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Article

Polypodium vulgare L.: polyphenolic profile, cytotoxicity and cytoprotective properties in different cell lines

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Abstract: *Pteridophytes*, represented by ferns and allies, are an important phytogenetic bridge between lower and higher plants (gymnosperms and angiosperms). Ferns have evolved independently of any other species in the plant kingdom being its secondary metabolism a reservoir of phytoconstituents characteristic of this taxon. The study of the possible medicinal uses of *Polypodium vulgare* L. (*Polypodiaceae*), PV, has increased particularly when in 2008 the European Medicines Agency published a monograph about the rhizome of this species. Thus, our objective is to provide scientific knowledge on the methanolic extract from the fronds of *P. vulgare* L., one of the main ferns described in the Prades Mountains, to contribute to the validation of certain traditional uses. Specifically, we have characterized the methanolic extract of PV fronds (PVM) by HPLC-DAD and investigated its potential cytotoxicity, phototoxicity, ROS production and protective effects against oxidative stress by using *in vitro* methods. Our results show that PVM is not cytotoxic against the different cell lines assessed, but we found potential cytoprotective and cellular repair activity in 3T3 fibroblast cells. This biological activity could be attributed to the high content of polyphenolic compounds; thus, this extract is positioned as a potential candidate for pharmaceutical uses.

Keywords: cytoprotection; cytotoxicity; ferns; oxidative stress; *Polypodiaceae*; *Polypodium vulgare* L.; medicinal plants

1. Introduction

Oxidative stress is characterized by an imbalance between pro-oxidant agents (free radicals, reactive oxygen species, ROS, or nitrogen species, RNS), generated by the metabolism itself or by the permanent exposure of the organism to the environment, and the system of antioxidant defence. According to its origin, this antioxidant system is classified as endogenous (enzymatic and non-enzymatic systems) or exogenous[1]. The exogenous antioxidant system was defined by Halliwell et al.[2] as *"any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate"*.

The inability to maintain an adequate redox state, either due to excess production of free radicals or an alteration of the antioxidant system, triggers oxidative damage that affects fundamental biological structures. This state is characterized by alterations in the different biological molecules, carbohydrates, proteins, lipids and mainly in DNA[3]. In this sense, studies have associated oxidative stress with the development of different neurodegenerative diseases, inflammatory bowel disease, cardiovascular diseases, metabolic diseases such as type 2 diabetes and even cancer[4,5]. The reduction of the

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incidence of cancer in population groups with diets rich in polyphenols compared to the absence or a mild diet rich in polyphenols is evidence for the chemopreventive action of plant-based food, as it is through the neutralization of part of the free radicals to the which is exposed our body[6].

Numerous investigations have established the link between sun exposure and skin alterations[7,8]. Today it is well documented that ultraviolet radiation affects animals in different causes, among which oxidative stress, inflammation, erythema, breakdown of the extracellular matrix, wrinkling and skin cancer. But the main effect of this ultraviolet irradiation is the increase in oxidative stress caused by the increase in ROS, which can lead to an imbalance in the endogenous antioxidant system[9]. Being ultraviolet irradiation the main etiology in the development of skin cancer in its two categorizations: melanoma and non-melanoma skin cancers[10]. In a retrospective study between 1970 and 2012 using German cancer registry data, Leiter et al.[11] observed that melanoma and non-melanoma skin cancers have increased because of repeated and inadequate sun exposure of individuals and thus to an increase in epithelial oxidative stress. The integumentary system, in addition to being the largest organ in the body, is the first physical barrier between the body and the external environment, hence its importance in its proper maintenance. Molecules of natural origin can modulate the behaviour of tumour cells by acting on different pathways of molecular signalling such as the topoisomerase inhibition (genistein[12]), kinase inhibition (apigenin[13]) and modulation of multidrug resistance (2',4',6'-tri*OH*-chalcone[14]), among other pathways[15]. The doxorubicin, paclitaxel, vinblastine, etoposide, irinotecan, gemcitabine and methotrexate are medically successful in anticancer therapy, for their security and efficacy, which are part of the list of anticancer agents provided or inspired by nature in recent years[16,17]. In addition, molecules of natural origin, as flavonoids for their safety and accessibility, can also be a potential inspiration to create an original therapeutic or preventive (chemopreventive) for cancer treatment and prevention respectively[18-20].

Ethnopharmacological investigations on traditional Chinese medicine have reported the therapeutic uses of ferns in modern medicine. For that purpose, different bioassays were performed as for example antioxidant (*Dryoathyrium boryanum* (Willd.) Ching (*Athyriaceae* family)[21]), acetycholinesterase inhibition (*Stenochlaena palustres* (Burm. f.) Bedd. (*Blechnaceae* family)[22]), tyrosinase inhibition (*Asplenium adiantum-nigrum* L. (*Aspleniaceae* family)[23]) and anti-tumour activity (*Davallia cylindrica* Ching (*Davalliaceae* family)[24]) attributed to some ferns.

The antibiotic activity of hundreds of ferns has also been evaluated in against a great diversity of bacteria[25,26]. Currently, the fern that presents a more detailed phytochemical characterization and evaluation for skin therapeutic uses such as photodermatosis, adjunctive treatment of melasma (as chemopreventive), vitiligo, psoriasis vulgaris and atopic dermatitis, is the aqueous extract of *Polypodium leucotomos* (PLE)[27,28]. This fern belongs to the *Polypodiaceae* family like the *Polypodium vulgare* L. (PV)[29,30].

PV, known commonly as "*polipodio*" in Spain for the shape of its fronds as feet (*poly:* many and *podos:* foot), is a fern of the leptosporangiate class belonging to the *Polypodiaceae* family[31-33]. Since 2008, the rhizome of PV has been accepted by European Medicines Agency (EMA) for its use as expectorant herbal medicine in cough and cold and in cases of occasional constipation[34]. PV has been used as medicinal plant in Europe since ancient times. As example, in the middle of the last century the use of PV rhizome infusion as expectorant or diuretic in traditional Polish medicine is reported[35]. Moreover, the fronds of PV have an ethnoveterinary use for treatment variolous, jaundice and parasitic diseases in Spain[36]. American Indians used PV root tea for the treatment of different kinds of pain[34]. *Polypodium leucotomos* (PL) rhizome has been described in Spain for relief of the symptoms associated with mild to moderate inflammatory processes of the skin for humans under the tradename Difur® as a traditional herbal medicine[37]. Due to the potent antioxidant activity of PLE tied as a chemopreventive agent[38], we think that

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PV (another fern of *Polypodiaceae* family) could have phytoconstituents with potential chemopreventive action.

In the present study we want to highlight the insufficient number of studies dealing with ferns in comparison with angiosperms to support their potential uses [24,39]. Hopefully, this study will be a turning point for the promotion of ethnopharmacological studies for validation in the future of the multiple traditional uses of this fern in Spain[40].

A **B**

Image 1. Photography of the face fronds (A) and underside frond (B) of fresh *Polypodium vulgare* L. (*Polypodiaceae*). Pictures were taken at Prades Mountains. The euro coin reflects the dimension of the frond (image 1B).

2. Results

2.1. Phytochemical characterization of PVM by liquid chromatography with diode-array detection (HPLC-DAD)

Different types of polyphenols were monitored in the extract. The extract proved to contain different types of phenolic acids and flavonoids, as observed in Table 1 (77823.7 mg/kg). However, the methanolic extract of fronds of *Polypodium vulgare* L. did not contain naringin, quercitrin, rosmarinic acid, cinnamic acid, eugenol and trans-cinnamaldheyde.

Table 1. Quantitative determination of metabolites in the methanolic extract *Polypodium vulgare* L. by HPLC-DAD reported at 272 nm.

N°	Phytochemicals	Quantity (mg/kg extract) [a]
1	Shikimic acid	5339.3 ± 70.6
2	Gallic acid	1791.3 ± 38.3
3	5-O-caffeoylquinic acid	256.5 ± 12.1
4	3-O-caffeoylquinic acid	58778.3 ± 417.7
5	(+)-Catechin hydrate	3879.8 ± 153.3
6	(-)-Epicatechin	7158.5 ± 88.8
7	Rutin	422.7 ± 30.4
8	Hyperoside	91.3 ± 11.7

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9	3,5-di-O-caffeoylquinic acid	106.0 ± 15.5
	Total content	77823.7 ± 838.4

[a]Results are expressed in mg/kg dry extract, n=3.

The major constituents in the PVM extract, as seen in Figure 1, were 3-O-caffeoylquinic acid (58778.3 mg/kg), epicatechin (7158.5 mg/kg) shikimic acid (5339.3 mg/kg) and catechin (3879.8 mg/kg) which were phenol acids. The peculiar secondary metabolites found were hyperoside and 3,5-di-O-caffeoylquinic acid, with low concentrations (91.3 and 106.0 mg/kg respectively).

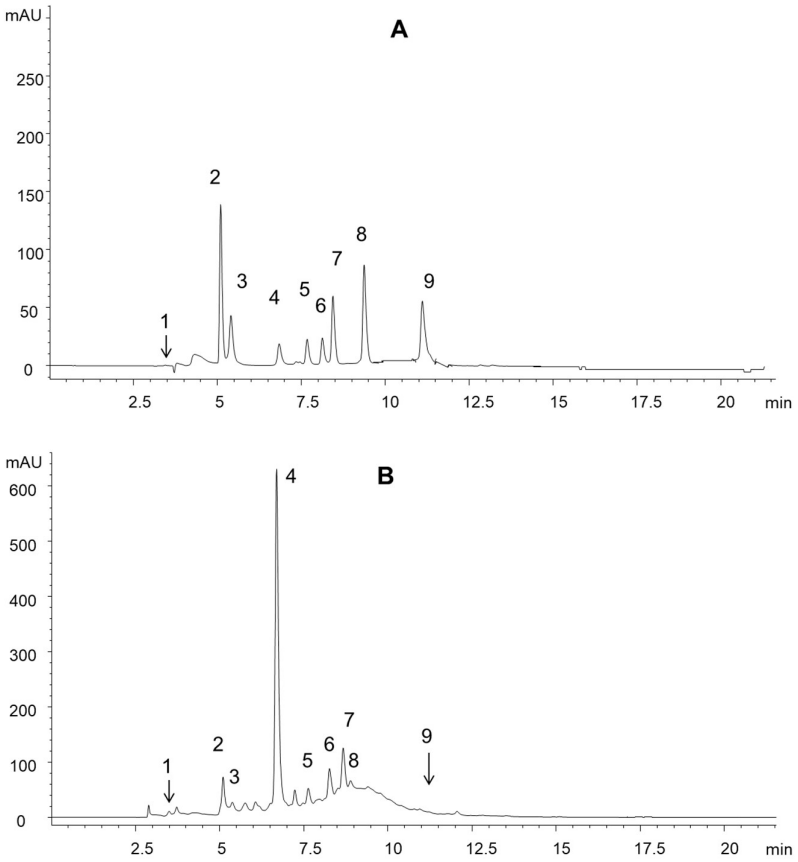

Figure 1. HPLC-DAD chromatograms reported only at 272 nm for sake of clarity and corresponding to A) standard mixture solution, B) extract of methanolic fronds extract of *Polypodium vulgare* L. List of compounds: 1 = shikimic acid, 2 = gallic acid, 3 = 5-O-caffeoylquinic acid, 4 = 3-O-caffeoylquinic acid, 5 = catechin, 6 = epicatechin, 7 = rutin, 8 = hyperoside, 9 = 3,5-di-O-caffeoylquinic acid.

2.2. In vitro cell assays

Cell viability obtained by the NRU method, show no significant differences between treated and untreated cells independently of the cell line studied (data not shown).

2.2.1. Cytotoxicity activity of PVM in non-tumoral and tumoral cell lines

A set of cytotoxic assays was carried out to determine the cytotoxic potential of different range concentrations of PVM (0.01, 0.1, 1 and 2 mg/mL PVM).

Figure 2 shows the cell viability obtained by the MTT assay for the different cell lines described here. First, we evaluated the cytotoxic activity of the PVM in 3T3 and HaCaT (Figure 2A and 2B) as a representation of non-tumoral cell lines. In both cell lines a marked increase in cytotoxicity was observed, at concentrations of 1 and 2 mg/mL PVM compared

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to 0.01 and 0.1 mg/mL PVM but with a slightly higher cytotoxicity activity of PVM in HaCaT (35.3%) than in 3T3 (46.4%) at 1 mg/mL PVM.

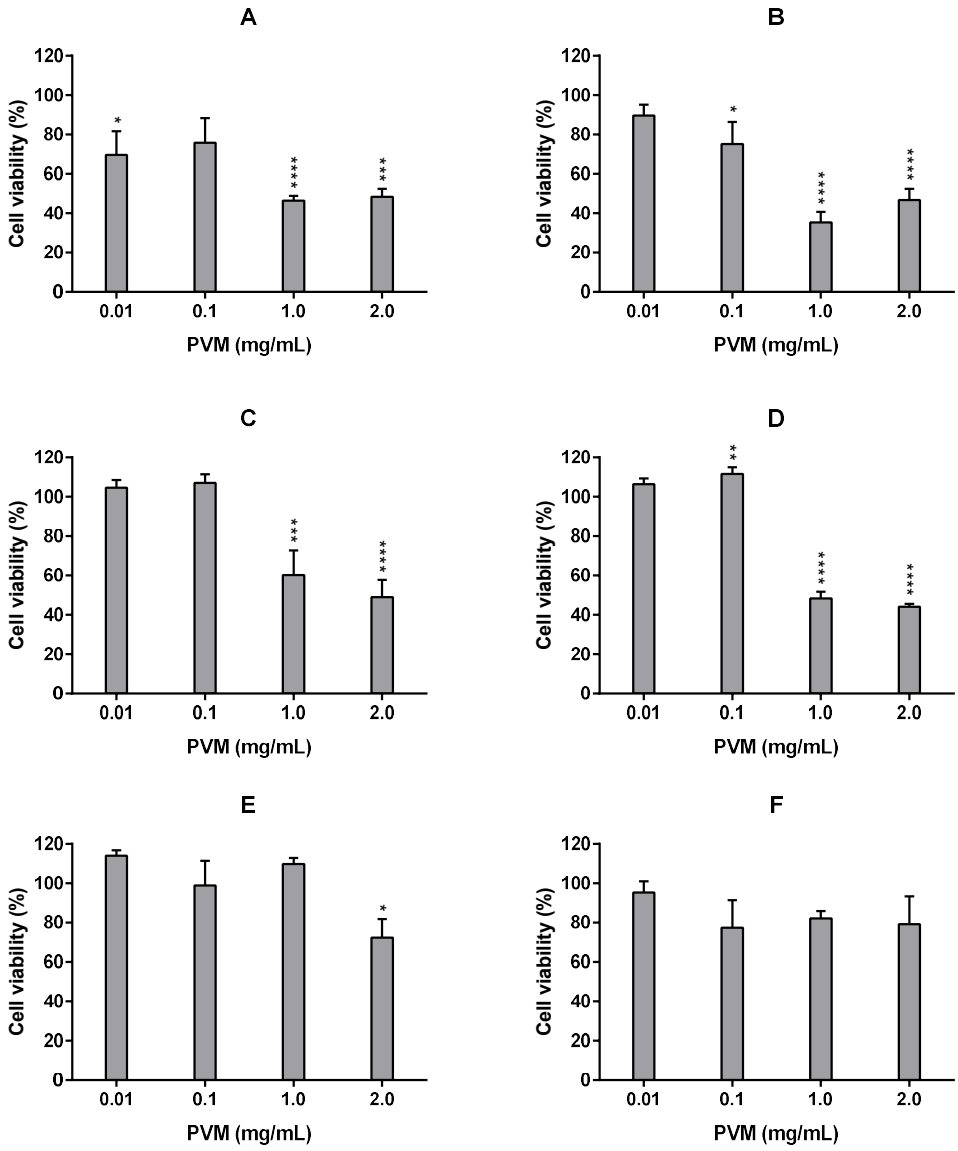

Figure 2. Cytotoxicity activity of PVM in 3T3 (A), HaCaT (B), HeLa (C), HepG2 (D), MCF-7 (E) and A549 (F) cell lines by MTT assay and expressed as percentage of cell viability respect to control cells. Results are expressed as mean ± standard error of n=3. Control cells were maintained only with culture medium. A two-way analysis of variance (ANOVA) and a Bonferroni *post hoc* assay have been performed. Statistical differences were considered as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$ compared with no treated cells (negative control).

We extended our PVM cytotoxicity study in tumoral cell lines: HeLa, HepG2, MCF-7 and A549. As we can observe in Figure 2C and 2D, PVM on HeLa and HepG2 cells presents a similar cytotoxic behaviour, presenting a significant decrease in cell viability at the highest concentration assessed, being this decrease slightly higher in HepG2 (44.2%) than HeLa (49.0%). No cytotoxic effects have been determined at 0.01 and 0.1 mg/mL of the extract.

In the case of MCF-7 and A549 cells (Figure 2E and 2F) no statistical differences among cell viability is observed at the different concentrations studied of PVM, although

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values show a slight decrease to 72.5% in MCF-7 at 2 mg/mL PVM and 77.3% in A549 at 0.1 mg/mL PVM.

The present results exhibited that, cytotoxicity effects only appear at 1 and 2 mg/mL PVM in 3T3, HaCaT, HeLa and HepG2.

2.2.2. Cytoprotective activity of PVM in 3T3 and HaCaT cell lines

Before the potential cytoprotective activity were studied, the deleterious effects of H_2O_2 in 3T3 and HaCaT were initially determined. From this previous assay (data do not show), we have established that cell viability obtained at 2 mM of H_2O_2 for 2.5 h (30.5% and 41.0% for 3T3 and HaCaT respectively) allows us to evaluate potential beneficial effects of PVM.

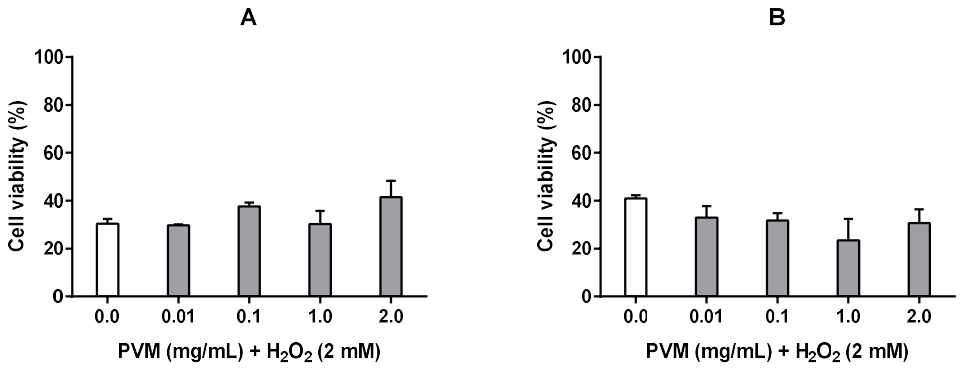

Figure 3. Cytoprotective activity of PVM in 3T3 (A) and HaCaT (B) cell lines for 2 mM H_2O_2 during 2.5 h by MTT assay and expressed as percentage of cell viability respect to untreated cells control. H_2O_2 cell viability was used as positive control. Results are expressed as mean ±- standard error of n=3 and n=2 respectively. A two-way analysis of variance (ANOVA) and a Bonferroni *post hoc* assay have been performed. No statistically significant differences were found.

As observed in Figure 3A, cell viability in 3T3 increases mainly in parallel to PVM concentration indicating some cytoprotective effect although not statistically significant, being this cytoprotecting activity of 18.9% and 26.5% at 0.1 and 2 mg/mL PVM, respectively. In the case of HaCaT (Figure 3B), no cytoprotective effect has been observed in any of the concentrations tested since an increase in the PVM concentration has not corresponded to an increase in cell viability.

2.2.3. Cellular repair activity of PVM in 3T3 tissue cell line

In the case of the cellular repair assay, we have used the same conditions of H_2O_2 as in cytoprotection assay (2 mM H_2O_2 for 2.5 h). As we can observe in Figure 4, there is a low increase in cell viability of 3T3 as PVM concentration rises, however, this discrete reparation effect is proportional to the concentration of the extract.

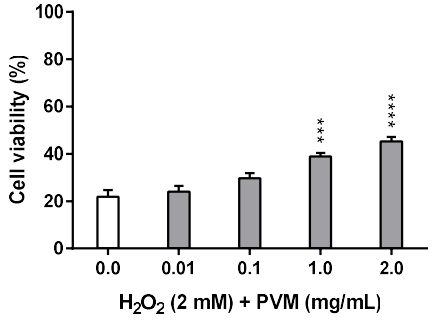

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Figure 4. Cellular repair activity of PVM in 3T3 cell line for 2 mM H_2O_2 during 2.5 h by MTT assay and expressed as percentage of cell viability respect to untreated cells control. H_2O_2 cell viability was used as positive control. Results are expressed as mean ±- standard error of n=3. A two-way analysis of variance (ANOVA) and a Bonferroni *post hoc* assay have been performed. Statistical differences were considered as follows: ***$p \leq 0.001$ and ****$p \leq 0.0001$ compared with positive control.

Table 2. Cellular repair activity of PVM in 3T3 cell line for 2 mM H_2O_2 during 2.5 h by MTT assay.

Concentration of PVM (mg/mL)	0.01	0.1	1	2
Cellular repair activity (%)[a]	8.5%	26.1%	43.6%	51.5%

[a]Percentage of cellular repair activity has been obtained from the following relation $[(CV_{PVM\text{-}H2O2} - CV_{H2O2}) / CV_{PVM\text{-}H2O2}] \times 100$.

2.2.4. Phototoxicity activity of PVM in 3T3 and HaCaT tissue cell lines

The validity of the assay has been determined by calculating the ratio of cell viability in irradiated respect to non-irradiated conditions of both negative and positive control cells (Table 3). As observed, a dose of 1.8 J/cm² of UVA light affects the viability of non-treated cells that decrease in both cell lines and indicating that 3T3 are much sensitive to light than HaCaT and that interpretation of data should be interpreted carefully. However, the effect of the photosensitizer CPZ is confirmed by the important drop of cell viability when exposed to UVA. Considering these ratios, the viability obtained when cells were exposed to UVA in the presence of PVM is considered.

Table 3. Comparison of cell viability of irradiated with non-irradiate controls[a].

Cell line	DMEM	CPZ
3T3	63.3%	26.9%
HaCaT	75.0%	13.7%

[a]Expressed as the percentage of cell viability of the irradiated control in relation to the corresponding non-irradiated.

In general, PVM do not show phototoxic behaviour in the conditions assayed here for 3T3 (Figure 5A) and HaCaT (Figure 5B), except at 0.1 mg/mL PVM. At this concentration, there is a decrease in viability when cells are exposed to light in a degree of 63.3% for 3T3 and 24.2% for HaCaT compared to the non-irradiated cells.

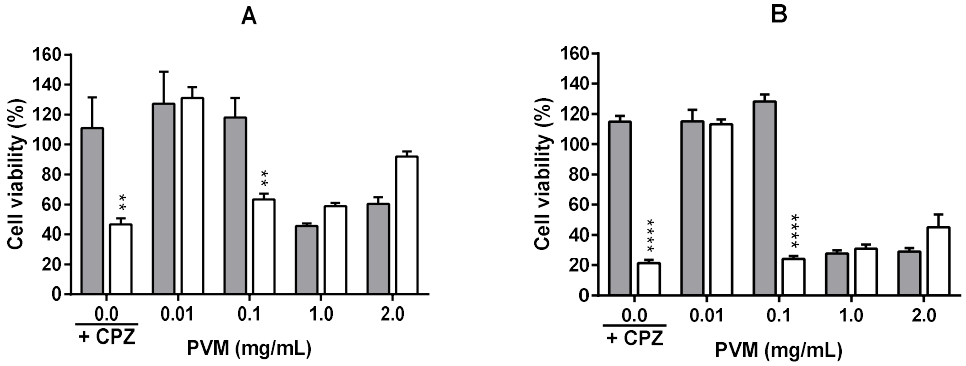

Figure 5. Phototoxicity activity of PVM in 3T3 (A) and HaCaT (B) cell lines by MTT assay and expressed as percentage of cell viability respect to the correspondent control cells. Chloropromazine cell viability was used as positive control. Gray columns correspond to cells non exposed to UVA light and white columns correspond to cells exposed to 1.8 J/cm² of UVA light. Results are expressed as mean ±- standard error of n=3. A two-way analysis of variance (ANOVA) and a Bonferroni *post*

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hoc assay have been performed. Statistical differences were considered as follows: $^{**}p \leq 0.01$ and $^{****}p \leq 0.0001$ compared with correspondence no irradiated/irradiated positive control.

2.2.5. Intracellular ROS induced by H_2O_2 of PVM in 3T3 and HaCaT cell lines

The production of ROS was explored by the fluorescence intensity with the DCF probe. As shown in Figure 6, for each cell line was obtained the same tendency of ROS production at 2 h, with similar pattern recorded at 1 h and 3 h (data not shown).

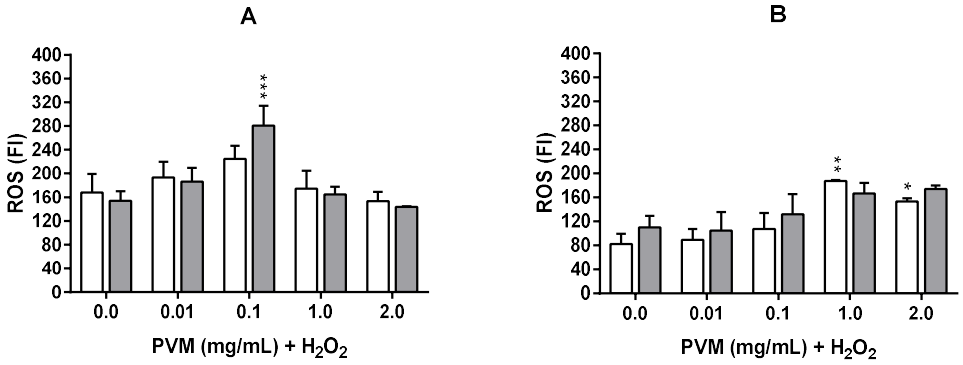

Figure 6. Intracellular ROS induced by 1 and 2 mM H_2O_2 for 2 h treatment with PVM in 3T3 (A) and HaCaT (B) cells. H_2O_2: positive control. White columns correspond to 1 mM H_2O_2 and gray columns correspond to 2 mM H_2O_2. Results are expressed as mean ±- standard error of n=3. A two-way analysis of variance (ANOVA) and a Bonferroni *post hoc* assay have been performed. Statistical differences were considered as follows: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ compared with the correspondent positive control.

The production of ROS in the conditions tested here are significantly different for both cell lines (3T3, Figure 6A and HaCaT Figure 6B). In the case of 3T3, positive controls show similar values of ROS production at the two concentrations of H_2O_2 but higher than those obtained in the case of HaCaT. This observation suggests that keratinocytes are less sensitive than 3T3.

Table 4. Intracellular ΔROS[a] induced by 1 and 2 mM H_2O_2 for 2 h respect 0 h at different concentrations of PVM in 3T3 and HaCaT.

Cell line	3T3				HaCaT			
Concentration of PVM (mg/mL)	**0.01**	**0.1**	**1**	**2**	**0.01**	**0.1**	**1**	**2**
1 mM H_2O_2	25.5	56.7	6.5	-14.7	6.8	24.77	105.1	70.9
2 mM H_2O_2	32.3	126.7	10.5	-10.7	-5.1	21.7	56.8	64.1

[a]Intracellular ΔROS cytoprotective activity has been obtained from the following relation $ROS_{PVM\ with\ DCF\text{-}H2O2}$ - $ROS_{DCF\text{-}H2O2}$ expressed as fluorescence intensity (FI).

In Table 4 we show the increment of ROS production in the different conditions. In the case of 3T3 there is a peak of intracellular ROS production in the presence of PVM at 0.1 mg/mL, which is more pronounced in cells treated with 2 mM H_2O_2, followed by an important reduction at 1 mg/mL and, finally, reverted at 2 mg/mL. This pattern is independent of the final concentration of H_2O_2. In contrast, the production of ROS is dose dependent in the case of HaCaT, except at 2 mg/mL PVM at 1 mM H_2O_2. One explanation to this different behavior between the two cell lines can be attributed to the presence of different protective antioxidant systems and that can also explain the ROS production showed by the positive controls (Figure 6).

3. Discussion

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Despite the significant biological activities attributed to *Polypodiaceae* family reported in various studies, such as antidiabetic activity (*Phymatopteris triloba* (Houtt.))[41], anti-skin tumor capability as example of anticancer activity (*Polypodium leucotomos*)[42] and anti-inflammatory activity (*Polypodium leucotomos*)[27], there are many other *Polypodiaceae* ferns not yet characterized or studied specifically from their potential medical uses. This is the case of *Polypodium vulgare* L. (PV). Currently the most studied *Polypodiaceae* fern is the *Polypodium leucotomos* (PL) because of the commercialization of its standardized aqueous extract fronds (PLE)[43]. As stated by Messeguer[44], the two major herbal drugs of PV fern are rhizome and fronds as determined in different species of *Polypodium* ferns reported by Liu[45]. In the present study we have obtained our methanol extract from the fronds of the fern. Some studies reported the isolated phytochemical composition of PV rhizome, describing the different types of phytoconstituents namely flavonoids as flavan-3-ol derivatives[46], triterpenoids hydrocarbons, triterpenoids alcohols of the cycloartane group, saponin glycosides (as osladin[47,48]), phytoecdysteroids[44] and others[49]. However, to date no published work or study has dealt with the composition of the PV fronds except Messeguer[44] that described the presence of some phytoecdysteroids. Moreover, it is required a precise phytocharacterization of the PV rhizome because of the large diversity of methodologies and solvents used to obtain the extract. For this reason, we agree with Liu et al.[45] and Sofiane et al.[50] that there is an obvious need for a chemical characterization and standardization of this rhizome extract that it is intended to be used as herbal drug. But to date few articles reporting the phytochemical-activity of this fern have been published. Among them, Sofiane et al.[50] describes antioxidant, antimicrobial and anti-inflammatory activities attributing these activities to different groups of phytoconstituents but unspecifying the plant drug used. Also, Glensk et al.[35] reports the antimicrobial activity of PV rhizome attributed to osladin. It is widely known that the production of secondary metabolites is a response to environmental conditions (environmental stress, period of the year, among other variables) to which the plant is exposed[51].To eliminate this variable, the fronds from which the PVM was obtained in the present study were collected at the same time of the year (November 2016). Using HPLC-DAD, we have determined a high number of polyphenolic substances and a small fraction of flavonoids (less than 15% of the total polyphenolic species) represented by (+)-catechin hydrate, (-)-epicatechin, rutin and hyperoside. This observation agrees with our previous study using thin layer chromatography (TLC)[23]. It is well known that flavonoids, due to their radical scavenging ability provided by its chemical structure described elsewhere[52-54], have a greater antioxidant capacity than certain phenolic acids[55]; however, flavonoids are residual components of our PVM extract so the antioxidant activity of PVM could be attributed to a synergy among all polyphenolic compounds. Other phenolics such as *p*-coumaric, ferulic, caffeic, vanillic and chlorogenic acids, were reported as the major polyphenol phytocompounds in PLE by Gombau et al.[56] and as Garcia et al.[29]. Another important aspect to consider is the synergy that the different phytoconstituents present in an extract against oxidative damage[57,58].

In traditional medicine, especially in the Asian continent, a variety of ferns have been used as remedies as the case of the Gu-Sui-Bu ferns[59]. However, other fern species contain ptaquiloside, such as *Pteridium aquilinum* (L.) Kuhn (*Dennstaedtiaceae* family), which can cause cancer[60,61]. Currently, the potential beneficial effects but also the adverse and undesirable ones of PVM are unknown. For this reason, the objective of the present work is to study the biological activity of the PVM including its cytotoxic behavior in non-tumoral (3T3 and HaCaT) and tumoral cells (HeLa, HepG2, MCF-7 and A549) as a first step to explore the potential use as anticancer remedy. The activity of the extract against radical species produced in various diseases and pathological entities is also studied by different assays. Four concentrations of the PVM extract (0.01, 0.1, 1 and 2 mg/mL) were selected in this preliminary study.

The possible use of natural fern extracts for anticancer therapy, as the case of some Asiatic fern species, has been demonstrated by the cytotoxic, pro-apoptotic or cell cycle-arresting effects of non-characterized plant extracts[62]. In our case, no relevant cytotoxic

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effects have been reported for the extract in the different cell lines, except in the case of the HaCaT cells, but only at the highest concentrations assayed. Our PVM phytochemical characterization (HPLC-DAD) indicates that epicatechin is the second major compound of the flavonoid components of the extract. Moreover, Cao et al.[24] described a strong cytotoxic behavior of an ethanol extract of the whole fern *Davallia cylindrica* Ching (*Davalliaceae* family) in A549 cells that was attributed to the high content of quercetin and its derivatives. Using HPLC-DAD we failed to detect quercitrin (a derivative sugar of quercetin), which can explain the absence of relevant cytotoxicity together with the *in chemico* antioxidant potency[23]. Considering these outcomes, we design some assays to evaluate the possible chemopreventive effect of our PVM extract in cancer[18] and topical uses, that is cytoprotection, ROS production and phototoxicity assays. For these assays, only 3T3 and HaCaT cells were used. Results obtained with these *in vitro* assays using these two cell lines open the possibility to validate the traditional use of this in the Sobrarbe region (Huesca)[40], mostly considering that fibroblasts, the most common cells in connective tissue, play a critical role in wound healing and keratinocytes form epidermis, which is a biological and physical barrier against injuries.

The potential protective effect of the PVM extract was assessed against hydrogen peroxide in 3T3 and HaCaT cells. Our results show that H_2O_2 causes slightly higher mortality in 3T3 (30.5% cell viability) than in HaCaT (41.0% cell viability) that can be explained by a higher antioxidant defense system on keratinocytes than fibroblasts[63]. This minor mortality of the positive control in HaCaT can explain that the extract failed to present cytoprotective capacity in such cell line. We only have observed a discrete cytoprotective effect of PVM in the 3T3 cells being the first report that deals with this kind of assays using a fern extract from the *Polypodiaceae* family. However, Gomes et al.[64] and Gombau et al.[56] have described the potential antioxidant activity of PLE, an extract of the fronds of a *Polypodiaceae* fern, by *in chemico* and *in vitro* methods. Considering our results of cytoprotection, the cellular repair test was only performed in the 3T3 cell line. This assay showed that there is a significant increase in cellular viability directly proportional to PVM concentration suggesting the capacity of the extract to induce cellular repair mechanisms.

Alonso-Lebrero et al.[65] reported that PLE was able to protect human fibroblast from cytoskeletal disarrangements induced by UVA light (1 J/cm^2). Moreover, Philips et al.[66] reported that a concentration lower than 0.1% PLE improves cellular membrane integrity and inhibits MMP-1 on fibroblast and keratinocytes thus suggesting its potential use in prevention on skin photoaging. However, before studying the potential photoprotective activity of the extract, we should discard any phototoxic reactions. In the present study, the determination of phototoxicity is based on the OECD TG 432[67], where the BALB/c 3T3 cell line has been replaced by NIH 3T3 and we included the HaCaT cell line and the determination of cell viability by MTT as previously reported by other authors as Baccarin et al.[68]. In general, we can conclude that the PVM extract is not phototoxic although the decrease in cell viability at 0.1 mg/mL PVM in both cell lines needs to be clarified. One explanation could arise from the direct toxic effects of UVA light over the cells that can be reverted by the presence of the extract at high concentrations but not at moderate ones as 0.1 mg/mL PVM. Further investigation should be conducted to explore the cellular mechanisms that are activated. Contact time of the extract also should be considered, thus in this phototoxicity test is one hour plus the time of UVA exposition, whereas in the rest of assays the extract remains approximately 24 h in contact with cells.

It is known that UV damages mitochondrial DNA[69], for this reason it would be interesting to also evaluate the potential phototoxic or photoprotective activity of the PVM by other assays such as the comet assay in a similar way as previously described for *Punica granatum* L. (*Lythraceae* family) seed oil nanoemulsion in HaCaT[68]. Currently, the mechanisms of PLE's preventive effect against UV-induced DNA damage, such as overexpression of the p53 gene, have already been described as chemopreventive agent[70]. However, the importance of carrying out the comet assay in PVM is justified by the classification of the fern species *Pteridium aquilinum* (L.) Kuhn (*Dennstaedtiaceae* family) in group 2B

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by the International Agency for Research on Cancer (IARC), as possibly carcinogenic to humans, consequence to its potential capacity for DNA damage mainly induced by ptaquiloside [71].

Hydrogen peroxide is an oxidative agent that promote the endogenous generation of ROS in diverse cell lines[72]. If ROS trigger cell death by dramatically toxic concentrations, the loss of mitochondrial functionality begins with the consequent apoptosis[73]. In our case, there is an increase of ROS production in both cell lines except at 2 mg/mL in the case of 3T3 which can be related to the not significant cell viability increase observed in irradiated 3T3 treated with this PVM concentration (see Table 4). This increase of ROS production observed here and, particularly in HaCaT cell line apart from the lowest concentration extract tested (0.01 mg/mL PVM), may be explained by the pro-oxidant effect of polyphenols[74]. The mechanism why this ROS can diminish cell viability needs to be clarified and further explored in other tumoral cell lines as a first step to better characterize chemotherapeutic potential of PVM[75].

4. Materials and Methods

We have designed the present work based on the experience of our group in the evaluation of the biological activity of both plant extracts and phytochemicals isolated from them[76,77]. Thus, considering FernBlock® as a reference of a protective fern against oxidative stress[65], we have evaluated the biological activity (cytotoxicity, cytoprotection, cellular repair, phototoxicity and ROS production) of the methanolic fronds extract of *Polypodium vulgare* L. (PVM) collected in the Prades Mountains. In parallel, we have also characterized the phytochemical composition of the fern extract by HPLC-DAD.

4.1. Chemicals and reagents

All reagents were of analytical grade. Trypan blue (0.4%) dye, hydrogen peroxide (H_2O_2) 30% w/w, 2,5-diphenyl-3-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), 2,7-dichlorodihydrofluorescein diacetate (DCF) and chlorpromazine hydrochloride (CPZ, CAS Nº 69-09-0) were supplied from Sigma-Aldrich (Madrid, Spain). Dulbecco's modified Eagle's medium (DMEM) with and without phenol red, fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin-ethylenediaminetetraacetic acid (EDTA) solution (170,000 U/L trypsin and 0.2 g/L EDTA) and penicillin-streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin) were acquired from Lonza (Verviers, Belgium). All analytical standards used for liquid chromatography analysis shikimic acid, gallic acid, 5-O-caffeoylquinic acid, 3-O-caffeoylquinic acid, (+)-catechin hydrate, (-)-epicatechin, rutin, hyperoside, naringin, quercitrin, 3,5-di-O-caffeoylquinic acid, rosmarinic acid, cinnamic acid, eugenol and trans-cinnamaldheyde were purchased from Sigma-Aldrich (Milan, Italy). The 75 cm^2 culture flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland). HyClone fetal bovine serum (FBS) was purchased from Thermo Scientific (Northumberland, UK).

4.2. Plant material

The fronds of *Polypodium vulgare* L. (PV) were collected from the Prades Mountains 41°17′34″N 1°02′42″E geographical coordinates (Tarragona, Spain); the authors previously verified that this species was reported in the selected area by *Banco de Datos de Biodiversidad de Cataluña*[78]. When the fronds were dried, a sample voucher was stored at Herbarium of Universidad San Jorge (Zaragoza, Spain), *Polypodium vulgare* L.: voucher nº 003-2016.

4.3. Preparation of methanolic fronds extract of Polypodium vulgare L. (PVM)

Powdered fronds of the plant material were macerated with methanol for 24 hours. After this, the methanolic extract was filtered using a Whatman Nº4 filter paper and to evaporate the solvent, a rotatory evaporator with a thermostatic bath at 30ºC was used. This process was repeated three times to obtain the correspondence exhaustion extract as described by Farràs et al. 2019[23]. Finally, extracts were conserved at -20ºC until we need.

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Homogenization of the plant extract with the corresponding culture medium was obtained by sonication.

4.4. Phytochemical characterization by liquid chromatography with diode-array detection (HPLC-DAD)

HPLC-DAD studies were performed using a Hewlett-Packard HP-1090 Series II (Palo Alto, CA, USA), equipped with a vacuum degasser, a binary pump, an autosampler and a model 1046A HP photodiode array detector (DAD) following a previous developed method with some modifications[79]. The chromatographic separation was accomplished on a Synergi Polar-RP C18 (4.6 mm x 250 mm, 4 μm) analytical column from Phenomenex (Chesire, UK). The column was preceded by a security cartridge. The mobile phase for HPLC-DAD (diode array detector) analyses was a mixture of (A) water with 0.1% formic acid (v/v) and (B) acetonitrile with 0.1% formic acid, flowing at 0.8 mL/minutes in gradient conditions: 0 minutes, 20% B; 0-15 minutes, 60 % B; 15-20 minutes, 60% B; 20-25 minutes, 20 % B, 25-30 minutes, 20% B. The column temperature was set at 30°C and the injection volume was 5 μL. UV spectra were recorded in the range 230-350 nm, where 230 nm was used for quantification of shikimic acid, 256 nm for rutin and hyperoside, 272 nm for gallic acid, 280 nm for (+)-catechin hydrate and (-)-epicatechin, 325 nm for 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid and 3,5-di-O-caffeoylquinic acid.

4.5. Cell culture

The mouse fibroblast cell line, NIH 3T3, and the spontaneously immortalized human keratinocyte cell line, HaCaT, were used in all *in vitro* experiments. In the case of cytotoxic assay, cell viability also was evaluated by the cervical cancer cell line HeLa, the liver cancer cell line HepG2, the breast cancer cell line MCF-7 and lung cancer cell line A549. 3T3 and A549 were purchased from Sigma-Aldrich as a worldwide provider of European Collection of Authenticated Cell Cultures (ECACC), whereas HaCaT, HeLa and MCF-7 were obtained from Eucellbank (Celltec-Universitat de Barcelona, Spain). HepG2 cell line was kindly donated by Dr. Borràs of Experimental Toxicology and Ecotoxicology Platform (UTOX-CERETOX) of Parc Científic of Universitat de Barcelona. Cells lines were maintained in Dulbecco's modified Eagle's medium (DMEM) and 4.5 g/L glucose red (DMEM) was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/mL:100 U/mL streptomycin-penicillin mixture (10% FBS-DMEM) at 37 °C in a 5% carbon dioxide (CO_2)-humidified incubator. Cells were routinely subcultured in 75 cm^2 flasks. When cells reached 80% of confluence, culture medium was removed, cells were rinsed with PBS and then detached by trypsinization (trypsin-EDTA). Then, 100 μL of the cell suspension (1 x 10^5 cells/mL) were seeded in 96 well microplates and incubated overnight (37°C and 5% CO_2). Cell density was previously established by the trypan blue (0.4%) dye exclusion method form the cell suspension obtained after trypsinization.

4.6. Determination of cell viability by NRU and MTT assays

Cell viability were determined by the NRU and MTT methods after treatments.

The Borenfreund and Puerner protocol for the determination of cell viability by NRU has been followed with some described adaptations [80]. Once the incubation time of the cells with the treatments had elapsed, the supernatant was extracted from each well and 100 μL of NR solution was applied (0.05 mg/mL in DMEM 0% FBS without phenol red). After three hours, the supernatant was removed by inversion from the plate and 100 μL of the developer NR solution was added. In the developer solution, the formaldehyde was replaced by an acidic ethanol solution as described by Riddell et al.[81]. The quantification of the remnant NR, which corresponds to the NRU bound to the lysosomes, is proportional to the viable cells[82]. After 5 to 10 minutes of shaking the plate, the absorbance was obtained at 550 nm, by means of the Tecan Sunrise microplate reader (Männedorf, Switzerland).

The MTT assay based on the experimental protocol of Mosmann[83] was used with the adaptations of Zanette et al.[84]; 100 μL of an MTT solution (0.5 mg/mL in 0% FBS-

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DMEM without phenol red) was added in each well following incubation of the plates for at least 3 h in cell culture incubation conditions (37°C and 5% CO_2). At the end of incubation, supernatant was removed and 100 µL of the organic dissolvent dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals[85]. The amount of soluble formazan is proportional to the number of cells with optimal mitochondrial activity[86]. Absorbance was measured at 550 nm using a Tecan Sunrise® microplate reader (Männedorf, Switzerland), previous homogenization of the well content by gently shaking each microplate during 5 minutes at 100 rpm/min.

Cell viability for NRU and MTT assays were calculated using the following equation:

$$Cell\ viability\ (\%) = ((A_{control} - A_{sample}) / A_{control}) \times 100\%$$

where $A_{control}$ and A_{sample} are the absorbance of the control and each sample, respectively.

4.6.1. Cytotoxicity activity of PVM in non-tumoral and tumoral cells lines

Non-tumoral (3T3 and HaCaT) and tumoral cells lines (HeLa, HepG2, MCF-7 and A549) were treated for 24 h (37°C and 5% CO_2) with increasing concentrations of methanolic extract 0.01, 0.1, 1 and 2 mg/mL PV in 5% FBS-DMEM. In each plate, untreated cells (maintained with culture medium) were included as negative controls. Cytotoxicity of PVM was determined by the NRU and MTT assays.

4.6.2. Cytoprotective and cellular repair activity of PVM in non-tumoral cell lines

In these experiments, oxidative stress was induced using hydrogen peroxide after treatment (cytoprotection) or before extract treatment (cellular reparation)[87].

4.6.2.1 Cytoprotective activity in 3T3 and HaCaT cell lines

Cells were pre-treated with 0.01, 0.1, 1 and 2 mg/mL PVM (100 µL) dissolved by 5% FBS-DMEM for 24 h following addition of H_2O_2 (in 5% FBS-DMEM) at a final concentration 2 mM for 2.5 h. Finally, cell viability was determined by NRU and MTT assay. In each microplate negative and positive controls were included. In this case, positive controls consist of cells treated by H_2O_2 at 2 mM during 2.5 h without previous pre-treatment with the extracts.

Cytoprotective activity was calculated as follows:

$$Cytoprotective\ activity\ (\%) = [(CV_{PVM\text{-}H2O2} - CV_{H2O2}) / CV_{PVM\text{-}H2O2}] \times 100$$

where *CV* is the cell viability for each condition described in the formula.

4.6.2.2. Cellular repair activity in 3T3 cells

18 h after the cell seeding the corresponding oxidative stress agent (100 µL H_2O_2 at 2 mM during 2.5 h) were applied in the cellular repair assay. Then, the same treatment concentrations of the extract in the previous cytoprotective assay were applied. After 24 h incubation, cell viability was assessed by NRU and MTT assays.

Cellular repair activity was calculated as follows:

$$Cellular\ repair\ activity\ (\%) = [(CV_{PVM\text{-}H2O2} - CV_{H2O2}) / CV_{PVM\text{-}H2O2}] \times 100$$

where *CV* is the cell viability for each condition described in the formula.

4.6.3. Phototoxicity activity in 3T3 and HaCaT cell lines

We followed the Organization for Economic Cooperation and Development (OECD) TG 432 (2019)[67] with some adaptions. Briefly, 3T3 and HaCaT cells were plated at a density of 1 x 10^5 cells/mL (100 µL) in a 96 well microplate in 10% FBS-DMEM for 24 h and then treated with 0.01, 0.1, 1 and 2 mg/mL PVM (100 µL). PVM samples were solubilized in a 0% FBS-DMEM without phenol red, while negative and positive controls consisted in cells no treated and treated with chloropromazine (37.5 µg/mL chlorpromazine) in 0% FBS-DMEM without phenol red, respectively. Cells were incubated for 1 hour and after that, one plate remain in the dark and other received 1.8 J/cm^2 of ultraviolet A (UVA) light. At the end of UVA light medium was replaced for 100 µL of fresh medium (10% FBS-DMEM) and cell viability was determined after 24 h of incubation by the NRU and MTT colorimetric assays.

Light exposure was performed in a photostability UV chamber (58 x 34 x 28 cm) equipped with three UVA lamps Actinic BL TL/TL-D/T5 (Philips®, 43 V, 352 nm, 15 W) as

described in Martínez et al.[88]. Dosage and time exposition of cells to UVA light was regularly settled thanks to a photoradiometer Delta OHM provided with a UVA probe (HD2302 - Italy). We followed the equation:

$$E\ (J/cm^2) = t\ (s)\ x\ P\ (W/cm^2)$$

where E stands for ultraviolet dose, t represents the time expressed in seconds and, finally, P is the lamp potency.

4.7. Intracellular reactive oxygen species (ROS) induced by H2O2 of PVM in 3T3 and HaCaT cell lines

The production of ROS generated by H_2O_2 over a range of time was determined according to Ferreira et al.[89], for ROS assay. After the incubation of the cells with the different concentrations of the extract for 24 h as reported in previous sections for *in vitro* assays, cells were washed twice with PBS and DCF (100 μM) was applied to each well for 45 minutes (37°C and 5% CO_2). DCF that has not penetrated cells was removed by washing twice with cell culture medium and then H_2O_2 (1 and 2 mM) was added to induce oxidative stress. The fluorescence intensity of the oxidized product of DCF was registered (($\lambda_{excitation}$ 480 nm; $\lambda_{emision}$ 530 nm) at 0, 1, 2 and 3 h by a plate reader ThermoFisher SCIENTIFIC VARIOSKAN LUX (ThermoFisher SCIENTIFIC, Waltham, Massachusetts, USA). Results were expressed as *Fluorescence Intensity (FI)* which have adimensional units. The $FI_{z\ h\ Vs\ 0\ h}$ were calculated as follows:

$$Fluorescence\ Intensity_{z\ h\ Vs\ 0\ h}\ (FI_{z\ h\ Vs\ 0\ h}) = [(FI_{z\ h} - FI_{0\ h}) / FI_{z\ h}]\ x\ 100$$

where $FI_{z\ h}$ is the intensity fluorescence at z h (z as 1 h, 2 h or 3 h) of incubation and $FI_{0\ h}$ the amount fluorescence intensity at 0 h.

The *FI* for each specific time was calculated using this formula:

$$FI = Fluorescence_{480\ nm\ (excitation)} / Fluorescence_{480\ nm\ (emission)}$$

The *ΔROS*, which have adimensional units for FI, was obtained using the following formula:

$$\Delta ROS_{H2O2} = ROS_{PVM\ with\ DCF\text{-}H2O2} - ROS_{DCF\text{-}H2O2}$$

4.8. Statistical analysis

All experiments were carried out in triplicates and almost 3 independent experiments were assayed, on different days, except for the cytoprotection PVM HaCaT against 2 mM H_2O_2 (2.5 h) MTT for which the results correspond to n=2 experiments, respectively. Statistical significance for MTT cell viability and fluorescence intensity was analysed by using GraphPad Prism version 7, San Diego, CA, USA. Data are presented as mean +/- standard error. Activities have been compared using a two-way analysis of variance (ANOVA) by Bonferroni. Statistical differences were considered as follows: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

5. Conclusions

In conclusion, PVM extract show biological activity at similar concentrations used in previous *in vitro* studios with PLE[43]. The antioxidant-prooxidant behavior described here with different assays, encourage us to continue PVM evaluation to discover and describe potential chemopreventive uses.

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Abreviations

3T3 = NIH 3T3	mouse fibroblast cell line
A549	lung cancer cell line
CO_2	carbon dioxide
CV	cell viability
CPZ	chlorpromazine hydrochloride
E	ultraviolet dose
EMA	European Medicines Agency
DCF	2,7-dichlorodihydrofluorescein diacetate
DMEM	dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ECACC	European Collection of Authenticated Cell Cultures
FBS	fetal bovine serum
FI	fluorescence intensity
HaCaT	spontaneously immortalized human keratinocyte cell line
HeLa	cervical cancer cell line
HepG2	liver cancer cell line
HPLC-DAD	liquid chromatography with diode-array detection
H_2O_2	hydrogen peroxide
MCF-7	breast cancer cell line
MTT	2,5-diphenyl-3-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide
NR	neutral red
NRU	neutral red uptake
OECD	economic cooperation and development
PBS	phosphate buffered saline
PL	*Polypodium leucotomos*
PLE	aqueous extract of *Polypodium leucotomos*
PV	*Polypodium vulgare* L.
PVM	methanolic extract of *Polypodium vulgare* L.
ROS	reactive oxygen species
RNS	reactive nitrogen species
TLC	thin layer chromatography
UV	ultraviolet
UVA	ultraviolet A

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