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Pharmacological Evaluation of Chlorogenic Acid in Animal Models of Migraine

by

Bisma Kalsoom

A thesis submitted in partial fulfillment for the degree of Master of Philosophy

in the

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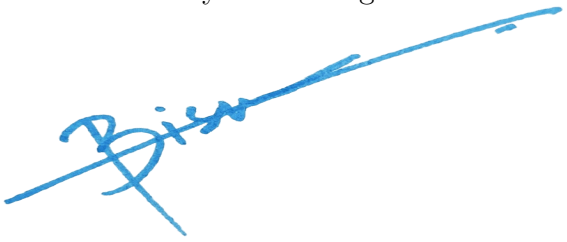
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Abstract

Migraine is a common and debilitating neurological disorder characterized by neuroinflammation, oxidative stress and central sensitization that is under-treated due to limited efficacy of medications and cardiovascular side effects. This study aimed to investigate the antimigraine effects of chlorogenic acid (CGA), a naturally occurring polyphenol known to have antioxidant and anti-inflammatory properties, in a migraine mouse models using nitroglycerin (NTG). The BALB/c mice were randomly divided among 6 groups of 10 mice per group: normal saline, NTG (10 mg/kg), NTG + zolmitriptan (5 mg/kg), and NTG + CGA (10, 50 or 100 mg/kg). To induce migraine-like symptoms, NTG was administered intraperitoneally every other day for 9 days. The CGA was administered as test compound daily 30 minutes before the administration of NTG.

Zolmitriptan was used as a standard drug. On days 0, 1, 5 and 9 behavioral assessments including the hot plate test, tail flick test, Von Frey filament test (periorbital and paw) and light aversion test were conducted. The cerebral cortex and trigeminal nucleus caudalis (TNC) were analysed to measure the expression of TNF-α and IL-1β, while the histopathology of the cerebral cortex was examined by Hematoxylin & Eosin staining. Treatment with NTG resulted in progressive thermal hyperalgesia, mechanical allodynia, and photophobia with significant increase in levels of both TNF-α and IL-1β in cortex and TNC and marked cortical vacuolation and disruption of tissue architecture. The protective effects of chlorogenic acid were dose-dependent, with the 50 mg/kg and 100 mg/kg treatment groups producing marked improvements in thermal hyperalgesia, thermal and mechanical allodynia, and photophobia compared with the nitroglycerin-treated group. Doses also significantly decreased elevated levels of the pro-inflammatory cytokines TNF-α and IL-1β in both cerebral cortex and trigeminal nucleus caudalis, and improved nitroglycerin induced histopathological changes in the cerebral cortex. In contrast, the 10 mg/kg dose produced comparatively modest protective effects. Overall, these results indicate that chlorogenic acid has a strong antimigraine effect against nitroglycerin induced migraine-like pathology by reducing the

behavioral abnormalities and neuroinflammation, suggesting chlorogenic acid is a potential therapeutic candidate for migraine treatment.

Contents

Author's Declaration iii

Plagiarism Undertaking iv

Acknowledgement v

Abstract vi

List of Figures xi

List of Tables xii

Abbreviations xiii

List of Symbols xiv

1 Introduction 1
- 1.1 General Background and Significance . . . 1
  - 1.1.1 Symptoms and Diagnosis of Migraine . . . 3
  - 1.1.2 Pathophysiology of Migraine . . . 5
  - 1.1.3 The Concept of Neuroprotection in Migraine . . . 7
- 1.2 Problem Statement: The Unmet Need . . . 8
- 1.3 Rationale for Investigating Natural Products: Chlorogenic Acid . . . 9
  - 1.3.1 Introduction to Chlorogenic Acid CGA . . . 10
- 1.4 Justification for the Mouse Model . . . 14
- 1.5 Research Gap, Questions and Hypothesis . . . 16
  - 1.5.1 Research Hypothesis . . . 17
- 1.6 Research Objectives . . . 17

2 Literature Review 18
- 2.1 Understanding Migraine Beyond Headache . . . 18
  - 2.1.1 Key Pathophysiological Mechanisms . . . 20
- 2.2 Current Pharmacological Management of Migraine and Unmet Needs 21
  - 2.2.1 Limitations and Gaps . . . 23
- 2.3 The Rationale for Neuroprotection in Migraine . . . 24

2.4 Chlorogenic Acid CGA: A Candidate Neuroprotective Agent 25
2.4.1 Established Pharmacological Activities Relevant to Migraine 30
2.5 Preclinical Models of Migraine: Evaluating Efficacy 31
2.6 Previous Studies on Polyphenols and Chlorogenic Acid in Pain and Migraine Models 32
2.7 Critical Summary and Identification of the Research Gap 34

3 Materials and Methods 36
3.1 Animals 36
3.2 Housing Condition 36
3.3 Food Consumption 37
3.4 Acclimatization 37
3.5 Mice Handling 38
3.6 Mice Bedding 39
3.7 Randomization 40
3.8 Sacrifice Procedure 40
3.8.1 Dislocation 41
3.8.2 Concussion 41
3.9 Chemicals and Drugs 41
3.10 Nitroglycerin-Induced Migraine Model 42
3.11 Behavioral Assessment 43
3.11.1 Tail flick Test 43
3.11.2 Light Aversion Test 43
3.11.3 Hot Plate Test 45
3.11.4 Von Frey Filament Test 45
3.12 Study design 48
3.12.1 Experimental Design and Groups Allocation 48
3.12.2 Drug Administration 49
3.12.3 Dose Preparation 50
3.13 Biochemical Analysis 53
3.13.1 Tissue Preparation 53
3.13.2 Biocinchoninic Assay 53
3.13.3 Enzyme Linked Immunosorbent Assay 54
3.13.3.1 Reagent Preparation 55
3.13.3.2 Assay Procedure 55
3.13.3.3 Absorbance Measurement and Quantification . . . 56
3.13.3.4 Quality Control 57
3.14 Histopathological Analysis H and E Staining 57
3.15 Statistical Analysis 58

4 Results 59
4.1 Behavioral Assessment 59
4.1.1 Effect of Chlorogenic Acid on Thermal Hyperalgesia (Hot Plate Test) 60

4.1.2 Effect of Chlorogenic Acid on Thermal Allodynia (Tail Flick Test) . . . 61
4.1.3 Effect of Chlorogenic Acid on Mechanical Allodynia (Von Frey Filament Test) . . . 62
4.1.3.1 Peripheral Mechanical Allodynia in Hind paw of mice . . . 62
4.1.3.2 Central Mechanical Allodynia in Periorbital Area of Mice . . . 63
4.1.4 Effect of Chlorogenic Acid on Photophobia (Light Aversion Test) . . . 64
4.2 Enzyme Linked Immunosorbent Assay Analysis of Mice Tissues . . . 66
4.3 Effect of Chlorogenic Acid on Tumor Necrosis Factor Alpha in Cortex and Trigeminal Nucleus Caudalis . . . 66
4.4 Effect of Chlorogenic Acid on Interleukin-1 Beta in Cortex and Trigeminal Nucleus Caudalis . . . 68
4.5 Hematoxylin and Eosin Staining for Identification of Morphological Changes . . . 71

5 Discussion **73**

6 Conclusion and Future Recommendations **79**
6.1 Conclusion . . . 79
6.2 Future Perspectives . . . 80
6.3 Limitations . . . 81

Bibliography **82**

List of Figures

1.1 The Involvement of Different Regions of Brain in Migraine 6
1.2 Pharmacological Profile of Chlorogenic Acid 11
1.3 Mechanism of Nitroglycerin Induced Migraine 15

2.1 Chemical Structure of Chlorogenic Acid 26
2.2 Metabolites of Chlorogenic acid 29

3.1 Mice Handling 39
3.2 Tail Flick Test of Mice 44
3.3 Light Aversion Test of Mice 44
3.4 Hot Plate Test 45
3.5 Von Frey Filament Test Kit 46
3.6 Von Frey Filament Test (Paw) in Mice 47
3.7 Von Frey Filament Test (Periorbital Area) in Mice 47
3.8 Study Design for In-vivo Analysis 48
3.9 Intraperitoneally Dose Administration in Mice 49
3.10 96 Well Plate for ELISA 55

4.1 Effect of CGA on Thermal Hyperalgesia (Hot Plate Test) 60
4.2 Effect of CGA on Thermal Allodynia (Tail Flick Test) 62
4.3 Effect of CGA on Mechanical Allodynia in Mice (Von Frey Filament test) 63
4.4 Effect of CGA on Mechanical Allodynia in Mice (Von Frey Filament test) 64
4.5 Effect of CGA on Photophobia in Mice (Time Spent in Light Area) 65
4.6 Effect of CGA on Photophobia in Mice (Time Spent in Dark Area) 65
4.7 Effect of CGA on Cortical TNF-α Levels 67
4.8 Effect of CGA on TNF-α Levels in TNC Region 67
4.9 Effect of CGA on Cortical IL-1 β 68
4.10 Effect of CGA on IL-1 β in TNC Region 70
4.11 Representative Histopathological Images of the Cerebral Cortex via H&E Staining 72

5.1 Proposed Mechanism of Chlorogenic Acid as Antimigraine Drug . . 78

List of Tables

2.1 Available Therapies for Migraine and Gap in Effectiveness 23

3.1 Dietary Composition 37
3.2 Dose Calculation of NTG, Zolmitriptan and CGA for Mice Model . 51
3.3 Preparation of Pharmacological Solutions and Treatments 52

Abbreviations

ANOVA	Analysis of Variance
BCA	Bicinchoninic Acid
CGA	Chlorogenic Acid
CGRP	Calcitonin-gene related peptide
CSD	cortical spreading depression
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
H&E	Hematoxylin and Eosin
HRP	Horseradish Peroxidase
i.p.	Intraperitoneal
IAEC	Institutional Animal Ethics recittee
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
LMIC	Low and middle income countries
NF-κB	Nuclear Factor Kappa B
NO	Nitric oxide
NTG	Nitroglycerine
SD	Standard Deviation
SEM	Standard Error of the Mean
TNC	Trigeminal Nucleus Caudalis
TNF-α	Tumor Necrosis Factor Alpha

List of Symbols

n	Number of animals per group
%	Percentage
mg	Milligram
μg	Microgram
pg	picogram
mg/kg	Milligram per kilogram
μL	Microliter
mL	Milliliter
cm	Centimeter
s	Second
°C	Degree Celsius
*	$P < 0.05$
**	$P < 0.001$
***	$P < 0.0001$
#	$P < 0.05$ vs control group
##	$P < 0.001$ vs control group
###	$P < 0.0001$ vs control group
±	Plus or minus (used with mean ± SD or mean ± SEM)

Chapter 1

Introduction

1.1 General Background and Significance

Migraine is a disabling neurological disorder which contributes to global burden of diseases. It is a complex recurrent neurological disorder, with moderate to severe headache occurring in attacks, often with a variety of symptoms including nausea and vomiting, photophobia, phonophobia, and in some people, transient aura. The traditional vascular theory of migraine no longer holds today, and migraine is now known to be a disorder of abnormal brain function with dynamic interaction between the hypothalamus, cerebral cortex, brainstem, thalamus and the Trigeminovascular system [1]. Because its symptoms are so frequent and often complicated, affecting physical, cognitive, emotional and social functioning, migraine has become one of the most important neurological disorders in the world, with a large contribution to the global burden of disease [2].

Migraine is a very common neurological disorder, with about 18% of women and 6-7% of men being affected [3]. On a larger scale, in 2021, an estimated 3.1 billion people or close to 40% of the world's population were affected by headache disorders. These disorders are among the top three most common neurological disorders in many age groups, starting around 5 years of age until the age of 80 [4]. Migraine is also known to be a significant cause of disability, especially for young

and middle-aged people. Migraine attacks are recurring and can significantly disrupt life and quality of life [5]. Although migraine has a significant burden in the world, it is often underdiagnosed and undertreated, especially in developing countries. This therapeutic imbalance contributes to the economic burden of the disease and to lower productivity in work and daily life due to the impact on health services utilization [6]. Beyond the impact of the individual attacks, the frequent occurrence of headache, together with the constant fear of another attack, can have a negative impact on family relationships, social activities and work [4].

A study which gave the first comprehensive nationwide prevalence data on headache disorders in Pakistan showed alarmingly high prevalence. Nationwide epidemiological data reveals a striking headache burden within Pakistan, with overall prevalence reaching 76.6%.

Specifically, age- and gender-adjusted rates show that 22.5% of the population suffers from migraine, 44.6% experience tension-type headaches, and 7.4% suffer from chronic headaches occurring on 15 or more days each month. These figures markedly surpass worldwide baseline estimates, underscoring the urgent necessity for healthcare providers and public health strategists to prioritize migraine management in the region [7].

These are significantly higher than the global averages which estimate the prevalence of migraine at around 11.6% worldwide, highlighting the fact that migraine is a significant public health problem in Pakistan and should be immediately addressed by policy makers and health care providers.

Globally, the overall ratio of female patients seeking care for migraine was 3:1, but in Pakistan the ratio was 1.5:1 (females: males), which was thought to be caused by cultural factors as women might be less likely to present to the hospital when accompanied by their husbands. The highest prevalence of migraine was in the 40-49 age group. The findings indicated a strong association between lower levels of education and poverty, and higher prevalence for migraine, in line with the results of other LMIC countries. There were significant differences among the provinces with regards to the proportion of tension-type headache and chronic

headache with higher rates reported in Punjab and Sindh respectively, and higher rates in Baluchistan, which could be due to differences in ethnic composition, environmental exposures, and access to health care [7].

The productivity losses are huge as 22.5% of the adults suffer from migraine which is the sixth largest cause of disability in the world [8]. This high prevalence of migraine in Pakistan has implications for clinical practice. Improved education of healthcare workers with a focus on headache diagnosis and management, specifically primary care physicians who are not well trained in headache diagnosis and are not always able to identify the type of headache and offer the proper treatment. Access to triptans and other migraine specific drugs are a huge hurdle in Pakistan, especially in rural areas where health care facility is not well developed. Raising awareness of migraine as a treatable medical problem, and not a trivial complaint, is crucial because many people may not seek medical attention because of lack of awareness or cultural beliefs [7].

The research is essential for ensuring the spotlight on healthcare facilities and the guidance and information to healthcare policy. A global burden study also pointed to the need for further research on the progression of migraine to chronic forms, as well as specific triggers, such as stress, sleep deprivation and diet. For Pakistan, research plays a crucial role in learning about the specific triggers, such as local cultural factors, dietary habits, environmental stressors including extreme heat exposure in the region, or the ubiquitous use of chai (black tea) [9].

1.1.1 Symptoms and Diagnosis of Migraine

Migraine is a complex neurological disorder and symptoms can range from mild to moderate to severe, with attacks often going through four stages. During the premonitory phase, which takes place several hours or days before the headache attacks, the patients experience mood changes, irritability, fatigue, food craving, repeated yawning, stiff neck, phonophobia, difficulty concentrating, thirst, frequent urination, and blurred vision. In the aura phase, fully reversible neurological deficits occur gradually over at least five minutes and usually last five to

sixty minutes. The most common symptom is visual aura which affects more than 90 per cent of cases and can be in the form of flashing lights or geometric patterns. Aura phase is reported to occur in one third of migraine patients. Sensory aura is usually manifested as numbness, which affects the body gradually, and speech and language aura as aphasia or word-finding difficulties. Motor aura is a weakness or paralysis characteristic of hemiplegic migraine, while brainstem aura symptoms include dysarthria, vertigo, tinnitus, ataxia and decreased consciousness [10]. The headache part of a migraine attack is usually considered the most debilitating.

It is accompanied by moderate to severe pain, typically one sided, and may be pulsating or throbbing, and may worsen with regular physical exercise. This period can also include nausea, vomiting, light sensitivity, and/or noises.

If there is a lack of treatment or effectiveness, migraines may last about 4-72 hours. After the headache subsides, patients can experience the postdrome, which can include fatigue, difficulty focusing, neck stiffness, or a general discomfort or "hangover" feeling. [11].

Migraine patients also suffer from hypersensitivity of the sensory system, which is reflected in the intensity of the pain on exposure to light. Some patients develop a sensitivity to sound (phonophobia) and others are hypersensitive to odors (osmophobia). In about two thirds of migraine patients, the pain is felt in areas that are usually not painful (e.g., brush hair, shave, shower, touch periorbital skin, wear eyeglasses and earrings) and occurs 1 to 2 hours after the onset of a headache [12].

The diagnosis of migraine is made mainly by careful history taking, which includes a detailed description of headache attacks, including details of frequency, duration, location, quality, associated symptoms, aggravating and relieving factors, age of onset, temporal pattern, premonitory symptoms and aura, drug use including overuse patterns, family history, menstrual relationship (where relevant), impact on daily functioning and trigger factors. The physical and neurological exam is conducted to look for any abnormalities that may indicate a secondary cause, including palpation over the temporal artery pulses, palpation over the occipital and supraorbital nerves, funduscopic exam, and attention to the neck and shoulders

[13]. Chronic tension-type headache has no features of migraine, is mild to moderate pain and is featureless. When history and examination indicate secondary causes, additional diagnostic evaluation with blood tests, brain imaging, imaging of the cervical spine, vascular imaging, lumbar puncture is indicated.

In patients with frequent headaches, especially in those taking ergotamines, triptans, opioids or combination analgesics regularly and using them more than 10 times/1 month or simple analgesics more than 15 times/1 month (diagnosis of medication overuse headache) there is a risk of developing chronic migraine [14].

1.1.2 Pathophysiology of Migraine

Migraine is a complex neurological disorder with abnormal sensory processing due to altered neuronal excitability, and dysfunction of interconnected regions of the brain, such as the hypothalamus, brainstem, cerebral cortex, thalamus and trigeminovascular system. [12].

Previous theories suggested that migraine pain was due to the vasodilatory effects of blood vessels in the brain and to sterile neurogenic inflammation caused by the release of neuropeptides from the trigeminal nerve endings. But, as more evidence has emerged, it has been shown that these vascular changes are not sufficient to explain migraine attacks; arterial dilatation does not reliably induce headache, nor is there a correlation with the intensity of the headache. Current knowledge thus establishes migraine as a disorder of brain function in which activation and sensitization of the trigeminovascular pathway, in addition to central pain processing alterations, play key roles in the initiation and perpetuation of migraine pain [15]. Peripheral sensitization and central sensitization have been linked to increase in nociceptive input to the trigeminal nucleus caudalis (TNC) and higher centers involved in pain processing, such as the thalamus and cerebral cortex [16]. Migraine is also considered a disorder in sensory processing that has an inherited basis, with the brain having a tendency to be more sensitive to repetitive sensory stimuli and to be less habituated [15]. The migraine brain does not adapt to repeated visual,

auditory and tactile stimuli, but instead responds in an exaggerated way, which leads to increased sensory sensitivity. In about one-third of patients, this abnormal neuronal activity causes migraine aura, and is linked to slowly propagating waves of neuronal and glial depolarization that are followed by a brief period of suppression of cortical activity known as cortical spreading depression (CSD). The role of CSD in the primary cause of migraine pain was once thought to be the main triggering event but there is now evidence that CSD is only one symptom of the underlying brain dysfunction and not the only event that triggers pain. [12].

Besides neuronal dysfunction, neuroinflammation and oxidative stress are also considered to be crucial in the migraine pathogenesis. Pro-inflammatory mediators like tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) are produced through the activation of inflammatory pathways like nuclear factor-kappa B (NF-κB) signalling pathway. These mediators can amplify the activation of these glial cells and the neuronal excitability, leading to peripheral and central sensitization. This inflammatory response could help perpetuate nociceptive transmission in the trigeminovascular system, and thus reinforce the chronicity and duration of migraine. Enhanced blood–brain barrier permeability in the medulla oblongata also may allow peripheral inflammatory mediators, such as IL-17A, to reach the trigeminal nucleus caudalis.

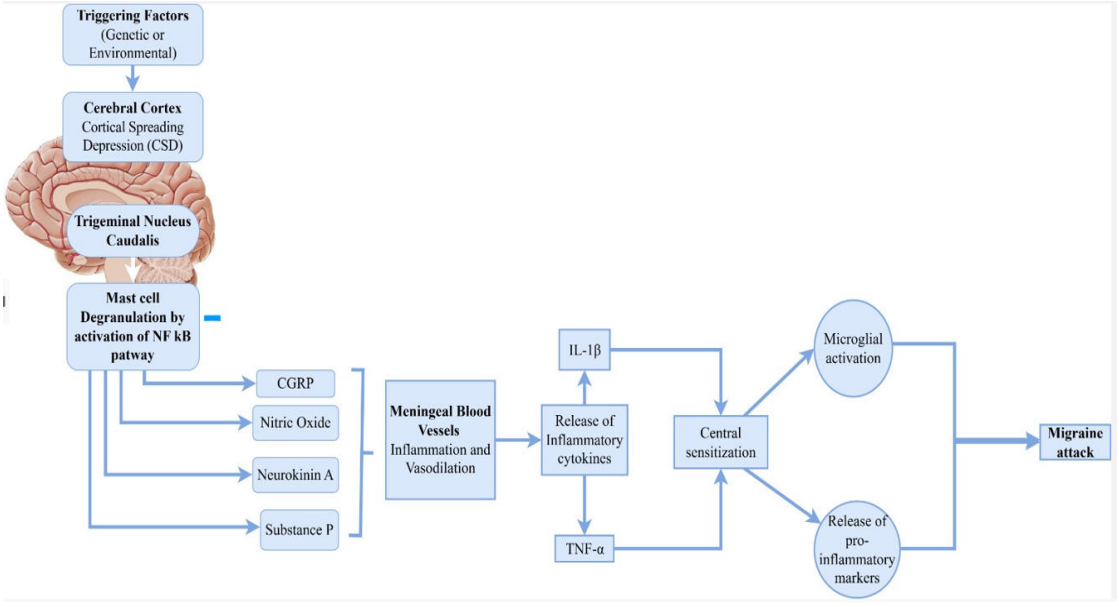


FIGURE 1.1: The Involvement of Different Regions of Brain in Migraine

They could trigger microglial activation and then the activation of the NF-κB pathway and its subsequent activation of neural inflammatory signaling. This cascade can lead to the upregulation of molecules involved in inflammation and migraine and further perpetuate central sensitization and migraine-related nociceptive signaling [17]. Further, nitric oxide (NO), a well-established experimental migraine trigger, has also been suggested to contribute to cognitive dysfunction and anxiety-like behaviour in chronic migraine, suggesting a multifactorial pathogenesis of migraine [18].

The figure-1 shows parts of brain involved in migraine pain involving cerebral cortex, Trigeminovascular system including the Trigeminal Nucleus Caudalis in the brain stem. The triggering factors cause release of neurons from cerebral cortex and activation of pathways in Trigeminal Nucleus Caudalis further causing release of CGRP, Nitric oxide, Neurokinin A and Substance P which causes vasodilation of meningeal blood vessels and release of inflammatory cytokines that causes central sensitization and migraine attack [17].

1.1.3 The Concept of Neuroprotection in Migraine

In the context of migraine, the concept of neuroprotection refers to strategies aimed at preventing the structural and functional brain reorganization that occurs as a result of recurring attacks [19]. Pathophysiological evidence shows that the migraine brain can undergo plastic changes over time, such as grey matter reduction in pain-processing areas (e.g., the anterior cingulate cortex and insula) or white matter integrity changes in the thalamocortical tract [15]. Neuroprotection seeks to shield the central nervous system from these long-term consequences and halt the progression from episodic to chronic migraine [20].

The effectiveness of neuroprotection in migraine can be discussed by linking with pathophysiology of migraine through following several mechanisms:

The cortical spreading depression (CSD) is the neurophysiological correlate of aura and involves a massive wave of neuronal and glial depolarization that releases

noxious mediators like glutamate, potassium, and nitric oxide [12]. These events cause significant metabolic stress and facilitate brain damage [15]. Neuroprotective interventions utilize drugs like topiramate, which may prevent the development of CSD by modulating ion channels and inhibiting glutamate release, thereby shielding the cortex from hyperexcitability [20].

Pathophysiological research shows that migraine triggers like nitroglycerin can increase BBB permeability, allowing peripheral cytokines such as IL-17A to infiltrate the medulla oblongata. This infiltration initiates microglia-mediated neuroinflammation and central sensitization. A primary neuroprotective strategy involves protecting the BBB to block this transport [17].

Chronic migraine is associated with cognitive impairments (such as memory loss and attention deficits) and anxiety, which are linked to the Nitric Oxide (NO) system. Recurrent attacks have been correlated with decreased hippocampal volume. Regulating NO pathways may offer a protective effect against these psychological sequelae by maintaining synaptic plasticity and preventing pain-induced memory deficits [18].

1.2 Problem Statement: The Unmet Need

Although research into migraine has progressed, existing treatments are not satisfactory for many sufferers.

Acute treatments such as triptans and nonsteroidal anti-inflammatory drugs (NSAIDs) are effective at relieving migraine attacks, but incompleteness of pain relief, contraindications to the use of these drugs, gastrointestinal side effects, and the risk of the development of medication-overuse headache (MOH) limit the use of these drugs. Acute antimigraine drugs (especially triptans) have been linked to headache chronicity and the risk of headache recurrence with repeated use [21]. A further drawback of existing acute treatments is the experimental evidence showing that chronic exposure to sumatriptan can lead to persistent hypersensitivity, a

phenomenon similar to medication overuse headache. The development of preventive therapies, including β-blockers, anticonvulsants, antidepressants and, recently, calcitonin gene-related peptide (CGRP) targeted monoclonal antibodies, has enhanced the management of migraine, but have a number of limitations including that their clinical responses are very different for patients, adherence to long-term prevention is often poor due to side-effects, many preventive agents don't offer satisfactory symptom control. Additionally, CGRP pathway-targeting treatments are costly and may not be available in low and middle income countries. Experimental studies have also revealed varying effectiveness of the available preventive agents, suggesting that the existing prophylactic measures are not completely effective, and that new therapeutic approaches are needed [22].

1.3 Rationale for Investigating Natural Products: Chlorogenic Acid

Herbal medications have a long history of use in the treatment of neurological diseases such as migraine. At Ayurvedic medicine, ginger was a common drug used for rheumatic pain, headache, indigestion and motion sickness [23]. Lavender oil has an extensive history of medicinal applications, including as an anticonvulsant, sedative and anxiolytic [24]. Citron was particularly used in ancient Persian medicine for headaches, and chamomile was traditionally used for insomnia, digestive issues and intermittent fever [25]. The toxic effects of ergot were not well known for centuries, but the plant was used as early as 600BC for abortions and to prevent postpartum haemorrhage [26].

Botanicals have regained a scientific interest as they could provide a number of benefits over traditional pharmaceuticals including their multi-target mechanisms of action. In migraine, it is a property that is especially applicable to the multifactorial nature of the disease and the roles of various processes such as cortical spreading depression, trigeminovascular activation, changes in serotonergic and dopaminergic function, rise in levels of calcitonin gene-related peptide (CGRP)

and oxidative stress. Therefore, one plant compound can act on multiple pathways associated with migraine. For instance, parthenolide, one of the bioactive components of Feverfew, has been reported to inhibit prostaglandin synthesis, vascular contractile mechanisms and serotonin release. Butterbur also contains compounds that have been linked to the inhibition of CRP release, petasin and isopetasin [27].

Secondly, plant based compounds usually display a better tolerability than prescription medications. The side effects of pharmaceutical interventions such as triptans, ergots and nonsteroidal anti-inflammatory drugs are severe, including gastrointestinal upset, chest tightness, cardiovascular effects, and medication overuse headaches [28]. On the other hand, a study that compared ginger powder to sumatriptan revealed that ginger was just as effective in relieving pain within two hours, but had way fewer side effects, only upset stomach was reported [23].

Third, antioxidant and anti-inflammatory effects are a key research interest today. Oxygen metabolism and mitochondrial functioning are involved in migraine pathophysiology and supplements with the ability to improve mitochondrial function have an effect, such as riboflavin, co-enzyme Q10 and alpha-lipoic acid [26]. Preclinical and clinical studies demonstrate that the major polyphenol compound of turmeric, curcumin, can decrease nitric oxide, calcitonin gene-related peptide and interleukin-6 and oxidative stress markers such as malondialdehyde. Furthermore, flavonoids found in chamomile, including apigenin, have anti-neuro-inflammatory effects in meningeal trigeminal nociceptors and inhibit cyclooxygenase-2 [27]. Thus, natural supplements are considered to provide a comprehensive and individual approach to migraine treatment, more large-scale, well-controlled studies using standardized extracts are needed to establish efficacy and safety longer term before they can be prescribed with confidence [26].

1.3.1 Introduction to Chlorogenic Acid CGA

Chlorogenic acid (CGA) is among one of the most prominent phenolics, and polyphenolic acid, that is formed by esterification of quinic acid and caffeic acid. It

is the most abundant of all the bioactive polyphenols in the human diet and is especially high in concentration in coffee, which contains up to 14% in dry weight, but the amount varies from 5.5% to 11.7% based on species and degree of maturity. Coffea canephora (Robusta and Conilon varieties) typically have higher content of chlorogenic acid than Coffea arabica. The amount of chlorogenic acid and caffeine vary greatly with the degree of maturation, with some genotypes having the highest levels reached at 60-80% maturity. CGA is also found in a variety of fruits and vegetables such as potatoes, apples, apricots, cherries, plums, and tomatoes and in beverages, like wine and tea [29].

CGA is structured as several isomers, 3-caffeoylquinic acid, 4-caffeoylquinic acid (cryptochlorogenic acid), 5-caffeoylquinic acid (neochlorogenic acid), and several di-caffeoylquinic acids, all of which are important for their biological activities [30].

CGA has a very well-defined and strongly studied pharmacological profile with several overlapping mechanisms, all of which address oxidative stress, inflammation, metabolic homeostasis and cell death pathways.

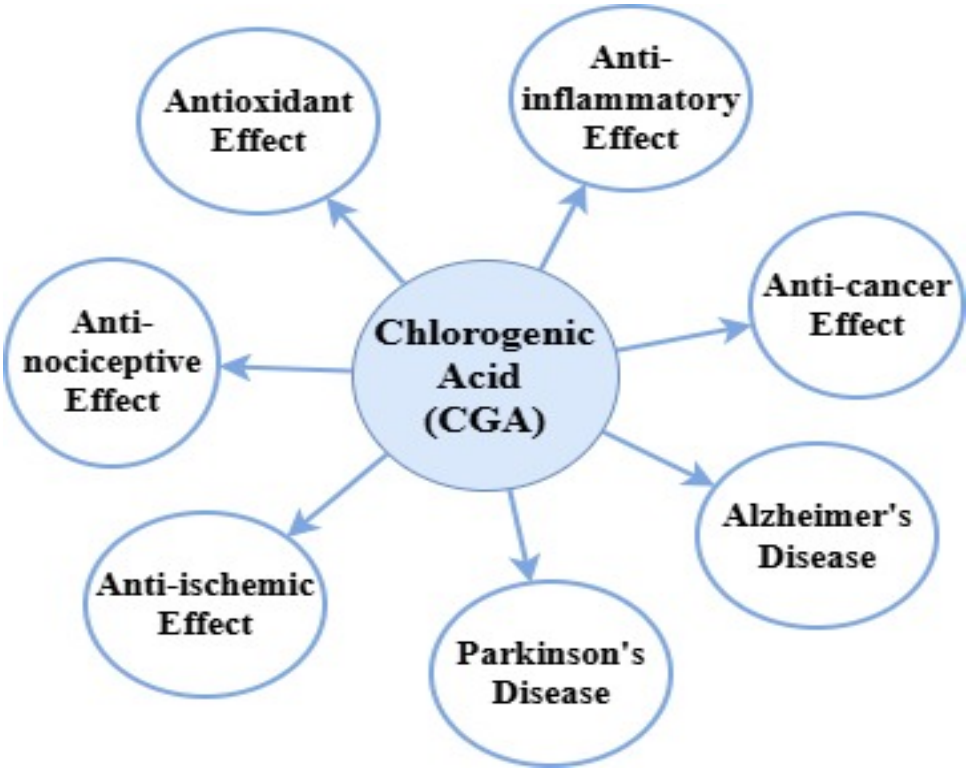


FIGURE 1.2: Pharmacological Profile of Chlorogenic Acid

The Figure-2 shows different pharmacological effects of chlorogenic acid compiled in a single flow chart. Different studies suggest that it shows anti-inflammatory and anti-oxidant effect showing the possibility to act in migraine pain pathways by acting on NF-kB pathway [30].

Chlorogenic acid is a very strong natural antioxidant that scavenges reactive oxygen species (ROS), increases the endogenous antioxidant defense mechanisms, activates the Nrf2 pathway and inhibits the activation of NF-kB pathway. CGA inhibits the generation of pro-inflammatory cytokines like TNF-α and IL-1β which downregulates neuronal sensitization and neuroinflammation that contribute to the migraine pathogenesis mechanism [31].

Chlorogenic acid has potent anti-inflammatory activity by blocking the NF-κB signaling pathway, thus decreasing the expression of pro-inflammatory cytokines like TNF-α and IL-1β, as well as inflammatory mediators like iNOS and NO [32]. Activation of these inflammatory pathways leads to neuroinflammation and sensitization of trigeminovascular nociceptive pathways in migraine pathogenesis, which is why chlorogenic acid could potentially help reduce migraine by inhibiting the inflammatory pathways [33].

In terms of bioavailability, CGA is well absorbed in human, with about 30% absorption from the small intestine and the rest is metabolized in the colon. Chlorogenic acid (CGA) is extensively biotransformed: approximately, one-third is absorbed directly in the upper gastrointestinal tract and the rest reaches the colon where it is metabolized by microfloral esterases into active metabolites including caffeic acid, quinic acid, ferulic acid, isoferulic acid, dihydrocaffeic acid and several phenolic and benzoic acid derivatives [34]. Preclinical studies have shown that CGA and its metabolites are able to penetrate the blood-brain barrier and have effects on the central nervous system. These metabolites and parent compounds are powerful antioxidants and anti-inflammatory agents that interfere with crucial inflammatory pathways (including NF-κB and MAPK pathways) and reduce pro-inflammatory mediators (including TNF-α, interleukins, and nitric oxide) [35, 36]. The time to maximum concentration varies between 0.6 to 1 hour for compounds

absorbed in the small intestine and 4.7 to 5.2 hours for colonic metabolites. The absolute bioavailability of CGA from green coffee extract is around 33% [37].

The CGA has a very good safety profile in several species. Acute toxicity studies showed that mice consumed CGA-enriched green coffee extract at 1 g/kg for two weeks without developing any side effects. The green coffee extract with 50% CGA did not show toxicity even after a single dose of 2 g/kg in rats, and daily CGA administration for 3-months at doses of 250, 500 and 1000 mg/kg also did not show any adverse effects in rats.

CGA consumption in humans is generally well tolerated, but moderate increases in plasma homocysteine levels of 12% and 11% have been reported with consumption of 2 g/day and 4 g/4 L water/day of black tea for 7 days respectively. Care must be taken in the concomitant use of CGA with some medications, as CGA can inhibit isoenzymes of cytochrome P450, and P-glycoprotein, and its long-term use should be conducted with caution until further studies are done on safety [32].

The oral bioavailability of CGA has been reported to be increased by 1.29-fold upon liposomal encapsulation and degradation is prevented during gastrointestinal digestion. The nanomicelles based on PLGA have been synthesized for achieving tissue residence time and sustained release of CGA. Furthermore, bovine serum albumin (BSA) assisted CGA silver nanoparticles has significant antioxidant and anti-neoplastic activity [29].

The CGA is a neuroprotective, systemic therapeutic agent with its primary mechanisms involving reduction of oxidative stress through its activation of Nrf2, suppression of neuroinflammation and systemic inflammation through its inhibition of NF-κB, MAPK and TLR4, prevention of apoptosis through its modulation of Bcl-2 family members and inhibition of caspases, preservation of synaptic function under pathological conditions, modulation of metabolic homeostasis through activation of AMPK and maintenance of gut barrier integrity and healthy gut microbiota. Due to its high bioavailability, crossing of the blood-brain barrier, excellent safety profile, and multi-target mechanisms, it is a very promising compound for long-term use in preventing and treating neurodegenerative diseases,

metabolic disorders, inflammatory diseases, cardiovascular diseases and even some types of cancers. Thus, it can be objectified as a leading compound in treatment and prevention of migraine.

1.4 Justification for the Mouse Model

Animal models are important for migraine studies because of the difficulty of studying the pathophysiology in humans. Human imaging studies may identify brain regions that are activated during migraine, but they cannot explain mechanisms at the cellular/molecular level. Animal models enable researchers to explore the sequence of events that trigger and sustain migraine attacks, directly assess the neuroinflammatory markers and release of neuropeptides from brain tissues, test potential therapeutic compounds before their application in humans and study the evolution of episodic to chronic migraine over time which are not feasible in humans [35].

One of the most popular and well-established migraine animal models is the nitroglycerin-induced migraine model. Nitroglycerin, a nitric oxide donor, causes a reproducible biphasic headache response in human subjects, with a headache response occurring immediately after infusion in both healthy volunteers and migraine patients, and a delayed migraine-like headache occurring 5 to 6 hours after infusion in migraine patients only, making this model attractive for use in translational research [17]. Systemic injection of NTG (10 mg/kg ip or sc) causes a potent spectrum of migraine-like symptoms and neuro-inflammatory processes in rodents. Both periorbital and hind paw mechanical allodynia is always seen when tested with von Frey filaments within the 60 to 120 minute timeframe following NTG administration [38]. Nitroglycerin (NTG) causes significant changes in the molecular structures of multiple brain regions related to pain processing [39]. An increase in levels of pro-inflammatory cytokines (such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α)) and changes in anti-inflammatory cytokines (interleukin-4 (IL-4) and interleukin-10 (IL-10)) have

been observed after NTG administration. Following NTG administration, both the expression of Iba1, a microglial marker, and microglial polarization toward a pro-inflammatory state is observed in the trigeminal nucleus caudalis (TNC). Moreover, TNC microglia activation leads to activation of the NLRP3 inflammasome, which triggers the maturation of IL-1β through activation of caspase-1.

The NF-κB signaling pathway is also activated following NTG exposure, along with an increase in expression and phosphorylation of the p65 subunit of NF-κB [40].

Neuropeptides such as calcitonin gene-related peptide, substance P and pituitary adenylate cyclase-activating polypeptide are increased. Importantly, the NTG model permits to differentiate episodic and chronic migraine states by different doses: a single dose of NTG will model episodic migraine state, whereas repeated doses of intermittent NTG every other day for 9 days will model a transition to chronic migraine state with sustained basal hypersensitivity. The model has been pharmacologically validated using standard migraine drugs such as sumatriptan, zolmitriptan, topiramate and olcegepant [41].

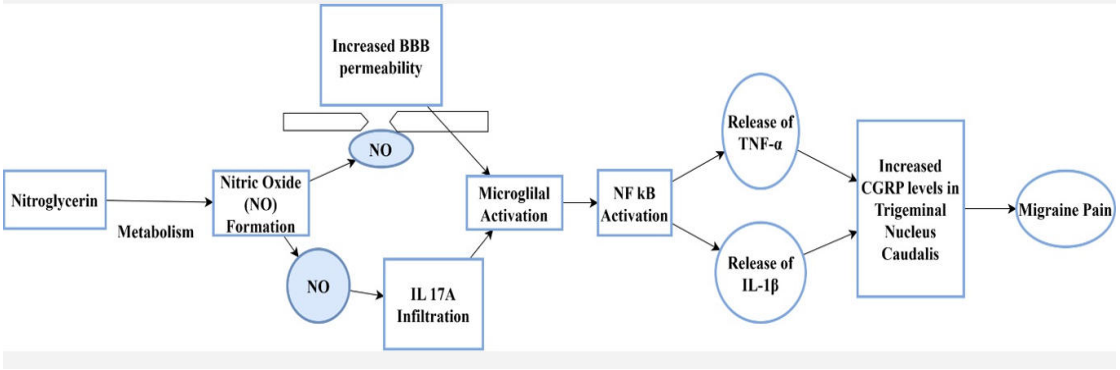


FIGURE 1.3: Mechanism of Nitroglycerin Induced Migraine

The figure 3 shows the mechanism of action of Nitroglycerin in inducing migraine. Nitroglycerin is first metabolized to Nitric Oxide (NO) which then causes infiltration of IL-17A into the microglial cells and causes activation of NF kB pathway that causes release of cytokines including TNF-α and IL-1β and release of Calcitonin Gene Related Peptide (CGRP) in the Trigeminal Nucleus Caudalis (TNC) region causing pain sensation and reduced threshold to pain [42, 43]. The NTG

model is a close parallel to the human condition, different models of NTG administration produce a migraine like state [44]. The model also enables the separation of episodic and chronic migraine states using different dosing regimens, a crucial issue since the research question is directed at assessing chlorogenic acid's effects in both acute and preventive treatment settings.

The NTG model has behavioral endpoints (mechanical allodynia, thermal hyperalgesia, light aversion) that are quantifiable and directly relate to clinical symptoms of migraine and can be objectively and reproducibly measured to evaluate efficacy of therapeutic interventions.

The model also produces strong and well characterized neuroinflammatory changes that are directly involved in chlorogenic acid's mechanism of action, such as activation of nuclear factor-kappa B pathway and increased levels of pro-inflammatory cytokines [45].

1.5 Research Gap, Questions and Hypothesis

The antioxidant, anti-inflammatory and neuroprotective activities of chlorogenic acid (CGA) have been comprehensively studied in various neurological disorders like Alzheimer's disease, Parkinson's disease, cerebral ischemia and other diseases, however, the efficacy of CGA in migraine has not been systematically explored.

The NTG-induced migraine model has been well validated for episodic and chronic migraine and shows many of the pathophysiological features associated with migraine, such as: mechanical allodynia, photophobia, neuronal activation, microglial activation, activation of the NF-κB pathway and increased levels of pro-inflammatory cytokines (TNF-α, Il-1β) and CGRP. With direct modulation of these pathways by suppressing NF-κB pathway, reducing inflammatory cytokines and oxidative stress and suppressing glial activation, there is significant mechanistic support for the anti-migraine activity of CGA. However, there are no studies yet to assess if systemic CGA administration is able to reduce NTG-induced migraine-like pain and neuroinflammation in either acute or chronic models. In addition, the

best dose-regimen and comparative effectiveness of CGA versus other medications used to treat migraine (both acute and preventative) are not known.

1.5.1 Research Hypothesis

Chronic treatment with chlorogenic acid will decrease migraine-like pain behaviours and neuroinflammatory changes caused by chronic nitroglycerin in mice models of migraine.

1.6 Research Objectives

To evaluate the anti migraine activity of chlorogenic acid (CGA) in a nitroglycerin (NTG) induced mice models of migraine, focusing on pain behavior and neuroinflammatory/oxidative mechanisms.

To evaluate the effect of chlorogenic acid pretreatment on NTG induces thermal hyperalgesia in mice models of migraine utilizing hot plate test.

To assess the effect of chlorogenic acid pretreatment on NTG-induced thermal allodynia in mice models of migraine using the Tail Flick test.

To assess mechanical allodynia induced by NTG (hind paw and periorbital regions) in mice models of migraine using von Frey filament test before and after chlorogenic acid pretreatment.

To evaluate the effect of chlorogenic acid pre-treatment on NTG induced photophobia in mice models of migraine using Light Aversion test.

To assess the anti-inflammatory effects of pre-treatment with chlorogenic acid in NTG-induced neuroinflammation in mice models of migraine by measuring the concentration of proinflammatory cytokines including IL-1 and TNF- in the cerebral cortex and trigeminal nucleus caudalis (TNC).

Chapter 2

Literature Review

2.1 Understanding Migraine Beyond Headache

Migraine is a chronic episodic neurological condition not just a common headache. It is officially described in the International Classification of Headache Disorders (ICHD-3) as a recurrent headache disorder with attacks of 4-72 hours. The headache phase is usually pulsating and has moderate to severe pain and is worsened by normal physical activity. Common symptoms associated with attacks include vomiting, nausea, sensitivity to sound (phonophobia) and sensitivity to light (photophobia) [10, 13].

Migraine is divided into four phases including premonitory phase, aura phase, headache phase and postdrome phase. Premonitory phase occurs a few hours to days before the onset of headache, symptoms may include fatigue, mood changes (depression or euphoria), yawning, food cravings, and stiff neck. While, Aura phase is present about one-quarter of the time and consists of fully reversible neurological symptoms. Most often there are visual disturbances (scotoma, fortification spectra) but sensory, speech or motor aura may occur. Aura is related to depolarization of neurons and glia (cortical spreading depression, CSD). Headache

phase is characterized by moderate to severe pain in head including sensory disturbances and feeling of nausea and vomiting. Postdrome phase is the phase in which patients may feel hangover, anorexia and tiredness [1].

Migraine is characterized into two types based on frequency of headaches.

Episodic migraine is characterized by 0-14 headache days per month. This is the more common type and is the form found in most people who suffer from migraines [46].

Chronic migraine is a severe, debilitating migraine condition according to ICHD-3 that is experienced 15 or more days a month, for at least three months, with 8 or more of those days being migrainous. CM is sometimes thought of as a "transformation" or progression of attacks, typically increasing in frequency over time, sometimes in association with medication overuse or other factors [46, 47].

Globally, migraine represents a major public health challenge, affecting over a billion individuals. World prevalence estimates indicate that roughly 11.6% of people, approximately one in every ten are impacted by the condition. Based on 2021 Global Burden of Disease assessments, global prevalent cases climbed past 1.16 billion, reflecting an approximate 58% expansion in disease burden relative to 1990 figures [48, 49].

Migraine is around 2-3 times more prevalent in females (13.8%) than males (6.9%). Prevalence is slightly higher in boys before puberty, and increases rapidly in girls after their first menses. This is thought to be due to hormonal changes, such as the effect of estrogen on pain processing pathways and neurotransmitter systems [10, 49].

Migraine affects all ages, but is most common age 25-55. It then slowly decreases from 55-60 years. This high point of the career phase allows for the greatest societal and economic value since there are important career and family demands [13].

Migraine is not evenly distributed around the world. There is considerable variation in prevalence estimates across regions: the highest reported are in Central

and South America (16.4%), and the lowest in North America (9.7%) and Asia (10.1%) [49]. Importantly, there is a positive correlation between a country's Socio-demographic Index (SDI) and its migraine burden such that high-SDI regions (Western Europe, North America) have the highest age-standardised rates. This could simply be a diagnostic awareness improvement and a lifestyle modification (stress, sedentarism) or an ageing population that is not a biological difference [48].

Interestingly, in high-income countries such as the US, there's a negative correlation with socioeconomic status, which shows the complex interplay of social determinants.

2.1.1 Key Pathophysiological Mechanisms

The prevailing theory now holds that migraine is a disorder of the brain, rather than of the vasculature and the key mechanisms include:

Cortical Spreading Depression (CSD) is a slowly moving sustained depolarization of neurons across the cortex.

This electrophysiologic finding, the CSD, is believed to be the neural correlate of the migraine aura and is believed to activate the trigeminovascular system [10].

This results in the release of interleukin 17A (IL 17A), which leads to vasodilation, plasma protein extravasation and mast cell degranulation further releasing inflammatory cytokines TNF-α and IL-1β leading to neurogenic inflammation which is an important mechanism responsible for pain.

The chronic or high level nociceptive input may result in central sensitization in the trigeminal nucleus caudalis. This leads to increased sensitivity to pain and non-painful stimuli, and is clinically apparent as allodynia (cutaneous) pain from non-painful stimuli such as touching the scalp [11].

2.2 Current Pharmacological Management of Migraine and Unmet Needs

Treatment for acute migraine is designed to quickly and effectively end the migraine attack, restore function and minimize recurrence with minimal side effects. Therapy is based on the severity of attacks, presence of accompanying symptoms (especially nausea/vomiting), escalation of pain symptoms and patient comorbidities [50, 51]. Drugs can be classified into two groups: nonspecific therapy and migraine specific therapy.

The non-specific agents are mainly prescribed for mild to moderate attacks and as adjunctive treatment. A combination of acetaminophen, aspirin and caffeine (AAC) has evidence of efficacy in severe migraine and is the first-line over the counter (OTC) treatment. The NSAIDs with good efficacy are ibuprofen (200-400 mg), naproxen sodium (500-550 mg), diclofenac potassium (50 mg) and acetylsalicylic acid (ASA, 500-1000 mg). Notably, the soluble or powder formulations are ideal for severe, rapidly increasing pain. Naproxen has a longer half-life and a favourable cardiovascular safety profile thus, is recommended as a preferred NSAIDs in many guidelines [51].

Metoclopramide (10 mg), prochlorperazine (10 mg) and domperidone are effective for associated nausea and also as primary migraine terminating agents, especially when administered through parenteral route [52]. The use of butalbital is contraindicated and the use of opioids (e.g., codeine, tramadol) is strongly discouraged. They are preferred as a last resort treatment when every other treatment fails or is contraindicated [50, 51].

There are 7 available triptans including rizatriptan, sumatriptan, eletriptan, zolmitriptan, almotriptan, naratriptan and frovatriptan that continue to be the treatment of choice for moderate-to-severe migraine. They work well, are safe to take and come in several different dosagecforms (tablet, orally disintegrating tablet, nasal spray, subcutaneous injection). Pharmacokinetic profiles guide selection based on onset of action describes sumatriptan, rizatriptan, eletriptan,

zolmitriptan and almotriptan as fast acting (30-60 mins) and suitable for fast-peaking attacks. While, naratriptan and frovatriptan are considered as slow acting. Subcutaneous sumatriptan (6 mg) is the fastest and most reliable, and indicated when fast onset is important or when oral absorption is difficult (e.g. vomiting). In patients with uncontrolled hypertension, coronary artery disease, stroke or hemiplegic migraine, triptans are contraindicated because of their vasoconstrictive effects [52].

Repurposed oral drugs from a variety of classes, with different evidence levels, had been used for decades to prevent migraine. These include following preventive therapies. Propranolol and timolol are FDA approved and have established efficacy for episodic migraine. They are especially beneficial when used in patients who have hypertension or angina pectoris as well.

They have contraindications in asthma, heart failure and insulin-dependent diabetes, and are associated with side effects, including fatigue, bradycardia and depression which reduces adherence [53, 54].

Topiramate is FDA-approved agent. Topiramate (target dose 100 mg/day) is an effective drug in decreasing migraine days in many patients (by ˜50%), but is known to cause cognitive dysfunction, weight loss, and teratogenicity [55, 56]. Amitriptyline is used widely off-label, particularly when insomnia is present or when there is comorbid depression. Low doses (10-25 mg at night) are well-tolerated, but are associated with anticholinergic effects (dry mouth, constipation, sedation) and weight gain [54]. Lisinopril and candesartan have been demonstrated to provide benefit in small, randomized controlled trials, appear well-tolerated and provide dual benefit in patients with hypertension. They have not been approved by the FDA for migraine and should be avoided during pregnancy [55].

OnabotulinumtoxinA is approved by FDA for chronic migraine (15 headache days/month) and is given every 12 weeks. It is effective for chronic migraine [51]. The most important recent breakthrough of the past decade has been the targeting of the calcitonin gene-related peptide (CGRP) pathway with monoclonal antibodies (mAbs). These agents are four in all: erenumab (CGRP-receptor antibody),

fremanezumab, galcanezumab and eptinezumab (CGRP-ligand antibodies). Results from a meta-analysis of 13 RCTs (6,979 patients) showed that monthly subcutaneous injection was effective in decreasing the number of monthly migraine days at week 4 and maintained this effect throughout the 12-week study [57].

2.2.1 Limitations and Gaps

Triptans may cause mild cognitive impairment, dizziness, CNS side effects (more common with frovatriptan, rizatriptan, zolmitriptan, eletriptan), non-coronary chest pain (may cause wariness to patients using a triptan again), and transient chest discomfort (unknown origin, not associated with cardiac ischemia, no clinical consequences) [58].

Topiramate can cause paresthesias, memory/concentration issues, glaucoma, hyperchloremic acidosis, nephrolithiasis and weight loss. In 10% of women of child bearing age, valproate causes gain of weight, drowsy feeling, thinning of hair and alopecia, tremor, GIT disturbance, dysfunction of bone marrow, pancreatitis, hepatitis, kidney problems, and polycystic ovaries.

TABLE 2.1: Available Therapies for Migraine and Gap in Effectiveness

Therapies	**Examples**	**Side effects/gaps in effectiveness**
Acute Therapies	Triptans, NSAIDs, opioids	Hepatotoxicity, asthma, rashes, GI bleeding, Respiratory depression, urinary retention [59, 60].
Preventive Therapies	Antidepressants	Sedation, hypotension, weight gain [61].
	Anticonvulsants	GI disorders, dizziness, blurred vision, diplopia, sedation [62].

The side effects of Amitriptyline include hypersomnolence (80%) and dry mouth (69%). Drowsy feeling, fatigue, lethargy, disorders of sleep, nightmares, depression, memory problems, hallucinations and reduced exercise tolerance are all side

effects of beta-blockers [56]. Verapamil causes constipation. Ergotamine is a non-selective serotonin, dopamine and adrenergic receptor stimulator (which causes more side effects, such as nausea and vomiting, peripheral and coronary vasoconstriction). Anti-emetics are needed for pretreatment with dihydroergotamine [58].

2.3 The Rationale for Neuroprotection in Migraine

Neuroinflammation is the activation of glial cells (mainly microglia, astrocytes) and the simultaneous production of inflammatory mediators in the central nervous system (CNS). Neuroinflammation in migraine is considered to be an inflammatory process, as opposed to systemic inflammation. Rather, it is activated by endogenous factors like cortical spreading depolarization (CSD), neuronal stress and tissue injury [63, 64].

Neurogenic inflammation is the activation of peripheral release of neuropeptides (such as CGRP, substance P) from sensory nerve terminals that leads to the vasodilation and plasma extravasation in the dura mater, whereas neuroinflammation is the larger, chronic immune response in the brain parenchyma involving glia, cytokines, and inflammatory signaling cascades [63, 65].

The cortical spreading depression (CSD) is a major trigger of neuroinflammation. CSD is a slow (2-6 mm/min) depolarizing wave of neurons and glia that is characterized by large inward and outward ionic fluxes (K^+ efflux, Na^+/Ca^{2+} influx), sustained suppression of the neuronal activity and metabolic stress [66, 67].

Nitric oxide (NO) is an important signaling molecule involved in the pathogenesis of migraine. Nitroglycerin (NTG) is an NO donor that is well established to reliably trigger migraine-like headaches in susceptible people and used as an experimental model. Administration of NTG in the rodents causes higher levels of IL-1β and TNF-α by microglial activation [18].

Tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) are among the key pro-inflammatory cytokines involved in neuroinflammation that have been considered to have a role in migraine and represent good candidate biomarkers of trigeminovascular inflammation. In both experimental models and patients with migraine, the increased levels of these cytokines are shown to promote neuronal hyperexcitability, activation of glial cells, increased blood–brain barrier permeability and peripheral and central sensitization that initiate and maintain migraine pain. The production of both TNF-α and IL-1β is principally regulated by activation of the nuclear factor-kappa B (NF-κB) pathway that is activated owing to oxidative stress and inflammatory mediators. One of the main upstream mechanisms involved in this inflammatory cascade is calcitonin gene-related peptide (CGRP), the primary neuropeptide secreted by activated trigeminal sensory neurons during migraine attacks. In addition to causing vasodilation and nociceptive transmission, excess release of CGRP activates satellite glial cells and immune cells in the trigeminal ganglion, which results in activation of NF-κB and up-regulation of the inflammatory cytokines, TNF-α and IL-1β.

In turn, these cytokines further boost the production and release of CGRP, thus establishing a positive feedback loop, which maintains neurogenic inflammation, increases trigeminovascular sensitization and plays a role in the chronification of migraine. Therefore, the concentration of TNF-α and IL-1β can be used as an important measure of the degree of neuroinflammation detected in the brain, and the inflammatory cascade involved in migraine pathogenesis [63, 68].

2.4 Chlorogenic Acid CGA: A Candidate Neuroprotective Agent

Chlorogenic acid (CGA) is among the most widely distributed phenolic compounds in the plant kingdom, and is known for its various biological activities and potential health implications. Its potent antioxidant, anti-inflammatory, antimicrobial, neuroprotective, and metabolic regulatory properties have led to significant research

interest in chlorogenic acid as a naturally occurring polyphenol. Coffee, fruits, vegetables and various medicinal plants are the main sources of the compound, which is part of the human diet. The understanding of the chemical structure and the natural occurrence of chlorogenic acid is essential to the understanding of its use as a nutraceutical, and its potential therapeutic uses [69]. Its modern IUPAC name is 3-O-caffeoylquinic acid or 5-O-caffeoylquinic acid and is an ester of caffeic acid with quinic acid. Payen first discovered a crystalline potassium caffeine chloroginate in green coffee beans in 1846, which led to the name "chlorogenic acid. Its name comes from the Greek word "chloros", meaning green, and the word "gennao", meaning to produce, because the compound produces a green color upon reacting with ammonia and oxygen, but it has nothing to do with chlorine [70, 71].

In 1932, Fischer and Dangschat deduced that chlorogenic acid was 3-caffeoylquinic acid. In 1976, the International Union of Pure and Applied Chemistry (IUPAC) decided to change the numbering of the atoms on the quinic acid ring and redesignated 3-caffeoylquinic acid as 5-O-caffeoylquinic acid (5-CQA). Although this official name change has been made, the older name, 3-caffeoylquinic acid, is still used in much of the literature and by many commercial suppliers [69, 70].

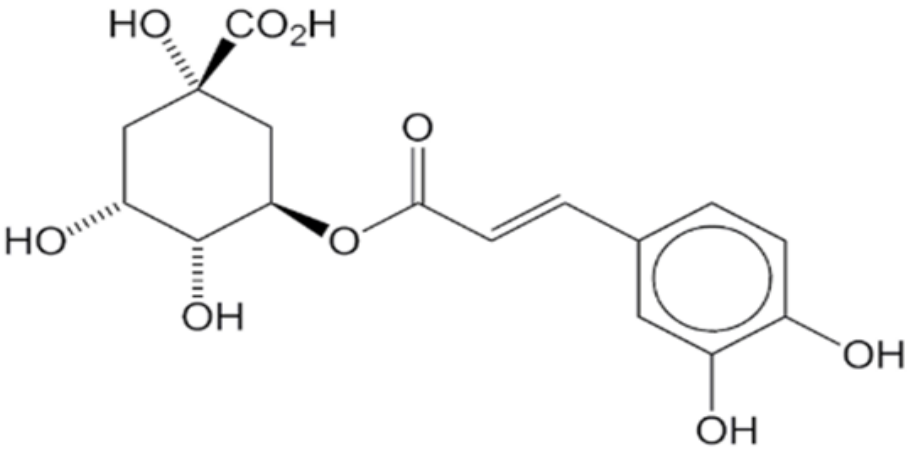


FIGURE 2.1: Chemical Structure of Chlorogenic Acid

Chlorogenic acid can be represented by its molecular formula, $C_{16}H_{18}O_9$, and has a molecular weight of 354.30 g/mol. It is composed of three strategically located

labile groups including ester bonds, unsaturated double bonds and dibasic phenols. The molecule has five hydroxyl groups and one carboxyl group that accounts for its biological reactivity [72, 73].

The Figure-3 shows the chemical structure of chlorogenic acid which contains two adjacent phenolic groups (Catechol) which makes it a potent hydrogen donor and anti-oxidant. It also contains ester linkages which make it more stable and increase bioavailability. The presence of carboxylic and hydroxyl group also makes chlorogenic acid more polar and act as anti-oxidant agent [32].

The chemical structure has a quinic acid, to which there is attached a caffeic acid residue, that is a phenylalanine compound formed as a result of the shikimic acid pathway in the aerobic respiration of plants. The hydroxyl (-OH), carboxyl (-COOH) and ortho-diphenol hydroxyl groups are the key functional groups that contribute to the bioactivity of chlorogenic acid. These groups allow chlorogenic acid to be involved in hydrogen atom transfer, free radical scavenging and metal chelation processes that are fundamental to its antioxidant activity [32, 74].

Chlorogenic acid is a member of a family of compounds called chlorogenic acids (CGAs), which are esters of different trans-hydroxycinnamic acid derivatives (most frequently caffeic acid, p-coumaric acid and ferulic acid) with quinic acid. This family contains many different compounds depending on the position, identity and number of the acyl residues [70, 75].

The solubility of Chlorogenic acid is high in polar solvents like water, ethanol, methanol, acetone and is insoluble in non-polar solvents like benzene, chloroform and petroleum ether. The order of the water solubility of chlorogenic acid isomers is 1-acyl $>$ 3-acyl $>$ 4-acyl $>$ 5-acyl, in which the more the free equatorial OH groups, the more soluble it is [70, 75]. One of the drawbacks of chlorogenic acid is its chemical instability. Chlorogenic acid is sensitive to temperature when heated in water, it undergoes isomerization, hydrolysis and transesterification. During roasting, chlorogenic acid is lost in coffee at the rate of 8-10% per 1% of dry matter lost, up to 95% during drastic roasting conditions. The intramolecular acyl migration and transesterification increase under basic conditions. Chlorogenic

acid can change trans-cis when exposed to UV light or visible light. The stability problems are significant in the fields of pharmaceutical formulation, where they require careful handling and the creation of more sophisticated delivery systems like liposomes, micells and nanoparticles [32].

The poor bioavailability of chlorogenic acid severely limits its clinical activity. To elucidate the potential health benefits of chlorogenic acid, and to develop safe and effective therapeutic strategies, it is important to understand the pharmacokinetic fate of this compound, i.e. its absorption, distribution, metabolism and excretion (ADME).

Chlorogenic acid (5-O-caffeoylquinic acid) has a molecular weight of 354.30 g/mol and the characteristics of five hydroxyl groups and one carboxyl group give the compound high water solubility, but also make it vulnerable to enzymatic and chemical degradation.

Caffeic acid and quinic acid bond is hydrolyzed by gastrointestinal tract and tissue esterases. Chlorogenic acid can degrade in presence of heat, light, pH conditions and enzymes [70, 76]. These properties combine to give low absolute bioavailability (the proportion of a dose given orally that appears in systemic circulation in its active form). The chlorogenic acid is absorbed in the intestine and blood [76].

Chlorogenic acid is absorbed in several locations throughout the gastrointestinal tract. Initial research in rats has shown that chlorogenic acid is absorbed as is in the stomach. But, most absorption takes place in the small intestine [72].

The absorption of chlorogenic acid is compared to its metabolite, caffeic acid, using a well-established human intestinal epithelial cell monolayer model and the uptake of chlorogenic acid is significantly lower than that of the metabolite, caffeic acid. Importantly, the uptake of caffeic acid was shown to be pH dependent in the extracellular compartment, but uptake of chlorogenic acid was not [76].

Hydrolysis of the ester bond is the main metabolic pathway of chlorogenic acid, resulting in caffeic acid and quinic acid. This reaction is catalyzed by Intestinal

esterases (in the brush border of enterocytes of small intestine), Microbial esterases (enzymes made by the bacteria in the colon, such as species of the genera Lactobacillus, Bifidobacterium and Clostridium) [76]. After absorption to the systemic circulation, chlorogenic acid and its metabolites bind to the plasma proteins, mainly to human serum albumin (HSA).

Moderate affinity binding (Kb ˜ 104 L/mol) implies that a considerable portion of the circulating chlorogenic acid is protein-bound, and this can extend the half-life of plasma (due to renal clearance) and reduce the amount of free drug in tissues available for distribution [77].

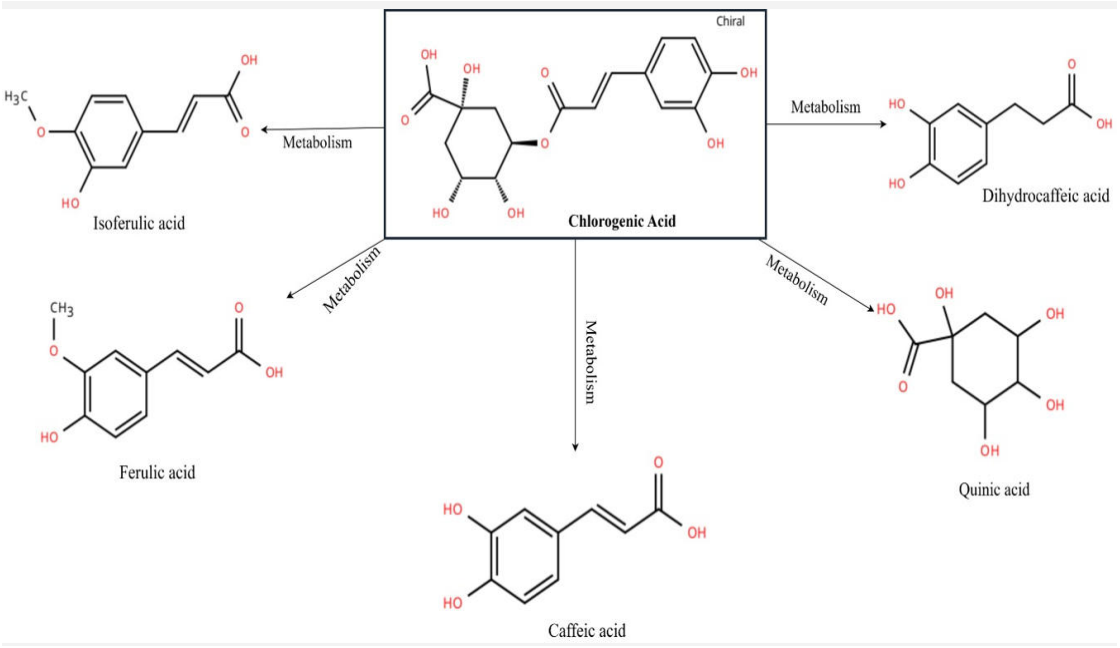


FIGURE 2.2: Metabolites of Chlorogenic acid

Chlorogenic acid is extensively metabolised in the body, with partial absorption in the small intestine or hydrolysis by the microorganisms present in the colon (e.g. Escherichia coli, Bifidobacterium spp. and Lactobacillus spp.) into important metabolites such as caffeic acid, quinic acid, ferulic acid, dihydrocaffeic acid and some derivatives of the phenylpropionate [34].

They are able to pass the blood brain barrier and produce strong neuroprotective, anti-inflammatory and antioxidant effects which have important therapeutic implications in the treatment of migraine. In particular, CGA and its metabolites have been shown to exert anti-inflammatory and antioxidant effects, by down-regulating the production of pro-inflammatory cytokines, including TNF-alpha,

IL-1beta and IL-6, down-regulating the NF-kappaB and MAPK signaling pathways, and scavenging ROS through the Nrf2 pathway, all of which have been implicated in migraine pathogenesis [37]. Chlorogenic acid and its metabolites can reach the brain tissues, which is consistent with the neuroprotective properties of this compound [78].

Renal excretion is the main route of elimination for chlorogenic acid and its metabolites. Most of the radioactivity recovered after oral exposure is found in the urine in the form of a variety of metabolites and conjugates [71]. A portion of chlorogenic acid and its metabolites is excreted in the bile and metabolites are also detected in the feces. Residence time of some metabolites in the body may be prolonged by the enterohepatic circulation [71].

2.4.1 Established Pharmacological Activities Relevant to Migraine

Chlorogenic acid (CGA) has several pharmacologic activities directly relevant to important pathophysiological mechanisms involved in migraine. These include strong antioxidant, anti-inflammatory, neuroprotective activity, modulation of pain pathways and regulation of vascular function [71]. A key underlying mechanism in the pathophysiology of migraine is neuroinflammation, which is characterized by activation of glial cells and the production of pro-inflammatory mediators. Chlorogenic acid has strong anti-inflammatory activities by several mechanisms at the molecular level [32]. Nitric oxide is an important player in the migraine pathogenesis. Nitroglycerin (NTG) is an NO donor that is able to produce migraine-like headache in susceptible people and is a standard experimental model of migraine. The inhibitory effects of chlorogenic acid on NO production have several mechanisms including inhibition of inducible nitric oxide synthase (iNOS) expression in activated macrophages, thus lowering NO production. chlorogenic acid blocks positive feedback loop of NO stimulations of CGRP release, which leads to the inhibition of iNOS [70]. The anti-inflammatory properties of chlorogenic acid target several aspects of the migraine pathophysiology including chlorogenic acid may

reduce the CSD-induced neuroinflammation by inhibiting activation of NF-κB. Chlorogenic acid has anti-inflammatory effects, which could lessen the production of peripheral and central sensitization, by decreasing the TNF-α and IL-1β levels. Chlorogenic acid reaches the colon in high concentrations, where it has anti-inflammatory effects on the colon, these effects, in turn, may have effects on migraine via the gut-brain axis.

2.5 Preclinical Models of Migraine: Evaluating Efficacy

The complex neurovascular, genetic and behavioral aspects of migraine make it impossible to fully simulate in silico or in vitro. Human studies are ethically and practically limited and cannot be used to examine the antimigraine effect of chlorogenic acid [79]. Thus, the use of animal models is ethical and enable to explore the pathway of disease completely as a depiction in human subjects (e.g., repeated nitroglycerin (NTG) used to induce chronic migraine in animal models) [15, 80]. The animals models also help in screening for possible toxicity and side-effects (e.g. coronary vasoconstriction from triptans) prior to first-in-human trials [35]. While human migraine can be triggered by various unknown, non-repeatable factors, genetic influences and environmental factors, the animal model enables systematic, controlled experimentation, a key to scientific validity. This controlled setting allows researchers to establish cause-and-effect relationships, such as showing that medicine overuse headache (MOH) like hypersensitivity that can occur with long-term treatment of sumatriptan or that light aversion does not occur when CGRP receptor blockade is used. This type of cause-effect relations cannot be established in human observational studies [21, 45].

Due to the difficulty of modelling this complex neurological disorder in animals, the development of effective migraine therapies has been severely hampered. Thus, different types of animal models have been designed in different articles that can mimic the pathophysiology of migraine among which nitroglycerin model is the

most similar model that depict symptoms of migraine clearly in animal as observed in human subjects. The NTG paradigm is among the most popular and translationally relevant paradigms. Nitroglycerin is a nitric oxide (NO) donor, which has been shown to definitely elicit headache in humans and migraine-like symptoms in migraine patients and is therefore a powerful human-relevant trigger. Acute, transitory episodes of pain have been produced in mice by an i.p. injection of NTG (usually 10 mg/kg). These consist of thermal hyperalgesia (hot plate test) and mechanical allodynia (von Frey filaments on the hind paw) [38]. In this model, neuronal activation markers such IL 17A are also elevated in the trigeminal nucleus caudalis (TNC) which is a crucial relay station in the brainstem that receives pain signals from the craniofacial region. Acute NTG model is sensitive to common abortive drugs, such as sumatriptan and zolmitriptan. Chronic intermittent NTG (e.g., 10 mg/kg i.p. every other day for 9-11 days) is administered [5, 21]. This has been beneficial in screening preventive therapies in this model. Tipton et al. (2016) showed that the known preventive drug propranolol also prevented acute and chronic NTG-induced hyperalgesia and that acute therapy with sumatriptan did not affect basal hypersensitivity, but actually produced a MOH-like state. Interestingly, in this model, the anticonvulsant valproate did not work, which indicates that the model is not fully predictive, and shows the heterogeneity of migraine. The EM model has higher neuronal activation (IL 17A) and microgliosis (Iba1) in the TNC, and the CM model has more activation in a higher-order pain modulation region, the anterior cingulate cortex (ACC) [21]. This implies a change in the migraine disease process from the brainstem to cortical regions.

2.6 Previous Studies on Polyphenols and Chlorogenic Acid in Pain and Migraine Models

There is also a good body of literature on other dietary polyphenols, with similar key mechanisms of action (antioxidant and anti-inflammatory) that are of direct

relevance to the pathophysiology of migraine. These studies provide a solid conceptual basis to anticipate a compound such as CGA to be effective. Resveratrol is well known and has been studied for its neuroprotective properties.

In particular, a comparative study reported that the protective effect of resveratrol, like that of CGA, against hydrogen peroxide (H_2O_2)-induced oxidative alterations is observed in slices of rat brain and this is a main factor causing cortical spreading depression and migraine pain [73].

Another extensively studied polyphenol compound, curcumin is found to be protective against liver injury caused by gentamicin through its ability to reduce oxidative stress, inflammation and apoptosis.

The anti-apoptotic and anti-inflammatory effects are of great relevance to migraine, as long and intense attacks may induce neuroinflammation and neuronal sensitization [31].

Like the CGA, quercetin has been studied for its protective properties with respect to oxidative damage. There is experimental evidence that it reduces the damages caused by diabetes in sciatic nerves, thus establishing its use as a potential agent in neuropathic pain models.

This is important because migraine is associated with acute pain, and when chronic, central sensitization occurs which shares characteristics with neuropathic pain [31].

All of these examples support the potential for systemic effects of dietary polyphenols, including neuroprotective, antioxidant, and anti-inflammatory activities, that would lend a strong rationale to investigate CGA in migraine, a disease characterized by neurogenic inflammation and oxidative stress.

Although none of the articles provided explores CGA in a classical migraine model (such as nitroglycerin or CGRP infusion), a significant and compelling amount of evidence supports the robust activity of CGA in pathways, pain models and molecular pathways closely connected to migraine pathology.

2.7 Critical Summary and Identification of the Research Gap

In recent years, migraine has been acknowledged as a complex neurobiological disorder in which neuroinflammation, oxidative stress, and central sensitization are key components in the initiation and progression of migraine. Significant evidence has emerged that activation of inflammatory signaling pathways, including the NF-κB pathway, results in secretion of pro-inflammatory cytokines including tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β). These cytokines are involved in the hyperexcitability of neurons, activation of glia and sensitization of trigeminovascular nociceptive pathways that allows the maintenance and spread of migraine pain. For this reason, both TNF-α and IL-1β are considered to be significant neuroinflammatory markers for the assessment of the inflammatory state of migraine. They are not just inflammatory mediators, but they are also involved in several upstream mediators that play a role in migraine pathogenesis such as oxidative stress, NO signaling and neuropeptides like CGRP.

While there is significant progress made in the understanding of the migraine pathophysiology, the therapeutic options available are still limited by poor efficacy, adverse effects, the fact that some medications lead to medication overuse headache, cardiovascular contraindications, and the high cost of recently developed targeted drugs. This has led to the increasing interest in naturally derived compounds which are multimodal and have both antioxidant and anti-neuroinflammatory properties.

One of these compounds, chlorogenic acid (CGA), has been found to possess strong antioxidant, anti-inflammatory and neuroprotective properties across various experimental models. In previous studies, it has been demonstrated that chlorogenic acid can inhibit NF-κB signaling pathway, reduce the production of pro-inflammatory cytokines such as IL-1β and TNF-α and suppress oxidative stress. Although these encouraging results have been found, and this treatment has been known to work in migraine, it has not been fully studied in this regard. Specifically,

very less is known about the effect of chlorogenic acid on reducing neuroinflammation in important brain regions involved in the pathogenesis of migraine, such as the cerebral cortex and the trigeminal nucleus caudalis (TNC), where inflammatory mediators play a role in the process of nociceptive processing and central sensitization. In addition, there are very few studies that have investigated the impact of chlorogenic acid on migraine-like changes in behavior, with histopathological analysis and inflammatory cytokine expression in a well-established experimental migraine model.

Thus, the neuroprotective effect of chlorogenic acid against nitroglycerin-induced migraine and its ability to modulate the levels of TNF-α and IL-1β in central regions involved in pain processing, along with its effect of improving behavioral and histopathological changes associated with migraine, remain unexplored. The aim of the present study is to fill this gap and explore the consequences of chlorogenic acid treatment on behavioral outcome, histopathology of cerebral cortex and trigeminal nucleus caudalis, as well as the expression of the neuroinflammatory markers, tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) in a mouse model of migraine induced by nitroglycerin (NTG). This study is designed to look at oxidative stress and neuroinflammatory pathways to provide additional data that make chlorogenic acid a potential therapeutic candidate for the management of migraine. This gap is logical and prospective and will provide a novel therapeutic approach to migraine through the translation of the known advantages of this dietary polyphenol.

Chapter 3

Materials and Methods

3.1 Animals

The investigation utilized healthy adult female BALB/c mice weighing between 20 and 25 g, obtained directly from the Capital University of Science and Technology (CUST) animal unit. Before assigning into groups, all mice were examined for any signs of illness or injury. Only healthy animals were included in the experiment.

The Experimental protocols were approved by Capital University of Science and Technology, Islamabad, Pakistan (Approval CUST/DERC/PH/ERC/PG/2026/026). All procedures were performed according to the recommendations of Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and the committee's instructions.

3.2 Housing Condition

Animals were kept in group housing in cages on vertical racks in an environmentally controlled animal facility. The room conditions were controlled using an automated timer system such that the temperature was maintained at 22 ± 2 °C,

relative humidity at 50–60% and 12 hours of light/dark cycle (08:00 to 20:00 h). Adequate ventilation and air exchange was maintained for a specific pathogen free environment.

Clean bedding materials (autoclaved wood shavings) were placed and changed at least every 48 hours to reduce the ammonia buildup, and to maintain cleanliness. Cages were regularly cleaned with 70% ethanol, and extra space was given to allow for the normal locomotor activity of the animals, avoiding overcrowding.

3.3 Food Consumption

The mice that were in this study were given a standard laboratory pellet diet, designed to provide the mice with their required daily intake of nutrients. The diet was prepared from ingredients that were locally available and can provide a good quality and reliable experimental outcome.

Table 3.1: Dietary Composition

Dietary Component	**Proportion (%)**	**Mass (Kg)**
Wheat Flour	50.0	2.50 Kg
Chokar (Bran)	15.0	750 g
Fish Meal	15.0	750 g
Dry Skimmed Milk	5.0	250 g
Vitamins/Minerals Mix	5.0	250 g

3.4 Acclimatization

Prior to initiating any experimental procedures, the animals underwent a 7-day adaptation period in standardized polycarbonate cages. Throughout this phase, mice were housed six per cage in a controlled environment maintained at 24 ± 1°C, 55–60% humidity, and a regulated 12-hour light and dark schedule (08:00 am to

08:00 pm) with continuous availability of food and water. Daily gentle handling (5–10 minutes per cage) was practiced to minimize stress and acclimatize the animals to routine testing.

Gentle handling was carried out daily for 5-10 minutes per cage to condition animals to handling. This led to a decrease in anxiety-related behaviors and helped to ease the handling of the animals during the next administration of the drug and behavioral testing. After acclimatization, mice were randomly allocated to experimental groups, based on their body weight, to ensure even distribution among experimental groups.

3.5 Mice Handling

All the procedures for handling of animals have been performed in accordance with the guidelines set by Ethics Committee of Capital University of Science and Technology (CUST), Islamabad. To minimize variation related to stress in the laboratory and animals were habituated for one week before starting the experimental protocols. The animals were observed for signs of stress, sickness and abnormal behavior each day during this period and were trained to be handled gently by the experimenter.

Mice were marked with a permanent non-toxic ink on their tail and cages were marked. The experimental allocation of animals was done by simple randomisation and balanced randomisation method. Mice were carefully handled by gripping the lower tail while letting them hold onto the wire cage cover to limit stress. The scruff was then placed between the thumb and forefinger and the tail inserted between the little finger and the thumb. This technique provided good restraint but good ventilation and without discomfort.

Food and water intake, activity (general), and grooming behavior were recorded daily for each animal throughout the experimental period, as well as weighing. In extreme cases of distress and/or ill health, animals were humanely slaughtered in accordance with recognized slaughtering procedures by cervical dislocation.



FIGURE 3.1: Mice Handling

3.6 Mice Bedding

Animals were kept in 30 x 20 x 15 cm enclosures containing a corn cob substrate. This was because it absorbs water, controls odor, has minimal dust and is not toxic, all characteristics that are important for maintaining health and reliability in experiments. It was chosen because it is very absorbent, effective for odor control, low in dust and non-toxic and was used to ensure animal health and experimental reliability. To support innate burrowing and nesting instincts, the enclosures were lined with a bedding depth of approximately 2–3 cm. Bedding was refreshed and cages were cleaned bi-daily to preserve cleanliness, inhibit ammonia

build-up, and curb microbial growth. Mice were moved in a gentle manner to clean cages, minimizing stress, for cage changes. All bedding material was autoclaved prior to use to be sterile and free from possible contaminants that might affect experimental results.

3.7 Randomization

Animals were randomly distributed between treatment groups before the beginning of the experiment to avoid any possible bias in the results of the experiments. Body weight stratification was combined with a computer-generated random list to evenly divide the rodents among experimental cohorts.

Individual tracking throughout the study period was maintained using distinct tail ring patterns applied with permanent, non-toxic ink. Animals within each cage were systematically assigned unique markings (ranging from single and multiple thin rings to wide bands and combination patterns). This allowed continuous monitoring and accurate record-keeping during routine drug administration and subsequent behavioral evaluations.

This identification process allowed them to monitor each animal throughout the experimental procedures, ensuring that accurate data could be collected and follow up studies of the behavioral and biochemical outcomes for each animal could be carried out.

3.8 Sacrifice Procedure

Following the experimental period, terminal procedures were conducted under controlled conditions in accordance with institutional animal ethics protocols to ensure minimal distress. Serum and brain specimens were subsequently harvested for downstream biochemical and histological assessments.

3.8.1 Dislocation

Euthanasia was primarily executed via cervical dislocation, selected for its rapid application and ability to preserve tissue integrity without anesthetic contamination during subsequent biochemical and histological assays. Trained experimenters applied firm pressure to the skull base while executing a quick, swift backward pull on the tail to disconnect the brainstem from the spinal cord. Immediate cessation of cardiac beat and respiratory movement confirmed death.

3.8.2 Concussion

In cases where immediate cervical dislocation could not be executed, physical stunning was performed first, followed by dislocation. Adopting this two-stage technique guaranteed humane animal sacrifice without compromising tissue integrity for downstream assays. The concussion technique consisted of a blow to the head with a stiff object that caused a traumatic injury to the brain and a loss of consciousness. Experienced staff carried out this technique to guarantee that it was applied correctly. Since the concussion, the cervical dislocation was immediately carried out to the point of irreversibly closing the vital functions: in international practice the concussion is a stunning technique and not a method of euthanasia. Presence of death was confirmed by absence of respiration, absence of heartbeat and absence of corneal reflexes. Brain tissues (cerebral cortex and brainstem) were collected immediately after euthanasia on ice, for biochemical and histopathological analyses.

3.9 Chemicals and Drugs

Analytical-grade chemicals and test compounds were procured through commercial distributors. To prevent degradation and maintain therapeutic potency, working preparations of nitroglycerin, zolmitriptan, and chlorogenic acid (CGA) were reconstituted daily immediately prior to animal administration.

The test compound (chlorogenic acid) was obtained from Sigma Aldrich, USA. Nitroglycerin was obtained from Wilson & Werrick Pharma, Islamabad. Zolmitriptan was obtained from Valor Pharmaceuticals, Islamabad. Normal Saline (0.9% NaCl) was obtained from UNISA Pharma, Peshawar. The DMSO was obtained from Sigma Aldrich, USA. The buffers for ELISA, Phosphate Buffered Saline and RIPA Buffer were both obtained from Sigma Aldrich, USA. The kits including ELISA Kit (Mouse IL-1β ELISA Kit and Mouse TNF-α ELISA Kit) and BCA Protein Assay Kit were obtained from Biosharp, China. Other reagents for H&E staining including Hematoxylin, Eosin, Xylene, Ethanol and Formalin were obtained from Sigma Aldrich, USA.

3.10 Nitroglycerin-Induced Migraine Model

Migraine like symptoms were induced in mice using a well-established nitroglycerin model that causes induction of key behavioral, neurochemical, and neuroinflammatory features of the human condition. Nitroglycerin causes induction of nitric oxide which further increases the CGRP by stimuation of NO-cGMP pathways and NF-κB pathways and causes migraine.

Nitroglycerin was dissolved in normal saline and administered intraperitoneally. Animals received an intraperitoneal (i.p.) dose of 10 mg/kg of body mass, with all injection volumes fixed at 10 mL/kg. Nitroglycerin was administered on alternative days for 09 days to establish a chronic migraine-like state. This repeated dosing produces increased CGRP production, alteration in glutaminergic neurons, neuroinflammation and behavioral changes that were observed during whole study.

Changes in the behavioral examination were observed on the day before administration of nitroglycerin, day-1, 5 and 9 following each nitroglycerin administration to assure the induction of migraine. Parameters assessed included thermal allodynia, mechanical allodynia and photophobia and overall behavioral reactivity. Observations were conducted 2 hours after administration of injection in a noise-free and well-lighted area. Light aversion (photophobia) and enhanced sensitivity

to thermal and mechanical stimuli represent characteristic behavioral responses in nitroglycerin-induced mouse models. To test preventive efficacy, CGA (10, 50, or 100 mg/kg) was administered 30 minutes before nitroglycerin challenging from day 1 through day 9. Controls received vehicle injections following an identical dosing scheme. Behavioral assays were performed on days 0, 1, 5, and 9 following induction to track acute responses and evaluate long-term preventive performance.

3.11 Behavioral Assessment

All the experimental groups were behaviorally tested for migraine-like symptoms. The assessments were conducted in a noise-free and well-lighted room from 09:00 to 17:00 to reduce the variability due to the circadian rhythm. Mice were acclimated to the experimental room for 30 minutes preceding every trial. Test sessions were arranged in ascending order of stress intensity to eliminate confounding carry-over influences between procedures.

3.11.1 Tail flick Test

It was done to analyze reaction or response to temperature change. 500ml water was added in a beaker and the temperature was set at 48°C±0.1°C keeping the beaker in a water bath. Then the tail of mice was submerged into the water for about 5cm covering the mice with a cotton towel. Time was noted until the mice show response to temperature by folding its tail or moving it. The experiment was continued for maximum of 60 seconds to avoid injury [81].

3.11.2 Light Aversion Test

This test was done to analyze the response to light. Light sensitivity of animals was accessed by using a light and dark box, each measuring 60cm × 60cm × 45 cm, which are linked together by an opening. Each mouse was tested individually

by allowing them to move in the box having two chambers; a completely bright having a light source and an entirely dark and closed chamber. A small opening between the chambers connected the two chambers. Animals were approached one at a time and observed for 5 - 10 minutes.

The time spent in each chamber and number of transitions from light to dark chamber was calculated [38].



FIGURE 3.2: Tail Flick Test of Mice



FIGURE 3.3: Light Aversion Test of Mice

3.11.3 Hot Plate Test

Hot Plate test was done analyze the response to temperature change. Mice were be placed on 52°C hot plate. The response latency to either a hind-paw lick or jump will be recorded. In the absence of a response, the animals were removed from the hot plate at 45 s to avoid tissue injury, and a 45-s latency was assigned as the response [41].

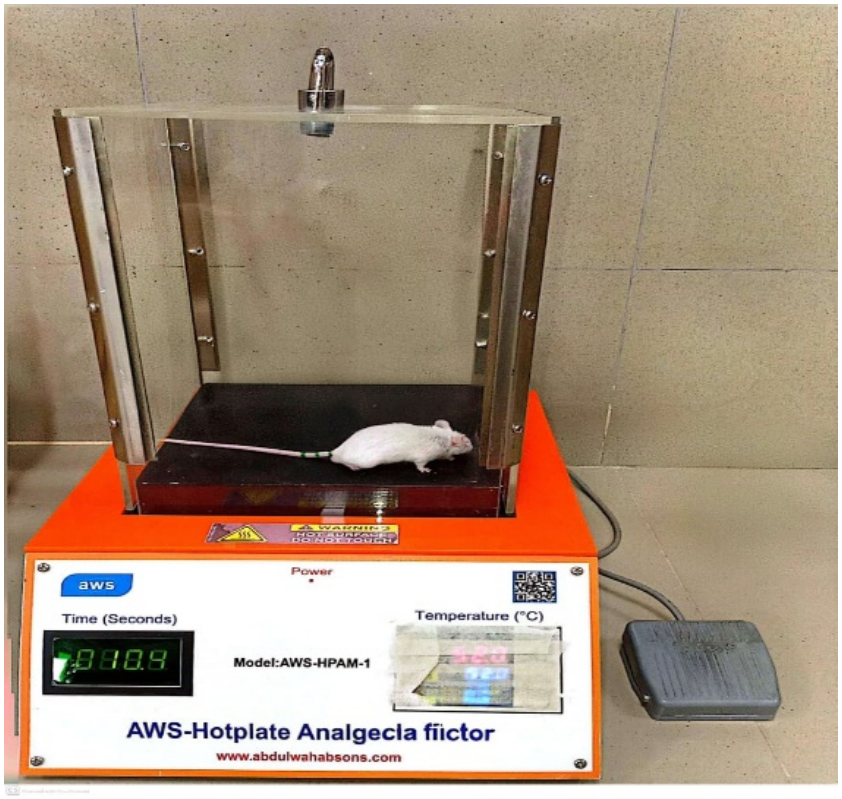


FIGURE 3.4: Hot Plate Test

3.11.4 Von Frey Filament Test

To analyze mechanical allodynia, the mice were placed on wire mesh with openings to provide a mechanical force against which response was calculated. The mice were acclimatized by providing 15 minutes to explore the space. The filaments were chosen in ascending order of thickness. Each mouse was touched with a filament 5 times with an interval of 30 seconds on the hind paw for central effects and paw licking and shaking response was observed. The test was considered

positive if mice showed response not less than 3 times out of 5 times. The mice were touched with a filament 5 times with interval of 2 minutes on the periorbital space for peripheral effects and head movements will be observed. The test was considered positive if mice showed response for not less than 3 time out of 5 times [38].



FIGURE 3.5: Von Frey Filament Test Kit

(Photography taken at Faculty of Pharmacy, Capital University of Science and Technology, Islamabad)



FIGURE 3.6: Von Frey Filament Test (Paw) in Mice

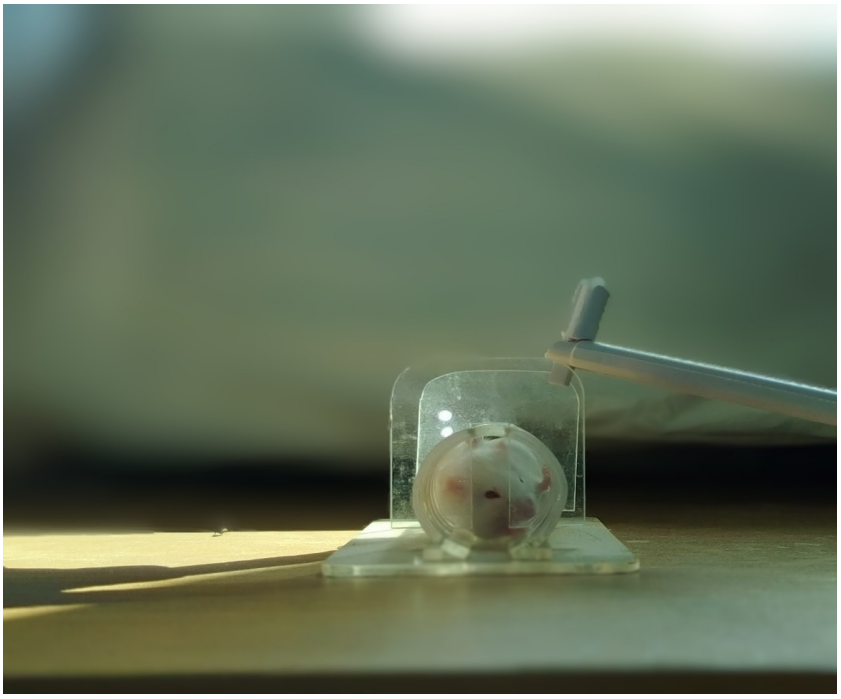


FIGURE 3.7: Von Frey Filament Test (Periorbital Area) in Mice

3.12 Study design

To investigate the anti-migraine potential of chlorogenic acid (CGA) in vivo, a nitroglycerin-induced mouse model was established. Following treatment regimens with CGA, functional parameters were tracked via behavioral assays, including the light aversion, von Frey, hot plate, and tail flick tests. Tissue harvested from the cerebral cortex was subsequently processed for histological structure (H&E) and biochemical profiling (BCA protein assay, alongside TNF-α and IL-1β ELISA kits).

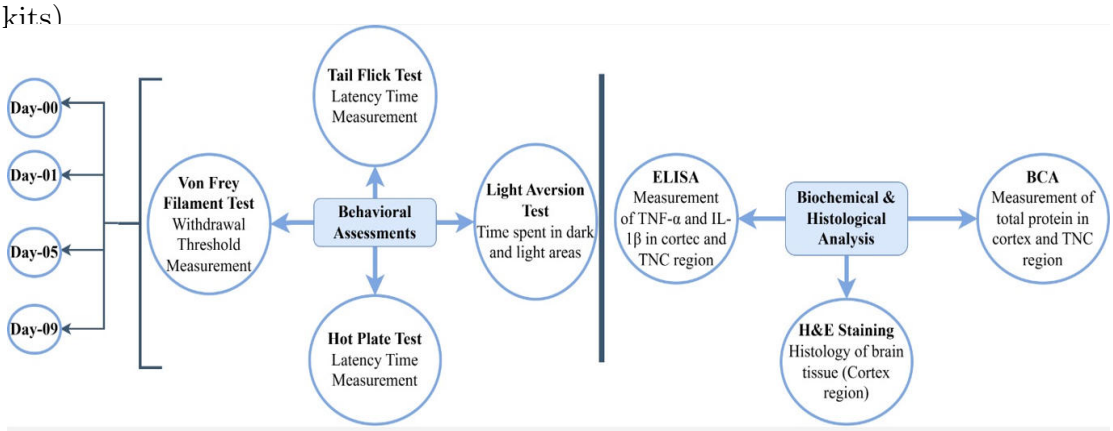


FIGURE 3.8: Study Design for In-vivo Analysis

The Figure-12 shows the whole study design of this project emphasizing on the behavioral tests including Von Frey Filament test, Hot Plate test, Light Aversion test and Tail Flick test and on biochemical and histological analysis of specific parts of brain (Cerebral Cortex and Trigeminal Nucleus Caudalis)

3.12.1 Experimental Design and Groups Allocation

Following the acclimatization period, mice were randomly assigned into six experimental groups (n = 10 per group) based on body weight to ensure uniform distribution. The study was designed to measure the neuro-protective effects of chlorogenic acid (CGA) at three different doses (10, 50 or 100 mg/kg) against Nitroglycerin-induced migraine-like symptoms, with Zolmitriptan (Zolmi) (5 mg/kg) serving as the positive control. The administration of all the drugs/treatments were through the intraperitoneal (i.p.) route. The study included a preventive protocol where Nitroglycerin was administered every alternative day for 09 days for induction of

migraine-like phenotype. Treatment with Zolmitriptan or CGA was injected from day 1 to day 9, 30 minutes prior to Nitroglycerin injection, for easily assessing the therapeutic effectiveness of CGA.

3.12.2 Drug Administration

To ensure accurate dosing of chlorogenic acid (CGA), zolmitriptan, and nitroglycerin, each mouse was weighed separately prior to pharmacological administration. All drugs were prepared in fresh solution every day to ensure stability and effectiveness.

Drugs were injected into the body by intraperitoneal (i.p.) route, using a sterile syringe (1 mL) equipped with a 26-gauge needle. All the treatments were given in the same volume of injection, 10 mL per kg BW.

To reduce the circadian variability, drug administration was performed in a sterile environment on the same time of the day (16:00 to 17:00 h).



FIGURE 3.9: Intraperitoneally Dose Administration in Mice

For the preventive protocol Nitroglycerin (10 mg/kg i.p.) was injected once every other day for 09 injections, which induced Migraine-like phenotype. CGA (10, 50 or 100 mg/kg, i.p.) or Zolmitriptan (5 mg/kg, i.p.) was given 30 minutes before injecting nitroglycerin, between day 1 and day 9. Vehicle control cohorts were administered matching volumes of normal saline on the same schedule.

3.12.3 Dose Preparation

All drug solution were prepared freshly before administration. The doses of drugs for individual mice were calculated according to the following formula:

$$Dose\ of\ Mouse\ (mg) = \ Dose\ level\ \left(\frac{mg}{kg}\right) \times Body\ weight\ of\ mouse\ (kg)$$

Individual body weights were documented prior to administration to personalize dosing, after which the appropriate injection volume (mL) was derived using:

$$Injection\ volume\ per\ mouse\ (mL) = Body\ weight\ (kg)\ ) \times \frac{10\ mL}{kg}$$

Pharmacological solutions were reconstituted to specific stock concentrations (mg/mL) ensuring that:

$$Concentration\ \left(\frac{mg}{mL}\right) = \frac{Dose\ per\ mouse\ (mg)}{Injection\ volume\ per\ mouse\ (mL)}$$

The vehicle group was injected with 10 mL/kg of a solution containing 20 µL DMSO and 980 µL normal saline. Nitroglycerine solution (10 mg/kg) was prepared by dissolving 50 mg (2% w/w) powder of nitroglycerin in 1ml Normal saline to obtain a concentration of 1mg/ml.

TABLE 3.2: Dose Calculation of NTG, Zolmitriptan and CGA for Mice Model

Group	**Compound**	**Dose (mg/kg)**	**Avg. Weight (kg)**	**Mouse Dose per Mouse (mg)**	**Total Mice**	**Frequency (Days)**	**Total Dose for Group (mg)**
I	Vehicle (20 μL DMSO + 980 μL normal saline)	10 ml/kg	0.025	0.25 ml	10	09 days	22.5 ml
II	Nitroglycerin	10 mg/kg	0.025	0.25 mg	10	05 days (Alt)	12.5 mg
III	Nitroglycerin	10 mg/kg	0.025	0.25 mg	10	05 days (Alt)	12.5 mg
	Zolmitriptan	5 mg/kg	0.025	0.12 mg	10	09 days	11.25 mg
IV	Nitroglycerin	10 mg/kg	0.025	0.25 mg	10	05 days (Alt)	12.5 mg
	CGA (Low)	10 mg/kg	0.025	0.25 mg	10	09 days	22.5 mg
V	Nitroglycerin	10 mg/kg	0.025	0.25 mg	10	05 days (Alt)	12.5 mg
	CGA (Medium)	50 mg/kg	0.025	1.25 mg	10	09 days	112.5 mg
VI	Nitroglycerin	10 mg/kg	0.025	0.25 mg	10	05 days (Alt)	12.5 mg
	CGA (High)	100 mg/kg	0.025	2.50 mg	10	09 days	225 mg

For CGA treatment groups, a specified quantity of CGA was first dissolved in 20 µL DMSO and then diluted with 980 µL normal saline to make final concentrations of 1 mg/mL (10 mg/kg), 5 mg/mL (50 mg/kg), and 10 mg/mL (100 mg/kg), respectively.

Zolmitriptan solution (5 mg/kg) was prepared by dissolving 0.5mg Zolmitriptan in 1ml Normal Saline to achieve concentration of 0.5 mg/ml.

Table 3.3: Preparation of Pharmacological Solutions and Treatments

Compound	**Dose**	**Concentration**	**Preparation**
Vehicle	10 mL/kg	-	20 μL DMSO + 980 μL normal saline (2% DMSO)
Nitroglycerin	10 mg/kg	1 mg/mL	50 mg 2% w/w Nitroglycerin dissolved in 1 mL normal saline
Zolmitriptan	5 mg/kg	0.5 mg/mL	0.5 mg Zolmitriptan dissolved in 1 mL normal saline
CGA (Low)	10 mg/kg	1 mg/mL	1 mg CGA in 20 μL DMSO + 9680 μL normal saline
CGA (Medium)	50 mg/kg	5 mg/mL	5 mg CGA in 20 μL DMSO + 980 μL normal saline
CGA (High)	100 mg/kg	10 mg/mL	10 mg CGA in 20 μL DMSO + 980 μL normal saline

All solutions were mixed in vortex mixer to make sure complete dissolution before administration.

The solutions were prepared in sterile glass vials and utilized within 24 hours of preparation.

All mice were injected intraperitoneally using a sterile syringe of 1mL consisting of a 26-guage needle and the volume of injection was set at 10mL/kg according to the body weight of mice.

3.13 Biochemical Analysis

3.13.1 Tissue Preparation

On day 09 animals underwent behavioral assessment and were then euthanized for brain tissue collection. Animals were slaughtered by cervical dislocation and all procedures were conducted in a controlled environment to reduce stress and maintain tissue integrity.

The skull was carefully opened for brain tissue collection and the whole brain was immediately removed. The brain was sliced under a stereomicroscope on an ice cold glass plate to dissect areas such as the cerebral cortex and brainstem. Dissected tissues were placed in ice cold phosgene oxime (dimethyl phosphate) buffered saline (PBS, pH 7.4) to wash away blood and debris. Biochemical samples were freezed in liquid nitrogen and kept at -80°C until further biochemical analysis. Tissues for histopathology were buried in 10% neutral buffered formalin at room temperature for 48 hours [82].

Brain tissue specimens were homogenized in chilled RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a 1:100 dilution of protease inhibitors. Mechanical homogenization was performed at 4°C using three 10-second pulses. The resulting homogenates underwent centrifugation at 14,000 rpm for 30 minutes at 4°C; the soluble protein supernatant was then isolated and preserved at -80°C for downstream assays [82].

3.13.2 Biocinchoninic Assay

Total protein content within cerebral cortex and trigeminal nucleus caudalis (TNC) homogenates was quantified using a commercial Bicinchoninic Acid (BCA) kit following supplier guidelines. The colorimetric principle relies on protein-driven reduction of Cu2+ to Cu+ under alkaline conditions, forming a violet complex detectable at 562 nm. Standard curves were established using serial BSA dilutions

(0–1000 µg/mL), and unknown protein concentrations were interpolated via linear regression analysis to normalize cytokine values.

Bovine serum albumin (BSA) standards were prepared at concentrations of 0, 25, 50, 100, 200, 400, 600, 800, and 1000 μg/mL to generate a standard curve. Reagent A and Reagent B were combined in a 50:1 ratio to make working reagent.

Each standard or sample was added in duplicate to 25 μL in a 96-well microplate and 200 μL of BCA working reagent added. The plate was shaken slightly to ensure good mixing and then incubated at 37° for 30 minutes.

The plate was then incubated, cooled to room temperature, and read in a microplate reader (Thermo Fisher Scientific, USA) at 562 nm. Total protein content was quantified by interpolating values against a linear regression standard curve, with final measurements reported as µg/mL.

$$\text{pg/mg protein} = \left[\frac{\text{Cytokine (pg/mL)}}{\text{Total protein } (\mu\text{g/mL})}\right] \times 1000 \tag{3.1}$$

3.13.3 Enzyme Linked Immunosorbent Assay

To quantify the levels of pro-inflammatory cytokines (TNF-α and IL-1β), the commercially available sandwich ELISA kits (Biosharp, China) were used to measure the levels of pro-inflammatory cytokines in cortex and brainstem tissue homogenates. ELISA (enzyme-linked immunosorbent assay) is a highly sensitive and specific immunological method based on the detection and quantification of antigens in biological samples with specific antibodies linked to an enzyme. The sandwich ELISA format uses an antigen capture antibody which is fixed to a solid matrix (microplate well plates) that binds the target antigen and then a secondary antibody, coupled with horseradish peroxidase (HRP), that recognizes the bound antigen gets bind to the antigen. The enzymatic reaction yields a colourimetric signal that is proportional to the level of the target antigen.

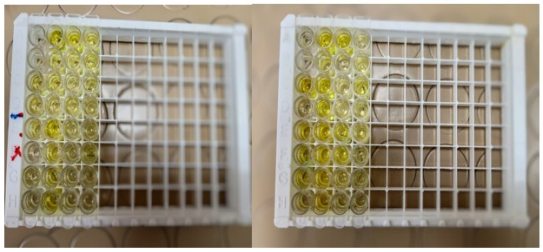


FIGURE 3.10: 96 Well Plate for ELISA

3.13.3.1 Reagent Preparation

All chemicals were allowed to cool at room temperature before use. The wash buffer (20× concentrate) was diluted with distilled water to make 1× wash buffer. Standards were prepared as per the manufacturer's instructions. Briefly, lyophilized standards were centrifuged briefly to collect the material at the bottom of the vial, and then the material was dissolved in standard diluent to get the highest concentration of the standard. Standard curves of 0 to 1000 pg/mL for both TNF-α and IL-1β were created by serial dilutions. To maintain absorbance readings within the dynamic range of the standard curve, tissue lysate samples were diluted two-fold (1:2) with working sample diluent prepared according to the manufacturer's protocol.

3.13.3.2 Assay Procedure

The ELISA procedure was performed according to the manufacturer's instructions using Biosharp Mouse TNF-α ELISA Kit (Cat. No. BSEM-004-96T) and Biosharp Mouse IL-1β ELISA Kit (Cat. No. BSEM-015-96T). Quantitative measurements

were performed in 96-well microtiter plates pre-functionalized with monoclonal capture antibodies specific to mouse TNF-α or IL-1β. All the samples and standards were conducted in duplicate to make sure accuracy and reproducibility of results. The standard, sample and control were pipetted into the appropriate wells (50 µL/well). This was gently tapped to promote mixing and then incubated at 37 °C for 30 minutes to allow antigen-antibody interactions. The plate was then aspirated and washed 5 times with 300 µL wash buffer with an automated microplate washer to completely remove unbound substances, after incubation. Each well was then filled with 50 L of HRP-conjugated detection antibody and incubated at 37 °C for 30 minutes. This step enabled the antigen to be captured by the detection antibody, creating a sandwich complex. The plate was then aspirated and washed 5 times with 300 µL of wash buffer to wash off any excess detection antibody after the incubation. Hydrogen peroxide (50 µL of chromogen solution A) and tetramethylbenzidine, TMB (50 µL of chromogen solution B) were added to each well for colour development. The microplate was subjected to mild shaking and incubated at 37 °C for 15 minutes away from light, during which HRP-mediated oxidation of TMB produced a visible blue color. The reaction was stopped after addition of 50 µL of stop solution (2 M HSO) leading to the change in colour from blue to yellow in the enzymatic reaction. The optical density was determined by the use of a microplate reader (Thermo Scientific Multiskan FC) at 450 nm. For both assays quantification of cytokine levels in hippocampal tissue, this procedure was rigorously followed to ensure consistent and reliable quantification of cytokines.

3.13.3.3 Absorbance Measurement and Quantification

Absorbance was measured at 450 nm using a microplate reader within 15 minutes of adding stop solution. A standard curve was generated by plotting the mean absorbance values of the standards against their respective concentrations. Standard curves were fitted using linear regression analysis to determine line equations and R^2 values. Concentration values for experimental samples were extracted from these curves using measured absorbance data and subsequently normalized to total protein content as quantified by the BCA assay.

3.13.3.4 Quality Control

Each assay was run twice and the CV for repeated assays was kept at less than 10%. Blank wells with diluent only were used to correct for background absorbance. The correlation coefficient (R2) value of the standard curve of ≥ 0.99 was regarded as acceptable for quantification.

3.14 Histopathological Analysis H and E Staining

Histopathological evaluation was performed on cortical tissue sections to assess neuronal morphology and tissue architecture following Nitroglycerin-induced migraine-like pathology and treatment with chlorogenic acid.

Brain tissues fixed in 10% neutral buffered formalin for 48 hours were processed for paraffin embedding. Tissues were dehydrated through a graded series of ethanol (70%, 80%, 95%, and 100%), cleared in xylene, and infiltrated with molten paraffin wax before being embedded in paraffin blocks. Coronal sections of 5 m thickness were cut using a rotary microtome, mounted on glass slides, and dried overnight at 37°C.

For HE staining, slides were deparaffinized in xylene, rehydrated through descending ethanol concentrations, and rinsed in distilled water. Tissues were immersed in Harris's hematoxylin for 5–7 minutes to stain nuclei, rinsed with tap water, and briefly differentiated in 1% acid alcohol (2–3 seconds). Nuclear bluing was performed using 0.2% ammonia water, after which cytoplasm was counterstained with 1% eosin Y for 30–60 seconds. The slides underwent dehydration in ascending ethanol concentrations, clearing with xylene, and mounting with a permanent medium. Microscopic evaluation was conducted under 10x and 40x magnification, with primary analysis directed toward the cerebral cortex [83].

3.15 Statistical Analysis

All dataset processing was conducted with GraphPad Prism version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). Values are reported as mean ± standard error of the mean (SEM) per treatment group. The Shapiro-Wilk test was applied to evaluate dataset normality and validate the use of parametric statistical evaluations.

For behavioral assessments (Hot-Plate test, Von Frey Filament test, Light Aversion test and Tail Flick test), two-way analysis of variance (ANOVA) was employed to evaluate the effects of treatment followed by Tukey's post-hoc test for multiple comparisons. This allowed assessment of the main effects of treatment and the interaction between treatment and groups. Group comparisons for biochemical parameters (TNF-α and IL-1β) were evaluated via two-way analysis of variance (ANOVA), with Tukey's post-hoc test applied for multiple pairwise comparisons. Differences were considered statistically significant at *p ¡ 0.05, **p ¡ 0.01, and ***p ¡ 0.001). A sample size of n = 10 rodents per group was maintained for all behavioral and biochemical evaluations. To minimize investigator bias, a double-blinded protocol was enforced during data collection and analysis.

Chapter 4

Results

4.1 Behavioral Assessment

The behavioral tests were conducted for Four days i.e. Day-0, 1, 5 and 9 of the experimental protocol to evaluate the neuroprotective effect of CGA in Nitroglycerin-induced migraine model of mice. Four validated tests were performed including Hot plate test, Tail flick test, Von Frey filament test and light aversion test in which hot plate test was to analyze thermal allodynia, tail flick to analyze response to change in temperature, Von Frey filament test to analyze mechanical allodynia and headache and Light aversion test to analyze photophobia or sensitivity to light. All parameters are measured as mean ± standard deviation (n=10 per group). Nitroglycerin (10 mg/kg i.p for 9 days on alternative days) served as migraine inducing agent, CGA was given in three different doses (10 mg/kg, 50 mg/kg or 100 mg/kg i.p daily) as test drug and Zolmitriptan (5mg/kg i.p daily) as a positive control.

4.1.1 Effect of Chlorogenic Acid on Thermal Hyperalgesia (Hot Plate Test)

Thermal nociceptive responses were similar across the experimental groups at baseline (Day 0). Thermal hyperalgesia was successfully induced in mice after the administration of nitroglycerin, with the mice showing a significant decrease in thermal nociceptive latency, as compared with the vehicle-treated group. Latency was reduced in the treatment period and remained so until the end of the experiment, while the vehicle-treated animals exhibited stable nociceptive responses across the experiment. The results showed that repeated nitroglycerin administration led to maintained thermal hypersensitivity, which was confirmed.

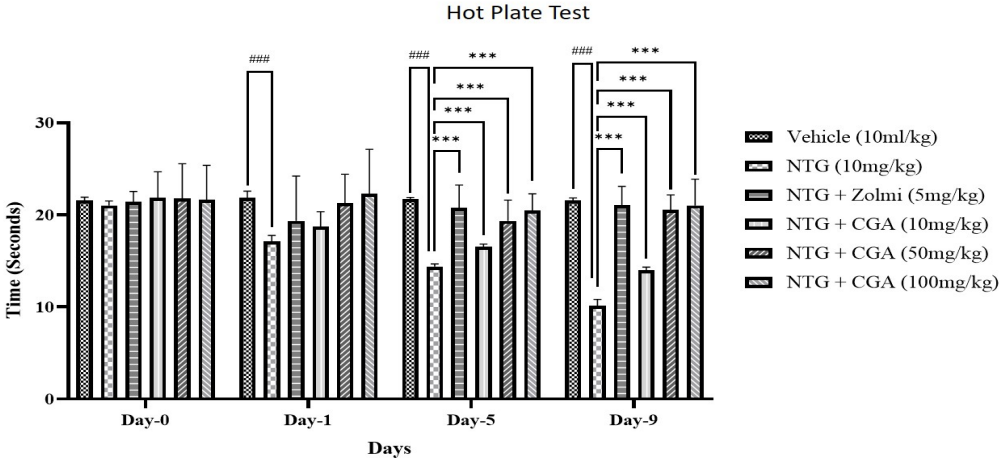


FIGURE 4.1: Effect of CGA on Thermal Hyperalgesia (Hot Plate Test)

Nitroglycerin induced thermal hyperalgesia was effectively reduced by pretreatment with zolmitriptan, as shown by the significant increase in thermal nociceptive latency compared with the nitroglycerin group. Throughout treatment, improvement on nociceptive responses was seen, indicating that antimigraine standard treatment was protective against central pain sensitization. In the same way, the nitroglycerin group showed a delay in thermal nociceptive latency when treated with chlorogenic acid at 10 mg/kg but, the high dose treatment with chlorogenic acid produced a higher improvement in thermal nociceptive responses than the low dose treatment, suggesting that with the high dose chlorogenic acid provides greater protection against nitroglycerin-induced thermal hypersensitivity. The 100

mg/kg dose of chlorogenic acid had the greatest effect on reducing response latency compared with the nitroglycerin group and the effect was close to that of the vehicle treated animals on thermal nociception. In conclusion, chlorogenic acid had a dose dependent protective effect against the thermal hyperalgesia caused by the nitroglycerin.

4.1.2 Effect of Chlorogenic Acid on Thermal Allodynia (Tail Flick Test)

At baseline (Day 0), no differences were found between experimental groups, which suggests that all groups started with similar sensitivity to thermal stimulation. After repeated nitroglycerin, the mice in the NTG group showed significant decrease in tail flick latency over time in comparison to vehicle treated mice, which suggests that thermal hyperalgesia was induced.

The vehicle treated animals had relatively consistent withdrawal latencies throughout the experimental period, in contrast. Zolmitriptan significantly reduced tail flick latency after the first dose of nitroglycerin and protected against the effect of nitroglycerin through the end of the experiment compared to the group not treated with the drug. The attenuation of NTG-induced thermal hypersensitivity was consistently more in the zolmitriptan-treated group, compared to the untreated NTG group, demonstrating effective attenuation of nitroglycerin-induced thermal hypersensitivity. Thus, thermal nociceptive responses were also better in the administration of chlorogenic acid group when compared to NTG group. The 10 mg/kg dose gave only minimal protection in the first few days of treatment, but the protection increased with the duration of the study. The treatment with chlorogenic acid at 50 mg/kg showed a greater effect in the tail flick latency than the low dose group, suggesting a greater protection against nitroglycerin-induced hyperalgesia. The most significant improvement was seen in the 100 mg/kg dose that showed withdrawal latencies approaching those of the vehicle-treated animals in all instances by the end of the experimental period. The results indicate that chlorogenic acid has a good anti-nociceptive effect and it was more effective at

higher doses and repeated administration to inhibit nitroglycerin induced thermal hyperalgesia.

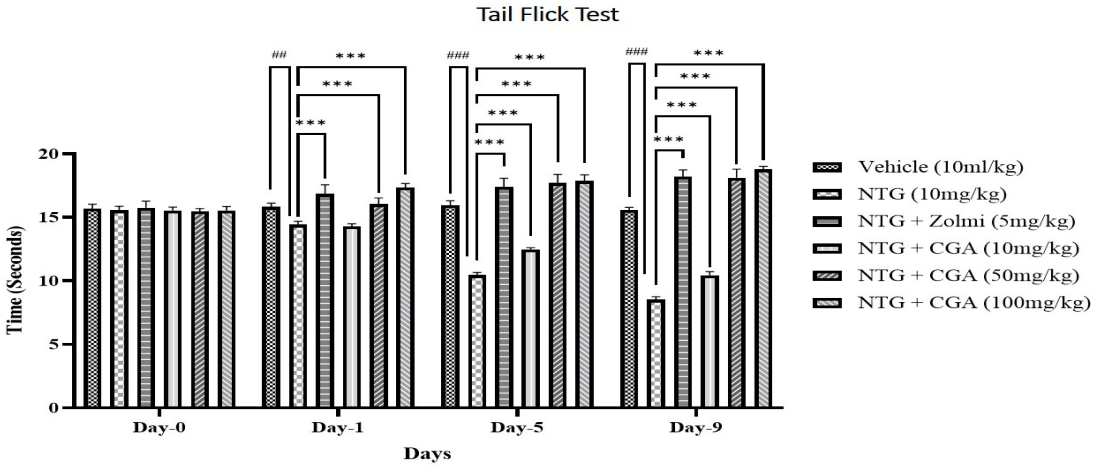


FIGURE 4.2: Effect of CGA on Thermal Allodynia (Tail Flick Test)

4.1.3 Effect of Chlorogenic Acid on Mechanical Allodynia (Von Frey Filament Test)

4.1.3.1 Peripheral Mechanical Allodynia in Hind paw of mice

On Day 0 (baseline), mechanical withdrawal threshold among experimental groups was not different. The NTG treated group showed a significant decrease in mechanical withdrawal threshold after multiple administrations of NTG as compared to th vehicle treated group, indicating successful induction of mechanical allodynia. The withdrawal threshold of the animals treated with the vehicles tended to remain stable while the animals treated with the drug showed a decrease during treatment, while the vehicle-treated animals showed a decrease that was more marked. The treatment with zolmitriptan significantly prevented nitroglycerin-induced mechanical hypersensitivity as evidenced by an increase in the paw withdrawal threshold score compared with the nitroglycerin group. In the same way, the mechanical withdrawal threshold was better in chlorogenic acid treated group versus the NTG group. While low dose (10 mg/kg) had only mild effect on the withdrawal threshold, the application of the highest dose of chlorogenic acid (50

mg/kg) led to a more significant reversal of mechanical allodynia. The highest dose (100 mg/kg) showed the best results in terms of paw withdrawal threshold and was invariably more protective against mechanical hypersensitivity induced by nitroglycerin than lower doses of treatment. There was a dose-dependent effect of chlorogenic acid on the reduction of nitroglycerin-induced mechanical allodynia in the hind paw in the overall group. Greatest therapeutic effect was seen in the high-dose chlorogenic acid group.

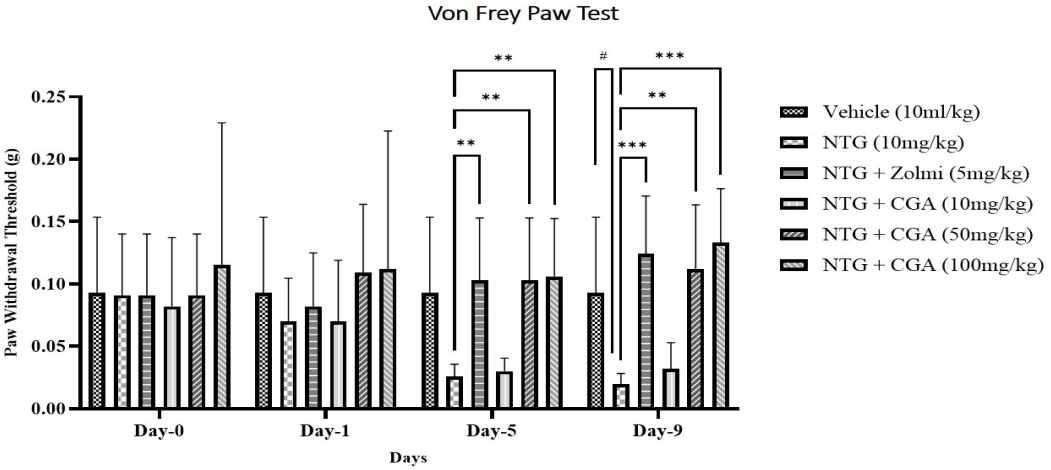


FIGURE 4.3: Effect of CGA on Mechanical Allodynia in Mice (Von Frey Filament test)

4.1.3.2 Central Mechanical Allodynia in Periorbital Area of Mice

The baseline withdrawal thresholds for the periorbital region were comparable across experimental groups, suggesting that mechanical sensitivity was similar prior to treatment. Cephalic mechanical allodynia was induced by repeated nitroglycerin administration which resulted in a large reduction in the periorbital withdrawal threshold when compared to the vehicle-treated group. This decrease in withdrawal threshold continued during the experimental period, while periorbital mechanical sensitivity of the vehicle-treated animals remained stable. The group that received zolmitriptan prior to the nitroglycerin exhibited a significantly higher periorbital withdrawal threshold than the group that received nitroglycerin alone, suggesting that zolmitriptan is effective in suppressing cephalic hypersensitivity induced by nitroglycerin. Treatment with chlorogenic acid also reduced

periorbital mechanical allodynia compared to the NTG group. Both the 50 and 10 mg/kg doses resulted in a greater protective effect against nitroglycerin hypersensitivity, and the 10 mg/kg dose produced a partial improvement in the withdrawal threshold. The greatest increase in periorbital withdrawal threshold and restoration of mechanical sensitivity were seen after treatment with chlorogenic acid (100 mg/kg). Taken together, the results showed that chlorogenic acid could successfully inhibit nitroglycerin-induced mechanical allodynia and that the higher the dose of chlorogenic acid treatment, the more effective it was.

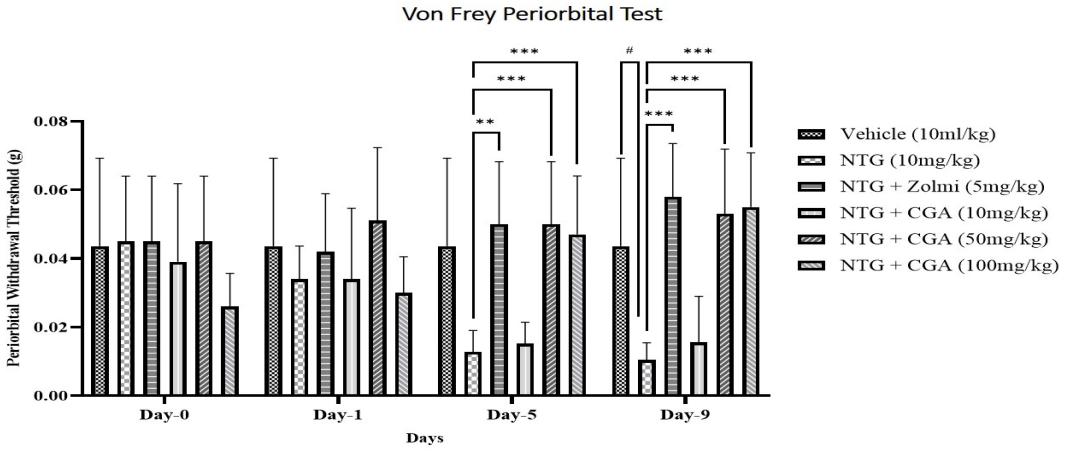


FIGURE 4.4: Effect of CGA on Mechanical Allodynia in Mice (Von Frey Filament test)

4.1.4 Effect of Chlorogenic Acid on Photophobia (Light Aversion Test)

All experimental groups were similar at baseline (Day 0), suggesting similar exploratory behavior prior to treatment. Mice receiving nitroglycerin showed a significant decrease in the amount of time spent in the light compartment and a corresponding increase in time spent in the dark compartment after repeated administration of nitroglycerin compared with the vehicle-treated group. These results show that the nitroglycerin-treated animals exhibited photophobia-like behavior, which is a successful induction of the behavior. Exploration behavior was relatively stable during the experimental period in the vehicle-treated group with no evident preference for either compartment.

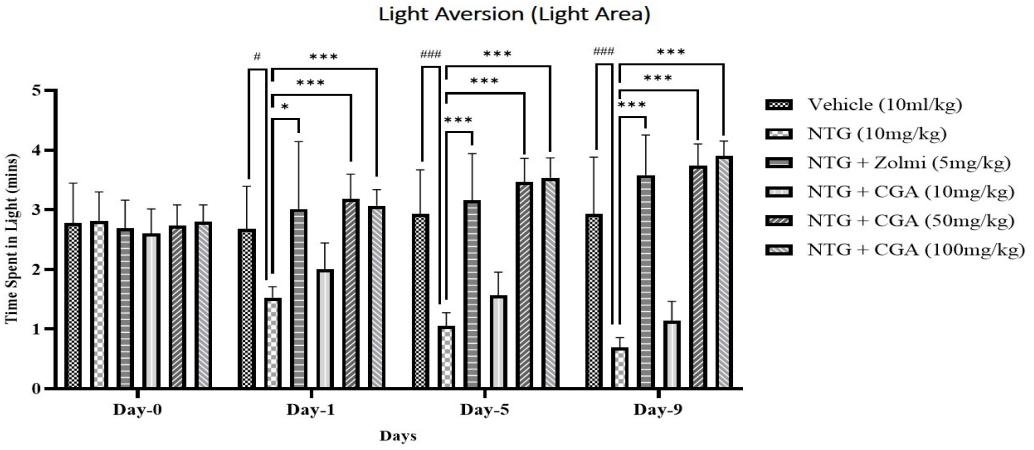


FIGURE 4.5: Effect of CGA on Photophobia in Mice (Time Spent in Light Area)

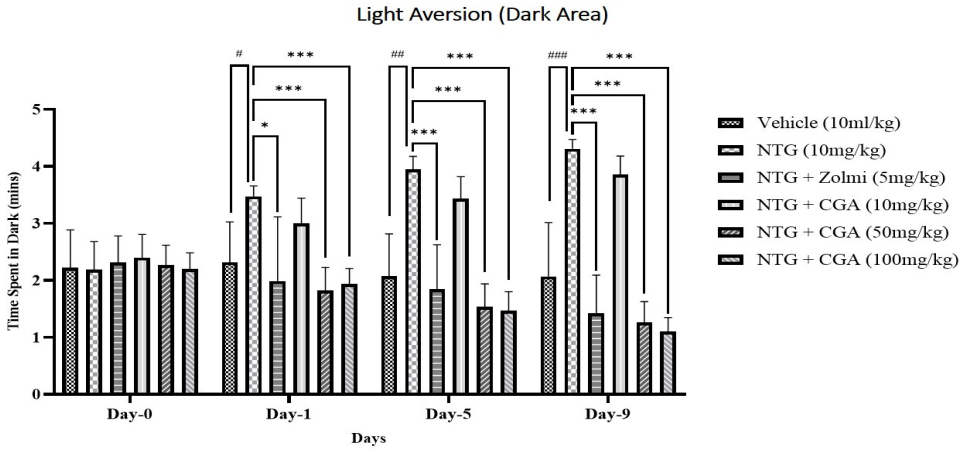


FIGURE 4.6: Effect of CGA on Photophobia in Mice (Time Spent in Dark Area)

Pretreatment with zolmitriptan significantly improved light aversion by increasing the amount of time observed in the light compartment and decreasing the amount of time observed in the dark compartment, compared with nitroglycerin treatment. The same improvements were seen in the groups treated with chlorogenic acid. The 10 mg/kg dose of chlorogenic acid was effective in recovering light aversion behaviour while the 50 mg/kg was effective in restoring the normal exploratory activity. The highest dose of chlorogenic acid (100 mg/kg) showed the greatest effect with more time spent in the light compartment, and a corresponding decrease in dark compartment time. In general, chlorogenic acid was able to significantly reduce the nitroglycerin-induced photophobia in a dose-dependent fashion, with

the most effective treatment dose producing effects close to those of the vehicle-treated animals.

4.2 Enzyme Linked Immunosorbent Assay Analysis of Mice Tissues

Total protein of the lysates of both cerebral cortex and TNC region was measured by BCA prior to performing ELISA to equalize the protein loading of all the samples and brain regions. We found that Nitroglycerin significantly decreased the total protein content in all brain regions as compared to the normal saline group, using BCA assay. Zolmitriptan demonstrated tissue proteins recovery. CGA treatment resulted in protein recovery in a dose dependent manner where the protein content recovered after 100 mg/kg dose of CGA was comparable to Zolmitriptan in most regions, whereas the 10 and 50 mg/kg doses of CGA gave moderate protection. The concentration of cytokines were normalized by the total level of protein in tissues and mentioned as pg/mg of protein to compensate for differences in tissue protein content because of neurodegeneration.

4.3 Effect of Chlorogenic Acid on Tumor Necrosis Factor Alpha in Cortex and Trigeminal Nucleus Caudalis

The above graph showed that the nitroglycerin (NTG) group had significantly elevated normalized cortical TNF-α levels, suggesting greater cortical neuroinflammation compared to the vehicle-treated group. The NTG group showed a significant increase in the expression of TNF-α when compared to the zolmitriptan group, which underlines a protective effect of zolmitriptan against nitroglycerin induced inflammatory changes. Similarly, the normalized level of TNF-α in the

cortex was decreased after chlorogenic acid treatment compared with NTG treated animals. The reduction seen with chlorogenic acid was dose dependent with 10 mg/kg showing a small attenuation of the expression of TNF-α and 50 mg/kg showing a larger attenuation. The highest dose group of chlorogenic acid (100 mg/kg) was the most effective in reducing the levels of the inflammatory marker, TNF-α in the cortex among the groups receiving chlorogenic acid and brought the levels of this inflammatory marker closer to the vehicle treated group. In conclusion, the effect of chlorogenic acid was found to be effective in reducing cortical neuroinflammation induced by nitroglycerin, with effects increasing with the dose of the chlorogenic acid.

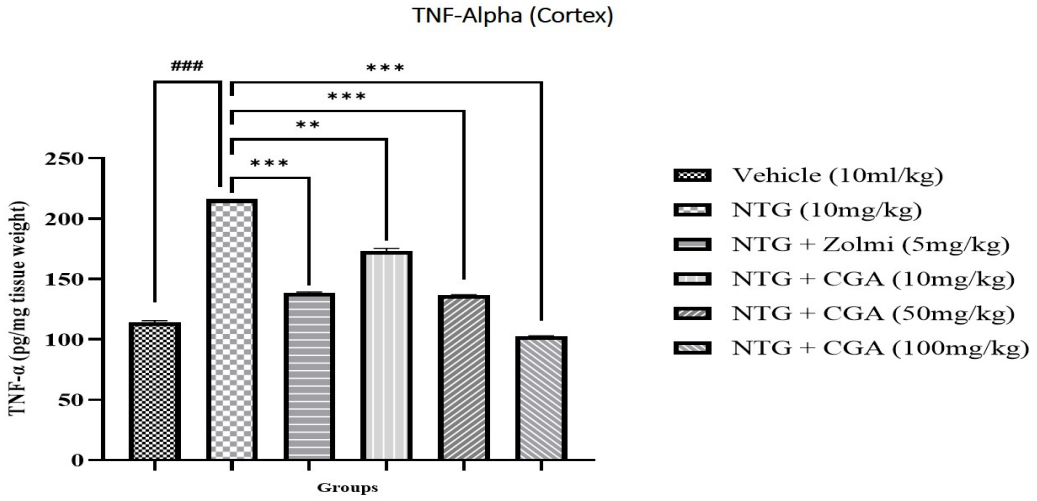


FIGURE 4.7: Effect of CGA on Cortical TNF-α Levels

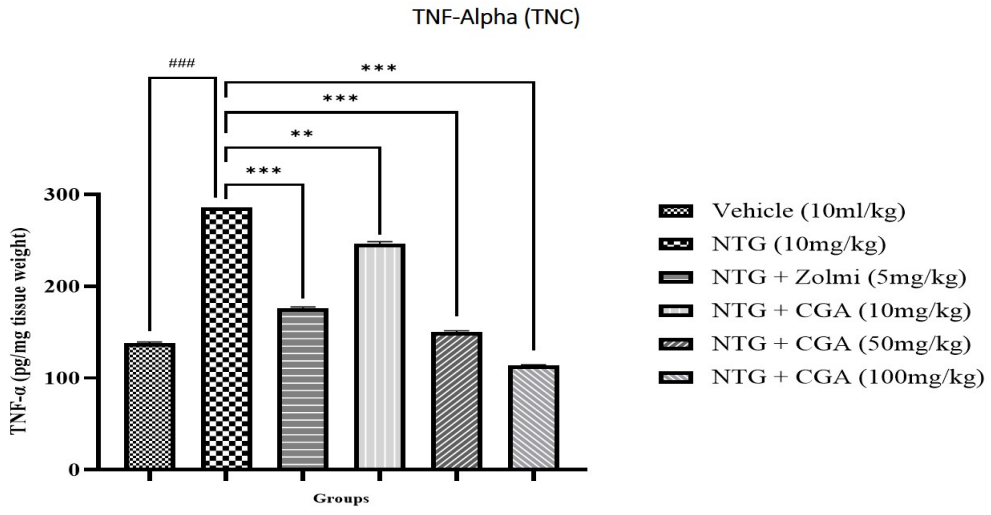


FIGURE 4.8: Effect of CGA on TNF-α Levels in TNC Region

The figure 18 shows that repeated administration of nitroglycerin induced a significant inflammatory response with normalized TNF-α levels in the TNC, compared with the vehicle-treated group, suggesting the induction of a pronounced inflammatory response following migraine induction. The zolmitriptan group showed a significantly greater reduction in TNF-α expression relative to the nitroglycerin-treated group, indicating zolmitriptan's ability to inhibit nitroglycerin-induced neuroinflammation in the TNC. Likewise, the increase of normalized TNF-α level compared to the NTG group was inhibited by chlorogenic acid treatment. The decreased expression of TNF-α was significant at all concentrations of chlorogenic acid, with a slight decrease seen in the 10 mg/kg dose, and a larger decrease seen in the 50 mg/kg dose, indicating a reduction in the inflammatory response. The 100 mg/kg group had the greatest decrease in normalized TNF-α levels and the levels approached those of vehicle treated animals. Overall, chlorogenic acid effectively reduced the nitroglycerin-induced neuroinflammation in a fashion that the highest dose shows the strongest anti-inflammatory property.

4.4 Effect of Chlorogenic Acid on Interleukin-1 Beta in Cortex and Trigeminal Nucleus Caudalis

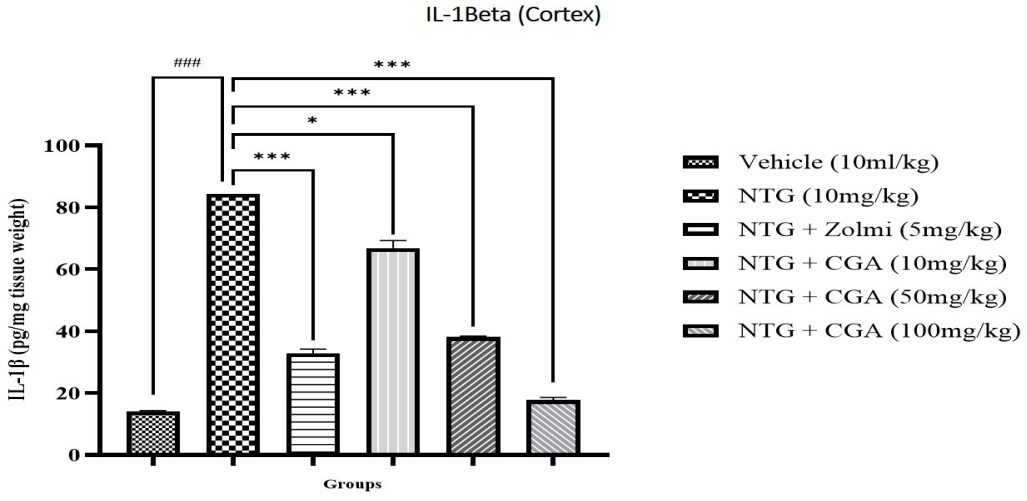


FIGURE 4.9: Effect of CGA on Cortical IL-1 β

The figure shows that mice treated with nitroglycerin showed a significantly higher level of normalized IL-1β in the cortex, which successfully produced a strong neuroinflammatory response.

The elevated expression of IL-1β in the NTG-treated group is a sign of activation of pro-inflammatory pathways in the cerebral cortex after migraine induction. Zolmitriptan significantly reduced the nitroglycerin-induced increase in normalized IL-1β level in the cortex, indicating that zolmitriptan effectively reduced nitroglycerin-induced cortical inflammation.

Similarly, the expression of IL1b in the cortex was decreased compared to NTG group after chlorogenic acid treatment, but in varying degrees, depending on the dose of the treatment. IL-1β expression was slightly inhibited at the 10 mg/kg dose, suggesting some protection against the inflammatory effects of nitroglycerin.

On the other hand, treatment with chlorogenic acid at 50 mg/kg led to more significant decreases in normalized cortical IL-1β levels, indicating its ability to effectively suppress the inflammatory response. The 100 mg/kg resulted in the greatest decrease in normalized cortical IL-1β levels in all chlorogenic acid-treated groups compared to the nitroglycerin-treated animals.

A dose-dependent decrease in expression of IL-1β was seen at this dose, with values becoming similar to the vehicle-treated group, suggesting significant protection against nitroglycerin-induced neuroinflammation.

Together, these results show that the expression of IL-1β in the cortex was effectively inhibited in a dose-dependent manner by the treatment with chlorogenic acid in nitroglycerin-induced increase of IL-1β expression in the cortex, with higher doses showing increased anti-inflammatory activity.

The IL1B reduction profile was similar after chlorogenic acid intervention to the standard intervention, thus suggesting its use in reducing inflammatory responses of the cortex involved in the pathogenesis of migraine.

The figure demonstrates that after repeated nitroglycerin treatment, normalized IL-1β levels were significantly higher in the trigeminal nucleus caudalis (TNC)

compared with the vehicle treated group, providing a successful induction of a strong inflammatory response in this area.

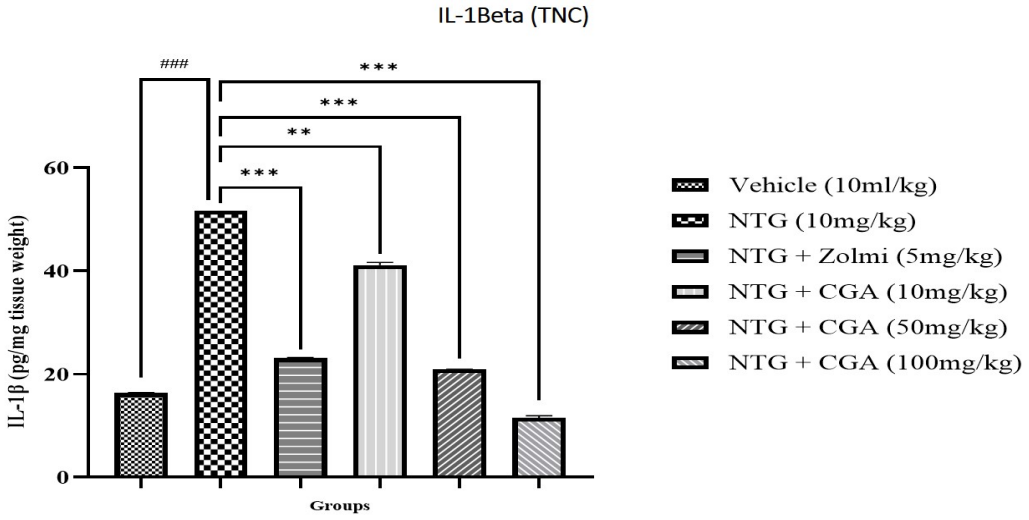


FIGURE 4.10: Effect of CGA on IL-1 β in TNC Region

The vehicle-treated group showed low basal IL-1β expression, which indicates that there were no inflammatory changes at physiological conditions. There was a significant reduction in IL-1β levels in the nitroglycerin pretreated group compared to the group that did not receive it, showing that zolmitriptan was effective at reducing IL-1β production in the nitroglycerin-induced neuroinflammation of the TNC. Likewise, administration of chlorogenic acid was found to efficiently inhibit IL-1β expression when compared to the NTG group. The 10 mg/kg dosage of the drug decreased the IL-1β level compared to the nitroglycerin treated animals, but not as significantly as the higher dosages of the drug. Repeated nitroglycerin administration led to upregulation of IL-1β, which was significantly reduced by treatment with chlorogenic acid (50 mg/kg). All the chlorogenic acid treated groups showed significant reduction in the normalized levels of IL-1β expression, with the 100 mg/kg dose having the highest reduction in the normalized levels of IL-1β expression as close to vehicle control. Overall, the results showed that chlorogenic acid is an effective intervention to reduce the nitroglycerin-induced increase in IL-1β in the trigeminal nucleus caudalis, with the highest dose demonstrating the most anti-inflammatory activity and response similar to that seen with the standard treatment, zolmitriptan.

4.5 Hematoxylin and Eosin Staining for Identification of Morphological Changes

Histological examination of H&E-stained sections of the cerebral cortex revealed preserved cortical architecture in the Control group, characterized by normal tissue organization with uniformly distributed cells and an intact neuropil, without apparent signs of degeneration or vacuolation.

In contrast, the Nitroglycerin (NTG, 10 mg/kg) group exhibited marked morphological alterations. The cortical tissue showed extensive vacuolation and spongiform changes, resulting in a loose and disrupted appearance of the neuropil. Cellular organization appeared disturbed, with reduced tissue compactness and evidence suggestive of neuronal degeneration and edema. These findings indicate substantial structural damage associated with NTG-induced neurotoxicity.

Treatment with Zolmitriptan (5 mg/kg) markedly attenuated these histopathological changes. Cortical sections demonstrated improved tissue integrity and a more organized cellular arrangement, with fewer vacuolated areas and restoration of normal architecture compared with the NTG group. Although mild alterations were still evident, overall morphology was considerably preserved.

Similarly, the CGA-treated group (100 mg/kg) showed notable protection against NTG-induced damage. The cerebral cortex exhibited improved structural organization with reduced vacuolation and better preservation of the neuropil compared with the diseased group. The cortical morphology appeared closer to that of the control group, suggesting that chlorogenic acid effectively mitigated NTG-induced histopathological alterations and exerted a neuroprotective effect.

Overall, NTG administration induced pronounced cortical degeneration characterized by vacuolation and disruption of tissue architecture, whereas treatment with zolmitriptan and chlorogenic acid substantially ameliorated these changes, with the CGA-treated group showing considerable restoration of normal cortical morphology.

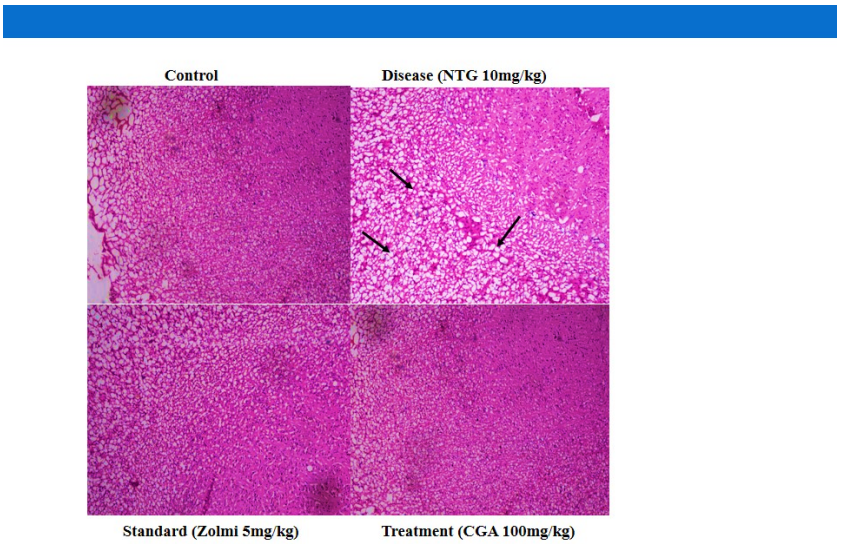


FIGURE 4.11: Representative Histopathological Images of the Cerebral Cortex via H&E Staining

The control group exhibited normal cortical architecture with intact neuropil and cellular organization. The NTG (10 mg/kg) group showed marked vacuolation and disruption of tissue architecture indicative of neurodegeneration. Treatment with zolmitriptan (5 mg/kg) and chlorogenic acid (100 mg/kg) attenuated these pathological changes, demonstrating reduced vacuolation and improved preservation of cortical morphology compared with the NTG group.

Chapter 5

Discussion

Our study emphasized on evaluation of anti-migraine activity of chlorogenic acid (CGA) against nitroglycerin (NTG)-induced migraine-like phenotype in mice, with particular focus on nociceptive behaviors, mechanical allodynia, photophobia-like behavior, and neuroinflammatory cytokine profiles. The results demonstrate that repeated NTG administration produced a consistent and progressive migraine-like phenotype across all behavioral endpoints, characterized by reduced thermal nociceptive latency, decreased mechanical withdrawal thresholds in both paw and periorbital regions, and increased light aversion. These behavioral changes were accompanied by significantly elevated TNF-α and IL-1β levels in both the cortex and trigeminal nucleus caudalis (TNC). Importantly, co-administration of chlorogenic acid produced dose-dependent protective effects across nearly all parameters, with the 50 mg/kg and 100 mg/kg doses showing efficacy comparable to or exceeding that of the reference drug zolmitriptan (5 mg/kg).

In the current study, thermal hyperalgesia was reproduced following repeated administration of nitroglycerin for nine days, with progressive decrease in thermal nociceptive thresholds in the hot plate and tail flick tests. The decreases in thermal nociceptive threshold was more marked in the tail flick test than in the hot plate test, indicating that nitroglycerin may have a greater effect on spinal nociceptive pathways. These results align with the central sensitization and increased

pain sensitivity that are known to occur with chronic migraine. Repeated nitroglycerin also significantly upregulated the pro-inflammatory cytokines TNF-α and IL-1β in both the cerebral cortex and the trigeminal nucleus caudalis (TNC) confirming the development of a strong neuroinflammatory response. These results were consistent with previous reports showing that nitroglycerin causes migraine-like pathophysiology by triggering inflammatory signaling pathways that lead to the release of pro-inflammatory mediators in areas of the brain that process pain. The protective effect of chlorogenic acid was observed in all the behaviour and biochemical parameters in the present study, which were in a dose dependent manner. The smallest dose (10 mg/kg) typically showed the smallest and relatively variable effect compared to the nitroglycerin group, whereas the 50 mg/kg and 100 mg/kg doses provided increasingly greater protection, often at levels similar to that of zolmitriptan. Thermal nociceptive responses were significantly enhanced, paw and periorbital withdrawal thresholds were increased and thermal hyperalgesia and mechanical allodynia induced by repeated nitroglycerin administration were significantly attenuated by the highest dose of chlorogenic acid. In addition, chlorogenic acid significantly lowered the amount of nitroglycerin-induced TNF-α and IL-1β increases in both the cerebral cortex and the TNC in a dose-dependent manner, thereby showing a dose-dependent anti-inflammatory effect on the brain. Taken together, these results indicate that the effect of chlorogenic acid is highly protective against the nitroglycerin induced migraine-like behaviours and neuroinflammatory alterations.

The neuroprotective effects of CGA observed in the present study are consistent with previous reports demonstrating that CGA possesses potent antioxidant and anti-inflammatory properties. Cantero-Téllez et al. (2025) demonstrated that systemic administration of CGA (100 mg/kg) significantly reduced lipid peroxidation and promoted an anti-inflammatory profile in the brain when co-administered with 3-nitropropionic acid (3-NP), a mitochondrial toxin that induces neurodegeneration. In their study, CGA significantly reduced lipid peroxidation in both the striatum and frontal cortex of 3-NP-treated mice. The authors attributed this neuroprotection to CGA's ability to scavenge hydroxyl radicals, peroxide radicals,

and superoxide ions, as well as its capacity to activate the Nrf2 pathway, promoting the upregulation of antioxidant enzymes [30]. The anti-inflammatory actions of CGA were also demonstrated by Cantero-Téllez et al. (2025), who found that CGA significantly reduced TNF-α levels in the striatum of 3-NP-treated mice. Similarly, IL-6 levels were significantly elevated in 3-NP-treated mice compared to controls in both the striatum and frontal cortex and CGA co-treatment kept IL-6 levels within the control range. These findings align with the present results, where CGA treatment reduced cortical and TNC TNF-α and IL-1β levels in a dose-dependent manner. The authors concluded that CGA may shift the balance of neuroinflammation towards an anti-inflammatory profile, highlighting the potential benefits of regular CGA consumption [30].

Anggreani and Lee (2017) reviewed the neuroprotective mechanisms of chlorogenic acid against Alzheimer's disease, focusing on its antioxidant activity in scavenging free radicals due to the presence of hydroxyl groups and double bond in its structure. The authors reported that CGA exhibits dose-dependent antioxidant activity in scavenging DPPH radicals, ABTS radicals, hydroxyl radicals, peroxyl radicals, and superoxide anions. In cell-based assays, CGA provided protective effects against cytotoxicity stimulated by various oxidative stressors including H_2O_2, methylglyoxal, and methylmercury. Pretreatment with CGA prevented H_2O_2-induced down-regulation of anti-apoptotic proteins Bcl-2 and Bcl-XL, reduced caspase-3 cleavage, and inhibited JNK and p38 MAPK activation in primary cortical neurons and PC12 cells. Furthermore, CGA also suppress the microglial activation stimulated by lipopolysaccharide (LPS) by decreasing the nitric oxide (NO) production and release of TNF-α thus, increasing the survival of dopaminergic neurons. These mechanisms are consistent with the present findings, where CGA treatment reduced neuroinflammation and preserved nociceptive function [29].

The neuroprotective effects of CGA observed in the present study can be attributed to several complementary mechanisms. CGA acts as a direct antioxidant, scavenging peroxide radicals, hydroxyl radicals and superoxide ions in a dose-dependent manner. This property is particularly relevant in the context of NTG-induced

migraine, as NTG administration leads to increased production of reactive oxygen species through nitric oxide-mediated pathways. By scavenging these reactive species, CGA may prevent the oxidative damage that contributes to neuronal sensitization and pain maintenance. Anggreani and Lee (2017) reported that CGA exhibits dose-dependent antioxidant activity in scavenging DPPH radicals, ABTS radicals, hydroxyl radicals, peroxyl radicals, and superoxide anions, with hydroxyl groups at 3- and 4-positions in the benzene ring being important in determining antioxidant activity [29].

CGA activates the Nrf2 pathway, promoting the upregulation of antioxidant enzymes including glutathione peroxidase, catalase, and superoxide dismutase. This mechanism was indirectly supported by the present study's biochemical data showing that CGA treatment restored antioxidant enzyme activity in NTG-treated mice [29].

Cantero-Téllez et al. (2025) noted that Activation of the Nrf2 pathway by chlorogenic acid (CGA) enhances the expression of endogenous antioxidant enzymes, thereby supporting mitochondrial stability and limiting mitochondrial damage and excessive reactive oxygen species (ROS) generation [30].

Singh et al. (2018) demonstrated that CGA treatment significantly increased the activity of SOD and catalase in MPTP-intoxicated mice, with significant increases observed in both the striatum and substantia nigra [77].

CGA exerts direct anti-inflammatory effects through inhibition of the NF-κB signaling pathway. NF-κB is a ubiquitous transcription factor that plays a central role in activating genes responsible for expressing pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and iNOS. CGA has been shown to prevent NF-κB translocation from the cytoplasm to the nucleus, thereby reducing the transcription of pro-inflammatory genes.

Singh et al. (2018) demonstrated that CGA significantly reduced NF-κB expression in MPTP-intoxicated mice, with significant effects observed in both the substantia nigra and striatum [77]. Mitrea et al. (2020) reported that CGA (100

mg/day) significantly inhibited NF-κB expression in inflamed skin and kidney, while the effects of CGA on NF-κB and pNF-κB were dose-dependent [31].

CGA may modulate glial cell activation. Neuroinflammation in pain states is driven, in part, by activation of microglia and astrocytes, which release pro-inflammatory cytokines and promote neuronal sensitization. Singh et al. (2018) reported that CGA reduced GFAP expression in MPTP-intoxicated mice, indicating attenuation of astrocyte activation [77].

A noteworthy finding of the present study is the dose-dependent nature of CGA's protective effects. The 10 mg/kg dose generally produced the weakest and most inconsistent improvement, while the 50 mg/kg and 100 mg/kg doses produced progressively greater protection. This dose-response relationship was evident across all behavioral endpoints and cytokine measures, supporting the conclusion that CGA's neuroprotective effects are concentration-dependent. The dose-dependent effects observed in the present study are comparable with previous researches demonstrating that CGA exerts antioxidant and anti-inflammatory effects in a dose-dependent manner. Mitrea et al. (2020) reported that CGA (100 mg/day) significantly decreased lipid peroxidation and inhibited NF-κB expression, while the effects of CGA on NF-κB and pNF-κB were dose-dependent. They observed that the administration of CGA (100 mg/day) significantly decreased COX-2 expression and NF-κB levels, while CGA (150 mg/day) had opposite effects compared to the carrageenan group, suggesting a possible inflammatory response to a higher concentration of CGA [31].

Singh et al. (2018) found that among different doses of CGA, 50 mg/kg body weight was found to be more effective compared to 25 and 100 mg/kg body weight doses of CGA as shown by different behavioral, biochemical, immunohistochemical, and qPCR analyses. They reported that 50 mg/kg CGA produced the most significant improvements in rotarod, hanging, and narrow beam walking tests, as well as the most significant reductions in MDA and nitrite levels and increases in SOD and catalase activities. The authors noted that 50 mg/kg CGA showed more efficacy than 100 mg/kg CGA in several parameters, suggesting an optimal dose

range for CGA's neuroprotective effects [77]. The finding that the 10 mg/kg dose of CGA was largely ineffective in preventing NTG-induced pain sensitization suggests a threshold effect, below which CGA does not provide meaningful protection. This observation has important implications for the potential clinical use of CGA, as it suggests that effective neuroprotection may require relatively high doses of the compound. Anggreani and Lee (2017) reported that CGA provides protective effects at concentrations as low as 0.1 μM, although 10 μM was required to achieve optimal protection in certain assays. The authors noted that the antioxidant and neuroprotective effects of CGA are dose-dependent, with higher concentrations generally providing greater protection [29].

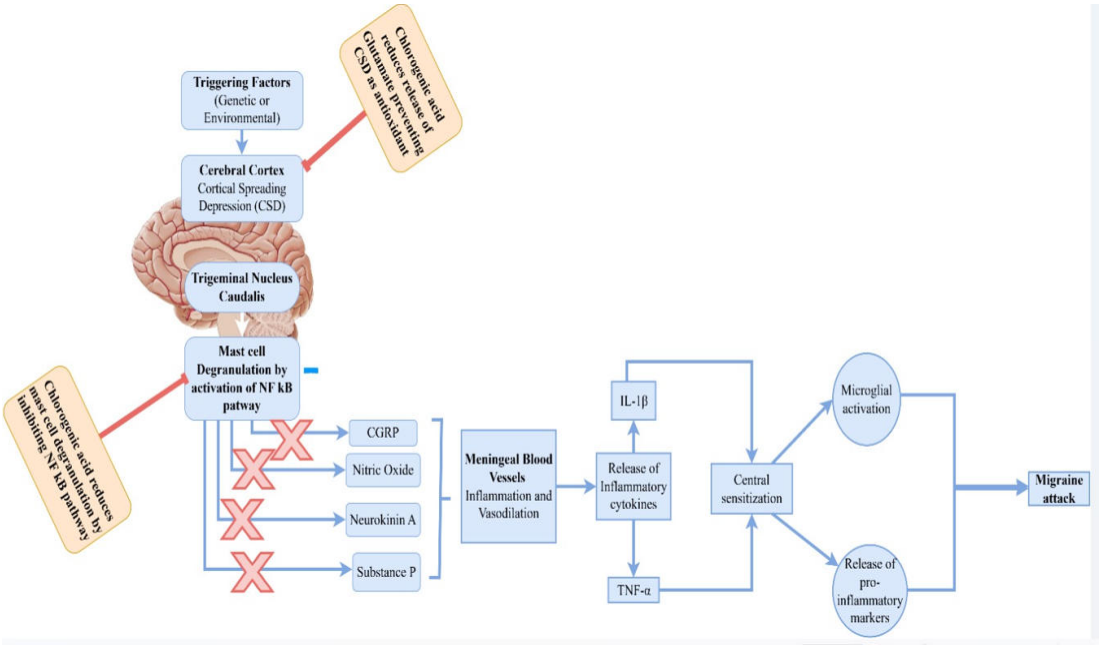


FIGURE 5.1: Proposed Mechanism of Chlorogenic Acid as Antimigraine Drug

The figure 26 shows the proposed mechanism by which chlorogenic acid act as antimigraine agent in mice. This diagram is aligned on the basis of previous studies which confirm its antioxidant and anti-inflammatory role by inhibition of NF kB pathway and indirectly inhibiting the release of inflammatory and proinflammatory cytokines involved in migraine pathophysiology [71, 78].

Chapter 6

Conclusion and Future Recommendations

6.1 Conclusion

In conclusion, the present study demonstrates that repeated NTG administration induces a comprehensive migraine-like phenotype in mice, characterized by thermal hyperalgesia, mechanical allodynia, photophobia, and elevated pro-inflammatory cytokine levels in the cortex and TNC. Chlorogenic acid treatment produced dose-dependent protection across all behavioral and biochemical endpoints showing a response seems to be delayed but the induction is slow due to involvement of transcription related signaling cascade and release of inflammatory cytokines that first lead to peripheral sensitization and then central sensitization which accounts for delayed progression of migraine like symptoms, with the 50 mg/kg and 100 mg/kg doses showing efficacy comparable to the reference drug zolmitriptan. The neuroprotective effects of CGA are likely occurring through the antioxidant and anti-inflammatory properties of CGA, including scavenging of reactive oxygen species and inhibition of NF-κB signaling. These findings are consistent with previous studies demonstrating that CGA reduces oxidative stress and neuroinflammation in various models of neurodegeneration. The dose-dependent

nature of CGA's protective effects, with the 50 mg/kg and 100 mg/kg doses showing significant efficacy while the 10 mg/kg dose was largely ineffective, suggests a threshold effect that has important implications for potential therapeutic use. Overall, these findings suggest that CGA may have potential as a preventive therapeutic agent for migraine and other pain conditions characterized by neuroinflammation and central sensitization.

6.2 Future Perspectives

Future studies with larger sample sizes are needed to confirm the cytokine findings and to permit more detailed mechanistic analyses. Power analysis should be conducted to determine appropriate sample sizes for detecting statistically significant differences in biochemical parameters. Future studies should investigate the effects of CGA on additional markers of neuroinflammation, including glial activation markers, oxidative stress markers such as lipid peroxidation products, and the expression of additional pro-inflammatory and anti-inflammatory cytokines. This would provide a more comprehensive understanding of the anti-inflammatory mechanisms of CGA. The effects of CGA on pain-related behaviors beyond thermal hyperalgesia, mechanical allodynia, and photophobia should be examined. Additional behavioral paradigms, including assessment of spontaneous pain, conditioned place preference/aversion, and open field and elevated plus maze tests for anxiety, would provide a more comprehensive characterization of the pain phenotype and the effects of CGA on affective pain processing. The pharmacokinetics of CGA in the brain should be investigated, including its ability to cross the blood-brain barrier and its concentration in relevant brain regions such as the cortex, TNC, and trigeminal ganglion. This would establish the relationship between brain CGA levels and neuroprotective effects and inform optimal dosing strategies. The effects of CGA should be evaluated in both male and female mice to investigate potential sex-dependent effects. Given the higher prevalence of migraine in women, understanding sex differences in the response to CGA would

have important clinical implications. Future studies should investigate the effects of CGA at different time points and durations of treatment to determine the optimal treatment regimen for migraine prevention. The present study used a preventive treatment protocol, but the effects of CGA on established pain states and on migraine chronification should also be evaluated. The molecular mechanisms underlying CGA's neuroprotective effects should be further elucidated. Studies examining the role of specific signaling pathways, including Nrf2, NF-κB, MAPK, and JNK pathways, as well as the role of specific receptors and ion channels in pain processing, would provide mechanistic insights. The potential synergistic effects of CGA with other dietary polyphenols or with conventional migraine treatments should be investigated. Given the complex etiology of migraine, combination therapies may provide greater efficacy than single compounds.

6.3 Limitations

While our study provides robust evidence for the neuroprotective effects of CGA against NTG-induced migraine-like phenotype, several limitations should be acknowledged. Our study did not observed the effects of CGA on additional markers of neuroinflammation, such as glial activation, oxidative stress, or the expression of other inflammatory mediators. Our study was conducted in BALB/c female mice only, thus the comparison of results based on gender or genetics can also be done which we lack in this study. Our study did not observed the effects of CGA on pain-related behaviors beyond the assessment of thermal hyperalgesia, mechanical allodynia, and photophobia. Additional behavioral paradigms to assess spontaneous pain, affective pain processing, and functional outcomes such as motor coordination and cognitive function would provide a more comprehensive characterization of the pain phenotype. Our study did not examined the pharmacokinetics of CGA in the brain, including its ability to cross the blood-brain barrier and its concentration in relevant brain regions.

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