EFFECT OF ORPHENADRINE–DICLOFENAC COMBINATION ON COX-2 EXPRESSION AND EXPLORATORY BEHAVIOUR IN ACETIC ACID–INDUCED NOCICEPTION IN MURINE MODELS

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BENIN CITY.

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A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY, FACULTY OF PHARMACY, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHARMACY (PHARM.D) DEGREE.

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CERTIFICATION

We the undersigned hereby certify that this work was carried out by **BEVERLY AWARITOMA OGBUEHI** with matriculation number **PHA1908557**, in the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City.

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DEDICATION

This project is dedicated God Almighty, whose grace, wisdom, and strength have guided me throughout this journey.

I also dedicate it to my family and friends for their constant love, prayers, and encouragement, and to my lecturers and mentors for their guidance and support in my academic pursuit.

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I sincerely give all thanks and glory to God Almighty for His abundant grace, wisdom, and strength that have guided me through every stage of this work and my academic journey. Without Him, none of this would have been possible.

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To my dad, Barr. Kelechi Ogbuehi and my mom, Mrs Sonia Ogbuehi**,** I owe my deepest gratitude. Thank you for your endless love, prayers, and sacrifices. To my siblings; Odera, Sharon, Obidike and Michelle**,** thank you for your encouragement and belief in me.

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Special thanks go to my biggest cheerleader, my best friend Great-Nelson. Thank you for always being there, for your understanding, encouragement, and care, which made this journey so much lighter. I am truly grateful to have you by my side.

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ABSTRACT

Background: Pain and inflammation remain major health concerns that reduce quality of life despite many available treatments. Diclofenac, a nonsteroidal anti-inflammatory drug, and orphenadrine, a centrally acting muscle relaxant, target different pain pathways. This study investigates whether their combined use enhances analgesic and anti-inflammatory effects, offering a safer and more effective approach to pain management.

Method: Twenty-four Swiss albino mice were divided into four groups to receive saline, diclofenac (50 mg/kg), orphenadrine (25 mg/kg) and ophenadrine-diclofenac (25 mg/kg-50 mg/kg). Analgesic effects were assessed using the acetic acid–induced writhing and hole-board tests, while COX inhibition was evaluated using the quantitative polymerase chain reaction assay. Data were expressed as Mean ± SEM and analyzed using one-way ANOVA, with significance set at $p < 0.05$.

Result: Diclofenac (1.67 ± 0.76), orphenadrine (2.17 ± 0.95), and their combination (0.00 ± 0.00) significantly reduced writhes compared to control (6.00 ± 1.53; $p < 0.05$). COX-2 levels were markedly lower in diclofenac (88.32 ± 1.18), orphenadrine (27.59 ± 2.26), and combination (68.30 ± 1.43) treated groups versus control (95.26 ± 1.88). In the hole-board test, the combination group (24.33 ± 1.59) maintained head dips comparable to the control group.

Conclusion: Diclofenac and orphenadrine provide complementary pain relief, with diclofenac acting peripherally and orphenadrine centrally. Their combination synergistically abolished writhing, balanced COX-2 activity, and preserved normal exploratory behaviour. These results highlight the diclofenac–orphenadrine combination as an effective multimodal analgesic that enhances pain control while supporting central function.

CHAPTER ONE

LITERATURE REVIEW

1.0 INTRODUCTION

Pain and inflammation are among the most prevalent clinical manifestations encountered in medicine and represent significant global health challenges. They not only signify tissue injury but also contribute substantially to disability, emotional distress, and socioeconomic burden (Goldberg & McGee, 2017; Miller & Malfait, 2020). At the cellular level, pain perception involves complex neurochemical interactions within the peripheral and central nervous systems, integrating nociceptive, emotional, and cognitive components (Basbaum *et al*., 2009; Dehghan *et al*., 2024). Inflammatory disorders such as arthritis and autoimmune diseases often underlie chronic pain conditions, and their pathogenesis is mediated through activation of molecular pathways including Toll-like receptor 4 (TLR4) and NF-κBsignaling cascades, which amplify the production of pro-inflammatory cytokines (Nathan, 2002; Saleh *et al*., 2021). In developing countries such as Nigeria, inadequate access to effective analgesics and limited pain management infrastructure further exacerbate the burden of pain and inflammatory diseases (Igbe *et al*., 2012).

Current pharmacologic strategies for managing pain and inflammation primarily rely on nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and centrally acting muscle relaxants (Freynhagen *et al*., 2016; Rainsford, 2013). NSAIDs such as diclofenac exert their effects through inhibition of cyclooxygenase (COX) enzymes, thereby reducing prostaglandin biosynthesis and peripheral sensitization (Smith *et al*., 2011; Brune &Patrignani, 2015). Diclofenac, in particular, acts as a nonselective COX inhibitor with additional ability to modulate macrophage peroxisome proliferator-activated receptor-γ (PPAR-γ) activity, leading to anti-inflammatory cytokine production (Ayoub *et al*., 2009). Despite their efficacy,

prolonged NSAID use is associated with gastrointestinal, renal, and cardiovascular toxicity (FitzGerald, 2004; Wallace, 2008). Opioid analgesics, though effective for severe pain, are limited by risks of dependence, tolerance, and respiratory depression (Volkow & McLellan, 2018).

Centrally acting muscle relaxants such as orphenadrine, tizanidine, and cyclobenzaprine are often employed in the management of musculoskeletal and neuropathic pain (Hoy, 2016). Orphenadrine, a derivative of diphenhydramine, exerts its analgesic effects via multiple mechanisms, including NMDA receptor antagonism and inhibition of central excitatory neurotransmission (Zhou *et al*., 2018). It also possesses antihistaminic and anticholinergic properties that contribute to its central modulatory effects on pain processing. The NMDA receptor plays a key role in central sensitization and chronic pain development; thus, its modulation represents a critical therapeutic target (Knabl *et al*., 2008; Taylor *et al*., 2016).

Given the limitations of monotherapy, contemporary pain research emphasizes multimodal analgesia, which involves the combination of drugs acting through complementary mechanisms to enhance efficacy while reducing toxicity (Kehlet & Dahl, 2018; White *et al*., 2017). This concept supports the concurrent use of peripherally acting NSAIDs and centrally acting agents to target both peripheral and central pain pathways. The combination of diclofenac and orphenadrine exemplifies this strategy—diclofenac acting through COX inhibition and suppression of prostaglandin synthesis (Rainsford, 2013), and orphenadrine modulating NMDA-mediated neurotransmission to reduce central pain amplification (Zhou *et al*., 2018; Hoy, 2016).

Recent clinical and preclinical studies have demonstrated improved analgesic efficacy of the diclofenac–orphenadrine combination compared with either drug alone, particularly in postoperative and musculoskeletal pain models (Tomic *et al*., 2019; Zeiner *et al*., 2023).

However, detailed mechanistic investigations elucidating their synergistic interaction, especially within neurobehavioral and biochemical paradigms, remain limited. Preclinical models such as the acetic acid-induced writhing test (Collier *et al*., 1968; Ribeiro *et al*., 2000) and hole-board test (File &Wardill, 1975; Takeda *et al*., 1998) are valuable tools for assessing both peripheral nociception and central emotional modulation of pain. Additionally, biochemical evaluation of COX enzyme activity offers mechanistic insight into prostaglandin-mediated inflammation and its inhibition by pharmacologic agents (Kang *et al*., 2007; Smith *et al*., 2011).

This study is therefore designed to investigate the analgesic and anti-inflammatory potential of diclofenac, orphenadrine, and their combination using behavioral and biochemical experimental models. By integrating neurochemical and pharmacological perspectives, the research aims to clarify whether their co-administration produces true synergism beyond additive analgesic effects. The findings are expected to contribute to the growing body of knowledge supporting multimodal analgesia and provide experimental justification for rational combination therapy in effective pain management.

1.1 Pain and Inflammation: An Overview

Pain is a multidimensional experience that encompasses both sensory and emotional components, typically associated with actual or potential tissue damage. It functions as a protective mechanism, alerting the organism to harmful stimuli and prompting avoidance or healing behaviors (Basbaum *et al*., 2009; Igbe *et al*., 2019). Based on duration, pain is broadly categorized as acute or chronic. Acute pain is transient and directly related to tissue injury or inflammation, subsiding once healing occurs. Chronic pain, however, persists beyond normal recovery time, often involving maladaptive neural plasticity that perpetuates pain even in the absence of injury (Anuge *et al*., 2018; Dehghan *et al*., 2024).

From a mechanistic perspective, pain can also be divided into nociceptive and neuropathic types. Nociceptive pain arises from activation of peripheral nociceptors by noxious stimuli, while neuropathic pain results from structural or functional alterations within the somatosensory pathways (Le Bars *et al*., 2001; Iniaghe *et al*., 2022). In clinical and experimental settings, these categories frequently overlap, emphasizing the complex neurochemical interplay between the peripheral and central nervous systems in pain perception (Sadler *et al*., 2022; Edosuyi *et al*., 2018).

The physiology of pain transmission begins with the activation of specialized sensory neurons—nociceptors—by thermal, mechanical, or chemical stimuli. These afferent signals are transmitted via myelinated Aδ and unmyelinated C fibers to the dorsal horn of the spinal cord, where they synapse with second-order neurons that ascend through the spinothalamic tract to higher brain centers for interpretation and modulation (Anthony *et al*., 2021; Dehghan *et al*., 2024). At the site of injury, inflammatory mediators such as prostaglandins, bradykinin, histamine, and cytokines sensitize nociceptors, a process termed peripheral sensitization. This sensitization lowers the activation threshold of nociceptors, producing hyperalgesia and tenderness around inflamed tissues (Erharuyi *et al*., 2015; Igbe&Edike, 2015). Among these mediators, prostaglandin E_2 (PGE_2) plays a pivotal role by enhancing the excitability of sensory neurons via activation of the cyclooxygenase (COX) pathway (Smith *et al*., 2011; Edosuyi *et al*., 2023).

Within the central nervous system (CNS), persistent or intense nociceptive input induces central sensitization, characterized by heightened responsiveness of dorsal horn neurons. This involves activation of glutamatergic and N-methyl-D-aspartate (NMDA) receptors, resulting in calcium influx and intracellular signaling that strengthen synaptic transmission (Zhou *et al*., 2018; Magaji *et al*., 2017). Dysregulation of these processes contributes to chronic pain states and is a key target for centrally acting analgesics and muscle relaxants such as orphenadrine

(Falodun *et al*., 2013; Tomic *et al*., 2019). Furthermore, descending modulatory pathways from supraspinal structures—mediated by serotonin, norepinephrine, dopamine, and GABA—can either inhibit or facilitate spinal pain transmission, depending on the balance between excitatory and inhibitory signals (Millan, 2002; Taylor *et al*., 2016; Knabl *et al*., 2008). Dysfunction in these pathways underlies the persistence of both nociceptive and affective dimensions of pain (Edosuyi *et al*., 2018; Anthony *et al*., 2021).

Inflammation, closely linked to pain, represents the body's innate defense response to infection, injury, or harmful stimuli. It involves a cascade of vascular and cellular events regulated by numerous mediators such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and prostaglandins, which collectively promote vascular permeability, leukocyte migration, and nociceptor sensitization (Nathan, 2002; Igbe *et al*., 2012; Saleh *et al*., 2021). While acute inflammation facilitates tissue repair and defense, chronic or uncontrolled inflammation leads to sustained pain, tissue damage, and pathological hypersensitivity (Omo-Erhabor& Edosuyi, 2024).

A key biochemical link between pain and inflammation lies in the cyclooxygenase (COX) pathway, which catalyzes the conversion of arachidonic acid to prostaglandins and thromboxanes (Kang *et al*., 2007; Smith *et al*., 2011). Two primary isoforms exist: COX-1, which is constitutively expressed and supports physiological processes such as gastric protection and platelet aggregation; and COX-2, an inducible form upregulated during inflammation, responsible for the synthesis of pro-inflammatory prostaglandins like PGE_2 (Rumzhum& Ammit, 2016; Brune &Patrignani, 2015). A third variant, COX-3, has also been identified as a COX-1 splice variant, sensitive to certain analgesics such as acetaminophen (Chandrasekharan *et al*., 2002). The inhibition of COX, particularly COX-2, constitutes the principal mechanism of nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, which alleviate pain and inflammation by suppressing prostaglandin synthesis (Rainsford,

2013; Hoy, 2016; Ayoub *et al*., 2009). Beyond COX inhibition, diclofenac also activates peroxisome proliferator-activated receptor-γ (PPAR-γ), enhancing anti-inflammatory cytokine production and modulating immune responses (Ayoub *et al*., 2009). These mechanisms underscore its clinical utility in both acute and chronic inflammatory pain states (McNicol *et al*., 2018; Zeiner *et al*., 2023).

In summary, pain and inflammation are intricately connected biological phenomena driven by complex interactions between peripheral and central pathways. The release of inflammatory mediators, activation of nociceptors, and sensitization of spinal neurons collectively shape the intensity and persistence of pain. The COX-prostaglandin pathway remains a central target for therapeutic intervention, with drugs such as diclofenac providing dual anti-inflammatory and analgesic actions. Understanding these mechanisms forms the foundation for exploring combination therapies—such as diclofenac with orphenadrine—that may offer enhanced modulation of both pain and inflammation through complementary peripheral and central mechanisms.

1.2 Neurochemical Basis of Pain and Analgesia

Pain perception arises from intricate neurochemical interactions that integrate peripheral nociceptive input with central processing mechanisms within the brain and spinal cord. This modulation involves a complex interplay between excitatory and inhibitory neurotransmitters, neuropeptides, inflammatory mediators, and oxidative signaling pathways (Basbaum *et al*., 2009; Dehghan *et al*., 2024).

Among these neurotransmitters, glutamate serves as the principal excitatory messenger within the central nervous system (CNS), mediating nociceptive transmission through ionotropic receptors such as N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Zhou *et al*., 2018). NMDA receptor

activation triggers calcium influx into postsynaptic neurons, promoting central sensitization—a key neuroplastic process underlying hyperalgesia and chronic pain states (Whiteside *et al*., 2016; Dehghan *et al*., 2024).

Conversely, inhibitory neurotransmitters such as γ-aminobutyric acid (GABA) maintain balance within pain circuits by suppressing excessive excitatory transmission. Disruption of GABAergic inhibition has been shown to contribute to neuropathic and inflammatory pain syndromes (Knabl *et al*., 2008). In addition, monoaminergic pathways involving serotonin (5-HT) and norepinephrine modulate pain perception through descending inhibitory tracts originating in the brainstem (Millan, 2002). Depending on receptor subtype and site of action, these neurotransmitters can either enhance or suppress nociceptive signaling, contributing to the dualistic nature of monoaminergic modulation (Anthony *et al*., 2021; Edosuyi *et al*., 2018).

Dopaminergic transmission also plays a significant role in the emotional and motivational aspects of pain. Alterations in dopamine signaling influence pain tolerance and reward-related components of analgesia (Taylor *et al*., 2016), thereby linking nociception with affective and behavioral responses (Iniaghe *et al*., 2017).

Neuropeptides such as substance P act as key mediators of pain facilitation. Released from primary afferent neurons, substance P binds to neurokinin-1 (NK1) receptors in the dorsal horn, amplifying nociceptive transmission and triggering neurogenic inflammation (Mantyh, 2002). Similarly, prostaglandins, particularly prostaglandin E_2 (PGE_2), play a crucial role in peripheral sensitization by lowering nociceptor thresholds and enhancing responses to mechanical or chemical stimuli (Smith *et al*., 2011). Prostaglandin synthesis is regulated by cyclooxygenase (COX) enzymes, particularly COX-1 and COX-2, which convert arachidonic

acid into prostanoid intermediates (Rumzhum & Ammit, 2016; Kang *et al*., 2007). Dysregulation of this pathway underlies the inflammatory component of pain.

Diclofenac, a widely used nonsteroidal anti-inflammatory drug (NSAID), alleviates pain primarily by inhibiting COX isoenzymes, thereby suppressing prostaglandin synthesis (Rainsford, 2013; McNicol *et al*., 2018). Beyond its COX blockade, diclofenac has been reported to activate peroxisome proliferator-activated receptor gamma (PPAR-γ), which contributes to the expression of anti-inflammatory cytokines and additional pain modulation mechanisms (Ayoub *et al*., 2009).

Orphenadrine, on the other hand, is a centrally acting skeletal muscle relaxant with notable analgesic and antihistaminic properties. It exerts its effects through NMDA receptor antagonism, which mitigates glutamate-mediated excitotoxicity and reduces central sensitization (Zeiner *et al*., 2023). Moreover, its anticholinergic and antihistaminic actions enhance comfort and sedation, further contributing to pain relief in muscle-related and postoperative pain conditions (Tomic *et al*., 2019).

Interestingly, accumulating evidence supports a bidirectional interaction between NMDA receptor activity and COX-mediated prostaglandin synthesis. Activation of NMDA receptors enhances COX-2 expression through calcium-dependent pathways, while COX-derived prostaglandins, in turn, potentiate NMDA receptor activity (Poligone& Baldwin, 2001; Zhu *et al*., 2015). This interdependence underscores the biological rationale for combination therapy involving orphenadrine and diclofenac. Such an approach targets both the excitatory glutamatergic and inflammatory prostaglandin pathways simultaneously, offering potential synergistic analgesic benefits (Brune &Patrignani, 2015; Kehlet & Dahl, 2018).

Furthermore, oxidative stress and neuroinflammation—mediated through the TLR4/NF-κBsignaling pathway—contribute significantly to the persistence of pain and reduced

responsiveness to analgesics (Saleh *et al*., 2021). By modulating these molecular cascades, combined NMDA antagonism and COX inhibition could dampen not only nociceptive transmission but also the underlying neuroinflammatory processes sustaining chronic pain.

1.3 Cyclooxygenase (COX) Enzymes

Cyclooxygenase (COX) enzymes, also known as prostaglandin endoperoxide synthases, are pivotal rate-limiting enzymes that catalyze the biotransformation of arachidonic acid into prostaglandin H_2 (PGH_2)—a crucial precursor for bioactive lipids such as prostaglandins, prostacyclin, and thromboxane (Smith *et al*., 2011). These prostanoids exert diverse physiological and pathological functions, including modulation of inflammation, pain perception, platelet aggregation, vascular tone, and maintenance of gastric and renal homeostasis (Wallace, 2008; Rumzhum& Ammit, 2016).

Three distinct isoforms of COX have been characterized: COX-1, COX-2, and COX-3. COX-1 is constitutively expressed in most tissues, where it synthesizes prostaglandins responsible for gastric mucosal defense, renal blood flow regulation, and platelet aggregation (Wallace, 2008). In contrast, COX-2 is an inducible isoform that is upregulated during tissue injury, inflammation, and pain, largely through the activation of transcription factors such as nuclear factor-kappa B (NF-κB) (Nathan, 2002; Poligone& Baldwin, 2001). Cytokines like interleukin-1β and tumor necrosis factor-alpha stimulate COX-2 expression, resulting in increased prostaglandin E_2 (PGE_2) synthesis that heightens nociceptor sensitivity and amplifies inflammatory signaling (Rumzhum& Ammit, 2016; Saleh *et al*., 2021).

COX-3, identified as a splice variant of the COX-1 gene, is primarily expressed in the cerebral cortex and is inhibited by analgesic drugs such as acetaminophen (Chandrasekharan *et al*., 2002; Botting, 2006). Although its exact physiological relevance remains under

investigation, COX-3 is thought to contribute to central mechanisms of fever regulation and nociception (Rummel *et al*., 2011).

The functional duality of COX enzymes underpins their significance in both health and disease. COX-1–derived prostanoids are vital for physiological homeostasis, while COX-2–mediated products are central to inflammation and hyperalgesia (Kang *et al*., 2007; Rumzhum& Ammit, 2016). Nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, exert their analgesic and anti-inflammatory actions by inhibiting COX activity, thereby reducing prostaglandin synthesis (Rainsford, 2013; McNicol *et al*., 2018). However, non-selective COX inhibition may lead to adverse gastrointestinal and renal effects resulting from suppression of protective COX-1–derived prostanoids (Wallace, 2008; Brune &Patrignani, 2015). Consequently, selective COX-2 inhibitors (coxibs) were developed to mitigate gastric toxicity while preserving anti-inflammatory efficacy, though their association with cardiovascular risks remains a clinical concern (FitzGerald, 2004).

Experimental findings further highlight a neurochemical interplay between COX activity and excitatory neurotransmission in pain modulation. COX-derived prostaglandins enhance glutamatergic signaling by potentiating NMDA receptor currents and calcium influx, promoting central sensitization and hyperalgesia (Zhu *et al*., 2015; Zhou *et al*., 2018). Conversely, activation of NMDA receptors can upregulate COX-2 expression through calcium-dependent pathways, forming a positive feedback loop that sustains inflammatory pain (Dehghan *et al*., 2024). This reciprocal relationship provides a mechanistic rationale for multimodal analgesia—combining COX inhibitors with NMDA receptor antagonists, such as diclofenac and orphenadrine, to achieve superior pain relief by targeting both peripheral and central mechanisms (Zeiner *et al*., 2023; Tomic *et al*., 2019; Kehlet & Dahl, 2018).

Preclinical studies involving plant-derived and synthetic agents have further reinforced the centrality of COX modulation in analgesic pharmacology. Extracts with anti-inflammatory activity, such as *Solenostemon monostachyus* and *Cussonia barteri*, have demonstrated significant analgesic effects in rodent models of acetic acid-induced writhing, indicating suppression of COX-mediated prostaglandin synthesis (Anuge *et al*., 2018; Igbe *et al*., 2019). Similarly, compounds with antioxidant properties—such as those from *Hunteria umbellata* and *Jatropha multifida*—attenuate pain through inhibition of oxidative stress and prostanoid generation (Edosuyi *et al*., 2018; Falodun *et al*., 2013; Erharuyi *et al*., 2015). These findings align with the established biochemical basis of COX inhibition and underscore the enzyme's role in both conventional NSAID action and the mechanistic exploration of novel analgesic therapies.

Collectively, COX enzymes represent critical pharmacological targets in experimental and clinical pain management. Their inhibition accounts for the therapeutic efficacy of diclofenac and forms the basis for combination therapies involving centrally acting agents such as orphenadrine. By concurrently modulating peripheral inflammation and central sensitization, such combinations may offer a more holistic approach to managing complex pain conditions.

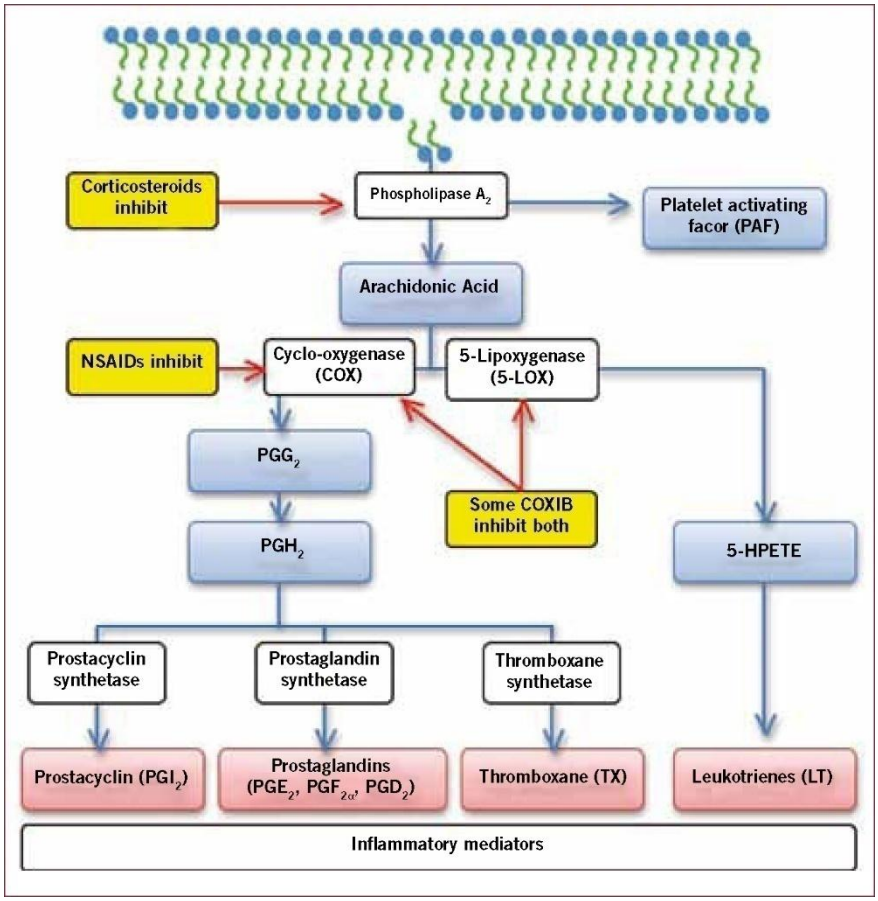

Fig 1.1 The eicosanoid cascade shows the metabolic pathways that result in the production of mediators involved in inflammation. Indicated are the sites of action of the major anti-inflammatory pharmaceutical agents

1.4 Diclofenac Sodium: Pharmacological Profile

Diclofenac sodium is a widely used non-steroidal anti-inflammatory drug (NSAID) belonging to the phenylacetic acid class, renowned for its potent analgesic, anti-inflammatory, and antipyretic activities. Its extensive use globally is attributed to its efficacy and well-characterized pharmacodynamic and pharmacokinetic profiles (Anuge *et al*., 2018; Rainsford, 2013). The therapeutic actions of diclofenac are primarily mediated through its inhibitory

effects on cyclooxygenase (COX) enzymes—specifically COX-1 and COX-2—which catalyze the conversion of arachidonic acid into prostaglandins, prostacyclins, and thromboxanes (Smith *et al*., 2011; Botting, 2006). These lipid mediators play crucial roles in pain perception, inflammation, and pyrexia. Consequently, diclofenac suppresses prostaglandin synthesis, thereby reducing peripheral inflammation, tissue edema, and pain hypersensitivity (Brune &Patrignani, 2015).

Beyond its classical peripheral actions, increasing evidence indicates that diclofenac also exerts significant central effects. It modulates nociceptive transmission within the spinal cord and brain by influencing prostaglandin synthesis and nitric oxide signaling in the central nervous system (Rummel *et al*., 2011; Edosuyi *et al*., 2023). The involvement of serotonergic and glutamatergic pathways in its analgesic mechanism has also been suggested, implying a broader neuromodulatory profile beyond COX inhibition (Anthony *et al*., 2021; Igbe *et al*., 2019). Through these multimodal mechanisms, diclofenac effectively mitigates both acute and chronic pain, aligning with evolving perspectives on integrated pain modulation (Basbaum *et al*., 2009; Dehghan *et al*., 2024).

Pharmacokinetically, diclofenac sodium is rapidly absorbed following oral or parenteral administration, achieving peak plasma concentrations within 1–2 hours (Hoy, 2016). It exhibits extensive plasma protein binding (>99%) and undergoes hepatic metabolism mainly via the cytochrome P450 2C9 pathway, followed by conjugation with glucuronic acid and sulfates (Edosuyi *et al*., 2022). The elimination half-life typically ranges between 1 and 2 hours, with metabolites excreted in urine and bile (McNicol *et al*., 2018). These characteristics contribute to predictable pharmacokinetics and make diclofenac suitable for diverse therapeutic settings, from acute postoperative pain to chronic inflammatory disorders (Freynhagen *et al*., 2016).

Experimental evidence corroborates diclofenac's potent analgesic and anti-inflammatory efficacy in standard nociceptive models. In the acetic acid-induced writhing test—a model that assesses peripheral antinociceptive activity—diclofenac significantly reduces the frequency of abdominal constrictions, indicating inhibition of prostaglandin-mediated nociceptive signaling (Collier *et al*., 1968; Ribeiro *et al*., 2000). Similarly, in central pain models such as the hot plate and formalin tests, diclofenac demonstrates both spinal and supraspinal analgesic actions (Le Bars *et al*., 2001; Barrot, 2012). Neurobehavioral assessments such as the hole-board test have also revealed that diclofenac-treated animals exhibit reduced anxiety-like behaviors, suggesting a potential modulatory role in pain-related affective responses (File &Wardill, 1975; Iniaghe *et al*., 2022). These findings align with the growing understanding that pain is not merely a sensory experience but also involves complex emotional and motivational dimensions (Taylor *et al*., 2016; Sadler *et al*., 2022).

Despite its proven therapeutic value, diclofenac is not devoid of adverse effects. Gastrointestinal mucosal injury, ulceration, and bleeding constitute major toxicological concerns, primarily resulting from COX-1 inhibition and the consequent reduction of protective prostaglandins in the gastric lining (Wallace, 2008). In addition, chronic use may lead to renal impairment due to decreased renal blood flow and sodium retention, while cardiovascular risks such as hypertension and myocardial infarction have been associated with sustained exposure (FitzGerald, 2004; Brune &Patrignani, 2015). These limitations have prompted research into strategies that can preserve analgesic efficacy while minimizing systemic toxicity (Nathan, 2002; Saleh *et al*., 2021).

One promising approach is combination therapy, which leverages multimodal mechanisms to achieve synergistic analgesia. When co-administered with centrally acting agents such as orphenadrine—a compound known for its NMDA receptor antagonism and anticholinergic properties—the analgesic efficacy of diclofenac may be potentiated through complementary

pathways (Zhou *et al*., 2018; Zeiner *et al*., 2023). Such combinations not only enhance pain relief but also permit dose reduction, thereby decreasing the risk of gastrointestinal and cardiovascular side effects (Kehlet & Dahl, 2018; Tomic *et al*., 2019). This pharmacological synergy underscores the clinical relevance of integrating peripherally and centrally acting drugs for optimized pain management (White *et al*., 2017; Rainsford, 2013).

Overall, diclofenac sodium remains a cornerstone in pain and inflammation management due to its multifaceted mechanisms, rapid absorption, and established clinical effectiveness. Ongoing research into its molecular interactions and potential synergistic combinations continues to refine its therapeutic application and safety profile, offering valuable insights for translational pain pharmacology.

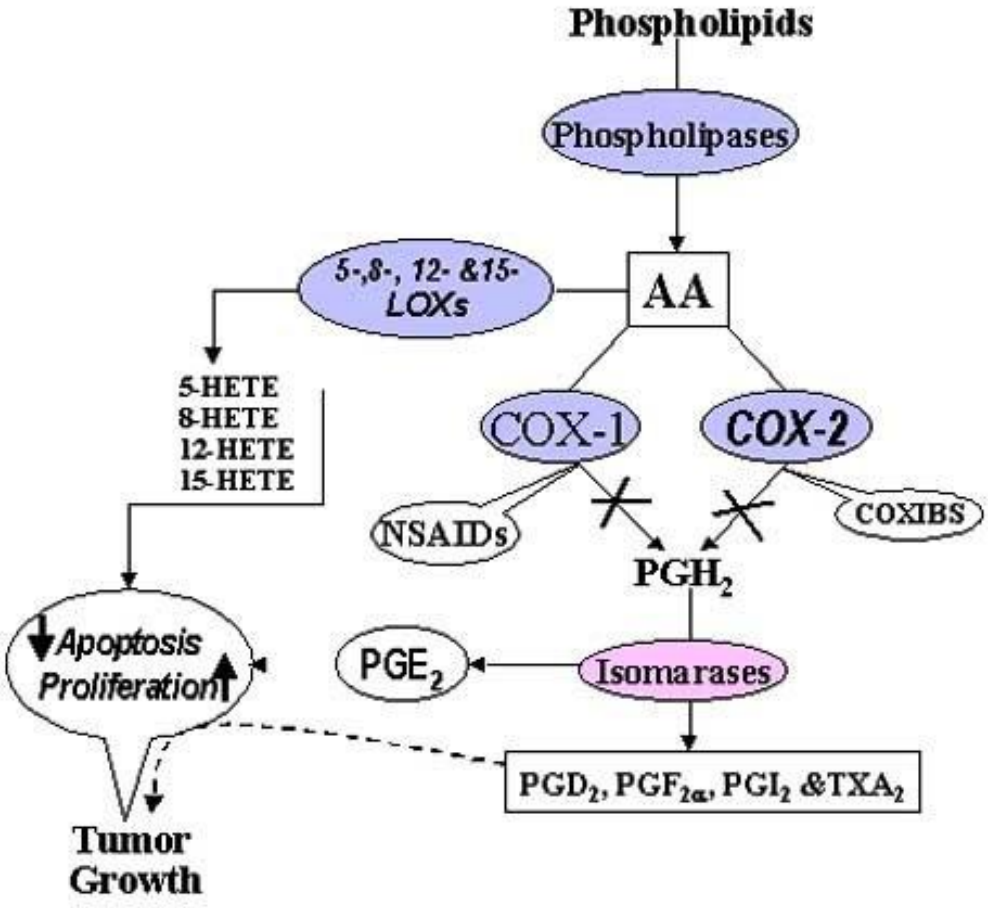

Fig 1.2 Mechanism of action of Diclofenac

1.5 Orphenadrine: Pharmacological Profile

Orphenadrine is a centrally acting skeletal muscle relaxant with additional anticholinergic and antihistaminic properties. Structurally related to diphenhydramine, it is commonly prescribed as an adjunctive agent for the management of painful musculoskeletal conditions, tension headaches, and postoperative muscle spasms (Zeiner *et al*., 2023). Beyond its established clinical applications, emerging evidence indicates that orphenadrine possesses intrinsic analgesic, neuroprotective, and antioxidant properties that may significantly extend its therapeutic potential (Tomic *et al*., 2019; Edosuyi *et al*., 2018).

The principal mechanism underlying orphenadrine's pharmacological action involves non-competitive antagonism of the N-methyl-D-aspartate (NMDA) receptor, which reduces excitatory glutamatergic neurotransmission and prevents central sensitization associated with chronic pain and muscle hyperactivity (Zhou *et al*., 2018; Dehghan *et al*., 2024). This NMDA receptor blockade helps modulate neuronal excitability, thereby attenuating hyperalgesia and allodynia. In addition, orphenadrine exerts notable antimuscarinic and H_1-receptor-blocking effects that contribute to its peripheral muscle relaxant and sedative properties (Erharuyi *et al*., 2015; Anuge *et al*., 2018). These multiple actions establish a multimodal pharmacological profile that distinguishes orphenadrine from peripherally acting relaxants and supports its role in integrated pain management strategies.

Experimental investigations have demonstrated that orphenadrine and other compounds acting on similar central pathways exhibit antioxidant and free radical–scavenging properties, thereby reducing oxidative stress–induced neuronal damage (Edosuyi *et al*., 2018; Saleh *et al*., 2021). Such antioxidant capacity is thought to contribute to the drug's neuroprotective potential, which complements its analgesic effect. Moreover, modulation of monoaminergic neurotransmission—particularly dopaminergic and serotonergic pathways—appears to play a

crucial role in orphenadrine's antinociceptive and mood-stabilizing effects (Okwuofu *et al*., 2021; Anthony *et al*., 2021). The interaction with serotonergic and opioidergic systems further aligns with known mechanisms of analgesic synergy observed in centrally acting agents (Edosuyi *et al*., 2018; Igbe *et al*., 2019).

Clinical evidence supports the use of orphenadrine in combination with non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac for enhanced postoperative pain control. Studies have shown that the diclofenac–orphenadrine combination provides superior analgesic efficacy compared with NSAID monotherapy, improving postoperative comfort without significantly increasing adverse effects (Tomic *et al*., 2019; Zeiner *et al*., 2023). This synergistic effect likely arises from the complementary mechanisms of cyclooxygenase inhibition and NMDA receptor antagonism, a principle consistent with multimodal analgesia approaches that aim to reduce opioid reliance and improve patient outcomes (Kehlet & Dahl, 2018; White *et al*., 2017).

Despite its long-standing clinical use, well-controlled studies assessing the stand-alone analgesic efficacy and mechanistic pathways of orphenadrine remain limited. Most available data stem from combination therapy trials, making it difficult to delineate its independent contribution to pain relief (Zeiner *et al*., 2023). Furthermore, interindividual variability in receptor subunit expression and genetic polymorphisms influencing NMDA or muscarinic receptor sensitivity may alter therapeutic responsiveness (Edosuyi *et al*., 2023; Omo-Erhabor& Edosuyi, 2024). Consequently, further mechanistic, pharmacogenomic, and behavioral research is warranted to clarify orphenadrine's precise molecular actions, optimize dosing strategies, and expand its utility in pain and anxiety modulation.

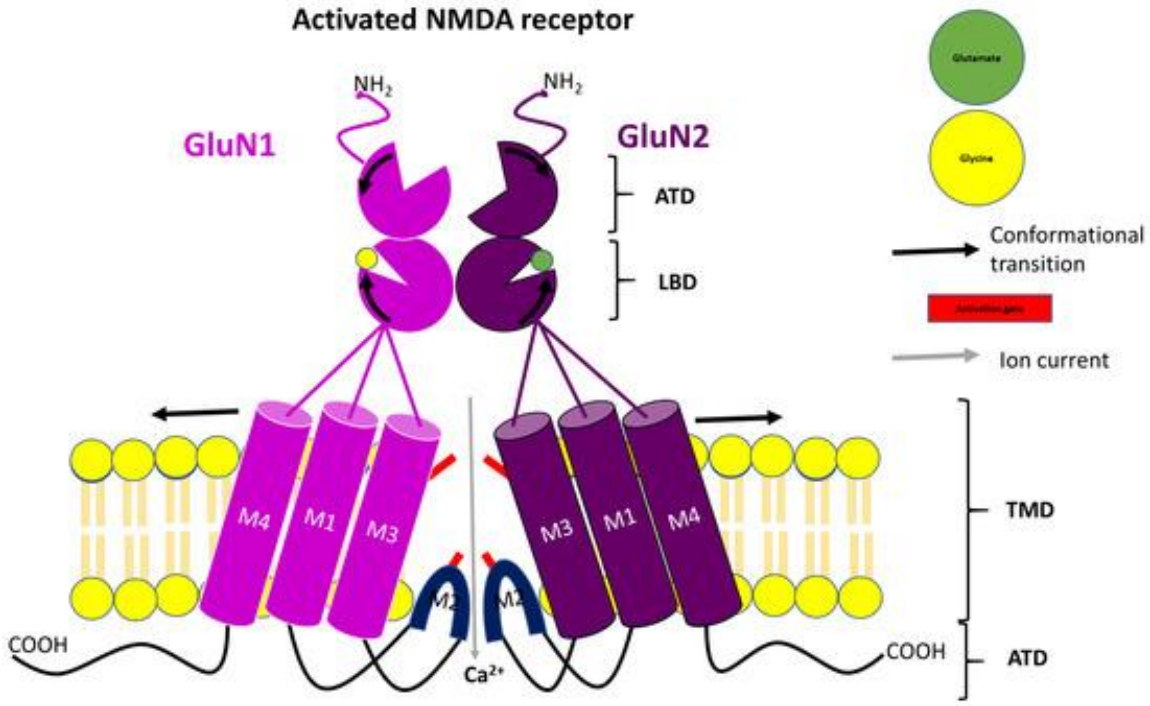

Fig 1.3 Schematic illustration of NMDA receptor activation (Egunlusi & Joubert, 2024)

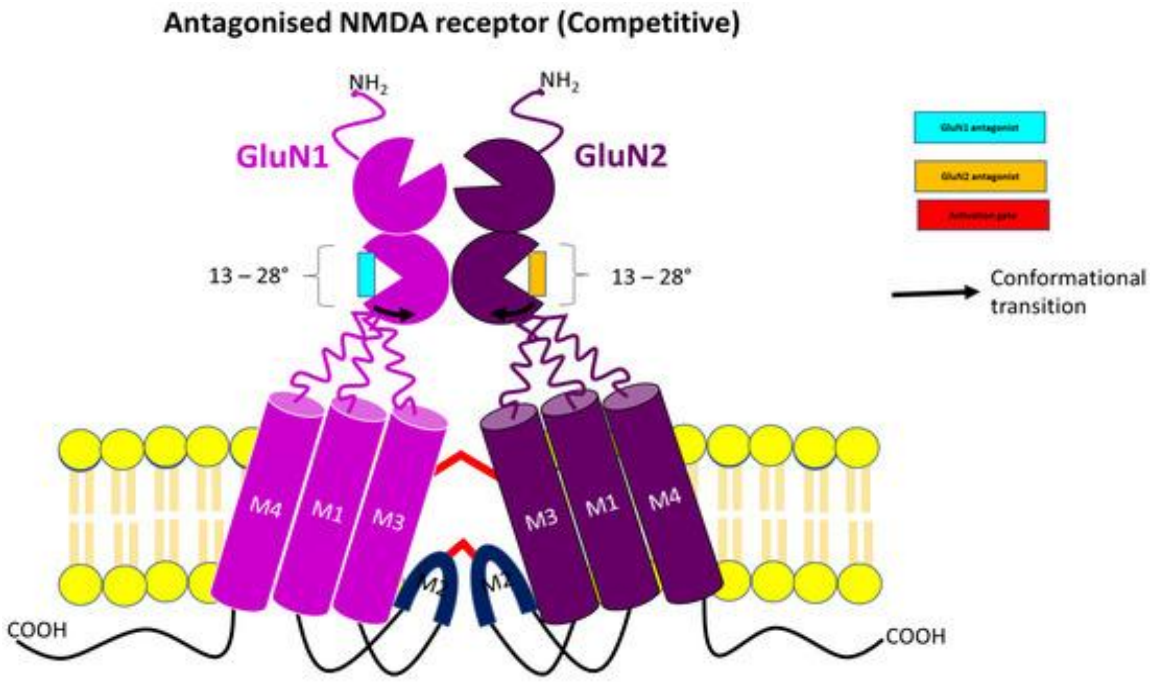

Fig 1.4: Schematic representation of competitive antagonism at NMDA receptor. (Egunlusi & Joubert, 2024)

1.6 Combined Use of Orphenadrine and Diclofenac

Modern pain management increasingly emphasizes multimodal analgesia, a strategy that targets multiple points along the pain pathway to enhance efficacy while reducing individual drug-related adverse effects (Kehlet & Dahl, 2018; White *et al*., 2017). Within this framework, the combination of a peripherally acting cyclooxygenase (COX) inhibitor, such as diclofenac, with a centrally acting agent like orphenadrine—a non-competitive NMDA receptor antagonist with anticholinergic properties—represents a rational and complementary therapeutic approach (Zeiner *et al*., 2023; Tomic *et al*., 2019). Diclofenac primarily mitigates the inflammatory, prostaglandin-mediated component of nociception, whereas orphenadrine modulates central sensitization, reduces muscle hypertonicity, and suppresses excitatory transmission that amplifies pain signals (Basbaum *et al*., 2009; Zhou *et al*., 2018).

From a pharmacodynamic perspective, this combination is particularly compelling. Diclofenac inhibits COX-1 and COX-2, reducing prostaglandin synthesis, peripheral sensitization, and inflammatory hyperalgesia (Hoy, 2016; Rainsford, 2013). Orphenadrine, through NMDA receptor antagonism and antimuscarinic activity, attenuates central excitatory pathways and muscle spasm, which frequently contribute to persistent pain (Zeiner *et al*., 2023). Notably, there is evidence of cross-talk between prostaglandin and NMDA signaling pathways, in which prostaglandins can potentiate NMDA receptor activity, and sustained NMDA activation may upregulate COX-2 expression, creating a feed-forward loop that perpetuates pain (Zhu *et al*., 2015). Targeting both peripheral prostaglandin production and central NMDA-driven sensitization may therefore result in additive or even synergistic analgesic effects beyond what either agent can achieve alone.

Pharmacokinetic considerations further support combination use. Diclofenac is rapidly absorbed, highly protein-bound, and metabolized hepatically primarily via conjugation (Hoy,

2016). Orphenadrine is similarly metabolized in the liver and distributes to the central nervous system due to its lipophilic nature (Tomic *et al*., 2019). Available clinical studies suggest minimal direct metabolic interaction between these drugs, supporting the feasibility of co-administration (Zeiner *et al*., 2023). Therapeutically, co-administration may allow dose-sparing, reducing diclofenac exposure while maintaining analgesic efficacy, which could lower the risk of gastrointestinal and cardiovascular adverse effects (McNicol *et al*., 2018; Rainsford, 2013). However, comprehensive pharmacokinetic–pharmacodynamic studies remain limited, leaving optimal dosing and interaction profiles to be fully clarified through future research.

Evidence from other multimodal analgesic pairings underscores the value of combining central and peripheral mechanisms. Combinations such as paracetamol with NSAIDs or NSAIDs with weak opioids consistently demonstrate superior pain relief compared with monotherapy and can reduce opioid requirements in acute settings (Freynhagen *et al*., 2016; White et al., 2017). Specifically, clinical studies of diclofenac plus orphenadrine in postoperative settings report enhanced analgesia relative to diclofenac alone or placebo, with acceptable short-term tolerability (Zeiner et al., 2023; Tomic et al., 2019). Nevertheless, most clinical data are derived from acute postoperative contexts, and controlled studies in chronic pain models remain scarce.

A major preclinical research gap exists regarding the mechanistic evaluation of diclofenac–orphenadrine combinations. Very few studies integrate behavioral assessments—such as the acetic acid-induced writhing test, hole-board exploration, or formalin assay—with biochemical endpoints including COX activity, prostaglandin levels, oxidative stress markers, and spinal NMDA/COX-2 expression (Barrot, 2012; Collier *et al*., 1968; File &Wardill, 1975). Quantifying these parameters would help determine whether observed analgesic effects are truly synergistic, additive, or driven by pharmacokinetic modulation. Dose-

response mapping and isobolographic analyses in animal models could further identify optimal therapeutic ratios for maximal efficacy with minimal adverse effects (Sadler *et al*., 2022; Le Bars *et al*., 2001).

In conclusion, diclofenac and orphenadrine represent a theoretically robust multimodal analgesic pairing, targeting peripheral inflammation and central sensitization simultaneously. Preliminary clinical evidence supports enhanced analgesic efficacy and acceptable tolerability, while pharmacokinetic profiles suggest feasible co-administration. However, rigorous preclinical studies combining behavioral and biochemical endpoints are necessary to fully elucidate mechanism, interaction type, and optimal dosing strategies to guide safe and effective translation into clinical practice.

1.7 Experimental Models of Analgesia

Animal models remain central to preclinical evaluation of analgesic and anti-inflammatory agents, offering insights into behavioral and biochemical mechanisms underlying pain modulation (Barrot, 2012; Basbaum *et al*., 2009). Pain is a multidimensional phenomenon encompassing sensory, emotional, and cognitive components, making it impossible for a single experimental model to fully capture its complexity. Therefore, researchers employ multiple complementary models targeting different aspects of nociception. Behavioral assays, such as the hole-board and acetic acid-induced writhing tests, assess observable responses to pain and drug effects, whereas biochemical assays, including cyclooxygenase (COX) inhibition studies, provide mechanistic insight at the molecular level (Saleh *et al*., 2021; Anthony *et al*., n.d.).

Ethical principles guide the use of experimental animals, with the “3Rs” framework—Replacement, Reduction, and Refinement—ensuring humane treatment while maintaining scientific validity (Russell and Burch, 1959). Translational relevance remains a key

consideration; animal models are continually refined to better mimic human pain conditions and pharmacodynamic responses (Barrot, 2012; Sadler *et al*., 2022). These models collectively provide a robust platform for evaluating compounds such as diclofenac sodium and orphenadrine, which modulate pain through both peripheral and central mechanisms (Zeiner *et al*., 2023).

1.7.1 Hole-Board Test

The hole-board test is widely used to assess exploratory behavior, anxiety, and central nervous system activity in rodents (Takeda *et al*., 1998). It measures head-dipping frequency and duration, behaviors sensitive to sedative, anxiolytic, and centrally acting analgesic agents (File and Wardill, 1975). Reduced head-dipping may indicate central depression or decreased exploratory drive, often associated with analgesia or sedation.

This model is particularly relevant when investigating centrally acting compounds like orphenadrine, which modulate NMDA receptor-mediated transmission and exhibit mild sedative effects (Zhou *et al*., 2018; Zeiner *et al*., 2023). The hole-board test complements peripheral pain models by providing data on spontaneous behavior and central sensory integration. The introduction of automated tracking systems has enhanced reproducibility and objectivity, further validating its use in neurobehavioral pharmacology (Magaji *et al*., 2017).

1.7.2 Acetic Acid–Induced Writhing Test

The acetic acid-induced writhing test is a classic model for evaluating peripheral analgesic activity. Intraperitoneal injection of dilute acetic acid induces abdominal constrictions, or "writhes," mediated by the release of prostaglandins, bradykinin, and other inflammatory mediators (Collier *et al*., 1968; Ribeiro *et al*., 2000). Agents that inhibit prostaglandin synthesis, such as NSAIDs, reduce writhing frequency, demonstrating peripheral antinociceptive efficacy (Rainsford, 2013; Brune and Patrignani, 2015).

Diclofenac sodium, a potent COX inhibitor, consistently demonstrates significant activity in this model, reflecting its clinical analgesic profile (McNicol *et al*., 2018; Hoy, 2016). However, the test does not differentiate between central and peripheral mechanisms, limiting its interpretive value for compounds like orphenadrine that act via both pathways (Zeiner *et al*., 2023). Despite this limitation, the writhing assay remains highly sensitive, reproducible, and suitable for preliminary screening of analgesic agents (Anuge *et al*., 2018).

1.7.3 Cyclooxygenase (COX) Inhibition Assays

COX inhibition assays provide complementary mechanistic data to behavioral outcomes. COX enzymes COX-1 and COX-2 catalyze the conversion of arachidonic acid to prostaglandins, which mediate inflammation and pain sensitization (Botting, 2006; Smith *et al*., 2011; Rainsford, 2013). Inhibition of COX activity serves as a direct indicator of analgesic and anti-inflammatory potential.

In vitro and ex vivo assays often quantify prostaglandin levels in tissue homogenates or plasma following drug administration. Diclofenac exhibits high affinity for both COX isoforms with modest COX-2 selectivity at therapeutic doses (Hoy, 2016; Brune and Patrignani, 2015). Recent studies suggest that combination therapies, such as diclofenac with orphenadrine, may enhance COX inhibition or prolong analgesic effects through

pharmacodynamic synergy (Tomic *et al*., 2019; Ayoub *et al*., 2009). Integrating behavioural and biochemical endpoints allows a holistic understanding of analgesic efficacy, highlighting both central and peripheral contributions to observed effects (Freynhagen *et al*., 2016).

1.8 Summary of Previous Findings

Over the years, diclofenac sodium has been widely recognized for its potent analgesic and anti-inflammatory effects. Its primary mechanism involves inhibition of cyclooxygenase (COX) enzymes, leading to reduced synthesis of prostaglandins, which are key mediators of pain and inflammation (Rainsford, 2013; Brune &Patrignani, 2015). Diclofenac acts both peripherally and centrally, modulating nociceptive pathways to alleviate pain in various clinical and experimental models (Hoy, 2016; McNicol *et al*., 2018). Experimental studies utilizing the acetic acid-induced writhing test and formalin test consistently demonstrate dose-dependent reductions in nociceptive behaviors and inflammatory markers following diclofenac administration (Collier *et al*., 1968; Ribeiro *et al*., 2000). Despite its efficacy, the clinical use of diclofenac is tempered by concerns over gastrointestinal and renal adverse effects, which has prompted interest in combination therapies to enhance analgesia while minimizing toxicity (Wallace, 2008; Zeiner *et al*., 2023).

Orphenadrine, in contrast, is a centrally acting muscle relaxant with weak antihistaminic and anticholinergic properties. While traditionally used for musculoskeletal conditions, recent evidence highlights its analgesic potential through NMDA receptor antagonism and modulation of dopaminergic and cholinergic neurotransmission (Freynhagen *et al*., 2016; Zhou *et al*., 2018). Animal studies employing behavioral paradigms such as the hole-board and hot plate tests indicate that orphenadrine produces moderate analgesia accompanied by sedation and reduced exploratory activity (File &Wardill, 1975; Tomic *et al*., 2019). Beyond

analgesia, orphenadrine has shown antioxidant and neuroprotective effects, suggesting possible benefits in chronic pain states where oxidative stress contributes to central sensitization (Whiteside *et al*., 2016).

The rationale for combining diclofenac with orphenadrine stems from the principle of multimodal analgesia, targeting both peripheral and central pathways to achieve additive or synergistic effects (Kehlet & Dahl, 2018; White *et al*., 2017). Clinically, fixed-dose formulations, such as diclofenac 75 mg plus orphenadrine 30 mg, have been employed in Europe for postoperative and musculoskeletal pain, demonstrating faster onset of action and greater analgesic intensity than diclofenac alone (Tomic *et al*., 2019; Zeiner *et al*., 2023). Nevertheless, the mechanistic basis for this combination remains underexplored, particularly regarding the interplay between COX inhibition and NMDA receptor modulation. Preclinical investigations have predominantly focused on behavioral endpoints, leaving gaps in understanding the biochemical and molecular correlates of combined therapy (Edosuyi *et al*., 2023).

In summary, diclofenac and orphenadrine exhibit complementary analgesic mechanisms, with diclofenac acting mainly peripherally via COX inhibition and orphenadrine centrally through NMDA receptor antagonism and neurotransmitter modulation. The limited integration of behavioral and biochemical assessments in combination therapy represents a significant research gap, which the present study seeks to address to provide a more comprehensive understanding of their synergistic analgesic potential.

1.9 Knowledge Gap and Justification for the Study

Although diclofenac, a potent non-steroidal anti-inflammatory drug (NSAID), and orphenadrine, a centrally acting muscle relaxant, are widely employed in clinical practice, significant gaps persist in the preclinical and mechanistic understanding of their analgesic

potential. Notably, the antinociceptive properties of orphenadrine beyond its muscle relaxant effects remain underexplored, with limited experimental evidence from standardized nociceptive models (Freynhagen *et al*., 2016; Zeiner *et al*., 2023). This paucity of data restricts comprehensive insights into its central analgesic mechanisms and its possible interaction with conventional NSAIDs.

Moreover, studies investigating the combined use of diclofenac and orphenadrine are scarce. Preclinical evaluations integrating peripheral and central analgesic pathways could elucidate potential synergistic or additive effects, yet such investigations remain largely absent (Tomic *et al*., 2019; Zeiner *et al*., 2023). Current literature also reveals a lack of correlation between behavioral outcomes, such as exploratory and nociceptive responses assessed using the hole-board and acetic acid-induced writhing tests, with biochemical endpoints like cyclooxygenase (COX) inhibition (Edosuyi *et al*., 2023; Anthony *et al*., 2023). This represents a critical gap in translating behavioral observations into mechanistic interpretations.

Bridging these gaps is essential for advancing both mechanistic and behavioral pharmacology. By concurrently evaluating neurobehavioral responses and biochemical markers, this study seeks to clarify the analgesic interplay between diclofenac and orphenadrine. The approach may uncover potential synergistic effects that optimize pain relief while minimizing adverse outcomes associated with non-selective COX inhibition or central side effects. Consequently, the study is justified as it could inform the rational development of safer, multimodal analgesic strategies, providing a preclinical foundation for enhanced clinical pain management.

1.10 Aim and Objectives

Aim:

To evaluate the analgesic potential and cyclooxygenase enzyme inhibitory activity of diclofenac, orphenadrine, and their combination using hole-board and acetic acid-induced writhing models in mice.

Specific Objectives

1. To determine the analgesic effects of diclofenac, orphenadrine, and their combination in acetic-acid induced mouse writhing
2. To assess the effect of diclofenac, orphenadrine, and their combination on exploratory behavior using the hole-board test, as an index of central modulation and anxiety-related activity.
3. To evaluate the effect of diclofenac, orphenadrine, and their combination on COX-2 gene expression.
4. To correlate behavioral outcomes with molecular findings, in order to identify whether the combination therapy preserves behavioral integrity while modulating COX-2 transcription.

CHAPTER TWO

2.0 MATERIALS AND METHODS

This chapter describes the experimental design, materials, animal models, and analytical procedures employed to evaluate the analgesic effects and cyclooxygenase (COX) enzyme inhibition of orphenadrine, diclofenac, and their combination in mice. The study integrated both behavioral and biochemical models to assess central and peripheral components of analgesia.

2.1 Materials

2.1.1 Drugs and Chemicals

The following drugs and reagents were used in this study: Diclofenac sodium (Voltaren®, Novartis), Orphenadrine citrate (Norflex®, Pfizer), Normal saline (0.9 % w/v NaCl; Bioflex®), Glacial acetic acid (BDH Chemicals, England), and reagents for the determination of cyclooxygenase (COX) enzyme activity. All chemicals and solvents were of analytical grade.

2.1.2 Apparatus and Equipment

Hole-board apparatus (wooden box with equidistant holes), transparent observation cages, 1 mL and 2 mL syringes with hypodermic needles, oral gavage tubes, analytical weighing balance, stopwatch/timer, centrifuge, spectrophotometer (for COX assay), sample bottles, glass beakers, filter paper, cotton wool, and disposable gloves were used throughout the experiment.

2.1.3 Experimental Animals

A total of twenty-four (24) healthy Swiss albino mice of either sex, weighing between 22 – 32 g, were used. The animals were obtained from the Animal House of the Department of Pharmacology, University of Benin, Benin City, Edo State, Nigeria.

They were housed in standard polypropylene cages under controlled laboratory conditions (temperature = 27 ± 2 °C; 12-hour light/dark cycle) and were provided standard pellet diet and water *ad libitum*. Animals were acclimatized for seven days before the commencement of experiments.

All procedures were conducted in accordance with international guidelines for the care and use of laboratory animals (NIH Publication No. 85-23, revised 2011). Ethical approval was obtained from the Departmental Ethics Committee, Faculty of Pharmacy, University of Benin, Benin City.

2.2 Method

The mice were randomly divided into four (4) groups of six (n = 6) animals each:

Group I (Control): Received normal saline (10 mL/kg, p.o.).

Group II (Standard): Received diclofenac sodium (50 mg/kg, i.p.) at a stock solution of 2.5mg/ml

Group III (Test): Received orphenadrine citrate (25 mg/kg, p.o.). at a stock solution of 1mg/ml

Group IV (Combination): Received orphenadrine citrate (25 mg/kg, p.o.) + diclofenac sodium (50 mg/kg, i.p.) at the same stock solutions respectively

2.2.1 Acetic Acid-Induced Writhing Test

Peripheral analgesic activity was assessed using the acetic acid-induced writhing method. Thirty (30) minutes after administration of the respective treatments, each mouse received 0.6 % v/v acetic acid (10 mL/kg, i.p.).

Immediately after injection, animals were placed individually in transparent observation cages, and the number of abdominal constrictions ("writhes") was recorded for 30 minutes, with counts taken at 5-minute intervals.

A reduction in the number of writhes compared with the control group indicated peripheral analgesic activity of the treatment.

2.2.2 Hole-Board Test

This test was used to evaluate the central activity of the drugs through exploratory and anxiety-related behavior. The test was carried out 24 hours after drug administration of the various treatment groups. Each mouse was placed individually on the hole-board apparatus, and the number of head dips into the holes was recorded for five (5) minutes.A reduction in head-dip frequency was interpreted as decreased exploratory activity, suggestive of central nervous system (CNS) depression or analgesia, while an increase implied CNS stimulation or anxiolysis.To remove olfactory and visual cues that may affect the exploratory behavior of subsequent mice, the surface of the hole-board apparatus was cleaned thoroughly with 50% ethanol after each mouse was scored. This ensured that behavioral responses were not influenced by scent trails or residues from previous animals. File and Wardill *et al*., 1975)

2.2.3 Sample Collection

All animals were euthanized by chloroform anesthesia following a 24-hour exposure to the different drug treatments. After the completion of 24-hour treatment period, all the animals

were humanely sacrificed while under chloroform anesthesia. The brains were excised and preserved in a 10% buffered formaldehyde solution for subsequent assays.

2.2.4 Polymerase chain reaction assay for COX-2 expression

Total RNA was isolated from brain samples using the protocols in the Quick-RNA Miniprep™ Kit (ZymoResearch). The DNA contaminant was removed with DNase I (NEB, Cat: M0303S). The DNA-free RNA was quantified using a spectrophotometer (A&E Lab, UK).Deoxyribonucleic acid (cDNA) conversion One (1 μg) of DNA-free RNA was converted to cDNA by reverse transcriptase using the cDNA synthesis kit (New England BioLabs).12The target gene was amplified with the OneTaqR2X master mix using the following primers (Inqaba Biotec, Hatfield, South Africa), endothelial nitric oxide synthase (eNOS), forward primer: TGGAGCGAGTTGTGGATTG reverse primer: CTACTGGGTCAAAGACAAGAGG, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward primer CTGGCAGCTCTTCTCAAAGC, reverse primer CCAGGTCATAGAGAGGCTCAA. Amplification was performed in a total of 0.025 ml of reaction mixture containing cDNA, primer (forward and reverse) and ready mix Taq PCR master mix. There was an initial denaturation at 95 ◦C for5 minutes, followed by 30cycles of amplification (denaturation at 95°C for 30 s, hardening for 30 s, and extension at 72 ◦C for 60 s) and ending with a final extension at 72°C for 10 minutes. The amplified genes were resolved on 1.0 % agarose gel. The GAPDH gene served as a 'housekeeping gene' and was used for the relative expression of each gene. The bands were quantified using the J® image software.

2.2.5 Data Analysis

All quantitative data were expressed as Mean ± Standard Error of Mean (SEM). Statistical analyses were performed using GraphPad Prism (version 10).

Data from behavioral tests were analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparisons test to determine pairwise differences between groups. Correlations between behavioral and biochemical outcomes were also assessed.

Differences were considered statistically significant at $p < 0.05$.

CHAPTER THREE

3.0. RESULTS

3.1 Comparison of the Acetic Acid Induced Mouse Writhing Test Across Groups

Figure 3.1 Shows the comparison of the mouse writhing test across groups over 30 minutes. A statistically significant decrease ($p<0.05$) in the number of writhes was observed when groups II (Diclofenac), III (Orphenadrine), and IV (Diclofenac+ Orphenadrine) with Mean±SEM (1.67±0.76, 2.17±0.95, and 0.00±0.00) at 5 minutes, when compared with the control group (6.00±1.53). Also, a significant decrease ($p<0.05$) in the number of writhes was observed when groups II and III (1.67±0.76, 2.17±0.95) were compared with group IV (0.00±0.00) at 5 minutes. A similar trend was observed across other time lines. At 30 minutes, comparison of group II (2.33±0.88) and III (9.67±3.71) revealed statistically significant ($p<0.05$) fewer writhes for group II. Group IV (1.2±0.58), when compared with groups II and III revealed statistically significantly ($p<0.05$) fewer writhes at 30 minutes.

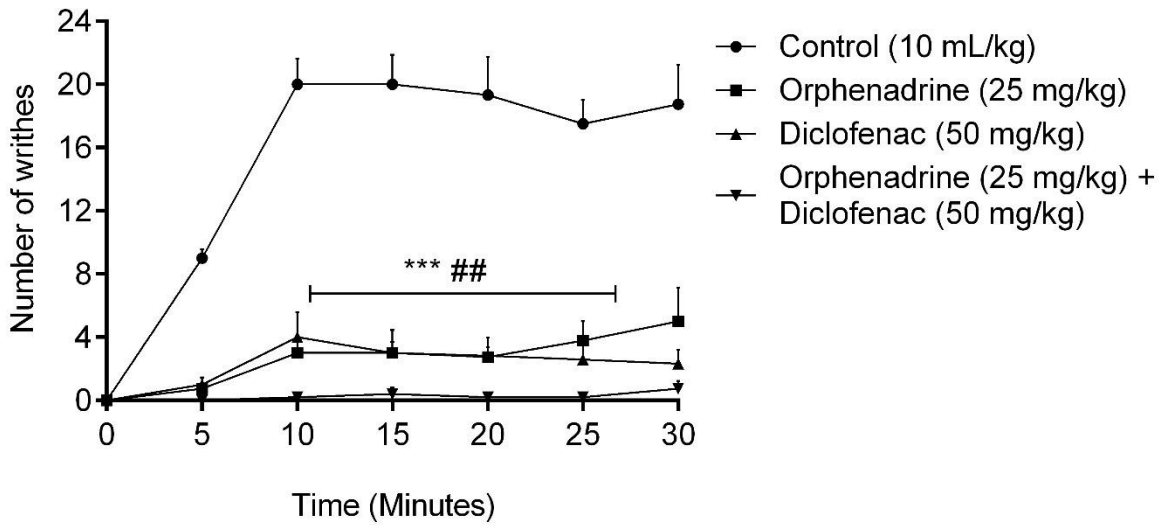

Figure 3.1 Comparison of the mouse writhing across groups.

Error bars represent Mean ± SEM (n=6). ***p<0.001 represents a significant difference from the control group, ##p<0.001 represents a significant difference from the orphenadrine + diclofenac group

3.2 Comparative Analysis Of COX-2 Levels Across Various Treatment Groups

Figure 3.2 Illustrates the comparative analysis of COX-2 levels across the experimental groups.

A one-way ANOVA revealed a statistically significant overall effect ($F(3,11)=295.1$, $p<0.0001$). Post-hoc comparisons showed a statistically significant decrease ($p<0.05$) in COX-2 levels in the orphenadrine group (27.59 ± 2.26) and the orphenadrine+diclofenac combination group (68.30 ± 1.43) when compared to the control group (95.26 ± 1.88). In contrast, the diclofenac-only group (88.32 ± 1.18) showed a smaller but still significant reduction compared to the control.

Furthermore, the orphenadrine+diclofenac combination resulted in a statistically significant difference ($p<0.05$) in COX-2 levels compared to both the Orphenadrine-only and Diclofenac-only groups.

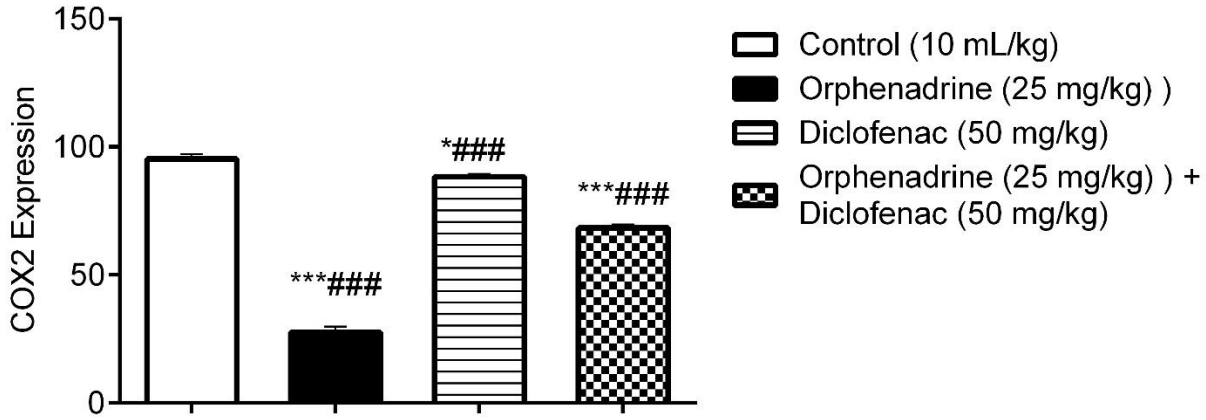

Figure 3.2: The graph depicts the expression levels of COX-2 across different treatment groups.

Data is presented as Mean ± SEM (n=6). Orphenadrine and combination groups showed a significant reduction (***$p<0.0001$) compared to control, while the diclofenac group showed a smaller but significant reduction (*$p<0.05$). The combination group was also significantly different from both individual drug groups (###$p<0.0001$).

3.3. Comparative Analysis of Exploratory Behaviour in the Hole Board Test Across Various Treatment Groups

Figure 3.3 Illustrates the effects of different drug treatments on exploratory behaviour, measured as the number of head dips, in the hole board test. A one-way ANOVA revealed a statistically significant overall effect of the treatments ($F(3,10) = 9.788$, $p = 0.0025$). Post-hoc comparisons demonstrated a statistically significant decrease ($p < 0.01$) in exploratory behaviour in the diclofenac-only group (15.67 ± 1.59) compared to the control group (22.50 ± 1.06). In contrast, the orphenadrine only group (18.13 ± 0.80) showed a non-significant reduction compared to the control.

The orphenadrine + diclofenac combination group (24.33 ± 1.59) was not significantly different from the control group. However, it resulted in a statistically significant increase ($p < 0.05$) in exploratory behaviour compared to the diclofenac-only group.

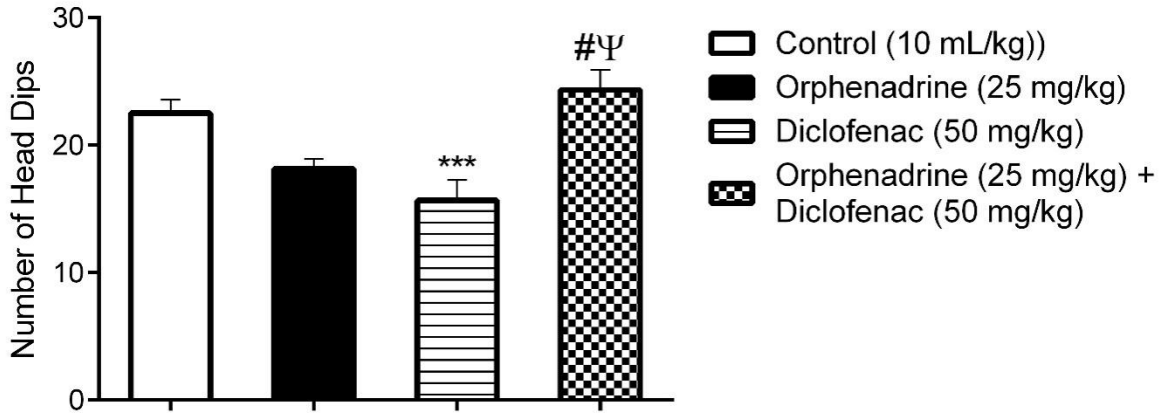

Figure 3.3 The bar graph shows the number of head dips across the different treatment groups.

Data are presented as Mean ± SEM (n=6). diclofenac (50 mg/kg) significantly suppressed exploratory behaviour compared to the control group (***$p < 0.001$). The orphenadrine + diclofenac combination was substantially different from the diclofenac alone group (#$p < 0.05$)

CHAPTER FOUR

4.0. DISCUSSION

The acetic acid–induced writhing test is a well-established and sensitive model for assessing peripheral analgesic activity in rodents. It measures abdominal constrictions (writhes) caused by visceral pain, which is caused by the release of inflammatory mediators of prostaglandins, bradykinin, substance P, and cytokines in the peritoneal cavity (Le Bars *et al*., 2001; Ribeiro *et al*., 2000). Writhing events are a good measure of the transmission of nociceptive information and the effectiveness of analgesic interventions, whether peripheral or central. In this study, treatment with diclofenac and orphenadrine as well as the combination of these two drugs were effective in reducing the number of writhes relative to the control group, and this demonstrates strong antinociceptive effects. This inhibitory effect was observed during the 30 minutes observation period with the combination group showing the greatest analgesic response. The strong action of diclofenac correlates with its known mechanism of action as a non-selective cyclooxygenase (COX-1 and COX-2) inhibitor which suppresses the production of peripheral prostaglandins and, therefore, the sensitization of nociceptors (Rainsford, 2013; Brune &Patrignani, 2015; Botting, 2006). Diclofenac is an effective inhibitor of abdominal constriction behaviours that are indicative of peripheral pain by attenuating prostanoid-mediated signalling. Orphenadrine also produced significant analgesia, and this indicates that it has mechanisms beyond what it is traditionally known to do, as a centrally acting muscle relaxant. Its antinociceptive action is probably mediated by a variety of mechanisms, such as the NMDA receptor antagonism, sodium channel blockage, anticholinergic activity, and antioxidant action, which all regulate central nociceptive transmission as well as peripheral inflammatory reactions (Zhou *et al*., 2018; Freynhagen *et al*., 2016).Surprisingly, the combination of diclofenac and orphenadrine was able to eliminate writhing behaviour entirely, indicating that there was a synergistic effect and not an additive

effect. This is probably the intersection of peripheral prostaglandin inhibition by diclofenac and central neuromodulatory and antioxidant effects of orphenadrine, which is an example of the principle of multimodal analgesia (White *et al*., 2017). Multimodal approaches take advantage of complementary processes across various stages of the pain pathway to increase analgesic effects and decrease adverse effects (Kehlet and Dahl, 2018). The synergism observed is translational. Fixed-dose diclofenac-orphenadrine combinations have been reported to have better analgesic effects, including faster onset, longer duration of effect, and less rescue analgesic use, than diclofenac monotherapy (Tomic *et al*., 2019; Zeiner *et al*., 2023). These clinical observations, coupled with the current preclinical results, support the possibility of therapeutic usefulness of this combination in the treatment of pain. The writhing test induced by acetic acid proved that both diclofenac and orphenadrine have a strong peripheral antinociceptive effect. Their combination, however, resulted in full suppression of writhing, which confirms the combination of central and peripheral mechanisms in pain modulation and supports the potential of the diclofenac-orphenadrine combination as a rational multimodal analgesic approach

Cyclooxygenase-2 (COX-2) is a key inducible enzyme of the prostaglandin biosynthesis pathway, which plays a key role in inflammation, pain regulation, and tissue homeostasis. In contrast to COX-1, which is constitutively expressed, COX-2 is regulated in a complex way by transcriptional, post-transcriptional, translational, and post-translational mechanisms, and it is regulated by inflammatory cytokines, oxidative stress, and other signalling molecules (Kang *et al*., 2007; Saleh *et al*., 2021). This multi-layered control means that changes in COX-2 mRNA expression do not always directly correspond to the changes in the activity of this enzyme or systemic protein levels. Thus, the evaluation of COX-2 gene expression and pharmacological interventions is an essential measure of the effect of drugs on the molecular mechanisms of inflammation. In the present research, the effect of diclofenac treatment on

COX-2 mRNA was observed to be increased, although the drug has a known inhibitory effect on the COX enzyme activity (Rainsford, 2013; Hoy, 2016). This paradox is explained by the feedback regulation: the inhibition of the synthesis of prostaglandins by diclofenac reduces the negative feedback on the activity of transcription factors like Nuclear Factor kappa-light-chain-enhancer of activated B cells, which is still active and leads to the compensatory increase in the expression of the COX-2 gene (Poligone& Baldwin, 2001; Brune &Patrignani, 2015). Therefore, high COX-2 transcripts indicate a transcriptional rebound, and not the inability of diclofenac to inhibit inflammation, because its enzymatic activity is inhibited effectively.On the other hand, the treatment with orphenadrine had a significant effect in suppressing the COX-2 mRNA expression in comparison to control. Orphenadrine is traditionally described as a centrally acting muscle relaxant, but it also has NMDA receptor antagonism, sodium channel blockage, and antioxidant properties, which suppress neuronal excitability and decrease oxidative stress, both of which are upstream activators of NF-kB and COX-2 transcription (Zeiner *et al*., 2023; Zhou *et al*., 2018). Orphenadrine indirectly stabilizes COX-2 expression by inhibiting these signalling pathways, indicating a transcriptional-level anti-inflammatory effect in addition to its neuromodulatory effects. Diclofenac and orphenadrine had a unique pattern of regulation. The COX-2 expression was lower than in the diclofenac-alone group, but it was higher than in the orphenadrine-alone group, suggesting an intermediate but coordinated response. Orphenadrine probably prevented the transcriptional rebound of diclofenac while diclofenac still inhibited enzyme activity. This dual-level modulation; diclofenac acting at the catalytic level and orphenadrine at the transcriptional level offers a viable molecular explanation of the synergistic analgesic action of the two in behavioural tests (Rainsford, 2013; Zeiner *et al*., 2023). These findings highlight the complexity of the COX-2 regulatory loop in inflammatory conditions. In normal circumstances, the production of prostaglandins through the COX-2 activity gives negative

feedback on transcription, which inhibits the production of the enzyme. This activity is inhibited pharmacologically, such as by diclofenac, and eliminates this feedback, leading to a compensatory transcriptional response. Agents such as orphenadrine, which stabilize upstream signalling pathways, inhibit this compensatory response, keeping COX-2 at lower levels of transcription. Therefore, the diclofenac/orphenadrine combination has a balanced COX-2 inhibitory effect on transcription and catalysis, which may maximize analgesic activity and reduce compensatory inflammation.

The hole-board test is a highly validated behavioural paradigm that is frequently used to measure exploratory behaviour and anxiety-like behaviour in rodents (File and Wardill, 1975; Takeda *et al*, 1998). In this test, the rate of dipping of the head in the holes of the perforated board is a sensitive index of both the exploratory drive and emotional reactivity, which is the interaction between curiosity and fear. The anxiolytic agents usually enhance the head-dipping behaviour, and the anxiogenic or sedative drugs decrease it, which is valuable in understanding how the central nervous system (CNS) is involved in arousal and motivation (Magaji *et al*., 2017). In the current experiment, the animals with diclofenac showed a significant decrease in head-dipping behaviour compared with the controls indicating an inhibition of exploratory drive. Although diclofenac is mainly a peripheral cyclooxygenase (COX) enzyme, some evidence suggests that it can penetrate the CNS and affect central production of prostaglandins (Rummel *et al*., 2011; Rainsford, 2013). On the other hand, there was a slight, nonsignificant decrease in exploratory behavior caused by the administration of orphenadrine. The diclofenac and orphenadrine combination was effective in restoring head-dipping behavior to the level of controls. This suggests a central protective effect of behavior, in which orphenadrine seems to offset the CNS dampening of diclofenac. The interaction between diclofenac and orphenadrine highlights a neurobehavioral synergy: while diclofenac exerts potent peripheral analgesic effects, it may inadvertently suppress

CNS arousal through prostaglandin inhibition. Orphenadrine, through its neuromodulatory properties, restores excitatory-inhibitory balance, maintaining normal exploratory behaviour. Incorporating a centrally active agent like orphenadrine could therefore enhance analgesic efficacy while preserving psychomotor function and behavioural vitality, improving tolerability in multimodal pain management. These preclinical findings are in line with clinical reports that reveal that diclofenac-orphenadrine combinations are more effective than diclofenac monotherapy in pain management, patient comfort, and functional recovery (Tomic *et al*., 2019; Zeiner *et al*., 2023). The present results can be regarded as an extension of this knowledge to the behavioral sphere, which indicates that this type of combination therapy can preserve the exploratory drive and retain the central responsiveness.

CHAPTER FIVE

5.0. CONCLUSION

This study shows that diclofenac and orphenadrine work together to provide powerful pain relief through complementary actions. Diclofenac primarily targets peripheral pain by inhibiting cyclooxygenase, while orphenadrine acts on central pathways, influencing NMDA receptors, sodium channels, and oxidative stress. When combined, they produced a synergistic effect, completely preventing writhing and balancing COX-2 activity at both the enzymatic and gene expression levels. Additionally, orphenadrine counteracted the subtle central dampening caused by diclofenac, allowing normal exploratory behaviour in the hole-board test. These findings suggest that the diclofenac–orphenadrine combination is a promising multimodal analgesic strategy, offering effective pain relief while supporting overall behavioural and neurological function.

5.1 Contributions to Knowledge

This study adds important insights to the understanding of multimodal pain therapy. It provides experimental evidence that combining diclofenac and orphenadrine produces not only additive analgesia but also a cooperative molecular interaction involving COX-2 regulation. The findings clarify why diclofenac may increase COX-2 gene expression despite enzyme inhibition—a response likely linked to feedback at the genetic level—and how orphenadrine can stabilize this process. By demonstrating this interplay, this study helps explain the pharmacological rationale behind their clinical co-administration.

Moreover, the research contributes to the understanding of orphenadrine's broader pharmacodynamic profile. Beyond its role as a muscle relaxant, the results suggest it has a modulatory effect on inflammatory signaling and central behavior, potentially involving oxidative and transcriptional pathways. This dual influence—on both the central nervous

system and molecular inflammation—strengthens its value as part of a combined analgesic approach.

Overall, this work bridges behavioral pharmacology and molecular findings, supporting a more integrated view of pain modulation that combines peripheral enzyme inhibition with central neural regulation.

5.2 Recommendations

The findings from this study suggest that combining diclofenac and orphenadrine offers a promising approach for managing inflammatory pain, particularly when both peripheral and central mechanisms are involved. Clinically, the combination could provide effective relief while preserving normal motor and cognitive function, making it a useful option for patients requiring sustained functionality during therapy.

Further studies are recommended to explore this interaction more deeply at the molecular level, including direct measurements of COX-2 protein and prostaglandin (PGE_2) levels. Long-term studies in chronic pain models could also help establish whether this synergy remains consistent over time. It would be beneficial to investigate the specific signaling pathways through which orphenadrine modulates COX expression, particularly in relation to oxidative stress and NF-κB activity. Finally, dose optimization and extended behavioral assessments could strengthen understanding of the safest and most effective therapeutic ratio.

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