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Incorporation of Curcumin in Bilayer Matrices to Reduce the Toxic Effects to Be Used for Wound-Healing Application

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ABSTRACT

Introduction: The self-assembly of hydrophobically modified polymers has become a research hotspot. Chronic, non-healing wounds place a significant burden on patients and healthcare systems.

Objective: The present work aims to create a poly (3hydroxybotyrate) (PHB) composite film containing curcumin that would effectively improve skin wound healing by antibacterial activity.

Methods: The PHB/curcumin films are devised using a solution casting method, and Chloroform is the main solvent in the work. FTIR spectroscopy analysis is used to prove the success of the insertion process between Cur and the PHB matrix. **Results:** The PHB/Curcumin film product is smooth and flexible without any defects. Long-term protection is provided by the composite film's sustained curcumin release. Within 15 hours, almost 90% of the curcumin in PHB/Curcumin films is released in a sustained way. Results from the MTT assay show that PHB/Curcumin film has lower cytotoxicity than that of free curcumin. Furthermore, Vivo experiments reveal that the PHB/Curcumin Films have a 95% healing rate and mature epithelialization on day 14 following surgery.

Conclusion: Due to the success of loading curcumin in a PHB film and proving the growth in the effectiveness of the method by increasing and prolonging the effect of the biological activity as an antibacterial and in helping tissues to heal wounds in addition to its rapid wound-healing effects, it could be concluded the study considers PHB/Curcumin films promising as a wound dressing agent in wound management.

Keywords: Poly (3-hydroxybutyrate), Curcumin, Wound Healing, Staphylococcus aureus, Antibacterial effect, Bio-films

INTRODUCTION

Skin is a flexible exterior tissue that covers the body of a vertebrate animal and performs activities such as controlling body temperature, receiving external stimuli, defending against harm, and excreting excessive water[(1)]. However, the skin may be wounded by scratching, bruising, or during surgery, in addition to immunodeficiency disease conditions such as diabetes mellitus. Regardless of whether the wound is acute or chronic, it may cause a wound, loss of body fluids, nutrients, and electrolytes, which may severely threaten the health of individuals [(2)]

. Thus, it is essential to promote wound-healing and restore skin functions through adopting diverse clinical treatment measures. Moreover, wounds can be colonized by many types of bacteria that cause inflammation and destruction of the surrounding tissues, and affect healing. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most commonly isolated organisms from wounds, for their capacity to acquire antibiotic resistance, colonization by these organisms which necessitates cautious treatment, and their connection with nosocomial infections[(3)].

Wounds are a risk factor for colonization with methicillin-resistant *S. aureus* (MRSA) as well as other multidrug-resistant organisms, especially in hospital environments [(4)]. On the other hand, Infections with antibiotic-resistant bacteria are related to prolonged hospitalization, increased morbidity, and mortality, and increased healthcare costs[(5)].

That is to say, wounds must be carefully dealt with to prevent the spread of microbes, consequently helping the patient recover and reducing healthcare costs through resorting to the use of medicinal plants to eliminate antibiotic-resistant bacteria and contribute to the repair of damaged skin tissues[(6)]. [(7)] state that curcumin is the main phytochemical of *Curcuma longa* L. rhizome with the common name of turmeric. Furthermore, turmeric is used as a spice in food and medicine to treat various diseases and kill antimicrobial-resistant bacteria. Turmeric inhibits bacterial biofilm formation. It blocks bacterial growth and prevents bacterial adhesion to host cell receptors through the bacterial quorum-sensing regulation system [(8)]. Besides, it can exert a synergistic antibacterial effect alongside other antibacterial substances. Although curcumin has high clinical applicability, it has low water solubility, absorbability, and metabolism have constrained its direct use in biomedicine [(9)]. Yet, it has been used to produce functional films mixed with various polymers such as cellulose, carrageenan, pecti, gelatin, and more for biomedical and food packaging applications. Bio-film, as [(10)] argue, is an emerging field that is possibly altering the treatment of diseases through drug delivery with curcumin. Moreover, the use of biodegradable polymers film, as a base for drug formulations, may only eliminate many side effects of the current compounds used in the

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manufacture of wound pads and introduce some novel characteristics as the continued effect of the drug [11]. It provides a regular release of drug substances from biodegradable microparticles and extended maintenance of necessary concentrations of an acting substance in the body or, locally, in a certain organ or tissue. However, if a biodegradable polymer such as poly(3-hydroxybutyrate) (PHB) is the base of the drug formulation, it is completely degraded after drug substance release. Then, biodegradation products are excreted from the body [12]. PHB is a biodegradable polyester produced by bacteria, for instance, Bacillus spp when they are stressed. Since PHB degrades fully in biotic environments and produces no hazardous byproducts, it has been recommended as a substitute for petroleum-derived plastics [13].

According to its biocompatibility, PHB can be used as an operative biological substance to activate the construction of body tissues or organs that are especially convenient in implantation medicine. A further important characteristic is that when PHB is used as an implantation material, the body does not produce any immune response and thus, accepts the implantation [14]. Due to a uniform rate of medication release and effective consumption, loading industrial or natural pharmaceutical compounds on films or wrapping them in safe chemicals reduces the side effects of the drugs and boosts their efficiency and stability. In light of this, a decrease in the issues that arise when curcumin is used in therapeutic applications is to be anticipated [15]. This leads to the main objective of this study which is to create a functional PHB-based film by combining it with Curcumin. To fulfill this aim, FT-IR analytical methods are used to characterize the bioactive film. Furthermore, the curcumin release profile and antibacterial activity in vitro and in vivo are examined.

MATERIALS AND METHODS

Chemicals and Biological Materials

First of all, the study purchased Poly (3hydroxybotyrate) (PHB) and Curcumin, from an American company known as Sigma-Aldrich. As for the chloroform, it was obtained from Daejung Chemicals and Metals company, Ltd., (Siheung, Korea). Additionally, Mueller Hinton Agar (MHA), McFarland standards Solution 0.5, and Trypticase Soy Broth (TSB) were acquired from Oxoid (Basingstone, UK). Last but not least, the *Staphylococcus aureus* strain was obtained from the specialized Al-Rasool Laboratory, approved by the Ministry of Health in Karbala, Iraq, and diagnosed by the VITEK system.

Methods

PHB/Curcumin Films Synthesis and Production

The present study uses a solution casting process to make the PHB/curcumin films [10]. One gram of curcumin is dissolved in 100 mL chloroform with vigorous stirring for 1.5 hours. Then, 3 g of PHB is added to the curcumin solution and combined for 18 hours with strong stirring using magnetic stirrers. The PHB/curcumin film solution is, then, cast on a leveled non-adhesive Teflon Film-coated glass plate, and the solvent is evaporated for 24 hours in a fume hood. Next, the dry film is scratched off the plate and placed in a humidity chamber set at 28°C for at least 48 hours. Finally, as a comparison, a tidy PHB film sans curcumin is also prepared.

In Vitro Curcumin Releasing Test

This part involves measuring the amount of curcumin released from the PHB/curcumin film into the water [16]. The test film (1.5 cm x 1.5 cm) was placed in 10 mL of distilled water and incubated at 37°C (3 times). At each predefined interval, a 2 mL solution sample was obtained and the absorbance of the sample was measured at 420 nm.

In Vitro Cytotoxicity of PHB/Curcumin Films

In vitro cytotoxicity of free curcumin and PHB/Curcumin films on Rat, embryonic skin fibroblasts were assessed using the MTT assay that was obtained from AL-Nahrain University. Sample solutions of free curcumin and PHB/Curcumin films were set with different units of density (1.5, 2.5, 5, 10, 20, 40 µg/mL). At a density of $1* 10^{5}$ cells/well, the cells were seeded in 96-well plates and incubated at 37°C and (5 %) CO2 for 24 h. Once incubation is done, cells were washed 3 times with PBS, and a 20 mL MTT reagent (5 mg/mL in PBS) was added to each well. For an additional 4 hours, the plates were incubated at 37 °C. Towards the end of the incubation period, the medium was removed, 150 mL DMSO was added to each well and the plates were incubated for 10 minutes at 37°C. The study, then, proceeded into mixing each sample again and reading the absorbance at 620 nm. The inhibition percentage of cell growth is calculated using the following formula: C =A-B/A*100 (Where C: Cytotoxicity, A: is the optical density of control, and B: is the optical density of test) [17].

Anti-Bacterial Activity

As an antibacterial action, the film was tested against the harmful bacteria *Staphylococcus aureus* obtained from the specialized medical laboratory and approved by the Ministry of Health, holy Karbala, Iraq. It was also isolated from infected wounds and diagnosed by the VITEK system. The antibacterial test was conducted according to the procedure described in the reports. The tested microorganisms were first injected under purifying conditions into TSB broth and incubated for 18

hours at 37°C. After adequately diluting the culture, 200 mL of the diluted inoculum (10^7–10^8 CFU/mL) was aseptically transferred to 50 mL of TSB broth with 200 mg of the film samples and incubated at 37°C for 14 hours with a shaking movement at 100 rpm so that the initial concentration of bacteria which is about 10^7 CFU/mL can be attained. The number of viable cells was assessed by diluting and plating samples on Mueller Hinton Agar plates at preset intervals [18].

Characterization of PHB /Curcumin Film by (FTIR) Spectroscopy

The study used FTIR spectroscopy (Shimadzu) to determine the functional groups present in the Curcumin (powders), PHB (powders), PHB films, and PHB/Curcumin films. Moreover, the FTIR spectra were measured in the range of 4000 to 600 cm−1 [18].

Ethical Consideration: The study was approved by the Ethics Board of Ahl Al Bayt University. The experiment was conducted under the supervision of a specialized doctor, Dr. Firas A. Ali, following the ethical controls for conducting experiments on laboratory animals (19).

In Vivo Wound-Healing Study

Female Sprague Dawley Rats (henceforth: S.D.R) weighing 250–270 g and at the age of ten weeks were housed in cages in the animal house and raised with water and commercial feed. A total of 24 S.D.R were separated into 4 groups (n = 6) in vivo experiments. Each animal was anesthetized using 70% chloroform. Then, Full-thickness circular excision wounds sized about 1 x 1 cm^2 area was created along the markings using a scalpel, toothed forceps, and scissors using similar procedures to those in the /excision wound model. The injured animals in group 1 received no treatment and were used as a control, while on the other hand, animals in groups 2, 3, and 4 were inoculated after three minutes from wounding with 50 µL suspension containing (1×10^6 CFU/mL) bioluminescent *Staphylococcus aureus* over each defined area containing the wounds with a pipette tip. The wounds of the animals in groups 3, as well as 4, were covered using PHB Film and PHB/Curcumin Film, respectively. The size of the injury was measured and photographed on days 0, 3, 7, 10, and 14 after treatment [20].

Statistical analysis

Statistical analysis of data was performed using SAS (Statistical Analysis System - version 9.1). One-way ANOVA and Least significant differences (LSD) post hoc test were performed to assess significant differences among means. $P < 0.05$ is considered statistically significant.

RESULTS

The Preparation of PHB /Curcumin Films Poly (hydroxybutyrate) based functional films

PHB/Curcumin Films Poly(hydroxybutyrate) based functional films combined with curcumin have been prepared using a Solution Casting Technique. The PHB/curcumin composite film was stretchy and highly transparent with bright yellow color (**Figure 1. A**). We also prepared PHB films without loading curcumin to use as a control (**Figure 1. B**).

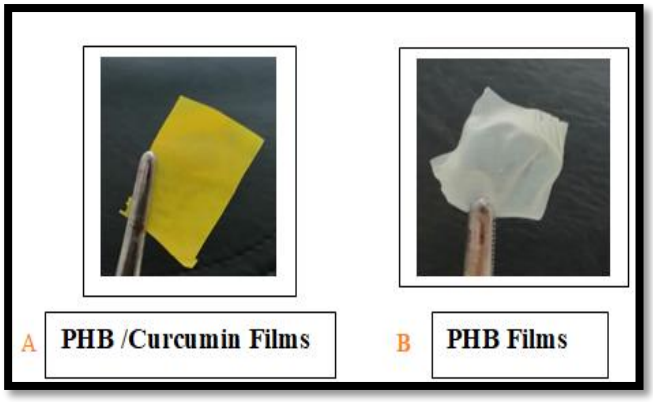

Figure (1): The appearance of the produced films **A.** PHB /Curcumin films. **B.** PHB films.

The Characterization of PHB /Curcumin Film by (FTIR) Spectroscopy

The FTIR spectra of curcumin (powders), PHB (powders), PHB films, and PHB/curcumin films are presented in **Figures 2, 3, 4, and 5** respectively. The functional groups of pure curcumin (powders) peak at 3502. 58 cm^{-1} be consistent with the phenolic –OH stretching vibration. However, the absorption peak at 1,627 cm−1 was attributed to C = O stretching. The peaks at 1500.67 cm^{-1} are consistent with C = C stretching in the benzene ring. The bands at 1273.06 cm^{-1} line with C – O of enol. Additionally, the bands in the region of 956.72–810.13 cm−1 belonged to C – H out-of-plane bending and aromatic stretching [18].

The structure of P3HB (powders) was characterized by Fourier-transformed infrared (FTIR) spectroscopy. Also, there were several major functional groups in the FTIR spectrum. The peaks at 3430 cm−1 matched the –OH of the absorption band at (2935.76, 1454.38, 1377.22) cm−1 being compatible with the C-H (alkane), CH2, and CH3, groups respectively. The next distinctive peaks: (1720.56) cm−1 are compatible with the long, short, and medium-chain. They show a solid characteristic of carbonyl expand vibrations of ester groups (C=O), and C-O (ester) at wave numbers of 1050 cm-1(Figure 3.3). All of the four major peaks were similar to the characteristics of polyhydroxy butyrate (PHB) [21].

The disappearance, diffraction, and reappearance of the peaks of the PHB spectrum after conversion are different, and this difference is evident in the emergence of new bonds to bind PHB molecules in the film texture. The characteristic peaks of curcumin and PHB accounted for the spectrum of PHB–curcumin films are caused by reaction and formation of new bonds. It should be noted here that in the spectrum of PHB–Cur film, the –OH stretching vibration of the film appeared in 3502.85 cm−1. Additionally, the peaks of curcumin were demonstrated (2974.33,1597.11,1508.38 and 1597.11) in PHB–Cur film.

In light of these results, it can be demonstrated that the hydrogen bonding between PHB and curcumin was produced in PHB–Cur films. In brief, PHB and Cur had been compounded together excellently.

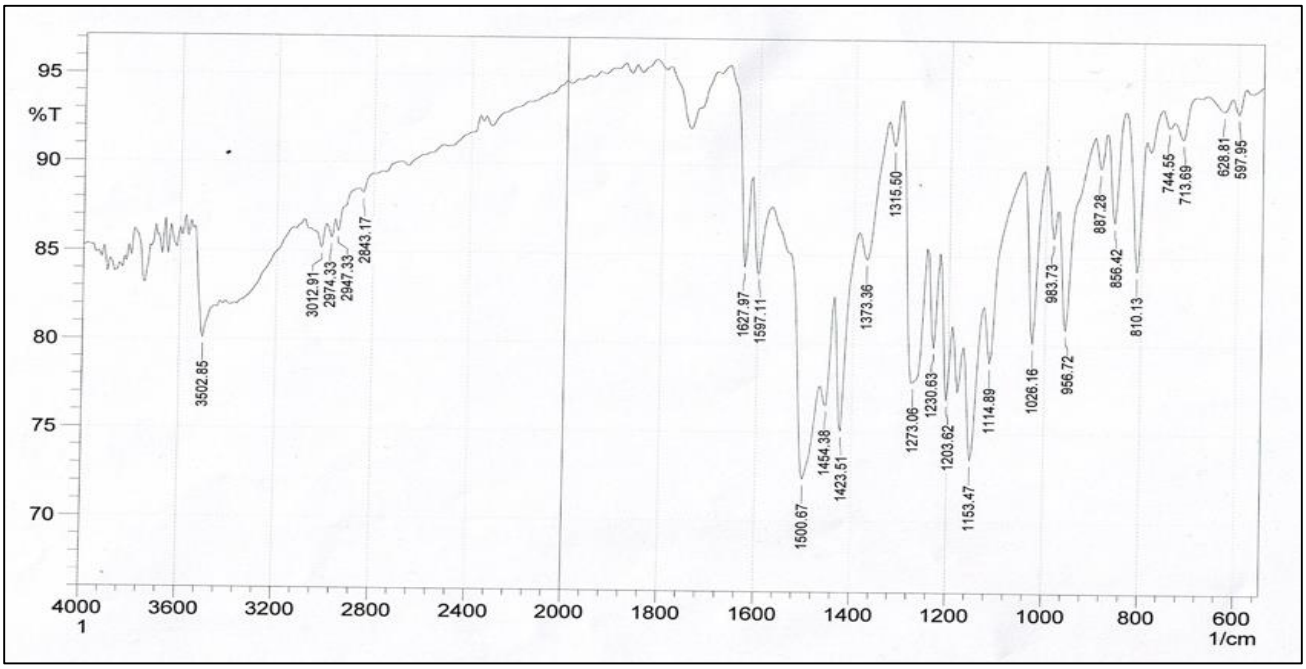

Figure: (2): FTIR spectra of curcumin (powders)

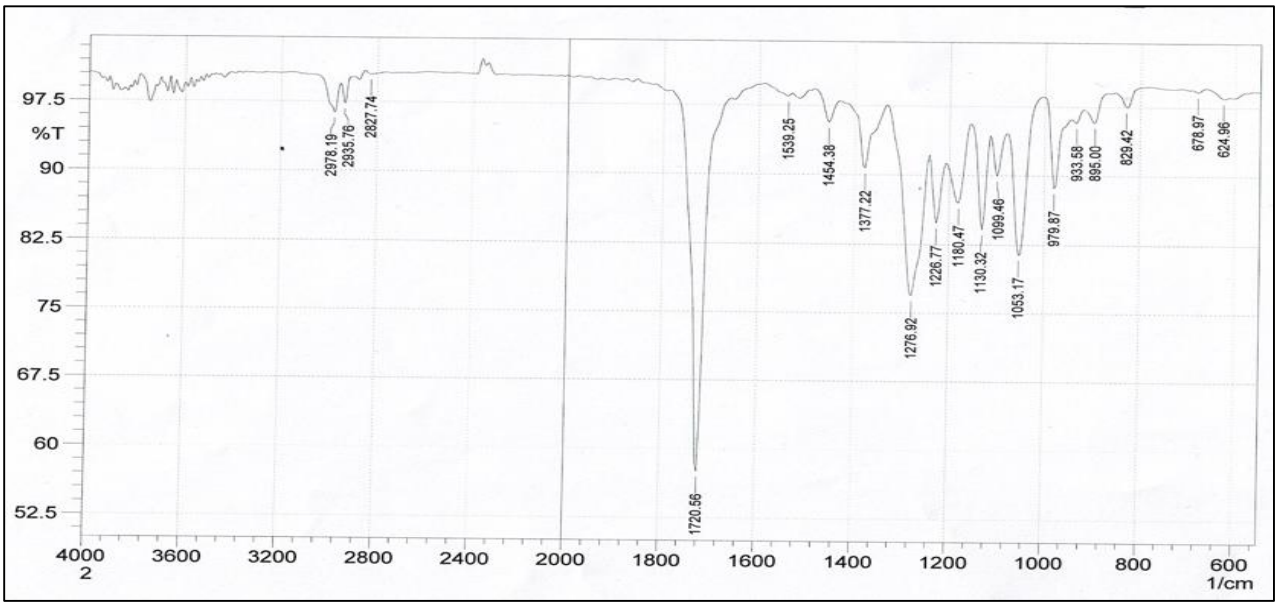

Figure (3): FTIR spectra of PHB (powders)

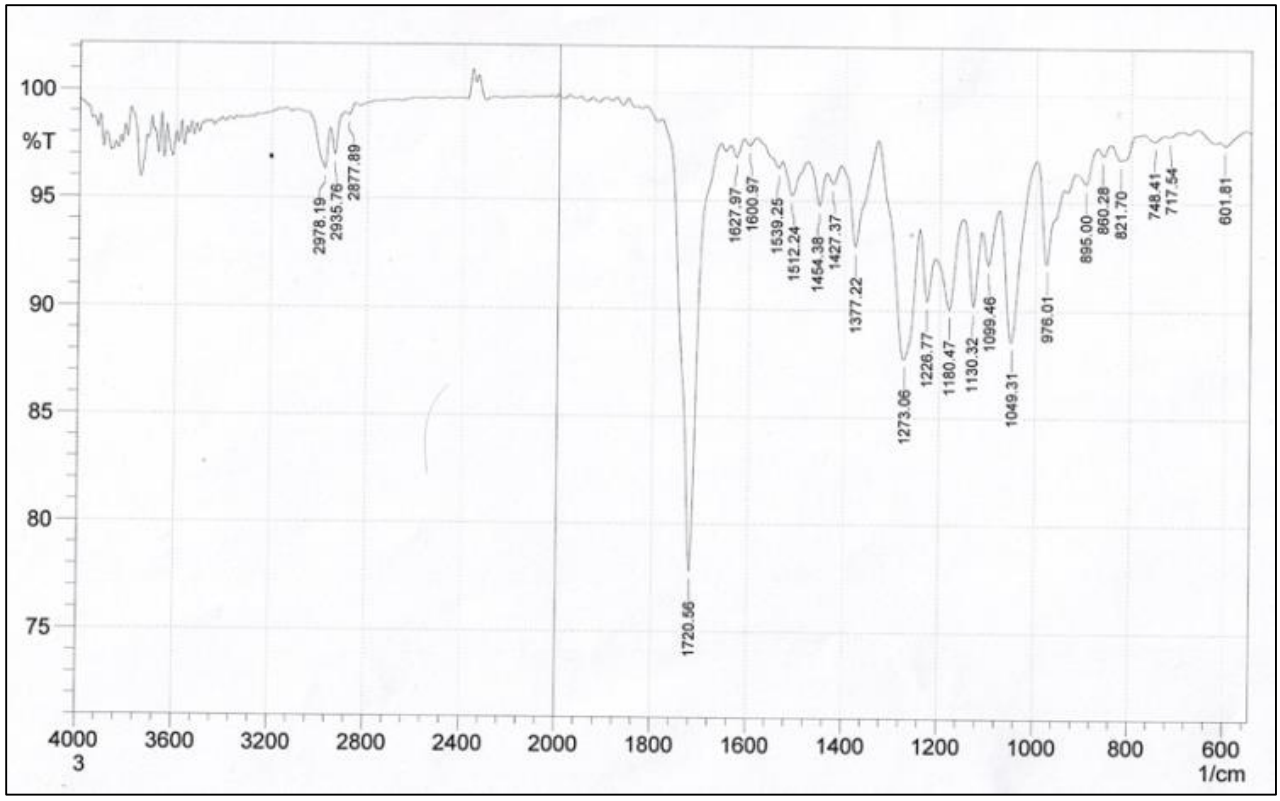

Figure (4): FTIR spectra of PHB films

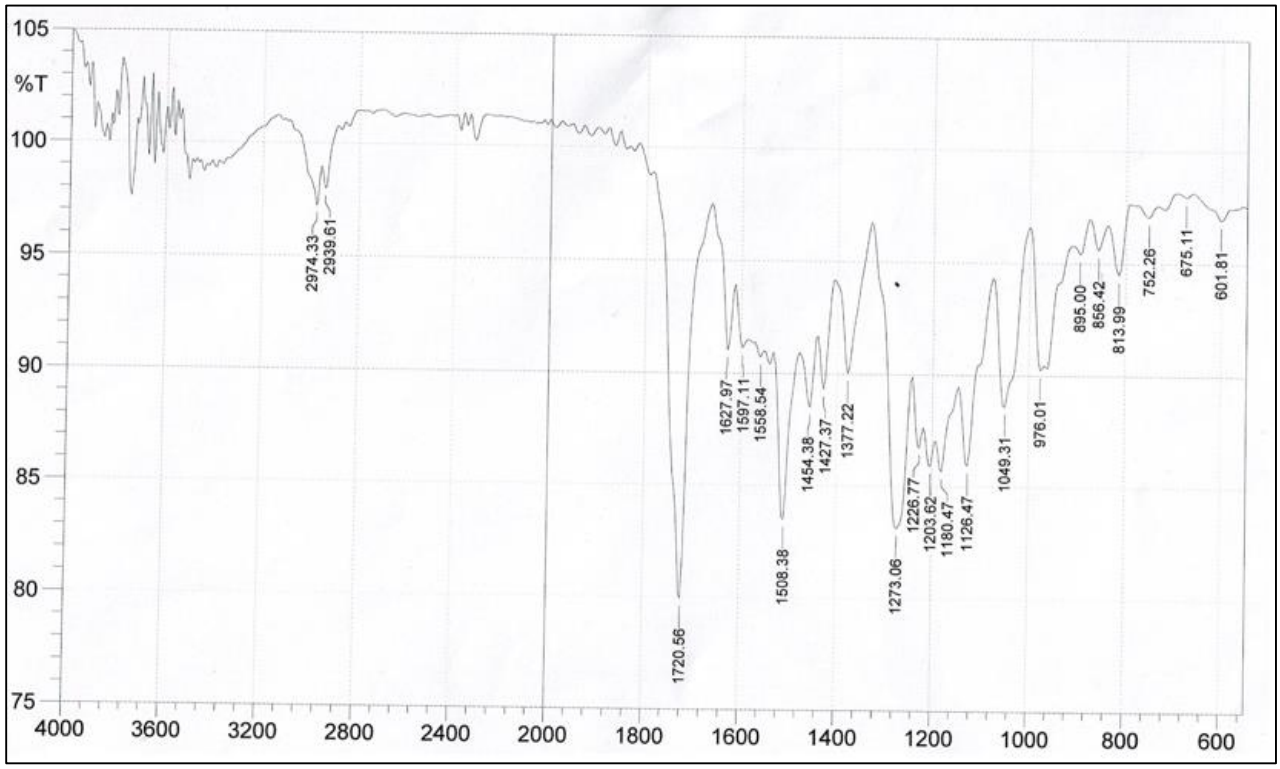

Figure (5): FTIR spectra of PHB/Curcumin films.

In Vitro Drug Release.

As illustrated in **Figures 6, and 7**, the curcumin was first liberated in large quantities from PHB/cur films after 3 hours. Within 15 hours, almost 90% of the curcumin in PHB/curcumin films was released in a sustained way. After 18 hours, free curcumin showed a rapid release pattern, with more than 98% of the curcumin being released into the aqueous medium.

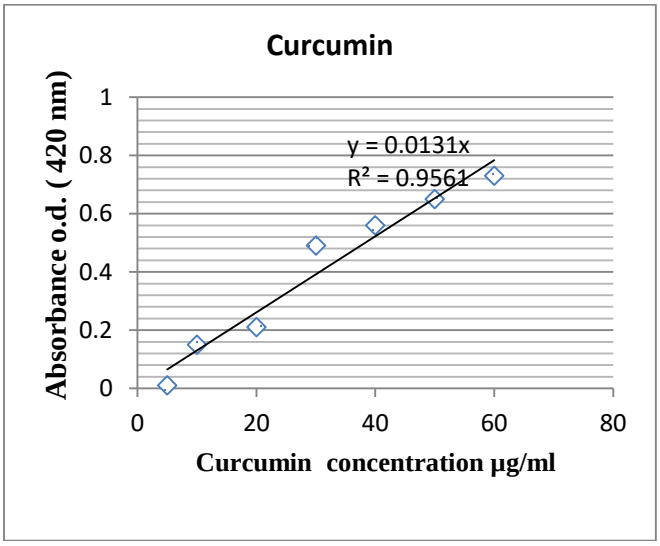

Figure (6): Calibration curve for curcumin at 425 nm.

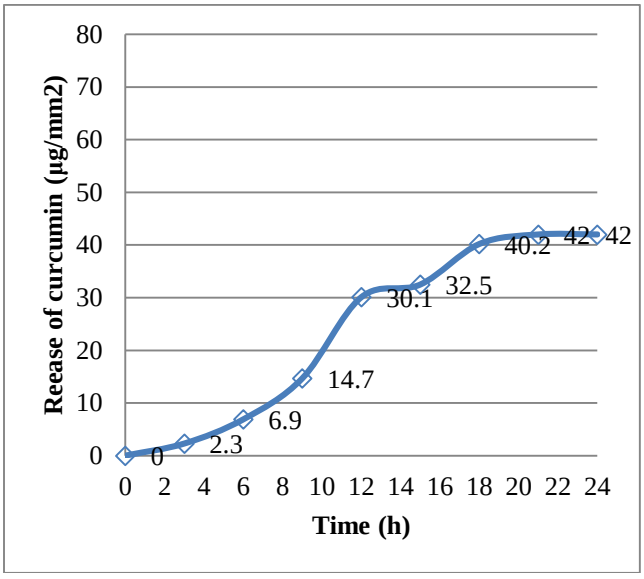

Figure (7): The releasing pattern of curcumin from the PHB /curcumin films.

In Vitro Cytotoxicity of PHB/Curcumin Films

To detect the results in vitro cytotoxicity of PHB/Curcumin Films on Rat embryonic skin fibroblasts, an MTT assay was performed and is shown in Figure 3.8. The results specified that PHB Films significantly reduced ($P<0.05$) the toxic effects of curcumin.

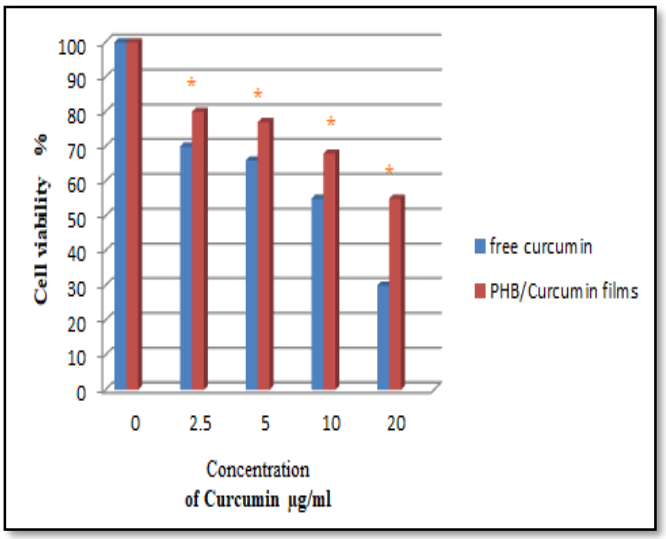

Figure (8): In vitro cytotoxicity of free Curcumin and PHB/Curcumin films against Rat embryonic skin fibroblasts. The data are presented as *SD (n = 1).

Antibacterial Activity

Using the CFU quantification method, the antibacterial behavior of the materials was assessed. In comparison to the control tube, the PHB (powders) and PHB films did not show any antibacterial activity, as shown in **Figure 9**. PHB/Curcumin films and Curcumin (powders) both could inhibit the growth of *S. aureus.*

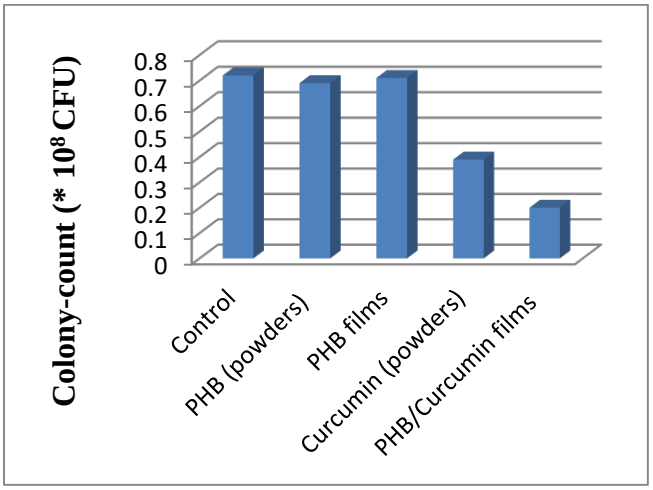

Figure (9): Antibacterial activity of the curcumin (powders), PHB (powders), PHB films, and PHB/Curcumin films against *Staphylococcus aureus.*

In vivo wound healing study. Images were taken to observe the degrees of change in the superficial skin wound areas, as shown in **Figure 10**. On day three; at the beginning of the animals' treatment, it was noticed that the wound surfacing on the animals in the PHB/Curcumin Film group had already dried due to the anti-inflammatory property of curcumin. On the other hand, the animals in the control group whose wounds were contaminated with S. aureus and the PHB film groups still exhibited some secretions. On day seven, some secretions could still be found on the wound surfacing on animals in the control and PHB film groups. On day ten, the superficial skin wound areas of all four groups began to show signs of healing, but in varying proportions. However, the wounds in the PHB/Curcumin Film and curcumin groups healed better than those in the other two groups. For the most part, the control group revealed slower wound healing than the other three groups during the observation period. That is to say, the wounds in the PHB/Curcumin Film group healed better. To further note, the study measured the wound areas of the selected rats and calculated the wound healing rate. **Figure 11** presents the average wound healing rate on day 14, after surgery, which was 80 % in group 1, 70 % in group 2 that was wound infected and did not receive any treatment, 75 % in the PHB film group, and 95% in the PHB/Curcumin film group.

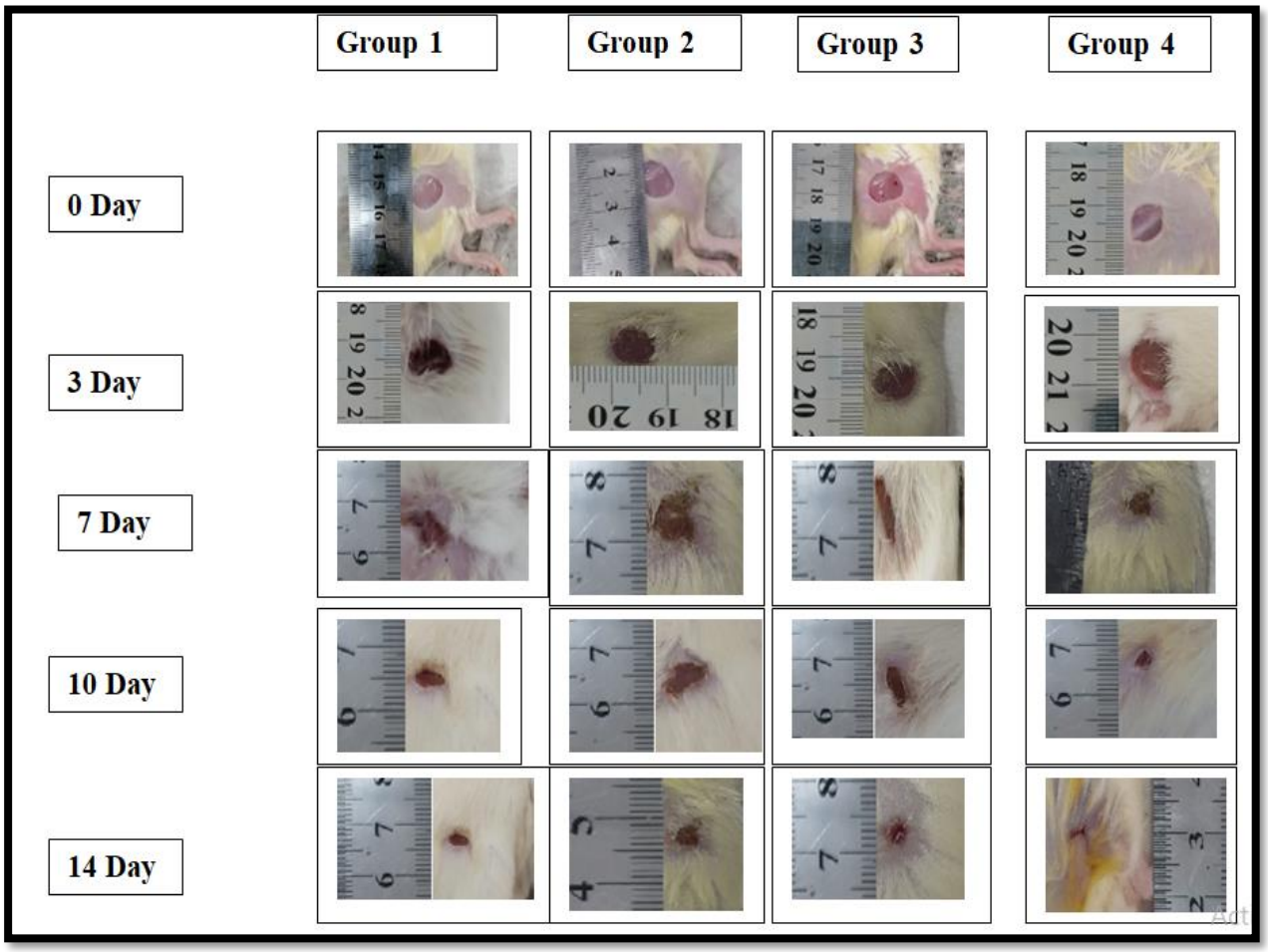

Figure (10): The pictures of wound surface healing over time in each group

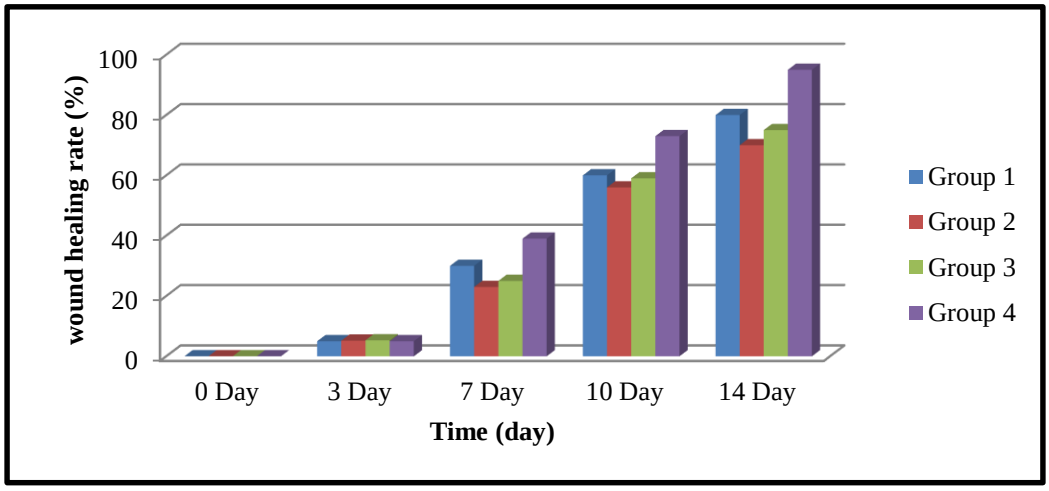

Figure (11): The percentage of wound closure versus healing time in each group.

DISCUSSION

Wound or damage healing is critical for postoperative skin function improvement. Various wound dressings, such as films, nanofibers, and hydrogels, have been extensively studied to promote wound healing. Histocompatibility and anti-infective characteristics are two of the most important advantages of injury dressings. [22]. Some antimicrobial techniques, such as the antibacterial medication curcumin, are used in skin dressings to suppress bacterial growth at the wound site to fulfill the dual objective of encouraging healing and fighting infection [23]. Moreover, different types of skin bandages have different benefits and drawbacks. Hydro-gel dressings, for instance, are convenient to use, specifically in filling wounds of all shapes and sizes, yet, however, they isolate the site from oxygen in the air which is not beneficial to cell and tissue regeneration and proliferation at the wound site [24]. Even though in quite a few studies, other fiber membranes use micropores to ensure the contact of the wound site with oxygen, it is often necessary to seal the composite membrane to the wound site to prevent it from falling off, which could, therefore, leads to an increased difficulty of surgery [25].

To find a proper solution to these problems, researchers have been investigating the composition and structure of skin dressings to improve the application potential of dressings. The present study developed a wound dressing comprising PHB /Curcumin Film. Similar to most wound dressings, PHB /Curcumin Film also exhibited anti-bacterial properties. Since bacterial multiplication is a key factor in bringing about wound infections, the antibacterial characteristic is very important for the application of film materials. Given that Staphylococcus aureus and *Pseudomonas aeruginosa* are two common infectious species of bacteria in the natural environment, the activities of PHB /Curcumin Films against *S. aureus* were evaluated in vitro [26]. The present study further used the CFU quantification method to evaluate the antibacterial behavior of the materials. Figure 7 shows that the PHB film did not exhibit noticeable antibacterial activity, in comparison with the Control group. Moreover, PHB /Curcumin Films and Curcumin groups both possessed a high ability to inhibit the growth of *S. aureus* bacteria, but lower than those of the Control group and those of the PHB film group [27].

It is important to note that curcumin's antibacterial mechanism has remained a mystery until now. According to some research studies, curcumin's antibacterial activity has been linked to Fts Z, which is a cytoskeletal protein that plays a vital role in the development of bacterial cells. Curcumin decreased S. aureus growth primarily by blocking the assembled FtsZ. It has the potential to harm bacterial membranes through increased permeability. Furthermore, it is believed that curcumin has antibacterial properties by clamping the bacterial cell wall, causing it to break, and then infiltrating the cell, rupturing the organelle's structure. [28]. The PHB /Curcumin film and Curcumin were a growth inhibition effect as shown in Fig 3.8. They were compared with tubes containing PHB film and control. This finding indicates that the antibacterial effect of PHB /Curcumin film could be accredited to curcumin, which is a renowned and effective antibacterial compound [29]. Additionally, due to the slow-release effect of PHB /Curcumin film, the film released curcumin slowly and continuously, which in turn provides long-lasting protection during the wound-healing process as presented in Fig 3.7. Apart from imparting anti-bacterial effects to skin dressing, it is also significant to design the dressing such that it is safe,

non-toxic, and does not stimulate allergic skin infections [30]. Many researchers use it in skin dressings to improve wound dressing biocompatibility and surge skin healing. Huan's earlier research shows that collagen can be used as a cell delivery vehicle and cell culture substrates [31 -32]. Thus, in this study, we incorporated Curcumin into PHB because it exhibits low antigenicity, water-soluble ability, nontoxic nature, and more compared to other natural polymers [33]. Regardless of the idea that PHB is only employed as a bandage matrix, it has garnered a lot of interest in the research of skin dressings, artificial organs, and contact lenses. This is because PHB is biodegradable by bacteria (because of its low strength). As a result, extra components are frequently added to improve these qualities. Chitosan, for instance, is utilized as a filler to improve the film-forming capabilities of PHB. [34]. In this study, we synthetic a bandage with antibacterial efficacy and appropriate biocompatibility, and the in vitro and in vivo results verified this hypothesis. The incorporation of Curcumin improved the film-forming properties of PHB [18]. The entire film may have sufficiently adhered to the wound surface. The results of the present study show no purulent manifestations, swelling, or redness during the examination time, indicating that it is safe for tissue usage. It further verifies that it does not trigger infections because it includes anti-bacterial action. According to antibacterial and drug-release studies, PHB-loaded curcumin particles displayed similar sustained drug-release behavior and considerable antibacterial activity in vitro. Last but not least, results from the MTT assay uncover that PHB /Curcumin film had lower cytotoxicity and increased cell growth than that of free curcumin, which is consistent with those from other studies [35].

CONCLUSION

Due to the success of loading curcumin in a PHB film and proving the growth in the effectiveness of the method by increasing and prolonging the effect of the biological activity as an antibacterial and in helping tissues to heal wounds in addition to its rapid wound-healing effects, It could be concluded the study considers PHB/Curcumin films promising as a wound dressing agent in wound management.

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Author contribution: Each of the researchers has a contribution rate equal to the rest of the researchers in the study.

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