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RESEARCH ARTICLE

IMPROVEMENT OF ANTIMICROBIAL AMINATED CHITOSAN/PVA BLENDS MEMBRANES FOR FOOD PACKAGING APPLICATIONS.

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Abstract

In this study, a new blend of aminated chitosan (Am)/Polyvinyl alcohol membranes was prepared and characterized for food packaging applications. The antibacterial activity of developed membranes was supported via the addition of chalcone to the film. Eight different composition membranes were prepared with different aminated chitosan and PVA ratio. Physical measurements such as water uptake, moisture content, and contact angle showing an increase in membranes hydrophilicity by increasing PVA content in membranes, on the other hand, it shows less hydrophilicity for membranes containing chalcone. Prepared membranes were characterizing using UV-vis spectroscopy, Fourier Transmission Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Thermal gravimetric analysis (TGA). Furthermore, the antibacterial activity of the blended films was evaluated against gram positive (*S. aureus* and *B. cereus*) and gram-negative bacteria (*E. coli* and *P. aeruginosa*). The results showed that these materials could be potentially used as antimicrobial films in packaging applications.

Introduction:-

Food packaging technology is continuously developing in response to increasing challenges from modern society. Significant current and future challenges to fast-moving consumer goods packaging include global markets, longer shelf life, legislation, convenience, safer and heal their food, environmental concerns, authenticity, and food waste [1]. Recently, the interest in active food packaging has been increasing significantly because of its ability to control the microbiological deterioration of perishable food products [2]. Many researchers have been directed to improve packaging strategies which can retain the active agent in the polymeric network and regulate its release [3─4]. It has been illustrated that active packaging containing antimicrobial compounds can delay, inhibit, or reduce the growth of food-borne microorganisms [2; 5─6]. Several methods can be used to combine antimicrobial agents into packaging systems such as: (i) sprayed or coated on food surfaces, (ii) volatilized from an insert or a sachet placed in the package into the head space, (iii) sprayed, coated or chemically bound to the surface of the packaging film, (iv) designed to occupy pores or channels within the film, or (v) formulated homogenously with the film polymers, [5─6].

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To reduce the environmental problem (that generated by using synthetic and non-biodegradable packaging films), biodegradable or edible packaging materials are supported to be developed, especially in the case of antimicrobial packaging [7─8]. In this respect, polysaccharides such as carboxymethyl cellulose (CMC), hydroxypropyl-cellulose (HPC), methylcellulose (MC), alginate (ALG), chitosan proteins such as soy protein isolates, corn zein, whey protein isolates or synthetic biodegradable polyesters such as polycaprolactone (PCL), have been used as main materials for manufacturing antimicrobial packaging films [3; 8─12].

In the case of antimicrobial agents that are encapsulated into packaging films, essential oils (EOs) and their derivatives are becoming more efficient as naturally derived antimicrobial agents [3; 13─14].

Chitin is mainly isolated from crustacean wastes [15]. It is tightly bound in complexes with other substances, like proteins and minerals. At the industrial scale, an acid treatment (decalcification) followed by an alkaline treatment (deproteination), or just in reversed order, and a decolorization step are used to obtain chitin. To prepare chitosan, an additional alkaline treatment is performed to deacetylate chitin. Different factors, like alkali concentration, incubation time, ratio chitin to alkali, temperature, atmosphere, type of chitin source (including polymorph type), particle size, heterogeneous/homogeneous N-deacetylation, and single or multiple processes play a role in the alkaline N-deacetylation of chitosan and thus affecting the properties of chitosan. Chitosan is biocompatible, biodegradable, nontoxic, antioxidant biopolymer. The unique properties of chitosan have driven the researchers to apply it in promising applications such as antimicrobial [16], antioxidant [17─19], water treatment [20], tissue engineering, wound dressing [21], gene and drug delivery [22], etc.

Chitosan, having cationic groups along the backbone, has been shown to have antimicrobial properties against bacteria, yeasts, moulds and fungi [23─25]. The mechanism through which chitosan acts as an antimicrobial compound is not entirely elucidated, however, two main hypotheses (I and II) have been proposed [26] whereas some authors have proposed a third one (III) [27]. These are:

(I) The polycationic nature (positive charge) of chitosan can conflicts with the bacterial metabolism by electrostatic interaction with Protein channels (negative charge) at the cell surface.
(II) Low molecular weight chitosan can enter to the cell's nucleus and block the transcription of RNA from DNA due to adsorption of DNA molecules.
(III) Chitosan functioning as a chelating agent of essential minerals.

Therefore, treatment with chitosan can offer protection against contamination and microbial spoilage. The good film forming properties of chitosan provides the production of coating material, films or membranes semi-permeable to gasses. [27]. In addition, chitosan has unique properties such as biocompatibility, non-toxicity biodegradability and it is renewable [28]. Furthermore, it is inexpensive and commercially available.

Chalcones (1, 3-diphenyl-propene-1-one) are members of the flavonoid family. Chalcones mainly can obtain from the natural and synthetic origin. It has been reviewed for their wide range of biological activities as antibacterial [29], anti-tumor [30], anti-inflammatory and antioxidant agents [31─32], etc. While studies on the bioavailability of heterocyclic chalcones of natural origin are limited, it has been reported that it has a wide range of biological activities, especially antibacterial [32], and antifungal activities [33]. To diversify the biological activities of conventional chalcones, a series of heterocyclic chalcone analogues in containing oxygen or nitrogen, as well as thiophene heterocycle, were synthesized.

In this study, antimicrobial membranes based on aminated chitosan/ PVA in collaborated with chalcone were prepared and characterized for food packaging applications.

Materials and methods:-

Materials:-

Shrimp shells were collected from wastes of seafood restaurants in Alexandria, Egypt. Polyvinyl alcohol (PVA), acetic acid (99.8%), hydrochloric acid (37%) and sodium hydroxide pellets (99–100%) were purchased from Sigma-Aldrich (Germany).Peptone, Yeast extract, and Ethanol (99.9%) were supplied from International Co. for Supp. & Med. Industries (Egypt).

Bacteria:-

Four bacterial strains were employed for assessing the antibacterial activity of the blend membranes. These included two Gram-negative strains (*E. coli* and *P. aeruginosa*) and two Gram-positive (*S. aureus* and *B. cereus*). The bacteria strains were refreshed through inoculating in Luria-Bertani broth (peptone 1%, NaCl 1%, yeast extract 0.5%, and pH 7±0.2) and were incubated overnight at 37°C and 150 rpm in a rotary shaker.

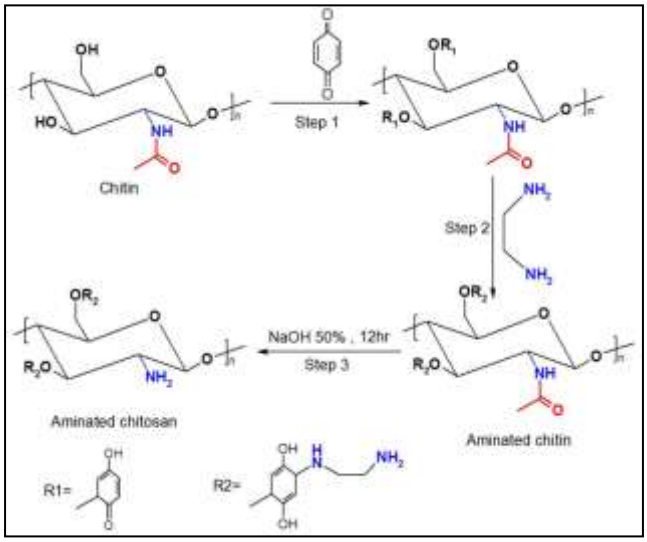
Methods:-

Preparation of aminated chitosan (AmCh):-

Aminated chitosan was prepared through three steps according to our previous work, see scheme 1 [34]. Activation step, 4 g of chitin was dispersed in 50 ml distilled water contained 8.3 mM of p- benzoquinone (PBQ) at pH 10 and stirred for 6 hrs at 60 ºC. The PBQ-conjugated chitin was filtrated and washed with distilled water to remove unreacted PBQ. Amination step, PBQ-conjugated chitin was dispersed in 50 ml of 8.3 mM of ethylenediamine (EDA) at 60 ºC and was stirred for 6 hrs. The amine chitin was separated and washed with distilled water to remove unreacted EDA. Final deacetylation step, the aminated chitin was treated with 50% aqueous solution of NaOH at 120–150 ºC for 6 hrs. The result aminated chitosan was separated and washed with distilled water to remove the excess of NaOH then dried in vacuum at 50 ºC oven overnight.

Table 1:- The ratio of Aminated chitosan (AmCs), poly (vinyl alcohol) (PVA), and the amount of chalcone (Ch) in different samples.

	Aminated chitosan (Am Cs)	PVA	Chalcone (Ch)
AmCs/PVA1	1 g	0.25 g	0.1 g
AmCs/PVA0.75	1 g	0.5 g	0.1 g
AmCs/PVA0.5	1 g	0.75 g	0.1 g
AmCs/PVA0.25	1 g	1.0 g	0.1 g
AmCs/Ch/PVA1	1 g	0.25 g	0.1 g
AmCs/Ch/PVA0.75	1 g	0.5 g	0.1 g
AmCs/Ch/PVA0.5	1 g	0.75 g	0.1 g
AmCs/Ch/PVA0.25	1 g	1.0 g	0.1 g

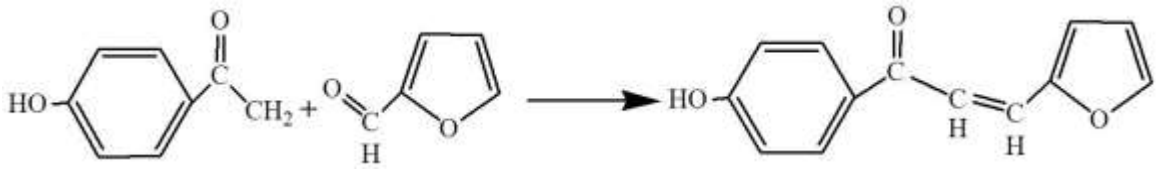


Scheme 1:- schematic preparation of aminated chitosan.

Preparation of chalcone:-

A chalcone derivative was prepared by a Claisen–Schmidt condensation [35] of the 2-Furaldehyde and 4′-Hydroxyacetophenone (Scheme 2). A solution of substituted 4′-Hydroxyacetophenone (5mM) and Furaldehyde (5mM) in methanol (15ml) was cooled to 5-10°C in an ice bath. The cooled solution was treated with adding a small portion of pulverized potassium hydroxide (10mM). The reaction mixture was magnetically stirred for 60 minutes and then left overnight or longer, monitored by thin layer chromatography using developing solvent n-hexane –

acetone (5:1). The resulting solution was diluted with ice water and carefully acidified using dilute hydrochloric acid. The chalcone which separated as a white solid was collected by filtration after washing with water and further purified by crystallization from methanol.



Scheme 2:- schematic preparation of chalcone.

Preparation of blend membranes

The polymer films were prepared by the casting method as follows: Aminated chitosan (Am Cs) solutions were prepared by dissolving AmCs in 1% aqueous acetic acid solution at room temperature with stirring. The PVA was suspended in hot water and stirred gently to form the polymer solution. Am Cs and PVA solutions were filtered through a piece of clothing with suitable mesh size to remove un dissolved particles, and then the solutions were carefully mixed at various ratios. The weight fraction of PVA was varied to obtain a series of blends with different PVA content in the final solution as listed in Table 1. The amount of chalcone (Ch) in various samples was 0.1 g under stirring. The blend solution was placed under vacuum to remove air bubbles. Then, the solution was poured out on a Petri dish. During next 48 hours at room temperature, the solvent evaporated completely. The dry membrane, which was separated from the Petri dish, was rinsed for approx. 30 s in the aqueous NaOH solution (1 M), thereby the traces of acetic acid (still adsorbed on the membrane) were removed thoroughly. The membrane was then washed for approx. 2 min in distilled water. Finally, the wet membrane was spread out and attached to the clean glass support with clamps and left to dry for 24 hours at room temperature.

Characterization:-

Physic-chemical characterization:-

Water uptake:-

Water uptake (%) estimation was performed by placing a weighed sample previously dried in distilled water For 6 hours to reach equilibrium swelling. The sample was then filtered off, carefully bolted with a filter paper and weighed. The water uptake, W, calculated by applying the following equation**;**

$$\boldsymbol{water\ uptake}\ \% = \frac{[\boldsymbol{M} - \boldsymbol{M0}]}{\boldsymbol{M0}} \times \mathbf{100}$$

Where **M** is the weight of the swelled sample and $\mathbf{M_0}$ is the weight of the dry sample.

Moisture content:-

Films were placed in humidity chambers with humidity ratio 80% overnight and then weighed before and after drying in an oven at 105 ^{o}C for 3 h. Water content was calculated as follows equation**.**

$$\boldsymbol{Moisture\ content}\ \% = \frac{[\boldsymbol{M} - \boldsymbol{M0}]}{\boldsymbol{M0}} \times \mathbf{100}$$

Where **M** is the weight of the sample before drying and $\mathbf{M_0}$ is the weight of the dry sample.

Surface roughness:-

The surface roughness of the substrate plays essential role in bacteria attachments. The average roughness of membranes was determined using surface roughness tester SJ- 201P, Japan. Membranes were fixed on a glass slide with a double-sided tape. Minimum sample dimensions were 20 mm × 20 mm. All results are the average of five measurements.

Contact angle measurements:-

Water contact angle determinations were done using (advanced Gonimeter model 500-F1) at room temperature in a sessile drop configuration (using Milli-Q water), linked with a video camera and image analysis software. At least, five droplet images were obtained and analyzed for each film.

UV-Vis Spectroscopic analysis:-
The electronic absorption spectra of blend membranes were recorded using spectrophotometer scanned from 200 - 900 nm.

Infrared spectroscopic analysis (FT-IR):-
The structures of blend membranes were investigated by FT-IR spectroscopic analysis using Fourier Transform Infrared Spectrophotometer (Shimadzu FTIR - 8400 S, Japan).

Thermal gravimetric analysis (TGA):-
Thermal analyzes of blend membranes were carried out using Thermal Gravimetric Analyzer (Shimadzu TGA –50, Japan).

Scanning electron microscope (SEM):-
Scanning of blend membranes were performed using Scanning Electron Microscope (Joel Jsm 6360LA, Japan).

Mechanical properties:-
The mechanical properties were done to characterize blend membranes and confirm reproducibility of the membrane formation technique. These properties include the membrane thickness which was obtained with an electronic digital micrometer, maximum stress and strain to failure. A method for testing the tensile properties of the film was done according to ASTM D- 882 standard method for examining tensile properties of paper using a constant rate of elongation apparatus. The instrument that used to verify these properties was an AG-1S, SHIMADZU.

Antibacterial activity:-
Broth evaluation method:-
Antimicrobial activity of blend membranes was measured according to the reported method [36]. In details, the bacteria were inoculated in a Luria- Bertani broth (LB broth) (1% peptone, 0.5% yeast extract, and 1% NaCl). The inoculation was incubated at 37 ºC for 24 hrs with shaking. The obtained bacterial suspension was diluted with the same LB media 100 times. 100 µl of bacteria suspension was cultured in a ten ml liquid peptone medium, which includes a portion of membranes (5 cm × 1 cm). The inoculated medium was maintained at 37 ºC for 24 hrs with shaking. The number of bacteria was counted by using the ultraviolet absorbance of culture medium at 620 nm.

Results and discussion:-
In this work, aminated chitosan/ PVA blend membranes were prepared in the presence and absent of chalcone and characterized from physic-chemical points of view.

Physicochemical analysis:-
Water uptake:-
Figure 1 demonstrates water sorption of aminated chitosan/ PVA blends membranes. Obtained result shows a dramatic increase of water absorption of tested membranes by increase PVA content of such membranes. The high affinity of PVA membranes was attributed to its hydroxyl groups that regularly distributed along chain backbone. Water sorption of membranes contains chalcone shows significant decrease that may be attributed to hydrophobic nature of chalcone.

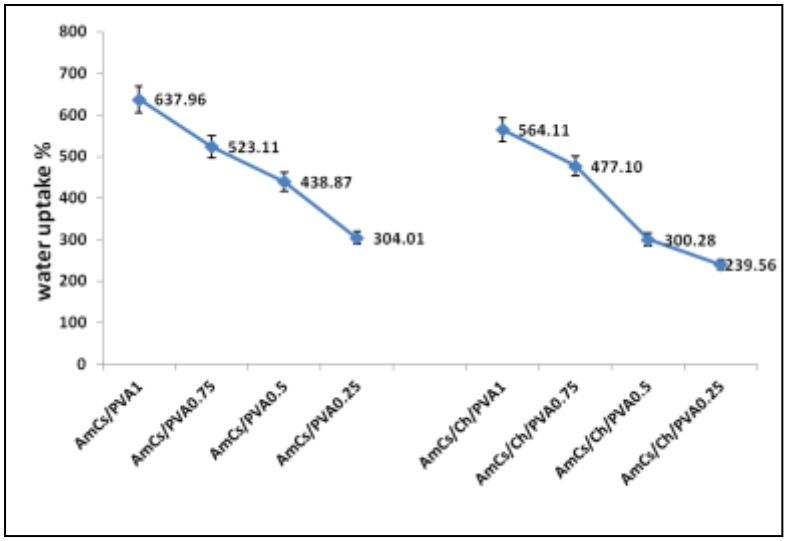


Figure 1:- water uptake of aminated chitosan/ PVA blends membranes.

Moisture content

The moisture content of membranes is a critical parameter for membranes employed in packaging applications. The presence of hydrophilic groups along polymer chains (hydroxyl group of PVA and hydroxyl and amine groups in aminated chitosan) allows membranes to trap water molecules from surround atmosphere. Figure 2 illustrates moisture content of different membranes. There is a general increase of hydrophilicity of membranes by increase PVA in blend ratio. Membranes hydrophilicity was decreased by loading with Chalcone.

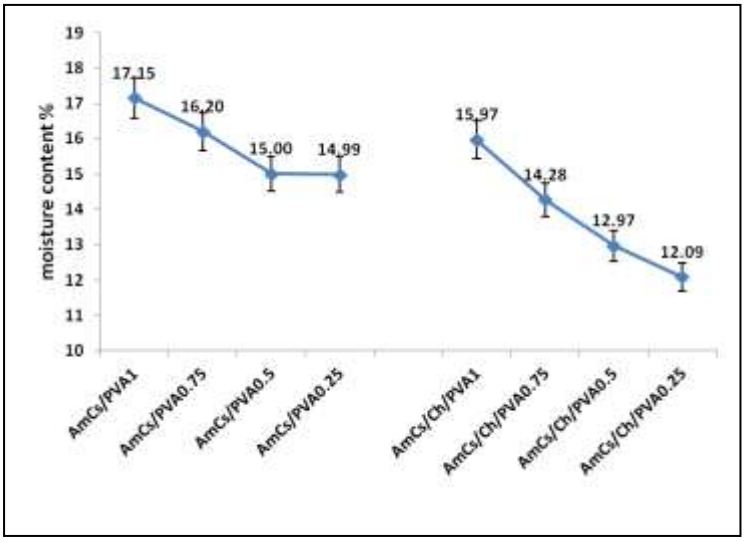


Figure 2:- moisture content of aminated chitosan/ PVA blends membranes.

Roughness:-

The surface roughness of packaging membranes has a great role in fixed attached microorganism on the surface. Figure 3 present surface roughness of aminated chitosan / PVA blend membranes. Increase aminated chitosan ratio in blend showing an increase of surface roughness. The figure shows a significant increase of surface roughness in membranes that contain chalcone may be attributed to the interaction of chalcone with the polymeric matrix.

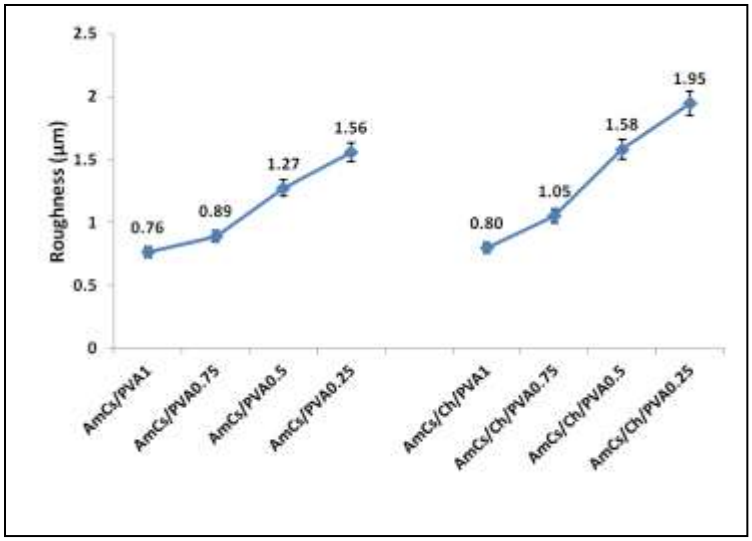


Figure 3:- Roughness measurments of aminated chitosan/ PVA blends membranes.

Wettability (contact angle measurement):-

Water contact angle with blend membranes was measured with advanced Gonimeter Model 500 F1 (figure 4). The contact angle test is used to consider the hydrophile or hydrophobe surface properties of blend membranes. There are increases in the contact angle by decreasing of PVA content in membranes. PVA membrane is showing more hydrophilic character than that of aminated chitosan [37]. It is clear that the increase of hydrophilic character of membranes was attributed to increase Aminated chitosan %. [38].

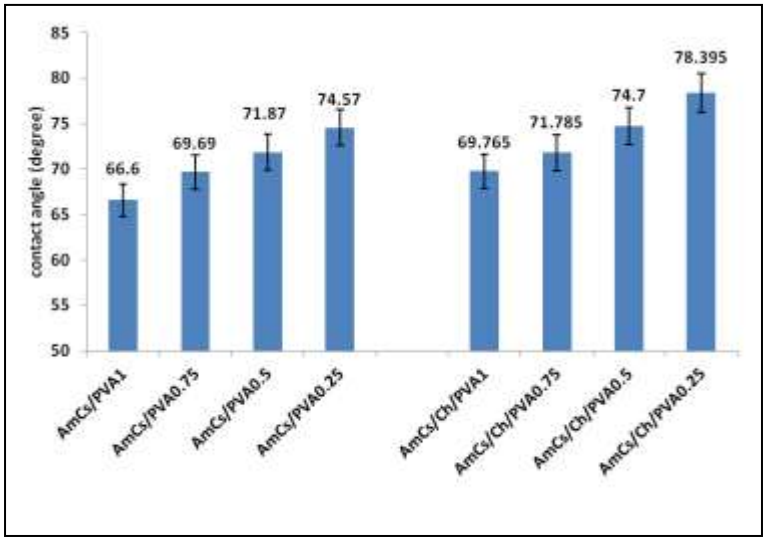


Figure 4:- contact angle measurements of aminated chitosan/ PVA blends membranes.

FT-IR:-

The FT-IR spectra of aminated chitosan and PVA blended films, in presence and absence of chalcone was done and represented in figure 5. There is no characteristic difference between them. PVA represent major peaks related to hydroxyl and acetate groups near that related to aminated chitosan peaks. More specifically, the broad band observed within 3550 and 3200 cm^{-1} is attributed to the stretching of OH group from the intramolecular and intermolecular hydrogen bonds. The vibrational band found between 2840 and 3000 cm^{-1} refers to the stretching of C-H of alkyl groups where the peaks within 1750 and 1735 cm^{-1} are due to the stretching C=O and C-O from acetate group remaining in PVA saponification reaction of polyvinyl acetate [39]. In the other hand, aminated chitosan exhibit characteristic polysaccharide bands at 890, 1020 and 1150 cm^{-1}, and high amino characteristic bands at around 3430, 1660 and 1560 cm^{-1} assigned to -OH stretching, amide I and amide II bands, respectively. According to Kumar et al. (2010), the peak at about 1250 cm^{-1} corresponds to the amino group vibration. [40─42].

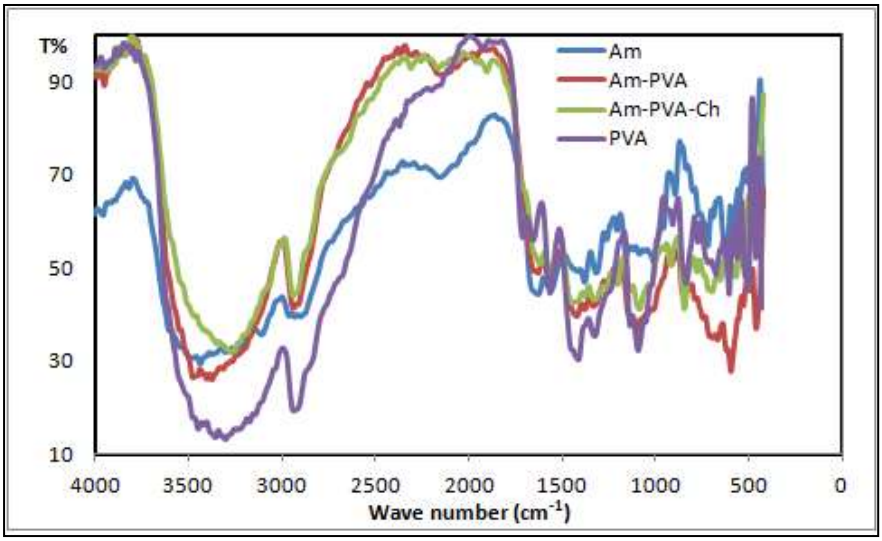


Figure 5:- FT-IR of aminated chitosan/ PVA blends membranes.

Electronic spectra:-

UV-VIS absorption of aminated chitosan/PVA blend films are shown in Fig.6 &7. The films showed an absorption band at 273- 280 nm which comes from unsaturated bonds (π-π*) mainly C=O and n-σ* mainly hydroxyl and amine groups of aminated chitosan [12; 41], which increased in absorption intensity when CH ratio increased. That lead to the promotion of the interactions between CH and PVA chains in the film. From the practical point of view, it is remarkable that blend films have a better barrier to ultraviolet light. One of the desired characteristics of packaging material is that it should protect food from the effects of light, especially UV radiation [43].

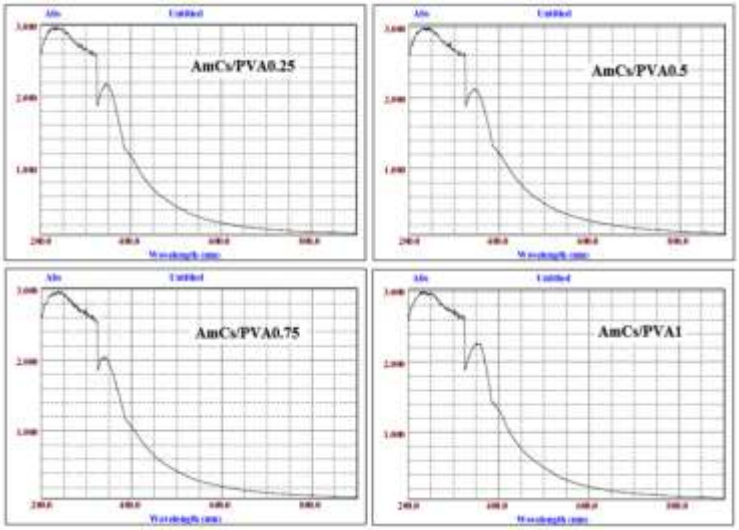


Figure 6:- Uv-Vis spectrum of aminated chitosan/ PVA blends membranes.

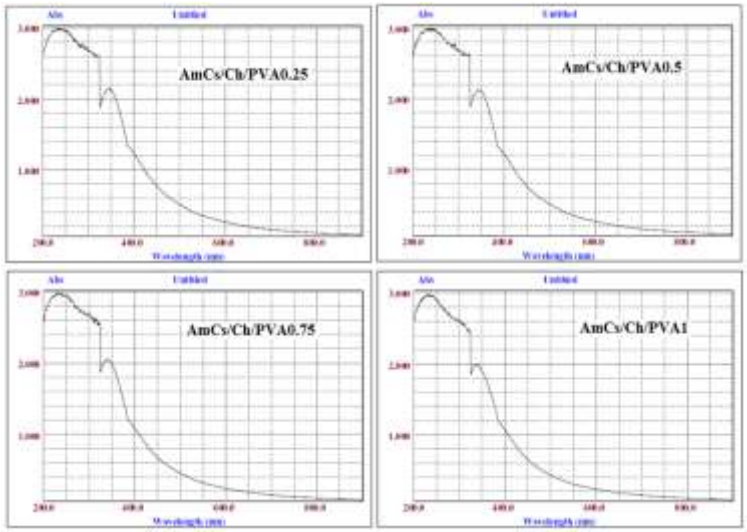


Figure 7:- Uv-Vis spectrum of aminated chitosan/ PVA/charcone blends membranes.

Scanning Electron Microscopy (SEM):-

The surface morphological structure of aminated chitosan/ PVA blend membranes was performed with scanning electron microscope. Figure 8 shows the microstructure of membrane surface. Aminated chitosan / PVA blend membranes have uniform surface without pore, holes or interface layers. [44] The blended films also exhibit smooth flat surface, in general, indicates the uniform distribution of AmCS and PVA molecules throughout the films. The formation of homogeneous blends of aminated chitosan and PVA was attributed to the interactions of hydrogen bonds between the Am Cs function groups (–OH and $–NH_2$ groups) and –OH groups in PVA. The addition of chalcone showing a significant increase of membrane roughness, this result is confirmed with that obtained from surface roughness measurement.

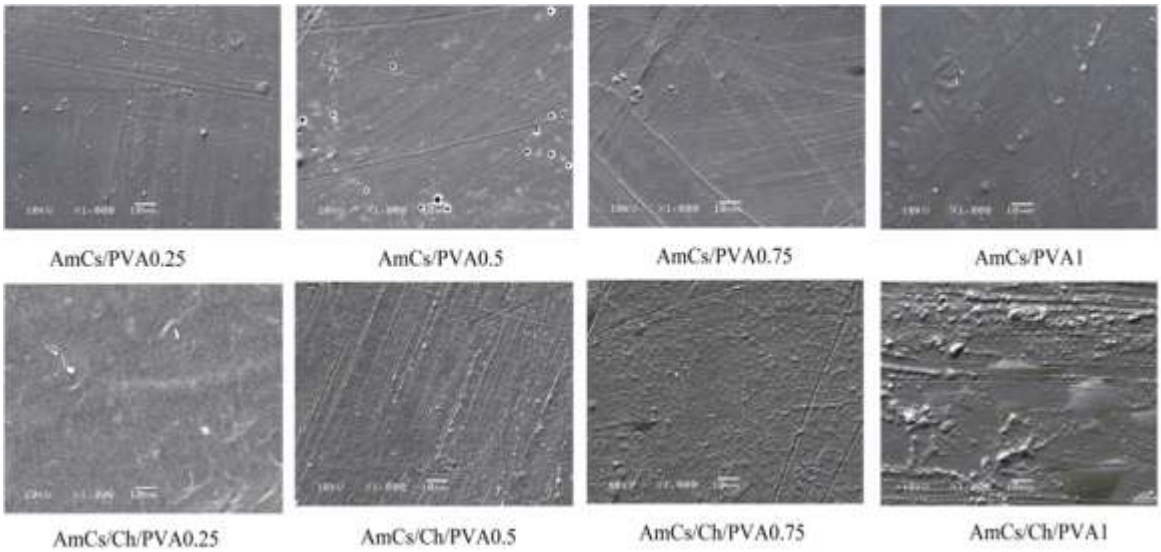


Figure 8:- SEM image of aminated chitosan/ PVA blends membranes.

Mechanical properties:-

Mechanical properties of aminated chitosan /PVA blended membranes with and without chalcone were determined at the breaking point of stretching pieces. Maximum stress σ_{max} (Nm^{-2}) was evaluated as the ratio of the stretching force divided by the cross-sectional area of broken membrane piece. The maximum strain λ_{max} was measured as the elongation ratio of the initial length of the test section. Results obtained are listed in Table 2.

Table 2:- Mechanical parameters of aminated chitosan / PVA blend membranes in presence and absence of chalcone.

	Max Force (N)	Max Dis (mm)	Max stress (N/mm^2)	Max strain (%)
Am Cs/PVA1	77.25	2.56	42.06	7.68
Am Cs/PVA0.75	72.34	2.14	26.78	6.42
Am Cs/PVA0.5	67.22	1.64	36.43	4.92
Am Cs/PVA0.25	35.21	1.35	29.34	4.05
Am Cs/Ch/PVA1	57.25	2.14	21.20	6.42
Am Cs/Ch/PVA0.75	46.36	1.95	41.21	5.85
Am Cs/Ch/PVA0.5	30.72	1.53	30.72	4.59
Am/Cs/Ch/PVA0.25	24.36	1.12	25.38	3.36

The obtained result demonstrate a promotion on mechanical properties of membranes by increasing PVA content in blend films; The same behavior was recorded by other authors working with blend films of PVA: CH, [43; 45]. Improvement of mechanical properties of aminated chitosan/PVA blend can be referring to the generation of a hydrogen bond between aminated chitosan function groups and PVA hydroxyl groups, loading of chalcone in membranes show the significant weakness of membranes. That may be attributed to distortion of inter hydrogen bond between polymer chains.

Thermal gravimetric analysis:-

Thermal gravimetric analyses of aminated chitosan/PVA blended membranes were carried out using TGA50 SHIMADZU JAPAN, Figure (9, 10), and the obtained data are shown in Table (3);

Table 3:- Thermal gravimetric data of aminated chitosan / PVA blends membranes in presence and absence of chalcone.

	Weight loss ambient – 200 ^oC	**T_{50}**
Am Cs/PVA1	15.79	344.97
Am Cs/PVA0.75	14.03	368.79
Am Cs/PVA0.5	11.73	362.16
Am Cs/PVA0.25	12.0	351.78
Am Cs/Ch/PVA1	14.56	368.12
Am Cs/Ch/PVA0.75	13.36	361.91
Am Cs/Ch/PVA0.5	12.43	357.34
Am/Cs/Ch/PVA0.25	9.87	351.89

The first decline from ambient temperature to about 200 ^oC can be attributed to the moisture content trapped inside polymeric chains. Going through bend membranes, we can recognize the decrease of moisture content in higher PVA material membranes that may be explained by the higher hydrophilic character of PVA than aminated chitosan. Obtained results confirm that previously recognized by moisture content test.

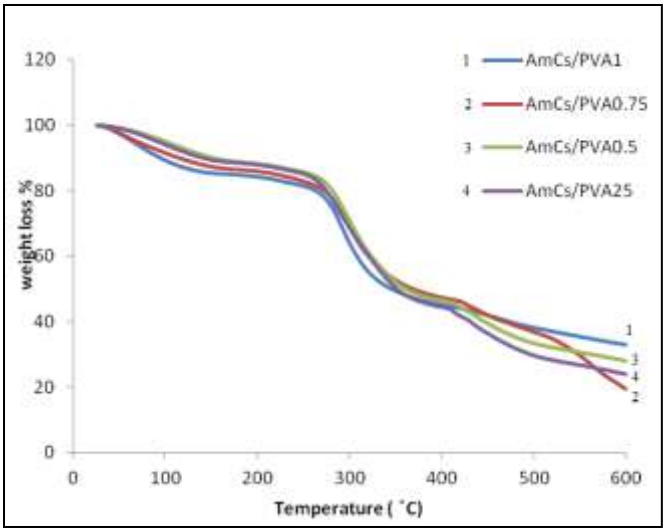


Figure 9:- TGA of aminated chitosan/ PVA blends membranes.

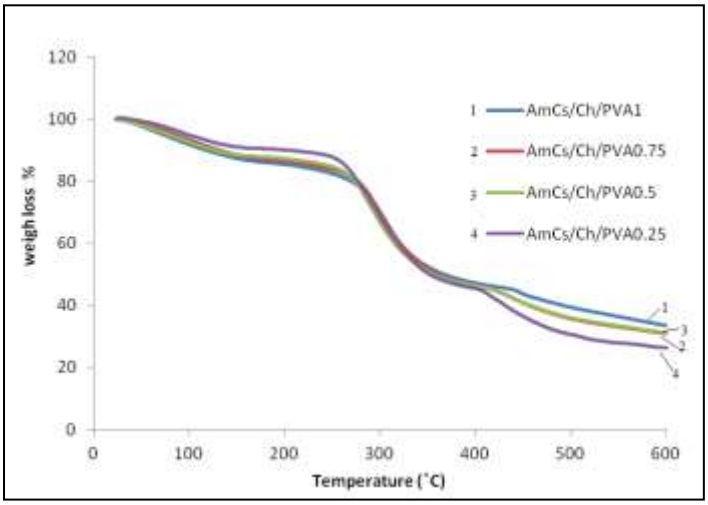


Figure 10:- TGA of aminated chitosan/ PVA/charcone blends membranes.

Second depression was between 220-380 °C. At this temperature, aminated chitosan pyranose ring and PVA backbone were started thermal degraded by some radical cleavage of it to form a crosslinked intermediate as [46]. Degradation of byproducts of PVA and aminated chitosan were done later at the higher temperature (from 380-600 ^{o}C) [32]. In between 200 and 300 ^{o}C, aminated chitosan showed less thermal stability than PVA [47]. This was cleary observed in membranes content chalcone.

Antibacterial evaluation:-

The antimicrobial activity of aminated chitosan/ PVA blend membranes, and that containing chalcone against several bacterial species have been recognized (figure 11) and is considered as one of the most important properties linked directly to their possible advanced bio-applications. This is reasonable, as chitosan has an innate antimicrobial characteristic itself due to its positively charged amino groups which interact with the negatively charged microbial cell membranes, leading to proteinaceous leakage and other intracellular constituents of the microorganisms [48]. Blended membranes demonstrated a moderated growth inhibition against tested bacterial. A significant increase of membrane activity can be recognized by increase aminated chitosan content.

As chalcone was added to membranes, antibacterial powerful was promoted and increased dramatically that can be associated with the activity of chalcone. Improvement of antibacterial activity by add chalcone was varied according to microorganism *B. cereus* inhibited by (17-29%), *S. aueus* (1.6-6.8%), *E.coli* (21-36 %) where *P.aeruginosa* (9-

15%). The molecular mechanism of action is not yet definitely understood. The synthesized heterocyclic chalcones display high antibacterial activities and may begin by damaging the cell wall of bacteria, which is naturally similar to the observed mechanism of a well-known cell membrane permeabilizer, polymyxin B [49].

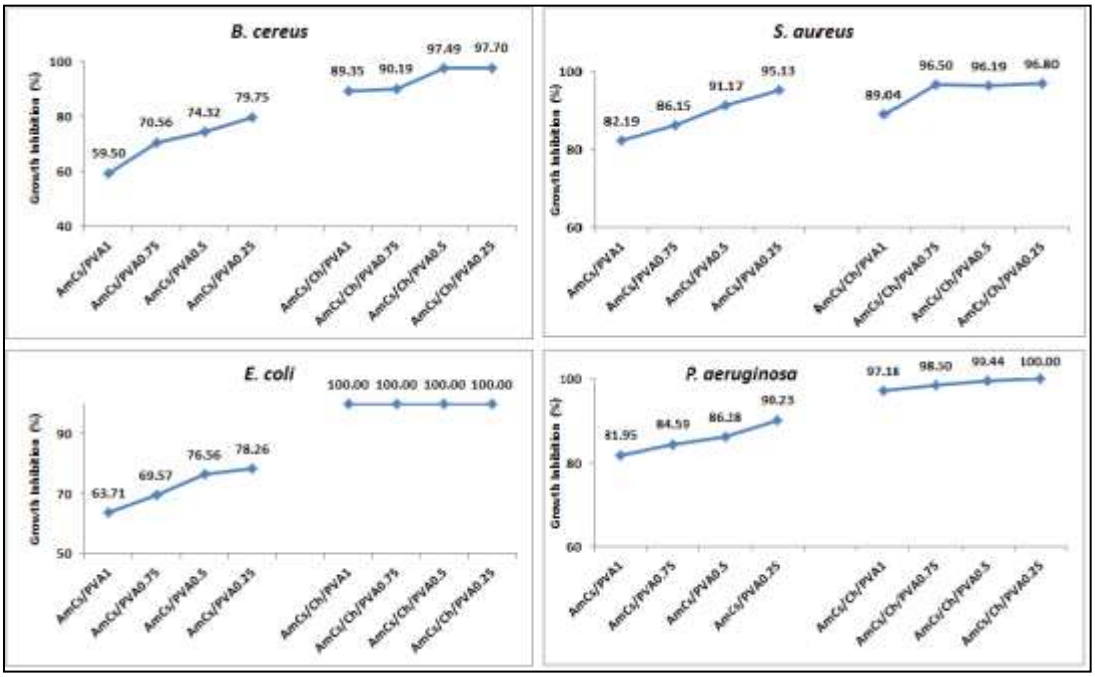


Figure 11:- Bacterial growth inhibition of different bacteria incubated with aminated chitosan/ PVA/charcone blends membranes.

Conclusion:-

Membranes of AmCs/PVA and AmCs/Ch/PVA were prepared. Results show remarked changes in their physicochemical properties. They can be summarized as follows:

- Hydrophilicity of blend membranes was evaluated through several parameters such as wettability, water uptake, and moisture content. Hydrophilicity was increased with PVA increase. The presence of chalcone in membranes reduce its hydrophilic character.
- The tensile analysis showed that both tensile modulus & tensile strength enhanced by increase PVA portion in blend membranes, while all mechanical parameters were reduced by loading Chalcone.
- SEM analysis revealed that the membranes exhibited some roughness of surface with the incorporation of Chalcone.
- The antibacterial of blended membranes showed increase antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Escherichia coli* by increase Am Cs portion. This effect was reinforced by loading Chalcone.

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