Osthole extracted from a citrus fruit that affects apoptosis on A549 cell line by histone deacetylase inhibition (HDACs)

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ABSTRACT

This study aims to investigate the interactions between osthole extracted from Egyptian citrus fruits as HDACs inhibitor by theoretical study and practically. Besides, osthole was assed as anti-cancer activity. In this study, osthole was extracted from the Egyptian citrus fruit and was characterized. The role of osthole as in vitro inhibitor of HDACs was estimated and evaluated the antitumor activity against human lung cancer cells (A549). Caspase-9 activity was detected. The results obtained from GC–MS indicate that the grapefruit showed the highest osthole concentration compared to the other citrus fruits. Moreover, the grapefruit osthole competitively inhibits HDACs. The inhibition constant value, (Ki=3.36 mM), indicates that osthole exerts an inhibitory effect upon HDACs activity. In vitro study of osthole could inhibit the growth of A549 cells that depend on time and concentration. It also induces apoptosis and causes an increase of caspase-9 by osthole. In conclusion, grapefruit osthole could induce the apoptosis in A549 lung cancer cells by inhibiting the histone deacetylase.

1. Introduction

Cancer is considered a fatal disease that is likely to be caused mainly by environmental factors, carcinogens that can be found in surrounding food, water, air, chemicals, and sunlight to which humans are exposed. The epithelial cells cover the skin, respiratory, digestive system, and metabolized ingested carcinogen. Based on the Egyptian National Population lung cancer is one of the most common cancers in Egypt. Lung cancer begins from the cells lining the bronchi or alveoli. The lung cancer cells invade the lymphatic vessels that begin to grow in the lymph nodes around the bronchi and in the mediastinum (the area between the 2 lungs). When lung cancer cells reach lymphocytes, they can spread to all parts of the body’s organ.

HDACs enzymes are acetyl group removal from the lysine residues on the N terminal of histone. HDACs have a role in gene regulation and expression by influencing the structure of compact chromatin. This causes changes in acetylation level and through it can influence the structure of chromatin compact. Thus, changes in the level of acetylation have been demonstrated by excessive gene expression in several cancer cell lines and tumor tissues.

In addition to that, HDACs contains target proteins, including proteins involved in tumor development and those that control cell cycle, programmed cell death, angiogenesis, and cell invasion. Therefore, HDACs inhibitors can affect multiple cells on their effects and mechanisms of action including arresting cell cycle, increase apoptosis, induction of autophagy, generation of free radicals, and angiogenesis. Since synthetic inhibitors are toxic to patients with no specificity for some HDACs enzymes, scientists have stimulated research on natural inhibitors. Coumarins are natural compounds found in many plants. Osthole (7-methoxy-8-(3-methyl-2-butanyl)-2H-1-benzopyran-2-one), is also one of coumarin that first derived from Cnidium plant.

As reported previously, the high content of osthole is found in the mature fruit of Cnidium monnieri. Furthermore, some medicinal plants like Angelica, Angelica, Citrus, Fructus Cnidii, and Clausena improves male function, enhance the immune system, and attenuate rheumatic disease and eliminating dampness; most of these medicinal properties are considered to attribute to bioactive components, osthole.

It has been reported that osthole has an antioxidant, anticancer, anti-inflammatory, and immunomodulatory character. The current study was undertaken to look for Egyptian osthole source as a potent inhibitor for HDACs to treat the invito lung cancer (A549). Osthole inhibited the migration and invasion of MCF-7 and MDA-MB-231 cells by suppression of matrix metalloproteinases (MMP) enzyme activities. Combination of osthole and...
platycodin D showed synergetic effect on inhibition of tumor cell proliferation and invasion of mammary carcinoma cells (MDA-MB-231 and 4T1) [15]. Osthole has been suggested to modulate PI3K/Akt signaling pathway leading to G2/M arrest and apoptosis in lung cancer A549 cells [16]. Chemically, Osthole has the potent characters to be an inhibitor for histone deacetylase as it contains a hydrophobic and aromatic group, which can act as capping agent for enzyme active site; zinc binding domain (ZBD), such ketone group or methoxy groups, which coordinates to the active site of Zn²⁺ ion.

2. Methods

2.1. Reagents

Standard osthole (M.wt = 244) and (2,2 diphenyl-1-picyrylhydrazyl) obtained from Sigma Aldrich Chemical Co. (St. Louis, Mo, U. S. A.). Caspase-9 colorimetric assay kit and histone deacetylase colorimetric assay kit were obtained from Biovision co. (U.S.A), ANNEXIN V–FITC apoptosis detection kit was purchased from Immunostep Co. (Spain), DMEM (Dulbecco’s Modified Eagle’s Medium with 4.5 g/L glucose and l-glutamine), Trypsin EDTA solution, fetal bovine serum, and Penicillin-streptomycin solution were purchased from (Cambrex, bioscience. Verviers, Belgium). Grapefruit (Citrus paradisi), orange (Citrus sinensis), and lemon (Citrus limon (L.)) were obtained from the local market, Tahta, Egypt.

2.2. Molecular docking software

Docking simulation was performed, (Molgero Virtual Docker 2008) (http://www.molgero.com/mvd-product.php) and (Thomson and Christensen 2006) a program for conformation of ligand bind to a macromolecule.

2.3. Extraction of osthole from Egyptian citrus fruits

Osthole was extracted from three types of citrus fruits: Grapefruit (Citrus paradisi), lemon (Citrus limon), and orange (Citrus sinensis) as described previously by [17] with some modifications. The raw plant materials were dried at 30 °C and finally ground to powder. Fifty grams of powdered fruits were defatted with 250 mL of petroleum ether in a Soxhlet apparatus to remove the pigments and lipids. The defatted fruits were re-extracted with 250 mL of acetone in a Soxhlet extractor for 12 h. The acetone extract was then concentrated by evaporating the solvent using a rotary evaporator under reduced pressure to get a yellow residue. The residue was dried and recrystallized in diluted ethanol (50 %), light yellow acicular crystals were obtained.

2.4. Identification of extracted osthole

The light yellow acicular crystals were blended with potassium bromide. The analysis was determined in the range between 4000 and 400 cm⁻¹ at a resolution of 4 cm⁻¹ using JASCO FT/IR-4100 spectrometer, Japan [18,19].

The samples were dissolved in methanol. The chemical composition of samples was performed using Trace GC DSQ II (Thermo Scientific, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 μm film thickness). Osthole extracts from citrus fruits were detected and quantified according to [20], using YL9100 HPLC.

2.5. Partial purification of HDACs from rat liver

Isolation and partial purification were performed according to [21].

2.6. Determination of HDACs activity in rat liver

The activity of HDACs was determined using a colorimetric assay kit (BioVision, USA, catalog K331–100).

2.7. Kinetic inhibition of HDACs by grapefruit osthole in rat liver

Two μl of different concentrations (2–10 mM) of grapefruit osthole were added to 83 μl of the enzyme then incubated for one hour. HDACs activity was assayed as mentioned previously. The kinematic measurements (Km and Vmax) and the inhibition constant (Ki) were determined from the Lineweaver Burk plot [22].

2.8. In vitro studies

2.8.1. Cell culture

A human lung cancer cell line (A549) was purchased from VACSERA CO. (Giza, Egypt) and cultured according to the standard protocol for cell culture. Cells were cultured in DMEM (Dulbecco modified Eagle) having 4.5 g/L of glucose and l-glutamine and 10% fetal bovine serum (FBS) and 1% of the penicillin-streptomycin solution. Initially, cells were seeded at a low density at 37 °C in a 5% CO₂ incubator with 95% humidity.

2.8.2. Cell viability assay

Cell proliferation (viability) was evaluated by MTT assay according to [23].

A549 cells were counted and then re-seeded in 96-well plates to final concentration 1 x 10⁵ cells/mL (3 x 10⁴/Well). Different concentration of grapefruit osthole extract ranged from (40.98 to 406.83 μMole) was treated in the cells. A parallel control experiment, using different concentrations of cisplatin (ranges from 6.6 to 40 μM) was run. The culture medium was removed from the 96 well microplates after 48 h of drug treatment and cells were washed twice gently by ice-cold PBS. 20 μl MTT (5 mg/mL) was added to each well. The microplate was incubated at 37 °C for 4 h in CO₂ incubator then medium/MTT was removed the 150 μl of DMSO was added to each well to dissolve the formazan produced. The microplate was incubated under shaking (at highest speed) for 15 min at 25 °C. Using a Bio-RAD microplate reader (Japan) was measured at 540 nm. The % of viability was calculated as follow:

% viability = sample absorbance/ control absorbance X 100

IC₅₀ = the concentration of grapefruit osthole that inhibits 50 % of A549 cell proliferation.

2.8.3. Determination of caspase-9 activity

The determination of caspase-9 activity was detected by using the Caspase-9 Colorimetric Assay Kit (BioVision, USA, cat.no. K119) [24].

Two million cells were cultivated in a 12-well plate for 24 h. Cells were treated with grapefruit osthole with different concentration from 94.26 to 377 μM for 24, 48 h [24]. Cells were suspended in 50 μL of lysis buffer and incubated for 10 min. Cells were centrifuged for 1 min. The supernatant (cytosolic extract) was transferred to a fresh tube. Fifty μL of 2X buffer was added to each samples μL of the 4 mM LEHD-pNA substrate was added and incubated at 37 °C for 2 h. The absorbance was detected at 405 nm using Bio-RAD microplate reader (Japan).

2.8.4. Apoptosis analysis by flow cytometry

Measurement of apoptosis was performed using ANNEXIN V-FITC APOPTOSIS DETECTION KIT (Immunostep, Spain) [25].

Two million cells were cultured in T-25 culture flask for 24 h. Cells were treated with osthole extract with different concentrations from 94.26 to 377 μMole. The culture medium was removed from flasks after 48 to 72 h and cells were washed gently
twice with ice-cold PBS, and resuspended in 1X Annexin-binding buffer at a concentration 1 × 10^6 cells/mL. Five µl of the Annexin V-FITC and 5 µl of PI were added to each 100 µl of cell suspension. The cells were incubated for 15 min at room temperature (25 °C) in the dark. Then, 400 µl of 1X Annexin-binding buffer was added. Samples were analyzed by flow cytometry (Beckman coulter, U.S. A.) within one hour.

2.8.5. Statistical analysis

The data were showed and analysed by using Excel Microsoft office and Graphpad prism followed by one way ANOVA follower by post hoc Dun can’s multiple range tests were done to evaluate the relative HDC activity with different concentration of osthol treatment. Finally, the data presented as Mean ± SD of repeated experiments.

3. Results

3.1. Molecular docking analysis of osthol with HDAC-like protein

The optimum molecular structure of the inhibitor was known by some experiments from semi-empirical calculations and conformational search. The native ligand has been removed from the active site and then was docked into the binding site of its enzyme to characterize the binding pocket of HDAC-like protein (the grid dimensions were: x = 67.71 Å, y=30.78 Å and z = 2.52 Å, with a grid spacing of 9 Å). The docked orientations with a root mean square deviation between the predicted conformer and the observed X-ray crystallographic conformer, 0.320 Å, were clustered together (superimposition) as shown in Fig. 1(a).

Docking calculations of osthol showed has 3H-bond interactions with the important amino acid residues, Tyr308, Gly154, and His183, with H-bond distances 2.73, 3.37 and 3.42 Å, respectively, (Fig. 1: b). This could explain the inhibition efficiency of osthol.

Also, the docking calculations showed that the phenolic moiety and the methyl group of osthol are close to Phe210 and His183 residues, respectively. The van der Waals forces were found responsible for the affinity of osthol with HDAC-like protein (Fig. 1: c). The surface representation of the minimum energy structure of the complex of osthol docked in the active site of the HDAC-like protein is shown from the molecular modeling study (Fig. 1: d). The calculated parameters of osthol molecule of molecular docking calculations are shown in (Table 1).

3.2. Identification of extract osthol

Osthol was extracted from three different citrus fruits by the method described previously. The osthol structure was identified using the FTIR spectrum and GC–MS.

FTIR spectrum showed a carbonyl (C=O) peak at 1747 and 1649 cm⁻¹, CO— stretching peak at 1048 and 1049 cm⁻¹ and aromatic
CC= peak at 1439 and 1451 cm⁻¹. These data confirm the skeleton of osthole in citrus fruits compared to standard as shown in (Fig. 2).

Further evidence of the examined compound was obtained from GC–MS that showed well-separated peaks with retention time 16.56 identical to the osthole standard solution. The molecular weight of osthole was 244 in grapefruit and lemon. Moreover, the appearance of the ion peak at 213 m/z is due to the cleavage of a methoxy moiety from the molecule. These data proposed the structure of osthole, with C₁₅H₁₆O₃ molecular formula and confirmed the presence of osthole in grapefruit and lemon only as shown in (Fig. 3).

Moreover, HPLC analysis indicated the presence of osthole in grapefruit and lemon with concentrations 8.6 and 0.286 mg/g dry weight, respectively. The chromatogram of standard osthole (Fig. 4, panel A), grapefruit osthole (Fig. 4, Panel B) and lemon osthole (Fig. 4, panel C) showed that osthole had retention time 6.5, 6.8 and 6, respectively.

### 3.3. Partial purification of HDACs

HDACs were partially purified from rat liver as shown in (Table 2). HDACs was purified about 4-fold starting from the crude homogenate to the final step. The 45 % ammonium sulfate fractionation yield was 111.76 units. The ammonium sulfate fraction retained approximately 27 % of the original activity (yield). The pellet of 25 % and 45 % ammonium sulfate had specific activities of 3.41 and 7.61 unit/mg protein, respectively. Also, the total protein content was estimated for each step giving 3.8, and 14.68 mg, respectively.

### 3.4. Kinetic inhibition of rat liver HDACs with grapefruit osthole

The activity of HDACs was detected in the presence of different grapefruit osthole concentrations ranged from 2 to 10 mM. As illustrated in (Fig. 5), the enzyme was inhibited by grapefruit osthole in a concentration-dependent manner. Grapefruit osthole was inhibited 50 % of HDACs at a concentration of 8.6 mMole. According to Lineweaver Burk plot, different concentrations of grapefruit osthole (4.3, 6, and 8.6 μMole) showed an inhibition pattern in which the lines intersected at the Y-axis. The Km of the enzyme acetylated poly lysin was changed (increased) and the Vmax value was unchanged (Fig. 6: A). Accordingly, the type of inhibition is competitive. The inhibition constant, Kᵢ value was found to be 3.36 mM. (Fig. 6: B).

### 3.5. Effect of grapefruit on A549 cell proliferation

A549 cell proliferation was determined by using MTT assay and compared with cisplatin as a positive control. The results showed that the viability of A549 cells significantly decreased by increasing grapefruit osthole concentrations with IC₅₀ 188.5 μMole (Fig. 7). HDAC activity was slightly inhibited in A549 cells treated with grapefruit osthole for 24 h compared to the untreated cells. When the cells were incubated with grapefruit osthole for 48 and 72 h, HDAC activity was significant (p < 0.01) decreased depending on increasing time and dose concentration as shown in (Fig. 8).

### Table 1

The calculated parameters, binding interaction (BE), Inhibition constant (Ki), molecular docking score (MDS), Rerank score (RS), Hydrogen bond interaction energy (HB), obtained from the molecular docking calculations.

| Parameter | BE  | Ki  | MDS  | RS  | HB  |
|-----------|-----|-----|------|-----|-----|
| Inhibitor (Osthole) | 6.9 | 3E-6 | -106.233 | -77.136 | -0.779 |
3.6. Effect of grapefruit osthole on Caspase-9 activity

The effect of grapefruit osthole on A549 cells caspase-9 activity was determined and compared with cisplatin as a positive control. Caspase-9 activity was increased in A549 cells treated with different concentrations (Fig. 9).

3.7. Apoptotic effect of grapefruit osthole

Flow cytometry analysis measured treatment of different concentrations of grapefruit osthole to A549 cells for 48 h (Fig. 4A, B). Compared to the control group the numbers of early and late apoptotic cells were significantly increased. The proportion of early and late apoptotic cells in the 188.5 μM treatment cells were about thirteen times higher than in cells free drug. The apoptosis treated cells were increased as a dose-dependent manner (Fig. 10).

4. Discussion

Regulating HDACS activity is very important for the epigenetic regulation of gene expression by studying its effects on the compact chromatin structure. Work by [26] revealed that the

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**Table 2**

Purification scheme of HDACs from rat liver.

| Step                              | Total Protein conc.(mg) | Total HDACs activity (μmol/min) | Specific activity (unit/ mg protein) | Yield (%) | fold1 |
|-----------------------------------|-------------------------|---------------------------------|--------------------------------------|-----------|-------|
| Crude                             | 194                     | 414.3                           | 2.14                                 | 100       | 1     |
| pellet of 25 % amm.sulfate        | 3.8                     | 12.94                           | 3.41                                 | 3.12      | 1.59  |
| pellet of 45 % amm.sulfate        | 14.68                   | 111.76                          | 7.61                                 | 26.98     | 3.55  |

**Fig. 4.** HPLC chromatogram of standard osthole (A), osthole extracted from grapefruit (B), and osthole extracted from lemon (C).
alterations level of acetylation and overexpression of HDACs have been detected in various cancer cell lines and tumor tissues. Consequently, this enzyme is an interesting target for the searching of antiproliferative drugs and it is not surprising that numerous approaches to the design of new antitumor agents were based on the search for HDACs inhibitors. It has been reported that many HDACS synthetic inhibitors have cytotoxic effects. These inhibitors have undesirable side effects associated with some chemotherapy drugs. In this manner, we increase the demand for novel antitumor drugs with low side effects. New insights are required to find potential natural inhibitors of HDACs may avoid such side effects [27,28].

Osthole, a naturally occurring compound, has a variety of pharmacological and biochemical uses that have potential therapeutic applications. It found mainly in 14 species of Umbelliferae family and 17 of the Rutaceae family [9]. One of the aims of this work was to show the effect of osthole on HDACs. To achieve this purpose, molecular docking studies were performed. Based on the results of molecular docking, it has been found that osthole showed favorable binding with HDAC-like protein.

Fig. 5. Inhibition of HDACs' activity by grapefruit osthole in rat liver. The data were expressed as mean ± SD of triplicate experiments.

Fig. 6. Inhibition study of rat liver HDACs (A), and Inhibition constant (K_i) (B) with grapefruit osthole.

Fig. 7. Effect of grapefruit osthole and cisplatin on A549 cell viability by the MTT assay. The data were expressed as Mean ± SD of separated triplicate experiments. The data were expressed as Mean ± SD of triplicated experiments.
Osthole was extracted from Egyptian citrus fruits including grapefruit, lemon, and orange. The structure of osthole was confirmed using different methods.

FTIR revealed that the presence of coumarinic carbonyl peak at 1747 cm\(^{-1}\), CO— stretching peak at 1048 cm\(^{-1}\) and aromatic CC peak at 1600 cm\(^{-1}\), confirms the skeleton of osthole in citrus fruits. This is in agreement with that previously described by [19] who suggested the presence of coumarinic carbonyl peak at 1721 cm\(^{-1}\) and C=C peak at 1606 cm\(^{-1}\) of osthole extracted from Persian Cnidium monnieri. In another study, osthole was extracted from prangos genus, showed the presence of coumarinic carbonyl peak at 1717 cm\(^{-1}\) and C=C peak at 1604 cm\(^{-1}\) [18].

Further evidence of the examined extracts was obtained from the GC–MS analysis which confirmed the FTIR data. The GC–MS analysis showed well-separated peaks with retention time 16.56 identical to the osthole standard solution with molecular weight 244 in grapefruit and lemon. Besides, the appearance of the ion peak at m/z 213 is due to the cleavage of a methoxy moiety from the molecule. The obtained data is inconsistent with the proposed structure of the known osthole with C\(_{15}\)H\(_{16}\)O\(_3\) molecular formula and confirmed the presence of osthole in tested citrus fruits except orange. These results are the same with obtained by [29].

The concentration of osthole in grapefruit and lemon extracts was determined by the HPLC. The concentrations of osthole in grapefruit and lemon were found to be 8.6 and 0.286 mg/g dry weights, respectively. The obtained data illustrated that the concentrations of osthole were higher than those obtained by [29] in which the concentrations of osthole in the peel of grapefruit and lemon were 12 and 7 µg/g dry weights, respectively. Another study reported that the concentration of osthole was found to be 20 mg/g powdered Cnidium monnieri fruit, which is considered to be the main source of osthole [30]. Our results might have a commercial application by finding a promising and cheap local source of osthole.

To compare the theoretical data obtained by the molecular docking with the experimental data, HDACs were isolated from rat liver and assayed in the presence and absence of grapefruit osthole. The rat liver HDACs’ activity was inhibited by grapefruit osthole. The inhibition showed a direct interaction between the enzyme and the grapefruit osthole with IC\(_{50}\) 8.6 mM of grapefruit osthole, whereas the results obtained by [31] suggested that the IC\(_{50}\) value of apigenin which is considered to be a natural inhibitor of HDACs was 32 mM.

The grapefruit osthole acts as a competitive inhibitor with the K\(_v\) value is 3.36 mM. This value is consistent with that obtained by molecular docking indicating that there is a resolvable aspect between the laboratory and theoretical data. Moreover, our result agrees with [32,33] in which Hela cell HDACs were inhibited by 2, pyrroloidinone-n butyric acid, topiramate, valproic acid and butyric acid with K\(_v\) 2.25, 2.22 and 0.51 mM, respectively. On another hand, the inhibition of butyric acid of HDACs was carried with K\(_v\) 60 mM [34].

Taken together, we demonstrate that osthole extracted from Egyptian grapefruit may act as a natural inhibitor for HDACs. This was supported by theoretical data provided by molecular docking as well as practical experiments.

Consequently, we evaluated the cytotoxic effect of grapefruit osthole against A549 lung cell line by MTT assay. The IC\(_{50}\) of grapefruit osthole in the cell line was 188.5 µM after 48 h of treatment. On the other hand, the IC\(_{50}\) of cisplatin, which was used as a reference drug, was 26.6 µM after 48 h of treatment. In parallel, the activity of HDAC was assayed in the tested cell line at different time points (24, 48, and 72 h) and the data showed that the enzyme activity decreased by 19.4 %, 47.1 %, and 54.5 %, respectively upon treatment with 188.5 µM of osthole. These findings are consistent with those previously obtained by [16].

The effect of osthole inhibitor on HDC is due to the hydrophobic nature of this inhibitor. This hydrophobicity is corresponded with the chemical feature of the capping group in known HDAC inhibitors that form hydrophobic contact with the surface residues at the edge of the active site [35]. This result was confirmed by our molecular docking by using EA DOCK software.

In order to investigate the apoptosis-inducing potentials of the grapefruit osthole, the caspase-9 activity was detected. The results showed that caspase-9 activity was increased by 16, 24, and 43 % compared to control after 24 h of treatment with grapefruit osthole. After 48 h of osthole treating, the activity of caspase-9 still elevated but slightly lower than that from at 24 h.

This is in agreement with that described by [36] who studied the effect of cinidium osthole on human carcinoma cell lines. They found that osthole could increase caspase-8 and -9 in H1299, PC3, and SKNM cells. Osthole induces apoptosis in these cell lines through both intrinsic and extrinsic pathway. In addition, [12] studied the effect of osthole on human breast cancer cell lines. They found that osthole induced apoptosis through increasing the activity of caspase-9 and caspase-3.

Consequently, flow cytometric analysis was used to discriminate between viable and apoptotic lung cancer cells after treatment. Egyptian grapefruit osthole induced apoptosis in lung cancer cells. We found that (4, 16, and 20 %) of cells in early apoptosis and (12.8, 27.4, and 46.3 %) of cells in late apoptosis. However, apoptotic cells increased with increasing time to 72 h (5,
26, and 4 %) in early apoptosis and (12.6, 27.5, and 81.7 %) of cells in late apoptosis.

In the previous studies, [16] studied the effect of osthol on the human lung cancer A549 cells and their results showed that 150µM of osthol induced about 17 % of cell in early apoptosis and about 20 % of cells in late apoptosis. The authors have attributed such findings to the role of osthol in the induction of G2/M arrest and apoptosis possibly by down-regulating Akt signaling in the investigated cell line. Moreover, [1,2] studied the effects of osthol on human breast cancer cells MDA-MB 435 for proliferation, cell cycle, and apoptosis. Their results showed that upon treating with 100 µM of osthol for 36 h, the apoptosis increased to 45 %.

These observations suggest that osthol may have an anticancer effect through inhibition of HDAC, which is involved in many pathways in cancer treatment such as activation of tumor suppressor gene (p53), cyclin-dependent kinase inhibitor (p21) and down-regulation of cyclin A correlated well with the decreased cyclin-dependent kinase 2 (Cdk2) activity leading to apoptosis and cell cycle arrest [37]. Therefore, it appears that apoptosis induced by increasing caspase 9 activity occurred via an intrinsic pathway in A549 cells.

**In conclusion**, the grapefruit osthol act as a natural inhibitor of HDACs with Ki 3.36 mM. Moreover, it considers a valuable therapeutic agent for lung cancer treatment. Further in vivo studies could confirm this data in the future.

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**Fig. 10.** Flow cytometric analysis of A549 cells treated with grapefruit osthol. Plots show Annexin V-FITC and propidium iodide stained A549 cells untreated (panel A) and treated (for 72 h) with cisplatin, 94.26, 188.5, and 377 µM of grapefruit osthol (panels B, C, D, and E, respectively).
Declarations of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00531.

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