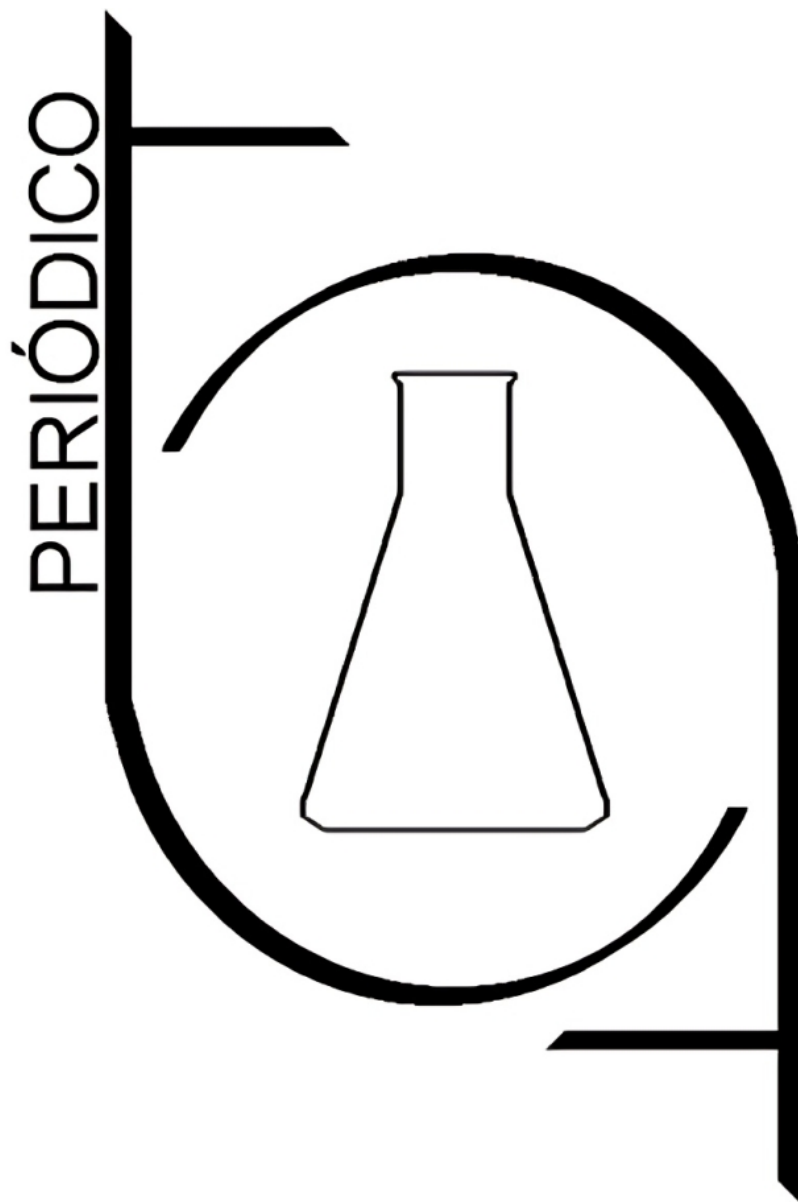


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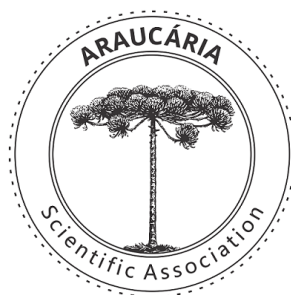
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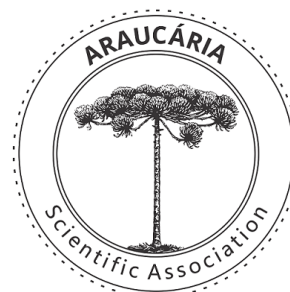
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## COMPOSIÇÃO QUÍMICA, DOCKING MOLECULAR E ATIVIDADE LARVICIDA DO ÓLEO ESSENCIAL DE *CITRUS X LIMON* (L.) OSBECK

## CHEMICAL COMPOSITION, MOLECULAR DOCKING AND LARVICIDAL ACTIVITY OF CITRUS X LIMON (L) OSBECK ESSENTIAL OIL

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## RESUMO

**Introdução:** O mosquito *Aedes aegypti* é o principal vetor de arboviroses de grande relevância para a saúde pública, incluindo dengue, Zika, chikungunya e febre amarela. Para combater a *Aedes aegypti* é utilizado larvicidas químicos, que apesar da eficácia, trazem riscos ambientais e podem ocasionar resistência. Óleos essenciais, como o de Citrus limon, surgem como alternativas sustentáveis, devido à sua atividade larvicida, baixa toxicidade, biodegradabilidade e complexidade química, que reduz a probabilidade de resistência.

**Objetivo:** O presente estudo descreve a composição química do óleo essencial destilado das cascas de Citrus x limon (L) Osbeck e avalia se ele tem atividade biológica contra larvas de terceiro estágio do *Aedes aegypti*.

**Métodos:** O óleo essencial foi extraído pelo método de hidrodestilação e seus compostos foram identificados por cromatografia gasosa acoplada à espectrometria de massas (CG/EM). O óleo foi analisado quanto a sua atividade larvicida, onde a concentração letal foi calculada pelo método Probit. O mecanismo de ação do óleo foi avaliado por docking molecular. **Resultados e Discussão:** A análises revelaram que o óleo é predominantemente composto por monoterpenos, com limoneno como o componente majoritário (75,77%),

seguido por o-cimeno (9,24%) e  $\beta$ -pineno (7,48%). Ademais, o óleo demonstrou atividade larvídica, com concentração letal de 44,11  $\mu\text{g/mL}$ . Estudos de docking molecular indicaram interações significativas entre os principais compostos e as proteínas-alvo, com afinidades de ligação variando de  $-5,97$  a  $-6,56$  kcal/mol para acetilcolinesterase e proteína transportadora de esteróis-2, sugerindo um mecanismo de ação múltiplo. **Conclusões:** Conclui-se, portanto, que o óleo é ativo e possui potencial para substituir larvicidas sintéticos.

**Palavras-chave:** *Compostos voláteis; monoterpenos; Aedes aegypti; limoneno*

## ABSTRACT

**Introduction:** The mosquito *Aedes aegypti* is the primary vector of arboviruses of major public health concern, including dengue, Zika, chikungunya, and yellow fever. Chemical larvicides are commonly used to control *Aedes aegypti*; however, despite their efficacy, they pose environmental risks and may lead to resistance. Essential oils, such as that from *Citrus limon*, have emerged as sustainable alternatives due to their larvicidal activity, low toxicity, biodegradability, and chemical complexity, which reduces the likelihood of resistance development. **Objective:** This study describes the chemical composition of the essential oil distilled from the peels of *Citrus x limon* (L.) Osbeck and evaluates its biological activity against third-instar larvae of *Aedes aegypti*. **Methods:** The essential oil was extracted by hydrodistillation, and its components were identified by gas chromatography coupled with mass spectrometry (GC/MS). Larvicidal activity was assessed, and the lethal concentration was determined using the Probit method. The oil's mechanism of action was investigated through molecular docking studies. **Results and Discussion:** Analyses revealed that the oil is predominantly composed of monoterpenes, with limonene as the major component (75.77%), followed by o-cymene (9.24%) and  $\beta$ -pinene (7.48%). Furthermore, the oil exhibited larvicidal activity, with a lethal concentration of 44.11  $\mu\text{g/mL}$ . Molecular docking studies showed significant interactions between the main compounds and target proteins, with binding affinities ranging from  $-5.97$  to  $-6.56$  kcal/mol for acetylcholinesterase and sterol carrier protein-2, suggesting a multi-target mechanism of action. **Conclusions:** The essential oil from *Citrus x limon* is therefore active and shows potential as a replacement for synthetic larvicides.

**Keywords:** *Volatile compounds; monoterpenes; Aedes aegypti; limonene.*

## 1. INTRODUÇÃO:

O *Aedes (A) aegypti* L. (Diptera: Culicidae) é um mosquito que transmite doenças virais graves, como dengue, Zika, chikungunya e febre amarela. É encontrado principalmente em regiões tropicais e subtropicais e apresenta notável adaptabilidade a ambientes urbanos, onde encontra condições favoráveis para o desenvolvimento de larvas, permitindo o ciclo de vida completo do mosquito (Zara, Santos, Fernandes-Oliveira, Carvalho, & Coelho, 2016). De acordo com os dados mais recentes do Ministério da Saúde do Brasil (Brasil, 2022), houve um aumento de 184,6% nos casos de dengue entre 2021 e 2022. Durante o mesmo período, os casos de chikungunya aumentaram 86,9% e os casos de Zika aumentaram 96,6%. A adaptabilidade reprodutiva do *Aedes aegypti* e seu potencial de transmissão de doenças virais graves ressaltam a importância das medidas de controle.

Algumas das abordagens contra a proliferação do *A. aegypti* e a consequente transmissão de doenças virais incluem o uso de vacinas, predadores biológicos e larvicidas químicos (Baldacchino *et al.*, 2015). Recentemente, uma vacina eficaz contra o vírus da dengue, a Qdenga®, foi aprovada para uso por

agências reguladoras em vários países, como a Agência Europeia de Medicamentos (Angelin *et al.*, 2023) e ANVISA (Brasil, 2023). No entanto, vacinas eficazes contra os outros vírus transmitidos pelo *A. aegypti* ainda estão sendo estudadas (Marin-Lopez, Wang, Jiang, Ledizet, & Fikrig, 2021; Wang, Marin-Lopez, Jiang, Ledizet, & Fikrig, 2020). O uso de predadores biológicos é uma estratégia ecológica para o controle do *A. aegypti*; dessa forma, existe a possibilidade de que a introdução dessas espécies cause desequilíbrio no ecossistema (Bowatte, Perera, Senevirathne, Meegaskumbura, & Meegaskumbura, 2013). Entre eles, o uso de larvicidas químicos tem sido o mais utilizado devido à sua eficiência, praticidade e controle direto das larvas e pupas do *A. aegypti*. Os principais larvicidas químicos utilizados no combate às larvas de *A. aegypti* incluem organofosforados como o temefós, fipronil e piretróides (Aldridge *et al.*, 2022; Farder-Gomes *et al.*, 2024; Kim, Khan, Estep, Cantrell, & Le, 2025). Contudo, o uso constante desses larvicidas podem causar impactos negativos. O uso frequente de pesticidas sintéticos pode causar contaminação ambiental, afetar organismos não-alvo (incluindo humanos e animais), e favorecer o surgimento de resistência nas populações de mosquitos (Farder-Gomes *et al.*, 2024; da Silva *et al.*, 2025; Sittichok *et al.*,

2024). Como exemplo de impacto negativo em humanos, o fipronil, mesmo em baixas concentrações, pode causar alterações comportamentais, danos ao intestino médio e morte celular nas larvas, além de afetar enzimas de desintoxicação (Farder-Gomes *et al.*, 2024). Como alternativa aos larvicidas químicos, o uso de óleos essenciais tem sido explorado.

Os óleos essenciais são substâncias naturais voláteis compostas por uma variedade de componentes, como terpenos, monoterpenos, compostos fenólicos e outros, cuja composição depende da planta de extração (Dhifi, Bellili, Jazi, Bahloul, & Mnif, 2016) e foi comprovado que alguns têm atividade larvicida (Gomes *et al.*, 2018, 2019, 2020, 2022). Os óleos essenciais oferecem uma abordagem não tóxica para o controle de larvas e biodegradabilidade, minimizando os impactos ambientais adversos, e têm uma baixa probabilidade de desenvolvimento de resistência de larvas devido à sua composição complexa (Nerio, Olivero-Verbel, & Stashenko, 2010). Essas propriedades os tornam mais vantajosos em comparação com os larvicidas sintéticos.

Embora os óleos essenciais apresentem vantagens promissoras em relação aos larvicidas sintéticos, seu uso como substituto parcial ou total requer mais informações sobre seus mecanismos de ação larvicida. A diversidade na composição dos óleos essenciais indica possíveis mecanismos múltiplos de ação, reduzindo a possibilidade de resistência das larvas (Dris, Tine-Djebbar, Bouabida, & Soltani, 2017). Para investigar esses mecanismos, a pesquisa de acoplamento molecular está surgindo como uma ferramenta eficaz para examinar as interações entre compostos de óleos essenciais e alvos de proteínas e proteínas relevantes para o crescimento larval.

O acoplamento molecular é uma técnica usada para prever a interação entre duas moléculas, como uma proteína e um ligante (Pagadala, Syed, & Tuszynski, 2017) envolvendo a simulação de como o ligante se encaixa no local ativo da proteína, formando um complexo estável. O processo usa algoritmos de computador para explorar todas as orientações e conformações possíveis do ligante, identificando a melhor combinação que maximiza a afinidade e a estabilidade do complexo (Guedes, de Magalhães, & Dardenne, 2014). Ao analisar os componentes do óleo por meio de acoplamento molecular, é possível analisar como eles agem nas proteínas que são cruciais para a sobrevivência das larvas. Esse entendimento é particularmente relevante para plantas com

potencial larvicida conhecido, como as plantas do gênero *Citrus*.

Entre as plantas com composições de maior interesse para o controle de larvas de *A. aegypti* está a *Citrus limon* L., também conhecida como limão. É uma planta da família *Rutaceae* e cultivada em várias partes do mundo, especialmente em países subtropicais (González-Molina, Domínguez-Perles, Moreno, & García-Viguera, 2010). O óleo essencial extraído dessa planta tem propriedades medicinais, dada a sua complexa composição de sesquiterpenos, terpenos, hidrocarbonetos aromáticos e derivados oxigenados. Entre suas aplicações estão ações antimicrobianas, antioxidantes, antidiabéticas, antivirais e larvicidas (Dosoky & Setzer, 2018).

Apesar da ação larvicida do óleo essencial de *Citrus limon* L., sua eficácia na eliminação das larvas de *Aedes aegypti* tem sido pouco explorada. Portanto, o presente estudo descreve a composição química do óleo essencial destilado das cascas de *Citrus x limon* (L) Osbeck e avalia se ele tem atividade biológica contra larvas de terceiro estágio do *Aedes aegypti*.

## 2. MATERIAL E MÉTODOS:

### 2.1. Obtenção e extração de óleo essencial

Partes estruturais e representativas (flores e frutos) da planta, medindo de 30 a 40 cm de tamanho, foram coletadas em uma área rural de São José de Ribamar-MA, Brasil, em janeiro de 2018, no período da manhã, nas coordenadas geográficas latitude 02°35'51,8"S e longitude 44°09'33,3"W para identificação botânica e extração do óleo essencial. Após essa coleta, o material foi certificado pelo Laboratório de Estudos Botânicos da Universidade Federal do Maranhão com o número 11169. Uma vez identificados, os frutos foram separados das demais partes, descascados com bisturi e armazenados em uma bandeja para a extração do óleo essencial.

A destilação do óleo foi realizada por hidrodestilação, e o rendimento médio foi calculado com base nas medições de densidade e no peso do material (Gomes *et al.*, 2020). Para a extração, as amostras foram pesadas (400 g), misturadas (4000 mL) com água destilada na proporção de 1:10, colocadas em um balão de fundo redondo acoplado a um extrator do tipo Clevenger e aquecidas a 100°C em uma manta de

aquecimento elétrico por 4 horas. Após esse tempo, o óleo destilado foi coletado e seco por percolação em solução de sulfato de sódio anidro (Synth, Diadema, Brasil) e armazenado em frascos de vidro âmbar a 4°C para evitar possíveis perdas de constituintes voláteis. A densidade foi medida em um picnômetro de 5 mL a uma temperatura de 25°C, e o material foi pesado em uma balança analítica (Gomes *et al.*, 2019, 2020).

## 2.2. Cromatografia Gasosa acoplada à Espectrometria de Massas

A identificação dos componentes do óleo destilado foi realizada por meio de um cromatógrafo a gás acoplado à espectrometria de massa, de acordo com o estudo realizado por Sousa e colegas (Sousa *et al.*, 2023). Para esse fim, as amostras foram analisadas em um cromatógrafo a gás (Shimadzu, Kyoto, Japão) acoplado a um espectrômetro de massa (QP2020AS), usando hélio como gás de arraste com uma taxa de fluxo na coluna de 2,5 mL/min; temperatura do injetor: 280°C, divisão 1:50; coluna capilar de silicone CarbolWax e metilfenil 5% (30m x 0,25mm x 0,25mm) e programa de temperatura do forno de 60°C a 280°C. No espectrômetro de massa, a temperatura foi ajustada para 280°C e os espectros foram registrados de 35 a 550 m/z. Foram injetadas alíquotas de 1 µL (injetor automático CP - 8410) das amostras diluídas em uma proporção de 20 µL em 1,5 mL de hexano. Os componentes foram identificados pela comparação de seus índices de retenção com dados obtidos de substâncias autênticas em bibliotecas de referência NIST14 e área de normalização.

## 2.3. Captura e reprodução de larvas de *Aedes aegypti*

A deposição dos ovos ocorreu em baldes de polietileno de 500 mL, onde foram adicionados água e dois pedaços de palitos de eucalipto durante os meses de janeiro e fevereiro de 2019. Após a deposição, os ovos foram colocados em um recipiente de 200mL com água mineral a uma temperatura de 31°C para a eclosão. Após a eclosão, as larvas foram mantidas em temperatura ambiente de 25 ± 2°C, umidade relativa de 70 a 80% e alimentadas com ração para cães até atingirem o terceiro estágio (Gomes *et al.*, 2019).

## 2.4. Ensaio larvicida

A realização desse ensaio seguiu a metodologia descrita pela Organização Mundial da Saúde (OMS) (World Health Organization, 2005). Inicialmente, dez larvas de terceiro estágio foram transferidas para copos descartáveis e expostas a três concentrações de óleo destilado (10, 50 e 100 µg/mL) por um período de 24 horas para verificar a presença ou ausência de atividade. Uma vez confirmada a atividade, os testes foram realizados em quintuplicata a partir do mesmo lote de larvas, onde dez larvas foram submetidas a dois tratamentos: um com cinco soluções de óleo destilado (50 a 90 µg/mL) preparadas a partir de sua dissolução em dimetilsulfóxido (DMSO) a 0,1% e o outro, um controle negativo apenas com solução de DMSO a 0,1%. Portanto, para verificar a atividade larvicida, o resultado da concentração letal (CL<sub>50</sub>) foi calculado e comparado com os critérios estabelecidos por Cheng *et al.* (2003) que consideram o óleo altamente ativo quando apresenta CL<sub>50</sub> < 50 µg/mL; ativo quando é < 100 µg/mL e inativo quando é > 100 µg/mL.

## 2.5. Avaliação *in silico*

### 2.5.1. Docking cego exploratório de proteína-ligante

O acoplamento cego foi utilizado para explorar minuciosamente as superfícies das proteínas acetilcolinesterase (ACE) e Sterol Carrier Protein-2 (SCP2) para identificar e prever bolsões. Essa abordagem foi escolhida porque a maioria dessas proteínas não foi estudada experimentalmente. A estrutura tridimensional da SCP2 foi baixada do banco de dados do Protein Data Bank (PDB ID: 1PZ4) e limpa usando o PyMOL Molecular Graphics System, versão 3.1.1 (Schrödinger, LLC.). A sequência de codificação (CDS) da acetilcolinesterase foi recuperada do banco de dados de nucleotídeos do National Center for Biotechnology Information (NCBI) (GenBank: ABN09910.1) e a estrutura da proteína foi prevista com o Protein Homology/analogY Recognition Engine V 2.2 (Phyre2.2) usando o modo de modelagem intensiva (Kelley, Mezulis, Yates, Wass, & Sternberg, 2015). O modelo foi considerado uma boa previsão quando apresentado com 75% ou mais dos resíduos modelados com mais de 90% de confiança e com pelo menos 30% de identidade do primeiro modelo classificado.

Posteriormente, as estruturas das proteínas foram preparadas com o



AutoDockTools (ADT) versão 1.5.7 (Morris *et al.*, 2009), com a adição de hidrogênios polares e cargas de Kollman. A grade de receptores foi determinada para cada proteína para compreender toda a estrutura da proteína. Os principais constituintes do óleo essencial de *Citrus limon* foram usados como ligantes, a saber, limoneno ( $C_{10}H_{16}$ ),  $\beta$ -pineno ( $C_{10}H_{16}$ ) e o-cimeno ( $C_{10}H_{14}$ ). Neste estudo, o temefós ( $C_{16}H_{20}O_6P_2S_3$ ) também foi usado como ligante devido à sua atividade larvídica. A adição de hidrogênios e a otimização da geometria usando o Universal Force Field (UFF) foram realizadas no software Avogadro (Hanwell *et al.*, 2012) e as cargas de Gasteiger foram adicionadas com ADT. As simulações de acoplamento cego proteína-ligante foram realizadas em triplicata com o AutoDock Vina 1.2.0 (Eberhardt, Santos-Martins, Tillack, & Forli, 2021).

### 2.5.2. Acoplamento específico de proteína-ligante

Os receptores e os ligantes foram preparados conforme descrito acima. O acoplamento molecular do local ativo foi realizado em triplicata usando o AutoDock Vina 1.2.0 (Eberhardt *et al.*, 2021). Os locais específicos previstos foram determinados com base nos resultados de acoplamento cego. Foram selecionados os locais com maior afinidade de ligação para cada ligante. Cada proteína tinha um ou dois locais diferentes para cada um dos compostos testados. Foram definidas grades para todos os locais com um tamanho de  $30 \times 30 \times 30$  Å e espaçamento de 0,375 Å, e a exaustão foi definida como 16. A eficiência do ligante (LE) representa os resultados de Vina da afinidade de ligação (Kcal/mol) normalizada pelo tamanho do composto (Hopkins, Groom, & Alex, 2004) e foi calculado com o RDKit, versão 2023.09.5. Todas as imagens foram geradas usando o BIOVIA Discovery Studio Visualizer, versão 24.1.0.23298 (Dassault Systèmes Corp).

### 2.5.3 Redocking molecular

O redocking foi feito apenas na proteína SCP-2 de *Aedes aegypti*, visto que para a acetilcolinesterase de *A. aegypti* não há estruturas cristalizadas disponíveis no PDB de organismos que sejam próximos. A estrutura cristalizada SCP-2 de *Aedes aegypti* está disponível no Protein Data Bank (PDB ID: 1PZ4) em complexo com ácido palmítico. O ligante co-cristalizado foi removido da estrutura proteica e convertido para

o formato .pdbqt usando o AutoDock Tools (versão 1.5.7) e em seguida o ligante passou por redocking na mesma posição. O grid box foi definido com base nas coordenadas do ligante na estrutura cristalizada. As simulações foram feitas com AutoDock Vina (versão 1.2.3). Os parâmetros usados foram dimensões da grid box ajustadas ao sítio de ligação (não padronizadas como  $40 \times 40 \times 40$  Å), espaçamento de 0,375 Å e exaustividade definida como 16. O procedimento foi repetido três vezes para verificar se os resultados eram consistentes. A pose com maior afinidade de ligação foi selecionada para cálculo do RMSD (Root Mean Square Deviation) entre essa pose e a posição do ligante na estrutura cristalizada utilizando o PyMOL Molecular Graphics System (versão 3.1.1, Schrödinger, LLC).

## 2.6. Tratamento estatístico

A análise Probit foi usada para calcular a  $CL_{50}$ , e os resultados foram apresentados como média, desvio padrão, limite de confiança superior e limite de confiança inferior, calculados usando o software Statistical Package for the Social Sciences (SPSS) com um nível de significância definido como  $p < 0,05$ .

## 3. RESULTADOS E DISCUSSÃO:

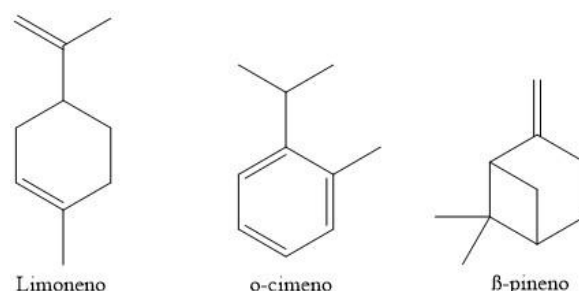
### 3.1. Extração do óleo e análise de composição

Para verificar o rendimento e identificar os componentes presentes no óleo destilado, realizamos a medição da densidade e a análise cromatográfica acoplada à espectrometria de massa (GC/MS). Por meio da extração, o óleo apresentou um rendimento e uma densidade de 2,45% (p/v) e 0,850 g/mL, respectivamente. A análise cromatográfica revelou a presença de 16 compostos (Tabela 1) com predominância de monoterpenos, entre os quais se destacaram o limoneno (75,77%), o-cimeno (9,24%) e o  $\beta$ -pineno (7,48%), cuja a estrutura apresentada na Figura 1. O limoneno possui uma ligação dupla e duas ramificações metila/isopropenil. O o-cimeno apresenta um anel benzênico ligado a um grupo metila e um grupo isopropila. Já o  $\beta$ -pineno, possui dois anéis fundidos, com um grupo metileno exocíclico e dois grupos metila.

Em nosso estudo atual, só foi possível identificar o limoneno, o composto predominante, conforme determinado pelos resultados da análise de GC/MS dos tempos de retenção, área

normalizada e comparação dos dados do cromatograma com os dados da biblioteca existente. Esse resultado é consistente com o estudo realizado por Gomes e colegas (2019) quando avaliaram a composição química do óleo essencial das cascas de *C. limon* obtidas em um bairro periférico do município de São Luís. Nesse estudo, os autores utilizaram os métodos de destilação e a identificação de compostos semelhantes ao que realizamos revelou a predominância do limoneno em frações superiores a 50%. Um estudo similar foi realizado por Paw *et al.* (2020) ao também estudar a composição de óleo essencial das cascas de *Citrus limon*. Todavia, diferente do nosso estudo, os autores identificaram que o componente majoritário, o limoneno, representava apenas 55,40 % da composição do óleo, enquanto em nosso estudo, foi encontrada concentrações mais elevadas de limoneno (75,77%). Outras diferenças em nossos estudos estão nos demais compostos majoritários. Enquanto em nosso estudo foi encontrado o-cimeno (9,24%) e o  $\beta$ -pineno (7,48%), o estudo de Paw *et al.* (2020) encontrou neral (10,39%), trans-verbenol (6,43%) e decanal (3,25%). Esse contraste pode ser explicado por fatores associados ao clima, solo, altitude ou variedade da planta (Luro *et al.*, 2020). Apesar disso, em ambos os estudos, o composto majoritário foi o limoneno, característico de espécies do gênero *Citrus*. Outros estudos realizados com métodos semelhantes também confirmaram a predominância do limoneno para outras espécies de *Citrus*. As espécies conhecidas são *C. limonia* (Gomes *et al.*, 2020), *C. aurantifolia* (Sousa *et al.*, 2023) e *C. sinensis* (Gomes *et al.*, 2021), que foram coletados de diferentes partes e obtiveram frações de 44,75%, 81,69% e 89,55%, respectivamente. Embora sejam de espécies diferentes, os resultados obtidos ainda são consistentes com as frações predominantes de limoneno em *Citrus* em geral, que variam de 30 a 97% (Mehl *et al.*, 2014; Simas, Amorim, Oliveira, Alviano, & Silva, 2015). Como observado, os resultados concordam com relação à predominância, mas diferem com relação às frações desse constituinte. Assim, inferimos que as diferenças observadas resultam de vários fatores, desde sazonalidade, tempo de extração, fatores abióticos até o simples redirecionamento das vias metabólicas da planta (Blank *et al.*, 2007; Cerqueira *et al.*, 2009; Figueiredo, Barroso, Pedro, & Scheffer, 2008; Gobbo-Neto & Lopes, 2007).

[Tabela 1]



**Figura 1.** Estruturas químicas dos principais componentes do óleo essencial de *Citrus x limon* (L.) Osbeck

### 3.2. Atividade larvicida

Para avaliar a atividade larvicida, dez larvas foram submetidas a dois tipos de tratamento: um experimental (concentrações de óleo variando de 50 a 90  $\mu\text{g/mL}$ ) e um controle negativo com DMSO 0,1%. Posteriormente, a  $\text{CL}_{50}$  foi calculada usando o método Probit e comparada com os parâmetros estabelecidos por Cheng *et al.* (2003). À medida que aumentamos a concentração de óleo, foi observado um aumento na mortalidade, levando a uma  $\text{CL}_{50}$  de 44,11 (Tabela 2). Comparando o resultado da  $\text{CL}_{50}$  com os critérios descritos por Cheng *et al.* (2003) para atividade, o óleo foi considerado ativo, pois apresentou uma  $\text{CL}_{50}$  inferior a 100  $\mu\text{g/mL}$ . Confirmamos a atividade larvicida do óleo essencial de *C. limon* (L.) Osbeck comparando a  $\text{CL}_{50}$  calculada com os critérios estabelecidos por Cheng *et al.* (2003). De acordo com esses autores, um óleo essencial é ativo contra larvas quando a  $\text{CL}_{50}$  é menor que 100  $\mu\text{g/mL}$ .

Apesar de não haver um critério padronizado estabelecido pela Organização Mundial da Saúde (OMS) para qualificar a atividade larvicida dos óleos essenciais, o resultado de nosso estudo está alinhado com estudos anteriores (Gomes *et al.*, 2019, 2020, 2022; Sousa *et al.*, 2023) que identificou o limoneno como o principal componente e o associou à atividade contra larvas de *Ae. aegypti* para *C. limon* e outras espécies de *Citrus*. Apesar das semelhanças nesses estudos em relação ao método de extração do óleo, ao estágio larval e à análise durante o verão, observamos uma variação significativa nos valores de  $\text{CL}_{50}$  para *C. limon* ( $\text{CL}_{50}$  = 15,48  $\mu\text{g/mL}$ ), *C. aurantifolia* ( $\text{CL}_{50}$  =

18,68 µg/mL) e *C. limonia* (CL<sub>50</sub> = 67,18 µg/mL) (Gomes *et al.*, 2019, 2020, 2022; Sousa *et al.*, 2023). Isso nos leva a inferir que a baixa letalidade observada em nosso estudo não está relacionada ao estágio larval ou ao momento da análise durante a estação (Cetin, Erler, & Yanikoglu, 2004; Fernandez *et al.*, 2014, 2018), mas sim a variações no período de desenvolvimento da planta e no ciclo circadiano (Gobbo-Neto & Lopes, 2007). Além disso, outro ponto a ser observado é o horário das coletas, pois elas ocorreram em horários diferentes, sendo que apenas a nossa foi coletada pela manhã.

### [Tabela 2]

A atividade larvicida do limoneno observada neste estudo pode estar relacionada à sua estrutura, fragrância e alterações histofisiológicas. Em um estudo conduzido por Santos *et al.* (2011) para avaliar a relação estrutura-atividade dos monoterpenos, os autores afirmaram que a potência larvicida é afetada pela estrutura do limoneno e que as ligações duplas endo e exo foram mais ativas do que as alterações estruturais decorrentes da adição de heteroátomos. O estudo conduzido por Rod (1997) atribui a atividade à forte fragrância do limoneno, que não apenas repele, mas também mata insetos que atacam as árvores. Enquanto isso, o estudo de Oliveira *et al.* (2021) que avaliaram os distúrbios histológicos, histoquímicos e energéticos causados pelo limoneno em larvas de *Ae. aegypti*, concluíram que esse composto afeta a capacidade de absorção de nutrientes, alterando a membrana peritrófica do intestino médio, inibindo assim a retenção de compostos vegetais isolados e causando a morte das larvas.

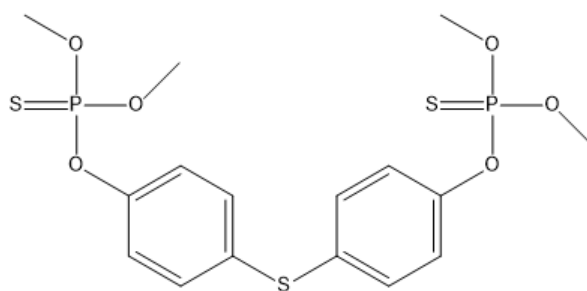
Assim, a presença do limoneno como composto predominante no óleo essencial das cascas de *C. limon* apenas indica que o óleo é um agente larvicida em potencial, mas não garante a eficácia na letalidade, pois há vários fatores que influenciam isso. Um estudo realizado por Lima *et al.* (2021) reforça essa hipótese ao demonstrar que o limoneno isoladamente apresenta maior atividade biológica que o óleo essencial de *Hedychium coronarium*, que contém limoneno (5,2%), β-pineno (17,0%) e p-cimeno (4,9%). Todavia, os autores reforçam a ideia de que a combinação dos constituintes do óleo pode potencializar o efeito biológico devido à sinergia. Esse sinergismo pode ocorrer devido à atuação dos componentes em múltiplos alvos celulares, à

facilitação da penetração de compostos mais ativos por moléculas menos ativas ou à modulação de mecanismos de resistência microbiana (Langeveld, Veldhuizen, & Burt, 2014). Por outro lado, nem todas as combinações são sinérgicas. Algumas podem ser aditivas ou até antagonista, dependendo das proporções e dos alvos biológicos (Bassolé & Juliani, 2012). Portanto, a identificação e otimização dessas interações são fundamentais para potencializar o uso de óleos essenciais.

### 3.3. Docking molecular

O acoplamento molecular concentrou-se apenas nos compostos predominantes do óleo, limoneno, β-pineno e o-cimeno, que foram selecionados por apresentarem uma maior concentração. Foi usado como controle positivo o temefós, cuja estrutura química é mostrado na Figura 2. As proteínas-alvo usadas foram a acetilcolinesterase (AChE) e a proteína transportadora de esterol-2 (SCP2). No acoplamento cego (Tabela 3), a AChE mostrou o temefós como o ligante com a maior afinidade (-7,02 kcal/mol), seguido pelo limoneno (-5,92 kcal/mol) e para a SCP-2 o β-pineno mostrou a maior afinidade (-6,48 kcal/mol), seguido pelo limoneno (-6,47 kcal/mol).

### [Tabela 3]



**Figura 2.** Estrutura química do temefós

No acoplamento específico no local ativo (Tabela 3), o temefós apresentou a maior afinidade de ligação (-7,78 kcal/mol) com a AChE, apesar de sua menor eficiência de ligação (-0,29). Entre os compostos do óleo, o o-cimeno apresentou a maior afinidade com a AChE (-6,14 kcal/mol). Para a SCP-2, o temefós apresentou a maior afinidade (-7,45 kcal/mol), enquanto os

compostos no óleo apresentaram valores semelhantes, variando de 6,56 a -6,39 kcal/mol. Em termos de eficiência de ligação, os compostos do óleo variaram de -0,64 a -0,66, mais altos do que o controle positivo temefós, que apresentou uma eficiência de ligação de -0,28.

Para validar o protocolo de docking molecular usado presente no estudo, foi feito um teste de redocking aplicado à proteína SCP-2 de *A. aegypti*, utilizando o ácido palmítico como ligante. O procedimento foi repetido três vezes para garantir convergência dos resultados. A pose com maior afinidade de ligação foi selecionada para cálculo do desvio quadrático médio (RMSD) em relação à posição original do ligante na estrutura cristalográfica, utilizando o software PyMOL. O valor de RMSD obtido foi de 2,179 Å. Esse valor, embora acima do limiar ideal de 2,0 Å (Wei *et al.*, 2022; Yang & Chen, 2004), encontra-se dentro da faixa aceitável para validação de protocolos de docking.

### [Tabela 3]

Os diagramas de interação 2D (Figura 3) mostram como os ligantes Temefós e o-cimeno se ligam às proteínas SCP2 e ACHE. Ambos os ligantes, Temefós e o-cimeno, foram selecionados para visualização dos tipos de ligação por terem as maiores afinidades de ligação com as enzimas. Na enzima ACHE, Temefós faz ligações de hidrogênio com TRP A:212 e GLY A:246, além de também ter interações polares com GLU A:215 e SER A:250. Já no SCP2, o Temefós possui ligações de hidrogênio com GLN A:25, interações  $\pi$ -cátion com ARG A:24, e interações hidrofóbicas com VAL A:26 e ILE A:19. O o-cimeno se liga com a enzima ACHE por meio de ligações hidrofóbicas com TYR A:456 e TRP A:212 e interações de Van der Waals com GLY A:246. Na SCP2, o-cimeno faz ligações hidrofóbicas LEU A:16, interações  $\pi$ - $\pi$  com PHE A:105, e interações de Van der Waals com ILE A:19. Essas ligações são principalmente hidrofóbicas e aromáticas, o que explica sua maior eficiência de ligação, superior ao Temefós. O o-cimeno é um monoterpene aromático, composto por um anel benzênico e cadeias laterais hidrocarbonadas (Balahbib *et al.*, 2021) que podem se ligar aos resíduos hidrofóbicos das proteínas por interações de Van der Waals e interações  $\pi$ - $\pi$  através do anel benzênico presente no o-cimeno e nas proteínas (Balahbib *et al.*, 2021; Tapala, Ndlangamandla, Ngoepe, & Clayton, 2024).

Alguns outros mecanismos larvicidas relatados para o o-cimeno são a ruptura da parede celular (Santos *et al.*, 2010), estresse oxidativo (Costa *et al.*, 2022), danos ao sistema digestivo (Yu, Wong, Ahmad, & Jantan, 2015) e ação sobre enzimas essenciais para a função larval, como a AChE (Waliwitiya, Kennedy, & Lowenberger, 2009), resultados que estão de acordo com os apresentados neste estudo. No entanto, além da interação com a AChE, observamos por meio de acoplamento molecular que o o-cimeno também tem uma forte afinidade de ligação com a SCP-2. Isso se deve à alta lipolicidade do o-cimeno, que se liga ao sítio ativo apolar da SCP-2, que é especializado no transporte de moléculas de lipídios, como o colesterol, um nutriente essencial para o crescimento e o desenvolvimento dos mosquitos (Larson, Wessely, Jiang, & Lan, 2014). Essa combinação de mecanismos larvicidas destaca o uso do o-cimeno como um larvicida natural e eficiente. Os mecanismos do limoneno, o principal composto do óleo essencial do *C. limon*, são semelhantes aos do o-cimeno, pois, além de causar danos ao sistema digestivo (Oliveira *et al.*, 2021), causam ruptura da parede celular devido à lipofilicidade (Santos *et al.*, 2011) e inibição da atividade da AChE (Botas *et al.*, 2017).

Além dos mecanismos já relatados, observamos, a partir do acoplamento molecular, que o limoneno também atua ligando a SCP-2, competindo pelos locais ativos com o colesterol. Embora tanto o o-cimeno quanto o limoneno tenham uma energia de ligação menor do que o temefós (controle positivo), esses compostos apresentaram melhor afinidade de ligação tanto para a AChE quanto para a SCP-2, sugerindo ligações mais estáveis e específicas. O SCP-2 é conhecido por ter locais de ligação hidrofóbica para interagir com lipídios (Larson *et al.*, 2014). O limoneno e o o-cimeno, por serem lipofílicos, formam interações hidrofóbicas com os locais ativos do SCP-2, possivelmente bloqueando o transporte de lipídios essenciais para o desenvolvimento das larvas (Du *et al.*, 2012). O mecanismo de inibição da enzima AChE se deve à presença de regiões apolares que interagem com os componentes do óleo (Steinberg, Mednick, Maddox, Rice, & Cramer, 1975) e também devido à presença de resíduos aromáticos (Krishna, Chavali, & Uberbacher, 2013). Embora o temefós seja amplamente usado como larvicida, sua menor afinidade de ligação indica que compostos como o limoneno e o o-cimeno são alternativas mais eficazes e possivelmente mais seguras para o meio ambiente. Os resultados indicam vários

mecanismos de reação tanto para o limoneno quanto para o o-cimeno, o que dificulta o desenvolvimento de resistência pelas larvas.

[Figura 3]

#### 4. CONCLUSÃO:

A destilação das cascas de *Citrus x limon* (L) Osbeck por hidrodestilação possibilitou a identificação da composição química do óleo e a avaliação de sua atividade larvica contra o *Aedes aegypti*. A partir dessas análises, descobrimos que o óleo é predominantemente composto de limoneno e apresentou atividade larvica contra o organismo testado. O mecanismo de ação dos componentes principais é semelhante ao dos larvicidas comerciais, com interação com a acetilcolinesterase e a proteína transportadora de esterol-2, inibindo tanto as funções neurotransmissoras quanto o transporte de colesterol. Portanto, esse óleo tem o potencial de atividade biológica para ser usado como substituto de larvicidas químicos ou como complemento a eles. Para validar sua aplicação em condições reais, recomenda-se a realização de testes de campo em diferentes ambientes, bem como estudos de toxicidade ambiental para avaliar possíveis impactos sobre organismos não-alvo e a persistência do produto no meio ambiente.

#### 5. DECLARAÇÕES

##### 5.1. Limitações do Estudo

Os resultados deste estudo são limitados em relação ao rendimento do óleo, ao período de desenvolvimento da planta e à sua aplicação apenas em um estudo in vitro; se aplicado a um sistema real, pode não produzir os mesmos resultados. Apesar de essas características afetarem a letalidade, elas não excluem seu potencial larvica. Além disso, de acordo com a literatura, os componentes estudados apresentam menor citotoxicidade do que os componentes sintéticos. Estudos comparativos mostram que o limoneno e outros terpenos possuem uma atividade citotóxica moderada, enquanto os temefós, um composto químico altamente utilizando como larvicida, exibe maior toxicidade em concentrações muito menores (Sarma, Adhikari, Mahanta, & Khanikar, 2019). Além disso, os óleos essenciais são biodegradáveis, menos persistentes no ambiente e geralmente considerados seguros para organismos não-alvo, incluindo humanos e

animais, reduzindo assim o risco de poluição ambiental e bioacumulação (Senthil-Nathan, 2020). Portanto, o óleo essencial destilado das cascas de *Citrus x limon* (L) Osbeck é um possível candidato, atuando como substituto de larvicidas sintéticos ou complementando-os

##### 5.2. Agradecimentos

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##### 5.3. Fontes de Financiamento

Os autores declaram que não receberam suporte financeiro de agências de fomento públicas, comerciais ou sem fins lucrativos para a realização desta pesquisa.

##### 5.4. Conflitos de Interesse

Os autores declaram não haver conflitos de interesse nem interesses concorrentes

##### 5.5. Disponibilidade de Dados

Todos os dados apresentados neste estudo estão disponíveis nas tabelas e figuras do manuscrito. Os dados brutos estão disponíveis mediante solicitação ao autor correspondente

##### 5.6. Contribuições dos Autores

Paulo Roberto Barros Gomes: Concepção e design (CD), coleta de dados (DC), análise e interpretação de dados (DAI), escrita do manuscrito (MW), aprovação final (FA).

Dionney Andrade de Sousa: Coleta de dados (DC), análise e interpretação de dados (DAI), revisão crítica (CR), aprovação final (FA).

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| Code | English                          | Português                        |
|------|----------------------------------|----------------------------------|
| CD   | Conception and Design            | Concepção e Design               |
| DC   | Data Collection                  | Coleta de Dados                  |
| DAI  | Data Analysis and Interpretation | Análise e Interpretação de Dados |
| MW   | Manuscript Writing               | Escrita do Manuscrito            |
| CR   | Critical Review                  | Revisão Crítica                  |
| FA   | Final Approval                   | Aprovação Final                  |

### 5.7. Declaração de Ferramentas de IA e Computacionais

Ferramenta: ChatGPT (OpenAI).  
Propósito: Apoio à redação científica, revisão gramatical e estilística, reformulação de trechos textuais. Extensão: Utilizado exclusivamente como ferramenta de assistência à escrita durante a preparação do manuscrito. Todas as alterações foram revisadas e aprovadas pelo autor correspondente. Nenhuma IA foi utilizada para análise de dados, testes estatísticos ou interpretação

### 5.8. Declaração de Integridade da Pesquisa

Os autores declaram que a pesquisa atende aos padrões de integridade de pesquisa. Marque tudo que se aplica.

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## 6. ESTUDOS RELACIONADOS COM HUMANOS E ANIMAIS

### 6.1. Aprovação do Comitê de Ética

Não aplicável.

### 6.2. Consentimento Informado

Não aplicável.

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**Tabela 1.** Identificação dos componentes presentes no óleo essencial de *Citrus limon*

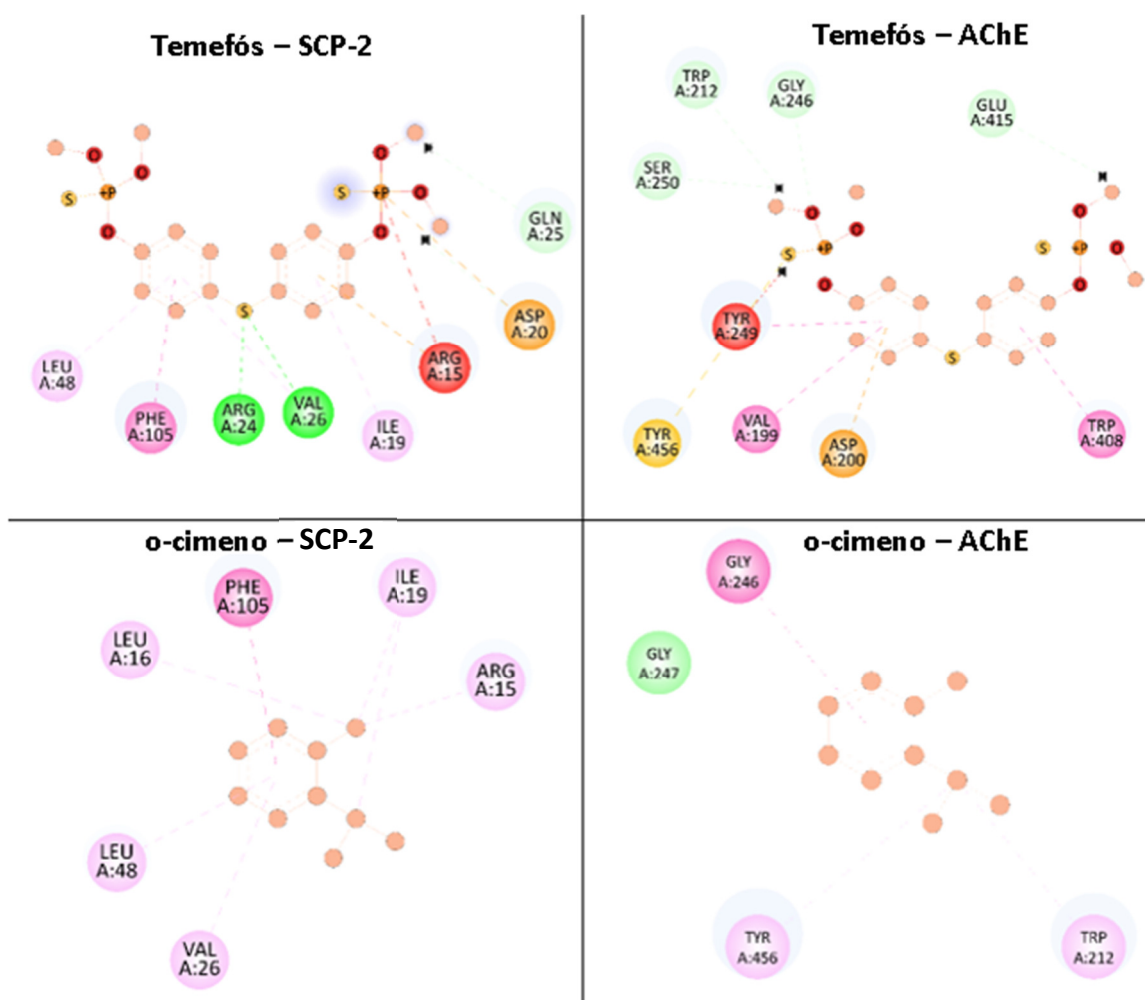
| Componentes                   | Tempo de retenção (min) | Retenção | Área normalizada (%) |
|-------------------------------|-------------------------|----------|----------------------|
| Cis-acetato-3-hexenila        | 3233                    | 992      | 0,07                 |
| $\alpha$ -Tujeno              | 3416                    | 902      | 0,23                 |
| $\alpha$ -Pineno              | 3508                    | 948      | 1,24                 |
| Tujene                        | 4027                    | 897      | 1,11                 |
| $\beta$ - Pineno              | 4080                    | 943      | 7,48                 |
| $\beta$ - Mirceno             | 4247                    | 958      | 1,26                 |
| Octanal                       | 4418                    | 1005     | 0,21                 |
| Butirato de 2-metil mirtenila | 4458                    | -        | 0,06                 |
| o-Cimeno                      | 4726                    | 1042     | 9,24                 |
| Limoneno                      | 4791                    | 1018     | 75,77                |
| 3-Careno                      | 5232                    | 948      | 0,17                 |
| Limoneno 1,2 - Epóxido        | 6350                    | 1031     | 0,53                 |
| Terpineno-4-ol                | 7010                    | 1137     | 0,39                 |
| $\alpha$ - Terpineol          | 7218                    | 1143     | 1,11                 |
| (E)- $\beta$ -farneseno       | 10343                   | 1440     | 0,04                 |
| $\alpha$ -Bergamoteno         | 10512                   | 1430     | 0,49                 |

**Tabela 2.** Atividade larvicida do óleo essencial em um período de 24 horas. Os dados de mortalidade foram expressos como média  $\pm$  desvio padrão.

| Concentração ( $\mu\text{g/mL}$ ) | Mortalidade (%) | CL <sub>50</sub> ( $\mu\text{g/mL}$ ) (IC 95%) | $\chi^2$ |
|-----------------------------------|-----------------|------------------------------------------------|----------|
| 50                                | 72 $\pm$ 0,83   | 44,11<br>(3,438 - 50,217)                      | 6,209    |
| 60                                | 82 $\pm$ 0,83   |                                                |          |
| 70                                | 88 $\pm$ 1,09   |                                                |          |
| 80                                | 98 $\pm$ 0,44   |                                                |          |
| 90                                | 100 $\pm$ 0,00  |                                                |          |
| Controle                          | 0,0 $\pm$ 0,00  |                                                |          |

**Tabela 3.** Pontuações do Autodock Vina para o docking molecular de local específico

| Composto        | Afinidade de ligação / Eficiência de ligação (kcal/mol) |                                         |
|-----------------|---------------------------------------------------------|-----------------------------------------|
|                 | Acetilcolinesterase                                     | Proteína transportadora de colesterol-2 |
| Limoneno        | -5,97 / -0,60                                           | -6,53 / -0,65                           |
| $\beta$ -pineno | -6,09 / -0,62                                           | -6,39 / -0,64                           |
| o-cimeno        | -6,14 / -0,61                                           | -6,56 / -0,66                           |
| Temefós         | -7,78 / -0,29                                           | -7,45 / -0,28                           |



**Figura 3.** Diagrama de interação 2D ilustrando a orientação dos ligantes Temefós e o-cimeno dentro da estrutura do SCP2 e AChE.

FORMAÇÃO DE BIOFILME, RESISTÊNCIA A ANTIBIÓTICOS E DETECÇÃO MOLECULAR DO GENE *rhII* EM ISOLADOS CLÍNICOS DE *Pseudomonas aeruginosa*BIOFILM FORMATION, ANTIBIOTIC RESISTANCE, AND MOLECULAR DETECTION OF THE *rhII* GENE IN CLINICAL ISOLATES OF *Pseudomonas aeruginosa*

تكوّن الأغشية الحيوية، ومقاومة المضادات الحيوية، والكشف الجزيئي عن جين *rhII* في العزلات السريرية لبكتيريا *Pseudomonas aeruginosa*

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## RESUMO

**Introdução:** *Pseudomonas aeruginosa* é um patógeno de origem hospitalar frequentemente envolvido em diversas infecções associadas à assistência à saúde, especialmente em pacientes imunocomprometidos. Sua capacidade de formar biofilme e desenvolver resistência a agentes antimicrobianos desempenha papel fundamental nas falhas terapêuticas. **Objetivo:** O presente estudo teve como objetivo avaliar fenotipicamente a formação de biofilme, identificar o padrão de suscetibilidade a antibióticos e detectar o gene *rhII* em isolados clínicos de *P. aeruginosa*. **Métodos:** Trinta e cinco amostras clínicas foram obtidas de diversas fontes, incluindo urina, swabs de feridas, swabs auriculares e amostras de queimaduras. A identificação dos isolados bacterianos foi realizada com base em métodos microbiológicos padrão e confirmada pelo sistema VITEK-2. O teste de difusão em disco de Kirby-Bauer foi empregado para determinar a suscetibilidade antimicrobiana. O ensaio em placa de cultura de tecidos (TCP) foi realizado para avaliar a capacidade de formação de biofilme, e a reação em cadeia da polimerase (PCR) foi utilizada para detectar a presença do gene *rhII*. A análise estatística utilizou o teste de Fisher ( $p < 0,05$ ). **Resultados:** Os isolados apresentaram resistência à ticarcilina, ceftriaxona e ciprofloxacina (100% cada). Novobiocina (96%), cloranfenicol (93%) e trimetoprima também apresentaram altas taxas de resistência (90%). Por outro lado, imipenem (9%) e amicacina (5%) apresentaram taxas de resistência mais baixas, indicando que esses antibióticos foram comparativamente mais eficazes. A análise do biofilme revelou que 60% dos isolados eram fortes produtores de biofilme, enquanto 40% apresentaram formação moderada de biofilme. Os testes de PCR revelaram que os isolados (100%) possuíam o gene *rhII*. **Conclusões:** Os isolados clínicos de *P. aeruginosa* demonstraram elevada resistência antimicrobiana, alta capacidade de formação de biofilme e presença universal do gene *rhII*. Esses resultados ressaltam a necessidade de associar abordagens fenotípicas e moleculares para melhor compreender e controlar as infecções causadas por esse microrganismo.

**Palavras-chave:** Detecção de quorum, *Pseudomonas aeruginosa*, Fatores de virulência, Infecções Associadas à Assistência à Saúde, Reação em cadeia da polimerase, Resistência a drogas.

## ABSTRACT

**Background:** *Pseudomonas aeruginosa* is a hospital-acquired pathogen that commonly causes healthcare-associated infections, particularly in immunocompromised patients. Its ability to form biofilms and develop resistance to antimicrobial agents plays a key role in treatment failure. **Purpose:** The study aimed to assess the phenotypic biofilm formation, identify the pattern of antibiotic susceptibility, and identify the *rhII* gene in clinical isolates of *P. aeruginosa*. **Methods:** Thirty-five clinical samples were obtained through various sources, including urine, wound swabs, ear swabs, and burn samples. Bacterial isolates were identified using standard microbiological methods and examined with the VITEK-2 system. Kirby-Bauer disk diffusion testing was done to determine antimicrobial susceptibility. A tissue culture plate (TCP) assay was conducted to assess biofilm-forming ability, and polymerase chain reaction (PCR) was performed to detect the *rhII* gene. The statistical analysis used Fisher's test ( $p < 0.05$ ). **Results:** The isolates were resistant to ticarcillin, ceftriaxone, and ciprofloxacin (100% each). Novobiocin (96%), chloramphenicol (93%), and trimethoprim also had high resistance rates (90%). On the

contrary, imipenem (9%) and amikacin (5%) showed lower resistance rates, indicating that these antibiotics were comparatively more effective. The analysis of biofilm revealed that 60% of the isolates were strong biofilm producers, while 40% formed moderate biofilms. PCR tests revealed that all isolates (100%) carried the *rhII* gene. **Conclusions:** *P. aeruginosa* clinical isolates displayed high antimicrobial resistance and biofilm-forming capacity, and the *rhII* gene was found in all isolates. These results highlight the need to consider a combination of both phenotypic and molecular approaches to gain more insight and manage infection by this organism.

**Keywords:** Quorum sensing, *Pseudomonas aeruginosa*, Virulence factors, Healthcare-Associated Infections, Polymerase Chain Reaction, Drug Resistance.

## المخلص

**الخلفية:** تُعدّ بكتيريا الزائفة الزنجارية من الممرضات الانتهازية المرتبطة بطيف واسع من العدوى المرتبطة بالرعاية الصحية، ولا سيما لدى المرضى ذوي المناعة الضعيفة. وتسهم قدرتها على تكوين الأغشية الحيوية وتطوير مقاومة مضادات الميكروبات إسهاماً كبيراً في فشل العلاج. **الهدف:** هدفت هذه الدراسة إلى تقييم تكوّن الأغشية الحيوية ظاهرياً، وتحديد أنماط الحساسية للمضادات الحيوية، والكشف عن وجود جين *rhII* في العزلات السريرية لبكتيريا الزائفة الزنجارية. **طرائق العمل:** جُمعت 35 عينة سريرية من مصادر مختلفة، شملت البول والجروح ومسحات الأذن والحروق. تم التعرف على العزلات البكتيرية باستخدام الطرق الميكروبيولوجية القياسية، وأُكِّد التشخيص باستخدام جهاز VITEK-2 كما أُجري اختبار الحساسية للمضادات الحيوية بطريقة انتشار الأقراص (Kirby-Bauer). وتم تقييم تكوّن الأغشية الحيوية باستخدام اختبار صفحية زراعة الأنسجة (TCP)، في حين تم الكشف عن جين *rhII* باستخدام تفاعل البوليميراز المتسلسل. استخدم التحليل الإحصائي اختبار فيشر ( $p < 0.05$ ). **النتائج:** أظهرت العزلات معدلات مقاومة مرتفعة لكلٍ من التيكارسيلين (100%)، والسيفترياكسون (100%)، والسبيروفلوكساسين (100%). كما سُجِّلَت معدلات مقاومة عالية للنوفلوبيوسين (96%)، والكلورامفينيكول (93%)، والتريميثوبريم (90%). في المقابل، لوحظت معدلات مقاومة منخفضة للإمبيبيديم (9%) والأميكاسين (5%)، مما يشير إلى فعاليتها النسبية. وكشف تحليل تكوّن الأغشية الحيوية أن 60% من العزلات كانت منتجة قوية، و40% منتجة متوسطة. كما أظهرت نتائج PCR أن جميع العزلات (100%) تحمل جين *rhII*. **الاستنتاجات:** أظهرت العزلات السريرية لـ *Pseudomonas aeruginosa* مستويات عالية من مقاومة المضادات الحيوية وقدرة على تكوين الأغشية الحيوية، مع وجود شامل لجين *rhII*. وتؤكد هذه النتائج أهمية دمج الطرائق الظاهرية والجزيئية لفهم أفضل وإدارة أكثر فاعلية للعدوى التي تسببها هذه البكتيريا.

**الكلمات المفتاحية:** استعمار النصاب، الزائفة الزنجارية، عوامل الضراوة، العدوى المرتبطة بالرعاية الصحية، تفاعل البوليميراز المتسلسل، مقاومة الأدوية.

## 1. INTRODUCTION:

*Pseudomonas aeruginosa* is a well-known opportunistic pathogen belonging to the family Pseudomonadaceae and is often associated with colonization in both humans and animals (Mohamed *et al.*, 2022). It is Gram-negative, non-fermentative, rod-shaped, and non-sporing, with a diameter of 0.5-0.8  $\mu\text{m}$  and a length of 1.5 -3.0  $\mu\text{m}$ . The organism is very common in natural habitats such as soil and water, and can also infect human and animal hosts. It is mostly aerobic, though it can grow under anaerobic conditions in the presence of nitrate. It moves using a polar flagellum (Spagnolo *et al.*, 2021).

The optimal temperature for this bacterium's growth is 37 °C, although it can grow up to 42 °C. In addition, *P. aeruginosa* is oxidase-positive, lactose-non-fermenting, and produces pigments, including pyocyanin, fluorescein, pyorubin, and pyomelanin; these pigments play an important role in its identification (Zavascki *et al.*, 2010).

Most *P. aeruginosa* strains possess virulence factors on their surface, enabling them to attach, colonize, and invade host tissues. The virulence factors stimulate cytokine secretion from injured tissues, which facilitates invasion and toxin secretion (Liao *et al.*, 2022). This bacterium can

invade numerous organs and suppress the immune system, leading to infections that cannot be fully cured. The main virulence factors contributing to bacterial colonization are the Lipopolysaccharide (LPS), flagella, and type IV pili (Saleh and Motib, 2023). In addition, other extracellular virulence factors include exotoxin A, alkaline proteases, elastases, pyocyanin, biofilm formation, and quorum sensing (Vadakkan *et al.*, 2024). Biofilm formation has been identified as a major virulence factor in many bacteria. It entails the creation of a well-organized community of microbes, deposited within a thick protective network. The major components of this matrix are extracellular polymeric substances (EPS), which include exopolysaccharides, proteins, nucleic acids, and other secreted molecules (Mann and Wozniak, 2012). Though biofilms were earlier viewed as simple aggregates of bacterial cells, it is now known to have a multifaceted, highly organized architecture. They are structured microcolonies linked together through pathways that facilitate the flow of water, nutrients, and waste products. This structural organization creates a heterogeneous microenvironment with nutrient and oxygen gradients (Hall-Stoodley *et al.*, 2004).

*P. aeruginosa* uses intrinsic, acquired, or adaptive resistance as its defense mechanism against antibiotics. In the intrinsic phase, the outer membrane is impermeable, efflux pumps are

expressed to expel antibiotics from cells, and enzymes that degrade antibiotics are produced. A horizontal shift of resistance genes or mutational changes might achieve the acquired phase (Artono *et al.*, 2025).

*Pseudomonas aeruginosa* shows resistance to a number of the most important antibiotic classes, including  $\beta$ -lactam antibiotics, aminoglycosides, and fluoroquinolones. Resistance to beta-lactams is primarily mediated by several mechanisms, including mutations in PBPs (penicillin-binding proteins) that limit drug binding, decreased permeability of the bacterial outer membrane, enhanced antibiotic efflux, and enzymatic degradation. Gene transfer also facilitates the acquisition of  $\beta$ -lactamase enzymes from other bacterial species, further contributing to resistance (Glen and Lamont, 2024). *P. aeruginosa* has been examined for innate causes that contribute to biofilm formation. *P. aeruginosa* DNA microarray analysis detected that a small fraction of the genome (approximately 1%) shows differential expression during biofilm growth, with about 0.5% of the genes being repressed and 0.5% of the genes being activated (Whiteley *et al.*, 2001). *Pseudomonas aeruginosa* isolates that carry the *lasR* and *rhlR* genes are believed to be the most critical factors that control the expression of virulence factors and biofilm formation. The *lasR* gene, especially, is positively regulated in biofilm formation, proteolytic, pyocyanin, and rhamnolipid biosynthesis. Similarly, the quorum-sensing regulator *rhlR* enhances protease and rhamnolipid synthesis (Elnegery *et al.*, 2021).

Despite the established role of quorum-sensing systems in regulating virulence and biofilm formation in *Pseudomonas aeruginosa*, there is a lack of local data on the coexistence of antimicrobial resistance with biofilm-forming ability and the presence of the *rhII* gene in clinical isolates. The combined assessment of these factors will provide a more holistic understanding of this organism's pathogenic behavior in the clinical setting. To this end, the study will explore trends in antibiotic resistance, biofilm-forming capacity, and *rhII* gene expression in clinical isolates of *Pseudomonas aeruginosa* using a hybrid methodology that combines phenotypic and molecular methods.

## 2. MATERIALS AND METHODS:

### 2.1. Study design and sample collection:

The 35 samples were collected from various clinical sources, including burn swabs, urine, stool, wound swabs, ear swabs, throat

swabs, and blood samples. All specimens were handled according to standard microbiological procedures (Wilson, 1996). The sample size was determined based on the available clinical isolates at the time of the study, and all samples were collected from patients with a confirmed or suspected bacterial infection.

### 2.2. Isolation and Identification

Clinical specimens were transported to the laboratory in transport media, inoculated onto a MacConkey's agar plate, and incubated at 37 °C for 18-24 hours. The primary diagnosis of isolates was made using different media and biochemical tests, as described by Figueroa-Bossi *et al.* (2022). The final diagnoses of the isolates were confirmed biochemically using the VITEK 2 automated system (GN ID card).

#### 2.2.1. GN-ID Identification through VITEK 2 Compact System

According to the manufacturer's instructions, the isolates were diagnosed using the VITEK-2 Compact system (bioMérieux, France) with GN ID cards.

### 2.3. Antimicrobial susceptibility testing

The Kirby-Bauer method was followed as described by Peterson *et al.* (1980) to determine antibiotic susceptibility to 8 antibiotics (ticarcillin, ceftriaxone, imipenem, novobiocin, amikacin, ciprofloxacin, trimethoprim, and chloramphenicol). A bacterial suspension was prepared by picking one to two well-isolated colonies from the original culture and placing them in a test tube containing 4 mL of normal saline to yield a suspension of moderate turbidity, as measured by DENSICHEK (0.5–0.63). A portion of the bacterial suspension was transferred and uniformly distributed on Mueller-Hinton agar using a sterile cotton swab, then incubated for 10 minutes. Next, the antibiotic discs were inoculated onto the agar surface with sterile forceps and pressed firmly to ensure adequate contact. The plates were then inverted and incubated at 37 °C for 18-24 hours. According to the Clinical and Laboratory Standards Institute (CLSI) standards, the diameter of the inhibition zone around antimicrobial discs was measured in millimeters (mm) using a metric ruler (Weinstein and Lewis, 2020). The sensitivity of the individual isolates was later established by comparing the observed inhibition zones with the standard criteria in the literature and categorizing them into three groups: susceptible, intermediate, and



resistant to the antimicrobial agent in question. All findings were read according to CLSI (2025) recommendations for standardized breakpoint values.

## 2.4. Biofilm assay

Each experiment was carried out three times, and the mean optical density (OD) was calculated. Negative control wells containing sterile broth have been added, and the cut-off optical density (OD<sub>c</sub>) was set at the average OD of the negative control plus 3 standard deviations. Table 1 summarizes the classification of biofilm formation depending on the OD values.

### 2.4.1. Tissue culture plate method (TCP)

The most commonly used method of biofilm assessment is the tissue culture plate (TCP) biofilm detection methodology, as identified by Christensen *et al.* (1985), and it is considered the standard method for assessing biofilm formation.

## 2.5. DNA Extraction

Genomic DNA was extracted using a commercially available extraction kit (Promega Genomic DNA Kit). A *Pseudomonas aeruginosa* suspension was prepared by inoculating Luria broth with the bacteria and incubating at 37 °C for 24 hr. DNA isolation was performed using a Promega kit. The entire process involved taking 1 ml of suspension and centrifuging it at 14000 rpm for 1 min. The supernatant is then discarded to obtain a lysate. 200 µL of GT solution is added for precipitation, and the precipitated cells are resuspended by vigorous shaking and incubated at room temperature for 5 minutes. Next, 200 µL of GB solution is added to the tube, mixed vigorously for 5 seconds, and incubated at 60 °C for at least 10 minutes. After lysis, 200 µL of absolute ethanol is added to the tube, and the mixture is immediately shaken vigorously for 10 seconds. The GD column tube was placed in a collection tube. The components were transferred to the GD column tube and centrifuged for 2 minutes. The collection tubes containing the flux were discarded.

400 µl of W1 was added to the GD column tube, then centrifuged for 30 seconds. The flux was discarded, and the GD column tube was returned to the 2 ml collection container. tube. 600 µL of wash buffer was added to the GD column tube, and the mixture was centrifuged for 30 seconds. The flow-through in the collection tube was discarded, and the GD column tube was placed back in the 2ml collection tube

tube and centrifuged again for 3 min. to dry the column matrix. Finally, the dried GD column tube was placed in a clean 1.5 ml centrifuge tube, and 100 µL elution buffer was added; the mixture was left for 3 min. Then, the DNA was centrifuged for 30 seconds to elute the target DNA solution, which was stored at -20 °C.

## 2.6. PCR amplification of *rhII*

The *rhII* gene, involved in biofilm formation in *Pseudomonas aeruginosa*, was detected by conventional PCR. The primers were designed by Alpha DNA (Canada) according to Edward *et al.* (2023) with the following sequences: The amplification of a 245 bp fragment was carried out using the forward primer (5'-CTCTCTGAATCGCTGGAAGG-3') and the reverse primer (5'-GCGAAGACTTCCTTGAGCAG-3'). PCR amplification of the *rhII* gene was conducted using the thermal cycling conditions listed in Table 2. The PCR products were subsequently amplified and subjected to electrophoresis on a 1% agarose gel to verify the expected fragment size. 4 µL of 10 mg/mL ethidium bromide was used to stain the gel, and 80 V was applied for 90 minutes. The bands of DNA were visualized using a UV transilluminator (Cleaver, UK) and recorded with a gel documentation system (Cleaver, UK). A 100 bp fragment was added to the dish to determine the size of the amplified fragments. PCR was performed in a 25 µL master mix containing primers and template DNA, with a total volume of 25 µL, under standard PCR conditions. The same reaction was used for the negative control, but without a DNA template.

## 2.7. Statistical analysis

Statistical analysis was performed using SPSS version 30 (IBM, USA). Categories were defined as means and percentages. Relationships between categories were analyzed using Fisher's exact test for small samples, and significance was determined at  $p < 0.05$ . Biofilm formation categories and the extent of antibiotic resistance were used to group the data in the current study. The *rhII* gene was detected in all 15 isolates, with a prevalence of 100%. The confidence interval (CI) was calculated using the Clopper-Person method.

# 3. RESULTS

## 3.1. Results

### 3.1.1. Isolation and identification of *P. aeruginosa*

A total of 35 clinical specimens were collected from different sources. Among these, 25 samples showed bacterial growth, while 10 did

not. Based on cultural and biochemical characteristics, 20 isolates were initially suspected to be *Pseudomonas* species. Based on VITEK-2 confirmation, 15 isolates were identified as *Pseudomonas aeruginosa*. These isolates formed non-lactose-fermenting colonies on MacConkey agar, produced pyocyanin pigment, were highly positive in oxidase and motility tests, and were strongly positive in the K/K reaction on triple sugar iron (TSI) medium (Brook and Frazier, 1995). On MacConkey agar, 15 isolates were identified as lactose non-fermenters, whereas 10 isolates were identified as other bacterial species. The identification was finally conducted by placing the samples in the automated VITEK-2 Compact system with GN-ID cards, which contain 47 biochemical tests and a single negative control well. The VITEK-2 system confirmed all suspected isolates, with identification confidence levels very good to excellent and probability values between 96% and 99%. Identification of isolates by sample source showed that the most frequent sources of *P. aeruginosa* were wound and burn specimens, followed by urine and ear swab samples.

### 3.1.2. Antibiotic susceptibility test

The antibiotic susceptibility of the 15 *Pseudomonas aeruginosa* clinical isolates was evaluated using the Kirby–Bauer disk diffusion method according to CLSI (2025) guidelines. Eight antibiotics representing various antimicrobial classes were evaluated. Table 3 shows the antimicrobial susceptibility profiles of the isolates. Notably, ticarcillin, ceftriaxone, and ciprofloxacin showed full resistance (100%), whereas relatively low rates of resistance were observed with imipenem (9%) and amikacin (5%). These results are illustrated graphically in Figure 1.

### 3.1.3. Biofilm formation in *P. aeruginosa* isolates.

All *Pseudomonas aeruginosa* isolates formed biofilms, as determined using the tissue culture plate (TCP) method. These isolates, 9 (60%) were strong biofilm producers, and 6 (40%) showed moderate biofilm-forming ability. None of the isolates was classified as weak or non-biofilm formers.

### 3.1.4. Biofilm formation and antimicrobial resistance

The results of the statistical analysis, according to Fisher's test, showed highly significant differences ( $P$ -Value = 0.004) between the biofilm formation categories and the extent of antibiotic resistance. The results showed that all isolates had a strong ability to form biofilms; 9 were completely resistant to antibiotics (100%), and none were sensitive. This indicates a strong

positive correlation between biofilm formation and the severity of antibiotic resistance, as shown in Table 5. Among isolates with moderate biofilm-forming capacity, 6 (66.7%) were resistant, and 3 (33.3%) were non-resistant. This indicates that biofilm formation intensity decreases with decreasing resistance.

### 3.1.5. *rhlI* Gene Detection

The *rhlI* gene was screened for using a predictive polymerase chain reaction (PCR) in all 15 *Pseudomonas aeruginosa* isolates. These outcomes revealed that no negative isolates were detected (100% positive), and each produced a clear 245 bp amplification band, as shown in Figure 2. The negative control used showed no band. The *rhlI* gene was detected in all 15 isolates, with a prevalence of 100%. The confidence interval (CI) calculated using the Clopper-Pearson method ranged from 78.2% to 100%, as shown in Table 6.

## 3.2. DISCUSSION

The obvious differences in resistance patterns suggest the presence of multiple resistance mechanisms underlying the isolates. The findings of this study are consistent with the prior literature on multidrug resistance in *Pseudomonas aeruginosa* (Bassetti *et al.*, 2018; El Zowalaty *et al.*, 2015). A combination of factors, such as reduced membrane permeability, activation of excretory pump systems, and the formation of antibiotic-inactivating enzymes, can explain this resistance. The high levels of resistance detected in this research have critical clinical implications, as they could limit the number of available treatment options and underscore the importance of ongoing antimicrobial surveillance.

The results showed that biofilm formation was a prevalent property of clinical isolates of *P. aeruginosa*, which can improve their survival and persistence in the clinical setting by increasing resistance. Biofilm development is one of the fundamental adaptive strategies by which bacteria can adhere to surfaces and grow within a self-assembled extracellular polymeric matrix of polysaccharides, proteins, and extracellular DNA. The matrix also provides resistance to environmental stresses and antimicrobial agents, which enhances bacterial resistance to antibiotics (Hall-Stoodley *et al.*, 2004; Mann and Wozniak, 2012; Kunwar *et al.*, 2021). The biofilm-forming capacity of the isolates was described in Table 4, and 60% and 40% were strong and moderate producers, respectively. This difference aligns with previous studies reporting heterogeneity in *P. aeruginosa* biofilm production among clinical

isolates (Chimi *et al.*, 2024). Biofilm formation is a highly controlled, dynamic process that generally undergoes phases of initial adhesion, microcolony development, maturation, and eventual dissemination, thereby supporting colonization of new environments (Hall-Stoodley *et al.*, 2004). The high rates of antibiotic resistance could be partly explained by the majority of strong biofilm-forming isolates in the present study, which form biofilms that serve as physical barriers, restricting antibiotic penetration and helping bacteria survive. These findings raise the possibility of a relationship between biofilm-forming capacity and antimicrobial resistance in the investigated isolates.

Ratajczak *et al.* (2021) mentioned that the majority of strains (73.6%) were strong biofilm producers, 17.0% were moderate, and 9.4% were weak biofilm producers. Both multiple antibiotic resistance and biofilm formation may complicate the treatment of *P. aeruginosa* infections. The present study showed that all *P. aeruginosa* strains isolated from clinical specimens formed biofilms *in vitro*. However, they differed in the degree of biofilm formation. Most strains showed strong biofilm-forming capacity (74.0%), while significantly fewer strains exhibited moderate (16.4%) and weak (9.6%) biofilm-forming potential. The study demonstrated that all *Pseudomonas aeruginosa* strains isolated from various clinical samples formed biofilms under laboratory conditions; however, they differed in the extent of biofilm formation. A 2018 study by da Costa Lima *et al.* estimated biofilm-forming ability and found that 77.5% of clinical *P. aeruginosa* strains were capable of forming biofilm. (Da Silva Carvalho & Perez, 2019) found that 86.5% of *P. aeruginosa* strains studied were classified as strong or moderate biofilm producers.

This observation indicates a high prevalence of this quorum-sensing gene among the clinical isolates studied. The *rhII* gene is another important component of the quorum-sensing system in *P. aeruginosa*, which controls the synthesis of signaling molecules involved in biofilm formation and the expression of several virulence factors. Quorum-sensing systems, such as *rhII*-mediated responses, allow bacterial communication and are critical for biofilm formation and maintenance (De Kievit *et al.*, 2001; Elnegery *et al.*, 2021). The prevalence of the *rhII* gene across all isolates is also consistent with earlier studies highlighting its role in biofilm formation and pathogenicity. Disruption of *rhII*-mediated signaling was shown to affect biofilm development and decrease bacterial virulence,

highlighting the importance of this functional process (Favre-Bonté *et al.*, 2003).

Nevertheless, the presence of the *rhII* gene may not always guarantee active quorum-sensing activity or direct participation in biofilm formation regulation. Further studies are needed to determine the functional role of gene expression levels in clinical conditions. Thus, its presence in detecting possible participation; further molecular research is required to clarify its specific role in biofilm formation and antimicrobial resistance.

## 4. CONCLUSIONS

The study demonstrates that biofilm-forming capability, antimicrobial resistance, and the ubiquitous presence of the *rhII* gene occur concomitantly in clinical isolates of *Pseudomonas aeruginosa*. These results indicate the demand for routine screening of biofilm-producing bacteria and quorum-sensing-related genes in hospital labs. Future studies should focus on the quantitative analysis of the *rhII* gene expression under different environmental and antimicrobial stress conditions.

## 5. DECLARATIONS

### 5.1. Study Limitation

The sample size used is relatively small, which might limit the scope of the findings. Also, an antimicrobial susceptibility test by disk diffusion was performed without confirmation by minimum inhibitory concentration (MIC). Bonferroni or other multiple-comparison correction was not applied to the eight antibiotic susceptibility comparisons. It was also restricted to *rhII* gene detection, with no evaluation of its expression level or functional activity. In addition, the analysis did not include other genes associated with quorum sensing and biofilm.

### 5.2. Acknowledgments

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### 5.3. Funding Sources

This research received no external funding. The authors funded this research.

#### 5.4. Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this manuscript.

#### 5.5. Data Availability

All data presented in this study are available in the manuscript tables and figures. Raw data are available upon request from the corresponding author.

#### 5.6. Author Contribution

The author confirms sole responsibility for the following: study conception and design, sample processing, laboratory analysis, data interpretation, and manuscript preparation.

| Code | English                          | Português                        |
|------|----------------------------------|----------------------------------|
| CD   | Conception and Design            | Concepção e Design               |
| DC   | Data Collection                  | Coleta de Dados                  |
| DAI  | Data Analysis and Interpretation | Análise e Interpretação de Dados |
| MW   | Manuscript Writing               | Escrita do Manuscrito            |
| CR   | Critical Review                  | Revisão Crítica                  |
| FA   | Final Approval                   | Aprovação Final                  |

#### 5.7. AI and Computational Tools Declaration

Tool: Grammarly (Grammarly Inc.), QuillBot (QuillBot Inc.), Turnitin (Turnitin LLC). Purpose: Grammarly was used for grammar and language editing. QuillBot was used for limited paraphrasing in the introduction section. Turnitin was used for plagiarism detection and similarity checking.

Extent: The use of AI tools was limited to language improvement and minor text refinement (less than 10% of the manuscript).

Human Verification: All outputs generated or modified using AI tools were carefully reviewed, validated, and substantially revised by the authors. No AI tools were used for data analysis, statistical testing, results interpretation, or scientific decision-making.

#### 5.8. Research Integrity Declaration

The author confirms that this research was conducted in accordance with accepted standards of scientific integrity. The data presented in this study are original, have not been fabricated or

manipulated, and accurately reflect the research findings. The manuscript has not been published previously and is not under consideration for publication elsewhere.

#### 5.9. Originality & Plagiarism Statement

The authors declare that this is an original manuscript and have attached the Turnitin similarity index, which was less than 20%.

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### 6. STUDIES INVOLVING HUMAN AND ANIMAL SUBJECTS

#### 6.1. Ethics Committee Approval

Ethical approval for this study was obtained from the ethics committee at the University of Alkafel, College of Medical and Health Technologies, with reference number HMT 05/2024 on April 8/2024

The requirement for informed consent was waived due to the study's design. All data were anonymized and handled in accordance with corporate confidentiality and data governance policies.

#### 6.2. INFORMED CONSENT

Informed consent was waived because the study was conducted on anonymized clinical isolates collected as part of routine diagnostic procedures, without direct patient intervention.

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**Table 1.** Classification of biofilm formation based on OD values (Christensen et al., 1985)

| Mean of OD value at 630 nm' | Biofilm formation |
|-----------------------------|-------------------|
| <0.120                      | Non               |
| 0.120-0.240                 | Moderate          |
| >0.240                      | High              |

**Table 2.** PCR program of (rhII) primer that applies in the thermocycler

| Gene        | Initial denaturati on | Denaturati on    | Annealing      | Extension       | No. of cycles | Final extension |
|-------------|-----------------------|------------------|----------------|-----------------|---------------|-----------------|
| <i>rhII</i> | 94 °C for 3 min       | 94 °C for 30 sec | 55.5 °C 30 sec | 72 °C for 1 min | 30            | 72 °C for 5 min |

**Table 3.** Antimicrobial susceptibility profile of *Pseudomonas aeruginosa* isolates (n=15)

| Antibiotic      | Abbreviate | Concentration (µg/disk) | Resistance No. (%) | Intermediated No. (%) | Sensitive No. (%) |
|-----------------|------------|-------------------------|--------------------|-----------------------|-------------------|
| Ticarcillin     | TIC        | 30                      | 100%               | 0%                    | 0%                |
| Ceftriaxone     | CRO        | 30                      | 100 %              | 0 %                   | 0%                |
| Imipenem        | IMP        | 10                      | 9%                 | 11 %                  | 80%               |
| Novobiocin      | NOV        | 30                      | 96 %               | 4 %                   | 0%                |
| Amikacin        | AK         | 10                      | 5%                 | 0%                    | 95%               |
| Ciprofloxacin   | CIP        | 10                      | 100 %              | 0 %                   | 0%                |
| Trimethoprim    | TMP        | 10                      | 90%                | 7%                    | 3%                |
| Chloramphenicol | CHL        | 30                      | 93 %               | 7 %                   | 0%                |

**Table 4.** Distribution of biofilm formation among *P. aeruginosa* isolates

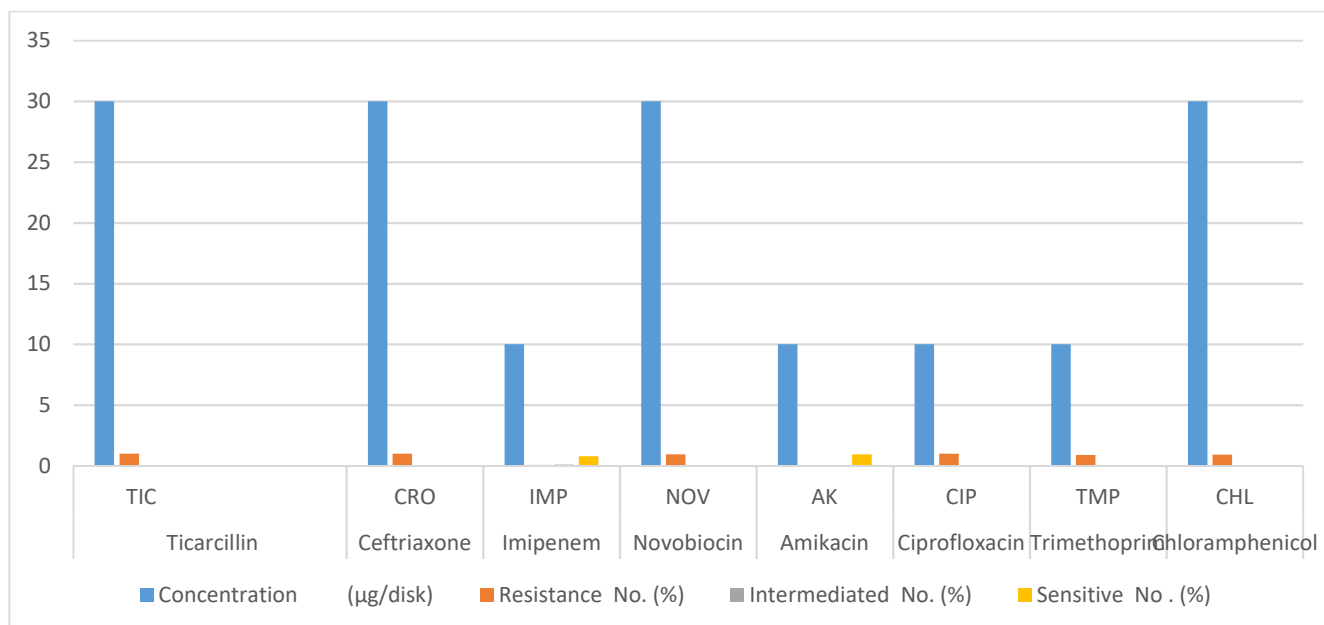
| Biofilm category | Number of isolates | Percentage (%) |
|------------------|--------------------|----------------|
| Strong           | 9                  | 60%            |
| Moderate         | 6                  | 40%            |
| Weak             | 0                  | 0%             |

**Table 5.** Association between biofilm formation and antimicrobial resistance among *Pseudomonas aeruginosa* isolates (n = 15)

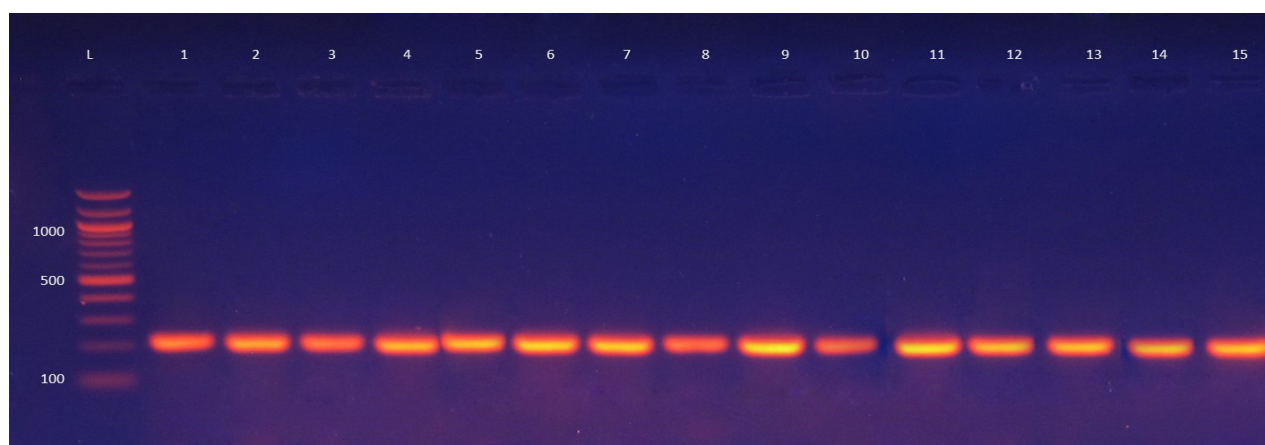
| Biofilm Category | Resistant N (%) | Non Resistant N (%) | Total | P-Value |
|------------------|-----------------|---------------------|-------|---------|
| Strong           | 9 (100%)        | 0                   | 9     | 0.004*  |
| Moderate         | 4 (66.7%)       | 2 (33.3%)           | 6     |         |
| Total            | 13              | 2                   | 15    |         |

\*P-Value calculated by using Fisher's exact test

| <b>Table 6. <i>rhII</i> gene prevalence among clinical isolates</b> |             |                                                       |
|---------------------------------------------------------------------|-------------|-------------------------------------------------------|
| Total number                                                        | Prevalence% | Interval coincident Clopper-Pearson95%<br>(78.2-100 ) |
| 15                                                                  | 100%        |                                                       |



**Figure 1.** Antimicrobial susceptibility profile of *P. aeruginosa* isolates



**Figure 2.** PCR amplification products of *P. aeruginosa* isolates amplified with *rhII* gene primers, yielding a 245 bp product. Lane (L), DNA molecular size marker (100-bp ladder). Lanes (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15) show positive results for the *rhII* gene.



URBANIZAÇÃO, CONSCIÊNCIA E A CRESCENTE CARGA DAS DOENÇAS ALÉRGICAS EM CRIANÇAS: UMA AVALIAÇÃO DE SAÚDE PÚBLICA EM OSH, QUIRGUIZSTÃO

URBANIZATION, AWARENESS, AND THE RISING BURDEN OF ALLERGIC DISEASES IN CHILDREN: A PUBLIC HEALTH ASSESSMENT FROM OSH, KYRGYZSTAN

УРБАНИЗАЦИЯ, ОСВЕДОМЛЕННОСТЬ И РОСТ БРЕМЕНИ АЛЛЕРГИЧЕСКИХ ЗАБОЛЕВАНИЙ У ДЕТЕЙ: ОЦЕНКА ОБЩЕСТВЕННОГО ЗДОРОВЬЯ В ГОРОДЕ ОШ, КЫРГЫЗСТАН

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## RESUMO

**Introdução:** As doenças alérgicas constituem uma crescente preocupação de saúde pública globalmente, impulsionadas pela urbanização, poluição ambiental e mudanças no estilo de vida. Compreender a consciência populacional e as tendências epidemiológicas de longo prazo é essencial para prevenção eficaz e organização dos cuidados de saúde. **Objetivo:** Determinar o papel da saúde pública no aprimoramento da organização dos cuidados médicos para doenças alérgicas na cidade de Osh, Quirguistão, considerando características epidemiológicas, acessibilidade e qualidade dos serviços médicos. **Métodos:** Foi conduzido um estudo transversal, incluindo uma pesquisa por questionário estruturado com 300 pacientes do Centro de Medicina Familiar de Osh (2023–2024) para avaliar a consciência sobre causas de alergia, fatores de risco, métodos de prevenção e fontes de informação. Paralelamente, foi realizada uma análise retrospectiva de dados oficiais de morbidade (2003–2023) para rinite alérgica, asma brônquica e dermatite atópica em diferentes faixas etárias. **Resultados:** A pesquisa revelou alta prevalência de doenças alérgicas (63,3% dos respondentes ou seus familiares). Poluição ambiental (25,0%), imunidade enfraquecida (21,7%) e hereditariedade (20,0%) foram identificados como os principais fatores de risco. A consciência sobre imunoterapia específica para alérgenos foi baixa (16,7%). Mídias sociais (30,0%), internet (28,3%) e médicos (26,7%) foram as principais fontes de informação. Os dados epidemiológicos mostraram uma tendência decrescente nas doenças alérgicas entre adultos ao longo de 20 anos, mas um aumento significativo entre crianças, especialmente para dermatite atópica e asma brônquica. **Discussão:** A divergência entre a melhoria dos resultados em adultos e o agravamento das tendências pediátricas destaca a necessidade de estratégias de saúde pública específicas por idade. A consciência limitada sobre métodos modernos de prevenção e o predomínio de fontes digitais de informação sublinham oportunidades para educação direcionada. **Conclusões:** Embora os resultados de saúde dos adultos

tenham melhorado, as crescentes taxas de alergia em crianças demandam intervenções urgentes de saúde pública. Fortalecer a alfabetização em saúde, aprimorar o papel dos provedores de cuidados primários e implementar programas ambientais e educacionais direcionados são essenciais.

**Palavras-chave:** *Saúde pública; asma brônquica; dermatite atópica; rinite alérgica; literacia em saúde; saúde infantil.*

## ABSTRACT

**Background:** Allergic diseases are a growing concern in global public health. Urbanization, environmental pollution, and lifestyle changes contribute to this rise. Understanding what the population knows and how disease rates change over time is necessary for better prevention and healthcare planning. **Aim:** This study aimed to assess the role of public health in improving medical care for allergic diseases in Osh, Kyrgyzstan. We considered local epidemiological patterns, service accessibility, and quality of care. **Methods:** We used a cross-sectional design. A structured questionnaire was given to 300 patients at the Osh Family Medicine Center during 2023–2024. It covered causes of allergies, risk factors, prevention methods, and information sources. In parallel, we analyzed official morbidity data from 2003 to 2023 for allergic rhinitis, bronchial asthma, and atopic dermatitis across age groups. **Results:** The survey found a high prevalence of allergic diseases – 63.3% of respondents or their family members had allergies. Main risk factors identified by respondents were environmental pollution (25.0%), weakened immunity (21.7%), and heredity (20.0%). Only 16.7% knew about allergen-specific immunotherapy. Leading information sources were social media (30.0%), the internet (28.3%), and doctors (26.7%). Epidemiological data showed a decrease in allergic diseases among adults over 20 years, but a significant increase among children, especially for atopic dermatitis and bronchial asthma. **Discussion:** Adult outcomes have improved, but childhood allergy rates are rising. This suggests a need for age-specific public health strategies. Limited awareness of modern prevention and the dominance of digital information sources point to opportunities for targeted education. **Conclusions:** Rising allergy rates in children require urgent public health interventions. Better health literacy, stronger primary care involvement, and targeted environmental and educational programs are needed.

**Keywords:** *Public health; bronchial asthma; atopic dermatitis; allergic rhinitis; health literacy; children's health.*

## АННОТАЦИЯ

**Введение:** Аллергические заболевания представляют собой растущую проблему общественного здравоохранения во всем мире, обусловленную урбанизацией, загрязнением окружающей среды и изменением образа жизни. Понимание информированности населения и долгосрочных эпидемиологических тенденций необходимо для эффективной профилактики и организации медицинской помощи. **Цель:** Определить роль общественного здоровья в совершенствовании организации медицинской помощи при аллергических заболеваниях на примере города Ош, Кыргызстан, с учетом эпидемиологических особенностей, доступности и качества медицинских услуг. **Методы:** Проведено поперечное исследование, включавшее анкетирование 300 пациентов Центра семейной медицины города Ош (2023–2024) для оценки информированности о причинах аллергии, факторах риска, методах профилактики и источниках информации. Параллельно проведен ретроспективный анализ официальных данных заболеваемости (2003–2023) по аллергическому риниту, бронхиальной астме и атопическому дерматиту в разных возрастных группах. **Результаты:** Анкетирование выявило высокую распространенность аллергических заболеваний (63,3% респондентов или членов их семей). Загрязнение окружающей среды (25,0%), ослабленный иммунитет (21,7%) и наследственность (20,0%) были определены как основные факторы риска. Осведомленность об аллерген-специфической иммунотерапии была низкой (16,7%). Основными источниками информации стали социальные сети (30,0%), интернет (28,3%) и врачи (26,7%). Эпидемиологические данные показали снижение заболеваемости аллергическими болезнями среди взрослых за 20 лет, но значительный рост среди детей, особенно по атопическому дерматиту и бронхиальной астме. **Обсуждение:** Расхождение между улучшением показателей у взрослых и ухудшением у детей подчеркивает необходимость возрастных стратегий общественного здоровья. Ограниченная осведомленность о современных методах профилактики и доминирование цифровых источников информации указывают на возможности для целенаправленного просвещения. **Выводы:** Несмотря на улучшение здоровья взрослых, рост аллергических заболеваний у детей требует неотложных мер общественного здравоохранения. Укрепление санитарной грамотности, усиление роли первичного звена и реализация целевых экологических и образовательных программ являются необходимыми.

**Ключевые слова:** *Общественное здоровье; бронхиальная астма; атопический дерматит;*

## 1. INTRODUCTION:

Allergic diseases are among the leading public health challenges. Worldwide, an estimated 30-40% of people suffer from some form of allergy (World Health Organization, 2021; Pawankar et al., 2020). Conditions like allergic rhinitis, bronchial asthma, atopic dermatitis, and food allergies lower quality of life and create large economic costs for health systems. According to the Global Burden of Disease Study, about 262 million people have asthma and more than 400 million have allergic rhinitis (GBD 2019 Asthma Collaborators, 2022). Atopic dermatitis is one of the most common chronic skin diseases in childhood; in some urban areas its prevalence exceeds 20% (Langan et al., 2020).

Several factors explain the rising allergy burden. Urbanization, environmental pollution, changes in diet, reduced microbial exposure (the “hygiene hypothesis”), and climate change all play a role (Akdis, 2021; Cecchi et al., 2021). Low- and middle-income countries are urbanizing quickly, often faster than their healthcare infrastructure can adapt (Serebrisky & Wohl, 2021).

Central Asia, including the Kyrgyz Republic, is no exception. Osh, the second largest city, illustrates these trends well. With a population of over 300,000, the city has high levels of particulate matter, seasonal pollen peaks, and dense motor traffic – all factors that increase the risk of respiratory and allergic diseases (Toktogulova et al., 2020; Semernik et al., 2023). Economic constraints also limit access to specialized allergy services and modern therapies, such as allergen-specific immunotherapy (Moldobaeva et al., 2022).

Public health strategies for allergic diseases emphasize primary prevention, early diagnosis, and patient education (GINA, 2021; EAACI, 2022). A key element is health literacy – the ability to obtain, understand, and use health information (Nutbeam & Lloyd, 2021). Poor health literacy is linked to worse disease control, more emergency visits, and underuse of preventive treatments (Wood et al., 2020). Yet few studies have examined public awareness of allergic diseases in Central Asian cities.

In Kyrgyzstan, most previous research

focused on clinical descriptions or simple epidemiological counts. Little attention has been given to population knowledge, attitudes, and practices about allergy prevention (Sulaimanov et al., 2024; Annayeva, 2025). No study has combined a community awareness survey with a long-term (20-year) trend analysis of allergic disease incidence in Osh. This gap makes it hard to design evidence-based public health interventions.

We therefore addressed two questions: (1) What is the level of public awareness about causes, risk factors, and prevention of allergic diseases among patients attending primary care in Osh?

(2) How have allergic disease incidence rates changed from 2003 to 2023 across different age groups, especially children? We hypothesized that despite high disease burden, public awareness of modern preventive methods is low, and the gap between adult improvement and pediatric worsening calls for targeted action.

### 1.1. Aims

The primary aim was to determine the role of public health in improving allergy care in Osh, taking into account epidemiological factors, accessibility, and service quality. Secondary aims were to measure public awareness, describe 20-year incidence trends by age, and develop practical recommendations for prevention and care.

## 2. MATERIALS AND METHODS:

### 2.1. Materials

#### 2.1.1. Study Design and Setting

We used a descriptive, cross-sectional, and analytical design. The study took place in Osh, Kyrgyz Republic, during 2023–2024. It had two parts: a patient survey and a retrospective analysis of official morbidity data over 20 years.

#### 2.1.2. Survey Participants

We enrolled 300 adult patients (≥18 years) who visited the Osh Family Medicine Center, a primary care facility serving a mixed urban population. Patients were recruited consecutively during routine visits. Inclusion criteria were age ≥18 years, ability to complete the questionnaire, and voluntary consent. Inability to complete the questionnaire was the only exclusion criterion.

### 2.1.3. Questionnaire Instrument

Our team developed a structured questionnaire based on relevant literature and international allergy guidelines (GINA, 2021; EAACI, 2022). We pilot-tested it on 30 patients to assess clarity and face validity, then made minor wording adjustments. The final version had four sections:

- Section A: age and gender.
- Section B: awareness of allergy causes and risk factors (multiple choice, multiple answers allowed).
- Section C: knowledge of allergy prevention methods.
- Section D: sources of information about allergic diseases.

Questionnaires were anonymous. Completion took about 10-15 minutes.

### 2.1.4. Epidemiological Data Source

We obtained official statistical data from the Center for Electronic Healthcare of the Ministry of Health of the Kyrgyz Republic for the period 2003–2023. The data covered three disease categories: allergic rhinitis (hay fever), bronchial asthma, and atopic dermatitis with related conditions. Incidence indicators were extracted separately for adults/adolescents ( $\geq 15$  years) and children under 14 years. Rates were calculated as absolute numbers and per 100,000 population.

## 2.2. Methods

### 2.2.1. Data Collection

Trained medical staff (interviewers) collected the surveys at the Osh Family Medicine Center from January 2023 to December 2024. After obtaining informed consent, interviewers helped participants complete the questionnaire to reduce literacy-related barriers.

### 2.2.2. Statistical Analysis

We used descriptive statistics: absolute frequencies ( $n$ ) and relative frequencies (%). For epidemiological data, we computed incidence rates per 100,000 population per year. Group comparisons (e.g., awareness by age) used chi-square tests, with significance set at  $\alpha = 0.05$ . All analyses were performed with SPSS version 29.0 (IBM Corp., Armonk, NY, USA).

### 2.2.3. Data Availability

Raw data are available from the corresponding author upon reasonable request.

Summary data are presented in the tables and figures of this manuscript.

## 3. RESULTS AND DISCUSSION:

### 3.1. Results

#### 3.1.1. Characteristics of respondents

Of the 300 participants, 180 (60.0%) were women and 120 (40.0%) were men. Most participants (245, 81.7%) were of working age (18-59 years). The largest age group was 30-44 years (95, 31.7%), followed by 45-59 years (80, 26.7%), 18-29 years (70, 23.3%), and  $\geq 60$  years (55, 18.3%).

#### 3.1.2. Prevalence of Allergic Diseases

A total of 190 respondents (63.3%) reported having an allergic disease themselves or in a family member. The remaining 110 (36.7%) reported no allergies. The difference was significant ( $\chi^2 = 21.3$ ,  $p < 0.001$ ).

#### 3.1.3. Awareness of Allergy Causes and Risk Factors

When asked about causes of allergies, the most frequent answers were environmental pollution (75, 25.0%), weakened immunity (65, 21.7%), heredity (60, 20.0%), poor diet (50, 16.7%), and frequent medication use (40, 13.3%). "Other" causes were mentioned by 10 (3.3%). The top three causes accounted for 66.7% of all responses.

Risk factor awareness showed a similar pattern (Table 1). Environmental pollution was named by 75 (25.0%), weakened immunity by 65 (21.7%), heredity by 60 (20.0%), poor diet by 50 (16.7%), and frequent medication use by 40 (13.3%). Other factors were named by 10 (3.3%).

**Table 1.** Awareness of allergy risk factors among survey participants ( $n=300$ ).

#### 3.1.4. Knowledge of Allergy Prevention Methods

Avoiding contact with allergens was the most recognized preventive measure (80, 26.7%), followed by regular cleaning (70, 23.3%). Only 50 (16.7%) mentioned allergen-specific immunotherapy (ASIT). Healthy eating was named by 45 (15.0%), and strengthening the immune system by 40 (13.3%). Other methods were noted by 15 (5.0%) (Table 2).

**Table 2.** Awareness of allergy prevention methods among survey participants ( $n=300$ ).

### 3.1.5. Sources of Information About Allergic Diseases

Social media was the most frequently cited source (90, 30.0%), followed by the internet (85, 28.3%), and doctors (80, 26.7%). Television accounted for 25 (8.3%), newspapers/magazines for 10 (3.3%), and other sources for 10 (3.3%) (Table 3). Digital sources (social media and the internet) accounted for 58.3%.

**Table 3.** Sources of information about allergic diseases among survey participants (n=300).

### 3.1.6. Long-term Trends in Allergic Disease Incidence (2003–2023)

The official data revealed opposite trends between adults and children (Table 4). Over the 20 years, incidence rates of allergic diseases decreased substantially among adults and adolescents, but increased among children.

- **Respiratory diseases:** Among adults/adolescents, the incidence rate fell from 18,055.9 per 100,000 in 2003 to 4,497.3 per 100,000 in 2023 (a 75.1% reduction). In children, the incidence rose from 5,244.7 to 15,936.5 per 100,000 (a 204% increase).
- **Allergic rhinitis:** Adult/adolescent rates declined from 384.8 to 68.3 per 100,000 (82.2% reduction). Child rates decreased from 81.0 to 64.7 per 100,000 (20.1% decrease), but there were fluctuations, with a higher rate in 2013.
- **Bronchial asthma:** Adult/adolescent rates declined from 142.3 to 60.7 per 100,000 (57.4% reduction). In children, rates increased from 41.5 to 82.8 per 100,000 (99.5% increase).
- **Atopic dermatitis and related conditions:** Adult/adolescent rates declined from 165.6 to 75.0 per 100,000 (54.7% reduction). In children, rates increased from 138.8 to 163.9 per 100,000 (18.1% increase).

The most concerning changes were for atopic dermatitis and bronchial asthma in children, where incidence more than doubled.

**Table 4.** Incidence of allergic diseases in Osh city (2003–2023), rates per 100,000 population.

## 3.2. Discussion

This study provides a combined picture of public awareness and long-term allergy trends in Osh. The results show a split pattern: adults have seen real improvements over 20 years, but children have not. At the same time, public knowledge about allergy causes is decent, but knowledge of modern prevention – especially allergen-specific immunotherapy – is low. People rely more on digital media than on doctors for information.

### 3.2.1. Public Awareness of Allergic Diseases

The 63.3% of respondents reporting allergies in themselves or family members is higher than the official rates in Table 4. This is likely because our survey captured both diagnosed and undiagnosed cases, plus family history. The gap suggests underdiagnosis or underreporting in routine statistics, which calls for better diagnostic capacity in primary care.

Most respondents correctly identified environmental pollution, weakened immunity, and heredity as major risk factors. This basic understanding is encouraging. Pollution, mentioned by a quarter of respondents, is a real concern in Osh's urban environment (Toktogulova et al., 2020). Heredity (20%) matches the well-known genetic component of atopy (Langan et al., 2020). However, fewer people recognized poor diet and frequent medication use as risk factors, suggesting areas where health education could be improved.

A critical knowledge gap is the low awareness of ASIT – only 16.7%. ASIT is a disease-modifying treatment that can prevent progression from allergic rhinitis to asthma and provide long-term tolerance (GINA, 2021; EAACI, 2022). The low awareness probably reflects limited patient education and poor availability of this therapy in Kyrgyzstan's public system (Moldobaeva et al., 2022). Both clinicians and the public need more information about ASIT.

### 3.2.2. Information Sources and Health Literacy

The finding that social media (30%) and the internet (28.3%) are the top information sources aligns with global trends in digital health information seeking (Wood et al., 2020). Digital platforms offer a wide reach but also carry the risk of misinformation. Only 26.7% of respondents cited doctors as a source. This suggests that clinical encounters are underused for patient education. Patients with allergic diseases need ongoing advice on trigger avoidance, medication adherence, and recognizing exacerbations.

Structured patient education in primary care could improve disease control (Nutbeam & Lloyd, 2021). Short educational sessions, written materials, and trusted digital channels (e.g., clinic social media) could help close the gap.

### **3.2.3. Divergent Epidemiological Trends: Adults vs. Children**

The drop in allergic disease rates among adults is a positive sign. Better medical care, more accurate diagnosis, and greater awareness of allergen avoidance likely contributed. For asthma, wider use of inhaled corticosteroids may have helped (GINA, 2021). For allergic rhinitis and atopic dermatitis, better treatments and self-management could explain the improvement.

By contrast, children have seen a clear rise in allergic diseases, especially atopic dermatitis and bronchial asthma. Atopic dermatitis in children went from 138.8 to 163.9 per 100,000; asthma from 41.5 to 82.8 per 100,000. This matches global reports of increasing childhood allergies in urbanizing settings (Akdis, 2021; Cecchi et al., 2021). Several factors may explain the increase. First, environmental exposures: Osh's growth has increased traffic emissions, construction dust, and industrial activity, raising particulate matter and allergen levels (Semernik et al., 2023). Children's developing immune systems are more vulnerable. Second, lifestyle changes: less physical activity, more time indoors, and dietary shifts toward processed foods may promote inflammation and reduce microbial exposure – the hygiene hypothesis (Serebrisky & Wohl, 2021). Third, early-life factors such as limited breastfeeding or inappropriate weaning could contribute, though we did not measure them. Fourth, increased awareness among pediatricians and parents might have led to better detection, but the consistency across multiple disease categories suggests a true epidemiological rise.

These divergent trends tell us that we cannot use the same public health strategy for children as for adults. Pediatric prevention needs its own focus.

### **3.2.4. Public Health Implications**

Our findings have several practical implications for Osh and similar Central Asian cities. First, the rising childhood allergy burden calls for targeted prevention programs. These could include environmental measures (monitoring and reducing air pollution, controlling indoor allergens in schools and kindergartens), early diagnosis (adding allergy screening to routine pediatric visits for children with a family history of atopy), and parental education about

allergen avoidance and weaning practices.

Second, limited awareness of modern prevention strategies (such as ASIT) suggests that continuing medical education for primary care physicians should include updates on allergy management. Referral pathways to allergists should be established where possible, and public funding for ASIT should be explored.

Third, the dominance of digital media offers an opportunity: health authorities can engage with the public through social media, using clinic-affiliated channels or trusted local influencers to share accurate content. Proactive digital health literacy campaigns are needed to counter misinformation.

Finally, progress in adult allergic disease control should not lead to complacency. Monitoring of emerging risks (e.g., climate-related pollen changes) and continued access to effective treatments are essential.

## **4. CONCLUSIONS:**

Allergic diseases are very common in Osh, especially in children. Adult rates have decreased by **57–82%** over the past 20 years (depending on the disease category), likely due to better medical care and increased awareness, likely due to better medical care and increased awareness. Child rates, however, have increased – most notably for atopic dermatitis and bronchial asthma. Public awareness of risk factors such as pollution and heredity is good, but knowledge of modern preventive methods (allergen-specific immunotherapy) remains low. Most people get information from digital media rather than doctors. The following priorities emerge for public health action:

- Strengthen primary prevention in children through environmental improvements (air quality monitoring, indoor allergen control) and parent education about early-life risk factors.
- Increase health literacy by incorporating allergy education into routine primary care consultations and using trusted digital platforms to spread evidence-based information.
- Improve access to modern therapies (allergen-specific immunotherapy) by raising clinician awareness and exploring sustainable funding mechanisms.
- Maintain adult disease control by preserving access to effective treatments and watching for emerging environmental threats.

Addressing the rising burden of childhood allergies will require coordinated work across the health, environmental, and education sectors. This study provides baseline data for targeted intervention design and highlights the important role of public health in Central Asian cities.

## 5. DECLARATIONS

### 5.1. Study Limitations

Several limitations should be considered. Our survey was done at a single family medicine center, so results may not apply to all of Osh. Convenience sampling may have introduced selection bias. We relied on self-reports, which are subject to recall and social desirability bias; we did not clinically confirm allergic diseases. The cross-sectional design does not allow causal inferences. The retrospective epidemiological analysis over 20 years may be affected by changes in diagnostic criteria and reporting practices. The questionnaire was not formally validated against a gold standard. We did not collect data on socioeconomic status, education level, or detailed environmental exposures, which could confound the results.

Additionally, the 2013 incidence data for several disease categories (e.g., respiratory diseases in adults, bronchial asthma and atopic dermatitis in children) show non-monotonic patterns that deviate from the overall 20-year trends. These anomalies likely reflect a systemic change in diagnostic criteria, disease coding (e.g., transition between ICD revisions), or data collection methodology within the Kyrgyz healthcare system around 2013, rather than true epidemiological fluctuations. Direct comparisons between 2003 and 2023 remain valid for assessing long-term trends, but intermediate year values should be interpreted with caution. This limitation is now explicitly acknowledged.

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### 5.3. Funding Source

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resources.

In accordance with the ethical guidelines of the Periódico Tchê Química, which do not allow donations from authors with manuscripts under evaluation (even when research funds are available), or in cases of authors' financial constraints, publication costs were fully absorbed by the journal under our Platinum Open Access policy, through the support of the Araucária Scientific Association (<https://acaria.org/>). This policy aims to ensure complete independence between the editorial process and any financial aspects, reinforcing our commitment to scientific integrity and equity in knowledge dissemination.

### 5.4. Conflicts of Interest

The authors declare no conflicts of interest.

### 5.5. Data Availability

Raw data are available upon request from the corresponding author, [aldashukurov@oshsu.kg](mailto:aldashukurov@oshsu.kg), due to participant confidentiality.

### 5.6. Author Contributions

Zarema A. Boronchieva: study conception and design, manuscript editing, supervision, final approval.

Yrysbek A. Aldashukurov: manuscript editing, final approval.

Aichurok S. Amirakulova: data collection, literature review, data analysis and interpretation, manuscript writing, final approval.

Turdaali Galalov: literature review, data processing, final approval.

Bektur A. Abdymanapov: literature review, data processing, final approval.

Orozgul Jalalova: literature review, data processing, final approval.

Nurgul A. Alimova: literature review, data processing, final approval.

### 5.7. AI and Computational Tools Declaration

After completing the full draft, the authors used ChatGPT 4.0 (OpenAI) only for minor grammar and language correction. The tool was applied to less than 10% of the text (mostly fixing awkward phrasing). No AI was used for data generation, statistical analysis, result interpretation, or scientific decision-making. All content was reviewed and approved by the

authors.

### 5.8. Research Integrity Declaration

The authors certify that this research meets the standards of research integrity: no data fabrication, no results falsification, no p-hacking or selective reporting, original work not previously published, and methods conducted ethically.

### 5.9. Originality & Plagiarism Statement

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## 6. STUDIES INVOLVING HUMAN AND

## ANIMAL SUBJECTS

### 6.1. Ethical Approval

This study was approved by the Ethics Committee of the Southern Branch of the Research Institute of Medical and Biological Problems, National Academy of Sciences of the Kyrgyz Republic (Protocol No. 6, dated February 14, 2023). The study was conducted in accordance with the Declaration of Helsinki. A digitized copy of the ethics approval letter is provided as Supplementary Material.

### 6.2. Informed Consent

Written informed consent was obtained from all 300 participants prior to data collection. Participants were informed about the study purpose, procedures, risks, and benefits. Data were collected anonymously, and all personal identifiers were removed to protect participant confidentiality.

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**Table 1.** Awareness of allergy risk factors among survey participants (n=300).

| Risk factor             | n          | % of total responses |
|-------------------------|------------|----------------------|
| Environmental pollution | 75         | 25.0                 |
| Weakened immunity       | 65         | 21.7                 |
| Heredity                | 60         | 20.0                 |
| Poor diet               | 50         | 16.7                 |
| Frequent medication use | 40         | 13.3                 |
| Other                   | 10         | 3.3                  |
| <b>Total</b>            | <b>300</b> | <b>100</b>           |

**Table 2.** Awareness of allergy prevention methods among survey participants (n=300).

| Prevention method               | n          | % of total responses |
|---------------------------------|------------|----------------------|
| Avoiding contact with allergens | 80         | 26.7                 |
| Regular cleaning of premises    | 70         | 23.3                 |
| Allergen-specific immunotherapy | 50         | 16.7                 |
| Healthy eating                  | 45         | 15.0                 |
| Strengthening the immune system | 40         | 13.3                 |
| Other                           | 15         | 5.0                  |
| <b>Total</b>                    | <b>300</b> | <b>100</b>           |

**Table 3.** Sources of information about allergic diseases among survey participants (n=300).

| Information source   | n          | % of total responses |
|----------------------|------------|----------------------|
| Social media         | 90         | 30.0                 |
| Internet             | 85         | 28.3                 |
| Doctors              | 80         | 26.7                 |
| Television           | 25         | 8.3                  |
| Newspapers/magazines | 10         | 3.3                  |
| Other                | 10         | 3.3                  |
| <b>Total</b>         | <b>300</b> | <b>100</b>           |

**Table 4.** Incidence of allergic diseases in Osh city (2003–2023), rates per 100,000 population.

| Disease class        | Year | Adults & adolescents | Children (<14 years) |
|----------------------|------|----------------------|----------------------|
| Respiratory diseases | 2003 | 18,055.9             | 5,244.7              |
|                      | 2013 | 26,103.5             | 3,719.8              |
|                      | 2023 | 4,497.3              | 15,936.5             |
| Allergic rhinitis    | 2003 | 384.8                | 81.0                 |
|                      | 2013 | 143.5                | 101.8                |
|                      | 2023 | 68.3                 | 64.7                 |
| Bronchial asthma     | 2003 | 142.3                | 41.5                 |
|                      | 2013 | 157.7                | 20.0                 |
|                      | 2023 | 60.7                 | 82.8                 |
| Atopic dermatitis    | 2003 | 165.6                | 138.8                |
|                      | 2013 | 52.2                 | 36.9                 |
|                      | 2023 | 75.0                 | 163.9                |

Source: Center for Electronic Healthcare, Ministry of Health of the Kyrgyz Republic.

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## SEVENTY YEARS OF THE BRAZILIAN FEDERAL COUNCIL OF CHEMISTRY (1956–2026): INSTITUTIONAL TRAJECTORY, PRESIDENCIES, AND PERSPECTIVES FOR THE CHEMISTRY PROFESSION

## SETENTA ANOS DO CONSELHO FEDERAL DE QUÍMICA (1956–2026): TRAJETÓRIA INSTITUCIONAL, PRESIDÊNCIAS E PERSPECTIVAS PARA A PROFISSÃO QUÍMICA

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## RESUMO

**Introdução:** Em 2026, o Sistema CFQ/CRQs completa 70 anos (Lei nº 2.800/1956), fiscalizando um setor responsável por cerca de 11% do PIB industrial, mas carecia de reconstrução cronológica baseada em seus registros primários oficiais. **Objetivo:** Reconstruir a gênese jurídica do Sistema, estabelecer a cronologia oficial documentada das presidências e discutir os desafios regulatórios atuais. **Métodos:** Pesquisa documental historiográfico-descritiva apoiada em fontes normativas e no acesso inédito ao registro interno "Galeria de Presidentes", fornecido pelo Gabinete da Presidência do CFQ em 2026. **Resultados:** Detalhou-se oito presidências efetivas e interinatos (1957–2026), fundamentando o exercício interino de Oscar Bergstron Lourenço (1963–1964) e a posse de Juvenal Dória em 1964. O Sistema hoje reúne 21 CRQs e mais de 230 mil registrados. Identificou-se subdocumentação da trajetória de Hebe Martelli (única mulher na presidência) e divergência em aberto sobre o número de CRQs criados em 1957. **Discussão:** A Resolução Normativa nº 36/1974 permanece como marco das atribuições. As normativas recentes retomam desafios históricos, como a atribuição a currículos híbridos e a segurança química como bem público, exigindo antecipação frente às transições energética, de materiais e digital. **Limitações:** A cronologia depende de registros institucionais não auditados independentemente em arquivo primário pelos autores. **Conclusões:** O acesso aos registros primários consolidou a memória documental dos setenta anos do Sistema, cujo legado reside na adaptação do arcabouço regulatório às transformações tecnológicas.

**Palavras-chave:** *Regulamentação profissional; Pesquisa documental; Resoluções Normativas; Governança de conselhos profissionais; Lei 2.800/1956.*

## ABSTRACT

**Background:** In 2026, the CFQ/CRQ System turns 70 (Law No. 2,800/1956), overseeing a sector responsible for ~11% of industrial GDP, but lacked a chronological reconstruction based on its own official primary records. **Aim:** To reconstruct the System's legal genesis, establish a documented official chronology of its presidencies, and discuss current regulatory challenges. **Methods:** Historiographical-descriptive documentary research based on normative sources and unprecedented access to the internal record "Galeria de Presidentes," provided by the CFQ Presidency Office in 2026. **Results:** Eight substantive presidencies and interim periods (1957–2026) were detailed, substantiating the interim exercise by Oscar Bergstron Lourenço (1963–1964) and Juvenal Dória's inauguration in 1964. The System now comprises 21 regional councils and over 230,000 registrants. Under-documentation of Hebe Martelli's trajectory (the only female president) and an open divergence regarding the number of regional councils created in 1957 were identified. **Discussion:** Normative Resolution No. 36/1974 remains the milestone for professional attributions. Recent regulations revisit historical challenges, such as attributing competencies to hybrid curricula and establishing chemical safety as a public good, requiring anticipation of energy, materials, and digital transitions. **Limitations:** The chronology relies on institutional records

not independently audited by the authors against primary archives. **Conclusions:** Access to primary records consolidated the documentary memory of the System's seventy years, whose legacy lies in adapting the regulatory framework to technological transformations.

**Keywords:** *Professional regulation; Documentary research; Normative resolutions; Council governance; Law 2,800/1956.*

## 1. INTRODUÇÃO

Profissões não nascem regulamentadas: elas se tornam regulamentadas. O caso brasileiro da Química é exemplar dessa distinção. Entre a criação dos primeiros cursos superiores de química industrial, nas décadas de 1910 e 1920, e a instalação efetiva de um órgão próprio de registro e fiscalização, decorreram cerca de quatro décadas de disputa institucional (Santos, Pinto, & Alencastro, 2006; Silva, Santos, & Afonso, 2006). O desfecho dessa disputa tem data: 18 de junho de 1956, quando a Lei nº 2.800 criou o Conselho Federal de Química (CFQ) e os Conselhos Regionais de Química (CRQs) (Brasil, 1956). A mesma data é hoje celebrada como Dia Nacional do Profissional da Química.

Em 2026, o Sistema CFQ/CRQs completa setenta anos. O aniversário foi assinalado por sessão especial do Senado Federal, por sessão solene na Câmara dos Deputados, pelo lançamento de selo e carimbo comemorativos pelos Correios e pela cunhagem de medalha pela Casa da Moeda do Brasil, além de ações institucionais reguladas pela própria autarquia (Correios, 2026; CFQ, 2026b; Senado Federal, 2026).

Até a presente atualização, a reconstrução da cronologia das presidências do CFQ dependia inteiramente de pesquisas em diversas fontes secundárias (RQI, 2020, 2023), que tornava mais difícil a pesquisa histórica por causar a busca em múltiplas fontes. O Gabinete da Presidência do CFQ, em resposta à solicitação formal dos autores, forneceu a relação oficial dos presidentes e um registro interno de atas de Reuniões Ordinárias (“Galeria de Presidentes”), permitindo preencher a linha do tempo do Conselho de Química (CFQ, Gabinete da Presidência, 2026).

### 1.1. Objetivos

O objetivo desta comunicação é reconstruir, a partir de fontes primárias verificáveis:

- (i) a gênese jurídica do Sistema;
- (ii) uma cronologia documentada das presidências do CFQ, apoiada nas

assinaturas apostas às Resoluções Normativas e nos registros de Reuniões Ordinárias fornecidos pelo próprio CFQ;

- (iii) a evolução do arcabouço que define as atribuições profissionais; e
- (iv) uma discussão franca dos desafios que o Sistema enfrenta em seu oitavo decênio. Onde a evidência ainda falta, isso é dito. Onde as fontes divergem, a divergência é registrada.

## 2. FONTES E MÉTODO

### 2.1. Fontes documentais

Trata-se de pesquisa documental de caráter historiográfico-descritivo, apoiada em quatro classes de fontes:

- Fontes primárias normativas: Lei nº 2.800/1956; Decreto-Lei nº 5.452/1943 (CLT); Decreto nº 24.693/1934; Decretos nos 85.877/1981 e 86.593/1981; e as Resoluções Normativas do CFQ, com atenção especial às assinaturas de presidente e secretário nelas apostas, que funcionam como testemunho datado do ocupante do cargo.
- Fontes primárias biográficas: a série “Pioneiros da Química” da Revista de Química Industrial (RQI), órgão da Associação Brasileira de Química, e os obituários e perfis institucionais publicados pelo próprio CFQ.
- Fontes contemporâneas: notas da Agência Senado, dos Correios e do CFQ relativas ao septuagenário (2026), além de dados setoriais da indústria química.
- Fonte institucional oficial (nova nesta atualização): a relação oficial de presidentes e o registro interno “Galeria de Presidentes”, fornecidos pelo Gabinete da Presidência do CFQ aos autores em 25 de junho de 2026, em resposta à solicitação protocolada em 22 de junho de 2026. O registro reúne datas de Reuniões Ordinárias (R.O.) do Plenário do CFQ, com

a identificação de quem exercia a Presidência em cada sessão — titular, vice-presidente ou secretário em exercício — desde 08 de julho de 1957 (CFQ, Gabinete da Presidência, 2026).

## 2.2. Critérios de evidência e limitações

O critério de evidência adotado foi conservador: preferiu-se a data de Reunião Ordinária (R.O.) ou a assinatura datada em ato normativo a qualquer menção secundária. Onde o registro interno da “Galeria de Presidentes” apresentou inconsistências internas prováveis — por exemplo, saltos numéricos de sessões incompatíveis com o intervalo de datas informado, ou uma data cujo ano diverge visivelmente da sequência cronológica das sessões vizinhas — isso é assinalado em nota de rodapé da Tabela 1, para transparência da reconstrução.

Uma limitação permanece: a documentação obtida não resolve a divergência entre a RQI (2023), segundo a qual a segunda Resolução Normativa do CFQ, de 1957, criava cinco Conselhos Regionais, e o histórico institucional do CRQ-X (s.d.), segundo o qual essa mesma Resolução dividiu o território nacional em sete regiões. O ponto permanece como pendência de pesquisa.

## 3. RESULTADOS E DISCUSSÃO

### 3.1. Resultados

#### 3.1.1. Antecedentes: da atividade sem profissão à profissão sem conselho

O Decreto nº 24.693, de 12 de julho de 1934, regulamentado pelo Decreto nº 57/1935, constitui a primeira regulamentação da profissão de químico no Brasil. Reconhecia atividades, mas não regulava efetivamente o exercício profissional: a condição de químico prendia-se ao exercício da atividade e não à formação de quem a exercia (RQI, 2023). O primeiro diploma legal a delimitar com maior clareza as atividades privativas da classe foi a Consolidação das Leis do Trabalho (Decreto-Lei nº 5.452, de 1º de maio de 1943), cuja Seção XIII do Capítulo I do Título III tratou do exercício da profissão de químico.

O problema, contudo, era estrutural: a CLT atribuiu a fiscalização a funcionários do Ministério do Trabalho, e não a profissionais da química. Na ausência de um conselho técnico próprio, a atividade acabava sendo exercida por profissionais de outras formações e mesmo por práticos sem diploma (RQI, 2023). Foi dessa insuficiência — e não de um impulso corporativo

abstrato — que nasceu a campanha pela criação de um órgão próprio, capitaneada, a partir de 1945, por entidades como o Sindicato dos Químicos do Rio de Janeiro e a Sociedade Brasileira de Química, com participação decisiva de Geraldo Mendes de Oliveira Castro (RQI, 2023).

#### 3.1.2. A Lei nº 2.800/1956 e a arquitetura do Sistema

Sancionada por Juscelino Kubitschek em 18 de junho de 1956, a Lei nº 2.800 transferiu aos Conselhos as atribuições de registro, fiscalização e imposição de penalidades até então previstas na CLT (Brasil, 1956, art. 1º). Conferiu ao CFQ e aos CRQs personalidade jurídica de direito público, autonomia administrativa e patrimonial (art. 2º), fixou a sede do federal no Distrito Federal (art. 3º) e definiu sua composição (art. 4º). Três elementos merecem destaque analítico:

- Composição técnica vinculante. O art. 5º impôs que, entre os nove conselheiros federais efetivos, houvesse no mínimo um terço de engenheiros químicos e um terço de químicos industriais, químicos industriais agrícolas ou químicos, além de um técnico químico.
- Mandato honorífico e trienal. O art. 7º estabeleceu que o mandato do presidente e dos conselheiros seria honorífico e de três anos, com renovação anual de um terço do colegiado — dispositivo cuja leitura, à luz da longevidade observada em algumas gestões, merece a atenção discutida na Seção 3.2.1.
- Escolha do presidente. Originalmente, o presidente era nomeado pelo Presidente da República a partir de lista tríplice organizada pelo próprio Conselho (art. 4º, alínea “a”). O Decreto nº 86.593/1981 transferiu essa escolha ao Ministro do Trabalho. Atualmente, a escolha cabe ao Plenário do Conselho.

O Decreto nº 85.877, de 7 de abril de 1981, veio estabelecer normas para a execução da Lei nº 2.800/1956, discriminando as atividades compreendidas no exercício da profissão, as privativas e as comuns a outras profissões. É ele, e não a lei de 1956, o instrumento que sustenta a maior parte da atuação fiscalizatória concreta.

A instalação efetiva ocorreu em 1957, no Rio de Janeiro, em sala cedida pelo Instituto Nacional



de Tecnologia. A carteira profissional de químico nº 1 do Brasil foi expedida a Geraldo Mendes de Oliveira Castro (RQI, 2023). Registra-se aqui uma divergência entre fontes, não resolvida pela documentação oficial obtida nesta atualização: segundo a RQI (2023), a segunda Resolução Normativa do CFQ, de 1957, criava cinco Conselhos Regionais; segundo o histórico institucional do CRQ-X (s.d.), a Resolução Normativa nº 2 dividiu o território nacional em sete regiões. As duas afirmações podem ser conciliáveis — cinco conselhos instalados em um desenho de sete regiões —, mas a documentação consultada não permite decidir.

A Tabela 1 sintetiza a reconstrução cronológica, agora fundamentada na relação oficial e no registro interno “Galeria de Presidentes” fornecidos pelo Gabinete da Presidência do CFQ (2026), que documentam, sessão a sessão, o exercício da Presidência desde 1957.

### 3.1.3. Perfis dos presidentes

#### 3.1.3.1. Geraldo Mendes de Oliveira Castro: o fundador



Foto: *Geraldo Mendes de Oliveira Castro* (1957 – 1963).

Adaptado de: <https://www.abq.org.br/rqi/2014/775/RQI-775-pagina22-Pioneiros-da-Quimica.pdf>. Imagem restaurada com IA.

Químico industrial formado em 1935 pela Escola Nacional de Química e especialista em borracha, Geraldo Mendes de Oliveira Castro foi um dos principais líderes da campanha pela regulamentação da profissão. Presidente do Conselho Federal de Química (CFQ) por dois mandatos consecutivos (1957–1963), fez da valorização do profissional da Química como agente do desenvolvimento científico e tecnológico a principal marca de sua gestão. Entre os primeiros atos normativos do Conselho, destacam-se a primeira Resolução Normativa, de 1957, que instituiu o Regimento Interno do CFQ, e a segunda Resolução Normativa, responsável pela criação dos primeiros Conselhos Regionais de Química (RQI, 2023). O registro oficial de Reuniões Ordinárias mostra uma vacância transitória do cargo em 1960, coberta pelo Secretário Ralphe Rezende Decourt e, na sequência, pelo Vice-Presidente Paschoal Senide, antes do retorno de Geraldo Mendes à Presidência, documentado até julho de 1963 (CFQ, Gabinete da Presidência, 2026).

#### 3.1.3.2. Oscar Bergstron Lourenço\*: a transição de 1963–1964.

O período entre 1963–1967 é, em parte, esclarecido pelo registro oficial de Reuniões Ordinárias: entre agosto de 1963 e abril de 1964, o Vice-Presidente Oscar Bergstron Lourenço exerceu interinamente a Presidência do CFQ (49ª a 54ª R.O.), na transição entre a gestão de Geraldo Mendes de Oliveira Castro e a de Juvenal Osório de Araújo Dória. Não há, nas fontes disponíveis, biografia publicada sobre Oscar Bergstron Lourenço; seu registro limita-se, por ora, às atas de sessão fornecidas pelo Gabinete da Presidência. Oscar foi Engenheiro químico, Livre Docente em Química Orgânica e Professor de física e química da Escola Politécnica da USP; Chefe do Departamento de Engenharia Química da Escola Politécnica da USP, 1966-1969.

\* Sem imagem localizada.

### 3.1.3.3. Juvenal Osório de Araújo Dória: a consolidação



Foto: Juvenal Osório Araújo Dória (1963 – 1970). Cortesia CFQ – 2026.

A fase inicial de estruturação do Sistema foi sucedida por um período de consolidação institucional sob a presidência de Juvenal Osório de Araújo Dória. Segundo presidente efetivo do CFQ, Dória já presidia sessões do Plenário a partir da 55ª Reunião Ordinária, por volta de 1964 — alterando a data de 1967 anteriormente atribuída à sua nomeação por fonte secundária (RQI, 2020), que provavelmente registrou um ato formal posterior ao início do exercício de fato do cargo. Dória havia exercido anteriormente a presidência do Conselho Regional de Química da 3ª Região, criado em 1957. Sua gestão é lembrada, sobretudo, pelo entendimento firmado com o Conselho Federal de Farmácia para definir os limites de atuação entre farmacêuticos e químicos, tema que, sob diferentes perspectivas, permanece atual (RQI, 2020). Também foi durante sua presidência que foi editada a Resolução Normativa nº 23/1969, ainda hoje considerada referência para a caracterização de departamentos químicos em indústrias e empresas comerciais sujeitos à responsabilidade técnica. Impedimentos pontuais de Dória em 1966 e 1967 foram cobertos pelo Secretário/Vice-Presidente Jorge da Cunha, em exercício da Presidência.

### 3.1.3.4. Peter Löwenberg e a Resolução Normativa nº 36/1974



Foto: Peter Löwenberg (1970 – 1976). Cortesia CFQ – 2026.

O processo de consolidação das atribuições profissionais alcançou um marco decisivo com a publicação da Resolução Normativa nº 36, de 25 de abril de 1974, assinada por Peter Löwenberg, que exerceu a Presidência em dois períodos (c. 1970–1972 e 1973–1976), com o Vice-Presidente Paulo Ribeiro em exercício interino na transição entre eles. Provavelmente o ato normativo mais influente da história do Sistema CFQ/CRQs, essa resolução substituiu a RN nº 26, revogou as RNs nº 5, 6, 7, 20 e 26 e reorganizou o exercício profissional a partir de um conjunto de dezesseis atividades, correlacionadas a três naturezas curriculares: “Química” (atividades 1 a 7), “Química Tecnológica” (atividades 1 a 13) e “Engenharia Química” (atividades 1 a 16), além das competências atribuídas ao Técnico em Química (CFQ, 1974). Foi também nessa resolução, especificamente na atividade nº 3, que passaram a constar como atribuições do profissional da Química a vistoria, a perícia, a avaliação, o arbitramento e a elaboração de pareceres, laudos e atestados, estabelecendo a base normativa da perícia química judicial e extrajudicial exercida no Brasil até os dias atuais.

### 3.1.3.5. Werner Gustav Krauledat e a abertura para novas modalidades



Foto: Werner Gustav Krauledat (1977 – 1978). Cortesia CFQ – 2026.

A evolução normativa do Sistema prosseguiu com a ampliação das categorias profissionais reconhecidas pelo CFQ. Nesse contexto, a Resolução Normativa nº 46, de 27 de janeiro de 1978, assinada durante a gestão de Werner Gustav Krauledat — iniciada em julho de 1977, após período de exercício interino do Vice-Presidente Clóvis Martins Ferreira —, exemplifica um movimento que se repetiria nas décadas seguintes: o reconhecimento de novas formações decorrentes do processo de diversificação industrial. Ao determinar o registro de Químicos de Alimentos, Tecnólogos de Alimentos e Engenheiros de Alimentos, o CFQ aplicou o art. 9º da RN nº 36, que autoriza a atribuição de competências a graduados em cursos com organização curricular semelhante, a uma profissão então considerada em “franco desenvolvimento” (CFQ, 1978).

### 3.1.3.6. Olavo Romanus



Foto: Olavo Romanus (1978 – 1979). Cortesia CFQ – 2026.

Prof. Olavo Romanus (1927–2011) nasceu em Curitiba e é um químico de formação marcada pela excelência e inovação. Graduado em Química Industrial e Engenharia Química pela Universidade do Paraná, complementou sua formação com pós-graduação no Instituto de Óleos do Ministério da Agricultura, no Rio de Janeiro, e estudos nos EUA, onde aprofundou conhecimento em Química Orgânica e Radioisótopos. Retornou ao Brasil por convicção de contribuir ao ensino; foi instrutor, professor titular e diretor da Escola de Química da Universidade Federal do Paraná, responsável pela modernização de laboratórios e pela transferência da escola para o Centro Politécnico. Seu trabalho pioneiro em cromatografia, com organização de cursos especializados, consolidou as bases analíticas na instituição. (CRQ-IX, 2011)

Além de sua atuação acadêmica, Romanus foi pesquisador no Instituto de Biologia do Paraná, colaborador na criação de laboratórios de Biologia Marinha na UFPR, membro dos Conselhos de Curadores e de Especialistas em Ensino de Química, e conselheiro federal. Presidiu a organização do I Simpósio Brasileiro de Cromatografia (1964) e foi batalhador incansável pela defesa da profissão química. Sua



preocupação com o meio ambiente o levou a organizar estudos sobre poluentes atmosféricos.

### 3.1.3.7. Hebe Helena Labarthe Martelli: a primeira mulher



Foto: Hebe Helena Labarthe Martelli (1979 – 1985). Cortesia CFQ – 2026.

A ampliação institucional promovida pelo Sistema coincidiu, em seguida, com um marco histórico na composição de sua liderança. Aprovada em primeiro lugar no curso de Química Industrial da Escola de Química da UFRJ — tornando-se a primeira mulher a alcançar essa colocação — e melhor aluna da turma formada em 1942, Hebe Helena Labarthe Martelli assumiu a presidência do CFQ em 10 de janeiro de 1979, após período de exercício interino do Vice-Presidente Olavo Romanus. Até 2026, permanece como a única mulher a ocupar o cargo. Sua primeira Resolução Normativa, a RN nº 49, de 21 de setembro de 1979, teve como objetivo uniformizar os processos de prestação de contas ao Tribunal de Contas da União. Durante sua gestão, também foram editadas a RN nº 66, que isentava do pagamento de anuidade profissionais em situação de vulnerabilidade, e a expansão dos Conselhos Regionais para Sergipe, Paraná e Ceará/Piauí, além da celebração, em 1982, do convênio com a Novacap para a construção da sede do CFQ em Brasília (CFQ, 2026a).

Do ponto de vista historiográfico, contudo, permanece uma assimetria digna de registro. A documentação disponível sobre Hebe Martelli é

substancialmente mais escassa do que aquela referente aos seus pares masculinos, e sua biografia institucional somente foi publicada pelo CFQ em julho de 2026, treze anos após seu falecimento, ocorrido em 2013. Essa subdocumentação das trajetórias femininas na Química brasileira constitui, por si só, um importante objeto de investigação ainda pendente.

### 3.1.3.8. Jesus Miguel Tajra Adad: trinta e três anos



Foto: Jesus Miguel Tajra Adad (1985 – 2018). Cortesia CFQ – 2026.

Na sequência, a presidência do CFQ passou a ser exercida por Jesus Miguel Tajra Adad, engenheiro químico e químico industrial formado pela UFPR, com mestrado em Engenharia Sanitária e especializações em Engenharia de Segurança do Trabalho e em Radioisótopos Aplicados à Bioquímica. À frente do Conselho entre 6 de março de 1985 e 2018 — tomando posse um dia após o registro de exercício interino do Vice-Presidente Roberto Hissa —, protagonizou a mais longa gestão da história do Sistema CFQ/CRQs. Foi durante esse período que a estrutura institucional atualmente existente se consolidou, que o número de Conselhos Regionais alcançou os atuais 21 e que a sede do CFQ foi transferida do Rio de Janeiro para Brasília. Em fevereiro de 2020, recebeu o título inédito de Conselheiro Emérito, falecendo em junho do mesmo ano (CFQ, 2020).

A duração de sua gestão constitui um fato histórico objetivo, e não um juízo de valor. Entretanto, esse período é indissociável das

discussões sobre governança desenvolvidas na Seção 3.2.1.

#### 3.1.3.9. José de Ribamar Oliveira Filho: a fase da modernização

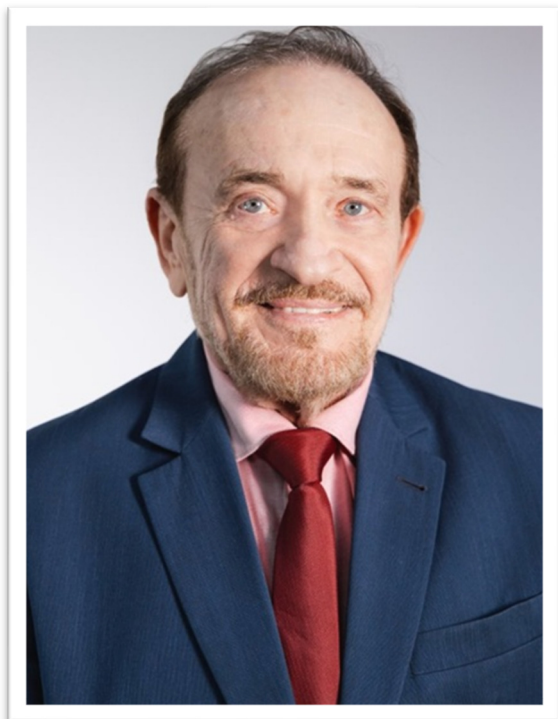


Foto: José de Ribamar Oliveira Filho (2018-Atual (2026+)).

Cortesia CFQ – 2026.

Doutor em Química Analítica pela Universidade de São Paulo (USP) e professor da Universidade Federal do Maranhão (UFMA), o Professor Dr. José de Ribamar Oliveira Filho foi eleito presidente do Conselho Federal de Química (CFQ) em 18 de abril de 2018 (R.O. nº 26.777, 607ª Reunião) e reconduzido, por aclamação, para o mandato 2024–2027. À frente da autarquia, liderou um amplo processo de modernização institucional, marcado pela implementação do Planejamento Estratégico 2018–2028, pela institucionalização do Colégio de Presidentes (Copresi), pela criação do Comitê de Relações Institucionais e Governamentais e pela atuação firme em defesa do Sistema CFQ/CRQs diante da Proposta de Emenda à Constituição nº 108/2019. Durante a pandemia de COVID-19, coordenou a iniciativa Química Solidária, responsável pela doação de mais de 100 mil litros de álcool e álcool em gel 70%, além de criar a Central de Informações da Química (CIQ). Sua gestão também fortaleceu a inserção internacional do Sistema, com participação em eventos como a COP28 (CFQ, 2024).

Esse compromisso com a valorização da Química e com a formação de profissionais éticos e socialmente responsáveis já era perceptível muito antes de sua atuação na presidência do CFQ. Um dos autores (Gomes), como ex-aluno da Universidade Federal do Maranhão, teve o privilégio de ser seu estudante nas disciplinas de Química Geral Experimental, em 2004.2, e Química do Meio Ambiente, em 2009.2. Suas aulas eram ministradas com elevado rigor científico, clareza e dedicação ao ensino, sempre acompanhadas de uma preocupação genuína com a formação integral dos futuros químicos. Nos momentos de convivência acadêmica, o Professor Ribamar frequentemente dialogava com os estudantes sobre a importância do Conselho Regional de Química para o exercício profissional. Essas conversas, conduzidas com entusiasmo e espírito formador, exerceram influência duradoura na trajetória acadêmica e profissional de um dos autores, constituindo um testemunho do legado de um professor que sempre compreendeu que formar químicos significava, acima de tudo, formar profissionais conscientes de sua responsabilidade perante a sociedade.

A identidade profissional do químico brasileiro não está na Lei nº 2.800: está na sedimentação das Resoluções Normativas. A Tabela 2 apresenta os marcos que, no entendimento dos autores, melhor explicam o desenho atual.

#### 3.1.4. O CFQ aos setenta anos: números e honrarias

O Sistema CFQ/CRQs reúne 21 Conselhos Regionais, cobrindo as 27 unidades da federação, e mais de 230 mil profissionais da química registrados (CFQ, 2026c; Senado Federal, 2026). Sua missão declarada é “*promover a atividade plena da Química, com vistas a contribuir para o desenvolvimento sustentável do país*” (CFQ, 2026c). O setor que fiscaliza tem peso macroeconômico: segundo dados da Abiquim divulgados em 2026, a indústria química brasileira registrou faturamento líquido de aproximadamente US\$ 158,6 bilhões em 2024 e responde por cerca de 11% do PIB industrial (Assembleia Legislativa de Goiás, 2026). Trata-se de estatística de fonte setorial, e deve ser lida com a cautela usual aplicável a números divulgados por associações de indústria.

O septuagenário foi marcado por sessão especial no Senado Federal em 18 de junho de 2026, decorrente do Requerimento nº 52/2026 (senador Sérgio Petecão); por sessão solene na

Câmara dos Deputados; pelo lançamento de selo personalizado e carimbo comemorativo dos Correios; e pela cunhagem, pela Casa da Moeda do Brasil, de medalha alusiva à data (Correios, 2026; CFQ, 2026b; Senado Federal, 2026).

## 3.2. Discussão

### 3.2.1. Formação profissional e o problema da atribuição

As RNs nº 342 e 343/2025 recolocaram, sob linguagem nova, a questão que a RN nº 36 tentara resolver em 1974: como atribuir competências legais a egressos de currículos cada vez mais heterogêneos. O problema agravou-se por dois vetores. O primeiro é a expansão acelerada de cursos na modalidade a distância em uma área cujo núcleo formativo é laboratorial e experimental. O segundo é o surgimento de formações híbridas (química computacional, ciência de dados aplicada à química, engenharia de bioprocessos) que não se acomodam com naturalidade nas três naturezas curriculares desenhadas em 1974.

### 3.2.2. Segurança química, produtos controlados e responsabilidade técnica

A RN nº 335/2025, ao disciplinar a Ficha com Dados de Segurança de Produtos Químicos (FDS) e a Ficha com Dados de Segurança de Resíduos Químicos Perigosos (FDSR), sinaliza um dos eixos mais promissores de atuação do Sistema: a segurança química como bem público. Aqui a responsabilidade técnica deixa de ser formalidade cartorial e passa a ser instrumento de proteção à vida — inclusive à vida do próprio trabalhador exposto. Um conselho profissional que leve a sério esse eixo tem, aí, sua justificativa social mais robusta.

### 3.2.3. Química verde, bioeconomia e o próximo decênio

O oitavo decênio do CFQ coincide com três transições simultâneas: a energética (biocombustíveis avançados, hidrogênio de baixo carbono, combustíveis sustentáveis de aviação), a de materiais (reciclagem química, recuperação de terras-raras e metais críticos, economia circular) e a digital (aprendizado de máquina aplicado à descoberta de materiais e ao controle de processos, laboratórios autônomos). Um conselho profissional que chegue tarde a essas fronteiras não apenas perde relevância: cria vazio de responsabilidade técnica exatamente onde o risco é maior.

## 4. CONCLUSÕES

Setenta anos depois da Lei Máter, o balanço é favorável e não precisa de retoques. O Sistema CFQ/CRQs deu à profissão química brasileira aquilo que ela não tinha em 1956: fronteira legal, órgão próprio de julgamento e um mecanismo de reconhecimento de novas modalidades capaz de acompanhar — ainda que com atraso — a diversificação da área. As trajetórias reunidas neste texto (Geraldo Mendes, Oscar Bergstron Lourenço, Juvenal Dória, Löwenberg, Krauledat, Hebe Martelli, Jesus Miguel e Ribamar Oliveira) são, cada uma a seu modo, capítulos dessa construção.

A colaboração direta do Gabinete da Presidência do CFQ permitiu, nesta atualização, preencher a lacuna cronológica de 1963–1967 anteriormente registrada: o período é hoje documentado por Reuniões Ordinárias datadas, com o exercício interino de Oscar Bergstron Lourenço (1963–1964) e a ascensão de Juvenal Dória à Presidência plena já por volta de 1964.

Duas tarefas permanecem propostas à comunidade. A primeira é historiográfica: resolver a divergência remanescente quanto ao número de Conselhos Regionais criados em 1957 (cinco ou sete) e resgatar trajetórias sub-registradas, a começar pela de Hebe Martelli. A segunda é política e técnica: enfrentar, com serenidade, o debate sobre o modelo de escolha dos dirigentes, e antecipar-se às transições energética, de materiais e digital, de modo que a responsabilidade técnica em química continue significando, no oitavo decênio, o que significou no primeiro.

É esse, e não a medalha, o patrimônio que se celebra.

## 5. DECLARAÇÕES

### 5.1. Limitações do Estudo

- Limitações metodológicas: a cronologia da Tabela 1 depende de registros institucionais fornecidos pelo Gabinete da Presidência do CFQ, não auditados pelos autores em arquivo primário independente; duas inconsistências tipográficas prováveis no documento-fonte foram identificadas e assinaladas, mas não resolvidas.
- Limitação de amostragem documental: a divergência entre RQI (2023) e CRQ-X (s.d.) quanto ao número de Conselhos Regionais criados em 1957 permanece não resolvida.

- Limitação de generalização: as biografias dos presidentes têm profundidade documentária desigual, refletindo assimetrias históricas de registro (notadamente no caso de Hebe Martelli e de Oscar Bergstron Lourenço), e não uma escolha editorial dos autores.
- Limitação de escopo: a discussão dos desafios contemporâneos do Sistema (Seção 3.2) é necessariamente seletiva e reflete a perspectiva dos autores como profissionais registrados no Sistema CFQ/CRQs.

## 5.2. Agradecimentos

Os autores agradecem ao Gabinete da Presidência do Conselho Federal de Química pelo fornecimento da relação oficial de presidentes e do registro interno “Galeria de Presidentes”, essenciais para a reconstrução da cronologia apresentada nesta comunicação; à Associação Científica Araucária pelo apoio editorial; e à Revista de Química Industrial (ABQ), cuja série “Pioneiros da Química” preserva parte substancial da memória aqui reconstruída.

Agradecimentos especiais a nossa colega Laila Kudsi - Analista da Assessoria de Comunicação, CFQ.

## 5.3. Fontes de Financiamento

Os autores financiaram esta pesquisa. Nenhum financiamento externo foi recebido.

## 5.4. Conflitos de Interesse

De Boni, L. A. B. é profissional da química registrado no CRQ-V e Editor-Gerente do Periódico Tchê Química. Não recebeu financiamento do Conselho Federal de Química nem de qualquer Conselho Regional de Química para a elaboração deste texto e não ocupa cargo eletivo no Sistema CFQ/CRQs.

Gomes, P.R.B é professor efetivo no Instituto Federal do Pará, Campus Paragominas. Não recebeu financiamento do Conselho Federal de Química nem de qualquer Conselho Regional de Química para a elaboração deste texto e não ocupa cargo eletivo no Sistema CFQ/CRQs.

## 5.5. Disponibilidade de Dados

Todas as fontes normativas e biográficas primárias citadas neste estudo são de acesso público e estão referenciadas na Seção 7. O registro interno “Galeria de Presidentes”, fornecido pelo Gabinete da Presidência do CFQ, está disponível mediante solicitação ao autor correspondente, sujeito à autorização do CFQ

para redistribuição.

## 5.6. Contribuições dos Autores

De Boni, L. A. B.: Concepção e Design (CD), Coleta de Dados (DC), Análise e Interpretação de Dados (DAI), Escrita do Manuscrito (MW), Aprovação Final (FA).  
Gomes, P. R. B.: Análise e Interpretação de Dados (DAI), Escrita do Manuscrito (MW), Aprovação Final (FA).

| Code | English                          | Português                        |
|------|----------------------------------|----------------------------------|
| CD   | Conception and Design            | Concepção e Design               |
| DC   | Data Collection                  | Coleta de Dados                  |
| DAI  | Data Analysis and Interpretation | Análise e Interpretação de Dados |
| MW   | Manuscript Writing               | Escrita do Manuscrito            |
| CR   | Critical Review                  | Revisão Crítica                  |
| FA   | Final Approval                   | Aprovação Final                  |

## 5.7. Declaração de Ferramentas de IA

Ferramenta: Claude (Anthropic). Propósito: apoio à verificação cruzada de datas entre fontes documentais fornecidas pelos autores, redação de revisões do manuscrito e reformatação conforme o template do periódico. Não foi utilizada para fabricação de dados, análise estatística ou interpretação de resultados históricos, que permanecem de responsabilidade exclusiva dos autores. Todo o conteúdo gerado foi revisado e substancialmente verificado pelo autor correspondente antes da submissão.

## 5.8. Declaração de Integridade da Pesquisa

Sem fabricação de dados; sem falsificação de resultados; sem relato seletivo; trabalho original; não publicado anteriormente; métodos conduzidos eticamente.

## 5.9. Declaração de Originalidade e Ausência de Plágio

Este manuscrito é trabalho original, não publicado anteriormente nem em avaliação simultânea em outro periódico. Todas as fontes estão devidamente citadas em formato APA. [A CONFIRMAR: execução de verificação de similaridade (ex.: Turnitin, iThenticate) e registro do índice obtido, antes da submissão final].

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## 6. ESTUDOS ENVOLVENDO SERES HUMANOS E ANIMAIS

Não aplicável. Trata-se de pesquisa documental histórico-institucional, baseada exclusivamente em fontes legais, normativas e institucionais de acesso público ou fornecidas formalmente pelo CFQ, sem coleta de dados de seres humanos ou animais e sem uso de informações pessoais sensíveis de indivíduos vivos além de fatos biográficos já publicados. Não houve, portanto, necessidade de aprovação por Comitê de Ética em Pesquisa.

### 6.1. Aprovação do Comitê de Ética

Não aplicável.

### 6.2. Consentimento Informado

Não aplicável.

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**Tabela 1.** Cronologia documentada das presidências do Conselho Federal de Química (1957–2026), com base na relação oficial e no registro interno “Galeria de Presidentes” (CFQ, Gabinete da Presidência, 2026).

| Período                 | Presidente / Exercício                                                              | Evidência documental (R.O. = Reunião Ordinária)                                                                                                                                                                                                                                                                                                                                                                                                                                               |
|-------------------------|-------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 08.07.1957 – c. 08.1963 | Geraldo Mendes de Oliveira Castro (fundador; dois mandatos consecutivos)            | Presidente na 1ª R.O. (08.07.1957), ainda presente na 25ª R.O. (29.05.1963) e na 48ª R.O. (03.07.1963). Vacância do cargo em 27.04.1960 (22ª R.O.), com o Secretário Ralpo Rezende Decourt em exercício, seguido do Vice-Presidente Paschoal Senide em exercício (23ª e 24ª R.O., 27.04–08.06.1960).                                                                                                                                                                                          |
| 14.08.1963 – 22.04.1964 | Oscar Bergstron Lourenço (Vice-Presidente, em exercício da Presidência)             | 49ª R.O. (14.08.1963) e 54ª R.O. (22.04.1964).                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| c. 1964 – 12.11.1969    | Juvenal Osório de Araújo Dória                                                      | Presidente na 55ª R.O. (logo após a 54ª R.O., c. 1964), com presença documentada até pelo menos a 95ª R.O. (12.11.1969). Impedimentos pontuais foram cobertos pelo Secretário Jorge da Cunha em exercício (65ª R.O., 09.03.1966; 74ª R.O., c. 1967; 77ª R.O., 25.10.1967) e, em 1969, pelo Vice-Presidente Peter Löwenberg em exercício (92ª R.O., c. 13.06.1969; 93ª R.O., 17.06.1969), com o retorno de Dória à Presidência registrado na 94ª R.O. (15.10.1969) e na 95ª R.O. (12.11.1969). |
| 23.04.1970 – 02.07.1972 | Peter Löwenberg (Vice-Presidente; depois Presidente)                                | Vice-Presidente em exercício da Presidência nas 99ª e 100ª R.O. (23.04 e 20.05.1970); assume como Presidente pleno na 101ª R.O. (24.06.1970). Mandato encerra-se por volta de 02.07.1972 (134ª R.O.), quando o Vice-Presidente Paulo Ribeiro passa a exercer a Presidência em razão do término do mandato.                                                                                                                                                                                    |
| 02.07.1972 – 12.09.1973 | Paulo Ribeiro (Vice-Presidente, em exercício; término do mandato de Löwenberg)      | 134ª R.O. (02.07.1972) e 135ª R.O. (15.08.1973).                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| 12.09.1973 – c. 1976    | Peter Löwenberg (segundo mandato)                                                   | Presidente na 136ª R.O. (12.09.1973); assina a RN nº 36, de 25.04.1974 (CFQ, 1974).                                                                                                                                                                                                                                                                                                                                                                                                           |
| c. 1976 – 15.07.1977    | Clóvis Martins Ferreira (Vice-Presidente, em exercício) / Werner Gustav Krauledat   | Clóvis Martins Ferreira em exercício na 171ª R.O. (08.10.1976); Krauledat assume como Presidente na 181ª R.O. (15.07.1977) e assina a RN nº 46, de 27.01.1978 (CFQ, 1978).                                                                                                                                                                                                                                                                                                                    |
| 1978 – 10.01.1979       | Olavo Romanus (Vice-Presidente, em exercício)                                       | 189ª R.O. (28.04.1978) e 197ª R.O. (15.12.1978).                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| 10.01.1979 – 05.03.1985 | Hebe Helena Labarthe Martelli (primeira e, até 2026, única mulher a presidir o CFQ) | Presidente na 198ª R.O. (10.01.1979) até pelo menos a 236ª R.O. (19.03.1982); breve exercício de Samuel Klein na 235ª R.O. (05.02.1982). Instala o CRQ-X em 23.11.1983 (CRQ-X, s.d.).                                                                                                                                                                                                                                                                                                         |
| 05.03.1985              | Roberto Hissa (Vice-Presidente, em exercício)                                       | Registro datado de 05.03.1985, véspera da posse de Jesus Miguel Tajra Adad.                                                                                                                                                                                                                                                                                                                                                                                                                   |
| 06.03.1985 – 2018       | Jesus Miguel Tajra Adad                                                             | Presidente na 269ª R.O. (06.03.1985); presença documentada em R.O. sucessivas até a 22.767ª R.O. (03.12.2014, mandato 2015–2018). 33 anos à frente da autarquia; primeiro Conselheiro Emérito do CFQ (CFQ, 2020).                                                                                                                                                                                                                                                                             |
| 18.04.2018 – atual      | José de Ribamar Oliveira Filho                                                      | Eleito na R.O. nº 26.777 (607ª Reunião, 18.04.2018), mandato 2018–2021; reconduzido por aclamação para o mandato 2024–2027 (CFQ, 2024).                                                                                                                                                                                                                                                                                                                                                       |

Notas: intervalos assinalados com “c.” são inferidos a partir da sequência de Reuniões Ordinárias vizinhas. O registro interno “Galeria de Presidentes” apresenta três prováveis inconsistências de transcrição, aqui assinaladas para transparência da reconstrução cronológica: (a) a 25ª R.O. é datada de 29.05.1963 e a 48ª R.O., de 03.07.1963 — um intervalo de cinco semanas para 23 sessões é incompatível com o ritmo observado no restante da série, sugerindo erro de digitação em um dos dois números de sessão ou datas; (b) a 74ª R.O. é datada de “14.06.1997” no documento original, o que é cronologicamente incompatível com a sequência (65ª R.O. em 1966; 77ª R.O. em 25.10.1967) — leitura mais provável: 14.06.1967; (c) a 92ª R.O. é datada de 13.08.1969 no documento original, data posterior à da 93ª R.O. (17.06.1969) — sequência cronologicamente incompatível, sugerindo erro de digitação no mês (leitura mais provável: 13.06.1969). Os três pontos foram comunicados ao Gabinete da Presidência do CFQ para confirmação em arquivo primário.

**Tabela 2. Marcos normativos selecionados da regulamentação da profissão química no Brasil.**

| Ano       | Marco                                                                                                                                                                                                        |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1934      | Decreto nº 24.693, regulamentado pelo Decreto nº 57/1935: primeira regulamentação da profissão de químico; institui a figura do “químico licenciado”.                                                        |
| 1943      | Decreto-Lei nº 5.452 (CLT), Título III, Cap. I, Seção XIII: primeira delimitação clara de atividades privativas; fiscalização a cargo do Ministério do Trabalho.                                             |
| 1956      | Lei nº 2.800: cria o CFQ e os CRQs e lhes transfere a fiscalização; 18 de junho torna-se o Dia Nacional do Profissional da Química.                                                                          |
| 1957      | Instalação do CFQ; RN nº 1 (regimento interno) e RN nº 2 (criação dos primeiros regionais). Ver a divergência de fontes discutida na Seção 3.1.2.                                                            |
| 1969      | RN nº 23: caracteriza, para os fins dos arts. 334-b e 341 da CLT, os departamentos químicos de indústrias e empresas comerciais sujeitos a direção e responsabilidade técnica.                               |
| 1974      | RN nº 36: elenco de 16 atividades profissionais; naturezas curriculares Química, Química Tecnológica e Engenharia Química; atribuições do Técnico Químico; consagra a perícia como atribuição (item 03).     |
| 1977–1984 | RN nº 44/1977 (agentes biológicos no controle de águas e efluentes); RN nº 46/1978 (alimentos); RN nº 76/1984 (revoga o parágrafo único do art. 2º da RN nº 36).                                             |
| 1981      | Decreto nº 85.877: regulamenta a Lei nº 2.800, discriminando atividades privativas e comuns. Decreto nº 86.593: transfere ao Ministro do Trabalho a escolha do presidente do CFQ.                            |
| 1987–1992 | RN nº 105/1987 e RN nº 122/1990 (pessoas jurídicas sujeitas a registro); RN nº 133/1992 (responsabilidade técnica).                                                                                          |
| 2004      | RN nº 194 (atualiza os arts. 8º e 9º da RN nº 36) e RN nº 198 (define as modalidades profissionais na área da Química).                                                                                      |
| 2019      | RN nº 287: disciplina a fiscalização, as penalidades e o processo administrativo em todo o Sistema.                                                                                                          |
| 2025      | RN nº 334 (responsabilidade técnica); RN nº 335 (FDS e Ficha com Dados de Segurança de Resíduos Químicos Perigosos); RNs nº 342 e 343 (concessão de atribuições a egressos de cursos superiores e técnicos). |
| 2026      | RN nº 344 (nova Carteira de Identidade do Profissional da Química); RN nº 346 (ações institucionais dos 70 anos do Sistema).                                                                                 |

Fonte: elaborado pelos autores a partir de Brasil (1934, 1943, 1956, 1981a, 1981b) e das Resoluções Normativas do CFQ (CRQ-IV, 2026).

## ASSOCIAÇÃO DA VARIAÇÃO DO GENE TSHB COM HIPERTIREOIDISMO EM MULHERES DA PROVÍNCIA DE MAYSAN

## THE ASSOCIATION OF TSHB GENE VARIATION WITH HYPERTHYROIDISM IN WOMEN OF MAYSAN PROVINCE

ارتباط التغيرات في جين TSHB بفرط نشاط الغدة الدرقية لدى نساء محافظة ميسان

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### RESUMO

**Introdução:** As disfunções da tireoide são condições complexas e poligênicas influenciadas por variantes genéticas. **Objetivo:** Avaliar, em caráter piloto e exploratório, a distribuição de uma substituição G>A observada na posição 113 do amplicon sequenciado do gene TSHB em mulheres iraquianas com hipertireoidismo. **Métodos:** Estudo caso-controle piloto com 15 mulheres com hipertireoidismo e 15 controles saudáveis, entre 18 e 45 anos. Um fragmento de 520 pares de bases do éxon 3 de TSHB foi amplificado por reação em cadeia da polimerase e sequenciado. As frequências genotípicas e alélicas foram descritas, e tabelas esparsas foram avaliadas pelo teste exato de Fisher. **Resultados:** As frequências dos genótipos GG, GA e AA foram 66,67%, 20,00% e 13,33% nos casos e 93,33%, 6,67% e 0% nos controles. O alelo A foi numericamente mais frequente nos casos (23,33% versus 3,33%). A análise assintótica produziu OR = 8,826 (IC95%: 1,012–76,963; P = 0,049), enquanto o teste exato de Fisher bilateral não atingiu significância estatística (P = 0,052). **Discussão:** A diferença observada constitui apenas uma tendência não significativa. O tamanho amostral reduzido, as células esparsas e os amplos intervalos de confiança limitam a precisão. O transcrito RefSeq revisado NM\_000549.5 foi adotado como referência para a nomenclatura; contudo, o número 113 foi contado a partir do início do amplicon de 520 pb, e não a partir do códon de iniciação ATG. Portanto, essa posição é apresentada apenas como coordenada do amplicon e não como nomenclatura HGVS c. **Conclusões:** Os resultados são geradores de hipótese e não confirmam associação entre a variante e hipertireoidismo. Estudos multicêntricos maiores, com fenotipagem bioquímica detalhada e nomenclatura HGVS validada, são necessários.

**Palavras-chave:** frequência alélica; variante genética; estudo caso-controle; hipertireoidismo; população iraquiana.

### ABSTRACT

**Background:** Thyroid disorders are complex polygenic conditions influenced by genetic variation. **Aim:** To conduct a pilot, exploratory assessment of a G>A substitution observed at position 113 of the sequenced TSHB amplicon in Iraqi women with hyperthyroidism. **Methods:** This pilot case-control study included 15 women with hyperthyroidism and 15 healthy controls aged 18–45 years. A 520-bp fragment of TSHB exon 3 was amplified by polymerase chain reaction and sequenced. Genotype and allele frequencies were described, and sparse contingency tables were evaluated using Fisher's exact test. **Results:** GG, GA, and AA genotype frequencies were 66.67%, 20.00%, and 13.33% in cases and 93.33%, 6.67%, and 0% in controls. The A allele was numerically more frequent in cases (23.33% vs. 3.33%). The asymptotic analysis yielded OR = 8.826 (95% CI: 1.012–76.963; P = 0.049), whereas the appropriate two-sided Fisher's exact test did not reach statistical significance (P = 0.052). **Discussion:** The observed difference represents a non-significant trend only. The small sample, sparse cells, and wide confidence interval substantially limit

precision. The reviewed RefSeq transcript NM\_000549.5 was selected as the reference sequence; however, position 113 was counted from the 5' end of the 520-bp amplicon rather than from the transcript ATG start codon. It is therefore reported only as an amplicon coordinate and not as HGVS c. nomenclature. **Conclusions:** These hypothesis-generating findings do not establish an association between the variant and hyperthyroidism. Larger multicenter studies with detailed biochemical phenotyping and validated HGVS nomenclature are required.

**Keywords:** allele frequency; genetic variant; case–control study; hyperthyroidism; Iraqi population.

## الملخص

**الخلفية:** تُعد اضطرابات الغدة الدرقية حالات معقدة متعددة الجينات تتأثر بالتغيرات الوراثية. **الهدف:** إجراء تقييم استطلاعي أولي للاستبدال G>A المرصود في الموضع 113 من قطعة جين TSHB التي جرى تسلسلها لدى نساء عراقيات مصابات بفرط نشاط الغدة الدرقية. **طرائق العمل:** شملت دراسة استطلاعية للحالات والشواهد 15 امرأة مصابة بفرط نشاط الغدة الدرقية و15 امرأة سليمة تراوحت أعمارهن بين 18 و45 عاماً. ضُخمت قطعة بطول 520 زوجاً قاعدياً من الإكسون الثالث لجين TSHB بتفاعل البوليميراز المتسلسل ثم سُلِّمَت. وُصفت تواترات الأنماط الجينية والأليلات، واستُخدم اختبار فيشر الدقيق للجدول ذات الخلايا قليلة العدد. **النتائج:** بلغت تواترات الأنماط GG وGA وAA لدى المصابات 66.67% و20.00% و13.33%، مقابل 93.33% و6.67% و0% لدى الشواهد. كان الأليل A أكثر تواتراً عددياً لدى المصابات (23.33% مقابل 3.33%). أعطى التحليل التقريبي OR = 8.826 (فاصل الثقة 95%: 1.012–76.963؛ P = 0.049)، في حين لم يبلغ اختبار فيشر الدقيق ثنائي الطرف مستوى الدلالة الإحصائية (P = 0.052). **المناقشة:** تمثل النتيجة اتجاهاً غير دال إحصائياً فقط، كما يحد صغر العينة وقلة أعداد بعض الخلايا واتساع فاصل الثقة من دقة التقدير. واعتمدت النسخة المرجعية المنقحة RefSeq NM\_000549.5 مرجعاً للتسمية؛ إلا أن الموضع 113 حُسب ابتداءً من الطرف 5' لقطعة التضخيم البالغ طولها 520 زوجاً قاعدياً، وليس من كودون البدء ATG في النسخة المرجعية. لذلك يُعرض هذا الموضع بوصفه إحداثياً داخل قطعة التضخيم فقط، ولا يُعد تسمية HGVS من نوع C. **الاستنتاجات:** لا تثبت هذه النتائج الاستطلاعية وجود ارتباط بين المتغير وفرط نشاط الغدة الدرقية، وتستلزم دراسات متعددة المراكز بعينات أكبر وتوصيف كيميائي حيوي مفصل وتسمية HGVS موثقة.

**الكلمات المفتاحية:** تواتر الأليل؛ متغير وراثي؛ دراسة الحالات والشواهد؛ فرط نشاط الغدة الدرقية؛ السكان العراقيون.

## 1. INTRODUCTION

The thyroid gland is a major endocrine organ located in the anterior neck and composed of two lobes connected by an isthmus. Follicular cells synthesize triiodothyronine and thyroxine, whereas parafollicular cells produce calcitonin. Thyroid hormone production is regulated through the hypothalamic–pituitary–thyroid axis (Anandkumar *et al.*, 2020; Al-Suhaimi and Khan, 2022; Yao *et al.*, 2022).

Thyroid-stimulating hormone (TSH) is a heterodimeric glycoprotein composed of a common alpha subunit and a hormone-specific beta subunit encoded by TSHB. Circulating TSH is widely used in the biochemical evaluation of thyroid function, and binding of TSH to its receptor on thyroid follicular cells promotes thyroid hormone synthesis (Ushakov, 2024; Yang *et al.*, 2024).

The TSHB gene is located at chromosome 1p13.2 and contains three exons, of which exons 2 and 3 encode the mature protein. Pathogenic TSHB variants reported in the literature predominantly impair hormone synthesis, secretion, dimerization, or receptor activation and are therefore mainly associated with central hypothyroidism rather than hyperthyroidism (Nicholas *et al.*, 2016; Persani and Bonomi, 2017; Talebi *et al.*, 2020).

Accordingly, a direct causal mechanism linking a TSHB coding variant to

hyperthyroidism has not been established. Hyperthyroidism is more commonly related to TSH-receptor autoantibodies or autonomous thyroid tissue. The present study was therefore designed as an exploratory association analysis and does not assume that the observed sequence change is causative.

The study specifically examined genotype and allele distributions for a G>A substitution observed at position 113 within the sequenced 520-bp amplicon. Because no reference-transcript accession was documented in the available laboratory record, this position is reported as an amplicon coordinate and should not be interpreted as validated HGVS coding-DNA nomenclature.

### 1.1. Aim

This pilot study aimed to explore whether the position-113 G>A substitution observed within the sequenced TSHB amplicon differed in frequency between Iraqi women with hyperthyroidism and healthy controls from Maysan Province.

## 2. MATERIALS AND METHODS

### 2.1. Study Population and Design

This pilot case–control study included 15 women with a clinical diagnosis of hyperthyroidism and 15 healthy controls aged

18–45 years. Participants were recruited in Maysan Governorate from 17 October 2024 to 25 May 2025, and blood samples were collected at Al-Sadr Hospital. The diagnosis recorded in the source dataset was based on hospital clinical and laboratory assessment; however, exact TSH/free-thyroxine thresholds, hyperthyroidism etiology, treatment status, and systematic exclusion criteria were not available for retrospective reporting. The sample size was determined by feasibility and available eligible participants; no a priori power calculation was performed. The study should therefore be interpreted as exploratory and hypothesis-generating.

## 2.2. Genomic DNA Extraction, PCR, and Genotyping

Genomic DNA was extracted from whole blood using the gSYNC™ DNA Extraction Kit (Geneaid, Taiwan). A 520-bp TSHB fragment was amplified with primers F: 5'-GGCTAAGCAATTCTTTCCAGT-3' and R: 5'-GCTCTCTAACGCCTGTGTAGG-3' (Özhan et al., 2017). PCR was performed in 25 µL containing 3.5 µL DNA, 13 µL Master Mix, 1 µL of each primer, and 6.5 µL nuclease-free water. Cycling comprised 95°C for 5 min; 35 cycles of 95°C for 1 min, 55–58°C for 40 s, and 72°C for 45 s; and 72°C for 10 min. Products were separated on 1.5% agarose gel and visualized under ultraviolet light. Chromatograms were aligned and genotypes assigned using Geneious version 10.1.3. The reviewed human TSHB RefSeq transcript NM\_000549.5 (protein accession NP\_000540.2) was selected as the reference transcript for nomenclature review. The reported number 113 was counted from the 5' end of the sequenced 520-bp PCR amplicon, not from the A of the transcript ATG initiation codon. Consequently, the observed change is reported as an amplicon-coordinate substitution (position-113 G>A) and not as HGVS c.113G>A.

## 2.3. Variant Nomenclature Verification

The reviewed reference transcript used for nomenclature assessment was NM\_000549.5, with NP\_000540.2 as the corresponding protein accession. HGVS coding-DNA numbering begins at the A of the ATG translation-initiation codon. Because the laboratory designation “position 113” was counted from the 5' end of the 520-bp PCR amplicon, it is not valid HGVS c. notation. Therefore, the previous c.113G>A and

p.Ala32Thr designations were withdrawn. The variant is described consistently throughout this manuscript as the position-113 G>A substitution within the sequenced TSHB amplicon. A definitive HGVS c. and p. designation can be assigned only after the original sequence files are aligned base-by-base to NM\_000549.5 and the exact transcript coordinate is confirmed.

## 2.4. Statistical Analysis

Data were analyzed using SPSS version 26 and MedCalc version 20.0111. Genotype distributions were summarized descriptively. Hardy–Weinberg calculations are reported as exploratory because of the small sample. For the sparse 2×2 allele table, the two-sided Fisher's exact test was used as the primary inferential test. The asymptotic Woolf/logit odds ratio and 95% confidence interval are retained as descriptive sensitivity estimates. Statistical significance was defined as  $P < 0.05$ . No adjustment for multiple comparisons was applied; therefore, all inferential findings are exploratory.

# 3. RESULTS AND DISCUSSION

## 3.1. Results

### 3.1.1. Identification of the Position-113 G>A Substitution

Multiple-sequence alignment of the sequenced TSHB amplicon revealed a G>A substitution at amplicon position 113 in women with hyperthyroidism (Figure 1). This coordinate is not presented as HGVS coding-DNA nomenclature because a reference transcript was not documented.

Sequence alignment revealed three genotypes at the studied site: GG, GA, and AA. A single yellow peak in the chromatogram indicated the wild-type genotype (GG), two overlapping red and yellow peaks indicated the heterozygous genotype (GA), and a single red peak corresponding to A indicated the homozygous variant genotype (AA) (Figure 2).

### 3.1.2. Genotype and Allele Frequencies

Three genotypes (GG, GA, and AA) were observed at the position-113 site. In the hyperthyroidism group, GG was the most frequent genotype (10/15; 66.67%), followed by GA (3/15; 20.00%) and AA (2/15; 13.33%). The corresponding control counts were 14, 1, and 0. Hardy–Weinberg calculations are shown descriptively in Table 1, but the small

group sizes limit their interpretation.

Genotype-level comparisons were not statistically significant. The A allele was numerically more frequent in cases than controls (7/30 [23.33%] vs. 1/30 [3.33%]). The asymptotic Woolf/logit analysis yielded OR = 8.826 (95% CI: 1.012–76.963; P = 0.049), but the two-sided Fisher's exact test, which is appropriate for the sparse allele table, did not reach the prespecified significance threshold (P = 0.052). The result is therefore reported as a non-significant trend and should be regarded as exploratory (Table 2).

### 3.1.3. Variant Annotation Status

The nucleotide substitution was detected reproducibly in the sequencing chromatograms. Nomenclature assessment used the reviewed TSHB transcript NM\_000549.5; however, the reported position 113 originated from the amplicon coordinate system rather than from ATG-based HGVS coding-DNA numbering. Accordingly, the change is reported as position-113 G>A within the sequenced amplicon, and no definitive HGVS c. or p. designation or amino-acid consequence is claimed.

### 3.2. Discussion

The A allele was numerically more frequent among women with hyperthyroidism; however, Fisher's exact test yielded P = 0.052. Thus, the present data do not demonstrate a statistically significant association at  $\alpha = 0.05$ . The direction and magnitude of the asymptotic odds ratio are unstable because the sample is small and one control cell contains only a single A allele.

The biological interpretation also requires caution. Published pathogenic TSHB variants primarily cause reduced TSH synthesis or function and central hypothyroidism, whereas hyperthyroidism is usually driven by TSH-receptor autoimmunity or autonomous thyroid tissue (Nicholas *et al.*, 2016; Persani and Bonomi, 2017; Talebi *et al.*, 2020). Therefore, the current observation cannot be interpreted through a simple loss-of-function mechanism, and a causal pathway linking this amplicon variant to hyperthyroidism remains unknown.

The reviewed RefSeq transcript NM\_000549.5 was used to assess

nomenclature. Because the original position 113 was counted from the start of the PCR amplicon rather than from the transcript ATG codon, the previous c.113G>A/p.Ala32Thr designation was not HGVS-compliant and was withdrawn. Functional interpretation should be reconsidered only after the original sequencing files are aligned to NM\_000549.5, and a transcript-based c. and p. annotation is established.

The control-group allele frequency could not be validly benchmarked against gnomAD, dbSNP, or ClinVar without an unambiguous transcript/genomic coordinate and strand orientation. Database comparison, rsID assignment, and ClinVar status must therefore follow sequence re-annotation rather than be inferred from the current amplicon coordinate.

Overall, the study provides preliminary local genotype counts but not confirmatory evidence of association. Replication requires larger multicenter cohorts, predefined biochemical diagnostic criteria, documentation of Graves' disease or other etiologies, treatment-status data, exact statistical testing, transcript-based HGVS annotation, and subsequent functional assays only if a protein-altering variant is confirmed.

## 4. CONCLUSIONS

This pilot study identified a G>A substitution at position 113 of the sequenced TSHB amplicon. The A allele was numerically more frequent in women with hyperthyroidism than in controls (23.33% vs. 3.33%), but the appropriate two-sided Fisher's exact test did not reach statistical significance (P = 0.052). Consequently, the finding is hypothesis-generating and does not establish an association or causal relationship. Nomenclature review used the RefSeq transcript NM\_000549.5 and confirmed that the laboratory number 113 was an amplicon coordinate, not an ATG-based HGVS c. coordinate; therefore, the prior c.113G>A/p.Ala32Thr designation and structural interpretation were withdrawn. Assignment of a definitive HGVS designation requires re-alignment of the original sequence files to NM\_000549.5. Larger, independently replicated studies with detailed biochemical phenotyping and validated HGVS annotation are required.

## 5. DECLARATIONS

### 5.1. Study Limitations

The principal limitations are the pilot sample size, recruitment from one province, absence of an a priori power calculation, sparse genotype cells, wide confidence intervals, no correction for multiple comparisons, incomplete biochemical diagnostic thresholds and disease-etiology data, unavailable treatment and exclusion-criterion details, analysis of a single amplicon site, absence of the original sequence-file re-alignment needed to convert the amplicon coordinate to an NM\_000549.5-based HGVS coordinate, and lack of functional validation. These limitations restrict precision, causal interpretation, reproducibility, and generalizability.

### 5.2. Acknowledgments

The authors acknowledge the staff of Al-Sadr Hospital in Maysan Province for their assistance with blood sample collection.

### 5.3. Funding Sources

The authors funded this research.

### 5.4. Conflicts of Interest

The authors declare no conflicts of interest and no competing interests.

### 5.5. Data Availability

All data presented in this study are available in the manuscript tables and figures. Raw data are available upon reasonable request from the corresponding author, subject to participant confidentiality and ethical restrictions.

### 5.6. Author Contributions

Yamama A. Oglu: CD, DC, DAI, MW, FA. Salah H. Faraj: CD, DAI, CR, FA.

| Code | English                          | Português                        |
|------|----------------------------------|----------------------------------|
| CD   | Conception and Design            | Concepção e Design               |
| DC   | Data Collection                  | Coleta de Dados                  |
| DAI  | Data Analysis and Interpretation | Análise e Interpretação de Dados |

| Code | English            | Português             |
|------|--------------------|-----------------------|
| MW   | Manuscript Writing | Escrita do Manuscrito |
| CR   | Critical Review    | Revisão Crítica       |
| FA   | Final Approval     | Aprovação Final       |

### 5.7. AI and Computational Tools Declaration

Tool: ChatGPT (OpenAI). Purpose: manuscript formatting, organization, and grammar/style improvement to comply with the journal template. All AI-assisted changes were reviewed and approved by the authors. No generative artificial intelligence was used for data fabrication, statistical testing, scientific interpretation, or independent scientific decision-making.

### 5.8. Research Integrity Declaration

The authors declare that this work is original, the methods were conducted ethically, and no data fabrication, results falsification, P-hacking, or selective reporting occurred.

### 5.9. Originality and Plagiarism Statement

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## 6. STUDIES INVOLVING HUMAN SUBJECTS

### 6.1. Ethics Committee Approval

The study involved human participants and was approved by the authorized ethics body of the College of Science, University of Misan (approval reference 756; 18 November 2024). The study complied with the Declaration of Helsinki. A digitized copy of the official approval letter must be uploaded as a separate supplementary submission file.

### 6.2. Informed Consent

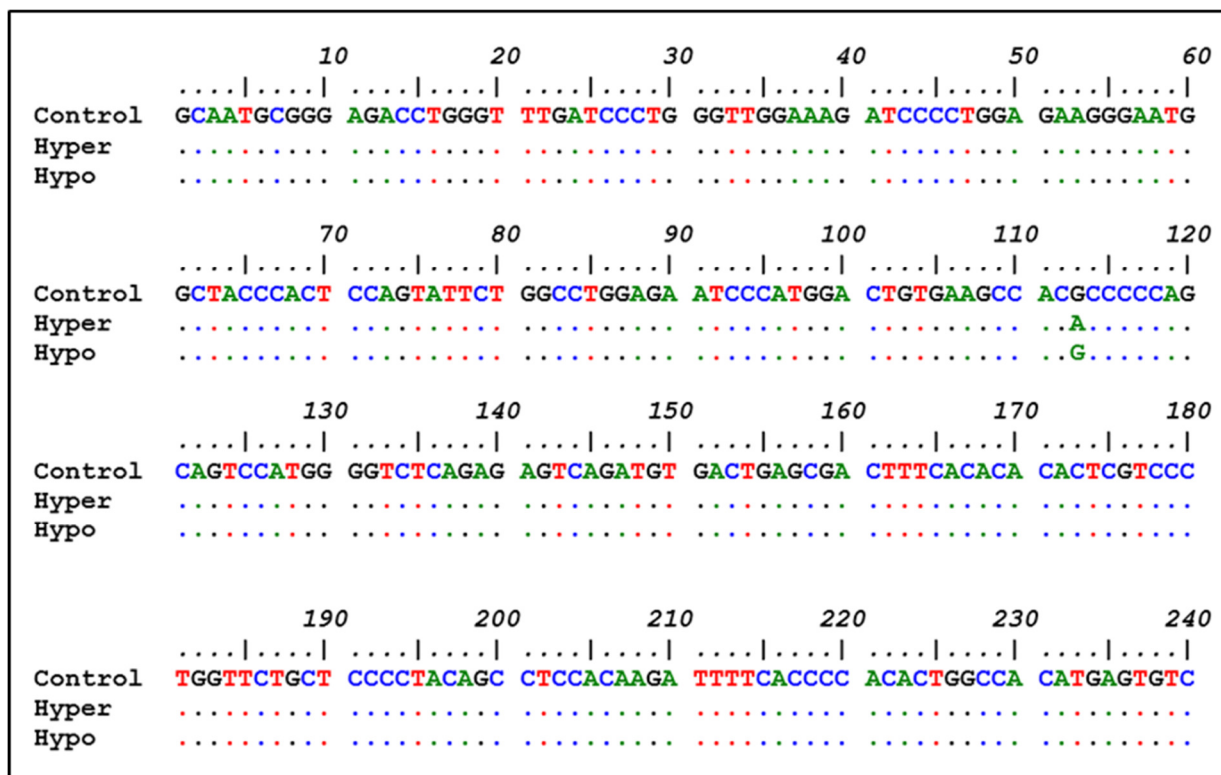
Written informed consent was obtained from all participants before blood sample collection. Data were handled without personal identifiers to protect participant confidentiality and privacy.

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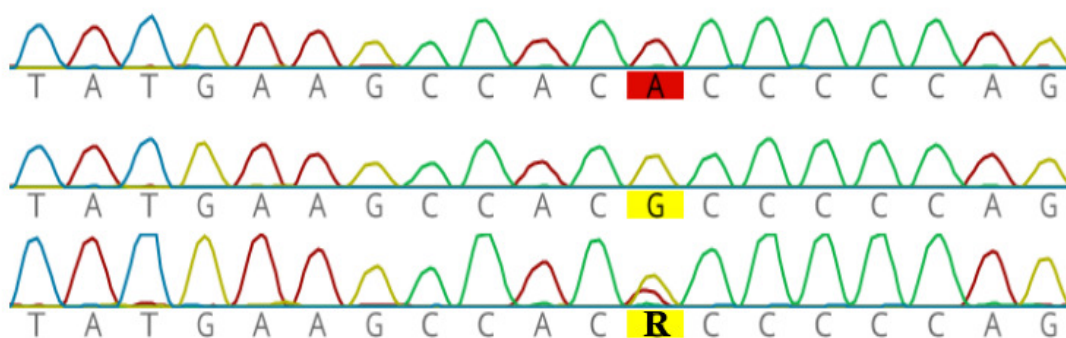
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**Figure 1.** Alignment of TSHB nucleotide sequences.



**Figure 2.** Sequencing chromatograms of the position-113 G>A substitution in the TSHB amplicon. The ambiguity code R indicates G/A.

**Table 1.** Observed and expected genotype counts at the position-113 G>A site in the hyperthyroidism and control groups

| Group          | Status   | GG    | GA   | AA   | $\chi^2$ | P-value |
|----------------|----------|-------|------|------|----------|---------|
| Cases          | Observed | 10    | 3    | 2    | 2.917    | 0.087   |
|                | Expected | 8.82  | 5.37 | 0.82 |          |         |
| Control        | Observed | 14    | 1    | 0    | 0.017    | 0.893   |
|                | Expected | 14.02 | 0.97 | 0.02 |          |         |
| Total observed |          | 24    | 4    | 2    |          |         |

HWE, Hardy–Weinberg equilibrium.

**Table 2.** Genotype and allele frequencies at the position-113 G>A site in the hyperthyroidism and control groups

| Genotype/allele | Hyper n (%) | Control n (%) | OR    | 95% CI        | P-value<br>(Fisher<br>exact where<br>applicable) | PF/EF      |
|-----------------|-------------|---------------|-------|---------------|--------------------------------------------------|------------|
| GG              | 10 (66.67%) | 14 (93.33%)   | 0.142 | 0.014–1.418   | 0.096                                            | PF = 0.858 |
| GA              | 3 (20.00%)  | 1 (6.67%)     | 3.500 | 0.320–38.233  | 0.304                                            | EF = 0.714 |
| AA              | 2 (13.33%)  | 0 (0.00%)     | 5.740 | 0.252–130.379 | 0.272                                            | EF = 0.825 |
| G               | 23 (76.67%) | 29 (96.67%)   | 0.113 | 0.012–0.988   | 0.052                                            | —          |
| A               | 7 (23.33%)  | 1 (3.33%)     | 8.826 | 1.012–76.963  | 0.052                                            | —          |

OR, odds ratio; CI, confidence interval; PF, preventive fraction; EF, etiological fraction. The allele-level P-value is the two-sided Fisher's exact result. Genotype-level estimates are exploratory; no multiple-comparison correction was applied.

## PEPTÍDEOS DERIVADOS DAS MITOCÔNDRIAS, GLICEMIA E NÍVEIS DE HEMOGLOBINA GLICADA EM HOMENS COM DIABETES MELLITUS TIPO 2, ATHEROSCLEROSE OU HIPERTENSÃO: ESTUDO TRANSVERSAL

### MITOCHONDRIAL-DERIVED PEPTIDES, BLOOD GLUCOSE, AND GLYCATED HEMOGLOBIN LEVELS IN MEN WITH TYPE 2 DIABETES, ATHEROSCLEROSIS, OR HYPERTENSION: A CROSS-SECTIONAL STUDY

قيم الببتيدات المشتقة من الميتوكوندريا، وسكر الدم، والهيموغلوبين السكري لدى الرجال المصابين بداء السكري من النوع الثاني أو تصلب الشرايين أو ارتفاع ضغط الدم: دراسة مقطعية

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#### RESUMO

**Introdução:** Os peptídeos derivados das mitocôndrias, incluindo a humanina e o peptídeo codificado pelo quadro aberto de leitura mitocondrial do RNA ribossômico 12S tipo c (MOTS-c), participam da resposta ao estresse celular e da regulação metabólica. **Objetivo:** Comparar as concentrações séricas de humanina e MOTS-c, hemoglobina glicada, glicemia de jejum, insulina e resistência à insulina entre homens saudáveis e homens com diabetes mellitus tipo 2, aterosclerose ou hipertensão. **Métodos:** Este estudo transversal incluiu 88 homens de 40–60 anos, recrutados entre dezembro de 2024 e outubro de 2025 e distribuídos em quatro grupos (n = 22 por grupo). A hemoglobina glicada foi medida em sangue total; glicose, insulina, humanina e MOTS-c foram determinadas no soro. A resistência à insulina foi estimada pelo índice HOMA-IR. As comparações foram realizadas por análise de variância de uma via seguida do teste post hoc da diferença mínima significativa. **Resultados:** A humanina não diferiu entre os grupos (P > 0,05). O MOTS-c foi significativamente menor nos três grupos de pacientes do que no controle (P < 0,001). A hemoglobina glicada, a glicemia de jejum e o HOMA-IR foram mais elevados no grupo com diabetes tipo 2; as concentrações de insulina também diferiram significativamente entre os grupos. **Discussão:** A redução do MOTS-c pode estar associada a alterações da homeostase mitocondrial e metabólica, enquanto a humanina circulante não diferenciou os grupos. **Conclusões:** Baixas concentrações de MOTS-c estiveram associadas a diabetes tipo 2, aterosclerose e hipertensão; entretanto, o desenho transversal, a amostra limitada e as análises não ajustadas impedem inferências causais e generalizações amplas.

**Palavras-chave:** humanina; MOTS-c; peptídeos derivados das mitocôndrias; resistência à insulina; hemoglobina glicada

#### ABSTRACT

**Background:** Mitochondrial-derived peptides, including humanin and mitochondrial open reading frame of the 12S ribosomal RNA type-c (MOTS-c), are bioactive microproteins involved in cellular stress responses and metabolic regulation. **Aim:** To compare serum humanin and MOTS-c, glycated hemoglobin, fasting blood glucose, insulin, and insulin resistance among healthy men and men with type 2 diabetes mellitus, atherosclerosis, or hypertension. **Methods:** This cross-sectional study included 88 men aged 40–60 years who were recruited between December 2024 and October 2025 and assigned to four groups (n = 22 each). Glycated hemoglobin was measured in whole blood; glucose, insulin, humanin, and MOTS-c were measured

in serum. Insulin resistance was estimated using the homeostatic model assessment of insulin resistance (HOMA-IR). Group differences were evaluated using one-way analysis of variance followed by the least significant difference post hoc test. **Results:** Humanin did not differ among groups [ $F(3, 84) = 1.013$ ,  $P = 0.391$ ]. MOTS-c was markedly lower in all patient groups than in controls [ $F(3, 84) = 113.339$ ,  $P < 0.001$ ]. Glycated hemoglobin, fasting glucose, and HOMA-IR were highest in the type 2 diabetes group, and insulin concentrations differed significantly among groups. **Discussion:** Reduced circulating MOTS-c may reflect altered mitochondrial and metabolic homeostasis, whereas circulating humanin did not discriminate among the groups. **Conclusions:** Lower MOTS-c concentrations were associated with type 2 diabetes, atherosclerosis, and hypertension. Because recruitment was site-based and the analyses were unadjusted, the findings are exploratory and do not establish causality.

**Keywords:** mitochondrial-derived peptides; humanin; MOTS-c; insulin resistance; glycated hemoglobin

## المخلص:

**الخلفية:** تُعدّ الببتيدات المشتقة من الميتوكوندريا، ومنها الهيومانين والببتيد MOTS-c، بروتينات دقيقة فعالة حيويًا تسهم في الاستجابة للإجهاد الخلوي وتنظيم الاستقلاب. **الهدف:** مقارنة تراكيز الهيومانين و MOTS-c في المصل، والهيوموغلوبين السكري، وسكر الدم الصائم، والإنسولين، ومقاومة الإنسولين بين رجال أصحاء ورجال مصابين بداء السكري من النوع الثاني أو تصلّب الشرايين أو ارتفاع ضغط الدم. **طرائق العمل:** شملت هذه الدراسة المقطعية 88 رجلاً تراوحت أعمارهم بين 40 و 60 عاماً، جرى استقطابهم خلال المدة من كانون الأول/ديسمبر 2024 إلى تشرين الأول/أكتوبر 2025، وقُسموا إلى أربع مجموعات بواقع 22 مشاركاً لكل مجموعة. قيس الهيموغلوبين السكري في الدم الكامل، في حين قيس كل من الجلوكوز والإنسولين والهيومانين و MOTS-c في المصل، وحُسبت مقاومة الإنسولين باستعمال مؤشر HOMA-IR. وقورنت المجموعات بتحليل التباين الأحادي متبوعاً باختبار أقل فرق معنوي. **النتائج:** لم تختلف تراكيز الهيومانين معنوياً بين المجموعات ( $P > 0.05$ )، بينما انخفضت تراكيز MOTS-c معنوياً في مجموعات المرضى الثلاث مقارنةً بالمجموعة الضابطة ( $P < 0.001$ ). وكانت قيم الهيموغلوبين السكري وسكر الدم الصائم و HOMA-IR الأعلى في مجموعة السكري من النوع الثاني، كما اختلفت تراكيز الإنسولين معنوياً بين المجموعات. **المناقشة:** قد يرتبط انخفاض MOTS-c باضطراب الاتزان الميتوكوندري والاستقلابي، في حين لم تميّز تراكيز الهيومانين الدورية بين المجموعات. **الاستنتاجات:** ارتبط انخفاض MOTS-c بداء السكري من النوع الثاني وتصلّب الشرايين وارتفاع ضغط الدم، إلا أن التصميم المقطعي وحجم العينة المحدود والتحليلات غير المعدلة لا تسمح بإثبات السببية أو التعميم الواسع.

**الكلمات المفتاحية:** الببتيدات المشتقة من الميتوكوندريا؛ الهيومانين؛ MOTS-c؛ مقاومة الإنسولين؛ الهيموغلوبين السكري.

## 1. INTRODUCTION:

Mitochondria coordinate cellular bioenergetics, redox balance, apoptosis, and stress signaling. Beyond adenosine triphosphate production, mitochondrial DNA encodes small biologically active peptides that participate in inter-organelle and systemic signaling (Saminathan *et al.*, 2022; Clemente-Suárez *et al.*, 2023). These mitochondrial-derived peptides include humanin, mitochondrial open reading frame of the 12S ribosomal RNA type-c (MOTS-c), small humanin-like peptides 1–6, SHMOOSE, and Gau (Saghatelian and Couso, 2015; Kumagai *et al.*, 2023).

Humanin is encoded within the mitochondrial MT-RNR2 region and was originally identified through its neuroprotective activity (Hashimoto *et al.*, 2001). It has antiapoptotic, cytoprotective, and metabolic effects, although circulating concentrations may vary with age, tissue activity, and disease state (Salemi *et al.*, 2020; Zuccato *et al.*, 2019; Coradduzza *et al.*, 2023). MOTS-c is encoded

within MT-RNR1 and can modulate cellular metabolism through pathways that include AMP-activated protein kinase; experimental evidence links it to improved glucose uptake, insulin sensitivity, and energy homeostasis (Lee *et al.*, 2015; Kim *et al.*, 2019; Merry *et al.*, 2020).

Type 2 diabetes mellitus is characterized by insulin resistance, compensatory hyperinsulinemia, and progressive  $\beta$ -cell dysfunction (Schleicher *et al.*, 2022). Mitochondrial-derived peptides have been implicated in pancreatic  $\beta$ -cell survival and metabolic regulation (Hoang *et al.*, 2010). In vascular disease, humanin may protect endothelial cells from oxidized low-density lipoprotein-related injury, whereas MOTS-c has been associated with metabolic and vascular homeostasis (Evangeline and Iyer, 2021; Zhu *et al.*, 2017; Li *et al.*, 2019). Clinical studies have reported altered circulating humanin or MOTS-c in diabetes and coronary artery disease, but findings are not uniform across populations and clinical phenotypes. Hypertension frequently coexists with dysglycemia and insulin

resistance, and elevated fasting glucose has been associated with a subsequent risk of hypertension, particularly in men (Ahn *et al.*, 2021). Because mitochondrial stress signaling contributes to metabolic and vascular homeostasis, altered circulating mitochondrial-derived peptides may also accompany hypertensive cardiometabolic phenotypes, although direct clinical evidence remains limited (Savvopoulos *et al.*, 2024).

Direct comparisons of both humanin and MOTS-c across type 2 diabetes, atherosclerosis, and hypertension using the same sampling and assay framework remain limited, particularly in Iraqi men. We hypothesized that circulating mitochondrial-derived peptides would differ between healthy controls and the three patient groups and would parallel disturbances in glycemic control and insulin resistance.

### 1.1. Aim

The objective of this study was to compare serum humanin and MOTS-c concentrations, glycated hemoglobin, fasting blood glucose, insulin, and homeostatic model assessment of insulin resistance (HOMA-IR) among healthy men and men with type 2 diabetes mellitus, atherosclerosis, or hypertension

## 2. MATERIALS AND METHODS:

### 2.1. Study Population

This cross-sectional study was conducted at the Center for Diabetes and Endocrinology, the Maysan Center for Cardiac Diseases and Surgery, Al-Taif Laboratory, and selected private clinics in Maysan, Iraq, from December 2024 to October 2025. Eligibility was restricted to men aged 40–60 years who belonged to one of four predefined clinical categories and provided written informed consent. The final analyzed sample comprised 88 participants: healthy controls (group A), type 2 diabetes mellitus (group B), atherosclerosis (group C), and hypertension (group D), with 22 participants in each group. All 88 enrolled participants were included in the reported analyses. No formal a priori power calculation was performed; the sample size reflected recruitment feasibility during the prespecified period, and the study should therefore be

regarded as exploratory.

Patient-group diagnoses were established by specialist physicians using medical history, clinical findings, and available medical records at the participating centers. Medication histories were recorded descriptively but were not used for stratification or covariate adjustment. Standardized eligibility, fasting blood collection, and laboratory processing procedures were applied to reduce measurement variability; however, the site-based non-random recruitment strategy may have introduced selection bias.

### 2.2. Blood Samples

Six milliliters of venous blood were collected from each participant after an 8-hour fast between 09:00 and 11:00. Two milliliters were placed in an ethylenediaminetetraacetic acid tube for glycated hemoglobin determination using the Huma Meter A1C reagent system (Human, Germany) according to Nathan *et al.* (2008). The remaining 4 mL were placed in a gel tube, allowed to clot for 15 minutes, and centrifuged at 3,000 rpm for 5 minutes. Serum glucose was measured using a BioSystems A15 automated spectrophotometer (Spain) according to standard clinical chemistry procedures. Residual serum was stored at –20 °C until analysis. Humanin and MOTS-c were measured using immunoassay kits supplied by MyBioSource (USA) and the ELISYS Uno system (Human, Germany), respectively. Insulin was measured using the AFIAS automated immunoassay system. HOMA-IR was calculated as fasting insulin ( $\mu\text{U/mL}$ )  $\times$  fasting glucose (mg/dL) / 405 (Matthews *et al.*, 1985).

### 2.3. Outcomes and Statistical Analysis

The primary biochemical outcomes were serum humanin and MOTS-c concentrations. Secondary outcomes were glycated hemoglobin, fasting glucose, insulin, and HOMA-IR. Quantitative variables were analyzed as continuous measures. Group differences were evaluated using one-way analysis of variance followed by the least significant difference post hoc test. Normality and homogeneity of variance were not formally assessed using the Shapiro-Wilk and Levene tests because archived participant-level diagnostic outputs were unavailable. Results are presented as mean  $\pm$  standard error together with 95% confidence intervals.



Omnibus effects are reported as  $F(3, 84)$ ,  $P$  values, and eta-squared ( $\eta^2$ ). All analyses were unadjusted because complete participant-level covariate data were not available for multivariable modeling; no subgroup or sensitivity analyses were performed. Statistical significance was defined as two-sided  $P < 0.05$ .

### 3. RESULTS AND DISCUSSION:

#### 3.1. Results

Serum humanin concentrations did not differ significantly among the control [ $3.66 \pm 0.55$  pg/mL; 95% confidence interval (CI), 2.52–4.80], type 2 diabetes [ $2.88 \pm 0.66$  pg/mL; 95% CI, 1.51–4.25], atherosclerosis [ $2.83 \pm 0.14$  pg/mL; 95% CI, 2.54–3.12], and hypertension groups [ $2.66 \pm 0.12$  pg/mL; 95% CI, 2.41–2.91;  $F(3, 84) = 1.013$ ,  $P = 0.391$ ,  $\eta^2 = 0.035$ ] (Figure 1; Table 1).

Serum MOTS-c concentrations were lower in the type 2 diabetes [ $17.77 \pm 1.53$  pg/mL; 95% CI, 14.59–20.95], atherosclerosis [ $19.78 \pm 3.07$  pg/mL; 95% CI, 13.40–26.16], and hypertension groups [ $18.27 \pm 2.29$  pg/mL; 95% CI, 13.51–23.03] than in the control group [ $99.27 \pm 6.36$  pg/mL; 95% CI, 86.04–112.50;  $F(3, 84) = 113.339$ ,  $P < 0.001$ ,  $\eta^2 = 0.802$ ]. The three patient groups did not differ significantly from one another (Figure 2; Table 1).

Glycated hemoglobin was highest in the type 2 diabetes group [ $9.21 \pm 0.46\%$ ; 95% CI, 8.25–10.17] and exceeded the values in the atherosclerosis [ $5.58 \pm 0.07\%$ ; 95% CI, 5.43–5.73], hypertension [ $5.55 \pm 0.06\%$ ; 95% CI, 5.43–5.67], and control groups [ $5.48 \pm 0.07\%$ ; 95% CI, 5.33–5.63;  $F(3, 84) = 58.676$ ,  $P < 0.001$ ,  $\eta^2 = 0.677$ ]. No significant differences were observed among the latter three groups (Figure 3; Table 1).

Fasting glucose differed significantly among groups [ $F(3, 84) = 25.989$ ,  $P < 0.001$ ,  $\eta^2 = 0.481$ ]. The type 2 diabetes group had the highest concentration [ $210.09 \pm 16.92$  mg/dL; 95% CI, 174.90–245.28], followed by the hypertension [ $134.23 \pm 14.54$  mg/dL; 95% CI, 103.99–164.47], atherosclerosis [ $94.23 \pm 2.72$  mg/dL; 95% CI, 88.57–99.89], and control groups [ $81.50 \pm 3.36$  mg/dL; 95% CI, 74.51–88.49]. Post hoc comparisons are summarized by the letter annotations in Figure 4.

Serum insulin concentrations differed significantly among groups [ $F(3, 84) = 4.965$ ,  $P = 0.003$ ,  $\eta^2 = 0.151$ ]. Mean concentrations were

$9.24 \pm 0.97$   $\mu\text{U/mL}$  (95% CI, 7.22–11.26) in controls,  $23.00 \pm 4.26$   $\mu\text{U/mL}$  (95% CI, 14.14–31.86) in type 2 diabetes,  $17.55 \pm 2.12$   $\mu\text{U/mL}$  (95% CI, 13.14–21.96) in atherosclerosis, and  $19.78 \pm 2.06$   $\mu\text{U/mL}$  (95% CI, 15.50–24.06) in hypertension. The post hoc pattern is shown in Figure 5.

HOMA-IR differed significantly among groups [ $F(3, 84) = 10.203$ ,  $P < 0.001$ ,  $\eta^2 = 0.267$ ]. Values were highest in type 2 diabetes [ $8.05 \pm 1.31$ ; 95% CI, 5.33–10.77], followed by hypertension [ $5.40 \pm 0.70$ ; 95% CI, 3.94–6.86], atherosclerosis [ $4.16 \pm 0.58$ ; 95% CI, 2.95–5.37], and controls [ $1.86 \pm 0.19$ ; 95% CI, 1.46–2.26]. The pairwise comparison pattern is presented in Figure 6.

#### 3.2. Discussion

The principal findings were a marked reduction in circulating MOTS-c across the three patient groups, no statistically significant group difference in humanin, and the greatest glycemic dysregulation and insulin resistance in men with type 2 diabetes. Because this was a cross-sectional, unadjusted comparison, these findings indicate associations rather than causal or predictive relationships.

The absence of a significant difference in humanin is consistent with the report by Conte *et al.* (2021), but contrasts with studies that observed lower circulating humanin in impaired fasting glucose or diabetes (Ramanjaneya *et al.*, 2019; Voigt and Jelinek, 2016). Exercise status, age, disease severity, assay characteristics, and tissue-specific activity may contribute to this heterogeneity. Humanin concentrations can vary with age and growth hormone/insulin-like growth factor-1 signaling, and circulating levels may not fully represent biological activity within target tissues (Gong *et al.*, 2014; Lee *et al.*, 2014; Boutari *et al.*, 2022). Evidence that resistance training can increase skeletal-muscle humanin levels in men with impaired glucose metabolism further supports exercise status as a potential source of between-study variation (Gidlund *et al.*, 2016).

Humanin has also been linked to vascular and oxidative-stress phenotypes. Lower circulating humanin has been associated with coronary endothelial dysfunction (Widmer *et al.*, 2013), whereas experimental ischemia/reperfusion studies have demonstrated changes in endogenous humanin

after injury (Thummasorn *et al.*, 2016). Increased tissue expression has been reported in mitochondrial disorders characterized by substantial oxidative stress (Kariya *et al.*, 2005). These context-dependent observations support cautious interpretation of a single serum measurement.

MOTS-c concentrations were substantially lower in men with type 2 diabetes, atherosclerosis, or hypertension than in controls. This pattern may reflect impaired mitochondrial stress signaling or reduced compensatory metabolic responses rather than a disease-specific effect. Reviews of diabetes-related evidence similarly describe reduced circulating MOTS-c in metabolic dysfunction, although stage of disease, obesity, and treatment may influence the measured concentration (Fang *et al.*, 2025).

The type 2 diabetes finding agrees with Ramanjaneya *et al.* (2019), who reported lower circulating MOTS-c in diabetes, and with Ikonomidis *et al.* (2020), who linked low MOTS-c to adverse cardiovascular phenotypes in patients with type 2 diabetes and coronary artery disease. The much larger difference between controls and all patient groups in the present study produced a large omnibus effect size; nevertheless, replication with independent cohorts and assay validation is required.

In coronary artery disease, lower MOTS-c has been associated with lesion severity and complexity (Yaşar *et al.*, 2022). Experimental studies also suggest that MOTS-c participates in mitochondrial dynamics, antioxidant defense, and exercise-related metabolic adaptation (Li *et al.*, 2019; Tang *et al.*, 2023; Yang *et al.*, 2021; Woodhead and Merry, 2021). Reduced expression in chronic metabolic stress has also been described (Liu *et al.*, 2019). These studies provide biologic plausibility, but they do not demonstrate that reduced MOTS-c caused the abnormalities observed in the present participants.

As expected, glycated hemoglobin, fasting glucose, and HOMA-IR were most markedly altered in the type 2 diabetes group. Recent evidence supports bidirectional crosstalk between impaired mitochondrial quality control and insulin resistance, whereby mitochondrial dysfunction may worsen insulin signaling, while hyperglycemia and other metabolic stressors can further compromise mitochondrial function (Chen *et al.*, 2025). Nevertheless, the cross-sectional design of the

present study precludes causal or directional inference. Accordingly, the lower MOTS-c concentrations observed alongside poorer glycemic indices should be interpreted as a group-level descriptive association rather than evidence of causality.

Previous experimental and clinical reports have linked mitochondrial-derived peptides to glucose homeostasis. Humanin-related peptides have altered metabolic profiles in obesity models (Mehta *et al.*, 2019), and combined humanin, MOTS-c, and SHLP2 treatment improved glucose homeostasis in diabetic mice (Paul *et al.*, 2026). Reviews and clinical studies have reported inverse associations of MOTS-c with fasting insulin, glycated hemoglobin, or insulin resistance, although findings are not uniform across comorbidity patterns (Miller *et al.*, 2020; Ramanjaneya *et al.*, 2019; Savvopoulos *et al.*, 2024).

The higher fasting glucose observed in the hypertension group is compatible with epidemiologic evidence linking dysglycemia to later hypertension risk, particularly in men (Ahn *et al.*, 2021). However, the current groups were not matched or adjusted for medication use, disease duration, adiposity, smoking, diet, or physical activity, and these factors could explain part of the between-group differences.

Insulin resistance is also relevant to atherosclerotic risk. In a prospective cohort of 929 adults, elevated insulin-resistance-related indices were associated with incident carotid plaques, including among individuals with comparatively favorable lipid or cardiovascular risk profiles (Liu *et al.*, 2026). This supports the clinical importance of insulin resistance, but it does not directly validate HOMA-IR or MOTS-c as diagnostic markers in the present sample.

Clinically, MOTS-c may merit further study as a complementary biomarker of cardiometabolic dysfunction, but the substantial between-study variability, limited assay standardization, and overlap among disease mechanisms preclude immediate diagnostic use. Future work should include women, wider age ranges, larger multicenter samples, detailed medication and lifestyle data, validated assay performance, and longitudinal follow-up to determine temporal relationships and clinical utility.

## 4. CONCLUSIONS

In this exploratory cross-sectional study, circulating MOTS-c was markedly lower in men with type 2 diabetes mellitus, atherosclerosis, or hypertension than in healthy controls, whereas humanin did not differ significantly among groups. Glycated hemoglobin, fasting glucose, and HOMA-IR were most abnormal in type 2 diabetes. These findings support an association between reduced MOTS-c and cardiometabolic dysfunction but do not establish causality or diagnostic performance. Larger longitudinal and multicenter studies that include women, broader age groups, assay validation, and adjustment for medication and lifestyle factors are needed.

## 5. DECLARATIONS

### 5.1. Study Limitations

The sample comprised only men aged 40–60 years from a single geographical area, which limits external validity. The modest sample size (22 participants per group) was based on recruitment feasibility rather than a formal a priori power calculation and may have limited detection of smaller effects, particularly for humanin. Recruitment was site-based and non-random; blinding and randomization were not applicable to group assignment, and laboratory blinding was not documented. Medication use, disease duration, adiposity, smoking, diet, physical activity, and other potential confounders were not controlled, and analyses were not adjusted. Formal archived outputs for distributional assumptions and sensitivity analyses were unavailable. The cross-sectional design precludes causal inference, and circulating humanin may not reflect tissue-level activity. In addition, six biochemical outcomes were evaluated using LSD post hoc comparisons without a multiplicity correction, which may have increased the family-wise risk of type I error.

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### 5.3. Funding Sources:

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### 5.4. Conflicts of Interest:

The authors declare that they have no financial, personal, or professional competing interests related to this work.

### 5.5. Data Availability:

Participant-level data are not publicly available because of ethical and regulatory requirements concerning privacy and confidentiality. De-identified aggregated data may be available from the corresponding author upon reasonable request and subject to institutional approval.

### 5.6. Author Contributions:

Inas Majid Mozan: CD, DC, DAI, MW, FA.

Zainab Abduljabber Ridha Al-Ali and Redha Alwan Hasan: CD, DAI, CR, FA.

| Code | English                          | Português                        |
|------|----------------------------------|----------------------------------|
| CD   | Conception and Design            | Concepção e Design               |
| DC   | Data Collection                  | Coleta de Dados                  |
| DAI  | Data Analysis and Interpretation | Análise e Interpretação de Dados |
| MW   | Manuscript Writing               | Escrita do Manuscrito            |
| CR   | Critical Review                  | Revisão Crítica                  |
| FA   | Final Approval                   | Aprovação Final                  |

### 5.7. AI and Computational Tools Declaration:

During manuscript preparation, the authors used ChatGPT (OpenAI, San Francisco, CA, USA) solely for language editing and readability. The tool was not used to generate study data, conduct laboratory procedures, perform the original statistical analysis, or determine scientific conclusions. All AI-assisted text was critically reviewed and edited by the authors, who verified the scientific content and accept full responsibility for the final manuscript.

### 5.8. Research Integrity Declaration:

The authors affirm that the study was conducted and reported according to accepted principles of accuracy, transparency, accountability, responsible data handling, and research integrity.

## 5.9. Originality and Similarity Statement:

The authors confirm that this manuscript is original, has not been published previously, and is not under consideration elsewhere. The manuscript was screened for text similarity before submission.

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## 6. STUDIES INVOLVING HUMAN SUBJECTS

### 6.1. Ethics Committee Approval

The study protocol was approved by the Training and Human Development Center, Misan Health Directorate (approval no. 770, dated 27 November 2024). All participant-related procedures complied with applicable institutional ethical requirements and the principles of the Declaration of Helsinki.

### 6.2. Informed Consent

All participants received an explanation of the study procedures, sample collection, and laboratory investigations. Written and signed informed consent was obtained before enrollment and before any study-specific procedure.

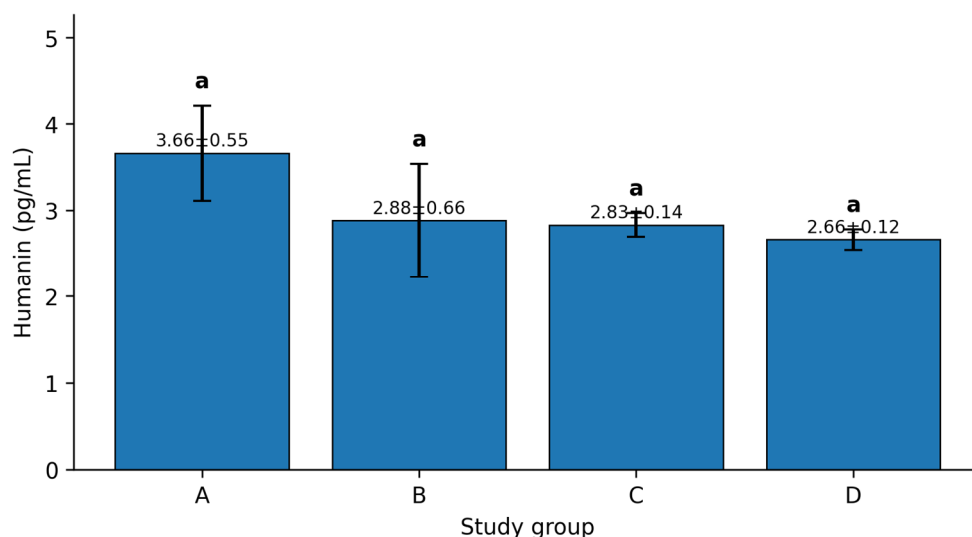
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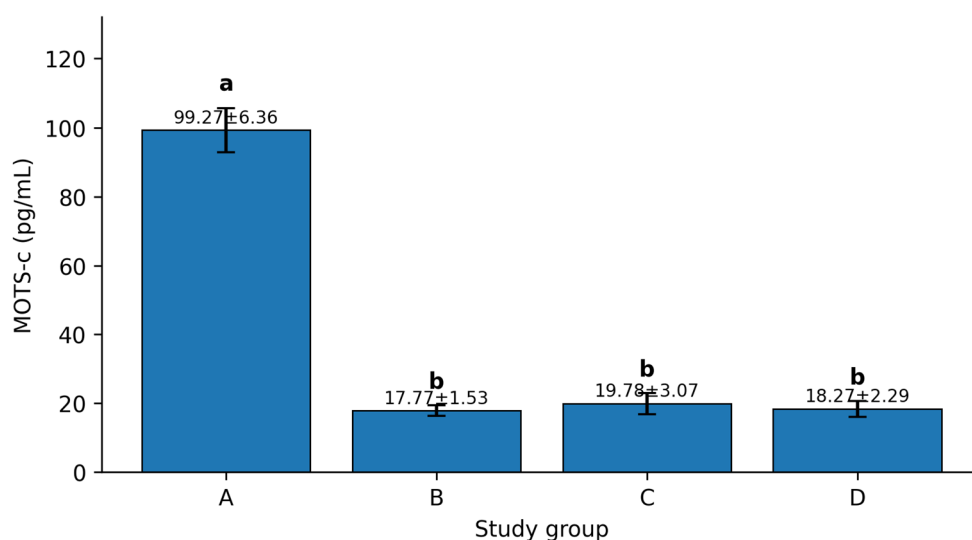
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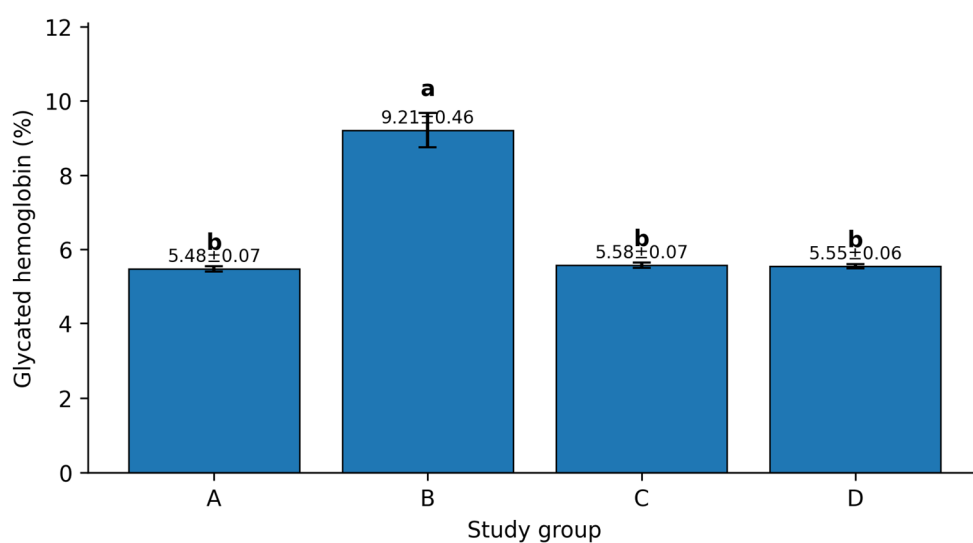


**Figure 1.** Serum humanin concentrations by study group. A, healthy control; B, type 2 diabetes mellitus; C, atherosclerosis; D, hypertension. Bars represent mean  $\pm$  standard error. Identical letters indicate no significant difference according to the least significant difference post hoc test ( $P > 0.05$ ).

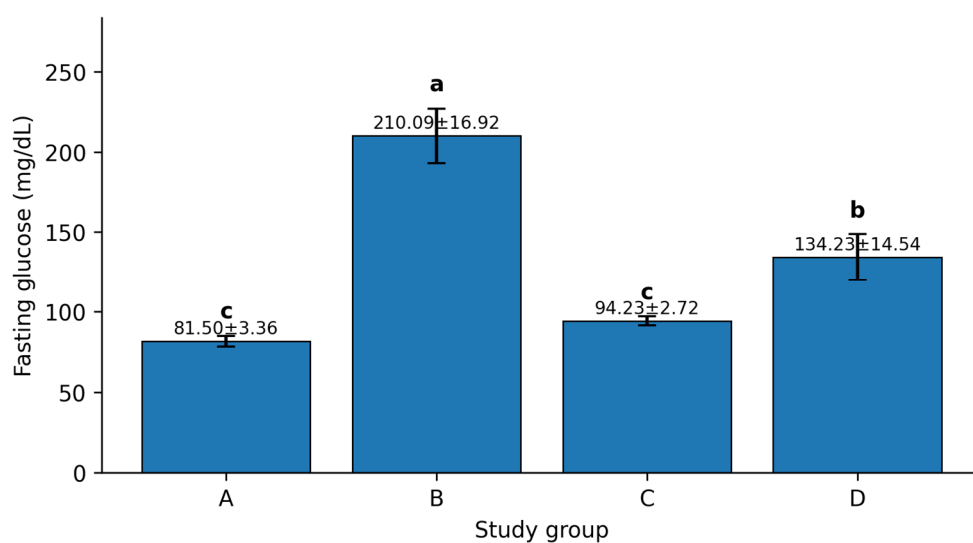


**Figure 2.** Serum MOTS-c concentrations by study group. A, healthy control; B, type 2 diabetes mellitus; C, atherosclerosis; D, hypertension. Bars represent mean  $\pm$  standard error. Different letters indicate significant pairwise differences, whereas identical letters indicate no significant difference ( $P < 0.05$ ).

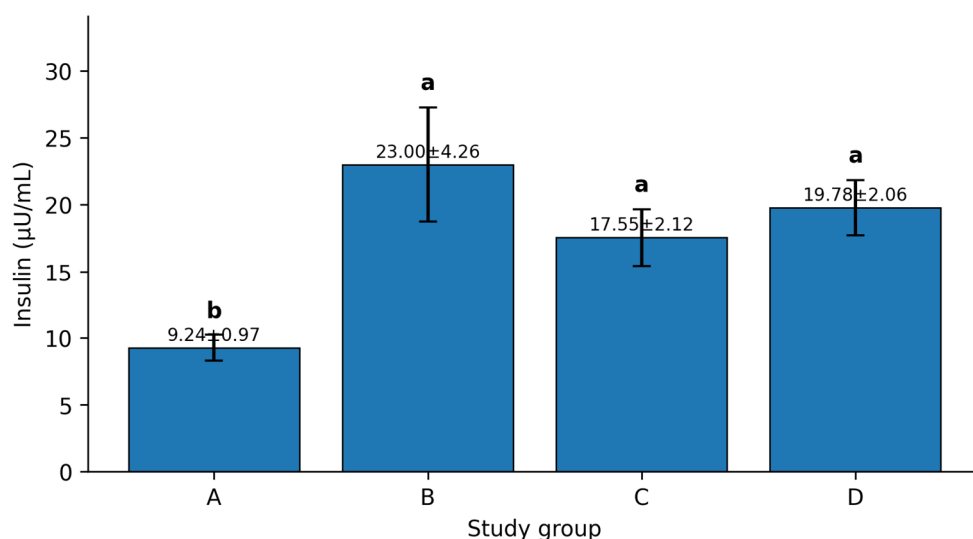




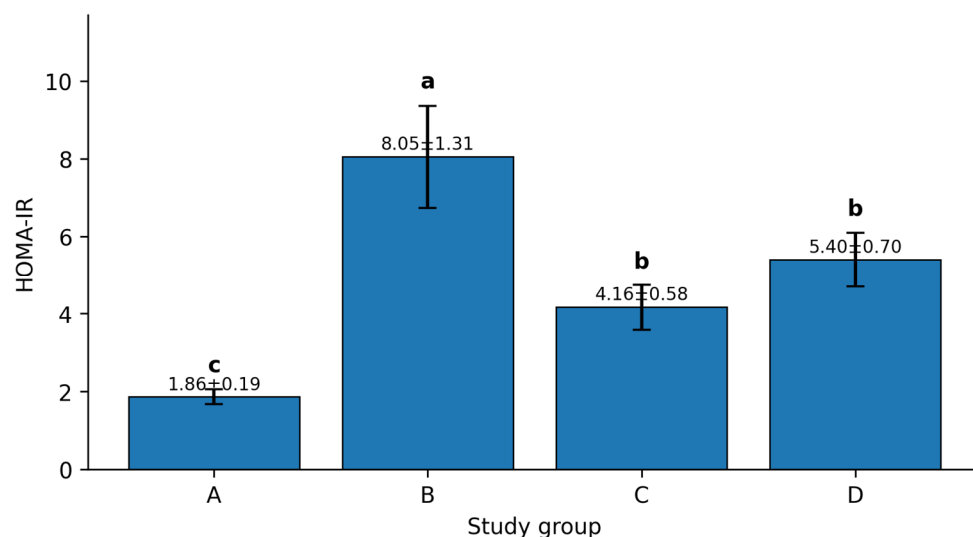
**Figure 3.** Glycated hemoglobin by study group. A, healthy control; B, type 2 diabetes mellitus; C, atherosclerosis; D, hypertension. Bars represent mean  $\pm$  standard error. Different letters indicate significant pairwise differences ( $P < 0.05$ ).



**Figure 4.** Fasting blood glucose by study group. A, healthy control; B, type 2 diabetes mellitus; C, atherosclerosis; D, hypertension. Bars represent mean  $\pm$  standard error. Letter annotations summarize least significant difference post hoc comparisons ( $P < 0.05$ ).



**Figure 5.** Serum insulin concentrations by study group. A, healthy control; B, type 2 diabetes mellitus; C, atherosclerosis; D, hypertension. Bars represent mean  $\pm$  standard error. Letter annotations summarize least significant difference post hoc comparisons ( $P < 0.05$ ).



**Figure 6.** Homeostatic model assessment of insulin resistance (HOMA-IR) by study group. A, healthy control; B, type 2 diabetes mellitus; C, atherosclerosis; D, hypertension. Bars represent mean  $\pm$  standard error. Letter annotations summarize least significant difference post hoc comparisons ( $P < 0.05$ ).

**Table 1.** One-way analysis of variance results for the six biochemical outcomes.

| Parameter | ANOVA df | F       | P value | $\eta^2$ |
|-----------|----------|---------|---------|----------|
| Humanin   | 3, 84    | 1.013   | 0.391   | 0.035    |
| MOTS-c    | 3, 84    | 113.339 | <0.001  | 0.802    |
| HbA1c     | 3, 84    | 58.676  | <0.001  | 0.677    |
| Glucose   | 3, 84    | 25.989  | <0.001  | 0.481    |
| Insulin   | 3, 84    | 4.965   | 0.003   | 0.151    |
| HOMA-IR   | 3, 84    | 10.203  | <0.001  | 0.267    |

For each outcome,  $df = 3, 84$ . Values are based on one-way analysis of variance;  $\eta^2$  denotes eta-squared effect size.  $P$  values below 0.001 are reported as  $P < 0.001$ . Pairwise comparisons were performed using the least significant difference post hoc test.

## SÍNTESE, CARACTERIZAÇÃO ESPECTROSCÓPICA E DOCKING MOLECULAR DE NOVOS HÍBRIDOS DE 1,4-DI-HIDROPIRIDINA-1,2,4-TRIAZOLIDINA-3-TIONA CONTRA A TIROSINA QUINASE EGFR

## SYNTHESIS, SPECTROSCOPIC CHARACTERIZATION, AND MOLECULAR DOCKING OF NOVEL 1,4-DIHYDROPYRIDINE-1,2,4-TRIAZOLIDINE-3-THIONE HYBRIDS AGAINST EGFR TYROSINE KINASE

تخليق وتوصيف طيفي والتحام جزيئي لمركبات هجينة جديدة من 1,4-ثنائي هيدروبييريدين-2,4-تريازوليدين-3-ثيون تجاه إنزيم تيروسين كيناز لمستقبل عامل نمو البشرة (EGFR)

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### RESUMO

**Introdução:** Sistemas heterocíclicos híbridos que combinam núcleos estruturalmente e farmacologicamente relevantes são de interesse em química medicinal, pois a integração de diferentes arcabouços pode ampliar a diversidade molecular e modificar o comportamento de ligação ao alvo. **Objetivo:** Sintetizar e caracterizar estruturalmente uma nova série de híbridos 1,4-di-hidropiridina-1,2,4-triazolidina-3-tiona e investigar suas interações previstas com o domínio tirosina-quinase do receptor do fator de crescimento epidérmico (EGFR). **Métodos:** Os compostos-alvo foram preparados por uma sequência de três etapas: condensação multicomponente do tipo Hantzsch, formilação de Vilsmeier-Haack e ciclização com tiosemicarbazida. Os intermediários e produtos finais foram caracterizados por espectroscopia no infravermelho com transformada de Fourier (FT-IR), ressonância magnética nuclear (RMN) de  $^1\text{H}$  e  $^{13}\text{C}$ , Distortionless Enhancement by Polarization Transfer (DEPT-135) e espectrometria de massas. O docking molecular foi realizado contra o domínio tirosina-quinase do EGFR obtido do Protein Data Bank (PDB ID: 6JWL), utilizando o software Molecular Operating Environment. **Resultados:** Os derivados-alvo foram obtidos com rendimentos moderados a bons, e os dados espectroscópicos foram consistentes com as estruturas propostas. Entre os compostos submetidos ao docking, M9 apresentou o escore previsto mais favorável ( $-15,91$  kcal/mol), seguido por M6, M3 e M12 ( $-14,64$ ,  $-14,49$  e  $-14,44$  kcal/mol, respectivamente). As poses previstas mostraram interações com resíduos do sítio ativo do EGFR, incluindo Lys745, Thr790 e Asp855. **Discussão:** As diferenças no comportamento de ligação previsto foram associadas à natureza e à posição dos substituintes arila. O derivado para-cloro M9 apresentou o escore de docking mais favorável nas condições computacionais aplicadas. **Conclusões:** Uma nova série de híbridos 1,4-di-hidropiridina-1,2,4-triazolidina-3-tiona foi sintetizada e caracterizada. M9 foi o candidato computacional mais bem classificado; entretanto, os resultados são preliminares e requerem confirmação por ensaios in vitro de inibição do EGFR, citotoxicidade e seletividade.

**Palavras-chave:** 1,4-di-hidropiridina; 1,2,4-triazolidina-3-tiona; híbridos heterocíclicos; reação de Vilsmeier-Haack; docking molecular; tirosina quinase EGFR; caracterização espectroscópica.

### ABSTRACT

**Background:** Hybrid heterocyclic systems that combine structurally and pharmacologically relevant scaffolds are of interest in medicinal chemistry because scaffold integration can expand molecular diversity and alter target-binding behavior. **Aim:** To synthesize and structurally characterize a new series of 1,4-dihydropyridine-1,2,4-triazolidine-3-thione hybrids and investigate their predicted interactions with the epidermal growth factor receptor (EGFR) tyrosine kinase domain. **Methods:** The target compounds were prepared through a three-step sequence involving Hantzsch-type multicomponent condensation, Vilsmeier-Haack formylation, and cyclization

with thiosemicarbazide. Intermediates and final products were characterized by Fourier-transform infrared (FT-IR) spectroscopy, proton and carbon-13 nuclear magnetic resonance ( $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR), Distortionless Enhancement by Polarization Transfer (DEPT-135), and mass spectrometry. Molecular docking was performed against the EGFR tyrosine kinase domain obtained from the Protein Data Bank (PDB ID: 6JWL) using Molecular Operating Environment software. **Results:** The target derivatives were obtained in moderate to good yields, and the spectroscopic data were consistent with the proposed structures. Among the docked compounds, M9 showed the most favorable predicted docking score ( $-15.91$  kcal/mol), followed by M6, M3, and M12 ( $-14.64$ ,  $-14.49$ , and  $-14.44$  kcal/mol, respectively). The predicted poses showed interactions with EGFR active-site residues, including Lys745, Thr790, and Asp855. **Discussion:** Differences in predicted binding behavior were associated with the nature and position of the aryl substituents. The para-chloro derivative M9 showed the most favorable docking score under the applied computational conditions. **Conclusions:** A new series of 1,4-dihydropyridine-1,2,4-triazolidine-3-thione hybrids was synthesized and structurally characterized. M9 was the highest-ranked computational candidate; however, the findings are preliminary and require confirmation through in vitro EGFR-inhibition, cytotoxicity, and selectivity studies.

**Keywords:** 1,4-Dihydropyridine; 1,2,4-triazolidine-3-thione; heterocyclic hybrids; Vilsmeier-Haack reaction; molecular docking; EGFR tyrosine kinase; spectroscopic characterization.

## المخلص

**الخلفية:** تحظى الأنظمة الحلقية غير المتجانسة الهجينة، التي تجمع بين هياكل بنائية ذات أهمية دوائية، باهتمام في الكيمياء الطبية؛ إذ يمكن أن يؤدي الدمج الهيكلي إلى تعزيز التنوع الجزيئي وتعديل سلوك الارتباط بالأهداف البيولوجية. **الهدف:** تخليق وتوصيف سلسلة جديدة من المركبات الهجينة التي تجمع بين نواة 1,4-ثنائي هيدروبيريدين ونواة 2,4-1-تريازوليدين-3-ثيون، ودراسة تفاعلاتها المتوقعة مع نطاق إنزيم تيروسين كيناز لمستقبل عامل نمو البشرية (EGFR). **طرائق العمل:** حُضِرَت المركبات المستهدفة عبر ثلاث خطوات شملت تكاثف هانتزش متعدد المكونات، وفورملة فيلسمير-هاك، والإغلاق الحلقي باستخدام الثيوسيميكاربازيد. وجرى توصيف المركبات الوسيطة والنهائية بمطيافية الأشعة تحت الحمراء بتحويل فورييه (FT-IR)، ومطيافية الرنين المغناطيسي النووي (NMR) للبروتون والكربون ( $^1\text{H}$ -NMR و  $^{13}\text{C}$ -NMR)، وتقنية تعزيز الاستقطاب عديم التشوه (DEPT-135)، ومطيافية الكتلة. وأجري الالتحام الجزيئي مع نطاق تيروسين كيناز لمستقبل EGFR (PDB ID: 6JWL) باستخدام برنامج Molecular Operating Environment. **النتائج:** تم الحصول على المشتقات المستهدفة بنواتج متوسطة إلى جيدة، وكانت البيانات الطيفية متوافقة مع التراكيب المقترحة. وأظهر المركب M9 أفضل درجة التلاحم متوقعة ( $-15.91$  كيلو كالوري/مول)، تلاه M6 و M3 و M12 ( $-14.64$ ،  $-14.49$ ، و  $-14.44$  كيلو كالوري/مول، على التوالي). وأظهرت أوضاع الالتحام المتوقعة تفاعلات مع بقايا في الموقع النشط لمستقبل EGFR، منها Lys745 و Thr790 و Asp855. **المناقشة:** ارتبطت الاختلافات في سلوك الارتباط المتوقع بطبيعة مستبدلات الأريل وموقعها، وكان مشتق الباراكور M9 صاحب أفضل درجة التلاحم ضمن الظروف الحسابية المطبقة. **الاستنتاجات:** تم تخليق وتوصيف سلسلة جديدة من هجائن 1،-4-ثنائي هيدروبيريدين-3-ثيون، و 2،4-1-تريازوليدين-3-ثيون. وكان M9 المرشح الحسابي الأعلى ترتيباً؛ إلا أن النتائج أولية وتتطلب تأكيداً باختبارات تثبيط EGFR والسمية الخلوية والانتقائية في المختبر.

**الكلمات المفتاحية:** 1,4-ثنائي هيدروبيريدين-3-ثيون، 2,4-1-تريازوليدين-3-ثيون؛ هجائن حلقية غير متجانسة؛ تفاعل فيلسمير-هاك؛ الالتحام الجزيئي؛ تيروسين كيناز مستقبل عامل نمو البشرية (EGFR)؛ التوصيف الطيفي.

## 1. INTRODUCTION

Heterocyclic compounds are organic cyclic systems containing at least one heteroatom within the ring structure. The most common heteroatoms are nitrogen, oxygen, and sulfur; however, heterocycles incorporating other elements, such as selenium (Abdel-Hafez, 2008), phosphorus, iron, and magnesium, have also been reported. These compounds represent one of the most prevalent and significant classes in organic chemistry, accounting for nearly half of the approximately 50 million organic compounds documented in the Chemical Abstracts database (Comins and Ma, 2016).

The importance of heterocyclic compounds arises from their broad biological and pharmacological relevance. They are key structural components of many pharmaceuticals, natural products, and biomolecules. In oncology, nitrogen-containing heterocycles are especially important because they provide scaffolds for

kinase-targeted agents, including inhibitors of epidermal growth factor receptor (EGFR) signaling (Vitaku, Smith, and Njardarson, 2014; Irfan *et al.*, 2025). Structurally, heterocycles are broadly classified into aromatic (Zhao *et al.*, 2017) and non-aromatic systems (Vitaku, Smith, and Njardarson, 2014). Many biologically active compounds contain five- or six-membered rings bearing one to three heteroatoms, either as isolated rings or fused systems such as the purine and pyrimidine bases in DNA (Kabir and Uzzaman, 2022). The clinical and pharmacological relevance of mutant-selective EGFR inhibition is exemplified by osimertinib, a third-generation irreversible inhibitor developed for sensitizing and T790M-resistant EGFR variants (Cross *et al.*, 2014).

Among heterocyclic systems, the pyridine ring has attracted considerable attention due to its versatile applications in medicinal and materials chemistry. Partial or complete reduction

of pyridine yields important derivatives such as piperidine, tetrahydropyridines, and dihydropyridines, each possessing distinct chemical and pharmacological properties. Notably, more than 85% of biologically active compounds either contain heterocyclic rings or are entirely heterocyclic in nature (Heravi, 2020), with representative examples including milrinone and amrinone (Chalán-Gualán *et al.*, 2022).

In particular, the 1,4-dihydropyridine ring is a six-membered heterocyclic system containing one nitrogen atom (Hussein and Al-Mathkuri, 2025) and is considered a crucial scaffold in medicinal chemistry. Its derivatives exhibit a wide spectrum of pharmacological activities, making them essential frameworks in drug design. Extensive research has demonstrated that dihydropyridine derivatives can interact with multiple biological targets, highlighting their therapeutic potential and importance in the development of modern pharmaceuticals (Ali and Al-Mathkuri, 2023).

Despite substantial progress in this field, structural modification of dihydropyridine systems to improve target engagement remains an active area of research (Lago *et al.*, 2023). One strategy is to incorporate an additional heterocyclic moiety into a dihydropyridine or dihydropyridinone core to expand chemical space and modulate target specificity (Faizan *et al.*, 2024). Thiosemicarbazone derivatives and their cyclized 1,2,4-triazolidine-3-thione products are attractive in this context because of their versatile reactivity and medicinal-chemistry relevance (Gričar *et al.*, 2025). However, their integration with dihydropyridinone scaffolds remains insufficiently explored. Recent analyses of catalytic 1,4-dihydropyridine synthesis further emphasize continued efforts to improve efficiency, sustainability, and structural diversity in this scaffold class (Soni *et al.*, 2025).

Although 1,4-dihydropyridine derivatives and 1,2,4-triazolidine-3-thione systems have each been investigated because of their pharmacological relevance, comparatively little attention has been given to hybrid structures that combine both motifs within one molecular framework; previous studies have mainly examined independently substituted dihydropyridines, thiosemicarbazones, or triazolidine derivatives (Patil *et al.*, 2019; Ramesh and Lalitha, 2016). The direct construction of 1,4-dihydropyridine–1,2,4-triazolidine-3-thione hybrids, particularly the effect of electron-withdrawing aryl substituents on synthesis, spectroscopic features, and predicted EGFR

binding, therefore represents a defined research gap.

In the present work, a new series of 1,4-dihydropyridine derivatives bearing a 1,2,4-triazolidine-3-thione moiety was synthesized through a sequential three-step route involving Hantzsch condensation, Vilsmeier–Haack formylation, and cyclization with thiosemicarbazide. The compounds contained p-NO<sub>2</sub>, p-Br, p-Cl, or o-Cl aryl substituents to examine their effects on reaction yield, spectroscopic behavior, and predicted molecular interactions. Molecular docking against the EGFR tyrosine kinase domain was used as a preliminary computational approach, consistent with prior EGFR-focused studies of dihydropyridine derivatives (Mansour *et al.*, 2022; Faizan *et al.*, 2024).

### 1.1. Aims

The main aim of this study was to synthesize and structurally characterize a new series of 1,4-dihydropyridine–1,2,4-triazolidine-3-thione hybrid derivatives and to investigate their predicted interactions with the EGFR tyrosine kinase domain using molecular docking.

The specific objectives were:

1. To prepare substituted 1,4,5,6-tetrahydropyridine derivatives through a Hantzsch-type multicomponent reaction.
2. To convert the synthesized intermediates into 5-formyl-1,4-dihydropyridine derivatives using Vilsmeier–Haack formylation.
3. To synthesize the corresponding 1,2,4-triazolidine-3-thione derivatives by reaction with thiosemicarbazide.
4. To characterize the synthesized compounds using FT-IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, DEPT-135, and mass spectrometry.
5. To examine the influence of p-NO<sub>2</sub>, p-Br, p-Cl, and o-Cl substituents on the synthetic yields and spectroscopic behavior of the compounds.
6. To evaluate the predicted binding modes and interaction profiles of the final derivatives within the EGFR tyrosine kinase active site.

## 2. MATERIALS AND METHODS

### 2.1. Materials

All chemicals and solvents used in this study were of analytical grade and were obtained from commercial suppliers, including Merck, BDH, and Sigma-Aldrich. The reagents included 4-nitrobenzaldehyde, 4-bromobenzaldehyde, 4-chlorobenzaldehyde, 2-chlorobenzaldehyde, Meldrum's acid, methyl acetoacetate, ammonium acetate, glacial acetic acid, dimethylformamide (DMF), phosphorus oxychloride ( $\text{POCl}_3$ ), thiosemicarbazide, sodium acetate, and anhydrous sodium sulfate. The solvents used included dichloromethane, ethanol, methanol, ethyl acetate, petroleum ether, and distilled water. Silica gel was used for column chromatography, and pre-coated silica gel 60  $\text{F}_{254}$  plates were used for thin-layer chromatography. Unless otherwise stated, all chemicals were used without further purification.

### 2.2. Instrumentation

Reaction progress was monitored by thin-layer chromatography (TLC) on pre-coated silica gel 60  $\text{F}_{254}$  plates, and spots were visualized under ultraviolet light. Column chromatography was performed using 60–120-mesh silica gel. Melting points were measured on a Stuart SMP11 apparatus and are uncorrected. Fourier-transform infrared (FT-IR) spectra were recorded on a Shimadzu IR Affinity-1 spectrophotometer using KBr pellets over 4000–400  $\text{cm}^{-1}$ . Proton and carbon-13 nuclear magnetic resonance ( $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR) spectra, including Distortionless Enhancement by Polarization Transfer (DEPT-135) experiments, were acquired on a Bruker spectrometer at 400 MHz for  $^1\text{H}$  and 101 MHz for  $^{13}\text{C}$ .  $\text{CDCl}_3$  and  $\text{DMSO}-d_6$  were used as deuterated solvents, with tetramethylsilane as the internal reference. Electrospray-ionization mass spectra were recorded on an AB SCIEX 3200 QTRAP instrument. Molecular structures and docking simulations were prepared and analyzed using Molecular Operating Environment (MOE), version 2014.

### 2.3. Methods

#### 2.3.1. Synthesis of methyl 2-methyl-4-(substituted phenyl)-6-oxo-1,4,5,6-tetrahydropyridine-3-carboxylate derivatives (M1, M4, M7, and M10)

The synthesis was performed using a Hantzsch-type multicomponent procedure adapted from Vijesh *et al.* (2011). Methyl

acetoacetate (1 mmol), the appropriate substituted benzaldehyde (1 mmol), Meldrum's acid (1 mmol), and ammonium acetate (1.5 mmol) were dissolved in glacial acetic acid (10 mL). The reaction mixture was heated under reflux at 120  $^\circ\text{C}$  for 12 h, as shown in Equation 1. Reaction progress was monitored by TLC using ethyl acetate/petroleum ether (1:3, v/v) as the mobile phase.

After completion, the reaction mixture was cooled to room temperature. For the 4-bromo, 4-chloro, and 2-chloro derivatives, the precipitated solids were collected by filtration, washed with cold ethanol, and recrystallized from methanol.

For the 4-nitro derivative, no precipitate formed upon cooling. The solvent was removed under reduced pressure, and the crude product was purified by column chromatography using ethyl acetate/petroleum ether. The isolated product was dried and characterized by FT-IR,  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, and DEPT-135 spectroscopy.

#### 2.3.2. Synthesis of methyl 6-chloro-4-(substituted phenyl)-5-formyl-2-methyl-1,4-dihydropyridine-3-carboxylate derivatives (M2, M5, M8, and M11)

The Vilsmeier–Haack formylation was performed using a procedure adapted from Armas *et al.* (2000). Dimethylformamide (1.24 mL, 16 mmol) in dichloromethane (4 mL) was added dropwise to phosphorus oxychloride (1.5 mL, 16 mmol) with continuous stirring at room temperature. The mixture was stirred for 30 min to generate the Vilsmeier–Haack reagent.

A solution of the appropriate methyl 2-methyl-4-(substituted phenyl)-6-oxo-1,4,5,6-tetrahydropyridine-3-carboxylate derivative (4 mmol) in dichloromethane (16 mL) was then added slowly. The resulting reaction mixture was stirred at room temperature for 24 h, as shown in Equation 2. Reaction progress was monitored by thin-layer chromatography using ethyl acetate/petroleum ether (1:2, v/v).

After completion, an aqueous sodium acetate solution, prepared by dissolving 16 g of sodium acetate in 24 mL of distilled water, was added slowly over 1 h with continuous stirring. The mixture was extracted three times with dichloromethane, and the combined organic layers were dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure.

The crude products were purified by column chromatography using ethyl acetate/petroleum ether (1:6, v/v) as the eluent. The purified derivatives were obtained as light-

yellow or yellow solids and were characterized by FT-IR,  $^1\text{H}$  NMR, and  $^{13}\text{C}$  NMR spectroscopy.

### 2.3.3. Synthesis of methyl 6-chloro-4-(substituted phenyl)-2-methyl-5-(5-thioxo-1,2,4-triazolidin-3-yl)-1,4-dihydropyridine-3-carboxylate derivatives (M3, M6, M9, and M12)

The cyclization procedure was adapted from reported thiosemicarbazone-to-triazolidine-3-thione transformations (Gričar *et al.*, 2025; Patil *et al.*, 2019; Ramesh and Lalitha, 2016). Thiosemicarbazide (1 mmol) and the appropriate methyl 6-chloro-4-(substituted phenyl)-5-formyl-2-methyl-1,4-dihydropyridine-3-carboxylate derivative (1 mmol) were dissolved in ethanol (5 mL) and distilled water (15 mL). The reaction mixture was heated under reflux at 80 °C for 12 h with continuous stirring, as shown in Equation 3.

Reaction progress was monitored by thin-layer chromatography using ethyl acetate/petroleum ether (1:3, v/v). After completion, the mixture was cooled to room temperature, and the resulting precipitate was collected by filtration, washed with cold ethanol, and dried.

The crude products were purified by column chromatography using ethyl acetate/petroleum ether (1:6, v/v) as the eluent. FT-IR,  $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR, and mass spectrometry were used to characterize the purified final compounds.

## 2.4. Molecular Docking Study

Molecular docking was conducted to examine the predicted binding modes of M3, M6, M9, and M12 within the EGFR tyrosine kinase domain, following a workflow comparable to that used for dihydropyridine-based EGFR ligands (Mansour *et al.*, 2022). The crystal structure of the L858R-mutant EGFR kinase domain in complex with osimertinib (AZD9291) was obtained from the Protein Data Bank (PDB ID: 6JWL; 2.55 Å resolution); the co-crystallized ligand is identified as YY3. The Protein Data Bank is the principal open repository for experimentally determined three-dimensional macromolecular structures (Burley *et al.*, 2019).

The receptor was prepared in MOE version 2014 by inspecting structural inconsistencies, removing water and non-essential crystallographic species, adding hydrogen atoms, assigning protonation states under physiological conditions, and minimizing the structure while preserving the crystallographic geometry. The co-crystallized osimertinib

molecule was retained to define the ATP-binding site and validate the protocol, then removed before docking the synthesized compounds. Ligands M3, M6, M9, and M12 were constructed, protonated, and energy-minimized in MOE.

The active site was defined from the position of co-crystallized osimertinib. Ligands were placed using Triangle Matcher, initially scored with London dG, and the 30 highest-ranked poses were subjected to rigid-receptor refinement and rescoring with GBVI/WSA dG. The five highest-ranked refined poses were retained for each ligand.

Protocol validation was performed by redocking co-crystallized osimertinib under the same placement, scoring, and refinement settings. Reproduction of the crystallographic orientation was assessed using the heavy-atom root-mean-square deviation (RMSD), with a predefined acceptance criterion of  $\text{RMSD} \leq 2.0$  Å. This cutoff is consistent with the commonly applied criterion for successful reproduction of crystallographic ligand poses in docking-validation studies (Warren *et al.*, 2006; Goullieux *et al.*, 2023).

The final poses of the synthesized compounds and reference ligand were evaluated using the GBVI/WSA dG score, pose RMSD, orientation in the ATP-binding pocket, hydrogen-bond interactions, and other polar or hydrophobic contacts with active-site residues.

## 3. RESULTS AND DISCUSSION

### 3.1. Results

The synthesized compounds (M1–M12) were obtained successfully. Their physical characteristics (color and melting point), reaction yields, molecular formulae, and chemical structures are summarized in Table 1.

#### 3.1.1. Spectroscopic Characterization of 1,4,5,6-Tetrahydropyridine Derivatives (M1, M4, M7, and M10)

##### 3.1.1.1. Methyl 2-methyl-4-(4-nitrophenyl)-6-oxo-1,4,5,6-tetrahydropyridine-3-carboxylate (M1)

Off-white; yield = 41%; m.p. = 202 °C (Table 1). FT-IR (KBr,  $\nu_{\text{max}}$ ,  $\text{cm}^{-1}$ ): 3466–3416 (N–H), 3116 (Ar–H), 2947 (aliphatic C–H), 1793 (C=O, ester), 1699 (C=O, amide), 1637–1599 (C=C, aromatic/olefinic), 1514 ( $\text{NO}_2$ , asym), 1350 ( $\text{NO}_2$ , sym), 1489–1450 (C–C, aliphatic), 1284 (C–N,  $\text{NO}_2$ ), 1201 (C–N, amide), 1178, 1109, 1085 (C–O, ester), 854–833 (Ar–C–H, out-of-plane).  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  10.06 (s, 1H, NH),



8.16 (d,  $J = 8.7$  Hz, 2H, Ar-H), 7.43 (d,  $J = 8.6$  Hz, 2H, Ar-H), 4.27 (d,  $J = 7.5$  Hz, 1H, CH), 3.53 (s, 3H, OCH<sub>3</sub>), 3.03 (dd,  $J = 16.4, 7.5$  Hz, 1H, CH<sub>2</sub>), 2.44 (d,  $J = 16.2$  Hz, 1H, CH<sub>2</sub>), 2.35 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>)  $\delta$  169.27, 166.73, 150.65, 149.57, 146.43, 128.04, 123.91, 103.49, 51.18, 39.52, 37.91, 37.40, 18.33.

**3.1.1.2. Methyl 4-(4-bromophenyl)-2-methyl-6-oxo-1,4,5,6-tetrahydropyridine-3-carboxylate (M4)**

Off-white; yield = 44.4%;  $R_f = 0.65$ ; m.p. = 187 °C (Table 1). FT-IR (KBr,  $\nu_{\max}$ , cm<sup>-1</sup>): 3464 (N-H), 3057 (Ar-H), 2951 (aliphatic C-H), 1708 (C=O, ester), 1689 (C=O, lactam), 1631 (C=C, DHP ring), 1539 (Ar C=C), 1489 (C=C), 1311 (C-N), 1226 (C-O), 1085 (C-O-C), 731 (Ar-H, out-of-plane), 630 (C-Br). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.47 (s, 1H, NH), 7.42 (d,  $J = 8.2$  Hz, 2H, Ar-H), 7.07 (d,  $J = 8.2$  Hz, 2H, Ar-H), 4.23 (d,  $J = 7.9$  Hz, 1H, CH), 3.67 (s, 3H, OCH<sub>3</sub>), 2.91 (dd,  $J = 16.5, 7.9$  Hz, 1H, CH<sub>2</sub>), 2.64 (d,  $J = 16.5$  Hz, 1H, CH<sub>2</sub>), 2.40 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  170.54, 167.06, 146.54, 140.97, 131.90, 128.46, 120.87, 106.68, 51.41, 37.89, 37.43, 19.14.

**3.1.1.3. Methyl 4-(4-chlorophenyl)-2-methyl-6-oxo-1,4,5,6-tetrahydropyridine-3-carboxylate (M7)**

Off-white; yield = 30%;  $R_f = 0.67$ ; m.p. = 173 °C (Table 1). FT-IR (KBr,  $\nu_{\max}$ , cm<sup>-1</sup>): 3475 (N-H), 3124 (Ar-H), 2947 (aliphatic C-H), 1747 (C=O, ester), 1697 (C=O, lactam), 1633 (C=C, DHP ring), 1558 (Ar C=C), 1489 (C=C), 1313 (C-N), 1222 (C-O), 1082 (C-O-C), 823 (Ar-H, out-of-plane), 651 (C-Cl). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.44 (s, 1H, NH), 7.24 (d,  $J = 8.4$  Hz, 2H, Ar-H), 7.12 (d,  $J = 8.3$  Hz, 2H, Ar-H), 4.22 (d,  $J = 7.7$  Hz, 1H, CH), 3.68 (s, 3H, OCH<sub>3</sub>), 2.92 (dd,  $J = 16.5, 7.7$  Hz, 1H, CH<sub>2</sub>), 2.64 (d,  $J = 16.5$  Hz, 1H, CH<sub>2</sub>), 2.43 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  170.85, 167.15, 146.72, 140.32, 132.80, 128.97, 128.12, 106.67, 51.56, 38.03, 37.26, 19.20.

**3.1.1.4. Methyl 4-(2-chlorophenyl)-2-methyl-6-oxo-1,4,5,6-tetrahydropyridine-3-carboxylate (M10)**

Off-white; yield = 42%;  $R_f = 0.67$ ; m.p. = 195 °C (Table 1). FT-IR (KBr,  $\nu_{\max}$ , cm<sup>-1</sup>): 3477 (N-H), 3057 (Ar-H), 2954 (aliphatic C-H), 1707 (C=O, ester), 1633 (C=C, DHP ring), 1539 (Ar C=C), 1487 (C=C), 1301 (C-N), 1190 (C-O), 1087 (C-O-C), 742 (Ar-H, out-of-plane), 671 (C-Cl). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  10.05 (s, 1H, NH), 7.51–7.44 (m, 1H, Ar-H), 7.26 (t,  $J = 4.8$  Hz, 2H, Ar-H), 7.08–7.01 (m, 1H, Ar-H), 4.50 (d,  $J = 8.0$  Hz, 1H, CH), 3.40 (s, 3H, OCH<sub>3</sub>), 3.01

(dd,  $J = 16.3, 8.0$  Hz, 1H, CH<sub>2</sub>), 2.42 (d,  $J = 16.3$  Hz, 1H, CH<sub>2</sub>), 2.29 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>)  $\delta$  169.57, 167.07, 150.43, 139.15, 132.78, 130.48, 129.11, 128.03, 127.76, 103.76, 51.59, 36.85, 35.30, 18.73.

**3.1.2. Spectroscopic Characterization of Methyl 6-chloro-4-(substituted phenyl)-5-formyl-2-methyl-1,4-dihydropyridine-3-carboxylate Derivatives (M2, M5, M8, and M11)**

**3.1.2.1. Methyl 6-chloro-5-formyl-2-methyl-4-(4-nitrophenyl)-1,4-dihydropyridine-3-carboxylate (M2)**

Light yellow; yield = 51.8%;  $R_f = 0.31$ ; m.p. = 151 °C (Table 1). FT-IR (KBr,  $\nu_{\max}$ , cm<sup>-1</sup>): 3479 (N-H), 3061 (Ar-H), 2951, 2922, 2860 (C-H), 1697 (C=O, ester), 1649 (C=O, CHO), 1606, 1512 (C=C), 1558, 1344 (NO<sub>2</sub>), 1294 (C-N), 1226, 1087 (C-O), 862, 692 (Ar-H). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  9.76 (s, 1H, CHO), 8.10 (d,  $J = 8.6$  Hz, 2H, Ar-H), 7.46 (d,  $J = 8.6$  Hz, 2H, Ar-H), 6.90 (s, 1H, NH), 5.22 (s, 1H, CH), 3.50 (s, 3H, OCH<sub>3</sub>), 2.45 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  187.19, 166.50, 152.03, 146.73, 144.66, 142.07, 128.82, 123.69, 112.12, 105.41, 51.61, 38.74, 19.08.

**3.1.2.2. Methyl 4-(4-bromophenyl)-6-chloro-5-formyl-2-methyl-1,4-dihydropyridine-3-carboxylate (M5)**

Light yellow; yield = 59.3%;  $R_f = 0.35$ ; m.p. = 141 °C (Table 1). FT-IR (KBr,  $\nu_{\max}$ , cm<sup>-1</sup>): 3464 (N-H), 3057 (Ar-H), 2951, 2920, 2848 (C-H), 1708 (C=O, ester), 1689 (C=O, CHO), 1600, 1539 (C=C), 1489 (C=C), 1296 (C-N), 1226, 1085 (C-O), 775, 731 (Ar-H), 630, 605 (C-Br). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  9.75 (s, 1H, CHO), 7.35 (d,  $J = 8.3$  Hz, 2H, Ar-H), 7.15 (d,  $J = 8.3$  Hz, 2H, Ar-H), 7.07 (s, 1H, NH), 5.09 (s, 1H, CH), 3.63 (s, 3H, OCH<sub>3</sub>), 2.41 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  187.65, 167.00, 144.09, 142.17, 129.69, 120.83, 112.74, 106.13, 51.63, 38.14, 19.06.

**3.1.2.3. Methyl 6-chloro-4-(4-chlorophenyl)-5-formyl-2-methyl-1,4-dihydropyridine-3-carboxylate (M8)**

Light yellow; yield = 50%;  $R_f = 0.37$ ; m.p. = 136 °C (Table 1). FT-IR (KBr,  $\nu_{\max}$ , cm<sup>-1</sup>): 3471 (N-H), 3062 (Ar-H), 2956, 2924, 2829 (C-H), 1710 (C=O, ester), 1689 (C=O, CHO), 1618, 1544 (C=C), 1487 (C=C), 1296 (C-N), 1226, 1085 (C-O), 804, 731 (Ar-H), 628, 455 (C-Cl). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  9.76 (s, 1H, CHO), 7.24–7.07 (m, 4H, Ar-H), 5.10 (s, 1H, CH), 3.63 (s, 3H, OCH<sub>3</sub>), 2.40 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  187.61, 166.95, 144.14, 143.53, 142.23, 132.56, 129.20, 128.45, 112.68, 106.15,

51.51, 38.00, 18.90.

**3.1.2.4. Methyl 6-chloro-4-(2-chlorophenyl)-5-formyl-2-methyl-1,4-dihydropyridine-3-carboxylate (M11)**

Yellow; yield = 58%; R<sub>f</sub> = 0.38; m.p. = 190 °C (Table 1). FT-IR (KBr, v<sub>max</sub>, cm<sup>-1</sup>): 3410 (N-H), 3057 (Ar-H), 2954, 2922, 2854 (C-H), 1707 (C=O, ester), 1633 (C=O, CHO), 1591, 1539 (C=C), 1487 (C=C), 1301 (C-N), 1230, 1087 (C-O), 831, 742 (Ar-H), 626, 453 (C-Cl). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.67 (s, 1H, CHO), 7.30 (d, J = 7.7 Hz, 2H, Ar-H), 7.20 (d, J = 6.5 Hz, 1H, Ar-H), 7.09 (t, J = 6.8 Hz, 1H, Ar-H), 7.02 (br s, 1H, NH), 5.39 (s, 1H, CH), 3.54 (s, 3H, OCH<sub>3</sub>), 2.27 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 187.56, 167.06, 143.60, 142.41, 133.26, 131.73, 129.86, 128.02, 126.73, 112.32, 105.94, 51.23, 37.76, 18.83.

**3.1.3. Spectroscopic Characterization of Methyl 6-chloro-4-(substituted phenyl)-2-methyl-5-(5-thioxo-1,2,4-triazolidin-3-yl)-1,4-dihydropyridine-3-carboxylate Derivatives (M3, M6, M9, and M12)**

The TLC profile before purification showed multiple spots, whereas the chromatographic fractions showed improved separation and purity (Figure 1).

**3.1.3.1. Methyl 6-chloro-2-methyl-4-(4-nitrophenyl)-5-(5-thioxo-1,2,4-triazolidin-3-yl)-1,4-dihydropyridine-3-carboxylate (M3)**

Orange; yield = 50%; R<sub>f</sub> = 0.20; m.p. = 226–228 °C (Table 1). FT-IR (KBr, v<sub>max</sub>, cm<sup>-1</sup>): 3545, 3419, 3317, 3236 (N-H, DHP and triazolidine rings), 3149 (Ar-H), 2947, 2918 (aliphatic C-H), 1639 (C=C), 1541 (NO<sub>2</sub>, asym), 1342 (NO<sub>2</sub>, sym), 1224 (C=S), 1103 (C-O-C). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ 11.22 (s, 1H, NH), 9.93 (s, 1H, NH), 8.12 (s, 1H, NH), 8.08 (d, J = 8.7 Hz, 2H, Ar-H), 8.03 (s, 1H, triazolidine CH), 7.57 (d, J = 8.7 Hz, 2H, Ar-H), 7.35 (s, 1H, DHP-NH), 5.25 (s, 1H, DHP-CH), 3.54 (s, 3H, OCH<sub>3</sub>), 2.28 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) δ 177.48, 167.14, 154.65, 147.10, 146.41, 140.64, 131.75, 129.45, 123.64, 107.63, 101.07, 51.29, 40.63, 18.63. MS (m/z): [M]<sup>+</sup> = 409.

**3.1.3.2. Methyl 4-(4-bromophenyl)-6-chloro-2-methyl-5-(5-thioxo-1,2,4-triazolidin-3-yl)-1,4-dihydropyridine-3-carboxylate (M6)**

Orange; yield = 45%; R<sub>f</sub> = 0.25; m.p. = 210–212 °C (Table 1). FT-IR (KBr, v<sub>max</sub>, cm<sup>-1</sup>): 3543, 3415, 3300 (N-H, DHP and triazolidine

rings), 3037 (Ar-H), 2956, 2850 (aliphatic C-H), 1735 (C=O, ester), 1681 (C=C), 1371 (C-N), 1222 (C=S), 1039 (C-O-C), 617–561 (C-Br). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ 11.23 (s, 1H, NH), 9.84 (s, 1H, NH), 8.12 (s, 1H, NH), 8.01 (s, 1H, triazolidine CH), 7.39 (d, J = 8.2 Hz, 2H, Ar-H), 7.25 (s, 1H, DHP-NH), 7.21 (d, J = 7.6 Hz, 2H, Ar-H), 5.07 (s, 1H, DHP-CH), 3.53 (s, 3H, OCH<sub>3</sub>), 2.26 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) δ 176.93, 166.91, 146.22, 146.07, 140.32, 131.48, 130.96, 130.80, 129.99, 119.32, 107.66, 101.20, 50.83, 40.66, 18.15. MS (m/z): [M]<sup>+</sup> = 443.

**3.1.3.3. Methyl 6-chloro-4-(4-chlorophenyl)-2-methyl-5-(5-thioxo-1,2,4-triazolidin-3-yl)-1,4-dihydropyridine-3-carboxylate (M9)**

Orange; yield = 62.3%; R<sub>f</sub> = 0.22; m.p. = 208–210 °C (Table 1). FT-IR (KBr, v<sub>max</sub>, cm<sup>-1</sup>): 3425, 3145 (N-H, DHP and triazolidine rings), 3024 (Ar-H), 2974 (aliphatic C-H), 1751 (C=O, ester), 1662 (C=C), 1371 (C-N), 1222 (C=S), 1093 (C-O-C), 553–495 (C-Cl). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ 11.23 (s, 1H, NH), 9.83 (s, 1H, NH), 8.11 (s, 1H, NH), 8.01 (s, 1H, triazolidine CH), 7.48 (s, 1H, DHP-NH), 7.26 (m, 4H, Ar-H), 5.08 (s, 1H, DHP-CH), 3.53 (s, 3H, OCH<sub>3</sub>), 2.26 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) δ 177.42, 167.35, 146.46, 146.21, 140.78, 131.20, 129.98, 128.32, 108.14, 107.59, 101.72, 51.23, 40.60, 18.57. MS (m/z): [M]<sup>+</sup> = 399.

**3.1.3.4. Methyl 6-chloro-4-(2-chlorophenyl)-2-methyl-5-(5-thioxo-1,2,4-triazolidin-3-yl)-1,4-dihydropyridine-3-carboxylate (M12)**

Orange; yield = 60%; R<sub>f</sub> = 0.22; m.p. = 210–212 °C (Table 1). FT-IR (KBr, v<sub>max</sub>, cm<sup>-1</sup>): 3414, 3336, 3163 (N-H, DHP and triazolidine rings), 3030 (Ar-H), 2972 (aliphatic C-H), 1735 (C=O, ester), 1668 (C=C), 1371 (C-N), 1224 (C=S), 1035 (C-O-C), 547–420 (C-Cl). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ 11.26 (s, 1H, NH), 9.94 (s, 1H, NH), 8.26 (s, 1H, NH), 8.07 (s, 1H, triazolidine CH), 7.36–7.19 (m, 4H, Ar-H), 7.16 (s, 1H, DHP-NH), 5.37 (s, 1H, DHP-CH), 3.51 (s, 3H, OCH<sub>3</sub>), 2.24 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) δ 177.29, 167.30, 146.14, 144.92, 141.54, 132.50, 131.36, 129.53, 128.61, 128.25, 107.82, 101.75, 51.06, 40.57, 18.43.

**3.1.4. Molecular Docking Results**

The docking protocol was evaluated by redocking the co-crystallized osimertinib into the active site of the L858R-mutant EGFR kinase domain. The highest-ranked redocked pose

reproduced the crystallographic orientation with a heavy-atom RMSD of 1.35 Å, which satisfied the predefined acceptance criterion of  $\text{RMSD} \leq 2.0$  Å. The corresponding GBVI/WSA dG score for osimertinib was  $-13.8$  kcal/mol (Table 2), supporting use of the validated protocol for docking M3, M6, M9, and M12. This cutoff is consistent with the commonly applied criterion for successful reproduction of crystallographic ligand poses in docking-validation studies (Warren *et al.*, 2006; Goullieux *et al.*, 2023).

Among the synthesized compounds, M9 showed the most favorable predicted GBVI/WSA dG score ( $-15.9135$  kcal/mol), followed by M6 ( $-14.6434$  kcal/mol), M3 ( $-14.4948$  kcal/mol), and M12 ( $-14.4427$  kcal/mol). M9 formed a predicted hydrogen-bond donor interaction with Asp855, M6 an acceptor interaction with Thr790, M3 acceptor interactions with Lys745 and Gly796, and M12 an acceptor interaction with Lys745 (Table 2).

The comparatively high interaction-energy magnitude observed for M12 was verified from the original MOE output. This value reflects a specific ligand–residue interaction and does not indicate superior overall binding, as the total docking score depends on multiple energetic and geometric contributions.

The redocking result ( $\text{RMSD} = 1.35$  Å) met the predefined validation criterion, and the reference-ligand score (GBVI/WSA dG =  $-13.8$  kcal/mol) enabled comparison with the synthesized compounds. Nevertheless, docking scores are computational ranking estimates and should not be interpreted as experimentally measured binding energies.

## 3.2. Discussion

### 3.2.1. Discussion of Step 1 (Hantzsch Reaction)

The Hantzsch-type multicomponent reaction afforded the expected 1,4,5,6-tetrahydropyridine derivatives M1, M4, M7, and M10 in moderate yields. The observed N–H, ester C=O, and lactam C=O bands, together with the aromatic, methine, methylene, methoxy, methyl, and N–H resonances, are consistent with Hantzsch-derived dihydropyridine systems reported previously (Vijesh *et al.*, 2011; Hussein and Al-Mathkuri, 2025). The nonequivalent CH<sub>2</sub> protons showed the expected diastereotopic splitting pattern, and the <sup>13</sup>C NMR and DEPT-135 spectra supported the assigned carbon environments. Because no mechanistic or purification data specifically explain the lower yield of M7, no causal explanation is proposed.

### 3.2.2. Discussion of Step 2 (Vilsmeier–Haack Formylation)

Vilsmeier–Haack formylation converted the tetrahydropyridine intermediates into the corresponding 5-formyl-1,4-dihydropyridine derivatives M2, M5, M8, and M11, in agreement with the transformation reported for related substrates (Armas *et al.*, 2000). The disappearance of the lactam carbonyl and CH<sub>2</sub> features, together with aldehydic <sup>1</sup>H signals at  $\delta$  9.67–9.76 ppm and <sup>13</sup>C signals near  $\delta$  187 ppm, supports successful formylation. The downfield aldehyde resonances are also consistent with established carbonyl-anisotropy effects in aromatic aldehydes and ketones (Abraham, Mobli, and Smith, 2003).

### 3.2.3. Discussion of Step 3 (Cyclization to Triazolidine Derivatives)

Reaction of the formylated intermediates with thiosemicarbazide afforded the proposed 1,2,4-triazolidine-3-thione derivatives M3, M6, M9, and M12. Loss of the aldehyde carbonyl and aldehydic proton signals, together with the appearance of a C=S band near  $1222\text{ cm}^{-1}$  and additional NH and ring-CH resonances, supports cyclization. Comparable thiosemicarbazone-to-triazolidine-3-thione transformations have been reported by Gričar *et al.* (2025), Patil *et al.* (2019), and Ramesh and Lalitha (2016). The downfield NH resonances in DMSO-d<sub>6</sub> are compatible with solvent and hydrogen-bonding effects described for amide-type protons (Abraham, Griffiths, and Perez, 2014).

The combined FT-IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, DEPT-135, and mass-spectral data support the proposed structures; however, two-dimensional NMR spectroscopy or single-crystal X-ray diffraction would provide more definitive structural and stereochemical confirmation. This limitation should be considered when interpreting the assignments.

### 3.2.4. Substituent Effects and Molecular Docking Analysis

The final compounds showed a narrow range of predicted docking scores, with M9 ranked highest, followed by M6, M3, and M12. The more favorable score of the para-chloro derivative M9 relative to the ortho-chloro derivative M12 is consistent with a possible positional effect on ligand accommodation, but the four-compound series is too small to establish a definitive structure–activity relationship. Similar EGFR-focused docking studies of dihydropyridine derivatives have also emphasized the influence of aryl substitution on predicted binding behavior

(Mansour *et al.*, 2022; Faizan *et al.*, 2024).

The predicted interaction profiles involved residues within the EGFR ATP-binding region. M3 showed acceptor contacts with Lys745 and Gly796, M6 with Thr790, M9 a donor contact with Asp855, and M12 an acceptor contact with Lys745. These contacts provide a structural basis for comparing the four poses, but the GBVI/WSA dG score should be interpreted as a ranking metric rather than a direct measure of binding affinity.

M9 combined the most negative docking score with a predicted interaction involving Asp855, whereas M6, M3, and M12 showed slightly less favorable scores and different contact patterns. This ranking is broadly consistent with prior computational work in which dihydropyridine derivatives occupied the EGFR ATP-binding pocket and interacted with catalytically relevant residues (Mansour *et al.*, 2022). Relative to the validated osimertinib reference score of  $-13.8$  kcal/mol (RMSD = 1.35 Å), all four synthesized compounds produced more negative GBVI/WSA dG scores under the applied protocol, with M9 showing the largest numerical difference. This comparison remains computational and does not establish superior experimental affinity or potency.

The substituent-dependent differences are therefore best viewed as preliminary qualitative observations. Additional analogs, independent docking validation, molecular dynamics simulations, and binding free-energy calculations would be needed to test whether the observed ranking is robust. Broader EGFR inhibitor programs likewise combine structural optimization with biochemical, cellular, and *in vivo* validation rather than relying on docking scores alone (Wang *et al.*, 2022).

Overall, the docking findings provide a hypothesis for subsequent experimental work but do not demonstrate EGFR inhibition or anticancer activity. Validation should include biochemical EGFR kinase assays, cell-based cytotoxicity testing, and selectivity assessment in non-malignant cells.

## 4. CONCLUSIONS

A new series of 1,4-dihydropyridine-1,2,4-triazolidine-3-thione hybrid derivatives was synthesized through Hantzsch-type condensation, Vilsmeier-Haack formylation, and cyclization with thiosemicarbazide. FT-IR,  $^1\text{H}$ -

NMR,  $^{13}\text{C}$ -NMR, DEPT-135, and mass-spectrometric data supported the proposed structures.

Molecular docking against the EGFR tyrosine kinase domain ranked M9 highest under the applied computational conditions (GBVI/WSA dG =  $-15.9135$  kcal/mol), followed by M6, M3, and M12. The compounds showed distinct predicted contacts within the EGFR binding pocket.

These computational results are preliminary and do not confirm EGFR inhibition or anticancer activity. The docking protocol was validated by redocking osimertinib (heavy-atom RMSD = 1.35 Å; GBVI/WSA dG =  $-13.8$  kcal/mol). The synthesized compounds, particularly M9, warrant further evaluation through *in vitro* EGFR kinase assays, cytotoxicity testing, selectivity assessment, and independent computational validation.

## 5. DECLARATIONS

### 5.1. Study Limitations

No *in vitro* or *in vivo* biological evaluation was performed; therefore, the docking results represent preliminary computational predictions only. EGFR kinase inhibition, cytotoxicity, cellular selectivity, and mechanism-based biological assays are required to validate the proposed activity.

Structural assignment of the final triazolidine-3-thione derivatives relied on FT-IR,  $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR, DEPT-135, and mass spectrometry. Two-dimensional NMR spectroscopy or single-crystal X-ray diffraction would provide more definitive structural and stereochemical confirmation.

The docking analysis used a single software platform, one receptor structure, and a limited compound series. Independent docking, molecular-dynamics simulations, and binding free-energy calculations would strengthen the computational conclusions. Moderate yields and the use of column chromatography for several compounds may also limit scalability.

Although the docking protocol met the predefined redocking criterion using osimertinib (RMSD = 1.35 Å), the analysis was limited to one software platform, one receptor structure, and a small compound series. Independent docking, molecular-dynamics simulations, binding free-energy calculations, and experimental EGFR-inhibition assays are therefore required to test the

robustness and biological relevance of the predicted ranking.

## 5.2. Acknowledgments

The authors would like to thank the Department of Chemistry, College of Science, University of Misan, for providing laboratory facilities.

## 5.3. Funding

The authors funded this research.

## 5.4. Conflicts of Interest

The authors declare that they have no competing interests.

## 5.5. Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

## 5.6. Author Contributions

Maryam Sabri Hashim: CD, DC, DAI, MW, FA.

Tahseen Saddam Fandi Al-Mathkuri: CD, DAI, CR, FA.

| Code | English                          | Português                        |
|------|----------------------------------|----------------------------------|
| CD   | Conception and Design            | Concepção e Design               |
| DC   | Data Collection                  | Coleta de Dados                  |
| DAI  | Data Analysis and Interpretation | Análise e Interpretação de Dados |
| MW   | Manuscript Writing               | Escrita do Manuscrito            |
| CR   | Critical Review                  | Revisão Crítica                  |
| FA   | Final Approval                   | Aprovação Final                  |

## 5.7. AI and Computational Tools Declaration

The authors used Molecular Operating Environment (MOE, version 2014; Chemical Computing Group Inc., Montreal, Canada) for molecular docking and ligand–protein interaction analysis. QuillBot was used only for language editing and readability improvement. The authors are responsible for all scientific content, data analysis, and interpretation.

## 5.8. Research Integrity Declaration

The authors confirm that this manuscript is original, has not been published previously, and is not under consideration for publication elsewhere.

## 5.9. Originality and Plagiarism Statement

This manuscript is original, has not been published previously, and is not under consideration for publication elsewhere. The manuscript was screened for textual similarity, and the similarity index was 9%, which is within the acceptable limit. All sources and previously published materials have been appropriately cited and acknowledged.

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## 6. STUDIES INVOLVING HUMAN AND ANIMAL SUBJECTS

### 6.1. Ethics Committee Approval

Not applicable.

### 6.2. Informed Consent

Not applicable.

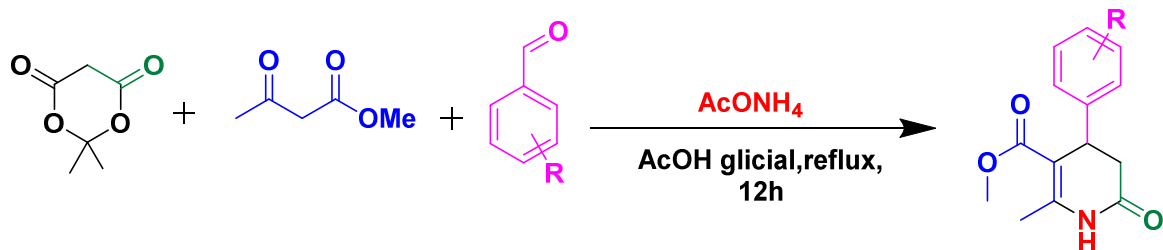
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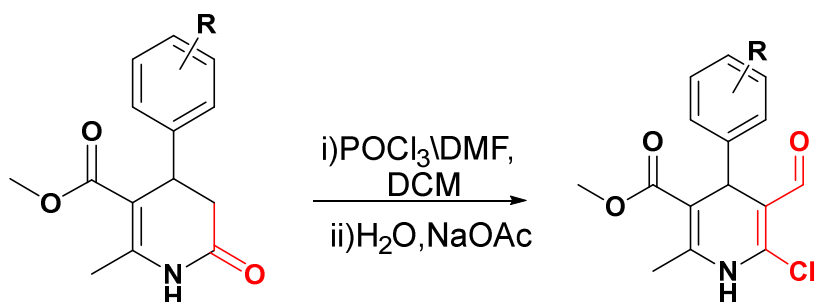
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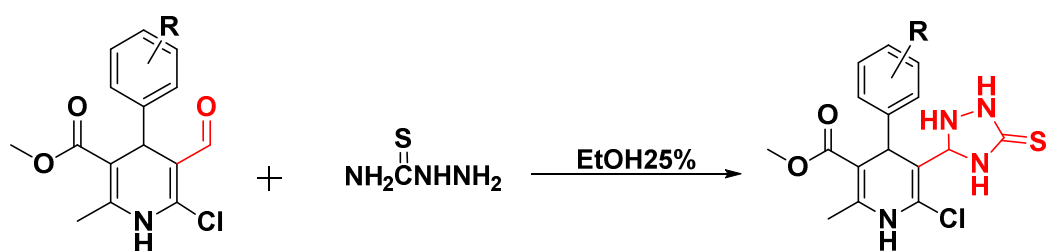
R=4-NO<sub>2</sub> , 4-Br , 4-Cl , 2-Cl  
M1    M4    M7    M10

**Equation 1.** Synthesis of 1,4,5,6-tetrahydropyridine-3-carboxylate derivatives.



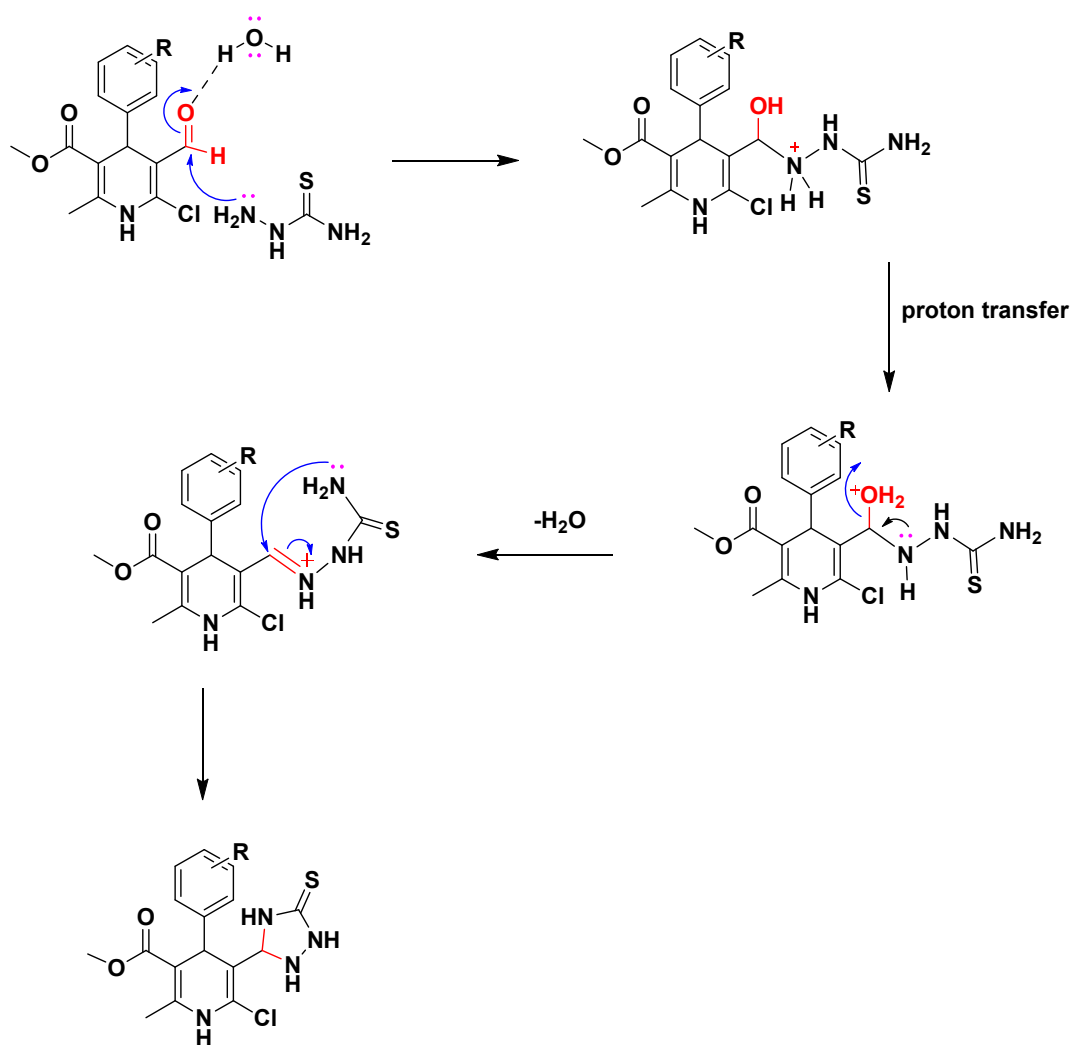
R= 4-NO<sub>2</sub> , 4-Br , 4-Cl , 2-Cl  
M2    M5    M8    M11

**Equation 2.** Synthesis of 5-formyl-1,4-dihydropyridine derivatives.

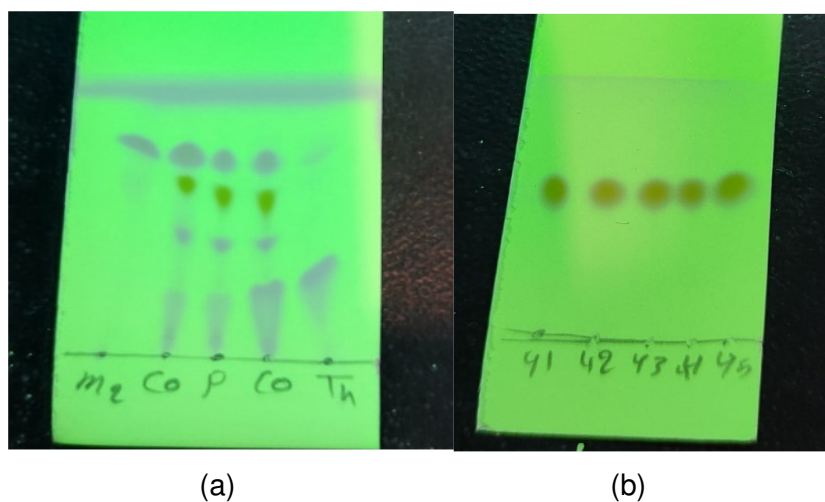


R= 4-NO<sub>2</sub> , 4-Br , 4-Cl , 2-Cl  
M3    M6    M9    M12

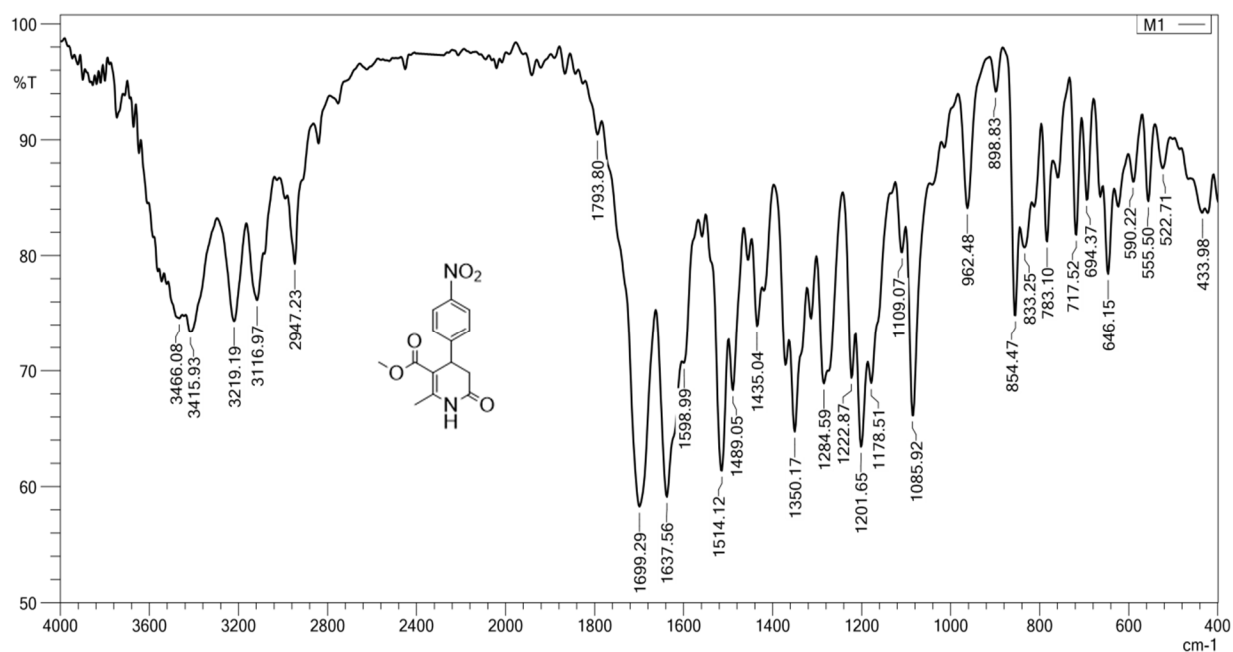
**Equation 3.** Synthesis of 1,4-dihydropyridine-1,2,4-triazolidine-3-thione derivatives.



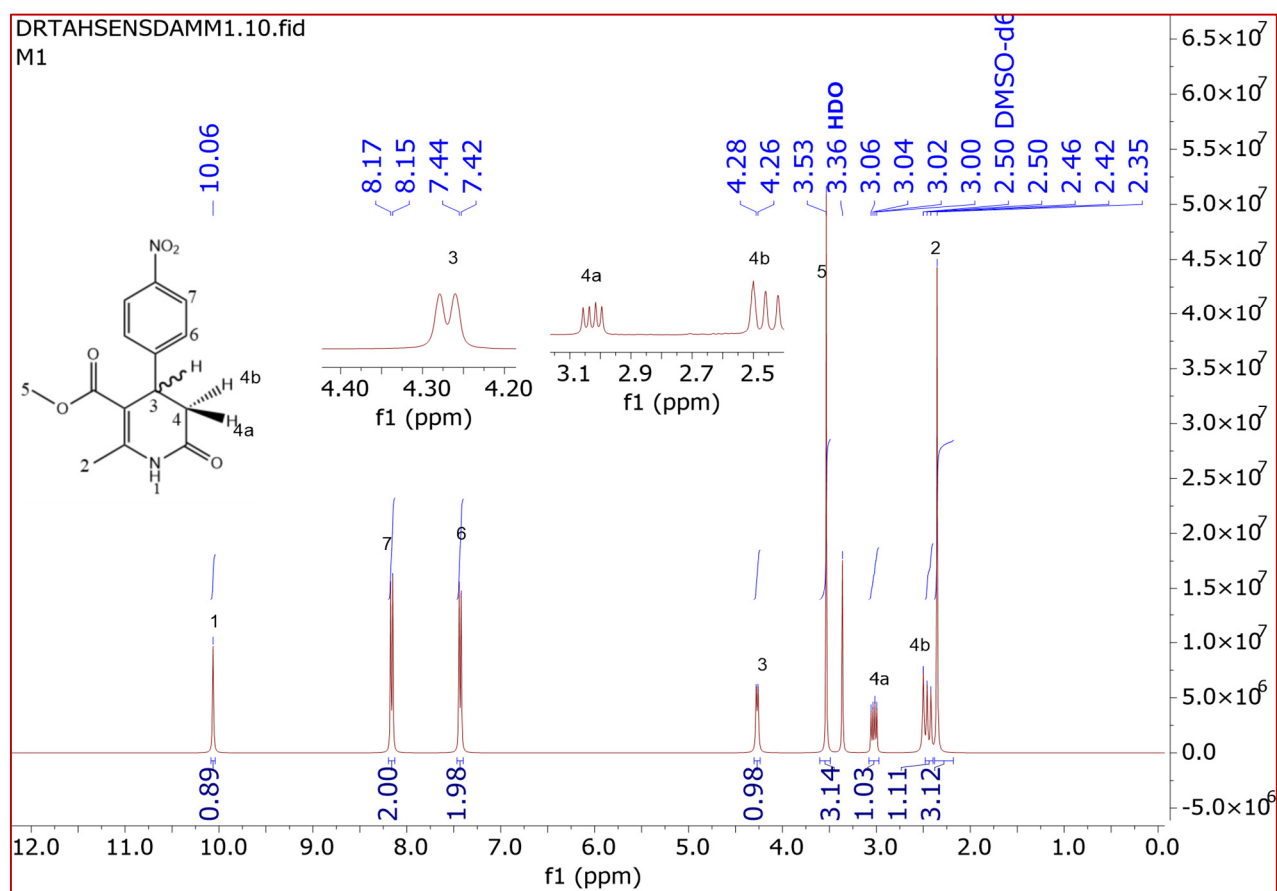
**Scheme 1.** Proposed mechanism for cyclization of the formylated intermediates with thiosemicarbazide to afford 1,2,4-triazolidine-3-thione derivatives.



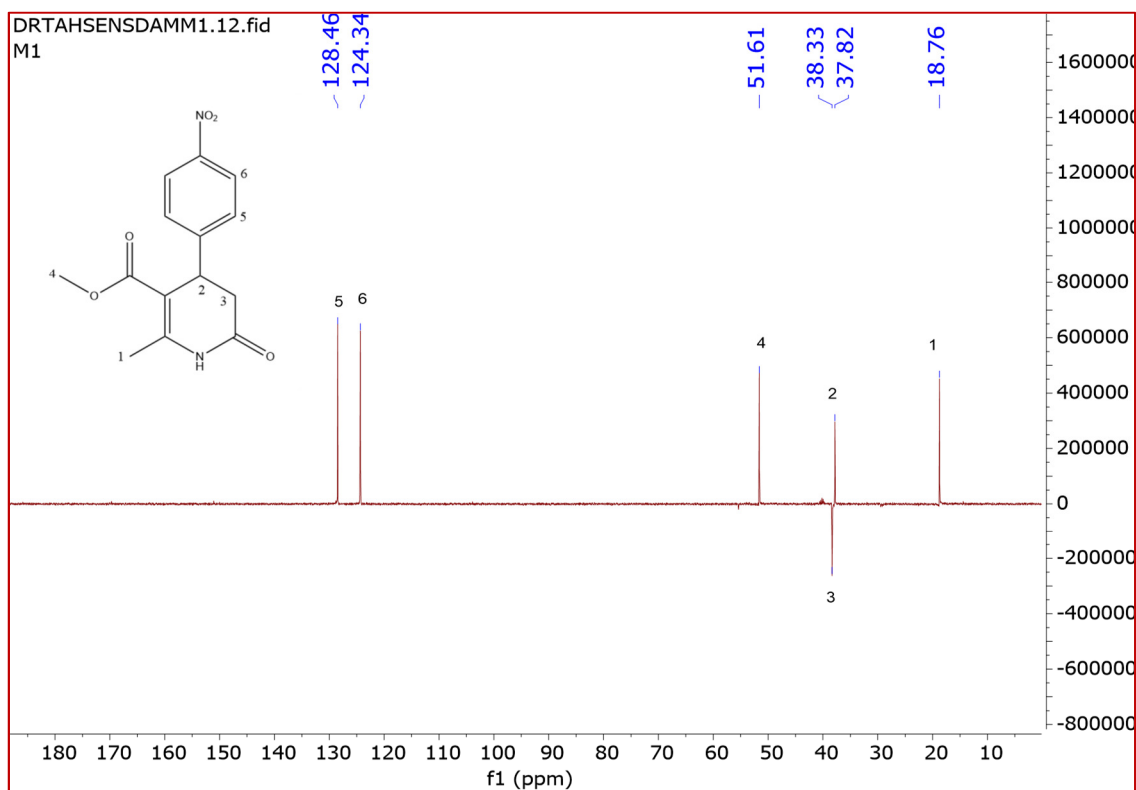
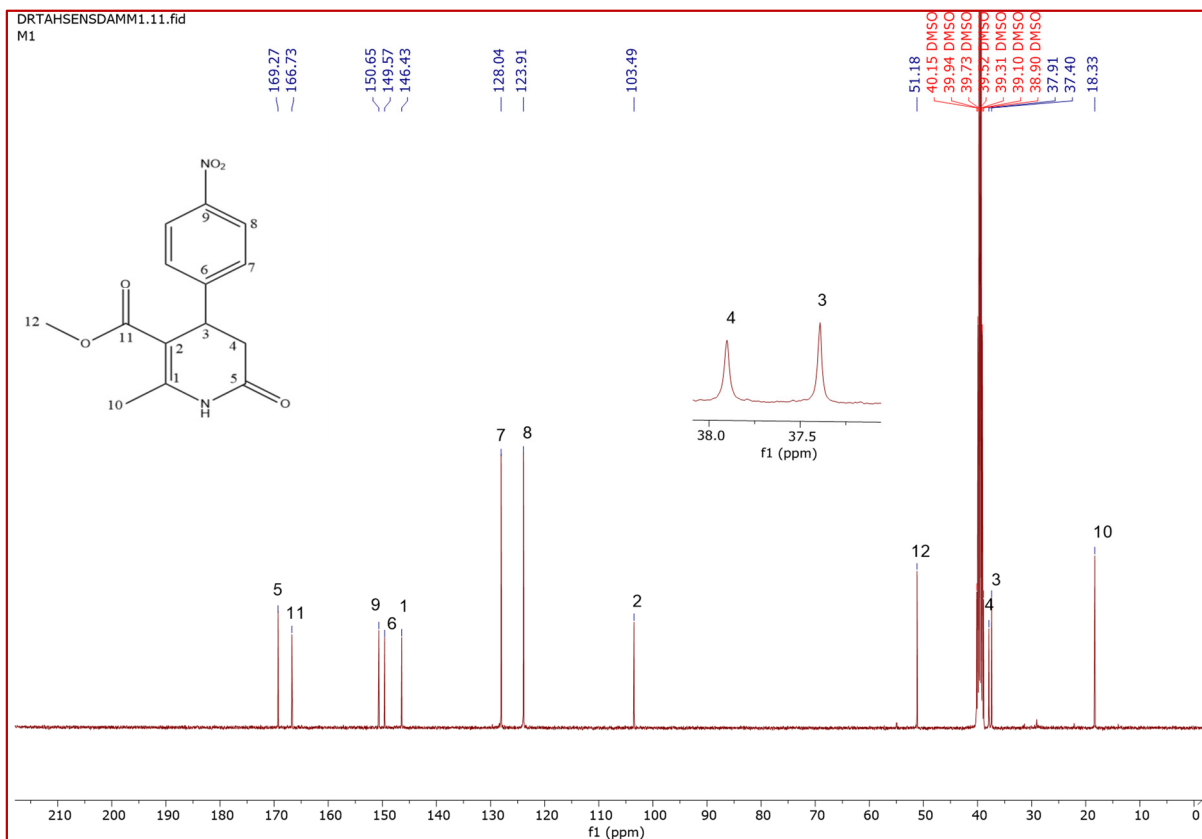
**Figure 1.** TLC analysis of compound M3 during step 3: (a) reaction-mixture profile before purification; (b) column-chromatography fractions obtained using ethyl acetate/petroleum ether (1:3, v/v).

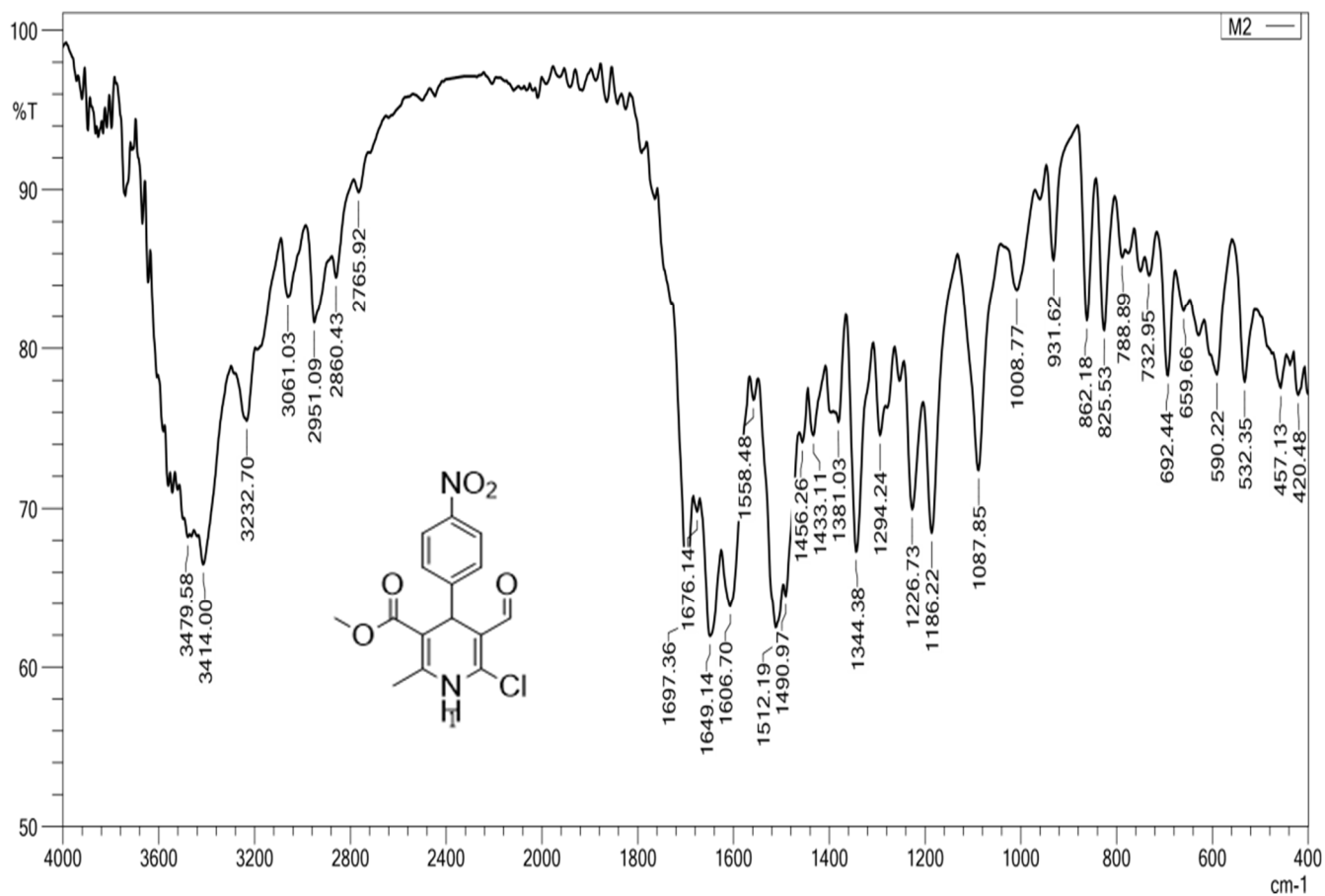


**Figure 2.** FT-IR spectrum of compound M1 (KBr, 4000–400 cm<sup>-1</sup>).

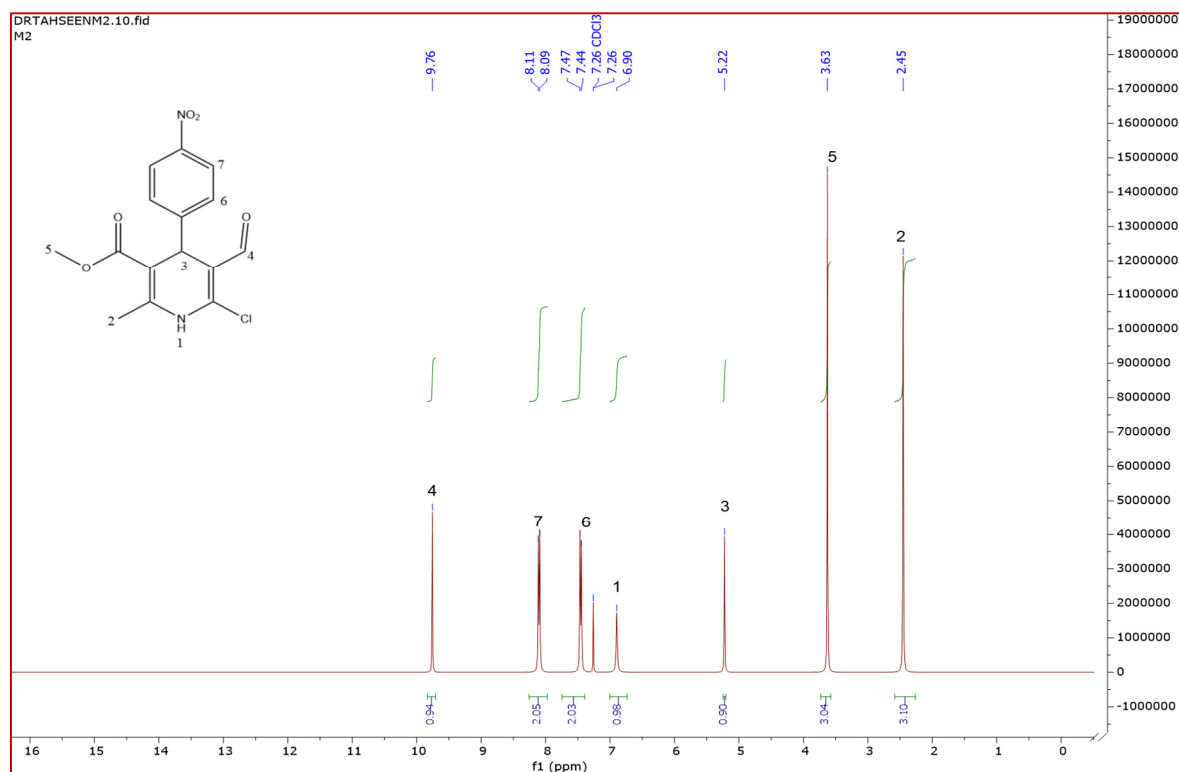


**Figure 3.** <sup>1</sup>H-NMR spectrum of compound M1 (400 MHz, DMSO-d<sub>6</sub>).

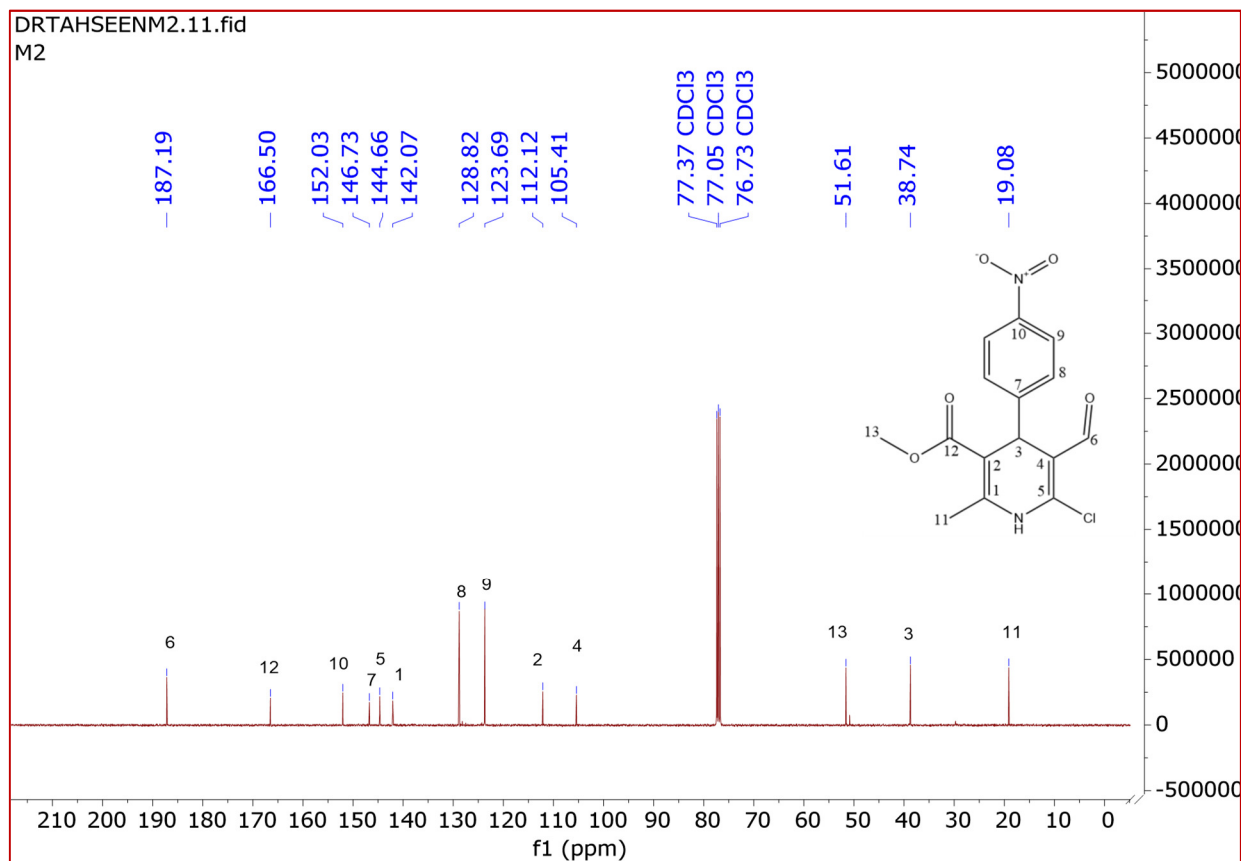




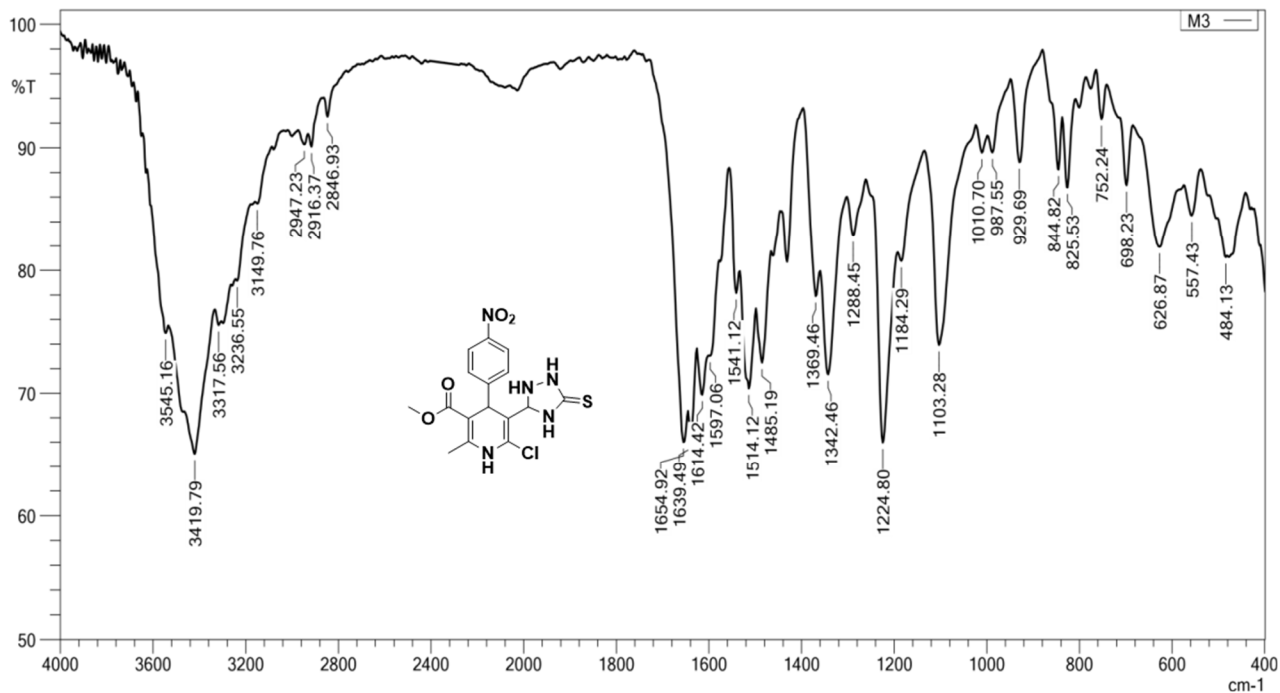
**Figure 6.** FT-IR spectrum of compound M2 (KBr, 4000–400 cm<sup>-1</sup>).



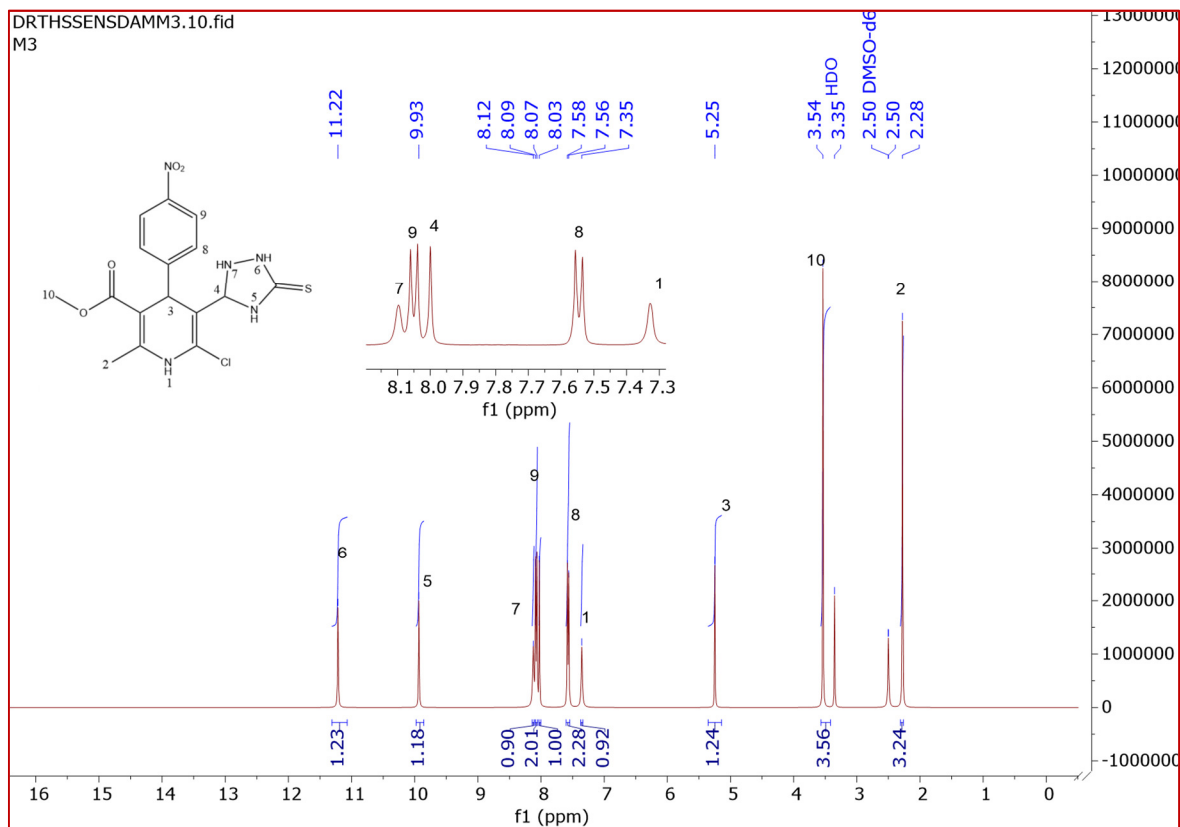
**Figure 7.** <sup>1</sup>H-NMR spectrum of compound M2 (400 MHz, CDCl<sub>3</sub>).



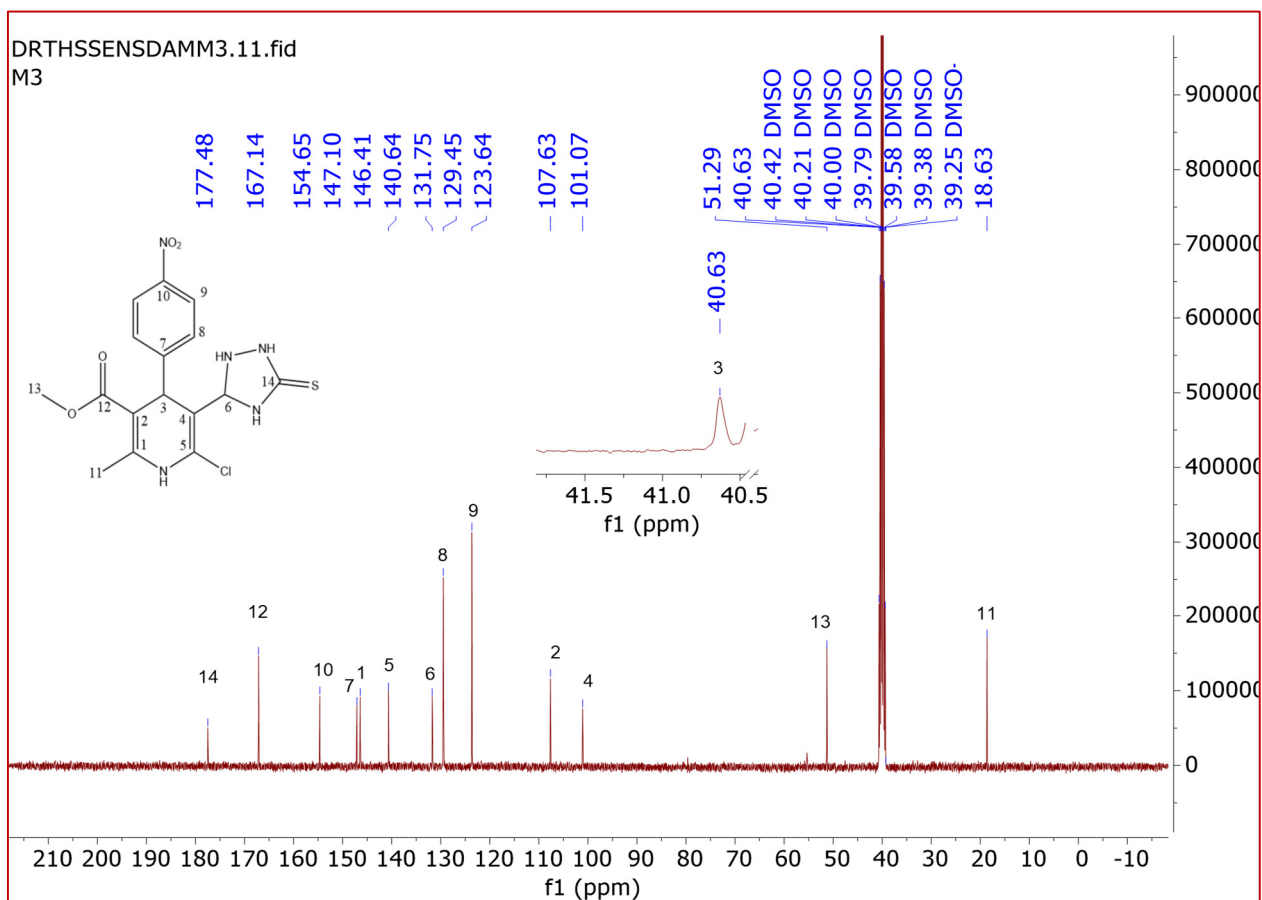
**Figure 8.**  $^{13}\text{C}$ -NMR spectrum of compound M2 (101 MHz,  $\text{CDCl}_3$ ).



**Figure 9.** FT-IR spectrum of compound M3 (KBr,  $4000\text{--}400\text{ cm}^{-1}$ ).

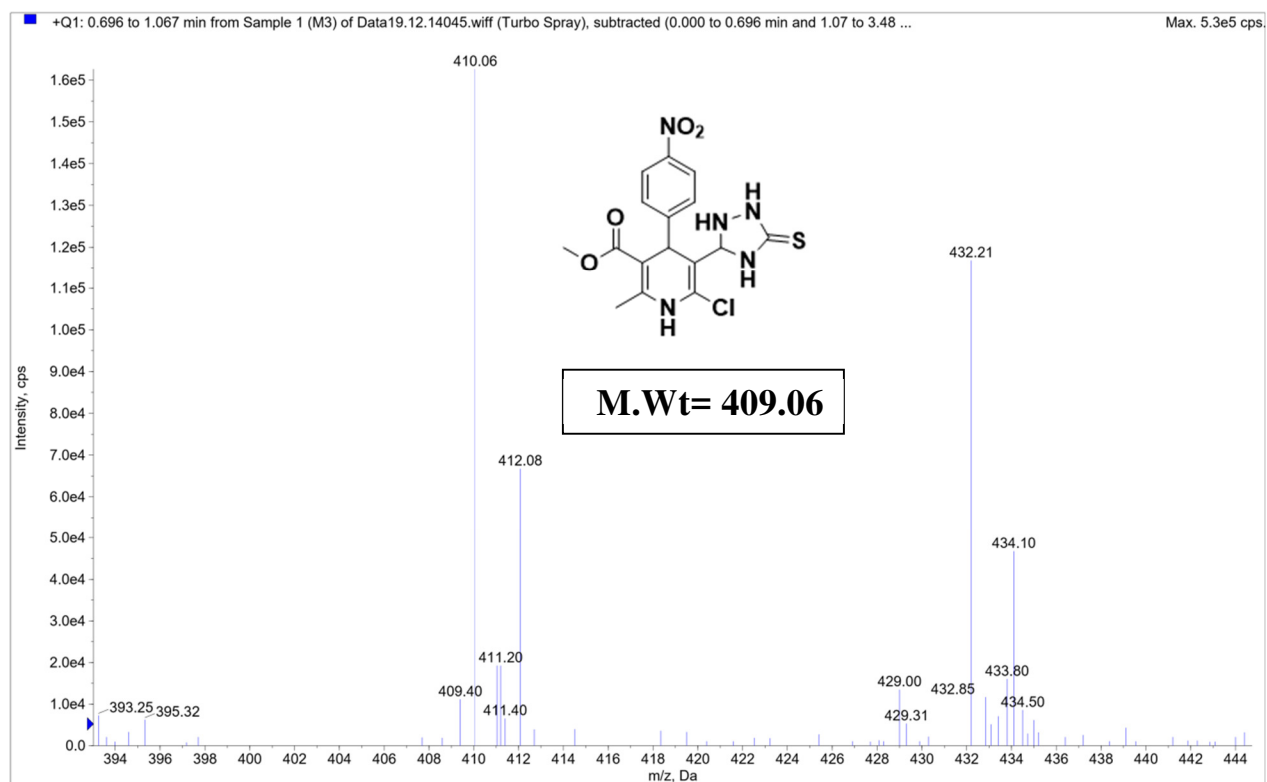


**Figure 10.** <sup>1</sup>H-NMR spectrum of compound M3 (400 MHz, DMSO-d<sub>6</sub>).



**Figure 11.** <sup>13</sup>C-NMR spectrum of compound M3 (101 MHz, DMSO-d<sub>6</sub>).

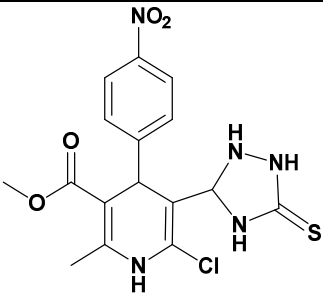
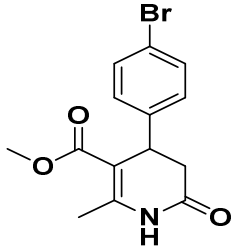
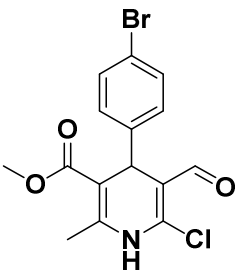
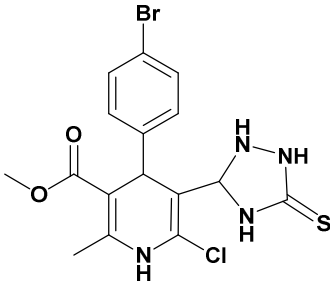
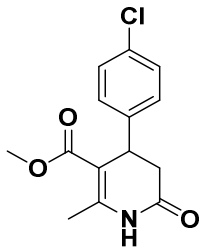
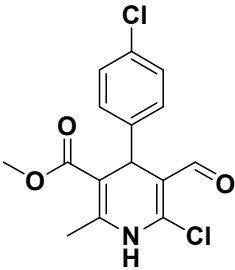




**Figure 12.** ESI mass spectrum of compound M3.

**Table 1.** Physical characteristics, reaction yields, molecular formulae, and structures of the synthesized compounds M1–M12.

| Compound | Structural formula | Molecular formula      | Color        | m.p. (°C) | Yield (%) |
|----------|--------------------|------------------------|--------------|-----------|-----------|
| M1       |                    | $C_{14}H_{14}N_2O_5$   | Off-white    | 202       | 41        |
| M2       |                    | $C_{15}H_{13}ClN_2O_5$ | Light yellow | 151       | 51.8      |

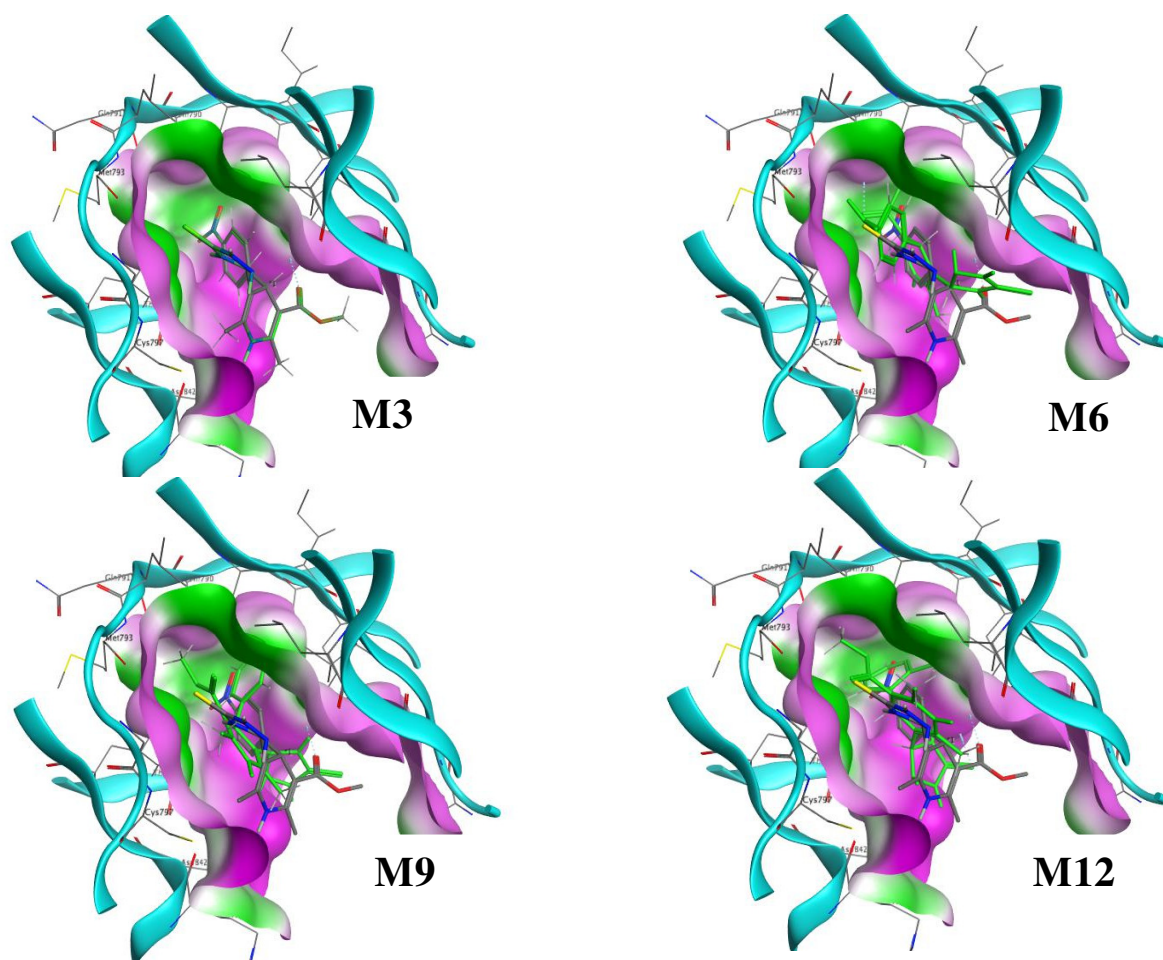
|    |                                                                                     |                           | 226–228      |            |
|----|-------------------------------------------------------------------------------------|---------------------------|--------------|------------|
| M3 |    | $C_{16}H_{16}ClN_5O_4S$   | Orange       | 50         |
| M4 |    | $C_{14}H_{14}BrNO_3$      | Off-white    | 187 44.4   |
| M5 |    | $C_{15}H_{13}BrClNO_3$    | Light yellow | 141 59.3   |
| M6 |   | $C_{16}H_{16}BrClN_4O_2S$ | Orange       | 210–212 45 |
| M7 |  | $C_{14}H_{14}ClNO_3$      | Off-white    | 173 30     |
| M8 |  | $C_{15}H_{13}Cl_2NO_3$    | Light yellow | 136 50     |

|     |  |                           |           |         |      |  |  |
|-----|--|---------------------------|-----------|---------|------|--|--|
|     |  |                           |           | 208–210 |      |  |  |
| M9  |  | $C_{16}H_{16}Cl_2N_4O_2S$ | Orange    |         | 62.3 |  |  |
| M10 |  | $C_{14}H_{14}ClNO_3$      | Off-white | 195     | 42   |  |  |
| M11 |  | $C_{15}H_{13}Cl_2NO_3$    | Yellow    | 190     | 58   |  |  |
|     |  |                           |           | 210–212 |      |  |  |
| M12 |  | $C_{16}H_{16}Cl_2N_4O_2S$ | Orange    |         | 60   |  |  |

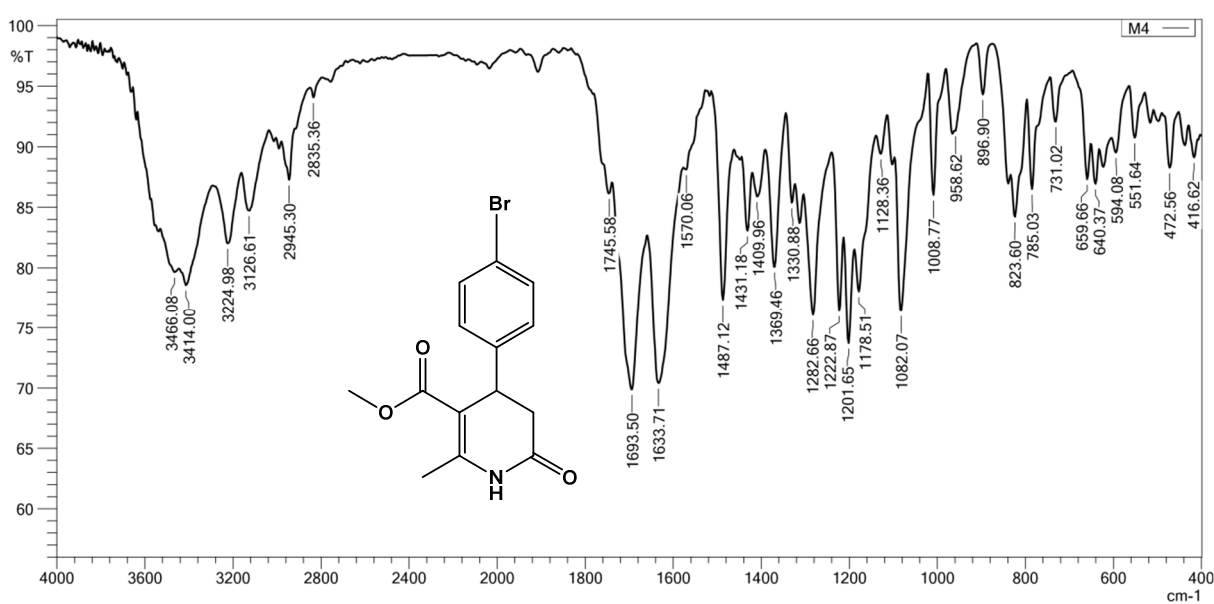
**Table 2.** Molecular docking scores, RMSD values, and major predicted interactions of osimertinib and compounds M3, M6, M9, and M12 within the EGFR tyrosine kinase active site (PDB ID: 6JWL).

| Compound    | GBVI/WSA<br>dG score<br>(kcal/mol) | RMSD<br>(Å) | Ligand<br>atom    | Receptor<br>atom/residue    | Interaction               | Distance<br>(Å) | Interaction<br>energy<br>(kcal/mol) |
|-------------|------------------------------------|-------------|-------------------|-----------------------------|---------------------------|-----------------|-------------------------------------|
| Osimertinib | -13.8                              | 1.35        | —                 | —                           | Reference<br>ligand       | —               | —                                   |
| M3          | -14.4948                           | 2.1930      | O (15);<br>S (27) | CE (Lys745);<br>CA (Gly796) | H-acceptor;<br>H-acceptor | 3.67; 4.25      | -1.3; -0.8                          |
| M6          | -14.6434                           | 1.4269      | O (15)            | OG (Thr790)                 | H-acceptor                | 3.29            | -0.9                                |
| M9          | -15.9135                           | 2.3085      | N (29)            | OD2 (Asp855)                | H-donor                   | 2.88            | -0.8                                |
| M12         | -14.4427                           | 1.4825      | S (27)            | NZ (Lys745)                 | H-acceptor                | 3.52            | -9.5                                |

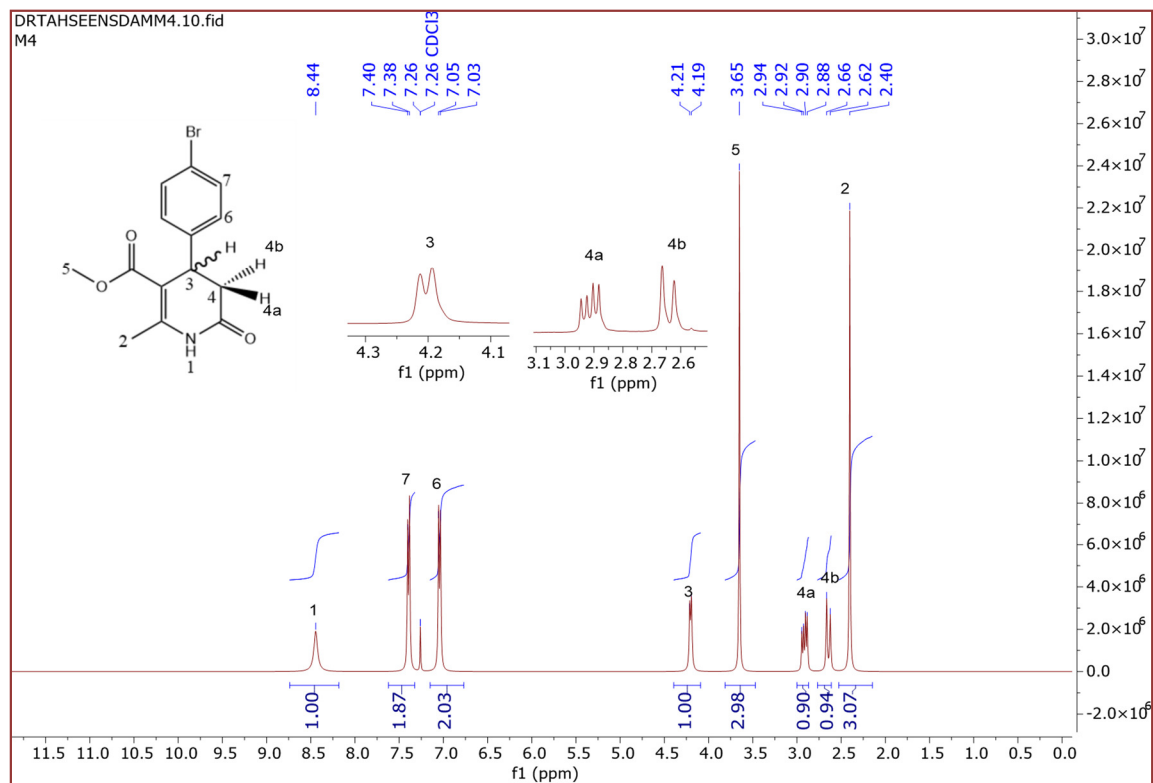




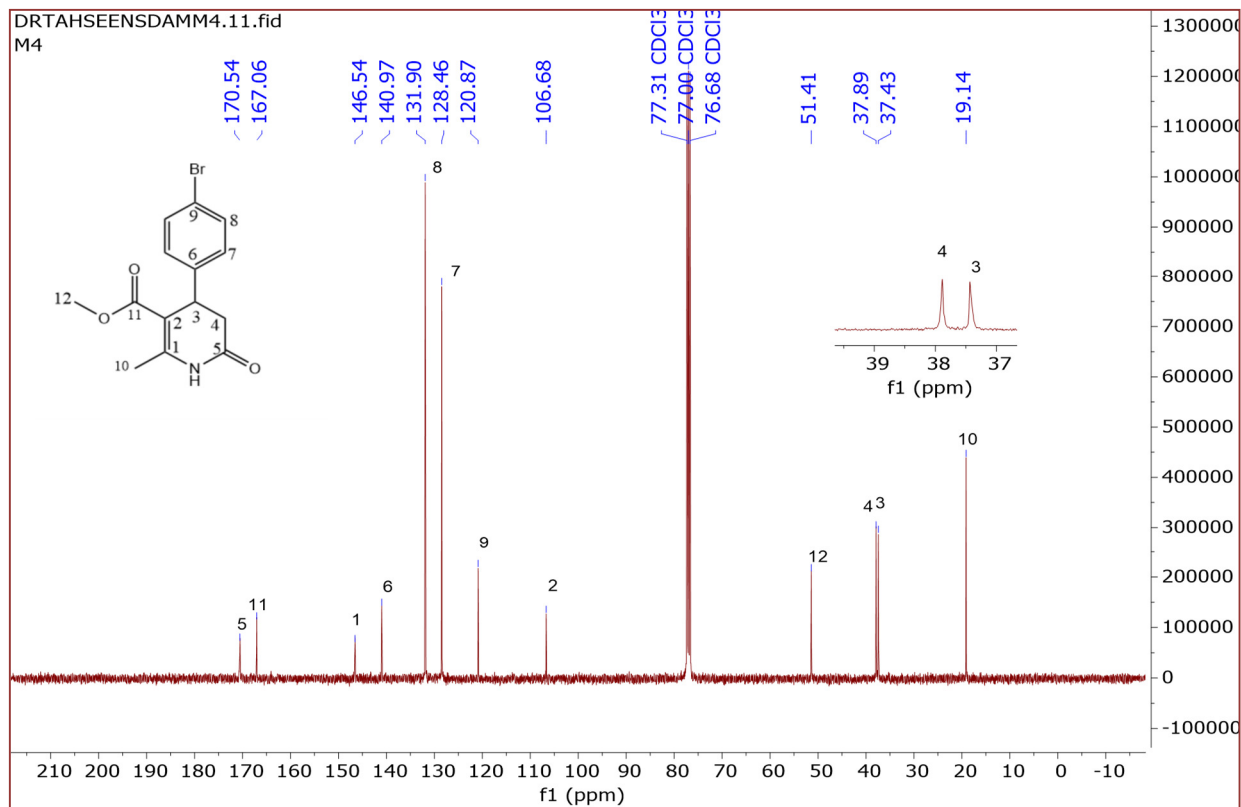
**Figure 14.** Surface representations of compounds M3, M6, M9, and M12 within the EGFR binding pocket, showing their predicted orientations and accommodation within the active site.



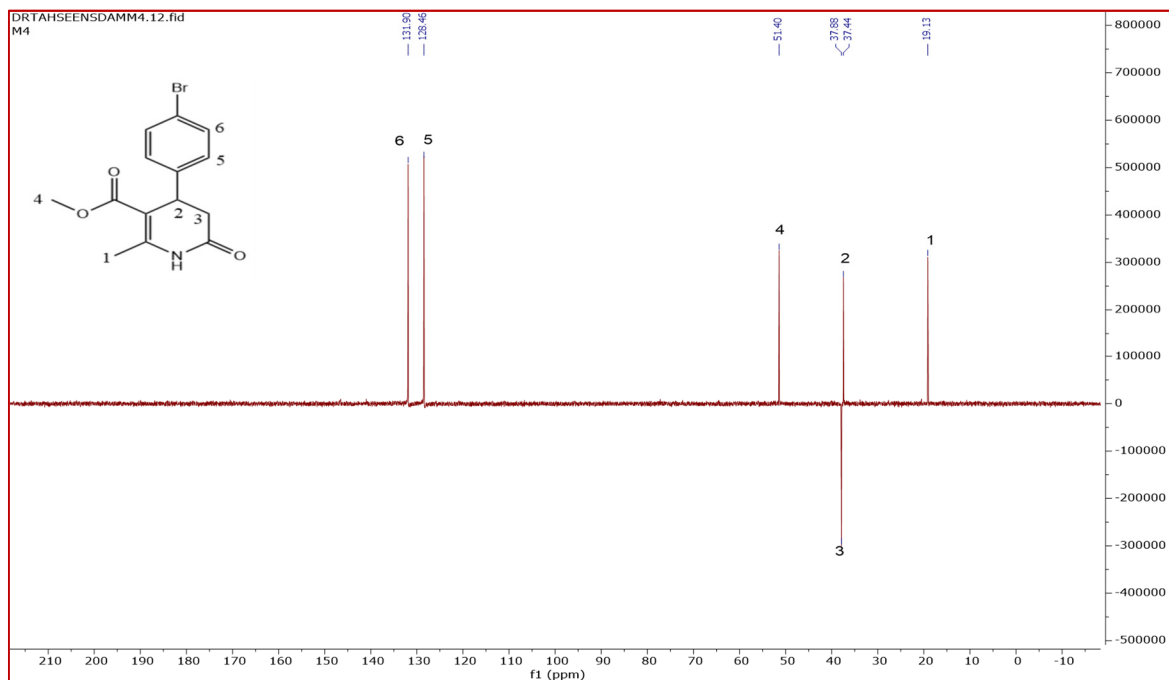
**Figure 15.** FT-IR spectrum of compound M4 (KBr, 4000–400  $\text{cm}^{-1}$ ).



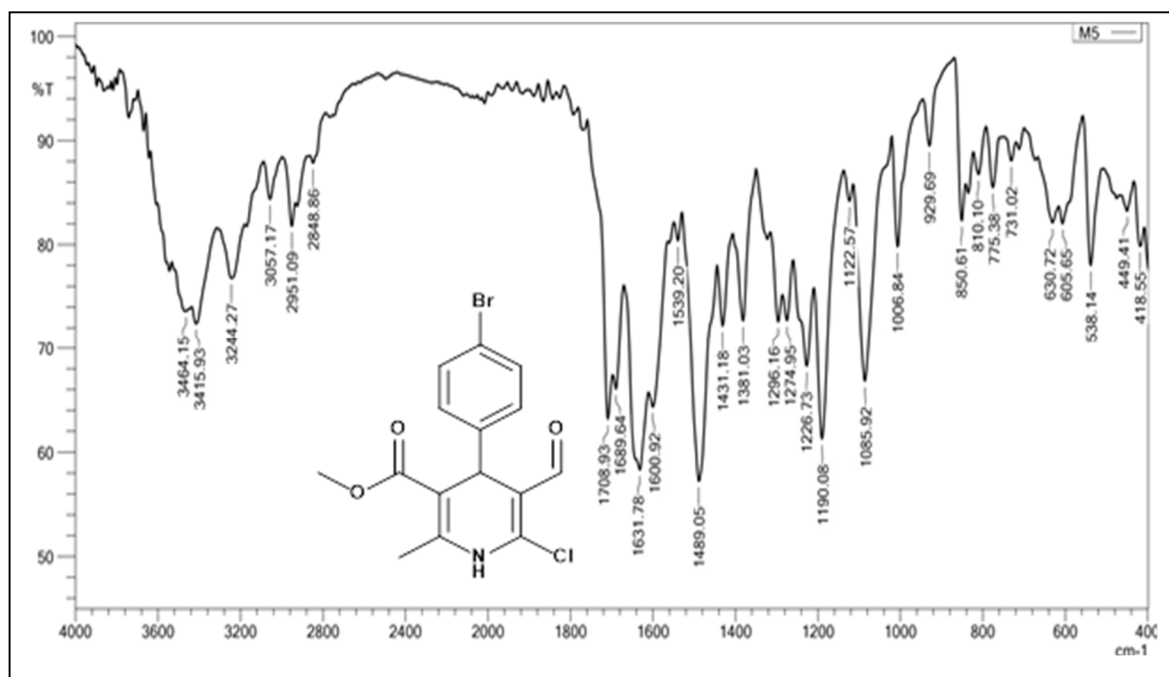
**Figure 16.**  $^1\text{H}$ -NMR spectrum of compound M4 (400 MHz,  $\text{CDCl}_3$ ).



**Figure 17.**  $^{13}\text{C}$ -NMR spectrum of compound M4 (101 MHz,  $\text{CDCl}_3$ ).

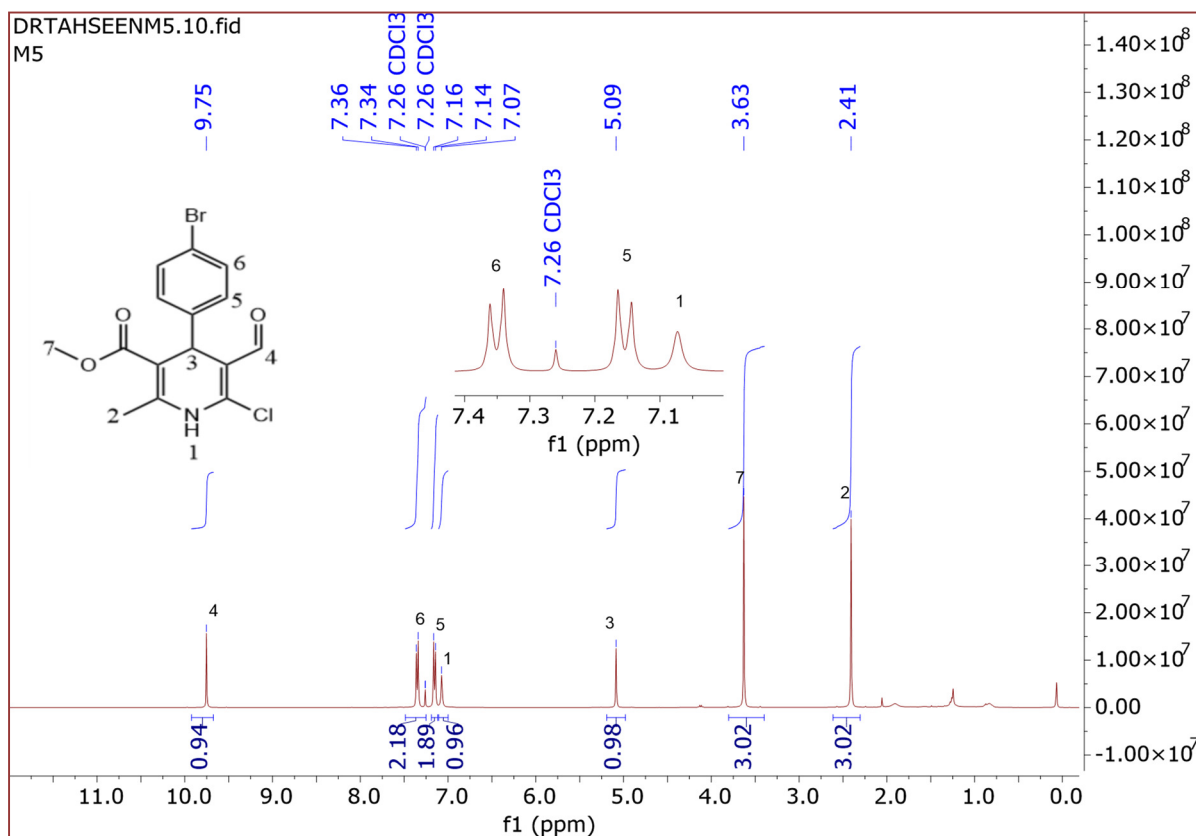


**Figure 18.** DEPT-135 spectrum of compound M4.

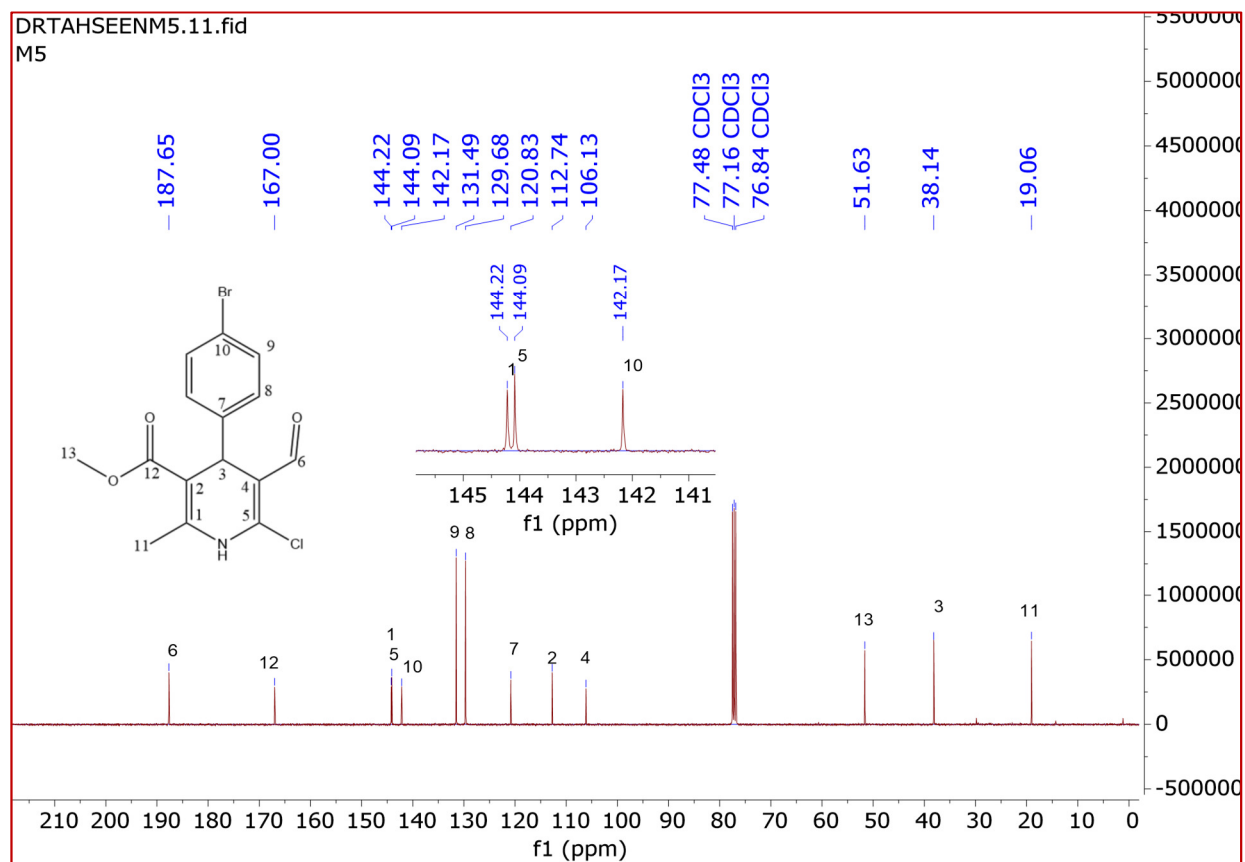


**Figure 19.** FT-IR spectrum of compound M5 (KBr, 4000–400  $\text{cm}^{-1}$ ).



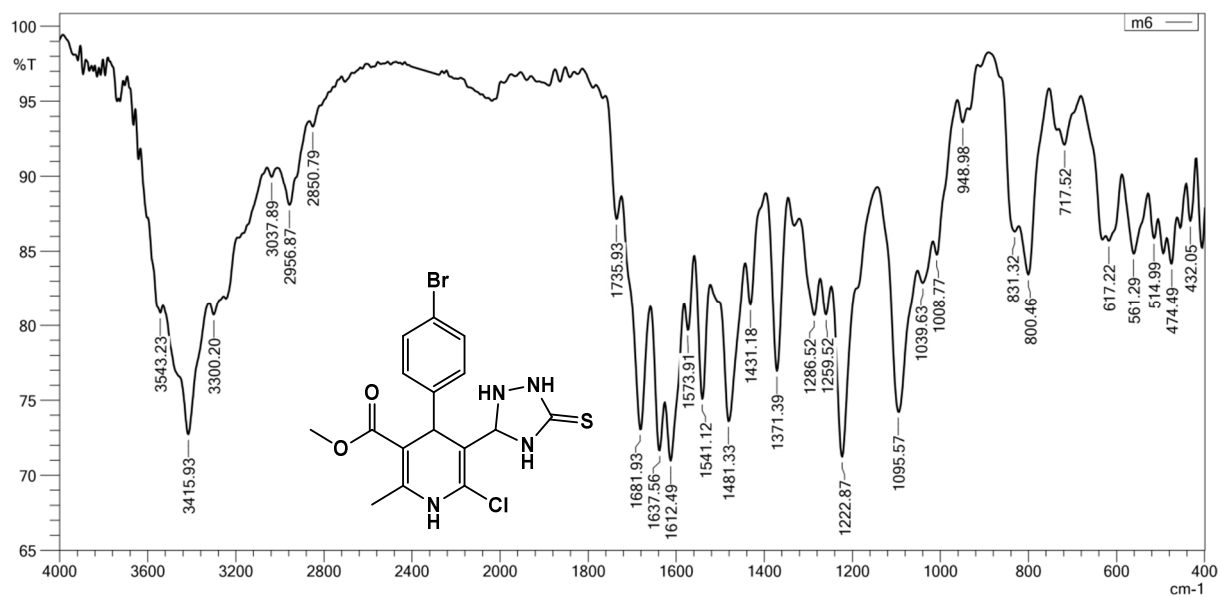


**Figure 20.**  $^1\text{H}$ -NMR spectrum of compound M5 (400 MHz,  $\text{CDCl}_3$ ).

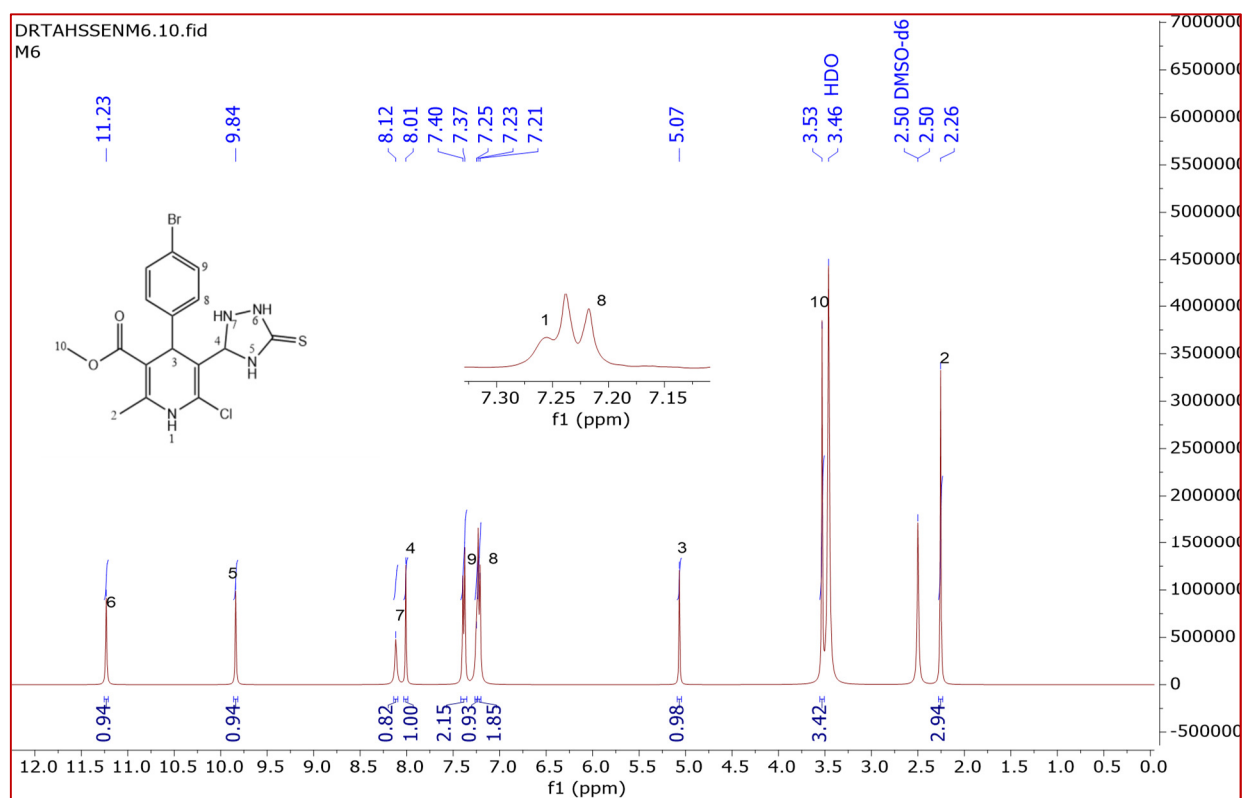


**Figure 21.**  $^{13}\text{C}$ -NMR spectrum of compound M5 (101 MHz,  $\text{CDCl}_3$ ).

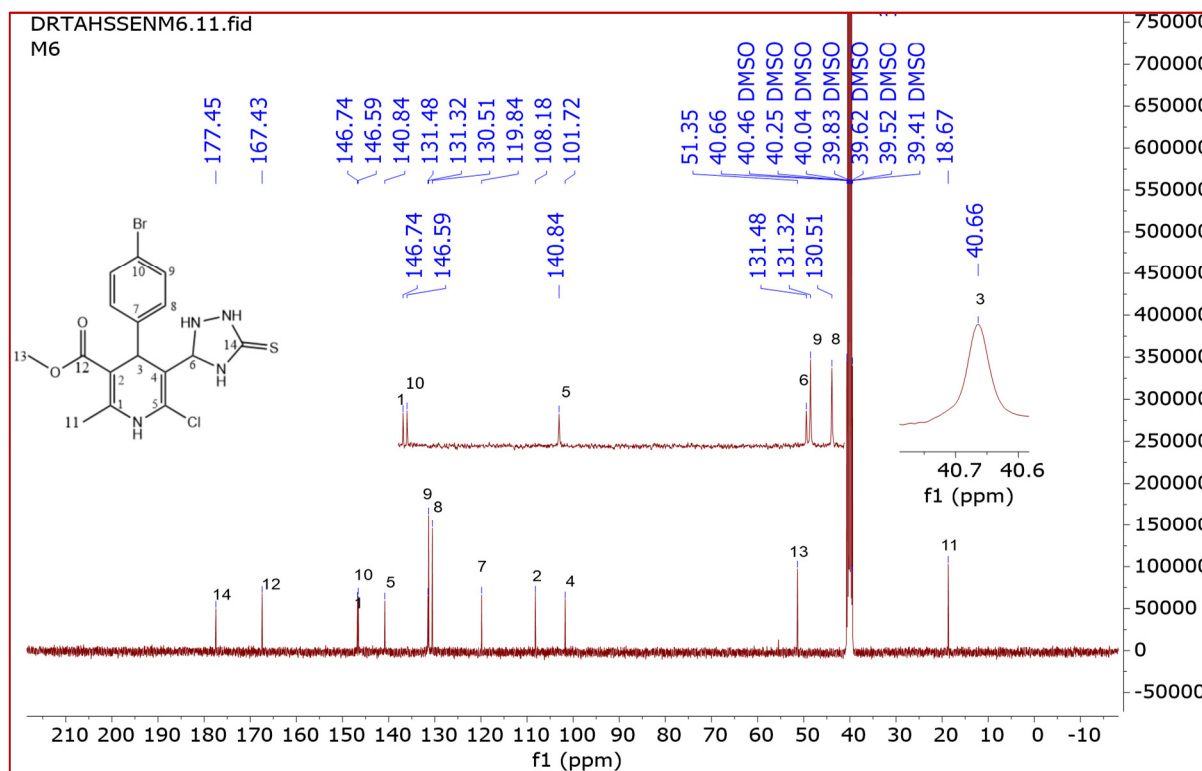




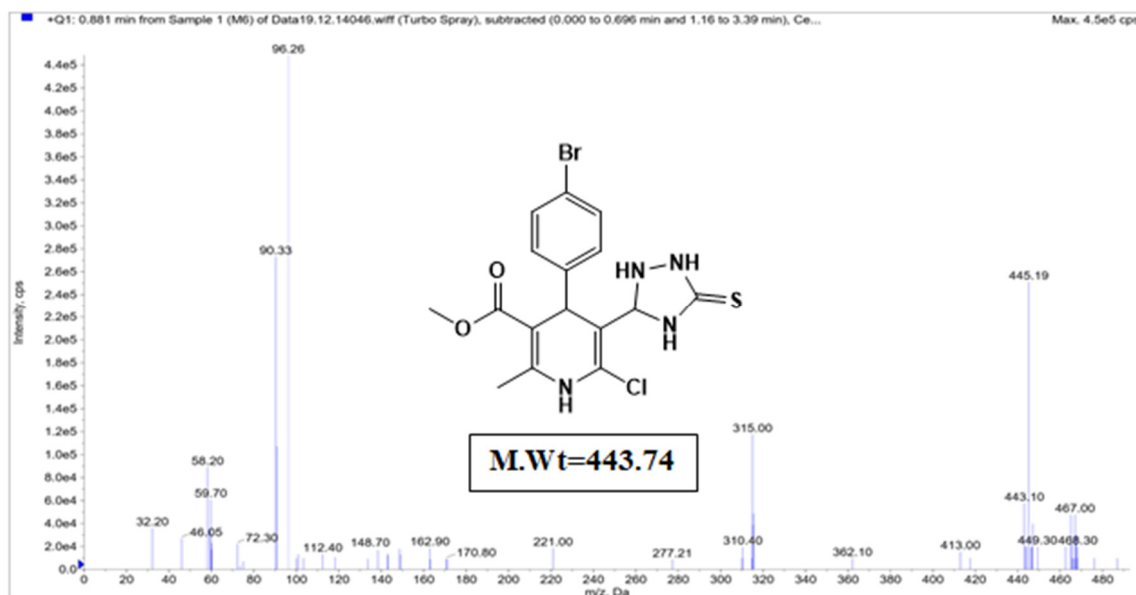
**Figure 22.** FT-IR spectrum of compound M6 (KBr, 4000–400  $\text{cm}^{-1}$ ).



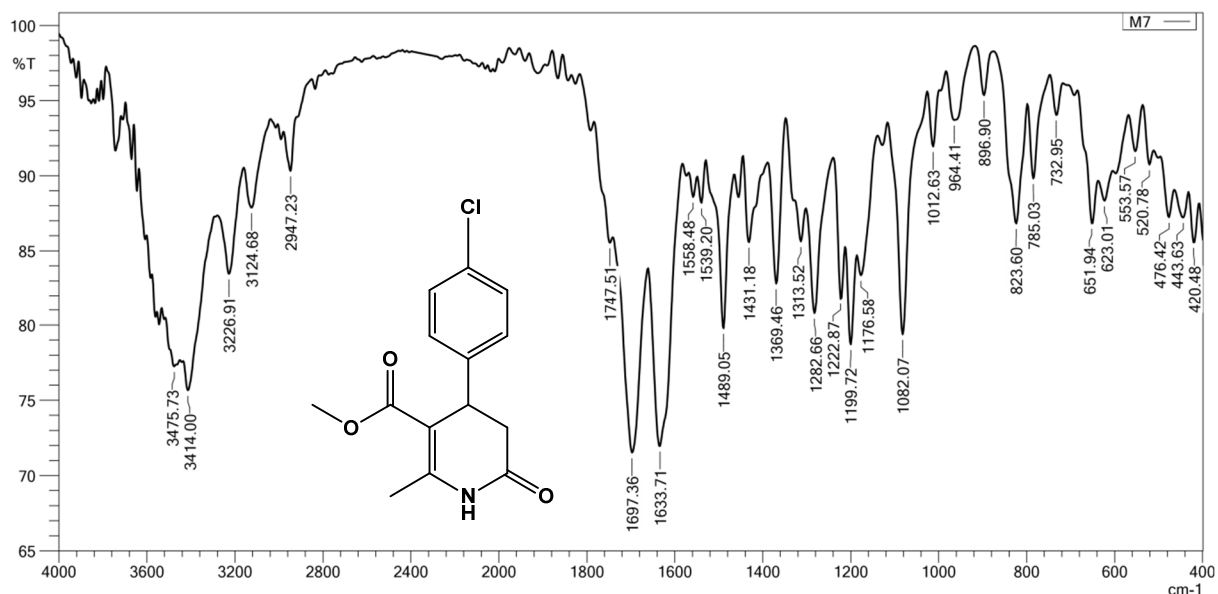
**Figure 23.**  $^1\text{H}$ -NMR spectrum of compound M6 (400 MHz,  $\text{DMSO-d}_6$ ).



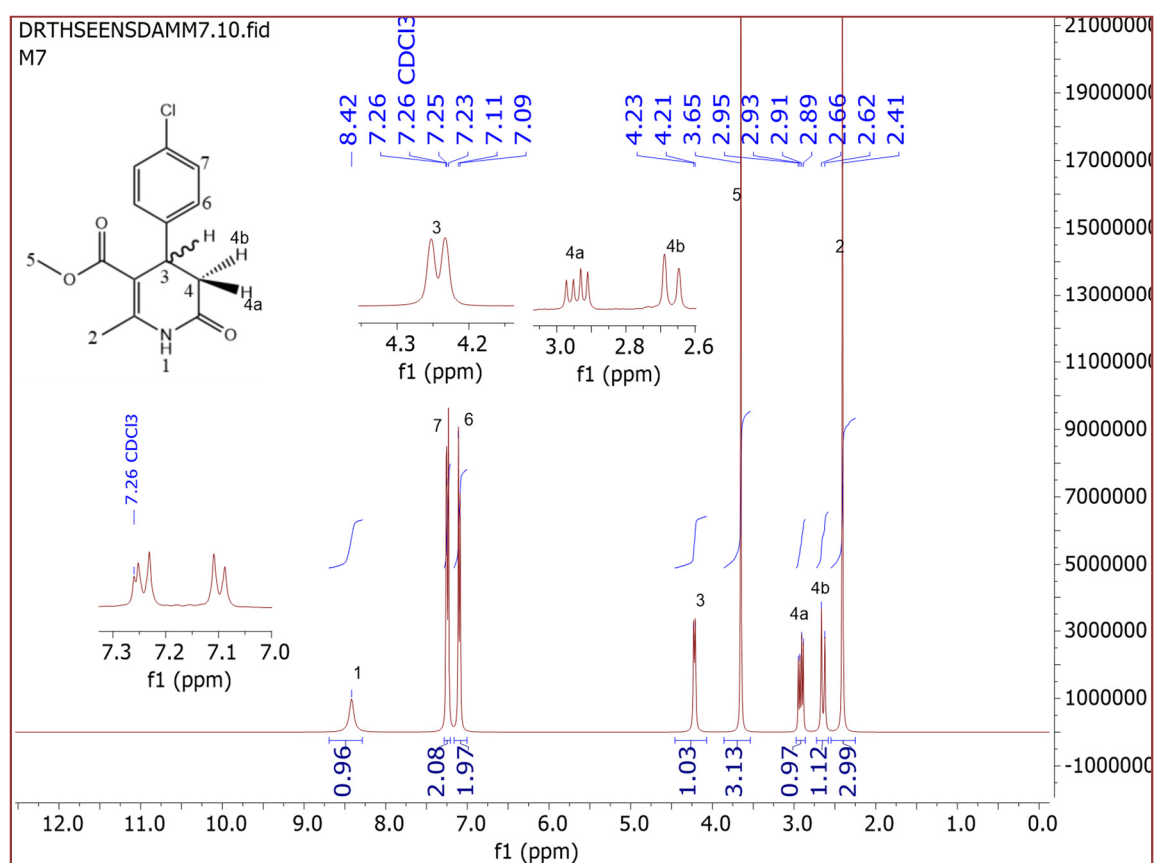
**Figure 24.**  $^{13}\text{C}$ -NMR spectrum of compound M6 (101 MHz,  $\text{DMSO-d}_6$ ).



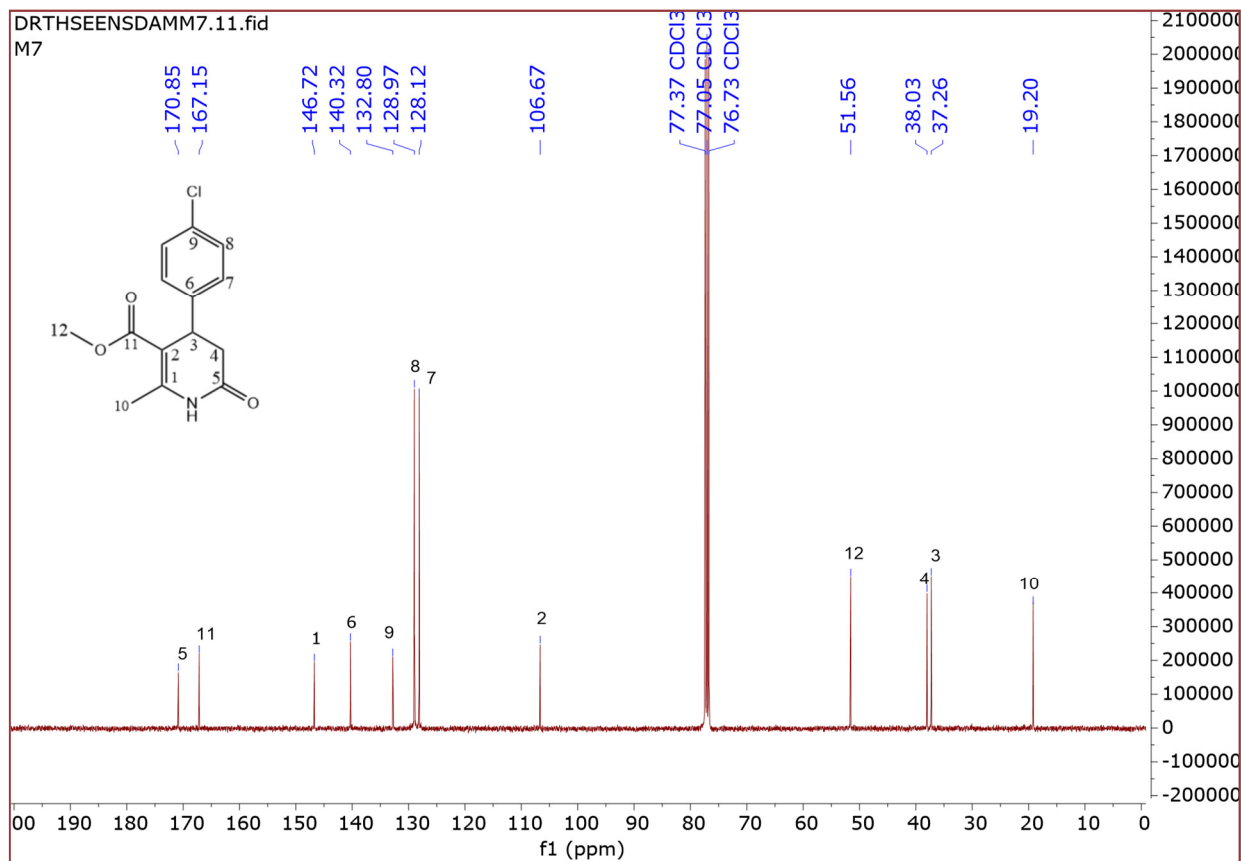
**Figure 25.** ESI mass spectrum of compound M6.



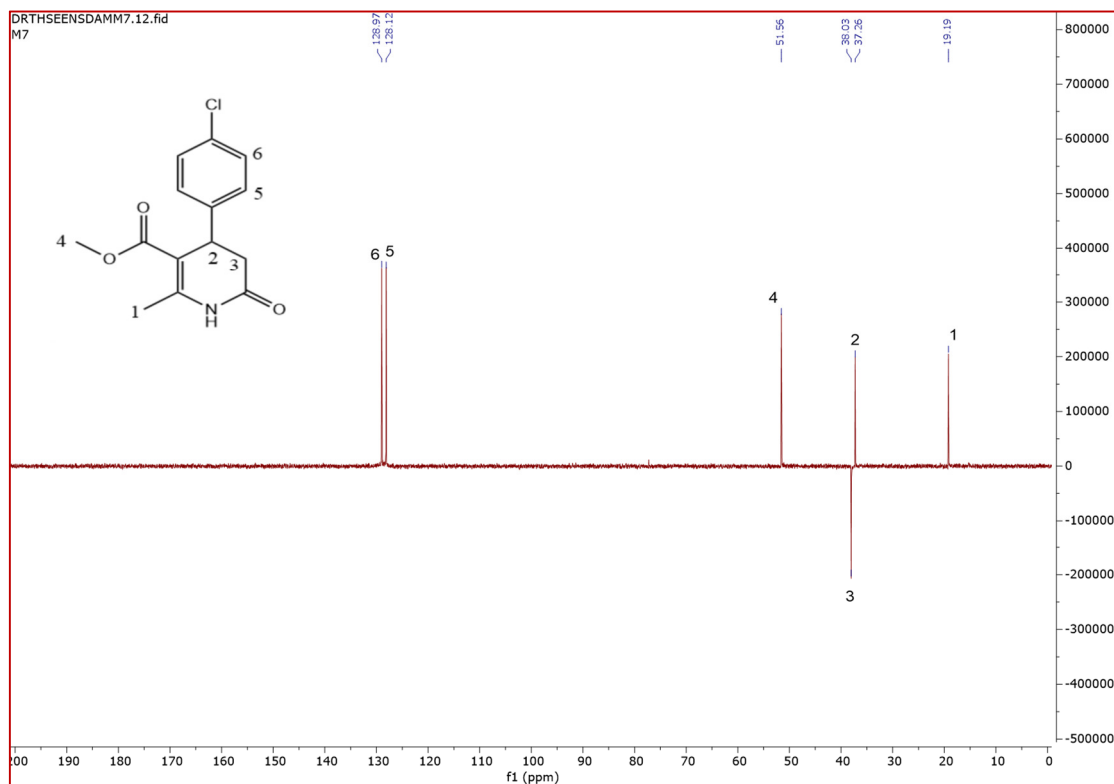
**Figure 26.** FT-IR spectrum of compound M7 (KBr, 4000–400  $\text{cm}^{-1}$ ).



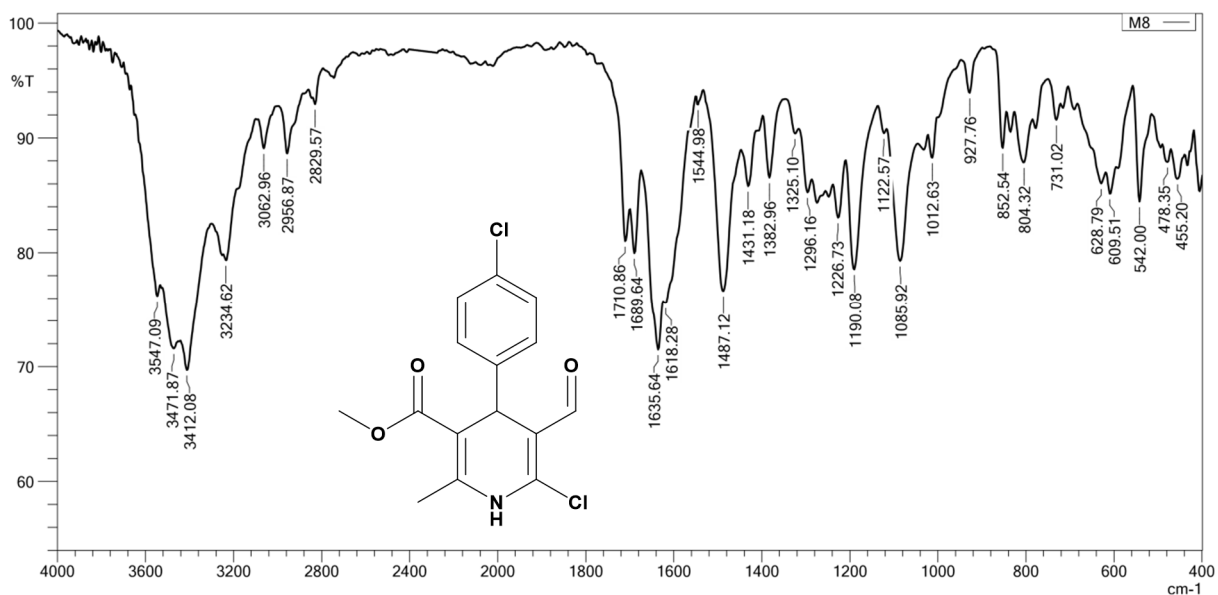
**Figure 27.**  $^1\text{H}$ -NMR spectrum of compound M7 (400 MHz,  $\text{CDCl}_3$ ).



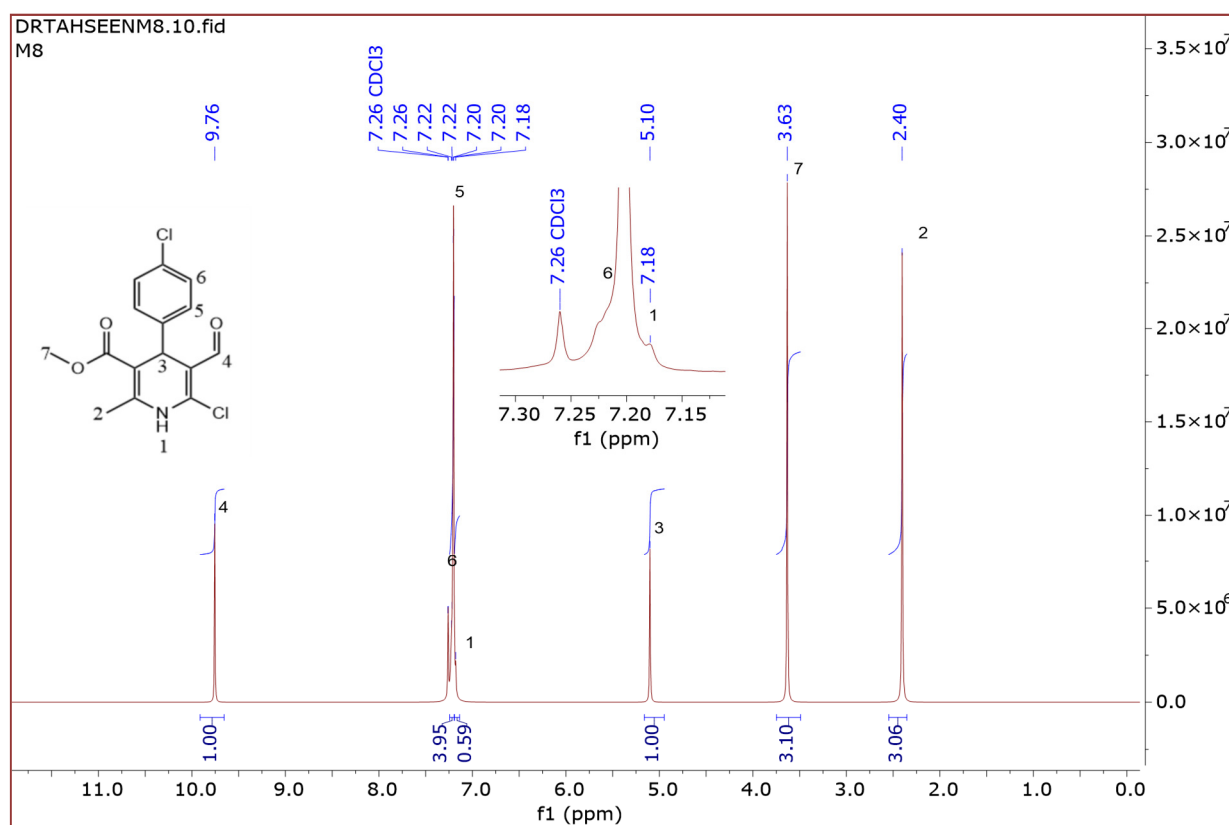
**Figure 28.**  $^{13}\text{C}$ -NMR spectrum of compound M7 (101 MHz,  $\text{CDCl}_3$ ).



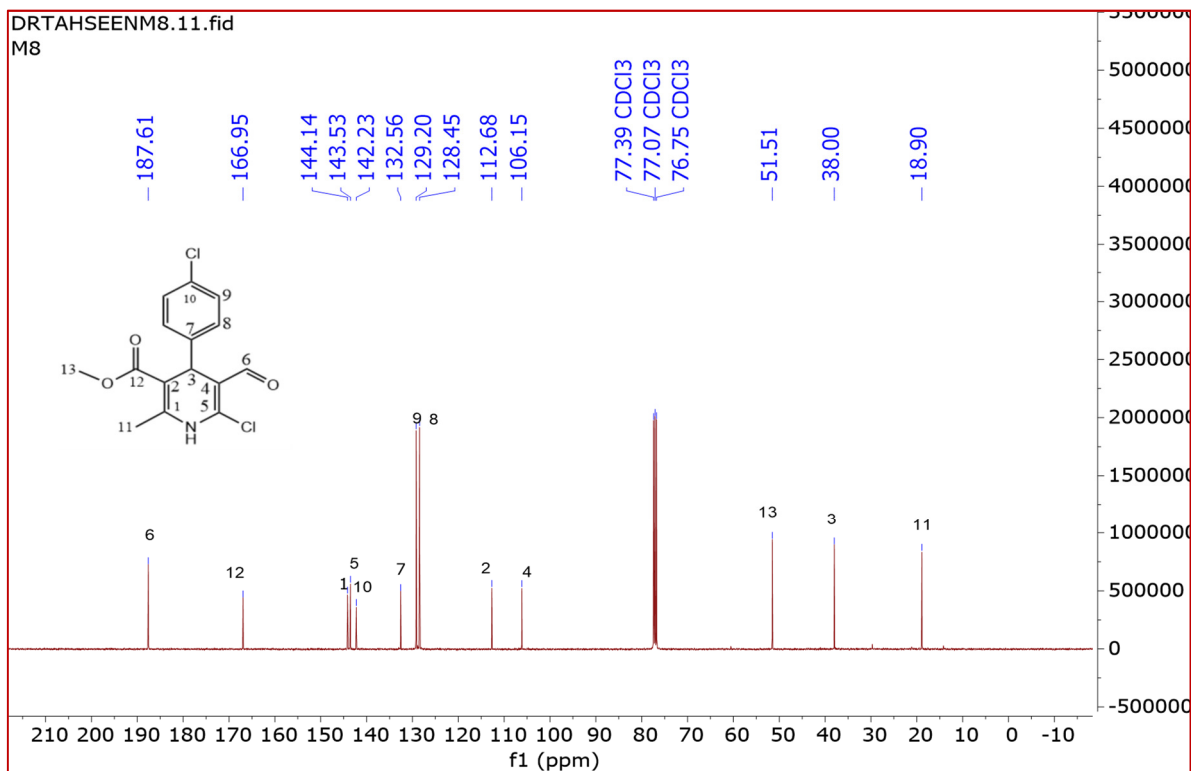
**Figure 29.** DEPT-135 spectrum of compound M7.



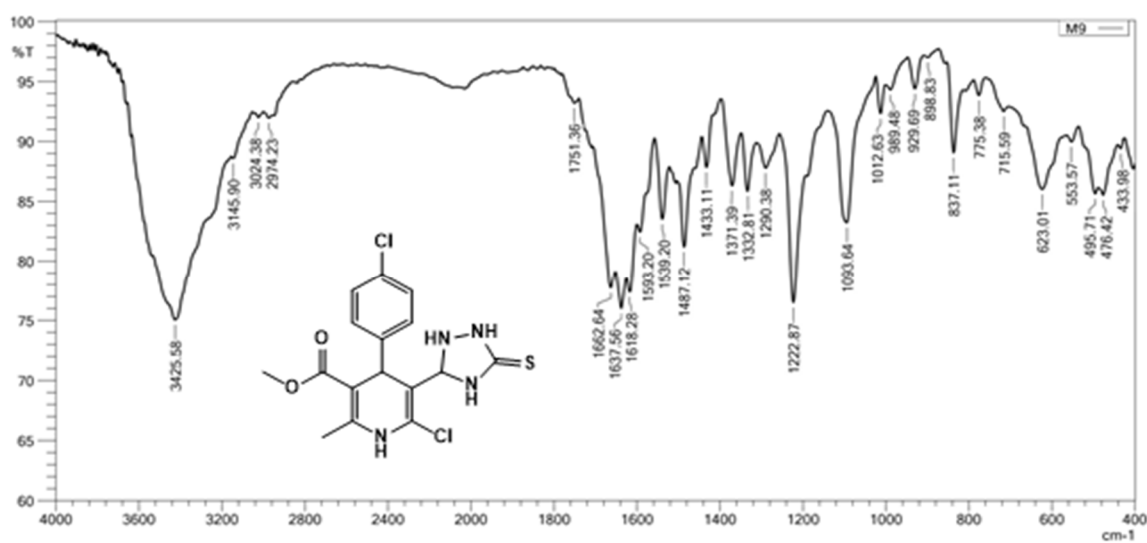
**Figure 30.** FT-IR spectrum of compound M8 (KBr, 4000–400 cm<sup>-1</sup>).



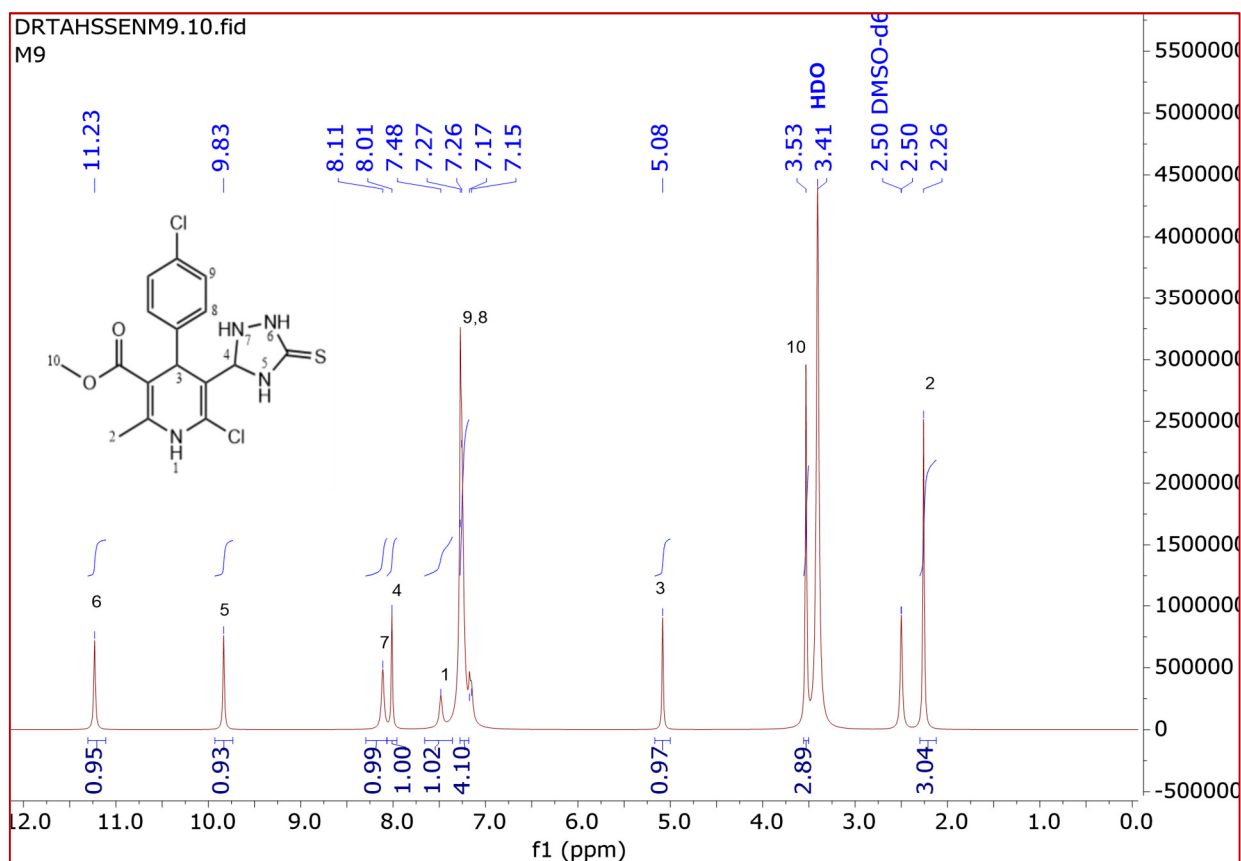
**Figure 31.** <sup>1</sup>H-NMR spectrum of compound M8 (400 MHz, CDCl<sub>3</sub>).



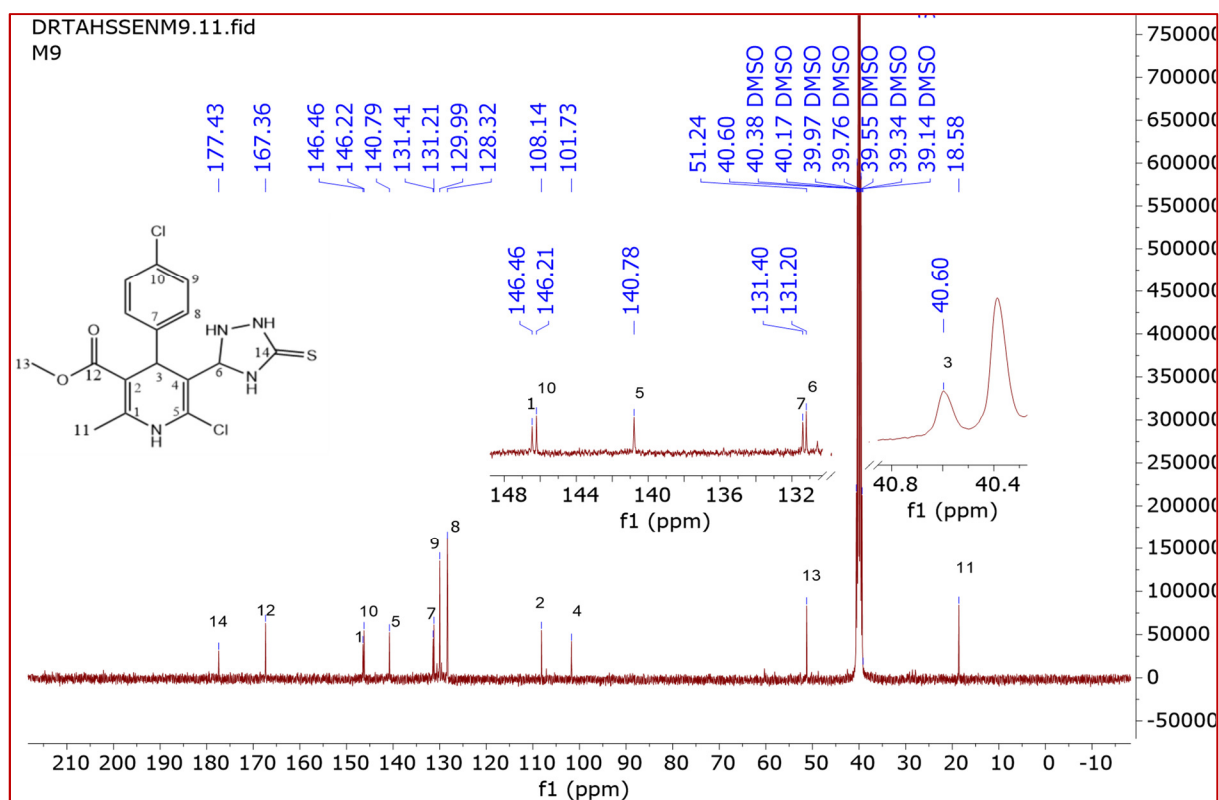
**Figure 32.**  $^{13}\text{C}$ -NMR spectrum of compound M8 (101 MHz,  $\text{CDCl}_3$ ).



**Figure 33.** FT-IR spectrum of compound M9 (KBr, 4000–400  $\text{cm}^{-1}$ ).

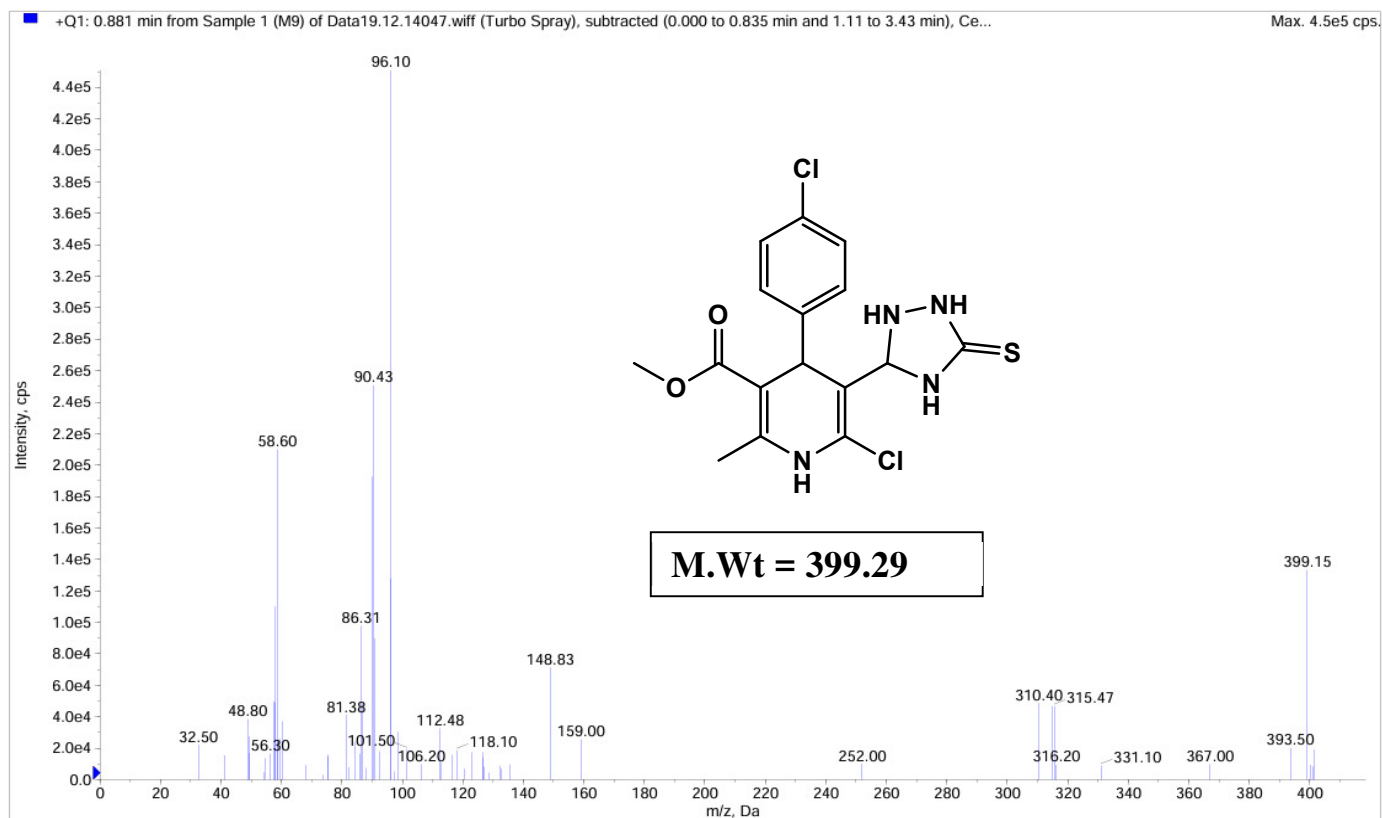


**Figure 34.**  $^1\text{H}$ -NMR spectrum of compound M9 (400 MHz,  $\text{DMSO-d}_6$ ).

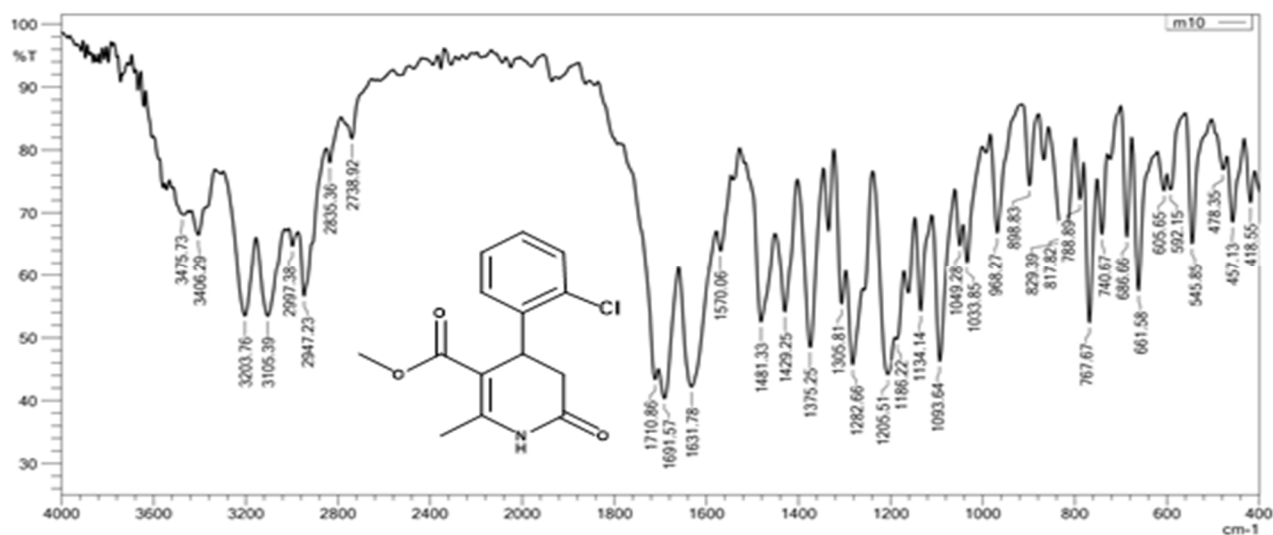


**Figure 35.**  $^{13}\text{C}$ -NMR spectrum of compound M9 (101 MHz,  $\text{DMSO-d}_6$ ).

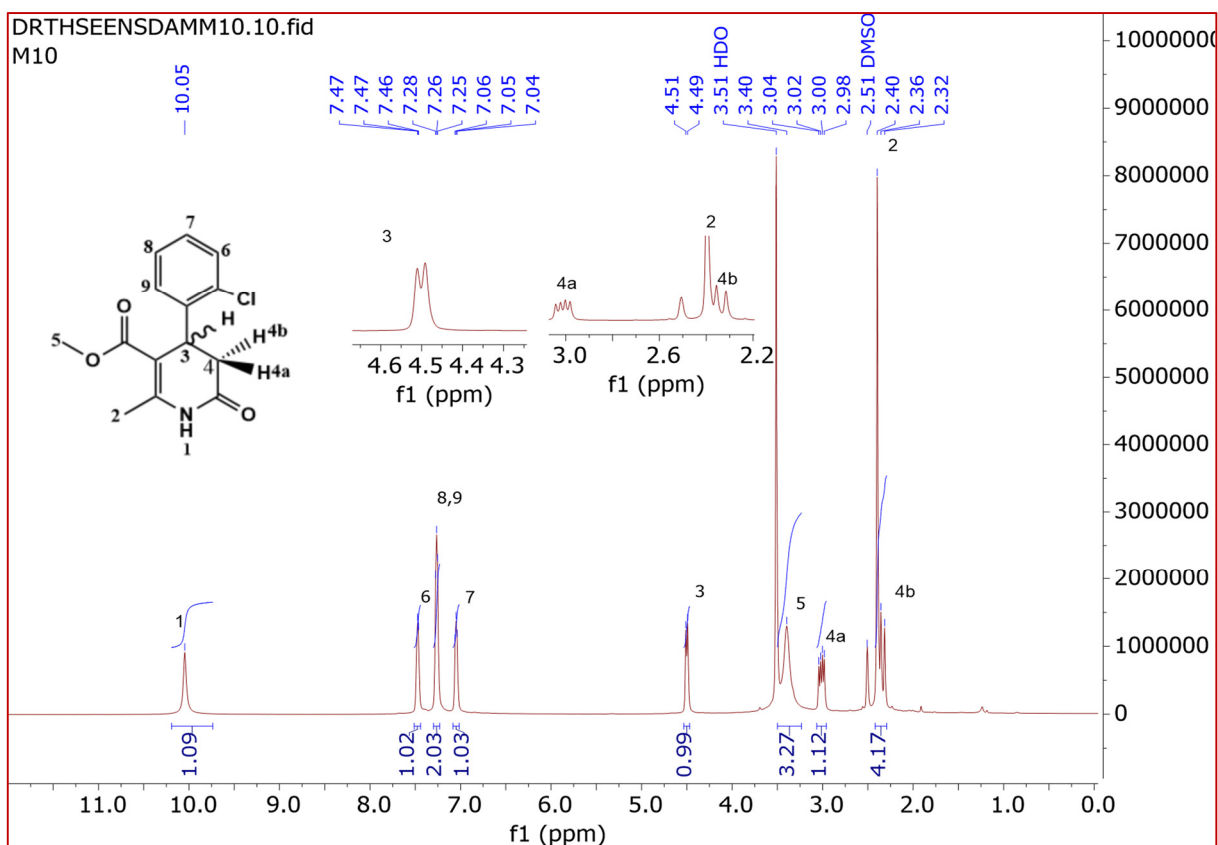




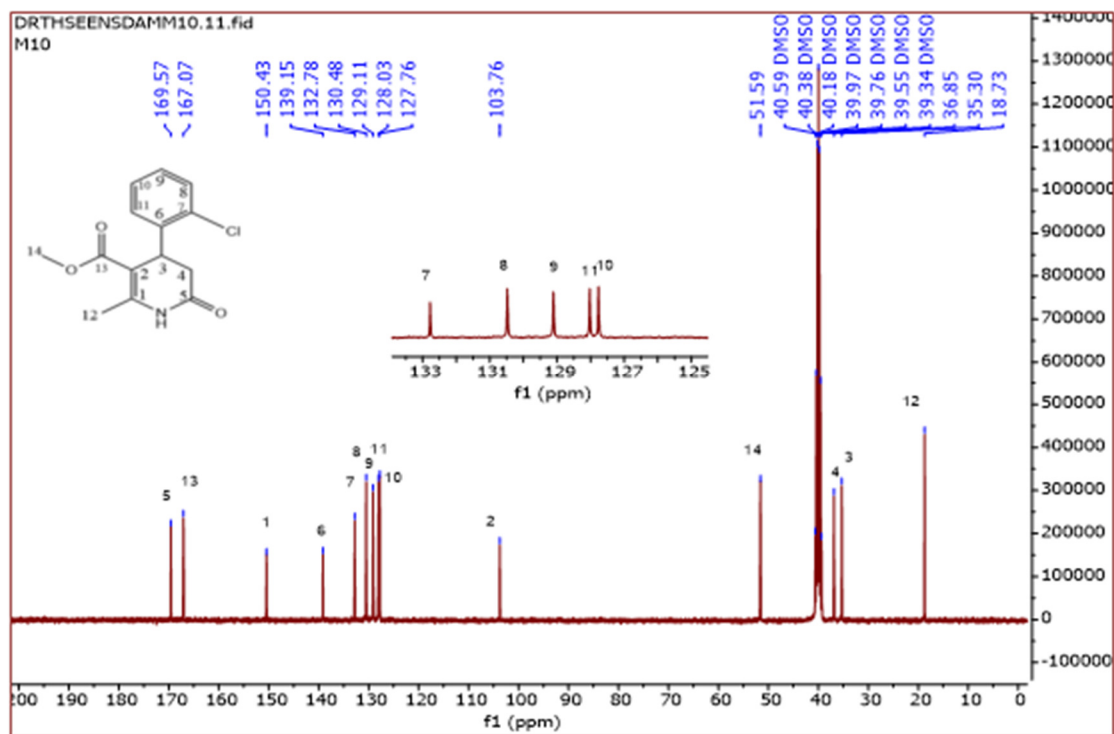
**Figure 36.** ESI mass spectrum of compound M9.



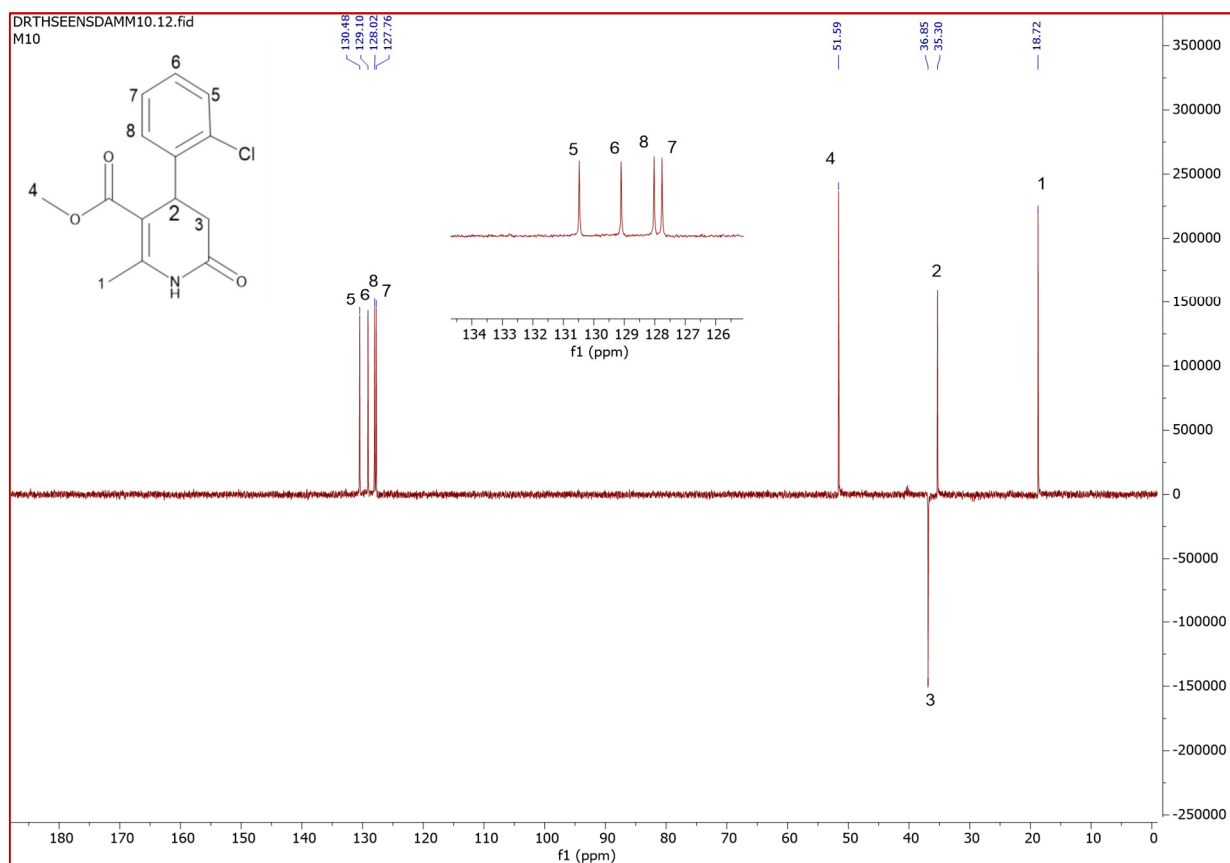
**Figure 37.** FT-IR spectrum of compound M10 (KBr, 4000–400 cm<sup>-1</sup>).



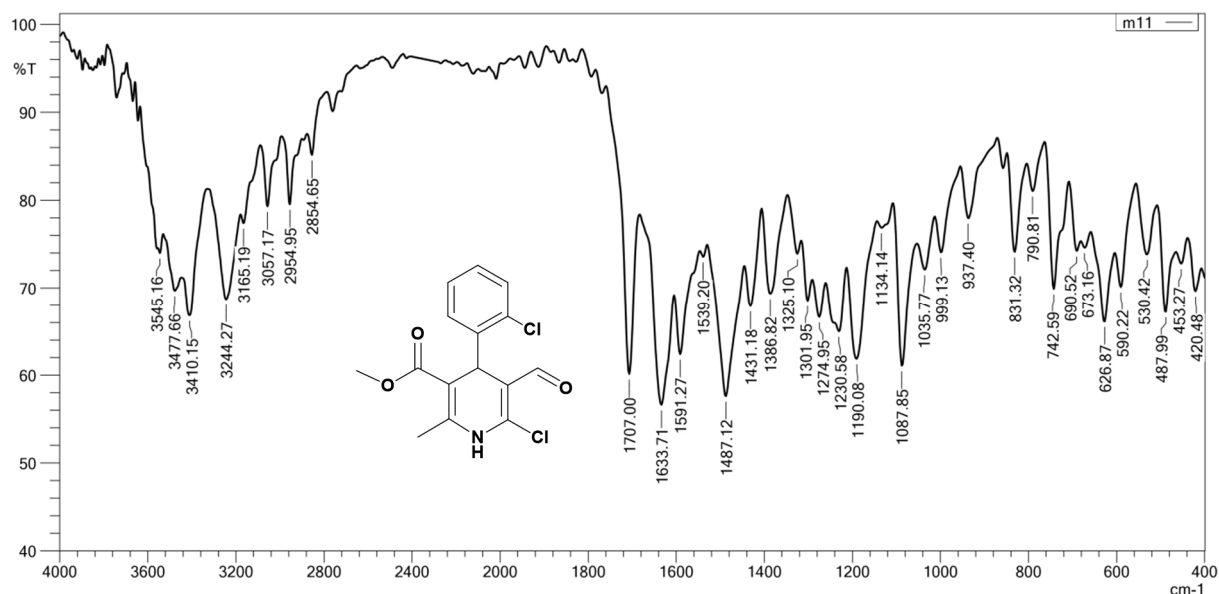
**Figure 38.**  $^1\text{H}$ -NMR spectrum of compound M10 (400 MHz,  $\text{DMSO-d}_6$ ).



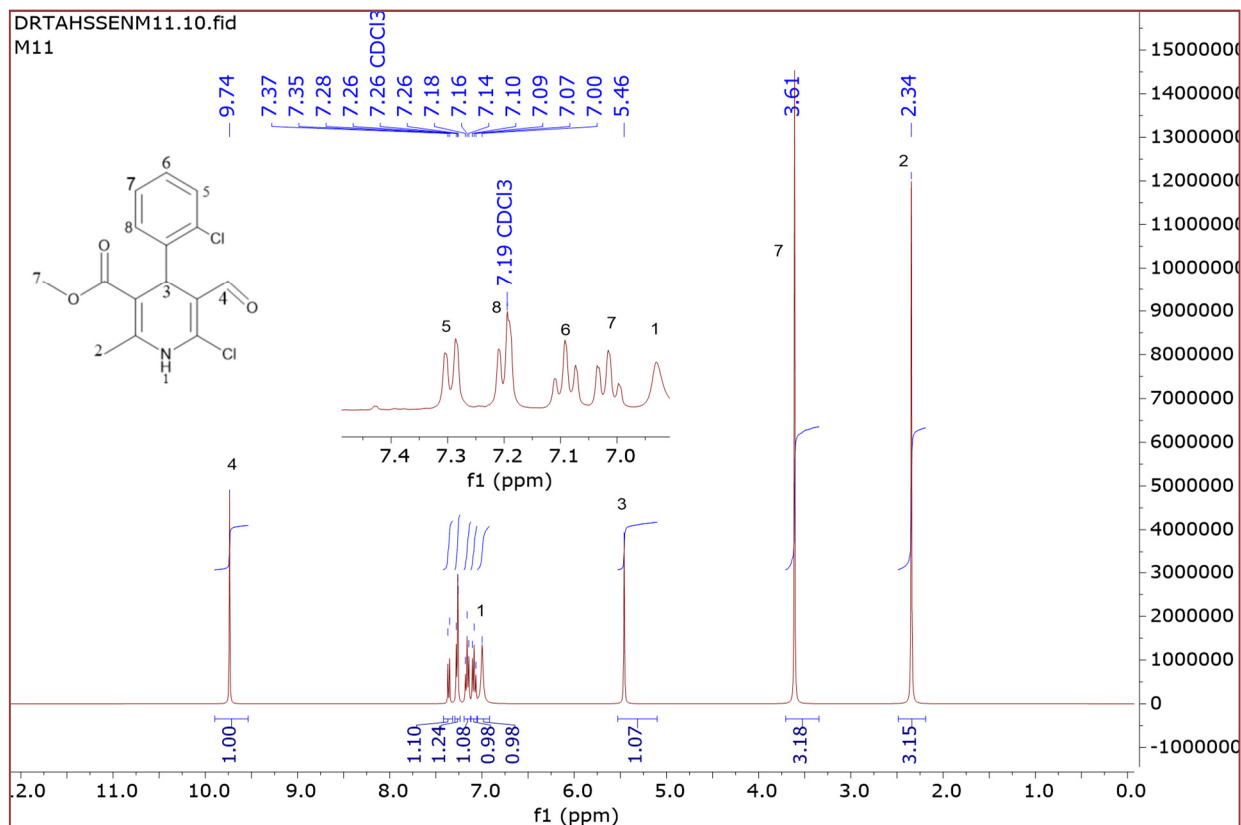
**Figure 39.**  $^{13}\text{C}$ -NMR spectrum of compound M10 (101 MHz,  $\text{DMSO-d}_6$ ).



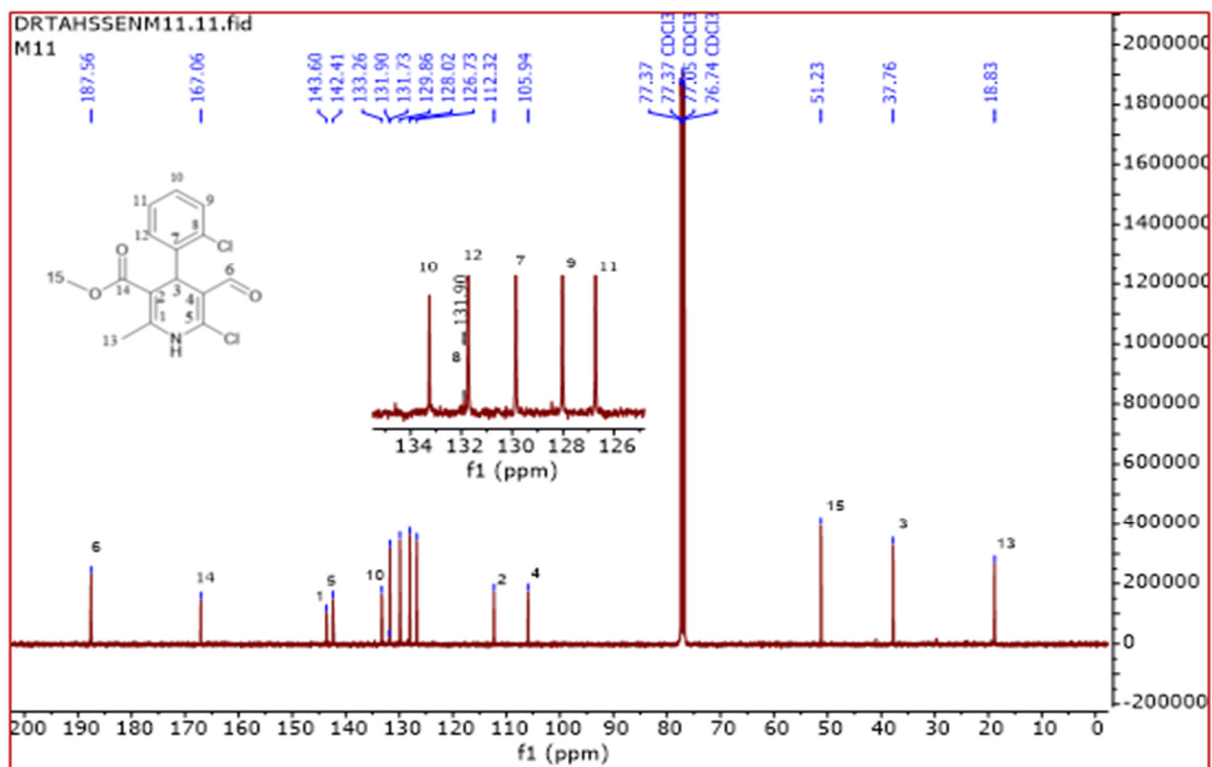
**Figure 40.** DEPT-135 spectrum of compound M10.



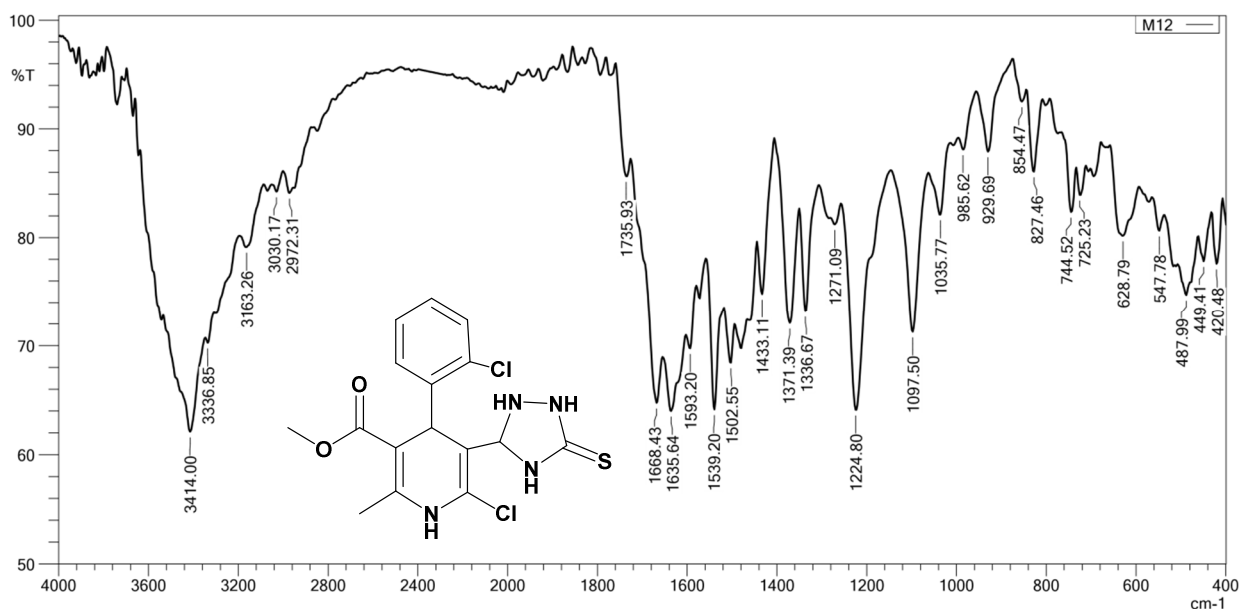
**Figure 41.** FT-IR spectrum of compound M11 (KBr, 4000–400  $\text{cm}^{-1}$ ).



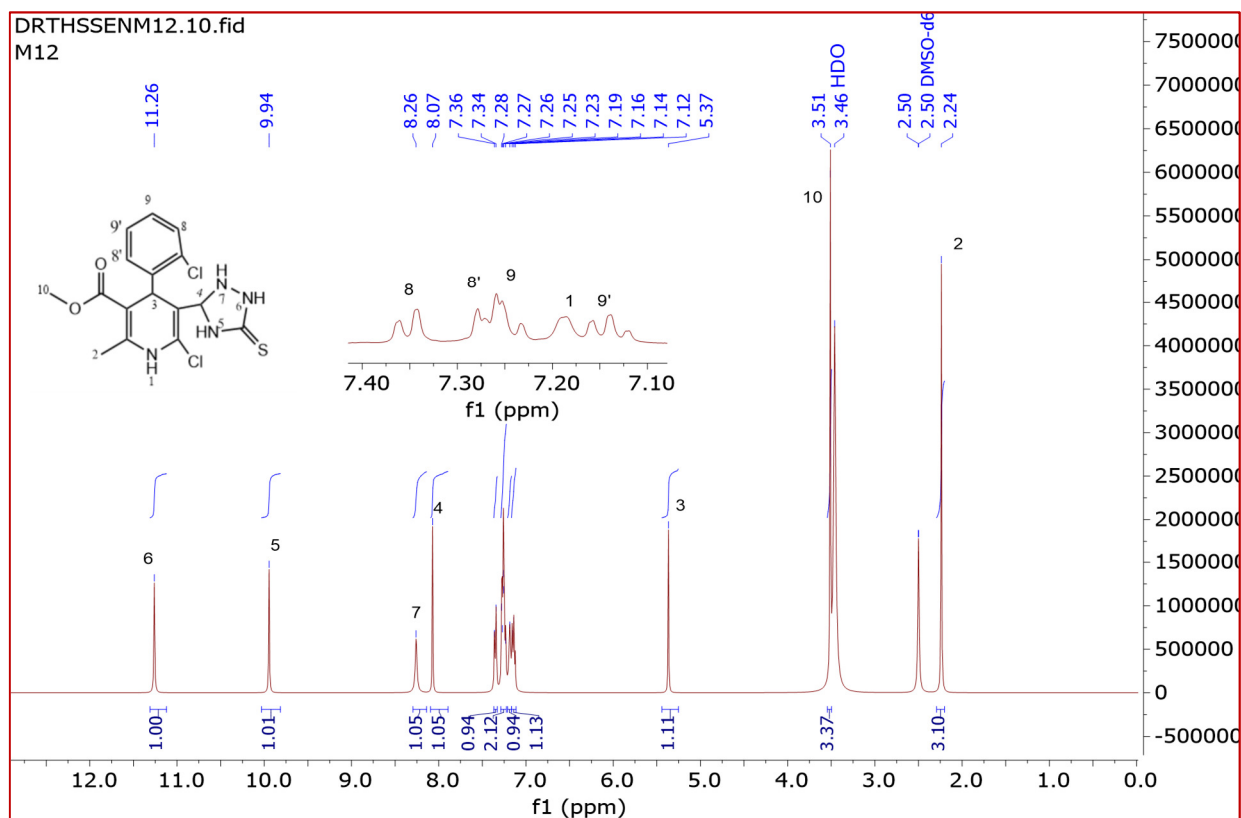
**Figure 42.**  $^1\text{H}$ -NMR spectrum of compound M11 (400 MHz,  $\text{CDCl}_3$ ).



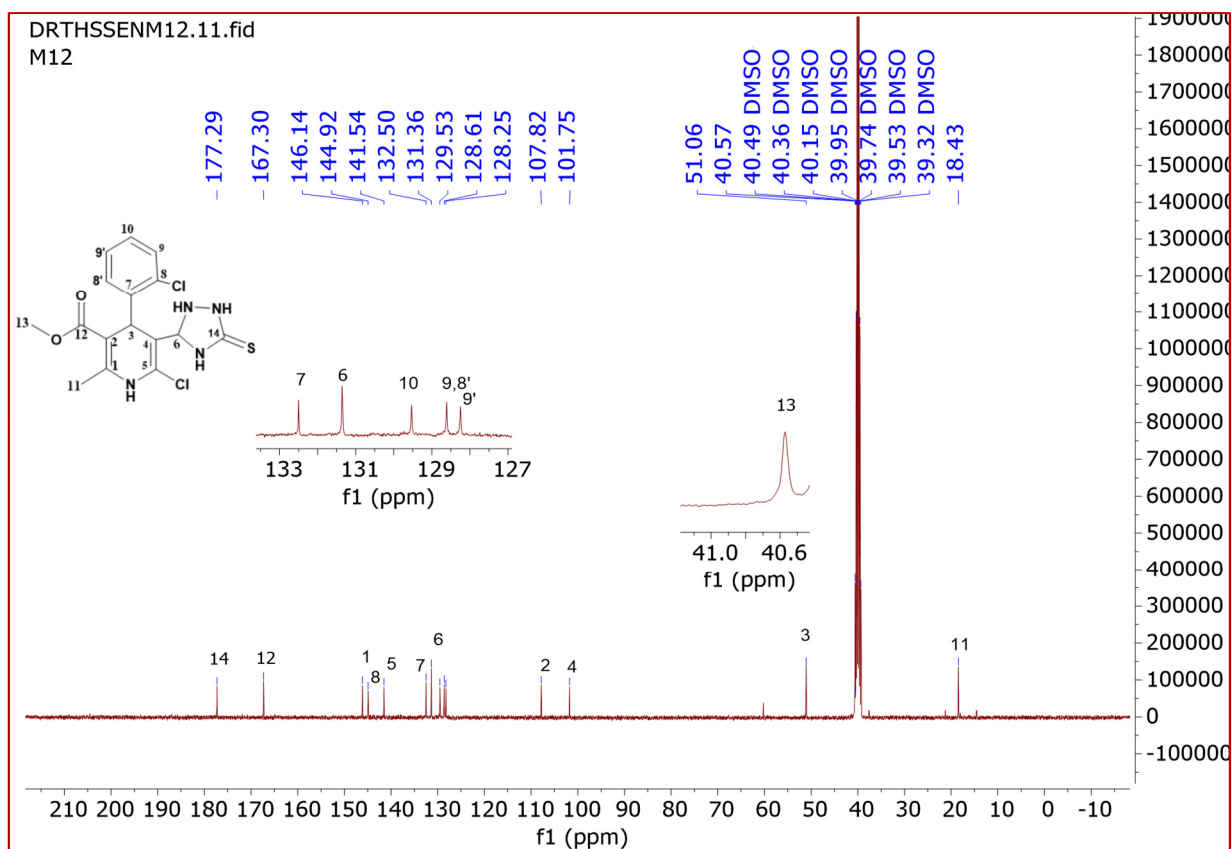
**Figure 43.**  $^{13}\text{C}$ -NMR spectrum of compound M11 (101 MHz,  $\text{CDCl}_3$ ).



**Figure 44.** FT-IR spectrum of compound M12 (KBr, 4000–400  $\text{cm}^{-1}$ ).



**Figure 45.**  $^1\text{H}$ -NMR spectrum of compound M12 (400 MHz,  $\text{DMSO-d}_6$ ).



**Figure 46.**  $^{13}\text{C}$ -NMR spectrum of compound M12 (101 MHz,  $\text{DMSO}-d_6$ ).



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