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ORIGINAL ARTICLE

# Antiproliferative effects of Norartocarpetin isoflavone in human lung carcinoma cells are mediated *via* targeting Ras/Raf/MAPK signalling pathway, mitochondrial mediated apoptosis, S-phase cell cycle arrest and suppression of cell migration and invasion

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## Summary

***Purpose:*** *The main purpose of the current research work was to investigate the anticancer activity of norartocarpetin - a plant derived isoflavone -in human lung carcinoma cells NCI-H460 and normal lung cell line MRC-9 along with studying its effects on cellular apoptosis, DNA damage, cell migration and invasion and Ras/Raf/MAPK signalling pathways.*

***Methods:*** *Cell proliferation was examined by CCK-8 assay while effects on apoptosis were evaluated by acridine orange (AO)/ethidium bormide (EB) staining and Comet assay using fluorescence microscopy. In vitro wound healing assay was used for checking the effects on cell migration and transwell assay for invasion while western blot was used to evaluate the effects on the expression of Ras/Raf/MAPK proteins.*

***Results:*** *The results showed that Norartocarpetin led to dose-dependent cytotoxic effects in NCI-H460 cells showing an $IC_{50}$ value of 22 μM while in normal lung cells, the cytotoxic effects were much lower as shown by higher $IC_{50}$ value of 85 μM. It also led to dose-dependent apoptosis and induced DNA damage as shown by fluorescence microscopy. This molecule also inhibited cell migration and invasion dose-dependently, along with inhibiting MMP-9 expression. Norartocarpetin treatment also led to inhibition of the expression of Ras/Raf/MAPK proteins and also caused S-phase cell cycle arrest in these cells.*

***Conclusion:*** *Norartocarpetin has a significant anticancer activity in lung carcinoma cells and these effects are mediated via targeting apoptosis, DNA damage, cell migration and invasion, cell cycle and inhibiting Ras/Raf/MAPK signalling pathways.*

***Key words:*** *norartocarpetin, lung carcinoma, apoptosis, DNA-damage, flow cytometry*

## Introduction

Lung cancer (LC) is a major cause of cancer-associated mortality, with around 1.5 million people yearly [1,2]. It has been extensively acknowledged that metastasis is a highlighted topic of research among the aggressive malignancies. Metastasis-related deaths in LC are more prevalent, about 90%, and nearly 70% of patients are found with distant spreading of cancer or local lymph node metastasis at the time of first diagnosis [3]. When normal lung epithelial cells undergo multiple genetic damages are finally transformed to uncontrolled proliferating cells with abnormal

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growth and aggressive behaviours. There are two major histological subtypes of LC: non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC); (NSCLC about 85% and SCLC about 15% of all lung cancer cases, respectively) [4,5]. In accordance with the histological classification, NSCLC type can further be classified into three subtypes: large-cell carcinoma (LCC), squamous cell carcinoma (SCC) and adenocarcinoma (AC) [4]. Classification of LC is more important for treatment strategy and predictive outcome. Currently different methods for LC treatment like radiation, surgery, target therapy, chemotherapy and combined chemotherapy and radiotherapy are used, but the clinical outcome is unsatisfactory due to drug resistance [6]. Various potential therapeutic agents have been discovered through investigation of natural products like plants, microorganisms, marine organisms and animals or natural product-derived lead compounds can serve as precursors for potential chemotherapeutic agents [7]. Among them, naturally-occurring isoflavones have received lots of attention because of their inhibiting influence on multiple common cancers [8-11]. Artocarpus species (Pacific or Asian tree crops) have revealed many pharmacological activities like antitumorigenic, antiinflammatory, antibacterial, tyrosinase inhibitory, antidiabetic, antiviral, antituberculosis, antioxidant, and antiplatelet [12-17]. So it is possible that some of these activities might be due to the presence of Norartocarpetin (2-(2,4-dihydroxyphenyl)-5,7- dihydroxy-4H-chromen-4- one) as this molecule is a major component in it. Thus, the current study was performed to investigate the antiproliferative effects of Norartocarpetin isoflavone in human LC cells along with evaluating its effects on Ras/Raf/MAPK signalling pathway, mitochondrial-mediated apoptosis, S-phase cell cycle arrest and suppression of cell migration and invasion.

## Methods

*Cell viability determination*

Cell viability of MRC-9 normal fibroblast lung cells and NCI-H460 human LC cells was performed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. MTS study is a cell titer 96 aqueous one solution cell multiplication/proliferation/viability assay. For seeding of both normal (MRC-9) as well as cancer cells (NCI-H460) 96-well plate were used. These cells were cultured at a density of $2\times10^5$ cells/well, separately. Firstly, MRC-9 normal fibroblast lung cells and NCI-H460 human LC cells were incubated for 24 h. After incubation these cells were exposed to varying concentrations of Norartocarpetin, i.e. 0, 3.12, 6.25, 12.5, 25, 50, and 100 µM. These doses of Norartocarpetin were added to the wells, followed by supplementary incubation for 24h. Subsequently, MTS solution was added to the wells according to the manufacturer guiding instructions, and finally absorbance at 490nm was measured by an ELISA microplate reader (ELX 800; Bio-Tek Instruments, Inc., Winooski, VT, USA).

*Apoptosis detection assays*

AO/EB staining and comet assay, using fluorescence microscopy, were used for apoptosis determination. NCI-H460 human LC cells in the logarithmic growth phase were processed with 0.25% trypsin. Dulbecco's modified Eagle's medium (DMEM) containing of 10% fetal calf serum (FCS) was added in each 96-well plate with 100 µl/well. Afterwards, cells were added to the wells at a density of $2\times10^5$ cells/ml, followed by incubation. Cells were treated with 0. 11, 22 and 44 µM of Norartocarpetin. After culturing for 24h, the samples were treated with 20 µl of trypsin. Suspensions (25 µl) were transferred to glass slides after cells had sloughed off. For AO/EB staining, dual fluorescent staining solution (1 µl) comprising of 100 µg/ml AO and 100 µg/ml EB was added to each well and then protected with a coverslip. After that, the morphology of apoptotic cells was studied using fluorescent microscope (OLYMPUS, Japan). The staining procedure was repeated at least 3 times.

For comet assay, about 180 µl of cell suspensions were mixed in 500 µl of 1% of agarose at 37°C. After mixing, the cells were instantly layered on custom frosted slides. To solidify the agarose these slides were placed on a chilled plate. The slides were then immersed in newly RIPA lysis buffer prepared with 2.5 M NaCl, 10 mM Tris-base, 0.1% sodium sarcosinate buffer solution with pH 12.3 at room temperature for one hour in the dark. After lysing, for salt equilibration and further DNA unwinding the slides were placed in 0.3 N NaOH, 1 mM EDTA buffer solution, which is an alkaline electrophoresis buffer with pH 12.3, for 30 min. At 20 V and 400 mA electrophoresis was performed for 10 minutes. In order to strengthen the DNA, the slides were submerged in a distilled water bath for 5 min at 37°C. Then, staining was performed with propidium iodide (PI) (at a concentration of 2.5 mg/ml) for 1/3 of an hour and before analysis with Axioscope fluorescent microscope equipped with a standard Photonics Camera, the slides were cover-slipped.

*Cell cycle analysis*

The NCI-H460 human LC cells were cultured at a density of $1\times10^5$ cells/ml in DMEM medium for 12h. These cells were treated with different concentrations, i.e. 0, 11, 22, and 44 µM of Norartocarpetin, and then collected. Before exposure to 15 µg/ml of RNase A, these cells were fixed with 70% ethanol. Further, these fixed cells were washed with phosphate buffered saline (PBS) and then stained with Annexin V/PI solution for staining at 20 µl and 20 µg/mL respectively. FACS Calibur flow cytometry (FACS Calibur; BD Biosciences) was used for the determination of different phases of cell cycle of NCI-H460 human lung carcinoma cells.

*Cell invasion assay*

Cell invasion ability of NCI-H460 human lung carcinoma cells after treatment with Norartocarpetin was done in transwell chambers with Matrigel. About 200 ml of cell culture were placed on the bottom chambers and only DMEM was placed in the upper chambers. Before the cells were fixed with methyl alcohol, they were incubated for 1 day and later were stained with crystal violet. For analysis inverted microscope was used to count the number of invaded cells at 200x magnification.

*Wound healing assay*

NCI-H460 human LC cells were treated with varying doses of Norartocarpetin (0, 11, 22, and 44 µM). Afterwards, DMEM was were completely discarded and NCI-H460 cells were washed with PBS. Sterile pipette tip was used to scratch a wound in each well and the cells were washed again with PBS. A picture was then captured before and after culturing of the cells for 24 h using an inverted microscope (Leica, Germany).

*Western blot analysis*

Treated NCI-H460 human LC cells were lysed with RIPA lysis buffer containing the protease inhibitor. About 40 µg of proteins from each sample were separated through electrophoresis via SDS-PAGE gels. Separation was followed by transference to PVDF (polyvinylidene difluoride) membrane. After transference, blocking of the membranes was performed at room temperature for 1 h using fat-free milk. Next, primary antibody treatment of NCI-H460 LC cells was done overnight at 4°C. Secondary antibody treatment was given to the cells followed by 24-h incubation. Finally, normalization was done by Actin and Odyssey Infrared Imaging System was used for band signal detection.

*Statistics*

Data from three or more independent experiments was presented as mean±standard deviation (SD). Multiple assessments for substantial differences among multiple groups were made using one-way analysis of variance (ANOVA), followed by individual comparisons with Scheffe's *post hoc* test. Statistical significance was set at $p<0.05$.

## Results

*Anti-proliferative effect of Norartocarpetin on NCI-H460 human lung carcinoma cells*

NCI-H460 human LC cells were treated with increasing concentrations of Norartocarpetin (Figure 1), ranging from 0–100 µM, for 48 h. MTS assay was used to study the cytotoxic effect of Norartocarpetin on NCI-H460 LC cells (Figure 2A). Norartocarpetin repressed the viability of NCI-H460 cells in a dose-dependent manner. Interestingly, compared to cancer cells, Norartocarpetin showed much lower or negligible effect on normal lung cells (Figure 2B).

*Norartocarpetin resulted in apoptotic cell death of NCI-H460 human lung carcinoma cells*

We next explored whether the antiproliferative effect of Norartocarpetin in NCI-H460 human LC cells was mediated via induction of apoptosis. For that we performed AO/EB staining and comet assay, using fluorescence microscopy. AO/EB staining showed that untreated cells had circular and intact nuclei, uniformly distributed at the centre of the cell. The NCI-H460-treated cells revealed that the early apoptotic cells were stained with yellow-green nucleus by AO staining and concen-

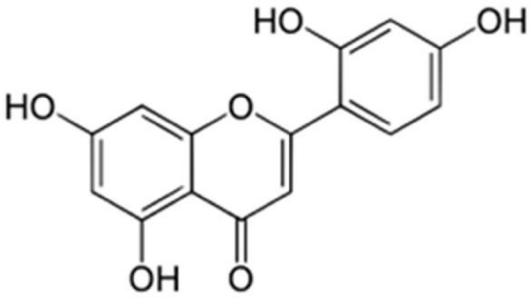


**Figure 1.** Chemical structure of norartocarpetin.

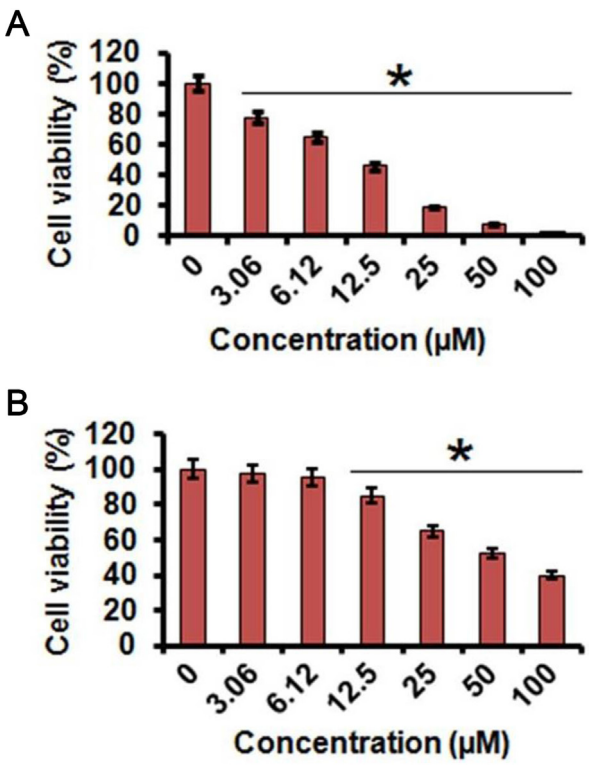


**Figure 2.** CCK-8 assay showing the effect of norartocarpetin on the viability of the human lung carcinoma cells (NCI-H460) **(A)** and normal lung cell line (MRC-9) **(B)**. The experiments were performed in triplicate and shown as mean ± SD (*$p<0.05$).

trated towards one side of the cell as granule or crescent, while late apoptotic cells were stained with orange nucleus by EB staining, the nuclei of these cells showed orange and yellow fluorescence by EB staining, indicating onset of apoptosis (Figure 3). Furthermore, the apoptosis of NCI-H460 LC cells by Norartocarpetin was examined by comet assay. Cultured NCI-H460 cells were examined after exposure with the test molecule at different concentrations, i.e. 0, 11, 22, and 44 μM, which revealed that the cells with fragmented nucleus and condensed chromatin, indicated nuclear damage, and thus DNA damage (Figure 4). Thus it was concluded that Norartocarpetin resulted in apoptotic cell death of NCI-H460 LC cells in a dose-dependent manner. In addition, western blot analysis was performed to check whether the antiproliferative effect of Norartocarpetin on NCI-H460 LC cells was due to apoptosis induction. What was revealed was that the expression of apoptosis-associated proteins changed signifi-

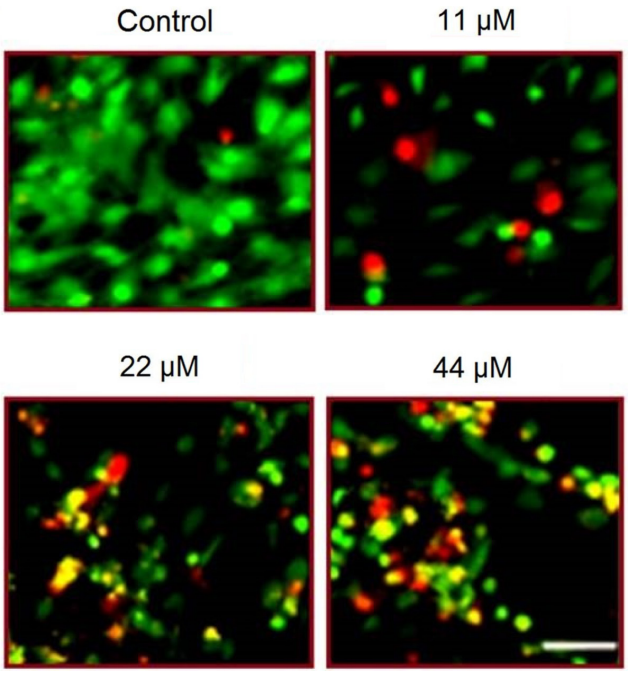


**Figure 3.** Induction of apoptosis in the human lung carcinoma cells (NCI-H460) by norartocarpetin used at varying doses as evidenced from the AO/EB staining. Orange and yellow colors at increased doses of the molecule indicate apoptotic cells. The experiments were performed in triplicate.

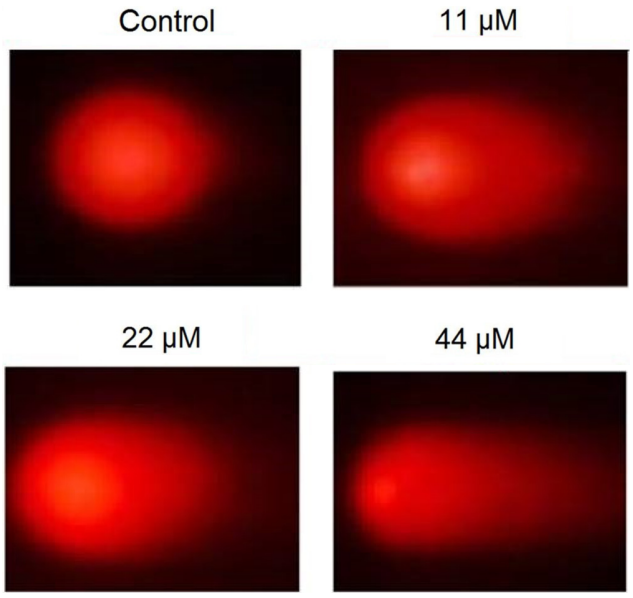


**Figure 4.** Comet assay using fluorescence microscopy showing the effect of increasing doses of norartocarpetin on the DNA damage in human lung carcinoma cells. The experiments were performed in triplicate.

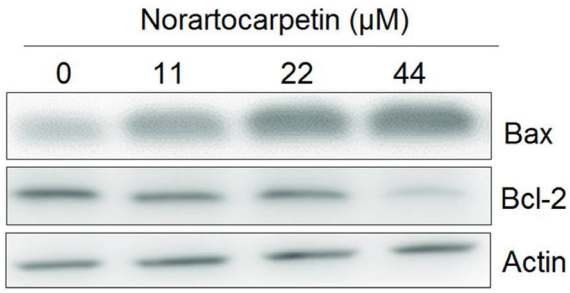


**Figure 5.** Effect of norartocarpetin on the expression of apoptosis associated proteins (Bcl-2 and Bax) as depicted by western blot analysis. The experiments were performed in triplicate.

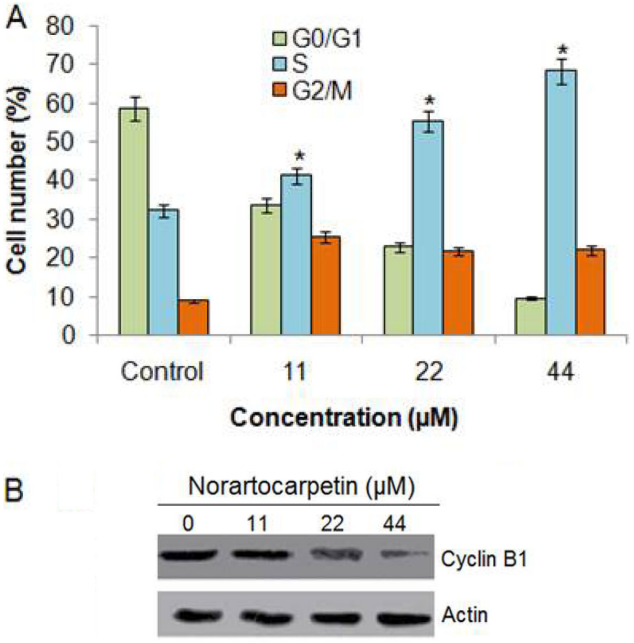


**Figure 6.** Effect of norartocarpetin on the cell cycle phase distribution of human lung carcinoma cells. **A:** showing that norartocarpetin led to S-phase cell cycle arrest. **B:** showing that norartocarpetin led to dose-dependent reduction in the expression of cyclin B-1. *p<0.05.

cantly, like increase in Bax and decrease in Bcl-2 expression in a dose-time dependent manner (Figure 5).

*Norartocarpetin resulted in S-phase cell cycle arrest of NCI-H460 human lung carcinoma cells*

The NCI-H460 LC cells were treated with different concentrations of Norartocarpetin and flow cytometry examined the distribution of NCI-H460 cells at each phase of the cell cycle. Remarkably, Norartocarpetin showed increase of S-phase cells. The percentage of S-phase NCI-H460 LC cells was 30%, 40%, 55% and 70% at 0, 11, 22 and 44 μM concentrations of Norartocarpetin, respectively. Simultaneously, the impact on G0/G1 and G2/M phases showed constant decline in cell number. Therefore, the above results are indicative of S-phase arrest of the NCI-H460 LC cells (Figure 6A). To assess the effects on S-phase cell cycle related proteins by the tested molecule, western blot analysis was performed which showed that the molecule was responsible for significant downregulation of the expression of cyclin B1 (Figure 6B).

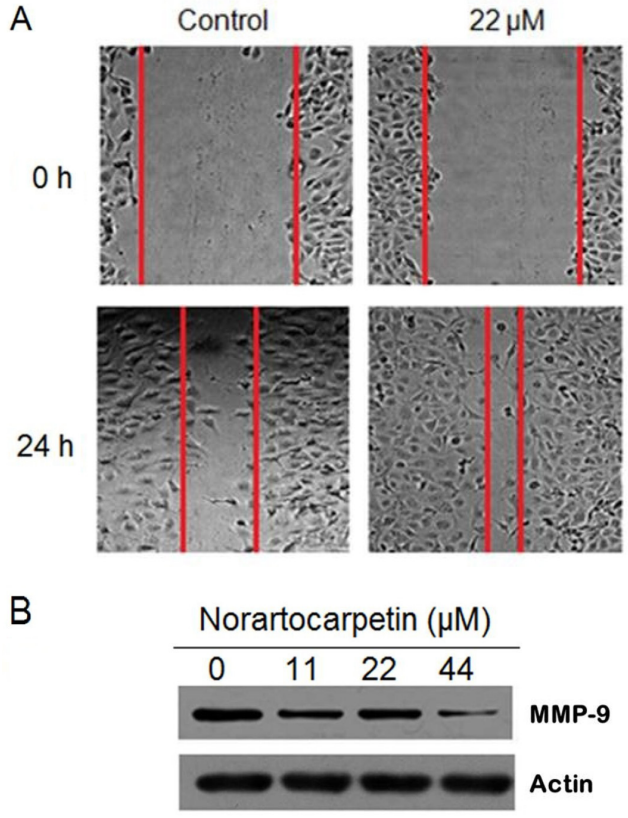


**Figure 7. A:** *In vitro* wound healing assay showing the effect of norartocarpetin on the cell migration in NCI-H460 human lung carcinoma cells; the molecule led to significant inhibition of cell migration. **B:** Effects of norartocarpetin on the expression of MMP-9, a key protein which has a significant role in cell migration.

*Norartocarpetin mediated inhibition on cell migration and invasion of NCI-H460 human lung carcinoma cells*

Next, we performed wound healing assay and transwell assay respectively to study the impact of Norartocarpetin on cell invasion and cell migra-

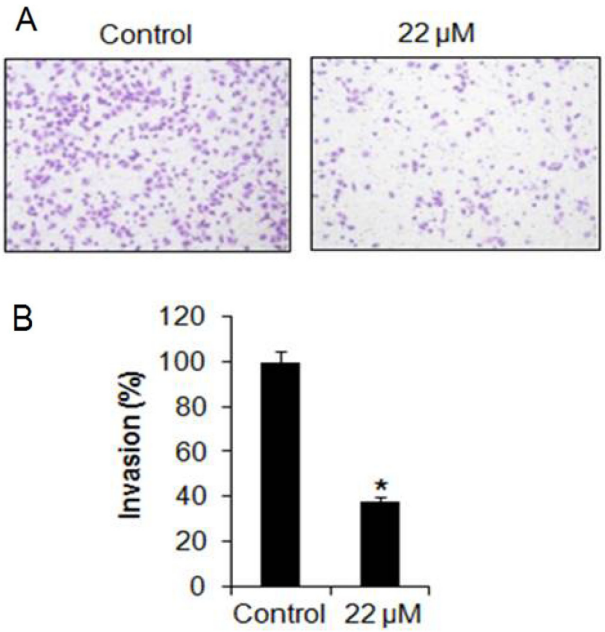


**Figure 8. A:** Matrigel assay showing the effect of norartocarpetin on the cell invasion in NCI-H460 human lung carcinoma cells; the molecule led to dose-dependent suppression of cell invasion. **B:** Graphical representation of invasion inhibition by norartocarpetin. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

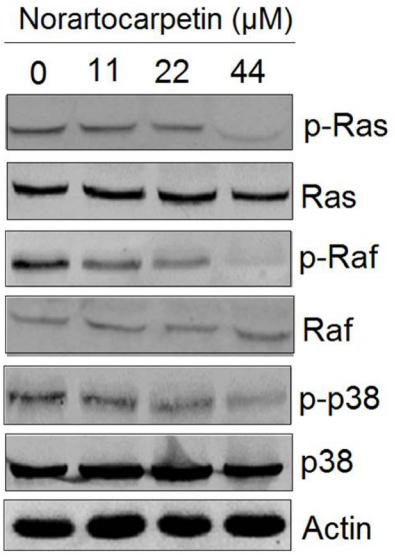


**Figure 9.** Effect of norartocarpetin on the Ras/Raf/MAPK signalling pathway as evaluated by western blot assay. The expression of p-RAS, p-RAF and p-P38 was significantly reduced, in contrast to the expressions of RAS, RAF and P38 which remained unchanged.

tion on NCI-H460 LC cells. Wound healing assay showed that the molecule decreased remarkably the cell invasion in NCI-H460 LC cells in a concentration-dependent manner (Figure 7A). Western blotting analysis also revealed that the expression of protein associated with cell migration (MMP-9) ability was retarded (Figure 7B). Furthermore, after exposure to varying doses of Norartocarpetin, there was a significant reduction (from 100% to about 40%) in the invasion of NCI-H460 human LC cells (Figure 8).

*Norartocarpetin targeting Ras/Raf/MAPK signalling pathway*

Western blotting was utilized to explore the effects of Norartocarpetin on Ras/Raf/MAPK signalling pathway in NCI-H460 LC cells. This pathway is important because it is involved in cell growth, cell division and cell differentiation. The results showed that the expression of proteins associated with this pathway were significantly hampered by the exposure of NCI-H460 LC cells to Norartocarpetin in a dose-dependent manner. The expression of p-RAS, p-RAF and p-P38 was significantly reduced, in contrast to the expressions of RAS, RAF and P38 (Figure 9), clearly indicating the fact that Norartocarpetin led to blockade of Ras/Raf/MAPK signalling pathway.

## Discussion

Besides recent improvements made in cancer treatment, international research level of LC falls substantially behind the publication outputs for other disease types (recent bibliometric investigation). Despite massive economic costs, poor early diagnosis and high death rates, least interest was shown in LC treatment till 2013 [18]. Novel drugs for cancer therapy are immediately required, particularly for LC, due to the inadequate efficiency from the currently available therapies, and the 5-year survival rates of LC patients are low owing to more relapsing chances [19]. Pharmacologically active compounds with hopes of better efficiency and lower toxicity from natural products have huge potential to be established as anticancer therapeutic agents [20]. We have demonstrated herein for the first time that Norartocarpetin, an isoflavones and plant-derived compound, has a promising ability to induce antiproliferative effects in human LC cells and these effects are mediated via targeting Ras/Raf/MAPK signalling pathway, mitochondrial mediated apoptosis, S-phase cell cycle arrest and suppression of cell migration and invasion. The antiproliferative property of Norartocarpetin on NCI-H460 LC cells and MRC-9 normal fibroblast lung cells was studied through MTS assay. MTS assay revealed that the target molecule retarded the proliferation of NCI-H460 cell line in a dose-dependent manner without halting MRC-9 cell proliferation, indicating selectivity. After cell proliferation assay, cells were prepared for apoptosis detection which was performed through AO/EB staining and comet assay using fluorescence microscopy. What was observed was that the untreated cells had intact and well established nucleus, however the impact of the tested molecule on the cells showed different results like as there were three types of cells observed: early apoptotic cells, with nucleus located to one side of the cell, late apoptotic cells, with nucleus totally assembled and necrotic cells, with unclear outline, near disintegration or dissolution of the nucleus. Apoptotic induction of the test molecule was further confirmed by western blotting assay which showed downregulation of Bcl-2 and upregulation of Bax. Furthermore, to predict the effect on the cycle of NCI-H460 cancer cells by Norartocarpetin, flow cytometry was used which revealed that the target molecule caused a significant increase in the number of S-phase cells and hence S-phase cell cycle arrest, also confirmed by western blotting that showed quite high inhibition of cyclin-B1. In addition, cell migration and invasion analyses were performed by wound healing and transwell assays, which showed dose-dependent inhibition of cell migration and invasion. Western blotting analysis revealed that the molecule significantly reduced the expression of p-RAS, p-RAF and p-P38, in contrast to the expressions of RAS, RAF and P38, thus clearly indicating blocking of Ras/Raf/MAPK signalling pathway. Hence, the above results make it clear that the tested molecule may act as a potential anticancer agent for lung cancer.

## Conclusion

The tested molecule showed significant antiproliferative effects in NCI-H460 human lung carcinoma cells. These antiproliferative effects were mediated via blocking of Ras/Raf/MAPK signalling pathway, mitochondrial mediated apoptosis, S-phase cell cycle arrest and suppression of cell migration and invasion. Thus, Norartocarpetin may be considered as novel therapeutic agent for lung cancer treatment.

## Conflict of interests

The authors declare no conflict of interests.

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