Pathology - Research and Practice 214 (2018) 30–37

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original article

Gallic acid modulates phenotypic behavior and gene expression in oral squamous cell carcinoma cells by interfering with leptin pathway

Eliane Macedo Sobrinho Santos[a,b], Rogério Gonçalves da Rocha[a], Hércules Otacílio Santos[c], Talita Antunes Guimarães[a], Carlos Alberto de Carvalho Fraga[c,d], Luiz Henrique da Silveira[a], Paulo Ricardo Batista[e], Paulo Sérgio Lopes de Oliveira[f], Geraldo Aclécio Melo[g], Sérgio Henrique Santos[h], Alfredo Maurício Batista de Paula[a], André Luiz Sena Guimarães[a,1], Lucyana Conceição Farias[a,*,1]

[a] Department of Dentistry, Universidade Estadual de Montes Claros, Minas Gerais, Brazil
[b] Instituto Federal do Norte de Minas Gerais – Campus Araçuaí, Minas Gerais, Brazil
[c] Instituto Federal do Norte de Minas Gerais – Campus Salinas, Minas Gerais, Brazil
[d] Universidade Federal de Alagoas, Arapiraca, Alagoas, Brazil
[e] Programa de Computação Científica – Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
[f] National Biosciences Laboratory (LNBio), Campinas, São Paulo, Brazil
[g] Department of Biology, Universidade Estadual de Montes Claros, Minas Gerais, Brazil
[h] Institute of Agricultural Sciences, Food Engineering College, Universidade Federal de Minas Gerais, Brazil

ARTICLE INFO

Keywords:
Leptin
Gallic acid
Oral squamous cell carcinoma
Cell proliferation
Cell migration
Angiogenesis

ABSTRACT

Gallic acid is a polyphenolic compost appointed to interfere with neoplastic cells behavior. Evidence suggests an important role of leptin in carcinogenesis pathways, inducing a proliferative phenotype. We investigated the potential of gallic acid to modulate leptin-induced cell proliferation and migration of oral squamous cell carcinoma cell lines. The gallic acid effect on leptin secretion by oral squamous cell carcinoma cells, as well as the underlying molecular mechanisms, was also assessed. For this, we performed proliferation, migration, immunocytochemical and qPCR assays. The expression levels of cell migration-related genes (MMP2, MMP9, Col1A1, and E-cadherin), angiogenesis (HIF-1α, mir210), leptin signaling (LepR, p44/42 MAPK), apoptosis (casp-3), and secreted leptin levels by oral squamous cell carcinoma cells were also measured. Gallic acid decreased proliferation and migration of leptin-treated oral squamous cell carcinoma cells, and reduced mRNA expression of MMP2, MMP9, Col1A1, mir210, but did not change HIF-1α. Gallic acid decreased levels of leptin secreted by oral squamous cell carcinoma cells, accordingly with downregulation of p44/42 MAPK expression. Thus, gallic acid appears to break down neoplastic phenotype of oral squamous cell carcinoma cells by interfering with leptin pathway.

1. Introduction

Leptin (Lep), a hormone secreted by adipose tissue, is known to be a component of the homeostatic loop of body weight regulation [1,2]. This hormone signaling can lead to the metabolic features associated with cancer malignancy, such as switching in cell energy balance from mitochondrial β-oxidation to the aerobic glycolytic pathway [3,4]. Furthermore, Lep provides the tumor microenvironment, mainly through its ability to potentiate both endothelial cells migration and angiogenesis and to sustain the recruitment of macrophages and monocytes, which in turn secrete vascular endothelial growth factor and proinflammatory cytokines [5]. Lep has been associated with increased risk of several cancers, as well as proliferative and anti-apoptotic effects on cancer cells [6,7].

Despite the scarcity of studies, Lep pathway has emerged as an important target, possibly involved in oral carcinogenesis. Surveys were conducted to assess serum Lep level in oral squamous cell carcinoma patients, suggesting that Lep can contribute to oral cancer-induced loss of body mass [8–10]. Besides, gene polymorphisms of Lep and its receptor LepR revealed an increased risk to oral carcinogenesis [11].

* Corresponding author at: Universidade Estadual de Montes Claros, Hospital Universitário Clemente de Faria, Laboratório de Pesquisa em Saúde, Avenida Cula Mangabeira, 562, Montes Claros, Minas Gerais, Zip code: 39401-001, Brazil.
E-mail address: lucyanacfarias@gmail.com (L.C. Farias).
[1] These authors have contributed equally to this work.

https://doi.org/10.1016/j.prp.2017.11.022
Received 28 February 2017; Received in revised form 16 November 2017; Accepted 28 November 2017

Therefore, Lep can be pointed as a possible target for new therapeutic investigations. Current strategies to inhibit Lep pathway, such as soluble Lep receptors (LRs), synthetic Lep-antagonists, and anti-LR monoclonal antibodies (anti-LR mAbs) [12] can be limited due to toxicity, as well as low efficacy.

Currently, there is substantial interest in understanding the therapeutic potential of herbal extracts in cancer. Several extracts have demonstrated activity against different kinds of cancer [13]. Gallic acid (3,4,5-trihydroxybenzoic acid; GA) is a polyphenol found widely in fruits and plants; it has been shown to have anticancer effects in human leukemia HL-60RG [14], lung cancer [15], stomach cancer, colon cancer [16], prostate cancer [17], melanoma [18] and esophageal cancer [19], pheochromocytoma [20], mouse leukemia WEHI-3 cells [21] and oral cancer [22,23]. In oral carcinogenesis, the real effect of GA on neoplastic cells is not well understood.

Given the above, we hypothesized that GA interacts with components of Lep signaling pathways, and it can interfere with the neoplastic role of this hormone. So, the study purpose was to investigate the potential of GA to modulate Lep-induced proliferation and migration of OSCC cell lines and the underlying molecular mechanisms. We also explored GA effect on Lep secretion by OSCC cells.

2. Material and methods

2.1. Bioinformatics analysis and in silico docking experiments

The hypothesis that GA interacts with Lep was first explored by *in silico* analysis, aiming to conduct the *in vitro* functional assays.

For the molecular docking simulation, the atomic coordinates of the GA structure were taken from the Zinc Database [24] under the code ZINC0000104, in the mol2 format. The structures of the human Lep and LepR were taken from the Protein Data Bank (PDB) codes 1ax8 [25] and 3v6o [26], respectively. Before our docking simulations, the protein structures were prepared with the addition of hydrogens taking into account the most likely protonation state of each titrable residue (considering a pH of 7.2), using the PROPKA program [27], inside the pdb2pqr program [28,29]. All molecular docking calculations were performed using the Autodock Vina program [30], in a two-step approach: *i.* a blind docking procedure and *ii.*a pocket search method. The blind docking procedure consisted of searching the entire protein surface to determine the potential binding pocket(s). This was achieved using the grid center as the center of each protein, using a grid size big enough to cover the entire protein surface. After finding the binding pockets, we centered the grid center within the discovered binding pocket where the best-scored conformation is located, and performed a more accurate search (the pocket search procedure), using the following parameters: energy_range = 10, num_modes = 20 and exhaustiveness = 800.

Investigation of putative target genes of GA was obtained from STITCH 3.1 (http://stitch.embl.de/) [31]. Briefly, this server predicts interactions of chemical compounds with proteins based on known interactions from metabolic pathways, crystal structures, binding experiments, and drug–target relationships. It also allows the use of information from phenotypic effects, text mining and chemical structure similarity in order to predict relations between chemicals.

2.2. Cell culture

Two human OSCC cell lines, SCC9 and SCC4 (CRL-1629 and CRL1624, ATCC cell bank, USA), were cultured in DMEM/Ham's F-12 (Gibco, USA), supplemented with 10% fetal bovine serum and 0.4 μg/ml hydrocortisone (Gibco, South America). All experiments were performed in triplicate and at least three independent experimental times.

2.3. Cell proliferation assay

Cell proliferation assay was performed as described before [32] with necessary adaptations. A density of 2×10^5 OSCC cells was plated in 60 mm dish and incubated at 37 °C for approximately 24 h to establish adherent monolayers. Then, cells were treated with 100 ng/ml of human recombinant leptin (Invitrogen, USA) and 10 μg/ml of GA (Sigma-Aldrich, USA) for 72 h. The GA concentration was previously defined through a dose-response curve in another study of our group [32]. The comparing groups, as Lep-treated cells, GA-treated cells and cells cultivated only in culture medium were included in study design. Cell proliferation was determined by trypan-blue exclusion. The proliferation assay was performed under normoxia, and mimicking hypoxia by the addition of 100 μM $CoCl_2$ (Sigma-Aldrich, USA).

2.4. Cell dead/viability assay

Acridine orange/ethidium bromide (AO/EB) staining was used to visualize dead and viable cells [33]. A volume of 25 μl of cell suspension (2.0×10^6cells/ml) was incubated with 1.0 μl of a solution containing 1 part of l00 μg/ml acridine orange in PBS; (AO, Sigma, St. Louis, USA) and 1 part of 100 μg/ml ethidium bromide in PBS (EB, Sigma, St. Louis, USA). The cell suspension was placed onto a microscopic slide and covered with a glass coverslip. Cells were observed in a fluorescence microscope FSX100 (Olympus, Center Valley, PA, USA). Intense EB staining (Ex360-370, Em420-460, filter DM400) indicates cell death, while intense AO (Ex460-495, Em510-550, filter DM505) indicate live cells.

2.5. Migration assay

Cell migration was assayed by *wound healing* method [34]. Briefly, at the full confluence, OSCC cells were scraped away horizontally using a 200 μl tip. Culture medium was then replaced by serum-free medium, adding 100 ng/ml leptin and/or10 μg/ml of GA for 72 h. The migration characteristic of cells treated with Lep and/or GA was also evaluated under hypoxia condition. In order to measure the wound covered area by migrating cells, images of the wounded cell monolayers were taken using an Olympus IX81 inverted microscope (Olympus, Center Valley, PA, USA) coupled to camera SC30 (Olympus, Center Valley, PA, USA) at 0 and 72 h after wounding.

2.6. Clonogenic assay

Treated or untreated OSCC cells were plated in 60 mm dish at a density of 1.0×10^2cells and maintained in culture for 14 days. Then, cells were fixed with 70% ethanol at 4 °C and stained for the counting of colonies formed. Colonies with over 50 cells were considered for analysis. Survival fraction (SF) was calculated as previously described [35].

2.7. Secreted leptin dosing

In all experimental groups, secreted Lep level by OSCC cells in culture medium, under normoxia condition, was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Leptin Human ELISA-LDN®). This ELISA kit shows a limit of quantification around 100 ng/ml. Secreted leptin dosing was performed as recommended by the commercial kit manufacturer. To this analysis, leptin dosage was adjusted by cell number in each group.

2.8. Immunocytochemical assay

Immunocytochemical was performed as described before [36] with necessary adaptations. A density of 2×10^4 OSCC cells was plated on coverslips and submitted to the experimental treatments. At the end of treatments, the cells were fixed with 70% ethanol for 30 min.

Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. The polyclonal antibodies LepR (obR; clone H-300, sc-8325, Santa Cruz Biotechnology, CA, USA), and p44/42 MAPK(ERK1/2) (Clone 137F5, Cell Signaling Technology, USA) were detected using LSAB kit (LSAB-Kit Plus Peroxidase, Dako, California, USA). Signals were developed with 3′3-diaminobenzidine-tetrahydrochloride and counterstained with Mayer's hematoxylin. For staining quantification, 10 fields were photographed at 400× magnification in a microscope FSX100 (Olympus, Center Valley, PA, USA). Cell counting was then performed in the software ImageJ (http://rsbweb.nih.gov/ij/).

2.9. Gene expression of LepR, HIF-1α, E-cadherin, Casp-3, MMP2, MMP9, Col1A1and mir-210

In order to assess the molecular effect of leptin and/or GA on gene expression in OSCC cells, we investigated, by qRT-PCR, cell migration-related genes (MMP2, MMP9, Col1A1, and E-cadherin), angiogenesis (HIF-1α, mir210), leptin signaling (LepR, p44/42 MAPK), and apoptosis (casp-3). Total RNA was isolated using Trizol (Invitrogen, USA). Reverse transcription was conducted from 1,5 μg of total RNA by Reverse Transcription Kit (Invitrogen, USA). Rt-synthesized cDNA was amplified using specific primers (Table 1, Supplementary materials), and SYBR Green following the manufacturer's instructions on a StepOne system (Applied Biosystems, Courtaboeuf, France). To mir210 analysis, qRT-PCR was performed using Taqman™ microRNA assay/000512 (Applied Biosystems, USA). The comparative C_t method was applied to analyses [37].

2.10. Statistical analyses

The analyses were carried out using SPSS 17.0 software. Statistical tests were selected according to the characteristics of the samples and the distribution of variables and probability values < 0.05 were considered statistically significant.

3. Results

3.1. In silico model shows possibility of interaction of gallic acid with leptin pathway

Our *in silico* analysis points to two possibilities of molecular interactions between GA and Lep. As an initial approach, we used molecular docking, focusing two possibilities of action: *i.* GA interacts directly with Lep; or *ii.* GA interacts with LepR.

Firstly, we considered the occurrence of a direct interaction between GA and Lep. To investigate this hypothesis, we performed molecular docking of GA against the structure of human Lep. We found three major clusters with a binding score between −4.5 to −4.8 kcal/mol. It is important to mention that none of them was located at the Lep/LepR binding interface (Fig. 1 – Model 1).

Another hypothesis tested was that GA could bind to LepR. To address this question, we docked the GA with the LepR structure. In contrast to the GA docking experiments over the Lep structure, the best-scored GA conformation obtained from blind docking over the entire LepR surface was located at the Lep binding interface (Fig. 1 – Model 2). A more accurate search addressing this particular cavity confirmed this result, obtaining the best-scored GA conformation with a binding score of −5.1 kcal/mol. In summary, these *in silico* results suggest that GA probably binds to LepR (at a region coincident to the Lep binding interface) and could interfere/impair the binding of Lep to the LepR.

The interaction network analyses of the putative proteins that interact with GA obtained from STITCH also guide our actions in choosing the proteins/genes evaluated in this study (Fig. 1). The 10 predicted proteins that interacted with GA were: ABCB1, MMP9, JUN, EIF2AK3, SULT1, ATM, PRKCA, CHUK, AKT1, MMP2.

These findings encouraged us to follow up with functional assays.

3.2. Gallic acid interferes with leptin-induced cell proliferation, cell death, and colony formation of OSCC cells

To assess the potential of gallic acid (GA) to interfere with leptin-induced cell proliferation, SCC-9 cells were treated with Lep and/or GA, under normoxia (Fig. 2A) and hypoxia (Fig. 2B) conditions. GA treatment was able to reduce cell proliferation, in both conditions, despite the proliferative effect induced by leptin treatment.

Accordingly with this result, in the GA-treated group, we identified an increased number of dead cells and higher levels of casp-3 mRNA comparing to leptin group (Fig. 2C). Cell survival fractional was also low in SCC-9 cells treated with GA regarding leptin-treated cells (Fig. 2D).

The results were similar in SCC-4 cells (Fig. 1, Supplementary material).

3.3. Gallic acid impairs cell migration in leptin-treated OSCC cells, reducing gene expression of MMP9, MMP2, and Col1A1

The behavior of migration of cells treated with Lep and/or GA was analyzed under normoxia (Fig. 3A) and hypoxia-mimicking (Fig. 3B). GA reduced cell migration significantly, in both normoxia and hypoxia conditions. Lep leads to the opposite effect of GA favoring cell migration. However, when OSCC cells were treated with Lep/GA under hypoxia condition, it was observed reduced migration and also an increase of cell-free area. It can be due to the induction of cell death by GA.

The Fig. 3C–F show expression of genes MMP9, MMP2, Col1A, and E-cadherin in SCC-9 cells treated with Lep and GA. It was observed a significant reduction in genes related to cellular migration, such as MMP2, MMP9, and Col1A1 in the group of cells treated with GA. The opposite effect on the expression of these genes was observed in the group Lep-treated OSCC cells. The expression of E-cadherin was increased in the groups treated with Lep and GA.

3.4. Gallic acid reduces mRNA expression of angiogenesis-related genes

The expression of mRNA of HIF-1α was not affected by the addition of the GA or Lep under normoxic conditions (Fig. 4A), but when the experiment was carried out in hypoxia conditions, GA and Lep seem to modify the expression of this transcript. GA impairs, and Lep stimulates the HIF-1α expression (Fig. 4B).

Mir-210 expression was lower in AG-treated OSCC cells (Fig. 4C).

3.5. Gallic acid impairs LepR expression and leptin secretion by OSCC cells, downregulating p44/42 MAPK expression

To verify if GA interferes with molecular components of leptin pathway, we assessed LepR expression, secreted Lep levels by OSCC cells, and MAPK expression.

Cells treated with GA show decreased LepR mRNA expression under hypoxia condition (Fig. 5A). However, this transcript was not different between experimental groups under normoxia (Fig. 5B).

GA treatment reduced Lep secreted level by OSCC cells into the culture medium (Fig. 5C) comparing to all groups. Immunocytochemical expression of LepR was lower in GA group than Lep group (Fig. 5D).

Interestingly, in GA-treated cells, showing lower secreted leptin levels, we identified a downregulation in p44/42 MAPK(ERK1/2) expression (Fig. 5E). This finding was consistent with the reduced proliferative behavior of cells in this group.

4. Discussion

GA has been shown to inhibit tumor growth and progression in some cancer types, especially due to its antioxidant activity [13–19]. Likewise, GA treatment inhibited proliferation, migration, and invasion

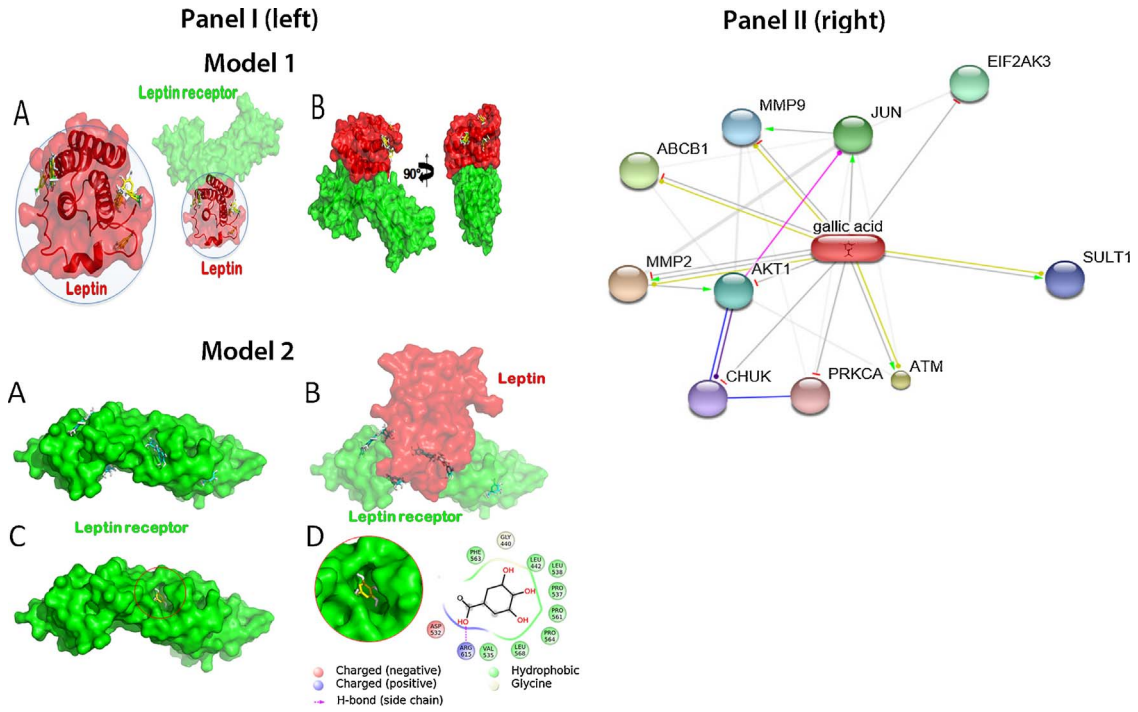

Fig. 1. Bioinformatics analyses showing the interaction between GA and Lep. **Panel I (left) – Model 1** shows a putative binding mode of GA in the human leptin structure (PDB ID 1ax8) achieved from blind docking experiments. In A, on the left, the superposition of the 20 best-scored GA docked conformations. On the right, the complex structure of leptin bound to its receptor. It is important to mention that none of the docked conformations was located in the leptin receptor binding site. In B, two different orientations of the leptin/leptin receptor complex with the predicted GA binding mode showing the three main clusters on the leptin structure surface. **Model 2** shows a putative binding mode of GA in the human leptin receptor structure from blind and site-directed docking experiments. All the docking experiments were performed with the leptin receptor structure in two steps: *i.* a blind docking scheme searching the entire leptin receptor surface; and *ii.* using a grid centered on the best-scored conformation of the gallic acid from the previous blind docking scheme. In A and B, the superposition of the 20 best-scored GA docked conformations. In B, it is also represented the structure of the leptin bound to its receptor (just for visualization pourposes). The best-scored docked conformations were all located in the leptin binding site on the Lep receptor surface. In C, is shown the best-scored docked conformation. In D, this conformation is zoomed and, on the right, the ligand-protein interaction diagram is shown. **Panel II (right).** Interaction network profile between GA and **predicted functional partners,** showing its action possibilities from STITCH program. Down-regulation is a red bar and up-regulation is a green arrow. Yellow circle represents that the directionality of the interaction is known, but the result of the interaction is unknown (*e.g.*, if it is up- or down-regulated). Black circle at both ends means some kind of interaction exists. The bar colors depend on the source of the data (in deep blue: binding; in blue: phenotype; in indigo blue: catalysis; in violet: post-translation; in black: reaction; and in yellow: expression.).

of OSCC cells [32]. However, the molecular pathways related to the antineoplastic effect of GA in oral cancer are still not well clarified. So, to understand this 'knowledge gap', initially, we designed an *in silico* model to study the interaction between GA and the Lep/LepR pathway. This particular molecular signaling was first considered as a possible targeted of GA, once Lep has emerged as an important target for cancer therapy. Furthermore, a bioinformatic survey showed an interacting network between obesity-related genes and oral cancer [38], and gene polymorphisms of Lep and its receptor LepR were also associated with increased risk to oral carcinogenesis [11].

A possible modulator role for GA, especially in the Lep signaling pathways, it was proposed by our *in silico* analyses (Fig. 1), which revealed that GA interacts positively or negatively with genes that are modulated by Lep, such as AKT, MMP2, and MMP9. Our molecular docking analysis suggests that GA could directly bind to LepR (coincident into the Lep binding site) and interfere/impair Lep binding to its receptor.

In the present study, we demonstrate for the first time that GA interferes with the pro-neoplastic effects of Lep on OSCC cells. Treatment with GA leads to the inhibition of leptin-induced cell proliferation in the immediate period and the late period, as shown in cell proliferation and clonogenic assay, respectively. A previous study indicated that treatment with GA resulted in the inhibition of cell proliferation, migration, and invasion in neoplastic cells [32]. Furthermore, GA induced apoptosis in oral cancer cells [39]. In our study, we show that GA increased cell death and caspase-3 mRNA expression, overlapping to the reducing in the frequency of dead cells associated with the action of Lep in OSCC cells. Similarly, GA was able to decrease cell migration, independently of treatment with Lep. These effects were associated with MMP-2, MMP-9, and Col1A1 expression. MMP-2 and MMP-9 play a critical role in cancer cell migration and invasion [40,41] and overexpression of both enzymes increases migration and invasion of cancer cells [42].

Mir210 overexpression, specifically under hypoxia, was found to affect many processes involved in tumor development, including the promotion of angiogenesis and a reduction in DNA repair capabilities [43]. In our study, mir210 was overexpressed in the group of cells treated with Lep, suggesting a significant relationship between Lep and mir210 to potentiate angiogenesis. The GA, in turn, appears to inhibit the expression of mir210 in Lep-treated cells.

The LepR mRNA expression under normoxia condition did not differ between the study groups. However, the immunocytochemistry expression of LepR protein was higher in the group treated with Lep being reduced after treatment with GA. These data suggest that expression of LepR protein can be a regulated phenomenon by posttranslational processing. Furthermore, these results strongly propose that the GA may be a significant anti-angiogenic agent acting through LepR and mir210, attenuating the neoplastic phenotype of OSCC cells.

These findings could be better evidenced in hypoxia condition in which GA alone or combined with Lep significantly decreased the HIF-1α and LepR expression, whereas treatment with Lep significantly increased this expression. Interestingly, the leptin, when combined with GA, is not able to counteract the GA effect on OSCC cells. Leptin expression can be up-regulated through HIF-1α, which controls tumor angiogenesis in many solid tumors [44]. Furthermore, leptin-receptor is activated directly by HIF-1α [45]. A previous study showed that GA inhibits HIF-1α and VEGF expression through blocking the

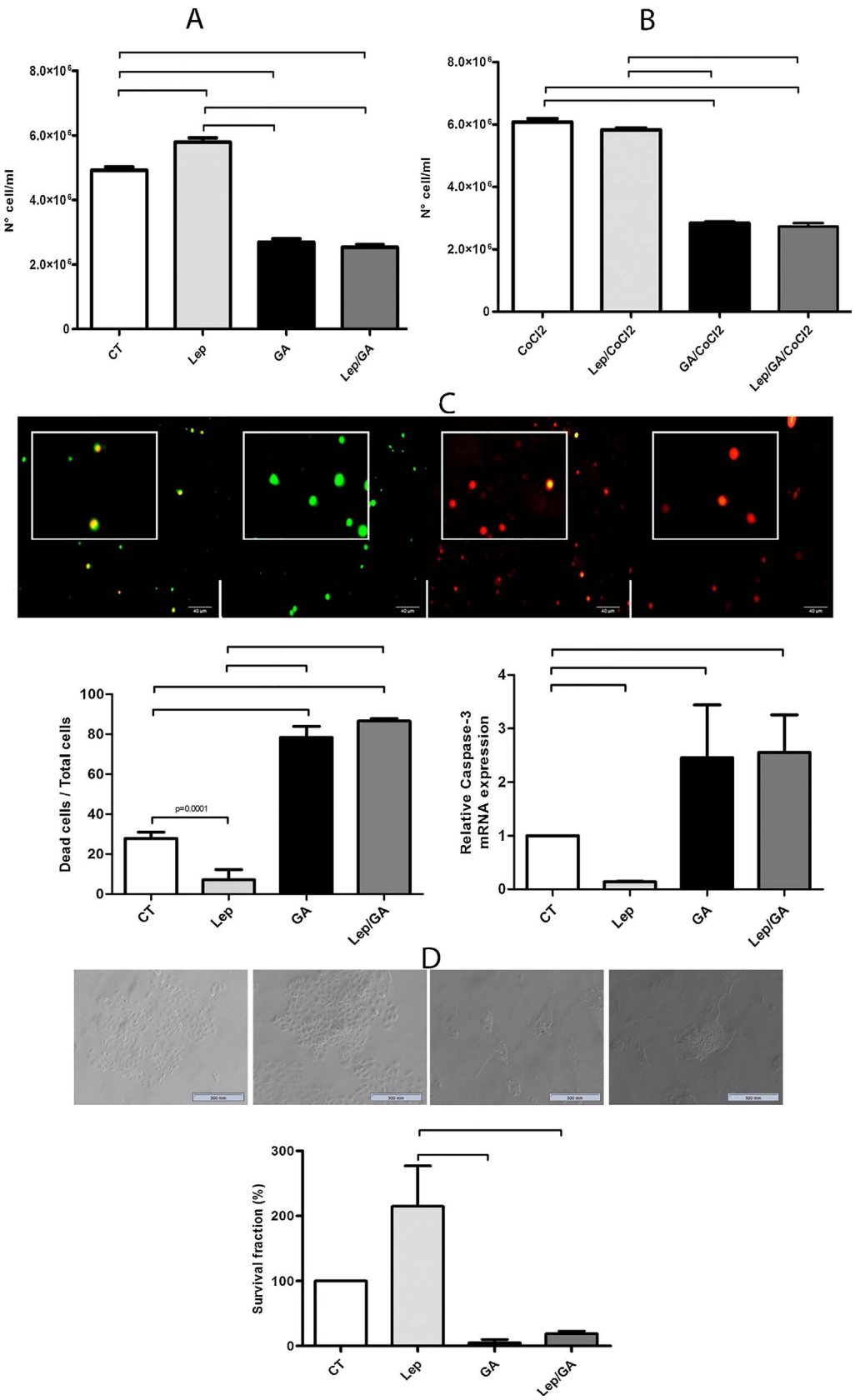

Fig. 2. Changes in proliferative and migratory behavior of SCC9 cell line following treatment with human recombinant leptin and/or GA for 72 h. Alterations in cell proliferation under normoxia (A) and hypoxia (B) conditions. (C) Colorimetric Assay by Acridine/Orange (A/O) and Ethidium Bromide (EB), indicating cell death or viability of leptin and/or GA-treated SCC9 cells. A/O is able to penetrate into cells emitting green fluorescence. EB emits red fluorescence due to intercalation with DNA only in cells showing changes in the plasma membrane. The graph also shows qRT-PCR results of caspase-3 mRNA in SCC-9 cells. (D) Colony formation assay. Graph and photography representatives of colony formation leptin and/or GA-treated SCC9 cells. Error bars represent standard error of the mean of two independent experiments, each consisting of three replicates. Significance was determined using ANOVA One-way (Symbol ⌐: $P < 0.05$ vs. the control). Lep: leptin-treated cells. GA: Gallic acid-treated cells.

phosphorylation of AKT [46], which is an essential protein of the Lep signaling pathway that leads to cell proliferation and migration.

The GA effect on inhibiting the Lep signaling pathway might be reinforced by our results of secreted Lep by OSCC cells. In the group treated with Lep/GA, secreted Lep amount reduced more than 50% when compared with the group treated with only Lep. In the group treated with GA, the amount of secreted Lep was minimal, much less than the amount of Lep emitted in the control group. These results are

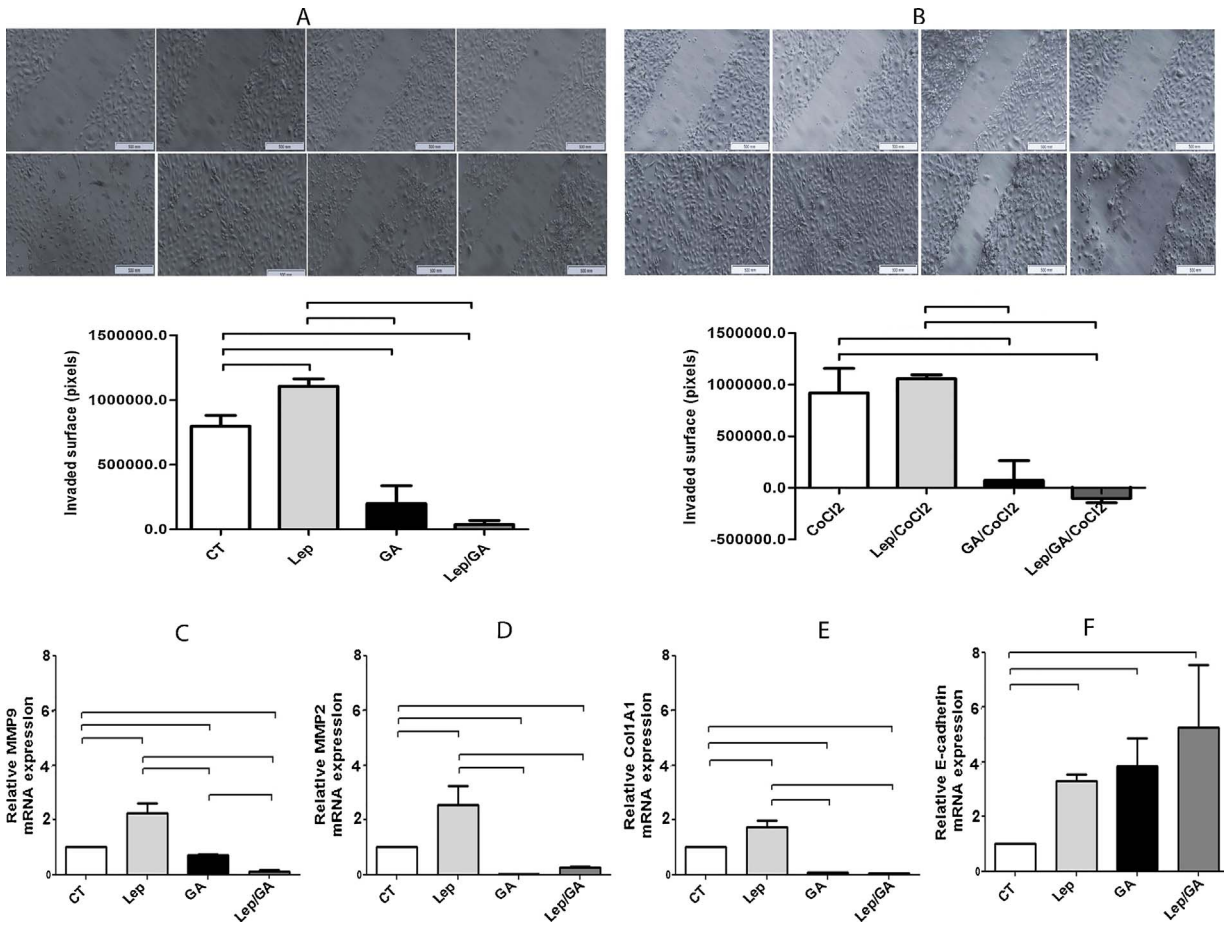

Fig. 3. Wound healing assay. It assesses the invaded area by SCC9 cells treated with Lep and/or GA under normoxia (A) and hypoxia (B) conditions. (C to F) mRNA expression of genes involved in the cell migration. In all analyses, gene expression is shown as the mean ± SE. All reactions were normalized to β-actin. MMP9 (C), MMP2 (D), Col1A1 (E), and E-cadherin (F). ANOVA One-way, symbol ⌐¬: $p < 0.05$.

supported by literature data showing that Lep mRNA expression and serum Lep levels were reduced following treatment with GA or derivatives [47,48].

Based on this evidence, along with modeling obtained from the analysis *in silico*, we suggested that both GA and Lep may compete or interact with a common signal intracellular pathway. As previously reported, in osteosarcoma cells, the chemopreventive activity of GA may be mediated by its ability to modulate the mitogen-activated protein kinase (MAPK) cascade [49]. Interestingly, the LepR is known to activate MAPK cascade after Lep binding [50,51]. In some cell types, the activation of MAPK cascade is crucial for induction of cell death, growth arrest and apoptosis [52,53]. Therefore, we show that GA appears to interfere with the Lep/LepR pathway and the Lep binding activity leading to harm the Lep anti-apoptotic activity in the OSCC cells. All these events mediated by GA and recombinant human Lep may be associated with a similar activation of MAPKs. In our study, expression of p44/42 MAPK (ERK1/2) in cells treated with GA and Lep confirms these reports.

We highlighted a novel role of GA as an inhibitor of secretion of Lep by OSCC cells, at the same time decreases the expression of LepR protein and increases the expression of caspase-3, possibly leading to the reducing of cell proliferation. Besides, GA can reduce Lep-induced

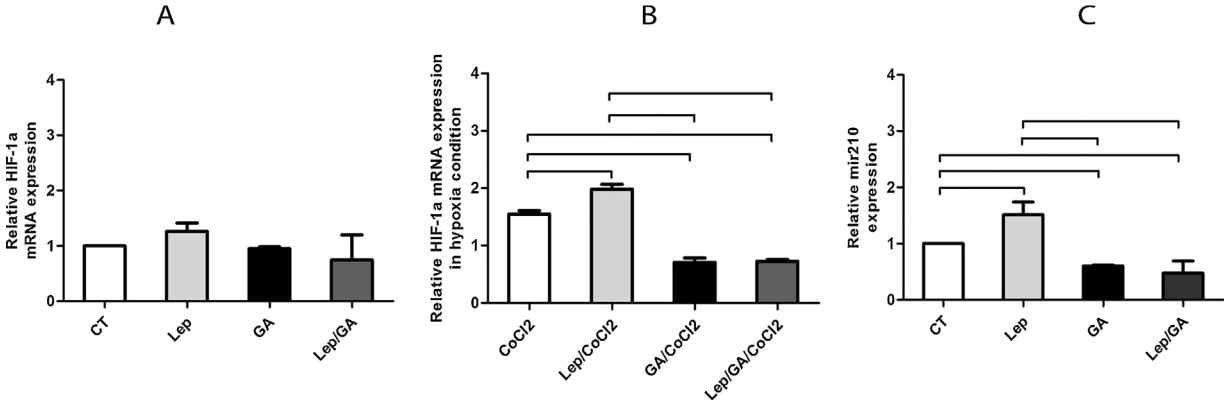

Fig. 4. mRNA expression of genes involved in angiogenesis in SCC-9 cells treated with Lep (Lep) and/or gallic acid (GA). HIF-1α under normoxia (A) and hypoxia (B) conditions, and mir210 (C). In all analyses, gene expression is shown as the mean ± SE. All reactions were normalized to β-actin.

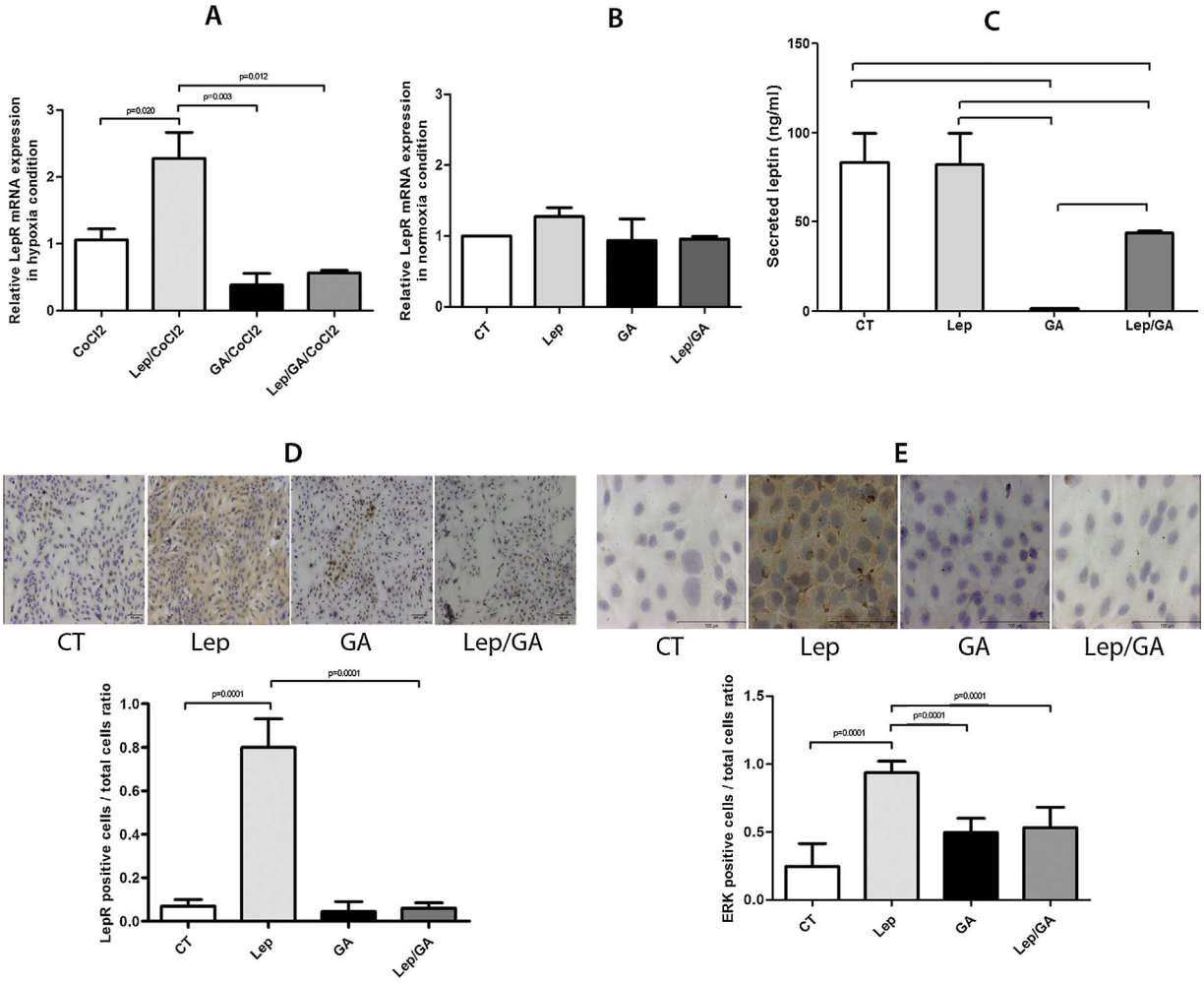

Fig. 5. mRNA expression of LepR under hypoxia (A) and normoxia (B) conditions in SCC-9 cells treated with Lep (Lep) and/or gallic acid (GA). In all analyses, gene expression is shown as the mean ± SE. All reactions were normalized to β-actin. (C) The level of Lep secreted by SCC-9 cells under the experimental tests. In groups treated with Lep and Lep/GA was added 100 ng/ml of human recombinant Lep; thus Lep levels in these groups are equivalent to secreted Lep and added recombinant Lep. Contrarily, in CT and GA groups, results show only secreted Lep. (D) Expression of LepR proteins in SCC-9 cells. (E) Immunohistochemical expression of p44/42 MAPK (ERK1/2) proteins in SCC-9. ANOVA One-way test; symbol ⌐¬: $p < 0.05$; control: CT.

migration and angiogenesis, through decreasing gene expression of mir210, MMP2, MMP9, Col1A1 and higher expression of E-cadherin.

Thus, we provide evidence that both GA and Lep play a role in the neoplastic phenotype and gene expression in OSCC cells. The GA-mediated activities suppress the proliferative and the anti-death roles promoted by Lep. In this scenario, GA acts as a potent antagonist of pro-carcinogenic activities of Lep and may represent an adjuvant substance for therapy of patients with OSCC.

Compliance with ethical standards

Funding

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Brazil. Dr. Guimarães and Dr. De Paula are research fellows of the CNPq. We thank Exame Ltda. Laboratory – Montes Claros/MG – Brazil.

Conflict of interest

All authors declare that no financial relationships exist regarding any of the products involved in this study.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors. This study involves using of commercially available immortalized cells.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2017.11.022.

References

[1] J.M. Friedman, J.L. Halaas, Leptin and the regulation of body weight in mammals, Nature 395 (6704) (1998) 763–770.
[2] J.K. Elmquist, E. Maratos-Flier, C.B. Saper, J.S. Flier, Unraveling the central nervous system pathways underlying responses to leptin, Nat. Neurosci. 1 (6) (1998) 445–450.
[3] S. Samuel-Mendelsohn, M. Inbar, E. Weiss-Messer, L. Niv-Spector, A. Gertler, R.J. Barkey, Leptin signaling and apoptotic effects in human prostate cancer cell lines, Prostate 71 (9) (2011) 929–945.
[4] C. Giordano, D. Vizza, S. Panza, I. Barone, D. Bonofiglio, M. Lanzino, D. Sisci, F. De Amicis, S.A. Fuqua, S. Catalano, et al., Leptin increases HER2 protein levels through a STAT3-mediated up-regulation of Hsp90 in breast cancer cells, Mol. Oncol. 7 (3) (2013) 379–391.
[5] S. Ando, S. Catalano, The multifactorial role of leptin in driving the breast cancer microenvironment, Nat. Rev. Endocrinol. 8 (5) (2012) 263–275.

[6] G.E. Carpagnano, A. Spanevello, C. Curci, F. Salerno, G.P. Palladino, O. Resta, G. Di Gioia, F. Carpagnano, M.P. Foschino Barbaro, IL-2, TNF-alpha, and leptin: local versus systemic concentrations in NSCLC patients, Oncol. Res. 16 (8) (2007) 375–381.
[7] K. Lang, J. Ratke, Leptin and Adiponectin: new players in the field of tumor cell and leukocyte migration, Cell Commun. Signal.: CCS 7 (2009) 27.
[8] Y. Ye, D. Dang, J. Zhang, C.T. Viet, D.K. Lam, J.C. Dolan, J.L. Gibbs, B.L. Schmidt, Nerve growth factor links oral cancer progression, pain, and cachexia, Mol. Cancer Ther. 10 (9) (2011) 1667–1676.
[9] H.P. Gharote, R.N. Mody, Estimation of serum leptin in oral squamous cell carcinoma, J. Oral Pathol. Med. 39 (1) (2010) 69–73.
[10] J. Kaur, R. Jacobs, Salivary and serum leptin levels in patients with squamous cell carcinoma of the buccal mucosa, Clin. Oral Investig. 20 (1) (2016) 39–42.
[11] S.R. Hussain, H. Naqvi, S. Gupta, A.A. Mahdi, P. Kumari, M. Waseem, M.K. Ahmad, A study on oncogenic role of leptin and leptin receptor in oral squamous cell, Tumour Biol.: J. Int. Soc. Oncodev. Biol. Med. 36 (8) (2015) 6515–6523.
[12] L. Taliaferro-Smith, A. Nagalingam, B.B. Knight, E. Oberlick, N.K. Saxena, D. Sharma, Integral role of PTP1B in adiponectin-mediated inhibition of oncogenic actions of leptin in breast carcinogenesis, Neoplasia 15 (1) (2013) 23–38.
[13] D.J. Newman, G.M. Cragg, Natural products as sources of new drugs over the last 25 years, J. Nat. Prod. 70 (3) (2007) 461–477.
[14] M. Inoue, R. Suzuki, T. Koide, N. Sakaguchi, Y. Ogihara, Y. Yabu, Antioxidant, gallic acid, induces apoptosis in HL-60RG cells, Biochem. Biophys. Res. Commun. 204 (2) (1994) 898–904.
[15] B.R. You, S.Z. Kim, S.H. Kim, W.H. Park, Gallic acid-induced lung cancer cell death is accompanied by ROS increase and glutathione depletion, Mol. Cell. Biochem. 357 (1-2) (2011) 295–303.
[16] K. Yoshioka, T. Kataoka, T. Hayashi, M. Hasegawa, Y. Ishi, H. Hibasami, Induction of apoptosis by gallic acid in human stomach cancer KATO III and colon adenocarcinoma COLO 205 cell lines, Oncol. Rep. 7 (6) (2000) 1221–1223.
[17] K. Raina, S. Rajamanickam, G. Deep, M. Singh, R. Agarwal, C. Agarwal, Chemopreventive effects of oral gallic acid feeding on tumor growth and progression in TRAMP mice, Mol. Cancer Ther. 7 (5) (2008) 1258–1267.
[18] C. Locatelli, P.C. Leal, R.A. Yunes, R.J. Nunes, T.B. Creczynski-Pasa, Gallic acid ester derivatives induce apoptosis and cell adhesion inhibition in melanoma cells: the relationship between free radical generation, glutathione depletion and cell death, Chem. Biol. Interact. 181 (2) (2009) 175–184.
[19] A. Faried, D. Kurnia, L.S. Faried, N. Usman, T. Miyazaki, H. Kato, H. Kuwano, Anticancer effects of gallic acid isolated from Indonesian herbal medicine, Phaleria macrocarpa (Scheff.) Boerl, on human cancer cell lines, Int. J. Oncol. 30 (3) (2007) 605–613.
[20] M.K. Kang, N.J. Kang, Y.J. Jang, K.W. Lee, H.J. Lee, Gallic acid induces neuronal cell death through activation of c-Jun N-terminal kinase and downregulation of Bcl-2, Ann. N. Y. Acad. Sci. 1171 (2009) 514–520.
[21] A. Serrano, C. Palacios, G. Roy, C. Cespon, M.L. Villar, M. Nocito, P. Gonzalez-Porque, Derivatives of gallic acid induce apoptosis in tumoral cell lines and inhibit lymphocyte proliferation, Arch. Biochem. Biophys. 350 (1) (1998) 49–54.
[22] S.W. Weng, S.C. Hsu, H.C. Liu, B.C. Ji, J.C. Lien, F.S. Yu, K.C. Liu, K.C. Lai, J.P. Lin, J.G. Chung, Gallic acid induces DNA damage and inhibits DNA repair-associated protein expression in human oral cancer SCC-4 cells, Anticancer Res. 35 (4) (2015) 2077–2084.
[23] C.L. Kuo, K.C. Lai, Y.S. Ma, S.W. Weng, J.P. Lin, J.G. Chung, Gallic acid inhibits migration and invasion of SCC-4 human oral cancer cells through actions of NF-kappaB, Ras and matrix metalloproteinase-2 and -9, Oncol. Rep. 32 (1) (2014) 355–361.
[24] J.J. Irwin, B.K. Shoichet, ZINC–a free database of commercially available compounds for virtual screening, J. Chem. Inf. Model. 45 (1) (2005) 177–182.
[25] F. Zhang, M.B. Basinski, J.M. Beals, S.L. Briggs, L.M. Churgay, D.K. Clawson, R.D. DiMarchi, T.C. Furman, J.E. Hale, H.M. Hsiung, et al., Crystal structure of the obese protein leptin-E100, Nature 387 (6629) (1997) 206–209.
[26] B. Carpenter, G.R. Hemsworth, Z. Wu, M. Maamra, C.J. Strasburger, R.J. Ross, P.J. Artymiuk, Structure of the human obesity receptor leptin-binding domain reveals the mechanism of leptin antagonism by a monoclonal antibody, Structure 20 (3) (2012) 487–497.
[27] H. Li, A.D. Robertson, J.H. Jensen, Very fast empirical prediction and rationalization of protein pKa values, Proteins 61 (4) (2005) 704–721.
[28] T.J. Dolinsky, P. Czodrowski, H. Li, J.E. Nielsen, J.H. Jensen, G. Klebe, N.A. Baker, PDB2PQR: expanding and upgrading automated preparation of biomolecular structures for molecular simulations, Nucleic Acids Res. 35 (2007) W522–525 Web Server issue.
[29] T.J. Dolinsky, J.E. Nielsen, J.A. McCammon, N.A. Baker, PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations, Nucleic Acids Res. 32 (2004) W665–667 Web Server issue.
[30] O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, J. Comput. Chem. 31 (2) (2010) 455–461.
[31] M. Kuhn, D. Szklarczyk, S. Pletscher-Frankild, T.H. Blicher, C. von Mering, L.J. Jensen, P. Bork, STITCH 4: integration of protein-chemical interactions with user data, Nucleic Acids Res. 42 (2014) D401–407 Database issue.
[32] T.A. Guimaraes, L.C. Farias, C.A. Fraga, J.D. Feltenberger, G.A. Melo, R.D. Coletta, S.H. Souza Santos, A.M. de Paula, A.L. Guimaraes, Evaluation of the antineoplastic activity of gallic acid in oral squamous cell carcinoma under hypoxic conditions, Anti-Cancer Drugs 27 (5) (2016) 407–416.
[33] S. Kasibhatla, G.P. Amarante-Mendes, D. Finucane, T. Brunner, E. Bossy-Wetzel, D.R. Green, Acridine orange/ethidium bromide (AO/EB) staining to detect apoptosis, CSH Protoc. 2006 (3) (2006).
[34] S.W. Chan, C.J. Lim, K. Guo, C.P. Ng, I. Lee, W. Hunziker, Q. Zeng, W. Hong, A role for TAZ in migration, invasion, and tumorigenesis of breast cancer cells, Cancer Res. 68 (8) (2008) 2592–2598.
[35] C. Petersen, S. Petersen, L. Milas, F.F. Lang, P.J. Tofilon, Enhancement of intrinsic tumor cell radiosensitivity induced by a selective cyclooxygenase-2 inhibitor, Clin. Cancer Res.: Off. J. Am. Assoc. Cancer Res. 6 (6) (2000) 2513–2520.
[36] T.A. Guimaraes, L.C. Farias, E.S. Santos, C.A. de Carvalho Fraga, L.A. Orsini, L. de Freitas Teles, J.D. Feltenberger, S.F. de Jesus, M.G. de Souza, S.H. Santos, et al., Metformin increases PDH and suppresses HIF-1alpha under hypoxic conditions and induces cell death in oral squamous cell carcinoma, Oncotarget 7 (34) (2016) 55057–55068.
[37] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (6) (2008) 1101–1108.
[38] E.M. Santos, H.O. Santos, S.N. Fonseca, T.A. Guimarães, C.A. de Carvalho Fraga, K.M. Jones, A.M. de Paula, S.H. Santos, A.L. Guimarães, L.C. Farias, Obesity-related genes and oral cancer: a bioinformatics approach and systematic review, J. Appl. Bioinform. Comput. Biol. 5 (1) (2016).
[39] Y.C. Lu, M.L. Lin, H.L. Su, S.S. Chen, ER-dependent Ca + +-mediated cytosolic ROS as an effector for induction of mitochondrial apoptotic and ATM-JNK signal pathways in gallic acid-treated human oral cancer cells, Anticancer Res. 36 (2) (2016) 697–705.
[40] M. Toth, A. Sohail, R. Fridman, Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography, Methods Mol. Biol. 878 (2012) 121–135.
[41] N.A. Sangle, L.J. Layfield, Telangiectatic osteosarcoma, Arch. Pathol. Lab. Med. 136 (5) (2012) 572–576.
[42] G.Z. Shi, Y. Yuan, G.J. Jiang, Z.J. Ge, J. Zhou, D.J. Gong, J. Tao, Y.F. Tan, S.D. Huang, PRAF3 induces apoptosis and inhibits migration and invasion in human esophageal squamous cell carcinoma, BMC Cancer 12 (2012) 97.
[43] M. Ivan, X. Huang, miR-210: fine-tuning the hypoxic response, Adv. Exp. Med. Biol. 772 (2014) 205–227.
[44] C. Garofalo, E. Surmacz, Leptin and cancer, J. Cell. Physiol. 207 (1) (2006) 12–22.
[45] H. Ren, L. Jia, T. Zhao, H. Zhang, J. Chen, S. Yang, J. Liu, M. Yu, J. Hao, Hypoxia inducible factor (HIF)-1alpha directly activates leptin receptor (Ob-R) in pancreatic cancer cells, Cancer Lett. 354 (1) (2014) 172–180.
[46] Z. He, A.Y. Chen, Y. Rojanasakul, G.O. Rankin, Y.C. Chen, Gallic acid, a phenolic compound, exerts anti-angiogenic effects via the PTEN/AKT/HIF-1alpha/VEGF signaling pathway in ovarian cancer cells, Oncol. Rep. 35 (1) (2016) 291–297.
[47] C.L. Hsu, G.C. Yen, Effect of gallic acid on high fat diet-induced dyslipidaemia, hepatosteatosis and oxidative stress in rats, Br. J. Nutr. 98 (4) (2007) 727–735.
[48] Y.K. Park, J. Lee, V.S. Hong, J.S. Choi, T.Y. Lee, B.C. Jang, Identification of KMU-3, a novel derivative of gallic acid, as an inhibitor of adipogenesis, PLoS One 9 (10) (2014) e109344.
[49] C.Z. Liang, X. Zhang, H. Li, Y.Q. Tao, L.J. Tao, Z.R. Yang, X.P. Zhou, Z.L. Shi, H.M. Tao, Gallic acid induces the apoptosis of human osteosarcoma cells in vitro and in vivo via the regulation of mitogen-activated protein kinase pathways, Cancer Biother. Radiopharm. 27 (10) (2012) 701–710.
[50] A. Bruno, S. Conus, I. Schmid, H.U. Simon, Apoptotic pathways are inhibited by leptin receptor activation in neutrophils, J. Immunol. 174 (12) (2005) 8090–8096.
[51] A. Bruno, E. Pace, P. Chanez, D. Gras, I. Vachier, G. Chiappara, M. La Guardia, S. Gerbino, M. Profita, M. Gjomarkaj, Leptin and leptin receptor expression in asthma, J. Allergy Clin. Immunol. 124 (2) (2009) 230–237 237 e231-234.
[52] Z. Xia, M. Dickens, J. Raingeaud, R.J. Davis, M.E. Greenberg, Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis, Science 270 (5240) (1995) 1326–1331.
[53] Y.H. Han, H.J. Moon, B.R. You, W.H. Park, The attenuation of MG132, a proteasome inhibitor, induced A549 lung cancer cell death by p38 inhibitor in ROS-independent manner, Oncol. Res. 18 (7) (2010) 315–322.