

ISOLATION AND CHARACTERISATION OF AR-TURMERONE FROM TURMERIC AND ANALYSING ITS BLOCKING EFFICIENCY IN MICROGLIAL CELL LINES

*Submitted in partial fulfilment of the requirements for the award of Master of
Science in Medical Biotechnology and Clinical Research*

By

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is a bonafide work of **Ms. BHARISHI S (42101002)** who carried out Project entitled **"ISOLATION AND CHARACTERIZATION OF AROMATIC TURMERONE FROM TURMERIC AND ANALYSING ITS BLOCKING EFFICIENCY IN MICROGLIAL CELL LINES"** under the supervision of **Dr. Y. BERYL VEDHA**, Assistant Professor (Research) from September 2023 to April 2024.

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DECLARATION

I, **BHARISHI S (42101002)** hereby declare that the Project Report entitled **"ISOLATION AND CHARACTERISATION OF AROMATIC TURMERONE FROM TURMERIC AND ANALYSING ITS BLOCKING EFFICIENCY ON MICROGLIAL CELL LINES"** done by me under the guidance of **Dr. Y. BERYL VEDHA, Assistant Professor (Research)** at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

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ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to **The Board of Management of Sathyabama Institute of Science and Technology**, Chennai, for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. A. DAYANANDAN, Professor (Research)**, Head of the Department, Centre for Molecular and Nanomedical sciences for providing me necessary support and details at the right time during the progressive reviews. I would also like to express my sincere and deep sense of gratitude for his constant support and encouragement during the advancement of my project work.

I am extremely grateful to **Dr. Y. BERYL VEDHA, Assistant Professor (Research)** my project guide for her valuable guidance, suggestions, and constant encouragement paved the way for the successful completion of my project work.

I am extremely thankful to **Dr. S. JOHNSON RETNARAJ SAMUEL, Associate Professor (Research)**, **Dr. S. JACKSON DURAIRAJ, Associate Professor (Research)** and **Dr. A. MADHAN KUMAR, Associate Professor (Research)** for their valuable suggestions and support for the successful completion of my project work.

I wish to express my sincere thanks to all Research Scholars, Teaching and Nonteaching staff members of the Centre for Molecular and Nanomedical sciences who were helpful in many ways for the completion of the project.

I thank my parents and family members for their continuous love and support for the completion of my project and master degree.

Bharishi S

ABSTRACT

Aromatic turmerone is one among the biologically active compound found in the perennial herb Turmeric, *Curcuma longa* L. (Zingiberaceae). Ar-turmerone a sesquiterpene compound, adds greatly to the benefits of the turmeric, is not a well researched topic as curcumin. The ar-turmerone adds greatly to the benefit of turmeric properties, it is also known to exert biological properties such as anti-inflammatory, microglial blocking ability, and neuroprotective property. These properties have been found to have a great impact on the suppression of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). In this project the isolation of ar-turmerone was carried out using extraction and phase separation techniques to obtain isolated ar-turmerone. The isolated compound was then characterized with different characterisation techniques such as TLC, HPLC, LC-MS, UV-Vis spectroscopy and FTIR. LPS induced microglial cells were used in cotreatment with ar-turmerone to understand it's blocking efficiency.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
APCI	Atmospheric Pressure Chemical Ionization
APS	Ammonium Per Sulphate
Ar-turmerone	Aromatic turmerone
A β	Amyloid-beta
B-ME	Beta-Mercaptoethanol
BSA	Bovine Serum Albumin
CNS	Central Nervous System
COX-2	Cyclooxygenase
DMAPP	Dimethylallyl Pyrophosphate
DMSO	Dimethyl Sulfoxide
ECI	Electronspray Ionization
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared Spectroscopy

HO-1	Heme Oxygenase-1
HPLC	High Performance Liquid Chromatography
iNOS	Inducible Nitric oxide synthase
IL-1 β	Interleukins-1 β
IL-6	Interleukin-6
JNK	Jun N-terminal kinase
LC-MS	Liquid Chromatography-Mass Spectrometry
LC50	Lethal Concentration 50
LPS	Lipopolysaccharide
MMP-9	Matrix Metalloproteinase
MMP+	1-methyl-4-phenylpyridine
Mn-EBDC	Manganese ethylene bisdithiocarbamate
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide)
NF-kB	Nuclear Factor kappa -light-chain-enhancer of activated B cells
NO	Nitric Oxide

PBST	Phosphate Buffered Saline with Tween 20
PD	Parkinson's Disease
PVDF	Polyvinylidene Difluoride
Rf	Retention factor
RPMI 1640	Roswell Park Memorial Institute 1640
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TEC	Tectorigenin
TGF- β	Transforming Growth Factor Beta
TLC	Thin Layer Chromatography
TNF- α	Tumour Necrosis Factor Alpha

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CHAPTER 1

INTRODUCTION

1.1. TURMERIC



Fig 1.1: Turmeric rhizome

India is referred to as the "Land of Species" since it is currently home to over 65 different kinds of medicinal plants and herbs. India, Sri Lanka, China, Pakistan, Haiti, Jamaica, Peru, Bangladesh, El Salvador, Thailand, Taiwan, and Indochina are among the countries where it is widely grown. As to a global assessment, with a more than 60% share of the nation's total production, Andhra Pradesh is India's top producer of turmeric. India accounts for about 80% of global output of turmeric, making it the world's top producer followed by China which accounts for around 8% of the world's total turmeric output, followed by Myanmar at 4%, Nigeria at 3%, and Bangladesh at 3%. Notably, India accounts for most of the world's turmeric production. As a spice, herb, and medicinal plant, turmeric is utilized. (Sehgal *et al.*, 2016; Masango, 2015).

In certain parts of the world, turmeric—a dried powder made from the rhizome of *Curcuma longa* L. has been used for centuries. It has been associated with a variety of biological benefits, such as anti-aging, anti-inflammatory, anti-cancer, antigrowth, anti-arthritic, anti-atherosclerotic, antidepressant, and wound healing as well as memory-boosting properties (Aggarwal *et al.*, 2013). Due to its high curcumin content, Indian turmeric is regarded as some of the best in the world. *Curcuma longa* L. is its biological name; it is also referred to as Haldi in Hindi and Haridra in Sanskrit. In the scientific past of the Indian subcontinent, it holds immense religious significance. Turmeric is associated with luck, fertility, and the sun in both Hinduism and Buddhism. Turmeric is an amazing remedy for a number of health conditions, including liver problems, chicken pox, smallpox, skin cancer, conjunctivitis, chronic anterior uveitis, urinary tract infections (Guenther & Althausen, 1949).

Turmeric is suitable for a range of biotechnical applications because of its anti-inflammatory, choleric, antibacterial, antioxidant, and antifungal qualities. In addition to these applications, turmeric is frequently used as an oleoresin, an essential oil, and a natural pigment in spices and curry powder (Viasan *et al.*, 1989).

Curcumin, one of the components of turmeric, has been well researched. Additionally, turmeric contains a variety of additional chemical entities that have been discovered recently (Aggarwal *et al.*, 2013). About 75 different chemicals make up turmeric's essential oil, some of which have been identified and some of which have not. Numerous studies on the chemical makeup of turmeric oil have been conducted (Garg *et al.*, 1999).

But turmeric also has additional bioactive substances that are also known to have pharmacological effects, such as polysaccharides, fragrant turmerone, and sesquiterpenes. Lesser-studied essential oil makes about 3–6% of the rhizomes' dry weight. Sesquiterpenes, such as α -turmerone, are the primary constituents of this volatile fraction (Yan *et al.*, 2013).

1.2. AROMATIC TURMERONE

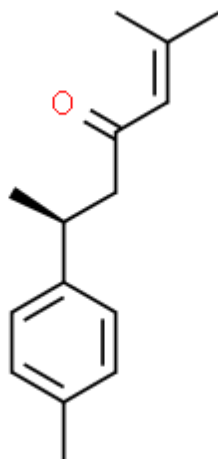


Fig 1.2: ar-turmerone (Pubchem)

Aromatic turmerone, commonly known as ar-turmerone or turmerone (-ar), is an intriguing sesquiterpenoid discovered in turmeric (*Curcuma longa* L.). Sesquiterpenoids are a kind of chemical substance composed of repeated units of a smaller molecule known as isoprene (Hucklenbroich *et al.*, 2014; Fluoria *et al.*, 2022). Aromatic turmerone is another important component, although its potential health benefits may be enhanced when combined with other curcuminoids found in turmeric. It adds greatly to turmeric's distinctive warm, spicy scent, which includes overtones of ginger and pepper. Beyond its lovely scent, aromatic turmerone has shown promise for human health. Early study indicates that it may have anti-inflammatory, neuroprotective, and potentially anticancer benefits (Sheikh *et al.*, 2023; Hucklenbroich *et al.*, 2014; Mythri & Bharath, 2012; Zha *et al.*, 2021). This fascinating chemical is isolated and characterized using techniques such as solvent extraction and GC-MS. Aromatic turmerone may be used as a natural flavoring agent in food and as a component of warm smells in perfumes, therefore its potential goes beyond culinary uses (Hucklenbroich *et al.*, 2014; Sheikh *et al.*, 2023). While much needs to be known, aromatic turmerone is a compelling chemical with a lovely scent and intriguing health effects, prompting more scientific research.

1.2.1. CHEMICAL COMPOSITION OF AROMATIC TURMERONE

Molecular formula: C₁₅H₂₂O.

Aromatic turmerone has a cyclic structure consisting of an aromatic ring and an oxygen-containing functional group (likely a ketone) (Hucklenbroich *et al.*, 2014).

1.2.2. BIOSYNTHESIS OF AROMATIC TURMERONE

Turmeric plants complicated metabolic processes facilitate the production of fragrant turmerone. This process is not completely defined yet, is anticipated to entail the following steps:

- The Mevalonic Acid Pathway: is the foundation to produce numerous terpenoids, including aromatic turmerone. It uses acetyl-CoA as a starting material to produce the five-carbon precursor molecule isopentenyl pyrophosphate.
- Dimethylallyl Pyrophosphate (DMAPP) Formation: IPP isomerizes to produce DMAPP.
- Tail-to-Tail Condensation: Multiple IPP and DMAPP units condense in a head-to-tail arrangement, with the exact mix dictating the terpenoid's ultimate structure. In the case of aromatic turmerone, the configuration of these precursors is still being studied.
- Cyclization and Functionalization: The condensed terpenoid precursor is cyclized and enzymatically modified to generate the final structure of aromatic turmerone, which includes the addition of the aromatic ring and an oxygen-containing functional group.

1.2.3. ENZYMES INVOLVED OF AROMATIC TURMERONE

While the enzymes involved in aromatic turmerone biosynthesis are not completely characterized, certain major enzymes usually seen in terpenoid biosynthesis are anticipated to play a role.

- Terpene Synthases: These enzymes catalyze the condensation of IPP and DMAPP units, resulting in the terpene skeleton.

- Cyclases: These enzymes catalyze the cyclization of the terpenoid precursor, resulting in the final ring shape.
- Oxidation/Reduction Enzymes: This class of enzymes introduces functional groups such as ketones or alcohols into terpenoid molecules.

1.2.4. EXPLORING THE POTENTIAL HEALTH BENEFITS OF AROMATIC TURMERONE

Aromatic turmerone, a fragrant chemical found in turmeric (*Curcuma longa* L.), has piqued researchers' interest not just for its pleasant perfume but also for possible health advantages. While turmeric has been used for medical purposes for millennia, research into the isolated effects of aromatic turmerone is still in its early stages. Here's a look at what science is discovering:

- Anti-inflammatory Potential: Turmeric's well-established anti-inflammatory qualities have sparked curiosity about the potential of its constituents, particularly fragrant turmerone. According to studies, aromatic turmerone may have anti-inflammatory properties, which correlate with its potential for treating illnesses such as chronic inflammation. However, further study is required to determine the precise processes by which aromatic turmerone performs these benefits (Hucklenbroich *et al.*, 2014).
- Neuroprotective potential: aromatic turmerone has a protective effect for brain cells. Some research indicate that it may have neuroprotective qualities, with possible effect neurodegenerative disorders such as Alzheimer's or Parkinson's. Further research is required to validate these preliminary findings and understand the underlying processes (Mythri & Bharath, 2012).
- Anti-Cancer Properties: Preliminary investigations indicate that aromatic turmerone may have anti-tumor properties, which require further exploration. Turmerones have therapeutic potential via altering many biological processes, including mutagenesis, oncogene expression, cell cycle control, apoptosis, tumorigenesis, and metastasis (Zha *et al.*, 2021; Nair *et al.*, 2019).
- Modulation of microglial activity: Microglia, immunological cells in the brain, have a double-edged sword effect. They can be useful for cleaning debris and pathogens, but prolonged activation can cause neuroinflammation. although not

complete inactivation, aromatic turmerone may have "blocking efficiency" - the capacity to inhibit microglial overactivation, thus fostering a more balanced response and lowering neuroinflammation. Further research is required to validate this topic (Perry *et al.*, 2010).

1.2.5. THE INTERACTION BETWEEN CURCUMINOIDS WITH AROMATIC TURMERONES

Turmeric contains several bioactive components, the most well-studied of which being curcumin.

Aromatic turmerone is another important factor, although its potential health benefits may be enhanced when combined with other curcuminoids found in turmeric.

Curcumin and aromatic turmerone may synergize through many mechanisms:

- Multiple Pathways: They may target diverse components of a disease process, resulting in a more holistic approach. Curcumin, for example, may target an inflammatory pathway, whereas aromatic turmerone focuses on cellular activities (Paul & Schaefer, 2013).
- Enhanced Absorption: Aromatic turmerone may boost curcumin's bioavailability (absorption), resulting in a larger overall benefit (Jiang & Gao, 2017).

1.3. MICROGLIAL CELLS ARE SENTINELS OF THE CENTRAL NERVOUS SYSTEM

Microglial cells, resident immunological protection of CNS, serve a variety of roles in brain health and function. These active cells are always monitoring their surroundings, ready to protect neurons from dangers and contribute to general neuronal health.

1.3.1. FUNCTIONS OF MICROGLIA

- Phagocytosis: Microglia serves as the CNS' sanitation squad. They continually search the brain parenchyma, recognizing and devouring cellular debris, infections, and apoptotic (death) neurons via a process known as phagocytosis.

This creates a clean environment devoid of hazardous substances, facilitating proper neural activity (Perry *et al.*, 2010).

- **Activating the Immune Response:** When the brain is exposed to stress, such as infection or damage, microglia are the first to respond. They get activated and release pro-inflammatory mediators, which draw immune cells from the periphery to confront the danger. This coordinated reaction is critical for eradicating infections and encouraging recovery (Hanisch & Kettenmann, 2007).
- **Neurotrophic Support:** Microglia not only defends the neuronal population, but they also nurture it. They release neurotrophic factors, chemical messengers that help neurons survive, develop, and differentiate. This supporting role is essential for sustaining a healthy and strong neural network (Liu *et al.*, 2021).
- **Synaptic Pruning:** Microglia helps to maintain the brain's dynamic character throughout development and maturity. They contribute to synaptic pruning, which is the act of selectively eliminating weak or unneeded connections between neurons. This exercise improves neuronal circuits, promotes effective neural transmission, and optimizes brain function (Schafer *et al.*, 2012).

1.3.2. THE DELICATE BALANCE: MICROGLIAL ACTIVATION AND NEURODEGENERATION

Microglial activation is a strictly controlled process. However, prolonged or excessive activation might have negative repercussions. In these situations, microglia release excessive pro-inflammatory mediators, which can harm healthy neurons and contribute to the progression of neurodegenerative illnesses like Alzheimer's and Parkinson's. Alzheimer's disease (AD) is a neurodegenerative illness characterized by gradual deterioration in cognitive function; similarly, Parkinson's disease (PD) has been significantly related to neuroinflammation caused by microglial activation. However, a treatment to slow or reverse the course of these illnesses remains elusive. Current research in the field of Alzheimer's disease reveals that age-dependent neuroinflammatory alterations have a substantial influence on neurogenesis and cognitive impairment. The innate immune response includes pro-inflammatory (M1) macrophage activation, which results in the production of cytokines, chemokines, and reactive intermediates. This is followed by a resolution

phase, which includes alternate activation of antiinflammatory signaling (M2a) and wound healing (M2c). It has been postulated that microglial activation characteristics are similar to macrophages and play an important role in controlling neurogenesis in the brain.

Microglial activation is a strictly controlled process. However, prolonged or excessive activation might have negative repercussions. In these situations, microglia release excessive pro-inflammatory mediators, which can harm healthy neurons and contribute to the progression of neurodegenerative illnesses like Alzheimer's and Parkinson's. Notably, as we age, microglia's activation profile shifts from M2 to M1 skew.

Furthermore, quantitative proteome analysis was performed to identify potential mediators involved in microglial activation because of degeneration, using techniques like SILAC. This method identified several novel proteins that may be involved in neurodegeneration-mediated microglial activation, a process considered to be critical to Parkinson's disease progression.

The overlap of data from both illnesses highlights the crucial significance that resident immune cells of the CNS, particularly microglia, play in initiating or aggravating neurodegeneration in several disorders. However, the precise mechanisms that trigger their activation in neurodegenerative disorders have yet to be identified. Identifying these pathways might lead to more effective therapy or prevention measures targeting CNS-resident immune responses in certain chronic disorders (Varnum & Ikezu, 2012; Zhou *et al.*, 2005).

1.3.3. THE TWO FACES OF MICROGLIAL ACTIVATION

Despite having different embryonic beginnings, microglia and resident tissue macrophages are connected. Microglial activation is widely categorized into two major phenotypes: M1- like activation and M2- like activation. Monocyte-derived macrophages are divided into four phenotypic subsets: M1, M2a, M2b, and M2c. Microglia may develop one of these distinct phenotypes as a result of their exposure to a variety of stimuli. M1 is a phagocytic condition characterized by the synthesis and release of pro-inflammatory cytokines (IL-1 β , IL-6, IL-23, TNF- α) and cytotoxic chemicals (oxygen-free radicals, nitric oxide (NO), and quinolinic acid). It is required

to eliminate infections and initiate immunological response in the brain. M2 is a phagocytic phenotype that causes anti-inflammatory responses (IL-10, TGF- β) or enhances tissue regeneration by increasing the proliferation of new glial cells and the development of scar tissue. It also helps neurons survive by increasing neurotrophic factor synthesis and neural resilience. A fast shift to M2 activation is critical for reducing inflammation and facilitating healing following an injury.

It is vital to remember that M1 and M2 indicate a range of activation patterns rather than distinct cell subtypes. Thus, a continuum of multiple intermediate phenotypes may exist, and microglial cells will flip between them depending on the nature of the stimulus that triggered the initial injury as well as subsequent events that influence phenotype. However, persistent or excessive M1 activation can be detrimental to neurodegeneration by encouraging neuronal death and tissue damage (Correale, 2014; Lull & Block, 2010).

CHAPTER 2

LITERATURE SURVEY

2.1. ISOLATION AND CHARACTERISATION OF AR-TURMERONE

Ar-turmerone is a curcumin derivative found in turmeric oleoresins. Ar-turmerone has a variety of pharmacological actions, including anticancer, antioxidant, antidepressant, antiepileptic, anti-dermatophyte, and antiplatelet properties. The goal of this study was to create a low-cost technique for isolating ar-turmerone from turmeric oleoresin using silica gel adsorbent.

Ar-turmerone was separated using silica gel adsorbent and eluted with solvents such as acetonitrile, 96% ethanol, ethyl acetate, toluene and n-hexane. In UV-visible spectra the peak was observed at 237nm. The FTIR spectrum revealed values at 2300 cm^{-1} and 2935 cm^{-1} for aromatic C=C stretching, 1445 cm^{-1} and 1735 cm^{-1} for C=O stretching, and 990 cm^{-1} and 1100 cm^{-1} for C-H bending. The GC-MS spectra revealed ar-turmerone (molecular weight 216) as the primary peak with a retention time of 12.884 minutes and a percentage area of 27.74%. To isolate ar-turmerone from turmeric oleoresin using silica gel as an adsorbent, a 1:3 ratio of turmeric oleoresin to silica gel was found to be optimal, with no effect on adsorption time. The ideal eluent for the elution step was 96% ethanol (Husein *et al.*, 2023).

2.2. AROMATIC TURMERONE AND MICROGLIAL CELLS

Aromatic turmerone, a chemical found in turmeric, has been extensively studied due to its possible medicinal benefits. Turmeric contains a variety of bioactive chemicals, including ar-turmerone, which has been demonstrated to have anti-inflammatory, antioxidant, and anticancer activities (Liu 2022; Shirsat 2022; Jayaprakasha 2005; Muhammad 2020). However, the isolation of ar-turmerone from turmeric has not received much attention in the literature. Additional study is required to investigate the isolation and possible uses of this chemical.

2.2.1. MICROGLIAL ACTIVATION AND NEUROINFLAMMATION

Various stimuli, such as lipopolysaccharide (LPS), amyloid-beta (A β) peptides, and HIV infection, can activate microglial cells, resulting in the production of proinflammatory cytokines and neuroinflammatory responses (Alvarez-Carbonell *et al.*, 2017; Fricker *et al.*, 2012; Ye *et al.*, 2017). These activated microglia have been linked to the aetiology of neurodegenerative illnesses such as Parkinson's and Alzheimer's, as well as the advancement of neuroinflammatory symptoms associated with HIV infection (Borrajó *et al.*, 2014; Ye *et al.*, 2017). However, the processes underpinning microglial activation and their potential regulation by bioactive chemicals, such as aromatic turmerone, have yet to be fully understood.

2.2.2. AROMATIC TURMERONE: A MODULATOR OF MICROGLIAL FUNCTION

Aromatic turmerone has been shown to have anti-inflammatory and anti-cancer properties by inhibiting NF- κ B activation and the expression of MMP-9 and COX-2 in cancer cells (Park *et al.*, 2012). These findings imply that aromatic turmerone has the ability to modulate inflammatory responses and cellular invasiveness, both of which are important characteristics of activated microglia. However, the direct effects of aromatic turmerone on microglial activation and neuroinflammatory pathways have not been extensively studied.

2.2.3. THE CYTOTOXIC AND GENOTOXIC EFFECTS OF AROMATIC TURMERONE

While aromatic turmerone has potential anti-inflammatory and anti-cancer properties, it has also been demonstrated to have cytotoxic and genotoxic effects on neuronal and neuroblastoma cells (Turkez *et al.*, 2014). These findings raise concerns regarding aromatic turmerone's safety profile and its possible effect on microglial cell survival and function. More study is needed to investigate the cytotoxic and genotoxic effects of aromatic turmerone on microglial cells, as well as to find the best dose for regulating microglial activities while minimizing cellular damage that microglial activation characteristics are like those of macrophages, and that activation plays an important role in controlling neurogenesis in the brain. During ageing, microglia convert from M2- to M1-skewed activation phenotypes. They have evaluated the neuroimmunological investigations that led to the identification of the

several microglial activation states in AD mice models. It will also examine how microglial activation affects neurogenesis in Alzheimer's disease and offer antiinflammatory drugs as promising treatment targets. Molecules such as interleukin-4 and CD200 have been identified as significant anti-inflammatory mediators in the control of neuroinflammation in the brain, and their therapeutic potential which is examined in depth.

2.3. MICROGLIA AND NEURODEGENERATION

2.3.1. MICROGLIAL ACTIVATION INDUCED BY NEURODEGENERATION IN PARKINSON'S DISEASE

The mechanism by which microglia get activated in Parkinson's disease is unknown. Preliminary studies using primary cell cultures treated with parkinsonian toxicants such as manganese ethylene bisdithiocarbamate (Mn-EBDC) and 1-methyl-4phenylpyridine (MPP+) show that these agents can cause DAergic (dopaminergic) neurodegeneration and subsequent microglial activation. Activated microglia increases the DAergic neurotoxicity generated by these toxicants. A study of neuron-microglia interactions identified components in conditioned media from a DAergic cell line treated with toxicants that potently activated microglia (Zhou *et al.*, 2005).

2.3.2. MICROGLIAL ACTIVATION PHENOTYPES ON NEURODEGENERATION IN ALZHEIMER'S DISEASE

While microglial activation characteristics are like those of macrophages, and that activation plays an important role in controlling neurogenesis in the brain. During ageing, microglia convert from M2- to M1-skewed activation phenotypes. They have evaluated the neuroimmunological investigations that led to the identification of several microglial activation states in AD mice models. It will also examine how microglial activation affects neurogenesis in Alzheimer's disease and offer antiinflammatory drugs as promising treatment targets. Molecules such as interleukin-4 and CD200 have been identified as significant anti-inflammatory mediators in the control of neuroinflammation in the brain, and their therapeutic potential which is examined in depth (Varnum & Ikezu, 2012).

CHAPTER 3

AIM AND OBJECTIVES

3.1. AIM

To isolate and characterize aromatic (ar-) turmerone from turmeric and analyse its blocking efficiency on microglial cell lines.

3.2. OBJECTIVES

- Isolation of Turmeric oil from Turmeric.
- Isolation of ar-turmerone from turmeric oil by Soxhlet extraction.
- Purification of turmeric oil using activated charcoal.
- Characterisation of ar-turmerone using TLC, HPLC, LC-MS, UV Spectroscopy and FTIR.
- To identify the blocking efficiency of ar-turmerone using microglial cell lines.

CHAPTER 4

MATERIALS AND METHODS

4.1. TURMERIC

Turmeric rhizome *Curcuma longa* was bought from a farm in Dindigul village. The turmeric rhizome was added to a grinder and grinded them to a fine powder(Fig 4.1). This turmeric powder was then kept in an airtight container, away from sunlight and moisture in a cool shady place. This helped the turmeric powder to preserve its freshness for a longer period.



Fig 4.1: Turmeric powder

4.2. ISOLATION OF AROMATIC TURMERONE

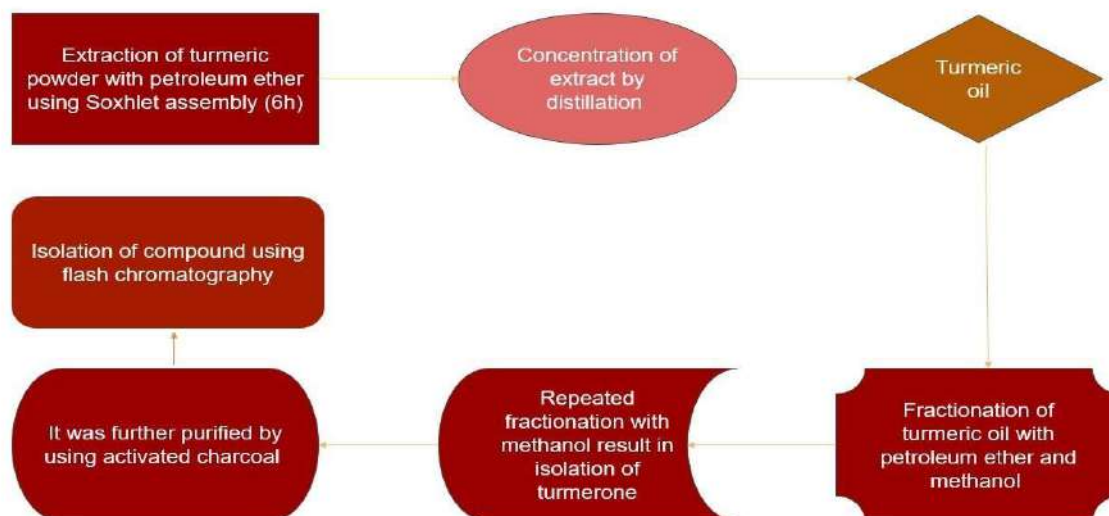


Fig 4.2: Work plan of isolation of ar-turmerone

4.2.2 SOXHLET EXTRACTION OF TURMERIC

Soxhlet extraction is a procedure for extracting nonvolatile and semi volatile organic compounds from solids such as soils, sludges, and wastes. The Soxhlet extraction process ensures intimate contact of the sample matrix with the extraction solvent.

4.2.2.1. Principle

Soxhlet extraction method uses a small amount of solvent and is very cost-effective. The Soxhlet extraction uses the solvent reflux and siphon principle to continuously extract the solid matter by pure solvent, which makes the solvent continuously extract the solid matter to pure solvent, which is highly efficient. The solid sample is placed on a thimble-shaped filter paper, positioned into Soxhlet extractor, and the device is assembled. The solvent is added to the solvent reservoir flask and mounted onto a heating mantle. After heating, the condensed vapors of the solvent interact with the sample powder, and the soluble part of the powder gets mixed with the solvent for extraction. When the solvent surface exceeds the maximum height of the siphon, the solvent containing the extract is siphoned back. The flask is repeated, extracting a portion of the material each time so that the solid material is constantly used as a pure solvent and the extracted material is concentrated in the flask.

4.2.2.2. Procedure

50g of turmeric powder (*Curcuma longa L.*) was extracted with 500ml of petroleum ether using soxhlet assembly for 12 hr. The extract obtained was then concentrated by using distillation. The solid material from the soxhlet assembly was extracted twice more with petroleum ether and the concentrated filtrates or extracts were combined. This extract was nothing but turmeric oil. Turmeric oil was then fractioned between petroleum ether and methanol using separating funnel. Repeated fractionation was carried out with methanol.

4.2.3 CHARCOAL PURIFICATION

Petroleum ether fraction was then treated with activated charcoal to remove any impurities present. In this, some amount of charcoal was taken in Whatman No 1 filter paper (110mm pore size) and the petroleum ether fraction was poured over it, until the colorless extract is obtained.

4.3. CHARACTERISATION

4.3.1. THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is an affinity-based method used to separate compounds in a mixture. TLC is a highly versatile separation method that is widely used for both qualitative and quantitative sample analysis. TLC can be used to analyse virtually any substance class, including pesticides, steroids, alkaloids, lipids, nucleotides, glycosides, carbohydrates, and fatty acids.

4.3.1.1. Principle

TLC is based on the classic chromatography principle where mixture components are separated between a fixed stationary phase and a liquid mobile phase by differential affinities between the two phases.

4.3.1.2. Procedure

Thin Layer Chromatography of isolated compounds was performed by the ascending technique. The process parameters include Silica gel 60F₂₅₄ pre-coated TLC plate (Merck) as adsorbent, Toluene: Ethyl acetate (93:7) as chromatography solvent. Vanillin sulfuric acid was used as spraying agent after the length 5.0 cm length and then heated in hot air oven for 5-10 mins.

4.3.1.3. Calculation

The retention factor (R_f) is used to measure the movement of compounds along the TLC plate. R_f is defined as the distance travelled by an individual component divided by the total distance travelled by the solvent. Its value is always between zero and one.

$R_f = \frac{\text{distance traveled by component}}{\text{distance traveled by solvent}}$

4.3.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High-performance liquid chromatography (HPLC) is a powerful separation technique used for the analysis of ions, proteins, and organic molecules. HPLC is based on the mechanisms of adsorption, partition, and ion exchange, which depend on the type of stationary phase used. The technique involves a solid stationary phase, usually enclosed in a stainless-steel column, and a liquid mobile phase. The molecules of interest are separated based on the difference in the relative distribution ratios of the solutes between the two phases. HPLC is widely used to assess the purity and to determine the content of potential pharmaceutical molecules. It can also be employed for the determination of enantiomeric composition, using the modified mobile phases or chiral stationary phases.

4.3.2.1. Principle

The high-performance liquid chromatography works on the principle that some components of the sample take longer than others to pass through the chromatography column. The time taken by the molecules depends on the affinity of the molecule with the mobile phase (liquid or gas) and the stationary phase (solid

or liquid). The ones with greater affinity with the stationary phase take longer to pass through the column and vice versa.

4.3.2.2. Procedure

The extraction of ar-turmerone was analysed by an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) with Zorbax SB-C18 column (100 Å, 3.5 µm, 150 mm length, 2.1mm diameter, Agilent Technologies, USA)). Mobilephase (A): ultrapure HPLC water; (B): acetonitrile. The flow rate was 1ml/min at gradient for the entire separation with column temperature at 25°C. Typically, 20µL of extracts obtained were injected into HPLC and analysed at 240nm.

4.3.1.3. Calculation

The concentration of the isolated compound using HPLC is calculated using the formula: $\text{Area of the sample} \div \text{Area of standard} \times \text{concentration of standard}$

4.3.3. LC-MS

The platform of analytical techniques is vast, and one such notable contribution to the field of diagnostics is by the LC-MS technology. This is basically a combination of a HPLC with MS, wherein the specificity of HPLC is coupled with the sensitivity of MS.

4.3.3.1. Principle

The LC-MS technology involves use of an HPLC, wherein the individual components in a mixture are first separated followed by ionization and separation of the ions on the basis of their mass/charge ratio. The separated ions are then directed to a photo or electron multiplier tube detector, which identifies and quantifies each ion. The ion source is an important component in any MS analysis, as this basically aids in efficient generation of ions for analysis. To ionize intact molecules, the ion source could be APCI (Atmospheric Pressure Chemical Ionization), ESI (Electrospray

ionization), etc. to name a few popular ones. The choice of ion source also depends on the chemical nature of the analyte of interest i.e. polar or non-polar.

The major advantages of this technology include sensitivity, specificity and precision as analysis is done at molecular level. Also, structural details of the analyte can be deciphered

4.3.3.2. Procedure

The petroleum ether extract was analysed by 1260 Infinity II Preparative LC/MSD System.

4.3.4. UV-VIS SPECTROSCOPY

UV-Vis Spectroscopy or Ultraviolet-visible spectroscopy or Ultraviolet-visible spectrophotometer (UV-Vis) is also called absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region.

4.3.4.1. Principle

When a specific wavelength of light hits a molecule, that molecule gets excited. Once the electron excites, it excites from the ground (lower) energy state to the higher energy state. When an electron jumps off, it absorbs light energy because electrons in the orbital at a lower energy state utilize energy to move to a higher energy level.

4.3.4.2. Procedure

The petroleum ether extract was analysed by Bio-Tek Epoch Microplate Spectrophotometer. Epoch is controlled by Gen6 Data Analysis Software, with simple programming and powerful data analysis. The petroleum ether extract was observed under UV-Vis spectrophotometer in the range of 200nm to 700nm.

4.3.5 FTIR

The main goal of the spectroscopy techniques is to measure how much light is absorbed in each wavelength shined on any material. This is easily done by using the dispersive spectroscopic technique by shining the beam of light on the material and measuring the amount of light absorbed and emitted. This technique is applied in various fields such as chemistry, geology, biology, and materials, etc. The technique is very popular because it can analyse all gas, liquid, and solid samples.

4.3.5.1. Principle

FTIR spectroscopy is based on the idea that the interference of radiation between the two beams yields an interferogram. The latter is a signal produced as a function of the path length change between the two beams reflected from two mirrors (resides in the interferometer block). The two domains of distance and frequency are interconvertible by the mathematical method of Fourier transformation hence the name Fourier transform infrared spectroscopy

4.3.5.2. Procedure

FTIR analysis shows that the compound is present in the petroleum extract. FTIR analysis was accomplished by using a PerkinElemer Fourier-transform infrared spectrophotometer with resolution 4cm^{-1} in the range of $400\text{-}4000\text{cm}^{-1}$. For FTIR analysis sample in liquid form was used.

4.4. ZEBRAFISH EMBRYO TOXICITY

It is an acute test that uses short-term exposure (96 hours) to a substance and determines the concentration that is lethal to 50% of zebrafish embryos (LC_{50}) as an indicator of acute fish toxicity.

4.4.1. PRINCIPLE

Newly fertilized zebrafish eggs are exposed to the test chemical for a period of 96 hrs. Every 24 hrs, up to four apical observations are recorded as indicators of lethality: (i) coagulation of fertilized eggs, (ii) lack of somite formation. (if) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heartbeat.

At the end of the exposure period, acute toxicity is determined based on a positive outcome in any of the four apical observations recorded, and the LC₅₀ is calculated.

4.4.2. DISTRIBUTION OF THE EGGS OVER 24 WELL PLATES

Eggs are distributed to well plates in the following numbers;

- 20 eggs on one plate for each test concentration
 - 20 eggs as solvent control on one plate (if necessary)
 - 20 eggs as positive control on one plate
 - 4 eggs in dilution water as internal plate control on each of the above plates ○
- 24 eggs in dilution water as the negative control on one plate.

4.4.3. PREPARATION OF E3 MEDIA (60X STOCK)

60x stock for 1litre

Sodium chloride (NaCl) – 17.4g

Potassium chloride (KCl) – 0.8g

Calcium chloride (CaCl₂, 2H₂O) – 2.9g

Magnesium chloride (MgCl₂, 6H₂O) – 4.88g

Check the pH for 7.2 and store it in refrigerator.

4.4.5. PREPARATION OF 1X E3 MEDIA

From 60x stock 16.5ml was added to 1 litre of autoclaved water. Then add 2 to 4µg of methylene blue to prevent fungal contamination and store it in refrigerator.

4.4.6. PROCEDURE

- Collect the fertilized embryos within 1-2hrs after mating and then gently collect the embryos from the tank
- Rinse the embryos with RO water 2 times and then transfer the embryos in E3 medium washed with 3 times to remove debris and excess mucus.
- Distribute the embryos in 24 well plate, 2µg, 4µg, 6µg, 8µg & 10µg of the test substance for ethanol extract. Ensure that the volume of the compound in each well is sufficient to cover the embryos adequately.
- Incubate the embryos under standard conditions, typically at controlled temperature 28-30°C.
- After 24hrs incubation observe the embryos under microscope to analyse the morphological changes such as developmental abnormalities, growth retardation, or mortality, using a standardized scoring system.

This toxicity assessment was strictly followed by OECD guidelines, for analysis the embryotoxicity of turmerone treated embryos at different concentrations 2µg, 4µg, 6µg, 8µg & 10µg.

4.4.7. CALCULATIONS

Apical observations were performed on each tested embryo, include coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat. These observations are used to determine lethality. Any positive outcome in one of these observations means that the zebrafish embryo is dead. Observations are recorded every 24 hrs, until the end of the test. Additionally, hatching is recorded in treatment and control groups daily starting from 48hrs.

4.5. MTT ASSAY

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium salt commonly used to measure cell viability, proliferation, and cytotoxicity. In live metabolically active cells, water-soluble MTT is reduced in the mitochondria by succinate dehydrogenase to insoluble purple formazan crystals.

The amount of formazan produced can be quantified spectrophotometrically (590nm) upon solubilization and is directly proportional to the number of viable cells in the culture.

The MTT assay is a safe, sensitive, and reliable indicator of cell viability and is preferred over other endpoint viability assays, such as the radioactive ³H-thymidine incorporation assay. Besides having a lengthy and tedious protocol, the health hazards of working with radioactive materials are well recognized, as are the accompanying economic considerations required for the appropriate handling and disposal of radioactive waste.

4.5.1. REAGENT PREPARATION

Prepare MTT solution

MTT is soluble in water (10 mg/ml), ethanol (20 mg/ml), and buffered salt solutions and culture media (5 mg/ml). We recommend using a 5 mg/ml solution in PBS. Mix by vortexing or sonication.

Prepare MTT solvent

PBS solvent

4.5.2. PROCEDURE

1. Cells were grown in RPMI 1640 media supplement with 10% FBS, collected using trypsin.
2. Cells were counted using a cell counter Countess F3.
3. Cells were seeded in the 96 well plate at the concentration of 10000 cells per well.
4. The cells were incubated at 37°C in CO₂ Incubator
5. After the cells were attached, the media was removed from the plate and washed with 1X PBS twice.
6. The sample was then added in triplicate along with 3% FBS in different concentrations from 1 µg - 20 µg (v/v).
7. Cells were incubated at 37°C in CO₂ Incubator for 24 hours.

8. For adherent cells, carefully aspirate the media.
9. Add 10 μ L of MTT solution into each well.
10. Incubate the plate at 37°C for 2 hours.
11. After incubation, add 100 μ L of DMSO to each well.
12. Wrap the plate in foil and keep the plate on the rack for 15 minutes. Occasionally, pipetting of the liquid may be required to fully dissolve the MTT formazan.
13. Read absorbance at OD=590nm. Read plate within 1 hour.

4.5.3. CALCULATION

1. Average the duplicate reading for each sample.
2. Subtract the culture medium background from your assay readings. This is the corrected absorbance.
3. Calculate percentage cytotoxicity with the following equation, using corrected absorbance: Percentage of cytotoxicity = $(100 \times (\text{control} - \text{sample}))$

4.6. ISOLATION OF PROTEIN SAMPLE

Beta-Mercaptoethanol (β -ME-reducing agent) is used for breaking disulfide bonds of protein. 2X sample buffers were brought to room temperature before use. After that, 2X sample buffer was added with 5% of Beta-Mercaptoethanol (reducing agent) which is freshly prepared. From the cells the media was removed and washed with 1X PBS twice. Now 100 μ l of sample buffer was added and scrapped using cell scraper. Then the samples were collected into a fresh 1.5ml vial which is then maintained at 4°C. Using pipette, the cells were homogenized by rapid pipetting for 2-3 minutes. After that samples were transferred into the dry bath at 95°C for 5 minutes, then allowed to cool and was stored at -20°C.

4.7. SDS-PAGE

SDS-PAGE is an analytical technique that is used to separate the proteins based on their molecular weight. Electrophoresis is the study of the movement of charge molecules in an electric field. Their rate of movement depends on the strength of the field, net charge, size and shape of the molecules, and also the ionic strength, viscosity, and temperature of the medium in which the molecules are moving. Polyacrylamide gels are widely used for separating larger molecules like proteins. Based on the resistance of the gel matrix proteins are separated viz., smaller proteins generally have less resistance as well as larger proteins have more resistance.

The molecular weight of the protein was estimated if they are subjected to electrophoresis in the presence of a detergent sodium dodecyl sulfate (SDS) and a reducing agent β Mercaptoethanol, it can bind with the protein backbone and denaturing effect of SDS is very strong and that disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. Mercaptoethanol assists the protein denaturation by reducing all disulfide bonds (both inter disulfide and intra-disulfide bonds). Polyacrylamide creates a mesh-like matrix that helps in the separation of proteins. During PAGE electrophoresis linear or primary structures of the proteins were stacked or arranged properly, once reached the separating gel, proteins started to separate based on their size.

4.7.1. MATERIALS

Eppendorf's; Micro-pipettes; Micro-tips; Centrifuge; Water bath; Vortex and Distilled water.

4.7.2. CHEMICAL PREPARATION OF SDS-PAGE

30% Acrylamide and 0.3% Bis acrylamide:

Acrylamide – 30g

N, N' Methylene Bisacrylamide - 0.3g

Makeup into 100ml with distilled water.

4X Separating Buffer:

Tris HCL - 18.2g

Distilled water - 60ml

pH was adjusted to 8.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4g

Makeup into 100ml with distilled water.

4X Stacking Buffer:

Tris Hcl - 6.05g

Distilled water - 40ml

pH was adjusted to 6.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4g

Makeup into 100ml with distilled water.

2X Sample Buffer:

Tris stacking buffer (pH 6.8) - 25ml

Sodium Dodecyl Sulphate (SDS) - 4g

20% Glycerol - 20ml

Makeup into 100ml with distilled water.

During protein sample preparation, 5% β Mercaptoethanol and during loading 0.001% (w/v) of Bromo-phenol blue was added.

10% Ammonium Per Sulphate (APS):

APS - 100mg

Makeup into 1ml with distilled water.

1X Running Buffer:

Tris Hcl - 3.94g

Glycine - 15.014g

SDS -1g

Makeup in 1L with distilled water.

Staining Solution:

Commassie Brilliant Blue R-250 - 200mg

Methanol - 50ml

Glacial Acetic acid - 7ml

Makeup into 100ml with distilled water.

The above mixture was filtered using Whatman No 1 filter paper.

Destaining Solution:

Methanol - 30ml

Glacial Acetic acid - 7ml

Makeup into 100ml with distilled water.

Table.1: Separating gel concentration

Stock Solution	5%	6%	7%	7.5%	8%	9%	10%	12%	13%	15%
30% Acrylamide + 0.3% Bis-acrylamide (ml)	5.0	6.0	7.0	7.5	8.0	9.0	10.0	12.0	13.0	15.0
4XTris Separating Buffer (ml) pH 8.8	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Distilled water (ml)	17.5	16.5	15.5	15.0	14.5	13.5	12.5	10.5	9.5	7.5
10%APS (μl)	100	100	100	100	100	100	100	100	100	100
TEMED (μl)	10	10	10	10	10	10	10	10	10	10

Table.2: Stacking gel concentration

Sl.no	Stock Solution	5ml	10ml	15ml	20ml
1.	30% Acrylamide + 0.3% Bis-acrylamide (ml)	0.65	2.5	3.75	5.0
2.	4X Tris Stacking Buffer (ml) pH 6.8	1.25	2.5	3.75	5.0
3.	Distilled water (ml)	3.05	5.0	7.5	10.0
4.	APS (μl)	100	100	100	100
5.	TEMED (μl)	10	10	10	10

4.7.3. PREPARATION OF GEL

The glass plates were cleaned by using ethanol and manually assembled. Glass plates were fixed in the proper equalized manner with each corner covered by using spacers so that they are used as a primary baseline on gel which helps in the process of agarose sealing or second level baseline system. 1% agarose was prepared and boiled until liquidy then corners were sealed in both surface and bottom area of the spacer. Separating gel was manually prepared and loaded on the gel carefully. The gel was overlaid with isopropyl alcohol so that it excludes the air. Then IPA was removed and washed with distilled water, after the drain stacking gel was prepared and added, instantly comb was fixed on the stacking gel. The gel was allowed a few minutes to solidify, then the comb was carefully removed, and wells manifestly washed with water to eliminates the bubbles, the bottom of the spacer was carefully removed, and the setup was fixed in the bottom chamber which carried 1X running buffer as well as an upper chamber.

4.7.4. SAMPLE LOADING AND ELECTROPHORESIS

A protein sample of 80µg and the appropriate molecular markers were loaded into the wells. The apparatus was connected to the power pack with proper polarity and 60v was applied. After electrophoresis, the glass plate assembly was taken from the buffer chamber. The spacer was gently twisted so that the upper glass plate pulled away from the gel.

4.7.5. STAINING AND DESTAINING THE GEL

The gel was carefully taken out from the glass plate and the stacking gel was removed by using a cleaver. The separating gel was removed by gently grasping the two corners of the gel and placed in the container containing the Coomassie brilliant blue R250 (stainer solution). The gel should be fully submerged in the staining solution for 60 minutes by gentle agitation on a Rocker. Then it was washed with distilled water and transferred to a destaining solution until protein bands are visualized clearly. Commonly, the first destainer wash is about 30 minutes, then the second wash is about overnight.

4.8. IMMUNOBLOTTING

4.8.1. CHEMICAL PREPARATION OF IMMUNOBLOTTING

10X Transfer Buffer (Stock)

Glycine- 72.1g

Tris-base – 15.2g

DH₂O- 500ml

The 10X transfer buffer was stored at room temperature, and during blotting the 1X transfer buffer was prepared from the 10X in the ratio of preparation viz.,

For a Total Volume of 1 litre

100ml- 10X Transfer Buffer

200ml- Methanol

700ml of DH₂O

4.8.2. RUNNING THE GEL

Prepare 12% SDS gel and place it in SDS-PAGE setup. Pour 1X running buffer (100ml of 10X running buffer + 900ml of distilled water) into the setup. A protein ladder of 5 μ l is loaded at the first lane of the gel for molecular weight determination. In other wells, the prepared protein samples were added (80 μ g/well) concentration. After loading all the samples, the setup started to run at a 60V unit. The samples were left to run till they reached the bottom line.

4.8.3. MEMBRANE TRANSFER

After the protein runs fully till the end, take off the glass slides from the setup and cut off the stacking gel. Then gently take the gel and keep it in the transfer butter for 5 minutes and simultaneously, cut the PVDF membrane by the same gel size. Soak the PVDF membrane in the methanol for 1–2 minutes for activation of the membrane. Then, keep the membrane in 1X transfer butter until preparing the sandwich for blotting.

Prepare blotting apparatus, sponge, and filter paper to arrange the sandwich setup and soak it in 1X transfer buffer before using.

4.8.4. BLOCKING

A blocking buffer was used to block all proteins which would bind with targetable and non targetable proteins. 5% of Bovine serum albumin (BSA) with 20ml of 1X TBST buffer was prepared for Blocking and let in for 2 hours.

4.8.5. PRIMARY ANTIBODY

The primary antibody was diluted in 5% BSA in the following dilutions given below and incubated overnight in cold conditions.

- Actin (produced from Rabbit polyclonal) - (1:5000 in 5%BSA).
- INOS (produced from Rabbit polyclonal) - (1:5000 in 5%BSA).
- HO-1 (produced from Rabbit polyclonal) - (1:2000 in 5%BSA).

After undergoing an incubation period overnight keep the membrane at room temperature for 10mins and start the washing process.

4.8.6. SECONDARY ANTIBODY

Secondary antibody (Anti Rabbit HRP (Produced in goat) was added in the 5% Bovine serum albumin (BSA) (1:10000 dilutions) and incubated for 1 hour at room temperature. (NOTE: starting from adding the secondary antibody, every step should be performed in dark condition as these antibodies are light sensitive). After undergoing a dark incubation period, start the washing process.

4.8.7. GEL IMAGING

Gel imaging has been carried out using the sophisticated BioRad ChemiDoc™ XRS+ imaging system for faster and more accurate western blot quantification. After the incubation with a secondary antibody for 2 hours has been completed, the PVDF membrane is washed with 1X PBST every 5 minutes for 5 times. The blot is visualized in the system using ECL as a substrate by introducing solution A and B in equal volume. The software used for imaging is BioRad image Lab 6.1 software.

4.9. STATISTICAL ANALYSIS

All statistical analysis was performed using the software GraphPad Prism v9.5.1. Ordinary one way ANOVA with Brown-Forsythe test was used to analyse the western blotting data $p < 0.05$ was considered as significant.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. ISOLATION OF AROMATIC TURMERONE

5.1.1. SOXHLET EXTRACTION OF TURMERIC OIL

50g of turmeric powder (*Curcuma longa* L.) was extracted with 500ml of petroleum ether in a Soxhlet assembly for 12 hours. The extract was then concentrated using distillation. The solid material in the Soxhlet assembly was extracted twice as much with petroleum ether, and the concentrated filtrates or extracts were mixed (Fig 5.1 a). This extract is nothing but turmeric oil. Turmeric oil was then fractionated into petroleum ether and methanol using a separating funnel (Fig 5.1 b).



Fig 5.1: a) Soxhlet extraction of turmeric b) Separation using separating funnel

Methanol was used for repeated fractionation with petroleum ether (Fig 5.2). A total of six repeated fractionations was done to obtain maximum separation. As a confirmatory step TLC was done at different stages of the phase separation.

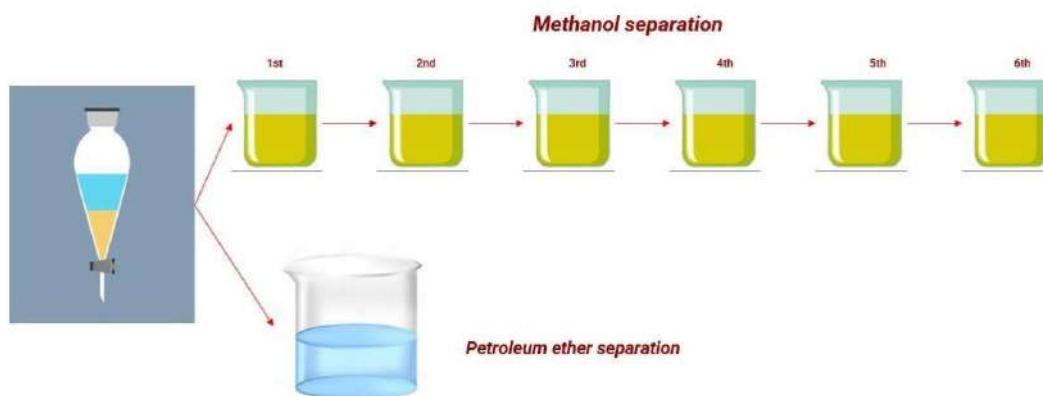


Fig 5.2: Schematic representation of separation and collection of extract using separating funnel

5.1.1. ACTIVATED CHARCOAL PURIFICATION

The petroleum ether fraction was treated with activated charcoal which resulted in partial purification of the extract. This extract was then desiccated and collected using ethanol for further assays.

5.2. CHARACTERISATION OF AR-TURMERONE

Characterisation of isolated compound was carried out with analytical techniques like TLC, HPLC, LC-MS, UV, and FTIR.

5.2.4. TLC

Thin Layer Chromatography of isolated compounds was performed by the ascending technique to analyse the presence of aromatic turmerone in turmeric extract. The extract showed violet spot at R_f of 0.84 with vanillin-sulfuric acid on heating. This spot matched with the standard ar-turmerone in the same mobile phase.

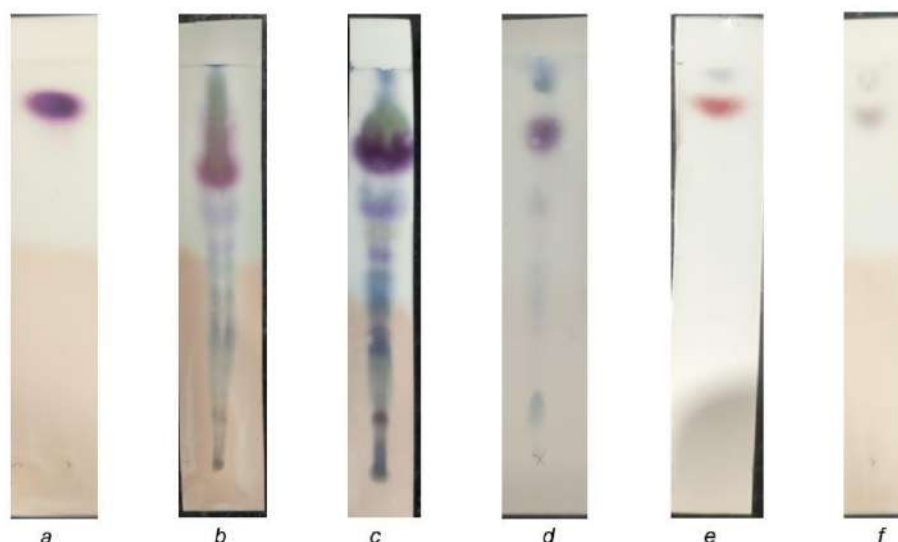


Fig 5.3: TLC of isolated compound at different stages (a) standard arturmerone (b) turmeric oil from Soxhlet extraction (c) petroleum ether fraction from phase separation (d, e) partially purified petroleum ether fraction with activated charcoal & preparative TLC fraction with ethanol (f) HPLC fraction of ar-turmerone

Preparative TLC at different stages of the isolation showed less visible spots, indicating the process of purification. The ar-turmerone spots are also observed at Rf of 0.72 (93:7 v/v) (Surwase *et al.*, 2013) and Rf of 0.43 in the ratio (8:2 v/v) (Husein *et al.*, 2023), Rf of 0.5 and 0.6 is observed in n-hexane: ethyl acetate (9.8:0.2 v/v) (Vikas *et al.*, 2007).

5.2.2. HPLC

The extraction performance of ar-turmerone was analysed with mobile phase (A): ultrapure HPLC water; (B): acetonitrile. The flow rate was 1ml/min at gradient for the entire separation with column temperature at 25°C. Typically, 20 µL of extracts obtained were injected into HPLC and analysed at 240nm. This HPLC performed for the presence of ar-turmerone in standard arturmerone (Fig 5.4 a) and standard ar-turmerone (Fig 5.4 b). Both the standard and turmeric extract showed a absorbance at 239nm.

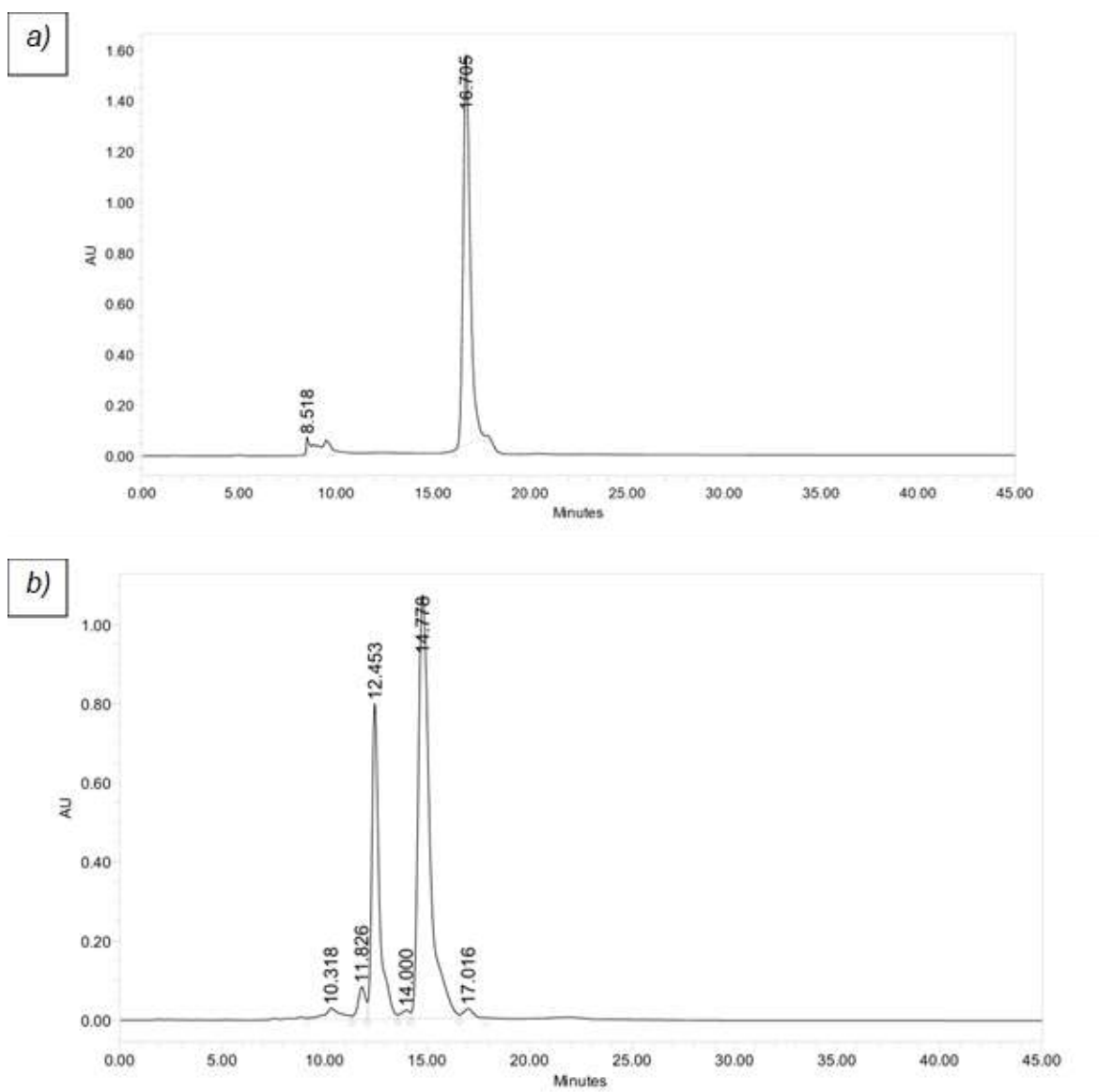


Fig 5.4: The HPLC data of a) standard *ar*-turmerone and b) isolated compound

Using the area of the peak of standard *ar*-turmerone and sample, the concentration was found to be an average of 0.5 μ g/ μ l.

5.2.3. LC-MS

The petroleum ether extract was analysed by 1260 Infinity II Preparative LC/MSD System(Fig 5.5).

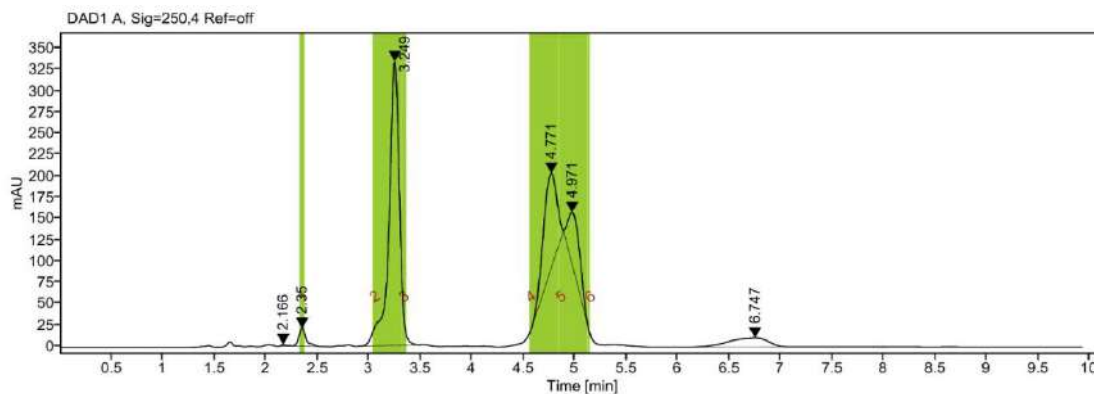


Fig 5.5: LC-MS chromatogram (Absorbance vs time in minutes) of isolated arturmerone.

5.2.4. UV-VIS SPECTROPHOTOMETER

The petroleum ether extract was analysed by BioTek Epoch Microplate Spectrophotometer. Epoch is controlled by Gen6 Data Analysis Software, with simple programming and powerful data analysis. The petroleum ether extract was observed under UV-Vis spectrophotometer in the range of 200nm to 700nm, where it showed maximum absorbance at 240nm.

While the mean maximum absorbance of ar-turmerone was observed at 233.5nm and 236nm (Surwase *et al.*, 2013), 237nm (Husein *et al.*, 2023).

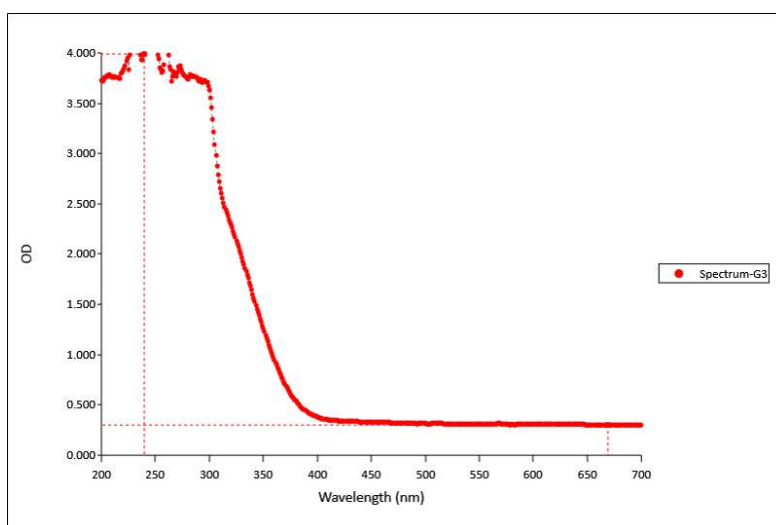


Fig 5.6: UV-Vis spectrum of petroleum ether extract.

5.2.5. FTIR

The FTIR spectrum for partially purified ar-turmerone showed bands of C=C-C aromatic ring & stretch vibration bands of C=O at 1300 to 1500 cm^{-1} , and bands of C-H at 2958 cm^{-1} (Fig 5.7). These results showed the typical functional groups found in ar-turmerone (Husein *et al.*, 2023).

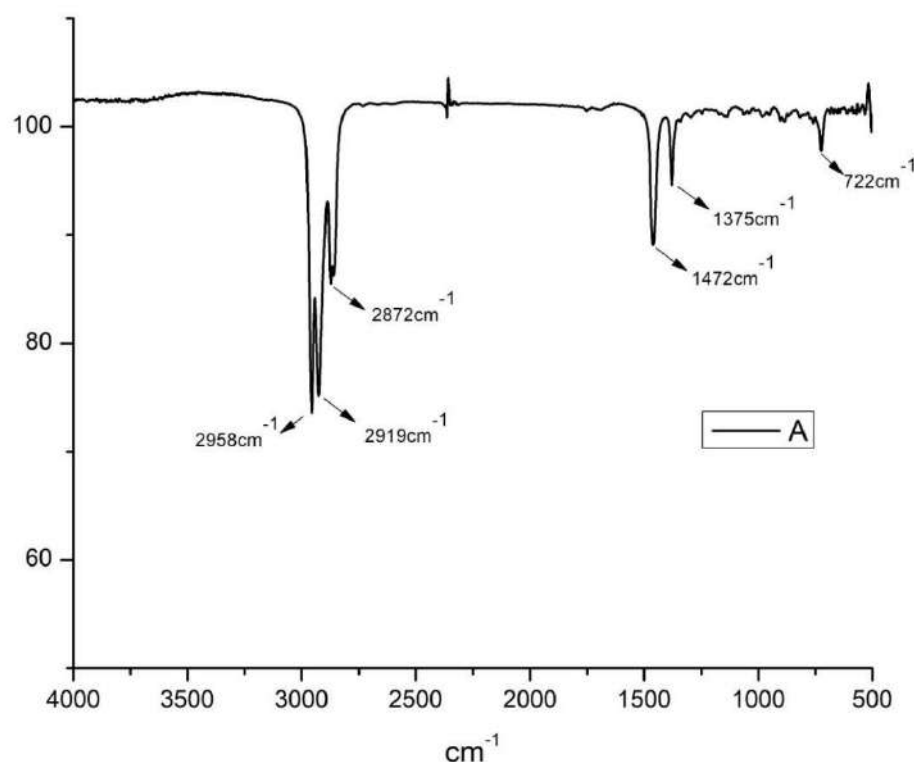


Fig 5.7: FTIR characterization of ar-turmerone extract

5.3. EMBRYO TOXICITY ASSAY

Embryotoxicity studies were carried out in zebrafish embryos as soon after the fertilization of embryos occurs. For analysis of embryotoxicity, ar-turmerone was treated with embryos in various concentrations - 2 μg , 4 μg , 6 μg , 8 μg and 10 μg . The compound exhibited the toxicity of ar-turmerone in embryo increases gradually with increase in concentration.

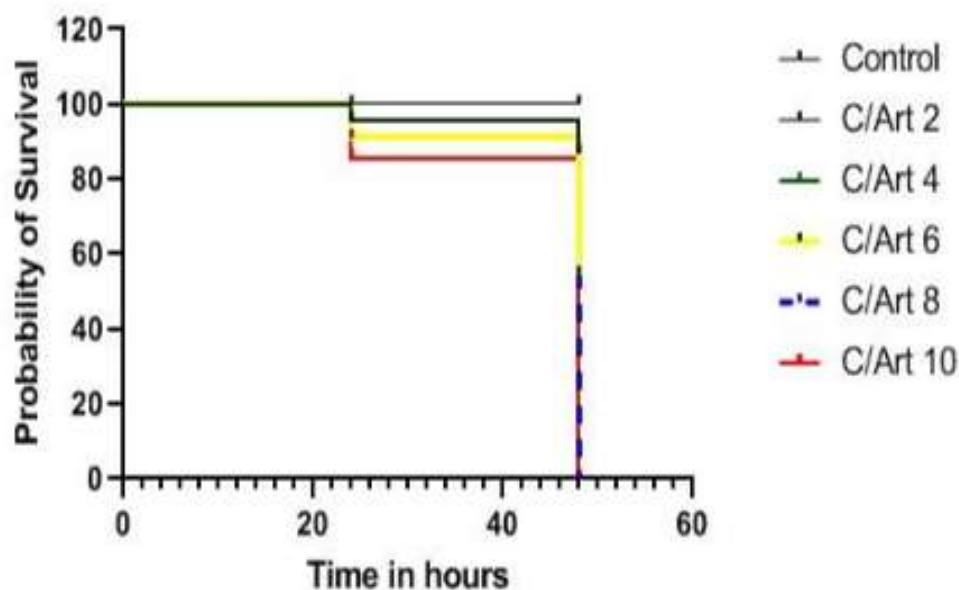


Fig 5.8: Survival graph of zebrafish embryos after exposure to aromatic turmerone

It is observed that concentration 8 μ g and 10 μ g showed complete death of embryos at 48 hrs while concentration 2 μ g is showed full survival at 48 hrs (Fig 5.8).

5.4. MTT ASSAY

Cytotoxicity of ar-turmerone was evaluated against microglial cell lines (N9). The following concentrations were used to examine the toxicity 1 μ g - 20 μ g and for control 3% RPMI 1640 media, 10% RPMI 1640 media were used. The compound exhibited a gradual increase in the cytotoxicity level ranging from 10 μ g - 20 μ g.

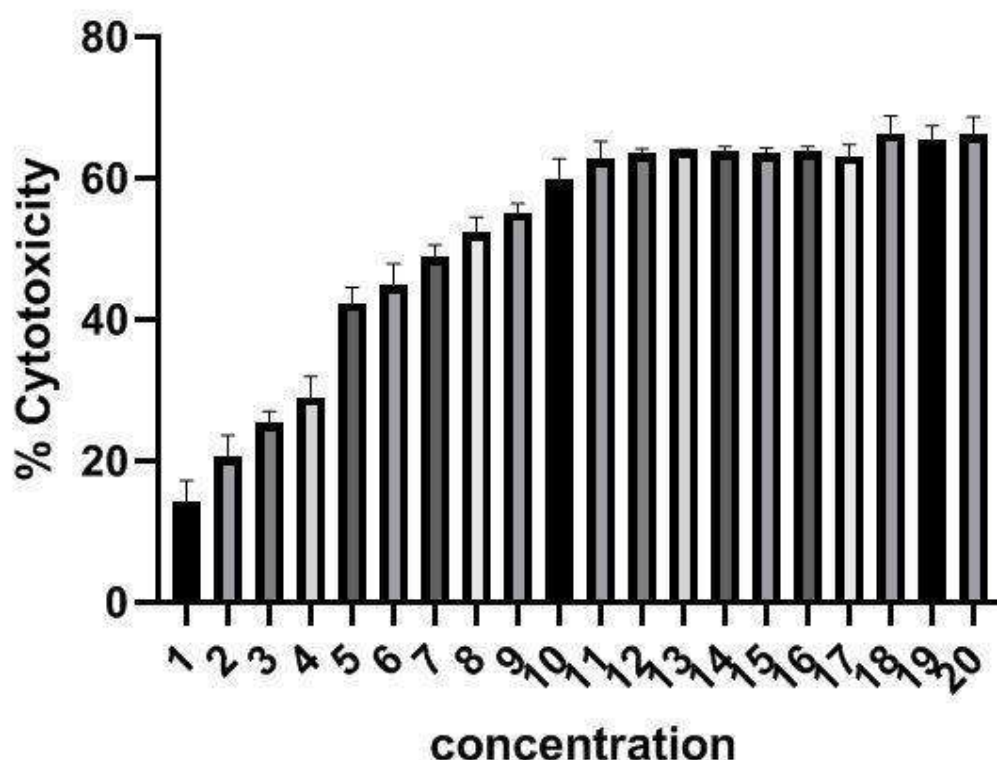


Fig 5.9: Graphical representation of cytotoxic effect of microglial cells on inducing ar-turmerone

5.5. GENE EXPRESSION ANALYSIS

The protein lysate was prepared from the following samples viz, control, arturmerone, ar-turmerone+LPS and LPS treated cells. The protein lysate was resolved in SDS-PAGE and western blotting was performed with the following antibodies Actin, iNOS and HO-1. Results show that the iNOS and HO-1 expression is increased in LPS treated and is suppressed by the co-treated (ar-turmerone+LPS) (Fig 5.10). The LPS treated shows increase in expression due to the increased oxidative stress while the co-treated (arturmerone+LPS) shows less expression due to blocking efficiency of arturmerone (Fig 5.10).

Tectorigenin (TEC), an isoflavone derived from medicinal plants, has been shown to reduce neuroinflammatory effects in lipopolysaccharide-treated BV-2 microglial and animal models. TEC reduced reactive oxygen species (ROS) formation, nitric oxide synthesis, and inflammatory mediators. It reduced inflammation in BV-2 cells by suppressing NF- κ B/ERK/JNK signalling pathways, attenuating microglial activation,

and inhibiting protein expression of toll-like receptor 4 and myeloid differentiation factor 88. TEC may reduce neuronal inflammation by decreasing inflammatory mediators such as iNOS, COX-2, TNF- α , and IL-6 via the NF- κ B/ERK/JNK signalling pathway. These data imply that TEC might be used as a neuroprotective medication to treat neuroinflammatory diseases (Lim *et al.*, 2018).

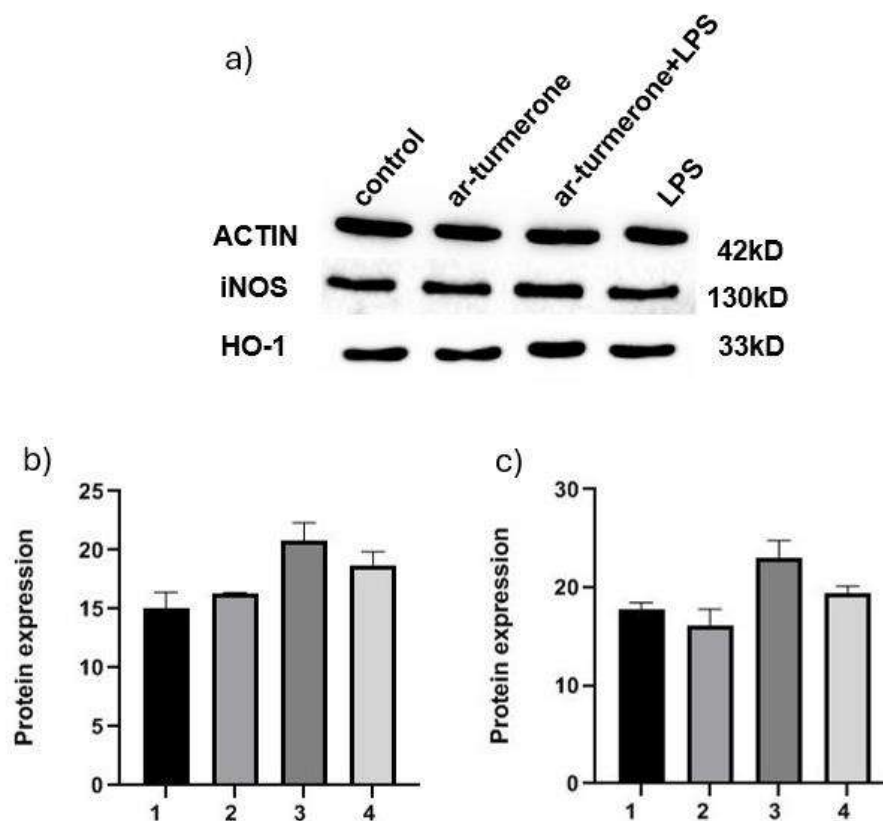


Fig 5.10: Gene expression analysis of b) iNOS and c) HO-1 upon exposure to LPS on N9 microglial cells

CHAPTER 6

SUMMARY AND CONCLUSION

The project aimed to isolate and characterize ar-turmerone from Turmeric and evaluate its blocking efficiency in microglial cell lines. The work involved extracting turmeric oil using Soxhlet extraction, isolating ar-turmerone, and purifying it with activated charcoal. Various analytical techniques such as TLC, HPLC, LC-MS, UVVis spectroscopy, and FTIR were employed to characterize the compound. The blocking efficiency of ar-turmerone in microglial cell lines was assessed by treating cells with LPS to induce stress and then treating them with ar-turmerone.

The results of the project demonstrated successful isolation and characterization of ar-turmerone from turmeric. The compound showed promising potential for its blocking efficiency in microglial cell lines, indicating its ability to potentially exert antiinflammatory and neuroprotective properties. The study provides valuable insights into the biological properties of ar-turmerone and its potential applications in the treatment of neurodegenerative diseases like Alzheimer's and Parkinson's. Overall, the project contributes to the understanding of the therapeutic benefits of arturmerone derived from turmeric.

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