Pursuance of Piper Nigrum and Cinnamomum Verum on Poly vinyl Alcohol-Starch Hydrogel Membrane

[1]Hafsa Iqbal, [1]Sarah Farrukh*, [1]Arshad Hussain, [1]Awais Hassan, [2]Sintayehu Mekuria Hailegiorgis
[1]School of Chemical and Materials Engineering (SCME), National University of Sciences & Technology (NUST), Sector H-12, 44000 Islamabad, Pakistan
[2]Chemical Engineering Department, Universiti Teknologi PETRONAS (UTP), Bander Seri Iskandar 32610, Perak Dural Ridzuan, Malaysia.
Sarah.farrukh@scme.nust.edu.pk*

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Summary: A series of poly vinyl alcohol (PVA)/starch hydrogel membranes (0gm, 0.1gm, 0.3gm, 0.5gm, 0.7gm, 1gm, 1.5gm) were prepared with varying contents of *piper nigrum* and *cinnamomum verum* by solution casting method. The comparison was made between the pure PVA/starch hydrogel and natural antimicrobial agents assembled hydrogel matrix membrane. Glutaraldehyde was added as a crosslinker. Whereas, Glycerin was incorporated as a plasticizer to increases the ductility of hydrogels membranes. The concentration of loaded natural agents affects the physical properties of the hydrogel determined by swelling behavior, gel fraction, moisture retention and water vapor transmission rate. The prepared membranes were characterized by scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR). SEM shows saturated and matrix structure of membrane which makes the tensile strength of fabricated membranes as high as 9.8 MPA, which suitable to be used in biomedical applications. Antimicrobial activity was investigated against *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* bacterial strains. The anti-fungal activity of these membranes was studied for *Aspergillus Flavus, Aspergillus Oryzae* and *Aspergillus Subolivaeus* fungus strains. Hydrogel membrane with 1.5g of natural antimicrobial agents has shown greater inhibitory activity. The results predict that; the membrane can be used as a wound dressing to provide a cushioning effect on the skin.

Keywords: Hydrogel, Antimicrobial activity, Polyvinyl alcohol, Black pepper, Cinnamon, Wound dressing.

Introduction

The process of repairing skin was considered as natural procedure which was taken into consideration by proposing ‘Three healing gestures”. These were the late medical words in 2000BC that describes the science of healing of a wound. It included three things, wash the injury, bandaging it and applying the dressing. Nowadays, there is great advancement on the healing bandages. As the late famous scientists Joseph Lister and Louis Pasteur concluded that a bacterium enters the wound from the outside source. This requires the washing of injuries and wounds rapidly with an antiseptic technique to kill bacteria on the surface, to avoid any infection [1].

Hydrogels are cross-linked three-dimensional structure of polymer, utilized in great range of applications such as cell culturing, regenerative medicine, agriculture, contact lenses, biosensor, drug delivery and tissue engineering. Wichterle and Lim in 1960 introduced hydrogel in medical application [2] while the Yannas group in 1980 initiated work on combining the hydrogels with naturally occurring polymers such as PVA and starch [3] and their work was focused on contact lenses by using poly (2-hydroxyethy methacrylate) (PHEMA). They are flabby in texture and have great water content [4]. Every year billions of people undergo chronic wounds which had long healing duration (<6 weeks) or can undergo perennial recurrence while acute wounds recovers in less time. The main problem was the healing of chronic wounds. According to statistics, it costs $13 to $15 billion for developing wound dressing globally [5].Some hydrogels have been commercialized such as “Geliperm®, Curasol® and Tegaderm®” under these brand names. Tegaderm was a thin urethane film while the Geliperm made up of agar material with acryl amide. These were synthesized by the chemical crosslinking of methylene bis acrylamide including some polysaccharide [6, 7].

With the emergence of new infectious bacteria and fungi, several infections have appeared and due to increase in microbial resistance there is requirement to use alternative natural antimicrobials. Selection based on their simplicity, non-toxicity and rapid process [8]. F. P. Mdoe *et al.*, [9]reported the antimicrobial activity of Cinnamomum osmophloeum as it consists of two components courmin and cimmaldehyde. Cimmaldehyde present in the plant up to 76% of the total constituents and was an active component for showing the antimicrobial activity in

***To whom all correspondence should be addressed.**

the cinnamon plant. T. K. Goswami *et al*[10] reported medicinal uses of black pepper as its inflammatory action, antipyretic and antimicrobial. Piperine pungent alkaloid is present in black pepper up to 17000-90000 ppm and responsible for antimicrobial activity [11].

In hydrogels the different sizes of antibiotic, loaded into the matrix, releasing their antibacterial effects; allow the usage of swollen or dried networks of polymers in wound dressing [12]. Hydroxyl hydrophilic groups attached in hydrogels provide the necessary dissolution between the crosslink networks. Water molecules present in hydrogels move freely, while the polymer matrix holds the water molecule together [13].

In 20th century, PVA poly (vinyl alcohol) emerged as artificially synthesized polymer worldwide [14]. It was a synthetic polymer which was biodegradable water–loving biopolymer and has wide range of applications in biomedical field, industry, fuel cell and separations process. PVA has some great properties such as film forming and structural strength [15, 16]. The thermal stability and mechanical strength of PVA can be enhanced by hybridization, polymerization and cross linking [15]. Previously, PVA was blended with alignate, polyvinyl pyrrolidone, starch, glucan, chitosan, dextran [18, 19]. Every blended component showed different properties [4]. Polysaccharides such as most abundant starch formed by network of glucose units' glycosidic bonds. Pristine starch lacks mechanical strength. However, its mechanical properties can be enhanced if blended with PVA. As PVA/starch blends along with glycerin as a plasticizer can enhance the tensile strength and rigidity [20].

Glutaraldehyde (GA) as a cross linking agent provides the fixation of cellular network. By cross linking with the proteins it was used to crosslink the carboxy peptidase crystals with enzymatic activity [21]. By the reaction of GA and OH present in hydrogels represent the acetal bridges formed. The addition of GA into the PVA hydrogels modifies the structure by formation of acetal bridges and covalent bonds thus reducing the flexibility [22].

By keeping all the facts in mind, it was planned to consider PVA in synthesis of biodegradable wound dressing with natural antimicrobial agents to protect the wound from any microbial infection. Use of turmeric in PVA/starch hydrogel has been reported by Awais *et al*[23]. Incorporating natural antimicrobial agents i.e. black pepper and cinnamon are important as they are compatible, non-toxic, environmental friendly and more effective in terms of antimicrobial activity, reported earlier.

Experimental

Materials

Polyvinyl alcohol with 1500 MW, Starch (corn) of extra pure quality, Glutaraldehyde 50% aqueous solution with 100.12 MW and Glycerin were purchased by Dae-Jung chemicals. Ethanol absolute was purchased by Reidal-De-Haen chemicals. Black Pepper and cinnamon were purchased locally. Distilled water was used throughout the experiment. In order to check antimicrobial activity nutrient Agar media (Oxoid Ltd, England) and LB broth (Merck Millipore Darmstadt Germany) used for culturing the bacteria. Potato dextrose agar was purchased from Oxoid Ltd to carry out antifungal activity procedure. All of the materials were purchased from Reliance scientific Ltd Pakistan.

Synthesis

Polymeric hydrogel membranes were prepared using the method applied by Kunal Pal *et al*(2006) [24] with more refinement. 10% w/v solution of PVA was prepared in 25 mL distilled water, heated for 2hr at constant stirring at 70°C. Separate solution of 7%w/v starch was prepared in 25mL distilled with constant stirring for 20 minutes at 100°C. Cinnamon and black pepper powder was passed through US mesh 100 mechanical sieve with opening of 0.149mm. Different contents of cinnamon and black pepper were added into the starch solution i.e. 0gm, 0.1gm, 0.3gm, 0.5gm, 0.7gm, 1gm, 1.5gm. Both solutions were mixed. Again, separately cross-linking solution was prepared by taking 5ml of ethanol with 0.25 mL of glutaraldehyde and 0.025mL of HCL and added into the hydrogel mixture. 1ml of glycerin was added afterwards as a plasticizer [25]. Hydrogel solution was sonicated by probe sonicator for 2h. According to solution casting method, the mixture was poured into the plastic Petri dishes and dried for 24h at room temperature. The dried hydrogel membranes were removed from the dishes and secured in air tight plastic pouches, the same procedure was followed for the preparation of hydrogel membrane without cinnamon and black pepper. Fabricated membranes were named as pure membrane (Mo), black pepper hydrogel membrane (M1) and cinnamon hydrogel membrane (M2).

Characterization of Hydrogels

Fourier Transforms Infrared Spectroscopy (FTIR)

Powdered material was characterized by making KBr pellets in a hydraulic press while the hydrogel membranes were directly placed in FT-IR (PerkinElmer, Spectrum™ 100) machine.

Scanning Electron Microscopy (SEM)

Images were taken at 20-5kV with required magnification. Samples were prepared by mounting them on metal studs by using double sided tape. They were kept in the desiccators to avoid sorption of moisture, analyzed in JEOL (JSM-6490A).

Tensile Strength Measurement

Membrane was cut into pieces of 2×1 cm dimension and analysis was performed at a stretching strain rate of 10 N/mm^2.Sample width was 0.136mm. Selected sample should not have any surface defect or notches at the edges. Samples were prepared according to ASTM D 638–14 standards. The samples were loaded into the clamps of the machine which holds the membrane edges smoothly [26]. The two clamps in opposite directions moved at constant rate with constant speed. The strain was measured till the membrane fractures, tested on Shimadzu Universal tensile test machine.

Antimicrobial Activity

Antimicrobial activity was determined by Agar disk diffusion method. Nutrient agar was used as media for culturing the bacteria and incubated in upright position at 37°C for 24h. *E coli* and *MRSA* strains have been prepared in broth medium was inoculated by using 1ml of microbial culture in 10ml of broth medium and kept on overnight shaking at 37°C. In order to check antibacterial activity, 10µL dose of broth solution with help of micro pipette was spread on the agar plates and membrane disks were placed on the test plates under laminar flow cabinet. The inoculated plates were incubated for 24h at 37°C.

Antibacterial activity was checked against Gram negative bacteria Escherichia Coli (DH5-Alpha strain) and Gram positive bacteria Methicillin-resistant Staphylococcus aureus (MRSA). While for antifungal activity, potato dextrose agar (PDA) was used as media. Antifungal Activity was checked against these three fungal strains from Aspergillus family, *Aspergillus Flavus*, *Aspergillus orzyae* and *Aspergillus subolivaeus*. The antimicrobial activity was assessed by measuring the inhibition zone diameter (IZD) around the disk. Ampicillin was used as positive control and PVA pure disc was as negative control.

Physical Property Characterization of Hydrogels

Water Vapor Transmission Rate

The sample contained 10ml de-ioned water with 29.5 mm bottle diameter. The hydrogel was capped on the mouth of the bottle sealed with help of Teflon tape, placed in an oven at 40 °C for 24h. The bottles were taken out and the water vapor transmission rate was measured by using this formula [27].

Water vapor transmission rate:

$$\frac{w_i - w_t}{A \times 24} \times 10^6 \; gm^{-2} h^{-1} \quad (1)$$

whereas, A was the area of the round bottle (mm), Wi and W_t were the weight of bottle before being placed in the oven and after being removed from the oven, respectively.

Moisture Retention Measurement

The hydrogel membrane was slit into the equal pieces and weigh at room temperature. They were placed in oven at 40° C for 5hr. Removed from oven and weight again. Moisture retention was found out by using this formula.

$$Rh\,(\%) = \frac{w_t}{w_i} \times 100\% \quad (2)$$

W_i was the initial weight and W_t was the final weight after taking out from the oven.

Swelling Behavior Measurement

Swelling behavior was investigated against simulated body fluid (SBF). The composition of SBF solution is given in Table-1.

Table-1: Composition of SBF solution.

Sr.No	Chemicals	Quantity (g)
1	NaCl	7.996g
2	$NaHCO_3$	0.350g
3	KCl	0224g
4	$K_2HPO_4.3H_2O$	0.228g
5	$MgCl_2.H_2O$	0.305g
6	$CaCl_2$	0.278g
7	Na_2SO_4	0.071g
8	$(CH_2OH)_3CNH_2$	6.057g

Hydrogel membrane was equally slit and placed in the oven to obtain constant weight. Then the samples were immersed in SBF solution for 24hr. The membranes were taken out and the excess water on the film was absorbed by the filter paper and again weight [27]. The swelling ratio was determined by the following equation

$$\text{Swelling Ratio} = \frac{w_s - w_d}{w_d} \times 100\% \quad (3)$$

whereas, W_d was the constant weight while the W_s was the weight of the swelled hydrogel.

Gel Fraction

Percentage of the initial sample weight over the remained sample weight was the gel fraction. To determine, gel fraction the hydrogel were slit in the equal pieces and weigh and kept in vacuum oven at 40°C to maintain constant weight. Then the weighted hydrogels were then immersed in water for 4 days at room temperature. After 4 days, the hydrogels were removed. Placed in vacuum oven and weighted by following formula,

$$\text{Gel fraction (\%)} = \frac{w_e}{w_o} \times 100\% \quad (4)$$

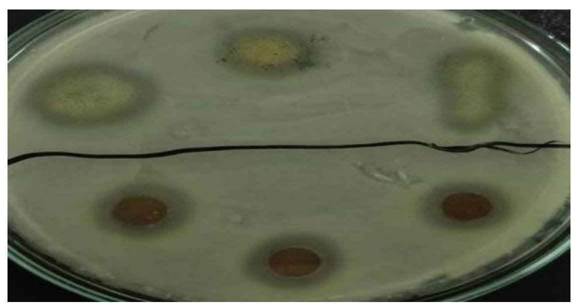
W_e weight of the hydrogel after the extraction and W_o was the constant weight.

Result and Discussion

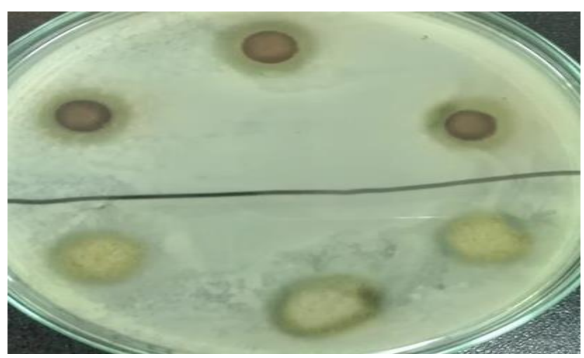
Antibacterial Activity

The optimum quantity of black pepper (M1) and cinnamon (M2) is 1.5gm on the basis of their activity and cytotoxicity. The neat membrane did not show any signs of antibacterial activity. In Fig. 1, membranes were tested against *DH5-alpha E coli* strain and *MRSA* strain While M1 (black pepper) showed best results as compared to M2 (cinnamon). Membranes were made of 6mm round equal disc pieces. The inhibition zones recorded in terms of M1 was 20mm and in M2 was 18mm at 1.5gm concentration of spices. Increase in concentration of cinnamon results in increase of antibacterial activity. The inhibition zones of the 1.5g and 1 g are almost comparable. Similar trend was observed for hydrogel containing black pepper as represented in Fig.2. This showed that the 1.5gm is the most suitable concentration of natural anti-microbial agents to be incorporated in polymer matrix.

The Gram-positive bacterium was less resistant than Gram-negative bacteria. The cell wall of Gram-positive cell consists of 90-95% of peptidoglycan. Hydrophobic compounds can penetrate through the cell wall of the Gram-positive bacteria and can act on the cell wall and deeper inside of the cytoplasm while the PVA hydrogel was hydrophilic, the bacterial structures may disrupt and results in the degradation of the cell wall by the actions of the membranes. Different compounds present in the natural antibacterial agents have different effects on the structure of the bacteria. The Gram –negative cell wall was more complicated, the peptidoglycan layer was much thinner than the Gram positive cell wall. The mechanism of antibacterial activity by black pepper and cinnamon depends upon the composition added. Both will have different mechanism in Gram –positive and Gram –negative bacteria. The functional groups present on the compounds have different activity. Cimmaldehyde had the capability to alter the lipid profile of the cell membrane. The phenols and mono-terpenes present in the natural compounds exhibit antifungal and antibacterial activity. The active agents in black pepper and cinnamon cause the leakage in the cell wall. The cells after some time start to disintegrate and collapse showing the activity of the agents against bacteria. This activity shows more effect on the Gram-positive bacteria than Gram-negative bacteria due to their major cell wall differences [28, 29].

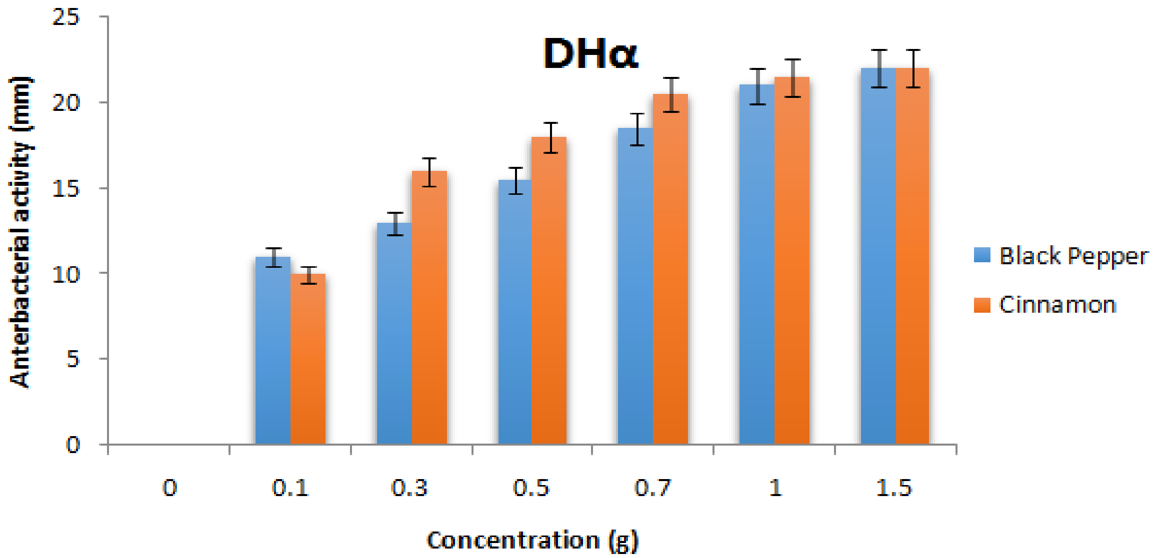


E coli



MRSA

Fig. 1: Antibacterial test plates for M1 and M2 hydrogel membranes against E.coli and MRSA.



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*[Smaller the error bars, lesser will be the deviation in duplicate reading. All the error bars were small]

Fig. 2: Graph for hydrogels against E.coli and MRSA Bacteria.

*Further testes have been performed on 1.5g incorporated black pepper and cinnamon.

Antifungal Activity was examined against the three fungal strains from Aspergillus family, *Aspergillus Flavus*, *Aspergillus orzyae and Aspergillus subolivaeus*. In which the *Aspergillus flavus* was resistant to antifungal activity while the other two were sensitive.

The hydrogels containing black pepper and cinnamon have significant results. In case of *Aspergillus subolivaeous* fungi M1 and M2 had great activity and shows inhibition zone up to 22mm and 17mm respectively shown in Fig 3 and 4. While *Aspergillus orzyae* shows 15mm inhibition zone in black pepper and 10mm in cinnamon. So, black pepper incorporated hydrogels have greater antifungal activity. In Fig5, graph was plotted between concentration and antifungal activity. The pure membrane did not show any antifungal activity. However, Black pepper and Cinnamon (as described earlier) had shown anti-fungal activity.

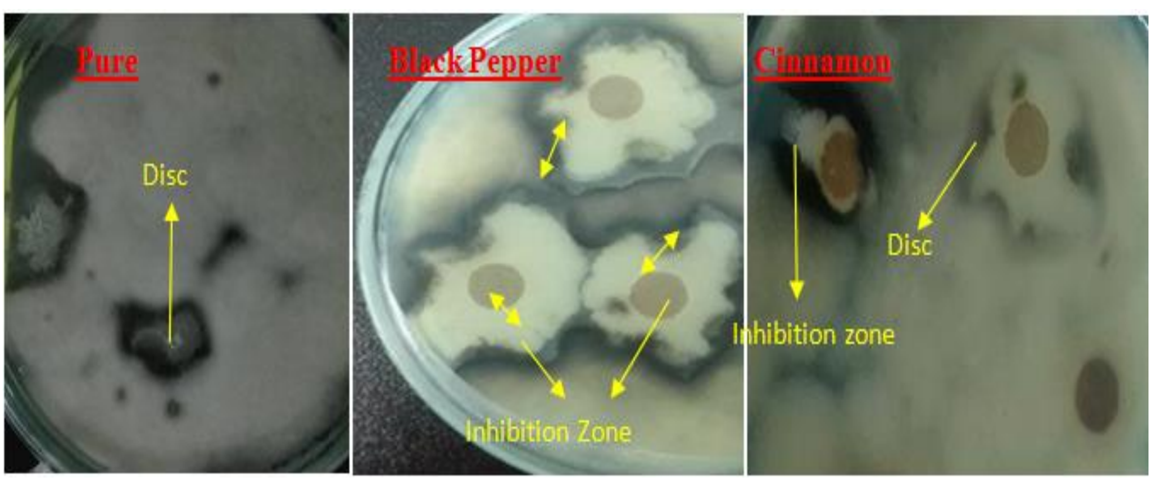


Fig. 3: Antifungal test plates for hydrogel membrane (pure (1), black pepper (2) and cinnamon (3) against Aspergillus Subolivaceus fungus.

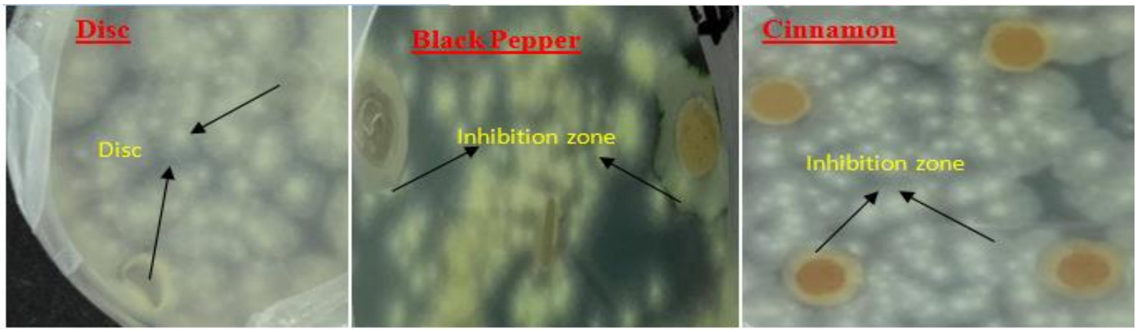


Fig. 4: Antifungal test plates for hydrogel membrane (pure, black pepper and cinnamon) against Aspergillus Oryzae fungus.

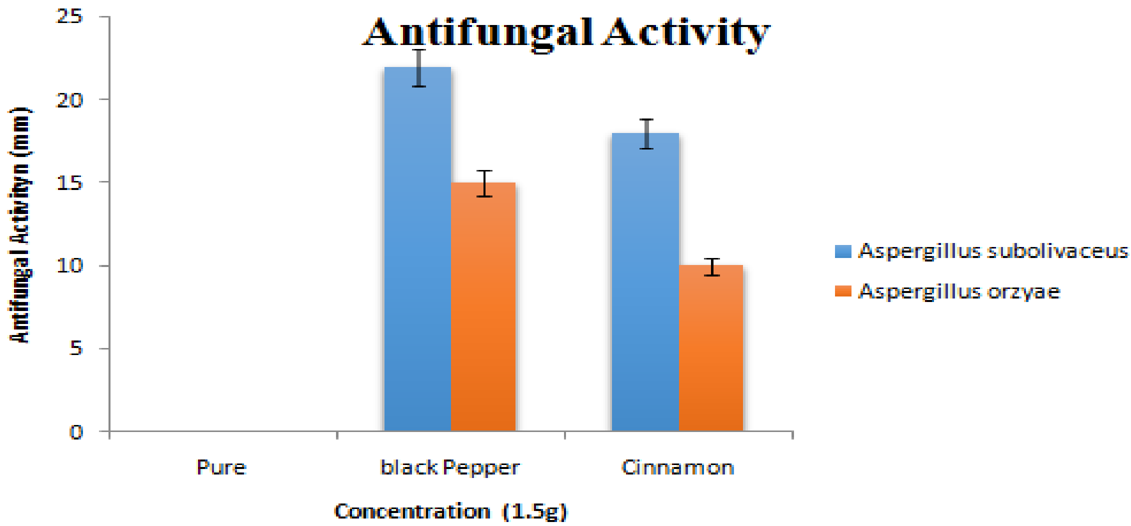


Fig. 5: Antifungal graph for hydrogels against Fungi strains.

*[Smaller the error bars, lesser will be the deviation in duplicate reading. All the error bars are small].

Table-2: Physical and mechanical properties of hydrogel membrane with and without natural antimicrobial agents

Membrane	WVTRH (g/m^{2h})	Swelling rate	Gel Fraction	UTS (N/mm^2)	Strain at break (%)	Rh (%)
Mo	74.16	140 ± 0.43	56.1 ± 0.98	19.5 ± 4.10	783.7 ± 0.67	96.89 ± 0.64
M1	72.4	122 ± 0.55	42.65 ± 0.63	9.5 ± 0.784	614.89 ± 0.92	96.65 ± 0.71
M2	73.19	132 ± 0.12	46.23 ± 0.56	9.38 ±0.337	320.9 ± 0.334	96.65 ± 0.62

Moisture Retention of Hydrogel Membrane

Pure hydrogel slightly showed the higher value because it had no other particles inside to stop the moisture from leaving, while the hydrogel membrane with cinnamon (M2) and black pepper (M1) has represented less value because presence of particles in the hydrogel structure restricts the evaporation of vapors (Table-2). Hydrogel membrane showed presence of moisture which was necessary for the healing of the wound.

Water Vapor Transmission Rate of Hydrogel Membrane

To overcome the loss of body fluids that occurs due to exudation and evaporation, hydrogel was prepared to maintain the moist environment in dressing and reduce the loss of body fluids. The water vapor transmission rate value of 53gm^2/his good in hydrogels. However, water loss from the 2nd and 3rd degree wounds is 178.55±4.5 g/m^{2}h and 143.2±4.5 g/m^{2}h [30]. The calculated values were shown in Table 2. The water vapor transmission rate depends upon the membrane thickness and the ratio of film area to water surface area. The Table 2 showed that Water vapor transmission rate of fabricated membranes is nearly same and have less effect by addition of natural antimicrobial agents [31].

Swelling Behavior

Swelling behavior of membranes was investigated in simulated body fluid (SBF) of pH 7.40. The hydrogels do not get dissolve into the solution because of the added cross-linking agent probably. Swelling increases, the separation between the polymer chains [32]. Absorption of water by M1 hydrogel membrane could be due to the presence of carbonyl group of peprine, –OH groups in polysaccharides. The pure PVA membrane Mo showed higher swelling rate than M1 and M2 (Table 2). This is due to absence of –OH groups in black pepper and cinnamon hydrogel (M1, M2) lacks the swelling ratio as compared to pure hydrogel (Mo) [23, 24]. However, M2 has more swelling rate as compared to M1, because of aldehyde group of cinnamon. But still the observed values were greater than 100% which helps the dressing to maintain moist environment to ease the healing of the wound.

Gel Fraction

To examine the cross linking, gel fraction was performed. If the hydrogel in water remains insoluble then it has perfect cross linking. Otherwise, the hydrogel immersed in water, usually gets dissolved. According to Table-2, addition of glutaraldehyde formed more entangled structure, induced interactions between the polymeric bonds and the functional groups. Whereas, Black pepper and cinnamon also got dissolved in water due to long immersion, causing leaching to give antibacterial activity [33].

Tensile Strength

For the evaluation of the mechanical properties, the tensile strength was measured for wound application. The tensile strength turned to be 19.5 MPa for neat PVA hydrogel while 9.5 MPa and 9.38 MPa for black pepper and cinnamon respectively, which is less than the failure strength of human skin (34 MPa) [24,34]. It will absorb the additional frictional stresses coming in day to day activities, without any fracture, providing safety to the injury. The Fig 6 showed that the values of pure hydrogel are higher than the incorporated membranes. Hydrogels can be affected by various factors such as temperature, pressure, branching, chain length and cross linking. The cross-linking effect is higher in pure hydrogel, it has smooth and plain morphology but the hydrogels with added agents lowers the values because it limits the chain mobility of the polymer matrix and gives the point for breakage. As the suspension did not get properly dissolve in PVA so it gave point for failure as seen in Fig 6 and values in Table-2.

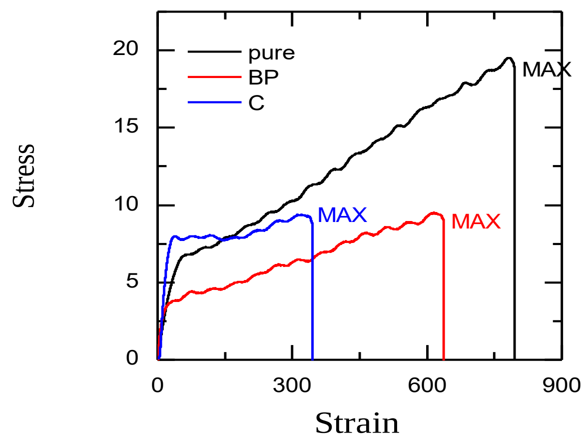


Fig. 6: Ultimate Tensile Strength (UTS) Graph for pure, black pepper and cinnamon hydrogel membranes (this Fig should be colored).

Scanning Electron Microscopy

The SEM images of fabricated membranes represent the morphology of the PVA hydrogel and agents incorporated in hydrogel membranes. At higher magnification, the structure was dense and no pores were found. It was extremely important because no pathogens can penetrate through the space and infect the wound. Starch and agents added were partially soluble in water. The particles were clearly seen in the pictures.

Fig. 7 (a) shows the morphology (surface and cross sectional view) of the neat PVA hydrogel. The membrane has dense, very smooth uniform surface. Starch can be seen over the smooth dense surface of membrane. The membrane has the cross sectional area width of 224.29nm.

Fig 7(b) and (c) shows the incorporation of micro-size particles of natural antibacterial agents in PVA-starch surface and cross section. The cross-sectional measurements of the membrane for black pepper and cinnamon were 208.48nm and 190.68nm respectively. The particles were equally distributed over the surface. Dense nature of membrane grips the particles and may get agglomerated. Particles do not have definite shape; they were scattered over the surface showing some roughness.

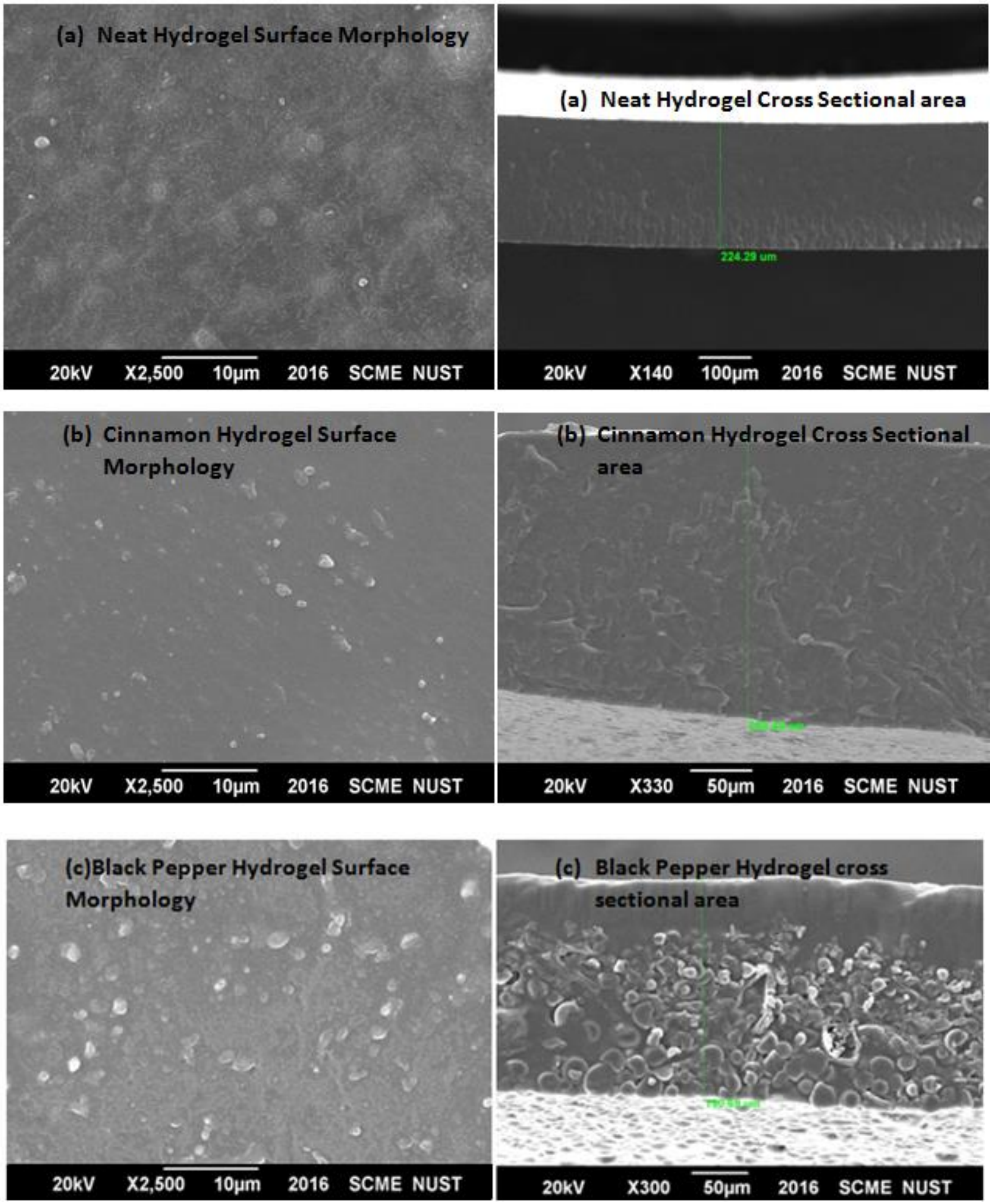
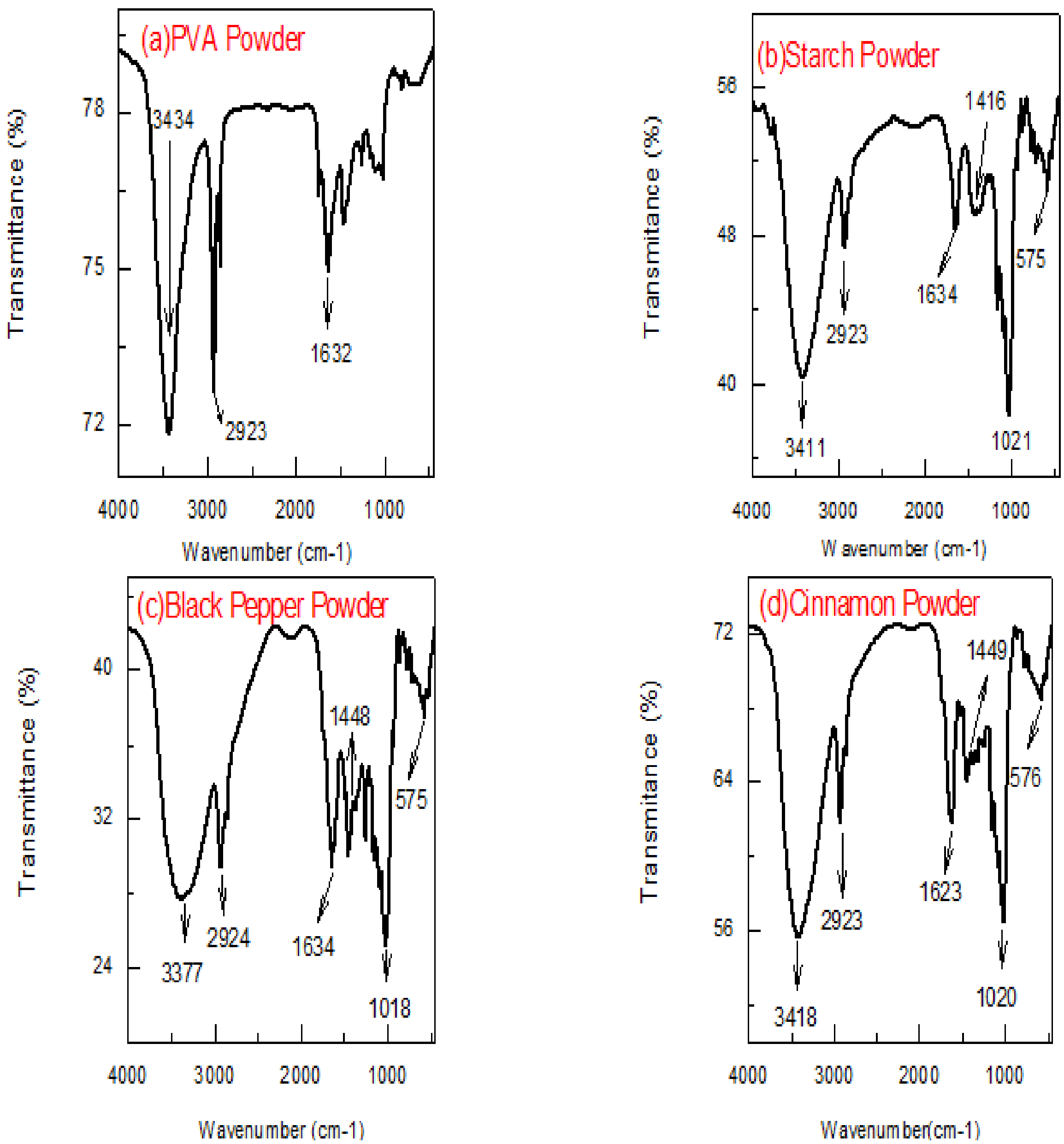


Fig. 7: SEM images of (a) neat hydrogel (b) Cinnamon Embedded in PVA Hydrogel (c) Black pepper hydrogel membranes.

Fourier Transforms Infrared Spectroscopy

Fig 8 represents the FTIR spectra of powder and fabricated membranes. PVA pure powder spectrum shows the peak related to acetate and hydroxyl groups. OH stretching shows the hydrogen bonds in the region 3000-3434 cm^{-1}. The alkyl group C-H Sp^3 hybridized carbon showed at 2923 cm^{-1} in PVA and starch refers to stretching and the vibrational bands formed in Fig 8 (a) (b). The 1620-1640cm^{-1} shows the C=O in powdered material such as PVA and starch. In starch 1020cm^{-1} peak corresponds to C-O and C-C bonds present.

The fabricated neat hydrogel and the hydrogel containing the natural antibacterial agents show the presence of hydroxyl groups at peaks 3440-3213cm^{-1}for hydrogel moisture absorption. As shown in Fig 8 (c, d, e) a large dip of hydroxyl groups is shown in both of the FTIR spectra of black pepper and cinnamon incorporated in hydrogel membrane. The added glutaraldehyde for cross linking process have peaks at 1690cm^{-1}for neat and 1721.19cm^{-1}for natural antibacterial agents incorporated membrane, showed the presence of C=O aldehyde group.



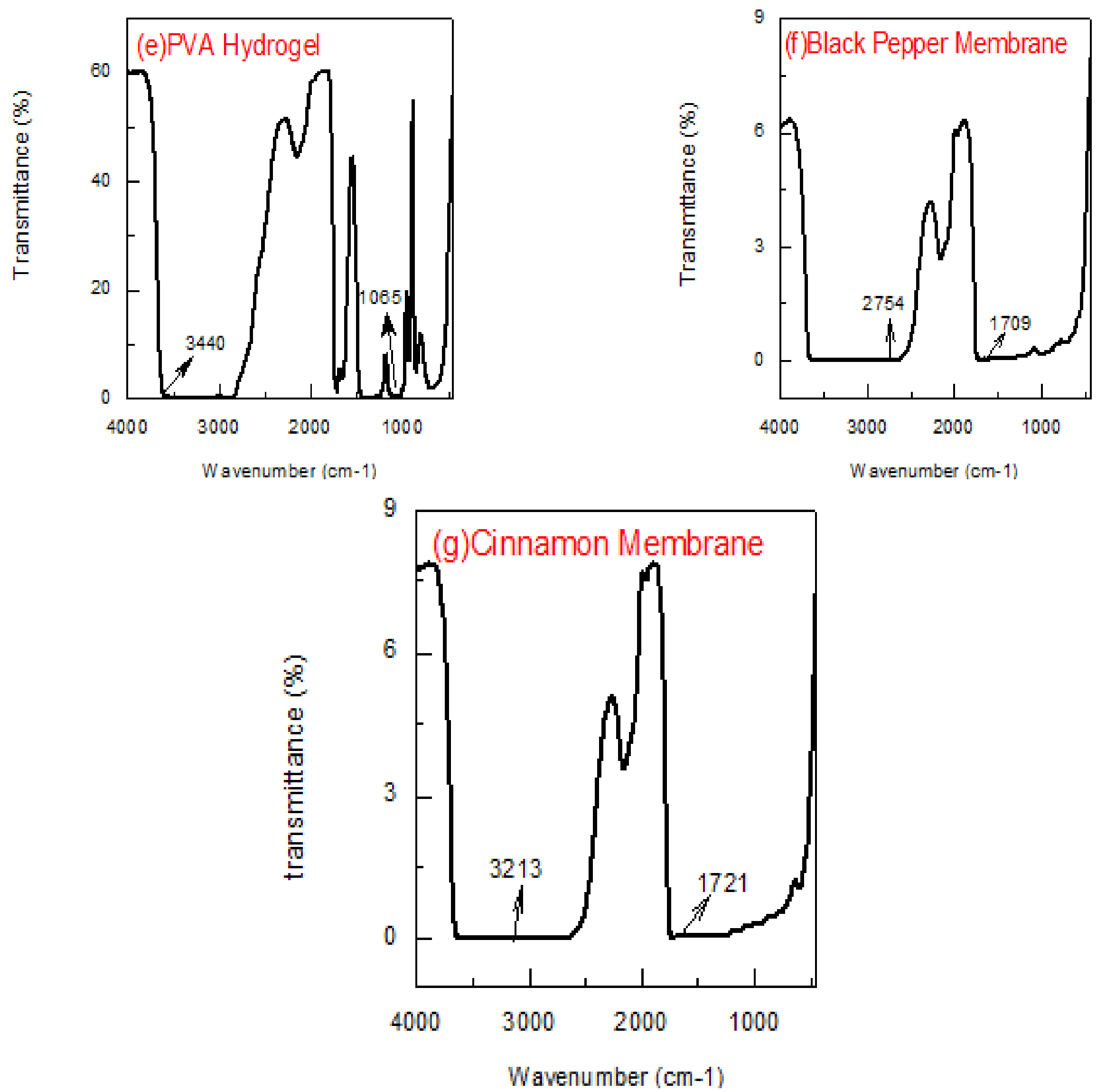


Fig. 8: FTIR spectra for (a) PVA powder (b) Starch(c)Black Pepper powder (d) Cinnamon powder(e) PVA Hydrogel (f) Black Pepper membrane (g) Cinnamon membrane.

Conclusion

The fabricated mix matrix hydrogel has required characteristics, which are essential in wound dressing application. Using all the biodegradable material as the natural polymers such as PVA, starch along with natural antimicrobial agents has surpassed synthetic polymers by increasing the therapeutic effect for wound dressing. However, 1.5g content of black pepper and cinnamon hydrogel showed the best antibacterial activity results.

Morphology studies explained the effective incorporation of macro sized particles in the interior of the polyvinyl alcohol matrix. The FTIR spectrum of the hydrogel membranes affirms the presence of –OH groups, which were mainly responsible for the hydrogel. Presence of aldehyde peak in hydrogel membranes confirms the presence of crosslinker glutaraldehyde as it did not let the hydrogel gets dissolved in any solution (pH:6-8). Addition of natural antimicrobial agents increases the physical properties such as moisture retention and water vapor transmission rate but relatively decrease the mechanical strength. Gram negative bacteria showed more resistant to the antibacterial agents, so inhibition zone was lesser than the Gram positive bacteria. Antifungal activity of M1 membrane showed highest activity against *Aspergillus Subolivaceus* while M2 had intermediate activity against *Aspergillus Orzyae*. Hence, the prepared hydrogel can be served for various biomedical applications as well as for the wound dressing application.

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