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Original Research Article

Anticancer and Antioxidant Effects of Sitagliptin and Linagliptin against Lung Cancer Cell Lines (*an In vitro Study*)

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ABSTRACT

Lung cancer has the highest global fatality rate of all cancers, and most existing therapies have a wide range of toxic effects. This necessitates searching for new medications with potential anticancer properties and a better safety profile against normal cells. Dipeptidyl peptidase-4 (DPP4) inhibitors have recently shown anticancer efficacy in various malignancies such as colorectal, prostate, and renal cancer. Therefore, this study investigated the anticancer activity of sitagliptin (SITA) and linagliptin (LINA) against the lung cancer cell line A549 alone and in combination with cisplatin (CP). A549 cells were divided into six groups: control (untreated cells), CP-treated cells, SITA-treated cells, LINA-treated cells, CP plus SITA-treated cells (ratio 1:1), and CP plus LINA-treated cells (ratio 1:1). After 72 hours of incubation, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test was used to determine cell viability and the concentration of 50% inhibition for cell viability (IC_{50}) for each group. A549 cells were later seeded in six flasks and treated with the resulting IC_{50}; cell pellets were collected and lysed to determine the malondialdehyde (MDA) level using ELISA kits. SITA and LINA therapy dramatically reduced A549 cell viability compared to the control ($P < 0.0001$), with results comparable to CP. When SITA and LINA were combined with CP, they demonstrated significantly higher anticancer efficacy than when used alone. Notably, both medicines lowered MDA levels when taken alone or in combination with CP. SITA and LINA showed promising anticancer and antioxidant activity against A549 cells. This may indicate a potential synergistic anticancer effect with CP.

***Keywords*:** sitagliptin, linagliptin, A549 cell line, cisplatin, antioxidant

Introduction

Lung cancer, also called pulmonary cancer, is a malignant tumor that develops in the lungs. It is characterized by the rapid and abnormal development of cells, resulting in tumor formation. These tumors progressively increase in number and size, obstructing the lung's capacity to efficiently exchange oxygen.[1]

In later progressive stages, lung cancer may metastasize to other body tissues, including adjacent lymph nodes or different regions in the lung and thoracic cavity, brain, skeletal system, nervous system, liver, bone, or adrenal glands. The median survival time following diagnosis was 13 months for individuals with non-metastatic lung cancer and five months for those with metastatic lung cancer.[2]

Lung cancer significantly contributes to morbidity and mortality worldwide, representing 12% of newly diagnosed cancers and causing 18% of yearly cancer-related deaths.[3] It has the most significant mortality globally due to its unfavorable prognosis.[4] The latest Global Cancer Observatory (GLOBOCAN) estimates indicated 2,206,771 new cases of lung cancer diagnosed worldwide in 2020; lung tumors were the leading cause of cancer incidence and mortality in men, with 1,435,943 new cases and 1,188,679 deaths.

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Among women, it ranked as the third most prevalent cause of cancer, with 770,828 incidents reported, and the second most common cause of cancer mortality, with 607,465 deaths following breast cancer.[5] Generally, lung cancer has two primary histologic types: small cell lung cancer (SCLC) comprises 15% of cases, whereas non-small cell lung cancer (NSCLC) makes up 85% of cases.[6]

Therapeutic options consist of one or more of the following: surgery, radiotherapy, immunotherapy, and chemotherapy, such as cisplatin (CP).[7] CP is a platinum-based drug typically given intravenously as the initial chemotherapy treatment for patients with various types of cancer.[8-10] Anticancer drugs induce oxidative stress in biological systems, producing lipid peroxidation and several electrophilic aldehydes. The consequences of oxidative stress can elevate the efficacy of anticancer therapies by inhibiting the proliferation of cancer cells.[11] Although CP monotherapy has demonstrated favorable clinical outcomes, numerous studies have documented significant adverse effects. Additionally, certain cancer patients have exhibited considerable drug resistance and toxicity. Therefore, novel formulations and combination therapies involving other medications have been examined to enhance CP's therapeutic effectiveness.

Recent studies have shown that dipeptidyl peptidase-4 (DPP4) inhibitors have notable anticancer effects on cancer cells, such as colon[12] and ovarian[13] cancer cells, particularly sitagliptin (SITA) and linagliptin (LINA).[14] Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are incretin hormones that enhance insulin secretion and are inactivated via the DPP4 enzyme.[15] DPP4 inhibitors block the effect of the DPP4 enzyme and extend incretin hormone activity. Consequently, insulin secretion after a meal increases significantly. In contrast, glucagon secretion is reduced.[16] The DPP4 enzyme, also known as Cluster of Differentiation 26 (CD26), is a transmembrane protein expressed by many normal cell types, with variable expression in cancers depending on type .[17] Some cancers, such as breast, melanoma, and endometrial cancers, are associated with

decreased DPP4 expression, suggesting its tumor-suppressing activity. Other, such as mesothelioma and renal, colon, and lung cancers, are associated with increased DPP4 expression, suggesting its tumor-activating role in these cancers.[18] DPP4 plays an important role in cancer biology as well as the progression and formation of metastases in malignant cells.[19,20] and the prognosis of cancer patients, [21] representing a helpful tumor marker and a novel therapeutic target for selected tumors.[22] Even though DPP4 inhibitors have been demonstrated to enhance the control of specific cancer types, including colon,[23] breast,[24] prostate,[25] kidney,[26] and colorectal cancers,[27] in addition to modifying the balance of oxidative stress during chemotherapy.[28] Direct comparative studies on the activity of SITA and LINA alone or in combination with standard chemotherapy against lung cancer cell lines A549 are limited. Additionally, the specific molecular mechanisms through which these drugs exert their anticancer effects, particularly in the lung tumor, still need to be fully understood. Exploring this gap could lead to a better understanding of the potential role of DPP-4 inhibitors in lung cancer treatment and might contribute to developing novel therapeutic strategies for lung cancer.
Therefore, this study aimed to evaluate the anticancer and antioxidant properties of SITA and LINA on the A549 lung cancer cell line.

Materials and Methods

Chemicals and cell line
A549 lung cancer cells were obtained from Basrah's Iraq Biotech Cell Bank Unit, passage number 20.
Generally, A459 cells were isolated from a human alveolar-cell carcinoma of a White, 58-year-old man in January 1972.
They underwent continuous *in vitro* propagation for over three years, resulting in over 1,000 cell generations widely studied as lung cancer models.[29]
Dimethyl sulfoxide (DMSO), MTT(3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) dye powder, and RIPA lysis buffer were obtained from Sigma, USA. 10% fetal bovine serum (FBS), phosphate buffer saline (PBS), and Roswell Park Memorial Institute-1640 (RPMI-1640) were obtained from Gibco, USA. Trypsin- EDTA was obtained from Capricorn, USA. The trypan-blue stain was obtained from Flow Laboratories, UK. Benzylpenicillin and streptomycin were obtained from Troge, Germany.CP was obtained from Pfizer, USA. SITA was obtained from Anhui Haikang Pharmaceutical CO, China. LINA was obtained from CHICO Pharmaceutical CO, China. A human MDA ELISA kit was obtained from the Bioassay Technology Laboratory in China.

Cell culture and MTT assay
The A549 cell lines were extracted using trypsin-EDTA as a proteolytic protein, PBS for rinsing the media, and FBS to deactivate trypsin, then cultured in a 96-well plate using RPMI-1640 liquid media with 100 units/mL penicillin, and 100 µg/mL streptomycin. The sample was allowed to incubate for 24 hours at 37 °C, 5% CO_2, and 95% humidity to encourage the formation of a single layer of cells (80% growth phase). Viable cells were counted using trypan blue dye. The previous medium was then substituted with 200 µL of the medium, including the test medicines and control group.[30] Six primary groups were utilized: control (untreated cells), CP-treated cells, SITA-treated cells, LINA-treated cells, CP plus SITA-treated cells (ratio 1:1), and CP plus LINA-treated cells (ratio 1:1). For each treated group, six concentrations (each with four replicates) were used:500, 250, 125, 62.5, 31.25, and 15.625 µg/mL. A blank (containing only medium) was used to evaluate the non-specific conversion of formazan and the tested drugs. After 72 hours of incubation, the (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide (MTT) test31 were used to determine cell viability and dose-effect curves were calculated by non-linear regression based on a four-parameter logistic Hill equation. The concentration of 50% inhibition for cell viability (IC_{50}) was calculated using GraphPadPrism10 for each group. The cell viability percentage was evaluated using the following formula:

$$\text{Cell viability\%} = \frac{\text{As}-\text{Ab}}{\text{Ac}-\text{Ab}} \times 100\%$$

Where As is the sample's absorbance, Ab is the blank's absorbance, and Ac is the control absorbance. All the determinations were done in four replicates.

Oxidative stress assay: ELISA measurement of MDA concentration
A549 cells were later seeded in six flasks (each with three replicates) and treated with the resulting IC_{50} for 36 hours. Cells were then extracted and centrifuged, and the supernatants were discarded. The pellets were collected and lysed by lysis buffer to obtain proteins and stored in a sterile Eppendorf tube, then frozen at -20°C to be used later for MDA measurement using the ELISA assay kit.
The human MDA ELISA kit, obtained from Bioassay Technology Laboratory Company (BT Lab) in Shanghai, China, was used to measure the MDA concentration in the samples. The experiment was conducted based on the BT LAB procedure following the company protocol provided (as a manual kit guideline). The standard stock solution was diluted at ratios ranging from 1:2 to 1:16. 50 µL of the standard was added to the standard well, 40 µL of the sample to the sample wells, and 10 µL of anti-MDA antibody and 50 µL of streptavidin-HRP to both the sample and standard wells. A sealant was used to cover the plate, and it was incubated at 37°C for one hour. The plate was repeatedly cleaned in the wash buffer. Each wash lasted between 30 seconds and one minute.
After dispensing 300 µL of wash buffer, each well received 50 µL of substrate solution A, followed by substrate solution B (per the kit manual guideline). A color change occurred upon applying the substrate solutions, directly correlated with the human MDA level. An absorbance reading was taken at 450 nm after the procedure was terminated by adding an acidic stop solution. A microplate reader (Thermo Fisher Scientific, USA) was configured to operate at a wavelength of 450 nm to determine the absorbance value of each well.

Statistical analysis
The data were collected and examined using GraphPad Prism Edition 10 and Microsoft Office Excel 2019. A one-way ANOVA test and post hoc (Tukey) were used to assess significant differences among the data means. A p-value of 0.05 or less indicated a statistically significant difference.

Results and Discussion

The main challenges in cancer management are the adverse effects of drugs and the development of resistance to therapy, which account for over 90% of deaths in patients receiving chemotherapeutics.[32]
Many therapeutic alternatives, such as combination therapy, may overcome these restrictions, enhancing the drug's therapeutic efficacy and safety and reducing the adverse effects of anticancer treatments by decreasing the dosage.[33,34]

MTT cell viability assay
This study aimed to assess the anti-cancer effects of SITA and LINA on A549 cancer cells, both alone and in combination with CP. To accomplish this, the viability and toxicity of the cancer cells were assessed using the MTT assay.

Cisplatin activity on the viability of the A549 cell line
The findings demonstrated a significant reduction in the viability of cells ($P < 0.0001$) for all concentrations (500, 250, 125, 62.5, 31.25, and 15.625 µg/mL) when compared with the control group, as seen in Figure 1.
CP anticancer activity has already been approved, and this chemotherapy is one of the standard medicines widely used for lung cancer treatment.[8] The primary biological target for CP is DNA.[35] The mechanism by which CP manifests its cytotoxicity toward tumor cells includes cellular intake and transport of CP to the nucleus, binding to DNA. This leads to the formation of intra-strand DNA adducts, preventing repair of the DNA, thus blocking DNA replication and transcription. It causes cell cycle arrest at S, G1, or G2-M, inhibiting cell growth and subsequently inducing apoptosis.[36]
The activity of sitagliptin or linagliptin on the viability of the A549 cell line

SITA demonstrated a significant decrease in the cell viability (P< 0.0001) for all concentrations (500, 250, 125, 62.5, 31.25, and 15.625 µg/mL) compared to the control group. LINA demonstrated a significant reduction in the cell viability (P < 0.0001) at concentrations of 500, 250, 125, 62.5 µg/mL, and (P < 0.05) at 31.25 and 15.625 µg/mL, compared to the control group, as shown in Figures 2 and 3, respectively.

A previous study found that expression of the DPP4 enzyme was significantly higher in lung adenocarcinoma than in normal lung tissue. This suggests that inhibiting an enzyme by DPP4 inhibitors can potentially suppress lung cancer growth.[37]

According to the current study findings, both SITA and LINA have shown anticancer activity, (significantly reducing the viability of A549 cells compared with the control using the MTT assay. These results are consistent with those published by Amritha *et al.*[23], who used the MTT assay to evaluate the anti-cancer effect of DPP4 inhibitors SITA and vildagliptin (VILDA) on colorectal cell lines (HT-29). They found that both drugs had significant anti-cancer properties compared to the control, which acted as a cytotoxic agent in tumor cells. SITA showed greater efficacy than VILDA in colon cancer cell lines. Similarly, a recent study found that diabetic people who received the DPP4 inhibitor SITA had enhanced overall survival following surgery for colorectal or lung cancer compared to patients who were undergoing alternative diabetic treatments.[38] Later studies have shown that LINA inhibited cell viability in HCT116 (colorectal) cancer cells by causing cell cycle arrest at the G2/M and S phases, inhibiting cell proliferation. *In vivo*, LINA can slow the growth of tumors by significantly decreasing the expression of Ki67, a nuclear protein found in all proliferating cells.

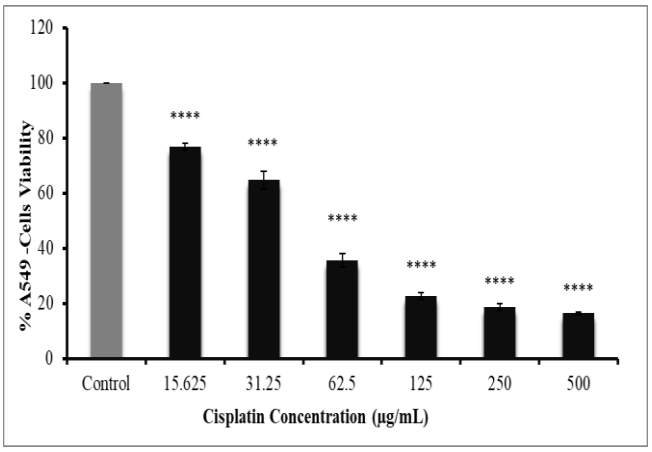

Figure 1: The effect of different cisplatin concentrations on the viability of the A549 cell line.
One-way ANOVA was used for the examination. Data are displayed as mean.
± SD, **** P < 0.0001, n= 4, incubation for 72 hours.

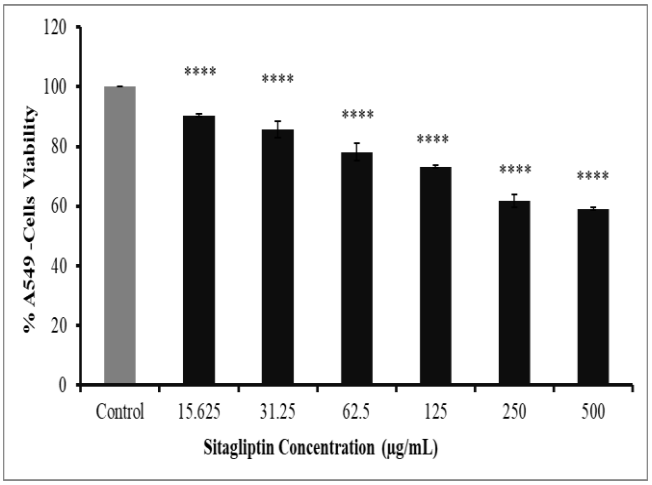

Figure 2: The effect of different concentrations of sitagliptin on the viability of the A549 cell line.

One-way ANOVA was used for the examination. Data are displayed as mean ± SD, **** P < 0.0001, n= 4, incubation for 72 hours.

This was seen in mice implanted with human colorectal cancer cells compared to the control group. This model is considered the most relevant in clinical settings for studying the impact of a compound on tumor development.[39,40]

Activity of a combination of cisplatin plus sitagliptin or linagliptin on the viability of the A549 cell line

In this part of the study, A549 cells were tested with a constant ratio (1:1) of CP plus SITA or LINA concentrations. The results showed that both combinations significantly reduced the cell's viability (P < 0.0001) for all concentrations compared with the control group, as seen in Figures 4 and 5, respectively.

Comparison between the activity of cisplatin alone against cisplatin and sitagliptin combination on the viability of the A549 cell line

As shown in Figure 6, the CP plus SITA combination reduced the viability of A549 cells at a high level of significance (P < 0.0001) at concentrations of 15.625, 31.25, and 62.5 µg/mL in comparison with cells exposed to CP alone.

However, compared to CP alone, the combination did not significantly alter cell viability (P > 0.05) at 125, 250, or 500 µg/mL concentrations.

Comparison between the activity of cisplatin alone against cisplatin and linagliptin combination on the viability of the A549 cell line.

As shown in Figure 7, the CP plus LINA combination reduced the viability of A549 cells at a high level of significance (P < 0.0001) at concentrations of 15.625, 31.25, and 62.5 µg/mL in comparison with cells exposed to CP alone. However, compared to CP alone, the combination did not significantly alter cell viability (P > 0.05) at 125, 250, or 500 µg/mL concentrations.

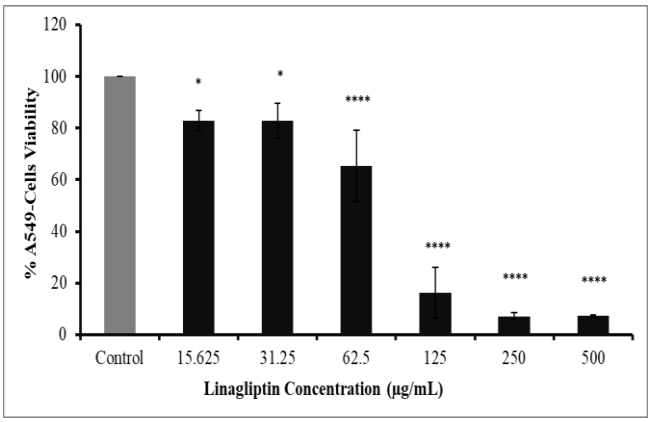

Figure 3: The effect of different concentrations of linagliptin on the viability of the A549 cell line.
One-way ANOVA was used for the examination. Data are displayed as mean ± SD. **** P < 0.0001, * P < 0.05, n= 4, incubation for 72 hours

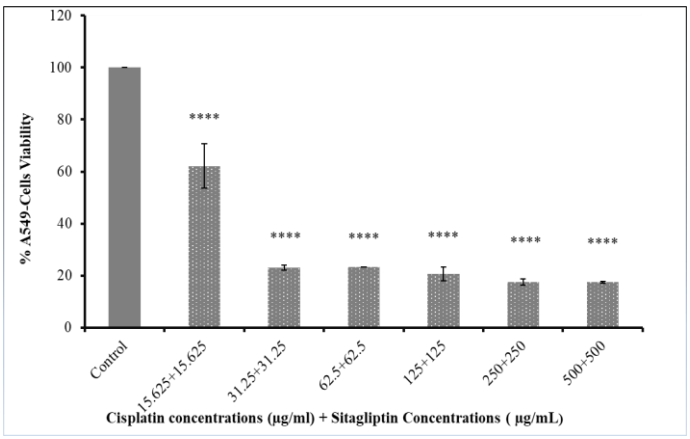

Figure 4: The effect of different concentrations of cisplatin plus sitagliptin on the viability of the A549 cell line.

One-way ANOVA was used for the examination. Data are displayed as mean ± SD, **** P < 0.0001, n= 4, incubation for 72 hours.

Combining SITA and LINA with CP significantly decreased the viability of A549 cells at low concentrations (15.625, 31.25, and 62.5 ug/mL) when compared with CP alone. This indicates a synergistic effect between CP and SITA and LINA, respectively, that increases cytotoxicity against cancer cells compared to that obtained when using CP alone. Previous studies have demonstrated the synergism between CP and DPP4 inhibitors, such as one that assessed the effect of SITA alone or in combination with paclitaxel on the development of ovarian cancer cells and the process of metastasis.[41]

Measurement of human MDA levels

The oxidative stress levels of the investigated drugs and combinations in this study were evaluated using the MDA assay in the tumor microenvironment.

Overproduction of reactive oxygen species (ROS) interrupts the antioxidant defense mechanisms and results in cellular oxidative stress,[42] which induces mitochondrial dysfunction, DNA damage, and cell death.[43] Oxidative stress has been associated with tumors' survival, growth, propagation, and angiogenesis. [44]

Cisplatin effects on MDA concentration

The findings demonstrated a significant elevation in MDA concentration (P < 0.0001) following treatment of A549 cells with an IC_{50} of CP compared to the control group, as seen in Figure 8.

Many studies have indicated that oxidative stress is one of the most critical mechanisms of CP cytotoxicity by increasing ROS directly or indirectly, leading to lipid peroxidation, increased MDA production, reduced glutathione (GSH) activity, and eventually inducing apoptosis.[45] In this study, the MDA level was measured to assess the level of oxidative stress. The findings demonstrated a significant elevation in MDA concentration (P < 0.0001) following treatment with the IC_{50} of CP alone compared to the control group, as seen in Figure 8, indicating CP's capability to induce oxidative stress. The findings are consistent with previous studies in which CP induced lipid peroxidation and significantly increased MDA levels in human leukemia (HL60) cells for all concentrations compared with the control.[46] However, the concentration of MDA in normal renal cells among mice subjected to CP treatment increased by 1.5 times, leading to kidney cell death.[47]

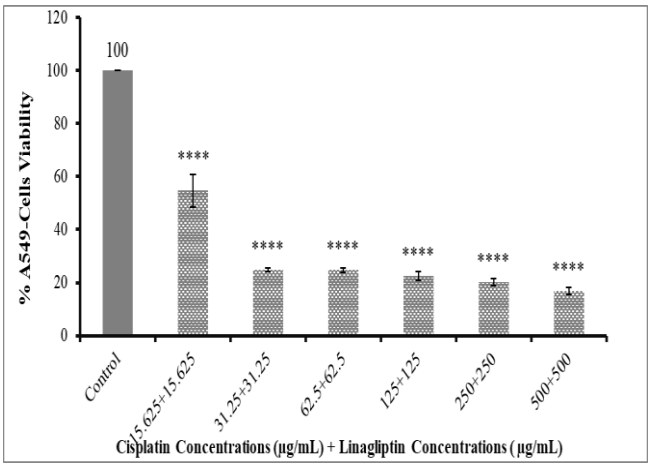

Figure 5: The effect of different concentrations of cisplatin plus linagliptin on the viability of the A549 cell line.

One-way ANOVA was used for the examination. Data are displayed as mean ± SD, **** P < 0.0001, n= 4, incubation for 72 hours.

Sitagliptin and linagliptin effects on MDA concentration

The findings indicated a significant reduction in MDA level (P < 0.0001) following treatment of A549 cells with IC_{50} of SITA and LINA compared to the control group. However, no significant difference was detected between SITA and LINA-treated cells, as seen in Figure 8.

The coadministration of CP with other drugs possessing antioxidant properties has decreased its toxicity. For example, DPP4 inhibitors (SITA and LINA) can enhance the function of nuclear factor erythroid 2-related factor 2 (Nrf2), a stimulator of diverse antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), which could be accountable for its antioxidant effect.[26,48]

Both SITA and LINA displayed a notable reduction in the MDA level of the A549 cells. Relative to the control group, these results aligned with the findings of earlier studies, which indicated the antioxidant effects of SITA and LINA in different tissues and conditions such as human mononuclear blood cells,[49] Parkinson's disease,[50] diabetic nephropathy,[51] and atherosclerosis. [52]

Comparison between the activity of cisplatin alone against cisplatin and sitagliptin or linagliptin combinations on the MDA concentration

When A549 cells were exposed to CP plus SITA or LINA IC_{50}, the amount of MDA in the cells was significantly lower (P< 0.0001) compared to cells only treated with CP. Additionally, no significant differences were seen between CP plus SITA and CP plus LINA-treated cells, as demonstrated in Figure 9.

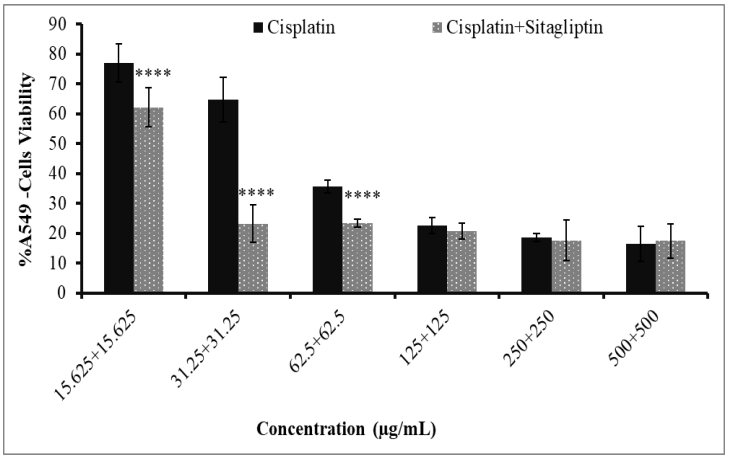

Figure 6: Comparison between the activity of cisplatin alone against cisplatin and sitagliptin combination on the viability of the A549 cell line.

One-way ANOVA was used for the examination. Data are displayed as mean ± SD, **** P< 0.0001, n= 4, incubation for 72 hours.

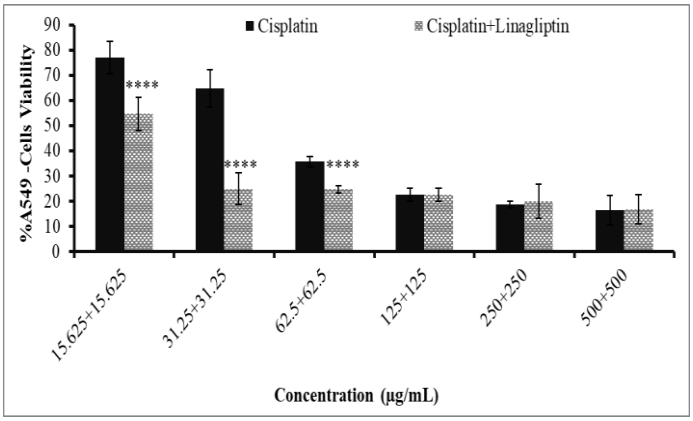

Figure 7: Comparison between the activity of cisplatin alone against cisplatin and linagliptin combination on the viability of the A549 cell line.

One-way ANOVA was used for the examination. Data are displayed as mean ± SD, **** P < 0.0001, n= 4, incubation for 72 hours.

The effect of the combination of CP plus SITA or LINA showed a notable reduction in the MDA concentration compared to the control and CP-treated groups. This demonstrated the capacity to reduce the oxidative stress of DPP4 inhibitors and consequently decrease CP toxicity and adverse effects. The results reported by Salama *et al.*[28] and Alameen *et al.*[53] showed that the combination of SITA with doxorubicin or CP led to a significant decrease in MDA levels compared to both the control group and the groups treated with doxorubicin or CP alone, which is consistent with the present research.

Conclusion

DPP4 inhibitors showed anticancer activity against A549 cells based on MTT assay results at concentrations of 500, 250, 125, 62.5, 31.25, and 15.625 µg/mL and, in combination with CP, synergistically inhibited the viability of A549 cells at low concentrations of 62.5, 31.25, and 15.625 µg/mL. This may help to decrease the required CP dosage and attenuate its adverse effects on humans during cancer chemotherapy protocols. Furthermore, based on MDA measurements, the antioxidant effect of DPP4 inhibitors on A549 cells indicated partial protection against oxidative stress for healthy cells.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

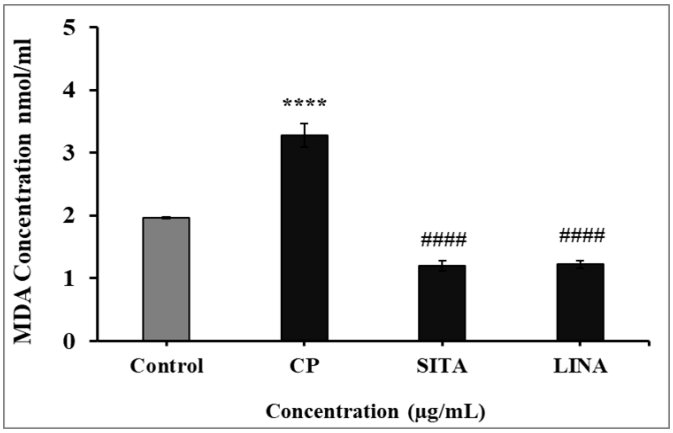

Figure 8: Cisplatin, Sitagliptin, and Linagliptin activity on MDA concentration in the A549 cell line.
One-way ANOVA was used for the examination. Data are displayed as mean ± SD.
**** P < 0.0001 compared to control.
P < 0.0001 compared to control.

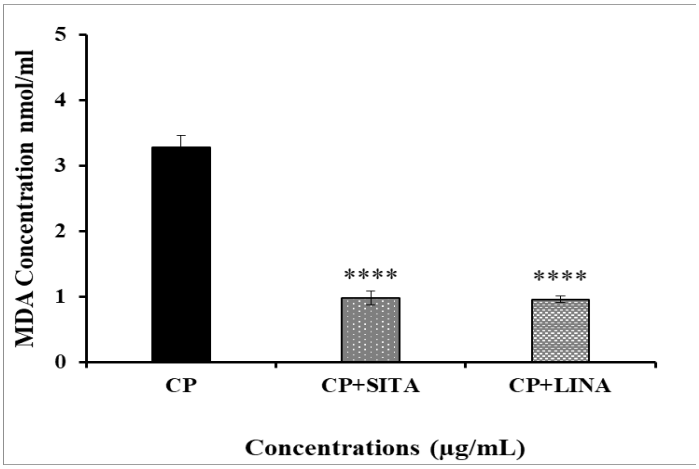

Figure 9: Comparison between the effect of cisplatin alone against cisplatin plus sitagliptin or linagliptin combinations on the MDA level in the A549 cell line.
One-way ANOVA was used for the examination. Data are displayed as mean ± SD.
**** P < 0.0001 Compared to CP-treated cells.

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