Preparation and characterization of active and intelligent PVA/Starch films using propolis extract and anthocyanin for food packaging applications.

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Dedication

This thesis is dedicated to my parents, daughter and husband.
For their endless love, support and
encouragement.

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Praise is due to **Allah** whose worth cannot be described by speakers, whose bounties cannot be counted by calculators, whom the height of intellectual courage cannot appreciate, and the diving's of understanding cannot reach; He for whose description no limit has been laid down, no eulogy exists, no time is ordained, and no duration is fixed.

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Pakeeza Mustafa

Abstract

Active and intelligent food packaging films have taken more importance over conventional packaging. The aim of this study was to develop active and intelligent food packaging films based on bio-degradable polymers (PVA and starch) incorporated with natural additives i.e. Propolis extract (PE) and Anthocyanin (ATH). Boric Acid (BA) and Glutaraldehyde (GA) were used as cross-linkers. These films are capable to resist against external bacterial attack by their anti-bacterial activity and color changing property due to change in pH.

The results proved the compatibility of films mixture using two cross-linkers separately i.e. BA and GA. The mechanical strength was also measured and highest values were achieved 6.1 MPa and 15.9 MPa for PSB5A20PE and PSGA5A20PE, respectively. Moreover, the maximum zone of inhabitation i.e. 21 mm and 15mm was also achieved against E. Coli and MRSA, respectively for PSB5A20PE. However, the maximum zone of inhibition of 25 mm and 20 mm against E. Coli and MRSA was achieved for PSGA5A20PE, respectively. Furthermore, all films had shown great color response against different pH ranging from 1 to 14. Physical tests i.e. water vapor transmission rate, moisture retention capability, swelling degree and biological leaching tests were also performed to evaluate the physical compatibility of these films with food. Finally, food spoilage test was performed using pasteurized milk to validate the applicability of these films' formulations. Films responded visibly by changing color and protected milk from spoilage through anti-bacterial property.

All results have shown that PSB5A20PE and PSGA5A20PE film formulations have performed best against all evaluation techniques. Hence, these bio-degradable active and intelligent films have potential to be used in food packaging applications.

Keywords: Propolis Extract; Anthocyanin; Polyvinyl Alcohol; Boric Acid; Glutaraldehyde; Active Packaging, Intelligent packaging films; food spoilage test.

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Abbreviations

Polyvinyl Alcohol	PVA/P
Starch	S
Boric Acid	BA/B
Glutaraldehyde	GA
Propolis Extract	PE
Anthocyanin	ATH/A
Escherichia coli	E. coli
Methicillin resistant Staphylococcus Aureus	MRSA

Chapter: 01

1. Introduction

The growing environmental and health concerns have shifted the market prevalence from conventional petroleum products to biodegradable food packaging materials [1, 2]. Food packaging market revolves around the consumer needs and preferences to get high quality food with maintained freshness and nutrients. For prolonged shelf-life and quality of food, active and intelligent packaging is the emerging concept in food industry [3]. Therefore, food packaging industry has a high demand of mechanically sound active and intelligent food packaging films.

1.1. **Bio-degradable food packaging**

Bio-degradable polymers are such materials which can be dis-integrated without harming the environment. They need proper temperature, moisture and oxygen for dis-integration. As a result, they do not transmit any kind of dangerous material into environment that can be harmful for ecological system. On the other hand, petroleum based plastic packaging has many advantages like ease in handling with good mechanical strength but they are not bio-degradable. Conventional plastic packaging is main reason for all kind pollution in water and land. So, it is the hour of need to research on more options for bio-degradable packaging that is more economical and eco-friendlier.

There are many bio-degradable polymers available in the market. Bio-degradable polymer are classified into three main types; a) Bio-polymers b) Synthetic polymer and c) microbial derived polymers[4]. Their classification is presented in figure: 1;

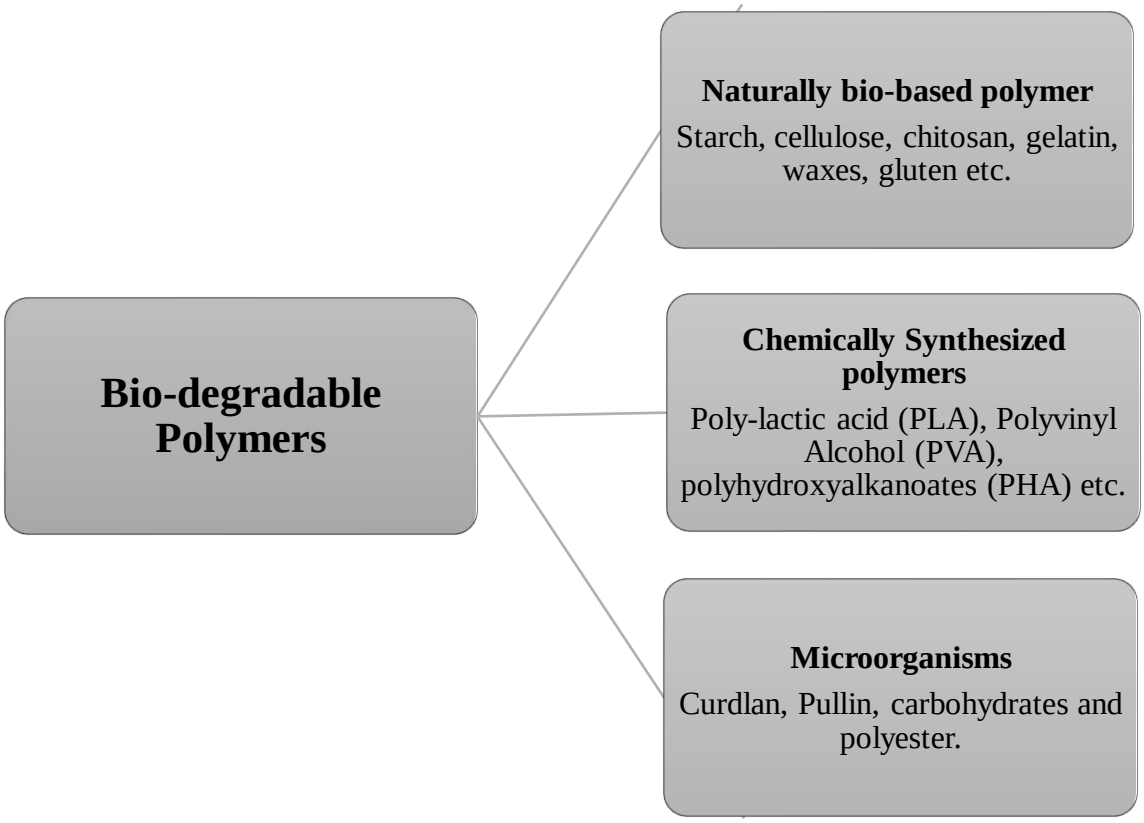

Figure 1: Classification of bio-degradable polymers[4].

Polymers like polyvinyl alcohol (PVA) and starch have very low market value because of poor mechanical and physical properties. PVA-Starch films are the appropriate substitute for conventional plastic packaging material owing to the excellent film-forming ability, gas and optic barrier, hydrophobicity, bio-degradability, high tensile strength and flexibility of PVA material[5]. However, to broaden the applications of PVA in the food packaging industry, its physical properties can be tailored by adding different cross-linkers. In literature, there are many cross-linkers that have been studied so far such as, malic acid, glyoxal, boric acid, Glutaraldehyde, hexamethylene diisocyanate and citric acid[6]. Both polymers are biodegradable and humidity sensitive materials, which cannot be used as a food packaging films without tailoring their properties [7]. For tailoring its mechanical strength and water resistant properties, different cross-linkers like boric acid, Citric acid, maleic acid, acetic acid and Glutaraldehyde etc. are incorporated [8].

The study done by Rohlmann et al, 2008 have proved that cross-linking of boric acid with PVA and starch have improved their mechanical, water resistant and thermal properties [9]. Furthermore, it does not have any harmful effect on human health. Owing to all these reasons, boric acid can be used as cross-linker for food packaging films.

Similarly for another study, Glutaraldehyde was used as cross-linker due to its ability to react in intermolecular spaces and increase the cross-link density[10]. As a result, more hydrophobic and stable films were produced.

Many base polymers are available to incorporate PE and ATH into the films matrix; such as Chitosan, poly lactic acid (PLA), poly vinyl alcohol (PVA) and starch [11]. Starch is the oldest base polymer used for packaging due to its bio-compatibility, bio-degradability and safe to the environment, but lacks in mechanical strength and water-resistant properties. However, these properties can be improved by blending it with other polymers like PVA [12].

1.2. Active packaging

Active food packaging refers to the anti-microbial packaging with the integrated active agent which hinders bacterial growth, protect food and extend shelf life [13]. Active packaging system aims to prevent food from external microbial attack and retard the growth of bacteria and pathogens. Anti-microbial activity in package material can be acquired by four different modes presented in figure: 2.

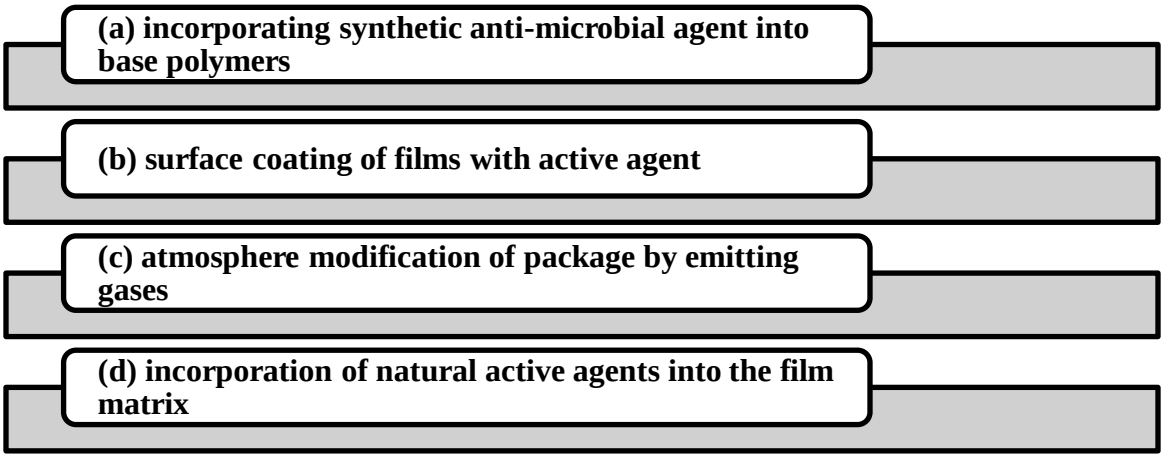

Figure 2: Different modes of active food packaging[14-16].

Active agents are divided into two categories;

(i) Conventional chemically synthesized active agents

(ii) Naturally extracted active agents derived from plants, animals and micro-organism.

Chemically synthesized active agents include types of organic acids and salts such as potassium sorbate, propionic acid, acetic acid, ascorbic acid, benzoic acid and alcohols. These agents are readily available in the market due to their low prices and affordability. However, naturally extracted active agents have attracted the consumer's interest due to their low toxicity compared to synthetic agents [17]. Naturally produce active agents are plants extracts (e.g. tea tree oil, rosemary oil), peptides and ninsin[18]. Other than natural sources nano-particles (silver, zinc, titanium and gold) are also incorporated into packaging films for anti-microbial activity[19]. The use of essential oil in food packaging is now limited due to undesirable secretion of a organoleptic agent, which changes color and texture of food[20]. Recently propolis (a by-product of beeswax) is used for antibacterial and anti-inflammatory purposes [21].

Propolis is the sticky resinous compound collected from beeswax, and is composed of 50% resinous flavonoids containing poly-phenols, 30% wax, 10% essential oil and remaining constitutes various organic components and pollen[22]. There are three different chemical compositions of extracted propolis; (i) Ethanol extracts of propolis (EEP) (ii) water extracted propolis (WEP) and (iii) volatile oil from propolis (VOP). EEP is mostly used in food packaging due to its anti-bacterial, anti-viral and anti-oxidant properties. The chemical composition of flavonoids present in PE mainly includes pinocembrin, quercetin, galanin, chrysin, kaempferol and naringenin [23]. PE can be incorporated into food packaging films as active agent due to its anti-bacterial properties.

1.3. Intelligent food packaging

Intelligent food packaging refers to on-time monitoring and an indication of any change happened to the pH of food during transportation and handling [24].

There are sensors, bar codes, time-temperature indicator, gas indicator and pH indicator. pH indicators are particularly applied for intelligent food packaging due to their availability and cost-effectiveness [25].

There are a large number of synthetic pH indicators that have been used for investigating freshness of food. For example, bromocresol green, bromothymol blue and polyanaline are used as pH indicator for checking the quality of food. However, these synthetic pigments have hazardous effect on human health and therefore; not suitable for food packaging[26].

Anthocyanin is the naturally occurring pigment which can be used as pH indicator due to its quick color change response and non-toxicity[25]. There are many plant sources of anthocyanin extracts such as purple sweet potato, red cabbage leaves and Roselle herb and Roselle anthocyanin[25, 27]. In this study, the ATH was extracted from red cabbage leaves and used as intelligent material in film preparation.

1.4. Applicability of active and intelligent food packaging

Food spoilage is the process in which food is decayed due to attack of external microbes and internally food borne pathogens. They can damage the taste, texture and nutrition of food. Therefore, several techniques are being used for quality control of food[11]. Food spoilage slightly lowers the pH of food, which can be easily measured by pH indicators like anthocyanin [28]. Similarly, the bacterial attack can be inhibited by incorporating active material into packaging films. Propolis extract is naturally extracted active agent which can inhibit bacterial growth without harming the environment. Therefore, anthocyanin and propolis being economical and organic sources are used in these packaging films, which make them different from conventional synthetic active and intelligent films.

1.5. Problem Statement

The emerging concept of active and intelligent bio-degradable food packaging has taken over the market demands for healthy and safe food with a prolonged shelf life [3].

- Use of bio-degradable polymers like PVA/Starch but they have minimum mechanical strength
- Tailoring of mechanical and physical properties of PVA/Films by adding different cross-linkers such as boric acid and glutaraldehyde
- Addition of naturally occurring organic components i.e. propolis extract and anthocyanin for active and intelligent packaging respectively

The focus of this study is to prepare, characterize and evaluate the multi-functional properties of these active and intelligent PVA/Starch films loaded with PE and Anth. Their functional groups and surface morphology was characterized using SEM and FTIR. Furthermore, water vapor transmission rate (WVTR), moisture retention capability and normalize swelling degree (NSD) were investigated to evaluate the extent of bonding between cross-linker and the rest of polymer chains. The pH test ranging from 2 to 14, was conducted to evaluate the color changing behavior of formulations. Anti-bacterial activity of formulated films was tested against two food born bacterial strains i.e. E.coli and MRSA. Finally, food spoilage test was conducted to practically monitor the color changing performance and anti-bacterial activity of these films.

1.6. Objectives

- Preparation of active and intelligent films using ATH and PE.
- Characterization of active and intelligent films using different physical and analytical techniques
- Evaluation of active and intelligent films in the basis of performance.
- Validation of active and intelligent films applicability in food industry through food spoilage tests.

Chapter: 02

2. Literature Review

2.1. Biodegradable food packaging polymers

Biodegradation is the chemical and biological process in which synthetic and natural polymers are dis-integrated and recycled into other productive elements. The most important factors which play important role in degradation are micro-organisms i.e. algae, fungi and several bacteria [29].

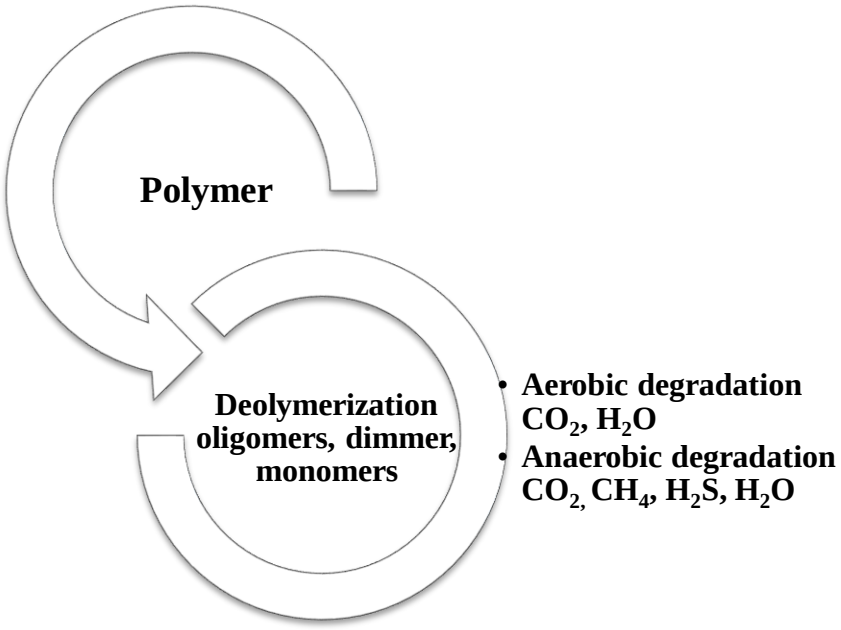

Figure 3: Bio-degradations scheme of polymers[30]

Bio-degradation is classified into two type;

Aerobic degradation (in the presence of oxygen)

$$Polymer + O_2 \rightarrow CO_2 + H_2O + Biomass + residue$$

Anaerobic degradation (in the absence of oxygen)

$$Polymer - O_2 \rightarrow CO_2 + CH_4 + H_2O + Biomass + residue$$

2.1.1. Xenobiotic Biodegradation process

Xenobiotic degradation is the process of dis-integration of made-made materials through bio-degradation process using micro-organisms. Bio-degradation of synthetic

polymer is very difficult process. Over many years, research have been made on bio-degradation of synthetic polymer through anaerobic digestion process. Such kind of degradation is present in under-water, landfills, sediments, sludge and water laden soil. Anaerobes use organic materials for bio-degradation such as, proteins, carbohydrates, lipid and many others[31].

2.1.2. Bio-degradation of aromatic compounds

Aromatic polymers are usually considered bio-synthetic in origin derived from organic compounds. Benzene, toluene, ethyl benzene, xylene and styrene etc. are produced in millions of tons annually in industries. They have wide usage in pharmaceutical industry, agrochemicals, polymers and many more[32]. Aromatic polymers are bio-degraded by both processes aerobic and anaerobic degradation. Aerobic degradation occurs in oxygenated aromatics such as benzene and naphthalene etc. Whereas, anaerobic degradation occurs in non-oxygenated aromatics such ethyl benzene, xylene, toluene etc[33].

2.1.3. Bio-degradation of Polymer blends

Synthetic polymers with their original form are very difficult to degrade and they keep on adding the waste material into the environment. Global environmental concerns are raising issues against recycling and degradation of plastic polymers. Blending synthetic polymers with purely bio-degradable organic polymers is the new trend. In which, bio-degradable polymers help the synthetic polymer to partially degrade into the environment through micro-organisms. If natural bio-degradable polymers are used independently, they have no effective use. Blending natural with synthetic polymers can enhance the mechanical and physical properties of the end product[34].

Starch being the natural polymer can be blended into many made polymers like polyvinyl alcohol, poly ethylene etc. These synthetic polymers are blended with starch are widely used in packaging industry due to their good bio-degradable properties with good mechanical and physical properties. Natural sources of starch are rice, potato, wheat, corn etc [35].

Starch/Polyethylene Blends

Polyethylene is widely used plastic in industry due to its good mechanical properties with degradability. Starch is added into polyethylene and an excellent blend can is prepared [35]. Starch being highly hydrophilic macro-molecule can be added in polyethylene blend to enhance it degradation properties. Polyethylene due to its huge molecular size is resistant to bio-degradation. Bio-degradation is related to molecular size, larger the size of molecule slow will be the degradation process. The starch/polyethylene blend compatibility is very poor due to the difference in their properties. So, this blend has poor degradation capability [35].

Starch/Polyester blend

Starch and polyester blend have excellent compatibility. They have very high bio-degradation capability. They have special types enzymes due to addition of starch which results into very good degradation. Other than degradation, the mechanical properties are also enhanced. Tensile strength is increase by 70% weight and % elongation is decreased[36].

Starch/PVA blend

Starch/PVA blend has been widely used in industry due to their availability and economical prices. PVA and starch both are hydrophilic in nature, therefore, they have very good compatibility with excellent bio-degradation properties. Addition of PVA into starch has also improved its mechanical properties. PVA/starch blend is a vastly studied and researched. It has many applications such as, films preparation for food industry[37].

2.2. Active Food Packaging

European Regulation no. 450/2009 has defined active food packaging as, “active materials and articlesre intended to extend the shelf-life or to maintain or improve the condition of packaged food; they are designed to deliberately incorporate components

that would release or absorb substances into or from the packaged food or the environment surrounding the food" [38].

2.2.1. Anti-microbial active food packaging

Active agents are those components which play important part in protecting the shelf-life and quality of packed food. These active agents are applied directly on surface of food or rather packed in package [39]. Commercially there are many active agents are available and manufactured by well-known companies. Some are tabulated in table: 1.

Table 1: Commercially available active agents[40].

Active agents	**Polymeric matrix**	**Applications in food industry**	**Commercial Names**
Wasabi extract	Encapsulation in cyclodextrin	Coated PET film, tablet	Wasapower™
Triclosan	Polymer, rubber	Food containers	Microban®
Sulphur dioxide	Laminated plastic sheet with Na2S2O5 Sheet or pad for postharvest	storage of grape fruits	Uvasy™
Silver substituted	Zeolite LLDPE, PE, PVE, rubber	Film, Wrap, milk containers, paperboard cartons	AgIon®, Zeomic™, Cleanaid™, Novaron®
Ethanol	Silicon dioxide	Sachet	Ethicap™
Chlorine dioxide	Polyolefin Film,	Sachet	MicroGarde™, Microsphere™

Active anti-microbial packaging is divided into four categories;

1) Food package can be incorporated with pads or sachet containing anti-microbial agents, which can on spot control and protect food by releasing anti-microbial agents[41].
2) Second method can be direct c0-extrusion of active component into the package material but due to high temperature there are chances of its lose, there are other methods like, use of solvent, solution casting and moulding, electrospinning and compounding. These methods have less active agent loss chances[42].
3) The other method can be direct coating of package material with active agents. These active agents can migrate to the surface of food. In this way they can protect food by increasing its shelf life and quality[43].
4) Anti-bacterial active agents like chitosan can be used in packaging material. Which are naturally active. So, they have two functions; packaging and protection[43].

Synthetic anti-microbial active agents

There are many active agents which are prepared synthetically such as, ethanol, carbon dioxide, silver ions, anti-biotics, zinc oxide and chlorine dioxide etc. They are tabulated in table: 2.

Table 2: Synthetic anti-microbial agents and their applicability[40, 44].

Active agent	**Attributes**
Silver zeolites	Films wrap, paperboard cartons
Silver	Masterbatch, sachet, trays and films
Glucose oxidase	Masterbatch
Triclosan	Plastic packaging
Ethanol vapor emitting	sachet

Sulphur dioxide	Laminated sheets and wraps
Chlorine dioxide	Sachet and film wraps

Natural anti-microbial active food packaging

Chitosan, essential oils, several proteins, lipids and fatty acids, plants extract and propolis are the naturally occurring active anti-microbial agents. They are widely used in the field of medicine and pharmaceutical. From few years they have been introduced in food packaging industry[45].

There are several essential oils, plants extract and naturally occurring polymers which can be added to base polymer of food packaging films. These active additives can act as anti-microbial agents in packaging films. Some of the naturally occurring active agents are tabulated in table: 3;

Table 3: Natural anti-bacterial active agents incorporated in base polymers for food packaging films.

Active Agents	**Film Matrix**	**References**
Tea tree oil and ginger	HPMC	[46]
Cinnamon, lemon, tea tree, bergamot and green tea tree	Chitosan	[46, 47]
Oregano, thyme, lemon grass and ginseng	alginate	[48]
Organum vulgare	Fish skin gelatine and chitosan	[49]
Oregano, lavender, lemon grass, bergamot	Gelatin	[50]

Oregano	Whey protein	[51]
Thyme, citronella, coriander	Hake protein	[52]

Addition of essential oils and other natural active ingredients into polymeric films through solution casting or extrusion method can change film properties. Many studies have proved that addition of natural active ingredients have improve the mechanical, physical and bio-active properties of packaging films[53].

Propolis as active anti-microbial agent in food packaging

Propolis is the natural occurring active agent collected from honey bee comb and widely used for healing purpose[54]. The main components of propolis are;

i. *Resins (50-70%)*
ii. *Oil and waxes (30-50%)*
iii. *Other chemical (15%-20%)*

Propolis has the wide diversity of chemical composition. It usually consist of four major chemical compositions [55];

iv. *Polyphenols (flavonoids, polyphenolic acid, polyphenolic ester, phenolic aldehydes)*
v. *Quinines, amino acids, amino acids and other inorganic components.*
vi. *Aromatic components and phenols*
vii. *Flavonoids and flavones.*

Green propolis is largely found in brazil. It is composed of large amount of phenolic compounds i.e. artepillin C, kaemoferide, druanin, ric acid, caffeic acid and other compounds like triterpene lupeol-3 and hexadecanoate[56]. Its molecular structure is presented below in figure: 4.

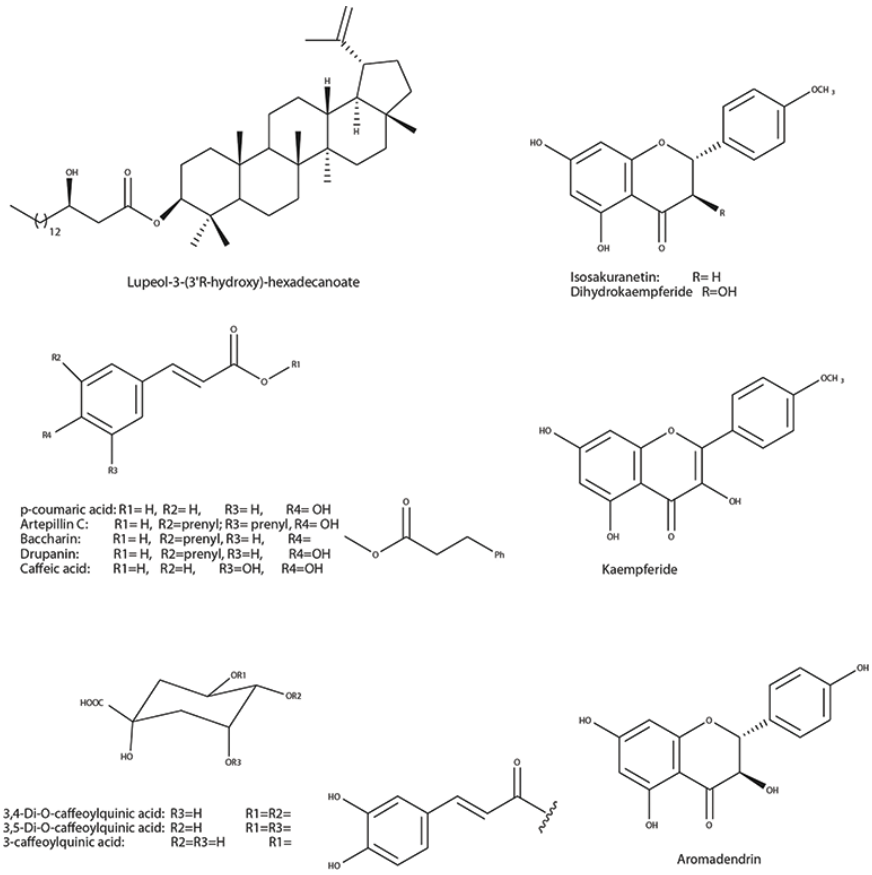

Figure 4: Molecular structures of compounds found in propolis[56]

2.2.2. Anti-oxidant agents for food packaging

In food packaging there are high chances of presence of oxygen into the package. Oxygen in food package can cause the lipid oxidation, support microbial growth and defoliate the colour and texture of food[57]. Hence, results in decrease of shelf life and quality of food. There are some anti-oxidant agents which can scavenge oxygen from the food packaging and protect food from microbial attack. They are basically divided into two classes

i. Dependent anti-oxidant devices
ii. Anti-oxidant packaging materials.

Dependant anti-oxidant devices includes pads, sachet, gels etc. which can be packed into food packaging and they act as oxygen scavenger[58].

Anti-oxidant packaging materials include essential oils and plant extracts, which are loaded into the base polymers of packaging films. They are added in the form of extrusion, electrospinning, as a solvent, solution casting or direct coating on the walls of packaging product[57].

2.2.3. CO_2 emitting/generating active agents for food packaging

In aerobic digestion CO_2 is generated to stop the process. Whereas, in anaerobic digestion CO_2 emitted to stop the process[59]. In many food packaging to avoid aerobic bio-degradation of food CO_2 is generated inside the package. Which can stop the growth of various bacteria and fungi which can grow in oxygenated environment. They are commercially available in the form of pads, sachet, gel and box system etc[44].

2.3. Intelligent Food Packaging

The commission of European community has define intelligent packaging as, "materials and articles that monitor the condition of packaged food or the environment surrounding the food" [60]**.** Another definition of intelligent was proposed, "a packaging system that is capable of carrying out intelligent functions (such as detecting, sensing, recording, tracing, communicating, and applying scientific logic) to facilitate decision making to extend shelf life, enhance safety, improve quality, provide information, and warn about possible problems" [61].

Active and intelligent packaging cannot be taken as mutual terminologies. Active packaging protect food against microbial attack. Whereas, intelligent packaging indicate and monitor the quality of food [62]. Intelligent packaging system consists of two major divisions; a) smart devices b) synthetic chemical and b) natural ingredients.

2.3.1. Smart Devices

Some of the smart devices used in intelligent packaging are summarized in tabulated form in table: 4.

Table 4: Smart devices used in intelligent packaging[63]

Intelligent smart devices	**Mechanism**	**Mode of information**	**Applications**
Time temperature indicators	Mechanical, chemical, enzymatic, microbiological	Storage conditions	Foods stored under chilled and frozen conditions
Gas indicators	Redox dyes, pH dyes, enzymes	Storage conditions, package leak	Foods stored in packages with required gas composition
Freshness indicators (e.g. microbial growth)	pH dyes; Dyes reacting with (non-volatile metabolites)	Microbial quality of food (i.e. spoilage)	Perishable foods such as meat, fish and poultry
Barcodes	Symbology Product and manufacturer information	Product identification, facilitating inventory control,	stock reordering, and checkout
Radio frequency identification tags	Radio waves	Product and manufacturer information	Product identification, supply chain management, asset tracking, security control

2.3.2. Synthetic materials for intelligent packaging

Synthetic materials for intelligent packaging include certain chemical indicators which upon changing pH change their colour. There are several researches present in the literature in which synthetic chemicals are used in packaging films for on time monitoring of food quality and measuring its freshness. CO_2, bromothymol blue, phenol red, bromocresol green are used in different types of food packaging as intelligent agents[64].

2.3.3. Natural materials for intelligent packaging

Anthocyanin is the natural pigment which is being used in several packaging films. Anthocyanin can be extract from many natural sources like fruits, vegetables, red beans coat, sweet potato, red cabbage, red rose etc.

Anthocyanin is derived from flavanol and its structure lacks ketone oxygen like flavylium ion. Molecular weight and empirical formula of flavylium ion of anthocyanin is 207.24724 g/mol and $C_{15}H_{11}O^+$ respectively. The red blue and purple color in plants is due to conjugated bonds of anthocyanins[65].

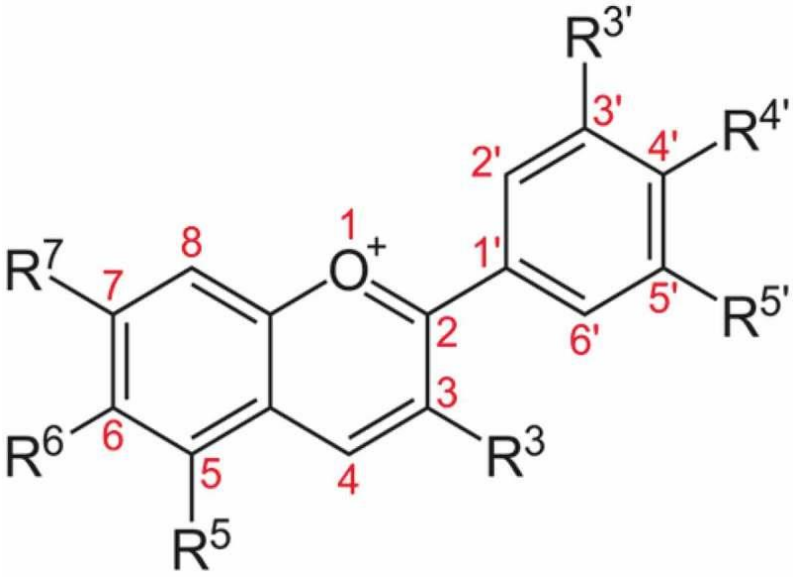

Figure 5: Molecular structure of Anthocyanin[65]

Chapter: 03

3. Materials and Methods

3.1. Materials

Polyvinyl alcohol (PVA), Starch, Boric acid, Glutaraldehyde and Hydrochloric acid are purchased from Sigma Aldrich USA. The red cabbage was bought from farmers market Islamabad, Pakistan for anthocyanin extraction. Propolis powder was purchased from Amazon. Nestle Milk Pack was used for food spoilage test. Distill water was used in all experimental work.

Bacterial strains of E. coli and MRSA were provided by the courtesy of Atta ur Rehman School of Applied Bio-sciences, National University of Science and Technology, Islamabad, Pakistan.

3.2. Active and Intelligent Films formulations preparations

3.2.1. Propolis extracts extraction

Propolis was extracted using the method available in literature with slight modifications [22]. 5 gm of Propolis powder was mixed with 50 ml of ethanol and water (7:3, ethanol: H_2O) solution. The mixture was further concentrated to half of its volume through rotary evaporator at 70 ^oC. Finally, concentrated mixture was prepared with the time limit of two hrs.

3.2.2. Anthocyanin Extraction

ATH was extracted from red cabbage leaves using pure ethanol as solvent. 150 gm of red cabbage leaves were crushed and mixed into 100 mL of pure ethanol solvent for pigment extraction. Afterwards, the mixture was filtered and the filtrate was centrifuged at 400 rpm for 10 minutes for further purification. Later, HCl and NaOH was added to the centrifuged mixture to maintain pH at 2 and was stored for 24 h at 5 ^oC. After 24 h the pH of the solution was maintained at 7 by adding NaOH and stored at 5 ^oC for further use [66].

3.2.3. Films formation

(a) Films preparation using Boric Acid as cross linker: Starch was added (1 g) in 10 mL of water and solution was prepared under continuous heating and stirring at

80° C. Similarly, PVA solution was prepared by dissolving 1g PVA in 10 mL water with continuous stirring at 95°C. After preparation of these solutions, 3:5 PVA-Starch solution was prepared and then mixed with 5mL of Ath extract. This solution was then mixed with 25mg of boric acid. Boric acid was added as cross-linker. The solution was then mixed with five different concentrations of PE ranging 0.2%, 2%, 5%, 10% and 20% as shown in Table: 5. As a result, eight different formulations were prepared and poured into petri dishes for film formation. Films are dried in vacuum oven for 24hrs at 60°C and finally stored in desiccator.

Table 5: Chemical Composition of Active and Intelligent Films using Boric acid as cross-linker

Films Formulations	**PVA**	**Starch**	**Boric Acid**	**Anthocyanin**	**Propolis Extract**
	P	**S**	**B**	**A**	**PE**
	mL	**mL**	**gm**	**mL**	**%**
PSB	6	10	0.3	0	0
PSB5A	6	10	0.3	5	0
PSB0.5PE	6	10	0.3	0	0.5
PSB5A0.5PE	6	10	0.3	5	0.5
PSB5A2PE	6	10	0.3	5	2
PSB5A5PE	6	10	0.3	5	5
PSB5A10PE	6	10	0.3	5	10
PSB5A20PE	6	10	0.3	5	20

(b) Films preparation using Glutaraldehyde as cross-linker: 1 gm of PVA in 10 mL of distill water, and 1 gm of starch /10 mL of distill water solutions were prepared separately. Afterward, PVA/Starch (3:5) solution was mixed and cross-linker GA (0.5 mL) with HCl (0.05mL) as catalyst was added. Furthermore, to make films active and intelligent different concentration of PE (0.5%, 2%, 5%, 10% and 20%) and 5 mL of ATH were added to form different formulations. Afterward, these film solutions were prepared using solution casting method and vacuum dried at 40^{o}C. Possible eight combinations of film formulations are enlisted in the table: 6.

Table 6: Chemical Composition of active and intelligent films using Glutaraldehyde as cross-linker

Films Formulations	**PVA**	**Starch**	**Glutaraldehyde**	**Anthocyanin**	**Propolis Extract**
	P	**S**	**GA**	**A**	**PE**
	mL	**mL**	**gm**	**mL**	**%**
PSGA	6	10	0.3	0	0
PSGA5A	6	10	0.3	5	0
PSGA0.5PE	6	10	0.3	0	0.5
PSGA5A0.5PE	6	10	0.3	5	0.5
PSGA5A2PE	6	10	0.3	5	2
PSGA5A5PE	6	10	0.3	5	5
PSGA5A10E	6	10	0.3	5	10
PSGA5A20PE	6	10	0.3	5	20

3.3. Scanning Electron microscopy

Scanning electron microscopy (SEM) analysis was performed to reveal the surface morphology of film formulations using JEOL SEM (JSM-64900). All film samples were coated using conductive gold sputter at 10 kV. The basic components of SEM are; electron gun, lenses, detector and vacuum chamber. Its main components are shown in Figure: 6 below;

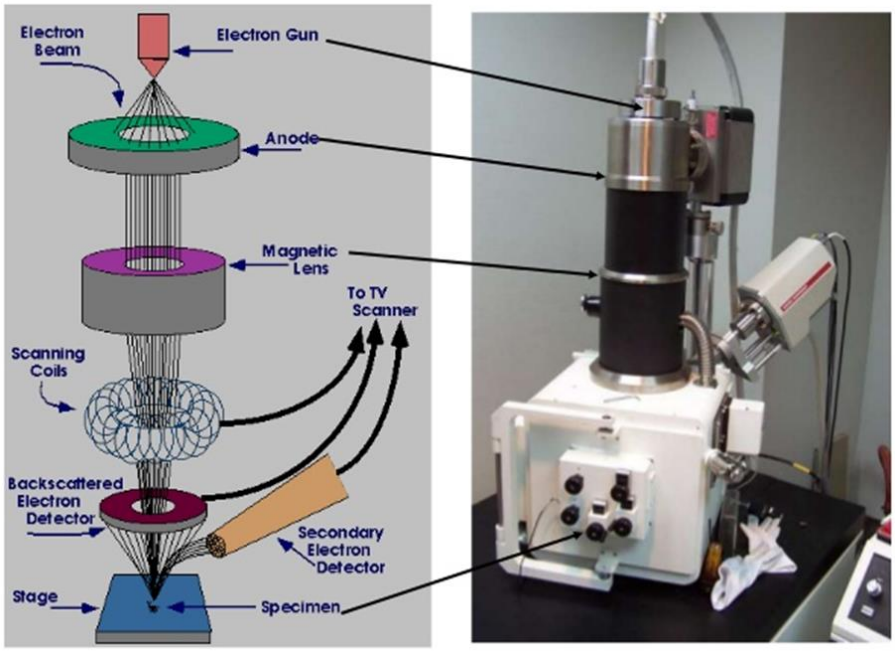

Figure 6: Scanning electron microscope

3.4. Fourier transmission infrared analysis

To study the inter-structural interactions and functional group attachment of PVA, Starch, Boric Acid, Glutaraldehyde, Anthocyanin and Propolis extract poly-phenols to the formulated films Fourier transform infrared (FTIR) analysis was performed. FTIR Perkin Elmer was used at a resolution of 4 cm^{-1} and wavenumber was ranged between 4000 cm^{-1} to 700 cm^{-1}. They key component of FTIR technique are displayed in figure: 7 below;

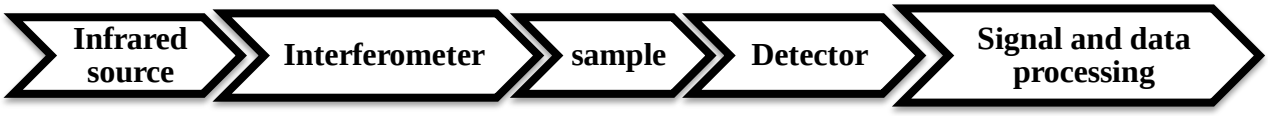

Figure 7: Key components of FTIR analysis

3.5. X-ray diffraction analysis

X-ray diffraction (XRD) analysis was performed by using STOE Germany. The 2θ was ranged from 10^0 to 70^0. The step size and scan ratio was 0.5 $step^{-1}$ and 0.01 sec^{-1}, respectively. The wavelength of Copper-K-α radiation was 1.54Å. There are three main components of XRD i.e. X-ray tube, sample holder and X-ray detector.

3.6. Mechanical Properties

Mechanical properties of active and intelligent films are associated with inter and intra molecular interaction between used material.

Universal testing machine (UTM) was used to calculate tensile strength and % elongation. Cross-head speed and grip separation was 50mm/min and 25mm respectively. Samples were cut into rectangular shape with dimension of 40 X 50 mm^2.

Tensile strength (TS) was calculated using following formula;

$$TS = \frac{F}{A_i} \quad \text{..... (1)}$$

F (N) is applied force and A_i (mm^2) is initial area of film sample.

% Elongation (%E) is calculated as;

$$\%\, E = \frac{l - l_i}{l_i}\, X\, 100 \quad \text{..... (2)}$$

l_i (mm) is the initial length of sample film, l(mm) is the final length of sample film.

3.7. Water vapor transmission rate (WVTR)

WVTR was calculated by using the standard method followed by the American standard of testing materials [67]. 10 mL of distill water was taken in a media bottle having a diameter of 29.5mm. The bottle was sealed with film using Teflon tape and the initial weight of the bottle was measured as W_i (gm). The bottle was then kept in the oven for 24 h at 40°C and again the weight of the bottle was measured as W_f (gm). WVTR was calculated by the formula given below;

$$WVTR = \frac{W_i - W_f}{A\ X\ 24}\ X\ 10^6 \quad \text{..... (3)}$$

A (mm^2) is the area of bottle.

3.8. Moisture Retention Capability test

Moisture retention capability test was performed to check the water vapor and moisture resistant properties of films formulations. For this purpose, films were cut into square (1 x1 cm^2) and weighed (W_i, gm). After that film samples were placed into the oven for 6 hrs at 60°C and weighed again (W_f, gm). Moisture retention capability was calculated by the formula given below;

$$Moisture\ retention\ capabity = \frac{W_f}{W_i}\ X\ 100 \quad \text{..... (4)}$$

3.9. Biological Leaching test (BLT)

BLT was conducted using UV-visible spectrophotometer GENESYS™ at wavelength 208 nm to measure the amount of additives i.e. ATH and PE that can be leached out of films. Heat methanol treatment method was followed with modifications [68]. Films were cut into square (1X1 cm^2) and dissolved into 10 mL methanol and continuously stirred for 2 h at 40°C. Afterwards these leached

out film samples were washed with deionized water and filtrate is then tested for UV spectroscopy.

3.10. Normalize Swelling degree (NSD)

NSD test was performed to check the performance of cross-linker and polymeric network formed by all components. This test was conducted by following the method used in literature[69]. Films were cut into square (20 X 20 mm^2) and then immersed into dimethyl sulfoxide (DMSO) solution for 2h. After that, this solution is filtered and the insoluble part leftover filter paper is weighed as W_1(gm). Then this filtered mass is dried in the oven for 6 h at 80^{o}C and weighed again as W_2 (gm). NSD was calculated by this formula;

$$NSD = \frac{W_i - W_f}{W_f X A} \ldots\ldots (5)$$

A (mm^2) is the area of film samples.

3.11. Colorimetric test

In order to check the colorimetric response of films formulation pH test was performed using the method followed in the literature [11]. Films formulations were cut into 10 X 10 mm^2 square samples. Eight different solutions were made having a pH range from 1 to 14. Afterward, film samples were dipped into different pH solutions and color change response was captured in the form of images taken by digital camera.

3.12. Anti-bacterial property

"Disc diffusion" method was employed to check the anti-bacterial activity of film formulations against two common food born bacterial strains i.e. Escherichia Coli (E. coli) and Methicillin-resistant Staphylococcus Aureus (MRSA) [70]. Ten discs (6mm diameter) were cut for each bacterial strain. PE concentration varies (0.5%, 2%, 5%, 10% and 20% PE) for each disc. Afterward, these discs were mounted on

Lysogeny broth (LB) medium with positive and negative control. Antibacterial activity of each disc was determined by measuring the diameter of zones of inhibition after 24 h and 48 h of each disc. Anti-bacterial activity images were also captured after 48 h.

3.13. Food spoilage test

Food spoilage test was performed to check the potential applicability of these active and intelligent films. Pasteurized milk pack (Milk pack) was purchased from the local market. Milk was poured into 10 mL voiles and sealed with square-cut films. The freshness of the milk was checked after 24 h and 48 h [7].

Chapter: 04

4. Results and Discussions

4.1. Surface morphology of films formulations

Surface morphology of different formulations added with Boric acid as cross-linker were studied using SEM. In Figure: 8 (a) PSB film has shown rough surface with un-mixed starch granules. Whereas in figure: 8(b & c), PSB5A and PSB0.5PE films have shown comparatively smoother surfaces. However, in figure: 8(d), PSB5A20PE film exhibited the smoothest surface accompanied with all formulations. Wrinkle-free and smooth surface may be the result of homogeneous mixing of PE's phenolic component and Anthocyanin into the PVA-Starch matrix [7].

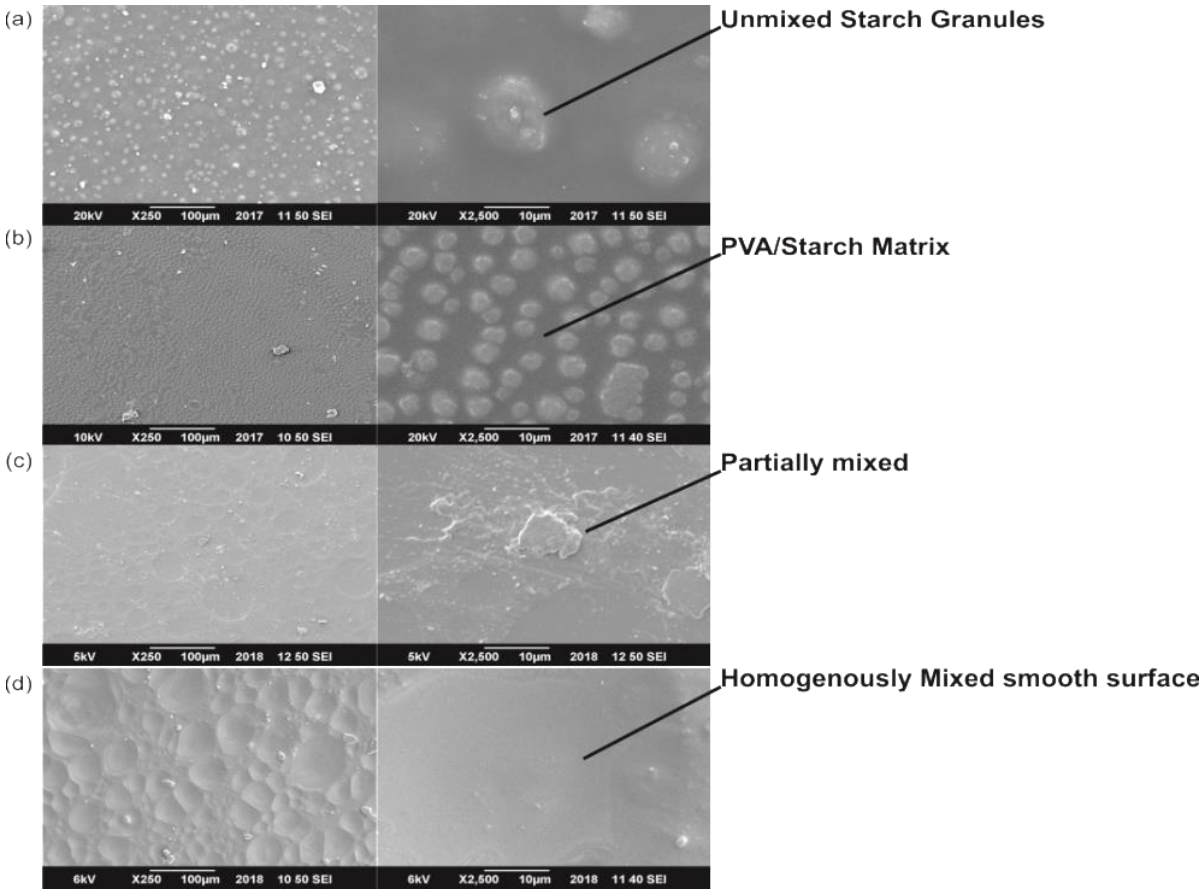

Figure 8: Surface morphology of different films formulations; (a) PSB (b) PSB5A (c) PSB0.5PE (d) PSB5A20PE

Figure:9, depicts the surface morphology of formulated films added with Glutaraldehyde as cross-linker using SEM. All films have different surfaces due to varying films compositions. In Figure: 9(a) PSGA has a rough surface with dispersed PVA molecules. When ATH is added into the film mixture, a partially mixed surface of PSGA5A was observed in Figure: 9(b). In Figure: 9(c) homogeneous surfaces are depicted whereas; in Figure 9(d) smooth homogeneous surface is shown. This could be attributed to the addition of a higher concentration of PE and ATH that facilitated the homogenous mixing of each component [71].

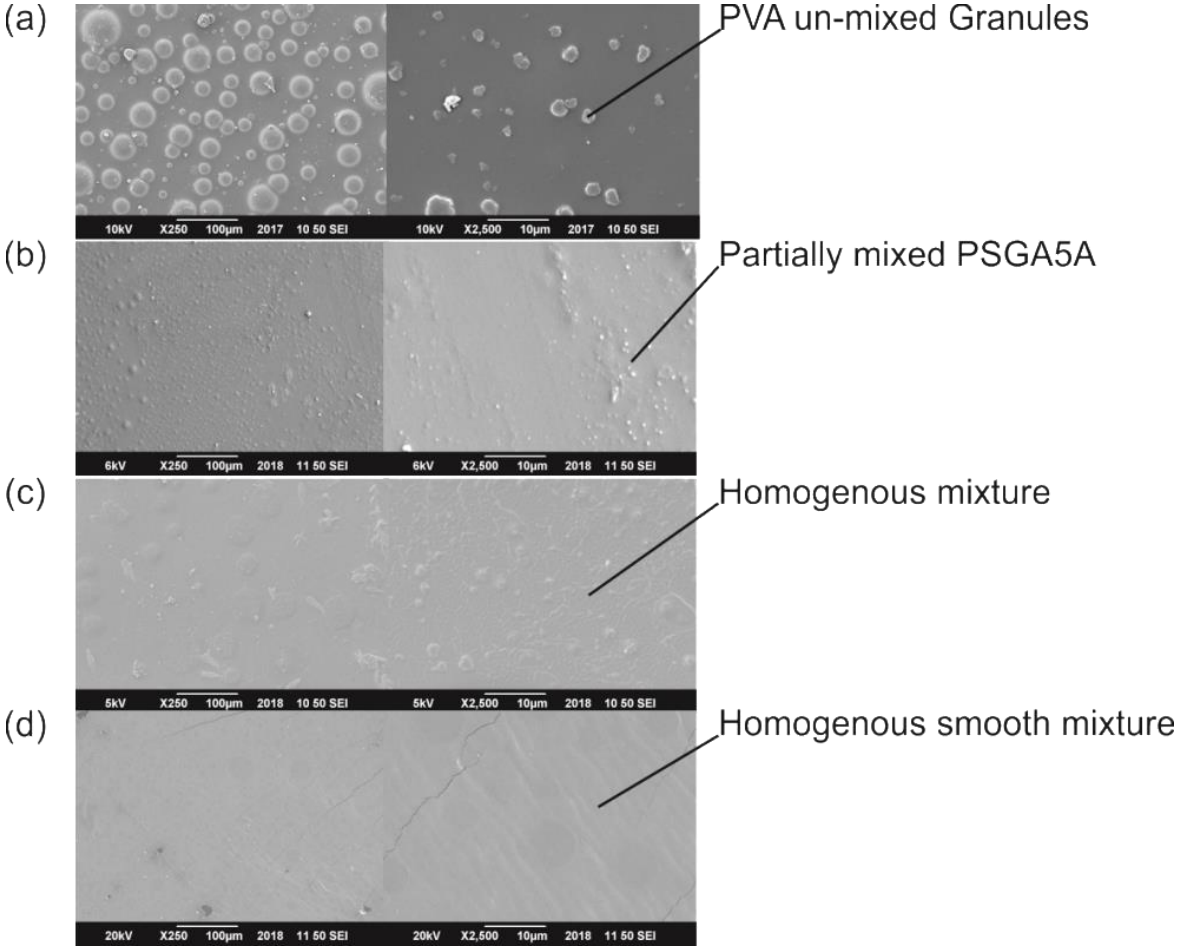

Figure 9: Surface morphology of different films formulations; (a) PSGA (b) PSGA5A (c) PSGA0.5PE (d) PSGA5A20PE

Hence, from figure:8 and figure: 9 it is clear that by adding different cross-linker i.e. BA and GA are not responsible for the change in surface morphology.

Whereas, increase in the concentration PE into films formulations effect the surface morphology and more smooth surfaces were observed.

4.2. Analytical Properties

4.2.1. FTIR analysis

FTIR characterization was performed to evaluate the molecular interaction of PE and ATH to PVA-Starch cross-linked with boric acid (BA) and Glutaraldehyde (GA). The frequency range was used between 4000 to 600 cm^{-1} and resolution was 4cm^{-1}. Spectra of each eight different formulations incorporated with PE and ATH are shown in Fig:10 and Fig: 11. Almost similar patterns were observed in each spectrum but their transmittance range varied.

In all formulations containing BA as cross-linker, FTIR spectra between 3550-3540 cm^{-1} is depicting the presence of OH group[72]. Asymmetric CH_2 methyl group is observed in all formulations at 2900 cm^{-1} [73]. The addition of ATH into films has observable peaks at 1710-1720 cm^{-1}. This is attributed to the presence of pyran ring of flavonoid group[12]. Furthermore, band stretching at 1650-1550 cm^{-1} is indicating the C=C bond of ATH aromatic ring stretching. This was observed in all films [74]. Peaks observed at 1143-1070 cm^{-1} are attributed towards the presence of phenol group attached with aromatic rings in PE and Anth[74].

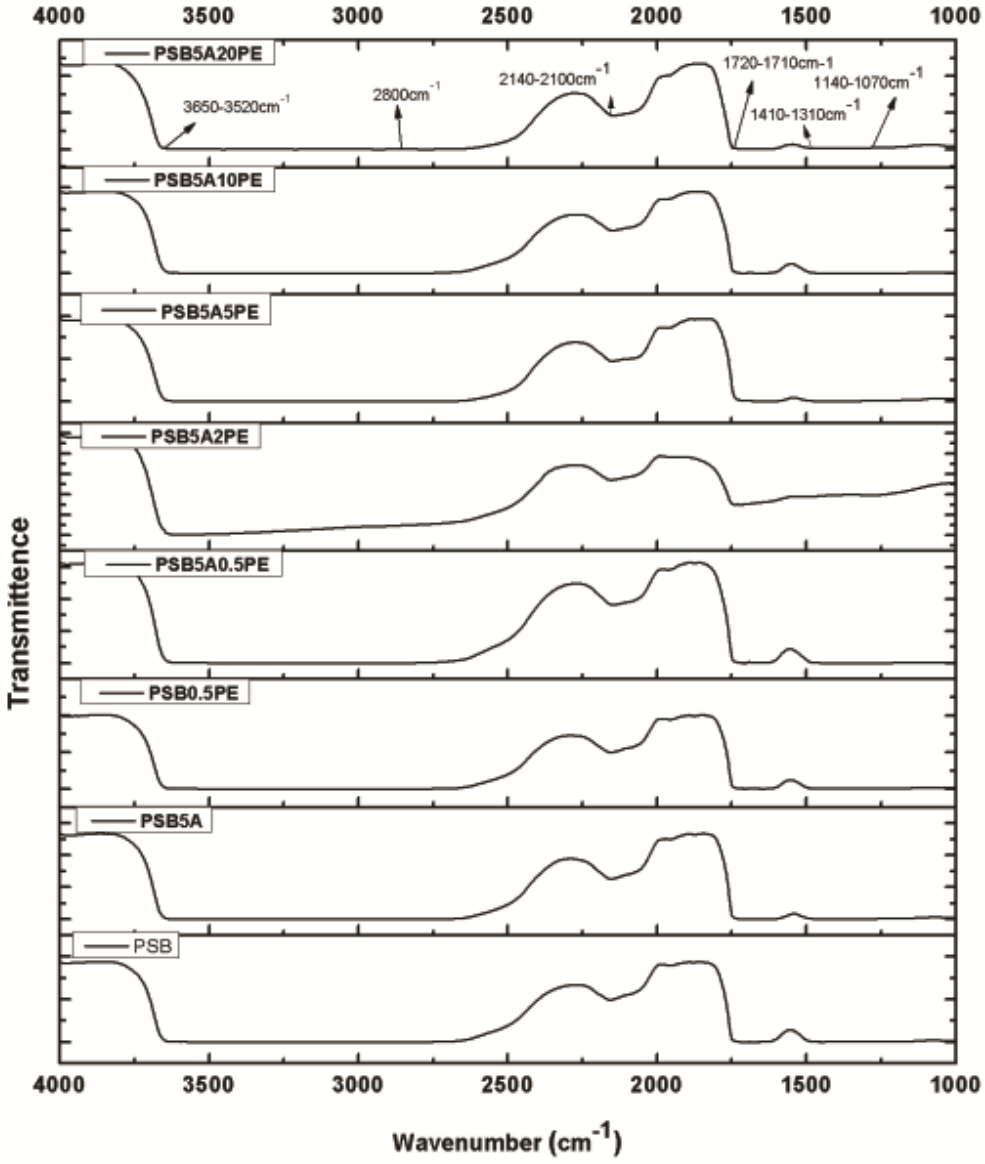

Figure 10: FTIR analysis of films formulations with Boric Acid as cross-linker

Similarly, in all spectra of films formulations containing GA as cross-linker absorption peaks between 3650cm^{-1} to 3550cm^{-1} of OH group were observed. It was attributed to the presence of PVA. Furthermore, absorption peaks at 1150cm^{-1} to 1080cm^{-1} confirms the presence of C-O group[75]. C-H bond stretching was observed at 2900cm^{-1} [76] in all spectra. A strong absorption band between

1650cm^{-1} to 1550cm^{-1} has shown the presence of aromatic ring of ATH and PE [77].

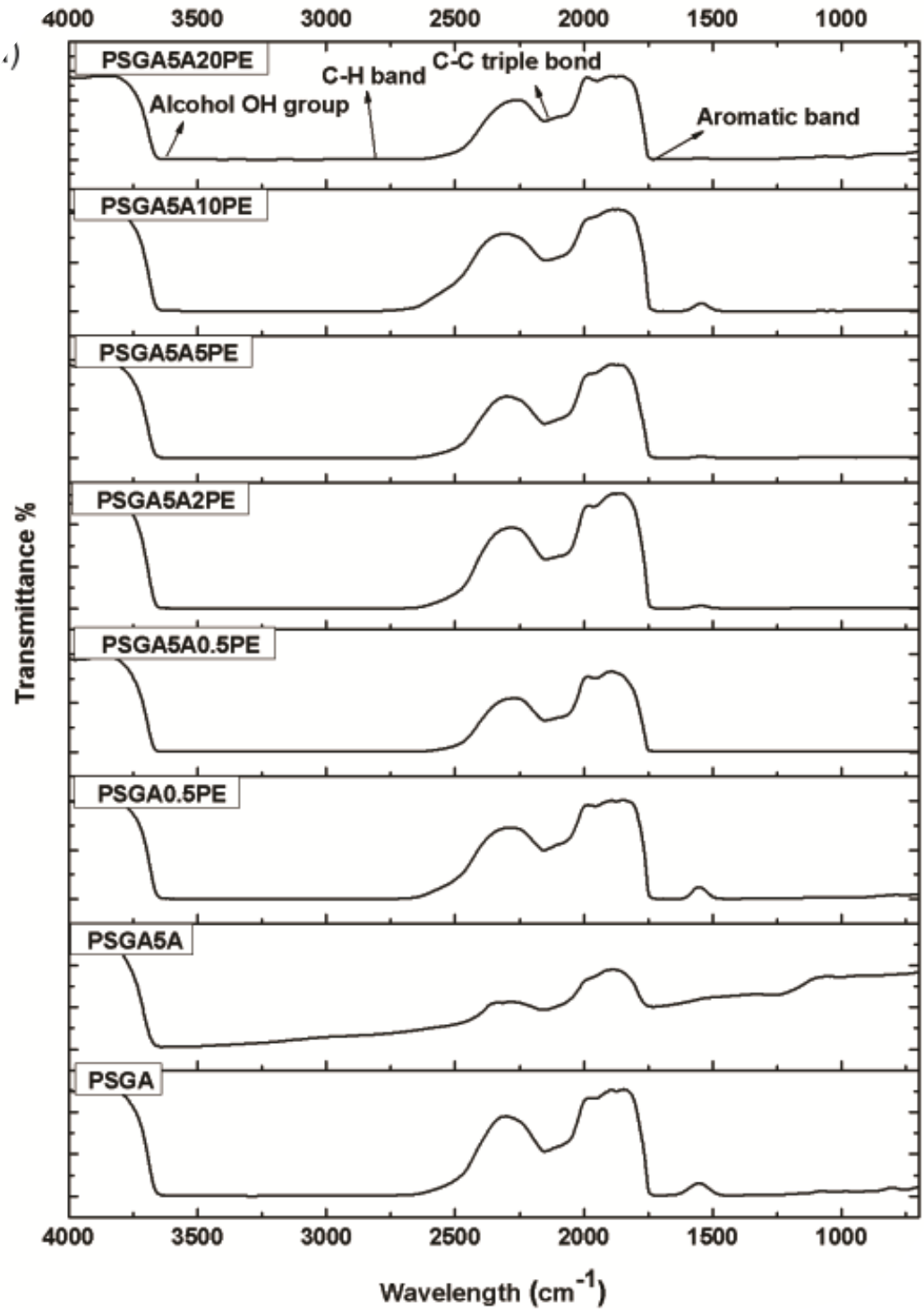

Figure 11: FTIR analysis of films formulation containing GA as cross-linker

Collectively, it confirms the functional group attachment of PE and ATH to the PVA-Starch film matrix containing BA and GA as cross-linkers.

4.2.2. XRD Analysis

XRD analysis for all formulations containing BA as cross-linker are presented in Figure: 12. Almost similar pattern was observed in all formulations. The characteristic diffraction peaks of starch were observed at 18^o, 22.5^o and 25^o. Peaks at 19^o, 21^o and 45^o are attributed to PVA [69]. When the concentration of PE is increased from 0.2% to 10%, the sharpness of the peaks at 17^o disappears. This can be the result of homogenous mixing of all components and cross-linker (BA). The starch molecules lost their crystallinity due to intensified interaction of cross-linker (boric acid) with starch and PVA [69]. Whereas, diffraction peak for PSB5A20PE has depicted peak shift from 45^o to 55^o. This peak shift can be the result of exceptional interaction between all molecules. Furthermore, this consequences in change of ionic radii of the molecules and results in peak shifts [78]

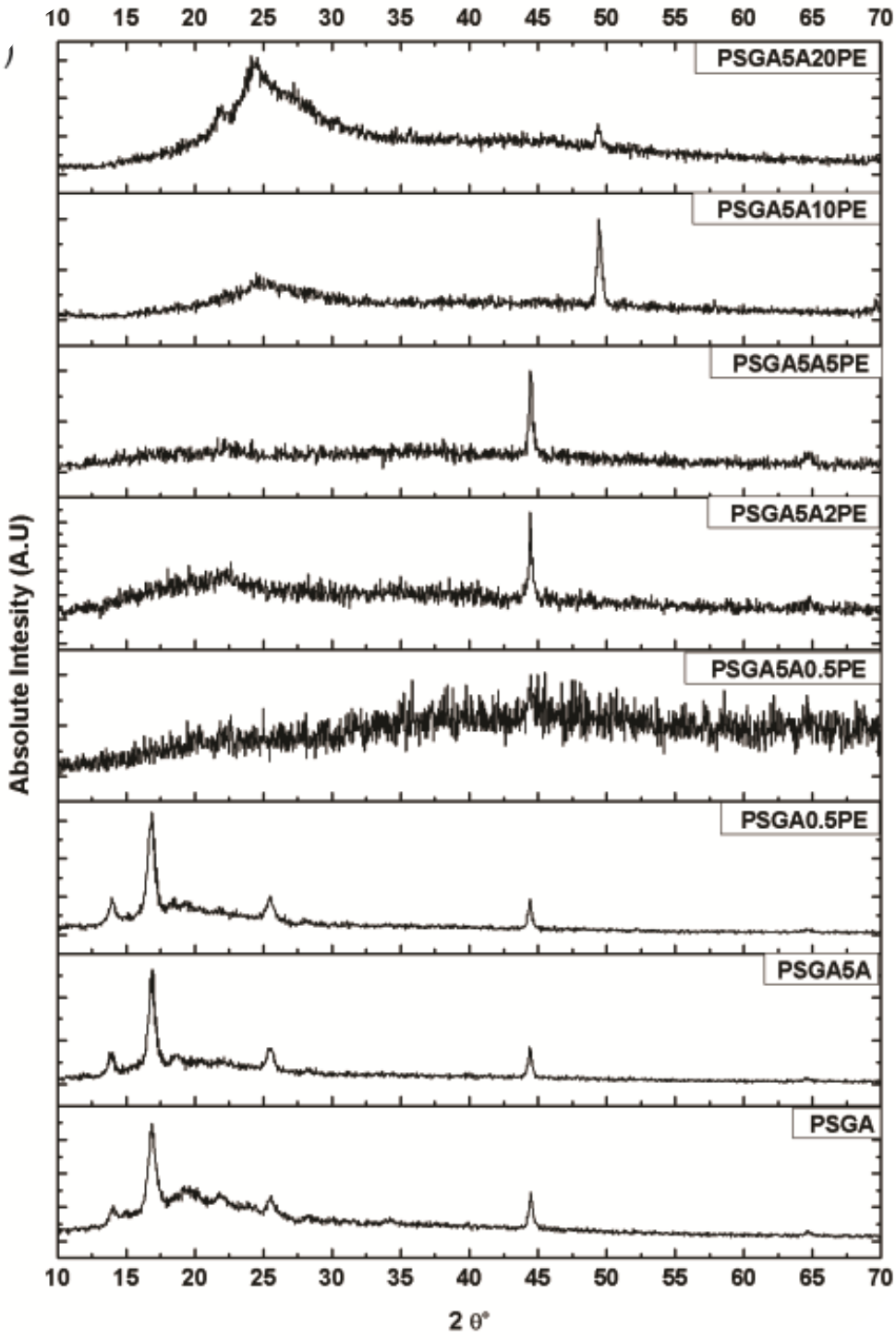

Figure 12: XRD analysis of films formulations containing BA as cross-linker

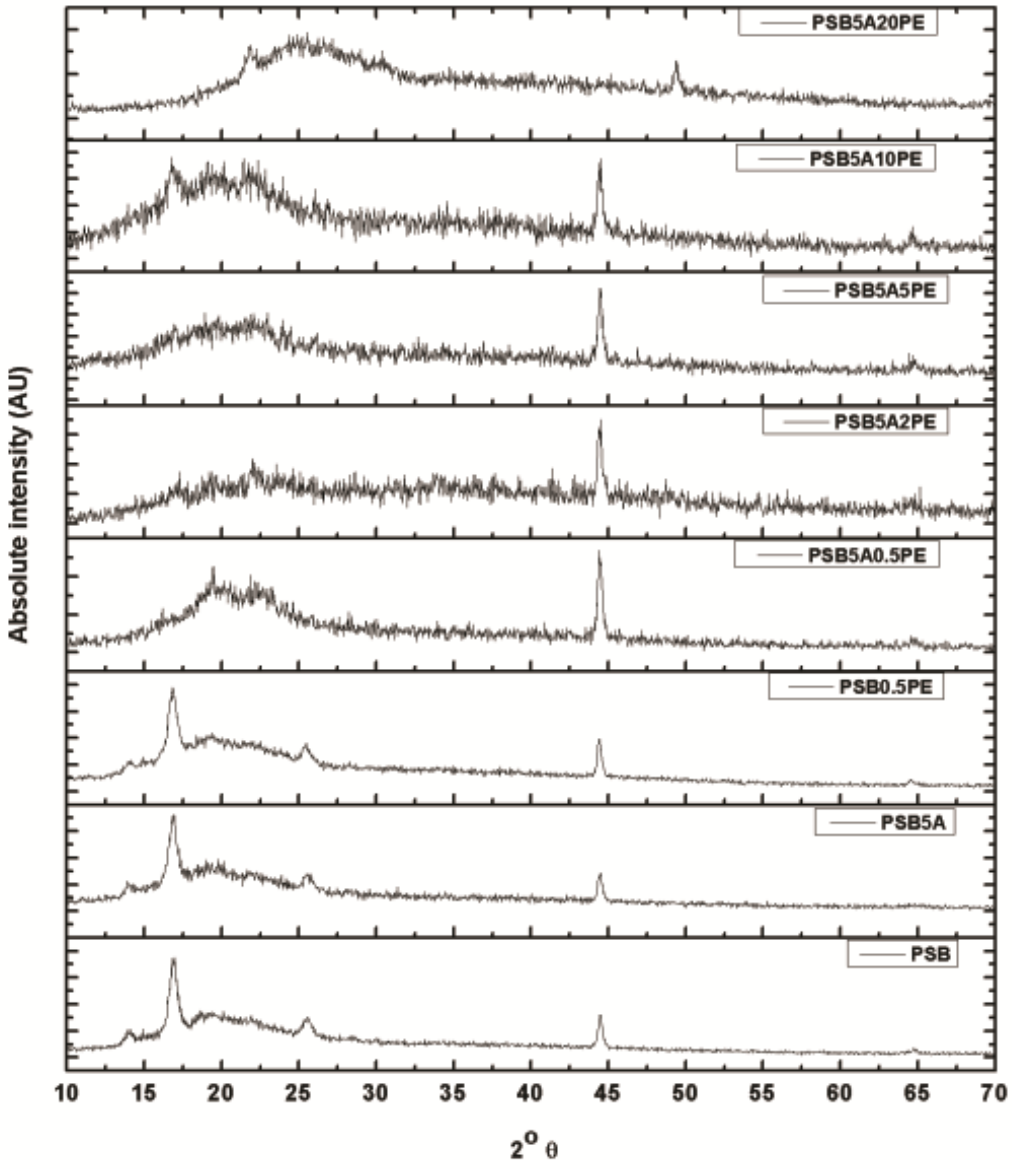

Figure 13: XRD analysis of films formulations containing GA as cross-linker

Figure: 13 depicts XRD analysis of films formulations containing GA as cross-linker. All formulations have a similar pattern with slight variations. In spectra PSGA, PSGA5A and PSGA5A0.5PE peaks at 18^o, 22.5^o and 25^o were diffraction peaks of starch. Similarly, the diffraction peaks at 19^o, 21^o and 45^o were attributed to the presence of PVA[69]. In film composition PSGA5A0.5PE, decrease in

sharpness of peaks as a result of homogenous mixing of ATH, PE, PVA, Starch and GA was observed. The crystallinity of starch molecules disappeared with addition of PE, ATH and proper interaction of cross-linker GA[69]. Peak shift from 45^{o} to 55^{o} is observed in PSGA5A20PE pertaining to the strong molecular interaction and change in ionic radii[78].Overall, it is observed that addition of cross-linkers (BA) and (GA) and additives i.e. PE and ATH has decreased the crystallinity of PVA and Starch. This is the proof of homogenous mixing of all components. Hence, better mixing results in increased mechanical strength, water resistant properties and physical structure of the formulated films.

4.3.Mechanical properties

Mechanical properties are very important for food packaging films. Tensile strength refers to the maximum strength of a film that can bear when an external force is applied. Whereas, % elongation refers to the maximum stretch a film can with-stand [79].

The tensile strength and % Elongation of films containing BA a as cross-linker are presented and compared in Figure: 14 and their values are tabulated in Table: 7.Tensile strength was found to be directly proportional to PE concentration. As PE concentration increases from 0.2% to 20%, an increase in tensile strength was observed. This can be attributed to the better interaction between cross-linked PVA-Starch , PE and ATH [74]. The poly-phenolic group present in PE and ATH perform hydrogen bonding with other components present in the formulated film. Hence, resulting in increased tensile strength.

% Elongation has a different trend as compared with tensile strength. Initially, it was enhanced when PE concentration is increased from 0.2% to 5%. Later, it started decreasing. This decrease can be attributed to the formation of crystalline structure between cross-linked PVA-Starch, PE and ATH by increasing the concentration of PE [80].

Table 7: Mechanical properties of films formulations containing BA as cross-linker

Films Formulations	Tensile Strength (MPa)	Standard Deviation	% Elongation	Standard Deviation
PSB	5.5	0.0015	4.9	0.00133
PSB5A	5.2	0.0014	4.5	0.00132
PSB0.5PE	5.15	0.0012	4.2	0.00125
PSB5A0.5PE	5.3	0.0009	4.3	0.00123
PSB5A2PE	5.35	0.01	4.3	0.00122
PSB5A5PE	5.5	0.0012	4.4	0.00114
PSB5A10PE	5.6	0.0011	3.1	0.00111
PSB5A20PE	6.1	0.0011	2.3	0.0009

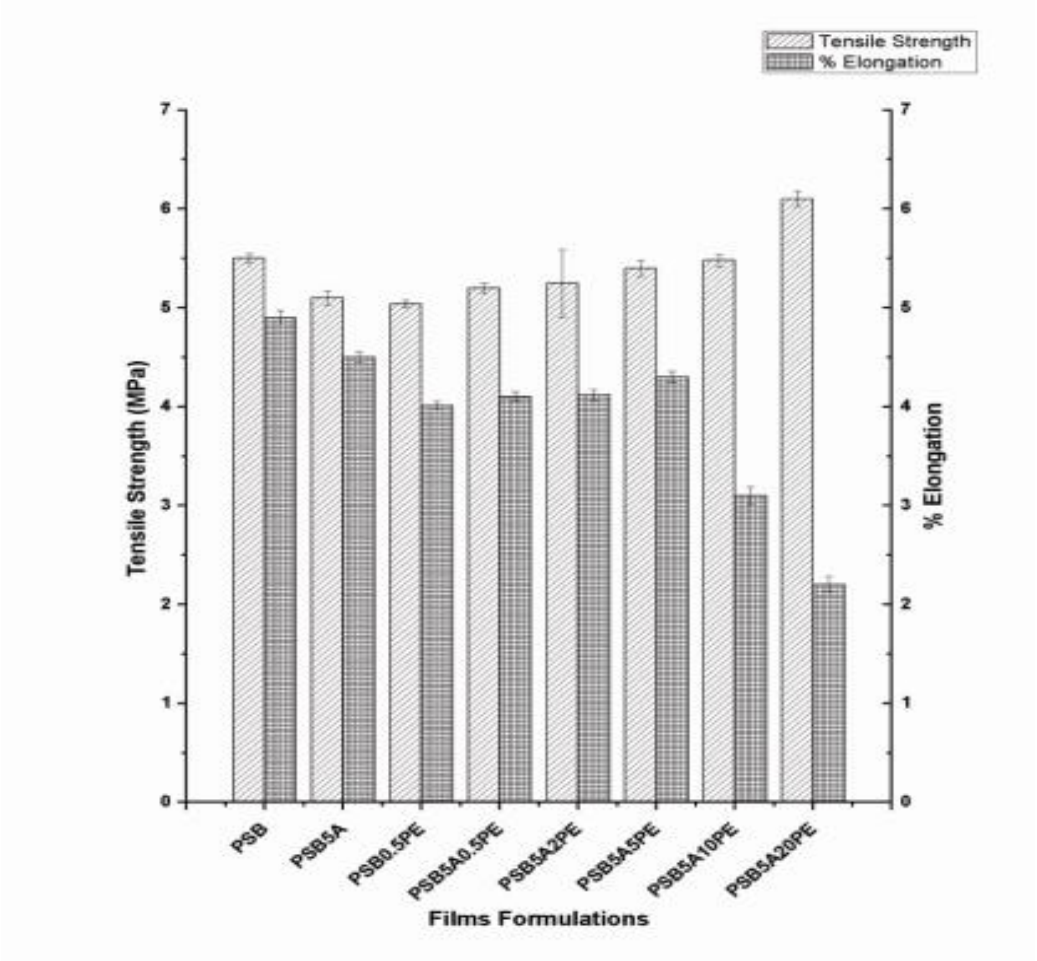

Figure 14: Mechanical Properties of films formulations containing BA as cross-linker

Figure: 15 depict the mechanical properties of all film formulations containing GA as cross-linker and their tensile strength and % elongation are tabulated in table: 8. It was noticed that tensile strength was enhanced by increasing the concentration of PE. On the other hand, percent elongation was decreased by increasing PE concentration. A possible reason for this decrease in % elongation could be the crystalline behavior of the films due to increased PE concentration which made them less flexible[81]. The other possible reason for the decrease in % elongation could be the cross-inking effect produced by increasing PE concentration, which has reduced inter-molecular spaces and increased tensile strength [82].

Table 8: Mechanical Properties of films formulations containing GA as cross-linker

Films Formulations	Tensile Strength (MPa)	Standard deviation	% Elongation	Standard deviation
PSGA	5	0.00016	2.2	0.000115
PSGA5A	5	0.000156	2.4	0.000113
PSGA0.5PE	4.9	0.000151	2.3	0.000113
PSGA5A0.5PE	6	0.000135	2.3	0.000112
PSGA5A2PE	6.9	0.000133	2.3	0.000112
PSGA5A5PE	9.9	0.000132	2.2	0.000111
PSGA5A10PE	13.5	0.000131	2.2	0.000110
PSGA5A20PE	15.9	0.000111	1.9	0.00011

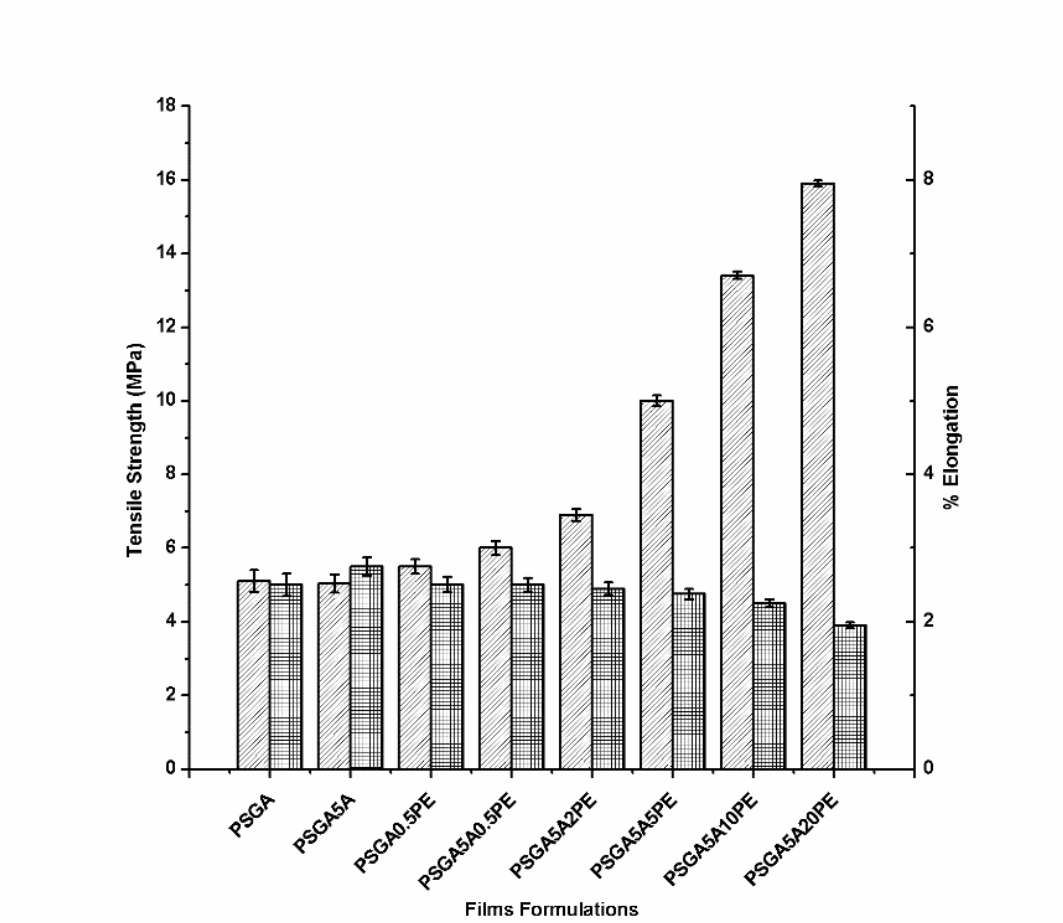

Figure 15: Mechanical Properties of films formulations using GA as cross-linker

In the literature, increased tensile strength with decreased % elongation has been reported several times with the addition of additives like essential oils, green tea extracts and cinnamon oil [83, 84]. Overall, PSGA5A20PE film formulation has the maximum tensile strength with minimum %elongation of 15.9 MPa and 1.9%, respectively.

4.4. Physical Properties of films formulations

4.4.1. WVTR

Water vapor transmission rate (WVTR) was measured to analyze the water resistance properties of PVA-Starch films. WVTR is the most important property in food packaging application due to interaction of packaging films with hydrophilic or hydrophobic environment. Cross-linkers, polymers, plasticizer, intermolecular interaction and other additives mainly affect this property[85].

Figure: 16 depicts the WVTR trend in these active and intelligent films and their values are tabulated in table: 9. In this study, PSB5A20PE has the minimum WVTR. Whereas, PSB has the maximum value of WVTR. The decrease is observed in the WVTR by increasing the amount of PE into the PVA-Starch cross-linked matrix. This decrease can be attributed to the hydrogen bonding (HB) between poly-phenolic component of PE and PVA-Starch cross-linked matrix. This interaction can lead to less availability of hydrogen molecule for hydrophilic interaction of water molecules [13, 86]. Moreover, the presence of resinous components, waxes and other essential oils in PE can also be the reason for this decrease. These components enhance the hydrophobicity of the films and better water resistant properties [87]. Similarly, phenolic content and flavonoids present in ATH have hydrogen bonding interactions with PVA-Starch(X) and PE. This results decrease in presence of hydrogen molecules reducing the WVTR [88]

Table 9: Water vapor transmission rate for films formulations containing BA as cross-linker

Films Formulations	WVTR (g/mm^2hr)	Standard Deviation
PSB	0.09	0.00015
PSB5A	0.088	0.00015
PSB0.5PE	0.075	0.00013
PSB5A0.5PE	0.06	0.00012
PSB5A2PE	0.059	0.00012
PSB5A5PE	00.057	0.00009

PSB5A10PE	0.057	0.000087
PSB5A20PE	0.056	0.00011

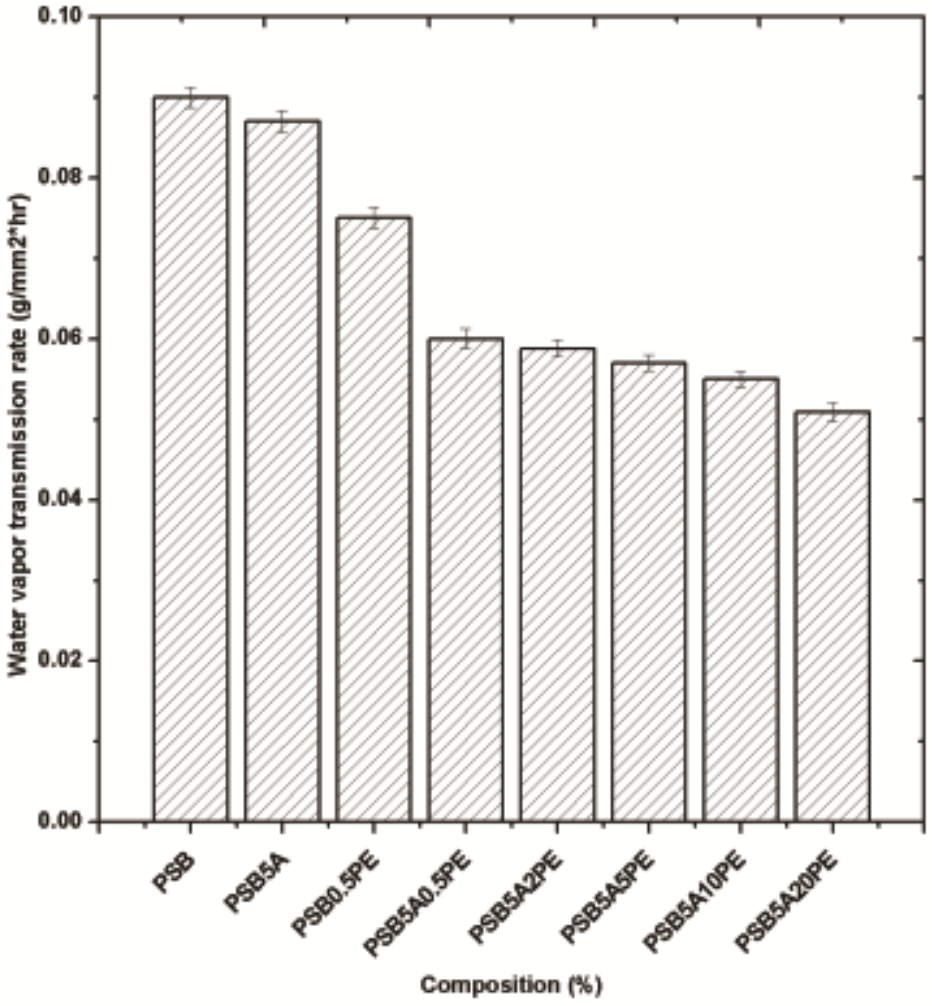

Figure 16: Water vapor transmission rate for films formulations containing BA as cross-linker

From figure: 17, it is depicted that film formulations containing GA as cr oss-linker without or with low PE concentrations have higher WVTR and their values are tabulated in table: 10. PSGA5A20E has the lowest WVTR. The possible reason for this lower rate with higher PE concentration in films could be the presence of phenolic component in PE and ATH fitted into PVA/Starch matrix

and enhanced intermolecular interaction and hydrogen bonding. Which consequently, hindered the escape of water vapor molecules from the films[84, 86]. The other reason for lower WVRT could be the presence of resinous components in PE which are hydrophobic in nature [87, 89].

Table 10: WVTR for films formulations containing GA as cross-linker

Films Formulations	**WVTR (g/mm^2hr)**	**Standard Deviation**
PSGA	0.10	0.00012
PSGA5A	0.099	0.00011
PSGA0.5PE	0.08	0.00014
PSGA5A0.5PE	0.075	0.00012
PSGA5A2PE	0.07	0.00011
PSGA5A5PE	0.043	0.00012
PSGA5A10PE	0.038	0.00011
PSGA5A20PE	0.029	0.00011

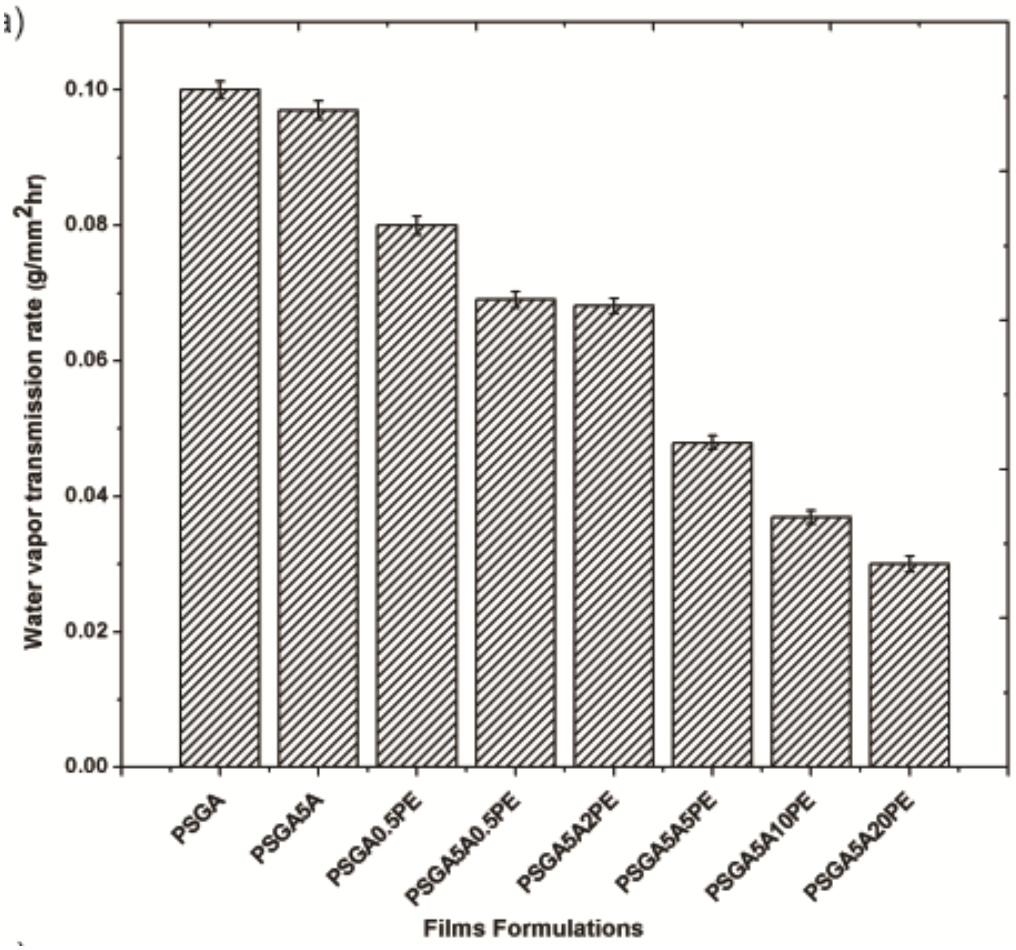

Figure 17: WVTR of films formulations using GA as cross-linker

Overall, PSGA5A20PE film formulations have shown the best results with of 0.0289 g/mm^{2}hr of WVTR in all films formulations. The reason behind low WVTR in GA as cross-linker containing films is their good mechanical strength.

4.4.2. Moisture Retention Capability test

Moisture retention capability is very important for food packaging films[85]. These tests were conducted to check the performance of PVA-Starch films containing BA as cross-linker. Result revealed that moisture retention capability enhanced with increase in concentration of active

component with in film formulations. In Figure: 18, PSB5A20PE has the maximum moisture retention capability of 96%. Their values of moisture retention capability are tabulated in table: 11. However, PSB has the minimum capability to retain moisture. Flores et al, 2007 have conducted a study and noticed that incorporation of naturally occurring antibacterial active component into bio-polymer matrix can encourage hydrogen bonding between Active agent and bio-polymer[90]. As a result, interaction of water with bio-polymer was inhibited and moisture will be retained inside the films. Same results were observed by Blasch et al, 2013 when potassium sorbate was added into films as an antibacterial agent and moisture retention capability was enhanced[91].[i]

Table 11: Moisture retention capability of films formulations containing BA as cross-linker

Films Formulations	Moisture retention capability (%)	Standard Deviation
PSB	91.5	0.5
PSB5A	91.7	0.54
PSB0.5PE	92	0.56
PSB5A0.5PE	92.5	0.55
PSB5A2PE	93	0.65
PSB5A5PE	94.5	0.6
PSB5A10PE	94.6	0.1
PSB5A20PE	95	0.5

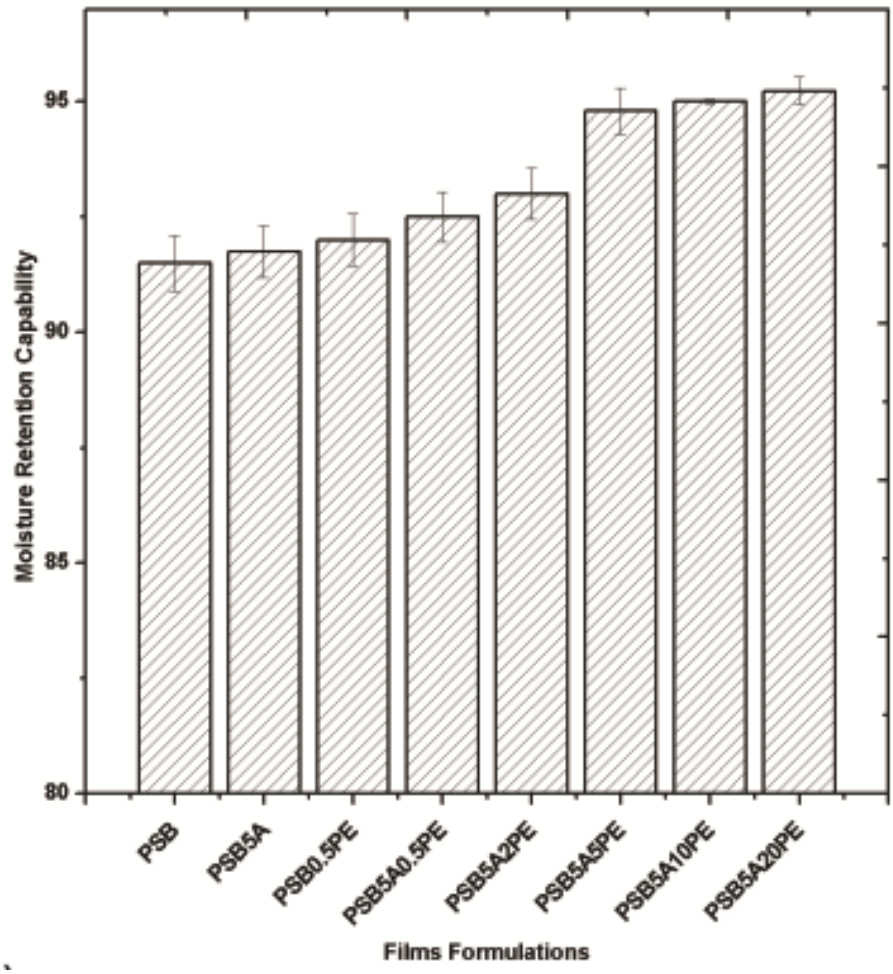

Figure 18: Moisture retention capability of films formulations containing BA as cross-linker

In Figure: 19, it is shown that PSGA5A20PE has the highest capability to retain moisture. Whereas, PSGA has the lowest capability to retain moisture. The reason behind this retention is the presence of the resinous and waxy component in PE which as a result, produced hydrophobic surface of films formulation. There are many studies reported in the literature in which different organic additives i.e. essential oil, bio-active components and tea tree oil were added to increase hydrophobic features [85, 90]. Same results were obtained, when synthetic active agent potassium sorbate was added and moisture retention capability was enhanced[91].

Table 12: Moisture retention capability of films formulations containing GA as cross-linker

Films Formulations	Moisture Retention Capability (%)	Standard Deviation
PSGA	91	0.85
PSGA5A	91.5	0.88
PSGA0.5PE	91.7	0.95
PSGA5A0.5PE	92	0.9
PSGA5A2PE	92.5	0.89
PSGA5A5PE	93	0.87
PSGA5A10PE	94.2	0.89
PSGA5A20PE	94.5	0.9

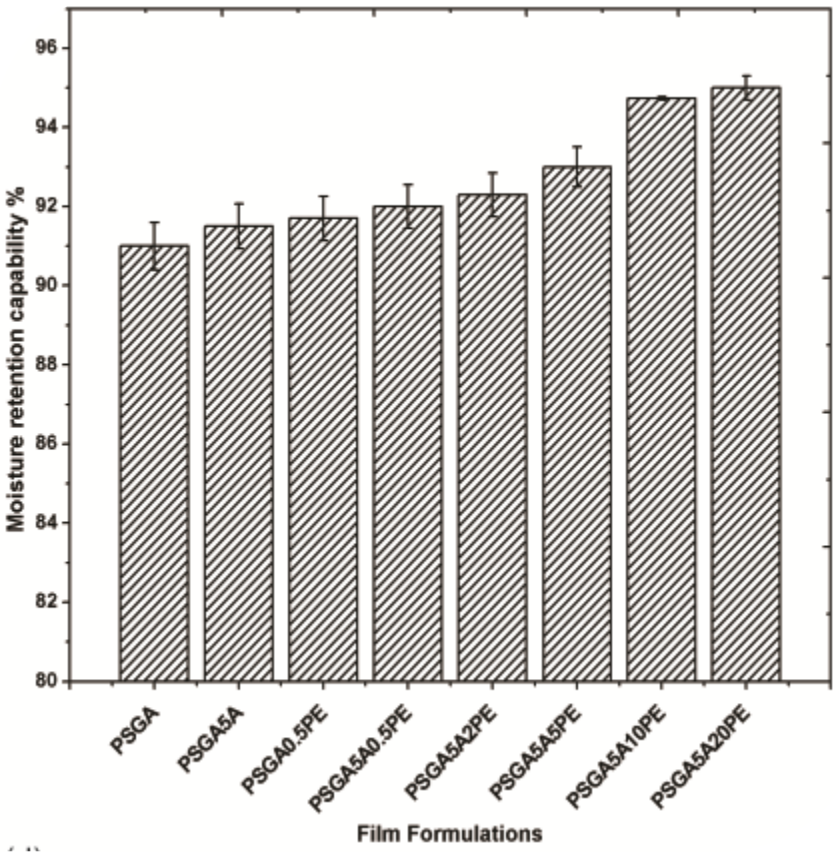

Figure 19: Moisture retention capability of films formulations containing GA as cross-linker

So, the moisture retention capability of film formulations was enhanced by the addition of higher concentration of PE in formulated films. Both films formulations have almost same moisture retention capability.

4.4.3. Normalize swelling Degree

NSD is referred to cross-linked density of polymeric network, which may include polymers, additives, cross-linkers, plasticizers and other component that can affect inter-molecular interaction. NSD and cross-linked density has an inverse relation i.e. higher the value of NSD lower will the cross-linked density [69].

It has been observed that PSB5A20PE has the maximum NSD value as shown in Figure: 20. Whereas, PSB has the lowest NSD value. This can be ascribed to the incorporation of PE into cross-linked PVA-Starch matrix. This incorporation has increased the chain-distance and resulted in higher value of NSD. Whereas, PSB films are cross-linked with BA and do not contain any additive. Hence, they have strong interaction with each other, resulting in high rigidity and less swelling degree[69].

Table 13: Normalize Swelling degree of films formulations containing BA as cross-linker

Films Formulations	NSD (cm^2)	Standard Deviation
PSB	0.0007	0.0000057
PSB5A	0.00069	0.0000056
PSB0.5PE	0.00071	0.0000057
PSB5A0.5PE	0.00072	0.0000055
PSB5A2PE	0.00075	0.0000054
PSB5A5PE	0.00078	0.0000054
PSB5A10PE	0.00082	0.0000052
PSB5A20PE	0.00099	0.0000049

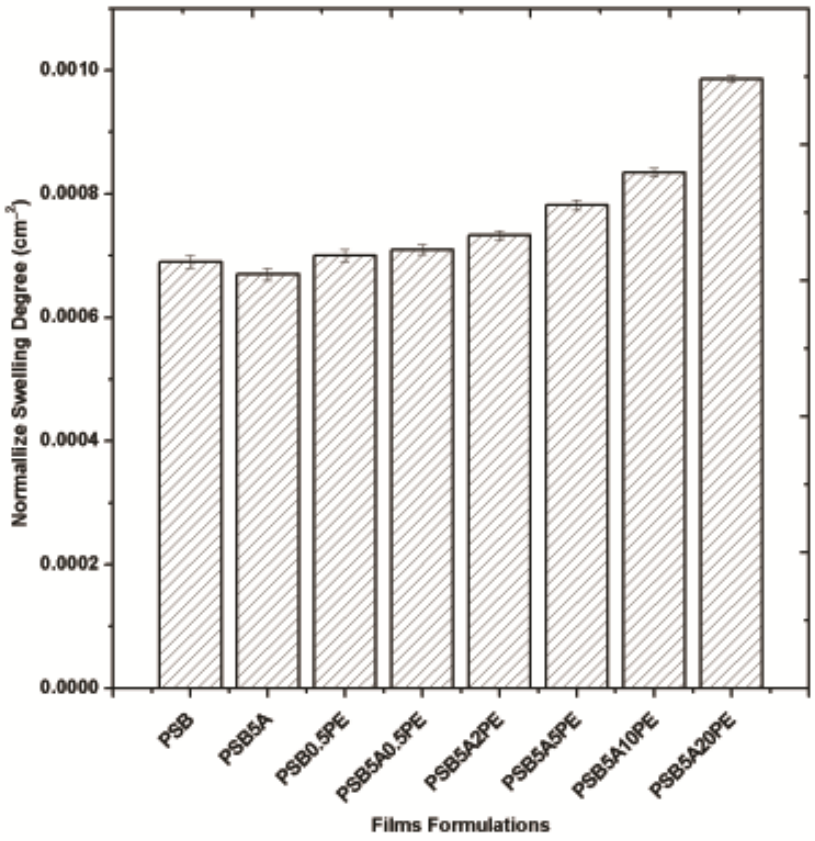

Figure 20: Normalize Swelling degree of films formulations containing BA as cross-linker

In Figure: 21, it was observed that PSGA5A20PE has the highest NSD value whereas; PSGA has the lowest value. The reason behind high NSD value could be the addition of ATH and PE as the incorporation of these additives result in an increase of chain distance, as well as molecular interaction. Resultantly, cross-linked density is decreased and swelling degree increased [69].

Table 14: Normalize Swelling Degree of films formulations containing GA as cross-linker

Films Formulations	Normalize swelling Degree (cm^2)	Standard Deviation
PSGA	0.0005	0.0000057
PSGA5A	0.00052	0.0000056
PSGA0.5PE	0.00056	0.0000057
PSGA5A0.5PE	0.0006	0.0000055
PSGA5A2PE	0.00068	0.0000054
PSGA5A5PE	0.00078	0.0000054
PSGA5A10PE	0.00083	0.0000052
PSGA5A20PE	0.0009	0.0000049

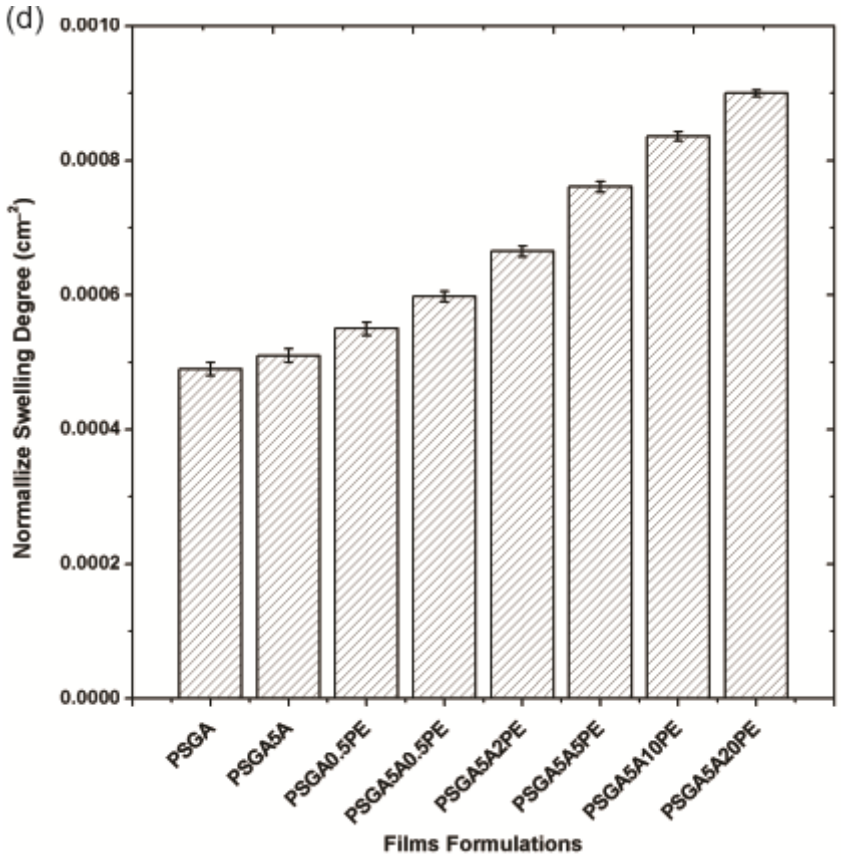

Figure 21: Normalize Swelling Degree of films formulations containing GA as cross-linker

Overall, NSD for both films' formulations containing BA and GA have maximum value is 0.0009 cm^2 for PSB5A20PE and PSGA5A20PE respectively. Which make no difference in films formulations by adding cross-linkers like BA and GA.

4.4.4. Biological Leaching Test

The BLT results for formulated films containing BA as cross-linker are presented in Figure: 22 and their values are tabulated in table: 15. Absorbance value was calculated from filtrate volume in which leached quantity was carried out. PSB5A20PE has the maximum absorbance value of 4.6 abs. Whereas, PSB has lowest absorbance value of 4.

Table 15: BLT for films formulations contain BA as cross-linker

Film Formulations	Absorbance value(abs)	Standard Deviation
PSB	4	0.095
PSB5A	4.2	0.09
PSB0.5PE	4.3	0.075
PSB5A0.5PE	4.5	0.08
PSB5A2PE	4.6	0.0099
PSB5A5PE	4.6	0.0097
PSB5A10PE	4.7	0.0098
PSB5A20PE	4.8	0.0009

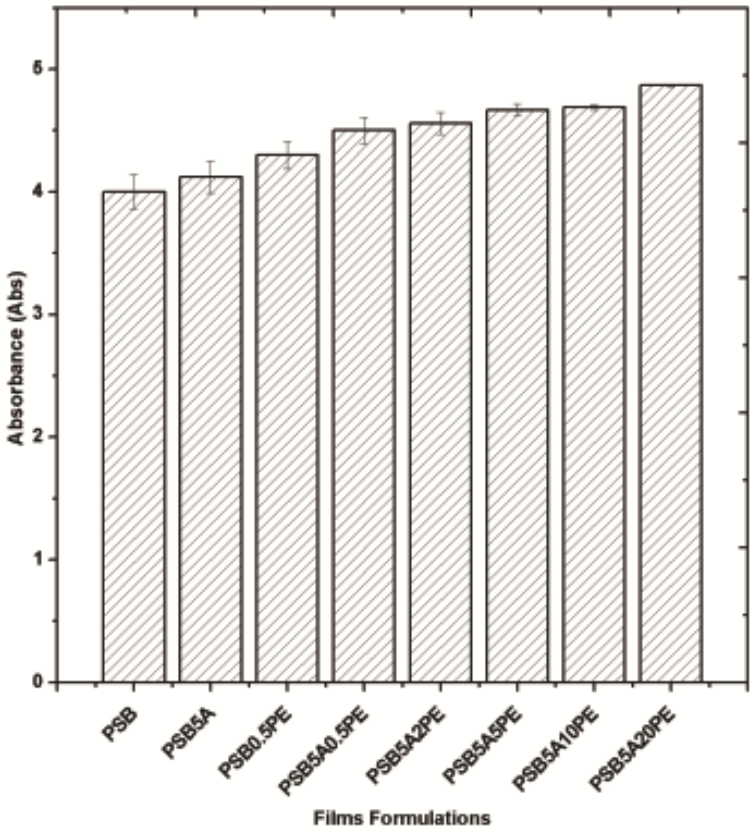

Figure 22: BLT for films formulations contain BA as cross-linker

In Figure: 23, BLT is presented for the film's formulations containing GA as cross-linker and their values are tabulated in table: 16. It is shown that PSGA5A20PE has the highest absorbance value of 4.9 abs whereas; PSGA has the lowest value of 4 abs. The highest absorbance value of PSGA5A20PE was due to the high concentration of PE used. Also, the chances of leaching augmented, as the amount was increased.

Table 16: BLT for films formulations contain GA as cross-linker

Films Formulations	**Absorbance value (abs)**	**Standard Deviation**
PSGA	4	1
PSGA5A	4.2	0.55
PSGA0.5PE	4.3	0.56
PSGA5A0.5PE	4.4	0.45
PSGA5A2PE	4.4	0.5
PSGA5A5PE	4.5	0.43
PSGA5A10PE	4.6	0.098
PSGA5A20PE	4.9	0.0009

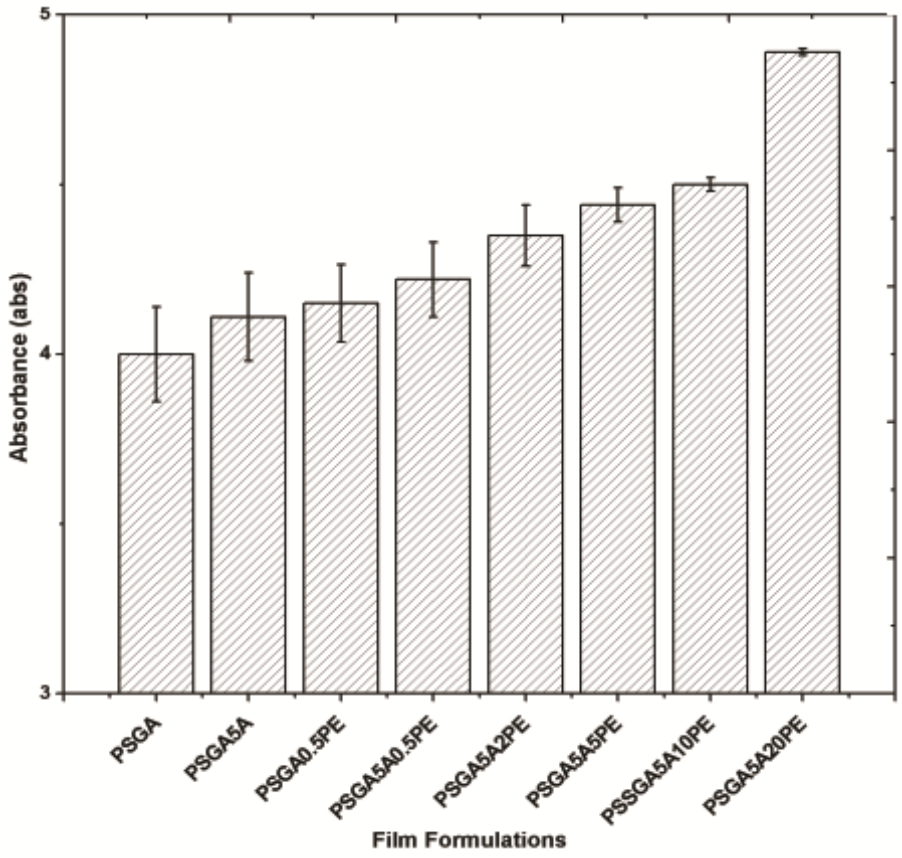

Figure 23: BLT for films formulations contain GA as cross-linker

It is understood that increasing the amount of PE can have greater chances of leaching out from the films. So, PSB5A20PE and PSGA5A20PE have the maximum leached out quantity i.e. 4.8 abs and 4.9 abs respectively. As, PE is a natural active agent so it has no effect on the environment, when leached out in minimum amount of absorbance value below 5abs [92].

4.5. Colorimetric Property test

Addition of ATH in PVA-Starch cross-linked matrix is responsible for color change in films at different pHs. Poly-phenolic pigments of plants are the major component in ATH, which can change color to red, blue, green and yellow with variation in pH [93]

The results of colorimetric response of films containing BA as cross-linker are presented in Figure 24. PSB5A20PE film was tested against the said pH range. From pH 1 to 5 (acidic) the color changed from reddish to blue, respectively. Whereas, blue color was noticed at pH 7(neutral). Furthermore, color changes from blue to green and then yellow when pH was ranging from 8 to 14, respectively.

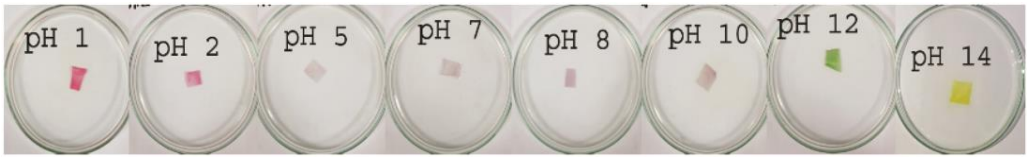

Figure 24: pH response of films formulations containing BA as cross-linker

Figure: 25 illustrates the varying color response of film formulations containing GA as cross-linker. The change in pH and dependence on pH was ascertained. The film used for this test was embedded with ATH and PE (PSGA5A20PE).

At lower pH (acidic) 1 to 5, film color changed into pink whereas; when pH increased it was converted into purple. At pH 7, the color of the film was light purple and when pH moved towards basicity the color film changed to blue-green. Finally, at pH 14 the color was changed into yellow.

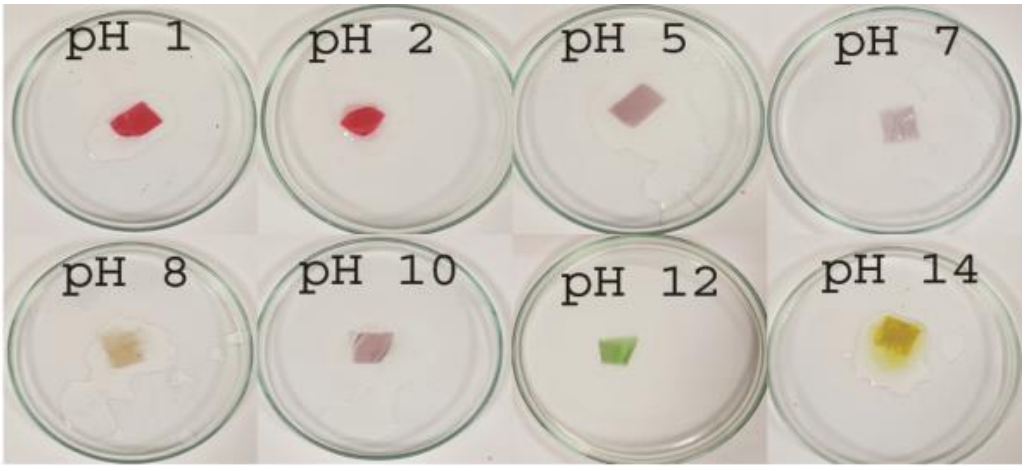

Figure 25: pH response of films formulations containing GA as cross-linker

These results are depicted to a mixture of four different molecular groups present in Anthocyanin i.e. flavonoid cation, carbinol pseudo-base, quinidol base and chalcone. Flavonoid cation concentration depict red color at lower pH (1-2), carbinol pseudo-base present purple color, quinidol base is responsible for green and chalcone is responsible for yellow color respectively[94]. The distinguishable color change due to presence of ATH in these films formulations makes films as an intelligent material. Food spoilage slightly lowers the pH that results into color change of films. Hence, addition of ATH into these films improves their applicability[7].

4.6. Anti-bacterial Property test

Anti-bacterial activity of films formulations was tested against two common food born bacterial strains i.e. E. coli and MRSA. Disc diffusion method was used to measure anti-bacterial property. The inhibition zones diameter is presented in graphical form and antibacterial activity is shown in image form.

Figure: 26, depicts the measurement of inhibition zones developed by PSB5A (with 0.2%, 2%, 5%, 10% and 20% of PE) films formulations containing BA as cross-linker. This activity was monitored for 48hrs. PSB5A20PE has shown maximum inhabitation zone of 22mm after 24hrs and 21mm after 48hrs against E.coli. Furthermore, maximum activity against MRSA was also depicted by PSB5A20PE i.e. 15mm dia after 24hrs and 14 mm dia after 48hrs. Hence, it is proved that by increasing the concentration of PE into film formulations increases antibacterial activity.

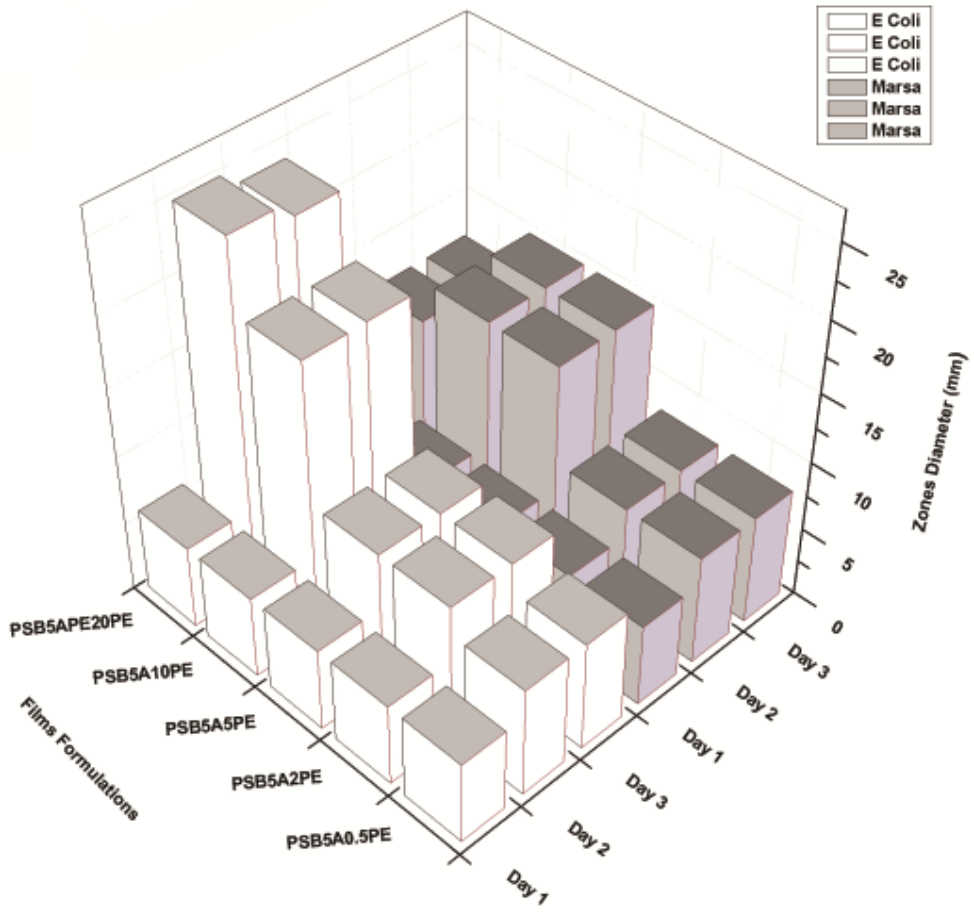

Figure 26: Zone of inhibition diameter for films formulations containing BA as cross-linker

In Figure: 27, PSB5A (with 0.2%, 2%, 5%, 10% and 20% PE) film discs were mounted on nutrient agar plate for disc diffusion procedure. Bacterial strains i.e. Escherichia Coli and MRSA are spread over these plates. The Figure 27, clearly suggests that active component (PE) properly diffused into agar medium and clear inhibition zones were observed. It was also noticed that by increasing the concentration of PE into PSB film matrix, inhibition zones were more prominent and larger in diameter.

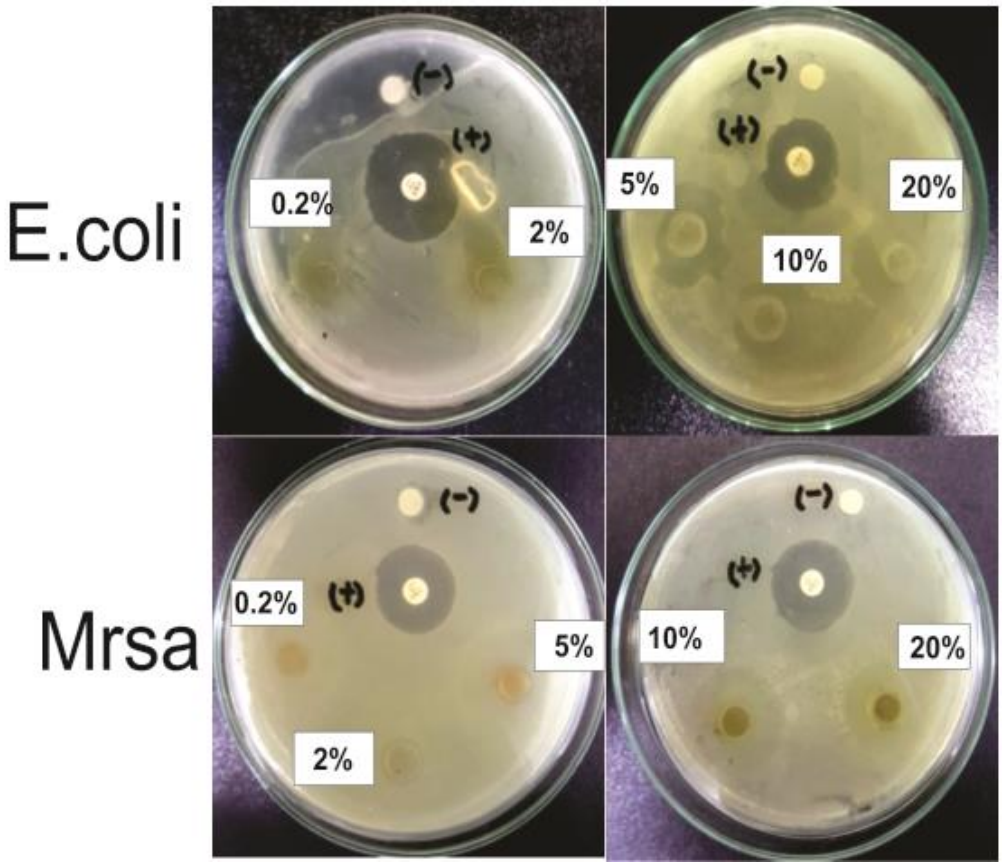

Figure 27: Disc diffusion presentation of anti-bacterial activity of films formulations containing BA as cross-linker

In Figure: 28, diameters of zones of inhibition are presented for the films formulations containing GA as cross-linker. The maximum inhibition zone was achieved by PSGA5A20PE i.e. 23mm after 24hrs and 23mm after 48hrs against E. coli and 19mm after 24hrs and 18mm after 48hrs against MRSA after 48 h respectively. So, it is clear that by increasing the amount of active agent i.e. PE enhanced anti-bacterial activity.

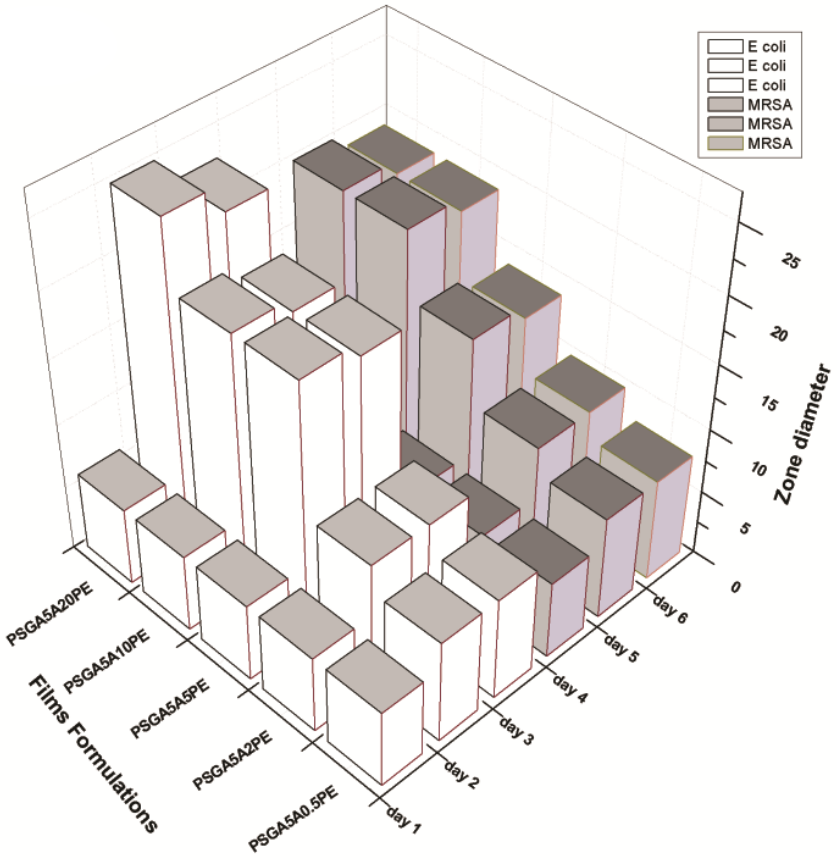

Figure 28: Zones of Inhibition diameter for anti-bacterial activity of films formulations containing GA as cross-linker

In Figure: 29, disc diffusion method was presented in which 6mm discs of PSGA5A (with 0.5%, 2%, 5%, 10% and 20% PE) were mounted on LB media. Clear zones of inhibition were observed when the concentration of active agent i.e. PE was increased. The highest activity against E coli and MRSA was shown by PSGA5A20PE.

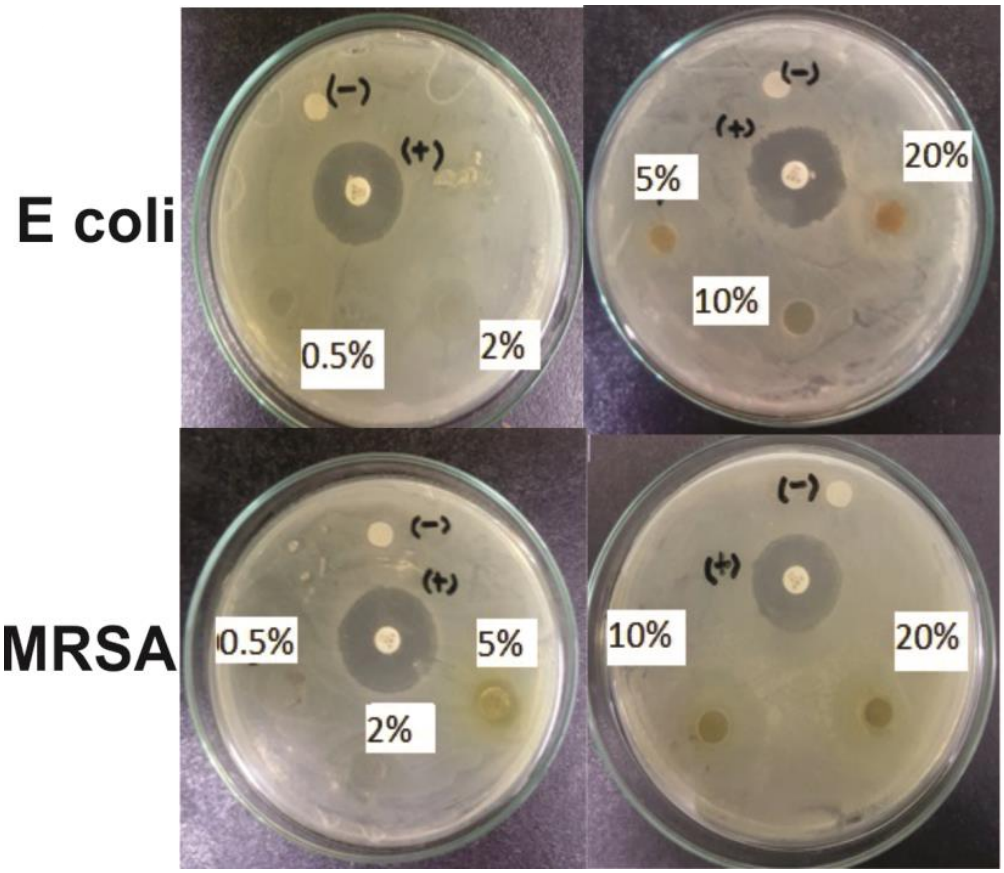

Figure 29: Disc diffusion presentation of anti-bacterial activity of films formulations containing GA as cross-linker

So, it is clear that by increasing the amount of active agent i.e. PE enhanced anti-bacterial activity. The anti-bacterial activity of PE can be explained on the basis of its chemical composition. It contains poly-phenols, flavonoids and aromatic rings. Such components are able to inhibit bacterial growth and kill bacterial cells[95]. As, propolis being organic component it can be used in food packaging due to its less harmful effects to the environment and human health [74].

4.7. Food Spoilage test

Food spoilage test was performed to check the applicability of prepared active and intelligent films.

The active and intelligent films formulations PSB, PSB5A10PE and PSB5A20PE was tested against pasteurized milk which had pH of 7. Films formulations with 20% PE were selected best as they had maximum anti-bacteria activity. Figure: 30 shows the results of spoiled milk after 48hrs for PSB and PSB5A10PE. Whereas, PSB5A20 PE has not spoiled the milk and protected against bacterial attack. It was clearly observed that PSB5A10PE has a color change from brown to reddish pink. This can be the result of change in pH of milk.

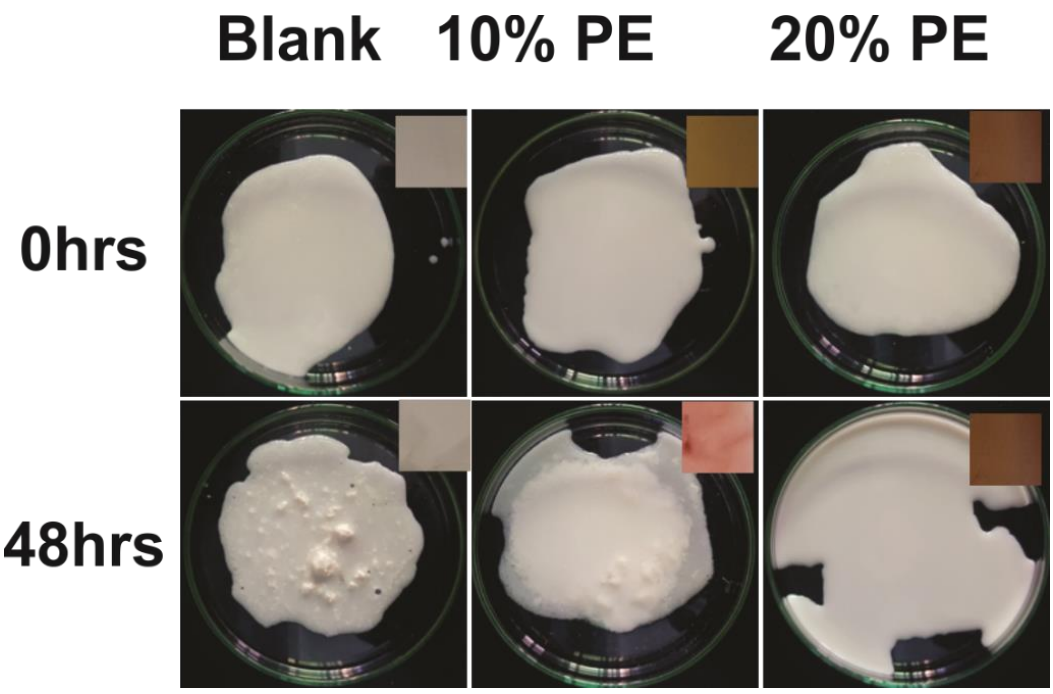

Figure 30: Food spoilage test of films formulations containing BA as cross-linker

Similarly, in Figure: 31, three different films i.e. PSGA, PSGA5A10PE and PSGA5A20PE were tested using pasteurized milk. Milk has pH 7 at normal conditions but spilt milk has pH 2. Therefore, after 48 h at 10^{o}C results were recorded and they suggested that films sample PSGA5A20PE has the maximum antibacterial activity. From Figure: 31, it is clear that blank sample with 0% PE has no antibacterial activity. Furthermore, PSGA5A10PE has the minimum anti-bacterial activity. The color of film changed from orange to pink due to decrease

in pH of spilt milk. PSGA5A20PE has the highest anti-bacterial activity with no spilt milk and color change.

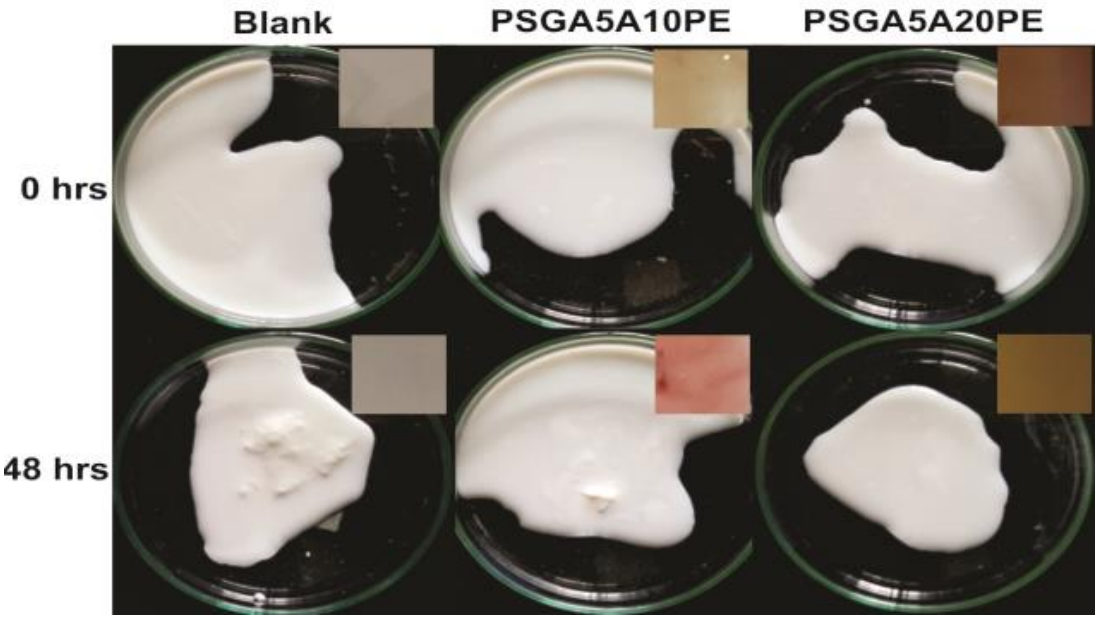

Figure 31: Food spoilage test of films formulations containing GA as cross-linker

Spoiled milk has slightly acidic pH ranging from 5.5 to 6 due to production of lactic acid after spoilage [7]. Whereas, the formation of lactic acid was inhibited with increase in the concentration of PE information up to 20% in both films i.e. PSB5A20PE and PSGA5A20PE. This stops the growth of milk born bacteria i.e. Staphylococcus aureus and Bacillus subtills [77]. Therefore, the color of film did not change and can be utilized effectively for enhancing shelf life of food.

Conclusion

This study was aimed to prepare biodegradable active and intelligent films containing BA and GA as cross-linkers, which are further evaluated and characterized by various tests. Firstly, surface morphology proved that increasing concentration of PE increased the homogeneity and smoothness of the surface regardless of the cross-linkers used. FTIR and XRD analysis further show that films mixture at higher concentration of PE had good molecular interaction and functional groups were properly attached. Similarly, by adding cross-linker the mechanical properties of films had been tailored in a positive way. Films containing GA have shown better mechanical strength with maximum tensile strength of 15.8 MPa and % elongation of 1.8%. Physical tests like WVTR, moisture retention capability, BLT and NSD had proved that molecules had hydrogen bonding and strong intermolecular interaction at higher concentration of PE. These film formulations had a quick and clear color change response to pH. Anti-bacterial activity test had exposed a high anti-bacterial activity against both E-coli and MRSA bacterial strains with an increase in PE concentration. Finally, food spoilage test had also shown bacterial attack retaliation at higher PE concentration and color change response as well. Overall, PSB5A20PE and PSGA5A20PE film formulation have depicted better results and are appropriate for biodegradable food packaging applications.

Future Scope

These active and intelligent films have broad spectrum of applications in food packaging industry. The features like anti-bacterial activity and color change property at different pH's make them suitable material to be used in food industries like milk, vegetable, fruit, meat and sea food etc.

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