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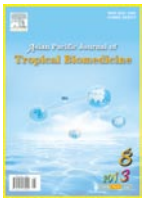

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Exploring a natural MDR reversal agent: potential of medicinal food supplement *Nerium oleander* leaf distillate

Meltem Demirel Kars[1,2*], Ufuk Gündüz[3], Kamil Üney[4], Ahmet Levent Baş[4]

[1]*Selçuk University, Sarayönü Vocational High School, 42430 Konya, Turkey*

[2]*Selçuk University, Advanced Technology Research and Application Center, 42075 Konya, Turkey*

[3]*Middle East Technical University, Department of Biological Sciences, 06531 Ankara, Turkey*

[4]*Selcuk University, Faculty of Veterinary Medicine, Department of Toxicology and Pharmacology, 42075, Konya, Turkey*

PEER REVIEW

Peer reviewer

Dr. Aytac Kocabas, Karamanoglu Mehmetbey University Department of Biological Sciences
Tel: +90 338 2262151
Fax: +90 338 2262150
E-mail: aytackocabas@kmu.edu.tr

Comments

The study applied novel method for chemical extraction from plants. It can be used to find out new compounds because, as indicated in the research, *N. oleander* leaf distillate is different from extracts obtained by infusion method in terms of composition and toxicity.

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ABSTRACT

Objective: To investigate the molecular effects of *Nerium oleander* leaf distillate on paclitaxel and vincristine resistant (MCF-7/Pac and MCF-7/Vinc) cells and sensitive (MCF-7/S) cell lines.
Methods: *Nerium oleander* (*N. oleander*) leaf extract was obtained by hydrodistillation method. The toxicological effects of *N. oleander* distillate, previously suggested as medicinal food supplement, on drug resistant cells were evaluated by XTT tests. MDR modulation potential of the plant material was evaluated by flow cytometry and fluorescent microscopy. Paclitaxel and vincristine were applied to the sublines in combination with *N. oleander* distillate.
Results: Fractional inhibitory indices show that *N. oleander* distillate did not increase the antiproliferative effects of anticancer drugs. *N. oleander* treatment in to MCF-7/Pac and MCF-7/Vinc did not inhibit P-gp activity and MDR1 gene expression level.
Conclusions: As a result it may be suggested that although *N. oleander* distillate has some medicinal effects as food supplement it may not be suitable as an MDR modulator for drug resistant breast cancer cells.

KEYWORDS
Nerium oleander, MCF-7, FRSA activity, MDR reversal, Medicinal food supplement

1. Introduction

Breast cancer is the cancer type mostly observed in women worldwide[1]. Chemotherapy is the major way of combating cancer cells. The drugs used for the therapy are named as anticancer drugs. Unresponsiveness to chemotherapy by patients or re-occurrence of the disease may be the outcomes of chemotherapy. To be successful in cancer therapy more than two drugs are sometimes used. However acquired drug resistance during chemotherapy limits the success of chemotherapy. This situation is known as multiple drug resistance (MDR)[2,3].

Resistance developed against chemotherapy causes an ineffective therapy period and progress of the disease[3]. There are various natural compounds being used in cancer therapy[4]. *Nerium oleander* L. (*N. oleander*) is an important plant used in medicine. *N. oleander* is a tree, middle in length 2-5 m, evergreen and with flower. This plant is actually a Mediterranean and tropical

*Corresponding author: Meltem Demirel Kars, Selcuk University, Advanced Technology Research and Application Center- Campus, KONYA-TURKEY.
E-mail: meltem@selcuk.edu.tr
Tel: 90-3322233438

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Asian tree[5]. Many parts of *N. oleander* are known to be poisonous for insects, animals and mankind[6]. Pharmacological activities of some active materials from *N. olaender* were determined previously[7]. The most important active ingredients of plant are known to be polysaccharides, cardenolides, glycosides and triterpenes. Total extract of the plant exhibited antinosiseptive, anti-inflammatory, antibacterial and anticancer effects[7,8]. Zhao *et al.* declared that the cardenolide compounds extracted from *N. oleander* exhibited MDR modulator effect on doxorubicin resistant ovarian carcinoma cell line 2780AD[9]. Bas *et al.* reported that *N. oleander* distillate may be used as dietary supplement to treat high blood cholesterol level in diabetic rats[10]. Yazihan *et al.* also suggested that *N. oleander* distillate is not toxic to non carcinoma cells[11].

In the present study, the effect of *N. oleander* distillate on paclitaxel and vincristine resistant (MCF-7/Pac, MCF-7/Vinc) and sensitive MCF-7/S cell lines has been investigated by fluorescent techniques and by RT-PCR. *N. oleander* leaf distillate was obtained by hydrodistillation method.

2. Materials and methods

2.1. Plants and preparation of distillate

N. oleander leaves were collected from Mersin region (Latitude: 36°47′42″ N Longitude: 34°37′04″ E) in April-May period. The plant was identified, and authenticated at the Department of Biology. Plant leaves were washed, chopped and boiled in water (100 g/1 000 mL) and then distillate (hydrodistillate) was collected. Distillate was lyophilized in different doses (FDT-8618 Freeze Dreyer, Operon, Korea). Dried extracts were stored at room temperature in dark vials until use to avoid dampening.

2.2. DPPH antioxidant activity determination

DPPH (2,2-diphenyl-1-picrylhydrazyl is a stable free radical that can abstract hydrogen atoms from phenolic antioxidants with by forming a colorless hydrazine (DPPH-H) [12]. Distillate was dissolved in methanol as 10 mg/mL concentration according to procedure[12]. The sample was diluted in between 2 mg/mL - 0.0156 mg/mL. Several dilutions of L-ascorbic acid (L-ASA, Sigma) and butylated hydroxy toluene (BHT, Sigma) which have high free radical scavenging activity were used as controls (200 μg/mL-1.56 μg/mL). A total of 3 mL of DPPH solution (20 mg/L) was added to the sample and mixed vigorously for 30 seconds. The mixture was kept at room temperature in dark for 30 min and then absorbance was read at 517 nm (Biochrom). The scavenging activity was determined by comparing the absorbance with the absorbance of the blank (100%) which only contains DPPH and solvent. The total free radical scavenging activity of distillate was given as the concentration of distillate that reduce 50 % of DPPH, IC_{50}. The assay was repeated for three times.

2.3. Cell lines

In order to test the effect of *N. oleander* distillate on cells, the drug resistant MCF-7 cell lines, models for drug resistant human mammary carcinoma, were used. The properties of the parental and previously established resistant sublines were described by Kars *et al*[13]. MCF-7/Pac is 150 fold and MCF-7/Vinc is 30 fold resistant to paclitaxel and vincristine respectively with respect to the sensitive cells MCF-7/S.

2.4. Cell proliferation assay

The effects of plant distillate on proliferation of sensitive and resistant MCF-7 cell lines were tested. Distillate was diluted horizontally through plate from 3 mg/mL concentration. Finally the cells were seeded in to 96-well microtiter plates (5×10^3 cells/well) and incubated for 72 h in medium containing dilutions of distillate. Dimethylthiazol-2-yl] 1-2,5-diphenyltetrazolium bromide based cytotoxicity kit (XTT reagent, Biological industries, Israel) was applied to form chromogenic formazan dye. After incubation at 37 °C for 4 h, optical density was measured at 490 nm with a 96-well plate reader (BioTek microplate reader). The inhibition of cell proliferation and IC_{50} values were determined.

2.5. Checkerboard combination assay

Checkerboard micro plate method was applied to study the effects of interactions between distillate and anticancer drugs on resistant MCF-7 cell lines[14]. The dilutions of anticancer drugs (A) were made in horizontal direction and the dilutions of distillate (B) vertically in microtiter plate. The cells were distributed to each well and incubated for 72 h at 37 °C. The cell growth was determined after XTT staining. Drug- distillate interaction was calculated by:

$FIC_A = IC_{50A}$ in combination / IC_{50A} alone

$FIC_B = IC_{50B}$ in combination / IC_{50B} alone where FIC is fractional inhibitory concentration

Fractional inhibitory index, $FIX = FIC_A + FIC_B$ gives the effect of combination of drug and distillate. It is accepted that if FIX value is 0.5-1, it is an additive effect. FIX value less than 0.5 is a synergism and if it is in between 1-2, the interaction is considered an indifferent effect while greater than 2 indicates antagonism[14].

2.6. Assay for the reversal of MDR in MCF-7 cell lines

Parental and resistant cell lines were trypsinized and

the cell concentration was adjusted to 2×106 cells/mL. The cells were suspended in serum free RPMI 1640 medium and distributed in 0.5 mL aliquots into 1.5 mL centrifuge tubes. *N. oleander* distillate was added (100 and 200 μg/mL) and samples were incubated for 10 min at room temperature (25 °C). Doxorubicin, P-gp substrate as fluorescent indicator, was added 10 μmol/L final concentration to samples and cells were incubated for 20 min at 37 °C. The cells were centrifuged, washed twice in 0.5 mL PBS and finally resuspended in 0.5 mL PBS for assay. The fluorescence of the cell population was measured using flow cytometry (BD FACS Calibur). Verapamil was used as positive control (40 μg/mL) in the doxorubicin exclusion assays. The fluorescent activities for the treated MCF-7 cell lines were calculated by comparing them with the fluorescent activities of the untreated cells. The ratio was calculated on the basis of the measured fluorescence intensities[13,14]. Affect of *N. oleander* on doxorubicin accumulation was also observed by fluorescent microscope. Cells were pelleted and tryphan blue (Sigma-Aldrich) viable cell count was performed under light microscope. MCF-7/S, MCF-7/Vinc, and MCF-7/Pac cells were seeded on to cover slips as 6×10^5 cells/coverslip, in 6 well plates and they were incubated in media overnight. Cells were washed with PBS for three times and incubated with verapamil and *N. oleander* distillate for 30 min. A control of non-treated cells was also run. Treated and nontreated cells were incubated with 3 μmol/L doxorubicin for 1 h in dark. Specimens were observed under an Olympus BX51 fluorescent microscope, 100 × objectives with green filter.

2.7. RNA isolation and RT-PCR

In order to determine the effect of *N. oleander* treatment on *MDR1* gene expression levels in drug resistant MCF-7 cell lines cells were evaluated. Total RNA was extracted using TRI Reagent (Sigma) after one week of treatments (100 and 200 μg/mL) according to the manufacturer's instructions. Absorbance values (260 nm, 280 nm) were measured for RNA quantification by spectrophotometry. cDNA synthesis was performed with 5 μg of total RNA, 20 pmol specific primers for *MDR1* and β-*microglobulin* and 40 units of M-MuLV Reverse Transcriptase according to the manufacturer's instructions (MBI Fermentas). cDNA was used as template for PCR reaction, 1 unit *Taq* DNA polymerase was used for 50 μL of reaction volume. *MDR1* specific primers were used for the expression analysis. *Beta-2 microglobulin* level was an inner standard to normalize the expression levels of genes[13]. PCR conditions were; initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 seconds, 30 cycles of annealing at 55 °C for 30 seconds (*beta-2 microglobulin*) and 56 °C for 25 seconds (*mdr1*), extension at 72 °C for 30 seconds and final extension at 72 °C for 10 min.

2.8. Statistics

The results of assays were subjected to two-tailed t-test by using SPSS Software (SPSS Inc., Illinois, USA to determine significant difference between means of groups (α=0.05).

3. Results

3.1. DPPH antioxidant activity determination

Free radical scavenging activity (FRSA) of the plant distillate has been determined by DPPH which is a stable highly colored free radical. The distillate has a considerable level of FRSA when compared to that of L-ascorbic acid (L-ASA) and BHT. According to IC_{50} values; *N. oleander* distillate, L-ASA and BHT have 53.80 μg/mL, 2.26 μg/mL and 2.45 μg/mL free radical scavenging activities respectively.

3.2. Cytotoxicity assay and combined application of distillate with anticancer agents

According to XTT tests, *N. oleander* distillate is not toxic for MCF-7/S, MCF-7/Pac and MCF-7/Vinc cells when *in vivo* concentration applied to animals are considered[10]. Also the effects of distillate on viability of all cell lines were similar (IC_{50} 0.22 mg/mL). To conclude, it has effects on the viability of all cell lines only at very high concentrations which may clinically be taken as overdose. The combined application of distillate with paclitaxel and vincristine together exerted antagonistic interaction (FIX >2) on the cell lines. On the other hand verapamil acts synergistically together with paclitaxel and vincristine on MCF-7 cell lines.

3.3. Affect of N. oleander on drug accumulation in MCF-7 cells

Fluorescent activitiy of the cells were determined by flow cytometry measurements. Mean fluorescent accumulation values and histograms (Figure 1) were determined for control and *N. oleander* applied resistant cells. The mean fluorescent accumulation (doxorubicin accumulation) and fluorescent activity alterations were determined and results were summarized in Table 1. Mean fluorescent values of vincristine and paclitaxel resistant cells were lower than the fluorescence accumulation of cells modified with verapamil. This result shows that *N. oleander* distillate does not inhibit P-gp activity when compared to verapamil. Flow cytometry results were confirmed with fluorescent microscope observations (Figure 2). MCF-7/Pac cells effluxed doxorubicin out by

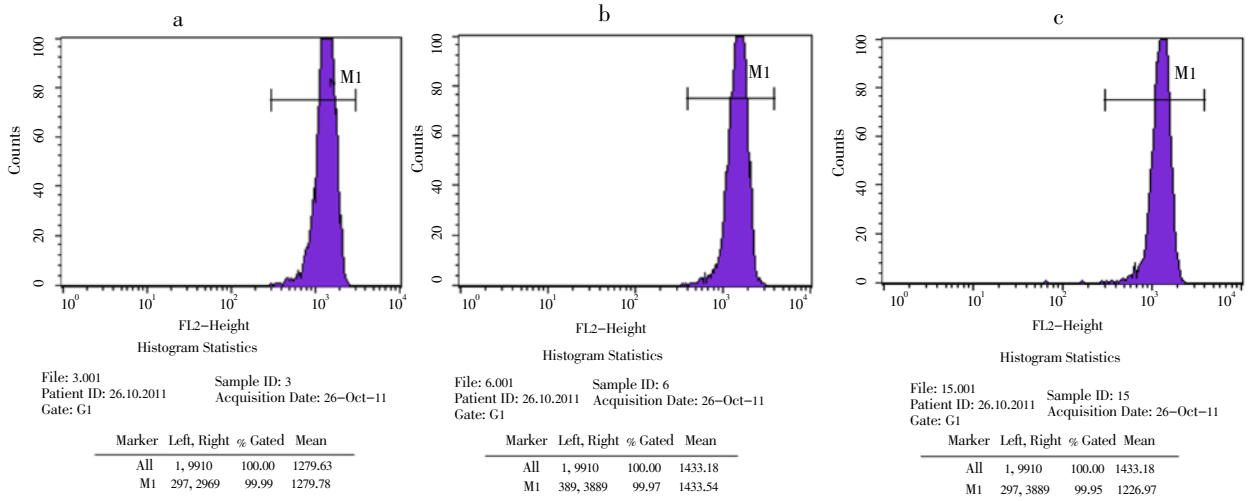

Figure 1. a: Fluorescent activity histogram of MCF-7/Vinc, b: Fluorescent histogram of MCF-7/Vinc treated with verapamil (40 μg/mL, c: Fluorescent histogram of MCF-7/Vinc treated with *N. oleander* (200 μg/mL distillate).

P-gp overexpression while MCF-7/S cells have drug accumulation. Verapamil application also resulted in doxorubicin accumulation in MCF/7 Pac cells which means P-gp is inhibited. However, *N. oleander* distillate treatment did not resulted in drug accumulation in the drug resistant cells.

Table 1

Fluorescent activity ratio (FAR) of cells after treatment.

Reagent	FAR ± SD	
	MCF-7/Pac	MCF-7/Vinc
Verapamil	1.05 ± 0.01	1.12 ± 0.03
Nerium oleander distillate	0.98 ± 0.04	0.99 ± 0.01

SD: standard deviation (P<0.05)

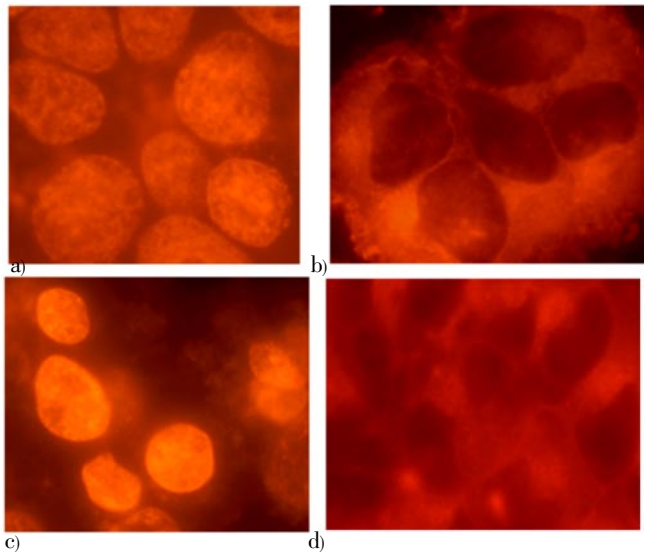

Figure 2. Doxorubicin accumulation was observed under 100 × objectives and green filter. a: MCF-7/S doxorubicin treatment, b: MCF-7/Pac doxorubicin treatment, c: MCF-7/Pac verapamil-doxorubicin treatment, d: MCF-7/Pac *N. oleander* distillate-doxorubicin treatment.

3.4. Determination of MDR1 gene expression levels

RNA isolation was performed from cells treated with *N. oleander* distillate concentrations lower than IC_{50} (100 ve 200 μg/mL. RT-PCR was performed from cDNA and expression values were evaluated (Figure 3) by Scion Image Software (Scion Corporation, USA). The densitometric results show *MDR1* gene is not expressed in sensitive cell line however the gene is overexpressed in resistant cells. However, *N. oleander* application did not affect *MDR1* gene expression levels in the drug resistant cell lines.

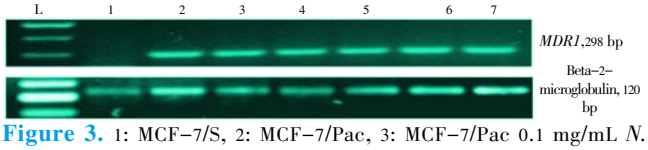

Figure 3. 1: MCF-7/S, 2: MCF-7/Pac, 3: MCF-7/Pac 0.1 mg/mL *N. oleander*, 4: MCF-7/Pac 0.2 mg/mL *N. oleander*, 5: MCF-7/Vinc, 6: MCF-7/Vinc 0.1 mg/mL *N. oleander*, 7: MCF-7/Vinc 0.2 mg/mL *N. oleander*.

4. Discussion

Cancer is a serious problem that treats human health. Although there is serious research to develop new therapeutic agents, cancer still affects people worldwide. Bacterial and plant cells are important natural sources for new anticancer drug development[15]. There are various plant types that take place in modern and traditional medicinal databases[16-19]. To obtain medicinal food supplement from *N. oleander* leaves a new oleander extraction method Distillation was used in this study finally the effects of distillate on MCF-7 cells were determined.

N. oleander, is used for treatment of some skin problems edema, leprosy as traditional medicine

application. Dosage of *N. oleander* distillate obtained by infusion is important due to its poisonous ingredients[20]. There is an extensive research about the effects of *N. oleander* distillate on cancer[20-21]. Hydrodistillation (an original method) was used to obtain leaf distillate from *N. oleander* in this study. The distillate was not toxic to mammary carcinoma MCF-7 cells according to the results which are very similar to the findings for liver cells HEP3B by Yazihan *et al*[11]. We found also that the distillate has antagonistic interaction with paclitaxel and vincristine which also show that it does not have toxic effect on cell proliferation. According to the previous studies, *N. oleander* distillate is not toxic to animal models, so *N. oleander* may be used in treatment of some diseases[11]. The effects of *N. oleander* distillate as food supplement on rats taking high fat diet were investigated also by the same group. It was found that *N. oleander* distillate may be used as medicinal food supplement for treatment of hypercholesterolemia in diabetic rats[10].

There are several pharmacological agents that reverse drug resistance to drugs, doxorubicin, vincristine and paclitaxel. They are named as modulators and they exert effect by interacting membrane lipids and inhibiting P-glycoprotein (MDR protein)[22,23]. So cell membrane is the very important target of MDR modulators. Zhao *et al*. reported that new cardenolide compounds obtained from *Nerium oleander* by infusion method reversed MDR resistance in 2780AD ovary cancer cell lines[9]. However *N. oleander* distillate did not exert any MDR modulator activity on drug resistant cells in our study. We can conclude that chemical compounds present in the distillate do not interact with cell membrane or P-gp. Effects of different distillate concentrations on *MDR1* gene expression was also investigated by RT-PCR. MCF-7/S cell line does not express *MDR1* gene and MCF-7/Pac and MCF-7/Vinc cell lines overexpress the P-gp encoding gene. *N. oleander* leaf distillate treatments did exert any change on *MDR1* gene expression level in drug resistant cells. It means that the distillate is not a *MDR1* gene and P-gp modulator. From these findings it can be concluded that *N. oleander* leaf distillate is not an effective drug resistance reversal agent.

To conclude, these findings indicate that *N. oleander* leaf distillate is different from extracts obtained by infusion method in terms of composition and toxicity. It is still a potential medicinal plant extract because it exhibits no toxic effects, considerable free radical scavenging activity and it improves cholesterol metabolism when taken as dietary supplement[10]. On the other hand, the composition and active components of the *N. oleander* leaf distillate should be clarified by chromatographic and spectroscopic methods. The toxicological and functional assessment of distillate should be made by using different concentrations of active ingredients on different types of cell lines and animal models.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Cancer is one of the worst disease threaten human health and life. Chemotherapy is applied for cure, nonetheless multiple drug resistance is one of the problem preventing effects of chemotherapy. Therefore there is always need for new chemicals making cell re-sensitive to chemotherapy.

Research frontiers

The presented study focused on re-sensitization of cancer cells against chemotherapy drugs. For this purpose, the extracts of *N. oleander* leaves are used. Besides its purpose, the extraction method is also novel and as indicated in the manuscript, it is a patentable method.

Related reports

Natural products obtained from plant parts are always in use in pharmaceutics and new chemicals are searched for novel applications. Moreover, some active materials of *N. oleander* have been proved as having pharmaceutical activities. Its extracted chemicals showed no MDR modulator effects, but can be used as dietary supplement.

Innovations and breakthroughs

N. oleander L. is an important plant used in medicine. Moreover, the cardenolide compounds extracted from *N. oleander* exhibited MDR modulator effect on doxorubicin resistant ovarian carcinoma cell line 2780AD. The present

study applied new and original extraction method.

Applications

Although, the distillate did not showed any effect on the MDR1 gene expression and P-gp activity, The study present novel method for extraction of plants.

Peer review

The study applied novel method for chemical extraction from plants and it can be used to find out new compounds because, as indicated in the research, *N. oleander* leaf distillate is different from extracts obtained by infusion method in terms of composition and toxicity.

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