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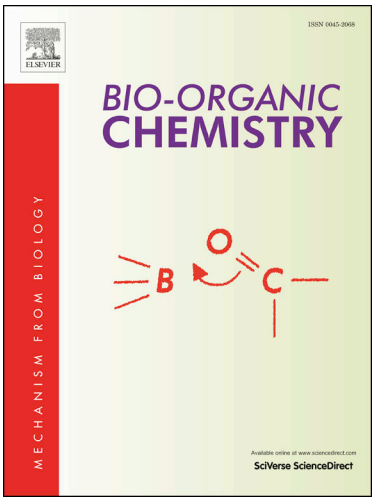

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Acovenoside A as a novel therapeutic approach to boost taxol and carboplatin apoptotic and antiproliferative activities in NSCLC: Interplay of miR-630/miR-181a and apoptosis genes

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Abstract:

The aim of the present study is to explore the potential anticancer effect of the cardenolide; acovenoside A against non-small cell lung cancer, understand its molecular mechanism in inducing apoptosis and show the effect of its combination with carboplatin and taxol. MTT assay showed that the combination of acovenoside A with taxol and carboplatin caused 78.9% cytotoxicity reflecting the synergistic effect. The triple combination showed the best growth inhibition efficiency where the number of cells at the G2/M phase was decreased and boosted up apoptotic and necrotic activity. The combination also showed the most remarkable increase in gene expression of Bax and p53 and the least level of Bcl2. The gene expression of miRNA181a and miRNA630 was significantly upregulated in cell lines treated with the combination. The present study has proven that the underlying mechanism of acovenoside A is partially attributed to the upregulation of miR-630 and miR-181a gene expressions which in turn targets the intrinsic apoptosis genes as p53, Bax and Bcl2 as well as caspase 3. The present study is the first to address the valuable effect of using acovenoside A together with carboplatin and taxol in the treatment of NSCLC *via* exerting apoptotic, antiproliferative, and cytotoxic effects.

Keywords: miRNA181a; miR-630; Bax; NSCLC; Acovenoside A.

1. Introduction:

Lung cancer is one of the most prevailed cancer types with a high incidence and mortality rate. Histologically, it is divided into two primary subtypes: non-small cell lung cancer (NSCLC), which affects 85% of patients, and small cell lung cancer (SCLC), which accounts for about 15% of cases [1]. The overall death rates of NSCLC are high and the possibility of cure is limited. Owing to the absence of effective biomarkers and diagnostic tools, most patients are diagnosed at the aggravated stages IIIB or IV which is accompanied by metastases and a bad prognosis. Lung cancer may arise from a variety of causes; smoking is the main risk factor for developing lung cancer [2, 3].

NSCLC treatment regimen comprises surgery, radiotherapy, and chemotherapy [2, 4]. Chemotherapeutic regimens are considered the primary therapeutic approach for lung cancer especially in early stages [5]. For NSCLC, taxol, carboplatin, cisplatin, and docetaxel are the most effective chemotherapeutic agents as they prevent the growth of cancer cells and increase their death [6-8]. Particularly, the taxol and carboplatin combination is one of the most commonly used efficient regimens where taxol interacts with tubulin polymers, inhibits cell proliferation in the G2/M phase, and causes cytotoxicity. In addition, carboplatin has the ability to alkylate DNA in malignant cells, causing structure disruption followed by the death of cancer cells [7, 9].

Unfortunately, the mechanism of action of these chemotherapeutic drugs is non-specific as they attack crucial cellular components and interfere with fundamental metabolic processes in both malignant and healthy cells, thus leading to a number of detrimental side effects [10]. Another frustrating point in the clinical utility of chemotherapeutic agents is cancer chemo-resistance which is provoked by diverse routes such as DNA repair, autophagy, drug efflux, metabolic alterations, and mitochondrial modifications. Newly diagnosed patients are responsive to chemotherapy, but quickly they acquire a resistance to it, which increases the likelihood of early relapse and aggressive metastases [11, 12]. However, the molecular mechanisms responsible for chemo-therapeutic resistance and metastasis are not fully explored till now [13].

MicroRNAs (miRNAs) which are identified as non-coding RNAs with nucleotide sequences of about 20 nucleotides; play a vital role in numerous biological processes [14]. In the realm of lung cancer, they have drawn extra attention where the aberrant miRNA participates in the onset, progression, and prognosis of the disease as well as regulates the response to chemotherapy [15-17]. These may be attributed to the involvement of miRNAs in cell differentiation, proliferation, apoptosis, cell cycle progression, invasion, and metastasis [18, 19]. Crucially, miRNAs might act as oncogenes or tumor suppressors and can be used as potential non-invasive biomarkers that may be helpful in diagnosis and prognosis [20]

The miR-181a (ENSG00000207759) is one of the highly conserved mature candidates of the miRNA181 family and is located on chromosome 1 (37.p5) [21]. It possesses a critical role in maturation, activation of immune cells, cellular invasion, and controlling inflammatory processes [22]. Furthermore, miR-181a was involved in apoptosis via modulating pro-apoptotic and anti-apoptotic markers. Based on its diverse functions in the apoptotic process, miR-181a may act as an oncogene and tumor suppressor [23-25]. Another noncoding miRNA investigated in the present study is miR-630 (ENSG00000283798) which is located on chromosome 15q24.1 [26]. It has twofold functions in the pathogenesis of human cancers where it acts as a tumor suppressor in esophageal squamous cell carcinoma [27], while acts as an oncogene in renal cell carcinoma [28]. Particularly in NSCLC, both miRNA 181a and miRNA630 deregulations modulate lung tumorgenesis, disease prognosis, and drug resistance mechanisms. Thus, exploring new drugs that can target the expression of these non-coding RNAs which are involved in disease progression, metastasis, and drug resistance may improve treatment strategies for lung cancer and improve survival rates.

Cardenolide glycosides are naturally occurring phytochemicals composed of a steroid skeleton attached to a sugar group at the third carbon and a lactone ring at the carbon 17 [29]. The usage of these glycosides is reputable in congestive heart failure and arrhythmias. Indeed, some of the cardiotonic steroids are produced endogenously in the human body, mediate several intracellular signaling pathways

and exert diverse physiological and pathophysiological functions [30]. Several studies reported that cardenolides interrupt Na+ /K+ -ATPase, activate the Ras-Raf-MAP kinase pathway and produce reactive oxygen species (ROS) with subsequent mitochondrial damage [29, 31]. Surprisingly, it was noted that declined levels of endogenous cardiotonic steroids result in tumor development such as leukemia/lymphoma and kidney/urinary tract cancers [32]. Although diverse pathways are revealed, it is still indefinite which one is responsible for the apoptotic effect.

Due to the encouraging results from preceding studies, the innovative use of cardiac glycosides as anticancer drugs in medical oncology has attracted a lot of consideration [33]. Acovenoside A is one of the cardenolide glycosides extracted from the pericarps of *Acokanthera oppositifolia* [34]. The anti-inflammatory and antioxidant activities of acovenoside A were reported in preceding studies [30, 35]. Besides, acovenoside A-induced cell death in NSCLC cell lines along with the production of ROS. Nonetheless, it was reported that ROS production is not a major contributor to acovenoside A-induced cell death [29, 31]. Thus, the actual molecular mechanisms underlying acovenoside A cytotoxicity in cancer cells have not been elucidated yet.

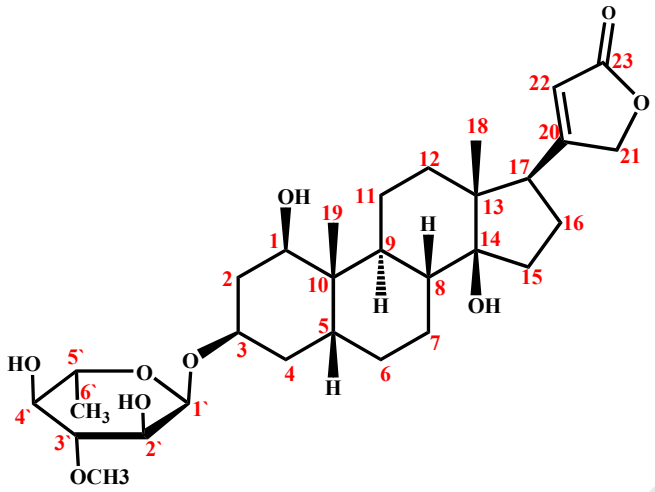
Collectively, the present study was conducted to explore the potential effect and the underlying molecular mechanism of acovenoside A in inducing apoptosis which consequently improves the therapeutic outcome, lessens metastasis and drug resistance. The current study also aimed to evaluate the effect of combining acovenoside A with carboplatin and taxol and whether this combination could improve apoptosis, inhibit cell proliferation, or halt drug resistance Furthermore, the study extended to investigate the effect of acovenoside A in modulating miR-181a and miR-630 which are implicated in NSCLC pathogenesis and drug resistance development in an attempt to explore phytochemicals which could control human epigenome.

2. Materials and Methods

2.1 Cell cultures and reagents

Human lung cancer cells (A549) were purchased from the Holding Company for Biological Products and Vaccines VACSERA (Giza, Egypt). A549 cells were grown in RPMI 1640 medium (Thermo Fisher Scientific Inc. (USA) supplemented with penicillin-streptomycin-amphotericin B cocktail (Sigma-Aldrich) in a 5% CO_2 humidified atmosphere at 37°C. The medium was changed every other day. Paclitaxel (Taxol, 6 mg/ml; Bristol-Myers Squibb, Egypt) and carboplatin (carboplatin 10 mg/mL; Mylan, France) were made in RPMI media. Stock cells were routinely grown as adherent monolayers and fed every alternate day and passaged when cells reached about 80% confluency.

Acovenoside A was isolated and identified by the authors from the chloroform fraction of the ethanolic extract of the pericarps of *Acokanthera oppositifolia* [34].



Acovenoside A

.2.2 Cell Viability Assay

The survival of cells was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. A549 cells were seeded into 96-well plates at a density of 1 X 10^5 cells/ml (100 ul/well) at 37°C for 24 hours to develop a complete monolayer sheet. The next day, cells were treated with DMSO or different concentrations of drugs and their combinations as shown in Table (1), then plate incubated at 37°C for 48 hours. Then 20 µl MTT metabolite solution (5mg/ml in PBS) (BIO BASIC CANADA INC) was added to each well and incubated for 4 hours at 37°C in CO_2 incubator. Cells were gently washed and incubated with DMSO for 15 min to dissolve the formazan crystals. Finally, the absorbance was read at 560nm and subtracted background at 620nm after brief premixing through a multi-plate ELISA reader (UV-VIS Quawell). The percentage of viable cells was calculated using the following equation:

Percent of viable cells (%) = (Optical density of sample/ Optical density of control) X 100

The results were expressed as the average of the three independent experiments.

2.3 Flow Cytometry Technique for cell growth assay

Cell growth assay was done using propidium iodide flow cytometry Kit (ab139418). A549 cells were cultured and allowed to adhere to the well walls overnight. Then cells were treated with taxol and carboplatin (IC_{50} doses that were used 66.07 ug/ml and 230.18 ug/ml, respectively), and acovenoside A (IC_{50} dose is 396 ug/ml), as well as their combinations for 48 hours and washed three times with ice-cold phosphate binding buffer (PBS). Cells were harvested from the treated and untreated samples, then they were centrifuged at 1200 rpm. Pellets were washed twice with PBS for 5 min then resuspended in 500µL of PBS and incubated with RNase A (0.1 mg/mL) for 30 min. After incubation, cells were stained with propidium iodide in the dark and incubated at room temperature for 45 min. The

stained cells were determined by flow cytometry (BD FACSCalibur; Becton Dickinson Biosciences, India).

2.4 Apoptosis Assay

To detect apoptotic cells, the FITC Annexin V Apoptosis detection kit was used (BioVision, Cat No: K101-25). The cultured cells were harvested and incubated with annexin V and PI staining for 15 min in the dark to identify cells in different phases. Flow cytometry was performed within 30 min using BD FACS Aria III flow cytometer to score annexin V positive cells. Cells that were stained with annexin V only were considered to be at the early stage of apoptosis. Additional exposure to propidium iodide (PI) made it possible to differentiate between the early apoptotic cells (annexin V-positive/PI-negative) from the late apoptotic cells (annexin V-positive and PI-positive).

2.5 RNA extraction & Real-time quantitative PCR (RT-qPCR) assay

A549 cells were treated with IC_{50} of taxol, carboplatin, and acovenoside A as well as their combinations. All of these drugs were incubated for 48 hours prior to RNA extraction. Total RNA was extracted using a miRNA extraction kit (Qiagen, Germany) as instructed in the manufacturer's protocol. RNA concentration and purity were evaluated using the NanoDrop 2000 c model (Thermo Fisher Scientific, USA). Then the extracted RNA was used for subsequent gene expression of p53, B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax), caspase 3, miR-181a, and miR-630. Of note, DNAase was added to ensure the purity of the extracted RNA.

Reverse Transcription (RT) of mRNAs as p53, caspase 3, Bax, and Bcl2 were performed using RT2 first strand kit (Qiagen, Germany) followed by RT-PCR using PCR Maxima SYBR Green kit (Thermo Fisher Scientific, USA) using the Rotor-Gene Q equipment (Germany) according to the manufacturer's protocol. PCR cycles were programmed as follows: 10 min at 95◦C, followed by 45 cycles for 15 Sec at 94◦C and 60 Sec at 58◦C. β-Actin was used as the housekeeping gene.

RT of miR-181a and miR-630 was done using miScript II RT Kit (Qiagen, Germany) was used according to the manufacturer's guidelines. Then miScript SYBR Green PCR kit was used for cDNA sample amplifications. PCR cycles were adjusted under the following conditions: 94◦C for 30 min, followed by 40 cycles at 95◦C for 15 Sec, 56◦C for 30 Sec, and 72◦C for 30 Sec. U6 was used as the housekeeping gene. Relative quantification analysis was done using the comparative CT ($2^{-\Delta\Delta CT}$) method. Primers are shown in Table (2).

2.6 Statistical analysis

Data were analyzed using Graph Pad Prism 8 software. Data are represented as mean ± SD of the triplicates of each experiment. One-way analysis of variance followed by Tukey's test for multiple comparisons was used for normally distributed quantitative variables. A p-value of less than 0.05 was considered statistically significant

3. Results

3.1 Effect of acovenoside A and its combination with taxol & carboplatin on cell viability and cell cytotoxicity by MTT assay

Each taxol, carboplatin, and acovenoside A glycoside were tested separately in a serial range of concentrations to assess their cytotoxic activities as shown in Table (3). The MTT assay revealed that all of these agents possess a direct cytotoxic effect on the A549 cancer cell line in a dose-dependent manner as shown in Figure (1). The IC_{50} of taxol, carboplatin, and acovenoside A glycoside were 66.07 ug/ml, 230.18 ug/ml, and 395.9 ug/ml, respectively. The former results showed that A549 cancer cells are most sensitive to taxol by 3.5 and 6-fold compared to individual treatment with each of carboplatin and acovenoside A glycoside, respectively.

Table 3 summarizes the effect of acovenoside A and its combination with taxol, and carboplatin on cell viability and cell cytotoxicity on A549 cells. Combination 5 (acovenoside A (396 ug/ml) + taxol (66.07ug/ml) + carboplatin (230.18ug/ml)) is the most potent reaching 78.9% cytotoxicity followed by combination 3 (acovenoside A (396 ug/ml) + taxol (66.07ug/ml)) and combination 4 (Acovenoside A (396 ug/ml) + carboplatin (230.18ug/ml)). That is why all the following tests focused on the effect of combinations 3,4 and 5.

3.2 Effect of acovenoside A and its combination with taxol & carboplatin on cell cycle progression in A549 cells using cell growth assay.

Cell cycle phase distribution was evaluated using flow cytometry to determine if the current combinations affect cycle kinetics and mediate cell growth inhibition. Acovenoside A displayed cell growth arrest at the G2/M phase where the percent of cells was higher in the G2/M phase concurrently with lower cells at G0/G1 and S phases compared with control cancer cells. However, the combination of acovenoside A with taxol showed significant cell growth arrest at G1 phase, where the percent of the cells at G1 phase was higher in this combination compared with the control cancer cells. While, the combination of acovenoside A with carboplatin displayed significant cell growth arrest at G1/S phases where the percent of cells at G1 and S phases were higher in this combination compared with control cancer cells. It is noteworthy that the triple combination of acovenoside A with taxol and carboplatin showed the best growth inhibition efficiency where the number of cells at the G2/M phase was almost zero recording 0.02% as shown in Table (4) and Figure (2).

3.3 Effect of acovenoside A and its combination with taxol & carboplatin on cellular apoptosis and necrosis induction in A549 Cell using annexin V FITC assay.

One of the fundamental properties of anticancer drugs is their capability to induce apoptosis and necrosis. Concerning classifying the apoptotic power of acovenoside A and its combinations, the combination of acovenoside A with taxol exhibited the uppermost apoptotic and necrotic activities followed by the combination of acovenoside A with carboplatin and then acovenoside A alone, where the total percent of dead cells reached 31.4%, 24.1%, and 12.7%, respectively. Interestingly, cell growth assay revealed that the triad of acovenoside A with taxol and carboplatin boost up apoptotic and necrotic activity compared with the other combinations, where the percent of early and late apoptotic cells were 7.9% and 20.1%, respectively with total apoptotic cells 39.2%. in addition, the percentage of necrotic cells was raised to 11.2% as shown in Table (5) and Figure (3).

3.4 Real-time quantitative PCR (RT-qPCR) assay for cell cycle-leading proteins

Based on the aforementioned results, the triad of acovenoside A with taxol and carboplatin exhibited the most cytotoxic activity against lung cancer cells. For a better understanding of the underlying mechanisms, the apoptosis-associated genes expression Bax, Bcl2, caspase 3, and p53 were investigated. The gene expression of Bax, caspase 3, and P53 were significantly up-regulated among the three drugs and their combinations compared with control A549 cells. Interestingly, the triplet of acovenoside A with taxol and carboplatin displayed the most remarkable increase in gene expression of Bax and p53 compared with other treatment groups. However, the triplet showed the second highest level of caspase3 gene expression preceded only by carboplatin-treated A549 cells. Regarding Bcl2, all treatment groups showed significant down-regulated levels compared with control non-treated cells. Favorably, the combination of acovenoside A along with taxol and carboplatin showed the least level of Bcl2 gene expression as shown in Figure (4).

3.5 Real-time quantitative PCR (RT-qPCR) assay for miRNA 181a and miRNA630

The present study revealed that the miR-181a was significantly up-regulated among all drugs and their combinations compared with control A549 lung cancer cells. At the same time, the extent of miR-181a up-regulation was variable among the three treatments and their combinations. Certainly, the highest miR-181a gene expression prevailed in cell lines treated with the triad of acovenoside A with taxol and carboplatin. Another non-coding RNA miR-630 was mostly significantly up-regulated in cell lines treated with the combination of acovenoside A with taxol and carboplatin compared with the control cell line. However, it showed the uppermost expression in NSCLC cells treated with acovenoside A together with taxol and carboplatin compared with other treatment regimens.

4. Discussion

In the present study, the MTT test was done to evaluate the cell viability and cytotoxicity of acovenoside A. It showed that usage of the triad acovenoside A, carboplatin, and taxol possesses lower cell vitality and a higher population of dead cells compared with the combination of carboplatin and taxol alone. Moreover, adding acovenoside A to each carboplatin and taxol individually showed significant potentiation in cell cytotoxicity and amelioration in cell viability. These findings suggested that the addition of acovenoside A promote the therapeutic efficiency of carboplatin and taxol treatment protocol by increasing their cytotoxic activity. Consistently, El Gaafary et al study reported the selective acovenoside A cytotoxicity where it showed lesser cytotoxicity in lung fibroblasts together with no prevailed cytotoxicity on blood mononuclear cells [29]. Other preceding literature disclosed versatile effects of cardiac glycosides, where they modulate several intracellular signaling routes involved in apoptosis, cellular proliferation, and control several transcriptional factors [30]. Importantly it was reported that cardenolides could modulate the expression and activity of enzymes that are responsible for drug metabolism and thus may change the pharmacokinetics of other drugs [30]. However, the underlying mechanisms of acovenoside A not fully understood yet.

Certainly, cell cycle progression is an essential target for chemotherapeutic agents [36]. In the present study, acovenoside A exerts cell growth arrest at the G2/M phase. These findings reflect the powerful effect of acovenoside A in disturbing the regulation of cells where it losses its ability to detect and repair gene damage. Furthermore, the addition of acovenoside A to either carboplatin or taxol accelerates the apoptotic process compared with each of them alone. In the same context, the triple combination showed a higher cell population at the G1/S phases concurrently with a lowered number of

cells in G2/M phase compared with each one separately. These results are consistent with previous preclinical and clinical studies which disclosed that combinations of phytochemicals with chemotherapeutics give better outcomes in fighting cancer by controlling signaling pathways responsible for stimulating cell cycle arrest, apoptosis, and hindering cell proliferation [37]. Collectively the present study demonstrated the cytotoxic effect of acovenoside A on A549 lung cancer cells where it showed significant apoptotic and antiproliferative effects. For a better understanding of the underlying mechanisms of the apoptotic effect of acovenoside A, the expression of apoptosis-related genes Bax, Bcl2, caspase 3, and P53 was investigated.

Despite p53 levels being low in normal cells, it exerts an essential function in controlling cell fate and managing stress events via cascades of post-translational modifications and binds to specific DNA sequences [38, 39]. In the current study, p53 was significantly up-regulated among the three drugs each alone and their combinations compared with control A549 cells. Interestingly, the triplet of acovenoside A with taxol and carboplatin displayed the most remarkable increase in gene expression p53 compared with other treatments group confirming the previously observed cytotoxic and apoptotic effects of acovenoside A. This is coherent with former reports which declared that P53 modifies cell cycle arrest at one of two major checkpoints either in G_1 near the border of S-phase or in G_2 prior to mitosis [40, 41]. The activated p53 signaling pathway coordinates with other transcription factors that finally suppress tumorigenesis. Moreover, preceding data demonstrated the involvement of the mutated form of p53 with diverse oncogenic features such as stimulated invasion, migration, angiogenesis, senescence, apoptosis, DNA repair, proliferation, and tissue remodeling. p53 is also implicated in the development of drug resistance, mitogenic defects, and genomic instability [42]. Definitely, disturbances of p53 gene expression are a common feature in the majority of human malignancies, so exploring novel p53-based chemotherapeutic agents such as acovenoside A is a highly attractive target.

The anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax are two genes that are markedly correlated with pathophysiological changes in cancer as they are encountered in cell suicide [43, 44]. The current study reported the up-regulated gene expression of Bax concurrently with the down-regulated gene expression of Bcl2 among the three drugs and their combinations compared with control A549 cells. Attractively, the triplet of acovenoside A with taxol and carboplatin achieved the most remarkable down-regulated Bcl2 gene expression along with up-regulated Bax gene expression compared with other treatment groups. These findings emphasize the hidden mechanisms of acovenoside A in fighting lung cancer. These findings are in harmony with a preceding report which addressed the mitotic arrest, and the disturbed cytokinesis action of acovenoside A, however, the present study is the first to link its activity with Bax and Bcl2 in NSCLC cell line [29]. Although availability of some drugs which have apoptotic effects, it is desirable to develop innovative treatments that can cover more dysregulated signaling pathways of apoptosis to achieve efficient and integral therapeutic protocol. Thus, adding acovenoside A to carboplatin and taxol augment the apoptotic effect and exerts better outcomes.

Caspases are considered key elements for the apoptogenic process where they cleave diverse cellular substrates which in turn disrupt cell structure and become susceptible to phagocytosis [45]. The ongoing study revealed that the gene expression of caspase 3 was highly up-regulated among the three drugs and their combinations compared with control A549 cells. Remarkably, the triplet of acovenoside A with taxol and carboplatin as well as the dual combination of acovenoside A and carboplatin recorded the greatest up-regulated caspase 3 gene expression compared with other treatment groups.These

findings point to the powerful effect of acovenoside A in boosting the cytotoxic effect of carboplatin and taxol combination via potentiaing the proteolytic effect of caspase 3. These results coincide with previous investigator who reported that acovenoside A distorted the mitochondrial membrane and aggravated oxidative stress leading to stimulation of canonical apoptosis through caspase 3 activation and DNA fragmentation [29].

In most cases, induction of apoptosis involves the activation of several coding genes as previously shown in the ongoing study; however, other non-coding genes interplay in the apoptosis signaling pathway. The present study revealed the significantly up-regulated miR-630 gene expression in all the treated cell lines compared with non-treated ones, in agreement with Chen et al who reported that cisplatin-treated NSCLC showed up-regulated miR-630 gene expression which confers better clinical outcomes [6]. Indeed, the loss of miR-630 is primarily linked with several disorders. This is consistent with a previous study that reports that the low level of miR-630 in liver cancer is accompanied by recurrence and low survival rates [46]. Favorably, the triplet combination of acovenoside A, carboplatin, and taxol showed the highest miR-630 gene expression compared with other treatments either singlet or combinations suggesting that miR630 may be one of the underlying mechanisms of acovenoside A chemotherapeutic effect.

Importantly, Song et al. reported low levels of miR-630 in NSCLC tissues as well as increased levels of miR-630 halt cellular proliferation, migration, and invasiveness [47]. Another study by Li et al. demonstrated that NSCLC cells with high expression levels of miR-630 exhibit suppressed resistance to chemotherapy and radiation [48]. Therefore, loss of miR-630 may be at least partially implicated in bad prognosis, low survival rate, and development of drug resistance in NSCLC. Crucially, the prevailed up-regulated levels of miR-630 with acovenoside A administration could confer better outcomes and higher survival rates without the development of drug resistance compared with the traditional combination of carboplatin and taxol. Furthermore, the present study hypothesizes that the modulatory effect of acovenoside A in up-regulating miR-630 may be the drive behind the observed up-regulated pro-apoptotic genes; p53, Bax, and caspase3 along with down-regulated Bcl2 in cells treated with acovenoside A. These postulations come in harmony with former studies which reported that suppressed miR-630 is associated with p53 inhibition in cervical cancer and associated with high levels of Bcl2 in NSCLC [6, 49].

miR-181a is another non-coding RNA that belongs to the miR-181 family and has a critical role in numerous cellular events, such as cell cytokinesis and proliferation as well as its regulation is implicated in various malignancies. Regarding NSCLC, Gao et al and Larroque-Cardoso et al reported the reduced levels of miR-181a [50, 51]. Herein, the gene expression of miR-181a was significantly up-regulated in A549 treated group with either acovenoside A, carboplatin, or taxol compared with non-treated cell lines. Significantly, the triple combination of these former agents displayed the highest expression of miR-181a which points to the synergism between these agents in the treatment of NSCLC. Consequently, the beneficial effect of acovenoside A may be attributed to its effect on up-regulating miR-181a where its loss provokes NSCLC pathogenesis. In an attempt to understand the molecular mechanism of miR-181a, Feng et al. reported the putative effect of miR-181a in targeting apoptotic genes where it enhances apoptosis via targeting p53, Bax, and Bcl-2 [52]. Accordingly, the observed up-regulation of miR-181a induced by acovenoside A may be responsible for the enhancement of pro-apoptotic genes and the suppression of the anti-apoptotic ones.

5. Conclusion and future prospects

The safety and efficacy of natural phytochemicals are the key rationales for their use as complementary and alternative therapies to treat diverse pathological conditions. The present study is the first to address the valuable effect of the usage of the cardiac glycoside; acovenoside A together with carboplatin and taxol in the treatment of NSCLC via exerting apoptotic, antiproliferative, and cytotoxic effects. The underlying mechanism of acovenoside A as adjuvant therapy is attributed to the up-regulation of miR-630 and miR-181a gene expressions which in turn targets the intrinsic apoptosis genes p53, Bax, and Bcl2 as well as caspase3. Indeed acovenoside A could overcome the developed carboplatin and taxol drug resistance, and improve prognosis and clinical outcomes. Prospective clinical studies will be needed to explore natural products that when administered in conjunction with conventional drugs, boost their therapeutic effect, improves clinical outcomes, and modulates the activity of metabolizing enzymes which may be responsible for the development of drug resistance.

Conflict of interest

The authors declare no conflict of interest.

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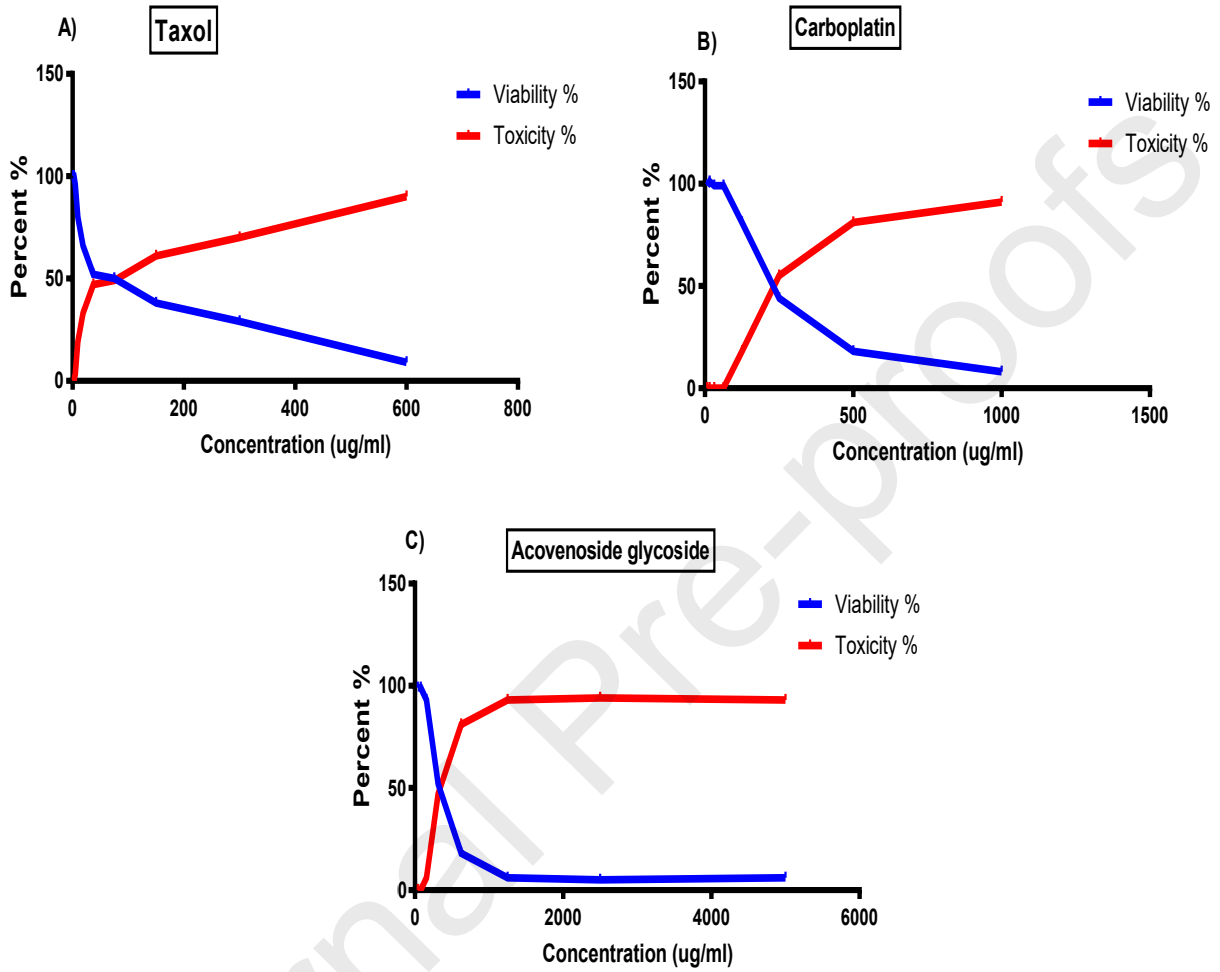

Figure 1: Effect of taxol, carboplatin, acovenoside A glycoside on cell viability and cytotoxicity in human lung cancer A549 cell line.

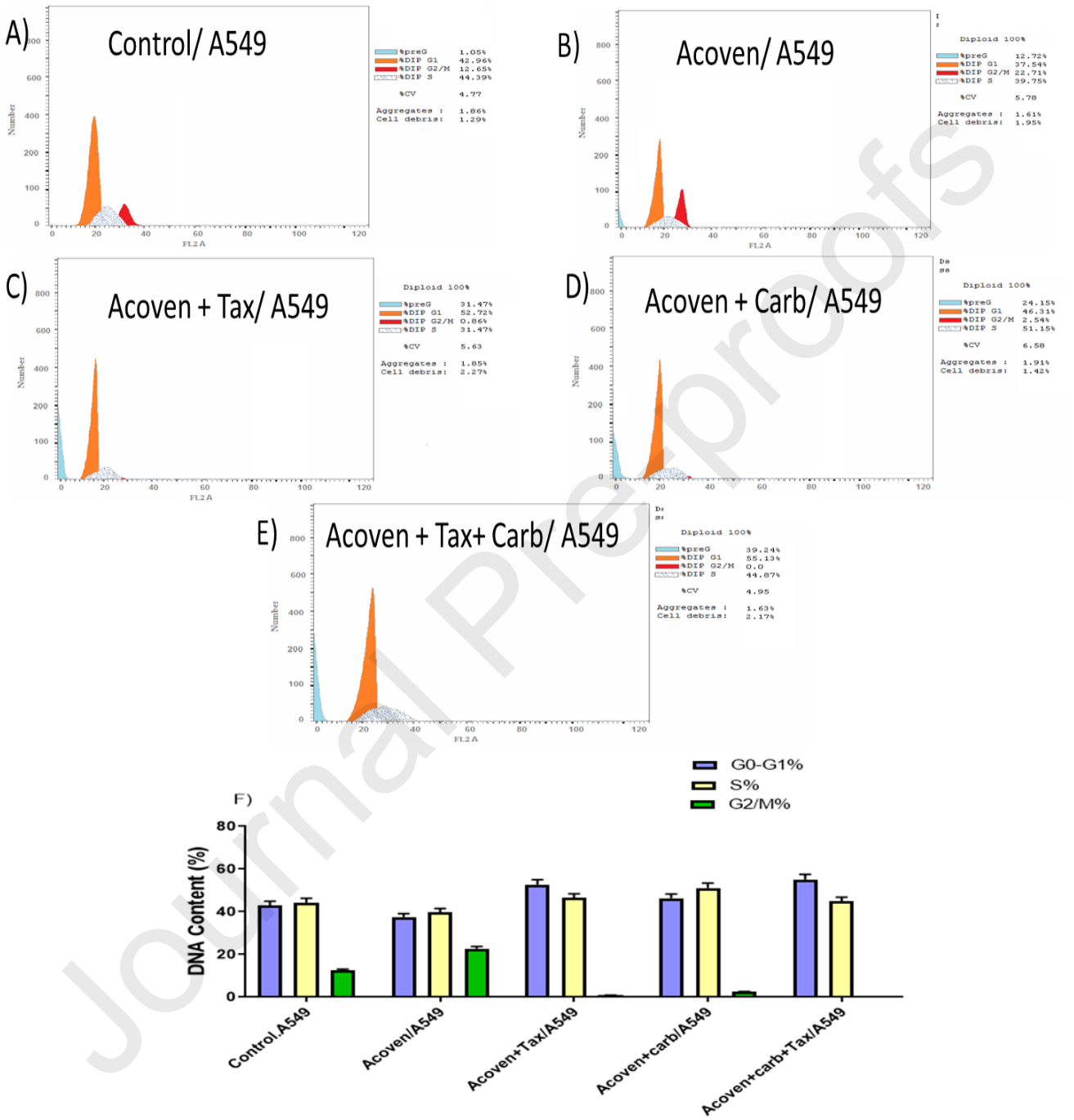

Figure 2: Flow cytometry analysis for cell cycle phase distribution beyond treatment with acovenoside A and its combination with taxol & carboplatin in human lung cancer A549 cell line. (A-E) Cellular DNA histograms displayed the cell cycle phases of A549 cells after treatment with

acovenoside A and its combination with taxol & carboplatin. (F) Bar graphs displayed the percentages of DNA in each cell cycle. Results obtained from three independent experiments and means were calculated. Acoven: Acovenoside A, Tax: Taxol, Carb: Carboplatin

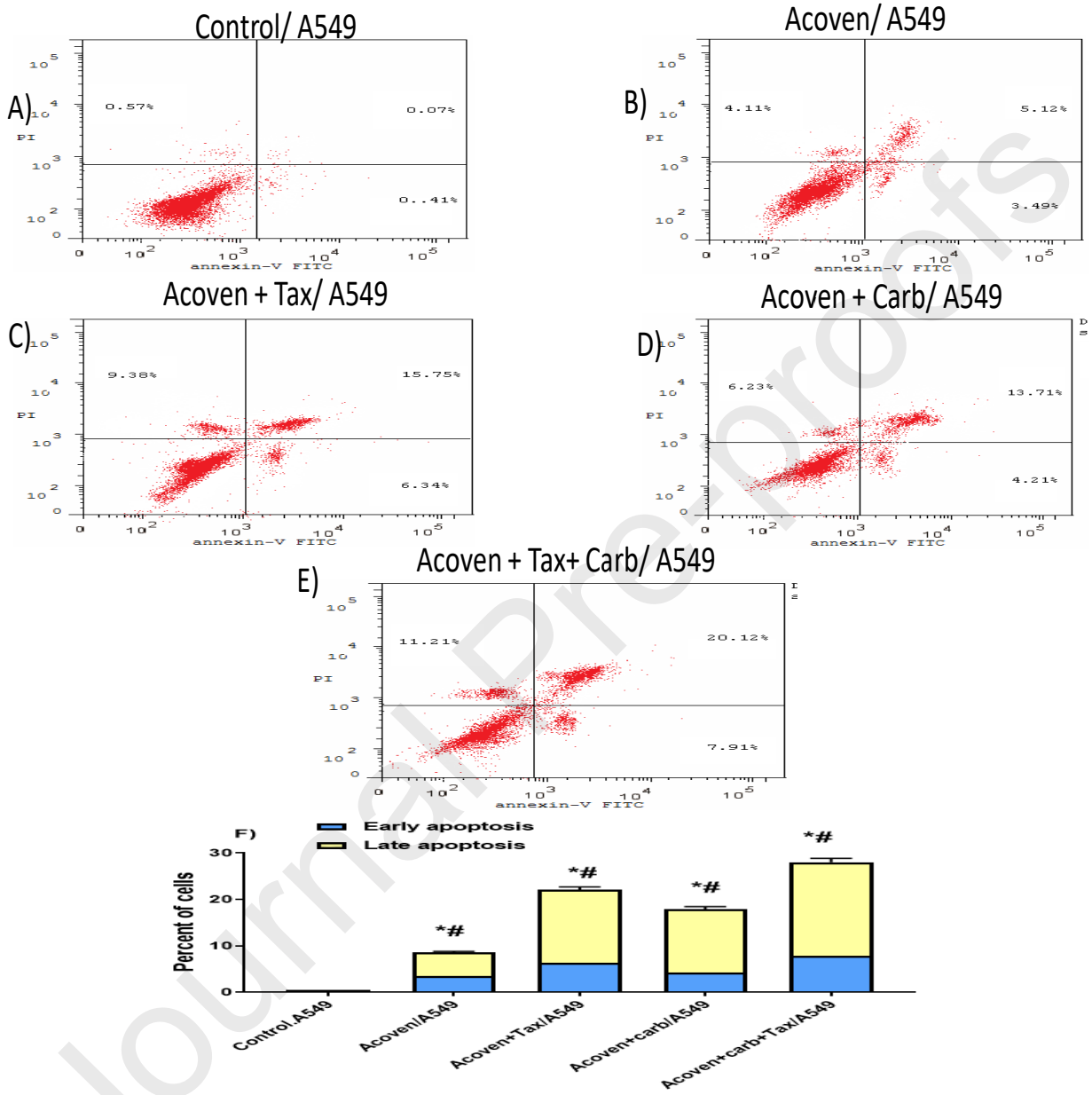

Figure 3: Effect of acovenoside A and its combination with taxol & carboplatin on cellular apoptosis and necrosis induction in human lung cancer A549 cells using annexin V FITC. (A-E) Dot plots representing the analysis of apoptosis induction on A549 cells after treatment with acovenoside A and its combination with taxol & carboplatin. (F) Bar graphs displayed the percentages of early and late apoptic cells. Results obtained from three independent experiments and mean was calculated. Comparisons were done between different treatment groups and Control A549 non treated cells. Statistical analysis was carried out using one-way ANOVA with Tukey's post hoc test for multiple

comparisons between groups * $p < 0.05$, and # $p < 0.01$. Acoven: Acovenoside A, Tax: Taxol, Carb: Carboplatin

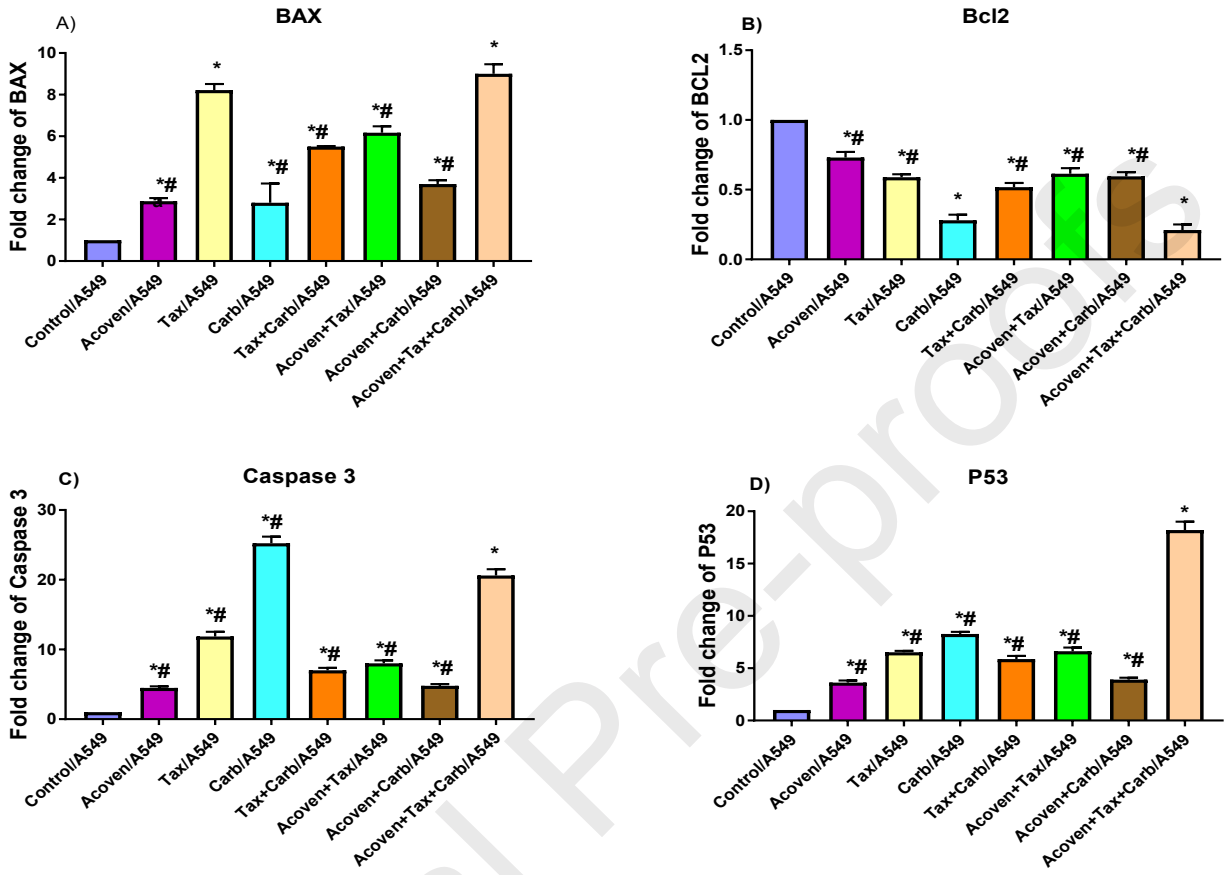

Figure 4: Effect of acovenoside A and its combination with taxol & carboplatin in human lung cancer A549 cell line on gene expression levels of Bax (A), Bcl2 (B), caspase3 (C) and P53 (D). Data are represented as mean ± SD. *Significance from control; # significance from triplet combination of acovenoside A, taxol and carboplatin. Acoven: Acovenoside A, Tax: Taxol, Carb: Carboplatin

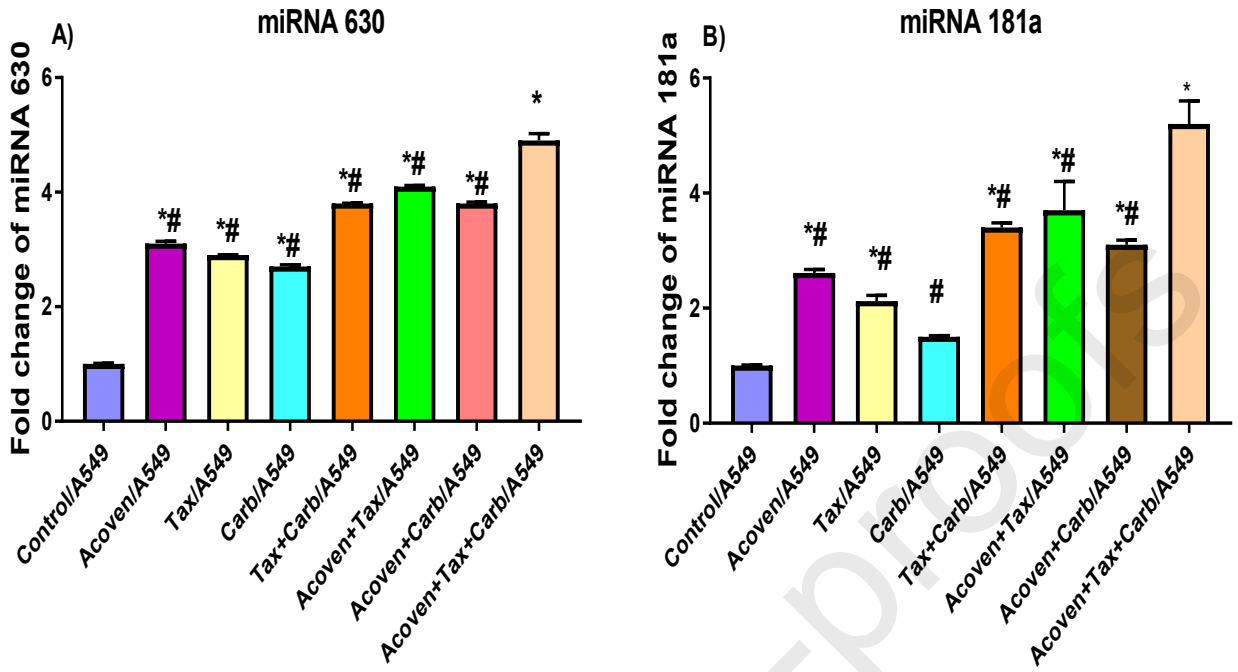

Figure 5: Effect of acovenoside A and its combination with taxol & carboplatin in human lung cancer A549 cell line on gene expression levels of miRNA 181a (A) and miRNA630 (B). Data are represented as mean ± SD. * Significance from control; # significance from triplet combination of acovenoside A, taxol and carboplatin. Acoven: Acovenoside A, Tax: Taxol, Carb: Carboplatin

Table 1: The different drug combinations and their concentrations.

Combinations	Drugs and their concentrations
Combination 1	IC50 of Taxol (66.07 ug/ml) + IC50 of carboplatin (230.18 ug/ml)
Combination 2	1/10 of IC50 of taxol (6.6 ug/ml) +1/10 of IC50 of carboplatin (23 ug/ml)
Combination	IC50 of Acovenoside A (396 ug/ml)+ IC50 of taxol (66.07ug/ml)

3	
Combination 4	IC50 of Acovenoside A (396 ug/ml) + IC50 of carboplatin (230.18ug/ml)
Combination 5	IC50 of Acovenoside A (396 ug/ml) + IC50 of Taxol (66.07ug/ml) + IC50 of carboplatin (230.18ug/ml)

Table 2: The list of primers used in real-time polymerase chain reaction.

Bax	Forward	5'-ATGTTTTCTGACGGCAACTTC-3'
	Reverse	5'-AGTCCAATGTCCAGCCCAT-3'

Bcl-2	Forward	5’-ATGTGTGTGGAGACCGTCAA-3’
	Reverse	5’-GCCGTACAGTTCCACAAAGG-3’
P53	Forward	5’-ATGTTTTGCCAACTGGCCAAG-3’
	Reverse	5’-TGAGCAGCGCTCATGGTG-3’
Caspase-3	Forward	5’-TGTTTGTGTGCTTCTGAGCC-3’
	Reverse	5’-CACGCCATGTCATCATCAAC-3’
β-actin	Forward	5′-TGACGTGGACATCCGCAAAG-3′
	Reverse	5′-CTGGAAGGTGGACAGCGAGG-3′.
miR-181a	Forward	5’-GCGGCGGAACATTCAACGCTGTC-3′
	Reverse	5′-ATCCAGTGCAGGGTCCGAGG-3′
miR-630	Forward	5’-TAAAGGAGGAAGATAAGG=3’
	Reverse	5’-GTAGCAGTGATAGGCATT-3’
U6	Forward	5’-CCTGCTTCGGCAGCACA-3’
	Reverse	5-‘TGGAACGCTTCACGAA-3’

Table (3): Effect of acovenoside A and its combination with taxol, and carboplatin on cell viability and cell cytotoxicity on human lung cancer A549 cell line.

ID	Conc.	Mean optical density (O.D)	Standard error of mean (SEM)	Viability %	Toxicity %	IC50

	ug/ml					
A549	-	0.315	0.0052	100	0	ug/ml
Taxol	600	0.028	0.0043	9.413	90.58	66.07
	300	0.089	0.0025	29.56	70.43	
	150	0.116	0.0046	38.53	61.46	
	75	0.152	0.0037	50.49	49.50	
	37.5	0.158	0.0020	52.60	47.39	
	18.75	0.2	0.0037	66.44	33.55	
	9.37	0.243	0.0043	80.73	19.26	
	4.68	0.290	0.0018	96.45	3.543	
	2.34	0.302	0.0028	100.4	0	
	1.17	0.302	0.0015	100.3	0	
Carboplatin	1000	0.024	0.0033	8.084	91.91	230.18
	500	0.056	0.0026	18.82	81.17	
	250	0.132	0.0044	44.07	55.92	
	125	0.245	0.0110	81.39	18.60	

	62.5	0.300	0.0026	99.88	0.110	
	31.25	0.300	0.0018	99.88	0.110	
	15.62	0.304	0.0027	101.1	0	
	7.81	0.299	0.0034	99.44	0.553	
Acovenoside A	5000	0.019	0.0005	6.031	93.96	395.9
	2500	0.018	0.0008	5.925	94.07	
	1250	0.021	0.0012	6.878	93.12	
	625	0.057	0.0052	18.30	81.69	
	312.5	0.164	0.0048	52.27	47.72	
	156.25	0.295	0.0058	93.86	6.137	
	78.12	0.314	0.0037	99.68	0.317	
	39.06	0.312	0.0072	99.04	0.952	
Drug combinations						

Combination 1: taxol and carboplatin at their IC_{50} dose; combination 2: 1/10 from IC_{50} of taxol (6.6 ug/ml) and 1/10 from IC_{50} of carboplatin (23 ug/ml); combination 3: acovenoside A +taxol at their IC_{50} dose; combination 4: acovenoside A + carboplatin at their IC_{50} dose; Combination 5: taxol+ acovenoside A + carboplatin at their IC_{50} dose.

Table 4: Effect of acovenoside A and its combination with taxol, and carboplatin on cell cycle progression in human lung cancer A549 cell line.

ID	%G0-G1	%S	%G2/M
Control/A549	42.96±1.89	44.39±1.95	12.65±0.56
Acovenoside A/A549	37.54±1.65 #	39.75±1.75 #	22.71±1.0 @
Acovenoside A +Taxol/A549	52.72±2.32 @	31.47±2.04	0.86±0.04 @
Acovenoside +Carboplatin/A549	46.31±2.04	51.15±2.25 @	2.54±0.11 @
Acovenoside + Carboplatin+ Taxol /A549	55.13±2.42 @	44.87±1.97	0.02 ±0.001 @

Data are represented as mean ± SD. Results were obtained from three independent experiments and the mean was calculated. Comparisons were done between different treatment groups and control A549 non-treated cells. Statistical analysis was carried out using one-way ANOVA with Tukey's post hoc test for multiple comparisons between groups * $p < 0.05$, # $p < 0.01$, and @ $p < 0.001$.

Table 5: Effect of acovenoside A and its combination with taxol, and carboplatin on cellular apoptosis and necrosis induction in human lung cancer A549 cell line

	Apoptosis			Necrosis
	Total	**Early**	**Late**	
Control/A549	1.05±0.04	0.41±0.02	0.07±0.003	0.57±0.02
Acovenoside A/A549	12.72±0.51 *#	3.49±0.14 *#	5.12±0.21 *#	4.11±0.16 *#
Acovenoside A +Taxol/A549	31.47±1.26 *#	6.34±0.25 *#	15.75±0.63 *#	9.38±0.37 *#
Acovenoside A+Carboplatin/A549	24.15±0.96 *#	4.21±0.17 *#	13.71±0.54 *#	6.23±0.25 *#

Acovenoside A +Carboplatin+Taxol/A549	39.24±1.57 *	7.91±0.32 *	20.12±0.8 *	11.21±0.45 *

Data are represented as mean ± SD. Results were obtained from three independent experiments and the mean was calculated. Comparisons were done between different treatment groups and Control A549 non-treated cells. Statistical analysis was carried out using one-way ANOVA with Tukey's post hoc test for multiple comparisons between groups * $p < 0.05$, and # $p < 0.01$. Acoven: Acovenoside A, Tax: Taxol, Carb: Carboplatin.

Highlights:

- Lung cancer is the most prevalent cause of cancer deaths all over the world.
- Cardenolides are natural phytochemicals that proven anticancer activity, however, their mechanism of action is still unclear
- Our study has proven that the underlying mechanism of acovenoside A is partially attributed to the up-regulation of miR-630 and miR-181a gene expressions.
- The miR-630 and miR-181in turn target the intrinsic apoptosis genes as p53, Bax and Bcl2 as well as caspase 3.
- The present study addressed the valuable effect of using acovenoside A together with carboplatin and taxol in the treatment of NSCLC *via* exerting apoptotic, antiproliferative, and cytotoxic effects