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Wound Dressing Based on PVA

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Abstract.

The number of patients with various types of skin wounds has increased due to the increase in population and the emergence of new skin diseases. So that other traditional dressings and techniques have fallen out of the reach of researchers and physicians and replaced with new ones. Nowadays, biopolymer polymeric scaffolds, especially nanofibers, are widely considered for engineering applications of skin tissue and wound coating. At first, GO was synthesized from graphite and then added to the extract of Nigella sativa and Arginine. FTIR, Zeta potential and SEM analysis were used to confirm the accuracy of the materials. The antibacterial properties of the materials were also evaluated by MIC, OD and Halo assay. The results showed that these materials are capable of inhibiting the growth of both groups of bacteria, Gram-positive and Gram-negative. Next, the polyvinyl alcohol solution was prepared and combined with Graphene oxide -Nigella sativa - Arginine to produce by the electro spinning process of nanofibers. FTIR analysis confirmed the presence of nanomaterials in the nanofibers produced. NIH 3T3 fibroblast cells were used to evaluate cell toxicity and viability by MTT method. The results indicate the non-toxicity of Graphene oxide -Nigella sativa -Arginine nanocomposite at concentration 2 micrograms per mL.

Keywords: Wound dressing; Nigella sativa; Graphene oxide; Arginine; Polyvinyl alcohol

1. Introduction

Recently, there have been lots of efforts and studies making different types of polymeric and biopolymeric dressings and have received much attention due to the importance of wound healing in deep injuries or wounds caused by certain diseases such as diabetes.

The skin is the outermost part of the body that is constantly exposed to physical injuries, burns, illness, and all kinds of accidents and skin injuries can have a huge impact on the patient physically, physiologically and psychologically. There are two major problems in the recovery of skin damage, including the presence of secretions and accumulation of microorganisms in the affected site. For this reason, it is essential to control the secretions as well as the fight against bacteria and microbes to heal perfectly. A highly repairable wound dressing requires various properties that are proper and nested pores. One of the best ways to achieve this is to fabricate nanofibers using an electrospinning process because of the unique advantages of this method such as high surface area to volume ratio, proper porosity and the ability to mimic collagen fibrous structures in the matrix environment. Extracellular has been the focus of many scientists.

Due to the increasing side effects of chemical drugs and the lack of treatment for many diseases, the use of herbal compounds and drugs has attracted the attention of researchers because they have fewer side effects and are more effective than most chemical drugs. Among these plants is the Nigella sativa, which has a rich medical history [1].

Polymers play an important role in modern industrial and environmental fields. In the last century, with the advances in chemistry and materials, different types of synthetic polymers have been introduced to the scientific community. Polymers are organic molecules with long chains at the nanoscale. The functional properties of synthetic polymers can be made by modifying the polymer synthesis method and can be modified according to the specific needs and biological applications to overcome the limitations of using natural polymers. The advantages of polymers include easy and low costs fabrication with high flexibility (applicable to different types of coating materials). Many biological and synthetic polymers with different properties have attracted the attention of researchers in the applications of medical devices, especially in wound dressings. In this study, Polyvinyl alcohol (PVA) was selected due to its special properties including biocompatibility and biodegradability, non-toxicity and non-sensitivity and good mechanical properties [2].

Controlled release of medicinal compounds applied to the dressing will greatly help the wound healing process as the drug must be released over time to maintain its long-lasting antibacterial and accelerating effects. In this study, graphene oxide was selected to meet this demand. Graphene oxide (GO) can be soluble in polar solvents such as water and its biodegradability has been proven in previous studies. [3]. The results of a study in 2014 showed a positive effect of GO on the tensile strength and antibacterial properties of a composite based on GO and PVA [4,5].

Other concerns of researchers and the medical community are the speed of wound healing in many cases. Scientists are looking for a drug to stimulate skin repair and speed up the operation. Arginine was selected as a stimulating agent for wound healing and was combined with an antibacterial drug that was the same as Nigella sativa. Also previous studies have shown the antibacterial effects of Arg on both groups of bacteria (Gram positive and Gram negative) [6].

Moreover, the controlled release of drugs on the wound and the presence of substances that accelerate the repair of damaged skin are the features of this wound dressing.

2. Materials and methods

2.1. Materials

Polyvinyl alcohol (PVA), Nigella sativa L., Graphite, phosphate buffer saline (PBS), Arginine (Arg, $C_6H_{14}N_4O_2$), Ethanol (C_2H_6O), Glyoxal ($C_2H_2O_2$), Sulfuric acid (H_2SO_4), Hydrogen peroxide (H_2O_2), Potassium permanganate ($KMnO_4$), Glycerol were supplied by Merck (Germany). The bacteria used in this study was obtained from Microbiological Resources Centre, Iranian Research Organization for Science and Technology (IROST), which include Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli).

2.2. Synthesis of Graphene Oxide

The method used to synthesize graphene oxide from graphite was the Hummer's method. In brief, to synthesize monolayer graphene oxide, first, pour 1 g of graphite into 20 mL of 98%

purity sulfuric acid (which is contained in an ice bath on a stirrer) until the graphite is completely dissolved by a magnet inside the sulfuric acid in low temperature. After about 30 minutes, add 3 g of potassium permanganate slowly and in the meantime, the solution turns to green. After about 30 minutes, 50 ml of distilled water is added dropwise to the solution. After 10 minutes, 100 mL of distilled water was added to the solution, after 30 minutes 35 mL of hydrogen peroxide was added dropwise, and after 24 hours the graphene oxide solution was removed from the heater stirrer. To powder the sample, the first centrifuge it, then place it in the refrigerator at -20 ° C until completely frozen, then put in the freeze dryer to make it powder [7].

2.3. Preparation of GO/Arg nanoparticles

At this stage, 75 mg of graphene oxide, which is prepared in the previous step, was added to 15 mL of distilled water and ultrasonic to obtain a solution of graphene oxide at a concentration of 5 mg / mL. In the next step, add 30 mg of arginine to the previous solution and finally, the GO/Arg nanocomposite is obtained at a concentration of 7 mg / mL [8].

2.4. Preparation of Nigella sativa solution

The seed extract, solidly prepared from the Institute of Medicinal Plants Research, first was weighed, poured into erlen, then 70% ethanol added to erlen. erlen was placed on a stirrer, inserted in the magnet, and closed with erlen's cork to dissolve the nigella sativa completely in alcohol [9].

2.5. Cultivation and preparation of microbial banks

For the culture of microbial strains, Muller Hinton broth culture medium was used for E.coli and S.aureus. After preparation of the culture medium, it was sterilized at autoclave at 121 ° C and pressure 112 bar for 45 minutes. After the culture medium had reached room temperature, 2 mL of culture medium containing E.coli was added to 50 mL fresh Müller Hinton broth and incubated at 30 ° C. After 1 day, 750 µl of cold sterilized glycerol was added and homogenized in 750 µl of bacteria cultured in 1.5 mL sterile microtube. The same method was used for S.aureus. Microtubes containing microbial suspension were stored at -70 ° C.

2.6. Determination of minimum inhibitory concentration of bacterial growth[1]

To determine the minimum inhibitory concentration, the bacterial overnight culture was carried out in Müller Hinton broth at 37 ° C. After this time, the optical density (OD600) of 0.5 McFarland solution produced using physiological serum and cultured bacteria (v / v ratio = 0.01) was set to 0.11. Then 100 µl of sterile culture medium was added to each well, and to all wells except the last well of all rows, 5 µl of 0.5 McFarland solution was added. Then, 100 µL of the first rows wells were removed and added to all the second rows wells. This process was repeated until the tenth wells of all rows, and finally, 100 µl was discarded from

[1] MIC

the tenth well. As a result, the concentration of samples from the first to tenth wells was 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512 and 1/1024 respectively. Eleventh well-containing bacteria and culture medium was considered as positive control and 12th well-containing culture medium as the negative control. For greater accuracy, the experiment was repeated 3 times for each sample and tests were performed on a separate plate for each bacterium. Plates were then incubated for 24 hours at 37° C. Finally, using ELISA, wells with minimum inhibitory concentration were obtained for each row and averaged from 3 replicates for each sample. This process was performed for both bacteria mentioned.

2.7. Fabrication of PVA-GO/Arg/NS nanocomposite

Based on the MIC test and according to the available literature, the information required for the electrospinning was extracted and the PVA-GO/Arg/NS nanocomposite was prepared as follows.

First, 0.4 g of PVA was dissolved in 4 mL of distilled water under stirring for 1 hour using a stirrer, and a homogeneous gel-free solution (w / v ratio of 0.1) was obtained. Then added 50 µl GO/Arg and 50 µl NS solution to PVA solution. The homogenate solution was poured into the syringe. The syringe was inserted into the apparatus and two positive and negative electrodes were attached to the collector to create an electric current in the polymer solution.

The voltage, solution injection rate, temperature, and needle to collector distance is 20 kV, 1mL/h, 28° C and 10 cm respectively. The sample Stored at room temperature for 24 h to ensure evaporation of any solvent.

2.8. Nanofibers crosslinking

The glyoxal was poured 50% (v / v) into a plate and placed on the desiccator. Then the nanofibers were split into smaller pieces, then separated from the foil and placed on a special filter. Filter was placed on a plate containing glyoxal. The cap of the container is impregnated with oil until the lid is firm and not moved. Insert the container into the oven and place at 50 ° C until the glyoxal has evaporated and sits on the pad to allow crosslinking and improve the mechanical strength of the pad. It should be noted that the duration of exposure to glyoxal vapor is very important.

2.9. Antibacterial properties characterization

To test the antibacterial properties of GO/Arg and GO/Arg/NS using disk diffusion method (DDM), both strains of the bacteria, S.aureus, were used as Gram-positive and E. coli as Gram-negative. Then the inhibition zone of GO/Arg and GO/Arg/Ns disk was compared with the gentamicin disk (Control disk). This test was repeated 3 times to increase accuracy.

2.10. Cell Toxicity (MTT assay)

To detect the toxicity or non-toxicity of these nanofibers, MTT assay was used. Thus, NIH 3T3 fibroblast cells were first embedded in 24-well cell culture plate. Then it was placed into the incubator for 24 hours to better adhere the cells to the bottom of the plate. The GO/Arg/NS solution was added to the culture well and in another well, DMEM medium containing 10% PBS and 1% penicillin streptomycin was added as a control. The cells were incubated for 24 h. Then the culture medium and cell samples were removed and 50 µl of MTT solution at 5 mg / mL concentration was added into each well. After 4 hours, the solution was removed from cells and 100 µl of dimethyl sulfoxide (DMSO) was added to

them to dissolve the created purple crystals. Then the plate was placed on Stirrer for 20 minutes. Then 100 µL was removed and its wavelength at 570 and 690 nm was obtained by ELISA reader. Wells containing more cells had higher optical density (OD) than wells containing fewer cells. Therefore, it is possible to identify wells with a higher cell number and compare them with the control sample.
The percentage of toxicity as well as cell viability were calculated based on the bellow equation [12].

$$\text{Cell toxicity } (\%) = 1 - \left(\frac{average\ of\ samples\ OD}{average\ of\ control\ OD} \right) \times 100 \quad (1)$$

2.11. Characterization

To study the surface morphology of the composite, scanning electronic microscopy (SEM, TeScan – Mira III) and transmission electron microscopy (TEM, Zeiss, EM10C, 80KV) were used, and to study the functional groups of each bonds, Fourier transform infrared spectroscopy (FTIR, Spectrum RX I, PerkinElmer) was used in the range of 450-4000 cm-1. Nanoparticles distribution was observed by scanning SEM Mapping and zeta potential analysis was used to evaluate the stability of the nanocomposites.

3. Results and discussion

3.1. Characterization of GO

3.1.1. Fourier transform infrared spectroscopy (FTIR)

FTIR is one of the common methods used to investigate the functional groups present in a material to prove the synthesis method. Figure 1 (a) shows the FTIR of graphene oxide. The presence of different types of reactive oxygen species in graphene oxide confirms the wide spectrum at 3436 cm-1 wavelengths and can indicate O-H, C-OH and water groups in this compound. Graphene oxide has distinct peaks in the range of 1054 cm-1 (C-O), 1384 cm-1(C-O-C), 1634 cm-1 (C = O). The presence of oxygenated functional groups indicates that graphite is well oxidized by oxidizing agents. By comparing this graph and the FTIR spectrum of graphene oxide obtained by other researchers, it is ensured that the material synthesized is graphene oxide [14,15,16].

3.1.2. SEM and TEM analysis

SEM and TEM images indicate the porosity in the graphene oxide plates. In this study, the morphology of synthesized graphene oxide was identified using SEM and TEM images. The SEM and TEM images of the synthesized graphene oxide are shown in Figures 2 (a) and 2 (b), respectively. In these figures, the various layers of synthesized graphene oxide are well visible, which is a reason for the good conversion of graphite to graphene oxide layers. The quality of the synthesized graphene oxide shown in Figure 2 (b), which is a single layer of GO, is also displayed [17].

3.1.3. Zeta potential

According to Table 1, the zeta potential in the 5 trials is about -31.42 to -34.72 mV. Comparing this result with the results of other researchers, indicated that the synthesized sample is electrostatically well and has good stability [18]

Table 1	
Test Number	Zeta potential(mV)
1	-31.42
2	-34.10
3	-34.72
4	-33.24
5	-32.89

3.2. Characterization of GO/Arg

3.2.1. FTIR

A comparison of the GO and GO/Arg diagrams shows that in addition to all GO functional groups being retained, the arginine functional groups have also been added to the GO diagram that Indicated the formation of GO-Arg bonds. According to Figure 1 (b), the peak at 1127 cm-1 represents the C-N bond, the peak at 1647 cm-1 represents the C=C bond, the peak at 3449 cm-1 represents the N-H bond, and the peak at 3799 cm-1 represents the O-H bond. By comparing this graph with the graphene oxide spectrum obtained by other researchers, it is concluded that the synthesized material is arginine graphene oxide [19].

3.2.2. SEM Mapping

Elemental analysis and surface structure indicate the presence of C, O, H and N elements in GO/Arg (Figure 3 (a)). Figure 3 (a-2), 3 (a-3), 3 (a-4) and 3 (a-5) show carbon, oxygen, hydrogen, and nitrogen respectively and Figure 3(a) shows that these materials are well dispersed and have a uniform structure. Due to these figures, the chemical composition of Arg, and comparisons with the results of other researchers, can prove that GO/Arg is well synthesized [20].

3.2.3. Zeta potential

According to Table 3, the zeta potential in the 5 trials is about -41.27 to -43.83 mV. Comparing this result with the results of other researchers, indicated that the synthesized sample is electrostatically well and has good stability [18].

Table 2	
Zeta potential (mV)	Test Number
-43.51	1
-42.72	2
-43.83	3
-42.39	4
-41.27	5

3.3. Characterization of the wound dressing

3.3.1. FTIR

FTIR analysis was used to confirm the binding of the synthesized nanocomposite to PVA. As shown in Figure 1 (c), the peak at 1239 cm-
1 represents the C-N bond, the peak at 1716 cm-1 represents the C=C bond, the peak at 2909 cm-1 represents the N-H bond and the peak at 3304 cm-1 indicates the O-H bond. Comparing this figure with other researchers' figures, indicated that the wound is well-synthesized with the mentioned compound [19, 21].

3.4. Disk diffusion

As shown in Figure 3, the antibacterial properties of the disks that impregnated with GO/Arg solution (No. 1), GO/Arg/NS (No. 2) and an antibiotic tablet (gentamicin) were used as a control. This test was performed once on Gram-positive bacteria and once on Gram-negative bacteria.

In Figure 3 (a), which relates to Gram-negative bacteria, the diameter of the inhibition zone for antibiotic disk is 2cm and for no2 disk is 1.7cm but for no1 disk antibacterial properties are negligible. In Figure 3 (b), which relates to Gram-positive bacteria, the diameter of the inhibition zone for the antibiotic disk is 2.5cm and for no2 disk is 2cm but for no1 disk antibacterial properties are negligible. Therefore, it can be concluded that the synthesized nanocomposite has a good antibacterial activity due to the presence of NS [24].

3.5. Minimum inhibitory concentration (MIC)

The antibacterial activity of the synthesized materials was evaluated by MIC method against two E.coli and S.aureus bacteria. The results of the evaluation of MIC test of GO/Arg and Go/Arg/NS nanocomposites on the two mentioned bacteria are shown in Table 3. This test was repeated three times for each nanocomposite. The following formulas can be used to calculate the percentage of MIC inhibition and inhibitory concentration [13]:

$$Inhibition\ (\%) = \frac{Sample\ absorption}{negative\ control\ absorption} \times 100 \quad (4)$$

$$Concentration\ of\ inhibition = \frac{Volume\ of\ sample \times Concentration\ of\ sample}{Total\ Volume} \quad (5)$$

According to Table 4, inhibition of GO/Arg/NS nanocomposite can be observed on both E.coli and S.aureus.

According to Table 3, the MIC of arginine GO/Arg/NS nanocomposite against both the above-mentioned bacteria is 0.91 mg /mL.

Table 3 minimum inhibitory concentration				
MIC of Gentamicin antibiotic	MIC of GO/Arg/NS	MIC of GO/Arg	MIC of GO	Bacteria
0.01	0.91	1.25	1.75	*S.aureus*
0.01	0.91	1.25	1.75	*E.coli*

Table 4 sample inhibition (%)		
GO/Arg/NS	GO/Arg	Bacteria
97.78	81.33	*S.aureus*
96.47	82.14	*E.coli*

3.6. Cell toxicity

The effect of GO/Arg/NS composite on the survival and proliferation of NHT3T3 fibroblast cells was performed by MTT test. Figure 4 shows the results of this test for this composite in different concentrations compared to the control, after 24 hours. It can be observed that in the treatment group with a concentration of 2 µg/mL, the survival rate is higher than the control state and other concentrations. It can be concluded that the GO/Arg/NS composite at a concentration of 2 µg/mL not only had no cell toxicity but also increased the growth and proliferation of fibroblast cells after 24 hours. At 20, 200, and 2000 µg/mL concentrations of this composite showed toxicity and, after 24h, destroyed fibroblast cells.

4. Conclusion

In the present work, graphene oxide was synthesized by a modified hummer method then Arginine and Nigella sativa extract were added. At each stage, the structure of the synthesized composite with different tests such as FTIR, SEM and TEM, Zetta potential and SEM mapping was examined, the results indicated that the composite was synthesized well. The results of tests such as the Disk diffusion and MTT assay proved the antibacterial properties of the composite. Comparing the synthesized wound dressing in this study with similar research in recent years, we conclude that the synthesized wound dressing has ideal properties for wound healing, which is shown in Table 5.

Table 5- Comparison between polyvinyl alcohol-based wound dressings

No	Year	Researchers	Materials	Inhibition zone diameter (cm)		Mechanical tensile Young module (MPa)	Wound healing (%) Viability of cells (%)	In vivo Wound healing
				Gram-	Gram+			
1	2010	Vicentini et al. [25]	PVA Chitosan ZnO nanoparticles	1.6	1.5	4.7	---	---

2	2011	Shalumon et al. [26]	PVA Sodium alginate ZnO nanoparticles	1.5	1.6	---	It has used another method for antibacterial properties	---
3	2012	Liu et al. [27]	PVA Chitosan Ag	1.19	1.28	56.7	---	---
4	2013	Zhou et al. [28]	PVA Silk fibroin Carboxyethyl chitosan	---	---	---	101% viability after 1 day	---
5	2017	Gutha et al. [29]	PVA Chitosan ZnO	1	1.7	---	150% viability after 7 days	No results have been reported, only animal model images are available
6	2018	Kheradvar et al. [30]	PVA Silk fibroin Aloe vera	---	---	---	107% viability after 3 days	---
7	2019	Wentao et al. [31]	PVA ZnO CuO Au	It has used another method for antibacterial properties	It has used another method for antibacterial properties	---	98% viability after 10 days 60% wound healing after 10 days	<100% after 10 days
8	2019	Huang et al. [32]	PVA Silk fibroin	---	---	0.2	43.2% viability after 5 days	45-53% after 5 days
9	2019	Ojah et al [33]	PVA Silk	1.2	1.1	12.2	---	---
10	2020	Bakhsheshi et al [34]	PVA Chitosan Silk protein sericin	0.62	0.6	5.56	110% viability after 3 days	It has used another method
*	2020	This research	PVA Graphene oxide Nigella sativa Arginine	2	1.7	5	127% viability after 1day 54% wound healing after 1 day	85.86 % after 14 days

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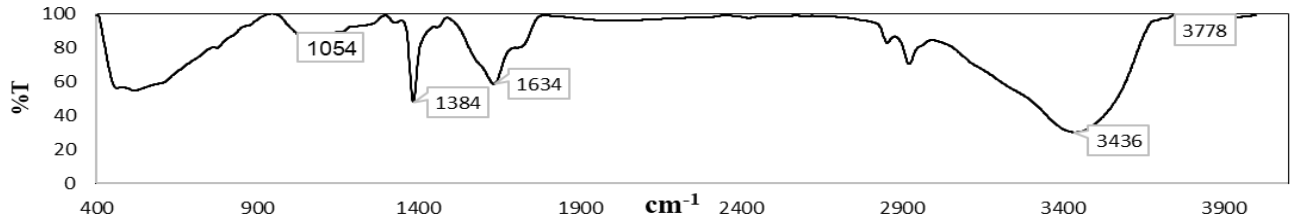


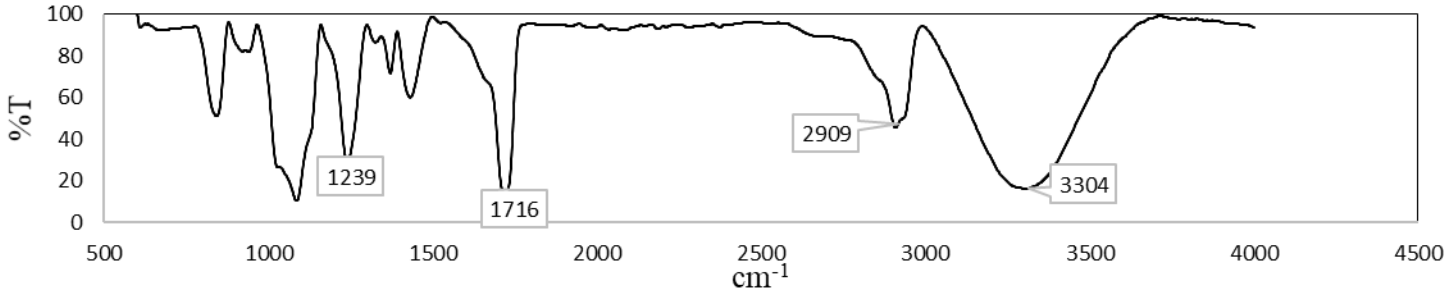
(a)

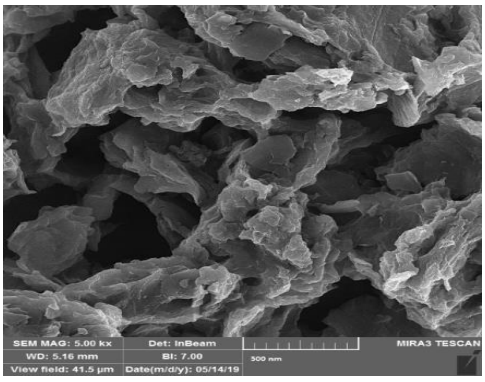
(b)

(c)

Figure 1- FTIR diagram of (a) GO (b) GO/Arg (c) Wound dressing

Figure 2- (a) SEM





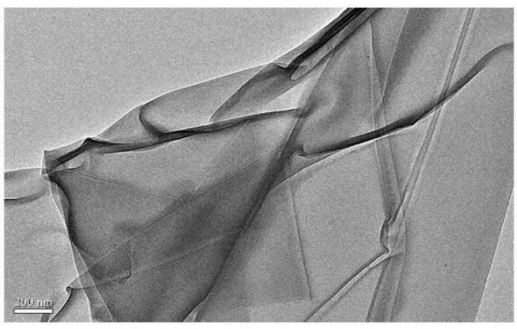


Image of GO (b) TEM
Image of GO

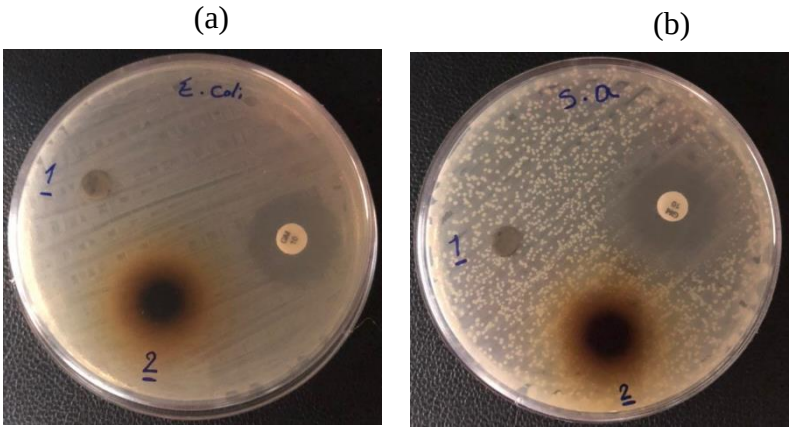


Figure 3- Disk diffusion (a) *E.coli* (b) *S.aureus*

Figure 4- Viability of composite at different concentration