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INVESTIGATION OF THE MUTAGENIC, CYTOTOXIC AND ANTIMICROBIAL EFFECT OF BIKAVERIN MYCOTOXIN

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Abstract

Bikaverin is a reddish pigment produced by different fungi species (*Mycogone jaapii, Verticillium agaricinum, Beauveria bassiana, Paecilomyces fumosoroseus, Polyporus sulphureus*), mainly of the *Fusarium* genus. Due to its pigment feature, bikaverin can be used as a dye in various fields in the industry. However, it is extremely important to study the mutagenic/genotoxic effects, cytotoxic effects and antimicrobial properties of bikaverin for application of industrial areas. In the study, the mutagenic, cytotoxic and antimicrobial effects of bikaverin were investigated. The mutagenic effect of bikaverin was studied with the Ames test. *Salmonella typhimurium* TA97a, TA98, TA100, TA102 and TA1535 strains were used in the test. Five different doses of bikaverin (0.075, 0.1, 0.2, 0.3 and 0.5 µg/plate) were tested against strains. It was determined that there was no mutagenic effect of bikaverin. The cytotoxicity of bikaverin was evaluated by MTT test on L929 fibroblast cell line. Bikaverin demonstrated no cytotoxic effect on L929 fibroblast cell line, according to cell viability calculations that showed >73% for all concentrations (1, 0.5, 0.4, 0.3, 0.2, 0.1, 0.075, 0.05, 0.025, 0.01, 0.005 and 0.001 µg/mL) examined. Bikaverin's IC50 value was determined to be 1.79±0.51 g/mL. The antimicrobial activity of the bikaverin was evaluated by using the microdilution method. Bikaverin was found to have antimicrobial effects on Methicillin resistant *Staphylococcus aureus*, Vancomycin resistant *Enterococcus faecalis*, *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Candida albicans* and *Candida krusei*, as MIC values ranged from 1.25 -5 µg/ mL.

Keywords: Ames. Antimicrobial Activity. Bikaverin. Cytotoxicity. Mutagenicity. Mycotoxin.

1. Introduction

Today, dyes are widely used in various industrial sectors such as textiles, pharmaceuticals, nutraceuticals, and most importantly, the food industry as a food additive (Valenzuela-Gloria et al. 2021). Some synthetic dyes are potentially carcinogenic, highly toxic, and cause allergic dermatitis, skin irritation and mutation in humans (Srivastava et al. 2004; Sinha et al. 2012). Synthetic dyes can pollute the environment, and cause adverse ecotoxicological effects and bioaccumulation in wildlife (Saha et al. 2010). For this reason, the preference of natural colorants as functional components are increasing in many areas (Dufossé et al. 2005; Yolmeh et al. 2014). Natural dyes are also preferred due to their environmental friendliness and high biodegradability (Nagia and EL-Mohamedy 2007). Natural pigments are used in many

products (such as baby formula, breakfast cereal, pasta, sauce, processed cheese, fruit juice, vitamin-enriched dairy products, and some energy drinks) (Nagpal et al. 2011).

There are various natural colorants obtained from plants, animals and microorganisms (Sequin-Frey 1981; Sarkar et al. 2017; Pal et al. 2018). Pigments obtained from microorganisms have many benefits such as low environmental impact, being more economical, and ease of use before, during and after processing compared to those obtained from plants and animals (Kumar et al. 2015). The natural pigments obtained from bacteria and fungi, besides being colorants, also have biological activity (Elkhateeb and Daba 2023). In addition, the ability of microorganisms to reproduce in a short time, the low cost of the nutrient media and the cheap way to obtain pigments have made microorganisms an important source in pigment production. These factors increase the use of microorganisms in pigment production (Seyedin et al. 2015).

Fungal pigments have the capacity to be an important source of biopigments due to their high yield potential and ease of extraction (Valenzuela-Gloria et al. 2021). Bikaverin is a reddish pigment produced by different fungi species (*Mycogone jaapii, Verticillium agaricinum, Beauveria bassiana, Paecilomyces fumosoroseus, Polyporus sulphureus*), mainly of the *Fusarium* genus (Hamill et al. 1969; Cornforth et al. 1971; Terashima et al. 1972; Bernardini et al. 1975; Deol et al. 1978; Osman and Valadon 1984). Although originally red, the color of bikaverin changes to blue after heat treatment by an unknown mechanism (Santos and Bicas 2021). This pigment is a tetracyclic polyketide that has antibacterial and anticancer activity (Son et al. 2008; Wiemann et al. 2009; Limon et al. 2010; Haidar et al. 2019).

There are many studies on the biological properties of bikaverin. Bikaverin exhibits a large spectrum of bioactivity; like antimicrobial effect against *Leishmania braziliensis* (Balan et al. 1970), nematicidal activity against *Bursaphelenchus xylophilus* (Kwon et al 2007), use of plant growing control (Son et al. 2008), effect against metastatic cancer cells (Zhan et al. 2007), antitumoral agent effect against different tumor cells (Fuska et al. 1975; Zhan et al. 2007), neuroprotective effect (Nirmaladevi et al. 2014), and effect against filamentous fungi (Cornforth et al. 1971). Norred et al. (1992) determined that the use of high dose bikaverin caused cell death on rat hepatocytes. As seen in these studies bikaverin can be used in many areas such as the food and textile industry, pharmaceutical and medical devices due to its pigment feature and biocompatibility properties. However, it is extremely important to study the mutagenic/genotoxic effects of bikaverin before using it in these areas.

Genotoxicity tests are used to determine the mutagenicity of physical and chemical agents (such as drugs, food additives, nanomaterials, newly synthesised chemicals). These tests are also applied to determine whether chemicals cause chromosomal abnormalities, DNA damage and to understand the mechanism of action (Şekeroğlu and Şekeroğlu 2011; Oğuz et al. 2013; Yüzbaşıoğlu et al. 2014). There are various *in vivo* and *in vitro* test systems applied in eukaryotic and noneukaryotic cells for the determination of genotoxic carcinogens. These tests include Ames test, Comet test, Chromosome abnormalities test, Sister chromatid exchange test and Micronucleus test. The Ames test is used as a screening test to determine the mutagenic potential of many environmental samples, such as new chemicals and drugs, and dyes, reagents, cosmetics, wastewater, pesticides, nanoparticles, and other substances that are readily soluble in a liquid suspension (Mortelmans and Zeiger 2000; Li et al. 2012; Vijay et al. 2018).

Cytotoxicity assay is one of the oldest *in vitro* methods used to measure the toxic effect of a substance on different tissues. *In vitro* cytotoxicity test is an essential tool in screening and evaluating the safety of a compound. various tests are used to determine cytotoxicity. Tests assess cellular viability, membrane stability, cell division, and metabolic activity are important in determining the response of cultured cells to treatment with suspected toxins. The cell viability determination test is the primary and most frequently used method among them. In the present study, this method was also used to determine the cytotoxic effect (Ghasemi et al. 2021).

Antimicrobial resistance is one of the most critical public health threats in terms of the treatment of infectious diseases today (Dadgostar 2019). Therefore, in the last two decades, natural antimicrobial agents such as plant extracts, essential oils, and microbial pigments have attracted attention from the scientific community because of their unique physicochemical properties and diverse biological activities (Angane et al. 2022).

In this study, the mutagenic, cytotoxic effect, and antimicrobial activity of bikaverin were examined.

2. Material and Methods

Preparation of the bikaverin

Bikaverin was purchased from GLPBIO (Cas no: 33390-21-5, isolated from *Fusarium oxysporum f.* sp. *lycopersici*) and 0.01 mg/ml stock solution of bikaverin was prepared in Dimethyl sulfoxide (DMSO). Five different concentrations (0.075, 0.1, 0.2, 0.3, and 0.5 µg/plate) for Ames test and twelve different concentrations (1, 0. 5, 0.4, 0.3, 0.2, 0.1, 0.075, 0.05, 0.025, 0.01, 0.005 and 0.001 µg/mL) for MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) were prepared with distilled water from the stock solution. Starting 5 µg/mL to 0,04 µg/mL twofold dilutions of bikaverin used to test for the antimicrobial activity.

Ames (Bacterial Reverse Mutation Test)

The potential mutagenicity of bikaverin was determined using bacterial reverse mutation assay according to the Organization for Economic Cooperation and Development (OECD) Guideline no 471 (OECD, 2020). *Salmonella typhimurium* strains (TA97a, TA98, TA100, TA102, and TA1535) which were used. These mutant strains contain different types of mutations in different parts of the histidine operon, and as a result, they cannot reproduce in the absence of histidine amino acid. 0.5, 0.3, 0.2, 0.1, 0.075 µg/plate doses were used to determine the mutagenicity of bikaverin. The test was run in three times with and without S9 for a period of 72 h at 37°C. In the test, 4-nitro-o-phenylenediamine for TA98 (2.5 µg/plate), sodium azide for TA100 and TA1535 (5 µg/plate), mitomycin C for TA102 (0.5 µg/plate), and 9-aminoacridine for TA97a (50 µg/plate) were used as positive controls without S9; 2-aminoflourene for all strains (10 µg/plate) was used as the positive control with S9. The mutagenicity of the test substance is determined according to the number of colonies formed on the plates. The test substance is considered mutagen when the number of colonies counted on the plate as a result of the Ames test is at least twice the number of colonies counted in the spontaneous control. However, if the number of colonies is less than twice the number of spontaneous control colonies, but there is a dose-related increase, the test substance is still mutagenic; test substance is considered weak mutagen if it is less than twice the spontaneous control, although a dose-dependent increase in colony count is observed (Maron and Ames 1983; Mahon et al. 1989; Cariello and Piegorsch 1996; Lee et al. 2020).

Statistical analysis for Ames test results

The results of Ames test were evaluated using analysis of variance (ANOVA) followed by the Tukey test. Values of $p < 0.05$ were considered statistically significant.

Determination of cytotoxicity

The L929 fibroblast cell line were seeded (5 x 10^4 cells/well) in a 96-well plate in 200 µL of Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with inactivated 10% Fetal Bovine Serum (FBS) and 100 U/mL of penicillin/streptomycin. After 24 h incubation (37°C in a humidified atmosphere containing 5% CO_2), the cells were treated with different concentrations of bikaverin (1, 0. 5, 0.4, 0.3, 0.2, 0.1, 0.075, 0.05, 0.025, 0.01, 0.005 and 0.001 µg/mL). DMSO (20%) was used as positive control and as a blank control, only cell medium was used. Treated cells were incubated in a humidified atmosphere containing 5% CO_2 for 24 h at 37°C. The cytotoxic effect of bikaverin against fibroblast cell line was determined by MTT assay. For MTT assay the medium was removed carefully from the wells after incubation. Each well was washed with Phosphate Buffered Saline (PBS) (1X) for 2-3 times and 50µl of MTT (1mg/ml) was added and the plates were incubated for 3-4 hrs at 37 °C in a humidified atmosphere containing 5% CO_2. After that, MTT solution was removed and 100 µL of isopropanol was transferred into the wells. Subsequently, the absorbance was measured in a microplate reader (ALLSHENG, AMR-100) at 570 nm wavelength. Cell viability (%) was calculated with the following equation (ISO 10993, 2014).

Cell viability (%)=100x OD1/OD2
OD1: Treated cells' optical density
OD2: Blank cells' optical density

Determination of Antimicrobial Activity

Antimicrobial activities of bikaverin were screened by microdilution method according to recommendations of CLSI reference methods for bacteria M07 (CLSI, 2018) and fungi M27 (CLSI, 2017). *Staphylococcus aureus* ATCC 25923, Methicillin resistant *S. aureus* ATCC 43300, Vancomycin resistant *Enterococcus faecalis* ATCC 51299, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, and *Candida krusei* ATCC 6258 strains were used in the study.

3. Results

Ames Test Results

The mutagenicity of bikaverin was determined by the Ames test (Table 1).

Table 1. Ames test results of bikaverin with (+S9) and without (-S9) metabolic activation.

Group	Dose (µg/plate)	S9	Number of colonies/plate (mean±SD)				
			TA 97a	TA98	TA100	TA102	TA1535
Bikaverin	0.075	-	78.67±8.39	17±4	95±15	240.33±25.50	10.33±1.53
	0.1	-	64.67±5.03	23.33±1.15	89.33±10.07	265±13.23	9.67±0.58
	0.2	-	68±7.21	18.05±1	82.67±4.04	271.67±18.93	12.33±1.53
	0.3	-	74.67±7.23	16.33±1.52	82.67±6.81	275.33±5.51	12.67±3.21
	0.5	-	87.33±1.15	21±2	85.33±12.34	243.67±24.58	10±1.73
Spontaneous control		-	87±4	23±2	96.33±20.08	281.33±12.22	14.33±1.15
Positive control		-	870±42.51*[a]	1,613.33±41.63*[b]	1,366.67±109.57*[c]	1,226.67±346.33*[d]	1,366.67±32.08*[c]
Bikaverin	0.075	+	124.0± 9.64	22.67±2.52	80.33± 8.5	237.33±23.19	10.33±4.62
	0.1	+	115.7± 9.45	26±2	74±1.73	261.33±2.31	11.33±2.31
	0.2	+	112.7±5.5	25.33±3.06	74.33±8.08	232.67±6.43	9.67±1.53
	0.3	+	113.7±10.2	24±2	88.33±7.23	250±11.36	12.67±2.89
	0.5	+	120.7± 2.51	24.67±3.51	77.33±7.09	253.33±3.51	14.67±1.53
Spontaneous control		+	131.7±27.5	25.33±3.79	85.33± 8.96	248.33±10.41	14.67±4.72
Positive control		+	1,026.7±55.1*[e]	663.3±61.1*[e]	1,274.67±49.37*[e]	1,465.33.0±52.05*[e]	1,677.33±48*[e]

a: 9-aminoacridine (50 µg/plate); b: 4-nitro-o-phenilendiamine (2,5 µg/plate); c: Sodium azide (5 µg/plate); d: Mitomycin-C (0,5 µg/plate); e: 2-aminoflourene (10 µg/plate). *Significantly different from the negative control, $p<0.05$

When the mean colony counts obtained as a result of three replicate experiments were statistically analyzed at the $p< 0.05$ level, no significance was observed. According to the test results, no increase or dose-dependent increase in colony numbers was observed compared to the spontaneous control. Accordingly, no mutagenic effect of bikaverin was detected at any concentration used (with or without S9).

MTT Assay

MTT test was performed to evaluate the cytotoxic effect of bikaverin on L929 fibroblast cell line. The results are shown in Figure 1.

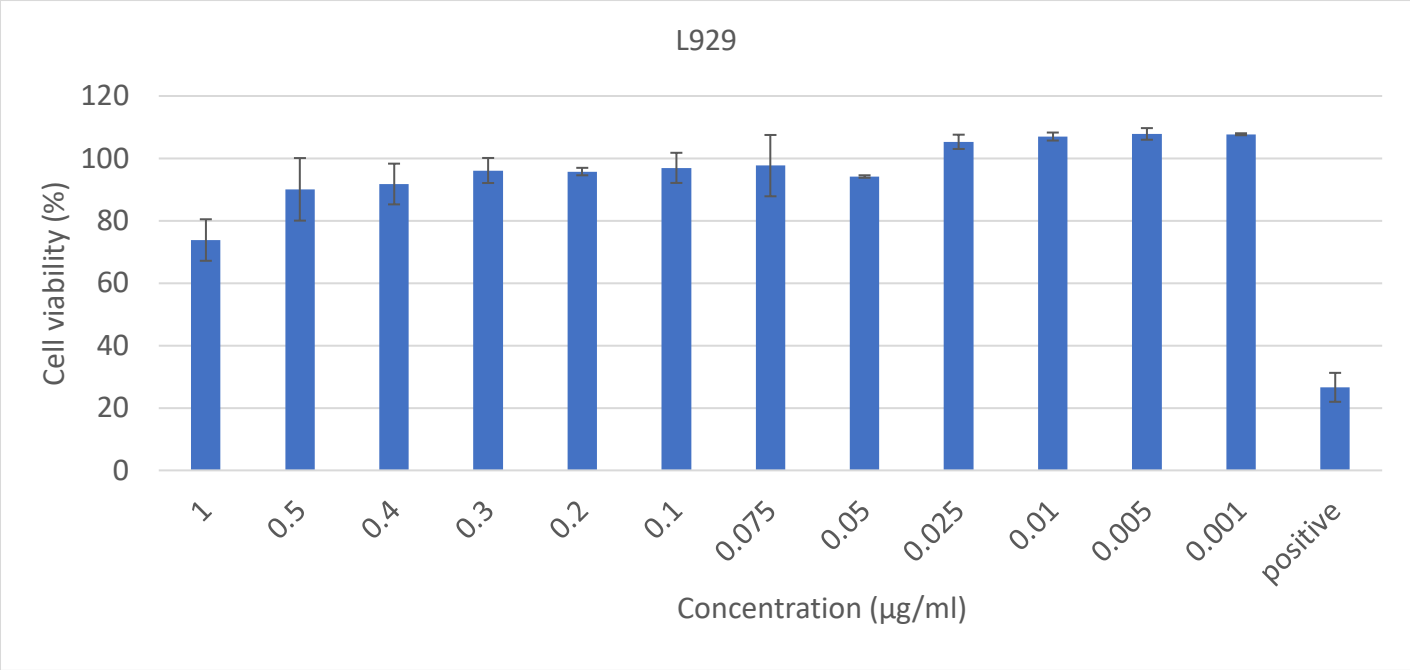

Figure 1. Cell viability percentages of treated concentrations of bikaverin on L929 cell line.

Cell viability was calculated as >73% for all concentrations tested, indicating that bikaverin had no cytotoxic effect on L929 fibroblast cell line. 50% Inhibitory Concentration (IC50) value of bikaverin was calculated as 1.79 ± 0.51 µg/mL.

Effect of bikaverin on *in vitro* viability of L929 fibroblast cell line at different concentrations and Standard deviation (SD) values are given in Table 2.

Table 2. Effect of bikaverin on *in vitro* viability of L929 fibroblast cell line at different concentrations and SD values.

Concentration (µg/ml)	1	0.5	0.4	0.3	0.2	0.1	0.075	0.05	0.025	0.01	0.005	0.001	positive
Actual Cell viability (%)													
Mean value	73.83	90.07	91.79	96.12	95.78	96.96	97.68	94.19	105.31	107.00	107.84	107.74	26.62
SD value	6.65	10.03	6.53	4.01	1.20	4.83	9.83	0.40	2.29	1.28	1.86	0.32	4.64

Antimicrobial Assay

The antimicrobial activity of the bikaverin was evaluated by using the microdilution method. Minimal Inhibitory Concentration (MIC) values of bikaverin against tested bacteria are given in Table 3. According to the obtained results MIC values ranged from 1,25-5 µg/ mL. Bikaverin was found to have antimicrobial effects on the studied microorganisms.

Table 3. MIC values of the bikaverin.

Organism	MIC Value
S. aureus	1.25 µg/mL
Methicillin resistant *S. aureus*	5 µg/mL
Vancomycin resistant *E. faecalis*	5 µg/mL
E. coli	> 5 µg/mL
K. pneumoniae	> 5 µg/mL
P. aeruginosa	> 5 µg/mL
C. albicans	5 µg/mL
C. krusei	2.5 µg/mL

4. Discussion

Bikaverin is a polyketide synthesised by various fungal species. The synthesis of these metabolites is catalysed by the Polyketide Synthase (PKS) family of enzymes. These enzymes consist of repetitive units such as ketosynthase, acyl transferase and acyl carrier protein (Hill 2006). These polyketides show clinically remarkable bioactivity due to their antimicrobial, anti-cancer and immunosuppressive properties (Santos et al. 2020, Zhao 2020). Polyketide is very important in the biological activity of bikaverin.

There are studies showing that the cytotoxic property of bikaverin is due to the inhibition of Casein Kinase 2 (CK2) enzyme (Nipun and Amin 2022). CK2 is an important enzyme involved in the fulfilment of vital functions such as proliferation, growth and regulation of metabolism of eukaryotic cells. Furthermore, this enzyme appears to be abnormally high in all types of cancer. In recent years, studies have referred to the effect of bikaverin on the down regulation of CK2 activity (Haidar et al. 2019).

There are many studies on the isolation and biological activities of bikaverin from various fungal species (Hinojosa-Ventura et al. 2019; Lebeau et al. 2019; Santos et al. 2020; Mohan et al. 2023). Deshmukh et al. (2014) were isolated bikaverin from *Fusarium* sp. (HKF15) and bikaverin was found active at 25 and 50 ppm against the *E. coli*, Metisilin esistant *S. aureus*, *P. aeruginosa* and Multi Drug Resistant *Enterococcus* and *Serratia* sp. In the study of Sondergaard et al. (2016) it was determined that bikaverin isolated from *Fusarium* strains had antibacterial activity on *Lactobacillus* and *Bifidobacterium* strains.

In our study, it was found that bikaverin had an antimicrobial effect on more microorganisms at a lower dose than the results of limited studies in the literature.

Hinojosa-Ventura et al. (2019) were isolated bikaverin from *Gibberella fujikuroi*. They also investigated the cytotoxic effect of bikaverin on L5178Y lymphoma cell line and its antitumor effect on BALB/c mice inoculated with L5178Y cell line. The results demonstrated the cytotoxic effect of bikaverin on cancer cell lines and its potential as an antitumoral compound in BALB/c mice inoculated with L5178Y lymphoma cell line.

In a study that tested the effect of bikaverin on cell viability in different cancer cell lines (MCF7, A427 and A431), it was determined that cell viability and cell proliferation were significantly reduced after 24 hours of application with 10 µM bikaverin (Haidar et al. 2019).

With IC50 values of 0.26, 0.43, 0.42, and 0.38 µM, respectively, bikaverin showed cytotoxic activities against the cancer cell lines MIA PaCa-2 (pancreatic carcinoma), NCI-H460 (non-small cell lung cancer), MCF-7 (human breast cancer), and SF-268 (human CNS cancer). This indicated selective cytotoxicity toward MIA PaCa-2 and NCI-H460. Moreover, EAC (Erlich ascites carcinoma), leukemia L5178, and sarcoma 37 cell lines were shown to be cytotoxic by bikaverin, which affects the precursor use of nucleic acid and protein synthesis (Hridoy et al. 2022).

Moreover, studies on non small cell lung (NCI-H460, IC50=0.43 µM), pancreatic (MIA Pa Ca-2, IC50=0.26 µM), breast (MCF-7, IC50=0.42 µM), and CNS glioma (SF-268, IC50=0.38 µM) cancers have examined the cytotoxic effects of bikaverin (Zhan et al. 2007).

When the anticancer studies with bikaverin and the results obtained in our study were compared, it was seen that bikaverin was effective on many cancer cells at doses much lower than the doses that were effective on healthy cells.

Bikaverin is known to have a wide range of biological activities. It is an excellent agent that can be used in the development of new therapeutic drugs and biosynthesis engineeringits because of anticancer, antifungal, antibiotic and immunomodulatory properties (Zhao et al. 2020).

In the presented study, it was determined that bikaverin has no mutagenic effect on mutant *Salmonella* strains at the applied doses. Also according to TS EN ISO 10993 and Food and Drug Administration (FDA) evaluation criteria, if the cell viability is over 70 and 50 percent, the applied chemical has no cytotoxic potential and is suitable for medical applications. We found that, it had antibacterial and antifungal effects on Methicillin resistant *S. aureus*, Vancomycin resistant *E. faecalis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *C. albicans* and *C. krusei* strains.

5. Conclusions

The advantage of bikaverin being a naturally derived pigment from fungi is that natural products are more reliable and preferable to synthetic products in commercial use. This study's results demonstrate additional properties of bikaverin as a potential industrial substance. Although many findings are pointing to the antimicrobial and anticytotoxic effects of bikaverin, more research about genotoxicity is needed on possible commercial uses of this natural chemical.

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