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ORIGINAL ARTICLE

Anticancer Activity of Chloroform Extract and Sub-fractions of *Nepeta deflersiana* on Human Breast and Lung Cancer Cells: An *In vitro* Cytotoxicity Assessment

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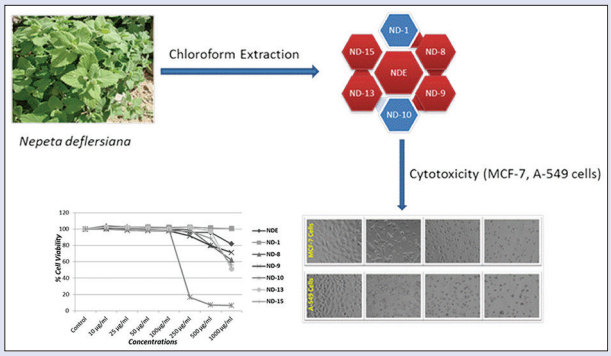
ABSTRACT

Background: Cancer is one of the major causes of death worldwide. The plant-derived natural products have received considerable attention in recent years due to their diverse pharmacological properties including anticancer effects. *Nepeta deflersiana* (ND) is used in the folk medicine as antiseptic, carminative, antimicrobial, antioxidant, and for treating rheumatic disorders. However, the anticancer activity of ND chloroform extract has not been explored so far. **Objectives:** The present study was aimed to investigate the anticancer activities of chloroform *Nepeta deflersiana* extract and various sub-fractions (ND-1–ND-15) of ND against human breast cancer cells (MCF-7) and human lung cancer cells (A-549). **Materials and Methods:** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and neutral red uptake assays, and cellular morphological alterations using phase contrast light microscope were studied. Cells were exposed with 10–1000 μg/ml of sub-fractions of ND for 24 h. **Results:** Results showed that selected sub-fractions of the chloroform extract significantly reduced the cell viability of MCF-7 and A-549 cells, and altered the cellular morphology in a concentration-dependent manner. Among the sub-fractions, ND-10 fraction showed relatively higher cytotoxicity compared to other fractions whereas, ND-1 did not cause any cytotoxicity even at higher concentrations. The A-549 cells were found to be more sensitive to growth inhibition by all the extracts as compared to the MCF-7 cells. **Conclusion:** The present study provides preliminary screening of anticancer activities of chloroform extract and sub-fractions of ND, which can be further used for the development of a potential therapeutic anticancer agent.

Key words: A-549 cells, anticancer, cytotoxicity, MCF-7 cells, *Nepeta deflersiana*

SUMMARY

- Nepeta deflersiana extract exhibit cytotoxicity and altered the cellular morphology. Sub-fractions of the chloroform extract of Nepeta deflersianareduced the cell viability of MCF-7 and A-549 cells. Among the sub-fractions, ND-10 fraction showed relatively higher cytotoxicity.The A-549 cells were found to be more sensitive as compared to the MCF-7 cells.



Abbreviations used: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NRU: Neutral red uptake; DMEM: Dulbecco's modified eagle medium; FBS: Fetal bovine serum; PBS: Phosphate buffer saline; DMSO: Dimethyl sulfoxide

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INTRODUCTION

Cancer is a life-threatening disease, accounted for 8.2 million deaths, in 2012.[1,2] According to World Health Organization, the annual cancer cases will rise from 14 million in 2012 to 22 million within next two decades.[2] It is second most occurring disease after cardiovascular disease and causes a great burden to both single human lives and the society as a whole.[3] Although there has been good progress in the development of prevention and treatment of cancer, the successful treatment of cancer still remains a challenge. Cancer cells often adapt to develop resistance to commonly used chemotherapeutic agents. Therefore, it is important to develop novel chemotherapeutic agents, which are more potent tumor-selective cytotoxic agents and can circumvent drug-resistant cancer cells. Natural products have for long played an important role in drug discovery, especially in the area of cancer pharmacology. Many natural or natural based anti-tumor drugs such as bleomycin, doxorubicin, mitomycin, vinblastine, vincristine, etoposide (VP16), topotecan, irinotecan, paclitaxel, and combretastatins have been clinically used in recent years.[4] *Nepeta* (family: Lamiaceae) is a large genus that is composed of about 250–300 annual and perennial species.[5] In the Kingdom of Saudi Arabia, there are two species of the genus *Nepeta*. *Nepeta deflersiana* (ND) is found in Saudi Arabia and Yemen, whereas *Nepeta sheilae* is found only in the northern Hijaz mountains of Saudi Arabia.[6]

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ND is a woody aromatic perennial herb with many erect, tall stems from the base that can be 50–80 cm.[7] Traditionally, it is used in the Yemeni folk medicine as an antiseptic agent in the treatment of wounds, carminative, as well as in the treatment of rheumatic disorders.[8] ND was found to be used as a tranquillizer and leaf decoction drunk with tea to relieve stomach problems and burns.[9-11] In general, the essential oils extracted from various *Nepeta* species have been a vital source of terpenoid and oxygenated terpenoid hydrocarbons. These compounds have been known for their biological activities such as sedative, diaphoretic, stomach tonic, antispasmodic, antipyretic, anti-viral, febrifuge, expectorant, diuretic, anti-inflammatory, antimicrobial, fungicidal, insecticidal, insect repellent, and antidote against snakes and scorpion bites.[12]

Despite the widespread traditional uses of ND, there are very few reports in the literature available regarding the therapeutic potential. Previous studies on Yemeni ND plant showed that the methanolic and aqueous extract exhibited anticancer, antimicrobial, and antioxidant activities,[13] while the essential oil showed strong antimicrobial activity against Gram-positive bacteria and moderate antioxidant activity.[14] However, the anticancer potential of ND chloroform extract has not yet been explored, and no report was found during our survey of current literature. Thus, the present investigations were carried out to study the anticancer activities of various sub-fractions of chloroform extracts of ND against human breast cancer cells (MCF-7) and human lung cancer cells (A-549).

MATERIALS AND METHODS

Chemicals and consumables

Dulbecco's modified eagle medium (DMEM) culture medium antibiotics-antimycotic solution, fetal bovine serum (FBS), and trypsin were purchased from Invitrogen, Life Sciences, USA. Consumables and culture wares used in the study were procured from Nunc, Denmark, EU. Chloroform and all other specified reagents and solvents were purchased from Sigma Chemical Company Pvt. Ltd. St. Louis, Missouri, USA.

Plant material

ND (Lamiaceae) plants were collected from Shaza mountains, Saudi Arabia [Figure 1]. Identification of the plant was done by Prof. Dr. Jakob Thomas, College of Science, King Saud University, Riyadh 11451, Saudi Arabia. A voucher specimen (#15797) was deposited in the herbarium of Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh 11495, Saudi Arabia.

Figure 1: *Nepeta deflersiana* plant growing in natural habitat

Extraction procedure

The fresh plant of ND (500 g) was washed with fresh water, air dried, cut with a mill grinder into fine pieces, and macerated with hexane (5 L) for 3 days at room temperature (25–27°C). After defatting, the residue was then macerated with chloroform (5 L) for 3 days. The process was repeated thrice. The extract was filtered by gravity, and the solvent was evaporated under reduced pressure using a rotary evaporator [Figure 2].

Fractionation of active plant extract

The chloroform extract obtained above was tested and found cytotoxic and thus warranted further investigation. The chloroform extract (approximately 2 g) was chromatographed on silica gel 60, 0.040–0.06 mm (Merck, Darmstadt, Germany, EU) (200 g). Gradient elution was carried out using various combinations (v\v) of hexane, chloroform, and methanol. The initial ratio of hexane: $CHCl_3$:MeOH, 9:1:0 (v\v\v) was followed by 4:1:0, 3:2:0, 2:3:0, 1:4:0, 0:1:0, 0:95:5, 0:9:1, 0:4:1, 0:3:2, and 0:2:3 (v\v\v). The volume of the solvent combination used in each gradient step was 500 ml. Eluents were collected in portions of 50 ml. Finally, the column was flushed with methanol. A small sample of each eluent was evaluated using thin layer chromatography and those eluents which showed similar chemical composition were combined and concentrated under vacuum to yield a total of 6 chloroform sub-fractions designated as ND-1, ND-8, ND-9, ND-10, ND-13, and ND-15 [Figure 2].

Cell culture

Human breast cancer cells (MCF-7) and human lung cancer cells (A-549) obtained from American Type Culture Collection (Manassas, Virginia, USA) were cultured in DMEM, supplemented with 10% FBS, 0.2% sodium bicarbonate and antibiotic antimycotic solution (×100, 1 ml/100 ml of medium). Cells were grown in 5% CO_2 at 37°C in the high humid atmosphere. Before the experiments, the viability of cells was assessed following the protocol.[15] MCF-7 and A-549 cells showing more than 95% cell viability and passage number between 12 and 14 were used in the present study.

Experimental design

MCF-7 and A-549 cells were exposed to various concentrations of chloroform *Nepeta deflersiana deflersiana* extract (NDE) and subfractions (ND-1, ND-8, ND-9, ND-10, ND-13, and ND-15) of ND (10–1000 µg/ml) for a period of 24 h. Following the exposures of NDE, ND-1, ND-8, ND-9,

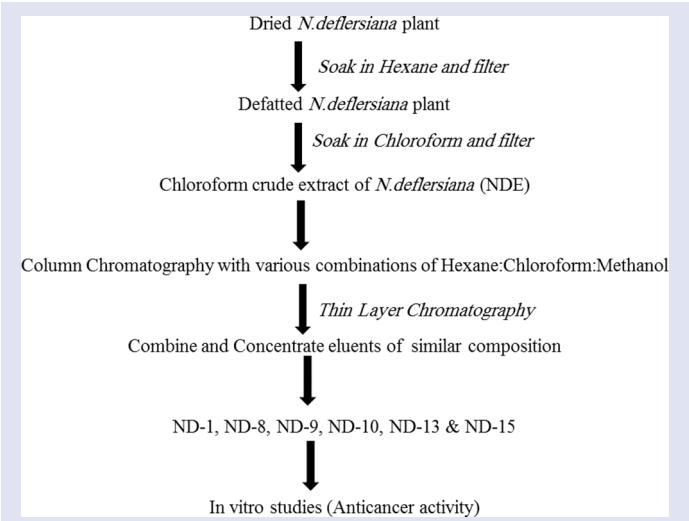

Figure 2: Flow diagram for preparation of chloroform extract and sub-fractions of *Nepeta deflersiana*

ND-10, ND-13, and ND-15, cells were subjected to assessment of cytotoxic responses using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and neutral red uptake (NRU) assays. Further, cellular morphological alterations induced by NDE, ND-1, ND-8, ND-9, ND-10, ND-13, and ND-15 were also studied.

Drug solutions

The extracts of ND were not completely soluble in aqueous medium solution; therefore, the stock solutions of all the extracts were prepared in dimethyl sulfoxide (DMSO) and diluted in culture medium to reach the desired concentrations. The concentration of DMSO in culture medium was not more that 0.1%, and this medium was used as a control.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Percentage cell viability was assessed using the MTT assay as described.[15] Briefly, cells (1×10^4) were allowed to adhere for 24 h in a CO_2 incubator at 37°C in 96 well culture plates. After the respective exposure, MTT (5 mg/ml of stock in phosphate-buffered saline [PBS]) was added (10 µl/well in 100 µl of cell suspension), and plates were incubated for 4 h. Then, supernatants were discarded, and 200 µl of DMSO was added to each well and mixed gently. The developed color was read at 550 nm using Multiwell Microplate Reader (Thermo Scientific, USA). Untreated sets were also run under identical conditions and served as control.

Neutral red uptake assay

NRU assay was carried out following the protocol as described.[16] Briefly, after the respective exposure, the medium was aspirated, and cells were washed twice with 1 mm phosphate buffer saline (PBS; pH = 7.4), and incubated for 3 h in a medium supplemented with neutral red (50 µg/ml). The medium was washed off rapidly with a solution containing 0.5% formaldehyde and 1% calcium chloride. Cells were subjected to further incubation for 20 min at 37°C in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. The plates were read at 540 nm using Multiwell Microplate Reader (Thermo Scientific, USA). The values were compared with the control sets run under identical conditions.

Morphological analysis

Morphological changes in MCF-7 and A-549 cells exposed to increasing concentrations (10–1000 µg/ml) of NDE, ND-1, ND-8, ND-9, ND-10, ND-13, and ND-15 were taken using an inverted phase contrast microscope (OLYMPUS CKX 41) at ×20.

Statistical analysis

The results are expressed as mean and standard error of means. One-way ANOVA was employed to detect differences between the groups of treated and control. The values showing $P < 0.05$ were considered as statistically significant.

RESULTS

In the present study, ND chloroform extract and sub-fractions were prepared and analyzed for their cytotoxic potential against human breast cancer (MCF-7) and human lung cancer (A-549) cell lines.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Cytotoxic effects of NDE, ND-1, ND-8, ND-9, ND-10, ND-13, and ND-15 extracts assessed by MTT assay in MCF-7 and A-549 cells exposed to various concentrations (10–1000 µg/ml) are summarized in Figures 3 and 4. Our MTT results showed that the extracts of ND-8, ND-9, ND-13, and ND-15 at 10, 25, 50, 100, and 250 µg/ml concentrations did not cause any significant decrease in the cell viability of MCF-7 and A-549 cells. Whereas, cell viability at 500 and 1000 µg/ml concentrations were recorded as 80% and 62% in ND-8, 79% and 71% in ND-9, 7% and 6% in ND-10, 97% and 51% in ND-13, 88% and 55% in ND-15 extract exposed to MCF-7 cells [Figure 3]. The cell viability in A-549 cell were recorded at 500 and 1000 µg/ml concentrations as 88% and 63% in ND-8, 64% and 47% in ND-9, 7% and 6% in ND-10, 89% and 51% in ND-13, 83% and 49% in ND-15 [Figure 4].

Neutral red uptake assay

The cytotoxic effects of NDE, ND-1, ND-8, ND-9, ND-10, ND-13, and ND-15 extracts assessed by NRU assay in MCF-7 and A-549 cells exposed to various concentrations (10–1000 µg/ml) are summarized in [Figures 5 and 6]. Similar kind of results by NRU assay was also found in all the cells exposed to various concentrations of extracts. The results showed that the ND-8, ND-9, ND-13, and ND-15 reduced the viability of MCF-7 cells and A-549 cells at 500 and 1000 µg/ml concentrations. The cell viability at 500 and 1000 µg/ml were recorded as 81% and 63% in ND-8, 81% and 63% in ND-9, 7% and 7% in ND-10, 96% and 52% in ND-13, and 88% and 55% in ND-15 extract, respectively [Figure 4].

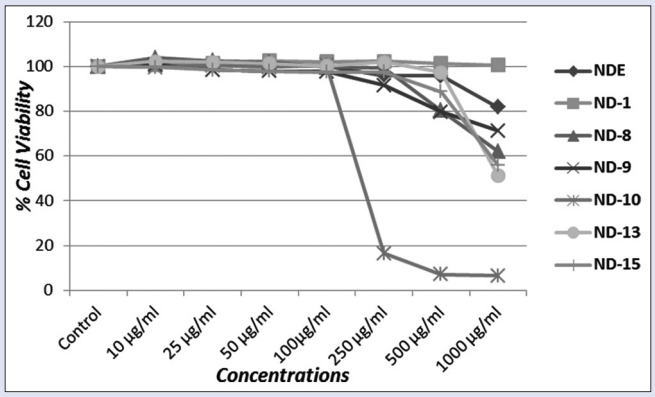

Figure 3: Cytotoxicity assessments of different fractions in MCF-7 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The cells were treated with various concentrations (10–1000 µg/ml) for 24 h. Values are mean ± standard error of three independent experiments

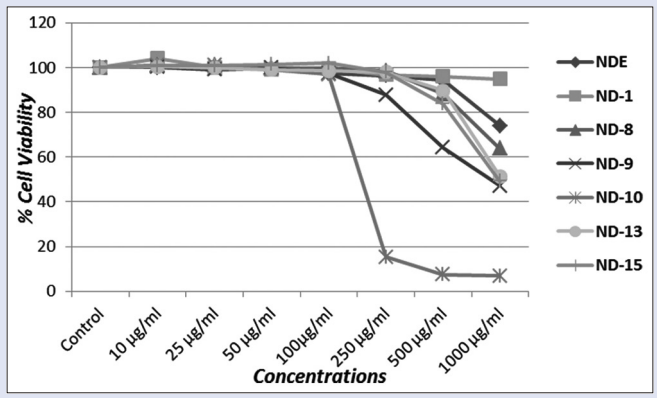

Figure 4: Cytotoxicity assessments of different fractions in A-549 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The cells were treated with various concentrations (10–1000 µg/ml) for 24 h. Values are mean ± standard error of three independent experiments

However, the decrease in cell viability of A-549 cells at 500 and 1000 μg/ml were recorded as 89%and 62% in ND-8, 65% and 48% in ND-9, 7% and 6% in ND-10, 87% and 50% in ND-13, and 84% and 48% in ND-15, respectively [Figure 5]. The ND-1 extract did not cause any cytotoxicity in both of cells exposed for 24 h. However, the ND-10 extract was found more toxic toward both the cells.

Morphological changes

The morphological changes in MCF-7 and A-549 cells exposed to various concentrations of NDE, ND-8, ND-9, ND-10, ND-13, and ND-15extracts are shown in Figures 7-12. Alterations in the morphology of MCF-7 and A-549 cells were observed under phase contrast inverted microscope. Results showed that MCF-7 and A-549 cells exposed to 500 and 1000 μg/ml concentrations of all extracts for 24 h reduced the normal morphology of the cells and cell adhesion capacity in compared to the control. Most of the cells at 1000 μg/ml of NDE lost their typical morphology and appeared smaller in size, shrunken, and rounded. However, at 250 μg/ml and lower concentrations of extracts did not cause any effects on the morphology of the cells [Figures 7-12].

DISCUSSION

The belief that natural medicines are much safer than synthetic drugs has caused exceptional growth in human exposure to natural products, as plants, phytotherapeutic agents, and phytopharmaceutical products. This fact has led to a resurgence of scientific interest in their biological effects. The second-hand metabolites produced in medicinal plants under biotic and abiotic conditions have many applications in the treatment of various human diseases. Recently reports have cited that plants and their components could act as tumor suppressor and apoptotic inducers in cancerous cells.[17-20] The medicinal paramount of genus *Nepeta* is attributed to the presence of essential oils and flavonoids,[21] which have potential roles in the treatment of human diseases. Some *Nepeta* species encompass cyclopentanoid iridoid derivatives such as monoterpenes, diterpenes, sesquiterpenes, and nepetalactones.[22-25] *Nepeta grandiflora* and *Nepeta clarkei* produce essential oils that contain (S)-germacrene D compound and other compounds.[26] The chemical compounds are present in the flowering tips of *Nepeta sp.* and aerial parts of *Nepeta persica* have sedative properties.[27] Several species of *Nepeta* are extensively used in folk medicine as an antispasmodic, antiseptic, antitussive, antiasthmatic, anti-inflammatory, diuretic, and expectorant.[5,28] Some species of *Nepeta* have antibacterial, antioxidant, antiviral, anticandidal, angiotensin-converting enzyme inhibitory activity, and fungicidal activities.[29-31] The essential oil of ND possesses antimicrobial and antioxidant activities because of the presence of monoterpenes.[14] Despite the widespread traditional use of ND, there is no report on the cytotoxicity of chloroform extract and its subfractions. Therefore,

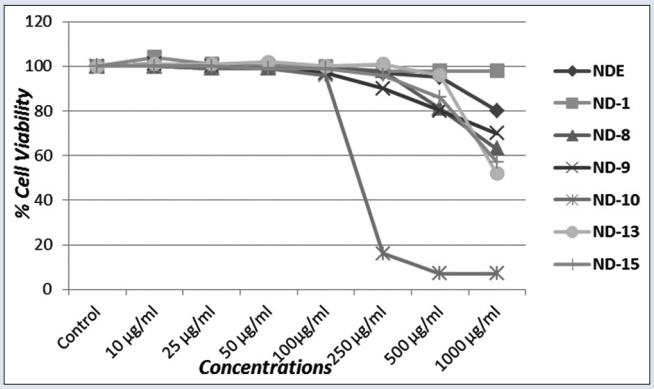

Figure 5: Cytotoxicity assessments of different fractions in MCF-7 cells by neutral red uptake assay. The cells were treated with various concentrations (10–1000 μg/ml) for 24 h. Values are mean ± standard error of three independent experiments

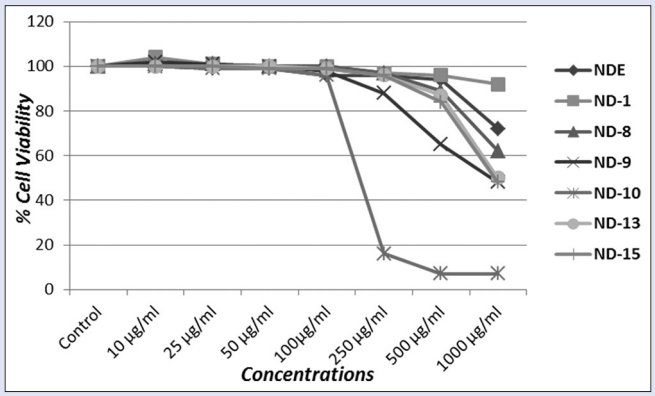

Figure 6: Cytotoxicity assessments of different fractions in A-549 cells by neutral red uptake assay. The cells were treated with various concentrations (10–1000 μg/ml) for 24 h. Values are mean ± standard error of three independent experiments

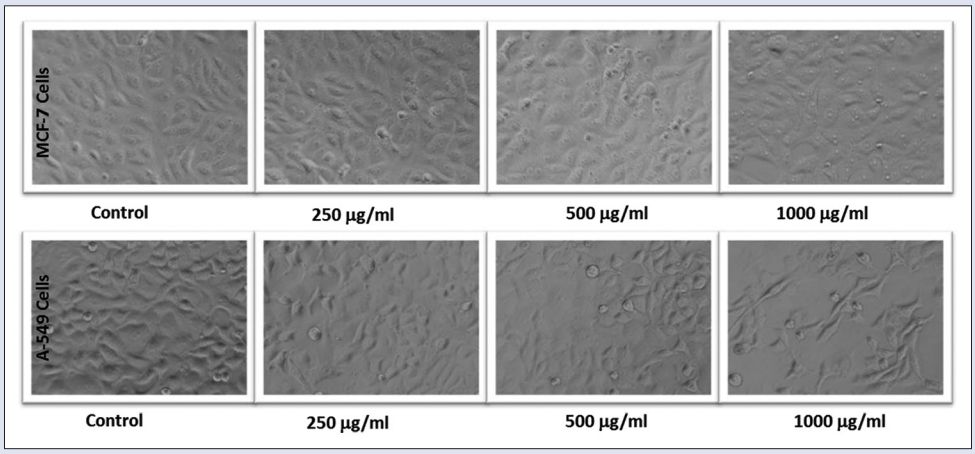

Figure 7: Morphological changes in MCF-7 and A-549 cells exposed to various concentrations of *Nepeta deflersiana deflersiana* extract for 24 h. Images were taken using an inverted phase contrast microscope at ×20

the present investigations were initiated to screen the cytotoxicity induced by ND in human breast cancer (MCF-7) and human lung cancer (A-549) cell lines. Our results show that ND chloroform extract and its subfractions induced cytotoxicity in MCF-7 and A-549 cell lines. It was found that the tested extracts significantly reduced cell viability in a concentration dependent manner. Among the sub-fractions the ND-10 fraction showed relatively higher cytotoxicity as compared to other fractions, whereas, ND-1 did not cause any cytotoxicity even at higher concentrations. The concentration of 500 µg/ml and above of ND was found to be cytotoxic whereas the concentration of 250 µg/ml and lower did not show any significant cell death in MCF-7 and A-549 cells. The *in vitro* concentrations used in this study were selected from the

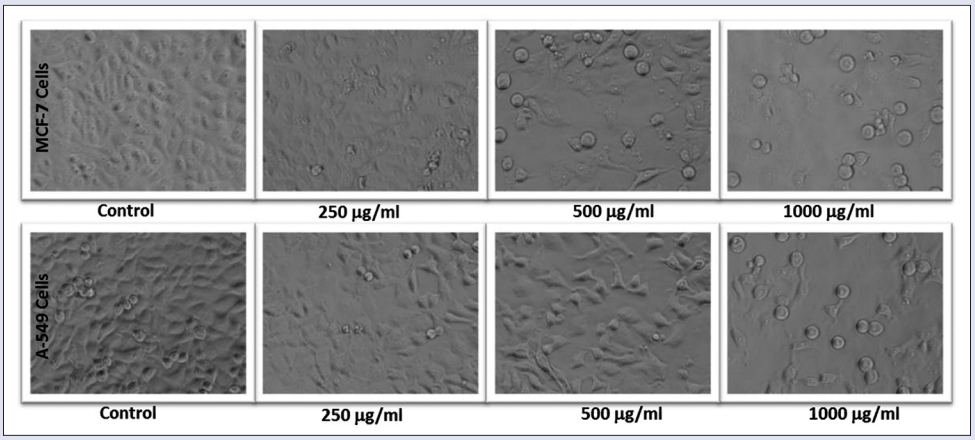

Figure 8: Morphological changes in MCF-7 and A-549 cells exposed to various concentrations of *Nepeta deflersiana*-8 for 24 h. Images were taken using an inverted phase contrast microscope at ×20

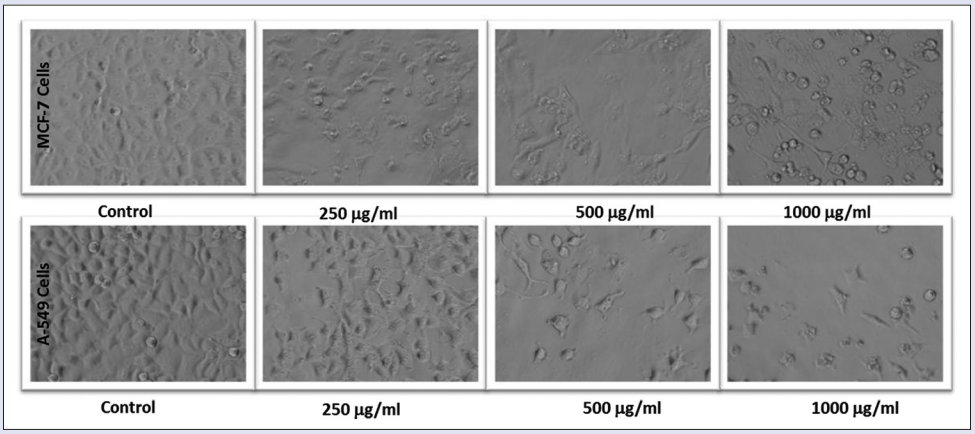

Figure 9: Morphological changes in MCF-7 and A-549 cells exposed to various concentrations of *Nepeta deflersiana*-9 for 24 h. Images were taken using an inverted phase contrast microscope at ×20

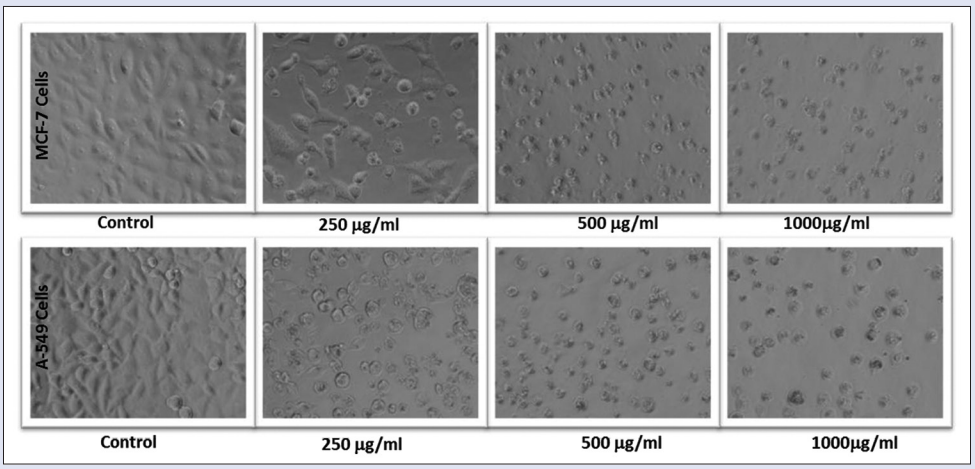

Figure 10: Morphological changes in MCF-7 and A-549 cells exposed to various concentrations of *Nepeta deflersiana*-10 for 24 h. Images were taken using an inverted phase contrast microscope at ×20

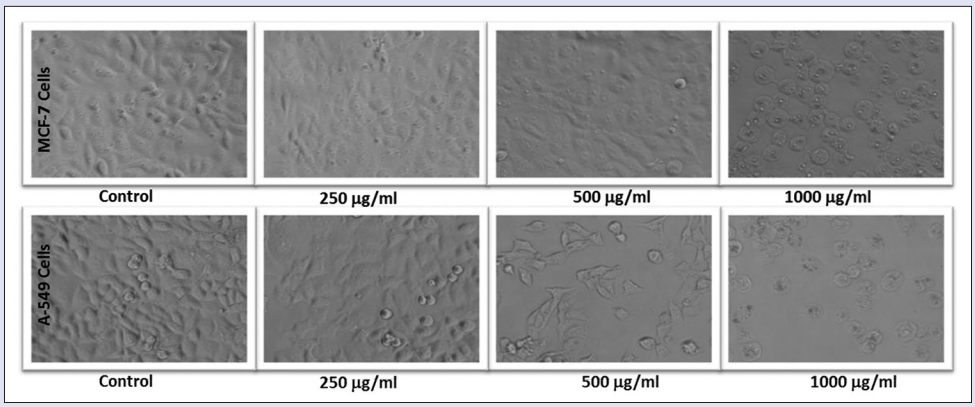

Figure 11: Morphological changes in MCF-7 and A-549 cells exposed to various concentrations of *Nepeta deflersiana*-13 for 24 h. Images were taken using an inverted phase contrast microscope at ×20

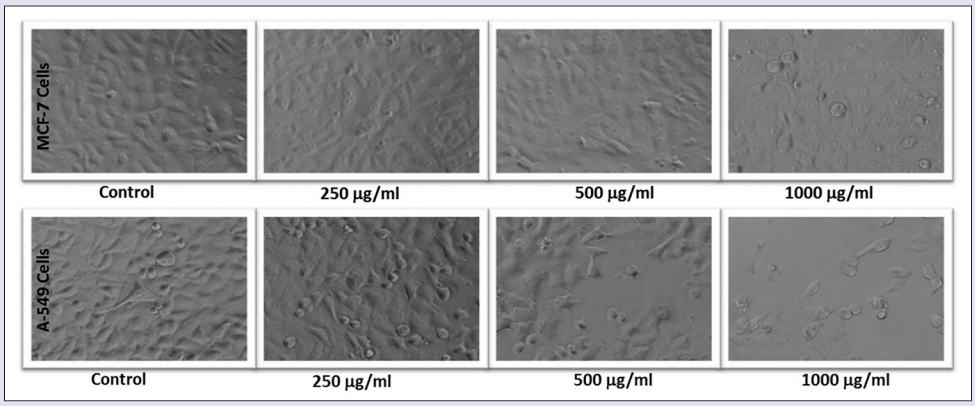

Figure 12: Morphological changes in MCF-7 and A-549 cells exposed to various concentrations of *Nepeta deflersiana*-15 for 24 h. Images were taken using an inverted phase contrast microscope at ×20

previous studies showing the toxicity in this range. The exposure of various natural products has also been investigated in this concentration range by various investigators in different *in vitro* models.[32-34] There are very few reports in the literature regarding the cytotoxicity of *Nepeta* species. In one of the studies, the methanolic leaf extract of ND was found cytotoxic with IC50 >50.[13] Cytotoxicity of *Nepeta cataria* and *N. cataria* var. citriodora essential oils were studied against human keratinocyte and bronchial epithelial cell lines by the MTT assay. The oils were cytotoxic to both keratinocytes and bronchial epithelial cells at IC50 values from 0.0012 to 0.015% (v/v).[35] Different extracts of *Nepeta juncea* were assayed for their cytotoxicity by brine shrimp bioassay. The H_2O fraction exhibited lower LD50 values (88.1253 mg/ml) as compared to the other fractions.[36] In another study, the essential oil of *Nepeta glomerata* was evaluated for its *in vitro* cytotoxic properties on two human cancer cell lines (C32: Amelanotic melanoma and ACHN: Renal cell adenocarcinoma) using the MTT test. The assay showed that the oil was more active against renal adenocarcinoma (ACHN) cell line (48% of inhibition of proliferation at 100 mg/ml) in comparison to amelanotic melanoma (C32; 28% of inhibition of proliferation at 100 mg/ml).[37] Our results are in agreement with these findings, where higher concentrations of *Nepeta sp*. were found to be more cytotoxic. Among the tested cell lines, the A-549 cells were more sensitive to all the screened extracts as compared to MCF-7 cells. The data from this study agreed with the previous findings, where the difference in sensitivities of different cells was found.[38] The morphological changes in the cells were also observed. The treated cells reduced the normal morphology and cell adhesion capacity as compared to control. The NDEs might promote cell detachment by interacting with intercellular junctions or extracellular matrix. The modification of cell surface might be due to the neoplastic transformation,[39] binding of plant lectins,[40] change of glycoprotein in cell surface,[41] and cell adhesion molecules[42] which might be correlated to the invasion of metastasis *in vivo*. Hynes[39] reported that fibronectin or large extracellular transformation sensitive protein was lost from the surface of transformed fibroblast due to alterations in intergrins. This loss might contribute to a decrease in cell-cell and cell-substrate adhesion[43] and leads to the reduction in cell attachment, as well as cell spreading for proliferation. Therefore, the NDE might be an independent cell inducer. The morphological changes in the cells were observed more prominent in treated cells showing extensive blebbing and vacuolation suggesting an autophagic mechanism of cell death.[44]

CONCLUSION

Our results demonstrated that NDEs significantly reduced cell viability in a concentration dependent manner and has potent anticancer activities *in vitro*. Therefore, it could be a potential source of pharmacologically active molecules for the development of the chemotherapeutic drug. Further studies are however required to determine the detailed and distinguishing feature of the intracellular pathway (s) involved in the mechanism of cytotoxicity and isolation of active compounds responsible for cytotoxicity.

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Conflicts of interest

There are no conflicts of interest.

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