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College of Veterinary Medicine

Comparison of the Pharmacological and Molecular Effects between Nimesulide and Aspirin in Mice

Taimaa Adlan Yahya

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Supervised by

Professor

Dr . Yaareb Jaafar Mousa

2025 A.D. **1446 A.H.**

Comparison of the Pharmacological and Molecular Effects between Nimesulide and Aspirin in Mice

A Dissertation Submitted

By

Taimaa Adlan Yahya

To

The council of the College of Veterinary Medicine

University of Mosul

In

Partial Fulfillment of the Requirements

For the Degree of Philosophy Doctorate

In

Veterinary Medicine / Veterinary Pharmacology

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Dr . Yaareb Jaafar Mousa

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

اللَّهُ لَا إِلَهَ إِلَّا هُوَ الْحَيُّ الْقَيُّومُ لَا تَأْخُذُهُ سِنَةٌ وَلَا نَوْمٌ لَهُ مَا فِي السَّمَاوَاتِ وَمَا فِي الْأَرْضِ مَنْ ذَا الَّذِي يَشْفَعُ عِنْدَهُ إِلَّا بِإِذْنِهِ يَعْلَمُ مَا بَيْنَ أَيْدِيهِمْ وَمَا خَلْفَهُمْ وَلَا يُحِيطُونَ بِشَيْءٍ مِنْ عِلْمِهِ إِلَّا بِمَا شَاءَ وَسِعَ كُرْسِيُّهُ السَّمَاوَاتِ وَالْأَرْضَ وَلاَ يَؤُودُهُ حِفْظُهُمَا وَهُوَ الْعَلِيُّ الْعَظِيمُ ﴾

صدق الله العظيم

سورة البقرة الاية (255)

Supervisor Certification

I certify that this dissertation entitled (**Comparison of the pharmacological and molecular effects between nimesulide and aspirin in mice**) was prepared under my supervision at the College of Veterinary Medicine / University of Mosul, as a partial fulfillment of the requirements for the degree of Ph.D. in Veterinary Medicine/Veterinary Pharmacology.

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Name: **Prof. Dr. Yaareb Jaafar Mousa**

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Name**: Prof. Dr.Raad Abdulghany Al-Sengery**

Date: / /2025

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We, the members of the Evaluation and Discussion Committee, have reviewed this dissertation and discussed the student **Taimaa Adlan Yahya** in its contents on / / 2025, and certify that the deserves the degree of Ph.D. in Veterinary Medicine/Veterinary Pharmacology .

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The College of Veterinary Medicine Council was met, the meeting, on / / 2025, and decided to award her a degree of Ph.D. in Veterinary pharmacology.

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Assistant Dean for Scientific Affairs

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Professor
Dr. Dhafer Mohammad Aziz
Dean

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taimaa

Abstract

The goal of this study is to compare the pharmacological (analgesic, antipyretic, and anti-inflammatory) and molecular effects between nimesulide (selective) and aspirin (non-selective) COX_2 inhibitors in mice.

The analgesic median effective doses (ED_{50s}) for nimesulide and aspirin in mice were 7.9 and 212.23 mg/kg given intramuscularly (IM) while the acute median lethal doses (LD_{50s}) were 181.7 and 1210.3 mg/kg, IM respectively determined by the up-and-down method. Furthermore, nimesulide was the safest one in mice according to the therapeutic index (TI) calculated (which was 23 for nimesulide and 6 for aspirin).

Nimesulide and aspirin when administered at doses of 15.8 and 424.5 mg/kg, IM respectively (which resemble the ED_{100} of both drugs) exert their analgesia in a dose- and time-dependent paradigm. The time of 30 min after injection bears a maximal analgesic efficacy for both drugs.

Nimesulide and aspirin (at doses of 15.8 and 424.5 mg/kg, IM) prevent the visceral pain (writhing reflex) induced by 1% acetic acid with a significant superiority of nimesulide (66%) over aspirin (46%) in comparison to the positive control (acetic acid) group.

The antipyretic effect of nimesulide was significantly more efficient than aspirin by decreasing the induced fever by baker's yeast (135 mg/kg, IP) along with all the measured times (1, 2, 3, and 4 h after baker's yeast induction).

Formalin (1%) induced inflammation and pain when injected in mice paw of the positive control group while nimesulide decreased the inflammation (0.5, 1, and 2 hours after formalin injection) and pain (through decreasing the numbers of paw lifting and licking during 30 minutes) in a good manner in comparison to aspirin.

Aspirin was the better medication as an anti-coagulant compared to nimesulide which was detected by measuring the prothrombin time in all the treated and control groups of mice.

High-performance liquid chromatography (HPLC) measurement of nimesulide plasma concentration (given at 15.8 mg/kg, IM) was higher compared to aspirin plasma concentration (424.5 mg/kg, IM) in mice at all the measured times of 0.5, 1, 2, 4, and 24 hours after injection.

Nimesulide pharmacokinetic variables were estimated to be as $AUC_{0-\infty}$ 169.18 µg×h /ml, $AUMC_{0-\infty}$ 2358.72 µg×h^2/ml, K_{el} 0.06 h^{-1}, C_{max} 14.62 µg, T_{max} 0.5 h, $t_{1/2\beta}$ 11.07 h, MRT 13.94 h, V_{ss} 1.49 L/kg, and Cl 0.09 L/h/kg, while aspirin pharmacokinetic parameters differs to be 82.31, 2428.32, 0.03, 4.35, 0.5, 21.25, 29.50, 158.12 and 5.16, respectively.

Both nimesulide (15.8 mg/kg, IM) and aspirin (424.5 mg/kg, IM) inhibited COX_2 activity through their decrease in COX_2 concentrations in plasma, liver, and kidney of mice with superior inhibition when administering nimesulide in comparison to the control (negative and positive) .

This study reports new pharmacological mechanisms for nimesulide and aspirin that contribute to their analgesic and anti-inflammatory effects by other important intracellular mechanisms.

Nimesulide than aspirin were significantly reducing caspase3 activity in the kidney, plasma and liver (anti-apoptotic and anti-inflammatory effects) when given for five consecutive days.

Further investigation at the molecular level revealed the ability of nimesulide and aspirin to inhibit the peroxisome proliferation-activated receptors-alpha (PPARα) which contributes to their anti-inflammatory and anti-nociceptive effects.

Other molecular assessments of nimesulide and aspirin pharmacological activity indicate their reduction of the COX_2 gene expression in the kidney of mice (down-regulation) after being given for five consecutive days with therapeutic doses of 15.8 and 424.5 mg/kg, IM, respectively which contribute to their efficacy as antipyretic, analgesic and anti-inflammatory drugs.

Our data demonstrate that nimesulide has better pharmacological properties (analgesic, antipyretic, and anti-inflammatory) than aspirin besides its superiority at the molecular level (caspase3, PPARα and COX_2 gene expression) in mice which makes it useful for practical use in the field of veterinary medicine.

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List of Abbreviation

Abbreviation	Full name
NSAIDs	Nonsteroidal anti-inflammatory drugs
PGs	Prostaglandins
TX	Thromboxane
AA	Arachidonic acid
COX	Cyclooxygenase enzyme
IL	Interleukin
Ph A2	Phospholipase A_2
LOX	Lipoxygenases
PPAR-α	Peroxisome proliferator-activated receptor alpha
NF-*K*b	Nuclear factor-*K*b
ASA	acetylsalicylic acid
UGT1A6	UDP-glucuronosyltransferase 1A6
CYP450	Cytochrome P450
NAT2	N-acetyl transferase 2
LDL	Low-density lipoprotein
GI	Gastrointestinal
IM	Intramuscular
IP	Intraperitoneal
ED_{50}	Analgesic median effected dose
LD_{50}	Lethal median effected dose
PT	Prothrombin time
HPLC	High-performance liquid chromatography
cDNA	Complementary DNA

Chapter One

Introduction

Historically, medicines were applied to relieve temperature , pain, and inflammation from plants or herbs known for centuries. The first record was about 3500 years ago in the Ebers papyrus. Hippocrates, Celsus, Dioscorides, Pliny the Elder, and Galen recommended salicylate decoctions for rheumatic pain (Vane, 2000). Edward Stone made probably the first “clinical trial” and found that 1.8 g of willow bark reduced fever in 50 patients (Stone, 1763; Edmundas, 2021). Felix Hoffmann, a chemist, prepared the first sample of pure acetylsalicylic acid in 1897. Dr. Heinrich tested acetylsalicylic acid in animals, found analgesic , antipyretic and anti-inflammatory properties, and marketed the product in 1899 under the trademark Aspirin (Sneader, 2000; Edmundas, 2021).

In the 19^{th}–20^{th} centuries promoted the development of non-steroidal anti-inflammatory drugs (NSAIDs) , most of which were initially organic acids, and later on non-acidic compounds were discovered (Prescott, 1979). It was agreed that aspirin was recognized as the progenitor of the pharmacotherapeutic class of NSAID, and the first in the class of medicine was phenylbutazone and indomethacin (Wright, 1993), continuing with other NSAIDs, including ibuprofen, diclofenac, naproxen, and piroxicam (Pasero and Marson, 2011) . The development of new NSAIDs and introduction to the market was derived from the discovery of the mechanism of action of NSAIDs based on the inhibition of prostaglandin biosynthesis through an arachidonic acid pathway. In 1971, Sir Vane confirmed that aspirin and NSAID-related drugs inhibit the formation of prostaglandins associated with fever, pain and inflammation, thus provided that a physiologic rationale for the use of NSAIDs in the managing of fever, pain and inflammation (Vane, 1971) .

The British pharmacologist John Robert shared the 1982 Nobel Prize in physiology with Swedish scientists Sune K. Bergström and Bengt I. Samuelsson for their discovered concerning prostaglandins and related biologically as active substances that effect on blood pressure, allergic reactions and body temperature

and other physiologic phenomena in mammals (Botting, 2010; Shampo *et al*., 2013). Since then, NSAIDs became first-choice drugs for the treatment of different fever, pain, and inflammatory conditions.

In animals, pain management was importance and the use of NSAIDs were increased dramatically in recent decades, and the use of NSAIDs in companion with animals is routine practice. However, all NSAIDs have the potential for other side effects that should be considered in the overall management of the inflammatory conditions .

In general, drugs with preferential activity against Cyclooxygenase-2 (COX-2) , which is the enzyme responsible for prostaglandin biosynthesis from arachidonic acid, may have fewer adverse effects due to COX-1 sparing activity . In dogs, favorable ratios have been reported for carprofen, deracoxib, meloxicam, robenacoxib and firocoxib, whereas unfavorable ratios have been reported for aspirin, vedaprofen and phenylbutazone. COX-2 inhibitor drugs are associated with less platelet aggregation and less gastrointestinal ulceration ; however, it may be an simplification to assume that COX-2 inhibition is without risk (Scott, 2021) .

Nimesulide (4-nitro-2-phenoxymethanesulfonanilide) is a non-acidic, NSAIDs with a preferential COX-2 inhibitor activity which decreased the production of prostaglandin (the chemical mediator responsible of pain, fever and inflammatory production). Nimesulide has more selectivity 20 times towards COX-2 than that of COX-1. Nimesulide is commonly used for the treatment of fever, pain and inflammatory conditions like arthritis with a relatively low risk for gastrointestinal side effects. (Cashman, 1996; Yuan *et al*., 2000; Botting, 2006; Gao *et al*., 2018; Caiazzo *et al*., 2019) .

Aspirin, (acetylsalicylic acid) on other hand, is a non-selective COX enzyme inhibitor. It has been used in human and veterinary medicine. It has the antipyretic, analgesic and anti-inflammatory properties of NSAIDs . The therapeutic effect of aspirin due to its ability to inhibit the production of prostaglandin. Aspirin binds covalently with COX-1 and COX-2, which leads to inhibition irreversibly of COX action, unlike other NSAIDs that bind reversibly with COX and cause wider side effects comparable to other ones (Cashman, 1996; Yuan *et al*., 2000; Bachert *et al*., 2005; Botting, 2006; Schror, 2009; Cooper and Voelker, 2012).

There are other mechanisms of nimesulide and aspirin to reduce inflammation and pain; these molecular mechanisms included their effects on caspase 3, apoptosis, peroxisome proliferation activating receptor (PPARα), and COX-2 gene expression (Xu *et al*., 1999; Fahmi *et al*., 2001; Mukherjee *et al*., 2001; Shaik *et al*., 2004; Kalajdzic *et al*., 2002; Zandbergen and Plutzky, 2007; Liang *et al*., 2014; Liu *et al*., 2017; Ferreira *et al*., 2021; Ozdemir *et al*., 2023).

The importance of our research lies in conducting scientific experiments to compare the pharmacological and molecular effects of nimesulide (selective) and aspirin (non-selective) COX-2 inhibitors in terms of the analgesic, antipyretic and anti-inflammatory efficacy, as well as the degree of safety of the drugs in mice, besides their pharmacokinetics and molecular effects for future use of these drugs in the field of veterinary medicine .

Specific aims and hypothesis :

1. Investigate the influences of selective and non-selective COX-2 inhibitors on the pharmacological effects in mice.

2. Assessment of the molecular effects of selective and non-selective COX-2 inhibitors in mice.

3. A comparative effect between nimesulide and aspirin on the inhibition of COX-2 concentration .

4. Measuring of nimesulide and aspirin pharmacokinetic variables.

5. Comparison of the effects of nimesulide and aspirin on apoptosis through their activity on caspase 3.

6. The comparison between nimesulide and aspirin in terms of their effects on the PPAR-α .

7. Measuring COX-2 gene expression due to administration of nimesulide and aspirin to conclude their influence upon the up- or down-regulation.

As for hypothesis related to the study ,the selective COX-2 inhibitors like nimesulide works by specifically inhibitors COX-2 while the non-selective COX-2 inhibitors such as aspirin ,cause non-specific inhibition of COX-1 and 2 which will affect their pharmacological (analgesic, antipyretic and anti-iflammatory properties) beside the molecular efficacy in mice.

To achieve these goals, a series of experiments were conducted:

1. Measuring the analgesic median-effective dose of nimesulide and aspirin in mice

2. Assessment the acute median lethal dose of nimesulide and aspirin in mice.

3. Evaluation the drug safety for nimesulide and aspirin in mice.

4. Measuring the relationship between these drugs and their Time- and dose dependant administration .

5. Comparative effect between nimesulide and aspirin for preventing of visceral pain induced by acetic acid.

6. Comparison between nimesulide and aspirin at the level of their antipyretic and anti-inflammatory effects in mice.

7. Comparative anti-coagulant activity between nimesulide and aspirin in mice.

8. Comparative measurement between nimesulide and aspirin pharmacokinetics in mice.

9. Evaluation of the degree of Nimesulide and Aspirin upon COX-2 inhibition in mice

10. Comparative efficacy between Nimesulide and Aspirin at their molecular level related to apoptosis , PPAR-α and COX-2 gene expression.

Chapter Two

Review of Literature

2-1:Overview on the Nonsteroidal anti-inflammatory drugs (NSAIDs) family

NSAIDs are the most prescribed drugs worldwide. It is estimated that 30 million people per day around the world take NSAIDs (Bhala *et al*., 2013). NSAIDs are used due to their potent antipyretic, anti-inflammatory and analgesic effects. Inhibition of the cyclooxygenase enzyme (COX), which is take the part in biosynthesis of prostaglandins (PGs) and thromboxane (TX), is the mechanism of action of NSAIDs . These PGs and TXs are important mediators of inflammation, pain, and fever. Inflammation has a major role in the pathophysiology of different diseases (Bacchi *et al*., 2012)

NSAIDs affect the action and synthesis of inflammatory mediators, including PGs, interleukin (IL)-2, IL-6, coagulation, cascade-derived peptides, and tumor necrosis factor (TNF). The synthesis of prostanoids (PGE_2, PGF_2 alpha, PGI_2, PGD_2, and thromboxne A2 (TXA_2) that are produced from arachidonic acid (AA) causes pain and inflammation. Arachidonic acid, mainly ground as esterified phosphatidylcholine and phosphatidylethanolamine phospholipids, forms in the membranes. AA is released from the cell membrane by phospholipase A_2 (PLA_2), which is the rate-limiting step for eicosanoids. COX enzymes convert arachidonic acid to PGs, prostacyclins, and TXs (Samad *et al*., 2002) .

This cascade starts with the formation of PGG_2, then PGH_2. Then PGH_2 is converted into numerous PG isoforms, such as PGD_2, PGE_2, PGF_2 α, PGI_2, or TXA_2, by tissue-specific isomerases. In other pathways, arachidonic acid is altered into leukotrienes by three forms of lipoxygenases (5-LOX, 12-LOX, and 15-LOX). Different kinds of cells produce leukotrienes, including white blood cells (leukocytes), mast cells, brain, spleen, lung, and heart. 12-LOX and 15-LOX are play roles in the production of lipoxins. There are three isoforms of Cyclooxygenase enzyme (Cox), namely COX-1, COX-2 and COX-3 (Birmingham and Buvanendran, 2014).

COX1 is a constitutive isoform, while the inducible isoform is COX-2 (Figure 1).

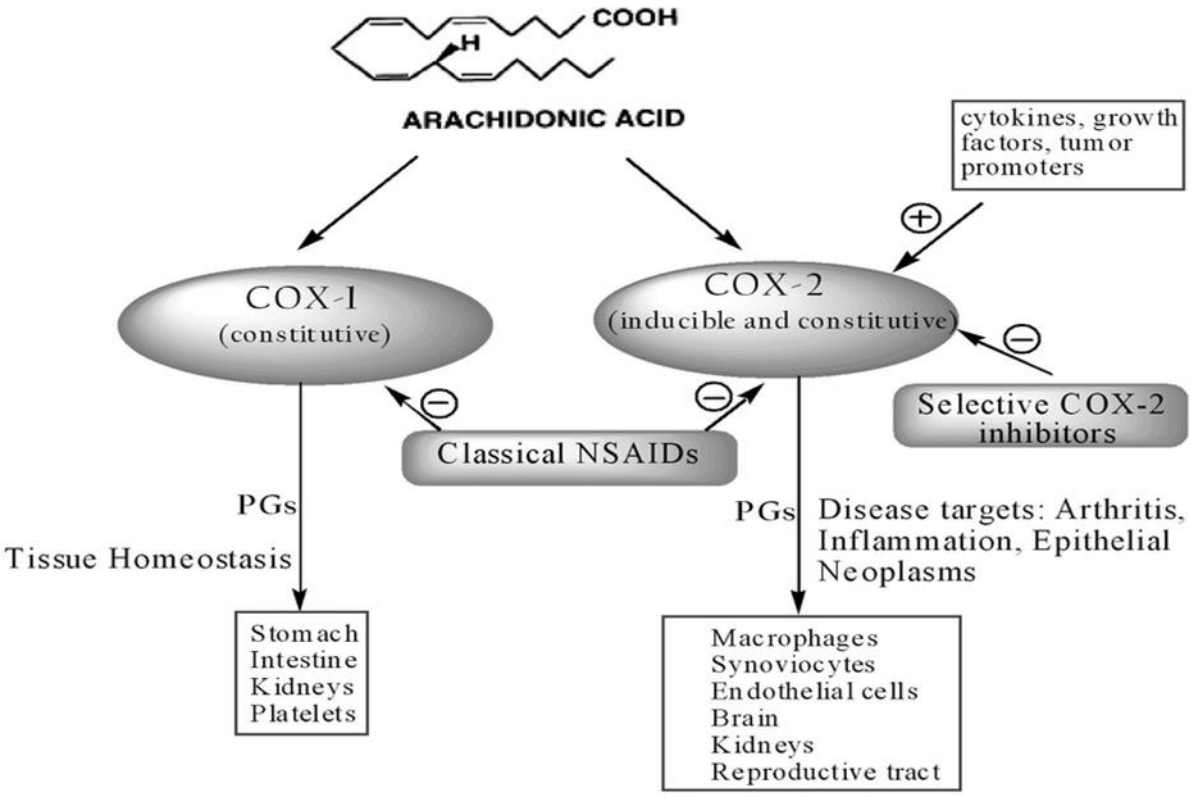


Figure 1: prostaglandins biosynthesis from arachidonic acid(Kasturi *et al*, 2019) .

COX : cyclooxygenase enzyme ; PG_S : prostaglandins .

Both isoforms are expressed in various tissues and at several levels, but they may also found within the same tissue. The COX-1 plays a role in the persistence of physiological events but found that the elevate expression level of COX1in different carcinomas, while the COX-2 is elevate in inflammatory conditions but the expression is low at physiological levels in some tissues such as the brain, uterus and kidney (Faki and Ayse , 2021).

In humans, COX-3 is expressed in the cerebral cortex and spinal cord and is found in the endothelial cells, heart and monocytes in smaller quantities, but in animals, COX-3 is expressed in the cerebral cortex of canine and in lesser amounts in other tissues analyzed . COX-3 play a role in the happened of pain, but its function is not fully understood yet. Acetaminophen prevents pain and fever and is an antipyretic/analgesic drug one of the world's most popular. It absences a clear mechanism of action, but it effects COX3 activity in the brain of dogs (Chandrasekharan *et al*., 2002; Zidar *et al*., 2009).

COX-1 is thought to be useful to the body's homeostasis with the maintain of mucosal epithelium, so inhibit of COX-1 leads to gastric ulcers (Buvanendran, 2012). On the other side, inhibition of COX-2 could only reduce the production of prostanoids such as PGE_2 and PGI_2, which are only produced in inflammatory and pathological condition, Therefore, there is a trend for clinical trials to use NSAIDs for selective COX-2 inhibition because of their superior safety profile (Agarwal *et al*., 2009) .

NSAIDs are routinely used for the treated of inflammation and pain associated with osteoarthritis in dogs and horses and for colic and laminitis in horses. Also, NSAIDs are used for the treated of perioperative pain in the animals. In veterinary medicine, NSAIDs also found that uses in the management of severe pain, and optimally in combination with opioid drugs. Also NSAIDs were used in conjunction with antimicrobial drugs for the treatment of acute respiratory diseases in cattle. Recently, NSAID has become important in the treatment and inhibition of cancer. In humans Epidemiologic studies show that aspirin used was associated with a significant decreased in the occurrence of colon cancer. Newer evidence suggested that the therapeutics effects of NSAIDs on colon cancer was mediate by the inhibition of COX-2, which may be up-regulated in several cancers. In veterinary medicine, nimesulide has been shown that have anticancer effects in neoplastic pancreatic cells through inhibiting the proliferation and apoptosis (Ferreira *et al*., 2021; Chu *et al*., 2018), also, piroxicam has been shown to decrease the size of tumors such as the transitional cell carcinoma in dogs. Specific COX-2 inhibitors may proved beneficial as a primary or adjunctive treatment in the management of cancer. (Edwards, 2021) (Figure 2):

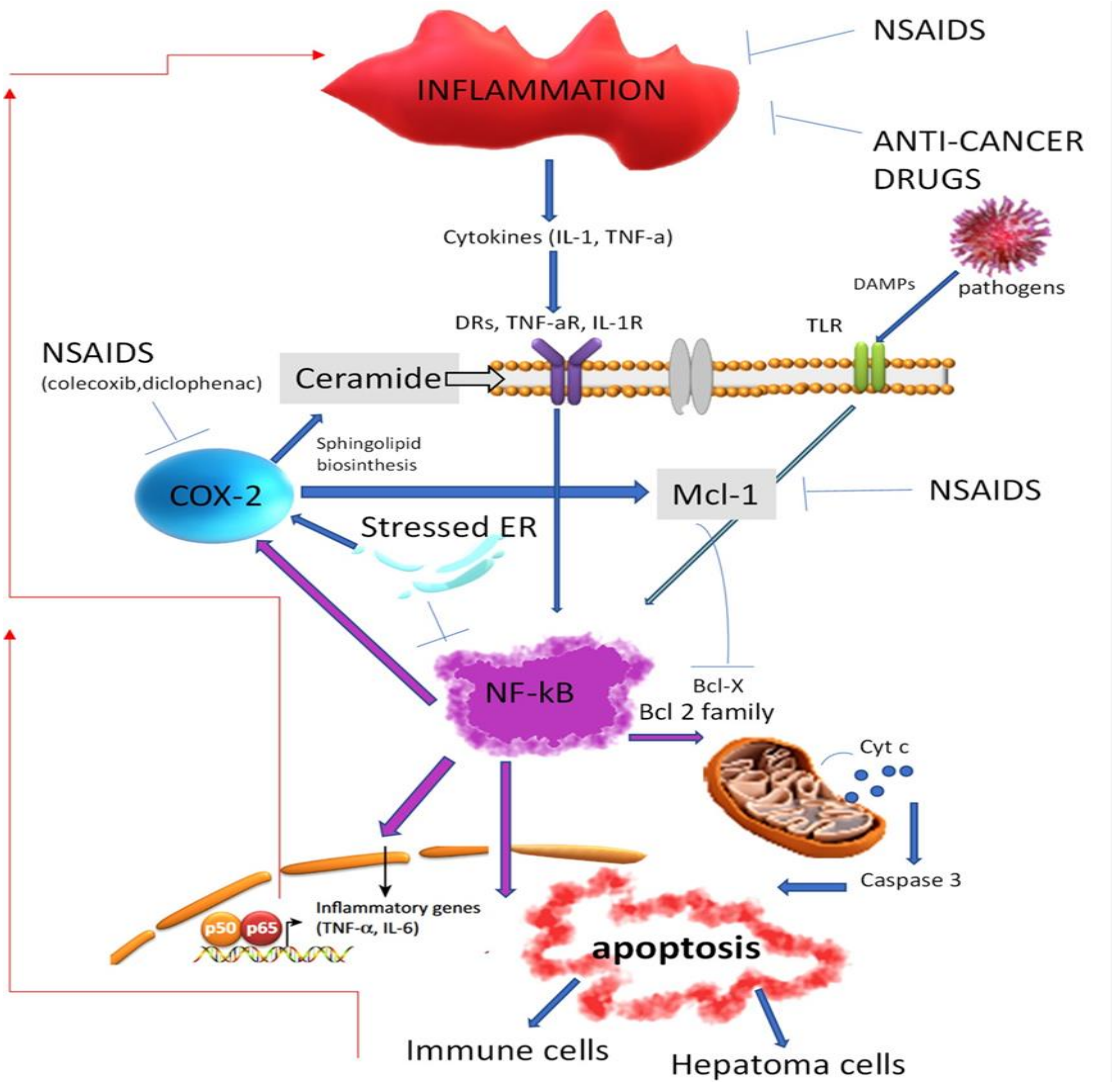


Figure 2: Effect of Cox2 on the cancer(De Re *et al*.,2022)

DRs, death receptors;
NSAIDs, non-steroidal anti-inflammatory drugs;
BCL-2, antiapoptotic
B-cell lymphoma;
NF-kB, nuclear factor-kB;
Cyt c, cytochrome C.
TNF-aR, Tumor necrosis factor receptor alfa;
IL-1R, Interleukin-1 receptor;
DAMPs, Damage-associated molecular patterns;
TLR, Toll like receptor;
COX-2, Cyclooxygenase-2;
ER, endoplasmic reticulum;
Mcl-1, Induced myeloid leukemia cell differentiation protein;
Bcl-x, Apoptosis regulator Bcl-extra;
IL-6, Interleukin 6

2-2:Nimesulide

2-2-1:Chemical name

N-4-Nitro-2-phenoxyphenylmethanesulfonamide (Caiazzo *et al*., 2019)

2-2-2: Molecular Formula

$C_{13}H_{12}N_2O_5S$

2-2-3: Chemical Structure

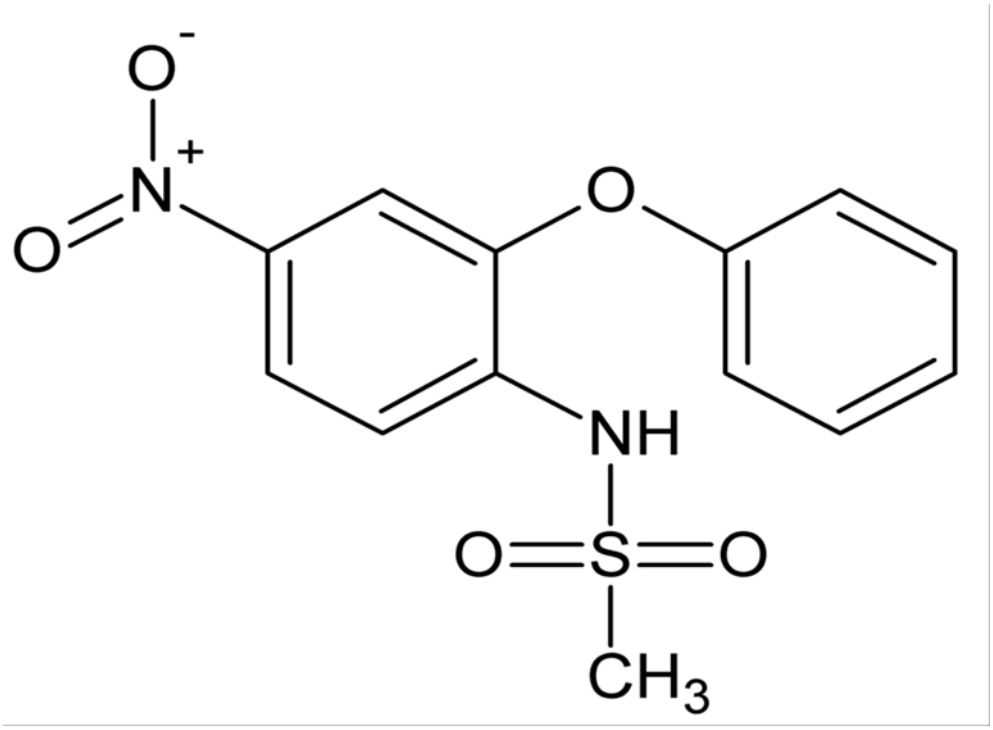


Figure 3: Chemical structure of nimesulide (Warner , 2001)

Nimesulide is one of the non-steroidal anti-inflammatory drugs (NSAIDs), belonging to the sulfonamide class. It is a light yellow crystalline powder that is practically odorless and is usually administered orally. Rectal administration of suppositories is also employ, although to a slight extent. A suspension of nimesulide and a topical formulation (gel) are also commercially available. Nimesulide has a selective inhibition of the COX-2 enzyme with negligible effects on the beneficial proteins needed for the kidney and gastrointestinal tract (Davis and Brogden, 1994; Bernareggi and Rainsford, 2005; Bahram *et al*., 2008; David *et al*., 2013).

Nimesulide was discover by Dr. George G.I. Moore (a medicinal-organic chemist) and Dr. Karl F. Swingle (a pharmacologist), Dr. Robert A. Scherrer (a medicinal chemist), and their colleagues at Riker Laboratories Inc. (Northridge, California, US) in 1971. After an initial unsuccessful observation on fluoroalkane-sulfonoanilides, the strategy was change , and they were found that incorporation of a 4-nitro group into the sulfonanilide structure gave a molecule, 4-Nitro-2-

phenoxyphenyl-methanesulfonamide, that showed good activity design. first used in Italy in 1985, and then has been widely used in Europe, Latin America, and Asia (Caiazzo *et al*., 2019). Nimesulide which introduced as an anti-inflammatory drug and COX-2 selective inhibitor since 1994 (Famaey, 1997).

2-2-4:The Mechanism of nimesulide

2-2-4-A : Mechanism of action of nimesulide on COX

Nimesulide has antipyretic, analgesic, and anti-inflammatory effects. It is a preferential COX-2 inhibitor, 20 times more selective than COX1 (Patrignani *et al*., 1997; Balaji *et al*., 2013), commonly used for treatment of fever, pain and inflammation with a low risk for the gastrointestinal tract, as demonstrated by numerous clinical trials comparison with other NSAIDs such as aspirin, piroxicam, naproxen, ibuprofen and indomethacin (Davis and Brogden, 1994; Bernareggi and Rainsford, 2005; Liang *et al*., 2014; Kress *et al*., 2016). In addition to the main mechanism for treating pain by inhibiting the generation of prostaglandins, nimesulide has other mechanisms to decrease pain by inhibiting the release of the oxidizers from activated neutrophils. It also has a scavenger effect on hypochloric acid, which, joint with the proteolytic enzymes produced by neutrophils during the inflammatory process, also diminution histamine release by mastoid cells and inhibited the production of the platelet-activating factor in basophils (Bocanegra *et al*., 2005).

It was used in treatment nociceptive and inflammatory conditions that are marked by hyperalgesia. Nimesulide was significantly more effective than rofecoxib and celecoxib agents in decreased the hindpaw hyperalgesia in rats and patients with rheumatoid arthritis. Nimesulide, diclofenac, celecoxib, and rofecoxib when used at a single oral dose decrease the inflammatory hyperalgesia, but only nimesulide was effective at 15 minutes after administration it. Nimesulide (100 mg) was significantly more effective than rofecoxib (25 mg). 100 mg of nimesulide was comparison with 500 mg of naproxen for relief of postoperative pain after the orthopedic surgery, also the single dose of nimesulide had more efficacy and rapid onset in the analgesic action than the single dose of celecoxib 200 mg in humans with osteoarthritis of the knee, nimesulide looks to be particularly effected and fast-acting (Bianchi and Broggini, 2002), and it was used

as a potent NSAID in condition of anti-inflammatory and antipyretic effects in dogs (Toutain *et al*., 2008). Nimesulide effects are unique to COX2 inhibitors and In the inflammation processes, nimesulide efficacy was dependent on a wide spectrum of actions, The effects of nimesulide combination with immune and non-immune cells,by a biochemical mechanism and inhibition of other inflammatory mediators that are produced in response to stimulation of cyclic-3,5'-adenosine monophosphate (cAMP); this mean that nimesulide has many factors to control the inflammation and pain.In general, nimesulide has relatively low effects on gastro-intestinal tract (GIT), which are related to its low inhibition of the physiologically important enzyme COX-1 in the GIT mucosa and it has important physicochemical properties (pKa of 6.5 and lipophilic), as well as inhibition mast cell-derived histamine and secretion of acid in the stomach. In contrast with coxibs, nimesulide has not been found to have toxicity of the cardiovascular system(Rainsford., 2006).

On the other hand, nimesulide has proven to be that more action and longer-lasting than paracetamol in inhibition fever in rats which induced by Brewer's yeast, also, other mechanisms of nimesulide were proven to strongly inhibit platelet aggregation in guinea pigs (in vitro) after administration both single dose and repeated it (once daily for 5 days) oral dosing (Ceserani *et al*., 1993; Maria *et al*., 2006). The effects of nimesulide as an anticoagulant were described in a previous study that proved that nimesulide inhibit platelet aggregation which induced by adrenaline and inhibit thromboxane A_2 at low concentrations (Sheikh *et al*., 1998). Other studies in rabbits suggest that the platelet inhibitory effects after using nimesulide through experimental ischaemia and infarction (Ahmed *et al*., 2015).

Also in a previous research in patients after extraction of impacted third molars with moderate to the severe pain, nimesulide and ibuprofen provided that their effective through 24-hour relief but the results suggest that nimesulide had a faster onset (<15 minutes) and was stronger than that of ibuprofen, so its outperform in the analgesic effect (Bocanegra *et al*., 2005).

As for the level of gene expression of COX-2, nimesulide inhibited cytokine-induced COX-2 expression at sub- and therapeutic concentrations (Fahmi *et al*., 2001) and reduces expression levels of COX-2 at both levels : the protein and

mRNA in mice, so a previous study suggested that selective COX-2 inhibitors can be potentially become part of the comprehensive treatment for the laryngeal squamous cell carcinoma (Liang *et al*., 2014); therefore, inhibition of the expression of COX-2 may play a major role in the process of antitumor (Chu *et al*., 2018).

2-2-4-B : Mechanism of action of nimesulide on caspase

NSAID inhibited caspases, and this effect was COX-independent; this is considered a new anti-inflammatory mechanism. Caspase is a cysteine-aspartic protease that its considered a new targets for some NSAIDs such as nimesulide, ibuprofen, aspirin, , ketorolac and naproxen. These NSAIDs during inflammation inhibited the caspase catalytic activity beside reducing cell death and induction of inflammatory cytokines (Mukherjee *et al*., 2001; Smith *et al*., 2017) (Figure 4).

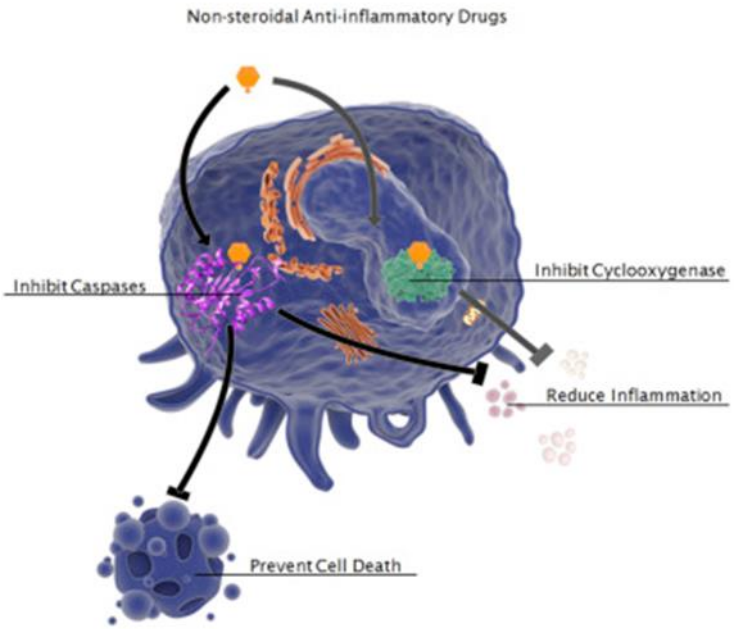


Figure 4: Effects of NSAIDs on the caspase (Smith *et al*, 2017)

There are two forms of caspases: initiator caspases (caspase-2, -8, -9, and -10) and executioner caspases (caspase-3, -6, and -7), when activated initiator caspases will be proteolytically activated executioner caspase, resulting the immunologically silent cell death through apoptosis (Lamkanf, 2011). (Figure 5)

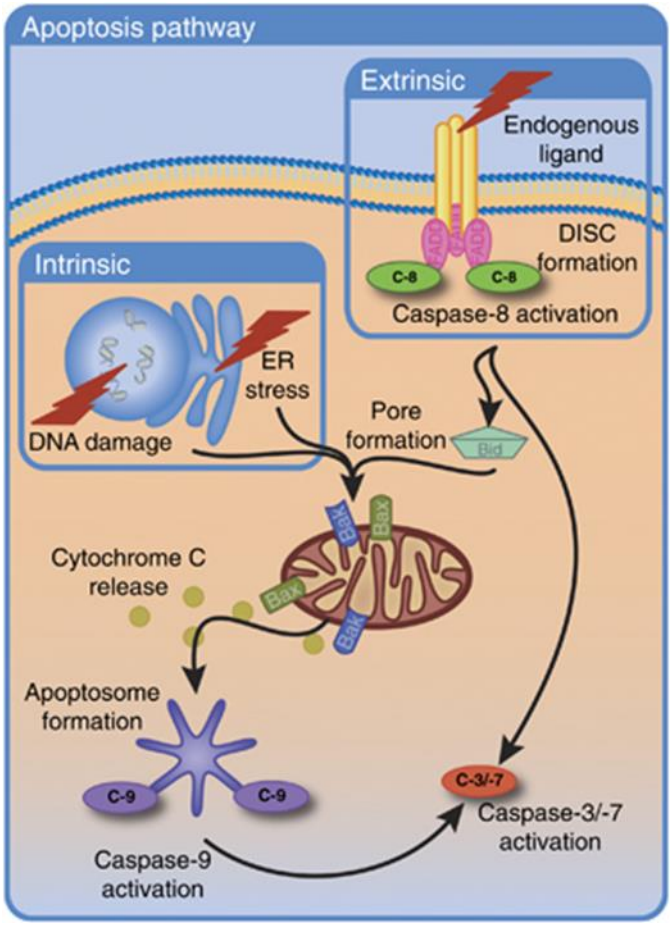


Figure 5: Apoptosis pathway (Karen *et al*., 2013)

Caspases have roles in cancer, rheumatoid arthritis, inflammation, and neurodegenerative diseases. The stimulation of inflammatory caspases result the production of active mediators, cytokines, and promotion of the immune response. Caspases are a family of genes which important for maintain homeostasis through regulation cell death and inflammation (McIlwain *et al*., 2013).

Nimesulide, a selective COX-2 inhibitor, and ibuprofen, a non-selective COX-1/COX-2 inhibitor, blocked the caspase-3 activation and apoptosis in the chondrocyte cultures. So, nimesulide may represent a preventive choice for osteoarthritis through blocking events of apoptosis in chondrocytes. Also, another study found that nimesulide decreased the cleavage of caspase-3 in 5 days and treated primary effusion lymphoma in humans (Mukherjee *et al*., 2001; Paul *et al*., 2011). Other studies found that nimesulide had a role in apoptosis through inhibition of prostaglandins, which modulate cell proliferation. Nimesulide acted to inhibition growth of lung tumors which associated with reduction of PGE_2 levels in mice (Shaik *et al*., 2004). Also, nimesulide was shown that it have anticancer effects in neoplastic pancreatic cells through inhibiting proliferation and apoptosis (Ferreira *et al*., 2021).

2-2-4-C : Mechanism of action of nimesulide on PPAR-α

Other molecular mechanism of nimesulide to exert effect as analgesic and anti-inflammatory drug by inhibition of peroxisome proliferator-activated receptor alpha (PPAR-α) mediated induction of COX-2 mRNA expression. PPARs were discover over a decade ago and were classify as nuclear receptor superfamily. Three types of PPAR have been discovered (PPAR-*α, γ, β/δ),* and this type of PPAR has been shown to play critical roles in the important diseases and conditions such as atherosclerosis, obesity, fertility, diabetes, and cancer. Evidence suggestion that PPAR*α* can treated the inflammation by multiple and different mechanisms (Cuzzocrea *et al*., 2008). Many researches have discovered that inhibition of PPAR-*α* and PPAR-*γ* exert as anti-inflammatory effects in vivo and in vitro. PPAR-α has a role in the inflammation through modulating of inflammation. Also, a previous study demonstrated that nimesulide in therapeutic concentrations treated synovial osteoarthritis in human by inhibiting PPAR-α (Chinetti *et al*., 2000; Kalajdzic *et al*., 2002; Delerive *et al*., 2001; Barbier,*et al*.,2004).

In addition to positively regulating gene expression of PPARs action, the effect on the PPARs can inhibition gene expression by negatively interfer with the activity of proinflammatory transcription factors, such as nuclear factor-*κ*B (NF-*κ*B) (Ren *et al*., 1996). Such transrepression mechanisms are likely to contribute in the anti-inflammatory actions of the PPARs (Figure 6).

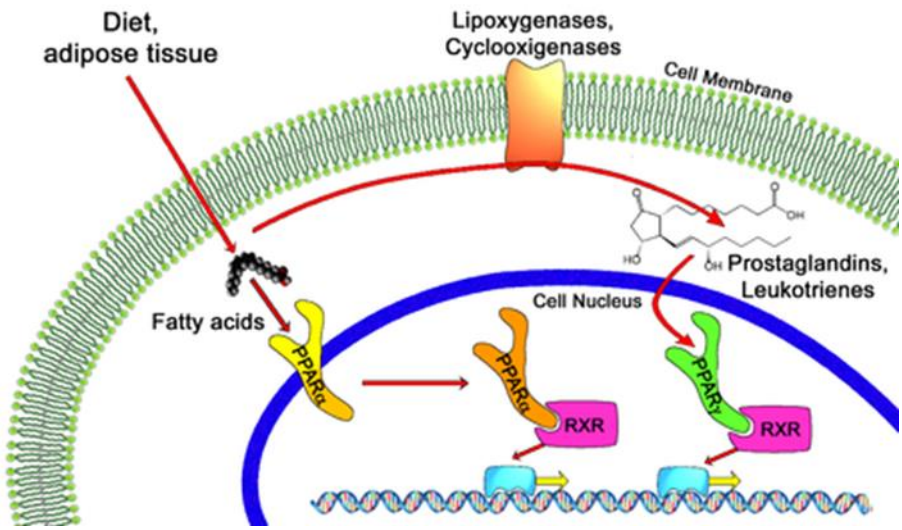


Figure 6: PPAR -alpha and -gamma pathways (Somoza, 2006) .

2-2-5:Pharmacokinetics of nimesulide

Nimesulide is a noncarboxylic, weakly acidic molecule with pKa = 6,4 lipophilicity (Demetris *et al*., 2007; Caiazzo *et al*., 2019). Rapidly solubility in water and completely absorbed from the stomach and small intestine after oral administration, and the drug has many active metabolites (M1: 4-amino-2-phenoxy-methanesulfonanilide), nimesulide was highly susceptible to oxidation by cytochrome P450 enzymes to form the reactive diiminoquinone intermediate (M2). The formation of M2 which is the mediated of P450 with 2C19 and 1A2 as the two principal P450 enzymes catalyzing M1 oxidation; P450-2C19, the mediated further metabolism of M1 to the amino hydroxynimesulide M3 and its diiminoquinone M4; the protein binding of nimesulide was about 97.5%, principally albumin (Li *et al*., 2009; Daived *et al*., 2013).

In humans, a single oral dose (100–200 mg) of nimesulide was rapidly metabolized and distributed throughout the body, with a volume of distribution of 0.18–0.35 L/kg , the parent drug and metabolites were rapidly cleared (31–152 mL/h/kg), dependent on the dose and period of oral drug ingestion; 1–3% of the dose is excrete unchanged in urine; about 70% of the metabolites are excrete mainly in the urine; and about 20% are excreted in the feces (Davis and Brogden, 1994; Bernareggi and Rainsford, 2005). Like several other NSAIDs, nimesulide is bound strongly with the plasma proteins, mainly albumin and to the lesser extent, to α1-acid glycoproteins and lipoproteins, but don't bound to red blood cells. The mean of plasma elimination half-life ($t_{1/2}$) the ranges from (1.80 to 4.73 h) (Bernareggi and Rainsford, 2005; Bjarnason *et al*., 2005).

In goats at doses of 4 mg/kg, BW given I.V. and I.M.; I.V administration of nimesulide had a rapidly distribution phase with a slower elimination phase; half-life during the distribution phase and elimination phase were 0.11 and 7.99 h, respectively; the steady-state volume of distribution of nimesulide was 0.64 L/kg; the total body clearance was 0.06 L/h/kg; and the mean residence time (MRT) was 11.72 h. In I.M. administration of nimesulide, the maximal plasma concentration was 2.83 Mg/mL, and achieved at 3.6 h (Tmax). A Plasma drug levels were measurable up to 72 h. After I.M. administration, the $t_{1/2}$ and MRT of nimesulide

were 1.63 and 1.73 times longer, respectively, than the I.V injection. The bioavailability of nimesulide was 68.25% after I.M. injection. These pharmacokinetic data advocate that nimesulide, which was given I.M injection, may be useful more than I.V. injection in the treatment of inflammatory cases in goats (Rao *et al*., 2007).

In mice, has been compared efficacy of I.M. injected of nimesulide with diclofenac, and to explain the pharmacokinetic shape of this formulation, analgesic action was tested by using an acetic acid writhing test and a tail-flick latency test. The results presented that the analgesic effects of nimesulide were significant higher than those of diclofenac in the writhing reflex and the tail-flick latency test, and the plasma level of nimesulide was measured by a HPLC method. A peak analgesic effect was detected between 60 and 120 min, while C_{max} (10.6 µg/ml) of nimesulide and T_{max} were got at 60 min. (Gupta *et al*., 1998).

In rats, nimesulide was evaluated for anti-inflammatory effects and compared with diclofenac effects in the same doses (1.5, 3, 6, 12.5, and 25 mg/kg). The anti-inflammatory action of nimesulide was superior than that of diclofenac in the carrageenan-induced in rat paw edema, the peak anti-inflammatory effect of nimesulide were detected between 2 and 3 h post-treatment which correlates well with the Tmax of 115 min and measure plasma concentration of nimesulide at several time by using HPLC after administration the dose at 25 mg kg, peak plasma concentration (C_{max}) was 23 µg/ml and $t_{1/2}$ was 4.2 h, Area Under Curve ($AUC_{0–6\ h}$) was detected as 83.31 µg/ml/h and noted that didn't find adverse effects or toxicity at the doses which administered, so previous study demonstrates that nimesulide, when injected I.M., may be outperform to other routes of administration when want fast onset of action with highly plasma concentration (Gupta *et al*., 1999)

2-2-6: Side effects of nimesulide

Long-term use of nimesulide may harm the liver or result in significant side effects, including:

Headache, dizziness, Skin rash, gastrointestinal upset, abdominal discomfort, diarrhea, nausea and vomiting, somnolence, peripheral edema, hypersensitivity reactions, increased liver enzymes and blood clotting problems (Bethesda, 2012).

2-2-7:Contraindications and interactions of nimesulide

1. In case of the respiratory diseases
2. Blood thinners
3. Antidiabetics (insulin)
4. Anti-epileptics
5. Drugs that influence the immune system
6. Antacids, and anti-HIV medications
7. Avoiding consume dairy products like milk, yogurt, or drinks with added calcium (Bethesda, 2012)

Nimesulide is safety to use for short term. When is used with long-term or in excessive, nimesulide could cause acute renal failure in individual cases. Extra use of nimesulide can cause kidney problems or liver damage (Ahmed *et al*.,2012) .

2-2-8: Toxicity of nimesulide

In the previous study mentioned that administered of nimesulide to the cat in a dose 100 mg per day orally separated into three doses for three days, The hematological found that there acute biliary injury and renal failure with elevated levels of alkaline phosphatase, bilirubin, γ-glutamyl transferase, , urea, and creatinine (Borku *et al*., 2008).

Other research tested nimesulide at 200 mg/kg b.w. for 14 days and found potential genotoxicity in rats (Debojyoti *et al*., 2013).

Also on the other study, the toxicity effect of nimesulide were tested compared with diclofenac in birds and showed safety and efficacy of nimesulide (injectable) in various animal models and compared to diclofenac, nimesulide-treated groups of Vanaraja (Synthetic female × Red Cornish) and PB1 poultry breeds didn't show any histopathological lesions, whereas diclofenac-treated birds were showed histopathological lesions in the kidney and liver at a dose of 5 mg/kg for each drug (Prakash *et al*., 2006).

2-2-9: Nimesulide during pregnancy

Nimesulide may cause renal failure in neonatal associated with consumption of nimesulide and modulated the genetic factors. Therefore, must be cautious use of nimesulide during the pregnancy period . (Benini *et al*., 2004)

2-3:Aspirin

2-3-1: Chemical name

Acetylsalicylic acid

2-3-2 : Molecular Formula

$C_9H_8O_4$

2-3-3: Chemical Structure

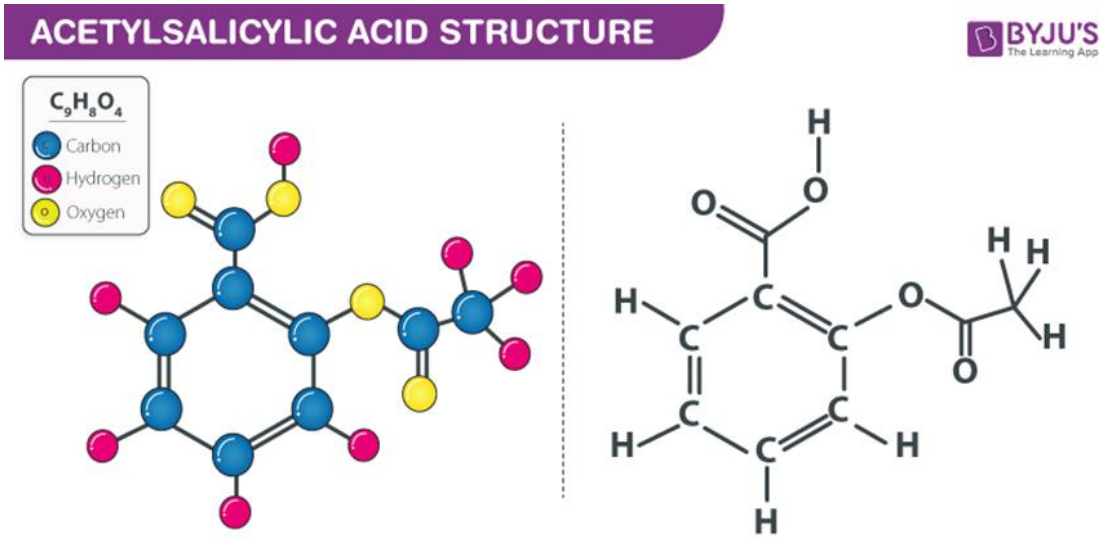


Figure 7: Chemical structure of Aspirin (Gawad *et al*.,2013; Alkhimova *et al*., 2022)

Aspirin (acetylsalicylic acid, ASA) was used as a therapeutic agent for over 100 years. It has the characters of non-steroidal anti-inflammatory drugs (NSAIDs) as anti-pyretic, analgesic, and anti-inflammatory drug, in addition to acting as an anticoagulant. This therapeutic effect of aspirin results from ability to inhibit prostaglandin and TXA2 production (Kunal *et al*., 2015**)**. The cyclooxygenase (COX) enzyme was responsible for the alteration of arachidonic acid to prostaglandin H_2 (PGH_2), which conceder a precursor to other prostaglandins. There are two isoforms of COX: COX-1 and COX-2. At general, COX-1 is the constitutive one, while COX-2 is an inducible form. Aspirin is bind covalently with both COX-1 and COX-2, and thus resulting in an irreversible inhibition of COX action, in contrast other NSAIDs that bind with COX reversibly (Bachert *et al*., 2005; Schror, 2009; Cooper and Voelker, 2012) . Also it can be used to prevented stroke , heart attack, and cancer (Flower, 2003).

Originally it was derived from extracts of the bark or leaves of the willow tree (genus *Salix*). The first person which described the synthesis of acetylsalicylic acid was believe to be a Frenchman, Charles Gerhardt, in his treatise on organic chemistry in 1853. Gerhardt attained this by reacting the sodium salt of salicylic acid with acetyl chloride. The first commercial preparation had to wait 50 years until the Bayer Company marketed acetyl salicylic acid as aspirin. Other early scintests in medicine to record the advantages of the willow bark and leaf were Hippocrates of Cos and the Roman Celsus. There are also articles of its use in

China as early as the 5th century in the Common Era. Before that, the Sumerians known the drug initiated before over 3500 years ago, and the ancient Egyptians used extracts of myrtle and willow leaves to calm joints (Maria *et al*., 2019)

Vane, Whittle, and their colleagues was develop the idea in 1980, when they revealed that aspirin and other NSAIDs caused noticeable inhibition of the synthesis of prostaglandins in both inflammatory secretion and gastric mucosa, with effect acute gastric damage (Whittle *et al*., 1980; Children, 2010; Yeomans, 2011).

Originally, aspirin was used as an anti-inflammatory and antipyretic drug, then became used to prevent cardiovascular and cerebrovascular diseases because it has anticoagulant properties. The story of aspirin continuously to today with increase the evidence of its chemopreventive effects against cancer, such as colorectal and other forms of cancer (Maria *et al*., 2019).

Veterinary forms of aspirin are used for pain, fever, and inflammation. Aspirin is used to relieve inflammation related with arthritis and joint problems in dogs and cats. For treating pain of the cattle, dogs, cats, and pigs: Aspirin is used to removal mild to moderate somatic pain, such as the pain after surgery, pain after dental procedures, and cystitis. Aspirin also used for treating fever of the cattle, dogs, cats, and pigs (Forsyth *et al*., 2024).

2-3-4: Mechanism of action of Aspirin

2-3-4-A : Mechanism of action of Aspirin on COX

Aspirin is a non-steroidal anti-inflammatory drug (NSAID) widely used, and it has several effects as antipyretic, analgesic, and anti-inflammatory through inhibition of COX enzymes (COX1 and COX2) and leading to a decrease in prostaglandin and prostacyclin (prostanoids) production, which potentiate the effects of other pro-inflammatory mediators such as histamine and 5-hydroxytryptamine. There is evidence suggested that the analgesic effect of aspirin occure due to a central mechanism (Al-Swayeh *et al*., 2000; Vergne *et al*., 2000; Jacob *et al*., 2012). COX-1 and COX-2 are expressed in various cell types, which contribute to prostanoid release during inflammation and normal physiologic events (Figure 8).

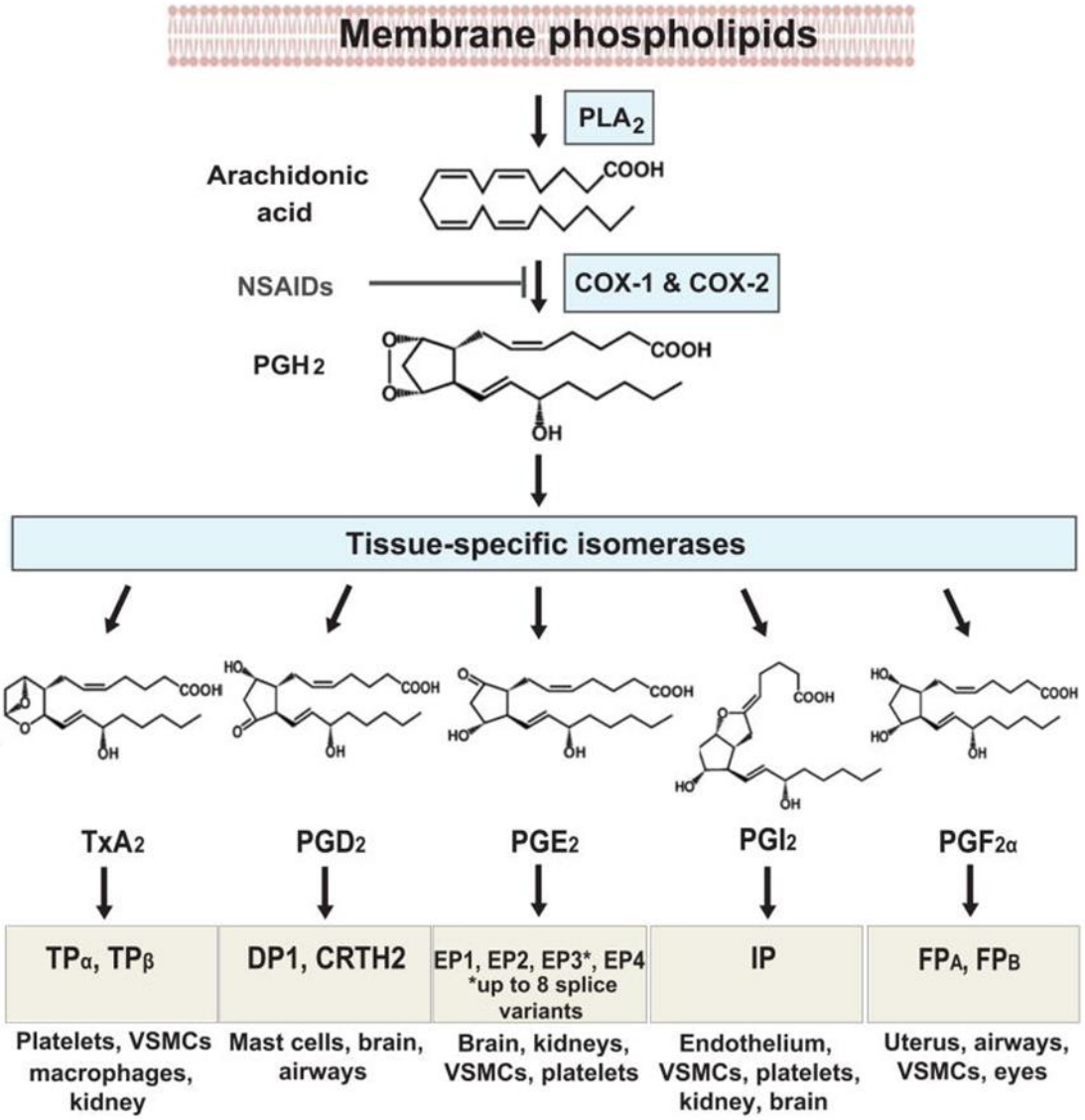


Figure 8: COX-1 and COX-2 are expressed in various cell types (Riciotti and Garret , 2011)

Aspirin acted on the acetylates hydroxyl group of the serine 530 residue in the active binding site of the COX enzyme. The acetyl group block arachidonic acid contact to the active binding site of the COX enzyme, lead to irreversible inhibition of the enzyme's activity. (Juan *et al*., 2019).

Inhibition the activity of the COX enzyme, which lead to the formation of prostaglandins (PGs) that cause inflammation, pain, swelling and fever. Inhibition this key enzyme in PG synthesis lead to prevented the production of physiologically important PGs in case where a non-selective COX inhibitor drug was administered. This PG protects from damage the stomach mucosa by hydrochloric acid and maintain kidney functions (Vane and Botting, 2003).

In a previous study, it was found that aspirin reduces the inflammation which caused by carrageenan injection in the hind paw of rats by inhibiting the hyperalgesia and it has an analgesic effect in the case of acetic acid-induced the abdominal contractions in the mice. It also reduces the late phase (but not early phase) of inflammation which induced by the formalin in the hind paw licking test in the mice (Al-Swayeh *et al*., 2000) and causes analgesia induced by a thermal method (hot plate) with a reduced the body temperature (Ohdo *et al*., 1995).

Aspirin has a mechanism to cause analgesic and anti-inflammatory effects, represented by inhibit of prostaglandin synthesis, but this alone is not enough to explain the anti-inflammatory efficiency of aspirin. Other mechanism was describe as induced by the production of lipoxins (aspirin-triggered lipoxins) from the arachidonic acid, lipoxins binding with G-protein-coupled receptors to exert action by resolving inflammation and acting as antioxidants and immunomodulators (Clària and Serhan, 1995; Cadavid, 2017), while the antipyretic effects of aspirin were exerted by their inhibition of the production of brain prostaglandin (Vane and Botting, 2003).

Aspirin has pharmacological effects as an antagonist of platelet; it has been availability for more than a century and now represent a mainstay in prevention and treatment of vascular events which include stroke, myocardial infarction, peripheral vascular obstruction, and sudden death. Aspirin irreversibly acetylates COX, weakening prostaglandin metabolite and thromboxane A2 (TXA_2) synthesis. As a result, inhibition of platelet aggregation occurs at low concentrations (Roth and Majerus, 1975).

Several mechanisms of the platelet inhibition through aspirin have been suggested, including the inhibition of the platelet activation by neutrophils and improved nitric oxide production. In addition, aspirin is prevented the progression of atherosclerosis through protection of low-density lipoprotein (LDL), cholesterol from oxidation and scavenging hydroxyl radicals (Becker and Frederick, 2006).

Other research mentioned that aspirin is commonly used as an anti-platelet agent. The anti-platelet effect of aspirin comes from a reduce the production of TXA_2. Aspirin acts as a cardioprotective agent through irreversible inhibition of

COX-1 and blockade the production of TXA_2 to prevent clots (Warner *et al*., 2011) (Figure 9)

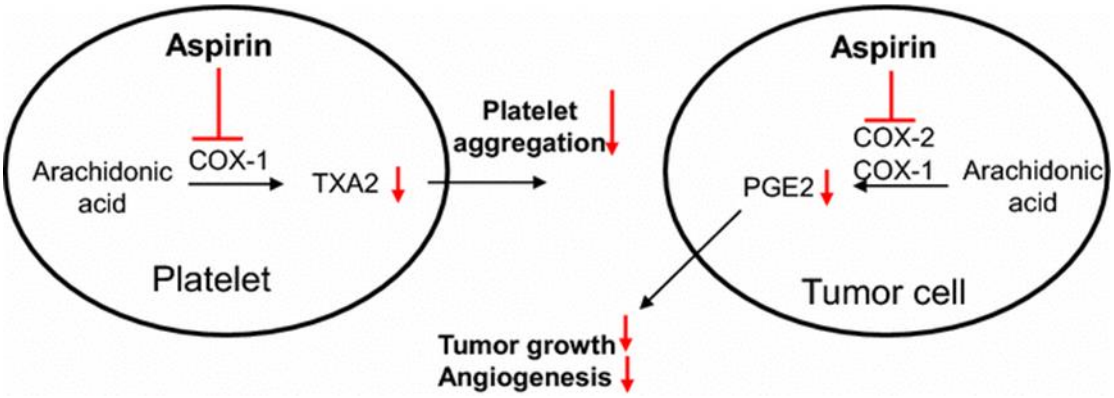


Figure 9: Action of low-dose aspirin in platelets and tumor cells (Junichi *et al*., 2016).

So, aspirin acts by inhibiting the COX , which causes suppression of prostaglandins and relieves inflammation, pain, and fever (Patel *et al*., 2018).

2-3-4-B : Mechanism of action of Aspirin on caspase-3

Recently, it was proven that aspirin is an inhibitor of caspase-3 (cysteine-aspartic proteases concede new targets for some NSAIDs) (Castaño *et al*., 1999; Mukherjee *et al*., 2001). Caspase-3 is a protein that play a role in achieving apoptosis. Excessive apoptosis occurs in preeclampsia due to the activation of caspase-8 in the extrinsic pathway, which followed by caspase-3 activation as an executioner caspase , which supports apoptosis in the trophoblast cells. (Akhmad *et al*., 2016).

Previous study mention that aspirin reduced caspase-3 activity in hepatocellular carcinoma (Feng *et al*., 2011); another research have proven that low dose of aspirin has an effect on the inhibition of caspase (3, 8, and 9); and trophoblast cell apoptosis pathways stay unclear (Akhmad *et al*., 2016). Also, aspirin significantly decreased the levels of pro-apoptotic proteins, caspase-3 in an induced diabetic neuropathy in rats, so, the research demonstrated that aspirin provided a significant reduction in apoptotic cell counts in diabetic neurons

(Ozdemir *et al*., 2023); further, it was proven that aspirin reduced the levels of apoptosis by decreasing caspase-3 cleaved (Liu *et al*., 2017).

2-3-4-C :Mechanism of action of Aspirin on PPARα

Peroxisome proliferator-activated receptor-alpha (PPARα) is a nuclear receptor act as a novel receptor of aspirin; there are three subtypes of PPARs (PPARα, γ, β/δ) (aPatel *et al*., 2018; bPatel *et al*., 2020). PPARα exert anti-inflammatory effects through regulating the expression of genes (Zandbergen and Plutzky, 2007) and proved in other research in rats that PPARs exert an anti-inflammatory effect by induced inflammation through carrageenan in the paws of rats (Ozdemir *et al*., 2001). In other study proved that aspirin bind with PPARα at the Tyr314 residue which its ligand-binding domain (aPatel *et al*., 2018) .

Previous study suggest that aspirin exerts its anti-inflammatory effect via suppressing COX-2 induction and reducing the synthesis of pro-inflammatory prostaglandin in mice with dose 10–30 mg/kg, orally rout and decreased colon cancer in humans via suppressing COX-2 expression (Xu *et al*., 1999). In mice, aspirin significantly inhibited cardiac fibrosis apoptosis and reduced the level of apoptotic markers (caspase-3 cleaved) (Liu *et al*., 2017).

In other research proved that aspirin when combine with cisplatin, inhibition COX-2 expression through inactivating NF-κB signaling in humans (Jiang *et al*., 2020). NF-κB is a proinflammatory signal pathway, the activation of NF-κB by proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα), and NF-κB has play a role in the expression of other pro-inflammatory genes, containing cytokines and chemokines (Lawrence, 2009).

2-3-5: Pharmacokinetics of aspirin

Aspirin is completely and rapidly absorbed when administered orally. It is a weak acid (pKa 3.5), but absorption may be variable dependent on the route of

administration and form of dosage, as well as other factors include the rate of tablet dissolution, gastric contents, gastric emptying time, and gastric pH. acetylsalicylic acid (Non-ionized) pass through the stomach lining by passive diffusion. Perfect absorption of salicylate in the stomach happens in the pH range of 2.15–4.10. Intestinal absorption of acetylsalicylic acid happens at a much faster rate.

Half of the ingestion dose is hydrolyzed to salicylic acid in the first hour after ingestion via esterases which found in the gastrointestinal tract. Peak plasma concentrations of salicylate happen between 1-2 hours after administration (Arif, H. and Aggarwal , 2023 ; Vane and Botting, 2003) . The absorption is directed proportional with the dose, and the absorption takes place to the lesser extent in the stomach and to the greater extent in the upper small intestine (Schror, 2009). The bioavailability of almost drug substances is greater in the gastrointestinal lumen when the drug is in the state of non-ionized. Time maximum of aspirin is 0.5 h and the concentration maximum is 5.43 (Kunal *et al*., 2015); protein binding of aspirin is 81.7% (Ghahramani *et al*., 1998).

Aspirin is distributed in to the body tissues shortly after administration. It crosses the placenta. The plasma contain high levels of salicylate, in addition to tissues such as synovial fluids, spinal and peritoneal, saliva and milk. after administration the drug, high concentrations of salicylate also are found in the kidney, liver, lung, and heart after dosing of aspirin, and minimal concentrations are found in sweat, feces, and bile. Therapeutic concentration of 50–90% of a salicylate binds to plasma proteins, particularly albumin, while acetylsalicylic acid binds little. Acetylsalicylic acid and acetylate can bind to numerous proteins, hormones, platelets, DNA, and hemoglobin (Vane and Botting, 2003). Salicylic acid was measured at 24 hours after a single dose of acetylsalicylic acid (Durlaza, 2015).

Aspirin is primarily metabolize in the liver, other tissues may be involved. The almost metabolites of acetylsalicylic acid are salicylic acid, salicyluric acid, the ether or phenolic glucuronide, and the ester or acyl glucuronide. A small portion is converted to gentisic acid (Vane and Botting, 2003) (Figure 10).

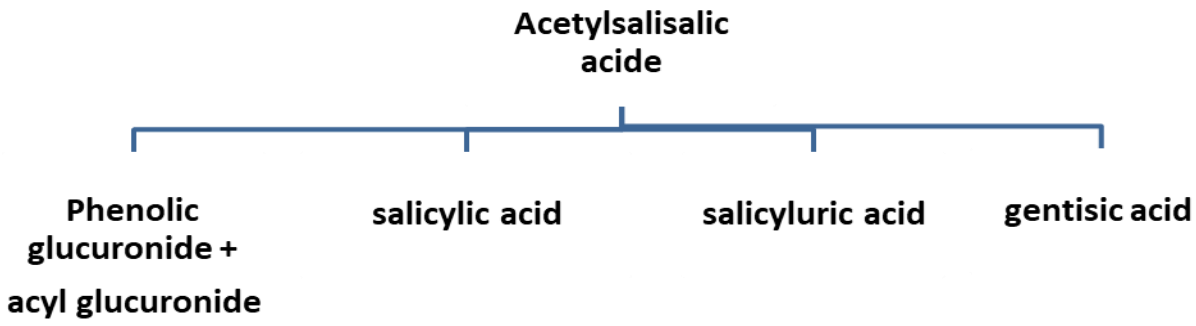


Figure 10:Aspirin metabolites (Vane and Botting, 2003).

Aspirin metabolize by UDP-glucuronosyltransferase 1A6 (UGT1A6), cytochrome P450 2C9 (CYP2C9), and N-acetyl transferase 2 (NAT2) produces slow-metabolizing enzymes. Aspirin different from other NSAIDs by binding covalently with both isoforms of COX (Ghahramani *et al*., 1998; Palikhe *et al*., 2011). Aspirin is deacetylated to the salicylic acid and then metabolize via glucuronidation, hydroxylation, and glycine conjugation, with CYP2C9 playing a main role in the metabolic process (Bigler *et al*., 2001).

The route of removal of salicylates occur mainly via the kidney, in the form of free salicylic acid, salicyluric acid, and, additionally, phenolic and acyl glucuronides. Can be found Salicylate soon in the urine after administration; however, all doses take about 48 hours to be completely excretion.

Level dose of values which conceder acute oral dose have been reported as over 1.0 g/kg in humans, cats, and dogs, 0.92 g/kg–1.48 g/kg in albino rats, 1.19 g/kg in guinea pigs, 1.1 g/kg in mice, and 1.8 g/kg in rabbit models (Vane and Botting, 2003).

2-3-6: Side effect of aspirin in human

Because of poor water solubility, so aspirin is taken orally. Some of the side effects of aspirin result from undissolved particles.

in the gastrointestinal mucosa, which contributed to ulcer and bleeding (Yeomans, 2011) and may cause:

1. Bleeding disorders
2. Uncontrolled highly blood pressure
3. stomach ulcers
4. kidney or Liver disease
5. Asthma
6. Nausea

2-3-7: Risks and precautions of aspirin usage

1. Don't recommend high-doses of aspirin through pregnancy.
2. Don't administer, in an allergy case, or with any other NSAID.
3. Not all strokes are caused by blood clots. In some state, aspirin may make a stroke worse; so, don't administer aspirin through a stroke, so you must diagnose the case before giving the drug.
4. Don't administer before surgical treatment (Alan, 2023)

2-3-8: side effects of aspirin in animal

1. Nausea
2. Decreased appetite
3. Intestinal irritation
4. Vomiting
5. Bleeding in the gastrointestinal tract sign include black or tarry stools, blood in the vomit; or red blood in stool .
6. Anemia can occur in severe bleeding condition.

In cats, aspirin may cause acidosis (the body fluids contains too much acid), this lead to depression, gastrointestinal upset, fever, and confusion. May be the effects continues longer period in pets with kidney or liver disease. However, the increase bleeding risk remains for about 7 to 10 days after the drug is stopped. So cats are very sensitive to aspirin (because slower aspirin clearance results mainly

from being deficient in glucuronyl transferase), so it is important to administer your cat cautiously because cats's metabolism systems for aspirin cause toxic effects more than a dog's. Liver and Kidney damage can occur, at 325 mg twice a day was lethal to cats (Michael, 2013; Lauren *et al*., 2023).

In dogs, toxic effects happened at dose of 100–300 mg/kg/day orally for 1–4 weeks, the signs may include vomiting, respiratory alkalosis, hyperpnea, metabolic acidosis, liver necrosis, gastric hemorrhage, fever and seizures may be seen because of the uncoupling oxidative phosphorylation, Renal deficiency is uncommon with salicylate toxicoses but may be developed (Sharon, 2010).

Toxic effects of aspirin in dogs happen due to uncoupling oxidative phosphorylation and disable of the Krebs cycle, which lead to organic dysfunction, however, don't found antidote for salicylate toxicity. Alkalinization of the urine is the main treatment for aspirin toxicity which lead to increase the rate of drug excretion (Amy, 2016).

2-3-9:Interaction of aspirin

Naproxen interfere with the aspirin inhibitory effect on the platelet aggregation and role of aspirin in cardioprotective effect (Capone *et al*.,2005), in contrast, acetaminophen is well tolerated and don't shown any gastrotoxicity effects when taken with aspirin, and neither interfere with effect of aspirin on the platelet aggregation (Gaziano and Gibson, 2006), in other research mention that celecoxib, Ibuprofen and rofecoxib inhibited the effect of aspirin, but not flurbiprofen or diclofenac . So the interactions with aspirin don't appear with all NSAIDs to the same level (Umar *et al*., 2004)

2-3-10:Aspirin usage in pregnancy

Using of aspirin through the pregnancy is associated with elevate postpartum bleeding, It may be causes hemorrhage of neonatal intracranial (Hastie *et al*., 2021).

Therapeutic dose of aspirin which used in human when it used in rats , didn't cause any embryotoxic or major deformities on the experimental animal but was responsible for ureteric dilatation of fetuses rate (Débora *et al*., 2002).

Other study provide that low-dose aspirin is useful for preeclampsia prevention through inhibiting of NF-κB in the cell of mice (Li *et al*., 2018).

Aspirin at adose 20 mg/kg effective in decreased high blood pressure in mice and reduced urinary protein level and urinary protein/creatinine percentage. also, aspirin elevate the weight of placenta and reduced the degree of placental lesions and kidney (Yongbing *et al*., 2022).

Chapter Three

Materials and methods

3-1: Experimental animals

The study used 313 albino Swiss mice weighing 21–34 g, with both sexes. The animals were raised in the animal house at the College of Veterinary Medicine, University of Mosul, under standard conditions with a 10/14 h light/dark cycle with 22±2ºC room temperature in a cage (10×20×40 cm). Given the food and water ad libitum at the laboratory according to standard protocol (all experiments were applied to mice aged 2 months) (Mohammad, 2000) .

3-2: Ethical approval

The study was standardized (the animal use and experimental design) through the care committee which affiliated to the Veterinary Medicine College / Mosul University (approval code no. UM.VET.2021.076).

3-3: Drugs and chemicals used

1. Nimesulide injection (10% Pharmaceuticals Instant, India) .
2. Aspirin injection (acetylsalicylate acid) (Sanofi, France) .
3. Distilled water
4. Physiological normal saline (Poli Farma, Turkey) .
5. Acetic acid (1% TEDIA, U.K) .
6. Formalin (1% BDH, U.K) .
7. Baker's yeast (Turkey)

3-4: The devices used

1. Hot plate , MS-H-PRO (DLAB , Germany) .
2. Centrifuge ,Chalice (Korea) .
3. Sensitive balance, (aeADAM , England)
4. Traditional balance (sf-400, China)
5. Digital Thermometer (China)
6. Digital caliper (INGCO , China)
7. Vortex (Thermofisher,U.S.A) .
8. High-Performance Liquid Chromatography (HPLC), (Shimadzu, Japan) .
9. ELISA device (ELKbiotech company, USA)
10. RT-PCR was performed on the MiniAmp PlusTM Thermocycler PCR, USA.

3-5: Kits used

1. Mouse COX-2 ELISA kit (Cataloge No SL 0731Mo, China)
2. Mouse Caspase-3 ELISA kit (Cataloge No SL0679Mo, China)

3. Mouse PPAR-α ELISA kit (Cataloge No ELK2009, China)
4. COX-2 Gen expression kit (Gena Bioscience, Germany) composed of :
    - Designed RT-PCR β-actin forward and reverse primer sequences (Bioron GmbH, Germany)
    - Designed primer for COX-2 gene (Macrogen company, Korea) .
    - The RT-PCR bands were 202 bp COX-2 cDNA (Bioron GmbH, Germany)

3-6: Drugs preparation

The doses of nimesulide (10%, Instant Pharmaceuticals, India) and aspirin (pure powder, Sanofi, France) were prepared and diluted with physiological normal saline and given by intramuscular (i.m.) injection with a volume of injection of 5 ml/kg of mice in all experiments.

3-7: Blood and tissue collection

Samples of blood were collected from mouse choroid venous plexuses of the eye (Igwebuike *et al*., 2011) in a capillary tube containing EDTA as anticoagulant. After incubating at room temperature at 10–20 minutes, centrifuged the tubes for 15 minutes at 3000 rpm. To obtain plasma sample. Tissue samples (liver and kidney) were cut, weighed and frozen at -20, pending homogenization after adding phosphate buffer solution (PBS) (PH= 7.4), to get supernatant after centrifuging for 15 minutes at 3000 rpm. Aliquot will assessed by using the ELISA assay.

3-8: The experiments

3-8-1:First experiment

3-8-1-A: Measuring the analgesic median effective dose (ED_{50}) of nimesulide by up- and -down method in mice (Dixon, 1980)

The ED_{50} of nimesulide was determined by using the thermal method (hot plate) at 56 ^{O}C (Hilde and Theo, 2004) and by using the up-and-down method. In our study was determine the initial doses depended on the preliminary and previous studies (Gupta *et al*., 2000). The increase and decrease in the dose were at a constant value of 3 mg/kg.

Five mice were used for determination of nimesulide ED_{50} , weighing 26–30 g, randomly chosen at age of 2 months. The initial dose of nimesulide was 10 mg/kg,i.m. and the later dose was 10 mg/kg,i.m. Animals were placed on the hot plate individually before administration the drug and recorde the response of latency time in seconds (first removal of fore or hind paw and licking and/or jumping) (Adzu *et al*., 2011), then recorded the latency after 30 min after injection of nimesulide by placing the same mice on the hot plate. The cut-off time for analgesia was 20 second (the maximum time permitte the animal to stay on the hot plate to avoid harm the paw tissue) (Mohammad *et al*., 2012). When the pain was occurring, the symbol O was assigned while the symbol X was assigned to analgesia, then repeating the doses injection up and down in constant dose for three animals after the first change of the analgesic effect.

The ED_{50} value was calculated according to the formula (Dixon,1980):
ED_{50}= Xf + Kd

Xf:Final dose used
K: Table value

d: Increase or decrease in the doses

3-8-1-B: Measuring the analgesic ED_{50} of aspirin by up- and -down method in mice (Dixon, 1980)

Eight mice were used for injected aspirin, weight (26-33) g, randomly chosen at age of 2 months. The initial dose of aspirin was 100 mg/kg,i.m., depending on preliminary and previous studies (Pong *et al*., 1985; Aubin *et al*., 1998). The later

dose of aspirin was 190 mg/kg.i.m. Animals were placed individually on the hot plate before administration of the drug and recorded the response as latency time in seconds (first removal of fore or hind paw and licking and/or jumping) (Adzu *et al*., 2011), then recorded latency after 30 min after injection of aspirin by placing the same mice on the hot plate. The cut-off time for analgesia was 20 s (the maximum time allowed the animal to stay on the hot plate to avoid damage to the paw tissue) (Mohammad *et al*., 2012). The decrease and increase in the dose were at a constant value of 30 mg/kg, and the ED_{50} of aspirin was determined as in the previous experiment. The ED_{50} value was calculated according to the formula (Dixon,1980):

as mention in the above experiment .

Figure 11: Hote plate apparatus

3-8-2: Second experiment

3-8-2-A: Measuring the acute median lethal dose (LD_{50}) of nimesulide by up- and -down method in mice (Dixon, 1980)

Five mice were used for measuring the LD_{50} of nimesulide, weighing 28–32 g, randomly chosen at age of 2 months. The initial dose of nimesulide was 200 mg/kg b.w., depending on the initial experiments. The later dose of nimesulide was 200 mg/kg. The constant dose of 60 mg/kg was used for up-and-down paradigm the symbol (X) was referred to death and the symbol (O) was assigned to alive mouse , then repeating of three animals after the first change from death to alive or vice versa LD_{50} was calculated according to the (Dixon, 1980) and by using the equation:

LD_{50}= Xf + Kd

Xf: Final dose
K: Table value

d: Increase or decrease in dose (constant)

3-8-2-B: Measuring the acute median lethal dose (LD_{50}) of aspirin by up- and -down method in mice (Dixon, 1980)

Five mice were used for measuring the LD_{50} of aspirin, weighing 26–30 g, randomly chosen at age of 2 months. The initial dose of aspirin was 1000 mg/k b.w., depending on the initial experiments. The later dose of aspirin was 1000 mg/kg. The constant dose of 300 mg/kg was used for up-and-down paradigm the symbol (X) was referred to death and the symbol (O) was assigned to alive mouse , then repeating of three animals after the first change from death to alive or vice versa LD_{50} was calculated according to the mentioned previously.

3-8-3: Third experiment

Determination of the drug safety of nimesulide and aspirin in mice

Determined the drug's safety of nimesulide and aspirin by using the equation of the Therapeutic index (T.I.) which depend on the results of the first and second experiments for each drugs as follows (Muller and Milton, 2012) :

$T.I.=LD_{50}/ED_{50}$

3-8-4: Fourth experiment

3-8-4-A: Time – response relationship of nimesulide in mice

Five mice were used for each group of time of nimesulide treatment , weight (25–30) g. Nimesulide was injected at a dose of ED_{100} at different times to determine the relationship between the response of the pharmacological effect at different times (15, 30, 60, 120 minutes) , so the analgesic effect was determined at different times by using a hot plate. Individually animals were placed on the hot plate before administration of the drug and recorder the response as latency time in seconds (first removal of fore or hind paw and licking and/or jumping) (Adzu *et al*., 2011), then recorder the latency after (15, 30, 60, 120 min) after injection of ED_{100} of nimesulide (15.8 mg/kg,i.m.) by placing the same mice on the hot plate. The cut-off time for analgesia was 20 s (the maximum time allowed the animal to stay on the hot plate to avoid damage to the paw tissue) (Mohammad *et al*., 2012).

3-8-4-B:Time – response relationship of Aspirin in mice

Five mice were used for each group of time of aspirin treatment, weight (25-29) g. Aspirin was injected at dose of ED_{100} at different times to determine the relationship between the response of the pharmacological effect at different times (15, 30, 60, 120 minutes), so the analgesic effect was determined at different times by using a hot plate. Animals were placed individually on the hot plate before administration of the drug and recorded the response as latency time in seconds (first removal of fore or hind paw and licking and/or jumping) (Adzu *et al*., 2011), then recorder the latency after (15, 30, 60, 120 min) after injection of ED_{100} of aspirin (424.5 mg/kg, i.m.) by placing the same mice on the hot plate. The cut-off time for analgesia was 20 s (the maximum time allowed the animal to stay on the hot plate to avoid damage to the paw tissue) (Mohammad *et al*., 2012).

3-8-5:Fifth experiment

3-8-5-A: Dose-response relationship of Nimesulide in mice

In this experiment, 15 mice were injected with nimesulide, i.m., weight (25–33 g), randomly chosen at age of 2 months, and divided into 3 groups (each group consists of 5 animals): the first group injected ED_{25} of nimesulide at a dose of 4 mg/kg; the second group injected 7.9 mg/kg(ED_{50}) and the third group injected 15.8 mg/kg. (ED_{100}), then assessing the analgesic effects of nimesulide after 30 min of injection (which is the optimal time found from the fourth experiment) by using the hot plate at 56 ^{O}C as in previous experiments (Mohammad *et al*., 2012).

3-8-5-B: Dose-response relationship of Aspirin in mice

Used in this experiment: 15 mice for injected aspirin, i.m., weight (24–33 g), randomly chosen at age of 2 months, divided into 3 groups (each group consists of 5 animals): the first group injected ED_{25} of aspirin at a dose of 106 mg/kg; the second group injected 212 mg/kg of (ED_{50}) and the third group injected 424.5 mg/kg of (ED_{100}). Then, assess the analgesic effects of aspirin after 30 min of injection (which is the optimal time found in the fourth experiment) by using the hot plate at 56 ^{O}C as in previous experiments (Mohammad *et al*., 2012).

3-8-6: Sixth experiment

Comparative effect of nimesulide and aspirin on visceral pain (writhing reflex) induced by acetic acid (chemical method) in mice

Three groups were used in this experiment, each consisting of five mice weight (22-33) g. as follows:

Group 1 was injected with acetic acid 1% (0.1 ml/10 g b.w. i.p.), which induced a writhing response (Mohammad *et al*., 2012a; Hijazi *et al*., 2017). Group 2 was injected with nimesulide at 15.8 mg/kg, i.m. and the group 3 was injected with aspirin at 424.5 mg/kg, i.m. before 30 minutes from injected acetic acid, then

recorded the time of writhing onset and the number of writhing through 30 minutes from injected acetic acid induction in mice (Gupta *et al*., 1998; Mousa *et al*., 2019).

Determined the percentage of the decrease in the number of writhing reflexes depended on the following equation: N Control – N Treatment /N Control x 100. (Adzu *et al*., 2001)

N: mean of the number of writhing reflex for each group

3-8-7: Seventh experiment

Comparison between nimesulide and aspirin at the level of antipyretic effect in mice

In this experiment, we used 4 groups (each group consisting of 5 mice), weight (21-31g) .

The first group was inject with normal saline i.p.,the second group was injected with baker's yeast (pyrogenic dose of 135 mg/kg dissolved in 5 ml of distal water to elevate the body temperature) (Jorgete *et al*., 2005), the third group injected with nimesulide (15.8 mg/kg, i.m.) after 30 minutes from baker's yeast injection and the fourth group was injected with aspirin (424.5 mg/kg, i.m.) after 30 minutes from the baker's yeast injection. All tested groups measured for the temperature before and after 1, 2, 3, and 4 h. the injection of baker's yeast.

3-8-8: Eighth experiment

Comparative anti-inflammatory effect between nimesulide and aspirin in mice

Twenty mice were divided into 4 groups, each consisting of 5 mice with a weight of (23–33g). Group 1 was injected with normal saline (negative control group) in the right paw, and the thickness of the paw was measured before and after injection in times (0.5, 1, and 2 h).

Group 2 was injected with formalin (0.02 ,1%) (positive control group) in the right paw to induce inflammation (Damas and Liégeois, 1999; Ardeshir *et al*., 2015) and measured the thickness of the paw before and after injection in times (0.5, 1, and 2) h.Group 3 was injected with nimesulide (15.8 mg/kg, i.m.) before 30 minutes of formalin injection (Gupta *et al*., 1999) and measured the thickness of the paw before and after the injected of formalin in times (0.5, 1, 2) while group 4 was injected with aspirin (424.5 mg/kg, i.m.) before 30 minutes of formalin injection. The thickness of the paw for all the groups was measured by using the digital caliper with the calculation of the time of onset and the number of paw lifting and licking or biting (Gupta *et al*., 1999; Bucan *et al*., 2022).

3-8-9: Ninth experiment

Comparative anti-coagulant effect between nimesulide and aspirin in mice

Fifteen of mice were divided into 3 groups, each consisting of 5 mice with a weighting (23–34g) .

Group 1 was injected with normal saline (negative control group) for five consecutive days. The second and third groups were injected with ED_{100} of nimesulide (15.8 mg/kg i.m.) and aspirin (424.5 mg/kg i.m.), respectively, for five consecutive days. After 30 minutes of the 5^{th} day of treatment , blood was drawn from the eye by a blue capillary tube, and prothrombin time (PT) was checked every 30 seconds and recorded the clotting occurring or not (Kagawa, 2002)

3-8-10: Tenth experiment

Detection of the nimesulide and aspirin concentration in plasma at different times using high-performance liquid chromatography (HPLC)

In this experiment, 30 mice at 2 months of age were divided into two groups, each consisting of 15 mice of different weights(24-32g). Nimesulide was injected into 3 of mice for each time at a dose of 15.8 mg/kg i.m., while the other 5 groups

of 3 mice for each time were treated with aspirin at a dose of 424.5 mg/kg i.m. The blood samples were collected from the two groups of nimesulide and aspirin at 0.5, 1, 2, 4, and 24 h . The plasma was gotten by centrifugation (4000 rpm for 15 minutes, Chalice, UK) of EDTA containing tubes (anticoagulant) after being incubated at room temperature for 10–20 minutes. The plasma samples were kept at -18 °C until assessment. The plasma samples were analyzed by using high-performance liquid chromatography (HPLC), Shimadzu, Japan) which contain an ultraviolet detector unit (Jaworowicz *et al*., 1999; Villa *et al*., 2007; Markku *et al*., 2008; Constuntinos and Paraskevas, 2011; Mi-Sun *et al*., 2012; Guillé *et al*., 2019)

Figure 12: device of HPLC(High-performance liquid chromatography)

3-8-10-1: Device settings

1. UV detector for nimesulide 300 nm and 238nm for aspirin
2. Flow rate 1.5 ml/min
3. Volume of injection 20 µl
4. Run time 10 min

3-8-10-2: Solutions used

1. Methanol(high purity for HPLC)
2. Acetonitrile (high purity for HPLC)
3. Phosphoric acid
4. Ultrapure water
5. Millipore filter paper 0.45 μm

3-8-10-3: Preparing multiple standards of nimesulide and aspirin

The nimesulide standards were made in concentrations of 10, 20, 40, 80, 160, and 320 μg/ml through diluting with the mobile phase composed of triethylamine (0.2%) and methanol at 1:1 volume:volume and pH 3 via using the phosphoric acid. The solution was filtered by using 0.45 μm filter paper (Millipore, England) and then underwent degassing (McMahon *et al*., 1998; Patel *et al*., 2000). Finally, the clear solution injected into a 20-μl volume and examined at a wavelength of 300 nm by using HPLC with a flow rate of 1.5 ml/min and a run time fixed for 10 min. while aspirin standards were made of 25, 50, 100, 200, 400, and 800 μg/ml in concentrations via diluting with a mobile phase containing acetonitrile and water at 35:65 volume:volume. The solution then filtered with degassing by used 0.45 μm filter paper, and the wavelength used was 238 nm with a flow rate of 1.5 ml/min and a run time fixed for 10 min (Ptáček *et al*., 2001; Peter *et al*., 2013).

Equation (y=a+bx) reflecta a simple linear regression that was estimated from the nimesulide and aspirin standards (R^2 = 0.9838) and (R^2 = 0.9994), respectively used for the calculation of individual nimesulide and aspirin concentration in plasma samples for two groups (Appendix 2 and 3) where ‘y’ represents the peak of area for the samples detected at 300 nm for nimesulide and 238 nm for aspirin by the HPLC, ‘a’ was the intercept (143080) for nimesulide and (841386) for aspirin, ‘b’ was the slope (15393) for nimesulide and (166368) for aspirin, and ‘x’ represents the nimesulide and aspirin concentration of unknown plasma samples.

3-6-10-4: Liquid-liquid extraction

The method of liquid-liquid extraction (LLE) was apply through using a simple, approved, and précised technique for nimesulide and aspirin extraction from plasma proteins (Ravi *et al*., 2017; Kim *et al*., 2022). The technique involved of liquid-liquid extraction via adding acetonitrile to the plasma sample at 1:1 v/v. After that, the mixture vortexed for up to 5 minutes for nimesulide and 1 minute for aspirin and centrifuged at 3500 rpm for 15 min. The resultant supernatant was filtered with degassing by used 0.45 μm filter paper. The sample was inject (20 μl) and analyzed by HPLC (combined with a UV chromatographic detector) at 300 and 238 nm, respectively.

3-8-11: Eleventh experiment

Comparison of nimesulide and aspirin pharmacokinetics in mice

A non-compartmental model used to acquire the pharmacokinetic variables for nimesulide and aspirin though used a PK Solver program plug-in in Excel (Zhang *et al*., 2010; Ravi *et al*., 2017). The variables of pharmacokinetics involved an area under the curve ($AUC_{0-\infty}$) (μg.h/ml), an area under the moment curve ($AUMC_{0-\infty}$) (μg.h^2/ml), mean residence time (MRT) (AUMC/AUC)(h), maximum concentration (C_{max})(μg), maximum time (T_{max})(h), half-life ($t_{1/2\beta}$)(h), volume of distribution (V_{ss}) [dose.AUMC/$(AUC)^2$](L/kg), elimination rate constant (K_{el})/ h , and total clearance (Cl) (dose/AUC)(L/h/kg).

3-8-12: Twelfth experiment

A comparative inhibition of nimesulide and aspirin COX-2 concentration in plasma, liver and kidney of mice

This experiment included 6 groups of mice (5 mice per group) weighing (23-32g). Normal saline was injected i.p. in the negative control group. The nimesulide-treated group received a dose of 15.8 mg/kg, i.m., while aspirin was injected at 424.5 mg/kg, i.m. The positive group was treated with acetic acid 1% (0.1 ml/10 g) i.p. for induction of COX-2; the other group injected by nimesulide

(15.8 mg/kg, i.m.)+acetic acid; and the last group was treated with aspirin (424.5 mg/kg, i.m.)+acetic acid.

Collected the blood samples After 30 minutes of being injected the drugs of the 5^{th} day of treatment (the treatment was for 5 consecutive days, one dose daily), then obtained from plasma and isolated the liver and kidney to estimate the COX-2 activity by using the enzyme-linked immunosorbent assay (ELISA) technique by a specified ELISA kit for mouse (with the catalog number SL0731Mo, China) to analyze the concentration of COX-2 in pg/ml. The absorbance of the drug was measured at 450 nm of COX-2 standards in pg/ml. Simple linear regression was obtained by the standard calibration curve, which was then applied ($y = 0.1034 + 0.0022 x$ with a correlation coefficient of $R^2 = 0.9988$) (appendix 4) .

The COX-2 concentration calibration was used on plasma samples of mice (Caiazza *et al*., 2019).

The concentration of COX-2 was measured in the plasma by collecting the whole blood in to the tubes containing an anticoagulant (EDTA), after being incubated at room temperature for 10–20 minutes, the tubes are centrifuged at 3000 rpm for 20 minutes, and then the supernatant was collected carefully as plasma samples, Liver and kidney samples are obtained by cutting, weighing, and freezing the tissues at -20 °C, then homogenized after adding PBS (pH 7.4) at 4 °C, collection of the supernatant was done after centrifuging at 3000 rpm for 20 min, and then measured by the ELISA technique (Li *et al*., 2017) .

3-8-13: Thirteenth experiment

Effect of nimesulide and aspirin on apoptosis in mice

Comparative effect of nimesulide and aspirin on the apoptosis was done via measuring the activity of Caspase-3 in plasma, liver and kidney . thirty mice weighing (22-30g). at 2 month age were divided into six groups, each group containing 5 animals. The control group injected normal saline , the second and third groups injected with the ED_{100} of nimesulide and aspirin i.m (15.8 and 424.5 mg/kg,i.m) respectively. The fourth group was injected with acetic acid (1% i.p)

while the fifth and sixth groups were injected with nimesulide+acetic acid and aspirin+acetic acid respectively. All groups treated for 5 consecutive days (one dose daily) then after 30 minute from injection in the fifth day, the blood was collected from the eye with the organs (liver and kidney). The sample were preserved in -20 to pending examination in laboratory by specialized ELISA kit for mouse Caspase-3 (Mukherjee *et al*.,2001) .

3-8-14: Fourteenth experiment

Effect of nimesulide and aspirin on PPAR-α in mice

The comparative effects between nimesulide and aspirin on the peroxisome proliferator-activated receptor (PPAR-α) by using thirty mice weighing (23-30g) at 2 month age were divided into 6 groups and each group consisted from 5 mice. The control group injected with normal saline, the second and third groups injected with the ED_{100} of nimesulide and aspirin (15.8 and 424.5 mg/kg,i.m) respectively. The fourth group was injected with acetic acid (1% i.p) while the fifth and sixth groups were injected with nimesulide+acetic acid and aspirin+acetic acid respectively. All groups were treated for 5 consecutive days (one dose daily) then after 30 minutes from injection in the fifth day, collected the blood from the eye with the organs (liver and kidney).The samples were preserved in -20 to pending examination in the laboratory by specialized ELISA kit for mouse PPAR-α (Ivan *et al*.,2006) .

3-8-15: Fifteenth experiment

Comparative effects of nimesulide and Aspirin on the level of COX-2 Gene expression in mice

This experiment weighted 30 mice (22-30g) were divided into 6 groups at 2 months of age, each group consisted of 5 animals. The control group injected with normal saline , the second and third groups injected with the ED_{100} of nimesulide and aspirin i.m (15.8, 424.5 mg/kg) respectively, the fourth group was injected

with acetic acid (1% i.p), fifth and sixth groups were injected with nimesulide+acetic acid and aspirin+acetic acid respectively, all groups were treated for 5 consecutive days (one dose daily) then after 30 minutes from injection in the fifth day, the blood was collected from the eye with the organs (liver and kidney). The samples the were in -20 to pending examination in the laboratory by specialized ELISA kit of COX-2 gene expression for the mouse.

3-8-15-1: mRNA extraction based on kit instructions (Gena Bioscience, Germany) for determination of COX-2 gene expression (Archer, 2017) (Figure 12,13)

1. Collection of kidney tissue sample (20–50 mg) from all groups mentioned above and placed it in a microcentrifuge tube. After that, we used the proper equipment to homogenize the sample after adding 300 µl of lysis buffer. After homogenizing the sample, add 200 µl of lysis buffer and vortexe for 15 to 30 seconds. Centrifugation was then used for 10 minutes at 10,000 grpm. As follows:
2. Using a 2 ml collection tube, add 100 µl of activation buffer to the spin column to activate it. Centrifugation was then used for 30 seconds at 10,000 g. The flow-through was discarded.
3. The obtained lysate was mixed with 300 µl of isopropanol and vortexed. The mixture was then put directly into the spin column. Centrifugation was then used for 30 seconds at 10,000 g. Lastly, the flow-through was discarded.
4. Applied 700 µl of the first washing buffer to the spin column. Centrifugation was applied at 10,000 g for 30 seconds. Then, discard the flow-through.
5. Applied 700 µl of second washing buffer to the spin column. Next, centrifugation was applied at 10,000 g for 30 sec. Then, discard the flow-through. After that, centrifugation was applied again at 10,000 g for 2 min to remove residual ethanol.
6. Place the spin column into a new DNAase- or RNAase-free microcentrifuge tube. I added 40–50 µl of elution buffer to the center of the column membrane. Then, it was incubated at room temperature for 1 minute. After that,

centrifugation was applied at 10,000 g for 1 minute to elute the mRNA. Finally, mRNA was stored at -20 °C for future use.

3-8-15-2: Measurement of COX-2 mRNA contents in the kidney tissue of mice

The primary step for determined the gene expression via reverse transcription polymerase chain reaction (RT-PCR) is the quantification of the extracted RNA, which was measured by using Qubit™ equipment (Qubit Fluorometer, Invitrogen, USA). A high-sensitivity kit was used for the procedure. Prepare the working solution put in a clean plastic tube. The final volume in each tube was 200 μL. The sample volume was 1–20 μL added to the 180–199 μL working solution, then mixed via vertexing for 2–3 seconds, At room temperature incubated for 2 minutes, All samples were measured by using a Qubit® fluorometer. The same quantitative procedure which used for RNA quantification also was used to quantify complementary DNA (cDNA) (Archer, 2017) .

3-8-15-3: Expression of COX-2 in the kidney tissue of mice:

1.The RT-PCR kit was provided by Bioron GmbH, Germany. One RT-PCR Master Mix is designed for all applications that require the reverse transcription of RNA into cDNA and subsequent PCR amplification. Only primers and sample RNA were added to the master mix.

2.We used β-actin as a housekeeping gene and loading control due to its general expression across all eukaryotic cell types to normalize the expression of the target gene (mRNA) levels between different samples.

3.We designed RT-PCR β-actin forward and reverse primer sequences as follow:

5’ TTGTGATGGACTCCGGAGAC 3’, 5’ TGATGTCACGCACGATTTCC 3’, respectively and F- 5’ CCCCTCTCTACGCATTCTGT 3’, R- 5’ TGGCAGAACGACTCGGTTAT3’ for COX-2 gene. All the primers were synthesized by a Macrogen company, Korea.

The total volume of the assay was 20 µl (10 µl One RT-PCR Master Mix + 0.5 µl of each primer (Forward and Reverse) + 2 µl RNA template + 7 µl Nuclease-free water).

4.According to the manufacturer's instructions, the following was the optimal reaction condition: reverse transcription at 55 °C for 30 min, denaturation of the RNA hybrid, and inactivation of reverse transcriptase at 95 °C for 3 min. PCR for 45 cycles, denaturation at 92 for 10 s, annealing at 60 °C for 10 s, extension at 72 °C for 20 s, final extension at 72 °C for 7 min.

5.RT-PCR was performed on the MiniAmp Plus™ Thermocycler PCR, USA.

6.The quantification of gene expressions was determined according to Archer (2017 and Rao (2013 methods using different equations (1, 2). The PCR threshold standard curve was based on an exponential model of the initial phase of a PCR run where template replication efficiency is constant from cycle to cycle.

7.Electrophoresis (Cleaver Scientific, England) was carried out on a 2% agarose gel containing gel-safe stain to separate charged DNA fragments according to their sizes.

8.The RT-PCR bands were 202 bp COX-2 cDNA and 186 bp β-actin.

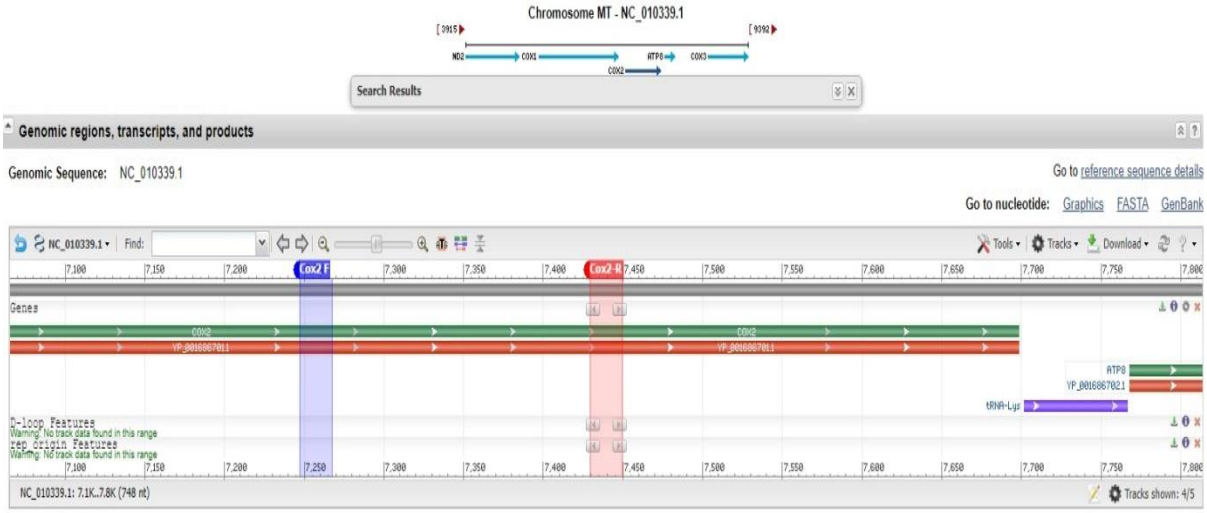


Figure 13: Nucleotide sequence of target COX-2 primers designed from the GenBank database

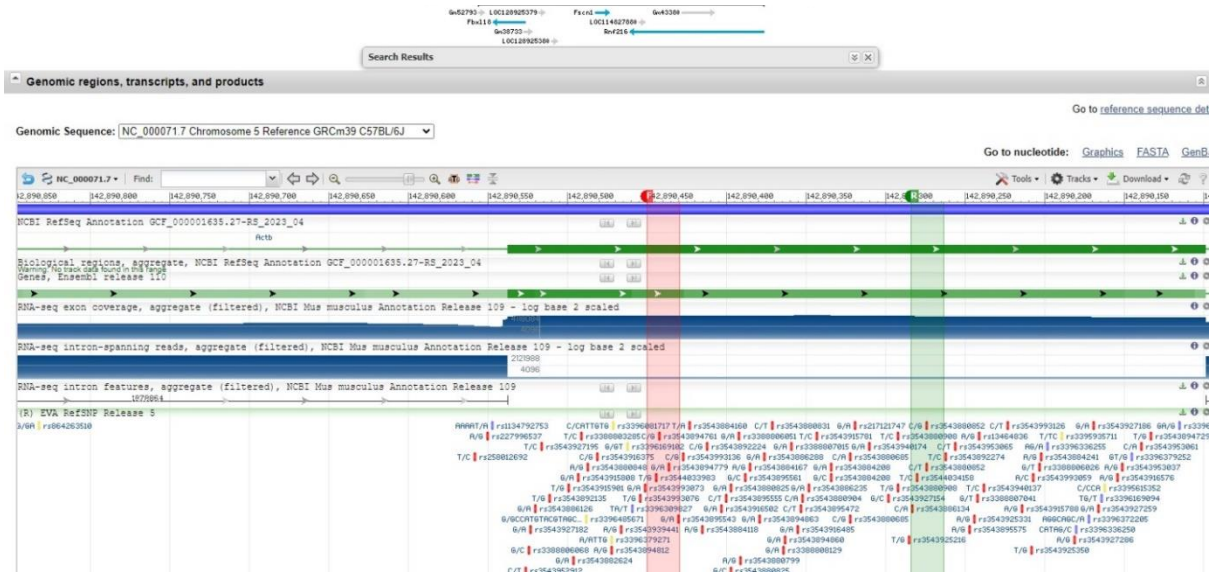
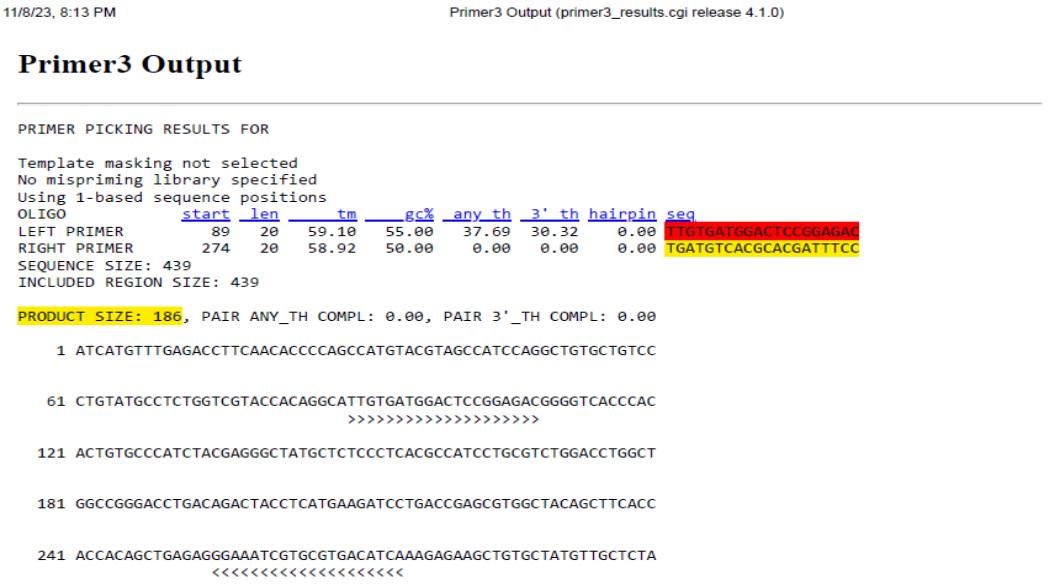


Figure 14: Nucleotide sequence of target β -actin primers designed from the GenBank database



11/8/23, 8:13 PM Primer3 Output (primer3_results.cgi release 4.1.0)

Primer3 Output

PRIMER PICKING RESULTS FOR

Template masking not selected
No mispriming library specified
Using 1-based sequence positions

OLIGO	start	len	tm	gc%	any_th	3'_th	hairpin	seq
LEFT PRIMER	89	20	59.10	55.00	37.69	30.32	0.00	TTGTGATGGACTCCGGAGAC
RIGHT PRIMER	274	20	58.92	50.00	0.00	0.00	0.00	TGATGTCACGCACGATTTCC

SEQUENCE SIZE: 439
INCLUDED REGION SIZE: 439

PRODUCT SIZE: 186, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

```
    1 ATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGTAGCCATCCAGGCTGTGCTGTCC

   61 CTGTATGCCTCTGGTCGTACCACAGGCATTGTGATGGACTCCGGAGACGGGGTCACCCAC
                                >>>>>>>>>>>>>>>>>>>>

  121 ACTGTGCCCATCTACGAGGGCTATGCTCTCCCTCACGCCATCCTGCGTCTGGACCTGGCT

  181 GGCCGGGACCTGACAGACTACCTCATGAAGATCCTGACCGAGCGTGGCTACAGCTTCACC

  241 ACCACAGCTGAGAGGGAAATCGTGCGTGACATCAAAGAGAAGCTGTGCTATGTTGCTCTA
                  <<<<<<<<<<<<<<<<<<<
```

Figure 15: Nucleotide sequence of target COX-2 primers designed from the GenBank database

3-9: Statistics

One-way analysis of variance (SPSS, version 16) was used for the statistical analysis of parametric data which was performed for multiple mean comparisons followed by the least significance difference (LSD), while the unpaired T-test was used to compare the means of two groups at significant level $p<0.05$ (Petrie and Watson, 2013) .

Chapter Four

Results

4-1:First experiment

4-1-A:Measuring the analgesic median effected dose ED_{50} of nimesulide by up- and -down method in mice

The analgesic ED_{50} value for nimesulide that produced analgesia in 50% of mice was 7.9 mg/kg, i.m. by using the up-and-down paradigm after injection of different doses from nimesulide to the numbers of mice and the range of the dosages used were 10-7 mg/kg (Table 1).

Table 1. Analgesic ED_{50} of nimesulide in mice

Variables	Nimesulide
ED_{50}=xf+(k×d)	7.9 mg/kg, i.m.
The first dose	10 mg/kg
The latest dose (xf)	10 mg/kg
Table value (k)	-0.701
± Dosage (d)	3 mg/kg
Range of the dosages	10-7=3 mg/kg
Overall mice used	5 (XOXOX)

The X symbol means analgesia while O indicates no analgesia

4-1-B: Measuring the analgesic median effected dose ED_{50} of aspirin by up- and -down method in mice

The analgesic ED_{50} value for aspirin that produced analgesia in 50% of mice was 212.23 mg/kg, i.m, using the up -and -down paradigm after injection different doses from aspirin to the number of mice and the range of the dosages used were 220-100 mg/kg (Table 2).

Table 2. Analgesic ED_{50} of aspirin in mice

Variables	Aspirin
ED_{50}=xf+(k×d)	212.23 mg/kg, i.m.
The first dose	100 mg/kg
The latest dose (xf)	190 mg/kg
Table value (k)	0.741
± Dosage (d)	30 mg/kg
Range of the dosages	220-100=120 mg/kg
Overall mice used	8 (OOOOXOXO)

The X symbol means analgesia while O indicates no analgesia

4-2:Second experiment

4-2-A: Measuring the acute median lethal dose (LD_{50}) of nimesulide by up- and -down method in mice

The acute LD_{50} value for nimesulide that killed half of the population of mice was 181.7 mg/kg, i.m., by using the up-and-down method, for the number of mice for range of the dosages were 260-140 mg/kg, the acute toxic signs represented by rapid breathing, increased movement, instability, shivering, limping, paralysis

and death during 24 h. Other signs of toxicity included seizures and spasms, (Table 3).

Table 3. Acute LD_{50} of nimesulide in mice

Variables	Nimesulide
LD_{50}=xf+(k×d)	181.7 mg/kg, i.m.
The first dose	200 mg/kg
The latest dose (xf)	200 mg/kg
Table value (k)	-0.305
± Dosage (d)	60 mg/kg
Range of the dosages	260-140=120 mg/kg
Onest of clinical toxic	1-3 min
Overall mice used	5 (OXXOX)

The X symbol means death while O indicates no death

4-2-B: Measuring the acute median lethal dose (LD_{50}) of aspirin by up- and -down method in mice

The acute LD_{50} value for aspirin that killed half of the population of mice was 1210.3 mg/kg, i.m, by using the up and down method, for the number of mice for range of the dosages were 1300-1000 mg/kg . and the acute toxic signs represented by rapid breathing, increased movement, instability, shiver, limping, abdominal cramps then death after 1 h , other animal showed the same signs and after 1 h appeared calm and motionless (Table 4).

Table 4. Acute LD_{50} of aspirin in mice

Variables	Aspirin

LD_{50}=xf+(k×d)	1210.3 mg/kg, i.m.
The first dose	1000 mg/kg
The latest dose (xf)	1000 mg/kg
Table value (k)	0.701
± Dosage (d)	300 mg/kg
Range of the dosages	1300-1000 mg/kg
Onest of toxicity	1-3 min
Overall mice used	5 (OXOXO)

The X symbol means death while O indicates no death

4-3: Third experiment

Determination of the drug safety of nimesulide and aspirin in mice

The drug safety of nimesulide and aspirin were determined by using equation of the therapeutic index (T.I.) which depend on the results of the first and second experiments:

T.I.=LD50/ED50

The results of the therapeutic index of nimesulide and aspirin were 23 and 6, respectively.(Table 5) .

Table 5: The drug safety of nimesulide and aspirin

Parameter	Nimesulide	Aspirin
T.I	23	6

T.I.=LD50/ED50

4-4: Fourth experiment

4-4-A:Time- response relationship of Nimesulide in mice

Led injection ED_{100} (15.8 mg/kg) from nimesulide at different times to determine the relationship between the response of the pharmacological effect at different times (15, 30, 60, 120) minutes, by using a hot plate (thermal method), after 15 min from injected nimesulide no analgesia was recorded but at other times (30,60 and 120) the results were (5, 4.4, 3.6) which represented period to respons to pain (latency time/second) respectively and the maximum analgesic effect (peak of time) of nimesulide at the time 30 minutes after injection , ED_{100} of nimesulide led to significant pain relief by prolonging the duration of the pain response (latency time) (first removal of fore or hind paw and licking and/or jumping) comparison with base line of same group (Table 6) (Figure 15)

4-4-B:Time- response relationship of Aspirin in mice

Led injection ED_{100} (424.5 mg/kg) from aspirin at different times to determine the relationship between the response of the pharmacological effect at different times (15, 30, 60, 120) minutes, by using a hot plate (thermal method) after 15 min from injected aspirin no analgesia was recorded but in other times the results were (4.4, 4.3, 3.5) which represented period to response to pain (latency time/ second) respectively and the maximum analgesic effect (peak of time) of aspirin at the time 30 minutes after injection , ED_{100} of aspirin led to significant pain relief by prolonging the duration of the pain response (latency time) (first removal of fore or hind paw and licking and/or jumping) comparison with base line of same group (Table 6,Figure 16).

Table 6: Time –response relationship of nimesulide and aspirin in mice

Groups	Latency time (second)				
	0	15 min	30 min	60min	120min
Nimesulide 15.8 mg/ kg, i.m	2.7±0.42	2.4±0.29	5±0.16$^{*\,a}$	4.4±0.48$^{*\,a}$	3.6±0.24^{*b}
Aspirin 424.5 mg/kg, i.m	2.6±0.37	2.4±0.17	4.4±0.40$^{*\,a}$	4.3±0.25$^{*\,a}$	3.5±0.25^{*b}

Numbers were as mean ± Std.E (5 mice /group)

*Differ significantly from the time baseline in the same group at $p<0.05$

^{a} Differ significantly from the time 15 min in the same group at $p<0.05$

^{b} Differ significantly from the time 30 min in the same group at $p<0.05$

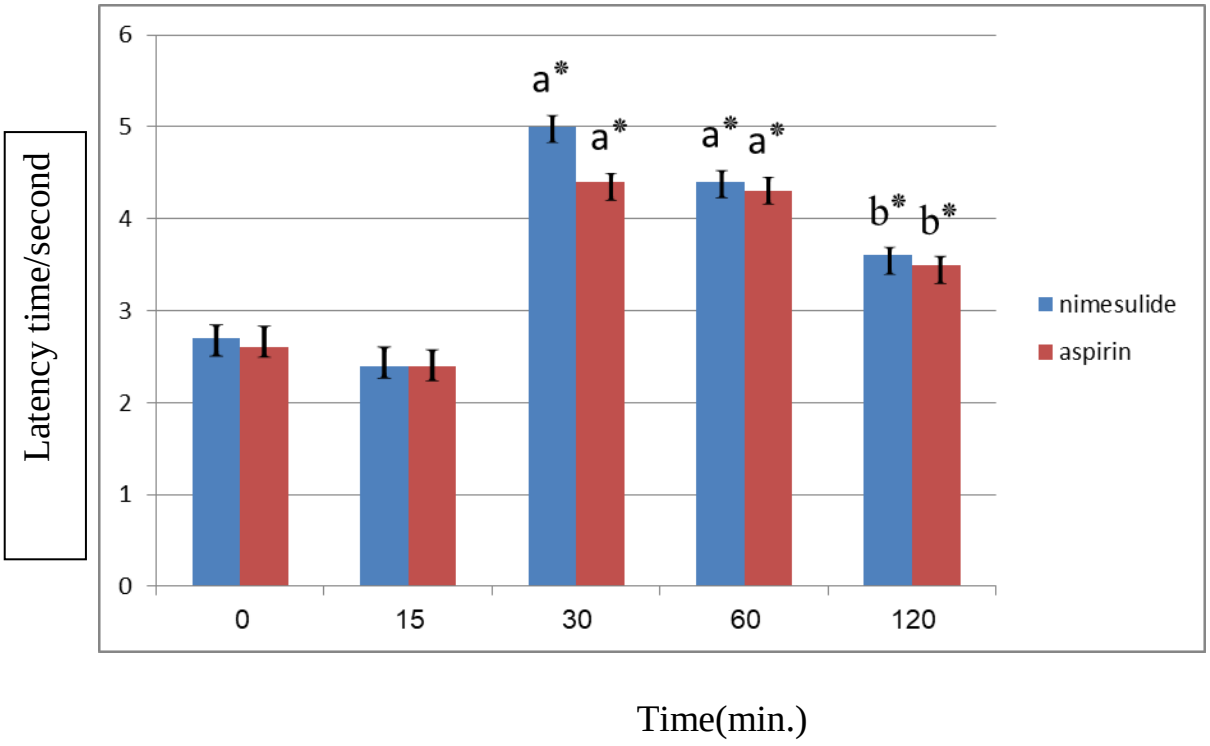


Figure 16: Time –response relationship of nimesulide and aspirin in mice

4-5: Fifth experiment

A:Dose – response relationship of Nimesulide in mice

Nimesulide exerted its effect in a dose-dependent manner. The determined ED_{50} and ED_{100} of nimesulide were 7.9 and 15.8 mg/kg i.m., respectively, which produced the analgesic response significantly in mice at (4, 4.6) second

respectively comparative with baseline for the same group all measured at 30 min after injection and this time was the best time for producing the analgesic effect which we got it from previous experiment, while ED_{25} (4 mg/kg) failed to relieve the pain in comparison with base line of same group, (Table 7, Figure 17).

Table 7: Dose-response relationship of nimesulide in mice

Dose (mg/kg, i.m.)	0 time	Latency time/second after 30 minutes from injection
Nimesulide		
4 (ED_{25})	2.20 ± 0.25	2.30 ± 0.20
7.9 (ED_{50})	2.60 ± 0.19	4.00 ± 0.42 ^{*,a}
15.8 (ED_{100})	2.50 ± 0.16	4.60 ± 0.43 ^{*,a}

Numbers were as mean ± Std.E (5 mice /group)

*Differ significantly from baseline in the same group at $p<0.05$

^{a} Differ significantly from the first dose (ED_{25}) of the drug at $p<0.05$

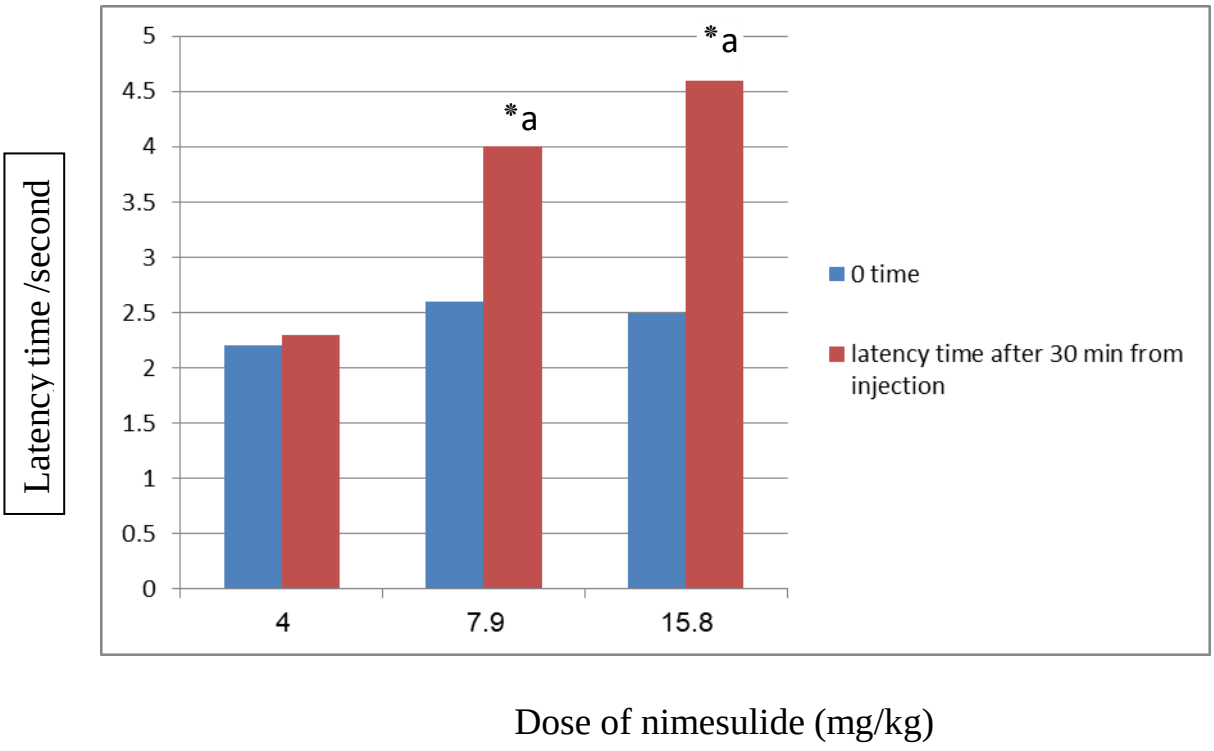


Figure 17: Dose-response relationship of nimesulide in mice

4-5-B: Dose – response relationship of aspirin in mice

Aspirin exerted its effect in a dose-dependent manner. The determined ED_{100} of aspirin was 424.5 mg/kg i.m., which produced the analgesic response significantly in mice 4.6 second comparative with 0 time for same group at 30 min and this time was the best time to analgesic effect which we got it from previous experience ,and the ED_{50} of aspirin was 212.23 which produced analgesic response in mice 2.8 second at 30 min comparison with 0 time , while ED_{25} (106.15 mg/kg) failed to relieve the pain comparison with base line of same group (Table 8, Figure 18).

Table 8: Dose-response relationship of aspirin in mice

Dose (mg/kg, i.m.)	0 time	Latency time/second after 30 minutes from injection
Aspirin		
106.15 (ED_{25})	2.60 ± 0.29	2.50 ± 0.28
212.23 (ED_{50})	2.20 ± 0.25	2.80 ± 0.20
424.5 (ED_{100})	2.50 ± 0.42	4.20 ± 0.34 ^{*,a,b}

Numbers were as mean ± Std.E (5 mice /group)

*Differ significantly from 0 time in the same group at $p<0.05$

^{a} Differ significantly from the first dose (ED_{25}) of the drug at $p<0.05$

^{b} Differ significantly from the second dose (ED_{50}) of the drug at $p<0.05$

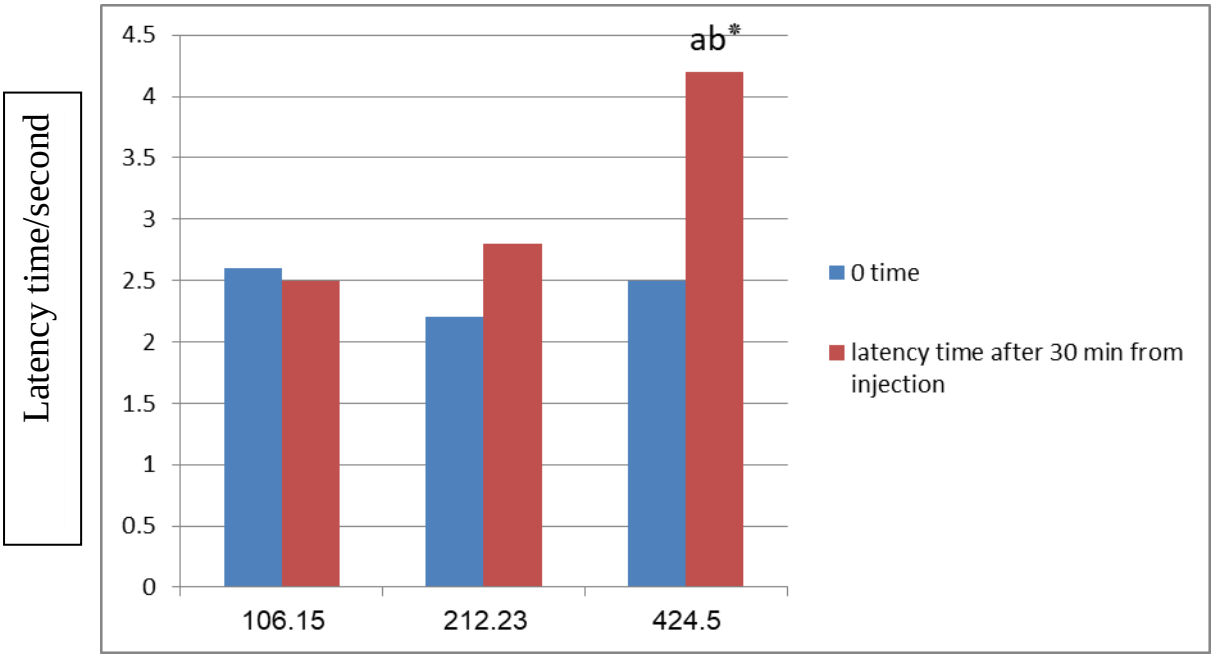


Figure 18 : Dose-response relationship of aspirin in mice

4-6: Sixth experiment

Comparative effect of nimesulide and aspirin on visceral pain (writhing reflex) induced by acetic acid (chemical method) in mice

Injection of the ED_{100} for individual nimesulide and aspirin (15.8 and 424.5 mg/kg i.m., respectively) before 30 minute from injection of acetic acid were caused significantly an increase in the onset of time and a reduce in the writhing number compared to a control group that injected with acetic acid (1%, 0.1ml/10gm), i.p. to induce writhing reflex and the recorded number of writhing reflex through 30 minute immediately after injection acetic acid which was represented by abdominal contraction, stretching forward with an arch, and extension of the hind limb, and it was 97.6 number of writhing reflex in control group (acetic acid) and the onset of time was 0.9, but when injected individual nimesulide and aspirin before 30 min from injection of acetic acid in the second and third group led to decrease in writhing number to 33 , 52.6 and increase the onset of time to 3.6, 4.4 min ,respectively. Also, the drugs produced a decrease in the percentage of writhing numbers to 66% and 46% for nimesulide and aspirin, respectively (Table 9,Figures 19,20,21).

Table 9: Determine the analgesic effect of nimesulide and aspirin on the level of visceral pain in mice

Treated groups	Time of onset (minute)	No. of writhing reflex /30 min	% inhibition in writhing No.
Control group(Acetic acid 1%)	0.90 ± 0.29	97.60 ± 4.04	0%
Nimesulide (15.8 mg/kg)	3.60 ± 0.58 *	33.00 ± 3.58 *	66 %
Aspirin (424.5 mg/kg)	4.40 ± 0.24 *	52.60 ± 3.75 *,a	46%

Numbers were as mean ± Std.E (5 mice /group)

*Differ significantly from control injected with acetic acid at $p<0.05$

[a] Differ significantly from the group injected with nimesulide at $p<0.05$

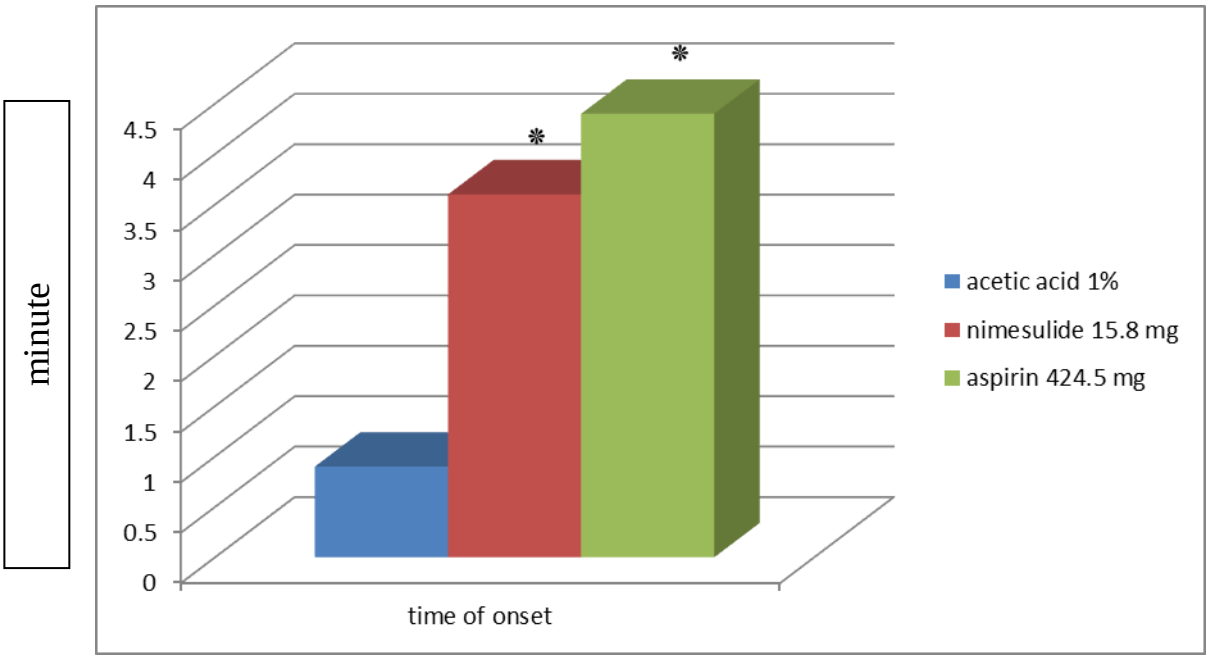


Figure 19: Analgesic effect of nimesulide and aspirin on the level of visceral pain in mice

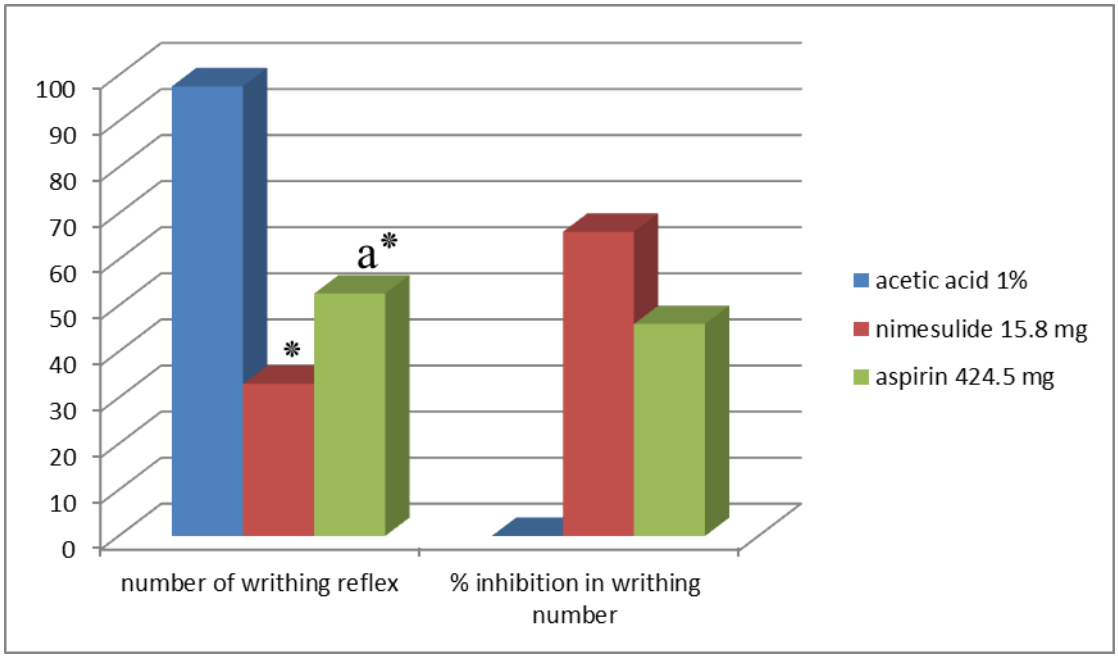


Figure 20: Analgesic effect of nimesulide and aspirin on the visceral pain in mice

Figure 21:Writhing reflex in mouse

4-7: Seventh experiment

Comparison between nimesulide and aspirin at the level of antipyretic effect in mice

Injection of baker's yeast (135 mg/kg, i.p) led to increase a temperature at time 1,2,3 and 4 h after injection in the positive control group in comparison with temperature in negative control group (normal saline) in mice, and noticed

significant difference in temperature at 2,3 and 4 h in the positive group in comparative with the baseline in the same group.

In third group which injected ED_{100} of nimesulide i.m after 30 min from injected baker's yeast i.p , the results showed a decrease in a temperature at time 1,2 ,3 and 4 h comparison with the baseline in the same group , and there was a significance difference at time 1,2,3 and 4 h comparative with positive control (baker's yeast).

In forth group which injected ED_{100} from aspirin i.m after 30 min from injected baker's yeast i.p , the results revealed a decrease in a temperature at time 1,2 and 3 h in comparison with the baseline in same group , and there was significant difference in a temperature at time 2 h in comparison with the positive control (baker's yeast).The antipyretic effect of nimesulide was more significantly efficient than that of aspirin in decreasing the temperature induced by baker's yeast (Table 10,Figure 22).

Table 10: Comparison of antipyretic effect between nimesulide and aspirin in mice

Groups	**Time (h)**								
	Before injection	**1**	**Change in tempreture after 1 h**	**2**	**Change in tempreture after 2 h**	**3**	**Change in tempreture after 3 h**	**4**	**Change in tempreture after 4 h**
Normal saline	**35.50**	**35.26 ± 0.47**	**0.24**	**34.80 ± 0.66**	**-0.7**	**35.02 ± 0.14**	**-0.48**	**35.10 ± 0.46**	**-0.4**
baker's **yeast**	**35.54**	**36.02 ± 0.40**	**0.48**	**36.14 ± 0.57 ***	**0.6**	**36.62 ± 0.40 ***	**1.08**	**37.12 ± 0.55 ***	**1.58**
Nimesulide + baker's **yeast**	**35.86**	**35.10 ± 0.37 [a]**	**-0.76**	**34.30 ± 0.28 [a]**	**-1.56**	**35.02 ± 0.38 [a]**	**-0.84**	**36.68 ± 0.66 [a]**	**-0.82**
Aspirin + baker's **yeast**	**37.00**	**36.12 ± 0.26**	**-0.88**	**35.90 ± 0.53 [a]**	**-1.1**	**36.92 ± 0.30**	**-0.08**	**37.28 ± .030**	**0.28**

Numbers were as mean ± Std.E (5 mice /group)

[*]Differ significantly from the normal saline group at the same time (p<0.05)

[a]Differ significantly from baker's yeast at the same time (p<0.05)

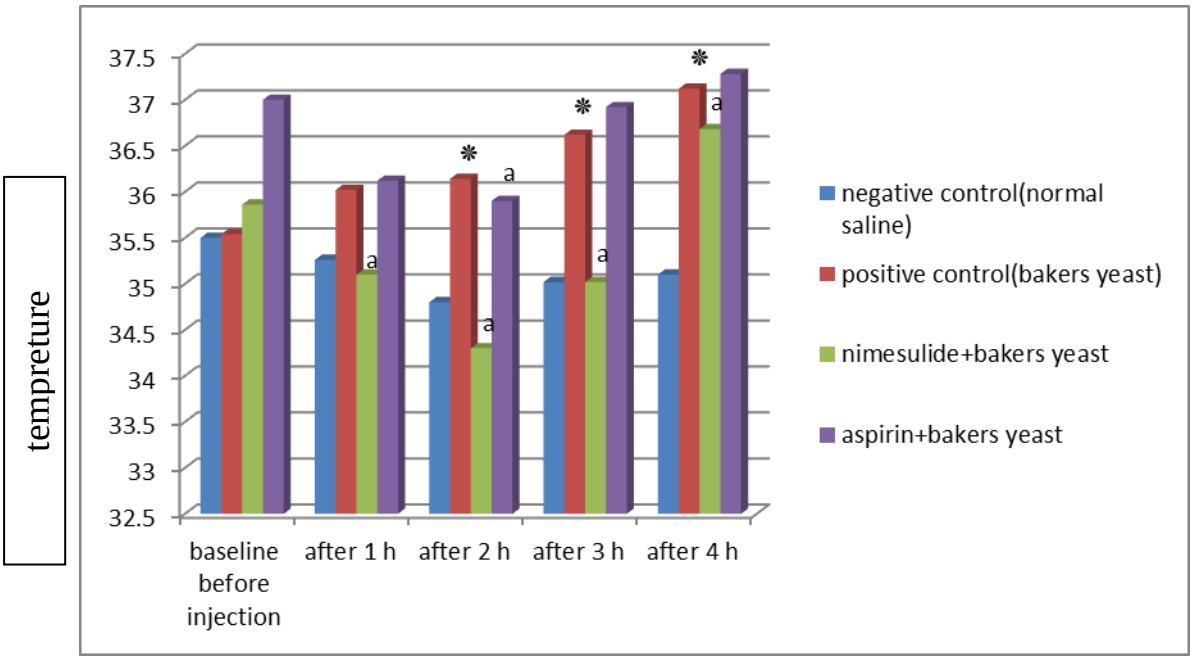


Figure 22: Comparison of antipyretic effect between nimesulide and aspirin in mice

4-8: Eighth experiment

Comparative anti-inflammatory effect between nimesulide and aspirin in mice

Injection of formalin at dose (0.02 ml /1%) in the right paw of mous in the positive control group led to induce

inflammation in the comparison with the negative control group (injected with normal saline), the second group (injected formalin) and fourth group (injected aspirin at ED $_{100}$ before 30 min from injected formalin 1%) differ significantly from the first group (injected normal saline) at time 0.5,1 and 2 h.

The results of third group which(injected nimesulide at ED_{100} before 30 min from injected formalin) were differ significantly from second group (formalin group) and fourth group (aspirin +formalin group) at time 0.5,1 and 2 h.

So nimesulide at ED_{100} 15.8 mg/kg decreased the inflammation in a good manner, unlike aspirin at ED_{100} 424.5 mg/kg at time 0.5, 1, and 2 hours after formalin injected (Table 11,Figure 23), The data also showed a reduction in the number of lifts and lickings of the paw during 30 minutes from injection of formalin which injected after 30 min from injection of nimesulide in third group and aspirin in fourth group in comparison with the positive control which injected formalin. The inhibition percentages of nimesulide and aspirin at ED_{100} in lifting and licking of a paw in mice were 58.22 and 55.40%, respectively (Table 11, Figure 23,24,25,26,27 and 28).

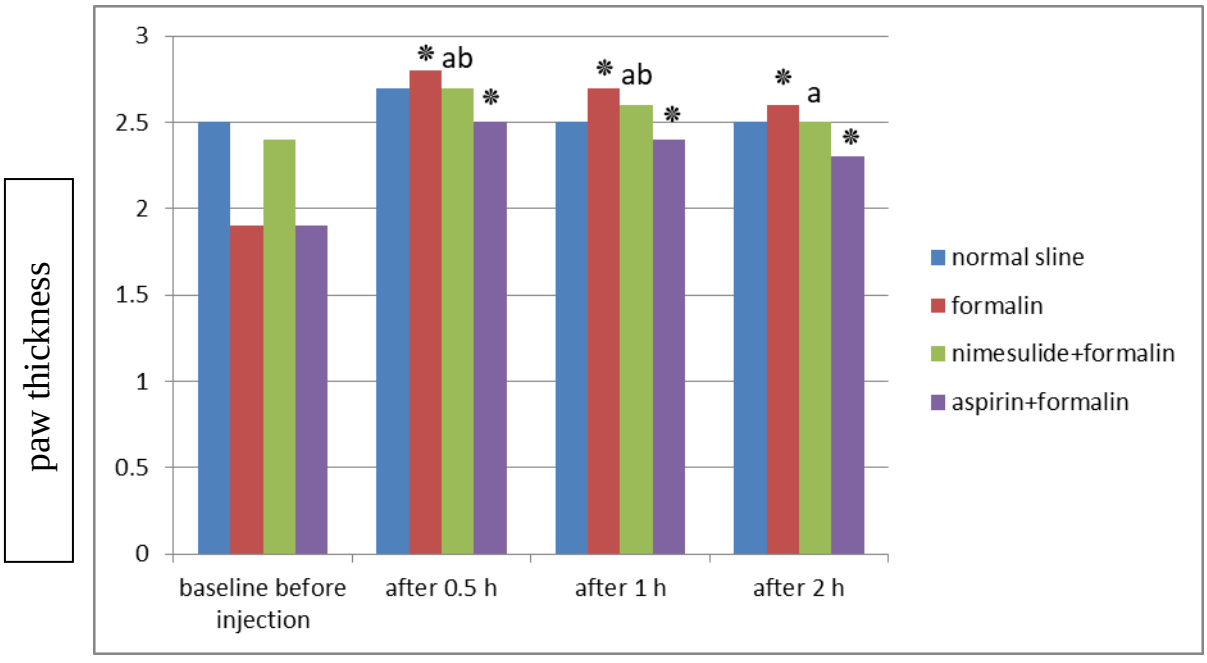


Figure 23: Comparative anti-inflammatory effect of nimesulide and aspirin in mice

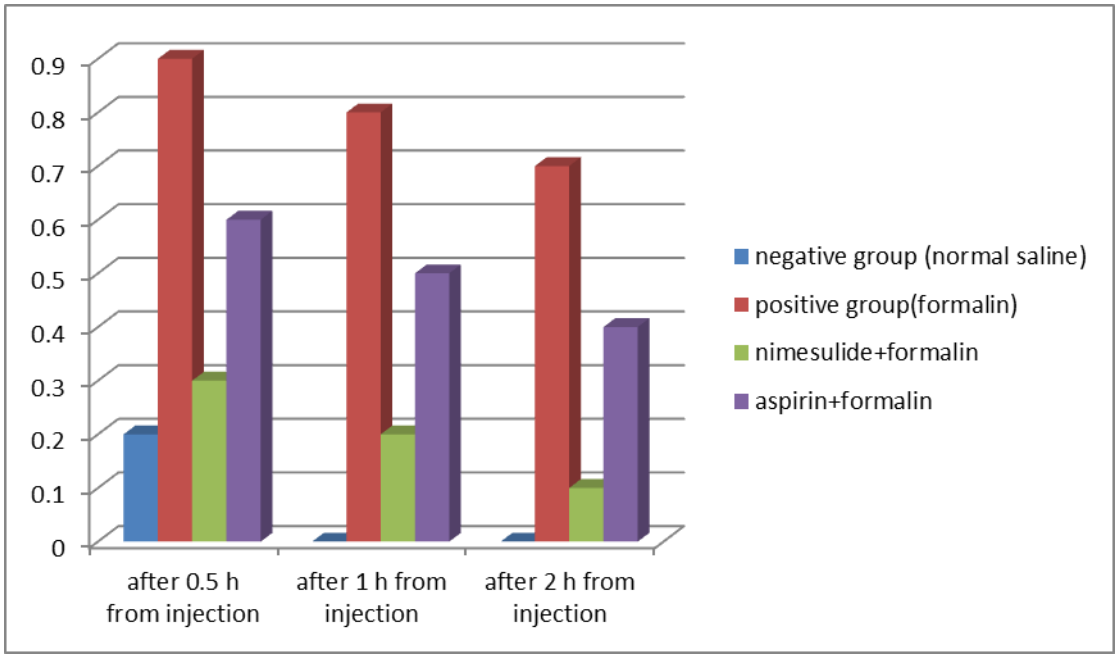


Figure 24:the difference in foot thickness (mm)

Table 11: Comparative anti-inflammatory effect of nimesulide and aspirin in mice

Groups	Paw thickness (mm)						
	Baseline	After 0.5 h	Change in thick of paw after 0.5 h	After 1 h	Change in thick of paw after 1 h	After 2 h	Change in thick of paw after 2 h

Normal saline	**2.50±0.06**	**2.70 ± 0.02**	**0.2**	**2.50 ± 0.02**	**0.0**	**2.50 ± 0.07**	**0.0**
Formalin	**1.90±0.04**	**2.80 ± 0.07***	**0.9**	**2.70 ± 0.04***	**0.8**	**2.60 ± 0.09***	**0.7**
Nimesulide + Formalin	**2.40±0.07**	**2.70 ± 0.02 ab**	**0.3**	**2.60 ± 0.05 ab**	**0.2**	**2.50 ± 0.06 a**	**0.1**
Aspirin + Formalin	**1.90±0.05**	**2.50 ± 0.05***	**0.6**	**2.40 ± 0.02***	**0.5**	**2.30 ± 0.13***	**0.4**

Numbers were as mean ± Std.E (5 mice /group) at p<0.05

*** Differ significantly from normal saline at p<0.05**

[a] Differ significantly from formalin group at p<0.05

[b] Differ significantly from group of aspirin + formalin at p<0.05

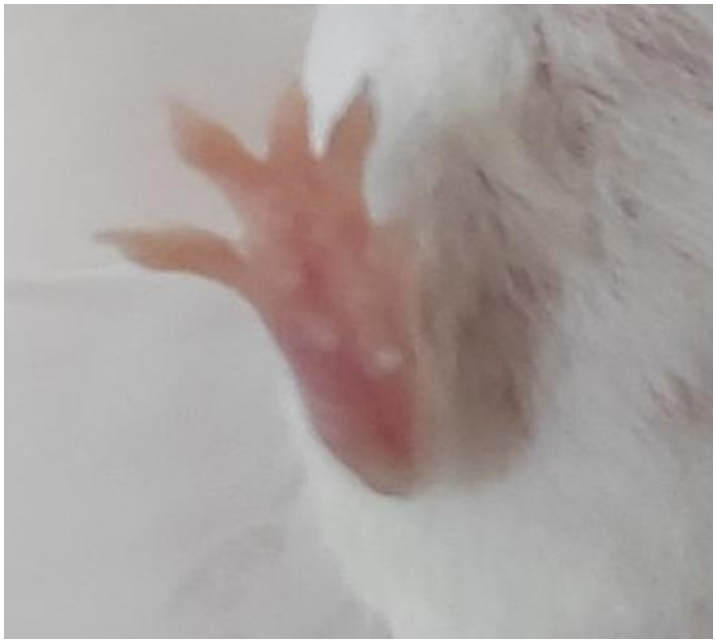


Figure 25: Normal paw of mouse before injection

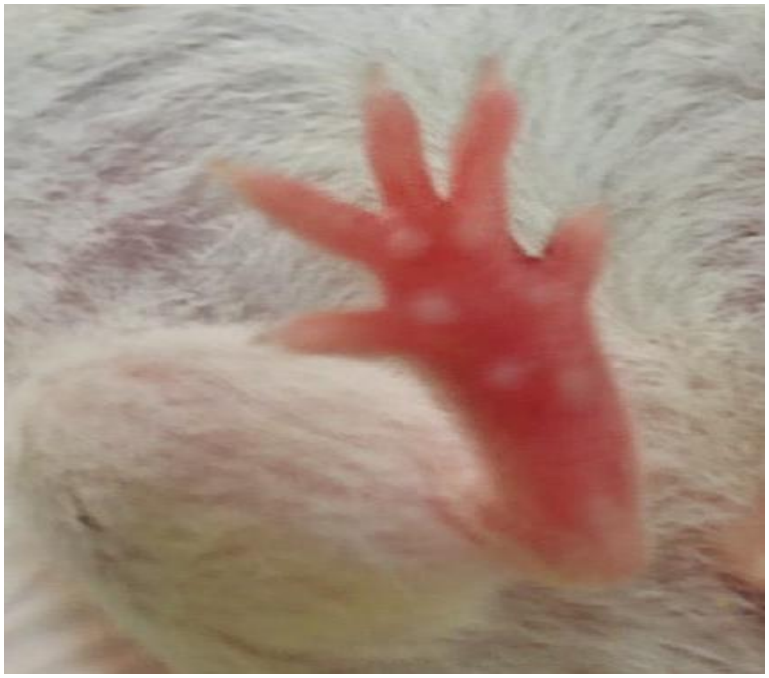


Figure 26: Paw of mouse after injection of formalin (1%)

Figure 27: Paw of mouse after injection of formalin (1%)

Table 12: Evaluation of the local analgesic effect of nimesulide and aspirin in mice

Groups	No. of lifting and licking the paw during 30 min	% of inhibition in lifting and licking the paw
Formalin	42.60±3.23	____

Nimesulide + Formalin	17.80±1.4 *	58 %
Aspirin + Formalin	19.00±2.07 *	55 %

Numbers were as mean ± Std.E (5 mice /group) at $p<0.05$

*Differe significantly from formalin group at $p<0.05$

% inhibition = N control – N treated / N control x 100

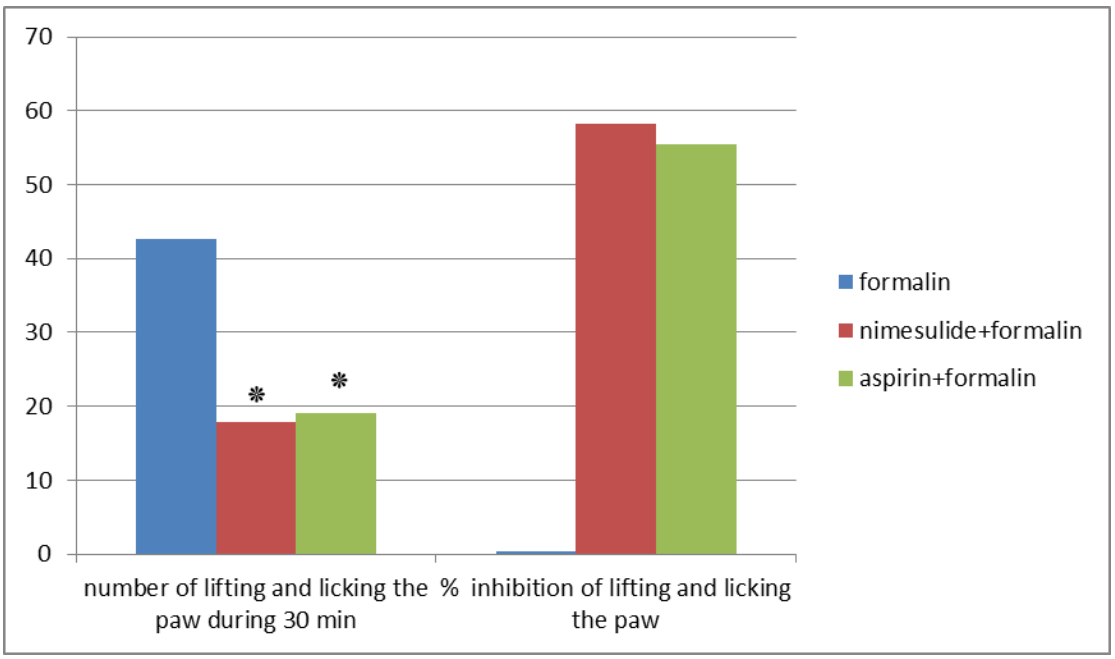


Figure 28: Evaluation of the local analgesic effect of nimesulide and aspirin in mice

4-9: Ninth experiment

Comparative anti-coagulant effect between nimesulide and aspirin in mice

Clotting time was measured by detecting prothrombin time in the control group (5 mice) injection with normal saline and injected the second and third groups injection with ED_{100} from nimesulide (15.8 mg/kg, i.m.) and aspirin (424.5 mg/kg, i.m.) respectively, for five consecutive days. Then, after 30 minutes from the injection in the five days, blood was drawn from the eye by a blue capillary tube and checked for prothrombin time (PT) every 30 s and recorded it when happened. The results in control group were 60 s for prothrombin time , while the second and third groups which injected ED_{100} from nimesulide and aspirin different significantly from control group and the results were 102 and 132 s , respectively (Table 13, Figure 28) .

Table 13: Effect anticoagulant of nimesulide and aspirin in mice

Groups	Prothrombin time (second)
Control group (normal saline)	60 ± 9.49
Nimesulide (15.8 mg/kg, i.m)	102.0 ± 15.3*
Aspirin (424.5 mg/kg, i.m)	132.0 ± 12.0 *

Numbers values as mean ± Std.E (5 mice/group)

* significantly different in comparison to the control group at $p<0.05$

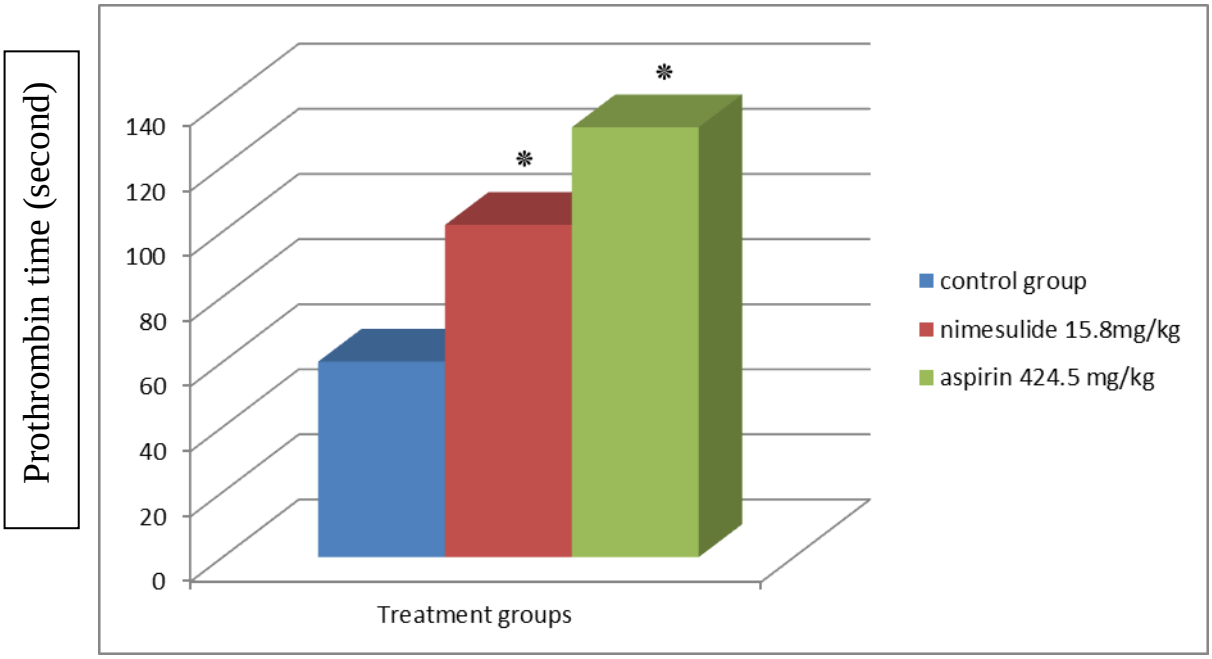


Figure 29: Anticoagulant effect of nimesulide and aspirin in mice

4-10: Tenth experiment

Detection of the nimesulide and aspirin concentration in plasma at different times using high-performance liquid chromatography (HPLC)

Nimesulide plasma concentration was measured with a device HPLC at dose 15.8 mg/kg, i.m in mice at times of 0.5, 1, 2, 4, and 24 hour after injection and the results were 14.62, 9.22, 9.88, 7.38 and 2.27 µg/ml, respectively, the highest concentration of nimesulide in plasma was after 0.5 h from injection .

Also the plasma concentration of aspirin was measured after administering a dose of 242.5 mg/kg, i.m in mice at times (0.5, 1, 2, 4, and 24 hour after injection)and the results were 4.35, 3.17, 2.54, 2.25 and 1.21 µg/ml, respectively, the highest concentration of aspirin in plasma was after 0.5 h from injection (Table 14) (Figure 29)

Table 14: The plasma concentration of individual nimesulide and aspirin in µg/ml of at different times measured

Time: Hour	Groups	
	Nimesulide	Aspirin
0.5	14.62±2.60	4.35±0.72*
1	9.22±0.91[a]	3.17±0.19*
2	9.88±0.25[a]	2.54±0.22*
4	7.38±1.43[a]	2.25±0.25*,[a]
24	2.27±0.48[a,b,c,d]	1.21±0.23[a,b,c]

Numbers represented as mean ± Std.E (3 mice/time)

*Differ significantly in comparative to the nimesulide group at $p<0.05$

[a] Differ significant from 0.5 h time in the same group at $p <0.05$

[b] Differ significant from 1 h time in the same group at $p <0.05$

[c] Differ significant from 2 h time in the same group at $p <0.05$

[d] Differ significant from 4 h time in the same group at $p <0.05$

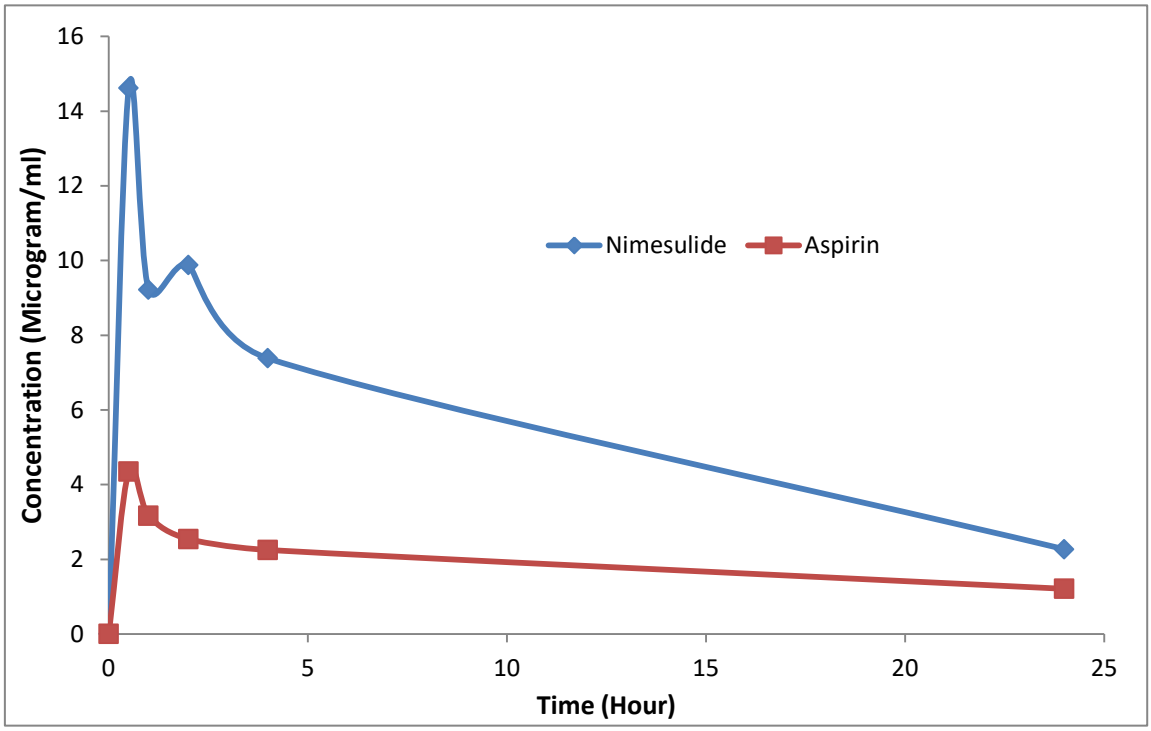


Figure 30: Comparative concentrations of nimesulide and aspirin in plasma of mice

4-11: Eleventh experiment

Comparison of nimesulide and aspirin pharmacokinetics in mice

Pharmacokinetic parameter for single dose of nimesulide (15.8 mg/kg,i.m) were ($AUC_{0-\infty}$ 169.18, $AUMC_{0-\infty}$ 2358.72, K_{el} 0.06, C_{max} 14.62, T_{max}0.5 , $t_{1/2\beta}$ 11.07, MRT 13.94, V_{ss} 1.49, and Cl 0.09 , while pharmacokinetic parameters for single dose of aspirin (424.5 mg/kg,i.m) were AUC 82.31, AUMC 2428.32, K_{CL} 0.03, C_{max} 4.35, T_{max} 0.5, $t_{1/2\beta}$ 21.25, V_{ss} 158.12, Cl 5.16 (Table 15).

Table 15: The pharmacokinetic variables of nimesulide and aspirin in mice

Variables	Groups	
	Nimesulide	Aspirin
$AUC_{0-\infty}$ (µg×h/ml)	169.18	82.31
$AUMC_{0-\infty}$ (µg×h^2/ml)	2358.72	2428.32
MRT (h)	13.94	29.50
$t_{1/2\beta}$ (h)	11.07	21.25
T_{max} (h)	0.5	0.5

C_{max} (µg)	14.62	4.35
Kel (h-1)	0.06	0.03
V_{ss} (L /kg)	1.49	158.12
Cl (L /h/kg)	0.09	5.16

Nimesulide was injected at 15.8 mg/kg, i.m. and aspirin at 424.5 mg/kg, i.m. in mice

Variables gained from non-compartment model of pharmacokinetic measurement by using PKSolver program built-in Microsoft office Excel

4-12: Twelfth experiment

A comparative inhibition of nimesulide and aspirin COX-2 concentration in plasma, liver and kidney of mice

Injection of nimesulide and aspirin individually at a dose 15.8, 424.5 mg/kg, i.m respectively, for a period of five consecutive days led to decrease in concentration of cyclooxygenase enzyme COX-2 in plasma , liver and kidney in mice .

The concentration of COX-2 in plasma of the negative control group was 753.59 pg/ml, while nimesulide and aspirin treated groups (at 15.8 and 424.5 mg/kg, i.m., respectively), decreased the COX-2 concentration to 680.44 and 688.43 pg/ml, respectively. The COX-2 concentration in the positive control group (1%, acetic acid) was 772.796 pg/ml while the group injected with nimesulide after 30 minute of acetic acid injection was significantly decreased COX-2 concentration to 588.59 pg/ml and the group of aspirin+acetic acid to 594.43 pg/ml.

The COX-2 concentration of the liver in the control group was 954.12 pg/ml, nimesulide and aspirin treated group decreased the enzyme activity to 922.06 and 931.10 pg/ml respectively. The positive control group was 980.23 pg/ml, while treated groups with nimesulide+ acetic acid and aspirin+acetic acid decrease concentration COX-2 to 905.05 and 924.57 pg/ml respectively.

The COX-2 concentration of the kidney in negative control group was 1028.22 pg/ml, treated group with nimesulide and aspirin decreased to 926.32 and 926.31 pg/ml, respectively. The positive control was increase the concentration of COX-2

to 1039.01 pg/ml, while treated groups with nimesulide+ acetic acid and aspirin+ acetic acid decreased significantly the concentration of COX-2 to 902.37 and 917.81 respectively (Table 16,Figures 31,32,33).

Table 16: COX-2 concentration in mice and inhibition it by nimesulide and aspirin administration

Groups	COX-2 concentration (pg/ml)		
	Plasma	Liver	Kidney
Non-inducible Mice			
Negative control-Normal saline	753.59 ± 88.01	954.12 ± 80.30	1028.22 ± 5058
Nimesulide+ NS	680.44 ± 68.88	922.06 ± 33.28	926.32 ± 92.15
Aspirin+ NS	688.43 ± 80.71	931.10 ± 56.71	926.31 ± 59.85
Inducible Mice			
Positive control-Acetic acid (AA)	772.80 ± 75.81	980.23 ± 48.77	1039.01 ± 43.18
Nimesulide+AA	588.59 ± 43.85*	905.05 ± 39.04	902.37 ± 39.94*
Aspirin+AA	594.43 ± 55.93*	924.57 ± 40.73	917.81 ± 38.97*

Numbers values as mean ± Std.E (5 mice/group)

* significantly difference in comparative to the positive control (acetic acid) group at $p<0.05$

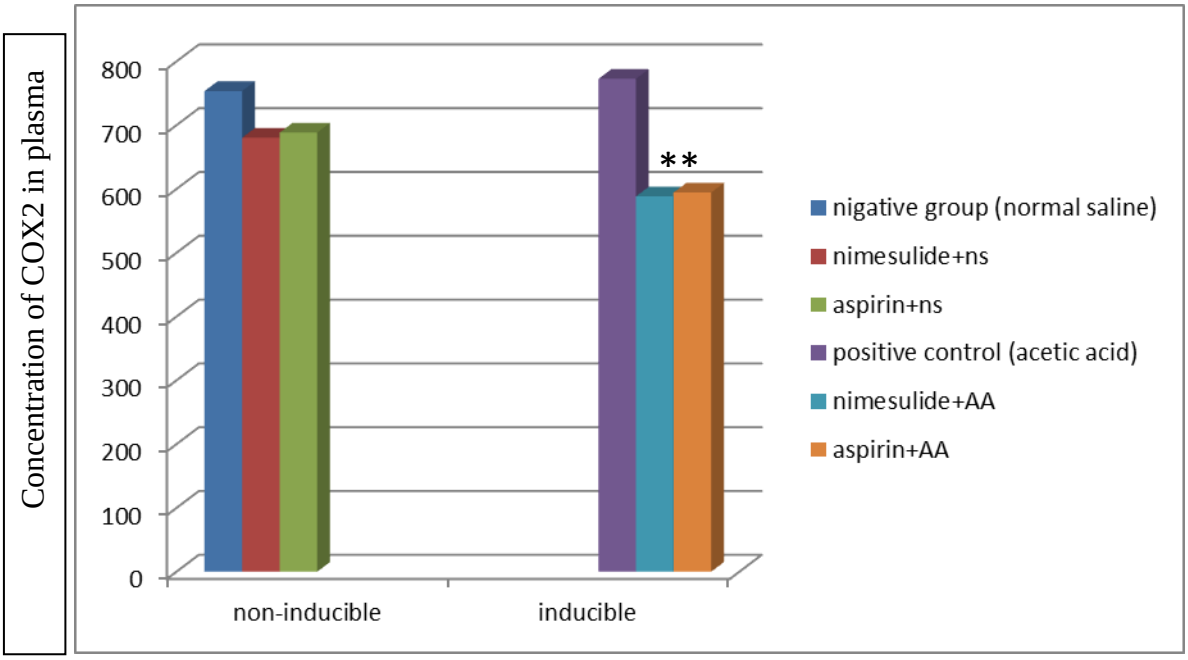


Figure 31: COX-2 concentration in plasma and inhibition it by nimesulide and aspirin administration in mice

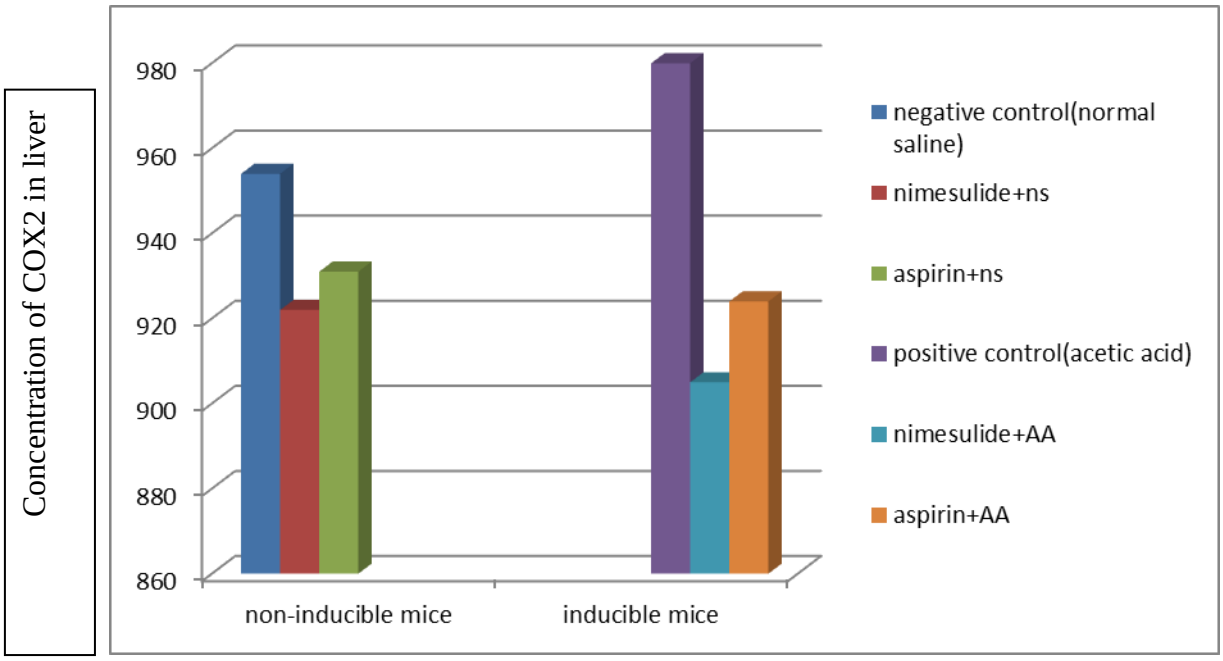


Figure 32 :COX-2 concentration in liver and inhibition it by nimesulide and aspirin administration in mice

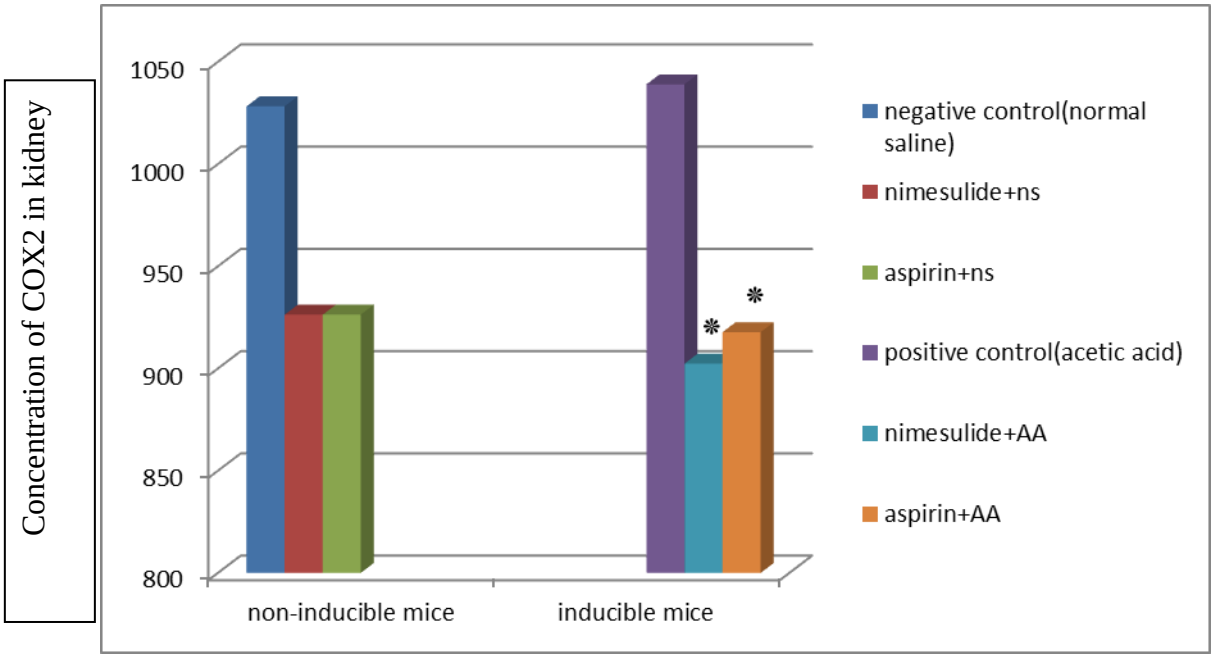


Figure 33:COX-2 concentration in kidney and inhibition it by nimesulide and aspirin administration in mice

4-13: Thirteenth experiment

Effect of Nimesulide and Aspirin on apoptosis in mice

Injection of nimesulide and aspirin individually at a dose 15.8, 424.5 mg/kg, i.m respectively, for a period of 5 consecutive days caused reduce in concentration of caspase-3 in plasma , liver and kidney in mice

The concentration of caspase-3 in plasma of the negative control group was 3.93 pg/ml, while nimesulide and aspirin treated groups (at 15.8 and 424.5 mg/kg, i.m., respectively), decreased the caspase-3 concentration to 3.56 and 3.80 pg/ml, respectively. The caspase-3 concentration in the positive control group (1%, acetic acid) was 4.37 pg/ml while the group injected with nimesulide after 30 minute of acetic acid injection was significantly decreased caspase-3 to 3.41 pg/ml and the group of aspirin+acetic acid to 3.78 pg/ml.

The caspase-3 concentration of the liver in the control group was 3.7 pg/ml, nimesulide and aspirin treated groups decreased the caspase-3 to 2.34 and 3.43 pg/ml respectively. The positive control group caspase-3 concentration was 4.04 pg/ml, while treated groups with nimesulide+acetic acid and aspirin+acetic acid decreased concentration of caspase-3 to 2.37 and 2.48 pg/ml respectively.

The caspase-3 concentration of the kidney in negative control group was 2.83 pg/ml, treated groups with nimesulide and aspirin decreased caspase-3 to 2.65 and 2.76 pg/ml, respectively. The positive control was increased the concentration of caspase-3 to 3.43 pg/ml, while treated groups with nimesulide+acetic acid and aspirin+acetic acid decreased the concentration of caspase-3 to 2.45 and 2.58 respectively (Table 17, Figures 34,35 and 36).

Nimesulide and aspirin inhibited significantly caspase-3 in kidney, liver and plasma in comparison with the control groups of normal saline (negative group) and positive group acetic acid of mice and these results expose the effect of nimesulide and aspirin on the apoptosis, because when inhibited caspase-3 lead to inhibited of apoptosis and nimesulide had more efficacy from aspirin in inhibition of caspase 3 at dose of ED_{100}.

Table 17: Effect of nimesulide and aspirin on the caspase-3 in kidney, liver and plasma in mice

Groups	Concentration of caspase-3(pg/ml) in kidney	Concentration of caspase-3(pg/ml) in liver	Concentration of caspase-3(pg/ml) in plasma
Non-inducible Mice			
Negative control normal saline(NS)	2.83 ± 0.15	3.7 ± 0.46	3.93 ± 0.80
Nimesulide + NS	2.65 ± 0.17	2.34 ± 0.27*	3.56 ±0.19

Aspirin + NS	2.76 ± 0.32	3.43 ± 0.20[a]	3.80 ± 0.25
Inducible Mice			
Positive control Acetic acid (AA)	3.43 ± 0.18	4.04 ± 0. 23	4.37 ± 0.25
Nimesulide + AA	2.45 ± 0.13[+]	2.37 ± 0.36[+]	3.41 ± 0.36[+]
Aspirin + AA	2.58 ± 0.20[+]	2.48 ± 0.17[+]	3.78 ± 0.18

Numbers were as mean ± Std.E (5 mice /group)

[*]Differ significantly from the normal saline group $p<0.05$

[+]Differ significantly from the acetic acid group $p<0.05$

[a]Differ significantly from the nimesulide group $p<0.05$

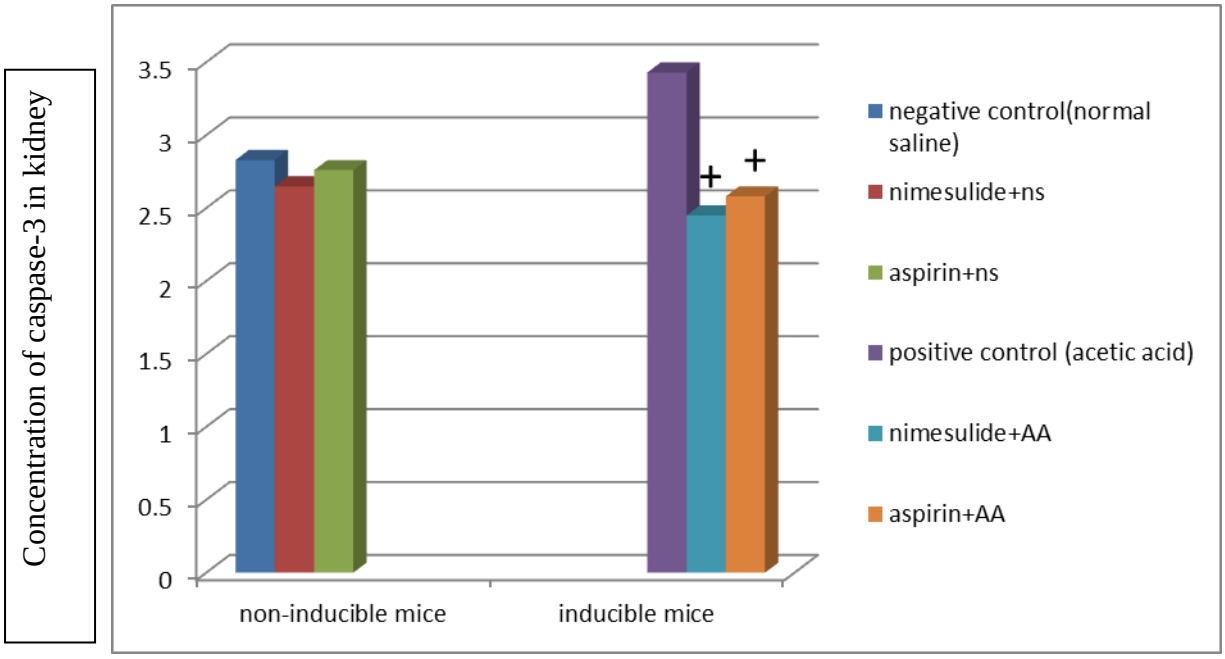


Figure 34: Effect of nimesulide and aspirin on the caspase-3 in kidney in mice

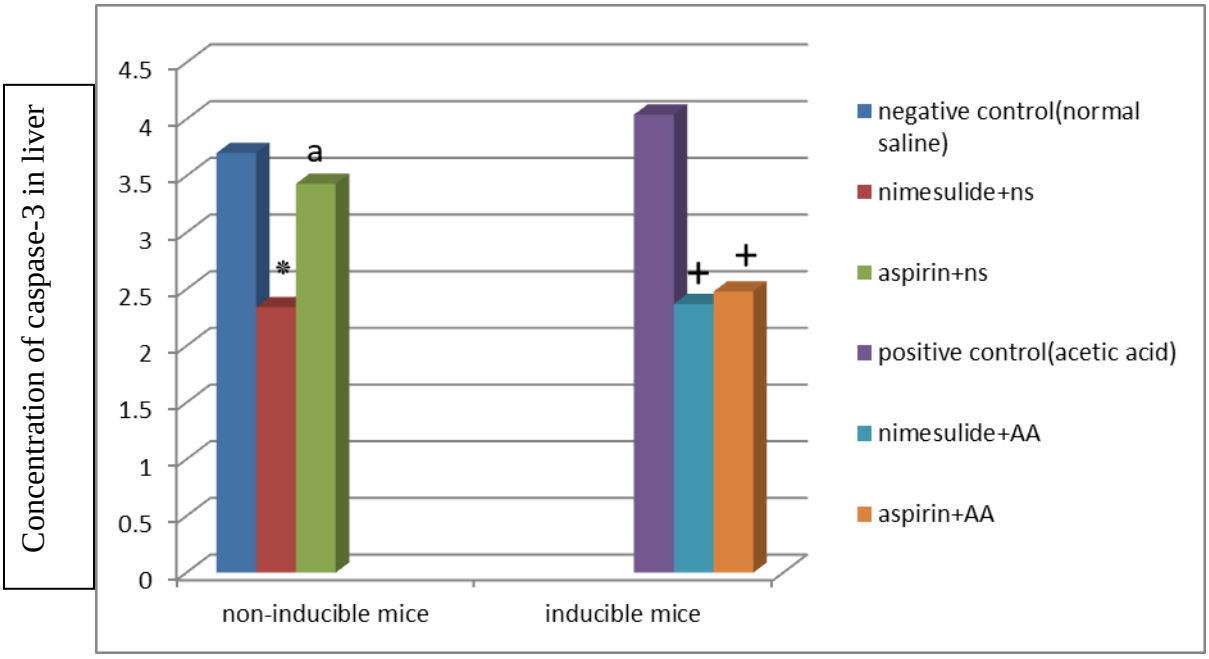


Figure 35: Effect of nimesulide and aspirin on the caspase-3 in liver in mice

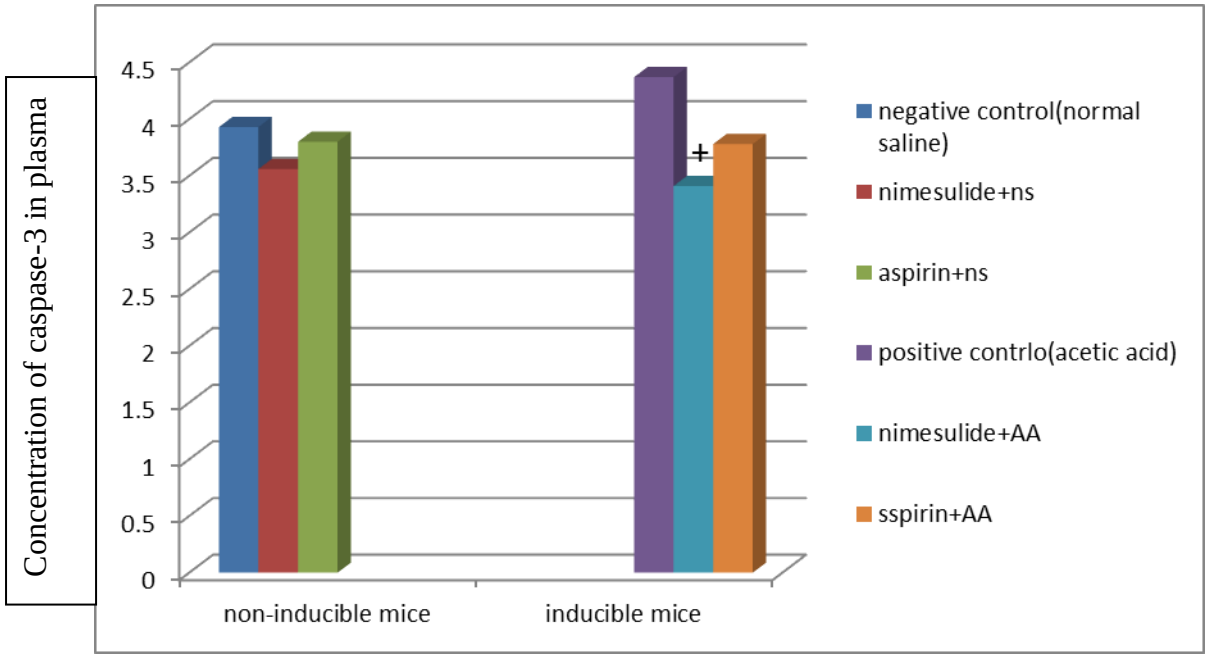


Figure 36: Effect of nimesulide and aspirin on the caspase-3 in plasma in mice

4-14: Fourteenth experiment

Effect of Nimesulide and Aspirin on PPAR-α in mice

Administration of nimesulide and aspirin individually at a dose 15.8, 424.5 mg/kg, i.m respectively for a period of 5 consecutive days reason decreased in concentration of PPAR-α in plasma , liver and kidney in mice

The concentration of PPAR-α in plasma of the negative control group was 18.67 pg/ml, while nimesulide and aspirin treated groups (at doses 15.8 and 424.5

mg/kg, i.m., respectively), significantly decreased the PPAR-α concentration to 9.99 and 9.71 pg/ml, respectively. The PPAR-α concentration in the positive control group (injected 1%, acetic acid) was 16.05 pg/ml while the groups which injected with nimesulide and aspirin after 30 minute of acetic acid injection was significantly decreased PPAR-α to 5.87 and 5.12 pg /ml respectively

The PPAR-α concentration of the liver in the control group was 365.21 pg/ml, nimesulide and aspirin treated groups significantly decreased the PPAR-α to 361.02 and 360.85 pg/ml, respectively the positive control group PPAR-α concentration was 363.11 pg/ml, while treated groups with nimesulide+ acetic acid and aspirin+acetic acid decreased concentration of PPAR-α to 352.34 and 356.43 pg/ml respectively.

The PPAR-α concentration of the kidney in the negative control group was 376.67 pg/ml, treated groups with nimesulide and aspirin decreased PPAR-α to 353 and 346.17 pg/ml, respectively. The concentration of PPAR-α in the positive control group was 349.89 pg/ml, while treated groups with nimesulide+acetic acid and aspirin+acetic acid significantly decreased the concentration of PPAR-α to 312.58 and 315.86 respectively.

Effect of nimesulide and aspirin on the PPAR-α –mediated induction of COX-2 expression was inhibited through therapeutic concentrations ED_{100} of nimesulide and aspirin which injected i.m. in mice and detected it in kidney, liver, and plasma via ELISA kit, nimesulide and aspirin act as ligand dependent receptor which resulted in suppression of PPAR-α (dependent transactivation of target genes COX-2), (Table 18, Figures 37,38 and 39).

Table 18: Effects of nimesulide and aspirin on the PPAR-α in kidney, liver, and plasma in mice

Treatment groups	Concentration PPAR-α (pg/ml) in kidney	Concentration PPAR-α (pg/ml) in liver	Concentration PPAR-α(pg/ml) in plasma
Non-inducible Mice			
Negative control (NS)	376.67 ± 27.19	365.21 ± 20.08	18.67 ± 4.74
Nimesulide + NS	353.00 ± 26.71	361.02± 23.91*	9.99 ± 1.50*
Aspirin + NS	346.17 ± 3.83	360.85 ± 8.56*	9.71± 1.30*
Inducible Mice			
Positive control (AA)	349.89 ± 25.29	363.11 ± 24.73	16.05 ± 2.61
Nimesulide + AA	312.58 ± 4.90+	352.24 ± 11.91	5.87± 0.33 +
Aspirin + AA	315.86 ± 23.85+	356.43 ± 20.41	5.12 ± 0.97 +

Numbers were as mean ± Std.E (5 mice /group)

* Differ significantly from the normal saline group at $p<0.05$

+ Differ significantly from the acetic acid group at $p<0.05$

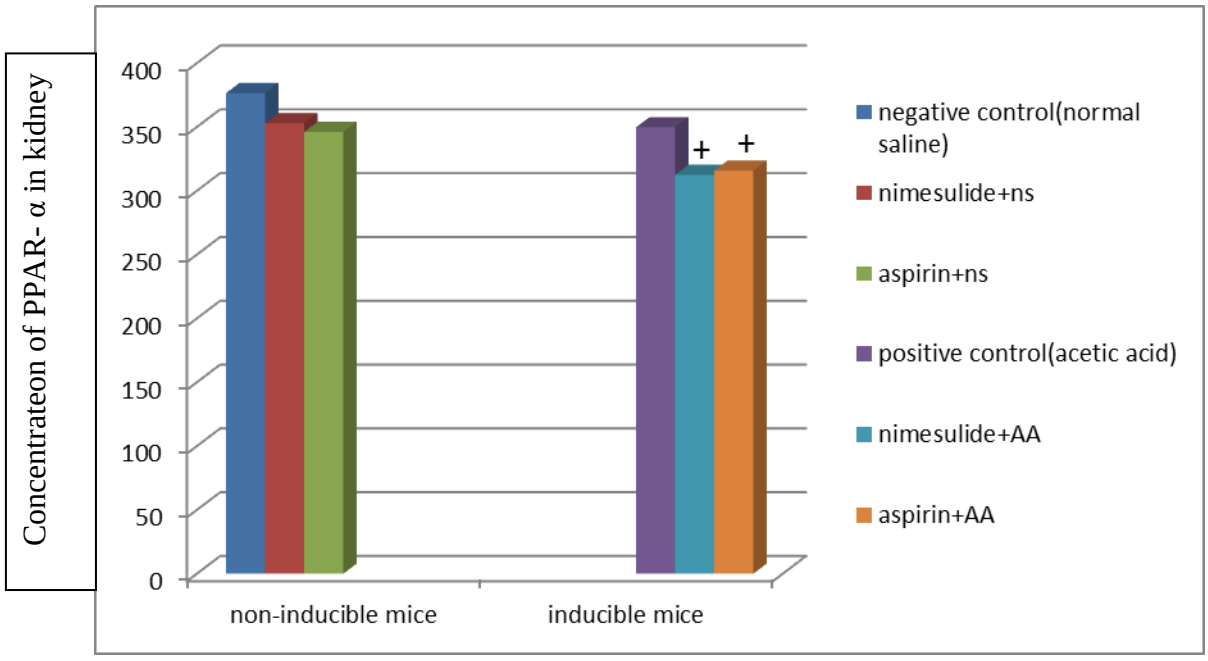


Figure 37: Effects of nimesulide and aspirin on the PPAR-α in kidney in mice

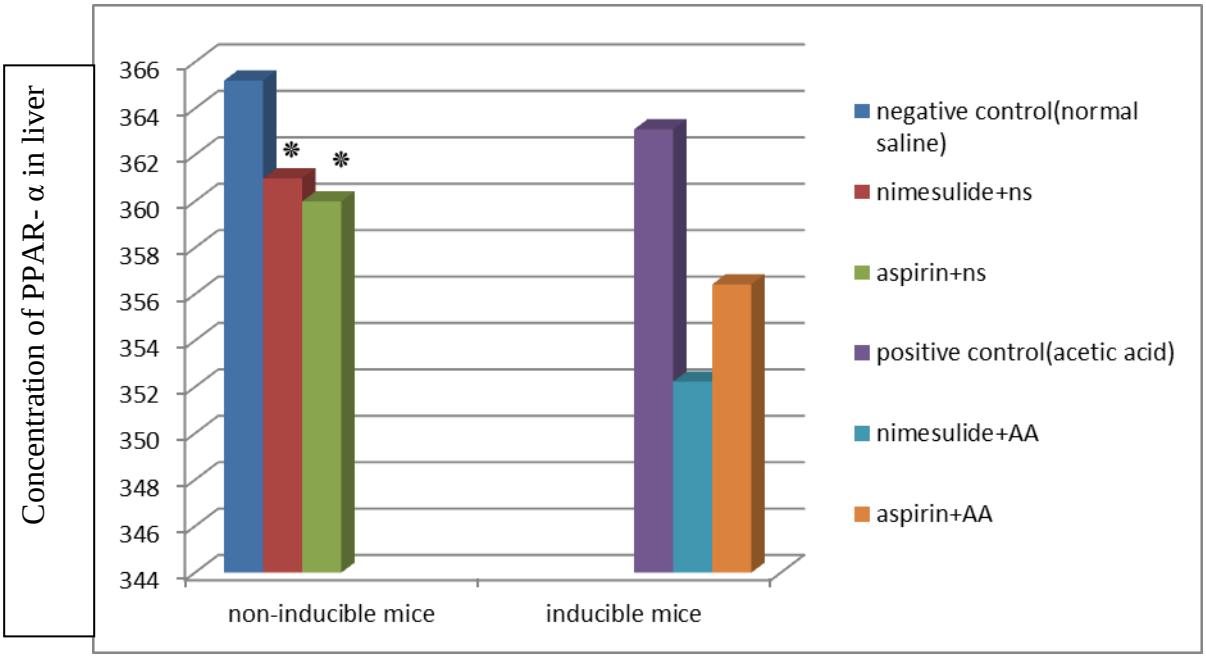


Figure 38: Effects of nimesulide and aspirin on the PPAR-α in liver in mice

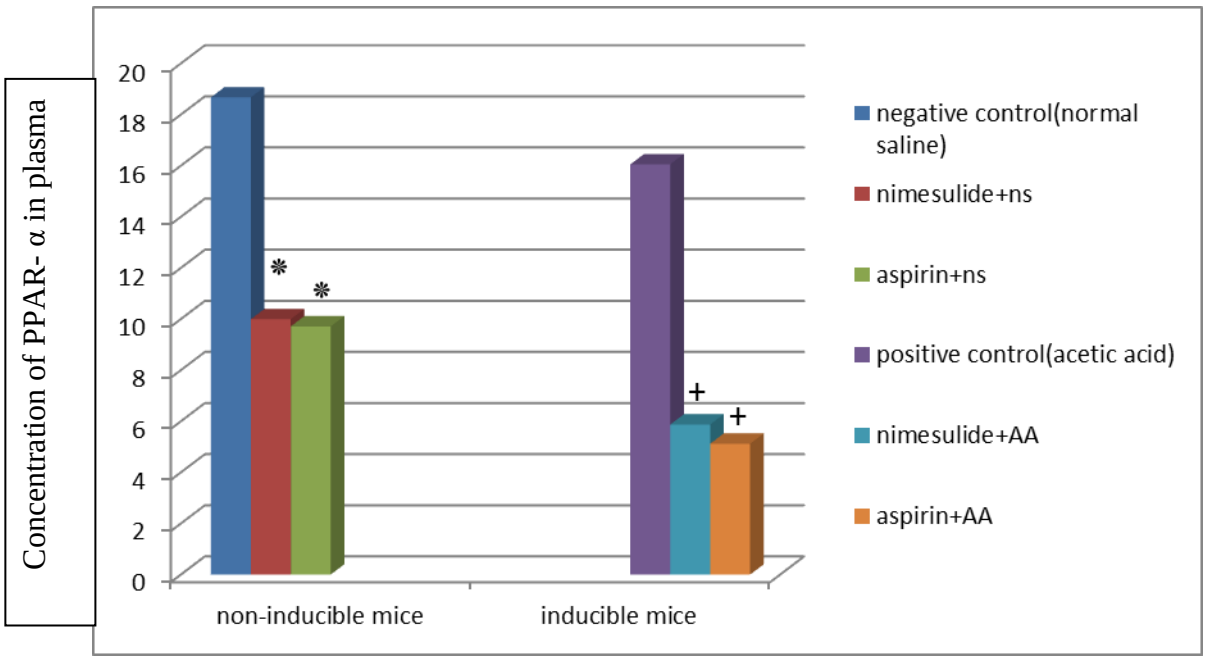


Figure 39: Effects of nimesulide and aspirin on the PPAR-α in plasma in mice

4-15: Fifteenth experiment

Comparative effects of nimesulide and Aspirin on the level of COX-2 Gene expression in mice

Consecutive administration of nimesulide and aspirin individually at a dose 15.8, 424.5 mg/kg i.m respectively for a period of 5 consecutive days led to decrease in COX-2 gene expression in kidney tissue of mice.

The gene expression of COX-2 in kidney tissue of the negative control group was 417 ng/µl, while nimesulide and aspirin treated groups (at doses 15.8 and 424.5 mg/kg, i.m., respectively), decreased the COX-2 gene expression to 412 and 410 ng/µl , respectively. The COX-2 gene expression in the positive control group (1%, acetic acid-AA, i.p) was 468 ng/µl while the groups which injected with nimesulide and aspirin after 30 minute of AA injection was decreased COX-2 gene expression to 390 and 400 ng/µl respectively.

Nimesulide and aspirin were suppressed the COX-2 gene expression through reducing the gene expression of COX-2 in comparative with positive and negative control group after treated the mice for 5 days (one dose daily) at ED_{100} for each drug , but nimesulide outperform the aspirin in decreasing COX-2 gene expression for positive groups which (induced inflammation with acetic acid), therefore nimesulide and aspirin in therapeutic doses decreased the COX-2 gene expression in kidney and caused down-regulation in gene expression (Table 19, Figures 40,41 and 42) .

Table 19: Effects of nimesulide and aspirin on COX-2 gene concentration in kidney tissue of mice

Trearment Groups	COX-2 gene concentration in kidney tissue (ng/µl)
Non-inducible mice	
Negative control-Normal saline (NS)	417 ± 30.90
Nimesulide	412 ± 3.61
Aspirin	410 ± 14.73
Inducible mice	
Positive control-Acetic acid (AA)	468 ± 48.12
Nimesulide + AA	390 ± 22.50*
Aspirin + AA	400 ± 4.04

Numbers were as mean ± Std.E (5 mice /group)

* Differ significantly from acetic acid group

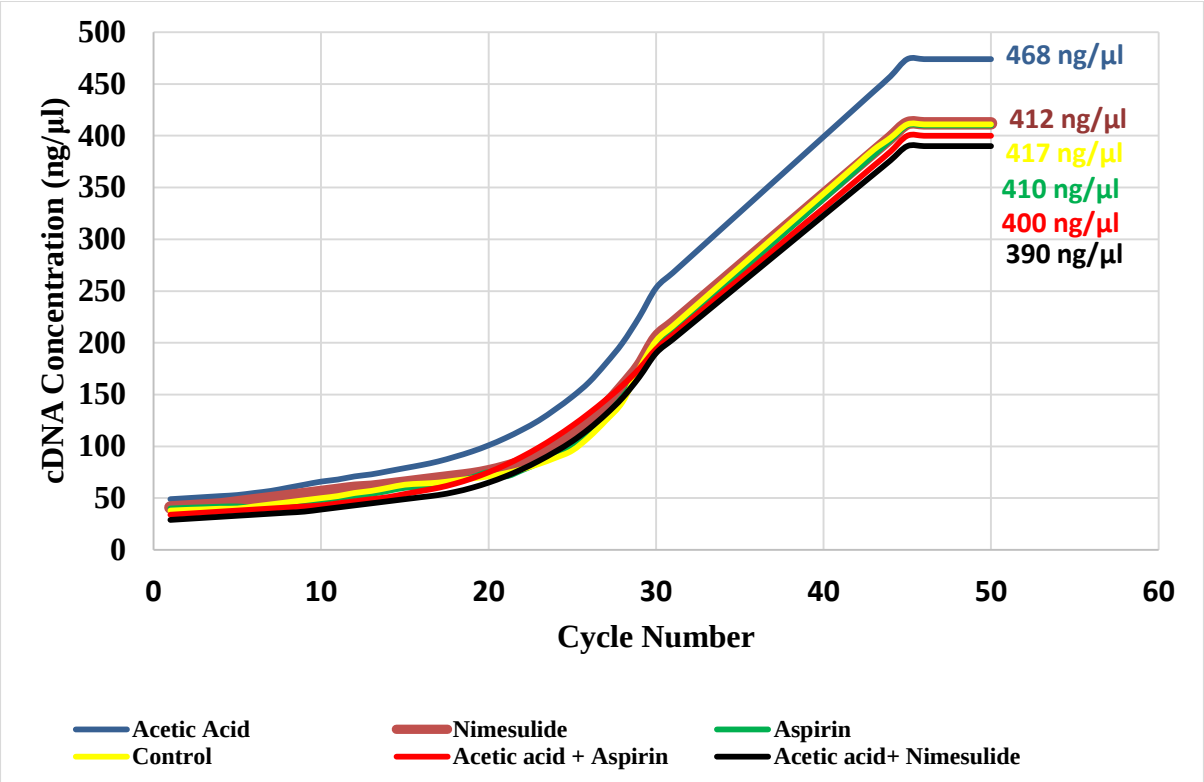


Figure 40: Comparison of COX-2 gene expressions using quantitative RT-PCR

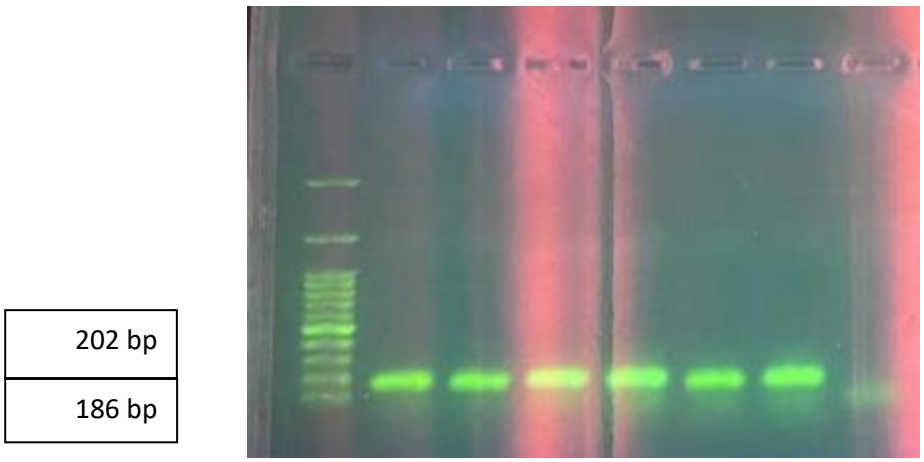
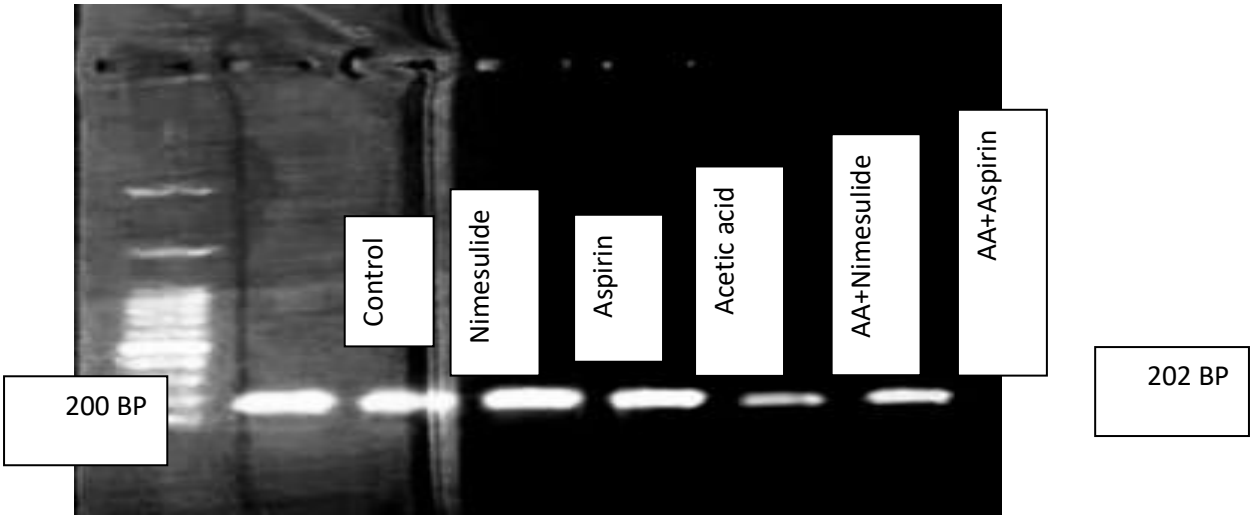


Figure (41): Positive β-actin located in lanes 1-2 (186 bp), positive COX-2 gene (202 bp)located in lanes 3-6, whereas negative controls are in lane 7. Lane M: 100 bp DNA ladder (Addbio company, Korea).



Figure(41): Kidney tissue COX-2 cDNA RT-PCR product electrophoresis

Chapter Five

Discussion

The present study aims to compare the pharmacological and molecular effects of nimesulide (selective) and aspirin (non-selective) COX_2 inhibitors in mice.

It focuses on the analgesic, antipyretic, and anti-inflammatory effects of nimesulide and aspirin and the degree of their drug safety in mice. In addition, a comparison between the two drugs at the level of pharmacokinetics indicate which is better in terms of future use in other animals, besides the molecular comparative activity on the level of caspase-3, peroxisome proliferator–activated receptor (PPAR-α), and COX_2 gene expression in mice. Also, our study aims to compare the degree of inhibition between nimesulide and aspirin regarding COX_2 concentration in plasma, liver, and kidney in mice.

The comparison between nimesulide and aspirin and their effect on apoptosis (by measuring caspase-3 activity in plasma, liver, and kidney) was applied here. Furthermore, the molecular mechanisms of nimesulide and aspirin to produce their

pharmacological effects through measuring the PPAR-α and COX_2 gene expression were also included.

Nimesulide, a non-steroidal anti-inflammatory drug (NSAID), has analgesic, antipyretic, and anti-inflammatory effects. It is a preferential COX_2 inhibitor (Patrignani *et al*., 1997; Lal *et al*., 2000; Balaji *et al*., 2013; Caiazzo *et al*., 2019). It acts via inhibited the production of prostaglandins produced via arachidonic acid by inhibit the COX_2, which leads to a decrease in pain, fever, and inflammation (Samad *et al*., 2002; Caiazzo *et al*., 2019).

Aspirin, on the other side, is a prototype of NSAIDs that has analgesic, antipyretic, and anti-inflammatory effects; it is a non-selective COX_2 inhibitor, and also acts as an anticoagulant. This therapeutic effect of aspirin results from its ability to inhibit both COX_1 and COX_2 (Cashman, 1996; Vane and Botting, 2003; Kunal *et al*., 2015**).**

In our study, the analgesic median effective dose (ED_{50}) was determined by using a hot plate (thermal methods to evaluate the analgesic efficacy of drugs in lab animals) for nimesulide (7.9 mg/kg, IM) which is less than aspirin (212.23 mg/kg, IM). So, the analgesic effect produced by nimesulide was in a small dose compared to aspirin. This finding was in accordance with precedent research in mice and rats (Pong *et al*., 1985; Gupta *et al*., 2000).

Nimesulide showed the ability to affect various mediators and intracellular pathways, and it has a unique multi-mechanism of action that makes it different from other NSAIDs, the mechanism of analgesia of nimesulide occurred by decreased the biosynthesis and accumulation of prostaglandins (PGs) by inhibition of the COX_2 which increased the pain threshold through their effects, especially on inducible COX_2. Nimesulide has another mechanism that induces its anti-nociceptive effect by inhibition of production and release of histamine, activation of nitric oxide synthesis, cytokine release, substance P production, and release (Kress *et al*., 2015). NSAIDs has been attributed to the peripheral inhibition of prostaglandin synthesis via the blockade of the enzyme cyclo-oxygenase and prevention of bradykinin-and cytokine-induced hyperalgesia via inhibition of tumor necrosis factor-alpha (TNF-α) release (Ferreira, 1993). However, it is becoming increasingly evident that NSAIDs exert their analgesic effect through a variety of other peripheral and central mechanisms (Cashman, 1996, Tassorelli *et*

al., 2003). The recent localization in the brain of the inducible cyclo-oxygenase isoform (cyclo-oxygenase-2) (Yamagata et al., 1993) provides an additional central mechanism of action. Cyclo-oxygenase-2, together with nitric oxide (NO), is likely to play an important role in chronic pain states, which are mediated by a constellation of changes, collectively termed as central sensitization (Woolf, 1983). Other research mentions that nimesulide decrease the release of histamine, which is produced by mastoid cells. This medicine also has a scavenger effect on hypochloric acid (Bocanegra *et al*., 2005).

Assessment of the acute median lethal dose (LD_{50}) of nimesulide in a previous study (1500 mg/kg in mice), while in our study, the LD_{50} in mice was 181.7 mg/kg, IM represented with rapid breathing, increased movement, instability, shivering, limping, paralysis and death during 24 h while for aspirin, the oral toxicity of aspirin in humans, dogs, cats, and albino rats varies in doses of 1000 mg and 920 mg in rats (Boyd, 1959; Gupta *et al*., 2000) and 1100 mg/kg in other research in mice (Thompson and Klotz, 1985), 1190 mg in guinea pigs, 1100 mg in mice and in dog and cat was 1000 mg (Boyd, 1959; Boyd, 1960), while in our study the LD_{50} in mice was 1210.3 mg/kg, IM with toxic signs represented by rapid breathing, increased movement, instability, shiver, limping, abdominal cramps then death after 1 h, other animal showed the same signs and after 1 h appeared calm and motionless. The reason for different results in our research related to LD_{50} of nimesulide and aspirin in comparison to another previous study may be a return to species and individual variation, drug manufacturing, and the route of administration.

The degree of safety of the drugs has been determined to be 23 for nimesulide and 6 for aspirin; this refers to the degree of safety for nimesulide being higher than aspirin. The reason for that is due to the large therapeutic window between the therapeutic dose and the toxic dose of the nimesulide.

In this study, the best time for pain relief (peak of time) was determined after injecting ED_{100} of individual nimesulide and aspirin at different times (15, 30, 60, 120 min) to elucidate the time-response relationship, and via using the hot plate (thermal method). The maximal time (Tmax) to produce better analgesia was 30 min after injection of both drugs. In a previous study, the Tmax of nimesulide was at 60 min in mice which approach to our results (Gupta *et al*., 1998). Another study

mentioned the time at 15 min in humans as the maximal time for producing analgesia caused by nimesulide (Bocanegra *et al*., 2005). On the other side, the best time of aspirin analgesia was determined in mice at 30 min which is closer to another study that found the time 20 min (500 mg/kg in humans) is the maximal time for producing analgesia (Cooper and Voelker, 2012). The difference in times may associated to variations in species, dose, and the route of administration.

When different multiple doses of nimesulide and aspirin (ED_{25}, ED_{50}, and ED_{100}) were administered for both drugs alone, the dose-response relationship concerning the analgesic effect was in a dependent manner. The results proved that the increased in the doses led to an increase in analgesia, which is similar to previous findings (Derry and Moore, 2012; Saghaei *et al*., 2012).

At the level of visceral pain which stimulated through acetic acid, the nimesulide and aspirin lowering the contractions of the abdomen (decreasing the writhing reflex). Nimesulide was superior to aspirin in reducing the number of writhing reflexes by 66% in comparison to aspirin (46%) and this results approach to previous studies proved that nimesulide outperformed diclofenac (non-selective COX_2 inhibitors like aspirin) in the analgesic effect at assayed writhing tests in mice. Also, other studies in mice and rats mentioned that nimesulide decreased the writhing reflex (Gupta *et al*., 1998; Ahmed *et al*., 2010) and aspirin decreased the writhing number in mice, which was stimulated via acetic acid with a percentage of 51% at a dose of 100 mg/kg (Chutha *et al*., 2008). A model of the visceral pain in mice through injection of acetic acid intraperitoneal (writhing test) was used in previous experiments with NSAIDs (nimesulide, ibuprofen, and celecoxib), which all were given orally. Simultaneous administration of guaifenesin with ibuprofen, nimesulide or celecoxib produced significant anti-nociceptive effects of visceral pain (Sliva *et al*., 2009), and this effect of nimesulide and aspirin on visceral analgesia returned to the roles of prostaglandins which are arachidonic acid-derived in their actions on afferent nociceptors and that prostaglandins were inhibited by nimesulide and aspirin. The administration of nimesulide and aspirin suppresses the PGG_2 and subsequently, PGH_2 as well as PGE_2, PGD_2, PGI_2, and TXA_2 which play roles in the peripheral somatosensory system located in skin, muscle, tendons, and visceral organs (Jang *et al*., 2020), this explains how

nimesulide and aspirin decreased the writhing reflex in mice as showed in our findings.

The antipyretic effect of nimesulide was significantly more efficient than aspirin in decreasing the fever induced by baker's yeast (135 mg/kg, i.p.) in overall measured times after injection (at 1, 2, 3, and 4 h) while aspirin has a good and significant antipyretic action after 2 h of baker's yeast injection. A previous study proved that nimesulide at a dose of 5 mg/kg had antipyretic and anti-inflammatory effects in dogs (Toutain *et al*., 2008). Other research mentions that nimesulide plays a larger role in the late phase of fever than that the first phase and that it outperforms indomethacin (non-selective COX_2 inhibitors) at dose of 10 mg/kg in guinea pigs (Steiner *et al*., 2001).

Also, the previous studies confirm that nimesulide differs in the scenario of antipyretic effects compared with non-selective COX_2 inhibitors (indomethacin) in rats, with superior additional mechanisms of nimesulide to reduce fever, including inhibition of TNF-α in plasma, which contributes to the antipyretic effect (Maria *et al*., 2006). Other research proved that nimesulide is more active and longer-lasting than paracetamol in inhibited the fever stimulated by Brewer's yeast in rats (Ceserani *et al*., 1993). Nimesulide produced a dose dependent antipyretic action, which was stronger than that of indomethacin and ibuprofen, and decreased dose dependently the increased brain prostaglandin E_2 levels, whereas it did not influence the expression of cyclooxygenase-2 mRNA. It inhibited markedly the enhanced brain cyclooxygenase activity, primarily cyclooxygenase-2, in vivo and dose dependently increased brain cyclooxygenase activity in vitro. These results suggest that the marked antipyretic action of nimesulide is primarily mediated through the selective inhibition of the activity of brain cyclooxygenase-2 induced fever (Taniguchi *et al*., 1997).

In prior research, it was shown that administration of aspirin during experimental infection of rabbits reduced the duration of fever and rectal temperature in mice (Kurosawa *et al*., 1987; Ohdo *et al*., 1995). Other research mention that single-dose of aspirin 500 and 1000 mg and acetaminophen 500 and 1000 mg were more effective against fever and other symptoms upper respiratory tract infection than placebo (Bachert *et al*., 2005) the mechanism of action of aspirin in decreased the fever by inhibited the activity of the enzyme

cyclooxygenase (COX) which leads to the formation of prostaglandins (PGs) that cause inflammation, swelling, pain and fever (Vane *et al*., 2003)

Also other study mention to the potent activity of nimesulide as an antipyretic in comparison to other NSAIDs, and the reason of these findings related to the majority of the drug inhibitory effect on the inducible COX_2 rather than the constitutive COX_1 (Steiner *et al*., 2001), and this results similar to our study which confirmed that nimesulide outperform of aspirin as antipyretic action.

In our study, we confirmed that nimesulide at a dose of ED_{100} has more efficacy in decreasing local inflammation in the paws of mice than aspirin at dose ED_{100} in times 0.5, 1, and 2 h after formalin injection, as well as the results showed a reduce in the number of lifts and lickings of the paw during 30 min. in comparison with the positive control (injected formalin) at percentages of 58 and 55% for nimesulide and aspirin, respectively.

The prostaglandin biosynthesis is the key mediator for causing inflammation which work as a pro-inflammatory mediator. Recent studies implicate that COX_2 induction is a critical event in inflammation (Vane *et al*., 1994). This theory has been supported through effective suppressing of inflammatory responses in experimental animals via selective COX_2 inhibitors (Seibert *et al*., 1994; Chan *et al*., 1995).

Nimesulide has reliable analgesic, antipyretic, and anti-inflammatory properties; therefore, it is used in nociceptive and inflammatory condition that are marked by hyperalgesia. Nimesulide appears to be fast-acting and particularly effective against inflammatory pain in comparison with celecoxib, diclofenac and rofecoxib in humans. The anti-hyperalgesic effect of the drug also was explored in patients with rheumatoid arthritis after administering a single oral dose which diminished the inflammatory hyperalgesia (Bianchi and Broggini, 2002; Kress *et al*., 2016).

Nimesulide was showed a significance analgesic effect in both the formalin test and the tail-flick. This drug proved to be effective in counteraction nitroglycerin-simulated hyperalgesia in both tests. The brain mapping of nuclei activate via the administration of nitroglycerin was showed that nimesulide pretreatment inhibited significantly neuronal activation in different regions, so conclusion that

nimesulide owns a strong anti-hyperalgesic action, therefore the mechanisms of action of nimesulide are in part has a central action (Tassorelli *et al*., 2003).

Nimesulide is a highly selective COX-2 inhibitor that inhibits PGE_2 by the block the enzyme COX-2 and prevents bradykinin and cytokine-stimulated hyperalgesia through inhibition of the release of tumor necrosis factor-alpha (TNF-α). So, it is become increasing evident that NSAIDs employ their analgesic effect by numerous mechanisms. Previous research suggests that the expression of COX_2 is found in the central nervous system, where COX_2 appears to have an important role in spinal pain transmission with nitric oxide. Nitroglycerin is a nitric oxide donor that stimulate a hyperalgesic condition, by mediated central mechanisms. Nimesulide is a preferential COX_2 inhibitor broadly used to treat the pain (Tassorelli *et al*., 2003), from properties of nimesulide, reduced TNF-α and neutrophil accumulation in the inflammatory exudate was reported, special inhibition of the production of COX_2 and other inflammatory mediators which production was controlled via stimulated of cyclic-3,5'-adenosine monophosphate (cAMP), this mean that nimesulide is a multi-factor drug in controlling the inflammation and pain (Rainsford, 2006).

As well as nimesulide has also proved that relative rapid onset of anti-hyperalgesic effect in human when compare with other NSAIDs such as celecoxib, diclofenac and rofecoxib; however, nimesulide can diminish inflammation and pain in formalin and other inflammatory tests (Bianchi and Broggini, 2002), previous results have shown that middle and high doses of nimesulide decreased mechanical pain (Tassorelli *et al*., 2003; Rainsford, 2006; Bianchi and Broggini, 2002).

Other studies reported the anti-inflammatory and analgesic activity of aspirin in rats and mice (Ohdo_*et al*., 1995; Al-Swayeh *et al*., 2000), but it caused negative effects on the stomach in mice by inhibition of protective prostaglandin mechanism (Darling *et al*., 2004) in contrary to nimesulide which has a good anti-inflammatory, analgesic, and antipyretic effectiveness, as well as having unique therapeutic and pharmacological activity with little toxicity to the gastrointestinal tract and kidney due to its selectivity to COX_2 (Bennett and Villa, 2000).

The adverse action of nimesulide is, in general, like that of other selective NSAIDs, has a relatively low side effect on the gastrointestinal tract (GIT), which

is associated with its low inhibitory effect of the important COX_1 needed to maintain the normal physiological integrity of the GIT and kidney (Rainsford, 2006).

The inhibition synthesis of prostaglandin represents the mechanism of action of aspirin as an analgesic, antipyretic, and anti-inflammatory, but this itself is not enough to explain the anti-inflammatory efficiency of aspirin, Other mechanism was defined as inducing the production of lipoxins (aspirin-triggered lipoxins) from the arachidonic acid via acetylation of the enzyme COX_2 and lipoxins binds with G-protein-coupled receptors to exert its action by resolving inflammation and acting as antioxidants and immunomodulators (Clària and Serhan, 1995; Cadavid, 2017).

The interpretation outperforming of nimesulide than aspirin as an anti-inflammatory may be due to have nimesulide deference mechanism to treat the inflammation such as decreased TNF-α and neutrophil accumulated in the inflammatory exudates, preferentially inhibition of the production of COX_2 and other inflammatory mediators whose production is controlled through stimulus of cAMP, also previous study proved that nimesulide at a therapeutic dose increased glucocorticoid receptor phosphorylation in cultured human synovial fibroblasts and activated of glucocorticoid receptor system (Di Battista *et al.*, 1999).

The results of the anticoagulant comparison trial between nimesulide and aspirin in mice which measured by detecting prothrombin time in the control and other treated groups with ED_{100} of nimesulide (15.8 mg/kg, IM) and aspirin (424.5 mg/kg, IM) respectively, for five consecutive days, the results shows that aspirin has efficient anticoagulant effect compared to nimesulide, this experiment proved that nimesulide (selective COX_2 inhibitors) is a preferential COX_2 inhibitor and it has some property against COX_1 activity, so nimesulide caused complete inhibition of COX_2 and a partial reduction of COX_1 activity. This confirm that the relative COX is selectivity of nimesulide, and this result corresponds to previous studies showing that nimesulide inhibited the platelet aggregation induce via adrenaline, it also inhibited the platelets biosynthesis of thromboxane A_2 (TXA_2) at low concentrations in humans (Saeed *et al*., 1998). Aspirin has been reported to be an irreversible inhibitor of both COXs; it inactivate platelet COX_1 irreversibly through acetylating a single serine residue in the enzyme, this action of aspirin may

responsible for its anti-platelet and anti-inflammatory action, also another study refers to the influence of aspirin and nimesulide on thromboxane inhibition in the percentage of 99% for aspirin and 43% for nimesulide (Roth *et al*., 1975; Kerola *et al*., 2008).

Other research proved that ibuprofen (one of the non-selective NSAIDs family) affects coagulation by mildly inhibiting platelet aggregation when taken for less than 10 days at the dose of 400 mg/kg unlikely to induce an adverse bleeding event in patients taking anticoagulants, so there may be an increased risk for bleeding (Flanagan, 2022).

This study measured nimesulide and aspirin plasma concentrations by high-performance liquid chromatography apparatus at 0.5, 1, 2, 4, and 24 hours after administration and found that maximal concentration (C_{max}) of nimesulide at a dose of ED_{100} in plasma was higher than aspirin (at ED_{100} dosing) as well as the AUC of nimesulide was more than aspirin at 0.5, 1, 2, 4, and 24 h time. This result is similar to previous research in humans (Toutain *et al*., 2001; Nagelschmitz *et al*., 2014) and in rats (Fu *et al*., 1991).

As well as the other parameters of nimesulide and aspirin were measured in this study, such as T_{max}, $t_{1/2\beta}$, and CL, which were also found in previous research similar to this study (Nagelschmitz *et al*., 2014; Wei *et al*., 2021). It may be interpreted that a higher concentration of nimesulide than aspirin may refer to its metabolites called 4-amino-2-phenoxy-methanesulfonanilide (M_1) which is an active constituent. Recently, a study revealed that M_1 is a stable metabolite that is high sensitive to easy oxidation via cytochrome P_{450} enzymes to produce reactive diiminoquinone intermediate (M_2). The formation of M_2 by cytochrome P_{450} 2C19 and 1A2 as the two principal enzymes stimulated M_1 oxidation; M_1 metabolic was irreversib inhibited by 2C19, but 1A2 activated it in a time-dependent manner. Exclusively 2C19 mediated further metabolic of M_1 to amino hydroxynimesulide M_3 and to diiminoquinone M_4. Another reason for the high concentration of nimesulide was the protein binding of nimesulide, which was about 97.5% (Li *et al*., 2009; David *et al*., 2013), while aspirin protein binding was 81.7% (Ghahramani *et al*., 1998). The volume of distribution of nimesulide was 1.49 L/kg smaller than that of aspirin (158.12 L/kg), and this interpreted a higher concentration of nimesulide more than aspirin in plasma. As revealed in this study,

the clearance of nimesulide (0.09 L/h/kg) is smaller than the clearance of aspirin (5.16 L/h/kg).

Metabolized aspirin by UDP-glucuronosyl transferase 1A6 (UGT1A6), cytochrome P450 2C9 (CYP2C9), and N-acetyl transferase 2 (NAT2) produce slow metabolizing enzymes, this interpretation may be the cause of the longer presence of aspirin in the body (21.25 h) while nimesulide stayed (11.07 h). Also, aspirin differs from other NSAIDs by binding covalently and irreversibly with both isoforms of COX (Ghahramani *et al*., 1998; Palikhe *et al*., 2011).

In previous research showed the pharmacokinetic parameters of nimesulide in dogs which were measured as C_{max} (4.7-5.78 ng/ml), T_{max} (1-4h), $t_{1/2}$ (4-6 h), AUC_0 (35-54.48 ng/mL/h), Vd (0.9-2.38 mL/kg), Cl 0.14-0.32 mL/kg/h) and the drug remained in high concentration until 12 h and then decreased dramatically at 24 h (Pérez, *et al*., 2019). Other research measured the pharmacokinetics of nimesulide to be as C_{max} (6.5 mg/ml), T_{max} (1-2h), Vd (0.39 mL/kg), Cl (31.106) mL/kg/h, and mentioned the effect of gender was a limited influence, children and elderly didn't difference from that healthy young individually in human (Bernareggi, 1998), and this results showed some difference in pharmacokinetics with our study that may be attributed to deference in an important factors such as the route of administration, amount of the dose administered and animal species.

Aspirin pharmacokinetic parameters as revealed by a previous study in human were as AUC_0 (98.5 mg/h/L), C_{max} (21.58 mg/L), T_{max} (0.66 h), Cl 5.5 L/h), $t_{1/2}$ (2.36 h) given in a dose of 500 mg/kg, IV, and some of these values were similar to our study (Nagelschmitz *et al*., 2013).

Nimesulide and aspirin have difference inhibitory action on COX_2 activity when given as a single injected dose of individual ED_{100} to treated the pain and induced inflammation (injection of acetic acid) which led to lowering the COX_2 level significantly when a treated group with nimesulide after 30 minutes from injection of acetic acid in plasma and kidney, also decreasing in the level of COX_2 significantly in the kidney and plasma of the group which treated with acetic acid after 30 minutes from injected aspirin. This experiment showed that nimesulide (selective COX_2 inhibitor) has more inhibitory effect of this enzyme than non-selective COX_2 inhibitor (aspirin). The previous study mentioned that nimesulide affected almost complete suppression of COX_2 activity and a partial reduced of

COX_1 activity in comparison to ibuprofen and aspirin (Markku *et al*., 2009). Furthermore, nimesulide is a preferential COX_2 inhibitor and 20 times more selective than COX_1 (Patrignani *et al*., 1997; Balaji *et al*., 2013).

NSAIDs inhibit the caspases, and this effect was COX-independent; this is consider a novel anti-inflammatory mechanism (Lamkanfi, 2011; Smith *et al*., 2017). Caspases have known roles in cancer, inflammation, rheumatoid arthritis, and neurodegenerative diseases. Caspases are a family of genes important for maintain homeostasis by regulation cell death and inflammation (McIlwain *et al*., 2013). Family of cysteine proteases that are centrally involved in cell death and inflammation responses, the molecular mechanisms that control caspase activation include the identification of additional inflammation and pathways that regulate activation of inflammatory caspases (Opdenbosch and Lamkanfi , 2019) so, induce inflammation lead to activated caspase-3 and this interpret in our study increased in caspase-3 when injected acetic acid.

Nimesulide has a protection effect in osteoarthritis by the inhibiting of apoptosis in chondrocytes by inhibition induction of caspase-3 activation (Mukherjee *et al*., 2001). In another study, nimesulide had a role in apoptosis through inhibition of prostaglandins, which showed modulate cell proliferation in lung tumors in mice (Shaik *et al*., 2004). Also, nimesulide decreased the cleavage of caspase-3 in 5 days and treated primary effusion lymphoma in humans, and a previous study mentioned that nimesulide and ibuprofen, beside that to their anti-inflammatory and analgesic effects, may be have a protective action in osteoarthritis by the inhibiting of apoptosis in chondrocytes (Mukherjee *et al*., 2001; Paul *et al*., 2011).

Low doses of aspirin showed the significant decrease in expression of TNF-α, caspase-3, and apoptotic index (Akhmad *et al*., 2016); other reports proved that aspirin reduced caspase-3 activation in the hepatocellular carcinoma, so aspirin has an anti-apoptotic effect through blocked caspase-3 in humans (Feng *et al*., 2011). In rats, The effect of aspirin on caspase-3 were represented by a decrease in its level in the neurons when used aspirin in combination with morphine as an adjuvant in diabetic neuropathy (Ozdemir *et al*., 2023) further, aspirin is decreased the level of apoptosis by decreasing cleaved caspase-3 (Liu *et al*., 2017).

In our study, nimesulide had more efficacy than aspirin in the suppression of caspase-3 in a dose of ED_{100}. Significantly nimesulide is inhibited caspase-3 in the liver in comparison with control groups of normal saline (negative control group) while nimesulide inhibited the caspase-3 in kidney, liver, and plasma significantly in comparison with the group of acetic acid (positive control group). Aspirin inhibited caspase-3 significantly in the kidney and liver in mice in an inducible group, and these results reveal the effects of nimesulide and aspirin as an anti-apoptotic drugs because caspase-3 is an important parameter for apoptosis induction.

All the above researches are in accordance with our current data findings, which proved that nimesulide and aspirin decreased caspase-3 and this contributed to anti-apoptosis activity. These results highlight these drugs in the treatment of cancer, however; nimesulide outperformed aspirin in inhibiting caspase-3 in the kidney, liver, and plasma in mice.

This study compared the inhibitory effect of nimesulide and aspirin on the peroxisome proliferator-activated receptor-alpha (PPAR-α) mediated induction of COX_2 expression and the data showed the inhibition of PPAR-α measured in kidney, liver, and plasma due to the injection of the therapeutic doses of nimesulide and aspirin.

Nimesulide and aspirin acted as ligand-dependent receptor which results the suppression of PPAR-α (dependent transactivation of target genes COX-2). Previous research mentions that the therapeutic dose of nimesulide which treats synovial osteoarthritis in human fibroblasts is mediated by its effects on the PPAR-induced COX_2 gene expression (Kalajdzic *et al*., 2002). Subtypes of PPAR have been shown to play critical roles in important diseases such as fertility, diabetes and cancer, The majority of researches focus on the roles of PPARs in inflammatory processes. Many studies have revealed that ligand of PPAR-α and PPAR-γ exert anti-inflammatory effect in vitro and in vivo, by using the carrageenan-induced paw edema model of inflammation, a recent study showed that these ligand affect on the the initiation phase but not the late phase of the inflammatory process, and exert roles as anti-inflammatory activity in different cell types via inhibition the expression of proinflammatory genes such as cytokines and metalloproteases (Delerive *et al*., 2001; Youssef and Badr, 2004; Zandbergen and

Plutzky, 2007). PPAR-α has a role in the inflammatory through modulating the inflammation. Other research mentions that binding with PPAR-α stimulated transcription of genes of the ω- and β-oxidation pathways that can catabolize the LTB_4 itself (Poynter and Daynes, 1998)

PPAR-α exerts anti-inflammatory effect by limits the inflammation (control of inflammation via reduced the activity of NF-κB) (Ren *et al*., 1996; Zandbergen and Plutzky, 2007).

In a previous study in vivo in mice showed that low dose of aspirin stimulated the expression of PPAR-α, whereas notes the opposite effect in high-dose of aspirin treatment (Tancevski *et al*., 2006).

Many studies showed evidence that binding ligand with PPAR-α have potent effects in regulating immune response including inflammation and cytokine production (Yang *et al*., 2008). In previous study demonstrate that murine lymphocytes (both B and T cells) express PPARα transcripts, contribute the blocking of the inflammatory response, PPARα and PPARγ ligands have been demonstrated to exert anti-inflammatory activities in macrophages by repressing the activities of several transcription factors in mice (Jones *et al*., 2002).

Also, it has been revealed that PPAR-α, δ, and γ play a important role in controlling the inflammatory response, and some exertion will result in the development of PPAR ligands as therapeutic agents for treatment of the inflammatory condition (Youssef and Badr, 2004).

Our study found that nimesulide and aspirin significantly affected the PPAR-α in the kidney and plasma (in the positive control group injected with acetic acid) while the results of the effect administration of nimesulide and aspirin on the PPAR-α in the liver and plasma were significant (in the negative group injected with normal saline). In general, nimesulide outperforms aspirin in decreased PPAR-α in mice. All those considered other molecular mechanisms of nimesulide and aspirin to exert an effect as anti-inflammatory and analgesic by inhibiting PPAR-α-mediated induction of COX_2 mRNA expression.

Nimesulide and aspirin were down-regulate the COX_2 gene expression via reducing the gene expression of COX_2 in comparative with the positive and

negative control groups after treated the mice for five consecutive days (one dose daily) at ED_{100} for each drug. Nimesulide outperformed aspirin in decreasing COX_2 gene expression for positive groups, which is inflammatory-induced by acetic acid, because nimesulide is a preferential COX_2 inhibitor (20 times more selective than COX_1 (Patrignani *et al*., 1997; Balaji *et al*., 2013). However, nimesulide and aspirin in therapeutic doses decreased the COX_2 gene expression in the kidney and caused down-regulation, which reasons enhance the pharmacological effects against inflammation, fever, and pain.

In previous studies, it was mentioned that nimesulide inhibits cytokine-induced COX_2 expression at therapeutic concentrations, nimesulide inhibited the IL-1 beta-induced COX_2 expression and protein at therapeutic and sub-therapeutic concentrations, while non-specific COX2 NSAIDs such as naproxen did not have an effect similar to nimesulide, Both drugs are suppresses PGE2 release via about 95% (Fahmi *et al*., 2001). Also, nimesulide reduced expression levels of COX_2 at both mRNA and protein levels, and with its inhibitory influence on the proliferation of cells, nimesulide could inhibit the development of the cell cycle; therefore, nimesulide-selective COX2 inhibitors could potentially become part of the comprehensive treatment for laryngeal cell carcinoma (Liang *et al*., 2016). Other research demonstrated that nimesulide-selective COX_2 inhibitors could play a role as antitumor. The mechanism of nimesulide acting as an anti-cancer by decreasing the expression of COX_2 in the pancreatic cancer cells and enhancing the expression of phosphatase and tensin homolog (PTEN), nimesulide acted to prevent tumor angiogenesis via decreasing the expression level of vascular endothelial growth factor (VEGF) (Chu *et al*., 2018).

Nimesulide was able to inhibited the proliferation of numerous types of cancer cells (Su and Chen, 2009), and previous studies believed that nimesulide inhibited growth of cancer cell and this independent of COX_2. (Zhong *et al*., 2012)

So this drug showed that it has anticancer effect in neoplastic pancreatic cells through inhibiting proliferation and apoptosis (Ferreira *et al*., 2021). However, COX_2-specific inhibitors may be a useful anti-tumor therapeutic option in pancreatic cancer (Okami *et al*., 2003).

The inflammation has been established to play a main role in the start, development, and diagnosis of cancer. The use of anti-inflammatory medicines reduce the occurrence and repetition of different cancers, in addition, used of anti-inflammatory drugs in combination with anti-cancer therapy (Rayburn *et al*., 2009).

Other studies demonstrated that the occurrence of colorectal, breast and lung cancers was reduced by use of aspirin and NSAIDs (Arun and Goss, 2004). Also, colorectal cancer patients who were used NSAID for long-term which dramatically lower the mortality rate than that patients which not used NSAID (Smalley and DuBois, 1997).

Previous studies have demonstrated that aspirin can reduce the short-term risk of colon tumors in patients with a prior history of cancer, The use of 300 mg or more of aspirin in a day for about 5 years was effective in primary prevention of colorectal cancer (Flossmann and Rothwell, 2007).

In general, COX_2 expression is up-regulated during the tumorigenic process, such as in hyperplasia and metastatic condition. COX_2 has been reported to be overexpressed in a different malignancies, especially prostate carcinoma, overexpressed of COX_2 was consistently in premalignant lesions such as prostatic intraepithelial neoplasia (Gallego *et al*., 2007).

Previous research evaluated the extent of the acetylation of COX_2 at serine-516 via aspirin; this use in the clinical studies will allow explanation of the mechanism of action of aspirin as an anticancer drug (Tacconelli *et al*., 2020). The role of COX_2 in the developed and progress of the tumors such as colorectal cancer (CRC) which suggested that through the finding expression of COX_2 is detectable in most colorectal cancer tissue (Patrignani and Patrono, 2016). The administration of NSAIDs, whose therapeutic efficacy primarily related to the inhibited of COX_2 activity, is related with anti-tumor effect in clinical trials ,epidemiologic studies, and animal experiments (Lecomte *et al*., 1994; Patrignani *et al*., 2014).

Aspirin at therapeutic concentrations act to inhibition of COX_2 mRNA and protein levels. In mice pretreated with aspirin (10 and 30 mg/kg), followed by challenge with lipopolysaccharide, COX-2 mRNA expression in peritoneal

macrophages was markedly suppressed. These findings suggest that salicylate exerts its anti-inflammatory action in part by suppressing COX-2 induction, thereby reducing the synthesis of pro-inflammatory prostaglandins (Xu *et al*.,1999).

Epidemiological observations in previous study explain that aspirin when used at low doses diminished colon cancer in humans (Thun *et al*., 1991). COX_2 overexpression in the colon epithelial and cancer cells has been linked with increased colon cancer growth (Tsujii *et al*., 1997). The useful effect of aspirin detected in the epidemiological studies was attributed in to the inhibiting of COX_2 activity which act as anti-colon cancer (Xu *et al*., 1999).

Aspirin has multiple molecular actions, it has the capability to control gene expressions of COX_2 in the cells which stimulated via various agonists by inhibition of kinases, at supra-pharmacological concentration, it depressions numerous kinases include IKKβ, thus suppression NF-κB (Cianferoni *et al*., 2001; Wu, 2003).

Another study reported that Aspirin at a low dose (3 mg/kg) decreased COX_2 mRNA level about 70% in mice after collecting Peritoneal macrophages and determining COX_2 mRNA levels (Hinz *et al*., 2000).

In previous study concluded that nimesulide, when used suitably, remain a specially safe and valuable choice for the treatment of numerous situations, such as acute inflammatory pain, because of the rapid onset of the analgesic action and the positive evidence-dependent on benefit profile (Kress *et al*., 2015).

Chapter Six

Conclusions and Recommendations

6-1: Conclusions

1. Nimesulide has more analgesic potency compared to aspirin as determined by their median effective doses (ED_{50}s) in mice.
2. The degree of nimesulide safety is more than aspirin in mice
3. Nimesulide and aspirin exert their analgesia in a dose- and time-dependent manner.
4. Nimesulide has superiority over aspirin in preventing the visceral pain in mice.
5. The analgesic, antipyretic and anti-inflammatory properties of nimesulide was significantly more efficient than aspirin in mice.
6. Aspirin was the better medication as an anti-coagulant compared to nimesulide in mice.
7. The plasma concentration of nimesulide reaches its higher level compared to aspirin both given at their pharmacological doses.
8. Pharmacokinetic parameters of nimesulide differs from aspirin in mice which reflect a better bioavailability and pharmacological efficacy.
9. Nimesulide is more efficient for inhibiting COX_2 activity in plasma, liver, and kidney of mice in comparison to aspirin.
10. Nimesulide was better than aspirin in reducing apoptosis as measured through caspase3 activity in mice.
11. Both nimesulide and aspirin have the ability to inhibit the peroxisome proliferation-activated receptors alpha (PPARα) at molecular level which contributes to their anti-inflammatory and anti-nociceptive properties.
12. Molecular assessments for nimesulide and aspirin indicates their ability to down-regulate of COX_2 gene expression in the kidney of mice.
13. The study demonstrate that nimesulide has better pharmacological properties (analgesic, antipyretic, and anti-inflammatory) than aspirin besides its superiority at the molecular level (caspase3, PPARα and COX_2 gene expression) in mice which makes it useful for practical use in the field of veterinary medicine.

6-2: Recommendations

1. Comparison of the pharmacological and molecular effects between nimesulide and aspirin in other animal models.
2. Comparison of the pharmacological and molecular effects between nimesulide and other non-selective COX_2 inhibitors in mice.
3. Comparative efficacy between nimesulide and selective COX_2 inhibitors in mice.

4. Effect of nimesulide and aspirin on the other types of peroxisome proliferation-activated receptors like β and γ receptors in mice.
5. Comparison between nimesulide and aspirin on the inflammatory mediators like prostaglandins and interleukins in mice.
6. Comparative pharmacological and molecular effects between nimesulide and aspirin on the level of COX_1 and COX_3 gene expression in mice.

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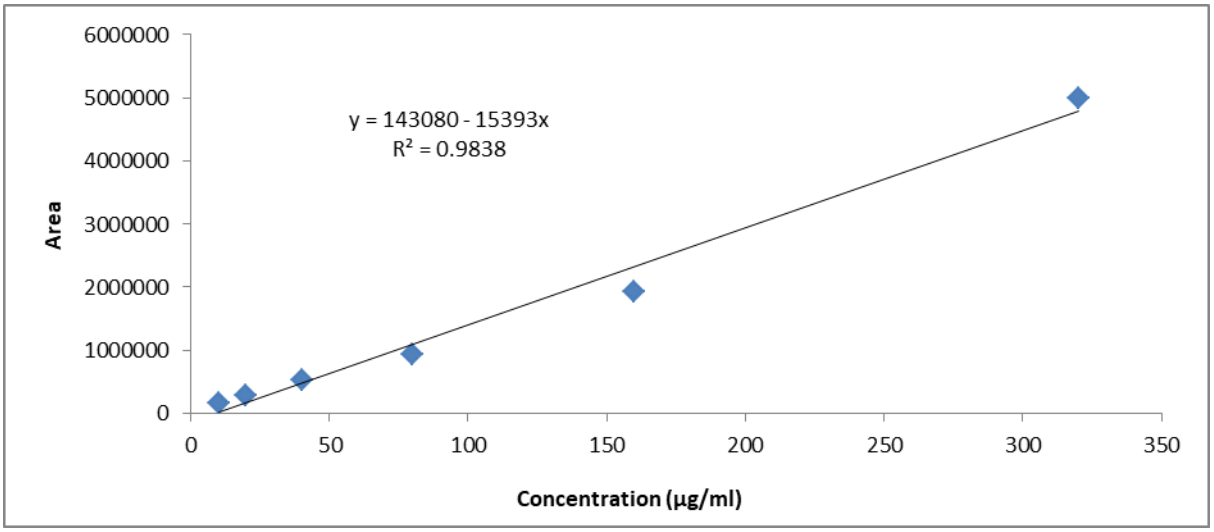
Appendix (1): Dixon table

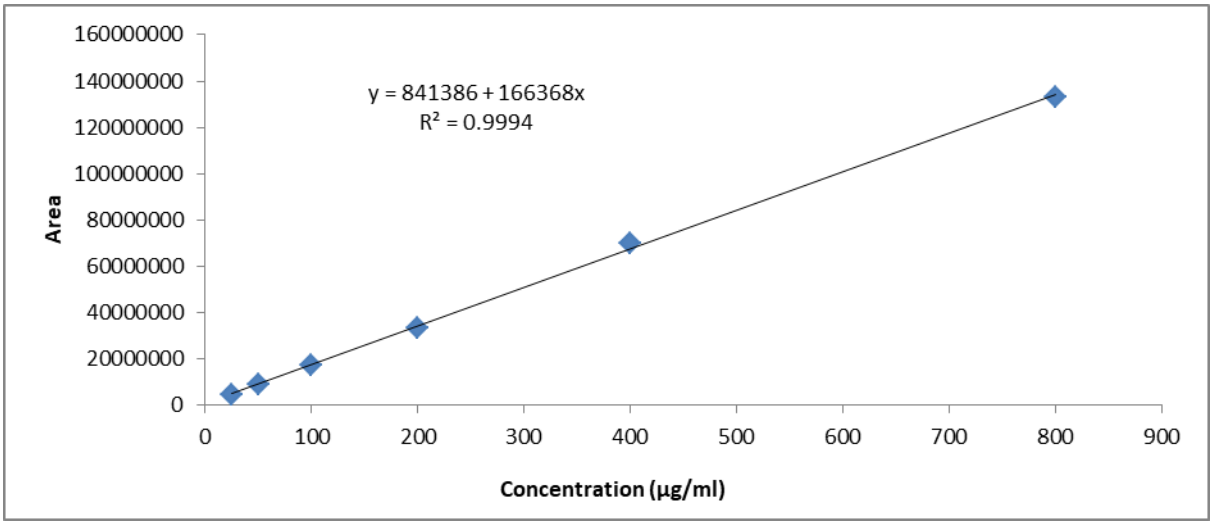
Second section from the series	K represent the series of tests that begin as follows					Stand Erro
	O	OO	OOO	OOOO		
XOOO	-0.157	-0.154	-0.154	-0.154	OXXX	0.61
XOOX	-0.878	-0.861	-0.860	-0.860	OXXO	
XOXO	0.701	0.737	0.741	0.741	OXOX	
XOXX	0.084	0.169	0.181	0.182	OXOO	
XXOO	0.305	0.372	0.380	0.381	OOXX	
XXOX	-0.305	-0.169	-0.144	-0.142	OOXO	
XXXO	1.288	1.500	1.544	1.549	OOOX	
XXXX	0.555	0.897	0.985	$^{1+}$1.000	OOOO	
	X	XX	XXX	XXXX	Second section from the series	
	-K represent the series of tests that begin as follows					

Appendix (2): Concentration of multiple Nimesulide standards measured by HPLC



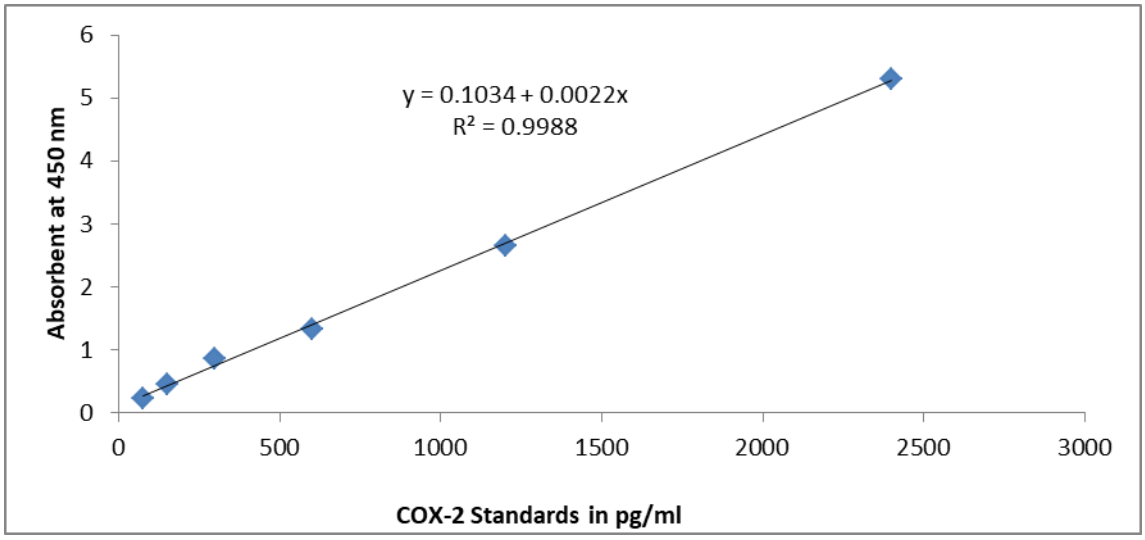
Nimesulide standards curve in µg/ml

Appendix (3): Concentration of multiple Aspirin standards measured by HPLC



Aspirin standards curve in µg/ml

Appendix (4): Concentration of multiple COX-2 standards measured by ELISA



Standards curve of COX-2 in pg/ml

Appendixe (5): The procedures of kits

Mouse Cyclooxygenase-2(COX-2) ELISA Kit and Mouse Caspase-3(Casp-3) ELISA Kit

Procedure

1- Dilution of Standards

Dilute the standard by small tubes first,then pipette the volume of 50ul from each tube to microplate well,each tube use two wells ,total ten wells.

2- In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40ul Sample dilution buffer and 10ul sample are added (dilution factor is 5).

Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking .Incubation: incubate 30 min at 37°Cafter sealed with Closure plate membrane. Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T and 20 times for 48T).Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5times Add 50 pl HRP-Conjugate reagent to each well except the blank control well.Incubation as described in Step 3.Washing as described in Step 5.Coloring: Add 50 pl Chromogen Solution A and 50 ill Chromogen Solution B to each well, mix with gently shaking and incubate at 37°C for 15 minutes. Please avoid light during coloring. Termination: add 50 ul stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

Appendix (6): Mouse PPARa(Peroxisome Proliferator Activated Receptor Alpha) ELISA Kit

Reagent Preparation

1-Bring all kit components and samples to room temperature (18-25°C) before use.

2-If the kit will not be used up in 1 time, please only take out strips and reagents for present experiment, and save the remaining strips and reagents as specified.

3-Dilute the 25 X Wash Buffer into 1 X Wash Buffer with double-distilled Water.

4-Standard Working Solution - Centrifuge the Standard at 1000 x g for 1 minute. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the Standard in the stock solution is 10 ng/mL. Please prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mix each tube thoroughly before the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.63 ng/mL, 0.32 ng/mL, 0.16 ng/mL, and the last EP tubes with Standard Diluent is the Blank as 0 ng/mL. In order to guarantee the experimental results validity,

please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as a Blank and do not pipette solution into it from the former tube.

5- 1x Biotinylated Antibody and 1 X Streptavidin-HRP - Briefly spin or centrifuge the stock

Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to the working concentration

-100fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

6-TMB Substrate Solution - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Samples Preparation

1-Equilibrate all materials and prepared reagents to room temperature prior to use. Prior to use, mix all reagents thoroughly taking care not to create any foam within the vials.

2-The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.

3-Please predict the concentration before assaying. If values for these are not within the range of the Standard curve, users must determine the optimal sample dilutions for their particular experiments.

Assay Procedure

1-Determine wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 100 uL each of Standard Working Solution (please refer to Reagent Preparation), or 100 uL of samples into the appropriate wells. Cover with the Plate Cover. Incubate for 80 minutes at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2-Pour out the liquid of each well. Aspirate the solution and wash with 200 uL of 1 x Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall of the wells to a void contamination.

(b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3-Add 100 uL of Biotinylated Antibody Working Solution to each well, cover the wells with the Plate

4-Repeat the aspiration, wash process for total 3 times as conducted in step 2.

5-Add 100 L of Streptavidin-HRP Working Solution to each well, cover the well with the plate sealer and incubate for 50 minutes at 37°C.

6-Repeat the aspiration, wash process for total 5 times as conducted in step 2.

7-Add 90 uL of TMB Substrate Solution to each well. Cover with a new Plate Cover, Incubate for 20 minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before 0D measurement.

8-Add 50 uL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.

9-Wipe off any drop of water and fingerprint on the bottom of the plate and confim there iS no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 mm immediately.

الخلاصة

كان الهدف من الدراسة الحالية هو مقارنة التأثيرات الدوائية (المسكنة للألم ، الخافضة للحرارة والمضادة للالتهاب) فضلا عن مقارنة التأثيرات الجزيئية بين الادوية المثبطة لأنزيم الاكسدة الحلقية 2 Cyclooxygenase 2 الانتقائية (النيميسولايد) والغير انتقائية (الاسبرين) في الفئران.

كانت الجرعة الفعالة الوسطية المسكنة للألم للنيميسولايد والاسبرين في الفئران هي 7,9 و212,23 ملغم/كغم في العضل بينما كانت الجرعة المميتة الوسطية الحادة 181,7 و1230,3 ملغم/كغم في العضل ، على التوالي والتي حددت باستخدام طريقة الصعود والنزول. علاوة على ذلك كان النيميسولايد الاكثر امانا في الفئران وفقا للمؤشر الدوائي Therapeutic index (والتي كانت 23 للنيميسولايد و 6 للأسبرين).

عمل النيميسولايد والاسبرين على تسكين الألم عند اعطائهما بجرع 15,8 و424,5 ملغم/كغم ، في العضل على التوالي (والتي تمثل الجرعة الفعالة في 100% من الفئران لهما) وكان هذا التسكين من الألم معتمدا على الجرعة والوقت اذ كان الوقت بعد 30 دقيقة من حقن هذه الادوية هو الوقت الأفضل للتسكين من الألم في كلا الدواءين.

منع النيميسولايد والاسبرين (بجرعة 15,7 و424,5 ملغم/كغم في العضل على التوالي) الالم الحشوي (منعكس التلوي) المحدث بحامض الخليك (1%) وتفوق النيميسولايد معنويا وبنسبة 66% عن الأسبرين بنسبة 46% بالمقارنة مع مجموعة السيطرة الموجبة (حامض الخليك).

كان التأثير الخافض للحرارة للنيميسولايد أكثر فعالية وبشكل معنوي مقارنة بالأسبرين من خلال تقليل الحرارة المحدثة باستخدام خميرة الخباز (135 ملغم/كغم ، في الخلب) خلال الأوقات المقاسة (1، 2، 3، و 4 ساعات بعد حقن خميرة الخباز).

سبب الفورمالين (1%) في حدوث التهاب وألم عند حقنه في مباطن قدم الفئران في مجموعة السيطرة الموجبة ، في حين أدى النيميسولايد إلى تقليل هذا الالتهاب (0,5 ، 1 و2 ساعة بعد حقن الفورمالين) والألم (من خلال تقليل عدد مرات رفع القدم ولعقها خلال 30 دقيقة) بشكل جيد مقارنة بالأسبرين.

كان الأسبرين هو الدواء الأفضل كمضاد للتخثر مقارنة بالنيميسولايد والذي تم استنتاجه عن طريق قياس زمن البروثرومبين في المجاميع المحقونة بالأدوية فضلا عن مجموعة السيطرة للفئران.

تم قياس تركيز النيميسولايد في بلازما الدم (عند اعطاءه بجرعة 15,8 ملغم/كغم ، في العضل) باستخدام جهاز الاستشراب السائل عالي الاداء HPLC وكان تركيزه أعلى مقارنةً بتركيز الأسبرين (المعطى بجرعة 424,5 ملغم/كغم ، في العضل) في الفئران وخلال جميع الأوقات المقاسة 0,5، 1، 2 ، 4 و 24 ساعة بعد الحقن.

تم تقدير معايير الحركية الدوائية للنيميسولايد في الفئران وكانت المنطقة تحت المنحنى 169,18 مايكروغرام×ساعة/مل ، المنطقة تحت المنحنى الاني 2358,72 مايكروغرام×الساعة^{2}/مل ، ثابت معدل الطرح 0,06 ساعة$^{-1}$ ، التركيز الاعلى 14,62 ، الوقت الاعلى 0,5 ساعة ، عمر النصف 11,07 ساعة ، معدل وقت البقاء 13,94 ساعة ، حجم الانتشار 1,49 لتر/كغم والتصفية الكلية 0,09 لتر/ساعة/كغم بينما اختلفت معايير الحركية الدوائية للأسبرين لتكون 82,31 ، 2428,32 ، 0,03 ، 4,35 ، 0,5 ، 21,25 ، 29,50 ، 158,12 و 5,16 على التوالي.

عمل كل من النيميسولايد (بجرعة 15,8 ملغم/كغم ، في العضل) والأسبرين (بجرعة 424,5 ملغم/كغم، في العضل) على تثبيط نشاط انزيم الاكسدة الحلقية 2 من خلال الانخفاض في تركيزه في بلازما الدم والكبد والكلى للفئران مع تفوق في التثبيط عند إعطاء النيميسولايد مقارنة مع المجاميع المحقونة بالأسبرين ومجموعتي السيطرة السالبة والموجبة.

سجلت هذه الدراسة ميكانيكية دوائية جديدة للنيميسولايد والأسبرين والتي تساهم في احداث تأثيرهما المسكن للألم والمضادة للالتهاب من خلال آليات خلوية مهمة أخرى.

كان النيميسولايد أفضل من الأسبرين في التقليل من نشاط انزيم الكاسبيز 3 Caspase 3 في بلازما الدم والكبد والكلى للفئران (تأثيرات مضادة للموت المبرمج للخلايا ومضادة للالتهاب) عند إعطائه لمدة خمسة أيام متتالية.

وثبط النيميسولايد والاسبرين على المستوى الجزيئي من مستقبلات البيروكسيسوم المتكاثرة-المنشطة الفا Peroxisome proliferator-activated receptor alpha والتي تعتبر ميكانيكية عمل اضافية تسهم في تأثيراتهما المضادة للالتهاب والمسكنة للألم.

وتشير القياسات الجزيئية الأخرى للفعالية الدوائية للنيميسولايد والأسبرين إلى الانخفاض في التعبير الجيني لأنزيم الاكسدة الحلقية 2 في كلية الفئران (قلة عدد المستقبلات down-regulation) بعد إعطائهما لمدة خمسة أيام متتالية وبجرع علاجية هي 15,8 و424,5 ملغم/كغم في العضل، على التوالي ، مما يعزز في كفاءتهم كأدوية مسكنة للألم وخافضة للحرارة ومضادة للالتهاب.

تشير بيانات هذه الدراسة إلى أن النيميسولايد يمتلك خصائص دوائية (مسكنة للألم ، خافض للحرارة ومضادة للالتهاب) وبشكل أفضل من الأسبرين فضلا عن تفوقه على المستوى الجزيئي (الكاسبيز3 ، مستقبلات البيروكسيسوم المتكاثرة-المنشطة الفا والتعبير الجيني لأنزيم الاكسدة الحلقية 2) في الفئران مما يجعله مفيدًا للاستخدام العملي في مجال الطب البيطري.

جامعة الموصل

كلية الطب البيطري

مقارنة التاثيرات الدوائية والجزيئية بين النيميسولايد والاسبرين في الفئران

تيماء عدلان يحيى

اطروحة دكتوراه

الطب البيطري / الأدوية البيطرية

بإشراف

الأستاذ الدكتور

يعرب جعفر موسى

مقارنة التاثيرات الدوائية والجزيئية بين النيميسولايد والاسبرين في الفئران

اطروحة تقدمت بها

تيماء عدلان يحيى

الى

مجلس كلية الطب البيطري في جامعة الموصل

وهي جزء من متطلبات نيل شهادة الدكتوراه فلسفة

في اختصاص الطب البيطري / الأدوية البيطرية

بإشراف

الأستاذ الدكتور

يعرب جعفر موسى