly inhibits both

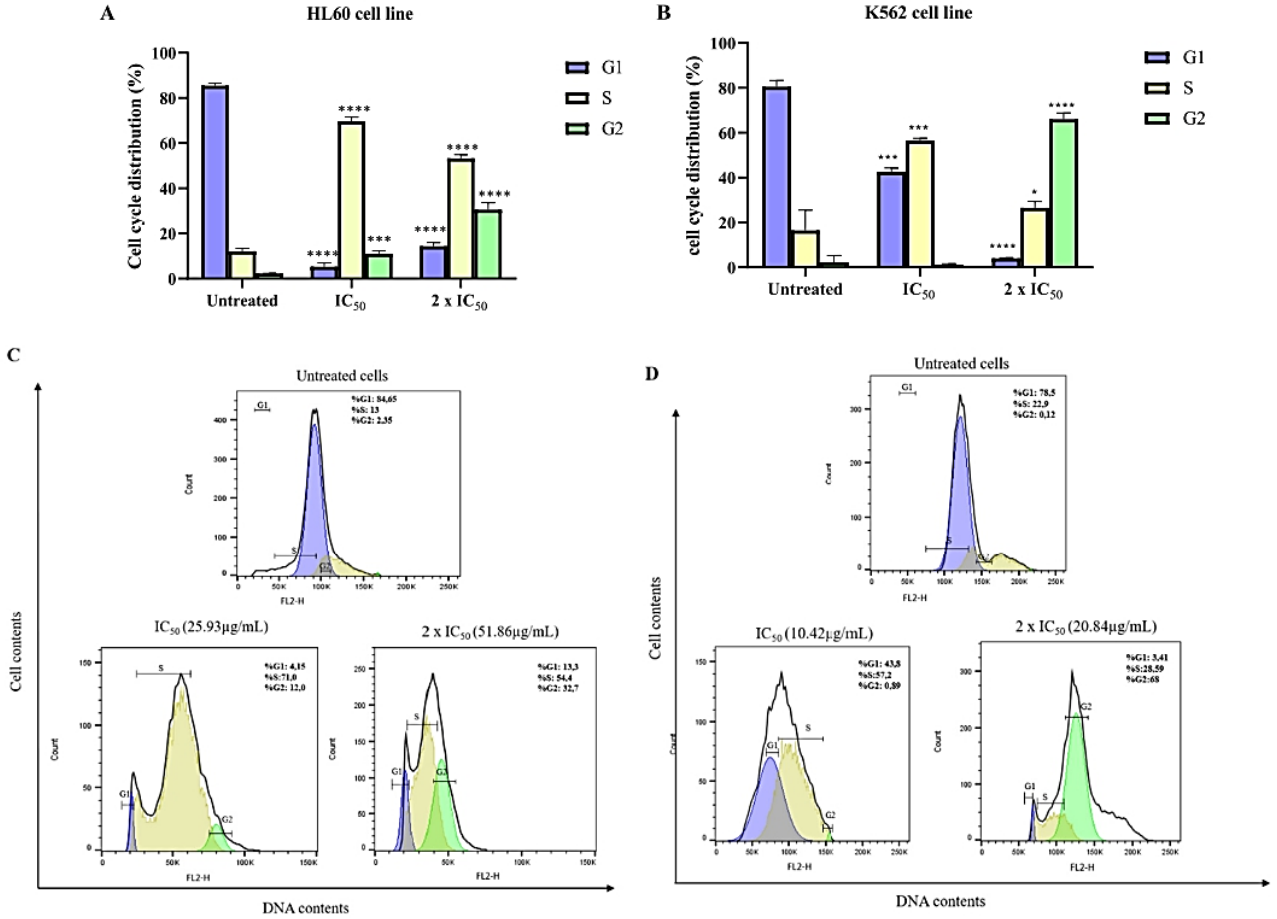

Fig. 4. Cell cycle analysis of cells treated with compound **3**. Analysis of the content distribution of the cell cycle phases in cell lines HL60 (A) and K562 (B) treated and not treated with compound **3** for 48 h. Representative cell cycle micrographs in HL60 (C) and K562 (D) cell lines, representing G_0, G_1 and G_2 cell populations with or without compound **3** treatment. Statistical significance was represented by asterisks; *$p < 0.05$ and ***$p < 0.001$.

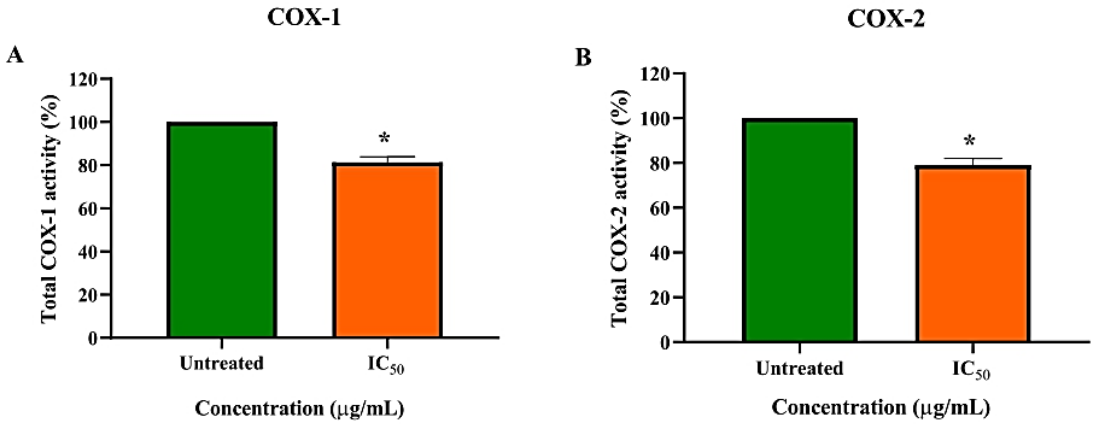

Fig. 5. Effect of compound **3** on total cyclooxygenase activity (%). The total activity of COX-1 (A) and COX-2 (B) was measured in HL60 cells by employing their respective supernatants post-lysis following treatment with IC_{50} (25.93 µg/mL) for 24 h. Statistical significance was represented by asterisks; *$p < 0.05$.

enzymes. Unlike COX-1, which is constitutive of various mammalian cells, COX-2 is not expressed in normal tissues and is only induced by inflammation or hypoxia [41]. Traditional non-steroidal anti-inflammatory drugs (NSAIDs) block both COX-1 and COX-2, reducing inflammation. However, blocking COX-1 can result in stomach pain since this enzyme is predominantly expressed in the gastrointestinal tract [41,42]. Compound **3** showed comparable inhibitory effects on both COX-1 and COX-2 enzymes, suggesting that it may have potential as a broad-spectrum inhibitor of prostaglandin synthesis. However, further studies are needed to assess its therapeutic potential and potential side effects.

The dysregulation of pro- and anti-inflammatory factors is

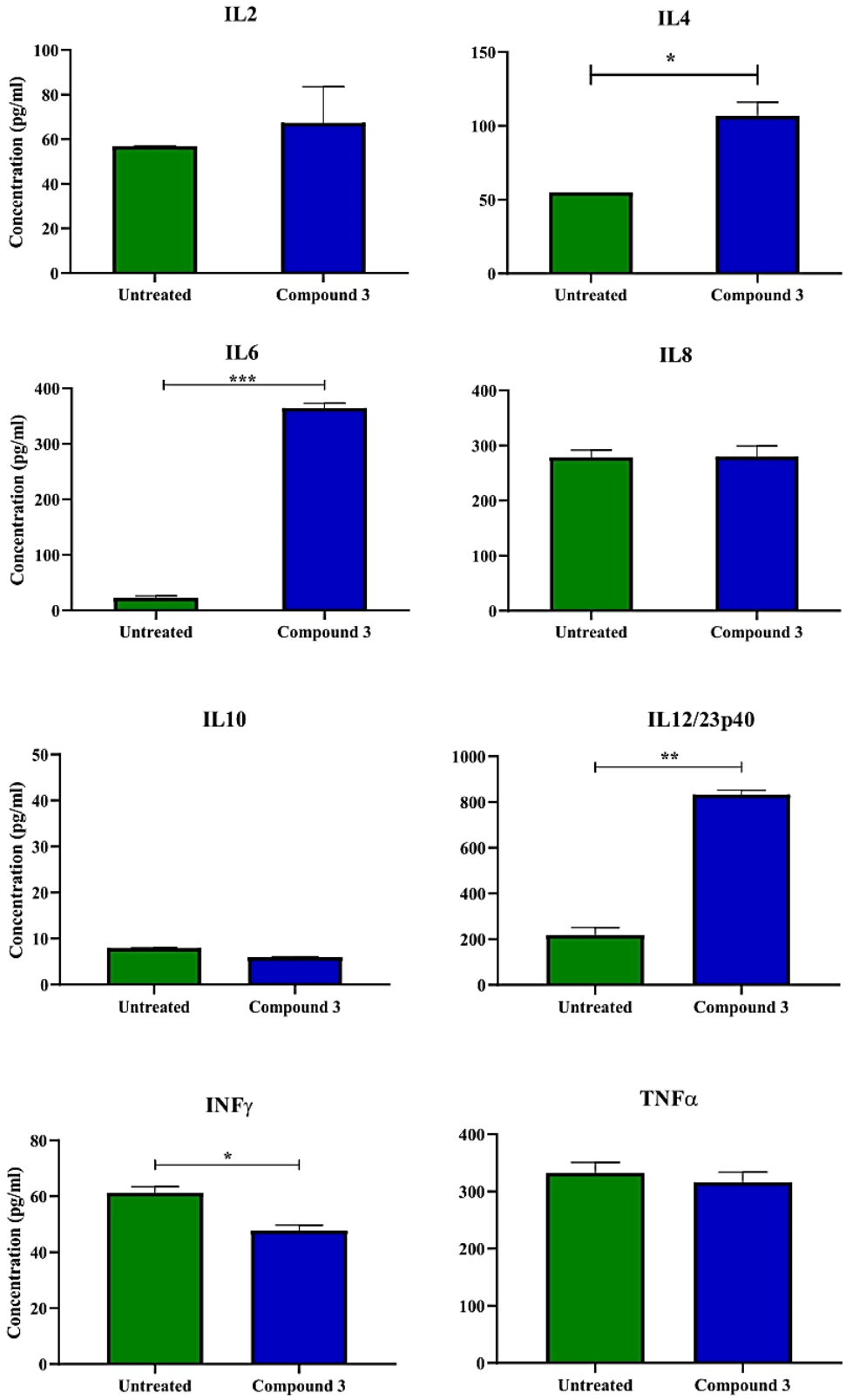

Fig. 6. Immunomodulatory effect of the compound **3**. The levels of cytokines IL2, IL4, IL6, IL8, IL10, IL12/23p40, IFNγ and TNFα were evaluated by ELISA in the supernatant of human PBMCs treated with the IC_{50} (25.93 μg/mL) concentration of the compound **3**. Statistical significance was represented by asterisks; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

implicated in the progression and promotion of myeloid malignancies [43]. In this study, we examined the immunomodulatory potential of compound **3**. Our findings indicate that this compound is capable of upregulating cytokines such as IL4, IL6, and IL12/23p40 and downregulating INFγ in human PBMCs.

Patients with pre-chemotherapy acute myeloid leukemia (AML) have decreased IL4 and increased INFγ levels compared to healthy controls, which may contribute to a poor prognosis [44]. In a murine model, IL4 stimulation increased macrophage-mediated phagocytosis of leukemic cells and suppressed the proliferation of granulocyte-macrophage colony-forming units, as observed in K562 cells in this study [45,46]. Conversely, excessive secretion of INFγ is associated with chronic myeloid leukemia (CML) progression by promoting leukemic stem cell proliferation and contributing to immune evasion through upregulation of PDL1 and PDL2 in these cells [47,48]. Therefore, our findings are promising as they indicate that compound **3** can balance both Th1-type and Th2-type immune responses by reducing INFγ levels and increasing IL4 secretion in myeloid leukemia.

Our findings also revealed a significant elevation of IL6 and IL12/23p40 levels in human PBMCs following treatment with compound **3**. Higher levels of IL6 have been reported in patients recently diagnosed with AML or CML as compared to healthy individuals, and the impact of this cytokine on cancer remains a matter of controversy [49,50]. Nevertheless, IL6 plays a crucial role as a regulator of the anti-tumor immune response by promoting the activation, trafficking, proliferation, and survival of cytotoxic T lymphocytes in the tumor microenvironment, which enables the control of malignant cells [51–53]. On the other hand, IL12 and IL23 are heterodimers that share the p40 subunit and exhibit a critical function in the fight against cancer cells by stimulating Th1 and Th17 immune responses, regulating natural killer cells, and enhancing CD8 + T lymphocyte function [54,55].

5. Conclusion

In conclusion, our findings highlight the selective cytotoxicity of a novel [2-(4-(2,5-dimethyl-1 H-pyrrol-1-yl) – 1 H-pyrazol-3-yl)pyridine] for myeloid leukemia. This compound also inhibits the total activity of cyclooxygenase 1 and 2, factors linked to the immunopathogenesis of cancer. Furthermore, the compound showed potential immunomodulatory activity on the cytokines IL4, IL6, IL12 and INFγ. The effectiveness of this compound can serve as a basis for future research in cancer therapy. However, further studies are needed to investigate the compound's cellular mechanism of action, its potential therapeutic benefits, and any possible side effects that may arise.

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CRediT authorship contribution statement

Carlos Eduardo de C. Alves: Project administration, manuscript writing, Writing – review & editing, Methodology, Investigation, Data analysis. **Alice de Freitas Gomes:** Methodology, Investigation, Formal analysis. **Regiane Costa de Oliveira:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Allyson Guimrães Costa:** Provision of study materials, Methodology. **Luiz Antônia Boechat:** provision of study materials. **André Corrêa de Oliveira:** Chemical data analysis, Formal analysis. **Georgyi Koidan:** Compound design and synthesis, provision of study materials. **Anastasiia N. Hurieva:** Compound design and synthesis, provision of study materials. **Sergii Zahoulko:** compound design and synthesis, provision of study materials. **Aleksandr Kostyuk:** compound design and synthesis, provision of study materials. **Gemilson Soares Pontes:** Project administration, Conceptualization, Funding acquisition, Investigation, Writing – review & editing, Formal analysis.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Supplementary materials

The following item is available, supplementary data: Spectra data of compound **2** and **3**.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114701.

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CAPÍTULO 3:

Artigo científico submetido ao *Investigational New Drugs Journal*:

Pharmacological assessment of the Antineoplastic and Immunomodulatory Properties of a new spiroindolone derivative (7',8'-Dimethoxy-1',3'-dimethyl-1,2,3',4'-tetrahydrospiro[indole-3,5'-pyrazolo[3,4-c]isoquinolin]-2-one) in Chronic Myeloid Leukemia

Pharmacological assessment of the Antineoplastic and Immunomodulatory Properties of a new spiroindolone derivative (7',8'-Dimethoxy-1',3'-dimethyl-1,2,3',4'-tetrahydrospiro[indole-3,5'-pyrazolo[3,4-c]isoquinolin]-2-one) in Chronic Myeloid Leukemia

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Abstract

The discovery and development of effective novel compounds is paramount in oncology for improving cancer therapy. In this study, we developed a new derivative of spiroindolone (7',8'-Dimethoxy-1',3'-dimethyl-1,2,3',4'-tetrahydrospiro[indole-3,5'- pyrazolo[3,4-c]isoquinolin]-2-one) and evaluated its anticancer- and immunomodulatory potential in a vitro model of chronic leukemia. We utilized the chronic leukemia cell line K562, as well as non-cancerous peripheral blood mononuclear cells (PBMC) and Vero cells (kidney epithelium of Cercopithecus aethiops). We assessed the cytotoxicity of the compound using the MTT assay, and performed cell cycle assays to determine its impact on different stages of the cell cycle. To evaluate its antineoplastic activity, we conducted a colony formation test to measure the effect of the compound on the clonal growth of cancer cells. Furthermore, we evaluated the immunomodulatory activity of the compound by measuring the levels of pro and anti-inflammatory cytokines. The study findings demonstrate that the spiroindolone-derived compound exerted noteworthy cytotoxic effects against K562 cells, with an IC50 value of 25.27 µg/mL. Additionally, it was observed that the compound inhibited the clonal proliferation of K562 cells while displaying minimal toxicity to normal cells. The compound exhibited its antiproliferative activity by inducing G2/M cell cycle arrest, preventing the entry of K562 cells into mitosis. Notably, the compound demonstrated an immunomodulatory effect by upregulating the production of cytokines IL-6 and IL-12/23p40. In conclusion, the spiroindolone-derived compound

evaluated in this study has demonstrated significant potential as a therapeutic agent for the treatment of chronic myeloid leukemia. Further investigations are warranted to explore its clinical applications.

Keywords: spiroindolones, derivative compound, anticancer, cytokines, myeloid leukemia

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1 Introduction

Cancer has been a major public health issue for many years and currently represents the second leading cause of mortality worldwide, following cardiovascular diseases [1]. Cancer is a disease characterized by uncontrolled cellular proliferation and the subsequent invasion of healthy tissues, resulting in significant damage to the individual's overall health [2]. In 2020, the World Health Organization (WHO) reported that there were 474,519 newly diagnosed cases of leukemia worldwide, and the disease accounted for 311,594 deaths [3]. Leukemias arise when blood cells undergo malignant transformation due to environmental or genetic factors, and it is classified as *either acute or chronic and as myelocytic or lymphocytic leukemia* [4].

Chronic myeloid leukemia (CML) is a hematological disorder that arises from the abnormal proliferation and accumulation of immature blood cells, known as blasts, in the bone marrow and blood [5]. This accumulation interferes with the normal production of healthy blood cells, leading to severe complications for the affected individual [5]. Its development has its main origin in mutations in the Philadelphia chromosome (Ph) and the resulting oncogene *BCR-ABL*$_1$ [6]. Treatment of CML consists of cell therapy or the administration of tyrosine kinase inhibitors (TKIs) [7]. Despite the efficacy of tyrosine kinase inhibitors (TKIs) in the treatment of CML, approximately 15-25% of patients develop resistance to these drugs, resulting in significant therapeutic challenges in controlling and curing the disease [8, 9]. Therefore, it is crucial to investigate new compounds that can effectively inhibit leukemic cells in combination with conventional therapies, while minimizing or reducing any potential toxicity to the patient.

Spiroindolone-based compounds have demonstrated significant potential as a basis for developing new drugs, due to their promising pharmaceutical activities. Spiroindolones is a class of compounds characterized by a chemical bond between two benzene rings that forms a spirocarbonyl (3-indolin-2-one). These compounds are of particular interest for the integration of heterocyclic systems to develop new antimalarial drugs, such as Cipargamin (KAE609) [10]. In addition, spiroindolone derivatives exhibit other biological activities, including anticancer, antimicrobial, antihyperglycemic, and antiviral action, among others [11–15]. Given their excellent therapeutic efficacy, the potential of spiroindolone derivatives compounds as anticancer agents warrant further exploration.

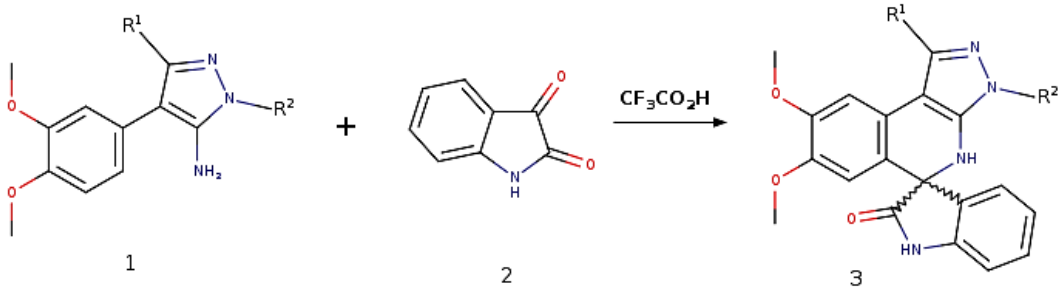
This study investigated the antileukemic and immunomodulatory pharmacological potential of a novel spiroindolone derivative, 7',8'-Dimethoxy-1',3'-dimethyl-1,2,3',4'-tetrahydrospiro[indole-3,5'-pyrazolo[3,4-c]isoquinolin]-2-one in the context of chronic myeloid leukemia.

2 Material and Methods

2.1. Chemical

2.1.1. General Experiments

Based on the modified Pictet-Spengler Reaction (PSR), we developed a convenient method of preparing pyrazolo[3,4-c]isoquinolines I (Figure 1). All reactions were performed in round bottomed flasks. All reagents and solvents were obtained from commercial suppliers and used without further purification unless otherwise specified. All reagents were weighed and handled in air at room temperature. Organic solutions were concentrated on a rotary evaporator at 23−35 °C. Melting points were determined with a capillary melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded with a 400 MHz spectrometer and are recorded in parts per million from internal tetramethylsilane on the δ scale and are referenced from the residual protons in the NMR solvent (DMSO: δ 2.50, CDCl3: δ 7.24). Data are reported as follows: chemical shift, multiplicity s = singlet, d = doublet, t = triplet, m = multiplet). Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded with a 100 MHz spectrophotometer and are recorded in parts per million from internal tetramethylsilane on the δ scale and are referenced from the carbon resonances in the NMR solvent (DMSO: δ 39.5). Data are reported as follows: chemical shift. Mass spectra were recorded on an 1100 LCMSD SL instrument (chemical ionization (APCI), electrospray ionization (ESI).



a: $R^1 = R^2 = H$; b: $R^1 = Me$, $R^2 = H$; c: $R^1 = R^2 = Me$; d: $R^1 = Me$, $R^2 = Ph$

Fig. 1 Synthesis of spiroindolones

2.1.2. General method for PSR cyclization of aminopyrazoles 1 with isatin

An equimolar amount of isatin was added to a solution of 3 mmol of suitable aminopyrazole 1 in 10 ml of trifluoroacetic acid. The reaction mixture was stirred and refluxed until the end of the reaction (1H NMR control). The solvent was evaporated to a volume of 2 ml, and an excess of 10% sodium bicarbonate water solution was added to the residue. After 12 hours, the reaction product is filtered off and crystallized from acetonitrile or ethanol.

2.1.3. Preparation of *1',3'-Dimethyl-7',8'-dimethoxy-1',3'-dimethyl-1,2,3',4'-tetrahydrospiro[indole-3,5'-pyrazolo[3,4-c] isoquinolin]-2-one (3c)*

The compound *1',3'-Dimethyl-7',8'-dimethoxy-1',3'-dimethyl-1,2,3',4'-tetrahydrospiro[indole-3,5'-pyrazolo[3,4-c] isoquinolin]-2-one (3c)* was obtained from aminopyrazole 1c and izatin. Yield 72%. Mp 211-212°C. ^{1}H NMR (400 MHz, DMSO-*d6*) δ 2,32 (3H, s, Me), 3,42 (3H, s, Me), 3,45 (3H, s, OMe), 3,79 (3H, s, OMe), 5,92 (1H, s), 6,91 (1H, s), 6,92 (1H, d, *J*=8), 6,98...7,01 (2H, m), 7,21 (1H, d, *J*=8), 7,29 (1H, t, *J*=8), 10,25 (1H, s, NH). ^{13}C NMR (126 MHz, DMSO-*d6*) δ 14.30, 33.70, 55.42, 55.95, 96.96, 104.67, 109.79, 110.33, 120.58, 122.46, 124.89, 125.51, 129.56, 134.48, 140.88, 141.49, 144.17, 145.18, 149.16, 177.73. LCMS (ESI) m/Z: found 377.0 [M+1], 378.0 [M+2] found 376.15.

2.2. Cell culture

The antiproliferative activity of the compound **3a** was evaluated in a chronic myeloid leukemia cell line (K562-ATCC® CCL-240^{TM}). Furthermore, the compound's cytotoxicity was evaluated for safety using both the Vero cell line and human peripheral blood mononuclear cells (PBMCs) obtained from human blood donors who provided informed consent to participate in the experimentation involving human subjects. Approval for the utilization of human PBMCs was granted by the Research Ethics Committee (REC) of the Foundation of Hematology and Hemotherapy Foundation of Amazonas State (HEMOAM), Brazil (approval number: 3.138.343; approved on February 8, 2019). K562 cells and PBMCs were cultured in RPMI medium (RPMI 1640/Gibco, Rockville, MD) supplemented with 10% activated fetal bovine serum (FBS; Gibco), 100 µg/ml penicillin and 100 µg/ml streptomycin. The Vero cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (FBS; Gibco) and 100 µg/ml streptomycin. All cells will be kept at 37°C in an atmosphere of 5% CO_2.

2.3. MTT cytotoxic assay

The cytotoxic activity of compound **3a** was assessed using the Methylthiazoletrazolium (MTT) assay, following the protocol previously described [16]. In summary, K562 and Vero cells were cultivated in 96-well plates ($1x10^5$ cells/well) and treated with different concentrations of compound (3-100µg/mL) and subsequently incubated for 24, 48 and 72h at 37ºC in an atmosphere of 5% CO_2. Human PBMCs were also cultured in a 96-well plate ($5x10^5$ cells/well) and treated with concentrations corresponding to IC_{50} and $2xIC_{50}$ of the compound for K562 cells. Untreated cells and those treated with 100% DMSO were used as negative and positive controls of the assay, respectively. After each incubation period, 10µl of an MTT solution (5mg/mL) was added to the wells and incubated for 4 h at the same condition described above. The reaction was stopped using 100µL of 0.1 NHCl in anhydrous isopropanol. Cell viability was assessed by measuring the absorbance at a wavelength of 570 nm using spectrophotometry. The relative viability of the cells were estimated using the following equation: (expressed as optical absorbance A_{570} of the treated sample) / (A_{570} of the untreated sample) × 100.

2.4. Colony test formation

To evaluate the ability of the compound **3a** to inhibit and/or reduce the clonal growth of K562 cells, the colony formation assay was used. Briefly, approximately $0.5x10^3$ of the cell line was cultured in a 12-well plate with the IC_{50} concentrations of the compound and with semi-solid methylcellulose (MethoCult 4230, StemCell Technologies Inc., Vancouver, BC, Canada). The plate was at 37ºC in an atmosphere of 5% CO_2. Colonies were detected after 8 to 10 days of culture with the addition of MTT reagent (1mg/ml) and the results were evaluated using ImageJ quantification software (US National Institute of Health, Bethesda, MD, USA).

2.5. Cell cycle assay

To assess the impact of compound **3a** on the cell cycle phases, K562 cells were cultured in a 6-well plate and treated with the compound concentration corresponding to its IC_{50} value for 24 hours at 37ºC in an atmosphere of 5% CO_2. After incubation, cells were collected, washed and fixed with 70% ethanol at 4°C

for 24h. After this period, they were washed with PBS (1x) and incubated with 100 µl of ribonuclease inhibitor (RNase A; 1mg/ml) and 100 µl of propidium iodide (PI, 400 µg/ml) at 37°C for 30 min. Cells were characterized by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA) using the doublet discrimination module and data will be acquired using Cell Quest software (BD Biosciences). The percentages of cells in phases S, G1, G2 and M were estimated using FlowJoTM software (Cytek® Bioscience Inc., EUA).

2.6. Cytokine dosage

To evaluate the immunomodulatory activity of the compound **3a**, human PBMCs from blood donors were isolated and plated at a density of $5x10^5$ cells per well in a 96-well plate. Then, the cells were treated with the concentration corresponding to IC_{50} value for K562 cells and incubated for 18 hours under conditions of 37°C temperature and 5% CO2. . After this period, the supernatants were collected. The levels of interleukin-2 (IL2), IL4, IL6, IL8, IL10, IL12, interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) were estimated using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (ImunoTools, Friesoythe, Germany). The assay was performed according to the manufacturer's instructions. The results of the concentrations of each cytokine were expressed in pg/mL.

2.7. Statistical analysis

Numerical variables were reported as mean ± standard deviation, while categorical variables were presented as absolute values (n) and relative frequencies (%). To evaluate the statistical significance of the results, Student's t-test, Mann-Whitney or ANOVA were employed as appropriate. Non-linear regression test was performed to estimate the IC_{50} of the compound. A p-value less than 0.05 was considered statistically significant. All experiments were conducted in triplicate to ensure the reliability of the findings.

3 Results

3.1. Cytotoxic effect of the spiroindolone-derived compound 3a

The compound **3a** had a significant cytotoxic effect on K562 cells at concentrations of 25 µg/mL ($p<0.05$), 50 µg/mL ($p<0.001$), and 100 µg/mL ($p<0.001$) for all three treatment periods (24h, 48h, and 72h) compared to untreated cells (control) (Figure 2.a). The compound exhibited an IC_{50} of 25.27µg/mL in K562 cells (Figure 2.b). On the other hand, no cytotoxic effect was observed on non-cancerous Vero cells and human PBMCs, except at the highest concentration of 100 µg/mL for Vero cells at 72h ($p<0.05$) (Figure 2.c and d).

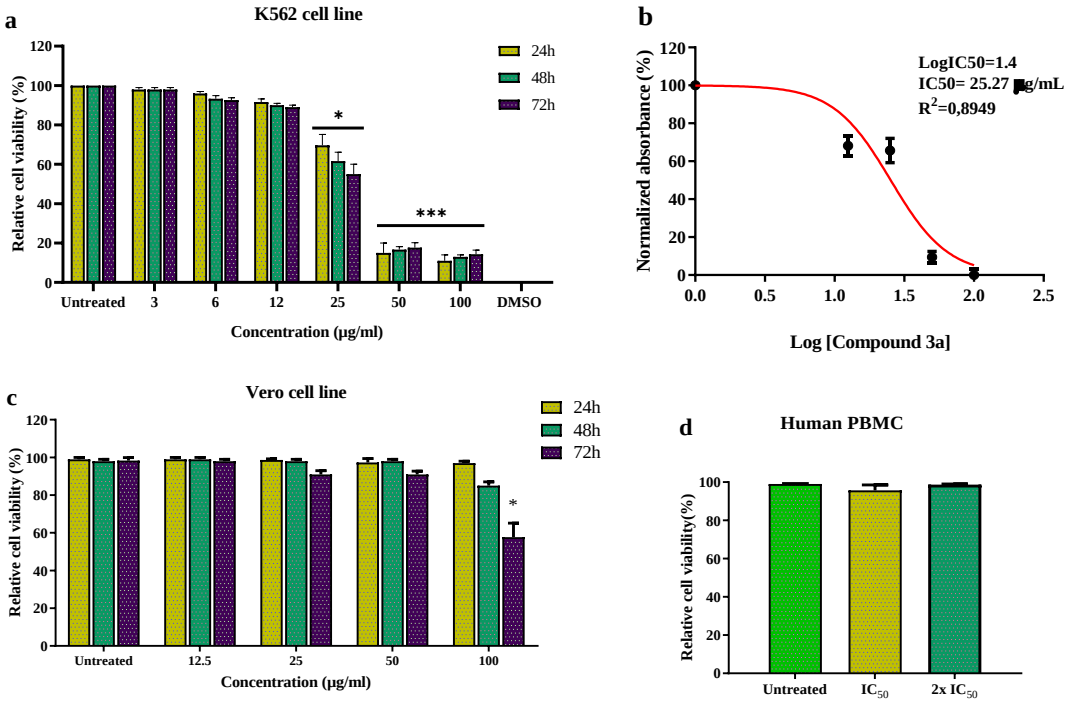


Fig. 2 Cytotoxic activity of compound 3a in cancerous and non-cancerous cells. Viability of K562 cells after treatment at concentrations of 3-100µg/mL in a kinetics of 24, 48 and 72h (a). Non-linear regression result and IC50 for K562 (b). Cell viability of non-cancerous cells, Vero (c) and PBMCs (d) after treatment with the compound 3a. Statistical significance was represented by asterisks; *p<0.05; ***p<0.001

3.2. Inhibition of colony formation

The colony formation assay was used in order to verify the effect of compound **3a** on the clonal proliferation of K562 leukemic cells. The findings of the study revealed a remarkable suppression (p<0.001) of the clonal growth of K562 cells by compound **3a**, leading to a decrease in colony formation by 61.5% when compared to the control group. (Figure 3.a). In addition, optical microscopy images demonstrated that the size of K562 colonies was reduced by the compound **3a** compared in comparison with the control (Figure 3.b).

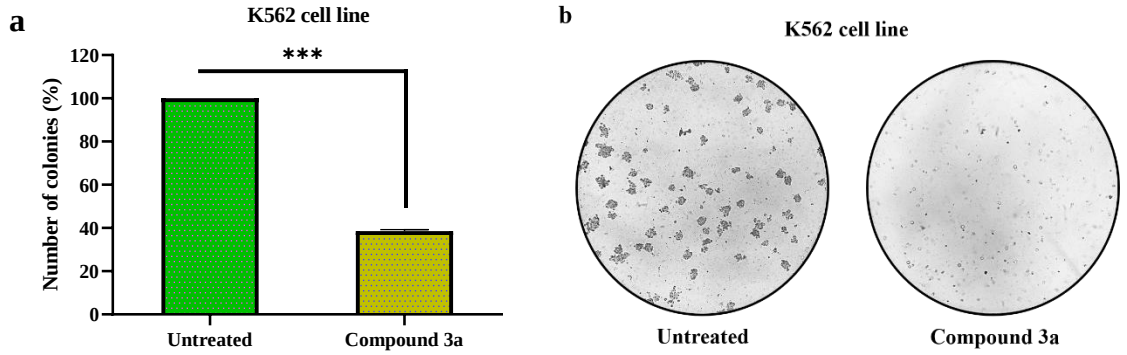


Fig. 3 Effect of the compound **3a** on the formation of colonies of K562 cells. Quantification of the number of colonies (%) in vitro of K562 cells after treatment with compound (a). Optical microscopy image (10x) representative of colony formation for K562 (b). Statistical significance was represented by asterisks; ***p<0.001

3.3. Effects of Compound 3a on Cell Cycle Progression

In order to examine the effect of compound **3a** on the cell cycle of K562 cells, cells were exposed to the compound's IC_{50} concentration (25.27 µg/mL) for 24 hours and then analyzed by flow cytometry. The results showed that the percentage of cells in the S phase significantly increased ($p<0.01$) after treatment with spiroindolone-derived compound **3a** (Figure 4). Additionally, an increase of 16.1% in the number of cells in the G2/M phase was observed. These results indicate that compound **3a** induces cell cycle arrest in both S and G2/M phases, with a greater proportion of cells arrested in the G2/M phase($p<0.001$).

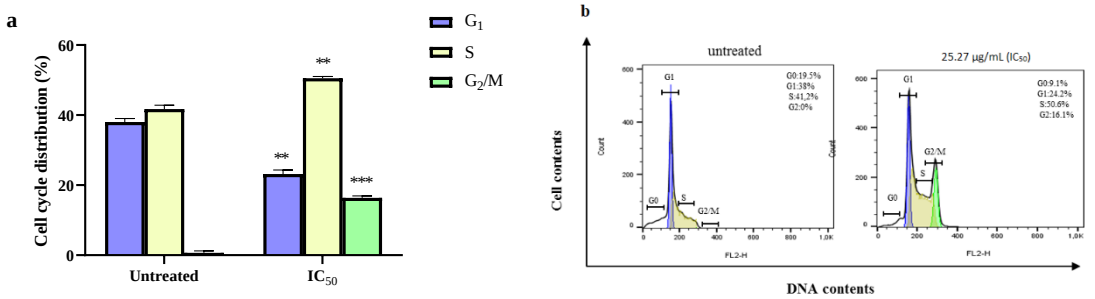


Fig. 4 Cell cycle analysis of K562 cells after treatment with **3a**. Result of the distribution of cell cycle arrest in untreated K562 (control) and treated with IC_{50} (25.27µg/mL) of compound (a). Representative cell cycle micrographs of susceptible K562 cells, representing G0, G1, and G2/M cell populations in treated and untreated cells (b). Statistical significance was represented by asterisks; **$p<0.01$

3.4. Immunomodulatory activity of compound 3a

To investigate the immunomodulatory activity of compound **3a**, human PBMCs were treated with the concentration of 25.27 µg/mL, which corresponds to the IC_{50} value for K562 cells. The results showed that the compound significantly upregulated the cytokines IL-6 ($p<0.01$) and IL-12/23p40 ($p<0.05$) compared to the control, which consisted of PBMCs without treatment (Figure 5). However, the compound 3a did not affect the levels of the other evaluated cytokines when compared to the control.

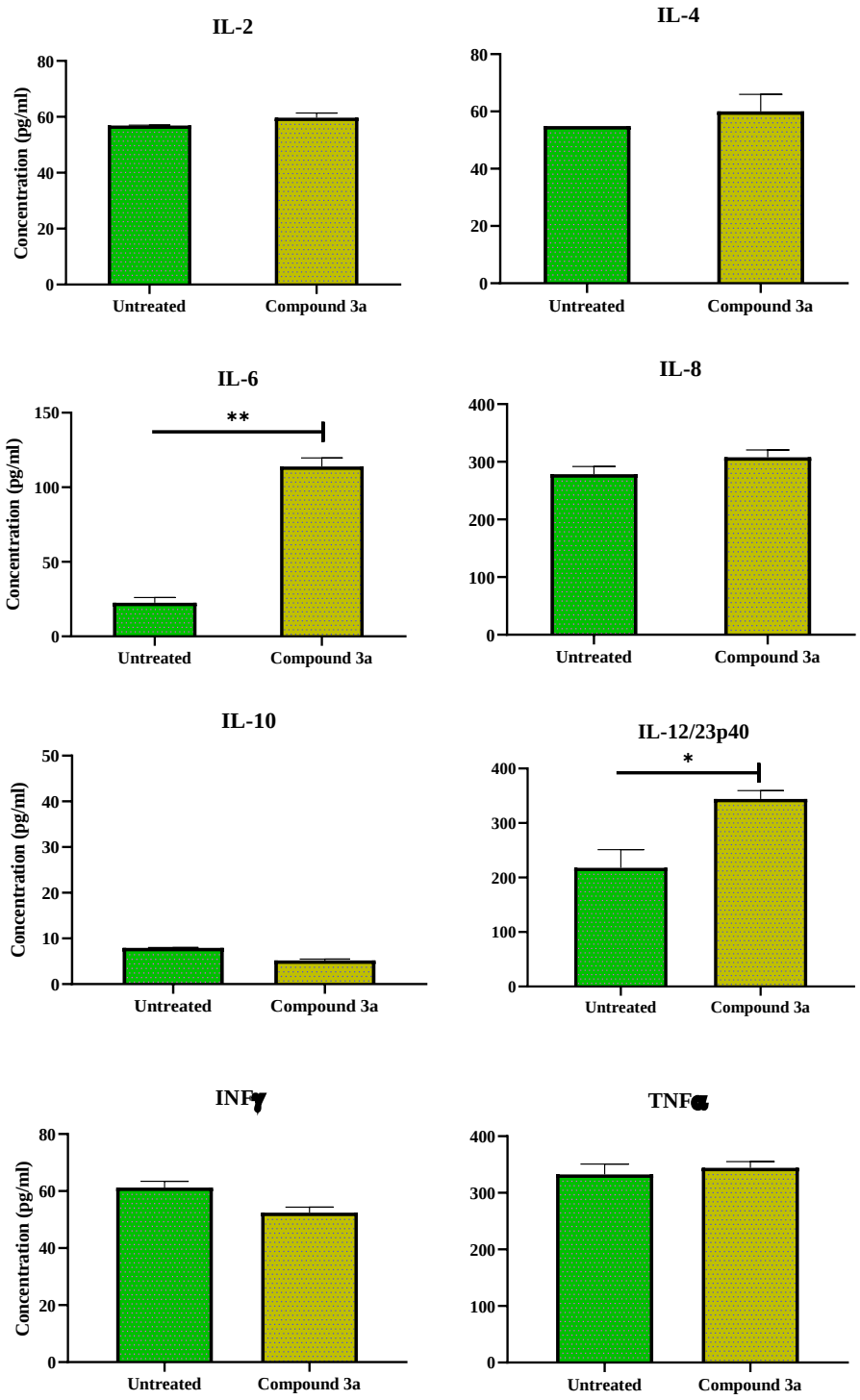


Fig. 5 Immunomodulatory effect of compound 3a. The levels of cytokines IL-2, IL-4, IL-6, IL-8, IL-10, IL-12/23p40, IFNγ and TNFα were evaluated by ELISA in the supernatant of humanized PBMCs treated with the IC_{50} of the compound **3a** (25.27μg/mL). Statistical significance was represented by asterisks; *p<0.05, **p<0.01

4 Discussion

Cancer is one of the greatest challenges for global public health and requires prophylactic and therapeutic actions with different degrees of complexity [17]. The search for new, more efficient and less invasive

chemotherapeutic compounds is the subject of several studies that seek to treat cancer patients. In addition, acting on the dynamics of immune modulation in the context of cancer is essential for the formulation of compounds that promote the control and inhibition of cancer cells [18]. This study demonstrated that the novel derivative of spiroindolone (compound **3a)** exhibits selective cytotoxicity activity against cancerous cells and had immunomodulatory potential.

The compound **3a** is synthesized from pyrazolo[3,4-c]isoquinolines via a modified Pictet-Spengler Reaction (PSR). Previous research has revealed that derivatives of pyrazolo[3,4-c]isoquinolines have anticancer properties combined with low toxicity, acting on the inhibition of protein kinases (PKs) in anaplastic lymphoma and on the inhibition of B-Raf in melanomas and cancers associated with this PK [19, 20]. Another study has also showed the inhibitory effect of PKs by pyrroloisoquinoline derivatives [21, 22]. These findings suggest that compound 3a may potentially exhibit activity against these proteins. However, in this study, we did not investigate the activity of compound 3a on PKs.

PKs are a group of intracellular enzymes that catalyze protein phosphorylation and are involved in signaling pathways that regulate malignant features in various cancers [23]. In CML, the BCR-ABL_1 kinase, produced by the homonymous oncogene, is the main driver of the disease [24]. Together with other PKs, it promotes cancer cells through the activation of pro-survival and pro-proliferation factors [24]. BCR-ABL_1 is also the main therapeutic target in the treatment of CML. Imatinib and other TKIs are used to treat CML and target BCR-ABL_1 by blocking the mitogenic and anti-apoptotic signaling pathways driven by this oncogene[25].

Upon analyzing the cell cycle, we found that the compound **3a** inhibits the proliferation of K562 cells by arresting the cell cycle at the S and G_2/M phases. This indicates that the compound prevents the cells from entering mitosis, a phase that requires coordinated participation of multiple proteins, including mitotic kinases [26, 27]. The *BCR-ABL* gene activates several signaling pathways, including the RAS-to-MAPK/ERK pathway, which is linked to the G_0/G_1 and G_2/M phases of the cell cycle [28]. Interference with the MAPK/ERK pathway can delay or inhibit entry into the mitotic phase [29]. These observations suggest that the compound **3a** may act on PKs linked to mitosis. To confirm and understand the mechanism of action, further analysis is required to comprehend the effect of the compound **3a** on PKs.

In the microenvironment of hematological neoplasms, different factors influence the promotion of the development and progression of malignant cells, including cytokines. Changes in the profile of these proteins can induce dedifferentiation of neoplastic cells and generate malignant phenotypes [30]. IL-6 is a cytokine that plays a crucial role in regulating various physiological processes, including immune response, inflammation, hematopoiesis, and acute phase response [31]. However, in CML, leukemic cells' overexpression of IL-6 can create an inflammatory microenvironment that supports tumor development [32].

A previous investigation demonstrated that IL-6 played a significant role in promoting tumor growth in CML by increasing the number and size of CML cell [33]**.** However, IL-6 plays a crucial role in regulating

the immune response to cancer by activating and mobilizing anticancer T lymphocytes, regulating lymphocyte function, and inducing apoptosis [34, 35]. L-6's multifaceted roles in regulating the immune response to cancer suggest it may serve as a potential target for cancer immunotherapy, despite its protumorigenic effects in certain contexts. Thus, IL-6 is a key mediator in the interplay between cancer cells and the immune system and an important target for developing cancer immunotherapies. Our findings revealed that compound 3a induces the upregulation of cytokines IL6 and IL-12/23p40 when compared to untreated cells, indicating its regulatory effect on this cytokine expression. However, we did not measure the apoptosis rates induced by Compound 3a, precluding any potential correlation between the upregulation of IL-6 and cell growth control. Further investigations employing compound 3a are necessary to determine this correlation.

Our results also showed that compound 3a induces the production of IL-12/23p40 cytokine. This cytokine is pro-inflammatory heterodimer proteins composed of the p40 subunit that play an important role in regulating effective anticancer immunity [36]. They act as a link between innate and adaptive immunity, promoting the recruitment and activity of natural killer (NK) cells and $CD8^+$ T cells and acting as a growth factor for B cells [36]. Some studies indicate that treatment with imatinib or some TKIs may directly affect the antineoplastic functions of NK cells [37, 38]. At this point, IL-12 has shown promise in cancer immunotherapy, both as a complementary therapy and in combination with other cytokines [39, 40]. Our findings showed that compound 3a can effectively upregulate the expression of IL-12/23p40, which consequently could enhance the activity of NK cells in CML, thereby exhibiting potential as a therapeutic agent for this disease. However, additional studies are required to elucidate the mechanistic basis of the immunomodulatory effects of this novel derivative of spiroindolone, specifically in the context of CML.

5 Conclusion

This study demonstrated that the novel derivative of spiroindolone exhibits selective cytotoxic activity against chronic myeloid leukemia cells, without harming non-cancerous cells. Moreover, the compound's ability to modulate the immune response makes it an attractive candidate to be explored pharmaceutically in prospective studies targeting new therapeutic strategies in the treatment of leukemias. Nonetheless, additional investigations are needed to unveil the underlying mechanisms that are accountable for the actions demonstrated by compound **3a**, as portrayed in this study.

Author contribution: Carlos Eduardo de C. Alves: project administration, manuscript writing, writing - review and editing, methodology, investigation, data analysis. Serge L. Bogza, Nathalie Bohdan and Alexander B. Rozhenko: compound design and synthesis, manuscript review. Alice de Freitas Gomes: methodology, investigation, formal analysis. Regiane Costa de Oliveira: methodology, investigation, formal analysis, writing - review. Renata Galvão de Azevedo and Larissa Raquel Silva: methodology, investigation, formal analysis. Anamika Dhyani: methodology, investigation, formal analysis, provision of resources for the study. Andriy Grafov: compound design and synthesis, provision of resources for the study. Gemilson Soares Pontes: project administration, conceptualization, funding acquisition, investigation, writing - review and editing, formal analysis.

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Data availability: data obtained and analyzed in this study are available from the corresponding author upon reasonable request.

6 Declarations

Conflict of interest: all authors declare no conflicts of interest

Competing interest: the authors have no relevant financial or non-financial interests to disclose.

Ethics approval: approval for the utilization of human PBMCs was granted by the Research Ethics Committee (REC) of the Foundation of Hematology and Hemotherapy Foundation of Amazonas State (HEMOAM), Brazil (approval number: 3.138.343; approved on February 8, 2019)

Consent to publish: all authors allowed the submission of the manuscript for publication.

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6. CONCLUSÃO

- Em conclusão, a nova síntese de pirimidina, o novo derivado de espiroindolonas e a fração enriquecida com polifenol extraída de *Myrtus communis* (PEMC) apresentam efeito de citotoxicidade seletiva contra linhagens celulares de leucemia, uma vez que não foi observada toxicidade para a células não cancerígenas;
- PEMC também revelou um alto potencial anti-inflamatório, antioxidante e cicatrizante;
- A nova síntese de pirimidina e o novo derivado de espiroindolonas apresentaram ainda efeito imunomodulador sobre a estimulação de citocinas em PBMCs humanas;
- Os compostos identificados neste estudo exibiram potenciais farmacológicos promissores como agentes antineoplásicos no tratamento de leucemias mieloides.

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