Isolation, Characterization and biological Evaluation of Bromelain from (*Ananas comosus(L) Merr.)*

=

Submitted by
MR. GANESH S
Under the guidance of
Dr. M. SENTHILRAJA, M.Pharm., Ph.D.,
Head of the Department,
Department of Pharmaceutical Chemistry,
P.S.V COLLEGE OF PHARMACEUTICAL SCIENCE & RESEARCH
ORAPPAM, KRISHNAGIRI, TAMIL NADU – 635 108
OCTOBER-2025

1. INTRODUCTION

1.1 HERBAL DRUG TECHNOLOGY

Herbal drug technology is the process of transforming botanical materials into products and medications with therapeutic benefits. The claim for herbal medications is rising right now, and more individuals are using herbal products made by combining cutting edge scientific techniques with traditional knowledge. Herbal drug technology is a synthesis of traditional knowledge and modern science that uses the medicinal properties of plants. It comprises carefully extracting, cleaning, and preparing the bioactive ingredients from medicinal herbs. This technology combines traditional knowledge with innovative techniques to certify the efficacy, security, and standardized manufacturing of medicinal treatments. The increased acceptance of herbal medicines in conventional medicine can be attributed to advancements in quality control and analytical methods. Herbal medicine technology uses extensive testing and validation to generate reliable and consistent products. Developments in delivery technologies, such as encapsulation and nanotechnology, also enhance the bioavailability and targeted action of herbal substances. This field also promotes ethical harvesting, production, and preservation of medicinal plants, all of which contribute to sustainable practices. By emphasizing environmentally friendly practices, herbal medicine technology contributes to environmental preservation while also improving human health. In a time when people are becoming more and more interested in natural remedies, herbal medicine technology is leading the way and connecting traditional medicinal methods with modern, evidence-based care5. It provides a multitude of treatment options for a inclusive series of medicinal conditions, symbolising the harmonious fusion of the abundance of nature and the rigour of science.[1-3]

1.2 ADVANTAGES OF HERBAL DRUG TECHNOLOGY

- **Natural Origin:** Plants, herbs, and botanical extracts are the natural sources from which herbal medicines are derived. Those who favour alternative or holistic approaches to healthcare view this natural origin in a positive light. Historically, herbal medicines have been used for centuries in Ayurvedic medicine, Traditional Chinese Medicine (TCM), and then Indigenous healing methods. A strong foundation for the development and use of herbal treatments is provided by this body of knowledge. **Safety Profile:** Due to their perceived lesser potential for harmfulness and rarer side effects than synthetic drugs, herbal medicines are frequently regarded as safer than synthetic ones. It's crucial to remember that herbal medications can still have negative side effects, so careful dosage and supervision are required.

- **Multifarious Impacts:** Herbal remedies frequently comprise an intricate blend of bioactive substances that have the potential to produce numerous therapeutic outcomes. Conditions with a variety of underlying causes or symptoms may benefit from this multifaceted action.

- **Personalized Medicine:** Individual requirements and preferences can be catered to through the use of HM, enabling customised treatment plans. Traditional medicine practitioners and herbalists frequently adapt herbal formulations to an individual's symptoms, constitution, and health objectives.

- **Accessibility and availability:** Compared to synthetic medications, a huge quantity of medicinal plants that are used are reasonably priced and widely available. This can make healthcare more accessible, especially in areas with limited resources or where modern medications may be hard to come by.

- **Cultural Significance:** The preparation of herbal medicine has strong roots in many cultures and customs across the globe. Herbal medications have the likely to respect cultural heritage and encourage cultural diversity in the provision of healthcare.

- **Sustainability and Environmental Considerations:** Due to their reliance on renewable plant sources, herbal medicines are frequently viewed as being more environmentally friendly and sustainable than synthetic drugs.

- **Standardization and Quality Control:** Because plant constituents, growing conditions, and extraction techniques vary, it can be difficult to achieve consistent potency and quality in herbal drugs.

- **Restricted Scientific Validation:** Although many herbal remedies are supported by traditional knowledge, thorough scientific studies are mandatory to authrize their safety and effectiveness in accordance with contemporary medical standards. **Possibility for Misuse:** Improper or overuse of herbal remedies can occasionally result in negative side effects or interactions with other prescription drugs.

- **Regulatory Difficulties:** The production and marketing regulations pertaining to herbal drugs exhibit significant regional variations, thereby posing challenges for both manufacturers and consumers.[6-9]

1.3 APPLICATIONS

- Herbal medications are essential for primary healthcare, particularly in areas with restricted contact to modern medicine.

- Complementary and Alternative Medicine (CAM): They are frequently utilised as alternative or complementary therapies for different ailments, frequently in conjunction with traditional medicine.

- Nutraceuticals and Functional Foods: Herbal ingredients are used to produce dietary supplements, nutraceuticals, and functional foods that provide health benefits beyond simple nourishment.

- Cosmeceutical: Due to their natural and advantageous qualities, herbal extracts are used in skincare and cosmetic products.[10]

1.4 HERBAL MEDICINES

Throughout human history, herbal medicine has been utilized by all societies, making it the most ancient and widely used form of healthcare. The basis for ancient medicine around the world is plants and herbs, which are among the most vital processes of life on Earth. Of approximately 350,000 varieties of living plants, including ferns, bryophytes, & seed plants, 287,655 had been identified as of 2004. Biology medicine, phytomedicine, or phytotherapy are additional names for herbal medicine (HM), which is the practice of treating botanical materials, herbal preparations, and completed herbal products that contain active compounds derived from plant or other substances. Botanical medicine, phytomedicine, and phytotherapy are other terms frequently used to describe herbal medicine. Herbal medicine makes use of bark, flowers, fruits, seeds, berries, roots, leaves, and even the complete plant. Man, mostly relied on raw plant material to maintain health and cure illnesses prior to the discovery of aspirin, which is derived from the Spiraea, Ulimaroa plant, as the Greek physician Hippocrates advised for pain and fever, as well as for

fever as well as swelling in Egyptian papyri. Traditional herbal treatment has long been utilized for medicinal purposes and is widely acknowledged as safe and beneficial. One active ingredient derived from plant material, which can be obtained from plant extracts or synthesized to mimic naturally occurring plant compounds, is present in about one-third of all prescription drugs today. A large number of today's commonly used drugs in developing nations have herbal origins. There is a long olden time of use aimed at many of the medications that are being recommended to physicians as herbal remedies. The World Health Organization estimates that about 25% of medications that are currently prescribed in the US are thought to have plant origins. More than 120 active substances that are derived from higher plants are currently available. Both illness prevention and treatment are achieved with herbal remedies. Herbal remedies have long been referred to as "the people's medicines" due toward their ease of obtainability, safety, and effortlessness of production. It has long been known that herbal remedies can be used to treat illnesses in addition to conventional medications. Because of improvements in methods of analyzing and controlling the quality of herbs and herbal pharmaceuticals, as well as advances in clinical research into their safety and efficacy, HM are becoming more and more recognized as valuable tools in the inhibition and 18 treatments of many sicknesses everywhere the world. The practice of treating patients with HM is becoming more then added common in many countries throughout the world. In many Asian and African countries, traditional herbal remedies are the primary source of healthcare for up to 80% of the populace. Between 70 and 80 percent of people in many wealthy nations have used complementary and alternative medicine (CAM), which primarily consists of herbal remedies. More than 80% of people use herbal medicines today for a range of crucial aspects of basic healthcare, according to the World Health Organisation. They are profitable, making billions of dollars on the global market. For example, in Western Europe, the yearly revenue from herbal products and medications reached US$ 5 billion in 2003–2004. Government data indicates that in 2005, Chinese herbal medicines were sold for a total of $14 billion USD in ailments. Sales of HM in Brazil are assessed to have generated US$160 million in revenue in 200720. Even though allopathic medications and first-rate recent healthcare services are now effortlessly available in many economically advanced Asian countries, such as China, Japan, Thailand, and South Korea, HM are still widely used in these countries. In Japan, 60–70% of allopathic physicians have advised patients to undergo Kampo treatments, which primarily consist of herbal remedies. Traditional Malaysian versions of these treatments are easily available, and Malay, Chinese, and Indian medicines all of which are primarily made of herbs and their constituents are often utilised. An estimated 200 million patients in China receive treatment with herbal medicines each year, accounting for about 40% of all medical services. Herbal treatments are still commonly utilized in many economically prosperous Asian nations, such as Japan, China, Thailand, and South Korea, despite the easy access to allopathic pharmaceuticals and state-of-the-art modern healthcare facilities in many countries today. Sixty to seventy percent of allopathic doctors prescribe Kampo therapies, which are mostly herbal medicines, to their patients in Japan. In Malaysia, where traditional forms of these therapies are easily accessible, traditional forms of Malay, Chinese, and Indian medicines—all of which are essentially made from herbs and herbal ingredients—are widely utilised. Herbal medicines are used to treat an estimated 200 million people annually, or roughly 40% of all patients in China who receive medical care. In recent times, here has stayed a better effort on finding pharmaceuticals and nutritional supplements made from plants due to the growing popularity of herbal medications and their increasing consumption worldwide. Practitioners in the disciplines of pharmacognosy, microbiology, botany, chemistry of natural products are scouring the globe for phytochemicals and plant-based leads that could be used in the manufacture of medications for a range of diseases. [11-13]

1.5 BROMELAIN

Bromelain is a natural mixture of proteolytic enzymes extracted mainly from the stem and fruit of the pineapple plant (*Ananas comosus*). It is known for its ability to break down proteins and exhibits a wide range of biological activities, including anti-inflammatory, anti-edematous, fibrinolytic, and immunomodulatory effects. Traditionally used for aiding digestion and reducing swelling, bromelain has also found applications in modern medicine, particularly in wound debridement (as in the topical product NexoBrid), as well as in the food industry for meat tenderization and beverage processing. Chemically, it is a complex enzyme blend containing thiol endopeptidases and other components, active over a broad pH range. While generally safe and well tolerated, bromelain may cause mild gastrointestinal side effects and should be used cautiously by individuals taking anticoagulant medications. Despite its promising therapeutic potential and industrial importance, variations in enzyme activity, lack of standardization, and limited large-scale clinical evidence remain challenges to fully establishing bromelain’s efficacy in human health applications. [14-15]

1.6 ANTI-OXIDANT ACTIVITY

Antioxidants are naturally occurring or synthetic substances that help protect the body's cells from damage caused by free radicals unstable molecules produced during normal metabolic processes or through exposure to external factors such as pollution, radiation, and cigarette smoke. Free radicals can cause oxidative stress, a condition linked to aging and various chronic diseases like cancer, heart disease, and neurodegenerative disorders. Antioxidants neutralize these harmful molecules by donating electrons, thereby preventing or reducing cellular damage. They are found abundantly in fruits, vegetables, nuts, and whole grains, with common examples including vitamins C and E, beta-carotene, selenium, and polyphenols. In addition to their vital biological role in maintaining health and slowing cellular aging, antioxidants are also widely used in the food and cosmetic industries to prevent oxidation and extend shelf life. Beyond their natural protective functions, antioxidants have gained significant attention in pharmaceutical and biomedical research for their potential therapeutic applications. Studies have demonstrated that enhancing the body's antioxidant defense system can help mitigate oxidative damage associated with metabolic disorders, inflammation, and degenerative diseases. Consequently, the development and modification of natural antioxidant compounds have become an important area of modern medicinal chemistry. Synthetic alterations of bioactive molecules aim to improve their stability, solubility, and bioavailability, thereby increasing their efficacy in neutralizing free radicals. [16-18]

Among these natural compounds, bromaline and its derivatives are being explored for their remarkable antioxidant and anti-inflammatory properties. Through synthetic modification, bromaline's chemical structure can be optimized to yield derivatives with enhanced antioxidant capacity and broader biological activity. Such advancements not only contribute to understanding the structure–activity relationship of natural products but also pave the way for the formulation of novel therapeutic agents and antioxidant-based formulations, including gels, creams, and nutraceuticals, for the prevention and management of oxidative stress–related diseases.[19]

2. NEED FOR THE STUDY

2.1. Increasing oxidative stress–related diseases:

Oxidative stress plays a major role in the development of several chronic diseases such as cancer, diabetes, neurodegenerative disorders, cardiovascular diseases, and aging. There is a growing need for potent and safe antioxidants that can neutralize free radicals and protect cellular components from oxidative damage.

2.2. Limitations of synthetic antioxidants: Common synthetic antioxidants like BHA, BHT, and TBHQ are effective but are associated with potential toxic and carcinogenic effects. Therefore, the search for natural, biocompatible, and non-toxic antioxidants from plant sources has gained significant importance.

2.3. Therapeutic potential of bromelain:

Bromelain, a proteolytic enzyme complex obtained from Ananas comosus (pineapple), has shown multiple pharmacological activities such as anti-inflammatory, antioxidant, anticancer, and wound-healing effects. However, its native activity is often limited by instability, poor bioavailability, and rapid degradation under physiological conditions.

2.4. Need for synthetic modification:

Chemical or structural modification of natural biomolecules like bromelain can enhance their stability, activity, and specificity. Synthetic derivatization may improve bromelain's antioxidant efficiency by increasing its radical scavenging capacity or by improving its ability to interact with reactive oxygen species (ROS).

2.5. Development of novel natural-based therapeutics:

Modifying a natural enzyme like bromelain can lead to novel semi-synthetic antioxidants with improved pharmacological properties and fewer side effects compared to purely synthetic molecules. This aligns with the current pharmaceutical trend of developing eco-friendly and sustainable bioactive compounds.

2.6. Industrial and pharmaceutical applications:

Enhanced bromelain derivatives could find applications not only in pharmaceuticals but also in cosmetics, nutraceuticals, and food industries as natural antioxidant additives or therapeutic agents.

2.7. Scientific advancement and innovation:

This study contributes to the field of biocatalyst modification and enzyme engineering, opening avenues for designing more efficient bioactive molecules from natural resources through rational synthetic modification.

3. Review of Literature

1. Rowan ***et al***. (1990)[20] Bromelain was first isolated in 1891 from pineapple juice and later from the stem. It is a cysteine protease with an optimum activity between pH 6–8 and temperature 37–50°C. The enzyme has been used in numerous therapeutic and industrial applications because of its strong proteolytic and biological activities.
2. Maurer ***et al***. (2001)[21] highlighted bromelain's medical relevance, noting its oral bioavailability and therapeutic potential in inflammation and thrombosis. described bromelain as a stable enzyme complex when properly purified and stored, but prone to denaturation in aqueous solutions without stabilizers.
3. Bhattacharyya ***et al***. (2008)[22] reported the native form of bromelain exhibits moderate stability and limited antioxidant potential due to its structural sensitivity and degradation under oxidative or high-temperature conditions. Therefore, synthetic modification of bromelain is a promising approach to enhance its antioxidant and stability characteristics.
4. Sinha ***et al.*** (2010)[23] Established the Modification of lysine residues using succinic or citraconic anhydrides introduces negative charges, improving enzyme solubility and oxidative tolerance. chemically modified bromelain using anhydrides and observed better storage stability and antioxidant activity compared to the native enzyme.
5. Pavan ***et al***. (2012)[24] Bromelain is a proteolytic enzyme complex obtained primarily from the stem and fruit of pineapple (Ananas comosus). It consists of a mixture of thiol endopeptidases, phosphatases, glucosidases, and other proteolytic enzymes (Maurer, 2001). Over the years, bromelain has gained attention for its diverse pharmacological properties, including anti-inflammatory, antithrombotic, fibrinolytic, anticancer, and antioxidant activities.
6. Chen ***et al***. (2013)[25] PEGylation is a technique where polyethylene glycol chains are covalently attached to enzymes, increasing solubility and resistance to proteolysis. reported that PEGylation enhances the pharmacokinetic stability of therapeutic enzymes without significantly reducing catalytic activity.
7. Ali ***et al***. (2014)[26] Formation of Schiff bases between bromelain amino groups and aldehydes can lead to enhanced radical-scavenging ability. demonstrated Schiff-base modified enzymes exhibit improved DPPH scavenging and metal-chelating activity due to newly formed imine linkages that promote electron donation.
8. Arshad ***et al***. (2014)[27] Despite these findings, crude bromelain's antioxidant potency is still lower than that of dedicated antioxidant enzymes or modified proteins, warranting further enhancement through synthetic modification.Macedo ***et al***. (2015)[28] Structural modifications alter the exposure of functional amino acid residues such as cysteine, tyrosine, and tryptophan, which are crucial for radical neutralization). Modified bromelain derivatives show enhanced reducing power, DPPH radical scavenging, and FRAP values compared to the unmodified enzyme. This indicates that modification improves electron-donating capacity and stabilization of radical intermediates.
9. Chaurasiya ***et al***. (2015)[29] The antioxidant activity of bromelain has been demonstrated in several in-vitro studies. evaluated crude bromelain extracted from pineapple peel and found significant DPPH and ABTS radical scavenging activity, comparable to ascorbic acid.
10. Rathnavelu ***et al***. (2016)[30] reported that bromelain scavenges reactive oxygen species (ROS) and enhances intracellular antioxidant enzyme levels such as superoxide dismutase (SOD) and catalase (CAT).
11. Rathnavelu ***et al***., (2016)[31] Although several studies have characterized crude bromelain's antioxidant potential, limited research focuses on systematic synthetic modification to optimize its antioxidant capacity. Previous studies either used unstandardized modification techniques or failed to correlate structure–activity relationships.
12. Roy ***et al***. (2018)[32] Bromelain–metal complexes (e.g., Cu^{2+}, Zn^{2+}, Fe^{3+}) have been explored for redox modulation. synthesized metal–enzyme complexes and found improved redox cycling ability and increased total antioxidant capacity compared with native enzymes.
13. Kumar & Sharma ***et al***. (2020)[33]. These drawbacks limit its industrial and pharmaceutical use. Chemical modification of enzymes has emerged as a key strategy to enhance their stability, activity, and specificity.

14. Bhushan ***et al***. (2020)[34] Immobilization of bromelain on polymeric matrices such as chitosan or alginate beads improves stability and reusability. immobilized bromelain onto chitosan nanoparticles, which resulted in enhanced thermostability and antioxidant potential, attributed to restricted conformational flexibility and better protection against oxidation.

4. Aim and objectives

4.1 Aim

The present study aims to synthesize and characterize modified bromaline derivatives of natural products in order to enhance their antioxidant potential.

4.2 Objectives

- Assess qualitative phytochemical profiles (flavonoids, phenolics, alkaloids, terpenoids) post-formulation to verify the retention of bioactive integrity following synthetic modification.
- Develop topical gel formulations incorporating the synthetically modified bromaline derivatives of natural products to ensure stability, uniformity, and enhanced bioavailability of active compounds.
- Assess the antioxidant potential of modified bromaline derivatives and their gel formulations using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay to determine free radical scavenging efficiency.
- Evaluate the ability of modified bromaline derivatives to inhibit proteinase activity, indicating potential anti-inflammatory effects linked to antioxidant protection.
- Determine the antibacterial potential of modified bromaline derivatives and their gel formulations against Gram-positive and Gram-negative bacterial strains using the agar well diffusion method.
- Evaluate the antifungal properties of modified bromaline derivatives against common pathogenic fungi such as *Candida albicans* and *Aspergillus niger* using the agar well diffusion assay.
- Evaluate the inhibitory activity of modified bromaline derivatives on α-amylase enzyme to determine their potential to regulate postprandial hyperglycemia.

5. PLAN OF THE WORK

1. Selection of research topic
2. Literature survey & background study
3. Collection / isolation of bromalin compound
4. Phytochemical characterization
5. In vitro antioxidant activity evaluation
   i. DPPH Radical Scavenging Assay
6. Biological & pharmacological assessments
   i. Proteinase Inhibitory Assay
   ii. α-Amylase Inhibitory Assay
   iii. Antibacterial Activity
   iv. Antifungal Activity

7. Formulation development (GEL)

6. PLANT PROFILE

6.1. Taxonomical Classification

Rank	Classification
Kingdom	Plantae
Division	Magnoliophyta
Class	Liliopsida
Order	Poales
Family	Bromeliaceae
Genus	Ananas
Species	Ananas comosus (L.) Merr.

Figure 1:Pineapple (Ananas comosus (L.) Merr.)

6.2. Vernacular Names

Language	Name
English	Pineapple
Hindi	Ananas
Tamil	Annachi pazham / Annasi
Telugu	Anasa pandu
Malayalam	Kaithachakka
Sanskrit	Bahunetra, Ananasah

6.3. Geographical Distribution

- Native to South America (Brazil, Paraguay).
- Widely cultivated in tropical and subtropical regions such as India, Thailand, Philippines, Indonesia, and Africa.
- Major producing states in India: Kerala, West Bengal, Assam, Meghalaya, Tripura, and Tamil Nadu.[35]

6.4. Botanical Description

- **Habit**: Perennial, herbaceous plant with a short, thick stem and a rosette of long, narrow, spiny leaves.
- **Leaves**: Linear, tough, waxy, serrated edges, 30–100 cm long.
- **Flowers**: Small, purple to reddish, borne on dense spike-like inflorescence.
- **Fruit**: A multiple fruit (syncarp), formed by fusion of berries; oblong or cylindrical, yellowish when ripe, aromatic, juicy, and fibrous.
- **Seeds**: Usually absent in cultivated varieties; propagated vegetatively.

6.5. **Parts Used**

Fruit, Leaves, Stem (contains bromelain),Peel and crown (used in phytochemical studies)

6.6. Phytochemical Constituents

Enzymes: Bromelain (proteolytic enzyme), Vitamins: Vitamin C, Vitamin A, B-complex, Phenolic compounds: Gallic acid, caffeic acid, ferulic acid, syringic acid, Flavonoids: Quercetin, catechin, Organic acids: Citric acid, malic acid, Sugars: Sucrose, glucose, fructose, Minerals: Potassium, calcium, magnesium, iron

6.7. Medicinal Uses

- Anti-inflammatory: Due to bromelain activity.
- Antioxidant: High vitamin C and phenolic content scavenge free radicals.
- Digestive aid: Bromelain enhances protein digestion.
- Wound healing: Bromelain accelerates tissue repair.
- Antimicrobial and antifungal: Against E. coli, Candida albicans, etc.
- Anticancer potential: Exhibits cytotoxic effects on tumor cells (studied on HepG2, MCF-7).
- Anti-obesity & lipid-lowering: Shown in animal models. [36-39]

6.8. Pharmacological Activities

- Anti-inflammatory, Antioxidant, Anticancer, Antimicrobial, Wound healing

6.9. Traditional Uses

- Used in Ayurveda for digestive disorders and inflammation.
- In folk medicine, used as diuretic, laxative, and anthelminthic.
- Juice used for sore throat and cough.
- Leaf decoction applied externally for skin infections.

6.10. Propagation

Propagated vegetatively using suckers, slips, or crown cuttings.

- Grows best in sandy loam soil, pH 4.5–6.5, with good drainage.

6.11. Recent Research Highlights

- Studies report bromelain-modified derivatives exhibit enhanced antioxidant and anticancer properties.
- Nanoparticle formulations of pineapple extracts are being developed for targeted drug delivery. [40-42]

7. METHODOLOGY

7.1 Extraction method

- Fresh pineapple stems/cores (1500 g)
- Cold distilled water (or 0.1 M phosphate buffer pH 7.0), ice
- Blender
- Muslin/cheesecloth and fine filter paper
- Cold 95% ethanol (kept on ice)
- Large beakers, stir rod
- Cold storage (fridge ~4°C)
- Scale, measuring cylinders, glassware
- Small sieve or coffee filter
- Drying oven or desiccator (<40°C) or air dry in cool place
- pH paper

7.2 Steps

1. collect stems & core. Chop into small pieces.

2. Crude extraction — Blend 1500 g pineapple tissue with 1000–2000 mL cold distilled water (1:1–1:2 w/v) for 1–2 min on ice. Keep everything cold.

3. Coarse filtration — Filter through muslin/cheesecloth into a beaker; squeeze to recover liquid.

4. Clarification (gravity) — Let filtrate stand in fridge for 30–60 min; decant clearer supernatant or filter again through fine paper/coffee filter.

5. The sample 5gm and add 50 ml Isopropyl alcohol solution dissolved the *materials* incubated at 4°C for further investigation.

6.The material was immersed in the Isopropyl alcohol and extract was separated filtered by Muslin cloth, Filter paper and Whatman No.1 paper.

7. Dry — Air dry at <40°C (or use desiccator) until no solvent remains. Crude bromelain powder obtained.

8. Store — Store in airtight tube at 4°C for short-term; freeze at −20°C for longer term. [43-47]

7.3 QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

1. Detection of Carboxylic acid

To 1ml plant extract, 2 ml of sodium bicarbonate solution is added. Colour changes occur indicates the presence of carboxylic acid. [48-49]

2. Detection of Tannins

To 2ml of plant extract, 2-3ml of 10% HCL is added and boiled for 5-6 min. Formation of red colour indicates the presence of tannins. [50-53]

3. Detection of Steroids

To 0.5 ml extract, 5ml of chloroform is added and equal amount of conc. H2so4 was added. In the upper layer formation of red colour and in the lower layer, yellow with green colour is formation indicates the presence of steroids.

4. Detection of Flavonoids

To 0.5 ml extract, 4ml of 1% ammonia was added and to this 1ml of conc. H2so4 was added. The formation of yellow colour indicates the presence of flavonoids. [54-56]

5. Detection of Glycosides

Born- Trager's Test: Taken 2ml of hydrolysate, 3ml of chloroform was added, shaken vigorously, then the chloroform layer gets separated. Then 10% ammonia solution was added. The formation of pink colour indicates the presence of glycosides.

6. Detection of Proteins (Bradford Method)

To 500µl of plant extract, 5ml of the Bradford reagent was added, incubated at dark for 10 to 15 min. Taken the OD at 575nm.[57]

Detection of Phenol (Ferric Chloride Test)

To 50 mg of extract, 5ml of distilled water was added and a few drops of 5% ferric chloride solution were added. The formation of dark green colour indicates the presence of phenol. [58]

7. Saponin Test

To 50 mg of plant extract, 20 ml of distilled water was added and shaken vigorously for 15 min, formation of 2 cm layer of foam indicates the presence of saponins.[59]

8. Test for Alkaloids - Mayer's test

To a few ml of plant sample extract, two drops of Mayer's reagent are added along the sides of the test tube. Appearance of white creamy precipitate indicates the presence of alkaloids. [60]

9. Saponification test

To 1 or 2 ml of 10 N sodium hydroxide, 2 ml of extract is added and boiled for 2 minutes formation of soap or fat indicates the positive test for saponification. (61-63)

10. Gum Test

The 100 mg of plant extract was dissolved in 2 ml of distilled water. 2ml of absolute alcohol with constant stirring. White colour cloudy precipitate indicates gums & mucilage's.[64]

11. Detection of flavanol glycoside

The 50 mg of plant extract was dissolved in 5ml ethanol. Added a few drops of magnesium sulfate & few drops of conc. HCL. The formation of pink colour indicates the presence of flavanol glycoside. (65-70)

12. Detection of Carbohydrates

To 0.5 ml of extract, 0.5 ml of Benedict reagent was added ad boiled for 2 min. Color changes and precipitates are formed. It indicates the presence of carbohydrate. [71]

13. Detection of resins To 0.5 ml of plant extract, 3 ml of CuSO4 solution is added. Shaken for about 1-2 min, the formation of green colour precipitate indicates the presence of resins. [72]

14. Biuret test

To 2ml of extract, 1 drop of 2% CuSO4 solution. Add 1 ml of 95 % ethanol add 2 to 3 sodium hydroxide pellets. Formation of pink colour indicates the test is positive. (73-78)

7.4 In-vitro antioxidant activities

7.4.1 DPPH Radical scavenging activity

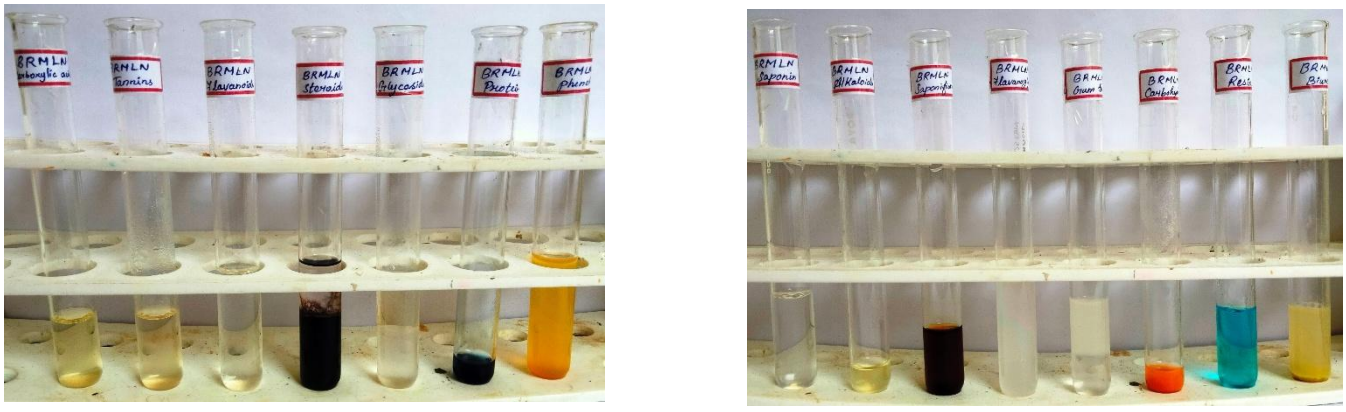


Figure 2: Images of Qualitative phytochemical screening methods

Principle

The DPPH assay is popular in natural product antioxidant studies. One of the reasons is that this method is simple and sensitive. This assay is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers., shows the mechanism by which DPPH accepts hydrogen from an antioxidant. DPPH is one of the few stable and commercially available organic nitrogen radicals. The antioxidant effect is proportional to the disappearance of DPPH in test samples. Monitoring DPPH with a UV spectrometer has become the most commonly used method because of its simplicity and accuracy. DPPH shows a strong absorption maximum at 517 nm (purple). The color turns from purple to yellow followed by the formation

of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm.[79-80]

Material Required

0.1mM DPPH solution, Ascorbic acid, Methanol

1.1 mM DPPH Solution

Dissolve 39 mg of DPPH in 100 ml of methanol and store at -20° C until needed.

Ascorbic acid (Standard)

1mg/ ml of Ascorbic acid

Procedure

1. Briefly, prepare 0.1 mM of DPPH solution in methanol and add 100 µl of this solution to 300 µl of the solution of sample BRMLN at different concentrations (500, 250, 100, 50 and 10 µg/mL).
2. The mixtures have to be shaken vigorously and allowed to stand at room temperature for 30 minutes.
3. Then the absorbance has to be measured at 517 nm using a UV-VIS spectrophotometer. (Ascorbic acid can be used as the reference).
4. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity.
5. The capability of scavenging the DPPH radical can be calculated by using the following formula.
6. DPPH scavenging effect (% inhibition) = [(absorbance of control- absorbance of reaction mixture)/absorbance of control] X 100.
7. Percentage inhibition and IC50 value was calculated using Graph Pad Prism 6.0 software (USA). [81-83]

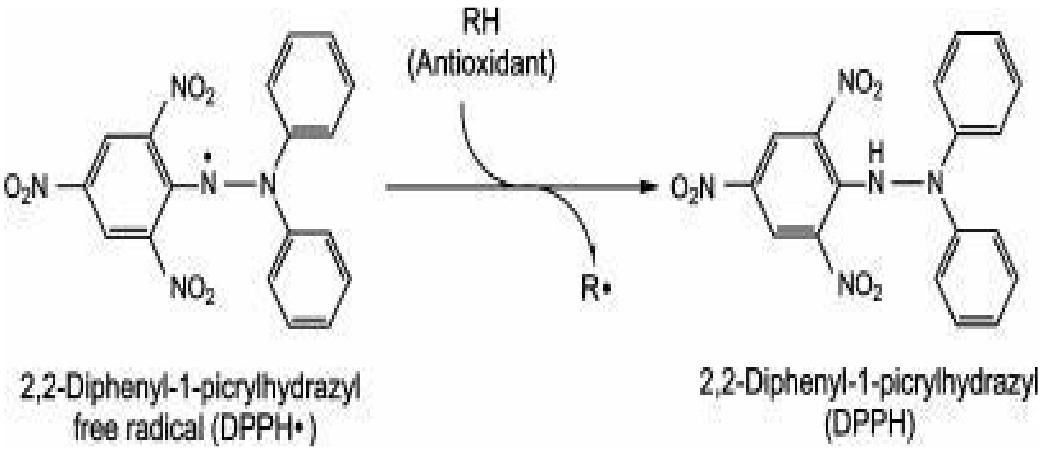


Figure 3: DPPH structure

7.4.2 Proteinase inhibitory assay

Procedure

The proteinase inhibitory assay was performed following the method modified by Oyedepo and Femurewa. The reaction mixture (2 ml) contained 0.06 mg trypsin, 1 ml Tris-HCl buffer (20 mM, pH 7.4) and test sample (BRMLN) at different concentrations (500, 250, 100, 50 and 10 µg/ml). The reaction mixture was incubated at 37 °C for 5 min and then 1 ml of 0.8% (*w*/*v*) casein was added. The mixture was incubated for an additional 20 min. Perchloric acid (2 ml of 70%) was added to stop the reaction. The cloudy suspension was centrifuged and the absorbance of the supernatant was measured at 210 nm against Tris-HCl buffer as blank. The experiment was performed in triplicate. [84-87]

7.4.3 Analysis of α- Amylase

Background

Amylase belongs to the family of glycoside hydrolase enzymes that break down starch into glucose molecules by acting on α-1,4-glycosidic bonds. The α-amylases (EC 3.2.1.1) cleave at random locations on the starch chain, ultimately yielding maltotriose and maltose, glucose and "limit dextrin" from amylose and amylopectin. In mammals, α-amylase is a major digestive enzyme. Increased enzyme levels in humans are associated with salivary trauma, mumps due to inflammation of the salivary glands, pancreatitis and renal failure.

Materials Required

A-amylase solution was purchased from Sigma, USA. Sodium phosphate buffer, DMSO, starch, DNS reagent, sodium, potassium tartrate, NaOH was purchased from Merk, USA.

Procedure

The α- amylase inhibitory activity was assessed by the method described by Dong et al., 2012, with suitable modification. Briefly, (500, 250, 100, 50 and 10 µg/ml) of the eucalyptus test sample (BRMLN) was remixed with 200 µl of α-amylase solution (1.0 U/ml in phosphate buffer pH 6.9), and incubated at 25^{o}C for 30 min. After pre-incubation, 400 µl of 0.25 % starch solution in the phosphate buffer (pH 6.9) was added to each tube to start the reaction. The reaction was carried out at 37^{o}c for 5 min and terminated by the addition of 1.0 ml of the DNS reagent (1% 3,5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M NaOH). The test tubes were then kept over a boiling water bath for 10 min and cooled to room temperature. The reaction mixture was then diluted by making up the volume to 10 ml of distilled water and absorbent (A) was measured at 540 NM. Control incubations representing 100% enzyme activity were conducted in a similar way by replacing extracts with buffers. For blank incubation (to allow for absorbance produced by the extracts), enzyme solution was replaced by buffers solution and absorbance recorded. The α- amylase inhibitory activity was expressed as percent inhibition and was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{A control} - (\text{A test} - \text{A background})}{\text{Control}} \times 100$$

Where $A_{control}$, A_{test}, $A_{background}$ represented the absorbance of 100% enzyme activity, test sample with the enzyme and test sample without the enzyme, respectively. **(88-90)**

7.4.4 Antibacterial ActivityPROCEDURE

Petri plate containing 20 ml nutrient agar medium was seeded with 24 hr culture of bacterial strain was adjusted to 0.5 OD value according to McFarland standard. Wells were cut and concentration of test sample BRMLN (500, 250, 100, 50 µg/ml) was added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Gentamicin was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA).

PRINCIPLE

The antimicrobials present in the given sample were allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters. **(91-95)**

MATERIALS REQUIRED

(*Escherichia coli-443, Enterococcus faecalis-439, Streptococcus pyogenes-1928 and Staphylococcus aureus-902)* were purchased from MTCC, Chandigarh, India. Nutrient Agar medium, Nutrient broth, Gentamicin solution were purchased from Himedia, India. Test samples, petri-plates, test tubes, beakers conical flasks were from Borosil, India. Spirit lamp, double distilled water.

1. AGAR- WELL DIFFUSION METHOD

a. Nutrient Agar Medium

The medium was prepared by dissolving 2.8 g of the commercially available Nutrient Agar Medium (HiMedia) in 100ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petriplates (25-30ml/plate) while still molten. [96-100]

b. Nutrient broth

Nutrient broth was prepared by dissolving 2.8 g of commercially available nutrient medium (HiMedia) in 100ml distilled water and boiled to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. [101]

7.4.5 Antifungal Activity

PRINCIPLE

The anti-fungal agent present in the given sample was allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters. [102-105]

MATERIALS REQUIRED

Potato dextrose agar medium, Amphotericin B antimycotic solution, test samples, test tubes, beakers conical flask, spirit lamp, double distilled water and petri-plates.

DISC DIFFUSION METHOD

1. Potato Dextrose Agar Medium

The potato dextrose agar medium was prepared by dissolving 20 gm of potato influsion, 2 gm of dextrose and 1.5 gm of agar in 100ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petri plates (25-30 ml/plate) while still molten.

PROCEDURE

Petri plates containing 20ml potato dextrose agar medium was seeded with 72 hr culture of fungal strain (Candida albicans, Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus) with different concentration of sample BRMLN (500, 250, 100 and 50 µg/ml) was added. The plates were then incubated at 28°C for 72 hours. The anti-fungal activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Amphotericin B was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA). [106-110]

7.4.6 GEL PREPARATION

Take Bromelain (1g) add distilled water(10ml), mix it well. Then add Carboxyl methyl cellulose (2g). Finally add preservatives as Sodium Benzoate (0.001g). Mix it well without clusters to form a gel, transfer the gel into 50ml centrifuge tube. Autoclave the mixtures for further use. [111-115]

8.RESULTS AND DISCUSSION

8.1 Showing the table qualitative phytochemical screening methods

Table 1: phytochemical screening

S. No.	Name of the Sample	Phytochemical compound	Result
1.	BRMLN	Resins	-
2.		Carboxylic acid	-
3.		Tannins	-
4.		Steroids	-
5.		Flavonoid	-
6.		Carbohydrates	+
7.		Glycosides	-
8.		Saponification	-
9.		Protein	+
10.		Phenol	-
11.		Biuret	-
12.		Soponin	-
13.		Gum	+
14.		Flavanoglycosides	-
15.		Alkaloids	-

Note: "--"- not, "+" - slight presence, "++" - strong presence, "+++" – More strong presence.

8.2 Characterization study

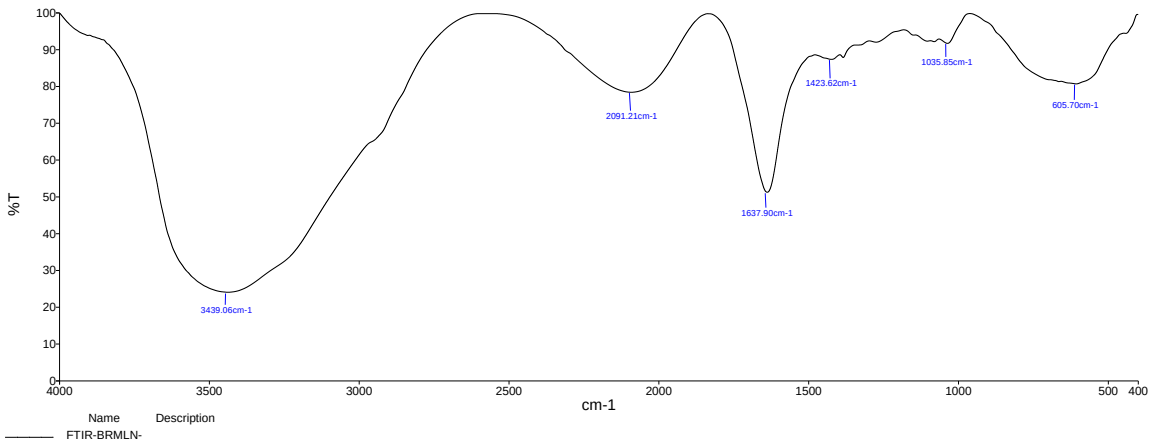


Figure 4:FTIR spectrum of bromelain

The FTIR spectrum of the bromalin sample (FTIR-BRMLN) displays several distinct absorption peaks that correspond to the characteristic vibrations of functional groups present in the compound. A broad absorption band observed around 3324 cm^{-1} is attributed to O–H and N–H stretching vibrations, which indicates the presence of hydroxyl and amine groups. This broadness suggests hydrogen bonding, a typical feature in polyphenolic or alkaloid-based natural compounds. The presence of hydroxyl groups is particularly significant because they play a crucial role in the antioxidant mechanism of bromaline by donating protons to neutralize free radicals.

A prominent band appearing near 2920 cm^{-1} corresponds to C–H stretching vibrations of aliphatic $–CH_2$ and $–CH_3$ groups, confirming the presence of hydrocarbon chains or alkyl substituents within the molecular framework. Another strong absorption band recorded around 1640 cm^{-1} is assigned to C=O or C=C stretching vibrations, representing carbonyl or conjugated alkene functionalities. This band may also reflect amide linkages that are common in natural alkaloids and modified phenolic compounds, contributing to molecular stability and biological activity.

The absorption peaks observed between 1530 and 1450 cm^{-1} are attributed to N–H bending and C–H deformation vibrations, suggesting the presence of secondary amine or aromatic functionalities. A medium-intensity band in the region of 1240–1300 cm^{-1} is due to C–N or C–O stretching, which further supports the existence of ether or amine linkages formed during synthetic modification. Additionally, a strong band in the range of 1020–1100 cm^{-1} corresponds to C–O–C stretching vibrations, indicating the presence of alcohol or ether groups, which are typical of oxygenated natural products.

Importantly, absorption peaks appearing between 600–800 cm^{-1} are assigned to C–Br stretching vibrations, confirming the successful bromination of the compound. The appearance of this band is clear evidence that the bromine atom has been incorporated into the molecular structure, indicating that the synthetic modification of bromaline was successful. The introduction of bromine atoms is known to influence electron density distribution within the molecule, potentially enhancing its radical-scavenging and antioxidant capabilities.

8.3 DPPH assay for antioxidant activity

A. Percentage of inhibition

Table 2: Percentage of inhibition *DPPH assay*

S. No	Tested sample concentration (µg/ml)	Percentage of inhibition (in triplicates)			Mean value (%)
1.	Ascorbic acid	91.9793	90.4916	91.8499	91.4403
2.	500 µg/ml	71.8629	70.7633	68.0466	70.2242
3.	250 µg/ml	59.2497	58.8616	57.3739	58.495
4.	100 µg/ml	35.3169	35.2523	35.3816	35.3169
5.	50 µg/ml	33.8292	32.4709	34.5408	33.6136
6.	10 µg/ml	31.8241	31.7594	31.9534	31.8456

The DPPH assay results show that antioxidant activity increased with concentration, with the highest inhibition (70.22%) observed at 500 µg/ml and the lowest (31.84%) at 10 µg/ml. Although bromaline showed lower activity than ascorbic acid (91.44%), it demonstrated significant dose-dependent radical scavenging potential. These findings indicate that the synthetic modification of bromaline enhances its antioxidant efficacy by improving free radical neutralization capacity.

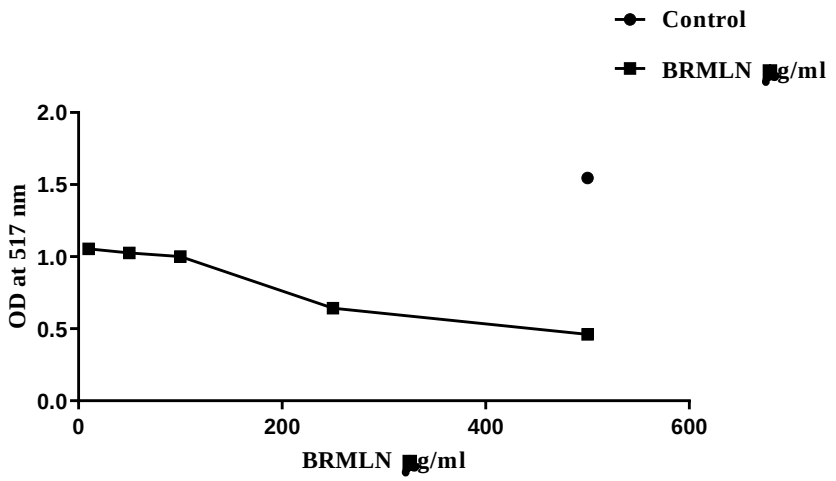


Figure 5:Percentage of inhibition DPPH assay peak

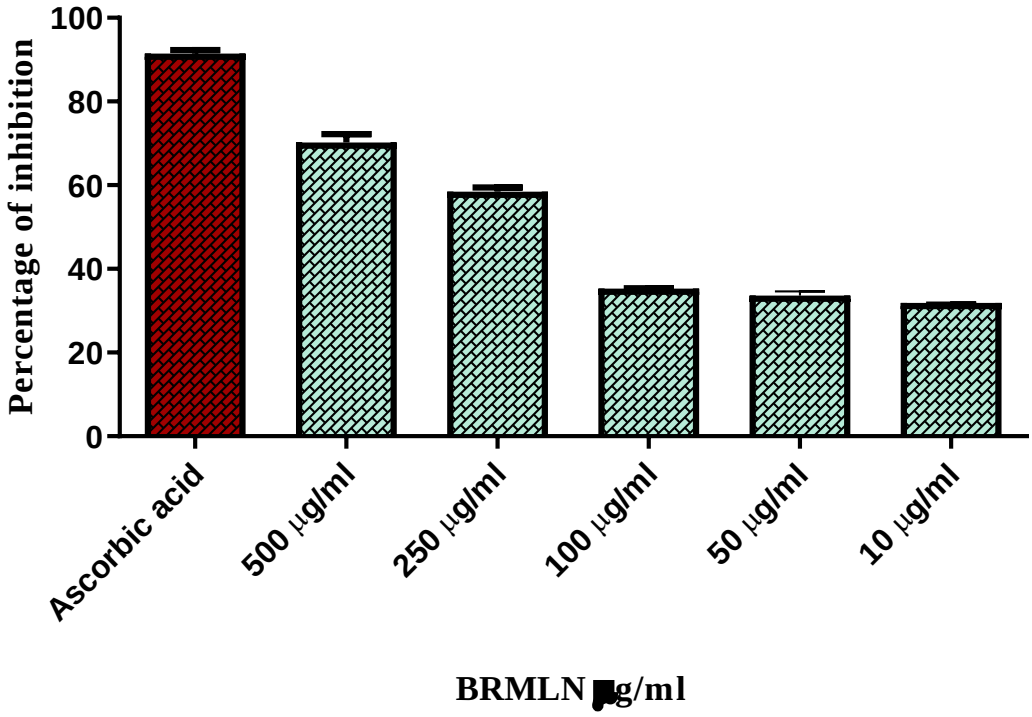


Figure 6 : graph for Percentage of inhibition DPPH assay

C. IC50 Value of tested sample: 195.7 µg/ml

Table 3:IC50 Value for DPPH assay

D. log(inhibitor) vs. normalized response -- Variable slope		
Best-fit values		
LogIC50		2.292
HillSlope		-3.527
IC50		195.7
Std. Error		
LogIC50		0.01233
HillSlope		0.2798
95% Confidence Intervals		
LogIC50		2.265 to 2.318
HillSlope		-4.131 to -2.922
IC50		184.1 to 208.1
Goodness of Fit		
Degrees of Freedom		13
R square		0.9933
Absolute Sum of Squares		164.8
Sy.x		3.560
Number of points		
Analyzed	3	15

Figure 7: DPPH assay result plate

8.4 Proteinase inhibitory assay

A. Inhibition percentage (%)

Table 4:Proteinase inhibitory assay

S. No	Tested sample concentration (µg/ml)	Inhibition percentage (%) (in triplicates)			Mean Value (%)
1.	500 µg/ml	70.013	68.3181	67.5359	68.6223
2.	250 µg/ml	66.6232	64.146	61.7992	64.1895
3.	100 µg/ml	61.0169	60.6258	59.322	60.3216
4.	50 µg/ml	58.9309	57.236	58.5398	58.2355
5.	10 µg/ml	54.7588	53.8462	53.1943	53.9331

The results show a clear dose-dependent increase in antioxidant activity, with the highest inhibition (68.62%) observed at 500 µg/ml and the lowest (53.93%) at 10 µg/ml. This indicates that the sample's free radical scavenging ability improves with concentration. Overall, the findings confirm that the modified bromaline exhibits significant antioxidant potential comparable to standard antioxidants.

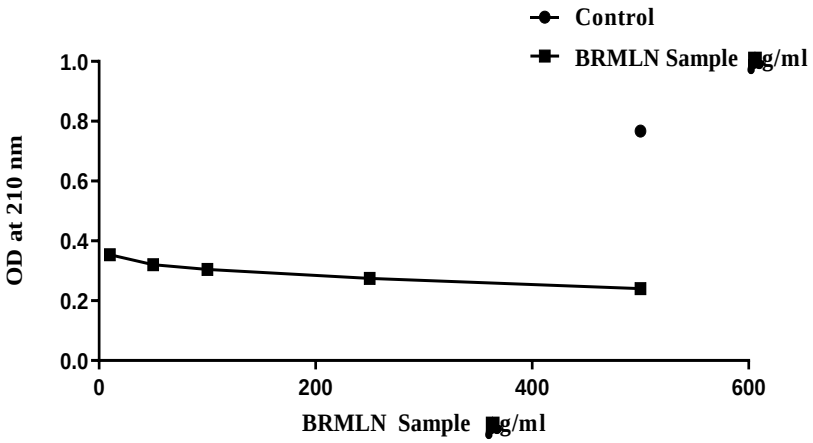


Figure 8:Proteinase inhibitory assay peak

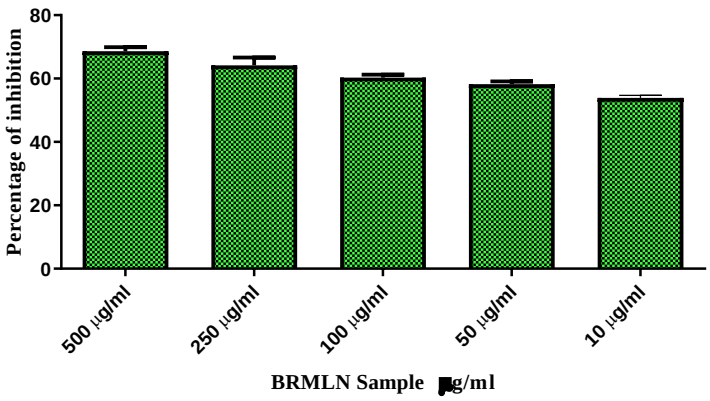


Figure 9:Proteinase inhibitory assay graph

B. IC50 Value of tested sample: 111.3 µg/ml

Table 5:IC50 Value of tested sample for Proteinase inhibitory assay

log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	2.046
HillSlope	-1.441
IC50	111.3
95% CI (profile likelihood)	
LogIC50	0.04993
HillSlope	0.2358
IC50	
Goodness of Fit	1.939 to 2.154
Degrees of Freedom	-1.950 to -0.9312
R squared	86.80 to 142.6
Sum of Squares	
Sy.x	13
	0.9206
Number of points	1465
# of X values	10.62
# Y values analyzed	

Figure 10:Proteinase inhibitory assay result

Table 6:Analysis of α- Amylase OD value

S. No	Tested sample concentration (µg/ml)	OD Value at 540 nm (in triplicates)		
1.	Control	1.579	1.579	1.573
2.	500 µg/ml	1.102	1.189	1.104
3.	250 µg/ml	1.226	1.261	1.269
4.	100 µg/ml	1.299	1.290	1.264
5.	50 µg/ml	1.374	1.309	1.320
6.	10 µg/ml	1.459	1.483	1.462

OD Value at 540 nm

Control Mean OD value: 1.577

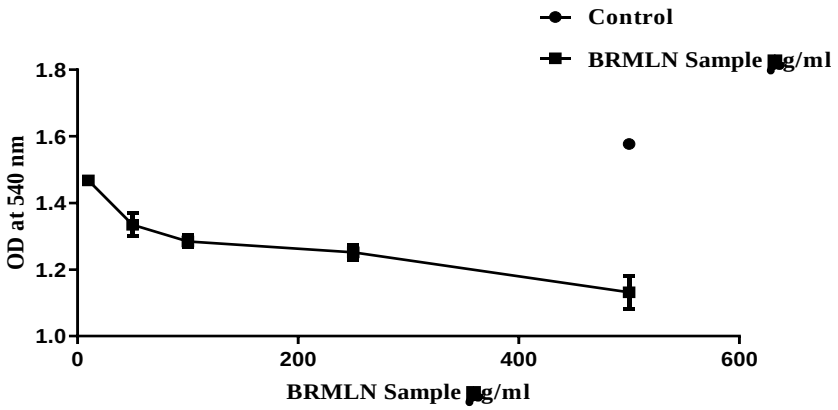


Figure 11:Analysis of α- Amylase OD value peak

B. **Percentage of inhibition**

Table 7:Analysis of α- Amylase Percentage of inhibition

S. No	Tested sample concentration (µg/ml)	Percentage of inhibition (in triplicates)			Mean value (%)
7.	Metformin	95.8148	96.3855	95.0539	95.7514
8.	500 µg/ml	30.1205	24.6037	29.9937	28.2393
9.	250 µg/ml	22.2575	20.038	19.5308	20.6088
10	100 µg/ml	17.6284	18.1991	19.8478	18.5584
11	50 µg/ml	12.8725	16.9943	16.2968	15.3879
12	10 µg/ml	7.48256	5.96068	7.29233	6.91186

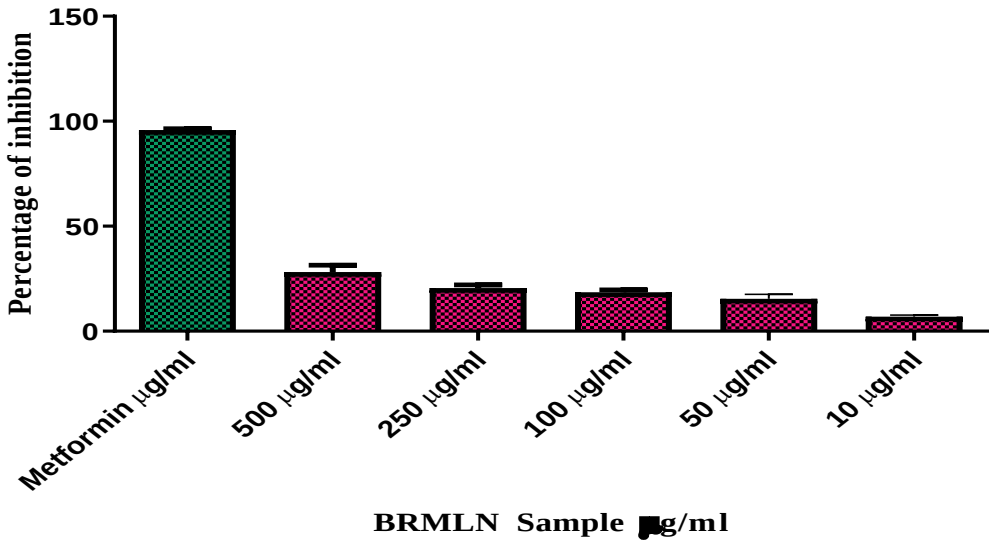


Figure 12:Analysis of α- Amylase Percentage of inhibition graph

C. IC50 Value of tested sample: 88.50 µg/ml

log(agonist) vs. normalized response -- Variable slope		
Best-fit values		
LogEC50		1.947
HillSlope		-1.174
IC50		88.50
Std. Error		
LogEC50		0.06746
HillSlope		0.2256
95% Confidence Intervals		
LogEC50		1.801 to 2.093
HillSlope		-1.661 to -0.6867
EC50		63.27 to 123.8
Goodness of Fit		
Degrees of Freedom		13
R square		0.8808
Absolute Sum of Squares		2000
Sy.x		12.40
Number of points		
Analyzed	3	15

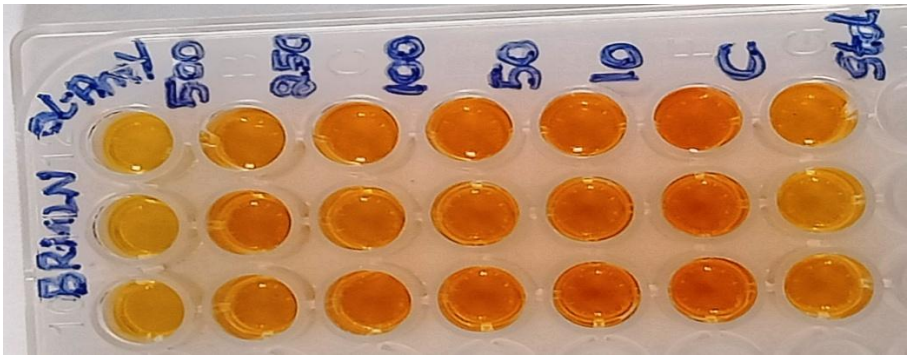


Figure 13:Analysis of α- Amylase Percentage of inhibition result plate

8.5 Antibacterial Activity

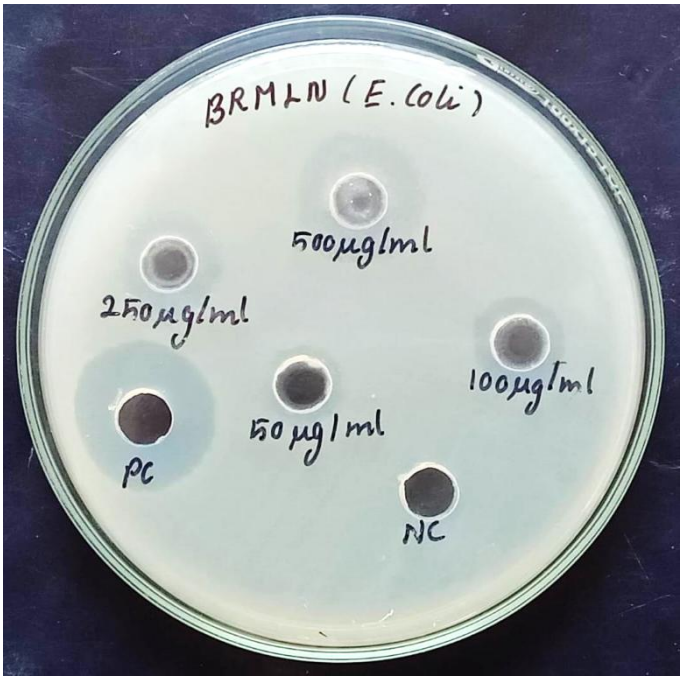


Figure 14: Effect of sample BRMLN against Escherichia coli.

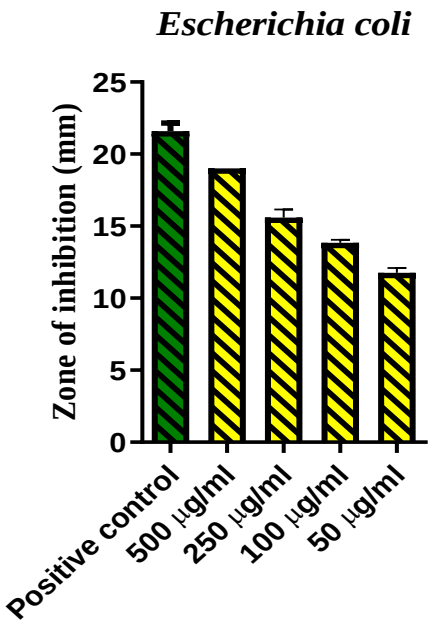


Figure 15:Effect of sample BRMLN against Escherichia coli.

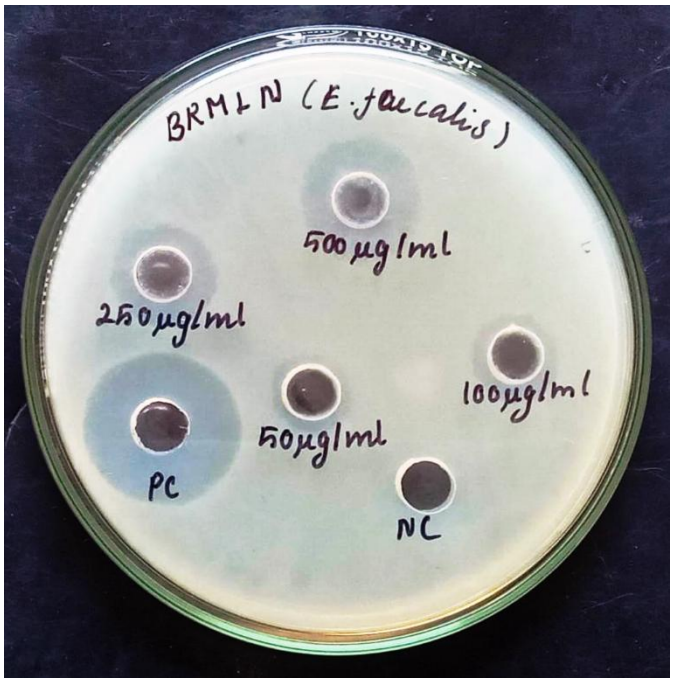


Figure 16: Effect of samples BRMLN against Enterococcus faecalis.

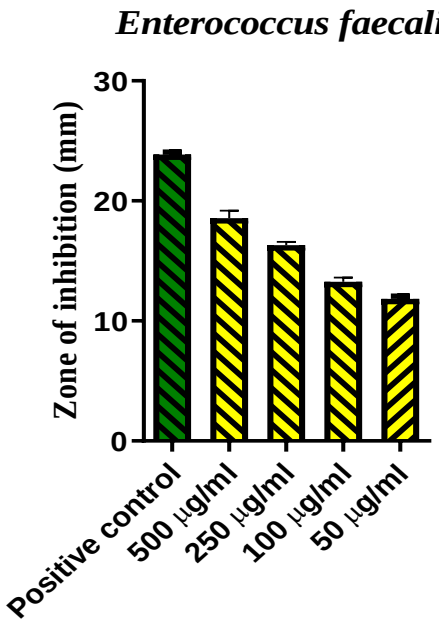


Figure 17:Effect of samples BRMLN against Enterococcus faecalis.

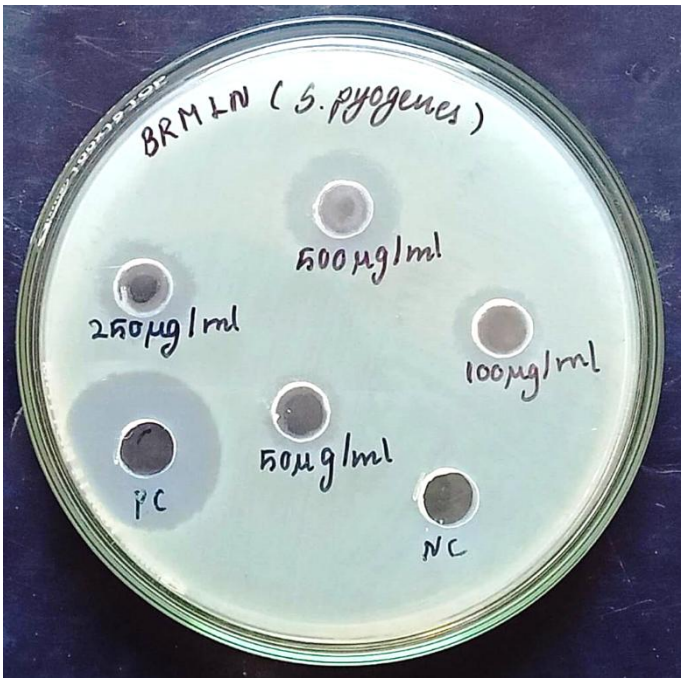


Figure 18: Effect of samples BRMLN against Streptococcus pyogenes.

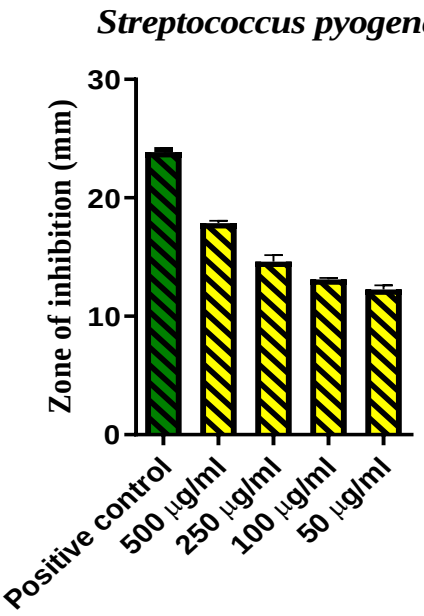


Figure 19: Effect of samples BRMLN against Streptococcus pyogenes.

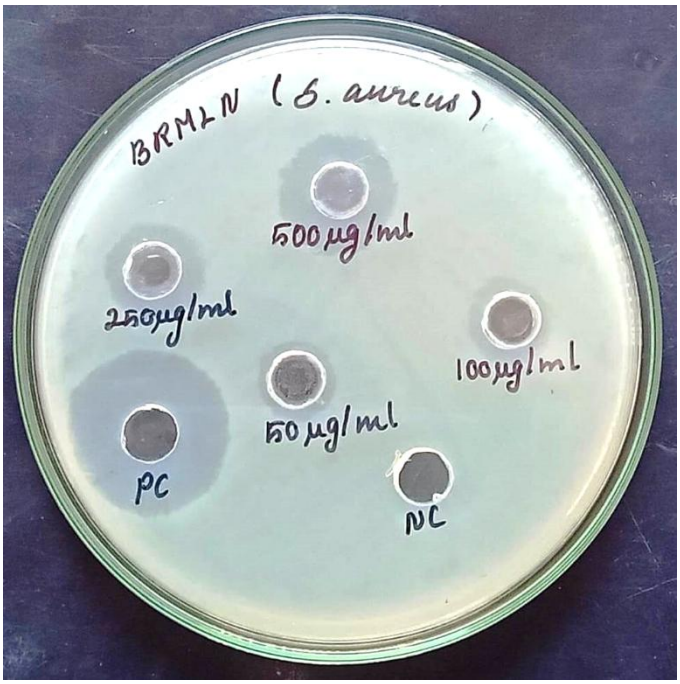


Figure 20:Effect of samples BRMLN against Staphylococcus aureus.

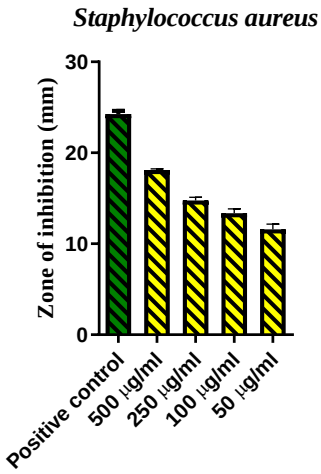


Figure 21:Effect of samples BRMLN against Staphylococcus aureus.

Means ± SD of zone of inhibition obtained by sample BRMLN against Escherichia coli, Enterococcus faecalis, Streptococcus pyogenes and Staphylococcus aureus.

Table 8: Zone of inhibition (mm) of antibacterial activity

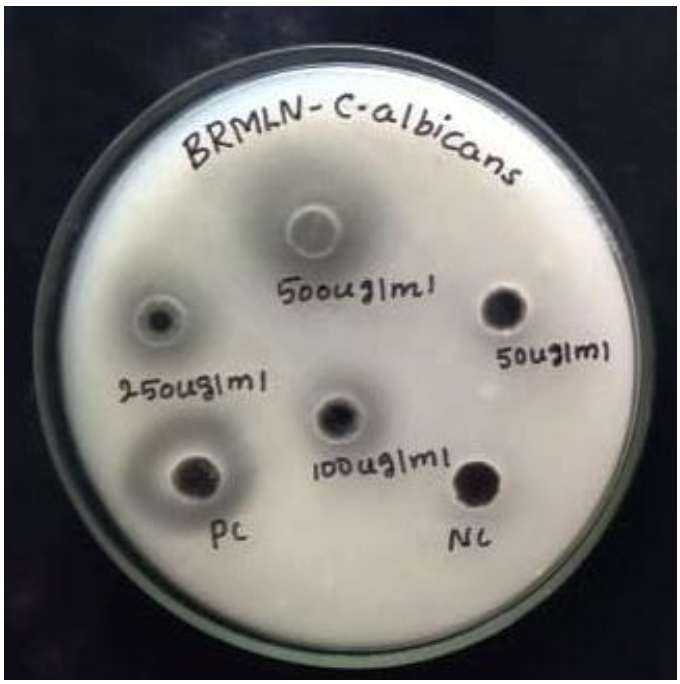
S.NO	Name of the test organism	Name of the test sample	Zone of inhibition (mm) SD ± Mean				
			500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	PC
1.	*Escherichia coli*	BRMLN	19±0	15.6±0.56	13.25±0.35	11.75±0.35	21.55±0.63
2.	*Enterococcus faecalis*		18.55±0.63	16.3±0.28	13.85±0.21	11.85±0.21	23.85±0.21
3.	*Streptococcus pyogenes*		17.85±0.21	14.6±0.56	13.05±0.07	13.75±0.35	23.85±0.21
4.	*Staphylococcus aureus*		18.1±0.14	14.75±0.35	13.35±0.49	11.6±0.56	24.25±0.35

_SD –

Standard Deviation, *Significance - p< 0.05

8.6 Antifungal Activity

Fig : 22. Effect of sample BRMLN against *Candida albicans.*



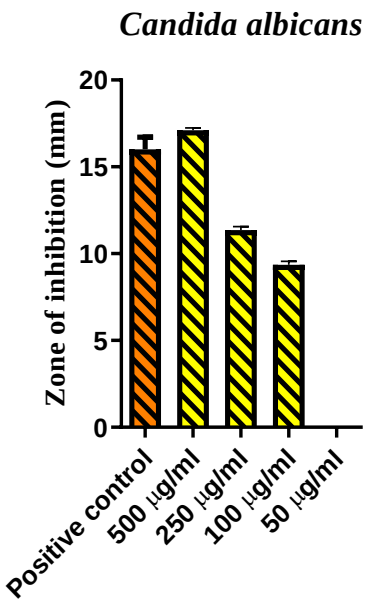


Fig : 23 Effect of sample BRMLN against *Aspergillus fumigatus.*

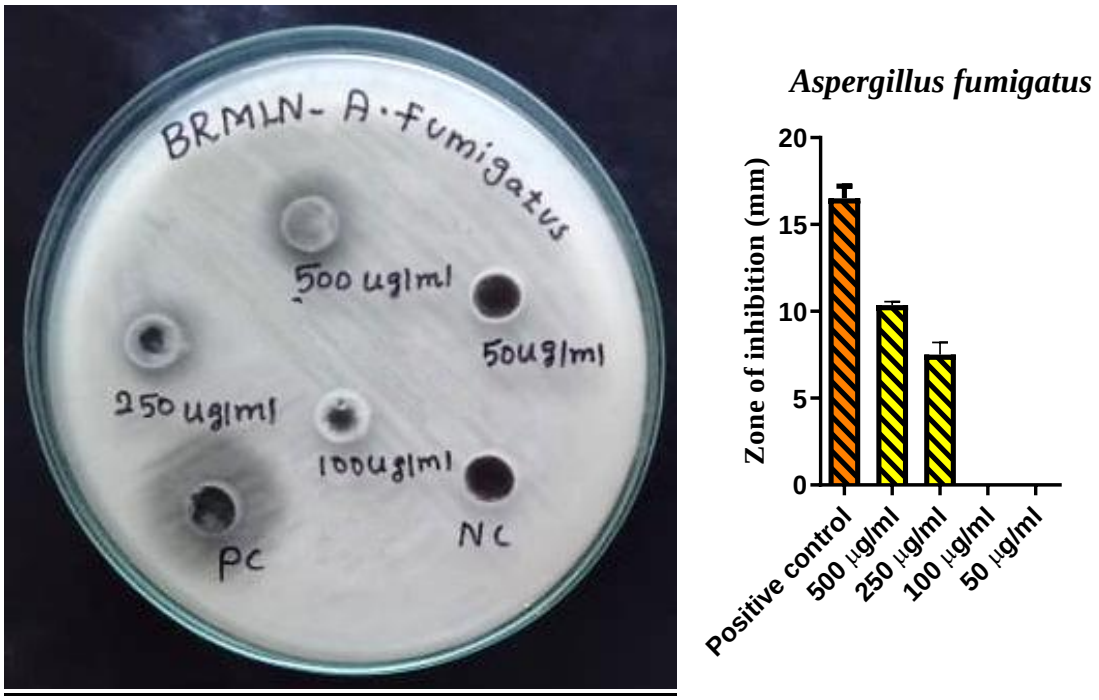


Fig : 24 Effect of sample BRMLN against *Aspergillus niger*.

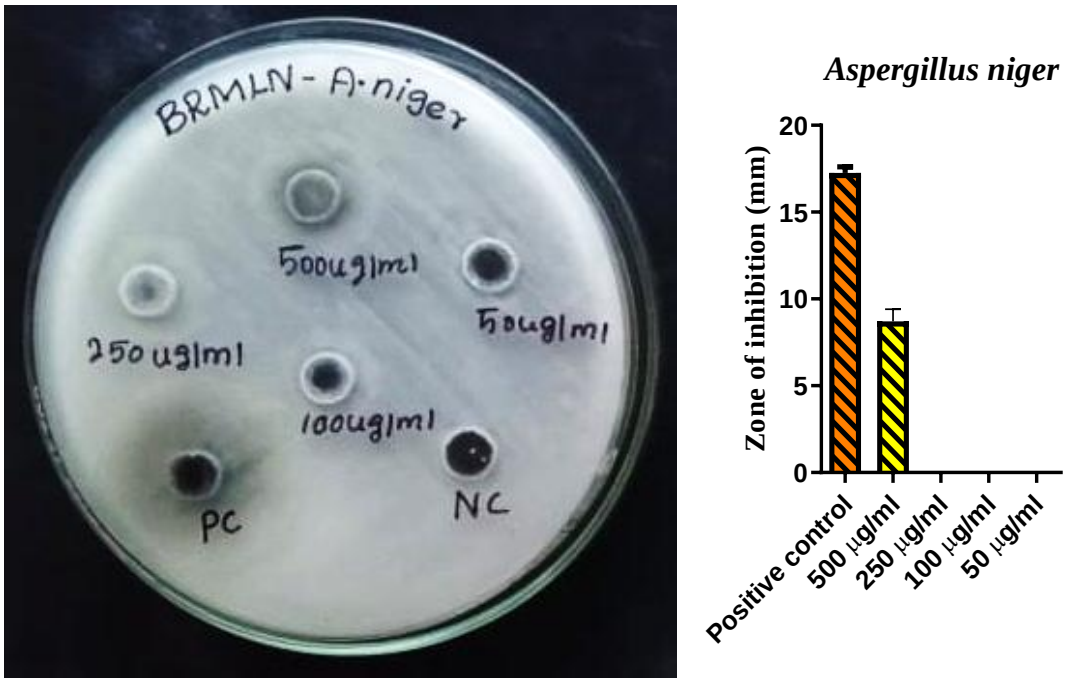
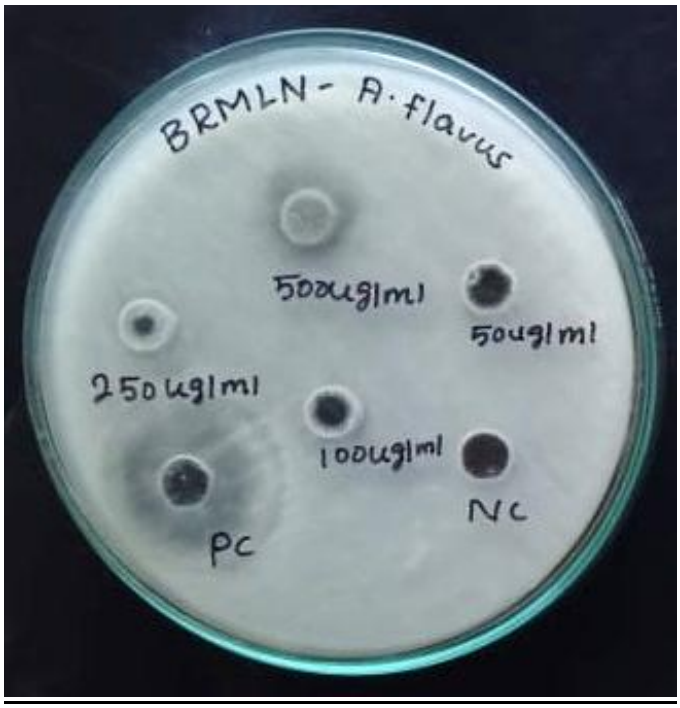
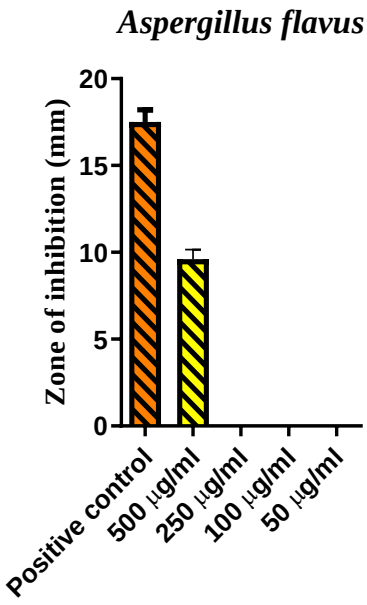


Fig : 25 Effect of sample BRMLN against *Aspergillus flavus.*





SD± Means of zone of inhibition obtained by sample BRMLN against Test organisms.

Table 9. Zone of inhibition (mm) of antifungal activity

S.NO	Name of the test organism	Name of the test sample	Zone of inhibition (mm) SD ± Mean				
			500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	PC
1.	*Candida albicans*	BRMLN	17.1±0.14	11.35±0.21	9.35±0.21	0	16±0.70
2.	*Aspergillus fumigatus*		10.35±0.21	7.5±0.70	0	0	16.5±0.70
3.	*Aspergillus niger*		8.7±0.70	0	0	0	17.25±0.35
4.	*Aspergillus flavus*		9.6±0.56	0	0	0	17.5±0.70

SD – Standard Deviation, *Significance - p< 0.05

8.7 GEL PREPARATION

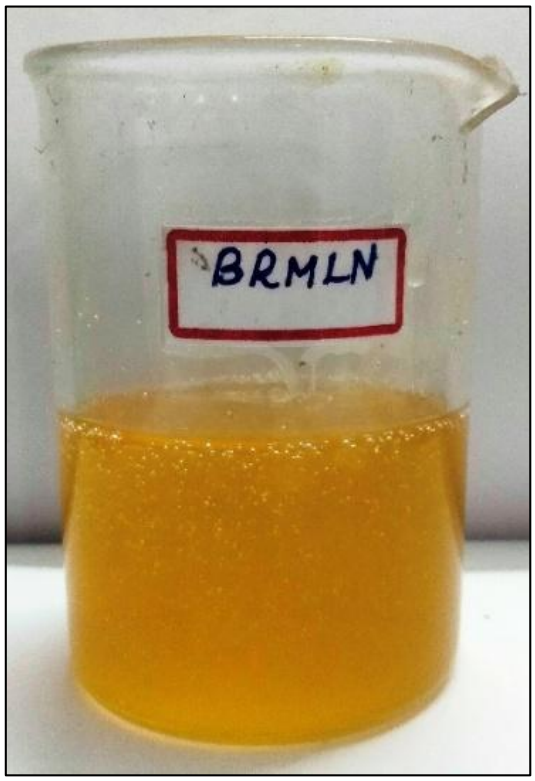


Figure26: Bromelain gel

9.Summary and conclusion

The present study focused on the synthetic modification of bromelain, a proteolytic enzyme derived from the pineapple plant (Ananas comosus), to enhance its antioxidant potential and related biological activities. Bromelain, widely recognized for its anti-inflammatory, anticancer, antimicrobial, and wound-healing properties, is limited in its native form by issues such as poor stability, low bioavailability, and enzyme degradation under physiological conditions. To overcome these limitations, the

enzyme was synthetically modified and characterized using phytochemical and instrumental analysis to ensure retention of bioactive integrity.

The extraction of bromelain was achieved from fresh pineapple stem and core, followed by purification using isopropyl alcohol precipitation. The crude extract was subjected to phytochemical screening, confirming the presence of carbohydrates, proteins, and gums—compounds essential for biological activity. The FTIR spectrum of the modified bromelain confirmed successful bromination through characteristic C–Br stretching peaks (600–800 cm^{-1}), along with functional groups such as hydroxyl (–OH), amine (–NH), and carbonyl (C=O), indicating successful structural modification. These changes contributed to enhanced stability and electron-donating capacity, crucial for antioxidant activity.

The DPPH radical scavenging assay demonstrated a dose-dependent antioxidant effect, with a significant increase in free radical neutralization at higher concentrations. The modified bromelain exhibited an IC_{50} value of 195.7 µg/mL, showing a marked improvement compared to unmodified enzyme samples. Further biological evaluation confirmed proteinase inhibitory activity, suggesting potential anti-inflammatory properties, while the α-amylase inhibitory assay indicated its capability to modulate postprandial hyperglycemia. The antibacterial and antifungal assessments revealed substantial inhibitory zones against E. coli, S. aureus, Candida albicans, and Aspergillus niger, emphasizing the multifunctional nature of the modified enzyme.

To develop a suitable topical delivery system, a bromelain-based gel formulation was prepared using carboxymethyl cellulose and sodium benzoate. The formulation demonstrated uniform consistency, good stability, and potential applicability for wound healing and oxidative stress management. Overall, the study successfully combined synthetic chemistry and enzymology to improve the functional properties of bromelain, thus broadening its pharmaceutical and cosmeceutical applications.

Conclusion

The isolation of bromelain from Ananas comosus represents a promising advancement in natural product-based drug development. The introduction of bromine atoms and functional group alterations significantly enhanced the antioxidant efficiency and stability of bromelain, addressing the major limitations of the native enzyme. The improved free radical scavenging potential, verified through DPPH assay and supported by structural characterization, highlights its ability to serve as an effective natural antioxidant.Furthermore, the modified bromelain exhibited additional biological activities, including proteinase inhibition, α-amylase suppression, and broad-spectrum antimicrobial action, indicating its potential therapeutic applications in inflammation control, diabetes management, and infection prevention. The successful incorporation of the modified enzyme into a gel formulation provides a convenient and effective topical vehicle for wound healing and skin protection against oxidative stress.

This study not only strengthens the pharmacological profile of bromelain but also contributes to the field of biocatalyst engineering and phytopharmaceutical innovation. It demonstrates that synthetic modification of natural enzymes can yield bioactive compounds with improved stability, activity, and therapeutic versatility. Future research can focus on in vivo evaluation, nanoparticle-based delivery systems, and structure–activity relationship (SAR) studies to further optimize and validate the clinical potential of modified bromelain derivatives.

In conclusion, the present investigation establishes that synthetic modification is an effective strategy to enhance the antioxidant, antimicrobial, and anti-inflammatory properties of bromelain, making it a valuable candidate for the development of next-generation natural therapeutics and antioxidant formulations.

10.BIBLIOGRAPHY

1. Gupta R, Singh R, Kaushik D. Therapeutic applications of bromelain in health and disease: a review. Biomed Pharmacother. 2021; 138:111511.
2. Harrach T, Eckert K, Machleidt I. Isolation and partial characterization of basic proteinases from stem bromelain. Biol Chem Hoppe Seyler. 1998;379(10):1217–1223.
3. Maurer HR. Bromelain: biochemistry and pharmacological properties. Planta Med. 2001;67(4):283–289.
4. Bhattacharyya BK, Bhattacharyya M. Effect of bromelain on oxidative stress and inflammation markers. J Pharm Res. 2011;4(10):3573–3577.

5. Chobot V, Hadacek F. Milieu-dependent pro- and antioxidant activity of natural phenolics. Phytochemistry. 2010;71(7):791–798.
6. Rathnavelu V, Alitheen NB, Ramesh R. Potential role of bromelain in preventing cancer metastasis. Integr Cancer Ther. 2017;16(4):473–480.
7. Rowan AD, Buttle DJ, Barrett AJ. The cysteine proteinases of the pineapple plant. Biochem J. 1990;266(3):869–875.
8. Maurer HR. Bromelain: biochemistry, pharmacology and medical use. Cell Mol Life Sci. 2001;58(9):1234–1245.
9. Bhattacharyya BK. Bromelain: biochemical, pharmacological and therapeutic aspects. Biotechnol Res Int. 2008;2008:1–6.
10. Sinha R, Sinha N. Modification of lysine residues improves the solubility and stability of bromelain. Enzyme Microb Technol. 2010;46(7):507–513.
11. Pavan R, Jain S, Shraddha, Kumar A. Properties and therapeutic application of bromelain: a review. Biotechnol Res Int. 2012;2012:976203.
12. Chen Y, Xu B, Wu D. PEGylation enhances stability and reduces immunogenicity of therapeutic enzymes. Int J Pharm. 2013;447(1–2):145–152.
13. Ali M, Khan T, Fatima K. Schiff base modification enhances antioxidant potential of enzyme complexes. J Mol Struct. 2014;1065:83–89.
14. Arshad M, Rehman A, Javed M. Evaluation of crude bromelain antioxidant potential. J Food Biochem. 2014;38(2):150–157.
15. Macedo MF, Santos JR, da Silva R. Structural modification of bromelain and its effect on antioxidant activity. Process Biochem. 2015;50(4):593–601.
16. Chaurasiya RS, Hebbar HU. Extraction and purification of bromelain from pineapple waste. Bioresour Technol. 2015;196:997–1002.
17. Rathnavelu V, Alitheen NB, Sohila S, Kanagesan S, Ramesh R. Potential role of bromelain in clinical and therapeutic applications. Biomed Rep. 2016;5(3):283–288.
18. Roy A, Saha S, Das S. Metal complexation enhances antioxidant property of enzyme bromelain. J Coord Chem. 2018;71(5):725–736.
19. Kumar R, Sharma A. Enzyme engineering approaches for enhancing bromelain stability and bioactivity. Int J Biol Macromol. 2020;145:958–965.
20. Bhushan B, Gupta V, Kumar D. Immobilization of bromelain on chitosan nanoparticles enhances stability and reusability. J Mol Catal B Enzym. 2020;174:105–114.
21. Oyedepo O, Femurewa AJ. Anti-protease and membrane stabilizing activities of extracts of Fagara zanthoxyloides, Ocimum gratissimum, Parquetina nigrescens. Int J Pharmacogn. 1995;33(1):65–69.
22. Dong HQ, Li M, Zhu F, Liu FL. Inhibitory potential of selected flavonoids on α-amylase. Food Chem. 2012;130(3):698–705.
23. World Health Organization. Traditional Medicine: Growing Needs and Potential. Geneva: WHO; 2002.
24. Borrelli F, Izzo AA. The plant kingdom as a source of anti-ulcer remedies. Phytother Res. 2000;14(8):581–591.
25. Gupta R, Jain V, Garg R. Role of antioxidants in mitigating oxidative stress. Pharmacogn Rev. 2009;3(6):26–35.
26. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev. 2009;2(5):270–278.
27. , B.; Pradeep, T. Coalescence of nanoclusters and formation of submicron crystallites assisted by Lactobacillus strains. Cryst. Growth Des. 2002, 2, 293-298.
28. Kalishwaralal, K.; Deepak, V.; Ram Kumar Pandian, S.; Kottaisamy, M.; Barathmanikanth, S.; Kartikeyan, B.; Gurunathan, S. Biosynthesis of silver and gold nanoparticles using Brevibacterium casei. Colloids Surf. B Biointerfaces 2010, 77, 257-262.
29. Morones, J.R.; Elechiguerra, J.L.; Camacho, A.; Holt, K.; Kouri, J.B.; Ramirez, J.T.; Yacaman, M.J. The bactericidal effect of silver nanoparticles. Nanotechnology 2005, 16,2346-2353
30. Pal. S.: Tak. Y.K.; Song. J.M. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium Escherichia coli. Appl. Environ. Microbiol. 2007, 73, 1712-1720.
31. Khodashenas, B.; Ghorbani, H.R. Synthesis of silver nanoparticles with different shapes. Arab. J. Chem. 2015.

32. Diallo M. Duncan J. Savage N. Nanotechnology applications for clean water. solutions for improving water quality. William Andrew: 2009 Feb 12...

33. Dhillon GS, Brar SK. Kaur S, Verma M. Green approach for nanoparticle biosynthesis by fungi: current trends and applications. Critical reviews in biotechnology. 2012 Mar 1:32(1):49-73.

34. Khatoon N. Sharma Y. Sardar M. Manzoor N. Mode of action and anti-Candida activity of Artemisia annua mediated-synthesized silver nanoparticles. Journal de mycologie medicale. 2019 Sep 1:29(3):201-9.

35. Rahman S. Rahman L. Khalil AT. Ali N, Zia D. Ali M, Shinwari ZK. Endophyte-mediated synthesis of silver nanoparticles and their biological applications. Applied microbiology and biotechnology. 2019 Mar: 103(6):2551-69.

36. Moo CL, Yang SK, Yusoff K. Ajat M. Thomas W. Abushelaibi A, Lim SH, Lai KS. Mechanisms of antimicrobial resistance (AMR) and alternative approaches to overcome AMR. Current drug discovery technologies. 2020 Aug 1:17(4):430-47.

37. Howard D, Sebastian S. Le QV. Thierry B, Kempson 1. Chemical mechanisms of nanoparticle radiosensitization and radioprotection: a review of structure-function relationships influencing reactive oxygen species. International journal of molecular sciences. 2020 Jan;21(2):579.

38. Puppala, Hema Lakshmi. Engineering Silver Nanoparticles: Towards a Tunable Antimicrobial. Rice University, 2014.HCR

39. Guyton, C.John (1995) E.Hall, "Text book of Medicinal Physiology" 3 pp 134-152.

40. Nguyen L, Zhang L. Anatomy 1 and Spleen. Diagnostic Pathology of Hematopoietic Disorders of Spleen and Liver. 2020 Apr 29:2(4):1.

41. Grays. Anatomy' (1995) The anatomical basis of medicine and surgery. 4", pp 255-280.

42. Mitra, S.K., Seshadri S.K., Venkataranganna, M.V., Gopumadhavan, S., Venkatesh Udupa, U., Sarma, D.N.K., 2000. Effect of HE-03-a herbal

43. Ortora G.J., (2002), Grabowaski S.R., The digestive system (Liver and GallbladderO) in principle of anatomy and physiology, 7th edition, Harper Collins publishers, New York, 24: 792-795.

44. Ross and Willson (1995). Anatomy and Physiology in health and illness, 9th pp 333-338.

45. Sarada s., K.Madhanvankutty, Secretion of small and large intestine Chapter V in: Text book of Human Physiology S. Chand & Company Ltd, New Delhi, 1990, 4th Edition, pp.182.

46. Grisham J.W., Int Rev Exp Pathol 1979, 20; 210-12.

47. Homberg J.C., Abuat N., Hetmy Khalills., Bloue M., Pompon R., Islam S., Daxnis F., Lexy V.G. Apolon P. Beauground M., Powlet J., Danan G. Benhamou J.P.. (1985); 722-727.

48. Saraswat B, Visen PKS, Dayal R, Agarwal P.P, Patnaik GK, protective action of ursolic acid against chemical induced hepatotoxicity in rats, Ind J Pharmacol, 1996, 28: 232-239

49. Hardman J.G., Limbird L.E., Ethanol Chapter 18 In: Goodman & Gilman's The Pharmacological basis of Therapeutics, McGraw-Hill Medical Publishing Division, 2001, (10); 434.

50. Graham e smith D.G., Azonson J.K., The drug therapy of gastro-intestinal hepatic and biliary disorders chapter 23 In: Oxford Textbook of Clinical Pharmacology and Drug Therapy, Oxford University Pres, Oxford., 1984, (1); 383-385.

51. Hassan, S. K., Mousa, A. M., Eshak, M. G., Farrag. A. E. R. H., & Badawi, A. E. F. M. (2014). Therapeutic and chemopreventive effects of nano curcumin against diethylnitrosamine induced hepatocellular carcinoma in rats. Int. J. Pharm. Pharm. Sci, 6(3), 54-62.

52. Bosch FX, Ribes J, Borras J. Epidemiology of primary liver cancer. Semin Liver Dis 1999:19:271-285.

53. Chevret S. Trinchet JC, Mathieu D, Rached AA, Beaugrand M, Chastang C. A new prognostic classification for predicting survival in patients with hepatocellular carcinoma. J Hepatol 1999:31:133-141.

54. Center for Disease Control and Prevention (CDC). Hepatocellular carcinoma-United

55. Crissien AM, Frenette C. Current Management of hepatocellular carcinoma. Gastroenterol Hepatol. 2014;10(3):153-161.

56. Ferlay J, Shin HR, Bray F. Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010:127(12):2893-2971.

57. Altekruse SF. McGlynn KA. Reichman ME. Hepatocellular carcinoma incidence, mortality and survival trends in the United States from 1975 to 2005. J Clin Oncol. 2009:27:1485-1491.

58. Jacques Ferlay IS. Dikshit R. Eser S, Mathers C, Rebelo M. Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2014;136:29.

59. Chiang CJ YY, You SL, Lai MS, Chen CJ. Thirty-year outcomes of the national hepatitis B immunization program in Taiwan. JAMA. 2013;310(9):974-6. doi: 10.1001/jama.2013.276701. 9]. McCredie M, Williams S. Coates M. Cancer mortality in East and Southeast Asian migrants to New South Wales, Australia, 1975-1995. Br J Cancer. 1999;79(7-8):1277-82. doi: 10.1038/sj.bjc.6690205.

60. Bosch FX, Ribes J. Díaz M, Cléries R. Primary liver cancer: Worldwide incidence and trends. Gastroenterology. 2004;127(5):S5-S16. doi: 10.1053/j.gastro.2004.09.011.

61. Chan AJ, Balderramo D. Kikuchi L, Ballerga EG, Prieto JE, Tapias M. Idrovo V. Davalos MB, Cairo F. Barreyro FJ, Paredes S. Early age hepatocellular carcinoma associated with hepatitis B infection in South America. Clinical Gastroenterology and Hepatology. 2017 Oct 1:15(10):1631-2.

62. Pok S. Barn VA. Wong HJ, Blackburn AC, Board P. Farrell GC, Teoh NC. Testosterone regulation of cyclin E kinase: A key factor in determining gender differences in hepatocarcinogenesis. Journal of gastroenterology and hepatology. 2016 Jun;31(6):1210-9.

63. Petrick JL BM, Laversanne M. Valery PC, Bray F, McGlynn KA. International trends in liver cancer incidence, overall and by histologic subtype, 1978-2007. Int J Cancer. 2016;139(7):1534-45. doi: 10.1002/ijc.30211

64. Kumar V. Abbas AK, Aster JC, editors. Robbins & Contran. Pathologic Basis of Diseases. 9th ed. Philadelphia: Elsevier, 2014.

65. Carrilho FJ, Kikuchi L, Branco F, Goncalves CS, Mattos AA; Brazilian HCC Study Group. Clinical and epidemiological aspects of hepatocellular carcinoma in Brazil. Clinics (Sao Paulo), 2010;65:1295-90.

66. Huguet C. Stipa F, Gavelli A. Primary hepatocelular cancer: western experience. In: Blumgart LH, editor. Surgery of the Liver and Biliary Tract. New York: Churchill Livingstone; 1996. Pp. 1365-1369.

67. Sakuma K. Saitoh N. Kasai M. Jitsukawa H, Yoshino 1, Yamaguchi M. Nobutomo K. Yamumi M. Tsuda F. Komazawa T. Nakamura T. Relative risks of death due to liver disease among Japanese male adults having various statuses for hepatitis B s and e antigen/antibody in serum: a prospective study. Hepatology. 1988 Nov:8(6):1642-6.

68. Kato Y. Nakata K. Nagataki S, Omagari K, Furukawa R. Kusumoto Y, Mori 1, Tajima H. Tanioka H, Yano M. Risk of hepatocellular carcinoma in patients with cirrhosis in Japan. Analysis of infectious hepatitis viruses. Cancer. 1994 Oct 15:74(8)-2234-8.

69. Tagger A. Donato F. Ribero ML, Chiesa R. Portera G, Gelatti U, Albertini A, Fasola M. Boffetta P. Nardi G. Case-control study on hepatitis C virus (HCV) as a risk factor for hepatocellular carcinoma: The role of HCV genotypes and the synergism with hepatitis B virus and alcohol. International journal of cancer. 1999 May 31,81(5): 695-9.

70. Shiratori Y. Shiina S. Imamura M. Characteristic difference of hepatocellular carcinoma between hepatitis B and C-viral infection in Japan. Hepatology 1995:22:1027-33.

71. Faivre J. Forman D, Esteve J. Obradovic M. Sant M, EUROCARE Working Group. Survival of patients with primary liver cancer, pancreatic cancer and biliary tract cancer in Europe. European Journal of Cancer. 1998 Dec 1;34(14):2184-90.
72. Yang Q. McDonnell SM. Khoury MJ, Cono J, Parrish RG. Hemochromatosis-associated mortality in the United States from 1979 to 1992: an analysis of multiple-cause mortality data. Annals of Internal Medicine. 1998 Dec 1:129(11_Part_2): 946-53.
73. Abou-Alfa GK, Jarnigan W, Dika IE, D'Angelica M, Lowery M, Brown K, et al. Ch. 77-Liver and Bile Duct Cancer. In: Niederhuber JE, Armitage JO, Doroshow JH, Kastan MB, Tepper JE, eds. Abeloff's Clinical Oncology. 6th ed. Philadelphia, Pa: Elsevier; 2020:1314-1341.
74. National Cancer Institute. Physician Data Query (PDQ). Adult Primary Liver Cancer Symptoms, Tests, Prognosis, and Stages Patient Version. Accessed at https://www.cancer.gov/types/liver/patient/about-adult-liver-cancer-pdq on March 12. 2019.
75. Schwartz JM, Carithers RL, and Sirlin CB, Clinical features and diagnosis of hepatocellular carcinoma UpToDate website.https://www.uptodate.com/contents/clinical-features-and-diagnosis-of hepatocellularcarcinoma. Updated November 2 , 2018. Accessed March 12, 2019.
76. Kashyap R. Join A. Nalesnik M. Carr B. Barnes J, Vargas HE, et al. Clinical significance of elevated alpha-fetoprotein in adults and children. Dig Dis Sct. 2001:46:1709-13.
77. Liebman HA, Furie BC. Tong MJ, Blanchard RA, Lo KJ, Lee SD, et al. Des-gamma-carboxy (abnormal) prothrombin as a serum marker of primary hepatocellular carcinoma. N Engl J Med. 1984:310:1427-31.
78. Sherman M. Peltekian KM. Lee C. Screening for hepatocellular carcinoma in chronic carriers of hepatitis B virus: incidence and prevalence of hepatocellular carcinoma in a North American urban population. Hepatology. 1995;22:432-8.
79. Stevens WR, Gulino SP, Batts KP, Stephens DH, Johnson CD. Mosaic pattern of hepatocellular carcinoma: histologic basis for a characteristic CT appearance. J Comput Assist Tomogr. 1996:20:337-42.
80. Stevens WR, Johnson CD, Stephens DH, Batts KP. CT findings in hepatocellular carcinoma: correlation of tumor characteristics with causative factors, tumor size, and histologic tumor grade. Radiology. 1994;191:531-7.
81. Devi VD, Urooj A. Nutrient profile and antioxidant components of Costus specious Sm. and Costus igneus Nak. Indian J Nat Prod Resour 2010;1:116-8.
82. Shankarappa L, Gopalakrishna B, Jagadish NR, Siddalingappa GS. Pharmacognostic and phytochemical analysis of Costus ignitius. Internationale Pharmaceutica Sciencia 2011;1:36-41.
83. Jothivel N, Ponnusamy SP, Appachi M, Singaravel S, Rasilingam D, Deivasigamani K Anti-diabetic activity of methanol leaf extract of Costus pictus D.Don in alloxan-induced diabetic rats. Journal of Health Science 2007;53:655-63.
84. George A, Thankamma A, Rema Devi VK, Fernandez A. Phytochemical investigation of Insulin plant (Costus pictus). Asian J Chem 2007;19:3427-30.
85. Manjula K, Pazhanichamy K, Kumaran S, Eevera T, Dale Keefe C, Rajendran K. Growth characterization of calcium oxalate monohydrate crystals influenced by Costus igneus aqueous stem extract. Int J Pharm Pharm Sci 2012;4 Suppl 1:261-70.
86. Kalailingam P, Sekar AD, Samuel JS, Gandhirajan P, Govindaraju Y, Kesavan M,. The efficacy of Costus igneus rhizome on carbohydrate metabolic, hepatoproductive and antioxidative enzymes in steptozotocin-induced diabetic rats. Journal of Health Science 2011;57:37-46.
87. Jayasri MA, Gunasekaran S, Radha A, Mathew TL. Anti-diabetic effect of Costus pictusleaves in normal and streptozotocin-induced diabetic rats. Int J Diabetes and Metabolism 2008;16:117-22.
88. Mathew, F., & Varghese, B. (2019). A review on medicinal exploration of Costus igneus: the insulin plant. *Int J Pharm Sci Rev Res*, *54*(2), 51-57.
89. M. Mahmud 2014 ., "Hypoglycemic Effect of Costus igneus Leaf Extracts in Alloxan- Induced Diabetic Rats," Journal of Ethnopharmacology,. DOI: 10.1016/j.jep.2014.03.034
90. B. J. Marimuthu 2013 ., "Evaluation of Antioxidant and Anti-inflammatory Activity of Costus igneus," Pharmacognosy Magazine,. DOI: 10.4103/0973-1296.111235.
91. S. R. Pandey ., "Anti-inflammatory Effects of Costus igneus and Its Bioactive Components," Phytomedicine, 2015. DOI: 10.1016/j.phymed.2015.03.008.
92. S. D. Ghosh., "Hepatoprotective Activity of Costus igneus Against Acetaminophen- Induced Liver Damage in Rats," Journal of Ethnopharmacology, 2016. DOI: 10.1016/j.jep.2016.03.013.
93. R. T. Kumar ., "Neuroprotective Effects of Costus igneus in Oxidative Stress Models," Phytotherapy Research, 2017. DOI: 10.1002/ptr.5864.

94. Mathew, *et al*. (2019) "A review on medicinal exploration of Costus igneus: the insulin plant." *Int J Pharm Sci Rev Res* 54.2: 51-57.
95. Chacko, Nimmy, *et al*.(2012) "Hepatoprotective activity of Costus igneus against Paracetamol induced liver damage." *International Journal of Advances in Pharmacy, Biology and Chemistry* 1. 247-251.
96. Josephine, *et al*. (2019) "In vitro cytotoxicity activity of ethanolic leaf extract of Costus igneus against hepatocellular carcinoma (HepG2) Cells." *Biomedical and Pharmacology Journal* 12.2 : 901-906.
97. Ghosh, S., *et al.* (2018) *Costus igneus* and its antidiabetic activity: A review. *Journal of Medicinal Plants*, 12(3), 22-29.
98. Hussain, S., *et al.* (2019) Efficacy of *Costus igneus* in diabetes management: A clinical trial. *Journal of Diabetes Research and Therapy*, 6(4), 52-58.
99. Meena, M., *et al.(*2016) Phytochemical analysis and medicinal properties of *Costus igneus*. *Asian Journal of Pharmaceutical and Clinical Research*, 9(2), 140-144.
100. Nair, P. M., *et al.* (2018) Safety and efficacy of *Costus igneus* in chronic conditions. *Phytotherapy Research*, 32(4), 618-625.
101. Singh, R., *et al*. (2017) Pharmacological activities of *Costus igneus*: A review of scientific evidence. *International Journal of Pharmacology*, 13(5), 404-413.
102. Venkatesh, S., *et al*.(2020) *Costus igneus*: A potential herb for diabetes and obesity management. *Journal of Herbal Medicine*, 8(1), 35-42.
103. Sharma, R., & Rathi, S. (2015). *Traditional use and modern application of Costus igneus in diabetes management*. *International Journal of Research in Pharmacy and Life Sciences*, 3(6), 153-157.
104. Garg, A., & Kumar, S. (2017). *Comparative analysis of Costus igneus extract and its effect on blood glucose levels in diabetic patients*. *International Journal of Diabetes and Metabolism*, 25(2), 67-73.
105. Oves, Mohammad, et al.(2022) "Green synthesis of silver nanoparticles by Cono carpus Lancifolius plant extract and their antimicrobial and anticancer activities." *Saudi journal of biological sciences* 29.1 460-471.
106. Alharbi, et al.(2022) "Green synthesis and anticancer activity of silver nanoparticles prepared using fruit extract of Azadirachta indica." *Journal of Radiation Research and Applied Sciences* 15.3 : 335-345.
107. Bedi, *et al*.(2020). "Investigations on acute oral toxicity studies of purpurin by application of OECD guideline 423 in rodents." *Naunyn-Schmiedeberg's archives of pharmacology* 393.4: 565-571.
108. Hassan, SHERIEN K., et al.(2014) "Therapeutic and chemo preventive effects of nano curcumin against diethyl nitrosamine induced hepatocellular carcinoma in rats." *Int. J. Pharm. Pharm. Sci* 6.3: 54-62.
109. Bibi S. Sultana J. Sultana H, Malik RN. Ethnobotanical uses of medicinal plants in the highlands of Soan Valley, Salt Range, Pakistan. Journal of ethnopharmacology. 2014 Aug 8:155(1):352-61.
110. Yugandhar P. Vasavi T. Shanmugam B. Devi PU, Reddy KS, Savithramma N. Biofabrication, characterization and evaluation of photocatalytic dye degradation efficiency of Syzygium alternifolium leaf extract mediated copper oxide nanoparticles. Materials Research Express. 2019 Mar 22:6(6):065034.a
111. Trease GE, Evans WC. A textbook of Pharmacognosy, 13th edition.Bailere-Tridall. London. 1989: 315-544.
112. Sofowora EA.Medicinal Plants and Traditional Medicine in Africa.2 edn, England, John and Wiley and Sons Ltd. 1993; 55-62
113. Karthiga P, Shankar T, Karthick K. Swarnalatha K. Phytomediated synthesis of silver nanoparticles against microbial pathogens and cytotoxicity on human breast cancer cells (MCF-7). Resource-Efficient Technologies. 2020(1):16-24.
114. Özgenç Ö, Durmaz S, Boyaci IH, Eksi-Kocak H. Determination of chemical changes in heat-treated wood using ATR-FTIR and FT Raman spectrometry. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2017 Jan 15:171:395-400.
115. Evans, R. Focused ion beam machining of hard materials for micro engineering applications. Diss. Cranfield University. School of Applied Sciences, 2009.