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Research article

Antioxidant Activity and Lethal 50 Doses of *Polyhydroxybutyrate* Nanoparticles (PHB-NPs)

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ABSTRACT

Preparing *Polyhydroxybutyrate* nanoparticles (PHB-NPs) in a safe, economical, and effective manner is one of the biggest challenges facing nanotechnology workers. The current study aims to find an effective and safe way to prepare PHB-NPs. Moreover, to find the antioxidant effect and lethal dose fifty (LD50) of prepared PHB-NPs. The PHB-NPs were prepared by exposing an emulsion of PHB (dissolve 0.5 mg of PHB in 25 ml of deionized distilled water) to ultrasound (4500 kh) for 25 seconds at pH 4, then the pH was elevated to 10 for 18 and after that reduced to 7.1. Anti-oxidant activity was measured by using using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging assay. The results were compared with ascorbic acid. Lethal dose 50 was measured to prepare PHB-NPs by using the linear assay. The results proved that the method used in the current study is an effective method in producing the PHB-NPs after testing it under the scanning electronic microscope (SAM), which showed that the obtained particles were nanoparticles (20-22 nm). The results showed the scavenger activity of PHB and PHB-NPs as compared with the scavenger activity of ascorbic acid. Moreover, the scavenger activity of PHB-NPs was higher than the Scavenger activity of PHB (200 and 100 µg/ml). The present study showed that 1500 mg/kg (PHB-NP/animal weight) killed 50 % of laboratory animals, and this percentage is very high, which confirms that PHB-NPs are a safe material. It can be concluded that the affectivity of the studied method in preparing PHB-NPs and these particles have antioxidant activity and are almost safe *in vivo*.

Keywords: Antioxidant, LD50, Nanoparticles, Polyhydroxybutyrate

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1. INTRODUCTION

Polyhydroxybutyrate nanoparticles (PHB-NPs) are nanoparticles prepared in different ways [1]. PHB is a type of polyester that can be produced by various microorganisms, offering an eco-friendly alternative to conventional plastics. These nanoparticles are typically used in the field of biotechnology and medicine. They have gained attention for their ability to encapsulate and deliver drugs or other therapeutic agents to specific target areas in the body, improving drug efficiency and reducing side effects [2]. PHB-NPs are advantageous because they are non-toxic, biodegradable and have a high drug-loading capacity [2]. They hold promise in applications such as cancer therapy, drug delivery, and tissue engineering, offering a sustainable solution to various biomedical challenges while minimizing environmental impact [3]. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them with antioxidants. Prolonged oxidative stress is implicated in various chronic diseases, including cancer, neurodegenerative disorders, and cardiovascular diseases. Antioxidants play a vital role in mitigating oxidative stress by scavenging ROS [4]. Different studies revealed that PHB possesses intrinsic antioxidant properties. These effectively scavenge ROS, reducing the oxidative burden on cells and tissues. PHB nanoparticles' ability to act as antioxidants is attributed to their chemical structure and

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unique physical properties, which enable them to interact with and neutralize ROS efficiently. The antioxidant properties of PHB nanoparticles hold immense potential for biomedical applications [5]. They can be incorporated into drug delivery systems, wound healing materials, and tissue engineering scaffolds to enhance therapeutic outcomes and minimize oxidative stress-related complications.

LD50 is a critical parameter used to assess the toxicity of a substance. It represents the dose at which 50% of the exposed population is expected to die. Evaluating the LD50 of PHB nanoparticles is crucial to establish safe exposure levels and ensure their responsible use in various applications. Assessing the LD50 of nanoparticles presents unique challenges due to their small size and potential for complex interactions within the biological system [6]. Rigorous testing protocols and comprehensive toxicity studies are required to accurately determine the LD50 of PHB nanoparticles. Preliminary findings indicate that PHB nanoparticles have a relatively low toxicity profile [7], but further investigations are needed to establish precise LD50 values.

The present study focused on the anti-oxidant effect of PHB nanoparticles by comparing their scavenger activity with the activity of ascorbic acid Moreover, the dose of PHB nanoparticles that killed 50 % of mice in the lab will be conducted in the current study to evaluate the toxicity of PHB nanoparticles to open the door for medical and in vivo application of this material.

2. MATERIALS AND METHODS

2.1. Synthesis of PHB Nanoparticles

Five hundred microliter of Polyhydroxybutyrate (PHB) (Sigma-Aldrich, USA) was added to 25 ml of deionized double distilled water (pH 4 by HCl, 1N). The mixture was exposed to 4500 kh for 25 seconds of ultra-sonication (SONOREX SUPER RK 156 BH). Then, the pH was adjusted to 10 by NaOH (1N). After mixing for 120 min at 21 ºC, the mixture was stored at 21 ºC for 18 h. After the period of incubation, the pH was readjusted to 7.1 by HCl (1 N). The scanned electron microscopy (ZEISS Ultra Plus SEM, Germany) was used to check the production of PHB nanoparticles [1].

2.2. Antioxidant Activity

Antioxidant activities of PHB and PHB nanoparticles were detected by using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging assay according to the procedure described by Kumar et al., (2008), as the following: five hundred of two-fold serial dilution from PHB, PHB nanoparticles and ascorbic acid (12.5, 25, 50 and 100µg/mL) were prepared in the test tubes reaction. Simultaneously, 3ml of methanol-DMSO mixture and 0.3ml of DPPH solution were added to each concentration. The tubes were incubated at 37ºC for 1 h. The radical scavenging activity of stable DPPH radicals was determined spectrophotometrically using a microtiter spectrophotometer. The colorimetric changed (from deep-violet to light-yellow) when DPPH reduction was measured at 517 nm. Ascorbic acid was used as a reference. The mixture of methanol-DMSO (3.3 mL) and DPPH (0.5 mL) serves as blank. The control solution was prepared by methanol-DMSO mixture (3.5 mL) and DPPH radical solution (0.3 mL). The scavenging activity percentage (AA%) was determined according to Mensor et al., (2001). $AA\% = 100 - \{\frac{(\text{Abs sample} - \text{Abs blank}) \times 100}{\text{Abs control}}\}$[8,9].

2.3. Half lethal dose 50 (LD_{50})

It is one of the common methods for measuring the toxicity of substances used for human or animal consumption. The principle of this method is based on the dose that causes the killing of 50% of the animals used in the experiment. The standard method of Abal et al. (2017) with little modification. All mice used in the experiment were weighed. Different amounts of PHB nanoparticles (1000, 500, 250, 125, 50 mg of PHB/kg of animal weight). The mice were grouped into five groups. Group A, Five mice were administrated orally with 1000 mg of PHB nanoparticles/kg of mouse. Group B, Five mice were administrated orally with 500 mg of PHB nanoparticles/kg of mouse. Group C, Five mice were administrated orally with 250 mg of PHB nanoparticles/kg of mouse. In Group D, Five mice were administrated orally with 125 mg of PHB nanoparticles/kg of mouse. In Group E, Five mice were administrated orally with 50 mg of PHB nanoparticles/kg of mouse. The mice were kept for 24 h at room temperature in clean polypropylene cages and fed on a standard antibiotic-free diet. The mice should be kept under watch within 24 h and within this time, the number of mice that died was recorded. The Percentage of death was calculated. The LD50 was calculated according to the graphic method of Abal et al. (2017) [10]. The x-axis represents the dose of PHB nanoparticles and Y axis represents the percentage of death. The dose that resulted in 50% of deaths represented the LD50. A similar method of calculation of LD50 was followed by administrating PHB nanoparticles intraperitoneally of mice to check which method is highly effective to measure LD50.

2.4. Statistical analysis

The statistical analysis and graphs were done by using Origin 8 software. The data was expressed as means ± SE. The differences were evaluated by using a student t-test and one-way ANOVA. Correlation coefficient values were also calculated. A value of $P<0.05$ was considered to be statistically significant.

3. RESULT

3.1. Preparation of PHB nanoparticles

The PHB nanoparticles prepared in the present study were analyzed by Scanning electron microscope (SEM) to determine the diameter and shape of the prepared nanoparticles. Fig. 1 showed that the prepared PBH were typically nanoparticles with irregular spherical shapes. The particles were very small in size. The results showed clearly that the range of diameter of PHB nanoparticles was from 22 nm to 20 nm.

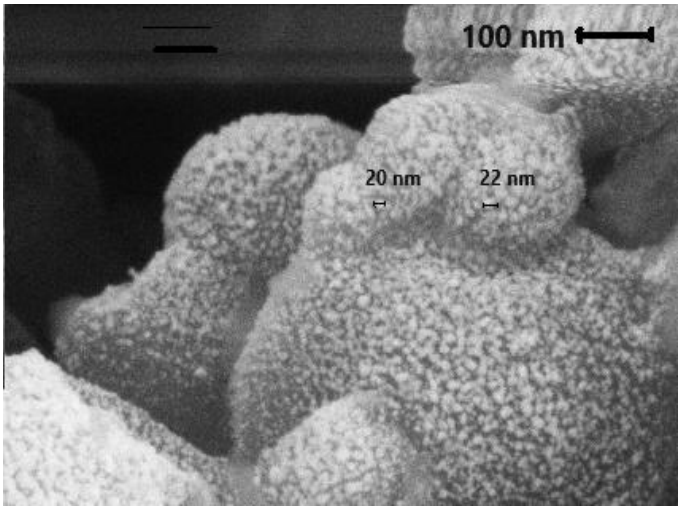

Fig. 1 PHB nanoparticles graph taken by Scanning electron microscope (SEM). The diameters of particles were ranged from 22-20 nm.

3.2. Antioxidant Activity

In current study, the antioxidant characteristic of PHB was evaluated in comparison with a substance previously known for its antioxidant capability, which is ascorbic acid (vitamin C). Fig 2 shows a comparison of different concentrations of ascorbic acid (vitamin C) with the same concentrations of PHB in terms of anti-oxidation. The results showed that there were no significant differences between vitamin C and PHB in terms of antioxidants in concentrations of 50 µg/ml, 25 µg/ml, and 12.5 µg/ml (P>0.05). Whereas, the results were significantly assessed using the student's T-test. The results showed that vitamin C has a greater capability as an antioxidant than PHB at concentrations of 200 µg/ml and 100 µg/ml (P<0.01 and P<0.05 respectively). This indicates the anti-oxidation property of the PHB especially in the last three concentrations.

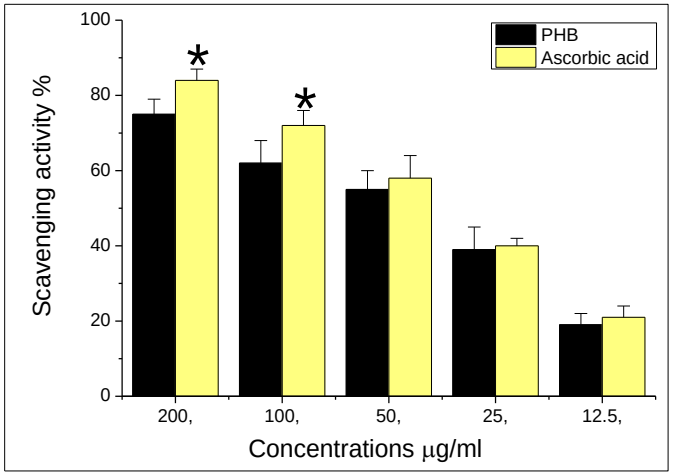

Fig 2. Percentage of antioxidant ability (Scavenging activity) of different concentrations of ascorbic acid (vitamin c) and PHB. *, P<0.05.

Fig. 3 shows a comparison of different concentrations of ascorbic acid (vitamin c) with the same concentrations of PHB nanoparticles in terms of anti-oxidation. The results showed that vitamin c has a greater antioxidant (Scavenging activity) than PHB nanoparticles at concentrations of 200 µg/ml (P<0.05), 100 µg/ml (P<0.05), and 25 µg/ml (P<0.01).

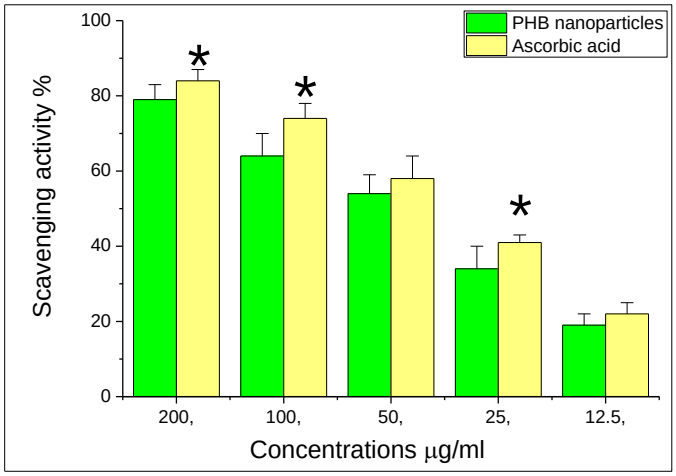

Fig. 3 Antioxidant ability (Scavenging activity) in percentage of different concentrations of ascorbic acid (vitamin c) and PHB nanoparticles. *, P<0.05.

Whereas, the results showed that there was no significant difference between vitamin C and PHB nanoparticles in terms of antioxidants in concentrations of 50 µg/ml and 12.5 µg/ml (P>0.05). This indicates the anti-oxidation property of the PHB nanoparticles in the concentration 50 µg/ml and 12.5 µg/ml. From the present results, it can be concluded that the PHB and PHB nanoparticles have antioxidant features.

In order to determine which form of PHB is more antioxidant PHB or PHB nanoparticles, a comparison was made between different concentrations of PHB and same concentrations of PHB nanoparticles in terms of antioxidant ability (scavenging activity). The results showed that PHB nanoparticles more antioxidant compared to the PHB at concentrations of 200 µg/ml and 100 µg/ml (P<0.05), while the results showed that the PHB were more antioxidant than PHB nanoparticles only at one concentration of 25 µg/ml. The results did not show any significant differences between the two substances (PHB and PHB nanoparticles) in two concentrations (50µg/ml and 12.5 µg/ml). Thus, it can be concluded that the PHB nanoparticles have a slightly more antioxidant ability than the PHB. This leads to the conclusion that the conversion of PHB to nanoparticles slightly increases its ability to absorb free radicals (anti-oxidation) and this gives more support to the safety of PHB nanoparticles to use *in vivo* Fig. 4.

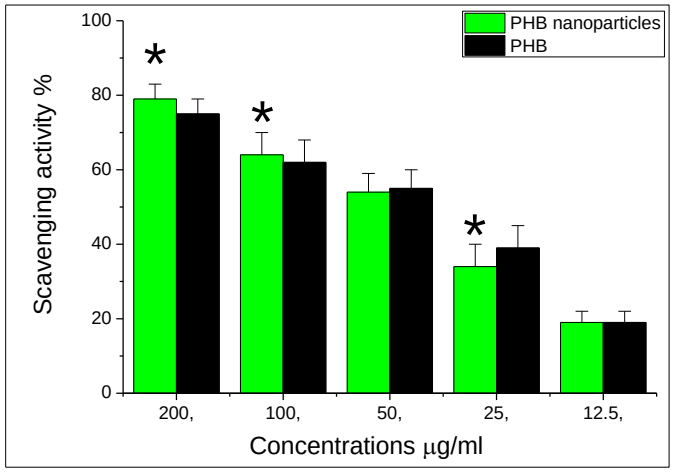

Fig 4. Comparison between antioxidant ability (scavenging activity) of PHB and PHB nanoparticles, *, P<0.05.

3.3. Half lethal dose 50 (LD_{50})

In the current study, the toxicity of PHB nanoparticles was assessed by investigating the lethal dose of fifty percent of laboratory mice (LD50). A nonlinear regression fitting procedure was used to determine the LD50 dose of PHB nanoparticles. Several methods have been used to administer PHB nanoparticles, as it was injected intraperitoneally, but no response was obtained even in high doses, as is the case with subcutaneous administration of PHB nanoparticles. However, when administered orally, a death rate was obtained. The highest mortality rate (75 %) in the dose 2000 mg/kg (weight of PHB/ weight of experimental mouse used in the experiment), but the low doses (125 mg/kg, 250 mg/kg, and 500 mg/kg) did not give any mortality in experimental mice. Fig 5 shows the relationship between mortality rate and PHB nanoparticle doses that are orally administered. Fig 5 shows that the dose of 1500 killed 50% of the mice used in the experiment, so this dose represents the lethal dose of fifty percent of the mice. This dose also can be called the median lethal dose or semi-lethal dose.

4. DISCUSSION

The preparation of Polyhydroxybutyrate (PHB) nanoparticles involves several steps to create these biodegradable and

biocompatible particles for various applications, including drug delivery and tissue engineering [1]. The manufacturing the safety and economic PHB nanoparticles will help in improving human life in terms of reflecting positively on public health and also in the safety industry. In the present study exposing the PHB to ultrasonic waves under the different levels of pH produces the PHB nanoparticles. To prove the production of PHB nanoparticles the production was examined under scanning electron microscopy technology (SAM). The results proved that the particles that produced were nanoparticles as the diameters of the particles were ranged from 20 to 22 nm. The use of SAM is one of the most important and accurate methods used to determine the sizes and shapes of nanoparticles obtained through practical experiments [11,12].

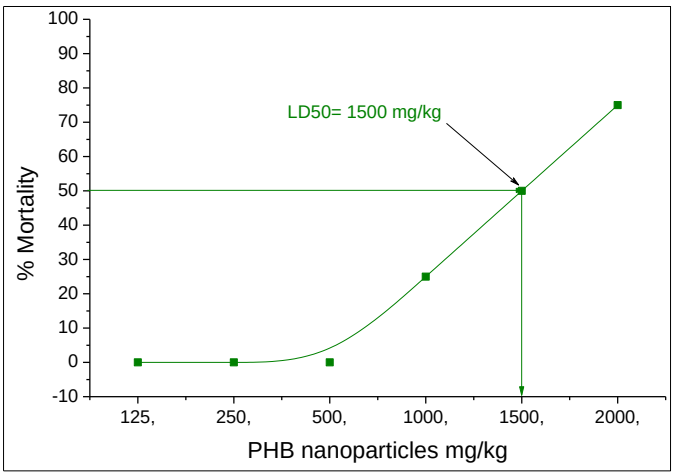

Fig 5. Nonlinear regression fitting procedure to determine LD50 that is showing dose response mortality curve of oral PHB nanoparticles in mice. Percentage lethality values were plotted against dose of the PHB nanoparticles.

In the present study, the antioxidant of PHB nanoparticles was measured and it was found that the PHB nanoparticles have antioxidant features more than PHB. The anti-oxidant features of PHB nanoparticles were measured in comparison with ascorbic acid (vitamin C) which acts as an antioxidant to protect cellular components from free radical damage. Ascorbic acid has been shown to scavenge free radicals directly in the aqueous phases of cells and the circulatory system. Ascorbic acid has also been proven to protect the membrane and other hydrophobic compartments from such damage by regenerating the antioxidant form of vitamin C [13]. The results of the present study proved evidence of the antioxidant property of PHB nanoparticles that raise the idea that PHB nanoparticles may have a positive role in human health in terms of scavenging the free radicals that play a certain role in several diseases, especially cancer, and others [14,15].

Studies on the anti-oxidative role of PHB or PHB nanoparticles are very few, and for this reason, we focused on it in the present study. It was found that Kang et al., (2012) evaluated the antioxidant activity of PHB via several ways in vitro, such as radical scavenging activities using electron spin resonance spectrometry, intracellular reactive oxygen species scavenging activity, and DNA damage assays. PHB evidenced profound scavenging activities on DPPH, alkyl, hydroxyl, and superoxide radicals. They found that the antioxidant activity of PHB was higher than that of ascorbic acid. Furthermore, PHB effectively inhibited H2O2-induced DNA damage. Their results indicate that PHB may prove useful as a novel natural marine antioxidant [16]. In the present study, we found that some concentrations of PHB were similar to the antioxidant activity of ascorbic acid. Müller-Santos et al., (2020) emphasized the role of the PHB in antioxidant processes, and this is consistent with the findings of the present study. Did not find any previous study focused on the antioxidant activity of PHB nanoparticles which is why, this study is considered a pioneer study in this field [17].

The safety of the PHB nanoparticles was checked by using the LD50 method. In the current study, it was found that the concentration that killed 50% of experimental mice was 1500 mg/kg and this amount is very high, which proves the safety of the PHB nanoparticle. LD50 is a statistically derived amount of a substance that can be expected to cause death in 50% of the animals when given by a specified route as a single dose and the animals observed for a specified time period. Although conducting routine acute toxicity testing in rodents has been criticized, it can serve useful functions and also have practical implications [18]. There is no information in the scientific published literature that calculates a mean LD50 and standard deviation for PHB nanoparticles orally to mice (or in any route of administration), using studies performed under good laboratory practice (GLP) or equivalent. As a measure of toxicity, LD50 is somewhat unreliable and results may vary greatly between testing facilities due to factors such as the genetic characteristics of the sample population, animal species tested, environmental factors, and mode of administration [19]. That is why, other tests highly required to judge the safety of PHB nanoparticles, the present study, it was used another method (micronucleus assay or using cell line or effect of this material on the liver and kidney of experimental animals) to find out the toxicity of PHB nanoparticles

4. CONCLUSION

Exposing PHB to ultrasonic waves under pH gradient represents a safe, economical, and effective method to produce PHB nanoparticles.

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Conflict of interest

The authors declare that they have no conflict of interests.

Ethical Approval

This review was approved by the Ethical Committee of the University of Baghdad, Baghdad, Iraq (No 974, 2021).

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