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Research Article

Piper nigrum: Therapeutic Potential of Leaves Extract in HNSCC

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Highlights

- *Piper nigrum* leaves extract (PNE) has anticancer properties in head and neck squamous cell carcinoma (HNSCC)
- PNE inhibited cell proliferation and migration
- PNE induced apoptosis and checkpoint responses in phase G1/G0 and G2/M
- PNE modulated the expression metalloproteinases, which elucidates the mechanisms inhibition of cell invasion and migration;

Abstract

Background

Piper nigrum L., commonly referred to as black pepper, is traditionally used in oriental and indigenous medicine for the treatment of various diseases associated with inflammation, such as asthma and cancer. Here we report that the leaves extract of *P. nigrum* L. is associated in the inflammatory responses and have antitumor activity in head and neck squamous cell carcinoma cells.

Objectives

The present study focused on investigating the potential antitumor effect of the leaves extract of *P*. *nigrum* L. on head and neck squamous cell carcinoma cell lines.

Methods

Two tumour cell lines (HEp-2 and SCC-25) were used and treated with different concentrations of *P*. *nigrum* L. leaf extract (10, 50 and 100 µg/mL) for 4 and 24 hours. After treatment, proliferation, cytotoxicity, migration, genotoxicity, apoptosis, cell cycle assays were performed, as well as gene and protein expression analysis.

Results

After 24 hours of treatment, the extract decreased cell proliferation and migratory, probably by inhibiting cell cycle division, arrest in G1/G0 (SCC-25) and G2/M (HEp-2). In addition, *P. nigrum* extract (PNE) induced cellular apoptosis through DNA damage and modulated the expression of genes and proteins involved in the PTGS2 inflammatory pathway. It seems that, PNE has an antiproliferative, antimigratory and anti-inflammatory effect, retaining the cell cycle, inducing DNA damage and cell death.

Conclusion

These findings suggest that *P. nigrum* leaf extract has synergistic effects between its metabolites and acts directly on tumorigenic cells by mechanisms related to antiproliferative, apoptotic and inflammatory pathways. These observations shed light on this total extract in understanding the mechanisms that may regulate the functions of different tumour.

Keywords: Cancer; Phytotherapy; Cell culture; Cell cycle; Apoptosis; Metalloproteinases

Introduction

Cancer is a set of diseases in which an individual's own cells have lost the ability to self-direct and grow uncontrollably, forming an abnormal tissue known as a tumour. The World Health Organization (WHO) reports that cancer is one of the leading causes of death, estimated at 9.6 million worldwide, with an increase in the frequency of cases in the last decade [1]. Among cancers, head and neck squamous cell carcinoma (HNSCC) is ranked as the sixth most common type of malignant tumour, with more than 650,000 new cases and 330,000 deaths each year [2].

Consumption of alcoholic beverages and recurrent use of tobacco after HPV infection are the main risk factors for HNSCC. Tobacco use has decreased globally in recent years [3]. Although modern treatments are effective against cancer, they are highly invasive, and the drugs used are often associated with adverse side effects. Thus, there is considerable interest in the development of natural, less toxic, and more effective drugs for the treatment of HNSCC [4]. Plant-derived compounds have been explored as therapeutic methods and potential anticancer agents without side effects and are highly tolerated by normal cells of the human body. In addition, phytomedicines have demonstrated a key role in drug discovery, as 50% of drugs approved by the Food and Drug Administration (FDA) are of natural origin [5].

Piper nigrum L., popularly known as black pepper, is one of the best-known species of the Piperaceae family [6]. This pepper has an important role in the global economic market, and it is medically important because it is a source of bioactive molecules with pharmacological properties that support its anticancer potential in different cell lines and animal models [7]. Its use is already described in oriental and indigenous medicine in the treatment of many diseases, such as inflammation and cancer. In addition, PNE has numerous pharmacological activities already reported, such as anti-inflammatory [8] and antitumorigenic [9] activities. Recently, scientific progress and technology, specifically pharmacological studies of plants, have led to the discovery of many new molecules and their mechanisms of action. In this research, the importance of these molecules and the ability of the extracts to act in synergy with the various components present in the plant have been highlighted. *P. nigrum* has well-known bioactive compounds in its different parts, such as stem and root, although little has been reported in the plant leaves that have potential anticancer activity [10].

HNSCC is also related to angiogenesis and inflammatory processes, with inflammation being a feature of cancer progression associated with several stages of tumorigenesis, such as cell transformation, promotion, survival, proliferation, invasion, angiogenesis and metastasis [11]. Some genes and proteins are being studied and related to these inflammatory processes, and recent studies have highlighted the role of cyclooxygenase-2 during tumorigenesis in HNSCC. The gene PTGS2 encodes this enzyme that converts arachidonic acid into prostaglandins (PG), including PGE2, a key mediator in inflammation [12].

Metalloproteinases (MMPs) proteolytic enzymes that are related to several physiological processes, but in cancer, they are associated with the degradation of most components of the extracellular matrix (ECM), "support" the structure of tissues and can be involved in the suppression of cell proliferation and migration in tumours [13]. The proteins that naturally inhibit the activity of MMPs are those encoded by tissue inhibitors of metalloproteinases (TIMPs), with four different important types that play a crucial role in the activation control process and function of MMP activities, which in tumour tissue is altered in favour of proteolysis [14]. In this research, we sought to evaluate the changes resulting from the action of the leaves extract of *P. nigrum*, exploring the antitumorigenic potential in head and neck cancer.

Materials and methods

Extract preparation

Leaves of *P. nigrum* were dried in stove at 45 ˚C for 48h. Dried leaves were powdered and exhaustively extracted by maceration for five days with 70% aqueous ethanol (500 g for 700 mL ethanol). After his period, the mixture was filtered and the solvent concentrated under vacuum to furnish the leaves extract of *Piper nigrum* (PNE).

The leaves extract (8385 µg/mL) was dissolved in DMSO to make a stock solution and stored at -4 ˚C. The prepared stock solution was further diluted in culture media upon use, as required for different assays. The final concentration of DMSO administered was maintained below 0.1% during the study course.

Positive control

The synthetic lignan, podophyllotoxin (Sigma), was used at a concentration of 10 ug/mL as a positive control for its numerous antitumor activities [15].

Cell lines

The HEp-2, SCC-25 cell line (human epidermoid carcinoma of the larynx and tongue) were purchased from ATCC and were cultivated in MEM-Earle and DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids and 1% antibiotic/antimycotic. They were kept at 37 ˚C below 5% carbon dioxide (CO_2) and 95% relative humidity.

MTS viability and Proliferation assay

The viability of tumour cells was assessed using (3-(4,5-dimethylthiazol-2-yl)3 carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium) (MTS reagent) (Abcam, ab197010, USA). Briefly, $3x10^3$ cells were introduced into 96-well plates with 200 µl de medium containing 10% FBS and treated with leaves extract under different concentration (10, 50 and 100 µg/mL), after that 20 µl of an MTS reagent was added to each well four hours after the end of the incubation (4 and 24 h) period following the manufacturer's instructions.

Migration Transwell assay

Cells were grown in 24-well culture plates with different treatments and incubated for 24 h (statistically significant time chosen for the other assays). Afterwards, cells were trypsinized and loaded in the amount of $3×10^4$ onto the Transwell inserts (BD - Biosciences San Jose, CA, USA) using 8µm pore filters in plates. In the upper portion of the chamber 200 µL MEM medium without FBS was added and in the lower portion 500 µL MEM 10 % FBS. The plate was incubated for 24h in a CO_2 oven at 37 ˚C. Fixation of the lower portion cells was performed with 4% paraformaldehyde in PBS for 30 min and stained with Crystal Violet solution for another 30min. The inserts were then photographed in four random fields.

Comet Assay

HEp-2, SCC-25 cells were cultured and treated with PNE in 6-well plates at a concentration of $5x10^5$ in complete medium, then the cells were trypsinized, centrifuged and the cell pellet mixed with low melting point (LMP) agarose and placed onto slides precoated with normal-melting-point agarose (NMP). After the material disposed on the slides, they were covered with coverslips and placed at 4 ˚C for 30 min. Once solidified, were removed from the slides and placed in glass vats for the membrane lysis step with the pH 10 lysis solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris, 35 mM Lauril, 10% DMSO and Triton- x100 1%), a 4 ˚C for 60 min. After lysing, the slides were placed in an electrophoresis with pH 13 running buffer (10N NaOH, 200 mM EDTA) and followed by a 20 min electrophoresis at 25V and 300mA. After the slides were washed with a neutralizing solution (0.4M Tris) at pH 7.5, fixed in absolute ethanol and after analysing the damage. In the staining step, each slide received 100 µL of the staining solution (GelRed®, 1M NaCl and distilled H_2O). and analysed under a fluorescence microscope.

Apoptosis analysis assay

The evaluation of cell death was performed with the Annexin V kit (PE Annexin apoptosis detection kit, BD, San Diego, USA) according to the manufacturer's protocol. The cells were cultured in 25 cm² culture flasks as described per treatment (10 and 100 µg/mL) for 24h, disaggregated and resuspended in Annexin V Binding Buffer at a concentration of $1×10^6$ cells/mL PE Annexin V (5 µL) and 7AAD (5 µL), incubated at room temperature in the dark for 15min. After this time, 400 µL of the 1X binding buffer was added and the percentage of apoptotic cells was analysed using a flow cytometer (GUAVA easyCyte ™, Millipore). Each test attempt 10,000 events per test sample.

Cell Cycle assay

For a cell cycle analysis, after 24h incubation with treatment (10 and 100 µg/mL), cells were trypsinized, fixed with 70% ethanol and incubated for one hour in 4 ˚C. After this, the cell pellet resuspended in 200 µl of the solution of the Guava Cell Cycle Reagent Kit (MILLIPORE, Temecula, USA) and then the reading was performed in the cytometer device, acquiring 5,000 events of each sample for investigation of the cell cycle using flow cytometer.

Western blot analysis

Expression of PTGS2, PTGER4 (1:500 Abcam, Cambridge, UK), MMP2 (1:500 ABclonal, Woburn, EUA) proteins were examined in HEp-2, SCC-25 cells cultivated for 24 h, by SDS-PAGE, then transferred to nitrocellulose paper (Immobilon 0.45 mm, Millipore) in a wet transfer apparatus (Bio-Rad). The blots were incubated with the respective secondary antibodies (1:1000) diluted in TBST-Tween 20 for one hour at room temperature, and membranes were developed using Clarity™ western ECL Substrate (Bio-Rad, USA) for the detection of reactive bands by chemiluminescence. For sample standardization, the same membranes were incubated with the anti-human β-actin antibody (Cell signalling Technology, Danvers, EUA). The densitometric analysis was performed by the Image J program (NIH, Maryland, EUA).

RNA extraction and reverse transcription

Total RNA was extracted from the HEp-2 and SCC-25 cell line after treatments for periods of 24 h using the RNeasy® Mini Kit (Qiagen, Germany). Complementary DNA (cDNA) was synthesized using High-Capacity cDNA-Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using 1µg of total RNA and stored at -20 ˚C following the manufacturer's protocols.

Quantitative real-time PCR

The expression of *PTGS2, PTER3, PTER4, MMP2, MMP9, TIMP1, TIMP2* genes was determined for Real Time PCR, performed in triplicate, on a Step One Plus System 2.2.3 using SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacture's recommendations. The endogenous GAPDH gene was used as reaction normalizers (Table 1 - Supplement 1). That experiment was performed for periods of 24 h. The data obtained from the PCR were analysed from the Ct (Cycle Threshold) values of each sample, the means of the triplicates calculated and applied the mathematical model $2^{(-\Delta\Delta Ct)}$.

PRIMER	SEQUENCES
PTGER3 ANTI-SENSE	5' TCTCCGTGTGTGTCTTGCAG 3'
PTGER3 SENSE	5' AGCTTATGGGGATCATGTGC 3'
PTGER4 ANTI-SENSE	5' CCAAACTTGGCTGATATAACTGG 3'
PTGER4 SENSE	5' CGAGATCCAGATGGTCATCTTAC 3'
MMP2 ANTI-SENSE	5' CCGTCAAAGGGGTATCCATC 3'
MMP2 SENSE	5' AAGTCTGGAGCGATGTGACC 3'
MMP9 ANTI-SENSE	5' ATTTCGACTCTCCACGCATC 3'
MMP9 SENSE	5' TTGTGCTCTTCCCTGGAGAC 3'
PTGS2 ANTI-SENSE	5' AGAAGGCTTCCCAGCTTTTG 3'
PTGS2 SENSE	5' ATTCCCTTCCTTCGAAATGC 3'
TIMP1 ANTI-SENSE	5' TTTTCAGAGCCTTGGAGGAG 3'
TIMP1 SENSE	5' ACTGTTGGCTGTGAGGAATG 3'
TIMP2 ANTI-SENSE	5' CTATATCCTTCTCAGGCCCTTTG 3'
TIMP2 SENSE	5' AGAAGGAAGTGGACTCTGGAAAC 3'

Table 1: Primer's sequencers.

Statistical analysis

Prisma® GraphPad version 8.0 software were used. The results were obtained as mean ± standard deviation (SD) and an analysis statistic was performed by analysis of variance for multiple comparisons (ANOVA) followed by post hoc Bonferroni or Dunnet's. Values less than at $p \leq 0.05$ were considered to indicate statistically calculated results. Data statistics for the Comet Assay were based on non-parametric Kruskal-Wallis post hoc analysis of variance at 5% probability level and as means were compared using the student t test at 5% probability level. Image J software was used to analyse western blot densitometry.

Results

The cellular morphology

The squamous cell carcinoma of the larynx (HEp-2) and tongue (SCC-25) tumorigenic cell lines were initially cultured and observed by phase contrast microscopy. HEp-2 cells (Fig. 1A) had a fusiform-appearing monolayer and central nucleus, with multiple nucleoli. The morphology of SCC-25 cells (Fig. 1C) was defined by small cells with scarce cytoplasm and large nuclei, with a shape varying from rounded to fusiform and a pattern of growth in groups, which is a characteristic of these cell lines. In both *P. nigrum* treated cells, there was no visible morphological change (Fig. 1B, D).

PNE modulates proliferative activity by affecting cell checkpoints and apoptosis

Treatment with PNE is related to the inhibition of tumour cell proliferation, cell death and the cell cycle checkpoint. We observed that after treatment at different concentrations over 4 and 24 hours, there was a reduction in the number of cells with proliferative activity and a decrease in cell viability (Fig. 2A-H) but no cytotoxicity. Statistical analysis showed that these differences were more significant ($p \leq 0.05$) at concentrations of 10 μg/ml and 100 μg/ml after 24 h of treatment with the PNE in both cell lines. Thus, the experiments were performed with 10 μg/mL for 24 hours of treatment, and for the flow cytometry analysis, we chose to use both concentrations for more complete data. This reduction occurred due to activation of apoptosis pathways (Fig. 2J) through DNA damage in cells (Fig. 3K-M). Furthermore, the treatment modified the cell cycle phases for the HEp-2 and SCC-25 cells, with checkpoints in the G1/G0 and G2/M phases, respectively (Fig. 2I). When cells were treated with podophyllotoxin, a positive control, they showed antiproliferative activity, DNA damage, cell death and cell cycle arrest in the G2/M phases, as described in the literature [16].

Antimigratory effect of PNE in head and neck carcinoma cells

To examine the antimigratory efficacy of the extract, we performed a Transwell migration assay. The results are shown in Figure 4, and PNE significantly inhibited the migration of HEp-2 (Fig.4A-C) and SCC-25 (Fig. 4D-F) cells. After treatment with podophyllotoxin, a positive control, a significant inhibitory effect on the migratory rate was observed in both tumour cell lines. These data indicate that PNE has antimigratory activity in head and neck carcinoma cells.

PNE modulates the gene and protein expression of the inflammatory pathway

Analysis of mRNA expression by real-time PCR showed that the genes of this inflammatory cascade were modulated after treatment with PNE (10 μg/mL). The *PTGS2* gene showed increased expression in cells after PNE administration and podophyllotoxin, and the latter also increased the expression of other genes in both cell lines, with the exception of MMPs in SCC-25 cells. In contrast, there was an increase in the expression of *PTGER4, TIMP1* and *TIMP2* and consequently a decrease in the expression of the metalloproteinases *MMP9* and *MMP2*. Protein expression analysis showed reduced levels of PTGS2 (Figure 5E, I), *PTGER4* (Figure 5F, J) and *MMP2* (Figure 5G, K) in the PNEtreated groups.

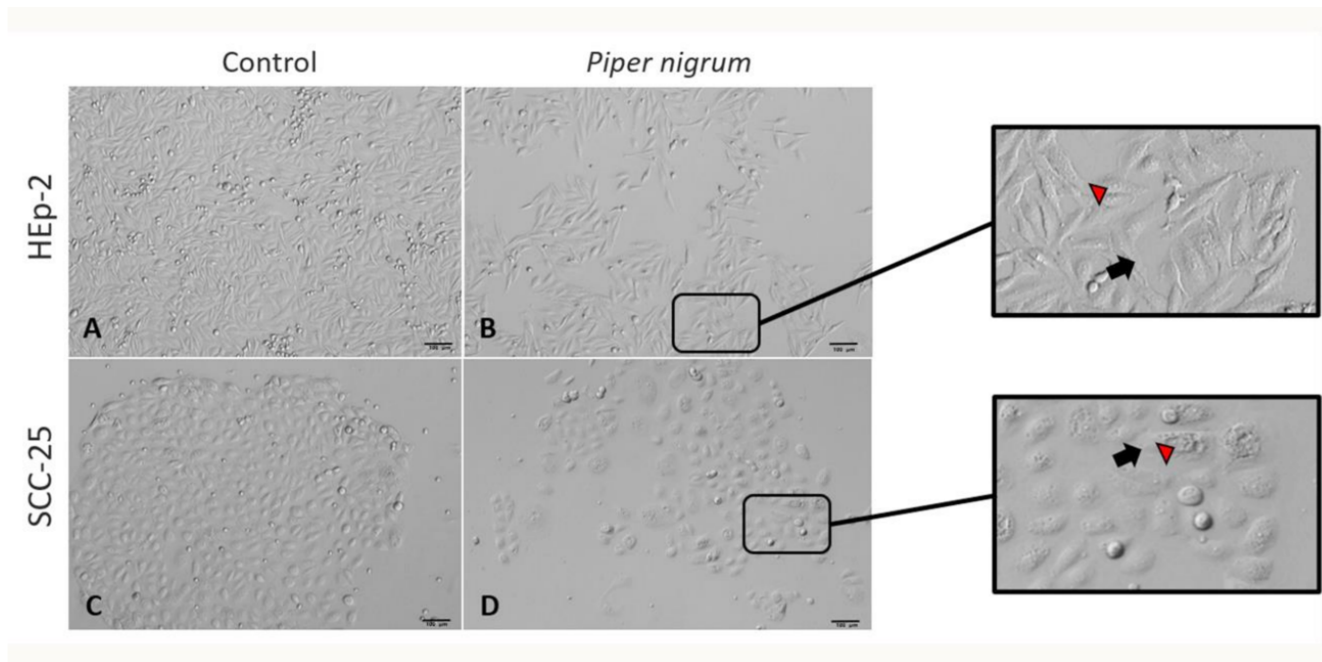

Figure 1: Morphological and proliferation analysis of PNE treatment on HEp-2 and SCC-25. The same morphology characters can be observed in control [A, C] and was not altered in [B, D] treated [10, 50 and 100 µg/mL]. Evidencing nuclei (red arrows) and cytoplasmic extensions (black arrows). Bars: 100µm.

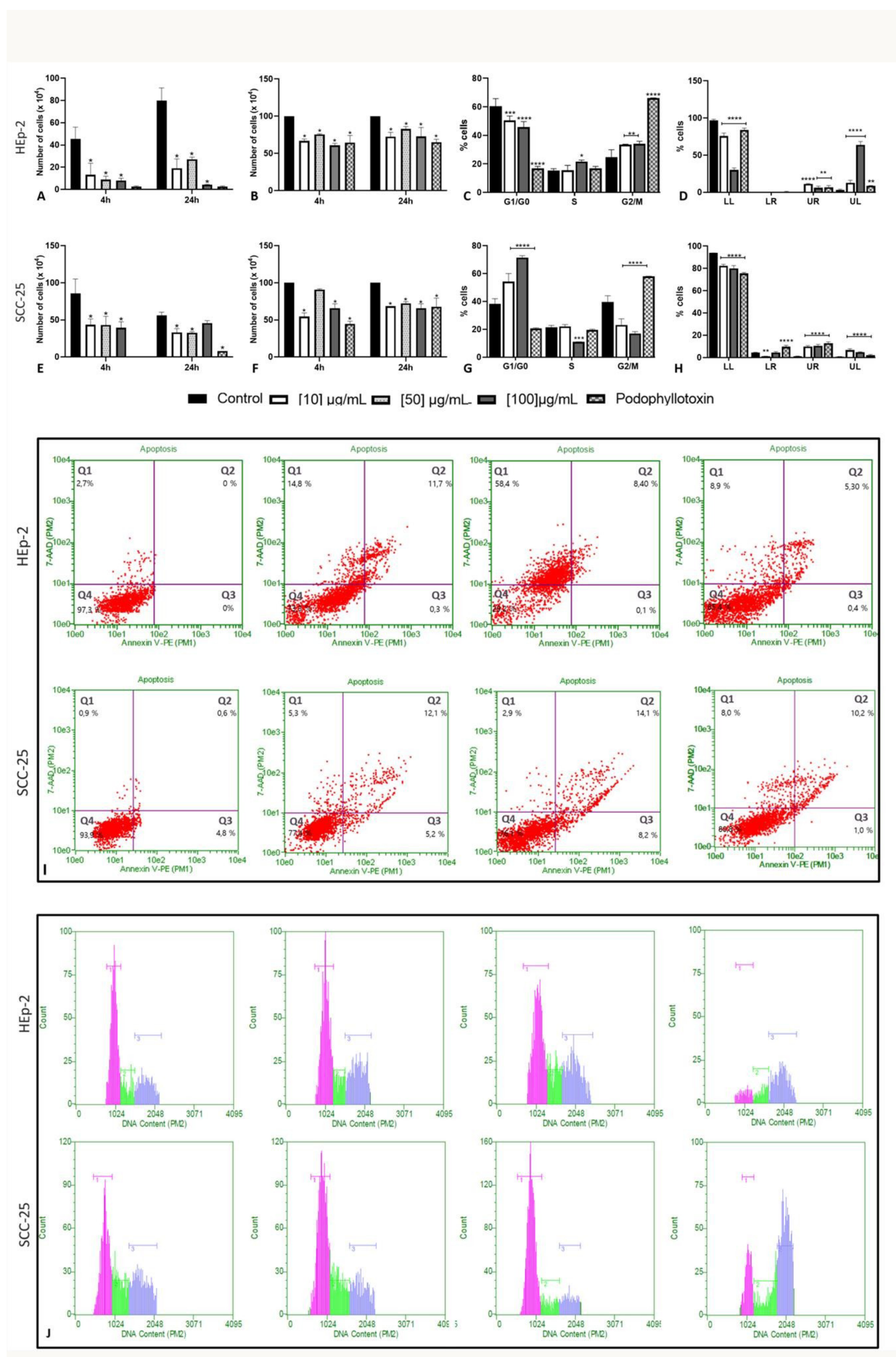

Figure 2: Analysis of proliferation, apoptosis, cell cycle and DNA damage in tumorigenic lines after 24h of treatment action. [A, B, E, F] Proliferation and cytotoxicity analyses. [D, H, I] Analyses of apoptosis. Dotplots with axis x= PE- ANXAV and y= 7-AAD. Analyses of cell cycle progression [C, G, J]. Bars: 5µm. Values P * =p< 0.05; ** =p< 0.01; *** =p<0.001; **** =p<0.0001 *vs control.

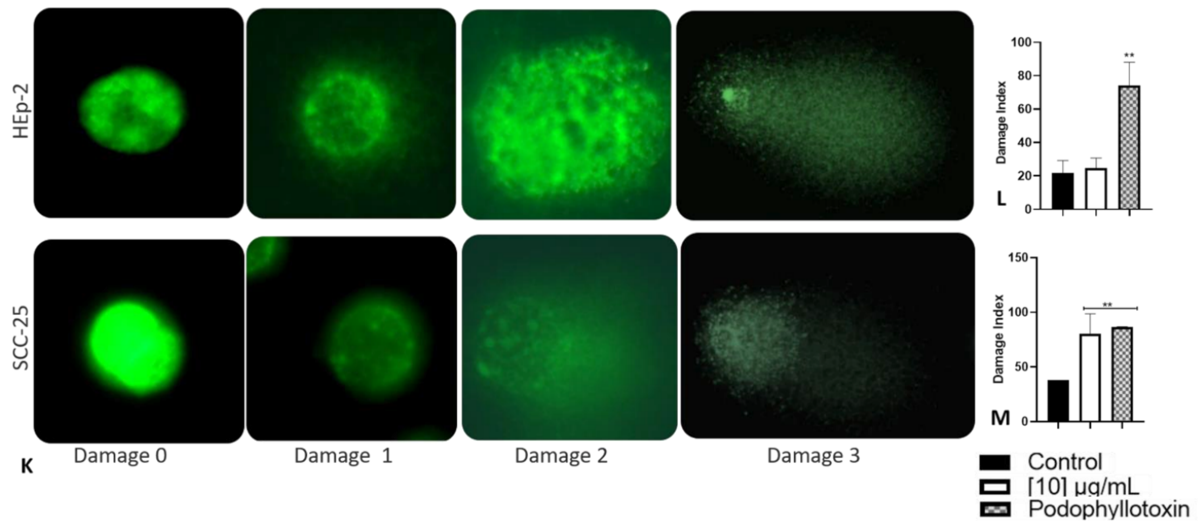

Figure 3: Analysis of DNA damage in tumorigenic lines after 24h of treatment action. Analyses of DNA damage [K-M]. Bars: 5µm. Values P * =p< 0.05; ** =p< 0.01; *vs control.

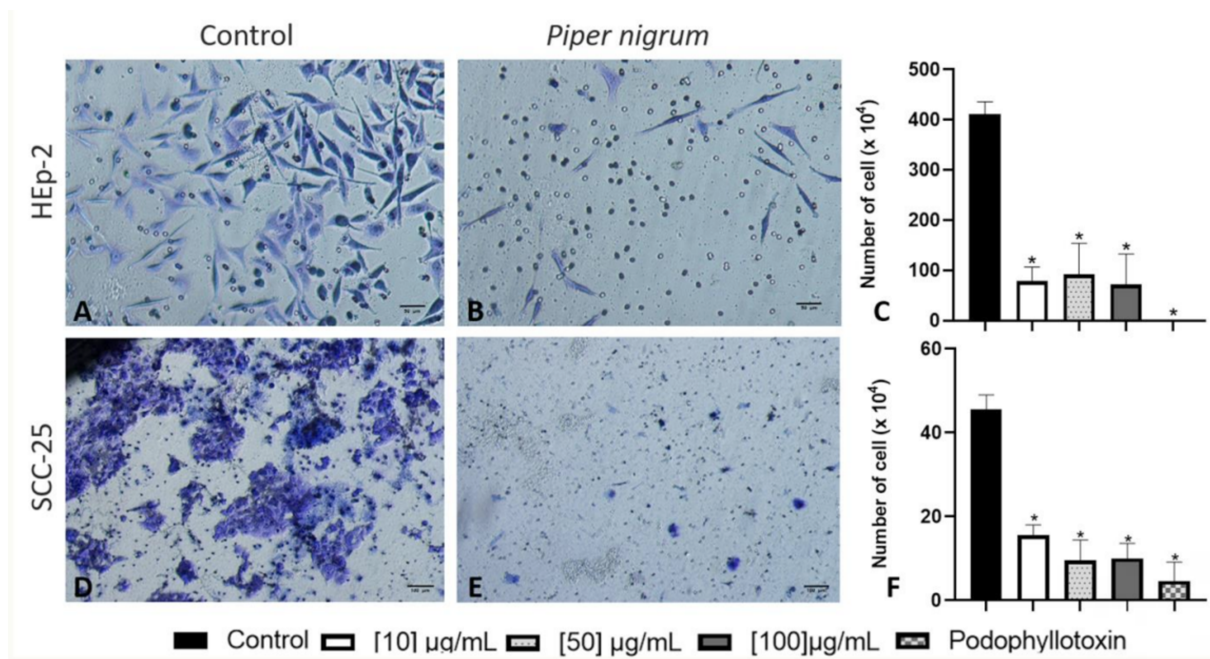

Figure 4: Effects of PNE extract treatments on cell migration. Phase contrast microscopy shows cell migration Transwell. Bars: 100 µm. After staining with crystal violet [A, B, D, E]. Statistical analysis of the quantification of the cells that migrated [C, F]. Values P < 0.05 vs. control.

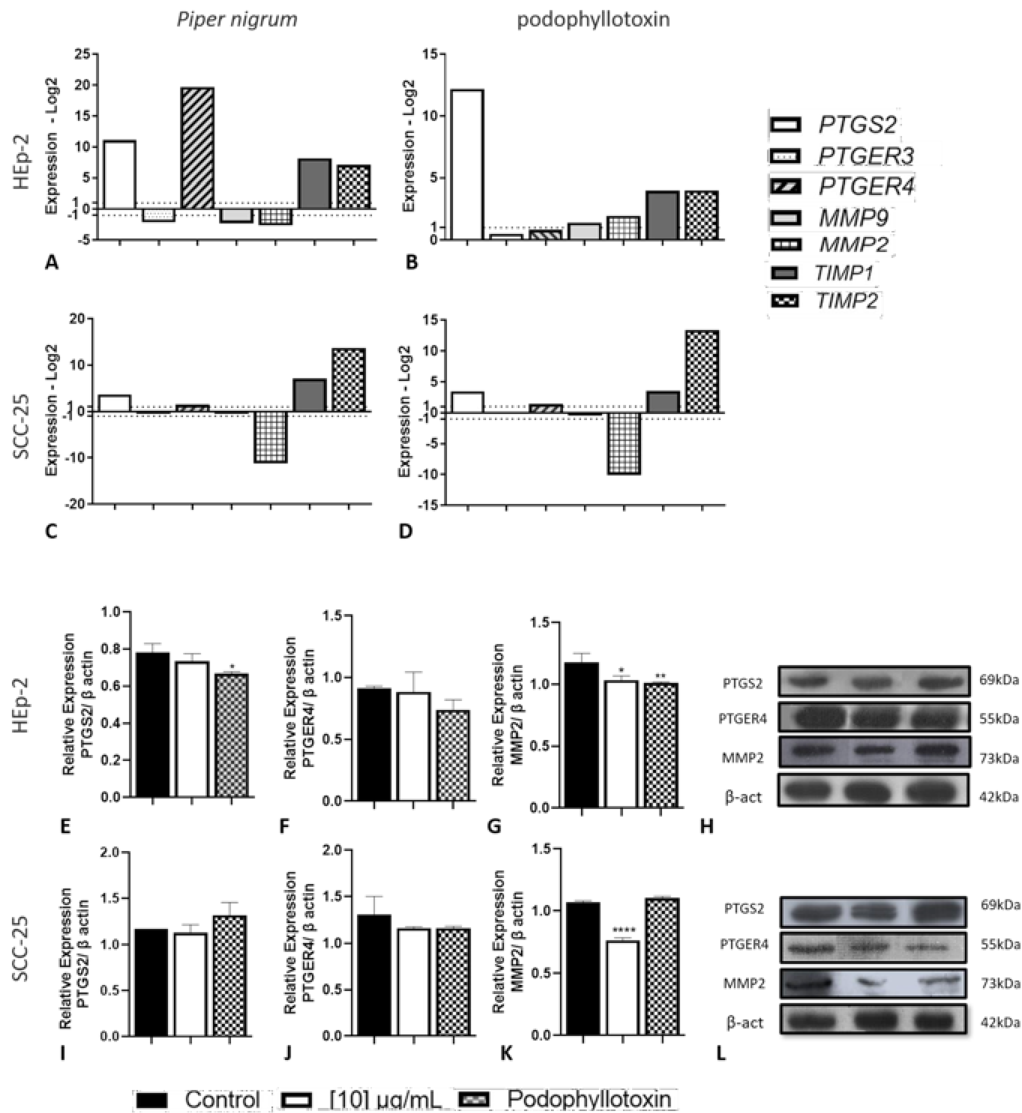

Figure 5: Expression of PTGS2 pathway genes by real-time PCR and western blot in tumor lines. [A-D] Gene expressions were analysed after treatments compared to control. The dashed line (≥ 1.0 or ≤ -1.0) equals the significant expression difference based on logarithm 2. [E-L] Expressions of PTGS2, PTGER4, MMP2 proteins were detected by western blot after 24h treatment; β-Actin used as endogenous control. Values P*** p < 0.01 and ****p < 0.001 vs. control.

Discussion

Although cancer treatment is widely studied, this disease remains a major challenge for uman medicine to date. Therefore, here, we investigated the potential mechanism of the PNE against squamous cell carcinoma of the head and neck. The extract is composed mainly by essential oil and others metabolites. The oil contains β-Caryophyllene (2.0 and 10.2%), Germacrene-D (4.6 and 56.7%), elemol (2.0 and 54.8%), nerolidol (2.1 and 22.3%) as the major metabolites in addition to other sesquiteres in a smaller proportion [17]. The components in higher proportion present important antitumoral properties as demonstrated by Francomano and collaborators (2019) in a review on biological properties of the β-Caryophyllene [18] and Sarvmeili, et al. (2016) to the β-Caryophyllene and Germacrene-D [19]. Therefore, in this study, the toxicity of essential oils and/or leaves extracts had an action on tumour cells where the proportion of these two components is high in leaves, in addition, these two sesquiteans are responsible for the observed activity. In our study, we observed

that cell proliferation decreased significantly after treatment with PNE, corroborates with a recent study in which the essential oil of Erythrin corallodendron leaves inhibited proliferation, migration and invasion in breast and leaf cell lines and *P. guineense* inhibited proliferation in ovarian cancer [20]. Other studies, such as by Abu and collaborators (2016), who when studying compounds from *Piper methysticum* differently observed a reduction in proliferation in breast tumour cells [21]. In the cell migration assay, we found that the treatment induced the anti-migratory effect on tumour cells, which corroborates with a recent study, where the compound (-) -kusunokinin, extracted from the seeds of *P. nigrum* decreased migration in breast cancer cells and inhibited tumour growth in vivo models [22].

In the comet assay, after administration of PNE to HEp-2 cells, there was no significant genotoxic effect, but PNE caused significant damage to SCC-25 cells, consistent with the results of Hang and collaborators (2018), these researchers examined head and neck tumorigenic cells treated with piperlongumine, an alkaloid isolated from the *P. longum* plant, and DNA damage and high levels of reactive oxygen species were observed in these cells. PNE in this study also triggered cell apoptosis in both tumour lines, with a decrease in viable cells and an increase in cells in late apoptosis and necrosis in the HEp-2 cell lines and in early and late apoptosis and necrosis in SCC-25 cells [23]. Recently, it has been demonstrated that total extract, piperine-free and two bioactive compounds (kusunokinin and piperlongumine) from the fruit of *P. nigrum* induced cellular apoptosis in breast tumour cells [9,24].

In this study, we observed that PNE induces cell cycle arrest in the G2/M and S phases of HEp-2 cells. Other studies examined the cytotoxic activity of the *P. nigrum* fruit extract and its ability to inhibit the cell cycle in the G2/M phase of breast cancer cells was affirmed [25], with similar results in oral squamous cell carcinoma [26]. In contrast to the results in SCC-25 cells, the treatment induced arrest in the G1/G0 phase of the cell cycle.

To evaluate the effect of PNE in head and neck cancer, we analysed the gene and protein expression of the cyclooxygenase-2 cascade (PTGS2), and our findings showed that the treatment positively affected this pathway in both tumour cell lines studied. The expression *PTGS2* gene increased in both tumour cell lines (HEp-2 and SCC-25) after treatment with the extract. However, it is important to note that *PTGS2* is present in laryngeal cancer, and its binding to receptors is associated with protumorigenic inflammatory responses and aggressiveness in oral squamous cell cancer [27].

Thus, our results showed that PTGER3 expression was reduced and PTGER4 expression was increased in both tumour cell lines, indicating that PTGS2 synthesized PGE2 and consequently activated only receptor 4, which is reported to be abundantly expressed in tongue carcinoma cells, and its hypermethylation is linked to recurrence in laryngeal cancer patients [28]. In particular, the results with PTGER3 corroborate the data found in recent studies by our group, which showed low expression after treatments with lignans and total extract of P. cubeba in SCC-25 cells [29].

The total extract of *P. nigrum*, promoted the mechanism of action of TIMPs studied in both tumour lines, as a consequence, there was a greater interaction with metalloproteinases (MMP9 and MMP2), which had an antagonistic response, reduced after the action of tissue inhibitors in HEp-2 and SCC-25 lines. These results confirm the biological mechanisms of TIMPs and their interactions with MMPs after PNE treatment. Several studies have reported the expression and role of MMP-9 and MMP-2 in aggressiveness and migration in head and neck cancer. In one of these studies, it was observed that in human oral cancer tissues the increased expression of these metalloproteinases was significantly associated with metastasis [30]. Thus, our results are promising as they demonstrate the effect of the treatment modulating the deregulated action of these cancer-associated proteolytic enzymes, and also correlate with our results on the antimigratory effect on tumour lines after treatment.

In the protein analysis, as expected, we observed a decreased trend of PTGS2, PTGER4 and MMP2 protein expression after treatment with the leaves extract of *P. Nigrum*, but with a significant difference only for MMP2 in tumour cells. As reported in a study investigating the ethanolic extract leaves of *O. sanctum*, it exerted anti-invasive effect on the squamous cells of the head and neck, attenuating the activity of MMP9 and MMP2, which are related to aggressiveness in this type of cancer [31].

Conclusion

In summary, PNE has anticancer effects, promoting antiproliferative activity and arresting the cell cycle by inducing DNA damage and cell death, in addition to inhibiting the PTGS2 pathway in the studied head and neck tumour cell lines. These findings indicate that the leaves of *P. nigrum*, being a source of compounds with antitumor activity, can act in synergism and be used for new therapeutic strategies in HNSCC.

Author's contributions

SOS: Conceptualization, Methodology, Analysis, Research, Writing. LPC, JPGZ, TH, RSL: Methodology. EHT and SMO: Resources. FCRL: supervised, Review, Editing and Resources.

Conflict of interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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