Evaluation of antimicrobial properties of derivative peptide of Naja naja snake's venom

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Abstract

Although most of the venoms and their derivative compounds have shown antimicrobial properties, most of them have not been studied to find such activities. In the world of pharmacology, along with invention and administration of new antibiotics, bacteria achieve new properties which allows them to become resistant to antibiotics; this proposes the issue of "antibiotic resistance", which motivates researchers to further study different natural resources in order to invent novel and effective antibiotics. Animal venoms have been in the center of attention because of their different observed effects, such as antibacterial effects. Venom is a very complex compound, consisting of different types of peptides and non-peptide materials with various activities. Few studies have been done to analyze antibacterial properties and purification of snake venoms. In this work, antibacterial effect of the derivative peptide of Cobra (Naja naja) snake against 4 bacteria (Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa) was studied and Minimal Inhibitory Concentration (MIC) was determined. At first, we quantified the intended dilution of lyophilized strains of bacteria and then diluted the peptide powder to the intended concentration. At first we added Mueller medium to all wells and then added peptide powder to the first well. We also added the provided concentration of bacteria to all of the wells. We evaluated light absorption using spectrophotometer after 16 hours of incubation at 37°C. Results of this analysis were compared to effects of 2 antibiotics (Ciprofloxacin and gentamycin). Results showed that the peptide derived from Naja snakes' venom has an antibacterial effect on gram-positive and gram-negative bacteria.

Key words: antimicrobial properties, peptide, Naja snake's venom

Introduction

Poisoning by snake venom is an important medical issue around the world and studying it is valuable. Millions of people get snakebite annually, and more than 100 thousand are killed by it. However, although snakebite is fatal, the venom has a natural biologic source that consists of compounds which have possible therapeutic values. Snake venom is a mixture of proteins, poly peptides, nucleotides, and non-organic compounds. Most of these proteins and poly peptides are in the monomer form, but those with complicated compounds show a high level of pharmacological activity [1].

Snake venom is used mainly for immobilizing and killing during hunting and it has a possible significance in defence. The fang is a shape shifted tooth of a snake, which has a groove (like an injection needle). In the viper family, fangs are bigger and are placed on the maxilla; they are moved by muscles, and while they are shorter in the Elapidae family such as cobras, they are constantly placed on maxilla. Hydrophis snakes have shorter fangs with some typical teeth on the maxilla [2, 3].

Semi-venomous snakes of the Colubrid family mostly have grooved fangs, which are placed at the posterior part of the maxilla. According to studies, a group of these peptides have shown good antimicrobial properties. These antimicrobial peptides (AMPs), which are generated by eukaryotes (e.g. mammals, amphibians, insects, and plants), are especially important because of their significant role in innate immunity. AMPs have a far-reaching activities against gram-positive and gram-negative bacteria, fungi, and viruses. They are also effective against multidrug resistant bacteria and have a low tendency to drug resistance [3]. Serious problems caused by multidrug resistant bacteria cause emergence of need for alternative therapeutics. AMPs are promising therapeutics as antimicrobial agents. Therefore, according to existing compounds in snake venoms, especially the cobra snake (Naja naja), studies in this field are essential so that we can use these derived compounds in the pharmacological and therapeutic fields [4-12].

Study Method

1. Growth mediums for identifying bacterium
Different mediums were used in this study, in order to identify bacteria, which have been mentioned below.

MacConkey agar growth medium
MacConkey agar growth medium consists of different compounds. These compounds are:

Table1: Constitutive compounds of agar growth medium.

No.	Compounds	Amount (gram)
1	Bacto-peptone	17
2	Proteose peptone	3
3	Baco lactose	10
4	Sodium chloride	5
5	Bacto-Bile Salt Mixture	1.5
6	Bacto-agar	13.5
7	Bacto-Neutral Red	0.03
8	Bacto-Crystal Violet	0.001

There are different instructions for preparing this medium. The instruction used to prepare this medium includes 50 grams in 1 liter of water. The intended amount of material is diluted in distilled water in an Erlenmeyer and then autoclaved. Mediums are divided into plates after becoming luke warm by heating. These plates then cross a flame so that the agar medium becomes solid. Plates are incubated at 37°C for 24 hours for the purpose of growing bacteria.

2. Triple sugar iron agar (TSI) medium
This medium is solid with light salmon and light brown granules, which becomes orange red by adding water to it.

Table 2: Constitutive compounds of TSI growth medium

No.	Compound	Amount (gram)	No.	Compound	Amount (gram)
1	Peptone from casein	10	7	D glucose	1
2	Peptone from meat	10	8	Ammonium iron citrate	0.5
3	Meat extract	3	9	Sodium thiosulfate	0.5
4	Yeast extract	3	10	Phenol red	0.024
5	Sodium chloride	5	11	Agar-agar	12
6	Sucrose	10	12	Lactose	10

The given instruction for preparing this medium is 56 grams in 1 liter of water. The intended amount of solid material is weighed and poured in an Erlenmeyer, then the required amount of distilled water is added. After that it is placed on a heater to boil. The liquid medium is divided into test tubes and then autoclaved. Tubes are placed obliquely so that a slant medium is obtained.

Using sterile microbiology loop once in order to culture bacteria in this medium, samples are picked up from colorless colonies on MacConkey medium and from colonies with black center on Salmonella-Shigella agar, are cultured deeply and are then taken out and cultured linearly on the slant. Cultured samples are incubated at 37°C for 12-24 hours.

3. Urea medium
Urea medium is prepared in two solid and liquid forms. Its compounds are shown in Table 2.3.

Table 3: Constitutive compounds of Urea culture medium

No.	Compounds	Amount (gram)
1	Yeast extract	0.1
2	Potassium dihydrogen phosphate	9.1
3	Di-sodium hydrogen phosphate	9.5
4	Urea	20
5	Phenol red	0.01

The given instructions from the company is 38.5 grams in 1 liter of distilled water and the medium should not be autoclaved. Small and narrow tubes are used to prepare a liquid medium. The intended number of small test tubes and distilled water are placed in an autoclave to be sterilized. The urea broth powder is dark purple before addition to water, while it becomes violet after being added to the water. The intended amount of the solid medium, which has been pre-weighed, is poured into an Erlenmeyer containing distilled water and then placed on a heater to be diluted. Then, the obtained liquid mediums are divided into tubes near the flame. Tubes are placed in a water containing beaker and then boiled in Bain Marie or on an alcohol burner for a 10 minute period. After cooling, the medium is available for being cultured. Samples are picked up from colorless colonies on the MacConkey agar and colonies with black center on Salmonella agar, using once, and then diluted in the liquid medium. The medium is incubated at 37°C for 12-24 hours. Urea positive samples in the urea broth medium turn the medium's color from light purple or pink into dark purple, while urea negative samples, such as Salmonella do not change the urea medium's color. In the solid urea medium, if the medium's color turns yellow, the urea is positive, and if it turns dark red or dark purple, the urea is negative.

Urease Test

The ability of a microorganism to synthesise from ammonium and CO2 can be found out by inoculating it to liquid and solid urea containing mediums. Microorganisms with the ability to synthesize this enzyme in large amounts can alkalinize the medium in a few hours; color changing will be apparent soon [58].

4. MR-VP (Methyl red-VoGes Proskauer) medium

This medium has bisque granules that turn orange by adding distilled water.

Table 4: Constitutive compounds of MR-VP culture medium

No.	Compound	Amount (gram)
1	Peptone from meat	1.7
2	D(+)glucose	2.5
3	Phosphate buffer	3.5

The given instruction by the company is 17 grams of medium in 1 liter of distilled water. After inoculation of bacteria in the medium and incubation at 37°C for 24 hours, 4-6 drops of alcoholic alpha-naphthol and 2 drops of KOH 40% are added to the tube per each drop of MR-VP medium. The tube is then shaken slightly so that oxygen reaches the culture medium. Existence of a red color on the surface of the medium indicates that the test is positive. This color usually appears 15-20 minutes after addition of barite indicator in the culture medium. Methyl red is added to the medium for MR test; red circle shows that the test is positive. MR-positive bacteria usually have negative VP and conversely, MR-negative bacteria usually have positive VP.

5. SIM medium

Table 5:

No.	Compound	Amount (gram)
1	Peptone from casein	20
2	Peptone from meat	6.6
3	Ammonium iron citrate	0.2
4	Sodium thiosulfate	0.2
5	Agar-agar	3

This medium is used with the purpose of analyzing production of H2S, production of indole, and motility in differentiation of enterobacter. The given instruction is 30 grams of medium in 1 liter of water. Samples are picked up from the culture using a sterile inoculating loop and then are inoculated deeply. If bacterium is immobile, its growth will happen along the inoculation line. H2S production is indicated by the medium's color turning black, where the bacterium has grown. In order to analyze indole production, the medium is coated with a layer of indole Co-Ax reagent. If indole has been produced, the indicator layer would turn violet.

6. SIM culture medium

This semisolid culture medium is used for recognizing three important properties of enterobacter (H2S production, indole production, and motility).

Hydrogen sulfide Production

A sterile inoculating loop is directly applied to the suspected colony; this is done by entering the colony for about 2CM and coming back in the same way. If microorganism can produce H2S, a black sediment will be observable in the medium.

Motility
Since the medium's agar concentration is low, if the bacterium is mobile, it will be able to move from the inoculated zone in all directions. If the medium becomes generally opaque, it indicates microorganism's motility. If microorganism is immobile, we will see an opaque part only in the track [13].

Indole production
Indole is the final product of metabolizing tryptophan amino acid, which is generated by tryptophanase enzyme's effect. After finishing incubation, some drops of Coax reagent, which has para-methyl aminobenzaldehyde in it, is added to the SIM culture media. If a red color exists, it will be formed constantly on the surface of the medium. Coax reagent is colorless; therefore, if test result is negative, it will not change in color [13].

EMB (Eosin methylene Blue Lactose Agar) culture media
The Eosin methylene blue medium is a culture medium which is used to insulate intestinal pathogenic basils, where their colonies are different from those that do not ferment lactose or sucrose. Some of the general types form mucoid colonies. Gram-positive bacteria do not grow on this medium due to existence of reagents; therefore, it's a selective medium. Identifying E.coli on this medium is available with small colonies and metallic polish. This bacterium's colonies are observed with a diameter of 2-3mm with a dark center [13]

Simmons Citrate Agar test
This medium contains cations, buffer, salt, citrate, and bromothymol blue as reagent, and the general color of it is green. The suspected microorganism colony is cultured on the surface, because the reaction needs oxygen. If the microorganism is able to metabolize citrate as a carbon source, the medium's color turns blue due to existence of bromothymol blue, and therefore, the test result is considered as positive. If the medium's color remains green, it means that the citrate has not been metabolized and PH has not changed; therefore, thetest result is negative [13].

Table 6: Biochemistry properties of E. coli

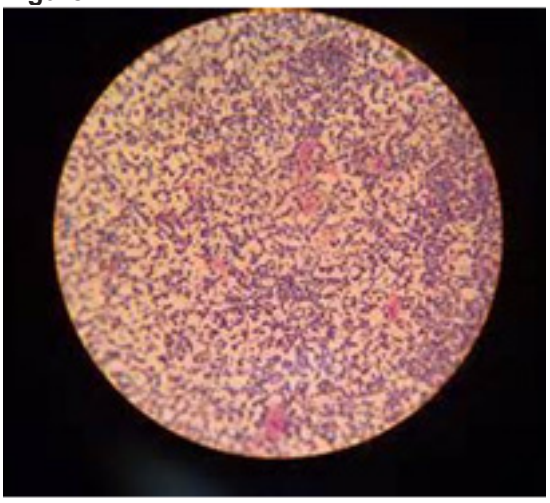
Test	Result
Indole	+
Methyl red	+
VP	-
Simmons Citrate	-
H_2S production in TSI	- (Except for some strains)

Blood agar culture medium
This is an enrichment media, which is used for proliferation and insulation of pathogenic bacteria, especially those that need nutrients for growing. In addition, existence of hemolysin in bacteria can be analyzed too. After autoclaving the basic medium, defibrinated blood of sheep is added to it with a ratio of 5 to 7; once its temperature reaches 50°C; then they are divided into sterile plates with a sterile condition [13].

Results

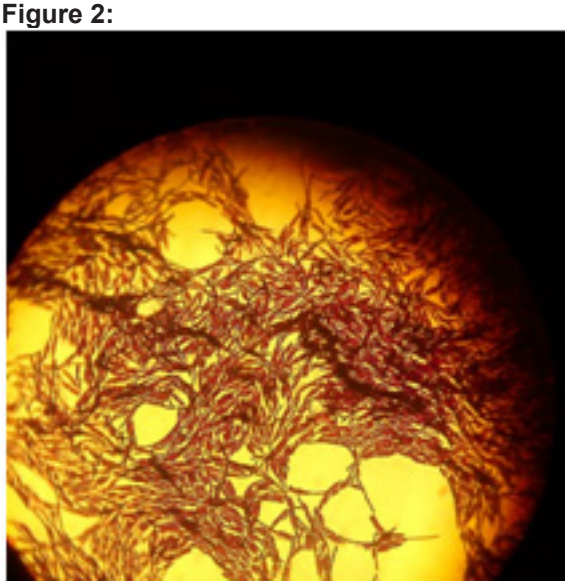
1. Gram staining
Staphylococcus aureus: Staphylococcus aureus was observed as a gram-positive (in purple) clustered cocci in gram staining.

Figure 1



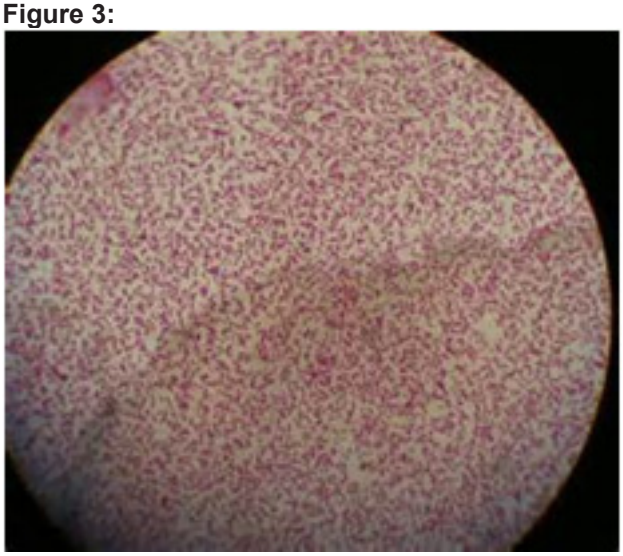
Bacillus subtilis: This is a gram-positive (in purple) basil, with approximate diameter of 1µ and length of 3-4µ.

Figure 2:



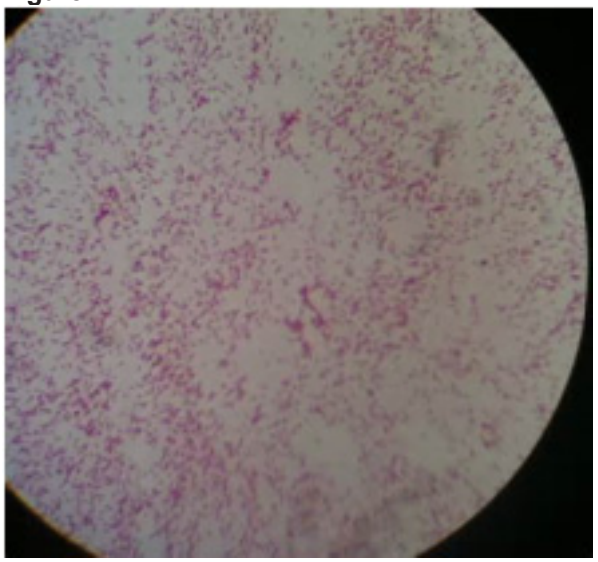
E.coli: These are gram-negative (in red) sporeless bacteria. Some have capsules. Their difference to enterobacteria is their size (which is usually 1.1-1.6 × 2-6µ).

Figure 3:



Pseudomonas aeruginosa: These are gram-negative (in red) bacteria with the size of 0.5-1µ × 3-4µ, which are formed as single, binary and sometimes, small chains.

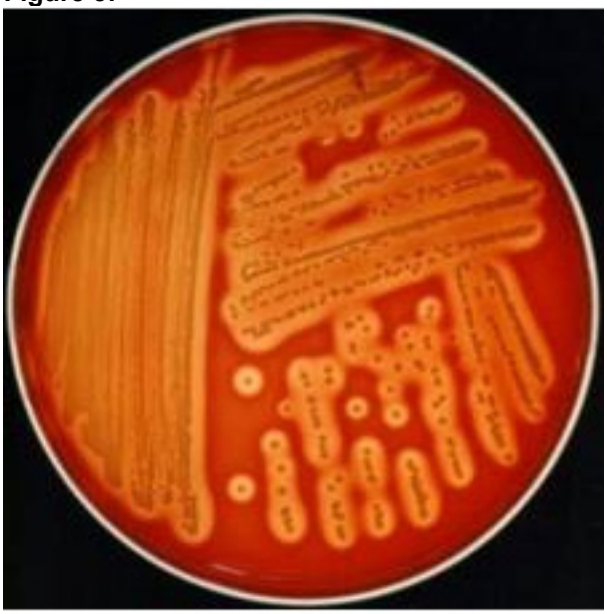
Figure 4:



Dedicated tests for identifying each bacterium was done after gram staining.

2. Analysis of dedicated biochemistry tests' results
Staphylococcus aureus: It generated beta hemolysis on blood agar culture medium. After 24 hours, spherical, convex, smooth, and regular margin colonies with size of 1-4 mm were generated.

Figure 5:



It is capable of fermenting mannitol sugar on agar mannitol salt medium, which turns its red color to yellow. Catalase and coagulase tests were positive for Staphylococcus aureus.

Bacillus subtilis: Colonies have a relatively big size (about 3-4mm) in blood agar culture media. Catalase and gelatin were positive.

E.coli: Results of Escherichia coli culture showed that this bacterium is indole positive, MR positive, VP negative, and citrate positive.

Pseudomonas aeruginosa: Pseudomonas aeruginosa results in generation of beta hemolysis on blood agar medium and generates flat and thin edged colonies with size of 3-5 mm after 24 hours. Its oxidase test was positive and biochemistry tests verified it.

3. Analyzing effect of different concentrations of peptide on aforementioned bacteria
Light absorbance of different samples were read by spectrophotometer machine and the following diagrams show their results.

As Diagram 1 shows, the studied peptide has shown antibacterial properties for Staphylococcus aureus at wavelength of 600nm and concentrations until 150µg.

As Diagram 2 shows, the studied peptide has shown antibacterial properties for Bacillus subtilis at wavelength of 600nm and concentrations ≤ 150µg.

Diagram 1: comparing light absorbance in different concentrations by (µg) in wavelength of 600nm for Staphylococcus aureus.

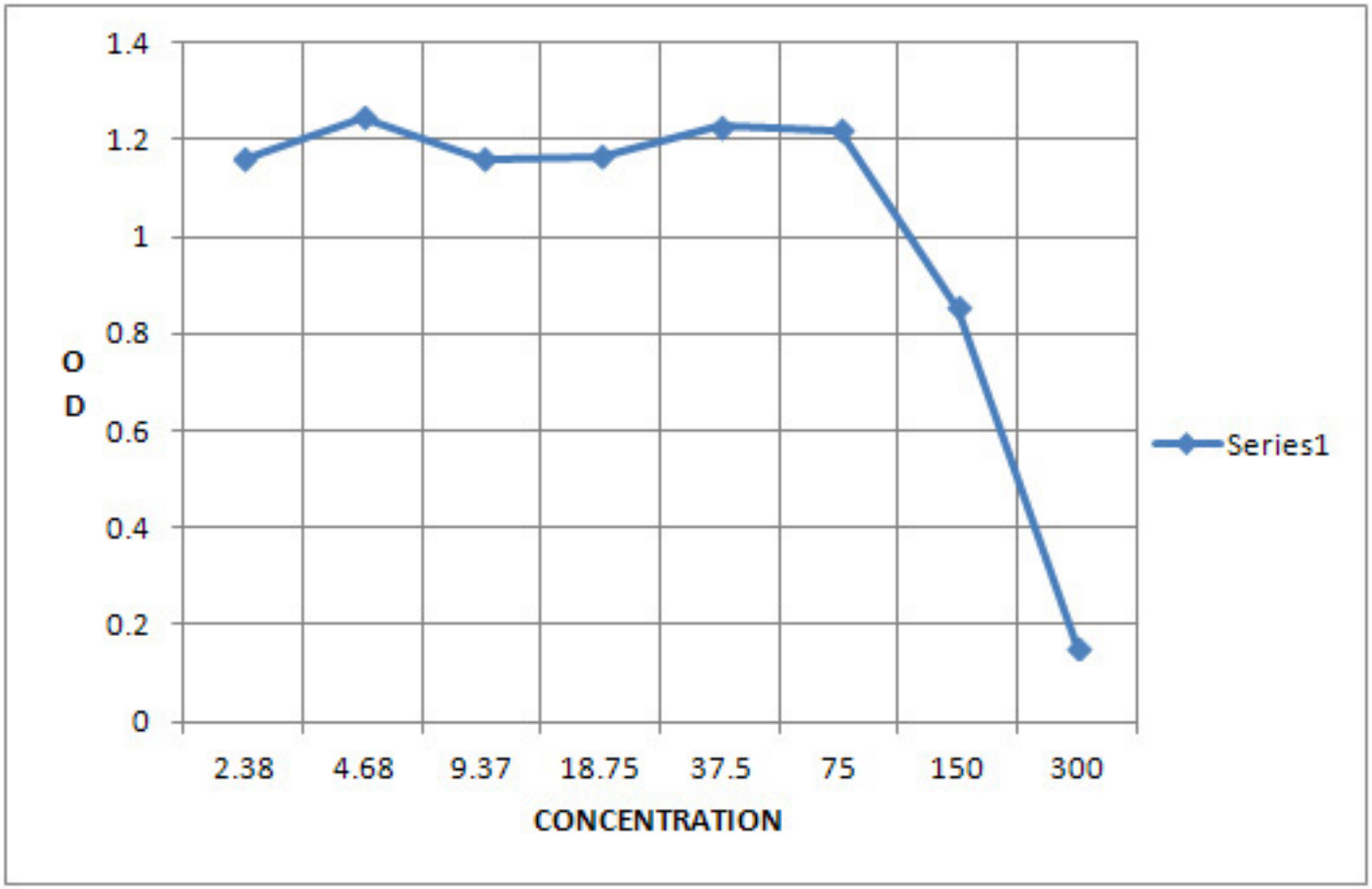
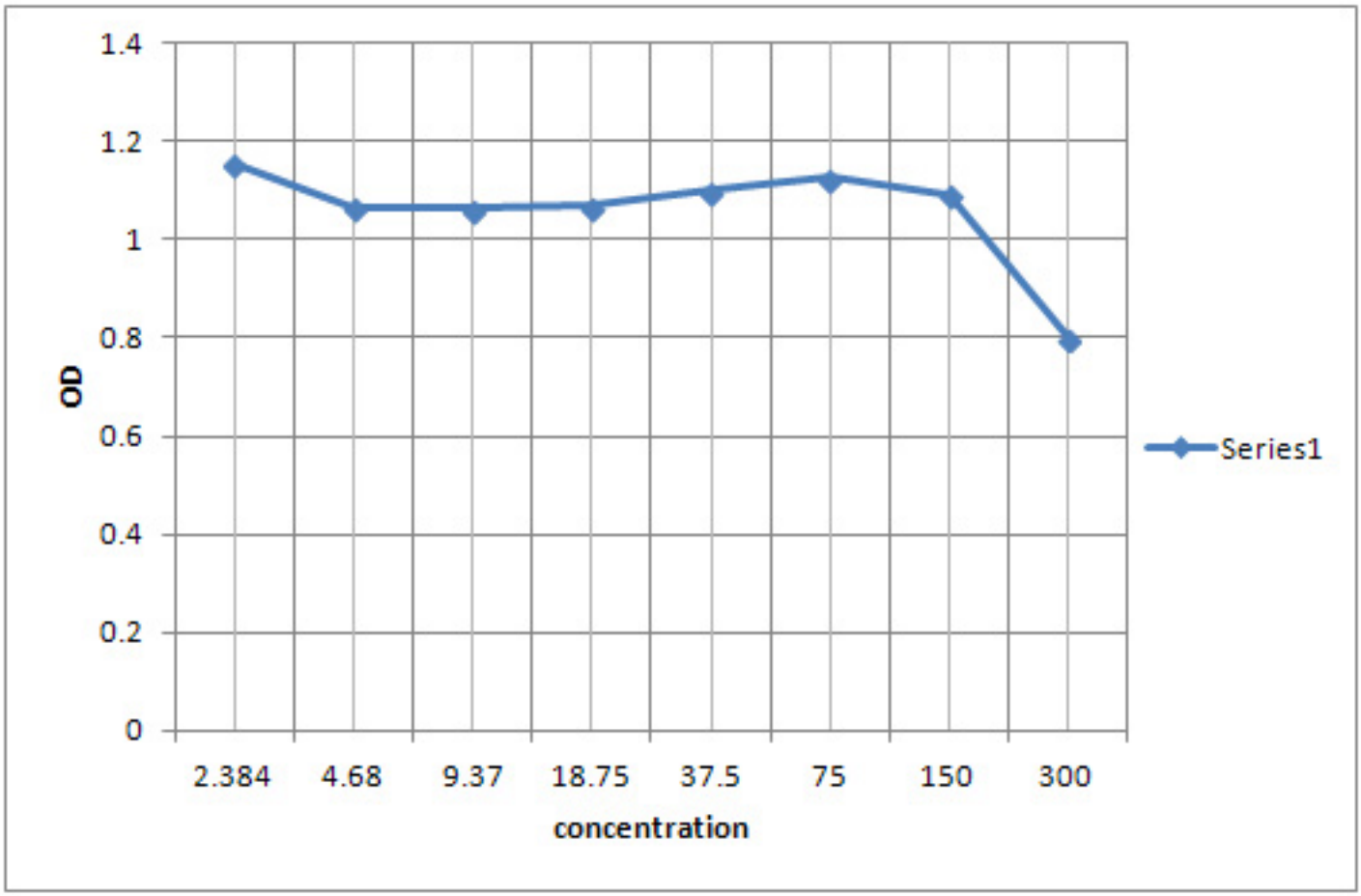
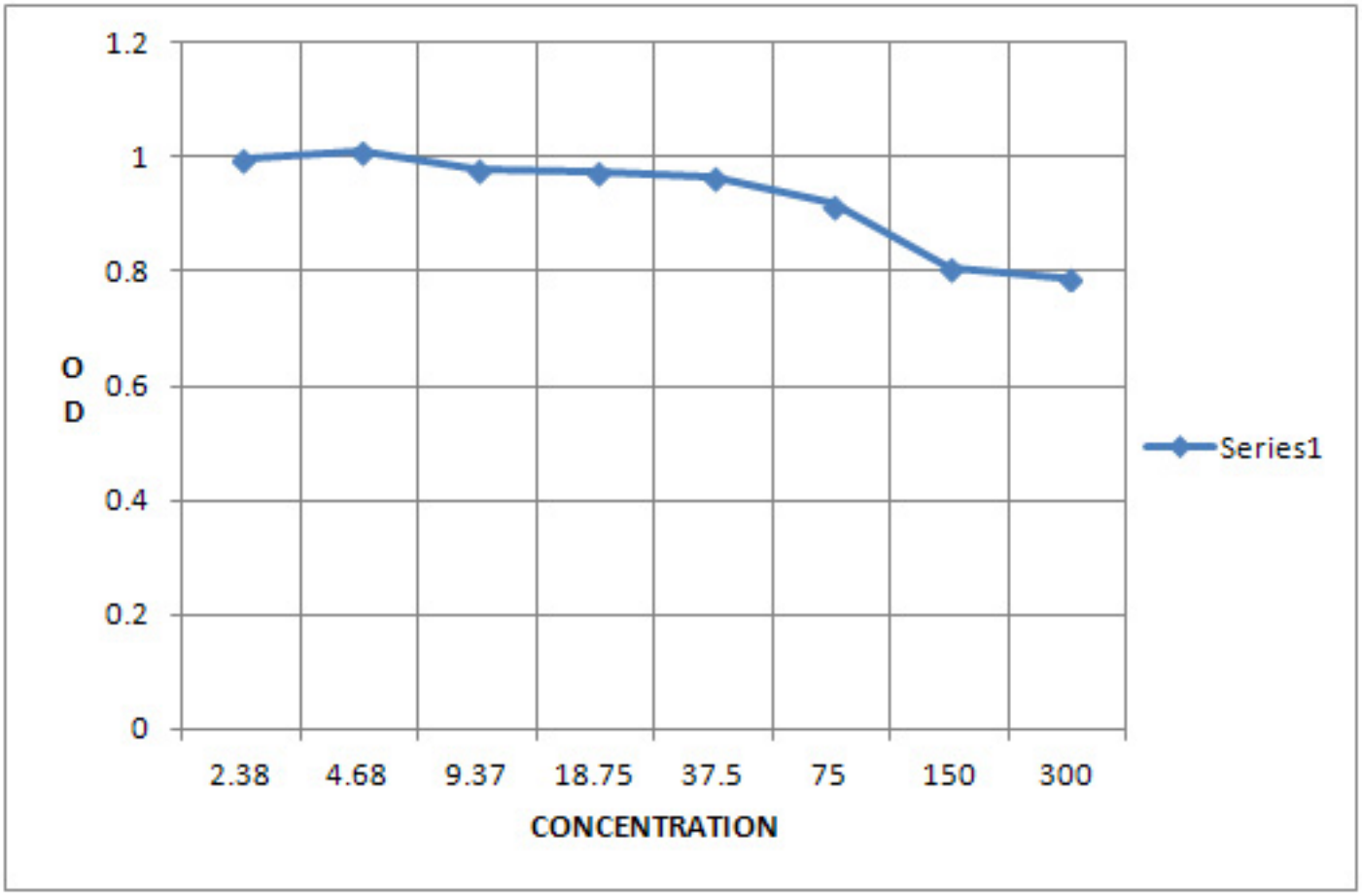


Diagram 2: comparing light absorbance in different concentrations by (µg) in wavelength of 600nm for Bacillus subtilis



As Diagram 3 shows, the studied peptide has shown antibacterial properties for E.coli at wavelength of 600nm and concentrations ≤ 75µg.

Diagram 3: comparing light absorbance in different concentrations by (µg) in wavelength of 600nm for E.coli.



As Diagram 4 shows, the studied peptide has shown antibacterial properties for Pseudomonas aeruginosa at wavelength of 600nm and concentrations ≤ 75µg.

We analyzed results once more at wavelength of 620nm:

As Diagram 5 shows, the studied peptide has shown antibacterial properties for Staphylococcus aureus at wavelength of 620nm and concentrations until 150µg.

Diagram 4: comparing light absorbance in different concentrations by (µg) in wavelength of 600nm for Pseudomonas aeruginosa.

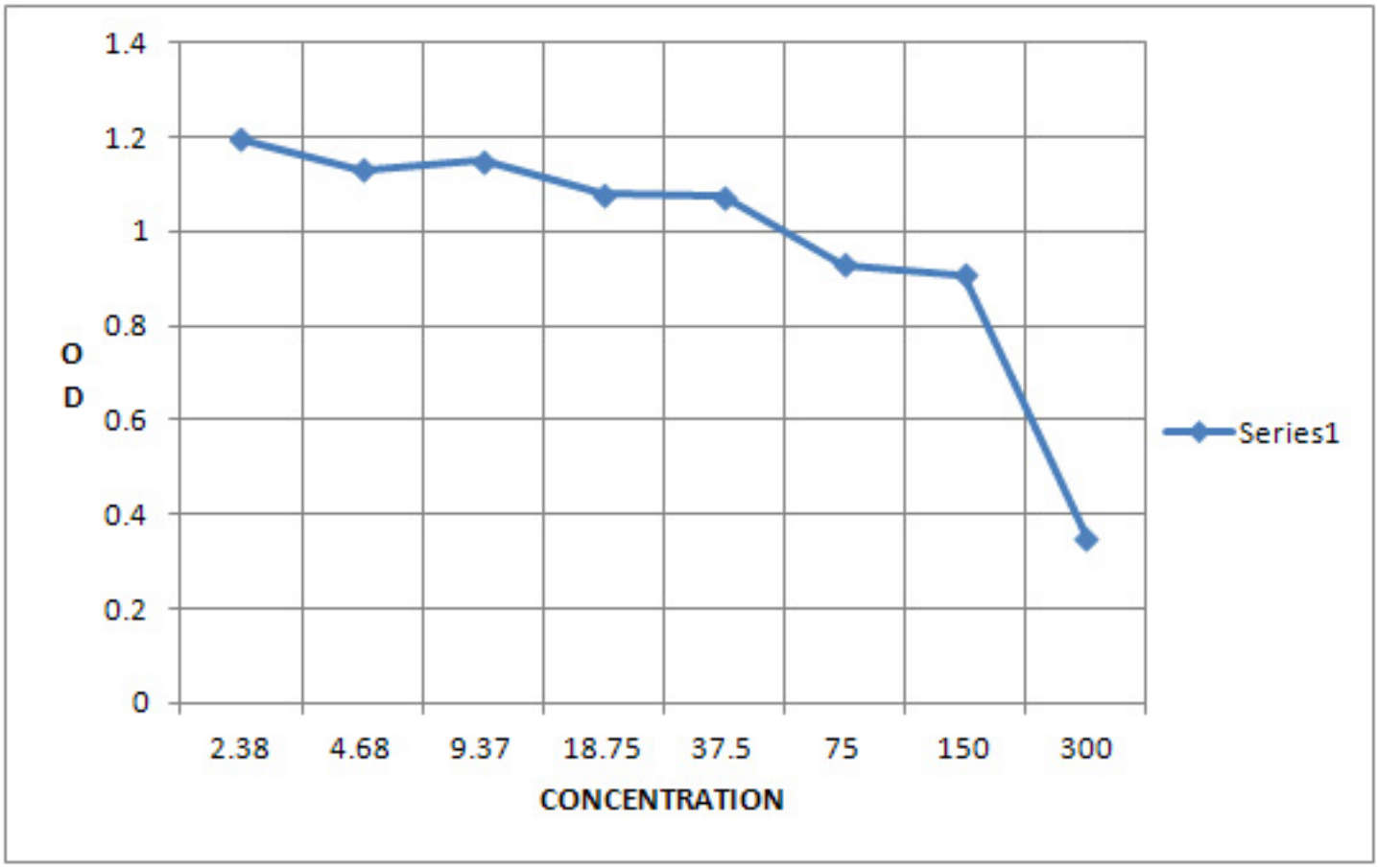
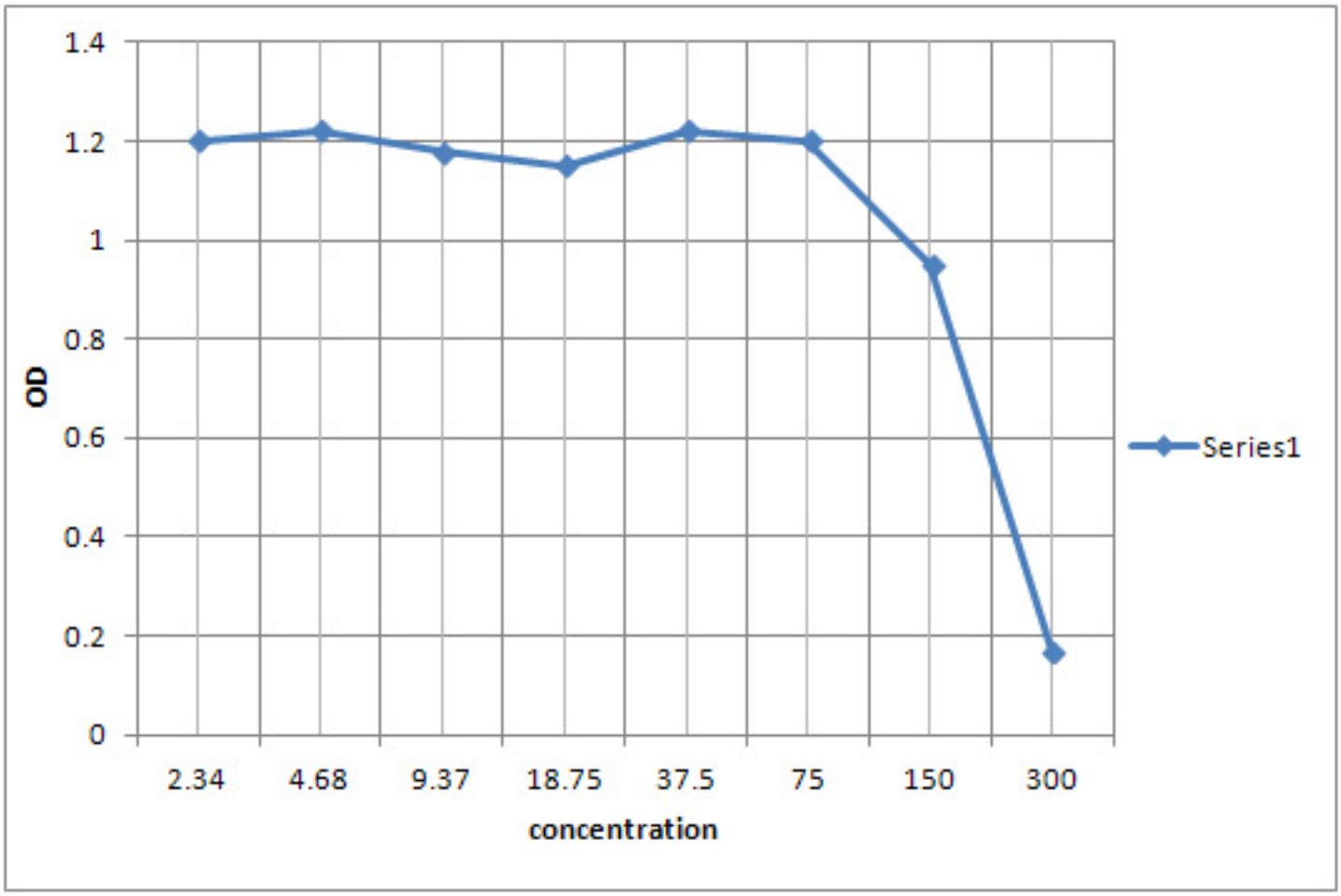
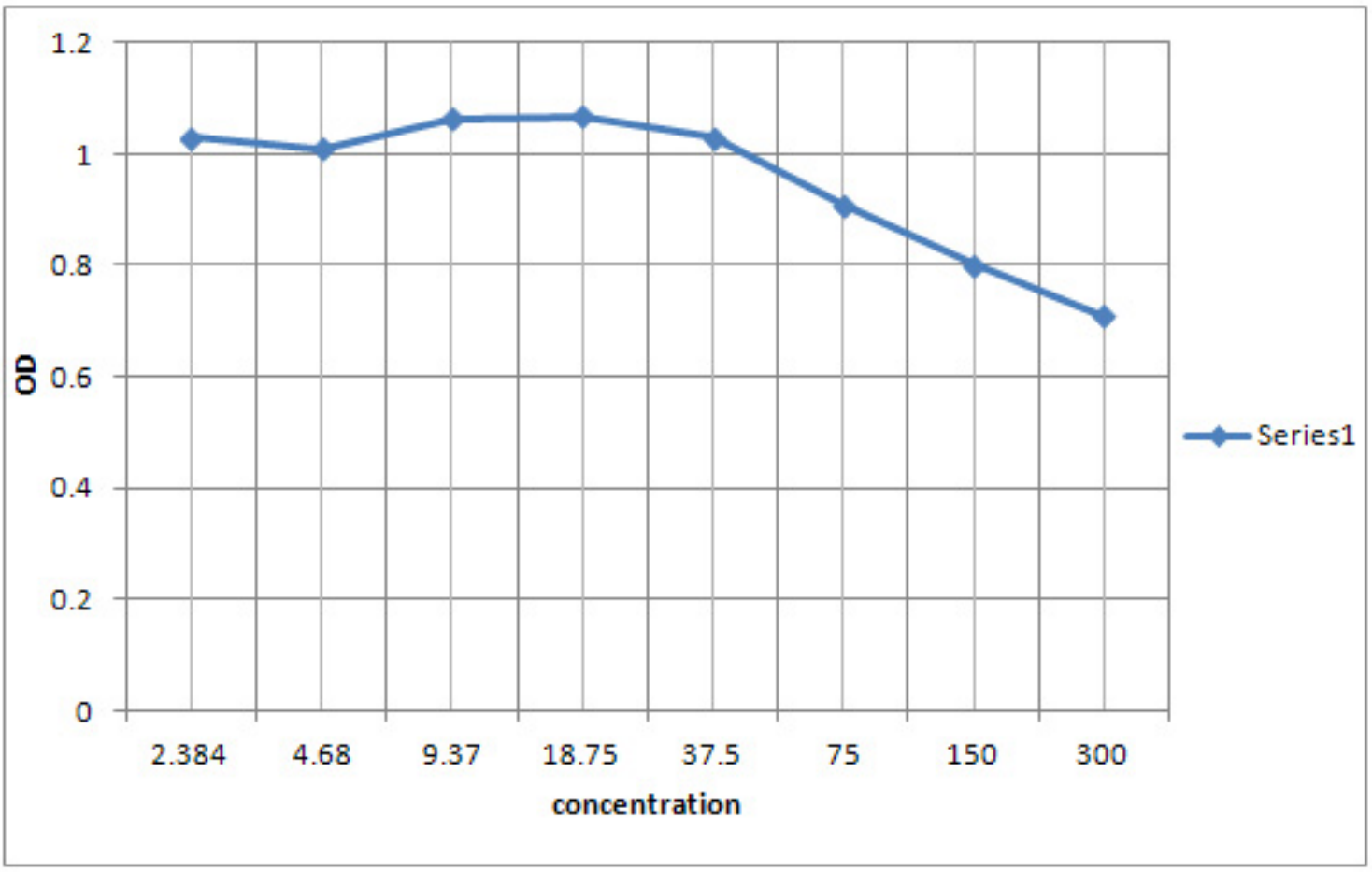


Diagram 5: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for Staphylococcus aureus.



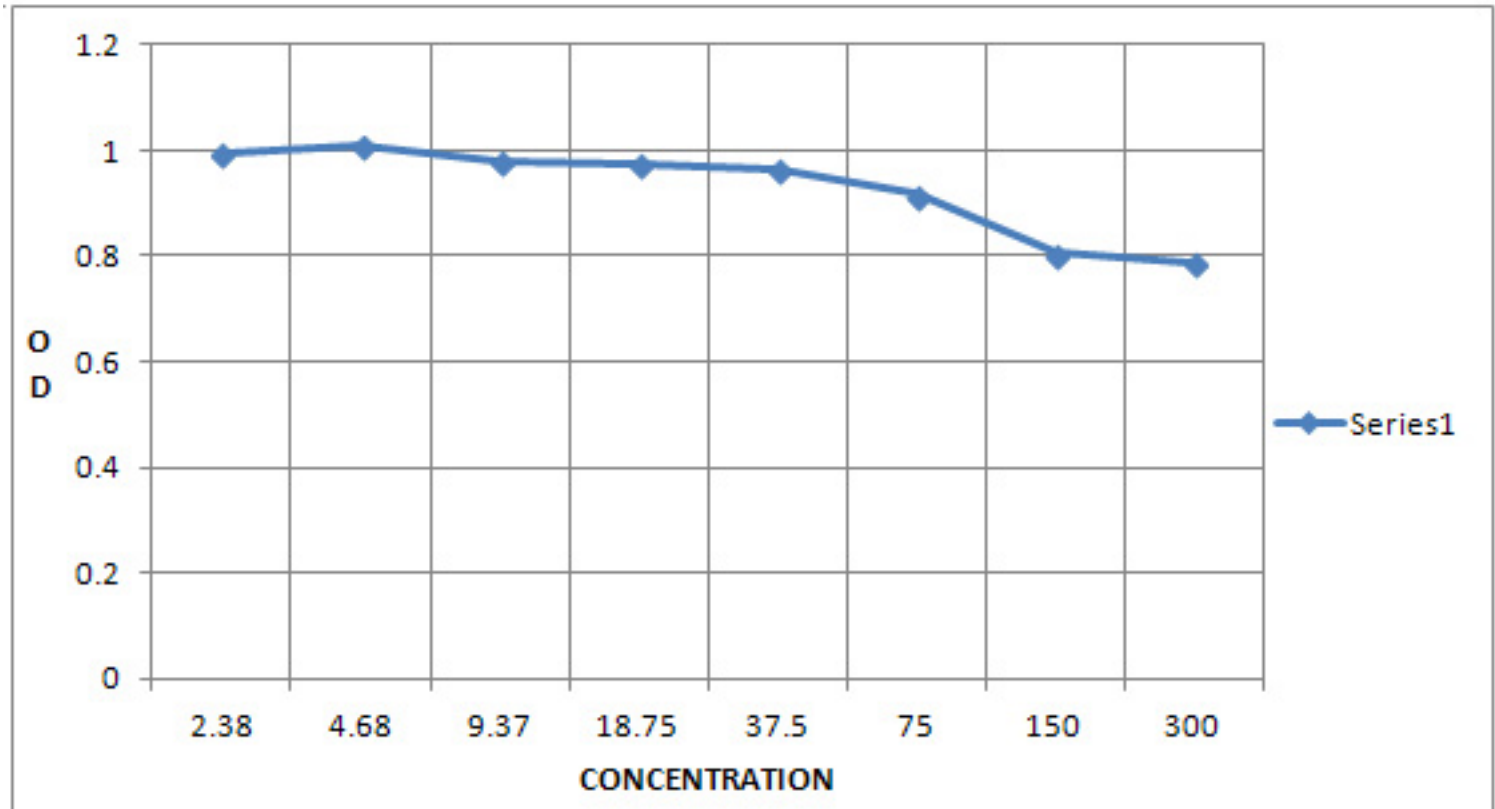
As Diagram 6 shows, the studied peptide has shown antibacterial properties for Bacillus subtilis at wavelength of 620nm and concentrations ≤ 150µg.

Diagram 6 comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for Bacillus subtilis.



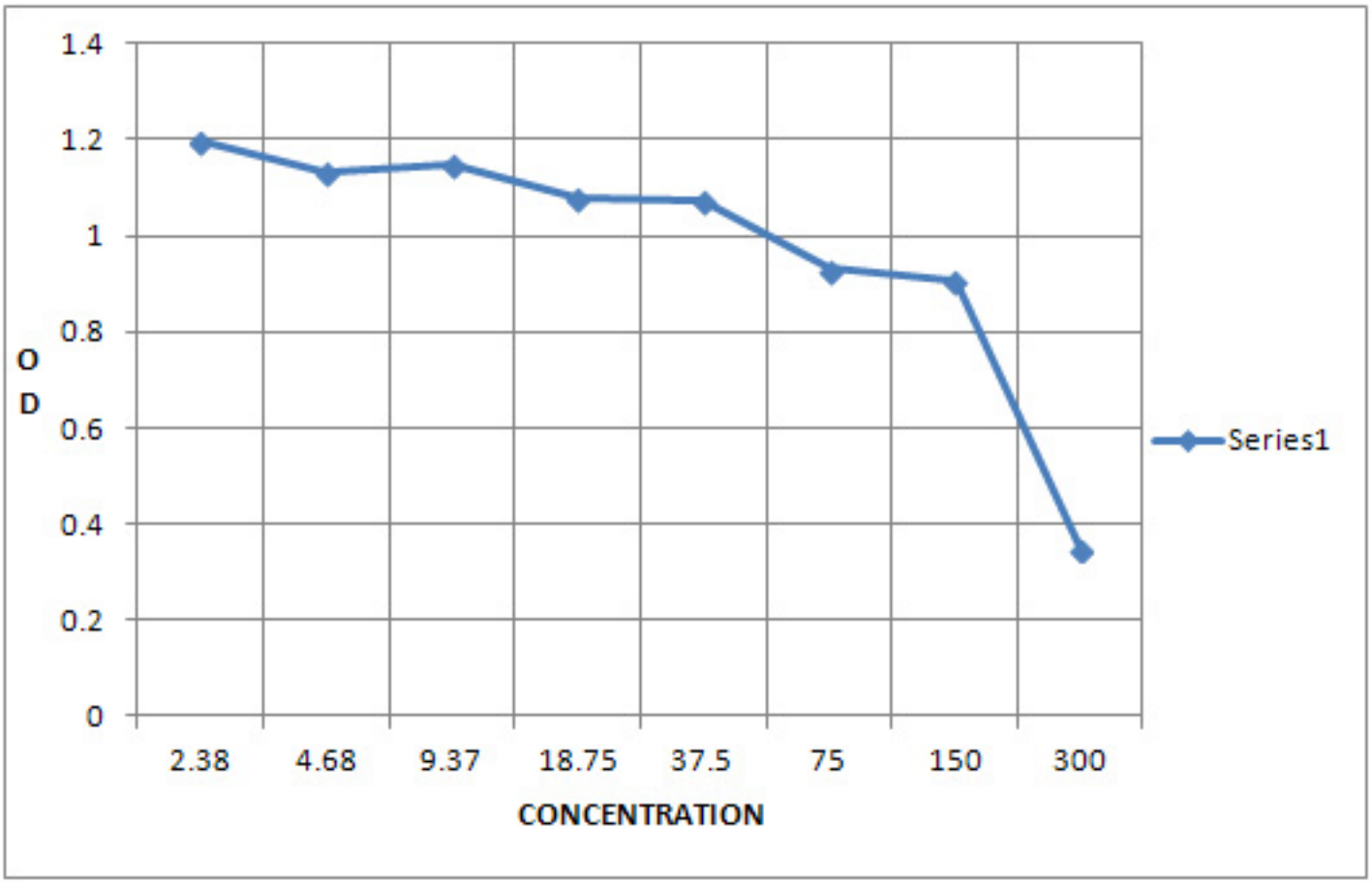
As Diagram 7 shows, the studied peptide has shown antibacterial properties for E. coli at wavelength of 620nm and concentrations ≤ 75µg.

Diagram 7: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for E. coli.



As Diagram 8 shows, the studied peptide has shown antibacterial properties for Pseudomonas aeruginosa at wavelength of 620nm and concentrations ≤ 75µg.

Diagram 8: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for Pseudomonas aeruginosa.



We studied minimal inhibitory concentrations at concentrations above 400, 500, and 600 except for 300. Obtained results are as follows (next page):

Diagram 9: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for Staphylococcus aureus.

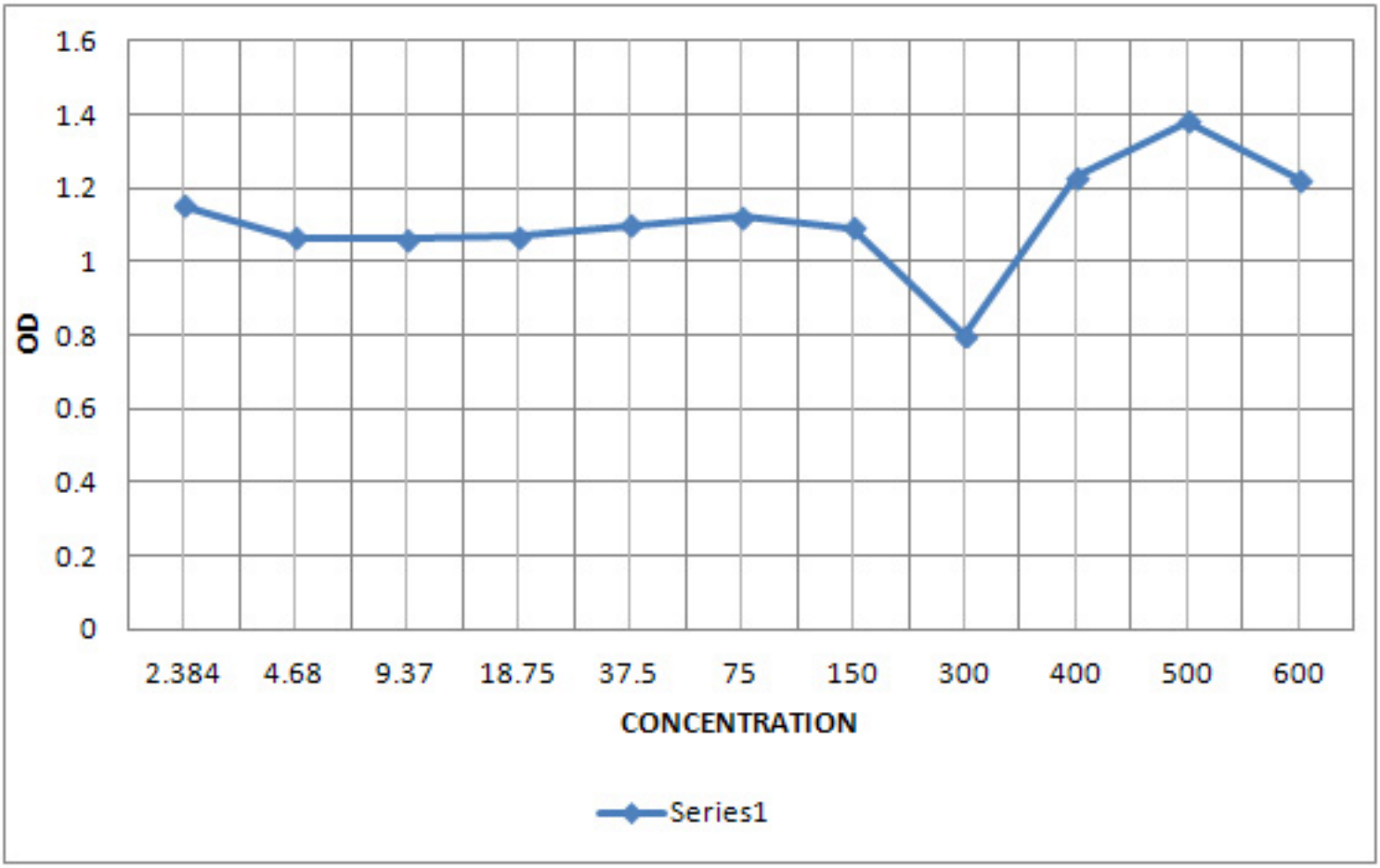


Diagram 10: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for Bacillus subtilis

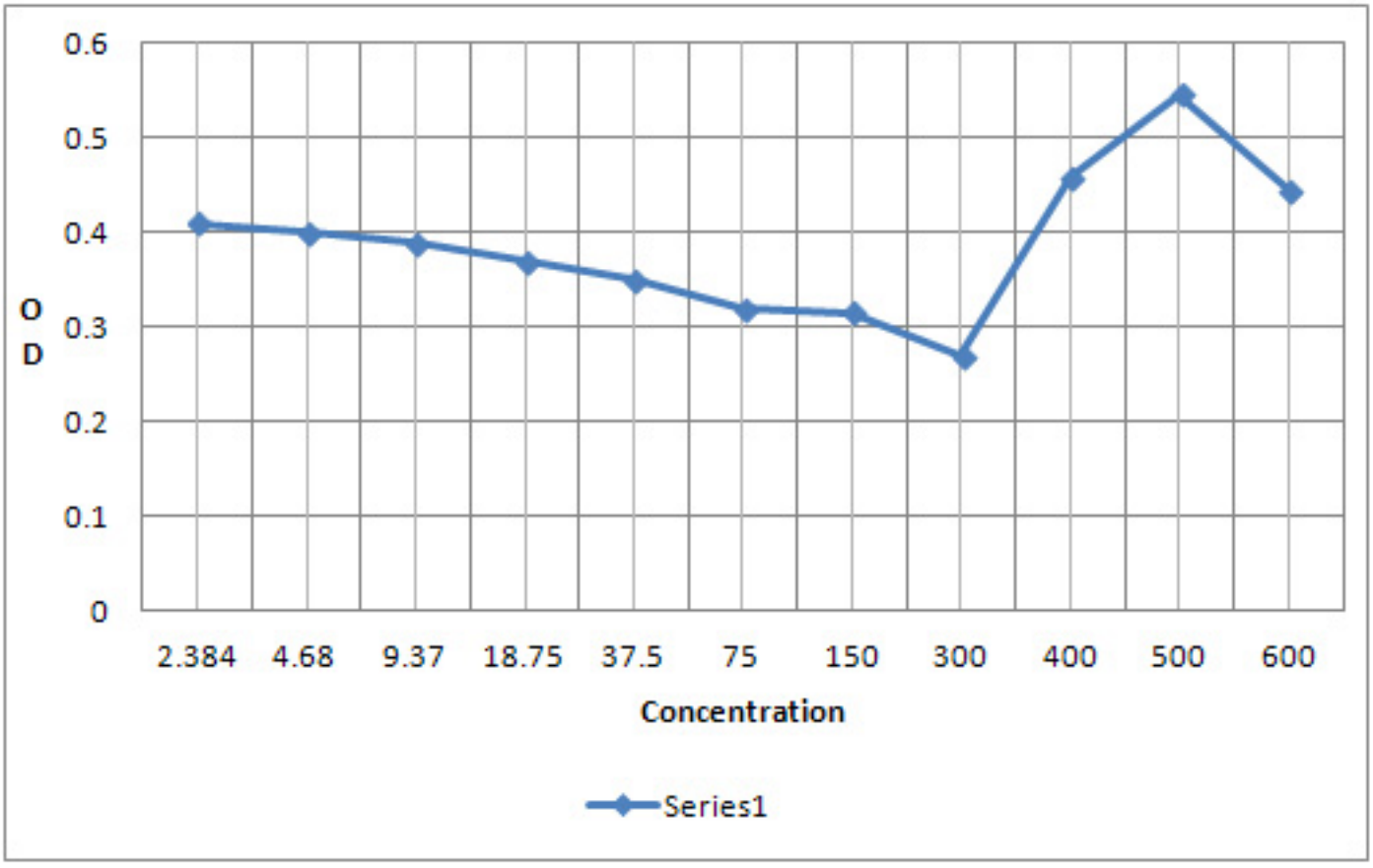


Diagram 11: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for E. coli.

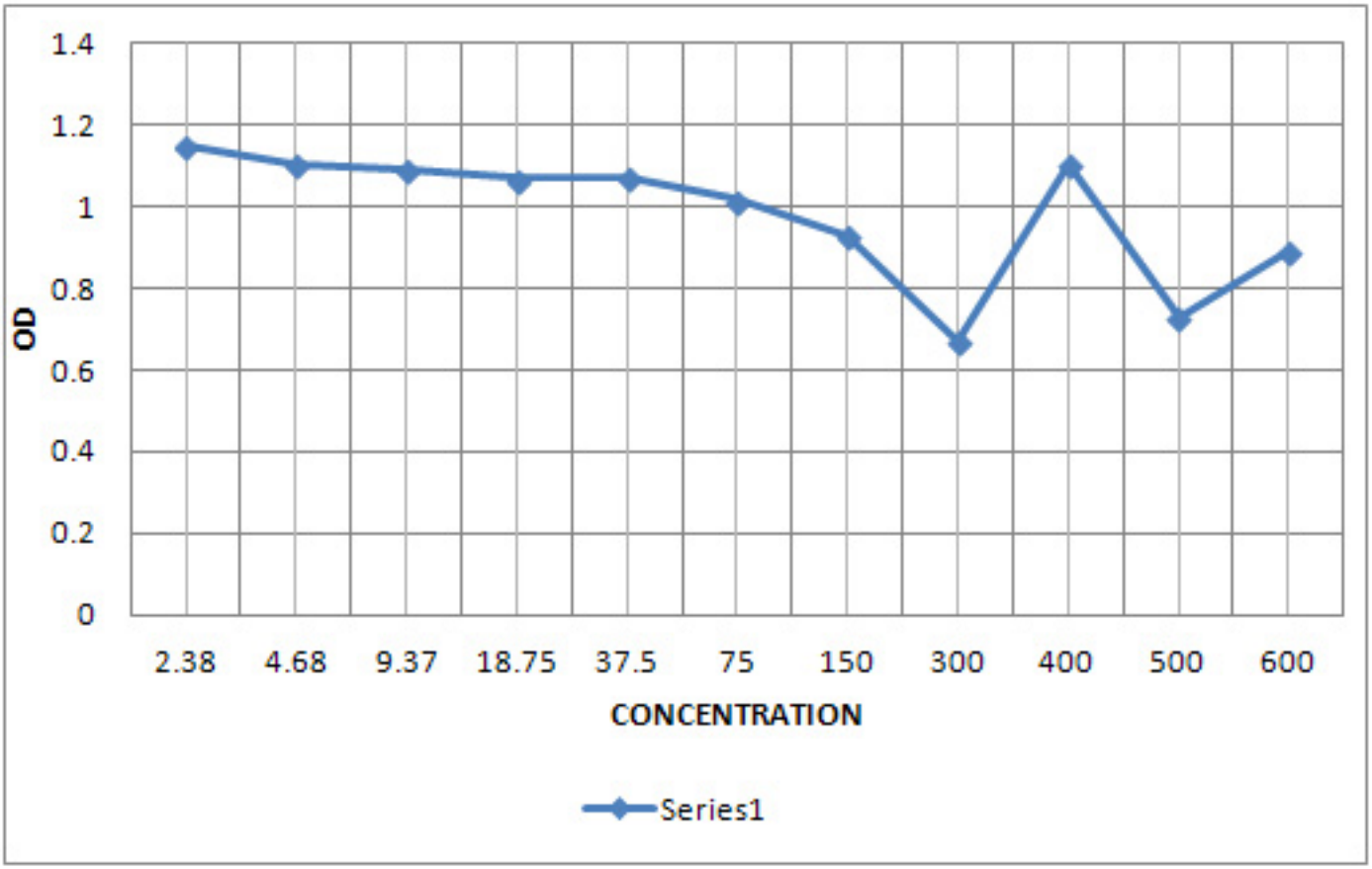
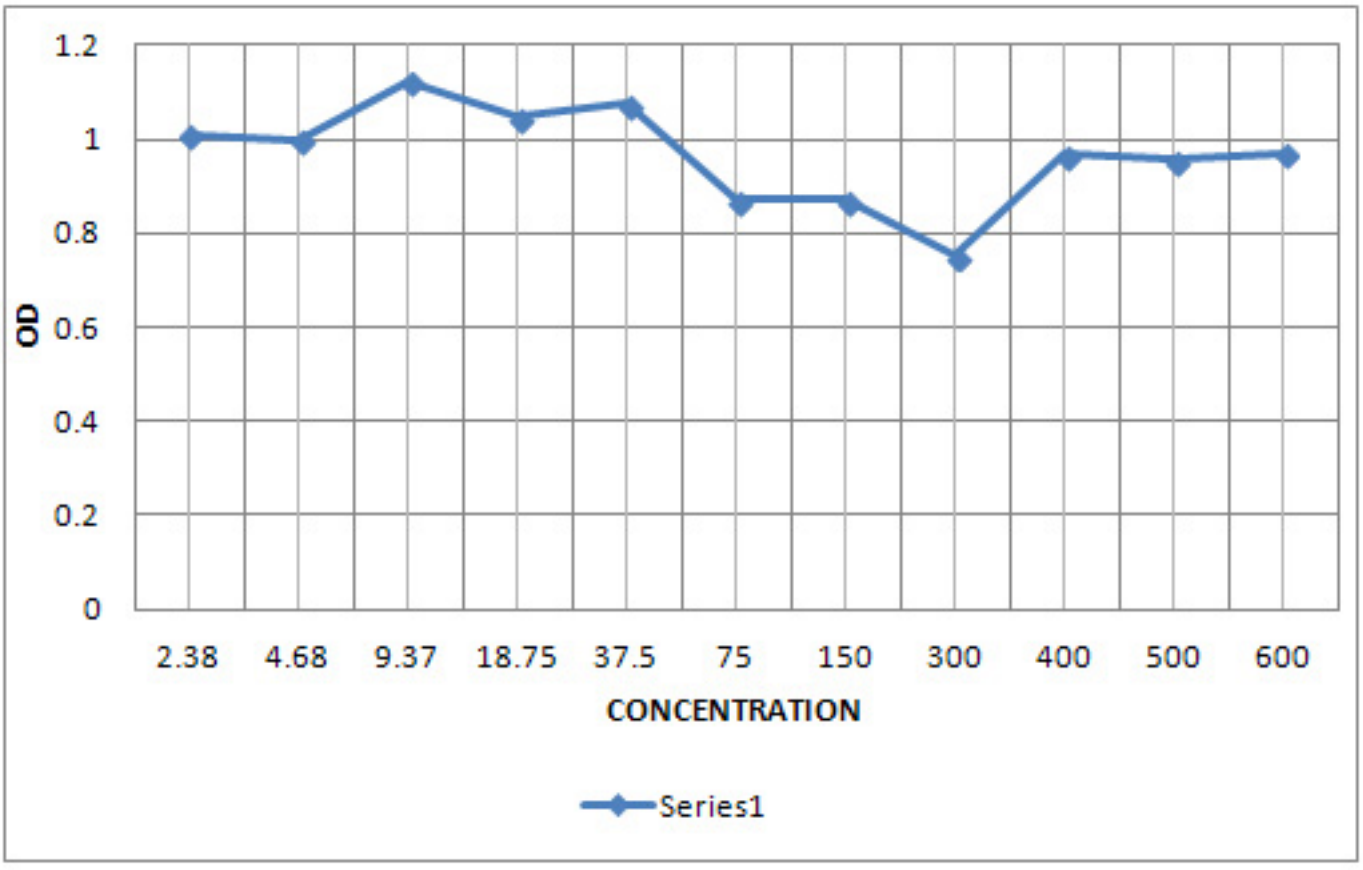


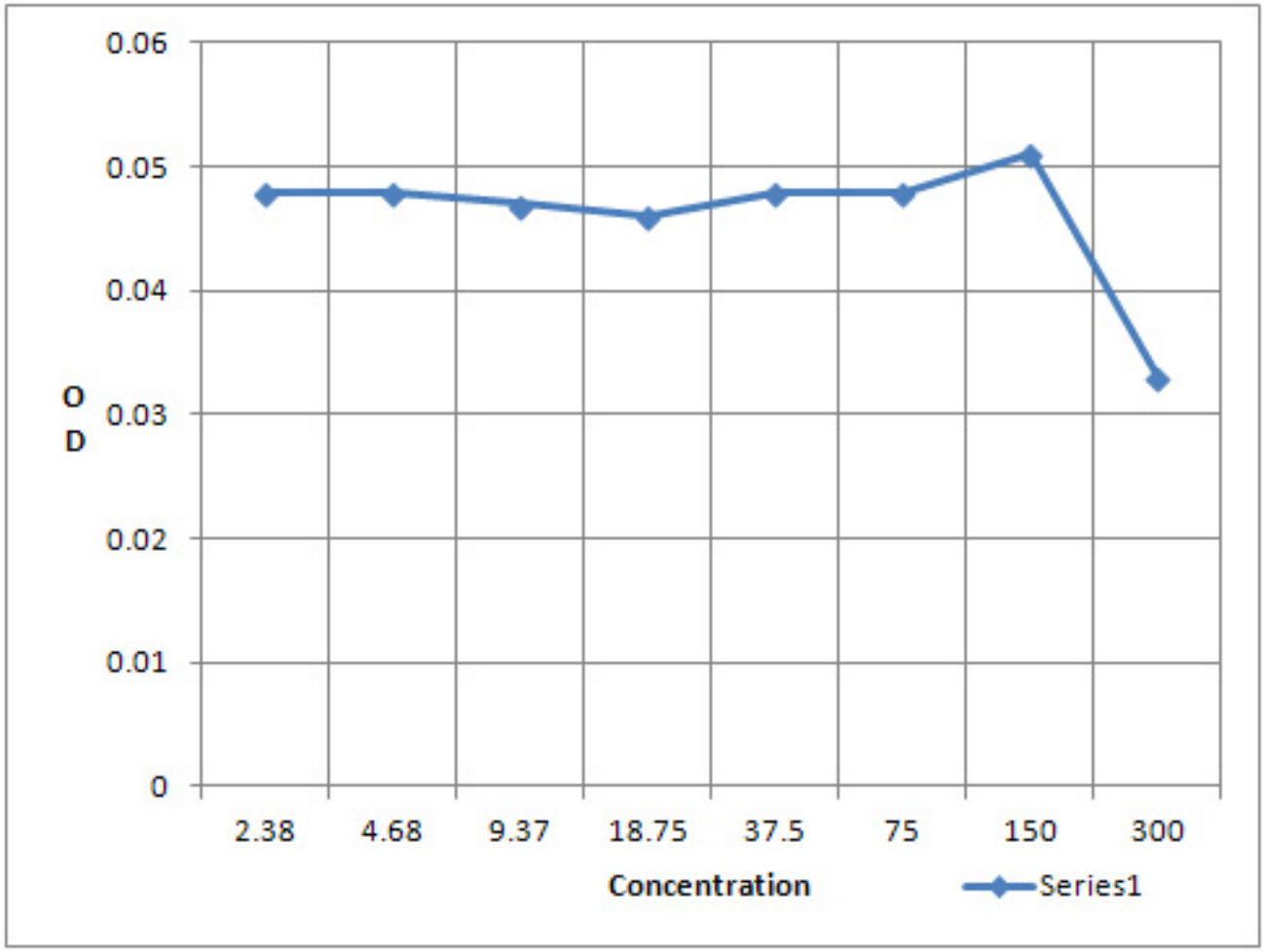
Diagram 12: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for Pseudomonas aeruginosa



We found out that the analyzed peptide does not show antimicrobial properties at concentrations more than 300µg. We compared results obtained from different concentrations of peptide to results obtained from antibiotic's effect, to see how much antibacterial effect the peptide has.

As you see in Diagram 13, antibiotic has shown antimicrobial properties at concentration of 300µg.

Diagram 13: comparing light absorbance in different concentrations by (µg) in wavelength of 600nm for Staphylococcus aureus



As you see in Diagram 14, antibiotic has shown antimicrobial properties at concentration of 300µg.

As you see in Diagram 15, antibiotic has shown antimicrobial properties at concentration of 300µg

Diagram 14 comparing light absorbance in different concentrations by (µg) in wavelength of 600nm for Bacillus subtilis

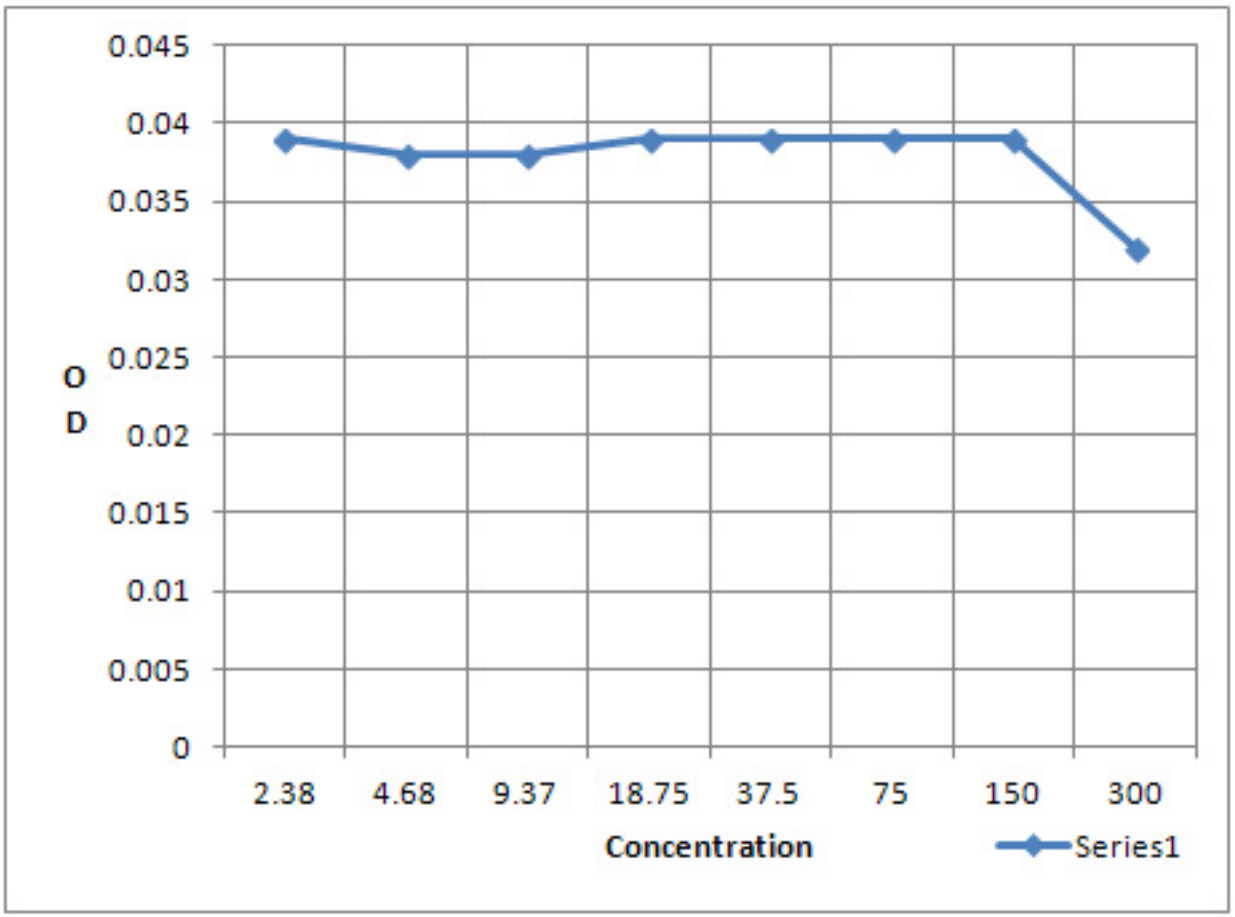
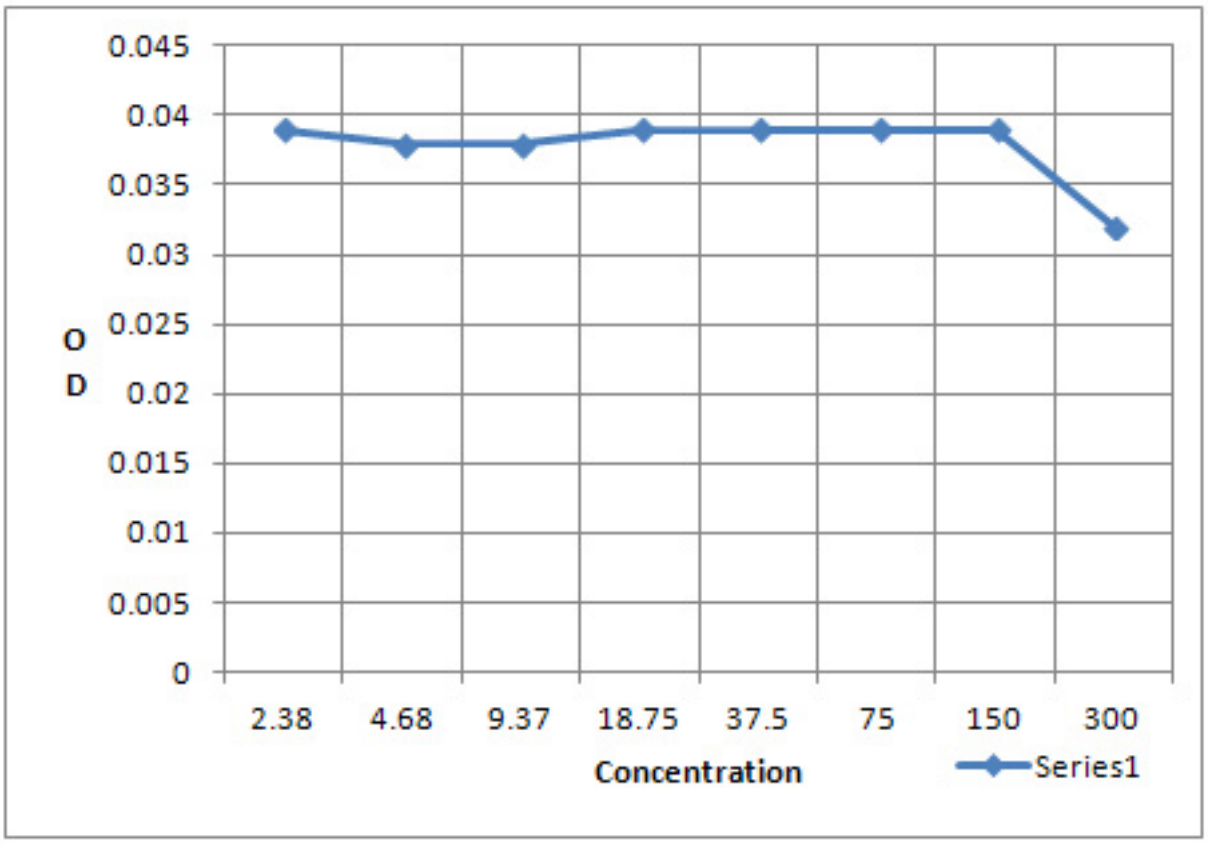
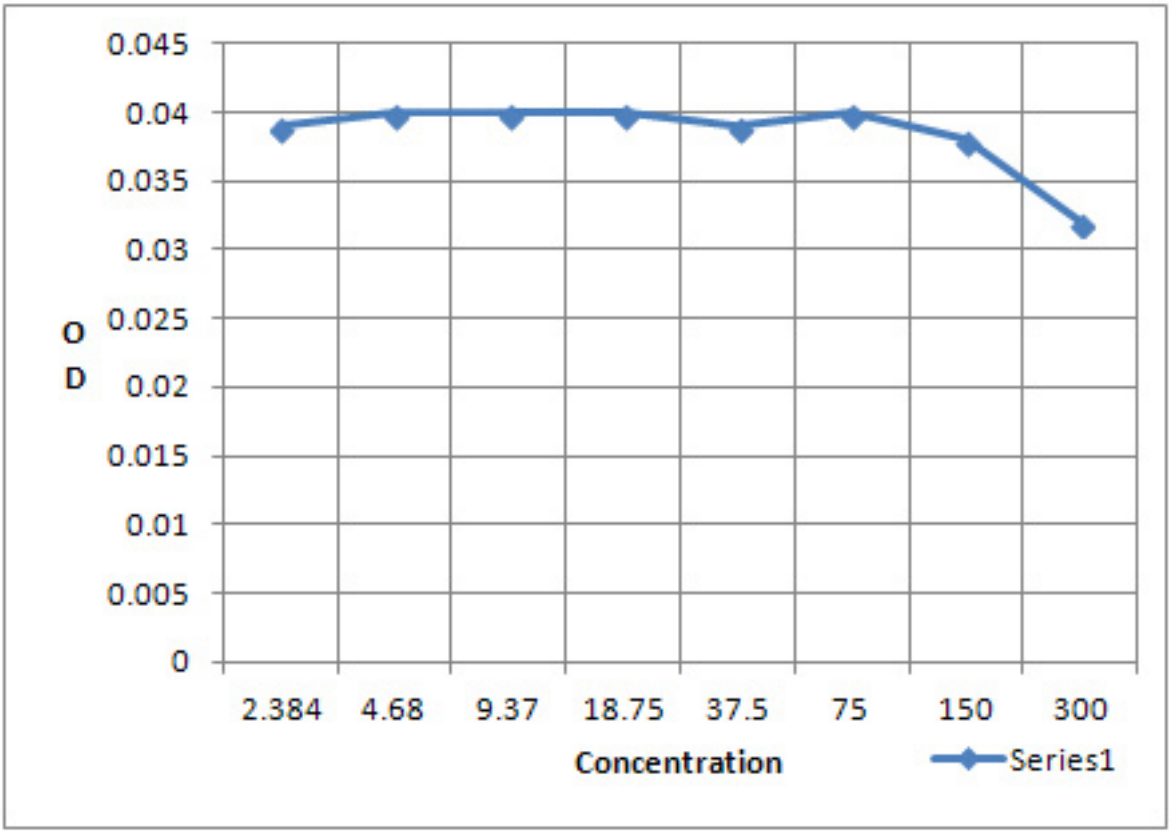


Diagram 15: comparing light absorbance in different concentrations by (µg) in wavelength of 600nm for E. coli.



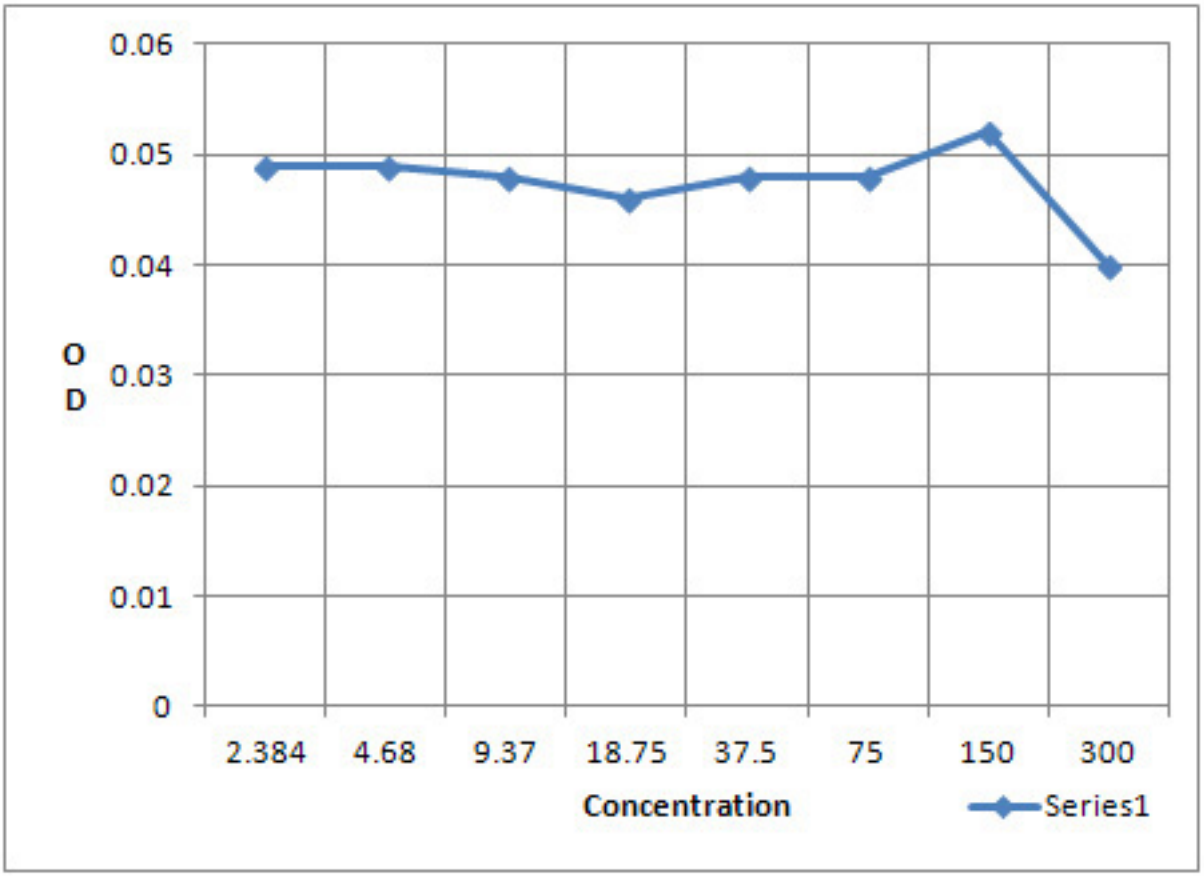
As you see in Diagram16, antibiotic has shown antimicrobial properties at concentration of 300µg.

Diagram 16: comparing light absorbance in different concentrations by (µg) in wavelength of 600nm for Pseudomonas aeruginosa



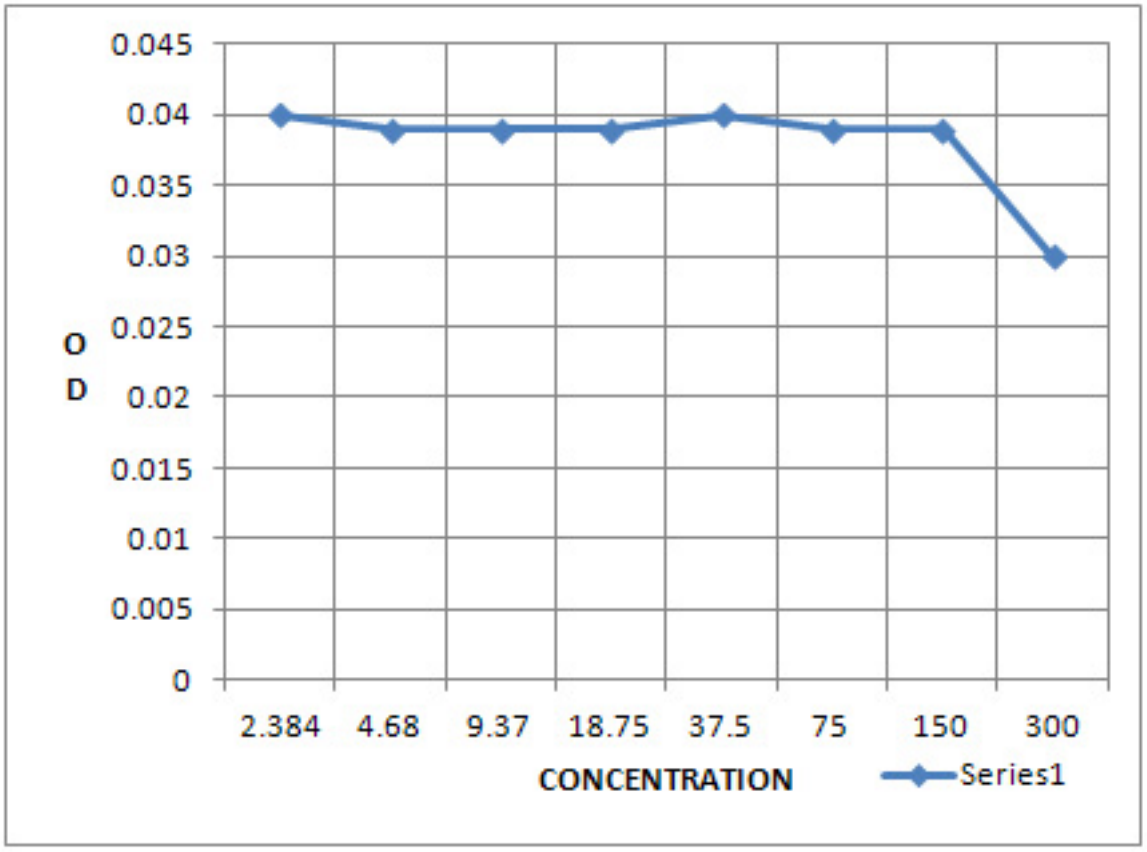
As you see in Diagram 17, antibiotic has shown antimicrobial properties at concentration of 300µg

Diagram 17: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for Staphylococcus aureus



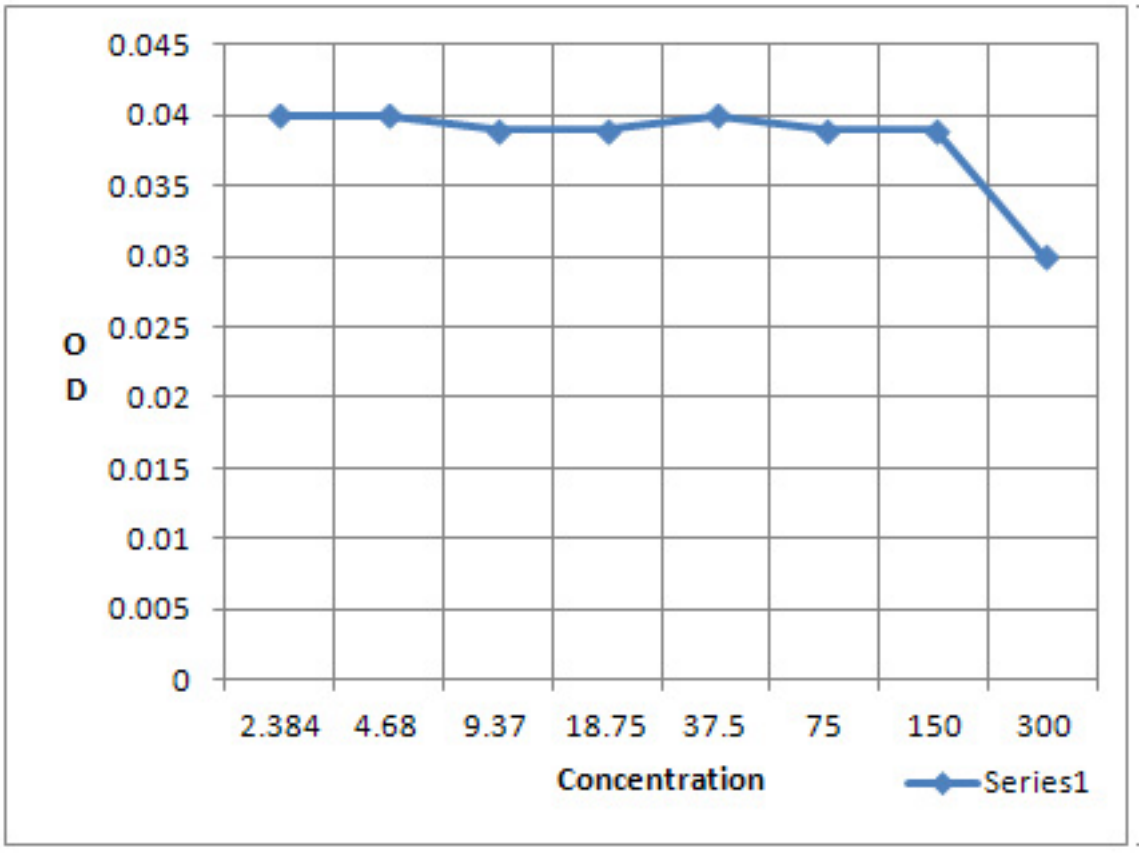
As you see in Diagram 18, antibiotic has shown antimicrobial properties at concentration of 300µg.

Diagram 18: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for Bacillus subtilis



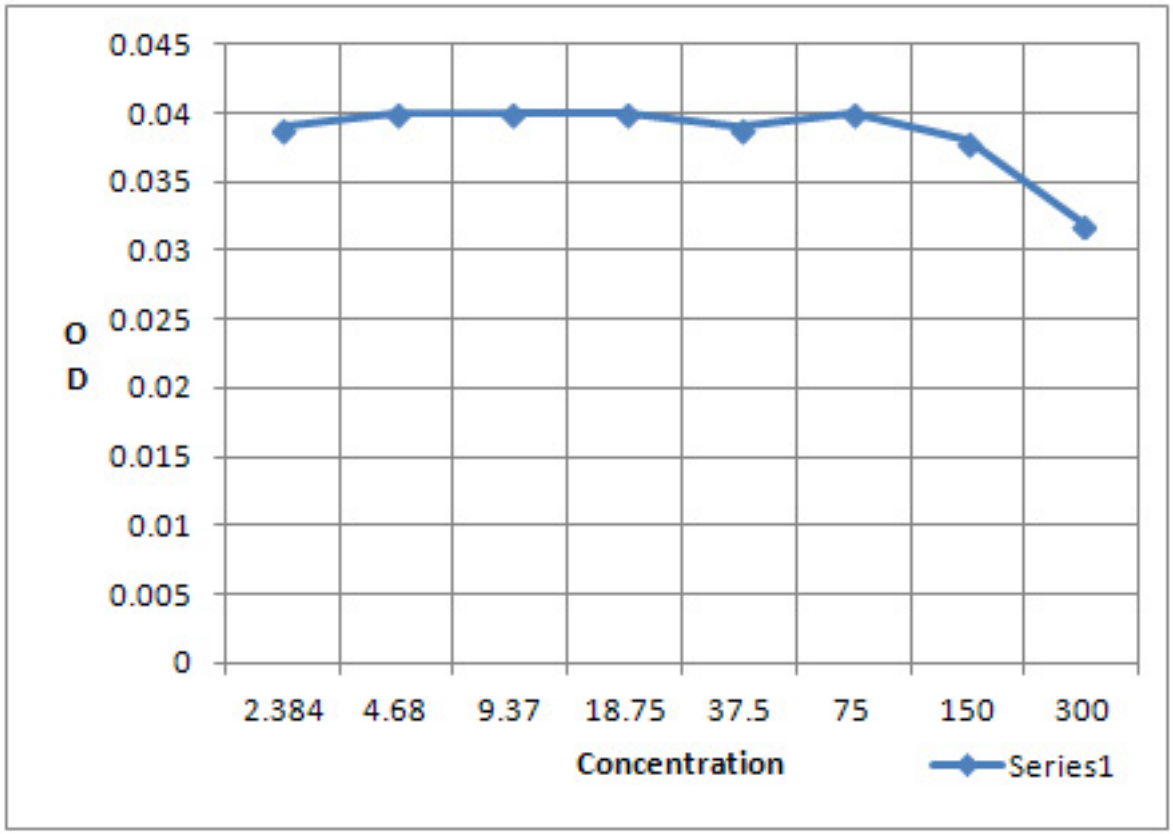
As you see in Diagram 19, antibiotic has shown antimicrobial properties at concentration of 300µg.

Diagram 19: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for E. coli



As you see in Diagram 20, antibiotic has shown antimicrobial properties at concentration of 300µg.

Diagram 20: comparing light absorbance in different concentrations by (µg) in wavelength of 620nm for Pseudomonas aeruginosa



Discussion

In this study, antimicrobial properties of the derivative peptide of Naja naja snake was examined. The studying peptide sequence includes 13 amino acids, of which their sequence is: DEQSTHGAYVWKL

D: aspartic acid, E: glutamic acid, Q: glutamine, S: serine, T: threonine, H: histidine, G: glycine, A: alanine, Y: tyrosine, V: valine, W: tryptophan, K: lysine, L: leucine, Alanine, valine, and leucine are hydrophobic amino acids in this structure; lysine and histidine have a positive charge and glycine has a role in peptide's flexibility.

Various studies about these peptides' effects have been carried out by researchers as mentioned below:

• Salehi et al. (2012) did a study named D28 peptide's antibacterial effects. Results of this study showed that the D28 peptide has an antimicrobial property against S. aureus.

• Gaiser et al. (2011) studied and examined production of antimicrobial peptides made by bacteria. These peptides showed a good antimicrobial property, due to their pure positive charge and the 3-dimension amphipathic structure.

• Shebl et al. (2012) studied derivative profile of various snakes and examined their antimicrobial and antiviral properties and found their effects relevant to their enzymes' activities. They also concluded that they had a good antimicrobial effect on gram-positives and gram-negatives.

• Samel et al. (2012) carried out a study on effect of derivative A2 phospholipase from some snake species and concluded that it had an antimicrobial effect on gram-positive and gram-negative bacteria.

• Zare et al. (2014) identified a new antimicrobial peptide from amphibians' dermal discharges, which showed its antimicrobial properties against gram-positive and gram-negative bacteria and fungi. Its major effect is against gram-negative bacteria. This peptide was named "Buforin-k".

• Zare et al. (2014) carried out a study on derivative peptide of coriander, which showed a good antimicrobial property against gram-positive (Staphylococcus aureus) and gram-negative (Klebsiella pneumonia) bacteria. It also didn't show hemolytic property against red blood cells. It was concluded that this peptide can be useful to be produced as a drug in treatment of diseases.

• Karbalaie Muhammad et al. (2011) synthesized a peptide that showed good antimicrobial and antineoplastic properties. They concluded that the synthesized peptide applies a good antimicrobial effect on gram-positive and gram-negative bacteria.

The present work examined antimicrobial properties of derivative peptide of cobra (Naja naja) snake's venom and showed that this peptide has antimicrobial effect with sequence of DEQSTHGAYVWKL against gram-positive and gram-negative bacteria, including Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa.

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