A STUDY TO EVALUATE AND ELUCIDATE THE SYNERGISTIC ANTI-CANCER AND ANTI-INFLAMMATORY EFFECTS OF CINNAMON IN CONJUNCTION WITH TARGETED BIOTHERAPEUTICS.

Thesis Submitted for the Award of the Degree of

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in

BIOCHEMISTRY

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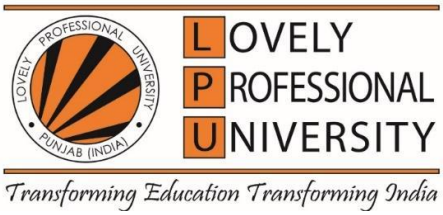

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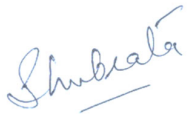
2025

DECLARATION

I, hereby declare that the presented work in the thesis entitled "**A STUDY TO EVALUATE AND ELUCIDATE THE SYNERGISTIC ANTI-CANCER AND ANTI-INFLAMMATORY EFFECTS OF CINNAMON IN CONJUNCTION WITH TARGETED BIOTHERAPEUTICS**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of **Dr. Minhaj Ahmad Khan**, working as **Associate Professor**, in the **Department of Biochemistry**, **School of Bioengineering and Biosciences, of Lovely Professional University, Punjab, India**. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "A STUDY TO EVALUATE AND ELUCIDATE THE SYNERGISTIC ANTI-CANCER AND ANTI-INFLAMMATORY EFFECTS OF CINNAMON IN CONJUNCTION WITH TARGETED BIOTHERAPEUTICS." submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Biochemistry, School of Bioengineering and Biosciences, is a research work carried out by Kum Shubrata Vijay Khedkar, Registration No.41800732, is bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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DEDICATION

This thesis is dedicated to me, for the countless hours of hard work, determination, and resilience. Through every challenge and triumph, I have grown and learned. This achievement is a testament to my unwavering commitment to my goals and dreams.

"Believe you can and you're halfway there." – Theodore Roosevelt

ABSTRACT

Tumor necrosis factor-alpha (TNF-α) is a pivotal pro-inflammatory cytokine implicated in various immune-mediated inflammatory diseases (IMID). Biological therapies targeting TNF-α, such as monoclonal antibodies like infliximab, have shown considerable success and are generally well-tolerated. However, some patients exhibit primary non-response or develop secondary resistance over time.

Angiogenesis, a complex process driven by numerous pro-angiogenic factors, is essential for cancer progression. Vascular endothelial growth factor (VEGF) is recognized as the most potent among these factors. Current therapies targeting VEGF receptors (VEGFR) and VEGFR tyrosine kinase inhibitors (RTKi), including bevacizumab, have demonstrated limited efficacy as monotherapies. Due to the intricate nature of angiogenesis, combinatorial, sequential, or synergistic therapeutic approaches are proposed to enhance treatment outcomes for resistant patients.

Cinnamon, traditionally used as a spice, is reported to have inhibitory properties against inflammation and cancer. Aqueous cinnamon extract (aCE) is reported to reduce levels of several pro-inflammatory and pro-angiogenic molecules. This study aimed to evaluate whether aCE synergizes with the anti-inflammatory biotherapeutic infliximab and the anti-angiogenic biotherapeutic bevacizumab. Additionally, it explored the differential effects of various cinnamon species on various factors and genes that play a role in exacerbating inflammation and angiogenesis.

This study was conducted in two major steps; first step was to show experimentally that cinnamon was demonstrating synergy, which was shown in an *in-vitro* assay. Subsequently, conditions demonstrating statistically significant synergy were selected to study the modulation of mRNA for genes involved in worsening the inflammatory and cancerous conditions. aCE and bioactives from three commonly used cinnamon species were used in the study. Relevant cell lines (L929 & U937 for Infliximab and HUVEC for bevacizumab) were used for synergy experiments with infliximab and bevacizumab, followed by analysis of mRNA levels under synergy conditions by qPCR. Not all aCE showed significant synergy or modulation of mRNA levels. Trans-cinnamaldehyde and *C. cassia* were significantly effective in inflammatory test system while, *C. zeylanicum* showed significantly in HUVEC cells.

Overall, this study demonstrated that aCE is a good candidate to be used in conjunction with biotherapeutic in chronic inflammatory conditions and cancer therapy thereby enhancing the efficacy of biotherapeutics.

Acknowledgment

The submission of this thesis marks the culmination of a long and eventful journey, filled with challenges, learning, and growth. This accomplishment would not have been possible without the steadfast support, guidance, and encouragement of many individuals, to whom I am sincerely grateful.

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Thank you,

Kum Shubrata Vijay Khedkar

List of Abbreviation

Abbreviation	Full form
aCE	Aqueous Cinnamon Extract
AD	Alzheimer Disease
Ang2	Angiopoietin 2
ANOVA	Analysis Of Variance
AP-1	Activator Protein 1
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
ATP5	ATP synthase subunit 5, mitochondrial
B2M	beta-2 Microglobulin
Bax	Bcl-2 Associated X-protein
BcL-2	B-cell lymphoma 2
BcL-xL	B-cell lymphoma-extra large
BMAB	Bevacizumab
CA	Cinnamic Acid
CAM	Complementary and Alternative Medicine
CAR-T	Chimeric Antigen Receptor T-cell therapy
CD	Crohn's disease
CDDP	Cis-diamminedichloroplatinum
CE	Cinnamon Extract
COX-2	Cyclooxygenase 2
CSWE	Cinnamon Subcritical Water Extract
CTLA-4	Cytotoxic T-lymphocyte-Associated Protein 4
CXCL5	C-X-C motif chemokine 5

CXCR1	C-X-C motif chemokine receptor 1
CXCR2	C-X-C motif chemokine receptor 2
CYC1	Cytochrome c1
DFS	Diclofenac Sodium
DMARDs	Disease-Modifying Anti-Rheumatic Drugs
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic Acid
EGCG	Epigallocatechin Gallate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMEM	Eagle's Minimum Essential Medium
ERK	Extracellular signal–Regulated Kinases
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GBM	Glioblastoma
G-CSF	Granulocyte Colony-Stimulating Factor
HCC	Hepatocellular Carcinoma
HI FBS	Heat Inactivated Fetal Bovine Serum
HIF-1α	Hypoxia Inducible Factor 1 subunit alpha
HK	House Keeping
HO-1	Heme Oxygenase-1
HPLC	High-Performance Liquid Chromatography

HUVEC	Human Umbilical Vein Endothelial Cells
IBD	Inflammatory Bowel Disease
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon Gamma
IL-6	Interleukin-6
IL-8	Interleukin-8
IMID	Immune-Mediated Inflammatory Diseases
IU	International Units
Jak2	Janus Kinase 2
JNK	c-Jun amino-terminal Kinases
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
mCRC	Metastatic Colorectal Cancer
MMP-1	Matrix Metallopeptidase 1
MMP-3	Matrix Metallopeptidase 3
MRSA	Methicillin-resistant *Staphylococcus aureus*
MyD88	Myeloid Differentiation primary response 88
NA	Not Applicable
NEB	New England Biolabs
NF-κB	Nuclear Factor-Kappa B
NHP	Natural Health Products
NK	Natural Killer
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
NSCLC	Non-Small Cell Lung Cancer
OS	Overall Survival

OSCC	Oral Squamous Cell Carcinoma
OTC	Oxytetracycline
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death 1
PDGF	Plate Derived Growth Factor
PD-L1	Programmed Death-Ligand 1
PFS	Progression-Free Survival
PI3K/Akt	Phosphatidylinositol 3-kinase/Protein Kinase B
PKB	Protein Kinase B
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RA	Rheumatoid Arthritis
RFS	Recurrence Free Survival
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RP-HPLC	Reverse Phase – High Performance Liquid Chromatography
RPL13A	Ribosomal Protein L13a
RPLP2	Ribosomal Protein Lateral Stalk Subunit P2
RPMI-1640	Roswell Park Memorial Institute-1640 medium
RT-qPCR	Reverse transcription, Quantitative Polymerase Chain Reaction
SD	Standard Deviation
SDHA	Succinate Dehydrogenase complex, subunit A
SOD	Superoxide dismutase
STAT-3	Signal Transducer and Activator of Transcription 3
TAE	Tris-acetate-EDTA

TCA	Trans-Cinnamaldehyde
TCM	Traditional Chinese Medicine
TIMP-1	Tissue Inhibitor of Metalloproteinase-1
TLR	Toll-Like Receptor
TME	Tumor Micro-Environment
TNF-α	Tumor Necrosis Factor, alpha
UC	Ulcerative Colitis
UHDLC- HRMS	Ultra-High-Performance Liquid Chromatography Coupled With High-Resolution Mass Spectrometry
UV	Ultraviolet
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
YWHA2	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

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CHAPTER 1
INTRODUCTION

1.1. CINNAMON AND ITS USES

Cinnamon (Cinnamomum spp., family Lauraceae) historically used as a spice and in preparation of perfume, is also known for its healing properties (Khedkar and Khan, 2023). Cinnamon is also referred as "ròu guì," which translates to "sweet wood" (Vinitha & Ballal, 2008; Hamidpour et al., 2015). There are over 250 species of cinnamon. Among them, *C. zeylanicum* also known as "true cinnamon," is mainly cultivated in south Asia (Figure. 1A), and another commonly used species is *C. aromaticum*, also known as *C. cassia*, is grown in east and southeast Asian countries (Figure. 1B). After drying, *C. cassia* forms a pen or quill shape with a single thick layer rolled into a curl, while *C. zeylanicum* consists of several thin slivers of cinnamon bark rolled together (Figure. 1B) (Hamidpour et al., 2015).

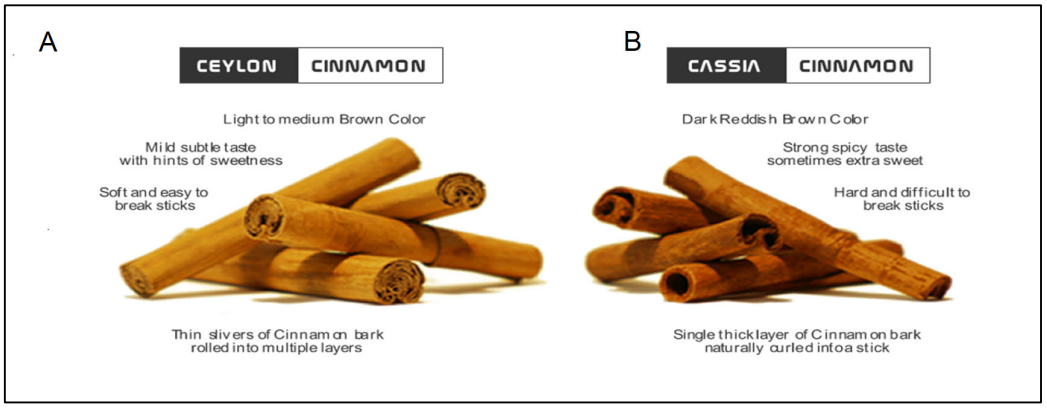


Figure 1: Cinnamon (A) *Cinnamomum zeylanicum* and, (B) *Cinnamomum cassia*. (Source: https://www.cinnamonzone.hk/cinnamon_varieties_a.html)

Cinnamon has been used traditionally in various cultures and medicinal systems, highlighting its long-standing significance as a versatile spice with medicinal benefits. Evidence of cinnamon cultivation and trade dates to ancient civilizations like Egypt, China, and India. In ancient Egypt, cinnamon was used in embalming due to its aromatic and preservative qualities (Ribeiro-Santos et al., 2017). In ancient India, it served as both a culinary spice and a traditional remedy for ailments such as colds, digestive problems, and menstrual pains (Ranasinghe et al., 2012).

The medicinal nature of cinnamon is because of its chemical composition, which includes active compounds like cinnamic acid, cinnamyl acetate, eugenol, caryophyllene oxide, L-bornyl acetate, trans-cinnamaldehyde, and terpinolene, among others. This diverse chemical profile makes cinnamon a widely cultivated plant known for its extensive medicinal applications throughout history (Rao and Gan, 2014). Traditionally, cinnamon has been used not only as a flavoring agent and mouth freshener due to its ability to combat oral health, but also for treating indigestion, relieving pain, and providing neuroprotective effects (Ullah & Hassan, 2022).

Cinnamon's ability to protect against nerve related disorders is mainly linked to cinnamaldehyde, which reportedly protects neurons from cell death due to free oxygen radicals (Angelopoulou et al., 2021). Additionally, cinnamon has shown potential in inhibiting angiogenesis in cancer cells by blocking commonly used angiogenic pathways (Hamidpour et al., 2015). Cinnamon also demonstrates antioxidant, antidiabetic, anti-inflammatory, antibacterial, lipid-lowering, and cardiovascular protective activities, as confirmed by various publications (Ranasinghe et al., 2012; Khedkar and Khan, 2023).

1.1.1. Traditional Uses in Different Cultures

In Traditional Chinese Medicine (TCM), cinnamon is believed to have carminative, antiseptic and astringent properties (Amitava Kabiraj and Deshmukh, 2024) . In Ayurvedic medicine, cinnamon, is highly regarded for its capability to balance the doshas, particularly trusna (related to thirst), vastiroga (bladder related diseases) and more. It is used to improve digestion, reduce inflammation, and enhance circulation (Panda et al., 2023).

In Middle Eastern cuisine, cinnamon is a key spice used in both savory dishes and desserts, adding warmth and depth of flavor in bakery and food items. In traditional Middle Eastern medicine, cinnamon is believed to aid in bloating and addressing nausea (Ahmad et al., 2019).

1.1.2. Traditional Uses in Indian Medicinal Systems

Cinnamon holds a prominent role in various Indian medicinal systems, including Ayurveda, Unani, Homeopathy, Allopathy, and other natural healing practices. Its extensive history of use in India spans thousands of years, with references in ancient texts such as the *Charaka Samhita* and *Sushruta Samhita*. The traditional applications of cinnamon in these systems reflect its versatility and therapeutic potential, ranging from digestive health to respiratory disorders. Modern research has validated many of these traditional uses and uncovered new therapeutic applications, highlighting cinnamon's relevance in contemporary healthcare. As interest in natural and integrative medicine grows, cinnamon remains a focus of ongoing research and innovation. This discussion covers cinnamon's traditional applications in Indian medicinal systems, its pharmacological properties, and its current significance in modern healthcare practices.

In Ayurveda, cinnamon is known for its warming qualities and is classified as a "Tikta" (bitter) and "Katu" (pungent) herb. It is believed to balance the "Vata" and "Kapha" doshas but may aggravate "Pitta" when consumed in excess. Cinnamon is used to stimulate digestion ("Agni"), relieve respiratory conditions, and improve circulation. It is frequently prescribed in formulations for colds, coughs, digestive issues, and menstrual irregularities (Pathak and Sharma, 2021).

In Unani medicine, cinnamon, referred to as "dalchini," is considered to have hot and dry properties. It helps stimulate appetite, useful in treating liver ailments, skin ailments, and relieving joint pains. Often combined with other herbs to enhance its effects, Unani practitioners also use cinnamon for cognitive enhancement and to relieve menstrual discomfort (Azmi et al., 2024)

Traditionally, cinnamon has been valued mainly for its culinary and flavoring properties. However, recent research has highlighted its therapeutic benefits, particularly in managing diabetes and metabolic disorders. Studies on cinnamon extracts and supplements suggest they can improve insulin sensitivity, lower blood sugar, and reduce cholesterol levels (Qin et al., 2010). Although more research is

needed to assess its *in vivo* performance, cinnamon shows promise as a CAM for managing diabetes (Khan et al., 2003).

Cinnamon's use in Indian medicinal systems demonstrates its diverse therapeutic potential. Modern research has confirmed many of these traditional uses and discovered new therapeutic roles for cinnamon. As interest in natural remedies and integrative healthcare grows, cinnamon continues to be explored in both traditional and modern contexts.

1.2. MEDICINAL PROPERTIES AND MODERN RESEARCH

1.2.1. Antioxidant Activity

Cinnamon contains a high concentration of polyphenols, especially cinnamaldehyde, which possess strong antioxidant effects. These compounds aid in fighting oxidative stress and may lower the risk of chronic conditions like cardiovascular disease and cancer. (Singh et al., 2021).

1.2.2. Antimicrobial Effects

Cinnamon is very effective in inhibiting the growth of a variety of bacteria and fungi. The antimicrobial properties are reported to be present in essential oils of cinnamon containing cinnamaldehyde, benzaldehyde and cinnamic acid. (Wang et al., 2020).

1.2.3. Anti-inflammatory Effects

Studies have shown that cinnamon has anti-inflammatory effects, which are linked to its ability to suppress cytokines and enzymes involved in inflammation. This makes it a promising therapeutic agent for conditions associated with chronic inflammation, such as arthritis and inflammatory bowel disease (Khedkar and Khan, 2023). Cinnamon bark contains key compounds like trans-cinnamaldehyde and p-cymene, which help reduce levels of cytokines, such as interleukin-8 (IL-8), that contribute to inflammation (Schink et al., 2018). Furthermore, cinnamon extracts have been found to improve intestinal barrier integrity and decrease inflammation in gut cells by inhibiting

various cytokines and interleukins. These findings suggest that cinnamon may be beneficial in managing gut-related inflammatory conditions (Kim and Kim, 2017).

1.2.4. Antidiabetic Effects

Cinnamon has been investigated for its role in improving insulin sensitivity and glucose metabolism, making it a promising adjunctive therapy for diabetes management. Cinnamon extracts have been shown to decrease fasting blood sugar levels and improve insulin sensitivity in both animal and human studies. (Khan et al., 2003).

1.2.5. Anticancer Effects

Plants have been extensively researched for their medicinal qualities, especially their anti-cancer properties. Additionally, studies have investigated the anti-cancer properties of marine plant life (Boopathy and Kathiresan, 2010). Plants are often preferred in the development of anticancer agents due to their rich diversity of potent bioactive compounds, lower toxicity, and fewer side effects compared to synthetic drugs. Some well-researched plants with known anticancer properties include turmeric, which contains the active compound curcumin; green tea, which is rich in catechins; and ginger, which contains gingerol (Ajanaku et al., 2022; Musial et al., 2020). These natural agents have proven effective in controlling tumor growth and migration.

Recent studies have underscored the potential of cinnamon as an anticancer agent (Caserta et al., 2023; Sadeghi et al., 2019). Cinnamon has demonstrated the ability to inhibit the growth of various human cancer cell lines. It has also shown promise in reducing the metastatic potential of transformed cells. One study investigated the impact of cinnamon extract (CE) on the SiHa cell line, which originate from human cervical tumors. The findings revealed that cinnamon effectively impeded the metastatic movement of these cancer cells, underscoring its potential as an anticancer agent for cervical cancer (Koppikar et al., 2010).

An expanding body of research emphasizes the value of cinnamon as a natural resource in the fight against cancer. Angiogenesis, the process by which tumors form new blood vessels which are required to supply nutrients and oxygen for tumor growth and migration. VEGF is one of the main factors driving angiogenesis (Lugano et al., 2020).

Various methods to inhibit VEGF are being explored in clinical trials. Existing anti-VEGF treatments are associated with negative effects such as high blood pressure, hemorrhage, and damage to gastric and intestinal tract (Falavarjani and Nguyen, 2013; Wang et al., 2024).

Given that natural products are safe for human consumption, there is renewed interest in identifying substances that inhibit VEGF activity. One study found that cinnamon is a natural source of dietary anti-VEGF compound (Lu et al., 2010). In endothelial cells, cinnamon, and its bioactives which includes procyanidins is reported to block receptor kinase activity and VEGF signaling.

1.2.6. Neuroprotective effects

Alzheimer's disease (AD) is an advancing neurodegenerative condition that worsens over time and currently this disease can only be managed but not cured. Disorientation, impaired language abilities, memory and recall, and poor judgment are some of the disease symptoms. Research has highlighted that the accumulation of soluble amyloid beta (Aβ) polypeptide assemblies is the major contributing factor in disease progression. Studies have indicated that natural compounds like cinnamon can inhibit the formation of Aβ oligomers and protects neuronal PC12 cells from Aβ-induced damage. Additionally, another study reported that when cinnamon extract was administered orally to a transgenic mice with AD, it resulted in reduction in plaque formation and improvements in memory and recall. (Frydman-Marom et al., 2011, Peterson et al., 2009).

1.3. CANCER AND INFLAMMATORY DISEASES – TREATMENT AND CHALLENGES

Cancer is marked by the unchecked growth and dissemination of abnormal cells. These cells have the potential to invade and damage healthy tissues, causing significant health issues. The primary causes of cancer include genetic mutations, environmental factors, and lifestyle choices. Common risk factors are excessive tobacco and alcohol consumption, poor lifestyle choices (food and exercise), and exposure to carcinogenic chemicals (Brennan and Smith, 2022). Treatment options for cancer vary depending on the type and stage of the disease and may require surgery, administration of

chemotherapeutic agents or radiation treatments, new age therapy like monoclonal antibodies and checkpoint inhibitors. Cancer progressions has various stage and detection at early stages tips the scale towards better recovery and survival (Patel et al., 2024).

There is a strong connection between cancer and inflammation, with chronic inflammation often playing a significant role in the initiation and progression of cancer. Inflammation serves as the body's natural response to injury or infection, involving the activation of immune cells, the release of signaling molecules, and changes in blood circulation. While this process is essential for healing, prolonged inflammation can result in tissue damage and create an environment conducive to cancer development (Colotta et al., 2009).

Chronic inflammation can result from persistent infections, autoimmune diseases, or long-term exposure to irritants. This sustained inflammatory state can cause DNA damage, promote cellular mutations, and support cancer growth and migration (Wang et al., 2024). Understanding the interplay between inflammation and cancer helps designing effective therapeutic molecules. Targeting inflammatory pathways can potentially reduce cancer risk and improve therapeutic outcomes (Mantovani et al., 2008). Additionally, combining anti-inflammatory or anti-cancer agents with CAM could enhance their efficacy and reduce side effects, offering a more comprehensive approach to treatment (Aggarwal and Gehlot, 2009).

1.3.1. Therapeutic Strategies in Cancer and Inflammatory Diseases

The treatment landscape for cancer and inflammatory diseases has advanced considerably with the development of various therapeutic strategies, including small molecule drugs, biotherapeutics, and CAM. Each of these approaches provides unique mechanisms of action and therapeutic potential, playing a vital role in the comprehensive management of these complex conditions.

A Small Molecule Drugs

Small molecule drugs are a key component in the treatment of cancer and inflammatory diseases because they can penetrate cells and specifically target intracellular processes (Figure. 2). These drugs generally have a low molecular weight, allowing them to

interact effectively with molecular targets involved in disease development. Examples include:

a) **Kinase Inhibitors:** These small molecules are essential in cancer therapy as they inhibit specific kinases that drive tumor cell proliferation and survival. For example, tyrosine kinase inhibitors like sorafenib and sunitinib have been used to treat various cancers. However, they lack selectivity and are often associated with several side effects (Elebiyo et al., 2022).

b) **Nonsteroidal Anti-Inflammatory Drugs (NSAIDs):** Widely utilized in managing inflammatory conditions, NSAIDs such as ibuprofen and aspirin exert their effects by inhibiting cyclooxygenase (COX) enzymes, thereby reducing inflammation and alleviating pain (Ghlichloo and Gerriets, 2023)

c) **Antimetabolites:** These drugs interfere with essential metabolic pathways, particularly nucleotide and nucleic acid synthesis, crucial for cell division. Methotrexate, for example, disrupts DNA synthesis and is employed in treating cancers like leukemia and autoimmune diseases such as RA (Chandraprasad et al., 2022)

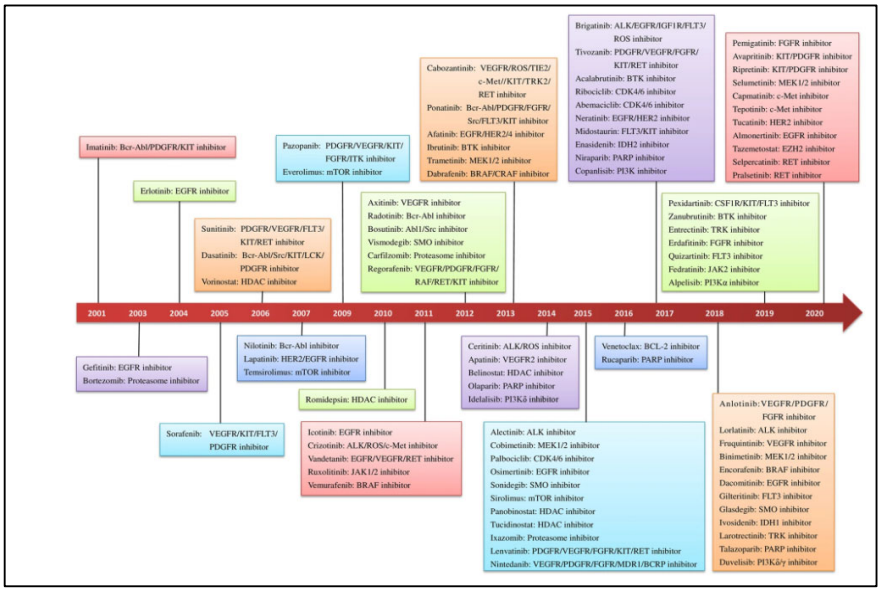


Figure 2: Small molecule drugs approved over the years (Source: Zhong et al., 2021)

B. Biotherapeutics

Biotherapeutics, derived from living organisms, offer innovative treatment modalities for cancer and inflammatory diseases. These products include monoclonal antibodies (Figure. 3), cytokines, and cell-based therapies, each designed to modulate specific biological pathways. Examples include (Papież and Krzyściak, 2021):

a) **Monoclonal Antibodies:** Engineered to target precise antigens on cancer cells or involved in cancer pathway or inflammatory mediators, monoclonal antibodies have transformed treatment outcomes. Trastuzumab (Herceptin), designed for HER2-positive breast cancer, and infliximab (Remicade), used in rheumatoid arthritis, exemplify the targeted approach of these therapies.

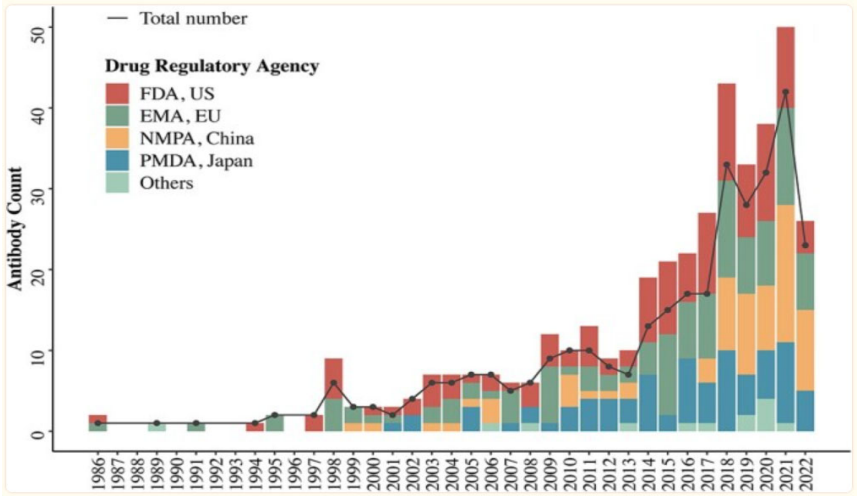


Figure 3: Timeline for the approval of monoclonal antibodies targeting cancer. (Source: Lyu et al., 2022)

b) **Cytokine Therapies:** Cytokines, such as interleukins and interferons, regulate immune responses and have therapeutic applications in cancers and viral infections. Interferon-alpha, for instance, stimulates immune activity against cancers like melanoma and renal cell carcinoma.

c) **Cell-Based Therapies:** Innovations like Chimeric Antigen Receptor T-cell therapy (CAR-T) harness the patient's immune cells, genetically modifying them to recognize and destroy cancer cells. This personalized approach has shown remarkable efficacy in hematologic malignancies

and is being investigated for broader applications in solid tumors (Figure. 4).

BRAND NAME	GENERIC NAME	TARGETED DISEASE
Kymriah™	tisagenlecleucel	Follicular Lymphoma, Diffuse Large B-cell Lymphoma, or Lymphoblastic Leukemia
Yescarta™	axicabtagene ciloleucel	Follicular Lymphoma or Diffuse Large B-cell Lymphoma
Tecartus™	brexucabtagene autoleucel	Mantle Cell Lymphoma or Acute Lymphoblastic Leukemia
Breyanzi®	lisocabtagene maraleucel	Large B-cell Lymphoma
Abecma®	idecabtagene vicleucel	Relapsed or Refractory Multiple Myeloma
Carvykti™	ciltacabtagene autoleucel	Relapsed or Refractory Multiple Myeloma

Figure 4: Approved CAR-T therapies. (Source: CAR-T Therapy: The Future of Regenerative Immunotherapy - OriGen Biomedical, December 2022)

C **Complementary and Alternative Medicine (CAM)**

CAM encompasses diverse practices and products that complement conventional medical treatments, offering patients additional avenues for managing symptoms and improving overall well-being. CAM therapies in cancer and inflammatory diseases include (Lin et al., 2019, Khedkar and Khan, 2023, Viscuse et al., 2017):

a) **Herbal Medicine:** Plant-derived substances such as turmeric (curcumin) and cinnamon possess bioactive compounds with potential anticancer and anti-inflammatory properties. Curcumin, for example, can be used effectively to manage oxidative and inflammatory conditions, suggesting therapeutic benefits beyond conventional treatments.

b) **Acupuncture:** Rooted in TCM, in acupuncture, thin needles are strategically inserted into specific body points to enhance energy flow and facilitate healing. This practice is increasingly integrated into cancer care to mitigate treatment-related symptoms and enhance quality of life.

c) **Mind-Body Therapies:** Techniques like yoga, meditation, etc. enable better mind-body connection, helps to achieve a stress free and relaxed

state of mind. These therapies are valuable adjuncts in managing chronic conditions, fostering a holistic approach to healing.

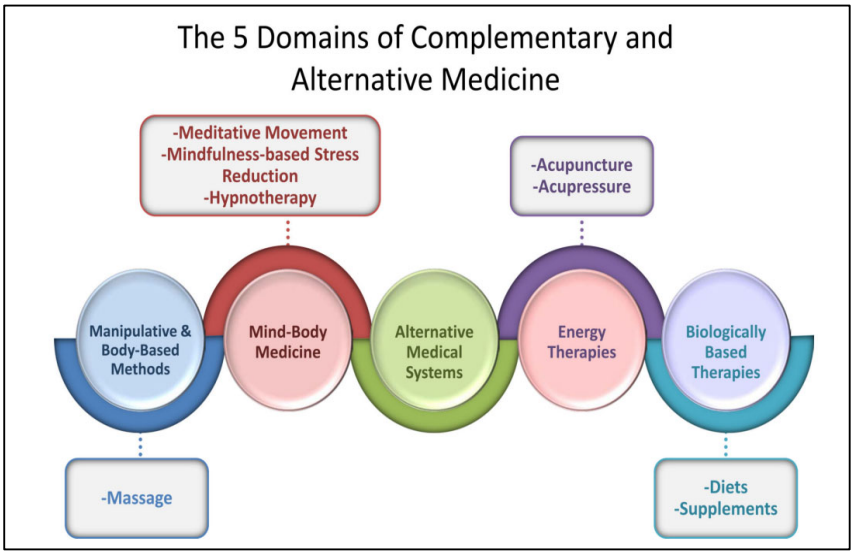


Figure 5: Domains of Complementary and Alternative Medicine. (Source: Viscuse et al., 2017)

CAM therapies are gaining recognition for their potential to complement conventional treatments, offering patients personalized options that address both physical and emotional aspects of their health.

1.3.2. Drug Resistance in Cancer and Inflammatory Diseases

Drug resistance poses a significant challenge in treating both cancer and inflammatory diseases. Cancer cells can become resistant to chemotherapy through various mechanisms, such as increased drug efflux pumps, changes in drug targets, and activation of survival signaling pathways (Gottesman, 2002). Similarly, in inflammatory conditions like Rheumatoid Arthritis (RA), patients may develop resistance to conventional Disease-Modifying Anti-Rheumatic Drugs (DMARDs) or biological therapies due to immune system dysregulation and the formation of autoantibodies (Khedkar and Khan, 2024).

1.3.3. Adverse Side Effects

The management of cancer and inflammatory diseases is often complicated by the occurrence of adverse side effects associated with treatment modalities. Conventional cancer treatments such as chemotherapy and radiation therapy often cause significant toxicity and adverse effects leading to symptoms such as nausea, vomiting, hair loss, and immunosuppression (Chabner and Roberts, 2005). Similarly, nonsteroidal anti-inflammatory drugs (NSAIDs) can cause gastrointestinal bleeding, renal impairment, and cardiovascular events (Vonkeman and van de Laar MA, 2010). Targeted therapies like monoclonal antibodies are also associated with adverse effects like high blood pressure and proteinuria (Lee et al., 2019).

1.3.4. High Cost of Biotherapeutics

The cost of biotherapeutic treatments for patients suffering from cancer and inflammatory diseases can be substantial, often leading to significant financial burdens. Here are some key points:

A. Cancer and Inflammatory Treatment Costs

a) ***Immunotherapy*:** Immunotherapy, a type of biotherapeutic treatment, has revolutionized cancer care but comes with high costs. For example, treatments like immune checkpoint inhibitors can cost between $100,000 and $200,000 per year (Pietrangelo, 2016). These costs can be prohibitive for many patients, especially those without comprehensive insurance coverage.

b) ***Targeted Therapies:*** Drugs like Herceptin, used for HER2-positive breast cancer, have dramatically improved survival rates but also come with high price tags. The annual cost of Herceptin can exceed $70,000 (Pietrangelo, 2016; Fang and Frosch, 2021). Treatments for inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease, often involve biologic agents. These drugs can cost between $10,000 and $30,000 annually (Winstead, 2022).

c) ***Economic Burden:*** The overall economic burden includes not only the direct costs of these medications but also indirect costs such as lost productivity and disability. For instance, the total annual healthcare

costs for inflammatory bowel disease in the U.S. were estimated to be around $8.5 billion in 2018 (Winstead, 2022).

B. Addressing the Costs

Efforts to reduce these costs include:

a) **Biosimilars:** Regulatory agencies are working to approve biosimilars, which are less expensive alternatives to existing biologics (Fang and Frosch, 2021; Winstead, 2022).
b) **Pricing Reforms:** Healthcare systems are implementing pricing reforms and reimbursement strategies to make these treatments more accessible (Fang and Frosch, 2021; Winstead, 2022).
c) **Research and Development:** Ongoing research into novel manufacturing technologies aims to reduce production costs and enhance affordability (Fang and Frosch, 2021; Winstead, 2022).

Addressing the high costs of biotherapeutic treatments requires a multifaceted approach involving various stakeholders across the healthcare continuum.

Cancer and inflammatory diseases often involve immune system dysregulation. While immunosuppression is beneficial in autoimmune and inflammatory conditions to reduce inflammation and tissue damage, it can also increase the risk of infections and malignancies. In cancer, immunosuppression allows tumor cells to evade immune surveillance and proliferate unchecked (Mellman et al., 2011). Despite advancements in drug discovery and development, there remains a significant unmet need for effective treatments, especially for cancers with poor prognosis or limited therapeutic options, such as pancreatic cancer and glioblastoma (Stupp et al., 2005; Conroy et al., 2011). Similarly, some inflammatory diseases, like systemic lupus erythematosus, still lack targeted therapies with favorable efficacy and safety profiles (Tsokos et al., 2016).

1.3.5. Herbal Medicines

Herbal medicines, including cinnamon, are often considered safe due to their natural origin and long history of use in traditional medicine systems. Cinnamon has been consumed for several decades for managing diseases with relatively few reports of adverse effects (Ranasinghe et al., 2012). As it has minimal side effects, it is a viable

choice for patients who suffer severe side effects from standard treatments. Unlike single-target drugs, many herbal medicines exert their effects through multiple mechanisms, targeting various pathways involved in cancer and inflammation. For example, cinnamaldehyde, cinnamic acid, and procyanidins present in cinnamon, exhibit antioxidant, anti-inflammatory, and anti-cancer properties. Its ability to modulate multiple targets makes it potentially effective against drug-resistant cancer cells and inflammatory pathways (Koppikar et al., 2010; Lu et al., 2010). CAMs are often used often integrated with conventional treatments to enhance effectiveness and reduce toxicity, showing synergistic effects. Studies have demonstrated synergistic interactions between cinnamon and chemotherapeutic agents, leading to improved anti-cancer activity and reduced side effects (Ranasinghe et al., 2012).

Herbs possess immunomodulatory properties, which can help restore immune homeostasis in cancer and inflammatory diseases. Cinnamon extracts enhance anti-tumor immune responses and suppress inflammation by inhibiting cytokines that promote inflammation and key signaling pathways (Zhao et al., 2021; Luo et al., 2023). Cinnamon's ability to modulate the immune system may help reduce infection risks and improve treatment results. Cancer and inflammatory diseases present complex challenges in modern medicine, including toxicity, drug resistance, high costs, and immunosuppression. Herbal medicines like cinnamon offer promising solutions to overcome these limitations by providing safer, multi-targeted, and cost-effective adjuncts to conventional therapies. The broad range of bioactive compounds found in cinnamon offers properties that fight tumors and decrease inflammation, making it an effective therapeutic agent. Additional research is necessary to fully understand its mechanisms, optimize dosage, and confirm its efficacy in clinical settings. Integrating herbal remedies like cinnamon into conventional healthcare practices could enhance treatment outcomes and improve patients' quality of life, particularly those with cancer and inflammatory diseases (Qin et al., 2004).

1.4. SYNERGY BETWEEN NATURAL COMPOUNDS AND THERAPEUTIC AGENTS

Combining natural compounds with western medicine offers an attractive strategy for treating chronic diseases and cancers (Lin et al., 2019). This approach aims to boost the effectiveness of therapeutic agents by utilizing the properties of natural compounds and providing a superior method for disease management. This section will briefly explore the mechanisms of synergy when using a natural compound along with conventional medicine.

A. Mechanisms of synergy

Natural compounds obtained from plants like cinnamon contain several bioactive constituents which act via multiple pathways to achieve synergistic effects (Rao and Gan, 2014).

a) **Modulation of Signaling Pathways:** Cinnamon can selectively influence critical signal transduction pathways involved in cancer and inflammation, such as the NF-κB and MAPK pathways (Khedkar and Khan, 2023). These pathways play an essential role in controlling immune responses, cell growth, programmed cell death, etc. within the human body. By inhibiting these pathways, cinnamon can reduce the overproduction of cytokines that exacerbate inflammation, thereby enhancing the effectiveness of therapies. Cinnamon has been shown to induce apoptosis in tumor cells and suppress tumor growth and survival by modulating various genes involved in the apoptosis pathway (Morsi et al., 2022).

b) **Reduction of Side Effects:** Natural products have shown promise in alleviating the side effects of cancer treatments. Compounds derived from plants, including flavonoids, alkaloids, and terpenoids, can boost the efficacy of conventional cancer therapies while minimizing adverse effects. For example, certain natural products can alleviate chemotherapy-induced gastrointestinal toxicity, hepatotoxicity, and neurotoxicity (Zhang et al., 2018). It has been observed that cancer patients undergoing chemotherapy who use natural products experience fewer adverse effects like nausea and vomiting, or sometimes no adverse

effects at all (Almatroodi et al., 2020). Additionally, the concurrent use of natural products with conventional therapies has been noted to improve patient tolerance and facilitate the practical, long-term treatment of diseases such as cancer, autoimmune diseases, arthritis, and gastrointestinal disorders (Alamgir, 2018; Prasher et al., 2020). By reducing the adverse effects, natural products help improve the quality of life for patients, particularly those receiving aggressive treatments, enabling them to adhere more strictly to their medical regimens. Furthermore, these natural compounds can enhance immune function and reducing symptoms like oral mucositis and diarrhea. However, more research is needed to fully understand their interactions with cancer medications and optimize their use in clinical settings (Zhang et al., 2018).

c) **Immune System Modulation**

Cinnamon contains immunomodulating compounds such as cinnamic acid that help strengthen the body's immune system, which is crucial for fighting cancer and inflammation. Numerous studies have shown that aqueous cinnamon extracts and bioactive components like cinnamaldehyde, cinnamic acid, procyanidins, and cinnamon essential oil can stimulate natural killer (NK) cells and other immune cells. These compounds influence cytokine production and modify the cytotoxicity of NK cells, thereby enhancing the body's defense mechanisms (Sharifi-Rad et al., 2021).

The combined use of curcumin with an immune checkpoint inhibitor, specifically an anti-Programmed Cell Death-1 (anti-PD-1) inhibitor, has been shown to produce a heightened anticancer effect in preclinical models by increasing the ability of immune cells to infiltrate the tumor microenvironment (Hayakawa et al., 2020). Natural compounds can also boost the effectiveness of immunotherapy by increasing the production of pro-inflammatory cytokines like interferon gamma (IFN-γ) and enhancing the cytotoxicity of NK cells (Luo et al., 2023).

By balancing anti- and pro-inflammatory signals and employing other immunomodulating strategies, cinnamon and its constituents help overcome tumor cell resistance mechanisms and improve the efficacy of anticancer therapies.

B. Direct Anti-Cancer and Anti-Inflammatory Effects

It has been established that cinnamon has direct anti-cancer and anti-inflammatory properties. It can induce apoptosis in cancer cells and inhibit angiogenesis and metastasis. Studies have shown that cinnamaldehyde can trigger apoptosis in breast cancer cells by stimulating the MAPK pathway and activating the caspase-8 pathway (Wu et al., 2005). Similarly, cinnamaldehyde has been found to promote apoptosis in the HL-60 leukemia cell line by generating reactive oxygen species through the activation of the mitochondrial pathway (Ka. H., et al., 2003).

The synergistic enhancement of therapeutic agents' effects following preconditioning with cinnamon extracts has also shown promising pharmacological outcomes. For instance, a study by Sandamali et al. demonstrated that rats pre-treated with cinnamaldehyde before receiving doxorubicin exhibited significantly improved efficacy of the latter. This was achieved through cinnamaldehyde's ability to decrease lipid peroxidation and myeloperoxidase activity, ultimately reducing doxorubicin-induced oxidative stress and inflammation in Wistar rats (Sandamali et al., 2021).

C. Synergistic mechanisms of cinnamon -

Cinnamon enhances the therapeutic index of biotherapeutics through several mechanisms:

a) **Antioxidant Properties:** Cinnamon contains bioactive compounds like cinnamaldehyde and procyanidins, which have strong antioxidant properties. These compounds help reduce oxidative stress, which can enhance the effectiveness of biotherapeutic agents by protecting cells from damage (Medagama, 2015).

b) **Anti-inflammatory Effects:** Cinnamon's anti-inflammatory properties help reduce inflammation, which is beneficial in treating both cancer and inflammatory diseases. By inhibiting pro-inflammatory cytokines

and signaling pathways, cinnamon can enhance the efficacy of biotherapeutics (Gu et al., 2022).

c) **Immune Modulation:** Cinnamon has immunomodulatory effects, meaning it can help regulate the immune system. This can enhance anti-tumor immune responses and improve the overall effectiveness of biotherapeutic treatments (Silva et al., 2022).

d) **Synergistic Interactions:** Studies have shown that cinnamon can interact synergistically with chemotherapeutic agents, leading to improved anti-cancer activity and reduced side effects. This means that when used together, cinnamon and biotherapeutics can be more effective than either treatment alone (Medagama, 2015).

e) **Multi-targeted Approach:** Unlike single-target drugs, cinnamon affects multiple pathways involved in disease processes. This multi-targeted approach can help overcome drug resistance and improve the overall therapeutic outcome (Gu et al., 2022).

By incorporating cinnamon into treatment regimens, it is possible to enhance the therapeutic index of biotherapeutics, making treatments more effective and potentially reducing side effects.

1.5. CASE STUDIES AND EXAMPLES

To further elucidate the benefits of combining natural products with various therapeutic agents, a few examples are discussed in this section.

a) Curcumin with chemotherapy

Curcumin, a bioactive compound derived from the Curcuma longa plant, shows great promise due to its anti-cancer, antioxidant, and antibacterial properties. When used in combination with chemotherapeutic agents like cisplatin, doxorubicin, and paclitaxel, curcumin has been shown to enhance therapeutic effects (Giordano and Tommonaro, 2019). Research indicates that curcumin amplifies the cytotoxic effects of cisplatin by modulating apoptosis and the NF-κB signaling pathway in lung cancer cells (Baharuddin et al., 2016). This synergistic effect not

only improves treatment outcomes but also reduces the required dosage of chemotherapeutic agents, thereby minimizing side effects.

b) Resveratrol with Cardiovascular Medications

Resveratrol, a natural compound found in red grapes and berries, is recognized for its significant cardiovascular benefits (Bonnefont-Rousselot D., 2016). When combined with statins, it has demonstrated synergistic effects, enhancing cholesterol reduction and cardiovascular protection through its antioxidant and anti-inflammatory properties. This combination not only amplifies the positive cardiovascular outcomes but also helps manage statin-induced myopathy, offering dual benefits in cardiovascular treatments (Soner and Sahin., 2014).

c) Green Tea Catechins with Anti-Cancer Agents

Catechin and epigallocatechin gallate (EGCG), bioactive compounds from green tea, have been researched in conjunction with anti-cancer agents like EGFR inhibitors and tyrosine kinase inhibitors (Ferrari et al., 2022). Studies on their role in tumor growth reduction have shown that EGCG enhances the anti-proliferative and pro-apoptotic effects of chemotherapeutic agents by regulating various signal transduction pathways, including MAPK and PI3K/Akt. This combination offers a promising approach to improve cancer therapy outcomes and overcome resistance factors (Ferrari et al., 2022)

d) Quercetin with Antibiotics

Quercetin, a flavonoid present in fruits and vegetables, exhibits synergistic effects with antibiotics, which is particularly important against multidrug-resistant bacterial species. Research has shown that quercetin enhances the efficacy of antibiotics such as lincosamide, ciprofloxacin, and erythromycin against resistant strains like MRSA (methicillin-resistant *Staphylococcus aureus*) (Jubair et al., 2021). This combined approach effectively addresses the challenge of increasing antibiotic resistance by boosting the bio efficacy of these antibiotics.

e) Ginsenosides with Immune Checkpoint Inhibitors

Ginsenosides, bioactive compounds derived from Panax ginseng, have demonstrated promising results in enhancing the effects of immune checkpoint inhibitors (ICIs) in cancer immunotherapy. These immunomodulatory effects are primarily due to alterations in cytokine release and increased T-cell proliferation (Tang et al., 2024). In animal models, combining ginsenosides with ICIs targeting PD-1/PD-L1 or CTLA-4 has been shown to boost antitumor effects and improve survival rates. This combined approach holds potential for improving immunotherapy outcomes in cancer patients (Yu et al., 2023).

f) Cinnamon with therapeutic agents

The idea of incorporating cinnamon with conventional therapies shows great potential in various medical fields. It enhances the effectiveness and activity of treatments while simultaneously reducing adverse effects. Numerous studies, including those by Silva et al. (2022), have highlighted the combined benefits of cinnamon and various biotherapeutic agents in treating conditions such as cancer, diabetes, neurodegenerative diseases, and inflammatory disorders.

In cancer research, bioactive compounds from cinnamon, such as cinnamaldehyde, have been shown to enhance the apoptotic effects of standard chemotherapeutic agents. For example, studies using breast cancer models demonstrated that combining cinnamaldehyde with oxaliplatin increased the rate of oxaliplatin-induced cancer cell apoptosis while reducing tumor cell survival rates (Wu et al. 2019). This combination therapy highlights cinnamon's role in boosting the effectiveness of conventional cancer treatments, thereby improving the prognosis for cancer patients.

In an *in vitro* study, cinnamon extracts were found to work synergistically with the monoclonal antibody infliximab. This effect is likely due to trans-cinnamaldehyde, which downregulates genes involved in signal transduction and apoptosis (Khedkar and Khan, 2024).

Cinnamon also shows promise in treating infectious diseases. For example, combining cinnamon bark oil with meropenem has been shown to enhance meropenem's potency by disrupting bacterial membranes and lowering the inhibitory concentration needed to control growth, highlighting its potential in antibiotic therapy, especially against

antibiotic resistance (Yang et al., 2017). Additionally, cinnamon oil combined with clotrimazole has been tested against multidrug-resistant dermatophytic infections. Clinical trials have demonstrated that this combination is more effective than clotrimazole alone, resulting in higher infection clearance rates and lower recurrence rates. The synergistic effect also reduces irritation, enhancing patient comfort (Mawra and Aziz, 2012). One study examined the effect of combining cinnamon with chloramphenicol and ampicillin, showing synergistic antibacterial activity against multi-drug resistant bacteria. This combination demonstrated potent bactericidal effects against *E. coli*, while cinnamon oil with streptomycin showed additive effects against various bacterial strains. This synergy emphasizes cinnamon oil's potential to reduce the minimum effective dose of drugs, offering enhanced antimicrobial efficacy compared to individual treatments (El Atki et al., 2019).

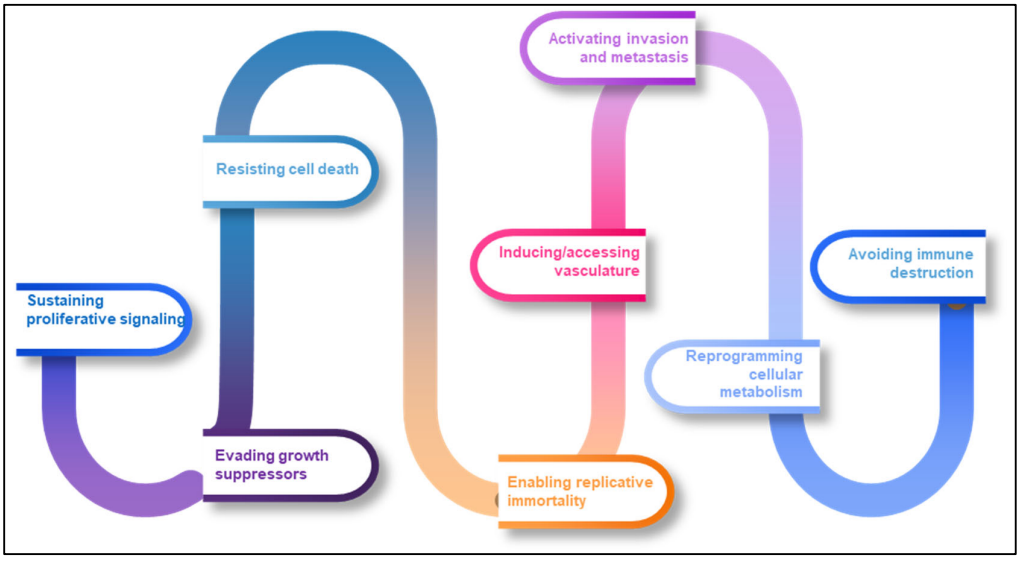
CHAPTER 2

REVIEW OF LITERATURE

2.1 Cancer, Inflammation and CAM

According to 2020 statistics, cancer is responsible for around 10 million deaths globally, with approximately double the number of reported cases worldwide (Doocey et al., 2022). Carcinogenesis is the gradual process where normal cells transform into cancerous cells, consisting of four stages: initiation, promotion, malignant transformation, and progression. The first phase of carcinogenesis, tumor initiation, is marked by changes in DNA (Deoxyribonucleic acid) brought about by physical or chemical carcinogenic stimuli (Cooper et al., 2000). Changes in DNA lead to oncogene-mediated activation or tumor suppressor gene-mediated suppression of various genes (Cooper et al., 2000; Vendramini-Costa, et al., 2012). During tumor promotion, cell proliferation and vascularization are increased, resulting in the formation of neoplastic cells. In the malignant conversion step, the preneoplastic cells transform into cells expressing malignant phenotypes. In the tumor progression stage, the tumor aggressively proliferates and invades other tissues, accumulating several different mutations (Vendramini-Costa, et al., 2012). Hanahan (Hanahan, 2022) reviewed the hallmarks of cancer, providing a detailed description of changes occurring in a cell moving from a normal to a transformed state (Figure. 6).

Figure. 6. Hallmark of cancer cells



Approximately 25% of cancer cases have a history of chronic inflammation or infectious diseases (Rossi et al., 2021; Greten and Grivennikov, 2019). Extrinsic factors, including smoking and infections, and intrinsic factors, such as tumor-associated inflammation, contribute to an inflammatory tumor microenvironment (TME) (Zhao et al., 2021). Additionally, changes in cell physiology and metabolism result in an increase in stress-related inflammatory markers (Ho et al., 2020). Cancer treatment using the conventional approach and biotherapeutics could also induce inflammation and have been the focus of extensive research in recent years (Greten and Grivennikov, 2019).

Inflammation is a protective response elicited by cells when tissue damage is caused by infection, trauma, exposure to a toxin, or physical or ischemic injury. A successful inflammatory response leads to homeostasis and inflammation resolution upon the removal of the stimulus (Zhao et al., 2021; Nazhvani et al., 2020). However, a standoff between the immune response and a stimulus that cannot be eradicated results in chronic inflammatory conditions (Greten and Grivennikov, 2019; Munn, 2017). In chronic inflammatory conditions, inflammatory mediators such as reactive oxygen species cause DNA damage by oxidation and facilitate spontaneous mutagenesis mediated by 8-oxo-7-hydro-2′-deoxyguanosine (Nakamura and Takada, 2021). The interplay between various pro-inflammatory factors in the TME contribute towards cancer development (Łukaszewicz-Zając and Mroczko (2021); Zhou et al., 2021). In recent years, extensive research has been devoted to exploring the link between inflammation and cancer (Zhao et al., 2021; Crusz and Balkwill FR, 2015; Lan et al., 2021).

One of the most well-studied genes involved in tumor initiation and progression is the mutated tumor suppressor gene *Tp53,* encoding the p53 protein which is involved in cell homeostasis (Marei et al., 2021). Wild type p53 suppresses tumor growth by activating factors responsible for DNA repair and inducing apoptosis and senescence (Marei et al., 2021), prevents oncogenic transformation in cultured cells, and p53 null mice are highly likely to develop tumors (Kennedy and Lowe, 2022). Several reports indicate the role of mutant p53 in TME. Bhatta et al. (Bhatta et al., 2021) have reported

that the presence of mutant p53 protein in the TME of non-transformed cells promotes tumor progression through cell reprogramming. The cross-talk between the TME and tumor is enhanced by mutant p53, resulting in the increased transcription of inflammatory messengers such as Nuclear Factor kappa B (NFκB) (Capaci et al., 2020). A mutated p53 protein results in unchecked NF-kB activity, causing increased expression of pro-inflammatory signaling molecules; the resulting TME is characterized by hypoxia and increased ROS (Reactive Oxygen Species) activity, further activating redox-sensitive factors such as Hypoxia-Inducible Factor (HIF-1α) (Greten and Grivennikov, 2019; Jing et al., 2019). Hypoxia also increases the levels of pro-inflammatory cytokines, including interleukin 6 (IL-6), tumor necrosis factor (TNF-α), and pro-angiogenic factors, including vascular endothelial growth factor (VEGF), resulting in chronic inflammation (Greten and Grivennikov, 2019, Jing et al., 2019). An interplay of factors, p53, along with increased IL-6, results in increased activity of signal transducer and activator of transcription (STAT-3), supporting tumor induction and providing a favorable environment for tumor growth (Shi and Jiang, 2021). Therefore, p53, IL-6, VEGF, and STAT3 are attractive therapeutic targets for cancer treatment (Almatroodi et al., 2021).

CAM is gaining attention among patients with cancer. Approximately 87% of the patients use at least one form of CAM, including herbal medicine, yoga, reiki, and naturopathy, to improve their overall quality of life (Balneaves et al., 2021). Furthermore, ~22% of patients are reported opting for herbal medicines along with conventional therapies (Asiimwe et al., 2021). Plant polyphenols are potent anti-cancer and anti-inflammatory agents and have been reported to reduce the side effects caused by chemotherapy and reverse prolonged treatment-induced chemoresistance (George et al., 2021). Even though several natural compounds such as curcumin, resveratrol, green tea extracts, quercetin, and lycopene have been used for clinical testing, the specific mechanisms and effects of these natural compounds remain largely unexplored and not well understood (Kubczak et al., 2021). Several widely used spices, such as curcumin (from turmeric, *Curcuma longa*) (Dini and Laneri, 2021; Tagde et al., 2021), eugenol (clove, *Syzygium aromaticum*) (Kammath et al., 2021), cinnamic acid, and trans-

cinnamaldehyde (*Cinnamomum spp.*) (Singh et al., 2021), have been identified to have anti-cancer, anti-inflammatory, and anti-oxidative properties (Moosavi-Movahedi, 2021).

2.2 Aqueous Cinnamon Extract

Cinnamon (*Cinnamomum* spp.) is a tropical plant belonging to the Lauraceae family and has been widely used as a spice, flavoring agent, and in the perfume industry since ancient times. *Cinnamomum* comprises ~250 species, of which true cinnamon or Ceylon cinnamon (*Cinnamomum verum*), Cassia cinnamon, or Chinese cinnamon (*Cinnamomum cassia*), Indonesian cassia (*Cinnamomum burmannii*), and Vietnamese cinnamon (*Cinnamomum loureiroi*) are most commonly used (Ribeiro-Santos et al., 2017). Increasing research on this plant has unraveled several benefits of cinnamon consumption. Not only cinnamon bark, but its flowers and leaves are also used for preparing extracts with different active compounds like cinnamaldehyde, eugenol, trans-cinnamyl acetate (Kawatra and Rajagopalan, 2015), and therapeutic properties. Apart from essential oils, the anti-inflammatory and anti-cancer therapeutic potentials of organic and aqueous extracts of cinnamon have also been reported (Kammath et al., 2021; Singh et al., 2021), and several extraction methods using various solvents have been developed over the years (Gilani and Najafpour, 2022; Rao et al., 2021; Souza et al., 2021). The aqueous polyphenol fraction has therapeutic potential in the treatment of periodontal disease to manage inflammation (Ben Lagha et al., 2021). In addition, several reports have confirmed the therapeutic potential of the aqueous cinnamon extract against cancer (Larasati et al., 2018; Sadeghi et al., 2019). The extraction method and type of solvent affect the recovery of polyphenols and, thus, the therapeutic properties of the cinnamon extract (Muhammad and Dewettinck, 2017).

Conventional methods like maceration, extraction chambers and percolation use organic solvents such as methanol and acetone for preparing plant extracts. However, these methods have a few drawbacks, they are not environmentally friendly, and it is crucial to ensure the removal of residual solvent from the final preparation before

testing its effects (Sadeghi et al., 2019). Therefore, many researchers now prefer the green extraction method, which aims to discover innovative extraction processes that are safe, yield high-quality extracts, and reduce energy consumption (Chemat et al., 2012). One of the principles of green extraction is using alternate solvents, principally water and agro-solvents also known as bio-solvents as they are produced from biological materials (for example, methyl esters of fatty acids in vegetable oil, ethanol from fermentation of sugar beet, glycerol from vegetable oil transesterification etc.), to minimize the release of toxic waste into the environment. Some of the green extraction methods (Figure. 7), and their advantages and limitations are captured in Table 1. Green extraction techniques use modern technologies and sometimes also referred to as technology assisted extraction methods (Gil-Martin et al., 2022). It should be noted that one technique may not suffice the extract requirements. A combination of various technology assisted green extraction methods can be used to achieve maximum yield of desired compounds (Panja, 2018; Gil-Martin et al., 2022).

Figure. 7. Conventional and Green extraction methods

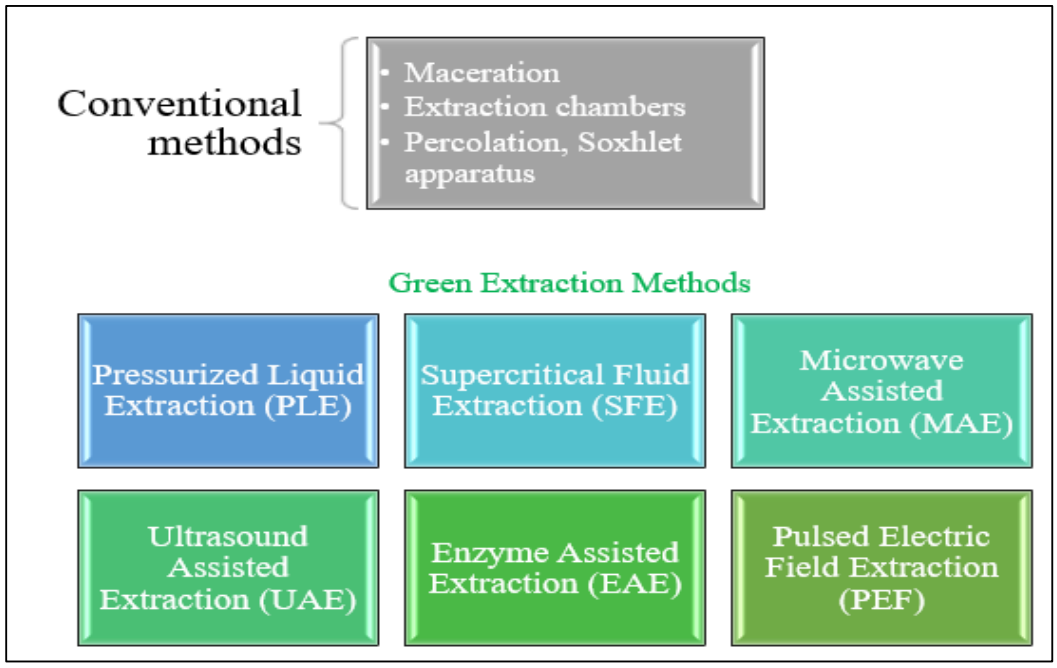


Table 1. Advantages and limitation of various green extraction methods

Extraction method	Extractants	Advantages	Limitations	Emerging Scenarios	References
Hot-Pressurized Liquid Extraction (HPLE) Pressurized hot water extraction (PHWE)	Water and aqueous alcohols (HPLE) and Water (PHWE)	• Recovery of polyphenols is higher. • Low solvent requirement	• Low selectivity • Instrumentation cost	Coupling with UAE and SFE	• Panja, 2018 • Chemat et al., 2019 • Kumar et al., 2021 • Gil-Martín et al., 2022
Supercritical fluid extraction (SFE)	Supercritical CO_2	• Suitable for extraction of non-polar or mid to low polar components	• Whole extracts cannot be achieved due to selectivity	Coupling with UAE	
Microwave Assisted Extraction (MAE)	Water, ethanol, methanol	• Most suitable for extraction of flavonoids and phenolic acids • Low solvent requirement	• Limitation of the technology as it is can be used for specific compounds	Vacuum Microwave Aqueous Assisted Extraction or VMAAE	
Ultrasound Assisted Extraction (UAE)	Water and aqueous alcohols	• Cost effective. • Good for thermolabile/unstable components	• High power UAE can cause degradation of desired species	Coupling with MAE and SFE	
Enzyme Assisted Extraction (EAE)	Water	• Low energy requirement • Reduced /No degradation of desired species as temperature working range is low.	• Time consuming	Coupling with high hydrostatic pressure (HHP)	
Pulsed Electric Field Extraction (PEF)	Not applicable	• Suitable for useful for thermolabile components	• Not yet identified	PEF with solvent extraction	

Subcritical water extraction, one of the green methods is performed at high temperatures (200°C) and pressure (1.5 Mpa), has gained considerable attention (Chemat et al., 2012; Castro-Puyana et al., 2017). During subcritical extraction, the

physicochemical properties of water, such as its dielectric constant, are significantly reduced (similar to ethanol), making it a suitable solvent for extracting low-polarity compounds (Chemat et al., 2012). Hydrodistillation is conventionally used to extract essential oils from cinnamon, and recent advances in this process include microwave-assisted hydrodistillation, which is energy-saving and environmentally friendly (Jeyarantnam et al., 2016; Gil-Martin et al., 2022). Aqueous cinnamon extract can be prepared by boiling, preparing a decoction, and either lyophilizing or using it as such after sterile filtration (Peterson et al., 2009; Kwon et al., 2010).

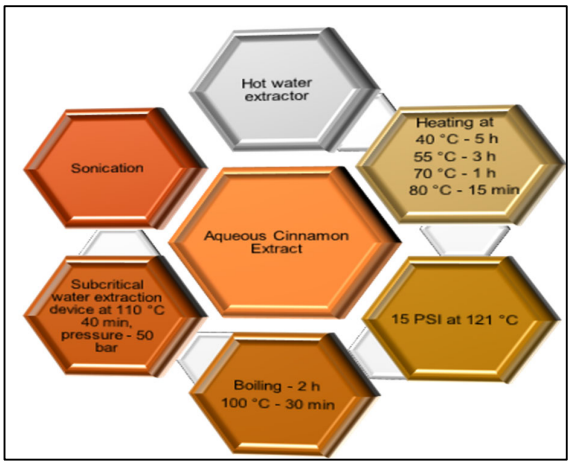
Aqueous cinnamon extract is extensively studied for its role in managing blood sugar levels and as an antioxidant (Silva et al., 2022; Yaseen and Mohammed, 2022; El-Ashmawy et al., 2022). Comprehensive information on the role of cinnamon in diabetes management has been described in a recent review article (Senevirathne et al., 2022). In addition, cinnamon also acts as an antioxidant, anti-inflammatory, anti-cancer, and wound healing agent. Cinnamon aqueous extract has been used as an anti-inflammatory agent in hepato-renal toxicity (Elshopakey and Elazab, 2021) and as an anti-proliferative agent in human prostate cancer cells (Gopalakrishnan et al., 2018). Aqueous CE can inhibit cancer growth (Zhang et al., 2017). As water is considered the most suitable form of solvent in terms of safety (Muzolf-Panek and Stuper-Szablewska, 2021), aqueous cinnamon extract (aCE) was used in this study.

2.3 Extraction methods

Figure 8 summarizes relatively simple aqueous extraction methods that could be carried out in laboratories with limited resources. However, the literature search revealed that even seemingly simple extraction methods involve several steps, including extracting at 40 °C, stirring rapidly for 10 min, centrifugation, chilling the beaker with the extract in an ice bath, and stirring, followed by filtration and lyophilization (Peterson et al., 2009), to a simple one-step extraction process, where the ground cinnamon powder was resuspended in sterile water (70 °C for 1 h), centrifuged, and used after filtration (Lu et al., 2010). A common feature of most extraction methods summarized in Table 2 is that the extract was lyophilized and stored at −70 °C to −80 °C (Singh et al., 2009; Lee et al., 2011; Hong et al., 2012). In most of the studies, ground/pulverized cinnamon was

used (Peterson et al., 2009; Kwon et al., 2009), while in some cases, the extract was prepared after soaking the bark in water (Hong et al., 2012).

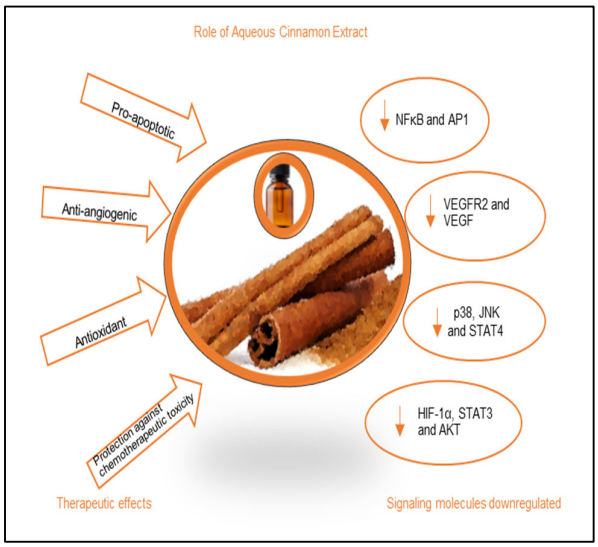
Figure. 8. Summary of the methods used for aqueous cinnamon extract preparation.



2.4 Role of aqueous cinnamon extract

Aqueous cinnamon extract has been reported to have anti-cancer (Singh et al., 2009; Kwon et al., 2009), anti-inflammatory [Ben Lagha et al., 2021; Lee et al., 2011), and anti-oxidant (Jiao et al., 2013) properties and exert these effects by various mechanisms, a few of which are described in this section and summarized in Figure. 9.

Figure 9. Role of aqueous cinnamon extract



2.5 Anti-cancer activity

Cinnamon extract is toxic to cancerous cells and inhibits tumor cell growth *in vitro* (Singh et al., 2009; Ariaee et al., 2014; Abd and Adzmi, 2017) . The cytotoxic effect of crude cinnamon aqueous extract was reported to be more potent than that of individual components such as cinnamaldehyde (Singh et al., 2009). In an *in vitro* study it was shown that aqueous cinnamon extract was toxic for oral squamous carcinoma cells (OSCC), reaching a 90% cytotoxicity with 10 mg/mL extract within 48 h. Moreover, treating OSCC with aqueous saffron and cinnamon extracts had a synergistic effect, with more cytotoxicity at lower concentrations (Nazhvani et al., 2020).

Like normal cells, tumor cells also need a way to get nutrients and a way to remove cellular metabolic waste. This is achieved by either utilizing the existing blood vessels or forming new ones (angiogenesis). The genes involved in angiogenesis are upregulated in the TME (Ansari et al., 2022). Aqueous cinnamon extract has been shown to downregulate the pro-angiogenic factors (Kwon et al., 2009), and exhibit anti-angiogenic properties (Zhang et al., 2017; Lu et al., 2010; Kwon et al., 2009). It exerts its effects at various stages of angiogenesis and modulates the expression of VEGF mediated by the inhibition of cyclooxygenase (Cox-2) in an *in vitro* as well as *in vivo* system (Kwon et al., 2009), and that of HIF-1α, STAT3, and Protein kinase B (AKT) in an *in vitro* system (Zhang et al., 2017). Cinnamaldehyde (Zhang et al., 2017) and procyanidin (Lu et al., 2010) were identified as active components in aqueous cinnamon extract, which inhibit VEGF expression and VEGFR2 signaling *in vitro*, respectively. An effective way to eliminate cancer cells is cytolysis which is mediated by CD8+ T cells. TME redesigns itself to evade the immune machinery and thus are refractory to the cytolytic CD8+ T cells. Kwon et al. (Kwon et al., 2009) have reported (*ex vivo* study) that cinnamon extracts enhance the cytolytic activities of CD8+ T cells which is mediated through perforin and granzymes.

NFκB regulates many factors that play a role in proliferation and survival of cells. Increase in NFκB results in an increase in anti-apoptotic molecules. Tumor cells increase their chances of survival by evading apoptosis; the mechanisms can vary from an increase in anti-apoptotic molecules like B-cell lymphoma 2 (BCL-2) and Bcl-2-like

protein 4 (Bax), downregulation of death receptors, or inactivation of caspase-8. Many plant extracts, including aqueous cinnamon extract, act as pro-apoptotic agents (Rajabi et al., 2021). One of the reported effects of the aqueous cinnamon extract both in *in vitro* and *in vivo* system was that it effectively inhibited the expression of pro-apoptotic genes, such as Bcl-2, BcL-xL, and Survivin, which was mediated via a decrease in NFκB and Activator Protein (AP-1) signaling (Kwon et al., 2010). Varalakshmi et al. (Varalakshmi et al., 2017) based on molecular docking studies have reported that Procyanidin B2 has inhibitory effect on NFκB and may act by inhibiting the translocation of this factor to nucleus. Another mechanism by which aqueous cinnamon extract facilitates its pro-apoptotic activity is by increasing intracellular calcium, leading to loss of membrane potential and, eventually, apoptosis. In an independent study, authors observed an increase in intracellular calcium and depolarization of mitochondrial membrane potential, when SiHa cells were treated with 80µg/mL of aqueous cinnamon extract (Koppikar et al., 2010).

Histone deacetylase family member 8 (HDAC8) is implicated in many cancers and an HDAC8 knockdown system showed decreased cancer progression of human colon, lung, and cervical cells. In an *in vitro* and *in silico* study, aqueous extract of cinnamon, cinnamic acid, cinnamyl alcohol and cinnamaldehyde, were all shown to bind to the HDAC8 enzyme. Whole extract was much more efficient in inhibiting the enzyme compared to purified components (Patil et al., 2017). Transformed cancerous cells are associated with increased activity of 26S proteasome, the protein degrading machinery. This increased activity facilitates cancer cell proliferation and survival. The aqueous cinnamon extract and one of its active components, procyanidin B2 were reported to have anti-protease activity (26S proteasome). These components inhibited the complete catalytic activity (all 3 activities of the 20S) and were selective for transformed cells only (Gopalakrishnan et al., 2018).

2.6 Anti-inflammatory activity

An infection-mediated immune response can be either innate when encountering the antigen for the first time or acquired when the response is generated from immunological memory (Miles and Calder, 2021). The immune response is identified as "good" inflammation, which leads to an antigen-specific T-cell response; however, prolonged, and uncontrolled inflammation can eventually lead to chronic inflammatory diseases and cancer. The activated T-cell response is characterized by the production of cytokines such as interferon (IFN-γ), interleukin 2 (IL2), and interleukin 4 (IL4) and modulating signaling molecules (Lee et al., 2011). In an immune response generated by activated T-cells, the aqueous cinnamon extract reduced the IFNγ levels and inhibited p38, c-Jun amino-terminal Kinases (JNK), Extracellular signal–Regulated Kinases (ERK), Signal Transducer and Activator of Transcription 4 (STAT4), and Signal Transducer And Activator Of Transcription 6 (STAT6), without affecting the inhibitor of kappa B (IkBα). Similarly, aqueous cinnamon extract downregulated Lipopolysaccharide (LPS)-induced TNF-α levels in serum by inhibiting the activation of p38, JNK/ERK1, and 2, and IkBα. LPS binds to receptors like toll like receptors which recognize unique microbial pattern and elicit an immune response. The response is mediated by NFκB and MAP kinase signal pathways. In this *in vivo* and *in vitro* study (Hong et al., 2012) cinnamon aqueous extract at low doses was able to decrease TNF-α and IL-6 level in the serum. Chronic inflammatory conditions are characterized IFN-γ driven initial response followed by release of other inflammatory mediators. Aqueous extract of cinnamon as well as its polyphenol fraction exert anti-inflammatory activities which result in decrease of IFN-γ, NFκB and MAP kinase signal pathways and expression of IL-6, IL8, and TNF-α. Another study using a cellular intestinal inflammation model revealed that aqueous cinnamon extract containing the active components cinnamic acid and cinnamaldehyde played a dual role in enhancing tight junction permeability and decreasing inflammatory modulators interleukin 6 (IL-6), interleukin 8 (IL8), TNF-α, and NF-κB. Cinnamon aqueous treated mice had better gut microbiota diversity than untreated counterpart (Kim and Kim , 2019).

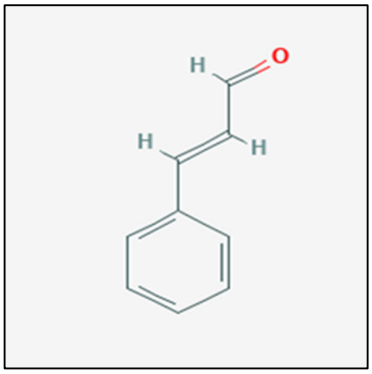
2.7 Antioxidant activity

One of the essential roles of aqueous cinnamon extract is its antioxidant activity. For cinnamon aqueous extract to be useful as CAM, in addition to its anti-cancer and anti-inflammatory role, the anti-oxidant role solidifies it as a CAM candidate. Effective chemotherapeutic agents, such as cis-diamminedichloroplatinum (CDDP), are associated with several side effects, one of which is increase in reactive oxygen species (ROS). Heme Oxygenase-1 (HO-1) is upregulated in the kidneys to counteract the oxidative stress. In an *in vitro* study, Vero cells were treated with CDDP to induce toxicity and treatment with aqueous cinnamon extract decreased CDDP-mediated ROS and helped counteracting oxidative stress and decrease apoptotic cell death. One of the ways by which the extract brings about this effect is by upregulation of Heme Oxygenase-1 (HO-1) transcript levels without interfering with CDDP activity (ElKady and Ramadan, 2016). In another example, acetaminophen, an antipyretic and analgesic drug, if abused beyond therapeutic doses is associated with acute renal toxicity which results due to increased oxidative stress from increased ROS. In-an *in vivo* study the aqueous cinnamon extract has been shown to protect against drug induced cell trauma and death in renal cells by limiting/decreasing lipid peroxidation and apoptosis (Abdeen et al., 2019). Small-molecule therapeutic agents are associated with acute toxicity when they exceed the recommended dose (ElKady and Ramadan, 2016; Abdeen et al., 2019), and the chances of toxicity are further increased when multiple therapeutic agents are administered together. Diclofenac sodium (DFS), a non-steroidal anti-inflammatory drug, is often consumed along with oxytetracycline (OTC) (an antibiotic), and the combination is associated with severe toxicities when abused. The toxicities are associated with increased ROS, decreased activity of antioxidant Superoxide dismutase (SOD), Reduced glutathione (GSH) etc. The antioxidant and anti-inflammatory properties of the aqueous cinnamon extract protected against the toxicity induced by DFS and OTC, individually or in combination, by increasing the pro-apoptotic factors, hepatic and renal caspase-3, and cyclooxygenase-II (Elshopakey and Elazab, 2021).

2.8 Active components in aqueous cinnamon extracts

A. Trans-cinnamaldehyde

Figure. 10 - Structure of trans cinnamaldehyde
(PubChem. Cinnamaldehyde | C9H8O).



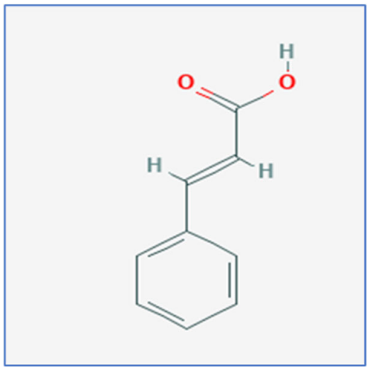
Cinnamaldehyde (cinnamic aldehyde), a phenylpropanoid (Figure. 10), occurs naturally as a trans-cinnamaldehyde and imparts the odor and flavor of cinnamon. Widely present in cinnamon essential oils, cinnamaldehyde is also present in the aqueous extract of cinnamon. Several studies have reported the anti-cancer and anti-inflammatory activities of cinnamaldehyde (Liu et al., 2020). The aqueous cinnamon extract containing cinnamaldehyde has been shown to exert higher cytotoxic activity than purified cinnamaldehyde against cancerous cell lines, suggesting that cinnamaldehyde, in conjunction with other polyphenols present in the aqueous cinnamon extract, can be cytotoxic to cancer cells. The aqueous cinnamon extract with cinnamaldehydes and polyphenols as the major bioactive compounds was cytotoxic to cancer cells. It downregulated human epidermal growth factor receptor-2 (EGFR-2) and matrix metalloproteinase (MMP-2) expression, affecting the invasion and metastasis of cervical cancer (Koppikar et al., 2010). Liao et al. (Liao et al., 2008) showed that blocking nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) degradation, thereby decreasing Intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), NF-κB signaling and induction of Nuclear factor erythroid 2-related factor 2 (Nrf2) -related genes (including HO-1), are the mechanisms by which aqueous cinnamon extract and purified cinnamaldehyde exert their effect on TNF-α-induced signaling in pre-treated TNF-α endothelial cells. Cabello et al. (Cabello et al., 2009), in their studies on A375 melanoma cells, demonstrated that cinnamaldehyde inhibited NF-κB constitutive and

TNF-α-induced transcriptional activity and suggested that Michael acceptor reactivity with this dietary electrophile is responsible for the observed effect.

Angiogenesis is central to the development and growth of tumor cells. Several small-molecule inhibitors and new age biological therapies are aimed at inhibiting angiogenesis. The aqueous cinnamon extract decreased VEGF, which was mediated by suppression of the HIF-1α gene via STAT3 and AKT. The decrease in VEGF and the resulting anti-angiogenic effects were attributed to cinnamaldehyde, a major component of the extract. Liu et al. (Liu et al., 2020) identified possible cinnamaldehyde targets in breast cancer using *in silico* and in an *in vitro* study and showed that cinnamaldehyde induced apoptosis, decreased proliferation, and reduced migration.

B. Cinnamic acid

Figure 11 - Structure of cinnamic acid
(PubChem. Cinnamic Acid|C9H8O2)

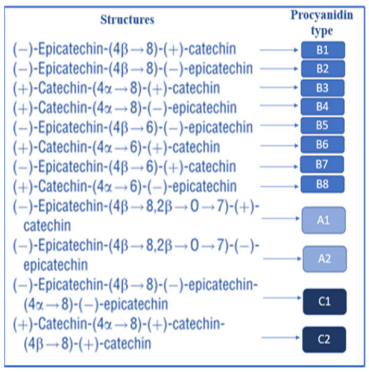


Cinnamic acid is a naturally occurring unsaturated carboxylic acid with a widely present trans isomer. Similar to other components of cinnamon, cinnamic acid (Figure. 11) is cytotoxic to cancerous cells. In an *in vitro* model, cinnamic acid showed anti-proliferative properties. It modulated the expression of *MMP2* and reduced the invasive capacity of cancer cells, suggesting its potential to reverse malignant human tumor cells to benign cells (Liu et al., 1995). Cancerous cells upregulate anti-apoptotic signaling cascades, leading to increased survival, which can be reversed by cinnamic acid. Niero and Machado-Santelli (Niero and Machado-Santelli , 2013) showed that cinnamic acid induced apoptosis in HT-144 human melanoma cells by upregulating the pro-apoptotic machinery, caspase-3 activity, Bax, and downregulating anti-apoptotic Bcl-2 protein.
Over the years, it has become apparent that overexpression of MMP proteins and malignant cancers are correlated. Yen et al. (Yen et al., 2011) demonstrated that cis and

trans stereoisomers of cinnamic acid inhibited MMP-2 and 9 activities, decreasing the invasive ability of A549 cells. Moreover, MMP downregulation can be linked to transcription factors, such as NF-κB, AP-1, and the Mitogen-activated protein kinases (MAPK) pathway (Tsai et al., 2013). Kwon et al. (Kwon et al., 2010) elucidated the anti-cancer activity of the cinnamon extract containing cinnamic acid and cinnamaldehyde as the major bioactive compounds in several transformed cell lines. Furthermore, in an intestinal epithelial cell and monocyte-macrophage co-culture model, a cinnamon water extract downregulated the inflammatory markers providing evidence for its anti-inflammatory activity. Even though the specific compound responsible for the anti-inflammatory effect was not identified in the study, cinnamic acid was one of the major bioactive compounds present in the extract.

C. Polyphenols

Figure 12 - Procyanidin dimers and structures.



Polyphenols, which are organic compounds found in high quantities in fruits, vegetables, tea, and spices, serve as secondary metabolites. They contribute to defense against pathogens and offer protection from ultraviolet radiation (Pandey and Rizvi, 2009). Procyanidins comprising (epi)catechin monomeric units (Figure. 12) protect against cancer and inflammation (Terra et al., 2011; Vetal et al., 2011). It has been reported that procyanidin tetramers and pentamers in *Cinnamomi cortex* (dried bark of *C. verum*) inhibited the enzyme activity regulated by Nrf2 in the A549 human lung cancer cell line (Ohnuma et al., 2011). Nrf2 is a crucial cytoprotective transcription factor, its overexpression induces resistance to chemotherapy, the most pressing dilemma in cancer therapy (Krajka-Kuźniak and Baer-Dubowska, 2021).

Angiogenesis is critical for cancer cells to flourish and propagate (Moyle et al., 2015). Of several molecules known to regulate angiogenesis, VEGF is the most important; it binds to its cognate receptors, VEGFR1 and VEGFR2, and initiates a signaling cascade leading to angiogenesis. Therefore, VEGF and its receptors are attractive targets for cancer therapy (Maslowska et al., 2015). The aqueous cinnamon extract could inhibit receptor kinase activity and downstream signaling of VEGFR2, suggesting the role of procyanidin trimers and tetramers in inhibiting VEGFR2 activity.

Proteosomes are another exciting target that has been identified for cancer therapy. Proteosome levels are elevated in tumor cells, which play an anti-apoptotic role. The procyanidin B2 component of the aqueous cinnamon extract was reported to selectively inhibit proteasome activity in cancer cells, decrease cell proliferation, and regulate genetic markers related to apoptosis and angiogenesis (Gopalakrishnan et al., 2018).
Cancer and inflammation are intricately linked; therefore, targeting inflammatory markers can help in chronic pathologic inflammation and in cancers where inflammatory molecules exacerbate cancer progression. NF-κB modulates the expression of several pro-inflammatory molecules and triggers inflammatory conditions. Targeting NF-κB using Cinnulin PF®, an aqueous cinnamon extract containing cinnamic acid, phenolic acids, flavonoids, and procyanidins, primarily as trimers and tetramers, decreased the production of inflammatory cytokines in a Lipopolysaccharide (LPS)-stimulated macrophage cell system, concomitantly with a significant reduction in NF-κB activity (60.1%) (Ben Lagha et al., 2021).

2.9 Biotherapeutics

In the last couple of decades several new modalities of treatment have been approved by the regulatory authorities. Biologics or biotherapeutics are therapeutic drugs produced by living source for example bacterial or mammalian cell lines. Biologics include, blood and blood factors, gene and cell therapy products, vaccines, monoclonal antibodies, proteins etc. Monoclonal antibodies have been very successful in treating chronic inflammations, cancers, and auto immunity (Johnson, 2018). Monoclonal antibodies bind and neutralize or block the target antigen thereby interfering in the

signaling process or marking the cell for destruction by immune cells (Kothari et al., 2024).

For instance, mAbs like rituximab target CD20 on B cells, flagging them for immune destruction, which is particularly useful in treating certain types of lymphoma (Caskey and Kuritzkes, 2022).Another example is trastuzumab, which binds to the HER2 receptor on breast cancer cells, blocking signals that promote cell growth (Singh et al., 2023).They can also block signals that promote cell growth, as seen with bevacizumab, which inhibits vascular endothelial growth factor (VEGF) to prevent blood vessel growth in tumors. Furthermore, some mAbs, like infliximab, are used to treat autoimmune disorders by targeting and neutralizing tumor necrosis factor-alpha (TNF-α), a substance involved in systemic inflammation (Caskey and Kuritzkes, 2022). These mechanisms make mAbs versatile and powerful tools in the treatment of various diseases, including cancer, autoimmune disorders, and infectious diseases. Infliximab and Bevacizumab cases are discussed in this section.

1. Chronic Inflammatory diseases and Infliximab

The immune system protects the host from various disease-causing agents, and one of the mechanisms by which it brings out the effect is by initiation of a protective response in the form of inflammation. However, autoimmune diseases such as Crohn's disease (CD), ulcerative colitis (UC), and rheumatoid arthritis (RA) set in when the host immune system turns against itself (Fugger et al., 2020). Approximately 5−8% of the world's population is affected by autoimmune diseases (Fugger et al., 2020), and approximately 80 autoimmune diseases are known so far (Khan et al., 2019). Several autoimmune diseases associated with chronic unresolved inflammation fall under the umbrella of immune-mediated inflammatory diseases (IMIDs) (David et al., 2018) and share common inflammatory pathways (Efthimiou and Markenson, 2005). Non-steroidal anti-inflammatory drugs, analgesics, and glucocorticoids represent the conventional treatment options, whereas biological agents represent a new line of therapy that has proven to be successful in managing IMIDs, such as ankylosing spondylitis (AS), Rheumatoid arthritis (RA), and psoriatic arthritis (PA) (Khan et al., 2019).

TNF-α is a key pro-inflammatory cytokine, and its irregular production is linked to various immune-mediated inflammatory diseases (IMIDs). Treatments targeting TNF-α include biological therapies such as monoclonal antibodies like infliximab and adalimumab, as well as Fc fusion proteins like etanercept (Fatima et al., 2023). These anti-TNF-α biological agents bind and neutralize TNF-α, which then cannot bind to its cognate receptor. Biological therapies have been successful and well-tolerated in most patients (Leone et al., 2023). Regulatory authorities recommend anti-TNF-α monoclonal antibody therapy over other conventional treatments due to its targeted mode of action. Although the cost associated with these biologics is high, the development and availability of biosimilars have benefited patients considerably, especially in developing countries (Marsal et al., 2022).

2. Infliximab Responders Vs Non Responders

Regrettably, some patients do not respond to therapy (primary non-responders) or may develop resistance to the biological agent over time (early and late secondary non-responders) (Vallejo-Yagüe et al., 2021). In primary non-responders, the failure to respond to treatment is associated with several factors, including the presence of genetic variants (single nucleotide polymorphisms) (Salvador-Martin et al., 2019) and differential expression (Wysocki and Paradowska-Gorycka, 2022) of genes that play critical roles in inflammatory pathways. One possible reason for the secondary non-response includes the formation of anti-drug antibodies. However, despite the development of anti-drug antibodies, patients continue to show a therapeutic response to the same biological agents (Leone et al., 2023). In non-responders, increasing the dose, switching, or swapping of different biologic agents have been reported. Clinical benefit has been observed in 50−70% of patients with increased amounts, while approximately 50% of patients have benefitted after switching the biologic. Some studies have also shown that combination therapy with immunosuppressants (initiated after non-response to higher doses of biologics) in non-responders was promising in half of the tested patients (Macaluso et al., 2018). Immunosuppressants and other small-molecule agents are associated with toxicity in patients. Therefore, in addition to the ongoing approaches, there is an

unmet requirement of identifying non-toxic molecules that work synergistically with anti-TNF-α monoclonal antibodies to initiate and sustain appropriate responses in biological non-responders to anti-TNF-α therapy.

3. Genes Differentially Regulated in Infliximab Non Responders

Infliximab is a chimeric antibody and bind to, both soluble and membrane bound. This binding results in neutralization of TNF-α and thus in preventing the activation of the downstream signaling pathways. Infliximab alters the expression of adhesion molecules, pro-apoptotic genes, and transcription factors (Danese, 2008). Toll-Like Receptors (TLRs) are pattern recognition receptors that are essential components of the innate immune system. TLR signal transduction is mediated via the MyD88-dependent and independent pathways, eventually activating NF-κB and releasing various pro-inflammatory cytokines (Zhao et al., 2021). Some studies have shown that TLRs and MMPs contribute to non-response (Meijer et al., 2007; Nie et al., 2022), the expression of which is modulated by infliximab. In a recent study, TLR-2 was identified as one of the markers of a non-response pathway (Nie et al., 2022). In another study, it was reported that infliximab decreased the expression of TLR-4 and TLR-5, the study indicated that higher TLR-4 and TLR-5 expression exacerbated inflammatory conditions in ankylosing spondylitis (Zhang et al., 2019). The pathogenesis of IBD is not entirely understood, and reports have indicated that a combination of factors is involved in disease manifestation, including the gut immune homeostasis. Environmental factors and genetic predispositions disrupt the balance between immune cells and intestinal microbes and contribute to an inflammatory environment. Cell adhesion molecules, such as ICAM-1 (required for leukocyte adhesion) and VCAM-1 (required for monocyte-endothelial cell interaction), along with other adhesion molecules, are involved in maintaining gut homeostasis, and their expression is elevated under inflammatory conditions (Okumura and Takeda, 2016; Gu et al., 2017). In active IBD, leukocytes and macrophages are recruited into the affected mucosa, which results in the production of a milieu of pro-inflammatory cytokines such as TNF-α, interferon (IFN)-γ, interleukin (IL)-6, and IL-1β. Infliximab treatment results in TNF-α neutralization,

thereby decreasing the levels of various pro-inflammatory cytokines, including that of TNF-α. Reports indicate that infliximab downregulates *ICAM-1* and *VCAM-1* mRNA levels in phytohemagglutinin and lipopolysaccharide-stimulated human peripheral blood leukocytes and U937 cells (Moriconi et al., 2011). Yarur et al. (Yarur et al., 2019) have shown that the levels of VCAM-1 and IL-6 were significantly higher in patients that were exposed to multiple anti-TNF-α agents. This observation is significant, as it shows that simply switching the anti-TNF-α agent in non-responders may not be beneficial. Therefore, new approaches should be identified.

Matrix Metalloproteinases (MMPs) are also upregulated under inflammatory conditions and have been reported to influence gut homeostasis. MMPs are involved in the degradation of the extracellular matrix, cytokines, and adhesion molecules. Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) neutralizes MMPs, and a balance between TIMP and MMP expression maintains MMP activity (Gao et al., 2007). Several MMPs, such as MMP1, 2, 3, and 7 are upregulated in IBD (Mariaule et al., 2021), which alters the MMP-TIMP ratio and further contributes to pathogenesis. Furthermore, in some patients, MMP3 cleaved IgG, thereby affecting the bioavailability of the anti-TNF-α agents and contributing to the lack of response (Barberio et al., 2020).

With increasing knowledge regarding the pathobiology of IMIDs, multiple cytokines and receptors have been identified as targets for therapy, which include members of the cytokine type 1 and 2 families, the transforming growth factor-β family, and TNF-α and its receptors (Takeuchi, 2022). The cytokine, IL-6, plays an important role in mediating host defense in response to tissue injury or infection, and its expression is controlled stringently. An aberrant increase in IL-6 level exacerbates inflammatory conditions (Tanaka et al., 2014). IL-6 binds to and activates the JAK2-STAT3 signaling pathway. Recently, inhibitors of the JAK-STAT pathway, such as tofacitinib, have been found to be effective against IBIDs (Kim and Kim, 2021). However, adverse events associated with tofacitinib such as pulmonary embolism and herpes zoster infection have been reported. Furthermore,

this inhibitor is not equally effective against different IMIDs (Cordes et al., 2020). Over the last decade, the effects of phytochemicals on inflammation have been investigated extensively with encouraging results. Moon et al. (Moon et al., 2021) have summarized the effects of a few phytochemicals, such as phenols, terpenic compounds, alkaloids, and others, which are effective in managing IMIDs. Interestingly, these compounds also affected the JAK-STAT pathway. The expression of the *Bcl-2* family of genes is mediated via the JAK-STAT pathway (Kaloni et al., 2023), and the ratio of Bax to Bcl-2 has been reported to be a predictor of infliximab response. High Bax to Bcl-2 ratio increases apoptosis, which is mediated by increased caspase 3 expression (Aghdaei et al., 2018). Targeting of cytokines and their signaling has been a successful approach, although it is associated with non-response in many patients (~40−50%). Furthermore, in IMIDs, a plethora of cytokines are responsible for the pathological conditions and targeting multiple cytokines/receptors simultaneously may improve the rate of remission. Many natural products possess anti-inflammatory properties and can be utilized as CAMs for the betterment and comfort of patients. Natural products can be used as a standalone treatment, where they have been proven to be associated with low toxicity, or as a supplement.

4. Use Cases of Complementary and Alternative Medicine for Chronic Inflammatory Diseases

Several reports have indicated that dietary products, including spices, are involved in the management of IMIDs (Holleran et al., 2020; Zhao et al., 2021). Cinnamon (Cinnamomum spp.) has been widely used as a spice and has many healing properties which includes managing conditions such as chronic inflammation (Hong et al., 2012; Khedkar and Khan, 2023). The extract from cinnamon has several other beneficial effects, such as having anticancer and antimicrobial effects (Chen et al., 2022; Sharifi-Rad et al., 2021). Some reports have indicated that cinnamon can decrease the levels of low-density lipoprotein, total cholesterol, and serum triglycerides in diabetic patients (Silva et al., 2022). Hall et al. (Hall et al.,

2017) published a study on the use of natural health products (NHP) in treating rheumatological conditions in which he reported that ~36% of the study participants (out of 1,063) used NHP. For some NHP users (42%), the NHP recommendations came from the physicians, indicating the acceptance of these complementary and alternative therapies by patients and physicians alike, albeit to a limited extent. Natural products, including cinnamon, have been reported to alter several signaling pathways (Long et al., 2023). *Cinnamomum verum* essential oil has demonstrated ability to decrease the expression of Toll-like receptor-4 (TLR-4) in an artificially induced colitis model and has proved beneficial in cases of inflammatory bowel disease (IBD). Similar results have been observed for curcumin and many other natural products (Dejban et al., 2021). In another study, aqueous cinnamon extract (aCE) partially controlled bowel symptoms by downregulating tryptophan hydroxylase 1 in a model of irritable bowel syndrome (Yu et. al., 2022). It has also been reported that cinnamon extract acts as a pro-apoptotic agent, downregulating BCL-2, BcL-xL and Survivin. A recently published single-center study explored the adjuvant effects of purple corn supplementation on IBD remission. The results showed that purple corn supplementation reduced the expression of inflammatory biomarkers in patients with CD undergoing infliximab treatment and improved the infliximab response. However, this effect was not observed in patients with UC (Liso et al., 2022). Unfortunately, such studies are rare.

5. Cancer, Angiogenesis and Bevacizumab

During embryonic development new blood vessels are formed by a process known as angiogenesis. In addition, physiological activities which require regeneration of internal lining such as menstruation or organ lining repair also see active angiogenesis. Under normal conditions new blood vessels formation is tightly regulated by factors which promote and suppress angiogenesis (Liu et al., 2023). However, under neoplastic conditions an angiogenic switch is seen which results in predomination of pro-angiogenic factors (Al-Abd et al., 2017). Hypoxia, genetic alterations, tumor associated inflammation are some of the causes of angiogenic

switch which results in increased vasculature. This vasculature plays a significant role in tumor growth and metastasis. Because of uncontrolled angiogenesis, the blood vessels in active neoplastic cells and tumor microenvironment (TME) are poorly developed resulting in irregular blood circulation and often contribute to hypoxia which leads to further increase in pro-angiogenic factors and thus angiogenesis (Lugano et al., 2019).

Angiogenesis is a multistep, elaborate process which is the result of action of multitude of pro-angiogenic factors. Primary factor responsible for angiogenesis is VEGF, others being fibroblast growth factor (FGF), plate derived growth factor (PDGF), angiopoietins (Angs), epidermal growth factors (EGF), HIF-1, granulocyte colony-stimulating factor (G-CSF), interleukin 8 (IL-8), ephrins, MMPs etc. (Haibe et al., 2020). Folkman in 1971 identified angiogenesis as a potential target to suppress tumor growth (Lopes-Coelho et al., 2021). Much later, in 2006 FDA approved anti-VEGF therapeutic monoclonal antibody Bevacizumab (Avastin) for nonsquamous, non-small cell lung cancer (NSCLC) to be given with carboplatin and paclitaxel (Cohen et al., 2007). Currently, therapeutics against vascular endothelial growth factor receptor (VEGFR), VEGFR tyrosine kinase (RTKi) are also available albeit these therapies including bevacizumab have shown limited success as monotherapy. Reports indicate that there are multiple reasons for the limited success of anti-angiogenic inhibitors as monotherapy and include activation of alternate angiogenic pathways driven by factors other than VEGF, vascular co-option (cancer cells use existing blood vessels), increased pro-angiogenic factors production caused by hypoxia amongst others (Lopes-Coelho et al., 2021 and Saman et al., 2020).

This research work focuses on therapeutic monoclonal antibody Bevacizumab (BMAB), that binds to VEGF-A. BMAB neutralizes VEGF-A and thus blocks VEGFR-1/VEGFR-2 and VEGF-A interaction. Interaction between VEGF and its cognate receptor results in increased cell survival and angiogenesis through activation of various pathways. In addition to angiogenesis, VEGF-A activates pathways for cancer cell division, migration, and invasiveness. VEGF-A signaling

also leads to increase immune tolerance. (Gerriets and Kasi, 2019, Garcia et al., 2020). Per the article published in 2020, approximately more than 3,500,000 patients have been treated with BMAB in either combination or mono therapy. BMAB remains a first line of therapy in combination with radiotherapy for metastatic colorectal cancer (mCRC) since its approval. Similarly, patients of NSCLC when treated with BMAB and carboplatin plus paclitaxel observed better progression-free survival (PFS) and overall survival (OS) benefits. By reducing the glucocorticoid requirement, BMAB treatment has improved the lifestyle quality for Glioblastoma (GBM) patients (Garcia et al., 2020). The specific actions that help in bringing out the effect of BMAB treatment include vessel depletion (apoptosis of endothelial cells in tumor) and vessel normalization which helps delivery of anti-tumor drug (Mao et al., 2023). BMAB is approved for treatment of NSCLC, cervical cancer, metastatic renal cell carcinoma (mRCC), mCRC, ovarian, fallopian tube, and primary peritoneal cancer, glioblastoma, and hepatocellular carcinoma (HCC). BMAB is used as monotherapy for glioblastoma, for all other indications it is used in combination with a chemotherapeutic agent (Gerriets and Kasi, 2019).

Reportedly, treatment with BMAB for many approved indications resulted in better PFS but did not have any significant effect on OS (Papachristos and Sivolapenko, 2020). These observations may result from the fact that mechanism involved in neo-angiogenesis are not fully understood and suppressing VEGF mediated angiogenesis results in activation of alternate angiogenic pathways like PDGF and FGF as seen in patients under BMAB therapy which showed increased FGF and PDGF levels in plasma. Furthermore, targeting VEGF and PDGF or FGF pathways simultaneously has shown better clinical outcomes and increased OS rate (Lopes-Coelho et al., 2021). According to another study BMAB treatment resulted in angiogenesis independent adaptive processes called as vessel co-option and vascular mimicry to fulfill their oxygen and nutrients need (Elebiyo et al., 2022). In addition to activation of alternate vascular options and alternate angiogenic pathways, increased pro-angiogenic and pro-inflammatory activity has also been reported to contribute to resistance to BMAB therapy (Lopes-Coelho et al., 2021).

It is well known that VEGF is the primary promoter of angiogenesis, and treatment with anti-angiogenic molecules will be a corner stone in cancer therapies. However, owing to the complexity of angiogenesis, many researchers propose that combinatorial, sequential, or synergistic approaches to anti-angiogenesis therapy may benefit the patients who become resistant to anti-angiogenesis therapy (Elebiyo et al., 2022). There have been clinical trials with combinatorial strategy where HCC and metastatic NSCLC patients were given BMAB, immunotherapeutic drug atezolizumab and chemotherapeutic drug and results showed better PFS and OS than with bevacizumab plus chemotherapy alone (Socinski et al., 2018).

6. Alternate Angiogenesis Pathways and Bevacizumab Resistance

In addition to the VEGF (Vascular Endothelial Growth Factor) pathway, several other pathways play crucial roles in angiogenesis. Here are some key alternative pathways:

a) **Angiopoietin-Tie Pathway**: Angiopoietins (ANG1 and ANG2) bind to the Tie2 receptor on endothelial cells. ANG1 supports the maturation and stabilization of blood vessels, whereas ANG2 can destabilize vessels, increasing their sensitivity to VEGF (Lopes-Coelho et al., 2021).

b) FGF (Fibroblast Growth Factor) Pathway: Fibroblast growth factors (FGFs), especially FGF1 and FGF2, are strong angiogenic agents. They interact with FGF receptors (FGFRs) on endothelial cells, stimulating their proliferation and migration (Ayoub et al., 2024).

c) **PDGF (Platelet-Derived Growth Factor) Pathway:** Platelet-derived growth factors (PDGFs), including PDGFA and PDGFB, bind to PDGF receptors (PDGFRs) and play a key role in attracting pericytes and smooth muscle cells to support the stabilization of newly formed blood vessels (Lugano et al., 2019).

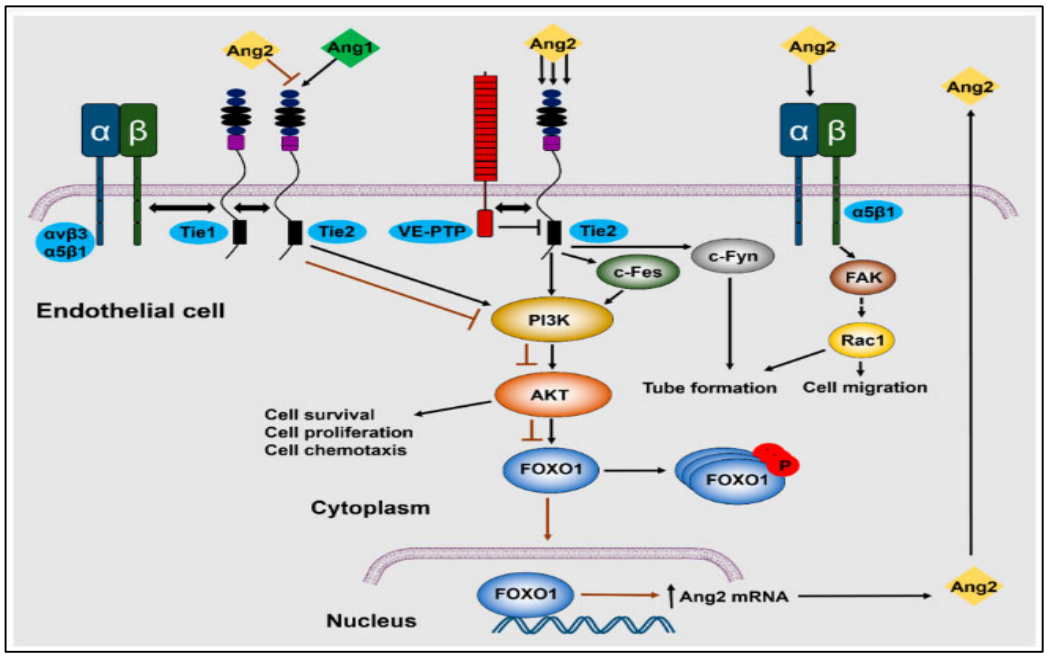
d) **HGF (Hepatocyte Growth Factor) Pathway:** Hepatocyte growth factor (HGF) interacts with the c-Met receptor, promoting the proliferation and migration of endothelial cells, which aids in the process of angiogenesis (Ayoub et al., 2024).

We examined the Angiopoietin-2 (Ang2) and Tie2 signaling pathway due to its crucial role in regulating vascular functions, especially in angiogenesis and vascular remodeling. Ang2, which interacts with the Tie2 receptor, can act as either an agonist

or antagonist, depending on the presence of other factors like VEGF. In the presence of VEGF, Ang2 facilitates angiogenesis by activating the Tie2 receptor, which in turn triggers downstream signaling pathways such as the PI3K/Akt pathway (Figure. 13). This activation promotes the survival and proliferation of endothelial cells, which are essential for the formation of new blood vessels (Kim et al., 2006)

Figure 13: Ang2 signaling pathway.

Reference: Akwii and Mikelis, 2021



When bevacizumab is present, the Angiopoietin-2 (Ang2) pathway experiences notable changes. Bevacizumab blocks VEGF signaling, a crucial factor in angiogenesis, thereby reducing the signals that typically encourage blood vessel formation and maintenance. This inhibition can lead to an increase in Ang2 as a compensatory response. Ang2, which binds to the Tie2 receptor, can then worsen vascular instability, and increase vascular permeability (Liu et al., 2022). This occurs because Ang2 can act as either an agonist or antagonist of Tie2, and without VEGF, it often promotes vessel regression and destabilization (Leong et al., 2020).

In inflamed or pathological environments, Ang2's role becomes even more intricate. For example, in the presence of inflammatory mediators or hypoxic conditions, Ang2 can switch from being an antagonist to an agonist of Tie2, thereby promoting

angiogenesis and vascular remodeling. This switch is influenced by the cleavage of the Tie1 receptor, which modulates Ang2's activity. Additionally, Ang2 interacts with integrins and other receptors, further influencing endothelial cell behavior and adding to the complexity of its role in vascular biology (Felcht et al., 2012).

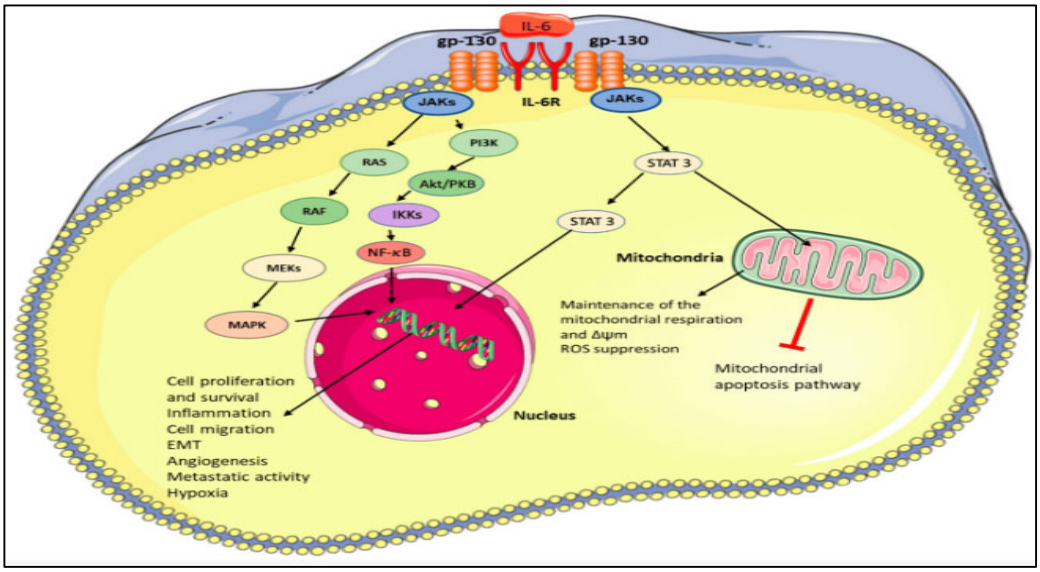
Understanding this context-dependent behavior of Ang2 is crucial for developing effective combination therapies. By targeting both VEGF and Ang2 pathways, it is possible to enhance the efficacy of anti-angiogenic treatments and overcome resistance mechanisms that tumors may develop in response to single-agent therapies (Akwii and Mikelis, 2021).

7. Other molecules contributing to angiogenesis.

C-X-C motif chemokine 5 (CXCL5), one of the several pro-inflammatory cytokine, is integral to the process of angiogenesis. It exerts its effects by binding to the CXCR2 receptor, initiating a series of signaling pathways that encourage the development of new blood vessels (Deng et al., 2022). In cancer, CXCL5 is frequently overexpressed, which enhances tumor angiogenesis, thereby providing the tumor with essential nutrients and oxygen for growth and metastasis. Moreover, CXCL5 can attract immune cells to the tumor microenvironment, promoting tumor progression and creating an immunosuppressive environment (Zhang et al., 2020). In one of the published reports, meta-analysis study revealed that higher CXCL5 is associated with poor OS, PFS and RFS in various types of cancers (Hu et al., 2018). One study reported that CXCL5 is upregulated in lung cancers; leads to increased expression of PD-L1 and also promotes neutrophil chemotaxis, which results in immune evasion by impairing functional activity of $CD8^+$ T-cells. Consequently, CXCL5 is being investigated as a potential therapeutic target in cancer therapy. Synergy between immune check point inhibitors and CXCL5 inhibitors have been tested *in vivo* and has given encouraging results (Sun et al., 2024).

IL-6, a versatile cytokine, can both promote and suppress inflammation. It directly influences cancer cells, aiding their growth and survival (Figure. 14). Higher levels of IL-6 in the blood have been linked to poorer outcomes for cancer patients, primarily due to its direct effects on cancer cells (Wang et al., 2017)

Figure 14: Influence of IL-6 signaling on cancer development and metastasis formation. Reference: Rašková et al., 2022

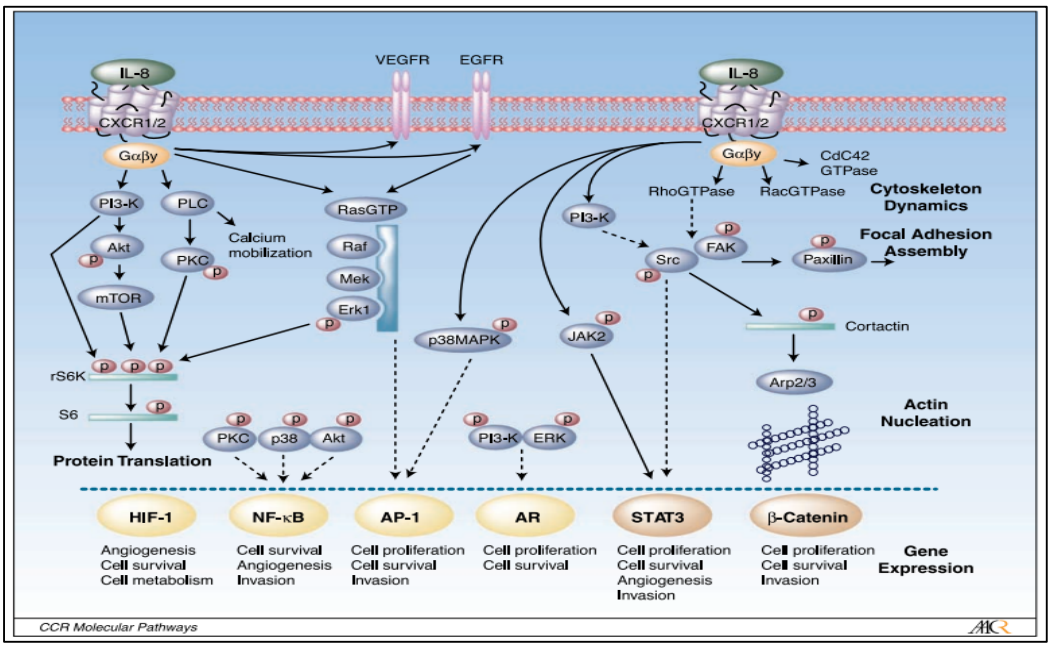


The alternate pathway for IL-6 results from the soluble interleukin-6 receptor (sIL-6R) and can contribute to tumor growth in cancers associated with chronic inflammation, like colitis-associated cancer. sIL-6R binds to circulating IL-6, forming a complex with gp130. This complex activates Janus kinases (JAKs), which initiate signaling pathways that promote cell growth, survival, and metastasis. One such pathway is the JAK/STAT3 pathway, which is strongly linked to cancer progression. IL-6 significantly contributes to the neoplastic microenvironment by influencing both tumor cell intrinsic signaling and immune cell function. Within tumor cells, activated STAT3 binds to regulatory DNA sequences, upregulating genes that promote cell proliferation, survival, and angiogenesis. These include cyclin D1 and cMYC (for cell growth), Bcl-XL and Bcl-2 (for cell survival), and VEGF (for blood vessel growth). Beyond its direct effects on tumor cells, IL-6 has a significant impact on the immune response. Although neutrophils, and other T cells subtypes immune cells seem to infiltrate the tumor, IL-6 can still impair their anti-tumor activity. This immune suppression is partially attributed to IL-6's ability to increase the number and activity of regulatory T cells and myeloid-derived suppressor cells. These immunosuppressive cell populations further contribute to the immunosuppressive phenotype of the neoplastic microenvironment, hindering

the effectiveness of anti-tumor immune responses (Rašková et al., 2022). After bevacizumab treatment, IL-6 levels can exhibit different patterns based on the condition being treated and the patient's response. In neovascular age-related macular degeneration (nAMD), IL-6 levels in the aqueous humor tend to increase post-treatment, leading to poorer response to bevacizumab (Connolly et al., 2024). In cancer therapy, particularly for metastatic renal cell carcinoma (mRCC), high baseline IL-6 levels have been linked to better OS when treated with bevacizumab and interferon-alpha (IFNα) simultaneously, although post-treatment IL-6 dynamics can vary. Generally, as an anti-VEGF therapy, bevacizumab can influence inflammatory pathways, causing fluctuations in IL-6 levels depending on the body's inflammatory response. Monitoring IL-6 levels is crucial for optimizing treatment strategies and improving therapy results (Nixon et al., 2021).

Interleukin-8 (IL-8), also known as CXCL8, is a chemokine with a significant role in both immune responses and cancer progression. It mainly functions to attract neutrophils to areas experiencing inflammation, but its functions extend to tumor biology, where it contributes to cancer development and metastasis. IL-8 is often upregulated in solid tumors such as prostate, and colorectal cancer, and is associated with poor patient outcomes (Waugh & Wilson, 2008). In cancer, IL-8 signaling is mediated through its receptors such as Interleukin-8 receptor A, which are expressed on both tumor cells and TME specific cells, such as endothelial cells. The activation of these receptors triggers several intracellular pathways, including PI3K/AKT, MAPK/ERK, and NF-κB, which promote tumor cell growth and migration (David et al., 2016) (Figure. 15). IL-8 also supports angiogenesis by upregulating pro-angiogenic factors like VEGF, facilitating the supply of nutrients to growing tumors (Sparmann & Bar-Sagi, 2004).

Figure 15: Signaling pathways activated by IL-8. Reference: Waugh & Wilson, 2008



Blocking VEGF signaling with bevacizumab can result in compensatory mechanisms, including increased expression of alternative pro-angiogenic factors like IL-8. Research suggests that tumors may upregulate IL-8 levels as a response to VEGF inhibition, potentially maintaining angiogenesis through alternative pathways and promoting tumor survival (Gyanchandani et al., 2013). This elevation in IL-8 can contribute to resistance against bevacizumab therapy by supporting new blood vessel formation and facilitating immune evasion and metastasis. In metastatic colorectal cancer (mCRC), IL-8 has been studied as a potential prognostic marker. Higher serum IL-8 levels have been linked to worse overall survival in patients receiving bevacizumab, suggesting that IL-8 could be useful in predicting treatment outcomes for mCRC patients. Elevated IL-8 may indicate a reduced effectiveness of bevacizumab therapy (Suenaga et al., 2020). It is interesting to note that IL-6 and IL-8 can both trigger STAT3 signaling via JAK2, and when this signaling occurs simultaneously, it leads to a marked increase in cell migration compared to when either IL-6 or IL-8 acts alone. The combined blockade of IL-6 and IL-8 receptors using Tocilizumab (an IL-6 receptor inhibitor) and Reparixin (an inhibitor of CXCR1) significantly lowers the expression of matrix metalloproteases

in a mouse model of MDA-MB-231 breast cancer, reducing the occurrence of liver and lung metastasis (Rašková et al., 2022).

Intercellular Cell. Adhesion Molecule-1 (ICAM-1) is a cell surface glycoprotein that is upregulated in numerous cancer types, including breast cancer, colorectal cancer, and melanoma. Under normal conditions it plays a critical role in various biological processes, including cell-cell interactions, cell signaling, and immune responses. Tumor cells also express ICAM-1, which affects their functions such as apoptosis, movement, invasion, and the formation of new blood vessels. The binding of ICAM-1 to its ligand can help tumor cells stick to the vascular endothelium, aiding in metastasis. The level of ICAM-1 expression is linked to the cancer's malignancy (Figure. 16a) (Qiu et al., 2022). Reports indicate pro-oncogenic role of ICAM-1 in colorectal cancer and a strong correlation between elevated ICAM-1 expression and poor patient outcomes (Lim et al.,2022). Studies done by Rosette et al. show that metastatic potential of five of the breast carcinoma cell lines significantly correlated with ICAM-1 expression (Rosette et al, 2005). Bevacizumab can increase ICAM-1 expression in tumor tissues. This rise in ICAM-1 is linked to greater infiltration of immune cells, suggesting a potential role in enhancing the immune response against tumors (Patrycja Nowak-Sliwinska et al.,2022). It seems that ICAM-1's role in cancer is multifaceted. It can both promote tumor growth and aid in host defense. The net effect depends on the specific cancer type and other factors in the tumor microenvironment. Understanding this complex interplay is crucial for developing effective cancer treatments (Haydinger et al., 2023). Targeted Therapies have emerged as promising strategies for treating ICAM-1-positive cancers. Chimeric Antigen Receptor (CAR) T-cell therapy, a revolutionary approach in cancer immunotherapy, has shown remarkable efficacy against ICAM-1-expressing tumors. Studies have demonstrated that ICAM-1-specific CAR-T cells can effectively target and eliminate breast cancer cells (Wei et al., 2020).

Vascular Cell Adhesion Molecule-1 (VCAM-1) is a protein expressed on endothelial cells and plays a crucial role in inflammation and immune responses (Figure. 16b). It also plays a significant role in cancer progression and metastasis. VCAM-I has been reported to facilitate trans-endothelial migration and subsequent metastasis. For

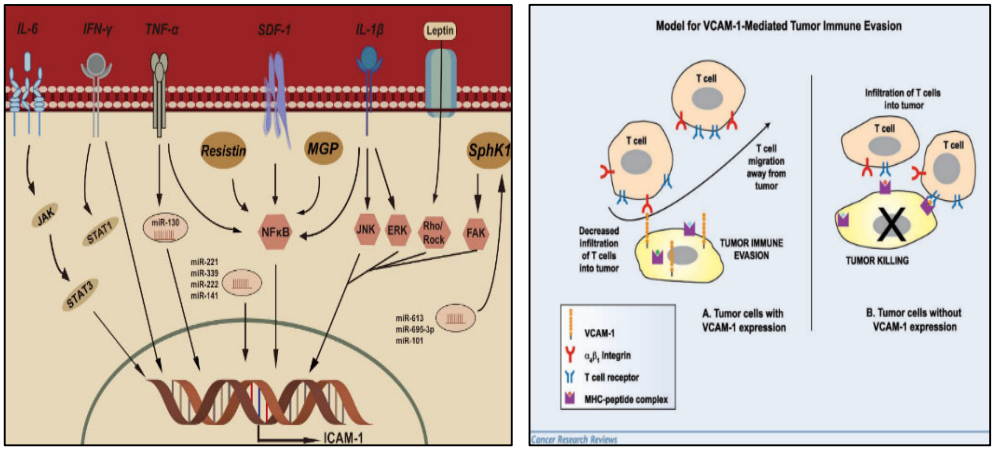
instance, in colorectal cancer, VCAM-1 has been shown to induce epithelial-mesenchymal transition (EMT), enhancing the invasive and metastatic potential of tumor cells (Zhang et al., 2020). Research has highlighted the significant role of VCAM-1 in cancer angiogenesis and reports indicate that higher VCAM-1 levels correlate with higher micro vessel density (Kong et.al., 2018). It has been reported that ICAM-1 and VCAM-1 expression in endothelial cells is modulated by JAK2/STAT3 pathway when activated by IL-6 in inflammatory vascular disease known as Buerger's disease (Z. Wei et al., 2018).

Figure 16a: A schematic diagram illustrating the potential mechanisms of ICAM-1 expression in CRC. Reference: Qiu et al., 2022

Figure 16b: Model for VCAM-1–mediated tumor immune evasion.

Reference: Wu, 2007

16a 16b



The JAK2/STAT3 pathway is a critical signaling cascade that transduces extracellular cytokine and growth factor signals to the nucleus. Aberrant activation of this pathway frequently occurs in diverse malignancies, contributing to tumorigenesis, angiogenesis, and metastasis (Ayele et al., 2022). The IL-6/JAK2/STAT3 axis is a well-characterized and pivotal signaling sequence in modulating cellular functions. STAT3 influences the expression of basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMP-2 and MMP-9), and VEGF, a key inducer of angiogenesis (Dutzmann et al., 2015). STAT3 also plays a global role in helping tumor cells adapt to hypoxic conditions by

spatially associating with HIF1α, which is necessary for the full transcriptional activation of HIF1α-regulated genes under low oxygen. Several drugs that inhibit STAT3 signaling have been approved by the FDA, such as JAK- inhibitor tofacitinib, and anti-IL-6 receptor therapeutic mAb, tocilizumab, for the treatment of rheumatoid arthritis conditions (Thomas et al., 2015).

8. Use Cases of Complementary and Alternative medicine for Cancer

In addition to ongoing efforts in developing combinatorial or synergistic therapies using immuno or chemo therapeutic drugs, there is considerable ongoing research on use of natural products in managing cancer. Norat et al. in 2015 published a (European Code against Cancer 4th Edition: Diet and cancer) report that highlighted the role of plant based products in managing cancers (Norat et al., 2015). Plant extracts rich in polyphenols (for example from grape seed extracts, berries extract etc.) have been shown to decrease angiogenesis by variety of mechanisms including decreased expression of VEGF, angiopoietins, and HIF-1. The mechanism is proposed to be via decrease in molecules like PI3K and ERK1/2 (Chojnacka and Lewandowska, 2019). Resveratrol has been shown to affect angiogenesis by inhibiting VEGF and FGF2 mediated MAPK activation (Mossenta et al., 2019). Curcumin present in turmeric is reported to have anti-angiogenic properties that are mediated via decrease in VEGF-A and MMP-9 expression. It is also reported to affect the NF-κB and Mammalian target of rapamycin (mTOR) pathway (Rajasekar et al., 2019). Several natural products have also been tested for their synergistic effect with VEGF inhibitors. Gao et al. demonstrated that curcumin and bevacizumab work synergistically to inhibit HCC in rats and this action is mediated by Kirsten rat sarcoma viral oncogene homolog (KRAS) pathway [Gao et al., 2014]. A study investigated the potential therapeutic benefits of *Cinnamomum japonicum* cinnamon extract in a cellular model of intestinal inflammation. The researchers found that cinnamon subcritical water extract (CSWE) significantly attenuated the expression of inflammatory mediators (IL-6, TNF-α, NF-κB). Furthermore, CSWE inhibited the phosphorylation of NF-κB pathway components, suggesting its ability to regulate inflammation-related signaling (Kim and Kim, 2019). Studies have shown that cinnamon can reduce ICAM-1 expression, which

is beneficial for managing inflammation and related conditions. For instance, a randomized controlled trial found that daily cinnamon supplementation significantly lowered ICAM-1 levels in patients with type 2 diabetes (Mirmiran et al., 2019). Our lab has also shown that aqueous extract of cinnamon as well as its purified bioactive, trans-cinnamaldehyde can decrease ICAM mRNA in an in vitro system (Khedkar and Khan, 2024).

CHAPTER 3

RESEARCH OBJECTIVES

3.1 Research Objectives

1. Identification of cinnamon species by HPLC and DNA based methods.
2. To study the synergistic effect of cinnamon in conjunction with anti-inflammation biotherapeutic infliximab in an *in vitro* assay
3. To study the synergistic effect of cinnamon in conjunction with anti-cancer bio therapeutic bevacizumab in an *in vitro* assay
4. To study the downstream signal transduction pathway modulation by qRT PCR

CHAPTER 4

SIGNIFICANCE OF THE STUDY

4.1 Significance of the study

Cancer and Inflammation remains a significant global health concern due to the challenges and side effects associated with conventional and targeted treatments, which often yield poor clinical outcomes for some patients. Targeted treatments using agents like monoclonal antibodies have revolutionized cancer therapy by specifically targeting cancer cells or their environment, resulting in lower toxicity compared to traditional chemotherapy (Brennan and Smith, 2022). However, issues such as resistance mechanisms and low complete response rates necessitate a combination approach to enhance biotherapy effectiveness (Mantovani et al., 2008).

The significance of this study lies in its ability to bridge traditional remedies and modern treatment strategies by leveraging the synergistic effects of cinnamon and biotherapeutics, particularly for cancer and chronic inflammatory conditions. This research goes beyond identifying the pharmacologically active components of cinnamon by focusing on the molecular interactions involved, laying the groundwork for a novel therapeutic paradigm that integrates natural compounds into precision medicine. Furthermore, the insights gained from this study could help develop unique treatment plans tailored to patient characteristics, leading to optimal therapeutic outcomes, fewer side effects, and improved quality of life. Based on preclinical research and the transition of these findings into clinical practice, the therapeutic potential of cinnamon as an adjuvant to biotherapeutic agents could enhance the quality of life for patients with complex and challenging conditions.

This research could significantly impact clinical practice in several ways:

A. **Enhanced Treatment Efficacy:** By demonstrating how cinnamon can boost the effectiveness of targeted biotherapeutics like monoclonal antibodies, this study could lead to more effective treatment protocols for cancer and inflammatory diseases.
B. **Reduced Drug Resistance:** The potential use of cinnamon as a complementary and alternative medicine (CAM) could help in reducing drug resistance, making treatments more sustainable over time.

C. **New Therapeutic Combinations:** The insights gained from this research might pave the way for new therapeutic combinations that could be more effective than current treatments, offering better outcomes for patients.
D. **Foundation for Clinical Trials:** The preclinical findings from this study could serve as a basis for future clinical trials, potentially leading to new, evidence-based treatment options in oncology and inflammatory diseases.

Overall, this research holds the promise of improving patient care by introducing more effective, personalized, and sustainable treatment strategies.

CHAPTER 5

MATERIALS & METHODS

5.1 Materials

A. Cinnamon

The powder of *Cinnamomum cassia* was acquired from a local distributor, namely Carmel Organics, located in Madhya Pradesh, India. The powder of C. loureiroi was sourced from a distributor in Kirkland, D.C, USA while that of *C. zeylanicum* was sourced from Sorich Organics, located in Noida, India. For HPLC the grape seed extract from Masquelier's TRU-OPCs (Nature's Way, WI, USA) was acquired online.

. **Figure 17: Cinnamon used in this study**

B. Monoclonal antibodies

Infliximab and Bevacizumab were procured from a nearby pharmacy located in India.

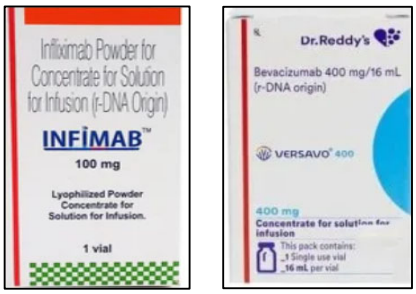


Figure 18. Infliximab and Bevacizumab

C. Cell lines and cell culture materials

a) Cell lines for Infliximab assay

The U937 (ATCC CRL-1593.2) cell line and L929 (ATCC CCL-1) cell lines were acquired from the American Type Culture Collection (ATCC) located in Manassas, Virginia, United States. The L929 cells were cultured in aseptic conditions using Eagle's minimal essential medium or EMEM (M4655, Sigma), whereas U937 cells

were cultured using RPMI-1640 or Roswell Park Memorial Institute-1640 medium (M8758, Sigma). 10% fetal bovine serum or FBS (10082-147, Gibco) as well as 1% streptomycin or penicillin from Sigma-Aldrich company (P4333) was used for enriching both the media. Both cultures were incubated at 37°C in an incubator which maintained 5% CO_2 and 95% relative humidity.

b) Cell lines for Bevacizumab assay

Cell culture reagents were obtained from Corning, located in New York, USA. The normal human primary umbilical vein endothelial cells (HUVEC) (PCS-100-010) cells were purchased from the ATCC (Manassas, VA, USA). HUVEC cells were cultured under aseptic conditions in endothelial cell basal medium with supplement mix (growth media) (C22210 and C39215; Promocell, Heidelberg, Germany). Growth media was supplemented with 1% antibiotic antimycotic Solution (A5955, Sigma). The cell cultures were maintained in a 37 °C incubator at 5% CO_2. For trypsin neutralization, endothelial basal media was mixed with 10% HI FBS (neutralization medium) (10082147; Thermo Fisher Scientific, Waltham, MA, USA) and 1% antibiotic antimycotic solution. Assay medium for dilution of bevacizumab, cinnamon extract and cells was prepared by mixing endothelial basal media with 2% HI FBS, 1% antibiotic antimycotic solution and 90 µg/mL heparin sodium (sc-203075, Santa Cruz Biotechnology, Dallas, Texas, USA).

D. <u>Solvents, reagents, and columns</u>

a) **DNA based identification**:

Genomic DNA was isolated using the NucleoSpin Plant II kit (Cat # 740770.250, Takara bio). TaqDNA polymerase (M0273), dNTP (N0447S) were from NEB. PCR consumables were from Thermofisher. Agarose was from Promega (V3125), Ethidium bromide from Thermofisher (17896), TE buffer from Sigma (93283), DNA ladder from NEB (N3231S and N05505), gel loading dye from NEB (B7024S) and TAE buffer from Invitrogen (15558-026). Primers were ordered from Bioserve India.

Table 2: Primer sequence for Cinnamon identification

	Primer sequences 5' to 3'
18S/5.8S	(F) TCATTGTCGTCCTAGAACCA
	(R) TCAAAGACTCGATGGTTCA

b) HPLC analysis:

Acetonitrile (HPLC grade) and Trifluoroacetate (HPLC grade) were procured from Fisher Scientific (United Kingdom). Column used for RP-HPLC was a C18 base deactivated reverse phase column [Prontosil 120-5-C18 H (250×4.6 mm); LC16242; 1747].

E. Reagents for synergy experiments

TNF-α (AB155699, Abcam, Waltham, MA, USA), VEGF165 (293-VE, R&D Systems, Minneapolis, USA), Caspase 3/7 glo reagent (G8090, Promega, Madison, WI, USA), trypsin-EDTA (25200-056, Gibco, Billings, MT, USA), eugenol (E51791, Sigma), trans-cinnamaldehyde (TCA; C80687, Sigma, Burlington, MA, USA), cinnamic acid (CA; 8.00235, Sigma), Alamar Blue (DAL1100, Invitrogen, Waltham, MA, USA), phosphate-buffered saline (PBS)-pH 7.2 (20012-027), sterile filters (0.4 µm pore size, HNWP04700, Merck Millipore Burlington, USA), and actinomycin D (A9415, Sigma).

F. RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Following the instructions provided by the manufacturer, the total RNA extraction was performed using RNeasy mini kit (74101, Qiagen, Hilden, Germany). To determine the RNA concentration, Take3 low-volume plates were utilized on a microplate reader. Protoscript II (E6560, NEB, Ipswich, Massachusetts, United States) was utilized in order to conduct cDNA synthesis in accordance with the product instructions. PowerUp™ SYBR™ Green master mix (A25742, Thermo Fisher Scientific, Waltham, Massachusetts, United States) was utilized for quantitative real-time PCR. Primers were ordered from Bioserve India.

G. Equipments Used

Table 3: List of equipments

Equipment used
CO_2 incubator (NUAIRE)
Biosafety cabinet (Lab concho)
Dual Zone refrigerators
PCR machine (Biorad)
Real-time PCR machine ABI7500 (Applied Biosystem)
Pipettes (Thermofisher)
Plate reader (Biorad)
Gel imager (Biorad)
GraphPad prism V10 for data analysis

5.2 Methods

A. Genomic DNA extraction and PCR

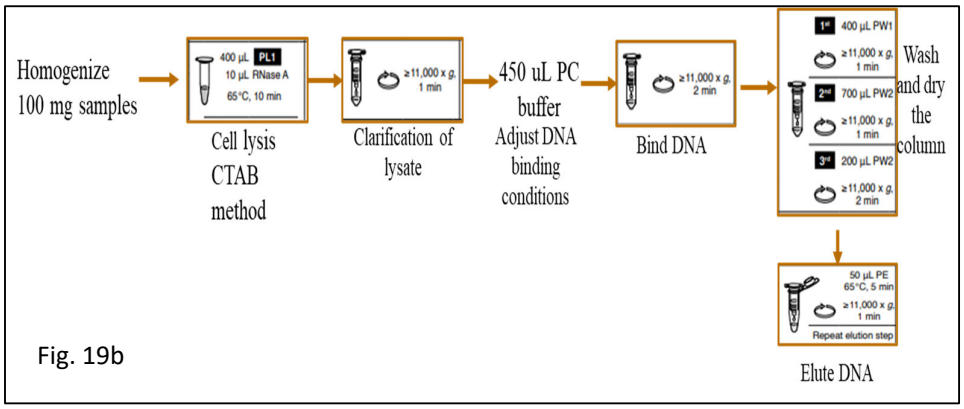


Figure 19: Flow chart for DNA extraction:

Step-by-step method -

1. Samples were homogenized by mechanical treatment (using Tissue Lyser II, 4–5 beads - diameter: 7 mm, 30 Hz, 1 min X 3)
2. Lysis buffer provided in the kit was used for DNA extraction
3. Filtration columns provided in the kit was used for clarifying the lysate.

4. Clarified flowthrough was mixed with binding buffer provided in the kit.
5. Above solution was then loaded to DNA binding column and spun, followed by washing of column. Genomic DNA at this step was bound to the column.
6. Sterile and nuclease free water was used for elution of genomic DNA.
7. 25 µL PCR reaction was setup for each sample. Negative control, without template, did not give any PCR product.
8. PCR product was run on a 2% agarose gel with 0.5mg/mL ethidium bromide. Gel was visualized in Bio-Rad gel doc system under UV light.

Table 4: PCR details –

1. 98° C – 5 mins
2. 98° C – 30 sec
3. 54° C – 30 sec — 30 cycles
4. 72° C – 30 sec
5. 72° C – 5 mins

	Sample	**NTC**
Materials	**Volume (in µL)**	**Volume (in µL)**
Template DNA	1.0	NA
Forward Primer (10 µM)	0.5	0.5
Reverse Primer (10 µM)	0.5	0.5
10X Standard Taq Reaction Buffer	2.5	2.5
10 mM dNTPs	0.5	0.5
Taq DNA Polymerase	0.125	0.125
Nuclease free water	19.875	19.875
Total reaction volume	**25.0**	**25.0**

Preparation of 1X TAE buffer (running buffer):

Added 100 mL of 10 X TAE buffer to 900 mL of Milli-Q-water.

Preparation of 2% Agarose Gel:

To prepare 2% gel, 1g agarose was resuspended in 50 mL running buffer. Agarose was melted using the water bath with frequent agitation. When the agarose solution

reaches to 50- 600, 80 µL of 0.625 mg/mL ethidium bromide solution equivalent to 0.5 µg/mL was added and mixed.

The agarose solution was then poured into a casting tray and allowed for solidification.

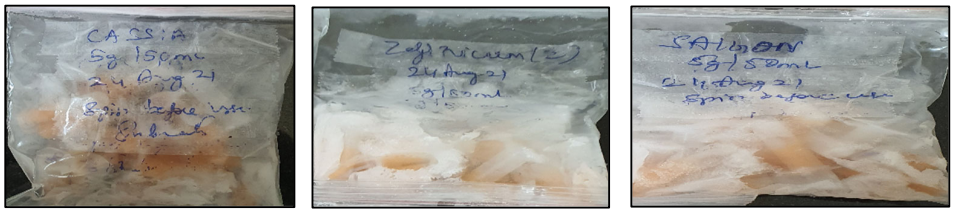
Agarose gel electrophoresis:

1. For electrophoresis, the buffer tank was filled with 1X TAE buffer (enough to fill the tank and immerse the gel).
2. 25 µL of each PCR amplification product were mixed with 5 µL of gel-loading dye purple (6X) and loaded into the wells of the gel.
3. 10 µL of 1kb plus or 100 bp DNA ladder was loaded in the gel.
4. The gel was allowed to run at 100 V until the dye purple front almost reached the end of the gel.
5. Gel was removed from the casting tray and observed in the Gel Imager system under UV light for the presence/absence of bands and the image was saved.

B. Preparation of aCE

5 g ground cinnamon (*C. zeylanicum, C. cassia, and C. loureiroi*) bark powder was dissolved in 50 mL water (70°C for 1 h) to obtain 100 mg/mL extracts. All extracts were centrifuged (12,000 rpm for 10 min) to remove the insoluble components. The supernatant was filtered (0.4 µm pore size), aliquoted, and stored at -80°C.

Figure 20: aCE aliquots



C. RP-HPLC characterization of cinnamon extracts

The aqueous cinnamon extract (aCE) was studied using reverse phase high-performance liquid chromatography (RP-HPLC). Grape seed extract from Masquelier's TRU-OPCs utilized as a qualitative reference for procyanidins, given its high procyanidin concentration. Purified trans-cinnamaldehyde, cinnamic acid and eugenol were used as reference. RP-HPLC conditions used are as follows -

Figure 21: RP-HPLC conditions

RP- HPLC Condition

Column: C18 base deactivated reverse phase column

[Prontosil 120-5-C18 H (250×4.6 mm); LC-16242; 1747]

Mobile phase A: Water + TFA (0.005% v/v)

Mobile Phase B: Acetonitrile (75% v/v) + TFA (0.005% v/v)

Run Time: 35 min

Flow rate: 1.0 mL/min

Column temperature: 25° ± 5

Sample temperature: 5° ± 3

Detection wavelength: 265 nm

Gradient Condition

Time (min)	Solution A (%)	Solution B (%)
0	75	25
1	75	25
21	62	38
30	60	40
35	60	40

D. *In vitro* cell based assay - Infliximab.

a) L929 and U937 cell viability assay

The L929 cells were cultured in a 75 cm2 flask in EMEM supplemented with 10% FBS. The assay medium contained 3% FBS in EMEM. The L929 cell suspension was prepared in assay media by trypsinizing the cell monolayer. Fifty microliters of 0.75 · 106 cells/mL (37,500 cells/well) were plated in each well of the 96-well plates. aCE was serially diluted to eight different concentrations ranging from 33.3 to 0.26 mg/mL (final concentrations). 50 µL of aCE and actinomycin D was added to treated and actinomycin control wells. Control wells without aCE or actinomycin D were also established.

The U937 cells were cultured in RPMI-1640 medium supplemented with 10% FBS. The assay medium contained 2% FBS. In total, 25 µL of 1.2 $X10^6$ cells/mL (30,000 cells per/well) was plated in each well of the 96-well plates. aCE was serially diluted to eight different concentrations ranging from 33.3 to 0.26 mg/mL (final concentrations). 25 µL of aCE was added to the treated wells. Control wells without aCE were also established.

The plates were incubated at 37°C in the presence of 5% CO2 for 18−20 h, followed by the addition of 20 µL Alamar Blue to the wells. The plates were further incubated for 6−7 h at 37°C in the presence of 5%. CO_2. The color change of alamar blue reagent was measured using a Synergy microplate reader (BioTek, Winooski, VT, U.S.) at 570 nm and 600 nm. The average absorbance of the cell culture medium alone (background) at 600 nm was subtracted from that at 570 nm. The background-

subtracted absorbance at 570 nm was plotted against the concentration of the test compound. Viability assays with TCA, CA, and eugenol were performed like that described above (the concentration range was 1–0.65 mg/mL).

b) aCE-infliximab synergy experiments

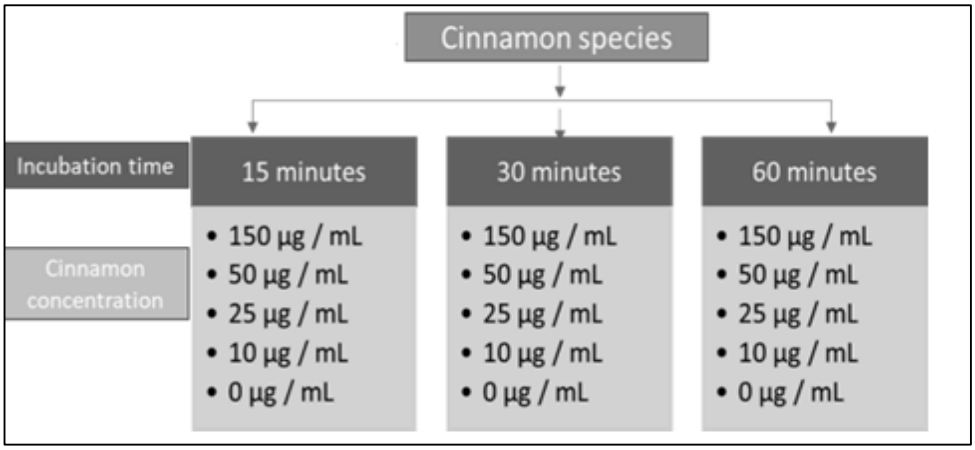


Figure 22: aCE incubation time optimization plan

The L929 cell suspension was prepared by trypsinizing the cell monolayers. In total, 25 µL of the cell suspension (1.5×10^6 cells/mL or 37500 cells/well) was added to each untreated, treated, TNF-α control and cell control labelled wells in a transparent 96-well microplate plate and incubated for 20 min at 37°C in an atmosphere of 5% CO_2. Twenty-five microliters of aCE or bioactives were added to the cells (labelled treated wells) to final concentrations of 150, 50, 25, and 10 µg/mL or 40, 32, 16, 8, and 4 µg/mL (in duplicates), respectively. Each set was incubated for 15, 30, or 60 min. Twenty-five microliters of the assay medium were added to the control wells (cell control, TNF-α control) and untreated wells in the assay plate. Simultaneously, 100 µL of eight different concentrations of infliximab (30 ng to 0.23 ng/mL, final concentrations) were added to the wells marked as treated and untreated on separate neutralization plate. One hundred microliters of TNF-α (20 IU/mL) was added to each treated, untreated, and TNF-α control well and mixed five times (this plate did not contain cells, only infliximab and TNFα in 1:1 volume ratio for neutralization) and incubated for 1 h at 37°C. One hundred microliters of the assay media were added to the TNF-α control wells and 200 µL to the cell control wells. After 15, 30, or 60 min of incubation of aCE with cells, 50 µL of the neutralization mixture was transferred to the wells in the assay plate

marked as treated and untreated. Fifty microliters of 4 µg/mL actinomycin D were added to the treated, untreated, and TNF-α control wells, and 100 µL assay medium was added to the control wells. The cell plates were incubated at 37°C in the presence of 5% CO2 for 18−20 h, followed by the addition of 20 µL alamar blue to the wells and further incubation for 6−7 h at 37°C in an atmosphere of 5%. CO2. The color change was read using a Synergy microplate reader (BioTek) at 570 nm and 600 nm. The average absorbance value of the cell culture medium alone (background) at 600 nm was subtracted from that of the experimental wells at 570 nm. The background-subtracted absorbance at 570 nm was plotted against the infliximab concentration to determine the shift in the half maximal effective concentration (EC_{50}) in the presence of aCE. Similar study was carried out for bioactives (15 minutes preincubation).

The U937 cell suspension was prepared by harvesting the cells, and then centrifuging at 125 rcf for 5 minutes, resuspending, and counting them in the assay medium. In total, 12.5 µL of the cell suspension (2.4×10^6 cells/mL or 30,000 cells/well) was added to each untreated, treated, TNF-α control and cell control wells in an opaque white 96-well microplate plate (assay plate) and incubated for 10 min at 37°C in an atmosphere of 5% CO_2. aCE (12.5 µL) was added to the cells (labelled treated wells) to final concentrations of 150, 50, 25, and 10 µg/mL, or 12.5 µL bioactives were added to cells to final concentrations of 40, 32, 16, 8, and 4 µg/mL (in duplicates). Each set was incubated for 15 min. The assay medium (12.5 µL) was added to the control wells (cell control, TNF-α control), and untreated wells in the assay plate. Simultaneously, 50 µL of eight different concentrations of infliximab (50 ng to 1.14 ng/mL) were added to the wells labelled as treated and untreated on a separate neutralization plate. Fifty microliters of TNF-α (20 IU/mL) was added to each treated, untreated, and TNF-α control wells and mixed five times (this plate did not contain cells, only infliximab and TNFα in 1:1 volume ratio for neutralization) and incubated for 1 h at 37°C. Fifty microliters of the assay media were added to the TNF-α control wells and 100 µL to the cell control wells. After incubation, 25 µL of the mixture was transferred to the wells marked as treated and untreated in the assay plate, and the plate was incubated for 2.5h at 37°C in the presence of 5% CO_2. Fifty microliters of the caspase 3/7 glo reagent were added to

the treated, untreated, TNF-α control and cell control wells, and the plates were further incubated at 30 minutes at room temperature before taking the reading. Luminescence was measured using a microplate reader.

c) Assay for Gene expression

L929 cells were added to 6-well plates (1×10^6 cells per well in 600 µL) in EMEM + 10% FBS (v/v) and incubated for ~18 h under sterile conditions. The spent medium was discarded, and the cells were rinsed with EMEM (200 µL/well). In a separate plate, infliximab EC100 (30 ng/mL, final concentration) was mixed with TNF-α (20 IU/mL, final concentration) and incubated for 1 h at 37°C. aCE samples and concentrations which showed significant synergy (p-value < 0.05) and reproducibility across days in the synergy experiments were selected for gene expression assays. The cells were preincubated with 300 µL of 50 and 25 µg/mL aCE (final concentrations) or 40 and 32 µg/mL (final concentrations) of bioactive for 15 min, followed by the addition of an equal volume of the neutralized TNF-α + infliximab (EC100 mix) and further incubated for 30, 60, or 120 min. Cell control and infliximab control wells (EC100 mix) were also set up. At each time point, the cells were harvested and used for RNA extraction and cDNA preparation. 120 min time point was selected based on data reproducibility. Each sample was analyzed in duplicates.

A U937 cell suspension was prepared in the assay medium and added to 6-well plates (1×10^6 cells per well in 600 µL). In a separate plate, infliximab EC100 (50 ng/mL, final concentration) was mixed with TNF-α (20 IU/mL, final concentration) and incubated for 1 h at 37°C. aCE samples and concentrations with a p-value < 0.0001 in the synergy experiments were selected for gene expression assays. Cells were preincubated with 300 µL of 50 and 25 µg/mL (final concentrations) aCE or 40 and 32 µg/mL (final concentrations) bioactive for 15 min, followed by the addition of 300 µL neutralized TNF-α + infliximab (EC100 mix) and further incubated for 120 min. Cell control and infliximab control (EC100 mix) wells were also set up. At each time point, the cells were harvested and used for RNA extraction and cDNA preparation. Each sample was analyzed in duplicate.

d) RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA from treated, untreated, and control samples was extracted using the RNeasy mini kit (74101, Qiagen, Hilder, Germany) according to the manufacturer's instructions, and the concentration was measured using Take3 low-volume plates on a microplate reader (BioTek). cDNA was prepared using Protoscript II per the product datasheet. qRT-PCR was performed using the PowerUp™ SYBR™ Green master mix on an ABI 7500 real-time PCR system. The conditions used were initial denaturation for 10 min at 95°C; primer annealing for 15 sec at 95°C, and elongation for 60 sec at 60°C for 40 cycles. A melting curve analysis was also performed where primer specificity was confirmed by the presence of a single peak at melting temperature. Housekeeping genes for data normalization were selected based on Norm Finder. The decrease or increase in TNF-α-induced gene expression was determined in the presence of infliximab, aCE, or bioactive compounds. Changes in gene expression were calculated in terms of fold induction with respect to the untreated cell control using the 2-ΔΔCT method. The level of gene expression in TNF-α-treated controls was considered 100%. An increase or decrease in expression after infliximab treatment was the second level of control. The effects of aCE or bioactive treatment in the presence of infliximab + TNFα (EC100) were considered significant when the expression levels of various genes differed significantly from those after the infliximab (EC100) treatment alone. The primers used in this study are listed below.

e) Housekeeping gene selection for L929 and U937 cells

Following genes were analyzed for both cell lines-

Figure 23: Screened housekeeping genes

Gene	Protein	Function
ATP5B	ATP synthase, H+ transporting mitochondrial F1 complex, β subunit	Produces ATP from adenosine diphosphate (ADP) in the presence of a proton gradient across the membrane; the β chain is the catalytic subunit
B2M	Beta-2-microglobulin	Beta-chain of major histocompatibility complex class I molecules
CYC1	Cytochrome c-1	Haem-containing component of the cytochrome b-c1 complex; transfers electrons to cytochrome c in mitochondrial respiratory chain
RPLP2	Ribosomal Protein Lateral Stalk Subunit P2	This gene encodes a ribosomal phosphoprotein that is a component of the 60S subunit.
RPL13A	Ribosomal protein L13a (Rpl13a)	Structural constituent of ribosome
SDHA	Succinate dehydrogenase complex, subunit A	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit involved in energy production and conversion
YWHA2	Tyrosine 3 monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Adapter protein implicated in the regulation of both general and specialized signaling pathways
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Catalysis of conversion of D-glyceraldehyde-3-phosphate to 3-phospho-D-glyceroyl phosphate

Relative fold gene expression after RT-qPCR was calculated using the 2–ΔΔCt method .

The calculation was done as follow:

ΔΔCt = ΔCt (treated sample) – ΔCt (untreated sample)

ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

Fold gene expression = 2^-(ΔΔCt)

Housekeeping genes – ATP5, B2M, CYC1, RPLP2, RPL13A, SDHA, YWHA2 and GAPDH were screened to find the best HK gene (whose expression remains unchanged across treatments)

f) Flow of Infliximab experiments-

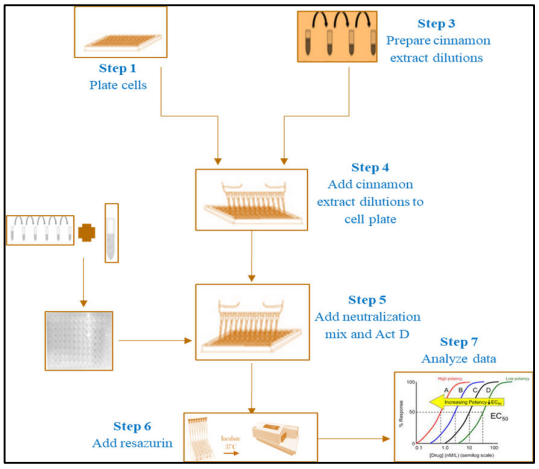


Figure 24: Flow of infliximab experiments

g) Statistical analysis

All experiments were performed at least three times unless stated otherwise. All samples were analyzed in duplicates. Means of more than two groups were compared using Brown-Forsythe and Welch One-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism version 10.0.0 for Windows. Statistical significance was set at $p < 0.05$. Data was analyzed using the GraphPad Prism software.

h) Primer sequences

Table 5A: Housekeeping gene primer sequences (U937)

Housekeeping genes	Primer sequences 5' to 3'
Human RPLP2	(F) TCTTGGACAGCGTGGGTATCGA
	(R) CAGCAGGTACACTGGCAAGCTT
Human GAPDH	(F) GTCTCCTCTGACTTCAACAGCG
	(R) ACCACCCTGTTGCTGTAGCCAA
ATP5F1B Human	(F) TCATGCTGAGGCTCCAGAGTTC
	(R) ACAGTCTTGCCAACTCCAGCAC
β2M Human	(F) CCACTGAAAAAGATGAGTATGCCT
	(R) CCAATCCAAATGCGGCATCTTCA
CYC1 Human	(F) CCAGATAGCCAAGGATGTGTGC
	(R) GACTGACCACTTGTGCCGCTTT
RPLP2 Human	(F) TCTTGGACAGCGTGGGTATCGA
	(R) CAGCAGGTACACTGGCAAGCTT
RPL13A Human	(F) CTCAAGGTGTTTGACGGCATCC
	(R) TACTTCCAGCCAACCTCGTGAG
SDHA Human	(F) GAGATGTGGTGTCTCGGTCCAT
	(R) GCTGTCTCTGAAATGCCAGGCA
YWHAZ Human	(F) ACCGTTACTTGGCTGAGGTTGC
	(R) CCCAGTCTGATAGGATGTGTTGG

Table 5B: Gene specific primer sequences (U937)

Genes	Primer sequences 5' to 3'
Human TLR2	(F) CTTCACTCAGGAGCAGCAAGCA
	(R) ACACCAGTGCTGTCCTGTGACA
Human TLR4	(F) CCCTGAGGCATTTAGGCAGCTA
	(R) AGGTAGAGAGGTGGCTTAGGCT
Human VCAM-1	(F) GATTCTGTGCCCACAGTAAGGC
	(R) TGGTCACAGAGCCACCTTCTTG
Human ICAM-1	(F) AGCGGCTGACGTGTGCAGTAAT
	(R) TCTGAGACCTCTGGCTTCGTCA
Human TIMP-1	(F) GGAGAGTGTCTGCGGATACTTC
	(R) GCAGGTAGTGATGTGCAAGAGTC
Human MMP 1	(F) ATGAAGCAGCCCAGATGTGGAG
	(R) TGGTCCACATCTGCTCTTGGCA
Human MMP 2	(F) CTGAAGGTGATGAAGCAGCC
	(R) AGTCCAAGAGAATGGCCGAG
Human STAT 3	(F) CTTTGAGACCGAGGTGTATCACC
	(R) GGTCAGCATGTTGTACCACAGG
Human JAK 2	(F) CCAGATGGAAACTGTTCGCTCAG
	(R) GAGGTTGGTACATCAGAAACACC
Human MyD88	(F) GAGGCTGAGAAGCCTTTACAGG
	(R) GCAGATGAAGGCATCGAAACGC
Human BCL-2	(F) ATCGCCCTGTGGATGACTGAGT
	(R) GCCAGGAGAAATCAAACAGAGGC
Mouse BCL-XL	(F) GCCACCTATCTGAATGACCACC
	(R) AGGAACCAGCGGTTGAAGCGC
Human Bax	(F) TCAGGATGCGTCCACCAAGAAG
	(R) TGTGTCCACGGCGGCAATCATC

Table 6A: Housekeeping gene primer sequences (L929):

Housekeeping genes	**All primer sequences 5' to 3'**
CYC1 Mouse	(F) CCATCTACACAGAAGTCTTGGAG
	(R) GCGTTTTCGATGGTCATGCTCTG
β2M Mouse	(F) ACAGTTCCACCCGCCTCACATT
	(R) TAGAAAGACCAGTCCTTGCTGAAG
ATP5F1B Mouse	(F) CTCTGACTGGTTTGACCGTTGC
	(R) TGGTAGCCTACAGCAGAAGGGA
β2M Mouse	(F) ACAGTTCCACCCGCCTCACATT
	(R) TAGAAAGACCAGTCCTTGCTGAAG
CYC1 Mouse	(F) CCATCTACACAGAAGTCTTGGAG
	(R) GCGTTTTCGATGGTCATGCTCTG
RPLP2 Mouse	(F) CTGCTCTCAAGGTTGTTCGGCT
	(R) CCTTCCGTTTCTCCTCCAGAGT
RPL13A Mouse	(F) GAACATTGAGGATGTCATCGCTC
	(R) GCAGAACCAGCAGCAGGTGCT
SDHA Mouse	(F) GAGATACGCACCTGTTGCCAAG
	(R) GGTAGACGTGATCTTTCTCAGGG
(YWHAZ) Mouse	(F) CAGAAGACGGAAGGTGCTGAGA
	(R) CTTTCTGGTTGCGAAGCATTGGG

	All primer sequences 5' to 3'
Mouse TLR2	(F) ACAGCAAGGTCTTCCTGGTTCC
	(R) GCTCCCTTACAGGCTGAGTTCT
Mouse TLR4	(F) AGCTTCTCCAATTTTTCAGAACTTC
	(R) TGAGAGGTGGTGTAAGCCATGC
Mouse VCAM-1	(F) GCTATGAGGATGGAAGACTCTGG
	(R) ACTTGTGCAGCCACCTGAGATC
Mouse ICAM-1	(F) AAACCAGACCCTGGAACTGCAC
	(R) GCCTGGCATTTCAGAGTCTGCT
Mouse TIMP-1	(F) TCTTGGTTCCCTGGCGTACTCT
	(R) GTGAGTGTCACTCTCCAGTTTGC
Mouse MMP 1	(F) AGGAAGGCGATATTGTGCTCTCC
	(R) TGGCTGGAAAGTGTGAGCAAGC
Mouse MMP 2	(F) CAAGGATGGACTCCTGGCACAT
	(R) TACTCGCCATCAGCGTTCCCAT
Mouse STAT 3	(F) AGGAGTCTAACAACGGCAGCCT
	(R) GTGGTACACCTCAGTCTCGAAG
Mouse JAK 2	(F) GCTACCAGATGGAAACTGTGCG
	(R) GCCTCTGTAATGTTGGTGAGATC
Mouse MyD88	(F) ACCTGTGTCTGGTCCATTGCCA
	(R) GCTGAGTGCAAACTTGGTCTGG
Mouse BCL-2	(F) CCTGTGGATGACTGAGTACCTG
	(R) AGCCAGGAGAAATCAAACAGAGG
Mouse BCL-XL	(F) GCCACCTATCTGAATGACCACC
	(R) AGGAACCAGCGGTTGAAGCGC
Mouse Bax	(F) AGGATGCGTCCACCAAGAAGCT
	(R) TCCGTGTCCACGTCAGCAATCA

Table 6B: Gene specific primer sequences (L929):

E. In vitro cell-based assay - Bevacizumab.

a) BMAB cell viability assay

HUVEC cells were cultured in a 75 cm^2 flask in growth medium. Cells were washed with DPBS and trypsinzed with cold trypsin-EDTA solution for 2-3 minutes. Post trypsinization, neutralization medium was added to neutralize trypsin and the cell suspension was spun at 220Xg for 5 minutes. Cell pellet was resuspended in assay medium and cell density was adjusted to at 0.9-1.0X10^5 cells/mL. 100 µL of 1.0X10^5 cells/mL (~10000 cells/well) were plated in each well of the clear 96-well plates. The plates were incubated at 37°C in the presence of 5% CO_2 for 5 hours. After 5 hours aCE was serially diluted to eight concentrations ranging from 28.57 to 0.22 mg/mL (final concentrations). 100 µL of aCE was added to treated wells. Control wells contained cells and 100 µL of assay medium. The plates were again incubated at 37°C in the presence of 5% CO_2 for 48 hours, followed by the addition of 20 µL Alamar Blue to the wells. The plates were further incubated for 6-7 hours at 37°C in the presence of 5%. CO_2. The alamar blue reagent color change was measured using a microplate reader at 570 nm and 600 nm. The average absorbance of the cell culture medium alone (background) at 600 nm was subtracted from that at 570 nm. The background-subtracted absorbance at 570 nm was plotted against the concentration of the test compound. Viability assays with bioactives was performed like that described above (the concentration range was 1000–0.064 µg/mL, final concentrations).

b) aCE and bevacizumab synergy experiments

HUVEC cell suspension was prepared as described above. 50 µL of the cell suspension (2 × 105 cells/mL or 10000 cells/well) was added to each treated, untreated and cell control wells in a transparent 96-well microplate plate and incubated at 37°C in the presence of 5% CO2 for 5 hours. After 5 hours, 50 µL of aCE or bioactives were added to the cells (labelled treated wells) to final concentrations of 10, 25, 50, and 100 µg/mL or 40, 32, 16, 8 and 4 µg/mL (final concentrations), respectively. Each set was incubated for 15 minutes. Based on Infliximab data a time course study was not performed for bevacizumab. 50 µL of the assay medium were added to the cell control wells and untreated wells in the

assay plate. Simultaneously, 100 µL of nine concentrations of bevacizumab (9 µg to 0.035 µg/mL, final concentrations) were added to the wells marked as treated and untreated on separate neutralization plate. 100 µL of VEGF165 (25 ng/mL, final concentration) was added to each treated and untreated wells and mixed five times (this plate did not contain cells, only bevacizumab and VEGF165 in 1:1 volume ratio for neutralization) and incubated for 2 hours at 37°C and 5% CO2. Two hundred microliters of the assay media were added to the cell control wells. After 15 minutes of incubation of aCE or bioactive with cells, 50 µL of the neutralization mixture was transferred to the wells in the assay plate marked as treated and untreated and 100 µL assay medium was added to the control wells. The cell plates were incubated at 37°C in the presence of 5% CO2 for 48 hours, followed by the addition of 20 µL alamar blue to the wells and further incubation for 6-7 hours at 37°C in an atmosphere of 5%. CO2. The color change was read using a microplate reader at 570 nm and 600 nm. The average absorbance value of the cell culture medium alone (background) at 600 nm was subtracted from that of the experimental wells at 570 nm. The background-subtracted absorbance at 570 nm was plotted against the bevacizumab concentration to determine the shift in the half maximal effective concentration (EC_{50}) in the presence of aCE. Similar study was carried out for bioactives (15 minutes preincubation).

c) Assay for Gene expression

HUVEC cells were added to 6-well plates (1 × 106 cells per well in 1 mL) in growth media and incubated for ~14-16 hours at 37°C and 5% CO_2. The spent medium was discarded, and the cells were rinsed with DPBS (200 µL/well). In a separate plate, bevacizumab EC100 (4.5 µg /mL, final concentration) was mixed with $VEGF_{165}$ (25 ng/mL, final concentration) and incubated for 2 hours at 37°C. aCE samples and concentrations which showed significant synergy (p-value < 0.05) and reproducibility across days in the synergy experiments were selected for gene expression assays. The cells were preincubated with 500 µL of 25 and 50 µg/mL aCE (final concentrations) or 40 and 32 µg/mL (final concentrations) of TCA for 15 min, followed by the addition of an equal volume of the neutralized VEGF165+bevacizumab (EC100 mix) and further incubated for 120 minutes. Cell

control, $VEGF_{165}$ control, and bevacizumab + $VEGF_{165}$ controls (EC100 mix) were also set up. At each time point, the cells were harvested and used for RNA extraction and cDNA preparation.

d) RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR): RNeasy mini kit was used to extract total RNA from all samples and low volume plate was used to determine the concentration of extracted RNA on a Synergy microplate reader (Take3 plate, BioTek). cDNA was prepared using Protoscript II and SYBR Green chemistry was used to perform qRT-PCR using the PowerUp™ SYBR™ Green master mix. Two step PCR was performed on an ABI 7500 real-time PCR system. Primer specificity was checked by melting curve analysis. Norm finder was used to selecting housekeeping genes. 2-ΔΔCT method was used to determine changes in gene expression as compared to cell control. $VEGF_{165}$ treated control wells represented 100% change in gene expression. The effects of aCE or bioactive treatment in the presence of bevacizumab + $VEGF_{165}$ (EC100) were considered significant when the expression levels of various genes differed significantly from those after the bevacizumab (EC100) treatment alone. The primers used in this study are listed in below.

e) Housekeeping gene selection for HUVEC cells.

Figure 25: Screened housekeeping gene

Gene	Protein	Function
ATP5B	ATP synthase, H+ transporting mitochondrial F1 complex, β subunit	Produces ATP from adenosine diphosphate (ADP) in the presence of a proton gradient across the membrane; the β chain is the catalytic subunit
B2M	Beta-2-microglobulin	Beta-chain of major histocompatibility complex class I molecules
CYC1	Cytochrome c-1	Haem-containing component of the cytochrome b-c1 complex; transfers electrons to cytochrome c in mitochondrial respiratory chain
RPLP2	Ribosomal Protein Lateral Stalk Subunit P2	This gene encodes a ribosomal phosphoprotein that is a component of the 60S subunit.
RPL13A	Ribosomal protein L13a (Rpl13a)	Structural constituent of ribosome
SDHA	Succinate dehydrogenase complex, subunit A	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit involved in energy production and conversion
YWHA2	Tyrosine 3 monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Adapter protein implicated in the regulation of both general and specialized signaling pathways
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Catalysis of conversion of D-glyceraldehyde-3-phosphate to 3-phospho-D-glyceroyl phosphate

Relative fold gene expression after RT-qPCR was calculated using 2–ΔΔCt method. The calculation was done as follows –

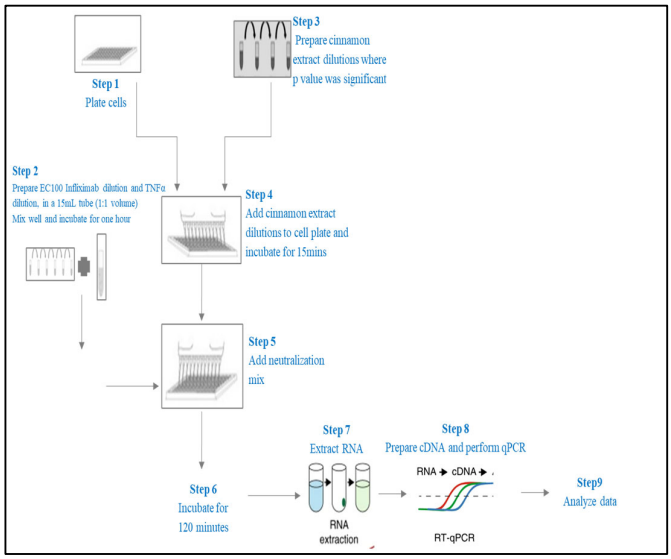
ΔΔCt = ΔCt (treated sample) – ΔCt (untreated sample)

ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

Fold gene expression = 2^-(ΔΔCt)

f) Flow of Bevacizumab experiments-

Figure 26: Flow of Bevacizumab experiment



g) Statistical analysis

All experiments were performed at least three times unless stated otherwise. All samples were analyzed in duplicates. Means of more than two groups were compared using Brown-Forsythe and Welch One-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism version 10.0.0 for Windows. Statistical significance was set at $p < 0.05$. Data was analyzed using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

h) Primer sequences

Table 7A: Housekeeping gene primer sequences (HUVEC).

Housekeeping genes	**Primer sequences 5' to 3'**
Human RPLP2	(F) TCTTGGACAGCGTGGGTATCGA
	(R) CAGCAGGTACACTGGCAAGCTT
Human GAPDH	(F) GTCTCCTCTGACTTCAACAGCG
	(R) ACCACCCTGTTGCTGTAGCCAA
ATP5F1B Human	(F) TCATGCTGAGGCTCCAGAGTTC
	(R) ACAGTCTTGCCAACTCCAGCAC
β2M Human	(F) CCACTGAAAAAGATGAGTATGCCT
	(R) CCAATCCAAATGCGGCATCTTCA
CYC1 Human	(F) CCAGATAGCCAAGGATGTGTGC
	(R) GACTGACCACTTGTGCCGCTTT
RPLP2 Human	(F) TCTTGGACAGCGTGGGTATCGA
	(R) CAGCAGGTACACTGGCAAGCTT
RPL13A Human	(F) CTCAAGGTGTTTGACGGCATCC
	(R) TACTTCCAGCCAACCTCGTGAG
SDHA Human	(F) GAGATGTGGTGTCTCGGTCCAT
	(R) GCTGTCTCTGAAATGCCAGGCA
YWHAZ Human	(F) ACCGTTACTTGGCTGAGGTTGC
	(R) CCCAGTCTGATAGGATGTGTTGG

Table 7B: Gene specific primer sequences (HUVEC).

	All primer sequences 5' to 3'
Human ANG2	(F) GTTTGATGCATGTGGTCCTTCC
	(R) CGAATAGCCTGAGCCTTTCCA
Human CXCL5	(F) TCTGCAAGTGTTCGCCATAGG
	(R) CAGTTTTCCTTGTTTCCACCGT
Human VCAM-1	(F) GATTCTGTGCCCACAGTAAGGC
	(R) TGGTCACAGAGCCACCTTCTTG
Human ICAM-1	(F) AGCGGCTGACGTGTGCAGTAAT
	(R) TCTGAGACCTCTGGCTTCGTCA
Human STAT 3	(F) CTTTGAGACCGAGGTGTATCACC
	(R) GGTCAGCATGTTGTACCACAGG
Human JAK 2	(F) CCAGATGGAAACTGTTCGCTCAG
	(R) GAGGTTGGTACATCAGAAACACC
Human IL8	(F) GAGAGTGATTGAGAGTGGACCAC
	(R) CACAACCCTCTGCACCCAGTTT
Human IL-6	(F) AGACAGCCACTCACCTCTTCAG
	(R) TTCTGCCAGTGCCTCTTTGCTG

CHAPTER 6

RESULTS & DISCUSSION

6.1 DNA based identification:

The DNA based identification of cinnamon species was performed using 18S and 5.8S specific primers, and the results were visualized on agarose gels stained with ethidium bromide. In the 0.8% agarose gel (Figure 27 and Table 8), lane 1 contained the 1KB ladder as a reference marker, while lanes 2, 3, and 4 showed the genomic DNA (gDNA) from *C. cassia*, *C. loureiroi*, and *C. zeylanicum*, respectively. For the PCR amplification, a 2% agarose gel was used to resolve the PCR products (Figure 28 and Table 9). Lane 1 displayed a band at approximately 357 bp corresponding to the *C. cassia* sample (GenBank: KX766398.1), lane 2 showed a band at approximately 356 bp for *C. loureiroi* (GenBank: MF110051.1), and lane 3 exhibited a band at approximately 348 bp for *C. zeylanicum* (GenBank: KX766399.1). Lane 4 contained the 100 bp ladder for size reference. These results confirm the identification of the cinnamon species based on their unique PCR product sizes.

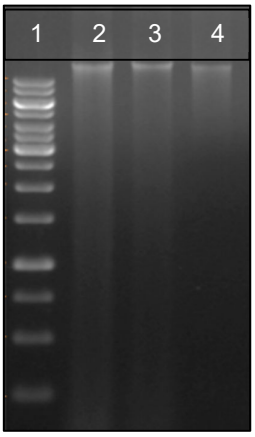


Figure. 27: Genomic DNA **(**0.8% agarose gel)

Table 8: Genomic DNA details

Lane number	Sample/Ladder
1	1KB Ladder (NEB) (10Kb to 100b)p
2	*C.cassia* gDNA
3	*C.loureiroi* gDNA
4	*C.zeylanicum* gDNA

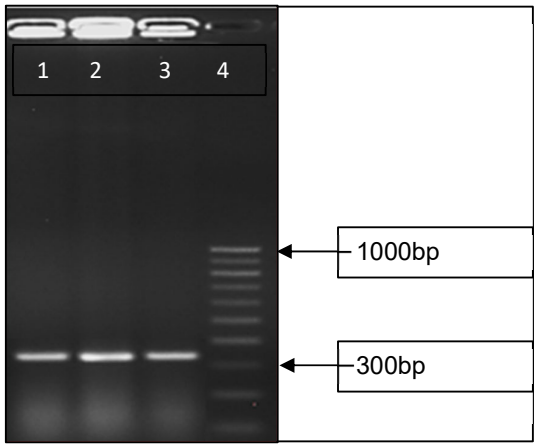


Figure. 28: PCR products (2% agarose gel)

Table 9: PCR result

Lane number	Figure 28 Sample/ Ladder	Expected size	Observed	GenBank Accession #
1	PCR product from *C. cassia* sample	357 bp	~357 bp	GenBank: KX766398.1
2	PCR product from *C. loureiroi* sample	356 bp	~356 bp	GenBank: MF110051.1
3	PCR product from *C. zeylanicum* sample	348 bp	~348 bp	GenBank: KX766399.1
4	100 bp ladder			

6.2 RP-HPLC results

The RP-HPLC analysis of *C. cassia, C. loureiroi and C. zeylanicum* aqueous cinnamon extracts (aCE) identified cinnamic acid and trans-cinnamaldehyde, with *C. cassia* aCE containing 0.22 mg/mL cinnamic acid and 10 mg/mL trans-cinnamaldehyde (Figure. 29), *C. loureiroi aCE* containing 3.2 mg/mL cinnamic acid and 7.81 mg/mL trans-cinnamaldehyde (Figure. 30), and *C. zeylanicum* aCE containing 19.81 mg/mL trans-cinnamaldehyde in their respective 100 mg/mL extract solutions Cinnamic acid was not detected in the extract (Figure. 31). A single peak was observed for each compound, with trans-cinnamaldehyde consistently showing a sharper peak and a rightward shift

compared to cinnamic acid. Purified trans-cinnamaldehyde, cinnamic acid and Masquelier's grape seed extract was used as reference materials.

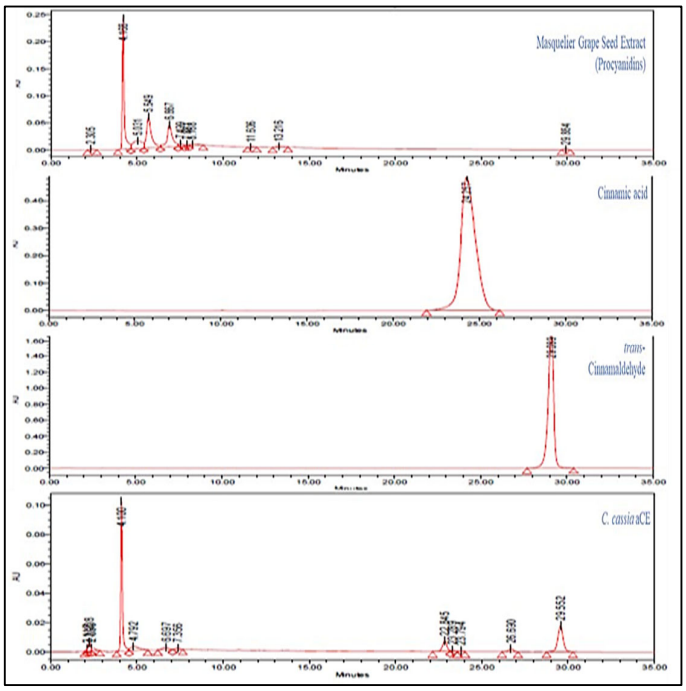


Figure 29*: RP-HPLC result of *C. cassia* aCE.

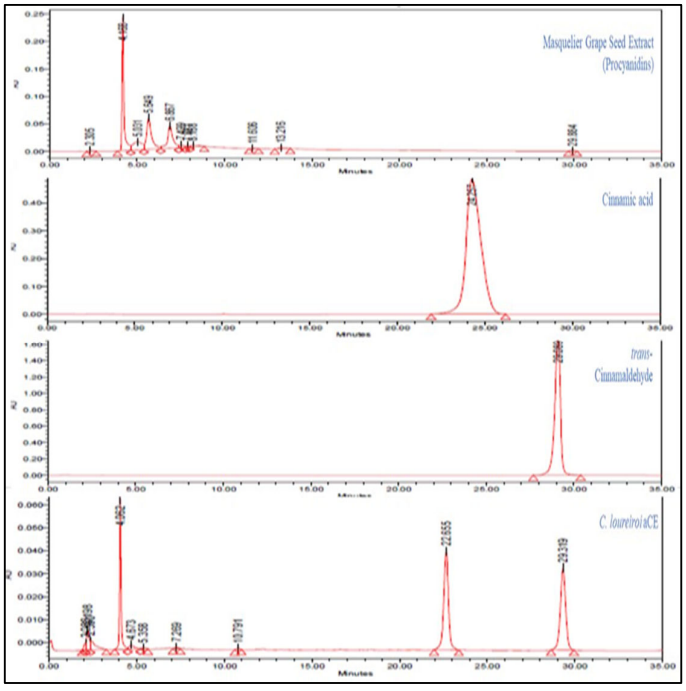


Figure 30*: RP-HPLC of *C. loureiroi* aCE.

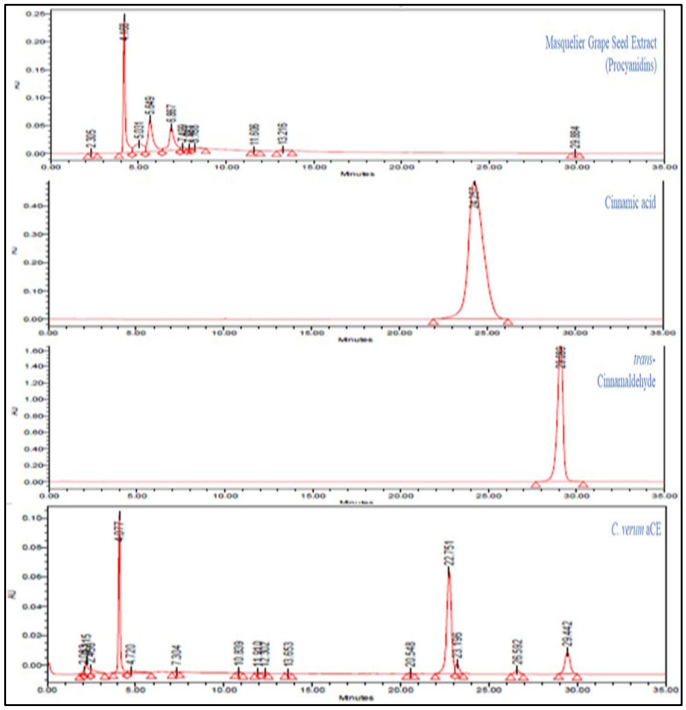
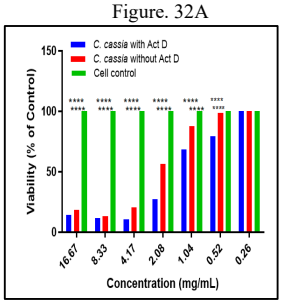


Figure 31*: RP-HPLC of *C. zeylanicum* aCE. *Refer annexure 1 for compiled image of aCE RP-HPLC data

6.3 *In vitro* assays with Infliximab and aCE

a. Effect of aCE on cell viability

Figure. 32A



First the concentration of cinnamon that was non-toxic to the cells was determined using Alamar Blue for detecting viability. As actinomycin D was required for the L929-based synergy experiments, cell death in the presence and absence of actinomycin D was also evaluated. A dose-dependent decrease in cell viability with increasing concentrations of aqueous cinnamon extracts (aCE) in both L929 and U937 cells were observed. In the L929 cells, *C. cassia, C. zeylanicum,* and *C. loureiroi* extracts significantly reduced viability (Figure 32A-C). Similar trends were noted in U937 cells (Figure 32D-F). The highest concentration of *C. cassia* aCE was excluded from analysis due to interference with the assay readout. Concentrations below 260 µg/mL were non-toxic to the cells. Similar experiment was performed with cinnamon bioactives,

Figure. 32B

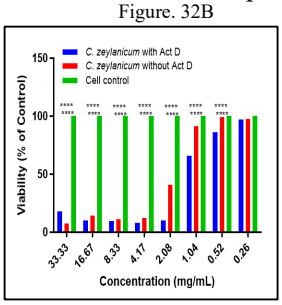
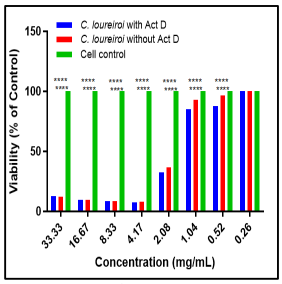


Figure. 32C



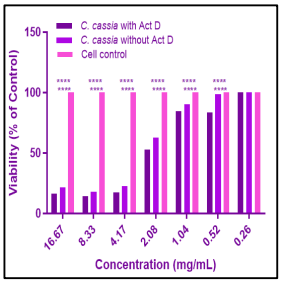


Figure. 32D

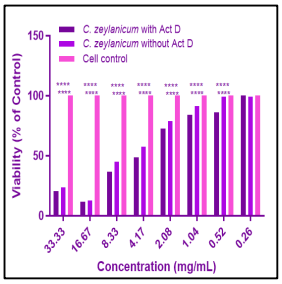


Figure. 32E

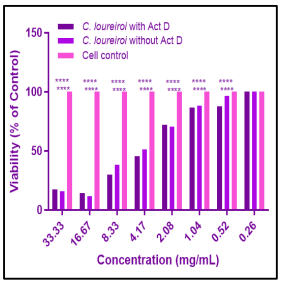


Figure. 32F

trans-cinnamaldehyde and cinnamic acid, and the cinnamon bioactive compounds were non-toxic at concentrations below 100 µg/mL. Four concentrations of aCE (150, 50, 25, and 10 µg/mL) and five concentrations of bioactive compounds (40, 32, 16, 8, and 4 µg/mL) were deemed safe for further synergy studies.

Figure 32. Cell viability after treatment with aqueous cinnamon extracts (aCE) and bioactives. L929 cells (A-C and G - H) and U937 cells (D - F and I - J) were treated with aCE from *C. cassia, C. zeylanicum,* and *C. loureiroi* (33.3 to 0.26 mg/mL) or trans-cinnamaldehyde (TCA) or cinnamic acid (CA), (1000 to 0.65 ug/mL) for 24 h. Cell viability was assessed using Alamar Blue, with results presented as mean ± SD from three independent experiments.

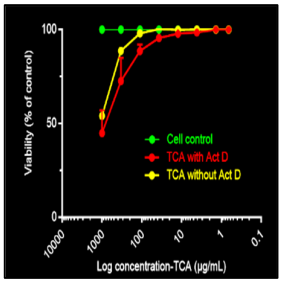


Figure. 32G

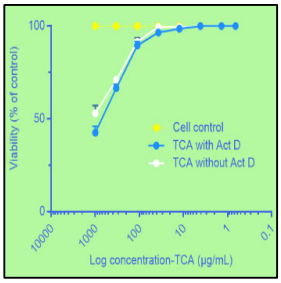


Figure. 32I

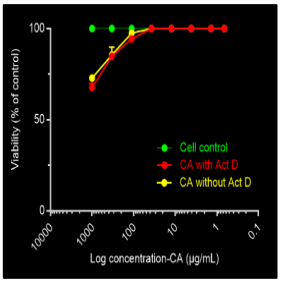


Figure. 32H

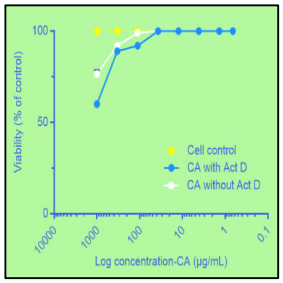


Figure. 32J

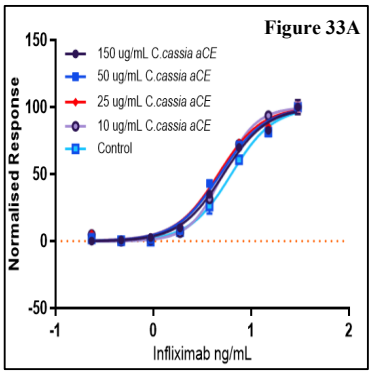
b. Synergy experiments with aCE

The results showed that preincubation of L929 cells with *C. cassia*, *C. loureiroi*, and *C. zeylanicum* aqueous extracts demonstrated synergistic effect with infliximab. The synergistic effect was time dependent, with 15 minutes

preincubation gave best results for *C. cassia* and *C. zeylanicum* while 30 minutes preincubation worked best for *C. loureiroi.*

i. Synergy experiment with *C.cassia* aCE in L929 cell line

The aCE of *C. cassia* demonstrated a significant synergistic effect ($p < 0.05$) with infliximab when preincubated for 15 min, with synergy observed across all tested concentrations (Table 10A and Figure. 33A). The EC_{50} of infliximab decreased in the presence of aCE, indicating enhanced activity. At 30 minutes preincubation, synergy was detected for some concentrations (Figure. 33B and C, Table 10B). The 60 min data showed high variability and changes in the slope of the dose-response curve, moreover it seems to interfere in infliximab activity as seen by increase in infliximab EC_{50}.



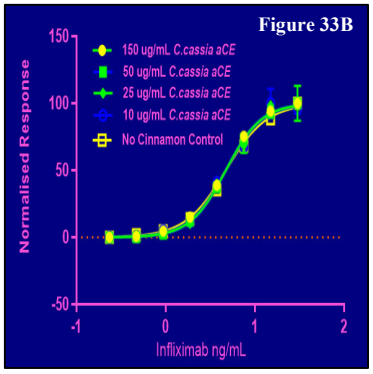


Figure 33. Synergy experiments with aCE in L929 cells. *C. cassia* showed synergy at all concentrations after 15 mins preincubation (A), synergy seen at 30 minutes for some concentrations and no synergy seen at 60 mins (B and C respectively) The changes in the EC_{50} values of infliximab in the L929 cell line following treatment with *C.cassia* aCE has been shown in Table 10. The control had an EC_{50} of 6.262 ng/mL. The addition of aCE at concentrations of 150 µg/mL, 50 µg/mL, 25 µg/mL, and 10 µg/mL significantly decreased the EC_{50} values to 5.203 ng/mL (0.0002), 4.828 ng/mL (0.0002), 4.618 ng/mL (<0.0001), and 5.115 ng/mL (<0.0001), respectively. These results indicate that *C. cassia* aCE improves the efficacy of infliximab at 15 minutes preincubation.

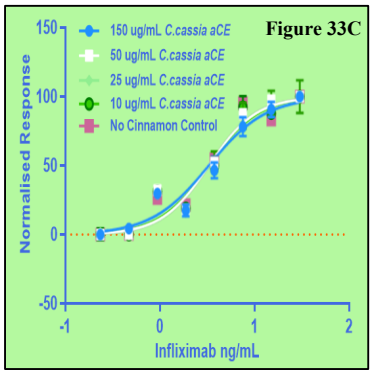


Table 10A. Changes in the EC_{50} and corresponding p-values after the *C. cassia* aCE synergy experiment (15 minutes preincubation) in the L929 cell line.

aCE concentration	Infliximab EC_{50} [ng/mL]	p-values
Control	6.262	NA
150 µg/mL	5.203	0.0002
50 µg/mL	4.828	0.0002
25 µg/mL	4.618	<0.0001
10 µg/mL	5.115	<0.0001

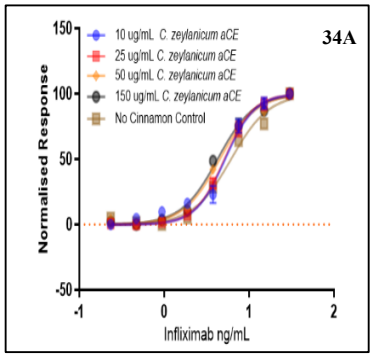
aCE, aqueous cinnamon extract; EC_{50}, half maximal effective concentration

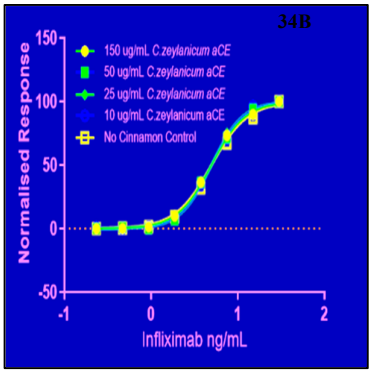
Table 10B. Changes in the EC_{50} after the *C. cassia* aCE synergy experiment (30 and 60 minutes preincubation) in the L929 cell line.

aCE concentration	Infliximab EC_{50} [ng/mL] and p-value 30 mins preincubation	Infliximab EC_{50} [ng/mL] and p-value 60 mins preincubation
Control	5.020	3.059
150 µg/mL	4.481(<0.0001)	3.659 (0.2383)
50 µg/mL	4.694 (0.0079)	3.201 (0.7677)
25 µg/mL	4.762 (0.2614)	3.307 (0.6162)
10 µg/mL	4.692 (0.2266)	3.226 (0.7405)

ii. Synergy experiment with *C. zeylanicum* aCE in L929 cell line*:*

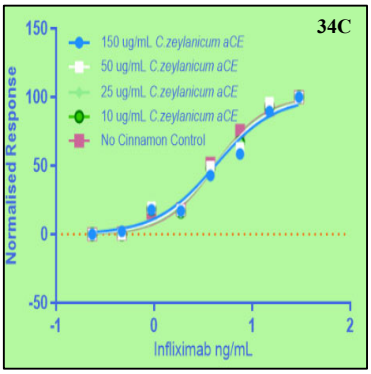
Similar to *C. cassia* aCE, *C. zeylanicum* aCE exhibited significant synergy ($p < 0.05$) at 15 minutes across all concentrations tested (Figure 34A and Table 11A). At 30 minutes preincubation no synergy was detected (Figure. 34B and 35C, Table 11B). The 60 min data showed high





variability and changes in the slope of the dose-response curve, moreover it seems to interfere in infliximab activity as seen by increase in infliximab EC_{50}.

Figure 34: Synergy experiments with aCE in L929 cells. *C. zeylanicum* showed synergy at all concentrations after 15 mins preincubation (A), no synergy seen at 30 and 60 mins (B and C respectively)



The changes in the EC_{50} values of infliximab in the L929 cell line following treatment with C.zeylanicum aCE has been shown in Table 11. The control had an EC_{50} of 6.117 ng/mL. The addition of aCE at concentrations of 150 µg/mL, 50 µg/mL, 25 µg/mL, and 10 µg/mL significantly decreased the EC_{50} values to 4.237 ng/mL (<0.0001), 4.507 ng/mL (<0.0001), 5.103 ng/mL (<0.0001), and 5.219 ng/mL (<0.0001), respectively. These results indicate that C. zeylanicum aCE improves the efficacy of infliximab at 15 minutes preincubation.

Table 11A. Changes in the EC_{50} and corresponding p-values after the *C. zeylanicum* aCE synergy experiment (at 15 minutes preincubation) in the L929 cell line.

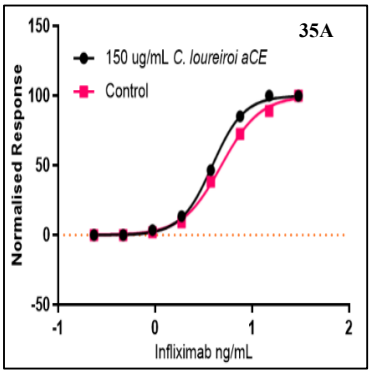
aCE concentration	Infliximab EC_{50} [ng/mL]	p- values
Control	6.117	NA
150 µg/mL	4.237	<0.0001
50 µg/mL	4.507	<0.0001
25 µg/mL	5.103	<0.0001
10 µg/mL	5.219	<0.0001

Table 11B. Changes in the EC_{50} after the *C. zeylanicum* aCE synergy experiment (30 and 60 minutes preincubation) in the L929 cell line. P-value were > 0.05 and observed results were not significant.

aCE concentration	Infliximab EC_{50} [ng/mL] and p-value 30 mins preincubation	Infliximab EC_{50} [ng/mL] and p-value 60 mins preincubation
Control	5.382	3.775
150 µg/mL	4.839 (0.2834)	4.781 (0.0852)
50 µg/mL	4.923 (0.3843)	4.153 (0.5133)
25 µg/mL	4.770 (0.2005)	4.165 (0.4600)
10 µg/mL	5.004 (0.4791)	4.169 (0.4864)

iii. Results with *C. loureiroi* aCE in L929 cell line.

The *C. loureiroi* aCE was the only extract that showed significant synergy ($p < 0.05$) after 30 min of preincubation. The synergy was observed only at the highest tested aCE concentration (150 µg/mL) (Figure 35A and Table 12A), other concentrations did not show synergy (Figure. 35B). No effect was seen at 15 and 60 minutes preincubation (Figure. 35C and 35D respectively and Table 12B)



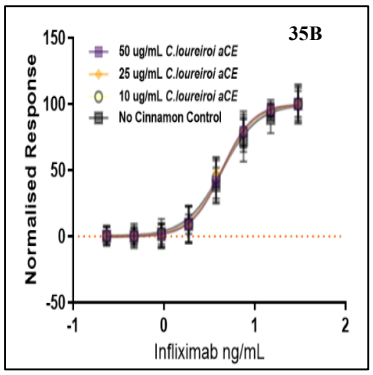


Figure 35: Synergy experiments with aCE in L929 cells. *C. loureiroi* showed synergy at highest tested concentrations after 30 mins preincubation (A), no synergy seen at other concentrations (B); 30 and 60 mins preincubation also did not show any synergy (C and D respectively)

The changes in the EC_{50} values of infliximab in the L929 cell line following treatment with *C.loureiroi* aCE has been shown in Table 12. The control had an EC_{50} of 4.815 ng/mL. The addition of aCE at concentrations of 150 µg/mL significantly decreased the EC_{50} values to 3.892 ng/mL (0.0343). 50 µg/mL, 25 µg/mL, and 10 µg/mL aCE did not decrease the EC_{50} values of infliximab (4.351 ng/mL, 4.072 ng/mL, and 4.252 ng/mL respectively)

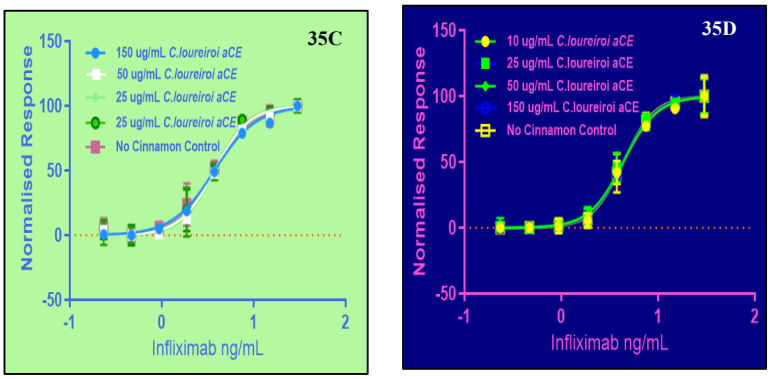


Table 12A. Changes in the EC_{50} and corresponding p-values after the *C. loureiroi* aCE synergy experiment in the L929 cell line.

aCE concentration	Infliximab EC_{50} [ng/mL]	p-values
Control	4.815	NA
150 µg/mL	3.892	0.0343
50 µg/mL	4.351	0.2926
25 µg/mL	4.072	0.0722
10 µg/mL	4.252	0.1803

Table 12B. Changes in the EC_{50} after the *C. loureiroi* aCE synergy experiment (15 and 60 minutes preincubation) in the L929 cell line.

aCE concentration	Infliximab EC_{50} [ng/mL] and p-value 15 mins preincubation	Infliximab EC_{50} [ng/mL] and p-value 60 mins preincubation
Control	4.342	3.341
150 µg/mL	4.522 (0.5243)	3.927 (0.0601)
50 µg/mL	4.304 (0.8921)	3.996 (0.0316)
25 µg/mL	4.189 (0.5936)	3.809 (0.1782)
10 µg/mL	4.500 (0.5966)	3.603 (0.4984)

iv. Synergy experiment with *C.cassia* aCE in U937 cell line.

Based on L929 synergy experiment data, for U937 synergy experiments only 15 minutes preincubation was used. The aCE of *C. cassia* exhibited a significant synergistic effect ($p < 0.05$) with infliximab in U937 cells when preincubated for 15 minutes (Figure 36, Table 13). The 150 µg/mL concentration was excluded due to unreliable data, but synergy was observed at all other tested concentrations of aCE.

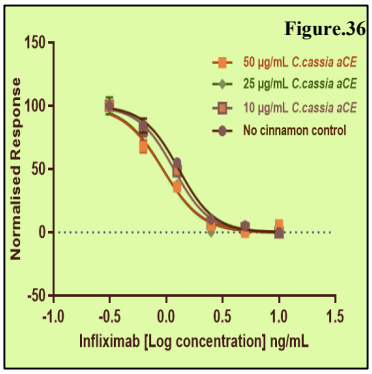


Figure 36. Synergy experiments with *C. cassia* aCE at 15 min preincubation in the U937 cell line.

The changes in the EC_{50} values and corresponding p-values for infliximab after treatment with varying concentrations of *C. cassia* aCE in the U937 cell line have been described in Table 4. The EC_{50} value for infliximab in the control group was 1.290 ng/mL. Treatment with *C. cassia* aCE resulted in a dose-dependent decrease in the EC_{50} values, indicating enhanced potency. At 50 µg/mL, the EC_{50} value dropped to 0.9294 ng/mL with a highly significant p-value of <0.0001. A concentration of 25 µg/mL led to an EC_{50} of 0.938 ng/mL, with a p-value of 0.0001, suggesting significant improvement in infliximab potency. At 10 µg/mL, the EC_{50} value was 1.042

ng/mL with a p-value of 0.0087, indicating a less pronounced but still significant effect.

Table 13. Changes in the EC_{50} and corresponding p-values after the *C. cassia* aCE synergy experiment in the U937 cell line.

aCE concentration	Infliximab EC_{50} [ng/mL]	p-values
Control	1.290	NA
50 µg/mL	0.9294	<0.0001
25 µg/mL	0.938	0.0001
10 µg/mL	1.042	0.0087

v. Synergy experiment with *C. zeylanicum* aCE in U937 cell line.

The aCE of *C. zeylanicum* exhibited a significant synergistic effect ($p < 0.05$) with infliximab in U937 cells when preincubated for 15 minutes (Figure 37, Table 14). The 150 µg/mL concentration was excluded due to unreliable data, but synergy was observed at all other tested concentrations of aCE.

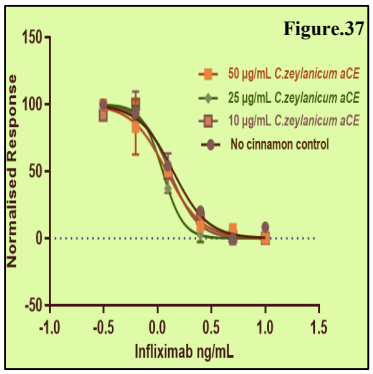


Figure 37. Synergy experiments with *C. zeylanicum* aCE at 15 min preincubation in the U937 cell line.

In the control group, the EC_{50} of infliximab was 1.397 ng/mL. Treatment with *C. zeylanicum* aCE led to a dose-dependent reduction in the EC_{50} values, reflecting an increase in infliximab potency. At 50 µg/mL, the EC_{50} was reduced to 1.125 ng/mL with a p-value of 0.0109, indicating a statistically significant enhancement. A lower concentration of 25 µg/mL resulted in an EC_{50} of 1.115 ng/mL and a highly significant p-value of 0.0001. At 10 µg/mL, the EC_{50} was 1.138 ng/mL with a p-value of 0.0167, showing a significant but less pronounced effect compared to higher concentrations.

Table 14. Changes in the EC_{50} and corresponding p-values after the *C. zeylanicum* aCE synergy experiment in the U937 cell line.

aCE concentration	Infliximab EC_{50} [ng/mL]	p-values
Control	1.397	NA
50 µg/mL	1.125	0.0109
25 µg/mL	1.115	0.0001
10 µg/mL	1.138	0.0167

vi. Synergy experiment with *C. loureiroi* aCE in U937 cell line.

C. loureiroi aCE did not exhibit synergy at any time point or concentration (Figure 38 and Table 15).

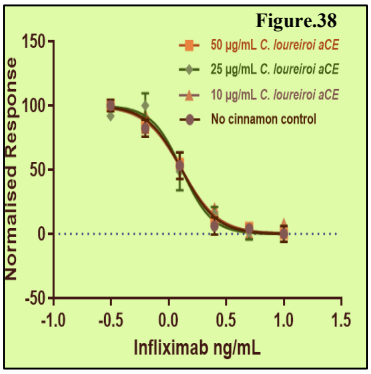


Figure 38. Synergy experiments with *C. loureiroi* aCE at 30 min preincubation in the U937 cell line.

Table 15. Changes in the EC_{50} and corresponding p-values after the *C. loureiroi* aCE synergy experiment in the U937 cell line

aCE concentration	Infliximab EC_{50} [ng/mL] and p-value 30 mins preincubation
Control	1.234
50 µg/mL	1.290 (0.5024)
25 µg/mL	1.294 (0.5959)
10 µg/mL	1.397 (0.0977)

c. Synergy experiments with purified cinnamon bioactives

Based on the recommendations received during SAS, we investigated the effects of selected purified compounds identified in our extract (through HPLC analysis). Major components identified in extracts were Cinnamic Acid and Trans-Cinnamaldehyde. The concentrations used in the study were as follows: Cinnamic Acid and Trans-Cinnamaldehyde at 40, 32, 16, 8, and 4 µg/mL.

i. Results with *Cinnamic Acid (CA) i*n L929 and U937 cell line.

- **L929 cell line.**

To investigate the components responsible for the observed synergy, two cinnamon bioactive compounds (CA and TCA) were tested. Cinnamic acid did not show any synergy in L929 cell line (Figure. 39 and Table 16).

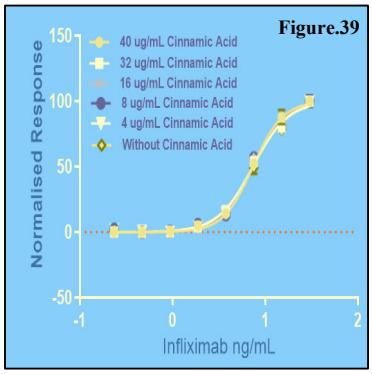


Figure 39: Synergy experiment with Cinnamic acid (CA) at 15 min preincubation in the L929 cell line.

Cinnamic acid concentration	Infliximab EC_{50} [ng/mL] and p-value 15 mins preincubation
Control	7.802
40 µg/mL	7.301 (0.0748)
32 µg/mL	7.209 (0.0869)
16 µg/mL	7.438 (0.2023)
8 µg/mL	7.546 (0.3682)

Table 16. Changes in the EC_{50} and corresponding p-values after the cinnamic acid (CA) synergy experiment in the L929 cell line

- **U937 cell line.**

Since no synergy was seen in L929 cell line, CA was not tested for synergy in U937 cell line.

ii. Results with Trans-cinnamaldehyde (TCA) in L929 and U937 cell line.

- **L929 cell line.**

To investigate whether TCA will act synergistically with infliximab, we tested different concentration of TCA in synergy experiment in L929 cell line first. Trans-cinnamaldehyde showed excellent and statistically significant synergy with infliximab (Figure. 40 and Table 17).

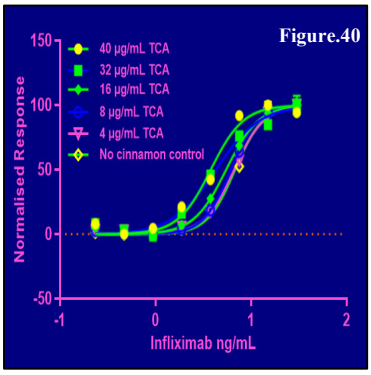


Figure 40. Synergy experiment with trans-cinnamaldehyde (TCA) at 15 min preincubation in the L929 cell line

The impact of varying concentrations of trans-cinnamaldehyde (TCA) on the EC_{50} values of infliximab in the L929 cell line has been shown in Table 17. The control had an EC_{50} of 6.800 ng/mL. At 40 µg/mL of TCA, the EC_{50} was significantly reduced to 3.777 ng/mL with a p-value of <0.0001, demonstrating a substantial synergistic effect. The EC_{50} continued to decrease with 32 µg/mL and 16 µg/mL of TCA, showing values of 4.275 ng/mL and 5.471 ng/mL, respectively, both with p-values <0.0001, reflecting strong statistical significance. At 8 µg/mL, the EC_{50} was 6.124 ng/mL with a p-value of 0.0099, indicating a notable effect, though less pronounced. Conversely, at 4 µg/mL, the EC_{50} value of 6.625 ng/mL with a p-value of 0.5145 suggests no significant effect compared to the control. These results suggest that higher concentrations of TCA are more effective in increasing infliximab's potency, but the effect diminishes at lower concentrations.

trans-cinnamaldehyde concentration	Infliximab EC_{50} [ng/mL]	p-values
Control	6.800	NA
40 µg/mL	3.777	<0.0001
32 µg/mL	4.275	<0.0001
16 µg/mL	5.471	<0.0001
8 µg/mL	6.124	0.0099
4 µg/mL	6.625	0.5145

Table 17. Changes in the EC_{50} and corresponding p-values after the trans-cinnamaldehyde (TCA) synergy experiment in the L929 cell line

- **U937 cell line.**

Based on encouraging results in L929 cell line, same concentration of TCA were tested in U937 cell line. In U937 cells, synergy between TCA and infliximab was observed at higher concentrations i.e., 40 and 32 µg/mL The changes in the EC_{50} values of infliximab in the U937 cell line following treatment with trans-cinnamaldehyde (TCA) has been shown in Table 18. The control had an EC_{50} of 1.234 ng/mL. Treatment with 40 µg/mL of TCA significantly reduced the EC_{50} to 0.9498 ng/mL ($p = 0.0007$), indicating a notable increase in infliximab's potency. Similarly, at 32 µg/mL of TCA, the EC_{50} was reduced to 0.9703 ng/mL with a p-value of 0.0008, also reflecting a significant synergistic effect. Remaining concentrations did not show any synergy in U937 cell line (Figure 41 and Table 18).

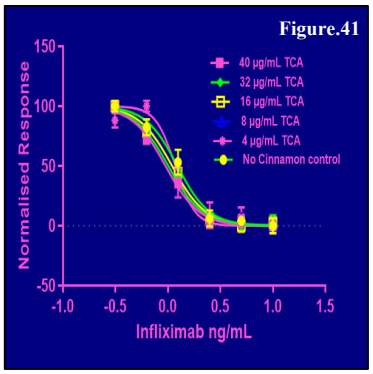


Figure 41. Synergy experiment with trans-cinnamaldehyde (TCA) at 15 min preincubation in the U937 cell line

Table 18. Changes in the EC_{50} and corresponding p-values after the TCA synergy experiment in the U937 cell line:

trans-cinnamaldehyde concentration	Infliximab EC_{50} [ng/mL]	p-values
Control	1.234	NA
40 µg/mL	0.9498	0.0007
32 µg/mL	0.9703	0.0008
16 µg/mL	1.097	0.1140
8 µg/mL	1.105	0.1304
4 µg/mL	1.177	0.5662

d. Synergy calculation and selection of cinnamon species for gene expression analysis

- **L929 cell line – Summary of synergy experiment data**

The relative potency of infliximab with aCE and bioactive compounds was calculated by considering the ratio of infliximab's EC50 without aCE/bioactive compound and that with aCE/bioactive compound and converting it to a

percentage. The highest synergy in the L929 assay system resulted in 125% relative potency (150 µg/mL *C. zeylanicum* aCE) of infliximab with aCE against 100% without aCE (Figure 42B).A relative potency of 120% was observed with 50 µg/mL *C. cassia* aCE (Figure 43A), 110% with 150 µg/mL *C. loureiroi* aCE (Figure 42C), and 180% with 40 µg/mL bioactive TCA (Figure 42D).

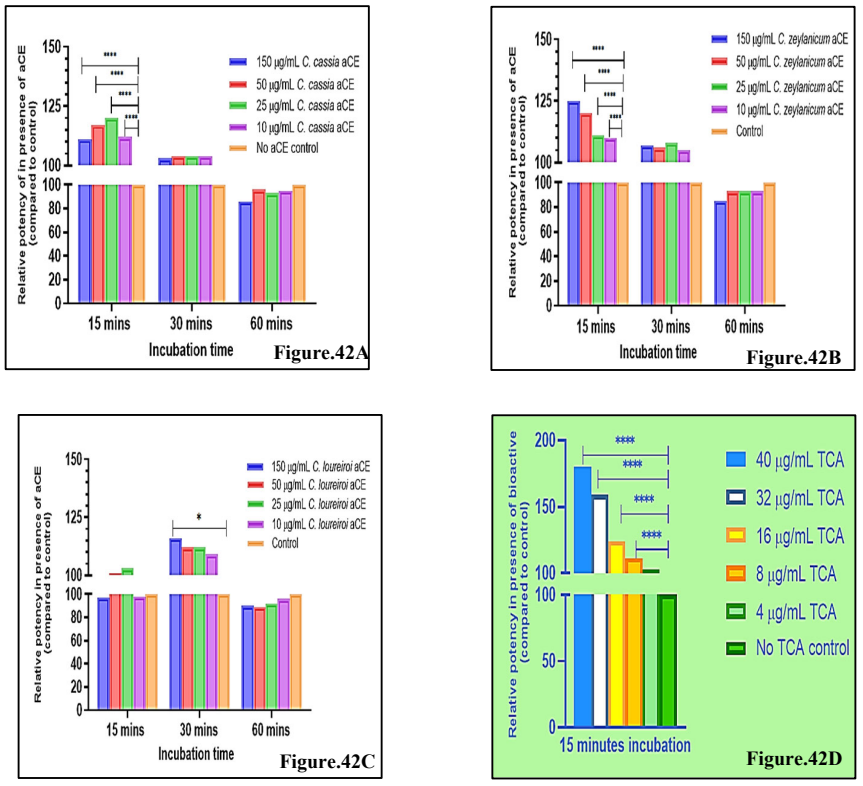


Figure 42. Effect of aCE on infliximab relative potency. (A) *C. cassia* aCE in L929 cells, (B) *C. zeylanicum* aCE in L929 cells, (C) *C. loureiroi* aCE in L929 cells, and (D) *TCA* in L929 cells with significance levels indicated: ****p < 0.0005, **p < 0.01, *p < 0.05.

- **U937 cell line – Summary of synergy experiment data**

In U937 cells, the highest relative potency of infliximab was 139% with 50 µg/mL *C. cassia* aCE (Figure 43A), 125% with 25 µg/mL *C. zeylanicum* aCE (Figure 43B), and 130% with 40 µg/mL TCA (Figure 43C).

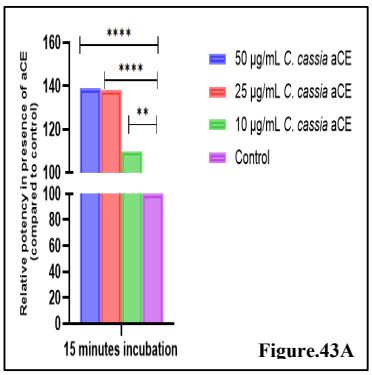


Figure.43A

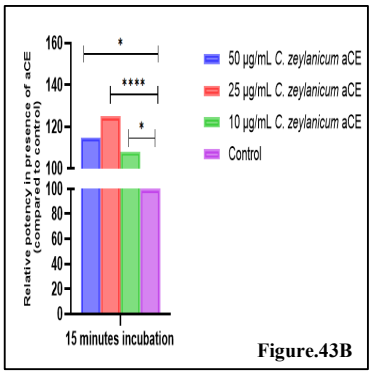


Figure.43B

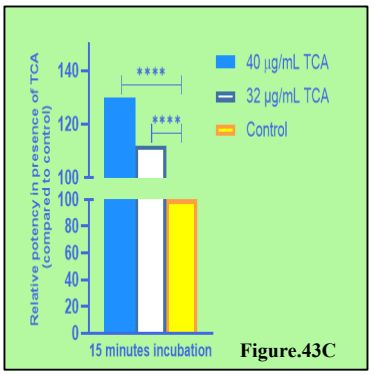


Figure.43C

Figure 43. Effect of aCE on infliximab relative potency. (A) *C. cassia* aCE in U937 cells, (B) *C. zeylanicum* aCE in L929 cells, and (C) *TCA* in U937 cells with significance levels indicated: ****p < 0.0005, **p < 0.01, *p < 0.05.

e. Selection of aCE and bioactive for gene expression studies –

● Based on the synergy experiment data, it was apparent that *C. cassia* and *C. zeylanicum* showed statistically significant synergy with infliximab. Only TCA showed significant synergy with infliximab and hence was by default chosen for gene expression studies.

● aCE concentrations 50 and 25 µg/mL were selected for the study because of more consistent data across days and cell lines.

● TCA concentration 40 and 32 µg/mL were selected for the study because of highly significant synergy with infliximab across days and cell lines.

6.4 Gene regulation analysis – aCE and infliximab synergy

This study aims at assessing the effect of aCE and infliximab synergism on genes typically upregulated in infliximab non responders**.**

Selected gene panel –

- Adhesion molecules panel: ICAM-1 and VCAM-1
- Innate immune response panel: TLR-2 and TLR-4
- Matrix remodelers panel: MMP-1, MMP-3, and TIMP-1
- Apoptotic gene panel: BcL-xL, BcL-2 and Bax
- Signaling molecules panel: Jak2, STAT3, and MyD88

1. **Results for housekeeping gene selection-**

- **L929 cell line**
  - Housekeeping genes – ATPS, B2M, CYC1, RPLP2, RPL13A, SDHA, YWHA2 and GAPDH were screened to find the best HK gene (whose expression remains unchanged across treatments)
  - The top three housekeeping genes identified through Normfinder analysis: YWHA2, CYC1, and B2M.
  - YWHA2 exhibited the highest stability with a value of 0.017, making it the best housekeeping gene for normalization, followed by CYC1 at 0.027 and B2M at 0.019.
  - The remaining genes showed higher stability values, with RPLP2 at 0.031, GAPDH at 0.030, SDHA at 0.027, ATPS at 0.034, and RPL13A at 0.036, indicating less consistency across treatments.
  - The combination of B2M and CYC1 was deemed the best for accurate normalization of gene expression data by Normfinder software. (Table 19).

Gene name	Stability value	Best gene	YWHA2
ATPS	0.034	Stability value	0.017
B2M	0.019		
CYC1	0.018	Best combination of two genes	CYC1 and B2M
RPLP2	0.031	Stability value for best combination of two genes	0.010
RPL13A	0.036		
SDHA	0.027		
YWHA2	0.017		
GAPDH	0.030		

Table 19: Housekeeping Gene Stability Analysis in L929 Cell Line

- **U937 cell line**
  - Housekeeping genes – ATPS, B2M, CYC1, RPLP2, RPL13A, ACTD, YWHA2 and GAPDH were screened to find the best HK gene (whose expression remains unchanged across treatments)
  - The top four housekeeping genes identified through Normfinder analysis: CYC1, RPLP2, RPL13A and GAPDH.
  - CYC1 exhibited the highest stability with a value of 0.015, making it the best housekeeping gene for normalization, followed by RPLP2 at 0.017, RPL13A at 0.018 and GAPDH at 0.019.
  - The remaining genes showed higher stability values, with ATPS at 0.032, B2M at 0.028, YWHA2 at 0.0065, and ACTD at 0.046, indicating less consistency across treatments.
  - The combination of RPLP2 and GAPDH was deemed the best for accurate normalization of gene expression data by Normfinder software. (Table 20).

Gene name	Stability value	Best gene	CYC1
ATPS	0.032	**Stability value**	0.015
B2M	0.028		
CYC1	0.015	**Best combination of two genes**	RPLP2 and GAPDH
RPLP2	0.017	**Stability value for best combination of two genes**	0.010
RPL13A	0.018		
YWHA2	0.065		
ACTD	0.046		
GAPDH	0.019		

Table 20: Housekeeping Gene Stability Assessment and Best Gene Combinations in the U937 Cell Line:

Based on the obtained results, Cytochrome C1 (CYC1) and b2-2-microglobulin (B2M) were used as housekeeping genes in L929 cells, while ribosomal protein lateral stalk subunit P2 (RPLP2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used for U937 cells.

2. Results for selected gene –

i. Adhesion molecules panel: ICAM-1 and VCAM-1

An increase in the level of *ICAM-1* and *VCAM-1* was observed with TNF-α treatment, which was reversed with infliximab treatment. Next, experiments were conducted to determine whether aCE or TCA could act synergistically to improve the effect of infliximab**.** *C. cassia* aCE and TCA worked synergistically with infliximab and downregulated the mRNA levels of ICAM-1 and VCAM-1 in both cell lines. Higher doses of *C. cassia* aCE (50 µg/mL) reduced the mRNA level of *ICAM-1* by an additional ~25.6−27.7% and that of *VCAM-1* by ~19% (Figures 44A (L929) and B (U937)). TCA (40 µg/mL) downregulated *ICAM-1* mRNA level by an additional ~18.9−24.1% and that of *VCAM-1* by ~31.9−41% (Figures 44C (L929) and D (U937)). *C. zeylanicum* did not show any synergistic effects (Figures 44E (L929) and F (U937)) in either cell line.

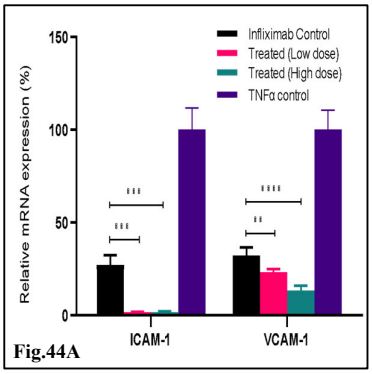


Fig.44A

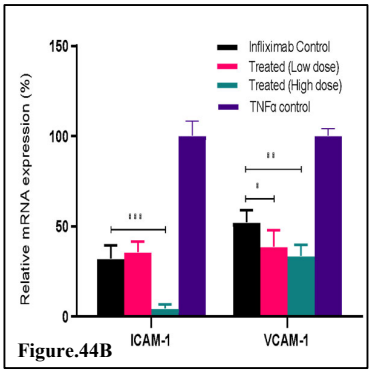


Figure.44B

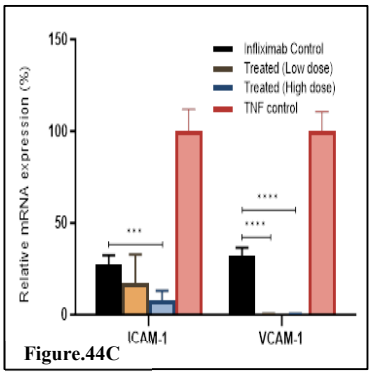


Figure.44C

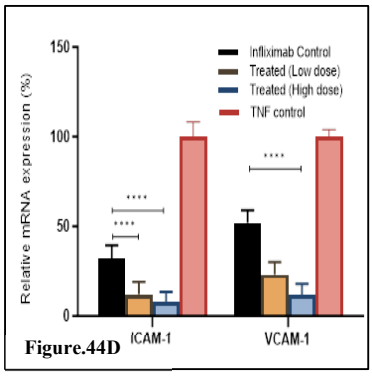


Figure.44D

Figure 44. High (50 µg/mL) and low (25 µg/mL) concentrations of *C. cassia* aCE (A (L929) and B (U937)) and high (40 µg/mL) and low (32 µg/mL) concentrations TCA (C (L929) and D (U937)) downregulated the mRNA levels of *ICAM-1* and *VCAM-1* synergistically with infliximab. *C. zeylanicum* aCE did not show any effect on the level of the selected mRNAs (E (L929) and F (U937)).

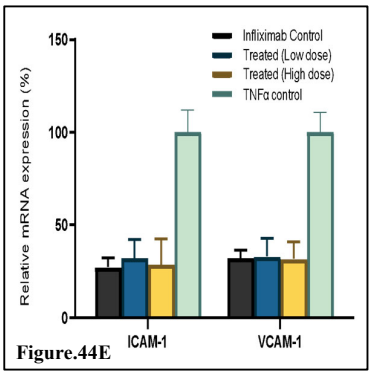


Figure.44E

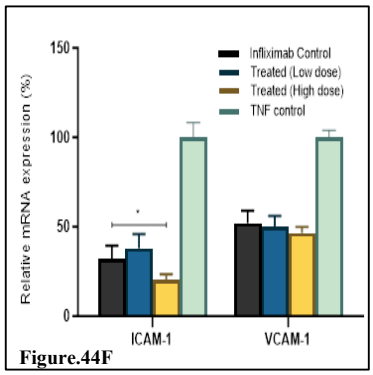


Figure.44F

ii. Innate immune response panel: TLR-2 and TLR-4

TLR-2 and *TLR-4* mRNA levels increased in the presence of TNF-α and decreased in the presence of infliximab. This decrease was more pronounced in the L929 (Figures 45A and C) cell line than in the U937 cells (Figures 45B and D). *C. cassia* aCE was better at downregulating the level of both Toll-like receptors than TCA (Figures 45C and D), and downregulated TLR-2 more than TLR-4. Higher doses of *C. cassia* aCE (50 µg/mL) reduced the mRNA level by an additional ~23.6−57.7% for TLR-2, and by 24.7–41.7% for *TLR-4* (Figures 45A (L929) and B (U937)). TCA (40 g/mL) downregulated *TLR-2* mRNA level by an additional 4–17.1% and *TLR-4* level by ~43.8–73.2% (Figures 45C (L929) and D (U937)). *C. zeylanicum* did not exhibit any synergistic effects (Figures 45E (L929) and F (U937)).

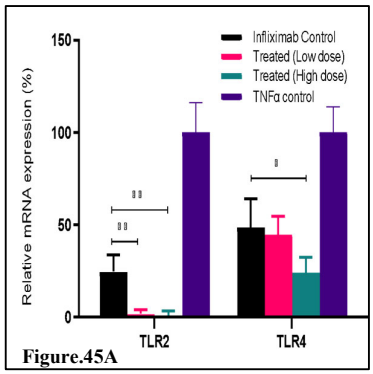


Figure.45A

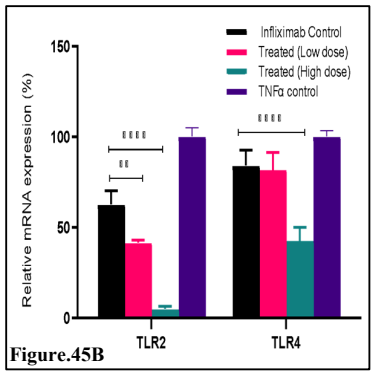


Figure.45B

Figures 45. *C. cassia* aCE (A (L929) and B (U937)) was better at downregulating the levels of both Toll-like receptors than TCA (C (L929) and D (U937)), TCA downregulated the level of TLR-4 more than TLR-2. Synergistic effects were more pronounced in L929 (A and C) than in U937 (B and D). *C. zeylanicum* aCE did not affect the level of the selected mRNAs (E (L929) and F (U937)).

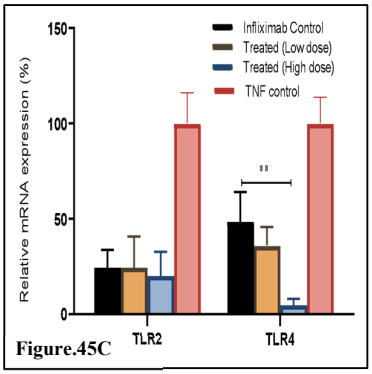


Figure.45C

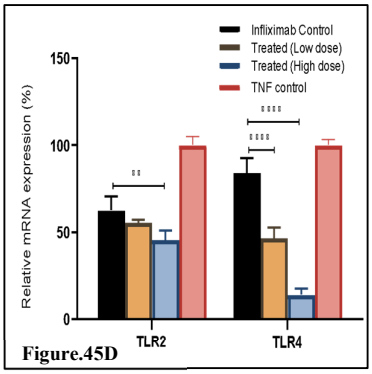


Figure.45D

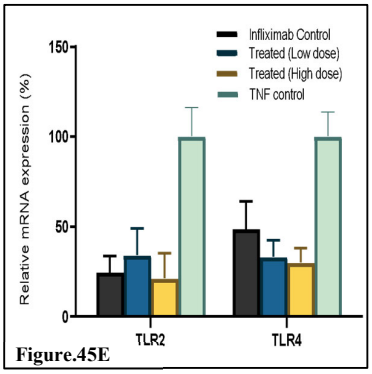


Figure.45E

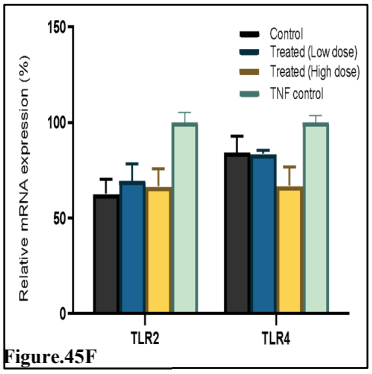


Figure.45F

iii. Matrix remodelers panel: MMP-1, MMP-3, and TIMP-1

We observed an increase in the mRNA levels of *MMP1, MMP3*, and *TIMP-1* upon TNF-α treatment. This increase was partially reversed by infliximab treatment (Figures 46A (L929) and B (U937)). Higher doses of C. cassia aCE (50 µg/mL) decreased *MMP1* level by an additional ~40.9−69.5%, *MMP3* level by ~53.3−60.4%, and *TIMP-1* level by ~54.3−62.9%. The other test samples (*C. zeylanicum* and TCA) did not show any synergistic effects with infliximab in either of the cell lines.

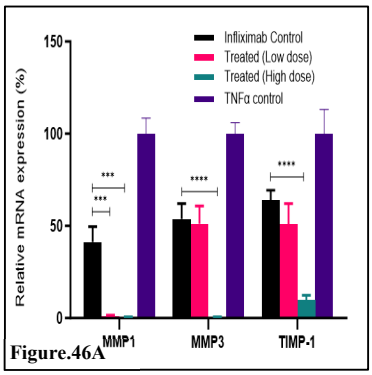


Figure.46A

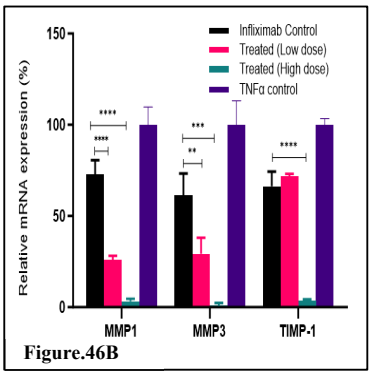


Figure.46B

Figure 46. *C. cassia* aCE was the only sample that showed synergistic activity with infliximab in downregulating *MMP1, MMP2,* and *TIMP-1* mRNAs (A (L929) and B (U937)).

iv. Apoptotic gene panel: BcL-xL, BcL-2 and Bax

BcL-xL and BcL-2 level increased after TNF-α treatment, which was downregulated in the presence of infliximab. *C. cassia aCE* and the bioactive TCA synergistically downregulated BcL-xL and BcL-2 level. Higher doses of *C. cassia* aCE (50 µg/mL) decreased the level of BcL-xL by an additional ~28.0–71.6% (Figures 47A (L929) and B (U937)), while TCA (40 µg/mL) reduced it by ~38.4–48.6% (Figures 48E (L929)and F (U937)). *C. zeylanicum* (50 µg/mL) downregulated BcL-xL by ~25.0–44.9% but did not affect BcL-2 level (Figures 48C (L929) and D (U937)). BcL-2 level was downregulated by an additional ~29.7–60.7% by 50 µg/mL *C. cassia* aCE (Figures 47A (L929) and B (U937)) and by ~53.8–56.1% by 40 µg/mL TCA (Figures 47E (L929) and F (U937)). *BAX,* a pro-apoptotic gene, was additionally upregulated in the presence of aCE (50 µg/mL) (Figures 35 A, B, C, and D). A ~16.5–708.3% increase in *BAX* mRNA was observed with *C. cassia* aCE (Figures 47A (L929) and B (U937)), whereas the extent of

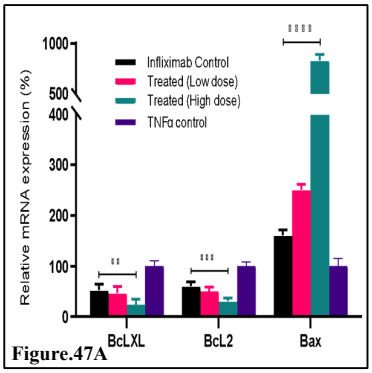


Figure.47A

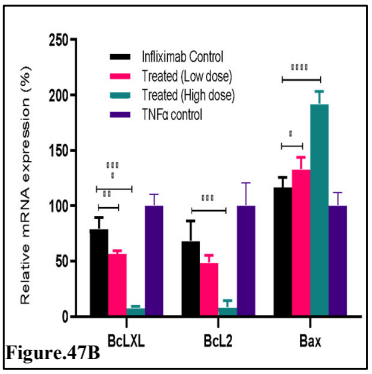


Figure.47B

upregulation was lower (~66.6 – 209.8%) with *C. zeylanicum* aCE (Figures 48C (L929) and D (U937)) and TCA (Figures 48E (L929) and F (U937)).

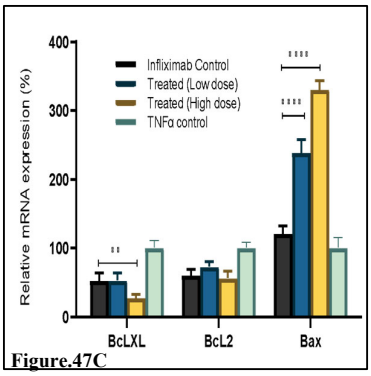


Figure.47C

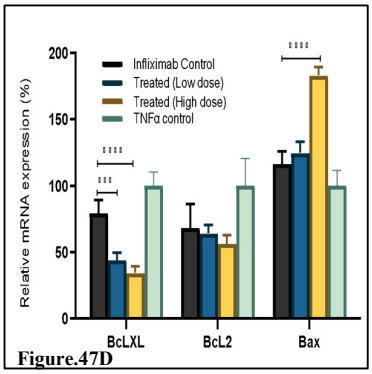


Figure.47D

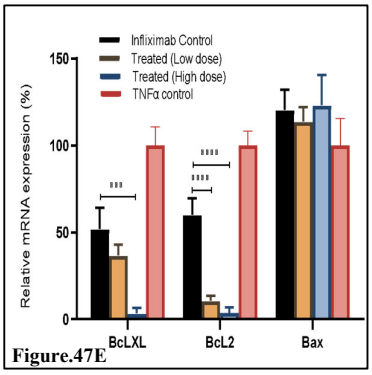


Figure.47E

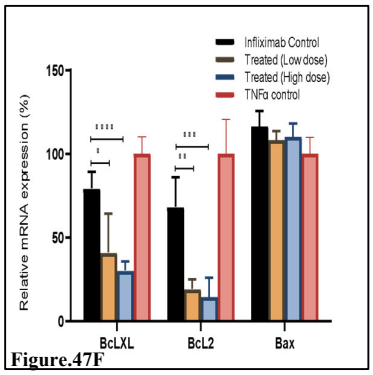


Figure.47F

Figure 47. TCA decreased the level of *Bcl-xL* and *Bcl-2* mRNAs. Downregulation was observed with both cell lines. *C. cassia* aCE in L929 (A) and U937 (B) and *C. zeylanicum* aCE in the L929 (C) and U937 cell lines (D). TCA was also effective in downregulating the *Bcl-xL* and *Bcl-2* mRNAs with an additive effect (Figure 47 E (L929) and F (U937)). The pro-apoptotic *BAX* mRNA was upregulated in the presence of infliximab and aCE (A, B, C, and D). TCA, however, did not affect *BAX* level (E and F).

v. Signaling molecules panel: Jak2, STAT3, and MyD88

Transcription factors such as MyD88, JAK2, and STAT3 are critical regulators of several signaling pathways such as for TLRs (MyD88), IL-6 and IL12 (JAK2 and STAT3) which play a key role in IBD. Hence, we assessed the synergistic

effects of aCE and infliximab on the level of these transcription factors. The *C. cassia* aCE synergistically downregulated MyD88 by ~45.8–61.8% (Figures 49A (L929) and B (U937)), while TCA downregulated it by ~16.5–50.2% (Figures 49E (L929) and F (U937)). *STAT3* was downregulated 25.3–50.4% by *C. cassia* aCE (Figures 49A (L929) and B (U937)) and by ~60.9–89.2% by TCA (Figures 49E (L929) and F (U937)). JAK2 was downregulated ~33.4–88.3% by *C. cassia* aCE (Figures 49A (L929) and B (U937)) and by ~42–79.4% by TCA (Figures 49E (L929) and F (U937)). The *C. zeylanicum* aCE did not show any additive effect on STAT3 levels. For other transcription factors, an additive effect was observed: ~30–34.7% for MyD88 and ~31.8–34.5% for TCA (Figures 49C (L929) and D (U937)).

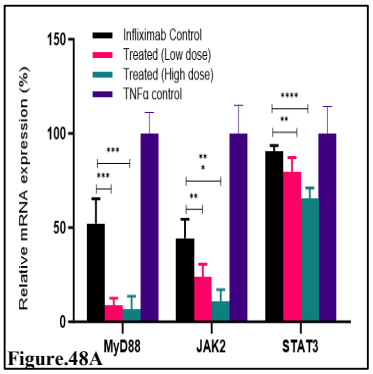


Figure.48A

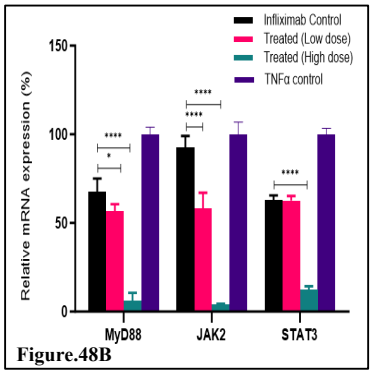


Figure.48B

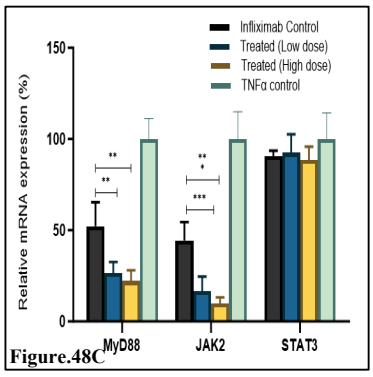


Figure.48C

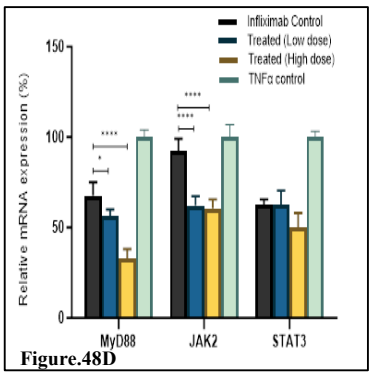


Figure.48D

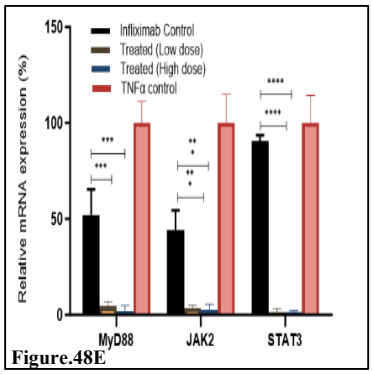


Figure.48E

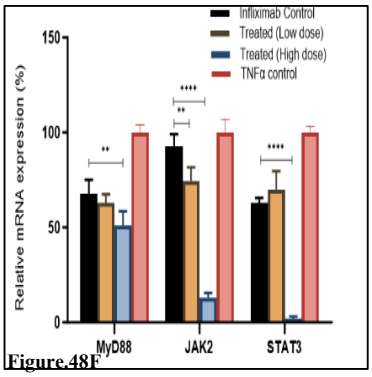


Figure.48F

Figure 48. Downregulation of MyD88 and JAK2 was observed with both *C. cassia* (A (L929) and B (U937)) and *C. zeylanicum* extracts (C (L929) and D (U937)) .*C. cassia* aCE was effective in downregulating *STAT3* (A (L929) and B (U937) synergistically, although *C. zeylanicum* did not affect *STAT3* mRNA level (C (L929) and D (U937)). TCA decreased *MyD88, JAK2*, and *STAT3* mRNA levels synergistically in both cell lines (E (L929) and F (U937)).

6.5 *In vitro* assays with Bevacizumab and aCE

Experiment plan – Based on the data obtained with infliximab synergy experiments, synergy experiments were performed using the following conditions -

i. *C.cassia* aCE and *C.zeylanicum* aCE
   - 150, 50, 25 and 10 µg/mL of aCE concentrations

ii. Bioactive, trans-cinnamaldehyde (TCA)
   - 40, 32, 16, 8 and 4 µg/mL of TCA concentrations

iii. 15 minutes preincubation

a. Effect of aCE on cell viability (HUVEC cells)

First the concentration of cinnamon that was non-toxic to the cells was determined using Alamar Blue for detecting viability. A dose-dependent decrease in cell viability with increasing concentrations of aqueous cinnamon extracts (aCE) in HUVEC cells were observed. *C. cassia, and C. zeylanicum* extracts significantly reduced viability (Figures 49A and B). The highest concentration of aCE was excluded from analysis due to interference with the assay readout. Concentrations below 220 mg/mL were non-toxic to the cells. Similar experiment was performed with cinnamon bioactives trans-cinnamaldehyde and it was non-toxic at concentrations below 100 µg/mL ((Figures 49C). Four concentrations of aCE (150, 50, 25, and 10 µg/mL) and five concentrations of bioactive compounds (40, 32, 16, 8, and 4 µg/mL) were deemed safe for further synergy studies.

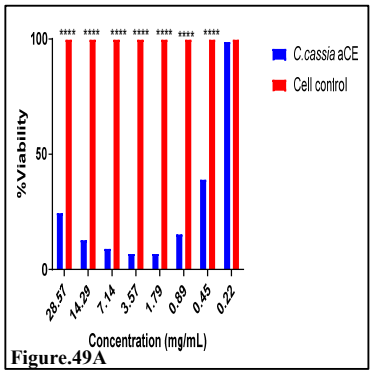


Figure.49A

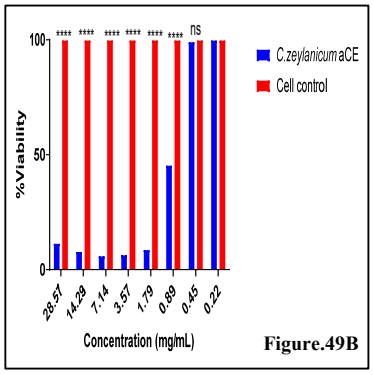


Figure.49B

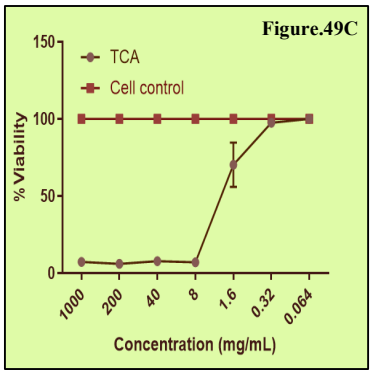


Figure.49C

Figure. 49: Cell viability after treatment with aCE and TCA. The HUVEC cells were treated with aCE of *Cinnamomum cassia* (A), *Cinnamomum zeylanicum* (B), and *TCA* (C) for 72 h, and viability was determined using the redox reagent, Alamar Blue. Experiments were performed thrice, and the data represent mean values.

b. Synergy experiment with aCE in HUVEC cells

i.* Synergy experiments with *C.zeylanicum aCE

C. zeylanicum aCE showed significant synergy (at 3 concentrations) when preincubated with HUVEC cells for 15 minutes as indicated by the EC_{50} shown

in Figure 50A and Table 21. At the lowest concentration of 10 µg/mL, the EC_{50} was 0.2207 µg/mL with a p-value of 0.2898 indicated lack of synergy. At 25 µg/mL, the EC_{50} decreased to 0.2054 µg/mL with a p-value 0.0045, at 50 µg/mL, the EC_{50} was 0.1752 µg/mL with a p-value of 0.0001, and at 150 µg/mL the EC_{50} was 0.1662 µg/mL, with a p-value < 0.0001, demonstrating significant synergy. The relative potency of bevacizumab with aCE was calculated by considering the ratio of bevacizumab's EC_{50} without aCE and that with aCE and converting it to a percentage. The highest synergy in the HUVEC assay system resulted in 137% relative potency (150 µg/mL *C. zeylanicum* aCE) of bevacizumab with aCE against 100% without aCE (Figure 50B).

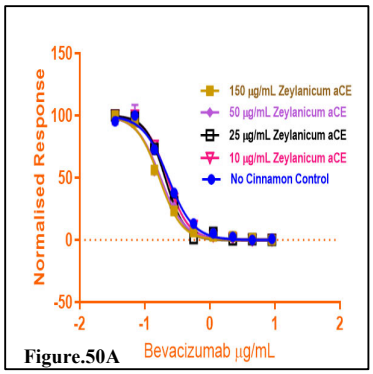


Figure.50A

Figure 50. Synergy experiments with aCE at 15 min preincubation in HUVEC cells. *C. zeylanicum* (A) showed a synergistic effect at 150, 50, and 25 µg/mL aCE concentration. The increases in the relative potency of bevacizumab in presence of *C. zeylanicum* aCE in HUVEC cells (B). p < 0.0005****, p < 0.01**, and p < 0.05*.

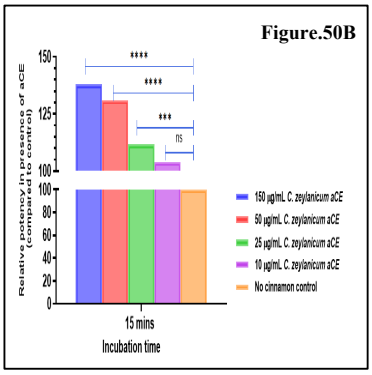


Figure.50B

Table 21: Changes in the EC_{50} and corresponding p-values after the *C. zeylanicum* aCE synergy experiment in the HUVEC cells

aCE concentration (µg/mL)	EC_{50} (µg/mL)	*p*-values
10	0.2207	0.2898
25	0.2054	0.0045
50	0.1752	0.0001
150	0.1662	<0.0001
Control	0.2293	NA

ii. Synergy experiments with *C. cassia* aCE

C. cassia aCE surprisingly did not show significant synergy when preincubated with HUVEC cells for 15 minutes as indicated by the EC_{50} shown in Figure 51 and Table 22. These result may indicate that either the C. cassia is not showing any synergy, or the selected concentrations were not optimal for this assay system. Since *C. zeylanicum* aCE showed significant synergy, further experiments were carried out with only *C. zeylanicum* aCE.

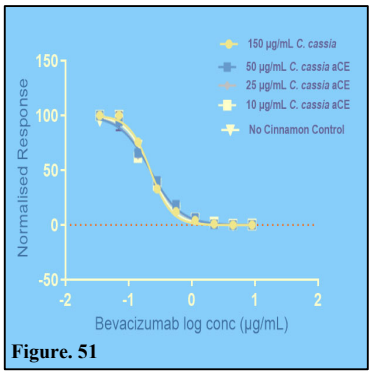


Figure. 51

aCE concentration (µg/mL)	EC_{50} (µg/mL)	*p*-values
10	0.2082	0.0684
25	0.2137	0.1616
50	0.2266	0.7868
150	0.2217	0.3819
Control	0.2293	NA

Figure 51. Synergy experiments with *C. cassia* aCE at 15 min preincubation in HUVEC cells. $p < 0.0005$****, $p < 0.01$**, and $p < 0.05$*.

Table 22: Changes in the EC_{50} and corresponding p-values after the *C. cassia* aCE synergy experiment in the HUVEC cells

iii. Synergy experiments with purified cinnamon bioactive – trans-cinnamaldehyde

The synergy experiments with trans-cinnamaldehyde (TCA) preincubated for 15 minutes in HUVEC cells demonstrated a consistent enhancement in the relative potency of bevacizumab across all tested concentrations as shown in Figure 52A and Table 23. The EC_{50} values decreased as the concentration of TCA increased, indicating an increased potency of bevacizumab. Specifically, at 4 µg/mL of TCA, the EC_{50} was 0.2326 µg/mL with a p-value of 0.0002; at 8 µg/mL, the EC_{50} was 0.2376 µg/mL with a p-value of 0.0001. At higher concentrations of 16 µg/mL, 32 µg/mL, and 40 µg/mL, the EC_{50} values were further reduced to 0.1875 µg/mL, 0.1780 µg/mL, and 0.1348 µg/mL, respectively, with p-values all below 0.0001. These results indicate that trans-cinnamaldehyde enhances the potency of

bevacizumab in HUVEC cells in a dose-dependent manner, with higher concentrations of TCA leading to a more significant reduction in the EC_{50} of bevacizumab. The relative potency of bevacizumab with TCA was calculated by considering the ratio of bevacizumab's EC_{50} without aCE and that with aCE and converting it to a percentage. The highest synergy in the HUVEC assay system resulted in 234% relative potency (40 µg/mL TCA) of bevacizumab with TCA against 100% without aCE (Figure 52B).

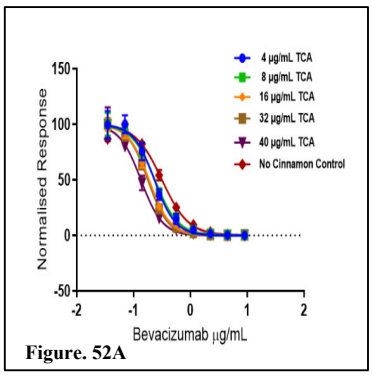


Figure. 52A

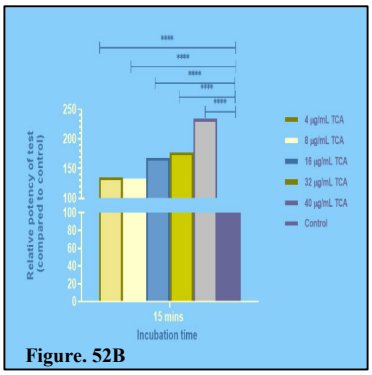


Figure. 52B

Figure 52. Synergy experiments with *trans* - cinnamaldehyde at 15 min preincubation in HUVEC cells. trans - cinnamaldehyde showed a synergistic effect at all tested concentrations (A). The increases in the relative potency of bevacizumab in presence of *C. zeylanicum* aCE in HUVEC cells (B). $p < 0.0005$****, $p < 0.01$**, and $p < 0.05$*.

Table 23: Changes in the EC_{50} and corresponding *p*-values after the *trans* - cinnamaldehyde synergy experiment in the HUVEC cells:

TCA Concentration (µg/mL)	EC_{50} (µg/mL)	P value
4	**0.2326**	**0.0002**
8	**0.2376**	**0.0001**
16	**0.1875**	**<0.0001**
32	**0.1780**	**<0.0001**
40	**0.1348**	**<0.0001**

6.6 Gene regulation analysis

This study aims at assessing the effect of aCE and bevacizumab synergism on genes typically involved in alternate angiogenesis pathways.

Selected gene includes –

- Adhesion molecules: ICAM-1 and VCAM-1
- Interleukins IL-6 and IL-8

- Alternate pathway molecule: Angiopoietin 2 (Ang2) and CXCL5, also known as epithelial neutrophil activating peptide (ENA-78)
- Signaling molecules: JAK2 and STAT3

1. **Results for housekeeping gene selection-**
   - Housekeeping genes – B2M, CYC1, RPLP2, YWHA2, ACTD, and GAPDH were screened to find the best HK gene (whose level remains unchanged across treatments)
   - The top three housekeeping genes identified through Normfinder analysis: CYC1, B2M, and YWHA2.
   - CYC1 exhibited the highest stability with a value of 0.036, making it the best housekeeping gene for normalization, followed by B2M at 0.038 and YWHA2 at 0.050.
   - The remaining genes showed higher stability values, with RPLP2 at 0.121, GAPDH at 0.054, and ACTD at 0.083, indicating less consistency across treatments.
   - The combination of CYC1 and YWHA2 was deemed the best for accurate normalization of gene level data by Normfinder software. (Table 24).

Table 24: Stability Values of Housekeeping Genes for RT-qPCR normalization in HUVEC cells

Gene name	Stability value	Best gene	CYC1
B2M	0.038	Stability value	0.036
CYC1	0.036		
RPLP2	0.121	Best combination of two genes	CYC1 and YWHA2
YWHA2	0.050	Stability value for best combination of two genes	0.028
GAPDH	0.054		
ACTD	0.083		

2. **Results for selected genes**

i. Adhesion molecules: ICAM-1 and VCAM-1

Downregulation of VCAM-1 and ICAM-1 was observed with both *C. zeylanicum* aCE and TCA (Figure 53A and B). *C. zeylanicum* aCE demonstrated effective downregulation of VCAM-1 and ICAM-1 only at the higher concentration of 50 µg/mL of the aCE. TCA also reduced VCAM-1 and ICAM-1 mRNA levels in the HUVEC cell line, with the higher concentration

of 40 µg/mL being more effective than 32 µg/mL. Notably, TCA showed a more pronounced downregulation of ICAM-1 compared to aCE. This suggests that while both *C. zeylanicum* aCE and TCA can downregulate these adhesion molecules, *C. zeylanicum* aCE is not able to bring ICAM-1 and VCAM-1 levels down to baseline while TCA had significant impact on ICAM-1 levels, decreasing its level closer to baseline.

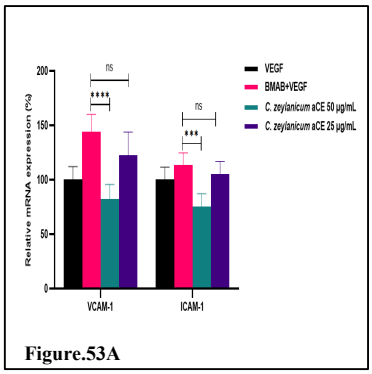


Figure.53A

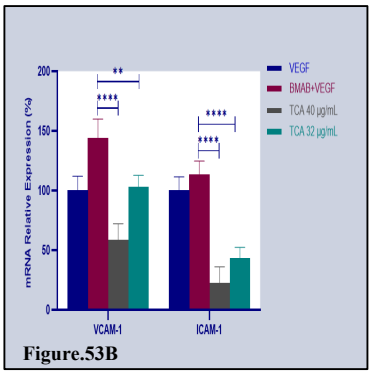


Figure.53B

Figure 53. High (50 µg/mL) concentrations of *C. zeylanicum* aCE (A) and, high (40 µg/mL) and low (32 µg/mL) concentrations TCA (B) downregulated the mRNA levels of *ICAM-1* and *VCAM-1*. Low (25 µg/mL) concentrations of *C. zeylanicum aCE* did not have any effect on *ICAM-1* and *VCAM-1* level.

ii. Interleukins: IL-6 and IL-8

Downregulation of IL-6 and IL-8 was observed with both *C. zeylanicum* aCE and TCA as shown in Figure 54A and B. *C. zeylanicum* aCE effectively downregulated IL-8, with 50 µg/mL showing greater efficacy compared to 25 µg/mL. TCA significantly decreased mRNA levels of IL-6 and IL-8 in the HUVEC cell line, with higher efficacy observed at 40 µg/mL compared to 32 µg/mL. Both doses of TCA markedly reduced IL-8 levels more effectively. Overall, these findings indicate that while both *C. zeylanicum* aCE and TCA can downregulate IL-6 and IL-8, TCA shows superior effect, especially in reducing IL-8 levels. This suggests that while both *C. zeylanicum* aCE and TCA can downregulate these IL-6 and IL-8, *C. zeylanicum* aCE was not able to bring IL-6 levels down to baseline while TCA had significant impact on both IL-6 and IL-8 levels, decreasing its mRNA closer to baseline.

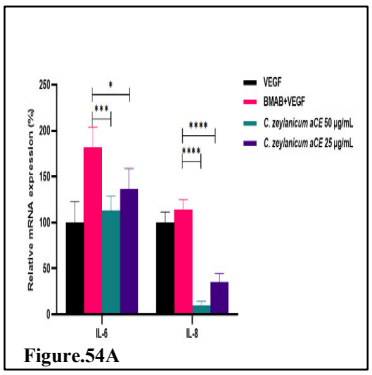


Figure.54A

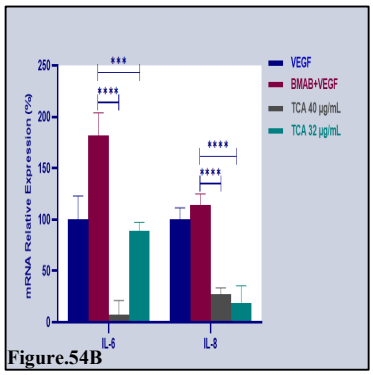


Figure.54B

Figure 54. High (50 µg/mL) and low (25 µg/mL) concentrations of *C. zeylanicum* aCE (A) and, high (40 µg/mL) and low (32 µg/mL) concentrations TCA (B) downregulated the mRNA levels of *IL-6* and *IL-8*.

iii. Alternate pathway molecule: Ang-2 and CXCL5

Downregulation of Ang2 and CXCL5 was observed with both *C. zeylanicum* aCE and TCA as shown in Figure 55A and B. *C. zeylanicum* aCE demonstrated effective downregulation of Ang2 synergistically only at the higher concentration of 50 µg/mL. For CXCL5, both concentrations of *C. zeylanicum* aCE (50 µg/mL and 25 µg/mL) reduced levels, with the higher dose showing a more pronounced effect. TCA significantly decreased mRNA levels of Ang2 and CXCL5 in the HUVEC cell line, with a particularly notable reduction in CXCL5 at both 40 µg/mL and 32 µg/mL. This indicates that TCA is more effective at downregulating CXCL5 compared to *C. zeylanicum* aCE. Overall, both treatments show potential for modulating Ang2 and CXCL5 level, with TCA exhibiting superior efficacy, especially for CXCL5.

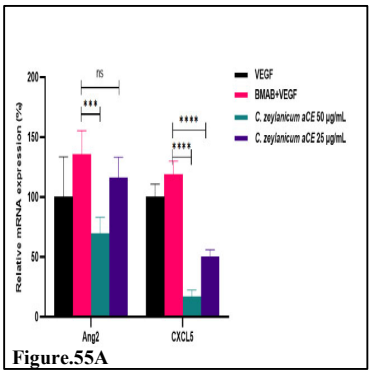


Figure.55A

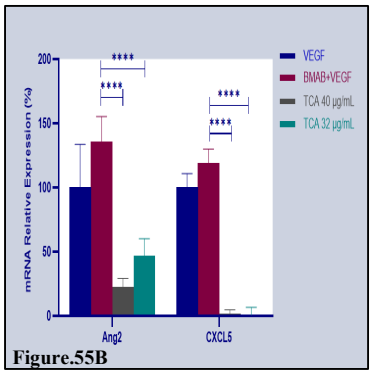


Figure.55B

Figure 55. High (50 µg/mL) and low (25 µg/mL) concentrations of *C. zeylanicum* aCE (A) and, high (40 µg/mL) and low (32 µg/mL) concentrations TCA (B) downregulated the mRNA levels of *Ang2 and CXCL5*.

iv. Signaling molecules: JAK2 and STAT3

Downregulation of JAK2 and STAT3 was observed with both *C. zeylanicum* aCE and TCA as shown in Figure 56 A and B. *C. zeylanicum* aCE was effective in downregulating JAK2, with the higher concentration of 50 µg/mL showing a more pronounced effect compared to 25 µg/mL. Although STAT3 levels were reduced with *C. zeylanicum* aCE, they did not reach basal levels. TCA significantly decreased the mRNA levels of both JAK2 and STAT3 in the HUVEC cell line. Both *C. zeylanicum* aCE and TCA show potential for modulating these critical signaling pathways, with TCA demonstrating superior efficacy, by bringing down the levels to baseline.

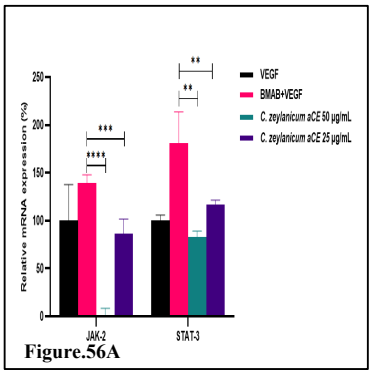


Figure.56A

Figure 56. High (50 µg/mL) and low (25 µg/mL) concentrations of *C. zeylanicum* aCE (A) downregulated the mRNA levels of *JAK2*. High (40 µg/mL) and low (32 µg/mL) concentrations TCA (B) downregulated the mRNA levels of both *JAK2 and STAT3.*

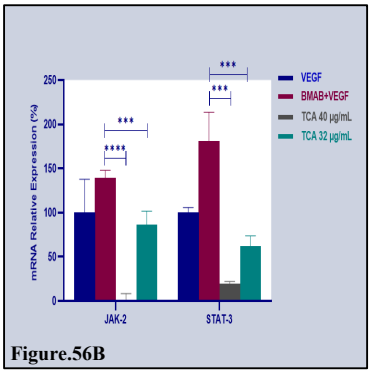


Figure.56B

6.7 Discussion

This study offers valuable insights into synergistic effects of cinnamon aqueous extract when used with the biotherapeutics infliximab and bevacizumab. The results align with earlier research on the bioactive properties of cinnamon, particularly in enhancing the efficacy of treatments for inflammatory diseases and cancer (Pan et al., 2024; Feltes et al., 2023).

i. DNA identification and RP-HPLC

The study successfully identified and characterized different *Cinnamomum* species using DNA-based methods and RP-HPLC analysis respectively, *C. cassia, C. zeylanicum, and C. loureiroi* were identified using Internal transcribed spacer (ITS) which is the spacer DNA situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome (Eui Jeong Doh et al., 2016). The aqueous extract (aCE) was then characterized using RP-HPLC, which revealed that major components of the extracts were trans cinnamaldehyde, cinnamic acid and possibly procyanidins. These findings align with previous metabolomic analyses, such as the UHPLC–HRMS study, which identified 36 metabolites across major cinnamon species, highlighting variations in compounds like cinnamaldehyde, methoxy cinnamaldehyde, and coumarin. The UHPLC–HRMS study emphasized that these phytochemical differences have a direct influence on the pharmacological potential of different Cinnamomum species (Wang et al., 2020).

ii. **Synergy experiments and gene regulation data-Infliximab**

One of the aim of this study was to understand whether CAM could act synergistically in infliximab non-response situations. Several herbal products have been reported to help manage chronic inflammatory conditions, such as IBD and RA (Long et al., 2023; Pegah Dejban et al., 2021) Cinnamon, which is known to possess several beneficial properties, including anti-inflammatory activity was selected for the study (Singh et al., 2021). Three cinnamon species, *C. cassia, C. zeylanicum*, and *C. loureiroi*, were selected for the study. These species vary in their chemical composition and contribute differently to various testing conditions and indications (Hayward et al., 2019; Chen et al., 2014). The species-specific effects were also assessed. A significant synergy between infliximab and the aCEs of *C. cassia* and *C. zeylanicum* was observed. Surprisingly, no synergy was observed between the biotherapeutic and the aCE of *C. loureiroi* in phase one of our study. The synergy of two bioactive cinnamon compounds (TCA and CA) was tested and observed that only TCA showed significant synergy with infliximab.

A synergistic effect was observed in two cell lines, L929 (a mouse fibroblast cell line) and U937 (a human monocyte cell line). In this *in vitro* assay, the cells were preincubated with aCE for different durations. The best experimental condition included preincubation for 15 min, followed by the addition of infliximab. A dose-dependent increase in synergy was observed from to 10−50 µg/mL aCE, as was evident from the reduction in the EC_{50} of infliximab. The data pertaining to 150 µg/mL aCE varied between *C. cassia* and *C. zeylanicum*. In terms of relative potency, we observed a 130% increase in the potency of infliximab in the presence of aCE and 180% in presence of TCA. Infliximab alters the level of adhesion molecules, pro-apoptotic genes, and transcription factors (Danese, 2008). Additionally, some studies have shown that TLRs and MMPs contribute to non-response (Meijer et al., 2007, Nie et al., 2022), the level of which is modulated by infliximab. Cinnamon extract and its bioactive compounds possess anti-inflammatory properties and downregulate the level of TLR, MyD88 (H. Zhao et al., 2021), JAK/STAT (Cheng et al., 2020), Bcl/BAX (Kubatka et al., 2020), ICAM-1, VCAM-1 (Liao et al., 2008), and MMPs

(Koppikar et al., 2010). The additive effects of infliximab and aCE and its bioactives on the level of adhesion molecules (ICAM-1 and VCAM-1), innate immune response receptors (TLR-2 and TLR-4), MMPs and TIMP (MMP1, MMP3, and TIMP-1), apoptosis-related genes (Bcl-2, BcL-xL, and BAX), and transcription factors (JAK2, STAT3, and MyD88) was investigated. 25 and 50 µg/mL aCE and 40 and 32 µg/mL bioactive TCA were selected for our phase two experiments to study the synergy between infliximab and aCE at the molecular level.

In a recent study, TLR-2 was identified as one of the markers of a non-response pathway (Nie et al., 2022) In another study, it was reported that infliximab decreased the level of TLR-4 and TLR-5, the study indicated that higher TLR-4 and TLR-5 level exacerbated inflammatory conditions in ankylosing spondylitis (Zhang et al., 2019). TLRs are pattern recognition receptors that are essential components of the innate immune system. TLR signal transduction is mediated via the MyD88-dependent and independent pathways, eventually activating NF-κB and releasing various pro-inflammatory cytokines (C. Zhao et al., 2021).

aCE synergistically downregulated the level of TLR-2 and TLR-4 in the presence of infliximab in this study. However, only *C. cassia* and TCA showed synergy, and the results in the monocytic U937 cell line were more pronounced than those in the L929 fibroblasts. The *C. zeylanicum* extract did not exhibit any synergistic effect in either cell line. Both C. cassia and C. zeylanicum extracts and TCA synergistically decreased MyD88 mRNA levels.

The pathogenesis of IBD is not entirely understood, and reports have indicated that a combination of factors is involved in disease manifestation, including the gut immune homeostasis. Environmental factors and genetic predispositions disrupt the balance between immune cells and intestinal microbes and contribute to an inflammatory environment. Cell adhesion molecules, such as ICAM-1 (required for leukocyte adhesion) and VCAM-1 (required for monocyte-endothelial cell interaction), along with other adhesion molecules, are involved in maintaining gut homeostasis, and their level is elevated under inflammatory conditions (OKUMURA and TAKEDA, 2016; Gu et al., 2017).

In active IBD, leukocytes and macrophages are recruited into the affected mucosa, which results in the production of a milieu of pro-inflammatory cytokines such as TNF-α, interferon (IFN)-γ, interleukin (IL)-6, and IL-1β. Infliximab treatment results in TNF-α neutralization, thereby decreasing the levels of various pro-inflammatory cytokines, including that of TNF-α. Reports indicate that infliximab downregulates ICAM-1 and VCAM-1 mRNA levels in phytohemagglutinin and lipopolysaccharide-stimulated human peripheral blood leukocytes and U937 cells (Moriconi et al., 2011). Yarur et al. (Yarur et al., 2019) have shown that the levels of VCAM-1 and IL-6 were significantly higher in patients that were exposed to multiple anti-TNF-α agents. This observation is significant, as it shows that simply switching the anti-TNF-α agent in non-responders may not be beneficial. Therefore, new approaches should be identified. This study showed that aCE from *C. cassia* and the bioactive TCA significantly decreased the mRNA level of ICAM-1 and VCAM-1 synergistically with infliximab in both cell lines.

MMPs are also upregulated under inflammatory conditions and have been reported to influence gut homeostasis. MMPs are involved in the degradation of the extracellular matrix, cytokines, and adhesion molecules. TIMP neutralizes MMPs, and a balance between TIMP and MMP level maintains MMP activity (Gao et al., 2007). Several MMPs, such as MMP1, 2, 3, and 7 are upregulated in IBD (Mariaule et al., 2021), which alters the MMP-TIMP ratio and further contributes to pathogenesis. Furthermore, in some patients, MMP3 cleaved IgG, thereby affecting the bioavailability of the anti-TNF-α agents and contributing to the lack of response (Barberio et al., 2019). The effects of aCE on the level of MMP1, MMP3, and TIMP-1 were tested. Only C. cassia aCE synergistically decreased the level of MMPs and TIMP-1 in both cell lines, albeit only at higher concentrations. Klimuk et al. (Piotr Adrian Klimiuk et al., 2004) have reported that initial infliximab infusion significantly reduces MMP1, 3, and 9 and TIMP-1 and 2 levels. Our observations agree with published data.

With increasing knowledge regarding the pathobiology of IMIDs, multiple cytokines and receptors have been identified as targets for therapy, which

include members of the cytokine type 1 and 2 families, the transforming growth factor-β family, and TNF-α and its receptors [Takeuchi, 2022]. The cytokine, IL-6, plays an important role in mediating host defense in response to tissue injury or infection, and its level is controlled stringently. An aberrant increase in IL-6 level exacerbates inflammatory conditions (Tanaka et al., 2014). IL-6 binds to and activates the JAK2-STAT3 signaling pathway. Recently, inhibitors of the JAK-STAT pathway, such as tofacitinib, have been found to be effective against IBIDs (Kim and Kim, 2021). However, adverse events associated with tofacitinib such as pulmonary embolism and herpes zoster infection have been reported. Furthermore, this inhibitor is not equally effective against different IMIDs (Cordes et al., 2020).

Over the last decade, the effects of phytochemicals on inflammation have been investigated extensively and the results are encouraging. Moon et al. (Moon et al., 2021) have summarized the effects of a few phytochemicals, such as phenolics, terpenoids, nitrogen-containing alkaloids, and sulfur-containing compounds, which are effective in managing IMIDs. Interestingly, these compounds also affected the JAK-STAT pathway. We demonstrated that *C. cassia* aCE and TCA, when supplemented with infliximab, reduced the mRNA levels of JAK2 and STAT3 in a dose-dependent manner. *C. zeylanicum* aCE does not affect STAT3 level. In addition to JAK-STAT, we checked the mRNA levels of the downstream anti-apoptotic genes, Bcl-2 and Bcl-xL, and the pro-apoptotic gene, BAX. We observed a decrease in Bcl-2 and Bcl-xL mRNA levels, whereas BAX mRNA levels were significantly and synergistically elevated. The level of the Bcl-2 family of genes is mediated via the JAK-STAT pathway (Kaloni et al., 2022), and the ratio of Bax to Bcl-2 has been reported to be a predictor of infliximab response. An increase in the ratio of Bax to Bcl-2 increases apoptosis, which is mediated by increased caspase 3 level (Aghdaei et al., 2018). *C. cassia* aCE showed a dose-dependent effect on all the selected apoptotic genes, whereas *C. zeylanicum* aCE did not affect Bcl-2, and TCA did not affect the BAX mRNA level.

iii. Bevacizumab and aCE

This part of the study addresses the unmet need of availability of CAM in cancers. Angiogenesis is a complex process which is the result of action of multitude of pro-angiogenic factors. In addition to ongoing efforts in developing combinatorial or synergistic therapies using immuno or chemo therapeutic drugs, there is considerable ongoing research on use of natural products in managing cancer.

iv. Synergy experiments and gene regulation data - Bevacizumab

Like infliximab synergy study, here we first checked the *C. zeylanicum, C. cassia* aCE and TCA concentrations that could reduce bevacizumab EC_{50}, followed by gene regulation studies using aCE and TCA concentrations that provided statistically significant observations. To our surprise, we did not observe any synergy with C. cassia aCE. This study focused on few molecules which are known to facilitate alternate angiogenesis pathways in presence of VEGF blockers. We examined the Angiopoietin-2 (Ang2) due to its crucial role in regulating vascular functions, especially in angiogenesis and vascular remodeling. It has been reported that in absence of VEGF, Ang2 levels are increased, and it can promote angiogenesis and vascular remodeling (Felcht et al., 2012).

In first part of this work, it was shown that aCE was able to down regulate a few apoptotic factors, signaling molecules etc. synergistically with infliximab. In case of bevacizumab the synergy was assessed in terms of reducing the level of factors involved in alternate angiogenic pathways. This meant bevacizumab treatment or absence of VEGF might result in upregulation of a particular pro-angiogenic molecule and aCE treatment should decrease its level thus working synergistically with bevacizumab to decrease angiogenesis.

The data generated with *C. zeylanicum* aCE showed that aCE could be used with bevacizumab to inhibit Ang2 levels. Not only did aCE decrease bevacizumab EC_{50} with highest synergy resulting in 137% relative potency (150 µg/mL C. zeylanicum aCE) of bevacizumab with aCE against 100% without aCE, but also decreased the level of Ang2 mRNA at higher (50 µg/mL) aCE

concentration. Similar observations were obtained with TCA, albeit TCA was much more effective than aCE. The highest synergy in the HUVEC assay system resulted in 234% relative potency (40 µg/mL TCA) of bevacizumab with TCA against 100% without aCE and both concentrations (40 and 32 µg/mL) were able to down-regulate Ang2 mRNA. It was observed that decrease in VEGF concentration in presence of bevacizumab lead to an increased Ang2 concentrations. As can be seen from the data, C. zeylanicum aCE and TCA both decreased Ang2 mRNA in an HUVEC based in vitro assay system. Bevacizumab has not been very effective as monotherapy and hence alternate options are being explored and *C. zeylanicum* aCE and TCA appear to be a suitable CAM candidate for same.

CXCL5, one of the several pro-inflammatory cytokines, is integral to the process of angiogenesis. In cancer, CXCL5 is frequently overexpressed, which enhances tumor angiogenesis, thereby providing the tumor with essential nutrients and oxygen for growth and metastasis. Moreover, CXCL5 can attract immune cells to the tumor microenvironment, promoting tumor progression and creating an immunosuppressive environment (W. Zhang et al., 2020). Consequently, CXCL5 is being investigated as a potential therapeutic target in cancer therapy (Sun et al., 2024). Both *C. zeylanicum* aCE and TCA were very effective in decreasing the level of CXCL5 mRNA. Bevacizumab treatment resulted in a moderate increase in CXCL5, which was downregulated to basal level by both aCE and TCA, indicating that their effect is much more direct on this molecule. Both in context of bevacizumab combined therapy and immune inhibitor check point combined therapy (Sun et al., 2024), CXCL5 appears to be an attractive target, which is effectively downregulated by aCE and TCA.

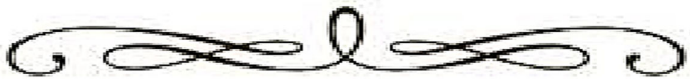
Higher levels of IL-6 in the blood have been linked to poorer outcomes for cancer patients, primarily due to its direct effects on cancer cells (Wang et al., 2017). IL-6 has been reported to activate JAK2/STAT3 pathways which are linked to cancer progression. Reports indicate that IL-6 levels influence the disease severity differently in different conditions and hence monitoring IL-6 levels is crucial for optimizing treatment strategies and improving patient outcomes (Nixon et al., 2021). In our study we observed that TCA decreased

IL-6 mRNA significantly, but *C. zeylanicum* aCE treatment did not have any effect on IL-6 mRNA. Additionally, aCE was not very effective in downregulating STAT3 mRNA to baseline levels. JAK2, STAT3 and IL-6 levels were significantly reduced by TCA.

IL-8 is another pro-tumorigenic cytokine that leads to epithelial to mesenchymal transition which eventually increases invasiveness of the tumor cells. IL8 is also reported to influence angiogenesis by increasing permeability of endothelial cell by disturbing gap junctions (Fousek K et al., 2021). Recently, it was reported that higher levels of aqueous IL-6 and IL-8 had poor response to bevacizumab treatment in neovascular age-related macular degeneration (nAMD) patients (Connolly E. et al., 2024). It appears that combined therapies (anti-VEGF, anti-IL-6 and IL8) would benefit nAMD patients. In this study it was observed that both TCA and aCE were decreased the level of IL-8 mRNA to base line levels.

CHAPTER 7

SUMMARY AND CONCLUSIONS



7.1 Summary and Conclusion

This study demonstrates the potent synergistic effects of cinnamon extracts, particularly *C. cassia*, *C. zeylanicum* and trans-cinnamaldehyde, when combined with infliximab and bevacizumab. The reduction in EC_{50} of both the biotherapeutic agents tested and the modulation of key inflammatory and apoptotic pathways highlight cinnamon's potential as an adjuvant in biotherapeutic treatments. These findings add to the existing research and offer promising avenues for improving the efficacy of treatments for chronic inflammatory diseases and cancer. Key highlights of the study are:

- As is shown in various literature, this study also highlights that cinnamon bioactive composition in extract varies with cinnamon species.
- Of the three species of extract tested, C. cassia, C. zeylanicum showed synergistic activity and thus the selection of cinnamon species as CAM will play significant role in the treatment outcome.
- aCE showed synergistic activity with biotherapeutic monoclonal antibodies Infliximab and Bevacizumab, which resulted in an increase in relative potency of the monoclonal antibodies. These results indicate that aCE when used as CAM may help lower the dose of the biotherapeutic.
- aCE can be used as CAM in infliximab non responders as it downregulates TLRs JAK2/STAT3 and MMPs mRNA, and thus help reduce the disease burden as well as improve patients' quality of life.
- aCE when used with bevacizumab downregulates alternate angiogenesis pathway (Ang 2 and CXCL5 mediated), key cytokines (IL-6 and IL8) and signaling molecules (JAK2 and stat3). Thus, when used as CAM should help during bevacizumab therapy by overcoming the resistance to monoclonal antibodies caused due to activation of alternate pathways.

The findings from this study suggest several important clinical implications. Firstly, the synergistic effects of aCE with infliximab and bevacizumab could enhance treatment efficacy, leading to better outcomes for patients who are non-responsive to monotherapies. This combination therapy might also reduce the likelihood of developing resistance to these treatments by targeting multiple pathways involved in

inflammation and angiogenesis. Additionally, the synergistic effects could allow for lower dosages of infliximab and bevacizumab, minimizing side effects and improving patient tolerance. The study indicates that cinnamon extracts and their bioactives could be used as CAM in both chronic inflammatory conditions and cancer therapy, broadening their clinical applications. The differential effects of various cinnamon species on pro-inflammatory and pro-angiogenic factors suggest the potential for personalized treatment plans based on the most effective species and bioactives for individual patients. Furthermore, using natural products like cinnamon extracts in combination with biotherapeutics could lower treatment costs, making therapies more accessible. Lastly, this research opens new avenues for exploring other natural compounds with similar synergistic effects, potentially leading to the discovery of new treatment combinations. These implications highlight the potential for this research to significantly impact clinical practices and improve patient outcomes in treating inflammatory diseases and cancer.

7.2 Limitations of the study

The study presents several limitations that warrant further exploration. One of the primary limitations is that it was conducted entirely *in vitro*, using cell lines such as L929, U937, and HUVEC. While these models are useful for studying cellular mechanisms, they lack the complexity of whole organisms. *In vivo* studies are needed to confirm the therapeutic potential of cinnamon extracts in conjunction with infliximab and bevacizumab in living systems, such as animal models or clinical trials in humans. Another limitation lies in the focus on the only two bioactives - trans-cinnamaldehyde (TCA) and cinnamic acid. Although TCA exhibited potent synergistic effects in both inflammation and cancer, cinnamon contains a variety of other bioactive compounds that were not fully explored in this study. Investigating whether other components contribute to the synergy or have their own therapeutic effects would provide a more comprehensive understanding of cinnamon's full potential.

While safe concentrations were established for in vitro experiments, the pharmacokinetics, metabolism, and bioavailability of these cinnamon extracts in humans remain unclear. This poses a challenge when translating these findings to clinical applications, especially regarding long-term usage and chronic diseases.

Lastly, while the study examined a range of genes involved in either inflammation or angiogenesis, the list was not exhaustive. We were also not able to study the effect of gene polymorphism of some of these tested genes. It is also important to see what aCE/TCA doses will help both responders and non-responders in case of infliximab therapy OR what concentration of aCE/TCA and bevacizumab will provide meaningful combination therapy in majority of patients. These aspects could provide additional insights into the therapeutic potential and safety of cinnamon extracts when used alongside biotherapeutic agents.

7.3 Recommendations for future research

Future research on the synergistic effects of cinnamon extracts in conjunction with biotherapeutics like infliximab and bevacizumab should aim to address several key areas for a more comprehensive understanding of their therapeutic potential.

Firstly, additional *in vitro* experiments using flow cytometry can be performed. Flow cytometry can be used to study the effects of aqueous cinnamon extract by analyzing cell cycle changes, detecting apoptosis, measuring mitochondrial membrane potential, and assessing reactive oxygen species production. Further to determine the impact of aqueous cinnamon extract on the migratory and invasive capabilities of HUVEC cells, trans well experiments can be performed.

In vivo studies are essential to validate the promising *in vitro* findings. Animal models can provide insights into the pharmacokinetics, metabolism, and bioavailability of cinnamon extracts, offering a better understanding of their efficacy and safety in real biological systems.

Expanding the range of bioactive compounds investigated is also crucial. While trans-cinnamaldehyde (TCA) showed significant synergy, other components present in cinnamon may also contribute to its therapeutic effects. Future studies should isolate and characterize these compounds to evaluate their individual and collective impacts on inflammation and angiogenesis. As is clear from the study that selection of cinnamon species will play a role in synergy or combinatorial therapy. Source of cinnamon, preparation of extract, storage of extract should be stringently followed to achieve desirable results.

Furthermore, examining the optimal dosing and administration routes of cinnamon extracts in conjunction with biotherapeutics will help establish practical applications in clinical settings. Research should also consider the long-term effects and safety of cinnamon supplementation, especially for patients undergoing chronic treatments with infliximab or bevacizumab.

Finally, conducting clinical trials with diverse populations will enhance the generalizability of findings and assess the effectiveness of cinnamon extracts as adjunct therapies in various inflammatory and cancer-related conditions.

By addressing these areas, future research can better inform clinical practices and optimize therapeutic strategies for enhanced patient outcomes.

CHAPTER 8

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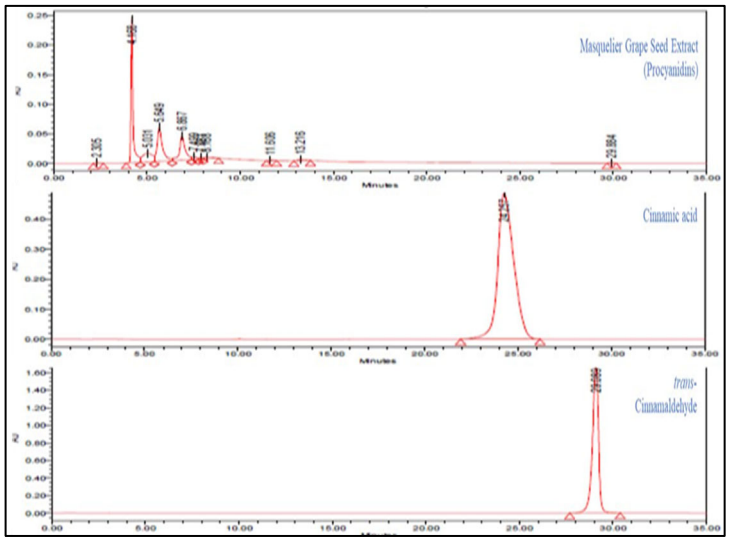
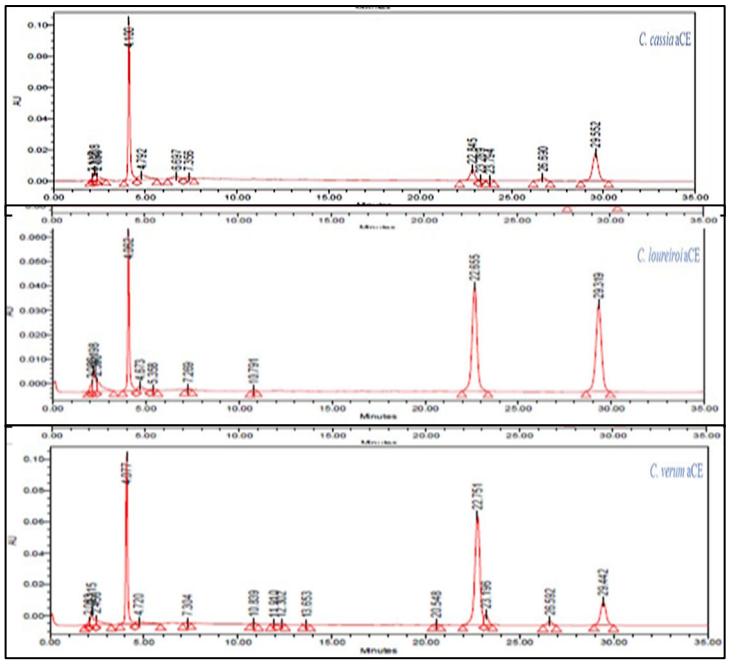
Annexure 1: RP-HPLC data

RP-HPLC of Purified controls and Masquelier grape extract



RP-HPLC of aCE.



BMC Complementary Medicine and Therapies

RESEARCH

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An in vitro study elucidating the synergistic effects of aqueous cinnamon extract and an anti-TNF-α biotherapeutic: implications for a complementary and alternative therapy for non-responders

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Abstract

Background Tumor necrosis factor-alpha (TNF-α) is a critical pro-inflammatory cytokine, and its abnormal production is associated with several immune mediated inflammatory diseases (IMID). Biological anti-TNF-α therapy includes treatment with monoclonal antibodies such as infliximab which have proven successful and are well-tolerated in most patients. Unfortunately, some patients may not respond to therapy (primary non-responders) or may lose sensitivity to the biological agent over time (early and late secondary non-responders). Natural products can reduce inflammation and act synergistically with small molecules or biologics, although evidence remains limited. This study aimed to investigate whether complementary and alternative medicine (CAM) could play a role in infliximab non-responders. Reportedly, cinnamon can help manage chronic inflammatory conditions owing to its anti-inflammatory properties.

Methods We studied the synergistic effects of cinnamon and infliximab in vitro using a two-step approach. First, we investigated whether cinnamon and infliximab act synergistically. Second, we selected conditions that supported statistically significant synergy with infliximab and studied the mRNA expression of several genes involved in non-response to infliximab. We used aqueous cinnamon extract (aCE) from *Cinnamomum cassia*, *Cinnamomum zeylanicum*, and *Cinnamomum loureiroi* and bioactive trans-cinnamaldehyde (TCA), cinnamic acid (CA), and eugenol to study the synergy between infliximab and aCE/bioactive compounds using bioassays in fibroblast (L929) and monocytic (U937) cell lines, followed by qPCR for molecular-level insights. TCA, *C. cassia* aCE, and *C. zeylanicum* aCE demonstrated a dose-dependent synergistic effect with infliximab. Moreover, we saw differential gene expression for adhesion molecules, apoptotic factors, signaling molecules, and matrix remodelers in presence and absence of aCE/bioactives.

Results CAM supplementation was most effective with *C. cassia* aCE, where a synergistic effect was observed for all the tested genes specifically for MMP-1, BcL-xL, Bax and JAK2, followed by TCA, which affected most of the tested genes except TLR-2, MMP1, MMP3, TIMP-1, and BAX, and *C. zeylanicum* aCE, which did not affect ICAM-1, VCAM-1, TLR-2, TLR-4, MMP1, MMP3, TIMP-1, and STAT3.

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Review Article

Aqueous Extract of Cinnamon (*Cinnamomum* spp.): Role in Cancer and Inflammation

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Cinnamon (*Cinnamomum* spp.; family Lauraceae), a plant widely used as a spice and flavoring agent and in the perfume industry, has high therapeutic value. However, the components and chemical properties of cinnamon extracts vary depending on the part of the plant, the method, and the solvent used for extraction. Green extraction methods using safe and green solvents have gained increased interest in recent years. Water is an environmentally friendly and safe green solvent widely used for preparing cinnamon extracts. This review focuses on the various preparation techniques for the aqueous extract of cinnamon, its major bioactive components, and their beneficial roles in different pathological conditions, specifically cancer and inflammation. The aqueous extract of cinnamon contains several bioactive compounds, such as cinnamaldehyde, cinnamic acid, and polyphenols, and exerts anticancer and anti-inflammatory properties by altering key apoptotic and angiogenic factors. The whole extract is a better anticancer and anti-inflammatory agent than the purified fractions, indicating a synergistic effect between various components. Studies have indicated that aqueous cinnamon extract has immense therapeutic potential, and to better understand its synergistic effects, extensive characterization of the aqueous extract and its potential to be used with other therapies should be explored.

1. Introduction

According to 2020 statistics, cancer accounts for approximately 10 million cancer-related deaths with approximately 19.3 million cancer cases worldwide [1]. Carcinogenesis is the progressive transformation of normal cells into neoplastic cells, comprising four phases: tumor initiation, tumor promotion, malignant conversion, and tumor progression. The first phase of carcinogenesis, tumor initiation, is marked by changes in DNA (deoxyribonucleic acid) brought about by physical or chemical carcinogenic stimuli [2]. Changes in DNA lead to oncogene-mediated activation or tumor suppressor gene-mediated suppression of various genes [2, 3]. During tumor promotion, genes involved in cell proliferation and vascularization are activated, resulting in the formation of neoplastic cells. In the malignant conversion step, the preneoplastic cells transform into cells expressing malignant phenotypes. In the tumor progression stage, the tumor aggressively proliferates and invades other tissues, accumulating several different mutations [3]. Hanahan [4] reviewed the hallmarks of cancer, providing a detailed description of changes occurring in a cell moving from a normal to a transformed state (Figure 1).

Approximately 25% of cancer cases have a history of chronic inflammation or infectious diseases [5, 6]. Extrinsic factors, including smoking and infections, and intrinsic factors, such as tumor-associated inflammation, contribute to an inflammatory tumor microenvironment (TME) [7]. Additionally, changes in cell physiology and metabolism result in an increase in stress-related inflammatory markers [8]. Cancer treatment using the conventional approach and biotherapeutics could also induce inflammation and has been extensively studied in recent years [6].

Inflammation is a protective response elicited by cells when tissue damage is caused by infection, trauma, exposure to a toxin, or physical or ischemic injury. A successful inflammatory response leads to homeostasis and inflammation resolution upon the removal of the stimulus [7, 9]. However,