



## Multi-target drug with potential applications: violacein in the spotlight

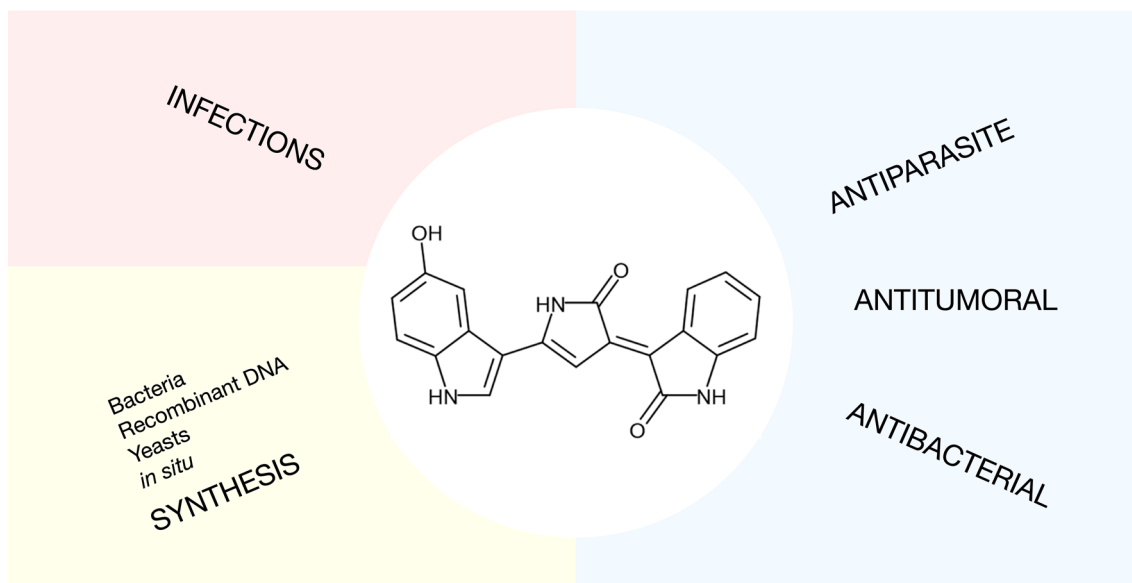
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### Abstract

The aim of the current review is to address updated research on a natural pigment called violacein, with emphasis on its production, biological activity and applications. New information about violacein's action mechanisms as antitumor agent and about its synergistic action in drug delivery systems has brought new alternatives for anticancer therapy. Thus, violacein is introduced as reliable drug capable of overcoming at least three cancer hallmarks, namely: proliferative signaling, cell death resistance and metastasis. In addition, antimicrobial effects on several microorganisms affecting humans and other animals turn violacein into an attractive drug to combat resistant pathogens. Emphasis is given to effects of violacein combined with different agents, such as antibiotics, anticancer agents and nanoparticles. Although violacein is well-known for many decades, it remains an attractive compound. Thus, research groups have been making continuous effort to help improving its production in recent years, which can surely enable its pharmaceutical and chemical application as multi-task compound, even in the cosmetics and food industries.

### Graphic abstract



**Keywords** Biological activities · *Chromobacterium violaceum* · Genome · Production · Violacein

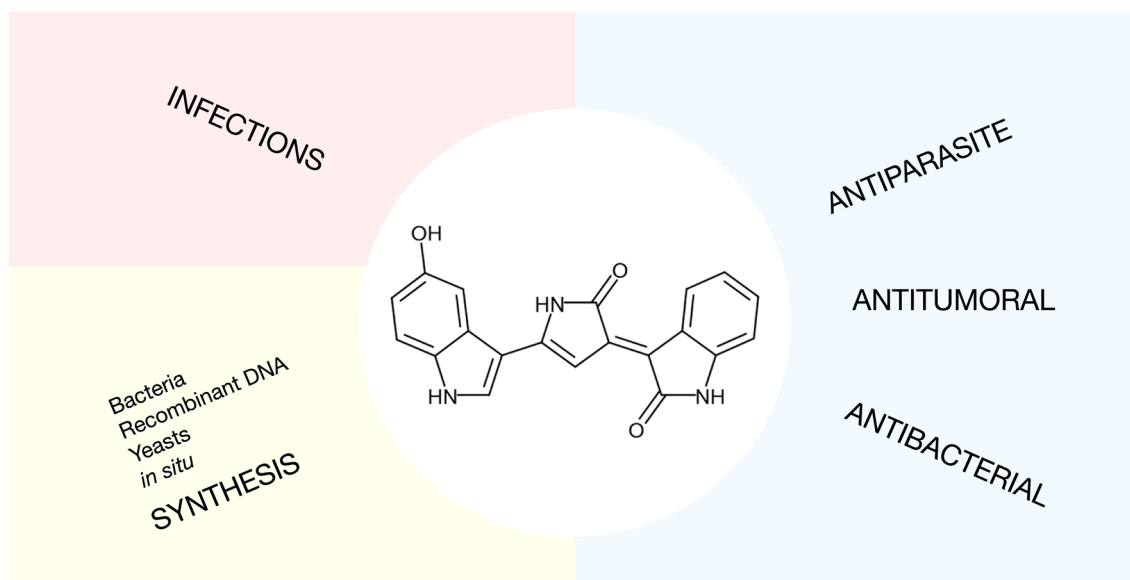
## Introduction

Nature has been a major source of compounds used for disease prevention and treatment purposes over the course of human history. Traditionally, marine and soil environments have provided several chemical scaffolds to help developing modern drugs, such as microbially and plant-derived metabolites (Harvey et al. 2015; Newman and Cragg 2016, 2020; Newman et al. 2000; Schreiber et al. 2002). Thus, bacterial strains are capable of synthesizing several secondary metabolites presenting biological activity (Numan et al. 2018). Among them, one finds violacein (Choi et al. 2021; Durán et al. 2012, 2016; Justo and Durán 2017), [3-(1,2-dihydro-5-(5-hydroxy-1*H*-indol-3-yl)-2-oxo-3*H*-pyrrol-3-ilydene)-1,3-dihydro-2*H*-indol-2-one] (Fig. 1), which is a natural purple pigment that triggers great interest due to its remarkable biological and physical properties. This pigment—which has molar mass of 343.3 amu—is insoluble in water, slightly soluble in ethanol, and soluble in methanol and dimethyl sulfoxide, as well as shows strong absorbance in the visible region of the spectrum due to resonance (Durán et al. 2007). Violacein's high hydrophobicity (octanol–water partitioning coefficient –  $\log P_{OW} = 3.34$ ) suggests that it is not secreted by bacteria into the environment (Choi et al. 2020).

Several violacein-producing microorganisms have been identified since it was discovered more than 130 years ago (Boisbaudran 1882). Among them, one finds species *Chromobacterium violaceum*, which is extensively investigated

as model system for violacein production, as well as species *Alteromonas*, *Janthinobacterium*, *Pseudoalteromonas*, *Duganella*, and *Collimonas* (Durán et al. 2016). In fact, bacterial species belonging to genus *Chromobacterium*, other than *C. violaceum*, have been used to synthesize several secondary metabolites with biocidal activity, such as the recently discovered *C. subtsugae*, *C. sphagni* and *C. vaccinii* (Blackburn et al. 2017; Martin and Soby 2016; Vöing et al. 2015). Several studies have recently reported new violacein sources. Two psychotropic bacterial strains capable of producing a mix of violacein and deoxyviolacein were isolated by Kuzyk et al. (2020) from Lake Winnipeg, Canada. Interestingly, Atalah et al. (2020) and Alem et al. (2020) have isolated and featured violacein produced by bacterial strains from extreme environments such as Antarctica. Recent literature has indicated increased number of studies describing violacein-producing bacteria featuring by whole genome sequencing to provide new information on violacein-producing bacteria (Bettina et al. 2018; Lamendella and Jude 2018; Xu et al. 2019). Table 1 shows a selection of violacein-producing strains, as well as the respective location bacteria were isolated from.

The first studies about violacein biosynthesis enabled observing that oxygenation in *C. violaceum* culture has reduced pigment production time (Tobie 1935). This finding was followed by the discovery that molecular oxygen and L-tryptophan were necessary to synthesize violacein (DeMoss and Evans 1959, 1960). From 1987 to 2000, there was great progress in the mechanistic aspects of violacein biosynthesis, such as the condensation of two L-tryptophan



**Fig. 1** Main topics covered in this review are synthetic biology violacein synthesis, using different bacteria, yeast or in situ processes. While violacein yielding species might provoke infections, violacein

shows many beneficial, and synergic effects and activities against tumors, microbes, and parasites, shown in light blue

**Table 1** Identified violacein-producing strains

Strain	Origin	References
<i>Chromobacterium violaceum</i>	Oil refinery soil in Malaysia	Ahmad et al. (2012)
<i>Chromobacterium amazonense</i> 56AF	Tropical freshwater lake	Santos et al. (2018)
<i>Chromobacterium subtsugae</i> MWU12-2387	Wild Cranberry Bog, Truro, Massachusetts	Vöing et al. (2017)
<i>Chromobacterium violaceum</i> ATCC 12,472	Freshwater in Malaysia	Brazilian National Genome Project Consortium (2003)
<i>Chromobacterium</i> sp. NIIST (MTCC5522)	Clay mine acidic sediment	Sasidharan et al. (2015)
<i>Duganella</i> sp.	Waterway in Eastern Pennsylvania	Lamendella and Jude (2018)
<i>Duganella violaceinigra</i> NI28	Forest soil	Choi et al. (2015a)
<i>Iodobacter</i> sp.	Hudson Valley watershed	Doing et al. (2018)
<i>Iodobacter</i> sp 7MAnt	King George Island, South Shetland, Antarctica	Atalah et al. (2020)
<i>Janthinobacterium lividum</i> MTR	Cajón del Maipo, Chile	Valdes et al. (2015)
<i>Janthinobacterium lividum</i>	Frogs from the USA, Switzerland and on captive frogs from Panama	Woodhams et al. (2018)
<i>Janthinobacterium svalbardensis</i> sp. nov	Island Spitsbergen in the Arctic	Avguštin et al. (2013)
<i>Janthinobacterium</i> sp. B9-8	Low temperature-sewage in China	Xu et al. (2019)
<i>Janthinobacterium</i> sp.	Hudson Valley watershed	Bettina et al. (2018)
<i>Janthinobacterium</i> sp. CG23_2	Cotton Glacier in the Antarctic Dry Valleys	Smith et al. (2016)
<i>Janthinobacterium</i> sp. Ant5-2	Proglacial Lake P9 at East Antarctica	Mojib et al. (2011)
<i>Janthinobacterium</i> sp. UV13	Lichen samples from Nebles Point, Collins Glacier (King George Island, Antarctica)	Alem et al. (2020)
<i>Janthinobacterium lividum</i> (P102), <i>Massilia violaceinigra</i> (P117)	Freshwater Lake Winnipeg	Kuzyk et al. (2020)
<i>Massilia</i> sp. NR 4-1	Topsoil under <i>Torreya nucifera</i>	Myeong et al. (2016)
<i>Massilia</i> sp. BS-1	Soil	Agematu et al. (2011)
<i>Pseudoalteromonas luteoviolacea</i> B (ATCC 29,581)	Surface seawater in Kinko Bay, Japanese Island Kyushu	Cress et al. (2013)
<i>Pseudoalteromonas luteoviolacea</i> S4054	Ocean surface waters and surface swabs of marine organisms	Thøgersen et al. (2016)
<i>Pseudoalteromonas</i> sp. 520P1	Pacific coast of Japan	Dang et al. (2014)
<i>Pseudoalteromonas amylolytica</i> sp. nov	Surface seawater of the Arabian Sea	Wu et al. (2017)
<i>Pseudoalteromonas ulvae</i> TC14	Mediterranean Sea	Aye et al. (2015)
<i>Pseudogulbenkiania ferrooxidans</i> EGD-HP2	Loktak Lake, India	Puranik et al. (2013)
<i>Chitinimonas</i> sp.	Hudson Valley waterways	Jude (2019)
<i>Collimonas</i> sp.	Coastal waters in Norway	Hakvåg et al. (2009)

molecules, molecular oxygen incorporation in pyrrolidone moiety, 5-hydroxy-L-tryptophan intermediacy, independent chromopyrrolic acid production, as well as oxygenases and NAD(P)H involvement in violacein biosynthesis. Moreover, violacein biosynthesis comprises the joint action of five enzymes, called VioA-VioE (Durán et al. 2016). These first studies have increased the interest of the scientific community in the field in developing efficient routes to enable the biosynthesis of this compound by using either natural or genetically modified microbial strains, as addressed later in the current study.

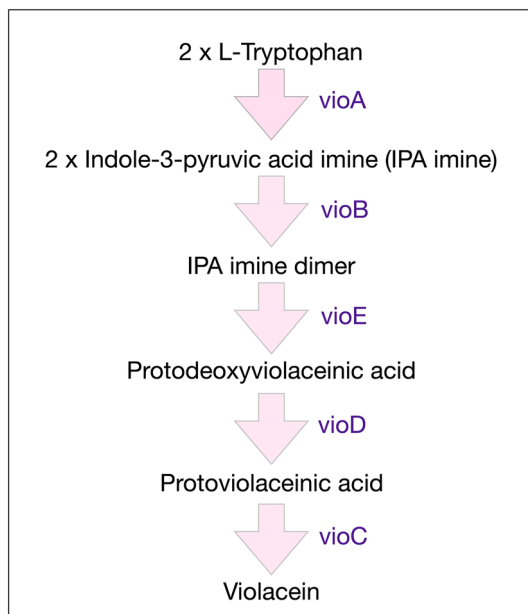
Extensive studies have reported violacein's pharmacological potential to be used as anticancer, antibacterial, antifungal, trypanocidal, antileishmanial, antinematode, antiulcerogenic, immunomodulatory and antiviral drug (Durán et al. 2016). There have been advances in understanding

violacein's action mechanisms and toxicity rates in the last years, and it contributed to improve its biological and pharmacological functionalities. In fact, there has been growing interest in investigating violacein and its activities in the last two decades, given the number of peer-reviewed articles published in the literature about it (Choi et al. 2021; Kothari et al. 2017). Although several anticancer, antiparasitic and antimicrobial properties of this purplish bisindole metabolite have been previously reviewed (Durán et al. 2016), the current study has described new features of this derivative and pointed out its value as chemotherapeutic agent. A promising application attributed to violacein lies on its potential to combat human multidrug resistant pathogens (Choi et al. 2017). In addition, violacein has been highlighted for its ability to interfere in different cancer hallmarks. It is also interesting emphasizing the symbiotic association between

violacein-producing skin bacteria and amphibians, a fact that substantiates the role played by this pigment in biodiversity. Finally, attempts to introduce violacein in engineering polymers have been reported, as well as its industrial applications, mainly as high value-added product. Therefore, the current review has addressed the main biological activities performed by violacein, as well as efforts focused on improving its production and some current applications. A brief outlook of the present review is illustrated in Fig. 1.

## Violacein biosynthesis

There are several ways to synthesize violacein, such as using bacteria, either wild or genetically modified strains (Park et al. 2021), yeasts (Chuang et al. 2018; Tong et al. 2021) or through synthesis performed in situ (Kanelli et al. 2018). L-Tryptophan is the substrate used to synthesize this secondary metabolite (Fig. 2), whereas the pathway counts on five enzymatic reactions and one consecutive non-enzymatic step (the sixth step) that completes the synthesis process. The secondary metabolism pathway strongly depends on oxidoreductive cofactors, such as



**Fig. 2** Violacein is synthesized from two tryptophan molecules in a six-step pathway that involves five enzymatic steps, and one non-enzymatic step (Hoshino 2011; Tong et al. 2021). Enzymatic oxidation reactions are FAD (vioA, vioC, and vioD), NAD(P)H (vioC and vioD), and heme-Fe(II) (VioB) dependent. Also, the last step involves oxygen-guided decarboxylation. It is important to state that the enzymatic steps start with the vioA, then vioB catalyzed reactions, follows with vioE reaction as the most important step, while vioD and vioC complete the pathway

FAD, NAD(P)H and heme-Fe(II) moieties in enzymes, as well as on oxygen supply at the final step.

Moreover, *C. violaceum* is the strain mostly investigated in the violacein-production field. Durán et al. (2016) have summarized violacein-producing microorganisms, both natural and genetically engineered ones. Aye et al. (2015) have investigated marine biofilm formation bacteria and found that species *Pseudoalteromonas ulvae* TC14 can produce violacein, just as other strains belonging to its genus; however, it does not produce *N*-acylhomoserine lactones (AHLs) necessary to regulate violacein production, although it is sensitive to exogenous AHLs, at least under planktonic conditions. Violacein production was upregulated by C6-, C12-, 3-oxo-C8- and 3-oxo-C12-HSLs (homoserine lactones) and downregulated by 3-oxo-C6-HSL and 3-oxo-C8-HSL. These exogenous AHLs regulate violacein production, as well as that of other phenotypes associated with biofilm formation. This finding suggests the presence of functional AHL LuxR-type receptor in TC14 (Aye et al. 2015).

It is known that violacein found in *C. violaceum* presents positive regulation through the *N*-acylhomoserine lactone CviI/R quorum sensing system, as well as negative regulation through undistinguished putative repressor. According to Devescovi et al. (2017), violacein biosynthesis was negatively controlled by a new suppressor protein, VioS, and positively controlled by the CviI/R system. VioS does not control the CviI/R system; besides violacein, VioS and quorum sensing antagonistically control other phenotypes.

*Saccharomyces cerevisiae* strains were genetically engineered in the last years to produce violacein in five-enzyme biosynthetic pathway. Lee et al. (2013) have sampled 100 clones from an expression library (3% of it); they transformed the clones, featured the promoters of five heterologous genes, as well as all pathway products (violacein, deoxyviolacein, and chromoviridians). Subsequently, regression model was created to predict violacein production in the rest of the library. The aforementioned authors used this method to double violacein yield by these strains. Chuang et al. (2018) have recently described a detailed protocol used to produce violacein in *S. cerevisiae* by using the Versatile Genetic Assembly System (VEGAS). They have successfully inserted all five necessary transcription units in the model. Moreover, Blount et al. (2018) used *S. cerevisiae* strains—whose natural chromosomes are replaced by designed synthetic ones—to insert an expression plasmid for violacein production purposes and used SCRaMbLE (Synthetic Chromosome Rearrangement and Modification by LoxP-mediated Evolution) to increase violacein yield. SCRaMbLE consists in the rearrangement of non-critical sequences in the synthetic genome, when yeasts are exposed to Cre in vivo, i.e., it is a black box procedure, according to which, rapid diversification takes place and generates several

phenotypes, some of them can lead to higher violacein yield (Blount et al. 2018).

On the other hand, violacein production in *E. coli* has also improved overtime. In fact, violacein production in *Escherichia coli* has gained room in the scientific field, since it is easily used, as well as presents high growth rate and protein yield. Jones et al. (2015) have created a T7 promoter library based on site-directed mutagenesis; then, they inserted all five violacein pathway genes under the control of this promoter library in *E. coli* specimens. The first analysis was applied to 4% of the library; results have shown 63-fold yield increase in this sample in comparison to the control. After fermentation optimization, titers were enhanced to approximately 2000 mg L<sup>-1</sup>, which corresponded to 2.6-fold improvement in titer and to 30-fold enhancement in yield in comparison to those in previous studies (Jones et al. 2015).

Kanelli et al. (2018) have optimized *Janthinobacterium lividum* fermentation conditions in order to maximize both violacein and biomass production. In addition, polyamide 6.6 fabrics were dyed by following three different methods, namely: simultaneous fermentation and dyeing (SFD), fabric incubation in bacterial culture after fermentation, or cell-free extract using in association with violacein. Among these methods, SFD presented maximum color change (1E) and strength (K/S), whereas dyeing has shown resistance to acid and alkaline perspiration or to water. Moreover, the fabric presented antimicrobial activity against yeasts (*Candida albicans*, *C. parapsilosis*, and *C. krusei*), as well as against bacteria (*E. coli*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus*—MRSA) (Kanelli et al. 2018).

Several advances have been made in elucidating violacein-related enzyme mechanism and structure. VioD—which is the enzyme capable of converting protodeoxyviolaceinic acid into protoviolaceinic acid from *Duganella sp.*—was expressed in *E. coli*. VioD was later purified and crystalized in order to generate X-ray diffraction (Ran et al. 2015). VioA—which catalyzes the first step in violacein synthesis, i.e., L-tryptophan conversion into the corresponding  $\alpha$ -imine—was also structurally and biochemically investigated to enable suggesting a mechanism for its activity (Füller et al. 2016). Enzyme kinetics of His<sup>163</sup>, Arg<sup>64</sup>, Lys<sup>269</sup>, Trp<sup>397</sup>, and Tyr<sup>309</sup> mutant proteins were functionally identified as crucial residues for VioA catalysis. In addition, synthetic biology approaches were used to engineer varying violacein pathways as promising strategy for novel bisindole therapeutics' synthesis. Accordingly, the authors proposed 7-aza-Trp, 1-methyl-Trp, 5-methyl-Trp, and 5-fluoro-Trp as interesting candidates for the synthesis of new violacein derivatives in vivo (Füller et al. 2016). Zhou et al. (2018) have also engineered *E. coli* to overexpress *vioABCDE* genes. VioE expression was reported to produce similar results to those reported by Fang et al. (2015, 2016), who overexpressed *serA*, *trpE<sup>abr</sup>*, *trpD*, and *vioABCDE* in *E. coli*.

Immanuel et al. (2018) used integrative approach based on the combination of constraints-based flux balance modeling to synthetic biology approach in order to optimize genetic changes to produce violacein in *E. coli*, in an efficient and scalable way.

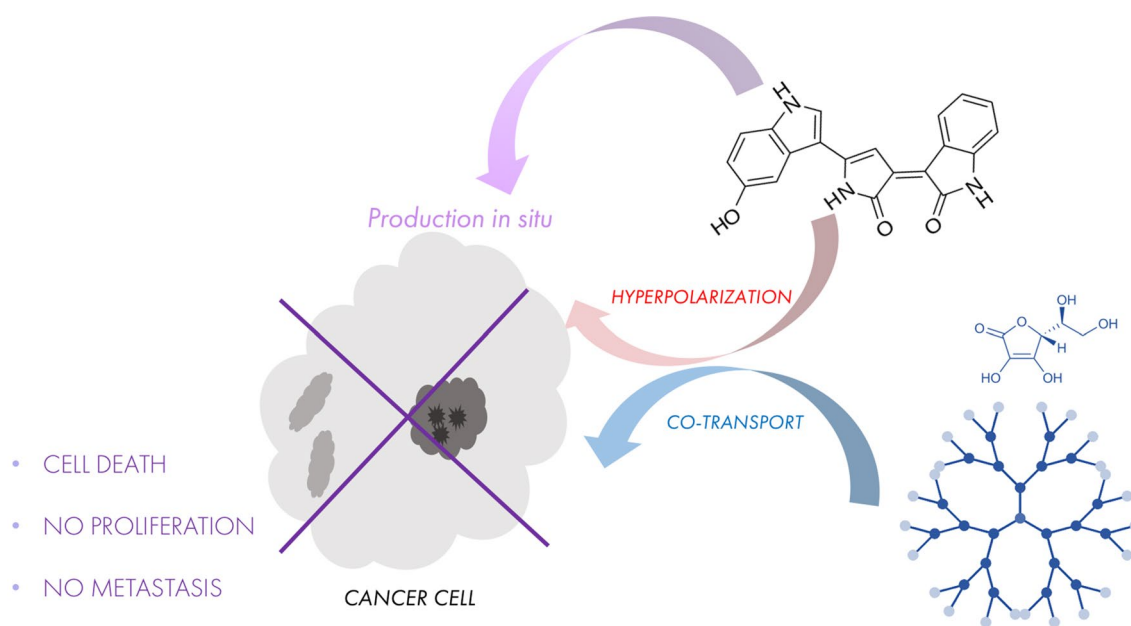
Recent article published by Tong et al. (2021) took into consideration potentially-harmful bacterial violacein-producing strains to suggest using genetically modified yeast species *Yarrowia lipolytica* in order to synthesize violacein. Interestingly, violacein production could increase to 70 mg L<sup>-1</sup>, and present approximately 5–10% of deoxyviolacein impurities, when the correct nitrogen and carbon supply, and medium pH were used.

Therefore, although synthetic biology solution applications have made great improvements in violacein synthesis, some issues remain unsolved, such as long production span of 100 h (or more), which requires adopting laborious purification methods.

## Violacein's antitumoral activity

Over the past two decades, several studies have reported violacein's antitumor property (Durán et al. 2016; Justo and Durán 2017); recently, a study has shown that this pigment is non-genotoxic (Alem et al. 2020). It is known that cytotoxic agents often induce cell death, partly due to reactive oxygen species (ROS) generation; thus, Leal et al. (2015) have investigated whether violacein affects cellular redox status. They observed that violacein was cytotoxic to CHO-K1 and MRC-5 (non-tumor), and to HeLa cell lines (tumor), although HeLa and MRC-5 cells were more sensitive to this pigment. In this study, no association between increased oxidative stress (ROS) and cell death induction was observed; however, enhanced mitochondrial membrane potential was observed (Fig. 3). Based on these findings, the aforementioned authors have stated that membrane hyperpolarization may be the main reason for violacein-induced cell death (Leal et al. 2015). Similar results were reported in Ehrlich ascites tumor cells, which presented fast ROS production due to violacein application (Bromberg et al. 2010). Although De Carvalho et al. (2006) identified ROS production after treating Caco-2 cells with violacein, the redox status of colorectal cancer cell line HT29 remained unchanged. Together, these findings have suggested that violacein has cell-type specific influence on redox status.

Given the poor violacein solubility in water—which can compromise its bioavailability and, consequently, lead to low effect on biological systems—some scholars have suggested using delivery devices loaded with violacein. Accordingly, our research group has described the synthesis of violacein inclusion complexes with beta-cyclodextrin ( $\beta$ -CD) and their influence on HL60 cell viability (De Azevedo et al. 2000;



**Fig. 3** Violacein affects tumor cells in many ways by stopping proliferation and metastasis and provoking apoptosis. Hyperpolarization and permeabilization of membranes were stressed as the most important events during violacein action. Some authors use violacein with

ascorbic acid loaded dendrimers. In addition, transfer of violacein biosynthetic cluster into an oncolytic bacteria strain led to an in situ production of violacein

Melo et al. 2003). Interestingly,  $IC_{50}$  value has dropped 15-fold when violacein was encapsulated in  $\beta$ -CD in comparison to values recorded for the violacein-free system (Melo et al. 2003). Fakhr et al. (2012) have also reported that violacein extracted from *Janthinobacterium lividum* DSM1522T presented enhanced cytotoxic activity when it was loaded on poly(amidoamine)—(PAMAM) in combination to ascorbic acid. According to the aforementioned study, human acute lymphoblastic leukemia cell line (Jurkat E6.1) was twofold more sensitive to violacein encapsulated in nanocomplex delivery system than that of the violacein-free system. Similar effect was also observed when apoptosis markers (caspases-3, -7 and -8) were analyzed; in other words, lower nanoencapsulated violacein dose was capable of activating caspases in comparison with violacein used in separate (Fakhr et al. 2012).

Another interesting aspect of violacein antitumor activity was reported by Hashimi et al. (2015), who evaluated the response of human cancer cell lines—such as A549 (lung), PC3 (prostate), HCT116 (colon), HT29 (colon), MCF-7 (breast), A431 (melanoma), HN5 (head and neck), and HeLa (cervix)—to violacein treatment under normoxic and hypoxic conditions. Almost all human tumor cells presented lower  $IC_{50}$  values under hypoxia, but HT29 was, by far, the most sensitive one—it recorded  $IC_{50}$  value 12.6-fold lower under hypoxia than the one recorded under normoxic conditions. Interestingly, the forementioned authors have found that  $IC_{50}$  values recorded for PC3 prostate cancer and A431

melanoma cells were not significantly affected by hypoxia (Hashimi et al. 2015).

### Violacein overcomes cancer hallmarks

Durán's group was the first to report violacein's anticancer property in 2003 (Melo et al. 2003). Since then, molecular mechanisms supporting the strong antitumor activity of this pigment have been investigated by different groups. In fact, cancer cells treated with violacein present broad spectrum of affected key signaling transduction mediators. Therefore, this compound is currently defined as cancer hallmark modulator. Along human tumor development, which is a multistep process, cells acquire biological plasticity; it was first defined by Hanahan and Weinberg as hallmarks of cancer (Hanahan and Weinberg 2000). These scholars have initially suggested six cancer cell abilities contributing to disease complexity, namely: supporting proliferative signaling, avoiding growth repressors, holding out cell death, enabling replicative immortality, causing angiogenesis, and invasion and metastasis activations. Eleven years later, two novel emerging hallmarks were included in the previous list, namely: deregulating cellular energetics and evading immune destruction (Hanahan and Weinberg 2011). The following paragraphs pinpoint how violacein overcomes some cancer hallmarks reported, so far: supporting proliferative signaling, holding out cell death, and activating metastasis and invasion.

**Violacein inhibits proliferative signaling** Healthy cells tightly control the synthesis and secretion of growth factors and, consequently, they ensure cell homeostasis, whereas cancer cells acquire the ability to continuously proliferate due to mutations and overactivation of key proteins capable of promoting cell cycle progression, such as receptor tyrosine kinase protein, mitogen-activated protein (MAP)-kinase route and PI3-kinase signaling circuitry comprising their key AKT signal transducer. Reports have shown that violacein treatment led to decreased AXL—which is a specific receptor of tyrosine kinase suppressor in melanoma and CD34<sup>+</sup>/c-Kit<sup>+</sup>/P-glycoprotein<sup>+</sup>/MRP1<sup>+</sup> TF1 leukemia progenitor cells (Liu et al. 2017; Queiroz et al. 2012). This kinase has been addressed as overexpressed in several human malignancy types, a fact that stimulates AKT, MAPK and FAK signaling pathways and evidences the role played by kinase in multiple oncogenic processes (Linger et al. 2013; Verma et al. 2011). Interestingly, AKT was inhibited by violacein in melanoma and colorectal cancer cases (Gonçalves et al. 2016; Kodach et al. 2006). According to Mojib et al. (2011), murine 2237 fibrosarcoma cells treated with violacein presented lower amounts of cyclin-dependent kinases (Cdk2, Cdk4 and Cdk6), key nuclear kinases accounting for cell cycle progression, and higher p53 and p21 (cell cycle blockers) levels. Accordingly, melanoma cell line (SKMEL-103) treated with violacein presented significantly decreased histone deacetylase-6 expression, which acts as proliferating activator in melanoma cells (Gonçalves et al. 2016). Masuelli et al. (2016) reported that violacein has inhibited head and neck cancer cell growth in vitro and in vivo.

**Violacein induces cell death** Although different cell death types have been described, apoptosis and autophagy are often the most explored ones. Violacein inhibits DAPK1, which contradicts both apoptosis and autophagic cell death in CD34<sup>+</sup>/c-Kit<sup>+</sup>/P-glycoprotein<sup>+</sup>/MRP1<sup>+</sup> TF1 leukemia progenitors (Queiroz et al. 2012). Solid and hematopoietic tumors subjected to violacein treatment presented decreased number of anti-apoptotic mediators and increased number of apoptotic mediators—Bcl2 and Bax, respectively (Alshatwi et al. 2016; Kodach et al. 2006; Mojib et al. 2011). Accordingly, Kodach et al. (2006) observed that colorectal cancer cells exposed to violacein presented increased p53 protein concentration, although this protein is often deleted or downregulated in cancer cells, as strategy to limit or circumvent apoptosis. More recently, it was reported that violacein was capable of inhibiting the autophagy process in melanoma cells, as evidenced by increased amount of p62 protein (Gonçalves et al. 2016). It is well-known that melanoma sustains high basal autophagy rate as cytoprotective strategy, since it allows cells to survive even under unfavorable environmental conditions, such as nutrient deprivation and/or the presence of cytotoxic materials, through recycling (Corazzari et al. 2013). Therefore, cell death due to apoptosis is

favored when violacein blocks autophagy. Findings reported by Gonçalves et al. (2016) have indicated that violacein can be useful in sensitizing cancer cells presenting high basal autophagy rate as drug-resistance strategy.

**Violacein inhibits metastasis and invasion** Metalloproteinases (MMPs) play essential role in metastatic processes, since metastasis requires extracellular matrix remodeling, since this matrix depends on MMPs (Conlon and Murray 2019). Platt et al. (2014) have shown that violacein was capable of decreasing MMP-2 and MMP-9 active forms. In addition, inflammatory chemokine secretion (CXCL12)—which is a positive cell migration and cancer metastasis modulator—has decreased after breast cancer cell line (MCF-7) treatment with violacein. This pigment has efficiently decreased the number of CXCR4 in the membrane through MMP-9 inhibition. Gonçalves et al. (2016) have also shown that violacein has significantly decreased the invasion capacity of a highly metastatic melanoma cell line (SKMEL-103) by using 3D culture model in matrigel.

In addition to investigate violacein's ability to change cancer hallmark levels, the study developed by Liu et al. (2017) has traced the proteomic profile of colorectal cancer cell line (HT29) response to two violacein doses (high and low). Results have shown that violacein inhibited cell growth in a concentration- and time-dependent manner. Increased violacein concentrations have led to vacuoles in the mitochondrion and membrane blebs, as shown by Transmission Electron Microscopy (TEM). Quantitative proteomic analysis has shown differential expression in 492 and 112 proteins from cells treated with high and low violacein concentrations, respectively. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis has shown that most of these differently expressed proteins were involved in 50 signaling pathways, such as ribosome, citrate cycle and RNA degradation; 10 of them were significantly enriched (Liu et al. 2017).

We have observed that purple pellet is obtained after cells treated with violacein are subjected to centrifugation, a fact that indicates interaction between this compound and the membrane. Therefore, violacein interaction with cell membrane models was investigated by using Langmuir monolayer selected lipids in order to validate our hypothesis. Extremely low violacein amounts have affected surface pressure-area isotherms, as well as the polarization-modulation reflection-absorption infrared spectra of lipid monolayers. These results have indicated significant violacein interaction with the membrane. Brewster Angle Microscopy (morphological analysis) monitoring has indicated that violacein at air–water interface was homogenized during its incorporation to lipids. Lipids acted on floating monolayer's viscoelastic and structural features over violacein interaction with them. These data helped better understanding violacein interaction at special membrane points (de Souza et al. 2017). Further studies

by Gupta et al. (2021) have shown that violacein interaction with lipid monolayer formed at the air–water interface depends on the electrostatic nature of lipids. Studies using X-ray reflectivity on solid supported lipid monolayer on hydrophilic substrate have shown significantly increased membrane thickness in zwitterionic and positively-charged lipids. Violacein has also induced changes in lipids tilt angle and in-plane ordering. Taken together, studies by de Souza et al. (2017) and Gupta et al. (2021) provided detailed information on how violacein induces the structural reorganization of lipid molecules, which can explain the mechanisms enabling its interaction with cell membranes. Interestingly, studies conducted with *Staphylococcus aureus* reported that violacein was capable of inducing cell permeabilization, which was followed by the emergence of visible discontinuities in the cytoplasmic membrane (Cauz et al. 2019). If one takes into account that violacein has broad capacity of modulating different signaling pathways, such as growth factor receptors, these results can be associated, at least in part, with violacein interactions with lipid surfaces.

## Antibacterial activity of violacein

Since the discovery of this purple bisindole derivative, different researchers have been carrying out studies to elucidate its antimicrobial effects on several microorganisms capable of causing severe infections to humans and to other animals. Outstanding antimicrobial activity against Gram-positive bacterial strains, such as *S. aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Vibrio cholerae*, and *Salmonella typhi*, has been reported. Cytoplasmic membrane disruption is a major mechanism assumingly associated with this activity. According to Cauz et al. (2019), liposomes made by commercial and bacterial phospholipids were disrupted after direct interaction with violacein, leading to their increased permeability. Moreover, a two-step process was suggested as accountable for violacein-induced antibacterial action (Aruldass et al. 2018). Firstly, membrane permeabilization induces proton, ion, ATP and protein leakage from cells. Finally, intracellular content loss leads to osmotic imbalance, which may induce cell death (Aruldass et al. 2018).

The emergence of multidrug resistant bacteria is a health issue worldwide; *S. aureus* is one of the most opportunistic pathogens associated with the development of multidrug resistance and hospital-associated infections (Cardozo et al. 2013). It is well-known that the number of MRSA infection cases has increased in the last few decades, and it was followed by increased resistance to current antibiotic therapies (Nathwani et al. 2014). Thus, new drugs capable of overcoming resistance issues are extremely desirable. Interestingly, violacein synthesized by *C. violaceum*

UTM5 was also effective against MRSA ATCC 43,300. It recorded minimum inhibitory concentration (MIC) value of  $15.6 \mu\text{g mL}^{-1}$ , which was twice the MIC value recorded for *S. aureus* ATCC 29,213 (Aruldass et al. 2015). Nevertheless, violacein has shown bacteriostatic activity against both *S. aureus* strains (ATCC 43,300 and ATCC 29,213); it recorded MIC value of  $3.9 \mu\text{g mL}^{-1}$ . Furthermore, fluorescent dyes used for membrane integrity evaluation and TEM analysis have confirmed membrane rupture as violacein's major antibacterial action mechanism (Aruldass et al. 2018).

Interestingly, violacein has also shown strong antimicrobial effect on *S. aureus* isolated from bovine mastitis, which is a chronic dairy farm-associated disease that typically presents multidrug resistance and leads to considerable economic losses (Cazotto et al. 2011). It is also important mentioning that, in addition to its antibacterial activity, violacein has shown synergistic effect, together with other antimicrobial compounds, on mastitis; this outcome suggested violacein use in combined treatments (Cazotto et al. 2011). Thus, it is reasonable prospecting violacein as antibiotic in vivo used to treat both human and animal diseases.

Several reports have shown synergistic effect of violacein associated with commercial antibiotics. Dodou et al. (2017) have shown the antimicrobial activity of violacein combined to other antibiotics against *S. epidermidis*. According to another study, violacein interacts with commercial antibiotics in a synergistic manner, and it enhances drug effectiveness (Priya et al. 2018). MIC recorded for violacein used in association with other commercial antibiotics against uropathogenic *E. coli* is summarized in Table 2. It was possible observing that violacein acts synergistically (> 55%) with commercial antibiotics and that it could be certainly used as antibiotic in association with other antimicrobial agents (Priya et al. 2018).

**Table 2** Minimum inhibitory concentration (MIC) of violacein and its combinations with commercial antibiotics against uropathogenic *E. coli*

Agent used ( $\mu\text{g}$ )	I	M	F	Note
Violacein	28.7	–	–	–
Ceftazidime	20.1	11.9	1.0	A
Aztreonam	3.9	1.7	0.48	S
Cefuroxime	12.3	4.2	0.41	S
Cefazolin	14.1	6.5	0.66	A
Tobramycin	11.7	3.6	0.52	A
Meropenem	3.7	1.9	0.18	S
Ampicillin/sulbactam	4.1	3.7	0.28	S
Cefepime	15.0	7.2	0.77	A
Amikacin	12.7	8.6	0.90	A

Modified from Priya et al. (2018)

I individual, M mixture, F fractional inhibitory concentration index, S synergistic, A additive



Subramaniam et al. (2014) have tested the activity of violacein-macrolide and violacein-aminoglycoside class combinations against major pathogens, such as *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *V. cholera*. These combinations have shown Fractional Inhibitory Concentration Indices (FICI) lower than 0.5, and it has evidenced synergistic effect (Subramaniam et al. 2014). According to this very same study, violacein-azithromycin and violacein-kanamycin association resulted in high synergistic effect (FICI=0.3) on *Salmonella enterica* serovar Typhi strain. Therefore, violacein can be used as potential synergistic antibiotic in combination to other antimicrobial agents.

Microbial growth in biofilm also plays important role in providing different defense mechanisms against microorganisms during infection processes. Besides preventing the access of immune cells to the infection site, biofilm matrix increases microorganisms' resistance to antimicrobial agents and contributes to infectious processes (Savage et al. 2013). Violacein has shown high antimicrobial activity in suppressing and killing *S. aureus* in biofilm formation processes and in planktonic cultures. Minimum bactericidal concentration (MBC) of  $5 \mu\text{g mL}^{-1}$  violacein has killed *S. aureus* after 3–4 h exposure, whereas MIC of  $1.25 \mu\text{g mL}^{-1}$  has suppressed bacterial growth within the first 8 h of interaction. Biofilm formation was also strongly inhibited at this very same violacein concentration. On the other hand, higher *S. aureus* resistance ( $40 \mu\text{g mL}^{-1}$ ) was observed in mature biofilm (Batista et al. 2017). These results suggest that increased bacterial metabolic activity (exponential growth phase) may favor violacein action, which appears to involve macromolecule synthesis. However, since violacein has also shown antimicrobial activity against *S. aureus* at all growth stages, although at different intensities, different mechanisms may have operated to explain violacein's action (Batista et al. 2017). Dodou et al. (2019) have also investigated the antimicrobial potential of violacein deriving from *C. violaceum* ATCC 12,472 against *S. epidermidis* biofilm. Violacein, at concentrations of  $20 \mu\text{g mL}^{-1}$  and  $160 \mu\text{g mL}^{-1}$ , was capable of inhibiting biofilm formation and eradicating mature biofilm, respectively. Moreover, these effects took place within less than 3 h of incubation.

Another interesting approach adopted to overcome multidrug resistance was reported by Mitchell's group. They used a combination of violacein—which is effective against Gram-positive bacteria—and *Bdellovibrio bacteriovorus* HD100—which predated on Gram-negative strains—as strategy against resistant mixed species communities (Im et al. 2017). These agents were used alone, in mixed bacterial cultures comprising Gram-positive and Gram-negative strains, but they were only effective against their respective strains. On the other hand, the combined treatment reduced by 84,500 times the total numbers of pathogens. Decrease by 99.98% in cell viability was observed after treatment

application to a mixed culture of *Acinetobacter baumannii*, *S. aureus*, *K. pneumoniae* and *Bacillus cereus* based on the association between violacein and *B. bacteriovorus*. On the other hand, reductions by only 19% and 68% were observed when violacein and *B. bacteriovorus* were used alone, respectively (Im et al. 2017).

Nanoparticles associated with drugs tend to increase their effectiveness, delivery and safety profiles. Accordingly, violacein deriving from *C. violaceum* and silver nanoparticles (AgNPs) deriving from *Fusarium oxysporum* have shown synergistic effect on several resistant microorganisms (Sarmiento et al. 2016). Arif et al. (2017) took advantage of AgNPs' therapeutic properties to synthesize violacein capped silver nanoparticles (vAgNPs), and compared their antimicrobial and antialgal properties to that of starch capped silver NPs (cAgNPs). MICs of vAgNPs were far lower than those recorded for cAgNPs, which showed 3–10 times higher therapeutic action against multidrug resistant bacteria (*P. aeruginosa*, *E. coli*, and *S. aureus*), and algae (*Dictyosphaerium* sp. strain DHM 1, *Pectinodesmus* sp. strain PHM 3 and *Dictyosphaerium* sp. strain DHM 2). Importantly, violacein capping seems to induce AgNPs' selectivity to Gram-positive bacteria. Thus, the aforementioned report has indicated a promising strategy to enhance not only AgNPs' stability and bioavailability, but also to modulate their therapeutic mechanism (Arif et al. 2017).

## Violacein effects on parasites and on their vectors

In 2000, WHO has estimated 12–15 million leishmaniasis cases worldwide, with approximately 70,000 deaths per year, as well as that 350 million individuals would be at risk of acquiring the infection in 89 countries (Torres-Guerrero et al. 2017). With respect to trypanosomiasis, approximately 8 million individuals are infected with *Trypanosoma cruzi* in America (*i.e.* Chagas disease) and more than 10,000 patients die of this infection on a yearly basis; whereas approximately 60 million people are at risk of getting infected with Human African trypanosomiasis (*i.e.* sleeping sickness). In addition, 212 million new malaria cases and approximately half a million death cases were estimated worldwide, among other parasitic diseases (Martin and Soby 2016; World Health Organization 2017). These data do not reflect small advances in therapies used to treat human parasites in the last decades, although most antiparasitic drugs were developed 25 to 40 years ago. Moreover, increased pathogen resistance to commercial drugs, restricted biocide activity, and undesirable secondary effects on humans during extended treatments (Islan et al. 2017) effectively contribute to increase the number of infected individuals.

The main interest in using violacein as antiparasitic molecule is based on its reported leishmanicidal, trypanocidal, antioxidant and antiprotozoal activities, among others (Leon et al. 2001; Matz et al. 2004). Since *Chromobacterium* spp. produces multiple biocide compounds, the role played by violacein as antiplasmodial and trypanocidal agent was investigated by cloning *vioABCDE* or *vioABCE* operons for violacein and deoxyviolacein, respectively, in several plasmids. This procedure was followed by *E. coli* BL21 transformation and by the expression of both molecules (Bilsland et al. 2018). Violacein  $IC_{50}$  was  $1.51 \pm 0.4 \mu\text{M}$ , which corresponded to half benznidazole's  $IC_{50}$ —benznidazole is the drug most often used to treat trypanosomiasis; its  $IC_{50}$  in *T. cruzi* (Tulahuen strain) is  $3.07 \pm 0.6 \mu\text{M}$ . The same experiment was performed with *Plasmodium falciparum*, wild-type (3D7 strain, sensitive to chloroquine) and chloroquine-resistant (W2 strain) strains; results have shown violacein  $IC_{50}$  values of  $0.4 \mu\text{M}$  and  $0.5 \mu\text{M}$ , respectively, whereas deoxyviolacein  $IC_{50}$  values were approximately  $11 \mu\text{M}$  and  $14 \mu\text{M}$ , respectively. Comparative violacein and deoxyviolacein toxicity analysis applied to *P. falciparum* and red blood cells has shown approximately 5 and 20 times more sensitivity to the parasite than the sensitivity recorded for mammalian cells, respectively (Bilsland et al. 2018). Similar results were previously reported for violacein extracts used against *P. falciparum* and *P. chabaudi* (Lopes et al. 2009). In addition, Canuto et al. (2019) reported ROS induction and changes in mitochondrial transmembrane using as potential action mechanisms.

Another study focused on investigating violacein's effect on *Acanthamoeba castellanii*—which is a protozoan species capable of causing severe diseases, such as amebic keratitis and granulomatous amebic encephalitis—has shown that  $1 \mu\text{M}$  of violacein was capable of producing 100% amoeba lysis, which was triggered by apoptotic mechanisms determined through TUNEL and caspase-3 assays (Matz et al. 2008).

Several pathologies affecting humans are caused by nematodes, such as angiostrongyliasis, ascariasis, enterobiasis, filariasis, hookworm and trichinosis, among others. The biocide effect of violacein on nematode species *Caenorhabditis elegans* was evaluated (Ballestriero et al. 2014). Besides *C. elegans* resistance to violacein,  $LC_{50} > 30 \mu\text{M}$  (although lower than that of common drugs), its accumulation in nematodes' intestine was considered the main cause for their increased mortality rate. The authors of the aforementioned study have speculated that violacein has compromised nematodes' immune system, which led to reduced ability to control bacterial concentrations in the gut and, consequently, to death (Ballestriero et al. 2014). Interestingly, they further showed that the investigated worms presented learned aversion to bacterial metabolites (Ballestriero et al. 2016). Yoon

et al. (2020) have recently reported that violacein is not only toxic to adults, but it also inhibits *C. elegans* larval development. In addition, matrophagy—i.e., mothers hold their eggs within their bodies until young larvae hatch inside them—happens to avoid some of the toxic effects of violacein. Moreover, the aforementioned study also helped elucidating that consumption of unsaturated fatty acids, such as oleate—which is produced by nematode mothers—has mitigated violacein toxicity (Yoon et al. 2020). If one takes into consideration mechanisms elicited by oleate in *C. elegans*, the study by Yoon et al. (2020) have also explained violacein activity against other pathologies. In another interesting study, violacein-5'-*O*-glucoside was produced by *E. coli* harboring the violacein biosynthetic gene cluster *vioABCDE* and expressing a *Bacillus* glycosyltransferase (*YjiC*) enzyme (Lee et al. 2019). This derivative increased violacein's solubility in water and presented effective anti-nematodal activity against the causative agent of pine wilt disease, namely *Bursaphelenchus xylophilus* (Lee et al. 2019).

Similarly, changes in midgut susceptibility to viruses and parasites in mosquitoes fed on violacein or violacein extracts were observed (Bahia et al. 2014; Ramirez et al. 2014). *Chromobacterium* spp. strains, such as *C. subtsugae*, *C. sphagni* and *C. vaccinii*, have shown insecticidal activity capable of killing *Aedes aegypti* and *Anopheles* spp. larvae, as well as vectors of *Plasmodium* spp., which is the etiological agent of malaria (Blackburn et al. 2017; Farrar et al. 2018; Vöing et al. 2015). For example, MWU205 strain produces approximately  $7.9 \times 10^{-6}$  ng/cell and  $1.3 \times 10^{-6}$  ng/cell extra- and intra-cellular of violacein, respectively. This amount of violacein is capable of killing diamondback moth (*Plutella xylostella*) larvae, although it only kills 40% and 50% of *A. aegypti* larvae 16 h and 72 h after insect hatching. However, *C. vaccinii* strains MWU300 and MWU328 are capable of killing *A. aegypti* larvae 16 h after hatching; this outcome is comparable to the mosquitocidal activity of *B. thuringiensis* IBL10003 toxin, which is often used as reference strain (Martin and Soby 2016).

Moreover, bacterial extracts of novel isolated *C. sphagni* strains IIBBL 14B-1 (NRRL B-67130) and 37-2 (NRRL B67131) were toxic to some lepidopteran insects (Blackburn et al. 2018). Gypsy moth (*Lymantria dispar*), diamondback moth (*Plutella xylostella*), tobacco hornworm (*Manduca sexta*), and cabbage looper (*Tricoplusia ni*) larvae fed on *C. sphagni* strain extracts presented decreased pupation, or larvae weight, or survival by approximately 30% to 40% (Blackburn et al. 2018). Moreover, Blackburn et al. (2018) and Martin et al. (2007) reported larvae inhibition in corn earworm (*Helicoverpa zea*), Colorado potato beetle (*Lepidotarsa decemlineata*), beet armyworm (*Spodoptera exigua*), fall armyworm (*Spodoptera frugiperda*), European corn borer (*Ostrinia nubilalis*), and tobacco budworm

(*Heliothis virescens*) specimens fed on *C. sphagni* IIBBL 14B-1 (NRRL B-67130) and 37-2 (NRRL B67131) cultures.

Violacein deriving from *C. violaceum*, and its combination to silver and gold nanoparticles (phytosynthesized), were reported for *P. falciparum* and *T. brucei* gambiense growth inhibition in vitro. Violacein association ( $IC_{50} = 51.8 \mu\text{g mL}^{-1}$ ) with silver nanoparticles has shown reduction by ~1.2-fold in  $IC_{50}$  values recorded for both parasites, and it slightly reduced the cytotoxicity in mammalian cells (peripheral blood mononuclear cells and cancer cell lines, such as HeLa and MCF7) (Rahul et al. 2015). However, previous reports have shown values close to  $2 \mu\text{g mL}^{-1}$  for *P. chabaudi chabaudi* and  $0.68 \mu\text{g mL}^{-1}$  for *P. falciparum* (Lopes et al. 2009). This difference could be associated with violacein purity level.

### Antifungal activity of violacein

Most studies focused on investigating violacein's antifungal activity were based on diseases affecting amphibians, such as chytridiomycosis (Scheele et al. 2019), or with fungi capable of damaging soybean crops.

Barreto et al. (2008) carried out a study with *C. violaceum*, which was used as model representative of the Amazonian biodiversity, against seven fungal pathogen strains affecting soybeans crops in Brazil. They used the paring method to analyze whether *C. violaceum* cells and metabolites, such as violacein, found in the supernatant of the investigated cultures were capable of inhibiting the selected fungal species growth. Seven-day inhibition results have shown that cells and supernatants of a whole variety of strains presented different antifungal activities against *Fusarium* sp., *Phomopsis* sp., *Corynespora* sp., *Aspergillus* sp., *Colletotric kikuchi* and *Cercospora kikuchi* strains (Barreto et al. 2008). The aforementioned study has emphasized the paramount importance of Brazilian biodiversity, since different *C. violaceum* strains were capable of acting against six different pathogenic fungal types that harm soybean tillage and cause significant economic losses in Brazil.

Another important environmental issue caused by fungi lies on the decrease and even extinction of different amphibian species worldwide, mainly due to chytridiomycosis, for which fungal pathogen *Batrachochytrium dendrobatidis* is the key agent (Brucker et al. 2008a, b; Harris et al. 2009; Sasidharan et al. 2015; Woodhams et al. 2018). Brucker et al. (2008b) have used *J. lividum* culture to investigate a mutualism mechanism observed in nature to inhibit *B. dendrobatidis* growth on amphibian skins. The study was based on the comparison between skin samples from salamander species *P. cinereus* to other organisms investigated in the literature. In addition, two main metabolites were isolated from *J. lividum*, namely: indole-3-carboxaldehyde

and violacein; they presented antifungal activity against *B. dendrobatidis*, even at relatively low concentrations (68.9 and  $1.82 \mu\text{M}$ , respectively), over a seven-day period-of-time (Brucker et al. 2008b).

Harris et al. (2009) conducted a study with another amphibian species and also found decreased mortality and morbidity rates associated with chytridiomycosis, due to the presence of *J. lividum*. Amphibians were divided into three major groups, namely: the ones only subjected to *B. dendrobatidis* zoospores, the ones only subjected to *J. lividum*, and the ones subjected to both. Swab collections were performed at different times to estimate violacein and *B. dendrobatidis* concentrations. Violacein was only found in frogs subjected to *J. lividum*, and it suggested that the additional presence of this bacterium can activate the production of secondary metabolites capable of protecting these amphibians (Harris et al. 2009).

Woodhams et al. (2018) have also investigated the important role played by symbiosis as defense mechanism in amphibian species. They used 20 amphibian species to investigate the presence of two metabolites—prodigiosin and violacein—and they tested their antifungal activity against *B. dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal). Results have shown violacein in six *J. lividum* isolates, as well as prodigiosin in five *Serratia plymuthica* and *S. marcescens* isolates. With respect to the antifungal activity of commercial metabolites, prodigiosin has significantly inhibited fungal growth—it recorded MIC values of 10 and  $50 \mu\text{M}$  for *Serratia plymuthica* and *S. marcescens* isolates, respectively,—whereas violacein MIC value recorded for Bsal and Bd was approximately  $15 \mu\text{M}$ . According to the aforementioned authors, even if the relative abundance of bacteria generating these protective secondary metabolites is low, lower than 1%, their prevalence is high (Woodhams et al. 2018). Thus, the presence of one, or more, bacteria in amphibians' skin can considerably decrease their chytridiomycosis-associated mortality rates.

Sasidharan et al. (2015) have isolated a new *Chromobacterium* sp. strain, NIIST (MTCC 5522), from clay mine acidic sediment, which was capable of producing large violacein amounts. The aims were to develop a high-yield violacein purification process and to test its antifungal activity. Fungi accountable for causing diseases in plants and humans, such as *Aspergillus flavus* MTCC 183, *Cryptococcus gastricus* MTCC 1715, and *Trichophyton rubrum* MTCC 296; as well as agriculturally significant fungi, such as *Fusarium oxysporum* MTCC 284, *Rhizoctonia solani* MTCC 4634, and *Penicillium expansum* MTCC 2006, were selected based on disc diffusion method. Violacein's antifungal potential was compared to that of commercial antifungals, such as bavistin and amphotericin B; violacein was more efficient against 4 of the 8 tested fungi. The aforementioned authors believe that violacein associated with

other compounds is a product with great potential to be used against fungal infections (Sasidharan et al. 2015).

Arif et al. (2017) have coupled silver nanoparticles to violacein (vAgNPs) and evaluated the pharmacological profile of this new molecule against different fungal species, such as *Aspergillus tamari*, *A. tubingensis* and *F. proliferatum*, as an attempt to solve the violacein solubility issue. Results have shown increased efficiency in vAgNPs' antifungal activity (10 mg and 1 mg) in comparison to that of starch capped AgNPs (cAgNPs). The overall susceptibility pattern of fungal species against vAgNPs presented the following order: *A. tamari* > *A. tubingensis* > *F. proliferatum* (Arif et al. 2017). Thus, although still modest, nanotechnology is an important tool used to help improving the antifungal activity of new or already used compounds in therapies.

## Inflammation

Despite the accumulating evidence suggesting that violacein has several pharmacologic effects, its anti-inflammatory and anti-oxidant activities have only recently started to be investigated in acute and chronic diseases (Durán et al. 2016; Justo and Durán 2017). Verinaud et al. (2015) were the first to report violacein's ability to protect mice from acute and chronic autoimmune inflammation. Violacein was capable of reducing the production of inflammatory cytokines such as IL-6 and CXCL1 when it was intraperitoneally administered to mice (3.5 mg kg<sup>-1</sup>) at the same time they received 1 µg LPS. Violacein has also reduced neutrophil infiltration in the peritoneal cavity; thus, no changes in T- and dendritic cell (DC) populations, or in the frequency of B cells, were observed. Curiously, serum IL-10 levels have increased after violacein treatment, and it suggested the modulatory effect of this bisindole on cytokine and chemokine. This outcome is consistent with violacein's ability to downregulate the interaction between inflammatory CXCL12 and CXCR4, which play important role in breast cancer cell adhesion and metastasis processes (Platt et al. 2014). Experimental autoimmune encephalomyelitis (EAE) was induced in mice to evaluate violacein's ability to counteract chronic inflammation progression. Treatment with 3.5 mg kg<sup>-1</sup> of violacein for 3 days has significantly reduced the clinical course of the disease, central nervous system (CNS) inflammation and cellular infiltration in mice. In addition, regulatory T cells (Tregs) play important role in controlling autoimmune inflammation processes (Sakaguchi et al. 2008; Thomé et al. 2013, 2014; Thornton and Shevach 1998). Increased Tregs frequency was also observed in mice treated with violacein. Most importantly, CNS gene expression profile has evidenced that violacein significantly induced increase in IL-10 and indoleamine 2,3-dioxygenase (IDO) expression levels, which appears to modulate DC and Tregs. Furthermore,

adoptive transfer of violacein-elicited Tregs (CD4<sup>+</sup>CD25<sup>+</sup>) has significantly reduced EAE, and it confirmed violacein's ability to suppress the disease.

As previously reported (Bromberg et al. 2010), Verinaud et al. (2015) have also found violacein toxicity at higher dose (7 mg kg<sup>-1</sup>). However, antimalarials also present toxicity; similar to chloroquine, violacein-induced DC immature phenotype may be an interesting approach to induce anti-inflammatory profile (Thomé et al. 2014). Interestingly, recent study has calculated violacein properties such as Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) (Verma and Pandey 2017). LD50 of 500 mg kg<sup>-1</sup> was observed, and it suggested that it may be safe for humans at doses lower than 300 mg kg<sup>-1</sup>. Furthermore, the aforementioned study has also shown that this compound follows the Lipinski's rule, a fact that suggests its potential to be used as drug.

Venegas et al. (2019) have recently attempted to clarify the role played by TNF-α in violacein activity, since violacein's ability to induce or suppress this cytokine remains unclear (Alshatwi et al. 2016; Antonisamy et al. 2014; Ferreira et al. 2004; Platt et al. 2014; Verinaud et al. 2015). These authors have used different immune cell lines and peripheral blood mononuclear cells (PMBCs) to show that violacein has immunostimulatory effect on different cell lines, as well as on PBMCs. Furthermore, TLR-transfected HEK-293 cells and molecular docking studies have clearly indicated that this effect could be associated with signaling through TLR8, thus stimulating pro-inflammatory response, which is consistent with previous reports (Alshatwi et al. 2016; Ferreira et al. 2004). Gene expression analysis has further suggested negative TLR signaling feedback mechanism, which could explain the anti-inflammatory effect observed in animal models (Antonisamy et al. 2014; Verinaud et al. 2015).

Several reports (reviewed in Durán et al. 2016; Justo and Durán 2017) have indicated that molecular mechanisms subjacent to violacein targets are diverse and suggested associations of multiple signaling pathways. Based on these previous studies, Verma and Pandey (2017) performed molecular docking analysis in violacein, MMP-2 and MMP-9 in order to investigate the inhibitory potential of this indole derivative. Matrix metalloproteinases play important role in cerebral ischemia, as well as in many other diseases, such as cancer, cardiovascular diseases and osteoarthritis (Dong et al. 2009; Lakhan et al. 2013; Romanic et al. 1998). The study by Verma and Pandey (2017) has clearly provided support for direct MMP-2 and MMP-9 inhibition by violacein—which recorded Ki values of 2.12 and 56.14 nM, respectively—and high binding energies. Most importantly, these Ki values are approximately 100 (MMP-2) and 6 times (MMP-9) lower than those recorded for resveratrol and quercetin in a study based on this very same approach, respectively (Pandey et al.

2012, 2015). In fact, several natural products have been suggested as MMP inhibitors to treat ischemic conditions (Lee et al. 2010; Sarkar et al. 2016). Thus, ADMET analysis, together with violacein's MMP inhibitory activity, supports its use to treat ischemic pathologies, as well as other potential inflammatory diseases.

## Violacein applications

Violacein is a purple pigment with potential to be used in a wide range of biological applications, such as photoprotective, antioxidant, anticancer, antiprotozoal and antibacterial agent (Berti et al. 2019; Choi et al. 2015b, 2017; Durán et al. 2016, 2020). However, one of the most interesting violacein applications lies on a natural process. Species *Duganella* spp. lives in the soil and in the rhizosphere of cultivated and wild olive trees in Southern Spain; it protects them from pathogens (Aranda et al. 2011). In addition, the combined antibiotic effect of violacein and *B. bacteriovorus* HD100 on Gram-positive MRSA was interesting, since they did not antagonize each other (Im et al. 2017). There was expressive reduction in bacterial viability when these agents were used in combination to one another, a fact that reinforced their prospective application against mixed species populations, as discussed above (Im et al. 2017). Nakazato et al. (2019) have recently suggested the combined use of violacein and biologically prepared silver nanoparticles (bio-AgNPs). Violacein-bioAgNPs have shown increased synergistic antimicrobial activity against *S. aureus* and *E. coli*. According to Choi et al. (2020), *C. violaceum* produced extracellular membrane vesicles that can be used to solubilize and transport violacein to other microorganisms, such as *S. aureus*, against which violacein has shown antibacterial activity; 90% of microorganisms died after 6 h of treatment with 3.1 mg L<sup>-1</sup> of violacein. Further studies conducted by Batista et al. (2020) have also shown that outer membrane vesicles produced by *C. violaceum* delivered violacein to mediate its toxicity at long distance. Furthermore, this membrane vesicle release was achieved by the concerted expression of two quorum sensing-regulated pathways, namely: violacein biosynthesis and VacJ/Yrb system. Thus, violacein can make its own secretion easier when membrane vesicle production decreases due to high cell density.

Swelling pectin-gelatin and TWEEN® 20 matrices loaded with violacein were developed by Berti et al. (2019) and tested against HCT116 colorectal cancer cells. Pectin-gelatin matrix had protective effect on the digestive tract, until the colon was reached, a fact that showed reduced cytotoxicity to cells. Polysorbate 20 microemulsions prepared with violacein avoided pigment precipitation inside the human body and effectively reduced colon cancer cell viability in a dose-dependent manner (Berti et al. 2019). An interesting

strategy was recently developed to overcome violacein's poor solubility in aqueous solutions and to reduce its toxicity to non-tumorigenic cells. Based on the interesting physico-chemical properties of ionic liquids (ILs) and on the likelihood of tailoring biological aspects, such as cytotoxicity and biodegradability, these ILs have shown important applications in several fields, such as biomedicine. Surface-active ionic liquids (SAILs) are an important class of ILs, which has surfactant properties and can be used to help developing drug delivery devices for hydrophobic molecules. According to Berti et al. (2020), SAILs based on cation 1-alkylimidazolium were used to dissolve violacein in micellar aqueous media [Viol-([C16Him]-S)]; then, this complex was used to develop an efficient hybrid solid lipid nanoparticle (SLN) carrier tailored with folate in order to target cancer cells. The uptake of these SLN was tested in cancer (HCT116 and HeLa) and non-tumorigenic (A549) cell lines; remarkably higher incorporation was observed in the former cells—this outcome is consistent with their higher expression of folate receptors. The study of Berti et al. (2020) has provided a new effective formulation for violacein delivery to cancer cells with potential application in therapy.

In addition, violacein and its derivative have shown interesting properties for cosmetic applications, mainly if one takes into consideration its antimicrobial protection property in decorative cosmetics and pharmaceutical products (Kallmayer et al. 2005). German pharmaceutical industry Beiersdorf has protected several cosmetic formulations, such as lipstick, gloss lip, paste, lipstick against herpes, mascaras, foundation, and nail polish, as well as healthcare emulsions and formulations for antimicrobial skin protection, deodorant, antiperspirant, and anti-dandruff shampoo, which were produced based on the addition of violacein, deoxyviolacein and/or violacein, and of their derivatives tri-acetyl-violacein and diacetyl-(di)-methyl-violacein and/or its furan analogs (Kallmayer et al. 2005). Violacein is also acknowledged as oxidase tyrosinase inhibitor, which has catalytic activity in melanin generation in skin pigmentation processes, such as freckles and spots (Hidachi et al. 2017). Satoshi and Takatoshi (1998) focused on producing a natural antimicrobial antioxidant to be applied on the skin. They proposed a process of constant large scale violacein production from *Chromobacterium* sp. or *Janthinobacterium* sp., and natural antioxidant and antimicrobial application as cosmetic products, such as gel, oil, surfactant, polyhydric alcohol, lower alcohol, perfume, pigment, dye antioxidant, ultraviolet absorber, ultraviolet scattering agent, purified water, moisturizing agent and cosmetic ingredients, among others (Satoshi and Takatoshi 1998). Another interesting violacein application was introduced in a Taiwan patent as environmentally friendly nail polish product and cuticle cream. The cosmetic and pharmaceutical industry has been making great effort in the last 20 to 30 years to reduce the use of synthetic

dyes capable of causing skin rashes and allergies (Rongzhen and Yuwen 2017). A World patent from 2012 has described the use of violacein isolated from *Corbetia marina* as dye additive in aquaculture to help increasing fish immunity (Tapia et al. 2012).

Kato et al. (1998) have acknowledged the dyeing or coloring additive properties of violacein and of its derivatives. Purple pigments dyed cotton fibers and silk fabric by dispersing the textile in a mix of cell body suspension comprising microorganisms deriving from *C. violaceum*, *A. luteoviolacea* or *J. lividum* S9601 strains. Accordingly, a violacein-AgNPs combination was recently used to dye silk composite with violacein presenting antimicrobial activity (Gao et al. 2019). Excellent antimicrobial activity was observed, since the investigated combination was capable of reducing by more than 99% the incidence of microorganisms, such as *S. aureus*, *E. coli* and *C. albicans*.

Gomez-Gomez et al. (2019) have shown that violacein production can be also controlled at quorum signaling molecule level. They used selenium nanoparticles (SeNPs) as quorum sensing-mediated violacein synthesis inhibitors in *C. violaceum*. Schaeffer et al. (2019) adopted a sustainable approach based on thermo-responsive system that combined sodium dodecyl sulfate surfactant to tetrabutylammonium chloride salt to enable solid-liquid violacein extraction from bio-engineered *Yarrowia lipolytica* yeast. This procedure was followed by 1:1 menthol:thymol hydrophobic eutectic solvent (HES) for violacein back-extraction, which also resulted in contaminant protein precipitation at the solvent interface. This thermo-responsive aqueous surfactant-based system is a reasonable alternative for the extraction of highly-hydrophobic value-added products.

Venil et al. (2015) have proposed the technique of new commercial violacein powder for application as pigment in jelly and yogurt. Products added with *C. violaceum* violet pigment remained stable for one storage month; most importantly, they achieved stability for 30 days at pH 7, and temperature ranging from 25 to 60 °C.

## Conclusions

Violacein is a multi-task compound used in food and cosmetics; however, given its potent anticancer, antiprotazoal and antimicrobial effects, it has huge potential to be deeply explored as potent drug, mainly if its side effects are mitigated and different methods focused on large-scale production are developed. In addition, further investigation is necessary to identify new violacein applications. For instance, Pauer et al. (2018) have recently reported violacein impact on gut microbiota. Treatment with low and high violacein doses has differentially shifted the

composition of bacterial communities, a fact that may benefit the hosts, such as the ones affected by syndromes associated with inflammatory diseases. Better understanding violacein activities and its effects on healthy microbiota will certainly provide the basis to the development of new therapeutic strategies. Another important research line focuses on improving violacein production. There is a vast literature on violacein production by using bacteria, recombinant DNA technology, yeast or even on its production in situ, which may be extremely useful to help controlling violacein load, as well as to direct its effects towards target cells. Other hot topics associated with violacein research comprise investigations on guided drug delivery, due to differential uptake by cancer cells, as well as on potentiating violacein effects through synergistic actions with other drugs, such as antibiotics. This biopigment is one of the promising agents used to combat multidrug-resistant pathogens, and it opens significant room to treat emerging diseases capable of overcoming traditional therapy. It is also worth mentioning the great advances in the knowledge about violacein mechanisms of action, mainly about its interaction with membranes to induce cell death. Furthermore, according to Choi et al. (2020), violacein is secreted within membrane vesicles of bacteria capable of producing it; these violacein-carrying vesicles show effective bactericidal activity. Finally, secondary metabolites may also play complex roles in microbial communities. Lozano et al. (2020) have recently reported violacein production by *C. violaceum* in response to sublethal hygromycin A concentrations deriving from *Streptomyces* sp., in a mechanism dependent on translational polypeptide elongation inhibition and on a previously unknown two-component regulatory system. Studies in this field not only bring insights into the dynamics of soil communities, but they also shed light on undesired effects of clinical antibiotic administration. However, there is yet much to be discovered for such great applications; thus, it is necessary performing extensive research about violacein effects to avoid cumulative and undesired reactions in humans and in the environment.

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## Declarations

**Conflict of interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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