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Study The Improvement Myco-manufacturing of Crud Bikaverin by Fusarium oxysporum with Evaluation The Antimicrobial Effectiveness

Ali J Al-Sa'ady
Biotechnology Dept. College of Science/ University of Baghdad
Baghdad/ Iraq
ali.reshak@sc.uobaghdad.edu.iq

Mohammed J Al-Obaidi
Tropical Biological Research Unit, College of Science/University of Baghdad
Baghdad/ Iraq
lafta@sc.uobaghdad.edu.iq

Ahmad Y Hanoon
Tropical Biological Research Unit, College of Science/University of Baghdad
Baghdad/ Iraq
ahmad.hanoon@sc.uobaghdad.edu.iq

Haider A Hassan
Biotechnology Dept. College of Science/University of Baghdad
Baghdad, Iraq
hayder.ahmed@sc.uobaghdad.edu.iq

***Abstract*- Bikaverin is a reddish pigment produced by a variety of fungal species, the majority of which belong to the Fusarium genus. This pigment has antibiotic activities against protozoa, bacteria, and fungus. This study investigated the toxicity of Bikaverin to some microbs, production, and characterization of the pigment from the fungus *Fusarium oxysporum*. The yield of total bikaverin in *F. oxysporum* shake flask culture was improved by optimizing cultivation variables such as complex medium, carbon supply, nitrogen source, temperature, medium pH, and incubation period. After 10 days and at 28°C in Potato dextrose broth [PDB] supplemented with 2% glucose, 2% corn step liquor, and a pH of 5.5, the maximum production of complete bikaverin was discovered. The isolates of more sensitive bacteria to bikaverin were *Staphylococcus aureus* and *S. typhi,* with inhibition zones 24 and 23 mm respectively, at a concentration of 75 µg/mL. The isolates more sensitive bacteria to bikaverin were *E. faecalis, E. coli* and *P. aeruginosa* while fungi was *C. albicans* with inhibition zones of 18, 22, 20 and 13 mm, respectively, at a concentration of 75 µg/mL. The results showed that the toxic antimicrobial activity of bikaverin was clear against fungi and bacteria.**

Keywords*— Optimization, Bikaverin, Toxicity, *Fusarium oxysporum

I. Introduction

Because of the numerous possibilities and applications that have been discussed, the production of pigments by fungi is a fascinating phenomenon [1]. The majority of fungal pigments produced are aromatic polyketides with medicinal and coloring properties, such as quinones, flavonoids, melanins, and azaphilones [2]. In addition to high levels of gibberellins, Gibberella fujikuroi produces a reddish polyketide pigment called Bikaverin [3]. *Fusarium fujikuroi* has taken over for *Gibberella fujikuroi*. Secondary metabolites produced by the fungus include moniliformin, beauvericin, fumonisin, fusarin C, neurosporaxanthin, and the red pigment Bikaverin, as well as its isomer, nor-Bikaverin [4]. Although the roles of the majority of secondary metabolites are unclear, it is widely assumed that pigments protect fungi against environmental stresses such as irradiation and oxidation, which restrict development, and that other chemicals contribute to fungal pathogenicity [5].

Several filamentous funguses produce polyketide-derived pigments, which have been studied for their biological activity and taxonomic significance. Polyketides are a type of secondary metabolite with a diverse set of structural and functional properties. These chemicals are of great interest due to their role in the medicinal fields. Because of their broad biological activity, they are economically, therapeutically, and industrially useful chemicals [3, 5].

A reddish pigment was discovered from cultures of *F. lycopersici* and *F. vasinfectum* sixty years ago, and *F. oxysporum*. It was given the name lycopersin because of its color [6]. Following that, a fungal vacuolation factor was identified in aging *F. oxysporum* cultures [4], and an antiprotozoal compound was isolated from *Gibberella fujikuroi* [7], now known as *F. fujikuroi*. Both metabolites were discovered to be the same substance, Bikaverin, after chemical analysis [1, 8]. This chemical was initially thought to be lycopersin [5], which was later confirmed by infrared spectroscopy [6]. The pigments of passiflorin [4] and mycogonin [9] were isolated from *F. oxysporum* passiflorae and *Mycogone jaapii*, respectively. The alternate names are still used in a current list of fungal mycotoxins, despite the fact that they all refer to the same chemical [3]. Only a few publications on Bikaverin were disseminated over the past 20 years, but the metabolite's synthesis and biological features have given more attention in recent years. The research on the genes and proteins involved in its biosynthesis and regulation, as well as the environmental factors that affect its

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production. The research was conducted on Bikaverin ever since it was discovered [5].

This study aimed to identify the best circumstances for the production of *F. oxysporum* Bikaverin and their potential to inhibit certain microorganisms..

II. Materials and methods

A. Fungal isolates

F. oxysporum isolates were obtained from the Tropical Biological Research Unit of the College of Science at Baghdad University. Samples were straightaway transported to the lab, these isolates were sub cultured and incubated at 28 degrees Celsius. The culture was maintained on a potato dextrose agar slant throughout the experiment. After the inoculation, slants were incubated for 5 days at 28°C with tubes kept at 4°C. The fungus was cultured on potato dextrose agar medium and then transferred to the culture broth for the inoculum preparation by punching out 4 mM2 of the agar plate culture with a sterile cutter. The inoculum was cultured for 5 days at room temperature in a 250mL flask with 50mL potato dextrose broth on a rotary shaker at 120 rpm.

B. The extraction and calculation of Bikaverin pigment

In Falcon tubes, samples were taken from shaking flasks and extracted with ethyl acetate (3 mL of solvent for every 3 mL of sample) using a vortex for 60 seconds. The samples were then centrifuged for 5 minutes at 10,000 rpm. The recovered organic layer was assessed using the spectrophotometric method by using a spectrophotometer to measure absorbance at 500 nm. The following equation was used to compute total Bikaverin:

$$A = \varepsilon \, L \, conc^{\,n} \qquad (1)$$

Where L is the length of the cell (1 cm), A is the absorbance at 500 nm, ε is the average molar absorptivity of all bikaverin (6,456 L/mol.cm2), and concn is the concentration (mol/L) of pigment [10].

C. Bikaverin Production on various kinds of complex media

MEB (20 g/L glucose; 20 g/L malt extract; 1 g/L peptone); SB (10 g/L glucose; 5 g/L peptone; 3 g/L yeast extract; 3 g/L malt extract); YPB (5 g/L peptone; 3 g/L yeast extract); The flask culture experiments were carried out in a 250 ml Erlenmeyer flask containing 100 ml of media and cultured for 8 days at 28 °C on a rotary shaker at 120 rpm after inoculating with 2 percent (v/v) of the seed culture. The ideal carbon supply, nitrogen source, mineral salts, and initial medium pH for the flask culture experiment were determined using the best complex media for the synthesis of the Bikaverin pigment [11].

D. The effect of carbon source

In the Potato dextrose broth, different carbon sources such as glucose, sucrose, mannose, maltose, galactose, and lactose were supplied independently to the final concentration of 2% (W/V) (PDB). The Bikaverin pigment was measured after the appropriate incubation period [10].

E. The effect of nitrogen source

Different nitrogen sources were supplemented to the final concentration of 2.0 percent (W/V) in the Potato dextrose broth, including peptone, urea, sodium nitrate ($NaNO^3$), yeast extract, $(NH_4)2SO_4$, NH_4CL, Ca (NO_3), $K(NO_3)$, and corn step liquor (PDB). The Bikaverin pigment was measured after the appropriate incubation period [11].

F. The influence of temperature

Incubation of the Potato dextrose broth (PDB) at various temperatures was used to investigate the influence of cultivation temperatures on Bikaverin pigment synthesis. The Bikaverin pigment was produced at 20, 25, 28, 30, 35, 40, 45, and 50 degrees Celsius, and then tested. This step's optimum temperature was recorded and used in following experiments [12].

G. The influence of pH

To examine the impact of pH, potato dextrose broth (PDB) was infected and incubated. Bikaverin pigment production was assessed after the pH of the aqueous solution was optimized by adding 2M HCl and 1M NaOH, bringing it from 2.0 to 9.0. The ideal pH for this procedure was noted and used in subsequent tests [11].

H. The effect of incubation time

Potato dextrose broth (PDB) was cultured for different time durations (1 to 14 days) and then analysed for Bikaverin pigment formation to find the best incubation period for pigment production [12].

I. Test the Antimicrobial activity to Bacteria and fungi

The pathogenic bacterial isolates were collected from the University of Baghdad's Department of Biotechnology/College of Science. Bacteria isolates included: *Salmonella typhi, Escherichia coli,* and *Pseudomonas aeruginosa* are Gram-negative bacteria, while *Enterococcus faecalis* and *Staphylococcus aureus* are Gram-positive bacteria, and *Candida albicans* is yeast. Bacteria were subculture onto nutritional agar media and the yeast onto PDA, and then incubated for 48 hours at 37°C for the bacteria and 28°C for the yeast, respectively. The cultures were refrigerated in preparation for future investigations.

J. The preparation of different concentrations of Bikaverin

Bikaverin concentrations of 25, 50, and 75 µg/ml were synthesized overnight using distilled water as the solvent. The antibiotic metronidazole was made with distilled water at a concentration of 50 µg/mL. As an antibacterial control, the antibiotic metronidazole was utilized [13].

K. Determination of Bikaverin activity by agar diffusion method

Twenty-five mL of Mueller-Hinton agar and Sabouraud dextrose agar in petri plates were employed for bacteria and Candida albicans, respectively, according to Obeidat et al. [14]. Agar media was inoculated with 48-hour-old microorganism strain cultures. Five wells (5 mm diameter) were cut into the agar with a cork-borer, and three wells were filled with 100 l of Bikaverin at concentrations of 25, 50, and 75 µg/mL. A hemacytometer was used to count cells after the inoculum size was calibrated to yield final inoculums of approximately 7106 cells/mL. Bacteria were incubated at 37°C for 48 hours, while yeast was incubated at 28°C. The diameter of the inhibition zone generated around the well was measured to determine antibacterial and antifungal activity. As a positive antimicrobial control, metronidazole (50µg/mL) was employed, while distilled water was used as a negative control.

III. Result and Discussion

The pigment formations in the fungi were found to be dependent on the preparation of the medium used in studies of Bikaverin biosynthesis in *F. oxysporum*. Most studies attempted to increase pigment production by growing plants in complex, rich media [12]. In this study, F. oxysporum was grown in a variety of nutrient media, including PDB, PGB, YMB, MEB, SB, and RM. The goal of this study was to find a good complex media for the production of Bikaverin by *F. oxysporum*. According to Figure 3-1-a, the Bikaverin pigment was present in the highest concentration in the Potato Dextrose Broth (PDB) medium (35.53 µg/ml) and the lowest concentration in the EMB medium (2.84 µg/ml). There was a moderate amount of pigment in the other media.

Figure (3-1-a): Bikaverin production by *F. oxysporum* using SmF, pH 5, incubation at 28 °C for 8 days.

The main difference between PDB and other media was that PDB had components like metal ions/or other micronutrients that were necessary for enzymes to function properly and increased Bikaverin synthesis. These findings suggested that PDB were effective in the synthesis of Bikaverin by *F. oxysporum*. Figure 1 depicts the influence of complex medium on fungal growth and pigment generation (3-1-b).

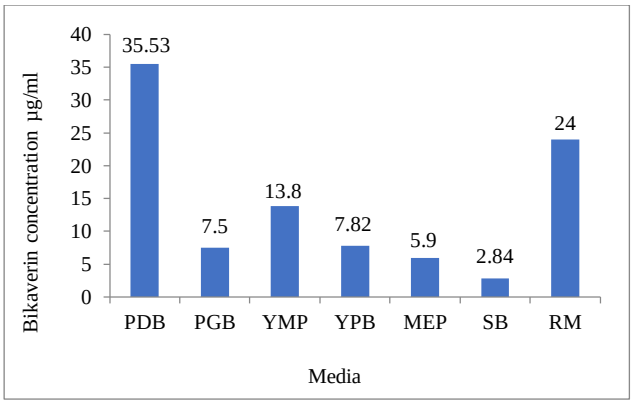

Figure (3-1-b): Effect of complex media on Bikaverin production by *F. oxysporum* using SmF, pH 5, incubation at 28 °C for 8 days.

These findings were similar to those of Premalatha *et al*. [11] who demonstrated that modifying the media components affects the selection of efficient media for Bikaverin synthesis by *F. oxysporum*.

A. The effect of carbon source

The effect of carbon sources on mycelial growth and the formation of Bikaverin pigment was investigated. *F. oxysporum* was grown for 8 days in PDB medium, which included 20 g/L of various carbon sources. As illustrated in figure 3-2, *F. oxysporum* may have employed a variety of carbon sources for pigment formation, based on the 6 types of carbon sources investigated. The glucose medium produced the most pigment (36.13 µg/ml), while maltose and lactose produced very little pigment. The manufacture of numerous secondary metabolites was hampered by glucose, which is normally a great carbon source for growth [12]. The impact of a carbon source on microbial growth and the production of pigment is shown in (Figure 3-2).

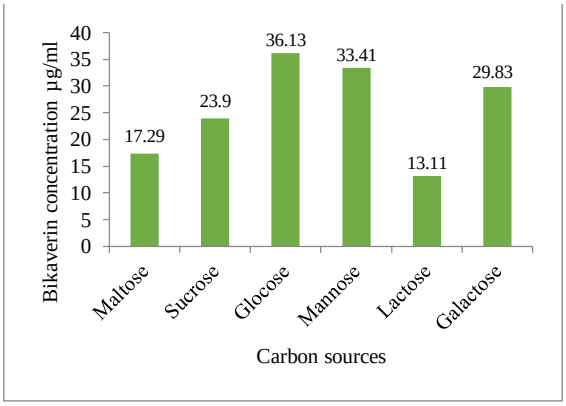

Figure (-3-2-): The effect of carbon source on Bikaverin production by *F. oxysporum* using SmF, pH 5 incubation at 28 °C for 8 days.

These results were similar to the results of Premalatha, et.al., [11], who found that the best carbon source for maximum Bikaverin production from *F. oxysporum* was glucose. Lebeau, et.al. [15], show that the better carbon source for Bikaverin production from *F. verticillioides* was glucose.

B. The effect of nitrogen source

For Bikaverin synthesis from *F. oxysporum*, different nitrogen sources such as peptone, urea, sodium nitrate ($NaNO_3$), yeast extract, $(NH_4)2SO_4$, NH_4CL, Ca (NO_3), $K(NO_3)$, and corn step liquor were added independently. It is generally known that different nitrogen sources have distinct impacts on microorganism growth and pigment generation in fermentation. Corn step liquor was shown to be the optimum nitrogen source for Bikaverin synthesis. Organic nitrogen sources generated more Bikaverin than other inorganic nitrogen sources, as shown in (Figure 3-3). It has been determined that a number of amino acids present in organic nitrogen sources are necessary for the synthesis of secondary metabolites. In fact, *F. oxysporum* fermentations in the presence of various amino acids can produce a variety of pigment derivatives with improved functional properties in the color range of orange-red to violet-red.

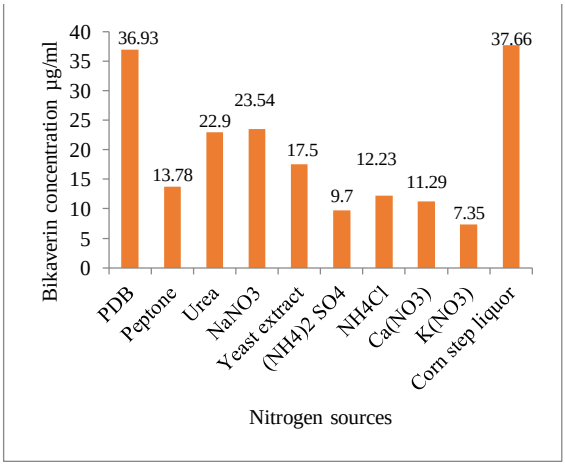

Figure (-3-3-): Effect of nitrogen source on Bikaverin production by *F. oxysporum* using SmF, pH 5, incubation at 28 °C for 8 days.

L-asparagine and L-arginine were substantially superior nitrogen sources for antibiotic production in *Cephalosporium acremonium* than ammonium compounds, according to Demain et al. [7]. Similarly, glutamate analogy such as L-glutamic acid, -monohydroxamate, and -benzyl-L-glutamate promoted penicillin production in *Penicillium chrysogenum*, however inorganic nitrogen sources such as ammonia inhibited this secondary metabolite formation. However, it is believed that the presence of salts containing zinc ions as well as nitrogen in both forms (NO_3 and NH_4 +) is required for the formation of toxin in Fusarium sp. (naphthazarins and fusaric acid) [8].

C. The effect of temperature

Temperature had a slight effect on the growth of the fungus. However, Bikaverin production was affected by temperature; with an increase in yield at 28°C. The highest yield of Bikaverin (37.87 g/ml) was found at a temperature 28°C. (Figure 3-4). This is comparable to how *F. solani* [16] produces Bikaverin and how *Monascus ruber* produces citrinin, a red pigment.

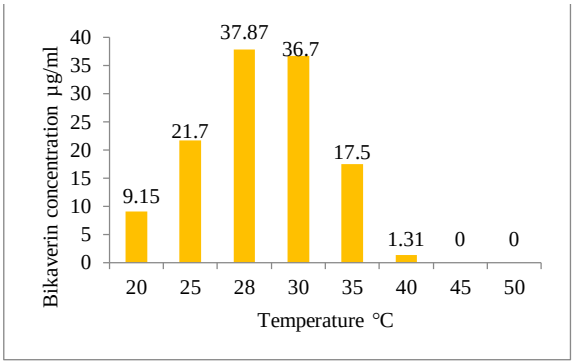

Figure (-3-4-): Effect of temperature on Bikaverin production by *F. oxysporum* using SmF, pH 5, incubation for 8 days.

The fungi preferred a temperature range of 22 to 28 °C for the production of pigment, with a maximum growth temperature of 28 °C. Other microbes produce secondary metabolites in the same way.

One of the critical parameters that determine the success of the SmF system is temperature. Some of the findings suggested that enzyme production correlated closely with fungal development, and that the optimum temperature for Bikaverin production by *F. oxysporum* is similar to the fungus's optimum temperature for growth. This finding was consistent with that of Premalatha et al. [11] who found that the highest levels of Bikaverin were achieved at temperatures that were optimal for fungal development in submerge fermentation.

D. The influence of pH

For an 8-day period, *F. oxysporum* was grown on PDB medium with 20 g/L glucose and 20 g/L corn step liquor to examine how the initial pH affected mycelial growth and the production of the Bikaverin pigment. At a pH value of 5.5, the maximum biomass output (38.64 µg/ml) was discovered. Figure 5 depicts the influence of pH on fungal growth and pigment synthesis. For *F. oxysporum*, the link between pigment synthesis and starting pH was detected between pH 2 and 9. This finding suggested that pH plays a role in pigment formation. The ideal pH for pigment synthesis (39.32 µg/ml) was noticed when the culture medium's pH was set to 5.5. In a related experiment, it was discovered that high levels of hydrogen ions in the medium (pH 4.5 and lower) and too much carbon were linked to the production of the antifungal drug Bikaverin. But when the culture medium's pH was increased to 7, fungus growth was slowed (Figure 3-5).

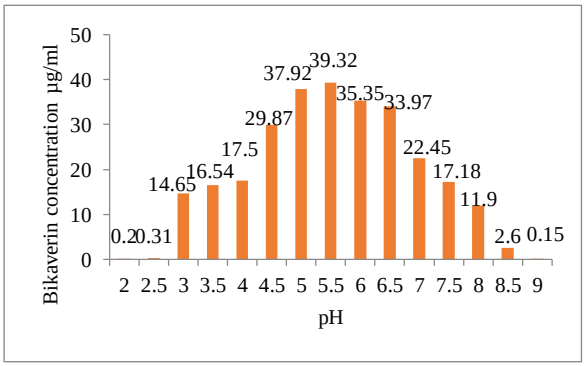

Figure (-3-5-): Effect of pH on Bikaverin production by *F. oxysporum* using SmF, incubation at 28 °C for 8 days.

The shape of the fungal mycelia under different initial pH values was a crucial factor in biomass accumulation and pigment development [6, 8, and 17]. The function of cell membranes, cell shape and structure, salt solubility, substrate ionic state, nutrient uptake, and product biosynthesis can all be affected by the pH of the medium. In general, cells can only expand within a certain pH range, and pH affects the synthesis of metabolites as well [18].

E. The effect of incubation time

By incubating the selected strain at various time intervals, the Bikaverin production parameter was optimized (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 days). To determine the ideal incubation time for the synthesis of Bikaverin, conical flasks were incubated for various amounts of time spaced by 24 hours at equal intervals. Incubation for 10 days yielded the highest pigment synthesis (65.55 µg/ml) (Figure 3-6).

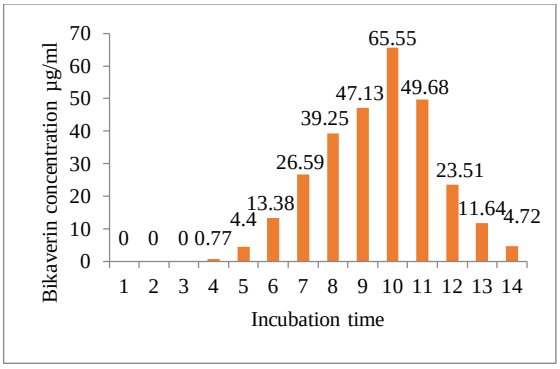

Figure (-3-6-): Effect of incubation time on Bikaverin production by *F. oxysporum* using SmF, pH 5.5, incubation at 28 °C.

The Bikaverin output decreased after 11 days. A reduction in the nutrients in the medium was the cause of the decline in Bikaverin production. Ahmed [13] and Obeidat et al. [14] calculated the incubation period for Bikaverin production by *F. oxysporum* to be 8 days [11].

F. Antimicrobial activity for Bikaverin

If the inhibition zone is greater than 10 mm, the substance is regarded to be an antibacterial active agent against bacteria and fungus. The effects of Bikaverin antibacterial and antifungal activities against fungi and bacteria are shown in Table (3-1). Bikaverin from F. oxysporum was found to be sensitive to the isolates. At 75 µg/mL, *Staphylococcus aureus* and *S. typhi* were more susceptible to Bikaverin than the other examined microbiological species, with inhibition zones of 24 mm and 23 mm, respectively. At a dose of 75 µg/mL, the inhibition zone of *E. faecalis* was 18 mm, whereas the inhibition zones of *E. coli*, *Candida albicans*, and *P. aeruginosa* were 22, 20, and 13 mm, respectively. In addition, as compared to the 75 g/mL concentration of Bikaverin, the other concentrations of Bikaverin showed minimal inhibitory zones. According to Muhammad & Muhammad, each concentration of Bikaverin (50 and 75 µg/mL) has antibacterial action against both bacterial and fungal isolates [16].

TABLE (-3-8-): ANTIMICROBIAL ACTIVITY OF BIKAVERIN ON THE BACTERIAL AND FUNGAL ISOLATES AT DIFFERENT CONCENTRATIONS BY WELL DIFFUSION TEST.

NO.	Isolates	Inhibition zone (mm) of Metronidazole (50 µg/mL) as control	Inhibition zone (mm) of Bikaverin 25 µg/mL	Inhibition zone (mm) of Bikaverin 50 µg/mL	Inhibition zone (mm) of Bikaverin 75 µg/mL
1	*C. albicans*	28	15	17	20
2	*S. aureus*	27	16	19	24
3	*E. faecalis*	22	11	15	18
4	*E. coli*	26	13	17	22
5	*S. typhi*	27	15	18	23
6	*P. aeruginosa*	13	8	10	13

Bikaverin is classified as a mycotoxin, however its toxicity varies greatly between organisms. Despite the fact that Bikaverin-producing *Fusarium* species are common phytopathogens in agriculture, there have been no reports of Bikaverin-contaminated products having a harmful impact on human health. According to an unscheduled DNA synthesis assay, Bikaverin is not genotoxic. However, it is not without risk, as many studies have revealed that it interacts with a variety of biological functions. Surprisingly, its biological characteristics could make it useful as an antibiotic or anticancer drug [1].

Although the biological function of Bikaverin in *Fusarium* is unknown, its antibiotic properties may help the producing fungi survive in competitive situations. Regardless of its natural function, its antimicrobial capabilities offer economic potential, particularly in the control of pathogenic protozoa and fungi, as well as in cancer treatment. Different research organizations are paying more attention to the biological effects of this metabolite, and more progress is expected in the near future [19, 20].

IV. CONCLUSIONS

The study concluded that after 10 days at 28°C in Potato Dextrose Broth [PDB] supplemented with 2% Glucose, 2% Corn Step Liquor, and a pH of 5.5, were the maximum production of Bikaverin. The isolates more sensitive bacteria to Bikaverin were Staphylococcus aureus and S. typhi, with inhibition zones 24 and 23 mm respectively, at a concentration of 75 µg/mL. The more sensitive fungi to Bikaverin were *E. faecalis*, *E. coli*, *C. albicans*, and *P. aeruginosa*, with inhibition zones 18, 22, 20 and 13 mm, respectively, at concentration of 75 µg/mL. Moreover, the toxic antimicrobial activity of Bikaverin improved against fungi and bacteria.

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