Enhancing the Functional Properties of Canola Protein-based Films Using Essential Oils and Polyvinyl alcohol

by

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Abstract

The growing demand for sustainable food packaging has intensified interest in biodegradable polymer films with enhanced functional and mechanical performance. Canola protein is a promising plant-based biopolymer; however, its application is limited by poor mechanical strength, water sensitivity, and limited antimicrobial activity. This thesis aimed to address these challenges by incorporating essential oils with antimicrobial properties into canola protein films and blending canola protein with polyvinyl alcohol (PVA) to improve film strength and stability. All films were fabricated using the solvent-casting method. Their mechanical properties, moisture-barrier performance, and thermal characteristics were evaluated. In the first study, two essential oils carvacrol and cinnamaldehyde were incorporated into canola protein-based films at various oil concentrations (0%, 1%, 3% and 5%, v/v) and proportions (0:1, 1:0, 1:1, 1:2, 2:1, 1:4 and 4:1). The objective of the first study was to explore the potential to develop an active canola protein-based films infused with carvacrol and cinnamaldehyde and to evaluate their influence on the films' properties. The addition of essential oils successfully imparted antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus.* The 3% oil concentration was found to be optimal, achieving good antimicrobial activity while having minimal negative effects on other film properties. In the second study, canola protein-PVA composite films were prepared at blend ratios of 100:0, 90:10, 80:20, 70:30, 60:40, and 0:100. The objective of the second study is to investigate the effect of PVA incorporation on the functional properties of the canola protein-based films. The addition of PVA enhanced both tensile strength and flexibility, with the 60:40 canola:PVA film showing the best performance, exhibiting approximately a 155% increase in tensile strength (TS) and a 106% increase in elongation at break (EB) compared to pure canola protein films (TS 1.73 ± 0.33 MPa, EB 57.53 ± 8.57 %). Although water vapor permeability was not significantly affected, a decreasing trend was observed with increasing PVA content. Collectively, the findings from both studies demonstrate that essential oil incorporation and PVA blending are effective strategies for enhancing the functionality of canola protein-based films. These results support the development of biodegradable packaging materials with improved antimicrobial properties and mechanical stability, contributing to more sustainable alternatives for food packaging applications.

Keywords: Canola protein, Essential oils, Carvacrol, Cinnamaldehyde, Polyvinylalcohol, Biopolymer-based packaging

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Dedication

To ALLAH SWT and to my family & children,

This thesis is as much yours as it is mine.

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List of abbreviations

AMPs – Antimicrobial peptides

CAR – Carvacrol

CIN – Cinnamaldehyde

CPI – Canola protein isolate

DH – Degree of hydrolysis

DMTMM – 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride

DSC – Differential scanning calorimetry

EAB – Elongation at break

EOS – Essential oils

FTIR – Fourier transform infrared spectroscopy

MW – Molecular weight

NCC – Nanocrystalline cellulose

PBAT – Poly(butylene adipate-co-terephthalate)

PVA – Polyvinyl alcohol

SEM – Scanning electron microscopy

TS – Tensile strength

WCA – Water contact angle

WVP – Water vapor permeability

YM – Young's modulus

Chapter 1: Introduction

1.1 Introduction

The development of effective food packaging materials is just as essential as the production of food itself. The primary objectives of packaging are to preserve food quality, ensure safety, and maintain shelf life until the product reaches the consumer (Kim et al., 2008). Common packaging materials include plastic, glass, paper, and metal (Noorbakhsh-Soltani, Zerafat, & Sabbaghi, 2018). Among these, plastic is the most widely used due to its practicality; however, it is also the most controversial because of its environmental impact.

Traditionally, plastic has been manufactured from oil and gas, which contribute to its advantageous properties, including high strength, flexibility, heat resistance, and excellent barrier performance against gases and moisture (Wang et al., 2019). These characteristics have made petroleum-based plastics a preferred material for food packaging. However, despite their critical function, plastic packaging is often discarded shortly after use, becoming a major source of environmental waste.

Improper management of plastic waste has led to its widespread presence in unintended environments. Plastic residues have been found along coastlines, in surface and groundwater, soil, sediment, air, food products, and even drinking water (Environment and Climate Change Canada, 2020). As the waste infiltrates the intricate ecosystem, it disrupts both terrestrial and aquatic habitats, negatively affecting biodiversity. Additionally, it has been shown to degrade soil health, thereby reducing agricultural productivity (Environment and Climate Change Canada, 2020). Beyond the waste management concerns, the production and incineration of conventional plastics also release substantial amounts of carbon dioxide (CO_2), a greenhouse gas that contributes significantly to global climate change. The effects of climate change, such as drought, storms, and extreme heat events, have serious implications for agriculture and livestock production (Ford et al., 2022).

In recent years, numerous innovative packaging materials with excellent biodegradability have been developed as alternatives to conventional plastic. Natural polymers such as polysaccharides, proteins, and lipids have attracted significant attention due to their functional properties, abundance, renewability, and non-toxicity (Jafarzadeh et al., 2020). Among these, proteins have emerged as promising candidates for biodegradable film production due to their ability to form

cohesive films with favorable mechanical, thermal, and barrier properties (Sudesh & Iwata, 2008). Building on this, canola protein emerges as a potential candidate due to its favorable intrinsic properties. Extracted from the by-product of canola oil production, canola protein has been demonstrated to produce films with good mechanical strength, thermal stability, and decent moisture permeability (Chang & Nickerson, 2014). A variety of strategies have been explored to improve the performance of canola protein-based films and bring them closer to the functionality of petroleum-based plastics. These include techniques such as layered film assembly, polymer blending, cross-linking, and nanomaterial reinforcement (Jafarzadeh et al., 2020).

Among these strategies, the development of composite films combining two or more polymers to achieve complementary properties has proven particularly effective in overcoming the limitations of single-component biopolymer films. One common approach involves blending a natural polymer, such as canola protein, with a synthetic but biodegradable polymer, such as polyvinyl alcohol (PVA). PVA is a water-soluble, semi-synthetic polymer known for its excellent film-forming ability, flexibility, and mechanical strength (Liu et al., 2022; Mallakpour & Rashidimoghadam, 2018). It is also approved for food-contact applications and is biodegradable under specific environmental conditions (Byrne et al., 2021; Government of Canada, 2023c). When incorporated into protein-based films, PVA has been shown to improve tensile strength, elongation at break, and moisture resistance, all of which are critical for practical food packaging applications (Alberto et al., 2020; X. Tian et al., 2023). In the context of canola protein, blending with PVA offers a promising improvement for the film performance, especially given canola's inherent brittleness and water sensitivity.

On the other hand, active packaging has become a growing area of research in response to concerns over foodborne illness, spoilage, and shelf-life stability. Unlike traditional packaging, which serves a passive role, active packaging systems are designed to interact with the food or its environment to produce beneficial effects. One key direction is the incorporation of antimicrobial agents into biodegradable film matrices. Compounds such as essential oils, nanoparticles, polysaccharides, peptides, and antioxidants have been integrated into packaging films to reduce microbial growth and improve product safety (Jafarzadeh et al., 2020). Among these, plant-derived essential oils, including cinnamaldehyde and carvacrol, have demonstrated strong antimicrobial activity against a broad range of foodborne pathogens and spoilage organisms. Their integration into biopolymer

films has yielded promising results in enhancing both the microbial safety and shelf-life of packaged foods. Thus, combining biodegradable and active packaging offers a synergistic solution that addresses environmental concerns while maintaining food quality and safety.

This study aims to develop an active, biodegradable packaging film using canola protein, polyvinyl alcohol (PVA), and two essential oils, cinnamaldehyde and carvacrol. The objective is to enhance antimicrobial activity and improve film performance while promoting the upcycling of agricultural by-products from the oil industry. By integrating sustainability and functionality, this research advances next-generation food packaging materials.

1.2. Hypothesis

1.2.1. Hypothesis – Study 1

The incorporation of carvacrol and cinnamaldehyde at different concentrations can imparts antimicrobial properties to canola protein-based packaging films while potentially enhance film flexibility. Furthermore, blending these EOS at different ratios may reduce the total oil required due to synergistic effects between carvacrol and cinnamaldehyde.

1.2.2. Hypothesis – Study 2

The incorporation of PVA can improve the the mechanical and barrier properties of canola protein-based packaging films.

1.3. Objectives

1.3.1. Objectives – Study 1

- To fabricate canola protein-based films incorporated with carvacrol and cinnamaldehyde at different concentrations and blending ratios.
- To evaluate the antimicrobial activity of the developed films against selected foodborne microorganisms.
- To identify optimal essential oil concentrations and ratios that provide effective antimicrobial performance while maintaining or improving film flexibility.
- To evaluate the mechanical performance, water barrier properties, and thermal stability of the developed canola protein films.

1.3.2. Objectives – Study 2

- To fabricate canola protein-based films with varying ratios of PVA.
- To evaluate the effect of PVA incorporation on the mechanical, moisture barrier and thermal properties of the blended films.
- To identify optimal canola:PVA ratios that enhance the overall performance of canola protein-based films.

Chapter 2: Literature Review

2.1. The Role and Environmental Impact of Conventional Plastic Food Packaging

Depending on its origin, plastic can be broadly categorized as either petroleum-based or bio-based (Sanyang et al., 2017). Petroleum-based plastic is synthesized from refined oil and natural gas through polymerization and polycondensation (Plastic Europe, 2022). Since its first application in the Second World War (Risch, 2009), conventional plastic has become indispensable in many industries, including agriculture, food packaging, aerospace, construction, automotive, healthcare, electronics, and manufacturing (British Plastic Federation, 2022).

The global production of plastics has grown exponentially, from 1.5 million metric tons in 1950 to approximately 322 million metric tons in 2015 (Plastic Europe, 2017). This number is forecasted to become 800 million metric tons in 2050 (Rouch, 2019). However, it was quickly discovered that the exceptional resilience of petroleum-based plastic also makes it highly resistant to microbial degradation. As a result, these materials are not compostable and are typically disposed of through incineration, which contributes to air pollution and greenhouse gas emissions. It is estimated that plastic production and incineration could generate up to 56 billion metric tons of CO_2 emissions between 2015 and 2050 (Hamilton et al., 2019). On the other hand, the recycling process for plastic remains challenging, as the material must be free of food residues or contaminants to preserve the quality of the recycled plastic. Only 9% of plastic waste is recycled (Government of Canada, 2022), while the majority ends up in landfills or enters the natural environment, contributing to severe pollution and posing significant threats to wildlife (United Nations Environment Programme, 2021). Over time, it became apparent that the production and use of synthetic plastic have a detrimental effect on mankind, wildlife, the natural environment, and the overall quality of life for current and future generations.

Food packaging is one of the major sectors that relies heavily on synthetic plastics due to their low cost, durability, and versatility. Packaging accounts for about 36% of all plastics produced, including disposable food and beverage containers used and discarded daily (United Nations Environment Programme, 2021). Common polymers used in food packaging, such as high-, low-, and very-low-density polyethylene, polyethylene terephthalate, polyvinyl chloride, polystyrene, and polypropylene, accounted for about 90% of the plastic used in this sector (Cazón & Vázquez, 2021; Plastics Europe, 2019). Over the past decade, the food industry has been under heavy

criticism from conscious consumers due to the growing awareness of plastic pollution and the massive volume of waste generated by single-use packaging. In response to public pressure and environmental concerns, governments around the world have introduced policies to reduce the use of conventional plastics. As of 2022, Canada implemented stricter regulations to limit the use of conventional plastic. These changes have accelerated the research and development of alternative packaging materials, particularly biodegradable plastics derived from renewable, more environmentally friendly sources.

2.2. Biopolymer-Based Plastic as an Answer to the Environmental Crisis

2.2.1 Definition and Classification of Biopolymers

The term "biopolymer" refers to polymers that are biodegradable and derived from biological sources (Amir et al., 2022). Based on their origin and production method, biopolymers can be broadly classified into two groups: natural biopolymers, which are directly extracted from biomass (e.g., starch, cellulose, proteins), and synthetic biopolymers, which are produced through biological processes or chemical synthesis (e.g., polylactic acid, PLA, polyhydroxyalkanoates, PHA) (Baranwal et al., 2022). Natural biopolymers are abundant, cost-effective, and highly biodegradable (Baranwal et al., 2022). They are often derived from agricultural or food industry by-products, contributing to waste reduction. However, their quality can be inconsistent, and they are moisture-sensitive with limited mechanical and barrier properties, which can limit their use in high-performance packaging applications (Baranwal et al., 2022). In contrast, synthetic bio-based biopolymers such as PLA and PHAs offer better mechanical strength, thermal stability, and processability (Baranwal et al., 2022). Despite these advantages, they are often more toxic, involve higher production costs, and exhibit limited biodegradability under natural environmental conditions (Baranwal et al., 2022).

Table 2.1: Comparison of natural and synthetic biopolymers for food packaging applications

	Natural biopolymers	**Synthetic polymers**	**References**
Sources	Naturally occurring, directly extracted from biomass (i.e., plants, animals, and microbes)	Produced via microbial fermentation or chemical synthesis	(Baranwal et al., 2022; Mojaveryazdi et al., 2014)
Examples	Starch, cellulose, chitosan, gelatin, soy protein, canola protein	Polylactic acid (PLA), polyhydroxyalkanoates (PHAs), bio-based polyethylene	
Biodegradability	Highly, biodegradable under natural conditions	Low, often require industrial composting (i.e., PLA)	
Cost and availability	Low-cost; widely available from agricultural by-products	Higher production cost; less commercially available in some regions	
Mechanical properties	Often brittle or weak without plasticizers or additional processing	Strong and flexible; comparable to conventional plastics	
Water resistance	Poor, especially in polysaccharides and proteins	Moderate to good, depending on polymer type	
Thermal stability	Limited; some denature at higher temperatures	High thermal stability; suitable for industrial processing	

Processing and Scalability	Often limited to lab or small pilot scale	Suitable for extrusion, molding, and large-scale production
Environmental impact	Low, reduces, and upcycles waste	Lower than fossil-based plastics but higher than natural biopolymer-based plastics

2.2.2 Natural Biopolymer-based Plastic

Natural biopolymers, such as carbohydrates, lipids, and proteins, are particularly attractive for food packaging applications due to their biocompatibility, biodegradability, renewability, and non-toxicity (Jeevahan & Chandrasekaran, 2019). These materials are widely available in nature and can often be sourced from agro-industrial by-products, offering a sustainable solution for both waste valorization and environmental impact reduction (Jeevahan & Chandrasekaran, 2019). As a result, considerable research has focused on developing biodegradable films from these natural biopolymers for food packaging applications.

2.2.2.1 Overview of Polysaccharides Usage in Packaging

Polysaccharides, also known as carbohydrates, are composed of long chains of monosaccharide units linked by glycosidic bonds (Amir et al., 2022). Major sources of polysaccharides include animals (chitin and chitosan), plants (starch, pectin, and cellulose), marine organisms (alginate, agar, and carrageenan), and microorganisms (pullulan, gellan, and xanthan gum) (Mohamed et al., 2020). Due to their natural abundance, biodegradability, film-forming ability, and compatibility with food systems, polysaccharides are considered highly promising materials for the development of biodegradable packaging films.

A key advantage of polysaccharides is their ability to form dense hydrogen-bonding networks, which confer excellent gas barrier properties, particularly against oxygen and carbon dioxide (Mohamed et al., 2020). This makes them especially suitable for applications such as fresh produce packaging, where reducing gas exchange is essential to delaying respiration, oxidation, and spoilage (Mohamed et al., 2020). Additionally, many polysaccharide-based films are transparent,

tasteless, odorless, and even edible, enhancing their suitability for direct food-contact applications (Mohamed et al., 2020).

Table 2.2: Commercial application of polysaccharide-based plastic

Polysaccharides	Commercial form	Brands	Applications	References
Starch	Thermoplastic blends	Mater-Bi®, Bioplast®, Solanyl® Glad®	Compostable and shopping bags, packaging, and tableware	(Bio Plast Limited, n.d.; Glad, n.d.; Material District, n.d.; Novamont, n.d.)
Cellulose	Regenerated cellulose films	NatureFlex™, Cellophane™	Food wraps and produce packaging	(Futamura, n.d.; NatureFlex, n.d.)

Despite these advantages, polysaccharide-based films face several challenges that limit their application in food packaging. Most notably, their strong hydrophilic nature is attributed to the abundance of hydroxyl groups in their structure. This leads to poor water vapor barrier properties and high moisture sensitivity, which can compromise the mechanical integrity of the films under humid conditions, making them unsuitable for packaging applications that require effective moisture protection (Zhao et al., 2021). To address this, various chemical modification techniques such as carboxymethylation, acetylation, silanization, esterification, urethanization, and amidation have been investigated at the laboratory scale to substitute the hydrophilic hydroxyl groups with more hydrophobic functional groups (Blohm & Heinze, 2019; Y. Zhang et al., 2025; Zhao et al., 2021).

In addition to poor moisture resistance, polysaccharide-based films often exhibit brittleness and limited flexibility, requiring the use of plasticizers such as polyols, lipids, and their derivatives to improve film flexibility and elongation (Sothornvit & Krochta, 2005). Further enhancements in film performance are commonly achieved through polymer blending, cross-linking, and nanomaterial reinforcement strategies, which improve mechanical strength, thermal stability, and barrier properties (Li et al., 2024; Zhao et al., 2021).

Among the various polysaccharides explored for biodegradable packaging, starch, cellulose, and its derivative, chitosan, and alginate are among the most widely studied due to their natural abundance, renewability, and film-forming capabilities.

Starch is a plant-derived polysaccharide composed primarily of two glucose polymers: amylose (linear chains of α-(1→4)-linked D-glucose) and amylopectin (highly branched chains with α-(1→4) and α-(1→6) linkages) (Zhao et al., 2021), which make up both crystalline and amorphous regions. It is derived from sources such as corn, potato, and cassava, and is widely regarded as a cost-effective material that can be easily processed into transparent films (Roveda et al., 2024; Zhao et al., 2021; F. Zhu, 2020). The ratio of amylose to amylopectin varies by source and significantly influences the film's mechanical and barrier properties (Cazón et al., 2017). Due to its semi-crystalline structure, starch can form films with good oxygen barrier performance and moderate mechanical strength (Cazón et al., 2017). However, native starch films are typically brittle and highly moisture-sensitive, limiting their packaging applications. As a result, plasticizers and further modification or blending are often required to enhance flexibility and improve resistance to water vapor (Cazón et al., 2017).

Cellulose is the most abundant natural polysaccharide on Earth and can be derived from a variety of sources, including plants, bacteria, fungi, and marine organisms (Li et al., 2022). Among these, cellulose extracted from agricultural residues has gained particular interest due to its sustainability and potential for waste valorization (Vigneswari et al., 2024). Structurally, cellulose consists of linear chains of β-(1→4)-linked D-glucose units, which align via extensive intra- and intermolecular hydrogen bonding into a structure that contains both crystalline and amorphous regions (Figueiredo et al., 2010; Q. Xu et al., 2016). Its high crystallinity and dense hydrogen-bonding network make it insoluble in water and any polar solvents (Cazón et al., 2017; Lindman et al., 2010). To overcome this, different chemical treatments were developed to solubilize cellulose, resulting in the production of cellulose derivatives such as carboxymethyl cellulose, methylcellulose, hydroxypropylmethyl cellulose, or hydroxypropyl cellulose (Bourtoom, 2008). Films produced from these cellulose derivatives are typically transparent, water-soluble, and flexible, with moderate mechanical strength and some resistance to lipid migration (Cazón et al., 2017; Dhall, 2013). However, their moisture sensitivity and mechanical properties often require further modification or reinforcement to meet packaging requirements. In recent years, cellulose

has also been processed into nanoscale materials, including cellulose nanocrystals and cellulose nanofibers, through mechanical, chemical, or enzymatic treatments (Nagarajan et al., 2021). These nanomaterials retain the inherent strength of cellulose and offer a high surface area, making them attractive as reinforcing agents in composite films. When incorporated into biopolymer matrices such as protein, starch, chitosan, or synthetic biopolymers, cellulose nanomaterials have been shown to significantly enhance mechanical strength, reduce water vapor permeability, and improve overall film integrity (J. Li et al., 2022). Their biodegradability and compatibility with other natural polymers make them ideal for sustainable, high-performance food packaging.

After cellulose, chitosan (1,4)-linked 2 amino-deoxy-β-D-glucan is the second most abundant polymer (Z. Liu et al., 2012; Van Den Broek et al., 2015). It is a natural polysaccharide derived from the deacetylation of chitin (poly (β-(1→4)-N-acetyl-D-glucosamine)), a structural component commonly found in the exoskeletons of crustaceans such as shrimp and crabs (Van Den Broek et al., 2015). The deacetylation process typically involves treating chitin with sodium hydroxide at high temperatures to remove acetyl groups. Chitosan is generally defined as chitin in which less than 50% of the original acetyl groups remain (Van Den Broek et al., 2015).

In food packaging applications, chitosan-based films are particularly valued for their ability to form thin, transparent layers with inherent bioactivity. One of chitosan's most notable characteristics is its inherent antimicrobial activity against bacteria, yeast, and fungi, which extends shelf life and enhances food safety. The antimicrobial effect is primarily attributed to the positively charged amino groups along the chitosan backbone, which interact with negatively charged components on microbial cell surfaces (Van Den Broek et al., 2015). Although the exact mode of action is not fully understood, several mechanisms have been proposed, including (1) electrostatic disruption of microbial membranes, (2) binding to microbial DNA and inhibiting RNA transcription, and (3) chelation of essential metal ions required for microbial metabolism (Benhabiles et al., 2012; Y. J. Jing et al., 2007; A. Martínez-Camacho et al., 2010). Due to its strong antimicrobial activity, considerable research has focused on developing active packaging systems based on chitosan (Charles et al., 2025).

In addition to its antimicrobial functionality, chitosan possesses excellent film-forming capabilities. Typically, chitosan films are prepared by the solvent-sting method, in which chitosan is dissolved in an acidic solution and cast onto a flat surface (Van Den Broek et al., 2015). The

physicochemical properties of the resulting films are influenced by a range of factors, including the intrinsic characteristics of chitosan such as its degree of deacetylation, molecular weight, and the type of acid solvent used, as well as external factors such as pH, molecular weight, plasticizer content, and storage conditions (Van Den Broek et al., 2015). Specifically, the water barrier and mechanical properties of chitosan are significantly affected by the degree of deacetylation, acid type, and pH (Kim et al., 2006; Van Den Broek et al., 2015). While chitosan-based films generally provide excellent oxygen barrier properties, their water vapor permeability is relatively high due to the polymer's hydrophilic nature (Cazón et al., 2019). This limitation can be addressed through various strategies aimed at enhancing moisture resistance, including the incorporation of hydrophobic additives (such as lipids, essential oils, and other polymers), chemical modifications (such as acylation, alkylation, esterification, etherification and amidation), crosslinking with suitable agents, and surface treatments (Cunha & Gandini, 2010; Oladzadabbasabadi et al., 2022). These approaches improve the hydrophobicity of chitosan films, making them more suitable for food packaging applications that require effective moisture barriers.

Agar is a natural hydrophilic polysaccharide extracted primarily from red seaweeds *(Rhodophyceae)*, with *Gelidium, Gracilaria*, and *Pterocladia* being the most prominent commercial sources (S. Roy & Rhim, 2019). It consists of alternating units of 3,6-anhydro-L-galactose and α-D-galactose, linked by β-(1→4) and α-(1→3) glycosidic bonds (M. Li et al., 2014). It is composed mainly of two fractions: agarose, the gelling component, and agaropectin, a non-gelling, highly substituted polysaccharide. Structurally, agar is composed of two main fractions: agarose and agaropectin (S. Roy, Chawla, et al., 2023). Agarose is a neutral, linear polymer responsible for agar's strong gel-forming properties, making it particularly suitable for applications such as films and coatings (S. Roy, Chawla, et al., 2023). In contrast, agaropectin contains numerous anionic functional groups, including sulfate, pyruvate, and uronic acids, which disrupt the regularity of the polymer chains and hinder gelation (S. Roy, Chawla, et al., 2023). As a result, agaropectin is generally considered less desirable for packaging applications and is often removed during processing to improve gel strength and film quality. Agarose is generally recognized as safe and is widely used in food applications, which has led to considerable interest in developing edible films from agar (Sadat Mostafavi & Zaeim, 2020). Agar-based films are typically transparent, flexible, and biodegradable, making them attractive for sustainable packaging (Sadat Mostafavi & Zaeim, 2020). However, like many polysaccharide-based materials,

agar films are brittle, moisture-sensitive, less mechanically robust than conventional plastics, and susceptible to microbial contamination (S. Roy, Chawla, et al., 2023). To enhance the functional properties of agar films, various modification strategies have been explored, including the incorporation of plasticizers (Madera-Santana et al., 2014), crosslinking agents (Belay et al., 2020), polymer blending (Rukmanikrishnan et al., 2021), nanomaterials (S. Roy & Rhim, 2019), and antimicrobial additives (W. Zhang & Rhim, 2022).

Table 2.3: Recent enhancement strategy for polysaccharide-based films

Polysaccharides	Source	Strategies	Components added	Improved properties	References
Starch	Plants, such as corn, rice, wheat, cassava, and potatoes	Polymers blending	Polyvinyl alcohol, polylactic acid, PBAT, polybutylene succinate	Mechanical, thermal, and antimicrobial properties	(Bouzidi et al., 2024; Dammak et al., 2020; Romainor et al., 2022; Shibasaki et al., 2024; Yusof et al., 2021)
		Hydrophobization via chemical addition or chemical modification	Waxes, oils, silica, anhydrides, alkyl halides, emulsions	Improved hydrophobicity	(Y. Cheng et al., 2024; S. Jain et al., 2018; X. Wang et al., 2020; Żołek-Tryznowska et al., 2024)
		Micro- or nanomaterials reinforcement	Microfibrillated cellulose, nanocrystalline cellulose, nano starch, chitosan nanoparticles	Improved mechanical strength, gas barrier and antimicrobial properties	(Q. Chen et al., 2020; Maloufi et al., 2025; Nazrin et al., 2020; Othman et al., 2019; K. Roy et al., 2020; Shapi'i et al., 2020)
		Crosslinking	Malic acid and succinic acid, adipic acid, citric acid	Tensile strength and moisture resistance	(Dai et al., 2019; Menzel, 2020; Thessrimuang & Prachayawarakorn, 2019)
		Antimicrobials	Essential oils, botanical extracts and peels, polyphenols, zinc oxide	Strong activity against *E. coli, S. aureus, C. albicans,* and *A. niger*	(L. Chen et al., 2023; Criollo-Feijoo et al., 2024; Guimarães et al., 2024; Hashem et al., 2023; Istiqomah et al., 2024a; K.

					et al., 2024; Y. Li et al., 2024)
Cellulose	Plants, mainly from wood and cotton, other sources include algae, vegetables, bacteria, and fungi.	Polymers blending	Polycaprolactone, resin, polylactic acid, polyvinyl alcohol	Mechanical and/or water barrier properties, UV-light barrier properties	(Amini et al., 2023; Kamthai & Magaraphan, 2019; Pan et al., 2022; Y. Wu, Qian, et al., 2018)
		Hydrophobization via chemical addition and chemical modifications	Waxes, phenolic acids, acyl chlorides, alkoxysilanes, polyalkylene glycols, and amine, isocyanate	Enhanced moisture resistance and/or mechanical properties	(LakshmiBalasubramaniam et al., 2022; W. Li et al., 2019; Z. Li et al., 2023; Yoshikawa et al., 2025; Y. Zhang et al., 2025; M. Zhu et al., 2022)
		Nanomaterials reinforcement	Zinc oxide nanoparticles, regular and modified cellulose nanomaterials	Improved mechanical strength, gas barrier, and antimicrobial properties	(Amini et al., 2023; Z. Li et al., 2023; Yi et al., 2020; Yoshikawa et al., 2025)
		Crosslinking	Gallic acid, vanillin, citric acid, DMTMM	Improved mechanical strength and gas barrier	(Beghetto et al., 2020; Plaeyao et al., 2025; Venkatesan et al., 2024; Yousefi et al., 2024)

		Antimicrobials	Polyethylene glycol, metallic nanoparticles, essential oils, and phenolic compounds	show strong activity against *E. coli, S. aureus, C. albicans,* and *A. niger*	(Chen et al., 2020; Ebrahimi et al., 2019; Maloufi et al., 2025; Plaeyao et al., 2025; Sayed et al., 2023)

2.2.2.2 Overview of Lipids Usage in Packaging

While biopolymer-based materials have been widely studied, their application in food packaging is often limited by poor water resistance. To address this drawback, researchers have increasingly turned to lipid-based materials as an effective strategy to improve the moisture-barrier properties of biopolymer-based packaging systems.

Lipids are a diverse class of hydrophobic molecules, mainly composed of carbon, hydrogen, and oxygen, with a small fraction also containing elements such as phosphorus, nitrogen, or sulfur (Zubair et al., 2021a). They can be broadly classified into three main categories: simple lipids (fats, oils, waxes), compound lipids (phospholipids and glycolipids), and derived lipids (including steroids, terpenes, and carotenoids) (Zubair et al., 2021). Among these, simple lipids, especially oils and waxes, are widely studied in food packaging systems, largely due to their accessibility and compatibility with natural polymers.

Although lipids offer excellent moisture-barrier properties, they are seldom used as the main ingredient in creating packaging films. Unlike polysaccharides or proteins, lipids lack the polymeric molecular structure required to form strong, cohesive networks (Yousuf et al., 2021). As a result, pure lipid films typically lack structural integrity, making them brittle, weak, and difficult to handle (Yousuf et al., 2021). As such, lipids are often incorporated into hydrocolloid-based films or applied as coatings, where the biopolymer provides structural support while lipids improve moisture resistance through their hydrophobicity (Yousuf et al., 2021). Common lipids used to enhance film hydrophobicity include natural waxes (such as candelilla wax, carnauba wax, and beeswax) and fatty acids (such as lauric, palmitic, oleic, linoleic, and stearic acids) due to their strong water-repelling properties (Balasubramaniam et al., 2020; Rhim & Shellhammer, 2005; X. Wu et al., 2023). When incorporated into common polysaccharide- or protein-based matrices such as starch, wheat gluten, whey protein, alginate, gelatine, and cellulose, these lipids have been shown to effectively enhance moisture barrier performance (Balasubramaniam et al., 2020; Butt et al., 2023; Nehchiri et al., 2021; Oliveira Filho et al., 2020; Schmidt et al., 2013; G. Shen et al., 2021; Zheng et al., 2022). This improvement is attributed to their nonpolar molecular structure, which repels water molecules, and their ability to reduce free volume and limit the availability of hydrophilic sites within the polymer network, thereby restricting water absorption and diffusion (Zubair et al., 2021). Factors affecting the hydrophobicity of lipids include their chemical

composition (such as chain length and degree of saturation), molecular structure, presence of functional groups, and interactions with other components in the film matrix (Zubair et al., 2021).

In addition to improving water barrier properties, lipids also exert a plasticizing effect, influencing the mechanical performance of films in various ways depending on their concentration and nature (L. S. Devi et al., 2024). Some studies have reported that lipid incorporation compromises mechanical strength (Rocca-Smith et al., 2016; Zahedi et al., 2010) while others have found improvements in flexibility and, in certain cases, tensile strength (Azevedo et al., 2019; Fangfang et al., 2020; Oliveira Filho et al., 2020). These contrasting outcomes are largely influenced by factors such as lipid type and concentration, lipid dispersion within the polymer matrix, the resulting film microstructure, and the compatibility between lipid and biopolymer phases (Zubair et al., 2021).

Typically, the addition of lipids disrupts the strong intermolecular interactions in biopolymer films by inserting between polymer chains and replacing robust polymer-polymer bonds with weaker lipid-polymer interactions (Fillery-Travis et al., 2000; Zubair et al., 2021). This substitution weakens the cohesive forces that maintain the film's structural integrity, commonly resulting in decreased tensile strength (Zubair et al., 2021). However, this mechanism can also contribute to increased polymer chain mobility and flexibility, as the rigid polymer bonds are replaced with more pliable lipid interactions (Shaw et al., 2002). In certain cases, lipids may also promote stronger intermolecular bonding, which can ultimately increase mechanical properties (Pérez-Gago & Krochta, 2001).

Over the past decade, there has been increasing interest in using packaging materials to extend the shelf life of food products and enhance their safety (Wyrwa & Barska, 2017). A major focus of this trend is the development of active packaging systems that incorporate antimicrobial agents into the packaging matrix (Amin et al., 2022). In this context, lipids, particularly essential oils, have received considerable attention due to their excellent antimicrobial properties. These plant-derived compounds exhibit strong antibacterial and antifungal activity and align with consumer demand for environmentally friendly, safe options (Sharma et al., 2021). Therefore, many studies have explored incorporating essential oils into hydrocolloid-based films to produce packaging materials that inhibit microbial growth without compromising environmental sustainability. Further details of this trend are discussed in the active packaging section.

2.2.2.3 Overview of Proteins Usage in Packaging

Proteins are complex macromolecules composed of long chains of amino acid monomers, arranged in hierarchical structural levels that influence their functional properties (Hadidi et al., 2022; Pollock, 2007; Rayment, 2003). The primary structure consists of linear amino acid sequences linked by peptide bonds, while the secondary and tertiary structures involve folding and coiling of the chains through hydrogen bonding, disulfide bridges, and ionic interactions, resulting in stable, compact configurations (Chen et al., 2019; Pollock, 2007; Rayment, 2003). The quaternary structure represents the final organization of multiple peptide chains into larger, functional protein assemblies (Pollock, 2007). This three-dimensional arrangement gives rise to either fibrous or globular forms, which directly affect the protein's techno-functional properties, such as solubility, emulsifying capacity, viscosity, and water- or oil-binding capacity (Hadidi et al., 2022; Pollock, 2007).

The structural complexity and functional versatility of proteins make them valuable components in the development of environmentally friendly and functional packaging materials. Protein-based food packaging films have been widely reported to exhibit better gas barrier and mechanical properties compared to their lipid- and polysaccharide-based counterparts, primarily due to the diverse chemical functionalities of amino acid residues within protein molecules (Bourtoom, 2008; Chen et al., 2019; Hadidi et al., 2022). However, a major drawback of protein films is their inherent brittleness and lack of flexibility, which limits their practical applications (Suhag et al., 2020). Therefore, plasticizers such as glycerol, sorbitol, Tween 80, and corn syrup are commonly incorporated into pure protein films to improve film flexibility (Suhag et al., 2020). Plasticizers are low-molecular-weight compounds that interact with polymer chains, disrupting strong intermolecular forces and enhancing chain mobility, thereby increasing film elasticity (Khalil et al., 2018). Among these, glycerol has been widely recognized as the most effective plasticizer for protein-based films due to its compatibility and performance (Audic & Chaufer, 2005).

However, despite the addition of plasticizers, the overall performance of pure protein films remains inferior to that of conventional petroleum-based plastics, particularly in terms of durability and moisture sensitivity. The hydrophilic nature of plasticizers can also further reduce the already limited moisture resistance of pure protein films. To overcome these limitations, recent strategies have focused on improving both the barrier and mechanical properties of protein films. These

include incorporating nanomaterials, polymers, hydrophobic compounds, and bioactive agents to reinforce the polymer matrix, improve structural integrity, and impart functional properties, such as antimicrobial activity (Hadidi et al., 2022).

2.3. Plant-Derived Proteins in Packaging Applications

Growing concerns regarding the cost, sustainability, ethical considerations, and health risks associated with animal-based materials have accelerated the shift toward plant proteins (Hadidi et al., 2022). Plant proteins offer advantages such as widespread availability, lower environmental impact, and consumer acceptance, particularly in the context of vegan, vegetarian, and clean-label trends (Sá et al., 2020). In the field of food packaging, common sources such as soy, wheat gluten, zein, pea, and rice proteins have been extensively studied, each offering distinct film-forming capabilities, mechanical behavior, and barrier properties, depending on their molecular structure and composition (Sá et al., 2020). In recent years, global sustainability initiatives have also emphasized the importance of waste valorization, such as transforming food processing byproducts and agricultural residues into value-added materials (Hadidi et al., 2022). This has led to a growing interest in underutilized plant sources, particularly those generated in large quantities by the agro-industrial sector, including oilseed meals (Sá et al., 2020). Oilseed meals, which remain after oil extraction from seeds, are notable for their high levels of residual proteins, fibers, and other bioactive compounds (Chmielewska et al., 2021). Among these, canola meal, a byproduct of canola oil production, stands out for its high protein content and widespread availability (Bandara et al., 2018). Utilizing canola byproducts offers significant potential to create sustainable, high-value products across diverse sectors, such as food packaging and bioplastic manufacturing (Bandara et al., 2018).

2.3.1. Canola Protein: A Promising But Underdeveloped Biopolymer for Packaging Applications

Brassica napus, commonly known as rapeseed, is an oilseed crop belonging to the Brassicaceae family, often characterized by its yellow flowers, leafy greens, and oil-rich seeds (Zohary et al., 2012). Canola is a variety of rapeseed that is bred specifically for the production of edible oil, with significantly reduced levels of erucic acid (<2%) and glucosinolates (<30 μmol/g), making it more palatable and suitable for human consumption (Cheung et al., 2015; Zohary et al., 2012). In Canada, canola is the leading oilseed crop with substantial economic significance. Over the past five years, Canadian oil production has ranged between 3.5 and 4.5 million metric tons (MMT),

contributing approximately 29.9 billion dollars annually to the economy (LMC International, 2020). In 2021 alone, Canada exported canola-related products valued at 14.4 billion dollars to major markets, including the United States, China, Mexico, Japan, and Europe (Statistics Canada, 2021). Globally, the canola oil market was estimated at 29.1 million tons in 2024 and is projected to reach 32.0 million tons by 2033, according to independent market research firms (IMARC, 2024).

The high production volume of canola oil, along with its increasing output over the years, underscores the substantial quantities of canola meal generated as a byproduct of oil extraction. After oil removal, the canola meal still contains about 40% protein (dry matter basis), indicating its considerable potential as a valuable source of plant protein (Radfar et al., 2017). Commercially, canola protein is typically obtained through alkaline extraction (Y. Zhang et al., 2018). In this process, the defatted canola meal is first treated with a basic solution, commonly sodium hydroxide, to solubilize the proteins, followed by centrifugation to remove insoluble residues (S. H. Tan et al., 2011). The solubilized proteins are then precipitated by adding an acid, such as hydrochloric or acetic acid, and further purified through repeated cycles of centrifugation and washing (S. H. Tan et al., 2011). This method can yield approximately 87.5% canola protein isolate (Klockeman et al., 1997). The resulting protein isolate is composed primarily of two storage proteins, cruciferin and napin, which together account for bout 70% and 30% of the total protein content, respectively (Cheung et al., 2015; Ntone et al., 2022). Both proteins differ in their amino acid composition, molecular configuration, size, and physicochemical properties (Campbell et al., 2016). Cruciferin is a salt-soluble protein with a higher molecular weight (12S, 240-300 kDa), while napin is water-soluble, smaller (4.5-10kDa) and stabilized by strong disulfide bonds that confer structural rigidity (Schwenke, 1994). As a result, cruciferin is more prone to conformational changes, whereas napin maintains its structure more readily under varying conditions (Schwenke, 1994). The functional properties of canola protein isolates can be tailored by adjusting the ratio of cruciferin to napin. For instance, isolates with a higher proportion of napin generally exhibit greater solubility compared to those dominated by cruciferin (Wanasundara et al., 2012). Therefore, understanding and controlling the composition of these protein fractions is key to optimizing the functionality of canola protein isolates for specific applications (Wanasundara et al., 2012).

Although canola protein has a well-balanced amino acid profile comparable to that of soy, its application in human nutrition is limited by the presence of anti-nutritional compounds such as glucosinolates and phytates (Y. Zhang et al., 2018). While additional processing technologies can be employed to reduce or eliminate these compounds, such methods often require more capital investment (Y. Zhang et al., 2018). Moreover, the commonly used alkaline extraction method can result in undesirable protein modifications, including denaturation, as the elevated pH disrupts the native protein structure (Gerzhova et al., 2015). The structural integrity of proteins obtained from conventional solvent-extracted canola meal is also often compromised by the harsh conditions of oil processing (Daun et al., 2011). During this process, canola seeds are typically subjected to high-temperature treatments, such as cooking and desolventizing, which denature native protein structures (Daun et al., 2011). Additionally, the use of strong chemical solvents, such as hexane, for residual oil removalurther promotes protein unfolding and aggregation, impairing functional properties such as solubility, emulsifying capacity, and gelation behavior (Daun et al., 2011; S. H. Tan et al., 2011). These challenges have prompted researchers to investigate alternative, value-added applications for canola protein beyond the food sector. Recent advancements have highlighted its potential as a sustainable material for various industrial applications, including adhesives, drug delivery systems, and biodegradable food packaging films (Bandara et al., 2018).

2.3.2. Recent Strategies on Enhancing Plant Protein-based Packaging Films

The functional limitations of protein-based packaging films, such as their weak mechanical properties and high water sensitivity, have posed challenges for their application in food packaging. Recent approaches include blending with other polymers, incorporation of nanomaterials, addition of active agents, and application of physical and chemical modifications, and the development of multilayer film systems has shown promise in addressing these challenges (He et al., 2025).

2.3.3. Compositing with Biodegradable Polymers

Blending plant proteins with other polymers is a strategy to address the inherent limitations of protein-based films by incorporating complementary natural or synthetic polymers. When blended, the intermolecular interactions between the protein and the added polymer can strengthen the film matrix, thereby improving cohesion and compatibility (He et al., 2025). These interactions not only enhance the structural integrity of the film but also contribute to improved mechanical

properties, flexibility, and barrier performance, making the films more suitable for practical applications in food packaging and other biodegradable materials (Pavon et al., 2020; Raj et al., 2024a; Sousa et al., 2018). However, there are several key considerations must be taken into account when selecting polymer blends, including blends miscibility, morphology and compatibility (Muthuraj et al., 2018; Olonisakin et al., 2025). Miscibility is a thermodynamic concept that refers to how well polymers mix within a blend, determined by the number of distinct phases present (Muthuraj et al., 2018). Depending on the extent of this mixing, polymer blends can be categorized as fully miscible, partially miscible, or completely immiscible (Muthuraj et al., 2018). A fully homogeneous polymer blend displays consistent composition and uniform properties across the entire material (Mural et al., 2018). However, since most polymer blends are thermodynamically immiscible, many research has focused on understanding their miscibility and internal structure (Arrighi et al., 2016; Mural et al., 2018; Muthuraj et al., 2018). Miscibility can be evaluated using several methods, including Scanning Electron Microscopy (SEM), Differential Scanning Calorimetry (DSC) and using thermodynamic calculations and models (Olonisakin et al., 2025). In SEM, the absence of phase separation indicates good miscibility (Olonisakin et al., 2024). In DSC, a homogenous blends exhibit a single glass transition temperature (T_g) whereas heterogenous material will have two distinguishable T_gs (Muthuraj et al., 2018). Thermodynamic approaches commonly use Gibbs free energy ($\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix}$) and Flory-Huggins parameter (χ) to determine the miscibility of the polymer blends where ΔH_{mix} and ΔS_{mix} are the change in enthalpy and change in entropy, respectively (Muthuraj et al., 2018). For miscible blends, ΔH_{mix} will be negative whereas immiscible blends typically have positive ΔH_{mix} (Muthuraj et al., 2018).

While miscibility provides a thermodynamic understanding of whether polymers can mix at the molecular level, morphology and compatibility determine how the blend behaves in practice. The morphology of a polymer blend such as phase continuity, domain size, and dispersion can directly influences blend properties (Starý, 2015). Immiscible blends often form heterogeneous morphologies with distinct dispersed and continuous phases, which can reduce interfacial adhesion and create weak points under stress (Starý, 2015). To overcome these limitations, compatibilization is employed to enhance the interactions between phases, refine the morphology, and improve stress transfer (Starý, 2015). Compatibilization is a strategy employed to improve the properties of immiscible and partially miscible polymer blends by modifying and reducing the

interfacial tension between their components (Muthuraj et al., 2018). Effective compatibilization can transform phase-separated structures into finer, more uniform morphologies, thereby increasing the blend's performance and functionality in applications such as packaging (Muthuraj et al., 2018; Starý, 2015). Compatibilization can be achieved through ex situ and in situ approaches. In ex situ (non-reactive) compatibilization, a pre-synthesized compatibilizer, such as a block or graft copolymer, is added to the blend to improve interfacial adhesion by reducing interfacial tension between the phases (Muthuraj et al., 2018). In contrast, in situ (reactive) compatibilization occurs during processing, where chemical reactions between blend components generate compatibilizing agents within the system, forming interfacial bonds and resulting in more stable and finely dispersed morphologies (Muthuraj et al., 2018).

Overall, the successful development of polymer blends depends on achieving a good balance between miscibility, morphology, and compatibility. By tailoring blend composition and employing appropriate compatibilization strategies, it is possible to enhance the functionality and reliability of biodegradable films for advanced packaging applications (Pavon et al., 2020; Raj et al., 2024b). This is especially critical when working with natural biodegradable polymers, such as plant proteins, polysaccharides, and other bio-based materials, which often have inherent limitations in mechanical strength, flexibility, or barrier properties.

Natural biodegradable polymers, such as chitosan, starch, alginate, pectin, and cellulose derivatives, are frequently used due to their biodegradability, renewability, and safety (K. C. de Souza et al., 2020; Hadidi et al., 2022). For example, chitosan offers good mechanical and antimicrobial properties, thermal, and barrier performance (Mujtaba et al., 2018). Starch and alginate, on the other hand, enhance flexibility and transparency while improving barrier properties (Mohamed et al., 2020). These natural polymers can work synergistically with plant proteins to improve the functionalities of protein-based films without compromising biodegradability. However, while polysaccharides can provide effective gas barrier properties, they are generally ineffective against moisture due to their hydrophilic nature (Hadidi et al., 2022).

Synthetic biodegradable polymers, such as polyvinyl alcohol (PVA), poly-ε-caprolactone (PCL), polybutylene adipate terephthalate (PBAT), polybutylene succinate (PBS), polyglycolic acid (PGA), and polylactic acid (PLA), are commonly used in food packaging (Agostinho et al., 2023; Kirac et al., 2022). Although these polymers can be synthetically produced from fossil fuel-derived

monomers, they are designed to be biodegradable under specific environmental conditions, such as industrial composting or microbial activity in soil or water (Agostinho et al., 2023). Because of their petrochemical origin, they exhibit mechanical and barrier properties comparable to conventional plastics, including strength, flexibility, and processability, which are essential for packaging performance (Agostinho et al., 2023). Compared with natural biodegradable polymers, synthetic biodegradable polymers offer greater esign flexibility, mechanical performance, and biocompatibility (S. Liu et al., 2020). As their fundamental building blocks, including monomer type, molecular weight, and polymer structure, can be controlled during synthesis, properties such as porosity, degradation rate, and elasticity can be tailored to a specific application (S. Liu et al., 2020). Additionally, these polymers are typically manufactured under controlled industrial conditions, ensuring high purity, batch-to-batch consistency, extended shelf life, and cost-effective scalability (S. Liu et al., 2020). These characteristics effectively overcome many limitations of natural biopolymers, such as compositional variability and weak mechanical properties (S. Liu et al., 2020). As a result, extensive research has focused on incorporating various biodegradable synthetic polymers into protein-based films to enhance their functional properties (He et al., 2025). Among these, polyvinyl alcohol (PVA) is one of the most widely studied. PVA is a synthetic, water-soluble polymer composed of a hydrocarbon backbone with hydroxyl groups on its side chains (Abdullah et al., 2017). It exhibits a semi-crystalline structure, characterized by small crystalline regions dispersed within a largely amorphous matrix, contributing to its strength and flexibility (Abdullah et al., 2017). PVA can be produced from polyvinyl acetate through the process of hydrolysis into varying degrees of hydrolysis (70%-99%) and molecular weights ranging from 20 x 10^3 to 400 x10^3 g/mol (Abdullah & Dong, 2020a). While PVAc is derived from petroleum-based sources, PVA itself is considered to exhibit “green” characteristics due to its water solubility and biodegradability, and compatibility with renewable biopolymers (Abdullah et al., 2017; Goodship & Jacobs, 2009a). In packaging applications, PVA has been reported to possess excellent mechanical strength, film-forming properties, and gas barrier properties, along with sufficient thermal stability (Abdullah et al., 2017; Othman, 2014). As a result, numerous studies have explored the development of packaging films based on either pure PVA or PVA blends with various biopolymers (Gaaz et al., 2015; Ghaderi et al., 2019; Kahvand & Fasihi, 2019; Lara et al., 2019; Loryuenyong et al., 2015). While pure PVA films exhibit superior mechanical properties and lower water vapor permeability compared to blended films (Cano et al., 2015), they

have limited biodegradability due to the absence of suitable environmental factors such as optimal pH, temperature, relative humidity, and the presence of specific microbial communities (Chiellini et al., 1999). One strategy to improve PVA's biodegradability and mechanical flexibility is to blend it with natural biopolymers, such as polysaccharides and proteins (Cano et al., 2015; Goodship & Jacobs, 2009a).

PVA has demonstrated strong potential to improve the functional properties of protein-based films, particularly strength and flexibility. For example, Chen et al., (2019) prepared films from cottonseed protein and PVA at different ratios (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9, wt.%). The study found that elongation at break and tensile strength increased from 2% and 3.5 MPa to 137% and 20.7 MPa, respectively, when the cottonseed protein-A mass ratio changed from 9:1 (wt.%) to 3:7 (wt.%) (Chen et al., 2019). Similarly, Wu et al., (2018) fabricated chitosan/PVA blend films and reported enhanced mechanical properties with increasing PVA content. However, both Chen et al., (2019)Wu et al., (2018) (2019) and Wu et al. (2018) reported that excessive incorporation of PVA led to phase separation within the film matrix, a common phenomenon in polymer blend systems arising from limited compatibility between the constituent polymers. Phase separation typically leads to morphological heterogeneity, which can disrupt the uniformity of the film's internal structure (Chen et al., 2019; Wu et al., 2018). This disruption negatively affects the film's overall performance, particularly its mechanical integrity and barrier properties. Therefore, determining an optimal blend ratio is essential to strike a balance between enhanced functionality and structural uniformity in blended films.

In addition to the blend ratio, the intrinsic properties of PVA(Abdullah et al., 2017; Limpan et al., 2010, Limpan et al.,, such as molecular weight and degree of hydrolysis, also play a crucial role in determining the final film characteristics (Abdullah et al., 2017; Limpan et al. 2010; Limpan et al. 2012). High molecular weight PVA contributes to increased tensile strength and film toughness due to its longer polymer chains and greater chain entanglement (Abdullah et al., 2017). However, it may also result in higher solution viscosity and reduced solubility, which can complicate processing and affect film uniformity (Abdullah et al., 2017). Conversely, low molecular weight PVA generally improves flexibility and processability, though often at the expense of mechanical strength (Abdullah et al., 2017). In terms of degree of hydrolysis, partially hydrolyzed PVA retains residual acetate groups from its precursor, polyvinyl acetate (Roohani et al., 2008). These acetate

groups limit intermolecular hydrogen bonding and reduce crystallinity, resulting in a more open structure that enhances water penetration (Abdullah et al., 2017). As a result, low degree of hydrolysis PVA typically exhibits poor water resistance and is less suitable for packaging film applications (Roohani et al., 2008). Regarding film flexibility, PVA can disrupt rigid protein-protein interactions such as disulfide bonds and hydrophobic associations by forming hydrogen bonds with polar functional groups in the protein matrix (Limpan et al., 2012). These hydrogen bonds are non-covalent and reversible, enabling them to break and reform under mechanical stress, which contributes to enhanced film ductility (X. Yu et al., 2021). The incorporation of hydrogen-bonding moieties is known to promote chain mobility and improve mechanical performance in polymer networks (Galuska et al., 2022; M. Y. Lee et al., 2020).

Limpan et al., (2012) investigated the effects of various degrees of hydrolysis (86%-99%) and molecular weights (27,000-120,000 g/mol) of PVA on the properties of fish protein-PVA blend films and reported a positive correlation between both DH and MW and TS and EB. Their findings showed that both higher DH and MW positively correlated with tensile strength and elongation at break. An increased degree of hydrolysis promotes the formation ofore hydroxyl groups along the PVA backbone, enhancing hydrogen bonding between PVA chains and leading to a denser, more cohesive film structure (Limpan et al., 2012). Similarly, higher MW facilitates stronger intermolecular interactions due to greater chain entanglement, thereby improving mechanical integrity and resistance to deformation (Limpan et al., 2012). However, these benefits must be balanced against the challenges of reduced solubility and increased viscosity during film formation (Limpan et al., 2012).

2.3.4. Reinforcement with Nanomaterials

To further enhance the performance of biopolymer films, nanomaterial reinforcement has emerged as a promising complementary approach. Unlike polymer blending, which improves film properties through the combination of compatible polymers, nanomaterial reinforcement involves the incorporation of nanoscale fillers, typically with at least one dimension below 100 nm, into the polymer matrix. Common examples include nanoclays, cellulose nanocrystals, carbon-based or metal-based nanoparticles (Emamhadi et al., 2020). These nanomaterials exhibit unique physicochemical properties, including high aspect ratios, large surface areas, and excellent mechanical strength (Barage et al., 2022). When uniformly dispersed, these nanofillers interact

with polymer chains via hydrogen bonding, electrostatic forces, or van der Waals interactions, resulting in a more compact and mechanically robust film structure. Compared to conventional blending, nanomaterial reinforcement offers greater improvements in barrier properties, thermal and mechanical stability, and imparts additional functionalities, such as antimicrobial activity (Emamhadi et al., 2020; J. S. Kim et al., 2020; Mei & Wang, 2020). Therefore, integrating nanomaterials represents a highly effective approach to further optimize the structural and functional performance of biodegradable films for advanced food packaging applications. Among the various nanomaterials investigated for reinforcing biopolymer films, nanoclays, cellulose-based nanomaterials, silver nanoparticles (AgNPs), and zinc oxide nanoparticles (ZnO NPs) have shown significant potential in enhancing the structural and functional performance of packaging systems (Barage et al., 2022).

Cellulose-based nanomaterials have gained considerable attention due to their abundance, renewability, and exceptional reinforcing capabilities. In particular, the remarkable rigidity and crystalline structure of cellulose make it a promising candidate for reinforcing biodegradable films (Rongpipi et al., 2019). Early research focused on extracting high-quality cellulose crystals from a wide range of natural sources, including plants, marine animals, and microorganisms (Rongpipi et al., 2019). As research progressed, it was discovered that reducing cellulose crystals to the nanoscale significantly enhanced their mobility and interfacial interaction, leading to the development of highly crystalline forms known as nanocrystalline cellulose (NCC), also referred to as cellulose nanocrystals (CNCs) or nanowhiskers (Sehaqui et al., 2011; Rongpipi et al., 2019). NCC can be isolated using various chemical methods, such as acid and enzymatic hydrolysis, or mechanical processes, such as grinding and sonication (Abe et al., 2007; Salas et al., 2014). Its high crystallinity and favorable aspect ratio (length-to-width) contribute to excellent mechanical strength and barrier properties in biopolymer films (Ferrer et al., 2017). The lightweight nature, high surface area, and enhanced mobility of NCC further facilitate strong interfacial bonding and effective dispersion within the polymer matrix (Ferrer et al., 2017; Mariano et al., 2014).

Numerous studies have reported positive outcomes from incorporating NCC into biopolymer-based films. For example, (Salari et al., 2018)Salari et al. (2018) incorporated NCC derived from bacterial cellulose at concentrations of 0, 2, 4, and 6% (w/w) into chitosan-based nanocomposite films using the solvent-casting method. Their results showed that increasing the NCC

concentration reduced water vapor permeability (WVP), moisture content (MC), and water solubility (WS), indicating enhanced moisture resistance. This improvement was attributed to additional hydrogen bonding between the hydroxyl groups of NCC and the chitosan matrix, which reinforced the polymer network (Salari et al., 2018). Similar enhancements in water barrier properties have been observed in other biopolymer films reinforced with NCC (Atef et al., 2014; L. Cao et al., 2019; Tieng Owi et al., 2019). It is worth noting that NCC is inherently hydrophilic due to its numerous surface hydroxyl groups (Ferrer et al., 2017). This characteristic presents a dual effect on the moisture sensitivity of biopolymer films. On one hand, when NCC is incorporated into hydrophilic matrices such as polysaccharides and proteins, its hydroxyl groups can form hydrogen bonds with the polymer chains, thereby becoming less available to interact with water (Atef et al., 2014). This can lead to reduced moisture sensitivity and improved water resistance.

On the other hand, the hydroxyl-rich surface of NCC can also attract and retain water molecules. As a result, the impact of NCC on the moisture barrier properties of films can depend on the nature of the polymer matrix, the degree of NCC dispersion, and the specific interactions within the composite (Ferrer et al., 2017). In contrast, this moisture-binding effect tends to be more pronounced when NCC is blended with hydrophobic matrices, where fewer interactions occur between NCC and the polymer, leaving more hydroxyl groups exposed to moisture (Ferrer et al., 2017). In addition to moisture resistance, Salari et al., (2018) also reported an increase in tensile strength and Young's modulus with NCC incorporation up to 4%, underscoring the reinforcing effect of NCC's rigid structure. However, further increases in NCC content led to a decline in mechanical properties, likely due to particle agglomeration. This highlights the importance of optimizing NCC concentration and ensuring uniform dispersion throughout the film. A corresponding decrease in elongation at break was also observed, which is consistent with the typical trade-off between strength and flexibility. Slight increases in film thickness and opacity were also observed with higher NCC content.

2.3.5. Research on Canola Protein-based Packaging Films

Research on canola protein bioplastic is very limited compared to other plant proteins, such as soy or zein (Zhang et al., 2017). Since protein-based plastics have low strength, flexibility, and moisture barrier properties, most of the work has focused on finding solutions for these issues. Shi

and Dumont (2014) prepared canola protein isolate-based films via the solvent-casting method with different plasticizers (glycerol, sodium dodecyl sulfate (SDS), and stearic acid (SA)). Jang et al. (2011) produced an edible rapeseed film using polysorbate 20 and gelatin, with sorbitol and sucrose as plasticizers, to improve mechanical properties. Manamperi et al. (2010) studied the effect of protein fractions on the properties of canola protein plastic. The authors reported that removing napin can reduce water affinity and improve the mechanical properties of the film (Manamperi et al., 2010). With the recent surge in nanomaterials, nanocomposite films from canola protein were studied. Dissanayake et al. (2023) prepared canola protein film via the solvent-casting method, reinforced with different levels (1%, 3%, and 5%) of pure NCC or modified NCC with oleic acid. The study's results showed that NCC has the potential to improve the mechanical and water-barrier properties of the film (Dissanayake et al., 2023). Chairez-Jimenez et al., (2024) incorporated graphite oxide nanoparticles into canola protein-based film and observed a significant increase in oxygen barrier. Building on these previous findings, this study explores the incorporation of polyvinyl alcohol and essential oils into canola protein matrices to further enhance their mechanical strength, barrier properties, and antimicrobial performance, aiming to develop a more functional and sustainable film for food packaging applications.

2.4. The Role of Antimicrobial Compounds in Packaging Systems

In recent years, biodegradable plastics have evolved from passive packaging materials to active systems capable of enhancing food safety and quality. A common strategy to achieve this is to incorporate antimicrobial compounds into the polymer matrix. Natural antimicrobials are increasingly preferred over synthetic alternatives because they are generally recognized as safe, non-toxic, and biocompatible, thereby reducing potential health risks and environmental impact (G. Chen & Liu, 2016).

The integration of these bioactive agents transforms conventional packaging into smart packaging systems. Smart packaging represents a new generation of food packaging technologies designed to go beyond mere containment and protection. It encompasses both active and intelligent packaging systems, each offering distinct functionalities (Biji et al., 2015). Active packaging interacts directly with the food or its headspace to improve preservation, such as by releasing

antimicrobial or antioxidant agents, scavenging oxygen, or regulating moisture to slow spoilage and extend shelf life (Biji et al., 2015; Du et al., 2023). In contrast, intelligent packaging monitors changes in the food or its environment and provides real-time information on freshness, safety, or storage conditions through indicators such as gas sensors, pH-sensitive color changes, time-temperature indicators, and more (Sohail et al., 2018; Yao et al., 2024). Building on these functionalities, smart packaging offers both protective and informative roles in modern food packaging systems, making it a key innovation.

Incorporating natural substances such as essential oils, antimicrobial peptides, and polysaccharides into biodegradable packaging can inhibit the growth of spoilage and pathogenic microorganisms, while also extending the shelf life of perishable products and preserving the environmental advantages of biodegradable materials (Abdollahzadeh et al., 2021; Kowalczyk et al., 2020).

2.4.1. The Incorporation of Essential Oils in Biodegradable Food Packaging

Among the various natural bioactive agents incorporated into biodegradable packaging, Essential oils (EOS) have gained particular attention due to their broad-spectrum antimicrobial and antioxidant properties. EOS are plant secondary metabolites, produced as part of the plant's defense mechanisms as a means to fight off microbial diseases (Biji et al., 2015). EOS can be produced from all parts of the plant, such as buds, leaves, stems, and so on (Bakkali et al., 2008). Terpenes, phenylpropanoids, and terpenoids are common EOS classes known for their potent antimicrobial activities. EOS are characterized by their volatility, odour, and excellent efficiency against viruses, bacteria, fungi, and insects (Bakkali et al., 2008). They are Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) in the USA and are therefore widely used across multiple industries, such as the pharmaceutical, cosmetic, perfume, and agricultural food industries (Bakkali et al., 2008). In the food industry, EOS are often used as preservatives and flavour additives; novel applications in packaging include their addition to biodegradable packaging materials to produce an interactive system (Bakkali et al., 2008; Maisanaba et al., 2017).

Despite these promising attributes, EOS application is often challenged by variability in performance, particularly in their antimicrobial efficacy. This inconsistency arises because the effectiveness of EOS is highly dependent on several external factors, including geographic origin, harvesting time, and processing methods, all of which directly influence their chemical composition and concentration of active compounds (Herchi et al., 2025; Reyes-Jurado et al.,

2014; Santos et al., 2022; Xiao et al., 2026). Plants cultivated in different geographic regions are exposed to diverse environmental conditions, including variations in soil composition, temperature, humidity, altitude, and sunlight intensity (Xiao et al., 2026). These factors influence plant metabolism and biosynthetic pathways, leading to differences in the production of key antimicrobial constituents (Dodoš et al., 2024). For example, Dodoš et al., (2024) examined the oil composition of various *Satureja montana L.* (winter savory) populations collected from different countries in the Western Balkans and found that their chemical profiles were influenced by the climatic conditions across geographic regions. Phenolic compounds, such as carvacrol and thymol, were more abundant in coastal populations where the climate is temperate with hot summers, while non-phenolic compounds, including geraniol and linalool, were more prominent in continental populations, which experience cooler conditions and warm summers (Dodoš et al., 2024). In addition to geographic influences, harvesting practices, particularly the timing of harvest can significantly impact EO yield and composition. Hazrati et al., (2022) observed that EOS content was the highest in *Salvia officinalis* (sage) harvested between 4:00-6:00 p.m. and lowest at 4:00-6:00 a.m., with differences in the relative abundance of key constituents such as cis-thujone, trans-thujone, 1,8-cineole, and camphor. Processing and extraction techniques also play a critical role in determining EO composition and functionality. Park et al., (2024) reported that different extraction methods, such as cold pressing and hydrodistillation, result in distinct volatile profiles in EOS derived from orange and tangor peel, primarily due to chemical transformations occurring during extraction. While certain compounds were influenced by the citrus species itself, the extraction method significantly altered the overall composition and, consequently, the functional properties of the oils (Park et al., 2024). Collectively, these findings highlight that the antimicrobial efficacy of EOs is highly sensitive, emphasizing the need to standardize cultivation, harvesting, and processing conditions to ensure consistent antimicrobial performance. As noted by Bakkali et al., (2008), obtaining a uniform essential oil composition requires careful control of multiple factors, including the plant organ, cultivation conditions, harvest timing, and extraction procedures; however, achieving such consistency is often challenging in practice.

Studies conducted to understand the working mechanisms of EOS, their compatibility in diverse biopolymer matrices, and their effect on food products have spanned across decades (Atarés & Chiralt, 2016; Basílico & Basílico, 1999; Bassolé et al., 2010; Cox et al., 2001; Juliani et al., 2002; Laorenza & Harnkarnsujarit, 2021a; Soofi et al., 2021). EOS from a wide variety of plants was

extracted, and their constituents were analyzed to gain insight into the negative interactions between these compounds and microorganisms under different packaging conditions. Standardized methods such as gas chromatography and mass spectrometry are typically used to analyze the chemical profile of EOS, which is found to contain an assortment of phenols, aldehydes, ketones, alcohols, esters, ethers, and hydrocarbons (Burt, 2004). About 20-70% of EOS were occupied by two to three dominating components, followed by other compounds in trace amounts (Bakkali et al., 2008). The antimicrobial properties of EOS are thought to be dependent on these major components (Bakkali et al., 2008). For example, thymol and carvacrol are thought to be responsible for the high antimicrobial activity in plants from the *Thymus* and *Origanum* species. At the same time, cinnamaldehyde, eugenol, linalool, and terpinene-4-ol are linked to the high antimicrobial properties of *Cinnamomum zeylanicum* (cinnamon), *Ocimum basilicum* (basil), and *Melaleuca alternifolia* (tea tree) (Bassolé et al., 2010; Bassolé & Juliani, 2012; Bouzouita et al., 2003; Prabuseenivasan et al., 2006). The problem is that, because EOS is a complex substance, pinpointing the source of its high antimicrobial activity can be difficult. As the constituents exhibit complex relationships, it might be unwise to single out the main compounds while neglecting the minor components. The question remains whether the high antimicrobial activity in EOS is due to shared interactions among its constituents or to a few dominant compounds. To answer this, principal substances such as carvacrol, thymol, cinnamaldehyde, eugenol, carvone, geraniol, eucalyptol, and so on were isolated from EOS and put into various biopolymer matrices as individuals or collective groups, where they exhibited good antimicrobial activities (Avila-Sosa et al., 2012; Bostancioğlu et al., 2012; Salgado et al., 2013; Synowiec et al., 2014). It was found that their efficiency in inhibiting microorganisms is limited to a threshold and increases with greater incorporation. However, in excess quantity, they can reduce the mechanical properties of the films (Ghasemlou et al., 2013). When EOS is considered as a whole, its constituents interactnd exhibit four distinct relationships: synergistic, antagonistic, additive, and indifferent (Delgado et al., 2004; Pei et al., 2009a). Among those, synergistic and additive relationships are more favourable, as the compounds interact to produce a combined effect that enhances antimicrobial properties, meaning even trace elements can contribute to antimicrobial activity through their interactions (Hoet et al., 2006; Santana-Rios et al., 2001). On the other hand, primary compounds from EOS were paired up (thymol/carvacrol, thymol/eugenol, cinnamaldehyde/carvacrol, cinnamaldehyde/thymol, cinnamaldehyde/eugenol, and more) and tested against multiple microorganisms to study their

relationship and efficiency (Lambert et al., 2001; Pei et al., 2009a; Rivas et al., 2010). In cases where synergism was observed between the compounds, the minimum effective dosage decreased. For example, Pei et al. (2009) paired carvacrol, thymol, cinnamaldehyde, and eugenol at different ratios (1:1, 1:4, and 1:8) and noted a 25% and 50% decrease in the threshold effective concentration against E. coli. Zhou et al., (2007) (2007) observed 25% and 50% reductions in the effective concentration against Salmonella when pairing carvacrol, thymol, and cinnamaldehyde at a 1:1 ratio. Under the synergistic effect, antimicrobial potency is observed at a much lower EOS dosage. This is an advantage for the application of active biopolymer films, as their mechanical properties can still be preserved. Nanoemulsions can be used to reduce the transfer of aromatic components of EOS from active packaging films into contact food; however, the stability of these emulsions remains questionable.

Primary compounds in EOS, such as thymol, carvacrol, cinnamaldehyde, eugenol, and others, have been widely studied due to their pronounced potency and versatile mechanisms of action against bacteria (Hyldgaard, Mygind & Meyer, 2012). Their mode of inactivation, including but not limited to, alternating cell morphology and membrane function, restricting cellular growth, enzyme and toxin production, and altering membrane electrochemical gradients and pH equilibrium (Hyldgaard, Mygind & Meyer, 2012). The multifaceted action mechanism of EOS, or its major components, renders microorganisms incapable of defending themselves and eventually leads to cell death (Hyldgaard, Mygind & Meyer, 2012). Therefore, EOS are effective against a wide spectrum of microorganisms, including both gram-positive (+) and gram-negative (-) bacteria (Smid & Gorris, 2020). Several studies hypothesized that gram (+) bacteria are more susceptible to EOS and their constituents due to the lack of a hydrophilic outer membrane that serves as an additional protective layer against hydrophobic substances like EOS (Ghasemlou et al., 2013; Gómez-Estaca et al., 2010; Z. Shen & Kamdem, 2015); however, an opposite trend was observed in certain studies where gram (+) bacteria showed more resistance to EOS or their components. This was explained by the presence of a thicker peptidoglycan membrane that limited the diffusion of antimicrobial substances (Burt, 2004; Ghasemlou et al., 2013). EOS is also effective against fungi such as yeast and mold; however, the sensitivity of these microorganisms to the antimicrobial substances in EOS compared to that of bacteria remains debatable.

Prior to selecting EOS for biopolymer film formulation, several factors, such as targeted microorganisms, antimicrobial compounds, matrix types, and end-use food products, need to be considered (Maisanaba et al., 2017). Some biopolymer matrices possess inherent antimicrobial properties that can be further enhanced by the addition of EOS or its major components. For example, Avila-Sosa et al., (2012) (2012) added oregano and cinnamon EOs to three different types of edible, biodegradable films made from amaranth, chitosan, and starch, and studied the films' inhibition against *A. niger* and *P. digitatum*. Chitosan-based films showed better antifungal properties at lower EOS concentrations, attributed to the compatibility between the matrix and EOS and to the good retention of active compounds, coupled with the inherent antimicrobial properties of chitosan (Avila-Sosa et al., 2012). After the process of fabrication, it is vital to start testing for the antimicrobial properties of biodegradable films since EOS constituents can gradually diffuse out of the matrices over time, leading to a reduction in film's functionality. Several studies were conducted on the migration of EOS components into biopolymer matrices and on the shelf-life stability of food products enclosed in active packaging films (Higueras et al., 2014; Kuorwel et al., 2013; V. G. L. Souza et al., 2018; Umaraw et al., 2020).

Antioxidants, a component of EOS that makes a major contribution to its antimicrobial activity, are also found in non-oil plants such as tea, coffee, cacao, fruits, and vegetables. They are composed of tocopherols, flavonoids, anthocyanins, and phenolic acids; their fundamental structures includebenzene rings and multiple hydroxyl groups (Tran, 2021). These hydroxyl groups are strong hydrogen donors with high radical-scavenging activity, which help stabilize chemical structures and thereby protect against many forms of deterioration (Tran, 2021). Solvent extraction is one of the most common methods for obtaining antioxidants from plants (Tran, 2021). However, due to concerns over pollution and toxicity, green solvents (i.e., water, vinegar, deep eutectic solvent, and more) and pre-treatments (microwave, pulsed electric field, pulsed Ohmic heating, and ultrasound) have been studied to reduce the use of toxic solvents (Barba et al., 2015; Casazza et al., 2012; Darra et al., 2012; Rajha et al., 2014; Yu et al., 2014). In food products, antioxidants' main usage is as preservatives. They can reduce lipid oxidation-caused rancidity in oils, retain nutrients, and maintain the organoleptic quality and freshness of food products (Morita et al., 2017; Wilson et al., 2017). Feridoni et al., (2020) (2020) assessed microbial growth (total volatile base nitrogen) and shelf-life stability (organoleptic and sensory parameters) of home-made chicken nuggets with added sour tea extracts (*Hibiscus sabdariffa* L.) encapsulated with

carboxymethylcellulose nanoparticles. The extract was mixed with wheat flour at different ratios and used to coat the chicken nuggets. The samples were deep-fried twice and refrigerated at 4°C for 9 days. The study found that lipid oxidation and microbial growth were lower and increased more slowly in samples infused with tea extracts than in control samples, suggesting that this was due to the antioxidant activity of anthocyanins and other phenolic compounds. Radi et al., (2017) prepared edible films made from gelatine, aloe vera, and tea extracts (green and black tea) and coated orange slices. The coatings include 1% pure gelatine, 1% gelatine with 50% and 100% aloe vera, and 5% and 10% tea extract. The slices were stored at 4°C for 17 days and assessed for quality and microbial growth. The result showed that the aloe vera and green tea duo has strong antimicrobial properties, with a lower viable count than aloe vera and black tea. This was explained by the higher antioxidant content in green tea, whereas black tea's antioxidants were oxidized during processing. The study concluded that the combination of aloe vera and green tea extract can yield a preservative effect in food products. As for the mechanism of action, antioxidants can permeate the cell membranes of microorganisms and inactivate the functions of intracellular components, which eventually leads to cell death (Gutiérrez-Larraínzar et al., 2012). The antimicrobial activity is attributed to the presence of free hydroxyl groups, though it can be inactivated if a proton or electron is lost (Prindle & Wright, 1977). It is worth noting that the antimicrobial potency of antioxidants can vary depending on the plant source and mayot be as effective as EOS, which contains antioxidants and other antimicrobial fatty acids (Ouattara et al., 1997). For example, Pires et al., (2022) (2022) investigated the freshness of poultry products encased in chitosan films infused with 1% (v/v) of six different plant hydroalcoholic (HAE) extracts (rosemary, green tea, black tea, ginger, kenaf, and sage). The film (5 x 18 cm^2) was used to wrap fresh, ground poultry meat, and the samples were stored at 40 ± 20 °C for 15 days. Pure extract and film's inhibition against *B. cereus* (gram +) and *S. Enterica* (gram -), total plate count, and total Coliform were evaluated. While the counts were lower in test samples than in control samples, only Gram+ bacteria were inactivated in both pure extract and films. Chitosan-kenaf and chitosan-sage showed better antibacterial activity, while chitosan-rosemary exhibited excellent protection against oxidation. In general, herbs and spices such as rosemary and sage are recognized for their strong antimicrobial properties.

2.4.2. Natural Antimicrobial Polysaccharides in Biodegradable Food Packaging

It has been established that some polysaccharides possess antimicrobial and anti-inflammatory properties and may aid the immune system (Y. T. Li et al., 2018; Perinelli et al., 2018; Y. Yu et al., 2018). The addition of antimicrobial polysaccharides into biopolymer matrices is considered an innovative approach, as it can improve the gas barrier properties of the films while enhancing antimicrobial activities (Bertuzzi et al., 2007; Z. Wang et al., 2018). Chitosan, dextran, hyaluronic acid, cellulose, and fucoidan are common natural antimicrobial polysaccharides that can be extracted from plants, animals, and microbes (Xia et al., 2021). Among them, chitosan has been extensively studied for possessing good film-forming and antimicrobial properties (Z. Wang et al., 2021). Additionally, antimicrobial polysaccharides can also be grafted with polypeptides to enhance the potency (Xia et al., 2021).

Chitosan is a derivative of the second most abundant polysaccharide, chitin, produced through the process of deacetylation (Ahmed & Ikram, 2017). Chitin can be found in terrestrial arthropods, marine crustaceans, and fungi, suggesting the abundance of its derivatives (Ahmed & Ikram, 2017). Extraction of chitosan is often performed by chemical or enzymatic methods, with NaOH as the conventional solvent for deacetylation (Ahmed & Ikram, 2017). Chitosan has a similar structure to chitin, differing only in the replacement of the $NHCOCH_3$ group by an amino group (NH2) (Jin et al., 2021). Therefore, it shares some characteristics of chitin, such as being insoluble in water and organic solvents but soluble in acidic solutions (Ahmed & Ikram, 2017). Chitosan has good film-forming properties, chemical stability, and reactivity; therefore, it is a good ingredient for the fabrication of packaging films (Dutta, Tripathi, & Dutta, 2012; Lago et al., 2014; Mujtaba et al., 2019). In fact, chitosan-based biodegradable films have been reported to have adequate mechanical properties and reduced permeability to gases such as carbon dioxide and oxygen (Cazón et al., 2017). However, the hydrophilic nature of chitosan makes the films vulnerable to moisture, which can lead to loss of structural integrity over time due to the penetration of moisture and water vapour (Aider, 2010). Adjusting the deacetylation degree, pH, solvent, or plasticizers, crosslinking, and complexing with other biopolymers (polysaccharides and proteins) are some methods to overcome this issue (Dutta et al., 2009; Elsabee & Abdou, 2013). For example, Batista et al., (2019) (2019) prepared protein-based films from fish myofibrillar protein with 20%, 30%, and 40% (w/w) chitosan using the solvent-casting method. Test films

exhibited better mechanical properties, thermal stability, solubility, and UV blocking than control films, thanks to the formation of new hydrogen bonds between the protein chitosan, which further stabilized the structure.

Another interesting property of chitosan is its broad-spectrum antimicrobial characteristics. Chitosan was found to be effective against microbial and fungal cells but not against mammalian cells; therefore, numerous studies were conducted to understand its mode of action, enhance its effects, and explore its applications (Martinez-Camacho et al., 2011). Two working mechanisms for chitosan were suggested: inactivation via its amino group and via molecular weight. In the first method, the positively charged amine side chain of chitosan binds to negatively charged species in the bacterial cell membrane. It increases permeability, opening a floodgate for external substances to enter the cell, resulting in swelling and, eventually, cell death through leakage of intracellular contents (Martinez-Camacho et al., 2011). Chitosan can also trap trace metals, such as iron, copper, and zinc, that are involved in toxin production and microbial growth (A. P. Martínez-Camacho et al., 2011). The second method attributed antimicrobial activity to chitosan's ability to infiltrate bacterial cell membranes and interact with intracellular components, such as DNA, thereby inhibiting reproduction (A. P. Martínez-Camacho et al., 2011). The degree of deacetylation and molecular weight have a significant impact on the polymer. A higher degree of acetylation corresponds to more positively charged surfaces, which facilitate bonding, whereas a lower molecular weight enables better cell permeability (Martinez-Camacho et al., 2011). In chitosan-based films, parameters such as concentration, molecular weight, source of chitosan, microbial type, pH, temperature, and so on are deciding factors in the film's antimicrobial ability (J. Li & Zhuang, 2020; Riaz et al., 2021). The antimicrobial activity of chitosan is only effective at a minimal threshold. Perdones et al., (2012) (2012) prepared two types of coatings, chitosan (1% w/w) and chitosan (1% w/w)-lemon EOS (3% w/w), for strawberries inoculated with the mold *Botrytis cinerea* and stored at 5 $^{\circ}$C for 10 days. Chitosan-lemon EOS duo showed higher antifungal activity and succeeded in reducing the rate of respiration, whereas chitosan coating did not show any important result, which was explained by the synergistic effect between chitosan and the EOS. A higher concentration of chitosan was suggested to improve the antifungal properties of chitosan coatings. Modification of chitosan (i.e., acylation, alkylation, phosphorylation, sulfation, and so on), film's surface (i.e., plasma treatment), and addition of EOS are techniques used to improve film's performance (Goy et al., 2009; Kong et al., 2010, Moradi et al., 2020). Moradi et all., (2020).

(2020) used cold plasma to alter the surface of a chitosan-low-density polyethylene (LDPE) bilayer film infused with 1%, 2%, and 3% (v/v) of summer savory EOS (*Satureja hortensis*). The films were used to cover polypropylene dishes containing chicken fillets, and samples were refrigerated at (4 ± 1 °C) for 13 days. Physicochemical analyses revealed that modified films had higher water vapour barrier properties and a reduced rate of oxygen diffusion. Additionally, test films containing 3% EOS showed the highest inhibition against both *E. coli* (gram -) and *S. aureus* (gram +) bacteria, while 1% was the minimum threshold, inhibiting only *S. aureus*.

2.4.3. Antimicrobial peptides (AMPs) in Biodegradable Food Packaging

In the search for natural antimicrobial compounds, it was found that certain bioactive peptides can manipulate or inhibit the growth of microorganisms (Santos et al., 2018a). Plants, animals, and microbes produce themselves as part of their defense mechanisms against pathogens and diseases, or to eliminate competition in the case of microbes (Santos et al., 2018a). AMPs display broad-spectrum activity against a wide range of microorganisms and exhibit good thermal stability; therefore, their application across multiple fields has been studied extensively (Phambu et al., 2017). In the food industry, antimicrobial peptides are mainly used as preservatives. Whether AMPs are produced inherently or as a threat response, their availability in nature is often limited, which hinders the study of these compounds (Santos et al., 2018a). Various synthesis techniques, including chemical, enzymatic, and biosynthetic methods, have been developed to facilitate the manufacture of AMPs for research purposes(Santos et al., 2018). Bacterial AMPs, also known as bacteriocins, are the first AMPs to be studied and continue to gain attention to this date thanks to their pronounced efficacy (Santos et al., 2018a). Innovative applications of bacteriocins in the food industry include their incorporation into biopolymer matrices for active packaging to reduce pathogen growth and extend product shelf life (Santos et al., 2018).

Nisin is a bacteriocin produced by Lactic acid bacteria (LAB), such as *Lactococcus lactis*, through fermentation, and is categorized as GRAS by the United States FDA for use in food products (Dillon & Board, 1994). It is a derivative of lanthionine antibiotics and is composed of 34 amino acid molecules with 5 cyclic rings (Khan & Oh, 2016; Van Kraaij et al., 1999). Nisin is well known for its safety, good thermal stability, and antimicrobial activity, making it an ideal preservative for pasteurized or canned food products (Santos et al., 2018). It is the polycyclic structure of nisin that shields the molecules from being denatured by protease enzyme and heat, which prolongs the

compound's presence. Nisin microbial inactivation mode includes binding to the pyrophosphate group of lipid II to interfere with cell wall synthesis and forming pores in the membrane, causing cell death by leakage of cellular contents (Hasper et al., 2006). A majority of gram-positive bacteria can be inactivated through this mechanism; however, it has little to no effect on gram-negative bacteria (Khan & Oh, 2016). This is because gram-negative bacteria have an additional outer membrane that prevents nisin penetration into the inner membrane, where lipid II is located (L. Zhou et al., 2016). Thus, chelators such as EDTA or Tween 80 are used in combination with nisin to help break down the outer membrane (Natrajan & Sheldon, 2000). When infused into the packaging material, the release and diffusion rates of nisin are relevant for its antimicrobial activity (Gharsallaoui et al., 2015). Biopolymer plastic offers better release due to its lower barrier properties compared to those of synthetic petroleum plastic (Gharsallaoui et al., 2015). Regarding diffusion rate, it was found that the gradual release of nisin over time, rather than rapid liberation in excess, can elicit less resistance from bacteria while prolonging the antimicrobial effect (Chi-Zhang et al., 2004). Film's properties (constituents and fabrication method), food products' properties (moisture content and pH), and storage conditions (time and temperature) can significantly affect the release rate of nisin (Gharsallaoui et al., 2015). An encapsulation system of nisin in different compounds can protect the molecules from losing their antimicrobial activity when interacting with food products (Gharsallaoui et al., 2015). For example, Liu et al., (2022) (2022) encapsulated nisin in zein-modified starch nanoparticles and tested the encapsulated compound's activity against *L. monocytogenes* in fresh cheese refrigerated at 40 ^{o}C for 2 weeks. Lower counts of *L. monocytogenes* were found when encapsulated nisin was used as opposed to regular nisin, though the counts began to soar for both compounds after the third day of storage.

Chapter 3: Valorization of Canola Protein into Antimicrobial Packaging Films Enhanced with Carvacrol and Cinnamaldehyde

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3.1. Abstract

Canola protein is an abundant, plant-derived biopolymer with desirable biodegradability, making it an attractive candidate for environmentally friendly packaging solutions. Despite these advantages, its practical use is limited by poor functional performance, low moisture resistance, and the lack of antimicrobial activity, which is an increasingly important attribute in the future trend of packaging technologies. To overcome these limitations, essential oils such as carvacrol and cinnamaldehyde were incorporated into canola protein films to enhance both barrier functionality and antimicrobial performance. These essential oils were selected for their well-documented antimicrobial efficacy and hydrophobic nature in biopolymer matrices. Canola protein-based essential oil films were prepared at various oil concentrations (0%, 1%, 3%, and 5%) and oil blend ratios (0:0, 1:0, 0:1, 1:1, 1:2, 2:1, 1:4, and 4:1) with glycerol as a plasticizer. The films were evaluated for their mechanical properties, moisture barrier performance, thermal behavior, surface characteristics, and antimicrobial activity. The addition of carvacrol and cinnamaldehyde resulted in strong antimicrobial effects The addition of essential oils successfully imparted antimicrobial activity against *Escherichia coli (E. coli)* and *Staphylococcus aureus (S.*

aureus) and altered the mechanical properties, moisture resistance, morphology, and thermal stability of the films. The 3% oil concentration was found to be optimal, achieving good antimicrobial activity while having minimal negative effects on other film properties. These results suggest that incorporating essential oils into canola protein films is a promising strategy for developing active packaging materials with good antimicrobial afficacy against common food-borne pathogen.

Keywords

Canola protein; essential oils; carvacrol; cinnamaldehyde; antimicrobial; mechanical properties

Abbreviations

CPI, canola protein isolate, EOs, essential oils, CAR, carvacrol, CIN, cinnamaldehyde, NCC, nanocrystalline cellulose

Author Contribution

Funds acquisition: Bandara N.; Conceptualization: Bandara N., Dissanayake T., and Dang A. Methods: Dang A., Adibi A., and Bandara N; Analysis: Dang A., Adibi A.; Writing the first draft: Dang A; Editing and reviewing: Bandara N. and Dang A. Research Supervision: Mekonnen T., Narvaez-Bravo C. and Bandara N.; Student academic supervision: Bandara N.

3.2. Introduction

In an era where sustainability is at the forefront of many manufacturing operations, plastic food packaging becomes a major environmental risk due to its poor decomposability and high disposal rates (Siddiqui et al., 2023). Conventional plastics such as polyethylene, polypropylene, polystyrene, poly(vinyl chloride), and poly(ethylene terephthalate) have been heavily criticized for their role in environmental pollution. This has led to the search for sustainable alternatives, with biopolymers emerging as a promising solution. Biopolymers are large macromolecules derived from natural or synthetic sources and can be degraded through biological processes (Deshmukh et al., 2017; Singh et al., 2021). Natural biopolymers such as proteins, starches, cellulose, and lipids are preferred as plastic materials due to their high degradability and renewability, with suitable physicochemical properties for plastic production (Guo et al., 2021; S. I. Hong et al., 2021; Istiqomah et al., 2024b; Pal & Agarwal, 2024; Rojas-Lema et al., 2021; Sun et al., 2023). Proteins, in particular, have attracted significant interest due to their ability to form films with strong, flexible, and oxygen-barrier properties, making them a potential candidate for sustainable plastic alternatives.

A valuable source of protein for biopolymer-based plastic production is the protein-rich by-products from agro-industrial waste. Agricultural industries, such as those involved in processing soy, corn, canola, wheat, and rice, generate substantial quantities of waste materials like oilseed meals, bran, and husks, which are often underutilized (Arzami et al., 2022; Getahun et al., 2024). These by-products contain valuable proteins that can be extracted and repurposed for the production of bioplastics. Canola *(Brassica napus L.)*, a widely cultivated oilseed crop, produces a large amount of protein-rich meal (36-39% protein on a wet basis) as a by-product of oil extraction (Evans et al., 2019). Between 2023 and 2024, global production of canola reached 89.33 MMT, yielding 34.31 MMT of oil and approximately 49.13 MMT of meal residue (United States Department of Agriculture, 2024c, 2024b, 2024a). This makes canola meal a good source of protein. On the other hand, an extensive review highlighted the potential use of canola protein for the manufacture of plastics(Bandara et al., 2018; Y. Zhang et al., 2018). Canola protein contains two major storage proteins, cruciferin and napin (Chang & Nickerson, 2014). Cruciferin, a 12S globulin, has a high molecular weight (300-310 kDa) and consists of multiple subunits, while napin, a 2S albumin, has a lower molecular weight (ranging from 12.5 to 14.5 kDa) (Aider & Barbana, 2011). The balanced composition of these proteins, with differing molecular weights,

enhances the film's properties, as cruciferin improves the mechanical strength of the matrix, and napin acts as a plasticizer, facilitating better flow and processability of the protein system (S. Li, Donner, et al., 2024). However, further enhancements are needed for canola protein-based plastics to function like conventional plastics, particularly for food packaging applications (Y. Zhang et al., 2018). Research efforts have focused on improving the properties of canola films through various techniques, including protein modification (Fetzer et al., 2021; Manamperi et al., 2010; Manamperi & Pryor, 2012), plasticization (Chang & Nickerson, 2014, 2015; Shi & Dumont, 2014), crosslinking (Chang & Nickerson, 2014; S. Li, Donner, et al., 2024; S. Li et al., 2017) and adding reinforcements such as cellulose fibers (S. Li et al., 2019) and nanomaterials (Chairez-Jimenez et al., 2024; Dissanayake et al., 2022; Osorio-Ruiz et al., 2019). Additionally, Li et al. (2019) successfully fabricated canola films using compression molding, demonstrating the potential for scale-up of canola-based plastics production from laboratory settings to an industrial level.

Many of these studies also focused on enhancing the moisture-barrier properties of canola films, which naturally have a high affinity for water. The addition of hydrophobic materials, such as plant-based essential oils (EOs), has shown considerable potential. EOs are secondary metabolites derived from various plant parts that protect against a range of microbial diseases (Ribeiro-Santos et al., 2017). Known for their strong hydrophobic properties and safety, EOs have been widely studied for their use in biopolymer-derived films (Asgher et al., 2020; Díez-Pascual & Cinelli, 2019; Hashim et al., 2021; Jamróz & Kopel, 2020; Muñoz-Gimena et al., 2023; Ribeiro-Santos et al., 2017; Zubair et al., 2022). Incorporating EOs into biopolymer-based films is a strategic approach to improve not only the film's physicochemical but also the antimicrobial properties (Zubair et al., 2022).

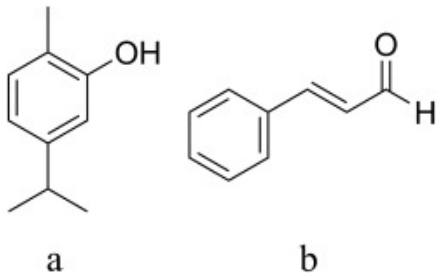

Figure 3.1: Carvacrol and cinnamaldehyde molecules. (a) Chemical structures of carvacrol and (b) cinnamaldehyde (Otoni et al., 2016).

EOs consist of a blend of more than 300 compounds (Bhavaniramya et al., 2019), including phenols, aldehydes, ketones, alcohols, esters, ethers, and hydrocarbons (Dhifi et al., 2016). Typically, two or three major components account for almost 20-70% of the oil's composition, and are key factors influencing the biological properties of EOs (Bakkali et al., 2008). Carvacrol (2-methyl-5-(1-methylethyl)-phenol) and cinnamaldehyde (3-phenyl-2-propenal) are major compounds found in common spices such as thyme, oregano, and cinnamon (Bhavaniramya et al., 2019). Carvacrol is a monoterpenoid phenol, while cinnamaldehyde is a terpene aldehyde; both are recognized as safe and are permissible to be used in food products by the U.S. Food and Drug Administration as well as the European Commission (European Commission, 2002; U.S. Food & Drug Administration, 2008). They are known for their potent antimicrobial activities against common foodborne bacteria, including Staphylococcus aureus, Escherichia (Bassolé & Juliani, 2012; Çakmak et al., 2020; Ed-Dra et al., 2021)al., 2020; Ed-Dra et al., 2021). When used in combination, carvacrol and cinnamaldehyde exhibit an additive effect, enhancing antimicrobial efficacy and lowering the required dosage compared to their individual application (Pei et al., 2009b). The duo also contains functional groups with antioxidant activity, which help prevent lipid oxidation. Therefore, the addition of EOs into biopolymer-based packaging is beneficial for inhibiting bacterial growth and preserving food products. In addition, EOs have been found to affect the mechanical properties and moisture barrier of biopolymer-based plastic (Tao et al., 2022a). Films incorporating EOs exhibit lower strength, greaterlexibility, and greater resistance to water vapour (Cai & Wang, 2021; Laorenza & Harnkarnsujarit, 2021b; Sung et al., 2014).

Although more progress has been made to improve the functionality of canola protein-based films, most existing studies primarily focus on mechanical and barrier enhancement through strategies such as plasticization, crosslinking, polymer blending and nanocomposite incorporation (Chairez-Jimenez et al., 2024; Chang & Nickerson, 2014; Dissanayake et al., 2022, Dissanayake et al., 2023; Li et al., 2017, Li et al., 2019, Li et al., 2024; Liu et al., 2025; Osorio-Ruiz et al., 2019). However, these approaches often enhance certain properties while also compromise others. Additionally, the antimicrobial functionality of canola protein-based bioplastics also remains largely unexplored (Bandara et al., 2018). Therefore, there is a need to develop systems that improve both the mechanical performance and functional activity of these materials. The incorporation of essential oils such as carvacrol and cinnamaldehyde can impart antimicrobial activity against foodborne pathogens while also improving film flexibility and reducing moisture sensitivity. However, the

use of high concentrations of essential oils may adversely affect mechanical strength and barrier properties. Consequently, combining antimicrobial agents may provide synergistic antimicrobial effects, enabling the use of lower concentrations of each compound while maintaining antimicrobial efficacy (Bassolé & Juliani, 2012; Ju et al., 2022). This approach may help minimize adverse impacts on the physicochemical properties of canola protein-based films, thereby enhancing their potential application in active food packaging systems.

In this study, canola protein-based films were prepared with different concentrations of carvacrol and cinnamaldehyde (0%, 1%, 3%, and 5%) at different ratios (0:0, 1:0, 0:1, 1:1, 1:2, 2:1, 1:4, and 4:1) using the solvent-casting method. Antimicrobial assay was conducted to assess the efficacy of the EOs against *E. coli* (ATCC 15597) and *S. aureus* (ATCC 25923). The objective of this study was to explore the potential to develop active canola protein-based films infused with carvacrol and cinnamaldehyde and to evaluate their influence on the films' properties. Specifically, this study investigates whether the incorporation and combination of these natural antimicrobial compounds can enhance the functional performance of canola protein-based films while also promote or minimize potential adverse impacts on their physicochemical characteristics.

3.3. Materials and Methods

3.3.1. Materials and chemicals

Canola meal was provided from Richardson International (Lethbridge, AB, Canada). NCC (off-white powder, moisture content 0–5 %, particle size <230 nm, crystallinity >70, surface charge <−31 mV) were provided by Richardson International (Lethbridge, AB, Canada) and InnoTech Alberta (Edmonton, AB, Canada), respectively, for research purposes. Carvacrol (purity > 98%, CAS: 499-75-2, Sigma-Aldrich, Darmstadt, Germany), trans-cinnamaldehyde (purity > 99%, CAS: 104-55-2, Sigma-Aldrich, Darmstadt, Germany), MacConkey agar, Manitol Salt agar, and Mueller-Hinton agar were purchased from Thermo Fisher Scientific (Oxford brand). *S. aureus* (serovar ATCC 25923) and *E. coli* (serovar ATCC 15597) were obtained from the culture collection of the Department of Food and Human Nutritional Sciences, University of Manitoba (Manitoba, Canada).

3.3.2. Canola protein extraction

Canola protein was extracted from defatted canola meal using the method outlined by Manamperi et al. (2010) and Dissanayake et al. (2023), with some modifications. Defatted canola meal was combined with deionized water at a 1:10 (w/v) ratio. The pH of the resulting mixture was adjusted to 12 with 3M NaOH, and the mixture was stirred at 300 rpm at room temperature for 30 minutes to solubilize the proteins. The suspension was then centrifuged at 7000 rpm for 20 minutes at 4 °C, and the supernatant containing the solubilized protein fraction was collected. The pH of the supernatant was adjusted to 4 with 3 M HCl to precipitate the protein, followed by centrifugation at 10,000 rpm for 20 minutes at 4 °C. The final product was freeze-dried and stored at -20 °C for further use.

3.3.3. Fabrication of canola protein films with carvacrol and cinnamaldehyde

Canola protein films were fabricated using the solvent-casting method, adapted from Agarwal et al. (2020) and Dissanayake et al. (2023) with some modifications. To prepare the film-forming solution, 3.5 g of canola protein isolate (5% w/v of the final volume) was dispersed in 50 mL of distilled water, and the pH was adjusted to 10 using 1.0 M NaOH. The mixture was stirred at 650 rpm for 30 minutes at room temperature, then heated in a water bath (VWR International, Cornelius, OR, USA) at 70 °C with continuous stirring at 70 rpm for 20 minutes. After cooling to room temperature, glycerol was incorporated as a plasticizer at 50% (w/w of protein). The dispersion was homogenized using a Fisherbrand 850 homogenizer (Fisher Scientific, ON, Canada) at 14,000 rpm for 5 minutes. Nanocrystalline cellulose (NCC) was introduced at 5% (w/w of protein), as this concentration yielded the best result according to Dissanayake et al. (2023). NCC was first dispersed in 20 mL of deionized water, sonicated for 2 minutes (60% amplitude, 20 kHz frequency, and 500 W power output), then added to the canola protein/glycerol mixture, followed by homogenization at 14,000 rpm for 5 minutes. For the films containing essential oils, CIN and CAR were added at different concentrations (0%, 1%, 3% and 5%, v/v of final volume) and ratios (0:0, 1:0, 0:1, 1:1, 1:2, 2:1, 1:4, and 4:1, v/v) and homogenized at 14,000 rpm for 5 minutes. A final sonication step was performed for 5 minutes under the same conditions to ensure uniform dispersion. The prepared film-forming solution was cast onto hexagonal polystyrene dishes at a casting volume of 0.43 mL/cm² and allowed to air-dry for 48 hours. Once dried, the films were removed and conditioned at 23 °C and 50% relative humidity for 48 hours before undergoing characterizations.

3.3.4. Preparation of bacterial cultures

Bacterial cultures were prepared with slight modifications (Hatab et al., 2016). *S. aureus* (serovar ATCC 25923) and *E. coli* (serovar ATCC 15597) cultures were maintained in Trypticase Soy broth, TSB (Difco, Becton Dickinson Co., Franklin Lakes, N.J., USA) containing 15% glycerol, and stored at -80 °C. *S. aureus S. aureus* strain was cultured on Mannitol Salt Agar, MSA (Thermo Scientific™ Oxoid™), while *E. coli* strain was cultured on MacConkey Agar (MAC; Thermo Scientific™ Oxoid™) plates at 37 °C for 24 hours. The bacterial suspensions were prepared by transferring 2 to 4 bacterial colonies from MSA and MAC to Mueller-Hinton broth (MHB, Oxoid Ltd., Basingstoke, U.K.).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined prior to disk diffusion assay to confirm the efficacy of carvacrol and cinnamaldehyde (see Appendix: Study 1 Supplementary Materials section, table S1 for data). The assay was performed according to the methods of Eloff (1998) and Hatab et al. (2016), with slight modifications. To prepare the bacterial inoculum, *S. aureus* and *E. coli* suspensions were adjusted to a final concentration of 10^8 CFU/mL in MHB by comparing their turbidity to that of a 0.5 McFarland Standard (Remel™, Thermo Fisher Scientific, Waltham, Mass., USA). The minimum concentration of Tween 80 required to stabilize EOs in MHB was determined by the absence of visible separation within 24 hours (Hatab et al., 2016). A final concentration of 6% (v/v) Tween 80 was selected as preliminary tests confirmed it had no inhibitory effect on bacterial growth. To determine the MIC and MBC, 100 µL of sterile MHB supplemented with 6% Tween 80 was transferred to the wells. Further, a serial dilution was performed by adding 50 µL of undiluted carvacrol or cinnamaldehyde to the first row of a 96-well microplate. The contents were mixed by pipetting, and 100 µL was transferred from the first row to the second row. This step was then repeated sequentially from row 2 to row 12, generating a series of wells with progressively decreasing essential oil concentrations. Finally, 20 µL of standardized bacterial inoculum (10^8 CFU/mL) was added to each well. All treatments were prepared in triplicate. Each plate has a negative control column containing sterile MHB with 6% Tween 80, and a positive control column containing sterile MHB with 6% Tween 80 and bacteria without essential oils. The plates were sealed with parafilm (Bemis Co. Inc., Neenah, WI, USA) and incubated at 37 °C for 24 hours. Following incubation, 15 µL of p-iodonitrotetrazolium chloride (INT; 0.2 mg/mL in 70% ethanol) was added to each well and further incubated at 37 °C for 1-2 hours to allow for color development.

Pink wells indicated active bacterial metabolism, whereas yellow wells indicated inhibition. The MIC was defined as the lowest essential oil concentration that did not cause a visible color change. To determine the MBC, 5 µL from each yellow-stained well was plated on MHA and incubated at 37 °C for 24 hours. The MBC was defined as the lowest essential oil concentration at which no visible colonies formed, indicating complete bactericidal activity. Plating was performed in duplicates to ensure reproducibility.

3.4. Characterization of canola-EOs films

3.4.1. Mechanical properties

Tensile strength (TS), Young's modulus (YM), and elongation percentage at break (EB) of the canola protein films were measured using Shimadzu AGS-X Universal Tester (Shimadzu America INC, Columbia, MD, USA) in accordance with the ASTM D882 (ASTM D882-18, 2018) standards. Film samples, after conditioning for 48 hours at 23°C and 50% RH, were precisely cut to dimensions of 70 mm (length) x 10 mm (width) and deformed under a tensile load of 500N at a crosshead speed of 100 mm/min until fracture. All samples were tested in quadruplicates, and the results for TS (MPa), YM (MPa), and EB (%) were reported as mean ± SD (standard deviation).

3.4.2. Water Vapour Permeability (WVP)

Moisture barrier properties of the films were assessed gravimetrically following the ASTM E96/E96M-16 method, with slight modifications (ASTM E96/E96M-16, 2016). Cylindrical cups (4.24 cm diameter and 4.02 cm height) were partially filled with distilled water up to ¾ of the volume. Circular film samples were carefully placed over the cup openings and sealed with glue. The combined weight of the cup, water, and film was recorded before placing the setup in a controlled chamber maintained at 50% relative humidity (RH) and 23 °C. The system's weight was measured at 2-hour intervals. Prior to testing, all film samples were preconditioned for 48 hours under the same temperature and humidity conditions. WVP of the films was calculated using the following equation:

$$WVP = \frac{Weight\ change\ (g)\ \times\ thickness\ of\ the\ film\ (mm)}{Area\ of\ the\ cup\ mouth\ (m^2)\ \times\ vapor\ pressure\ difference\ (kPa)\ \times\ time\ (hours)}$$

3.4.3. Water Solubility (WS)

Small film samples (2 cm × 2 cm) were initially dried in an oven at 105 °C for 24 hours to determine their initial dry weight (*W* initial weight - *W* first dried weight). Following this, the samples were submerged in distilled water at 25 °C for 24 hours, continuously stirred in a water bath. After soaking, the films were dried again under the same oven conditions to obtain their final dry weight (*W* first dried weight - *W* second dried weight). The water solubility of the films was then calculated using the following equation:

$$Water\ solubility\ \% = \left(\frac{W_{initial\ dry\ weight} - W_{final\ dry\ weight}}{W_{initial\ dry\ weight}} \right) \times 100$$

3.4.5. Water contact angle (WCA)

The surface wettability of canola protein films and distilled water was examined using a contact angle goniometer (Model No. 200-00-115, Rame-Hart Inc., Mountain Lake, NJ, USA) and DROPimage Standard software. For each sample, 10 measurements were taken at 0.01 seconds intervals, and the average of the acceptable angles was used for analysis.

3.4.6. Differential Scanning Calorimetry (DSC)

DSC analysis was conducted on canola protein powder and film samples to evaluate their thermal properties using a DSC 2500 (TA Instruments, New Castle, DE, USA). Samples (~5-10 mg) in T-zero pans will be heated from 5 to 350 °C at a rate of 10 °C/min. A temperature (°C) vs heat flow (mW) graph was used to analyze the thermal properties. Graphs were obtained from Origin Pro 2025 software.

3.4.7. Scanning Electron Microscopy (SEM)

The microstructure of the canola films was observed using a nanoscale scanning electron microscope (Quanta 650 FEG, FEI Company, Hillsboro, OR, USA) at an accelerating voltage of 10.0 kV. Prior to analysis, the films were cut to 1 cm × 1 cm and coated with 60% gold (Au) and 40% lead (Pd) using a Denton vacuum to facilitate electron conductivity. Film surfaces were observed at ×1000 magnification.

3.4.8. Fourier transform infrared spectroscopy (FTIR)

Canola protein-PVA composite films were characterized using Attenuated Total Reflectance-Fourier transformed infrared spectroscopy (ATR-FTIR) to identify functional groups and assess

molecular interactions within the film matrix. Spectral data were collected over the range of 400-4000 cm^{-1} with a resolution of 4 cm^{-1} for 120 scans using a spectrometer (Invenio S, Bruker, Leipzig, Germany). The IR spectra were analyzed and graphed using OriginPro 2025 software (OriginLab Corporation, Northampton, MA, USA).

3.4.9. Disk diffusion assay

The disk diffusion assay was performed according to Dissanayake et al. (2022) with minor modifications. Prior to testing, the total plate count was conducted on canola protein to assess microbial load, and packaging films were Ultraviolet (UV) treated for 15 minutes to ensure sterility and to avoid potential interference in the disk diffusion assay. *E. coli* and *S. aureus* stock solutions (10^8 CFU/mL) were streaked onto MacConkey and Mannitol Salt agar plates, respectively, and incubated at 37 °C for 24 hours. Film samples were cut into 5 mm circular discs, placed at the center of the Mueller-Hinton agar plates, and incubated at 37 °C for 24 hours. The diameter of the inhibition zones (cm) was then measured using a caliper.

3.4.10. Statistical Analysis

A completely randomized design (CRD) with a two-factorial arrangement was used. The two factors considered were the concentration and ratio of carvacrol and cinnamaldehyde, with four levels (0%, 1%, 3%, and 5%) and eight levels (0:0, 0:1, 1:0, 1:1, 1:2, 2:1, 1:4, 4:1), respectively. The samples were analyzed in triplicate using Minitab 2024 (Minitab LLC, State College, Pennsylvania, USA), and figures and illustrations were created using Origin Pro 2025 (OriginLab Corporation, Northampton, MA, USA). A two-way ANOVA (analysis of variance) was conducted to evaluate the main effects of oil concentration and oil proportion, as well as their interaction on the dependent variables. Additionally, a one-way ANOVA was conducted to compare the control group with the 3% oil group that yielded the best overall result. Mean comparisons were carried out using Tukey's test. All treatments were conducted in triplicate, and data were presented as mean ± standard deviation (SD).

3.5. Results and Discussion

3.5.1. Mechanical properties analysis of canola protein-EOs composite film

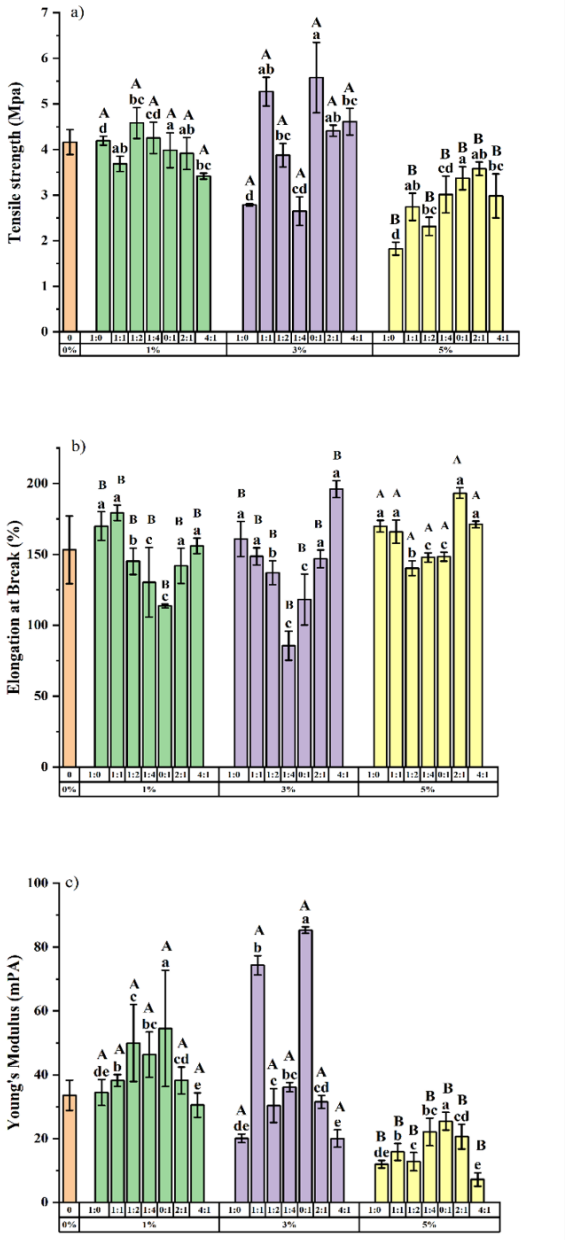

Figure 3.2: a) TS (MPa) b) EAB (%), and c) YM (MPa) of canola protein films with 0%, 1%, 3% and 5% (v/v) of carvacrol (CAR) and cinnamaldehyde (CIN) at 0:0, 1:0, 0:1, 1:2, 1:4, 2:1, and 4:1 ratios. All the values were reported as mean ± SD, and five specimens per film were tested. Uppercase letters indicate statistical differences among oil concentrations; lowercase letters indicate statistical differences among oil ratios.

Film thickness ranged from 0.29 mm to 0.45 mm, exhibiting a positive correlation with increasing EOs concentrations and aligning with previous research findings (Agarwal et al., 2020; Dhumal et al., 2019; Papadaki et al., 2024; Y. Zhou et al., 2021). This effect is likely due to the integration of oil droplets within the protein matrix during homogenization (Noshirvani et al., 2017; Y. Zhou et al., 2021).

Mechanical properties are crucial for assessing the performance of packaging materials, which directly impact their potential applications in food packaging, and are measured as TS, EAB, and YM. The resulting increase in solid content can promote more chemical interactions, potentially leading to higher force-at-break values (Chu et al., 2020; Cuq et al., 1996). Figure 2. illustrates the TS (MPa), EAB (%), and YM (MPa) values of films at 50% RH and 23 ºC. The control film exhibited a TS of 4.16 ± 0.27 MPa, a YM of 153.24 ± 23.86 MPa, and an EAB of 33.60 ± 4.73 %. Variations in oil concentration, oil ratios, and their interactions significantly affected the mechanical properties of canola protein films ($p < 0.01$). Notably, EAB exhibited an inverse relationship with both TS and YM. The lack of a consistent trend across all parameters could be attributed to the influence of both the oil blend concentration and the different fractions used. At 3% oil, various oil ratios slightly improved TS and YM values compared with pure canola films ($p < 0.05$).

Previous studies have reported different trends regarding the impact of EOs on the mechanical properties of protein films. For example, Amjadi et al. (2021), Mohammadi et al. (2020), and Xu et al. (2019) observed an inverse relationship between TS and EAB upon the addition of EOs, while Kaur & Santhiya (2021), Sajimon et al. (2023), and Wang et al. (2023) found a positive correlation. Higher TS and YM values, coupled with lower EAB values, could indicate a more reinforced structure, possibly facilitated by the crosslinking of the matrix components and the EOs (Choi et al., 2018; Lamp et al., 2022).

CIN is capable of forming a crosslink with canola protein through Schiff base reaction (Cao et al., 2022; Chen et al., 2017; Higueras et al., 2015), where the carbonyl (C=O) in the aldehyde group of CIN reacts with the amino group (N-H) in protein (Chen et al., 2018). Additionally, the formation of hydrogen and covalent bonds from the phenolic hydroxyl groups in CAR and NCC effectively reinforces and anchors the film's structure (Dissanayake et al., 2023; Lahmar et al., 2018; Nisar et al., 2018). However, at higher oil concentrations, an opposing trend is typically

observed, in which TS decreases, and EAB increases due to the plasticizing effect of EOs. This occurred as strong intermolecular protein-protein interactions (covalent bonds, hydrogen bonds, hydrophobic interactions, and electrostatic forces) were replaced by weaker protein-oil interactions (J. H. Kim et al., 2016; Papadaki et al., 2024). Protein-oil interactions primarily involve non-covalent bonding, acting as a plasticizer between protein chains, reducing friction and enhancing chain mobility (Lamp et al., 2022). The increase in mobility allows the chains to move more freely under stress, resulting in more flexible films (Lamp et al., 2022). In this study, films with 5% oil incorporation showed significantly lower YM values than control films, indicating greater flexibility but a corresponding reduction in TS. Furthermore, visible oil leakage was observed, suggesting excessive oil addition and saturation of binding sites, ultimately compromising the structural integrity and mechanical performance. The varying effects of EOs on protein films can be attributed to their distinct physicochemical properties, including physical state, viscosity, solubility, hydrophobicity, and oxidation potential (A. Devi & Khatkar, 2016; Laorenza & Harnkarnsujarit, 2021c).

In addition to incorporating individual oils, blends of these compounds have been used to produce active biopolymer-based packaging films with enhanced antimicrobial properties. For example, Pires et al. (2013) investigated the effects of different EOs, including citronella, tarragon, thyme, and coriander, on hake protein films. Their findings indicated that while TS ($p > 0.05$) remained largely unchanged, films containing coriander or citronella oil exhibited significantly higher elongation at break ($p < 0.05$). Additionally, films containing tarragon or coriander oils exhibited greater elasticity compared to those containing thyme oil. The observed increase in EAB in films containing citronella was attributed to the crosslinking of protein chains facilitated by β-citronella, along with the presence of Δ^3 -carene in the oil (Pires et al., 2013).

The rationale for using EO blends is primarily to achieve a synergistic antimicrobial effect against a wide spectrum of bacteria and fungi in food, rather than using a single oil (Ju et al., 2022). However, research on the impact of oil blend composition and proportion on canola protein films remains limited. Furthermore, the extent to which variations in oil fractions influence mechanical properties is not well understood. A deeper understanding of EOs concentrations and their interactions is essential for optimizing the mechanical performance and antimicrobial effectiveness of canola protein-based films.

3.5.2. FTIR characterization of canola protein-EOs composite films

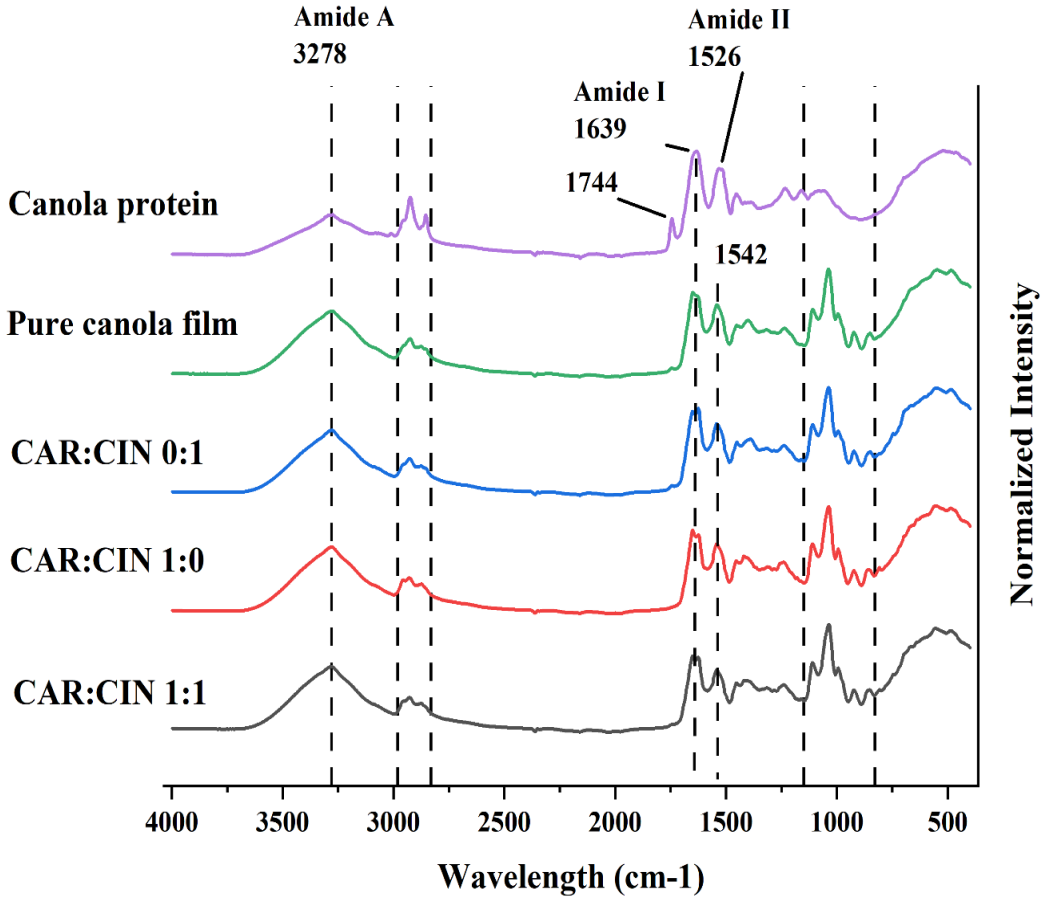

Figure 3.3: FTIR spectra of CPI powder and canola protein films with 0% and 3% (v/v) of carvacrol (CAR) and cinnamaldehyde (CIN) at CAR:CIN 0:1, 1:0 and 1:1 ratios.

FTIR analysis was used to identify functional groups in the film matrix and to assess structural modifications resulting from component interactions. Canola protein contains a diverse range of functional groups that contribute to its structural and chemical properties, influencing its film-forming ability and interactions with incorporated compounds. The presence of amide groups (-CONH-), primarily in peptide bonds, is a key feature of the protein structure, with characteristic Amide I (1600-1800 cm^{-1}, C=O stretching vibrations), Amide II (1470-1570 cm^{-1}, N-H bending vibration and C-N stretching vibration), and Amide A (3300-3500 cm^{-1}, stretching vibrations of O-H) bands in FTIR spectra (Ji et al., 2020; S. Li et al., 2017; Stani et al., 2020). Under this context, the absorption peaks of Amide I are identified at 1633 cm^{-1}, Amide II at 1533 cm^{-1}, and Amide A at 3280 cm^{-1} (Figure 3). A distinct absorption peak was observed at 1744 cm^{-1}, corresponding to

the C=O stretching of the ester carbonyl group (Weal et al., 2024), likely coming from residual canola oil that was not completely removed during defatting and extraction. Additionally, the cluster of peaks spanning 2850 cm^{-1} to 2980 cm^{-1} corresponds to the asymmetric and symmetric stretching of C-H groups on proteins (S. Li et al., 2017; C. Pires et al., 2013).

Changes in peak characteristics, such as shifts or the disappearance of specific bands, indicate molecular interactions among the film's constituents (Raza et al., 2021). In this study, the FTIR spectra of the sample films exhibited characteristic peaks similar to those of canola protein powder, with slight variations. Notably, all films showed an increase in the intensity of the Amide A region, likely due to the introduction of additional O-H groups from glycerol, NCC, carvacrol, and cinnamaldehyde. This increase, coupled with a reduction in intensity within the 2850–2980 cm^{-1} region, which corresponds to C-H stretching vibrations from hydrophobic interactions, suggests an overall enhancement in film hydrophilicity (Tao et al., 2022).

In the spectral range of 1600-1700 cm^{-1}, characteristic peaks associated with the aromatic C=C or C=O bonds are reported for cinnamaldehyde and carvacrol (Canales et al., 2019). The addition of EOs likely led to peak splitting at 1639 cm^{-1}, indicating possible interactions between the EOs and canola protein (Karabasannavar et al., 2017). In the Amide II region, a shift from 1526 cm^{-1} to 1542 cm^{-1}, accompanied by a slight reduction in peak intensity, likely reflects interactions between amino groups in canola protein and NCC, glycerol, carvacrol, and cinnamaldehyde. Additionally, the emergence of a new cluster of peaks between 800 and 1100 cm^{-1}, corresponding to C–C and C–O stretching vibrations of glycerol and the C–O bonds of NCC, suggests successful incorporation of these components into the protein matrix (Acquah et al., 2020; Calderón-Vergara et al., 2020; Dissanayake et al., 2022).

3.5.3. Moisture barrier properties of canola protein-PVA composite films

3.5.3.1. Water solubility

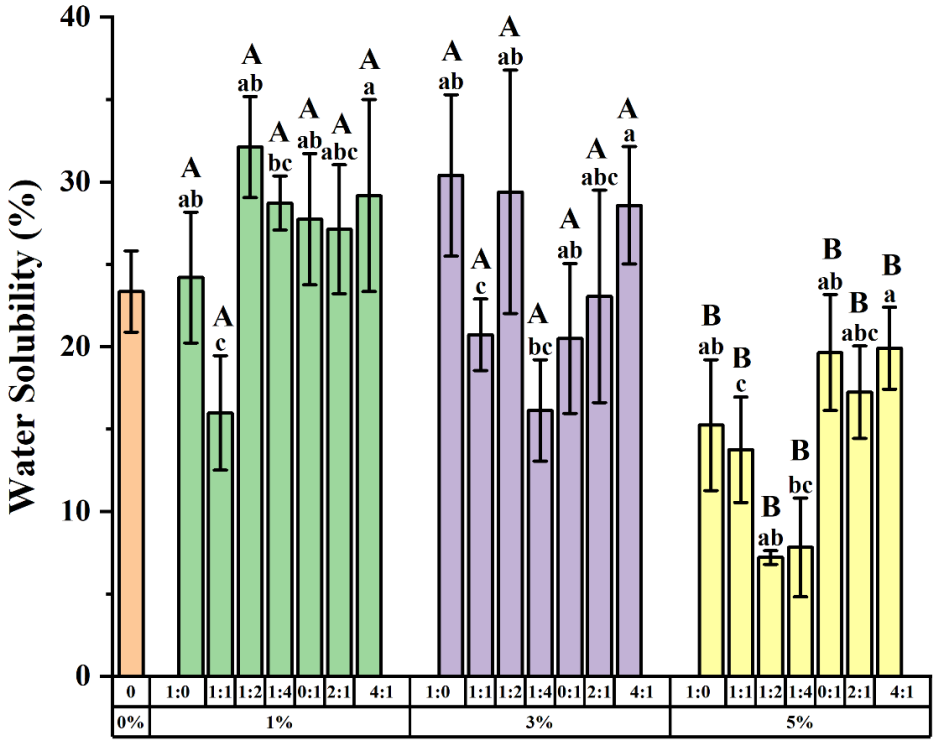

Figure 3.4: Water solubility (%) canola protein films prepared with 0%, 1%, 3% and 5% (v/v) of CAR and CIN at CAR: CIN 0:0, 1:0, 0:1, 1:2, 1:4, 2:1, and 4:1 ratios. Uppercase letters indicate statistical differences among oil concentrations; lowercase letters indicate statistical differences among oil ratios.

Determining water solubility is crucial for understanding material's water resistance and biodegradability (Haghighatpanah et al., 2022). In this study, the WS of the control film was measured at 23.36 ± 2.47 % while the WS of the experimental films ranged from 7.22 ± 0.41 % to 32.13 ± 3.07 %. The water solubility of canola protein films was significantly influenced by the incorporation of CIN and CAR at different concentrations and ratios ($p<0.05$). However, no significant difference was detected between the 3% essential oil films and the control ($p > 0.05$). At 1% and 3% EOs, WS values ranged from 15.98 ± 3.48 % to 32.13 ± 3.07 %, with no significant difference between these concentrations. However, at 5% EOs, WS decreased significantly (7.22 ± 0.41 % to 19.92 ± 2.48 %). The lack of differences at lower EOs concentrations may be attributed to their plasticizing effect as EOs molecules can disrupt protein aggregation, forming a looser matrix that facilitates water penetration (Do Socorro Rocha Bastos et al., 2016). This structural

modification increases the availability of hydrophilic groups, thereby enhancing water affinity. Similar findings were reported in whey protein-based films, where EOs incorporation led to higher WS due to limited EOs solubility and the formation of micropores that promote water absorption (Amjadi et al., 2021; Socaciu et al., 2020).

At 5% EOs concentration, the reduction in water solubility can be attributed to the predominance of hydrophobic interactions between EOs molecules and the protein network, which decreases the matrix's affinity for water. The oil may also act as a hydrophobic barrier, further limiting water penetration and solubilization. Similar tendencies have been reported in previous studies (Asyila Marzlan et al., 2022; Cheng et al., 2021; Kurtfaki & Yildirim-Yalcin, 2023; Wang et al., 2022). Liu et al., (2024) added thyme essential oil to soy protein films at different amount (0.6, 1.2, 1.8, and 2.4g/100g film-forming solution) and noticed a reduction in WS with increasing oil concentration due to EOs limiting the formation of hydrophilic bonds between water molecules and OH group in the matrices (Vilas Dhumal et al., 2019; Wang et al., 2022). Additionally, although different CAR: CIN ratios resulted in minor variations within each EOs concentration, no distinct trend emerged, indicating that total EOs concentration played a more dominant role in modulating water solubility than the specific EOs ratios.

3.5.3.2. Water vapour permeability

Table 3.1: Water vapour permeability (g mm/m^2 kPa h), water solubility (%), and water contact angle (^{o}) of canola protein films with different concentrations and proportions of carvacrol and cinnamaldehyde.

Sample(s)	Oil ratio CAR: CIN	WVP (g mm/m^2 kPa h)	WS (%)	Water contact angle (^{o})
Control, 0% oil	0	0.09 ± 0.01	23.36 ± 2.47	34.39 ± 6.98
1% oil	1:0	0.07 ± 0.00^{ABab}	24.21 ± 3.96^{Aab}	38.64 ± 2.78^{Abc}
	1:1	0.08 ± 0.01 ^{ABab}	15.98 ± 3.48^{Ac}	41.36 ± 2.24 ^{Aab}
	1:2	0.08 ± 0.00 ^{ABa}	32.13 ± 3.07^{Aab}	41.53 ± 6.34 ^{Aa}
	1:4	0.08 ± 0.01 ^{ABab}	28.73 ± 1.64^{Abc}	38.28 ± 2.62 ^{Ac}
	0:1	0.08 ± 0.01 ^{ABab}	27.74 ± 3.99^{Aab}	62.57 ± 0.32 ^{Aa}

	2:1	0.07 ± 0.01^{ABab}	27.13 ± 3.91^{Aabc}	46.27 ± 3.11^{Aabc}
	4:1	0.07 ± 0.01^{ABb}	29.18 ± 5.82^{Aa}	46.54 ± 1.47^{Aab}
3% oil	1:0	0.08 ± 0.01^{Bab}	30.40 ± 4.90^{Aab}	35.28 ± 2.25^{Cbc}
	1:1	0.07 ± 0.00^{Bab}	20.72 ± 2.17^{Ac}	37.40 ± 1.89^{Cab}
	1:2	0.07 ± 0.00^{Ba}	29.41 ± 7.39^{Aab}	44.65 ± 3.06^{Ca}
	1:4	0.08 ± 0.00^{Bab}	16.14 ± 3.07^{Abc}	23.16 ± 0.01^{Cc}
	0:1	0.07 ± 0.01^{Bab}	20.50 ± 4.56^{Aab}	29.62 ± 2.93^{Ca}
	2:1	0.07 ± 0.01^{Bab}	23.06 ± 6.44^{Aabc}	31.08 ± 2.33^{Cabc}
	4:1	0.07 ± 0.00^{Bb}	28.59 ± 3.55^{Aa}	29.93 ± 4.62^{Cab}
5% oil	1:0	0.08 ± 0.01^{Aab}	15.25 ± 3.97^{Bab}	34.98 ± 1.85^{Bbc}
	1:1	0.08 ± 0.00^{Aab}	13.75 ± 3.20^{Bc}	42.04 ± 1.45^{Bab}
	1:2	0.10 ± 0.05^{Aa}	7.22 ± 0.41^{Bab}	39.54 ± 4.19^{Ba}
	1:4	0.07 ± 0.01^{Aab}	7.82 ± 3.00^{Bbc}	40.20 ± 2.07^{Bc}
	0:1	0.08 ± 0.01^{Aab}	19.65 ± 3.52^{Bab}	32.77 ± 3.46^{Ba}
	2:1	0.08 ± 0.01^{Aab}	17.24 ± 2.80^{Babc}	35.10 ± 4.63^{Babc}
	4:1	0.07 ± 0.01^{Ab}	19.92 ± 2.48^{Ba}	44.06 ± 4.07^{Bab}

All values were presented as mean ± SD. Different letters after mean values in columns indicate significant differences at $p < 0.05$. Uppercase letters indicate statistical differences among oil concentrations; lowercase letters indicate statistical differences among oil ratios.

The incorporation of hydrophobic substances into protein films reduces their inherent water affinity, as assessed by the WVP index (Cheng et al., 2021). WVP measures the rate at which water vapour passes through a material, with lower WVP values indicating a stronger water barrier (Cheng et al., 2021). In this study, oil concentration had a significant impact on the WVP of the canola films ($p<0.05$), whereas oil proportions did not ($p>0.05$).

It was expected that adding EOs would reduce WVP values due to their hydrophobic nature (Yang et al., 2024). Results revealed that WVP was only slightly reduced, with no significant difference in the quantity of the EOs blend incorporated ($p>0.05$). Similarly, there was no significant difference between the 3% essential oil films and neat protein films ($p > 0.05$). This finding is consistent with several studies that reported no change or an increase in WVP upon the addition of EOs (Dissanayake et al., 2022; Ghoshal & Shivani, 2022; Hajirostamloo et al., 2023; Song et al., 2015; Zhou et al., 2021). This was attributed to the formation of micropores in the structure

induced by EOs, which created additional spaces within the films and enabledfaster water diffusion (Amjadi et al., 2021; Zhou et al., 2021). Additionally, film thickness and RH can affect WVP values (Mchugh et al., 1993; Tao et al., 2022b). Santos Paglione et al., (2019) have reported that encapsulated EOs reduce WVP more effectively than free EOs when added to protein films, suggesting stronger interactions with the film matrix. However, the requirements for high or low WVP depend on the intended end-use of the packaging film. For example, semipermeable packaging film can be used for fruits and vegetables, as the exchange of water between them, mediated by the film, can help keep them fresher for longer. A low WVP is desirable when the goal is to reduce the growth of bacteria, yeast, and mold, which can cause spoilage or foodborne illness (Hajirostamloo et al., 2023).

3.5.3.3. Water contact angle (WCA)

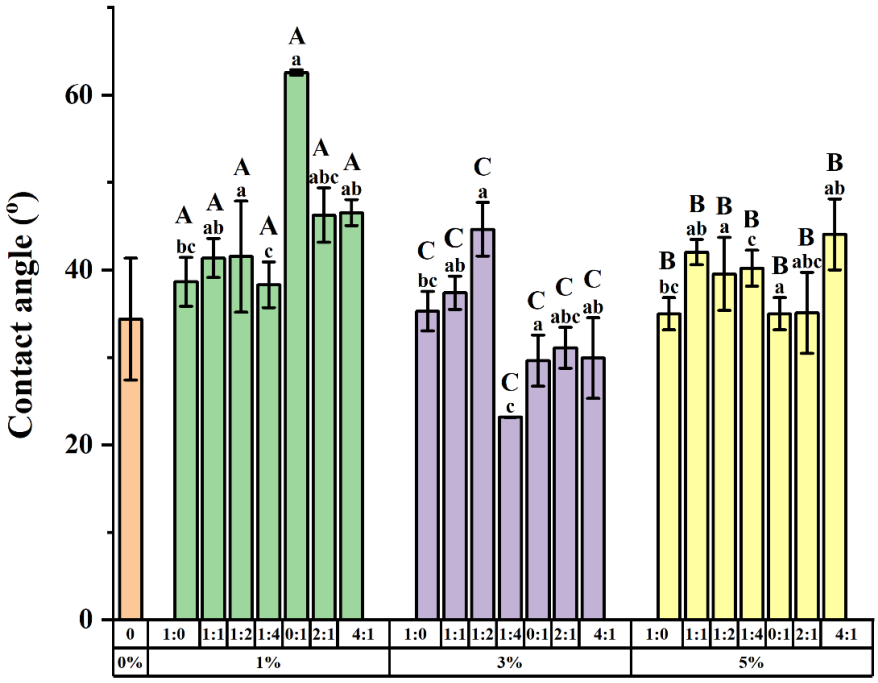

Figure 3.5: Contact angle (°) of canola protein films prepared with 0%, 1%, 3% and 5% (v/v) of CAR and CIN at CAR:CIN 0:0, 1:0, 0:1, 1:2, 1:4, 2:1, and 4:1 ratios. Uppercase letters indicate statistical differences among oil concentrations; lowercase letters indicate statistical differences among oil ratios.

The water contact angle test is crucial for evaluating the wettability of canola protein film samples for food packaging applications. This test involves measuring the angle formed between a water

droplet and the film's surface, with the contact angle providing insight into the material's affinity for water (Karbowiak et al., 2006). A contact angle of 90° or higher indicates a hydrophobic surface, whereas values below 90° suggest hydrophilicity, as evidenced byncreased spreading of the water droplet on the material (Selva et al., 2023).

Contact angle measurements of canola protein films incorporating CAR and CIN showed a decreasing trend, indicating enhanced hydrophilicity. At 3% oil, the ratio of carvacrol to cinnamaldehyde had a significant effect on contact angles compared with the control group ($p < 0.05$). The water contact angle of canola protein film is 34.39 ± 6.98 º, confirming a hydrophilic nature. Among the films with different oil concentrations, those containing 1% oil exhibited the lowest surface water affinity, albeit still within the hydrophilic range. The pure CIN film exhibited a maximum contact angle of 62.57 ± 0.32 °, which aligns closely with findings from a previous study (Dissanayake et al., 2022). However, films containing 3% and 5% oil exhibited significantly lower values, with maximum values of 44.65 ± 3.06 º and 44.06 ± 4.07 º, respectively. This trend suggests that essential oils alter the surface properties of the film matrix, thereby increasing surface wettability. This effect is likely due to the interactions between the EOs and the protein matrix, which influenced the exposure of polar functional groups on the film surface (Cheng, Kandula, et al., 2024; Cheng, Velez, et al., 2024; Munhuweyi et al., 2017; Roy et al., 2023). A similar trend was reported by Nostro et al. (2012) in poly(ethylene-co-vinyl acetate) films prepared with 3.5 wt% and 7 wt% CAR and CIN by compression molding. Their study also observed a decrease in contact angles with increasing oil concentration. While EOs are generally hydrophobic, the presence of aldehyde and hydroxyl functional groups in CAR and CIN may facilitate hydrogen bonding with water molecules, thereby increasing surface wettability. Furthermore, the heterogeneous dispersion of EOs in films can also affect the distribution of polar and non-polar functional groups in the film's surface (Cheng, Kandula, et al., 2024). Finally, another potential factor influencing surface properties is glycerol migration during drying, which can increase hydrophilicity as a portion of the plasticizer migrates to the film's surface over time (Chairez-Jimenez et al., 2024; Dissanayake et al., 2022; Dissanayake et al., 2023). Glycerol, being highly hydrophilic, can form a moisture-attracting layer on the surface, further lowering the contact angle. This migration effect may be more pronounced in films with higher oil content, as changes in the film matrix structure resulting from oil phase separation could facilitate plasticizer movement.

3.5.4. Surface morphology of canola protein-based EO composite films

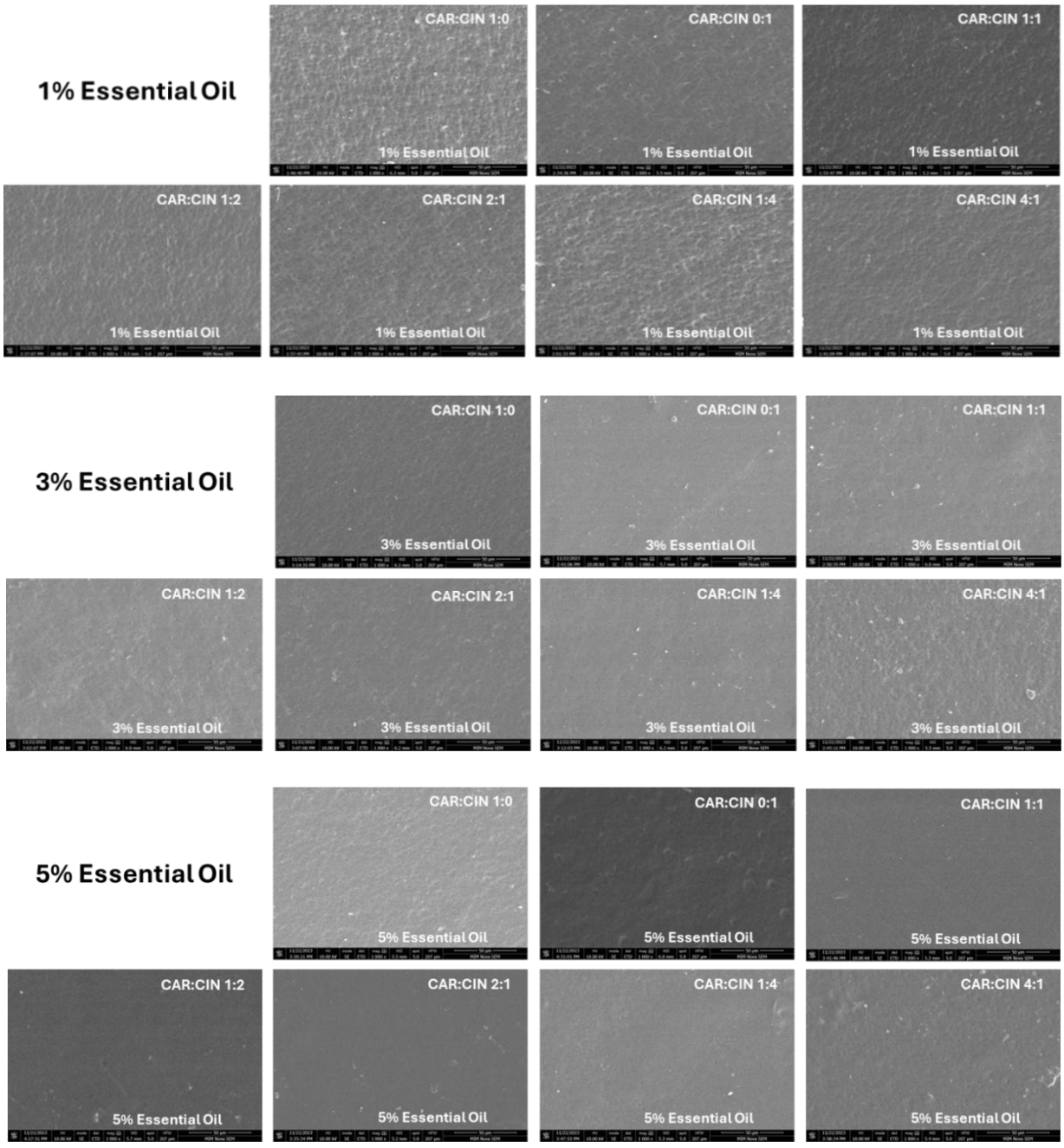

Figure 3.6: SEM images of the surface of canola protein films with 0%, 1%, 3% and 5% (v/v) of CAR and CIN at CAR:CIN 0:0, 1:0, 0:1, 1:2, 1:4, 2:1 and 4:1 ratios (x1000 magnification).

The surface morphology of canola films is shown in Figure 6. The control film showed a relatively smooth surface, whereas EOs incorporation led to a rougher, heterogeneous surface. Samples

containing 5% oil exhibited a slight reduction in surface roughness compared with those containing 1% or 3% oil. Surface heterogeneity is frequently observed in composite films when there is limited compatibility between the composite and the film matrix. Such heterogeneity reflects the EOs embedded in the film matrix (Niknam et al., 2023).

Surface roughness often develops during drying and manifests as microaggregates or indentations in the final film, influencing its interactions with external factors such as moisture and gases. As water evaporates, the solution viscosity increases, leading to phase changes. Several phenomena, including flocculation, aggregation, or coalescence of molecules, can occur, negatively impacting the microstructure (Chu et al., 2020; Galus, 2018). Coalescence is often associated with lipids and occurs when small oil droplets combine to form larger droplets as they integrate into the matrix (Van Aken, 2004). Strategies to minimize coalescence could involve refining the formulation or adjusting processing conditions. Understanding and improving the film's microstructure can enhance the properties of canola films, making them more versatile and effective for diverse industrial applications. Additionally, the type of oil used, or its combination, did not improve smoothness, and incorporating 5% essential oils resulted in significant oil leakage.

3.5.5. Thermal properties of canola protein-based EO composite films

Table 3.2: Thermal analysis of canola protein-based EO composite films: onset temperature (°C), melting temperature (°C), and enthalpy (J/g).

Sample(s)	Oil ratio CAR: CIN	Extrapolated onset temperature (°C)	Protein degradation temperature (°C)	Enthalpy (J/g)
Control, 0% oil	0	239.40	241.50	44.99
1% oil	1:0	248.60	251.17	53.00
	1:1	232.70	234.28	65.93
	1:2	234.53	235.95	65.00
	1:4	231.16	232.58	64.08
	0:1	249.75	249.90	49.40

	2:1	236.69	238.88	47.61
	4:1	244.30	245.38	57.60
3% oil	1:0	248.85	249.76	60.89
	1:1	248.80	250.35	55.54
	1:2	251.42	251.86	58.27
	1:4	250.07	251.48	50.35
	0:1	249.00	250.09	55.99
	2:1	253.58	254.81	50.42
	4:1	259.19	256.64	52.50
5% oil	1:0	253.34	255.21	45.16
	1:1	257.99	259.21	51.14
	1:2	256.41	257.37	51.86
	1:4	258.99	254.80	49.93
	0:1	253.91	254.40	51.67
	2:1	254.61	255.80	49.97
	4:1	255.45	256.78	49.23

Differential Scanning Calorimetry was used to characterize the thermal behaviour of canola protein films incorporated with carvacrol and cinnamaldehyde, providing insights into their thermal stability and degradation processes (Table 2). The thermograms of all films showed a single endothermic peak, corresponding to the denaturation of canola protein (Dissanayake et al., 2023). Pure canola film showed a denaturation temperature of 241.5 ± 3.9 ^{o}C, whereas films containing EOs exhibited a denaturation temperature ranging from 234.3 ± 1.6 ^{o}C to 259.2 ± 3.2 ^{o}C. The addition of EOs resulted in a slight increase in the denaturation temperature of the canola

protein films, with oil concentration as an influencing factor ($p < 0.05$). This suggests that higher EOs concentrations may improve thermal stability. The improved stability observed with cinnamaldehyde may be attributed to its ability to form Schiff bases with amino groups in the protein matrix, thereby reinforcing the structure through covalent and hydrogen bonding interactions (M. Cao et al., 2022). On the other hand, carvacrol primarily enhances intermolecular interactions, which may also contribute to increased thermal resistance.

3.5.6. Antimicrobial properties of canola protein-EO-based composite films

Table 3.3: Antimicrobial properties of canola protein films incorporated with carvacrol (CAR) and cinnamaldehyde (CIN).

Sample(s)	Oil ratio CAR:CIN	Zone of inhibition against *E. coli* (cm)	Zone of inhibition in against *S. aureus* (cm)
Control, 0% oil	0	0 ± 0	0 ± 0
1% oil	1:0	0 ± 0^{Ba}	0 ± 0^{Cab}
	0:1	0 ± 0^{Ba}	0 ± 0^{Cd}
	1:1	0 ± 0^{Ba}	0 ± 0^{Cb}
	1:2	0 ± 0^{Ba}	0 ± 0^{Cbc}
	2:1	0 ± 0^{Ba}	0 ± 0^{Cbc}
	1:4	0 ± 0^{Ba}	0 ± 0^{Ccd}
	4:1	0 ± 0^{Ba}	0 ± 0^{Ca}
3% oil	1:0	0.89 ± 0.03^{Aa}	1.69 ± 0.08^{Bab}
	0:1	0.63 ± 0.97^{Aa}	0.97 ± 0.63^{Bd}
	1:1	0.82 ± 0.00^{Aa}	1.72 ± 0.10^{Bb}
	1:2	0.27 ± 0.47^{Aa}	1.43 ± 0.21^{Bbc}
	2:1	0.54 ± 0.47^{Aa}	1.29 ± 0.07^{Bbc}
	1:4	0.72 ± 0.02^{Aa}	0.92 ± 0.06^{Bcd}
	4:1	1.04 ± 0.19^{Aa}	1.68 ± 0.10^{Ba}
5% oil	1:0	0.81 ± 0.03^{Aa}	1.98 ± 0.15^{Aab}
	0:1	1.08 ± 0.14^{Aa}	1.54 ± 0.27^{Ad}
	1:1	0.82 ± 0.10^{Aa}	1.77 ± 0.14^{Ab}
	1:2	0.79 ± 0.18^{Aa}	1.69 ± 0.16^{Abc}
	2:1	0.81 ± 0.08^{Aa}	1.84 ± 0.15^{Abc}
	1:4	0.65 ± 0.57^{Aa}	1.84 ± 0.28^{Acd}
	4:1	0.78 ± 0.07^{Aa}	2.51 ± 0.10^{Aa}

The disk diffusion assay is a widely used method to assess the antimicrobial potency of EOs and their diffusion. The size and clarity of the zone of inhibition (ZOI) serve as a combined measure of diffusion dynamics and antimicrobial bioactivity (Barnard, 2019). Larger and more distinct zones generally indicate stronger antimicrobial activity and more effective EO release, whereas smaller or less distinct zones may suggest restricted diffusion, weaker bioactivity, or partial microbial resistance. Depending on the size of the ZOI, the bacteria can be categorized as susceptible, intermediate, or resistant (Clinical and Laboratory Standards Institute, 2025).

In the control films, the ZOI was not observed, confirming the absence of antimicrobial activity without the addition of essential oils. Similarly, all the films infused with 1% oil also failed to exhibit antimicrobial activity against the tested *E. coli* and *S. aureus* strains (see Table 3 and Appendix: Study 1 Supplementary Materials section, Table S2 for data). This lack of inhibition could be attributed to the low concentration of essential oil, which is insufficient to reach the minimum inhibitory concentration (MIC) or the minimum bactericidal concentration (MBC) required to suppress or eliminate bacterial growth (see Appendix: Study 1 Supplementary Materials section, Table S2). Since the essential oil content at 1% falls below both thresholds, it does not provide a strong enough antimicrobial effect, resulting in the lack of observable ZOI. These findings are consistent with those of Seydim and Sarikus (2006), who developed whey protein-based edible films incorporating oregano, rosemary, and garlic EOs at concentrations of 1%, 2%, 3%, and 4% (w/v) and reported no inhibition at 1% against *E. coli O157:H7, L. monocytogenes, S. aureus*, and *S. enteritidis*. This highlights the importance of selecting EOs levels in films to meet or exceed MIC and MBC values to achieve effective microbial control.

Films containing 3% EOs displayed a modest antimicrobial effect, ranging from none to small to relatively large ZOI, suggesting that this concentration is approaching the MIC/MBC thresholds (Table 3). In comparison, the 5% EOs films exhibited the strongest antimicrobial activity, characterized by a higher frequency of larger ZOI, indicating more efficient diffusion and greater bioactivity of EOs, sufficient to exceed the MIC/MBC for the tested microorganisms (Table 3). Both carvacrol and cinnamaldehyde showed strong antimicrobial activity against *S. aureus*, with statistically significant differences observed across individual oil concentrations and their combinations ($p < 0.05$). In comparison, their efficacy against *E. coli* was lower, with oil concentration as the significant factor ($p < 0.05$). Although the MIC values of carvacrol and

cinnamaldehyde were identical for both *E. coli* and *S. aureus* (10.42 µL/mL), the MBC value of carvacrol was notably higher (83.33 µL/mL) than that of cinnamaldehyde (20.83 µL/mL), indicating stronger bactericidal activity for cinnamaldehyde (Appendix: Study 1 Supplementary Materials section, Table S2). When combined at a 1:1 ratio, the mixture exhibited the same MBC as cinnamaldehyde for both microorganisms (20.83 µL/mL), suggesting that cinnamaldehyde is the main contributor to the bactericidal effect in the mixture, allowing the blend to achieve effective killing at lower concentrations than carvacrol alone (Appendix: Study 1 Supplementary Materials section, Table S2).

The observed differences in EOs efficacy are influenced by bacterial cell structure and composition. Gram-positive bacteria, such as S. aureus, have a relatively simple peptidoglycan-rich cell wall, which allows lipophilic EO components to penetrate and disrupt the cytoplasmic membrane, leading to effective antimicrobial activity (Silhavy et al., 2010; Gonon et al., 2023; Bassolé & Juliani, 2012). In contrast, Gram-negative bacteria possess an additional outer membrane consisting of a phospholipid bilayer anchored by lipopolysaccharides, which acts as a barrier to hydrophobic compounds and reduces susceptibility to EOs (Nazzaro et al., 2013; Perumal et al., 2022). These structural differences help explain the stronger activity observed against *S. aureus* compared to *E. coli.*

These findings are consistent with those of Yu et al. (2023), who formulated zein-based films incorporating cinnamaldehyde, carvacrol, and eugenol at 1%, 3%, and 5% (v/v) with different plasticizers. Their results indicated no antimicrobial activity against *E. coli* at 1% EOs, with inhibition observed at 3% and 5%, depending on the plasticizer used. Additionally, their study demonstrated that cinnamaldehyde and carvacrol were highly effective against Gram-positive bacteria compared to Gram-negative bacteria, reinforcing the trends observed in this study.

In addition to bacterial cell structure, the chemical composition of the EOs also influences their antimicrobial potency and activity (Silhavy et al., 2010). Essential oils rich in aldehydes or phenols, such as cinnamaldehyde and carvacrol, are particularly well known for their strong antibacterial properties (Bassolé & Juliani, 2012). Cinnamaldehyde interacts with cellular proteins, including enzymes, structural proteins, and transport proteins, leading to inhibition of enzyme activity through active site blockage or conformational changes (Wendakoon & Morihiko, 1995; Zhou et al., 2007). This interference disrupts metabolic pathways and destabilizes structural

proteins, ultimately impairing essential cellular processes, inhibiting microbial growth, and resulting in cell death (Wendakoon & Morihiko, 1995; Zhou et al., 2007) Similarly, carvacrol targets the bacterial cell membrane via its phenolic structure, disrupting the phospholipid bilayer, increasing membrane permeability, and causing leakage of critical intracellular components such as ATP (Bassolé & Juliani, 2012). When combined, carvacrol and cinnamaldehyde can exert a synergistic antimicrobial effect that varies with the microorganism (Bassolé & Juliani, 2012; Lambert et al., 2001; Zhou et al., 2007). The synergy arises from their complementary modes of action: carvacrol primarily compromises membrane integrity, while cinnamaldehyde targetsntracellular components (Zhou et al., 2007). This interaction is particularly pronounced in Gram-positive bacteria, which are generally more susceptible to membrane-disrupting compounds (Zhou et al., 2007).

While the chemical composition and antimicrobial mechanisms of EOs are fundamental to their activity, the efficacy of films incorporating these oils is not determined solely by their intrinsic properties. It is also influenced by factors related to the film matrix and the experimental environment, including diffusion rate, chemical composition, microbial characteristics, and environmental conditions (Bassolé & Juliani, 2012; Pei et al., 2009; Yu et al., 2023; Zhou et al., 2007). Among these, diffusion capability is particularly crucial, as it affects the release of carvacrol and cinnamaldehyde from the protein network into the surrounding medium and their subsequent interaction with bacterial cells. Strong protein-oil interactions can hinder the mobility of the essential oils, slowing down their release and reducing their bioavailability, thereby limiting their antimicrobial efficacy (Yu et al., 2023).

The application of EOs also faces biological and chemical challenges. A major limitation is the high variability in antimicrobial response, observed not only among microbial species but also among strains within the same species (Nazzaro et al., 2013). For example, De Martino et al. (2009) observed that two strains of *Bacillus cereus* (4384 and 4313) exhibited different susceptibility when exposed to the same essential oil. Such strain-specific differences likely reflect underlying genetic variation influencing susceptibility, highlight the complexity of EOs-bacteria interactions, and demonstrate that antimicrobial efficacy is not uniform (Aguilar-Veloz et al., 2020; Sandoval-Contreras et al., 2017). Aside from genetic diversity, environmental factors such as pH, temperature, nutrient availability, and the composition of the surrounding medium can also

modulate the antimicrobial activity of EOs (Aguilar-Veloz et al., 2020; Sandoval-Contreras et al., 2017). Additionally, variability is inherent to the oils themselves, as EOs' composition, yield, and efficacy are strongly influenced by plant species, geographic origin, cultivation conditions, harvest time, extraction methods, and the specific plant part utilized. EOs derived from leaves, flowers, seeds, or roots can vary in their chemical profiles and bioactive compound concentration, leading to inconsistent antimicrobial outcomes (Aguilar-Veloz et al., 2020; Azmir et al., 2013; Joas et al., 2012; Kumari et al., 2014). Collectively, these variations complicate the interpretation of antimicrobial assays for EOs. Therefore, no standardized breakpoints are currently available for categorizing bacterial susceptibility to EOs in disk diffusion assays, mainly because ZOI reproducibility is difficult to standardize.

In food packaging applications, the hydrophobic and volatile nature of EOs limits their stability and uniform distribution in the film matrix, posing significant challenges. In particular, in canola protein-based packaging films, the protein matrix has limited compatibility with hydrophobic EOs, resulting in uneven dispersion or phase separation. This can lead to localized regions of high or low EOs concentration, which not only compromises antimicrobial efficacy but may also affect the mechanical and barrier properties of the film. Furthermore, the volatility of EOs can result in substantial loss during film drying and storage, especially when exposed to heat, light, or oxygen (Royo et al., 2010). Sensory considerations and potential toxicity further constrain the use of EOs in food packaging, as their incorporation can impart strong flavors, aromas, or off-notes that may be undesirable for certain food products. Moreover, some EOs components may interact with food constituents or migrate into the food at concentrations that raise safety concerns, restricting the levels that can be applied (El Bourakadi et al., 2024). However, this is not the case for carvacrol and cinnamaldehyde, which are generally recognized as safe (GRAS) for food use (Food and Drug Administration, 2025). Overall, these challenges highlight the need for strategies that improve EOs stability, controlled release, and compatibility within the packaging film matrix (Amin et al., 2022; Hossen et al., 2024). Approaches such as encapsulation, emulsification, or the use of carriers and co-polymers have been explored to enhance EOs retention and ensure uniform distribution (Kashiri et al., 2017; Niu et al., 2018; Xue et al., 2019). By optimizing film formulation and processing conditions, it is possible to achieve a balance between antimicrobial activity and mechanical properties.

3.5.7. Interpretation of EOs influence on film properties

Across all tested properties, these observations suggest that essential oil concentration had a more pronounced effect on the functional and antimicrobial properties of canola protein-based films, including antimicrobial activity, mechanical performance, and moisture resistance. On the contrary, the proportion of carvacrol to cinnamaldehyde had a relatively limited or unclear impact. The lack of clear effects from varying oil ratios may be due to differences in the chemical composition and molecular properties of the oils, which can influence their binding, distribution, and activity within the protein matrix, as well as oil volatilization and uneven dispersion during film formation. No consistent trend was observed across the characterizations, and anticipated synergistic antimicrobial effects of combining carvacrol and cinnamaldehyde were not evident. These results highlight the challenges of incorporating multiple essential oils into canola protein-based films and emphasize the role of oil concentration in determining film properties.

3.6. Conclusion

This study investigated the incorporation of carvacrol and cinnamaldehyde into canola protein-based films to improve their functional performance and introduce antimicrobial activity. The addition of these essential oils at varying concentrations and blend ratios effectively enhanced the films' antimicrobial properties. However, higher concentrations of essential oils were found to negatively impact film integrity, resulting in diminished mechanical strength and reduced moisture resistance. These findings underscore the importance of optimizing both the concentration and ratio of essential oils to balance bioactivity with structural performance. Overall, the results demonstrate that carvacrol and cinnamaldehyde can be effectively used to develop active, biodegradable canola protein films with strong potential for food packaging applications. Future research should address long-term film stability, interactions with packaged foods, and biodegradability in the natural environment.

3.7. References

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Chapter 4: Canola Protein-polyvinyl alcohol (PVA) polymer blending improves mechanical and barrier properties of canola protein-based films

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4.1 Abstract

Canola protein, a renewable plant-based biopolymer, holds promising potential for sustainable packaging applications due to its biodegradability and high availability. However, canola protein films suffer from drawbacks, including brittleness, low flexibility, and poor moisture resistance. To address these challenges, polyvinyl alcohol (PVA), a synthetic yet biodegradable polymer with excellent mechanical strength and thermal stability, was incorporated to develop composite films. Canola protein-based PVA films were prepared at various blend ratios (100:0, 90:10, 80:20, 70:30, 60:40, and 0:100, w/w) with glycerol as a plasticizer. The films were evaluated for their physicochemical properties, including mechanical strength, elongation at break, moisture barrier performance, surface wettability, thermal behavior, and chemical structure. Among the blends, the 60:40 canola protein–PVA film exhibited optimal mechanical performance, with an approximate 155% increase in tensile strength and a 106% increase in elongation at break compared to pure canola protein films. Although water vapor permeability was not significantly affected, a decreasing trend with higher PVA content suggested improved barrier potential. These results indicate that blending canola protein with PVA is an effective strategy to enhance the functionality

of protein-based films, offering a promising approach for the development of biodegradable, high-performance packaging materials.

Keywords

Canola protein; polyvinyl alcohol, biopolymer packaging

Abbreviations

PVA, polyvinyl alcohol, DH, degree of hydrolysis, MW, molecular weight

Author Contribution

Funds acquisition: Bandara N.; Conceptualization: Bandara N. and Dang A. Methods: Dang A. and Abeysinghe V.; Analysis: Dang A. and Abeysinghe V.; Writing the first draft: Dang A; Editing and reviewing: Bandara N. and Dang A.

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4.2. Introduction

As industries strive to transition to more sustainable practices, the potential to repurpose industrial byproducts into valuable materials has attracted growing interest. Industrial byproducts often ended up in landfills due to several factors, including a lack of awareness about their potential applications, challenges in processing and modifying these materials, and the availability of cheaper alternatives (Bawab et al., 2022; Hossain et al., 2024; Mutwedu et al., 2022). This has led to significant waste and missed opportunities to create high-value products from resources that would otherwise be discarded (Narisetty et al., 2022). By addressing these barriers, industries can maximize the full potential of such byproducts, contributing to both economic and environmental sustainability (Narisetty et al., 2022). Building on this idea, a key area of focus is plastic packaging, which accounts for a significant portion of global plastic waste (Amaral-Zettler et al., 2015). Due to its durability, versatility, and cost-effectiveness, plastic has become deeply embedded in modern life, especially in consumer products. Approximately 64% of grocery store items are packaged in plastic, primarily food and beverages (Government of Canada, 2023a). However, most conventional plastic is produced from petroleum-based materials, making it non-biodegradable and capable of persisting in the environment for decades. This leads to its accumulation in landfills, rivers, and oceans, contributing to widespread environmental pollution (Meijer et al., 2021). In 2020, over a quarter of the litter found on Canadian shorelines was food and beverage plastic packaging (Government of Canada, 2023b). As industries seek to adopt more sustainable practices, there is a growing interest in exploring alternatives to traditional plastic packaging that align with environmental goals. A promising solution lies in developing biopolymer-based materials derived from renewable resources, which are often biodegradable. These materials offer a sustainable alternative to conventional plastics, addressing both environmental concerns and consumer demand for eco-friendly products. By utilizing feedstocks from byproducts, such as plant-derived proteins, polysaccharides, and lipids, biopolymer-based packaging offers a solution to minimize waste while maintaining functionality and performance. As such, biopolymer-based packaging has been gaining recognition as a promising alternative due to its renewability and ability to replicate many desirable characteristics of plastic, such as flexibility, durability, and barrier properties.

Among various plant-derived feedstock, proteins have demonstrated significant potential for packaging development due to their favourable properties. Canola protein, derived as a byproduct of canola oil production, has garnered considerable attention in this regard. Traditionally, canola

protein has been used as animal feed due to its reduced quality and functionality, often caused by heat damage during oil extraction (Chmielewska et al., 2020). Despite these limitations, its unique chemical composition has enabled novel applications in sustainable packaging (Chairez-Jimenez et al., 2024; Chang & Nickerson, 2014; Dissanayake et al., 2023; Jang et al., 2011; S. Li, Donner, et al., 2024; Osorio-Ruiz et al., 2019). Canola protein is primarily composed of two major storage proteins: cruciferin and napin, which collectively account for 85%-90% of its total protein content (Campbell et al., 2016). Cruciferin, a 12S globulin, constitutes approximately 60% of canola protein and is rich in essential amino acids such as glutamic acid, aspartic acid, and leucine (Hoglund et al., 1992; Klockeman et al., 1997). Known for its excellent film-forming capabilities, cruciferin is crucial for creating strong, flexible films. Napin, a 2S albumin protein, accounts for about 20% of the total protein content and is characterized by its high content of sulfur-containing amino acids, such as cysteine, which can form disulfide bonds and thereby enhance film stability (Klockeman et al., 1997). Together, cruciferin and napin form a stable, partially functional film with good properties. However, films made from canola protein alone often lack the mechanical strength and durability required for practical applications (Dissanayake et al., 2023). Furthermore, both cruciferin and napin are known for their water-binding capabilities (Klockeman et al., 1997), which can affect film performance under varying environmental conditions. To address these limitations, composite film development has been explored to enhance the functional properties of canola protein-based films. By incorporating additional materials, the mechanical strength, flexibility, and overall performance of the resulting films can be improved significantly (Dissanayake et al., 2023). Ideally, these materials should be degradable, strong, and flexible to align with sustainability goals. Among potential blending agents, synthetic polymers have shown promise for addressing the structural and functional limitations of canola protein. One such polymer of interest is polyvinyl alcohol (PVA), a water-soluble synthetic polymer widely recognized for its excellent mechanical strength, flexibility, and film-forming capabilities (Abdullah et al., 2017). PVA has a hydrocarbon backbone and contains 1,2-diols and 1,3-diols in its side chains, which contribute to its high structural integrity (Abdullah & Dong, 2020b; Mousa et al., 2016). It is typically produced by hydrolyzing polyvinyl acetate to varying degrees of hydrolysis (70%-99%) and molecular weights ranging from 20×10^3 to 400×10^3 g/mol (Abdullah & Dong, 2020a). Additionally, PVA is biodegradable under specific conditions, making it a

suitable candidate for developing environmentally friendly packaging materials (Abdullah & Dong, 2020b).

Due to its high durability and compatibility with other polymers, PVA has been extensively incorporated into biocomposite films to enhance their structural properties (Abdullah & Dong, 2020a; Espinosa et al., 2019; Lee et al., 2021; Mohammed et al., 2023; Osolnik et al., 2025; Shahi et al., 2020; Tan et al., 2021; Wen et al., 2020; Yeşilyurt et al., 2025). In protein-based films, PVA enhances mechanical performance, addressing the inherent lack of strength in these materials (Liu et al., 2017; Tian et al., 2023). The degree of hydrolysis (DH) and molecular weight (MW) of PVA significantly affectthe mechanical properties of the film (Limpan et al., 2012). Higher DH increases tensile strength but reduces flexibility, while lower DH improves flexibility at the expense of strength (Limpan et al., 2012). Similarly, high-MW PVA enhances strength and durability, whereas low-MW PVA improves flexibility and processability (Limpan et al., 2012). Based on this, medium-MW PVA may offer an optimal balance between strength and elongation. By adjusting PVA concentration, DH, and molecular weight, protein films can be optimized for various applications.

Despite the established benefits of PVA in biocomposite films, its integration with canola protein remains largely unexplored, presenting a valuable research opportunity. Combining the biodegradability of canola protein with the structural strength of PVA may offer a promising solution for developing functional, eco-friendly composite films. This study hypothesizes that the addition of PVA can enhance the films' functional properties, particularly by improving the mechanical performance. Therefore, the objective of this study is to develop canola protein-PVA composite films at varying ratios and evaluate the effect of PVA incorporation on their mechanical and barrier properties as well as thermal stability, and to assess their potential for eco-friendly packaging applications. The films were prepared at five canola protein-to PVA ratios (100:0, 90:10, 80:20, 70:30, and 60:40, w/w) by the solvent-casting method. The findings from this study aim to support the development of sustainable packaging materials by providing insights into the performance potential of canola protein-PVA composite films.

4.3. Materials and methods

4.3.1. Materials and chemicals

Canola meal was provided by Richardson International (Lethbridge, AB, Canada). Medium molecular weight PVA, 80,000 g/mol, ≥ 98% purity, 98-99% hydrolyzed, glycerol (≥ 99.5% purity), NaOH (≥ 97.0% purity), HCl (12N) were purchased from Fisher Scientific (Ottawa, ON, Canada). Unless otherwise specified, all other chemicals were purchased from Fisher Scientific, Ottawa, ON, Canada.

4.3.2. Canola protein extraction

Canola protein was extracted from defatted canola meal using a method outlined by Manamperi et al. (2010) and Dissanayake et al. (2023), with some modifications. Defatted canola meal was mixed with mili-Q water at a 1:10 (w/v) ratio. The pH of the solution was adjusted to 12 with 3 M NaOH, and the mixture was stirred for 30 minutes. Then the mixture was centrifuged at 10,000 g 4 °C for 15 minutes. The supernatant was collected and adjusted to pH 4 with 3 M HCl. The solution was then centrifuged at 10,000g for 15 minutes at 4 °C, freeze-dried, and stored at -20 °C until further use.

4.3.3. Preparation of canola protein solution

The canola protein solution was prepared following the method adapted from Dissanayake et al. (2023), with some modifications. Various amounts of canola protein isolate were dispersed in 40 mL of distilled water, and the pH was adjusted to 10 with 1 M NaOH. The mixture was stirred at 650 rpm for 30 minutes at room temperature, then heated in a water bath (VWR International, Cornelius, OR, USA) at 70 °C with continuous stirring at 70 rpm for 20 minutes. After cooling to room temperature, glycerol (50% w/w of protein) was incorporated as a plasticizer, and the dispersion was homogenized using a Fisherbrand 850 homogenizer (Fisher Scientific, ON, Canada) at 14,000 rpm for 5 minutes.

4.3.4. Preparation of PVA solution

To prepare the PVA solution, different mass of PVA with an average molecular weight of 80,000 g/mol was dissolved in 30 mL of distilled water at 90 °C under magnetic stirring at 650 rpm for 45 minutes. Once fully dissolved, the solution was cooled to room temperature, and glycerol (50% w/w of PVA) was added. The solution was magnetically stirred at 450 rpm for 1 hour to ensure thorough mixing.

4.3.5. Fabrication of canola protein-PVA blend films

The composite film preparation followed the method described by Dissanayake et al. (2023), with a few modifications. The prepared PVA solution was added to the canola protein solution according to the formulation ratios listed in Table 1. Then the combined solution was homogenized at 14,000 rpm for 5 minutes and subsequently sonicated (60% amplitude, 20 kHz frequency, and 500 W power output) for 5 minutes. The resulting film-forming solution was cast onto hexagonal polystyrene dishes (0.43 ml/cm^2) and air-dried for 3 days. Prior to characterization, all films were conditioned at 50 % relative humidity (RH) and 23 °C (ASTM D882-18, 2018).

Table 4.1: Compositions of Canola protein-PVA films

Sample(s)	Total solid mass (g)	Canola protein solution			PVA solution			Total volume (mL)
		Canola protein (g)	Water (mL)	Glycerol (%, w/w)	PVA (g)	Water (mL)	Glycerol (%, w/w)	
Control, CP: PVA 100:0	3.5	3.5	70	50	0	0	50	70
CP: PVA 90:10	3.5	3.15	40	50	0.35	30	50	70
CP: PVA 80:20	3.5	2.8	40	50	0.7	30	50	70
CP: PVA 70:30	3.5	2.45	40	50	1.05	30	50	70
CP: PVA 60:40	3.5	2.1	40	50	1.4	30	50	70
CP: PVA 0:100	3.5	0	0	50	3.5	70	50	70

4.4. Characterization of canola-PVA films

4.4.1. Mechanical properties

Tensile strength (TS), Young's modulus (YM), and elongation percentage at break (EB) of the canola protein films were measured using the Instron 68TM-50 universal testing machine (Illinois Tool Works Inc., MA, USA) with a 5000 N load cell. in accordance with the ASTM D882 (ASTM D882-18, 2018) standards. Film samples, after conditioning for 48 hours at 23 °C and 50% RH, were precisely cut to dimensions of 70 mm (length) x 10 mm (width) and deformed under a tensile load of 500N at a crosshead speed of 5 mm/min until fracture. All samples were tested in quadruplicates, and the results for TS (MPa), YM (MPa), and EB (%) were reported as mean ± SD (standard deviation).

4.4.2. Water Vapour Permeability (WVP)

Moisture barrier properties of the films were assessed gravimetrically following the ASTM E96/E96M-16 method, with slight modifications (ASTM E96/E96M-16, 2016). Cylindrical cups (4.24 cm diameter and 4.02 cm height) were partially filled with distilled water up to 3/4 of the volume. Circular film samples were placed over the cup openings and sealed with glue. The combined weight of the cup, water, and film was recorded before placing the setup in a controlled chamber maintained at 50% RH and 23 °C. The weight of the system was measured at 2 hours. Prior to testing, all film samples were preconditioned for 48 hours under the same temperature and humidity conditions. WVP of the films was measured using the following equation:

$$WVP = \frac{Weight\ change\ (g)\ \times\ thickness\ of\ the\ film\ (mm)}{Area\ of\ the\ cup\ mouth\ (m^2)\ \times\ vapor\ pressure\ difference\ (kPa)\ \times\ time\ (hours)}$$

4.4.3. Water Solubility (WS)

Film samples (2 cm × 2 cm) were dried in an oven at 105 °C for 24 hours to determine their initial dry weight (*W* initial weight - *W* first dried weight). Following this, the samples were submerged in distilled water at 25 °C for 24 hours, continuously stirred in a water bath. After soaking, the films were dried again under the same oven conditions to obtain their final dry weight (*W* first dried weight - *W* second dried weight). The water solubility of the films was then calculated using the following equation.

$$Water\ solubility\ \% = \left(\frac{W_{initial\ dry\ weight} - W_{final\ dry\ weight}}{W_{initial\ dry\ weight}} \right) \times 100$$

4.4.4. Water contact angle (WCA)

The surface wettability of canola protein-PVA films in contact with distilled water was evaluated using a contact angle goniometer (Model No. 200-00-115, Rame-hart Inc., Mountain Lake, NJ, USA) and DROPimage Standard software. For each sample, 10 measurements were taken at 0.01 s intervals, and the average of the acceptable angles was used for analysis.

4.4.5. Differential Scanning Calorimetry (DSC)

DSC analysis was conducted on canola protein powder and film samples to evaluate their thermal properties using the DSC 2500 (TA Instruments, New Castle, DE, USA). Samples (~5-10 mg) in T-zero pans were heated from 5 °C to 350 °C at a heating rate of 10 °C/min. A temperature (°C) vs. heat flow (mW) graph was used to analyze the thermal properties. Graphs were generated using Origin Pro 2025.

4.4.6. Scanning Electron Microscopy (SEM)

The surface uniformity of canola protein-PVA films was observed using a nano scanning electron microscopy (Quanta 650 FEG, FEI Company, Hillsboro, OR, USA) at an intensity of 10.0 kV. During sample preparation, the films were cut into 1 cm × 1 cm squares and coated with 60% gold (Au) and 40% lead (Pd) using a Denton vacuum to facilitate electron conductivity. Film surfaces were observed at ×2000 magnification.

4.4.7. Fourier transform infrared spectroscopy (FTIR)

Canola protein-PVA composite films were characterized using Attenuated Total Reflectance-Fourier transformed infrared spectroscopy (ATR-FTIR) to identify functional groups and assess molecular interactions within the film matrix. Spectral data were collected over the range of 400-4000 cm^{-1} with a resolution of 4 cm^{-1} for 120 scans using a spectrometer (Invenio S, Bruker, Leipzig, Germany). The IR spectra were analyzed and graphed using OriginPro 2025 software (OriginLab Corporation, Northampton, MA, USA).

4.4.8. Statistical Analysis

The samples were analyzed in triplicate using Minitab 2024 (Minitab LLC, State College, Pennsylvania, USA), and figures and illustrations were created using Origin Pro 2025 (OriginLab Corporation, Northampton, MA, USA). One-way ANOVA (analysis of variance) was conducted to evaluate the effect of PVA on canola protein films.

4.5. Results and discussions

4.5.1. Mechanical properties analysis of canola protein-PVA composite films

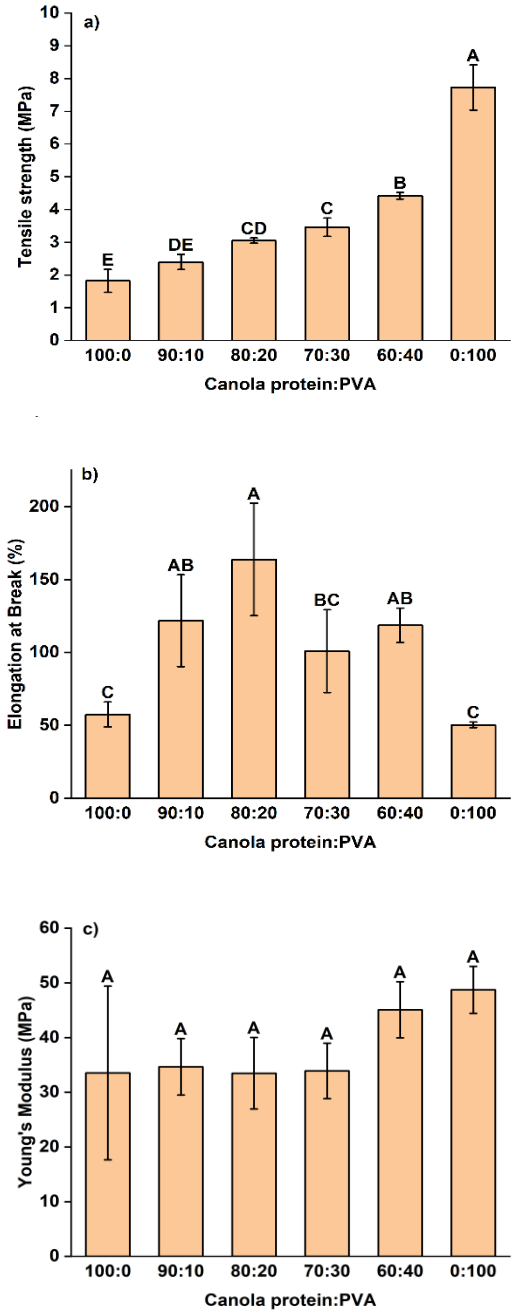

Figure 4.1: a) Tensile strength (MPa), b) elongation at break (%), and c) Young's modulus (MPa) of canola-PVA film blends (100:0, 90:10, 80:20, 70:30, 60:40, and 0:100, w/w) at 50% RH and 23 °C. All the values were reported as mean ± SD, and five specimens per film were tested.

Tensile strength (TS), elongation at break (EB), and Young's modulus (YM) are critical parameters in measuring the performance of biopolymer films for packaging applications. Figure 1. a) illustrates the tensile strength of canola protein-PVA composite films at varying blend ratios. Pure PVA film (0:100) exhibited the highest TS 7.87 ± 0.71 MPa compared to all other formulations, which is attributed to PVA's crystalline structure, strong intermolecular hydrogen bonding, and relatively high molecular weight (Teodorescu et al., 2019). Although this value is lower than those reported in the literature for PVA films (Iritani et al., 2021; Limpan et al., 2012; Soydan et al., 2024), the discrepancy in tensile strength may stem from differences in film formulations and processing conditions. Factors such as the degree of hydrolysis, molecular weight, pH, concentration, film thickness, presence or absence of plasticizers, and drying conditions can influence the final mechanical strength of PVA films. In contrast, the pure canola protein film (100:0) demonstrated the lowest TS value (1.73 ± 0.33 MPa), consistent with previously reported values (Chairez-Jimenez et al., 2024; Chang & Nickerson, 2014; Dissanayake et al., 2023; Shi & Dumont, 2014). The inherently low tensile strength of neat canola protein films can be attributed to their limited formation of cohesive network, a consequence of protein denaturation caused by thermal or chemical processes such as oil extraction and film fabrication (Bandara et al., 2018; Zhang et al., 2018). The incorporation of PVA had a significant effect on the mechanical properties of canola protein films ($p < 0.05$). As the PVA content increased (i.e., from 90:10 to 60:40 protein-to-PVA ratio), the TS rose from 2.40 MPa to 4.42 MPa, reflecting a positive relationship. This finding aligns with established research on protein-PVA composite films where PVA interacted with canola protein via hydrogen bonds, dipole-dipole attractions and entanglement of polymer chains, creating a more cohesive network (Limpan et al., 2010; Liu et al., 2017; Tian et al., 2023; Yan et al., 2023). Additionally, PVA's semicrystalline domains also provide structural reinforcement, further enhancing the TS.

Elongation at break (EB) is a key indicator of a film's flexibility and ductility. The data in Figure 1b) demonstrate that blending canola protein with PVA significantly influenced the extensibility of the resulting films. Neat canola protein and PVA films demonstrated similar poor flexibility, with EB values of 57.53 ± 8.57 % and 50.49 ± 1.83 %, respectively. The low extensibility of the pure canola protein film reflects the brittle nature of protein-based matrices, which are prone to breaking under stress due to strong protein-protein interactions and limited molecular flexibility (Bandara et al., 2018; Zhang et al., 2018). Interestingly, the pure PVA film in this study displayed

one of the lowest EB values, a result that contrasts with PVA's typical properties as a flexible polymer. This may be attributed to the migration of glycerol, a commonly observed phenomenon in biopolymer-based composite films, which also helps reduce the EB of the films (Castro et al., 2023; Zhang et al., 2021). Glycerol is commonly used as a plasticizer in protein-based films to enhance flexibility by disrupting intermolecular interactions between protein chains (Zhang et al., 2021). However, glycerol tends to migrate from the film's interior to its surface over time, compromising the film's mechanical strength. This migration is primarily driven by two factors: the film's large surface area, which facilitates glycerol diffusion, and internal phase separation between protein-rich and glycerol-rich regions, which creates heterogeneity that promotes uneven distribution and glycerol loss (Tian et al., 2018; Zhang et al., 2021). Furthermore, drying and storage conditions also drive glycerol molecules toward the film surface (Bagheri et al., 2019; Zhang et al., 2021).

Additionally, the high degree of hydrolysis (≥ 98%) and medium molecular weight of the PVA used, both of which tend to increase crystallinity and intermolecular hydrogen bonding, thereby reducing polymer chain mobility (Teodorescu et al., 2019). On the other hand, blended canola protein-PVA films exhibited EB values ranging from 100% to 163%, indicating good flexibility. These results are consistent with previous findings (Yan et al., 2023), which suggest that incorporating PVA significantly improves film ductility. This enhancement is likely attributed to the inherent flexibility of PVA chains and their ability to disrupt the rigid structure of the canola protein matrix. Similarly, (Lara et al., 2019) reported a linear increase in EB values when whey protein isolate-PVA blend films were prepared at 90:10, 80:20, and 70:30 ratios. The authors concluded that PVA may act as a plasticizing agent within the protein network, reducing internal stresses and enhancing polymer chain mobility during tensile deformation (Lara et al., 2019). However, in this study, beyond a certain threshold, such as the 80:20 protein-to-PVA ratio, further increases in PVA content appeared to reduce flexibility. This reduction may be attributed to potential phase separation or increased stiffness resulting from dominant PVA-PVA interactions.

Figure 1c) demonstrated the Young's modulus values of protein-PVA composite films, a critical parameter reflecting the stiffness of a material and its resistance to elastic deformation under applied stress (Long et al., 2022; Svennersten et al., 2011). Although the YM values ranged from approximately 33 MPa to 49 MPa across different formulations, there were no significant

differences among the samples. This suggests that varying the PVA ratio did not significantly affect the overall stiffness of canola protein films. One possible explanation is that the PVA concentration in the blends was insufficient to affect film rigidity. While films with higher PVA content showed slightly higher numerical values, likely due to PVA's semicrystalline structure and strong intermolecular forces (Teodorescu et al., 2019), these differences were not statistically significant, indicating a uniform elastic response across the blends. Furthermore, hydrogen bonding between canola protein and PVA may have formed a stable matrix that maintained similar stiffness across compositions. Overall, the mechanical behavior of the canola protein based-PVA films is largely influenced by the intrinsic characteristics of PVA, particularly its degree of hydrolysis (DH) and molecular weight (MW), both of which play important roles in determining film strength and stiffness. (Limpan et al., 2012) investigated the effect of various DH (86%-99%) and MW (27,000-120,000 g/mol) of PVA on the properties of fish protein-PVA blend films and reported a positive correlation between both DH and MW with TS and EB. The author found PVA with a higher degree of hydrolysis contains a greater proportion of hydroxyl groups, which facilitates extensive intermolecular hydrogen bonding, particularly between PVA chains. This leads to a denser, more compact film structure, thereby enhancing tensile strength (Limpan et al., 2012). Similarly, higher molecular weight corresponds to longer polymer chains, which allow for increased chain entanglement and stronger intermolecular interactions, thereby improving film integrity and resistance to deformation (Limpan et al., 2012). In terms of flexibility, PVA can disrupt rigid protein-protein interactions such as disulfide bonds and hydrophobic associations by introducing dynamic hydrogen bonding between its hydroxyl groups and polar functional groups (e.g., amino, carbonyl, and carboxyl) of canola protein (Limpan et al., 2012). These hydrogen bonds are non-covalent and reversible, allowing them to break and reform under mechanical stress, thus enhancing the film's ductility (Yu et al., 2021). The incorporation of hydrogen-bonding moieties has been shown to improve mechanical performance in polymer networks by promoting chain mobility (Galuska et al., 2022; Lee et al., 2020). The PVA utilized in this study had a molecular weight of approximately 80,000 g/mol, which is classified as medium molecular weight (Thermo Scientific Chemicals, n.d.). At this range, PVA is expected to exhibit a moderate degree of chain entanglement that contributes to a balance between structural strength and flexibility.

4.5.2. FTIR characterization of canola protein-PVA composite films

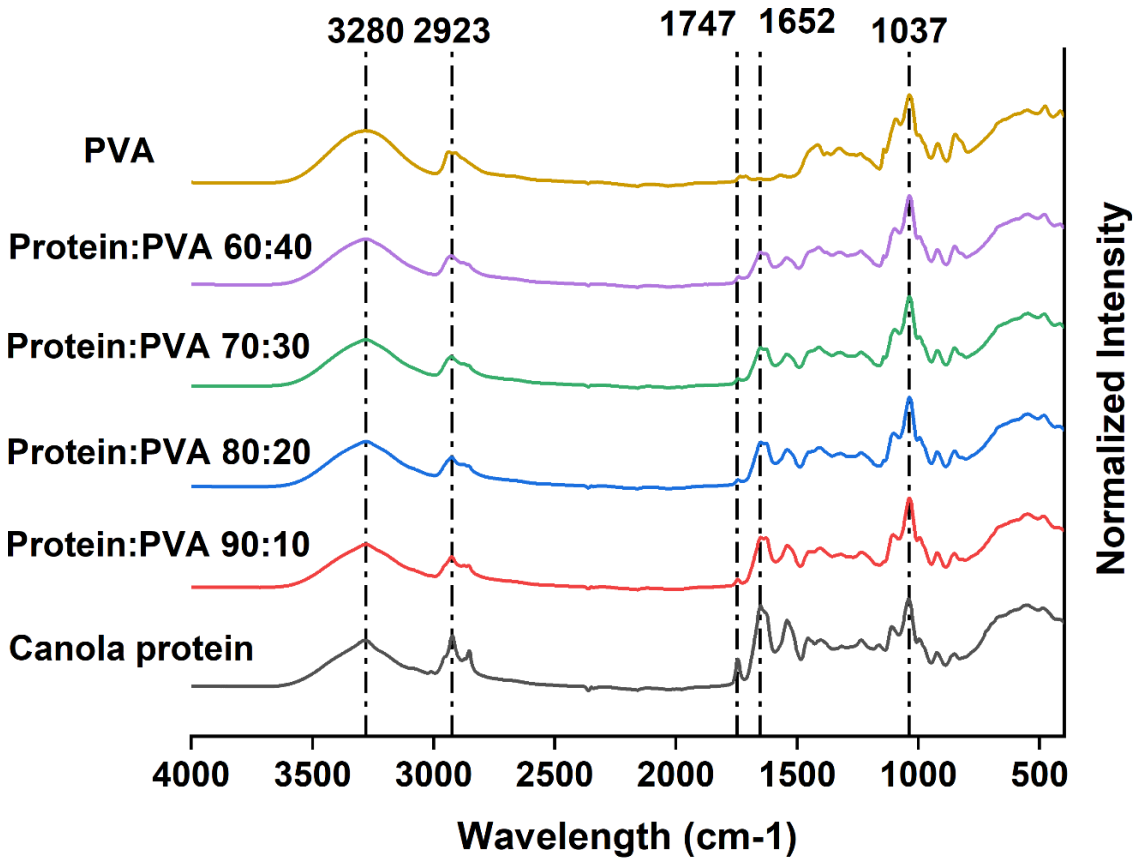

Figure 4.2: FTIR spectra of protein-PVA composite films (100:0, 90:10, 80:20, 70:30, 60:40, and 0:100, w/w)

The FTIR spectra presented in Figure. provide critical insights into the molecular interactions within the canola protein-based PVA composite films. In the pure canola protein film, a sharp absorption band around 3280 cm^{-1} is observed, which corresponds to the stretching vibrations of N-H bonds in the protein backbone, as well as O-H groups from protein side chains and bound water (Lara et al., 2019). The cluster of peaks spanning 2980 cm^{-1} to 2828 cm^{-1} corresponds to the stretching of C-H groups in proteins (S. Li et al., 2017; C. Pires et al., 2013). The presence of amide groups (-CONH-) is a key feature of the protein structure, with characteristic peaks include the Amide I band (1747 cm^{-1} and 1652 cm^{-1}), associated with C=O stretching of peptide linkages and carboxyl groups, and the Amide II band (1540 cm^{-1}), related to N-H bending and C-N stretching (Ji et al., 2020; Schrader, 1995; Stani et al., 2020). A distinct peak at 1747 cm^{-1} is also noted, attributed to C=O stretching of free carboxyl groups present in canola protein (Weal et al.,

2024). In contrast, the FTIR spectrum of the pure PVA film shows a broad O-H stretching band between 3500-3000 cm^{-1}, resulting from extensive intermolecular and intramolecular hydrogen bonding within the polymer and absorbed water (Biranje et al., 2019). Other characteristic PVA peaks include C-H stretching vibrations between 2980-2828 cm^{-1}, C-H bending at 915 cm^{-1} and 850 cm^{-1}, confirming the presence of aliphatic and hydroxyl-rich structures typical of PVA (Limpan et al., 2010).

As PVA is incrementally incorporated into the canola protein matrix, the FTIR spectra of the composite films begin to exhibit changes in peak intensities, suggesting molecular interactions. The O-H/N-H stretching band around 3280 cm^{-1} broadens and loses sharpness, reflecting new hydrogen bonds formed between the hydroxyl groups of PVA and the N-H or carboxyl groups of the protein (Limpan et al., 2010). These interactions disrupt the original protein-protein hydrogen-bonding network and promote new intermolecular interactions that enhance compatibility between the two polymers (Limpan et al., 2010). Simultaneously, a gradual reduction in the peak intensities of the Amide I and II bands is observed in the blend films, suggesting that these protein functional groups are increasingly involved in interactions with PVA, thereby reducing their spectral visibility. Moreover, the disappearance or significant weakening of the 1747 cm^{-1} peak in the blended films suggests that carboxyl groups of canola protein are engaged in hydrogen bonding with the hydroxyl groups of PVA and glycerol, further supporting the formation of an integrated matrix (Dissanayake et al., 2023; Weal et al., 2024). Additionally, a consistent peak at 1037 cm^{-1} in all spectra is attributed to the presence of glycerol (Acquah et al., 2020).

4.5.3. Moisture barrier properties of canola protein-PVA composite films

4.5.3.1 Water vapour permeability (WVP)

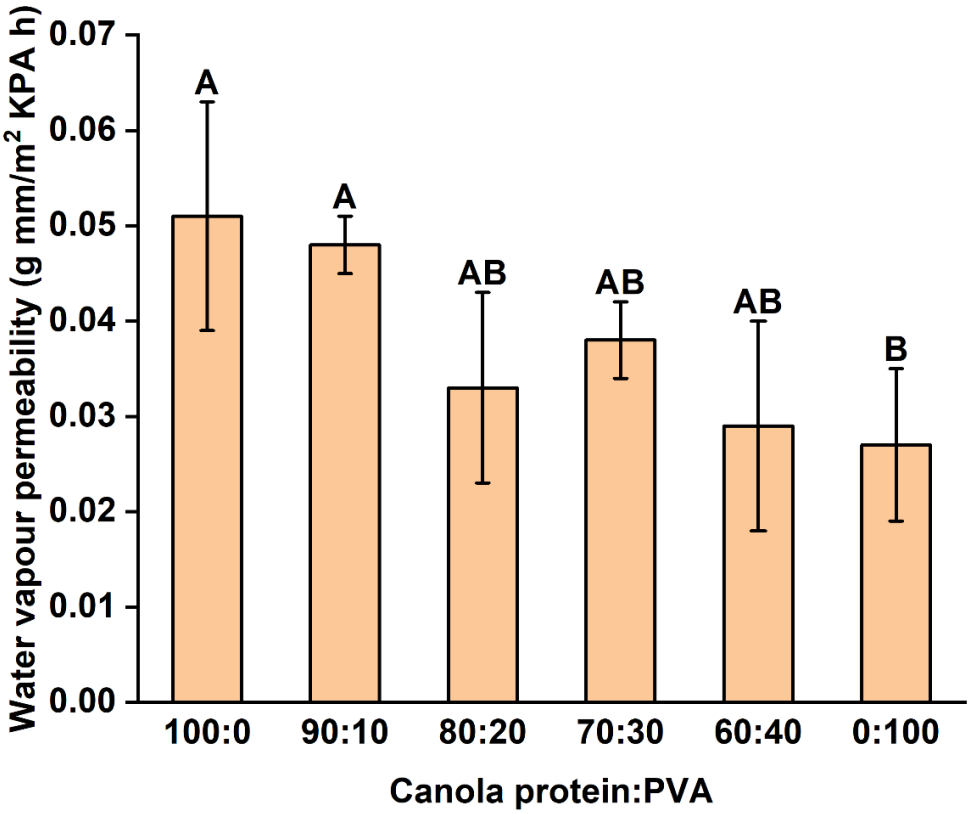

Figure 4.3: Water vapour permeability (g mm/m^2 KPa h) of protein-PVA composite films (100:0, 90:10, 80:20, 70:30, 60:40, and 0:100, w/w).

Table 4.2: Water vapour permeability (g mm/m^2 kPa h), water solubility (%), water contact angle (^{o}) and degradation temperature (^{o}C) of protein-PVA composite films (100:0, 90:10, 80:20, 70:30, 60:40, and 0:100, w/w)

Canola protein: PVA	WVP (g mm/m^2 kPa h)	WS (%)	Water contact angle (^{o})
100:0	0.05 ± 0.01^{A}	23.87 ± 4.05^{B}	59.64 ± 2.06^{A}
90:10	0.05 ± 0.00^{A}	36.38 ± 6.444^{AB}	19.78 ± 0.76^{C}
80:20	0.03 ± 0.01^{AB}	35.55 ± 3.50^{AB}	38.03 ± 1.58^{B}
70:30	0.04 ± 0.00^{AB}	47.23 ± 5.61^{A}	55.60 ± 4.08^{A}
60:40	0.03 ± 0.01^{AB}	27.29 ± 6.22^{B}	35.99 ± 0.52^{B}
0:100	0.03 ± 0.08^{B}	34.31 ± 2.10^{B}	36.24 ± 7.27^{B}

Water vapor permeability is a critical property to evaluate in packaging materials, particularly given their frequent exposure to humid conditions. Assessing WVP is crucial for evaluating the film's effectiveness in preventing water vapor transmission. This is especially important in food packaging, where controlling moisture is essential for maintaining product quality and extending shelf life (Durmaz & Aytac, 2019).

Among the tested films, pure canola protein films exhibited the highest WVP (0.05 ± 0.01 g mm/m^2 kPa h), albeit slightly lower than previously reported values ranging from 0.08 to 0.10 g mm/m^2 kPa h (Chairez-Jimenez et al., 2024; Dissanayake et al., 2022, Dissanayake et al., 2023). The relatively high moisture permeability can be attributed to the abundance of hydrophilic functional groups in canola protein (such as -OH, -COOH, and -NH_2), which facilitates water absorption and vapor diffusion through the film matrix (Limpan et al., 2012). In contrast, pure PVA films showed the lowest WVP at 0.03 ± 0.08 g mm/m^2 kPa h. Despite containing primarily hydrophilic hydroxyl groups, PVA's semicrystalline structure resulted in a denser and more cohesive network that can limit water vapor permeability (Alberto et al., 2020).

A gradual decrease in WVP was observed as the PVA proportion increased, although this difference was not statistically significant. Nonetheless, this suggests that blending with a higher

PVA concentration could improve the water vapor barrier performance of canola protein films. This reduction in WVP may result from molecular interactions between PVA and protein molecules, particularly through hydrogen bonding (Limpan et al., 2012), which increases the tortuosity of diffusion pathways (Sarfraz et al., 2020). As the polymer network becomes more compact and well-structured, water vapor molecules must follow a longer, more tortuous path to diffuse through the film, effectively slowing down permeation (Dissanayake et al., 2023). Similar trends have been observed in soy protein- and starch-based composite films, in which the incorporation of PVA effectively reduced WVP through enhanced polymer cohesion and matrix densification (Alberto et al., 2020; Yan et al., 2023). The degree of hydrolysis and molecular weight of PVA influence the WVP of the films. (Limpan et al., 2012) reported that PVA with higher DH and MW can increase WVP due to the introduction of more amorphous regions and free volume within the polymer matrix, creating pathways for moisture diffusion. The study suggested that selecting PVA with suitable DH and MW can help reduce the WVP of protein-based films.

4.5.3.2. Water solubility (WS)

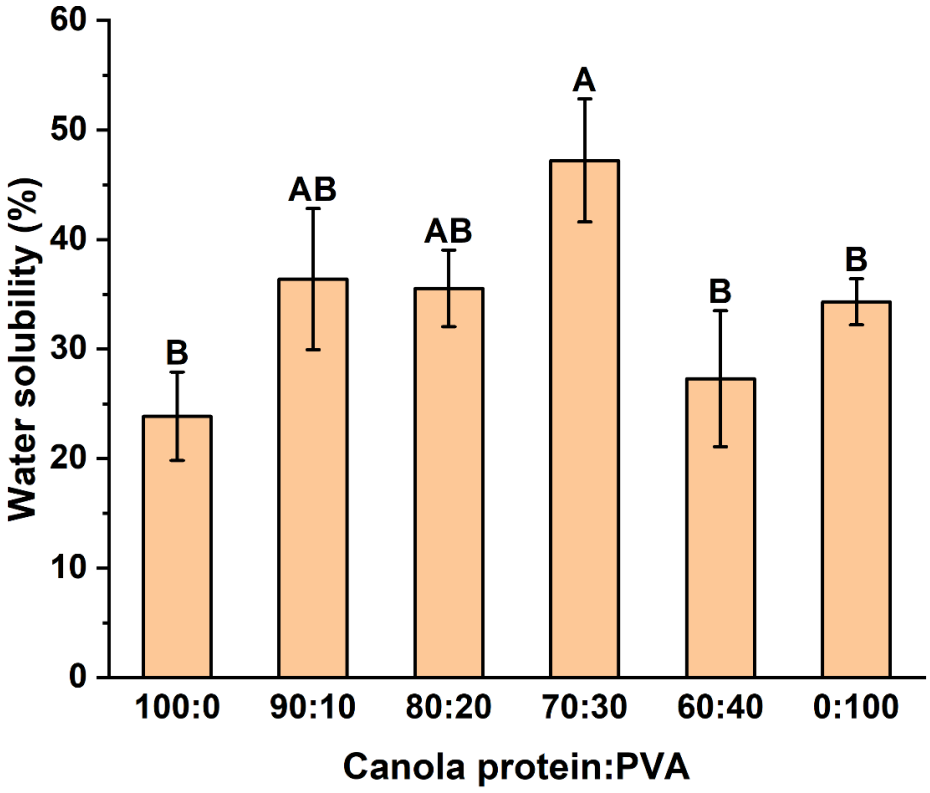

Figure 4.4. Water solubility (%) of protein-PVA composite films (100:0, 90:10, 80:20, 70:30, 60:40, and 0:100, w/w).

Water solubility data for canola protein-PVA films are presented in Table 2. Neat canola protein and neat PVA films exhibited a solubility of 23.87% ± 4.05 and 34.31% ± 2.10, respectively, aligning with values previously reported in literatures (Chairez-Jimenez et al., 2024; Dissanayake et al., 2022, Dissanayake et al., 2023; Limpan et al., 2012). The incorporation of PVA significantly affected the WS of the films ($p < 0.05$). Among the blends, only the 70:30 canola protein-PVA formulation showed a significant increase in water solubility compared to pure canola protein and pure PVA films, reaching approximately 47%. This indicates that at this specific ratio, PVA incorporation effectively enhances solubility, likely due to increased hydrophilicity and partial disruption of the protein network. In contrast, the 90:10 and 80:20 blends, while showing slightly higher solubility (35-37%), were not significantly different. Interestingly, the 60:40 blend remained statistically similar to pure canola protein and pure PVA films. These results indicate

that PVA's effect on water solubility is formulation-dependent and becomes more pronounced at certain ratios. The increase in solubility is likely due to the hydrophilic hydroxyl groups in PVA, which enhance water interaction within the film matrix (Chan et al., 1999). Similar findings have been reported in previous studies, in which the incorporation of PVA into protein composite films increased water solubility (Dong et al., 2024; Limpan et al., 2010). However, some studies have reported the opposite trend, in which the incorporation of PVA decreased water solubility (Dou et al., 2015; Fernandes et al., 2022; Lara et al., 2019). This has been attributed to a denser film structure, in which strong hydrogen bonding between PVA and the protein matrix limits the availability of free hydroxyl groups (Fernandes et al., 2022). As a result, fewer sites are available for interaction with water molecules, thereby restricting water penetration and reducing overall solubility (Fernandes et al., 2022).

These contrasting observations in film properties highlight the influence of PVA's intrinsic physicochemical characteristics, including degree of hydrolysis, molecular weight, and crystallinity (Goodship & Jacobs, 2009b). The relatively low solubility observed in the pure PVA film in this study can be attributed primarily to its high degree of hydrolysis and medium molecular weight, both of which reduce polymer-water interactions and enhance resistance to dissolution (Goodship & Jacobs, 2009b). The degree of hydrolysis plays a critical role in modulating PVA's water solubility. As the DH increases, the acetate groups are replaced by hydroxyl groups, which enhances the polymer's ability to form intra- and intermolecular hydrogen bonds (Harpaz et al., 2019). While hydroxyl groups are typically hydrophilic, their extensive hydrogen bonding within and between PVA chains reduces the availability of interaction sites for water molecules, thereby diminishing water uptake and solubility (Harpaz et al., 2019; N. Jain et al., 2017). In this study, the PVA used was ≥ 98% hydrolyzed, which is known to exhibit poor solubility in water below 80 °C due to its dense hydrogen-bonding network (Teodorescu et al., 2019). Additionally, molecular weight influences water uptake into the polymer matrix, with a linear relationship observed(Goodship & Jacobs, 2009; Harpaz et al., 2019). Higher molecular weight corresponds to longer polymer chains, which leads to greater chain entanglement and stronger intermolecular interactions (An, 2022; Chan et al., 1999). These structural features can limit water penetration, thereby decreasing solubility. In contrast, lower molecular weight polymers have shorter chains with fewer entanglements and weaker molecular interactions, leading to increased solubility (An, 2022).

Aside from intrinsic material properties, processing conditions during film formation also significantly influence the final solubility. Under slow drying conditions, PVA chains have more time to align and form microcrystalline regions (F. Y. Jing & Zhang, 2022). These crystalline domains act as physical crosslinks within the amorphous matrix, hindering the penetration and diffusion of water molecules (F. Y. Jing & Zhang, 2022). The extent of crystallinity is affected by drying conditions, such as temperature, humidity, and the rate of water evaporation, as well as the presence of additives, such as plasticizers (F. Y. Jing & Zhang, 2022; Packter & Nerurkar, 1968; F. Wu et al., 2023).

4.5.3.3. Water contact angle (WCA)

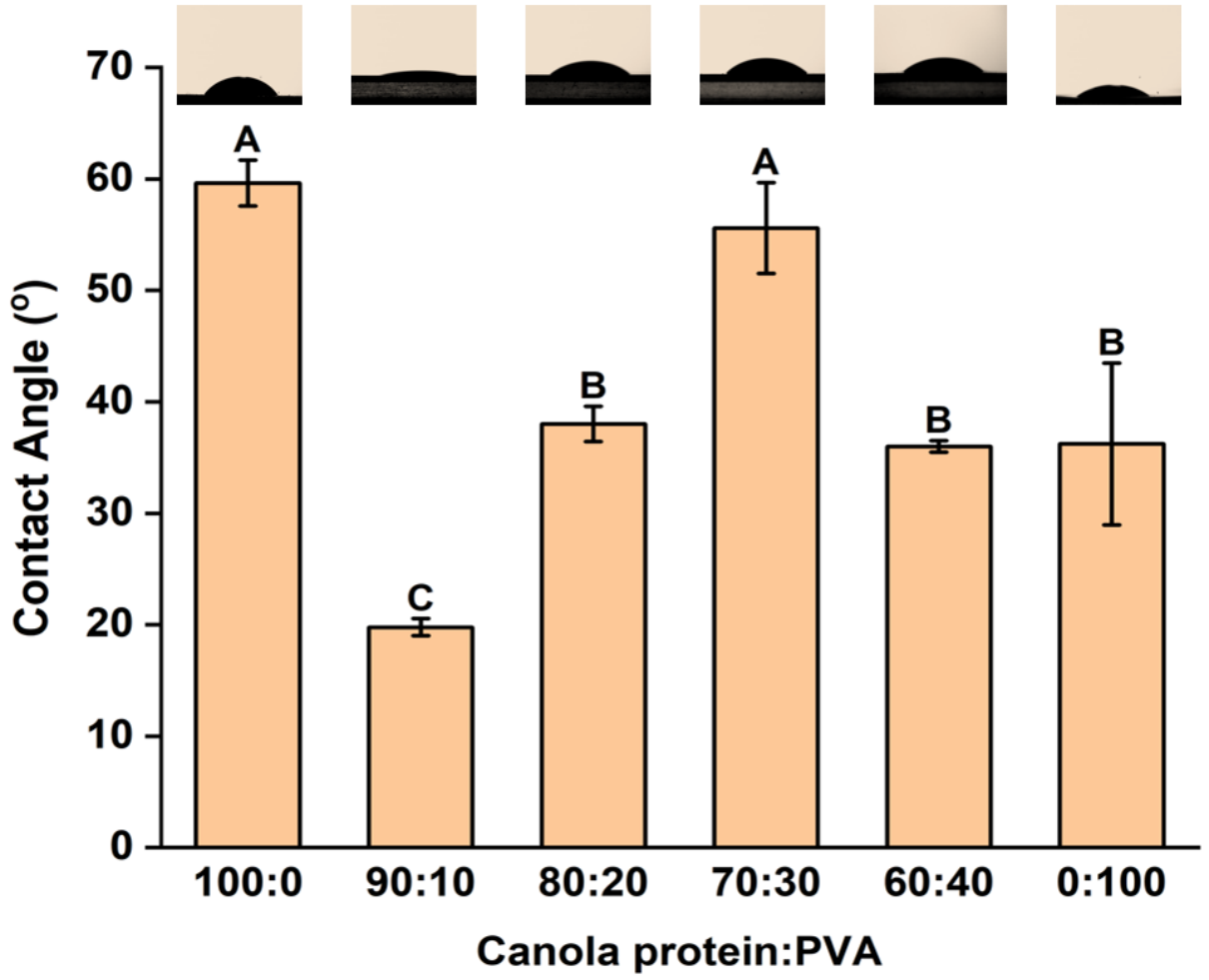

Figure 4.5. Contact angle (°) of protein-PVA composite films (100:0, 90:10, 80:20, 70:30, 60:40, and 0:100, w/w).

The water contact angle test is crucial for evaluating the wettability of canola protein film samples for food packaging applications. The contact angle measurements for films prepared with varying

canola protein-to-PVA ratios are presented in the Table 2. The results reveal that PVA significantly influences surface wettability ($p < 0.05$). According to Vogler (1998), surfaces with contact angles greater than 65^o are considered hydrophobic, while those below this threshold are hydrophilic. The neat canola protein film exhibited the highest contact angle (59.64 ± 2.06 ^{o}), indicating a moderately low wettability, though still falls below the hydrophobic threshold. This is consistent with previously reported results (Dissanayake et al., 2022) and is likely due to the surface orientation of non-polar amino acid residues. On the other hand, the pure PVA film showed a contact angle of 36.24 ± 7.27 ^{o}, within the reference range of 40 ^{o}-55 ^{o} (Kim et al., 2015; Wu et al., 2020; Yang et al., 2020), indicating a hydrophilic nature. Among the blends, the 70:30 formulation showed a contact angle (55.60 ± 4.08 ^{o}) similar to that of the pure canola protein film. However, all other formulations exhibited lower contact angles, ranging from 19 ^{o} to 38 ^{o}, indicating a further shift toward hydrophilicity.

The incorporation of PVA did not increase surface hydrophobicity in the canola protein-based PVA composite films, which can be attributed to PVA's intrinsic hydrophilic nature. PVA is rich in hydroxyl groups, which have a strong affinity for water molecules and readily promote hydrogen bonding (J. G. Hong et al., 2015). The hydrophilicity of neat PVA films has been well-documented, and in some cases, PVA films have demonstrated even higher surface hydrophilicity than neat biopolymer films (Ahn et al., 2024; Al-Tayyar et al., 2020; Jayasekara et al., 2004; Liu et al., 2022; Park et al., 2023; Yan et al., 2021). When blended with biopolymers, PVA can increase the density of hydrophilic groups within the film matrix and potentially at the film surface, thereby enhancing the surface wettability of the composite films (Al-Tayyar et al., 2020; Patil et al., 2021; F. Xu et al., 2015).

In addition to the inherent hydrophilicity introduced by PVA, glycerol migration toward the film surface further contributed to the observed decrease in contact angle. Over time, hydrophilic plasticizers such as glycerol tend to migrate toward the film surface due to their weaker secondary interactions with the polymer chains (Eslami et al., 2023; Zhang et al., 2021). This migration often occurs during the drying process, resulting in a surface layer enriched with hydroxyl groups capable of forming hydrogen bonds with water at the air-film interface, thereby significantly lowering the contact angle (Basiak et al., 2018; Chairez-Jimenez et al., 2024; Dissanayake et al., 2022, Dissanayake et al., 2023). Factors influencing glycerol migration include drying and storage

conditions, the compatibility between glycerol and the polymer network, and the cohesiveness of the film matrix (Bagheri et al., 2019; Vieira et al., 2011; Zhang et al., 2021).

4.4.4.4. Surface morphology of protein-PVA composite films

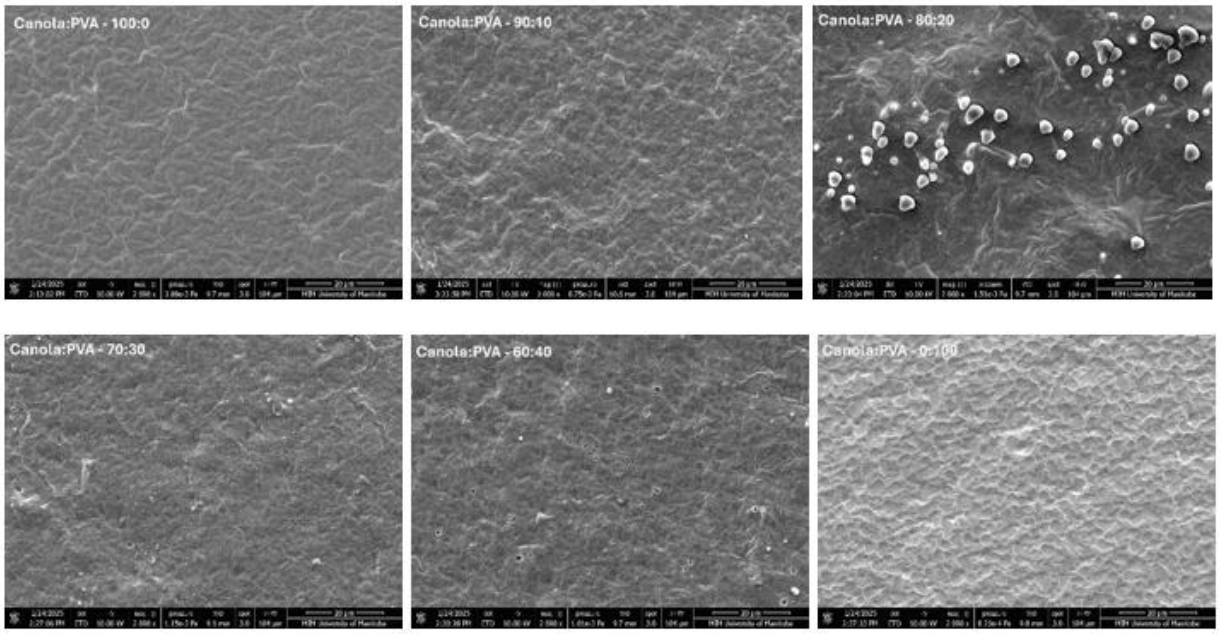

Figure 4.6. SEM images of the surface of canola protein- PVA blended films (100:0, 90:10, 80:20, 70:30, 60:40, and 0:100, w/w) at x2000 magnification.

Surface morphology of canola-PVA blend films was analyzed using SEM at x2000 magnification to evaluate the influence of PVA (Figure). The neat canola protein film exhibited a relatively smooth and homogeneous surface. This smoothness may be attributed to the homogeneity of the pure canola protein network and its ability to form a compact structure during film casting and drying. Similarly, the pure PVA film exhibited a continuous, seemingly homogeneous surface.

In contrast, the incorporation of PVA into the canola matrix noticeably altered the surface characteristics of the films. As the PVA content increased from 0% to 40%, the films became rougher with more heterogeneous surfaces. SEM images revealed phase-separation features, including microstructural discontinuities and surface irregularities, particularly pronounced in the 80:20, 70:30, and 60:40 films. Among them, the 80:20 film exhibited the most pronounced surface heterogeneity, with visible PVA particulate aggregates across the film surface. The 70:30 and 60:40 films exhibited noticeable surface pores, suggesting disruption of the matrix continuity.

Similar findings have been reported in other protein-PVA systems. (W. Yan et al., 2023) observed that incorporating increasing concentrations of PVA (0.2–0.8% w/v) into soy protein films led to increased surface heterogeneity, whereas neat films remained relatively uniform. The study attributed the phase separation to the semicrystalline nature of PVA and its limited compatibility with the protein matrix at high concentration. In another study, Balavairavan et al. (2023) developed PVA-corn protein blend films (5–25% wt.). They reported greater phase separation at higher protein content, demonstrating that limited polymer compatibility is a key factor contributing to the structural irregularities observed in the films.

Segregative phase separation in aqueous biopolymer systems, such as between PVA and protein, is a common phenomenon primarily driven by thermodynamic incompatibility (Hu et al., 2024). Thermodynamic incompatibility refers to an energetically unfavorable condition, such as the blending of polymers with limited miscibility, causing them to separate into distinct phases and self-associate (Grinberg & Tolstoguzov, 1997). This typically occurs when there are significant structural and chemical differences between the polymers (Grinberg & Tolstoguzov, 1997). In this case, PVA is a semicrystalline polymer with hydrophilic domains, while proteins are predominantly amorphous with both hydrophilic and hydrophobic regions (Assender & Windle, 1998; Cháirez-Jiménez et al., 2023; Zhang et al., 2018). At lower PVA concentrations, there is sufficient protein-PVA interactions, which help to maintain a relatively homogeneous matrix. However, as PVA content increases, PVA-PVA interactions become more dominant, reducing the compatibility with the protein matrix. This shift promotes phase separation, resulting in microstructural discontinuities and the formation of PVA-rich and protein-rich domains. As a result, SEM images reveal surface roughness, pores, and particulate aggregates. These morphological changes reflect the limited miscibility between PVA and the protein and may significantly affect the functional characteristics of the resulting films, including mechanical strength and barrier performance.

4.4.4.5. Thermal properties of protein-PVA composite films

Table 4.3: Thermal analysis of canola protein-PVA films: onset temperature (ºC), melting temperature (ºC), and enthalpy (J/g)

Canola protein: PVA	Extrapolated onset temperature (ºC)	Melting temperature (ºC)	Enthalpy (J/g)
100:0	247.69	248.97	43.741
90:10	247.88	249.70	48.012
80:20	233.41	235.16	78.020
70:30	256.30	257.72	47.735
60:40	222.98	224.05	111.153
0:100	290.13	290.13	27.881

The differential scanning calorimetry results are presented in Table 3, revealing notable changes in the thermal properties of canola protein-based films upon incorporation of PVA. The extrapolated onset temperature, peak temperature, and enthalpy provide insight into the thermal transitions, molecular interactions, and overall thermal stability of the films.

The neat PVA film exhibited the highest melting temperature of 290.13 ºC, consistent with PVA's known thermal stability, given its semicrystalline nature (Pugar et al., 2024). The neat canola protein film exhibited a similar melting temperature of 248.97 ºC, within the range of 230 ºC - 280 ºC reported in the literature (Chairez-Jimenez et al., 2024; Dissanayake et al., 2022, Dissanayake et al., 2023). Incorporating PVA significantly influenced the thermal properties of the canola protein-based films ($p < 0.05$), although no consistent trend was observed across the blends. The melting temperatures and enthalpy values varied, with the 70:30 canola protein-to-PVA blend showing the highest melting temperature of 257.72 ºC, likely due to better hydrogen bonding and network formation between the components. In contrast, the 80:20 and 60:40 ratios exhibited reduced melting temperatures (235.16 ºC and 224.05 ºC, respectively), potentially indicating partial phase separation and weaker interactions between the components. Phase separation may result in distinct protein and PVA domains, reducing crystallinity and thermal stability compared to the neat films (Naderizadeh et al., 2019; Suleiman et al., 2024). The blending process may also disrupt crystalline regions, increasing the proportion of less stable amorphous

structures (Naderizadeh et al., 2019; Suleiman et al., 2024). Furthermore, the presence of glycerol can also reduce crystallinity and lower thermal stability (Luo et al., 2012; Suleiman et al., 2024).

On another note, other studies have reported enhanced thermal stability in blended films, which they attribute to strong hydrogen bonding between polymer components (Tanwar et al., 2021; Yu et al., 2022). These interactions promote greater cohesion within the film matrix, resulting in improved compatibility and a more uniform polymer network that resists thermal degradation more effectively (Tanwar et al., 2021; Yu et al., 2022).

4.6. Conclusion

This study examined the potential of PVA to improve the functional properties of canola protein-based films, particularly mechanical strength, water vapor barrier performance, and thermal stability. Blending canola protein with varying concentrations of PVA resulted in significant improvements in tensile strength and flexibility, helping to overcome the inherent brittleness of pure protein films. PVA also showed potential in reducing water vapor permeability. The degree of hydrolysis and molecular weight of PVA influenced film performance, underscoring the importance of selecting appropriate PVA types to optimize film characteristics. However, several challenges were also identified with PVA usage. At higher PVA blend ratios, phase separation may occur, compromising film uniformity and stability. Furthermore, due to its hydrophilic nature, PVA does not significantly reduce moisture sensitivity. Although performance was slightly improved, the barrier properties of canola protein-PVA films remain inferior to those of conventional plastics. Additionally, since PVA is synthetic and not fully biodegradable under natural conditions, its incorporation may compromise the biocomposite's overall environmental sustainability. Future research should include long-term biodegradability studies and life cycle assessments to better evaluate the environmental impact and practical potential of protein-PVA films.

4.7. References

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Chapter 5: Conclusions and Future Research Directions

This thesis investigated the development and characterization of canola protein-based biodegradable films enhanced through two complementary strategies: 1) incorporation of essential oils carvacrol and cinnamaldehyde, and 2) blending with PVA.

5.1. Summary of the findings of 1st study – addition of two essential oils, carvacrol and cinnamaldehyde:

- The mechanical properties of the films were affected by the addition of essential oils. Increasing oil concentration led to modest reductions in both tensile strength and elongation at break.
- Water solubility also showed a slight decline at the 5% oil concentration compared to the 1% and 3% levels, with values ranging from 7-20% at 5% oil, 16-30% at 3% oil, and 16-29% at 1% oil.
- No significant differences were observed in water vapour permeability, as all films displayed values within the range of 0.06-0.09 g mm/m^2 kPa h.
- Water contact angle measurements indicated a slight decrease with increasing oil concentration. The control film had a contact angle of approximately 34º. Films containing 1% oil exhibited contact angles between 38° and 62°, those with 3% oil ranged from 23° to 44°, and films with 5% oil ranged from 32° to 44°, reflecting minor changes in surface hydrophobicity.
- DSC showed no notable changes in thermal properties, with protein denaturation temperatures ranging from 232-260 °C. The control film showed a denaturation temperature of 241.50°C.
- Antimicrobial analysis showed that the control and 1% oil films had no inhibitory effect. Films with 3% oil produced inhibition zones of 0.26-1.03 cm against *E. coli* and 0.9-1.6 cm against *S. aureus*. The 5% oil films exhibited the strongest antimicrobial performance, with inhibition zones ranging from 0.6 cm to 1.0 cm for *E. coli* and 1.5 cm to 2.5 cm for *S. aureus*. No clear trend was observed regarding the effect of oil ratios. Both carvacrol and cinnamaldehyde displayed greater antimicrobial activity against *S. aureus* (gram-positive) than against E. coli (gram-negative). Cinnamaldehyde demonstrated higher bactericidal

activity against both *E. coli* and *S. aureus* than carvacrol, as indicated by its lower minimum bactericidal concentration (20.83 µL/mL vs. 83.33 µL/mL, respectively).

Overall, both carvacrol and cinnamaldehyde improved antimicrobial activity and influenced the physical characteristics of the films. However, it is critical to select the correct amount, as the matrix has limited miscibility. Additionally, a clear synergistic effect was not observed when combining the essential oils.

5.2. Summary of the findings of 2nd study - addition of PVA:

- Tensile strength increased gradually with higher PVA content. Pure canola protein film exhibited the lowest strength (1.733 ± 0.333 MPa), while the 60:40 film, which contained the highest proportion of PVA, showed the greatest tensile strength among the blended films (4.423 ± 0.098 MPa). In terms of elongation at break, the pure canola protein film demonstrated limited flexibility (57.525 ± 8.536%), whereas the 80:20 film reached 163.818 ± 38.619%. No substantial changes in Young's modulus were observed across all film formulations.
- Water solubility remained largely unchanged except for the 70:30 film, which exhibited significantly higher solubility compared to the pure canola protein film (47.231 ± 5.612% vs. 23.364 ± 2.468%, respectively). This increase is likely due to the introduction of additional hydroxyl groups from PVA into the polymer matrix.
- No significant differences were observed in water vapour permeability, although a slight decreasing trend was observed. All values ranged between 0.03 and 0.05 g mm/m^2 kPa h.
- Water contact angle decreased for all blended films compared to the pure canola protein film, except for the 70:30 formulation, which remained unchanged. Overall, the values ranged from approximately 20 to 60, indicating high surface hydrophilicity.
- The pure PVA film exhibited the highest thermal denaturation temperature of 290.13 °C. The pure canola protein films and all blended films showed denaturation temperatures ranging from approximately 235 to 258 °C.

The second study demonstrated that canola protein is compatible with PVA, as its incorporation can enhance the strength and flexibility of canola protein films. However, no significant change in water resistance was observed, and surface hydrophilicity increased slightly due to the introduction of additional hydroxyl (OH) groups into the matrix.

5.3. Future research directions:

The present study primarily focused on enhancing the water barrier, antimicrobial, and mechanical properties of canola protein-based films. While some improvements were observed in antimicrobial properties, the water barrier, and mechanical properties, overall enhancements remained limited, highlighting the need for further research to optimize film performance.

One promising approach is to encapsulate essential oils in nanocarriers to enhance their incorporation into the film matrix. As observed in the first study, higher concentrations EOS can lead to film heterogeneity and oil leakage. Encapsulation could improve the stability and miscibility of the oils during film formation, prevent premature volatilization, and thereby maintain the structural integrity of the films while enhancing antimicrobial efficacy. Furthermore, future studies should investigate the release kinetics of essential oils from the films. Understanding the rate and pattern of oil release is essential for balancing sustained antimicrobial activity with the mechanical and barrier properties of the films.

Another important avenue for future research is the assessment of film biodegradability. While canola protein, EOS, and PVA are inherently biodegradable, understanding their real-time degradation rates is critical. Evaluating biodegradability under various environmental conditions will provide valuable insights into the environmental impact and overall sustainability of these active packaging materials.

Finally, exploring extrusion as a film fabrication method instead of solvent casting could bring the research closer to industrial applications. Extrusion mimics large-scale production conditions and may influence the mechanical, barrier, and functional properties of the films, making it an important consideration for future studies aiming to scale up these biodegradable packaging materials.

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Appendix: Study 1 Supplementary Materials

Table S1: Minimum inhibitory concentration (µg/µL) and minimum bactericidal concentration (µg/µL) of carvacrol and cinnamaldehyde against *S. aureus* and *E. coli* in MHB at pH 7 and 37°C.

Essential oil	Row	Essential oil concentration (µL/mL)	*E. coli* (ATCC 15597) MIC (µL/mL)	*E. coli* (ATCC 15597) MBC (µL/mL)	*S. aureus* (ATCC 25923) MIC (µL/mL)	*S. aureus* (ATCC 25923) MBC (µL/mL)
Carvacrol only	A	333.33				
	B	166.67				
	C	83.33				
	D	41.67				
	E	20.83		83.33		83.33
	F	10.42	10.42		10.42	
	G	5.21				
	H	2.60				
Cinnamaldehyde only	A	333.33				
	B	166.67				
	C	83.33				
	D	41.67				
	E	20.83		20.83		20.83
	F	10.42	10.42		10.42	
	G	5.21				
	H	2.60				

Carvacrol & Cinnamaldehyde 1:1	A	333.33	20.83	20.83	20.83	20.83
	B	166.67				
	C	83.33				
	D	41.67				
	E	20.83				
	F	10.42				
	G	5.21				
	H	2.60				

Sample calculation of MIC of cinnamaldehyde for *E.coli and S.aureus*

In row A, 50 µL of cinnamaldehyde was added.

Therefore, the concentration of cinnamaldehyde at row A (µL/mL) =

$$\frac{\text{Volume of cinnamaldehyde at row A (µL)}}{Total\ volume\ (\mu L)} \text{ x } \frac{1000\ \mu L}{mL} = \frac{50}{150}\ x\ 1000 = 333.33\ (\mu L/mL)$$

Since 100 µL (or 0.1 mL) of the mixture in row A was transferred to row B, the concentration of cinnamaldehyde at row B (µL/mL) =

$$\frac{\text{Concentration of cinnamaldehyde at row A(µL/mL) x 0.1 (mL)}}{Total\ volume\ (\mu L)} \text{ x } \frac{1000\ \mu L}{mL}$$

$$= \frac{333.33 \text{ x } 0.1 \text{ x } 1000}{200} = 33.33\ (\mu L/mL).$$

The same calculation was applied to calculate the concentrations of cinnamaldehyde from rows C to H.

MIC of cinnamaldehyde (µL/mL) = Concentration of cinnamaldehyde at row F (µL/mL) =

$$\frac{Concentration\ of\ cinnamaldehyde\ at\ row\ E\ (\mu L/mL)\ x\ 0.1\ (mL)}{Total\ volume\ (\mu L)} \text{ x } \frac{1000\ \mu L}{mL}$$

$$= \frac{20.83\ x\ 0.1\ x\ 1000}{200} = 10.42\ (\mu L/mL).$$

MBC of cinnamaldehyde l (µL/mL) = Concentration of cinnamaldehyde at row E (µL/mL) =

$$\frac{Concentration\ of\ cinnamaldehyde\ at\ row\ D\ (\mu L/mL)\ x\ 0.1\ (mL)}{Total\ volume\ (\mu L)} \text{ x } \frac{1000\ \mu L}{mL}$$

$$= \frac{41.67\ x\ 0.1\ x\ 1000}{200} = 20.83\ (\mu L/mL).$$

Table S2: Comparison of prepared EO concentration ([Oil], uL/mL) and equivalent MIC (µL/mL) and MBC (µL/mL) levels for *E. coli* and *S. aureus.*

Oil levels (%, v/v)	**V_{EO} added (mL, v/v)**	**[Oil] (uL/mL)**	**[Oil] versus cinnamaldehyde MIC of *E. coli* and *S. aureus***	**[Oil] versus cinnamaldehyde MBC of *E. coli* and *S. aureus***	**[Oil] versus carvacrol MIC of *E. coli* and *S. aureus***	**[Oil] versus carvacrol de MBC of *E. coli* and *S. aureus***
1	0.7	10.00	Slightly below	Below	Slightly below	Slightly below
3	2.1	30.00	Higher	Higher	Below	Below
5	3.5	50.00	Higher	Higher	Below	Below

Sample calculation of oil concentrations:

Volume of EO (µL) added at 1%, 3% and 5% (v/v):

0.7 mL = 700 µL

2.1 mL = 2100 µL

3.5 mL = 3500 µL

Oil concentration (uL/mL) = $\frac{Oil\ volume\ (\mu L)}{Total\ volume\ (mL)}$

Therefore, oil concentration (uL/mL) at 1% = $\frac{700\ \mu L}{70\ mL}$ = 10.00 (uL/mL)

Similarly, oil concentration (uL/mL) at 3% = $\frac{2100\ \mu L}{70\ mL}$ = 30.00 (uL/mL)

And oil concentration (uL/mL) at 5% = $\frac{3500\ \mu L}{70\ mL}$ = 50.00 (uL/mL)