IN SILICO AND IN VIVO ASSESSMENT OF THE INDIVIDUAL AND COMBINED EFFECTS OF ORPHENADRINE AND DICLOFENAC ON ACUTE NOCICEPTION AND ANXIETY-LIKE BEHAVIOURS IN MICE: POSSIBLE ROLE ON SEROTONERGIC PATHWAY

BY

IREDIA ISAAC MARVELLOUS

PHA1908522

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY,

FACULTY OF PHARMACY,

UNIVERSITY OF BENIN,

BENIN CITY.

NOVEMBER, 2025.

IN SILICO AND IN VIVO ASSESSMENT OF THE INDIVIDUAL AND COMBINED EFFECTS OF ORPHENADRINE AND DICLOFENAC ON ACUTE NOCICEPTION AND ANXIETY-LIKE BEHAVIOURS IN MICE: POSSIBLE ROLE ON SEROTONERGIC PATHWAY

BY

IREDIA ISAAC MARVELLOUS

PHA1908522

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.

NOVEMBER, 2025.

CERTIFICATION

We the undersigned hereby certify that this work was carried out by IREDIA ISAAC MARVELLOUS with matriculation number PHA1908522, in the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City.

Dr. Osazee Edosuyi (Project Supervisor)	**Date**
Dr. Adaeze P. Uchendu (Ag. Head of Department)	**Date**

DEDICATION

I dedicate this project to God Almighty. Also, I dedicate this project to my Aunt of blessed memory, Late Supr. Linda.

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to the people who supported and guided me throughout this project.

First and foremost, My appreciation goes to my supervisor, Dr. Osazee Edosuyi, for his exceptional guidance, expertise, and continuous encouragement. His insightful critiques and academic support significantly shaped the direction and quality of this project. Thank you for always making time for discussions and for sharing your knowledge.

Secondly, I appreciate the Head of Department of Pharmacology, Dr. (Mrs) Adaeze Phina Uchendu, for her services and academic support in making this a success.

My sincere appreciation goes to my parents (Mr and Mrs Iredia), Your unwavering love, financial support, encouragement, and countless sacrifices provided the foundation for everything I pursue. Your belief in me was the constant driving force that enabled the successful completion of this project.

I would also like to acknowledge my aunt, Mrs Osarugue Tracy for her prayers, moral and financial assistance throughout my academic journey. God bless you ma.

I would also like to acknowledge my aunt, Mrs Precious Ikponmwosa for her moral and financial assistance throughout my academic journey.

Finally, I am incredibly thankful for my project partners; Nelson, Beverly and Onyedika and also my colleagues Raphael, Efua, Theophilus, Imade, Naga, Bernice, My G7 squad, Dahlia, Joan, Andra and many others. Your constant companionship, willingness to listen, and timely humor provided the necessary relief and balance during the challenging periods of this project. Thank you for making this journey a memorable one.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Pain is a multifaceted phenomenon that encompasses both sensory and emotional elements and is often linked with anxiety. This research investigated the effects of diclofenac, orphenadrine, and their combination on pain perception and anxiety-related behaviours in mice.

Twenty-four albino mice weighing 22–32 g were randomly assigned to four groups and administered saline (10 mL/kg, control), diclofenac (50 mg/kg, intraperitoneally), orphenadrine (25 mg/kg, orally), and orphenadrine (25mg/kg) + diclofenac (50mg/kg). Anxiety-like behaviour was evaluated using the Hole Board Apparatus, while analgesic activity was measured using the Formalin-Induced Paw-Licking Test. *In silico* studies were also carried out to test for serotonergic activity of the drugs.

In the formalin test, diclofenac significantly decreased paw-licking time in both the early and late phases (*$p < 0.05$), demonstrating strong peripheral analgesic activity. Orphenadrine showed a moderate reduction in nociceptive behaviour, while the combined treatment produced the greatest analgesic effect (***$p < 0.001$ vs. control, #$p < 0.05$ vs. orphenadrine), indicating a possible synergistic interaction between the two agents.

Mice treated with orphenadrine and the orphenadrine + diclofenac combination exhibited a significant reduction (***$p < 0.001$) in the number of head dips, indicating a marked suppression of exploratory activity and demonstrated anxiolytic or sedative-like effects. Diclofenac alone produced a mild, non-significant decrease in head dips, implying minimal behavioural alteration. The combined treatment resulted in a slightly lower exploratory response than orphenadrine alone, demonstrating an additive or synergistic behavioural effect when both drugs are administered together. The docking study demonstrated that both orphenadrine and diclofenac can interact with the serotonin receptor binding pocket through hydrophobic, hydrogen-bond, and π–π stacking interactions thereby confirming its serotonergic activity.

In conclusion, orphenadrine + diclofenac combination produced complementary actions, resulting in greater and more balanced attenuation of both pain and anxiety. These results suggest that co-administration of a peripherally acting drug with a centrally acting agent may offer a superior therapeutic strategy for managing pain.

CHAPTER ONE

1.0 INTRODUCTION

Pain is a complex sensory and emotional experience associated with actual or potential tissue damage. It serves a protective biological function, but when prolonged, it becomes maladaptive and contributes to suffering and disability. Inflammation, often accompanying pain, involves a cascade of biochemical and cellular events mediated by prostaglandins, cytokines, histamine, and serotonin (5-hydroxytryptamine, 5-HT), which collectively sensitize nociceptors and modulate central processing of pain signals (Sommer, 2009). The interrelationship between peripheral inflammatory mechanisms and central neurotransmission is central to understanding the pharmacology of analgesic agents.

Experimental animal models remain indispensable in elucidating the mechanisms of analgesia and inflammation. The formalin (paw-licking) test and hole-board test are two well-established models for assessing both nociceptive and behavioral effects of pharmacological agents. The formalin test produces a biphasic response: an early neurogenic phase, reflecting direct activation of nociceptors, and a late inflammatory phase, associated with tissue inflammation and central sensitization (Hunskaar & Hole, 1987). The hole-board test, on the other hand, measures exploratory behavior such as head dipping, rearing, and locomotor activity, serving as an index of anxiety or sedation that may accompany analgesic or centrally acting drugs (Brown *et al.*, 2008). When used together, these models help differentiate true analgesic actions from non-specific motor or sedative effects.

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), exerts analgesic and anti-inflammatory effects primarily through inhibition of cyclooxygenase (COX)

enzymes, reducing prostaglandin synthesis and nociceptor sensitization (Gan, 2010). Orphenadrine, a centrally acting muscle relaxant structurally related to diphenhydramine, possesses anticholinergic, antihistaminic, and NMDA receptor antagonist properties that contribute to its analgesic activity (Driessen *et al*., 2013). Clinically, the combination of orphenadrine and diclofenac is prescribed for musculoskeletal pain to provide both anti-inflammatory and muscle-relaxant benefits. However, despite widespread clinical use, the neurochemical basis of their combined analgesic and behavioral effects, particularly involving serotonergic mechanisms, remains poorly understood.

Serotonin plays a multifaceted role in pain modulation, exhibiting both pronociceptive and antinociceptive effects depending on receptor subtype, site of action, and pain state. In the periphery, serotonin released from platelets contributes to inflammation and pain sensitization, whereas in descending spinal pathways it can enhance or inhibit nociceptive transmission (Sommer, 2009). Thus, understanding how analgesic agents modulate serotonergic activity is vital for clarifying their mechanisms of action and side effect profiles.

Research in Nigeria and other developing regions has contributed valuable data on natural and synthetic analgesics using murine models. Igbe *et al*. (2009) demonstrated that the aqueous extract of *Hunteria umbellata* fruit pulp produced significant analgesic and antipyretic effects in mice and rabbits, as evidenced by reduced acetic acid–induced writhing and fever responses. These findings support the relevance of peripheral analgesic mechanisms in phytotherapeutic studies. However, Igbe *et al.* (2009) did not explore central neurotransmitter modulation, leaving open questions about how such agents—and by extension, drugs like orphenadrine and diclofenac—might influence central pain pathways such as those mediated by serotonin.

Given this background, the current study seeks to investigate the effects of orphenadrine, diclofenac, and their combination on behavioral and serotonergic parameters in murine models. By integrating data from the paw-licking and hole-board tests with biochemical measures of serotonin, the study aims to clarify whether the combined therapy acts synergistically or additively, and whether serotonergic modulation underlies its analgesic effects.

1.1 Overview of Pain

Pain is a multidimensional and protective biological experience that signals potential or actual tissue damage. It involves sensory, emotional, cognitive, and social components that together influence the perception of noxious stimuli *(Woolf, 2011)*. When pain becomes chronic or persistent, it loses its adaptive purpose and contributes to pathological states that impair quality of life. Although conceptually distinct, pain and inflammation are closely interlinked; inflammation sensitizes peripheral nociceptors and amplifies pain perception, while sustained pain can, in turn, enhance inflammatory signaling (*Vanderwall & Milligan, 2019)*.

Both phenomena share overlapping biochemical pathways involving prostaglandins, histamine, bradykinin, cytokines, and monoamines such as serotonin *(Sommer, 2009)*. Understanding this bidirectional relationship provides the foundation for exploring the mechanisms through which pharmacological agents such as orphenadrine**,** diclofenac, and their combination modulate pain perception and inflammatory responses.

1.2 Experimental Models of Pain and Behaviour in Rodents

Animal models play a pivotal role in preclinical pain research by providing reproducible and quantifiable measures of nociception, inflammation, and behavioral

responses to pharmacological interventions. Among the available laboratory animals, rodents (mice and rats) are most commonly employed due to their manageable size, rapid breeding, and well-characterized neuroanatomical and biochemical pathways *(*Le Bars *et al., 2001)*.

These models allow the investigation of both peripheral and central mechanisms of analgesia, and when coupled with biochemical assays, they provide valuable insight into drug action at multiple physiological levels (Curatolo *et al., 2023)*. The formalin-induced paw-licking test and the hole-board exploratory behavior test are two of the most widely used paradigms for assessing nociceptive response and central nervous system (CNS) activity. Each model contributes complementary information—pain intensity, inflammation, exploratory drive, anxiety, and motor coordination—which together help to characterize the pharmacodynamic profile of candidate analgesic compounds.

In the context of the present research, these models are employed to evaluate the analgesic and behavioral effects of orphenadrine, diclofenac, and their combination, and to explore their relationship with serotonergic neurotransmission as a biochemical correlate of central pain modulation.

1.2.1 The Paw-Licking (Formalin-Induced Pain) Test

The formalin-induced paw-licking test is a validated model for assessing nociceptive and inflammatory pain *(*Tjølsen *et al., 1992)*. It is particularly useful for distinguishing between neurogenic and inflammatory components of pain, a feature that makes it superior to acute thermal models like the tail-flick or hot-plate tests, which primarily assess reflexive pain responses *(*Hunskaar & Hole*, 1987)*.

In this assay, a small volume (20–50 μL) of dilute formalin (1–5%) is injected subcutaneously into the plantar surface of a rodent's hind paw. The ensuing pain behavior follows a biphasic pattern:

Phase 1 (0–5 minutes post-injection): Represents direct activation of nociceptive C-fibers by formalin, leading to an immediate pain response. It is primarily neurogenic and mediated by direct stimulation of transient receptor potential (TRP) channels and nociceptive afferents *(*Shibata *et al., 1989)*.

Phase 2 (15–60 minutes post-injection): Reflects the inflammatory response resulting from tissue injury, release of prostaglandins, bradykinin, histamine, and serotonin, and subsequent central sensitization within the dorsal horn neurons *(Woolf, 2011)*.

The total time spent licking, biting, or lifting the injected paw serves as a quantitative measure of pain intensity. Analgesic agents are classified based on their ability to suppress one or both phases.

- Centrally acting drugs (e.g., opioids, NMDA antagonists) typically inhibit both phases, reflecting action at the spinal or supraspinal levels.
- Peripherally acting agents (e.g., NSAIDs like diclofenac) mainly reduce the second phase by blocking prostaglandin synthesis and inflammatory signaling *(*Curatolo *et al., 2023)*.

Diclofenac, a potent nonselective COX inhibitor, decreases formalin-induced licking duration, confirming its inhibition of peripheral prostaglandin-mediated hyperalgesia *(Wautier & Wautier, 2023)*. Orphenadrine, by contrast, exhibits central neuromodulatory effects, including weak NMDA antagonism and modulation of descending inhibitory pathways involving serotonin and noradrenaline *(*Hao *et al.,*

2023). Therefore, orphenadrine's action could extend across both phases, complementing the peripheral effect of diclofenac when used in combination.

The formalin test has also been used to validate natural and synthetic analgesic agents. Igbe *et al*. (2009) demonstrated that aqueous extracts of *Hunteria umbellata* significantly reduced both phases of formalin-induced pain, suggesting the involvement of both peripheral anti-inflammatory and central analgesic mechanisms *(*Igbe *et al., 2009)*. This finding provides a comparative framework for interpreting combination therapy, as agents targeting distinct sites—peripheral prostaglandin suppression and central serotonergic modulation—often exhibit additive or synergistic effects.

1.2.2 The Hole-Board Test

The hole-board test is a sensitive and widely accepted model for evaluating exploratory behavior, anxiolytic or anxiogenic activity, and sedative effects in rodents *(File & Wardill, 1975)*. The apparatus consists of a flat platform (usually 40–60 cm square) with regularly spaced holes, each approximately 3 cm in diameter. Rodents are placed individually on the board, and their behavior is recorded for 5–10 minutes. The number and duration of head dips into the holes, along with locomotor activity, are measured as indices of exploratory motivation and emotional reactivity (Brown *et al., 2018)*.

This model is particularly valuable in pharmacological testing because changes in exploratory activity can indicate CNS side effects that may confound pain assessment in other models. For example, a significant reduction in head-dipping behavior may suggest sedation, motor impairment, or anxiety, while increased head-dipping may reflect anxiolytic or stimulant properties *(Vogel, 2023)*.

In analgesic studies, the hole-board test serves a complementary role to nociceptive assays such as the formalin test. It helps to distinguish whether a reduction in pain-related behavior arises from genuine analgesia or from non-specific CNS depression. This distinction is especially crucial when evaluating centrally acting drugs such as orphenadrine, which possesses anticholinergic, antihistaminic, and mild dopaminergic antagonistic properties that could affect locomotion and alertness *(*Hao *et al., 2023)*.

Moreover, exploratory behavior is closely linked to serotonergic neurotransmission, particularly in brain regions such as the hippocampus and prefrontal cortex. Increased serotonin activity, especially via 5-HT_1A and 5-HT_7 receptor pathways, promotes exploratory behavior and reduces anxiety, whereas reduced 5-HT signaling or overactivation of 5-HT_2A and 5-HT_3 receptors tends to suppress exploration and induce anxiety-like responses (Sommer*, 2009;* Hao *et al., 2023)*. Consequently, monitoring behavioral changes in the hole-board test alongside biochemical quantification of serotonin provides an indirect yet informative measure of central serotonergic modulation by test compounds.

1.2.3 Integration of Behavioral and Biochemical Analyses

The integration of behavioral and biochemical endpoints enhances the interpretative value of preclinical pain studies. While behavioral tests reflect the functional outcome of nociceptive and affective processing, biochemical assays elucidate the underlying neurochemical mechanisms.

Quantification of serotonin (5-HT) levels in discrete brain regions—such as the hypothalamus, midbrain, and prefrontal cortex—using enzyme-linked immunosorbent assay (ELISA) or high-performance liquid chromatography (HPLC) provides insight into the involvement of central monoaminergic pathways *(Vogel, 2023;* Hao *et al.,*

2023). Reduced 5-HT levels typically indicate diminished descending inhibition and heightened nociception, whereas normalization or elevation following treatment suggests restored inhibitory tone (Sommer*, 2009)*.

Parallel measurement of prostaglandin E_2 (PGE_2) and cytokines (e.g., TNF-α, IL-1β, IL-6) in paw tissue or plasma helps to delineate peripheral anti-inflammatory activity. NSAIDs like diclofenac markedly reduce PGE_2 concentrations, while drugs influencing central pathways may exert secondary effects on cytokine production via neuroimmune modulation *(Wautier & Wautier, 2023)*

Integrating these parameters allows classification of analgesic mechanisms into three categories:

Peripheral inhibition of inflammation,

Central neuromodulation, and combined or synergistic activity when both pathways are targeted simultaneously.

This multidimensional approach aligns with current recommendations in translational pain research, emphasizing mechanistic specificity over simple behavioral outcomes *(*Curatolo *et al., 2023;* Woolf, *2011;* Sommer*, 2009)*. It also supports the rationale for measuring serotonin alongside behavioral assays in the present study, as alterations in 5-HT concentrations provide a neurochemical correlate of drug-induced analgesia.

The formalin-induced paw-licking and hole-board exploratory behavior tests together provide a robust framework for evaluating the analgesic potential and central effects of pharmacological agents. The former distinguishes between peripheral and central phases of nociception, while the latter assesses general activity and emotional reactivity, ensuring observed analgesia is not secondary to sedation or motor deficits.

In this study, these models are used to assess the analgesic and behavioral profiles of orphenadrine, diclofenac, and their combination, while concurrent quantification of serotonin levels serves to clarify the neurochemical mechanisms underlying observed effects. The dual assessment of behavioral and biochemical outcomes enhances the mechanistic understanding of drug interactions and offers a comprehensive perspective on pain modulation.

1.3 Pharmacological Overview of the Test Drugs

Effective management of pain and inflammation often requires pharmacological agents that target both peripheral and central mechanisms of nociception. The two test drugs examined in this study — diclofenac sodium and orphenadrine citrate — represent pharmacologically distinct yet complementary classes of therapeutic agents. Diclofenac acts mainly by inhibiting prostaglandin biosynthesis through cyclooxygenase (COX) blockade, thereby exerting potent anti-inflammatory and analgesic effects. Orphenadrine, on the other hand, is a centrally acting muscle relaxant and analgesic that modulates cholinergic, histaminergic, and NMDA receptor activity (Hao *et al., 2023)*.

Understanding the chemical composition, molecular structure, and pharmacological profiles of these drugs is critical in explaining their mechanisms of action and potential synergistic interactions when co-administered.

1.3.1 Orphenadrine

Chemical Structure and Properties

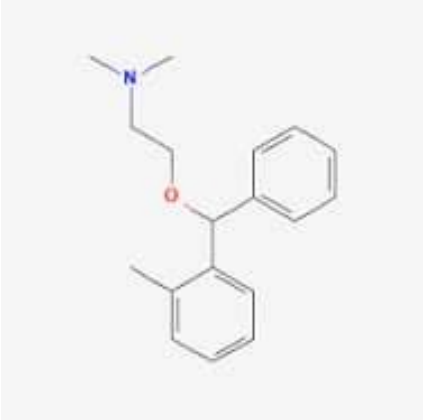


Fig 1.1 *Structure of Orphenadrine (PUBMED)*

Chemically, orphenadrine ($C_{18}H_{23}NO$) is an ethanolamine derivative belonging to the diphenhydramine class of compounds. Structurally, it consists of a 2-methylbenzhydryl moiety linked to an N,N-dimethyl-2-aminoethanol side chain *(Rosenbaum, 1955)*. The molecule possesses a chiral center at the benzhydryl carbon but is used clinically as a racemic mixture. The presence of aromatic rings confers high lipophilicity, which enhances penetration across the blood–brain barrier (BBB), contributing to its central nervous system (CNS) activity.

Orphenadrine citrate, the commonly used salt form, appears as a white crystalline powder with high solubility in water and ethanol. The pKa of its amino group is approximately 9.2, indicating that it exists largely in ionized form at physiological pH but retains sufficient lipophilic balance to permit membrane diffusion *(DrugBank, 2024)*.

This amphipathic nature (having both hydrophilic and hydrophobic regions) is essential for its interaction with multiple receptor sites, including muscarinic, histaminic, and NMDA receptors. The structural similarity to diphenhydramine underlies its antihistaminic and anticholinergic effects, while the aromatic ring system

is thought to mediate NMDA receptor antagonism through steric and electronic interactions *(*Reis & Farias*, 2017)*.

Mechanism of Action

The pharmacological activity of orphenadrine arises from its multi-receptor interactions:

1. Antimuscarinic Activity:

Orphenadrine is a competitive antagonist of central and peripheral muscarinic acetylcholine receptors (M_1–M_5). Inhibition of cholinergic signaling at the spinal and supraspinal levels leads to muscle relaxation and suppression of motor neuron hyperactivity *(*Brunton *et al., 2018)*.

2. Antihistaminic Effect:

The compound antagonizes H_1 histamine receptors, producing mild sedation and decreasing histamine-induced excitation. This contributes to a reduction in the anxiety and tension commonly associated with musculoskeletal pain (Reis & Farias*, 2017)*.

3. NMDA Receptor Antagonism:

Orphenadrine acts as a non-competitive NMDA receptor antagonist, blocking glutamate's excitatory effects on pain transmission pathways. By preventing calcium influx and neuronal hyperexcitability, it helps suppress central sensitization, a key factor in chronic pain development *(*Curatolo *et al., 2023)*.

4. Monoaminergic Modulation:

Evidence suggests that orphenadrine enhances serotonergic (5-HT) and noradrenergic activity in descending pain-inhibitory pathways, thereby strengthening endogenous analgesia *(*Hao *et al., 2023)*. This aligns with its proposed interaction with serotonin-mediated antinociceptive mechanisms relevant to this study.

Pharmacodynamics

Pharmacodynamically, orphenadrine depresses spinal polysynaptic reflexes and reduces skeletal muscle tone without impairing voluntary muscle function *(*Goodman & Gilman*, 2018)*. Its analgesic activity is partly independent of its muscle relaxant action and is related to NMDA and 5-HT modulation.

Animal studies, including those by Igbe *et al*. (2009), have demonstrated that orphenadrine and related compounds reduce both neurogenic and inflammatory pain responses, as seen in the formalin and paw-licking tests *(*Igbe *et al., 2009)*. These findings support its dual action on peripheral and central pain mechanisms.

Pharmacokinetics

Orphenadrine is rapidly absorbed after oral administration, with peak plasma levels occurring within 2–3 hours. Its oral bioavailability exceeds 85%, reflecting efficient gastrointestinal absorption *(DrugBank, 2024)*. The drug's lipophilicity ensures broad tissue distribution and significant CNS penetration.

Metabolism occurs primarily in the liver via CYP2B6 and CYP2D6 enzymes, yielding N-demethylated and hydroxylated metabolites, which are excreted renally. The elimination half-life is 14-16 hours, allowing twice-daily dosing.

Food does not significantly affect absorption, but hepatic impairment may prolong elimination. The drug's steady-state distribution in neural tissues contributes to its prolonged analgesic effect despite moderate plasma clearance.

Toxicology and Adverse Effects

At therapeutic doses, orphenadrine is generally safe and well tolerated. However, due to its anticholinergic properties, side effects such as dry mouth, blurred vision, constipation, urinary retention, and tachycardia are relatively common *(*Brunton *et al., 2018)*.

CNS adverse effects may include dizziness, insomnia, tremor, or confusion, especially in elderly patients or when co-administered with other CNS depressants. High doses can produce anticholinergic delirium, hallucinations, and cardiac arrhythmias *(*Goodman & Gilman*, 2018)*.

Toxicological studies in rodents indicate that chronic exposure to large doses may elevate liver enzymes and induce mild hepatocellular vacuolation, but these effects are reversible *(*Vogel*, 2023)*. The therapeutic index of orphenadrine is relatively wide, and serious toxicity is uncommon when used appropriately.

1.3.2 Diclofenac

Chemical Structure and Properties

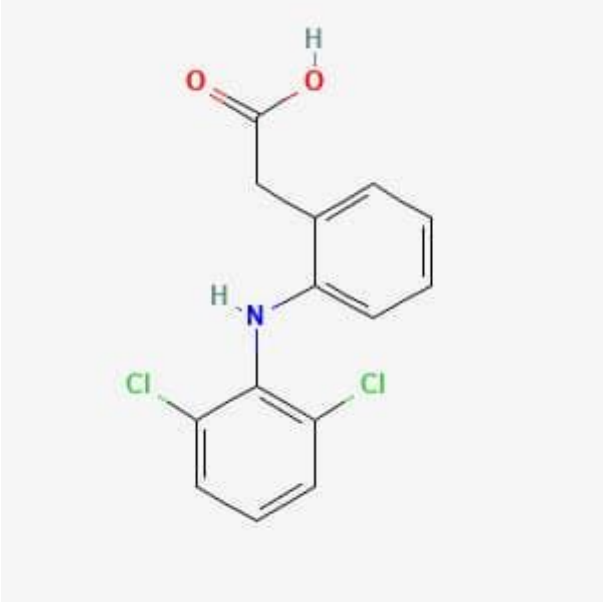


Fig 1.2 *Structure of Diclofenac (PUBMED)*

Diclofenac ($C_{14}H_{11}Cl_2NO_2$) is a phenylacetic acid derivative structurally characterized by a 2,6-dichloroaniline moiety linked to an acetic acid group through an amino bond *(*Vane & Botting, *1998)*. The molecule contains two electron-withdrawing chlorine atoms, which increase its acidic strength and lipophilicity, thereby enhancing tissue penetration and COX enzyme affinity *(*Brunton *et al., 2018)*.

Diclofenac sodium and diclofenac potassium are the most common pharmaceutical forms. The sodium salt is used for oral and parenteral formulations due to its stability, while the potassium salt offers faster dissolution and onset of action.

The drug appears as a white to faintly yellow crystalline powder, practically insoluble in water but soluble in organic solvents. The pKa (4.0) of its carboxylic acid group allows it to exist primarily in the ionized state at physiological pH, favoring COX active-site binding through ionic interactions.

Diclofenac's structural configuration permits hydrogen bonding and hydrophobic interactions within the COX enzyme pocket, accounting for its potent inhibition of prostaglandin synthesis *(*Curatolo *et al., 2023)*.

Mechanism of Action

1. Cyclooxygenase Inhibition:

Diclofenac inhibits both COX-1 and COX-2 isoenzymes, thereby preventing the conversion of arachidonic acid to prostaglandins, prostacyclins, and thromboxanes *(Curatolo et al., 2023)*. The suppression of PGE_2 synthesis reduces peripheral nociceptor sensitization and inflammation.

2. Selective COX-2 Preference:

Although classified as a non-selective NSAID, diclofenac demonstrates moderate

COX-2 selectivity, contributing to its strong anti-inflammatory potency and somewhat improved gastrointestinal safety compared to older NSAIDs *(Brunton et al., 2018)*.

3. Modulation of Ion Channels and NMDA Receptors:

Diclofenac also stabilizes neuronal membranes by inhibiting voltage-gated sodium and potassium channels, which may suppress central pain transmission. Some studies suggest a weak NMDA receptor inhibitory effect, enhancing its analgesic profile *(Ayoub et al., 2018)*.

4. Serotonin and Cytokine Interaction:

By reducing proinflammatory cytokines such as TNF-α and IL-1β, diclofenac indirectly normalizes serotonergic neurotransmission, potentially restoring descending pain inhibition *(*Sommer, *2009)*.

Pharmacodynamics

Diclofenac exhibits analgesic, anti-inflammatory, and antipyretic properties. It reduces pain and edema in both acute and chronic inflammation by suppressing prostaglandin-mediated sensitization. In experimental models such as the formalin-induced paw-licking test, diclofenac predominantly inhibits the second (inflammatory) phase, demonstrating its strong peripheral anti-inflammatory effect *(Hunskaar & Hole, 1987; Igbe et al., 2009)*.

Pharmacokinetics

Diclofenac is rapidly absorbed after oral administration, achieving peak plasma concentrations within 1–2 hours. Despite high absorption, extensive first-pass metabolism reduces bioavailability to 50–60% *(Goodman & Gilman, 2018)*.

The drug is >99% protein-bound, distributes extensively into inflamed tissues, and crosses the placenta but not the blood–brain barrier efficiently. It is metabolized by CYP2C9 into hydroxylated metabolites and excreted via urine and bile. The elimination half-life averages 1–2 hours, but tissue retention accounts for prolonged activity.

Toxicology and Adverse Effects

Common adverse effects include dyspepsia, nausea, and epigastric pain, primarily due to COX-1 inhibition and loss of gastric mucosal protection. Chronic or high-dose use may lead to ulceration, gastrointestinal bleeding, hepatotoxicity, and renal impairment *(Brunton et al., 2018)*.

Diclofenac may also cause elevated transaminases, fluid retention, or hypertension, especially in susceptible individuals. Rare events include hepatic necrosis and cardiovascular thrombotic events *(*Wautier & Wautier*, 2023)*. Toxicological animal studies have shown dose-dependent gastric erosion and hepatic cellular injury.

1.3.3 Combination of Orphenadrine and Diclofenac

Chemical and Pharmacological Rationale

The orphenadrine–diclofenac combination is formulated to provide multimodal analgesia—targeting both peripheral inflammation (via diclofenac) and central pain modulation (via orphenadrine). Marketed products such as *Norflex® Plus* and *Neurodiclo®* are based on this synergy *(*Chandrasekhar *et al., 2012)*.

Diclofenac's carboxylic acid moiety does not chemically interact with orphenadrine's tertiary amine group, minimizing direct physicochemical incompatibility.

Pharmacologically, however, the two act at distinct but complementary sites within the pain pathway.

Mechanistic Synergy

The combination achieves:

- Suppression of prostaglandin synthesis (diclofenac)
- Blockade of NMDA-mediated excitatory transmission (orphenadrine)
- Enhancement of serotonergic inhibition (both drugs indirectly)

  This integrated mechanism reduces nociceptor sensitization and central hyperexcitability, leading to a broader and longer-lasting analgesic effect *(Hao et al., 2023)*.

Pharmacokinetic and Toxicological Considerations

Both agents undergo hepatic metabolism via distinct CYP pathways—CYP2C9 (diclofenac) and CYP2B6/2D6 (orphenadrine)—reducing metabolic competition. Combined use allows for lower individual doses, decreasing systemic toxicity. Adverse effects are generally mild and include drowsiness or gastrointestinal discomfort *(DrugBank, 2024)*.

Animal studies report no significant hepatotoxic or nephrotoxic potentiation when the drugs are co-administered within therapeutic ranges *(Vogel, 2023)*. Nonetheless, long-term human use requires monitoring of hepatic and renal function.

Therapeutic Implications

The combination of orphenadrine and diclofenac presents a rational approach for treating pain conditions with both inflammatory and neuromuscular components. In preclinical models such as the paw-licking and hole-board tests, it allows assessment of behavioral responses and neurochemical markers, such as serotonin levels, offering insights into the interplay between peripheral and central analgesic mechanisms.

1.4 Serotonergic Modulation of Pain and Analgesic Mechanisms

The serotonergic system plays a crucial role in the modulation of nociception, influencing both the inhibition and facilitation of pain signals at multiple levels of the central nervous system. Serotonin (5-hydroxytryptamine; 5-HT) functions not merely as a neurotransmitter but as a neuromodulator that integrates sensory, emotional, and autonomic aspects of pain. It acts through a complex network of receptor subtypes distributed in the spinal cord, brainstem, limbic system, and peripheral tissues, which together determine the net outcome on pain perception *(*Milan*, 2002;* Hao *et al., 2023)*.

1.4.1 Biosynthesis and Distribution of Serotonin

Serotonin is synthesized from the amino acid **L-tryptophan** via two key enzymatic steps:

1. Tryptophan hydroxylase (TPH) converts tryptophan to 5-hydroxytryptophan (5-HTP), and
2. Aromatic L-amino acid decarboxylase (AADC) converts 5-HTP to serotonin (5-HT).

Two isoforms of TPH exist: TPH1, predominantly expressed in peripheral tissues such as enterochromaffin cells of the gut, and TPH2, localized primarily in neurons of the brainstem raphe nuclei *(*Walther & Bader*, 2003)*. The serotonergic neurons of the dorsal and median raphe nuclei project extensively to the spinal dorsal horn, thalamus, hypothalamus, and cerebral cortex, forming the anatomical basis for serotonin's descending control of nociceptive transmission *(Sommer, 2009)*.

In the periphery, serotonin is stored in platelets and mast cells, released during tissue injury or inflammation, and acts on local nociceptors to modulate pain sensitivity. This dual localization — central and peripheral — underscores the complexity of serotonergic control of pain.

1.4.2 Serotonin Receptor Subtypes and Pain Pathways

To date, seven major classes of serotonin receptors (5-HT_1 to 5-HT_7) and multiple subtypes (e.g., 5-HT_1A, 5-HT_2A, 5-HT_3, 5-HT_7) have been identified. Each receptor subtype couples to distinct intracellular signaling pathways, conferring either pro-nociceptive or anti-nociceptive actions depending on its location and activation state *(Millan, 2002)*.

- 5-HT_1A and 5-HT_1B/$_1$D receptors:

  These receptors are Gi/o-protein coupled, leading to inhibition of adenylate cyclase, decreased cAMP, and neuronal hyperpolarization via potassium efflux. Their activation in the spinal dorsal horn and brainstem produces analgesia by inhibiting presynaptic neurotransmitter release *(Bardin, 2011)*

- 5-$HT_2A/_2C$ receptors:

  These are Gq-coupled receptors that activate phospholipase C, increasing intracellular calcium and protein kinase C activity. Activation may cause facilitatory or pronociceptive effects, particularly when expressed in spinal interneurons or peripheral sensory neurons *(Sommer, 2009)*.

- 5-HT_3 receptors:

  These ligand-gated ion channels mediate rapid depolarization of neurons. They are implicated in both pain facilitation and inhibition, depending on circuit context. At the spinal level, 5-HT_3 activation can enhance inhibitory interneuron output, but excessive activation contributes to hyperalgesia *(Bourgoin, 2010)*.

- 5-HT_7 receptors:

  These are G-protein-coupled, increasing cAMP and promoting neuronal inhibition in dorsal horn circuits. Agonists of 5-HT_7 receptors consistently exhibit antinociceptive activity in inflammatory and neuropathic pain models *(Hao et al., 2023)*.

The diversity of receptor types, their localization, and the physiological state of the system collectively determine whether serotonin acts as a pain inhibitor or facilitator.

1.5 Mechanisms of Pain Assessment Using Behavioral Models

Pain assessment in experimental animals requires valid, reproducible, and ethically sound paradigms that capture both the sensory and affective-motivational components of nociception. Unlike reflexive responses that measure only withdrawal thresholds, behavioral models permit the evaluation of complex central processes underlying pain perception, emotional state, and analgesic efficacy. Among these, the hole-board and

formalin-induced paw-licking tests are two of the most widely used and physiologically relevant assays in neuropharmacological research *(Bardin, 2011)*.

These models allow investigation of both peripheral and central analgesic mechanisms, providing insight into serotonergic modulation, prostaglandin involvement, and descending inhibitory pathways. The combination of behavioral observation and neurochemical measurement thus yields a multidimensional understanding of analgesic drug action.

1.5.1 Principles of Behavioral Pain Models

Pain in animals cannot be directly verbalized; it is inferred from observable, quantifiable behaviours such as licking, biting, flinching, vocalization, or exploratory suppression. Behavioural assays exploit the fact that nociceptive stimuli evoke stereotyped responses mediated by defined neural circuits *(Mogil, 2009)*

An ideal model should exhibit:

- Predictive validity, correlating with clinical analgesic effectiveness;
- Construct validity, reflecting known physiological pathways; and
- Reliability and reproducibility, ensuring consistent results across animals and trials *(Le Bars et al., 2001)*.

The hole-board test primarily assesses exploratory behaviour and anxiety-related locomotion, which can be secondarily modulated by analgesic or sedative drugs, while the formalin (paw-licking) test directly measures biphasic nociceptive responses representing both peripheral and central sensitization.

1.5.2 The Hole-Board Test

a. Principle and Methodology

The hole-board apparatus consists of a flat platform perforated with evenly spaced holes. When placed on the board, rodents exhibit spontaneous head-dipping behaviour—an expression of exploratory curiosity dependent on cortical and limbic activity (Takeda *et al., 1998)*.

The number and duration of head dips serve as indices of the animal's emotional reactivity and locomotor activity. Analgesic or anxiolytic drugs tend to increase head-dipping frequency, reflecting reduced pain or anxiety, whereas sedatives or CNS depressants suppress exploration *(*Stankevicius *et al., 2008)*.

1.5.3 The Formalin-Induced Paw-Licking Test

a. Principle and Phases

The formalin test, first described by Dubuisson and Dennis (1977), is one of the most sensitive and informative models of tonic and inflammatory pain. A small volume (usually 0.05 mL) of dilute formalin (2–5%) is injected subcutaneously into the plantar surface of the hind paw, producing a biphasic nociceptive response characterized by paw licking, shaking, and biting *(Dubuisson & Dennis, 1977; Tjølsen et al., 1992)*.

- **Phase I (neurogenic pain)**: Occurs within 0–5 min post-injection due to direct activation of C-fibers and local depolarization of nociceptors.

- **Phase II (inflammatory pain)**: Occurs from 15–60 min post-injection and is driven by **inflammatory mediators** such as prostaglandins, bradykinin, and serotonin.

The intensity and duration of paw-licking behaviour during these phases provide quantitative indices of nociceptive processing.

1.5.4 Integration of Hole-Board and Formalin Tests

The dual application of these models provides a comprehensive behavioural assessment of analgesia:

- The hole-board evaluates the affective-motivational and central exploratory components of pain.
- The formalin test quantifies direct nociceptive behaviour reflecting both peripheral and central mechanisms.

Together, they permit differentiation between true analgesic activity and sedation, and enable correlation with biochemical indices such as serotonin concentration and prostaglandin levels.

By integrating these endpoints, the study achieves both behavioural and neurochemical validation of the proposed drug mechanisms. This approach aligns with modern neuropharmacological paradigms emphasizing translational relevance between preclinical models and human pain syndromes *(*Mogil, *2009;* Hao *et al., 2023)*.

1.6 Justification of study

Pain and anxiety often occur together as comorbid conditions, significantly affecting patients' quality of life and complicating treatment outcomes. Conventional therapy frequently requires the use of multiple agents, such as analgesics and anxiolytics, which may lead to unwanted adverse effects or potential drug interactions. Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is widely used for its potent peripheral analgesic and anti-inflammatory properties but shows limited action on the central nervous system. Conversely, Orphenadrine is a centrally acting muscle relaxant possessing anticholinergic, antihistaminic, and NMDA receptor antagonist properties, suggesting potential modulatory effects on both pain and anxiety.

Exploring the combination of Orphenadrine and Diclofenac is therefore of pharmacological importance. Their concurrent use may produce synergistic or complementary actions that enhance therapeutic efficacy while minimizing adverse effects. Furthermore, the serotonin (5-HT) pathway plays a critical role in both pain modulation and anxiety regulation. Investigating the potential involvement of this pathway could provide mechanistic insight into how these drugs exert their effects.

By integrating in silico molecular docking analysis and in vivo behavioural assays, this study aims to bridge the gap between computational predictions and biological outcomes. The findings may contribute to the development of improved multimodal analgesic strategies and offer a scientific basis for rational drug combination in the management of pain associated with anxiety disorders.

1.7 Aims and Objectives

1.7.1 Aim of the Study

The aim of this study is to evaluate the individual and combined effects of Orphenadrine and Diclofenac on acute nociception and anxiety-like behaviours in mice, and to investigate the possible involvement of the serotonin pathway in their mechanism of action using in silico studies.

1.7.2 Specific Objectives

The specific objectives of this study are to:

1. Assess the acute antinociceptive (analgesic) effects of Orphenadrine, Diclofenac, and their combination using the paw-licking test in mice.

2. Evaluate the behavioural effects of Orphenadrine, Diclofenac, and their combination using the hole-board assay.

3. Conduct in silico molecular docking studies to predict and compare the binding affinities of Orphenadrine and Diclofenac with serotonin-related receptor targets.

4. Compare the individual and combined pharmacological outcomes to identify possible synergistic interactions between Orphenadrine and Diclofenac.

1.8 Significance of the Study

This study holds considerable scientific and clinical importance as it explores the therapeutic potential of combining Orphenadrine and Diclofenac in the management of pain and anxiety-related disorders. Pain and anxiety frequently coexist, and their overlapping neurochemical pathways—particularly involving serotonin—make it

necessary to explore treatment options that can simultaneously address both conditions.

By assessing the effects of Orphenadrine and Diclofenac individually and in combination, this research provides valuable insight into the possible synergistic interactions between a centrally acting agent and a peripherally acting analgesic. Such findings could lead to enhanced analgesic efficacy, reduced dosage requirements, and minimization of side effects associated with higher single-drug doses.

Furthermore, evaluating the role of the serotonin pathway contributes to a better understanding of the neurochemical basis of pain–anxiety comorbidity. The in silico docking studies also offer predictive information about drug–receptor interactions, which may guide future drug design and development.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Drugs and Chemicals

The following drugs and reagents were used for the study:

Diclofenac sodium (Voltaren, Novartis™), Orphenadrine citrate (Norflex, Pfizer™), Formalin solution (1% v/v in saline), Normal saline (0.9% NaCl), Chloroform (BDH chemicals), Distilled water, Reagent for Serotonin(5HT) assay, 10% buffered formaldehyde solution.

All chemicals and reagents were of analytical grade and used as supplied without further purification. Drug doses and concentrations were prepared freshly on the day of the experiment based on the required dose per kilogram of body weight.

2.1.2 Apparatus and Equipment

The following apparatus and equipment were utilized throughout the study:

Analytical weighing balance, Orogastric tubes (size 18G), Sterile syringes (1 ml), hypodermic needles (for intraperitoneal injection), Stopwatch/Timer, Centrifuge (for tissue homogenization), Beakers and measuring cylinders, Universal and plain sample bottles, Wooden hole-board apparatus (40 × 40 cm) with 16 holes (3 cm diameter each), Cage racks and animal cages, Non-toxic animal marker kit (4 colour), observation chamber (for formalin test), Personal protective equipment (gloves, lab coats, masks), Filter paper and cotton wool.

All instruments were calibrated and disinfected before and after use to ensure experimental accuracy and hygiene.

Study Area and Design

The area for this study was conducted in the Department of Pharmacology, Faculty of Pharmacy, University of Benin, Benin City, Edo State.

This antinociceptive study employed a murine model. Animals were administered standard doses of orphenadrine, diclofenac, and orphenadrine-diclofenac combination. The hole board assay was also conducted.

Afterwards, animals were humanely sacrificed and organs harvested.

Inclusion and Exclusion Criteria

Healthy mice weighing an average of 25 g with no obvious signs of illness, distress, and physical deformity were used for this study.

Pregnant or lactating females, wounded mice, or any form of infection, distress, or abnormality, weighing less than 20 g or above 32 g were excluded from this study.

2.1.3 Experimental Animals

A total of twenty-four (24) healthy adult Swiss albino mice of either sex, weighing between 22-32g, were used for this experiment. The animals were bred and kept in the Animal House of the Department of Pharmacology and Toxicology, University of Benin, Benin City, Edo State. The mice were allowed to acclimatize for two weeks before the commencement of the experiments.

During acclimatization, the mice were housed in clean, well-ventilated polypropylene cages with wood shavings as bedding. Environmental conditions were maintained at a temperature of 22 ± 2°C, and a 12-hour light/dark cycle. They were fed with standard pellet diet and had free access to clean drinking water *ad libitum.*

All experimental procedures were conducted in accordance with the Internationally accepted guidelines for laboratory animal care and use. were approved by the Institutional Animal Ethics Committee.

2.2 Methods

2.2.1 Experimental Design and Drug Administration

The mice were randomly divided into four experimental groups, each containing six (6) animals. The animals were marked on their tails using non-toxic marker to facilitate identification (I–IIIII and One unmarked).

The control group received normal saline at a dose of 10 ml/kg body weight, administered orally with the aid of an orogastric tube.

The second group (Standard drug) received diclofenac sodium at a dose of 50 mg/kg, administered intraperitoneally using a 1 ml syringe.

The third group (Test drug) received orphenadrine citrate at a dose of 25 mg/kg body weight, administered orally using an orogastric tube.

The fourth group received a combination of orphenadrine (25 mg/kg, oral) and diclofenac (50 mg/kg, intraperitoneal).

Each mouse was identified using a non-toxic marker on the tail for individual recognition. All administrations were performed once daily for 24 hours

2.2.2 Formalin-Induced Paw Licking Test and Post-Treatment Holeboard

Twenty-four hours after the baseline Holeboard test, Drug administration and the formalin test were carried out as follows:

Drug Administration: Each group received their respective treatment as detailed above.

Formalin Test: After the 30-minute absorption period, each mouse received a 20 µL of 1% formalin solution into the plantar surface of the right hind paw. The mouse was then immediately placed in a transparent observation chamber to facilitate observation (Hunskaar *et al, 1985).*

The total duration of paw licking or biting behaviour was recorded using a stopwatch during two distinct phases (Igbe *et. al*, 2009).

1. The early (neurogenic) phase, occurring 0–5 minutes after injection.
2. The late (inflammatory) phase, occurring 15–30 minutes post-injection.

After completion of the observation period, the animals were returned to their cages.

2.2.3 Hole-Board Test

Prior to drug administration, baseline anxiety-like behaviour was assessed using the holeboard apparatus. The apparatus consisted of a wooden board (40 cm × 40 cm) with sixteen equidistant holes (3 cm in diameter), elevated 25 cm above the ground. Each mouse was gently placed in the centre of the board and allowed to explore freely for five (5) minutes. The number of head dips—defined as the insertion of the snout and eyes below the plane of the board—was manually counted. (File, S.E., & Wardill, A. G. 1975)

Between trials, the surface of the apparatus was cleaned with 70% ethanol to eliminate scent cues that might affect subsequent observations.

2.2.4 Sample Collection

After completion of behavioral assessments, all animals were euthanized using chloroform anesthesia. The brain, kidney, heart and liver were excised, weighed, and examined for any visible abnormalities. The tissues were preserved in 10% buffered formaldehyde solution for subsequent biochemical analysis.

2.2.5. Biochemical Assay

The activity of Serotonin (5HT) in tissue homogenates was determined spectrophotometrically using a commercially available assay kit following the manufacturer's instructions.

2.2.6. Data Analysis

All data were expressed as Mean ± Standard Error of the Mean (SEM). Statistical analyses were performed using GraphPad Prism software (version [insert version]). Data from the formalin test were analyzed using one-way Analysis of Variance (ANOVA).

Data from the EPM test (baseline vs. post-treatment) were analyzed using two-way ANOVA with treatment and time as factors, followed by appropriate post-hoc analysis.

Data from the serotonin (5HT) assay were analyzed using one-way ANOVA.

A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

CHAPTER THREE

3.0 RESULTS

3.1 Formalin-Induced Paw Licking Assay

The formalin-induced paw licking assay was performed to evaluate the analgesic efficacy of the test compounds and their combination during the early (neurogenic) and late (inflammatory) pain phases. The findings are presented in Table 3.1.

The control group displayed vigorous and prolonged paw licking, recording mean durations of 84.9 ± 4.0 s at 5 min and 110.7 ± 15.9 s at 15 min.

Orphenadrine (25 mg/kg) produced a moderate attenuation, lowering licking times to 60.2 ± 4.3 s and 91.5 ± 10.1 s at the 5- and 15-min marks, respectively.

Diclofenac (50 mg/kg) elicited a stronger effect, significantly reducing licking to 52.9 ± 17.5 s at 5 min and 39.2 ± 17.2 s at 15 min (*$p < 0.05$ vs. control).

The combination of orphenadrine and diclofenac (25 + 50 mg/kg) yielded the most striking analgesia, with licking times of 32.5 ± 8.2 s at 5 min and 1.4 ± 1.4 s at 15 min (*$p < 0.001$, #$p < 0.05$ vs. control and orphenadrine alone, respectively).

Table 3.1: Effect of orphenadrine, diclofenac, and their combination on paw licking duration (Mean ± SEM) in the formalin-induced test.

Groups	Dose	5minutes	15minutes
Control	10 ml/kg	84.9±4.0	110.7±15.9
Orphenadrine	25 mg/kg	60.2±4.3	91.5±10.1
Diclofenac	50 mg/kg	52.9±17.5	39.2±17.2*
Orphenadrine + Diclofenac	25 mg/kg + 50 mg/kg	32.5±8.2*	1.4±1.4***#

*Compared to Control, # Compared to Orphenadrine alone.

3.2 Hole Board Assay:

Figure 3.2 presents the effects of the different drug treatments on exploratory behaviour, assessed by the number of head dips in the hole board test. Results from a one-way ANOVA showed a significant overall difference among the treatment groups ($F(3,10) = 9.788$, $p = 0.0025$)

Post-hoc analysis revealed that there was a statistically significant difference in exploratory behavior among the treatment groups. When compared to the control group (15.00 ± 0.73), the orphenadrine treated group (8.00 ± 0.61) and the orphenadrine + diclofenac combination group (8.20 ± 0.53) showed significant effects (***$p < 0.001$), whereas the diclofenac only group (10.50 ± 0.69) showed no significant difference ($p > 0.05$). These findings suggest that orphenadrine reduces exploratory activity, possibly through a CNS depressant or anxiolytic-like mechanism, while diclofenac does not alter this behavior.

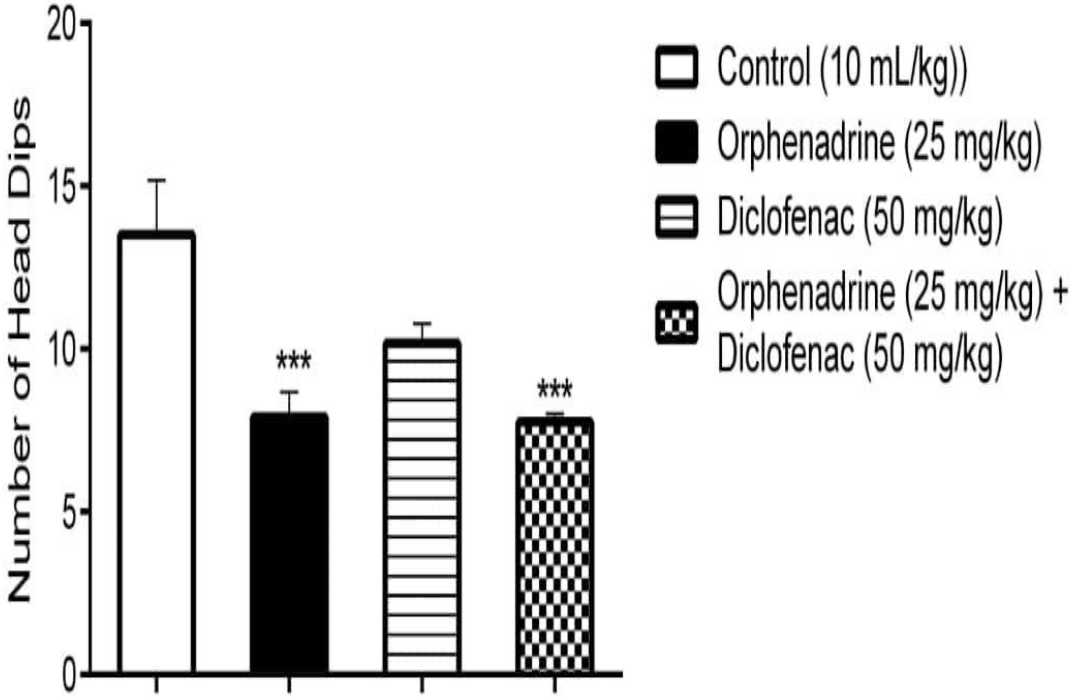


Figure 3.1: The bar graph shows the number of head dips across the different treatment groups.

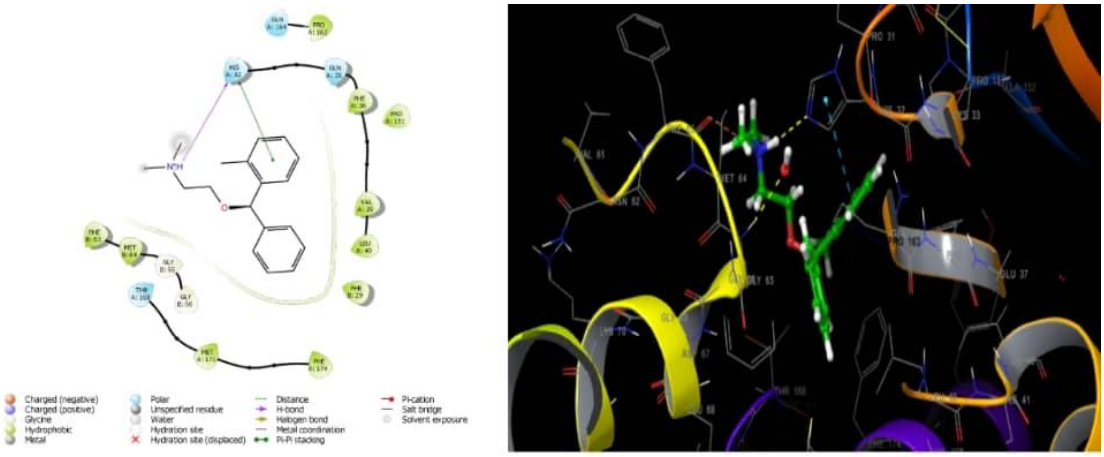
Data are presented as Mean ± SEM (n=6). Diclofenac (50 mg/kg) showed no significant difference compared to the Control group ($p > 0.05$). The Ophenadrine + Diclofenac combination was substantially different from the diclofenac alone group (***$p > 0.001$)

3.3 Serotonin *In silico* Assay:

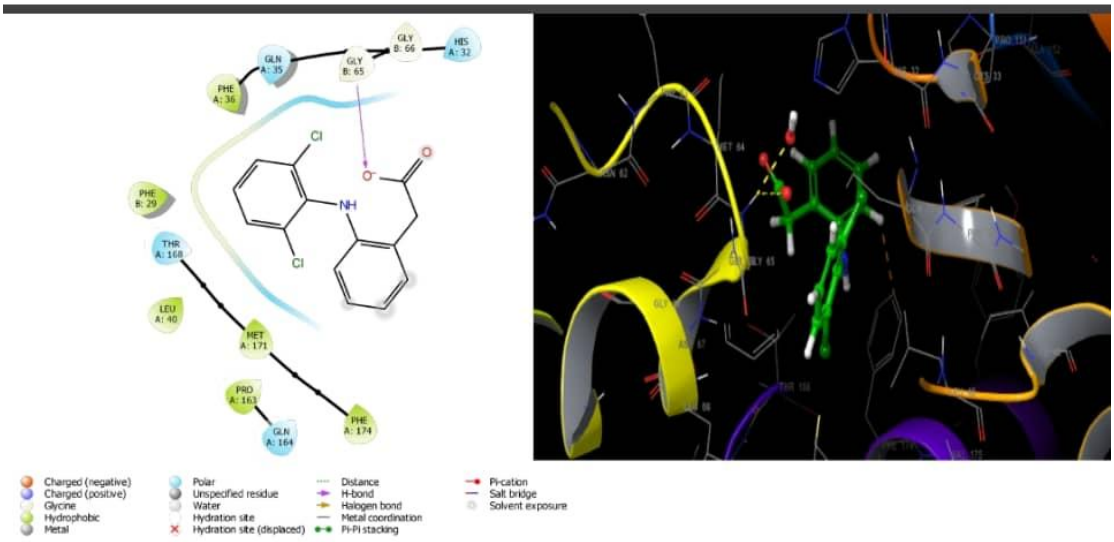
The figure below is a Molecular docking studies showing that both Orphenadrine and Diclofenac interacted strongly with residues within the binding pocket of the serotonin receptor. Orphenadrine formed key hydrogen bonds and π–π stacking interactions with residues such as Phe, Tyr, and Glu, indicating stable aromatic and polar associations. Diclofenac displayed similar affinity, forming hydrogen bonds and hydrophobic contacts with residues including Phe, Gly, Gln, and His. The 3D docking visualization confirmed proper fitting of both ligands into the receptor cavity, suggesting strong binding stability and favorable binding energies.

Table 3.2: Binding interaction, binding residues, docking implication and effect relevance of orphenadrine and diclofenac.

Features	Orphenadrine	Diclofenac
Binding Interaction	H-bonds + π-π + hydrophobic	H-bonds + hydrophobic
Binding residues	PHE, TYR, GLU, ASN, SER, LEU	PHE, GLN, THR, LEU, PRO
Docking implication	Stronger aromatic interaction potential	Moderate, mainly hydrophobic anchoring
Effect relevance	Alter serotonin levels by receptor interaction	Indirectly influence serotonin pathways



2D and 3D interacting plots between Orphenadrine with residues at the binding pocket of serotonin receptor



2D and 3D interacting plots between diclofenac with residues at the binding pocket of serotonin receptor

Fig 3.2: *Docking interaction of orphenadrine and diclofenac*

CHAPTER FOUR

4.0 DISCUSSION

The paw-licking test is a standard method used to assess the analgesic effects of drugs. It consists of two separate phases that indicate different pain mechanisms. The early phase (0–5 minutes) corresponds to neurogenic pain which is caused by the direct activation of nociceptors by the injected chemical. The late phase (15–30 minutes) reflects inflammatory pain, which arises from the release of inflammatory mediators like prostaglandins, histamine, and serotonin.*(Experimental methods in pharmacology).*

In this experiment, administration of orphenadrine, diclofenac, and their combination (orphenadrine + diclofenac) led to a reduction in paw-licking duration when compared with the control group confirming their analgesic properties. During the early phase, both orphenadrine (25 mg/kg) and diclofenac (50 mg/kg) produced moderate analgesic effects, whereas their combination (orphenadrine (25 mg/kg) and diclofenac (50 mg/kg)) produced a marked reduction in licking time, signifying an enhanced early-phase pain-relieving response. This is because the central analgesic action of orphenadrine complemented the mild central and peripheral effects of diclofenac. This interaction enhanced the suppression of neurogenic pain, leading to stronger and faster analgesic effect at the initial phase of the response.

In the late phase, the combined treatment of Orphenadrine and Diclofenac produced a highly significant decrease in paw-licking duration ($p < 0.001$) relative to both the control and Orphenadrine alone, indicating a synergistic interaction. This implies that the combination provides both central and peripheral analgesic effects. The central anticholinergic and antihistaminic actions of Orphenadrine may have amplified the anti-inflammatory and prostaglandin-inhibitory properties of diclofenac, leading to superior relieve during the second phase.

The synergistic effect observed with orphenadrine and diclofenac is biologically plausible, as both drugs act through different paths in the nociceptive pathway. While diclofenac has been suggested to primarily inhibit cyclooxygenase enzymes and reduce peripheral prostaglandin synthesis, orphenadrine dampens nociceptive signaling pathway and NMDA antagonism as reported by Desaphy *et al.*, (2009). Our results also agree with previous preclinical studies, such as those of Hunskaar and colleagues (2009), which showed that orphenadrine produced antinociception in hot-plate and formalin models and potentiated the effects of other analgesics. Clinically, fixed-dose combinations of diclofenac and orphenadrine have demonstrated superior pain relief and reduced need for opioids in postoperative settings compared to diclofenac alone, further supporting the translational relevance of our findings.

Nevertheless, some studies contrast with our observations. Friedman *et al*. (2018) found that adding orphenadrine to naproxen did not significantly improve pain or functional outcomes in patients with acute low back pain. Similarly, systematic reviews of skeletal muscle relaxants highlight that their analgesic efficacy in humans is inconsistent and often condition-dependent (Cashin *et al*., 2021). These discrepancies may reflect differences in pain models, species variability, dosing regimens, and the fact that human pain syndromes are more complex than acute nociception induced in mice.

Taken together, the findings of this study support the antinociceptive potential of orphenadrine and suggest that its combination with diclofenac yields greater analgesic efficacy than monotherapy. While these results are encouraging and align with much of the preclinical and some clinical evidence, caution is warranted in extrapolating directly to clinical practice, especially in light of studies that question the additive benefit of muscle relaxants in pain management.

The Hole-Board Assay is a standard behavioral test used to quantify exploratory behavior in rodents, with the number of head dips serving as a primary measure of exploration and curiosity (Mora, S.*, et al*., 1998). A significant reduction in head dipping is typically interpreted as a decrease in exploratory drive, often attributed to sedation or increased anxiety-like behavior (Redolat, *et al*. 2000).

The results showed a contrast in the central nervous system effects of the two drugs. The control group established the baseline level of exploration with an increased number of head dips. The administration of orphenadrine (25 mg/kg) resulted in a highly statistically significant reduction (*) in exploratory activity. orphenadrine is classified as a skeletal muscle relaxant with potent anticholinergic and antihistaminic actions (Brogden, *et al.* 1975). Since both these mechanisms are strongly associated with central nervous system depression and sedation, the observed reduction in head dipping is most likely a direct consequence of orphenadrine's sedative effect, leading to decreased motor output and reduced head dips.

Diclofenac (50 mg/kg), an NSAID primarily known for its peripheral anti-inflammatory and analgesic properties, caused a numerical decrease in head dips, but this reduction lacked statistical significance when compared to the control group (as indicated by the absence of an asterisk). This finding indicates that, at the tested dose, diclofenac does not meaningfully alter exploratory behavior in the Hole-Board Assay. While NSAIDs can penetrate the blood-brain barrier and have been implicated in modulating neuroinflammation, the lack of a significant behavioral effect here suggests its influence on core exploratory drive is negligible.

The combination of orphenadrine (25 mg/kg) + diclofenac (50 mg/kg) produced a result that was statistically significant compared to the Control, but quantitatively identical to the orphenadrine-alone group. It demonstrates a clear lack of additive or synergistic effect between the two drugs in this behavioral assay. Since diclofenac

showed no significant effect alone, and the combination's outcome mirrored that of orphenadrine alone, the data confirm that the behavioral effect of the combination is entirely dictated by the dominant, centrally-acting sedative properties of orphenadrine. These findings confirm the potent CNS depressant action of orphenadrine as a major factor in reducing exploratory behavior. The lack of statistical effect for diclofenac alone, coupled with the absence of potentiation in the combination group, indicates that any potential central action of diclofenac is behaviorally inactive or sub-threshold at the dose tested, having no remarkable impact on the exploratory drive measured in the Hole-Board Assay.

Future research must first establish whether the reduction in head dips is due to sedation or genuine changes in exploratory behavior. The most critical step is the Open Field Test (OFT), which would confirm sedation or motor impairment by measuring the Total Distance Traveled (Redolat, *et al.* 2000). Finally, to investigate if diclofenac possesses a hidden, synergistic potential that was simply masked by the current dose of orphenadrine, a Dose-Response Study is necessary. Repeating the Hole-Board Assay with a significantly Lower Dose of orphenadrine (e.g., 5 mg/kg and 10 mg/kg) both alone and in combination with the same diclofenac dose (50 mg/kg) would be beneficial. If a lower dose of orphenadrine yields a sub-maximal reduction in head dips, the addition of diclofenac might then produce a statistically significant additive effect, providing evidence for combined central activity at non-sedating doses.

The present study employed *in silico* molecular docking to investigate the binding interactions of orphenadrine and diclofenac with the serotonin receptor. Molecular docking is a computational technique used to predict how a small molecule fits into the binding site of a target protein. This computational approach provides insight into

how these compounds may modulate serotonergic activity, potentially explaining their observed analgesic and behavioural effects in experimental models.

Docking results revealed that both orphenadrine and diclofenac occupy the binding pocket of the serotonin receptor, forming stabilizing interactions with several key amino acid residues. The 2D and 3D interaction plots showed that orphenadrine formed multiple hydrogen bonds, hydrophobic interactions, and π–π stacking with residues such as Phe, Leu, Tyr, and Gln, indicating a strong binding orientation within the receptor cavity. Diclofenac, on the other hand, engaged primarily through hydrophobic contacts and a few hydrogen bonds involving residues like Phe, Leu, Gln, and Thr. These interaction profiles suggest that while both compounds exhibit affinity for the serotonin receptor, orphenadrine demonstrates a stronger and more stable binding pose.

The aromatic structure of orphenadrine allows π–π interactions with phenylalanine and tyrosine residues in the receptor pocket. Such interactions are essential for stabilizing ligand–receptor complexes, particularly in G-protein coupled receptors (GPCRs) like serotonin receptors (Wang *et al*., 2013). Additionally, orphenadrine's tertiary amine group contributes to hydrogen bonding with polar residues, further enhancing its affinity.

Diclofenac's interaction was characterized by extensive hydrophobic anchoring, reflecting its planar aromatic structure and lipophilic nature. Although it exhibited fewer hydrogen bonds, its chlorine substituents facilitated van der Waals contacts within the receptor cavity. Similar findings were reported by Tian *et al*. (2013), where diclofenac was observed to interact non-specifically with PDZ domains through hydrophobic forces.

Collectively, these observations suggest that orphenadrine may exhibit higher receptor affinity than diclofenac, consistent with the more diverse array of stabilizing

interactions in its docking pose. This could explain why, in the paw-licking and hole-board tests, orphenadrine and its combination with diclofenac produced a more pronounced reduction in nociceptive and exploratory responses confirming their synergistic effect.

Despite the promising binding results, molecular docking represents a static model of receptor–ligand interaction and does not account for receptor flexibility or dynamic solvent effects. Consequently, the predicted binding affinities should be interpreted qualitatively rather than quantitatively. Moreover, in the absence of subtype-selective receptor structures (e.g., 5-HT_1A vs. 5-HT_2A), the specific binding subtype remains uncertain. Future research should examine subtype selectivity into other serotonin receptor (e.g., 5-HT_2A, 5-HT_7) to evaluate whether binding is selective or broad, which has implications for therapeutic/side-effect profiles.

CHAPTER FIVE

5.0 CONCLUSION, CONTRIBUTION TO KNOWLEDGE AND RECOMMENDATION

5.1 CONCLUSION

This research examined the behavioural influence of diclofenac, orphenadrine, and their combination on anxiety and pain-related responses in mice, using the Hole board and the Formalin-Induced Paw Licking Test. Diclofenac showed no significant effect in exploratory behaviour in the hole board assay and strong analgesic activity in the formalin test. Orphenadrine, a centrally acting muscle relaxant and NMDA receptor antagonist, produced modest analgesic effects but exhibited signs of anxiety-like behaviour in the hole board, possibly due to its anticholinergic and dopaminergic actions. Interestingly, the combination of diclofenac and orphenadrine resulted in a synergistic effect, amplifying analgesic responses and showed a slight significant difference in the hole board compared to orphenadrine alone thereby demonstrating synergistic effect. The docking study demonstrated that both orphenadrine and diclofenac can interact with the serotonin receptor binding pocket through hydrophobic, hydrogen-bond, and π–π stacking interactions thereby confirming its possible serotonergic activity. Orphenadrine exhibited stronger and more diverse binding patterns, suggesting higher affinity and a greater potential for serotonergic modulation. Diclofenac also showed stable hydrophobic interactions, supporting the hypothesis of a complementary or synergistic mechanism when used in combination.

5.2 CONTRIBUTION TO KNOWLEDGE

This study offers several significant and original insights into behavioural pharmacology and the relationship between pain and anxiety. It provides experimental evidence that diclofenac, in addition to its well-established anti-inflammatory properties, exhibits mild anxiolytic-like effects in mice. Furthermore, the findings

reveal the dual nature of orphenadrine—while it exerts central analgesic actions through NMDA receptor antagonism and serotonin receptor, it may also provoke mild anxiolytic and sedative responses. This behavioural profile emphasizes the importance of assessing both the pain-relieving and emotional effects of centrally acting drugs, particularly those with anticholinergic activity. The combined administration of diclofenac and orphenadrine produced a synergistic interaction, enhancing analgesic efficacy and promoting a more stable behavioural outcome. This observed synergy reinforces the principle of multimodal analgesia, demonstrating that agents targeting different pathways—both peripheral and central—can work together to achieve improved therapeutic benefits with fewer adverse effects.

5.3 RECOMMENDATION

Building upon these results, several recommendations are suggested for future research and potential clinical translation. Further studies should explore the molecular and neurochemical bases of the observed synergistic interaction, with particular attention to subtype selectivity into other serotonin receptor (e.g., 5-HT_2A, 5-HT_7) to evaluate whether binding is selective or broad, dopaminergic pathways, and cytokine regulation in brain regions involved in pain and anxiety processing. Dose-response analyses are also necessary to determine the most effective combination ratios that enhance therapeutic efficacy while reducing adverse effects such as sedation or anticholinergic toxicity, thereby ensuring clinical safety. Additionally, future research should include both male and female animals from different strains to evaluate possible sex-related and genetic variations in drug response, improving the reliability and applicability of the findings.

REFERENCES

Albadrany, Y. M., Naser, A. S., & Shaban, K. A. (2024). Detection of potential effects of orphenadrine upon anesthesia with propofol and/or thiopental in mice. Iraqi Journal of Veterinary Sciences, 38(1), 239-243.

Baggio, C. H., et al. (2009). The antinociceptive effect of diclofenac, paracetamol, and their combination in mice: an isobolographic analysis. European Journal of Pharmacology, 618(1-3), 51-57.

Casarrubea, M., Sorbera, F., Santangelo, A., & Crescimanno, G. (2012). The effects of diazepam on the behavioral structure of the rat's response to pain in the hot-plate test: anxiolysis vs. pain modulation. Neuropharmacology, 63(2), 310-321.

Chiappini, S., Mosca, A., Miuli, A., Semeraro, F. M., Mancusi, G., Santovito, M. C., ... & Di Giannantonio, M. (2022). Misuse of anticholinergic medications: a systematic review. Biomedicines, 10(2), 355.

Edosuyi, O., Igbe, I., & Iniaghe, L. O. (2018). Antinociceptive and antioxidant activities of Hunteria umbellata stem bark: possible role of the serotonergic, opioidergic and dopaminergic pathways. Journal of Complementary and Integrative Medicine, 15(1).

Egunlusi, A. O., & Joubert, J. (2024). NMDA receptor antagonists: emerging insights into molecular mechanisms and clinical applications in neurological disorders. Pharmaceuticals, 17(5), 639.

Ennaceur, A., & Chazot, P. L. (2014). The Hole-Board apparatus: an updated review on its performance in animal models of anxiety and memory. Behavioural Brain Research, 264, 1-25.

Falodun, A. B. I. O. D. U. N., Igbe, I. G. H. O. D. A. R. O., Erharuyi, O., & Agbanyim, O. J. (2013). Chemical characterization, anti-inflammatory, and analgesic properties of Jatropha multifida root bark. Journal of Applied Sciences and Environmental Management, 17(3), 357-362.

Gan, T. J. (2010). Diclofenac: an update on its mechanism of action and safety profile. Current medical research and opinion, 26(7), 1715-1731.

Gómez-Gonzalo, M. (2025). Astrocytes in Rodent Anxiety-Related Behavior: Role of Calcium and Beyond. International Journal of Molecular Sciences, 26(6), 2774.

Hunskaar, S., Berge, O. G., & Hole, K. (1985). Antinociceptive effects of orphenadrine citrate in mice. European journal of pharmacology, 111(2), 221-226.

Igbe, I., & Edike, T. (2015). In vivo antinociceptive activity of the aqueous leaf extract of Voacanga africana Stapf (Apocynaceae) in mice. Journal of Science and Practice of Pharmacy, 2(1), 51-54.

Javed, M. A., Bibi, S., Jan, M. S., Ikram, M., Zaidi, A., Farooq, U., ... & Rashid, U. (2022). Diclofenac derivatives as concomitant inhibitors of cholinesterase, monoamine oxidase, cyclooxygenase-2, and 5-lipoxygenase for the treatment of Alzheimer's disease: synthesis, pharmacology, toxicity, and docking studies. RSC advances, 12(35), 22503-22517.

Kahraman, N. H., & Tunç, S. K. (2025). Comparative evaluation of the effects of diclofenac sodium and vitamin D supplementation on symptoms in individuals with myofascial pain and vitamin D deficiency: a randomized controlled clinical trial. BMC Oral Health, 25(1), 1383.

Kapo, S. M., Rakanović-Todić, M., Burnazović-Ristić, L., Kusturica, J., Ćesić, A. K., Ademović, E., ... & Aganović-Mušinović, I. (2023). Analgesic and anti-inflammatory effects of diclofenac and ketoprofen patches in two different rat models of acute inflammation. Journal of King Saud University-Science, 35(1), 102394.

Kristiansen, K. (2004). Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. Pharmacology & Therapeutics, 103(1), 21–80.

Mehta, K., Murugan, S., Krishnan, M., Kumar, S., & Puranpanda, M. (2024). Comparative efficacy of pre-emptive Paracetamol, Ibuprofen, and Diclofenac for post-operative pain relief in surgical extractions. Frontiers in Health Informatics, 13(6).

Millan, M. J. (2002). Descending control of pain. Progress in Neurobiology, 66(6), 355–474.

Moore, R. A., Derry, S., McQuay, H. J., & Wiffen, P. J. (2011). Single-dose oral analgesics for acute postoperative pain in adults. Cochrane Database of Systematic Reviews, (9).

Naser, A. S., Albadrany, Y. M., & Abdullah, M. A. (2024). Evaluation of the advantages of orphenadrine in anaesthesia caused by ketamine in mice. Iraqi Journal of Veterinary Sciences, 38(1), 233-238.

Pacifici, G. M. (2024). Clinical Pharmacology of Diclofenac. Biomedical Journal of Scientific & Technical Research, 56(5), 48609-48621.

Saindane, R. A., & Thombre, N. A. (2025). Concurrent use of skeletal muscle relaxants and NSAIDs in low back pain management: A critical review of current evidence and future directions. The Thai Journal of Pharmaceutical Sciences, 49(2), 5.

Salari, M., Ziaeefar, P., Rezaei, B., Alikhani, A., Pourani, M. R., Dashti, T., ... & Abdollahimajd, F. (2024). Cutaneous adverse reactions associated with medications for movement disorders: a narrative review. Iranian Journal of Dermatology, 27(4), 251-268.

Silva, J., Todorovic, D., & Fiala, K. (2024). Skeletal Muscle Relaxants and Acute Pain. In Perioperative Pain Management: A Clinical Guide (pp. 655-678). Cham: Springer Nature Switzerland.

Singh, H. (2022). Single Dose Diclofenac and Tamsulosin Effectiveness in Patients Undergoing Double J-Stent Removal Under Local Anaesthesia.

Sommer, C. (2004). Serotonin in pain and analgesia: actions in the periphery. Molecular Neurobiology, 30(2), 117–125

Tjølsen, A., Berge, O. G., Hunskaar, S., Rosland, J. H., & Hole, K. (1992). The formalin test: an evaluation of the method. Pain, 51(1), 5-17.

Tomic, J., Wallner, J., Mischak, I., Sendlhofer, G., Zemann, W., Schanbacher, M., ... & Zrnc, T. A. (2022). Intravenous ibuprofen versus diclofenac plus orphenadrine in orthognathic surgery: a prospective, randomized, double-blind, controlled clinical study. Clinical Oral Investigations, 26(5), 4117-4125.

Ushkalova, E. A., Zyryanov, S. K., & Zatolochina, K. E. (2020). The fixed combination of diclofenac and orphenadrine in the treatment of acute pain syndromes. Neurology, Neuropsychiatry, Psychosomatics, 12(1), 100-104.

Van Niekerk, B. (2023). Evaluation of the muscle relaxant orphenadrine in third molar surgery (Doctoral dissertation, University of the Western Cape).

Wang, C., Jiang, Y., Ma, J., Wu, H., Wacker, D., Katritch, V., ... & Stevens, R. C. (2013). Structural basis for molecular recognition at serotonin receptors. Science, 340(6132), 610–614.