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Bacterioruberin from *Haloferax mediterranei* Triggers Cytotoxic and Pro-Oxidant Effects in Different Solid Tumour Models, Effectively Targeting P-gp-Resistant Lung Cancer Cells

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Abstract

Bacterioruberin (BR), a C₅₀ carotenoid produced by halophilic archaea, is emerging as a bioactive molecule with potential anticancer relevance, but its activity in solid tumour and multidrug-resistant (MDR) models remains poorly defined. This in vitro study evaluated the cytotoxic, antiproliferative and growth-inhibitory effects of a chemically characterized bacterioruberin-rich carotenoid extract (BRCE) from *Haloferax (H.) mediterranei* in A549 lung adenocarcinoma, BT-549 triple-negative breast cancer and WM115 melanoma cells, as well as in paired sensitive/multidrug resistant (MDR) lung cancer models. Metabolic activity and proliferation were assessed by thiazolyl blue tetrazolium bromide (MTT) and carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) assays, intracellular reactive oxygen species (ROS) by 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) staining, and apoptosis-associated morphology by acridine orange/ethidium bromide (AO/EB) staining. Growth inhibition in A549/A549-CDR2 and NCI-H460/NCI-H460/R cells was analysed by sulforhodamine B (SRB) assay, while P-glycoprotein (P-gp) function and expression were examined using Rhodamine 123 (Rh123) accumulation and Western blotting. BRCE reduced metabolic activity and proliferation in a concentration- and time-dependent manner, increased intracellular ROS levels, and induced apoptosis-associated morphological changes in A549 cells. In MDR models, BRCE retained comparable growth-inhibitory activity in sensitive and resistant cells and partially attenuated P-gp-related drug efflux. These findings support further mechanistic investigation of BR in solid tumour and MDR cancer models.

Keywords: apoptosis; bacterioruberin; *Haloferax mediterranei*; multidrug resistance; oxidative stress; P-glycoprotein



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1. Introduction

Cancer remains a major global health challenge and a leading cause of mortality, with the efficacy of treatment in solid tumours often limited by marked genetic and phenotypic heterogeneity, metastatic progression and, critically, the emergence of drug resistance [1–3]. These factors substantially reduce the long-term efficacy of current therapies, even in the era of targeted agents and immunotherapy, and underscore the need for novel anticancer tools capable of selectively eliminating tumour cells while minimizing systemic toxicity. Among the major clinical barriers in oncology, multidrug resistance (MDR), often associated with the overexpression of ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp), encoded by the *ABCB1* gene, represents a major obstacle to effective chemotherapy by promoting active drug efflux, reducing intracellular drug accumulation and ultimately leading to treatment failure [4,5].

Against this backdrop, natural products have played a pivotal role in anticancer drug discovery, providing a rich source of structurally diverse molecules with pleiotropic biological effects and generally favourable toxicity profiles [6–9]. Among them, carotenoids are increasingly recognized due to their antioxidant, anti-inflammatory, antiproliferative, proapoptotic and antimetastatic properties, as well as their ability to modulate key signalling pathways involved in cancer development and progression [10]. Notably, beyond their classical antioxidant activity, carotenoids can also act as pro-oxidant agents under specific cellular conditions, especially in cancer cells characterized by elevated basal reactive oxygen species (ROS) levels, thereby promoting oxidative stress-mediated cytotoxicity [11–13].

In recent years, extremophilic microorganisms have emerged as a promising but underexplored source of novel carotenoids and other bioactive compounds. Among them, halophilic archaea are a major source of a unique class of C₅₀ carotenoids, predominantly bacterioruberin (BR; 2,2'-bis[3-hydroxy-3-methylbutyl]-3,4,3',4'-tetrahydro-1,2,1',2'-tetrahydro- γ,γ -carotene-1,1'-diol) and related derivatives, which constitute the major carotenoid fraction in haloarchaeal membranes [14]. BR has attracted particular interest due to its distinctive structure and biological properties. Its extended polyene chain with 13 conjugated double bonds and four hydroxyl groups confers high antioxidant capacity and strong membrane affinity [15,16]. Functionally, BR contributes to cellular adaptation to extreme environments by stabilizing membranes and protecting against oxidative stress, UV radiation and high salinity [17,18]. These features have driven increasing interest in BR and BR-rich carotenoid extracts (BRCEs) as potential bioactive compounds for biomedical applications, particularly in oxidative stress-related diseases and cancer. Consistent with this, recent studies have reported selective cytotoxic effects of BRCEs against several cancer cell lines, with minimal toxicity in non-transformed cells [19–22]. However, the anticancer potential of these compounds remains poorly characterized, particularly in the context of MDR phenotypes.

Given this background, we investigate the anticancer activity of BRCEs derived from *Haloferax (H.) mediterranei* in representative solid tumour cell lines, including non-small cell lung adenocarcinoma (A549), triple-negative breast cancer (BT-549) and primary melanoma (WM115). These cell lines were selected to encompass tumour models of distinct tissue origin and biological background, thereby allowing BRCE activity to be evaluated across different solid tumour contexts. In addition, we evaluated two paired lung cancer models comprising sensitive cells and their P-gp-overexpressing MDR derivatives (A549-CDR2 vs. A549 and NCI-H460/R vs. NCI-H460), enabling direct comparison between drug-sensitive and resistant phenotypes. Our results provide novel insights into the potential of BR as selective anticancer agents capable of targeting both sensitive and MDR tumour phenotypes.

2. Results

2.1. Cytotoxic Activity of BRCE in Human Cancer Cell Lines

The cytotoxic effects of BRCE were initially evaluated in A549, BT-549 and WM115 human cancer cell lines using the MTT assay at 24, 48 and 72 h of exposure (Figure 1). BRCE caused a concentration- and time-dependent reduction in cell metabolic activity in all three models, with a progressive decrease in IC_{50} values as the exposure time increased (Table 1).

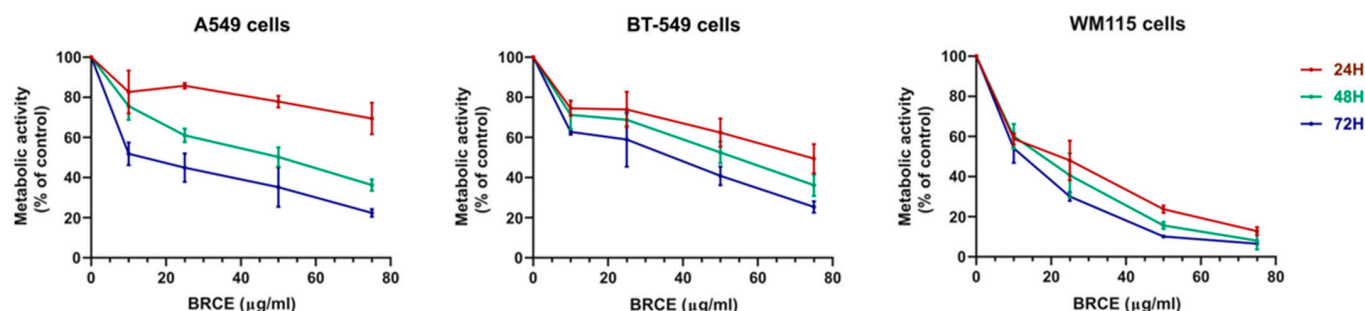


Figure 1. Cytotoxic effect of bacterioruberin-rich carotenoid extract (BRCE) on A549, BT-549 and WM115 cells. Cellular metabolic activity was assessed in A549, BT-549 and WM115 cells treated with increasing concentrations of BRCE (0, 10, 25, 50 and 75 $\mu\text{g/mL}$) for 24, 48 and 72 h using the thiazolyl blue tetrazolium bromide (MTT) assay. Results are expressed as percentage of metabolic activity relative to untreated controls. Error bars represent standard deviations (SD).

Table 1. IC_{50} values of bacterioruberin-rich carotenoid extract (BRCE) for A549, BT-549 and WM115 cells.

Time (h)	A549 Cells		BT-549 Cells		WM115 Cells	
	IC_{50} ($\mu\text{g/mL}$)	R^2	IC_{50} ($\mu\text{g/mL}$)	R^2	IC_{50} ($\mu\text{g/mL}$)	R^2
24	>75.00 *	0.5862	69.82 ± 8.03	0.7914	16.97 ± 1.60	0.9544
48	42.22 ± 2.74	0.9529	46.30 ± 4.40	0.8961	13.87 ± 1.55	0.9486
72	18.39 ± 2.32	0.8907	28.47 ± 3.19	0.8967	10.39 ± 1.06	0.9651

IC_{50} values (mean $\pm$ SD) were calculated from dose–response curves obtained after 24, 48 and 72 h of treatment with BRCE (0–75 $\mu\text{g/mL}$). R^2 values correspond to nonlinear regression analysis. * The IC_{50} was not reached within the concentration range tested (up to 75 $\mu\text{g/mL}$) and is therefore reported as >75 $\mu\text{g/mL}$.

Although significant effects were already observed at 24 h, a longer exposure period appeared to enhance the cytotoxic response consistently across the three cell lines analysed. An incubation period of 48 h was selected for subsequent analysis since the effect on metabolic activity was already quite pronounced in the three cell lines and thus 72 h incubation was not considered necessary to observe a stronger effect. Three BRCE concentrations were chosen for each cell line, including one below, one close to and one above the corresponding IC_{50} value, to assess potential concentration-dependent effects.

2.2. Effect of BRCE on Cancer Cell Proliferation

Exposure to BRCE caused a significant reduction in cell proliferation in all three cancer cell lines after 48 h of treatment (Figure 2A,B). This effect was concentration-dependent when compared with untreated control cells.

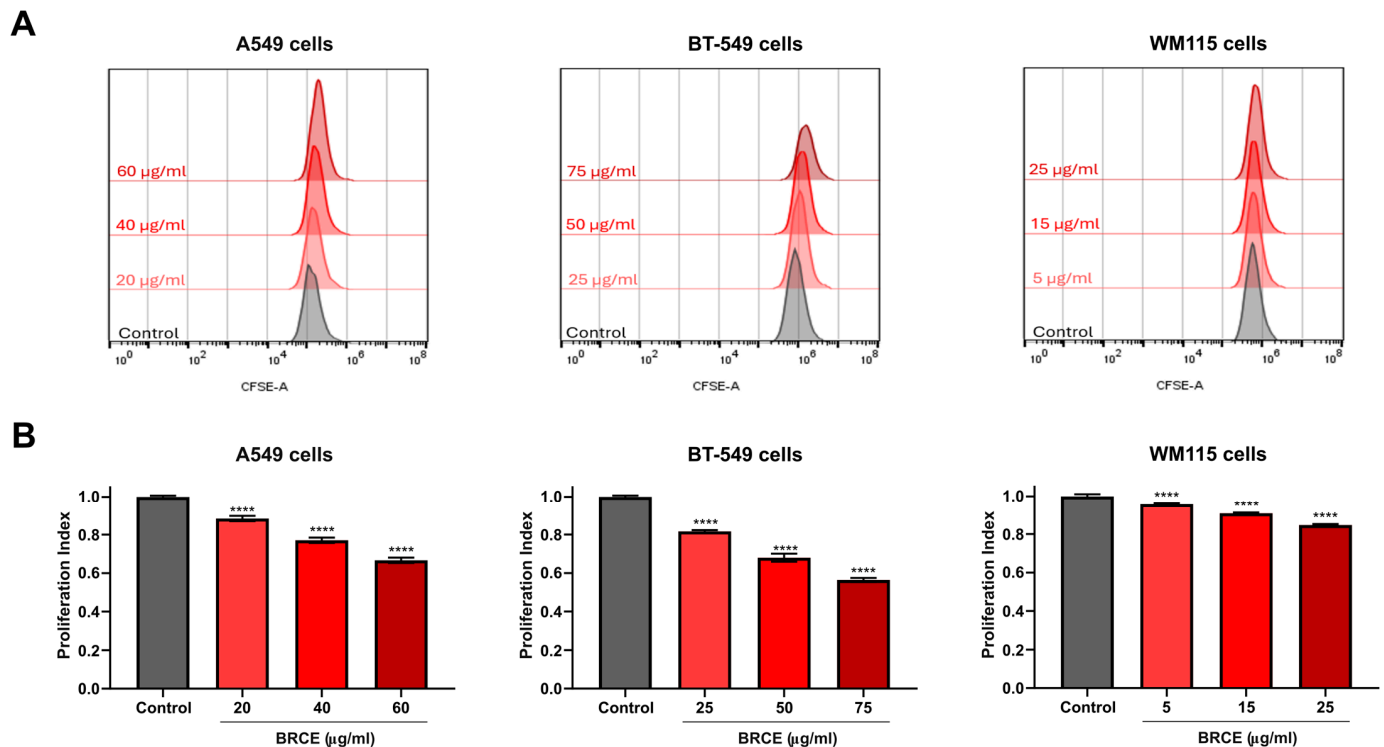


Figure 2. Effect of bacterioruberin-rich carotenoid extract (BRCE) on cell proliferation in A549, BT-549 and WM115 cells after 48 h of treatment. (A) Representative histograms showing the mean fluorescence intensity (MFI) of carboxyfluorescein succinimidyl ester (CFSE) measured by flow cytometry in A549, BT-549 and WM115 cells treated with increasing concentrations of BRCE. Because CFSE fluorescence is progressively diluted with each cell division, greater fluorescence retention and a rightward shift in the distribution indicate reduced proliferation, whereas a leftward shift reflects greater dye dilution and a higher number of cell divisions. BRCE treatment produced a concentration-dependent increase in CFSE retention, consistent with reduced cell proliferation. (B) Proliferation indexes derived from CFSE dilution analysis. Data are presented as mean $\pm$ SD. Statistical significance is indicated as **** $p \leq 0.0001$ versus untreated control.

In A549 cells, BRCE caused a progressive reduction in the proliferation index, decreasing from control values close to 1 to approximately 0.65 at the highest concentration (60 $\mu\text{g/mL}$). A similar pattern was observed in BT-549 cells, where BRCE treatment (25–75 $\mu\text{g/mL}$) resulted in a marked decrease in proliferation, reaching values around 0.55 at 75 $\mu\text{g/mL}$. In WM115 cells, lower BRCE concentrations (5–25 $\mu\text{g/mL}$) also produced a concentration-dependent and statistically significant reduction in proliferation index, although the magnitude of the effect was more moderate, with values around 0.85 at the highest concentration.

2.3. Intracellular ROS Levels in BRCE-Treated Cancer Cells

A significant increase in ROS levels was observed following BRCE treatment in all three tumour cell lines after 48 h of exposure (Figure 3A,B). This effect was clearly concentration-dependent, with progressively higher ROS accumulation observed at increasing BRCE concentrations.

In all three cancer cell lines, BRCE induced a strong, concentration-dependent increase in intracellular ROS levels. In A549 cells, ROS levels increased approximately 4-fold at 20 $\mu\text{g/mL}$, 7-fold at 40 $\mu\text{g/mL}$ and up to 10-fold at 60 $\mu\text{g/mL}$ compared with control cells. A similar trend was observed in BT-549 cells, where ROS levels reached approximately 6-fold, 10-fold and 14-fold of control values following treatment with 25, 50 and 75 $\mu\text{g/mL}$.

BRCE, respectively. In WM115 cells, treatment with 5, 15 and 25 $\mu\text{g/mL}$ BRCE resulted in increased ROS levels of approximately 2-fold, 5-fold and 9-fold relative to control cells.

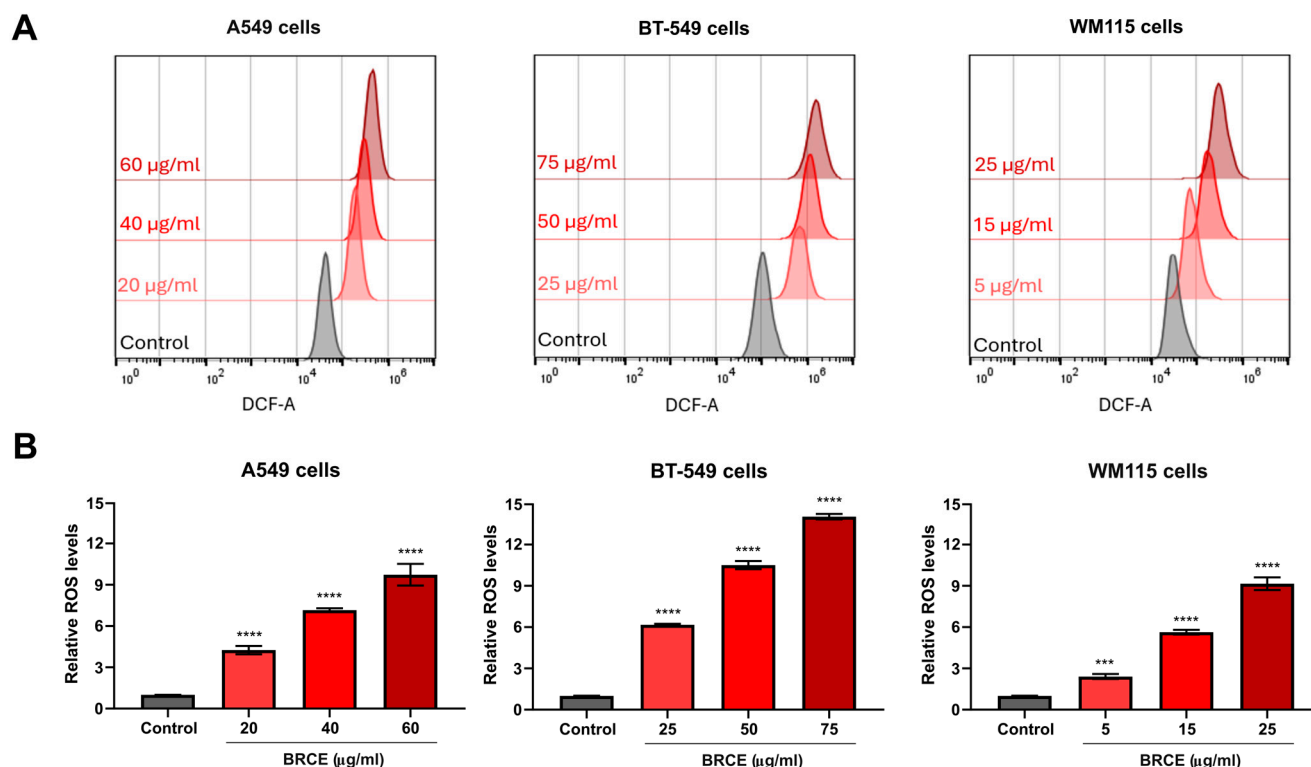


Figure 3. Relative quantification of intracellular reactive oxygen species (ROS) in A549, BT-549 and WM115 cells after 48 h of bacterioruberin-rich carotenoid extract (BRCE) treatment. (A) Representative histograms showing the mean fluorescence intensity (MFI) of 2',7'-dichlorofluorescein (DCF) measured by flow cytometry in A549, BT-549 and WM115 cells treated with increasing concentrations of BRCE. (B) Bar graphs illustrating the relative intracellular ROS levels, expressed as MFI normalized to untreated control cells. Data are presented as mean $\pm$ SD. Differences were estimated by comparison with control conditions. Statistical significance is indicated as follows: *** $p \leq 0.001$; **** $p \leq 0.0001$.

2.4. Apoptosis Analysis in BRCE-Treated A549 Cells

Treatment with BRCE triggered a concentration-dependent increase in apoptotic cell populations in A549 lung cancer cells, accompanied by a corresponding decrease in viable cells, as revealed by AO/EB dual staining (Figure 4). Untreated control cells exhibited uniform green fluorescence (Figure 4A), indicative of a predominantly viable population. Following BRCE treatment, early apoptotic cells were identified by green fluorescence with nuclear condensation (Figure 4B). Late apoptotic cells displayed orange/red fluorescence and clearly fragmented nuclei (Figure 4C–E), with nuclear fragmentation highlighted in Figure 4F. Bright-field optical microscopy revealed progressive morphological changes in treated cells (Figure 4G). Control cells maintained typical epithelial morphology and strong adherence to the substrate, while BRCE exposure led to reduced cell density, loss of adherence, and cell shrinkage and rounding. Quantitative analysis of live, apoptotic and dead cells, based on AO/EB staining, confirmed the concentration-dependent proapoptotic effect of BRCE (Figure 4H). Control cells were predominantly viable (>97%), with negligible apoptotic or dead populations. At low BRCE concentrations (1.172 $\mu\text{g/mL}$), viability remained high and apoptotic cells represented less than 2% of the population. From 2.344 $\mu\text{g/mL}$ onwards, a marked increase in apoptosis was observed, with apoptotic populations ranging between approximately 7 to 14%. This trend intensified at intermediate

concentrations (4.688 and 9.375 $\mu\text{g/mL}$), where apoptotic cells accounted for up to 23% and 35% of the total population, respectively. At higher concentrations (18.75–75 $\mu\text{g/mL}$), apoptosis became the predominant cellular outcome, reaching values above 40–60%, while the proportion of viable cells decreased accordingly.

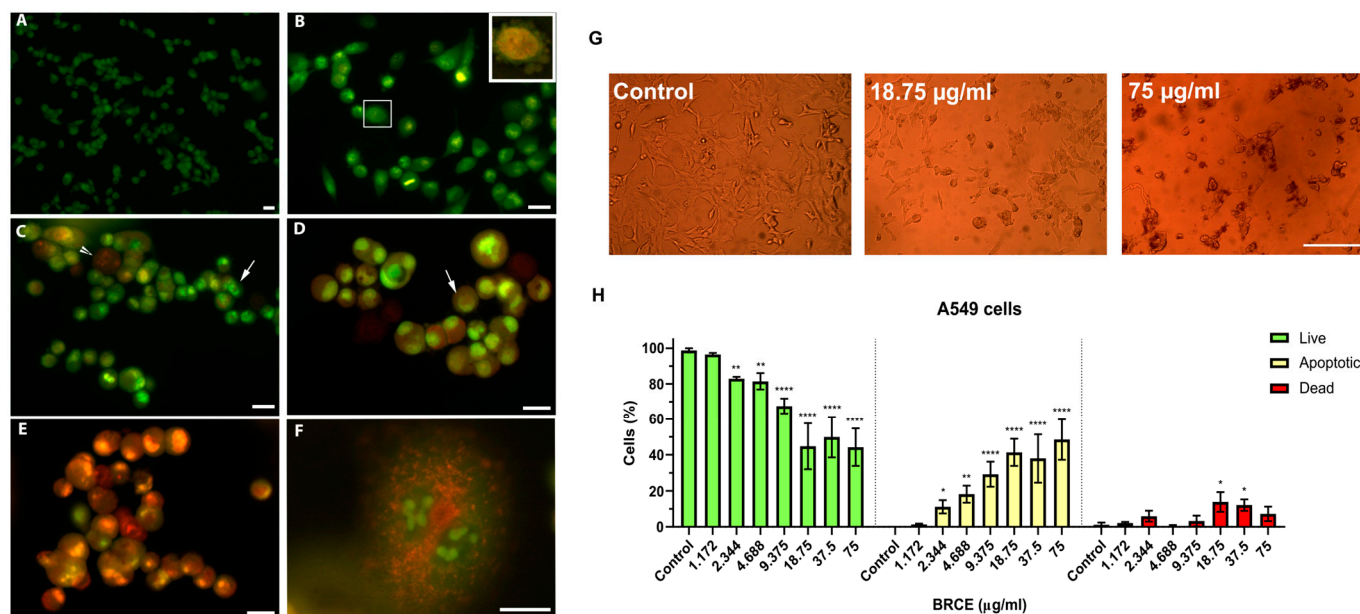


Figure 4. Induction of apoptosis in A549 lung cancer cells following bacterioruberin-rich carotenoid extract (BRCE) treatment. (A) Representative fluorescence microscopy images showing live cells stained with acridine orange (AO). (B) Early apoptotic cells exhibiting chromatin condensation and membrane blebbing. (C–E) Late apoptotic cells displaying orange/red fluorescence due to ethidium bromide (EB) uptake. Arrowhead points to a necrotic cell. Arrows indicate fragmented nuclei. (F) Nuclear fragmentation in late apoptotic cells. Arrows indicate fragmented nuclei. (G) Representative bright-field microscopy images of A549 cells under control conditions and after treatment with 18.75 and 75 $\mu\text{g/mL}$ BRCE for 48 h, showing morphological alterations and loss of cell adherence. (H) Quantification of live, apoptotic and dead cell populations expressed as percentage of total cells after 48 h of BRCE treatment based on AO/EB staining. Data are presented as mean $\pm$ SD. Differences were estimated by comparison with control conditions. Statistical significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$. Scale bars: (A–E), 20 μm ; (F), 5 μm ; (G), 100 μm .

2.5. BRCE Retains Cytotoxic Activity in MDR Lung Cancer Cells

The cytotoxic response of sensitive and MDR lung cancer cell lines to conventional chemotherapeutic agents and BRCE was evaluated using the SRB assay after 48 h of treatment (Figure 5A–D). As expected, MDR cell lines exhibited a marked reduction in sensitivity to their corresponding reference cytotoxic drugs (Table 2). Indeed, A549-CDR2 cells showed a significantly higher GI_{50} value for PTX compared to parental A549 cells (171.39 ± 26.85 nM vs. 7.48 ± 0.96 nM), resulting in an RF of 22.92 ± 4.65 (Figure 5A,B). Similarly, NCI-H460/R cells displayed strong resistance to DOX, with a GI_{50} of 1304.35 ± 125.00 nM compared to 63.76 ± 4.13 nM in sensitive NCI-H460 cells (RF = 20.46 ± 2.37) (Figure 5C,D). In contrast, BRCE induced comparable cytotoxic effects in both sensitive and resistant phenotypes (Table 2). The GI_{50} values for BRCE were similar between A549 and A549-CDR2 cells (38.18 ± 6.41 $\mu\text{g/mL}$ and 36.96 ± 5.90 $\mu\text{g/mL}$, respectively), resulting in an RF close to one (0.97 ± 0.22) (Figure 5A,B). Likewise, NCI-H460 and NCI-H460/R cells exhibited similar sensitivity to BRCE, with GI_{50} values of 56.04 ± 5.90 $\mu\text{g/mL}$ and 42.01 ± 5.14 $\mu\text{g/mL}$, respectively (RF = 0.75 ± 0.12) (Figure 5C,D). Vehicle controls (DMSO) did not affect cell growth in any condition, with viability values consistently above 98% (Figure 5A,C).

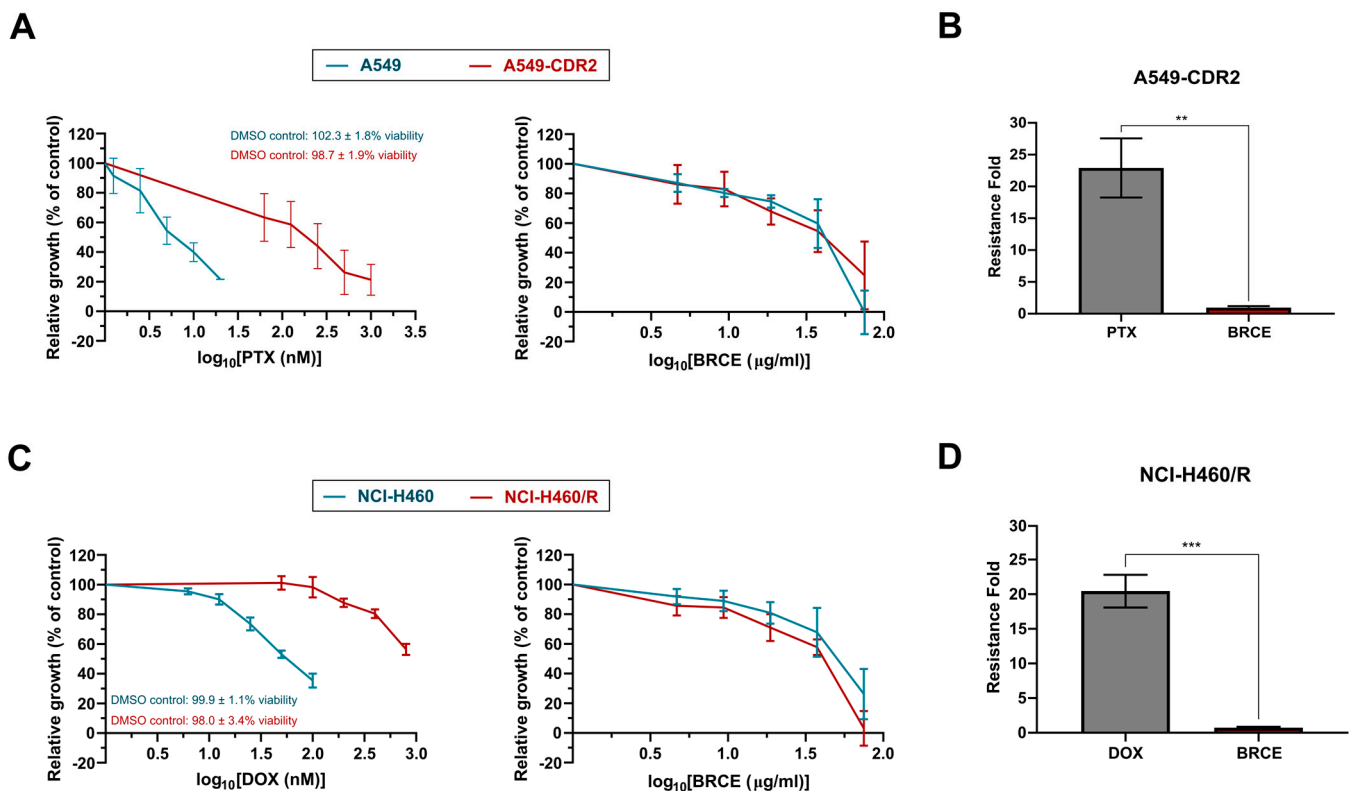


Figure 5. Growth-inhibitory effect of bacterioruberin-rich carotenoid extract (BRCE) on multidrug-resistant (MDR) lung cancer cells. **(A)** Left: Dose–response curves of A549 (sensitive) and A549-CDR2 (MDR) cells treated with paclitaxel (PTX) for 48 h. Vehicle controls (DMSO) did not affect cell growth, which remained above 98.7% of untreated control values at the highest concentration. Right: Dose–response curves of A549 and A549-CDR2 cells treated with BRCE for 48 h. **(B)** Resistance fold values for A549-CDR2 cells treated with PTX and BRCE. **(C)** Left: Dose–response curves of NCI-H460 (sensitive) and NCI-H460/R (MDR) cells treated with doxorubicin (DOX) for 48 h. Vehicle controls did not affect cell growth, which remained above 98.0% of untreated control values at the highest concentration. Right: Dose–response curves of NCI-H460 and NCI-H460/R cells treated with BRCE for 48 h. **(D)** Resistance fold values for NCI-H460/R cells treated with DOX and BRCE. Data are presented as mean $\pm$ SD. Statistical significance is indicated as follows: ** $p \leq 0.01$; *** $p \leq 0.001$.

Table 2. Growth inhibition (GI_{50}) and resistance fold (RF) values in sensitive and multidrug-resistant (MDR) lung cancer cell lines.

Cells	Phenotype	Drug	GI_{50}	R^2	RF
A549	Sensitive	PTX	7.48 ± 0.96 nM	0.8807	-
		BRCE	38.18 ± 6.41 μ g/mL	0.8063	-
A549-CDR2	MDR	PTX	171.39 ± 26.85 nM	0.8217	22.92 ± 4.65
		BRCE	36.96 ± 5.90 μ g/mL	0.8034	0.97 ± 0.22
NCI-H460	Sensitive	DOX	63.76 ± 4.13 nM	0.9612	-
		BRCE	56.04 ± 5.90 μ g/mL	0.9077	-
NCI-H460/R	MDR	DOX	1304.35 ± 125.00 nM	0.8922	20.46 ± 2.37
		BRCE	42.01 ± 5.14 μ g/mL	0.8602	0.75 ± 0.12

GI_{50} values (mean $\pm$ SD) of paclitaxel (PTX), doxorubicin (DOX) and bacterioruberin-rich carotenoid extract (BRCE) in sensitive (A549 and NCI-H460) and MDR (A549-CDR2 and NCI-H460/R) cell lines after 48 h of treatment. R^2 values were obtained from nonlinear regression analysis. RF indicates the degree of resistance in the MDR cells relative to the corresponding sensitive parental cell line.

2.6. BRCE Modulates P-gp Functional Activity in MDR Lung Cancer Cells

The effect of BRCE on P-gp functional activity was subsequently evaluated in MDR lung cancer cell lines using the Rh123 accumulation assay (Figure 6A,B). Verapamil (20 μ M) was used as a positive control for P-gp inhibition and sensitive cell lines were included as negative controls. In sensitive parental cell lines, Rh123 accumulation was markedly higher than in their resistant counterparts, indicating a higher P-gp activity in the MDR phenotypes under basal conditions. In both A549-CDR2 and NCI-H460/R cells, treatment with verapamil led to a marked increase in intracellular Rh123 accumulation, confirming effective inhibition of P-gp-mediated efflux. BRCE treatment also modulated Rh123 accumulation in a concentration-dependent manner, with a statistically significant increase in intracellular fluorescence at concentrations of both $0.5 \times \text{GI}_{50}$ and GI_{50} in both MDR cell lines, suggesting that BRCE can inhibit P-gp-mediated efflux.

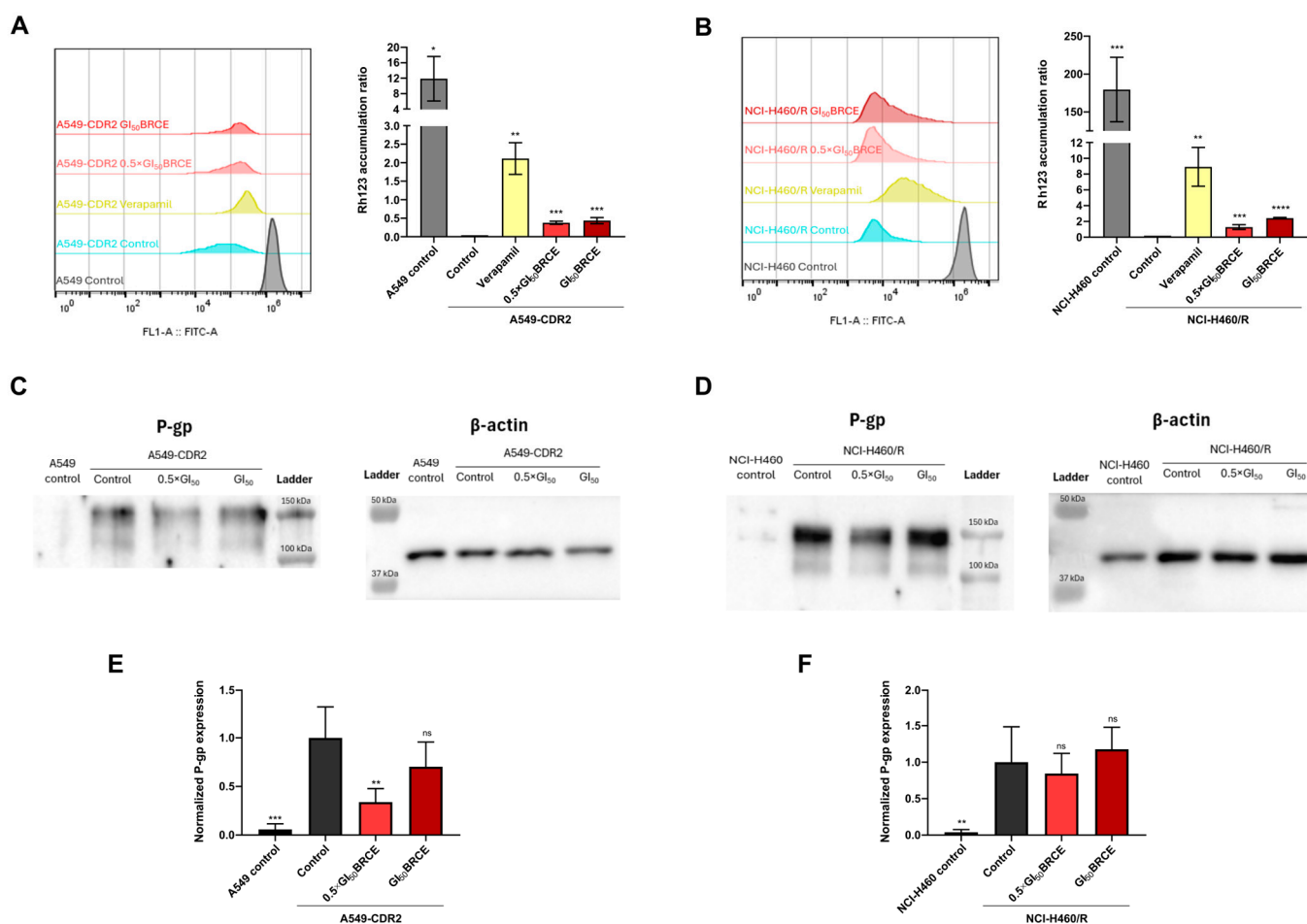


Figure 6. Effect of bacterioruberin-rich carotenoid extract (BRCE) on P-glycoprotein (P-gp) activity and expression in multidrug-resistant (MDR) lung cancer cells. (A) Left: Representative histograms showing Rhodamine 123 (Rh123) median fluorescence intensity (MFI) in A549 parental cells and A549-CDR2 cells under control conditions and after treatment with verapamil, $0.5 \times \text{GI}_{50}$ BRCE and GI_{50} BRCE for 1 h. Right: Bar graphs illustrating the Rh123 accumulation ratios, expressed as MFI normalized to untreated A549-CDR2 cells. (B) Left: Representative histograms showing Rh123 MFI in NCI-H460 parental cells and NCI-H460/R cells under the same experimental conditions. Right: Bar graphs illustrating the Rh123 accumulation ratios normalized to untreated NCI-H460/R cells. (C) Western blot membranes showing P-gp and β -actin protein expression in A549 and A549-CDR2 cells following BRCE treatment, including molecular weight markers. (D) Western blot membrane showing P-gp and β -actin expression in NCI-H460 and NCI-H460/R cells following BRCE treatment,

including molecular weight markers. (E) Densitometric analysis of normalized P-gp expression in A549-CDR2 cells relative to β -actin. (F) Densitometric analysis of normalized P-gp expression in NCI-H460/R cells relative to β -actin. Data are presented as mean $\pm$ SD. Differences were estimated by comparison with untreated MDR cells. Statistical significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns, not significant.

Western blot analysis was performed to assess the effect of BRCE on P-gp protein expression levels (Figure 6C–F). As expected, in the sensitive phenotypes, P-gp expression was negligible while in the MDR cells there was a considerable increase in P-gp expression. In A549-CDR2 cells, a significant decrease in P-gp expression was observed following treatment with $0.5 \times \text{GI}_{50}$ BRCE ($p < 0.01$), whereas treatment with GI_{50} BRCE showed a P-gp expression level similar to untreated cells (Figure 6E). In NCI-H460/R cells, a similar trend was observed, with a small reduction in P-gp expression in cells treated with $0.5 \times \text{GI}_{50}$ but an increase with the GI_{50} concentration, although these differences did not reach statistical significance (Figure 6F).

3. Discussion

The present study demonstrates that BRCE obtained from *H. mediterranei* exerts broad cytotoxic and antiproliferative activity across representative lung, breast and melanoma models, reducing metabolic activity and proliferation while promoting intracellular ROS accumulation and apoptosis-associated changes. Most notably, BRCE retained comparable growth-inhibitory activity in parental and P-gp-overexpressing MDR lung cancer cells and attenuated P-gp-mediated efflux. These findings extend the previously reported activity of BRCE and identify its preserved efficacy in MDR models as the principal novel contribution of this study.

Our findings are in line with previous reports describing the cytotoxic and antiproliferative activity of BR and BR-rich extracts derived from different haloarchaeal species. BRCE from *H. mediterranei* has previously shown cytotoxic and antiproliferative effects in breast cancer and haematological models, whereas carotenoid extracts from other haloarchaea have reduced viability and induced apoptosis in several cancer cell types [19–22]. The present results therefore support the broad cytotoxic activity of BR-rich extracts and extend it to lung and melanoma models.

Among the tested cell lines, WM115 cells exhibited higher sensitivity, with a marked decrease in metabolic activity observed at low concentrations after 24 h of exposure, whereas A549 and BT-549 cells showed a comparatively more moderate response, suggesting cell-type-dependent susceptibility to BRCE. This pattern may be related to their distinct cellular origins: BT-549 and A549 are epithelial carcinoma-derived cell lines, whereas WM115 originates from primary melanoma and is derived from melanocytes [23–26]. Such lineage-specific differences in gene expression, signalling pathways and cellular physiology may account for the differential sensitivity observed [27,28].

Previously reported IC_{50} values for BRCE in triple-negative breast cancer cell lines include $73.2 \pm 0.2 \mu\text{g/mL}$ in MDA-MB-231 and $58.23 \pm 0.1 \mu\text{g/mL}$ in MDA-MB-468 at 30 h [21]. In agreement with these findings, the IC_{50} obtained in BT-549 cells in the present study ($69.82 \pm 8.03 \mu\text{g/mL}$ at 24 h), which are also classified as triple-negative breast cancer cells, falls within a comparable range [23]. This concordance suggests that BRCE exerts a consistent cytotoxic effect across triple-negative breast cancer models, supporting a potentially shared vulnerability of this subtype to BRCE treatment.

The cytotoxic effect became more evident at 48 h and showed only a slight additional increase at 72 h. For this reason, 48 h was selected for subsequent analyses, as it provided a robust and reproducible reduction in metabolic activity while minimizing potential confounding effects associated with prolonged culture. Importantly, although the MTT

assay is commonly used as an indirect estimate of cell viability, it primarily measures cellular metabolic activity rather than direct cell number or proliferative capacity. Therefore, it may overestimate viable cells under conditions where mitochondrial function remains partially active despite the initiation of cell death pathways or metabolic alterations [29]. In addition, the observed reductions in MTT signal likely reflect both decreased proliferation and early cytotoxic effects, supporting the use of complementary assays to better define its mechanism of action.

In this context, and to determine whether the observed reduction in metabolic activity was associated with impaired proliferative capacity, the effect of BRCE on cell proliferation was further investigated by the CFDA-SE assay. BRCE treatment resulted in a marked reduction in cell proliferation in a concentration-dependent manner, confirming its antiproliferative effect. Interestingly, despite exhibiting higher sensitivity to BRCE in terms of IC_{50} , WM115 cells showed a comparatively weaker antiproliferative response than A549 and BT-549 cells. This apparent discrepancy may be explained by the intrinsically lower basal proliferation rate of WM115 cells, as primary melanoma cells often display slower cell cycle progression compared to carcinoma-derived cell lines [30–32]. Consequently, the relative reduction in proliferation when normalized to untreated controls may appear less pronounced, even when overall viability is strongly affected. Furthermore, this observation may indicate that the relative contribution of metabolic impairment, apoptosis and antiproliferative effects to the BRCE response differs among cell lines according to their intrinsic biological characteristics. In line with this interpretation, previous studies have shown that BRCE can impair proliferation through cell-cycle perturbation, and similar antiproliferative effects have been described for other carotenoids [10,19,33].

BRCE treatment induced a marked and concentration-dependent increase in intracellular ROS levels in all three cancer types analysed, indicating that ROS accumulation is a consistent cellular response to BRCE. A similar effect was previously observed in myeloid leukaemia models [19]. Although BR is canonically described as a potent antioxidant, these results suggest that it may exert context-dependent pro-oxidant effects [13,34,35]. Indeed, several carotenoids have been reported to increase intracellular ROS in tumour models, in association with growth inhibition and apoptosis [11]. This dual redox behaviour supports the notion that compounds such as BR can act as antioxidants under physiological conditions but switch to pro-oxidant activity in the oxidative tumour microenvironment, potentially contributing to tumour selectivity [12]. In line with this hypothesis, BRCE has shown preferential cytotoxicity toward leukaemia cells compared with peripheral blood mononuclear cells (PBMCs) from healthy donors [19]. Similarly, BRCE also displayed selectivity for breast cancer cells over the non-tumorigenic mammary epithelial cell line 184A1 [21]. Moreover, carotenoid extracts from *Natrialba* sp. M6 have been reported to exhibit greater selectivity than the classical chemotherapeutic agent 5-fluorouracil in several cancer models [22]. Together with the reported photoprotective and cytoprotective effects of BRCE in PBMCs from healthy donors, these observations reinforce the concept that BR-rich extracts may possess a functionally selective redox activity that favours tumour targeting while sparing non-transformed cells [18].

Alongside the marked reduction in metabolic activity and the increase in intracellular ROS observed after BRCE treatment, a clear apoptosis-associated response was also detected in A549 cells. AO/EB staining revealed a concentration-dependent increase in apoptotic cells, characterised by chromatin condensation, membrane blebbing, and nuclear fragmentation. These changes, together with the progressive loss of adherence and cell shrinkage observed by light microscopy, are consistent with an apoptosis-associated phenotype [36] and agree with the proapoptotic activity reported for other carotenoids in lung cancer models [33,37]. Although ROS accumulation and apoptosis-associated changes were

observed concurrently, the present data do not establish a causal relationship between these responses. Oxidative stress may contribute to the apoptotic response because excessive ROS can disrupt mitochondrial homeostasis and activate apoptotic signalling [38,39]. However, BRCE may exert pleiotropic effects, and ROS cannot be considered the predominant or exclusive cause of the observed apoptotic phenotype based on the present findings.

Although the apoptotic fractions observed at BRCE concentrations ranging from 18.75 to 75 µg/mL were of a similar magnitude, these results should be interpreted considering the different information provided by each assay. The MTT assay reflects the combined effects of reduced metabolic activity, inhibition of proliferation, and cell death, whereas AO/EB staining measures the relative proportions of viable, apoptotic, and dead cells among the cells present and analysed at a single experimental endpoint. Therefore, these assays are not expected to show a direct quantitative correlation. Moreover, the progressive loss of cell adherence observed at high BRCE concentrations suggests that severely affected cells may have detached before staining and may consequently have been underrepresented in the AO/EB analysis. The CFSE assay further demonstrated that BRCE markedly inhibited A549 cell proliferation, indicating that the reduction in MTT signal results from both antiproliferative and cytotoxic effects. The relatively similar apoptotic fractions observed between 18.75 and 75 µg/mL may also suggest that apoptosis had reached a near-maximal detectable level after 48 h, while additional increases in BRCE concentration mainly affected metabolic activity and proliferative capacity. However, the present data do not allow us to conclude that a distinct cytostatic cell subpopulation exists.

Taken together, these findings support the cytotoxic and growth-inhibitory potential of BRCE in drug-sensitive models. However, its relevance as a candidate anticancer agent also depends on whether this activity is preserved in the context of therapy-driven MDR, a major cause of treatment failure in lung and other cancers [40]. To address this, we evaluated cell growth by SRB assay in two established paired lung cancer MDR models, A549 versus A549-CDR2 and NCI-H460 versus NCI-H460/R, in which the resistant derivatives overexpress P-gp [41,42]. The marked resistance of A549-CDR2 cells to PTX and of NCI-H460/R cells to DOX confirmed the MDR phenotypes under our experimental conditions.

Against this background, the behaviour of BRCE was particularly noteworthy. In contrast to PTX and DOX, BRCE produced comparable growth inhibition in parental and resistant cells in both lung cancer model pairs, yielding resistant fold values close to 1. Thus, no relevant cross-resistance to BRCE was detected in either A549-CDR2 or NCI-H460/R cells, despite their MDR phenotype and P-gp overexpression. This finding suggests that the mechanisms responsible for resistance to conventional chemotherapeutics in these models do not substantially impair BRCE activity. One possible explanation is that BRCE activity may be less efficiently counteracted by P-gp-mediated efflux than that of conventional chemotherapeutics. This may be related to the highly hydrophobic nature of carotenoids and their strong interaction with lipid membranes, which may also underlie pleiotropic effects on transporter function and membrane organisation, as proposed for other carotenoids [43]. In addition, given that oxidative stress has been associated with changes in P-gp regulation, the BRCE-induced increase in intracellular ROS could potentially contribute to the modulation of P-gp-related responses [44].

Methodologically, the SRB assay provides a robust basis for these comparisons because it reflects cellular biomass rather than mitochondrial metabolic activity, which is particularly relevant in MDR settings where metabolic rewiring may confound metabolism-based assays [45,46]. Moreover, the BRCE-induced reduction in viability observed in A549 cells was comparable when measured by MTT and SRB, consistent with previous reports showing good agreement between both assays in cytotoxicity studies [47]. Together, these observations support the robustness of the resistant fold estimates.

Given the central role of efflux transporters in MDR, we next examined whether BRCE affects P-gp activity in the resistant lung cancer models. To evaluate acute functional inhibition of P-gp, both BRCE and verapamil were applied for only 1 h before Rh123 accumulation analysis, a time window suitable for detecting rapid changes in transporter activity [48]. As expected, Rh123 accumulation assays showed lower basal dye retention in A549-CDR2 and NCI-H460/R cells than in their parental counterparts, consistent with increased efflux activity in the MDR cell lines. Verapamil, a first-generation P-gp inhibitor, was used as a positive control and produced a marked increase in intracellular Rh123 accumulation, confirming effective inhibition of transporter-mediated efflux.

BRCE also increased Rh123 retention in both MDR models in a concentration-dependent manner. These results indicate that BRCE can reduce P-gp functional activity in resistant lung cancer cells. Comparable effects have been described for other carotenoids, such as β -carotene and fucoxanthin, which have been reported to increase intracellular retention of P-gp substrates and to promote reversal of the MDR phenotype in resistant cancer models [49–51]. This finding provides a plausible mechanistic basis for the preserved activity of BRCE in MDR cells. By partially attenuating P-gp-mediated efflux, BRCE may remain active in resistant cells in which conventional chemotherapeutics such as PTX or DOX lose efficacy.

Classical P-gp inhibitors have shown limited clinical applicability because of toxicity and pharmacokinetic interactions [52–54]. In this context, BRCE may be of interest not only as a bioactive compound with cytotoxic and growth-inhibitory effects but also as a potential adjuvant for sensitising resistant tumour cells to chemotherapy, as has also been proposed for other carotenoids including lutein or β -carotene [49,55,56]. Further studies will be required to determine whether this effect translates into enhanced responses in combination settings.

At the level of protein expression, Western blotting confirmed that P-gp abundance was much lower, almost undetectable, in the parental sensitive lines than in their MDR counterparts, consistent with the resistant phenotype. In A549-CDR2 cells, BRCE significantly reduced P-gp abundance at $0.5 \times GI_{50}$, whereas levels at GI_{50} were comparable to those in untreated cells. In NCI-H460/R cells, a small reduction was observed at $0.5 \times GI_{50}$, followed by an increase at GI_{50} , although neither change reached statistical significance. These cell-line-specific and non-monotonic patterns may reflect differences in the regulation of P-gp associated with their distinct cellular backgrounds and resistance-selection histories. P-gp abundance can be dynamically regulated at transcriptional, post-transcriptional and protein-stability levels, and its response to BRCE exposure may therefore differ between models [44,57,58]. Importantly, BRCE increased Rh123 accumulation in both resistant cell lines, indicating reduced P-gp-mediated efflux despite the different changes observed in protein abundance. Thus, the functional modulation of P-gp by BRCE does not appear to depend exclusively on a reduction in its expression. Overall, these findings suggest that BRCE modulates P-gp in a concentration- and cell-line-dependent manner, in agreement with previous reports describing carotenoid-mediated modulation of ABC transporters [59–61].

Integrating the present results with mechanisms described for other carotenoids, we propose a working model in which BRCE exerts a pleiotropic cytotoxic effect that is not substantially impaired by the P-gp-associated MDR phenotype. BRCE-induced ROS accumulation could plausibly contribute to the modulation of stress and survival signalling, mitochondrial dysfunction and cell-cycle inhibition. In A549 lung cancer cells, lutein-induced growth inhibition and apoptosis have been associated with modulation of the PI3K/AKT/mTOR pathway [33], whereas ROS-dependent regulation of MAPK and PI3K/AKT signalling has been reported in glioma cells treated with fucoxanthin [62]. Fucoxanthin has also been associated with loss of mitochondrial membrane potential and

apoptosis in glioblastoma cells [63]. In other tumour models, carotenoid-induced mitochondrial apoptosis has involved changes in the Bax/Bcl-2 balance, cytochrome c release and caspase activation [64], while both p53-dependent and p53-independent responses have been described in A549 and SKOV3 cells treated with crocetin [65]. These mechanisms could contribute to the antiproliferative and apoptosis-associated responses observed here and are consistent with the cell-cycle perturbation previously reported for BRCE [19], although the specific mitochondrial and signalling events remain hypothetical for the present models.

In MDR cells, these intracellular effects may operate in parallel with the partial attenuation of P-gp-mediated efflux observed in both resistant cell lines. Similar modulation of P-gp function has been reported for β -carotene in P-gp-overexpressing lung cancer cells [49], whereas fucoxanthin has been shown to sensitise resistant cancer cells through the combined induction of apoptosis and modulation of MDR-associated proteins [51]. However, because the changes in P-gp protein abundance were concentration- and cell-line-dependent, the preserved activity of BRCE cannot be explained exclusively by P-gp downregulation. Its comparable activity in sensitive and resistant cells may therefore result from the combination of intracellular pro-oxidant, antiproliferative and apoptosis-associated effects with functional modulation of P-gp, which together are not efficiently counteracted by transporter overexpression.

Several aspects should be taken into consideration when interpreting the present findings. First, the preparation used here corresponds to a BR-rich extract rather than purified BR, and therefore the contribution of minor carotenoids cannot be excluded. In addition, this study was limited to in vitro models, so further in vivo investigations will be necessary to evaluate pharmacokinetics, pharmacodynamics and systemic safety. Given the intrinsic hydrophobicity of carotenoids, delivery strategies to improve stability and bioavailability should also be explored [66]. Finally, additional studies will be needed to clarify the molecular basis of BRCE activity, its effects on tumour heterogeneity and adaptive resistance, and its potential usefulness either as an antitumour agent or as an adjuvant in MDR settings.

4. Materials and Methods

4.1. BRCE Preparation

BRCEs were obtained from cultures of *H. mediterranei* R-4, originally obtained from the American Type Culture Collection (ATCC 33500; Manassas, VA, USA), as previously described [19,21,67]. Briefly, harvested biomass was resuspended in pure acetone of high-performance liquid chromatography (HPLC)-grade (Panreac AppliChem, Panreac Quimica, Barcelona, Spain) at a ratio of 1 mL per 10 mL of initial culture volume and incubated overnight at 4 °C under continuous agitation. The acetone phase containing the extracted carotenoids was subsequently recovered by filtration. The concentration of the BRCE was determined spectrophotometrically using a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Optical density (OD) was measured at 494 nm, corresponding to the absorption maximum of BR [68,69]. Extract concentration was calculated using an extinction coefficient, ϵ (1%), of 2540 according to the following equation [67]:

$$\text{BRCE}(\mu\text{g/mL}) = \frac{\text{OD}_{494}}{2540} \times 10^4$$

After that, acetone was removed using a rotary evaporator (Rotavapor RV 3 V, IKA, Staufen, Germany) and the dried extract was dissolved in Roswell Park Memorial Institute medium 1640 (RPMI-1640) culture medium by sonication, filtered through 0.2 μm membranes and adjusted to a final concentration of 150 $\mu\text{g/mL}$. BRCE stock solutions

were immediately divided into single-use aliquots, protected from light, stored at 4 °C and used within one week. Each aliquot was used only once to avoid repeated handling and temperature fluctuations.

The extraction procedure and the characteristic carotenoid profile of the resulting BRCE were previously established by HPLC and triple quadrupole liquid chromatography–mass spectrometry (LC–MS/MS) [70]. This initial analytical characterisation identified BR as the predominant carotenoid, accounting for a relative abundance of $75.5 \pm 1.9\%$, together with minor carotenoids and carotenoid precursors. Representative LC–MS/MS data from this characterisation are provided in Figure S1. Subsequent BRCE preparations used in the present study were obtained following the same standardised extraction procedure.

4.2. Cells and Culture Conditions

The human non-small cell lung cancer cell line A549 (lung adenocarcinoma) was obtained from the ATCC, whereas WM115 (primary melanoma) and BT-549 (triple-negative breast cancer) cells were kindly provided by Dr. Berta López Sánchez-Laorden (Alicante, Spain). MDR cell line variants were generated by collaborators of our research group, through prolonged exposure to low, increasing concentrations of chemotherapeutic agents, leading to the selection of stable drug-resistant phenotypes. The paclitaxel-resistant A549-CDR2 cell line was generated in the laboratory of Dr. Maria Helena Vasconcelos (Porto, Portugal) [41,71]. The doxorubicin-resistant NCI-H460/R cell line (large cell lung carcinoma), together with its parental sensitive counterpart, was kindly provided by Dr. Milica Pešić (Belgrade, Serbia) [42,72].

A549 and WM115 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Biowest, Nuaille, France), NCI-H460 cells were cultured in RPMI-1640 medium (Biowest), and BT-549 cells were grown in a 1:1 mixture of DMEM and Ham's F-12 Nutrient Mixture (DMEM/F12; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). All media were supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific), 1% penicillin/streptomycin (Biowest) and 1% L-glutamine (Biowest), with the addition of 10 µg/mL insulin (Roche, Basel, Switzerland) for BT-549 cells. The resistant cell lines, NCI-H460/R and A549-CDR2, were treated with 100 nM doxorubicin (DOX; Merck Life Science, Darmstadt, Germany) and 16.2 nM paclitaxel (PTX; Merck Life Science), respectively, every 2 weeks in order to maintain cell resistance. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and were routinely subcultured upon reaching 70–80% confluence.

Cell morphology was routinely monitored using an inverted light microscope (Leica DMIRB, Wetzlar, Germany). Cell number and viability were determined by Trypan blue exclusion using a haemocytometer (Trypan Blue reagent; Sigma-Aldrich, St. Louis, MO, USA). All experiments were conducted using cells in the exponential phase of growth with viability consistently exceeding 90%. For experimental assays, cells were seeded at a density of 2.5×10^4 cells/mL.

For BRCE treatments, the extract was diluted in RPMI-1640. Accordingly, A549 and WM115 cells were seeded in a 1:1 (v/v) mixture of DMEM and RPMI-1640, BT-549 cells in a 1:1 (v/v) mixture of DMEM/F12 and RPMI-1640, and NCI-H460 cells in RPMI-1640 alone. In all cases, untreated control groups were exposed to the same culture medium conditions as their corresponding treated groups.

4.3. Cytotoxicity Assays in Solid Tumour Models—MTT

Cell metabolic activity was assessed using the thiazolyl blue tetrazolium bromide (MTT) assay [73]. A549, BT-549 and WM115 cells were seeded in 96-well plates and allowed to adhere overnight prior to treatment. Cells were then exposed to increasing concentrations

of BRCE (10, 25, 50 and 75 $\mu\text{g/mL}$) for 24, 48 and 72 h, according to previous work [19]. At the end of each treatment period, MTT solution (5 mg/mL; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was added to each well to reach a final concentration of 0.5 mg/mL, followed by incubation at 37 °C for 4 h. The culture medium was subsequently removed and formazan crystals were solubilized by adding 100 μL of dimethyl sulfoxide (DMSO; Corning, NY, USA) per well. OD was measured at 600 nm using an LT-5000MS microplate reader (LabTech, Sorisole, Italy). All experimental conditions were tested in triplicate and cell metabolic activity was calculated as a percentage relative to untreated control. IC_{50} (half-maximal inhibitory concentration) values, defined as the concentration of BRCE required to reduce cell metabolic activity by 50% relative to untreated control cells, were determined from dose–response curves.

4.4. Cell Proliferation Rates Quantification by Flow Cytometry

Cell proliferation was analysed using carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) staining [74]. Cells were collected, stained with 5 μM CFDA-SE (Thermo Fisher Scientific) in PBS 1X supplemented with 10% FBS for 5 min in the dark at room temperature and washed three times. Cells were then seeded in 48-well plates at a density of 2.5×10^4 cells/mL and allowed to adhere overnight. Subsequently, cells were treated for 48 h with BRCE at concentrations determined from prior MTT assays. After treatment, cells were harvested and washed and the median fluorescence intensity (MFI) of carboxyfluorescein succinimidyl ester (CFSE) was measured by spectral flow cytometry (Northern Lights, Cytex, Fremont, CA, USA) and analysed using FlowJo v.10.10.0 software (FlowJo LLC, Ashland, OR, USA). As CFSE fluorescence is diluted during successive cell divisions, higher fluorescence retention was interpreted as reduced proliferative activity. All conditions were tested in triplicate. The proliferation index was expressed as the ratio of MFI in treated cells relative to untreated control cells.

4.5. ROS Quantification by Flow Cytometry

Intracellular ROS levels were quantified using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) [75]. A549, BT-549 and WM115 cells were harvested, washed and stained with 5 μM H_2DCFDA (Cayman Chemical, Ann Arbor, MI, USA) in PBS 1X for 30 min at 37 °C in the dark. After staining, cells were washed three times to remove excess probe and seeded in 48-well plates. Cells were allowed to adhere overnight and subsequently treated for 48 h with BRCE at concentrations determined from preliminary MTT assays. Following treatment, cells were collected, washed and measured by spectral flow cytometry (Northern Lights, Cytex). Data were analysed using FlowJo v.10.10.0 software (FlowJo LLC) and intracellular ROS levels were expressed as MFI relative to untreated control cells ($\text{MFI}_{\text{Control}} = 1$). All experimental conditions were performed in triplicate.

4.6. Detection of Cell Death and Assessment of Cell Membrane Integrity by Fluorescence Microscopy Following AO/EB Dual Staining

Acridine orange/ethidium bromide (AO/EB) dual staining was performed to evaluate cell death in A549 cells following BRCE treatment [76]. Cells were allowed to adhere overnight before treatment with serial dilutions of BRCE, ranging from 1.172 to 75 $\mu\text{g/mL}$, according to previous work [19]. After 48 h of treatment, cells were harvested, washed three times and incubated with a staining solution containing 5 μM AO (Sigma-Aldrich) and 5 μM EB (Sigma-Aldrich) in PBS 1X for 20 s at room temperature. Samples were immediately mounted on glass slides and observed under an epifluorescence microscope (Leica DM750, Leica, Wetzlar, Germany) with a blue excitation filter ($\lambda = 470\text{--}490$ nm). Imaging was performed within 10 min to minimize photobleaching. For each experimental condition, a minimum of 300 cells were counted to ensure statistical reliability.

4.7. Cytotoxicity Assays in MDR Models—SRB

Cell growth and collateral sensitivity in MDR models were evaluated using the sulforhodamine B (SRB) assay [77]. Sensitive and MDR counterpart pairs of cell lines (A549 vs. A549-CDR2 and NCI-H460 vs. NCI-H460/R) were seeded in 96-well plates and allowed to adhere overnight in medium supplemented with 5% FBS to limit excessive proliferation. In these cell line pairs, no additional glutamine or antibiotics were added, to avoid potential interference with MDR-associated responses.

After cell attachment, a subset of plates was fixed immediately to obtain time-zero controls (T_0), while the remaining plates were treated with BRCE using five serial 1:2 dilutions ranging from 75 to 4.6875 $\mu\text{g/mL}$ and incubated for 48 h. In parallel, standard chemotherapeutic agents were used as positive controls and to calculate growth inhibition parameters. PTX was used for the A549/A549-CDR2 pair (A549: 20–1.25 nM; A549-CDR2: 1000–62.5 nM), and DOX for the NCI-H460/NCI-H460/R pair (NCI-H460: 100–6.25 nM; NCI-H460/R: 800–50 nM), as these drugs were those originally employed to generate the corresponding resistant phenotypes. Since chemotherapeutic drugs were dissolved in DMSO, vehicle controls were included at the highest DMSO concentration used.

At the end of the treatment period, cells were fixed with 10% (*w/v*) ice-cold trichloroacetic acid (TCA; Merck Life Science), washed and stained with 0.4% (*w/v*) SRB (Merck Life Science) in acetic acid (Merck Life Science). Unbound dye was removed by washing with 1% (*v/v*) acetic acid and protein-bound SRB was solubilized in 10 mM Tris-base solution (Merck Life Science). Absorbance was measured at 510 nm in a microplate reader (Synergy Mx; Biotek Instruments Inc., Winooski, VT, USA) using the Gen5 software (version 1.04.5, Biotek Instruments Inc.). All experimental conditions were performed in triplicate.

Growth inhibition (GI) percentage was calculated according to the National Cancer Institute (NCI) algorithm using absorbance values at T_0 , untreated control (C) and treated cells (T), as follows [78]:

$$\text{GI}(\%) = \begin{cases} \frac{T-T_0}{C-T_0} \times 100, & \text{if } T \geq T_0 \\ \frac{T-T_0}{T_0} \times 100, & \text{if } T < T_0 \end{cases}$$

GI_{50} (50% growth inhibition) values were obtained from concentration–response curves, and resistance fold (RF) was calculated as the ratio between GI_{50} values of resistant and sensitive cell lines, and the corresponding standard deviation (SD) was estimated according to the following equations:

$$\text{RF} = \frac{\text{GI}_{50}^{\text{Resistant}}}{\text{GI}_{50}^{\text{Sensitive}}}$$

$$\text{SD}_{\text{RF}} = \text{RF} \times \sqrt{\left(\frac{\text{SD}^{\text{Resistant}}}{\text{GI}_{50}^{\text{Resistant}}}\right)^2 + \left(\frac{\text{SD}^{\text{Sensitive}}}{\text{GI}_{50}^{\text{Sensitive}}}\right)^2}$$

4.8. Assessment of P-gp Functional Activity by Flow Cytometry

P-gp functional activity and its modulation by BRCE were evaluated by the Rhodamine 123 (Rh123) accumulation assay in MDR cell lines [79]. The corresponding drug-sensitive parental cell lines were analysed in parallel as functional controls.

MDR and sensitive counterpart cell lines were seeded at 5×10^4 cells/mL in 96-well plates and allowed to adhere overnight. Cells were then treated with BRCE at the concentrations previously determined by the SRB assay (GI_{50} and $0.5 \times \text{GI}_{50}$), or with

verapamil (20 μ M; Sigma-Aldrich) as a positive control for P-gp inhibition [80]. Treatments were carried out for 1 h at 37 °C.

Following pre-treatment, Rh123 (Sigma-Aldrich) was added to each well at a final concentration of 1 μ M and cells were incubated for an additional 1 h at 37 °C in the dark. After incubation, cells were washed and harvested and the MFI was immediately analysed by flow cytometry (BD Accuri C6 Plus, BD Biosciences, San Jose, CA, USA). All experimental conditions were performed in triplicate. P-gp functional inhibition was quantified by calculating the Rh123 accumulation ratio, defined as the relative increase in intracellular Rh123 fluorescence in treated MDR cells compared with untreated MDR controls, after subtraction of the corresponding unstained background signals:

$$\text{Rh123 accumulation ratio} = \frac{\text{MFI}_{\text{Treated}}^{\text{Rh123}^+} - \text{MFI}_{\text{Treated}}^{\text{Unstained}}}{\text{MFI}_{\text{Resistant control}}^{\text{Rh123}^+} - \text{MFI}_{\text{Resistant control}}^{\text{Unstained}}} - 1$$

4.9. P-gp Expression Analysis—Protein Extraction and Western Blotting

The MDR cell line pairs were plated in 6-well plates at a density of 5×10^4 cells/mL and allowed to adhere overnight. Cells were then treated with the GI_{50} and $0.5 \times \text{GI}_{50}$ concentrations of BRCE for 48 h. Following treatment, cells were collected and centrifuged at $250 \times g$ for 5 min at 4 °C, and subsequently lysed in Wynman's buffer (1% NP-40, 0.1 M Tris-HCl pH 8.0, 0.15 M NaCl, 5 mM EDTA) supplemented with EDTA-free protease inhibitors (Roche) and phosphatase inhibitors (Merck Life Science) for 30 min at 4 °C under gentle agitation.

Protein lysates were obtained after centrifugation at $14,000 \times g$ for 10 min at 4 °C and stored at -20 °C until analysis. Total protein concentration was determined using a modified Lowry method (DCTM Protein Assay Kit, Bio-Rad, Hercules, CA, USA) with a bovine serum albumin (BSA) standard curve. The absorbance was analysed with the Gen5 software on the microplate reader Synergy Mx, at 488 nm excitation and read at 655 nm emission. Protein samples (10 μ g per condition) were mixed with $4 \times$ SDS sample buffer (Laemmli-type) and denatured at 95 °C for 5 min prior to electrophoresis. A final volume of 20 μ L was loaded per lane.

Proteins were separated on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Buckinghamshire, UK) for 2 h using a Mini Trans-Blot Cell system (Bio-Rad). Membranes were stained with Ponceau S solution (PanReac AppliChem, Barcelona, Spain) to confirm equal protein transfer and subsequently blocked in Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5% (*w/v*) non-fat dry milk for 2 h at room temperature.

Membranes were incubated for 90 min at room temperature with primary antibodies against P-gp (1:500; sc-55510) and β -actin (1:2000; sc-47778) (Santa Cruz Biotechnology, Dallas, TX, USA). After washing with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG secondary antibody (1:2000; NA931V, Cytiva, Marlborough, MA, USA) for 1 h at room temperature. Immunoreactive bands were detected using enhanced chemiluminescence reagents (ECL Western Blotting Detection Reagents, Cytiva) and visualized on Amersham Hyperfilm ECL (Cytiva) using a Fuji Medical Film Processor (FPM-100A; Fuji Photo Film Co., Tokyo, Japan).

Blots were digitized using a GS-800 calibrated densitometer (Bio-Rad) and band intensities were quantified with Image Lab software version 6.0.1 (Bio-Rad). The molecular weights of detected proteins were estimated using a PageRuler Plus Prestained Protein Ladder (10–250 kDa; Thermo Fisher Scientific). P-gp expression levels were normalized to β -actin and expressed as relative protein levels.

4.10. Statistical Analysis

Statistical analyses were performed using Prism 8.0.2 software (GraphPad Software Inc., San Diego, CA, USA). Initially, outliers were identified using the Rout method. The normality of the data was then assessed with the Shapiro–Wilk test. If the data followed a normal distribution, differences from control conditions were estimated using an analysis of variance (ANOVA) test with uncorrected Fisher’s least significant difference (LSD) test. If the data did not follow a normal distribution, differences were estimated using a Kruskal–Wallis test. IC₅₀ and GI₅₀ values were calculated by nonlinear regression. A *p*-value less than 0.05 was considered statistically significant. All experiments were repeated at least three independent times.

5. Conclusions

BRCE obtained from *H. mediterranei* exhibits broad in vitro cytotoxic and antiproliferative activity in lung, breast, and melanoma cancer models, reducing metabolic activity and proliferation while promoting ROS accumulation and apoptosis-associated changes. Importantly, BRCE retains comparable growth-inhibitory activity in P-gp-overexpressing MDR lung cancer cells, in which conventional chemotherapeutics lose activity, and attenuates P-gp-mediated efflux. These findings support a working model in which BRCE acts through coordinated pro-oxidant, antiproliferative and apoptosis-associated effects, potentially involving the modulation of cellular stress and survival signalling and mitochondrial dysfunction. In MDR cells, these intracellular effects may operate in parallel with functional modulation of P-gp. Overall, BRCE emerges as a promising carotenoid-based antitumour candidate whose pleiotropic activity is not substantially impaired by the P-gp-associated MDR phenotype. Future work should address its molecular targets, translational formulation strategies, and in vivo efficacy and safety.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27156658/s1>.

Author Contributions: A.B.-M. carried out the experiments, data analysis and interpretation. S.P.-G. and P.M.-P. carried out data analysis and interpretation. A.N.-S. and Y.S. carried out some of the experiments, data analysis and interpretation. M.M.-G., C.P.-S. and R.R. were involved in some experiments. M.G., M.H.V., R.M.M.-E. and J.M.S.-O. were involved in conceptualization, project administration, supervision and data interpretation. The first draft of the manuscript was written by A.B.-M. and all authors commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the Ethics Committee of the University of Alicante (reference no. UA-2025-05-28_2; approval date: 29 May 2025). The study did not involve human participants, identifiable human material, or animals. All experiments were performed using established cell lines obtained from commercial sources (ATCC) or kindly provided by other research groups, and were conducted in accordance with the principles of Good Cell and Tissue Culture Practice 2.0 (GCCP 2.0).

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw flow cytometry data generated in this study are deposited in the Repository of the University of Alicante (RUA) at: <http://hdl.handle.net/10045/163851> (accessed on 15 July 2026).

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ABC	ATP-binding cassette
ABCB1	ATP-binding cassette subfamily B member 1
AO	Acridine orange
ATCC	American Type Culture Collection
BR	Bacterioruberin
BRCE	Bacterioruberin-rich carotenoid extract
BSA	Bovine serum albumin
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CFSE	Carboxyfluorescein succinimidyl ester
DCF	2',7'-Dichlorofluorescein
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GCCP	Good Cell and Tissue Culture Practice
GI	Growth inhibition
GI ₅₀	50% growth inhibition
H ₂ DCFDA	2',7'-Dichlorodihydrofluorescein diacetate
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IC ₅₀	Half-maximal inhibitory concentration
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
MDR	Multidrug resistance
MFI	Median fluorescence intensity
MTT	Thiazolyl blue tetrazolium bromide
NCI	National Cancer Institute
OD	Optical density
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
P-gp	P-glycoprotein
PTX	Paclitaxel
RF	Resistance fold
Rh123	Rhodamine 123
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute medium 1640
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SRB	Sulforhodamine B
TBS-T	Tris-buffered saline with Tween 20

TCA Trichloroacetic acid
UV-Vis Ultraviolet–visible

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