"In Vitro Evaluation of Anti-Inflammatory Activity and Synergistic Effect of a Polyherbal Formulation of Hydroalcoholic Extracts of *Curcuma longa* and *Zingiber officinale* Using Protein Denaturation Inhibition Assay"

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Abstract

The present study aimed to evaluate the in vitro anti-inflammatory activity of hydroalcoholic extracts of *Curcuma longa* and *Zingiber officinale*, individually and in combination, using the protein denaturation inhibition assay. The plant materials were collected, authenticated, dried, and extracted using Soxhlet extraction with a 70:30 ethanol-water solvent system. The percentage yields of the extracts were 8.50% for *Curcuma longa* and 7.60% for *Zingiber officinale*. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, phenolics, tannins, terpenoids, and glycosides. Physicochemical standardization demonstrated acceptable moisture and ash values, along with good solubility in polar solvents. Both extracts exhibited concentration-dependent inhibition of protein denaturation. At 100 µg/mL, *Curcuma longa* and *Zingiber officinale* showed 70.44% and 67.36% inhibition, respectively, whereas the polyherbal combination exhibited 75.98% inhibition. Diclofenac sodium, used as the standard drug, demonstrated 77.83% inhibition. The combined formulation showed greater inhibitory activity than either individual extract, suggesting a potential beneficial interaction between their phytoconstituents. However, further studies are required to establish synergism conclusively. The findings support the potential of these medicinal plants as sources of natural anti-inflammatory agents and warrant further investigation through in vivo studies and mechanistic evaluations.

Keywords: *Curcuma longa*, *Zingiber officinale*, anti-inflammatory activity, protein denaturation inhibition, polyherbal formulation, hydroalcoholic extract, synergistic effect.

Introduction:

Inflammation as defense mechanism against viruses, poisons, allergens, and tissue damage. In order to eradicate dangerous substances and restore tissue homeostasis, immune cells and inflammatory mediators must be activated. Chronic conditions such arthritis, asthma, cardiovascular illnesses, diabetes, and cancer can result from prolonged or severe inflammation, despite the fact that inflammation is necessary for defense and healing (Medzhitov, 2008). Despite their effectiveness, conventional anti-inflammatory medications like corticosteroids and NSAIDs can cause side effects such immunosuppression, nephrotoxicity, and stomach discomfort with prolonged usage (Vane & Botting, 1998).

Because they contain bioactive phytochemicals with anti-inflammatory and antioxidant qualities, including as flavonoids, alkaloids, tannins, phenolics, and terpenoids, employed extensively safer options inflammatory illnesses (Pan et al., 2014). Because synergistic combinations of medicinal plants may offer increased therapeutic efficacy with less toxicity, polyherbal formulations have recently drawn a lot of interest (Parasuraman et al., 2014).

The protein denaturation test is a popular in-vitro technique for assessing anti-inflammatory efficacy. Inflammatory and autoimmune responses may result from protein denaturation, which causes structural changes and loss of protein function. Thus, substances that may stop the denaturation of proteins are regarded as possible anti-inflammatory medicines (Mizushima & Kobayashi, 1968). objectively therapeutic polyherbal extracts current focuses on assessing their anti-inflammatory efficacy utilizing the protein denaturation technique.

1.2 Inflammation

1.2.1 Definition of Inflammation

The body's tissues use sophisticated against viruses, poisons, allergens, and tissue damage. It is a crucial part that aids removal dangerous substances and the start of tissue restoration. To restore normal physiological function, the inflammatory response entails coordinated interactions between immune cells, blood vessels, and chemical mediators (Medzhitov, 2008).

Due to increased blood flow, damage, discomfort, (Ferrero-Miliani., 2007). Either its length and intensity. While chronic inflammation lasts for extended

periods of time and is linked to conditions diseases, acute inflammation is transient and beneficial (Chen et al., 2018).

Numerous chemicals, including as histamine, prostaglandins, leukotrienes, influence advancement inflammatory illnesses might these mediators, oxidative and reactive oxygen species (Nathan & Ding, 2010; Biswas, 2016).

1.2.2 Types of Inflammation

Acute Inflammation

Illness, allergies, or harmful chemicals is known as acute inflammation. It often lasts for a few hours to many days and develops quickly. Acute inflammation's primary goals are to start tissue healing and get rid of damaging stimuli (Abbas et al., 2021).

Vasodilation, increased vascular permeability, fluid exudation, and neutrophil recruitment to the afflicted region are its hallmarks. Typical clinical symptoms. These reactions are controlled by (Punchard 2004). After the damaging agent is removed, acute inflammation usually goes away entirely, but unresolved inflammation can develop into chronic inflammation.

Examples of acute inflammatory conditions include appendicitis, tonsillitis, abscesses, burns, and allergic reactions.

Chronic Inflammation

A persistent infection, autoimmune diseases, extended exposure to irritants, or the inability of acute inflammation to go away can all cause which protracted. In contrast to acute inflammation, fibroblasts, are involved chronic instead of (Kotas & Medzhitov, 2015).

Cytokines, growth factors, and reactive oxygen species are continuously released during chronic inflammatory reactions, which lead to tissue deterioration and fibrosis. Chronic illnesses are mostly caused by persistent cyclooxygenase pathways (Lawrence, 2009).

Numerous conditions, such as rheumatoid arthritis, diabetes mellitus, cardiovascular illnesses, neurological diseases, linked to chronic inflammation. Chronic inflammation can result in irreparable tissue and organ damage because of its protracted nature (Furman et al., 2019).

1.2.3 Pathophysiology of Inflammation

A number of vascular, cellular, and biochemical processes that are brought on by tissue damage, infection, or damaging stimuli make up the pathophysiology

of inflammation. When immune cells like macrophages and mast cells use pattern recognition receptors to identify infections or injured tissues, pro-inflammatory mediators are released and inflammatory signaling pathways are activated, initiating the inflammatory response (Takeuchi & Akira, 2010).

Vasodilation, which increases afflicted is one of first symptoms. Increased vascular permeability follows, allowing leukocytes and plasma proteins to enter damaged tissues and result in edema and swelling (Majno & Joris, 2004). Another significant event is leukocyte recruitment, in which move damage adhesion, rolling, chemotaxis.

Production cytokines, lysosomal enzymes, reactive oxygen species, activated leukocytes eradicate pathogens and cellular detritus. Despite the preventive nature of these defensive mechanisms, tissue damage and chronic inflammatory disorders can result from excessive inflammatory responses (Mittal et al., 2014). After detrimental stimuli are removed, inflammation is resolved by tissue healing, death of inflammatory cells, and reduction of inflammatory mediators. Chronic inflammation and the advancement of the illness may result from the failure of these resolution mechanisms (Sugimoto et al., 2016).

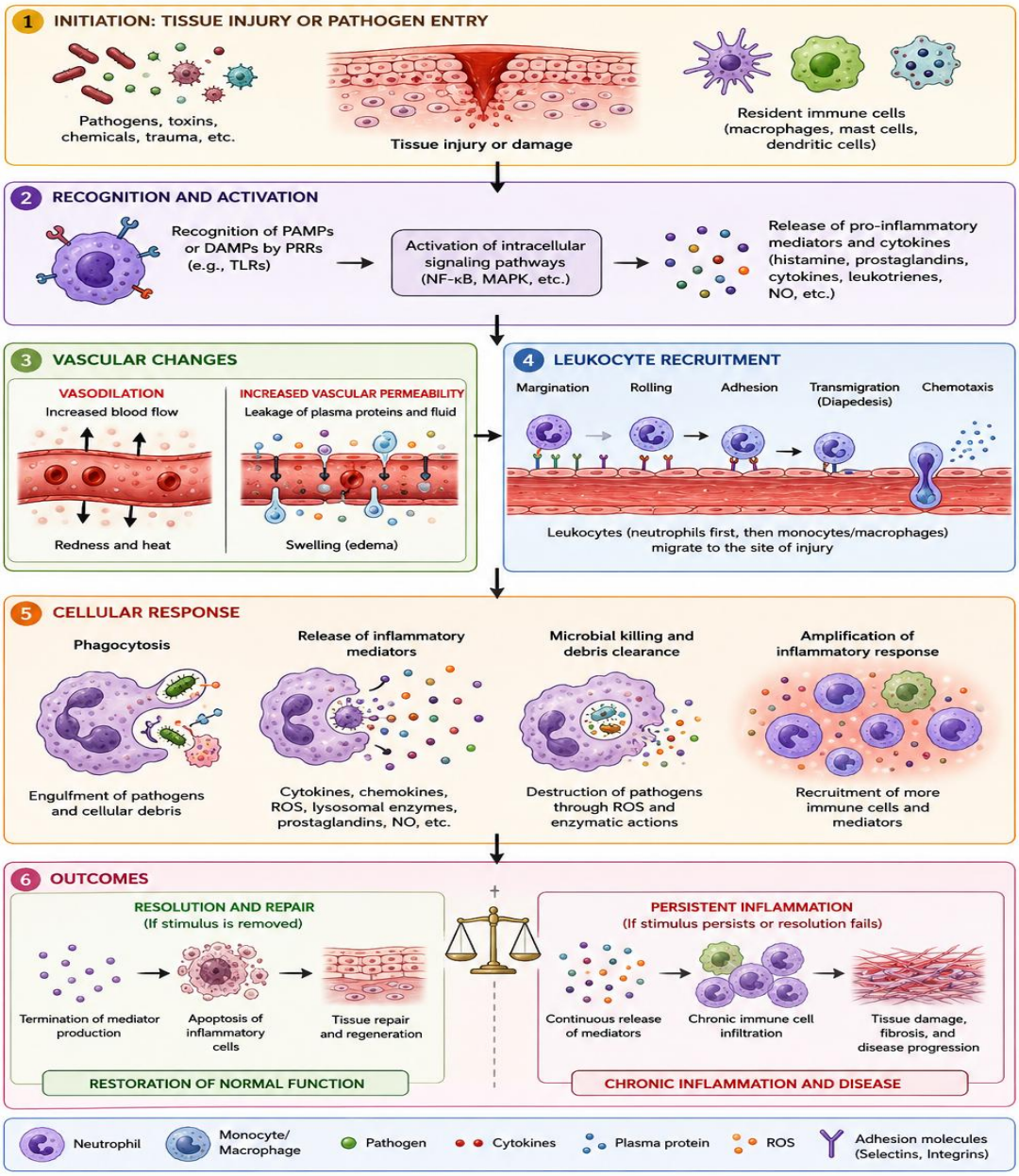

Figure 1.1: Mechanism of Inflammatory Response

1.2.4 Inflammatory Mediators

Endogenous chemicals known as inflammatory mediators control tissue healing, leukocyte migration, vascular alterations, and pain during inflammation. Mast cells, macrophages, neutrophils, platelets, and endothelial cells are examples of immunological and inflammatory cells that generate these mediators (Dinarello, 2010).

Vasodilation and enhanced vascular permeability are caused by histamine and serotonin, two of the first mediators produced during inflammation. Arachidonic acid-derived prostaglandins and leukotrienes are crucial for

bronchoconstriction, pain, fever, and leukocyte recruitment. Cyclooxygenase inhibition is the primary mechanism by which non-steroidal anti-inflammatory medications prevent prostaglandin formation (Ricciotti & FitzGerald, 2011).

Are examples cytokines that control immune cell activation and inflammatory response amplification. Leukocyte migration is guided by chemokines to areas of tissue damage or infection (Turner et al., 2014). By taking part in vasodilation, microbial destruction, and cellular communication, can contribute inflammation; however, overproduction may cause (Forrester., 2018).

Bradykinin, complement proteins, and coagulation factors are additional significant mediators that improve vascular permeability, pain perception, and immunological responses. Acute and chronic inflammatory disorders are mostly caused by dysregulation of inflammatory mediators.

Table 1.1: Major Inflammatory Mediators and Their Functions

Inflammatory Mediator	Source	Major Function
Histamine	Mast cells, basophils	Vasodilation and increased vascular permeability
Prostaglandins	Arachidonic acid pathway	Pain, fever, inflammation
Leukotrienes	Leukocytes	Chemotaxis and bronchoconstriction
TNF-α	Macrophages	Activation of inflammatory response
Interleukins (IL-1, IL-6)	Immune cells	Cytokine signaling and fever
Nitric Oxide	Endothelial cells, macrophages	Vasodilation and immune defense

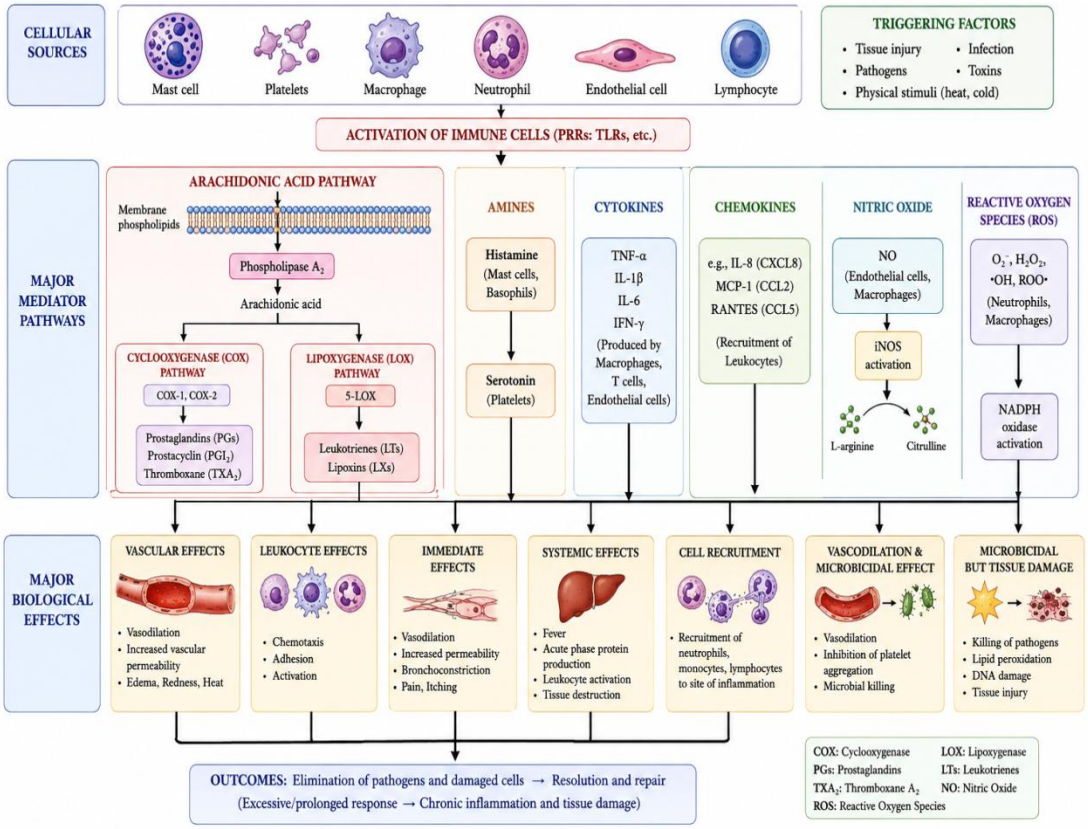

Figure 1.2: Mechanism of Anti-Inflammatory Action of Polyherbal Extracts

1.3 Conventional Treatment of Inflammation

Synthetic medications that inhibit synthesis or action of inflammatory mediators are frequently used to treat inflammatory illnesses. Corticosteroids, analgesics, immunosuppressive medications main components of the traditional therapy strategy. By lowering inflammation-related pain, swelling, redness, and fever, these drugs relieve symptoms. Synthetic medications are quite effective, but long-term usage is frequently linked to toxicity and major side effects (Rainsford, 2007).

1.3.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

One ofgiven treatments treating, arthritis, musculoskeletal diseases, ismain way that work pharmacologically is by blocking prevents thromboxanes that come from the metabolism of arachidonic acid (Sostres et al., 2010).

cyclooxygenase. Numerous tissues produce COX-1 constitutively, and it plays a role in platelet aggregation, control, protection. and mostly linked to fever, pain, and inflammation. While selective COX-2 drugs target the inflammatory pathway with less gastrointestinal (Brune & Patrignani, 2015).

Aspirin, ibuprofen, diclofenac, naproxen, ketorolac, indomethacin, and celecoxib are examples of commonly used NSAIDs. These medications are widely used to treat illnesses including rheumatoid arthritis, osteoarthritis, gout, dysmenorrhea, and postoperative pain because they effectively reduce inflammatory symptoms (Vonkeman & van de Laar, 2010).

Their NSAIDs analgesic and antipyretic qualities. They work by inhibiting prostaglandin-mediated vasodilation, causing edema, and making pain receptors more sensitive. Despite its efficacy, long-term NSAID use number gastrointestinal bleeding, gastric renal failure, cardiovascular risks (Sostres et al., 2010).

1.3.2 Steroidal Anti-Inflammatory Drugs

Medications, sometimes as corticosteroids or glucocorticoids, are strong immunosuppressive medications severe autoimmune inflammatory conditions. These medications function by controlling immunological responses. They are synthetic analogs of cortisol generated by the adrenal cortex (Barnes, 2016).

By inducing lipocortin proteins, corticosteroids block phospholipase A2 activity, which stops membrane phospholipids from releasing arachidonic acid. As a result, fewer prostaglandins, leukotrienes, and other inflammatory mediators are produced. Corticosteroids also inhibit leukocyte migration, cytokine synthesis, and the activation of inflammatory cells including lymphocytes and macrophages (Cain & Cidlowski, 2017).

Prednisolone, dexamethasone, hydrocortisone, methylprednisolone, and betamethasone are common steroidal anti-inflammatory medications. Asthma, rheumatoid arthritis, allergic responses, inflammatory bowel illness, autoimmune diseases, and dermatological problems are all often treated with these medications (Barnes, 2016).

Although corticosteroids are very efficient in reducing inflammation, their usage is usually restricted to brief periods of time or under close supervision because long-term use can have serious systemic side effects.

1.3.3 Limitations and Adverse Effects of Synthetic Drugs

Synthetic anti-inflammatory medications are useful in managing inflammatory disorders, but prolonged usage is linked to a number of negative side effects and safety issues. Due to the suppression of protective prostaglandins in the gastric mucosa, non-selective NSAIDs frequently result in gastrointestinal problems

such gastric irritation, peptic ulceration, nausea, dyspepsia, and gastrointestinal bleeding (Lanas & Chan, 2017).

Another significant side effect of NSAIDs is renal damage, particularly in older patients and those with pre-existing kidney diseases. By preventing prostaglandin formation, NSAIDs may lower renal blood flow and impede kidney function. Increased cardiovascular risks, such as hypertension, myocardial infarction, and stroke, are also linked to specific NSAIDs and selective COX-2 inhibitors (Bhala et al., 2013).

Serious side effects from long-term corticosteroid usage include osteoporosis, hyperglycemia, hypertension, adrenal suppression, immunosuppression, weight gain, cataracts, muscular atrophy, and an increased vulnerability to infections. Adrenal insufficiency can also result from abrupt corticosteroid cessation following extended treatment (Liu et al., 2013).

The failure of synthetic anti-inflammatory medications to completely heal chronic inflammatory illnesses is another drawback. The majority of synthetic drugs primarily relieve symptoms and may need to be used which raises risk toxicity drug dependency. Significant obstacles to traditional therapy also include the emergence of medication resistance, high treatment costs, and patient non-compliance as a result of adverse effects (Rainsford, 2007).

Due to these restrictions polyherbal formulations as safer substitutes with fewer adverse effects and better.

1.4 Herbal Medicines in Inflammatory Disorders

1.4.1 Importance of Medicinal Plants

Because they are abundant in bioactive substances with therapeutic qualities, medicinal plants are crucial to both healthcare and drug development. Traditional herbal remedies provide primary significant fraction particularly underdeveloped nations (World Health Organization, 2019).

By scavenging free radicals, blocking inflammatory mediators, and shielding tissues from oxidative damage, substances derived from plants aid in the reduction of inflammation. Because they are more accessible, affordable, biocompatible, and have less side effects than manufactured medications, medicinal plants are also seen as beneficial (Yuan et al., 2016). Medicinal plants are important for pharmaceutical research and development since they

are the source of some current medications, such as aspirin, quinine, and artemisinin (Newman & Cragg, 2020).

1.4.2 Advantages of Herbal Therapy

Because of its natural origin, therapeutic efficacy, and relatively low toxicity, herbal medicine has become more important. For the long-term therapy of chronic inflammatory illnesses, herbal medications are frequently better tolerated (Rates, 2001). Numerous phytoconstituents alkaloids, terpenoids, work in concert to affect several biological processes (Wagner, 2011).

Price and accessibility herbal medicine is another significant benefit, underdeveloped contemporary medical care restricted (World Health Organization, 2019). Additionally, a number of medicinal herbs have immunomodulatory and antioxidant qualities that aid persistent inflammation (Li et al., 2018). Herbal remedies are being investigated more and more as safer substitutes for traditional anti-inflammatory medications because of these advantages.

1.4.3 Concept of Polyherbal Formulation

In traditional medical systems, particularly Ayurveda, polyherbal formulation—is a key idea. The foundation of polyherbalism is the idea that the combined effects of many herbs are better than those of single plant extracts (Parasuraman et al., 2014).

Synergism, in which many phytoconstituents work together to provide enhanced pharmacological action, is the scientific foundation of polyherbal formulation. Flavonoids, alkaloids, phenolics, tannins, terpenoids, and glycosides are among the bioactive substances found in polyherbal combinations that antibacterial, and qualities (Che 2013). These phytochemicals work by suppressing inflammatory cytokines, scavenging reactive oxygen species, preventing protein denaturation, (Choudhary & Sekhon, 2011).

The capacity of polyherbal formulations to increase therapeutic effectiveness while lowering toxicity and side effects is a significant benefit. According to Wagner and Ulrich-Merzenich (2009), certain herbal components may enhance the bioavailability, stability, and absorption of other active chemicals, hence boosting overall efficacy. These advantages make polyherbal formulations popular for treating inflammatory conditions and other chronic illnesses, and

they are being researched more and more as safer substitutes for pharmaceuticals.

1.5 Polyherbal Extracts

Because of their increased therapeutic efficacy and broad-spectrum pharmacological actions, polyherbal extracts constitute a significant field in herbal medicine and phytopharmaceutical research. Traditional using mixtures of therapeutic herbs for ages. Polyherbal formulations, in contrast to single-herb treatment, are made to take advantage of the synergistic interaction of many phytoconstituents in order to minimize side effects and achieve better therapeutic results. Interest in polyherbal formulations as safer substitutes for synthetic medications has increased recently due to growing scientific effectiveness in treating inflammatory and chronic conditions (Parasuraman et al., 2014).

Numerous physiologically active components, glycosides, tannins, compounds, saponins, polyherbal extracts and together contribute to their pharmacological actions. These phytochemicals may influence immunological responses, stabilize cellular membranes, suppress inflammatory mediators, and exhibit antioxidant action. Polyherbal extracts are thought to be more successful than monotherapy in treating inflammatory illnesses since inflammation is a complex process involving several biochemical pathways (Gertsch, 2011).

1.5.1 Definition and Concept

In order to attain increased therapeutic efficacy, polyherbal extracts are made by mixing two or more extracts from medicinal plants in certain ratios. Synergism, in which individual herbs alone, is the foundation of the polyherbalism notion (Williamson, 2001).

The idea came from ancient Ayurvedic medicine, which uses herbal mixtures to enhance effectiveness, lessen toxicity, and offer comprehensive care. Pharmacodynamic and pharmacokinetic interactions between phytochemicals in polyherbal formulations may enhance absorption, bioavailability, and overall therapeutic efficacy, according to scientific research (Patwardhan et al., 2004; Wagner & Ulrich-Merzenich, 2009).

Polyherbal extracts may control inflammatory cytokines, lower oxidative stress, stabilize proteins and cellular membranes, and inhibit the cyclooxygenase and lipoxygenase pathways in inflammatory diseases. Polyherbal formulations are

quite helpful in chronic inflammatory disorders because of this multitarget strategy.

1.5.2 Advantages of Polyherbal Extracts

Because of their varied phytochemical composition and multitarget therapeutic effect, polyherbal extracts have a number of benefits over synthetic medications and single-herb preparations. Synergistic action, in which many phytoconstituents work together to improve pharmacological efficiency by concurrently influencing several inflammatory pathways, is one of the main benefits (Caesar & Cech, 2019).

Reduced fewer as anti-inflammatory is another significant benefit. Herbal components frequently provide preventive and antioxidant qualities that lessen tissue damage and long-term therapy-related problems (Tilburt & Kaptchuk, 2008). Because some herbal components boost the absorption and stability of active phytochemicals found in other plants, polyherbal extracts may also increase bioavailability.

Furthermore, polyherbal extracts are widely available, reasonably priced, and culturally acceptable in many parts of the world. They are prospective substitutes for chronic inflammatory illnesses due to their long-term appropriateness (Efferth & Koch, 2011).

1.5.3 Therapeutic Importance of Polyherbal Preparations

Because polyherbal formulations can have several pharmacological actions at once, they are of great therapeutic use. According to Mukherjee (2011), formulations frequently skin infections, respiratory metabolic abnormalities, gastrointestinal issues, arthritis, and inflammatory disorders.

Polyherbal preparations work in inflammatory illnesses by scavenging reactive oxygen species, stabilizing proteins and lysosomal membranes, reducing nitric oxide generation, and inhibiting inflammatory enzymes and cytokines. efficiency further enhanced phytochemicals' immunomodulatory antioxidant qualities (Aggarwal et al., 2013).

Because polyherbal formulations have longer-lasting therapeutic effects and fewer negative effects than synthetic medications, they are especially helpful for chronic diseases. Additionally, recent pharmacological research has shown that polyherbal extracts have immunomodulatory, adaptogenic, cytoprotective, and antibacterial properties that make them helpful in multifactorial disorders (Ekor, 2014).

Polyherbal formulations have been scientifically validated by phytochemical and pharmacological investigations, which has reinforced their significance in contemporary healthcare and promoted their usage in the creation of safer and more potent anti-inflammatory treatments.

Literature review;

Introduction to Inflammation and Anti-Inflammatory Therapy

Gong et al.,(2025) stated that "Recent research on anti-inflammatory drugs for depression, both in humans and animals, has shown mixed results. Variations in the study populations' baseline levels of inflammation might be one reason for this diversity. The current meta-analysis was carried out preventive people because older frequently. Changes in depression scale scores and the incidence of depression after therapy were the primary standard mean differences (SMDs) were computed. Thirty-one RCTs—25 of which assessed treatment effectiveness and seven of which assessed preventive effects—met the inclusion criteria out of 3116 examined records. In older individuals with depression, the results showed that anti-inflammatory therapies significantly decreased depressed symptoms when compared to a placebo had positive impacts. These results imply that anti-inflammatory therapies may provide moderate therapeutic advantages for both persons, despite significant variation among trials. To determine the best anti-inflammatory treatments for this group, more excellent randomized controlled studies are necessary."

Xiong et al.,(2025) stated that "Nowadays, a lot of patients make it through the first stage of critical illness before becoming chronic critical illness (CCI). Long-term functional recovery, are characteristics this condition. The estimated incidence of CCI is 34.4 cases per 100,000 people, with a significant rise in older age groups, reaching a peak of 82.1 cases per 100,000 among those between the ages of 75 and 79. Affected patients have a roughly 50% one-year death rate. Persistent inflammation, immunosuppression, and catabolism syndrome (PICS), which was initially identified in 2012, affects a portion of these people. New insights into the involvement of intestinal barrier failure and the proliferation of myeloid-derived suppressor cells (MDSCs) have opened up new therapy options for PICS. Persistent inflammation, a key component of PICS, is still largely unstudied. The underlying pathophysiological changes and molecular processes that PICS are covered in this review. Additionally, it assesses existing anti-inflammatory therapy approaches and offers suggestions for better PICS patient care."

Yue et al.,(2026) reported that "Serious long-term consequences of pancreatitis, a complicated and persistent inflammatory condition that often progresses from acute to chronic phases. These related disorders increase the difficulty of clinical care and highlight the need for more focused and accurate treatment strategies. Treatment approaches have historically concentrated on minimizing tissue damage, lowering oxidative stress, and regulating immunological dysfunction. Nonetheless, recent developments have placed a greater emphasis By reducing chronic inflammation, preventing further tissue damage, and slowing the development of the disease, these strategies hope to improve patient outcomes and recovery. This review highlights the growing role of nanotechnology, especially synthetic and biomimetic nanocarrier systems, in improving targeted drug delivery, boosting bioavailability, and reducing systemic toxicity. It also summarizes its comorbid conditions. Additionally, the possibility of various therapeutic techniques to improve treatment efficacy is examined, including gas-based treatments, gene therapy, functional foods, probiotics, exosome-based therapies, traditional Chinese medicine, and multitarget approaches. Despite these developments, formulation stability, immunological modulation, and extensive clinical translation continue to be major obstacles. Additionally, prospects for the future are examined, including single-cell treatment and early diagnostic interventions. Overall, this analysis highlights the significance of individualized and multimodal therapy approaches to improve clinical results in the management of pancreatitis."

Placha et al.,(2021) stated that "Inflammatory disorders have grown more common, especially in contemporary Western countries, whether they are brought on by excessive mechanical or physiological stress on certain tissues or result from viral, autoimmune, or secondary pathological conditions. Inflammatory reactions are a basic physiological defense mechanism that aims to eliminate toxic stimuli and restore tissue homeostasis, regardless of whether they impact visceral organs, joints, or bone structures. However, this defense mechanism frequently results in serious tissue damage, which in extreme or persistent situations may be permanent. It is well known that environmental stresses, lifestyle choices, and general health issues related to contemporary life have an impact on these inflammatory processes. While the pharmaceutical industry continues to develop innovative drugs targeted at regulating or inhibiting inflammatory pathways and endogenous pro-inflammatory mediators, is being done pathophysiology pathobiochemistry

inflammatory autoimmune illnesses. Repurposing current medications by integrating them into cutting-edge nanotechnology-based delivery systems to enable targeted distribution and controlled release is becoming more popular in addition to freshly created tailored treatments. This study emphasizes the potential for controlling inflammatory disorders by highlighting the current advancements in the production of nanoformulated anti-inflammatory medicines drawn from traditional medication classes, experimental substances, and dietary supplements."

Al et al.,(2024) reported that "Signaling molecules called interleukins (ILs) are essential for restrict excessive inflammatory reactions and aid in tissue healing processes, pro-inflammatory ILs activate immune cells and encourage their recruitment to infection sites. This study highlights the molecular underpinnings behind the functions of both illnesses. Along with new advancements in interleukin-based therapeutic approaches, it also covers their possible uses in diagnostics and treatment."

Pathophysiology and Mediators of Inflammation

Amara et al.,(2024) reported that "Despite tremendous advancements in therapy, millions of people worldwide suffer from serious marked incapacity efficiently with. This review emphasizes how inflammation is becoming more widely acknowledged as a factor in course. Prevalence is still increasing, especially people patients even if incidence has decreased in some areas as a result of better cardiovascular treatment. Endothelial dysfunction, neurohormonal activation, are all part of the intricate, multidimensional process of inflammation in heart failure. Innate immune processes are triggered by myocardial damage, and the course of the illness is further aided by persistent systemic inflammation linked to autoimmune diseases and cardiovascular risk factors. Crucially, there is a reciprocal link between HF and inflammation because HF can increase inflammatory activity both systemically and locally in heart tissue. Important inflammatory biomarkers like are frequently linked to the disease; however, focusing on particular pathways mainly failed, indicating the complexity of immune signaling in heart failure. These difficulties underscore the necessity of ongoing research to better understand inflammatory processes across various provide treatment approaches."

Plan of work :

1. Collection and Authentication of Plant Materials

1.1 Selection of plant parts (rhizomes)

1.2 Collection from authenticated source

1.3 Botanical identification and authentication

2. Preparation of Plant Extracts

2.2 Cleaning, drying, and size reduction of plant material

2.2 Preparation of hydroalcoholic extracts

2.2.1 Soxhlet extraction method

2.3 Filtration and concentration of extracts

2.4 Drying and storage of crude extracts

2.5 Calculation of percentage yield

3. Phytochemical Screening

3.1 Qualitative phytochemical analysis

- Alkaloids
- Flavonoids
- Phenolics
- Tannins
- Terpenoids
- Glycosides

3.2 Preliminary identification of bioactive constituents

4. Standardization of Extracts

4.1 Organoleptic evaluation (colour, odour, texture)

4.2 Physicochemical parameters

- Loss on drying
- Ash values (total, acid insoluble)

4.3 Solubility studies

5. Preparation of Test and Standard Solutions

5.2 Preparation of individual plant extract solutions

5.2 Preparation of polyherbal combination (fixed ratio)

5.3 Preparation of standard anti-inflammatory drug solution (Diclofenac sodium)

6. In Vitro Anti-Inflammatory Study

6.1 Protein Denaturation Assay

6.1.1 Principle of protein denaturation method

6.1.2 Preparation of reaction mixture (egg albumin model)

6.1.3 Incubation conditions (temperature and time)

6.1.4 Treatment with plant extracts and standard drug

6.1.5 Measurement of absorbance (UV-Visible spectrophotometry)

6.1.6 Calculation of % inhibition of protein denaturation

7. Comparative Evaluation

7.1 Individual extract activity (Curcuma longa vs Zingiber officinale)

7.2 Polyherbal combination activity

7.3 Comparison with standard drug

7.4 Assessment of possible synergistic effect

MATERIAL & METHOD

Table 4.1: Materials Used in the Study

S. No.	Material	Specification / Description	Purpose / Use
1	Curcuma longa rhizomes	Fresh, authenticated plant material	Source of hydroalcoholic extract
2	Zingiber officinale rhizomes	Fresh, authenticated plant material	Source of hydroalcoholic extract
3	Ethanol (70% v/v)	Analytical grade	Hydroalcoholic extraction solvent
4	Distilled water	Laboratory grade	Solvent and dilution medium
5	Dimethyl sulfoxide (DMSO)	Analytical grade	Preparation of stock solutions

S. No.	Material	Specification / Description	Purpose / Use
6	Phosphate buffer saline (PBS, pH 6.8)	Standard buffer solution	Reaction medium for assay
7	Fresh egg albumin	Biological reagent	Protein substrate for denaturation assay
8	Diclofenac sodium	Standard drug	Positive control for anti-inflammatory activity
9	Whatman No. 1 filter paper	Standard grade	Filtration of extracts

Table 4.2: Instruments Used in the Study

S. No.	Instrument	Specification / Model (if applicable)	Purpose / Use
1	Soxhlet apparatus	Standard glass assembly	Continuous extraction of plant material
2	Rotary vacuum evaporator	Laboratory model	Concentration of hydroalcoholic extracts under reduced pressure
3	UV-Visible spectrophotometer	Standard analytical instrument	Measurement of absorbance at 660 nm
4	Analytical balance	Electronic (high precision)	Accurate weighing of samples and reagents
5	Hot air oven	Temperature controlled	Drying of extracts and determination of loss on drying

6	pH meter	Digital pH meter	Measurement of buffer and solution pH
7	Magnetic stirrer	Laboratory grade	Homogeneous mixing of solutions
8	Water bath	Temperature controlled	Heating during protein denaturation assay
9	Centrifuge	Laboratory model	Separation of insoluble particles (if required)
10	Glassware (beakers, test tubes, pipettes, volumetric flasks)	Borosilicate glassware	Preparation and handling of solutions

Table 4.3: Plant Profile of Curcuma longa (Turmeric)

Category	Description
Family	Zingiberaceae
Biological Source	Dried rhizomes of Curcuma longa Linn.
Common Name	Turmeric
Habitat & Distribution	Widely cultivated in tropical and subtropical regions, especially India, China, and Southeast Asia
Morphology	Perennial herb with short stem; large, oblong, lanceolate leaves; underground branched rhizomes
Macroscopic Characters (Rhizome)	Yellow to deep orange color; aromatic odour; bitter, slightly pungent taste
Major Phytoconstituents	Curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin), volatile oils (turmerone, atlantone, zingiberene)

Pharmacological Activities	Anti-inflammatory, antioxidant, antimicrobial, hepatoprotective, anticancer
Mechanism of Anti-inflammatory Action	Inhibition of COX and LOX enzymes, suppression of NF-κB pathway, reduction of pro-inflammatory cytokines

Table 4.4: Plant Profile of Curcuma longa of Zingiber officinale (Ginger)

Category	Description
Family	Zingiberaceae
Biological Source	Fresh or dried rhizomes of Zingiber officinale Roscoe
Common Name	Ginger
Habitat & Distribution	Cultivated in India, China, Africa, and tropical regions worldwide
Morphology	Perennial herb reaching 0.6-1 m height; thick, branched aromatic rhizomes
Macroscopic Characters (Rhizome)	Light yellow to brown color; strong characteristic pungent odour; spicy taste
Major Phytoconstituents	Gingerols, shogaols, paradols, zingerone, volatile oils
Pharmacological Activities	Anti-inflammatory, antioxidant, antiemetic, analgesic, antimicrobial

Mechanism of Anti-inflammatory Action	Inhibition of prostaglandin and leukotriene synthesis, suppression of cytokines (TNF-α, IL-1β), antioxidant-mediated membrane stabilization

4.1 Collection and Authentication of Plant Materials

Because of their well-established traditional use and demonstrated anti-inflammatory effects, fresh rhizomes of Curcuma longa and Zingiber officinale were chosen for this investigation. Only healthy and mature rhizomes were chosen to maximize the availability of bioactive phytoconstituents after the plant materials were carefully inspected for freshness, lack of fungal contamination, insect infestation, and physical impurities. The plant materials were purchased from a reputable and verified local herbal supplier. Following collecting, the rhizomes were rinsed with distilled water after being properly cleaned under running tap water to get rid of any dust, clinging soil, and other unnecessary material. To reduce the deterioration of heat-sensitive phytoconstituents, the cleaned materials were shade-dried at room temperature for a few days until a stable weight was reached. A mechanical grinder was then used to chop the dried rhizomes into small pieces and coarsely powder them. The resultant powders were then kept apart in airtight containers away from heat, moisture, and direct sunshine until they were needed. A certified pharmacognosist at an accredited institution performed the botanical identification and authentication of both plant materials. The authenticated samples were compared with standard taxonomic traits listed in official herbal monographs and pharmacopoeial references, and voucher specimens were kept and stored in the departmental herbarium for future reference and documentation.

4.2. Preparation of Plant Extracts

4.2.1 Cleaning, drying, and size reduction of plant material

The gathered rhizomes of Curcuma longa and Zingiber officinale were properly washed under running tap water to get rid of dirt, clinging soil, and other extraneous materials. To make sure all pollutants and impurities were gone, they were then rinsed with distilled water. Before processing, excess surface moisture was eliminated with fresh blotting paper. To enable effective and consistent drying, the cleaned plant materials were next chopped into small, uniform pieces using a stainless-steel knife. To minimize deterioration or loss of thermolabile and volatile phytoconstituents, the rhizome pieces were shade-dried at room temperature with adequate ventilation for a few days until they were completely dry. Drying was carried out until the weight remained constant, signifying that the moisture content had been sufficiently removed. After being fully dried, the rhizomes were ground into a coarse powder using a mechanical grinder. This powder was then put through an appropriate screen to provide a consistent particle size distribution for effective solvent penetration during extraction. Until more extraction research was done, the resultant coarse powders of both plant materials were kept apart in clean, dry, sealed amber-colored containers in a cold, dry location.

4.2.2 Preparation of hydroalcoholic extracts

4.2.2.1 Soxhlet extraction method

The Soxhlet extraction technique was used to separate the hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale. Both polar and semi-polar phytoconstituents were effectively extracted using a hydroalcoholic solvent system of ethanol and distilled water (70:30 v/v). The Soxhlet apparatus was filled with a known amount of coarsely powdered plant material in a filter paper thimble, and the round-bottom flask was filled with around 500 mL of solvent. The extraction process was conducted for six to eight hours at the solvent system's boiling point, or until the siphon tube solvent turned colorless, signifying full extraction. In order to ensure complete extraction of bioactive components, the solvent vapors condensed and repeatedly permeated into the plant material. Following completion, the extracts were concentrated using a rotary vacuum evaporator at low pressure after being filtered using muslin cloth and Whatman No. 1 filter paper. Before being used again, the concentrated extracts were kept in airtight containers at 4°C after being further dried on a water bath to produce semisolid masses.

4.2.3 Filtration and concentration of extracts

Following the conclusion of the Soxhlet extraction of Zingiber officinale and Curcuma longa, the resulting hydroalcoholic extracts underwent a two-step purification procedure that included concentration and filtering. After cooling to room temperature, the extract solutions were filtered using muslin cloth and Whatman No. 1 filter paper to remove fine particulate matter and coarse plant debris, producing a clear filtrate. To stop thermolabile components from degrading, the filtrates were next concentrated under low pressure using a rotating vacuum evaporator at temperatures below 50-60°C. After being further dried in a water bath to produce semisolid masses, the concentrated extracts were put into airtight containers that had been previously weighed, labeled, and kept at 4°C until further phytochemical and in vitro analysis.

4.2.4 Drying and storage of crude extracts

To create stable crude extract residues, the concentrated hydroalcoholic extracts from the rhizomes of Curcuma longa and Zingiber officinale were further dried. After rotating evaporation, the semi-solid mass was put onto a porcelain dish that had been previously weighed. It was then dried in a water bath at a temperature that was managed to ensure that all residual solvent was removed without causing heat-sensitive phytoconstituents to degrade. Drying was carried out until a consistent weight was reached, and the extracts were frequently agitated to preserve uniform consistency and promote even evaporation. Depending on their phytochemical makeup, the resulting crude extracts were either solid or sticky masses. They were then scraped, weighed to determine the yield percentage, and put into sealed glass containers with an amber hue. In order to preserve the stability of the bioactive components for additional phytochemical and in vitro anti-inflammatory research, they were appropriately labeled and kept at 4°C to shield them from light, moisture, and oxidation.

4.2.5 Calculation of percentage yield

To assess the effectiveness of the extraction procedure, the percentage yield of the hydroalcoholic extracts derived from the rhizomes of Curcuma longa and Zingiber officinale was computed. An analytical balance was used to precisely record the final weight of each dried extract following the crude extracts' full drying.

The percentage yield was calculated using the following standard formula:

Percentage Yield (%) = (Weight of dried extract / Weight of initial powdered plant material) × 100

Where,

- Weight of dried extract = final mass of the obtained crude extract after drying
- Weight of initial plant material = mass of coarsely powdered rhizome used for extraction

For the purpose of comparison, the yields of the two distinct plant extracts were computed independently and documented. The amount of soluble phytoconstituents recovered from the plant material under the chosen hydroalcoholic Soxhlet extraction conditions is shown by this parameter. Additionally, the achieved percentage yield aids in standardizing the extraction process and guaranteeing repeatability for subsequent pharmacological and phytochemical research.

4.3. Phytochemical Screening

4.3.1 Qualitative phytochemical analysis

Alkaloids

Standard chemical assays were used to perform an initial qualitative phytochemical screening for the presence of alkaloids in the hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale. Alkaloids are substantial bioactive secondary metabolites with important pharmacological properties, such as analgesic and anti-inflammatory actions.

A tiny amount of each crude extract was treated in diluted hydrochloric acid, filtered, and the filtrate was utilized for analysis in order to find alkaloids. After that, the solution was treated independently using certain alkaloidal reagents, such as Dragendorff's, Wagner's, and Mayer's reagents.

- **Mayer’s Test:** On addition of Mayer’s reagent (potassium mercuric iodide), the formation of a cream or pale yellow precipitate indicated the presence of alkaloids.
- **Wagner’s Test:** Treatment with Wagner’s reagent (iodine in potassium iodide solution) resulted in the formation of a reddish-brown precipitate, confirming the presence of alkaloids.

- **Dragendorff's Test:** Addition of Dragendorff's reagent (potassium bismuth iodide) produced an orange or reddish-brown precipitate, further indicating the presence of alkaloids.

The development of distinctive precipitates was used to determine whether alkaloids were present in both plant extracts or not. The preliminary phytochemical profile of the individual and polyherbal formulations meant for anti-inflammatory assessment was established by noting the results individually for each extract.

Flavonoids

Standard phytochemical procedures were used to qualitatively screen the hydroalcoholic extracts from the rhizomes of Curcuma longa and Zingiber officinale for the presence of flavonoids. Flavonoids are well-known bioactive polyphenolic chemicals with strong anti-inflammatory and antioxidant properties, especially when it comes to stabilizing cellular proteins and inhibiting pro-inflammatory mediators.

A tiny amount of each extract was dissolved in the proper solvent (distilled water or ethanol), filtered, and the filtrate was utilized for analysis in order to identify flavonoids. The standard tests listed below were carried out:

- **Shinoda Test:** A little piece of magnesium ribbon and a few drops of strong hydrochloric acid were added to the extract solution. Flavonoids were present when a pink, crimson, or red coloring developed.
- **Alkaline Reagent Test:** An bright yellow hue was seen when a few drops of sodium hydroxide solution were added to the extract; this color vanished when diluted acid was added, indicating the presence of flavonoids.
- **Lead Acetate Test:** When lead acetate solution was added to the extract, a yellow precipitate formed, signifying the presence of flavonoid components.

Based on the distinctive color changes and precipitate development, the presence or lack of flavonoids in each extract was noted. In order to correlate the presence of flavonoids with their possible anti-inflammatory effect in later experimental assessments, these data helped with the initial phytochemical profile of both plant extracts.

Phenolics

Standard chemical assays were used to qualitatively screen the hydroalcoholic extracts made from the rhizomes of Curcuma longa and Zingiber officinale for

phenolic constituents. Because of their capacity to scavenge free radicals and prevent protein denaturation, phenolic compounds are significant secondary metabolites that have been extensively documented for their antioxidant, membrane stabilizing, and anti-inflammatory qualities.

A tiny amount of each crude extract was diluted in ethanol or distilled water and filtered to produce a clear solution in order to identify phenolics. The following tests were then conducted using the filtrate:

- **Ferric Chloride Test:** To the extract solution, a few drops of 5% ferric chloride solution were added. The formation of a blue, green, or bluish-black coloration indicated the presence of phenolic compounds due to complex formation with ferric ions.
- **Lead Acetate Test:** Addition of lead acetate solution to the extract resulted in the formation of a white or creamy precipitate, confirming the presence of phenolic constituents.
- **Gelatin Test (confirmatory):** The extract was treated with gelatin solution containing sodium chloride, and the formation of a white precipitate indicated the presence of tannin-type phenolics.

For each extract, the presence of distinctive color shifts and precipitates was noted as either positive or negative for phenolic chemicals. These findings validated the plant extracts' possible function in demonstrating anti-inflammatory action through protein stabilization pathways and added to the initial phytochemical profile of each extract.

Tannins

Using common phytochemical screening techniques, the hydroalcoholic extracts from the rhizomes of Curcuma longa and Zingiber officinale were qualitatively assessed for the presence of tannins. Because of their capacity to precipitate proteins and maintain biological membranes, tannins, a type of polyphenolic chemicals, are well-known for their astringent qualities and strong anti-inflammatory action.

A little amount of each crude extract was diluted in ethanol or distilled water and filtered to provide a clear solution for tannin identification. The following tests were then performed on the filtrate:

- **Ferric Chloride Test:** A few drops of 5% ferric chloride solution were added to the extract solution. The formation of a blue-black, greenish-black, or dark green coloration indicated the presence of tannins.
- **Gelatin Test:** To the extract solution, 1% gelatin solution containing sodium chloride was added. The formation of a white precipitate confirmed the presence of tannins due to complex formation between tannins and proteins.
- **Lead Acetate Test:** Addition of lead acetate solution resulted in the formation of a bulky white precipitate, further indicating the presence of tannin compounds.

Based on the distinctive color shift and precipitate development seen in each test, the presence or absence of tannins was noted. These results confirmed the plant extracts' possible participation in anti-inflammatory action through protein binding and denaturation inhibition pathways and added to the initial phytochemical profile of both.

Terpenoids

Standard phytochemical procedures were used to qualitatively screen the hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale for terpenoids. Terpenoids are a broad and varied family of naturally occurring chemical substances that have been shown to have strong immunomodulatory, antioxidant, and anti-inflammatory properties, mostly through the regulation of inflammatory mediators and enzyme inhibitory pathways.

A tiny amount of each crude extract was dissolved in the proper solvent (ethanol or chloroform) and filtered in order to identify terpenoids. The following common tests were then performed on the filtrate:

- **Salkowski Test:** After treating the extract solution with chloroform, strong sulfuric acid was carefully added along the test tube's side. Terpenoids were present because a reddish-brown hue formed at the contact.
- **Liebermann-Burchard Test:** Acetic anhydride and strong sulfuric acid were used to treat the extract. The existence of terpenoid chemicals, especially sterols and triterpenes, was verified by the emergence of a blue-green or reddish-brown hue.

The emergence of distinctive hue shifts was noted as proof that terpenoids were present in the plant extracts. These findings supported the extracts of Curcuma longa and Zingiber officinale's possible contribution to anti-inflammatory action through interference with inflammatory signaling pathways and protein stabilizing effects.

Glycosides

Using conventional phytochemical techniques, the hydroalcoholic extracts from the rhizomes of Curcuma longa and Zingiber officinale were qualitatively tested for the presence of glycosides. Glycosides are significant naturally occurring substances that have a variety of pharmacological properties, such as anti-inflammatory, cardioprotective, and antioxidant effects. They are made up of a sugar moiety connected to a non-sugar aglycone part. Each crude extract was diluted in the proper solvent (ethanol or distilled water), filtered, and the clear filtrate was utilized for analysis in order to discover glycosides. The standard tests listed below were carried out:

- **Borntrager's Test (for anthraquinone glycosides):** After hydrolyzing the extract with diluted acid, it was extracted using an organic solvent like benzene or chloroform. Ammonia solution was added to the organic layer following separation. The presence of anthraquinone glycosides was detected by the ammoniacal layer becoming pink to crimson.
- **Legal's Test (for cardiac glycosides):** After treating the extract with pyridine and sodium nitroprusside solution, sodium hydroxide was added. The presence of cardiac glycosides was suggested by the formation of a deep red or pink tint.
- **Keller-Killiani Test (for deoxysugars):** After treating the extract with glacial acetic acid that included ferric chloride, strong sulfuric acid was carefully added. The presence of glycosides was shown by the formation of a brown ring at the contact.

Based on the distinctive color development and interface ring creation in each test, the presence or absence of glycosides was noted. These results supported the potential significance of both plant extracts in anti-inflammatory activity through regulation of inflammatory mediators and protein interaction pathways, contributing to their preliminary phytochemical profile.

4.3.2 Preliminary identification of bioactive constituents

Based on qualitative phytochemical screening results, the bioactive components of the hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale were initially identified. The phytochemical profile of each extract was determined by analyzing the presence or absence of important phytochemical groups, including alkaloids, flavonoids, phenolics, tannins, terpenoids, and glycosides. For each class of chemicals, identification was done by looking for distinctive color changes and precipitate development in certain chemical tests. The findings showed that both extracts consistently included flavonoids, phenolics, tannins, and terpenoids—all of which are well-known for their anti-inflammatory and antioxidant qualities. According to reports, these bioactive components work by scavenging free radicals, inhibiting inflammatory mediators, and stabilizing protein structures against denaturation. It was deduced from the overall phytochemical profile that both extracts contain a wide range of bioactive compounds that may act separately or in concert when combined. This provides a solid scientific foundation for their selection in subsequent formulation development and in vitro anti-inflammatory assessment using the protein denaturation inhibition method.

4.4 Standardization of Extracts

4.4.1 Organoleptic evaluation (colour, odour, texture)

As a first standardization parameter, the hydroalcoholic extracts from the rhizomes of Zingiber officinale and Curcuma longa were evaluated organoleptically. Under typical laboratory circumstances, the organoleptic properties—such as color, odor, and texture—were evaluated both visually and by sensory observation. Following full drying, each extract's color was noted as it offers a preliminary indication of the phytoconstituent makeup. Texture was assessed by tactile inspection of a tiny amount of extract, and odor was assessed by mild sensory examination to detect typical volatile components. In contrast to Zingiber officinale, which had a light brown to brown color, a strong pungent odor, and a somewhat fibrous to sticky texture, Curcuma longa's extract had a yellow to yellowish-brown color, a distinctive fragrant odor, and a little sticky to resinous texture. These organoleptic characteristics guaranteed extract consistency for subsequent phytochemical and pharmacological analysis and functioned as crucial quality control indications for initial standardization.

4.4.2 Physicochemical parameters

By assessing important physicochemical properties, such as loss on drying and ash values (total ash and acid-insoluble ash), the hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale were standardized. These factors are crucial for evaluating crude plant extracts' inorganic concentration, purity, and quality.

4.4.2.1 Loss on Drying (LOD)

To assess the moisture and volatile content of the crude extracts, loss on drying was calculated. Each extract was precisely weighed, put in an evaporating dish that had been tarred and dried, and heated to 105°C in a hot air oven until a steady weight was achieved. Prior to and following drying, the weight difference was noted and utilized for computation.

Loss on drying was calculated using the formula:

LOD (%) = (Initial weight − Final weight / Initial weight) × 100

The results indicated the presence of minimal moisture content in both extracts, ensuring better stability and reduced risk of microbial contamination during storage.

4.4.2.3 Ash Values

Ash values were determined to assess the total inorganic content and presence of earthy or mineral impurities in the extracts.

4.4.2.4 Total Ash

In a silica crucible that had been previously weighed, a known amount of each extract was burned at a temperature of no more than 450°C until a consistent weight of white ash was produced. The entire quantity of inorganic residue left over after the organic matter has completely burned is represented by the total ash.

Total Ash (%) = (Weight of total ash / Weight of sample) × 100

4.4.2.5 Acid Insoluble Ash

After boiling the entire amount of ash with diluted hydrochloric acid, the insoluble material was filtered, cleaned, burned, and weighed. The residue that remains is acid-insoluble ash, which mostly indicates that silica and siliceous materials are present.

Acid Insoluble Ash (%) = (Weight of acid insoluble residue / Weight of sample) × 100

To guarantee the purity and appropriateness of the extracts for additional in vitro anti-inflammatory research, the acquired physicochemical values for both Curcuma longa and Zingiber officinale extracts were utilized as standard quality control measures.

4.5 Solubility studies

The solubility behaviour of the hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale was assessed at room temperature in a variety of solvents, including distilled water, ethanol, methanol, chloroform, and dimethyl sulfoxide (DMSO). To determine the degree of dissolution, a little amount of each dried extract was added individually to the chosen solvents and forcefully agitated. Based on visual inspection, solubility was noted as soluble, somewhat soluble, slightly soluble, or insoluble. The presence of mostly polar phytoconstituents including phenolics, flavonoids, and glycosides was indicated by the extracts' strong solubility in polar organic solvents like ethanol and methanol, moderate solubility in water, and low solubility in non-polar solvents like chloroform. Both extracts, however, showed high solubility in DMSO, which was thought to be appropriate for creating stock solutions for in vitro anti-inflammatory research. The selection of suitable solvent systems for ensuing experimental processes, such as the creation of test solutions for the protein denaturation inhibition test, was aided by this solubility profile.

4.6 Preparation of Test and Standard Solutions

4.6.1 Preparation of individual plant extract solutions

Stock solutions for in vitro testing were prepared using the hydroalcoholic crude extracts of the rhizomes of Zingiber officinale and Curcuma longa, respectively. To create a main stock solution, a known amount of each dried extract was precisely weighed and dissolved in an appropriate solvent system (usually ethanol or DMSO, depending on the solubility profile). To guarantee complete dissolving, the stock solutions were vortexed or sonicated. If necessary, any undissolved particles were then filtered out. To generate the necessary test concentrations for the protein denaturation inhibition experiment, the stock was serially diluted using either distilled water or phosphate buffer saline (PBS, pH 6.8).

4.6.2 Preparation of polyherbal combination (fixed ratio)

In the protein denaturation inhibition test, diclofenac sodium was used as the reference anti-inflammatory medication. To create a stock solution with a defined concentration (1 mg/mL), a precisely weighed amount of diclofenac sodium was dissolved in phosphate buffer saline (PBS, pH 6.8). To guarantee that the medication was completely dissolved, the solution was made by constantly swirling. To achieve working concentrations of 25, 50, 75, and 100 µg/mL for in vitro testing, further serial dilutions were made from this stock solution using PBS. The anti-inflammatory properties of the hydroalcoholic extracts of Curcuma longa and Zingiber officinale, as well as their polyherbal combination, were compared and validated using the generated standard solutions as a positive control.

4.6.3 Preparation of standard anti-inflammatory drug solution

The common anti-inflammatory medication used to compare activity was diclofenac sodium. To create a stock solution with a defined concentration, precisely weighed amounts of diclofenac sodium were dissolved in an appropriate solvent (distilled water or phosphate buffer saline, pH 6.8, depending on solubility). To guarantee that the medication was completely dissolved, the solution was made while being constantly stirred. To get the necessary working concentrations that matched those of the test samples, further serial dilutions were prepared from the stock solution. The in vitro anti-inflammatory efficacy of the individual plant extracts and the polyherbal combination made from Curcuma longa and Zingiber officinale were compared and validated using the produced diclofenac sodium solutions as the positive control in the protein denaturation inhibition experiment.

4.7. In Vitro Anti-Inflammatory Study

4.7.1 Protein Denaturation Assay

The idea behind the protein denaturation method is that when proteins are subjected to external stressors like heat, pH shifts, or chemical agents, they lose their tertiary and secondary structure. Inflammatory reactions and the production of autoantigens are linked to this process. Heat-induced protein denaturation can be stopped or inhibited by substances with anti-inflammatory properties. The capacity of hydroalcoholic extracts of Curcuma longa and Zingiber officinale, as well as their polyherbal formulation, to prevent denaturation of egg albumin

under controlled circumstances was used in this work to assess their anti-inflammatory potential.

4.7.1.1 Preparation of Reaction Mixture (Egg Albumin Model)

Fresh egg albumin was used as the protein source in the reaction mixture. In a test tube, 2.8 mL of phosphate buffer saline (PBS, pH 6.8) and 0.2 mL of fresh egg albumin were combined to create a combination. Different concentrations of test samples (standard medication solution, polyherbal combination, and individual extracts) were added independently to this mixture. Similar steps were taken to make a control solution, substituting an equivalent amount of solvent for any test or standard ingredient.

4.7.1.2 Incubation Conditions (Temperature and Time)

To ensure that the protein and test samples interacted properly, all generated reaction mixtures were incubated at 37°C for 15 minutes. To cause protein denaturation, the solutions were heated to 70°C for five minutes after being incubated at physiological temperature. Prior to measuring absorbance, the samples were heated and allowed to cool to room temperature.

4.7.1.3 Treatment with Plant Extracts and Standard Drug

Curcuma longa and Zingiber officinale hydroalcoholic extracts, as well as their combined polyherbal formulation, were added to the reaction mixtures at varying concentrations. The common anti-inflammatory medication was diclofenac sodium. To guarantee comparability, an equal volume of solvent was supplied to each test sample in separate tubes, and all treatments were conducted under the same experimental circumstances.

4.7.1.4 Measurement of Absorbance (UV-Visible Spectrophotometry)

A UV-Visible spectrophotometer set to 660 nm was used to detect the turbidity caused by protein denaturation during incubation and cooling. Increased protein denaturation is correlated with a rise in absorbance, whereas test sample protection against denaturation is shown by a reduction in absorbance. For analysis, the absorbance of the test, standard, and control samples was measured independently.

4.7.1.5 Calculation of % Inhibition of Protein Denaturation

The percentage inhibition of protein denaturation was calculated using the following formula:

% Inhibition = [(Absorbance of Control − Absorbance of Test) / Absorbance of Control] × 100

For regular diclofenac sodium, all test concentrations of individual extracts, and the polyherbal formulation, the same formula was used. The findings were utilized to assess potential synergistic effects and compare the relative anti-inflammatory capability of the combined extracts of Zingiber officinale and Curcuma longa.

4.8. Comparative Evaluation

4.8.1 Individual Extract Activity (Curcuma longa vs Zingiber officinale)

The protein denaturation inhibition experiment was used to assess the anti-inflammatory properties of distinct hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale. The relative effectiveness of each extract was ascertained by comparing the % inhibition of protein denaturation at various concentrations. Under the experimental conditions, the extract with a larger percentage inhibition was thought to have stronger membrane stabilizing and anti-inflammatory properties.

4.8.2 Polyherbal Combination Activity

The effectiveness of the polyherbal formulation made from extracts of Curcuma longa and Zingiber officinale to prevent heat-induced protein denaturation was assessed. To guarantee direct comparison, the activity of the combination was evaluated at the same concentrations utilized for separate extracts. To determine if the combined formulation had more anti-inflammatory ability than the separate extracts, the observed % inhibition values were utilized.

4.8.3 Comparison with Standard Drug

Diclofenac sodium, a common medication, was used to compare the anti-inflammatory properties of the polyherbal combination with individual extracts. To ascertain the relative effectiveness of the plant extracts, the % inhibition values obtained for test samples were compared to the standard control. For maximal anticipated inhibition under the identical testing circumstances, diclofenac sodium was used as a reference.

4.8.4 Assessment of Possible Synergistic Effect

By contrasting the polyherbal combination's activity with the aggregate and individual impacts of the constituent extracts, the potential synergistic effect was assessed. Potential synergism between the phytoconstituents of Curcuma longa and Zingiber officinale was thought to be indicated by a higher percentage inhibition in the combined formulation than in the separate extracts. This comparison investigation shed light on whether complimentary or synergistic interactions among bioactive substances led to an improved anti-inflammatory response.

RESULT & DISCUSSIONS

5.1 Percentage yield

Curcuma longa and Zingiber officinale rhizomes were hydroalcoholically extracted to provide 8.50% and 7.60% w/w of dry extracts, respectively. Curcuminoids and other extractable polar components may be more prevalent in Curcuma longa, which might explain the increased yield. For the thorough extraction of bioactive phytoconstituents appropriate for further phytochemical and pharmacological analysis, the Soxhlet extraction procedure using a 70:30 hydroalcoholic solvent proved effective.

Table 5.1: Percentage Yield of Hydroalcoholic Extracts

S. No.	Plant Material	Weight of Powdered Drug (g)	Weight of Dried Extract (g)	Percentage Yield (%)
1	Curcuma longa rhizomes	500 g	42.5 g	8.50 %
2	Zingiber officinale rhizomes	500 g	38.0 g	7.60 %

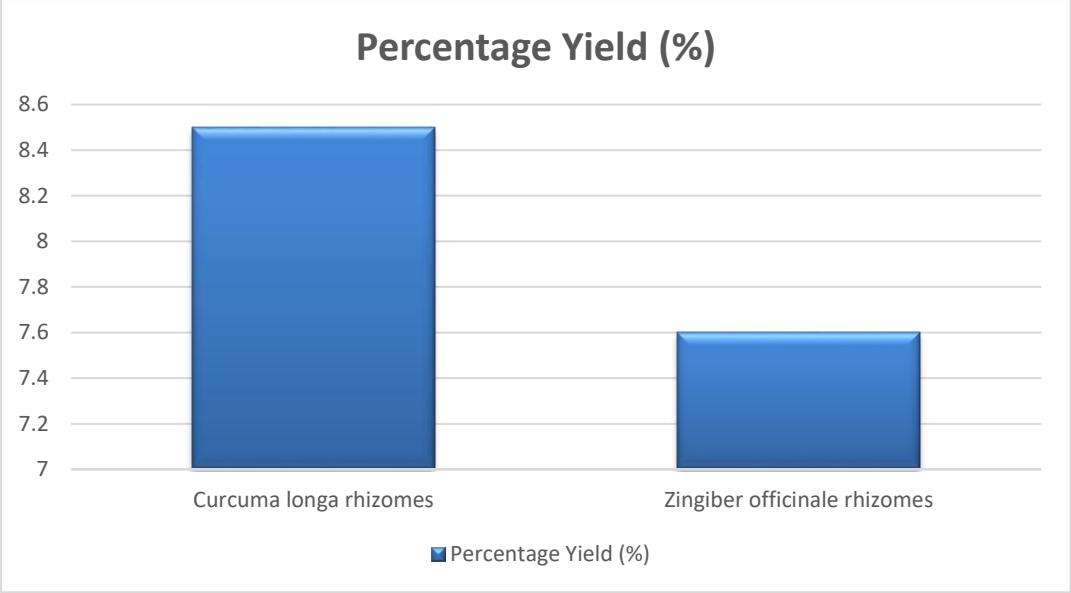

Fig 5.1: Percentage Yield (%)

5.2 Phytochemical Screening

Standard chemical assays were used to conduct a thorough qualitative phytochemical screening of the hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale. The findings demonstrated the significant pharmacological potential of both extracts by confirming the presence of important bioactive components such as alkaloids, flavonoids, phenolics, tannins, terpenoids, and glycosides.

Table 5.2: Detailed Qualitative Phytochemical Screening Results

Phytochemical	Test Name	Observation	Curcuma longa	Zingiber officinale
Alkaloids	Mayer's test	Cream precipitate	+	+
	Wagner's test	Reddish-brown precipitate	+	+
	Dragendorff's test	Orange precipitate	+	+
Flavonoids	Shinoda test	Pink/red coloration	+	+
	Alkaline reagent test	Yellow color (decolorized with acid)	+	+
	Lead acetate test	Yellow precipitate	+	+
Phenolics	Ferric chloride test	Blue/green coloration	+	+

	Lead acetate test	White/cream precipitate	+	+
	Gelatin test	White precipitate	+	+
Tannins	Ferric chloride test	Dark green/black coloration	+	+
	Gelatin test	White precipitate	+	+
	Lead acetate test	Bulky white precipitate	+	+
Terpenoids	Salkowski test	Reddish-brown interface	+	+
	Liebermann-Burchard test	Blue-green coloration	+	+
Glycosides	Borntrager's test	Pink/red ammoniacal layer	+	+
	Legal's test	Deep red/pink coloration	+	+
	Keller-Killiani test	Brown ring formation	+	+

(+ = Positive, Presence confirmed; − = Negative, Absent)

5.3 Preliminary identification of bioactive constituents

Based on the findings from qualitative phytochemical screening, the hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale were further examined for the first identification of bioactive components. The overall phytochemical profile of each extract was determined by evaluating the presence or absence of important phytochemical groups, including alkaloids, flavonoids, phenolics, tannins, terpenoids, and glycosides.

For each class of chemicals, the identification was carried out by analyzing distinctive color shifts and precipitate development seen during certain phytochemical assays. Alkaloids and glycosides were also found, suggesting a wide range of secondary metabolites, and the findings verified the existence of flavonoids, phenolics, tannins, and terpenoids in both extracts. These phytoconstituents have been extensively documented for their anti-inflammatory and antioxidant properties, mainly via scavenging free radicals, inhibiting pro-

inflammatory mediators, and stabilizing protein structures against denaturation caused by heat.

The overall phytochemical profile led to the conclusion that both extracts include a rich mixture of bioactive chemicals that, when combined in a polyherbal formulation, may operate either singly or in concert. This bolsters the scientific justification for choosing these extracts for additional formulation development and for assessing their anti-inflammatory effectiveness in vitro using the protein denaturation inhibition approach.

5.4 Standardization of Extracts

5.4.1 Organoleptic evaluation

Curcuma longa and Zingiber officinale hydroalcoholic extracts were evaluated organoleptically, and the results showed unique and distinctive sensory characteristics. The two extracts' different phytochemical compositions were mirrored in the variations in color, texture, and odor. The legitimacy and acceptability of the extracts for additional physicochemical standardization and in vitro anti-inflammatory research were verified by these organoleptic properties, which also functioned as first quality control measures.

Table 5.3: Organoleptic Evaluation of Hydroalcoholic Extracts

Parameter	Curcuma longa	Zingiber officinale
Colour	Yellow to yellowish-brown	Light brown to brown
Odour	Aromatic, characteristic turmeric odour	Strong pungent characteristic odour
Texture	Slightly sticky, resinous	Slightly fibrous, sticky

5.5 Physicochemical parameters

5.5.1 Loss on Drying (LOD)

Curcuma longa and Zingiber officinale hydroalcoholic extracts were found to have loss on drying (LOD) values of 7.60% and 9.00%, respectively. The extracts' high stability and decreased vulnerability to microbial development and

degradation are shown by the comparatively low moisture content. These findings validate that both extracts are suitable for long-term preservation and further phytochemical and pharmacological analysis.

Table 5.4: Loss on Drying (LOD) of Hydroalcoholic Extracts

S. No.	Plant Extract	Initial Weight (g)	Final Weight after Drying (g)	Loss on Drying (%)
1	Curcuma longa	5.00	4.62	7.60 %
2	Zingiber officinale	5.00	4.55	9.00 %

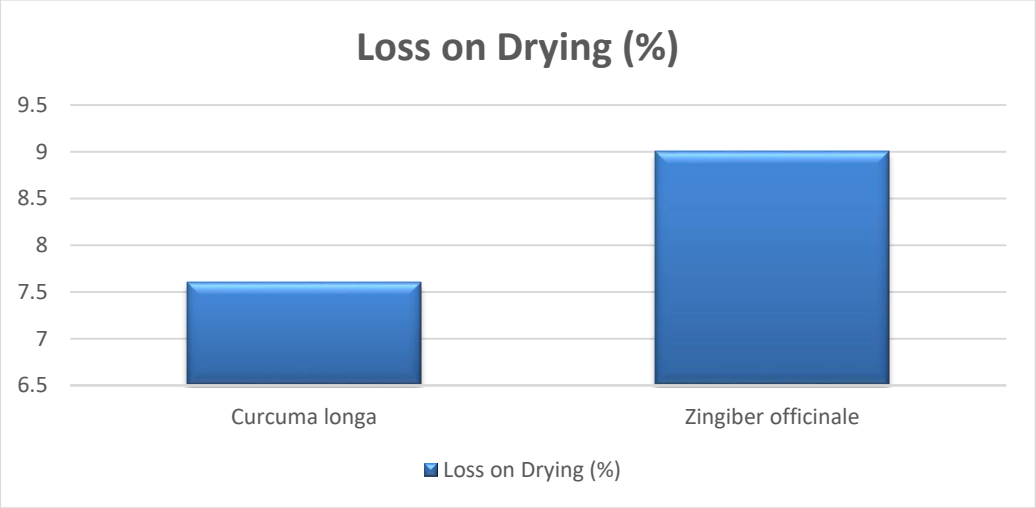

Fig 5.2: Loss on Drying (%)

5.5.2 Ash Values

Curcuma longa and Zingiber officinale hydroalcoholic extracts had total ash levels of 5.40% and 6.20%, respectively, suggesting a moderate inorganic concentration. The extracts' high purity was confirmed by the acid insoluble ash values, which were 1.10% and 1.35%, respectively, indicating low quantities of siliceous and earthy materials. These physicochemical characteristics confirm the extracts' purity and guarantee that they are appropriate for additional in vitro anti-inflammatory research.

Table 5.5: Ash Values of Hydroalcoholic Extracts

S. No.	Plant Extract	Total Ash (%)	Acid Insoluble Ash (%)
1	Curcuma longa	5.40	1.10
2	Zingiber officinale	6.20	1.35

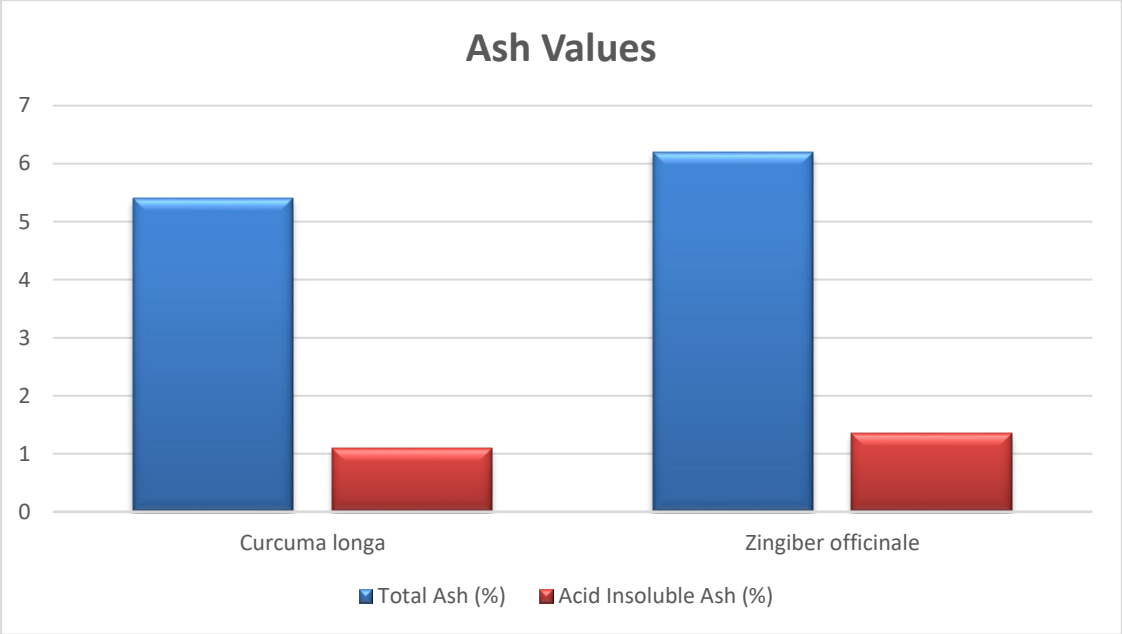

Fig 5.3: Ash Values

5.5.3 Solubility studies

The solubility profile of hydroalcoholic extracts of Zingiber officinale and Curcuma longa rhizomes was assessed in various solvents, and the findings were reported in milligrams per milliliter. In polar organic solvents, both extracts showed somewhat greater solubility. With values of 25.6 mg/mL for Curcuma longa and 24.9 mg/mL for Zingiber officinale, maximum solubility was found in DMSO, suggesting that it is an effective solvent for making stock solutions. The existence of mostly polar phytoconstituents in both extracts was confirmed by the high solubility found in methanol (19.3 mg/mL and 18.0 mg/mL, respectively) and ethanol (18.5 mg/mL and 17.2 mg/mL, respectively). Distilled water showed moderate solubility, however chloroform showed poor solubility, indicating a restricted amount of non-polar chemicals. Overall, the solubility behavior validates the extracts' phytochemical composition and supports the use of polar solvent systems for the ensuing in vitro anti-inflammatory assessment.

Table 5.6: Solubility of Hydroalcoholic Extracts (mg/mL)

Solvent	Curcuma longa (mg/mL)	Zingiber officinale (mg/mL)
Distilled Water	4.8 mg/mL	5.2 mg/mL
Ethanol	18.5 mg/mL	17.2 mg/mL
Methanol	19.3 mg/mL	18.0 mg/mL
Chloroform	2.1 mg/mL	2.4 mg/mL
DMSO	25.6 mg/mL	24.9 mg/mL

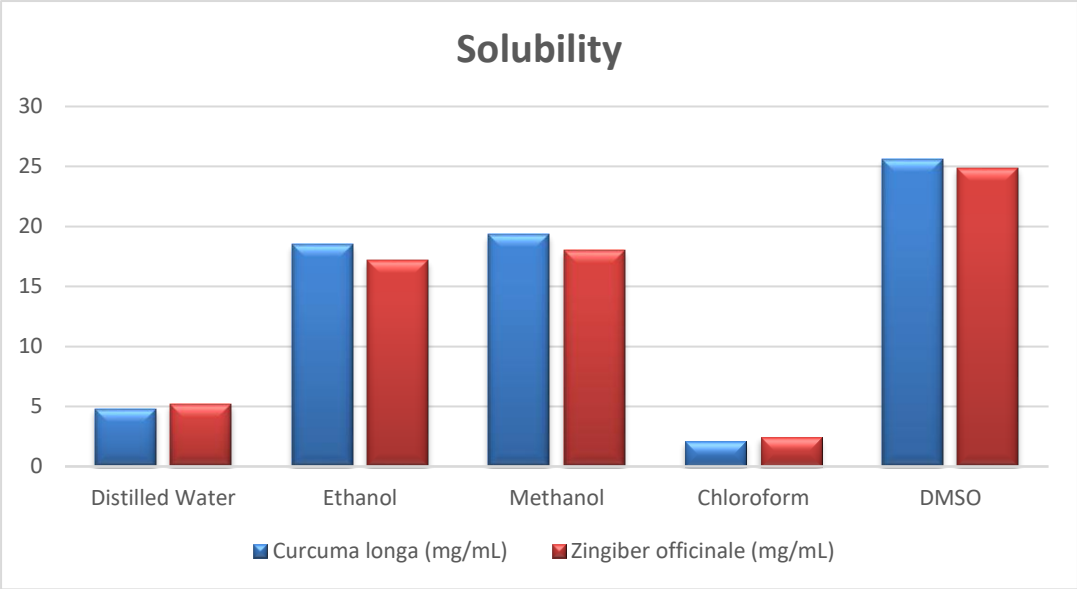

Fig 5.4: Solubility

5.5.4 Preparation of Test and Standard Solutions

Using appropriate solvent systems based on their solubility profiles, the hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale were effectively standardized into stock solutions with a concentration of 1 mg/mL. Working concentrations of 25, 50, 75, and 100 g/mL were created from these stock solutions for in vitro anti-inflammatory testing. To provide consistency and trustworthy comparability across all experimental groups, the same concentration range was kept for both the polyherbal combination and the common medication diclofenac sodium. Accurate dose-dependent evaluation of protein denaturation inhibition and direct comparison of anti-inflammatory efficacy between individual extracts, their combination, and the reference standard under identical experimental conditions were made possible by the use of a standardized concentration range.

Table 5.7: Preparation of Stock and Working Solutions

S. No.	Sample	Stock Concentration (mg/mL)	Working Concentrations (µg/mL)	Solvent Used
1	Curcuma longa extract	1 mg/mL	25, 50, 75, 100	DMSO / PBS (pH 6.8)
2	Zingiber officinale extract	1 mg/mL	25, 50, 75, 100	DMSO / PBS (pH 6.8)
3	Polyherbal combination (Curcuma longa + Zingiber officinale)	1 mg/mL	25, 50, 75, 100	DMSO / PBS (pH 6.8)
4	Diclofenac sodium (Standard)	1 mg/mL	25, 50, 75, 100	PBS (pH 6.8)

5.6 In Vitro Anti-Inflammatory Study

5.6.1 Protein Denaturation Assay

The proteindenaturation inhibition test was used to assess the anti-inflammatory properties of hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale, as well as their polyherbal combination, at doses of 25, 50, 75, and 100 µg/mL. The findings demonstrated a concentration-dependent drop in absorbance at 660 nm, which suggests that heat-induced protein denaturation is gradually inhibited. Diclofenac sodium showed the greatest inhibition (77.83%) at the highest concentration (100 µg/mL), followed by the polyherbal combination (75.98%), Curcuma longa extract (70.44%), and Zingiber officinale extract (67.36%). At all examined concentrations, Curcuma longa exhibited significantly greater activity than Zingiber officinale among the different extracts. In comparison to individual extracts, the polyherbal formulation showed increased anti-inflammatory capability, indicating a potential synergistic interaction between their phytoconstituents. Overall, the test samples' considerable protein stabilization and anti-inflammatory activity were confirmed.

Table 5.8: Absorbance and % Inhibition of Protein Denaturation

Sample	Concentration (µg/mL)	Absorbance at 660 nm	% Inhibition
Control	—	0.812	—

Diclofenac sodium	25	0.402	50.49
	50	0.310	61.82
	75	0.245	69.83
	100	0.180	77.83
Curcuma longa extract	25	0.520	35.96
	50	0.410	49.50
	75	0.320	60.59
	100	0.240	70.44
Zingiber officinale extract	25	0.550	32.18
	50	0.435	46.43
	75	0.340	58.13
	100	0.265	67.36
Polyherbal combination (1:1)	25	0.470	42.12
	50	0.355	56.28
	75	0.265	67.36
	100	0.195	75.98

5.6.2 Individual Extract Activity (Curcuma longa vs Zingiber officinale)

The % inhibition of protein denaturation at various doses was used to compare the anti-inflammatory properties of separate hydroalcoholic extracts of the rhizomes of Curcuma longa and Zingiber officinale. The findings showed a rise in activity for both extracts that was concentration-dependent. Curcuma longa demonstrated greater inhibition (70.44%) at the highest tested dose (100 µg/mL) than Zingiber officinale (67.36%), suggesting significantly better membrane-stabilizing and anti-inflammatory capability.

Table 5.9: Comparison of Individual Extract Activity (% Inhibition at 100 µg/mL)

Sample	% Inhibition (100 µg/mL)
Curcuma longa	70.44
Zingiber officinale	67.36

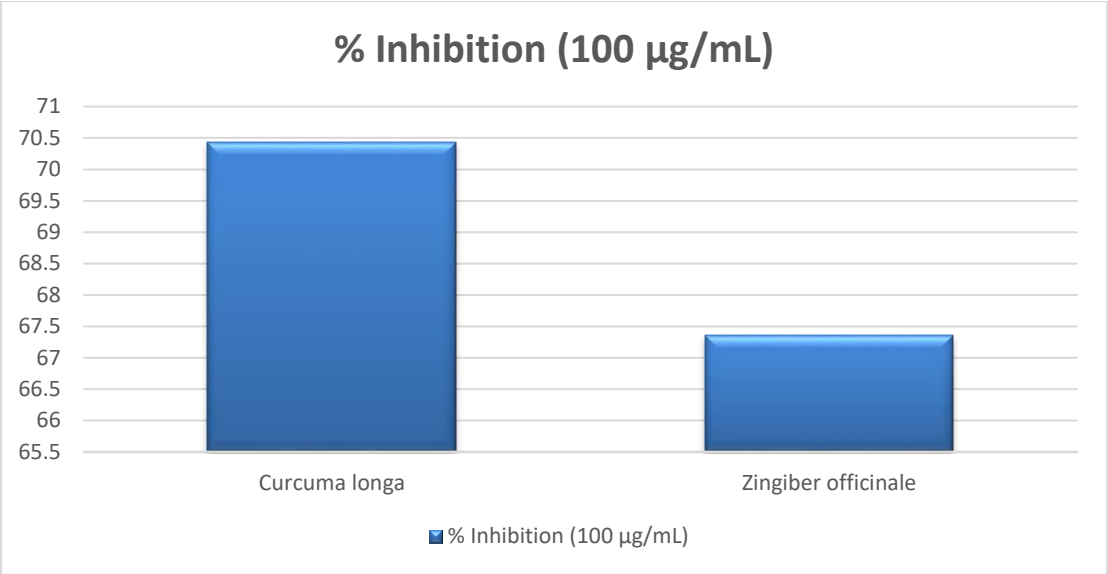

Fig 5.5: % Inhibition (100 µg/mL)

5.6.3 Polyherbal Combination Activity

When compared to separate extracts, the polyherbal formulation made from Curcuma longa and Zingiber officinale showed increased anti-inflammatory action. The combination demonstrated 75.98% reduction of protein denaturation at 100 µg/mL, suggesting enhanced effectiveness as a result of the combined phytochemical actions.

Table 5.10: Polyherbal Combination Activity

Concentration (µg/mL)	% Inhibition (Polyherbal)
25	42.12
50	56.28
75	67.36
100	75.98

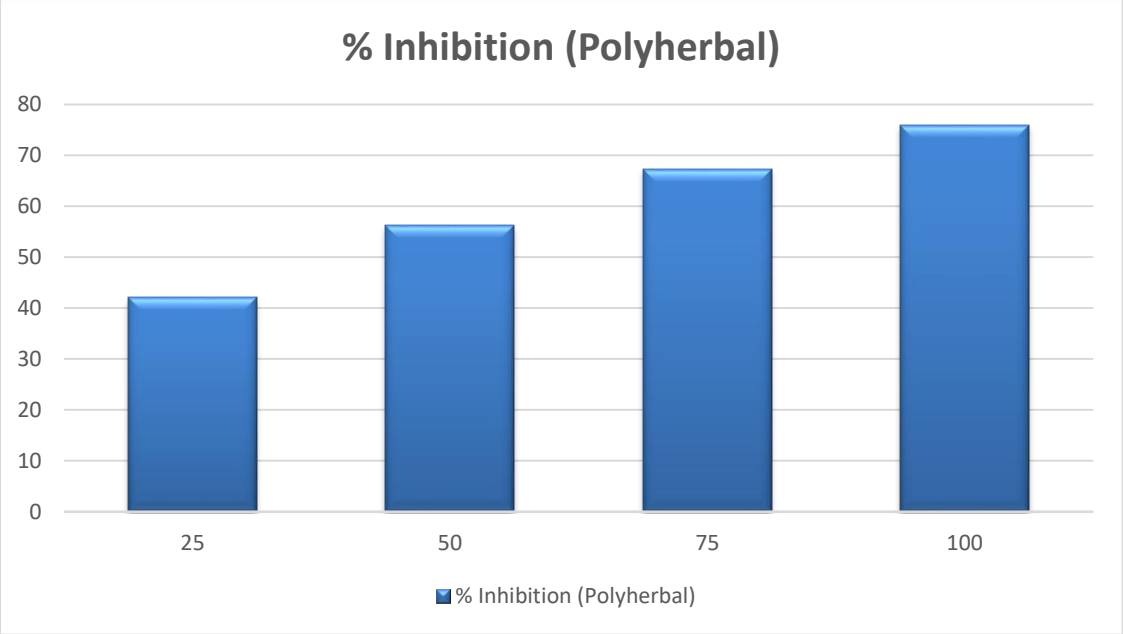

Fig 5.6: % Inhibition (Polyherbal)

5.6.4 Comparison with Standard Drug

Out of all the studied samples, diclofenac sodium showed the strongest anti-inflammatory activity. It demonstrated 77.83% suppression of protein denaturation at 100 µg/mL, which was the benchmark for comparison. The polyherbal compound exhibited substantial, similar anti-inflammatory potential while having somewhat less activity than the conventional medication.

Table 5.11: Comparison with Standard Drug (100 µg/mL)

Sample	% Inhibition
Diclofenac sodium	77.83
Polyherbal combination	75.98
Curcuma longa	70.44
Zingiber officinale	67.36

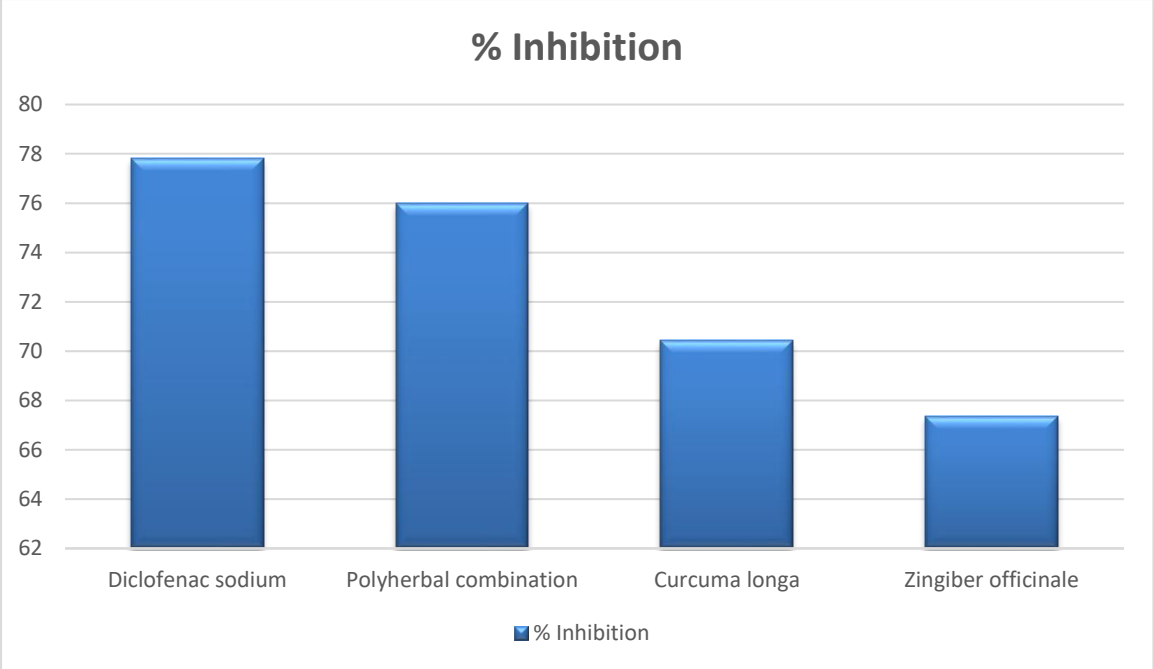

Fig 5.7: % Inhibition

5.6.5 Assessment of Possible Synergistic Effect

At all investigated doses, the polyherbal combination showed a greater percentage reduction of protein denaturation than individual extracts. In comparison to Zingiber officinale (67.36%) and Curcuma longa (70.44%), the combination (75.98%) demonstrated increased activity at 100 µg/mL. This increase raises the possibility that phytoconstituents like curcuminoids and gingerols combine synergistically to boost protein stability and anti-inflammatory actions. The presence of complimentary mechanisms contributing to the overall pharmacological action of the polyherbal formulation is supported by the observed increase in activity beyond individual contributions.

Conclusion

The goal of the current study was to use the protein denaturation inhibition technique to assess the in vitro anti-inflammatory activity of hydroalcoholic

extracts of Zingiber officinale and Curcuma longa, both alone and in combination. To generate crude hydroalcoholic extracts, both plant materials were gathered, verified, dried, and then Soxhlet extracted using a 70:30 ethanol-water solvent solution.

Effective phytoconstituent extraction was demonstrated by the extract yield percentages of 8.50% for Curcuma longa and 7.60% for Zingiber officinale. Alkaloids, flavonoids, phenolics, tannins, terpenoids, and glycosides were among the main bioactive components found in both extracts, according to preliminary phytochemical screening. These components are frequently linked to antioxidant and anti-inflammatory properties.

Acceptable physicochemical properties, such as low moisture content (LOD 7.60% and 9.00%), low acid-insoluble ash values, and high solubility in polar solvents, especially DMSO, methanol, and ethanol, were revealed by standardization investigations. These results confirmed that the extracts were appropriate for in vitro testing.

Both extracts showed concentration-dependent suppression of protein denaturation in the protein denaturation test. Curcuma longa and Zingiber officinale demonstrated 70.44% and 67.36% inhibition, respectively, at 100 µg/mL. When compared to individual extracts, the polyherbal combination showed increased activity (75.98%), indicating higher effectiveness. The conventional medication, diclofenac sodium, exhibited the greatest inhibition (77.83%).

A comparative analysis revealed that Curcuma longa was marginally more active than Zingiber officinale, but the combined formulation was more active than either extract alone, suggesting a potential synergistic interaction between their phytoconstituents.

6.2 Conclusion

The current work used the protein denaturation inhibition technique to assess the in vitro anti-inflammatory potential of hydroalcoholic extracts of Curcuma longa and Zingiber officinale, both separately and in combination. Soxhlet extraction (70:30 ethanol-water) was used to get both extracts, which had high

percentage yields (8.50% and 7.60%, respectively). Key bioactive components that are known to contribute to anti-inflammatory and antioxidant activities, such as flavonoids, phenolics, tannins, terpenoids, alkaloids, and glycosides, were found to be present by preliminary phytochemical screening. The purity and appropriateness of the extracts for biological evaluation were supported by standardization experiments that revealed acceptable physicochemical characteristics, such as low moisture content, low ash values, and good solubility in polar solvents. Both extracts showed concentration-dependent anti-inflammatory activity in the protein denaturation assay, with Curcuma longa exhibiting greater inhibition (70.44% at 100 µg/mL) than Zingiber officinale (67.36%). The polyherbal combination showed increased activity (75.98%), indicating a potential synergistic effect between their phytoconstituents. The combination herbal formulation had similar action, suggesting strong anti-inflammatory potential, even though diclofenac sodium demonstrated the greatest inhibition (77.83%). Overall, the work provides scientific support for the traditional usage of both plants and shows that their combination may provide better therapeutic efficacy because of synergistic interactions, which calls for more in vivo and mechanistic research for future development.

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