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Research paper

Pholiotic acid promotes apoptosis in human metastatic melanoma cells

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ABSTRACT

Mushrooms produce a great variety of secondary metabolites that can be successful in both prevention and treatment of various cancers. In particular, higher Basidiomycete mushrooms contain various types of biologically active low-molecular compounds in fruiting bodies with suggested anticarcinogenic effects. The polyamine analogue {(2R)-2-[(S)-3-hydroxy-3-methylglutaryloxy] putrescine dicinnamamide} indicated with the name pholiotic acid, isolated for the first time by us from the fruiting bodies of the Basidiomycete *Pholiota spumosa* (Fr.) Sing. (Strophariaceae), inhibited the viability of human prostate cancer cells, such as other polyamine synthetic analogues that have shown antitumor activity in several types of cancer, including melanoma. Melanoma is an aggressive skin cancer that can metastasize to other organs and presents a high resistance to conventional therapies. In light of these considerations, the present study was therefore designed to assess whether this putrescine derivative could inhibit the growth of human metastatic melanoma cell lines, M14 and A2058. The results obtained demonstrate that this natural compound, at 12.5–50 μM concentration, was able to reduce cell viability of both cancer cells inducing cell death by intrinsic apoptotic pathway that probably involves PTEN activity, inhibition of Hsp70 expression and reactive oxygen species production. On the other hand, the increased expression of enzymes involved in polyamine catabolism trigger apoptotic cell death leading to polyamine depletion and generation of reactive oxygen species as by-products. In conclusion, these findings, starting point for further investigation, implement available our data to support pholiotic acid as an attractive potential chemopreventive agent, and provide a basis for further research into the use of this polyamine derivative as potential anticancer agent for melanoma in combination with existing therapies to improve treatment efficacy and overcome the obstacle of drug resistance.

1. Introduction

The plant kingdom is an unlimited source of biologically active substances with therapeutic potential [1–5]. In particular, these vegetable molecules are proving to be promising structures for developing novel anticancer drugs [1,5]. Tumor is the second leading cause of death in industrialized countries after cardiovascular diseases [6]. Anticancer therapy such us chemotherapy is not always effective and has numerous adverse effects. Therefore, the application of integrative therapies also of vegetable origin can be important. Phytochemicals present different advantages: some of them exhibit favorable anti-cancer activity for their ability to act on multiple molecular targets and would provide new core structures in the drug discovery. In fact, for their anti-inflammatory, antioxidant, antiproliferative and proapoptotic properties, they could be considered new agents able to counteract the incidence and mortality of cancer [7].

Medicinal mushrooms have been used in different traditional medicine for several centuries to different affections including cancer [8] and are widely used in East Asia in complementary cancer care. This has aroused particular interest in the Western countries [8]. Mushrooms have an important role as a nutraceutical resource for their content in proteins, vitamins, minerals and carbohydrates [9]. Moreover, mushrooms produce a great variety of secondary metabolites that can be successful in both prevention and treatment of various cancers [9]. In particular, higher Basidiomycete mushrooms contain various types of biologically active low-molecular compounds in fruiting bodies with

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suggested anticarcinogenic effects [10]. Various compounds affect signaling pathways of tumor-specific proliferation, regulation of apoptosis, cancer-specific metabolism, angiogenesis and metastasis [11]. The polyamine analog {(2R)-2-[(S)-3-hydroxy-3-methylglutaryloxy] putrescine dicinnamamide} (Fig. 1), indicated with the name pholiotic acid, with the related cinnamic acid amides (referred to with the name "dicinnamides") isolated for the first time by us from the fruiting bodies of the Basidiomycete *Pholiota spumosa* (Fr.) Sing. (Strophariaceae) [12], are the first examples of cinnamic acid amides occurring in the fungal kingdom, and therefore considered a new class of fungal metabolites [12]. Interestingly, we evidenced that this molecule inhibited the viability of human prostate cancer cells [12], such as other polyamine analogues that have shown antitumor activity in several types of cancer, including melanoma [13,14]. Cutaneous melanoma is an aggressive skin cancer that can metastasize to other organs [15]. It presents a high resistance to conventional therapies. In fact, melanoma cells often become resistant to traditional chemotherapeutic drugs and radiation therapy, this last one widely used as a non-invasive approach in half of cancer patients [15]. Today, immunotherapies, such as ipilimumab, nivolumab and pembrolizumab, are habitual used as adjuvant and neoadjuvant treatments, and have improved previously poor survival of melanoma patients. However, these drugs are correlated with severe side effects. Moreover, there is a risk of resistance promptly after treatment for different months [15]. Therefore, the exploration of novel secondary metabolites with therapeutic and preventive efficacy has become an important objective also in melanoma research. In light of these considerations, it could be postulated that pholiotic acid might have growth inhibition effect on melanoma. However, there is no such a study reported. In order to test this hypothesis, the present study was therefore designed to assess whether this putrescine derivative could inhibit human metastatic melanoma cell growth *in vitro*, and to elucidate its potential mechanism of anticancer activity.

2. Materials and methods

2.1. Chemicals

All chemicals were of commercial quality and were used as received. They were purchased from Sigma Aldrich Co (St. Louis, USA) and GIBCO BRL Life Technologies (Grand Island, NY, USA). Pholiotic acid {(2R)-2-[(S)-3-hydroxy-3-methylglutaryloxy] putrescine dicinnamamide} (Fig. 1) was isolated from the fruiting bodies of the Basidiomycete *Pholiota spumosa* (Fr.) Sing. (Strophariaceae). General experimental details have been reported previously [12].

2.2. Cell culture and treatments

M14 and A2058 human melanoma cell lines were obtained from American Type Culture Collection. Normal human non-immortalised buccal fibroblast cells, kindly donated by Institute IGB, CNR (Naples, Italy). The cells were grown as previously reported [16], and were plated at a constant density to obtain identical experimental conditions in the different tests. In the MTT assay, cancer cells were plated at 6×10^3 cells per well in a 96-well flat-bottomed 200 μL microplate, and at 2×10^4 cells per well for normal human non-immortalised buccal fibroblast cells, in a 96-well flat-bottomed 200 μl microplate. In other tests, cells were plated at 8×10^5 cells (2 mL) per 35 mm culture dish. After 24 h incubation at 37 °C under a humidified 5 % carbon dioxide to allow cell attachment, the cells were treated with different concentrations of pholiotic acid (6.25–50 μM), and incubated for 72 h under the same conditions. In preliminary experiments, a time course was performed. At 72 h it exhibited the highest effect, at least for the parameters examined by us. In addition, only in MTT test, caspase-3 activity, COMET assay and reactive oxygen species assay both melanoma cells were also treated by compound (12.5–50 μM) with antioxidant N-acetyl cysteine, NAC (500 μM) for 72 h. The concentrations used in the different assays were chosen since no significant effect of the pure compound has ever been observed at lower dosage. Stock solution of pholiotic acid was prepared in dimethylsulfoxide (DMSO) and the final concentration of this solvent was kept constant at 0.25 %. Control cells received DMSO alone.

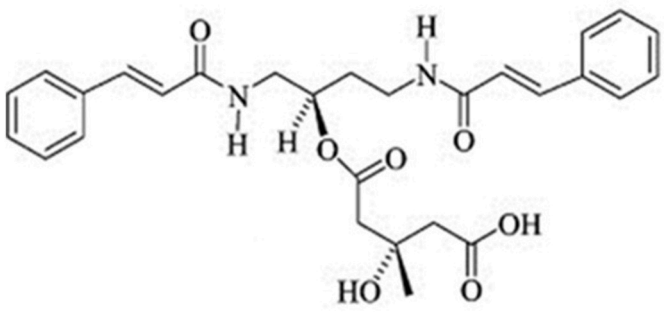

Fig. 1. Structure of Pholiotic acid {(2R)-2-[(S)-3-hydroxy-3-methylglutaryloxy] putrescine dicinnamamide}.

2.2.1. MTT bioassay

MTT assay was performed as described previously [17].

2.2.2. Lactate dehydrogenase (LDH) release

LDH activity was measured in the culture medium and in the cellular lysates as previously reported [17].

2.2.3. Activity of Caspase-3

The activity of caspase-3 was determined by using the Caspase colorimetric assay Kit (SIGMA RBI, St. Louis, MS, USA), as previously described [17]. The total protein content was measured according to Bradford (1976) [18].

2.2.4. DNA analysis by COMET assay

The presence of DNA fragmentation was examined by COMET assay as previously reported [17].

2.2.5. Western blot analysis

The expression of cleaved caspase-9, cleaved caspase-3, PTEN, Hsp70, Bcl-2, Bax and superoxide dismutase (SOD) proteins was evaluated by Western blot analysis, as previously described [17]. The antibodies of these proteins and α-tubulin were diluted in TBST.

2.2.6. Reactive oxygen species assay

ROS determination was performed as previously described [17].

2.2.7. Measurement of GSH levels

GSH levels were measured as described previously [17].

2.2.8. Statistical analysis

Representative data from three independent experiments, performed in quadruplicate, are shown and quantified, and represented as mean ± SD. Results were analyzed as previously described [17]. *Significant vs. control untreated cells ($p < 0.001$).

3. Results

Here we show that pholiotic acid (Fig. 1) has a potential antigrowth activity. The results, summarized in Fig. 2, show that this natural compound exhibited comparable inhibitory effect on both human melanoma cell lines, M14 and A2058. Interestingly, the same amount of pholiotic acid was ineffective on normal cells (Fig. 2). Encouraged by these results, we next assessed the mechanisms by which this compound may inhibit cell viability. No statistically significant increase in LDH release, used as an indicator of membrane integrity, was observed after

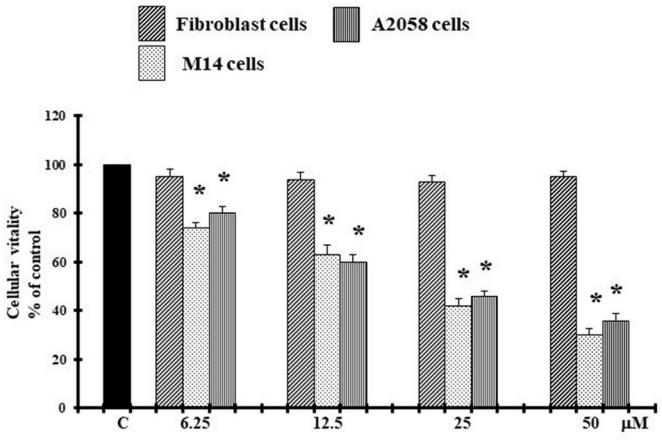

Fig. 2. Cell viability of melanoma and non-cancer cells control and treated with different concentrations of pholiotic acid for 72 h *Significant vs. control untreated cells ($p < 0.001$).

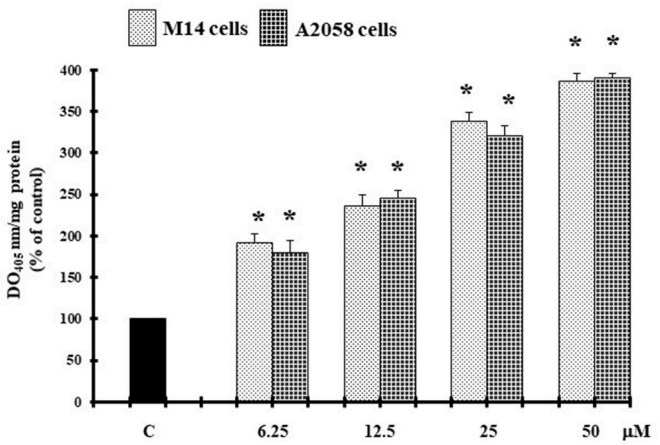

Fig. 3. Caspase-3 activity of melanoma cells control and treated with different concentrations of pholiotic acid for 72 h *Significant vs. control untreated cells ($p < 0.001$).

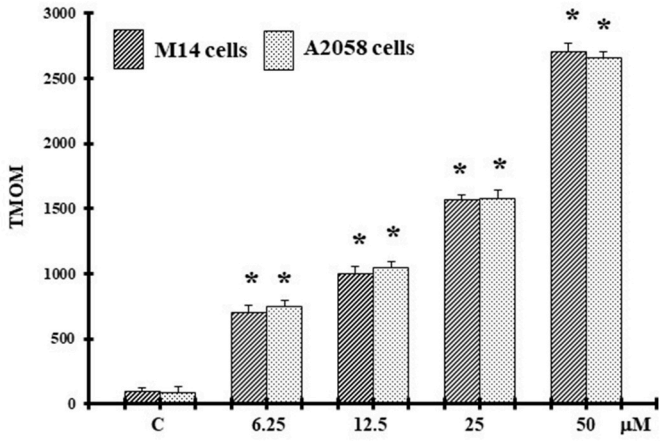

Fig. 4. Analysis of genomic DNA in melanoma cells control and treated with different concentrations of pholiotic acid for 72 h *Significant vs. control untreated cells ($p < 0.001$).

the treatment with pholiotic acid also at 50 μM concentration (Table 1). Alternatively, in the same conditions the activity of caspase-3 significantly and in a dose dependent manner resulted increased (Fig. 3). Nuclear DNA was analyzed using the COMET assay, a method useful for detecting apoptosis [19]. Quantification of data is reported as TMOM (tail moment) in Fig. 4. TMOM is defined as the product of the percentage of DNA in the tail of the comet and TD value, which is obtained calculating the distance between the center of mass of the comet head and the center of mass of the tail. According caspase-3 activity results, we found an increase in TMOM value in both cancer cell lines (Fig. 4). Cleaved caspase-9 and cleaved caspase-3, determined by Western blot, resulted also increased by exposure to our compound, in particular at 12.5–50 μM concentration (Fig. 5AB).

The expression levels of members of the BCL-2 family were analyzed to determine the effects of pholiotic acid treatment on the intrinsic pathway of apoptosis in melanoma cells. It is interesting that we observed its capacity to shift the Bax/Bcl-2 ratio in favor of apoptosis (Fig. 6AB).

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a highly effective tumor suppressor that is commonly mutated in melanoma. Loss of PTEN activity through mutation, deletion or reduced expression has been shown to play an important role in melanoma development [20]. PTEN plays an important role in antagonising the AKT (phosphatidylinositol 3-kinase (PI3K)/protein kinase B) pathway, a pathway critical to cell growth, survival and migration [21]. PTEN has been suggested to downregulate the level of antiapoptotic protein Bcl-2 [21]. To test the effects of our compound on PTEN, the levels of this protein were measured in both cancer cells. The treatment with different concentrations of pholiotic acid significantly affected PTEN protein. In particular, at 50 μM concentration PTEN levels increased by 3-fold in both metastatic melanoma cells (Fig. 5AB), suggesting that the potential proapoptotic and antitumor activity of pholiotic acid could be partially mediated by increasing PTEN expression. In addition, a possible relationship between Hsp70 expression and cell death was investigated. The data, reported in Fig. 5AB, show a decrease in the expression of this protein in a concentration dependent manner.

Increased generation of ROS, induced by a variety of stimuli, has been shown to be involved in apoptosis induction of tumor cells [22]. Therefore, we tested also intracellular ROS levels, employing an oxidation-sensitive fluorescent probe DCFH-DA. DCFH-DA can be taken up into cells, and then oxidized by ROS to its fluorescent derivative DCF. As shown in Fig. 7A, both melanoma cells treated with our natural polyamine analogue had an increase of fluorescence intensity and in particular, at the highest concentration 50 μM when compared to control. These results are correlated to a down regulation of SOD (Fig. 7B), an antioxidant enzyme [23], and with a significant depletion of GSH content (Fig. 7C), permitting to hypothesize that pholiotic acid exerts effects on redox homeostasis in our experimental conditions. To verify the role of ROS in pholiotic acid-induced apoptosis cell death, metastatic melanoma cells were treated by the compound (12.5–50 μM) with the antioxidant NAC (500 μM) for 72 h, following which cell viability, caspase-3 activity, DNA fragmentation and reactive oxygen species assay were determined. NAC control group did not show any significant change in the tested parameters (data not shown). The co-treatment, in part restored cell viability (Fig. 8A) and reduced caspase-3 activity

Table 1
LDH release, expressed as percentage of LDH released into the cell medium with respect to total LDH, in melanoma cell lines treated with pholiotic acid.

Treatments	% LDH released	
	M14	**A2058**
Control	4.3 ± 0.5	5.0 ± 0.8
Pholiotic acid		
6.25 μM	4.1 ± 0.8	4.3 ± 0.6
12.5 μM	4.7 ± 0.5	5.3 ± 0.7
25 μM	5.1 ± 0.8	4.6 ± 0.5
50 μM	3.9 ± 0.6	3.9 ± 0.5

The values are the mean ± SD of three experiments, performed in quadruplicate.

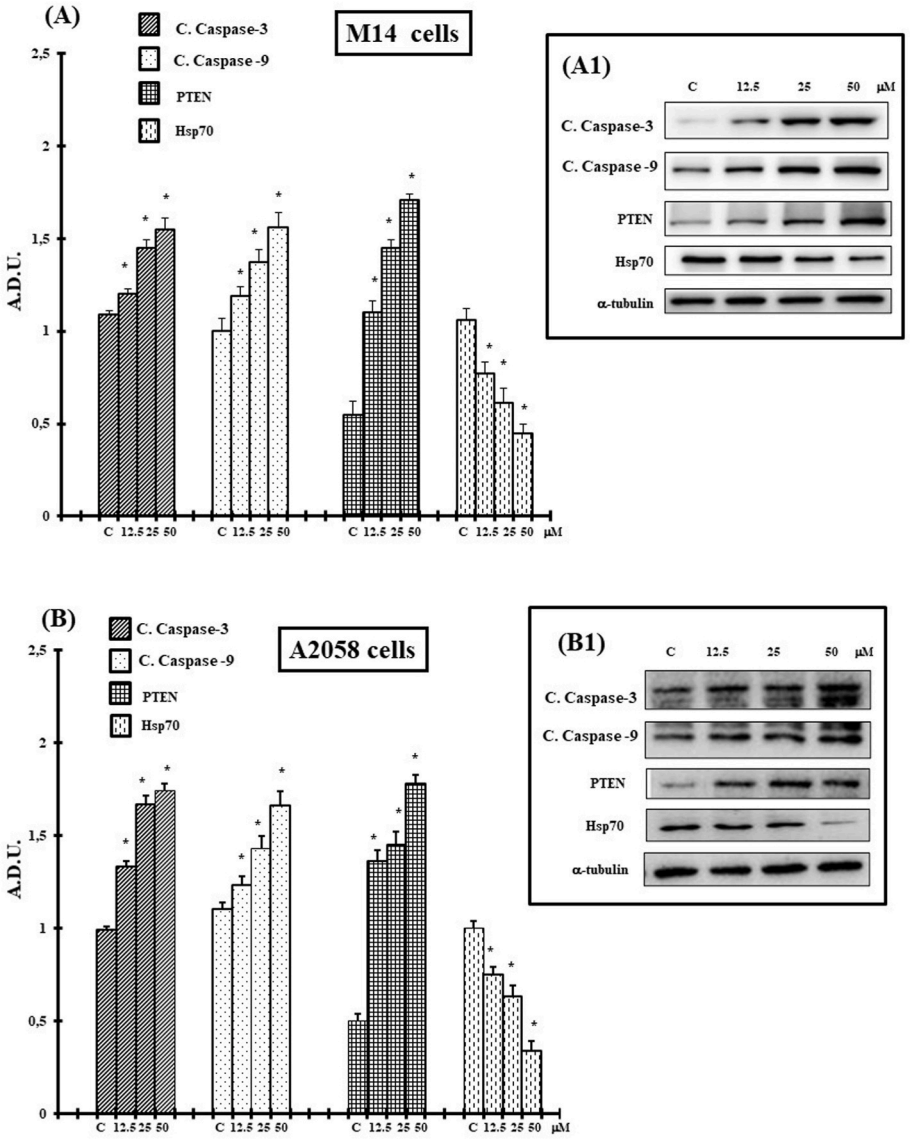

Fig. 5. AB Levels of cleaved caspase-9, cleaved caspase-3, PTEN and Hsp70 proteins in melanoma cells M14 (A) and A2058 (B) control and treated with different concentrations of pholiotic acid for 72 h. Representative blots are reported (A1, B1). *Significant vs. control untreated cells ($p < 0.001$).

(Fig. 8B), TMOM values (Fig. 8C) and green fluorescence (Fig. 9), suggesting that the increase in ROS generation, at least in part, is involved in pholiotic acid-mediated apoptosis.

4. Discussion

Melanoma is the most invasive and deadly forms of skin cancer. In the metastatic stage it becomes very refractory to conventional therapies [24]. The frequency of this cancer is incessantly increasing all over the world [25], therefore there is an urgent need for new drugs or adjuvant therapies to improve patient survival and health quality [26]. Polyamines (putrescine, spermidine and spermine), synthesized by all cells, are organic cations that play a key role in cell growth [27]. Clinical evidences strongly suggest that the influence of polyamines on cell development is greatest in cancer cells because polyamine pools of tumors are significantly higher than those of the normal tissues [28]. In particular, polyamines were proposed as potential marker of cell proliferation and differentiation in melanoma [29,30]. Polyamine catabolism is often reduced in cancer cells. The activity of the biosynthesis enzyme ornithine decarboxylase (ODC) was found higher in various tumor types compared to normal tissues [28], and it has been reported that the activation of polyamine catabolism increases the effect of chemotherapeutics in cancer cells [28,31]. On the other hand, the activation of this metabolic pathway produces substances that might cause apoptosis in cancer cells [32]. Chemical compounds, able to restore the level of polyamine catabolism in cancer, could become potential antineoplastic agents. Various polyamine analogues have been designed and tested for their therapeutic potential in different tumors [31]. These molecules are similar in structure to the natural polyamines but cannot mimic their functions that are essential for cellular growth and differentiation [33]. For example, N1, N11-Diethylnorspermine was shown as a strong apoptotic agent in melanoma cells [34] through the activation of polyamine catabolism and suppressing the polyamine biosynthesis. Moreover, this spermine derivative was able to increase the efficiency of different therapeutic drugs and prevent its drug-induced resistance in tumor cells [31]. Interestingly, our results consistent with previous studies [12], suggest that the potential anticancer activity of pholiotic acid, belonging to a new class of fungal metabolites [12], is related to its characteristic molecular structure including the polyamine putrescine and phenolic substituents like plant amides, that are of primary importance for their biological roles [12]. In fact, this secondary metabolite reduced the viability of cancer cells but not of normal

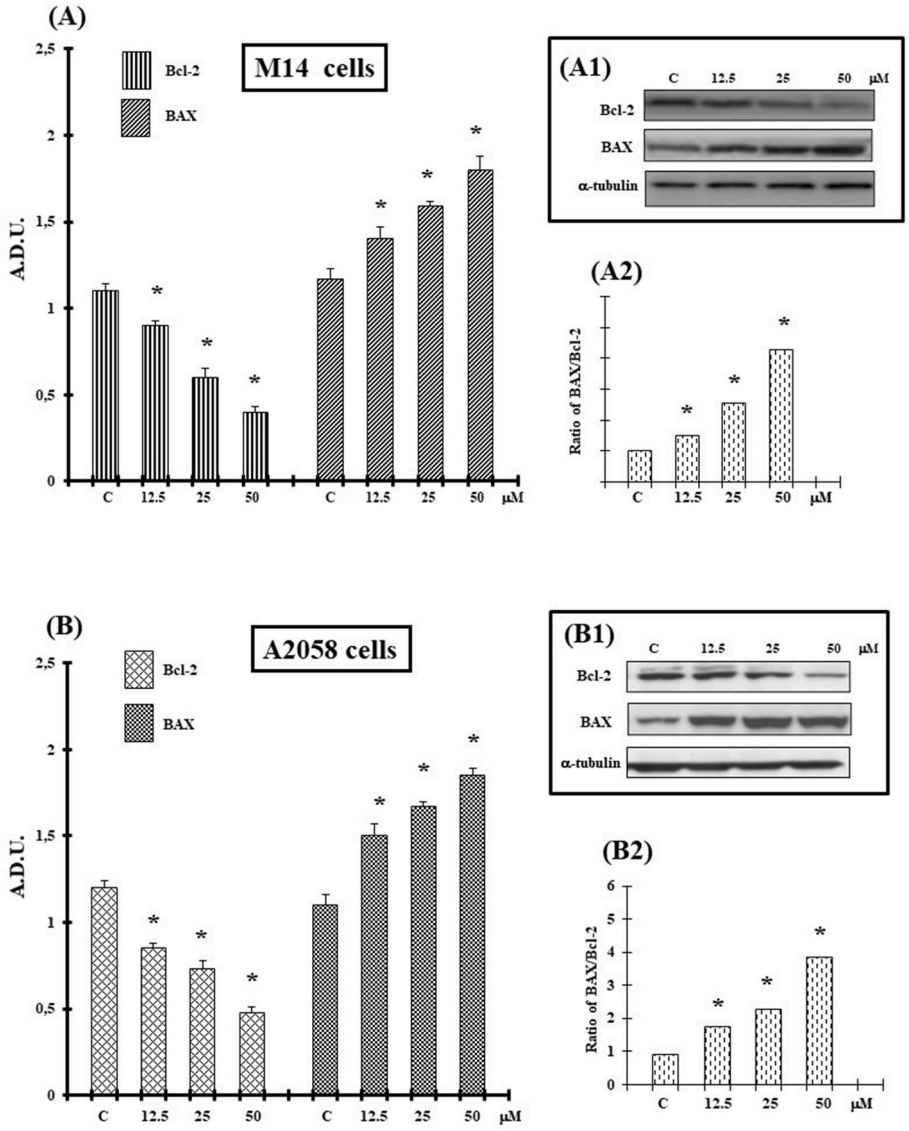

Fig. 6. AB Levels of Bcl-2 and Bax proteins in melanoma cells M14 (A) and A2058 (B) control and treated with different concentrations of pholiotic acid for 72 h. Representative blots (A1, B1) and Bax/Bcl-2 ratio (A2, B2) was reported *Significant vs. control untreated cells ($p < 0.001$).

fibroblasts (Fig. 2), and its selective effect was comparable in both metastatic melanoma cell lines (Fig. 2). Based on our data this molecule seem to be nontoxic in *vivo* and clinically. If these results are demonstrated *in vivo*, this natural polyamine analogue has high potential as an integrative support to anti-melanoma conventional cancer agents. The hypothesize that pholiotic acid in part affect melanoma cells probably with the same mechanism explained by the synthetic analogues of polyamines in cancer cells [31], prompts us to perform further experiments to understand the type of cell death evoked by this natural polyamine analogue in our experimental conditions.

Apoptosis, a programmed cell death mechanism, can be considered as the natural barrage that restricts the survival and spread of cells in cancer process. Apoptosis functions to maintain the homeostasis of various cell processes and avoidance of the apoptotic pathway is associated with the uncontrolled proliferation of melanoma cells [35,36]. Like other cancer cells, melanoma cells can self-destruct through this cell death [35,36]. This process is mediated by both the initiator and the effector proenzyme, caspases, and their release of a proteolytic cascade ultimately results in cell death [35,36]. Caspase-3 belonging to caspase family, is recognized as an important marker of apoptosis [37]. In the intrinsic apoptosis pathway, Bax is inserted into the mitochondrial membrane forming channels, where the cytochrome-c molecules escape from the inside of the mitochondria, leading to the formation of apoptosomes. During this process, procaspase-9 is activated. Caspase-9 cleaves procaspase-3, producing the proteolytic degradation of its target substrate, leading to cell apoptosis [37]. The results obtained allowed us to suppose that pholiotic acid is able to trigger apoptotic cell death in both melanoma cancer cells. In fact, the raise of cleaved caspase-9 and cleaved caspase-3 expression (Fig. 5AB), accompanied by an increase in caspase-3 enzyme activity (Fig. 3) and a high DNA fragmentation (Fig. 4), not correlated to LDH release (Table 1), a marker of membrane breakdown, occurred in both melanoma cells exposed to this natural molecule. On the other hand, necrosis is the uncontrolled cell death induced by external factors producing the cell membrane rupture causing spillage of the cell content [38]. Moreover, pholiotic acid up-regulated the protein expression of Bax and down-regulated the expression of Bcl-2 (Fig. 6AB). These results, observable in both cell lines (Fig. 6AB), further suggest that this natural putrescine analogue might have effects on activating intrinsic mitochondrial apoptotic pathway in melanoma cells, and permit to hypothesize a possible use of pholiotic acid to overcome drug-induced resistance in cancer cells. On the other hand, overexpression of Bcl-2 in cancer cells was reported to be a

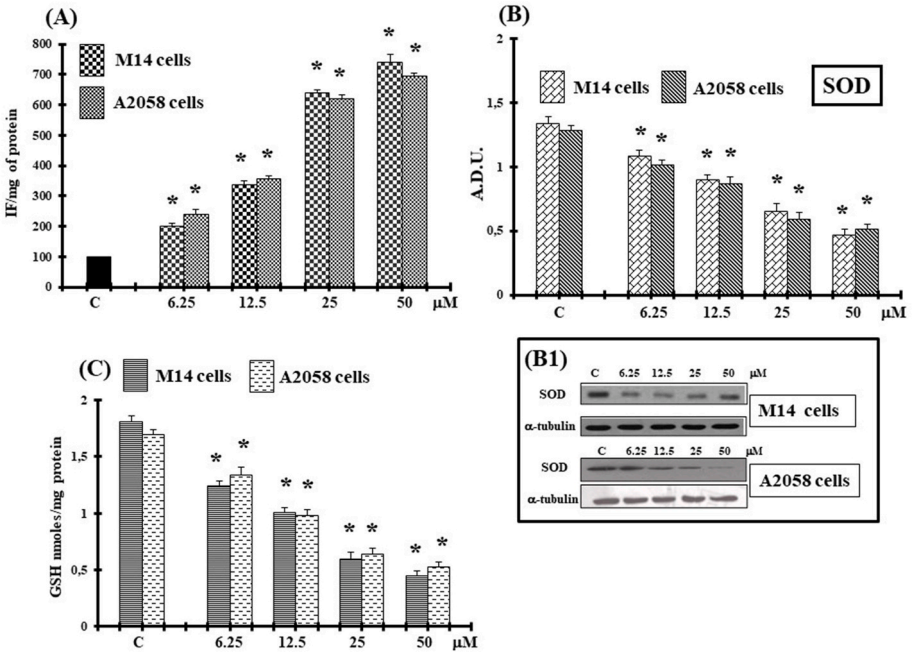

Fig. 7. ABC ROS determination (A), SOD protein expression (B and B1) and GSH levels (C) in melanoma cells control and treated with different concentrations of pholiotic acid for 72 h *Significant vs. control untreated cells ($p < 0.001$).

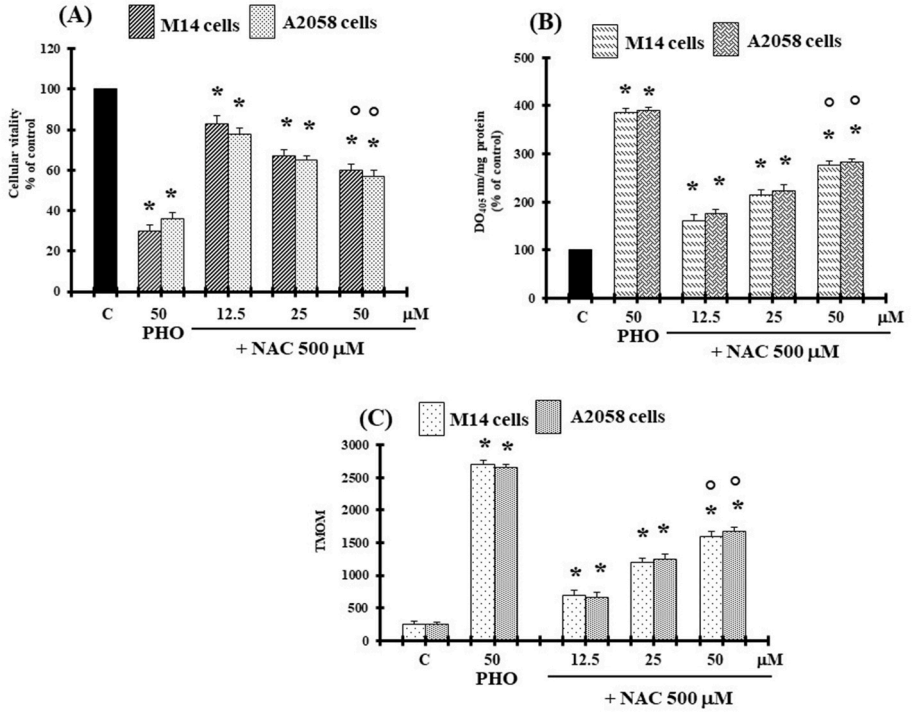

Fig. 8. ABC Cell viability (A), Caspase-3 activity (B) and COMET assay (C) in control and treated melanoma cells for 72 h with pholiotic acid (12.5–50 μM)-NAC (500 μM) or with pholiotic acid (PHO) (50 μM) alone. *Significant vs. control untreated cells ($p < 0.001$). ◦Significant vs. cells treated with pholiotic acid (50 μM) ($p < 0.001$).

resistance factor against chemotherapy and radiation [39].

The Hsp70 operates as a protective molecule. This protein, acting as chaperone stabilizing protein structure of de novo synthesized proteins, can be beneficial for the whole organism, if it is expressed in normal cells. However, in cancer cells, this chaperone, contributing to keeping cancerous cells alive, is a negative prognostic marker [40]. In some tumors, the expression of Hsp70 is abnormally high, and it may participate in oncogenesis and in resistance to chemotherapy [40]. In particular, some literature data show that this chaperone is overexpressed in melanoma. It plays a role in melanoma progression/prognosis, and its high levels may be associated with drug resistant melanoma [41,42]. It has been suggested that in melanoma cells its tumorigenic potential seems to correlate with its capacity to impair apoptosis [41]. High Hsp70 expression prevents the apoptotic pathway at various levels. It has been

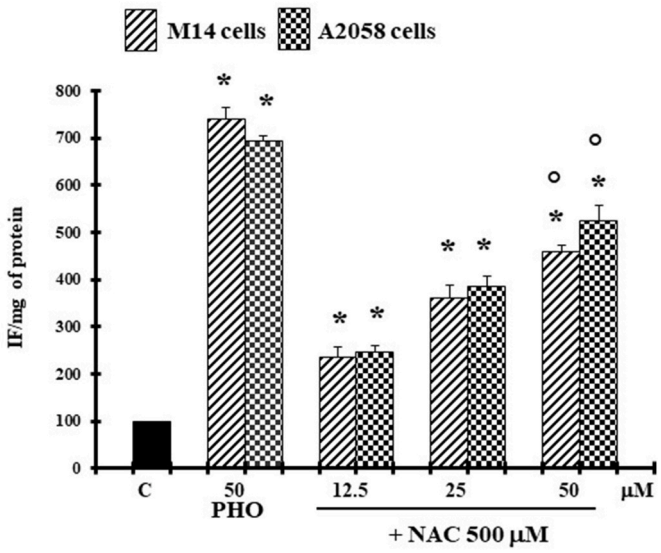

Fig. 9. ROS determination in control and treated melanoma cells for 72 h with pholiotic acid (12.5–50 μM)-NAC (500 μM) or with pholiotic acid (PHO) (50 μM) alone. *Significant vs. control untreated cells ($p < 0.001$). °Significant vs. cells treated with pholiotic acid (50 μM) ($p < 0.001$).

shown to modular some transcription factors correlated with the expression of the Bcl-2 family [40,43]. In addition, it has been suggested that Hsp70 may inhibit apoptosis downstream of the release of cytochrome *c* and upstream of the activation of caspase-3. Hsp70 has been demonstrated to directly bind to apoptosis protease-activating factor-1 (Apaf-1), thereby preventing the recruitment of procaspase-9 to the apoptosome [40,43]. Consequently, Hsp70 inhibitors show tumor selective cytotoxicity, with limited toxicity in non-transformed cells [41]. Interestingly, recent experimental evidences [44] show a synergistic effect of combining Hsp70 inhibitors with binimetinib and trametinib, that have produced modest results in patients with NRAS-mutant melanoma, when administered as single agents. Consequently, it has been suggested that Hsp70 is an interesting therapeutic target for melanoma [44]. In tumor cells, the crosstalk between Hsps and polyamines leads to tumor progression in different cancer types [45]. Interruption of this interaction might be beneficial for the treatment of various tumors. It was observed that polyamine depletion in cancer cells resulted in inhibition of Hsp70 expression [45]. Therefore, it has been suggested that a combined treatment of Hsp70 inhibitors and polyamine antagonists might be an excellent strategy [46]. In this context, the idea was born to investigate the role of our natural polyamine analogue on the expression of this chaperone in melanoma cells. Pholiotic acid, possibly inducing depletion of polyamines through their catabolism, as reported for other polyamine analogues [46], inhibited Hsp70 expression (Fig. 5 AB). We supposed that this inhibitory activity, at least in part, is correlated with an increase of caspase-3 enzyme activity (Fig. 3), a high DNA fragmentation (Fig. 4) and an increase in Bax (Fig. 6AB), and caspase-9 proteins (Fig. 5AB), in conjunction with the more pronounced decrease in Bcl-2 (Fig. 6AB). Therefore, these data that reinforce the existence of a linkage between polyamines, Hsp70 expression and cancer cell demise, permit to hypothesize that the reduction of Hsp70 levels, evidenced in our experimental conditions (Fig. 5AB), may contribute to induce apoptosis by the intrinsic or mitochondrial apoptotic pathway in human metastatic melanoma cell lines, M14 and A2058, suggesting its possible use in combination therapies that will have considerable clinical promise [31].

PTEN is a tumour suppressor, frequently lost or mutated in melanoma [47]. PTEN is a phosphatase that plays an important role in antagonising the AKT pathway, that is critical to cell growth, survival and migration. In fact, it is a major negative regulator of the AKT pathway, in that it catalyses the dephosphorylation of PIP3 to PIP2, decreasing levels of activated AKT, that act on a variety of downstream targets, including mTORC1, that control processes such as cell cycle progression and cell survival [47]. In view of increasing knowledge of the interplay between polyamine metabolism and the pathway PTEN–PI3K–mTOR complex 1 (mTORC1), the significant increase of PTEN expression (Fig. 5AB), evoked by our polyamine analogue acquire more value, and further supports the possibility of clinical use of pholiotic acid for treating melanoma.

It is widely known, that oxidative stress can be one of the critical factors in promoting cellular death. While a moderate level of ROS provokes DNA mutations and triggers tumorigenesis, too high ROS level is generally detrimental to the intracellular environment, as it activates signaling pathways of programmed cell death [48–50]. Although increasing ROS generation has recently been considered a valuable system by which to kill cancer cells, this might be inadequate due to cancer cell adaptation strategies [46]. It has been reported that some polyamine analogues overcame resistance against Paclitaxel treatment in cancer cells via activating polyamine catabolism and consequently increasing ROS production [46]. On the other hand, it was well established that the increased expression of enzymes involved in polyamine catabolism trigger apoptotic cell death leading to polyamine depletion and generation of ROS as by-products [31,32,46]. Our preclinical evidences, which of course require further studies, permit to suppose that pholiotic acid could act in this via like some other polyamine analogues [31,32,46]. The hypothesis that the activity of this fungal secondary metabolite could be attributable, at least in part, to increased polyamines catabolism is sustained by the increase of ROS production (Fig. 7A), correlated to a reduction of SOD enzyme expression (Fig. 7B), evidenced in our experimental conditions. On the other hand, the incubation with pholiotic acid and NAC, an antioxidant drug, in part reduced ROS production (Fig. 9) and increased cell vitality (Fig. 8A). Contextually, the co-treatment resulted in a reduction of caspase-3 activity (Fig. 8B) and DNA damage in both metastatic melanoma cell lines (Fig. 8C).

Moreover, consistent with these findings, pholiotic acid was able to induce GSH depletion (Fig. 7C). In fact, it is known that GSH oxidation is a factor involved in the intrinsic apoptosis pathway, which is associated with mitochondria [51]. This observation further leads us to think that the increased ROS production might have contributed to the inhibition of cell growth observed in melanoma cells following pholiotic acid-exposure. Also these pre-clinical evidences suggest that research on this polyamine analogue can be extremely valuable as an alternative to traditional chemotherapy to improve efficacy and reduce severity of side effects.

5. Conclusion

In conclusion, these findings implement available our data to support pholiotic acid as an attractive potential chemopreventive agent and provide a basis for further research into the use of this polyamine derivative as a potential agent to combinate with existing melanoma therapies to improve treatment efficacy and overcome the obstacle of drug resistance. They might suggest hope for improvement as a new therapeutic path to human cutaneous melanoma. In particular considering the medical, social and financial effects of melanoma treatment, as well as mortality from it, the use of the hypothesized synergism, can be a significant improvement in the quality of life of patients that present resistance to chemotherapy and radiation.

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CRediT authorship contribution statement

R. Avola: Formal analysis, Data curation, Conceptualization. **A.C.E. Graziano:** Formal analysis, Data curation. **A. Madrid:** Supervision, Conceptualization. **M. Clericuzio:** Supervision, Conceptualization. **V. Cardile:** Supervision, Data curation, Conceptualization. **A. Russo:** Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing 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.

Data availability

No data was used for the research described in the article.

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References

[1] J. Fernández, B. Silván, R. Entrialgo-Cadierno, C.J. Villar, R. Capasso, J.A. Uranga, F. Lombó, R. Abalo, Antiproliferative and palliative activity of flavonoids in colorectal cancer, Biomed. Pharmacother. 143 (2021) 112241, https://doi.org/10.1016/j.biopha.2021.112241.

[2] I. Cerqua, K. Neukirch, M. Terlizzi, E. Granato, E. Caiazzo, C. Cicala, A. Ialenti, R. Capasso, O. Werz, R. Sorrentino, D. Seraphin, J.J. Helesbeux, G. Cirino, A. Koeberle, F. Roviezzo, A. Rossi, A vitamin E long-chain metabolite and the inspired drug candidate α-amplexichromanol relieve asthma features in an experimental model of allergen sensitization, Pharmacol. Res. 181 (2022) 106250, https://doi.org/10.1016/j.phrs.2022.106250.

[3] E.G. Ferrarini, R.S. Paes, G.M. Baldasso, P.M. de Assis, M.C. Gouvêa, P. Cicco, N.R. B. Raposo, R. Capasso, E.L.G. Moreira, R.C. Dutra, Broad-spectrum cannabis oil ameliorates reserpine-induced fibromyalgia model in mice, Biomed. Pharmacother. 154 (2022) 113552, https://doi.org/10.1016/j.biopha.2022.113552.

[4] Y. Açar, D. Ağagündüz, P. De Cicco, R. Capasso, Flavonoids: their putative neurologic roles, epigenetic changes, and gut microbiota alterations in Parkinson's disease, 2023 Parkinson's disease, Biomed. Pharmacother. 168 (2023) 115788, https://doi.org/10.1016/j.biopha.2023.115788.

[5] T.Ö. Şahin, B. Yılmaz, N. Yeşilyurt, D. Cicia, A. Szymanowska, P. Amero, D. Ağagündüz, R. Capasso, Recent insights into the nutritional immunomodulation of cancer- related microRNAs, Phytother Res. 37 (2023) 4375–4397, https://doi.org/10.1002/ptr.7937.

[6] Global Burden of Disease Cancer Collaboration, C. Fitzmaurice, C. Allen, R. M. Barber, L. Barregard, Z.A. Bhutta, H. Brenner, et al., Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 32 cancer groups, 1990 to 2015: a systematic analysis for the Global Burden of Disease Study, JAMA Oncol. 3 (2017) 524–548, https://doi.org/10.1001/jamaoncol.2016.568.

[7] G.B. da Silva, M.A. Yamauchi, D. Zanini, M.D. Bagatini, Novel possibility for cutaneous melanoma treatment by means of rosmarinic acid action on purinergic signaling, Purinergic Signal. 18 (2022) 61–81, https://doi.org/10.1007/s11302-021-09821-7.

[8] S.P. Wasser, Medicinal mushroom science: history, current status, future trends, and unsolved problems, Int. J. Med. Mushrooms 1 (2010) 1–16. https://doi:10.1615/IntJMedMushr.v12.i1.10.

[9] M. Jeitler, A. Michalsen, D. Frings, M. Hübner, M. Fischer, D.A. Koppold-Liebscher, V. Murthy, C.S. Kessler, Significance of medicinal mushrooms in integrative Oncology: a narrative review, Front. Pharmacol. 11 (2020) 580656. https://doi:10.3389/fphar.2020.580656.eCollection2020.

[10] S.P. Wasser, Medicinal mushrooms in human clinical studies. Part I. Anticancer, oncoimmunological, and immunomodulatory activities: a review, Int. J. Med. Mushrooms 19 (2017) 279–317. https://doi:10.1615/IntJMedMushrooms.v19.i4.10.

[11] A. Blagodatski, M. Yatsunskaya, V. Mikhailova, V. Tiasto, A. Kagansky, V. L. Katanaev, Medicinal mushrooms as an attractive new source of natural compounds for future cancer therapy, Oncotarget 9 (2018) 29259–29274. https://doi:10.18632/oncotarget.25660.

[12] M. Clericuzio, S. Tabasso, J.A. Garbarino, M. Piovano, V. Cardile, A. Russo, G. Vidari, Non-phenolic dicinnamamides from *Pholiota Spumosa*: isolation, synthesis and antitumour activity, Eur. J. Org. Chem. 33 (2007) 5551–5559, https://doi.org/10.1002/ejoc.200700558.

[13] D.L. Kramer, S. Vujcic, P. Diegelman, J. Alderfer, J.T. Miller, J.D. Black, R. J. Bergeron, C.W. Porter, Polyamine analogue induction of the p53-p21WAF1/CIP1-Rb pathway and G1 arrest in human melanoma cells, Cancer Res. 59 (1999) 1278–1286.

[14] B. Provenzano, A. Lentini, R. Tatti, A. De Martino, I. Borromeo, C. Mischiati, G. Feriotto, C. Forni, C. Tabolacci, S. Beninati, Evaluation of polyamines as marker of melanoma cell proliferation and differentiation by an improved high-performance liquid chromatographic method, Amino Acids 51 (2019) 1623–1631. https://doi:10.1007/s00726-019-02799-y.

[15] S.-Y. Hwang, J.-I. Chae, A.-W. Kwak, M.-H. Lee, J.-H. Shim, Alternative options for skin cancer therapy via regulation of AKT and related signaling pathways, Int. J. Math. Stat. 21 (2020) 6869, https://doi.org/10.3390/ijms21186869.

[16] C. Formisano, D. Rigano, A. Russo, V. Cardile, S. Caggia, N. Apostolides Arnold, A. Mari, S. Piacente, S. Rosselli, F. Senatore, M. Bruno, Phytochemical profile and apoptotic activity of *Onopordum cynarocephalum*, Planta Med. 78 (2012) 1651–1660, https://doi.org/10.1055/s-0032-1315259.

[17] A. Russo, A. Graziano, M. Bruno, V. Cardile, D. Rigano, Apoptosis induction of essential oils from Artemisia arborescens L. in human prostate cancer cells, J. Ethnopharmacol. 303 (2023) 115929. https://doi:10.1016/j.jep.2022.115929.

[18] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[19] V. Cardile, A.C.E. Graziano, R. Avola, A. Madrid, A. Russo, Physodic acid sensitizes LNCaP prostate cancer cells to TRAIL-induced apoptosis, Toxicol. Vitro 84 (2022) 105432. https://doi:10.1016/j.tiv.2022.105432.

[20] A.E. Russo, E. Torrisi, Y. Bevelacqua, R. Perrotta, M. Libra, J.A. McCubrey, D. A. Spandidos, F. Stivala, G. Malaponte, Melanoma: molecular pathogenesis and emerging target therapies, Int. J. Oncol. 34 (2009) 1481–1489. https://doi:10.3892/ijo_00000277.

[21] H. Wu, V. Goel, F.G. Haluska, PTEN signaling pathways in melanoma, Oncogene 22 (2003) 3113–3122, https://doi.org/10.1038/sj.onc.1206451.

[22] C.Y. Chen, C.Y. Yen, H.R. Wang, H.P. Yang, J.Y. Tang, H.W. Huang, et al., Tenuifolide B from *Cinnamomum tenuifolium* stem selectively inhibits proliferation of oral cancer cells via apoptosis, ROS generation, mitochondrial depolarization, and DNA damage, Toxins 8 (2016) 319. https://doi:10.3390/toxins8110319.

[23] J. Fang, T. Seki, H. Maeda, Therapeutic strategies by modulating oxygen stress in cancer and inflammation, Adv. Drug. Deliv. Rev. Rev. 61 (2009) 290–302, https://doi.org/10.1016/j.addr.2009.02.005.

[24] K.D. Miller, L. Nogueira, A.B. Mariotto, J.H. Rowland, K.R. Yabroff, C.M. Alfano, D. V.M. Ahmedin Jemal, L. Joan, M.D. Kramer, L.R. Siegel, Cancer treatment and survivorship statistics, 2019, CA A Cancer J. Clin. 69 (2019) 363–385, https://doi.org/10.3322/caac.21565.

[25] IARC - International Agency of Research in Cancer, Global Burden of Cutaneous Melanoma in 2020 and Projections to 2040, 2022. Available in: https://www.iarc.who.int/news-events/global-burden-of-cutaneous-melanoma-in-2020-and-projections-to2040/#:~.

[26] D. Schadendorf, A.C.J. van Akkooi, C. Berking, K.G. Griewank, R. Gutzmer, A. Hauschild, A. Stang, A. Roesch, S. Ugurel, Melanoma, Lancet 392 (2018) 971–984, https://doi.org/10.1016/S0140-6736(18)31559-9.

[27] S. Coni, L. Di Magno, S.M. Serrao, Y. Kanamori, E. Agostinelli, G. Canettieri, Polyamine metabolism as a therapeutic target in hedgehog-driven basal cell carcinoma and medulloblastoma, Cells 8 (2019) 150, https://doi.org/10.3390/cells8020150.

[28] R.A. Casero, T. Murray Stewart, A.E. Pegg, Polyamine metabolism and cancer: treatments, challenges and opportunities, Nat. Rev. Cancer 18 (2018) 681–695, https://doi.org/10.1038/s41568-018-0050-3.

[29] B. Provenzano, A. Lentini, R. Tatti, A. De Martino, I. Borromeo, C. Mischiati, G. Feriotto, C. Forni, C. Tabolacci, S. Beninati, Evaluation of polyamines as marker of melanoma cell proliferation and differentiation by an improved high-performance liquid chromatographic method, Amino Acids 51 (2019) 1623–1631, https://doi.org/10.1007/s00726-019-02799-y.

[30] I.N. Sari, T. Setiawan, K.S. Kim, Y.T. Wijaya, K. Won Cho, H.Y. Kwon, Metabolism and function of polyamines in cancer progression, Cancer Lett. 519 (2021) 91–104. https://doi:10.1016/j.canlet.2021.06.020.

[31] Z. Akyol, A. Çoker-Gürkan, E.D. Arisan, P. Obakan-Yerlikaya, N. Palavan-Ünsal, DENSpm overcame Bcl-2 mediated resistance against Paclitaxel treatment in MCF-7 breast cancer cells via activating polyamine catabolic machinery, Biomed. Pharmacother. 84 (2016) 2029–2041. https://doi:10.1016/j.biopha.2016.11.016.

[32] E.V. Neborak, A.B. Kaldybayeva, L. Bey, A.Y. Malmakova, A.S. Tveritinova, A. Hilal, V.K. Yu, M.V. Ploskonos, M.V. Komarova, E. Agostinelli, D.D. Zhdanov, Anticancer cytotoxic activity of bispidine derivatives associated with the increasing catabolism of polyamines, Molecules 27 (2022) 3872. https://doi:10.3390/molecules27123872.

[33] K.A. Perevoshchikova, E.A. Eshtukova-Shcheglova, O.V. Markov, A.V. Markov, I. V. Chernikov, M.A. Maslov, M.A. Zenkova, Symmetric lipophilic polyamines exhibiting antitumor activity, Bioorg. Med. Chem. 76 (2022) 117089. https://doi:10.1016/j.bmc.2022.117089.

[34] Y. Chen, D.L. Kramer, P. Diegelman, S. Vujcic, C.W. Porter, Apoptotic signaling in polyamine analogue-treated SK-MEL-28 human melanoma cells, Cancer Res. 61 (2001) 6437–6444.

[35] M.S. Soengas, S.W. Lowe, Apoptosis and melanoma chemoresistance, Oncogene 22 (2003) 3138–3151. https://doi:10.1038/sj.onc.1206454.

[36] P. Lodeserto, M. Rossi, P. Blasi, G. Farruggia, I. Orienti, Nanospermidine in combination with nanofenretinide induces cell death in neuroblastoma cell lines, Pharmaceutics 14 (2022) 1215, https://doi.org/10.3390/pharmaceutics14061215.

[37] M. Asadi, S. Taghizadeh, E. Kaviani, O. Vakili, M. Taheri-Anganeh, M. Tahamtan, A. Savardashtaki, Caspase-3: structure, function, and biotechnological aspects,

Biotechnol. Appl. Biochem. 69 (2022) 1633–1645, https://doi.org/10.1002/bab.2233.
[38] M.S. D'Arcy, Cell death: a review of the major forms of apoptosis, necrosis and autophagy, Cell Biol. Int. 43 (2019) 582–592, https://doi.org/10.1002/cbin.11137.
[39] A. Basu, The interplay between apoptosis and cellular senescence: Bcl-2 family proteins as targets for cancer therapy, Pharmacol. Therapeut. 230 (2022) 107943, https://doi.org/10.1016/j.pharmthera.2021.107943.
[40] A.R. Goloudina, O.N. Demidov, C. Garrido, Inhibition of HSP70: a challenging anticancer strategy, Cancer Lett. 325 (2012) 117–124. https://doi:10.1016/j.canlet.2012.06.003.
[41] A. Budina-Kolomets, M.R. Webster, J.I. Leu, M. Jennis, C. Krepler, A. Guerrini, A. V. Kossenkov, W. Xu, G. Karakousis, L. Schuchter, R.K. Amaravadi, H. Wu, X. Yin, Q. Liu, Y. Lu, G.B. Mills, X. Xu, D.L. George, A.T. Weeraratna, M.E. Murphy, HSP70 inhibition limits FAK-dependent invasion and enhances the response to melanoma treatment with BRAF inhibitors, Cancer Res. 76 (2016) 2720–2730. https://doi:10.1158/0008-5472.CAN-15-2137.
[42] K.A. Parker, S. Glaysher, M. Polak, F.G. Gabriel, P. Johnson, L.A. Knight, M. Poole, A. Narayanan, J. Hurren, I.A. Cree, The molecular basis of the chemo sensitivity of metastatic cutaneous melanoma to chemotherapy, J. Clin. Pathol. 63 (2010) 1012–1020. https://doi:10.1136/jcp.2010.080119.
[43] M.E. Murphy, The HSP70 family and cancer, Carcinogenesis 34 (2013) 1181–1188, https://doi.org/10.1093/carcin/bgt111.
[44] J.L.D. Parris, T. Barnoud, J.I.-J. Leu, J.C. Leung, W. Ma, N.A. Kirven, A.N.R. Poli, A. V. Kossenkov, Q. Liu, J.M. Salvino, D.L. George, A.T. Weeraratna, Q. Chen, M. E. Murphy, HSP70 inhibition blocks adaptive resistance and synergizes with MEK inhibition for the treatment of *NRAS*-mutant melanoma, Cancer Res. Commun. 1 (2021). https://doi:10.1158/2767-9764.CRC-21-0033.
[45] X.H. Makhoba, R. Ragno, A. Kaiser, E. Agostinelli, An undefined interaction between polyamines and heat shock proteins leads to cellular protection in plasmodium falciparum and proliferating cells in various organisms, Molecules 28 (2023) 1686, https://doi.org/10.3390/molecules28041686.
[46] E.V. Neborak, A.B. Kaldybayeva, L. Bey, A.Y. Malmakova, A.S. Tveritinova, A. Hilal, V.K. Yu, M.V. Ploskonos, M.V. Komarova, E. Agostinelli, et al., Anticancer cytotoxic activity of bispidine derivatives associated with the increasing catabolism of polyamines, Molecules 27 (2022) 3872, https://doi.org/10.3390/molecules27123872.
[47] S. Chagani, M.P. De Macedo, F. Carapeto, F. Wang, D.M. Marzese, K. Wani, L. E. Haydu, W. Peng, G.T. Ong, S.E. Warren, J.M. Beechem, D.S.B. Hoon, G.B. Mills, M.T. Tetzlaff, A.J. Lazar, L.N. Kwong, M.A. Davies, Multiplatform analysis of intratumoral PTEN heterogeneity in melanoma, J. Invest. Dermatol. 143 (2023) 1779–1787.e1. https://doi:10.1016/j.jid.2023.01.034.
[48] B. Halliwell, Oxidative stress and cancer: have we moved forward? Biochem. J. 401 (2007) 1–11.
[49] E. Isacescu, P. Chiroi, O. Zanoaga, A. Nutu, L. Budisan, R. Pirlog, A.G. Atanasov, I. Berindan-Neagoe, Melanoma cellular signaling transduction pathways targeted by polyphenols action mechanisms, Antioxidants 12 (2023) 407, https://doi.org/10.3390/antiox12020407.
[50] G. Cappelli, D. Giovannini, L. Vilardo, A. Basso, I. Iannetti, M. Massa, G. Ruberto, R. Muir, C. Pastore, I. D'Agnano, et al., *Cinnamomum zeylanicum* blume essential oil inhibits metastatic melanoma cell proliferation by triggering an incomplete tumour cell stress response, Int. J. Mol. Sci. 24 (2023) 5698, https://doi.org/10.3390/ijms24065698.
[51] M.L. Circu, T.Y. Aw, Glutathione and modulation of cell apoptosis, Biochim. Biophys. Acta 1823 (2012) 1767–1777. https://doi:10.1016/j.bbamcr.2012.06.019.