

Antimicrobial and Haemostatic Effect of Chitosan/ Polyacrylic Acid Hybrid Membranes

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CHITOSAN/ polyacrylic acid membranes containing different amounts of Al₂(SO₄) and/or TiO₂ were prepared. The prepared membranes were characterized by measuring mechanical properties, such as tensile strength and elongation at break, swelling properties, antimicrobial and blood clotting. The results obtained indicate that the presence of Al₂(SO₄) and TiO₂ in the membrane formulations has an incremental effect on the antimicrobial properties and blood clotting in albino rates.

Keywords: Chitosan, Acrylic acid, Antibacterial, Blood clotting and Membrane.

Wounds are defined as skin defect caused by mechanical, thermal, electrical and chemical injuries. Many types of wounds occur in everyday life⁽¹⁾. Most of the soldiers in the war field are facing many problems and losing their lives due to overflow of blood. People can save their lives if the blood clots easily at that moment. The US army uses a new high performance bandage for blood stopping, to save injured people on the battlefield⁽²⁾.

Wound dressings are materials used to cover the wounds. The principle functions of wound dressings are to avoid strikethrough, and to protect the wounds site from bacterial contamination and further physical damage to the tissue. Some of the main functions of wound dressing are:

- The ability to absorb fluid from a highly exuding wound.
- Microbial control.
- Physical barrier, to separate the wound surface from atmosphere and from bacterial contamination.
- Stop bleeding as early as possible to prevent blood loss.
- Wound healing acceleration.

Coagulation is the process by which blood forms clots. The main biological purpose of blood coagulation is the formation of an obstacle to prevent blood

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loss. Different methods used TiO_2 nanotubes in the enhancement of blood clotting for the control of hemorrhage⁽²⁾. The TiO_2 nanotubes appear to act as scaffold fibrin formation. The results suggest that application of TiO_2 nanotubes functionalized bandage could be used to help stop hemorrhage. Previous experiments show that the aluminophosphate containing Ca^{2+} ions can clot blood. Data suggest that Ca^{2+} containing microporous aluminophosphate may be useful as inexpensive human blood clotting agents⁽³⁾.

The aim of the present work is to prepare membranes have some functional properties that help wound healing acceleration and blood clotting, to achieve this goal, the work is designed to include: the preparation of chitosan/polyacrylic acid copolymer membranes. The prepared samples loaded with different concentrations of active ingredients such as aluminum sulfate $\text{Al}_2(\text{SO}_4)_3$ and mixture of aluminum sulfate with titanium dioxide. The prepared membranes were characterized by measuring physical and mechanical properties, swelling, antimicrobial, water uptake as well as blood clotting.

Experimental

Materials

Acrylic acid obtained from Merk-Schuchart, Germany, was used without further purification. Chitosan, high molecular weight as determined by Brookfield viscometer (800.00 cps in 1% w/w Chitosan aqueous 1% w/w acetic acid at 25°C), degree of deacetylation 87%⁽⁴⁾ was kindly supplied by Aldrich Chemical Company (Germany). N, N-methylene bisacrylamide, ammonium persulfate were from Aldrich Chemical Company, Inc., Germany. Aluminum sulfate and titanium dioxide and all other chemicals were laboratory grade reagents.

Preparation of copolymer membrane

The copolymer membranes were prepared by a bulk polymerization method. A typical copolymerization procedure was described as follows⁽⁵⁻⁷⁾: 1% Chitosan was dissolved with stirring into 20% acrylic acid solution in water at room temperature. The solution was filtered through a glass sinter filter (coarse grade) to remove insoluble impurities.

For preparation of the membranes, N, N-methylene bisacrylamide (crosslinker), ammonium persulphate and tetramethyl-ethylene diamine were then added to the solution to initiate the polymerization. To the prepared solution, a calculated amount of blood clotting ingredient [TiO_2 or $\text{Al}_2(\text{SO}_4)_3$ or $\text{Al}_2(\text{SO}_4)_3/\text{TiO}_2$ (1:1) by weight] was added with stirring. The solution was placed in water bath at 70°C for 2 hr. The solution was transferred to the mold, where it was left for 1 hr to complete polymerization. After that, the samples were allowed to swell in water to remove most of the water soluble component remained, and then vacuum dried.

*Evaluation of prepared copolymer membranes**Mechanical characteristics*

The sheets prepared for mechanical tests were cut into four individual dumb-bell-shaped specimens by a steel die of constant width (4mm). The thickness of the test specimens was determined by a gauge calibrated in hundredths of a millimeter. A working part of size 15mm was chosen for each test specimen. The mechanical properties (e.g. tensile strength, elongation at break and Young's modulus of the investigated specimens were determined according to standard methods using an electronic Zwick testing machine, model 1425, in accordance with ASTM.

Swelling studies

Equilibrium degree of swelling (EDS) was determined gravimetrically. The test samples dried to constant weight were immersed in water at room temperature, for 24hr. The excess water was removed with a filter paper and the samples were weighed. The EDS was calculated, using the following equation^(8,9).

$$\text{EDS \%} = \frac{(W_s - W_d)}{W_d} \times 100$$

where W_s and W_d represent the weight of the swollen and dry sample, respectively.

Estimation of antimicrobial activity

The antimicrobial activity was measured according to the Diffusion Disk Method⁽¹⁰⁻¹³⁾, at Dairy Microbiology, Dairy Department, *Bacillus cereus* B-3711(G+) and *Bacillus subtilis* (G+) were provided by the Northern Regional Research Laboratory Illinois, USA (NRRL). *Listeria monocytogenes* (G-) 598 was provided by the Department of Food Science, University of Massachusetts, Amherst MA, USA. *Escherichia coli* 0157: H7 (G-) and *Staphylococcus aureus* (G+) were isolated and serologically identified by Dairy Microbiological Lab., National Research Center. *Yersenia enterocolitica* (G-) were obtained from Hungarian National Collection of Medical Bacteria, OKI, Gyaliut 2-6, H-1966 Budapest, Hungary. *Aspergillus niger*, *Pseudomonas aeruginosa* (G+) and *Candida albicans* were provided by the Institute of Applied Microbiology, University of Tokyo, Japan.

The pathogenic strains were cultured in tryptone soya broth for 24 hr at 37°C for activation. Measuring method of antimicrobial was carried out according to Kim & Kim⁽¹⁴⁾. Melted nutrient agar medium was transferred to the Petri dishes and allowed to solidify. An aliquot of 0.2 ml of each active pathogenic strain suspension was transferred to plates and spreaded uniformly over the agar surface with a sterile bent glass rod. Plates were dried at 37°C for 1 hr and discs of 0.5 cm diameter from each sample were placed on agar medium, the plates were then incubated at 37°C for 24hr. The inhibition zone formed around each well was measured in mm.

Determination of coagulation time

This test was carried out in Pharmacology Department, Medical Research Division, at National Research Centre. The coagulation effect of the prepared samples was measured according to determination of the percentage of change in coagulation time to control in male albino rats. The test was carried out as follows: animals were divided into groups, each one contains five animals shaving of the skin of back for each rat (3cm x cm) and wounds were done to the skin for each rat. The samples were applied for 3 min to the shaved skin. After the end of absorption time the clotting time was measured by stop watch with the whole blood come out from the wound ⁽¹⁵⁾.

Results and Discussion*Mechanical properties of copolymer membranes*

The stress-strain curves of H₂O/AA/chitosan [80/20/1(w/w/w)] samples loaded with different concentrations of active ingredients such as aluminum sulfate Al₂(SO₄)₃ and mixture of aluminum sulfate with titanium dioxide [Al₂(SO₄)₃/TiO₂ (1: 1) by weight] are shown in Fig.1(a and b) respectively. From this figure, it is clear that the stiffness as governed by the slope of the initial linear part increases with increasing the concentration of Al₂(SO₄)₃ or Al₂(SO₄)₃/TiO₂ that act as reinforcing agent. Consequently, elongation at break decreases. This improvement may be due to their good dispersion and good adhesion with the matrix. Moreover a slight increase in Young's modulus attained after incorporation of aluminum sulfate alone or mixture of aluminum sulfate with titanium dioxide, while the elongation decreased. On the other hand, The effect of incorporating aluminum sulfate alone or mixture of aluminum sulfate with titanium dioxide on modulus at 100% elongation (M 100 %), tensile strength, elongation at break and young's modulus are shown in Fig. 2(a, b and c); a remarkable increase in these properties was observed. The addition of these additives gradually increases the tensile strength and modulus at 100% elongation until maximum is attained at 1g of this agent as shown in Fig. 2 (a, b and c). Further increase lead to a decrease in these properties. It can be seen that the value of tensile strength and modulus at 100% elongation of samples loaded with Al₂(SO₄)₃/TiO₂ is more than that loaded with Al₂(SO₄)₃ alone and the blank sample. This increase could be attributable to improved interfacial bonding between the active ingredients (Al₂(SO₄)₃/TiO₂) and the matrix. Therefore the dispersion becomes good and the properties improve in presence of aluminum sulfate with titanium dioxide.

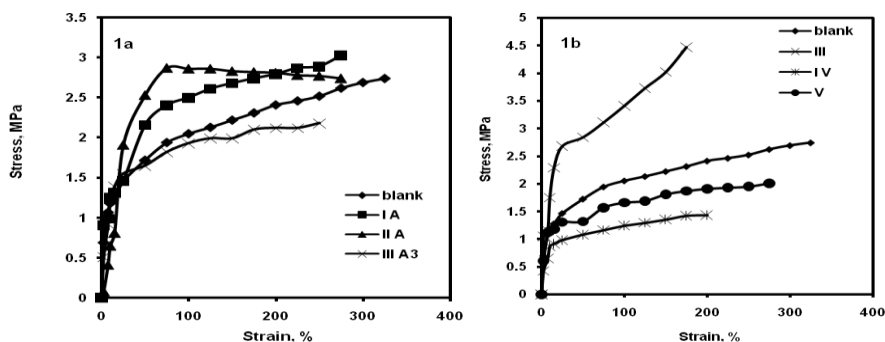


Fig. 1. Stress-strain curves of H₂O/AA/chitosan [80/20/1(w/w/w)] loaded with different concentrations of coagulating agent: (a) aluminium sulfate (Al₂(SO₄)₃) (b) aluminum sulfate with titanium dioxide (Al₂(SO₄)₃/TiO₂).

Blank : H₂O/AA/chitosan [80/20/1(w/w/w)]

III : blank+1 g [Al₂(SO₄)₃+TiO₂(1:1 by weight)] IA : blank + 1 g Al₂(SO₄)₃

IV : blank + 2 g [Al₂(SO₄)₃+TiO₂(1:1 by weight)] IIA : blank + 2 g Al₂(SO₄)₃

V : Blank +3 g [Al₂(SO₄)₃+TiO₂ (1:1 by weight)] IIIA : Blank + 3 g Al₂(SO₄)₃

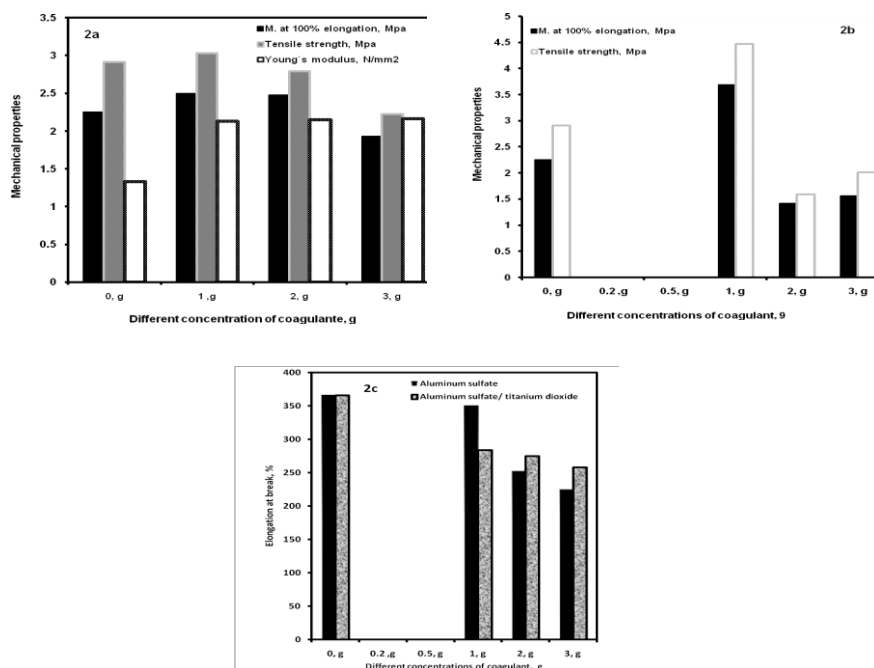


Fig. 2. Mechanical properties H₂O/AA/chitosan [80/20/1(w/w/w)] loaded with different concentrations of coagulating agent: (a) aluminium sulfate (Al₂(SO₄)₃) (b) aluminum sulfate with titanium dioxide (Al₂(SO₄)₃/TiO₂) and (c) elongation at break, % for investigated coagulants.

Swelling properties

As shown in Fig. 3 (a, b) with the increase of aluminum sulfate content, the degree of swelling of test samples H₂O/AA/chitosan [80/20/1(w/w/w)] in water decreases, which may be due to the decreased mobility of polymer chains in the presence of aluminum sulfate, then swelling increases at 3g of aluminum sulfate. On the other hand, the degree of swelling of H₂O/AA/chitosan [80/20/1(w/w/w)] samples increases in the presence of Al₂(SO₄)₃/TiO₂. The ionic interaction between both polymers may cause changes in porosity of the network, thus the value of water uptake varied as shown in Fig. 3b^(16,17). From the previous results, it is observed that the sample of H₂O/AA/chitosan/ 3g of Al₂(SO₄)₃/TiO₂ has the highest value of swelling equilibrium. So, this amount of coagulant is the most suitable as wound dressing, it is one of the most promising medical applications. Figure 4 (a, b) shows the relationship of swelling degree and time up to 96hr. It is clear from the figure that loading the samples of H₂O/AA/chitosan [80/20/1(w/w/w)] with 1g or 2g of Al₂(SO₄) and Al₂(SO₄)₃/TiO₂ as coagulating agent leads to reduction of water up-take propensity as contrasted with the blank or the higher concentrations of coagulating agent. All samples showed fast absorption of water within the initial 24 hr, followed by gradual decrease until achieving a saturated point (stable behavior). The efficient reinforcement of the samples under investigation is visible from the swelling degree graph⁽¹⁸⁾.

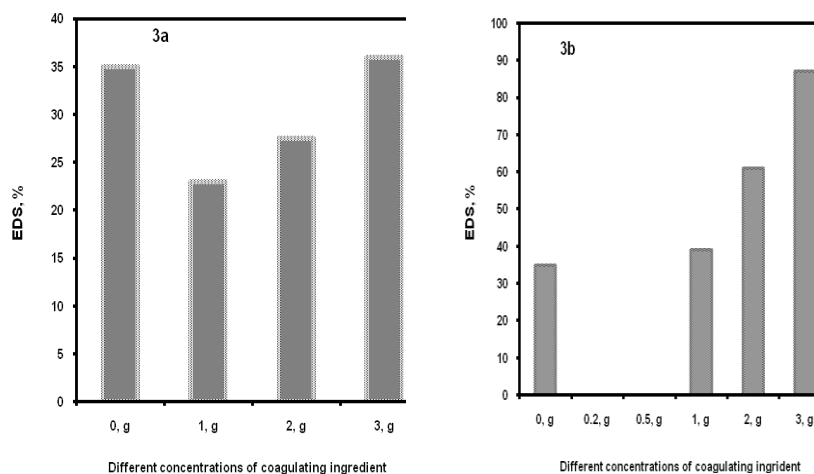


Fig. 3. The equilibrium degree of water uptake % of [H₂O/AA/chitosan (80/20/1)] loaded with different concentrations of coagulating agent: (a) aluminium sulfate (Al₂(SO₄)₃) (b) aluminum sulfate with titanium dioxide (Al₂(SO₄)₃/TiO₂).

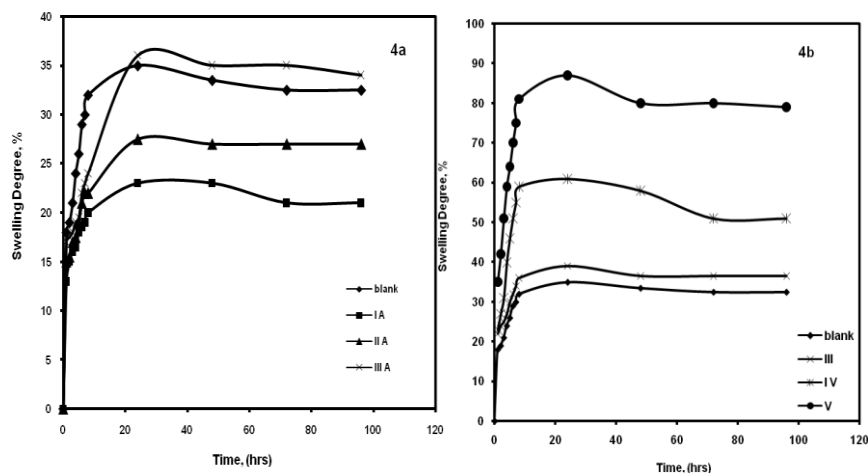


Fig. 4. The relation between swelling degree and time for H₂O/AA/chitosan [80/20/1(w/w/w)] loaded with different concentrations of coagulating agent: (a) aluminium sulfate (Al₂(SO₄)₃) (b) aluminum sulfate with titanium dioxide (Al₂(SO₄)₃/TiO₂).

Blank : H₂O/AA/chitosan [80/20/1(w/w/w)]

III : blank+1 g [Al₂(SO₄)₃+TiO₂(1:1 by weight)]

IV : blank + 2 g [Al₂(SO₄)₃+TiO₂(1:1 by weight)]

V : Blank +3 g [Al₂(SO₄)₃+TiO₂(1:1 by weight)]

IA : blank + 1 g Al₂(SO₄)₃

IIA : blank + 2 g Al₂(SO₄)₃

IIIA : Blank + 3 g Al₂(SO₄)₃

To investigate more precisely the effect of inter-copolymer compound formation on the release of coagulating ingredient, the results were analyzed according to the following equation⁽¹⁹⁾.

$$\frac{M_t}{M} = K t^n$$

where M_t/M is amount of coagulant (%) released at time t (h), n is a diffusion exponent and K is the apparent release rate (%/h). From the plot of $\ln(M_t/M)$ versus $\ln t$ as shown in Fig. 5, kinetic parameters, n and K , were calculated and listed in Table 1. The determination coefficient (r) was calculated from the coordination of the start line. The presence of TiO₂ in copolymer matrix reduces the crosslinking density of the compound. This leads to more free volume in the copolymer network and consequently; more water can be absorbed⁽²⁰⁾. This can be attributed to the presence of the pores inside the formulation⁽²¹⁾.

The antimicrobial activity

Data in Fig. 6 showed that the different copolymer membranes which contained different concentration of coagulating agents had higher inhibitory effect with variable spectrum activity against the tested pathogenic strains (Gram+, Gram- bacteria and fungi). It is clear that there is a marginal effect of

the coagulants ($\text{Al}_2(\text{SO}_4)_3$ and/ or TiO_2) on the inhibition zone (mm) as shown by Table 2. This can be attributed to that the antibacterial effect could be influenced by the presence of chitosan only in the membranes formulations which have almost a fixed amount of chitosan^(22,23).

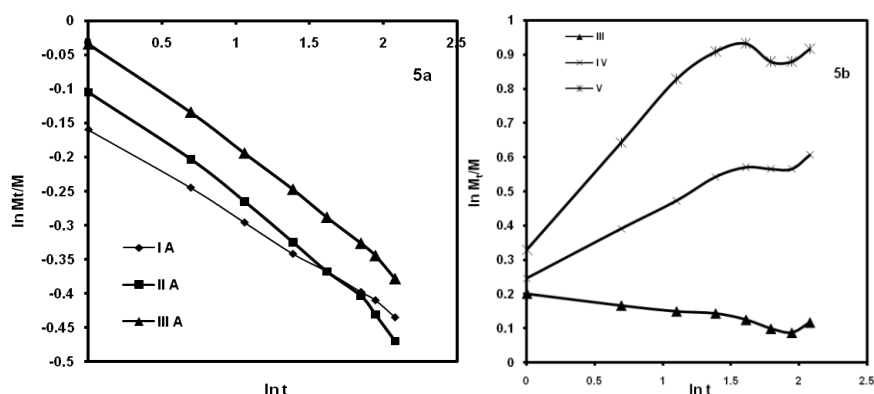


Fig. 5. Plot of $\ln M_t/M$ against time : (a) aluminium sulfate ($\text{Al}_2(\text{SO}_4)_3$) (b) aluminium sulfate with titanium dioxide ($\text{Al}_2(\text{SO}_4)_3/\text{TiO}_2$).

Blank : $\text{H}_2\text{O}/\text{AA}/\text{chitosan}$ [80/20/1(w/w/w)]

III : blank + 1 g [$\text{Al}_2(\text{SO}_4)_3 + \text{TiO}_2$ (1:1 by weight)] IA : blank + 1 g $\text{Al}_2(\text{SO}_4)_3$

IV : blank + 2 g [$\text{Al}_2(\text{SO}_4)_3 + \text{TiO}_2$ (1:1 by weight)] IIA : blank + 2 g $\text{Al}_2(\text{SO}_4)_3$

V : Blank + 3 g [$\text{Al}_2(\text{SO}_4)_3 + \text{TiO}_2$ (1:1 by weight)] IIIA : Blank + 3 g $\text{Al}_2(\text{SO}_4)_3$.

TABLE 1. The value of kinetic constants (K), release exponents (n) and determination coefficients (r) following linear regression of release data of coagulants from $\text{H}_2\text{O}/\text{AA}/\text{chitosan}$ [80/20/1(w/w/w)] inter polymer complex films.

Copolymer/coagulant	n	K	r
$\text{H}_2\text{O}/\text{AA}/\text{chitosan}/1\text{g } \text{Al}_2(\text{SO}_4)_3$	-0.131	- 0.158	0.999
$\text{H}_2\text{O}/\text{AA}/\text{chitosan}/2\text{g } \text{Al}_2(\text{SO}_4)_3$	- 0.158	-0,1008	0.997
$\text{H}_2\text{O}/\text{AA}/\text{chitosan}/3\text{g } \text{Al}_2(\text{SO}_4)_3$	-0.157	-0.0303	0.999
$\text{H}_2\text{O}/\text{AA}/\text{chitosan}/1\text{g } \text{Al}_2(\text{SO}_4)_3/\text{TiO}_2$	-0.0446	0.1993	0.993
$\text{H}_2\text{O}/\text{AA}/\text{chitosan}/2\text{g } \text{Al}_2(\text{SO}_4)_3/\text{TiO}_2$	0.206	0.246	0.999
$\text{H}_2\text{O}/\text{AA}/\text{chitosan}/3\text{g } \text{Al}_2(\text{SO}_4)_3/\text{TiO}_2$	0.389	0.354	0.988

Blood coagulation

Table 3 (a, b) shows the effect of concentration of aluminum sulfate and aluminum sulfate plus titanium dioxide as active ingredients on the coagulation time in male albino rates; this effect was determined by the percentage change in comparison with control. These data suggest that aluminum sulfate and titanium oxide may be useful as inexpensive human blood clotting agents. It is well noticed that the use of aluminum sulfate and titanium oxide in preparation of membranes was useful in clotting blood when applied to an open wound. Chitosan a compound that is naturally occurring in shrimp shells may be the right

material to slow or stop the flow of blood, beside its antimicrobial action. Results show that copolymer membranes reduced the time taken for the blood to begin clotting by 13 % - 17 %.

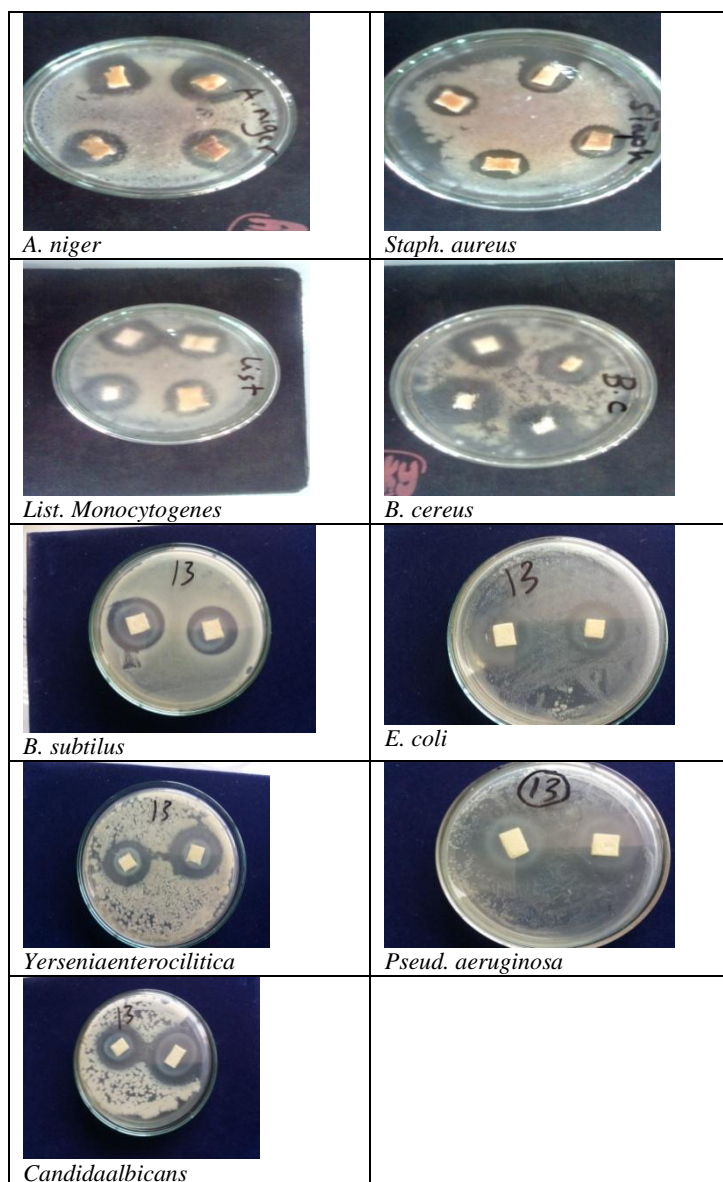


Fig. 6. Antimicrobial testes of copolymer films against nine strains measured by diffusion disk method.

Conclusion

Blood clotting experiments showed that the proposed crosslinked copolymer in form of thin membranes in presence of $\text{Al}_2(\text{SO}_4)_3/\text{TiO}_2$ reduces the blood clotting time in albino rates. The results suggest that application of aluminum sulfate and titanium oxide could be used to help stem or stop hemorrhage. Incorporation of chitosan in the copolymer solution helps to accelerate wound healing and increase the antimicrobial properties of the membranes. The membrane form of crosslinked copolymer is useful in clotting blood through the absorption of fluids from wounds.

TABLE 2. Diameter of inhibition zone (mm) by different treatments of membranes .

Pathogenic Strains	BLANK	III	IV	V	I A	II A	III A
Inhibition Zone (mm for 0.5 cm sample)							
<i>Pseud. aeruginosa</i>	25	27	28	29	25	29	28
<i>Staph. aureus</i>	24	20	25	22	30	25	23
<i>E. coli</i>	20	29	26	26	25	32	27
<i>Listeria monoytogenes</i>	27	21	30	29	27	28	25
<i>B. cerues</i>	30	27	27	29	22	30	29
<i>B. subtilus</i>	25	15	25	25	30	28	20
<i>Yerseniaenter-ocilitica</i>	29	28	31	26	26	28	25
<i>A. niger</i>	25	23	28	29	25	25	23
<i>Candidaalbicans</i>	30	30	27	33	27	31	28

Blank : $\text{H}_2\text{O}/\text{AA}/\text{chitosan}$ [80/20/1(w/w/w)]

III : blank+1 g [$\text{Al}_2(\text{SO}_4)_3+\text{TiO}_2$ (1:1 by weight)]

IV : blank + 2 g [$\text{Al}_2(\text{SO}_4)_3+\text{TiO}_2$ (1:1 by weight)]

V : Blank +3 g [$\text{Al}_2(\text{SO}_4)_3+\text{TiO}_2$ (1:1 by weight)]

IA : blank + 1 g $\text{Al}_2(\text{SO}_4)_3$

IIA : blank + 2 g $\text{Al}_2(\text{SO}_4)_3$

IIIA : Blank + 3 g $\text{Al}_2(\text{SO}_4)_3$

TABLE 3. Effect of Al₂(SO₄)₃ and/or TiO₂ used in film preparation on coagulation time in male albino rates.

(a)

Al ₂ (SO ₄) ₃ + TiO ₂ concentration in 100 g copolymer solution	Coagulation time (% change to control)
1	13.3
2	13.3
3	14.8

H₂O/AA/chitosan [80/20/1(w/w/w)]

(b)

Al ₂ (SO ₄) ₃ concentration in 100 g copolymer solution	Coagulation time (% change to control)
1	15.3
2	16.2
3	17.0

H₂O/AA/chitosan [80/20/1(w/w/w)]

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مقاومة البكتريا وتجلط الدم للافلام المحضرة من الكيتوزان وعديد الاكريليك

فكري عطا الله ، منال البيسي ، أشرف ابو عقيل ، أماني سليم** ، سلوي الصباغ* ، كوثر الشافعي*** ، هدى السيد** و سناء الصاوي*
شعبة بحوث الصناعات النسجية ، * قسم الملونات و البوليمرات ، ** الشعبة الطبية،
و*** قسم الألبان والميكروبيولوجي - المركز القومي للبحوث- القاهرة - مصر.

يهدف هذا البحث إلى تحضير أفلام من الكيتوزان وعديد الاكريليك التي تحتوي على كميات مختلفة من كبريتات الالومنيوم وثاني اكسيد التانتاليوم بغرض مقاومتها للبكتريا وتجلط الدم وقد تم بالفعل تجربتها على فئران الالبينو.

اوضحت النتائج أن وجود كبريتات الالومنيوم/ثاني اكسيد التانتاليوم يؤدي إلى زيادة مقاومة البكتريا وكذلك إلى زيادة تجلط الدم مما يمنع نرف الجروح.

تم تقييم الخواص الميكانيكية للافلام المحضرة مثل قوة الشد والاستطالة والخواص الفيزيائية مثل امتصاص الماء.

