



**UNIVERSITÀ  
DI FOGGIA**

PhD in

***“Experimental and Regenerative Medicine”***

*XXIX cycle*

*“In vitro study on anticancer properties of Curcumin and  
Genistein in Squamous Cell Carcinoma of Tongue”*

***Tutor***

*Prof. Nazzeno Capitanio*

***PhD Student***

*Dr. Fatima Ardito*

Lasciare un sogno in un cassetto è sempre un peccato.  
Per questo ho cercato di realizzarlo per te.  
Anna, questo titolo è anche tuo.

*“Mi ricordo ancora quell’orizzonte ampio e senza punti  
di riferimento, in cui solo il sole faceva da limite  
all’infinito.*

*In quel momento capii che ciò che conta  
di fronte a tanta libertà del mare non è avere una nave,  
ma un posto dove andare, un porto,  
un sogno che valga tutta quell’acqua da attraversare”*

A. D’Avenia

## Abstract

**Background:** The squamous cell carcinoma of the tongue (TSCC) is the most frequent cancer of oral cavity and is extremely aggressive and characterized by poor prognosis. It is a complex disease to be treated and therapies in use have led to mediocre results and many side effects. Some facts suggest that natural essences can support traditional cancer therapy, carrying out a synergistic function with chemotherapy. Curcumin and genistein have anticancer properties in many tumors but their action on the tongue carcinoma is not entirely clear and many other investigations are necessary.

**Methods:** In this study, we evaluated the effects of curcumin on TSCC cells using different concentrations (1, 5, 10, 20 and 50  $\mu\text{M}$ ) and 3 different treatment times (24, 48 and 72 hours). Furthermore, 20, 50 and 100  $\mu\text{M}$  of genistein are used at the same time points. The inhibition of adhesion, proliferation, viability, migration and tumorigenesis were studied. Calcium Colorimetric Assay Kit, ATP-measurement methods, LDH Cytotoxicity Assay, quantitative determination of intracellular ROS level, analysis of  $\Delta\Psi\text{m}$  by fluorescence assay were used to determinate biochemical changes responsible for cellular injury.

**Results:** We monitored in real time the growth kinetics for 72 hours after treatment and all CI values analyzed. The adhesion interval was 0-10 hours.

We found a halving of cell adhesion after treatment with 5  $\mu\text{M}$  of curcumin, a ~ 25% reduction after treatment with 1  $\mu\text{M}$  of curcumin. Furthermore, adhesion of TSCC cells treated with 50  $\mu\text{M}$  of curcumin was reduced by 75% compared to control.

Even, the down-regulation of integrin expression supports the theory goes that curcumin inhibits adhesion of the tongue cancer cells.

The proliferation is reduced by 50% after treatment with 5-10 $\mu\text{M}$  curcumin at all time points considered and IC50 value is ~ 10  $\mu\text{M}$ . Curcumin reduces vitality, migration and progression of TSCC cells and it promotes apoptosis and inhibits tumorigenesis. In fact, PAR 4 is increased after curcumin treatments while Survivin and Oct4 decreased.

While, cell adhesion of TSCC cells is inhibited especially between 20 and 50  $\mu\text{M}$  of genistein treatment. Proliferation is reduced by 50% for treatments with 20  $\mu\text{M}$  at 24 hours, with 20 or 50  $\mu\text{M}$  at 48 and 50  $\mu\text{M}$  at 72 hours ( $p < 0.0001$ ). The viability and migration appeared to be reduced with high significance ( $p < 0.001$ ). Genistein down-regulated vitronectin, oct4 and survivin. It was showed an increase of intracellular  $\text{Ca}^{2+}$  and LDH release while there was a reduction of ATP levels after treatments of

curcumin and genistein. Also ROS and  $\Delta\Psi_m$  were reduced after treatments. These biochemical changes have confirmed the cell damage and consequently apoptosis after treatments.

**Conclusions:** These results suggest the possible use of curcumin and genistein as anticancer agents in TSCC. In vivo studies are needed to confirm these data and to be able to manufacture a suitable delivery system that is acting directly in the tumor site.

# CONTENTS

<b>1. INTRODUCTION.....</b>	<b>1</b>
1.1 - Tongue cancer: generality and treatments.....	1
1.2 - Nutraceuticals: new era of medicine and health.....	3
1.3 - Biological, therapeutic and anticancer properties of curcumin.....	5
1.3.1 Chemical's nature and generality of curcumin.....	5
1.3.2 Anticancer activity .....	9
1.3.3 Curcumin as alternative treatment option for Oral cancer .....	11
1.4 - Genistein: a multimechanistic anticancer agent from soya .....	14
1.4.1 What it is, uses, how it works.....	14
1.4.2 Anticancer therapeutic potential of soy isoflavones genistein .....	16
1.4.3 Genistein and Oral cancer: current status .....	18
<b>2. AIM OF THE STUDY .....</b>	<b>21</b>
<b>3. MATERIALS AND METHODS .....</b>	<b>23</b>
3.1 – Cell culture and treatment .....	23
3.2 – xCELLigence system.....	23
3.2.1 Cell adhesion.....	25
3.2.2 Cell proliferation and estimate IC50 values .....	25
3.3 – MTT assay .....	26
3.4 – Trypan blue exclusion test of cell viability .....	26
3.5 – Fluorescence cell viability.....	26
3.6 – Scratch assay .....	26
3.7 – Western blotting analysis .....	27
3.8 – Measurement of intracellular calcium.....	27
3.9 – Measurement of total cellular ATP .....	27
3.10 – Measurement of LDH release.....	28

3.11 – Analysis of $\Delta\Psi_m$ by fluorescence assay .....	28
3.12 – Quantitative determination of intracellular ROS level .....	29
3.13 – Statistical analysis .....	29
<b>4. RESULTS .....</b>	<b>30</b>
4.1 – Evaluation of cell adhesion .....	30
4.1.1 Effects of curcumin on cell adhesion .....	30
4.1.2 Effects of genistein on cell adhesion.....	33
4.2 – Cell proliferation and half maximal inhibitory concentration (IC <sub>50</sub> ).....	35
4.2.1 Effects of curcumin on cell proliferation and IC <sub>50</sub> determination.....	35
4.2.2 Effects of genistein on cell proliferation and IC <sub>50</sub> determination .....	37
4.3 – Inhibition on cell viability .....	40
4.3.1 Effects of curcumin on cell viability .....	40
4.3.2 Effects of genistein on cell viability.....	41
4.4 – Determination of cell migration rate .....	42
4.4.1 Curcumin inhibits cell migration.....	42
4.4.2 Genistein inhibits cell migration .....	43
4.5 – Effects on apoptosis and tumorigenesis.....	44
4.5.1 Curcumin induces apoptosis and reduces tumorigenesis .....	44
4.5.2 Genistein induces apoptosis and reduces tumorigenesis .....	45
4.6 – Biochemical changes in cellular injury .....	47
4.6.1 Increases in intracellular calcium ion concentration after treatments with curcumin and genistein.....	47
4.6.2 Curcumin and genistein induces ATP release from TSCC cells.....	47
4.6.3 The LDH is released after curcumin and genistein treatments.....	48
4.6.4 Effects of curcumin and genistein on $\Delta\Psi_m$ assessed by the fluorescent probe TMRE.....	49

4.6.5 Determination of ROS levels in TSCC cell lines after curcumin and genistein treatments .....	51
<b>5. DISCUSSION .....</b>	<b>53</b>
<b>6. CONCLUSION.....</b>	<b>63</b>
<b>7. REFERENCES.....</b>	<b>64</b>

# 1. INTRODUCTION

## 1.1- Tongue cancer: generality and treatments

Oral squamous cell carcinoma (OSCC) is the eighth most common cancer in males worldwide whereas in females it is relatively rare and not ranked among the top ten cancers [1, 2].

While the incidence and mortality rates vary widely among different populations, OSCC is generally found to be more common in developing countries [1, 3]. The higher incidence and low survival rates have been reported from India, Taiwan, Pakistan and Hungary [4-6].

The recent data available in GLOBOCAN 2012 report shows an incidence of 529,500 cases, 292,300 deaths in 2012 accounting for about 3.8% of all cancer cases and 3.6% of cancer death [7]. Moreover, it is predicted to rise by 62% to 856,000 cases by 2035 because of changes in demographics [8]. These figures give a clear indication that oral cancer remains a huge global burden and the rising rates are a cause of concern.

OSCC is a major subtype of head and neck squamous cell carcinoma (HNSCC) which displays many pathological differences to cancers found at other sites in the head and neck region. OSCC has been studied separately from other subtypes of HNSCC and this is because of the risk factors which are specific to oral cavity [9, 10].

OSCC is a malignancy of the oral squamous epithelium and includes tumors found in tongue, lip, gingival, palate, floor of mouth and buccal mucosa [11].

One of the most common site for intraoral carcinoma is the tongue. These tumors most frequently arise from the lateral margins of the oral tongue. The majority of tongue cancers are squamous cell carcinoma (TSCC) well differentiated, while poorly differentiated varieties are rare [12].

These arise from the lining that covers the muscles of the tongue. Studies shows that tongue cancer usually occurs after the age of 40, with men affected more than women (10%) [13].

The condition may appear as a lump, white or red spot or ulcer. Pain is a relatively late occurrence as a rule. Though exact etiology of tongue cancer is still unknown, several risk factors have been identified.

Chewing tobacco or betel nuts is very common in parts of Asia and is known to be an important cause of oral cancer including tongue [14].

The tongue is divided into two parts for the purpose of diagnosis and treatment of tongue tumors. The first is the oral or mobile tongue (front two-thirds of tongue), cancers that develop in this part of the tongue come under a group of cancers called mouth cancer. The other is the base of the tongue (back third of tongue), cancers that develop in this part are called oropharyngeal cancers. Tumors of each of these areas present slightly differently and are treated differently [14].

The most common protocol of treatment of TSCC are surgical resection, resection of floor of mouth musculature, removal of bone and associated muscle attachments and the sacrifice of both sensory and motor cranial nerves along with or without neck dissection [15].

Radiotherapy is an alternative to surgery in small and intermediate size tumors of the anterior tongue. Radiation therapy is usually started 4–6 weeks after the surgery.

Medical oncologists administer chemotherapy when the oral tongue cancer has spread to lymph nodes or other organs in the body, usually in stage II cancers after surgery and in stage III and IV cancers after radiotherapy. Chemotherapy is commonly administered intravenously and/or intramuscularly or orally. These cytotoxic drugs, such as cisplatin or carboplatin, destroy cancer cells [16].

However, there are many different types of biological therapies like monoclonal antibodies (cetuximab and zalutumumab) and cancer growth blockers. Both monoclonal antibodies block the epidermal growth factor receptors (EGFR) on cancer cells [17].

Problems usually may be related to surgical treatment of the primary tumor or radiation treatment or chemotherapy.

Tongue resection causes significant mobility problems and dramatic changes in articulation, as well as aesthetics, swallowing, nutritional status and psychosocial function.

Radiation treatment causes acute mucosal changes, erythema and edema to frank necrosis and breakdown (mucositis), candidiasis, loss of taste or altered taste, dysphagia, xerostomia [18, 19].

Currently, there are no treatments that are highly effective. In addition, they have many side effects.

## **1.2 - Nutraceuticals: new era of medicine and health**

Nutraceutical is a term derived from *nutrition* and *pharmaceutics*. It was coined in 1989 by Dr. Stephen DeFelice, founder and chairman of the Foundation for Innovation in Medicine, an American organization located in Cranford, New Jersey. He defined nutraceuticals as “*as foods, food ingredients or dietary supplements that demonstrate specific health or medical benefits including the prevention and treatment of disease beyond basic nutritional functions*”[20].

The philosophy behind nutraceuticals is to focus on prevention, according to Hippocrates, known as the father of medicine, who said “Let food be thy medicine and medicine be thy food”. In a few last years, nutraceutical medicine was considered as new branch of Complementary and Alternative Medicine.

The plant products have been defined as food, food supplement, functional food and nutraceuticals, depending upon its isolation step.

Pure extracted phytomolecule is named as nutraceuticals, whereas semi-purified plant product, not taken as regular food, is named as functional food [21].

Food supplements are those products which can be taken regularly as food to maintain the general health.

In the US, the term *nutraceutical* products are regulated as drugs, food ingredients and dietary supplements. The term is not defined the same in different countries, but is usually defined as a product isolated from foods that is generally sold

in medicinal forms not usually associated with food. A nutraceutical product may be defined as a substance, which has physiological benefit or provides protection against chronic diseases [20]. Nutraceuticals may be used to improve health, delay the aging process, prevent chronic diseases, increase life expectancy, or support the structure or function of the body [22].

The use of nutraceuticals, as an attempt to accomplish desirable therapeutic outcomes with reduced side effects, as compared with other therapeutic agents met with great monetary success [23].

The nutraceuticals have shown different mechanism of actions at different cellular levels. Most of them have emerged as a versatile source of antioxidants affecting the signaling pathway related to redox mediated transcription factors.

Initial in vitro studies found that nutraceuticals may reduce the tumorigenic actions of carcinogens, blocking their mutagenic activity and suppressing cell proliferation [24].

In addition, nutraceuticals may also be helpful in reducing toxicity, associated with chemotherapy and radiation therapy, and may lead to better life conditions by reducing cancer cachexia [25].

## 1.3 – Biological, therapeutic and anticancer properties of curcumin

### 1.3.1 Chemical's nature and generality of curcumin

Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-dione] is one of nutraceuticals most studied in recent decades for its many properties.

It is the main biologically active curcuminoid of the *Curcuma longa*, an herbaceous perennial plant belonging to the ginger family (*Zingiberaceae*) [26].

*Curcuma longa*, commonly known as turmeric, is native to South Asia, India and Indonesia and mainly grown in South India [27, 28].

The root and rhizome (underground stem) of the *Curcuma longa* is crushed and powdered into ground turmeric. Ground Turmeric is used worldwide as a seasoning and as the main ingredient in curry.

Curry contains about 2% of curcumin which was first identified in 1910 by *Milobedzka et al* [29].

Curcumin is responsible for the yellow color of the spice as well as the majority of turmeric's therapeutic effects [28, 30]. The other two curcuminoids obtained from *Curcuma longa* are demethoxycurcumin (DMC) and bis-demethoxy curcumin (BDMC; *Fig.1*).

In addition, turmeric also contains volatile oils (zingiberone, atlantone, tumerone, etc.), sugars, resins and proteins.

The curcuminoids consist of two methoxylated phenols connected through two  $\alpha$ ,  $\beta$  unsaturated carbonyl groups. Curcumin is rich in terpene derivatives and contains mainly monocyclic sesquiterpenes and oxygenated derivatives (turmerone, zingibrene) [31].

Flavonoids are also abundant. The rhizome contains 3-5% of curcuminoids and 2-7% of essential oil [32, 33].

Curcumin dissolves difficultly in water whereas it is quite soluble in organic solvents such as dimethyl sulfoxide, ethanol, methanol or acetone and has a melting

point of 183°C. Spectrophotometrically, curcumin presents a maximum absorption in methanol at 430 nm, it absorbs maximally between 415 and 420 nm in acetone, and a 1% solution of curcumin has 1650 absorbance units [34].

However, other than curcumin, turmeric contains no known agents with anti-inflammatory and anti-proliferative activity [35].

Following its extraction and purification, curcumin is used for its attributed medicinal properties as a natural treatment for numerous diseases. In Ayurvedic medicine, curcumin has been used for centuries for its medicinal properties and has been administered through various routes, including topically, orally and by inhalation [36]. It is well known that the curcumin exerts certain antioxidant, analgesic, anti-inflammatory and antimalarial properties [36-41].

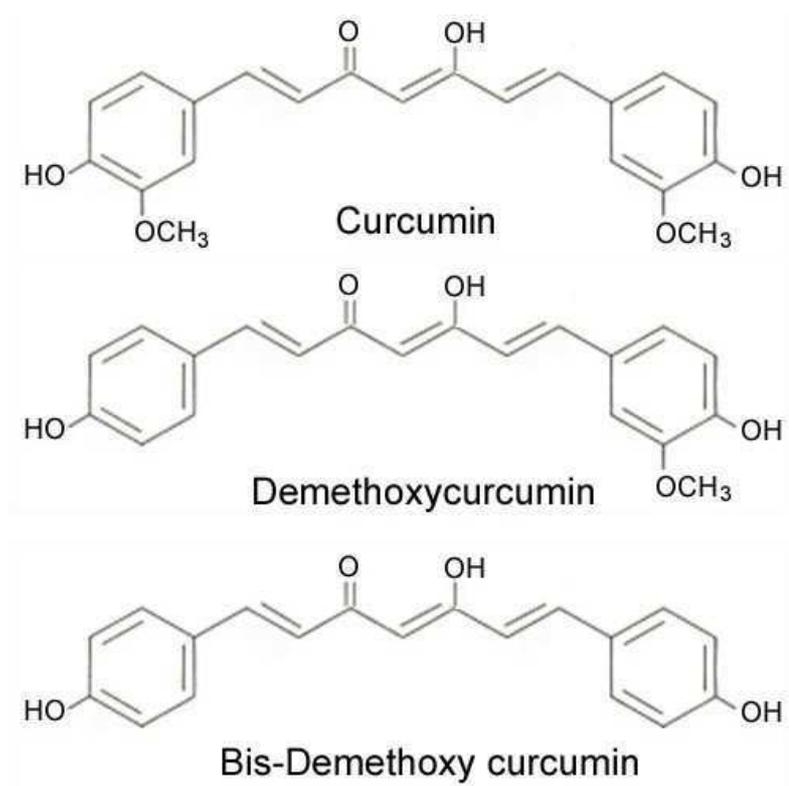


Figure 1. Chemical structures of curcumin, demethoxycurcumin, and bis- demetoxy curcumin

Molecular studies indicated that Curcumin blocks the activation of factors or enzymes present in human cells able to trigger the inflammatory response.

For instance, *Surt et al* revealed that curcumin is able to inhibit the activity and induced expression of cyclooxygenase-2 (COX-2) in various cell lines and animal models [42, 43].

Topical application of curcumin inhibits the lipopolysaccharide [44]-mediated induction of COX-2 expression. This effect, rather than the catalytic inhibition of COX, may contribute towards the reduced formation of prostaglandin E2 (PGE2), while in macrophages not stimulated by LPS, curcumin increases the levels of COX-2 [45].

However, curcumoids exert a significant inhibitory effect on the peroxidase activity of COX-1, but not that of COX-2. In addition, curcumin and the curcumoids markedly inhibit the activity of 5-lipoxygenase (5-LOX), as curcumin interferes with the metabolism of AA by blocking cytosolic phospholipase A2 phosphorylation, and thus reducing the expression of COX-2 and inhibiting the catalytic activities of 5-LOX. These activities may explain the anti-inflammatory action of curcumin and the curcumoids in general [45].

Curcumin has been shown to suppress the activation of NF-kB, an inducible transcription factor that regulates the expression of genes involved in inflammation, cellular proliferation and cell survival [46, 47].

Curcumin inhibitory effect on the NF-kB pathway is central to providing the compound with its anti-inflammatory properties. Curcumin blocks the Ikk-mediated phosphorylation and degradation of Ikb $\alpha$ , thus NF-kB remains bound to Ikb $\alpha$  in the cytoplasm and is not able to enter the nucleus to activate transcription [48].

However, the antioxidant activities of curcuminoids are a result of their chemical composition. Curcumin inhibits lipid peroxidation using linoleate, a polyunsaturated fatty acid that is able to oxidize and form fatty acid radicals. It has been shown that curcumin downregulates the iNOS activity in macrophages, thus reducing the amount of reactive oxygen species (ROS) generated in response to oxidative stress [49].

Furthermore, curcumin is considered to be pharmacologically safe and is classed as safe for human consumption by the US Food and Drug Administration [38]. It is widely consumed as a condiment without any known side effects.

The oral bioavailability of curcumin is reduced because of its instability in the intestinal pH, the low absorption, low water solubility, a fast metabolism and rapid elimination [50].

Thus, continuous research on curcumin found some possible ways to overcome these problems. To increase the bioavailability, longer circulation, better permeability, and resistance to metabolic processes of curcumin several formulations have been prepared which include nanoparticles, liposomes, micelles, and phospholipid complexes [51].

Examples are the bioenhancer, natural molecules used in combination with a substance to facilitate passage through cellular membranes. In this manner, they increase the absorption of curcumin by the cells. Among the bioenhancer studied to curcumin there are piperine, quercetin or eugenol. The main bioenhancer of curcumin is piperine, an alkaloid extracted from black pepper.

In addition, the absorption of curcumin is greater when taken with meals. The half-life of curcumin in circulation varies between two and eight hours. Curcumin remains little in the circle because it is conjugated with glucuronic acid or a sulfate group curcumin glucuronide and sulfate or reduced to esaidrocurcumina in the liver. The biological activity of these metabolites of curcumin is less than that exerted by curcumin unchanged [51].

Human clinical studies with high doses (2-12 grams) of curcumin showed few side effects such as nausea and diarrhea [52].

Recently, it was found that curcumin alters the metabolism in relation to the iron through chelation and suppressing hepcidin causing a potential iron deficiency [53].

People with gallstones should not take curcumin as it may cause the onset of biliary colic or other complications [54].

### 1.3.2 Anticancer activity

Curcumin performs several activities as potential anticancer remedies, both in a chemopreventive and directly therapeutic way. Although the results have been obtained in animal models, the curcumin is active in many other different models and the dosages are comparable to those used by humans. Both in vitro and in vivo studies have showed that curcumin prevents carcinogenesis in two main levels: angiogenesis and tumor growth [55].

Turmeric and curcuminoids act on the process of tumor angiogenesis through multiple and interdependent processes:

- Action at the level of transcription factors NF-kB, AP-1, associated with inflammatory processes, and Egr-1 (this action has attenuated the expression of IL-8 cell lines and prevented the induction of VEGF synthesis);
- Inhibition of angiogenesis mediated by nitric oxide and iNOS
- Inhibition of COX-2 and LOX
- Action at the level of angiogenic factors: VEGF, the main factor for migration, sprouting, survival, and proliferation during angiogenesis, and basic fibroblast growth factor (bFG)
- Action at the level of stability and coherence of the extracellular matrix (ECM), with downregulation of MMP2 (matrix metalloproteinase-2) and MMP9, and upregulation of TIMP1 (tissue inhibitor of metalloproteinase-1). It also interferes with the release of angiogenic factors stored in the ECM [55, 56].

Curcumin induces cell death in several animal and human cell lines, such as leukemia, melanoma, carcinomas of the breast, lung, colon, kidney, ovarian and hepatocellular and it appears to act by mechanisms both caspase-dependent and independent (mitochondrial) [55-57]; these are both related to the presence of p53 and its absence. Some data have shown a biphasic action of curcumin which acts on the proteasome, with an activation at lower doses and with inhibition at higher doses.

Since inhibition of proteasome leads to apoptosis, and its stimulation leads to cell survival, it is possible that curcumin causes apoptosis or survival depending on the dosage used.

In addition, turmeric at different doses may also affect the type of cell death: low doses lead to oxidative stress and apoptosis, while higher doses lead to reduced production of ROS, reduction of ATP and death by necrosis [58].

Curcumin seems to be able to cause cell death also of various cell lines resistant to apoptosis, probably by activating cell death mechanisms different from apoptosis, such as mitotic catastrophe that is characterized by aberrant mitosis and formation of multinucleated and giant cells. The mitotic catastrophe caused by curcumin is linked to the reduction of gene expression of various apoptotic inhibitor proteins [59], in particular of survival [60].

Furthermore, in a study of a few years ago, the authors have demonstrated that the curcumin administration was able to significantly reduce the levels of the cell cycle regulators CDK4 and cyclinD1 as the expression of p53, which is an upstream regulator of the CDK4-cyclinD1 complex [61].

Recently *Villianou et al* have discussed on the ability of curcumin to cause apoptosis in tumor cells by inducing severe ER stress (that has an important role in the apoptosis process) and on recent data have suggested that curcumin may act by suppressing the Sp-1 activation that when inhibited could prevent cancer formation, migration, and invasion [62].

Consistent with the *in vitro* data, *in vivo* administration of curcumin inhibits growth of human breast cancer cells through demethylation of DLC1 promoter [63].

*Liu et al* demonstrated that in MDA-MB-361 cells, curcumin down-regulates the expression of Sp1 to inhibit the expression of DNA methyltransferase 1, thus subsequently reducing hypermethylation of DLC1 promoter to induce DLC1 expression [63].

Curcumin is a potent suppressor of cell viability and invasion. Curcumin effectively inhibited the proliferation of melanoma cells *in vitro* and *in vivo* [64].

In fact, curcumin is able to suppress cell invasion, arresting the cancer cells at G2/M phase of the cell cycle, and inducing autophagy because curcumin suppressed the activation of AKT, mTOR and P70S6K proteins [64].

Curcumin seems to be a good adjuvant drug also for therapy of prostate cancer. In a recent study, the authors demonstrated that curcumin induces apoptosis in prostate cancer cell up-regulating Bax and down-regulating Bcl-2 [65].

The anti-cancer properties of curcumin have been very studied in lung cancer. Recently, *Jiao et al* showed that curcumin inhibits lung cancer metastasis via constructing a miRNA-transcription factor (TF)-target gene network [66].

Furthermore, *Wang et al* demonstrated that curcumin inhibits the migration and metastasis of pancreatic cancer cells by reducing the mesenchymal characteristics of cancer-associated fibroblasts (CAFs), which reverses the epithelial-to-mesenchymal transition [67] phenotypes of pancreatic cancer cells [68].

### **1.3.3 Curcumin as alternative treatment option for Oral cancer**

The activity of curcumin has been investigated in a number of HNSCC cell lines, including CAL27, CCL23, UM-SCC1 and UMSCC14A [69]. The growth suppression effect was represented primarily by the effect of curcumin on the NF- $\kappa$ B signaling pathway. Curcumin caused a reduction in the expression of NF- $\kappa$ B and, in addition, inhibited its nuclear localization.

*Chakravarti et al* indicated that curcumin is able to suppress the growth of immortalized oral mucosal epithelial cells and OSCC cells, while exerting minimal effects on normal oral epithelial cells [70].

Additionally, in a study using a mouse model of SCC-1 tumors, curcumin showed to decrease COX-2 expression and inhibit EGFR phosphorylation[71].

*Rinaldi et al* studied how curcumin modulates expression and function of carcinogen-metabolizing enzymes in OSCC [72]. Results of their metabolism studies showed that curcumin significantly inhibited CYP1A1-mediated benzo(a)pyrene diol

bioactivation in OSCC and because CYP1A1 is one of the primary carcinogen-activating enzymes in oral mucosa, the use of curcumin as an oral cavity chemopreventive agent could have significant clinical impact via its ability to inhibit carcinogen bioactivation.

Furthermore, an *in vivo* study showed that curcumin (0.1, 1.0, 10.0  $\mu\text{M}$ ) induced significant dose-dependent inhibition in both cell growth as well as cell proliferation in an OSCC cell line, SCC25 [73].

Another paper showed the effect of curcumin and irradiation in PE/CA-PJ15 oral squamous cell carcinoma [74]. Curcumin was administered at doses of 3, 3.75, 4.50 and 5.25  $\mu\text{M}$  in PE/CA-PJ15 cultures irradiated with different doses (1, 2.5 and 5 Gy), followed by evaluation of the effects upon cell viability after 24, 48 and 72 h, based on the MTT colorimetric test. The application of curcumin to the PECA/PJ15 tumor cells during 24, 48 and 72 h of incubation without irradiation exerted an inhibitor effect upon cell viability. The curcumin concentration at which the inhibition of cell viability proved maximum was 5.25  $\mu\text{M}$ , with statistically significant differences for 24 h ( $p = 0.002$ ), 48 h ( $p < 0.001$ ) and 72 h of incubation ( $p < 0.001$ ). In contrast, the combination of curcumin and irradiation exerted a synergic effect-the greatest effects in relation to cell viability being recorded with a curcumin concentration of 3.75  $\mu\text{M}$  and 5 Gy of irradiation, in the studied cell line.

Furthermore, curcumin shows anticancer activity against OSCC via both autophagy and apoptosis [75].

*Xiao et al* demonstrated that curcumin inhibited OSCC cells (SCC-9 cells) proliferation through up-regulating miR-9 expression, and suppressing Wnt/ $\beta$ -catenin signaling by increasing the expression levels of the GSK-3 $\beta$ , phosphorylated GSK-3 $\beta$  and  $\beta$ -catenin, and decreasing the cyclin D1 level [76]. Additionally, the up-regulation of miR-9 by curcumin in SCC-9 cells was significantly inhibited by delivering anti-miR-9 but not control oligonucleotides. Downregulation of miR-9 by anti-miR-9 not only attenuated the growth-suppressive effects of curcumin on SCC-9 cells, but also re-activated Wnt/ $\beta$ -catenin signaling that was inhibited by curcumin [76].

Curcumin is known to inhibit growth, invasion and metastasis by downregulating EGFR expression in some cancer cells. *Zhen et al* revealed that curcumin reduced SCC-25 cells proliferation and invasion through inhibiting the phosphorylation of EGFR and EGFR downstream signaling molecules Akt, ERK1/2 and STAT3 [77].

*Lee et al* demonstrated that curcumin treatment not only decreased the expression of MMP-2 and MMP-9 to inhibit invasiveness in oral cancer but also modulated the expression of EMT markers, such as Snail, Twist, and E-cadherin, and induced p53 expression that is crucial to EMT repression [78].

Numerous studies have demonstrated that, *in vivo*, curcumin exerts growth suppressive effects, using nude mouse xenograft models [79, 80].

Curcumin may also have a potential application as an enhancer of radiation therapy. *Rao et al* compared the effects of curcumin and single-dose radiation alone and in combination in the HNSCC cell lines SCC-1, SCC-9, A431 and KB [81]. The results demonstrated that curcumin inhibited HNSCC cell growth and augmented the effect of radiation *in vitro* and *in vivo*. The underlying mechanism may have involved the inhibition of COX-2 expression and EGFR phosphorylation.

In a recent study *in vitro*, the authors confirmed that curcumin used in combination with AG490, a JAK-2 inhibitor, reduced the expression of JAK-2/STAT-3 in laryngeal squamous cell carcinoma. In particular, the expression of JAK-2, p-STAT3, MMP-2 and VEGF at the protein levels were decreased [82].

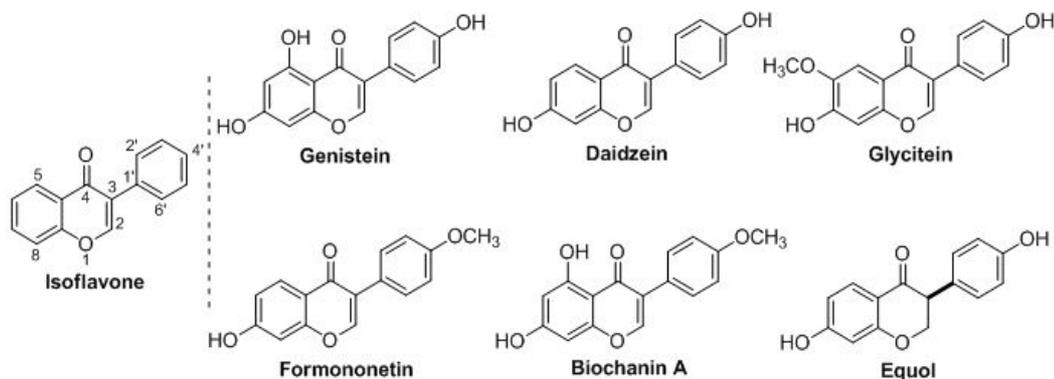
Intracellular copper levels have been reported to correlate with tumor pathogenesis and affect the sensitivity of cancer cells to cytotoxic chemotherapy. *Lee et al* hypothesized that intracellular copper levels may affect the sensitivity of oral cancer cells to curcumin [83]. In the end supplemental copper also inhibited migration of curcumin-treated cells with enhanced level of E-cadherin and decreased vimentin, indications of suppressed epithelial-mesenchymal transition. Early apoptosis was observed in combined treatment but not in treatment with curcumin or copper alone. They suggested a new strategy for cancer therapy [83].

## 1.4- Genistein: a multimechanistic anticancer agent from soya

### 1.4.1 What it is, uses, how it works

The genistein [5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one] has been extracted 1899 by dyer's broom, *Genista tinctoria* and it has been chemically synthesized for the first time in 1928 and, subsequently, with other methods [84-87].

The genistein is a component of isoflavones family like daidzein, glycitein, formononetin, biochanin A and equol (*Fig. 2*). The first three can be found in high quantity in soy and its derivatives such as tofu, soy milk and soy flour. Even legumes like "lupini", fava beans, chickpeas contain mostly genistein although in lower quantity than soy [88]. Whilst formononetin and biochanin A are present in red shamrock and can be completely demethylated by the body in genistein and daidzein.



**Figure 2. Chemical structures of isoflavones genistein, daidzein, glycitein, formononectin, biochanin A and equol**

The genistein has a bitter taste and is feebly soluble in water which strongly limits its bioavailability, while it has a high solubility in polar solvent like dimethyl sulfoxide (DMSO), acetone [89] and ethanol [90-92].

The absorption by intestine is characterized by the split of a sugar molecule from isoflavone glycoside genistein, so that it is obtained aglycone isoflavone genistein, a biologically active form [93].

However, the intestine hydrolysis by  $\beta$ -glycosidase enzymes is influenced by individual susceptibility and by individual gastroenteric apparatus; it may happen that part of genistein contained in food is not converted in biologically active form, therefore it is not absorbed. For this reason, very often it is better to bypass the intestine digestion giving aglycone genistein as pure active principle easing in this way its bond to estrogen receptors [94, 95].

As matter of fact, the genistein show an affinity of 87% for the ER $\beta$  and 4% for the ER $\alpha$  [96].

Furthermore, genistein it is part of phytoestrogens family because it has an estrogen-like structure, mostly similar to 17  $\beta$ -estradiol, which gives many of the typical properties of estrogen hormones [96].

Due to its chemical nature genistein can bond even to other molecular targets like tyrosine kinase protein (PTK), DNA topoisomerase and telomerase behaving as inhibitor [97].

*Akiyama and al* demonstrate the inhibitor activity of genestein on PTK; particularly they found an inhibition of EGFR kinase with IC<sub>50</sub> 0.7  $\mu$ g/ml [98].

Genistein has many properties. Different studies showed that genistein helps the rise of vitamin D receptors which has a protective action on the bones, decreasing the bone loss caused by estrogens shortage and it enhances significantly the bone mineral density[99-101].

Genistein can prevent in the cardiovascular system the risk of cardio circulation diseases, due to the antiplatelet effect and to its capacity to decrease the level of cholesterol in the blood, reducing the level of LDL cholesterol and increasing the level of HDL cholesterol [102, 103].

Several advantages have been found taking genistein in active form in order to relieve the menopause symptoms such as heat flushes and night perspiration [104] ,

it decreases as well the aging process of the skin due to its antioxidant property [105, 106].

A large body of evidence suggests that genistein possesses many physiological and pharmacological properties that make this molecule a potential agent for the prevention and treatment of a number of chronic diseases. Growing evidence suggests that genistein could act as a vasodilating, anti-thrombotic, and anti-atherosclerotic agent, exerting these effects through different mechanisms of action [107].

In the last few years, many studies demonstrated good effects of genistein on Alzheimer's disease (AD). AD is a devastating brain disorder characterized by an increased level of amyloid-beta ( $A\beta$ ) peptide deposition and neuronal cell death leading to an impairment of learning and thinking skills.

Genistein has been mainly focused because of its potential on improvement of  $A\beta$ -induced impairment and its antioxidant capacity to scavenge the free radicals produced in Alzheimer's disease [108]. It can also directly interact with the targeted signaling proteins and stabilize their activity to prevent AD. Therefore, genistein may prevent hypoxia/ischemic-induced neuronal apoptosis that is mediated by alterations in GluR2 expression and voltage-activated potassium currents [109].

The data collected in several studies do not show any relevant collateral effects of genistein. Only in some specific study on murine models using high dosage it has been found a low toxicity in ovary and fertility but there are not sufficient studies to demonstrate the negative effects on reproduction and foetal developing in humans [94, 110].

#### **1.4.2 Anticancer therapeutic potential of soy isoflavones genistein**

In Asian countries like China and Japan, several epidemiological studies have shown a lower incidence of breast cancer and prostate cancer than the rest of the peoples of the world [111, 112] probably because these people routinely use foods containing soy and therefore genistein.

Many studies have confirmed the potential of genistein as antitumor substance showing that this molecule has pleiotropic effects [113].

*Russo et al* showed that genistein has a dual function. At low concentrations has chemopreventive and radiopreventive properties, while treatments with concentrations greater than 10  $\mu\text{M}$  lead inhibition of the PTK, and then a secure antitumor action [96, 114].

*Song et al* showed that treatment with genistein can significantly reduce liver damage consequent to the Co-gamma radiation in cancer patients [115].

A recent study shows that the treatment with genistein concentrations below 1,5  $\mu\text{M}$  may has radioprotective action preventing apoptosis, DNA damage, chromosomal aberrations, while for treatments greater than 20  $\mu\text{M}$  has been shown that genistein it can be also considered as a good adjuvant for antitumor therapy [116].

In tumor pathologies, the main actions of genistein are to reduce cell proliferation and induce apoptosis. In breast cancer the ERs showed an important role in tumor progression and in vitro studies showed that treatment with genistein induce a reduction of the expression of HER2, EGFR, Abl, Src and inhibit of NF-kB signaling pathway [117]. However, the action of genistein is dependent on ER $\alpha$ /ER $\beta$  Ratio [118].

The epithelia-mesenchymal transition [67] is an important process of migration and tumor progression. *Kim et al* showed that genistein is able to reduce the migration of ovarian tumor cells down-regulating TGF- $\beta$  signal and the most important promoters EMT, such as bisphenol A (BPA) and Nonylphenol [119]. Moreover, genistein is able to block growth and cell cycle in ovarian cancer by inhibiting the expression of VEGF [120].

Genistein inhibits cell proliferation also in colon cancer through inactivation of PI3K/Akt pathway. This is because genistein promotes the FOXO3 activities by inhibiting EGF [120]. In addition, it induces apoptosis through signaling pathway dependent on p53 [120-122].

In other tumors, such as prostate, lung and bladder, genistein promotes apoptosis by NF-kB down-regulation [123-125].

Genistein seems to have extraordinary effects against breast cancer. *Zhao et al* investigated genistein-induced regulation of the cancerous inhibitor of protein phosphatase 2A (CIP2A), a new oncogene frequently overexpressed in breast cancer [126].

Their results identified CIP2A as a functional target of genistein and they demonstrated that modulation of E2F1-mediated transcriptional regulation of CIP2A contributes to its downregulation [126].

Therefore, the literature confirms that genistein may have effects both in radiotherapy and chemotherapy and it can be a good adjuvant in the therapy of various cancers.

### **1.3.3 Genistein and Oral cancer: current status**

The association of OSCC and genistein appears for the first time in a paper of 1996 in which the authors used two OSCC cell lines (SCCKN and SCCTF), one HNSCC cell lines (ACCS) and one fibroblasts primary cells lines obtained by gum tissue [127].

In this paper, they exploited the ability of genistein to inhibit the protein tyrosine kinase demonstrating that fibroblast-derived motility factor (FDMF) can in any case stimulate cell motility through the activation of tyrosines phosphorylation [98]. However, they did not evaluated the effects that genistein can have on OSCC cells but only its capacity to inhibit the protein tyrosine kinase, characteristic already known for years [98].

*Alhansan et al* studied the in vitro effects of genistein on OSCC, using the cell line HN4 treated with 25 and 50  $\mu\text{M}$  of genistein for 1, 2 and 3 days [128]. They observed a down-regulation of Cdk1 and Cyclin B1, dose- and time-dependent effects of p21<sup>WAF1</sup> and an up-regulation of Cdc25C during the first and the second days. At the third day genistein completely degraded Cdc25C.

In addition, Bcl-2 was down-regulated, while Bax was up-regulated. The authors, in this case, suggested that genistein causes a cell cycle arrest in S/G2-M and induces apoptosis, confirming the anti-tumor activity of the genistein in OSCC.

*Myoung et al* studied the in vivo effects of genistein [129]. In this paper, they used HSC-3 cell line and they treated it with 27.3 µg/ml of genistein. Then, they induced cancer inoculating this tumor cell line and subsequently treating the OSCC with 0.5µg/kg of genistein. The results obtained have suggested that genistein reduces cell migration and therefore the ability to metastasize and it also reduces angiogenesis because down regulated VEGF mRNA expression.

*Wall et al* showed that genistein has protective effects for oral cavity because within the saliva there is a hydrolysis of the glucoside to the active form of flavonoid aglycone. Moreover, there is a reduction of cell proliferation in SCC-P cell line of OSCC later to treatment with genistein and other flavonoids [130, 131].

However, an in vivo study on hamsters with oral cancer showed the results which were totally different compared to other conducted previously. In fact, *Yang et al* have demonstrated the effects of genistein on oral carcinogenesis but they did not show variations of the tumor compared to an untreated control macroscopically [132].

In addition, they used DMBA, a chemical inducer of cancer, and showed that it worked together with genistein. For this, they believed that genistein promotes carcinogenesis of oral cancer but none reported the same observations.

An interesting study showed the effects that the three most important isoflavones, genistein, biochanin A and daidzein, have in the treatment of OSCC. The squamous cell carcinoma lines of tongue, SCC 15 and SCC25, were treated with 20, 50 and 100 µM of these isoflavones. The authors found a decrease in cellular phosphorylation pathways of ERK and Akt, and they concluded that genistein and biochanin are good therapeutic agents for the treatment of OSCC because they seem to inhibit cell proliferation [133].

The combination of genistein and anticancer drugs seems to be a winning strategy. Cetuximab is a chimeric monoclonal IgG directed against the epidermal

growth factor receptor (EGFR) and administered mainly for the treatment of HNSCC [134, 135].

*Park et al* tried to treat OSCC in vitro and then in vivo with a mix of cetuximab and genistein [136]. In vitro they obtained a down expression of EGFR, p-EGFR and p-Akt with a reduction of proliferation and induction of apoptosis. While, in vivo experiments showed an inhibition of tumor cell proliferation and a rather significant reduction in microvessel density,  $p > 0.05$ .

Several studies showed that the proteins extracted from soybeans contain not only a combination of isoflavones, including genistein, but also molecules that can act in synergy with them to inhibit tumor growth. In particular, *Kingsley et al* were able to isolate soy protein [137] and treat CAL27 and SCC25 cell lines achieving good results [138].

It appears that SPE are able to inhibit the proliferation of these cells down-regulating the expression of the mRNA, up-regulating caspase 2 and 8 causing cellular apoptosis. All the results obtained suggest that SPE have a potent chemopreventive and chemotherapeutic effect in OSCC.

To potentiate the anti-tumor effects of genistein, many researchers are focusing on the construction of delivery systems capable of acting directly on the tumor. A very interesting study is those of *Gavin et al*, which devised an adhesive mucus system to be inserted in the oral cavity in an area where the tumor is macroscopically evident. This tablet containing genistein was completely biocompatible and gave also excellent therapeutic results in the tumor mass present [139].

This opened an entirely new scenario to improve cancer therapies anti OSCC.

## 2. AIM OF THE STUDY

One of the most common site for oral carcinoma is the tongue (TSCC).

TSCC is a multi-factorial disease affected by various genetic alterations and environmental factors and for this reason, its diagnosis is very difficult especially at the early stage of disease.

In terms of treatment, surgery remains the best option for TSCC patients. Although chemotherapy and radiotherapy are applied in combination with surgery for treating late-stage tumors, five year survival rates are disappointingly low with a high possibility of recurrence. All treatments have side effects. Some are temporary but some may be permanent. Surgery to the tongue can cause problems with your speech and changes in eating and drinking.

Radiotherapy to the head and neck area can cause several side effects including a dry, sore mouth and taste changes. Side effects of cancer drugs used in chemotherapy are well known.

Therefore, it is urgent to find the best treatment approaches that can improve the effectiveness and reduce side effects.

Nutraceuticals are mostly phytochemicals derived from dietary or medicinal plants. They may have chemopreventive and anticancer activities as already suggested by epidemiologic and animal model studies. Many nutraceuticals are currently being investigated for their promising anticarcinogenic properties.

Curcumin is a natural component of the rhizome of turmeric (*Curcuma longa*) and one of the most powerful chemopreventive and anticancer agents. Its biological effects range from antioxidant, anti-inflammatory to inhibition of angiogenesis and is also shown to possess specific antitumoral activity. While, Genistein is a soy-derived isoflavone and phytoestrogen with antineoplastic activity. Genistein binds to and inhibits protein-tyrosine kinase, thereby disrupting signal transduction and inducing cell differentiation.

Recently, many researchers are studying the effects of these nutraceuticals on oral cancer but there is no data to fully clarify the role of curcumin and genistein for TSCC, the most aggressive among OSCC tumors.

This in vitro study has the aim to evaluate the cellular and metabolic alterations following administration of curcumin and genistein at various concentrations and at three different time points.

The ultimate goal is therefore to give an initial scientific contribution in order to start future in vivo experiments. Above all, we believe we can create delivery systems that allow these substances to act directly in situ, whereas TSCC is located in a place of easy access.

## 3. MATERIALS AND METHODS

### 3.1- Cell culture and treatment

The cell lines PE/CA-PJ15, PE/CA-PJ49, HSC-3 have been isolated from human Oral Squamous Cell Carcinoma of the tongue (TSCC). All lines were obtained from the European Collection of Cell Cultures (ECACC). Dulbecco's modified Eagle's medium with 4 500 mg/L glucose was used for PE/CA-PJ15 and PE/CA-PJ49 cells, while RPMI 1640 was used for HSC-3 cells. Both culture media were supplemented with 10% fetal bovine serum, L-glutamine (2 mM) and penicillin–streptomycin (100 U/ml). All cell lines were maintained at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. All these reagents were purchased from Sigma-Aldrich (Sigma Aldrich, MO, USA).

Curcumin (Santa Cruz Biotechnology Inc, CA, USA) was prepared by dissolving it in dimethylsulfoxide (DMSO) at a stock concentration of 5 mM and serial dilutions 1, 5, 10, 20 and 50µM of curcumin were prepared in culture medium. The cell lines treated for 24, 48 and 72 hours at the concentrations indicated above.

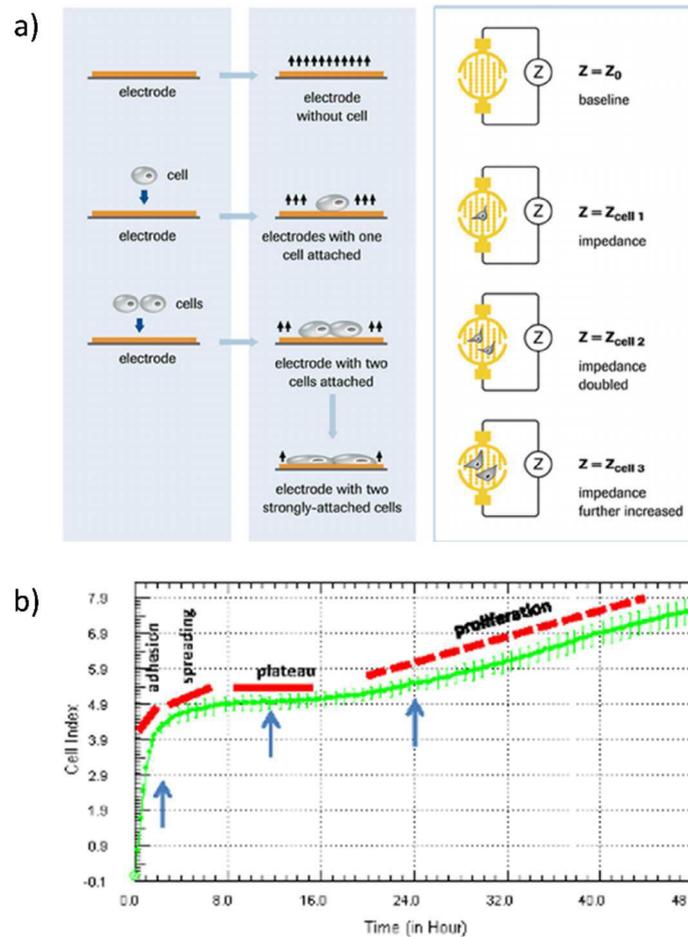
Genistein (Abcam, Cambridge, UK) was dissolved in DMSO at a stock concentration of 5 mM and we have prepared serial diluted at 20, 50 and 100 µM in culture medium. The cell lines treated for 24, 48 and 72 hours.

### 3.2 - xCELLigence system

The xCELLigence DP Real-Time Cell Analyzer is used for real-time monitoring of cell adhesion, proliferation, cytotoxicity, and migration. This system is based on recording the electronic impedance with a unitless parameter called Cell Index (CI). The CI calculation is based on the following formula:  $CI = (Z_i - Z_0)/15\zeta\zeta$  where  $Z_i$  is the impedance at the start of the experiment (*Fig. 3 a*). Thus, CI is a self-calibrated value derived from the ratio of measured impedances. When many cells are attached on the surface of a particular E-Plate the CI gets high. *Figure 3 b* shows an example of Cell Index curve.

The initial period of the Cell Index curve reveals the initial phase of cell adhesion and spreading, followed by a plateau phase prior to a gradual period of proliferation.

The arrows represent potential time points for drug treatment to evaluate adhesion and proliferation phases of the cells.



**Figure 3. xCELLigence RTCA Technology.** Schematic illustration of the impedance measurement principle (a). The baseline impedance is measured in the absence of cells. If more cells attach to the electrodes or if cells are spreading, there will be an increase in electrical impedance, leading to higher CI values. The CI decreases, when cells detach and/or die off. Increases in cell density correspond to increased impedance ( $Z$ ). Interpretation of xCELLigence Cell Index curves (b). The initial period of the Cell Index curve reveals the initial phase of cell adhesion, followed by a plateau phase prior to a gradual period of proliferation. The arrows represent potential time points for drug treatment.

For this reason, we have used it to determine the variation of cell adhesion after curcumin treatments. The RTCA System was used accordingly to the manufacturer's instructions.

### **3.2.1 Cell adhesion**

The cell lines PE/CA-PJ15, PE/CA-PJ49, HSC-3 were washed with PBS and treated with 0.05% trypsin/EDTA (Sigma Aldrich, MO, USA). Cells were resuspended in media after 2 min and they were counted by using a hemocytometer. Then,  $5 \times 10^4$  cells per well were seeded in triplicate inside E-plate with 1, 5, 10, 20 and  $50 \mu\text{M}$  of curcumin. Cells without curcumin treatments has considered as negative control and they were treated with DMSO as vehicle. To valuate effects of cell adhesion, genistein added to cells in their adhesion phase. The concentrations used are the same previously indicated. Cells were monitored for 72 hours.

We have individuated the cell adhesion phase in the initial part of Cell Index curve (0-10 hours).

### **3.2.2 Cell proliferation and estimate IC50 values**

Curcumin and genistein added to cells after their adhesion phases on E-plate, previously individuated. The concentrations used are the same previously indicated. IC50 values of curcumin and genistein treatments at 24, 48 and 72 hours obtained using the software of the xCELLigence RTCA DP system that uses mathematical algorithms described and validated in several studies [140, 141]. They were expressed as the mean (M)  $\pm$  SEM (n=3).

### **3.3 - MTT assay**

Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Massachusetts, USA) used to valuate cell viability of PE/CA-PJ15, PE/CA-PJ49, HSC-3 cells after treatments of curcumin and genistein. We seeded  $5 \times 10^4$  cells in a total volume of  $250 \mu\text{l}$ /well in a 96-well plate with 1, 5, 10, 20, and  $50 \mu\text{M}$  of curcumin and 20, 50 and  $100 \mu\text{M}$  of genistein for 24, 48 and 72 hours. After,  $100 \mu\text{L}$  of fresh culture medium and  $10 \mu\text{l}$  of 12 mM MTT stock solution added for each well. Cells allowed to incubate for 4 hours at  $37^\circ\text{C}$ . For each well, SDS-HCl solution (10

mL of 0.01 M HCl to 1 gm of SDS) were added for an incubation period of 12 hours in a humidified chamber at 37°C. Absorbance was read at 570 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

### **3.4 - Trypan blue exclusion test of cell viability**

In the trypan blue exclusion method 0.1 ml of the PE/CA-PJ15, PE/CA-PJ49, HSC-3 cell suspensions and 0.1 ml of 0.4% trypan blue solution were mixed. After 10 minutes, the cells were counted automatically with JuLI™ FL (NanoEntek, CA, USA), and all blue and damaged cells were counted as dead. The cell count was performed on all the cells treated with different concentrations of curcumin (1, 5, 10, 20 and 50µM) and genistein (20, 50 and 100µM) at the three different time points (24, 48 and 72 h).

### **3.5 - Fluorescence cell viability**

ReadyProbes® Cell Viability Imaging Kit (Invitrogen, CA, USA) was used to determine the viability of cells. NucBlue® Live reagent stains in blue the nuclei of all cells while Propidium iodide stains in red the nuclei of dead cells with compromised plasma membrane. The images were acquired with fluorescent microscope EVOS™ FL Cell Imaging System (Thermo Fisher Scientific, Massachusetts, USA). Fluorescence test was performed on all the cells treated at three time points and with the same concentrations of curcumin (1, 5, 10, 20 and 50µM) and genistein (20, 50 and 100µM).

### **3.6 - Scratch assay**

A monolayer of each cell lines was scaped with a p200 pipet tip and it was washed twice with DPSB 1X (Life Technologies, Gibco) to remove the debris. Then, cells were treated with IC50 dose of genistein at 24 hours. Untreated cells were used

as control. Initially, we acquired the first image (T0) and the subsequent after 1 hour (T1), 2 hours (T2), 3 hours (T3), 5 hours (T4), 6 hours (T5) and 24 hours (T6) after treating it. ImageJ software was used to calculate the gap size of wound and to analyze all acquired images. GraphPad Prism 7 software was used for statistical evaluation.

### **3.7 - Western blotting analysis**

After treatments of genistein at the same concentrations and at the same time point previously used, cells were lysated to obtain proteins. They were measured and, then, separated by 15% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, California, USA). 5% Bovine Serum Albumin (BSA) was used for 1 hour as blocking solution.

Membranes were incubated with integrin  $\beta$ 1 (1:5000; BD Biosciences), PAR 4 (1:200; Santa Cruz Biotechnology), vitronectin (1:150; BD Biosciences), OCT4 (1:700; Novus Biologicals), survivin (1:1000; Cell Signaling Technology), and  $\beta$ -actin (1:5000; Sigma Aldrich), overnight at 4°C. Then, peroxidase-conjugated secondary antibody was used (1:2500; Santa Cruz Biotechnology). Signals were acquired with enhanced chemiluminescence kit (Clarity™ Western ECL Substrate, Bio-Rad). UVP ChemiDoc-It®TS2 Imaging System was used.

### **3.8 - Measurement of intracellular calcium**

Calcium Colorimetric Assay Kit (Sigma Aldrich) was based on determination of the chromogenic complex formed between calcium ions and o-cresolphthalein, which was measured at 575 nm and is proportional to the concentration of calcium ions present. The samples were treated according to instructions of the kit manufacturer.

### **3.9 - Measurement of total cellular ATP**

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega) is an ATP-measurement method, which signals the presence of metabolically active cells.

CellTiter-Glo® Reagent added directly to  $5 \times 10^4$  cells treated and not treated with concentrations of curcumin and genistein previously indicated. An ATP standard curve was used immediately prior to adding the CellTiter-Glo® Reagent because endogenous ATPase enzymes found in sera may reduce ATP levels. The homogeneous “add-mix-measure” format resulted in cell lysis and generation of a luminescent signal proportional to the amount of ATP present which was measured with Fluoroskan Ascent FL (Thermo Fisher Scientific).

### **3.10 - Measurement of LDH release**

The Thermo Scientific™ Pierce™ LDH Cytotoxicity Assay Kit is a colorimetric method for quantifying the lactate dehydrogenase (LDH), a cytosolic enzyme released into the cell culture media as a result of the plasma membrane damages.

Extracellular LDH in the media can be quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD<sup>+</sup> reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt to a red formazan product that can be measured at 490nm. The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity.

Cultured cells were incubated with concentrations of curcumin and genistein previously indicated. The LDH released into the medium was transferred to a new plate and mixed with Reaction Mixture. After a 30 minute room temperature incubation, reactions was stopped by adding Stop Solution. Absorbance at 490nm and 680nm was measured using a plate-reading spectrophotometer to determine LDH activity.

### **3.11 - Analysis of $\Delta\Psi_m$ by fluorescence assay**

Cells treated and not treated with IC50 values of curcumin and genistein at 24 hours were dyed with 1 $\mu$ M of Tetramethylrhodamine, Ethyl Ester, Perchlorate

(TMRE) for 30 minutes, washed by PBS and the images were acquired with fluorescent microscope EVOS™ FL Cell Imaging System and the red fluorescence was measured using ImageJ software.

### **3.12 - Quantitative determination of intracellular ROS level**

Quantitative analysis of ROS level was determined using the cell permeant probe 2'-7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA). The ROS-dependent oxidation of the fluorescent probe was acquired with fluorescent microscope EVOS™ FL Cell Imaging System and the green fluorescence was measured using ImageJ software.

### **3.13 - Statistical analysis**

The unpaired Student's *t*-test and one-way ANOVA followed by Newman–Keuls tests were used to compare continuous variables. All the data are expressed as the mean ± standard error mean; a *p* value < 0.05, *p* < 0.01, *p* < 0.001, *p* < 0.0001 were accepted as statistically significant.

## 4. RESULTS

### 4.1 - Evaluation of cell adhesion

#### 4.1.1 Effects of curcumin on cell adhesion

The PE/CA-PJ15, PE/CA-PJ49 and HSC-3 cell lines were seeded in E-Plate of xCELLigence system with 1, 5, 10, 20 and 50  $\mu\text{M}$  of curcumin to verify some variation in adherence of treated cells compared to untreated ones (*Fig.4a, b, c*).

Curcumin has been put in the E-plate at time 0, at the same time to the cell seeding.

We monitored in real time the growth kinetics for 72 hours after treatment and all CI values analyzed. The adhesion interval was 0-10 hours.

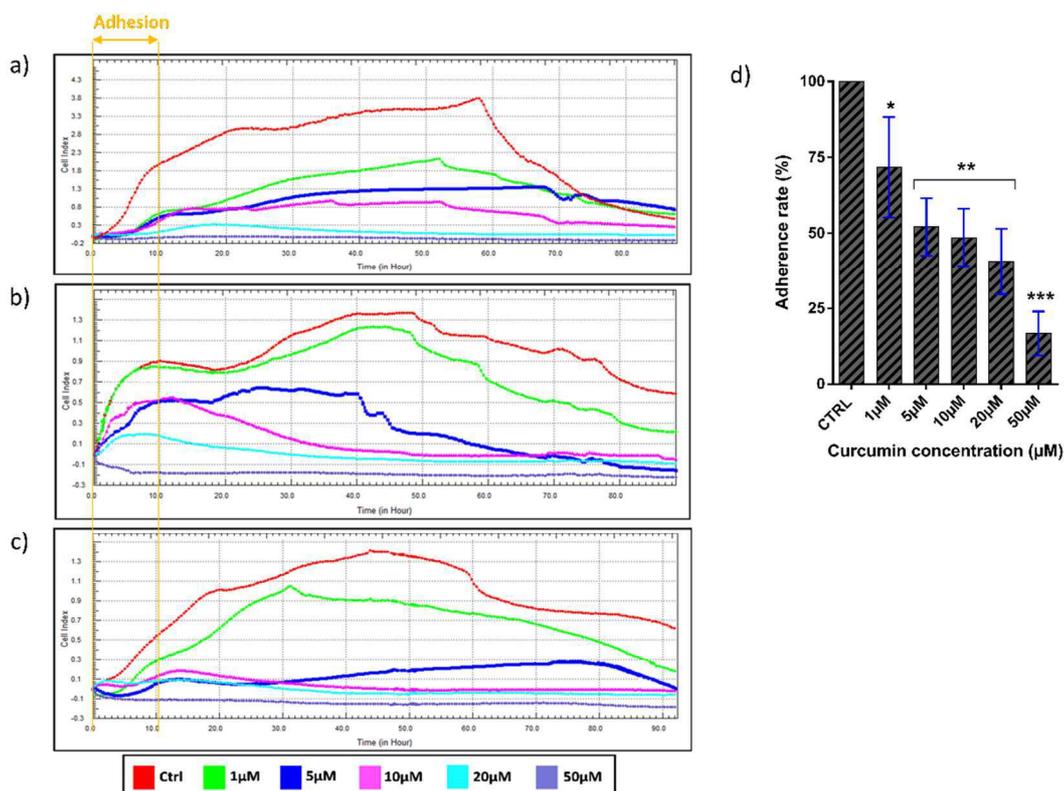
The resulting curves (*Fig.4a, b, c*) show a difference of adhesion between control and treated cells. The reduction of adhesion is proportionally increased respect to the concentration of curcumin used for all TSCC lines.

The CI values are considerably reduced in all conditions analyzed, even considering the limitations of xCELLigence system, such as the normal reduction in cell growth space for long analysis times. However, the results obtained confirm that the increase in curcumin concentrations leads to a proportional reduction of cell adhesion, even for very long treatments.

In the evaluation of adherence rate, we converted the CI values in percent values in order to obtain a better indication of the reduction of cell adhesion, considering the control value of CI, at each time point, as the maximum value of adhesion at 10 hours (*Fig. 4 d*).

In particular, CI values showed a ~ 25% reduction of cell adhesion in TSCC cells treated with 1 $\mu\text{M}$  of curcumin, at 10 hours (*Fig. 4d*). We have a significance compared to control of  $p < 0.5$ . While, cells treated with 5 $\mu\text{M}$  of curcumin showed a 50% reduction of adhesion compared to control.

Furthermore, adhesion of TSCC cells treated with 50  $\mu\text{M}$  of curcumin was reduced by 75% compared to control (*Fig.4f*). This data showed a high significance,  $p < 0.001$ .

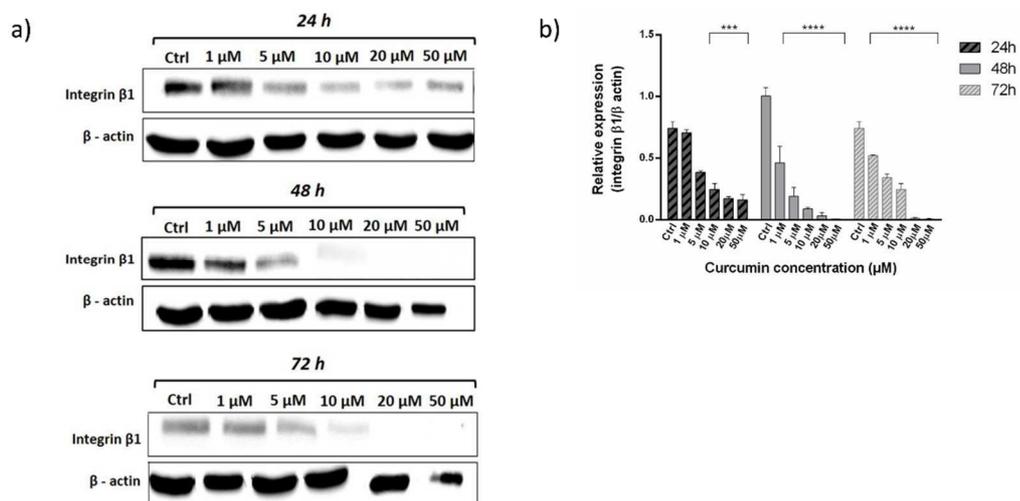


**Figure 4. Curcumin inhibited adhesion of tongue cancer cells.** Different Cell Index curves are obtained from 3 tongue carcinoma cells types. All cells treated with different concentration of curcumin 1, 5, 10, 20 and 50  $\mu\text{M}$ . We monitored the cells for 72 hours. The adhesion phase is identified between 0-10 hours for all cell lines. The adhesion of (a) HSC-3 cells, (b) PE/CA-PJ15 cells and (c) PE/CA-PJ49 cells is shown. Cells untreated used as control (red curves). The interval of adhesion is delimited by a line at 10 hours after treatment. The results are the mean CI for three replicates  $\pm$  SD. CI values is measured and it was converted in % rate using untreated cells as control (100% adhesion) (d). Student's t-test: \* $p < 0.5$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

To better understand the effect of curcumin on cell adhesion, we evaluated the expression of integrin  $\beta 1$ , an integral membrane glycoprotein that binds the extracellular matrix proteins, and which plays a key role in cell adhesion. In this case, we evaluated this adhesion molecule for even longer of treatments. *Figure 5a, b* show

the reduction of integrin expression by 50% in cells treated with 10  $\mu\text{M}$  of curcumin after 24 hours compared to control.

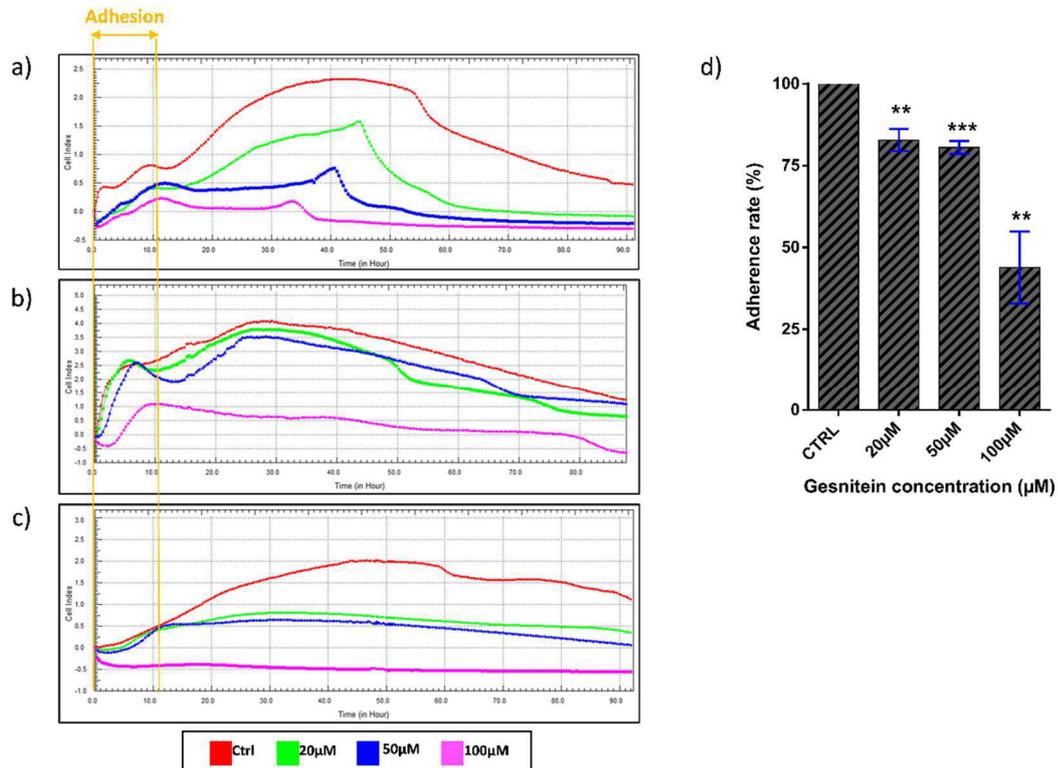
While, after 48 hours the same reduction is present for cells treated with about 5  $\mu\text{M}$  of curcumin. Instead, at 72 hours after treatment the values are significantly reduced compared to the treatments after 24 and 48 hours. In particular, a reduction of 50% adhesion relative to the control is evident in cells treated with 5  $\mu\text{M}$  of compound. In addition, we have seen a great reduction of integrin expression in all the cells treated with 20 and 50  $\mu\text{M}$  of curcumin to 48 hours and 72 hours after treatment (*Figure 5a, b*).



**Figure 5. Expression of integrin  $\beta 1$ .** Downregulation of integrin  $\beta 1$  expression by 50% in cells treated with 10  $\mu\text{M}$  of curcumin after 24 hours compared to control (**a**), (**b**). A great reduction of integrin expression is at 20 and 50  $\mu\text{M}$  concentration of curcumin for 48 hours and 72 hours to treatments (**a**), (**b**).

## 4.1.2 Effects of genistein on cell adhesion

PE/CA-PJ15, PE/CA-PJ49 and HSC-3 cell lines were treated with 20, 50 and 100 $\mu$ M of genistein during cell adhesion phase (0-10 h). Through xCELLigence system, we monitored adhesion kinetics in real time of treated cells and we used untreated cells as control. The adhesion intervals are indicated in *Figure 6 a, b, c*.



**Figure 6. Genistein inhibited adhesion of tongue cancer cells.** All cell lines treated with 20, 50 and 100  $\mu$ M of genistein. The adhesion of (a) HSC-3 cells, (b) PE/CA-PJ15 cells and (c) PE/CA-PJ49 cells is shown. Cells untreated used as control (red curves). We monitored in real time the adhesion for 24, 48 and 72 hours after treatment and all CI values of all TSCC cells (d) are shown. The interval of adhesion is delimited by a line at 10 hours after treatment. The results are the mean CI for three replicates  $\pm$  SD. Student's t-test: \*\*p < 0.01, \*\*\*p < 0.001.

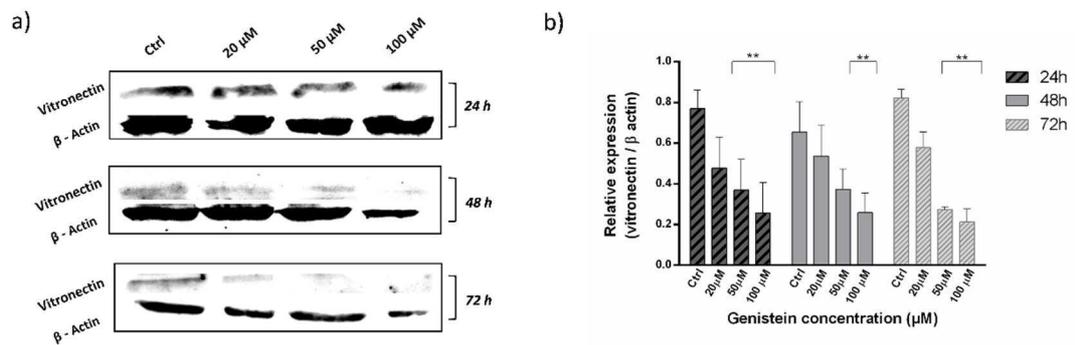
CI values were taken at 10 hours and adhesion curves were analyzed. Data obtained show clear effects of genistein on all cells used during adhesion interval.

The CI values show that the adhesion of TSCC treated with 20  $\mu$ M of genistein appears to be reduced by 25% compared to the same untreated cells, p < 0,01 (*Fig.6d*).

The same reduction was also proved after treatment with 50  $\mu\text{M}$  of genistein,  $p < 0,001$  (Fig.6d).

While, the CI values of TSCC cells treated with 100  $\mu\text{M}$  of genistein they show a great reduction of cell adhesion (Fig.6 d).

In fact, the adhesion is reduced by approximately 60% compared to untreated control, with a significance  $p < 0.01$  (Fig.6 d).



**Figure 7. Inhibition of vitronectin expression.** Western blotting shows the inhibition of vitronectin expression after genistein treatment. Vitronectin is especially downregulate at 48 hours with 100  $\mu\text{M}$  of genistein and at 72 hours with 50 and 100  $\mu\text{M}$  of genistein (a). In Figures is shown significance degrees of tests used  $**p < 0.005$  (b).

To evaluate the effects of genistein on cell adhesion of tongue carcinoma cells, we also studied the expression levels of the vitronectin protein.

It is a glycoprotein found mainly in the extracellular matrix and promotes cell adhesion and spreading.

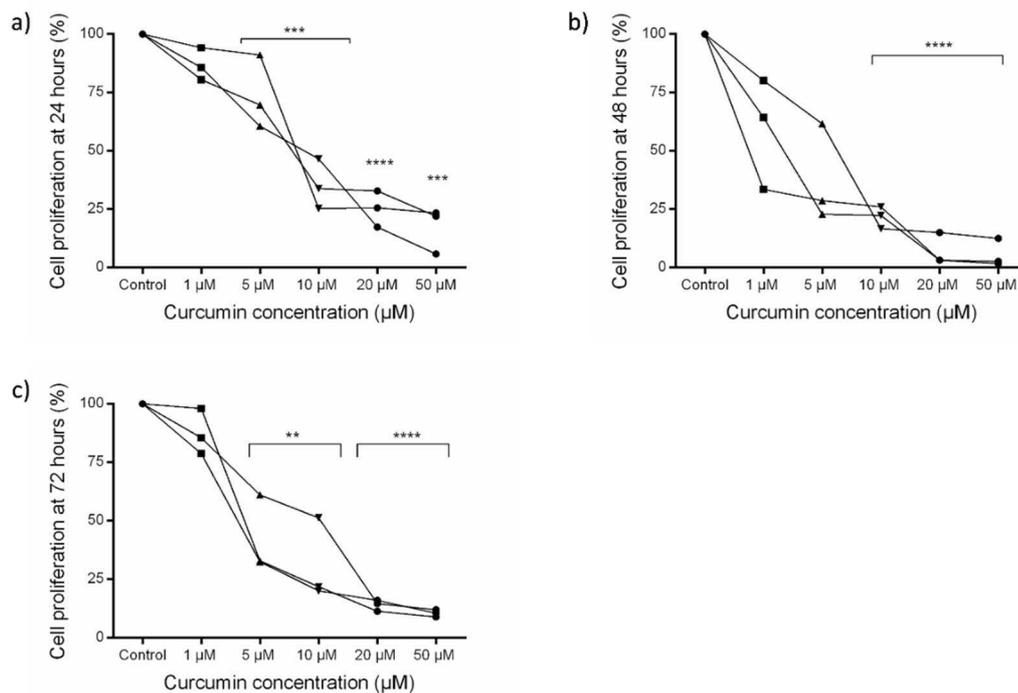
From the western blotting performed on protein lysates of the 3 cell types used, we noticed that the expression of vitronectin seems to be reduced as a result of treatments with increasing concentrations of genistein, especially those long-lasting.

In fact, at 48 hours after treatment with 100  $\mu\text{M}$  of genistein vitronectin seems to be little expressed and unexpressed almost for treatments with 50 and 100  $\mu\text{M}$  of genistein to 72 hours (Fig.7 a, b).

## 4.2 - Cell proliferation and half maximal inhibitory concentration (IC50)

### 4.2.1 Effects of curcumin on cell proliferation and IC50 determination

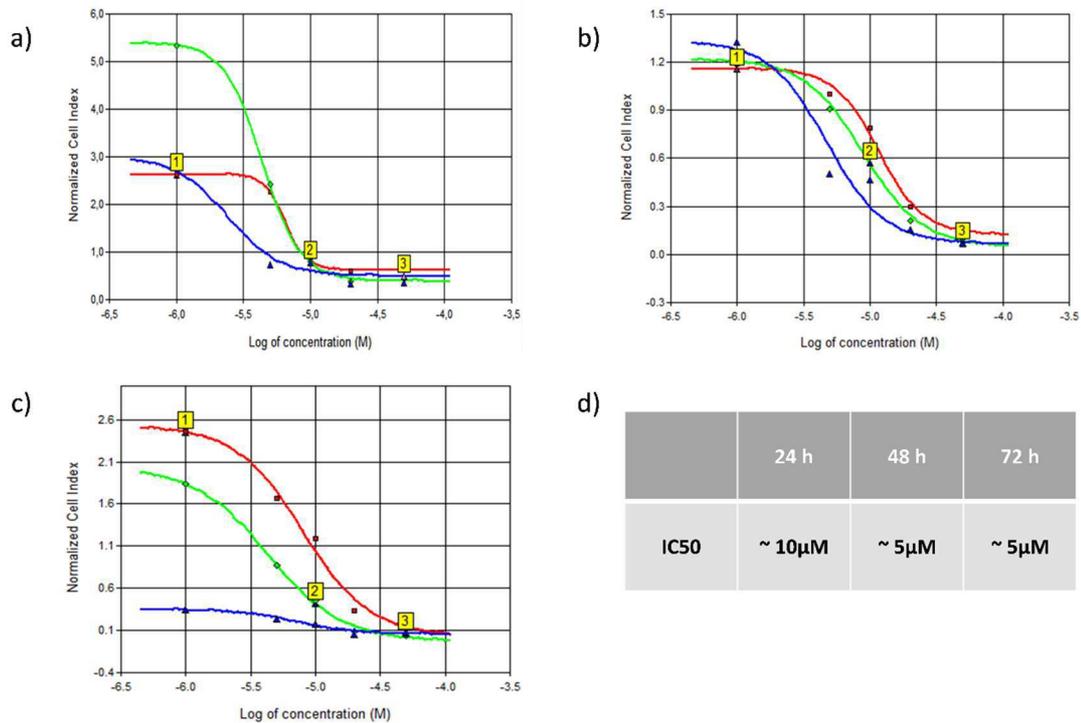
xCELLigence system was also used to evaluate the effect of the nutraceutical on tongue carcinoma cells. Proliferation rate (%) shows a decrease by 50% compared to control at 24 hours for cells treated with concentration of curcumin about 5-10  $\mu\text{M}$  with  $p < 0,001$  (Fig. 8a). There is only about 25% of tumor cell proliferation compared to the control after treatment with 20  $\mu\text{M}$  of curcumin with  $p < 0,0001$  (Fig. 8a). Even with 50  $\mu\text{M}$  of curcumin ( $p < 0,001$ ), the proliferation is less than 25 ( $p < 0,001$ ) (Fig. 8a).



**Figure 8. Curcumin dose-dependent effects on tongue carcinoma.** We added curcumin to all cells in proliferation phase. Curcumin inhibitory effects at 24 hours (a), 48 hours (b) and 72 hours (c) in all cell lines with  $**p < 0.05$ ,  $***p < 0.001$ ,  $**** p < 0.0001$ .

Furthermore, treatments at 48 hours, with 1-10  $\mu\text{M}$  of curcumin, have shown a reduction of the proliferation of 50% compared to control (*Fig. 8b*). The reduction of cell proliferation observed between 10  $\mu\text{M}$  and 50  $\mu\text{M}$  is highly significant compared to the control ( $p < 0,0001$ ) (*Fig. 8b*).

All CI values were used for the calculation of half maximal inhibitory concentration (IC<sub>50</sub>) by RTCA Software 1.2 (*Fig. 9 a, b, c*). IC<sub>50</sub> value was expressed as the mean (M)  $\pm$  S.E.M. (n=3).



**Figure 9. Determination of IC<sub>50</sub> value.** Dose-response curves are shown for HSC-3 cells (a), PE/CA-PJ15 cells (b) and PE/CA-PJ49 cells (c). In each graph, the yellow square with the number 1, 2 and 3 indicates respectively IC<sub>50</sub> values at 24, 48 and 72 hours. IC<sub>50</sub> values are expressed as the mean (M)  $\pm$  S.E.M. (n=3) and they are shown as the average of all IC<sub>50</sub> values of the cell lines used at each time point (c). Square R of IC<sub>50</sub> was 0.99.

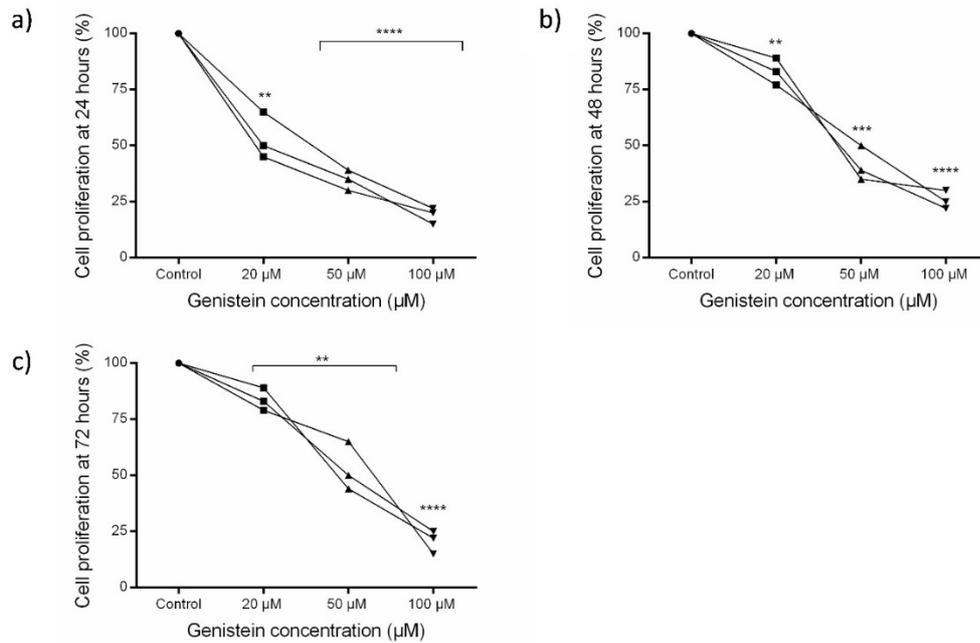
In *Figure 9 d*, we reported the IC<sub>50</sub> values obtained from the averages of the IC<sub>50</sub> values of the individual cell lines for each time point.

IC50 at 24 hours post treatment was around 10  $\mu\text{M}$ , while at 48 and 72 hours after the treatment, it was around 5  $\mu\text{M}$  (*Fig. 9d*). For the calculation of all IC50, the square R was 0.99.

#### 4.2.2 Effects of genistein on cell proliferation and IC50 determination

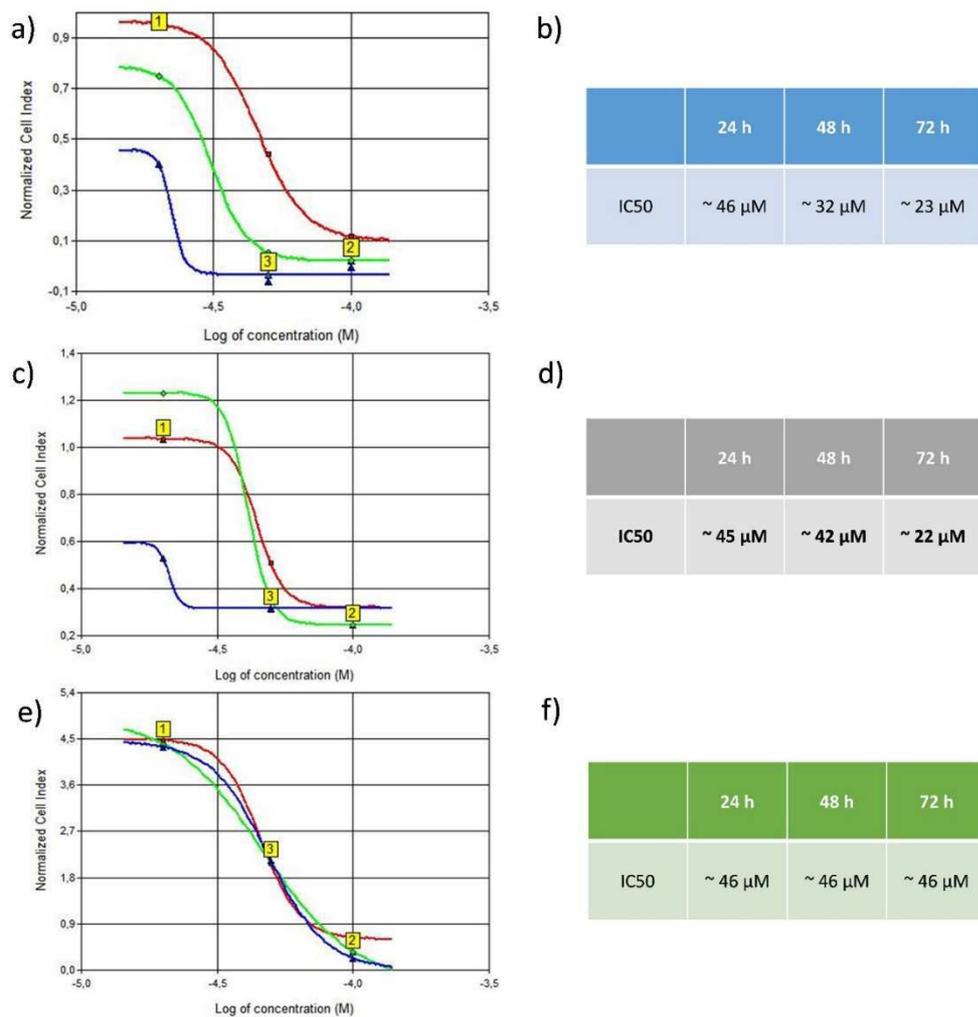
Genistein has been added to all cancer cell lines in cell proliferation phase. All cells were monitored in real time and all IC values were taken at 24, 48 and 72 hours. In addition, they were converted into percentage values and we used the ANOVA as statistical tests to evaluate the cell proliferation percentage.

We found a 50% significant reduction of cell proliferation for all cells treated with 20  $\mu\text{M}$  of genistein after 24 hours of treatment (*Fig. 10a*).



**Figure 10. Variation in cell proliferation rate (%).** CI values was measured and it was converted in % rate using untreated cells as control (100% adhesion). (a) It shows a 50% reduction of adhesion post treatment with 20  $\mu\text{M}$  at 24 hours. (b) The same reduction is present for treatment between 20 and 50  $\mu\text{M}$  of genistein at 48 hours, while 50  $\mu\text{M}$  of genistein at 72 hours (c). In Figures is shown significance degrees of tests used (\*\*p < 0.005, \*\*\*p < 0.001, \*\*\*\*p < 0.0001).

While, the same reduction have been found at 48 hours in a concentration range of between 20 and 50  $\mu\text{M}$  genistein (*Fig. 10b*). Instead, cellular proliferation seems to be reduced by 50% compared to the untreated control to 72 hours for all the cells treated with 50  $\mu\text{M}$  of genistein (*Fig. 10c*). In all cell lines, 100  $\mu\text{M}$  of genistein resulted in a reduction of about 25% of proliferation at all time points considered. All the data were considered statistically significant for  $p < 0.005$ ,  $p < 0.001$ ,  $p < 0.0001$  (*Fig. 10 a, b, c*).



**Figure 11. IC50 values.** Dose-response curves are shown for HSC-3 cells (a), PE/CA-PJ15 cells (c) and PE/CA-PJ49 cells (e). In each graph, the yellow square with the number 1, 2 and 3 indicates respectively IC50 values at 24, 48 and 72 hours. IC50 values are expressed as the mean (M)  $\pm$  S.E.M. (n=3) and they are shown as the average of all IC50 values of HSC-3 cells (b), PE/CA-PJ15 cells (d), PE/CA-PJ49 cells (f) at each time point. Square R of IC50 was 0.99.

From the growth curves obtained by treating cells with 20, 50 and 100  $\mu\text{M}$  of genistein, CI values were used to calculate the value of IC 50 to 24, 48 and 72 hours.

In particular, it found that at 24 hours, the IC50 value is about 46  $\mu\text{M}$  for all cell lines used in mean (*Fig. 11 a, b, c, d, e, f*). While for treatments to 48 hours, the average IC50 value of the 3 cell lines used was around 40  $\mu\text{M}$  in mean (*Fig. 11 a, b, c, d, e, f*).

However, there is a slight discrepancy between the lines for 72 hours treatments. In fact, for HSC-3 and PE/CA-PJ15 the IC50 value seems to be around 22  $\mu\text{M}$  (*Fig. 11 b, d*). Only for PE/CA-PJ49 this value is around 46  $\mu\text{M}$ . This is probably because this line shows a higher resistance to the treatment with genistein than the other (*Fig. 11 e, f*).

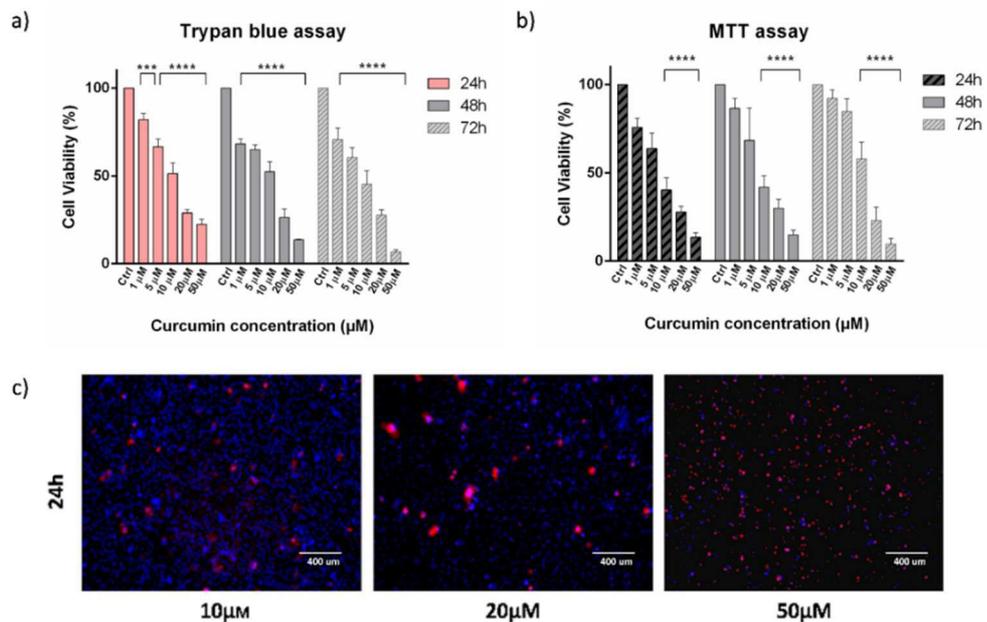
## 4.3 - Inhibition on cell viability

### 4.3.1 Effects of curcumin on cell viability

We observed a 50% reduction of viability in cells treated with 5-10  $\mu\text{M}$  of curcumin at 24 hours after treatment (*Fig.12 a, b*). While, the same reduction is observed for the concentrations between 5-20  $\mu\text{M}$  of curcumin at 48 hours and about 10-20  $\mu\text{M}$  at 72 hours (*Fig.12 a, b*).

We assessed cell viability fluorescence with ReadyProbes®Cell Viability Imaging Kit, where Hoechst stained the nuclei of all live cells and propidium iodide stained only the nuclei of cells with compromised plasma membrane integrity.

*Figure 12 c* show the important effects of curcumin at concentrations of 10, 20 and 50  $\mu\text{M}$  at 24 hours for all cells used. Data show the same effects evaluated in the previous tests and, in particular, at 50  $\mu\text{M}$  of curcumin we noticed huge reductions in cell viability for all cells and at all time points.

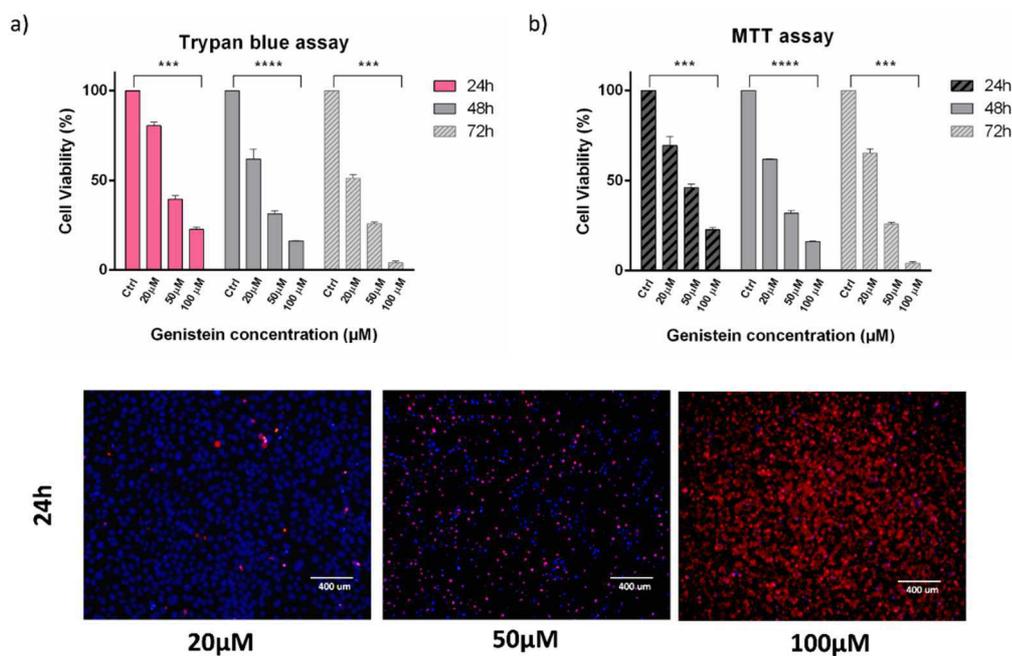


**Figure 12. Curcumin reduces the viability of tongue cancer cells.** Trypan blue test (a), MTT assay (b) and Fluorescence assay (c) show a proportional reduction in cell viability with increasing concentrations of curcumin. Results were performed at least three times and they are presented as the means  $\pm$  SD of all cells used. The figure (c) is the result of the fluorescence test on the HSC-3 cells.

### 4.3.2 Effects of genistein on cell viability

For a better evaluation of genistein on cell viability we used three simple test: the MTT assay, the Trypan blue assay and fluorescence assay. In all assays, we noticed that genistein change cell viability already 24 hours after treatment.

In fact, we noticed a reduction of about 50% of cell viability in each treated cell line with concentrations between 20 and 50  $\mu\text{M}$  at 24, 48 and 72 hours (*Fig. 13 a*). Trypan blue assay and fluorescence assay confirmed almost all the data obtained with the MTT assay (*Fig. 13 b, c*). All the data were considered statistically significant for  $p < 0.001$  and  $p < 0.0001$ .



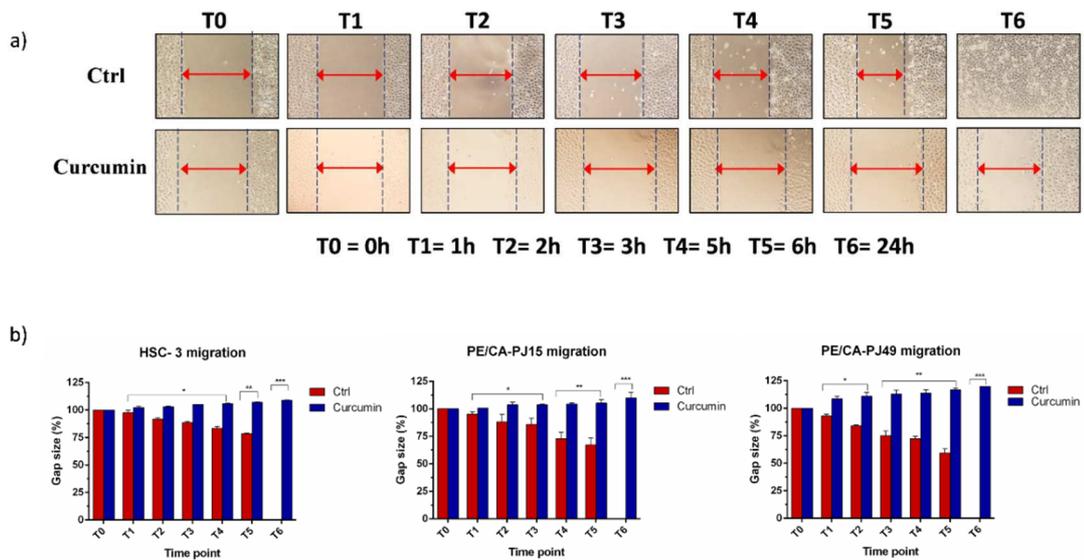
**Figure 13. Genistein reduces the viability of tongue cancer cells.** Trypan blue test (a) MTT assay (b), and fluorescence assay (c) show a proportional reduction in cell viability with increasing concentrations of genistein. Results were performed at least three times and they are presented as the means  $\pm$  SD, and statistically significant for \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$ . The figure (c) is the result of the fluorescence test on the HSC-3 cells.

## 4.4 - Determination of cell migration rate

### 4.4.1 Curcumin inhibits cell migration

To evaluate cell migration, we have captured images at the beginning of the treatment with 10  $\mu\text{M}$  of curcumin (T0) and at intervals (T1, T2, T3, T4, T5 and T6) during the migration of the cells to close the scratch, and we have compared the images to determine the rate of cell migration (*Fig.14a*). The concentration of curcumin used was the average IC50 value at 24 hours, previously estimated.

All experiments were performed at least three times and the results are presented as means  $\pm$  SD. We have noticed a significant increase of the gap size directly proportional to the increase of the treatment time with curcumin, while it decreases in the untreated control (*Fig.14a, b*).



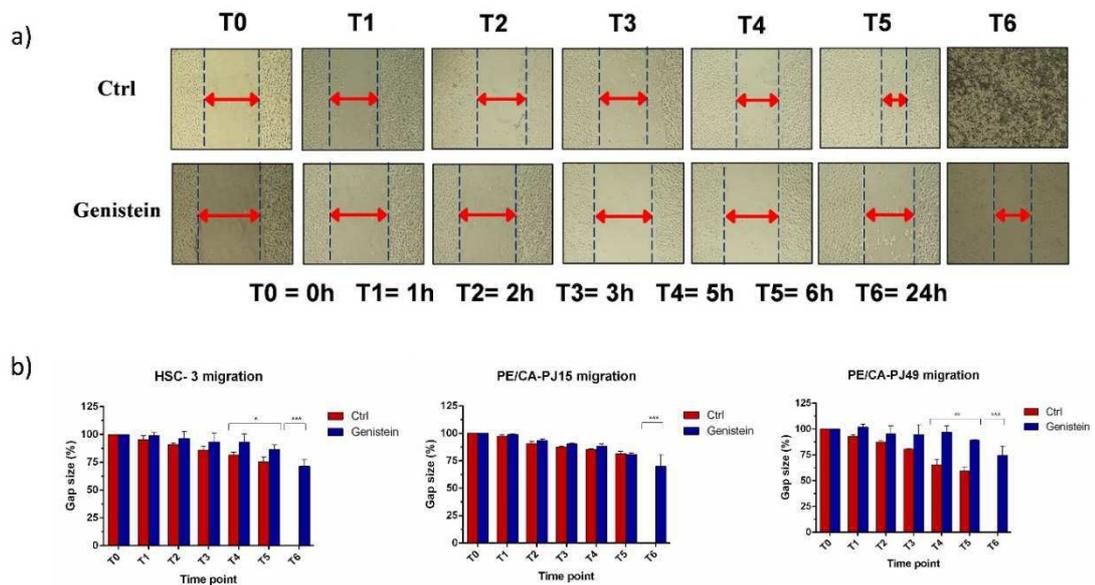
**Figure 14. Effects on cell migration.** Significant increase of the gap size is most evident from T3-T4 in tongue carcinoma cells compared to control untreated (Ctrl) (a). The figure (a) is the result of scratch assay on the PE/CA-PJ49 cells. All experiments were performed at least three times and results are presented as the means  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  (b).

The increase is most evident from T3-T4 onwards and it is present in all of tongue carcinoma cells. For T4 and T5 times we have a very high significance compared to control ( $p < 0.05$  and  $p < 0.01$ ) for all cell lines (*Fig.14b*). Instead, the wound treatment

with curcumin after 24 hours (T6) prevents the complete healing of the wound, while it disappears completely in the untreated control ( $p < 0.001$ ) (Fig.14a, b).

#### 4.4.2 Genistein inhibits cell migration

To evaluate if genistein is able to reduce the migration of the tongue cancer cells, we used the Scratch assay. Tongue cells were treated with IC50 value at 24 hours, previously calculated (10  $\mu$ M). The control was not undergone to any treatment. The treated cells migrate very little in the area cutted (Fig.15a).



**Figure 15. Effects of genistein on cell migration.** Significant increase of the gap size is most evident from T4 in tongue carcinoma cells compared to control untreated (Ctrl) (a). The figure (a) is the result of scratch assay on the PE/CA-PJ49 cells. All experiments were performed at least three times and results are presented as the means  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  (b).

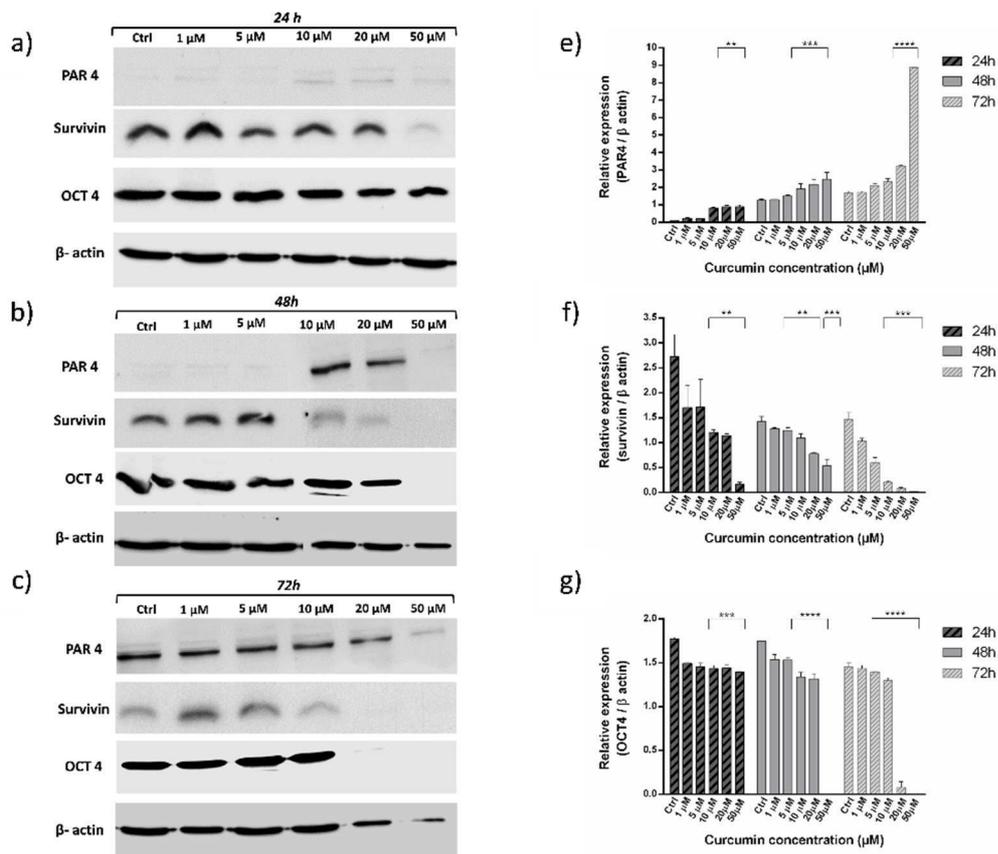
The most significant effects are evident starting from T4 and, specifically, the wound of the treated cells does not show any healing after 24 hours (Fig.15a). While, the untreated control showed an increasing level of healing of the wound during the considered times and total after 24 hours (Fig.15a). Evaluations to the timing T4, T5 and T6 were the most significant with  $p$  value  $< 0.05$ ,  $p < 0.01$ ,  $p < 0.001$  (Fig.15b).

## 4.5 - Effects on apoptosis and tumorigenesis

### 4.5.1 Curcumin induces apoptosis and reduces tumorigenesis

After treating tongue cancer cells with different curcumin concentrations, we evaluated the expression of PAR 4, OCT4 and survivin (Fig. 16 a, b, c).

We noticed an increased expression of PAR4, pro-apoptotic protein, for concentration higher than 10  $\mu\text{M}$  at 24 and 48 hours and for all treatments at 72 hours (Fig. 16 a, b, c, d). This demonstrates, probably, that curcumin induces apoptosis in cells treated with 10  $\mu\text{M}$  already after 24 hours and that possible apoptosis is more evident after 72 hours of treatment (Fig. 16 a, c, d).



**Figure 16. Curcumin reduces tumorigenesis and promotes apoptosis.** Variation in level expression of PAR4, survivin and OCT4 show at 24 hours (a), 48 hours (b) and 72 hours (c). Curcumin inhibits survivin and OCT4 but up regulates PAR4 (d), (e), (f).

In addition, there is an inhibition of OCT4 with 50  $\mu\text{M}$  of curcumin at 24 hours and with 20 and 50  $\mu\text{M}$  at 72 hours, which demonstrates that curcumin can inhibit tumorigenesis (Fig. 16 c, f).

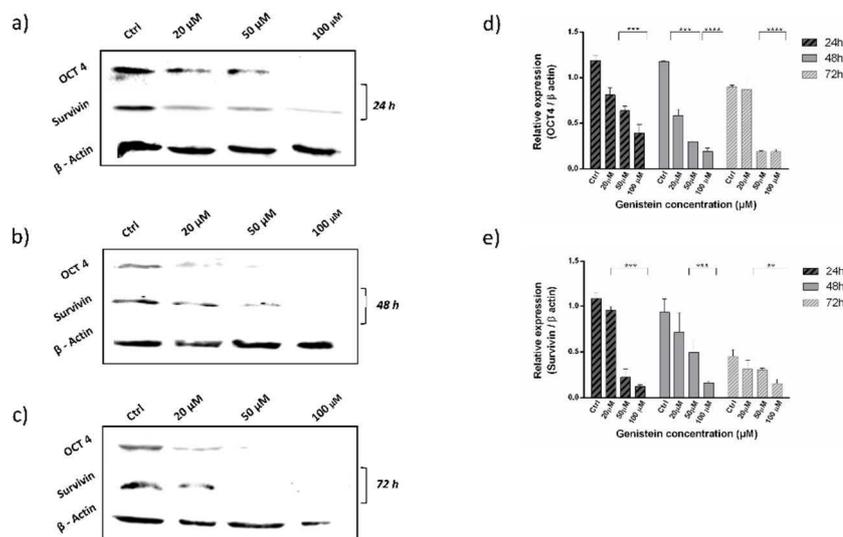
Furthermore, curcumin downregulates expression of survivin with concentrations of 50  $\mu\text{M}$  at 24 hours, greater than 10  $\mu\text{M}$  after 48 hours and at all concentrations at 72 hours. There is a total inhibition for concentrations greater than 20  $\mu\text{M}$  for 72 hours (Fig. 16 a, b, c, d, e).

#### 4.5.2 Genistein induces apoptosis and reduces tumorigenesis

After treating cancer cells of the tongue with different concentrations of genistein, we evaluated the expression of OCT4 and survivin. OCT4 is a known protein because its overexpression promotes tumorigenesis in different types of cells.

In tongue cancer cells, genistein appears to have an action on the expression of OCT4 only after high concentrations treatments.

In fact, Figure 17 shows a reduction in those cells who were treated with 50 and 100  $\mu\text{M}$  of genistein for each time point considered.



**Figure 17. Genistein reduces tumorigenesis and promotes apoptosis.** Variation in level expression of OCT4 and surviving show. Genistein inhibits survivin and OCT4 at all time points considered (a), (b), (c). The significance degrees of test used is \*\*p < 0.005, \*\*\*p < 0.001, \*\*\*\*p < 0.0001 (d), (e).

Moreover, expression of survivin appears to be extremely reduced as a result of treatments with concentrations of 50 and 100  $\mu\text{M}$  at 24 hours (*Figure 17 a, e*). This seems to be confirmed at all time points considered and, especially, at a concentration of 100  $\mu\text{M}$  there is almost a complete inhibition (*Fig. 17 a, b, c, d, e*).

## **4.6 – Biochemical changes in cellular injury**

### **4.6.1 Increases in intracellular calcium ion concentration after treatments with curcumin and genistein**

TSCC cells were treated with the same concentrations of curcumin and genistein previously used.

After 24 hours, we noticed an increase in intracellular  $\text{Ca}^{2+}$  in all treated cells on the contrary, controls showed normal values ( $\sim 0.1 \mu\text{g}/\mu\text{l}$ ) (*Fig. 18 a, d*).

We got a fold change (FC) of  $\sim 0.4$  for treatments with 5 and 10  $\mu\text{M}$  of curcumin compared to control ( $p < 0.1$ ) (*Fig. 18 a*). While, FC was 0.8 for treatment with 50  $\mu\text{M}$  of curcumin ( $p < 0.01$ ) (*Fig. 18 a*).

In addition, cells treated with genistein showed an increased  $\text{Ca}^{2+}$  compared to the control (*Fig. 18 d*). The FC for IC50 genistein treatments at 24 h was  $\sim 0.8$ , while it was 1.5 with 100  $\mu\text{M}$  of genistein ( $p < 0.1$ ).

### **4.6.2 Curcumin and genistein induces ATP release from TSCC cells**

The ATP levels measured after treatment with curcumin and genistein at 24 hours showed a significant reduction of energy levels (*Fig. 18 b, e*).

Notably, 50  $\mu\text{M}$  of curcumin have reduced ATP levels of  $\sim 94\%$  compared to the control, with a high significance  $p < 0.001$  (*Fig. 18 b*). Even in the other treatments, ATP levels are reduced compared to the control with  $p < 0.01$  and  $p < 0.001$  (*Fig. 18 b*).

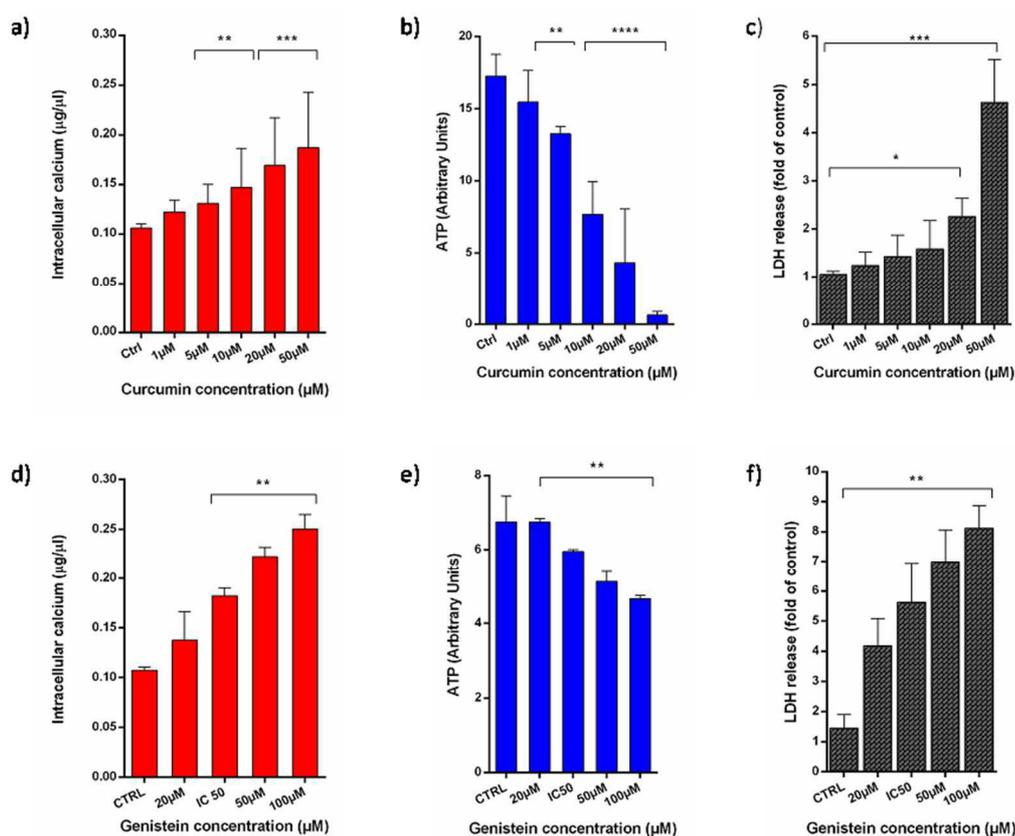
TSCC cells were also treated with genistein. The ATP levels were reduced for all treatments, with  $p < 0.1$  (*Fig. 18 e*). In particular, the cells treated with 100  $\mu\text{M}$  of genistein had a reduction in ATP levels of 35% compared to control (*Fig. 18 e*).

### 4.6.3 The LDH is released after curcumin and genistein treatments

Cultured cells were incubated with the same concentrations of curcumin and genistein previously indicated. The quantity of LDH released after treatments was higher than to untreated control (*Fig. 18 c, f*).

For treatments with 1, 5, 10 and 20  $\mu\text{M}$  of curcumin, we noticed an increasing release of LDH, proportional to the concentrations of nutraceutical utilised ( $p < 0.5$ ) (*Fig. 18 c*).

For treatments with 20  $\mu\text{M}$  of curcumin, the amount of LDH released was twice, compared to control. While, it is increased by  $\sim 4$  times for treatments with 50  $\mu\text{M}$  of curcumin ( $p < 0.01$ ) (*Fig. 18 c*).



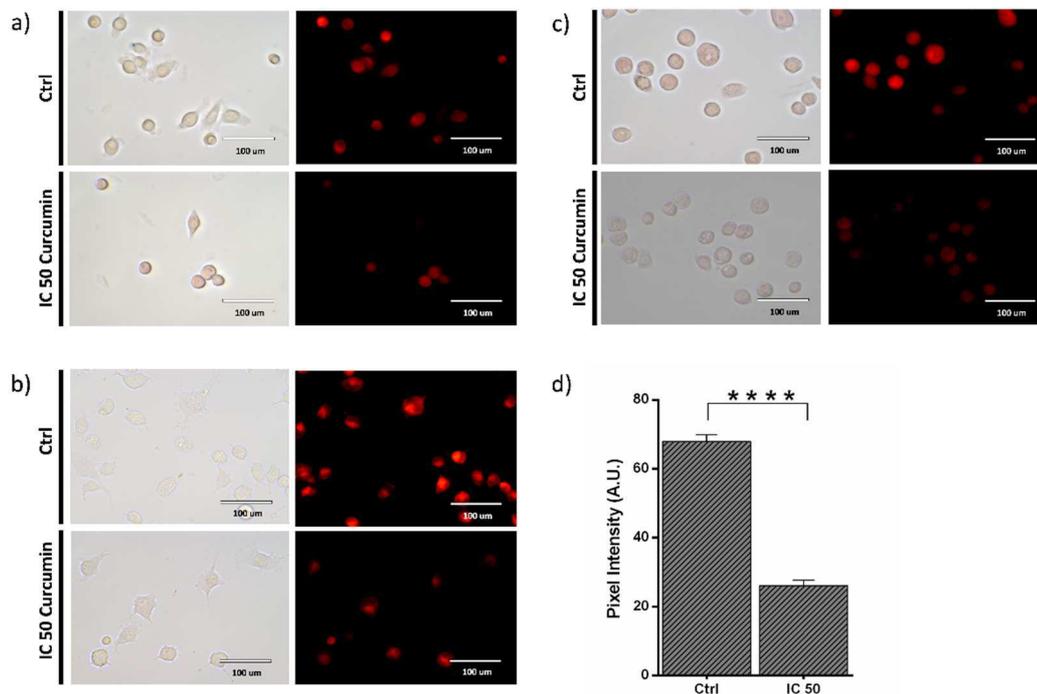
**Figure 18. Curcumin and genistein induce the cell damage and they disrupt the normal homeostasis of treated cells.** Increase of intracellular calcium (a)(d), reduction of ATP levels (b) (e) and increase of LDH release (c) (f) after treatment of curcumin and genistein. The significance degrees of tests used are \*\* $p < 0.005$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  (d), (e).

However, the release of LDH was remarkable with treatments with genistein ( $p < 0.01$ ) (Fig. 18 f). Already with 1  $\mu\text{M}$  of genistein, the value of LDH released was 4 times more high than the control, up to a maximum of about 8 times more for cells treated with 100  $\mu\text{M}$  of genistein (Fig. 18 f).

#### 4.6.4 Effects of curcumin and genistein on $\Delta\Psi\text{m}$ assessed by the fluorescent probe TMRE

PE/CA-PJ15, PE/CA-PJ49 and HSC-3 treated with curcumin and genistein IC50 values at 24 hours, as previously calculated. Untreated cells have been used as control. Using TMRE probe, we evaluated the mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ) of TSCC cells treated with curcumin and genistein. The analysis was done using ImageJ software.

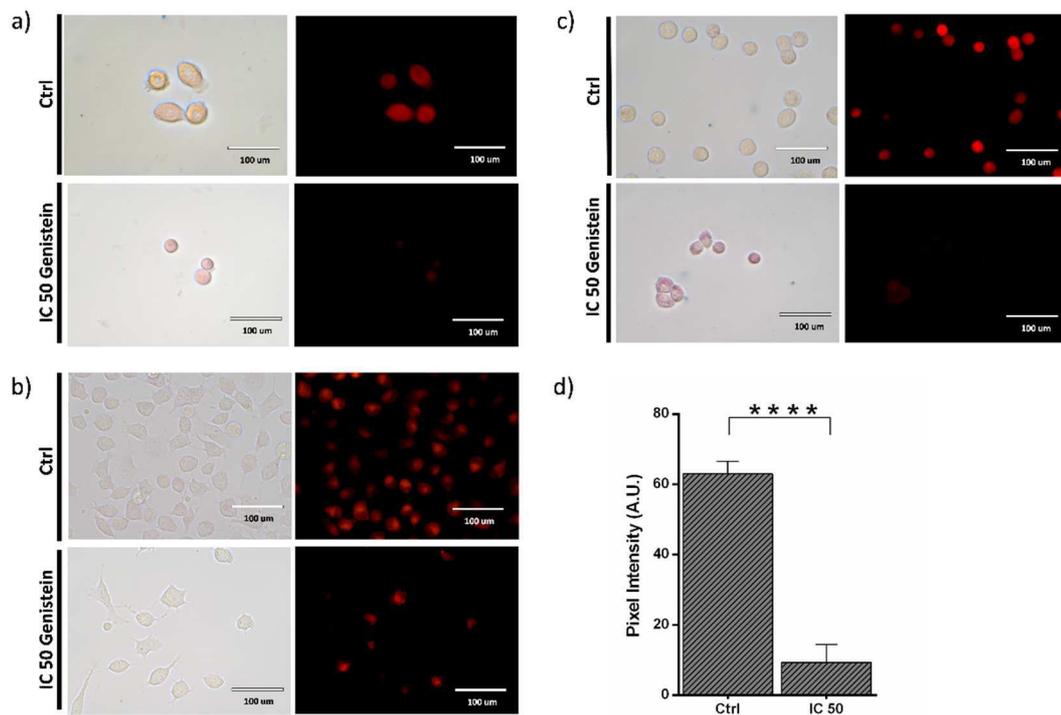
The Figures 19, 20 show a different pixel intensity (A.U.) treated cells compared to controls ( $p < 0.0001$ ).



**Figure 19. Curcumin reduces  $\Delta\Psi\text{m}$  of TSCC cells.** PE/CA-PJ49 (a), PE/CA-PJ15 (b) and HSC-3 (c) untreated and after treatment of curcumin IC50 value. The graph bars in the bottom panel show the quantitative analysis of  $\Delta\Psi\text{m}$ -related TMRE fluorescence (d). Student's t-test: \*\*\*\* $p < 0.0001$  (d).

In fact, the cells treated with curcumin have a lower affinity to TMRE (Fig. 19 a, b, c, d). TMRE-fluorescence of the cells treated with IC50 value of curcumin is reduced by ~ 75% compared to untreated cells (Fig. 19 d).

Instead, TSCC cells treated with genistein have a higher reduction of  $\Delta\Psi_m$  compared to the control (Fig. 20 a, b, c, d). It is ~ 80% reduced compared to the control (Fig. 20 d).



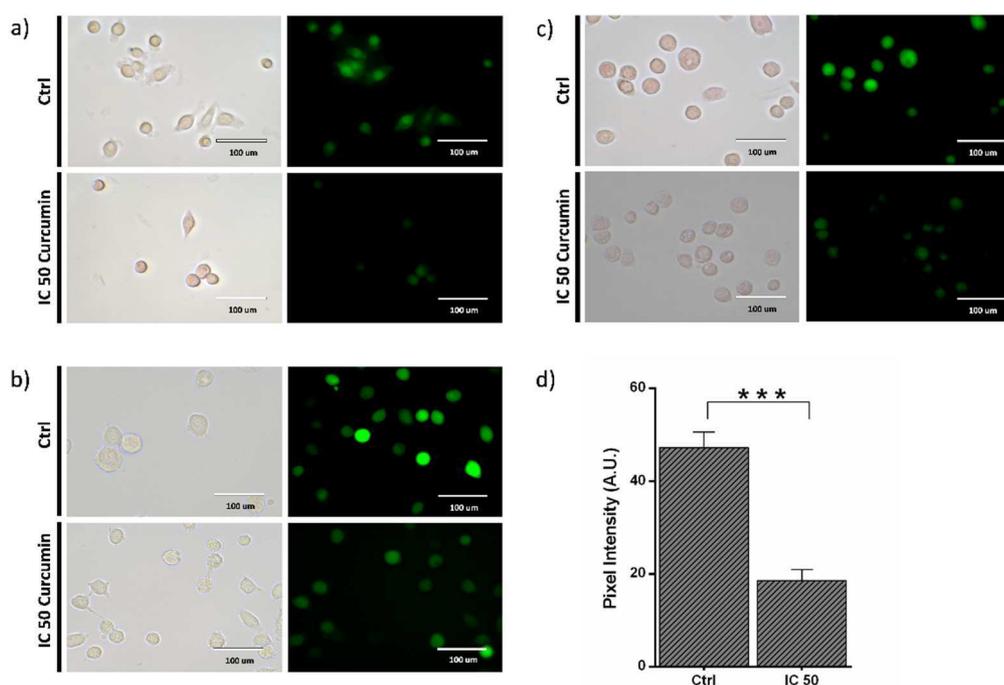
**Figure 20. Effect of genistein on  $\Delta\Psi_m$  in TSCC cells.** PE/CA-PJ49 (a), PE/CA-PJ15 (b) and HSC-3 (c) untreated and after treatment of genistein IC50 value. The graph bars in the bottom panel show the quantitative analysis of  $\Delta\Psi_m$ -related TMRE fluorescence, \*\*\*\*p < 0.0001 (d).

#### 4.6.5 Determination of ROS levels in TSCC cell lines after curcumin and genistein treatments

DCF-fluorescence is a commonly used probe to assess the intracellular redox state.

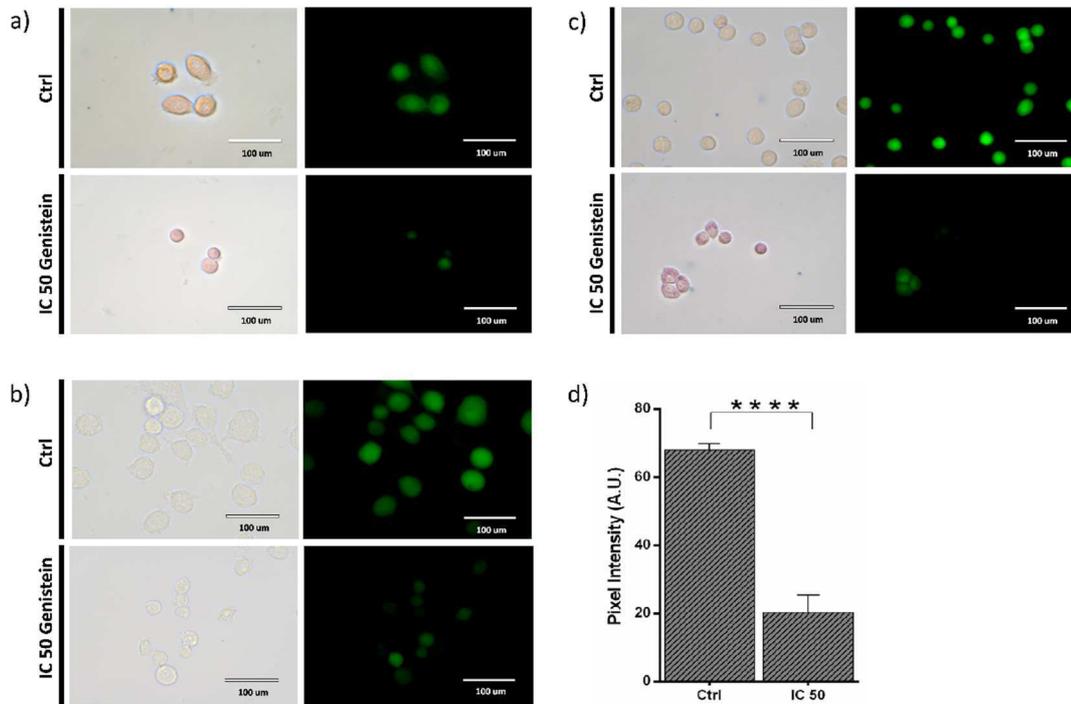
Figure 21 a, b, c shows PE/CA-PJ15, PE/CA-PJ49 and HSC-3 cells treated and untreated with IC50 value of curcumin.

Figure 21 d shows ImageJ analysis of DCF-fluorescence for all cells treated (Pixel Intensity, A.U.). In all cell lines curcumin appears to reduce the levels of ROS (Figure 21 a, b, c, d). In fact, Figure 21 d shows a halving of fluorescence intensity of cells treated with IC 50 value of curcumin compared to controls. ( $p < 0.001$ ). Our data show a reduction in DCF-fluorescence of about 50% in cells treated with IC50 value of curcumin at 24 hours, compared to untreated.



**Figure 21. Intracellular ROS content.** Representative images of PE/CA-PJ49 (a), PE/CA-PJ15 (b) and HSC-3 (c) untreated and treatment with curcumin IC50 value. The graph displays the statistical analysis of the DCF-related mean pixel intensity (d). Significance was calculated with Student's t-test,  $n = 3$  under each condition,  $***p < 0.001$  (d).

In *Figure 22* the reduction of ROS is more significant after treatment with genistein ( $p < 0.0001$ ). DCF-fluorescence of cells treated with IC50 value of genistein is reduced by  $\sim 75\%$  compared to untreated cells (*Figure 22 d*).



**Figure 22. Effect of genistein on ROS content.** PE/CA-PJ49 (a), PE/CA-PJ15 (b) and HSC-3 (c) untreated and treatment with genistein IC50 value. Representative images of three experiments are shown, scale bar 100  $\mu\text{m}$ . The graph bars in the bottom panel show the quantitative analysis of ROS fluorescence (d). Student's t-test: \*\*\*\* $p < 0.0001$  (d).

## 5. DISCUSSION

Surgery, radiation and chemotherapy are the most widely used treatments in head and neck tumors. Despite the use of modern techniques and treatments, the survival rate of patients at 5 years after diagnosis has not changed significantly [142].

For these reason, scientific attention has been focused in recent decades on possible use of natural substances, well know as nutraceuticals, how adjuvant therapy.

Curcumin is a vegetal extract for long used in Asian countries to treat inflammation, liver disorders and rheumatic diseases and, in recent years, it has been confirmed as an antiseptic, analgesic, anti-inflammatory and anti-malarials agent.

To date, literature data demonstrate that curcumin has an antiproliferative effect on tumor cells of breast, lung, prostate [143-145].

The molecular basis of its antitumor effect are attributed to inhibition of transcription factors, growth regulators, adhesion molecules and the genes involved in apoptosis [146].

So, curcumin appears to have a strong inhibitory power against adhesion, proliferation, viability, migration, invasion and apoptosis [147].

In recent decades, many studies demonstrate the powerful action of curcumin in head and neck tumors. *Borges et al* summarize all studies conducted to date on the action of curcumin on head and neck cancer [148]. They clarify that curcumin inhibits cell proliferation and promotes apoptosis. However, in literature there are not many studies to clarify the effect of curcumin on the tongue.

In recent years, scientific community seems to be fascinated by the remarkable properties that natural substances possess and especially by genistein.

It is an isoflavone isolated in 1899 from dyeing of genistra, characterized in 1926 e synthesized for the first time in 1928. It is well known to be present mainly in soybeans, it has a structure similar to estrogen and this characteristic seems to play an important role against the menopause symptoms [99].

Notable are also its antitumor effects, demonstrated especially in breast cancer [126, 149, 150] and in prostate cancer [151, 152].

Even for OSCC, there are studies on genistein action, but they investigate the inhibitory activity of the protein tyrosine kinase [127], its action on tumor angiogenesis [129] and its chemopreventive effects when used with quercetin [130, 131], and biochanin A [133] which are other isoflavones.

*Park et al* have carried out an in vitro study on oral cancer cells, HSC-3 and KB, validating their results on nude mice. They demonstrated that genistein and cetuximab have a synergistic action in the inhibition of EGFR signaling pathway [136].

In literature, there are no papers that demonstrate the effects of genistein, as only treatment, against the tongue carcinoma, considered the most common cancer and the most aggressive of the oral cancers.

In this study, we treated 3 TSCC cell lines with curcumin concentrations of 1, 5, 10, 20 and 50  $\mu\text{M}$  for 72 hours and in vitro effects have shown a dose-dependent correlation. Furthermore, we treated cells with 3 concentrations of genistein, 20, 50 and 100  $\mu\text{M}$ . The choice of concentrations was made by studying the literature data and, in particular, inspired by the work of *Johnson et al*, even if the genistein was used with the biochanin A, the results of oral carcinoma cells were good.

We monitored in real time the growth kinetics for 72 hours after treatment and all CI values analyzed. The adhesion interval was 0-10 hours for both nutraceuticals.

In particular, CI values showed a ~ 25% reduction of cell adhesion in TSCC cells treated with 1  $\mu\text{M}$  of curcumin, at 10 hours. We found a halving of cell adhesion after treatment with 5  $\mu\text{M}$  of curcumin. Furthermore, adhesion of TSCC cells treated with 50  $\mu\text{M}$  of curcumin was reduced by 75% compared to control (*Fig.4 f*). This data showed a high significance,  $p < 0.001$ .

These data demonstrate that curcumin has a strong inhibitory power on adhesion in vitro in the tongue cancer at concentrations even lower than 10  $\mu\text{M}$ .

Even, the integrin expression supports the theory goes that curcumin inhibits adhesion of the tongue cancer cells.

In this study, our goal was to show how genistein inhibits adhesion, proliferation, viability and migration of cancer cells of the tongue. It is known that alterations in

cell adhesion affect the ability of cells to move. In particular, cancer cells should be able to move and migrate in order to spread, and cell adhesion plays an important role in regulating cell movements and the spread of metastases [153].

Our data show that cell adhesion is reduced by almost 25% compared to the control, at genistein concentrations of 20 and 50  $\mu\text{M}$ .

While, the CI values of TSCC cells treated with 100  $\mu\text{M}$  of genistein they show a great reduction of cell adhesion by approximately 60% compared to untreated control, with a significance  $p < 0.01$  (*Fig.6 d*).

This would show that the adhesion of the tongue cancer cells is greatly compromised by treatment with genistein.

*Kingsley et al* have shown that the adhesion of cells of OSCC (CAL 27 and SCC25 cell lines) was clearly reduced when cells were treated with soy protein extract [137] belonging to different isoflavones including genistein [138].

In addition, to confirm the strong action of genistein on adhesion, we also evaluated the expression of vitronectin, famous adhesion protein. Western blotting analysis demonstrates that the inhibition of the vitronectin after treatment with genistein is dose-dependent and it is also downregulated by treatment at 48 and 72 hours with high concentrations (50-100  $\mu\text{M}$ )

Inhibitory effects of genistein are also confirmed by *Skogseth et al*. They demonstrate that genistein is able to reduce the adhesion of prostate cancer cells because it inhibits many adhesion proteins. This is probably because it is a strong inhibitor of tyrosine kinases [154].

Even for *Haier et al* genistein has strong cell adhesion inhibition capacity in colon carcinoma cells. Indeed it also seems to inhibit the expression of vitronectin in HT-29 cell lines [155].

In our study, we also evaluated the effects of curcumin and genistein on cell proliferation. Also in this case, we obtained a reduction of 50% in cell proliferation after treatment with 5-10 $\mu\text{M}$  curcumin at all time points considered. We had showed a strong reduction of proliferation with high significance ( $p < 0.0001$ ) for all tongue

cancer cells treated with concentrations between 20 and 50 $\mu$ M of genistein at all time points.

*Davis et al* showed that genistein also has antiproliferative action on prostate tumor cells [156].

While *Alhasan et al* confirm that genistein has a strong antiproliferative effect in OSCC HN4 cell line treated with 50 $\mu$ M of genistein, even favoring an arrest of the cell cycle and promote apoptosis [128].

In addition, we used the Cell Index values (CI) obtained with the xCELLigence system to calculate the IC50 values of curcumin and genistein at 24, 48 and 72 hours.

IC50 value for curcumin is around 5-10  $\mu$ M of concentration at all time points.

*Xi et al* identified the same IC50 for all head and neck cancers [157], even *Sivanantham et al* noted a dose-depend growth inhibition on cells [158]. However, IC50 of the other tumors are much higher. In fact, 50% inhibiting concentration (IC50) of curcumin in human liver cancer HepG2 cells was  $23.15 \pm 0.37 \mu\text{mol/l}$  [159].

In all cell lines, in average, we have found about 46  $\mu$ M at 24 hours and about 40 $\mu$ M at 48 hours for genistein. However, we have obtained discordant IC50 values at 72 hours in each cell line used. This is probably because PE/CA-PJ49 cells are more aggressive than the other two cell lines used.

*Johnson et al* affirm that the IC50 value for the SCC15 and SCC25 cell lines, OSCC cells, is approximately 50  $\mu$ M [133]. The same IC50 value was attributed to breast cancer cells [160].

All tests done to evaluate the viability have shown that it acts with large inhibitory power and it causes a halved of viability compared to the control for 5-10  $\mu$ M curcumin concentrations at 24 hours and for lower concentrations of 5-20  $\mu$ M of curcumin at 48 hours and about 10-20  $\mu$ M at 72 hours.

In addition, we showed that the tongue carcinoma cells reduce their viability by 50% when treated with concentrations between 20 and 50  $\mu$ M of genistein, almost at all time points.

Even *Alhansa et al* have reported a significant reduction of the vitality and growth of oral squamous cell carcinoma cells after treatment with genistein [128].

*Ye et al* also argue that genistein inhibits the viability and proliferation of cells of Head and Neck cancer by inducing also apoptosis [161].

Scratch assay allowed us to evaluate the effects on cell migration considering various treatment times. After treating the cells with 10  $\mu$ M of curcumin (the mean value of IC50 at 24 hours) of the scratch size is increased proportionally to the increase of incubation time and, above all after 24 hours did not undergo healing contrary to control. In this way, we have demonstrate that curcumin has a big effect on the migration of tumor cells to the tongue and this effect has also for very long treatment times (as 24 hours).

For genistein, one of most significant data, among those obtained, relates to cell migration. In fact, the treatment of all cells with IC50 value of genistein at 24 hours show a very significant reduction of cell migration after 24 hours,  $p < 0.001$ . This shows that genistein can inhibit tongue carcinoma cells migration and so it can have a big power in inhibition of metastasis processes. This power of genistein seems to be confirmed for ovarian cancer and in OSCC [119, 129].

In addition, treatment with curcumin have been shown to inhibit tumorigenesis and promote cell apoptosis.

PAR4 is a pro-apoptotic and tumor suppressor protein that selectively induces apoptosis in cancer cells by activating the extrinsic mechanisms [162]. For this reason, PAR 4 is an attractive therapeutic option. We have demonstrated that level expression of PAR 4 increase after curcumin treatment in tongue carcinoma cells and then curcumin promotes apoptosis.

Survivin is a member of the inhibitor of apoptosis family and it considered an oncogene due its overexpression in cancer cells, making them able to escape to apoptotic stimuli and conventional therapies [163]. Its overexpression is especially know in oral cancer [164].

This study demonstrates that curcumin action leads to reduction of survivin after 24 hours of treatment, and this shows that curcumin induces apoptosis in tongue cancer cells.

OCT4 is a known protein because its overexpression promotes tumorigenesis in different cell types [165], including that of cervical cancer. In tongue cancer cells, turmeric appears to have an action on OCT 4 expression only after long treatments at high concentrations. This shows that it acts on tumorigenesis but it does not act very effectively on OCT 4 pathway. Therefore, we should study how it acts on tumorigenesis more vigorously.

Furthermore, we have shown that genistein is able to strongly inhibits, with treatment more than 50  $\mu\text{M}$ , the expression of OCT4 and survivin in tongue cancer cells, thus reducing the tumorigenesis enough to be considered a good agent against kind of tumor.

This inhibitory action of OCT4 expression seems to be also confirmed in embryonal carcinoma [166, 167], while *Tian et al* have shown that genistein downregulate the expression of survivin in H446 small-cell lung cancer cells [168].

As known, the cell damage is an event able to modify normal cellular homeostasis, leading to effects of varying degrees [169-171].

Among the causes, there are substances considered toxic to cells. So, we have investigated whether curcumin and genistein can be considered as substances which could cause irreversible damage in TSCC cells.

For this reason, we measured the intracellular concentration of  $\text{Ca}^{2+}$  in these cells after administration of 1, 5, 10, 20 and 50  $\mu\text{M}$  of curcumin and 20, 50 and 100  $\mu\text{M}$  of genistein, in addition to the IC50 value at 24 hours, previously calculated. We used samples treated with only vehicles of curcumin and genistein, as controls. For both nutraceuticals, we found an increase in intracellular calcium.

Usually, it is primarily sequestered by endoplasmic reticulum, and to a lesser extend, by mitochondria [172].

The Fould change (FC) found was of  $\sim 0.4$  for treatments with 5 and 10  $\mu\text{M}$  of curcumin, compared to the control, and 0.8 for 50  $\mu\text{M}$  of curcumin. While, the FC was 1.5 for treatments with 100  $\mu\text{M}$  of genistein.

Therefore, this important accumulation determines an opening of the mitochondrial permeability transition pore (MPTP) generating numerous severe mitochondrial damage.

An increase in  $\text{Ca}^{2+}$  is able to generate the activation of phospholipases (which break down membrane phospholipids), proteases (which break down proteins), endonucleases (responsible for DNA and chromatin fragmentation) and ATPase (depletes ATP even further).

Numerous facts show that the increase of intracellular  $\text{Ca}^{2+}$  activates caspases, which trigger apoptosis directly [172].

Certainly, another cause of cellular death is certainly the decrease in ATP levels. In our study, we have shown that ATP was reduced by ~ 94% in the treatment with 50  $\mu\text{M}$  of curcumin and 35% with 100  $\mu\text{M}$  of genistein, compared to control.

The decrease of ATP levels can be considered a cause of cell death because it blocks the transport of electrons in the oxidative phosphorylation. Usually, distinct anticancer drugs reduce the intracellular concentration of ATP [173]. Indeed, as apoptosis progresses, intracellular ATP concentrations decrease.

*Hosseinzadehdehkordi et al* have investigated the effects of curcumin on lung cancer cell lines and they also have studied intracellular ATP content [174]. It seems that curcumin exert synergistic effects in order to reduce the ATP level and induce apoptosis.

However, *Szkudelska et al* have revealed the potent ability of genistein to reduce ATP via attenuation of the metabolic activity of mitochondria [175].

Lactate dehydrogenase (LDH) is a cytosolic enzyme and is released into extracellular space when plasma membrane is damaged [176, 177]. The amount of LDH released is indicative of cytotoxicity.

In our study, curcumin and genistein treatments have resulted in a significant increase in membrane permeability to LDH. The LDH is increased by ~ 4 times for treatments with 50  $\mu\text{M}$  of curcumin ( $p < 0.01$ ).

With genistein treatments, the level of LDH released was 4 times more high than the control, up to a maximum of about 8 times more for cells treated with 100  $\mu$ M of genistein.

*Grabowski et al* have demonstrated, for the first time, that genistein inhibits activities of LDH [178].

*Wu et al* have evaluated the anticancer effect of curcumin on lung cancer cells. They reported a strong damage to the plasma membrane and an increase in intracellular calcium [179].

Furthermore, *Yeh et al* have demonstrated that genistein induces apoptosis in human hepatocellular carcinomas via endoplasmic reticulum stress, mitochondrial insult and damages on plasma membrane [180].

Mitochondrial membrane potential ( $\Delta\Psi_m$ ) is highly interlinked to many mitochondrial processes. The  $\Delta\Psi_m$  controls ATP synthesis, generation of ROS, mitochondrial calcium sequestration, import of proteins into the mitochondrion and dynamics of mitochondrial membranes [181].

The TMRE is a fluorescent probe used to quantify the  $\Delta\Psi_m$ . In our study, we noticed a depolarization of the mitochondrial potential for both treatments, with curcumin and genistein. TMRE-fluorescence of the cells treated with IC50 value of curcumin is reduced by  $\sim 75\%$  compared to untreated cells. While, TMRE-fluorescence of the cells treated with IC50 value of genistein is reduced by  $\sim 80\%$ , compared to untreated cells.

The loss of  $\Delta\Psi_m$  causes an increasing in  $\text{Ca}^{2+}$  in mitochondria and, consequently, release of cytochrome c, inducing apoptosis. In fact, the dissipation of the  $\Delta\Psi_m$  is a clear characteristic of apoptosis.

*Singh et al* demonstrated that curcumin induces apoptosis in breast cancer cells, following a sharp decline in the  $\Delta\Psi_m$  [182].

*Antosiak et al* showed a sharp drop in the  $\Delta\Psi_m$  in ovarian cancer cells treated with genistein [183]. A drastic decrease in fluorescence has been evidenced at 48 hours after treatment for concentrations between 50 and 90  $\mu$ M genistein.

The oxidative stress has a key role in cancer. Reactive oxygen species (ROS) are produced mostly in mitochondria [184]. A possible accumulation of ROS within the cells can cause serious damage [185].

In this study, we quantified ROS in TSCC cells after treatment with nutraceuticals, using a fluorescent probe known as DCF.

Our data show a reduction in DCF-fluorescence of about 50% in cells treated with IC50 value of curcumin at 24 hours, compared to untreated. While, DCF-fluorescence of cells treated with IC50 value of genistein is reduced by ~ 75% compared to untreated cells

From our data, it is assumed that these nutraceuticals have the power to reduce oxidative stress, closely related to the decrease in mitochondrial membrane potential.

This is probably because both retain antioxidant properties, performing a cytotoxic effect for TSCC cells. Previous studies confirm this capacity.

*Serrano et al* have investigated the effects of genistein on breast cancer [186]. In their study they reached the conclusion that genistein protects cells from oxidative damage but leads to a damage of the mitochondrial membrane.

However, regarding the curcumin, there are conflicting data about its action on ROS in cancer, in literature [187, 188].

Probably, curcumin and genistein have cytotoxic effects on cells of TSCC, but they seem to retain their antioxidant action.

The oral bioavailability of curcumin is reduced because of its instability in the intestinal pH, the low absorption, low water solubility, a fast metabolism and rapid elimination [50].

To overcome these problems, studies are in progress to make nanoparticles, liposomes, micelles, and phospholipid complexes with curcumin [51].

Examples are the bioenhancer as piperine, an alkaloid extracted from black pepper able to facilitate passage through cellular membranes, increasing absorption of curcumin by the cells.

*Zhang et al* developed a co-delivery system for doxorubicin (Dox), a pro-apoptotic drug, and curcumin in pH-sensitive nanoparticles (NPs) constituted with

amphiphilic poly( $\beta$ -amino ester) copolymer, a promising strategy to effectively inhibit cancer and cell progression in a synergistic manner [189].

Phospholipid complexation is one of the most prevalent strategies to improve the solubility of drug, increase drug-loading capacity and control the release rate of drug. It is well known that phospholipid is a vital component of cell membrane with good biocompatibility/biodegradability and low toxicity. At present, drug-phospholipid complex has received significant attention. *Xie et al* developed A novel CUR-soybean phosphatidylcholine (SPC) complex and CUR-SPC complex self-assembled nanoparticles (CUR-SPC NPs), prepared by a co-solvent method and a nanoprecipitation method [190].

Also for genistein there are many problems of biodisponibility. Furthermore, to obtain the optimal absorption it is better to bypass the intestine digestion giving aglycone genistein as pure active principle.

To potentiate the anti-tumor effects of genistein, many researchers are focusing on the construction of delivery systems capable of acting directly on the tumor. A very interesting study is those of *Gavin et al*, which devised an adhesive mucus system to be inserted in the oral cavity in an area where the tumor is macroscopically evident. This tablet containing genistein was completely biocompatible and gave also excellent therapeutic results in the tumor mass present [139].

This opened an entirely new scenario to improve cancer therapies anti OSCC. Therefore, we believe that curcumin and genistein may be used for the treatment of TSCC, utilizing them as adjuvants along with chemotherapeutic drugs. Additionally, recent studies are well hoped for their possible in situ use to overcome the difficulties of intestinal absorption.

## 6. CONCLUSION

Tongue cancer is a form of cancer characterized by flat squamous cells on the surface of the tongue (TSCC).

Treatment for tongue cancer typically involves surgery to remove the cancer. Chemotherapy, radiation therapy and targeted drug therapy may also be recommended.

Treatment for advanced tongue cancers can impact your ability to speak and eat.

However, there are still no therapy without serious side effects for patients. Curcumin is a nutraceutical with many beneficial effects for human cells and many studies have shown its effects against various tumors. While, genistein is a natural isoflavone whose effects have been fully studied in many neoplastic diseases. However, genistein's antitumor properties have been evaluated, especially in combination with other isoflavones, against oral squamous cell carcinoma and there are no data in literature that have clarified its effect, in particular in the tongue carcinoma.

In our study, we can confirm that curcumin and genistein act on tongue cancer cells by inhibiting efficaciously cell viability, proliferation, adhesion, migration and tumorigenesis.

This shows that they have excellent anti-cancer properties that can be exploited for this type of tumor.

Our data suggest that curcumin and genistein can induce biological and structural damages in TSCC cells, causing apoptosis.

Therefore, future in vivo studies are needed to validate these results. In addition, we believe that these nutraceuticals can be easily applied in situ, since the carcinoma of the tongue is localized in an easy access area, in this way it would be possible to increase the bioavailability of curcumin and genistein.

## 7. REFERENCES

1. Petersen, P.E., *Oral cancer prevention and control--the approach of the World Health Organization*. Oral Oncol, 2009. **45**(4-5): p. 454-60.
2. Jemal, A., et al., *Global cancer statistics*. CA Cancer J Clin, 2011. **61**(2): p. 69-90.
3. de Camargo Cancela, M., D.L. de Souza, and M.P. Curado, *International incidence of oropharyngeal cancer: a population-based study*. Oral Oncol, 2012. **48**(6): p. 484-90.
4. de Camargo Cancela, M., et al., *Oral cavity cancer in developed and in developing countries: population-based incidence*. Head Neck, 2010. **32**(3): p. 357-67.
5. Liu, S.Y., et al., *Surgical outcomes and prognostic factors of oral cancer associated with betel quid chewing and tobacco smoking in Taiwan*. Oral Oncol, 2010. **46**(4): p. 276-82.
6. Nemes, J.A., et al., *Oral cancer report from Northeastern Hungary*. Pathol Oncol Res, 2008. **14**(1): p. 85-92.
7. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012*. Int J Cancer, 2015. **136**(5): p. E359-86.
8. Shield, K.D., et al., *The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012*. CA Cancer J Clin, 2017. **67**(1): p. 51-64.
9. Bertolus, C., et al., *Clinical relevance of systematic human papillomavirus (HPV) diagnosis in oral squamous cell carcinoma*. Infect Agent Cancer, 2012. **7**(1): p. 13.
10. Johnson, N.W., P. Jayasekara, and A.A. Amarasinghe, *Squamous cell carcinoma and precursor lesions of the oral cavity: epidemiology and aetiology*. Periodontol 2000, 2011. **57**(1): p. 19-37.
11. Estilo, C.L., et al., *Oral tongue cancer gene expression profiling: Identification of novel potential prognosticators by oligonucleotide microarray analysis*. BMC Cancer, 2009. **9**: p. 11.
12. Watkinson JC, G.M., Wilson JA *Stell and Marans head and neck surgery, 4th edn. Reed Educational and Professional Publishing Ltd., Oxford, pp 288–301, 68–73. 2000.*
13. Mc Gregor IA, M.G.F.T., *In: Cancers of the face and mouth: pathology and management for surgeons, 1st edn. Longman Group Limited, New York, Churchill Livingstone, pp 474, 477. 1986.*
14. Tongue, T.c.o.C.o.t., *Its various causes along with symptoms and Treatments. (Internet). Cancer-Info-Guide.com/ (updated 2008) Web. <http://www.cancer-infoguide.com/tonque-cancer.Html>. Accessed 20 Feb 2009. 2008.*
15. Zandoni, D.K., et al., *A Proposal to Redefine Close Surgical Margins in Squamous Cell Carcinoma of the Oral Tongue*. JAMA Otolaryngol Head Neck Surg, 2017.
16. Zhang, P., et al., *xCT expression modulates cisplatin resistance in Tca8113 tongue carcinoma cells*. Oncol Lett, 2016. **12**(1): p. 307-314.
17. Ansell, A., et al., *Epidermal growth factor is a potential biomarker for poor cetuximab response in tongue cancer cells*. J Oral Pathol Med, 2016. **45**(1): p. 9-16.
18. Elad, S. and Y. Zadik, *Chronic oral mucositis after radiotherapy to the head and neck: a new insight*. Support Care Cancer, 2016. **24**(11): p. 4825-30.

19. Lubbers, H.T., A.L. Kruse, and D.A. Ettlin, *Postradiation xerostomia and oral pain*. J Am Dent Assoc, 2014. **145**(9): p. 964-5.
20. Kalra, E.K., *Nutraceutical--definition and introduction*. AAPS PharmSci, 2003. **5**(3): p. E25.
21. Roudebush, P., D.J. Davenport, and B.J. Novotny, *The use of nutraceuticals in cancer therapy*. Vet Clin North Am Small Anim Pract, 2004. **34**(1): p. 249-69, viii.
22. Zhao, J., *Nutraceuticals, nutritional therapy, phytonutrients, and phytotherapy for improvement of human health: a perspective on plant biotechnology application*. Recent Pat Biotechnol, 2007. **1**(1): p. 75-97.
23. Whitman, M., *Understanding the perceived need for complementary and alternative nutraceuticals: lifestyle issues*. Clin J Oncol Nurs, 2001. **5**(5): p. 190-4.
24. Surh, Y.J., *Cancer chemoprevention with dietary phytochemicals*. Nat Rev Cancer, 2003. **3**(10): p. 768-80.
25. Grimble, R.F., *Nutritional therapy for cancer cachexia*. Gut, 2003. **52**(10): p. 1391-2.
26. Takahashi, M., et al., *Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) blocks the chemotaxis of neutrophils by inhibiting signal transduction through IL-8 receptors*. Mediators Inflamm, 2007. **2007**: p. 10767.
27. Jurenka, J.S., *Anti-inflammatory properties of curcumin, a major constituent of Curcuma longa: a review of preclinical and clinical research*. Altern Med Rev, 2009. **14**(2): p. 141-53.
28. Wilken, R., et al., *Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma*. Mol Cancer, 2011. **10**: p. 12.
29. Miłobędzka, J.v.K., St.; Lampe, V., *Zur Kenntnis des Curcumins*. Berichte der deutschen chemischen Gesellschaft, 1910. **43** (2): p. 2163-70.
30. Sharma, R.A., A.J. Gescher, and W.P. Steward, *Curcumin: the story so far*. Eur J Cancer, 2005. **41**(13): p. 1955-68.
31. Aggarwal, B.B., L. Deb, and S. Prasad, *Curcumin differs from tetrahydrocurcumin for molecular targets, signaling pathways and cellular responses*. Molecules, 2014. **20**(1): p. 185-205.
32. Masuda, T., et al., *Chemical studies on antioxidant mechanism of curcuminoid: analysis of radical reaction products from curcumin*. J Agric Food Chem, 1999. **47**(1): p. 71-7.
33. Wang, Y.J., et al., *Stability of curcumin in buffer solutions and characterization of its degradation products*. J Pharm Biomed Anal, 1997. **15**(12): p. 1867-76.
34. Prasad, K., et al., *Prevention of Hypercholesterolemic Atherosclerosis by Garlic, an Antioxidant*. J Cardiovasc Pharmacol Ther, 1997. **2**(4): p. 309-320.
35. Sandur, S.K., et al., *Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin and turmerones differentially regulate anti-inflammatory and anti-proliferative responses through a ROS-independent mechanism*. Carcinogenesis, 2007. **28**(8): p. 1765-73.
36. Gupta, S.C., G. Kismali, and B.B. Aggarwal, *Curcumin, a component of turmeric: from farm to pharmacy*. Biofactors, 2013. **39**(1): p. 2-13.
37. Kumar, A., et al., *Conundrum and therapeutic potential of curcumin in drug delivery*. Crit Rev Ther Drug Carrier Syst, 2010. **27**(4): p. 279-312.

38. Ammon, H.P. and M.A. Wahl, *Pharmacology of Curcuma longa*. *Planta Med*, 1991. **57**(1): p. 1-7.
39. Lev-Ari, S., et al., *Curcumin synergistically potentiates the growth-inhibitory and pro-apoptotic effects of celecoxib in osteoarthritis synovial adherent cells*. *Rheumatology (Oxford)*, 2006. **45**(2): p. 171-7.
40. Bhullar, K.S., et al., *Curcumin and its carbocyclic analogs: structure-activity in relation to antioxidant and selected biological properties*. *Molecules*, 2013. **18**(5): p. 5389-404.
41. Somchit, M., et al., *Demethoxycurcumin from Curcuma longa rhizome suppresses iNOS induction in an in vitro inflamed human intestinal mucosa model*. *Asian Pac J Cancer Prev*, 2014. **15**(4): p. 1807-10.
42. Surh, Y.J. and K.S. Chun, *Cancer chemopreventive effects of curcumin*. *Adv Exp Med Biol*, 2007. **595**: p. 149-72.
43. Surh, Y.J., et al., *Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation*. *Mutat Res*, 2001. **480-481**: p. 243-68.
44. Trump, B.F., et al., *Cell calcium, cell injury and cell death*. *Environ Health Perspect*, 1984. **57**: p. 281-7.
45. Hong, J., et al., *Modulation of arachidonic acid metabolism by curcumin and related beta-diketone derivatives: effects on cytosolic phospholipase A(2), cyclooxygenases and 5-lipoxygenase*. *Carcinogenesis*, 2004. **25**(9): p. 1671-9.
46. Li, W., N.C. Suwanwela, and S. Patumraj, *Curcumin by down-regulating NF-kB and elevating Nrf2, reduces brain edema and neurological dysfunction after cerebral I/R*. *Microvasc Res*, 2016. **106**: p. 117-27.
47. Fan, Z., et al., *Anti-inflammatory and antioxidant effects of curcumin on acute lung injury in a rodent model of intestinal ischemia reperfusion by inhibiting the pathway of NF-Kb*. *Int J Clin Exp Pathol*, 2015. **8**(4): p. 3451-9.
48. Basha, R., et al., *Small molecule tolfenamic acid and dietary spice curcumin treatment enhances antiproliferative effect in pancreatic cancer cells via suppressing Sp1, disrupting NF-kB translocation to nucleus and cell cycle phase distribution*. *J Nutr Biochem*, 2016. **31**: p. 77-87.
49. Huang, S.L., et al., *Curcuminoids Modulate the PKCdelta/NADPH Oxidase/Reactive Oxygen Species Signaling Pathway and Suppress Matrix Invasion during Monocyte-Macrophage Differentiation*. *J Agric Food Chem*, 2015. **63**(40): p. 8838-48.
50. Pulido-Moran, M., et al., *Curcumin and Health*. *Molecules*, 2016. **21**(3): p. 264.
51. Prasad, S., A.K. Tyagi, and B.B. Aggarwal, *Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice*. *Cancer Res Treat*, 2014. **46**(1): p. 2-18.
52. Hsu, C.H. and A.L. Cheng, *Clinical studies with curcumin*. *Adv Exp Med Biol*, 2007. **595**: p. 471-80.
53. Jiao, Y., et al., *Curcumin, a cancer chemopreventive and chemotherapeutic agent, is a biologically active iron chelator*. *Blood*, 2009. **113**(2): p. 462-9.
54. Rasyid, A. and A. Lelo, *The effect of curcumin and placebo on human gall-bladder function: an ultrasound study*. *Aliment Pharmacol Ther*, 1999. **13**(2): p. 245-9.

55. Maheshwari, R.K., et al., *Multiple biological activities of curcumin: a short review*. Life Sci, 2006. **78**(18): p. 2081-7.
56. Yance, D.R., Jr. and S.M. Sagar, *Targeting angiogenesis with integrative cancer therapies*. Integr Cancer Ther, 2006. **5**(1): p. 9-29.
57. Karunagaran, D., R. Rashmi, and T.R. Kumar, *Induction of apoptosis by curcumin and its implications for cancer therapy*. Curr Cancer Drug Targets, 2005. **5**(2): p. 117-29.
58. Helson, L., *Curcumin (diferuloylmethane) delivery methods: a review*. Biofactors, 2013. **39**(1): p. 21-6.
59. Remaschi, G., et al., *A rare cause of osteomyelitis with a rare genetic mutation: when Mycobacterium doesn't only mean tuberculosis*. Int J Tuberc Lung Dis, 2015. **19**(8): p. 999-1000.
60. Salvoli, S., et al., *Curcumin in cell death processes: a challenge for CAM of age-related pathologies*. Evid Based Complement Alternat Med, 2007. **4**(2): p. 181-90.
61. Yang, J.Y., et al., *Curcumin Inhibits STAT3 Signaling in the Colon of Dextran Sulfate Sodium-treated Mice*. J Cancer Prev, 2013. **18**(2): p. 186-91.
62. Vallianou, N.G., et al., *Potential anticancer properties and mechanisms of action of curcumin*. Anticancer Res, 2015. **35**(2): p. 645-51.
63. Liu, Y., et al., *Curcumin inhibits growth of human breast cancer cells through demethylation of DLC1 promoter*. Mol Cell Biochem, 2017. **425**(1-2): p. 47-58.
64. Zhao, G., et al., *Curcumin induces autophagy, inhibits proliferation and invasion by downregulating AKT/mTOR signaling pathway in human melanoma cells*. Oncol Rep, 2016. **35**(2): p. 1065-74.
65. Yang, J., et al., *Effect of curcumin on Bcl-2 and Bax expression in nude mice prostate cancer*. Int J Clin Exp Pathol, 2015. **8**(8): p. 9272-8.
66. Jiao, D.M., et al., *Exploration of inhibitory mechanisms of curcumin in lung cancer metastasis using a miRNA- transcription factor-target gene network*. PLoS One, 2017. **12**(2): p. e0172470.
67. Memtsa, P.T., et al., *Validity and reliability of the Greek version of the xerostomia questionnaire in head and neck cancer patients*. Support Care Cancer, 2017. **25**(3): p. 847-853.
68. Wang, Q., et al., *Curcumin suppresses epithelial-to-mesenchymal transition and metastasis of pancreatic cancer cells by inhibiting cancer-associated fibroblasts*. Am J Cancer Res, 2017. **7**(1): p. 125-133.
69. LoTempio, M.M., et al., *Curcumin suppresses growth of head and neck squamous cell carcinoma*. Clin Cancer Res, 2005. **11**(19 Pt 1): p. 6994-7002.
70. Chakravarti, N., et al., *Differential inhibition of protein translation machinery by curcumin in normal, immortalized, and malignant oral epithelial cells*. Cancer Prev Res (Phila), 2010. **3**(3): p. 331-8.
71. Khafif, A., et al., *Curcumin: a potential radio-enhancer in head and neck cancer*. Laryngoscope, 2009. **119**(10): p. 2019-26.
72. Rinaldi, A.L., et al., *Curcumin activates the aryl hydrocarbon receptor yet significantly inhibits (-)-benzo(a)pyrene-7R-trans-7,8-dihydrodiol bioactivation in oral squamous cell carcinoma cells and oral mucosa*. Cancer Res, 2002. **62**(19): p. 5451-6.

73. Elattar, T.M. and A.S. Virji, *The inhibitory effect of curcumin, genistein, quercetin and cisplatin on the growth of oral cancer cells in vitro*. *Anticancer Res*, 2000. **20**(3A): p. 1733-8.
74. Lopez-Jornet, P., F. Camacho-Alonso, and F. Gomez-Garcia, *Effect of curcumin and irradiation in PE/CA-PJ15 oral squamous cell carcinoma*. *Acta Odontol Scand*, 2011. **69**(5): p. 269-73.
75. Kim, J.Y., et al., *Curcumin-induced autophagy contributes to the decreased survival of oral cancer cells*. *Arch Oral Biol*, 2012. **57**(8): p. 1018-25.
76. Xiao, C., et al., *Curcumin inhibits oral squamous cell carcinoma SCC-9 cells proliferation by regulating miR-9 expression*. *Biochem Biophys Res Commun*, 2014. **454**(4): p. 576-80.
77. Zhen, L., et al., *Curcumin inhibits oral squamous cell carcinoma proliferation and invasion via EGFR signaling pathways*. *Int J Clin Exp Pathol*, 2014. **7**(10): p. 6438-46.
78. Lee, A.Y., et al., *Curcumin Inhibits Invasiveness and Epithelial-Mesenchymal Transition in Oral Squamous Cell Carcinoma Through Reducing Matrix Metalloproteinase 2, 9 and Modulating p53-E-Cadherin Pathway*. *Integr Cancer Ther*, 2015. **14**(5): p. 484-90.
79. Li, N., et al., *Inhibition of 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamsters by tea and curcumin*. *Carcinogenesis*, 2002. **23**(8): p. 1307-13.
80. Lin, Y.C., et al., *Therapeutic efficacy evaluation of curcumin on human oral squamous cell carcinoma xenograft using multimodalities of molecular imaging*. *Am J Chin Med*, 2010. **38**(2): p. 343-58.
81. Rao, C.V., B. Simi, and B.S. Reddy, *Inhibition by dietary curcumin of azoxymethane-induced ornithine decarboxylase, tyrosine protein kinase, arachidonic acid metabolism and aberrant crypt foci formation in the rat colon*. *Carcinogenesis*, 1993. **14**(11): p. 2219-25.
82. Hu, A., et al., *Curcumin suppresses invasiveness and vasculogenic mimicry of squamous cell carcinoma of the larynx through the inhibition of JAK-2/STAT-3 signaling pathway*. *Am J Cancer Res*, 2015. **5**(1): p. 278-88.
83. Lee, H.M., et al., *Copper supplementation amplifies the anti-tumor effect of curcumin in oral cancer cells*. *Phytomedicine*, 2016. **23**(12): p. 1535-1544.
84. Kochs, G. and H. Grisebach, *Enzymic synthesis of isoflavones*. *Eur J Biochem*, 1986. **155**(2): p. 311-8.
85. Fukui, K. and M. Nakayama, *A new method for the synthesis of isoflavones*. *Bull Chem Soc Jpn*, 1965. **38**(10): p. 1803-4.
86. Baker, W. and W.D. Ollis, *A new synthesis of isoflavones*. *Nature*, 1952. **169**(4304): p. 706.
87. Sathe, V.R. and K. Venkataraman, *A new reaction for the synthesis of chromones and isoflavones*. *Curr Sci*, 1949. **18**(10): p. 373.
88. Murphy, P.A., et al., *Group B saponins in soy products in the U.S. Department of Agriculture--Iowa State University isoflavone database and their comparison with isoflavone contents*. *J Agric Food Chem*, 2008. **56**(18): p. 8534-40.

89. Smets, L.A., et al., *BCL-2 expression and mitochondrial activity in leukemic cells with different sensitivity to glucocorticoid-induced apoptosis*. Blood, 1994. **84**(5): p. 1613-9.
90. Aymeric, L., et al., *Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity*. Cancer Res, 2010. **70**(3): p. 855-8.
91. Anjani, G., et al., *Solubilization of genistein by caseinate micellar system*. J Oleo Sci, 2014. **63**(4): p. 413-22.
92. Buddhiranon, S. and T. Kyu, *Solubilization of genistein in poly(oxyethylene) through eutectic crystal melting*. J Phys Chem B, 2012. **116**(27): p. 7795-802.
93. Ganai, A.A. and H. Farooqi, *Bioactivity of genistein: A review of in vitro and in vivo studies*. Biomed Pharmacother, 2015. **76**: p. 30-8.
94. Jefferson, W.N. and C.J. Williams, *Circulating levels of genistein in the neonate, apart from dose and route, predict future adverse female reproductive outcomes*. Reprod Toxicol, 2011. **31**(3): p. 272-9.
95. Yoon, K., et al., *Estrogenic endocrine-disrupting chemicals: molecular mechanisms of actions on putative human diseases*. J Toxicol Environ Health B Crit Rev, 2014. **17**(3): p. 127-74.
96. Spagnuolo, C., et al., *Genistein and cancer: current status, challenges, and future directions*. Adv Nutr, 2015. **6**(4): p. 408-19.
97. Xiong, P., et al., *Design, Synthesis, and Evaluation of Genistein Analogues as Anti-Cancer Agents*. Anticancer Agents Med Chem, 2015. **15**(9): p. 1197-203.
98. Akiyama, T., et al., *Genistein, a specific inhibitor of tyrosine-specific protein kinases*. J Biol Chem, 1987. **262**(12): p. 5592-5.
99. Marini, H., et al., *Effects of the phytoestrogen genistein on bone metabolism in osteopenic postmenopausal women: a randomized trial*. Ann Intern Med, 2007. **146**(12): p. 839-47.
100. Abdi, F., et al., *Effects of phytoestrogens on bone mineral density during the menopause transition: a systematic review of randomized, controlled trials*. Climacteric, 2016. **19**(6): p. 535-545.
101. Park, C.Y. and C.M. Weaver, *Vitamin D interactions with soy isoflavones on bone after menopause: a review*. Nutrients, 2012. **4**(11): p. 1610-21.
102. Meyer, M.R. and M. Barton, *Estrogens and Coronary Artery Disease: New Clinical Perspectives*. Adv Pharmacol, 2016. **77**: p. 307-60.
103. Carmignani, L.O., et al., *The effect of soy dietary supplement and low dose of hormone therapy on main cardiovascular health biomarkers: a randomized controlled trial*. Rev Bras Ginecol Obstet, 2014. **36**(6): p. 251-8.
104. Bitto, A., et al., *Visfatin correlates with hot flashes in postmenopausal women with metabolic syndrome: effects of genistein*. Endocrine, 2017. **55**(3): p. 899-906.
105. Ganesan, P. and D.K. Choi, *Current application of phytocompound-based nanocosmeceuticals for beauty and skin therapy*. Int J Nanomedicine, 2016. **11**: p. 1987-2007.
106. Terra, V.A., et al., *Genistein prevents ultraviolet B radiation-induced nitrosative skin injury and promotes cell proliferation*. J Photochem Photobiol B, 2015. **144**: p. 20-7.
107. Sureda, A., et al., *Hypotensive effects of genistein: From chemistry to medicine*. Chem Biol Interact, 2017. **268**: p. 37-46.

108. Devi, K.P., et al., *Molecular and Therapeutic Targets of Genistein in Alzheimer's Disease*. Mol Neurobiol, 2016.
109. Wang, Y.X., et al., *Genistein inhibits hypoxia, ischemic-induced death, and apoptosis in PC12 cells*. Environ Toxicol Pharmacol, 2017. **50**: p. 227-233.
110. Saleh, D.O. and D.F. Mansour, *Ovario-protective effects of genistein against cyclophosphamide toxicity in rats: Role of anti-mullerian hormone and oestradiol*. Eur J Pharmacol, 2016. **789**: p. 163-71.
111. Yan, L. and E.L. Spitznagel, *Soy consumption and prostate cancer risk in men: a revisit of a meta-analysis*. Am J Clin Nutr, 2009. **89**(4): p. 1155-63.
112. Maskarinec, G., et al., *Soy Food Intake and Biomarkers of Breast Cancer Risk: Possible Difference in Asian Women?* Nutr Cancer, 2017. **69**(1): p. 146-153.
113. Russo, M., et al., *Understanding genistein in cancer: The "good" and the "bad" effects: A review*. Food Chem, 2016. **196**: p. 589-600.
114. Russo, M., et al., *Phytochemicals in cancer prevention and therapy: truth or dare?* Toxins (Basel), 2010. **2**(4): p. 517-51.
115. Song, L.H., H.L. Yan, and D.L. Cai, *Protective effects of soybean isoflavone against gamma-irradiation induced damages in mice*. J Radiat Res, 2006. **47**(2): p. 157-65.
116. Song, L., et al., *Radioprotective effects of genistein on HL-7702 cells via the inhibition of apoptosis and DNA damage*. Cancer Lett, 2015. **366**(1): p. 100-11.
117. Obiorah, I.E., P. Fan, and V.C. Jordan, *Breast cancer cell apoptosis with phytoestrogens is dependent on an estrogen-deprived state*. Cancer Prev Res (Phila), 2014. **7**(9): p. 939-49.
118. Pons, D.G., et al., *The Phytoestrogen Genistein Affects Breast Cancer Cells Treatment Depending on the ERalpha/ERbeta Ratio*. J Cell Biochem, 2016. **117**(1): p. 218-29.
119. Kim, Y.S., K.C. Choi, and K.A. Hwang, *Genistein suppressed epithelial-mesenchymal transition and migration efficacies of BG-1 ovarian cancer cells activated by estrogenic chemicals via estrogen receptor pathway and downregulation of TGF-beta signaling pathway*. Phytomedicine, 2015. **22**(11): p. 993-9.
120. Luo, H., et al., *Inhibition of cell growth and VEGF expression in ovarian cancer cells by flavonoids*. Nutr Cancer, 2008. **60**(6): p. 800-9.
121. Zhu, J., et al., *Genistein induces apoptosis by stabilizing intracellular p53 protein through an APE1-mediated pathway*. Free Radic Biol Med, 2015. **86**: p. 209-18.
122. Qin, J., et al., *Genistein induces activation of the mitochondrial apoptosis pathway by inhibiting phosphorylation of Akt in colorectal cancer cells*. Pharm Biol, 2016. **54**(1): p. 74-9.
123. Gadgeel, S.M., et al., *Genistein enhances the effect of epidermal growth factor receptor tyrosine kinase inhibitors and inhibits nuclear factor kappa B in nonsmall cell lung cancer cell lines*. Cancer, 2009. **115**(10): p. 2165-76.
124. Wang, Y., et al., *Genistein sensitizes bladder cancer cells to HCPT treatment in vitro and in vivo via ATM/NF-kappaB/IKK pathway-induced apoptosis*. PLoS One, 2013. **8**(1): p. e50175.
125. Adjakly, M., et al., *Genistein and daidzein: different molecular effects on prostate cancer*. Anticancer Res, 2013. **33**(1): p. 39-44.

126. Zhao, Q., et al., *Genistein targets the cancerous inhibitor of PP2A to induce growth inhibition and apoptosis in breast cancer cells*. *Int J Oncol*, 2016. **49**(3): p. 1203-10.
127. Sugiura, T., et al., *Effects of human fibroblasts on invasiveness of oral cancer cells in vitro: isolation of a chemotactic factor from human fibroblasts*. *Int J Cancer*, 1996. **68**(6): p. 774-81.
128. Alhasan, S.A., J.F. Ensley, and F.H. Sarkar, *Genistein induced molecular changes in a squamous cell carcinoma of the head and neck cell line*. *Int J Oncol*, 2000. **16**(2): p. 333-8.
129. Myoung, H., et al., *Anti-cancer effect of genistein in oral squamous cell carcinoma with respect to angiogenesis and in vitro invasion*. *Cancer Sci*, 2003. **94**(2): p. 215-20.
130. Walle, T., et al., *Flavonoid glucosides are hydrolyzed and thus activated in the oral cavity in humans*. *J Nutr*, 2005. **135**(1): p. 48-52.
131. Browning, A.M., U.K. Walle, and T. Walle, *Flavonoid glycosides inhibit oral cancer cell proliferation--role of cellular uptake and hydrolysis to the aglycones*. *J Pharm Pharmacol*, 2005. **57**(8): p. 1037-42.
132. Yang, Y., Z.T. Zhou, and J.P. Ge, *Effect of genistein on DMBA-induced oral carcinogenesis in hamster*. *Carcinogenesis*, 2006. **27**(3): p. 578-83.
133. Johnson, T.L., et al., *Inhibition of Cell Proliferation and MAP Kinase and Akt Pathways in Oral Squamous cell Carcinoma by Genistein and Biochanin A*. *Evid Based Complement Alternat Med*, 2010. **7**(3): p. 351-8.
134. Naruse, T., et al., *Cetuximab for the treatment of locally advanced and recurrent/metastatic oral cancer: An investigation of distant metastasis*. *Mol Clin Oncol*, 2016. **5**(2): p. 246-252.
135. Lyu, J., et al., *Predictive value of pAKT/PTEN expression in oral squamous cell carcinoma treated with cetuximab-based chemotherapy*. *Oral Surg Oral Med Oral Pathol Oral Radiol*, 2016. **121**(1): p. 67-72.
136. Park, S.J., et al., *Combined cetuximab and genistein treatment shows additive anti-cancer effect on oral squamous cell carcinoma*. *Cancer Lett*, 2010. **292**(1): p. 54-63.
137. Vainshtein, J.M., et al., *Impact of xerostomia on dysphagia after chemotherapy-intensity-modulated radiotherapy for oropharyngeal cancer: Prospective longitudinal study*. *Head Neck*, 2016. **38 Suppl 1**: p. E1605-12.
138. Kingsley, K., et al., *Soy protein extract (SPE) exhibits differential in vitro cell proliferation effects in oral cancer and normal cell lines*. *J Diet Suppl*, 2011. **8**(2): p. 169-88.
139. Gavin, A., et al., *Layered nanoemulsions as mucoadhesive buccal systems for controlled delivery of oral cancer therapeutics*. *Int J Nanomedicine*, 2015. **10**: p. 1569-84.
140. Pan, T., et al., *In vitro cytotoxicity assessment based on KC(50) with real-time cell analyzer (RTCA) assay*. *Comput Biol Chem*, 2013. **47**: p. 113-20.
141. Zhang, M., et al., *Measuring cytotoxicity: a new perspective on LC50*. *Anticancer Res*, 2007. **27**(1A): p. 35-8.
142. Pezzuto, F., et al., *Update on Head and Neck Cancer: Current Knowledge on Epidemiology, Risk Factors, Molecular Features and Novel Therapies*. *Oncology*, 2015. **89**(3): p. 125-36.

143. Jiang, M., et al., *Curcumin induces cell death and restores tamoxifen sensitivity in the antiestrogen-resistant breast cancer cell lines MCF-7/LCC2 and MCF-7/LCC9*. *Molecules*, 2013. **18**(1): p. 701-20.
144. Tsai, J.R., et al., *Curcumin Inhibits Non-Small Cell Lung Cancer Cells Metastasis through the Adiponectin/NF-kappaB/MMPs Signaling Pathway*. *PLoS One*, 2015. **10**(12): p. e0144462.
145. Zhou, D.Y., et al., *Pyridine analogues of curcumin exhibit high activity for inhibiting CWR-22Rv1 human prostate cancer cell growth and androgen receptor activation*. *Oncol Lett*, 2016. **11**(6): p. 4160-4166.
146. Aggarwal, B.B., A. Kumar, and A.C. Bharti, *Anticancer potential of curcumin: preclinical and clinical studies*. *Anticancer Res*, 2003. **23**(1A): p. 363-98.
147. Mareel, M. and I. Madani, *Tumour-associated host cells participating at invasion and metastasis : targets for therapy?* *Acta Chir Belg*, 2006. **106**(6): p. 635-40.
148. Borges, G.A., et al., *In vivo and in vitro effects of curcumin on head and neck carcinoma: a systematic review*. *J Oral Pathol Med*, 2017. **46**(1): p. 3-20.
149. Avci, C.B., et al., *Genistein-induced mir-23b expression inhibits the growth of breast cancer cells*. *Contemp Oncol (Pozn)*, 2015. **19**(1): p. 32-5.
150. Orlando, L., P. Schiavone, and S. Cinieri, *Genistein: the future of prevention and treatment of breast cancer?* *Cancer Biol Ther*, 2011. **11**(10): p. 918-20.
151. Pavese, J.M., S.N. Krishna, and R.C. Bergan, *Genistein inhibits human prostate cancer cell detachment, invasion, and metastasis*. *Am J Clin Nutr*, 2014. **100** Suppl 1: p. 431S-6S.
152. Chiyomaru, T., et al., *Genistein inhibits prostate cancer cell growth by targeting miR-34a and oncogenic HOTAIR*. *PLoS One*, 2013. **8**(8): p. e70372.
153. Khalili, A.A. and M.R. Ahmad, *A Review of Cell Adhesion Studies for Biomedical and Biological Applications*. *Int J Mol Sci*, 2015. **16**(8): p. 18149-84.
154. Skogseth, H., et al., *Tyrosine kinase inhibitors alter adhesivity of prostatic cancer cells to extracellular matrix components*. *APMIS*, 2006. **114**(3): p. 225-33.
155. Haier, J., M. Nasralla, and G.L. Nicolson, *Influence of phosphotyrosine kinase inhibitors on adhesive properties of highly and poorly metastatic HT-29 colon carcinoma cells to collagen*. *Int J Colorectal Dis*, 1999. **14**(2): p. 119-27.
156. Davis, J.N., O. Kucuk, and F.H. Sarkar, *Expression of prostate-specific antigen is transcriptionally regulated by genistein in prostate cancer cells*. *Mol Carcinog*, 2002. **34**(2): p. 91-101.
157. Xi, Y., et al., *Induction of BCL2-Interacting Killer, BIK, is Mediated for Anti-Cancer Activity of Curcumin in Human Head and Neck Squamous Cell Carcinoma Cells*. *J Cancer*, 2015. **6**(4): p. 327-32.
158. Sivanantham, B., S. Sethuraman, and U.M. Krishnan, *Combinatorial Effects of Curcumin with an Anti-Neoplastic Agent on Head and Neck Squamous Cell Carcinoma Through the Regulation of EGFR-ERK1/2 and Apoptotic Signaling Pathways*. *ACS Comb Sci*, 2016. **18**(1): p. 22-35.
159. Jiang, J., et al., *Curcumin disturbed cell-cycle distribution of HepG2 cells via cytoskeletal arrangement*. *Scanning*, 2013. **35**(4): p. 253-60.
160. Scherbakov, A.M. and O.E. Andreeva, *Apigenin Inhibits Growth of Breast Cancer Cells: The Role of ERalpha and HER2/neu*. *Acta Naturae*, 2015. **7**(3): p. 133-9.

161. Ye, F., et al., *Inhibition of cyclooxygenase-2 activity in head and neck cancer cells by genistein*. *Cancer Lett*, 2004. **211**(1): p. 39-46.
162. Burikhanov, R., et al., *Novel mechanism of apoptosis resistance in cancer mediated by extracellular PAR-4*. *Cancer Res*, 2013. **73**(2): p. 1011-9.
163. Zhang, W., et al., *Targeting of Survivin Pathways by YM155 Inhibits Cell Death and Invasion in Oral Squamous Cell Carcinoma Cells*. *Cell Physiol Biochem*, 2016. **38**(6): p. 2426-37.
164. Mishra, R., et al., *High expression of survivin and its splice variants survivin DeltaEx3 and survivin 2 B in oral cancers*. *Oral Surg Oral Med Oral Pathol Oral Radiol*, 2015. **120**(4): p. 497-507.
165. Yuan, F., et al., *Expression of Oct4 in HCC and modulation to wnt/beta-catenin and TGF-beta signal pathways*. *Mol Cell Biochem*, 2010. **343**(1-2): p. 155-62.
166. Regenbrecht, C.R., et al., *The molecular basis of genistein-induced mitotic arrest and exit of self-renewal in embryonal carcinoma and primary cancer cell lines*. *BMC Med Genomics*, 2008. **1**: p. 49.
167. Chen, Z., et al., *Oct4, a novel marker for human gastric cancer*. *J Surg Oncol*, 2009. **99**(7): p. 414-9.
168. Tian, T., et al., *Genistein exhibits anti-cancer effects via down-regulating FoxM1 in H446 small-cell lung cancer cells*. *Tumour Biol*, 2014. **35**(5): p. 4137-45.
169. Goncalves, A.P., et al., *Extracellular calcium triggers unique transcriptional programs and modulates staurosporine-induced cell death in Neurospora crassa*. *Microb Cell*, 2014. **1**(9): p. 289-302.
170. Farber, J.L., *The role of calcium ions in toxic cell injury*. *Environ Health Perspect*, 1990. **84**: p. 107-11.
171. Dong, Z., et al., *Calcium in cell injury and death*. *Annu Rev Pathol*, 2006. **1**: p. 405-34.
172. Bravo-Sagua, R., et al., *Calcium Transport and Signaling in Mitochondria*. *Compr Physiol*, 2017. **7**(2): p. 623-634.
173. Martins, I., et al., *Chemotherapy induces ATP release from tumor cells*. *Cell Cycle*, 2009. **8**(22): p. 3723-8.
174. Hosseinzadehdehkordi, M., A. Adelinik, and A. Tashakor, *Dual effect of curcumin targets reactive oxygen species, adenosine triphosphate contents and intermediate steps of mitochondria-mediated apoptosis in lung cancer cell lines*. *Eur J Pharmacol*, 2015. **769**: p. 203-10.
175. Szkudelska, K., L. Nogowski, and T. Szkudelski, *Resveratrol and genistein as adenosine triphosphate-depleting agents in fat cells*. *Metabolism*, 2011. **60**(5): p. 720-9.
176. Hamann, K., et al., *Hydralazine inhibits compression and acrolein-mediated injuries in ex vivo spinal cord*. *J Neurochem*, 2008. **104**(3): p. 708-18.
177. Burd, J.F. and M. Usategui-Gomez, *A colorimetric assay for serum lactate dehydrogenase*. *Clin Chim Acta*, 1973. **46**(3): p. 223-7.
178. Grabowski, M., et al., *Genistein inhibits activities of methylenetetrahydrofolate reductase and lactate dehydrogenase, enzymes which use NADH as a substrate*. *Biochem Biophys Res Commun*, 2015. **465**(3): p. 363-7.

179. Wu, S.H., et al., *Curcumin induces apoptosis in human non-small cell lung cancer NCI-H460 cells through ER stress and caspase cascade- and mitochondria-dependent pathways*. *Anticancer Res*, 2010. **30**(6): p. 2125-33.
180. Yeh, T.C., et al., *Genistein induces apoptosis in human hepatocellular carcinomas via interaction of endoplasmic reticulum stress and mitochondrial insult*. *Biochem Pharmacol*, 2007. **73**(6): p. 782-92.
181. Van Blerkom, J., P. Davis, and S. Alexander, *Inner mitochondrial membrane potential ( $\Delta\psi$ ), cytoplasmic ATP content and free  $Ca^{2+}$  levels in metaphase II mouse oocytes*. *Hum Reprod*, 2003. **18**(11): p. 2429-40.
182. Singh, D.V., et al., *Curcumin conjugates induce apoptosis via a mitochondrion dependent pathway in MCF-7 and MDA-MB-231 cell lines*. *Asian Pac J Cancer Prev*, 2013. **14**(10): p. 5797-804.
183. Antosiak, A., et al., *Cytotoxic activity of genistein-8-C-glucoside from *Lupinus luteus* L. and genistein against human SK-OV-3 ovarian carcinoma cell line*. *Med Chem Res*, 2017. **26**(1): p. 64-73.
184. Behrend, L., G. Henderson, and R.M. Zwacka, *Reactive oxygen species in oncogenic transformation*. *Biochem Soc Trans*, 2003. **31**(Pt 6): p. 1441-4.
185. Pelicano, H., D. Carney, and P. Huang, *ROS stress in cancer cells and therapeutic implications*. *Drug Resist Updat*, 2004. **7**(2): p. 97-110.
186. Nadal-Serrano, M., et al., *Genistein modulates oxidative stress in breast cancer cell lines according to ERalpha/ERbeta ratio: effects on mitochondrial functionality, sirtuins, uncoupling protein 2 and antioxidant enzymes*. *Int J Biochem Cell Biol*, 2013. **45**(9): p. 2045-51.
187. Zhang, J., et al., *Curcumin derivative WZ35 efficiently suppresses colon cancer progression through inducing ROS production and ER stress-dependent apoptosis*. *Am J Cancer Res*, 2017. **7**(2): p. 275-288.
188. Park, W., et al., *New perspectives of curcumin in cancer prevention*. *Cancer Prev Res (Phila)*, 2013. **6**(5): p. 387-400.
189. Zhang, J., et al., *pH-sensitive polymeric nanoparticles for co-delivery of doxorubicin and curcumin to treat cancer via enhanced pro-apoptotic and anti-angiogenic activities*. *Acta Biomater*, 2017.
190. Xie, J., et al., *Design of a novel curcumin-soybean phosphatidylcholine complex-based targeted drug delivery systems*. *Drug Deliv*, 2017. **24**(1): p. 707-719.

## Research Activity – Publications

### Articles

1. Perrone D., **ARDITO F**, Giannatempo G, Dioguardi M, Troiano G, Lo Russo L., De Lillo A, Laino L., Lo Muzio L (2015). Biological and therapeutic activities, and anticancer properties of curcumin (Review). EXPERIMENTAL AND THERAPEUTIC MEDICINE, vol. 10; p. 1615-1623, ISSN: 1792-0981
2. **ARDITO F**, Pellegrino MR, Perrone D, Cocco A, Lo Muzio L. In vitro study on anticancer properties of genistein in tongue cancer. Accepted for publication on OncoTarget and therapy.
3. **ARDITO F**, Pellegrino MR, Perrone D, Cocco A, Lo Muzio L. Biochemical and structural changes in cellular injury: Genistein in Tongue Cancer therapy. Accepted for publication on Current Topics in Medical Chemistry.
4. **ARDITO F** , Perrone D, Troiano G, Giuliani M, Lo Muzio L. Effects of curcumin on squamous cell carcinoma of tongue: an in vitro study. Submitted to BMC Cancer.

### Poster

1. **Genistein as drug for oral squamous cell carcinoma of tongue: anticancer properties and metabolic profiling.** Ardito F, Perrone D, Troiano G, Di Fede O, Mascitti M, Lo Muzio L. - XXIV Congresso nazionale, Collegio docenti universitari di discipline odontostomatologiche. 6-8/04/2017 Centro congressi San Raffaele, Milano
2. ***In vitro effects of curcumin on squamous cell carcinoma of tongue.*** Perrone D, Ardito F, Panzarella V, Campisi G, Laino L, Lo Muzio L - XXIV Congresso nazionale, Collegio docenti universitari di discipline odontostomatologiche. 6-8/04/2017 Centro congressi San Raffaele, Milano
3. ***Synergic effects of curcumin and polydatin on MG63-osteosarcoma cells proliferation are mediated through down-regulation of Survivin*** - G. Troiano, F. Ardito, D. Perrone, N. Termine, L. Lo Muzio - XIII Congresso Nazionale Società Italiana di Patologia e Medicina Orale (SIPMO) – 8,9,10/10/2015 Department of Biomedical and Neuromotor Sciences - University of Bologna

## RINGRAZIAMENTI

*Dire "grazie" non è un obbligo, ma un dovere che noi abbiamo verso noi stessi. Del resto, Fred De Witt Van Amburgh dice che "Nessuno è più povero di colui che non ha gratitudine. La gratitudine è una moneta che possiamo coniare da soli e spendere senza timore di fallimento".*

*Questi anni di dottorato sono stati un costante flusso di esperienze che hanno suscitato in me emozioni di natura molto diversa e per questo ringraziare è doveroso..*

*Il mio grazie va al Professor Lorenzo Lo Muzio per avermi insegnato che le piccole cose, quelle più semplici, sono le più importanti, come i silenzi che spesso valgono più di mille gesti e parole.*

*Un ringraziamento va anche al mio Tutor di dottorato, il Professor Nazzareno Capitano, per aver reso il mio percorso unico.*

*È risaputo che quando una persona fa qualcosa per noi, compreso offrirci una spalla su cui piangere nei momenti bui, allora ci avviciniamo di più a lei. Per questo, a Donatella e Bruna va il mio grazie per avermi sostenuta nei momenti difficili e per aver passato insieme molti momenti felici.*

*Il più grande dei ringraziamenti va alla mia famiglia perché, come dice Papa Francesco, "la famiglia è una fabbrica di speranza di vita e di risurrezione". Grazie per avermi insegnato che tutte le difficoltà si superano con l'amore e che l'odio non supera nessuna difficoltà. Il vostro amore mi ha fatto superare tutti gli ostacoli e mi ha aiutata ad andare avanti, sempre.*

*Grazie anche al mio fidanzato, perché camminare insieme è stato per me motivo di forza, così come il tuo essere sempre pronto a darmi la mano, ad abbracciarmi e a correre in mio aiuto.*

*Ringrazio i miei numerosi Angeli che vegliano costantemente su di me e che gioiscono con me.*

*Per ultimo ringrazio Colui che guida la mia vita ogni giorno e Coei che intercede per appianare i miei sentieri.*

*Sono caduta mille volte e persa altrettante ma è grazie a Voi se sono così forte che nemmeno io saprei come distruggermi.*