Original Article

Apigenin and Carbenoxolone Co-Treatment Induces Potent Cytotoxicity in CAL-27 Oral Squamous Cell Carcinoma: A Morphological and Dose-Response Study

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ABSTRACT

Background: Oral squamous cell carcinoma is a major health burden across the globe due to its widespread incidence, limited effective therapeutics, and high recurrence rates. Natural and synthetic compounds such as Apigenin (API) and Carbenoxolone (CBX) have shown significant antitumor potential against various cancer types. The current study aimed to evaluate the anti-proliferative potential of API and CBX, alone or in combination in the CAL-27 oral cancer cell line.

Methods: MTT assay was used to analyze the cytotoxic potential of API, CBX, and their combination in the CAL-27 cell line. Differential Interference Contrast (DIC) microscopy was performed to identify alterations in cellular morphology after treatment.

Results: The half maximal inhibitory concentrations for API and CBX were calculated as 50 µM and 200 µM respectively. Significantly greater inhibition of 86.37% (p-value <0.001) was observed after combination treatment in the CAL-27 cell line. Microscopic examination revealed cell death, cell shrinkage and detachment, and membrane damage in combination-treated cells compared with control and single agent treatment groups.

Conclusion: Treatment with API and CBX exhibited decreased cell viability, and their combination further increased anti-proliferative effects in CAL-27 cells, suggesting that these compounds in combination are potential candidates for anticancer therapy in OSCC.

Keywords: *Apigenin, Carbenoxolone, Cytotoxicity, Combination therapy, Cytology, Oral squamous cell carcinoma.*

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INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is one of the most prevalent malignancies of the oral cavity with significant impact on the global health burden, especially in the South Asia, where its prevalence continues to rise at a substantial rate.[1,2] Socioeconomic disparities, tobacco consumption, betel nut use, alcohol exposure, poor oral hygiene, poor nutritional status, and infection with tumorigenic HPV genotype strongly influence disease impact.[3-4]

Moreover, demographic transitions underscore a significant disparity and pronounced rise in OSCC within the male population, younger age groups, and underserved population, across intraoral regions, including buccal, labial mucosa, alveolar ridge, tongue, hard palate, retromolar trigone, and floor of the mouth.[5,6] Oral cancer has been shown to exhibit drug resistance towards various chemotherapeutic drugs, resulting in decreased treatment efficacy. Hence, there is a dire need to develop more effective and novel anticancer agents. While

advancements in surgical and anticancer therapy continue, the treatment efficacy remains insufficient with undesired side effects, which stresses the necessity for developing newer therapies with optimized safety and efficacy profiles.

Bioactive phytochemicals have drawn interest as putative anticancer agents owing to their biodiversity and lower toxicity. Flavonoids comprise a key category of phytochemicals with anticancer properties.[7,8] Apigenin (API), a naturally derived flavonoid, has been extensively documented to attenuate cancer cell proliferation and enhance tumor cell death across diverse cancer types.[9] Its tumor-suppressive activity has been linked with restriction of cellular growth, attenuation of metastatic capacity, and induction of structural and morphological alterations in malignant cells by disrupting key signaling pathways such as PI3K/Akt, MAPK, and p53.[10]

Carbenoxolone (CBX), a derivative of glycyrrhetinic acid extracted from the liquorice plant and a gap junction inhibitor, is known to have a broad pharmacological profile, including anti-inflammatory, antiviral, anticancer, antibacterial, anti-fibrotic, and neuroprotective.[11,12] As proven from the previous studies that showed that CBX can decrease the cell viability by inducing apoptosis in multiple cancer cell lines.[13-15].

Although API and CBX have individually demonstrated anticancer activity in various malignancies, comparative studies examining their cytotoxic effects alongside morphological changes in OSCC cell lines are scarce. Combining both compounds can enhance their anticancer effect by simultaneously inhibiting proliferation and inducing cell death. Additionally, CBX-induced membrane and mitochondrial destabilization may sensitize cancer cells to API-mediated anti-proliferative signaling, resulting in synergistic or additive anticancer effects in OSCC. CAL-27 (tongue squamous cell carcinoma) cell line used in this study was selected because it is one of the most reported cell lines in the literature and is well-characterized at the molecular level. Compared to other oral cancer cell lines, it provides stable proliferation kinetics and reliable dose-response outcomes. CAL-27 exhibits an overactive PI3K/AKT pathway and is a suitable cell line for the investigation of cytotoxicity, proliferation, and migration.[16,17] Hence, the current study aimed to evaluate the cytotoxic effects of API and CBX in the OSCC (CAL-27) cell line using the MTT assay and to characterize treatment-induced morphological alterations via DIC microscopy.

METHODOLOGY

Cell Culture

The human oral squamous cell carcinoma cell line CAL-27 was used in this study. It was an in vitro experimental study conducted at Multidisciplinary Research Lab 1 (MDRL-1), Ziauddin University, Karachi, Pakistan. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2 and sub-cultured at 80-90% confluency. Cells were treated with trypsin-EDTA. The cell line at passage number 7 was used for the study. The cell line was previously tested for mycoplasma contamination using DAPI staining and was found to be mycoplasma negative.

Reagents

API (A167323) and CBX (C4790) were obtained from Sigma- Aldrich (USA). Trypsin-EDTA (25200-056), Dulbecco's Modified Eagle Medium (11995-065), Foetal Bovine Serum (10270-106), Dimethyl Sulfoxide (137285925), MTT dye (M6494), Phosphate Buffered Saline (10010023), and Penicillin-Streptomycin (15070063) were obtained from Thermo Fisher Scientific (USA).

Cell Viability Assay

Cell viability was assessed using the MTT assay. CAL-27 cells were plated in 96-well plates at a density of 8×10^3 cells per well, and subjected to overnight incubation for cell

adherence. Different treatment concentrations of CBX (300 µM, 150 µM, 75 µM) and API (200 µM, 100 µM, 50 µM, 25 µM) were added. Combination treatment was performed using half maximal inhibitory concentrations of 50 µM for API and 200 µM for CBX for 48 hours. Following compound exposure, the MTT solution (0.5 mg/mL) was dispensed into each well and cultured for 3 h at 37°C to allow formation of formazan crystals. Removal of cell culture media was done, and crystals were solubilized in Dimethyl Sulfoxide (DMSO). To rule out vehicle (DMSO) induced cytotoxicity, vehicle control wells received the same amount of DMSO as the compound-treated wells. Furthermore, the final concentration of DMSO was ≤0.25% across all treatment groups, which is below the cytotoxic threshold for cell lines. Absorbance was determined at 570 nm using a MultiSkan Sky microplate reader. Relative cell viability was calculated using untreated control cells as a baseline. All experiments were conducted in triplicate and replicated independently three times.

Coefficient of Drug Interaction (CDI) for the half maximal inhibitory concentrations of 50 µM for API and 200 µM for CBX was determined by using fractional viability using the percent inhibition formula:

- **Fractional Viability** = 1 − (Cell Death (%) / 100)

- **CDI** = Combination of Drug A & B / Drug A x Drug B

Differential Interference Contrast (DIC) Microscopy

The cells were plated at a density of 50,000 cells per well in a 24-well plate. Cells were incubated for 24 hours before treatment to induce morphological changes. Afterwards, the compounds API, CBX, and their co-treatment were introduced for 48 hours. Post treatment, cells were rinsed with PBS, and DIC microscopy was used to assess and document morphological changes. Imaging was done using a Nikon Eclipse Ts2 microscope at 10x magnification using Nikon NIS-Elements imaging software.

Statistical Analysis

Analysis was done using SPSS version 24.0. The graphs were made using the same software. Percent inhibition in the control, API, CBX, and co-treatment groups was expressed as mean ± standard deviation. Normality of the data distribution was assessed using the Shapiro-Wilk test. For comparison among multiple groups, one-way analysis of variance (ANOVA) was applied, followed by Tukey's post hoc test for pairwise comparison, along with 95% confidence interval (CI) for the estimation of the magnitude and precision of the treatment effects. A p-value of less than 0.05 was considered significant.

RESULTS

API and CBX Reduce CAL-27 Cell Viability in a Dose-Dependent Manner

Viability of CAL-27 cells following treatment with API and CBX was evaluated using the MTT assay. Both compounds exhibited a reduction in cell viability in a dose-dependent manner as compared to untreated (DMSO vehicle control) CAL-27 cells.

Treatment with API resulted in growth inhibition of approximately 11.52±3.99 (95%CI=1.58-21.45), 51.16±1.850 (95%CI=46.57-55.76), 69.23±1.950 (95%CI= 64.38-74.07), and 70.48±2.17 (95%CI= 65.08-75.88) at 25 µM, 50 µM, 100 µM, and 200 µM, respectively, after 48 hours (Figure 1A). These doses and times were selected after a literature review and a pilot MTT assay. Approximately 50% inhibition of cell viability was observed at 50 µM, indicating the half-maximal inhibitory concentration of API in CAL-27 cells.

CBX treatment also induced a dose-dependent reduction in cell viability. Growth inhibition values of approximately 3.1652±2.20 (95%CI=-2.31-8.65), 10.39566±0.52, (95%CI= 9.09-11.69), and 80.6341±3.60, (95%CI= 71.69-89.57) were observed at 75 µM, 150 µM, and

300 µM (Figure 1B). Concentrations below 75 µM did not inhibit the cell growth, and were therefore not reported. The estimated half-maximal inhibitory concentration of CBX was 200µM. To evaluate potential combined effects, CAL-27 cells were co-treated with API (50 µM) and CBX (200 µM). This combination treatment produced the greater reduction in cell viability of 86.37±1.40 (95%CI= 82.87-89.85), exceeding the inhibitory effects observed with individual treatments (Figure 1C). The combination of API and CBX half maximal inhibitory concentration showed synergistic/enhanced effect after calculation (Table 1). The statistical analysis for inter and intra group comparisons and multiple comparisons between compounds are depicted in Table 2 and 3.

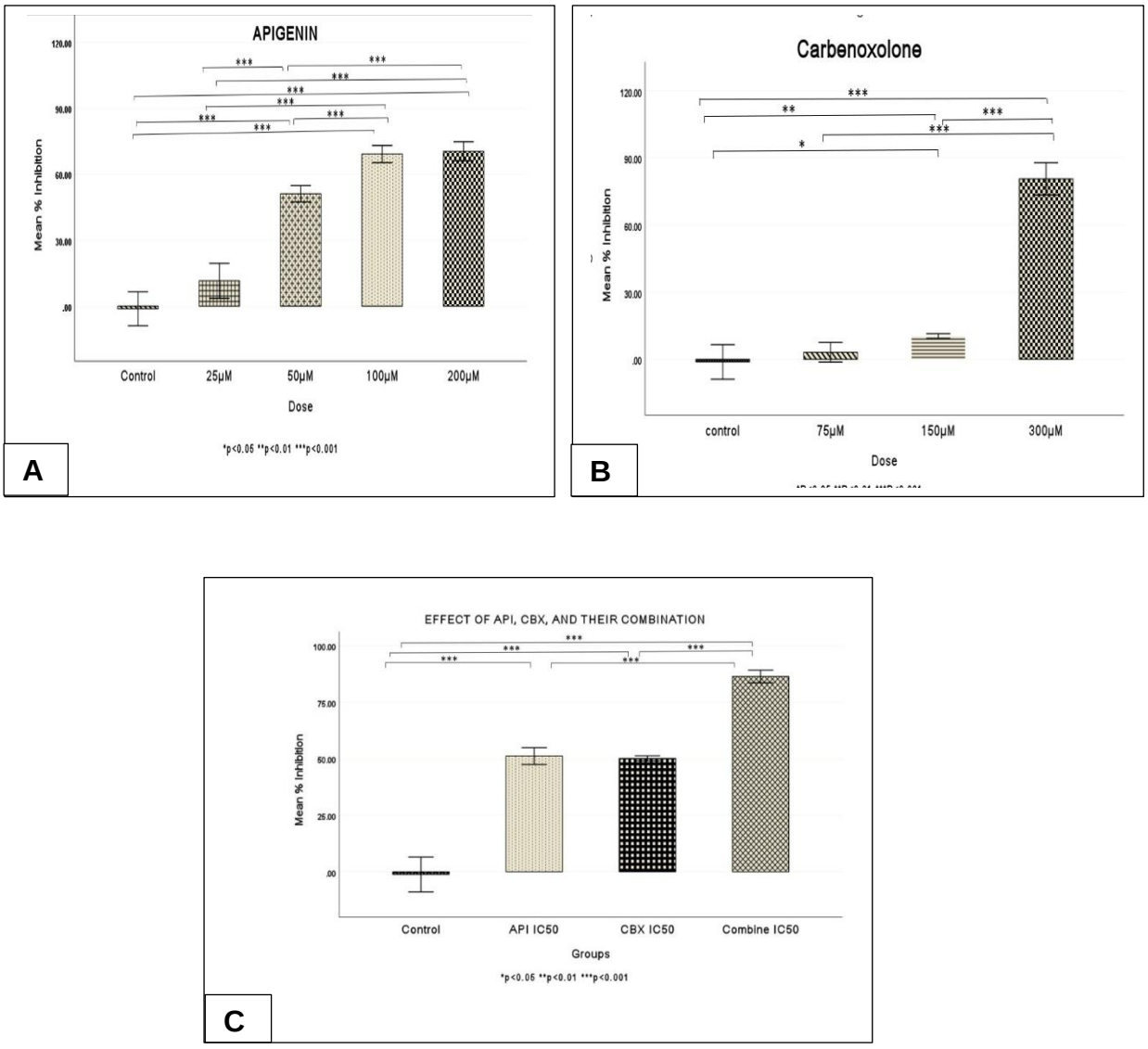

Figure 1: Dose-dependent cytotoxicity effects of Carbenoxolone, Apigenin, and their combination in CAL-27 cells (A)percentage inhibition at varying API concentrations (B)percentage inhibition at varying CBX concentrations, (C) percentage inhibition at the half maximal inhibitory concentration of API, CBX, and their combination, with maximal inhibition observed at combine half maximal inhibitory concentration. Data are presented as mean ± SD of three independent experiments. Statistical analysis was performed using One-way ANOVA followed by Tukey's post hoc multiple comparison test. Differences compared among various groups were observed at $p < 0.05$, $p < 0.01$, and $p< 0.001$.

Table-1: Coefficient of Drug Interaction (CDI) Analysis Using Fractional Viability

Combination Dose	% Cell Death	% Cell Viability	Fractional Viability	CDI Values	Interpretation
CBX 200µM	50%	50%	0.50	0.558	Synergistic Effect
API 50µM	51.16%	48.84%	48.84		

Table-2 One-Way ANOVA Analysis of Apigenin and Carbenoxolone-Induced Inhibition

ANOVA (APIGENIN)			
	df	**F-statistic**	**Significance**
Between Groups	4		
Within Groups	10	386.626374	<.001
Total	14		
ANOVA (Carbenoxolone)			
	df	**F-statistic**	**Sig.**
Between Groups	3		
Within Groups	8	540.801070	<.001
Total	11		

Table-3 Multiple Comparison for Apigenin and Carbenoxolone (Post Hoc Tukey Test)

	Group-wise Comparison		**Significance**
Apigenin	**Control**	**API 25 µM**	0.002
		API 50 µM	<.001
		API 100 µM	<.001
		API 200 µM	<.001
	25 µM	**API 50 µM**	<.001
		API 100 µM	<.001
		API 200 µM	<.001
	50 µM	**API 100 µM**	<.001
		API 200 µM	<.001
Carbenoxolone	**Control**	**CBX 150 µM**	0.005
		CBX 300 µM	<.001
	75 µM	**CBX 300 µM**	<.001
	150 µM	**CBX 300 µM**	<.001

Morphological Alterations Observed by DIC Microscopy

DIC microscopy was used to evaluate the significant morphological changes in the CAL-27 cell line, which showed a notable difference between the treated and untreated groups. In the untreated group (control), the cells displayed typical epithelial morphology, with adherent cells and cellular contact, all in a healthy, proliferative condition without any cellular stress (Figure 2A). On the other hand, the API-treated group showed enhanced morphological changes, indicating decreased cellular health, including early signs of cell death, cell rounding, cellular shrinkage, cytoplasmic condensation, membrane disruption, and cell detachment from the cell culture surface (Figure 2B).

Likewise, CBX-treated cells showed marked cellular changes (Figure 2C). Signs of cellular stress, including cell rounding, irregular cell boundaries, reduced cell count, and loss of adhesion to the cell culture surface, indicate cell death. Remarkably, combined treatment with API and CBX (Figure 2D) appeared to cause the most pronounced morphological damage among all groups. The cells showed extensive damage to cell membranes, loss of adherence, and pronounced cytoplasmic shrinkage. Cellular fragmentation and lysis were also observed post-treatment, indicating cell death. All treatment groups exhibited cytotoxic effects, however the most notable decrease in cell viability and structural integrity was observed in the combination treatment group as compared to the control.

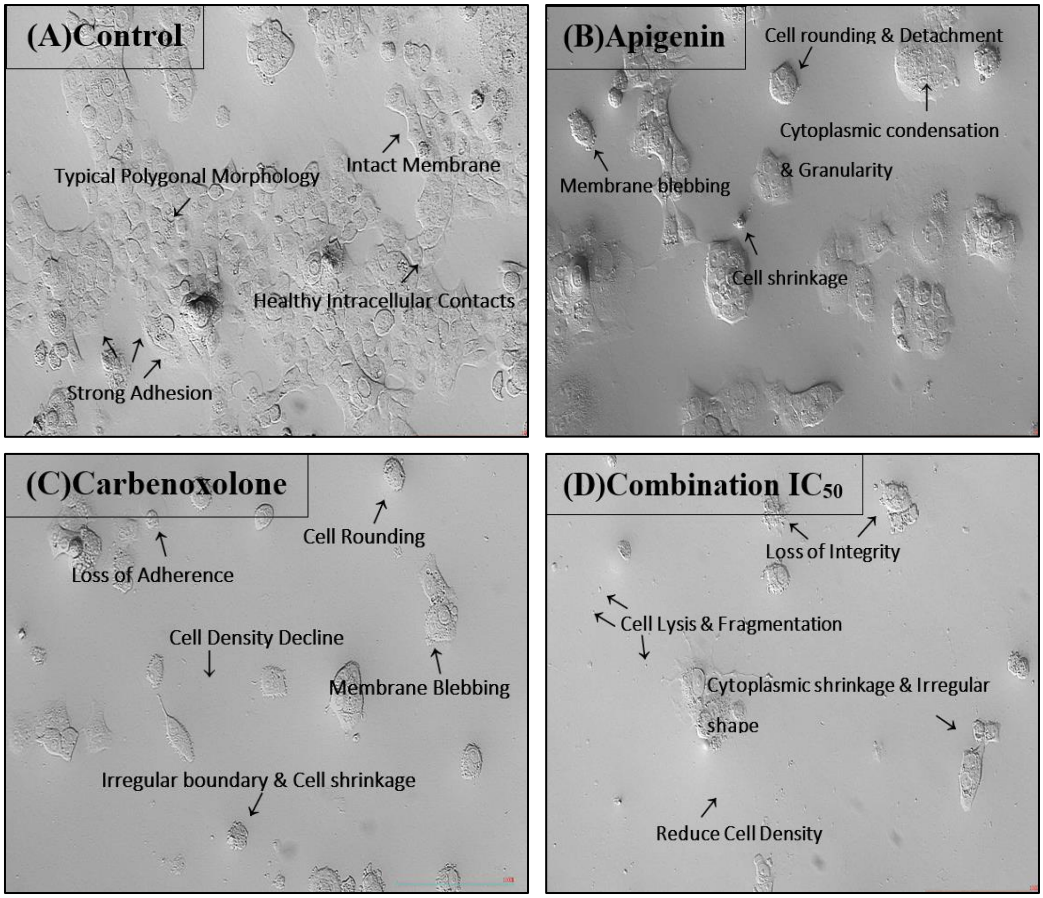

Figure 2: CAL-27 cells after treatment for 48 hours as observed by differential interference contrast (DIC) microscopy. Cells treated with API, CBX, and their combination displayed cytotoxic effects, scale bar 50µm.

DISCUSSION

API is widely considered a natural compound and a flavonoid widely considered as anticancer. The research findings of this study are consistent with results reported in the literature showing a dose-dependent effect. Decreased HSC-3 tongue carcinoma cell line proliferation following API exposure demonstrated its capacity to inhibit tumor cell growth even at comparatively low concentrations. In gastric carcinoma cell lines, API was found to decrease cell viability via dysregulating cell survival pathways and induction of apoptosis. The similar inhibitory pattern seen in this study is aligned with the broad anticancer activity of API against epithelial cancers.[18]

Comparatively lower sensitivity was observed in the CBX group against the CAL-27 cell line. Similar findings were also observed against K562 leukemia cells, where the compound induced proliferation reduction at around 150µM concentration. The comparatively higher half-maximal inhibitory concentration observed in the CBX group in this study may reflect tumor-specific responses toward drug treatment, influenced by cancer type and molecular background.[19] In another study, CBX exhibited anticancer effects against small cell lung carcinoma (SCLC) cells at concentrations ranging from 0-100µM by targeting pannexin 1, which in turn suppressed the extracellular ATP levels.[20]

The combination therapy showed enhanced efficacy compared with individual groups, indicating that combination strategies targeting complementary cellular pathways are effective approaches in cancer therapy. API and CBX target complementary oncogenic pathways and cause enhanced cell death. As per evidence that, API targets the cancer cell survival pathways such as PI3K/AKT, and CBX acts at the intercellular level by inhibiting gap junctions, simultaneously targeting intracellular survival pathways and intercellular communication, and metabolic pathways can produce a multi-targeted anticancer effect.[21,22]

Despite the antitumor potential of API and CBX, their efficacy across different cancers varies. For instance, an API's ability to induce apoptosis can vary depending on signaling pathway modulations within specific tumors.[23] Similarly, the differences in sensitivity observed with CBX at high concentrations in certain suspension cancer cell lines, compared with lower sensitivity in solid tumors, highlight tumor-specific effects. CBX can also function as a channel blocker, suppressing cancer proliferation.[24]

Morphological evaluation further supported the cytotoxic findings. Untreated CAL-27 cells maintained epithelial morphology, whereas treated cells displayed structural alterations consistent with cellular stress and death. Similar morphological changes following API treatment have been reported in endometrial carcinoma cells, in which cell shrinkage, loss of adhesion, and reduced spreading were associated with apoptotic processes.[25] The Same morphological alteration was seen in the leukemia model after treatment of the leukemic cells with CBX[19]. In the combination treatment group, the most significant structural damage was seen which intensified cellular damage. Therefore, supporting the findings of reduced cell viability in the MTT assay.

The selective toxicity is crucial for the creation of anticancer drugs. The prior experimental evidence supports API's minimal cytotoxicity in non-malignant cellular systems, and it was seen in the retina of rats both in vivo and in vitro.[26] Similar effects of CBX have been reported to modulate intracellular signaling in normal endothelial cells without inducing irreversible cytotoxic damage.[27] These findings show the potential therapeutic relevance of API and CBX as supplementary anti-tumor candidates with acceptable safety profiles, although confirmation in OSCC-specific normal cell models remains necessary.

Collective findings of the present study suggest that API functions as a potent growth inhibitor in OSCC cells, while CBX contributes additional cytotoxic stress that enhances overall treatment

efficacy when used in combination. Such multi-agent strategies may represent a promising approach for improving therapeutic responses in oral cancer while minimizing drug-associated cytotoxicity.

Limitations

The use of a single OSCC cell line may not fully reflect the effect of compounds, and results may not apply to other oral cancer subtypes. Furthermore, the study lacks critical molecular analysis, such as apoptotic evaluation, ROS production studies, and gene expression analysis. These parameters could have provided a deeper understanding of the molecular mechanism of action of these compounds. Furthermore, in vivo animal studies were also not conducted.

CONCLUSION

This study highlights the significant anticancer effects of API and CBX in the CAL-27, a representative cell line of OSCC. API exhibited higher potency as compared to CBX, and their combination resulted in enhanced anti-proliferative effects. Combination treatment also resulted in structural alterations as well as significant cell death, as evident by microscopic examination. These findings support the combined therapeutic potential of API and CBX in OSCC.

Author Contributions

AM: Conception & Design, Data Collection, Data Analysis & Interpretation, Manuscript Writing, Critical Revision and Final Approval
RI: Conception & Design, Data Collection, Data Analysis & Interpretation, Critical Revision and Final Approval
SH: Critical Revision and Final Approval
AB: Critical Revision and Final Approval
AH: Data Analysis & Interpretation, Critical Revision and Final Approval

Ethical Approval

This study is approved by the Ethical Review Committee of Ziauddin University, Karachi, Pakistan **(Ref: 7740923ABPHY)**

Conflict of Interests

The authors proclaim no potential bias.

Data Availability

Data will be available upon request.

Funding Source

No sources.

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