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Potent anticancer effects of vitamin C and vitamin C-rich fruit extract (*Citrus sinensis* L.) on human lung cancer cells

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ABSTRACT

Introduction: Studies have shown that synthetic agents are connected with some complications. This work was designed to study the effects of vitamin C and *Citrus sinensis* fruit extract (CSFE) on viability, DNA synthesis, and apoptosis stimulation in human lung cancer cells (COR-L105).

Methods: The total contents of the phenolics, flavonoids, and ascorbic acid in CSFE were assessed through the Folin-Ciocalteu, aluminum chloride, and dinitrophenyl hydrazine methods, respectively. The cytotoxicity of vitamin C and CSFE on COR-L105 cells was evaluated by the cell viability assay. Measurement of the DNA synthesis was done through the BrdU solution assay. The expression levels of some apoptosis regulatory genes were also evaluated.

Results: The total phenolic, flavonoids, and ascorbic acid contents of CSFE were 94.31 ± 2.27 gallic acid equivalents (mg/g) of dry extract, 63.26 ± 2.86 quercetin equivalents (mg/g) of dry extract, and 59 mg/L, respectively. The 50% cytotoxicity concentration (CC_{50}) values of vitamin C and CSFE on cancer cells were 54.6 and 82.7.6 µg/mL, respectively. Vitamin C and CSFE dose-dependently declined the amount of DNA production in the cancer cells. The expression levels of caspase-3 and Bax genes were markedly ($P<0.001$) elevated by vitamin C and CSFE, while they reduced the level of Bcl-2 gene ($P<0.05$).

Conclusion: The findings showed the potent anticancer effects of vitamin C and CSFE against human lung cancer cell lines. DNA synthesis reduction and apoptosis induction can be considered as possible mechanisms of action. However, further surveys are necessary to clarify the accurate mechanism and their efficacy.

Implication for health policy/practice/research/medical education:
The results of the current investigation revealed the high anticancer potency of vitamin C and *Citrus sinensis* against human lung cancer cell lines. Although more studies are required, however, they can be considered new agents for cancer therapy.

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Introduction

Lung cancer is considered the second most prevalent cancer in humans (1,2). At present, the use of some synthetic drugs, e.g., cisplatin, paclitaxel, carboplatin, docetaxel, and gemcitabine is the preferred chemotherapy for lung cancer (3,4). However, recent studies have shown that these agents are connected with some complications (4). Therefore, finding a new drug or complementary drug for existing drugs with high efficiency and low side effects has attracted the attention of many researchers.

Vitamin C (ascorbic acid) is described as one of the most useful and safe dietary supplements in the health system (5). Vitamin C as a critical antioxidant and radical scavenger agent plays crucial roles in some important procedures, e.g., tissue repairing, stimulation of neurotransmitters secretion, and immune system improvement (6). It has been reported that vitamin C plays an important role in protecting against peroxidative injury and free radicals due to its acidic properties (7). Vitamin C is also able to inhibit the production of carcinogenic nitrosamines via the reduction of nitrates through the NADH-dependent system (8). Previous investigations have also shown that vitamin C exhibits a promising cytotoxic effect on cancer cells through pro-oxidant properties, inhibition of cells,

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and cell replication (9).

Today, it has been recommended that high nutritional ingestion of vegetables and fruits may prevent a minimum of 20% of cancers (10,11). This protective outcome in fruits and vegetables is mainly originated by biologically active compounds such as flavonoids which have the ability to interact in the development and cancer progression (12). These compounds have the capacity to control and prevent cancers through inhibiting carcinogen stimulation, provoking carcinogen detoxification, stimulating apoptosis, and inhibiting free radical species, cell cycle, cell proliferation, and hormones (13).

Citrus sinensis L. or orange (Rutaceae family) is one of the most consumed fruits in the Mediterranean region, rich in flavonoids. *C. sinensis* is considered one of the main herbs containing vitamin C with various therapeutic effects such as antidepressant and anxiety, anti-inflammatory, anticancer, and antimicrobial properties (14). Reviews have also shown that *C. sinensis* extracts or its juice possess promising *in vitro* effects against a number of cancer cell lines (15). This work was designed to study the effects of vitamin C and *C. sinensis* fruit extract (CSFE) on viability, DNA synthesis, and apoptosis stimulation in human lung cancer cells (COR-L105).

Materials and Methods

Plant materials

The fruit peel of *C. sinensis* was obtained from the North of Iran (Babol, Mazandaran province, Iran) in December 2022. The identity of the materials was approved and kept at the herbarium of the Faculty of Pharmacy, Eastern Mediterranean University, North Cyprus (Herbarium No. 2022-8-658).

Extraction

Extraction was done by maceration methods; briefly, 150 g of fruit peel was dried, powdered, and soaked in 450 ml of 80% methanol for 72 hours in a dark place. After filtering the extract with filter paper (Whatman No.1), it was concentrated under a vacuum below 50°C using a rotary evaporator (Heidolph, VE-11) (16).

Phytochemical analysis

Phytochemical assays for evaluating the active phytochemical compounds, e.g., flavonoids, terpenoids, alkaloids, and saponins were performed based on the standard procedures, e.g., Mg and HCl, Mayer and Dragendorff's reagents, 1% gelatin, and suds production according to the methods described Evans (17,18).

Total phenolic and flavonoids contents

The amounts of the phenolic (PC) and flavonoid (FC) contents were assessed through the Folin-Ciocalteu and aluminium chloride colorimetric process based on the approaches reported elsewhere, respectively (19,20). The values of PC and FC were indicated as gallic acid (as standard) equivalents (GAE) mg/g of dry extract and quercetin equivalents (QE) mg/g of dry extract, respectively.

Measurement of ascorbic acid

The content was assessed through dinitrophenyl hydrazine as described previously. To do this, CSFE (75 µL) was mixed with 2, 4-dinitrophenylhydrazine thiourea copper (II) sulfate solution (80 µL) and after adding the 65% sulfuric acid, the combination was kept at 37°C for 30 minutes. The optical density was recorded at 520 nm with a spectrophotometer (21).

Cell culture

COR-L105 cancer cells from the European Collection of Authenticated Cell Culture (ECACC, 92031918) and normal lung fibroblast (THLE-3) cells were prepared from ATCC (RL11233), and were cultured in DMEM, improved with penicillin/streptomycin (100 µg/mL) and 10% FCS at 37 °C in 5% CO_2.

Cytotoxic assay

Initially, 100 µL of cell suspension (1×10^5/mL) was added to each well of the 96-culture plate, containing vitamin C (>99%, Sigma-Aldrich, Germany) and CSFE at concentrations of 5-200 µg/mL and were incubated at 24°C for 48 hours. After adding 10 µl of MTT solution (Sigma-Aldrich, Germany), the plate was kept in the incubator for 4 hours. Followed by adding DMSO (100 µL) to the wells, their absorbance was read by the ELISA reader at 570 nm, and the 50% cytotoxic concentrations (CC_{50} values) were evaluated (22).

Measurement of DNA synthesis

To do this, cells were treated with vitamin C, CSFE (1/2 CC_{50} and CC_{50}), and Dox (1 mM) in 96-well culture plates for 48 hours based on the cell proliferation ELISA BrdU kit (Roche, Germany) using the instruction of producer.

Examining the gene expression of caspase-3, Bcl2 and Bax

At first, the whole RNA was isolated from the culture specimens by means of an RNeasy kit based on the kit protocol (Qiagen, USA). Next, the RNA was reverse transcribed according to the commercial kit (Fermentas, USA). All amplification DNAs were assessed by SYBR green. Finally, $\Delta\Delta Ct^{-2}$ was calculated using CA, Hercules, and Rad-Bio optical system software (iQTM5); the housekeeping gene was beta-actin. β-actin was applied to normalize the mRNA levels of the tested samples (23). The sequence of primers used for real-time PCR (5'-3') was as follows:

Bax F: GGCTGGACACTGGACTTCCT
R: GGTGAGGACTCCAGCCACAA
Bcl-2 F: CATGCAAGAGGGAAACACCAGAA
R: GTGCTTTGCATTCTTGGA TGAGGG
Caspase-3 F: TTCATTATTCAGGCCTGCCGAGG
R: TTCTGACAGGCCATGTCATCCTCA

β-actin F: GTGACGTGACATCCGTAAAGA
R: GCCGACTCATCGTACTCCGT

Statistical analysis

Tests were performed in triplicate. SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA) was used for analysis. $P<0.05$ was described as a statistically significant level. One-way analysis of variance (ANOVA) and Tukey and Dunn's post hoc tests were used to compare the effects of extract concentrations.

Results

Phytochemical analysis and total polyphenols and flavonoids contents

Phytochemical investigation of CSFE confirmed the presence of flavonoids, phenols, terpenoids, saponins, tannins, and quinones. The total phenolics, flavonoids, and ascorbic acid contents of CSFE were 94.31 ± 2.27 mg GAE/g DW, 63.26 ± 2.86 mg QE)/g DW, and 59 mg/L, respectively.

Cell viability

According to the findings of MTT assay, the viability of cancer cells of COR-L105 was dose-dependently declined ($P<0.01$) after treatment with vitamin C and CSFE. The analyzed CC_{50} values of vitamin C and CSFE were 54.6 and 82.7.6 µg/mL for COR-L105 cells, respectively; these values for normal MC3T3 cells were 119.4 and >200 µg/mL, respectively (Figure 1).

Measurement of DNA synthesis

Treatment of cells with vitamin C and CSFE dose-dependently declined the amount of DNA production in the cancer cell line; the maximum level of reduction in DNA proliferation was found 40.4, and 47.9 for vitamin C and CSFE at CC_{50}, respectively (Figure 2).

Assessing the apoptosis-regulatory genes expression

The expression level of caspase-3 and Bax gene was markedly ($P<0.001$) elevated after treatment with vitamin C and CSFE ranging from 1.39 to 2.66-fold change mean of control, while reduced the expression level of Bcl-2 gene ($P<0.05$) (Figure 3).

Discussion

At present, some synthetic drugs, e.g., cisplatin, paclitaxel, carboplatin, docetaxel, and gemcitabine are the preferred chemotherapy for lung cancer (3,4). However, recent studies have shown that these agents are connected with some complications (4). Therefore, finding a new drug or complementary drug for the existing ones with high efficiency and low side effects has attracted the attention of many researchers. This work was designed to study the effects of vitamin C and CSFE on viability, DNA synthesis, and apoptosis stimulation in human lung cancer cells. We found the presence of flavonoids, phenols, terpenoids, saponins, tannins, and quinones; the total phenolics, flavonoids, and ascorbic acid content of CSFE were 94.31 ± 2.27 mg GAE/g DW, 63.26 ± 2.86 mg QE)/g DW, and 59 mg/L, respectively. In line with our results, Ashari et al showed that the total phenols in Iranian orange juice specimens were vary ranging from

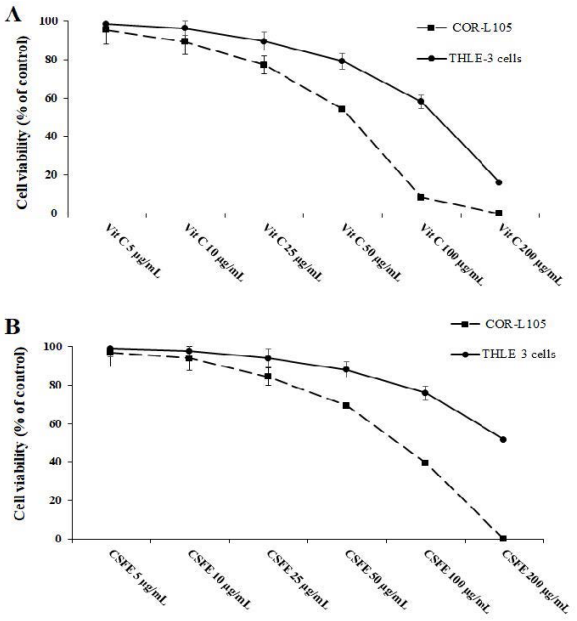

Figure 1. Effect of vitamin C (A) and Citrus sinensis fruit extract (B) on the viability of cancer (COR-L105) and normal (THLE-3) cells. n=3 (Mean ± SD).

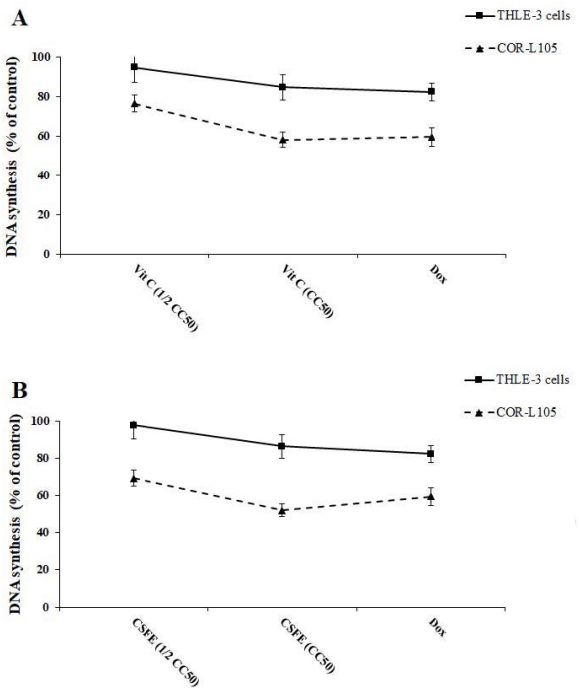

Figure 2. Effect of vitamin C (A) and *Citrus sinensis* fruit extract (B) (1/2 CC_{50} and CC_{50}) and 1 µM doxorubicin (DOX) on inhibition of DNA production of cancer cells of COR-L105 cancer cells and normal fibroblast (THLE-3) cells. n=3 (Mean ± SD).

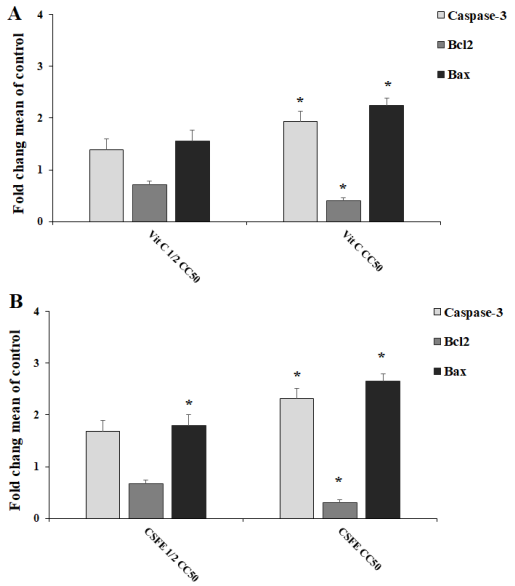

Figure 3. Effect of vitamin C (A) and *Citrus sinensis* fruit extract (B) (1/2 CC_{50} and CC_{50}) on the level of the apoptotic gene in COR-L105 cancer cells. Mean ± SD (n = 3). * $P < 0.001$ in comparison with non-treated cells of the control group.

28.39 to 114.20 mg GAE/L. They reported that the total flavonoids of these extracts were ranging from 12.53 to 32.62 mg QE/L); the ascorbic acid content ranged from 29.95 to 93.08 mg/L (24). Previous studies showed that the flavonoid and polyphenolic compounds derivate from *Citrus* fruits displayed anticancer effects on various cancer cell lines (e.g., MCF-7 and HepG2) through carcinogen detoxification, free radical scavenging, cell cycle suppression, cell multiplying inhibiting, induction of apoptosis, decreasing oncogene activity, and preventing metastasis (25).

Our results indicated that the viability of cancer cells of COR-L105 was dose-dependently declined after treatment with vitamin C and CSFE. Several investigations demonstrated the promising cytotoxic effects of vitamin C alone or along with chemotherapeutics against different cancer cell types, e.g., colon cancer, ovarian, leukemia, prostate, melanoma, pancreatic, and breast cancer cell lines with the effective concentration ranging from 0.05 to 3.53 mg/mL (26). With respect to the anticancer effects of *Citrus* herbs, reviews have shown the promising anticancer of *Citrus* spp., against human leukemia (HL-60) and breast adenocarcinoma (26). Shirisha et al (2019) eported that orange peel extract (OPE) had moderate *in vitro* toxicity, high regenerative ability, and variations in nuclear morphology in L-929 cancer cell lines like to apoptotic cells. They also reported that OPE exhibited promising anticancer in mice with Dalton lymphoma ascites (DLA) tumors by increasing the average life span and improving the level of the antioxidant enzyme and hematological parameters (27). Magalhães et al demonstrated that the *C. sinensis* essential oil markedly reduced the cell viability of CCD-1059Sk tumor cells up to 50% with the CC_{50} value of 272.6 µg/mL (28). Previously, it has been reported that *C. sinensis* extract due to having the contents of both vitamin C and flavonoids showed anticancer properties through its antioxidant activity, inhibiting peroxidation and ROS generation, anti-inflammatory effects, elevated manganese-dependent superoxide dismutase expression and controlling the factors of apoptosis (29).

Considering the anticancer effects of vitamin C, studies have reported that this compound displayed its anticancer effects by provoking the oxidative stress caused by H2O2, elevating the cell cycle arrest, reducing the level of ATP, upregulation of p53, disrupting mitochondrial function, and inhibiting of antioxidant gene expression NrF-2 (30,31). On the other hand, flavonoids, have the capacity to control and prevent cancers by inhibiting carcinogen stimulation, provoking carcinogen detoxification and apoptosis stimulation, and inhibiting free radical species, cell cycle, cell proliferation, and hormones (25).

We also revealed that the treatment of cells with vitamin C and CSFE, dose-dependently declined the amount of DNA production in some cancer cell lines. The potent effects of vitamin C on bleomycin have been reported to be by changing its cytotoxicity into DNA injury and making it more sensitive to manipulations of DNA repair in H460 and A549 cancer cells (32). In addition, it has been proven that vitamin C exhibits its anticancer effects through ROS-mediated mechanisms and effects on DNA demethylases (33). We reported that the expression levels of caspase-3 and Bax gene were markedly elevated after treatment with vitamin C and CSFE while reducing the expression level of Bcl-2 gene. Vitamin C motivates caspase apoptosis in some human cancer cells (33). Vitamin C is able to prompt apoptosis in colon cancer cells via the elevation of the calcium influx, as well as the expression of Bax gene (34).

Conclusion

This in vitro study, in line with other studies, showed the potent anticancer effects of vitamin C and CSFE as a main vitamin C-rich plant against lung cancer cells through DNA synthesis reduction; apoptosis induction can also be considered as a possible mechanism of action. However, further surveys are necessary to clarify the accurate mechanism and their efficacies in animal models and next in clinical trials.

Authors' contribution

MK was involved in data collection, data analysis, and paper revision. MK designed the study and wrote the manuscript draft. All read the final version and confirmed its publication

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

The protocol of this work was confirmed by the Ethical

Committee of the Faculty of Pharmacy, Eastern Mediterranean University, Famagusta, North Cyprus (No. 16700658).

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