SİDAS MEDYA
Akademik Gıda®
ISSN Online: 2148-015X
http://dergipark.gov.tr/akademik-gida

Akademik Gıda 18(3) (2020) 241-246, DOI: 10.24323/akademik-gida.XXXXXX

Research Paper / Araştırma Makalesi

Antibacterial and Anticancer Activities of Violacein Extracted Through Ultrasound-Assisted Extraction Method

Çağdaş Deniz Periz[1], Seyhan Ulusoy[1] ✉, Gülgün Tınaz[2], Turgut Şekerler[3]

[1]*Suleyman Demirel University, Department of Biology, Isparta, Turkey*
[2]*Marmara University, Department of Basic Pharmaceutical Sciences, Faculty of Pharmacy, Istanbul, Turkey*
[3]*Marmara University, Department of Biochemistry, Faculty of Pharmacy, Istanbul, Turkey*

Received (Geliş Tarihi): 31.05.2020, Accepted (Kabul Tarihi): 10.09.2020
✉ *Corresponding author (Yazışmalardan Sorumlu Yazar): seyhanulusoy@sdu.edu.tr (S. Ulusoy)*
+90 246 211 40 68 +90 246 211 43 99

ABSTRACT

Violacein is a natural violet pigment produced by various bacterial species, especially by the Gram-negative bacteria *Chromobacterium violaceum.* Violacein has antibacterial, antifungal, antioxidant and anticancer activities and has various applications in food, cosmetic, textile and pharmaceutical industries. In this study, violacein was extracted from the *Chromobacterium violaceum* culture through ultrasonic-assisted (UA) and microwave-assisted (MA) extraction methods using methanol, ethanol, acetone, and ethyl acetate as solvent. Violacein was quantified using High Performance Liquid Chromatography (HPLC). Then, antibacterial and anticancer activities of these extracts were investigated. In this study, for the first time, the violacein extract was obtained by using UA and MA extraction methods. The highest violacein concentration could be achieved by UAE method with methanol. It was found out that violacein extracts had an antibacterial effect against *Staphylococcus aureus*, *S. aureus* (MRSA) and *Bacillus cereus*. The violacein extract had a strong anticancer effect on cervical cancer (HeLa) cells. Finally, the UA and MA extraction methods were successfully applied to bacterial cultures in order to extract the violacein.

Keywords: Violacein, Extraction, Antibacterial, Anticancer

Ultrason Destekli Ekstraksiyon Yöntemi ile Elde Edilen Violasinin Antibakteriyel ve Antikanser Aktiviteleri

ÖZ

Viyolasin, özellikle Gram-negatif bakteri *Chromobacterium violaceum* tarafından üretilen ve antibakteriyel, antifungal, antioksidan ve antikanser etkilere sahip çeşitli bakteri türleri tarafından üretilen doğal mor bir pigmenttir. Bu çalışmada, viyolasin, Ultrason destekli (UA) ve mikrodalga destekli (MA) ekstraksiyon prosedürleri ile *C. violaceum* suşundan metanol, etanol, aseton ve etil asetat kullanılarak ekstrakte edilmiştir. Elde edilen viyolasin miktarı, yüksek performanslı sıvı kromatografisi (HPLC) kullanılarak belirlenmiştir. Daha sonra bu ekstraktların antibakteriyel ve antikanser aktiviteleri incelenmiştir. Bu çalışmada, viyolasin ilk kez UA ve MA ekstraksiyon yöntemleri kullanılarak elde edilmiştir. Viyolasin ekstrelerinin *Staphylococcus aureus*, *S. aureus* (MRSA) ve *Bacillus cereus*'a karşı antibakteriyel aktiviteye sahip olduğu belirlenmiştir. Viyolasin ekstresi rahim ağzı kanseri hücreleri (HeLa) üzerinde güçlü anti-kanser etkisi göstermiştir. Sonuçta, viyolasini ekstrakte etmek için, UA ve MA ekstraksiyon yöntemleri bakteri kültürlerine başarıyla uygulanmıştır.

Anahtar Kelimeler: Viyolasin, Ekstraksiyon, Antibakteriyel, Antikanser

INTRODUCTION

There is a great interest for using natural pigments in the food, textile, cosmetic and pharmaceutical industries. Numerous molecules (pigments etc.) waiting to be discovered have the potential to be used for new treatment methods and the production of alternative drugs. Violacein which is one of those molecules is the bisindole antibiotic, which is produced by a number of bacterial species [1-4], including strains of *Chromobacterium* [1, 2], *Janthinobacterium* [4] and *Duganella* [4]. Violacein, a violet pigment, has attracted much attention due to its pharmacological properties and bioactivities [5, 6]. Violacein pigment has several biological characteristics (including antifungal, antitumoral, antiparasitic, antiprotozoal, antioxidant, antiviral, and antibacterial activities) and it has gained increasing importance in food, medicine, cosmetics and textiles for various applications [7-14]. A study demonstrated that evaluation of the powdered violacein by spray drying mode and its potential application in food [14]. Violacein is primarily active against Gram-positive strains [1, 8, 15] however, is generally ineffective against Gram-negative strains.

Natural active molecules are important sources for drug development. There are several extraction procedures for extraction of these valuable natural compounds from plants and the other resources. Traditional extraction techniques are time-consuming and require relatively large quantities of solvents [16]. Microwave-assisted and ultrasonic-assisted extraction methods have been used for the extraction of bioactive compounds in order to shorten the extraction time, lower solvent consumption, increase extraction yields and improve the quality of the extracts [17-18].

In this study, violacein was firstly extracted with UA and MA extraction methods from *C. violaceum* ATCC 12472. The extract's antibacterial and cytotoxic activities was evaluated against Gram-positive (*S. aureus, Bacillus cereus, S. aureus MRSA*), and Gram-negative (*P. aeruginosa, E. coli*).

MATERIALS AND METHODS

Chemicals, Bacterial Strains and Media

Methanol, ethanol, ethyl acetate, and acetone were purchased from Merck. Violacein was purchased from Sigma. All other reagents and chemicals used were of analytical reagent grade.

Staphylococcus aureus ATCC 25923*, Bacillus cereus* ATCC 11778*, Pseudomonas aeruginosa* ATCC 27853*, Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 43300 and *Chromobacterium violaceum* ATCC 12472 which were used in the study were obtained from the bacterial culture collection of the Department of Biology, the Faculty of Arts and Sciences in Süleyman Demirel University. Bacterial strains were maintained Luria Bertani (LB) agar (Difco).

Violacein Extraction

Overnight culture of *C. violaceum* ATCC 12472 was inoculated into fresh LB and grown at 120 rpm and 35°C for 24 hours. The culture was centrifuged and the violacein in the pellet was extracted using Ultrasound assisted extraction (UAE) method for 20 minutes method (Bandelin, Sonorex, RK-100) or Microwave assisted extraction (MAE) method (2450 MHz, 30°C for 20 minutes) with solvent (ethanol/ methanol/ acetone/ ethyl acetate). The extraction was repeated five times and the supernatant was combined. The resulting solvent was evaporated at 45°C under vacuum by a rotary evaporator. The residue was carefully dissolved in dimethyl sulfoxide (DMSO) and filtered through a 0.45µm PVDF syringe filter and stored at 4°C prior to analysis.

HPLC Analysis of the Violacein and Deoxyviolacein

Violacein was quantified through Shimadzu's (a Japanese brand) HPLC system [19]. The HPLC system is comprised of a system control unit (SCL 10AVP), pump (LC-10ADVP), diode array detector (SPD-M10AVP), manual injector, column oven (CTO-10ACVP) and degasser (DGU-14A). The chromatographic method was performed by using isocratic system composed of methanol-water (70 : 30 v/v) with a flow rate of 1 mL/min. A reversed-phase column Agilent zorbax XDB-C18, (5µ, 4.6 mm ID× 250 mm) was used and the column temperature was maintained at 30°C and mobile phase consists of methanol-water (70:30 v/v). Violacein and deoxyviolacein were detected at its absorbance maximum (585 nm).

Antibacterial Effects of Violacein Extracts

The antibacterial activities of the violacein extracts were evaluated by Agar well diffusion method [20], against *S. aureus* ATCC 25923*, B. cereus* ATCC 11778*, P. aeruginosa* ATCC 27853*, E. coli* ATCC 25922, *S. aureus* ATCC 43300. In this method, 100 µL of standardized inoculum of each test bacterium was mixed with soft agar (0.5%) and the mixture was transferred to plates prepoured with 1.5% agar. 8 mm diameter well was cut from the agar using a sterile cork-borer; subsequently each well was filled with 50 µL of the violacein extracts and incubated at 35°C for 24 h. Clear inhibition zones around wells indicated the presence of antibacterial activity. Each assay was repeated three times.

Cytotoxicity Assays of Violacein Extract

To evaluate the cytotoxic effects of violacein, on NIH (Mouse embryonic fibroblast cells) and HeLa (human cervical adenocarcinoma) cells were cultured in flasks containing DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin) (Gibco) in a CO_2 incubator at 37°C. For the cytotoxicity

assays, cells were seeded (3×10^5 cells mL^{-1}/well) in 12-well plates for 24h and exposed to 0.04–4 µM violacein for 24 and 48h. Cell viability was monitored using the Real-Time Cell Analyzer (RTCA) station (Roche Diagnostics) instrument at 15-minute time intervals for up to 72 hours.

Statistical Analysis

Each assay was repeated three times. Microsoft Excel was applied for the data analysis. The values are presented as a mean ±standard deviation.

RESULTS

The purpose of this study was to evaluate the UA and MA extraction procedures, that were not employed to extract violacein before, including the variations of organic solvents (methanol, ethanol, ethyl acetate and acetone). Violacein and deoxyviolacein were quantified by high performance liquid chromatography–diode array detection (HPLC–DAD) (Table1). Figure 1 presents a HPLC chromatogram of the UA extracted samples. Results for MAE and UAE of the violacein are shown in Table 1. Better results were obtained with UAE (methanol). Using external calibration, the concentration of the violacein and deoxyviolacein in the extract samles were determined as 4.7±0.1-82.0±7.7 0 µg violacein/mg extract, 0.67±0.002-20.7±1.2 µg deoxyviolacein/mg extract respectively (Table1).

Table 1. The violacein and deoxyviolacein concentration quantified with HPLC related to methanol, ethanol, ethyl acetate and acetone extracts by ultrasonic and microwave-assisted extraction (µg violacein/mg extract)*

Samples	Violacein	Deoxyviolacein
	µg Violacein/mg extract	µg Violacein/mg extract
Methanol UAE	82.0±7.70	20.7±1.20
Ethanol UAE	48.5±1.50	15.8±3.70
Acetone UAE	60.8±2.10	15.9±1.50
Ethyl Acetate UAE	4.70±0.12	0.67±0.02
Methanol MAE	68.8±4.40	17.2±2.30
Ethanol MAE	44.5±1.00	11.9±1.10
Acetone MAE	49.8±3.80	15.7±1.20
Etil Acetate MAE	5.90±0.10	3.40±0.10

*: UAE: Ultrasound assisted extraction method, MAE: Microwave assisted extraction method

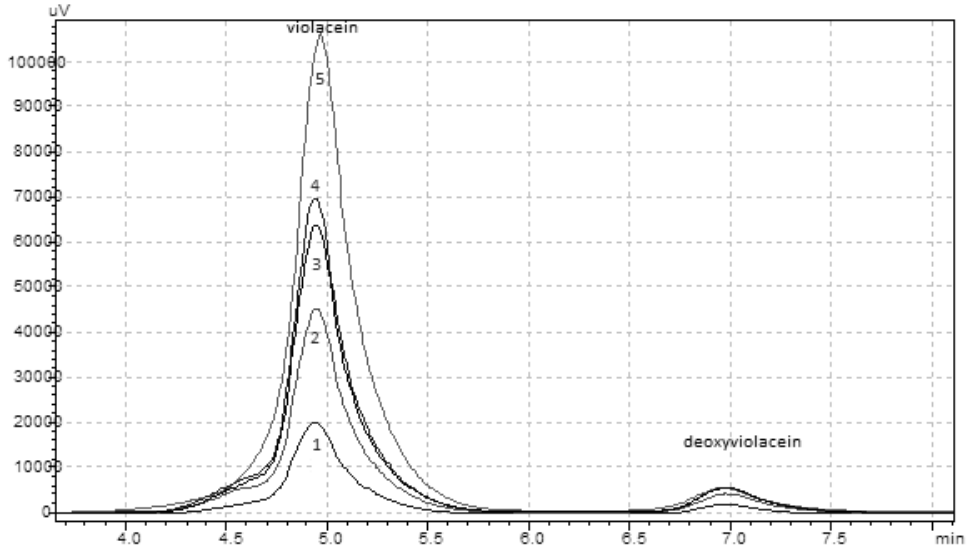

Figure 1. HPLC chromatogram of violacein and deoxyviolacein extracted with ultrasound assisted extraction method 1: Ethyl acetate extract, 2: Violacein standard, 3: Ethanol extract, 4: Acetone extract, 4:Methanol extract.

The UA and MA extracted violacein have been tested for in vitro antibacterial activity and demonstrated to have potential antibacterial effect (Table 2). The results of this study showed that, the zone of inhibition (mm) for the UA and MA extracted violacein varied from (8.5±0.6mm) to (15.5±1.1mm) as compared to Tobramycin.

Table 2. The inhibition zone diameters of the UA and MA extracted violacein samples obtained by agar well diffusion (UA: ultrasound assisted, MA: microwave assisted, E: ethanol, M: methanol, EA: Ethyl acetate, A: acetone), *: No inhibitory effect.

	Inhibition zone diameters (mm)								
Strains	UA-E	MW-E	UA-M	MW-M	UA-EA	MW-EA	UA-A	MW-A	Tobramycin (10µg)
S. aureus ATCC *25923*	15.5±1.1	9.5±0.7	13±1.0	9±0.8	*	*	*	11±0.9	13.0±0.0
S. aureus ATCC 43300	11±0.9	11.5±1.0	13±1.0	12.5±1.0	*	*	*	10.5±0.8	12.7±0.57
B. cereus ATCC 11778	8.5±0.6	*	*	9±0.8	9±0.7	*	11±0.9	*	11.3±0.57
P. aeruginosa ATCC *27853*	*	*	*	*	*	*	*	*	15.0±1.00
E. coli ATCC 25922	*	*	*	*	*	*	*	*	12.3±0.57

Among all the tested strains, the UA extracted violacein had highest antibacterial activity against the *S. aureus* ATCC 25923 followed by *S. aureus* ATCC 43300. *B. cereus* ATCC 11778 showed poor sensitivity to the UA and MA extracted violacein as compare to the other Gram-positive bacteria. But UA and MA extracted violacein did not show any antibacterial activity against *P. aeruginosa, E. coli*.

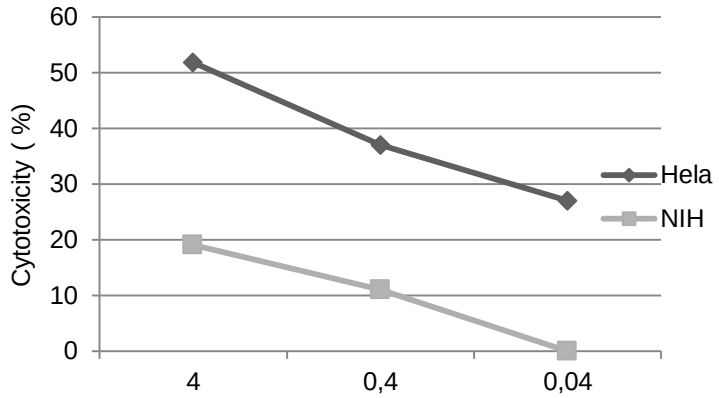

Figure 2. *In vitro* cytotoxicity of UA extacted violacein extracts (4, 0.4 and 0.04 µm) against HeLa and NIH cells.

The cytotoxic efficacy of the UA extacted violacein was evaluated by cell viability in HeLa and NIH cell lines by using real time cell analyzer xCELLigence method. The cells were tested with a range of violacein concentrations (0–10 µM) based on previous reports [5]. The UAE violacein reduced cell growth in a dose-dependent manner from 0.04 to 4 µM, respectively. Significant cytotoxic effect was observed for 4µM the UAE violacein, which inhibited cell viability by 51.8% where NIH cell growth was inhibited by 19% (Figure 2). These results suggest that the UAE violacein potentiates the antiproliferative activity at very low concentrations.

DISCUSSION

Violacein is described as a natural antibiotic that has crucial biologic activities and pharmacological properties. We extracted the highest violacein concentration by using UAE method with methanol (Table1). The antibacterial activity of violacein against different bacteria has been studied by several research groups [1,12, 21, 22]. It was found out in a variety of antimicrobial tests conducted before that while the violacein had the ability to inhibit the growth of Gram-positive bacteria, it had quite a little effect on Gram-negative bacteria [10, 12, 23, 24]. The result of this study was in conformity with the previous studies. In this study the UA extracted violacein had highest antibacterial activity against Gram-positive strains (Table 2). This result can be explained by that the outer membrane of Gram-negative strains protects cell while Gram-positive bacteria, with no outer membrane, have no protection and are susceptible to effects of violacein [25].

In general, the cytotoxicity of violacein for tumor cells is observed in the range of 1–5µM [5, 26, 27], findings that are in agreement with the data obtained in our work. In this study significant cytotoxic effect was observed for 4µM the UA extracted violacein, which inhibited cell viability by 51.8%.

If compared to conventional extraction methods, the UAE and MAE techniques are both requires short in time and the smaller solvent consumption. On the basis of the results of this study, the UAE provided better efficient violacein extraction than the MAE method.

CONCLUSION

Due to the pharmacological potential of violacein, the UAE method can be considered as an easy and economic tool for the extraction of violacein from bacterial culture.

ACKNOWLEDGEMENT

We would like to thank the Scientific Research Projects Unit of Süleyman Demirel University for supporting this study with the project number 4365-YL1-15.

Conflict of Interest

The authors confirm that this article content has no conflict of interest.

REFERENCES

[1] Choi, S.Y., Kim, S., Lyuck, S., Kim, S.B., Mitchell, R.J. (2015). High-level production of violacein by the newly *isolated Duganella violaceinigra* str. NI28 and its impact on *Staphylococcus aureus*. *Scientific Reports*, 5, 15598.

[2] Moss, M.O., Ryall, C. (1981). Distribution of chromobacteria in a lowland river. *Microbial Ecology*, 7(2), 139-149.

[3] Lichstein, H.C., Van De Sand, V.F. (1945). Violacein, an antibiotic pigment produced by *Chromobacterium violaceum*. *The Journal of Infectious Diseases*, 76(1), 47-51.

[4] Jude, B.A., Tanner, J., Koko, T., McLaughlin, E.C. (2012). Analysis, characterization, and synthesis of violacein from Janthinobacterium isolate extracts. In *Abstracts of Papers of the American Chemical Society* (Vol. 244). 1155 16TH ST, NW, Washıngton, DC 20036 USA: Amer. Chemical Soc.

[5] De Carvalho, D.D., Costa, F.T., Duran, N., Haun, M. (2006). Cytotoxic activity of violacein in human colon cancer cells. *Toxicology in Vitro*, 20(8), 1514-1521.

[6] Durán, N., Justo, G.Z., Durán, M., Brocchi, M., Cordi, L., Tasic, G.R., Castro, L., Nakazato, G. (2016). Advances in *Chromobacterium violaceum* and properties of violacein-Its main secondary metabolite: A review. *Biotechnology Advances*, 34(5), 1030-1045.

[7] Durán, N., Menck, C.F. (2001). Chromobacterium violaceum: a review of pharmacological and industiral perspectives. *Critical Reviews in Microbiology*, 27(3), 201-222.

[8] Nakamura, Y., Asada, C., Sawada, T. (2003). Production of antibacterial violet pigment by psychrotropic bacterium RT102 strain. *Biotechnology and Bioprocess Engineering*, 8(1), 37-40.

[9] Bromberg, N., Durán, N. (2001). Violacein transformation by peroxidases and oxidases: implications on its biological properties. *Journal of Molecular Catalysis B: Enzymatic*, 11(4-6), 463-467.

[10] Subramaniam, S., Ravi, V., Sivasubramanian, A. (2014). Synergistic antimicrobial profiling of violacein with commercial antibiotics against pathogenic micro-organisms. *Pharmaceutical Biology*, 52(1), 86-90.

[11] Mendes, A.S., de Carvalho, J.E., Duarte, M.C., Durán, N., Bruns, R.E. (2001). Factorial design and response surface optimization of crude violacein for *Chromobacterium violaceum* production. *Biotechnology Letters*, 23(23), 1963-1969.

[12] Durán, N., Justo, G.Z., Ferreira, C.V., Melo, P.S., Cordi, L., Martins, D. (2007). Violacein: properties and biological activities. *Biotechnology and Applied Biochemistry*, 48(3), 127-133.

[13] Durán, M., Ponezi, A.N., Faljoni-Alario, A., Teixeira, M.F., Justo, G.Z., Durán, N. (2012). Potential applications of violacein: a microbial pigment. *Medicinal Chemistry Research*, 21(7): 1524-1532.

[14] Venil, C.K., Aruldass, C.A., Abd Halim, M.H., Khasim, A.R., Zakaria, Z.A., Ahmad, W.A. (2015). Spray drying of violet pigment from *Chromobacterium violaceum* UTM 5 and its application in food model systems. *International Biodeterioration & Biodegradation*, 102, 324-329.

[15] Nakamura, Y., Sawada, T., Morita, Y., Tamiya, E. (2002). Isolation of a psychrotrophic bacterium from the organic residue of a water tank keeping rainbow trout and antibacterial effect of violet pigment produced from the strain. *Biochemical Engineering Journal*, 12(1), 79-86.

[16] Zhang, Q.W., Lin, L.G., Ye, W.C. (2018). Techniques for extraction and isolation of natural products: a comprehensive review. *Chinese Medicine*, 13(1), 20.

[17] Kaufmann, B., Christen, P. (2002). Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*, 13(2), 105-113.

[18] Wang, L., Weller, C.L. (2006). Recent advances in extraction of nutraceuticals from plants. *Trends in Food Science & Technology*, 17(6), 300-312.

[19] Blosser, R.S., Gray, K.M. (2000). Extraction of violacein from *Chromobacterium violaceum* provides a new quantitative bioassay for N-acyl homoserine lactone autoinducers. *Journal of Microbiological Methods*, *40*(1), 47-55.

[20] Holder, I.A., Boyce, S.T. (1994). Agar well diffusion assay testing of bacterial susceptibility to various antimicrobials in concentrations non-toxic for human cells in culture. *Burns*, 20(5), 426-429.

[21] Batista, A.H., Moreira, A.C., De Carvalho, R.M., Sales, G.W., Nogueira, P.C., Grangeiro, T.B., Medeiros S.C., Silveira E.V., Nogueira, N.A. (2017). Antimicrobial effects of violacein against planktonic cells and biofilms of *Staphylococcus aureus*. *Molecules*, 22(10), 1534.

[22] Cazoto, L.L., Martins, D., Ribeiro, M.G., Durán, N., Nakazato, G. (2011). Antibacterial activity of violacein against Staphylococcus aureus isolated from bovine mastitis. *The Journal of Antibiotics*, 64(5), 395-397.

[23] Aranda, S., Montes-Borrego, M., Landa, B.B. (2011). Purple-pigmented violacein-producing Duganella spp. inhabit the rhizosphere of wild and

cultivated olives in southern Spain. *Microbial Ecology*, 62(2), 446-459.

[24] Im, H., Choi, S.Y., Son, S., Mitchell, R.J. (2017). Combined application of bacterial predation and violacein to kill polymicrobial pathogenic communities. *Scientific Reports*, 7(1), 1-10.

[25] Choi, S.Y., Lim, S., Cho, G., Kwon, J., Mun, W., Im, H., Mitchell, R.J. (2020). *Chromobacterium violaceum* delivers violacein, a hydrophobic antibiotic, to other microbes in membrane vesicles. *Environmental Microbiology*, 22(2), 705-713.

[26] Saraiva, V.S., Marshall, J.C., Cools-Lartigue, J., Burnier Jr, M.N. (2004). Cytotoxic effects of violacein in human uveal melanoma cell lines. *Melanoma Research*, 14(5), 421-424.

[27] Bromberg, N., Dreyfuss, J.L., Regatieri, C.V., Palladino, M.V., Duran, N., Nader, H.B., Justo, G.Z. (2010). Growth inhibition and pro-apoptotic activity of violacein in Ehrlich ascites tumor. *Chemico-biological Interactions*, 186(1), 43-52.