

Synthesis, *In Vitro*, and *In Silico* Anti-Inflammatory Screening of Orthocetamol Azo Derivatives

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Abstract—Chronic inflammation drives various non-communicable diseases, necessitating the search for novel therapeutic agents. Integrating heteroatoms in the inhibitor drug design enhances potency by enabling multifaceted interactions with the target cell receptor. Herein, a new series of orthocetamol azo derivatives, featuring a nitrogen-based moiety ($-N=N-$), was synthesized via the diazotization of orthocetamol with substituted aniline derivatives, yielded 31–95%. The *in vitro* anti-inflammatory evaluation on orthocetamol azo derivatives showed a minimum $49.4 \pm 1.3\%$ NO inhibition against LPS-induced RAW 264.7 macrophages at $50 \mu\text{M}$. Notably, *para*-bromo substituted compound exhibited the most potent NO inhibition of $92.8 \pm 0.3\%$, with an IC_{50} value of $16.0 \pm 0.6 \mu\text{M}$, comparable to the positive control, Dexamethasone ($16.9 \pm 2.2 \mu\text{M}$). *In silico* molecular docking analysis of the *p*-Br orthocetamol azo against the COX-2 enzyme (PDB ID: 5IKR), a key regulator often associated with NO production pathways, revealed a binding affinity of -7.2 kcal/mol driven by a stable hydrogen bond with TYR355 and a predicted isoform-selective π -alkyl interaction with VAL523. While these computational insights suggest a potential preference for COX-2, further pharmacokinetic profiles of *p*-Br orthocetamol azo confirmed its druglikeness through adherence to the Lipinski rule, in addition to an optimal balance of ADMET properties. The finding suggested *p*-Br orthocetamol azo could serve as a promising therapeutic candidate, warranting further *in vitro* enzymatic assays to experimentally validate its COX-1/COX-2 selectivity profile in anti-inflammatory drug development.

Keywords: orthocetamol, regioisomer, nitric oxide production inhibitory assay, MTT cell viability assay, COX-2 enzyme

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INTRODUCTION

Inflammation is a fundamental protective immune response that shields the body from harmful stimuli, such as pathogens, damaged cells, or irritants. This complex process is initiated by various mediators, including prostaglandins and histamine, which trigger key physiological responses like pain, increased blood flow, enhanced vascular permeability, and the recruitment of leukocytes to the site of injury [1]. However, after a prolonged inflammation, the excessive formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can lead to the destruction of DNA,

protein nitration, and cells, increasing the risk of chronic inflammatory diseases such as cardiovascular diseases, cancer, diabetes mellitus, and autoimmune disorders [2, 3]. To counter this, anti-inflammatory drugs, particularly non-steroidal anti-inflammatory drugs (NSAIDs), are commonly used. NSAIDs, a diverse group of non-opioid medications, play a crucial role in alleviating symptoms by inhibiting the production of nitric oxide (NO), superoxide anion (O_2^-), and cyclooxygenase (COX) enzymes, thereby reducing inflammatory mediators [4]. The concurrent utilization of non-opioid analgesics, mainly paracetamol, and non-steroidal anti-inflammatory drugs (NSAIDs) constitutes

a highly prevalent multimodal strategy in the clinical setting [5].

It is a well-known fact that paracetamol has been widely used as an analgesic and antipyretic drug [6]. However, its regioisomer, orthocetamol, has received comparatively little attention despite emerging evidence of pharmacological potential, including hepatoprotective activity and antidote efficacy against paracetamol-induced toxicity [7]. This limited exploration may be attributed to the early clinical success and widespread acceptance of paracetamol, which reduced interest in its isomers, as well as concerns that orthocetamol could undergo metabolic pathways leading to toxic intermediates such as 2-acetamidohydroquinone [8]. Nevertheless, systematic studies on structural modifications of orthocetamol are limited, making derivatives synthesis and SAR analysis a clear avenue for novel work. Orthocetamol, also known as 2-acetamidophenol, is an aromatic molecule similar to salicylic acid [9], whereby its structure forms almost planar, endless ribbons that are held together by hydrogen bonding between the hydroxyl and carbonyl groups of adjacent molecules [10]. Scientists believed that orthocetamol has similar antipyretic and analgesic properties to paracetamol [11].

Akin to paracetamol, the hydroxyl group on the phenyl ring of the orthocetamol make it eligible for the electrophilic aromatic substitution, i.e., azo coupling reactions. The hydroxyl allows the aromatic ring to function as a nucleophile and attack the electrophilic diazonium cation, resulting in the formation of azo compounds [12]. Azo compounds, recognized by their $-N=N-$ group, are a key type of organic colorants [13]. This reaction is popular because azo compounds are cheap to produce, straightforward to work with, versatile, and have excellent optical and thermal properties [14]. Plus, azo compounds have shown various biological benefits, including anticancer [15], anti-diabetic [16], antioxidant [17], and anti-inflammatory [18] effects. The incorporation of nitrogen-containing scaffolds, such as the azo moiety ($-N=N-$), onto orthocetamol follows the strategic approach in the development of bioactive compounds due to the ability of nitrogen atoms to increase overall compound affinity and lipophilicity, allowing easy diffusion and subsequently enhancing the inhibition action [19, 20].

In this research, orthocetamol azo compounds **1–19** were synthesized via the diazotization of substituted aniline derivatives and coupled with orthocetamol,

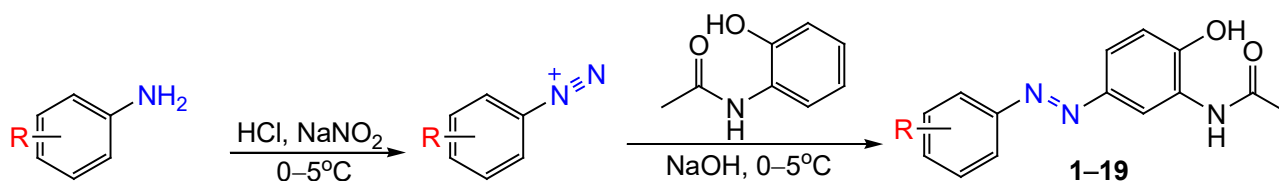
followed by evaluation of their inflammatory properties. The anti-inflammatory properties were tested using the Nitric Oxide (NO) production inhibitory assay in LPS-stimulated RAW 264.7 macrophages with Dexamethasone as the positive control. The NO assay was used to indicate suppression of inflammation-related NO production, a key signaling molecule in the inflammatory response, using Griess reagent [21–25]. Subsequently, to provide a structural hypothesis for the observed bioactivity on the most potent lead compound, *in silico* molecular docking analysis was performed using AutodockVina software [26, 27] against the COX-2 enzyme to elucidate potential binding modes and intermolecular interactions. Furthermore, the pharmacokinetic profile and druglikeness were assessed through ADMET [28–30] screening to evaluate the compounds' bioavailability and suitability as a potential drug.

RESULTS AND DISCUSSION

Chemistry. Orthocetamol azo derivatives **1–19** were successfully synthesized by first introducing NaNO_2 with a series of aniline derivatives in HCl under the temperature of 0–5°C for 10–15 min to form diazonium salt. The formation of diazonium salt was confirmed by the turning of white potassium iodide paper to blue color [18]. The temperature was kept below 5°C throughout the reaction because diazonium salt is very unstable, which arises from the weak N_2^+ leaving group, which can be released suddenly, resulting in the breakdown of the intermediate or hazardous side reactions [31]. The targeted compounds **1–19** were formed in low to high yields of 31–95% via diazo-coupling of diazonium salt reacting with orthocetamol, whose nucleophilic hydroxyl groups enabled azobenzene formation [32]. Since the coupling reagent is a phenolic compound, the reaction occurred in slightly basic conditions. The synthetic pathway of **1–19** is illustrated in Scheme 1.

The formation of compounds **1–19** was elucidated using FT-IR, ^1H NMR and ^{13}C NMR spectroscopies. In the FTIR spectra, the presence of a new bonding of azo, $\text{N}=\text{N}$ bond, was indicated by the appearance of a peak at 1541–1420 cm^{-1} , which further validated the occurrence of azo-coupling reaction [33, 34]. Absorption bands at 3409–3255 cm^{-1} indicated the presence of the N-H group, a characteristic of amide [35]. The peaks at 3096–2937 cm^{-1} were attributed to the O–H group. Usually, the frequency of the OH group is at 3600–3200 cm^{-1} [36]; however, the intermolecular hydrogen bonds in **1–19**

Scheme 1.



R = H (**1**), *o*-F (**2**), *m*-F (**3**), *p*-F (**4**), *o*-Cl (**5**), *m*-Cl (**6**), *p*-Cl (**7**), *o*-Br (**8**), *m*-Br (**9**), *p*-Br (**10**), *o*-I (**11**), *m*-I (**12**), *p*-I (**13**), *o*-Me (**14**), *m*-Me (**15**), *p*-Me (**16**), *o*-OMe (**17**), *m*-OMe (**18**), *p*-OMe (**19**).

may have caused the shifting, leading to a decrease in intensity and frequency [37]. Strong absorption bands at 1679–1593 cm^{−1} corresponded to C=O amide of the orthoacetamol [38], while aromatic carbon (C=C) appeared at 1585–1608 cm^{−1} [39].

In ¹H NMR spectra, singlet peaks at the most downfield region of 9.31–10.93 ppm and 9.34–10.98 ppm were attributed to the NH group of amides and the OH group, respectively. This is due to the deshielding effect of the electronegative atom, directly bonded to the protons, pulling electron density away [40]. Meanwhile, multiple peaks appeared in the 7.00–8.54 ppm region, attributed to aromatic protons on benzene rings, while singlet peaks at around 2.13–2.14 ppm were attributed to the methyl group [28]. The high shielding effect makes the chemical shift of the methyl (CH₃) substituted aniline protons move towards upfield [41]. On the other hand, in the spectrum of methyl series (**14–16**), the singlet peak observed at 2.37–2.41 ppm attributed to the methyl group from the aniline is more downfield than the single peak methyl of amide at δ 2.14 ppm due to the presence of high electronegative oxygen that pulled the electron density away [42]. Furthermore, the singlet peak at 3.84–3.93 ppm in the spectrum of methoxy series (**17–19**) attributed to methoxy appeared slightly upfield region due to electron donating effect of the oxygen and another singlet peak at 2.13–2.14 ppm was assigned to methyl of amide for the series due to the electron-donating nature of the methyl group and the partial shielding effect of the adjacent carbonyl [43].

As for ¹³C NMR, the spectra of compounds **1–19** showed an amide carbonyl peak at 168.2–169.7 ppm in the most downfield region because of the deshielding effect [42]. In contrast, methyl peaks of amide appeared at the most upfield region at 17.57–24.36 ppm as they experience less of the deshielding effect from the

electron-withdrawing amide group. This reduces electron density around the methyl group, leading to a stronger magnetic field and higher energy state, culminating in an upfield shift [41]. Moreover, the two peaks attributed to C–N=N (azo) were shown at 150.0–153.1 ppm and 144.7–144.8 ppm, while C–O was observed at the region of 148.1–162.3 ppm. The downfield resonance was caused by carbon atoms next to electronegative nitrogen and oxygen atoms, resulting in increased deshielding [44]. The halogenated orthoacetamol azo derivatives (**2–13**) possessed an extra peak at 159.9–164.2 ppm (C–F), 128.0–134.8 ppm (C–Cl), 116.0–125.5 ppm (C–Br) and 95.41–101.6 ppm (C–I). When comparing the chemical shifts of CF, CCl, CBr, and CI, the peaks move progressively upfield as the halogen atom increases in size and decreases in electronegativity [45], which might be due to the spin-orbital heavy-atom on the light-atom (SO-HALA) effect [46]. Whereas methyl substituted orthoacetamol azo (**14–16**) showed a peak at 137.3–141.1 ppm, representing the methyl group (CCH₃) and methoxy substituted orthoacetamol azo (**17–19**) showed a peak at 54.5–56.0 ppm, attributed to the methoxy group (COCH₃).

***In vitro* anti-inflammatory analysis.** Prior to evaluating nitric oxide (NO) inhibition, cell viability assays were performed to confirm that any reduction in NO was due to specific inhibition and not general cytotoxicity. As tabulated in Table 1, all compounds **1–19** maintained cell viability above 80% at the concentration of 50 μM. Fluorinated derivatives showed slightly lower viability, consistent with the previous findings reported that fluorine substitution can increase cytotoxicity [47]. Given their acceptable cell viability, all compounds **1–19** were subjected to the NO inhibition assays.

NO is a key inflammatory mediator produced by activated macrophages through the enzyme inducible

Table 1. Cell viability and NO inhibition against LPS-induced RAW 264.7 macrophages at 50 μ M

Compound	R	Cell viability, %	NO inhibition, %
1	H	100	54.8 $\pm$ 0.5
2	<i>o</i> -F	97.5 $\pm$ 2.8	49.4 $\pm$ 1.6
3	<i>m</i> -F	98.9 $\pm$ 2.7	49.4 $\pm$ 1.3
4	<i>p</i> -F	100	53.9 $\pm$ 0.5
5	<i>o</i> -Cl	100	54.9 $\pm$ 1.9
6	<i>m</i> -Cl	97.8 $\pm$ 1.2	55.8 $\pm$ 1.4
7	<i>p</i> -Cl	100	60.2 $\pm$ 1.2
8	<i>o</i> -Br	100	60.5 $\pm$ 0.4
9	<i>m</i> -Br	100	57.7 $\pm$ 2.8
10	<i>p</i> -Br	100	92.8 $\pm$ 0.3
11	<i>o</i> -I	100	55.6 $\pm$ 0.3
12	<i>m</i> -I	100	55.1 $\pm$ 2.1
13	<i>p</i> -I	100	65.7 $\pm$ 1.8
14	<i>o</i> -CH ₃	100	54.8 $\pm$ 3.8
15	<i>m</i> -CH ₃	100	53.2 $\pm$ 2.0
16	<i>p</i> -CH ₃	100	65.3 $\pm$ 1.1
17	<i>o</i> -OCH ₃	100	60.6 $\pm$ 0.5
18	<i>m</i> -OCH ₃	100	59.9 $\pm$ 0.4
19	<i>p</i> -OCH ₃	100	68.2 $\pm$ 0.5

nitric oxide synthase (iNOS), in response to inflammatory stimuli such as LPS [48]. While physiologically important in vasodilation and immune defense, its excessive production during inflammation contributes to tissue damage [49]. Therefore, the inhibition of NO production serves as a key marker of anti-inflammatory potential.

The compounds demonstrated varying inhibitory effects on NO inhibitory activities, ranging from 49.4 $\pm$ 1.3 to 92.8 $\pm$ 0.3% inhibition at 50 μ M. Four compounds, **10** (*p*-Br), **13** (*p*-I), **16** (*p*-CH₃) and **19** (*p*-OCH₃), exhibited stronger activities with inhibition exceeding 65%. Among these, compound **10** (*p*-Br) demonstrated the highest activity, inhibiting 92.8 $\pm$ 0.3% NO production. This was significantly stronger than the *ortho*- and *meta*-halogenated analogues and suggests that *para*-bromination optimally modulates the molecule's electronic and steric properties for cellular targets such as iNOS or signaling pathways upstream of NO release. The potency of compound **10** (*p*-Br) may be attributed to bromine's optimal balance of size and polarizability, which fosters stronger van der Waals interactions and enhances fit within hydrophobic regions of the iNOS active site or regulatory proteins involved in NO production [50, 51]. In contrast, the larger iodine atom in **13** (*p*-I) likely introduces steric hindrance, interfering with

optimal binding [52]. Thus, I-substituted compounds **11**–**13** exhibited lower NO inhibition activities. Furthermore, it was observed that *para*-substituted derivatives such as compounds **4** (*p*-F), **7** (*p*-Cl), **10** (*p*-Br), **13** (*p*-I), **16** (*p*-CH₃), and **19** (*p*-OCH₃) consistently exhibited higher NO inhibition activities compared to their *ortho*- or *meta*-substituted compounds. This is attributed to the *para*-substitution, which minimizes steric hindrance, thereby allowing the molecule to adopt a favorable planar conformation for iNOS binding [53].

Following the initial NO inhibition screening, a prioritization threshold of 65% was established to identify

Table 2. Nitric oxide inhibitory effects of potential compounds and dexamethasone^a

Compound	R	IC ₅₀ ^b , μ M
10	<i>p</i> -Br	16.0 $\pm$ 0.6
13	<i>p</i> -I	33.5 $\pm$ 0.5
16	<i>p</i> -CH ₃	38.9 $\pm$ 1.0
19	<i>p</i> -OCH ₃	39.5 $\pm$ 0.3
Dexamethasone	–	16.9 $\pm$ 2.2

^a Data are presented as mean $\pm$ SD from three independent experiments.

^b Values with significant differences at $p < 0.05$.

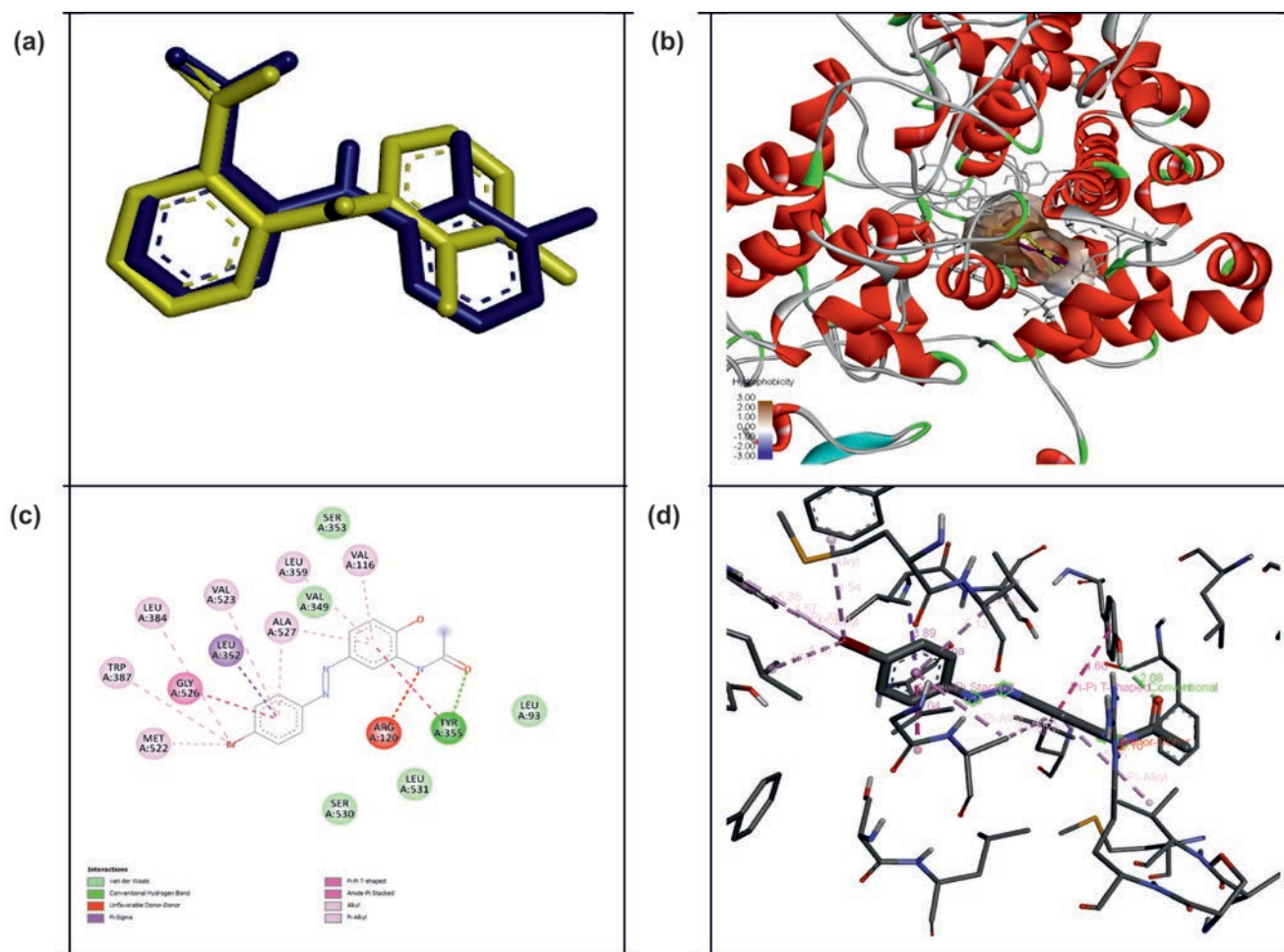


Fig. 1. (a) Superimposition of redocking mefenamic acid (blue stick model) and mefenamic acid crystal (yellow stick model); (b) compound **10** (*p*-Br) and mefenamic acid within the hydrophobic pocket of COX-2 (PDB ID: 5IKR); (c) the 2D schematic diagram of residues in the binding site of COX-2 (PDB ID: 5IKR) exhibiting hydrogen bond and hydrophobic interaction with compound **10** (*p*-Br); (d) The distances between hydrogen bonds and hydrophobic interactions involving amino acid residues of COX-2 (PDB ID: 5IKR) were visualized and analyzed using Discovery Studio 4.0.

the most potent candidates for further evaluation. The most active candidates, **10** (*p*-Br), **13** (*p*-I), **16** (*p*-CH₃), and **19** (*p*-OCH₃), which met the criterion, were further subjected to IC₅₀ values determination. As tabulated in Table 2, compound **10** (*p*-Br) showed the strongest inhibitory effect with an IC₅₀ of 16.0 ± 0.6 μM, which was comparable to that of the standard drug Dexamethasone (IC₅₀ = 16.9 ± 2.2 μM). In contrast, compounds **13** (*p*-I), **16** (*p*-CH₃), and **19** (*p*-OCH₃) exhibited weaker inhibition activities, with higher IC₅₀ values of 33.5, 38.9, and 39.5 μM, respectively.

Molecular docking. After the *in vitro* study, orthocetamol azo **10** (*p*-Br) was identified as the

promising lead compound, portraying an inhibitory effect comparable to Dexamethasone. Consequently, it was subjected to molecular docking analysis to understand the degree of interactions that contributed to the activity at the molecular level. Based on literature precedents, inhibiting enzymes such as COX-2 expression in LPS-induced RAW 264.7 macrophages had reduced the inflammation, thus contributing to suppression of LPS-induced ERK, JNK, and nuclear factor-κB (NF-κB) activation [54]. Thereby, the inhibition of COX-2 is considered an effective approach to treat inflammatory disease by playing a role in the mechanism of NO production inhibition. Hence, in this study, COX-2 (PDB ID: 5IKR) was selected as the

target receptor, and redocking of the inhibitor, mefenamic acid, present in the COX-2 was performed to verify the docking parameters used, ensuring the reproducibility of the docking outputs. The orientation of the best docking pose of the inhibitor was superimposed with the original crystal structure of the inhibitor at the binding site of the crystal structure. In COX-2, the root mean square derivative (RMSD) value between the top-ranked docked mefenamic acid conformation and the crystal pose of mefenamic acid was found to be 1.8690 Å (Fig. 1a). Moreover, it was observed that the docked conformation of the inhibitor (mefenamic acid) had a similar orientation to the crystallized inhibitor in the crystal structure. The redocking study suggests that AutoDock Vina has high accuracy in terms of predicting the binding interactions of the ligand of interest with the crystal structure (COX-2).

Under these validated docking parameters described, orthocetamol azo **10** (*p*-Br) showed commendable binding affinity for COX-2, with a binding energy of -7.2 kcal/mol. The hydrogen bond between the carboxyl group in **10** (*p*-Br) with TYR355 (2.08 Å) in the COX-2 binding pocket was proposed to make the key contribution to the inhibitory activity (Fig. 1c). Like most effective NSAID drugs, TYR355 serves as the primary contact point. TYR355 and ARG120 are an important amino acid that constitutes the catalytic gate of the COX enzyme [55], at which the occupancy of this site sterically hinders the entry of arachidonic acid, causing reduced prostaglandin production. While an unfavorable donor-donor interaction was noted with ARG120, a residue traditionally essential for COX-1 anchoring [56, 57], this repulsion likely facilitates the deep insertion of the **10** (*p*-Br) aromatic ring into the hydrophobic side pocket of COX-2 instead. This is evidenced by the presence of a π -alkyl interaction with VAL523, a residue unique to the COX-2 isoform [58], confirming the compound's potential selectivity toward COX-2. Besides, the analysis on compound **10** (*p*-Br) with COX-2 via Discovery Studio® 4.0 revealed additional van der Waals, π -sigma, π - π T-shaped, and alkyl interaction appeared between the ligand and receptor (Fig. 1c). It is worth mentioning that **10** (*p*-Br) also demonstrated an interaction with the amino acid residue SER530, mirroring the binding interactions previously reported in crystal structures of clinically established NSAIDs like diclofenac and lumiracoxib complexed with murine COX-2 [59]. However, despite these computational insights suggesting a potential preference for COX-2, an experimental *in vitro*

enzymatic study is necessary to validate these findings and definitively establish the COX-1/COX-2 selectivity profile. Collectively, the discussed interactions combined with the high binding affinity provide a structural rationale for the potent anti-inflammatory activity observed in the *in vitro* NO inhibition assay, characterizing **10** (*p*-Br) as a promising lead anti-inflammatory agent.

Druglikeness properties screening. The druglikeness of the synthesized orthocetamol azo derivatives **1–19** was computationally screened using SwissADME and pkCMS platforms, and was compared with the standard, Dexamethasone and Sulfasalazine. Sulfasalazine is an FDA-approved anti-inflammatory drug to treat the joints and gastrointestinal tract, including Crohn's disease and ulcerative colitis [60]. Sulfasalazine was selected due to its structural similarity, particularly the presence of azo, carbonyl, and hydroxyl functional groups.

Preliminary assessment of druglikeness confirmed that the entire series **1–19** strictly adheres to Lipinski's Rule of Five (Ro5) criteria. All derivatives occupied a favorable physicochemical range, characterized by molecular weights below 500 g/mol, lipophilicity ($\log P$) values under 5, and hydrogen bond donor and acceptor count well within the established thresholds [28]. The *in silico* ADMET screening revealed that analogues **1–19** demonstrated better Human Intestinal Absorption (HIA), ranging from 86.22 to 90.66%, significantly outperforming Sulfasalazine (64.51%). Moreover, compound **1–19** exhibits favorable moderate to high Caco-2 cell permeability ($\log P_{app}$) of 0.5 to 0.9 [61]. Besides, the low to moderate blood-brain barrier (BBB) permeability values ($\log BB < 1$) [62] of **1–19** suggest negligible central nervous system penetration and a reduced risk of neurotoxicity [63], which is a desirable characteristic for a peripheral anti-inflammatory agent [64]. Regarding metabolism, while most derivatives were predicted to be inhibitors of major CYP450 isoforms, compounds **1–19** exhibited favorable clearance rates of 0.72 to 1.39 $\log (\text{min}^{-1} \cdot \text{kg}^{-1})$ and high half-life profiles, suggesting sustained therapeutic coverage and reduced dosing frequency [65]. Despite most derivatives being predicted to be safe, compound **1**, fluorinated series (**2–4**), methoxy series (**17–19**) and standard Sulfasalazine were flagged as toxic in AMES mutagenicity.

Notably, compound **10** (*p*-Br) demonstrated an optimal balance of these properties as tabulated in Table 3. It fully complies with Lipinski's criteria ($M = 334.17$ g/mol, $\log P = 3.29$) and exhibits high intestinal absorption

Table 3. Pharmacokinetic and toxicity profile of compound **10** (*p*-Br) and reference standards

Parameter	10	Dexamethasone	Sulfasalazine
Molecular weight, g/mol	334.17	392.46	398.39
Lipophilicity (log <i>P</i>)	3.29	2.15	2.22
Intestinal absorption, %	87.66	80.49	64.51
BBB permeability (log BB)	−0.45	−0.85	−1.76
AMES mutagenesis	Safe	Safe	Toxic
Half-life profile	High	Low	High

(87.66%) alongside moderate Caco-2 permeability (0.655). Crucially, its BBB permeability (−0.45) indicates it is non-permeable to the CNS, and unlike fluorinated substituted (**1–4**) and methoxy substituted (**17–19**) derivatives in the series. Moreover, compound **10** (*p*-Br) was also flagged as safe in the AMES mutagenicity assessment. These results, supported by the promising *in vitro* data, reinforce the potential of **10** (*p*-Br) as a promising lead candidate for peripheral anti-inflammatory development. Comprehensive Lipinski and ADMET data for the full series (**1–19**) are provided in Tables S1 and S2 (see Supplementary Information).

CONCLUSIONS

A series of orthocetamol azo **1–19** was successfully synthesized and evaluated for anti-inflammatory potential. Primary *in vitro* screening against LPS-induced RAW 264.7 macrophages identified the four high-potency candidates of **10** (*p*-Br), **13** (*p*-I), **16** (*p*-CH₃), and **19** (*p*-OCH₃), all bearing a substituent at the *para*-position, has met the prioritization threshold of >65% NO inhibition and proceeded for IC₅₀ determination. Notably, among these, compound **10** (*p*-Br) emerged as a promising lead by exhibiting exceptional potency inhibition with 92.8%, and an IC₅₀ value of 16.0±0.6 μM, comparable to standard Dexamethasone, 16.9±2.2 μM. The *in silico* molecular docking further evidenced the structural rationale of **10** (*p*-Br) through the stable hydrogen bonding with TYR355 residue, a construction site of the COX-2, alongside a predicted isoform-selective π -alkyl interaction with VAL523 pronounced with a binding affinity of −7.2 kcal/mol. Furthermore, the observed donor-donor repulsion with ARG120 suggests a reduced affinity for the COX-1 anchor, potentially enhancing its safety profile. While these *in silico* results offer a structural basis for COX-2 preference, further *in vitro* enzymatic inhibition assays are required to confirm this selectivity profile experimentally. Coupled

with a favorable pharmacokinetic profile of ADMET for bioavailability and strict compliance with Lipinski's Rule of Five, these findings characterize compound **10** (*p*-Br) as a promising lead for the development of potential selective anti-inflammatory agents.

EXPERIMENTAL

All reagents and chemicals were used without further purification. Melting points were determined using an open tube capillary (Stuart SMP3) without any corrections. Infrared (IR) spectra were obtained using a Thermo Fisher Scientific Smart Omni Transmission Nicolet IS10 Fourier Transform Infrared Spectrometer (FTIR). ¹H NMR and ¹³C NMR spectra were recorded on a JEOL ECA 500 spectrometer operating at 500 MHz (¹H) and 125 MHz (¹³C), with chemical shifts referenced to DMSO-*d*₆ or CDCl₃ as the standard. Elemental CHNS analysis was conducted using the CHNS Analyzer THERMO-FLASH EA 1112 Series.

General procedure for the synthesis of orthocetamol azo derivatives (1–19**).** A solution of substituted aniline derivatives (1 mmol) with HCl (6 mL, 2 M) in water (10 mL) was cooled in an ice bath (0–5°C) with constant stirring. Sodium nitrite solution (5 mL, 1 M) was added slowly while maintaining the temperature. The reaction was monitored for 10–15 min using potassium iodide paper to ensure the formation of diazonium salt [18]. A solution of orthocetamol (12 mmol) in NaOH (2 mL, 2 M) was added dropwise to the reaction mixture. The pH of the solution was adjusted with NaOH (2 M) solution until pH 10–12. The reaction mixture was stirred and monitored using TLC with ethyl acetate as eluent. Upon completion, the reaction was acidified using 50% HCl to adjust the pH to 2–3 to promote precipitation. The mixture was filtered and rinsed with cold distilled water. The solid obtained was dried in vacuum and recrystallized with hot hexane to give orthocetamol azo derivatives **1–19** in 31–95% yield.

(*E*)-*N*-[2-Hydroxy-5-(phenyldiazenyl)phenyl]-acetamide (1). Yield 41%, pale yellowish-brown solid, mp 189–190°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1094 (C–O), 1276 (C–N), 1421 (N=N), 1587 (Ar–C), 1641 (C=O), 3062 (OH), 3374 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 7.04 d (1H, J 8.5 Hz), 7.50 t (1H, J 7.3 Hz), 7.56 t (2H, J 7.3 Hz), 7.60 d (1H, J 8.5 Hz), 7.81 d (2H, J 9.0 Hz), 8.48 s (1H), 9.36 s (1H, OH), 10.78 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 24.3 (C–CH₃), 115.6, 115.9, 121.8, 122.6, 127.7, 129.8, 131.1, 145.4, 151.6, 152.6, 169.6 (C=O).

(*E*)-*N*-{5-[(2-Fluorophenyl)diazenyl]-2-hydroxyphenyl}acetamide (2). Yield 54%, yellowish orange solid, mp 178–179°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1095 (C–O), 1287 (C–N), 1429 (N=N), 1652 (C=O), 3033 (OH), 3408 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 7.07 d (1H, J 8.4 Hz), 7.31 t (1H, J 7.2 Hz), 7.45 t (1H, J 9.5 Hz), 7.53 s (1H), 7.61–7.69 m (2H), 8.50 s (1H), 9.38 s (1H, OH), 10.93 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 24.3 (C–CH₃), 115.8 d ($^2J_{\text{CF}}$ 27.6 Hz), 117.6 ($^2J_{\text{CF}}$ 19.3 Hz), 118.0, 122.3, 125.4, 127.8, 132.8 d ($^3J_{\text{CF}}$ 8.4 Hz), 140.5 d ($^3J_{\text{CF}}$ 7.3 Hz), 145.7, 152.2, 159.4 d ($^1J_{\text{CF}}$ 252.0 Hz), 169.7 (C=O).

(*E*)-*N*-{5-[(3-Fluorophenyl)diazenyl]-2-hydroxyphenyl}acetamidephenol (3). Yield 39%, pale yellowish-brown solid, mp 201–202°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1098 (C–O), 1276 (C–N), 1424 (N=N), 1587 (C=O), 3079 (OH), 3265 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.15 s (3H), 7.07 t (1H, J 9.5 Hz), 7.35 d (1H, J 8.6 Hz), 7.54–7.58 m (1H), 7.62 d (2H, J 6.3 Hz), 7.71 t (2H, J 8.9 Hz), 8.51 d (1H, J 8.0 Hz), 9.36 s (1H, OH), 10.95 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 24.3 (C–CH₃), 107.6 d ($^3J_{\text{CF}}$ 21.5 Hz), 107.8, 115.7 d ($^2J_{\text{CF}}$ 27.6 Hz), 117.5 d ($^2J_{\text{CF}}$ 21.6 Hz), 122.4, 123.0, 127.8, 131.6 d ($^3J_{\text{CF}}$ 8.4 Hz), 145.2, 152.2, 154.3, 163.3 d ($^1J_{\text{CF}}$ 244.7 Hz), 169.6 (C=O).

(*E*)-*N*-{5-[(4-Fluorophenyl)diazenyl]-2-hydroxyphenyl}acetamide (4). Yield 72%, brown solid, mp 197–198°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1090 (C–O), 1276 (C–N), 1420 (N=N), 1587 (Ar–C), 1652 (C=O), 3057 (OH), 3395 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 7.04 d (1H, J 8.6 Hz), 7.38 d (2H, J 8.7, 2.5 Hz), 7.58 d (1H, J 8.6, 2.3 Hz), 7.86–7.89 m (2H), 8.47 d (1H, J 1.7 Hz), 9.35 s (1H, OH), 10.81 s (1H, NH). ^{13}C NMR spectrum (125 MHz,

DMSO- d_6), δ_{C} , ppm: 24.3 (C–CH₃), 115.6, 115.9, 116.8 d ($^2J_{\text{CF}}$ 22.3 Hz), 117.0, 121.8, 124.8 d ($^3J_{\text{CF}}$ 8.4 Hz), 127.7, 145.2, 149.3, 151.7, 163.8 ($^1J_{\text{CF}}$ 247.1 Hz), 169.6 (C=O).

(*E*)-*N*-{5-[(2-Chlorophenyl)diazenyl]-2-hydroxyphenyl}acetamide (5). Yield 40%, orange solid, mp 211–212°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1097 (C–O), 1286 (C–N), 1458 (N=N), 1588 (Ar–C), 1662 (C=O), 3066 (OH), 3405 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 7.07 d (1H, J 8.6 Hz), 7.46 t. d (2H, J 14.9, 7.4 Hz), 7.60–7.66 m (3H), 8.52 s (1H), 9.38 s (1H, OH), 10.97 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 24.3 (C–CH₃), 116.0, 116.3, 118.1, 122.3, 127.8, 128.5, 131.1, 132.2, 133.7, 145.7, 148.6, 152.4, 169.7 (C=O).

(*E*)-*N*-{5-[(3-Chlorophenyl)diazenyl]-2-hydroxyphenyl}acetamide (6). Yield 37%, orange solid, mp 223–224°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1099 (C–O), 1278 (C–N), 1423 (N=N), 1584 (Ar–C), 1613 (C=O), 3073 (OH), 3256 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 7.06 d (1H, J 8.6 Hz), 7.54–7.63 m (3H), 7.79 t (2H, J 7.2 Hz), 8.49 s (1H), 9.35 s (1H, OH), 10.96 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 24.3 (C–CH₃), 115.6, 115.9, 121.0, 122.5, 122.7, 127.8, 130.5, 131.7, 134.6, 145.2, 152.3, 153.7, 169.6 (C=O).

(*E*)-*N*-{5-[(4-Chlorophenyl)diazenyl]-2-hydroxyphenyl}acetamide (7). Yield 81%, yellow solid, mp 242–243°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1089 (C–O), 1265 (C–N), 1387 (N=N), 1586 (Ar–C), 1650 (C=O), 3035 (OH), 3333 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 7.05 d (1H, J 8.6 Hz), 7.59–7.62 m (3H), 7.83 d (2H, J 8.6 Hz), 8.49 s (1H), 9.35 s (1H, OH), 10.87 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 23.8 (C–CH₃), 115.1, 115.4, 121.6, 123.8, 127.2, 129.4, 134.9, 144.7, 150.7, 151.4, 169.1 (C=O).

(*E*)-*N*-{5-[(2-Bromophenyl)diazenyl]-2-hydroxyphenyl}acetamide (8). Yield 55%, orange solid, mp 205–206°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1095 (C–O), 1284 (C–N), 1456 (N=N), 1586 (Ar–C), 1657 (C=O), 3060 (OH), 3393 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 7.07 d (1H, J 8.60 Hz), 7.40–7.42 m (1H), 7.49 t (1H, J 7.4 Hz), 7.59 d (1H, J 8.0 Hz), 7.63 d (1H, J 10.9 Hz), 7.83 d (1H, J 7.4 Hz), 8.52 s (1H), 9.37 s (1H, OH), 10.92 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 24.32 (C–CH₃), 116.05, 116.64, 118.28, 122.03, 124.36, 127.82,

129.14, 132.39, 134.13, 145.65, 149.61, 152.37, 169.70 (C=O).

(E)-N-{5-[(3-Bromophenyl)diazenyl]-2-hydroxyphenyl}acetamide (9). Yield 49%, brown solid, mp 215–216°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1095 (C–O), 1284 (C–N), 1456 (N=N), 1586 (Ar–C), 1661 (C=O), 3062 (OH), 3372 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 7.07 d (1H, J 8.6 Hz), 7.40–7.42 m (1H), 7.49 t (1H, J 7.4 Hz), 7.59 d (1H, J 8.0 Hz), 7.63 d (1H, J 10.9 Hz), 7.83 d (1H, J 7.4 Hz), 8.52 s (1H), 9.37 s (1H, OH), 10.92 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 24.4 (C–CH₃), 115.6, 115.9, 122.5, 123.0, 123.2, 123.7, 127.8, 132.0, 133.4, 145.2, 152.3, 153.8, 169.6 (C=O).

(E)-N-{5-[(4-Bromophenyl)diazenyl]-2-hydroxyphenyl}acetamide (10). Yield 63%, brownish orange solid, mp 216–217°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1099 (C–O), 1275 (C–N), 1457 (N=N), 1586 (Ar–C), 1645 (C=O), 3072 (OH), 3391 (NH). ^1H NMR spectrum (500 MHz, CDCl₃), δ , ppm: 2.29 s (3H), 7.65 d (1H, J 8.6 Hz), 7.70 d (1H, J 8.6 Hz), 7.77 d (2H, J 8.6 Hz), 7.82 d (2H, J 8.6 Hz), 7.91 s (1H), 8.27 s (1H, NH), 9.09 s (1H, OH). ^{13}C NMR spectrum (125 MHz, CDCl₃), δ_{C} , ppm: 25.0 (C–CH₃), 116.3, 123.5, 124.5, 125.3, 128.9, 132.4, 133.0, 135.6, 145.8, 146.8, 148.0, 151.3, 168.6 (C=O).

(E)-N-[2-Hydroxy-5-[(2-iodophenyl)diazenyl]-phenyl]acetamide (11). Yield 71%, yellowish orange solid, mp 223–224°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1097 (C–O), 1281 (C–N), 1457 (N=N), 1588 (Ar–C), 1657 (C=O), 3053 (OH), 3389 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 7.07 d (1H, J 8.6 Hz), 7.22–7.26 m (1H), 7.48–7.53 m (2H), 7.63 d. d (1H, J 8.6, 2.3 Hz), 8.06 d (1H, J 8.6 Hz), 8.54 s (1H), 9.37 s (1H, OH), 10.91 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 24.3 (C–CH₃), 102.2, 116.0, 117.1, 117.6, 121.7, 127.8, 129.8, 132.6, 140.2, 145.4, 151.5, 152.3, 169.6 (C=O).

(E)-N-[2-Hydroxy-5-[(3-iodophenyl)diazenyl]-phenyl]acetamide (12). Yield 50%, yellowish brown solid, mp 212–213°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1093 (C–O), 1277 (C–N), 1421 (N=N), 1589 (Ar–C), 1644 (C=O), 3059 (OH), 3376 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 7.05 d (1H, J 8.6 Hz), 7.37 t (1H, J 7.6 Hz), 7.62 d (1H, J 7.6 Hz), 7.85 d (2H, J 7.6 Hz), 8.08 s (1H), 8.49 s (1H), 9.35 s (1H, OH), 10.91 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 24.4 (C–CH₃), 95.9, 115.6, 115.9,

122.5, 123.6, 127.8, 129.6, 132.0, 139.3, 145.2, 152.2, 153.6, 169.6 (C=O).

(E)-N-[2-Hydroxy-5-[(4-iodophenyl)diazenyl]-phenyl]acetamide (13). Yield 64%, brown solid, mp 219–220°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1096 (C–O), 1277 (C–N), 1423 (N=N), 1590 (Ar–C), 1643 (C=O), 3096 (OH), 3313 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.13 s (3H), 7.04 d (1H, J 8.6 Hz), 7.59 d (3H, J 6.9 Hz), 7.92 d (2H, J 8.6 Hz), 8.48 s (1H), 9.34 s (1H, OH), 10.86 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 24.4 (C–CH₃), 98.0, 115.7, 115.9, 122.1, 124.6, 124.7, 127.8, 138.8, 138.9, 145.3, 152.0, 169.6 (C=O).

(E)-N-[2-Hydroxy-5-(*o*-tolyl diazenyl)phenyl]acetamide (14). Yield 47%, brown solid, mp 189–190°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1091 (C–O), 1276 (C–N), 1426 (N=N), 1594 (Ar–C), 1646 (C=O), 3036 (OH), 3380 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 2.63 s (3H), 7.05 d (1H, J 8.6 Hz), 7.27–7.30 m (1H), 7.38 d (2H, J 5.2 Hz), 7.51 d (1H, J 8.0 Hz), 7.59 d. d (1H, J 8.6, 2.3 Hz), 8.49 s (1H), 9.36 s (1H, OH), 10.74 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 17.6 (C–CH₃), 24.3 (C–CH₃), 115.5, 116.0, 116.1, 121.4, 127.1, 127.7, 130.9, 131.8, 137.3, 146.0, 150.6, 151.5, 169.7 (C=O).

(E)-N-[2-Hydroxy-5-(*m*-tolyl diazenyl)phenyl]acetamide (15). Yield 31%, yellow solid, mp 219–220°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1096 (C–O), 1277 (C–N), 1482 (N=N), 1585 (Ar–C), 1610 (C=O), 3086 (OH), 3263 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.14 s (3H), 2.40 s (3H), 7.04 d (1H, J 8.6 Hz), 7.31 d (1H, J 6.9 Hz), 7.43 d (1H, J 15.5 Hz), 7.60 d (2H, J 8.6 Hz), 7.63 s (1H), 8.47 s (1H), 9.34 s (1H, OH), 10.76 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 21.4 (C–CH₃), 24.4 (C–CH₃), 115.7, 115.9, 120.3, 121.7, 122.7, 127.7, 129.7, 131.7, 139.3, 145.5, 151.6, 152.7, 169.6 (C=O).

(E)-N-[2-Hydroxy-5-(*p*-tolyl diazenyl)phenyl]acetamide (16). Yield 66%, yellow solid, mp 236–237°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 096 (C–O), 1283 (C–N), 1423 (N=N), 1591 (Ar–C), 1658 (C=O), 3030 (OH), 3409 (NH). ^1H NMR spectrum (500 MHz, DMSO- d_6), δ , ppm: 2.08 s (3H), 2.38 s (3H), 7.04 d (1H, J 5.7 Hz), 7.36 d (2H, J 6.3 Hz), 7.57 d (1H, J 8.60 Hz), 7.73 d (2H, J 8.0 Hz), 8.45 s (1H), 9.34 s (1H, OH), 10.73 s (1H, NH). ^{13}C NMR spectrum (125 MHz, DMSO- d_6), δ_{C} , ppm: 21.5 (C–CH₃), 24.3 (C–CH₃), 115.6, 115.9, 121.6, 122.7, 127.7, 130.4, 141.1, 145.4, 150.7, 151.4, 169.6, 207.0 (C=O).

(*E*)-*N*-{2-Hydroxy-5-[(2-methoxyphenyl)diazenyl]-phenyl}acetamide (17). Yield 95%, brownish-orange solid, mp 196–197°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1095 (C–O), 1278 (C–N), 1485 (N=N), 1588 (Ar–C), 1656 (C=O), 2937 (OH), 3356 (NH). ^1H NMR spectrum (500 MHz, $\text{DMSO}-d_6$), δ , ppm: 2.14 s (3H), 3.93 s (3H), 7.01–7.05 m (2H), 7.22 d (1H, J 8.6 Hz), 7.46 q (2H, J 7.6 Hz), 7.55 d (1H, J 8.6 Hz), 8.38 s (1H, NH), 9.39 s (1H, OH). ^{13}C NMR spectrum (125 MHz, $\text{DMSO}-d_6$), δ_{C} , ppm: 23.8 (C–CH₃), 56.0 (C–OCH₃), 113.3, 115.4, 115.5, 116.3, 120.6, 121.3, 127.1, 131.9, 141.7, 145.5, 151.0, 156.2, 169.2 (C=O).

(*E*)-*N*-{2-Hydroxy-5-[(3-methoxyphenyl)diazenyl]-phenyl}acetamide (18). Yield 62%, brownish-orange solid, mp 171–172°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1100 (C–O), 1278 (C–N), 1482 (N=N), 1587 (Ar–C), 1679 (C=O), 3073 (OH), 3262 (NH). ^1H NMR spectrum (500 MHz, $\text{DMSO}-d_6$), δ , ppm: 2.14 s (3H), 3.84 s (3H), 7.04 d (1H, J 2.3 Hz), 7.07 d (1H, J 7.4 Hz), 7.33 t (1H, J 2.3 Hz), 7.45 m (2H), 7.59 d (1H, J 8.6 Hz), 8.47 s (1H), 9.35 s (1H, OH), 10.82 s (1H, NH). ^{13}C NMR spectrum (125 MHz, $\text{DMSO}-d_6$), δ_{C} , ppm: 23.0 (C–CH₃), 54.5 (C–OCH₃), 104.8, 114.4, 114.5, 114.9, 116.0, 120.5, 126.3, 129.3, 143.9, 150.4, 152.5, 159.2, 168.2 (C=O).

(*E*)-*N*-{2-Hydroxy-5-[(4-methoxyphenyl)diazenyl]-phenyl}acetamide (19). Yield 82%, yellow solid, mp 206–207°C. IR spectrum (ATR), $\nu_{\max}$, cm^{-1} : 1095 (C–O), 1239 (C–N), 1416 (N=N), 1548 (Ar–C), 1593 (C=O), 3076 (OH), 3268 (NH). ^1H NMR spectrum (500 MHz, $\text{DMSO}-d_6$), δ , ppm: 2.14 s (3H), 3.84 s (3H), 7.02 d (1H, J 8.6 Hz), 7.09 d (2H, J 9.2 Hz), 7.53 d (1H, J 8.6 Hz), 7.81 d (2H, J 9.2 Hz), 8.41 s (1H), 9.35 s (1H, OH), 10.67 s (1H, NH). ^{13}C NMR spectrum (125 MHz, $\text{DMSO}-d_6$), δ_{C} , ppm: 23.8 (C–CH₃), 55.6 (C–OCH₃), 114.5, 115.0, 115.4, 120.7, 124.0, 127.1, 144.9, 146.3, 150.5, 161.3, 169.1 (C=O).

MTT cell viability assay. The murine macrophage cell line, RAW 264.7 (TIB-71), was obtained from American Type Culture Collection (ATCC) and was cultivated in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo-Fisher Scientific) supplemented with 10% fetal bovine serum (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO₂. The reduction of pale-yellow substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan product was performed to measure the cell viability according to a previous literature procedure with some modifications [22]. Briefly, RAW 264.7 cells were seeded

(2×10^6 cells/mL) and incubated overnight at 37°C and 5% CO₂, followed by treatment of lipopolysaccharide (LPS; Merck) (5 $\mu\text{g/mL}$) in the presence of compounds or in the absence of compounds (negative control) for 24 h. After that, 100 μL of medium was removed, and 10 μL of 5 mg/mL MTT was added and further incubated for 3 h. The medium was then replaced by DMSO before measuring the absorbance at 540 nm. The percentage of cell viability was calculated as $A_1/A_0 \times 100\%$, where A_1 is the absorbance of treated cells, and A_0 is the absorbance of the negative control [23].

Nitric oxide production inhibitory assay.

Compounds that maintained at least 80–90% cell viability in preliminary MTT cell viability assay were considered for NO evaluation to ensure that any reduction in NO levels was due to anti-inflammatory activity rather than cell death [24]. Griess assay was performed to evaluate the inhibition effect of non-toxic compounds against nitric oxide (NO) production in LPS-induced RAW 264.7 cells according to previously published literature with some modifications. Briefly, RAW 264.7 cells (2×10^6 cells/mL) were seeded for 24 h before being treated with LPS (5 $\mu\text{g/mL}$) and compounds for 24 h [3]. Then, 100 μL of the cell supernatant was aliquoted and added to 100 μL of Griess reagent. The plates were incubated for 10 min at room temperature, followed by absorbance measurement at 550 nm. Dexamethasone was used as a positive control, and the percentage of NO production inhibition was calculated as

$$\frac{A_0 - A_1}{A_0} \times 100,$$

where A_1 is the absorbance of treated cells, and A_0 is the absorbance of the negative control [25]. A primary screening threshold of 65% NO inhibition was established to identify the most potent candidates, and those that met the criterion were proceeded for IC₅₀ determination.

Statistical analysis. The data were shown as mean $\pm$ SD from three independent experiments. The statistical analyses were carried out by one-way analysis of variance (ANOVA), followed by Tukey's test in Graph Pad Prism version 10 at a 5% significance level.

Molecular docking. The 2D structure of selected compound **10** (*p*-Br) was drawn using Discovery Studio® 4.0 (Accelrys, San Diego, USA) and saved in PDB format, followed by structural optimization using DS 4.0 via the smart minimizer algorithm. The crystal structure of

COX-2 (PDB ID:5IKR) enzyme was downloaded from the Protein Data Bank (PDB). Hydrogen atoms were added to the COX-2 protein structure using AutoDockTools [26], and the docking was performed using AutoDock vina [27]. The grid box for COX-2 was set to 30 by 30 by 30 with 1.0 Å spacing. The grid box was set to be 1.0 Å so that all the residues were available in an equal-opportunity zone for ligand binding. The docked ligand-binding sites were ranked according to their binding energies. The highest binding affinities were selected, and the binding interactions were visualized using Discovery Studio® 4.0 (Accelrys, USA). This ligand conformation was carefully inspected, analyzed, and discussed. To verify the docking parameters, redocking of the inhibitors, mefenamic acid, present in the COX-2 crystal protein structure, was done following the previously set parameters.

***In silico* ADMET prediction and druglikeness evaluation.** The druglikeness and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties of orthocetamol azo derivatives **1–19** were computationally assessed to predict their potential pharmacokinetic profile and anti-inflammatory activity [28]. This *in silico* screening, which includes an initial evaluation using Lipinski's Rule of Five, was performed using SwissADME and pkCSM tools [29], providing crucial insights into their suitability as therapeutic candidates, helping to streamline the identification of promising compounds for further in-depth *in vitro* and *in vivo* studies [30].

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CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

SUPPLEMENTARY INFORMATION

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