Therapeutic Effect of Luteolin Natural Extract Versus its Nanoparticles on Oral Squamous Cell Carcinoma Cell Line: in Vitro Study

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Abstract

Oral squamous cell carcinoma still has no satisfactory treatment. Luteolin is a natural flavonoid, exhibiting anti-cancer activity. Nanomedicine has been implemented in cancer therapy, utilizing the benefits of nanoparticles. In this context, this study was conducted to examine whether luteolin and nano-luteolin exert an inhibitory effect on oral squamous cell carcinoma cell line; SCC-25 by inducing apoptosis, and to assess whether nano-luteolin is more efficient compared to luteolin and the gold-standard; 5-fluorouracil. nano-luteolin was prepared and has fulfilled the criteria of nanoparticles. The cytotoxicity of luteolin, nano-luteolin, and 5-fluorouracil were assessed at three-time intervals using the MTT assay. IC50 doses of each intervention were applied to SCC-25, then caspase-3 levels were assessed at three-time intervals, using Real-Time polymerase chain reaction. Our results demonstrated dose- and time-dependent reduction in the % viabilities and IC50 values when SCC-25 treated with luteolin, nano-luteolin, and 5-fluorouracil. Among all the groups, nano-luteolin expressed the highest levels of caspase-3 (nano-luteolin > 5-fluorouracil > luteolin) and its highest value was at 48 h. In conclusion, both luteolin and nano-luteolin exerted cytotoxic effect and have succeeded to induce apoptosis against SCC-25 cells, with superior effect offered by nano-luteolin compared to luteolin and even 5-fluorouracil.

Keywords: *Luteolin, Nano-luteolin, Nanoparticles, Oral squamous cell carcinoma, SCC-25, Caspase-3, Apoptosis, Cytotoxicity*

Introduction

According to the International Agency for Research on Cancer (IARC) in 2018, the annual incidence of oral cancer (ICD C00-06 code: Lip, oral cavity) is about 354,000 cases, worldwide [(1)]. The primary modalities for oral squamous cell carcinoma (OSCC) are surgical intervention, radio- and chemotherapy. Unfortunately, the estimated 5-year survival rate remains 50% [(2)]. This unsatisfactory prognosis might be due to the ability of conventional therapies to work well for some time and eventually develop serious complications, primarily cancer resistance and non-selective toxicity [(3, 4)]. Hence, the scientists face a challenge to identify innovative therapeutic approaches destroying cancerous cells professionally and avoiding such major problems for cancer patients.

The naturally-occurring flavonoids have been reported to offer a good safety profile; damaging cancerous cells selectively [(5)]. Luteolin (Lut), a 3',4',5,7-tetrahydroxyflavone, is isolated from a wide range of fruits, vegetables, and medicinal herbs [(6-9)]. Lut has exhibited a wide range of activities, including anti-cancerous effects [(9, 10)]. However, poor solubility and fairly low bioavailability might hinder the pharmacological potential of Lut [(11)]. Nanotechnology has been reported to improve drug delivery, pharmacokinetics, and pharmacodynamic profiles, besides their ability to enhance the bioavailability of

poorly water-soluble drugs [12].

To the best of our knowledge, few studies were conducted to examine the effect of Lut in OSCC in the available English literature, although it was adequately examined in other cancers. Even more, the current study was the "pioneer" to investigate the effect of nano-luteolin (NLut) toward OSCC cells. The present work was designed to assess the inhibitory effect of Lut and NLut, compared to the gold standard 5-fluorouracil (5-FU), on OSCC cell line by inducing apoptosis, using microculture tetrazolium (MTT) assay and real-time quantitative polymerase chain reaction (RT-qPCR). By engaging the superlative properties of nanotechnology to the natural extract Lut, it seems very interesting to determine whether its nanoparticles' (NPs) has advantages superior to Lut in inducing apoptosis on OSCC.

Materials and Methods

Materials

The OSCC cell line (SCC-25) was purchased from the ATCC (# CRL-1628) (Manassas, VA, USA). 1:1 RPMI 1640 and 10% FBS were purchased from Gibco™; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). L-glutamine dissolved in DMSO (Product # C6164), Lut (Product # L9283), 5-FU (Product # F6627–1G), PLA-PEG polymer (Product # 659657), 1% PVA solution (Product # P1763), 5 mg/ml MTT reagent (Product # M-5655) and MTT Solubilization Solution (Product # M-8910) were purchased from Sigma-Aldrich (Louis, MO, USA). RNeasy Protect Mini Kit (Product # 74126) was purchased from Qiagen (Venlo, Netherlands), iScript™ One-Step RT-PCR Kit with SYBR® Green (Product # 170-8892) was purchased from the Bio-Rad Laboratories (Hercules, CA, USA). Primers: CASP3 (NM_004346.4) and beta-actin (ACTB) (NM_001101.3).

Methods

The entire procedure was carried out in the National Research Centre and the Confirmatory Diagnostic Unit of the Research and Development sector, VACSERA, Cairo, Egypt.

Preparation of NLut

NLut in the current study was formulated according to *Majumdar and colleagues*, using the emulsion solvent evaporation technique [13]. A diblock copolymer polylactic acid (PLA), polyethylene glycol ether (PEG), was utilized for encapsulation of Lut (100 mg in 60 mL MeOH and 1 gm in 10 mL MeOH, respectively). The mixture of Lut-PLA-PEG-OMe solution was added carefully to polyvinyl alcohol (PVA) emulsifier (200 ml of 1% w/v). The mixture was continuously stirred for one day, and centrifuged (6000 rpm). Then, at the bottom, the unencapsulated Lut precipitate was accumulated, and the supernatant was filtered (Millex® Syringe Filter: cut-off 0.20 μm) and purified (Amicon® Ultra -15 Centrifugal Filter: cut-off 30,000), to remove unencapsulated Lut. The mixture was sonicated for 30 minutes. Ultimately, pure and homogenous NLut was obtained.

MTT Cytotoxicity Assay and IC50 Value Determination

The effect of Lut, NLut, and 5-FU on cell viability of SCC-25 cells was assessed using the MTT assay after 24 h, 48 h, and 72 h of treatment to assess the cell viability. The half-maximal inhibitory concentration (IC50) value was calculated and used for the following investigation [14].

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

In this work, performed to determine the mRNA levels of caspase-3 (CASP3) expression in the SCC-25 treated cells was detected using the RT-qPCR assay. The mRNA level of the housekeeping gene; beta-actin (ACTB) was used as a reference to normalize the values [15]. The treated SCC-25 cells in the presence or absence of interventions were harvested. Total RNA was extracted using RNeasy Protect Mini Kit. Then, the reverse transcription was generated using iScript™ One-Step RT-PCR Kit with SYBR® Green. The results were analyzed using Corbett Rotor-Gene rotary analyzer PCR 5-PLEX with Q-6000 Series (Software 1.7, Build

87). The used primer sequences: CASP3, forward 5'-TTCATTATTCAGGCCTGCCGAGG-3'and reverse 5'-TTCTGACAGGCCATGTCATCCTCA-3'; and ACTB, an endogenous control, forward 5'-GTGACATCCACACCCAGAGG-3' and reverse 5'-ACAGGATGTCAAAACTGCCC-3'. The relative expression levels of CASP3 were calculated and normalized to ACTB in each sample using a double delta crossing threshold (ΔΔCT) method [16].

Statistical Analysis

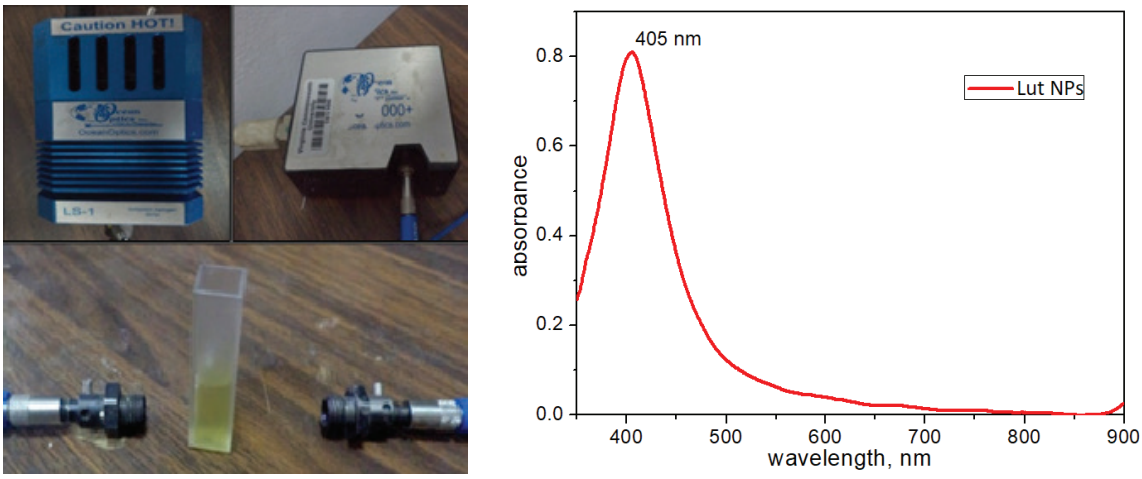
Statistical analysis was then performed using a commercially available software program (SPSS 18; SPSS, Chicago, IL, USA). Values were presented as mean ± standard deviation (SD) of ≥ 3 independent experiments for each group. Data were explored for normality using the Kolmogorov-Smirnov test. Since data were parametric, a one-way analysis of variance (ANOVA) test was used to compare all groups and to compare different observation times within the same group. This was followed by Tukey's post hoc test when ANOVA revealed a significant difference. The level of statistical significance was set at $P < 0.05$.

Results and Discussion

Characterization of NLut

To ensure that the prepared NLut has fulfilled the criteria of NPs, it was characterized to identify their size and shape using Visible Near Infra-Red (Vis-NIR) spectroscopy (Ocean Optics; Model LS-1). It consists of a Tungsten halogen light source; a versatile white-light source optimized for Vis-NIR (360 nm - 2000 nm) range and a sensor connected to the computer that detects the change in the amount of emitted light passed through NLut solution. The light source and the sensor were connected through two optical fibers and the cuvette containing NLut solution was placed in-between these fibers (Figure 1a). Data were recorded using special computer software (Ocean Optics Software) and the absorption rate curve was drawn (Figure 1b). The optical absorption spectrum of the NLut solution represents the surface characteristic of NLut, which shift to longer wavelengths with increasing particle size. The absorption rate of the NLut was with a maximum peak of 405 nm, indicating spherical NPs.

Interestingly, a diblock copolymer; PLA-PEG is already approved by the FDA, because it is biocompatible, biodegradable, and safe. Besides, this polymer can increase drug loading and encapsulation efficiency and reduce drug resistance [17, 18]. Moreover, PLA-PEG has a core-shell structure with amphiphilic molecular properties. This allows the hydrophilic PEG to diffuse into a shell of spherical particles, and the hydrophobic PLA to cohere and enwrap the same hydrophobic target drug to form the inner core "nucleus" of the spherical particles [13, 19].



(a) (b)

Figure 1. Vis-NIR Spectroscopy: (a) Tungsten Halogen Light Source, Sensor and Cuvette containing NLut solution placed in-between two optical fibers; (b) Absorption rate curve of NLut.

Cell Viability and Cytotoxicity Determination

The summary of % viability results in different experimental groups was demonstrated (Figure 2). Among all groups, the group treated with 100 µg/ml of NLut at 72 h recorded the lowest % viability (12.7302 %), however, the group treated with 0.01 µg/ml of Lut at 24 h recorded the highest % viability (86.1195 %). In the present study, % viability and the IC50 values revealed an overall time-dependent decline in SCC-25 treated with the different interventions. Besides, the results showed a considerable dose-dependent reduction in the % viability in the different experimental groups.

The IC50 doses for the different study groups were estimated. Statistical results of IC50 values in different experimental groups are summarized and represented graphically (Table 1,2, Figure 3). Interestingly, our results pointed out that NLut has provided the greatest cytotoxicity against OSCC cells, which even exceeded that of 5-FU when compared with Lut. Such results suggested that the increased duration as well as the higher doses of both Lut and NLut could promote the desired cytotoxicity against OSCC cells.

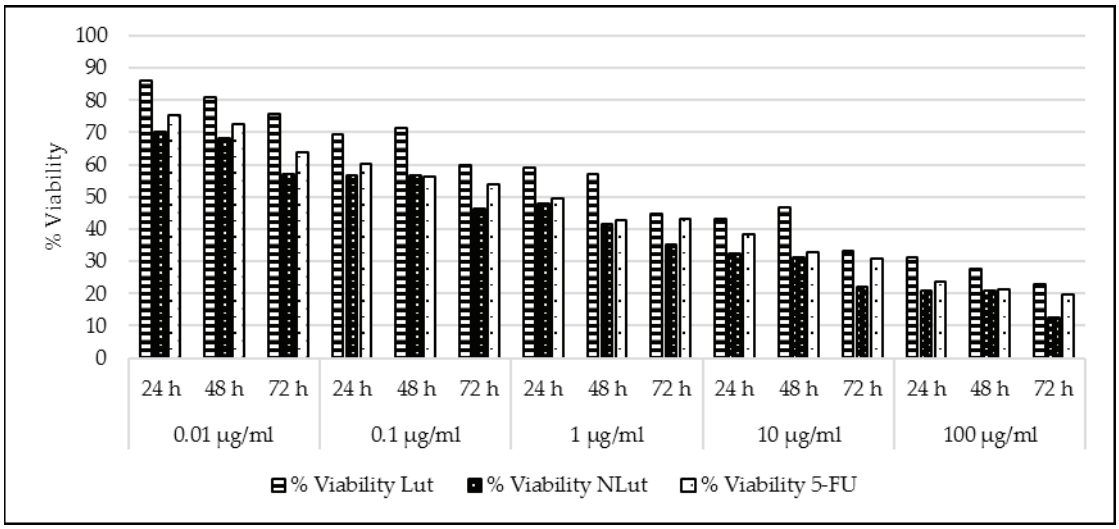


Figure 2. A clustered column chart comparing the % viability across the different groups.

Table 1. Summary of IC50 values ± SD in different experimental groups with their *P*-values and pairwise comparisons at different durations.

Intervention	IC50 results (µg/ml)			P-value
	24 h	48 h	72 h	
Lut	3.71a ± 0.6	3.32a ± 0.51	0.62 b ± 0.15	0.00 *
NLut	0.44 a ± 0.12	0.28 b ± 0.07	0.04 c ± 0.01	0.00 *
5-FU	0.89 a ± 0.25	0.41 b ± 0.11	0.2 b ± 0.04	0.0001 *

Significance level $P < 0.05$, * Significant. In Tukey's post hoc test: means sharing the same superscript letter are not significantly different.

Table 2. Summary of IC50 values ± SD in different experimental groups with their *P*-values and pairwise comparisons of different interventions.

Duration	IC50 results (µg/ml)			P-value
	Lut	NLut	5-FU	
24 h	3.71 a ± 0.6	0.44 b ± 0.12	0.89 b ± 0.25	0.00 *
48 h	3.32 a ± 0.51	0.28 b ± 0.07	0.41 b ± 0.11	0.00 *
72 h	0.62 a ± 0.15	0.04 c ± 0.01	0.2 b ± 0.04	0.00 *

Significance level $P < 0.05$, * Significant. In Tukey's post hoc test: means sharing the same superscript letter are not significantly different.

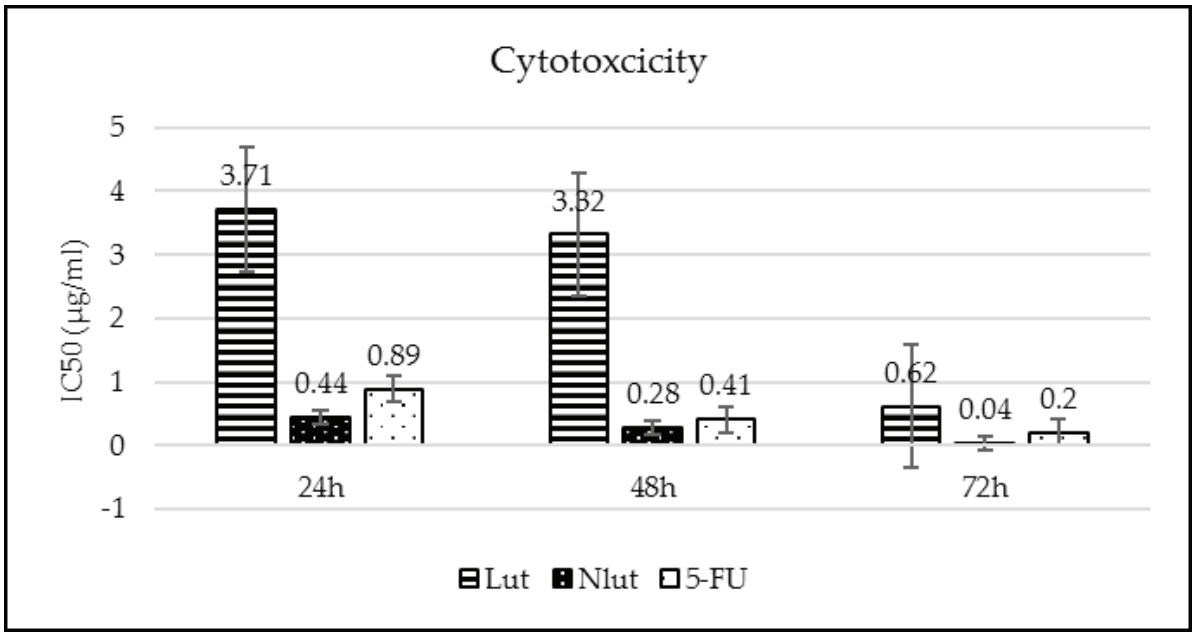


Figure 3. Column chart illustrating the different IC50 values of each group.

Detection of CASP3 Gene Expression

CASP3 is a critical effector enzyme in the caspase cascade, that acts as a junction between the intrinsic and extrinsic apoptotic pathways. Ultimately, the activated CASP3 can lead to the same execution pathway [20]. The CASP3 results for the different study groups were estimated (Table 3,4, Figure 4). All the treated groups showed upregulation of CASP3 gene expression relative to control. The fold-changes in both Lut and 5-FU treated groups were presented in a time-dependent manner, with the highest values appeared at 72 h, except in NLut groups at which the highest value was presented at 48 h. Among all groups, the highest values were offered by NLut, followed by 5-FU, then Lut.

Table 3. Summary of CASP3 levels ± SD in different experimental groups with their *P*-values and pairwise comparisons at different durations.

Intervention	CASP3 results (fold-changes)			P-value
	24 h	48 h	72 h	
Lut	6.49 ± 1.18	7.11 ± 1.43	7.47 ± 1.56	0.55 NS
NLut	10.78 b ± 2.51	19.42 a ± 4.61	17.65 a ± 4.17	0.0099 *
5-FU	8.02 b ± 1.67	9.74 a,b ± 2.08	12.21 a ± 3.13	0.046 *

Significance level $P < 0.05$, * Significant, ^{NS} Non-significant. In Tukey's post hoc test: means sharing the same superscript letter are not significantly different.

Table 4. Summary of CASP3 levels ± SD in different experimental groups with their *P*-values and pairwise comparisons of different interventions.

Duration	CASP3 results (fold-changes)			P-value
	Lut	NLut	5-FU	
24 h	6.49 b ± 1.18	10.78 a ± 2.51	8.02 a,b ± 1.67	0.0176 *
48 h	7.11 b ± 1.43	19.42 a ± 4.61	9.74 b ± 2.08	0.0001 *
72 h	7.47 b± 1.56	17.65 a ± 4.17	12.21 b ± 3.13	0.0009 *

Significance level $P < 0.05$, * Significant. In Tukey's post hoc test: means sharing the same superscript letter are not significantly different.

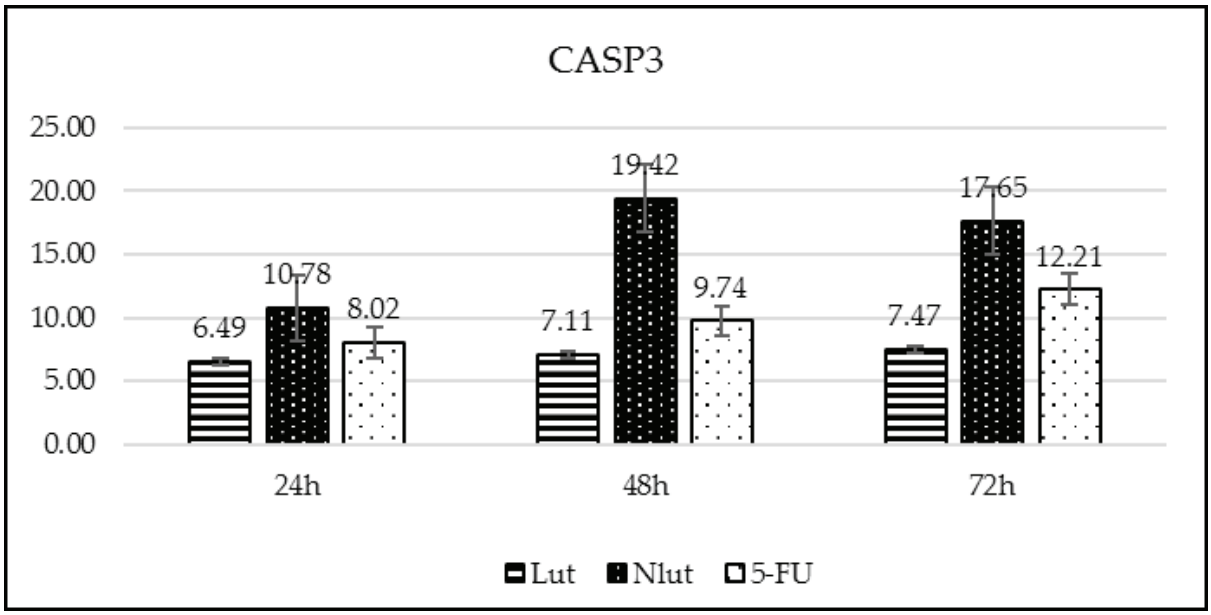


Figure 4. Column chart illustrating a summary of CASP3 results.

The success of Lut to upregulate CASP3 levels in all treated SCC-25 groups support its use as a potential chemotherapeutic candidate. Meanwhile, in this work comparing between levels of CASP3 of the different interventions at the same duration, Lut revealed the lowest values. This might be linked to the structural features of Lut, including hydroxyl groups moieties and 2−3 carbon double bond, which affect its biochemical and biological features [21]. Also, the chemical structure offers Lut with differential binding abilities which could be one of the possible reasons behind its anti-cancer activity.

5-FU has higher cytotoxic activity against OSCC cells than that of Lut, though having several adverse effects; mainly toxicity to normal cells and resistance which limit its use. On the other hand, although Lut showed lowered cytotoxicity compared to 5-FU, it has a safe nature and fewer adverse effects. In this light, to circumvent the drawbacks of 5-FU alongside the limited cytotoxic effect of Lut, NLut was prepared and used in this study. Thereby, NLut combines the advantages of Lut natural extract with the advantages of nanotechnology.

Superiorly, in this work NLut offered the highest levels of CASP3 expression (NLut > 5-FU > Lut), supporting its use as a novel chemotherapeutic agent against SCC-25 cells. Among NLut-treated groups, the highest level of CASP3 expression was found at 48 h (19.42-fold). Noteworthy, this result advocates NLut to be the drug of choice to treat OSCC cells with a recommended IC50 dose at 48 h (NLut: 0.28 µg/ml), to get the maximal apoptotic rate in the lowest possible time.

The supreme results of NLut in this work might be referred to: *first*; the characteristics of NPs which offer optimal size, shape, and efficient surface-to-volume ratio, with unique physicochemical and even biological features than that of the raw Lut. Besides, the global reduction of the amount of drug. *Second*; encapsulation properties of hydrophobic Lut into amphiphilic PLA-PEG nanocarrier as an attempt to make the drug hydrophilic. These NPs, which comprise a hydrophobic core and a hydrophilic shell, are excellent candidates for carrying highly hydrophobic chemotherapeutic agents [22]. *Third*: the ultrasonication can de-agglomerate and disperse NPs in aqueous-based media, improving the homogeneity and stability of the suspension, hence provides an open way for synthesizing such nano-sized core/shell structures [23]. *Fourth*: nanoencapsulation offers packaging to enhance cellular uptake, which mainly contributed to NLut's higher cytotoxicity [19, 24]. Consequently, NLut with several promising qualities could provide an alternative therapy to overcome the drawbacks of the naturally-occurring drug; Lut, as well as the undesired effects of the conventional chemotherapeutic drug; 5-FU.

Conclusion

Our result offers Lut and NLut as potential chemotherapeutics for OSCC treatment. NLut revealed the upper hand over Lut and even exceeding 5-FU, in inducing apoptosis of neoplastic cells. These data highlight NLut to be a drug of choice to treat OSCC, using the recommended dose and duration (NLut: 0.28 µg/ml for 48 h). Although of its drawbacks, the potent cytotoxic effect of 5-FU against cancer cells cannot be ignored. In this light, the combination between 5-FU and the "supreme" NLut could provide a compound utilizing their benefits, alongside avoiding the side effects of 5-FU, via reducing its dose. Such an adjuvant compound could fight the cancerous cells more efficiently. Furthermore, utilizing the ability of Lut to enhance the sensitivity of tumor cells toward other anti-cancer drugs could be beneficial to combine Lut with 5-FU, offering another alternative therapy with a lower dose and associated toxicity.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethical Clearance: Taken from Research Ethics Committee, Faculty of Dentistry, Cairo University.

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