IMMUNOMODULATORY ACTIVITY OF SYNTHETIC AURONE DERIVATIVES, AZAAURONES, IN LPS-STIMULATED RAW 246.7 MACROPHAGE-LIKE CELLS

BY

Zahrah R. Alrayes

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Thesis committee:

Dr. Anthony L. Farone, Committee Chair
Dr. Mary Farone, Committee Member
Dr. Justin Miller, Committee Member
Dr. Paul Kline, Committee Member

DEDICATION

In loving memory of my beloved father the legal counsel Rabie Alrayes, I humbly dedicate this remarkable achievement. His unwavering support, guidance, and inspiration have shaped me intothe person I am today.

Also, to the unwavering support and love of my mother Thabyah Alrayes, who has been my pillarof strength throughout this journey. her sacrifices, encouragement, and belief in my abilities have propelled me forward, even in the face of challenges.

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Abstract

Inflammation, a fundamental biological response, is essential for various physiological processes. However, dysregulated or chronic inflammation is linked to numerous pathological conditions. This thesis delves into the unexplored realm of azaaurone derivatives, nitrogen-substituted versions of aurones, investigating their potential as anti-inflammatory agents. The research comprises two distinct chapters, each shedding light on different facets of the anti-inflammatory properties of azaaurones. In the first experiment, titled "Evaluation of Twenty-Seven Azaaurone Derivatives as Potential Anti-Inflammatory Agents," a screening of 27 azaaurones for cytotoxicity in RAW 264.7 cells, a murine macrophage-like cell model, was conducted. Four promising derivatives (AAA2Me, AAA3Br, AAAPh and AAA3Me) were identified as showing minimal cytotoxicity. Subsequent treatments of the macrophage-like cells demonstrated a dose-dependent reduction in nitrite production, with AAAPh and AAA3Me exhibiting notable inhibitory effects. Importantly, azaaurones displayed anti-inflammatory potential at concentrations approximately 40 times lower than aurones, highlighting their considerable bioactivity. In the second experiment, titled "The Effects of the Synthetic Azaaurone (Z)-1-Acetyl-2-Benzylideneindolin-3-one on iNOS, NF-κB, and MAPK Pathways in LPS-Stimulated RAW 264.7 Cells," the focus shifted to (Z)-1-Acetyl-2-Benzylideneindolin-3-one and its derivatives. The study assessed iNOS protein expression, NF-κB and MAPK pathway activation, and TNF-α secretion in LPS-stimulated RAW 264.7 cells. While azaaurones did not significantly reduce iNOS protein expression, they demonstrated promising effects

on inflammation-associated signaling pathways. Notably, AAA2Me showed a significant reduction in p38 protein expression, indicating a potential mechanism of action. Additionally, a trend towards decreased pp38 and reduced TNF-α secretion by multiple derivatives further supports their anti-inflammatory efficacy. These findings collectively underscore the considerable anti-inflammatory potential of azaaurone derivatives, positioning them as promising candidates for further drug development. The significant inhibition observed at low concentrations, coupled with insights into their molecular interactions and impact on key signaling pathways, provides a solid foundation for future research and therapeutic applications targeting inflammatory responses. The potential of azaaurones to address chronic inflammation and associated diseases opens up new possibilities for advancing drug discovery and improving clinical outcomes.

TABLE OF CONTENTS

Contents

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Inflammation is a complex biological response that involves a delicate interplay between various elements, including immune cells, blood vessels, and molecular mediators (Abdulkhaleq et al., 2018; Isailovic et al., 2015; Medzhitov, 2008; Meizlish et al., 2021). This intricate process is essential for two main purposes: resolving and repairing damagedtissue and neutralizing foreign organisms or materials (Lawrence and Gilroy, 2007; Naqviet al., 2022). Furthermore, inflammation is a pivotal player in the development and progression of non-infectious diseases, such as diabetes mellitus (DM), chronic obstructive pulmonary disease (COPD), rheumatoid arthritis (RA), and cardiovascular diseases (CVDs) (Cazzola et al., 2023; Zhong and Shi, 2019). In the realm of inflammation, we cancategorize the responses into three distinct categories based on their cause, duration, and severity (Medzhitov, 2008; Zhong and Shi, 2019). (i) Acute Inflammation: this type of inflammation initiates promptly in response to tissue damage, injury, infection with a pathogen, or exposure to toxins. Acute inflammation is usually of short duration, lasting from a few hours to a few days (Lawrence and Gilroy, 2007; Parisien et al., 2022). (ii) Subacute Inflammation: it represents a transitional phase between acute and chronic inflammation. It typically occurs when acute inflammation persists for a longer duration, generally lasting between 2 to 6 weeks (Sugimoto et al., 2016; Elgazzar, 2023). (iii) Chronic Inflammation: chronic inflammation is characterized by an ongoing and prolonged inflammatory response that the body fails to resolve, persisting for months to years (Lawrence and Gilroy, 2007). Prolonged inflammation is associated with more severe

clinical outcomes and is often linked to various chronic diseases (Furman et al., 2019). Understanding the different facets of inflammation is vital not only for comprehending itsrole in health and disease but also for developing strategies to manage and modulate theseresponses for therapeutic purposes (Fioranelli et al., 2021). It underscores the importance of balancing the body's inflammatory reactions to maintain overall well-being (Medzhitov,2008; Zhong and Shi, 2019).

Pathogen-associated molecular patterns (PAMPs), which are specific molecular structures on the surface of microbial pathogens, are recognized by the cells of the innate immune system via their intracellular or surface-expressed pattern recognition receptors (PRRs) and can initiate inflammation. Inflammation can be initiated when the cells of theinnate immune system recognize the invading microbial pathogens via their intracellular or surface-expressed pattern recognition receptors (PRRs) that detect specific PAMPS on the surfaces of microbial pathogens (Jang et al., 2015; Li and Wu, 2021) (Figure 1).

PAMPs include various molecules, such as microbial nucleic acids, Gram-negative bacterial lipopolysaccharides (LPS), and microbial carbohydrates (Newton and Dixit, 2012). When host PRRs bind to microbial PAMPs, the release of pro-inflammatory cytokines like TNF-α, chemokines, and IL-1β is triggered. This activation occurs throughthe Nuclear Factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, leading to the recruitment of immune cell subtypes and further cytokine release, intensifying the inflammatory response (Ginwala et al., 2019) (Figure 1).

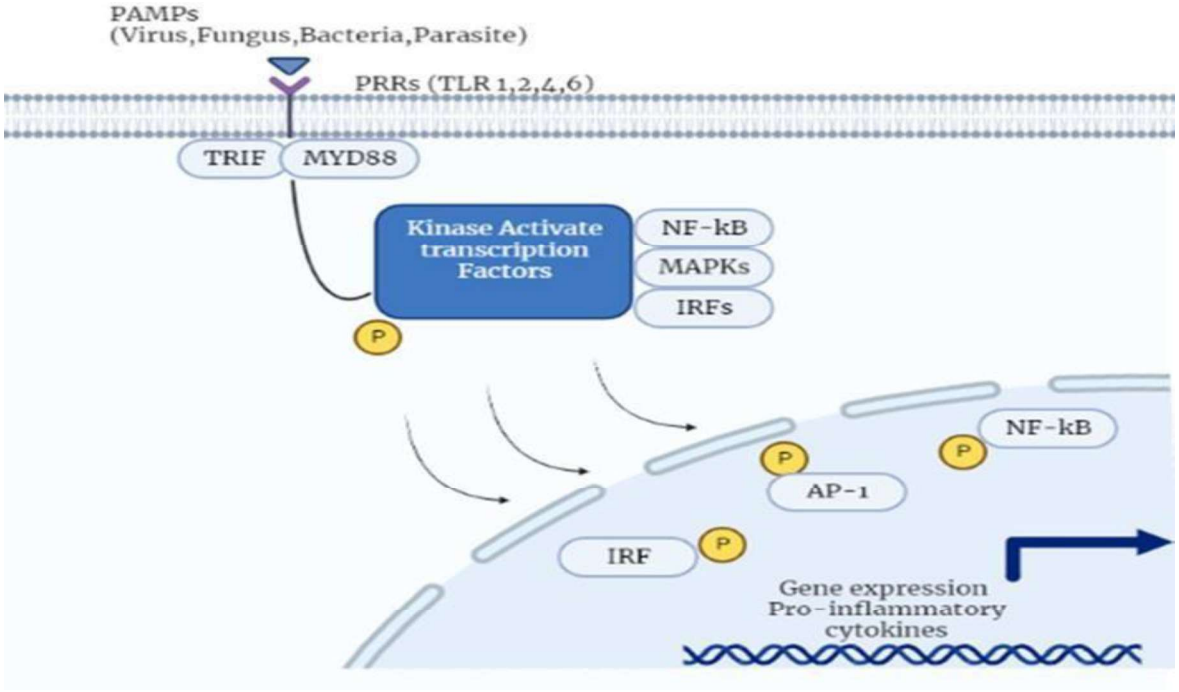


Figure 1: NF-κB and MAPK activation via pAMP -PPR interaction.

NF-κB, a transcription factor, plays a multifaceted role in various cellular activities, encompassing DNA transcription, cell growth, cell division, immune activation (Albensi, 2019), as well as neural conduction and memory (Kaltschmidt and Kaltschmidt, 2009). Activation of NF-κB can occur through either of two major signaling pathways: the canonical and noncanonical pathways, both of which are crucial for regulating inflammatory and immune responses (Liu et al., 2017). The canonical NF-κB pathway responds to signals from a range of cell surface receptors, including PRRs like Toll-like receptors (TLRs), cytokine receptors such as TNF receptors (TNFRs), T cell receptors (TCRs), and B cell receptors (BCRs) (Zhang and Sun, 2015). Following receptor

activation, downstream signaling leads to the phosphorylation of IκBα by the multi-subunitIκB kinase (IKK) complex, marking it for proteasomal degradation (Hinz and Scheidereit,2014; Antonia et al., 2021). This results in the release of p65/NF-κB, which translocates into the nucleus, promoting the transcription of inflammatory genes (Antonia et al., 2021)(Figure 2).

Additionally, TLR activation can also trigger the activation of the mitogen- activated protein kinase (MAPK) signaling cascade. MAPK activation leads to the phosphorylation of p38, which then translocates into the nucleus to regulate gene transcription (Figure 2). Both MAPK and NF-κB can work together or independently following an inflammatory stimulus to induce the expression of inflammatory genes. In some tissues, p38 has been demonstrated to promote NF-κB expression (Saha et al., 2007).While inflammation is essential for clearing infections and repairing tissue damage following injury, chronic inflammation can be detrimental, and effective therapeuticinterventions for managing chronic inflammation remain a challenge.

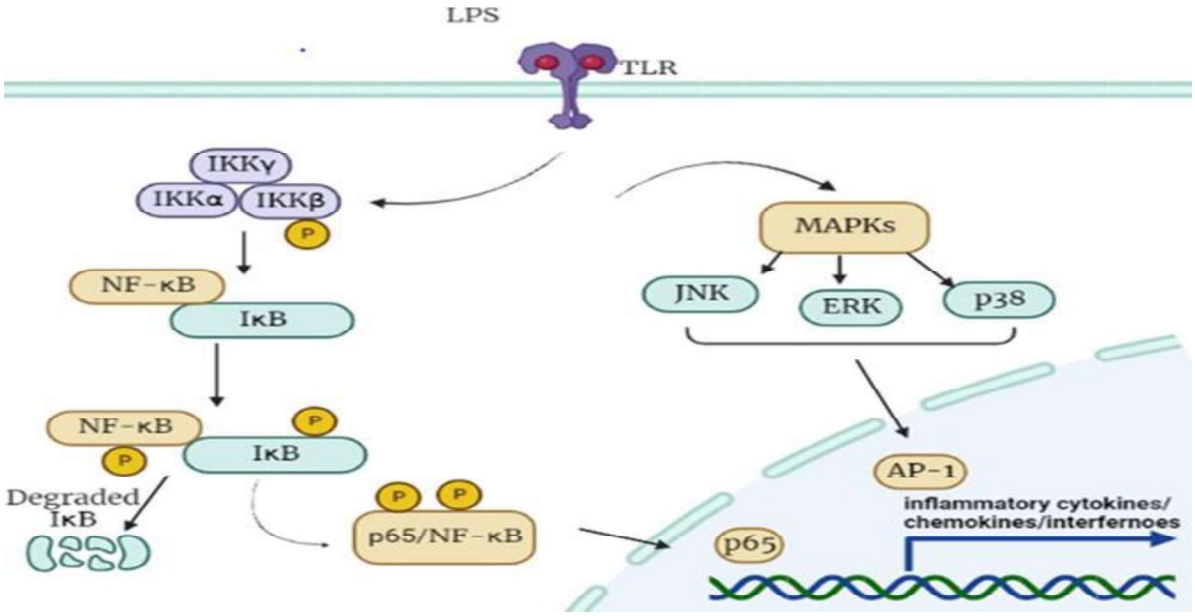


Figure 2: The role of Nuclear Factor κB and MAPK in inflammatory cascade.

Various naturally occurring compounds have been explored as potentialtherapeutics to control inflammation, some of which have included flavonoids (Chen et al.,2023). Flavonoids are considered an important group of natural products that have been documented to suppress inflammatory response *in vitro* and *in vivo*. Flavonoids are knownto have anti-inflammatory, antioxidant, antimutagenic, antibacterial, and anticancer properties (Kim et al., 2021; Kumar and Pandey, 2013). *In vitro* studies have shown flavonoids can suppress the release of pro-inflammatory cytokines such as TNF-α, IL-6, and COX-2 via suppression of the MAPK and NF-κB pathways and even alleviatemyocardial injury (Garg et al., 2020; Ginwala et al., 2019; Malik et al., 2017). Activationof NF-κB also leads to production of nitric oxide (NO). The production of NO is a representative mediator of inflammatory responses and results from expression of

inducible nitric oxide synthase (iNOS) (Arias-Salvatierra et al., 2011). In a feed-forward loop, greater iNOS production leads to increase NO production which further drives NF- κB transcriptional activity and subsequent increases in inflammatory response (Baig et al.,2015).

Naturally occurring compounds, including flavonoids, have gained attention as potential therapeutic agents for managing inflammation (Ferraz et al., 2020; Aghababaei and Hadidi, 2023). Flavonoids are secondary metabolites found in fruits, vegetables, herbs,stems, cereals, nuts, flowers, and seeds (Santos et al., 2017). Over 10,000 flavonoid compounds have been identified (Ullah et al., 2020). These compounds have shown favorable effects on diseases like cardiovascular disease, cancer, and neurodegenerative conditions (García-Lafuente et al., 2009). Their health-promoting properties have known for their ability to suppress inflammation, exhibit various beneficial properties, such as anti-inflammatory, antioxidant, antimutagenic, antibacterial, anticancer effects and their ability to regulate key cellular enzyme function (Kumar and Pandey, 2013; Panche et al.,2016; Karak, 2019; Ullah et al., 2020; Chen et al., 2023).

In vitro studies, flavonoids have demonstrated the ability to inhibit the release of pro-inflammatory cytokines like TNF-α, IL-6, and COX-2 by suppressing the MAPK andNF-κB pathways. Additionally, they have shown promise in alleviating myocardial injury(Malik et al., 2017; Ginwala et al., 2019; Garg et al., 2020). Activation of NF-κB also leadsto the production of NO, which is a key mediator of inflammatory responses. NO is generated through the expression of iNOS (Arias-Salvatierra et al., 2011). In a feedback loop, increased iNOS production leads to greater NO production, further enhancing NF- κB transcriptional activity and subsequently amplifying the inflammatory response (Baig

et al., 2015). The ability of flavonoids to modulate these pathways and reduce inflammation makes them an interesting area of study for potential therapeutic interventions (Ullah et al.,2020; Al-Khayri et al., 2022; Melrose, 2023).

Flavonoids exhibit a distinctive structural composition, characterized as diphenylpropanes (Mutha et al., 2021; Chen et al., 2023). Their structure comprises three rings: A, B, and C, as depicted in Figure 3. In this structure, rings A and B are both benzenerings, while ring C is a pyran ring, which is a six-carbon ring containing one oxygen atom(Kumar and Pandey, 2013).

Flavonoids are categorized into three major classes based on the attachment site ofthe phenyl ring to the pyran ring: (i) Flavonoids: the phenyl ring is attached to the C-2 position of the pyran ring. (ii) Isoflavonoids: the phenyl ring is attached to the C-3 positionof the pyran ring. (iii) Neoflavonoids: the phenyl ring is attached to the C-4 position of thepyran ring (Menezes et al., 2016). These three primary classes of flavonoids further branchinto various subclasses, depending on the degree of oxidation and unsaturation in the pyranring (Santos-Buelga and Feliciano, 2017).

Flavonoids also encompass structural variations, such as chalcones and aurones, which may either have an open pyran ring or lack the benzopyran core, respectively (Obaidet al., 2021). The diversity in flavonoid structures is attributed to the different patterns of chemical substitution in the phenylchromanone unit and pyran ring (Aneklaphakij et al., 2021). This diversity arises through processes like hydroxylation, methoxylation, and glycosylation, leading to numerous derivatives within each flavonoid subclass (Menezes et al., 2016) (Figure 4). This structural diversity contributes to the varied biological activities and potential therapeutic properties of flavonoids (Aneklaphakij et al., 2021).

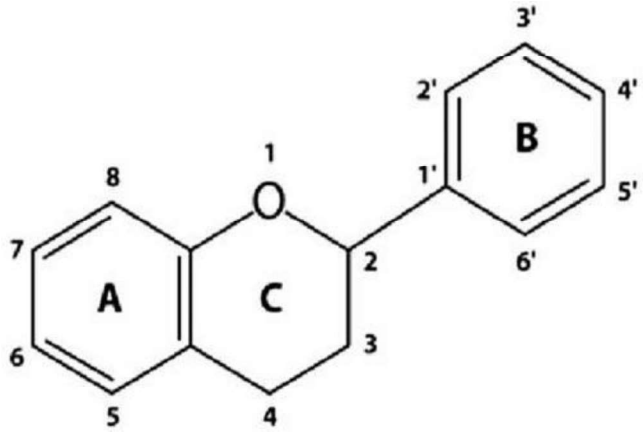


Figure 3: Basic chemical structure of flavonoids (Chen et al., 2023).

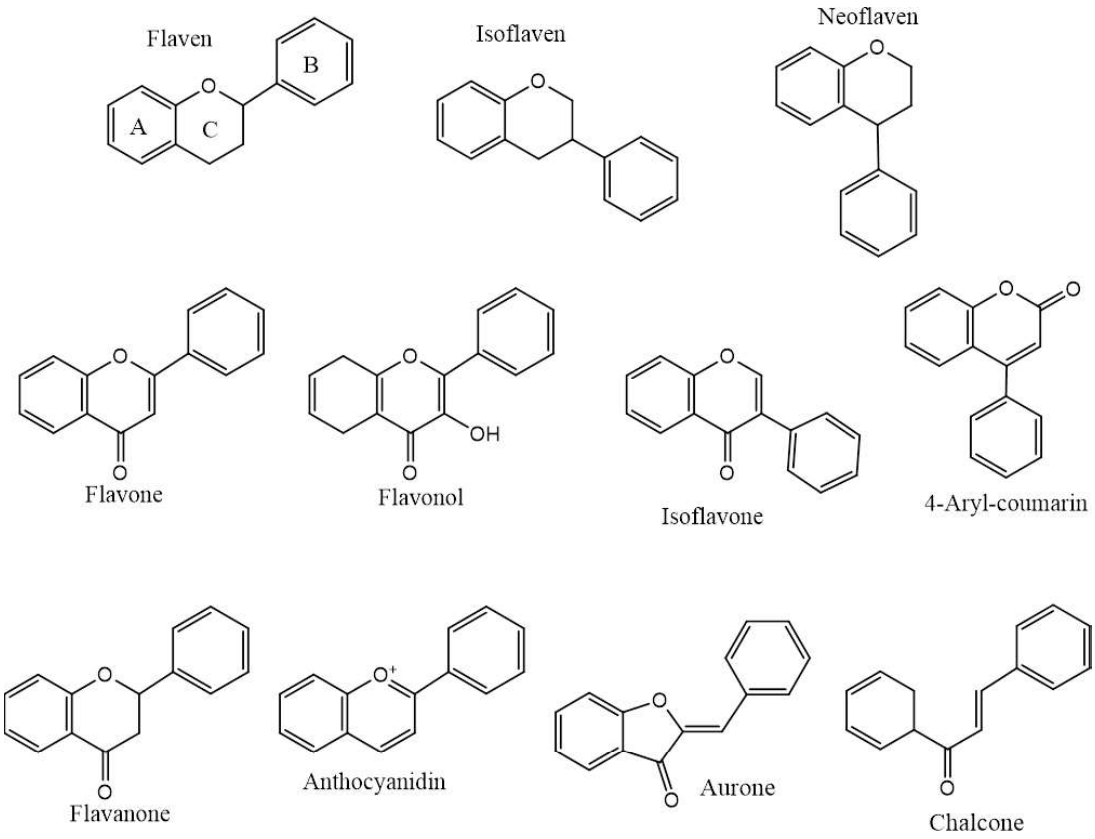


Figure 4: Minor classes of flavonoids: Flavanones, Flavonols, Isoflavones, Flavones, Anthocyanins Falven, Isoflaven, Newflaven, 4-Aryl-coumarin, Aurone, and Chalcone *(*Brodowska, 2017*)*.

Flavonoids can be synthesized through chemical processes involving the condensation of 2'-hydroxyacetophenones with benzaldehydes (Khdera et al., 2022). This condensation can occur under either acidic conditions to produce chalcones or basic conditions to yield flavanones (Marais et al., 2006; Pereira et al., 2023). Synthetic flavonoids, such as 2'-hydroxychalcones and 2',5'-dihydroxychalcones, have been reported to exhibit anti-inflammatory activity (Won et al., 2005).

Aurones, a subset of flavonoids, are isomeric to flavones, characterized by the replacement of the pyran ring with a furanone ring and the presence of an exocyclic alkene. Aurones have been found to offer various health benefits (Alsayari et al., 2019). The name "aurone" is derived from the Latin word "aurum," signifying gold, owing to the golden yellow color exhibited by aurones. This vibrant yellow color is responsible for the bright hues of the flowers in plants that contain aurones (Boumendjel, 2003; Zwergel et al., 2012;Hassan et al., 2018). Aurones are present in several plant families, including Scrophulariaceae and Compositae, as well as in seedling leaves, wood, bark, and even certain marine organisms (Mazziotti et al., 2022). They are considered phytoalexins, usedby plants to combat microbial infections (Suresh Kumar, 2014). Chemically, aurones existin the thermodynamically more favorable (Z)-configuration (Zwergel et al., 2012; Lazinskiet al., 2022). It has been reported that stable 6'-deoxychalcones are the native substrates forthe biosynthesis of aurones (Miosic et al., 2013).

The most well-known natural aurone is aureusidin, also referred to as tetrahydroxy aurone, as shown in Figure 5 (Sui et al., 2021). It has been isolated from various *Cyperus* species. Aureusidin can occur in two forms: the 6-glucosidic form, known as aureusin, andthe 4-glucosidic form, referred to as cernuoside (Andersen and Jordheim, 2010). Aurones are typically found with hydroxylation, methoxylation, or glycosylation on the aryl rings, and to a much lesser extent, as biaurones (Boumendjel, 2003; Yang et al., 2023).

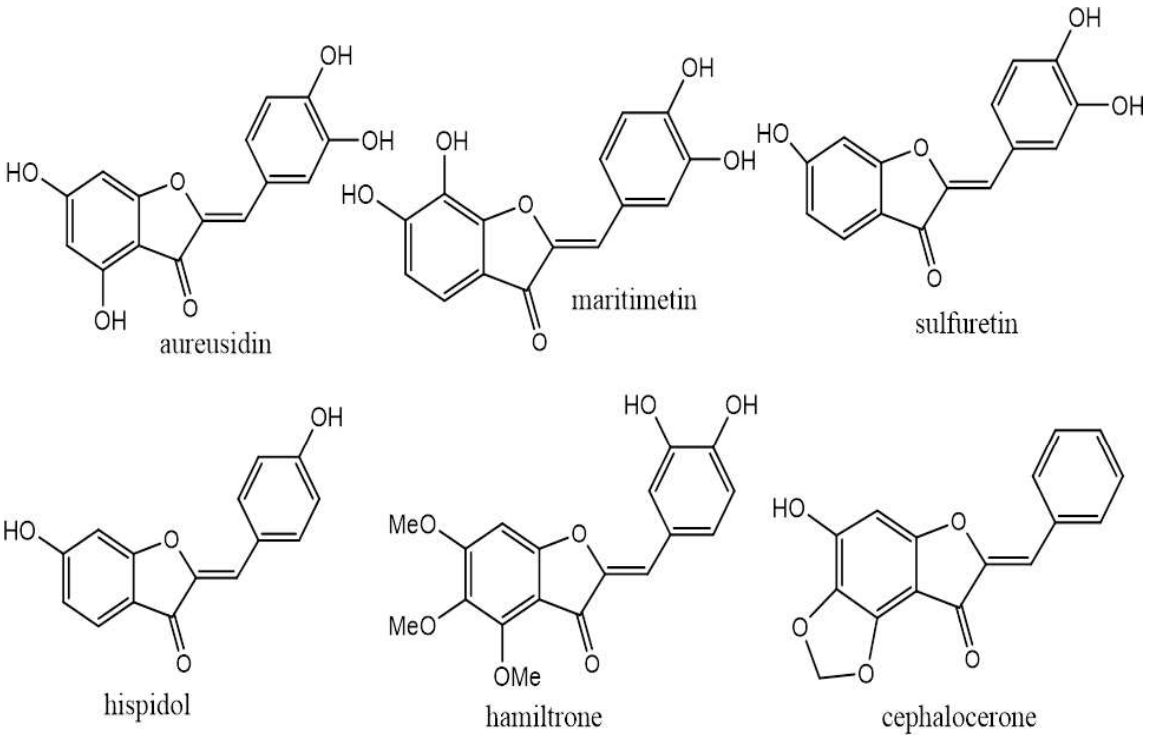


Figure 5: Structures of natural aurones.

The synthesis of aurones includes two common methods: i) Aldol-LikeCondensation: includes an aldol-like condensation of benzofuran-3(2H)-ones with benzaldehydes, which has been described in various studies (Varma and Varma, 1992; Venkateswarlu et al., 2004; Hawkins and Handy, 2013), and ii) Oxidative Cyclization: which is the oxidative cyclization of 2'-hydroxychalcones, as reported in studies like (Venkateswarlu et al., 2004; Moussouni et al., 2010).

In recent years, additional methods based on palladium, rhodium, copper, and cesium-catalyzed cyclization have been developed (Sui et al., 2021). In 2013, Liu and

colleagues introduced a novel approach for preparing aurone derivatives in good to high yields (Liu et al., 2013). They employed an intramolecular 5-exo cyclization of 2- alkynoylphenol using PBu3 in place of a transition metal catalyst (Figure 6 and Figure 7).This method offered an alternative way to synthesize aurones (Huang et al., 2007; Shin etal., 2011; Haudecoeur and Boumendjel, 2012).

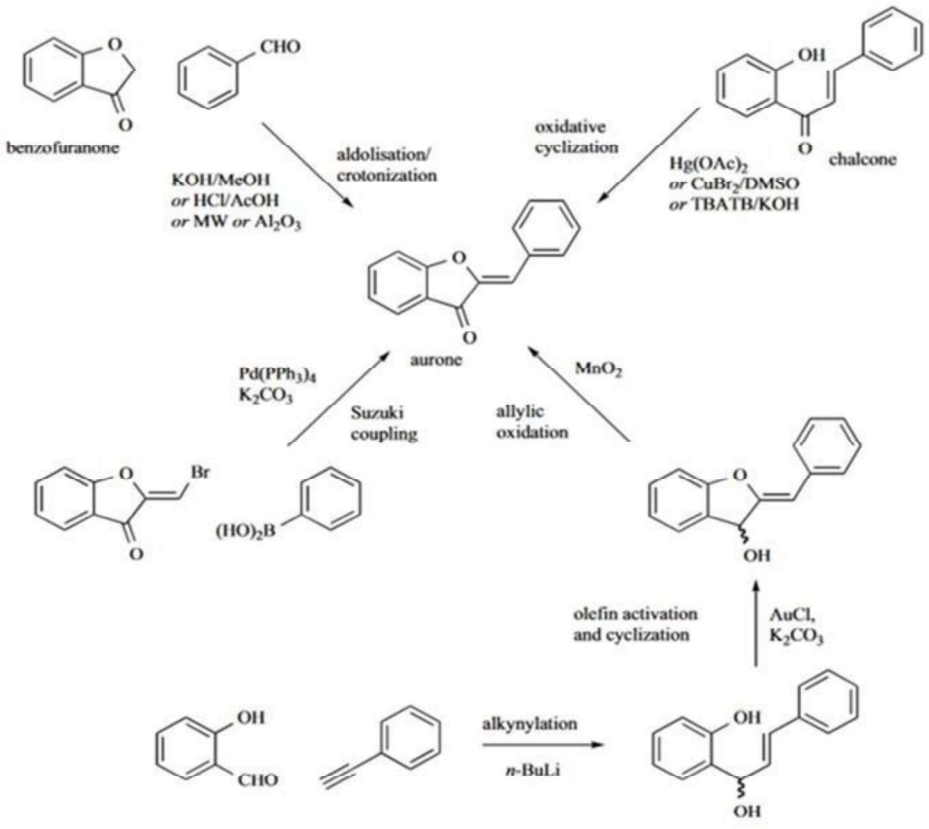
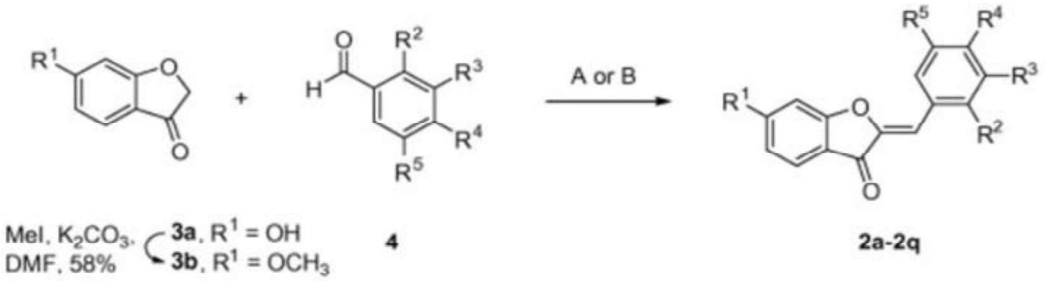


Figure 6: Most common methods for aurone synthesis (Haudecoeur and Boumendjel, 2012).



Compounds	R_1	R_2	R_3	R_4	R_5
2a	OH	OH	H	H	H
2c	OH	H	H	OH	H
2g	OH	H	OCH3	H	H
2j	OH	H	OCH3	H	OCH3
2m	OCH3	H	H	OH	H
2o	OCH3	H	OH	OH	H
2h	OH	H	H	OCH3	H

Figure 7: Aurone synthesis methods: (A): 12N HCl, ethanol, 60–70°C; (B) 50% KOH,methanol, 60°C (Shin et al., 2011).

Both natural aurones and their synthetic analogs have displayed a diverse range of biological activities, encompassing anticancer (e.g., maritimetin), antioxidant (e.g., aureusidin), antidiabetic (e.g., sulfuretin), and antifungal (e.g., hispidol) actions (Sui et al.,2021). These compounds have also inhibited the production of NO through the suppressionof nitric oxide synthase, cyclooxygenase-2, and other crucial cytokines involved in the inflammatory cascade (Liu et al., 2018). For instance, aurone analogs, such as hydroxyphenyl **(79a-79c)**, phenyl **(79d)**, and heteroaryl **(79e-79f)**, have exhibited a significant dose-dependent inhibition of iNOS expression in LPS-stimulated RAW 264.7 macrophage cells (Wang et al., 2017; Sui et al., 2021). Figure 8 provides examples of

aurone analogs with inhibitory effects on NO production. Additionally, Shin et al. demonstrated the impact of synthetic sulfuretin derivatives on NO production and prostaglandin (PGE2) in LPS-stimulated RAW 264.7 macrophages, as illustrated in Figure8 (Shin et al., 2011). These findings underscore the potential therapeutic applications of aurones and their analogs in managing inflammatory and related conditions.

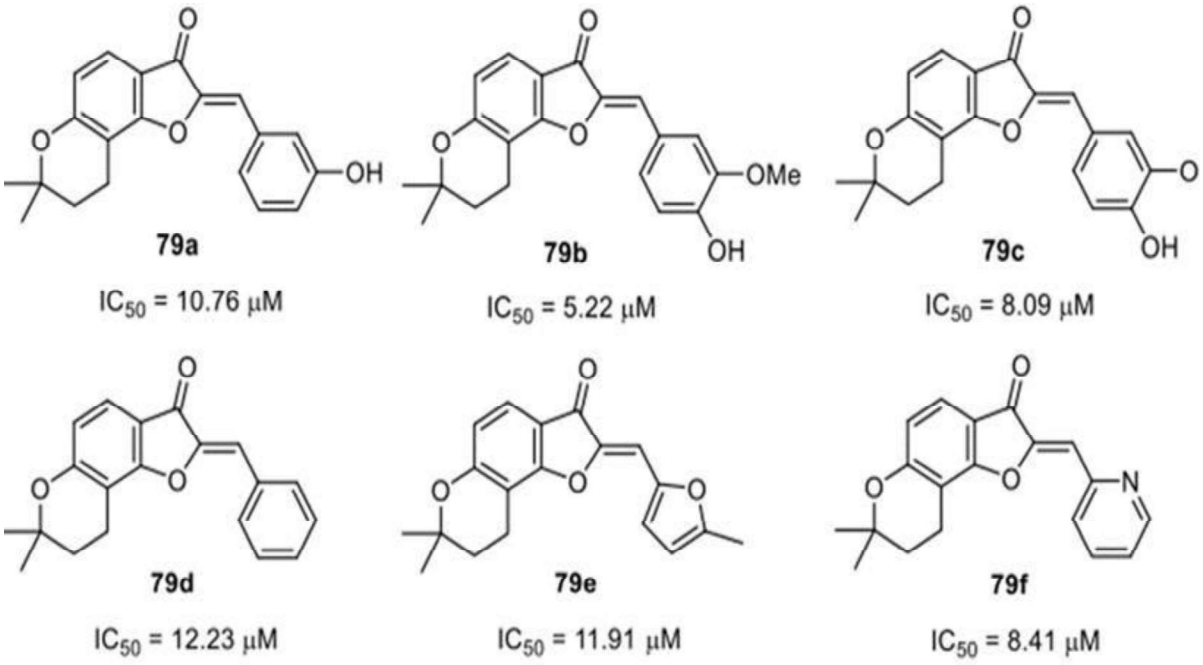


Figure 8: Dihydropyranoaurone structures with inhibitory effects on NO (Wang, 2017).

The structure of these compounds appears to play a significant role in their abilityto regulate the production of inflammatory mediators. For example, it has been observed

that compounds **2a, 2c**, and **2g-2j**, which contain a hydroxy substituent at the C-6 position, displayed more effective suppression of PGE2 formation compared to sulfuretin. Conversely, sulfuretin was less effective than C-6 methoxy-substituted aurones like **2m** and **2o** in inhibiting the generation of NO. Notably, in LPS-induced RAW 264.7 cells, compounds **2h-2j** and **2o** exhibited stronger inhibitory effects than sulfuretin on the production of both NO and PGE2, as summarized in Table 1 (Shin et al., 2011). This underscores how the specific structural features of these compounds can influence their impact on inflammatory mediator production.

Table 1: Inhibitory effect of aurones on the cytotoxicity, NO, and PGE2 generation in vitro in LPS-induced RAW 267.4 cells (Shin et al., 2011).

Compound	NO Production (μM)	PGE2(μM)	Cytotoxicity (%)
2a	46.29 ± 9.45	1.80 ± 0.12	131.40 ± 2.49
2c	38.70 ± 3.73	1.06 ± 0.13	99.79 ± 1.93
2g	23.38 ± 13.03	3.79 ± 0.22	32.85 ± 2.53
2j	22.64 ± 9.87	1.67 ± 0.09	55.11 ± 4.58
2m	10.53 ± 6.60	35.82 ± 2.23	115.47 ± 14.63
2o	9.30 ± 3.87	4.90 ± 0.38	77.43 ± 11.58
2h	23.51 ± 5.90	2.00 ± 0.11	74.84 ± 2.09

In a study conducted by Lee et al. (2012), the effects of sulfuretin were assessedboth in vitro on TNF-α-treated human rheumatoid fibroblast-like synoviocytes (FLS) and

in vivo on collagen-induced arthritis (CIA) mice. The results indicated that sulfuretin had a beneficial impact, as it reduced chemokine synthesis, matrix metalloproteinase release, and TNF-α-induced cell proliferation in rheumatoid FLS in vitro. Early treatment with sulfuretin also prevented joint degradation in CIA-affected mice, leading to a reduced cumulative disease incidence and the absence of disease characteristics, as measured by hind paw thickness, radiological and histopathological findings, and inflammatory cytokine levels. Notably, sulfuretin treatment significantly decreased synovial inflammation and joint damage in mice with developed arthritis. This protective effect of sulfuretin was attributed to the inhibition of the NF-κB signaling pathway, both in vitro and in vivo (Lee et al., 2012). In another study by Park et al. (2017), the synthetic aurone (z)-2-((5-(hydroxymethyl) furan-2-yl) methylene) benzofuran-3(2H)-one (9067) was found to inhibit the LPS-induced secretion of pro-inflammatory cytokines (TNF-α, IL-1β,and IL-8) in THP-1 cells, as depicted in Figure 9. This suggests its potential as an anti- inflammatory agent (Park et al., 2017).

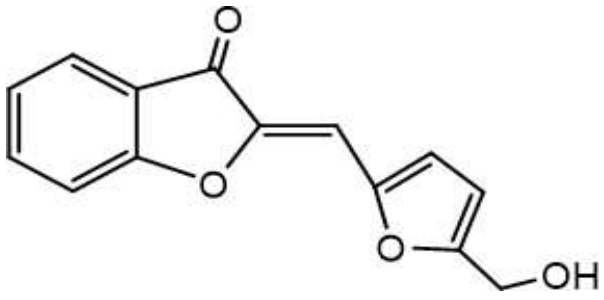


Figure 9: Structure of aurone 9067, (Z)-2-((5-(hydroxymethyl) furan-2-yl) methylene) benzofuran-3(2H)-one (Park et al., 2017).

The aurone 9067 was found to suppress IKK-β activation in the human THP-1 cellline, resulting in reduced p65 phosphorylation and Iκβα degradation. This, in turn, inhibited the translocation of p65 and the expression of the TNF-α gene transactivation in the RAW 264.7 cell line, as well as the expression of TNF-α itself, as demonstrated by qRT-PCR (Park et al., 2017).

Apart from modifying the phenyl and benzofuranone rings of the aurone system, there are related systems where the intracyclic oxygen is substituted with other heteroatoms. A common variation is the azaaurones, in which the oxygen in ring C of the aurone nucleus is replaced by a nitrogen atom. Azaaurones are isomeric analogs of auronesand result from this substitution, as shown in Figure 10 (Souard et al., 2010).

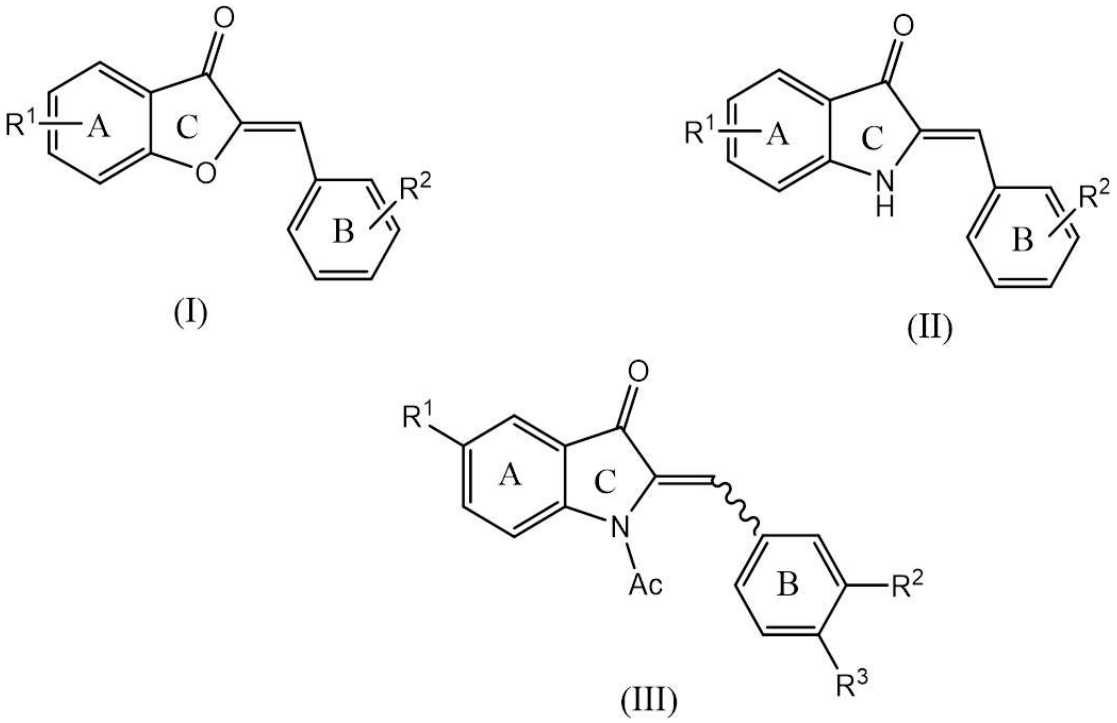


Figure 10: Chemical structure of an (I) aurone, (II) azaaurone B and (III) N-acetylazaaurone.

Several studies have highlighted the enhanced activity of azaaurones compared to their corresponding aurones, underscoring the benefits of replacing oxygen with nitrogen. For example, Tóth et al. (2020) described azaaurone derivatives that exhibit targeted toxicity against multi-drug resistant (MDR) cells, presenting a new class of MDR-selectiveanticancer drugs. Their investigation of the structure-activity relationship (SAR) revealed that replacing intracyclic oxygen with nitrogen in aurones leads to selective toxicity.

In addition, Zhang et al., 2018 reported the effectiveness of azaaurone derivatives against HepG2 and HeLa cancer cell lines. Azaaurones have also been explored for their

potential as anti-leishmanial, anti-plasmodial, and anti-bacterial agents, as documented in studies by (Souard et al., 2010; Soria-Carrasco et al., 2014; Carrasco et al., 2016).

Furthermore, (Campanico et al., 2019) demonstrated that azaaurones and their N- acetyl counterparts represent a new chemotype that can inhibit the growth of *Mycobacterium tuberculosis*. Notably, N-acetylazaaurones **10a, 10i**, and **10j** displayed excellent activity against MDR and extensively-drug resistant (XDR)-TB from clinical isolates, with Minimum Inhibitory Concentration (MIC)99 values in the sub-micromolar range. These findings are illustrated in Figure 11 and summarized in Table 2 (Campanico et al., 2019).

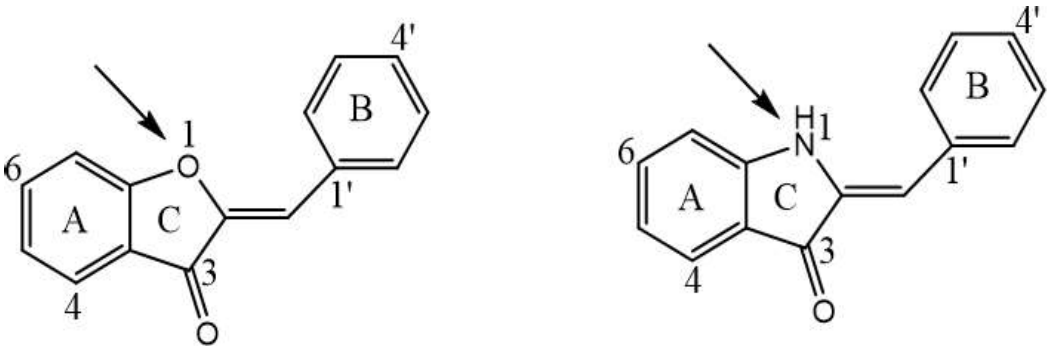
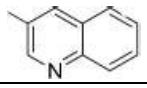
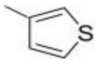


Figure 11: Chemical structure of aurone and 1-azaaurone. Arrows highlight the chemical substitution in ring C.

Table 2: The cytotoxicity and in vitro antimycobacterial activity of N-acetylazaaurone derivatives 10a, i and j. (Campanico et al., 2019).

Compounds	R^1	R^2	R^3	MIC_{99}
10a	H	H	Br	0.37
10i	H	H		0.736
10j	H	H		0.6

The pharmaceutical industry is currently facing a significant demand for the development of new drugs, particularly those with anti-inflammatory properties that can mitigate the adverse effects commonly associated with existing treatments, such as hepaticand renal toxicity and the risk of cardiovascular disease. The primary goal of this study was to investigate aurone analogs to pinpoint the essential structural components requiredfor anti-inflammatory activity. Specifically, the study centered on targeted aurone analog structures, chosen based on the isosteric substitution of the intracyclic oxygen with N-H (azaaurone), and the introduction of various substituents at the B-ring. Research efforts were dedicated to identification of azaaurones with anti-inflammatory properties. By establishing structure-activity relationships between these novel azaaurones and their anti-inflammatory effects, it becomes feasible to design more potent molecules with this therapeutic profile.

CHAPTER 2

EVALUATION OF TWENTY-SEVEN AZAAURONE DERIVATIVESAS POTENTIAL ANTI-INFLAMMATORY AGENTS

1. Introduction

Inflammation is a fundamental process essential for the clearance of infective agents and the response to other inflammatory triggers, playing a pivotal role in tissue repair in affected areas (Fioranelli et al., 2021; Aggarwal and Gehlot, 2009; Verri et al., 2012). In response to infection or tissue damage, non-specific innate immune cells, including macrophages, neutrophils, dendritic cells, mast cells, basophils, eosinophils, andnatural killer (NK) cells, are recruited (Marshall et al., 2018). This non-specific innate immune response is followed by signaling to the adaptive immune system, which then works to clear the infection and facilitate tissue repair. The regulation of inflammation is of paramount importance in the treatment of infectious diseases, autoimmune conditions, chronic ailments, and tissue injuries.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have traditionally been a popularchoice for reducing inflammation. However, the use of NSAIDs is associated with a rangeof side effects, including cardiovascular adverse effects, nephrotoxicity, and gastrointestinal toxicity (Fracasso et al., 1992; Heerdink et al., 1998; Nagappan et al., 2014;Wongrakpanich et al., 2018). While monoclonal antibodies targeting molecules like TNF-α, CD20, or the IL-6 receptor have significantly improved the prognosis of patients with inflammatory disorders, particularly rheumatoid arthritis, these treatments also come withan increased risk of serious infections (Navarro-Millán and Curtis, 2013; J. Li et al., 2021).

Therefore, despite advancements in the management of inflammation, there remains a pressing need for the development of safe and effective therapeutic interventions with minimal adverse side effects.

Natural products have long been a cornerstone of medical therapy for various ailments (Atanasov et al., 2021). Notably, between 1981 and 2010, the U.S. Food and DrugAdministration (FDA) approved a range of anti-inflammatory medications, and a significant portion of these drugs comprised naturally derived compounds and theirderivatives, accounting for 34% of the approved anti-inflammatory drugs (Newman and Cragg, 2012). The vast reservoir of natural products includes more than 7000 flavonoids, which have been extensively investigated and found to possess a plethora of beneficial properties. These properties encompass anti-viral, anti-inflammatory, anti-mutagenic, anti-allergic, anti-diabetic, anti-cancer, antioxidant, anti-aging, and cardio-protective activities(Galati and O'Brien, 2004; Thilakarathna and Rupasinghe, 2013; Yao et al., 2004).

Flavonoids are phytochemical compounds classified as aromatic polyphenols, primarily characterized by their core structure, 2-phenylchromen-4-one (also referred to as 2-phenyl-1-benzopyran-4-one) (Marais et al., 2006). These compounds possess a relatively low molecular weight and are composed of a C6-C3-C6 ring system (Figure 1).The bioactivity of flavonoids is closely connected to their structural conformation, with specific attention to features such as the carbonyl group, which is associated with their inflammatory properties (De Souza Farias et al., 2021).

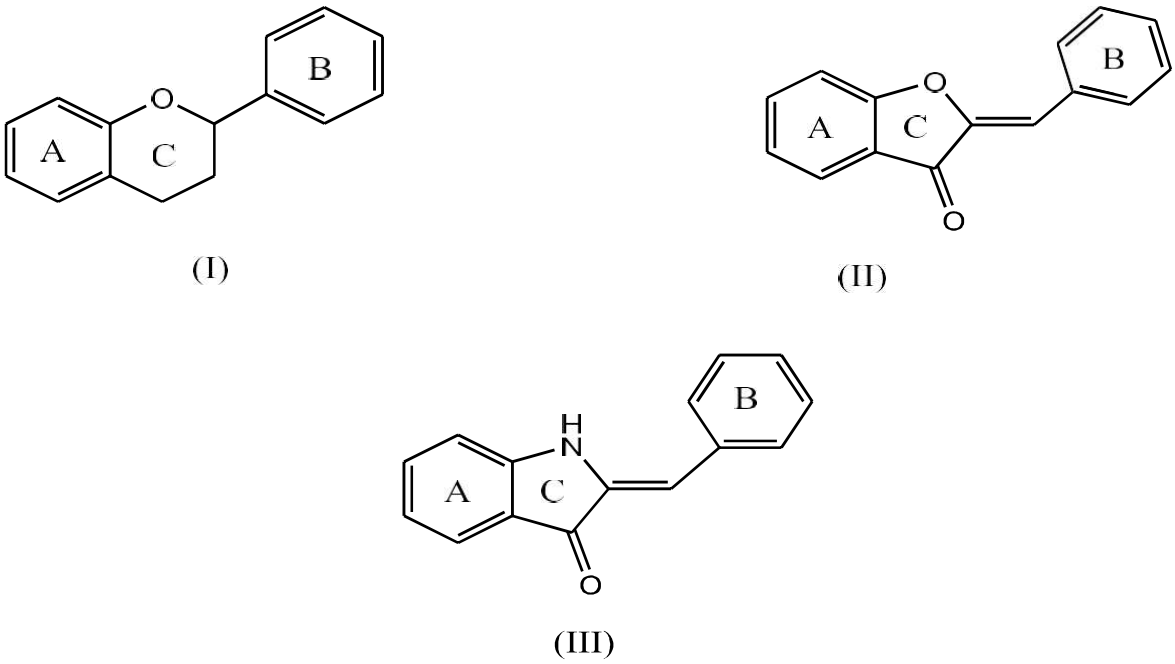


Figure 12: Structures of common flavonoids.

I. Chemical structure of the most common flavonoids, characterized by a fifteen-carbon skeletal structure consisting of two benzene rings (A and B) attached to a heterocyclic pyrene ring (C).
II. Chemical structure of aurones, a group of flavonoids with a distinct structure.
III. Chemical structure of azaaurones, which feature the replacement of an oxygen atom with an N-H group, creating a variation in the flavonoid structure).

The bioactivity of flavonoids is intricately linked to their structural conformation. For instance, the inflammatory properties associated with flavonoids are often contingent on the presence and position of glycosylated hydroxyl groups, with the carbonyl group located on C-4 playing a significant role (Lago et al., 2014; Theoharides et al., 2001). Moreover, flavonoids possessing specific structural features, including the C-ring 2,3 double bond, A-ring 5,7 hydroxyl groups, and B-ring 4'- or 3',4'-hydroxyl groups, such asapigenin and quercetin, have demonstrated the capacity to inhibit the expression of key proinflammatory enzymes and cytokines (Kim et al., 2004).

The simultaneous presence of hydroxy and methoxy groups in position 6 of both Aand B rings of flavonoids plays a crucial role in achieving potent anti-inflammatory effects.This combination is instrumental in maximizing effectiveness while minimizing potentialtoxicity. These effects are attributed to the electron-donating properties of hydroxy and methoxy groups on both the A and B rings, as supported by studies (Moon et al., 2015; Wang et al., 2017; Mazziotti et al., 2022).

Aurones, a subgroup of flavonoids responsible for the golden-yellow pigment in certain flowers within the Antirrhinum (Scrophulariaceae) and *Cosmos bipinnatus* (Asteraceae) families, have exhibited a range of beneficial properties, including anticancer,antimicrobial, and anti-inflammatory effects (Bandgar et al., 2010; D. Yang et al., 2020). The creation of synthetic aurones, alongside natural derivatives, underscores the importance of diverse substitution patterns in achieving meaningful efficacy. Among thesederivatives, azaaurones, characterized by the substitution of an N-H group for the intracyclic oxygen of aurones, have garnered attention (Campanico et al., 2019; Kumar etal., 2020).

Previous research has unveiled the significant anti-malarial, anti-leishmanial, anti-bacterial, and selective anti-cancer properties of azaaurones, potentially attributable to the nitrogen molecule substitution (Campanico et al., 2019; Carrasco et al., 2016; Souard et al., 2010; Tóth et al., 2020). However, the anti-inflammatory potential of azaaurones remains unexplored. In the present study, we screened 27 azaaurones, identifying five compounds with low cytotoxicity. We investigated their anti-inflammatory properties

using murine macrophage-like RAW 264.7 cell lines, aiming to assess their suitability as anti-inflammatory agents.

2. Material and methods

2.1. Reagents

The RAW 264.7 (ATCC TIB 71) cell line was procured from the American Type Culture Collection (Manassas, VA, USA). Various reagents and materials were obtained from specific sources: lipopolysaccharide (LPS; Salmonella enterica serotype typhimurium), dexamethasone, dimethyl sulfoxide (DMSO), Dulbecco's modified eagle medium (DMEM), and Griess reagent (modified) were sourced from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin/streptomycin, and Gibco™ Trypsin- EDTA (0.25-0.05%) were acquired from Fisher Scientific (Pittsburgh, PA, USA). The alamarBlue® reagent was purchased from Invitrogen Thermo Fisher Scientific (Waltham,MA, USA). ELISA kits and associated reagents were obtained from R&D Systems(Minneapolis, MN, USA).

2.2. Synthesis and characterization of azaaurone derivatives

The acetylated azaaurone (AAA) compounds chosen for this study were selected to investigate two primary aspects: substitutions on the exocyclic aryl ring and the significance of the acetyl group attached to the nitrogen. By replacing the intracyclic oxygen atom with its isosteric equivalent N-H group and introducing various substituents to the B-ring, several active analogs could be generated. The variations made in the aryl ring of azaaurones encompassed changes in size, position, polarization, electron- withdrawing or donating characteristics, and hydrogen-bonding properties of these groups.

The substitutions included halogen, methyl, nitro, cyano, hydroxyl, and methoxy groups. These compounds could be easily deacetylated by using methanolic sodium hydroxide or aqueous ammonia in tetrahydrofuran to yield azaaurones (AA). Interestingly, in the case of the reaction involving pyridine-2-carboxaldehyde, the condensation process directly yielded the azaaurone compound rather than the acetylated product. This synthesis approach allowed for the creation of a diverse set of azaaurone analogs.

2.3. Maintenance and Differentiation of the RAW264.7 cell line

RAW 264.7 cells were maintained in DMEM medium supplemented with 10% heat- inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. These cells were maintained at 37°C in an atmosphere with 5% CO_2. Trypsin-EDTA was employed to detach the cells, and their concentration was adjusted to $5x10^5$ cells/mL. The cells were then seeded into 96-well plates (Denville/Thomas Scientific, Swedesboro, NJ, USA) and incubated for 12-16 hours or until they reached approximately 90% confluence. To removeundifferentiated cells, the monolayers were washed with phosphate-buffered saline (PBS)before each assay.

2.4. AlmarBlue® Cell Viability Assay

In this experimental procedure, a 10 mM stock solution of azaaurone derivatives in DMSOwas prepared and subsequently diluted in culture medium to create five serial dilutions with concentrations of 10 µM, 5 µM, 2.5 µM, 1.25 µM, and 0.62 µM. RAW-264.7 cells were used when they reached a confluence of 60-80%. These cells were plated in microplates and pretreated with above mentioned concentrations of azaaurone derivatives or 15 µM of dexamethasone for 1 hour. After this pretreatment, the cells were stimulated

with 1 µg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with5% CO_2. As a positive control, dexamethasone, a synthetic glucocorticoid, was used. Dexamethasone is known to inhibit cytokine gene transcription by repressing the activationof nuclear factor-κB (NF-κB) (Morgan and Davis 2017).

Both untreated cells and DMSO (0.1%) were tested as negative controls. Intriplicate, the culture medium was replaced with the various serial dilutions of the differentazaaurone derivatives and incubated for 24 hours. Freshly prepared culture medium was mixed with alamarBlue® reagent (10%) by gentle shaking, and then 200 µL of this mixturewas added to each well of the microplate. The microplate was further incubated for 4 hoursat 37°C in the dark.

To assess cell viability, measurements were taken in relative fluorescent units (RFU) using SpectraMax M2e (Molecular Devices Inc., Sunnyvale, CA, USA) or Clario Star microplate readers (BMG Labtech Inc, Weston Parkway, NC, USA) at an excitation wavelength of 570 nm and an emission wavelength of 590 nm. The calculation for percent(%) viability was determined using the following equation:

% Viability = (RFU of dilution / RFU of control) x 100.

2.5. Anti-inflammatory Activity by Reactive Nitrogen Species (RNS) Assay

To assess the anti-inflammatory activity related to the generation of Reactive Nitrogen Species (RNS), the measurement of nitric oxide (NO) production in RAW-264.7 cells at 90% confluence treated with azaaurones was conducted, following a previously establishedprotocol (Shin et al., 2011; Park et al., 2017).

The quantification of NO levels in the cell supernatants was performed using a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, USA) and following the manufacturer's instructions. This assay is commonly used to estimate the levels of nitrate and nitrite, which are indicative of NO production and, by extension, the potential anti-inflammatory effects of the tested compounds.

2.6. IC_{50} Evaluation

Cells were treated with two-fold dilutions of azaaurone analogs (10 μM, 5 μM, 2.5 μM, M, 0.625 μM) for a 24-hour duration. Following this treatment, cell viability was evaluated using the alarmarBlue® assay. The obtained data, representing the response of the cells to different compound concentrations, were then used to generate a dose-responsecurve. To model the dose-response relationship and determine the IC50 values (the concentration at which 50% inhibition is achieved), a Four-Parameter Logistic model wasemployed. Non-linear regression analysis, specifically comparing inhibition to the logarithm of the compound concentration, was conducted using GraphPad Prism 9.0 software by GraphPad Software Inc. (Boston, MA, USA).

2.7. Statistical Analyses

The obtained results were subjected to statistical analysis using GraphPad Prism, version 9.0, developed by GraphPad Software Inc. (Boston, MA, USA). To assess the impact of treatment, the treated groups were compared to the control groups. The results are presented as mean values accompanied by the standard error of the mean (SEM) to providea measure of the data precision and variation. The experiments were conducted in triplicateand repeated three times to ensure the reliability of the findings. For comparing groups,

statistical tests such as unpaired t-tests and one-way analysis of variance (ANOVA) were applied. A significance level of $P < 0.05$ was used as the threshold to determine whether observed differences were statistically significant. Results with a P-value below 0.05 were considered as having significant differences.

3. Results

3.1. Azaaurone derivative synthesis and characterization

The compounds chosen for this study were selected to investigate two primary aspects: 1) substitutions on the exocyclic aryl ring and 2) the significance of the acetyl group attachedto the nitrogen. Souard et al. (2010) noted that by replacing the intracyclic oxygen atom with its isosteric equivalent N-H group and introducing various substituents to the B-ring,several active analogs could be generated (Souard et al., 2010).

The variations made in the aryl ring of azaaurones encompassed changes in size, position, polarization, electron-withdrawing or donating characteristics, and hydrogen- bonding properties of these groups. The substitutions included halogen, methyl, nitro, cyano, hydroxyl, and methoxy groups. Additionally, a few heteroaromatic compounds were included in the study. In total, 27 azaaurone analogs were synthesized following a standard methodology, commencing with a piperidine-catalyzed condensation reaction between an acetylated oxyindole and an aldehyde to produce acetyl azaaurones (AAA) (Figure 13) (Carrasco et al., 2016; Boumendjel, 2003).

These compounds could be easily deacetylated by using methanolic sodium hydroxide or aqueous ammonia in tetrahydrofuran to yield azaaurones (AA). Interestingly,in the case of the reaction involving pyridine-2-carboxaldehyde, the condensation process

directly yielded the azaaurone compound rather than the acetylated product. This synthesis approach allowed for the creation of a diverse set of azaaurone analogs, which were subsequently evaluated for their potential biological activities, as described in the study.

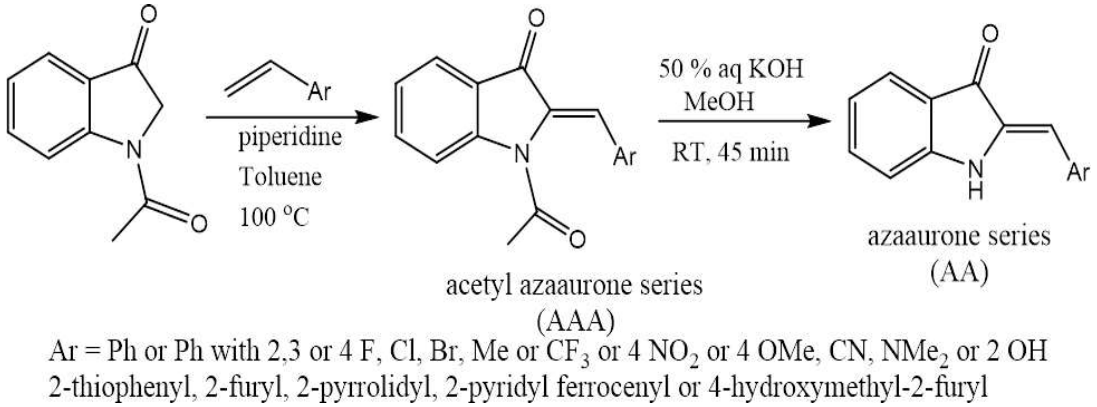


Figure 13: Schematic diagram of azaaurone derivative synthesis.

3.2.Cytotoxicity of azaaurone analogs on murine macrophage-like RAW 264.7 cells

Before assessing the impact of azaaurone analogs on the production of pro-inflammatory mediators, the study initially conducted cell viability assessments using alamarBlue®. Alamar Blue was chosen as the cell viability assay due to its high sensitivity, non-toxic nature, non-radioactive properties, and compatibility with various cell models (Rampersad, 2012). It is important to confirm cell viability to ensure that any observed inflammatory response is not solely attributed to cell death caused by the compounds.

In the primary screening of the 27 synthesized azaaurones, it was observed that 17 derivatives (Figure 14; AAAPh, AAA2F, AAA2OH, AAA2Me, AAA3F, AAA3Br, AAA3Me, AAA4Cl, AAA4Br, AAA4Me, AAA4CF3, AAA4CN, AAA4NMe2, AAAthio, AAAfuryl, AAApyrro, and AAAferro) induced less than 10% cell death in RAW 264.7 cells (Figures 17-18). Further viability assays were conducted for all 27 derivatives at both10 μM and 100 μM concentrations in the presence of LPS. However, it was observed thatRAW 264.7 cells treated with azaaurone derivatives at 100 μM in the presence of LPS showed less than 90% viability. Therefore, the 10 μM concentration was chosen as the highest concentration for subsequent assays in RAW 264.7 cells.

Pretreatment of cells with the deacetylated azaaurones AAPh, AA2Me, AA3Me, and AA3Br (Figure 15) at 100 μM, followed by 1μg/mL LPS, had a toxic effect on cell viability compared to the LPS control (Figure 19). Nevertheless, pretreatment of cells withthe same compounds at 10 μM had no significant effect relative to the LPS control. RAW 264.7 cells pretreated with both 100 and 10 μM concentrations of aurones Ph and 3Me in the presence of LPS resulted in less than 10% cell death (Figure 20), while treatment withboth 100 and 10 μM concentrations of the aurone 3Br led to more than 90% cell death in RAW 264.7 cells (structure of the parent aurones shown in Figure 16). It is important to note that DMSO (used as a vehicle control) showed no effect on viability after 4 hours of treatment on RAW 264.7 cells. These findings demonstrate the importance of selecting appropriate compound concentrations to ensure that cell viability is maintained during theassays.

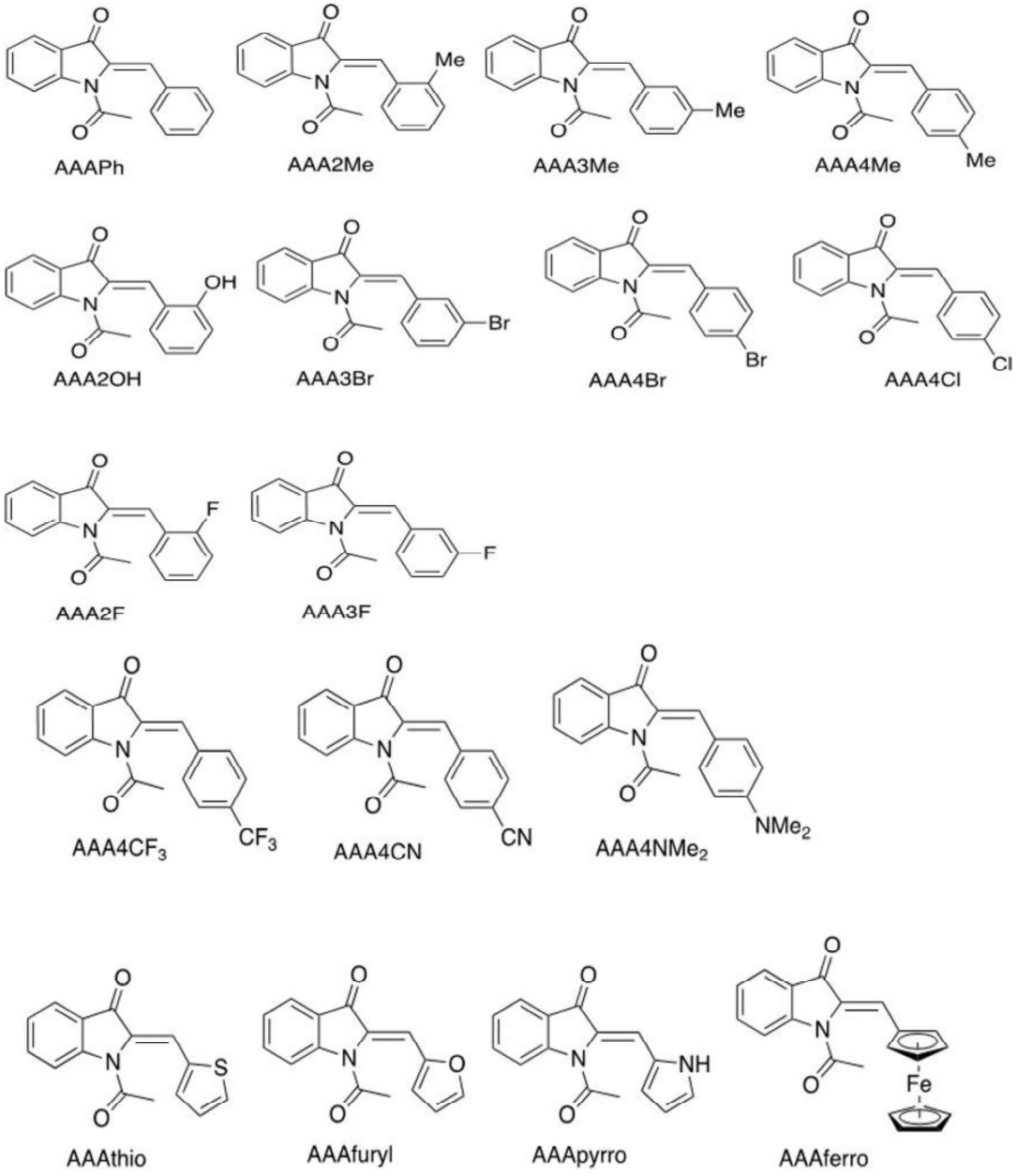


Figure 14: The structure of 17 acetylated azaaurone derivatives chosen for this studyfollowing cell viability testing.

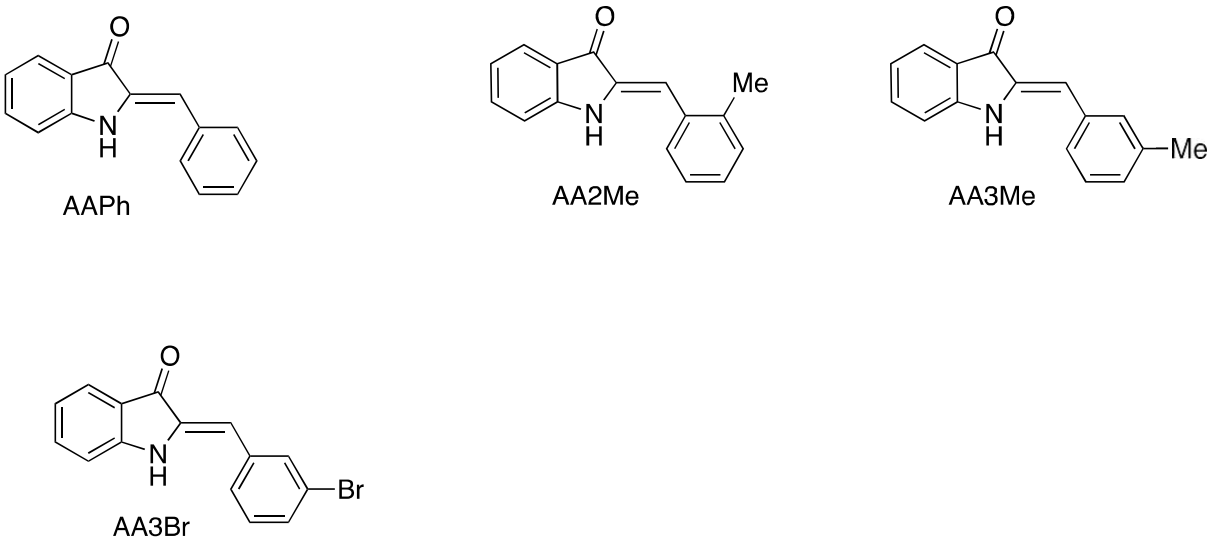


Figure 15: The structure of four deacetylated azaaurone derivatives.

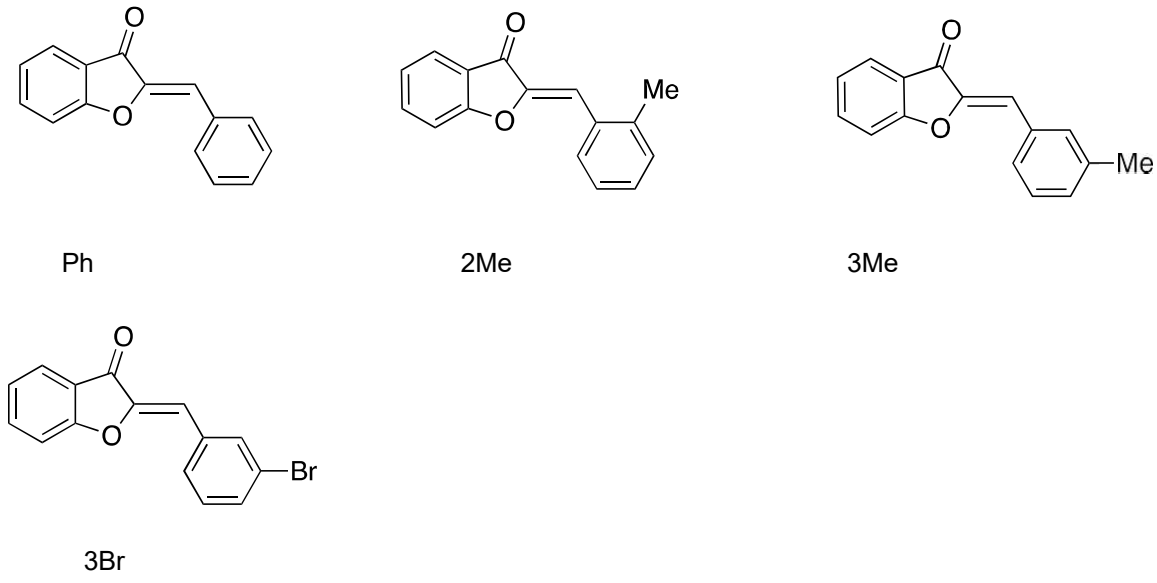


Figure 16: The structure of four parent aurone derivatives.

Table 3: Cell viability values after treating with the selected azaaurones derivatives.

Compound	Cell Viability (%)	
	100 μM	10 μM
AAAPh	-	105.3±3.259
AAA2Me	-	93.43±1.77
AAA3Me	-	90.74±0.918
AAA3Br	-	92.70±1.048
AAPh	-	71.3±6.740
AA2Me	-	46.1±4.282
AA3Me	-	104.9±1.150
AA3Br	-	113.5±4.854
Ph	121.2±3.191	121.6±3.260
2Me	84.8±2.606	103.5±0.5941
3Me	114.5±3.602	120.6±5.131
3Br	-	-

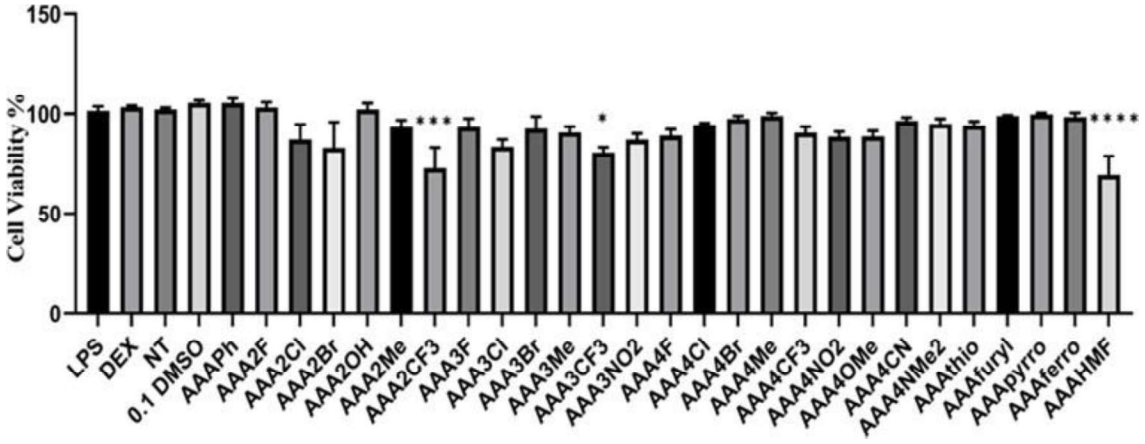


Figure 17: Cytotoxicity of 27 azaaurones for RAW 264.7 macrophages.

RAW 264.7 cells were pretreated with 10 μM of azaaurone derivatives or 15 μM of dexamethasone (DEX) for 1 hour. After this pretreatment, the cells were stimulated with 1 μg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with 5% CO_2. DMSO (0.1%) was the vehicle control and NT was the no treatment control.Treatment groups were all tested for relative viability using the Alamar Blue assay. The results are reported as the mean ± SEM as derived from a minimum of three independent experiments, each performed in triplicate, totaling n=9 data points for each condition. Statistical significance is indicated as follows: *P < 0.05, ***P < 0.001, ****P < 0.0001. These significance levels represent comparisons with untreated cells (NT control group).

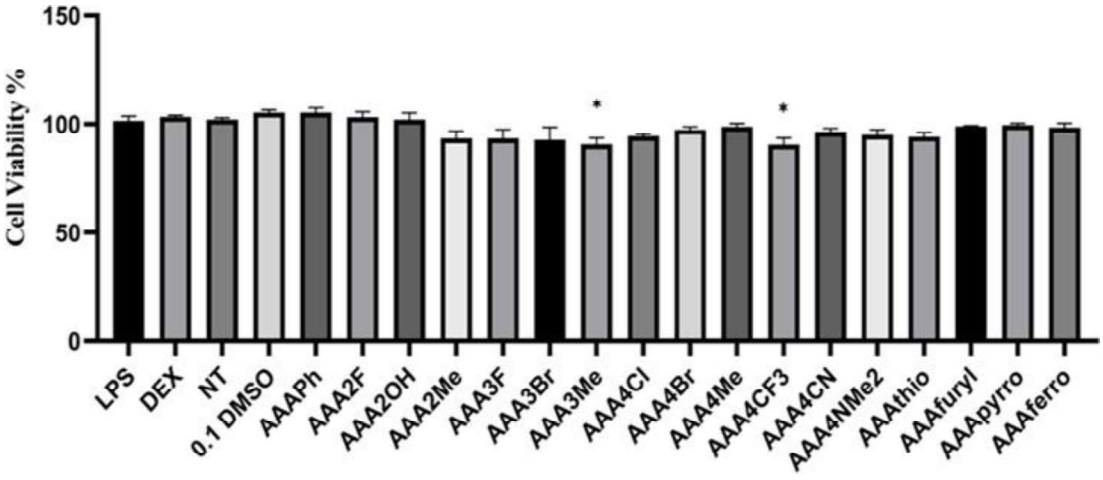


Figure 18: Cytotoxicity of 17 azaaurones for RAW 264.7 macrophages.

RAW 264.7 cells were pretreated with 10 µM of azaaurone derivatives or 15 µM of DEX for 1 hour. After this pretreatment, the cells were stimulated with 1 µg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with 5% CO_2. DMSO (0.1%) was the vehicle control and NT was the no treatment control. Treatment groups were all tested for relative viability using the Alamar Blue assay. The results are reported as the mean ± SEM derived from a minimum of three independent experiments, each performed in triplicate, totaling n=9 data points for each condition. Statistical significance is indicated as follows: *P < 0.05. These significance levels represent comparisons with untreated cells(NT control group).

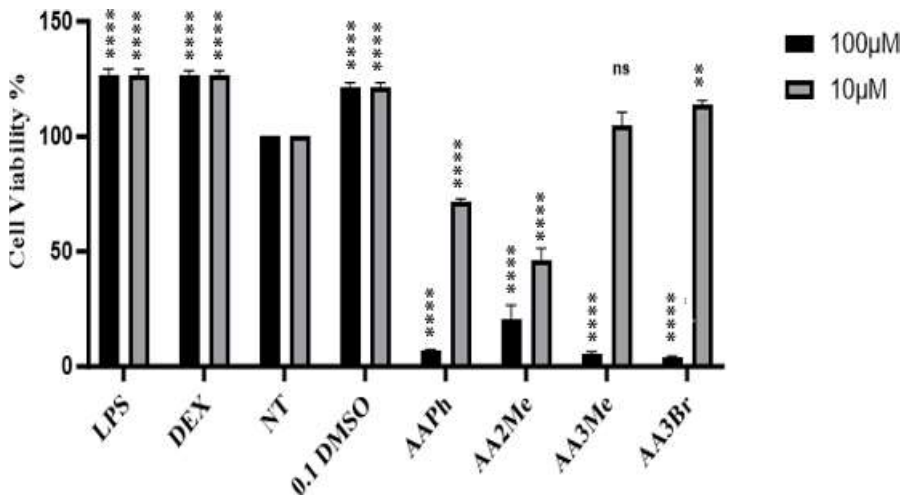


Figure 19: Cytotoxicity of deacetylated azaaurones for RAW 264.7 macrophages.

RAW 264.7 cells were pretreated with 100 and 10 μM of deacetylated azaaurone derivatives or 15 μM of DEX for 1 hour. After this pretreatment, the cells were stimulatedwith 1 μg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with5% CO_2. DMSO (0.1%) was the vehicle control and NT was the no treatment control. Treatment groups were all tested for relative viability using the Alamar Blue assay. The results are reported as the mean ± SEM derived from a minimum of three independent experiments, each performed in triplicate, totaling n=9 data points for each condition. Statistical significance is indicated as follows: **P < 0.01, ****P < 0.0001. Thesesignificance levels represent comparisons with untreated cells (NT control group).

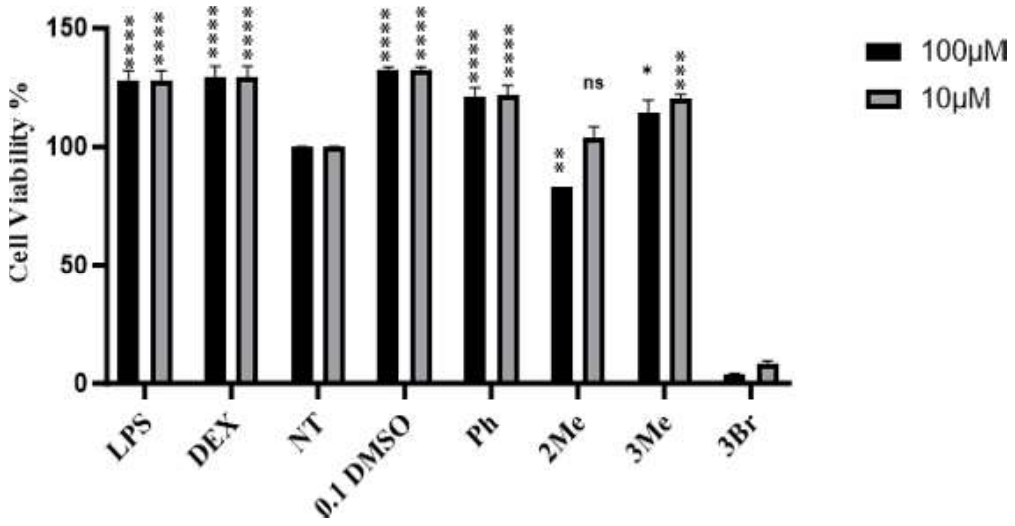


Figure 20: Cytotoxicity of aurones for RAW 264.7 macrophages.

RAW 264.7 cells were pretreated with 100 and 10 µM of aurones derivatives or 15 µM ofDEX for 1 hour. After this pretreatment, the cells were stimulated with 1 µg/mL of bacterialLPS and incubated for 24 hours at 37°C in an environment with 5% CO_2. DMSO (0.1%) was the vehicle control and NT was the no treatment control. Treatment groups were all tested for relative viability using the Alamar Blue assay. The results are reported as the mean ± SEM derived from a minimum of three independent experiments, each performedin triplicate, totaling n=9 data points for each condition. Statistical significance is indicatedas follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. These significance levelsrepresent comparisons with untreated cells (NT control group).

3.3.Azaaurone Analogs Significantly Suppress the NO Response in LPS-induced RAW 264.7 Cells

The effects of various compounds, including azaaurone derivatives (AAAPh, AAA2Me, AAA3Me, AAA3Br), deacetylated derivatives (AAPh, AA2Me, AA3Me, AA3Br), and parent aurones (Ph, 2Me, 3Me, 3Br), on nitric oxide (NO) production were investigated. The azaaurone derivatives (AAAPh, AAA2Me, AAA3Me, and AAA3Br) exhibited a notable dose-dependent reduction in NO production in LPS-stimulated RAW 264.7 cells, as illustrated in Figures 21-24.

It is noteworthy that the use of DMSO (negative control) had no significant impacton NO production in LPS-treated RAW 264.7 cells at all tested concentrations. In contrast, dexamethasone, utilized as a positive control, displayed significant inhibition of NO production. Specifically, dexamethasone achieved a 51.5% reduction in NO production ata concentration of 15 μM, consistent with prior reports (Park et al., 2016), as depicted in Figures 21A, 22A, 23A, and 24A. Furthermore, dexamethasone was associated with 38.3%and 34.8% inhibition of NO production at a concentration of 15 μM, as shown in (Figures22-24). These dexamethasone results serve as a comparison to highlight the potential of the tested azaaurone derivatives to also effectively modulate NO production in LPS- stimulated cells, signifying their potential anti-inflammatory properties.

Cells treated with the acetylated azaaurone (AAAPh) significantly suppressed LPS-induced NO production at concentrations ranging from 10 μM to 0.625 μM. The inhibition reached 62.4% ($P \leq 0.001$ or $P < 0.0001$) with an IC_{50} value of 1.422 μM (Figures 21A and21B; Table 4). However, the deacetylated azaaurone (AAPh) significantly inhibited LPS- induced NO production at concentrations from 5 μM to 0.625 μM by 40.8% ($P < 0.0001$) (Figure 25). The parent analog (Ph aurone) significantly suppressed LPS-induced NO production at the concentration of 100 μM ($P < 0.0001$) (Figure 26).

The methylated azaaurone (AAA2Me) significantly suppressed LPS-induced NO production at concentrations of 10 μM, 5 μM, and 2.5 μM, resulting in 64.9% inhibition ($P < 0.01$ and $P < 0.001$), similar to AAAPh, but with an increased IC_{50} value of 2.092 μM (Figures 22A and 22B; Table 4). In contrast, NO production was suppressed by only 48.9%in the presence of the deacetylated AA2Me at concentrations from 5 μM to 0.625 μM ($P <$

0.0001) (Figure 25). The 2Me parent aurone did not exhibit inhibitory effects on LPS- induced NO production (Figure 26).

The azaaurone (AAA3Me) significantly inhibited NO production by 49.5% in LPS-induced RAW 264.7 cells at concentrations ranging from 10 μM to 0.625 μM ($P< 0.0001$)with an IC_{50} of 1.856 μM (Figures 23A and 23B; Table 4). Moreover, the deacetylated azaaurone (AA3Me) also similarly significantly suppressed NO production by 46.6% ($P<0.0001$) (Figure 27). No effect was observed with the 3Me parent aurone (Figure 26).

Finally, the azaaurone (AAA3Br) significantly suppressed NO production at concentrations of 10 μM and 5 μM by 64.5% ($P< 0.0001$). The IC_{50} value was calculated as 3.936 μM for AAA3Br (Figures 24A and 24B; Table 4). Furthermore, the deacetylated azaaurone (AA3Br) inhibited NO production by 63.7% ($P< 0.0001$) (Figure 27). The parent aurone 3Br significantly suppressed NO production at only the 1.25 μM and 0.625 μM concentrations. These results demonstrate the differential effects of the tested azaaurone derivatives; both acetylated and deacetylated, as well as parent aurones on LPS-induced NO production in RAW 264.7 cells. The data highlight the potential of these azaaurone compounds to modulate NO production, which is indicative of their anti-inflammatory properties.

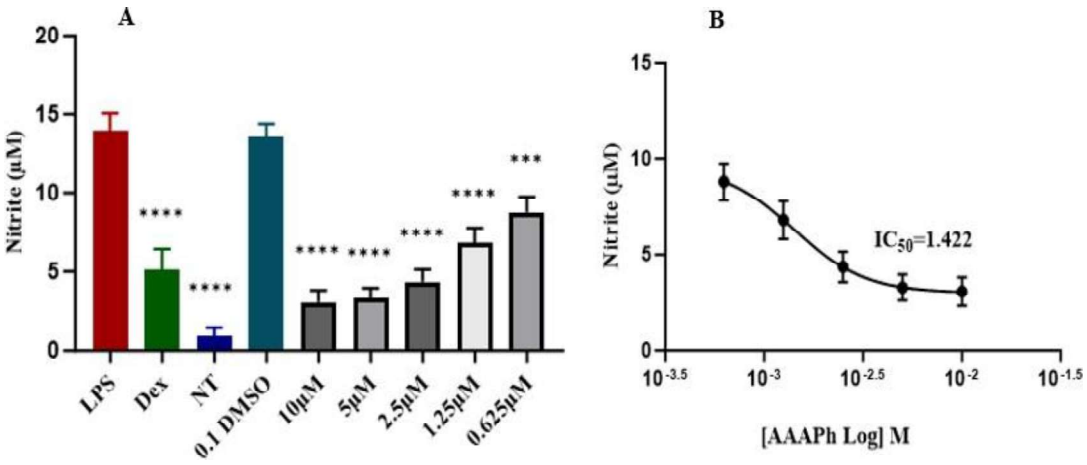


Figure 21: Azaaurone AAAPh inhibits NO production.

RAW 264.7 cells were pretreated with 10 µM of azaaurone derivatives or 15 µM of DEX for 1 hour. After this pretreatment, the cells were stimulated with 1 µg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with 5% CO_2. DMSO (0.1%) was the vehicle control and NT was the no treatment control. (A) NO production in RAW 264.7 cells was measured by Griess assay. (B) The IC_{50} value of AAAPh was calculated from the dose-response curve as 1.422. The results are presented as the mean ± SEM from three independent experiments, each conducted in triplicate, for a total of n=9 data points for each condition. Significance is reported as follows: ***P ≤ 0.001, ****P < 0.0001. TheseSignificance levels indicate comparisons with the LPS-treated groups.

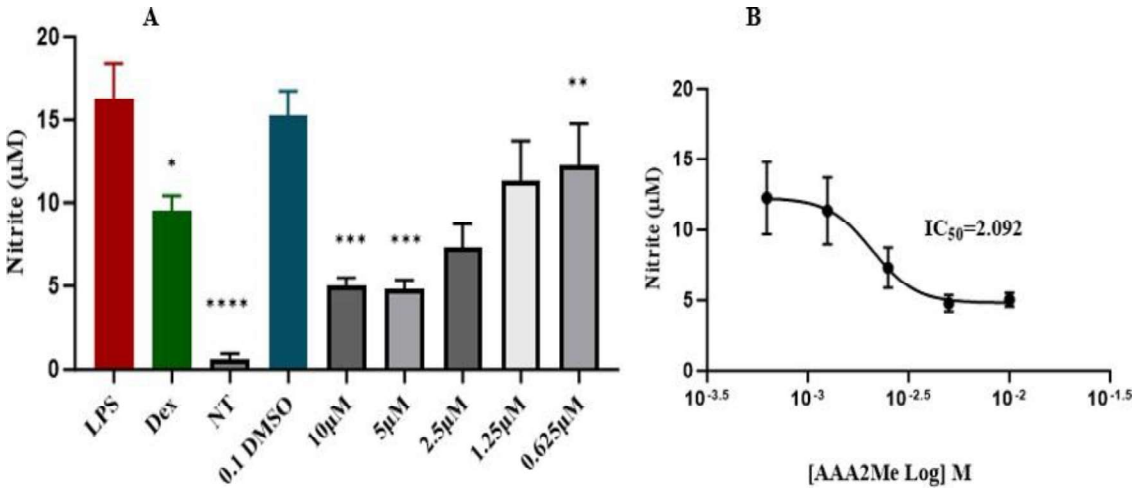


Figure 22: Azaaurone AAA2Me inhibits NO production.

RAW 264.7 cells were pretreated with 10 µM of azaaurone derivatives or 15 µM of DEXfor 1 hour. After this pretreatment, the cells were stimulated with 1 µg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with 5% CO_2. DMSO (0.1%) is the vehicle control and NT is the no treatment control. (A) NO production in RAW 264.7 cells was measured by Griess assay. The IC_{50} value of AAA2Me was calculated from the dose-response curve as 2.092. (B). The results are presented as the mean ± SEMfrom three independent experiments, each conducted in triplicate, for a total of n=9 data points for each condition. Significance is reported as follows: *P < 0.05, **P < 0.01, ***P ≤ 0.001, ****P < 0.0001. Significance levels indicate comparisons with the LPS-treated groups.

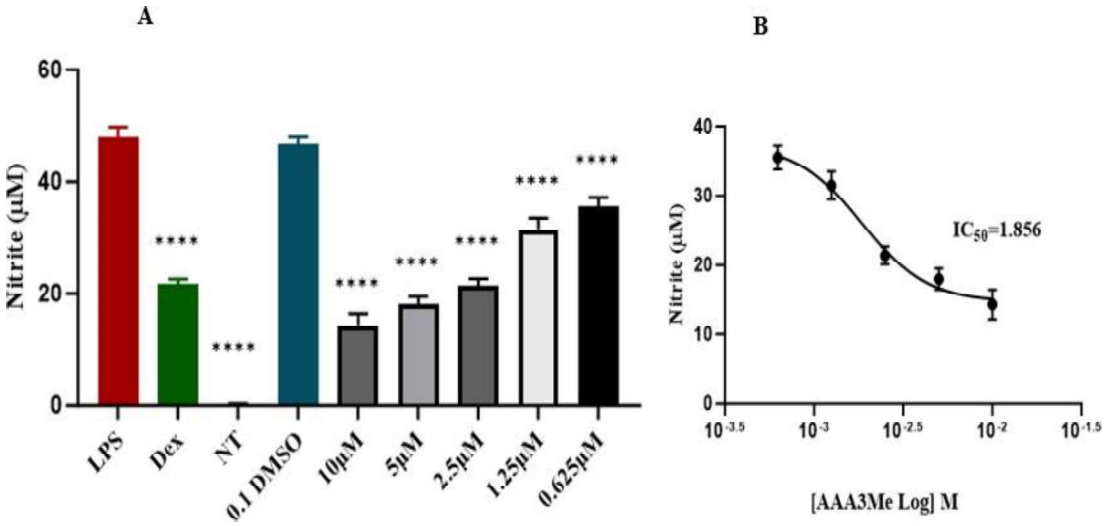


Figure 23: Azaaurone AAA3Me inhibits NO production.

RAW 264.7 cells were pretreated with 10 µM of azaaurone derivatives or 15 µM of DEX for 1 hour. After this pretreatment, the cells were stimulated with 1 µg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with 5% CO_2. DMSO (0.1%) was the vehicle control and NT was the no treatment control. (A) NO production in RAW 264.7 cells was measured by Griess assay. (B) The IC_{50} value of AAA3Me was calculated from the dose-response curve as 1.856. The results are presented as the mean ± SEM from threeindependent experiments, each conducted in triplicate, for a total of n=9 data points for each condition. Significance is reported as follows: ****P < 0.0001. Significance levels indicate comparisons with the LPS-treated groups.

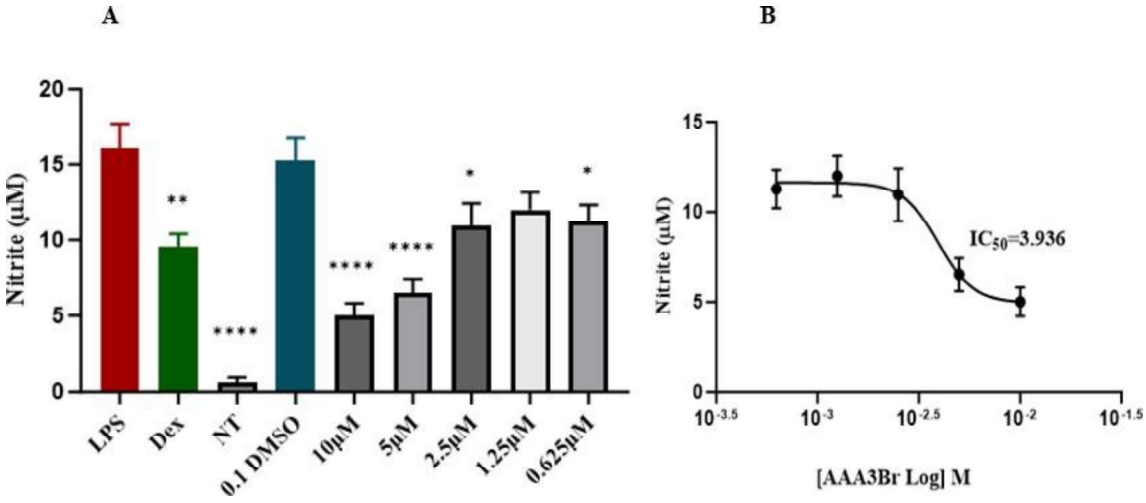


Figure24: Azaaurone AAA3Br inhibits NO production.

RAW 264.7 cells were pretreated with 10 µM of azaaurone derivatives or 15 µM of DEX for 1 hour. After this pretreatment, the cells were stimulated with 1 µg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with 5% CO_2. DMSO (0.1%) was the vehicle control and NT was the no treatment control. (A) NO production in RAW 264.7 cells was measured by Griess assay. (B) The IC_{50} value of AAA3Br was calculated from the dose-response curve as 3.936. The results are presented as the mean ± SEM from threeindependent experiments, each conducted in triplicate, for a total of n=9 data points for each condition. Significance is reported as follows: *P < 0.05, **P < 0.01, ****P < 0.0001.Significance levels indicate comparisons with the LPS-treated groups.

Table 4: NO production using Griess Reaction assay values after treating with the selected azaaurones derivatives.

-	Concentrations						-
Compound	100 µM	10 µM	5 µM	2.5 µM	1.25 µM	0.625 µM	IC_{50}
AAAPh	-	3.063±10.92	3.284±10.70	4.354±9.634	6.800±7.188	8.795±5.192	1.422
AAA2Me	-	5.45±11.23	4.787±11.49	7.302±8.973	11.36±4.919	12.23±4.042	2.092
AAA3Me	-	14.30±15.11	17.99±13.45	21.37±11.92	31.48±7.373	35.53±5.547	1.856
AAA3Br	-	5.019±11.04	6.533±9.525	10.96±5.101	12.00±4.064	11.27±4.791	3.936
AAPh	-	-	16.224±5.23	16.99±4.62	30.15±11.45	35.00±6.601	1.436
AA2Me	-	-	15.38±6.22	18.05±2.56	26.6514.95	24.88±6.72	2.397
AA3Me	-	13.89±7.71	21.09±2.52	24.13±7.48	26.35±5.25	25.46±6.14	10.46
AA3Br	-	11.09±3.51	15.63±5.97	16.03±5.57	15.95.2.65	16.79±4.82	37.21
Ph	4.097±1.75	16.63±7.2	30.98±7.12	31.27±7.42	31.67±7.82	32.67±8.81	9.622
2Me	25.63±1.77	22.41±1.44	24.98±1.12	22.13±1.72	26.67±2.81	28.96±5.11	1.208
3Me	16.07±7.78	23.09±0.76	22.54±1.31	24.49±0.63	25.32±1.46	24.69±0.83	-
3Br	-	-	-	-	33.56±9.71	35.75±11.85	-

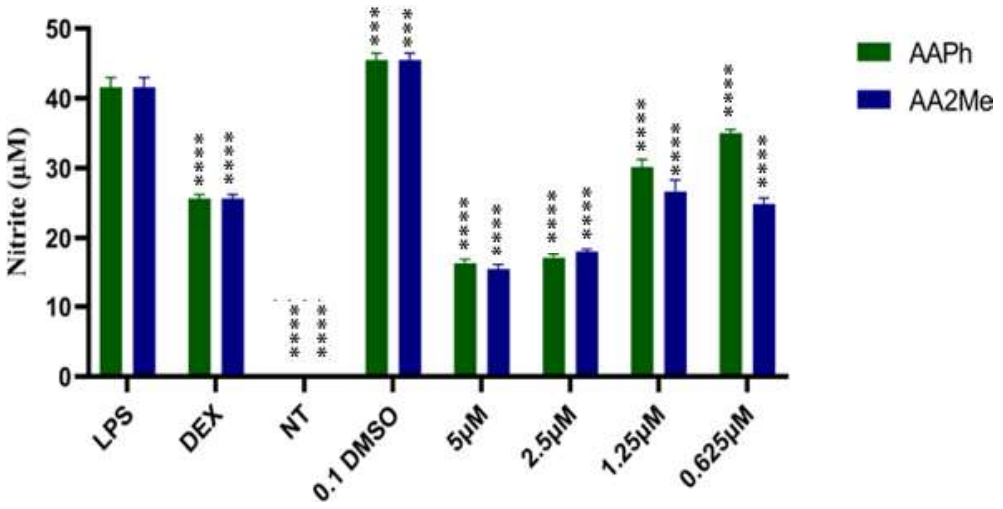


Figure 25: Deacetylated AAPh and AA2Me inhibit NO production.

RAW 264.7 cells were pretreated with 10 µM of deacetylated derivatives or 15 µM of DEX for 1 hour. After this pretreatment, the cells were stimulated with 1 µg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with 5% CO_2. DMSO (0.1%) was the vehicle control and NT was the no treatment control. Nitrite concentrations in cell growth medium were tested 24 h post LPS by Griess assay as an indirect measurement of NO production. The results are presented as the mean ± SEM from three independent experiments, each conducted in triplicate, for a total of n=9 datapoints for each condition. Significance is reported as follows: ***P ≤ 0.001, ****P < 0.0001. Significance levels indicate comparisons with the LPS-treated groups.

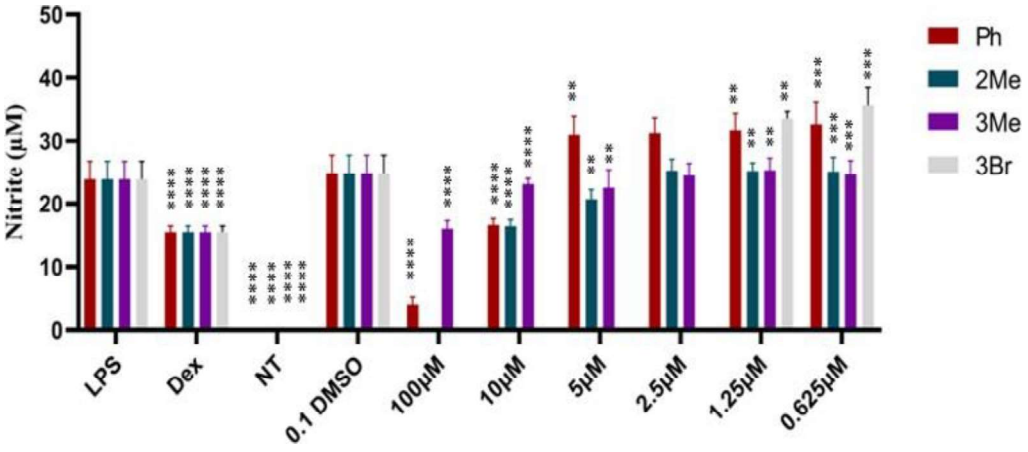


Figure 26: Aurones Ph, 2Me, 3Me and 3Br inhibit NO production.

RAW 264.7 cells were pretreated with 10 µM of aurone derivatives or 15 µM of DEX for1 hour. After this pretreatment, the cells were stimulated with 1 µg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with 5% CO_2. DMSO (0.1%) was the vehicle control and NT was the no treatment control. Nitrite concentrations in cell growth medium were tested 24 h post LPS by Griess assay as an indirect measurement of NO production. The results are presented as the mean ± SEM from three independent experiments, each conducted in triplicate, for a total of n=9 data points for each condition. Significance is reported as follows: **P < 0.01, ***P ≤ 0.001, ****P < 0.0001.Significance levels indicate comparisons with the LPS-treated groups.

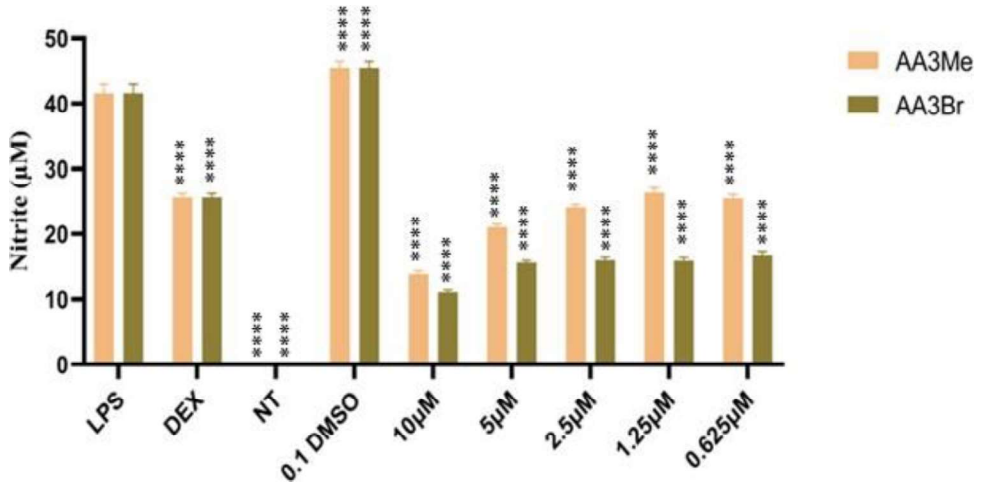


Figure 27: Deacetylated AA3Me and AA3Br inhibit NO production.

RAW 264.7 cells were pretreated with 10 µM of azaaurone derivatives or 15 µM of DEXfor 1 hour. After this pretreatment, the cells were stimulated with 1 µg/mL of bacterial LPS and incubated for 24 hours at 37°C in an environment with 5% CO_2. DMSO (0.1%) was the vehicle control and NT was the no treatment control. Nitrite concentrations in cell growth medium were tested 24 h post LPS by Griess assay as an indirect measurement of NO production. The results are presented as the mean SEM from three independent experiments, each conducted in triplicate, for a total of n=9 data points for each condition. Significance is reported as follows: ****P < 0.0001. Significance levels indicate comparisons with the LPS-treated groups.

4. Discussion

The present study explored the anti-inflammatory potential of azaauronederivatives, compounds belonging to the flavonoid family that can be found in fruits and flowers (Sadighara et al., 2012). It is noteworthy that many flavonoids have exhibited various pharmacological activities, including antioxidant and anti-inflammatory effects (Kim et al., 2021; Park et al., 2017). From the initial screening of 27 azaaurones, four specific azaaurones (AAA2Me, AAA3Br, AAAPh, and AAA3Me) were identified as promising candidates for further evaluation. These compounds exhibited potent and dose-dependent inhibitory effects on the production of cytokines and NO by a macrophage-likecell line when stimulated with LPS, while maintaining minimal cell toxicity for those cells. These results underscore the pharmacological potential of azaaurone derivatives in modulating inflammatory responses. Aurones and their derivatives have been recognized for their diverse biological activities, encompassing anti-cancer, antibacterial, and anti- parasitic properties (Tóth et al., 2020; Campanico et al., 2019). Aurones are a subgroup of flavonoids distinguished by their heterocyclic ring structures containing at least twoelements within the ring (Mazziotti et al., 2022). The biological activities of these compounds may be linked to their specific structural conformations (De Souza Farias et al., 2021).

Azaaurones represent isosteric equivalents of aurones, with the critical distinction being the replacement of the intracyclic oxygen atom with an N-H group, rendering them

more bioactive compared to aurones (Souard et al., 2010; Campanico et al., 2019; Tóth etal., 2020). This substitution has been demonstrated to enhance anti-plasmodial activity compared to aurones (Souard et al., 2010; Carrasco et al., 2016; Tóth et al., 2020). SAR analyses have shown that substituting the intracyclic oxygen in aurones with a nitrogen atom leads to increased activity and selective toxicity (Tóth et al. 2020; Nyembe et al., 2023). The presence of the nitrogen atom allows for further extension and branching withdifferent substituents, potentially facilitating interactions with biologically active sites (Lawson et al., 2003). The structural conformation of these compounds plays a pivotal rolein influencing bioactivity, which is thought to be primarily driven by substituents at the A-ring or B-ring of the overall structure (Yang et al., 2020). Previous studies have documented the potent anti-inflammatory effects of aurone derivatives in various cell lines,primarily through the inhibition of specific signaling pathways (Park et al., 2017; Ren et al., 2020). Furthermore, the analysis of structure-activity relationships has emphasized thesignificance of substitution patterns in positions 4 and 6 of the benzofuran ring. (Gerby etal., 2007 ; Zhang et al., 2012; Zhang et al., 2018; Mazziotti et al., 2022).

In the current study, it was demonstrated that azaaurones exhibited significantly higher bioactivity compared to parent aurone structures. Specifically, certain azaaurones (AAAPh and AAA3Me) displayed remarkable inhibition of nitrite production when used to pretreat RAW 264.7 cells followed by exposure to the inflammatory molecule LPS. Concentrations as low as 2.5 μM resulted in a reduction of nitrite production by more than50% in LPS-stimulated cells compared to the control or other azaaurones. Additionally, deacetylated azaaurones AAPh and AA2Me also led to a more than 50% reduction in nitriteproduction when used at a concentration of 2.5 μM in LPS-stimulated RAW 264.7 cells.

Moreover, this current study supports the robust anti-inflammatory properties of aurone derivatives. For example, Bandgar et al. (2010) reported potent inhibition of TNF-α and IL-6 production in human THP-1 macrophage-like cells exposed to a series of 2,2-bisaminomethylated aurone analogs. In the present study, exposure to azaaurones AAAPhand AAA3Me also resulted in lower TNF-α production compared to other azaaurones andthe parent aurones. The deacetylated aurones (AAPh, AA2Me, AA3Br, and to a lesser extent AA3Me) displayed similar nitrite inhibition at a concentration of 2.5 μM. The mostpotent inhibition of nitrite production in RAW 264.7 cells was observed when the parent aurones (Ph and 2Me) were used at the maximum tested concentration of 100 μM.

The data from this study suggest that acetylated azaaurone derivatives exhibit greater bioactivity at concentrations 40 times lower than the parent aurone structures. Furthermore, the presence of an intracyclic oxygen atom substituted with an N-H group inazaaurones leads to greater bioactivity, as demonstrated by significant inhibition of nitriteand TNF-α in LPS-stimulated RAW 264.7 cells. The findings also support previous reports highlighting the importance of specific substituents on the B-ring and the ethyl group at the C-4 position in promoting greater bioactivity (Souard et al., 2010; Mazziotti et al., 2022). In inference, azaaurone derivatives represent promising candidates for further exploration as potential anti-inflammatory agents, and their superior bioactivity relative toparent aurone structures is a noteworthy finding. These compounds hold significantpotential for the development of novel therapeutics targeting inflammatory responses. Further investigations into the precise mechanisms underlying their anti-inflammatory properties and their therapeutic potential are warranted.

5. Conclusion

In conclusion, the findings of this study underscore the promising anti- inflammatory potential of azaaurone derivatives, which belong to the flavonoid family. Theresults indicate that these compounds possess significant bioactivity and demonstrate a dose-dependent inhibition of pro-inflammatory cytokine and NO production when RAW 264.7 cells are stimulated with LPS, all while exhibiting minimal cell toxicity. These observations highlight the therapeutic promise of azaaurone derivatives in the modulationof inflammatory responses.

It is essential to note that aurones and their derivatives have previously been recognized for their diverse pharmacological properties, including anti-cancer, antibacterial, and anti-parasitic effects. The structural conformation of these compounds significantly influences their bioactivity, with the presence of specific substituents at the A-ring or B-ring being a critical factor. Additionally, the substitution of the intracyclic oxygen atom with an N-H group in azaaurones has been shown to enhance their bioactivitycompared to aurones, as reported in the literature. This substitution provides the opportunity for further modification and branching of substituents, potentially facilitating interactions with biologically active sites.

The current study has demonstrated that azaaurones exhibit superior bioactivity compared to parent aurone structures. Specifically, several azaaurones displayed remarkable inhibition of nitrite production, with a concentration of 2.5 μM leading to morethan a 50% reduction in nitrite production in LPS-stimulated RAW 264.7 cells. The deacetylated aurones also exhibited substantial nitrite inhibition at this concentration.

Notably, the presence of an intracyclic oxygen atom substituted with an N-H group in azaaurones was associated with greater bioactivity, as indicated by the significant inhibition of nitrite and TNF-α production in LPS-stimulated RAW 264.7 cells.

Considering these findings, azaaurone derivatives hold great promise for further exploration as potential anti-inflammatory agents. Their ability to exhibit superior bioactivity at significantly lower concentrations than parent aurone structures is a noteworthy discovery. These compounds present a compelling avenue for the development of novel therapeutic interventions targeting inflammatory responses.

CHAPTER 3

THE EFFECTS OF THE SYNTHETIC AZAAURONE, (Z)-1- ACETYL-2-BENZYLIDENEINDOLIN-3-ONE WITH SUBSTITUTESON iNOS, NF-κB AND MAPK PATHWAYS IN LPS-STIMULATED MURINE MACROPHAGE-LIKE RAW 264.7

1. Introduction

Inflammation, a complicated biological response orchestrated by the immunesystem, serves as a protective mechanism triggered by various factors like cell damage, toxins, pathogens, irritants, and other compounds (Chen et al., 2018; Han and Hyun, 2023). This response manifests as swelling, redness, pain, fever, and impaired tissue role (Chenet al., 2018; Takahashi et al., 2019). While inflammation is crucial for host defense, chronicinflammation underlies several diseases, including endometriosis, asthma, atherosclerosis,obesity, psoriasis, and rheumatoid arthritis (RA) (Henein et al., 2022; Li et al., 2022;Nowowiejska et al., 2022). Therefore, the control of unusual inflammatory reactions issupreme for the treatment and prevention of inflammatory ailments (Yang and He, 2022).

Macrophages play a pivotal role in systemic inflammation, associated with meta-inflammation and inflammageing (Qu et al., 2022). Numerous experimental models havebeen devised over the years to facilitate the development of novel anti-inflammatory drugs,with in vitro models of RAW 264.7 cells now being widely utilized (Facchin et al., 2022).The mouse macrophage cell line RAW 264.7, transformed by the Abelson leukemia virus,necessitates lipopolysaccharide (LPS) for full activation (Raschke et al., 1978). Assessment of inflammatory mediators and pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukins (IL)-1β and IL6, along with inflammatorymediators like PGE2 and nitric oxide, is considered vital in these models (Zhang and An,

2007; Kany 2019). Therefore, LPS-stimulated RAW 264.7 macrophages serve as aclassical inflammatory cell model for assessing the anti-inflammatory activity andmechanisms of action of drugs (Hartley et al., 2008; Facchin et al., 2022; Qu et al., 2022).

Nitric oxide synthase (iNOS), an enzymatic catalyst, naturally produces nitric oxide(NO), a critical second messenger involved in a myriad of physiological and biological processes, including neurotransmission and inflammation (Król and Kepinska, 2020). Notably, NO contributes to the regulation of blood vessel dilation, blood pressure control, and exhibits various vasoprotective and anti-atherosclerotic effects (Forstermann and Sessa, 2012). Under nonpathological conditions, NO plays an anti-inflammatory role, for example, inhibiting leukocyte adhesion to vascular endothelium (Hocher et al., 2004). However, under pathological conditions, NO can function as an inflammatory signaling molecule (Tripathi et al., 2007). The nuclear factor-kappa B (NF-κB) signaling pathway plays a pivotal role as one of the primary responders to harmful stimuli (Singh and Singh 2020). Activation of NF-κB leads to the transcription of various inflammatory genes involved in immune responses, inflammation, and stress responses, including TNF-α, iNOS, and cyclooxygenase (COX-2) (Natarajan et al. 2018). It is noteworthy that NO production and NF-κB signaling are intricately interconnected, with NO sustaining NF-κBactivation (Clancy et al., 2004), while NF-κB, in turn, promotes iNOS protein expression (Arias-Salvatierra et al., 2011; Jia et al., 2013).

Inflammation activation is also associated with the initiation of the MAPK signaling pathway, resulting in increased expression of various inflammatory genes (Chenet al., 2018). Similarly, NO production promotes the activation of the MAPK cell signalingpathway (Kim and Choi, 2010). Elevated NO production augments macrophage activation

and polarization through the MAPK signaling pathway, implicating MAPK in response to inflammatory stimuli (Yao et al., 2022).

Inflammation involves the activation of four key signaling pathways, namely: (1) Ikappa B kinase (IκB)/nuclear factor kappa B (NF-κB); (2) mitogen-activated protein kinase(MAPK); (3) phosphoinositide 3-kinase; and (4) Janus kinase (JAK) signal transducer andactivator of transcription (STAT) signaling pathways (Yeung et al., 2018; Zhao et al., 2021;Ghelani et al., 2022). These pathways play pivotal roles in the release of inflammatory mediators and pro-inflammatory cytokines (Kany et al., 2019).

Furthermore, the initiation of the MAPK signaling pathway is intricately associatedwith the activation of inflammation, leading to the heightened expression of various inflammatory genes (Chen et al., 2018). Similarly, the production of NO impacts the MAPK cell signaling pathway (Kim and Choi, 2010). The upregulated production of NO not only enhances macrophage activation but also influences macrophage polarization through the MAPK signaling pathway, underscoring the crucial role of MAPK in the cellular response to inflammatory stimuli (Yao et al., 2022). This interconnected network of signaling pathways highlights their intricate involvement in the regulation of inflammation and immune responses.

As a part of our studies with azaaurones, we observed that five azaaurones, namely(Z)-1-acetyl-2-benzylideneindolin-3-one (AAAPh) and four of its derivatives (AAA2Me, AAA4CN, AAA3Br, AAA3Me) displayed anti-inflammatory properties in LPS-stimulated RAW 264.7 cells, as evidenced by a notable reduction in NO production. These azaaurone derivatives exhibited inhibitory effects on NO production at concentrations significantly lower than traditional aurones, all while inducing minimal cytotoxicity. Previous studies

have also highlighted the anti-inflammatory potential of aurone derivatives through the inhibition of iNOS expression (Park et al., 2017). iNOS expression is tightly regulated bythe transcriptional activity of NF-κB (Lamon et al., 2010; Jia et al., 2013). During the course of an inflammatory response, the MAPK/NF-κB signaling pathway is activated, and subsequent nuclear translocation of NF-κB promotes the expression of anti-apoptotic factors, cell cycle regulators, cytokines, chemokines, and adhesion molecules (Zhang et al.,2021). These factors are crucial for the survival, activation, and differentiation of both innate and adaptive immune cells, and their activities are tightly modulated by iNOS production (Lamon et al., 2010; Park et al., 2017).

Building upon these findings, the objective of this study was to investigate whetherthe inhibition of NO production by azaaurones is accompanied by a reduction in iNOS protein expression, achieved through inhibition of the MAPK/NF-κB signaling pathway.

2.Materials and Methods

2.1 Reagents

The RAW 264.7 (ATCC TIB 71) cell line was acquired from the American Type Culture Collection (Manassas, VA, USA). Key reagents and materials used in this study were sourced from reputable suppliers as follows: Lipopolysaccharide (LPS; Salmonella *enterica* serotype *typhimurium*), dexamethasone, dimethyl sulfoxide (DMSO), Bay 11- 7082, and Dulbecco's modified eagle medium (DMEM) were acquired from Sigma- Aldrich (St. Louis, MO, USA). Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) were obtained from Thermo Scientific (Waltham, MA, USA). Fetal bovine serum (FBS), penicillin/streptomycin, enhanced chemiluminescence luminol (ECL) substrate, sodium

pyrophosphate, SDS-PAGE gels, nitrocellulose membranes, and Gibco™ Trypsin-EDTA(0.25-0.05%) were sourced from Thermo Fisher Scientific (Pittsburgh, PA, USA). Enzyme-linked Immunosorbent Assay (ELISA) kits and associated reagents were procuredfrom R&D Systems (Minneapolis, MN, USA). The alamarBlue® reagent was purchased from Invitrogen Thermo Fisher Scientific (Pittsburgh, PA, USA). Bovine serum albumin (BSA) was obtained from EMD Millipore (Billerica, MA, USA). The radio- immunoprecipitation assay (RIPA) lysis and extraction buffer were obtained from G- Bioscience (St. Louis, MO, USA).

2.2 Maintenance and Differentiation of the RAW 264.7 Cell Line

RAW 264.7 cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% complement-inactivated FBS and 1% penicillin/streptomycin solution. These cells were incubated at 37°C in a 5% CO_2 atmosphere. Subsequently, the cells were detached from the culture flasks using trypsin- EDTA, and their concentration was adjusted to $5x10^5$ cells/mL. The cells were then incubated for 12-16 hours or until they reached a confluence of 90% in 96-well microplates(Denville/Thomas Scientific; Swedesboro, NJ, USA). Prior to each assay, the cells were washed with phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) to eliminate undifferentiated cells.

2.3 Cell Viability Assay

Five serial dilutions of azaaurone derivatives were prepared from a 10 mM stock solution in DMSO, resulting in the following concentrations: 10 mM, 5 mM, 2.5 mM, 1.25

mM, and 0.62 mM. RAW 264.7 cells were plated into 96-well microplates, and when they reached 60-80% confluence, they were pretreated with one of the five serial dilutions of azaaurone derivatives or 15 µM of dexamethasone for 1 hour. Subsequently, the cells were stimulated with 1 µg/mL of LPS and further incubated for an additional 24 hours at 37°C in a 5% CO_2 environment.

Fresh culture medium containing alamarBlue® reagent (at a final concentration of10%) was prepared, and 100 µL of this medium was added to each well in the microplate. The microplate was then incubated for 3.5 to 4 hours at 37°C. Cell viability was assessed by measuring relative fluorescent units (RFU) using either a SpectraMax M2e microplate reader from Molecular Devices Inc. (Sunnyvale, CA, USA) or a Clario Star microplate reader from BMG Labtech Inc. (Weston Parkway, NC, USA). The measurements were taken at an excitation wavelength of 570 nm and an emission wavelength of 590 nm, and the percent cell viability was calculated using the provided equation:

% Viability= (RFU of dilution/ RFU of control) x 100.

2.4 Cytokine response by ELISA assay

When RAW-264.7 cells reached 60-80% confluence, they were seeded into microplates and subjected to the following treatments: pretreatment with 15 µM ofdexamethasone (utilized as a positive control) or pretreatment with the five serial dilutions of azaaurone derivatives, specifically at concentrations of 10 µM, 5 µM, 2.5 µM, 1.25 µM,and 0.62 µM. Following this pretreatment, the cells were stimulated with 1 µg/ml of LPS and subsequently incubated for 24 hours at 37°C in a 5% CO_2 environment. As negative controls, both untreated cells and cells treated with DMSO were included in the

experimental setup. To assess the levels of mouse TNF-α cytokine in the culture supernatants, an ELISA assay was conducted according to the manufacturer's instructions.

2.5 Antibodies

Western blot analysis employed the following antibodies, which were obtained from Cell Signaling Technology (Denver, MA, USA): iNOS (D6B6S), p65 (D14E12), Phosphorylated p65 (Ser536; 93H1), p38 (D13E1), Phosphorylated p38 (Thr180/Tyr182; D3F9), Anti-rabbit IgG, HRP-linked (7074). Additionally, Actin was detected usingantibodies from Sigma (St. Louis, MO, USA) and Santa Cruz Biotechnology (Dallas, TX,USA).

2.6 Western Blot Analysis

The Western blot procedure was performed as previously described (Park et al., 2017; Ghosh et al., 2019) with modifications as follows. RAW 264.7 cells were pre-treatedwith varying concentrations of azaaurone or 10 μM of Bay 11-7082 for 1 hour prior to stimulation with 1 μg/ml of LPS for 15 minutes. Cell lysis was carried out using Radio- Immunoprecipitation Assay (RIPA) lysis and extraction buffer, which includes a proteaseand phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). To standardize protein content across all samples, cell lysates were appropriately diluted withRIPA lysis solution. Protein concentrations were determined using a Pierce™ BCA ProteinAssay kit (Thermo Scientific, Waltham, MA, USA). The lysates were adjusted to a concentration of 10μg and mixed with a sample loading solution as per the manufacturer'sinstructions. The protein samples were loaded into a 4-12%, 1.0mm Tris-Glycine Mini Gel (Novex™, Thermo Scientific, Waltham, MA, USA) and electrophoresed at 100 volts for

45 minutes to 1 h. 1X Tris-glycine was used as the running buffer (Thermo Scientific, Waltham, MA, USA).

Following electrophoresis, the separated proteins were transferred from the SDS- PAGE gel to a 0.2 μm PVDF membrane (Thermo Scientific, Waltham, MA, USA) using 1X transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) at 25V for 1.5h. Subsequently, the PVDF membrane was washed 3x for 5 minutes each with 5% BSA in 1X Tris-buffered saline with 0.1% Tween-20, and then was blocked for 30-60 min in 5% BSA in 1X Tris-buffered saline with 0.1% Tween-20 or 5% non-dry milk in 1X Tris- buffered saline with 0.1% Tween-20 at room temperature. The blots were then incubated at 4°C overnight with primary antibodies in the cold room. Afterward, they were incubatedfor 30 min to 1 h with secondary antibodies that were HRP-conjugated. To develop the membranes, an enhanced chemiluminescence (ECL) substrate was employed, and images were captured using a ChemiDoc XRS+ system chemiluminescence imager (Bio-Rad, Hercules, CA, USA). The Western blot band intensity was quantified using Image Lab software (Bio-Rad, Hercules, CA, USA).

2.7 Statistical Analyses

Statistical analyses of the results were performed using GraphPad Prism version 9.0 (GraphPad Software Inc., Boston, MA, USA). Treatment groups were compared with control groups, and mean values along with standard error (SE) were reported. The experiments were conducted in triplicate, and statistical significance was determined using one-way analysis of variance (ANOVA) and an unpaired t-test. A P-value less than 0.05 was considered statistically significant.

3 Results

The impact of selected azaaurone derivatives on the NF-κB signaling pathway in LPS-stimulated RAW 264.7 cells was assessed in comparison to the potent IKK inhibitor,Bay 11-7082 (Bay). Cells were pre-treated with azaaurones or Bay for 1 hour followed by LPS treatment. No significant differences in iNOS protein expression were observed among the no-treatment (NT) control, LPS alone treatment, or Bay-treated cells (Figure 28A-D). However, a non-significant increase in iNOS expression was noted in LPS pre- treated RAW 264.7 cells exposed to the aurone derivatives. Specifically, a non-significantincrease in iNOS expression was observed in cells treated with AAA2Me and AAA3Br at a concentration of 1.2 μM compared to NT, LPS, or Bay. Similarly, a slight increase in iNOS expression was detected in cells treated with AAA4CN at 0.6 μM ($P < 0.1$; Figure 28B). Additionally, a non-significant increase in iNOS protein expression was observed in cells treated with AAA3Me at a concentration of 1.2 μM (Figure 28C). Similarly, AAAPh- treated cells exhibited a non-significant increase in iNOS expression at concentrations of
0.6 μM and 1.2 μM (Figure 28D). No reduction in iNOS expression was observed in cellstreated with AAAPh at any concentration (Figure 28D).

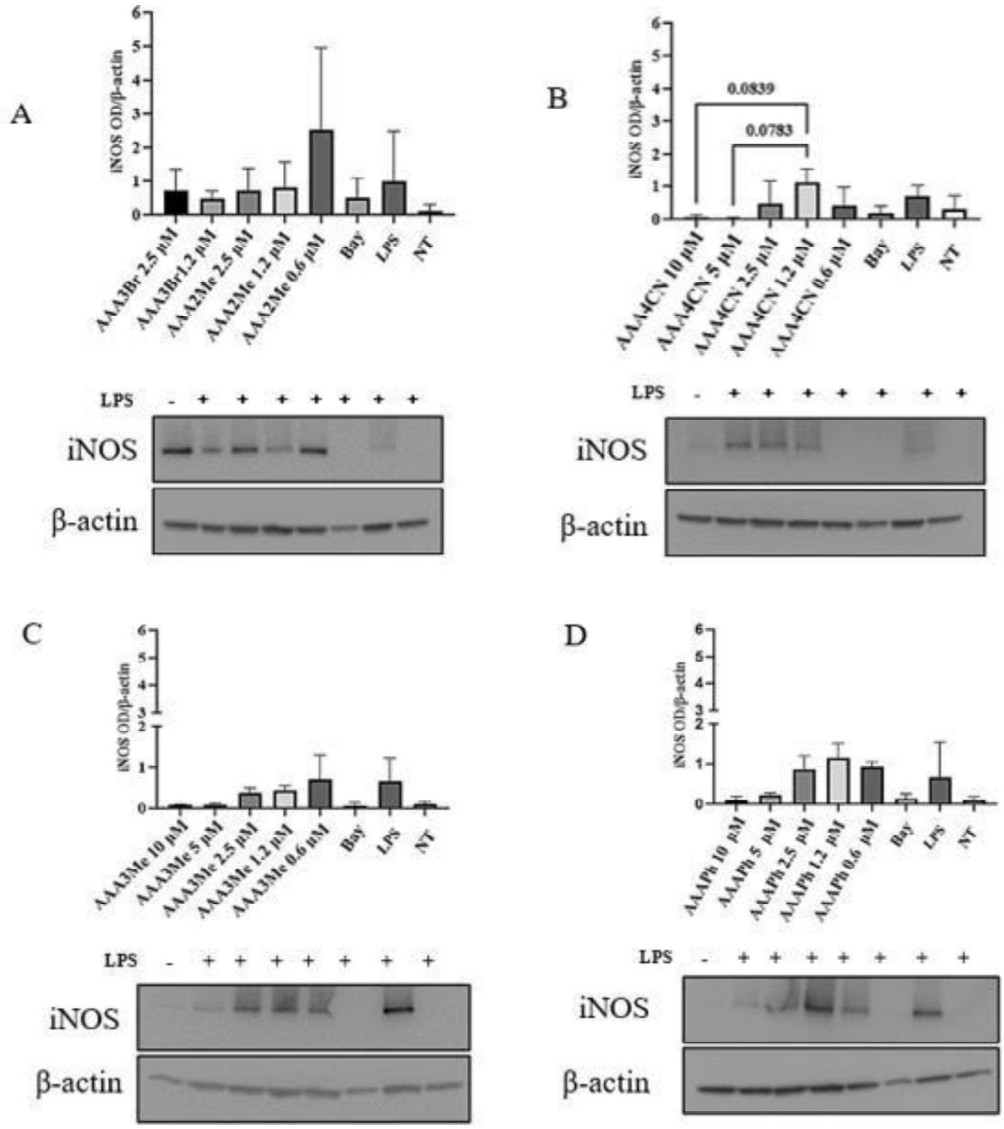


Figure 28: Levels of iNOS protein expression and Western blots of RAW 264.7 cellspre-treated with azaaurones or Bay 11-7082 for 1 hour prior to LPS stimulation.

iNOS and actin as measured by quantification of Western blot band intensity (top panel) and representative Western blots with β-actin as the control (bottom panel) of cells treated with azaaurone derivatives. RAW 264.7 cells were pre-treated with dilutions of azaaurone derivatives or Bay (10 μM) for 1 hour prior to stimulation withof LPS (1 μg/mL) for 24 h. NT is a no treatment control. (A) AAA2Me and AAA3Br,
(B) AAA4CN (C) AAA3Me, (D) AAAPh. Panel B shows the statistical significance between the no-treatment (NT) and LPS control and 0.6 μM of azaaurone AAA4CN.

To assess the inhibitory effect of azaaurones on NF-κB signaling, protein expression of p65 and phosphorylated p65 (pp65) was evaluated. AAAPh significantly reduced p65 and pp65 protein expression in LPS-stimulated RAW 264.7 cells (Figure 29A- C), while AAA3Me moderately reduced p65 and pp65 (Figure 30A-C). The MAPK signaling pathway, a potent inflammatory pathway affecting NF-κB signaling, was also evaluated for changes in p38 activation through Western blot analysis. AAAPh had no effect on p38 or pp38 (Figure 31A-C). Both AAA2Me and AA2Me reduced p38 and pp38(Figures 32A-C). Although AAA3Me had no effect on p38, it reduced pp38 protein expression (Figures 33A-C), while AAA3Br reduced both p38 and pp38 proteins (Figure 34A-C).

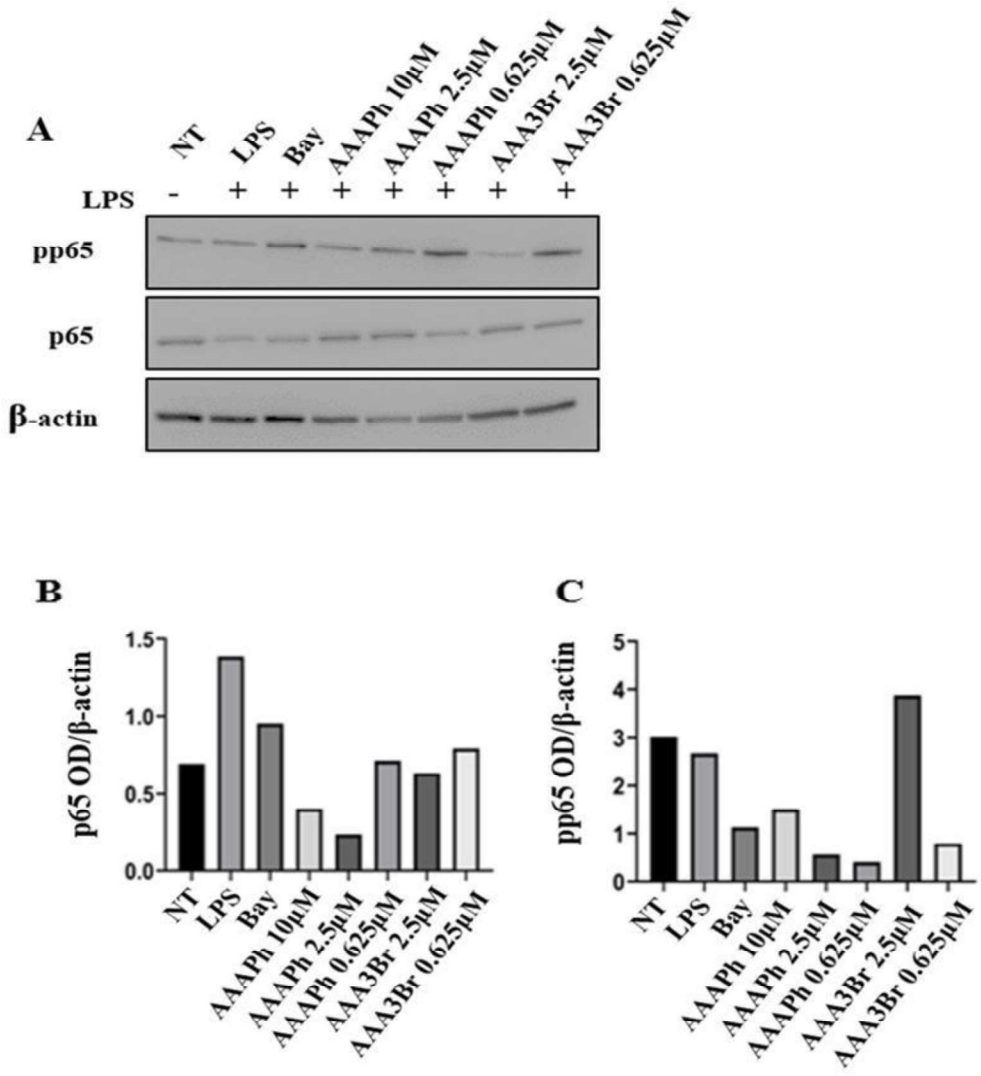


Figure 29: Inhibition of p65 and pp65 protein expression following pre-treatment with dilutions of AAAPh, AAA3Br or Bay (10 μM) prior to LPS (1 μg/mL) stimulation.

RAW 264.7 cells were pretreated with dilutions of azaaurone derivatives or Bay (10 μM) for 1 hour prior to stimulation with of LPS (1 μg/mL) for 15 min. (A) Images of blot for phosphorylated p65 (Ser536) and p65, (B) Corresponding quantification for p65 in RAW 264.7 from Western blot (C) Corresponding quantification for pp65 in RAW 264.7 from Western blot.

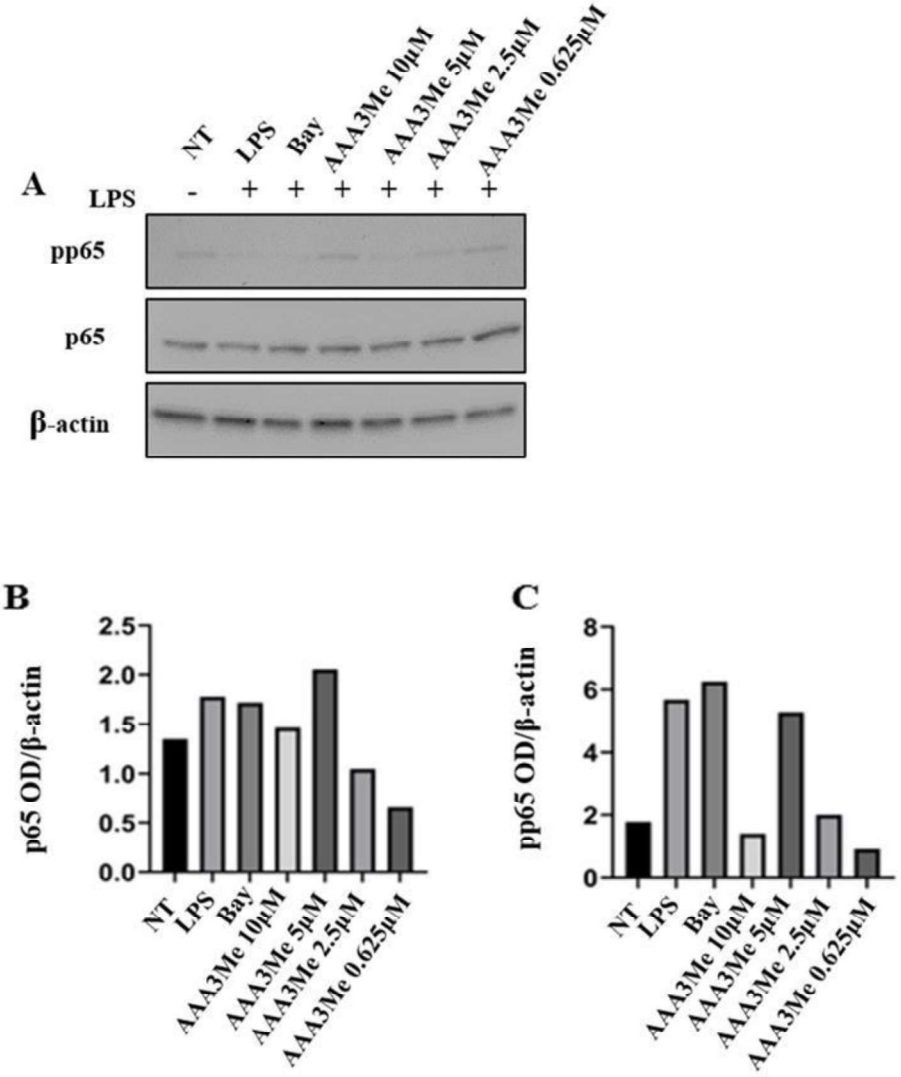


Figure 30: Inhibition of p65 and pp65 protein expression following pre-treatment with dilutions of AAA3Me or 10 µM Bay prior to LPS (1 µg/mL) stimulation.

RAW 264.7 cells were pretreated with dilutions of azaaurone derivatives or Bay (10 µM) for 1 hour prior to stimulation with of LPS (1 µg/mL) for 15 min**.** (A) Images of Western blots for phosphorylated p65 (Ser536) and p65. (B) Corresponding quantification for p65 in RAW 264.7 cells from the Western blot. (C) Corresponding quantification for pp65 in RAW 264.7 from the Western blot.

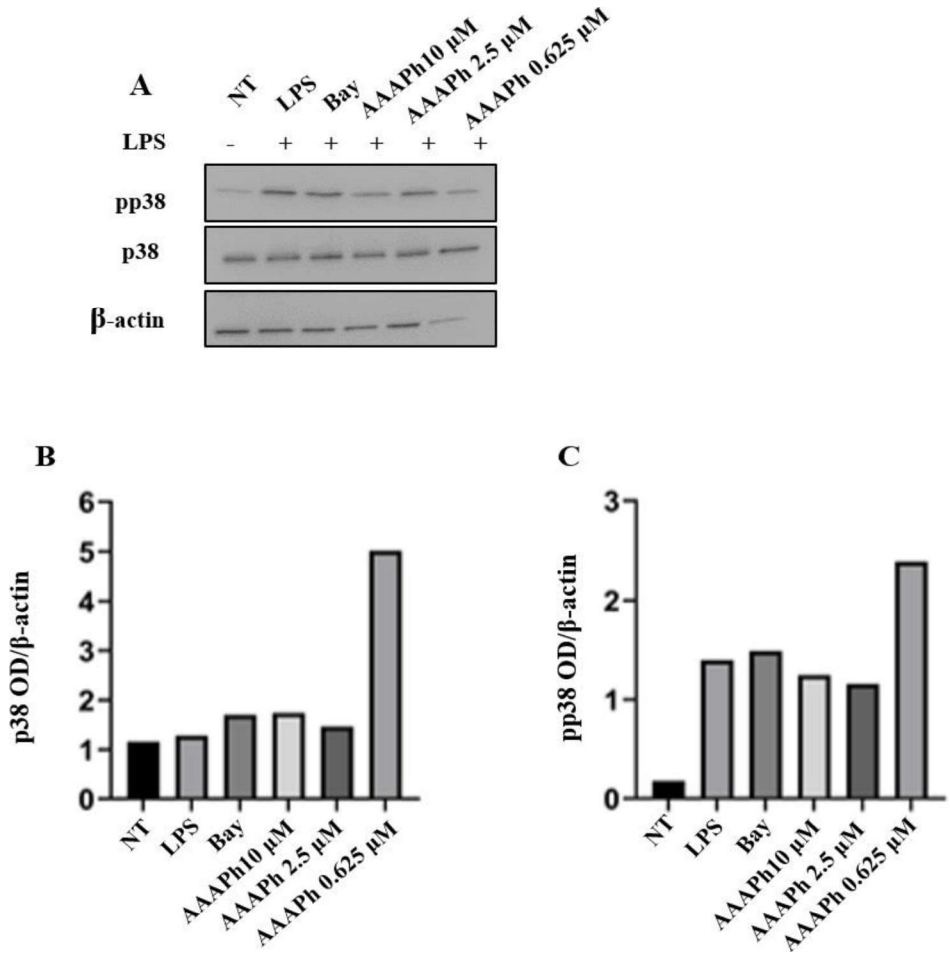


Figure 31: Inhibition of p38 and pp38 protein expression following pre-treatment with dilutions of AAAPh or Bay (10 µM) prior to LPS (1 µg/mL) stimulation.

RAW 264.7 cells were pretreated with dilutions of azaaurone derivatives or Bay (10 µM) for 1 hour prior to stimulation with of LPS (1 µg/mL) for 15 min. (A) Images of Western blots of phosphorylated p38 (Thr180/Tyr182) and p38. (B) Corresponding quantification for p38 in RAW 264.7 cells from Western blot. (C) Corresponding quantification for pp38in RAW 264.7 cells from Western blot.

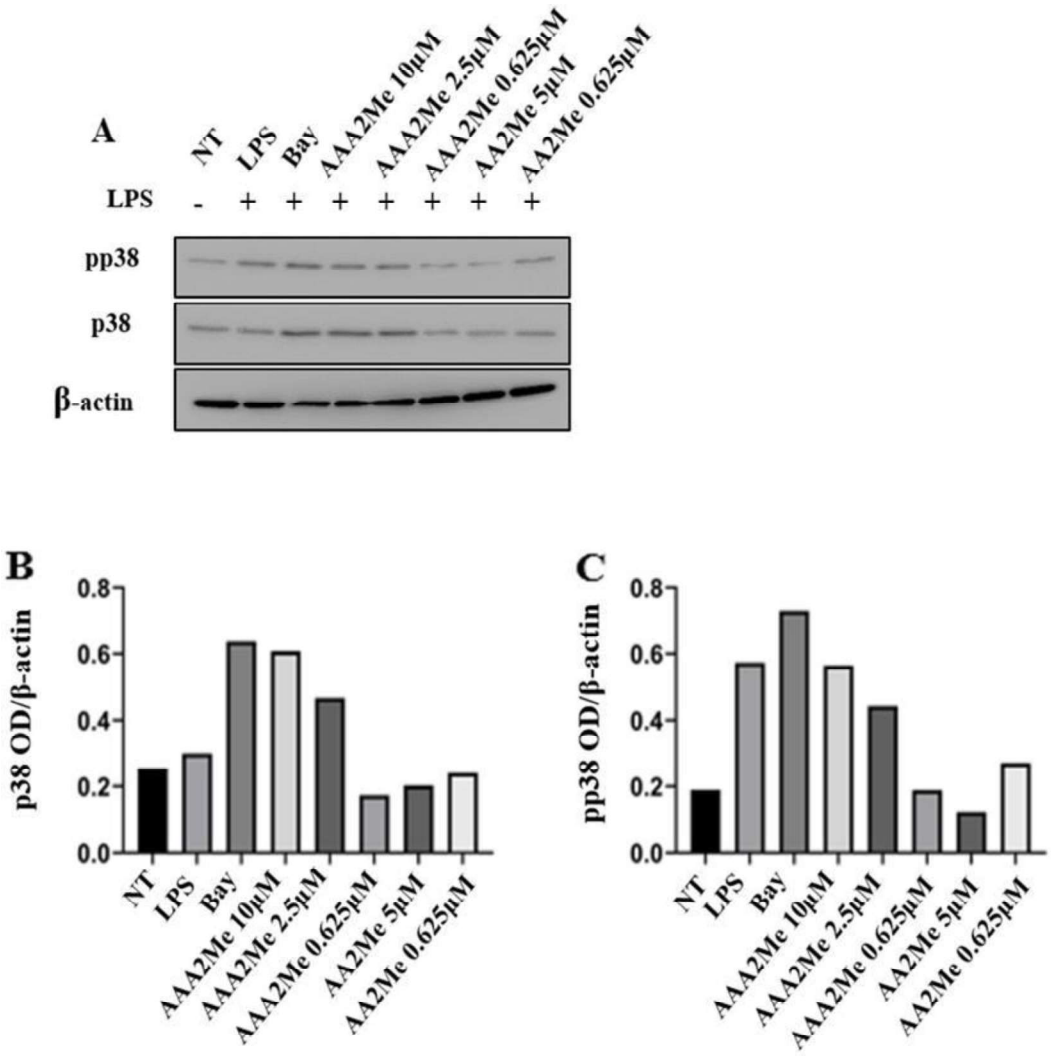


Figure 32: Inhibition of p38 and pp38 protein expression following pre-treatment with dilutions of AAA2Me, AA2Me, or Bay (10 μM) prior to LPS (1 μg/mL) stimulation.

RAW 264.7 cells were pretreated with dilutions of azaaurone derivatives or Bay (10 μM) for 1 hour prior to stimulation with of LPS (1 μg/mL) for 15 min. (A) Images of Western blots of phosphorylated p38 (Thr180/Tyr182) and p38. (B) Corresponding quantification for p38 in RAW 264.7 cells from Western blot. (C) Corresponding quantification for pp38in RAW 264.7 cells from Western blot.

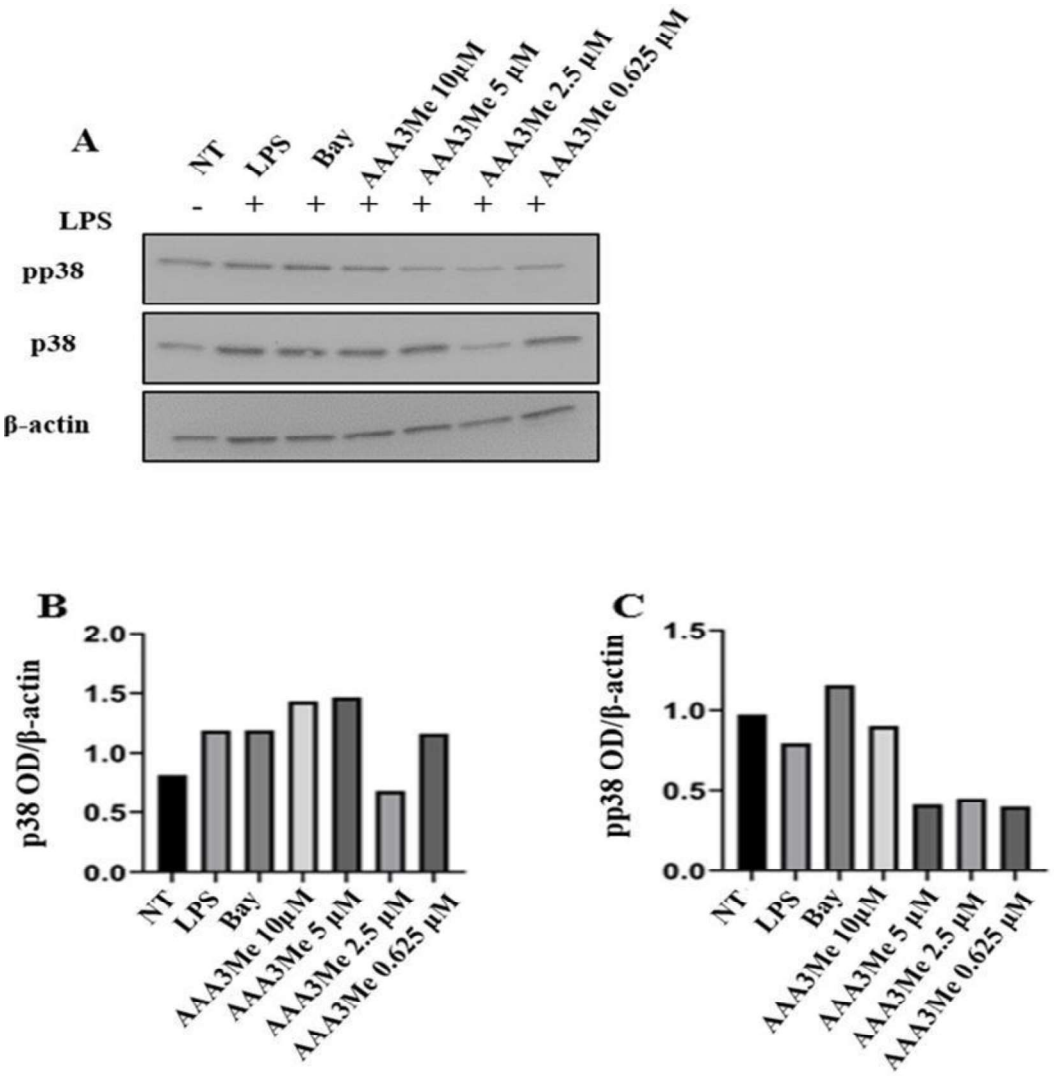


Figure 33: Inhibition of p38 and pp38 protein expression following pre-treatment with dilutions of AAA3Me or Bay (10 μM) prior to LPS (1 μg/mL) stimulation.

RAW 264.7 cells were pretreated with dilutions of azaaurone derivatives or Bay (10 μM) for 1 hour prior to stimulation with of LPS (1 μg/mL) for 15 min. (A) Images of Western blots of phosphorylated p38 (Thr180/Tyr182) and p38. (B) Corresponding quantification for p38 in RAW 264.7 cells from Western blot. (C) Corresponding quantification for pp38in RAW 264.7 cells from Western blot.

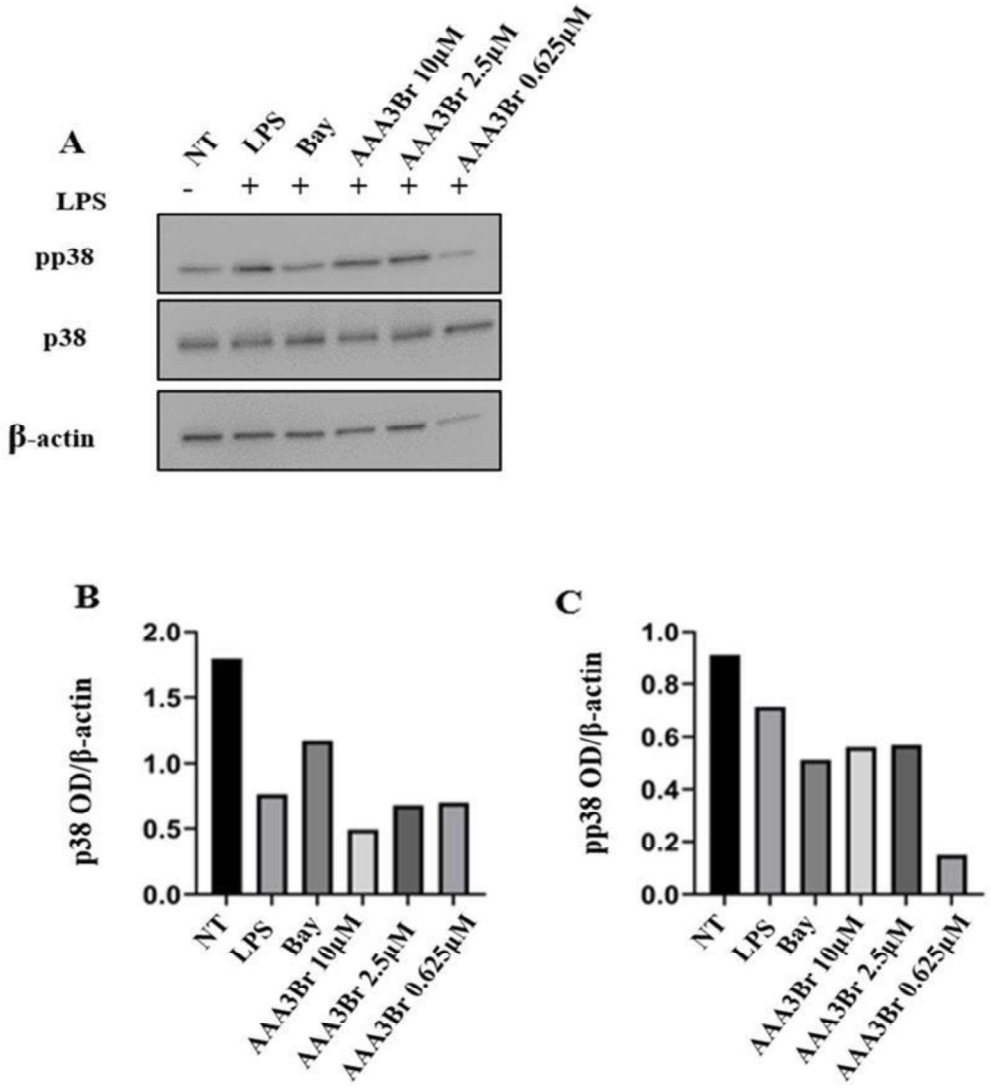


Figure 34: Inhibition of p38 and pp38 protein expression following pre-treatment with dilutions of AAA3Br or Bay (10 μM) prior to LPS (1 μg/mL) stimulation.

RAW 264.7 cells were pretreated with dilutions of azaaurone derivatives or Bay (10 μM)for 1 hour prior to stimulation with of LPS (1 μg/mL) for 15 min. (A) Images of Western blots of phosphorylated p38 (Thr180/Tyr182) and p38. (B) Corresponding quantification for p38 in RAW 264.7 cells from Western blot. (C) Corresponding quantification for pp38in RAW 264.7 cells from Western blot.

TNF-α, a potent inflammatory cytokine produced by macrophages, was measured to further understand the impact of azaaurone exposure. A significant increase in TNF-α was observed in response to LPS, validating the cell line's response to treatment compared to untreated (NT) cells. A dose-dependent inhibition of TNF-α secretion was noted in LPS-stimulated cells treated with (A) AAAPh, (B) AAA2Me, (C) AAA3Me, and (D) AAA3Br (Figure 35A-D). Previous studies have reported the inhibitory effects of various aurone derivatives on TNF-α secretion, corroborating the findings in the present study (Park et al.,2017; Bandgar et al., 2010). The data suggest a potential increase in the expression of anti-inflammatory factors leading to the inhibition of TNF-α production.

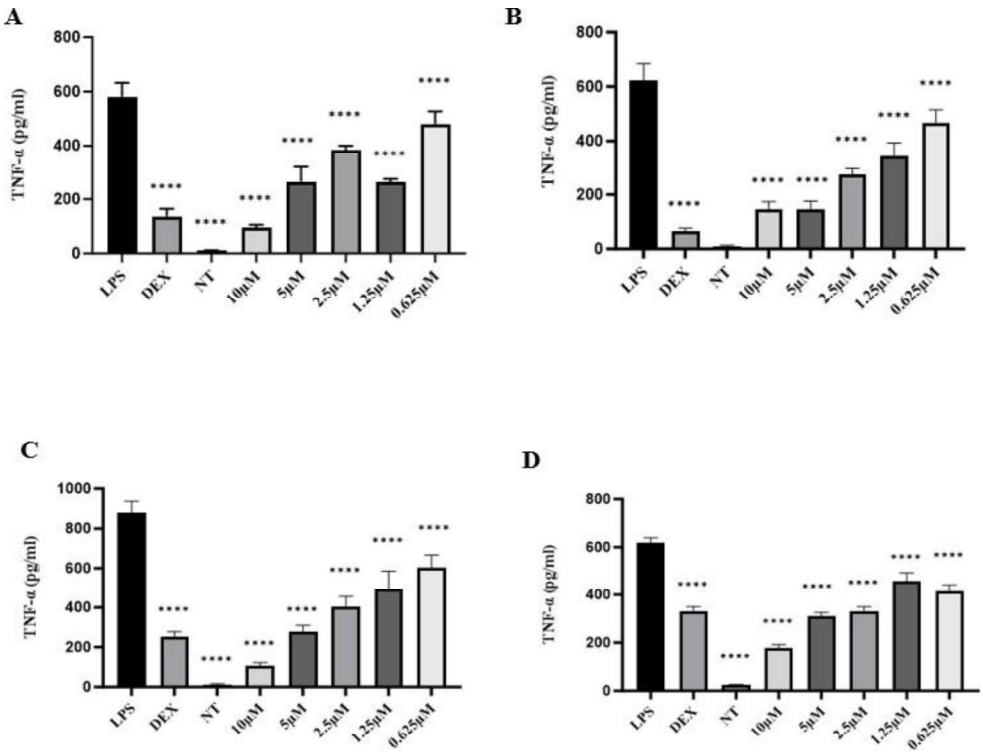


Figure 35: Secreted TNF-alpha in the presence of dilutions of azaaurone derivatives and controls (NT - no treatment, 1 µg/mL LPS, or 15 µM Dex) as quantified by ELISA.

RAW 264.7 cells were pretreated with 15 µM DEX, or 10, 5, 2.5, 1.25 and 0.625µMof azaaurone derivatives for 1 h and stimulated with 1 µg/ml of LPS for 4 h. The concentration of TNF-α in supernatants was tested by ELISA. (A) AAAPh, (B) AAA2Me, (C) AAA3Me, (D) AAA3Br. Results are presented as the mean± SEM of3 independent experiments performed in triplicate, n=9. Significance is reported as ****P<0.0001 compared with LPS-treated groups.

4 Discussion

We have demonstrated the potent anti-inflammatory function of aurone derivatives,as evidenced by the inhibition of LPS-induced NO production, a process primarily mediated by iNOS. Previous work (Park et al. 2017) suggested that aurone derivatives inhibit NO production potentially through the inhibition of NF-κB signaling. The NF-κB pathway is activated by intracellular activation of IKK, leading to the phosphorylation of IκBα and subsequent proteasome degradation. The degradation of phosphorylated IκBα promotes the release of RelA and p50, which translocate into the nucleus to facilitate genetranscription (Liu et al. 2017). Various aurone derivatives have also been reported to inhibitTNF-α secretion, with effects observed at concentrations as low as 1 μM (Bandgar et al. 2010; Park et al. 2017). These effects on TNF-α may involve direct actions on TNF-α itself,including reduced mRNA expression and promoter activity following aurone treatment (Park et al. 2017).

Despite the previously reported reduction in NO production, no significant decreasein iNOS protein expression was observed in our study. This discrepancy may be explainedby the primary function of aurone derivatives to inhibit secretion rather than reducing totalprotein levels in the cell. Three-dimensional quantitative structure–activity relationships (QSAR) and docking analysis have revealed specific amino acid interactions in the azaaurone-protein interaction. For instance, anti-malarial aurones have been shown to competitively inhibit the quinol oxidation site of heme, blocking electron transfer and disrupting ATP synthesis (Hadni and Elhallaoui 2020). While a reduction in protein function may not correlate with a decrease in total protein, the observed negative change

in iNOS protein expression suggests a potential inhibition of iNOS enzymatic function by azaaurones. iNOS production is regulated by the NF-κB cell signaling pathway (Jia et al. 2013), and conversely, NO can negatively regulate the NF-κB pathway (Katsuyama et al. 1998).

In contrast to prior reports, we did not observe a significant increase in iNOS production in LPS-treated cells (Watters et al. 2002; Park et al. 2012). This discrepancy may be attributed to differences in the duration and concentration of LPS treatment. No significant differences in iNOS production were observed in cells treated with Bay 11– 7082 compared to untreated control cells. Bay 11–7082, known as a broad-spectrum inhibitor, has various targets, including inhibition of NO, TNF-α, and prostaglandin E(2) (Lee et al. 2012; Moon, Kim, and Kim 2015). Future studies should assess not only iNOSprotein expression but also the stepwise activation or inhibition of NF-κB cell signaling- associated proteins. This will provide insights into the mechanisms through which azaaurones drive the inhibition of LPS-stimulated NO production.

5 Conclusions

In conclusion, our study demonstrates that azaaurone derivatives exhibit potent anti-inflammatory properties, as evidenced by the significant inhibition of LPS-induced NO production in RAW 264.7 macrophage cells. Despite no significant decrease in iNOSprotein expression, the observed reduction in NO production suggests that azaaurones potentially inhibit iNOS enzymatic function. These findings align with previous reports indicating that aurone derivatives, through their interaction with specific amino acids, maydisrupt key cellular processes, such as competitive inhibition of the quinol oxidation site

of heme. Moreover, azaaurones displayed inhibitory effects on the NF-κB signalingpathway, a crucial regulator of inflammatory responses. AAAPh significantly reduced protein expression of both p65 and phosphorylated p65 (pp65), indicating its potential to modulate NF-κB-mediated gene transcription. Interestingly, while there was no significant change in iNOS expression, azaaurones demonstrated a dose-dependent inhibition of tumornecrosis factor-alpha (TNF-α) secretion, further supporting their anti-inflammatory efficacy.

CHAPTER 4

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Our comprehensive exploration of azaaurone derivatives has unveiled their potentanti-inflammatory effects, marking a significant stride towards understanding their therapeutic potential. In the first set of experiments, azaaurones demonstrated a dose- dependent inhibition of pro-inflammatory cytokines and nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells. Strikingly, these compounds exhibited superior bioactivity compared to parent aurone structures, with specific modifications, particularlythe substitution of an intracyclic oxygen atom with an N-H group, enhancing their efficacy.

Although the second investigation did not reveal a significant decrease in induciblenitric oxide synthase (iNOS) protein expression in response to azaaurone treatment, the nuanced findings suggest a potential inhibition of iNOS enzymatic function by azaaurones. Notably, azaaurones displayed inhibitory effects on the NF-κB signaling pathway, a criticalregulator of inflammatory responses. AAAPh, in particular, significantly reduced protein expression of p65 and phosphorylated p65 (pp65), indicating its potential to modulate NF-κB-mediated gene transcription. However, this study's divergence from some prior findingsemphasizes the nuanced response influenced by experimental conditions, urging caution ininterpreting results.

Looking ahead, future investigations should delve into the precise mechanisms through which azaaurones modulate NF-κB signaling and their impact on other key components of the inflammatory response. The observed dose-dependent inhibition of

tumor necrosis factor-alpha (TNF-α) secretion further supports the anti-inflammatory efficacy of azaaurones. Understanding the structural basis of azaaurone-protein interactions through quantitative structure-activity relationships holds promise for unveiling novel therapeutic avenues. Overall, our study contributes significant insights intothe anti-inflammatory potential of azaaurones, paving the way for future research aimed at unraveling their therapeutic applications in the context of chronic inflammation and relateddiseases.

Future Directions

Our research, in line with previous studies, affirms the anti-inflammatory effects of aurones. Notably, we have demonstrated that azaaurone derivatives exhibit even more potent anti-inflammatory effects on LPS-stimulated immune cells. The robustness of current investigations lies in the use of a relevant immune stimulatory marker, LPS, and immune cell lines capable of eliciting inflammatory responses. However, caution iswarranted in generalizing these findings across all immune cell subtypes or antigens.

Future studies should address critical aspects to deepen our understanding of azaaurone impact on immune responses. Employing RNA-sequencing techniques can provide valuable insights into the modulation of mRNA expression of inflammatory markers in macrophages, offering a more comprehensive view of the molecular changes induced by azaaurones. Additionally, evaluating the effect of azaaurones on the efficiency of antigen presentation between innate and adaptive immune cells will contribute to elucidating their broader immunomodulatory effects.

Furthermore, various disease models for inflammatory conditions, including arthritis, ankylosing spondylitis, diabetic-associated ulcers, and rheumatoid arthritis, present avenues for assessing the potential therapeutic efficacy of azaaurones in vivo. Investigating how azaaurones influence disease progression in these inflammatory conditions can provide valuable insights that may translate into novel therapeutic strategies. This knowledge holds promise for individuals grappling with chronic inflammation or inflammatory diseases, potentially offering new avenues for effective intervention and management.

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