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ORIGINAL ARTICLE

Impact of Portuguese propolis on keratinocyte proliferation, migration and ROS protection: Significance for applications in skin products

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Abstract

Objective: Propolis has been used since antiquity, but recent reports of its biological properties hint that it could be employed as a topical pharmaceutical and cosmetic ingredient. This work aims to probe the action of Portuguese propolis extracts on skin cells, providing mechanistic insights into its mode of action and preliminarily assessing its applicability as a skin repair ingredient.

Methods: The total phenolic content of propolis extracts was measured by the Folin Ciocalteu method. The cytotoxic effect of propolis extracts in human keratinocytes was determined and non-cytotoxic concentrations of the extracts were used to study the impact on collective cell migration, cell cycle and intracellular ROS levels.

Results: o significant impact was observed in collective cell migration, but one of the extracts mildly increased G2 phase while reducing the % of sub-G1 at a non-cytotoxic concentration. The two extracts with higher phenolic content strongly prevented intracellular cellular ROS accumulation upon exposure to TBHP. Collectively, these results indicate that the putative beneficial effects of propolis extracts in skin repair may not be attributable to induction of collective cell migration but could be partially ascribed to the protection from oxidative stress, which could act in synergy with its well-known antimicrobial activity.

Conclusion: These data support the applicability of this material in topical and cosmetic formulations and further in vivo assays should be conducted to fully characterize its efficacy and safety.

KEYWORDS

cell culture, chemical analysis, intracellular ROS, propolis, safety testing, skin repair

Résumé

Objectif: la propolis est utilisée depuis l'Antiquité, mais des rapports récents sur ses propriétés biologiques suggèrent qu'elle pourrait être utilisée comme ingrédient pharmaceutique et cosmétique topique. Ce travail de recherche vise à explorer l'effet d'extraits de propolis portugaise sur les cellules cutanées, en fournissant des informations sur le plan mécanique relatives à son mode d'action et en évaluant de manière préliminaire son applicabilité en tant qu'ingrédient de réparation cutanée.

Méthodes: la teneur en substance phénolique totale d'extraits de propolis a été mesurée par la méthode de Folin-Ciocalteu. L'effet cytotoxique d'extraits de propolis dans les kératinocytes humains a été déterminé, et des concentrations non cytotoxiques de ces extraits ont été utilisées pour étudier l'impact sur la migration cellulaire collective, le cycle cellulaire et les taux de ROS intracellulaires.

Résultats: un impact significatif a été observé sur la migration cellulaire collective, mais l'un des extraits a légèrement augmenté la phase G2 tout en réduisant le % de sub-G1 à une concentration non cytotoxique. Les deux extraits présentant une teneur phénolique plus élevée ont fortement prévenu l'accumulation de ROS intracellulaires lors de l'exposition à l'hydroperoxyde de tert-butyle (TBHP). Collectivement, ces résultats indiquent que les effets bénéfiques présumés des extraits de propolis dans la réparation cutanée pourraient ne pas être attribuables à l'induction de la migration cellulaire collective, mais partiellement à la protection contre le stress oxydatif, qui pourrait agir en synergie avec son activité antimicrobienne bien connue.

Conclusion: ces données étayent l'applicabilité de cette substance dans les formulations topiques et cosmétiques, et des tests *in vivo* supplémentaires doivent être réalisés afin de caractériser plus précisément son efficacité et sa sécurité d'emploi.

INTRODUCTION

Propolis, sometimes named bee glue, has been used empirically since antiquity, but only recently has been more thoroughly investigated. This resinous biomaterial is produced by honey bees (*Apis mellifera*) using materials obtained from the flora of the region and altered by enzymes contained in their saliva. Its composition is variable according to its provenance, but normally contains around 50% resin and balsam, 30% wax and 10% essential and aromatic oils [1,2]. The bees make use of the antibacterial and antimycotic properties of propolis to keep the hive free from harmful microorganisms [3]. There are numerous references in the literature to other biological properties attributed to propolis, such as antioxidant [4], anti-inflammatory [5] and skin regenerating activities [6], which are suggestive of its applicability in topical and cosmetic formulations.

Methodologies based on cell cultures are especially suitable in the initial stages of safety assessment of topical formulations. This is of particular relevance for cosmetic formulations, as the EU has banned animal testing of cosmetic ingredients since 2013 [7]. This framework has stemmed the development of alternative methods for the safety assessment of topical ingredients and in vitro approaches based on cell culture have risen to prominence [8]. Many of these methodologies can also be highly relevant in efficacy testing of skin repair ingredients [9] since the impact in cellular mechanisms such as proliferation or migration can be more easily dissected in vitro.

Human in vivo trials are, undoubtedly, the most relevant assays. However, due to ethical considerations and since they can be costly and time consuming, it is advantageous to employ them only in advanced stages of research. It should be added that most in vivo human models for skin repair are invasive, since they inevitably involve inflicting a wound in order to observe healing [10]. The tape

stripping approach is relatively mild, but in the blister model a skin damage is caused by chemical vesicants [11] or by application of negative pressure for 3–4 h to separate the epidermis from the dermis [12]. In other models abrasion is achieved using a surgical brush, microdermabrasion device or laser [10]. Punch biopsy models are based in wounds that can be precisely controlled in size and depth, however, since the dermal layer is breached, biopsies must be performed under local anaesthetic [13].

Under these constraints, cell viability/proliferation, migration and intracellular ROS accumulation assays conducted in keratinocytes or fibroblasts can be suitable to probe new topical and cosmetic ingredients and address mechanistic questions related not only to biocompatibility, but also to putative skin tissue repair properties [14] [15]. Nevertheless, it can be challenging to extrapolate from in vitro to an in vivo skin regeneration scenario [16] [17].

This work aims at probing specific properties of Portuguese propolis extracts that might be relevant for their applicability as skin repairing ingredients, by dissecting their biological activities on skin cells in vitro.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), penicillin–streptomycin solution, crystal violet, RNase A, DAPI were purchased from Sigma-Aldrich (St Louis, MO, USA). Dimethylsulfoxide (DMSO), propidium iodide (PI), ethanol and acetic acid were purchased from Merck (Darmstadt, Germany). Acetic acid glacial and NaCl were purchased from Panreac (Barcelona, Spain). The $DCFH_2$-DA fluorescent probe was acquired from Invitrogen™ Molecular Probes™ (Oregon, EUA).

Acetonitrile, ethanol absolute and formic acid were purchased from Riedel-de Haën (Seelze, Germany).

Preparation of propolis extracts and wax fraction

Crude Portuguese propolis from different locations was supplied by different producers and kept stored at −20°C until further usage. Extracts EP4 and EP5 were prepared using propolis from the central region of Portugal (Caramulo) and extract EPM1 was prepared using a mixture of propolis from the central and northern region of Portugal. Propolis extracts were prepared according to the procedure described by Loebler et al. [18] Briefly, a representative sample of crude propolis was extracted (1:10, w/v) with 96% ethanol (extracts EP4 and EP5) or 70% ethanol (extract EPM1) in an ultrasonic bath (Sonorex-SK-100) during 20 min and kept in the dark for 24 h, at room temperature (20 to 22°C). The resulting suspensions were filtered, and the procedure was repeated with the part trapped in the filter. Finally, the filtrated solutions were combined, cooled at −18°C during 12 h to precipitate waxes that were co-dissolved with the phenolic components, and the waxes were recovered by filtration. This wax fraction is a homogeneous, semisolid material, that still retains some liquid extract containing phenolic components. The wax fraction from EP4 (EPC4) was spread in a Petri dish and left at room temperature for 7 days, to evaporate the residual solvent. The dry weight of EP4 before and after the de-waxing operation and of the de-waxed EP5 and EPM1 was determined by eliminating the solvent from a 10 ml aliquot, at 50°C and reduced pressure (Buchi rotative evaporator). The EP4 wax extraction yield was evaluated by determining the dry weight of the extract before and after the de-waxing operation and corresponded to 1.1 g of wax/100 ml of extract and 6.9% (w/w) of the crude propolis. Propolis ethanolic extracts were stored in glass containers at −18°C, until chromatographic analysis. Extracts EP5 and EPM1 were evaporated to dryness at 50°C and reduced pressure (Buchi rotative evaporator). The wax fraction (EPC4) and the dry ethanolic extracts (EP5 and EPM1) were stored at −18°C until subsequent assays.

The wax fraction (EPC4) was dissolved in DMSO and diluted in 10% (v/v) DMSO in PBS. The two propolis ethanolic extracts (EPM1 and EP5) were initially dissolved in propylene glycol (PPG) and further diluted in 10% (v/v) PPG in PBS. Control conditions corresponding to vehicle (10% PPG or 10% DMSO in PBS) alone were used as indicated. All experiments were performed after an incubation of 24 h with the propolis extract in 10% FBS complete media, unless stated.

Total phenolic content and chromatographic analysis of the propolis extracts

The total phenolic content of propolis extracts was determined using the Folin–Ciocalteu method according to Loebler et al. [18]. Results were expressed as milligram gallic acid equivalents (GAE) per gram of dry extract.

Prior to the chromatographic analysis the ethanolic propolis extracts were subject to a SPE cleaning step to remove residual waxes that might interfere with this analysis. The ethanolic extracts (2 ml) were diluted with water (1:5) and applied to a solid phase extraction C18 columns

(500 mg/3 ml, Ref. 7020–03, J.T. Baker). The adsorbed components were eluted with 10 ml of distilled water and 10 ml of ethanol. The extracts were combined, filtered was through a 0.22 µm filter (Whatman® GD/X) and injected in a HPLC system (SpectraSystem, Thermo), equipped with a diode array detector (DAD), and a Thermo C-18 column. The eluents used were 0.1% formic acid (solvent A) and a mixture of 90% acetonitrile +9.9% water +0.1% formic acid (solvent B). Elution with a flow rate of 0.8 ml/min, started with an isocratic period of 10 min with 2% solvent B, and increased to 98% solvent B during 80 min, followed by a re-equilibration step of 10 min to reach the initial condition. The injection volume was of 20 µl and spectra acquisition was made in the range of 190 nm to 700 nm with selective detection at 280 nm, 320 nm and 360 nm. Identification of the main components of the extract containing different classes of phenolic compounds was performed through the detected peaks and comparison of their UV spectra with those of representative standards analysed in equivalent conditions and by comparison of retention times and UV spectra with literature data. A qualitative evaluation of the different phenolic compounds present in the propolis extract was performed by adding the relative chromatographic areas of the compounds with the same functional group.

2D and 3D HaCaT cell culture

The human keratinocyte cell line HaCaT was obtained from ATCC. Cells were kept in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cultures were kept at 37°C, under a 5% CO_2 humidified atmosphere. To generate HaCaT 3D cultures, 96 well plates were coated with 1% agar to generate a conical bottom and 1.5 $\times 10^4$ cells were added to each well. Cultures were incubated for 48 h to obtain spheroids.

Cell viability assays

The impact of the propolis extracts (wax fraction and ethanolic extracts) on cell viability was determined by the crystal violet (CV) assay. Briefly, 5 $\times 10^3$ cells were cultured in 200 µl of complete medium in 96-well plates. The cells were grown for 24 h and then exposed to different concentrations of propolis extracts or vehicle controls for a 24 h-period. Propolis extracts were tested for solubility in culture media to determine the maximum soluble concentration that could be used in the cell viability assay. The CV assay was carried out according to previously described protocols [19,20]. Three independent experiments were performed, each including four replicate cultures.

Propidium iodide (10 µg/ml) was added to 3D cell cultures previously incubated with extracts and controls, and incubated for 20 min at 37°C, under a 5% CO_2 humidified atmosphere. Spheroid image acquisition was performed on a wide-field Zeiss Axiobserver microscope with a x10 objective using ZEN software. The average PI fluorescence intensity was measured using ZEN software.

TABLE 1 Total phenolic content of propolis extracts

Propolis extract	Phenolic compounds (mg GAE/g dry extract)
EPC4	79.6 ± 3.2
EP5	302.1 ± 6.6
EPM1	313.7 ± 12.5

Cell cycle progression of HaCaT cells treated with the propolis extracts

Cell cycle progression upon incubation with the indicated concentration of propolis extracts and vehicle controls was analysed by propidium iodide staining of fixed cells [21,22]. Briefly, 7.5 $\times 10^4$ HaCaT cells were seeded in 6-well plates. After 24 h cells were treated with the various extracts for 72 h. Cells were then detached using 5 mM EDTA in PBS and fixed with 80% ethanol. After treatment with RNase A (20 µg/ml) and staining with PI (10 µg/ml) the cellular DNA content was evaluated by flow cytometry using a FACSCalibur flow cytometer (BD). Data acquisition and analysis were performed using CellQuest software (BD) and FlowJo (Tree Star), respectively. Two independent experiments were performed.

In vitro wound healing assay

The impact of the selected propolis extracts on in vitro collective cell migration was assessed by the standard wound healing assay (WHA) [23]. Briefly, 9 $\times 10^4$ cells were seeded on 12 well plates so cells would achieve confluence 48 h later. At this point, each well was scratched using a 200 µl pipette tip, wells were washed twice with PBS to remove detached cells and cell debris. Cells were kept in 2% FBS media containing the propolis extracts or vehicle controls. The distance between the two limits of the scratch was monitored using a wide field BX51 fluorescent Olympus microscope with a 10× objective. Image analysis was performed with ImageJ (National Institutes of Health). At each time point, two photos of each scratch were taken and three representative measures of wound width were performed per image. Each assay was performed with intern duplicates and 3 independent experiments were performed.

Impact of propolis extracts on intracellular reactive oxygen species

Overall intracellular reactive oxygen species (ROS) levels were measured using a $DCFH_2$-DA fluorescence probe [24]. In short, 1.2×10^4 HaCaT cells were seeded on a black 96-well plate with clear bottom, in 200 µl of complete media. Cells were incubated with the extracts or controls for 18 h. After this time interval, the cells subjected to an oxidative challenge were incubated with 1 mM TBHP for 2 h All wells were gently washed with PBS at 37°C and after the addition of 50 µl of PBS, fluorescence reading was performed using a fluorimeter with 460/40 nm excitation and 528/20 nm emission filters (BioTek Synergy HT) to control for background fluorescence. After a 30 min incubation at 37°C, under a 5% CO_2 humidified atmosphere with 10 µM $DCFH_2$-DA, fluorescence was measured using the same filters. To normalize for cellular content of each well, a CV assay was carried out. Three independent experiments were performed, each including four internal replicates.

Statistical analysis

The results shown correspond to mean values and respective standard deviations (SD). Statistical analyses were

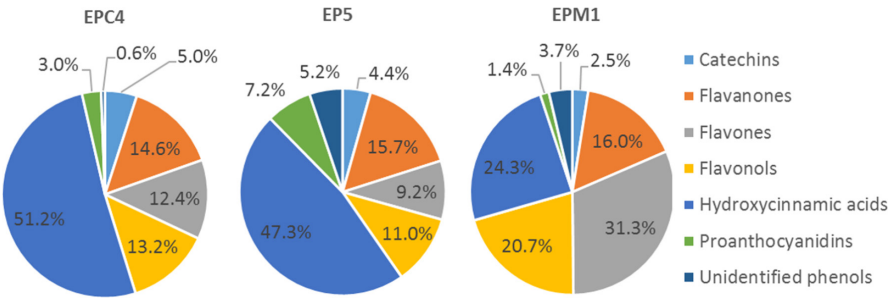

FIGURE 1 Distributions of the main phenolic compounds present in the propolis extracts according to their relative chromatographic areas [Colour figure can be viewed at wileyonlinelibrary.com]

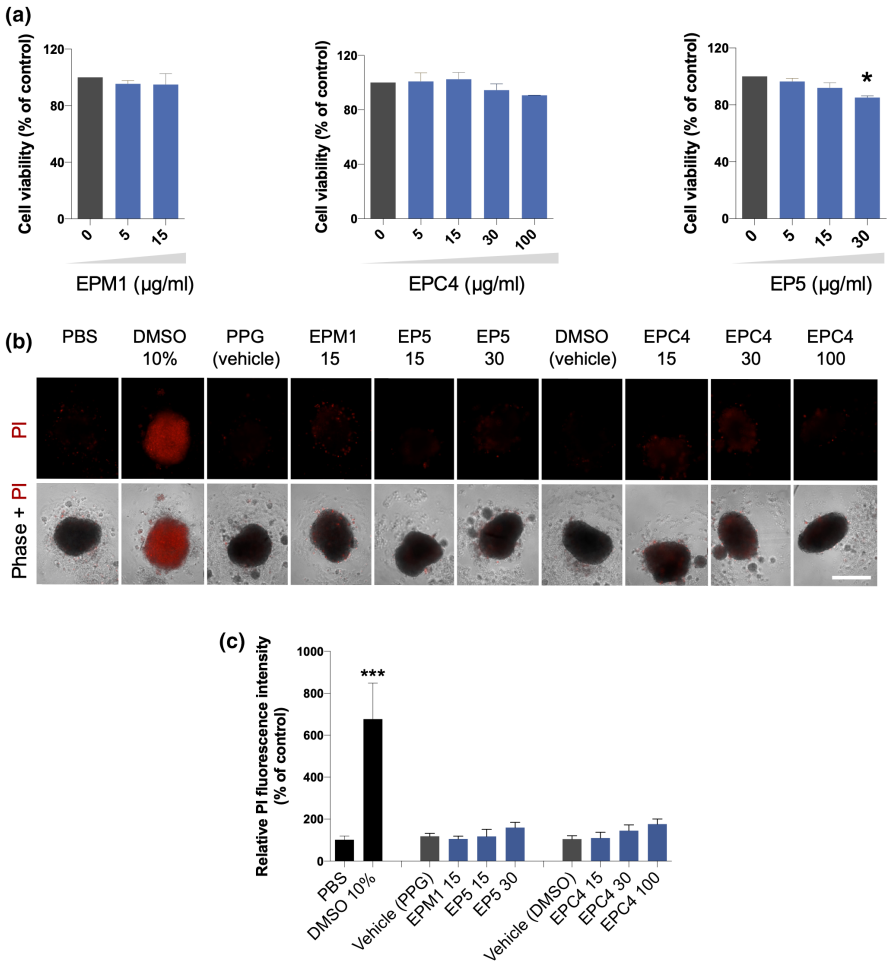

FIGURE 2 Cell viability was measured in (a) 2D and (b and c) 3D cell cultures with crystal violet and propidium iodide assays, respectively. Cells were incubated for 24 h with the indicated propolis extracts controls, or vehicles. (b) Representative images of 3D HaCaT cultures treated with the indicated compounds or extracts. Vehicle controls were PPG for EPM1 and EP5 and DMSO for EPC4. DMSO at a final concentration of 10% was used a cell death inducer. Summary results are shown as (a) mean cell viability percentage or (b) relative PI fluorescent intensity ± SD from 3 independent experiments. *$p < 0.05$, *** $p < 0.001$ (2-way ANOVA, Tukeys's multiple comparison test compared to vehicle-treated cells). Scale bar 200 µm [Colour figure can be viewed at wileyonlinelibrary.com]

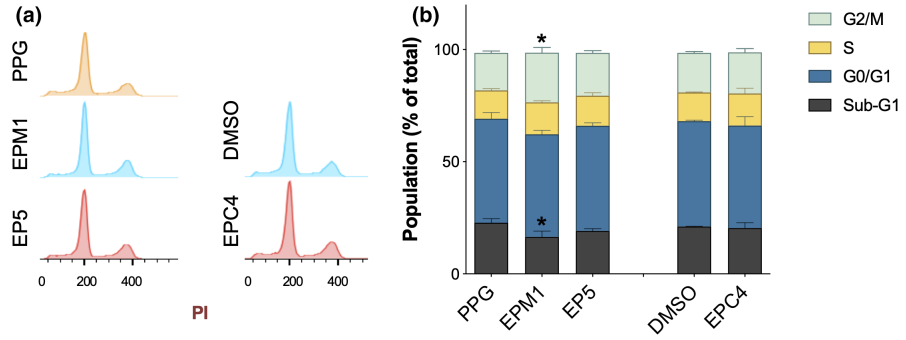

FIGURE 3 The cell cycle distribution after a 24 h incubation with the propolis extracts (15 μg/mL) was determined by flow cytometry analysis of PI-stained cells. (a) Representative flow cytometry histograms. (b) Summarized results are shown as mean percentage of sub-G1, G0/G1, S and G2/M populations ±SD. Data from two independent assays with 3 replicates each. *$p < 0.05$ (2-way ANOVA, Tukeys's multiple comparison test compared to vehicle treated cells) [Colour figure can be viewed at wileyonlinelibrary.com]

performed using the GraphPad Prism statistical analysis software. $p < 0.05$ was considered statistically significant (represented as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$).

RESULTS

Phenolic profile of the propolis ethanolic extracts

The total phenolic content of the propolis extracts used in the subsequent experiments ranged from 79.6 ± 3.2 mg GAE/g (EPC4) to 313.7 ± 12.5 mg GAE/g (EPM1) (Table 1), being the phenolic content of the wax fraction (EPC4) much lower than the phenolic content of the ethanolic extracts. A residual fraction of the ethanolic extract is retained during wax precipitation and separation. Therefore, EPC4 presents a residual fraction of the phenolic compounds from the ethanolic extract from which the wax sample was isolated (EP4). Thus, although presenting a similar qualitative phenolic composition, the total phenolic content of EPC4 was much lower (~24%) than the phenolic content of EP4.

As mentioned above the wax fraction (EPC4) and the extract from which this fraction was isolated (EP4), have the same phenolic profile, since part of the ethanolic extract was retained during wax precipitation and separation. The propolis extracts presented as main components phenolic acids such as ferulic acid, *p*-coumaric acid and the corresponding esters, and flavonoids such as pinocembrin, pinobanksin, chrysin and galangin. The flavonoid fraction included flavones, flavanones and flavonols, and their hydroxylated and methoxylated derivatives (data not shown).

The main phenolics in extracts EP4/EPC4 and EP5 were hydroxycinnamic acids followed by flavanones, flavonols and flavones while extract EPM1 presented higher contents of flavonoids (68%) with predominance of flavones and derivatives (Figure 1).

Cell viability of HaCaT cells treated with the propolis extracts

The cytotoxicity of the propolis extracts, was assessed in vitro using HaCaT cells, a commonly used human keratinocyte cell line for the evaluation of the safety and activity of compounds with potential application in skin products. Both standard 2D and 3D (spheroids) cultures were used for cell viability assessment. As shown in Figure 2, none of the propolis extracts tested had considerable cytotoxicity towards HaCaT cells during a 24 h incubation period in the range of concentrations tested in either 2D (Figure 2a) or 3D (Figure 2b,c) culture conditions. Higher concentrations were not used, since the extracts were not soluble. For this reason, all the subsequent assays were performed with 15 μg/mL of extract, since this concentration did not significantly reduce cell viability for a 24 h-period in any of the conditions tested.

Cell cycle progression of HaCaT cells treated with the propolis extracts

Considering the essential role of cell proliferation and survival in the wound repair process, the impact of the extracts in cell cycle progression was evaluated by measuring the cellular DNA content by flow cytometry in cells incubated with the extracts for 72 h. This long exposure time allows for the detection of effects on cell cycle progression and long-term cell survival. Non-cytotoxic concentrations determined by the crystal violet assay were used.

One of the studied propolis extracts (EPM1) lead to a small but significant accumulation of cells at G2/M phase and to a reduction of cells at the sub-G1 phase, when

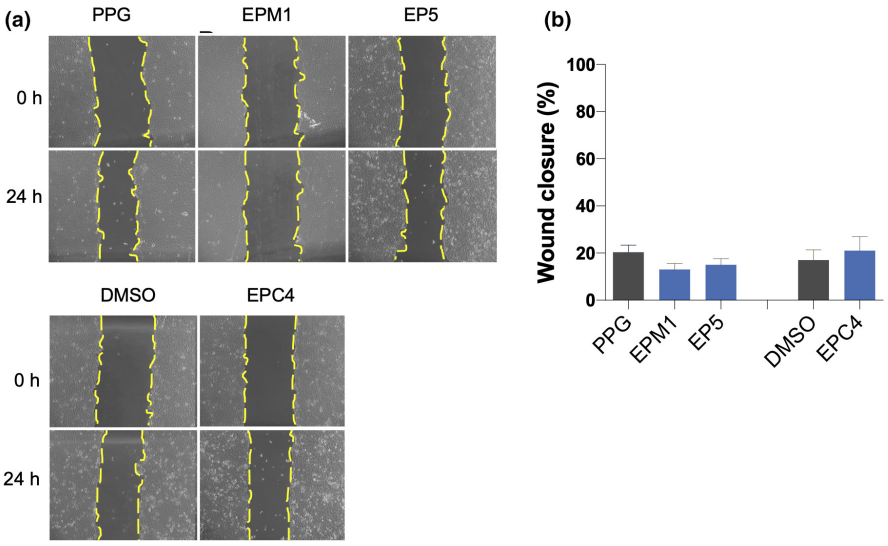

FIGURE 4 Collective cell migration was measured after a 24 h incubation with the propolis extracts (15 μg/mL) using the wound healing assay. (a) Representative images of wound size over time. (b) Summarized results are shown as mean percentage of wound closure ±SD. Data from three independent assays with 3 replicates each (2-way ANOVA, Tukeys's multiple comparison test compared to vehicle-treated cells) [Colour figure can be viewed at wileyonlinelibrary.com]

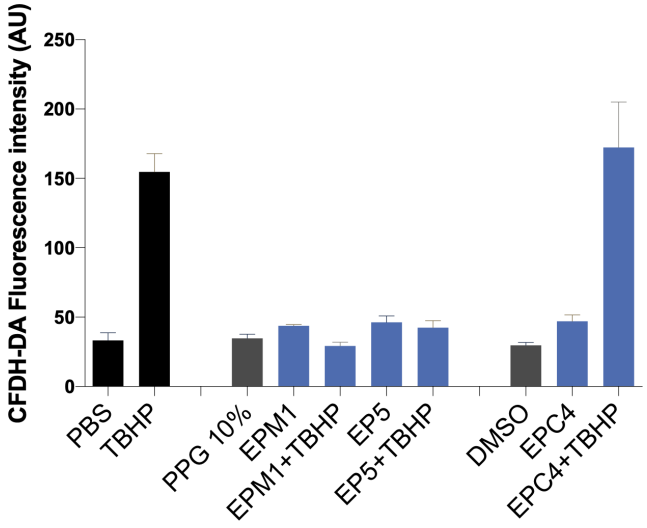

FIGURE 5 Intracellular ROS levels were measured after a 24 h incubation with the propolis extracts (15 μg/mL) and vehicle controls using $DCFH_2$-DA probe. The effects of the extracts when a strong pro-oxidant (TBHP) was used are also shown. Summarized results are shown as mean fluorescence intensity ±SD. Data from two independent assays with 4 replicates each. ****$p < 0.0001$ (2-way ANOVA, Tukeys's multiple comparison test compared to TBHP-treated cells) [Colour figure can be viewed at wileyonlinelibrary.com]

compared to the vehicle control (Figure 3). Overall, these results suggest that one of the analysed propolis extracts only mildly affects the proliferation of HaCaT cells while providing some resistance against cell death as measured by sub-G1 population.

In vitro wound healing assay

Since collective cell migration plays a central part in in vivo wound healing, the effect of propolis extracts was evaluated in vitro by a wound healing assay. A low FBS concentration was used to reduce the interference of cell proliferation in the assay, allowing a more accurate determination of cell migration as measured by wound closure rate. The results obtained suggest that none of the extracts had a significant effect on the collective motility of the cells after 24 h (Figure 4), 48 or 72 h (data not shown).

Impact of propolis extracts on intracellular reactive oxygen species

As propolis extracts are known for their antioxidant properties [4] and these can be of relevance for the skin repairing properties ascribed to these extracts, intracellular ROS levels were evaluated. The fluorescence intensity measured by the $DCFH_2$-DA assay in HaCaT cells following a 24 h period of exposure to the extracts was measured with and without a challenge with a strong oxidant (TBHP) (Figure 5). The basal ROS levels were not altered by any of the extracts. Despite that, the propolis extracts EPM1 and EP5 strongly prevented the increase in intracellular ROS accumulation upon a challenge with TBHP. The wax fraction EPC4 did not revert the oxidative effect of TBHP. In summary, these results suggest a significant antioxidant effect of the propolis extracts EPM1 and EP5.

DISCUSSION

This study aimed to evaluate the biological properties of Portuguese propolis extracts and their potential use as ingredients in skin repairing formulations. By using a strategy combining different in vitro assays conducted in HaCaT cells, a preliminary safety and efficacy assessment of the extracts was conducted.

The phenolic profiles of the propolis extracts were typical from the propolis of Mediterranean countries, as described by Falcão et al. [25] or Pellati et al. [26]. The composition of propolis depends on the flora of the areas where it was collected and, therefore, these different qualitative compositions may be related mainly to the different geographical origins of propolis. Moreover, the difference observed between the composition of the EP4/EPC4 and EP5 extracts and the EPM1 extract, can also be related to the different solvent concentrations used in the extraction procedures.

Our data show the absence of cytotoxic effects induced by the three propolis extracts (EPM1, EPC4, and EP5) in HaCaT cells in concentrations up to 15 µg/ml in both 2D and 3D culture conditions. The wax fraction EPC4 extract did not exhibit considerable cytotoxicity up to 100 µg/ml, while ethanolic extract EP5 was only mildly toxic at 30 µg/ml. The extracts are not soluble at higher concentrations, which could allow for maybe more conclusive information regarding their cytotoxic effects. Nevertheless, others report similar results when testing propolis extracts in the same cell lines, and in two studies at concentrations above 20 µg/mL a significant cytotoxic effect was observed [27,28]. In another study using an in vitro model of human dermal fibroblasts [29], the results are in line with those observed in our work: absent or low cytotoxicity at up to 200 µg/ml of ethanolic propolis extract. The disparate reports of several propolis extracts regarding cellular cytotoxicity could be partially explained by the different extraction methods and different chemical composition of this material of natural origin, since they are complex mixtures mainly composed of flavonoids and phenolic acids, which are well known to have bioactivity [30].

To the best of our knowledge, the impact of propolis extracts in cell cycle progression in HaCaT cells is addressed for the first time in this study. Our results indicate that the propolis extract with the highest total phenolic content (EPM1) was able to mildly promote HaCaT cell proliferation and reduce cell death induction based on the observed small increase of cells in G2/M phase and reduction of the sub-G1 population, respectively. These observations suggest that propolis extracts may provide beneficial outcomes in a wound healing or skin repair context, where cells generally are required to proliferate as part of the damage repair process.

Regarding collective cellular migration, our results indicate that the studied propolis extracts at non-cytotoxic concentrations do not induce any significant alteration in cell motility. Martinotti and his collaborators showed that low doses of propolis extracts were able to promote collective migration of HaCaT cells [31]. In another different in vitro model using human dermal fibroblasts, propolis extracts induced a dose-dependent increase in cellular migration with much higher doses of propolis extract than those here evaluated [32]. Having this in mind, the differences in the observed effects of propolis extracts might be strongly dependent on the concentration applied as well as in the chemical composition of the propolis extracts. Ideally, if an application in topical formulations is envisioned, the extract composition should be fully characterized, and as reproducible as possible from batch to batch. However, it is well known that one of the limitations when using materials of natural origin is that they contain a wide variety of compounds (including possible contaminants), as well as inter-batch variability.

Importantly, we observe that propolis extracts EPM1 and EP5 had a strong ability to counteract the oxidant activity of the organic peroxide TBHP, by preventing the rise of intracellular ROS. The antioxidant properties of propolis extracts are in agreement with previous studies involving propolis extracts. In studies conducted on HaCaT cells where oxidative stress was induced by UV irradiation, the treatment with propolis extracts decreased ROS levels in a dose-dependent manner to levels similar to the control sample [27,33]. The absence of a ROS accumulation prevention with EPC4 is likely linked to the much lower concentration of total phenols present in that extract. This information is crucial to guide future propolis extract production to be used in skin formulations.

Overall, the results obtained in our study confirm the potential application of propolis extracts from Portuguese provenience as active ingredients for skin-directed formulations, mainly due to their non-cytotoxic and antioxidant properties.

CONCLUSIONS

Propolis is widely portrayed as having cutaneous regenerative properties. This process is multifactorial and involves a series of mechanisms. According to the results obtained in this study, the protective properties of the tested Portuguese propolis extracts in skin regeneration might be mainly associated to its antioxidative protection activity. A synergic effect between these effects and the antimicrobial activity of propolis is very likely to occur, benefiting wound healing. Further studies should evaluate other possible biological actions, such as immuno-stimulation.

AUTHOR CONTRIBUTIONS

Conceptualization, N.S., A.S.F. and C.R.; methodology, N.S., M.G., M.N., N.A., M.P.D, E.M., A.S.F.; formal analysis, N.S., A.S.F. and C.R.; data curation, N.S and M.M..; writing original draft preparation C.R., N.S., N.A.

and M.M.; writing review and editing, N.S., C.R., A.S.F., M.N., E.M., M.M., M.P.D., M.G., A.R.B; supervision, N.S., A.S.F. and C.R.; project administration N.S., A.S.F. and C.R..; funding acquisition, N.S., A.S.F. and C.R. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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