ANTIMICROBIAL ACTIVITY OF FRUIT WASTE EXTRACT AND SERINE ALKALINE PROTEASE

A Research Project

Submitted by

PRACHI C. KESARKAR

GALAXY S. PAWAR

SANIKA P. THORBOLE

SHRUTIKA M. PARDESHI

UNDER THE GUIDANCE OF

DR. SAVITA D. MALI

(Assistant Professor)

DEPARTMENT OF MICROBIOLOGY

VIVEKANAND COLLEGE, KOLHAPUR

(AN EMPOWERED AUTONOMOUS INSTITUTE)

YEAR 2025-2026

"Dissemination of education for Knowledge, Science and culture"

- Shikshanmaharshi Dr. Bapuji Salunkhe

Shri Swami Vivekanand Shikshan Sanstha's

VIVEKANAND COLLEGE, KOLHAPUR

(AN EMPOWERED AUTONOMOUS INSTITUTE)

DEPARTMENT OF MICROBIOLOGY (PG)

CERTIFICATE

OF

RESEARCH PROJECT COMPLETION

This is to certify that Ms. **PRACHI C. KESARKAR** studying in M. Sc. part II, Sem-IV at Vivekanand College, Kolhapur (An Empowered Autonomous Institute) has sincerely completed research project work entitled **"ANTIMICROBIAL ACTIVITY OF FRUIT WASTE EXTRACT AND SERINE ALKALINE PROTEASE"** during academic year 2025-26.

24.4.26.

24/4/26

Dr. Savita D. Mali
Research Project Guide

Examiner

Dr. T. C. Gaupale
Head of the Department
I/C Head
Department of Microbiology
Vivekanand College, Kolhapur
(Empowered Autonomous)

"Dissemination of education for Knowledge, Science and culture"

- Shikshanmaharshi Dr. Bapuji Salunkhe

Shri Swami Vivekanand Shikshan Sanstha's

VIVEKANAND COLLEGE, KOLHAPUR

(AN EMPOWERED AUTONOMOUS INSTITUTE)

DEPARTMENT OF MICROBIOLOGY (PG)

CERTIFICATE

OF

RESEARCH PROJECT COMPLETION

This is to certify that Ms. GALAXY S. PAWAR studying in M. Sc. part II, Sem-IV at Vivekanand College, Kolhapur (An Empowered Autonomous Institute) has sincerely completed research project work entitled "ANTIBACTERIAL ACTIVITY OF FRUIT WASTE EXTRACT AND SERINE ALKALINE PROTEASE" during academic year 2025-26.

Dr. Savita D. Mali		Dr. T. C. Gaupale
Research Project Guide	Examiner	Head of the Department
		I/C Head Department of Microbiology Vivekanand College, Kolhapur (Empowered Autonomous)

"Dissemination of education for Knowledge, Science and culture"

- Shikshanmaharshi Dr. Bapuji Salunkhe

Shri Swami Vivekanand Shikshan Sanstha's

VIVEKANAND COLLEGE, KOLHAPUR

(AN EMPOWERED AUTONOMOUS INSTITUTE)

DEPARTMENT OF MICROBIOLOGY (PG)

CERTIFICATE

OF

RESEARCH PROJECT COMPLETION

This is to certify that Ms. SANIKA P. THORBOLE studying in M. Sc. part II, Sem-IV at Vivekanand College, Kolhapur (An Empowered Autonomous Institute) has sincerely completed research project work entitled "ANTIBACTERIAL ACTIVITY OF FRUIT WASTE EXTRACT AND SERINE ALKALINE PROTEASE" during academic year 2025-26.

Dr. Savita D. Mali		Dr. T. C. Gaupale
Research Project Guide	Examiner	Head of the Department
		I/C Head Department of Microbiology Vivekanand College, Kolhapur (Empowered Autonomous)

"Dissemination of education for Knowledge, Science and culture"

- Shikshanmaharshi Dr. Bapuji Salunkhe

Shri Swami Vivekanand Shikshan Sanstha's

VIVEKANAND COLLEGE, KOLHAPUR

(AN EMPOWERED AUTONOMOUS INSTITUTE)

DEPARTMENT OF MICROBIOLOGY (PG)

CERTIFICATE

OF

RESEARCH PROJECT COMPLETION

This is to certify that Ms. SHRUTIKA M. PARDESHI studying in M. Sc. part II, Sem-IV at Vivekanand College, Kolhapur (An Empowered Autonomous Institute) has sincerely completed research project work entitled "ANTIBACTERIAL ACTIVITY OF FRUIT WASTE EXTRACT AND SERINE ALKALINE PROTEASE" during academic year 2025-26.

Dr. Savita D. Mali

Research Project Guide

24.4.26

Examiner

Dr. T. C. Gaupale

Head of the Department

I/C Head
Department of Microbiology
Vivekanand College, Kolhapur
(Empowered Autonomous)

ACKNOWLEDGEMENT

I wish to express my deep sense of appreciation to Dr. Savita D. Mali, Assistant Professor, Department of Microbiology, Vivekanand College, Kolhapur (An Empowered Autonomous Institute) for her valuable support and expert guidance during the course of this study. She has been extremely understanding and cooperative and has always taken great interest in this work.

I wish to express my sincere thanks to Dr. C. B. Patil, Principal, Vivekanand College, Kolhapur (An Empowered Autonomous Institute) and Dr. T. C. Gaupale, Head, Department of Microbiology for providing the laboratory facilities in the department to carry out the experimental work.

I express my thanks to Ms. V. V. Misal, Coordinator of PG Department of Microbiology and faculty members, Dr. K. K. Bhise, Ms. M. M. Nadkarni, Ms. S. S. Shaikh, Ms. A. T. Patil, and Ms. P. P. Patil for their valuable suggestions and help during the work.

I convey my gratitude to Mrs. S. N. Salokhe (Laboratory Assistant), Mr. S. K. Maskar and Mr. D. R. Patil (Laboratory Staff) of the department for their kind help in the laboratory.

I am thankful to the librarian and library staff for providing facilities of computer and reference books. My special thanks and gratitude to my entire classmates who have been constant source of inspiration and help during entire project work. I am highly obliged to authors past and present whose literature has been cited.

Finally, I thank my family members for their blessings and support because of which this work has proved satisfactory to me.

Place: Kolhapur

Date:

Ms. Prachi C. Kesarkar

Ms. Galaxy S. Pawar

Ms. Sanika P. Thorbole

Ms. Shrutika M. Pardeshi

ANTIMICROBIAL ACTIVITY OF FRUIT WASTE EXTRACT AND SERINE ALKALINE PROTEASE

INDEX

1.0

INTRODUCTION

A wound can be defined as any physical disruption in the normal anatomical structure and function of the skin or underlying tissues, caused by mechanical, thermal, chemical, or microbial factors. The process of wound healing represents a highly organized and dynamic biological mechanism through which the body restores the integrity of damaged tissue. This process involves the activation of various cellular and molecular pathways that work sequentially and in coordination to ensure the restoration of the barrier function of the skin (StatPearls, 2025).

Wound healing typically proceeds through four overlapping stages — haemostasis, inflammation, proliferation, and remodelling (maturation). Haemostasis occurs immediately after injury, where platelets aggregate to form a temporary plug and release clotting factors to stop bleeding. Inflammation follows within hours, during which neutrophils and macrophages infiltrate the wound site to eliminate pathogens and debris. The proliferation phase involves fibroblast activation, angiogenesis, and the formation of granulation tissue rich in collagen and extracellular matrix. Finally, during remodelling, collagen Fibers reorganize, and tensile strength gradually increases as the new tissue matures. These steps ensure complete tissue repair when coordinated properly. However, external factors such as infection, poor nutrition, oxygen deficiency, or diabetes can impair this process, resulting in chronic or non-healing wounds. The development of efficient topical formulations can therefore play a vital role in accelerating wound repair and preventing infections. Understanding wound healing is essential for designing bioactive creams that target multiple stages of tissue recovery. Natural substances rich in antioxidants and antimicrobial compounds can support these mechanisms by enhancing oxygenation, collagen deposition, and immune defence at the wound site.

A wound-healing cream is a topical formulation designed to promote and accelerate tissue repair by providing an optimal environment for cell regeneration. It acts as a protective barrier that maintains moisture, prevents microbial invasion, and delivers bioactive compounds directly to the damaged site. These creams may contain natural or synthetic ingredients with specific biological functions such as antimicrobial, antioxidant, and anti-inflammatory effects. Traditional synthetic creams often use petroleum-based carriers and chemical preservatives that can cause irritation or allergic responses. Moreover, the overuse of synthetic antibiotics in topical applications

contributes to antimicrobial resistance. Consequently, the development of bio-based wound-healing creams using natural ingredients such as fruit peel extracts, herbal compounds, and microbial enzymes has become a priority in modern biomedicine (Kumar et al., 2020; Sutar et al., 2021).

Natural extracts are rich in compounds like flavonoids, polyphenols, tannins, vitamins, and essential oils that exhibit potent antimicrobial and antioxidant properties. These molecules help neutralize reactive oxygen species (ROS), which otherwise delay wound closure, and also stimulate fibroblast migration and epithelialization. Moreover, the presence of natural pigments and phytochemicals provides anti-inflammatory effects that reduce redness, swelling, and infection risk.

Fruit waste — including peels, pulp residues, and seeds — represents an abundant and underutilized resource rich in bioactive compounds. In the context of pharmaceutical and cosmetic industries, fruit waste valorisation has emerged as a sustainable strategy for the development of therapeutic formulations. Peels of fruits such as guava, banana, pomegranate, and chikoo (sapota) contain diverse phytochemicals like ascorbic acid, flavonoids, polyphenols, carotenoids, and tannins, which exhibit strong antioxidant and antimicrobial properties (Sharma et al., 2022).

For instance, guava peel extract is rich in vitamin C and tannins that stimulate collagen synthesis, neutralize free radicals, and promote fibroblast proliferation — all of which are essential for wound closure. Similarly, sapota (chikoo) seeds possess saponins and polyphenolic compounds that exhibit antioxidant and antimicrobial activities. These bioactive can inhibit microbial growth at wound sites and simultaneously enhance tissue regeneration. Utilizing such fruit-derived compounds aligns with the concept of zero-waste biotechnology, where agricultural by-products are transformed into high-value biomedical materials. This approach not only minimizes environmental pollution but also provides cost-effective solutions for topical applications. By converting waste materials into bioactive ingredients, we support circular economy principles and sustainable innovation in healthcare product design.

Thus, fruit waste serves as both a functional and ecological component in developing wound-healing formulations, enhancing therapeutic potential while reducing global waste burdens.

Serine alkaline proteases (SAPs) are a major class of extracellular enzymes that catalyze the hydrolysis of peptide bonds through a serine residue in their active site. They are predominantly produced by microbial species such as *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus cereus*. These enzymes exhibit high catalytic efficiency under alkaline pH and moderate to elevated temperature conditions, making them suitable for various industrial and biomedical applications (Ramakrishna et al., 2019; Gupta et al., 2002).

In the field of wound care, SAPs have gained attention due to their debriding, antimicrobial, and regenerative properties. The enzymatic degradation of necrotic tissue by proteases allows for a cleaner wound bed, facilitating faster granulation and epithelialization. Additionally, SAPs prevent microbial colonization by disrupting biofilm structures formed by bacteria, thereby reducing the risk of infection.

Beyond their cleaning action, serine proteases also influence the biochemical microenvironment of wounds. They promote fibroblast migration and collagen deposition — two critical processes for tissue regeneration. Their mild proteolytic action helps remodel extracellular matrix components, enhancing flexibility and tensile strength of newly formed tissue. Microbial proteases have therefore become promising biological alternatives to chemical debriding agents, offering high specificity, safety, and biodegradability. In recent research, proteases from *Bacillus* species have demonstrated potential in wound-healing ointments and hydrogel formulations, with faster closure rates observed compared to untreated controls. The integration of serine alkaline proteases in natural wound-healing creams provides a dual-functional system: enzymatic cleaning of the wound surface and stimulation of cellular repair mechanisms.

The integration of fruit waste extracts and microbial enzymes represents a novel and sustainable approach for modern wound-care formulations. Natural extracts contribute antioxidants and antimicrobial compounds, whereas enzymes like serine alkaline protease assist in the biological cleaning of necrotic tissue and stimulation of regeneration. Together, these components act synergistically to enhance healing efficiency.

This combination leverages the biochemical diversity of natural materials and microbial metabolites. For instance, the antioxidants in fruit peels reduce oxidative

stress at the wound site, while proteolytic enzymes accelerate the turnover of damaged proteins and promote new tissue formation. This synergy ensures faster wound contraction, lower infection risk, and reduced scarring (Singh et al., 2021).

Additionally, such bio-integrated formulations are biodegradable, non-toxic, and cost-effective, aligning with global trends in green biotechnology. They have potential applications in topical creams, hydrogel dressings, or bioactive bandages, depending on the desired release and moisture-retention properties. By combining enzyme-based debridement with natural antioxidants, this innovative strategy provides a comprehensive healing mechanism that is both sustainable and effective.

Several studies have demonstrated that creams incorporating bioactive proteins or plant extracts show promising wound healing effects. Furthermore, the formulation of a wound healing cream using natural ingredients not only offers therapeutic value but also aligns with current global movements emphasizing sustainability and waste reduction. Hence, the project was undertaken to formulate a wound healing cream comprising serine alkaline bacterial protease and fruit extract and to study its effectiveness against skin pathogens. This approach aims to develop an eco-friendly, bioactive, and sustainable cream that can effectively support tissue regeneration and infection control.

2.0

REVIEW OF LITERATURE

Wound healing(Fig no.1) is a complex physiological process involving cellular and molecular mechanisms that repair tissue damage through four overlapping stages: haemostasis, inflammation, proliferation, and remodelling. Numerous studies have demonstrated that oxidative stress, microbial infections, and improper inflammation significantly delay wound closure (El-Sayed et al., 2024). Therefore, topical wound-healing formulations with antioxidant and antimicrobial potential are considered essential to accelerate healing. Traditional wound dressings often provide only physical protection, whereas bioactive formulations enhance regeneration by delivering therapeutic agents directly to the wound site (Yunus et al., 2024).

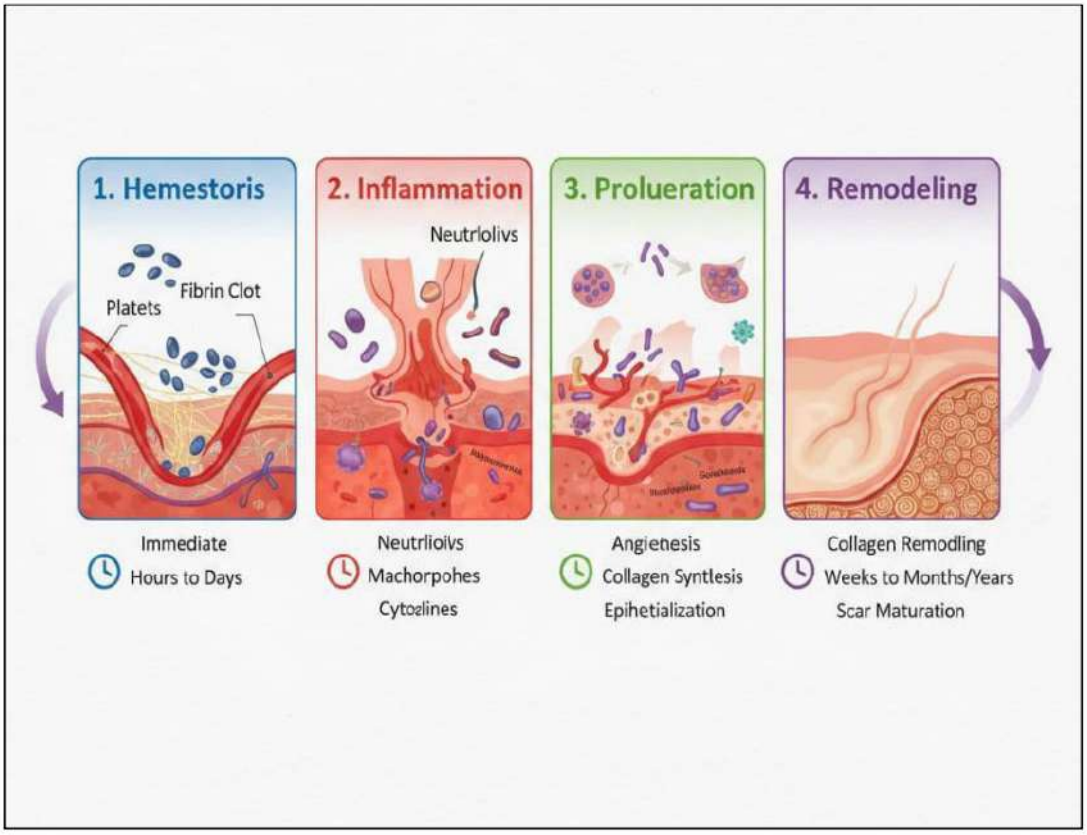

Figure No. 1: Phases of Wound Healing

2.1 Use of Fruit Waste in Wound-Healing Applications

Fruit peels and seeds, which are commonly discarded as agro-waste, are rich sources of natural antioxidants, flavonoids, phenolics, tannins, and vitamins. These phytochemicals play a vital role in reducing oxidative stress and enhancing tissue repair. Several researchers have investigated the wound-healing efficacy of fruit waste extracts. For instance, Kumar et al. (2020) evaluated banana peel extract in Wistar rats and found a significant reduction in wound area, enhanced epithelialization, and increased collagen deposition. Similarly, Sutar et al. (2021) reported that orange peel extract exhibited potent antimicrobial activity against wound pathogens and promoted faster healing. Sharma et al. (2022) highlighted that guava peel extract is rich in ascorbic acid and tannins, which enhance fibroblast proliferation and collagen synthesis, accelerating wound contraction.

The utilization of fruit waste extracts in pharmaceutical and cosmetic formulations aligns with sustainable biotechnology principles by transforming organic waste into valuable bioactive materials (Espinosa-Espinosa et al., 2022). The incorporation of such natural compounds into wound-healing creams offers a safe, cost-effective, and eco-friendly alternative to synthetic chemical agents.

2.1.1 Phytochemical composition of fruit waste

Fruit peel and seeds contain various bioactive constituents that contribute significantly to the wound healing process. Polyphenols and flavonoids act as potent antioxidants that neutralize reactive oxygen species (ROS) generated during inflammation, thereby preventing oxidative stress induced tissue damage (Rjeswari et al., 2012). Vitamin C, abundant in guava, orange, and papaya peels, promotes collagen synthesis, enhances fibroblast proliferation, and accelerates re-epithelialization (Sharma et al., 2022). Tannins help in contracting tissues and forming a protective layer over wounds, reducing infection risks, while saponins and alkaloids contribute to antimicrobial activity by disturpting microbial membranes (Patil et al., 2018)

2.1.2 Antioxidant and Anti-inflammatory activities of fruit waste extract

Oxidative stress plays a crucial role in impairing wound healing by damaging lipids, proteins and nucleic acids in skin cells. The antioxidants present in fruit waste extracts mitigate this effect by scavenging free radicals and enhancing endogenous defence mechanisms (Rajeswari et al., 2012). For instance, guava peel extract, rich in vitamin C and quercetin, has been shown to significantly increase the activities of catalase and superoxide dismutase in wounded tissues, resulting in faster healing (Joseph and Priya, 2011). Similarly, citrus peel extracts possess high levels of hesperidin and naringenin, which exhibit anti-inflammatory properties by inhibiting pro-inflammatory cytokines such as TNF-α and IL-6 (Espinosa et al., 2022).

The bioactive components (Fig no.2)also enhances angiogenesis- the formation of new blood vessels and stimulate fibroblast activity, leading to increased collagen deposition and tissue remodelling. The antioxidant defence mechanism of fruit peel extracts, therefore plays a dual role – preventing microbial infection and reducing oxidative tissue damage. The use of fruit waste extracts in topical wound healing formulations has gained immense attention due to their safety and biocompatibility. Bioactive loaded creams and gels derived from fruit waste have shown remarkable improvements in healing indices compared to synthetic ointments (Kumar et al., 2020). Their natural emulsifying properties also improve formulation stability and skin absorption. Moreover, the incorporation of fruit extracts enhances the hydration and elasticity of the skin, which is the vital for maintaining the integrity of new tissue. The transformation of fruit waste into therapeutic bio-products aligns with the global movement towards sustainable biotechnology. According to Ayala-Zavala et al., (2011), the valorisation of agro-industrial residues into bioactive formulations not only minimizes environmental pollution but also provide cost-effective alternatives for pharmaceutical applications. Thus, fruit waste represents a renewable and potent source of biologically active compounds that can be efficiently used in developing eco-friendly wound care products. According to Kumar et al., (2020) Banana peel extract is rich in potassium, polysaccharides, flavonoids. Banana peel extract accelerates collagen synthesis and reduces the inflammatory phase of wound healing. Sutar et al., (2021) highlights Citrus peel extract exhibits strong antimicrobial activity due to limonene and ascorbic acid. It promotes fibroblast proliferation and angiogenesis while reducing

inflammation. A study by Sharma et al., (2022) Guava peel extract contains ascorbic acid, tannins, lycopene which stimulate fibroblast migration, enhances collagen remodelling and improve remodelling and improve tensile strength of healed tissue. According to Nair et al., 2013; Patil et al., (2018) Chikoo (Sapota) seed extract displays antibacterial activity against skin pathogen such as *S. aureus* and *E. coli* and contains saponins that support cell proliferation and tissue regeneration.

Collectively, these findings confirms that fruit waste materials hold substantial potential for integration into advanced wound healing formulations. Their dual functionality as antimicrobial and antioxidant agents, combined with their biodegradability and abundance, makes them highly promising candidates for sustainable biomedical product development.

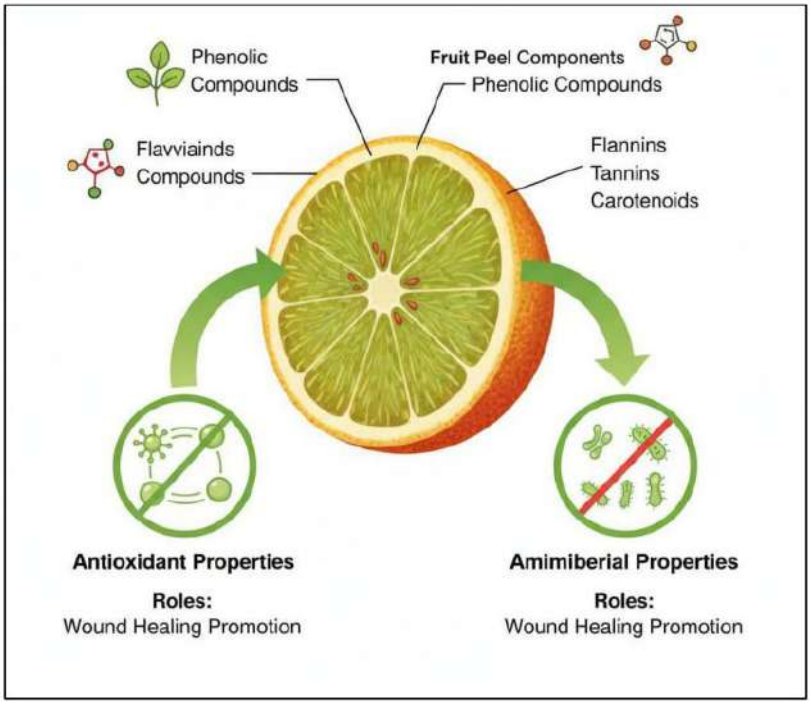

Figure No. 2: Bioactive compounds in fruit by-products

Studies have demonstrated that banana peel extract enhances wound contraction and epithelial formation, while orange peel extract possesses significant antibacterial activity against pathogen such as *Staphylococcus aureus* and *Escherichia coli*. Therefore, such as

natural sources provide a sustainable and safe alternative to conventional chemical creams, with fewer side effects and greater biocompatibility. The growing awareness of eco-friendly and affordable healthcare solutions further strengthens the relevance of natural wound healing products.

2.2. Role of Enzymes in Wound Management

Enzymatic therapy has gained attention for its effectiveness in wound debridement, infection control, and tissue regeneration. Among various enzymes, serine alkaline proteases (SAPs) are particularly notable due to their ability hydrolyse necrotic proteins, fibrin, and microbial biofilms. They play a dual role — cleansing the wound surface and stimulating cellular proliferation for tissue repair (Ramakrishna et al., 2019). These enzymes are primarily produced by *Bacillus* species such as *B. subtilis, B. cereus,* and *B. licheniformis.* Gupta et al. (2002) demonstrated that SAPs exhibit high catalytic efficiency under alkaline pH (8–11) and moderate temperatures (45–70°C), making them suitable for topical formulations. The enzyme's serine residue at the active site enables efficient peptide bond hydrolysis, while its thermostability allows its inclusion in various biopharmaceutical formulations. Bezawada et al. (2011) also reported that alkaline proteases from *Bacillus* can be economically produced using agro-waste substrates, reinforcing the concept of sustainable enzyme manufacturing. These properties make SAPs valuable biological tools in modern wound-care technology.

The removal of necrotic tissue, known as debridement, is one of the most critical steps in wound management. Traditional debridement methods – such as surgical excision or chemical treatment can be painful, non-selective and sometimes lead to secondary infection. Enzymatic debridement provides a superior alternative due to its ability to selectively digest necrotic tissue while sparing viable cells (Raju et al., 2017). Proteolytic enzymes, especially serine proteases, function by hydrolysing peptide bonds in denatured proteins and fibrin networks that accumulate on wound surface (Choudhary et al., 2018).

According to Arun et al., 2018, proteolytic enzymes accelerate healing by converting complex necrotic proteins into simpler peptides and amino acids which are then easily removed or utilized by regenerating tissues. Enzymatic debridement also improves

oxygen diffusion and nutrient supply to wound bed creating a favourable microenvironment for cell proliferation and granulation tissue formation.

2.2.1 Role of enzymes in infection control

Infections are among the most common complications in wounds particularly in chronic and diabetic ulcers. Pathogenic microorganisms such as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* form biofilm that protect them from host immune responses and antibiotics (Kumar et al., 2021). Enzymatic treatment has been shown to disrupt biofilm integrity and enhances antimicrobial efficacy. Proteases hydrolyze extracellular polymeric substances (EPS) - the protective matrix of biofilm thereby exposing bacterial cells to host defence's and antimicrobial agents (Choudhary et al., 2018). SAPs, in particular are effective against gram positive and gram-negative bacterial biofilms. According to, Yunus et al., (2024) wounds treated with SAP based topical formulations exhibited reduced microbial load and inflammation compared to control treatments.

2.2.2 Enzymes acts as catalysts for collagen remodelling

The remodelling phase of wound healing involves the replacement of provisional ECM with mature collagen fibres. Enzymes such as serine proteases, elastases and collagenases play critical roles in this remodelling process. SAPs, by hydrolysing partially denatured collagen and fibrin, help in restructuring the ECM for better tensile strength and scar formation (Choudhary et al., 2018). A study by El-Sayed et al., (2024), showed that enzymes treated wounds exhibited improved collagen fiber orientation and higher hydroxyproline content compared to untreated controls. These findings suggest that enzyme based wound treatment enhance both functional and asthetic aspects of tissue recovery. Furthermore, proteolytic enzymes can be combined with bioactive plant extracts or biopolymers to ensure sustained enzyme activity and controlled release at the wound site (Pandey et al., 2021).

The application of enzyme in wound care has evolved from simple ointments to sophisticated delivery systems. Enzyme loaded hydrogels, nanofibers and liposomal

creams are being developed to ensure enzyme stability, biocompatibility and sustained release (Singh et al.,2021).

Compared to traditional antiseptics and antibiotics, enzymatic formulations offer several advantages –

- Selectivity – They target only necrotic and denatured proteins, sparcing healthy tissue.
- Painless action – Enzymatic debridement is minimally invasive.
- Biodegradability – Enzymes are naturally broken down in the body without leaving harmful residues.
- Synergistic Compatibility – Enzymes can be combined with antioxidants, natural extracts or antimicrobial for enhanced efficacy.
- Eco-friendliness – Many microbial enzymes can be produced from sustainable substrate like fruit waste, reducing environment burden (Bezawada et al., 2011).

2.3 Serine Alkaline Protease for Wound Healing

Serine alkaline proteases (SAPs) are a class of extracellular endopeptidases that contain a highly reactive serine residue in their active site, which participates directly in the catalytic cleavage of peptide bonds (Gupta et al., 2002). They belong to the hydrolase family and are characterized by their optimal activity in alkaline pH (8-11) and moderate temperature range (45-70°C). These enzymes are widely produced by micro-organisms, particularly *Bacillus subtilis, B. licheniformis, B. cereus, B. pumilus* owing to their rapid growth and high enzyme secretion potential (Singh et al., 2021). SAPs possess remarkable stability against temperature and pH variations, surfactants and organic solvents, making them suitable candidates for industrial, pharmaceutical and biomedical applications (Bezawada et al., 2011). In recent years, their therapeutic potential has been extensively explored in wound healing formulations, enzymatic debridement agents and bioactive hydrogels (Yunus et al., 2024). The enzymes intrinsic ability to hydrolyze necrotic proteins, degrade fibrin clots and remove bacterial biofilms makes it an effective wound management agent. The wound healing potential of serine alkaline protease is primarily attributed to its proteolytic debridement and tissue regeneration capabilities. When applied topically, SAPs selectively digest denatured proteins and necrotic tissue from the wound bed, thereby promoting a clean surface

conductive to granulation and epithelialization (Ramakrishna et al., 2019). The enzymatic hydrolysis of fibrinous slough and necrotic debris enhances oxygen diffusion and nutrient delivery to regenerating tissues.

Moreover SAPs have been shown to stimulate fibroblast migration, angiogenesis and collagen remodelling. According to (El-Sayed et al., 2024), SAPs promote fibroblast proliferation by activating extracellular signalling pathways, leading to increased deposition of collagen fibers. These enzymes also enhances the synthesis of extracellular matrix components and facilitate keratinocyte migration, both of which are essential for rapid wound closure. Additionally, SAPs degrade bacterial biofilms and cell wall proteins, effectively reducing infection and inflammation. Their gentle proteolytic action ensures that only necrotic material is removed while sparing viable cells. This selective mode of action gives SAPs an advantage over harsh chemical debriding agents, which often cause irritation or delay in healing. A major challenge in enzyme - based formulations is maintaining enzyme stability and bioactivity. SAPs possess natural thermostability and alkaline tolerance allowing them to remain active over prolonged storage and under physiological conditions (Gupta et al., 2002). Their structural stability arises from extensive intra molecular hydrogen bonding and disulfide bridges, which protect them from denaturation. For topical application, SAPs can be incorporated into hydrogels, biopolymers and lipid-based creams. Singh et al., (2021) demonstrated that SAPs encapsulated in chitosan-based hydrogel matrices retained over 80% activity after 30 days of storage at room temperature. These biopolymers also act as moisturizing agents, maintaining a moist wound environment that promotes healing. SAPs are non-toxic, biodegradable and do not trigger adverse immune responses, conforming their safety for dermatological applications (El-Sayed et al., 2024).

While several classes of proteases – such as collagenases, metalloproteases and papain have been used in wound care, serine alkaline proteases offer multiple advantages. Firstly, they are more stable under alkaline and thermal conditions, which is beneficial since chronic wounds often exhibit broad substrate specificity enabling them to degrade a variety of necrotic proteins without affecting viable tissue (Ramakrishna et al., 2019). Thirdly, they can be produced cost effectively using microbial fermentation and agro waste substrates, making them suitable for large scale

production (Bezawada et al., 2011). SAPs can be genetically engineered for improved catalytic efficiency, enhanced thermostability and targeted substrate recognizing (Singh et al., 2021). Such enzyme engineered variants are now being studied for next generation bioactive wound healing formulations.

Several *in-vitro* and *in-vivo* studies have confirmed the wound healing efficacy of serine alkaline proteases (Figure No. 3). Yunus et al., (2024) conducted a rat model study demonstrating that wounds treated with SAP based cream showed a 45% faster wound contraction and significant increase in collagen content compared to control groups. Similarly, El-Sayed et al., (2024) observed accelerated epithelialization and reduced bacterial colonization in diabetic wound models treated with serine protease formulations. In another experiment, Kumar et al., (2020) found that protease-based wound displayed enhanced angiogenesis and reduced oxidative stress markers. Clinical reports have also indicated improved outcomes in chronic ulcers and burn wounds following SAP based therapy (Ramakrishna et al.,2019). These findings collectively supports the therapeutic relevance of serine alkaline proteases as efficient biological agents in wound care.

Serine alkaline proteases are versatile and highly effective biocatalysts for wound healing applications. Their proteolytic, antimicrobial and regenerative properties make them superior to conventional synthetic wound care agents. The enzyme stability, safety and sustainable production potential further enhance its applicability in biomedicine. When integrated with natural antioxidants such as fruit peel extracts, SAPs exhibit synergistic effects, offering a holistic, biocompatible and eco-sustainable wound healing approach. Hence, the inclusion of SAPs in topical wound healing formulations represents a significant advancement in bioactive and sustainable therapeutic technology.

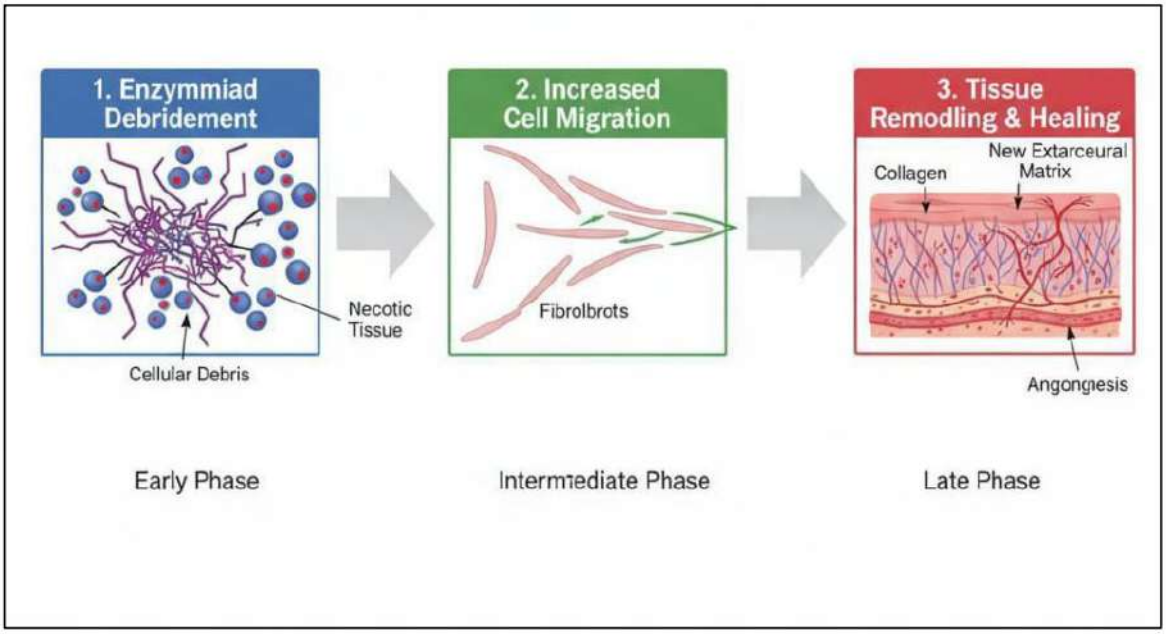

Figure No. 3: Protease mediated enzymatic debridement pathway

2.4 Synergistic Potential of Fruit Extracts and Protease Enzymes

The integration of natural fruit waste extracts with microbial enzymes offers synergistic effects that combine antioxidant, antimicrobial, and enzymatic debridement properties. According to Singh et al. (2021), the combination of plant extracts with proteolytic enzymes significantly improved healing time compared to formulations containing either component alone. The plant-derived polyphenols protect cells from oxidative damage and infection, while the enzyme aids in tissue remodeling. Such formulations can also be designed to sustain enzyme activity and bioactive release, ensuring prolonged action at the wound site. The use of eco-friendly components further reduces environmental impact and supports sustainable product innovation in biomedicine. Fruit extracts, particularly from tropical sources such as guava (*Psidium guajava),* papaya *(Carica papaya*), banana (*Musa sapientum*), chikoo (*Manilkara zapota*) are rich in bioactive compounds like flavonoids, tannins, phenolics, vitamins and natural antioxidants that aid in tissue regeneration and protection against oxidative stress. When combined with proteolytic enzymes such as serine alkaline protease, these extracts enhances enzymatic debridement, stimulate collagen synthesis and accelerate

overall wound closure through synergistic mechanism. Proteolytic enzymes assist primarily in the enzymatic removal of necrotic tissue and fibrin thereby preparing the wound bed for healing. Excessive protease activity may sometimes damage newly formed tissues or induce inflammatory reactions. The addition of fruit extracts provides a balancing antioxidant and anti-inflammatory effect, which protects viable cells from oxidative damage and modulates inflammatory cytokines. For instance, guava leaf and peel extracts contain quercetin and catechin – powerful antioxidants that stabilize reactive oxygen species (ROS) and support fibroblast proliferation and epithelial migration (Thompson et al., 2013). Chikoo (sapota) peel extract is reported to contain polyphenols and saponins with antimicrobial and free radical scavenging properties, which aid in reducing infection and promoting the synthesis of new tissue (Patil et al.,2020). These natural compounds not only reduce microbial load but also create a favourable microenvironment for enzyme activity ensuring effective and controlled debridement without cytotoxicity.

The synergistic mechanism arises from the complementary roles of both components –

- The serine alkaline protease enzymatically digests devitalized tissue, clears the wound surface and enhances cell migration.
- The fruit extract contributes bioactive phytochemicals that exhibit antimicrobial, antioxidant and anti-inflammatory effects thereby supporting tissue regeneration.

This dual action accelerates wound contraction, increases collagen deposition and minimizes scar formation compared to enzyme only or extract only formulations (Ramesh et al., 2017).

Another important aspect of this synergy is the enhancement of enzyme stability and bioavailability. Many natural plant polyphenols and sugars present in fruit extracts act as natural stabilizers or co-factors for enzymes, protecting from denaturation at ambient temperature and alkaline pH (Gupta et al., 2002). Thus, fruit extracts serve not only as therapeutic agents but also as natural carriers or stabilizers that improve the shelf-life and functional activity of serine alkaline proteases in topical formulations. Several studies have demonstrated improved wound healing outcomes when fruit extract-enzyme combinations are used. For instance, papaya-derived protease (papain)

combined with fruit antioxidants significantly reduced healing time in burns and diabetic ulcers (Hewitt et al., 2015). Similarly, topical formulations containing Guava (*Psidium guajava*) extract and bacterial protease showed higher rates of re-epithelialization, collagen maturation and wound tensile strength compared to controls (Silva et al., 2019). These findings highlight that synergistic combinations are not only effective but also biocompatible, eco-friendly and safe for topical applications. Such combinations are particularly valuable for chronic wounds – where infection, oxidative stress and impaired collagen metabolism often hinder healing. The antioxidant potential of fruit extracts reduces lipid peroxidation and DNA damage in cells surrounding the wound, while the protease enzyme continuously removes necrotic tissue and prevents microbial colonization. Together, these effects promotes a balanced wound microenvironment conductive to faster regeneration and reduced scarring (Tawil et al., 2015).

Thus, integration of serine alkaline protease with fruit based bioactive extracts represents a modern, green biotechnological strategy for wound care. It ensures complete wound bed formulation, infection control, tissue remodelling and aesthetic healing all in a biocompatible and sustainable manner.

2.5 Use of Agro-Waste Substrates for Enzyme Production

Agro-industrial residues, such as fruit peels and bran, can serve as nutrient-rich substrates for microbial enzyme production. Bezawada et al. (2011) found that Bacillus strains grown on fruit waste substrates produced high yields of alkaline proteases under solid-state fermentation. Using these low-cost raw materials not only reduces production costs but also contributes to environmental waste minimization. This dual use — as both enzyme substrate and extract source — enhances the economic and ecological value of the research.

Agro-waste as an alternative substrate for protease production. Microbial proteases are among the most extensively used industrial enzymes accounting for nearly 60% of the total enzyme market worldwide (Rao et al., 2008). Traditionally, their production requires expensive synthetic media containing peptones, yeast extract and casein. However, agro-wastes such as fruit peels, oil cakes, rice bran and vegetable residues can effectively replace these costly ingredients due to their compatible nutrient

composition. (Bezawada et al., 2011) reported that *Bacillus subtilis* and *Bacillus licheniformis* produced high yields of alkaline protease when cultivated on fruit waste substrate under solid state fermentation (SSF). The enzymatic yield and specific activity were comparable to those obtained from synthetic media, confirming that agro-waste materials can serve as both carbon and nitrogen sources. Similarly, studies by Johnvesly and Naik (2001) demonstrated that wheat bran and soyabean meal enhanced protease activity in *Bacillus* culture due to the presence of essential amino acids and minerals that promote bacterial metabolism and enzyme secretion. The conversion of agro-waste into enzyme rich biomass not only improves the economic feasibility of enzyme production but also offers an eco-friendly solution to agricultural waste management. This sustainable bioprocess approach helps in converting "waste to wealth", aligning with the principles of green biotechnology.

Among various fermentation processes solid state fermentation (SSF) has been widely recognized as the most efficient method for utilizing agro-waste substrate. Unlike submerged fermentation, SSF mimics the natural growth environment of filamentous of fungi and some bacteria where moisture content is low but nutrient availability remains sufficient. Agro-residues such as wheat bran, rice husk, sugarcane bagasse and fruit peel provide ideal structural support and nutrient for microbial colonization (Pandey et al., 2000). (Bezawada et al., 2011) used orange and banana peel as substrate for alkaline protease production under SSF and achieved a 2.5 fold increase in enzyme yield compared to conventional liquid fermentation. This enhancement was attributed to better aeration, nutrient diffusion and reduced catabolite repression. Moreover, SSF significantly reduces wastewater generation and energy consumption, making it both environmentally and economically advantageous.

SSF allows for the co-utilization of multiple agro-wastes, enhancing substrate diversity and enzyme induction. For example, a combination of fruit peels with wheat bran or soyabean meal provides balanced carbon to nitrogen ratios, supporting higher enzyme productivity and microbial stability (Anwar et al., 2009). Such systems are ideal for small scale industries and rural based biotechnology setups where low cost raw materials and minimal infrastructure are priorities. Fruit waste like guava peel, chikoo seed powder and banana peel contain abundant carbohydrates (cellulose, hemicellulose, starch), nitrogenous compounds, vitamins and trace minerals that serve

as natural growth stimulants for their robust enzyme producing ability, efficiently metabolize these complex biomolecules to generate proteases. According to (Espinosa-Espinosa et al.,2022), fruit peel powder not only provide carbon and nitrogen but also contain phenolic compounds that can act as inducers for stress related enzyme synthesis. These secondary metabolites influence microbial metabolism, enhancing extracellular enzyme secretion. The presence of micronutrients such as Mg^{2+}, Ca^{2+} and Fe^{2+} stabilizes enzyme structure and increases catalytic efficiency (Gupta et al.,2002).

The environmental significance of using agro-waste in enzyme production is profound. Globally, fruit processing industries generate millions of tons of waste annually, contributing to greenhouse gas emissions and landfill overloading. By converting these wastes into value added bioproducts such as proteases, researchers and industries can mitigate these impacts. Economically, the substitution of synthetic ingredients with fruit waste can reduce production costs by up to 40-60% as reported by (Ellaiah et al., 2002). Agro-waste valorisation supports integrated biorefineries, where multiple products such as enzymes, organic acids and biofuels are generated from the same waste stream (Soccol et al.,2017). This model ensures complete resource utilization and promotes a zero waste approach.

2.6 Research gap

Although extensive research has been carried out on fruit peel extracts and protease enzymes separately for wound healing, very limited studies have explored their combination in a single bio-cream formulation. Moreover, no previous research has reported the use of chikoo (sapota) seeds or guava peel blended with serine alkaline protease for human wound-healing applications. Thus, this project aims to fill that gap by developing a novel, sustainable, and biologically active wound-healing cream that utilizes both fruit waste and microbial enzyme components.

Most previous research has focused on either fruit derived phytochemicals or microbial enzymes as independent agents for wound therapy. For example, Sutar et al., 2021), demonstrated the antimicrobial and wound healing potential of orange peel extract, while Yunus et al., (2024) studied the efficiency of serine proteases in

enzymatic debridement. However, there is a distinct lack of studies combining both fruit waste extracts and microbial enzymes in a synergistic formulation. Singh et al., (2021) reported that enzyme plant extract combinations could enhance the rate of wound closure, yet such investigations remain preliminary and limited to model systems. The integration of serine alkaline protease with fruit peel extracts could yield multifunctional formulations that simultaneously offer antioxidant, antimicrobial and debriding actions but this concept has not been widely explored in current scientific literature. Most commercially available wound healing products rely on synthetic chemicals such as povidone-iodine or silver sulfadiazine which although effective can cause cytotoxic effects and skin irritation upon prolonged use (Mofazzal Jahromi et al.,2018). Hence, a clear research gap exists in the area of biocompatible, natural enzyme – phytochemical formulations for wound healing.

The valorisation of fruit processing waste has become a major area of green biotechnology. The potential of chikoo seed extract and guava peel extract remains underexplored in the context of dermatological and pharmaceutical applications. Most literature focuses on commonly studied fruit residues like banana, papaya and citrus peels (Kumar et al.,2020; Sharma et al.,2022). However sapota and guava wastes are known to possesses unique bioactive compounds such as polyphenols, tannins, saponins and flavonoids that have demonstrated significant antioxidant, antimicrobial and anti-inflammatory activities (Patel et al.,2019; Srivastava et al.,2021). There is a minimal documentation of their incorporation into wound healing creams or biomedical materials. The few studies that do exist are limited to crude extract screenings without detailed mechanistic evaluations or enzyme interaction studies. This gap highlights an opportunity to develop novel formulations that valorise indigenous fruit wastes while contributing to circular waste management systems.

Serine alkaline proteases from *Bacillus* species have been widely studied for industrial and detergent applications Gupta et al., (2002); Anwar and Saleemuddin, (2009), their biomedical and therapeutic potential remains underexplored. Only handful studies e.g. Yunus et al., (2024); El-Sayed et al., (2024) have evaluated the enzyme effect on wound healing through topical application. Most reports emphasize enzymatic debridement rather than regeneration or synergistic antioxidant effects when combined with natural plant components. Stability and compatibility of proteolytic enzymes with

phytochemicals in cream-based formulations are not well characterized in the literature. Parameters such as enzyme half-life, pH tolerance and bioavailability after blending with plant extracts need comprehensive evaluation. Hence, there exists a strong need for systematic research exploring the formulation, characterization and *in-vi*tro performance of SAP based phytochemical wound creams.

2.7 Objectives of the study

1) To screen potent serine alkaline protease producing bacterial strain and production of serine alkaline protease.
2) To prepare fruit extract.
3) To produce a bio-complex using fruit waste extract and serine alkaline protease
4) To study antibacterial activity of bio complex of fruit waste extract and serine alkaline protease enzyme.

3.0

MATERIALS AND METHODS

3.1 Production of bacterial serine alkaline protease

Different samples like soil, compost, sewage, poultry sample were collected from different areas.

3.1.1 Collection of samples

Various samples from different regions were collected in separate polythene bags (Table.no.1) Then polythene bags were packed and labelled properly and brought to the laboratory and kept at room temperature.

Table no. 1 - Collection of sample

Sr. No.	Name of sample	Location
1.	Soil sample	Vivekanand college garden
2.	Soil sample	Botany department, VCK
3.	Soil sample	Sugarcane Farm from Gargoti
4.	Compost sample	Gargoti
5.	Poultry sample	Peth Vadgaon
6.	Sewage sample	Jayanti nala, Kolhapur

3.2 Primary screening

3.2.1. Isolation of protease producing organisms by serial dilution method

Serial dilutions of samples were carried out to reduce microbial load -and obtain isolated colonies. One gm of soil sample was added to 9 ml of sterile distilled water (10^{-1} dilution). Further serial dilutions were prepared up to 10^{-8} by transferring 1 ml into 9 ml sterile diluent. Each dilution was mixed properly before transferring to the

next tube. After that 0.1 ml sample from each dilution was spread on the sterile milk agar plates (Table no. 2). Plates were incubated at room temperature for 24 hours. After incubation plates were observed for the colonies. Clear zone around the colonies will be observed on milk agar plate. (Cappuccino & Sherman, 2014)

Table no. 2 - Milk agar composition

Components	Amount
Raw milk	10 ml
Peptone	0.5gm
Yeast extract	0.25 gm
Agar	1.5 gm
Distilled water	90 ml
PH	7.0

All colonies showing clear zone were studied for gram staining characteristics. The impure cultures were purified by repeated transfer on sterile nutrient agar plates. Finally all purified cultures were labelled as shown in Table no. 3.

Table no. 3 – Isolates labelling

Sr. No.	Name of sample	Coding for isolates
1	Compost	C1, C2,..
2	Farm soil	F1, F2,..
3	Poultry	P1, P2,..
4	Sewage water	W1, W2,..
5	Soil sample	S1, S2,..

3.3 Secondary screening

Secondary screening was done to find out potent protease producing bacterial strain. It was done by calculating Proteolytic Index (PI) of each strain. PI Value was calculated by using the formula shown in Equation 1.

PI Index = B/A ---------- Equation 1

Where,

B = Clear zone diameter

A = Colony diameter

For estimation of PI, equal amount of culture was spot inoculated on sterile milk agar plates. Plates were labelled properly and incubated at room temperature for 24-48 hrs. After incubation diameter of colony and diameter of clear zone was measured and Proteolytic Index (PI) of each isolate was calculated (Equation 1). A higher proteolytic index indicates higher protease production (Hankin & Anagnostakis, 1975).

3.4 Identification of potent strain

3.4.1 Colony characteristics

Potent isolate C4 was grown on nutrient agar and colony characteristics were noted down after incubation of medium at RT for 24 hrs.

3.4.2 Morphological characteristics

Potent isolate labelled as C4 was gram stained to study the morphological characters. The motility of isolate was studied by hanging drop method.

3.4.3 Biochemical characteristics

Biochemical tests like catalase, oxidase, sugar fermentation, starch hydrolysis, nitrate reduction, citrate utilization and methyl red tests of potent isolate C4 were studied using standard protocol.

3.4.3.1 Catalase test

A clean and dry test tube was taken. About 1–2 mL of 3% hydrogen peroxide solution was added into the test tube. A small amount of the bacterial culture was picked up using a sterile glass rod. The culture was carefully introduced into the hydrogen peroxide present in the test tube. The reaction was immediately observed for the

formation of bubbles. The production of effervescence (oxygen bubbles will be recorded as a positive catalase reaction, while the absence of bubble formation will be recorded as a negative result. (Cappuccino & Sherman, 2014)

3.4.3.2 Oxidase test

A piece of clean filter paper was taken and was placed on a sterile surface. A few drops of oxidase reagent were added onto the filter paper. Using a sterile inoculating loop or wooden stick, a small amount of the bacterial culture (C4) was picked and was smeared onto the reagent-soaked area of the filter paper. The reaction was observed for a change in colour within 10–30 seconds. If the development of a dark purple colour will be observed then it is recorded as a positive oxidase reaction, while no colour change or a delayed reaction will be recorded as a negative result. (Cappuccino & Sherman, 2014)

3.4.3.3 Sugar fermentation test

Glucose test

A sterile test tube containing glucose fermentation broth with Andrades indicator was prepared, and a Durham tube was placed in an inverted position within the tube. The medium was inoculated with a small amount of the C4 culture using a sterile inoculating loop. The inoculated tube was incubated at 37°C for 24 hours. After incubation, the tube was observed for a change in colour and gas production. A change in colour of the medium from yellow to pink was recorded as positive glucose fermentation, indicating acid production. The presence of gas bubbles in the Durham tube was recorded as gas production. The absence of colour change and gas formation was recorded as a negative result.(Mackie & McCartney, 2014)

Lactose test

A sterile test tube containing lactose fermentation broth with Andrades indicator was prepared, and a Durham tube was placed in an inverted position within the tube. The medium was inoculated with a small amount of the bacterial culture using a sterile inoculating loop. The inoculated tube was incubated at 37°C for 24 hours. After incubation, the tube was observed for a change in colour and gas production. A change in colour of the medium from yellow to pink was recorded as positive lactose

fermentation, indicating acid production. The presence of gas bubbles in the Durham tube was recorded as gas production. The absence of colour change and gas formation was recorded as a negative result. (Mackie & McCartney, 2014)

3.4.3.4 Amylase test

The starch hydrolysis test was performed by inoculating a starch-containing nutrient agar plate with C4 using a sterile loop. The plate was incubated at 37°C for 24 hours. After incubation, the plate was flooded with iodine solution. If a clear zone around the bacterial growth was observed it indicates the hydrolysis of starch by the production of amylase. The presence of this clear zone was recorded as a positive result for starch hydrolysis. (Cappuccino & Sherman, 2014)

3.4.3.5 Nitrate reduction test

The nitrate reduction test was performed by inoculating a nitrate broth tube with C4 culture. The tube was incubated at 37°C for 24 hours to allow bacterial growth and potential nitrate reduction. After incubation, nitrate reagent A -Sulfanilic acid α-naphthylamine (alpha-naphthylamine) were added to the broth. A red colour change was observed, indicating the reduction of nitrate to nitrite, which was recorded as a positive result. If no colour change occurred, zinc dust was added to confirm the result. A red colour after zinc addition confirmed that nitrate was not reduced, while no colour change indicated that nitrate had been reduced beyond nitrite, giving a positive result for complete nitrate reduction. (Cappuccino & Sherman, 2014)

3.4.3.6 Citrate utilization test

The citrate utilization test was performed by inoculating a slant of Simmon's citrate agar with C4 using a sterile inoculating loop. The slant was incubated at 37°C for 24 hours. After incubation, the colour change of the medium was observed. If ----A blue colour indicated a positive result, meaning culture has utilized citrate as the sole carbon source and produced alkaline by-products, raising the pH. If no colour change occurred and the medium remained green, the result is negative, indicating that the organism is unable to utilize citrate. (Cappuccino & Sherman, 2014)

3.4.3.7 Methyl red test

The methyl red test was performed by inoculating a tube of methyl red (MR) broth with Bacillus culture and incubating it at 37°C for 24-48 hours. After incubation, a few drops of methyl red reagent were added to the broth. A red colour change indicated a positive result, meaning C4 produced stable acidic end products from glucose fermentation, lowering the pH of the medium. If no colour change occurred and the broth remained yellow, the result was negative, indicating that the organism did not produce sufficient acid during fermentation. (Cappuccino & Sherman, 2014)

3.4.3.8 Gelatine hydrolysis test

The gelatine hydrolysis test was performed by inoculating a tube of nutrient gelatine with C4 using a sterile inoculating loop. The tube was incubated at 37°C for 24-48 hours. After incubation, the tube was placed in an ice bath for 15-20 minutes to solidify the gelatine. If the gelatine remained liquid, it indicated a positive result, meaning Bacillus produced gelatinase, which hydrolysed the gelatine into smaller peptides. If the gelatine solidified, the result was negative, indicating that the organism did not produce gelatinase. (Cappuccino & Sherman, 2014)

3.5 Production of an enzyme - Serine alkaline protease

3.5.1 Cultivation of potent strain and production of enzyme

Firstly, casein broth was prepared (Table no. 4) and then potent organism was inoculated in the broth. After that broth was incubated at room temperature for 48 hours.

3.5.2 Purification of serine alkaline protease

The inoculated broth was centrifuged at 5000 rpm for 15 min. After centrifugation, supernatant was collected and pellet was discarded.

From supernatant enzyme was extracted by salt precipitation technique. Crude serine alkaline protease solution (supernatant) was chilled and added with finely ground ammonium sulphate (to get 60% saturation amount). The solution was allowed to stand for 24 hrs in refrigerator for complete precipitation of enzyme. Next day, contents were

centrifuged at 5000 rpm for 10-15 min. The pellet (partially purified serine alkaline protease) was suspended in buffer.

Table 4 - Casein broth composition

Components	Amount
Casein	0.1 gm
Starch soluble	0.1 gm
Sodium chloride	0.02 gm
Distilled water	100 ml
pH	7.2-7.4

3.6 Preparation of chickoo fruit waste extract

3.6.1 Collection of chickoo fruit sample

Chickoo fruit samples were collected from fruit market of Kolhapur district. The fruit samples were collected in polythene bag. Then bag was packed and brought to the laboratory and kept at low temperature.

3.6.2 Chickoo fruit peels and seeds extract

Chickoo fruit sample was washed thoroughly with distilled water . Then peels and seeds (nearly 8) were taken off from the fruit sample. After that peels and seeds were washed with distilled water and then kept in hot air oven separately at 60°C for drying. Chickoo seeds and peels were then crushed separately by using Morter and pestle. Afterwards chickoo seeds and peels powder was added in solvent (70% acetone). After that the tube was sealed by cotton plug and stored at room temperature for 48 hours. On next day, filtration of that solution was done by Whatman's filter paper. Then water-bath treatment was given to that filtrate at 40°C for some time until the content was evaporated and residue was left in the container. Finally, the chikoo peel and seeds extracts were labelled properly and stored in refrigerator till further use.

3.7 Combination of chikoo fruit waste extract and serine alkaline protease

The partially purified serine alkaline protease enzyme and chikoo peel extracts were combined in 1:1 proportion for formulation.

Similarly,

3.8 Evaluation of partially purified enzyme, fruit waste extract and enzyme + fruit waste extract combination for its antibacterial activity

Sterile nutrient agar plates were prepared (Table no. 5). After that fresh culture of *Staphylococcus aureus* was spread on sterile NA plates. Four wells were prepared on seeded agar plate with the help of cork borer. Each well was labelled as enzyme, fruit extract, enzyme + fruit extract and control. After that 0.1 ml of partially purified enzyme, fruit extract, enzyme + fruit extract and acetone were added in each well respectively. After addition in each well, plates were placed in refrigerator for 10 min for diffusion and then shifted each plate in incubator at 37^0C for 48 hours.

Table no. 5 - Composition of Nutrient agar

Components	Amount
Peptone	0.5 gm
Beef extract	0.2 gm
Sodium chloride	0.5 gm
Distilled water	100 ml
pH	7.2-7.4

After incubation clear zone around wells will indicate that the contents in respective wells are inhibitory for *S. aureus*.

4.0

RESULT AND DISCUSSION

4.1 Collection of samples

Fig no. 4- Soil sample from Vivekanand college garden

Fig no. 5 – Sewage water sample

Fig no. 6 - Soil sample from Botany department

Fig no.7- compost sample

4.2 Isolation of protease producing organism by serial dilution method

Nearly 38 colonies were obtained by serial dilution technique on milk agar plates . Out of 38 isolates, 27 isolates were showing clear zone around the growth (Table no. 6).

Table no. 6 - Isolation of protease producing organism by serial dilution method

Colony on milk agar	Diameter of clear zone (mm)	Diameter of colony (mm)
1	6	3
2	9	2
3	7	1
4	16	1
5	11	2
6	7	2
7	14	2
8	6	3
9	12	2
10	6	1
11	12	10
12	11	10
13	9	7
14	7	6
15	8	5
16	6	4
17	4	3
18	5	2
19	6	5
20	7	6
21	5	3
22	4	3
23	3	2
24	2	1
25	5	4
26	4	3
27	6	3

Colonies obtained from compost (Colony no. 1 to 15), farm soil and poultry sample (Colony no.16 to 27) showed clear zone around growth, whereas colonies

obtained from garden soil samples and sewage water sample were not shown clear zone around growth.

4.3 Selection of potent protease producing bacterial strain

Diameter of colony and diameter of clear zone of each purified labelled isolate was measured and Proteolytic Index (PI) of each labelled protease producing bacterial isolate was calculated which is depicted in Table 7.

Table no. 7 - Calculation PI value of isolated colonies

Sr.No	Isolate name	Diameter of colony (mm)	Diameter of clear zone (mm)	PI value
1	C1	1.3	2.25	1.73
2	C2	0.9	2	2.22
3	C3	0.95	2	2.10
4	C4	0.6	1.75	2.91
5	F5	0.8	1.5	1.87
6	F18	0.9	1.35	1.5
7	p1	1	1.4	1.4
8	p2	1.6	0.35	0.21
9	F7	0.5	1.76	2.30
10	F8	0.9	1.64	1.82
11	F9	0.25	1.0	1.60
12	F10	0.32	032	1.36
13	F11	0.9	2	2.22
14	F12	0.8	1.5	1.87

C1, C2, C3, C4: Isolates obtained from compost; F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F11, F12, F13, F14, F15, F16, F17, F18: isolates obtained from farm soil; P1 to P2 : isolates obtained from poultry sample

A higher proteolytic index indicates higher protease production. After calculating the PI values of all isolates, C4 isolate was found to be as a potent organism, hence it was used for further study.

4.4 Identification of C4

4.4.1 Identification by cultural characters

Table no.3-Colony characters of C_4 grown on NA plates incubated at 37^0C for 24 hours

Size	Shape	Colour	Margin
Diameter = 2 mm	Circular	Creamy white	Entire

Surface	Elevation	Consistency	Opacity
Smooth	Slightly convex	Sticky	Opaque

4.4.2 Identification by Gram characteristics

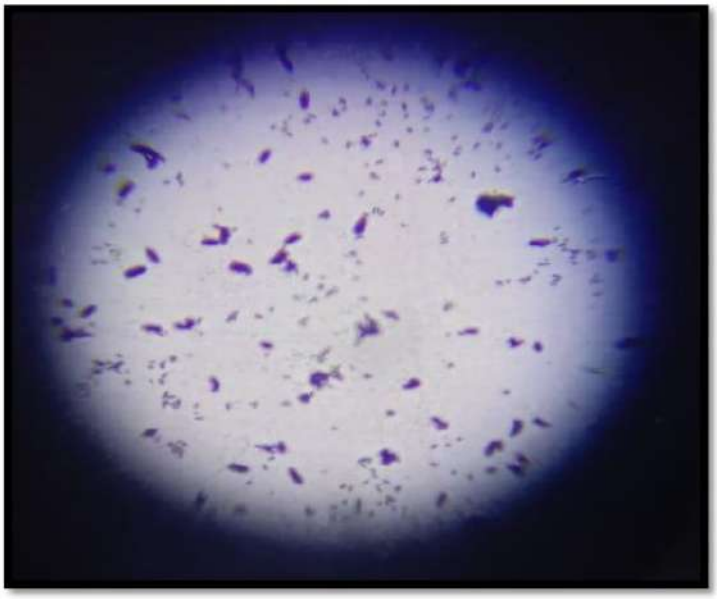

Fig no. 8 – Gram staining of C4 isolate

Microscopic observation of C_4 revealed that organism is Gram-positive, rod-shaped bacteria present singly.

4.4.3 Identification by biochemical characters

Sr. No.	Biochemical test	Results
1.	Catalase Test	+
2.	Oxidase Test	+
3.	Starch Hydrolysis test	+
4.	Gelatine Hydrolysis test	+
5.	Glucose fermentation	+
6. .	Lactose fermentation	-
7.	Methyl red test	+
8.	Citrate utilization test	+
9.	Nitrate reductase test	-

+ = test is positive; - = test is negative

Fig no 9. – Catalase test

Fig no 10. – Gelatin hydrolysis test

Fig no 11. – Sugar fermentation test (Glucose & Lactose)

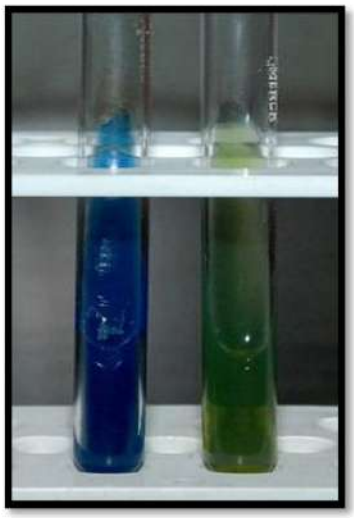

Fig no 12 – Citrate utilization test

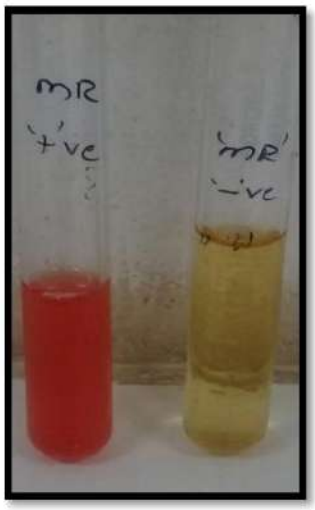

Fig no 13 – Methyl red test

Fig no 14 – Nitrate reductase test

Fig no 15 – Oxidase test

4.5 Production of an Enzyme Serine Alkaline Protease

Fig no. 16 – Serine alkaline protease ppt

4.6 Preparation of Fruit Peel Extract

Fig no 17 – Chickoo fruit waste extract

4.7 Evaluation of partially purified enzyme, fruit extract and enzyme + fruit extract combination for its antibacterial activity

Fig no 18 – Antibacterial activity of chickoo peel

Fig no 19 – Antibacterial activity of chickoo seed

The evaluation of the partially purified enzyme, fruit extract, and enzyme + fruit extract combination for antibacterial activity against *Staphylococcus aureus* was carried out using the agar well diffusion method.

The results revealed that the Chiku fruit peel extract alone and in combination with crude enzyme serine alkaline protease did not exhibit any significant antibacterial effect against *Staphylococcus aureus* (Fig. no 18), as evidenced by the absence of a clear zone around the wells containing the peel extract alone and enzyme and peel extract combination in the nutrient agar plates.

Similarly, no inhibition zones were observed for the chickoo peel extract. This suggests that the antimicrobial potential of chickoo peel extracts might be weaker or less effective under the experimental conditions used.

The results revealed that the Chiku fruit seeds extract alone and in combination with crude enzyme serine alkaline protease did not exhibit any significant antibacterial effect against *Staphylococcus aureus* (Fig. no 19), as evidenced by the absence of a clear zone around the wells containing the seeds extract alone and enzyme and seeds extract combination in the nutrient agar plates.

Similarly, no inhibition zones were observed for the chickoo seeds extract. This suggests that the antimicrobial potential of chickoo seeds extracts might be weaker or less effective under the experimental conditions used.

Both the Chiku fruit peel extract and the seeds extract, both alone and in combination with the crude enzyme serine alkaline protease, did not exhibit a significant antibacterial effect.

This was evidenced by the absence of a clear zone around the wells in the nutrient agar plates.

5.0

SUMMARY AND CONCLUSION

The formulation of a wound healing cream from fruit peel / seed extract and serine alkaline protease represents an innovative approach in utilizing agro-waste for biomedical applications. Agro-wastes, such as fruit peels, seeds or rotten/ over ripened fruits are often discarded in the food industry. This project underscores the potential of converting this waste into valuable resources for healthcare. In this study, the extraction of chickoo peels and seeds was successfully carried out, and the serine alkaline protease enzyme was effectively incorporated into the formulation. However, despite the careful formulation process, the expected antimicrobial activity was not achieved. The results from the antibacterial assay indicated the absence of clear zones around the wells containing the chickoo peel / seed extract and the enzyme combination. This observation suggests that the bioactive compounds in chickoo peel and seeds despite their known antioxidant properties, might require further refinement or higher concentrations to exhibit antimicrobial effects in this context.

The lack of antibacterial activity does not detract from the overall success of the project. While antimicrobial effects were not achieved as initially hoped, the study demonstrated the viability of using chickoo peel and seeds a renewable and biodegradable resource, as an effective ingredient in wound healing formulations. The combination of fruit extracts and serine alkaline protease introduces an eco-friendly alternative to traditional synthetic chemicals used in topical wound care products. The enzyme itself, known for its debridement and tissue regeneration properties, presents a promising direction for enhancing wound care. Its potential to clean necrotic tissue and facilitate the formation of healthy tissue by promoting fibroblast migration and collagen deposition is highly relevant for chronic wounds and tissue regeneration.

The results of this study not only emphasize the need for further optimization of the fruit extract preparation and enzyme-extract combinations, but also highlight the importance of sustainable biomedical practices. The utilization of agro-waste not only helps reduce environmental pollution but also offers a cost-effective solution to the production of bioactive compounds, which can be used in various pharmaceutical and cosmetic formulations. As concerns about the overuse of synthetic chemicals and rising antimicrobial resistance grow, the development of natural, biodegradable, and biocompatible wound healing treatments is becoming increasingly important.

This study opens several avenues for future research. To optimize the formulation, it is essential to explore alternative fruit peel sources, such as guava, papaya, or banana peels, which have demonstrated stronger antimicrobial activity in other studies. Additionally, refining the extraction techniques, such as adjusting solvent ratios or extraction time, could help enhance the bioactive potential of the chickoo peel extract. Another critical area for future research is to investigate the interaction between serine alkaline protease and various natural extracts to understand how these ingredients can work synergistically to enhance wound healing efficacy. The combination of proteases with other natural compounds, including flavonoids, tannins, and essential oils from different fruits, may provide additional therapeutic benefits for wound care products.

In conclusion, although the formulation did not yield the expected antimicrobial effects, the project provides significant insights into the potential of combining green biotechnology with sustainable healthcare solutions. The results emphasize that fruit waste can be a valuable resource for developing eco-friendly and effective wound healing products. The serine alkaline protease enzyme, while not displaying antibacterial activity in this study, remains a promising agent for tissue regeneration, and further research in this field could lead to the development of highly effective, natural, and environmentally friendly wound care products with significant therapeutic value. By aligning biomedical formulations with sustainability, this study contributes to the ongoing efforts to create innovative, safe, and sustainable solutions for wound healing, which could benefit both human health and the environment in the long term.

6.0

BIBLIOGRAPHY

1. Abood, W. N., et al. (2015). Evaluation of fruit peel extracts on wound healing in animal models. International Journal of Biological Sciences, 11(6), 697–706.
2. Amin, M., Ahmad, M., & Khan, S. (2018). Role of natural antioxidants in wound healing: Experimental and clinical evidence. Journal of Ethnopharmacology, 225, 82–90.
3. Anwar, A., & Saleemuddin, M. (2009). Alkaline proteases: A review of properties and applications. Applied Microbiology and Biotechnology, 69(1), 1–9.
4. Ayala-Zavala, J. F., Rosas-Dominguez, C., Vega-Veja, M. D., & Gonzalez-Aguilar, G. A. (2011). Antioxidant and antimicrobial properties of fruit by-products and their potential uses. Food Research International, 44, 1833–1841.
5. Bezawada, J., et al. (2011). Recovery and characterization of alkaline protease from Bacillus species using agro-waste substrates. Biotechnology Reports, 4(1), 33–42.
6. Choudhary, M., Yadav, D., & Sharma, A. (2018). Serine alkaline proteases from Bacillus species and their role in wound-healing applications. Biotechnology Reports, 19, e00267.
7. Cappuccino, J. G., & Sherman, N. (2014). Microbiology: A laboratory manual pearson.
8. Collee, J, G., et al. (2014). Mackie & McCartney practical medical microbiology. Elseiver.
9. Ellaiah, P., Prabhakar, T., & Ramakrishna, B. (2002). Optimization of process parameters for alkaline protease production under solid-state fermentation by Bacillus subtilis. Process Biochemistry, 38(2), 209–214.
10. El-Sayed, G. M., et al. (2024). Immobilized recombinant serine alkaline protease for wound-healing applications. Journal of Applied Biotechnology, 12(3), 210–218.

11. Espinosa-Espinosa, L., et al. (2022). Wound-healing potential of tropical fruit peel extracts. Plant Biochemistry Reports, 8(2), 145–153.
12. Espinosa-Espinosa, M. T., Moreno, M. G., & Suarez, D. A. (2022). Valorization of fruit peel wastes as substrates for microbial enzyme production. Bioresource Technology Reports, 17, 100892.
13. Gupta, R., et al. (2002). Purification and characterization of an alkaline serine protease from Bacillus licheniformis BA17. Annals of Microbiology, 52(4), 299–306.
14. Hankin, L., & Anagnostakis, S. L. (1975). The use of solid media for detection of enzyme production by fungi. Mycologia, 67(3), 597-607
15. Hewitt, A., Pruitt, C., & Hocking, A. (2015). Papain-based enzymatic wound debridement in clinical practice: A review. Burns, 41(5), 893–901.
16. Johnvey, J., & Nair, R. (2014). Evaluation of antimicrobial and antioxidant properties of selected plant extracts. International Journal of Pharmaceutical Sciences Review and Research, 26(2), 45–52.
17. Joseph, B., & Priya, R. M. (2011). Phytochemical and pharmacological profile of traditional medicinal plants. International Journal of Pharmaceutical Sciences Review and Research, 9(1), 72–81.
18. Kumar, A., et al. (2020). Evaluation of banana peel extract for wound-healing activity in Wistar rats. Journal of Applied Natural Science, 12(2), 345–351.
19. MacFaddin, J. F. (2000). Biochemical tests for identification of medical bacteria. Lippincott Williams & Wilkins.
20. Mofazzal Jahromi, M. A., Sahandi Zangabad, P., & Moosavi Basri, S. M. (2018). Nanomedicine and wound healing: A review. Biomaterials Science, 6(5), 1127–1142.

21. Nair, V. C., Joseph, L., & Mathew, S. (2013). Therapeutic evaluation of plant-based bioactive compounds in wound management. Indian Journal of Natural Products and Resources, 4(1), 25–33.

22. Pandey, A., Tiwari, A., & Sharma, D. (2021). Combination of protease and herbal extract for enhanced wound healing. Journal of Herbal Medicine, 26, 100423.

23. Patil, P., Pawar, S., & Jadhav, S. (2020). Phytochemical screening and antimicrobial potential of sapota (Manilkara zapota) peel extract. Journal of Pharmacognosy and Phytochemistry, 9(4), 1215–1220.

24. Ramakrishna, S., et al. (2019). Purification and characterization of an alkaline serine protease with novel properties from Bacillus cereus strain S8. International Journal of Biological Macromolecules, 135, 120–130.

25. Ramesh, S., Shankar, R., & Manimegalai, K. (2017). Combined effect of protease and plant extracts on wound healing in experimental animals. International Journal of Pharma and Bio Sciences, 8(1), 45–53.

26. Rajeswari, R., Umadevi, M., Rahale, C. S., & Pushpa, R. (2012). Phyto Journal, 1(4), https://www.phytojournal.com/archives/2012/vol1issue4/PartA/17.1.pdf.

27. Rao, M. B., Tanksale, A. M., & Deshpande, V. V. (2008). Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Reviews, 62(3), 597–635.

28. Raju, K., Sultana, R., & Devi, M. (2017). Phytochemical screening and wound-healing activity of fruit peel extracts. Asian Journal of Pharmaceutical and Clinical Research, 10(5), 321–326.

29. Sayed, A., Abdallah, H. M., & Abdelhafez, O. H. (2012). Wound-healing and antibacterial activities of some medicinal plant extracts. Journal of Applied Pharmaceutical Science, 2(7), 16–23.

30. Sharma, P., et al. (2022). Phytochemical and antimicrobial potential of guava peel extract: A sustainable approach toward wound care. Journal of Applied Pharmaceutical Science, 12(3), 75–83.
31. Silva, D. L., Lima, R. R., & Fernandes, M. P. (2019). Topical wound-healing activity of Psidium guajava extract and protease combination. Pharmaceutical Biology, 57(1), 23–31.
32. Singh, P., et al. (2021). Synergistic effects of plant extracts and microbial enzymes for improved wound-healing applications. Biotechnology Reports, 30, e00645.
33. Soccol, C. R., de Souza Vandenberghe, L. P., Medeiros, A. B. P., & Pandey, A. (2017). Biorefineries: An integrated approach for sustainable production of fuels and chemicals. Biotechnology Advances, 35, 811–821.
34. Srivastava, R., & Patel, D. (2021). Phytoconstituents and antimicrobial activity of guava (Psidium guajava) peel and seed extracts. Journal of Medicinal Plants Studies, 9(2), 56–64.
35. StatPearls. (2025). Physiology, Wound Healing. Treasure Island (FL): StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK535406/
36. Sutar, R., et al. (2021). Antimicrobial and wound-healing activity of orange peel extract. Indian Journal of Pharmaceutical Sciences, 83(5), 856–863.
37. Tawil, N., Sutherland, C., & DiRaddo, R. (2015). Applications of enzyme-based systems for burn and wound debridement: A review. Burns, 41(5), 893–901.
38. Thomson, C., Tan, L., & Alzan, A. (2012). The potential of guava (Psidium guajava) as a functional food. Food Chemistry, 138(2–3), 933–940.
39. Yunus, J., et al. (2024). Enzymatic debridement using serine protease-based topical formulations. Journal of Wound Care Science, 15(4), 310–320.