UNIVERSIDADE SÃO FRANCISCO
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EFEITOS DA MIRISTICINA NO MECANISMO DE REVERSÃO DA RESISTÊNCIA A MÚLTIPLOS FÁRMACOS COMUNS À QUIMIOTERAPIA PARA TRATAMENTO DO CÂNCER

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“Em algum lugar, algo incrível está esperando para ser descoberto. ”

Carl Sagan

RESUMO

O câncer representa um dos principais problemas de saúde pública no mundo e é uma das causas mais frequentes de morte precoce. Atualmente os protocolos de tratamento disponíveis envolvem a combinação do uso de radioterapia, cirurgia e quimioterapia, com objetivo de curar, paliar ou melhorar a qualidade de vida do paciente portador da doença. Muitos dos medicamentos utilizados na terapêutica atual têm sua origem nos produtos naturais, que representam uma fonte promissora de novos fármacos, justificando a busca constante por novas moléculas dessa fonte. Contudo, no que se refere à quimioterapia, ainda há uma grande barreira representada pela resistência a múltiplos fármacos (multidrug resistance – MDR). Um dos principais mecanismos envolvidos na MDR é o aumento da expressão de proteínas transportadoras de membrana, que causam o efluxo da droga antineoplásica para o meio extracelular, resultando em redução da sua atividade, e consequentemente, da eficácia do tratamento. Este trabalho buscou avaliar a capacidade da miristicina, um composto de origem natural conhecido por sua ação antiproliferativa, antimicrobiana, antioxidante, entre outros, em inibir a atividade da glicoproteína-P (P-gp), uma das principais proteínas envolvidas na MDR, e reverter o fenômeno da resistência ao tratamento com quimioterápicos. Para isto, foram utilizadas técnicas de cultivo celular bidimensionais e tridimensionais, nas quais células de linhagem de ovário NCI/ADR-RES resistentes a múltiplos fármacos e que superexpressam P-gp foram tratadas com miristicina em associação com os quimioterápicos cisplatina e docetaxel. Além disso, foram utilizadas ferramentas computacionais para avaliar a ligação entre a miristicina e a P-gp, bem como investigar sua biodisponibilidade. Após a análise do ensaio viabilidade em formato bidimensional, verificou-se que a concentração de quimioterápicos necessária para reduzir a viabilidade celular foi menor quando associada com a miristicina. Este efeito foi reproduzido no ensaio em formato tridimensional, mostrando que as concentrações encontradas inicialmente possuem a mesma eficácia quando aplicadas a uma estrutura mais complexa e semelhante a um tumor real. As concentrações de cisplatina e docetaxel associadas à miristicina foram reduzidas em 32,56% e 73,33%, respectivamente, em relação à dose de tratamento isolada. Os resultados do *docking molecular* mostraram que a miristicina é capaz de se ligar à P-gp e potencialmente inibir sua atividade extrusora. A molécula também se encaixou nos 5 parâmetros de Lipinski, indicando que ela possui boa biodisponibilidade, e, portanto, seria uma boa candidata a fármaco.

Palavras-chave: Câncer. Produtos naturais. Miristicina. MDR. P-gp. Quimioterapia.

ABSTRACT

Cancer represents one of the main public health problems in the world and is one of the most frequent causes of early death. Currently, the available treatment protocols involve the combination of radiotherapy, surgery and chemotherapy, with the aim of curing, palliating or improving the quality of life of patients with the disease. Many of the drugs used in current therapy have their origin in natural products, which represent a promising source of new drugs, justifying the constant search for new molecules from this source. However, with regard to chemotherapy, there is still a major barrier represented by multidrug resistance (MDR). One of the main mechanisms involved in MDR is the increase in the expression of membrane transport proteins, which cause the efflux of the antineoplastic drug to the extracellular environment, resulting in a reduction in its activity and, consequently, in the effectiveness of the treatment. This work sought to evaluate the ability of myristicin (a compound of natural origin known for its antiproliferative, antimicrobial, hepatoprotective action, among others), to inhibit the activity of P-glycoprotein or P-gp (one of the main proteins involved in MDR) and reverse the phenomenon of resistance to treatment. For this, 2D and 3D cell culture techniques were used, in which NCI/ADR-RES ovarian lineage cells resistant to multiple drugs and overexpressing P-gp were treated with myristicin in association with the chemotherapeutic agents cisplatin and docetaxel. Furthermore, computational tools were used to evaluate the link between myristicin and P-gp, as well as to investigate its bioavailability. After analyzing the viability assay in two-dimensional format, it was found that the concentration of chemotherapeutic drugs required to reduce cell viability was lower when associated with myristicin. This effect was reproduced in the assay in three-dimensional format, showing that the concentrations found initially have the same effectiveness when applied to a more complex structure and more similar to a real tumor. Myristicin-associated cisplatin and docetaxel concentrations were reduced by 32.56% and 73.33%, respectively, relative to the treatment dose alone. Molecular docking results showed that myristicin is capable of binding to P-gp and potentially inhibiting its extruding activity. The molecule also fit Lipinski's 5 parameters, indicating that it has good bioavailability, and therefore would be a good drug candidate.

Keywords: *MDR. Natural products. Cancer. Myristicin. Chemotherapy.*

LISTA DE SÍMBOLOS E ABREVIAÇÕES

INCA: Instituto Nacional do Câncer

MDR: *Multidrug resistance* – fenômeno de resistência a múltiplos fármacos

P-gp: glicoproteína-P

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SUMÁRIO

1. INTRODUÇÃO

Câncer é um termo que se refere a um grupo de doenças causadas pela transformação de células saudáveis em células malignas. Essa mudança ocorre devido a mutações genéticas que ocasionam estimulação de crescimento, evasão de supressores de crescimento, resistência à apoptose, indução de angiogênese e ativação de invasão de outros tecidos (metástase) (Horne, Pollick e Henner, 2014). Na maioria dos países, a doença é uma das principais causas de morte antes dos 70 anos de idade. Segundo o Global Cancer Observatory (Globocan), em todo o mundo, estima-se que 19,3 milhões novos casos de câncer (18,1 milhões excluindo câncer de pele não melanoma) e quase 10,0 milhões de mortes por câncer (9,9 milhões excluindo câncer de pele não melanoma) ocorreram em 2020. Os tipos mais incidentes no mundo são o câncer de pulmão, o câncer de mama, câncer de cólon e reto, e câncer de próstata (Sung et al., 2021). A estimativa para o Brasil no período de 2023 a 2025 é de que ocorrerão 704 mil casos novos de câncer por ano (Figura 1), o qual o mais incidente será o câncer de pele não melanoma, (220 mil), seguido pelos cânceres de mama (74 mil), próstata (72 mil), cólon e reto (46 mil), pulmão (32 mil) e estômago (21 mil) (INCA, 2023).

Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2023 por sexo, exceto pele não melanoma*

Localização Primária	Casos	%			Localização Primária	Casos	%
Próstata	71.730	30,0%	Homens	Mulheres	Mama feminina	73.610	30,1%
Cólon e reto	21.970	9,2%			Cólon e reto	23.660	9,7%
Traqueia, brônquio e pulmão	18.020	7,5%			Colo do útero	17.010	7,0%
Estômago	13.340	5,6%			Traqueia, brônquio e pulmão	14.540	6,0%
Cavidade oral	10.900	4,6%			Glândula tireoide	14.160	5,8%
Esôfago	8.200	3,4%			Estômago	8.140	3,3%
Bexiga	7.870	3,3%			Corpo do útero	7.840	3,2%
Laringe	6.570	2,7%			Ovário	7.310	3,0%
Linfoma não Hodgkin	6.420	2,7%			Pâncreas	5.690	2,3%
Fígado	6.390	2,7%			Linfoma não Hodgkin	5.620	2,3%

*Números arredondados para múltiplos de 10.

FIGURA 1. Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para cada ano do triênio 2023-2025 por sexo, exceto pele não melanoma. Fonte: INCA, 2023.

O tratamento do câncer baseado na quimioterapia busca reduzir o tamanho do tumor ou eliminá-lo por completo, mas também pode ser empregado com finalidade paliativa. As drogas citostáticas têm como alvo o ciclo celular. Essas drogas interferem na proliferação celular ao direcionar o DNA ou RNA celular e seu metabolismo, entretanto, inúmeros efeitos colaterais podem ser observados nos pacientes em tratamento quimioterápico, e estes apresentam diferentes níveis de intensidade: leve (grau 1), moderado (grau 2), grave (grau 3) e incapacitante ou potencialmente fatal (grau 4). Os sintomas imediatos são mais frequentes em tecidos como trato gastrointestinal, pele e cabelo, medula óssea e sangue (Schirrmacher, 2019). Estes sintomas incluem mielossupressão com leucopenia, trombocitopenia, anemia, alopecia. No trato gastrointestinal, são frequentes os sintomas leves como náuseas e vômitos, mas podem haver complicações como mucosite oral e gastrointestinal, que podem levar a quadros de à anorexia, má absorção de nutrientes, perda de peso, anemia, fadiga e aumento do risco de sepse (Caley and Jones, 2012; Nurgali; Jagoe; Abalo, 2018;). Embora os efeitos adversos sejam mais comumente visualizados nestes tecidos, todos os órgãos do corpo podem ser afetados, incluindo órgãos essenciais, como coração, pulmões e cérebro. Podem ser observados cardiomiopatia congestiva e fibrose pulmonar. A neurotoxicidade de graus 3 e 4 pode induzir sonolência, parestesia, paralisia, ataxia, espasmos e coma. Além disso, os efeitos crônicos da quimioterapia incluem resistência a drogas, carcinogenicidade (por exemplo, linfoma não-Hodgkin e a leucemia mielóide aguda) e infertilidade (menopausa prematura e disfunção gonadal) (Caley and Jones 2012; Schirrmacher, 2019).

Além dos efeitos adversos que estes quimioterápicos provocam nos pacientes, existe outra grande barreira no tratamento quimioterápico, representada pela resistência a múltiplos fármacos (MDR). A MDR ocorre quando, após iniciar o uso de medicamentos antineoplásicos, as células cancerígenas passam a adquirir mecanismos de resistência que ocasionam a diminuição da eficácia do tratamento em 90% dos casos (Molnar et al., 2010). Estes mecanismos, na maioria dos casos, estão relacionados à diminuição da concentração intracelular da droga. Os ativos atingem o ambiente intracelular através de canais transportadores da membrana plasmática. Assim, mutações que alteram a atividade destas proteínas, ou aumentam a sua expressão, podem causar o aumento do efluxo da droga para o meio extracelular, reduzindo o seu efeito (Rocha, 2015).

A proteína mais frequentemente associada à MDR é a chamada glicoproteína-P, ou P-gp. Ela faz parte de um grupo de proteínas transportadoras de membrana que realizam o transporte de substâncias através da hidrólise do ATP. A superexpressão da P-gp está relacionada ao fracasso terapêutico de diversos tipos de tumores (Molnar et al., 2010).

Devido a tais barreiras no uso de quimioterápicos, é constante a pesquisa em busca de novas moléculas para o tratamento do câncer. Nesse contexto, as fontes naturais são de grande importância para obtenção de novas substâncias, visto que cerca de 40 a 70% das novas moléculas aprovadas para uso nas últimas décadas são de origem natural (Newmann and Cragg, 2020, Kumar and Jaitak, 2019).

Diversas classes de compostos naturais foram estudadas ao longo dos últimos anos e apresentaram resposta positiva na modulação da quimiorresistência. A origem de tais classes varia desde plantas até microorganismos e fontes marinhas. Os mecanismos responsáveis pela atividade da maioria destes compostos estão relacionados à capacidade de bloquear a P-gp e outros canais de membrana, além de reduzir a expressão destas proteínas, resultando em efeito sinérgico quando associados a medicamentos antineoplásicos. As principais classes que apresentaram tal efeito positivo são: carotenoides, flavonóides, alcaloides, esteróides cardiotônicos, cumarinas, peptídeos e terpenóides. Estes estudos mostram que, tal como no cenário geral da terapia medicamentosa, os produtos naturais são uma fonte de grande importância na obtenção de substâncias com potencial para reverter quadros de câncer resistente à quimioterapia (Kumar and Jaitak, 2019; Molnar et al., 2010).

A substância estudada neste trabalho é denominada miristicina. Trata-se de um alilbenzeno de origem natural encontrado em diversas especiarias, mas principalmente na noz-moscada (*Myristica fragrans*). Esta semente era utilizada nos tempos antigos tratar ansiedade, cólicas estomacais, náuseas e diarreia, mas também como conservante de alimentos. Nas décadas de 1960 e 1970 foi utilizada como droga pela cultura hippie devido aos seus efeitos psicodélicos. Atualmente, a noz-moscada é utilizada pela indústria alimentícia como flavorizante de alimentos. Estudos conduzidos com a miristicina nas últimas décadas demonstram que ela apresenta importantes atividades biológicas, como antimicrobiana, antioxidante, anti-inflamatória e inseticida. Além disso, foi evidenciada a atividade antiproliferativa em linhagens celulares não-resistentes (Seneme et al., 2021).

Embora alguns estudos tenham abordado a atividade antiproliferativa da miristicina, nenhum deles investigou a sua associação com quimioterápicos, bem como sua ação no mecanismo de reversão da MDR. Contudo, uma molécula estruturalmente semelhante à miristicina, denominada apiol, foi avaliada recentemente pelo nosso grupo frente à sua capacidade de reverter a MDR e de potencializar a ação de quimioterápicos já amplamente utilizados (Lima et al., 2020). O apiol por si só não apresentou efeito citostático significativo em linhagens de células tumorais resistentes. Entretanto, a sua associação aos quimioterápicos vincristina e doxorrubicina apresentou um efeito sinérgico, ou seja, a ação citostática dos medicamentos foi potencializada pelo apiol. Este mecanismo estaria relacionado à afinidade da molécula ao sítio ativo da P-gp, antagonizando a sua ação de promover efluxo da droga para o meio extracelular (Lima et al., 2020).

Os resultados encontrados para o apiol demonstram um potencial promissor em relação à miristicina, pois dada a sua semelhança estrutural (a diferença se dá pela ausência de um grupo metoxila, conforme figura 2), seria esperado que ela fosse capaz de inibir a P-gp e, consequentemente, reverter a MDR. Estes achados incentivaram nosso grupo de pesquisa a iniciar os estudos acerca do potencial farmacológico e terapêutico da miristicina, bem como de investigação *in vitro* e *in silico* dos seus efeitos no mecanismo de reversão da resistência a múltiplos fármacos comuns à quimioterapia para tratamento do câncer.

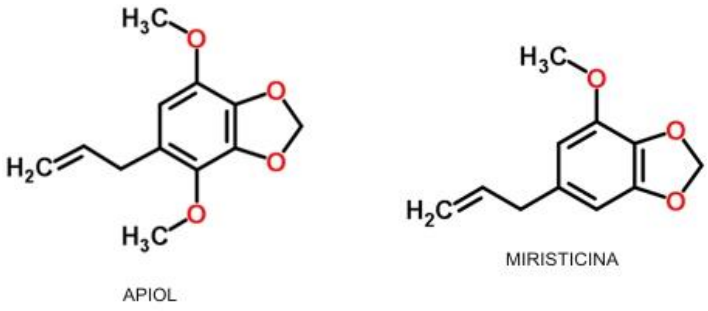

FIGURA 2. Fórmulas estruturais do apiol e miristicina. Fonte: https://www.sigmaaldrich.com/brazil.html

2. OBJETIVOS

2.1 Objetivo Geral

Investigar os efeitos *in vitro* da miristicina no mecanismo de reversão da resistência a múltiplos fármacos comuns à quimioterapia para tratamento do câncer.

2.2 Objetivos Específicos

- Averiguar o potencial terapêutico e farmacológico da miristicina;
- Investigar o potencial citostático da miristicina;
- Avaliar os efeitos da associação entre miristicina e quimioterápicos padrão (docetaxel e cisplatina) na viabilidade de células tumorais resistentes em modelo de cultivo celular tridimensional;
- Identificar o alvo molecular da miristicina na proteína P-gp em modelo *in silico*;
- Avaliar propriedades físico-químicas da molécula para predizer sua biodisponibilidade, através de ferramenta computacional.

3. CAPITULO 1: Artigo Publicado

Pharmacological and Therapeutic Potential of Myristicin: A Literature Review

O artigo apresentado neste capítulo é uma revisão bibliográfica acerca do potencial terapêutico da miristicina. O objetivo da pesquisa foi investigar todos os efeitos biológicos encontrados para a molécula e divulgados em literatura nos últimos 10 anos (2012-2021), em especial o antiproliferativo. Este artigo foi publicado em 2021 na revista *Molecules* (IF = 4,927).

molecules

MDPI

Review

Pharmacological and Therapeutic Potential of Myristicin: A Literature Review

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Abstract: Natural products have been used by humanity for many centuries to treat various illnesses and with the advancement of technology, it became possible to isolate the substances responsible for the beneficial effects of these products, as well as to understand their mechanisms. In this context, myristicin, a substance of natural origin, has shown several promising activities in a large number of in vitro and in vivo studies carried out. This molecule is found in plants such as nutmeg, parsley, carrots, peppers, and several species endemic to the Asian continent. The purpose of this review article is to discuss data published in the last 10 years at Pubmed, Lilacs and Scielo databases, reporting beneficial effects, toxicity and promising data of myristicin for its future use in medicine. From 94 articles found in the literature, 68 were included. Exclusion criteria took into account articles whose tested extracts did not have myristicin as one of the major compounds.

Keywords: myristicin; nutmeg; natural products; bioactive compounds; therapeutic properties

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1. Introduction

Biodiversity is the variability of all living beings in the biosphere, in its entirety. Such beings become renewable sources of substances that can originate various products for human consumption, including medicines for the treatment of various pathologies. The main producers of these substances are plants, microorganisms, marine algae, among others, which over thousands of years of evolution, were capable of adaptations that made them capable of acquiring biological activities of various types [1].

Data show that there are still few drug discovery programs based on natural products in pharmaceutical companies, although they are a promising source of new drugs [2]. Even so, drugs produced from natural substances are numerous, since those obtained from natural sources represent about 70% of all drugs approved for therapeutic use in the last four decades [3].

Natural compounds have been one of the main sources of the production of medicines since the beginning of time, giving rise to drugs of different therapeutic classes. One of the main examples is the discovery of penicillin in 1928, by the researcher Alexander Fleming, whose research with fungi of the *Penicillium* genus culminated in the discovery of a compound with an antibacterial activity. This compound was called penicillin and is currently used to treat infections caused by bacteria of the *Staphylococcus genus* [4]. Another natural compound of great importance is quinine, derived from the bark of the quinine. Initially this herb was consumed by the indigenous people of the Amazon region. This plant has been used for decades to treat malaria, and this substance gave rise to other drugs

to treat the disease, such as chloroquine [2]. *Arnica montana* plant species, also widely used in Brazil for many years, has anti-inflammatory, analgesic and healing actions that improve or prevent injuries, and currently its extract can be found in ointments and gels produced by the pharmaceutical industry [5]. Therefore, since the main source of new medications are natural products, it is necessary to carry out research to discover new treatments from sources that have been little explored.

In this work, we will discuss a substance called myristicin. It was first discovered in the seed of nutmeg (*Myristica fragrans*), and was described in the French colonies in the mid-18th century, on the Maluku islands [6]. In addition to the high concentration in this seed, myristicin can also be found in cinnamon, parsley, some types of pepper and other spices native to Asia. Nutmeg was used in ancient times (in India and other regions of Asia) to treat anxiety, stomach cramps, nausea and diarrhea [7]. In addition, it has been described as a food preservative, as it has antimicrobial activities, and it is currently used as a flavoring agent by the food industry [8]. When used in very high amounts, myristicin can have toxic effects, leading to liver degeneration and mental confusion, as it is toxic to the central nervous system. It is believed that myristicin is in the main responsible for the benefits described with the use of nutmeg, as well as for its toxic effects, since it is the largest compound present in this spice [7].

Several preliminary studies have been conducted with myristicin over the last few years, demonstrating that it has promising biological activities, but it is still little explored. Thus, considering the ethnopharmacology of myristicin, as well as the importance of natural products as a source of new drugs, there is an urgent need to investigate scientific data about its properties, which may justify its use as a therapeutic substance in addition to arousing scientific interest in continuing the investigation of its pharmacological properties.

2. Results and Discussion

2.1. Metabolization and Toxicity of Myristicin

In the 1960s and 1970s, nutmeg was used as a psychedelic drug by the hippie culture, but it was abandoned due to the headache it caused in users. The main toxic activity of nuts occurs in the central nervous system, and is directly linked to the high concentrations of myristicin (1-allyl-5-methoxy-3,4-methylenedioxybenzene), although there may be synergistic effects with the other components [9,10]. The psychedelic effects of myristicin are thought to be related to its active amphetamine-derived metabolite. Furthermore, myristicin is slightly capable of inhibiting the enzyme monoamine oxidase (MAO), which would cause pro-serotoninergic effects and cardiovascular symptoms. Studies have shown that myristicin is able to promote anxiogenesis and affect motor actions and it is suggested that it is able to modulate GABA receptors, possibly acting as an antagonist, generating anxiety [11–14]. Myristicin is metabolized in the liver by enzymes of the cytochrome P450 complex. Its hepatic biotransformation generates metabolites that remain active and may be responsible for its toxicity. In phase 1 metabolism, the main active metabolites are 1'-hydroxymyristicin and 5-allyl-1-methoxy-2,3-dihydroxybenzene. It has also been reported that myristicin can be converted to an amphetamine-like metabolite: 3-methoxy-4,5-methylenedioxy-amphetamine (MMDA), known for its psychedelic effects (Figure 1). The main enzyme responsible for its bioactivation is CYP1A1. Therefore, inducing substances of the CYP1A group may facilitate the formation of reactive metabolites and increase the risk of toxicity related to myristicin. However, myristicin was also evaluated for its likely ability to inhibit CYP complex enzymes, and the results showed that myristicin exerts some inhibitory effect on human CYP2E1, CYP2C19, and CYP1A2 but exerts the strongest inhibitory effect on CYP1A2 [9,10,13].

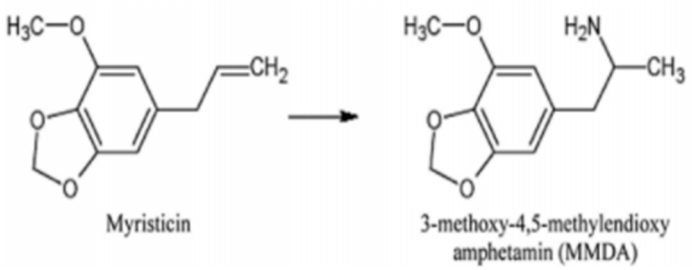

Figure 1. Molecular structure of myristicin and its active metabolite MMDA [9].

After phase 2 metabolism, myristicin metabolites are able to form complexes with endogenous N-acetylcysteine, forming the myristicin-NAC complex, as well as glutathione, forming the myristicin-GSH complex. These complexes are mostly eliminated in urine [9,10].

The toxic effects of myristicin are dose-dependent. Neuropsychological symptoms usually develop after consuming 10 to 15 g of nutmeg, or about 400 mg of myristicin (corresponding to 6–7 mg/kg). Anticholinergic effects occur after ingestion of 25 to 28 g of nutmeg. Symptoms may appear 3 to 6 h after administration and persist for up to 72 h. The main symptoms reported are: dry mouth, facial flushing, blurred vision, hypertension, tachycardia, psychomotor agitation and restlessness; in more severe cases, patients develop delusions, dissociative episodes and visual, auditory or tactile hallucinations [11,13,14].

Although potentially serious, cases of accidental poisoning are relatively rare, as it is necessary to ingest a large amount of nutmeg to reach a plasma concentration of myristicin capable of promoting such effects. Only one fatal case of poisoning was reported for a boy, aged 8, who ate two nutmegs and passed into a comatose condition [15]. In a case study, a 29-year-old male patient who had ingested about 3 to 4 whole walnuts (18 to 28 g) presented unmotivated laughter, disorganized speech and psychomotor agitation, evolving to remission in the following days. Another case study describes the intoxication of a normally developing 3-month-old infant who presented repeated episodes of afebrile status epilepticus after ingestion of nutmeg. Phenytoin, phenobarbital and midazolam were administered to control the seizures, and the patient progressed to improvement, with no new epileptic episodes [11,14].

From these data, it is possible to observe that myristicin and its active metabolites have psychoactive activity, which is mainly responsible for its toxicity. However, due to this activity, it can also be explored as a potential therapeutic agent to treat central nervous system pathologies [11,14].

2.2. Antioxidant Activity

Investigation studies of antioxidant activity were conducted with myristicin isolated or present in extracts and essential oils obtained from the following plant species: *Dorema glabrum* (14% myristicin); *Naturtium officinale* (57% myristicin); *Athamanta turbith* (52% myristicin), *Heracleum transcaucasicum* and *Heracleum anisactis* (containing 96.87% and 95.15% myristicin respectively) and *Myristica fragrans* (containing 8% to 11% myristicin) [16–22].

The results obtained showed that, in vivo, isolated myristicin (high purity) was able to increase the concentration and activity of antioxidant enzymes: catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase, as well as decreasing the levels of lipid peroxidation (Figure 2). The essential oils containing myristicin also showed good antioxidant activity in vitro, with the exception of the oil from the plant *Athamanta turbith*, whose activity was relatively low [16–22].

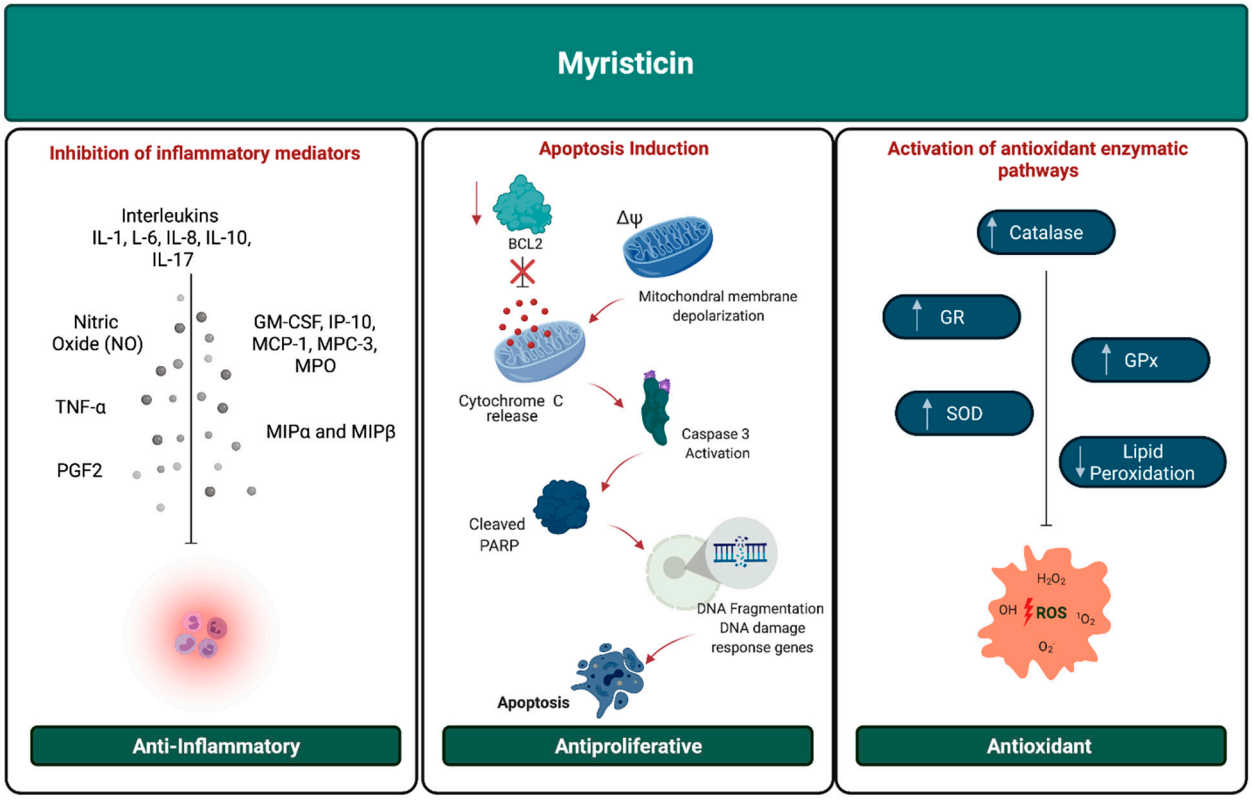

Figure 2. Graphical representation of the anti-inflammatory, antiproliferative and antioxidant pathway mechanisms induced by myristicin.

One of the studies used *Myristica fragrans* (nutmeg) essential oil containing myristicin, the same oil without myristicin, and also myristicin alone. In addition to the antioxidant activity, this study provided information about a possible sun protection factor, through tests of light absorption and reactions with free radicals in vitro. The results showed that the essential oil without myristicin had minimal protection and antioxidant activity and the oil containing myristicin had moderate activity. Isolated myristicin, on the other hand, had the highest protective and antioxidant activity, which indicates that it is the substance responsible for this biological effect in nutmeg oil [23].

However, few studies indicate that myristicin is a promising antioxidant substance, which justifies further tests to understand its mechanism of action.

2.3. Anti-Inflammatory and Analgesic Activity

The anti-inflammatory potential of myristicin has been extensively studied in recent years. Tests to investigate this property were developed in vitro and in vivo and were conducted for isolated myristicin, as well as oils and plant extracts containing the substance in different concentrations. The main plant species used in such studies were *Trachydium roylei*, *Cinnamomum syntoc*, *Pycnocycla bashagardiana*, *Perilla frutescens*, *Myristica fragrans* (nutmeg), *Illicium lanceolatum*, *Piper chaba*, *Piper sarmentosum*, *Piper interruptum* (peppers), *Plumbago indica* and *Zingiber officinale* (ginger). In general, the tests show that myristicin is a potent anti-inflammatory. Several studies report that it is able to inhibit the production of prostaglandins (PGE2), one of the main substances involved in the inflammatory process. This activity was studied in vitro, using techniques such as Western Blotting, PCR and ELISA, and also in vivo, using paw and ear edema reduction assays in mice. Furthermore, a molecular docking study revealed that myristicin would be able to non-selectively inhibit the cyclooxygenase 2 (COX-2) enzyme, which is one of those responsible for the production of prostaglandins. However, it did not show this activity at mRNA and protein levels when treated in human liver cancer cells [24–28].

The anti-inflammatory activity of myristicin can also occur through other pathways (Figure 2). This molecule is also capable of inhibiting several cytokines and mediators responsible for the chemotaxis of the inflammatory process, such as: tumor necrosis factor alpha (TNF-a), interleukins (IL-1, IL-6, IL-8, IL-10 and IL-17), nitric oxide (NO), macrophage inflammatory proteins (MIP-1α r MIP-1β), colony stimulating factor (GM-CSF), IP-10, MCP-1 and MCP-3 and myeloperoxidase (MPO). This inhibition occurs both at the protein level and at the mRNA regulation level. In vitro studies have shown that the inhibition of these cytokines was able to block the migration and growth of neutrophils and macrophages, while in vivo, it promoted a reduction in mice paw edema [16,24,29–34].

The analgesic action of myristicin has also been evaluated. Tests conducted with *Pycnocycla bashagardiana* essential oil containing myristicin did not result in analgesic activity in hot plate tests with mice, despite its good anti-inflammatory action (reduction of paw edema). The essential oil of *Illicium lanceolatum*, in addition to its anti-inflammatory activity in vivo (reduction of ear edema), also showed reduced writhing in mice after pain induction by acetic acid, indicating a possible analgesic action. In this case, however, the author attributes the activity to the association between myristicin and other components of the essential oil [29,33].

Although many results were obtained through tests with essential oils containing other substances that can contribute to the anti-inflammatory action, myristicin was the major component in most of them. From these results, its anti-inflammatory activity in several pathways of the inflammation process is remarkable.

2.4. Antiproliferative Activity

The antiproliferative activity of myristicin has been studied in recent years. Literature data report that myristicin is responsible for the anticancer activity of some medicinal plants and is a cancer chemopreventive agent [35–38].

Athamanta sicula crude extract and isolated myristicin were tested in vitro for their antiproliferative activity, at a concentration of 100 µg/mL, against K-562 (human chronic myeloid leukemia), NCI-H460 (human non-small cell lung adenocarcinoma) and MCF-7 (human breast adenocarcinoma) cells using the methyltetrazolium (MTT) assay. The extracts and isolated myristicin showed significant antiproliferative activity in the tested cancer cell lines, with inhibition of 50% to 100% of cells at different concentrations. Other assays were used to investigate the mechanisms of growth inhibition, and it was concluded that myristicin induced cell apoptosis through changes in mitochondrial membrane potential, cytochrome C release, caspase-3 activation, PARP cleavage and fragmentation of DNA. Gene expression profiling revealed a general down-regulation of DNA damage response genes after exposure to myristicin [35,38].

Exposure of the KB cell line (human oral epidermal carcinoma) with a variable concentration of *Myristica fragrans* extract (nutmeg) resulted in a concentration-dependent inhibition of cell proliferation, suggesting that the nutmeg extract inhibited the proliferation of KB cells. The extract was able to reduce the expression of the bcl-2 gene in cells, diminishing the expression of this protein and inducing early and late apoptosis. Furthermore, the cells shrank and showed morphological changes when analyzed under a microscope. Cancer cells, however, exhibit resistance to apoptosis in order to sustain their uncontrolled proliferation, and therefore any compound that modulates apoptosis is desirable as a plausible cancer chemotherapy agent [37].

Pure and partially purified myristicin obtained from *Myristica fragrans* were tested against human rhabdomyosarcoma (RD) cells in vitro. At lower concentrations and in the first 24 h of treatment, cell growth inhibition had a significant difference: the partially purified extract showed a greater inhibitory activity. However, after 48 h of treatment and at concentrations above 125 µg/mL, both extracts showed a similar inhibitory activity. The highest rate of inhibition was 82.3%, reported at the concentration of 500 µg/mL of pure myristicin. Therefore, it is suggested that the extraction method may interfere with the

biological effect; however, myristicin showed cytotoxic/antiproliferative activity for the studied strain [39].

The essential oil of *Myristica fragrans* containing 32% myristicin was able to induce a significant reduction in human colorectal adenocarcinoma cells (Caco-2) cell viability at the concentration of 250 μg/mL. Furthermore, myristicin isolated from the oil showed an IC50 value of 146 μg/mL, indicating that it could be the substance responsible for the cytotoxic activity of the oil [36].

Pure myristicin is also capable of inhibiting the growth of AA8 and EM9 ovarian cells. Cell viability assays were performed after treatment with different concentrations of myristicin (from 50 to 2000 μM) for 24 h, using the MTT assay protocol. The results showed a reduction in viability. Other assays were carried out, and the results showed that myristicin induced cell apoptosis through the activation of caspases (as already reported by other authors) in both strains, but mainly in EM9. However, it was not able to induce DNA damage [40].

One of the in vitro studies compared the cytotoxicity of myristicin and its active metabolite, 1′-hydroxymyristicin, against HepG2 cells, a human hepatocellular carcinoma line. Cells exposed to myristicin for 24 h did not show a significant reduction in cell viability. In contrast, cells exposed to 1′-hydroxymyristicin, in the same concentration range, showed a dramatic reduction in viability in the MTT test. A significant increase in the number of apoptotic cells (both in the early and late stages of apoptosis) was observed in cells exposed to 1′-hydroxymyristicin. These results indicate that the active metabolite of myristicin is possibly more cytotoxic and apoptotic than the substance itself [41].

Benjakul extract, a traditional medicine composed of extracts of *Piper chaba, Piper sarmentosum*, *Piper interruptum*, *Plumbago indica* and *Zingiber officinale*, which contains 3.5 mg/g of myristicin, was tested for its antiproliferative activity against human small cell lung cancer (NCI-H1688) and non-tumor human lung fibroblast cell line (MRC-5). In vitro assays have shown that benjakul is selective and can kill cancer cells of the NCI-H1688 lineage more than non-tumor cells (MRC-5). However, the isolated myristicin showed a low toxicity to the cell lines [42].

In addition to the products mentioned, a study carried out tests on the antiproliferative activity of essential oils obtained from flowering aerial parts (containing 16.5% of myristicin) and ripe fruits (containing 15.3% of myristicin) of the *Echinophora spinosa* plant. Both oils tested were toxic to U937 cells, but the fruit oil was much more cytotoxic. Although myristicin may have contributed to the cytotoxicity of the oils, the difference between the results was attributed to other components [43].

Through these data, it is not possible to conclusively establish the antiproliferative activity of myristicin. Although some of the studies presented have shown that it is capable of inducing cellular mechanisms that lead to apoptosis (Figure 2), other articles have shown that it was not able to reduce cell viability in some cell lines. Therefore, further studies are needed to prove its effectiveness, covering several cell lines, and carrying out more detailed studies to elucidate the mechanisms of action of the substance. Above all, it is important that further research is carried out with isolated or purified myristicin, to eliminate interference from other compounds present in the analyzed plant extracts and essential oils.

2.5. Antimicrobial Activity

The antimicrobial activity of myristicin has been widely studied in the last decade, but there are still divergences regarding its in vitro effects and mechanisms of action.

Among the substances investigated, the essential oils of *Myristica fragrans* (nutmeg), *Heracleum transcaucasicum*, *Heracleum anisactis*, *Anethum graveolens* (dill), *Apium nodiflorum*, *Petroselinum crispum* (parsley), *Pycnocycla bashagardiana* and *Piper sarmentosum*, all containing high concentrations of myristicin, ranging between 12% and 96% of the composition, are noteworthy. In addition, crude extracts of Athamanta sicula and isolated myristicin with a high degree of purity were tested. The inhibition of growth promoted by these

substances was evaluated by means of disk diffusion assays, microdilution, determination of the minimum inhibitory concentration (MIC) and in silico assays. Different species of bacteria and fungi were tested [8,22,35,44–52].

Some studies showed that the essential oils of *Heracleum transcaucasicum* and *Heracleum anisactis* (containing 96.87% and 95.15% of myristicin, respectively), the *Athamanta sicula* plant extract, as well as the myristicin isolated from the plant, showed weak or absent activity against the species tested: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Candida tropicalis*. In a study that tested the essential oil of nutmeg with different concentrations of myristicin, it was found that those with higher amounts (ranging from 26% to 38%) had no inhibitory effect against *Escherichia coli, Aspergillus fumigatus*, and methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and were slightly active against *Cryptococcus neoformans* [8,22,35,44].

In a study carried out to evaluate the fungicidal activity on several species, essential oils and *Apium nodiflorum* extracts containing 29% of myristicin were tested. The results showed a variability of inhibition among all strains of fungi tested, being especially active against dermatophytes. Moreover, for *Cryptococcus neoformans*, there was significant activity. For *Aspergillus* spp., the oil proved to be less effective. However, this activity was attributed to a synergistic effect between myristicin and dilapiol, another substance present in the plant [46].

Other studies showed that the essential oil of nutmeg (*Myristica fragrans*) containing only 10% of myristicin was able to strongly inhibit the growth of the fungi *Aspergillus flavus* and *Aspergillus ochraceus*. The essential oil of the *Pycnocycla bashagardiana* plant containing 39% myristicin exhibited strong antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli* and *Candida albicans*. Essential oils of dill (*Anethum graveolens*) and parsley (*Petroselinum crispum*), containing from 28% to 42% of myristicin, were able to inhibit the following microorganisms: *Escherichia coli*, *Staphylococcus albus*, *Bacillus mesentericus* and *Aspergillus flavus*. The essential oil of parsley (*Petroselinum crispum*) containing 14% of myristicin showed fungistatic and fungicidal activity against *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus versicolor*, *Penicillium funiculosum*, *Penicillium ochrochloron*, *Penicillium verrucosum* and *Trichoderma viride*, and inhibited the growth of bacteria *Bacillus cereus, Enterobacter cloacae, Escherichia coli, Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Staphylococcus aureus* with varying degrees of sensitivity. A study that aimed to investigate the activity of myristicin in combating acne tested the extract and essential oil of nutmeg (*Myristica fragrans*) against the bacteria *Cutibacterium acnes* and *Staphylococcus aureus*, and presented a good antibacterial effect against both [26,46–49].

Myristicin isolated from the essential oil of *Piper sarmentosum* (representing about 81% to 83% of its composition) was able to inhibit the proliferation of *Escherichia coli* in vitro. The study that demonstrated this activity also revealed that myristicin was able to inhibit, in vitro, the activity of the GTPase enzyme, interfering with a fundamental step for cell division [50].

A computer assay performed with myristicin tested its ability to inhibit the multi-drug resistant bacterial strains growth: *Bacillus anthracis*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Mycobacterium tuberculosis*. The results obtained showed that myristicin would be effective against *Streptococcus pneumoniae*, as it would be able to inhibit the bacterial folic acid biosynthesis dihydropteroate synthase enzyme (DHPS) [51].

Myristicin was also evaluated for its ability to protect food against aflatoxins produced by certain fungi. In this study, the essential oil of nutmeg containing 21% of myristicin was used, which was able to inhibit the growth of the strain of *Aspergillus flavus* that produced the most aflatoxin in vitro. Furthermore, it was shown that the oil caused a decrease in the ergosterol content of the fungus's plasma membrane, which caused cellular ion leakage [52].

After surveying these data, it is possible to conclude that myristicin may have selective antimicrobial activity on some species (Table 1, Figure 2). However, many of the results (positive or negative for antimicrobial activity) observed in the studies can be attributed to the interaction between myristicin and other compounds, as they can either potentiate or inhibit its effect. Therefore, it is necessary to carry out further studies with the isolated molecule to assess its antiproliferative potential against each species, as well as elucidating the mechanisms by which it can inhibit growth or destroy microbial cells.

Table 1. The main biological activities of myristicin and its known mechanisms.

Biological Activity	Mechanisms	Species/Cell Lines
Antioxidant	Increases the concentration of catalase, superoxide dismutase, glutathione peroxidase glutathione reductase and decreases levels of lipid peroxidation	-
Anti-inflammatory	Inhibits PGE2, COX-2, tumor necrosis factor alpha (TNF-a), interleukins (IL-1, IL-6, IL-8, IL-10 and IL-17), nitric oxide (NO), macrophage inflammatory proteins (MIP-1α r MIP 1β), colony stimulating factor (GM-CSF), IP-10, MCP-1, MCP-3 and myeloperoxidase (MPO)	RAW 264.7, A549, HEK293, HL-60 and human fibroblast cells
Antiproliferative	Induces cell apoptosis through changes in mitochondrial membrane potential, cytochrome C release, caspase-3 activation, PARP cleavage, fragmentation of DNA, down-regulation of DNA damage response genes and reduces the expression of bcl-2 gene	K-562, NCI-H460, MCF-7, KB cell line, RD cells, Caco-2, AA8 and EM9, HepG2, NCI-H1688, MRC-5, U937
Antimicrobial	Inhibition of the polymerization of FtsZ, of the enzyme dihydropteroate synthases (DHPSs) and of the GTPase enzyme	Fungi: *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus versicolor*, *Penicillium funiculosum*, *Penicillium ochrochloron*, *Penicillium verrucosum*, *Trichoderma viride* Bacteria: *Bacillus cereus*, *Enterobacter cloacae*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus*, *Cutibacterium acnes*
Insecticide and larvicide	Inhibition of the CYP450 enzyme and acetylcholinesterase in insects	*Liposcelis bostrychophila* and *Lasioderma serricorne*, *Culex pipiens* (larva), *Aedes aegypti*, *Euschistus heros*, *Culex quinquefasciatus* (larva), *Spodoptera littoralis* (larva), *Musca domestica* (adult), and *Spodoptera littoralis*, *Trichoplusia ni*, *Tribolium castaneum*, *Lasioderma serricorne*, *Liposcelis bostrychophila* and *Microcerotermes beesoni*

2.6. Insecticide and Larvicide Activity

The insecticidal and larvicide activities of myristicin were the properties described in recent years that showed the most satisfactory results in vivo and in vitro. The materials used in the research were essential oils extracted from plants such as *Piper aduncum*, *Echinophora spinosa*, *Clausena anisum-olens*, *Helosciadium nodiflorum*, *Ligusticum pteridophyllum*, *Trachyspermum ammi*, *Smyrnium olusatrum*, *Pimpinella anisum*, *Myristica fragrans* (nutmeg), *Ligusticum jeholense* or isolated myristicin [53–60].

In general, myristicin had a potentially toxic effect on the studied insect species: Liposcelis bostrychophila and Lasioderma serricorne, *Culex pipiens* (larva), *Aedes aegypti*, *Euschistus heros*, *Culex quinquefasciatus* (larva), *Spodoptera littoralis* (larva), *Musca domestica* (adult), and *Spodoptera littoralis*, *Trichoplusia ni*, *Tribolium castaneum*, *Lasioderma serricorne*, *Liposcelis bostrychophila* and *Microcerotermes beesoni*. Several researches have shown the

inhibition of the enzyme acetylcholinesterase and cytochrome detoxifying enzymes. This inhibition blocks the transmission of nerve impulses in the insect, causing paralysis and death [53,56–60].

The essential oil obtained from the plant *Clausena anisum-olens* and the myristicin isolated from this oil showed contact toxicity against both adult insects species *Liposcelis bostrychophila* and *Lasioderma serricorne*. The isolated myristicin showed 92% repellency against *Liposcelis bostrychophila* and 68% repellency against *Lasioderma serricorne*, while the plant's essential oil showed only 32% to 38% of its toxic effect [53].

The essential oil of the *Piper aduncum plant*, which has 30% myristicin, showed histopathological toxicity against the insect *Euschistus heros*, with cytological changes and tissue ruptures, characterizing an increase in mitochondria population and a loss of glycogen and lipids. The salivary glands, as well as the midgut, are affected by the oil, showing an insecticidal activity [56].

The insecticidal property of *Echinophora spinosa* roots' and leaves' essential oils containing 47% and 2.7% of myristicin, respectively, were evaluated against larvae of the species *Culex quinquefasciatus*, *Spodoptera littoralis* and adult insects of *Musca domestica*. Insects were subjected to various concentrations of the oils to determine the LC50 (50% lethal concentration). The results show a greater efficacy of the root, as the LC50 was lower, indicating that myristicin may be the substance responsible for the effect [57]. Another study was conducted with the same insect species and used *Helosciadium nodiflorum* as a source of myristicin. In this case, the plant contained 35% myristicin, and was subjected to the hydrodistillation process, obtaining an essential oil that was then tested on insects. The result showed that myristicin has a toxic effect on insects, and its mechanism was attributed to synergistic effects between myristicin and other components [58].

Essential oils of plant species *Helosciadium nodiflorum* collected from different localities (containing 49% and 24% of myristicin), as well as the isolated substance, were also investigated for insecticidal activity against *Trichoplusia ni*. The essential oil had a stronger toxic effect than myristicin alone; however, among the compounds tested in isolation, myristicin was the most potent. Its toxicity is a consequence of inhibition of the CYP450 enzyme in insects [61].

Adult insects of *Tribolium castaneum*, *Lasioderma serricone* and *Liposcelis bostrychophila* were exposed to essential oil containing 90% myristicin, fluid extract containing 48% of it, as well as myristicin isolated from the *Ligusticum pteridophyllum* plant. All tested components showed insecticidal activity, but myristicin alone exhibited a more potent action as the LD50 value was lower [62].

The insecticidal activity of the essential oil of nutmeg containing 6% myristicin was evaluated against termite *Microcerotermes beesoni*. The results showed that the LC50 value of the essential fruit oil is 28.6 mg. Treatment for 14 days with 5 mg of myristicin resulted in 100% mortality [50]. The myristicin found in the roots and rhizomes of *Ligusticum jeholense* showed contact toxicity and repellency. When in contact with *Tribolium castaneum* and *Lesiodwema serricorne*, myristicin exerted its insecticidal and repellent effect on target insects [51].

Pure myristicin, *Peperomia borbonensis* essential oil (containing 39% of the substance) and a mixture of myristicin and elemicin (which are the main components of the oil) insecticidal activity was evaluated against *Bactrocera cucurbitae* insects. The oil showed a neurotoxic effect as a consequence. Soon after contact with the association of myristicin and elemicin, the flies had convulsions and were knocked down. Isolated myristicin has led to only 40% mortality. Thus, it is noted that myristicin has insecticide properties for the studied species, but it is enhanced when there is the presence of other components of the plant's essential oil [63].

Studies conducted with essential oils (containing 20.39% myristicin) and isolated myristicin obtained from *IIIicium henryi* root bark revealed insecticidal activity against *Liposcelis bostrychophila* lice. The oils and isolated myristicin showed strong contact and fumigant toxicity for insects and myristicin was the most potent compound [64].

Essential oils from plants of the *Apiaceae* family, with a 99% myristicin presence, were examined as larvicides for the Asian tiger mosquito species (*Aedes albopictus*). The research showed a 95% mortality result for mosquito larvae treated with a concentration of 0.1 mg/mL of oil [65].

In a research to evaluate the larvicidal activity against *Culex quinquefasciatus* larvae, essential oils from *Sison amomum* and *Echinophora spinosa* (with 41% myristicin) were used, as well as isolated myristicin, and also oils that did not contain myristicin obtained from *Heracleum sphondylium*, *Heracleum sphondylium subsp. ternatum* and *Trachyspemum ammi*. The study showed that among all the oils tested, the second most toxic was the one containing myristicin, and isolated myristicin also has a potential for larvicidal capacity [66].

An in vivo study, which evidenced the larvicidal activity of myristin against *Culex pipiens* larvae, reports that myristicin had a potent toxic activity for the larvae. The test to verify the insecticidal effects of myristicin isolated from nutmeg essential oil against *Culex pipiens* and *Aedes aegypti* insects were also carried out. The study performed was a vapor toxicity test in adult mosquitoes. Myristicin had a more potent larvicidal capacity than oil against the investigated insect. The *Culex pipiens* mosquito is more susceptible to the activity of both compared to *Aedes aegypti* [54,55].

According to the data presented, we conclude that myristicin is a natural substance, with insecticidal and larvicide capacity being an alternative to chemical products that are also used for the same purposes.

2.7. Other Activities

Figure 3 and Table 1 summarizes the main biological acitivities of myristicin and its mechanism of action studied until now. There are published studies on other biological activities of myristicin, but little is reported in the literature. However, they point out promising paths for new therapeutic properties, and that is why it is relevant to continue studying them.

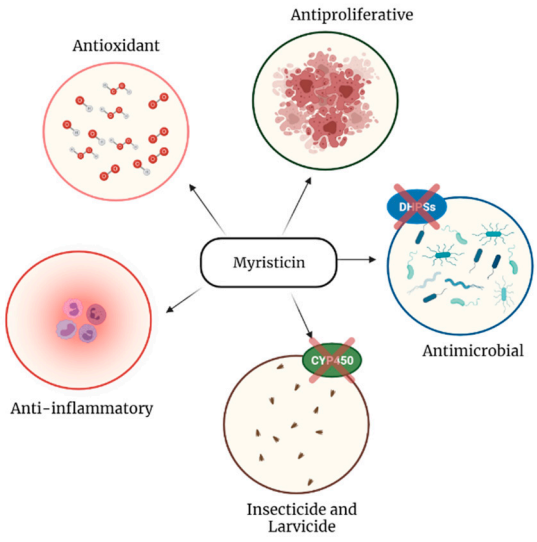

Figure 3. Graphical representation of the main biological activities of myristicin.

A publication on the aqueous extract of the aerial part of parsley (*Petroselinum crispum*) sought to investigate the antihypertensive activity of the plant. In vivo studies were performed with male albino rats, and an in vitro study used isolated thoracic aorta rings. The results show a potent vasorelaxant activity in aortic vascular rings, while in animals the extract induced a decrease in blood pressure parameters. More detailed studies showed that there was a blockage of calcium channels present in the vascular wall, but also suggest that other pathways may be involved in the antihypertensive effect such as, for example, increased nitric oxide synthesis [67].

The ability of myristicin to protect neurons from hypoxia-induced injuries was investigated. To conduct these assays, rat dorsal root ganglion (DRG) neurons were used. The results showed that myristicin reduced the viability of neurons when exposed to concentrations greater than 50 mM. However, at lower concentrations, it significantly increased cell viability in neurons when exposed to hypoxia, as it protected against hypoxic injury, not causing apoptosis. Complementary trials showed that myristicin decreased cleaved caspase-3 and bcl-2 levels in these hypoxia-induced neurons. Therefore, it was observed that it can reverse hypoxia-induced apoptosis in DRG neurons, affecting protein expression levels of molecules associated with apoptosis. Furthermore, myristicin decreased the malondialdehyde (MDA) content and the release of lactate dehydrogenase (LDH) enzymes, and positively regulates the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX). These enzymes are involved in the hypoxia process, and this modulation may have been responsible for the protective effect of myristicin [68].

A study conducted with myristicin isolated from the leaves of *Perilla frutescens* sought to investigate whether it induces MUC5AC gene expression and mucin production by airway epithelial cells. Tests were then performed with NCI-H292 cells (mucoepidermoid lung carcinoma), and the results showed that myristicin significantly inhibited gene expression and production of MUC5AC from NCI-H292 cells. Furthermore, it suppressed the production of mucin protein MUC5AC induced byepidermal growth factor (EGF), although it did not affect TNF-α-induced mucin protein MUC5AC production [69].

The pro-sexual effects of myristicin were also investigated. A study conducted with an aqueous extract of *Piper auritum* on Wistar rats showed that, in males with delayed ejaculation, the extract stimulated ejaculatory behavior and recovered the electromyographic activity of the pelvic muscles, participating in seminal emission and ejaculation. The most relevant action provoked by the PA was to increase the number of GMPEs (genital motor ejaculation pattern), thus restoring the ejaculatory capacity. The pro-sexual effects of *Piper auritum* produced on ejaculatory function are related to the participation of several neurotransmitter systems, and with a prominent role of 5-HT1A serotonin receptors. Although several other compounds are present in the extract, myristicin has a pro-serotoninergic action, indicating that it may be responsible for the action shown in the study [13,70].

An interesting publication discusses the anticonvulsant and inhibitory effects on glial activation of *Myristica fragrans* (nutmeg) extract. This material, containing about 11% myristicin, was tested in male NMRI rats that were induced to have seizures. Behavioral studies have shown that pretreatment with nutmeg extract effectively reduced seizure behavior, decreased cell death in the hypothalamus and improved glial activation [71].

Still regarding the actions of myristicin on the central nervous system, a study on the antidepressant potential of *Myristica fragrans* was published. Male Wistar rats were submitted to tests for analysis of antidepressant activity, using imipramine as a control and nutmeg extract as a test. Myristicin was not quantified in this extract; however, as it is the major chemical component of seeds, it is possible to correlate its presence with the results obtained. The extract exhibited an action very similar to the group treated with imipramine, demonstrating the potential antidepressant activity of the species [72].

Interestingly, the effects of myristicin on appetite were assessed through inhalation. In this study, male ddY mice inhaled essential oil of nutmeg and myristicin isolated from the oil. In both cases, there was an increase in appetite and weight gain in mice. However, this effect was lost when the compounds were administered daily to mice (after 8 days) [73].

Another study sought to assess the anti-obesity effect of nutmeg. The essential oil of *Myristica fragrans* showed a high binding activity to the CB1 cannabinoid receptor. Blocking this receptor reduces appetite and stimulates lipid metabolism, which would cause the anti-obesity effect. However, the author relates this activity to a synergism between myristicin and other compounds found in nutmeg essential oil [74].

The hepatocarcinogenic effects of myristicin were also evaluated by a study mentioned by Chen et al. (2016). For this, medium white turkey eggs with 22- to 24-day-old fetuses received myristicin injections (25.50 mg/egg), and measurement of DNA strand breaks

in fetal livers was performed. The 50 mg/egg dose induced a significant increase in DNA support breaks, in addition to reducing cell viability by 50%. This result indicates a genotoxic and carcinogenic potential [75].

A study published in 2013 sought to assess the molluscicidal activity of myristicin. To evaluate this effect, the snail vector *Lymnaea acuminata* was exposed to isolated myristicin, and to combinations of nutmeg and myristicin with piperonyl butoxide (PB) as a synergist. Both myristicin alone and nutmeg powder treatment were more potent when administered together with PB. Noteworthy, myristicin alone also showed molluscicidal activity [76].

These studies, despite dealing with little-explored activities of myristicin, demonstrate how much of this molecule's potential can still be explored through further research in various applications.

2.8. Future Perspectives

Myristicin is a molecule that is still poorly studied and is still not used in therapy, but the few available data point to a promising therapeutic potential. Considering the need of the pharmaceutical industry worldwide to obtain new treatments for diseases such as cancer and infections, it becomes relevant to clinically evaluate the use of substances that have shown therapeutic potential in preliminary studies. We encourage researchers to explore this promising molecule and carry out more detailed studies about its mechanism of action, especially on its anti-inflammatory, antiproliferative and antioxidant activites.

3. Materials and Methods

The study was carried out through a literature review. Pubmed, Lilacs and Scielo platforms were used to search for articles from the last 10 years, using the keywords myristicin and therapeutic properties. Through these platforms, 94 articles were found that conducted biological activity assays involving myristicin. However, only those in which the amount of myristicin in the sample was relevant were selected. A total of 68 references were included in this review.

4. Conclusions

Myristicin is an alkylbenzene present in the roots, fruits and aerial parts of several plant species used for centuries by countless populations, both as food and as natural medicine treatments. After this survey of literature data, it is evident that this is a promising molecule, with several properties, such as antimicrobial, insecticide, larvicide, psychoactive and therapeutics, among which the following stand out: antioxidant, anti-inflammatory and antiproliferative. These data demonstrate myristicin is a great potential to be explored in medicine to be used as a clinical treatment for pathologies, justifying the conduct of new studies focusing on its mechanism of action.

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4. CAPÍTULO 2: Artigo Publicado

Effects of Myristicin in Association with Chemotherapies on the Reversal of the Multidrug Resistance (MDR) Mechanism in Cancer.

O artigo deste capítulo apresenta resultados obtidos pelo nosso grupo de pesquisa acerca dos efeitos da associação entre miristicina e quimioterápicos estabelecidos na clínica em linhagem celular que superexpressa a P-gp. Ademais, como objetivos desta dissertação, foi realizado estudo *in silico* para identificar o alvo molecular da miristicina na proteína P-gp e investigar o perfil de biodisponibilidade da molécula. Trata-se, portanto, da investigação preliminar do mecanismo de ação deste composto. Este artigo foi publicado em 2022 na revista *Pharmaceuticals* (IF = 5,215).

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MDPI

Article

Effects of Myristicin in Association with Chemotherapies on the Reversal of the Multidrug Resistance (MDR) Mechanism in Cancer

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Abstract: A range of drugs used in cancer treatment comes from natural sources. However, chemotherapy has been facing a major challenge related to multidrug resistance (MDR), a mechanism that results in a decrease in the intracellular concentration of chemotherapeutic agents, resulting in reduced treatment efficacy. The protein most frequently related to this effect is P-glycoprotein (P-gp), which is responsible for promoting drug efflux into the extracellular environment. Myristicin is a natural compound isolated from nutmeg and has antiproliferative activity, which has been reported in the literature. The present study aimed to evaluate the effect of the association between myristicin and chemotherapeutic agents on the NCI/ADR-RES ovarian tumor lineage that presents a phenotype of multidrug resistance by overexpression of P-gp. It was observed that myristicin showed no cytotoxic activity for this cell line, since its IC50 was >1 mM. When myristicin was associated with the chemotherapeutic agents cisplatin and docetaxel, it potentiated their cytotoxic effects, a result evidenced by the decrease in their IC50 of 32.88% and 75.46%, respectively. Studies conducted in silico indicated that myristicin is able to bind and block the main protein responsible for MDR, P-glycoprotein. In addition, the molecule fits five of the pharmacokinetic parameters established by Lipinski, indicating good membrane permeability and bioavailability. Our hypothesis is that, by blocking the extrusion of chemotherapeutic agents, it allows these agents to freely enter cells and perform their functions, stopping the cell cycle. Considering the great impasse in the chemotherapeutic treatment of cancer that is the MDR acquired by tumor cells, investigating effective targets to circumvent this resistance remains a major challenge that needs to be addressed. Therefore, this study encourages further investigation of myristicin as a potential reverser of MDR.

Keywords: multidrug resistance; glycoprotein-P; myristicin; cisplatin; docetaxel

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1. Introduction

Cancer is the main public health problem in the world. In most countries, it is the leading cause of death before age 70. The Global Cancer Observatory (GLOBOCAN) estimates that, in 2020 alone, there were 19.3 million new cases of cancer (18.1 million excluding non-melanoma skin cancer) and almost 10 million deaths due to the disease. The most common is breast cancer with an estimated 2.3 million new cases (11.7%), followed by lung (11.4%), colorectal (10.0%), prostate (7.3%) and stomach (5.6%) cancer. Studies indicate that, in 2040, there may be a 47% increase in cases compared to 2020, reaching 28.4 million cases [1].

Cancer treatment seeks to cure, prolong and improve the patient's quality of life. Each type of cancer has a different clinical protocol, defined according to the location and tumor stage at diagnosis. The most frequently used therapy for the treatment of cancer is chemotherapy. The drugs used directly in the cell, interfere in processes of different phases of the cell cycle, and, therefore, they do not have great selectivity. Due to the low specificity, one of the main difficulties with this therapy is its ability to damage normal cells, causing high levels of toxicity to the patient's organism and resulting in several side effects [2–4].

Despite the availability of several drugs of different pharmacological classes, there is a major barrier in chemotherapy treatment represented by multidrug resistance (MDR). MDR occurs when, after starting the use of anticancer drugs, the cancer cells acquire resistance mechanisms that cause a decrease in treatment effectiveness in 90% of cases. These mechanisms can be classified into two groups: the classical pathway and the non-classical pathway. The non-classical pathway is characterized by mechanisms related to cellular metabolism (GST proteins, topoisomerase, growth factors) that alter the mechanism of drugs or interfere with their effect. The classical pathway is related to decreased intracellular drug concentration. The drug reaches the intracellular environment through plasma membrane transport channels. Therefore, mutations that alter the activities of these proteins, or increase their expression, can cause an increased efflux of drug into the extracellular environment, reducing its effects [5].

The main group of transport proteins related to MDR is called the ABC family (from ATP-binding cassette protein). These proteins transport substances through the plasma membrane by the hydrolysis of ATP, reducing its concentration intracellularly. The main representative is called MDR1, also known as P-glycoprotein (P-gp), and its overexpression is related to the failure of treatments for different types of tumors [5].

Studies conducted with several substances already used in therapy, such as calcium channel blockers, immunosuppressants and antimalarials, showed toxicities and little significant reversal of the MDR. Several classes of natural compounds have been studied over the last few years and showed a positive response in the modulation of chemoresistance. The origin of such classes varies from plants to microorganisms and marine sources. The mechanisms responsible for the activity of most of these compounds are related to the ability to block P-gp and other membrane channels, in addition to reducing the expression of these proteins, resulting in a synergistic effect when associated with drug antineoplastics. The main classes that show such a positive effect are carotenoids (xanthines, lycopene, lutein), flavonoids (rutin, quercetin, chalcones, among others), alkaloids (indoles, steroids, piperidine derivatives, quinolines), cardiotonic steroids, coumarins, peptides and terpenoids. These studies show that, as in the general setting of therapy with medicinal products, natural products are a source of great importance in obtaining substances with the potential to reverse chemotherapy-resistant cancers [5,6]. For this reason, compounds of natural origin have been researched not only in the search for new antineoplastics but also to assess their MDR modulating activity [6].

Myristicin (1-allyl-3,4-methylenedioxy-5-methoxybenzene) is an active natural substance from the alkylbenzene family, mainly found in nutmeg (*Myristica fragrans*). It is also present in parsley (*Petroselinum crispum*), in black pepper (*Piper nigrum*), carrots (Umbelliferae family) and plants of the Apiaceae family. Historically, nutmeg has been used to treat illness such as cholera, diarrhea, stomach cramps, nausea and anxiety. It is believed that myristicin is the compound responsible for the benefits of nutmeg, since it is the main compound in this spice. Several studies have been conducted with this molecule in the last decades, demonstrating some biological activities with therapeutic potential. These studies show that the myristicin has anti-inflammatory and antioxidant properties, antimicrobial activity against pathogenic bacteria and fungi, insecticide and larvicide effects and also antiproliferative activity against several cancer cell lines [7].

Although some studies have addressed the antiproliferative activity of myristicin, none of them investigated its potential mechanism of MDR reversal. Considering the

similarity of the myristicin molecular structure with compound apiole recently studied by our research group (Figure 1) and whose results indicated it is a potential reverser of MDR [8], the aim of this research was to investigate the effects of myristicin in association with chemotherapeutic agents in a multidrug-resistant cell line, as well as its binding affinity for P-gp efflux pump.

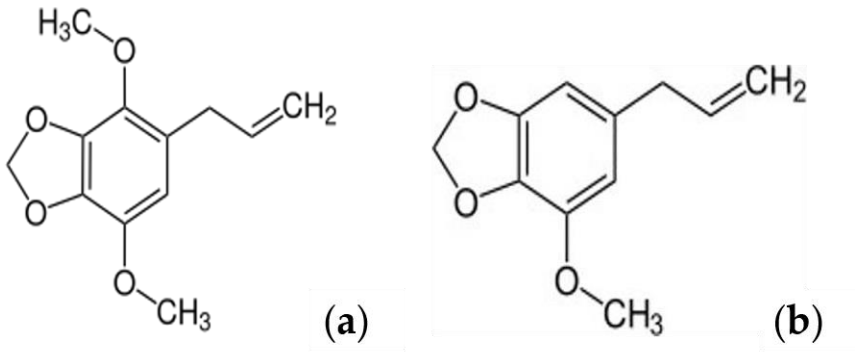


Figure 1. Molecular structure of the compounds apiole (**a**) and myristicin (**b**).

2. Results

2.1. Association of Myristicin with Cisplatin and Docetaxel on NCI/ADR-RES Cell Line

The cytotoxic activity of the myristicin compound, as well as the chemotherapeutic agents cisplatin and docetaxel, was evaluated against the NCI-ADR/RES tumor line. From the curve obtained, IC50 values were calculated. The myristicin compound did not show cytotoxic activity, since its IC50 was >1 mM for this strain (Table 1). The chemotherapy drugs cisplatin and docetaxel, when administered alone, presented an IC50 of 215.60 ± 6.36 and 15.04 ± 1.36 μM, respectively (Tables 1 and 2).

Table 1. Cytotoxic activity expressed as IC50 (μM) of myristicin and cisplatin and their association in the NCI/ADR-RES [a] lineage after 48 h of exposure.

	Myristicin	Cisplatin	Myristicin 1 mM + Cisplatin	Myristicin 500 μM + Cisplatin	Myristicin 100 μM + Cisplatin
IC50 [b]	>1000	215.60 ± 6.36	144.70 ± 2.44 **	176.15 ± 21.61	259.86 ± 5.82
CRI [c]	n.a.	n.a.	1.49	1.22	0.67

[a] NCI/ADR-RES: multidrug-resistant ovarian adenocarcinoma cell line; [b] IC50: sample concentration required (μM) to inhibit 50% of cell viability and calculated by non-linear regression analysis using ORIGIN 7.5® (OriginLab Corporation, Northampton, MA, USA); [c] CRI: concentration reduction index calculated as IC50 cisplatin/IC50 cisplatin + myristicin; n.a.: not applied. Statistical analysis by one-way ANOVA followed by Tukey's test (** $p < 0.01$ related to cisplatin). The experiments were performed in biological triplicate and experimental duplicate. Concentration tested: 1.6–1000 μM (myristicin) and 5–332 μM (cisplatin).

Table 2. Cytotoxic activity expressed as IC50 (μM) of myristicin and docetaxel and their association in the NCI/ADR-RES [a] lineage after 48 h of exposure.

	Myristicin	Docetaxel	Myristicin 1 mM + Docetaxel	Myristicin 500 μM + Docetaxel	Myristicin 100 μM + Docetaxel
IC50 [b]	>1000	15.04 ± 1.36	3.69 ± 0.00 **	10.90 ± 0.04 *	22.23 ± 2.78
CRI [c]	n.a.	n.a.	4.08	1.38	0.83

[a] NCI/ADR-RES: multidrug-resistant ovarian adenocarcinoma cell line; [b] IC50: sample concentration required (μM) to inhibit 50% of cell viability and calculated by non-linear regression analysis using ORIGIN 7.5® (OriginLab Corporation, Northampton, MA, USA); [c] CRI: concentration reduction index calculated as IC50 docetaxel/IC50 docetaxel + myristicin; n.a.: not applied. Statistical analysis by one-way ANOVA followed by Tukey's test (** $p < 0.01$; * $p < 0.05$, related to docetaxel). The

experiments were performed in biological triplicate and experimental duplicate. Concentration tested: 1.6–1000 μM (myristicin) and 0.4–25 μM (docetaxel).

Although it did not show cytotoxic activity in isolation (Figure 2) in the resistant tumor line, myristicin corroborated the potentiation of the effect of chemotherapeutics (Figures 3 and 4), demonstrated by the reduction of IC50 values found for them when they were associated with myristicin (Tables 1 and 2). With regard to cisplatin, this reduction was significant for the highest concentration of myristicin associated with the chemotherapy agent (1 mM). For docetaxel, it was observed that the two highest concentrations (500 μM and 1 mM) reduced the IC50 value. At the concentration of 1 mM, the CRI obtained for cisplatin was 1.49, which means that myristicin potentiated the effect of this chemotherapy agent by 1.49 times and reduced the IC50 value by 32.88%. At the same concentration, the CRI obtained for docetaxel was 4.08, demonstrating that myristicin potentiated the effect of this drug by 4.08 times and reduced the IC50 value by 75.46%. These CRI values above 1 indicate that there was synergism between myristicin and the chemotherapeutic agents used. At the lowest concentration, myristicin presented CRI < 1 for cisplatin and docetaxel, but this apparent worsening of effect was not statistically significant. Figures 3 and 4 show that the effect of association of 100 μM myristicin and chemotherapeutics was not different from the effect of the chemotherapeutics alone.

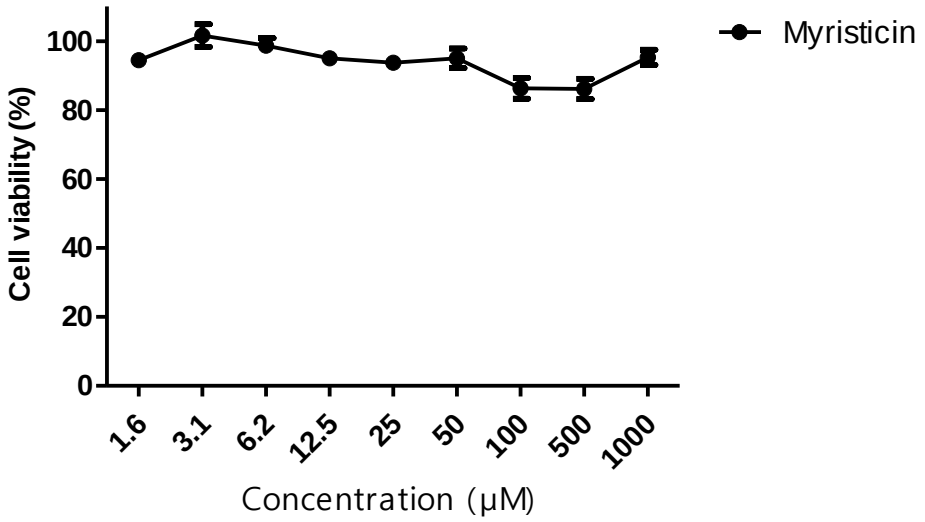


Figure 2. Cytotoxic activity of the myristicin compound. Assay performed with the NCI/ADR-RES tumor line, relating the percentage of cell viability versus concentration of myristicin, after 48 h of incubation.

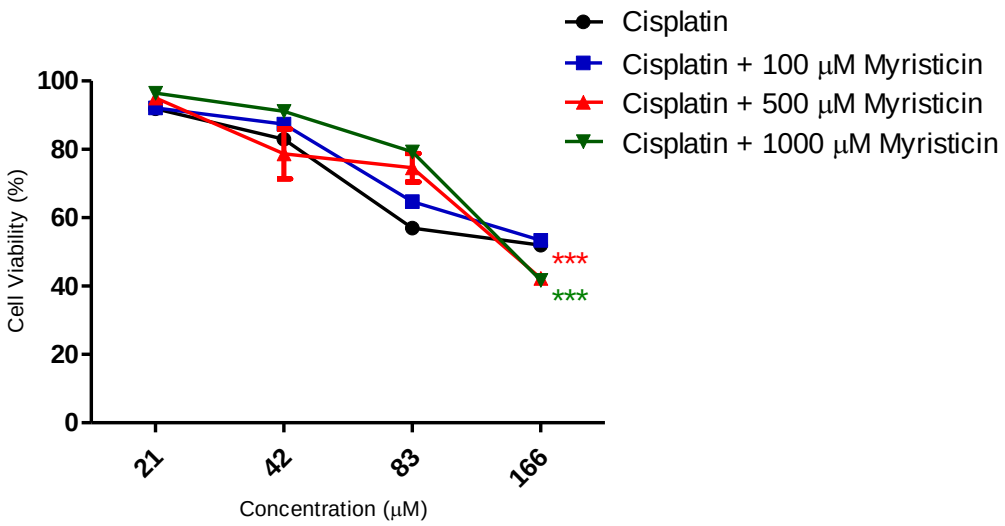


Figure 3. Cytotoxic activity of the compound myristicin in association with the chemotherapy drug cisplatin. Assay performed with the NCI/ADR-RES tumor line, relating the percentage of cell viability versus concentration of cisplatin, after 48 h of incubation.

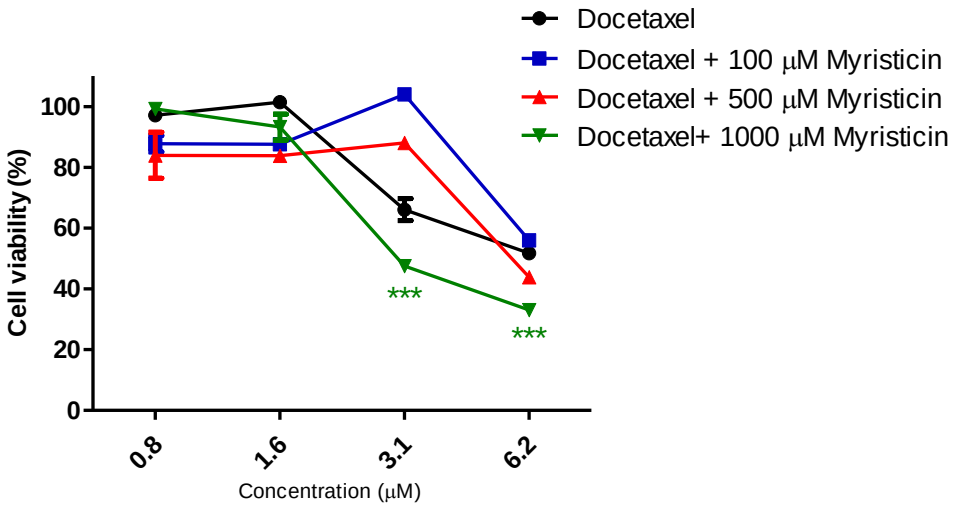


Figure 4. Cytotoxic activity of the compound myristicin in association with the chemotherapy drug docetaxel. Assay performed with the NCI/ADR-RES tumor line, relating the percentage of cell viability versus concentration of docetaxel, after 48 h of incubation.

Considering the relevant IC50 and CRI values obtained for the highest concentration evaluated, there was an urgent need to prove whether the effect of the association between myristicin and chemotherapeutics was synergistic. The analysis of isobolograms was used to evaluate the interactions between myristicin and chemotherapeutics (Figure 5). The isobol represented in blue in the graph indicates the predictive additive effect of the association between myristicin and chemotherapeutics. The curve obtained below this isobol and represented in red indicates that the real effect of this association was synergistic, which means the resulting action is greater than the simple sum of the isolated effects of each one of them.

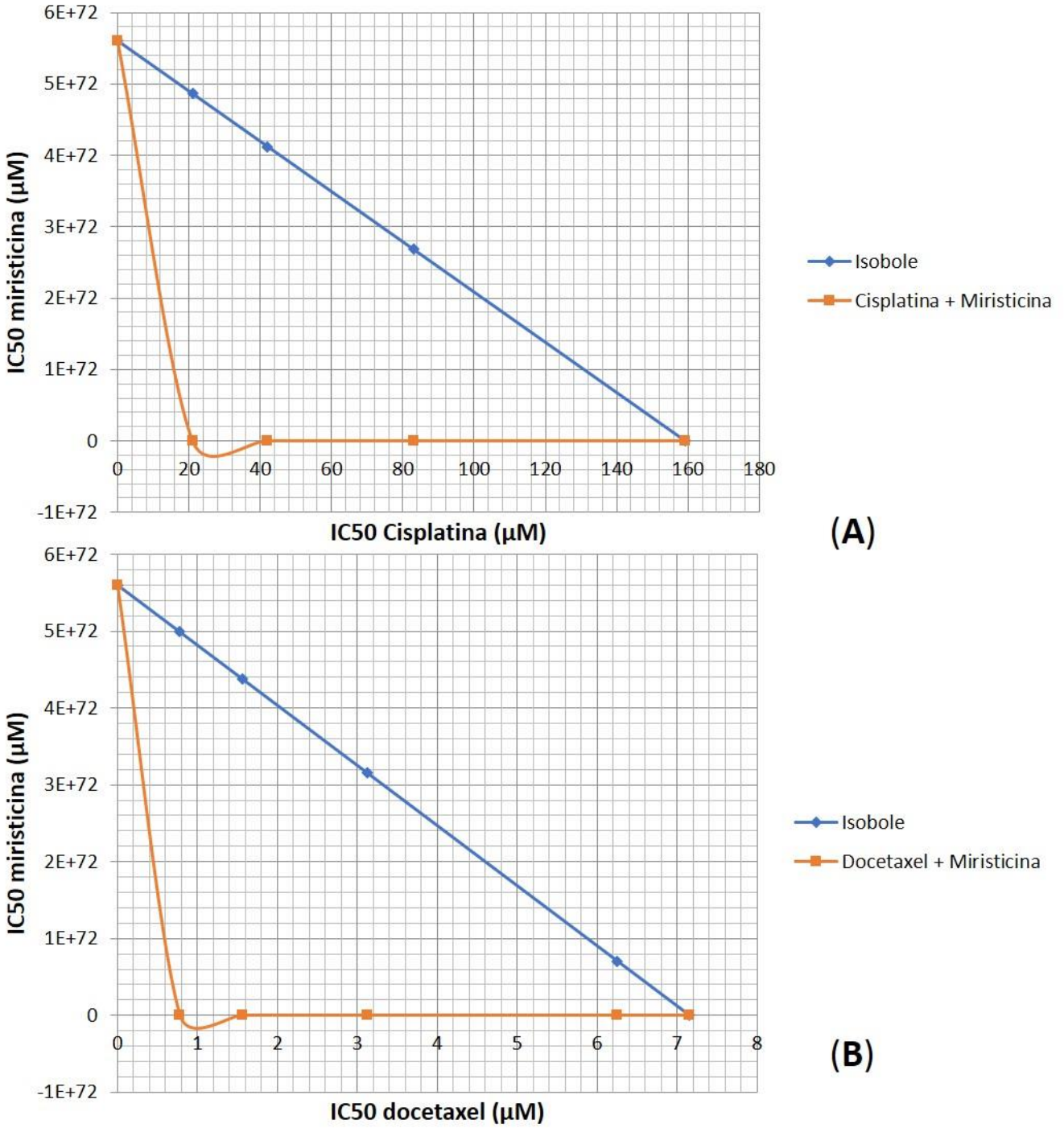
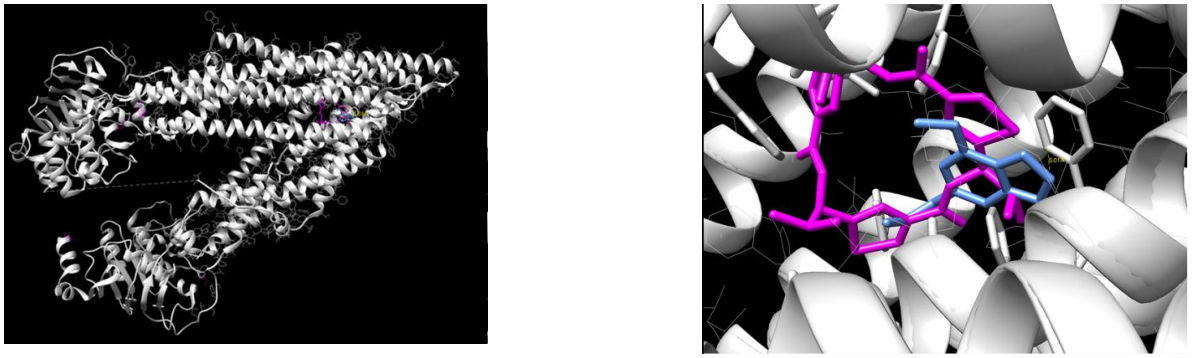


Figure 5. Isobolograms representing the synergistic interaction between myristicin and the chemotherapeutic agents cisplatin (**A**) and docetaxel (**B**) in the NCI/ADR-RES multidrug-resistant ovarian line after 48 h of incubation.

2.2. Molecular Docking

Molecular docking was performed to evaluate the binding capacity of myristicin to P-gp. The result demonstrated that the myristicin molecule is able to bind at the center of the P-gp action site (Figure 6). This binding occurred in a very similar way to its natural ligand using a binding energy of −6.81 kcal/mol, which is considered an adequate value for a stable binding.



(**A**) (**B**)

Figure 6. Graphic representation of the binding mode of P-glycoprotein and myristicin (**A**). Detail of the protein showing its sites occupied by myristicin and the natural ligand (**B**). The ligands are represented by pink (natural ligand) and blue (myristicin); P-gp is represented bygray.

The chemical name of the ligand is (4R,11R,18R)-4,11,18-tri(propan-2-yl)-6,13,20-triselena-3,10,17,22,23,24-hexaazatetracyclo[17.2.1.1~5,8~.1~12,15~]tetracosa-1(21),5(24),7,12(23),14,19(22)-hexaene-2,9,16-trione.). Through the UCSF Chimera program, it was possible to verify which myristicin residues are able to bind to P-gp amino acids. The strongest binding found was between the C11 ligand of myristicin and the amino acid Phe 728 of P-gp, with a distance of 5.01 Å. This distance is very similar to that of the natural P-gp ligand with this amino acid. In addition, because it is a connection between two rings, it is considered very strong.

2.3. In Silico PK

According to the study led by Lipinski, there are five aspects of the molecule that must be considered to predict its permeability through biological membranes and, therefore, assess its pharmacokinetics and bioavailability [9]. Table 3 shows data obtained from myristicin and the values recommended by the rule.

Table 3. Predicted pharmacokinetic parameters for myristicin compared to Lipinski's criteria.

Parameter	Criteria	Myristicin
LogP	2 a 5	2.44
Molecular Weight	<500 g/mol	192.21 g/mol
Hydrogen-bond acceptors (HBAs)	<10	3
Hydrogen-bond donors (HBDs)	<5	0
Number of rotatable bonds	<10	3
Topological polar surface area (TPSA)	40 a 100 Â²	27.70

The logP parameter is also called the oil–water partition coefficient. The ideal parameter for a drug is to have a logP lower than 5. Therefore, the logP of myristicin indicates that it has a good affinity for oil and water, favoring permeation through plasma membranes and reflecting good gastrointestinal absorption. In addition, drugs must have a molecular weight of less than 500 g/mol to have good permeability, as a very bulky molecule is more difficult to transport. Myristicin has a suitable molecular weight. Interactions with hydrogen occur mainly in aqueous media. The more ionic bonds the molecule makes with water, the more unfavorable its transport through membranes (which have the lipid component). According to Lipinski, the molecule must have less than 5 donors and less than 10 hydrogen acceptors to be a good drug; myristicin presented 0 and 3, respectively, fitting these parameters. The number of rotatable bonds can influence the bioavailability and binding potency, as the molecule must assume a fixed conformation to

pass through membranes. The fewer rotatable bonds, the stiffer the molecule. A good drug candidate should have less than 10. Myristicin has only three, which is within the proper parameters.

3. Discussion

Cancer remains among the most serious diseases, although its treatment options are well established. There are many types of cancer treatment, depending on the type and at what stage it is. Chemotherapy is often used to treat cancer and well-designed drug delivery regimens have been effective in treating cancer and causing fewer adverse effects [10]. In some cases, the treatment plan may use a combination of methods to have maximum therapeutic effectiveness [11].

Platinum-derived chemotherapeutics are used as the main treatment for ovarian cancer despite their serious adverse effects and development of resistance. In clinical trials, cisplatin is often selected because of its strong antitumor activity, but its adverse effects include renal toxicity, nausea and vomiting. Therefore, to avoid renal toxicity, urine volumes must be monitored, and large-dose infusion is mandatory in cisplatin-based chemotherapy. The molecular mechanism of cisplatin-induced apoptosis involves activation of tumor protein 53 (p53), phosphorylation of the activator protein component (AP-1) leading to cell cycle arrest through stimulation of p21 and downregulation of cyclins and cyclin-dependent kinases [12].

Docetaxel is a semi-synthetic taxane that inhibits microtubule depolymerization, arresting cells in the G2/M phase of the cell cycle and induces bcl-2 phosphorylation, thus promoting a cascade of events that ultimately leads to apoptotic cell death [13]. It is approved for the treatment of breast and lung cancer and is indicated for the treatment of metastatic ovarian carcinoma after failure of first-line chemotherapy. Docetaxel is an important anticancer drug that can induce hypersensitivity reactions, such as blood hypereosinophilia, leading to deleterious treatment interruptions. Blood hypereosinophilia can be a potentially lethal biological sign of late visceral hypersensitivity reactions [14].

Although most ovarian tumors initially respond to chemotherapy, tumors often arise as a result of the expansion of clones with innate or acquired resistance, which later develop into recurrent tumors [15]. Certain tumor cells acquire a chemotherapy-resistant phenotype, resulting in treatment difficulties [10]. Statistical data show that more than 90% of cancer patient mortality is attributed to drug resistance [16]. Multidrug resistance (MDR) is a prominent mechanism of resistance to clinically approved therapies in ovarian cancer patients, and P-gp is one of the best-studied proteins involved in MDR.

Historically, natural products have played a key role in drug discovery, especially for cancer and infectious diseases [17]. The data show that there are still few drug discovery programs based on natural products in pharmaceutical companies, although they are a promising source of new drugs. Even so, drugs produced from natural substances are numerous, as they represent about 70% of all drugs approved for therapeutic use in the last four decades. Natural compounds have been one of the main sources of drug production since the beginning of time, giving rise to drugs of different therapeutic classes. Therefore, since the main source of new medicines are natural products, it is necessary to carry out research to discover new treatments from sources that are little explored [18].

The interest in investigating the in vitro effects of the association between myristicin and chemotherapeutics in an ovarian tumor cell line resistant to multiple drugs arose from the results obtained and published by our group for the apiole molecule, which differs from myristicin only by the presence of one more methoxy group [8]. It is a phenylpropanoid found mainly in parsley (Apiaceae) and in species of the families Lauraceae and Piperaceae. Apiole alone has no significant cytostatic effect on cell lines of resistant tumors. However, its association with the chemotherapy drugs vincristine and doxorubicin presented a synergistic effect, and this mechanism would be related to the affinity of the molecule to the active site of P-gp, antagonizing its action to promote drug efflux into the extracellular environment [8].

It has already been described in the literature that a higher number of methoxy groups that accept hydrogen bonds at the terminal of phenolic rings is favorable for the inhibitory activity of P-gp. Studies suggest that this chemical group acts as an additional acceptor of hydrogen bonding, implying a greater affinity for the active site of P-gp and resulting in potent inhibition of this protein [19]. However, although myristicin contains one methoxy group less than apiole, the binding energy observed in the in silico study was more negative than that demonstrated by apiole, suggesting that the binding is even more stable.

The molecular docking results indicated that myristicin is able to bind and block P-gp. This mechanism may be related to the improvement in the efficacy of chemotherapeutic agents, as it would allow a reduction in the dose of drugs and limit their cytotoxicity.

Another important tool for the study of drug interactions has been the construction of isobologram-type graphs. These are constructed from the IC50 values by generating the response curves and are used to prove synergism between molecules. The probability of interaction occurring after combining drugs increases, which can affect effectiveness and cause safety issues. If the combination of clinical drugs is much more effective than the sum of their individual effects, synergism between the drugs results, but if the therapeutic effect is weakened, the effect is one of antagonism. Synergy means that two or more components are mixed together and the effect is greater than the sum of the effects of the individual components when applied alone, thus producing the effect "1 + 1 > 2". Within the scope of isobolographic analysis, antagonism occurs when the IC50 is greater than the expected concentrations of drugs A and B that are necessary to produce the target effect [20].

The results obtained in this study show that myristicin at a concentration of 1 mM potentiated the cytotoxic effects of chemotherapeutic agents, as evidenced by the decrease in IC50 values obtained and CRI values greater than 1. This effect is synergistic, since the effect of the association is greater than the sum of the effects of individual components when applied alone. It is believed that this potentiation of chemotherapeutic effects is primarily due to the blockage of the MDR-related efflux pump. Clinical co-administration of drugs that inhibit the efflux promoted by transmembrane proteins in combination with anticancer drugs is considered a treatment modality to overcome MDR in anticancer therapy [21]. Our hypothesis for myristicin action is that, by blocking the extrusion of chemotherapeutic agents, it allows these agents to freely enter cells and perform their functions, stopping the cell cycle.

Considering the potential clinical use of myristicin in association with chemotherapeutic drugs, we evaluated in silico some parameters for druglikeness according Lipinski's criteria. For a molecule to be considered a good candidate for a drug, it must present at least four values within the recommended parameters [10]. Through the computational model used, it was possible to verify that myristicin fits in five of these parameters, with the exception of TPSA. Therefore, this study indicates that myristicin has adequate pharmacokinetics to become a drug, as it has good permeability through membranes and consequent bioavailability.

4. Material and Methods

4.1. Compound

The compound myristicin was purchased at Sigma-Aldrich (Burlington, MA, USA) using the code 09237.

4.2. Cell Culture

The resistant ovarian tumor cell line (NCI/ADR) was obtained from the National Cancer Institute at Frederick MA-USA. Stock cultures were grown in complete medium: RPMI 1640 medium (Sigma-Aldrich, Burlington, MA, USA) supplemented with 5% fetal

bovine serum (LGC Biotecnologia, Cotia, SP, Brazil) and 1% penicillin:streptomycin (LGC Biotecnologia, Cotia, SP, Brazil) mixture (1000 U·mL^{-1}:1000 μg·mL^{-1}) at 37 °C with 5% CO_2.

4.3. Cytotoxicity Assay of Myristicin and the Chemotherapeutic Agents Cisplatin and Docetaxel

For this test, the colorimetric method 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, Burlington, MA, USA) was used, which indirectly evaluates the cell viability by the mitochondrial enzymatic activity of living cells. Briefly, 5000 cells were inoculated in 100 μL of complete medium in each well of the 96 wells, which was incubated for 24 hours at 37 °C in a 5% CO_2 atmosphere and a humid environment. After 24 hours, myristicin was diluted in DMSO (Synth, Diadema, SP, Brazil) stock solution at a concentration of 0.1 g/mL, (100 μL/well) in triplicate, and, subsequently, the plate was incubated for 48 h at 37 °C in atmosphere of 5% CO_2 and humid environment. As a positive control, the chemotherapeutic agents, cisplatin (C-platin, Blau Farmaceutica, Cotia, SP, Brazil) at concentrations from 10 to 333 μM and docetaxel (Eurofarma, São Paulo, SP, Brazil) at concentrations from 0.8 to 25 μM (100 μL/compartment), were tested in triplicate. These concentrations were based on previous studies conducted by the research group. After 48 h of treatment, the treated cells were then stained with MTT. After 4 h (incubation period), the dye was solubilized with DMSO, and the absorbance data were analyzed in a microplate reader (Promega) and compiled in the elaboration of graphs relating the percentage of cell growth with the concentration of the sample. Through the Origin® software, the linear regression of the curves obtained with the averages of the percentage of viable cells in comparison to the DMSO negative control and the IC50 was calculated (concentration that reduces 50% of the cell viability). This parameter is used to determine the cytotoxic potency of the sample.

4.4. Association Assay between Myristicin and Chemotherapeutic Agents (Cisplatin and Docetaxel)

After obtaining the IC50 values of myristicin and the chemotherapeutic agents docetaxel and cisplatin, the cells received joint treatments with myristicin–docetaxel and myristicin–cisplatin, respectively, during 48 h of incubation and, at the end of this treatment time, the cells were stained with MTT and absorbance data were analyzed and compiled into graphs relating the percentage of cell growth to the concentration of the sample, as already described.

4.5. Molecular Docking

The myristicin molecule was designed using the OpenBabel tool, considering 3D parameters and pH 7. The result was converted to mol.2 format for use in docking. The target protein, P-glycoprotein, was selected from the RCSB PDB (Protein Data Bank), access code 3G60, chain A. Finally, the protein and the ligand were submitted to the SwissDock platform, which was able to predict the binding energy and position of myristicin in relation to P-gp.

The UCSF Chimera 1.15 program was used for data processing in order to verify the positioning of the molecule in P-gp and the overlap with the ligand. This program was also used to calculate the binding in the P-gp amino acids and the distance between the ligand and the protein.

4.6. In Silico PK

Pharmacokinetic parameters were calculated based on the molecular structure of myristicin and compared to the criteria established by Lipinski. The Molinspiration platform [22] was used to design the molecule and then obtain parameters related to physicochemical properties, solubility, lipophilicity.

5. Conclusions

Myristicin alone has no cytotoxic effect toward the resistant ovarian tumor lineage NCI/ADR-RES, but it promotes a synergistic effect when associated with the chemotherapy drugs cisplatin and docetaxel, reducing the chemotherapeutic concentration necessary to cause a 50% decrease in cell viability.

Considering the great obstacle in the chemotherapeutic treatment of cancer that is MDR acquired by tumor cells, investigating effective targets to circumvent this resistance remains an important challenge that needs to be solved. Therefore, this study encourages the continuation of the investigation of myristicin as a potential compound for the reversal of MDR.

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5. CAPÍTULO 3: Artigo em Elaboração

Investigation of the effect of myristicin associated with chemotherapeutics in a three-dimensional model of multidrug resistant cell culture.

Este capítulo compreende os resultados acerca da investigação dos efeitos farmacológicos da miristicina na reversão do mecanismo de reversão da MDR através de ensaio em cultura tridimensional de células, que mimetiza mais fidedignamente um tumor, por induzir interações célula-célula e célula-matriz e permitir a disposição das células em camadas, conferindo barreira biológica à difusão de fármacos. Pretende-se, ainda, investigar o mecanismo de ação desta associação miristicina-quimioterápicos nos processos de morte celular programada, ciclo celular e no mecanismo de extrusão de fármacos pela P-gp. Para fins de redação da presente dissertação, foi considerado o template da revista Natural products Research, no estilo *Short communication*.

Investigation of the effect of myristicin associated with chemotherapeutics in a three-dimensional model of multidrug resistant cell culture.

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Investigation of the effect of myristicin associated with chemotherapeutics in a three-dimensional model of multidrug resistant cell culture.

Cancer represents one of the main public health problems in the world and is one of the most frequent causes of early death. Many of the drugs used in current therapy have their origin in natural products, which represent a promising source of new drugs. However, there is still a major barrier to treatment represented by multidrug resistance (MDR). One of the main mechanisms involved in MDR is the increase in P-glycoprotein expression, resulting in reduced treatment efficacy. This work sought to evaluate the ability of myristicin (a compound of natural origin known for many biological effects) to inhibit P-gp activity. To find out, 3D cell culture techniques were used, in which multidrug resistant ovarian cells (NCI/ADR-RES) were treated with myristicin in association with chemotherapeutic drugs. The results demonstrated that myristicin is able to reduce the dose of chemotherapeutic drugs necessary to control the proliferation of the cell line in a three-dimensional model.

Keywords: MDR; natural products; cancer; myristicin; chemotherapy

1. Introduction

Cancer is one of the main health problems in the world. Worldwide, an estimated 19.3 million new cancer cases (18.1 million excluding non-melanoma skin cancer) and nearly 10.0 million cancer deaths (9.9 million excluding non-melanoma skin cancer) occurred in 2020 [1]. In the case of anticancer therapy, most of the drugs introduced in therapy in recent decades have their origin in natural products [2].

Despite the availability of several treatments, there is a phenomenon called multidrug resistance (MDR) that occurs when, after initiating the use of anticancer drugs, cancer cells start to acquire resistance mechanisms that cause a decrease in the effectiveness of the treatment in 90% of cases. The protein most often associated with MDR is called P-glycoprotein, or P-gp, and

it promotes the exit of drugs to the extracellular environment. Several classes of natural compounds have been studied over the last few years and have shown a positive response in modulating chemoresistance [3].

In this work, we evaluated the ability of myristicin to potentiate the effect of standard chemotherapeutic drugs used in the treatment of ovarian cancer. Myristicin is a molecule of natural origin that is known for its anti-inflammatory and antioxidant properties, antimicrobial activity against pathogenic bacteria and fungi, insecticidal and larvicidal effects and also antiproliferative activity against various cancer cell lines [4]. In previous studies, the association of myristicin and two chemotherapeutic drugs (cisplatin and docetaxel) was performed in a two-dimensional cell culture format, using a resistant cancer cell line, and promising results were obtained. [5].

However, there is a more effective cell culture technique, which is the three-dimensional format. Compared to 2D cell culture models, 3D culture is able to accurately mimic some characteristics of solid tumors, such as their spatial architecture, physiological responses, secretion of chemical mediators, gene expression patterns, and drug resistance mechanisms [6].

In this work, a technique called spheroids, which consists of a three-dimensional arrangement composed of three layers, was used. The outermost zone is the proliferative zone, where cells are quickly multiplying. The middle layer is called the quiescence zone, where cells are metabolically active but not multiplying. And the innermost layer is the necrotic zone, an area of hypoxia and poor in nutrients, where cells have entered the process of cell death. [6].

Therefore, the present work aims to investigate whether the results obtained in the 2D culture [5] will be reproduced using the 3D culture technique.

2. Results and Discussion

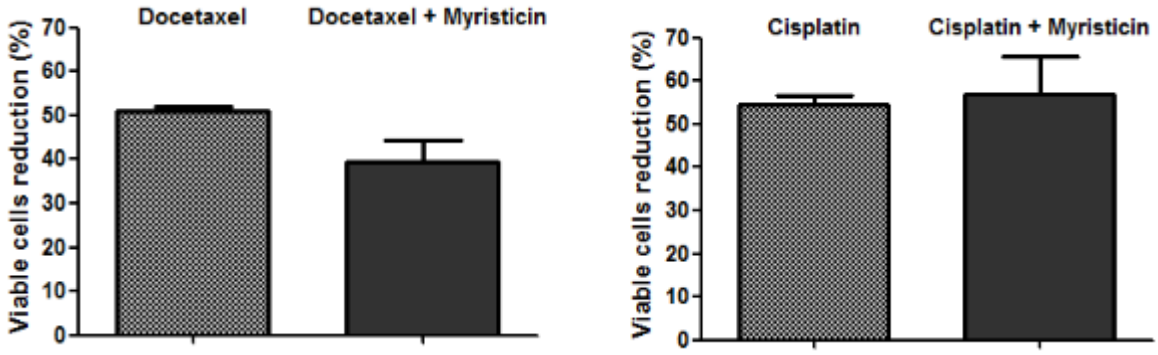
The spheroid formation assay was used to assess the ability of myristicin to inhibit the replication of tumor cell lines and prevent spheroid formation, that is, the generation of a new tumor.

The association of myristicin with **cisplatin** already proved to be very promising. Based on the previous studies conducted in bidimensional model of cell culture [5], the IC_{50} of cisplatin was 215µM when isolated, and 145µM when associated with 1000µM of myristicin. The results obtained for **docetaxel** association also demonstrated improved efficacy. In the same study [5], the IC_{50} of docetaxel alone was 15µM and 4µM when associated with 1000µM of myristicin. So based on this study, the concentrations were chosen to treat the tridimensional spheroids. Also, the tests were conducted on the same cell line, NCI/ADR-RES, which is a resistant ovarian cancer cell line that expresses high levels of the Multi-Drug Resistance 1 (MDR1) protein – also known as P-glycoprotein.

As a result, we found that when associated to myristicin, the cisplatin concentration was reduced in 32.56%, the docetaxel concentration was reduced in 73.33% and the cytotoxic effect was maintained (Figure 1 A and B).

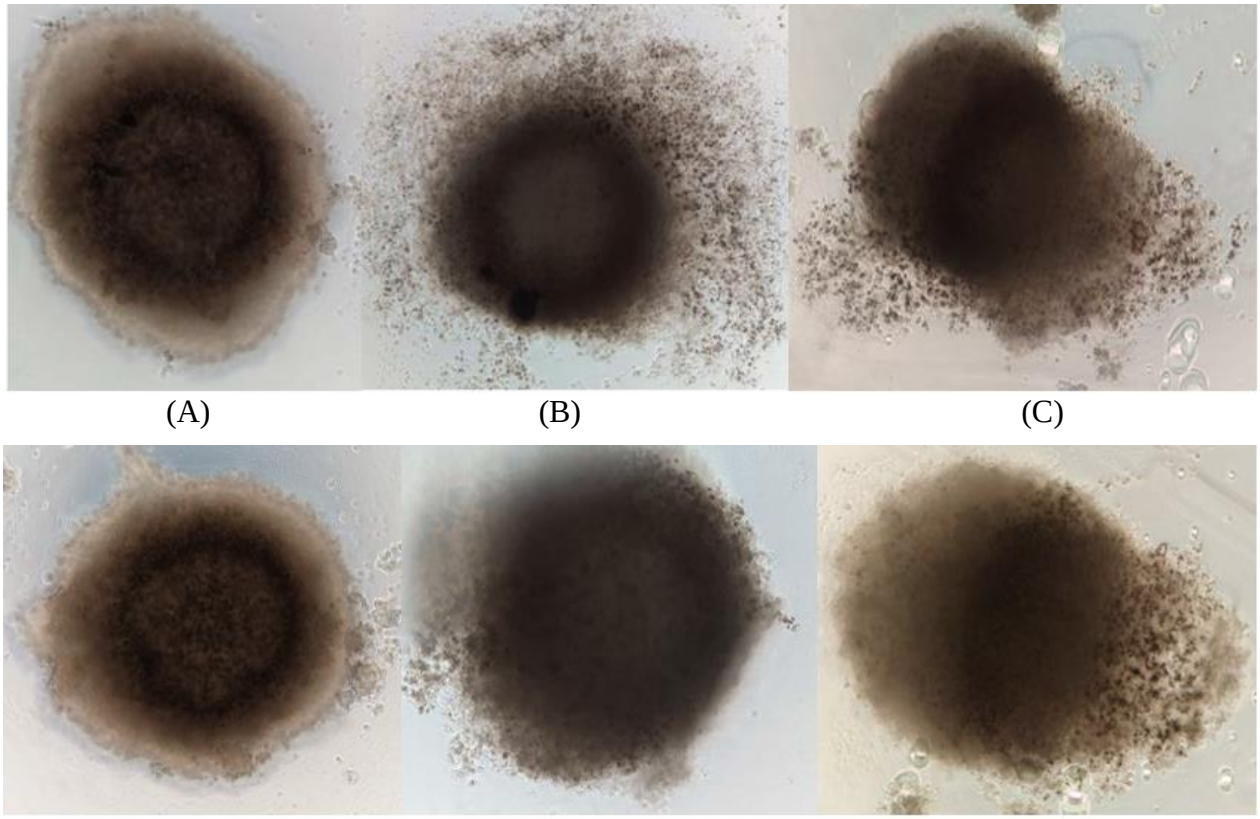
In addition to promoting a reduction in cell viability in a very similar way, the associations proved to be more capable of interfering with the arrangement of the spheroid layers. In the images below, we notice that the untreated spheroid has the three layers of the structure well defined (Figure 2 A and D). In the spheroids treated only with cisplatin and docetaxel, there were changes mainly in the proliferative zone, which is the most external and permeable layer (Figure 2 B and E, respectively). However, in the spheroids treated with the association between myristicin-chemotherapeutic drugs, the entire spheroid was affected, so that it was no longer

possible to visualize any layer separately (Figure 2 C and F). It shows that myristicin is highly capable of permeating the matrix of a solid tumor and reaching its cells.



(A) (B)

Figure 1. Reduction of cell viability promoted by docetaxel 15µM and by the association docetaxel 4µM – myristicin 1000µM. T-test (A). Reduction of cell viability promoted by cisplatin 215 µM and by the association cisplatin 145 µM – myristicin 1000µM. T-test (B).



(A) (B) (C)

(D) (E) (F)

Figure 2. Images of the spheroids after 48 hours without treatment (A and D); images after 48 hours of treatment with docetaxel (B) and association of myristicin-docetaxel (C); 48 hours after treatment with cisplatin (E) and association of myristicin-cisplatin (F).

These results in three-dimensional model corroborated the previous study conducted in bidimensional cell culture model. Both doses of cysplatin and docetaxel (alone and associated with myristicin) were able to reduce approximately 50% of cell proliferation, as in the 2D assay. This means that the IC_{50} doses found in 2D were able to permeate the solid tumor layers of the 3D spheroids. Once again our results prove that the association with myristicin may implicate in reduction of chemotherapeutic drugs concentration, which brings great benefit as it is possible to maitain efficacy and minimize side effects.

The culture of cells in 2D format is limited, once we study the drug association effect directly on the cell in a single layer, which is very far from the complexity of the structure of a real tumor. In this regard, the results obtained with the 3D culture are promising. They demonstrate that myristicin is able to permeate all layers of the spheroid, as it was shown in the images of the treatment. The association also promotes a reduction in cell viability even when they are arranged in a complex format, similar to what would be seen in a real tumor, so it is expected that myristicin would be able to reduce tumor size and bring back success in the treatment of patients with resistant cancer. These results encourage further research with myristicin, with the aim of better detailing its mechanism of action and its effects on living organisms.

3. Experimental

The resistant ovarian tumor cell line (NCI/ADR) was obtained from the National Cancer Institute at Frederick MA-USA. Stock cultures were grown in complete medium: RPMI 1640

medium (Sigma-Aldrich, Burlington, MA, USA) supplemented with 5% bovine serum (LGC Biotecnologia, Cotia, SP, Brazil) and 1% penicillin:streptomycin (LGC Biotecnologia, Cotia, SP, Brazil) at 37 °C with 5% CO2, in a humid environment. Magnetic nanoparticles (1 μL for each 1000 cells) were added to the cells to induce the formation of a spheroid. After 48 hours of incubation, the magnetic drive was removed and the cells were treated with DMSO (Synth, Diadema, SP, Brazil), myristicin and chemotherapy drugs (docetaxel and cisplatin) for 48 hours. After the treatment, the MTT salt (MTT, Sigma-Aldrich, Burlington, MA, USA) was added for analysis of cell viability by colorimetry, and the reading was performed in a Glomax microplate reader (Promega) at 576nm. Results were expressed as standard deviation (SD) of two independent experiments conducted in duplicate. Statistical analyses were performed with the GraphPad Prism 5 software. T-test was applied.

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6. CONCLUSÃO

Os efeitos biológicos da miristicina são bem relatados na literatura. Dentre eles, destacam-se as atividades antimicrobiana, antioxidante, anti-inflamatória, inseticida e larvicida. Além disso, ela apresenta atividade antiproliferativa frente a várias linhagens celulares. Contudo, não havia, até então, estudo acerca da ação citostática da miristicina em linhagem tumoral resistente e na reversão da MDR.

Os resultados prévios obtidos pelo grupo de pesquisa demonstram que a miristicina sozinha não é capaz de promover efeito citostático na linhagem tumoral resistente avaliada (NCI/ADR-RES); contudo, esta substância promove efeito sinérgico quando associada aos quimioterápicos cisplatina e docetaxel. Neste trabalho, os estudos preliminares de investigação do mecanismo de ação *in silico* demonstraram que ela seria capaz de promover bloqueio da P-gp, que é uma proteína de efluxo relacionada à MDR. Por se ligar nesta proteína, a miristicina impediria que os quimioterápicos sejam bombeados para o meio extracelular, diminuindo a concentração quimioterapêutica necessária para reduzir 50% da viabilidade celular. A molécula também se encaixou nos 5 parâmetros de Lipinski, indicando que ela possui boa biodisponibilidade, e, portanto, seria uma boa candidata a fármaco.

Além de investigar o mecanismo de ação e a farmacocinética da molécula, também foi avaliado se o resultado da atividade citostática encontrado previamente em modelo bidimensional de cultivo celular seria capaz de ser reproduzido em um formato tridimensional, que assemelha-se a um tumor real. Os resultados deste estudo mostraram que a miristicina é capaz de permear as membranas de um tumor sólido, possibilitando reduzir a dose dos quimioterápicos e manter a eficácia.

Considerando o grande obstáculo no tratamento quimioterápico do câncer que é a MDR adquirida por células tumorais, investigar alvos eficazes para contornar essa resistência continua sendo um importante desafio a ser superado. Portanto, este estudo encoraja a continuação da investigação da miristicina como um potencial composto para a reversão da MDR.

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