Effect of Quercetin loaded Chitosan Nanoparticles and 5-Fluorouracil loaded Chitosan nanoparticles on Tongue Squamous Cell Carcinoma Cell Line

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Abstract

Background: Anticancer medications often have a limited therapeutic index, multiple drug resistance (MDR), and severe side effects. As a result, novel therapeutic techniques have been examined, and the most promising one is naturally obtained substances with known anticarcinogenic activities.

Aim: To assess the effect of quercetin-loaded chitosan nanoparticles (Qu-ChNPs), 5-fluorouracil-loaded chitosan nanoparticles (5-FU-ChNPs) and dual quercetin and 5-fluorouracil-loaded chitosan nanoparticles (Qu and (5-FU)-ChNPs) on tongue squamous cell carcinoma (TSCC) cell line.

Materials and Methods: Tongue squamous cell carcinoma (TSCC) cell lines (SCC-25) were used. ChNPs, Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNPs were prepared and characterized using TEM and SEM. Qu-ChNPs, (5-FU)-ChNPs, dual Qu and (5-FU)-ChNPs and the gold standard 5-FU were assessed using the MTT viability assay followed by the IC50 calculation. Using the IC50 doses, cell cycle and apoptosis analysis in different study groups were assessed using flow cytometry followed by immunocytochemistry of caspase 3 to visualize apoptotic cells. Furthermore, invasion and migration abilities of TSCC cells were assessed using wound healing assay.

Results: This study revealed that that the different experimental groups demonstrated dose dependent reduction in their % viability and IC50 values when treated with Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNPs and have promising effects against TSCC cell lines. A cytotoxic, cell cycle arrest, apoptotic, and antimigratory effects were detected towards treated cells rather than untreated cells. Interestingly, the results of the present study pointed out that the dual Qu and 5-FU-ChNPs have provided the greatest cytotoxicity against cancerous cells and the Qu-ChNPs have also provided more cytotoxic effect compared with (5-FU)-ChNPs and even the gold standard 5-FU

against cancerous cells. Moreover, the levels of caspase 3 were elevated in the treated TSCC cells groups compared to untreated cells.

Conclusion: Chitosan NPs make really good prospects for use in TSCC therapy as a carrier and the Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNPs have promising effects against TSCC cell lines.

These drug-loaded NPs can be useful means for treatment of tongue cancer as they exert a beneficial antiproliferative, apoptotic, and antimigratory effects against TSCC cells and the combination of Qu and 5-FU in chitosan NPs yields a powerful synergistic effect against TSCC cell line.

Keywords: Cell Lines, Chitosan, Nanoparticles, Quercetin, Tongue squamous cell carcinoma, Five-fluorouracil

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most prevalent malignant tumors in humans.[1] Oral SCC is the 16th most frequent cancer in the world, affecting about 377,000 people each year, with men accounting for 70% of cases.[2] Oral cancer is more common in Arab nations and in western and Southeast Asia. While this kind of cancer is not frequent in the Arab Gulf, Saudi Arabia and Yemen are prominent exceptions.[3] In Egypt, the incidence rate of oral cancer is approximate ranges from 1.4 to 2 per 100.000 persons.[4]

The most common OSCC site is tongue squamous cell carcinoma (TSCC), which is highly aggressive and can lead to lymph node or distant organ metastases, lowering the patient's overall survival rate. The study of TSCC has moved to the next level with research into the development of targeted therapeutics.[5]

For the therapy of OSCC, a range of options are available. Excision/resection, radiotherapy, systemic cytotoxic chemotherapy, or a combination of these techniques are among the options.[6] Chemotherapy has been the standard treatment for both localized and metastatic cancers until now. However, it is not particularly successful due to various constraints (low aqueous solubility, lack of anticancer drug selectivity, multidrug resistance, and so on).[7]

Five-fluorouracil (5-FU) is an anticancer medication licensed by the Food and Drug Administration (FDA) of the United States for the treatment of several solid malignancies, it has a poor pharmacokinetics profile, though.[8] The agent 5-FU produces serious health consequences due to its narrow treatment window. As a result, targeting 5-FU therapy in oncologic tissues is a critical challenge in order to optimize their pharmacodynamics and facilitate its selective deposition in cancerous cells over time.[9]

Low apparent permeability, poor bioavailability, and poor aqueous solubility represented limitations with most cytotoxic drugs. Nanotechnology-drug delivery was created to address these difficulties. Enhanced therapeutic benefits reduced harmful influence of the medication payloads by optimizing their pharmacodynamics, lengthy circulatory half-lives, enhanced impact on entrance and retention, medication safety, and patient acceptance are all advantages of nanoparticle-based drug delivery therapy. These nanocarriers are a promising prospect and offer a one-of-a-kind platform capable of replacing existing chemotherapy treatments.[10]

To achieve targeted delivery, various medications can be incorporated into nanoparticles (NPs). Biodegradable, biocompatible, less toxic, and easy to make, chitosan nanoparticles (ChNPs) are a promising medication transport system. Chitosan is a natural biomaterial that may be simply synthesized and characterized to get the necessary outcomes and has received certification from GRAS (Generally Recognized as Safe by the United States Food and Drug Administration [US FDA]).[11]

In general, biological active compounds from plants, herbs, or by-products of the agriculture industry are a rich source of compounds (polyphenols, anthocyanins, flavonoids like quercetin (Qu), and many others) that can be applied to treat a number of diseases, suggesting their use in pharmaceutical and cosmetic formulations, as well as nutraceutical supplies.[12]

Quercetin is a flavonoid that is common in plants and has received a great deal of interest because of its antioxidant and chemopreventive properties. Due to its limited water solubility and instability, Qu has an extremely low bioavailability. Recent researches, on the other hand, have revealed successful encapsulation of Qu in ChNPs to target the tumor microenvironment and have shown that Qu-ChNPs are more effective in cancer therapy.[13] Quercetin also enhances the impact of the chemopreventive medication 5-FU on cancer cell line growth suppression and apoptosis in a synergistic manner.[14]

Aim of the Work

This study aims to: Assess the effect of quercetin-loaded chitosan nanoparticles (Qu-ChNPs), 5-FU-loaded chitosan nanoparticles (5-FU-ChNPs) and dual drug-loaded chitosan nanoparticles (Qu and (5-FU)-ChNPs) on tongue squamous cell carcinoma (TSCC) cell line.

Materials and Methods

All the steps of the current experiment were carried out in Confirmatory Diagnostic Unit, veterinary serum & vaccine research institute, Cairo, Egypt (VACSERA-Egypt)

Materials:

1. Cell Lines: Tongue squamous cell carcinoma cell lines (SCC-25) (product# CRL-1628) were purchased from the American type culture collection (ATCC)[1] through veterinary serum & vaccine research institute (VACSERA)[2], supplied with compatible nutrient media.

2. Interventions: Chitosan, quercetin and Five-FU ware purchased from Sigma-Aldrich Company[3]

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Methods:

The tongue squamous cell carcinoma (TSCC) cell line (SCC-25) was sub-cultured to obtain 5 groups, which were subjected to 5-FU (as a positive control), Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNPs in addition to one group not subjected to any of these compounds (as a negative control (NC). First of all, Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNPs were prepared and characterized. Second, the study groups were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assay followed by the half-maximal inhibitory concentration (IC50) calculation. Then, the cell cycle and apoptosis analysis in the different groups were evaluated by flow cytometry using the IC50 dose, and immunocytochemistry (ICC) for visualization of apoptosis by caspase3 marker was used. Finally, cell invasion and migration were assessed through wound healing assay.

Results

1. Characterization of Nanoparticles

The morphology and size of the NPs were examined employing transmission electron microscope (TEM) and scanning electron microscope (SEM). All samples show the existence of cluster centers of sphere-shaped nano dimensional particles. The sizes of the blank ChNPs, Qu-ChNPs, and (5-FU)-ChNPs were in the range of 26.23-34.22 nm while the dual Qu and (5FU)-ChNPs were between 29.75-42.22 nm. The TEM and SEM micrographs of the ChNPs and Qu-ChNPs were shown in figs. (1-4).

2. Assessment of Cell Cytotoxicity using MTT Assay

2.1. Effect of Concentration:

To concentrate on the cytotoxic effect of a specific concentration, the half maximal inhibitory concentration (IC50) that was determined as the concentration needed to reduce the growth of tumor cells in culture by 50% relative to the untreated cells.

After 24 hours of in vitro testing against TSCC cell lines, the anticancer activity of the Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNPs was assessed. Their IC50 values were calculated from a graph of cell viability obtained over a range of doses between 0.01 and 100 g/ml. The IC50 was calculated using five serial doses with a wide range of doses (100, 10, 1, 0.1, and 0.01µg/ml) against the cell lines.

There was a significant difference between different groups ($p<0.001$). The highest value of viability was found in 0.01 µg/ml, followed by 0.1 µg/ml, then 1 µg/ml, and 10 µg/ml, while the lowest value was found in 100 µg/ml. Post hoc pairwise comparisons showed that there was no significant difference 0.1 and 0.01 ($p>0.05$). In addition, they showed other groups to be significantly different from each other ($p<0.001$) as shown in table (1)

2.2. IC50:

Mean and standard deviation (SD) values of IC50 for various groups were displayed in table (2)

There was a significant difference between various groups ($p<0.001$). The highest value was found in 5FU (10.11±0.89), followed by (5-FU)-ChNPs (8.48±0.52) then Qu-ChNPs (7.48±1.36), while the lowest value was found in Dual Qu and (5-FU)-ChNPs (5.75±0.17). Post hoc pairwise comparisons showed 5FU to have significantly higher value than other groups ($p<0.001$). In addition, they showed Dual Qu and (5-FU)-ChNPs to have significantly lower value than other groups ($p<0.001$). In other words, the highest cytotoxic potential was detected in the dual Qu and (5-FU)-ChNPs group which represented by the lowest IC50 value followed by Qu-ChNPs then (5-FU)-ChNPs while the lowest cytotoxic potential was found in 5FU.

3. Cell cycle analysis

The effects of Qu-ChNPs, (5-FU)-ChNPs and dual Qu and (5-FU)-ChNPs on cell cycle phases were investigated by utilizing PI staining in FACS analysis. This assay measured the percentage of cells in each phase of the cell cycle and recorded it.

The TSCC cells were given the IC50 of these medications for 24 hours in order to conduct this analysis, and then they were stained with PI. The results of flow cytometric analysis showed an increase of cells in the G1/S phase in cells treated with Qu-ChNPs and in the dual Qu and (5-FU)-ChNPs. Additionally, the data showed that the increase of cells in the G2/M phase in cells treated with 5-FU (Positive control) and (5-FU)-ChNPs as shown in table (3)

In the untreated cells, there were 1.69% of cells in the pre-G1 phase (apoptotic cells), and 24.15% in cells treated with 5-FU, 26.83% in cells treated with (5-FU)-ChNPs, 29.41% in cells treated with Qu-ChNPs and 36.25% in the cells treated with dual Qu and (5-FU)-ChNPs at their IC50.

There was a significant difference between different groups ($p<0.001$). The highest value was found in Dual Qu and (5-FU)-ChNPs (36.25±0.41), followed by Qu-ChNPs (29.41±0.45), then (5-FU)-ChNPs (26.83±0.30), and 5-FU (24.15±0.25), while the lowest value was found in negative control (1.69±0.06). Various groups had significantly different values from one another, according to post hoc pairwise comparisons (p 0.001). Mean and standard deviation (SD) values of pre-G1 (%) for various groups were displayed in table (4)

Flow cytometric images showing representative histogram of DNA content in the untreated TSCC cells and TSCC cells treated with IC50 concentration of different groups were demonstrated in figs. (5-9).

4. Cell apoptosis analysis

4.1 Assessment of apoptosis using flow cytometric analysis by annexin V-FITC/PI:

To examine if apoptosis is the cause of the suppression of cell growth induced by Qu-ChNPs, (5-FU)-ChNPs and dual Qu and (5-FU)-ChNPs, flow cytometry assay was performed.

After applying the IC50 concentration of the 5-FU, Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNPs for 24 hours on OSCC-25 cells, the results demonstrated that exposure to all of these chemicals significantly decreased the fraction of cells in the viable cell category.

These were accompanied by a rise in the percentage of cells undergoing early and late apoptosis when compared to control groups. The total percentage of apoptotic cells showed highly significant values in all study groups in comparison to control. The same trend in the percentage of early and late apoptosis as well as necrosis was observed among the studied groups. The results are represented in table (5) and figs. (10,11).

For the visualization of apoptosis, a monoclonal antibody against caspase3 was used as shown in figs. (12-16).

In total apoptosis, there was a significant difference among various groups ($p<0.05$). The highest value was found in Dual Qu and (5-FU)-ChNPs (11.98±9.53), followed by Qu-ChNPs (9.47±6.65), then (5-FU)-ChNPs (9.34±3.56), and 5-FU (6.54±4.07), while the lowest value was found in negative control (0.32±0.18). Negative control had a significantly lower value compared to the other groups, according to post hoc pairwise comparisons (p 0.001). Mean and standard deviation (SD) values of apoptotic cells (%) for different groups were presented in table (6).

4.2. Immunocytochemistry

For the visualization of apoptosis induced by Qu-ChNPs, (5-FU)-ChNPs and dual Qu and (5-FU)-ChNPs on apoptotic pathway, the activity of caspase-3 in Qu-ChNPs, (5-FU)-ChNPs and dual Qu and (5-FU)-ChNPs treated SCC cells was examined.

The intensity score and percentage of positive caspase-3 in TSCC cells were recorded in table (7).

Mean and standard deviation (SD) values of Caspase-3 positive cells (%) for different groups were presented in table (8).

There was a significant difference between various groups ($p<0.001$). The highest value was found in dual Qu and (5-FU)-ChNPs (25.10±0.25), followed by Qu-ChNPs (21.81±0.48), then (5-FU)-ChNPs (20.73±0.30), and 5-FU (14.95±0.14), while the lowest value was found in Control (0.09±0.03). Various groups had significantly different values between them according to post hoc pairwise comparisons (p 0.001).

As shown in figs. (12-16), the Caspase3 expression was abnormally increased in treated TSCC cells relative to the control cells. The expression of caspase-3 was in the cytoplasm and nucleus. Caspase-3 was expressed following the treatment with Qu-ChNPs, (5-FU)-ChNPs and dual Qu and (5-FU)-ChNPs.

V.5. Cell Migration Assay (Wound Healing Assay)

To investigate the anti-migratory property of Qu-ChNPs, (5-FU)-ChNPs and dual Qu and (5-FU)-ChNPs on TSCC cells, the IC50 concentration of them in inhibiting TSCC cell migration was studied using migration culture dish inserts wound healing assay. The migration of the treated cells was inhibited compared to the untreated cells as shown in figs. (17-22)

Mean and standard deviation (SD) values of wound closure (%) for various groups were displayed in table (9)

There was a significant difference between various groups ($p<0.001$). The highest value was found in negative control (97.63±0.56), followed by 5-FU (65.70±0.37), then (5-FU)-ChNPs (59.11±0.34), and Qu-ChNPs (56.52±0.31), while the lowest value was found in Dual Qu and (5-FU)-ChNPs (49.93±0.55). Various groups had significantly different values between them according to post hoc pairwise comparisons (p 0.001).

These findings therefore highlighted that these drugs could inhibit the in vitro motility of tongue TSCC cells.

Combining, these observations indicate that the combined drug-loaded chitosan nanoparticles may possess the most anti-migratory property and thus, prevent the spread of cancer cells along with its anti-apoptotic nature.

In summary, the (5-FU)-ChNPs, Qu-ChNPs and dual Qu and (5-FU)-ChNPs were created and used in the TSCC therapy for site-specific, prolonged medication delivery. The results of this study showed that by comparing the results of cytotoxicity, cell cycle and apoptosis, and wound healing assay, in treated groups in compared to untreated control groups, the results reported that the (5-FU)-ChNPs, Qu-ChNPs and dual Qu and (5-FU)-ChNPs could have effective and promising functions as an anticancer and in site-specific and sustained drug delivery against TSCC.

Discussion

In spite of the advances in earlier diagnosis of cancer and treatment strategies, numerous malignancies are still detected and managed at late stages, with poor overall survival and high mortality rates, due to the restricted selection of therapy methods, which traditionally include surgery, radiotherapy, and/or chemotherapy. As a result, novel therapeutic techniques have been examined, and naturally occurring compounds with recognized anticancer properties are the most attractive.[15]

Katz and Baltz. (2016) suggested that natural compounds have a longstanding history and a bright future as sources of therapeutic effective medications.[16] The natural agent quercetin can perform anticancer effect because it can suppress the progression of angiogenesis and metastatic process, alter cell cycle progress, reduce cell growth, induce apoptosis, and alter autophagy in certain malignancies.[17]

For almost 50 years, 5-FU has been applied to the systemic therapy of cancer patients. In both adjuvant and metastatic disease situations, 5-FU is the main backbone of combination chemotherapy for patients with certain tumors like colorectal cancer. The link between 5-FU plasma levels and biological consequences, including toxicity, is well-established. Severe toxicity caused by 5-FU-based chemotherapy, such as febrile neutropenia, nausea/vomiting, and diarrhea, place a considerable clinical and financial burden on the healthcare system, resulting in hospitalization and increased morbidity and mortality.[18] So, this study was performed and directed toward this point of research.

The drawbacks of 5-FU in cancer treatment can also be mitigated by using a targeted drug delivery platform to reduce drug dosage. As a result, 5-FU has been employed in a variety of platforms and solid supports, including chitosan.[19] In light of this, the current study was designed to utilize the obvious advantages of 5-FU with chitosan NPs in the therapy of TSCC by creating (5-FU)-ChNPs

Furthermore, medication combinations or other medicines with fewer or milder side effects would be good candidates for reducing the toxicity of first-line drugs like 5-FU. For these goals, bioactive nutraceuticals or powerful food components like Qu may be considered useful for these purposes.[20] Synergistic actions of Qu with antitumor, bactericidal, antidiabetics, and anti-inflammatory drugs make it a prospective option for the development of new forms of treatment for different diseases with less adverse effects and higher efficacy.[21]

To our knowledge, no experiments in the English literature have investigated the anticancer activity of 5FU-ChNPs and Qu-ChNPs in TSCC. Despite the fact that they had never been tested on the TSCC cell line, only a few researches had looked at their impact on other cell lines.[22] In this study, (5-FU)-ChNPs, Qu-ChNPs, and dual Qu and (5-FU)-ChNPs were examined their inhibitory effect on TSCC cell line by arresting cell cycle, inducing apoptosis and inhibiting cells migration, and to assess if one compound had more efficient anticancer activity than other on TSCC cell.

Therefore, in this study, (5-FU)-ChNPs, Qu-ChNPs, and dual Qu and (5-FU)-ChNPs were synthetized by combining the 5-FU and Qu materials with ChNPs separately and in combination, and tested their effects in TSCC cells. To compare the effects of (5-FU)-ChNPs, Qu-ChNPs, and dual Qu and (5-FU)-ChNPs on the TSCC cell line, 5-FU was utilized as the gold standard. This is due to its widespread clinical use in the treatment of several cancers and its role as a major chemotherapeutic for OSCC.[23],[24]

This study showed that NP drug release systems that function as drug carriers and sustained delivery techniques not only increase the 5-FU's effectiveness but also reduce its negative impacts. This is

in consistence with the study of Cheng et al. (2012) which showed this 5-FU loaded Galactosylated chitosan nanoparticles improve the efficacy of the 5-FU and reduce their side effects in liver cancer mouse model.[25]

In the current study, cell survival and cytotoxicity effect were estimated by MTT assay which is one of the viability procedures that relies on viable cells converting substrates into a colorimetric molecules, the MTT assay is still one of the best adaptable and widely used assays. This result confirmed Kumar et al. (2018) who suggested that this assay is highly accurate and linear up to ∼106 cells per well.[26]. Each drug in this study was applied using five different doses, in order to exactly determine the dose sufficiently needed to provide the desired cytotoxic effect on cancerous cells according to the (VACSERA-Egypt) protocol.

In this study, the IC50 values were detected for each drug to determine the specific doses used for the cell cycle and apoptosis assay and wound healing assay. The highest value was found in free 5-FU while the lowest value was found in dual Qu and (5-FU)-ChNPs These results were in line with Roshanazadeh et al. (2021) who confirmed that the value of IC50 was reduced when Qu combined with 5-FU compared with the IC50 of the free 5-FU and concluded that Qu act synergistically with 5-FU as a chemo sensitizing agent, lowering the 5-FU dose while simultaneously inhibiting breast cancer cell proliferation and migration.[20]

In the current study, the possible cytotoxicity of (5-FU)-ChNPs on TSCC cells was assessed by MTT assay. Results of the present work showed that the (5-FU)-ChNPs had antiproliferative effect against TSCC. This finding is consistent with the finding of Cavalli et al. (2014) who illustrated that (5-FU)-ChNPs were effective in reducing tumor cell proliferation against human colon adenocarcinoma and human prostate carcinoma cell lines.[27]

The results of this study exhibited enhanced antiproliferative efficacy of Qu-ChNPs against TSCC cells which was evaluated by MTT assay. This finding is consistent with Baksi et al. (2018) who revealed that Qu-ChNPs exhibited more efficient anti-cancer action on human pulmonary cancer cell lines A549 and breast cancer cell lines.[13]

The results of this study revealed that combination of 5-FU and one of the potent antitumor natural products like Qu when loaded as drug-polymer delivery system enhanced the therapeutic efficacy in TSCC cell line compared to a single individual agent. This finding is consistent with Ni et al. (2019) who declared that the 5-fluorouracil- and curcumin-loaded dual drug-polymer delivery system enhanced the therapeutic efficacy in hepatocarcinoma cell line compared to a single individual agent.[28]

This is also in agreement with David et al. (2015) who attempted to improve the effectiveness of 5-FU, a cytotoxic medication utilized in in chemotherapy. They created a dual medication including 5-FU and Qu as an antioxidant, and then provided this treatment along with ChNPs. They showed that combining an antioxidant chemical, such as Qu, with 5-FU would increase 5-FU encapsulation and hasten its delivery.[29]

As a main target of cancer therapy, apoptotic potentiality induced by (5-FU)-ChNPs, Qu-ChNPs, and dual Qu and (5-FU)-ChNPs on the TSCC cells were studied using annexin V-FITC/PI assay to distinguish between apoptotic and necrotic cell death. Negative control cells showed stain negativity for annexin V suggesting live cells while, (5-FU)-ChNPs, Qu-ChNPs, and dual Qu and (5-FU)-ChNPs as well as 5-FU treated cells showed stain positivity for both annexin V and annexin V-/PI which indicates early and late apoptosis, respectively.

The present results revealed that (5-FU)-ChNPs, Qu-ChNPs, and dual Qu and (5-FU)-ChNPs as well as 5-FU decreased TSCC cells viability and increased the percentage of total apoptotic cells. Moreover, there was a significant elevation in necrotic cell death when compared with unexposed cells. These findings supported that results of David et al. (2015) who confirmed a significant toxicity and increased apoptosis and necrosis of these nanoparticles against pancreatic cancer cells.[29]

One of the major strategies of cancer therapeutics is induction of apoptosis in cancer cells. The present results revealed that (5-FU)-ChNPs induced apoptotic cell death in TSCC cells. This is in line with Deveci et al. (2018) who illustrated that 5-FU increased the formation of intracellular ROS, depolarized the mitochondria, triggered apoptosis, and activated caspase 3 and 9 in MCF-7 human breast cancer cells.[30]

Results of the present work illustrated that the action in tumor inhibition of Qu-ChNPs resulted from induction of cell cycle arrest and apoptosis. This is in accordance with Rashedi et al. (2019) who revealed that colon cancer inhibition in wistar rats by Qu-ChNPs resulted from induction of cell cycle arrest and apoptosis.[31]

Moreover, regarding the apoptosis effect of Qu-ChNPs on tumor cells (TSCC) in this study, there was a significant statistical elevation in tumor cells that had undergone apoptosis in the treatment group compared to untreated group ($P = <0.001$) This is might be justified by Liu et al. 2012 who stated that the capability of Qu to induce apoptosis in human prostate cancer PC-3 Cells is via the activation of proapoptotic proteins like caspase-3, -8, and bid, and through the endoplasmic reticulum stress.[32] Fortunately, in the present study the significant levels of caspase 3 expression denoted active apoptosis and thereby, confirmed the effectiveness of the used interventions.

In the current study, the combination of 5-FU and Qu demonstrated induction of apoptosis on TSCC. The administration of Qu with 5-FU dramatically increased the cytotoxicity and apoptosis cellular responses, even at low concentrations of 5-FU. This shows that when 5-FU paired with Qu, the cells could be more sensitive to 5-FU. The current findings proved that Qu and 5-FU work synergistically to trigger apoptotic response in TSCC. This data revealed that combining Qu with 5-FU would be a more effective therapeutic approach for the treatment of tongue cancer. This is in consistent with Xin et al. (2012) who reported similar result in esophageal cancer.[33]

In contrast to this study, Li et al. (2014) found that Qu impairs the therapeutic benefits of 5-FU and other anti-neoplastic medicines in ovarian cancer cells by minimizing ROS damage at low doses.[34] This may be attributed to the different technique used or to the different type of cell line. However, Wu et al. (2018) proved that Qu has potent

anticancer effects through the activation of a ROS-dependent apoptotic pathway in MCF-7 cells.[35] Results of the present work supported these potent apoptotic effects.

Apoptosis can be detected using a variety of techniques. The activation of cysteine proteases (caspases) is the most well-known biochemical marker of both early and late phases of apoptosis. Active caspase-3 detection in cells and tissues is a crucial approach for detecting apoptosis triggered by a range of apoptotic signals. Immunohistochemistry, immunoblotting for active caspase-3, colorimetric techniques are the most used assays for assessing caspase-3 activation.[36] So, in this study, additional validation of apoptosis was performed by Immunocytochemistry of caspase-3

Regarding the current results, caspase 3 showed high activity among all study groups. The highest value was found in dual Qu and (5-FU)-ChNPs, followed by Qu-ChNPs, then (5-FU)-ChNPs, and 5-FU, while the lowest value was found in control group. This indicates that caspase 3 induced apoptosis in exposed TSCC cells.

The current results of caspase 3 expression in immunohistochemistry technique detected many morphological phenotypes of apoptotic cells in the treated groups which represent the different stages of apoptosis. This finding was also reported by Helmi et al. (2012) who recorded similar results.[37]

Helmi et al. (2012) clarified that the first phase of apoptosis is associated with an early step of caspase-3 induction in the cytoplasm, characterized by usual nuclear structure. The second phase, which is marked by excessive nuclear and cytoplasmic staining, can signify the progression of increased cytoplasmic caspase-3 induction and its subsequent translocation to the nucleus. The third phase is consistent with the usual representation of the late phase of apoptosis, marked by the cell's disintegration into apoptotic fragments and nuclear and cytoplasmic condensation.[37]

The current study provided evidence of the antimigratory effects of 5-FU-ChNPs against TSCC cells. This is in accordance with Wang et al. (2021) who suggested that by targeting MARCH1 (play a role as an oncogene), 5FU prevented colorectal cancer cells from growth, migrating, and invading their surrounding tissue, and the subsequent knockdown of the PI3K/AKT pathway resulting in slowed EMT process.[38]

Results of the present work also illustrated that the Qu-ChNPs prevented the in vitro motility of TSCC cells. This is could be attributed to the effect of Qu on cell migration as explained by Chen et al. (2013) who proved that Qu efficiently suppresses SCC-25 cells' migration and invasion in vitro.[39]

Furthermore, Zhao et al. (2019) illustrates that in oral cancer cells, Qu administration increased microRNA-16 (miR-16) expression while inhibiting Homeobox A10 (HOXA10) expression. By modulating miR-16 and HOXA10, Qu acted as an anti-metastatic agent in oral cancer, highlighting Qu as a promising onco-therapeutic agent.[40] In addition, Kim et al. (2020) explained that Qu can reduce the survival and metastatic capacity of OSCC cells via the epithelial-to-mesenchymal transition (EMT)-mediated route., specifically Slug.[41]

The current results revealed that the Qu and 5-FU had a good synergistic effect and the dual Qu and (5-FU)-ChNPs induced anti-migratory effect against TSCC cells. This could be explained by Roshanazadeh et al. (2021) who illustrated that Qu synergistically potentiates the anti-metastatic effect of 5-FU against the breast cancer cell line.[20]

Therefore, 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 Qu could provide a compound utilizing their benefits, alongside avoiding the side effects of 5FU via reducing its dose. Such adjuvant compound could fight the cancerous cells more efficiently. Furthermore, admixing Qu with 5-FU could offer another alternative therapy with lower cost.

Overall, based on these data and the previous research works, this study underlined that it is quite clear that the Qu-ChNPs has important anticancer potentials against TSCC making it a promising chemopreventive and chemotherapeutic agent for oral cancer treatment and the combination of Qu with 5-FU has potent synergistic anticancer effect; this is likely to be confirmed by further investigations that will lead to their use in cancer therapy.

Conclusion

Chitosan NPs are good candidates for being employed as a carrier in TSCC therapy and the Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNPs have cytotoxic effect against TSCC cell line. The Qu-ChNPs have superior cytotoxic effect than that of (5-FU)-ChNPs and even than the gold standard 5-FU. Furthermore, the combination of Qu and 5-FU in chitosan nanoparticles yields a synergistic effect against TSCC cell line and the dual Qu and (5-FU)-ChNPs have the most potent cytotoxic effect of all the studied compounds. The Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNP succeed to increase the caspase 3 levels, which denotes induction of apoptosis. In addition, they reduce the invasion and migration abilities of TSCC cell line. The findings of this study may offer some useful insight for creating efficient preventive approaches and innovative treatment plans to enhance human health.

Recommendation

- The Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNPs worth further investigation for their potential chemotherapeutic use against TSCC as well as their side effects in experimental and then in human studies.
- The mechanistic pathways and the molecular targets of Qu-ChNPs, (5-FU)-ChNPs, and dual Qu and (5-FU)-ChNPs that mediate their anticancer effect are needed to be further elucidated.
- The co-delivery of Qu and 5-FU in chitosan nanoparticles, as a combined anticancer therapy, require further elucidation.

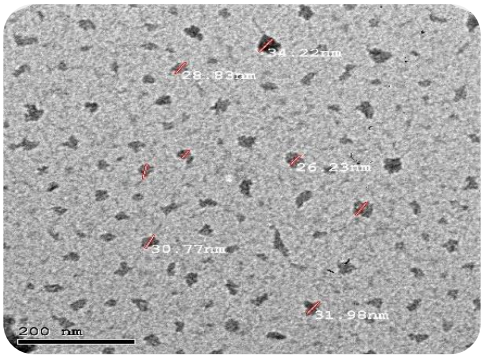
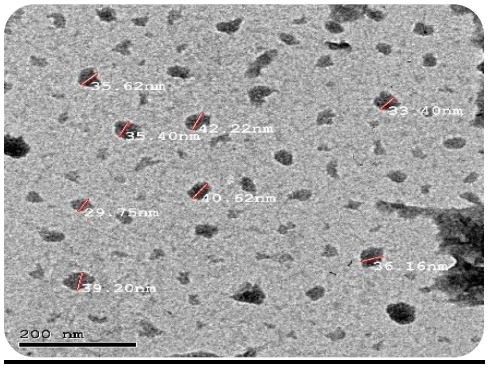


Figure 1.



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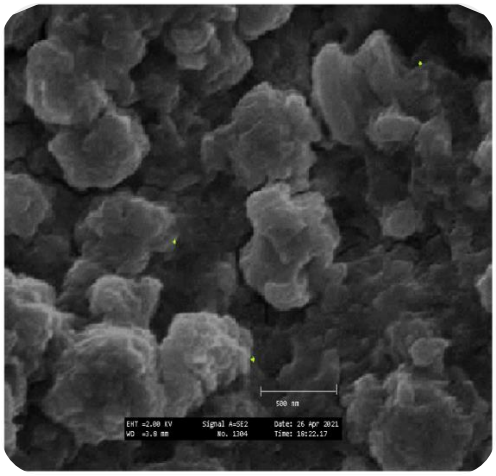

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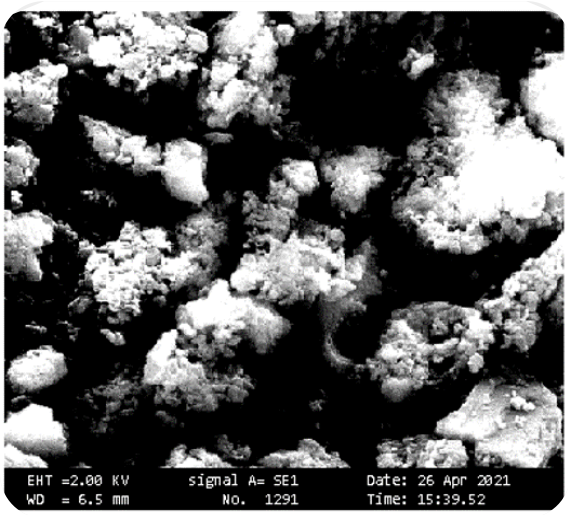

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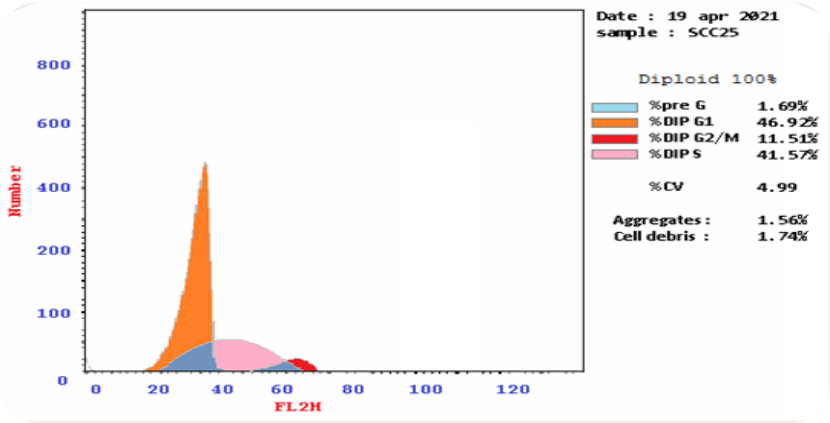

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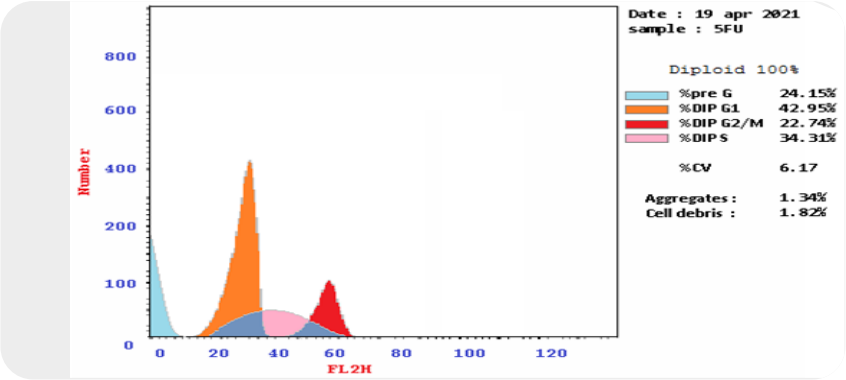

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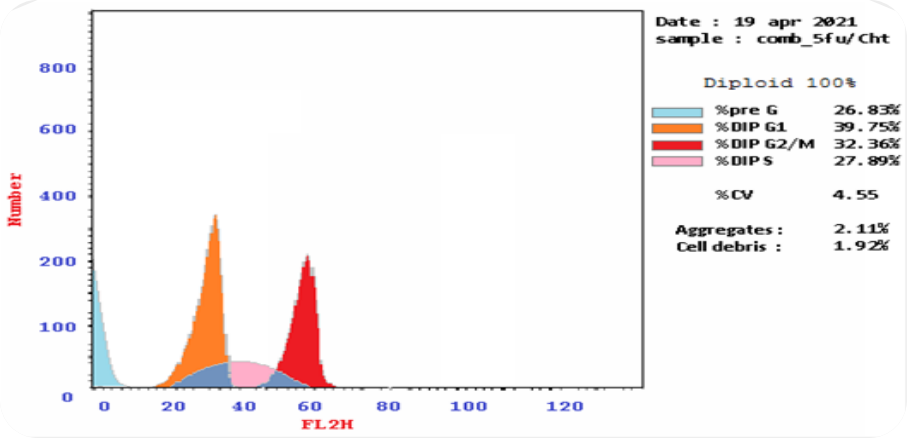

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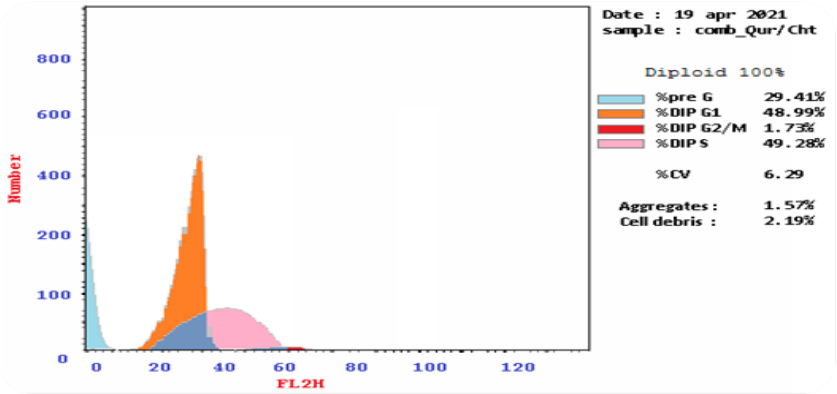

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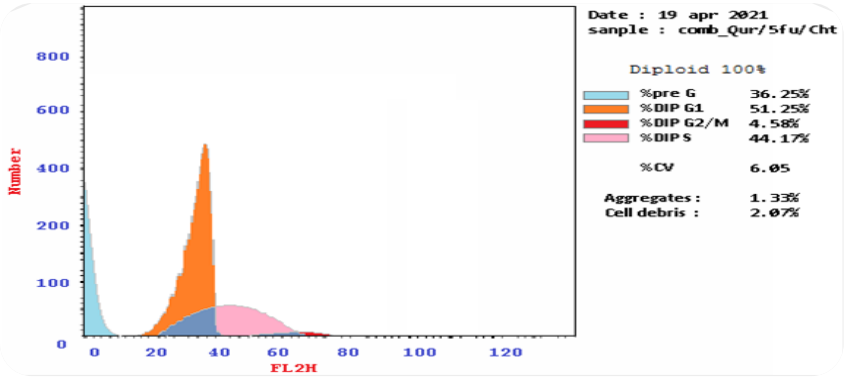

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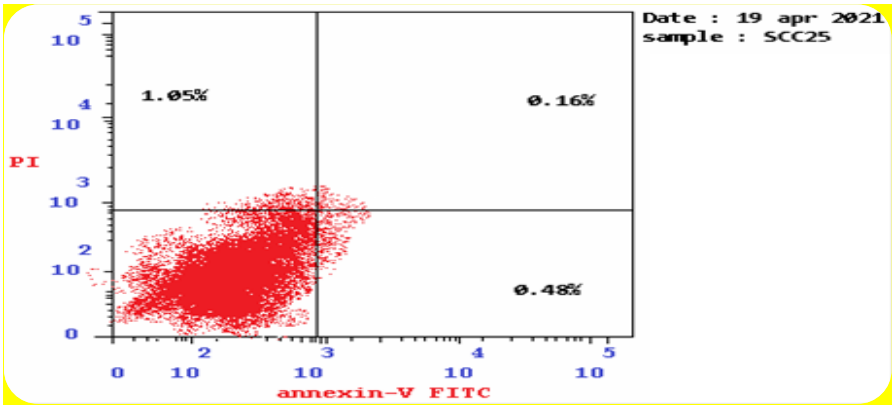

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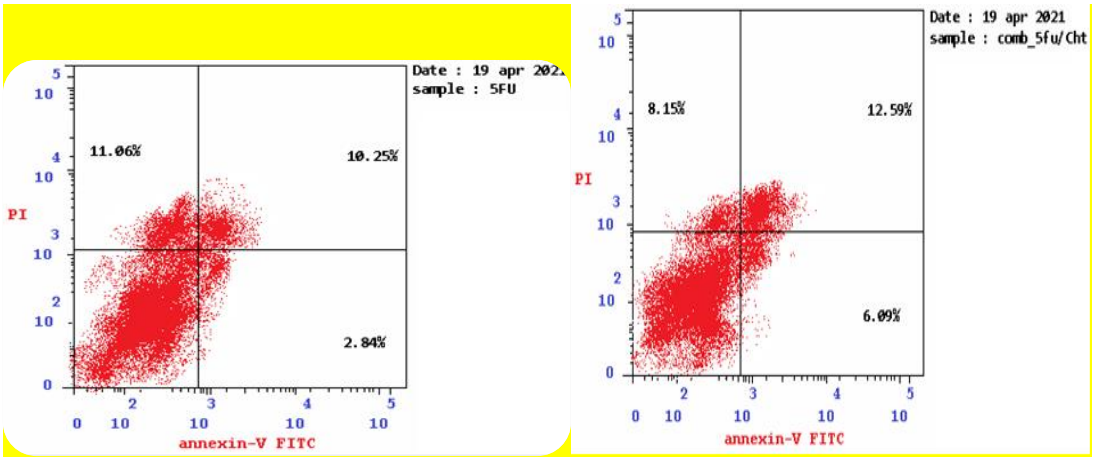

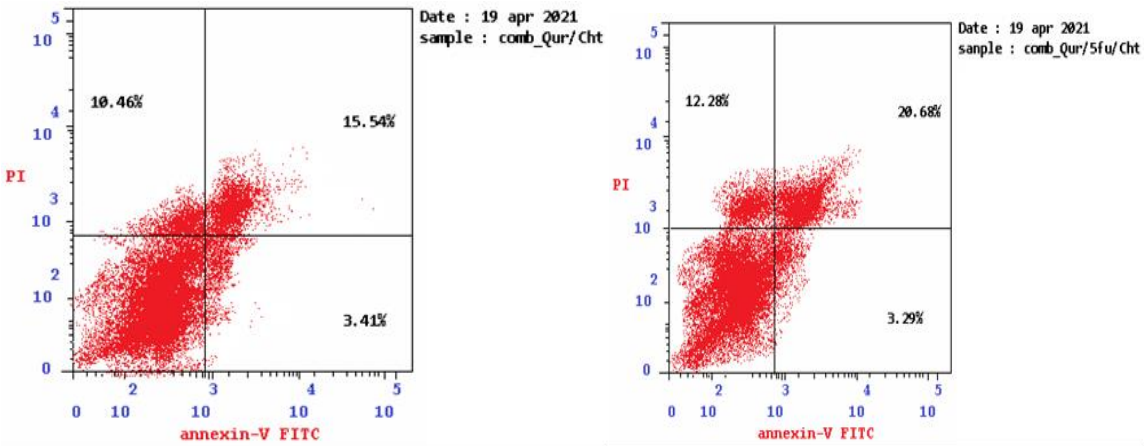

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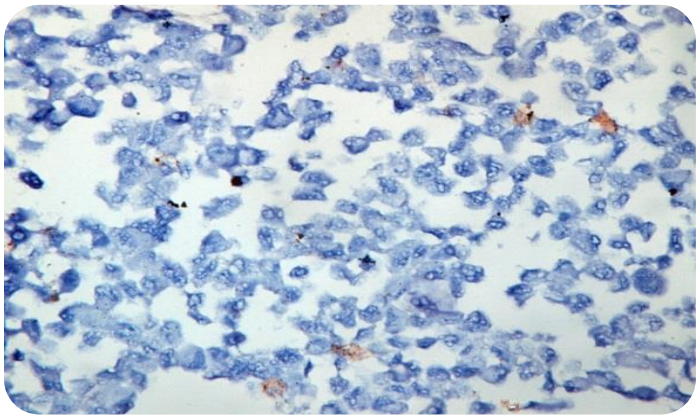

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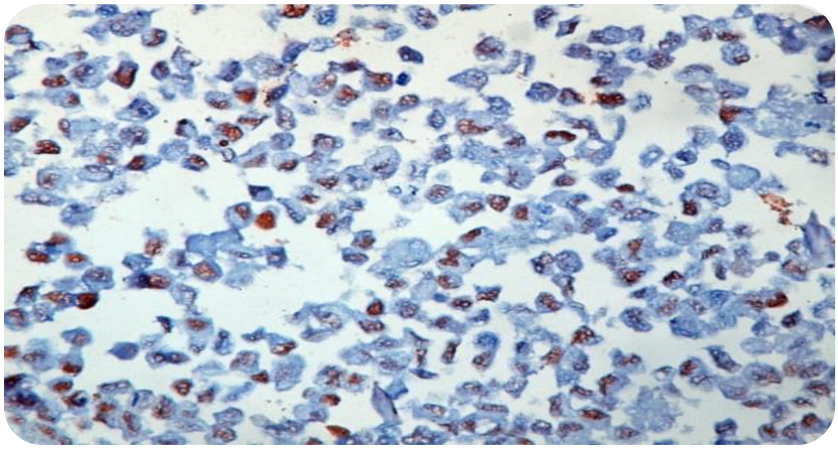

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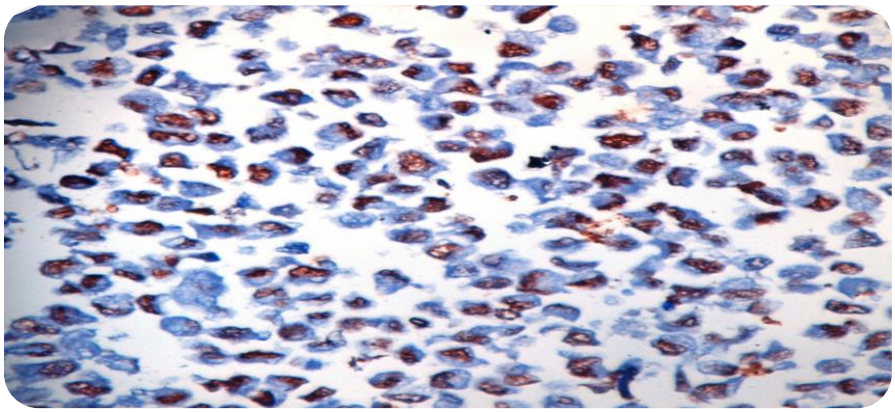

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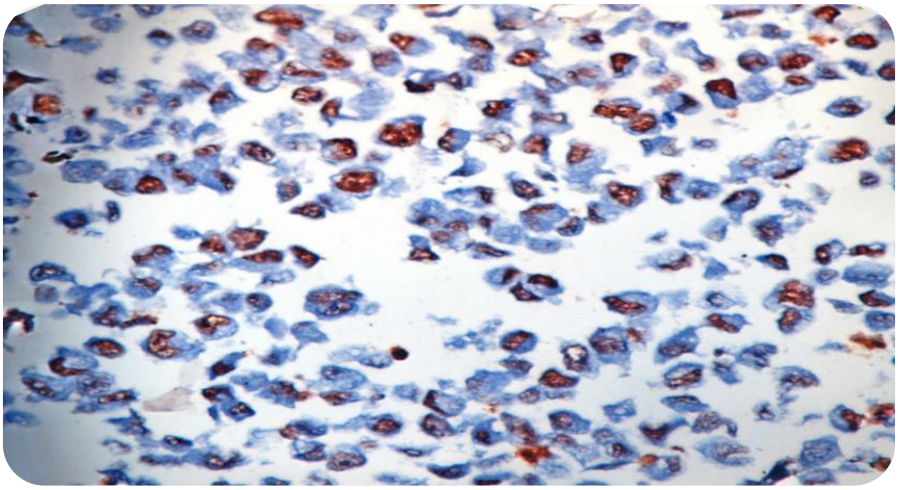

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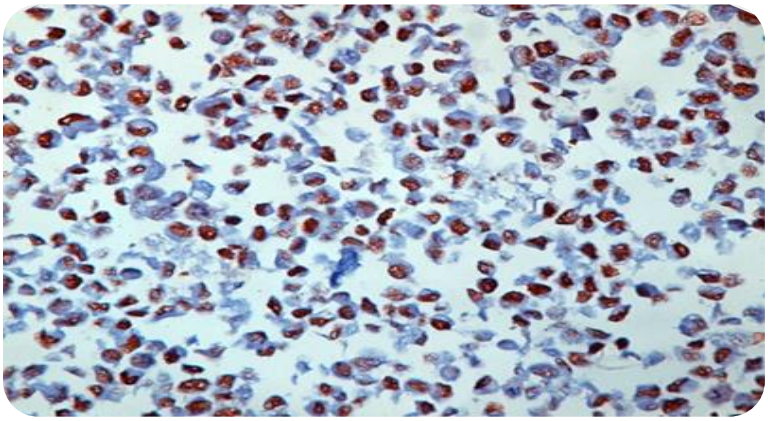

Figure 16.

Figure 17.

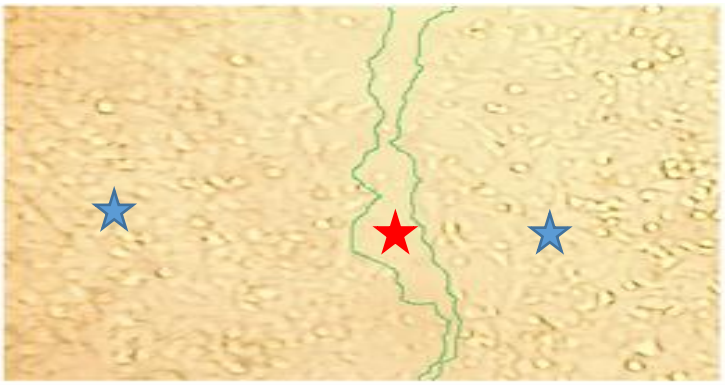

Figure 18.

Figure 19.

Figure 20.

Figure 21.

Figure 22.

Figure 1. Photomicrograph of chitosan nanoparticles observed by TEM

Figure 2. Photomicrograph of dual Qu and (5-FU)-ChNPs observed by TEM

Figure 3. Photomicrograph of chitosan nanoparticles observed by SEM

Figure 4. Photomicrograph of dual Qu and (5-FU)-ChNPs observed by SEM

Figure 5. Flow cytometric image showing representative histogram of DNA content in the untreated TSCC cells. The relative nuclear DNA amount and cell count are represented by the horizontal and vertical axes, respectively

Figure 6. Flow cytometric image showing representative histogram of DNA content in the TSCC cells treated with IC50 concentration of 5-FU. The relative nuclear DNA amount and cell count are represented by the horizontal and vertical axes, respectively

Figure 7. Flow cytometric image showing representative histogram of DNA content in the TSCC cells treated with IC50 concentration of (5-FU)-ChNPs. The relative nuclear DNA amount and cell count are represented by the horizontal and vertical axes, respectively

F**igure 8.** Flow cytometric image showing representative histogram of DNA content in the TSCC cells treated with IC50 concentration of Qu-ChNPs. The relative nuclear DNA amount and cell count are represented by the horizontal and vertical axes, respectively

Figure 9. Flow cytometric image showing representative histogram of DNA content in the TSCC cells treated with IC50 concentration of dual Qu and (5-FU)-ChNPs. The relative nuclear DNA amount and cell count are represented by the horizontal and vertical axes, respectively

Figure 10. Dot plot presentation of flow cytometric analysis of untreated TSCC cells showing quantitative analysis of apoptotic cells using the annexin V/PI double staining assay

Figure 11. Dot plot presentation of flow cytometric analysis of TSCC cells treated with IC50 concentration of (a): 5-FU, (b): 5-FU-ChNPs, (c): Qu-ChNPs, (d): dual Qu and (5-FU)-ChNPs showing quantitative analysis of apoptotic cells using the annexin V/PI double staining assay

Figure 12. Immunohistochemical expression of caspase 3 in the cytoplasm and nuclei of untreated control TSCC cells (DAB, ABC ×400)

Figure 13. Immunohistochemical expression of caspase 3 in the cytoplasm and nuclei of 5-FU-treated TSCC cells (DAB, ABC ×400)

Figure 14. Immunohistochemical expression of caspase 3 in the cytoplasm and nuclei of (5-FU- ChNPs)-treated TSCC cells (DAB, ABC ×400)

Figure 15. Immunohistochemical expression of caspase 3 in the cytoplasm and nuclei of (Qu-ChNPs)-treated TSCC cells (DAB, ABC ×400)

Figure 16. Immunohistochemical expression of caspase 3 in the cytoplasm and nuclei of (dual 5-FU and Qu-ChNPs)-treated TSCC cells (DAB, ABC ×400)

Figure 17. Cell migration determined by wound healing assay: quantitative evaluation of cell migration and invasion expressed as the percentage of the wound area covered by the untreated TSCC cells (control) at time of the insert removal. (Original magnification, ×200)

Figure 18. Cell migration determined by wound healing assay: quantitative evaluation of cell migration and invasion expressed as the percentage of the wound area covered by the untreated TSCC cells (control) after 24hours of the insert removal. Cells (blue stars) have higher migration ability into the wound area or a cell free gap (red star) compared to treated control groups. (Original magnification, ×200)

Figure 19. Cell migration determined by wound healing assay: quantitative evaluation of cell migration and invasion expressed as the percentage of the wound area covered by the 5fu-treated cells after 24hours of the insert

removal. Cells (blue stars) have limited migration ability into the wound area or a cell free gap (red star) compared to untreated control group. (Original magnification, ×200)

Figure 20. Cell migration determined by wound healing assay: quantitative evaluation of cell migration and invasion expressed as the percentage of the wound area covered by the (5-FU)-ChNPs-treated cells after 24hours of the insert removal. Cells (blue stars) have limited migration ability into the wound area or a cell free gap (red star) compared to untreated control group. (Original magnification, ×200)

Figure 21. Cell migration determined by wound healing assay: quantitative evaluation of cell migration and invasion expressed as the percentage of the wound area covered by the Qu-ChNPs-treated cells after 24hours of the insert removal. Cells (blue stars) have limited migration ability into the wound area or a cell free gap (red star) compared to untreated control group. (Original magnification, ×200)

Figure 22. Cell migration determined by wound healing assay: quantitative evaluation of cell migration and invasion expressed as the percentage of the wound area covered by the dual Qu and (5-FU)-ChNPs-treated cells after 24hours of the insert removal. Cells (blue stars) have limited migration ability into the wound area or a cell free gap (red star) compared to untreated control group. (Original magnification, ×200)

Table 1.

	Cell viability (%) (mean±SD)					p-value
Concentration (µg/ml)	100	10	1	0.1	0.01	
5-FU	10.36 ±0.40D	49.22± 0.40C	87.46 ±0.35B	99.33 ±0.28A	100.00 ±0.00A	<0.001*
5-FU-ChNPs	9.95 ±0.82D	46.32 ±2.16C	86.46 ±0.53B	99.05 ±0.31A	100.00 ±0.00A	<0.001*
Qu-ChNPs	18.88 ±0.89E	48.45 ±1.15D	84.11 ±0.93C	95.09 ±0.03A	99.81 ±0.15A	<0.001*
Dual Qu and (5-FU)-ChNPs	7.45 ±0.07D	37.45 ±0.27C	88.72 ±0.20B	99.90 ±0.00A	100.00 ±0.00A	<0.001*

*Means with different superscript letters within the same horizontal row are significantly different while means with same superscript letters within the same horizontal row are non-significantly different, *; significant ($p \leq 0.05$) ns; non-significant ($p>0.05$)*

Table 2.

IC 50 (mean±SD)				p-value
5-FU	(5-FU)-ChNPs	Qu-ChNPs	Dual Qu and (5-FU)-ChNPs	
10.11 ± 0.89^{A}	8.48 ± 0.52^{B}	7.48 ± 1.36^{B}	5.75 ± 0.17^{C}	**<0.001***

*Means with different superscript letters within the same horizontal row are significantly different while means with same superscript letters within the same horizontal row are non-significantly different, *; significant ($p \leq 0.05$) ns; non-significant ($p>0.05$)*

Table 3.

ser	Sample data		Results DNA content SCC-090				
	code	IC50	%G0-G1	%S	%G2/M	%Pre-G1	Comment

		ug/ml					
1	Dual Qu and (5-FU)-ChNPs	5.75	51.25	44.17	4.58	36.25	cell growth arrest@G1/S
2	Qu-ChNPs	7.48	48.99	49.28	1.73	29.41	cell growth arrest@G1/S
3	(5-FU)-ChNPs	8.48	39.75	27.89	32.36	26.83	cell growth arrest@G2/M
4	5-FU (Positive control)	10.11	42.95	34.31	22.74	24.15	cell growth arrest@ G2/M
-	control		46.92	41.57	11.51	1.69	---

Table 4.

Pre-G1 (%) (mean±SD)					p-value
Negative control	5-FU	(5-FU)-ChNPs	Qu-ChNPs	Dual Qu and (5-FU)-ChNPs	
1.69±0.06E	24.15±0.25D	26.83±0.30C	29.41±0.45B	36.25±0.41A	<0.001*

*Means with different superscript letters within the same horizontal row are significantly different while means with same superscript letters within the same horizontal row are non-significantly different, *; significant ($p \leq 0.05$) ns; non-significant ($p>0.05$)*

Table 5.

s	code	IC50 ug/ml	Apoptosis			Necrosis
			Total	Early	Late	
1	Dual Qu and (5-FU)-ChNPs	5.75	23.97	3.29	20.68	12.28
2	Qu-ChNPs	7.48	18.95	3.41	15.54	10.46
3	(5-FU)-ChNPs	8.48	18.68	6.09	12.59	8.15
4	5-FU (Positive control)	10.11	13.09	2.84	10.25	11.06
--- control	0.64 0.48 0.16	1.05				

Table 6.

Apoptosis	Apoptotic cells (%) (mean±SD)					p-value
	Negative control	5-FU	(5-FU)-ChNPs	Qu-ChNPs	Dual Qu and (5-FU)-ChNPs	
Early	0.48 ±0.05D	2.84 ±0.08C	6.09 ±0.06A	3.41 ±0.04B	3.29 ±0.06B	<0.001*
Late	0.16 ±0.06E	10.25 ±0.54D	12.59 ±0.24C	15.54 ±0.34B	20.68 ±0.48A	<0.001*
Total	0.32 ±0.18B	6.54 ±4.07A	9.34 ±3.56A	9.47 ±6.65A	11.98 ±9.53A	0.018*

*Means with different superscript letters within the same horizontal row are significantly different while means with same superscript letters within the same horizontal row are non-significantly different, *; significant ($p \leq 0.05$) ns; non-significant ($p>0.05$))*

Table 7.

Samples	Fields	Caspase-3 % of positive cells	Score (Caspase-3 Analysis by IHC profiler)
Control	1	0.13	Negative
	2	0.05	Negative
	3	0.09	Negative
	4	0.12	Low positive
	5	0.07	Negative
5FU	1	15.02	Positive
	2	14.98	Positive
	3	14.87	High positive
	4	15.13	Positive
	5	14.76	Positive
(5-FU)-ChNPs	1	20.71	High positive
	2	20.56	Positive
	3	21.07	Positive
	4	20.97	Positive
	5	20.34	High positive
Qu-ChNPs	1	22.23	Positive
	2	21.95	High positive
	3	20.99	High positive
	4	21.87	Positive
	5	22.00	High positive
Dual Qu and (5-FU)-ChNPs	1	25.31	High positive
	2	25.24	Positive
	3	24.76	High positive
	4	24.89	High positive
	5	25.29	Positive

Table 8.

Caspase-3 positive cells (%) (mean±SD)					p-value
Negative control	5FU	5FU-ChNPs	Qu-ChNPs	Dual Qu and 5FU-ChNPs	
0.09±0.03E	14.95±0.14D	20.73±0.30C	21.81±0.48B	25.10±0.25A	<0.001*

*Means with different superscript letters within the same horizontal row are significantly different while means with same superscript letters within the same horizontal row are non-significantly different, *; significant ($p \leq 0.05$) ns; non-significant ($p>0.05$)*

Table 9.

Wound closure (%) (mean±SD)					p-value
Negative control	5-FU	(5-FU)-ChNPs	Qu-ChNPs	Dual Qu and (5-FU)-ChNPs	
97.63±0.56A	65.70±0.37B	59.11±0.34C	56.52±0.31D	49.93±0.55E	<0.001*

*Means with different superscript letters within the same horizontal row are significantly different while means with same superscript letters within the same horizontal row are non-significantly different, *; significant ($p \leq 0.05$) ns; non-significant ($p>0.05$)*

Table (1): Mean and standard deviation (SD) values of cell viability Percentage (%) for different concentrations of the experimental groups

Table (2): Mean and standard deviation (SD) values of IC50 for various groups

Table (3): The distribution pattern of cells within various cell cycle phases in the different study groups

Table (4): Mean and standard deviation (SD) values of Pre-G1 Percentage (%) for various groups

Table (5): The average percentage of apoptotic and necrotic cells in the different study groups

Table (6): Mean and standard deviation (SD) values of the percentage (%) of apoptotic cells for different groups

Table (7): Protein expression of caspase-3 in TSCC represented by the percentage of positive cells and score analysis by IHC profiler of Image J

Table (8): Mean and standard deviation (SD) values of Caspase-3 positive cells Percentage (%) for different groups

Table (9): Mean and standard deviation (SD) values of wound closure Percentage (%) for different

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