

Cytotoxic effects of leaf, stem and root extracts of *Nerium oleander* on leukemia cell lines and role of the p-glycoprotein in this effect

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Nerium oleander (No), is a toxic plant. In recent studies, it was determined that the extracts of this plant are effective to treat some types of cancer, but these studies are limited and do not include human leukemia. In the present study, firstly we aimed to investigate *in vitro* the cytotoxic effects of No on the HL60 and K562 leukemia cell lines. The cells were incubated with six different concentrations of each three extracts. MTT assay was employed as a cytotoxicity test. It was observed that concentrations of 1000, 500 and 50 µg/ml from each extract possess marked antileukemic effects. No leaf and root extracts were seen to be more cytotoxic than the stem extract according to LC₅₀.

Secondly, in order to understand the role of P-gp in cytotoxicity, P-gp levels of K562 resistant and sensitive cells were measured by flow cytometry before treatment extracts, and then, the cells were incubated with No leaf, stem and root extracts in 500 µg/ml concentrations overnight. After incubation, measurements showed decreased levels of P-gp in the cells. Hence, it is possible to think contributes to their cytotoxic effects that inhibiting of the P-gp pump by No extracts on leukemia cell lines.

Key words: *Nerium oleander*, P-glycoprotein, cytotoxicity, leukemia cell line, MTT

INTRODUCTION

Nerium oleander(No), an ornamental plant from the Apocynaceae family, is widely distributed in subtropical Asia and Mediterranean countries (el-Shazly et al., 1996; Begum et al., 1997, 1999; Dongarra et al., 2003). No flowers and leaves to be used as folk medicine¹ for the treatment of a wide variety of diseases including infection, malaria, autoimmunity, abscesses, asthma, allergy, eczema, dysmenorrhea, epilepsy, HIV and cancers (Newman et al., 2001; Erdemoglu et al., 2003). No that is a poisonous plant was responsible from % 27 pediatric poisoning during 1972-1978 in Australia. No leaves contain cardiac glycosides like oleandrin, oleandrigenin, digoxin, digitonin, digitoxigenin, nerizoside, neritaloside, odoroside (Trease and Evans, 2002). These cardiac glycosides have been used in the treatment of heart diseases in Russia and China for years (Huang, 1999).

After reports of breast tumor regression in Scandinavian patients taking cardiac glycosides, their anticancer effects noticed (Stenkvist, 1999). Interestingly, anecdotal evidence has emerged from suggesting that may produce beneficial side effects in patients with leiomyosarcoma, Ewing's sarcoma, prostate cancer and breast cancer. Therefore, there is growing interest in evaluating No products and possibly other cardiac glycosides as antineoplastic

¹ it is informed in ethernet page followed to <http://www.ozelle.com>

agents (Stenkvist, 1999; Haux et al., 2001; Lin et al., 2004; Moxnes, 2004).

There are only a few studies about the effects of No extracts and their active components on cancer cells. Water-soluble extract from No called Anvirzel developed by Dr. Huseyin Ziya Ozel¹ who is a Turkish physician and patented in docket number 95 S-0316 by FDA in the United States, is now undergoing clinical evaluation as a treatment for cancer. It is known to contain a complex mixture of polysaccharides and oleandrin (Newman et al., 2001). The investigations conducted within the last few years has shown that oleandrin produces apoptosis in prostate tumor cells and that this effect is mediated through inhibition of Na⁺, K⁺-ATPase (McConkey et al., 2000). Cardiac glycosides, such as oleandrin, digoxin or ouabain inhibit the membrane Na⁺, K⁺-ATPase pump that, in Ca⁺⁺ exchange. In another study, they have demonstrated that, *in vitro*, cardiac glycosides may inhibit fibroblast growth factor-2 (FGF-2) export through membrane interaction with Na⁺, K⁺-ATPase the pump on two human prostate cancer cell lines (Smith et al., 2001).

Like Na⁺, K⁺-ATPase pump, there are also other pumps located in the cell membrane which are affected by cardiac glycosides. One of these pumps is a member of the ABC (ATP-binding cassette) transporter family and highly conserved known as P-glycoprotein (P-gp) is overexpressed in many multidrug-resistant cancer cell lines (Bosh et al., 1996; Gottesman, 1996; Fu et al., 2000; Lehne, 2000; Simon et al., 2001). Numerous studies indicate that digoxin efflux is mediated by P-gp in the small intestine and nephron (Cavet et al., 1996; Johansson et al., 2001; Pauli-Magnus et al., 2001; Mikkaichi et al., 2004). However, No components may also affect P-glycoprotein of ATP-dependent efflux membrane transporter function and cause interactions with its constituents that might be P-gp substrates, similar to digoxin. Because P-gp plays an important role in pharmacokinetics such as excretion of xenobiotics and multidrug resistance, an understanding of the factors regulating its function and expression is necessitate.

Therefore, in the present report *in vitro* cytotoxic effects of No leaf, stem and root extracts on the HL60 and K562 leukemic cell lines and the role of P-gp in this cytotoxicity were investigated.

MATERIALS AND METHODS

Plant material

No leaves were collected in 2000 from Antalya, southern Turkey, from its natural environment (Identified by Filiz Meriçli).

Preparation of the plant extract

The dried leaves, stems and roots of plant were extracted separately in a soxhlet apparatus with petroleum ether and ethanol respectively. Each extract was evaporated under vacuum and dissolved in dimethylsulfoxide (DMSO) to a concentration of 50 mg/ml and stored at 4°C. Then they were diluted to different concentrations to be used in the cytotoxic assays.

Cell culture

K562 and HL60 cells were purchased from American Type Culture Collection (ATCC, MD, U.S.A.). The cell lines were grown and maintained in a humidified incubator at 37 °C and in 5% CO₂ atmosphere. Leukemia cells (K562, HL60) were suspended at 1 x 10⁵ cells/ml in IMDM (Sigma) that contained 10% fetal calf serum (FCS) (Sigma), gentamycin, L-glutamin and sodium bicarbonate. Then 90 µl of this suspension were dispensed into 96-well round-bottom plates which contained 10 µl of extract dilutions. Wells that did not contain extracts as a positive control and only medium as a negative control were used (Mossman, 1983).

Cytotoxicity assay

In vitro chemosensitivity of leukemic cell lines was determined using this short-term cell culture assay based on the principle that cells surviving the extract exposure are capable of reducing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to a dark formazan by mitochondrial dehydrogenase. Lyophilized No was prepared as a stock solution 50 µg/ml in DMSO. 10 µl of each No extracts (1000, 500, 50, 5, 0.5, 0.05 µg/ml) were added in 96-well microculture plate. 90 µl cell suspension was plated to the wells at 10⁵ cells/ml including three control wells. The plates were incubated for 48 hours at 37 °C in a humidified incubator with 5% CO₂. Then 10 µl MTT (Sigma) (5 mg/ml) solution in phosphate buffered saline (PBS) was added to each wells and the plates were incubated 4 hours at 37 °C. The plate was centrifuged and then removed from the medium, and acidified isopropanol in 10% sodium doedocil sulphate (SDS) solution was added to the wells to solubilize the MTT crystals. Afterwards, plates were left in the dark room overnight, and optical density (OD) was measured with 570 nm test wavelength and a 620 nm reference wavelength with an ELISA multiwell spectrophotometer. Cytotoxicity index (CI) was calculated to following formula.

$$CI \% (\text{Cytotoxicity index}) = 1 - \frac{\text{OD treated wells}}{\text{OD control wells}}$$

OD control wells X 100. Also, LC_{50} (the concentration of the extract that killed 50 % cells) was calculated from dose-response curves.

P-gp determination

Flow cytometric analysis of P-gp was performed using a direct immunofluorescence staining technique with antibody UIC2 (Coulter). Briefly, 5×10^5 cells incubated with FITC conjugated antibody UIC2 at 4°C for 30 min. Matched isotype controls of Mouse IgG2a at the same protein concentration were used. After washing, cells were resuspended in PBS and were analysed on

flow cytometer (COULTER Epics MCL). Results were expressed as the ratio of the mean fluorescence of antibody labelled cells divided by that of the isotype control (Boutonnat et al., 1998). P-gp levels were measured before and after K562 cells incubated with No leaf, stem and root extracts of 500 µg/ml for a night.

Statistical analysis

Results were expressed as the mean \pm standard deviation (SD). Statistical differences were assessed by the Student's unpaired t-test, with $p < 0.05$ as statistically significant.

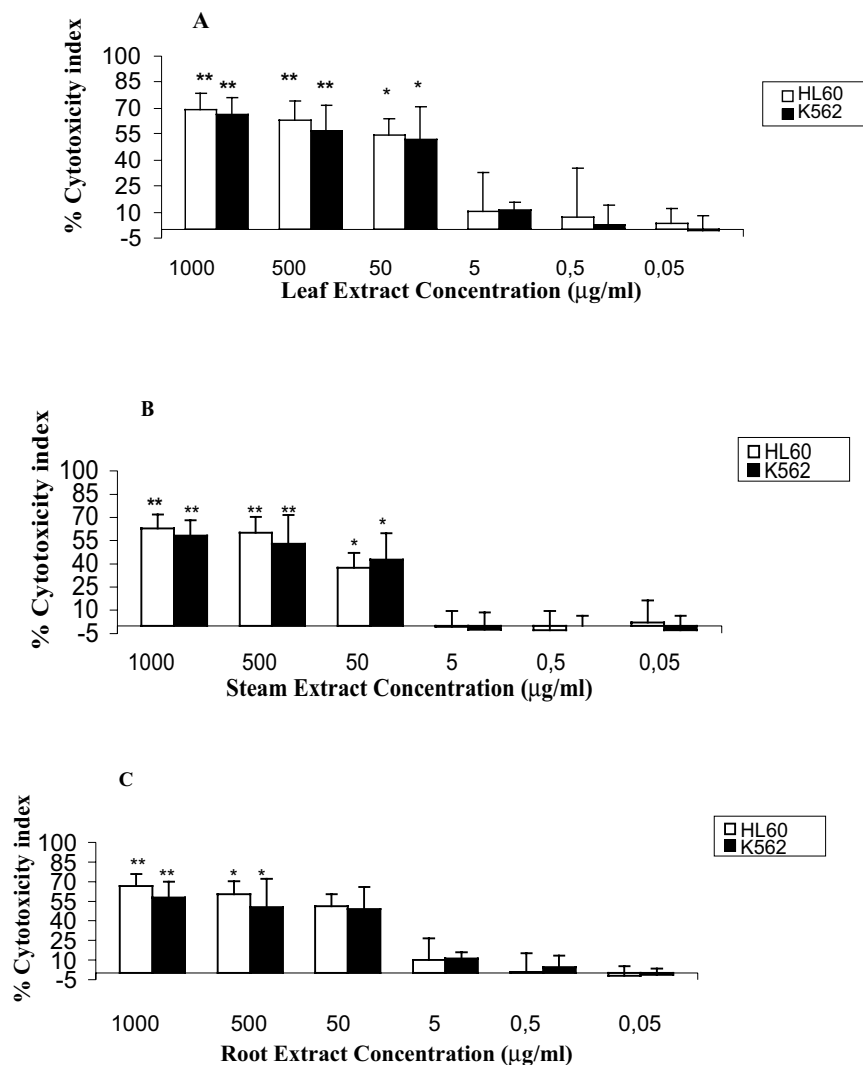


Figure 1. Effects of N.oleander extracts on proliferation of HL 60 and K 562 cells. The three graphs shows cytotoxic effects of leaf, stem and root extracts, respectively A,B,C. Cells were incubated with increasing concentrations (0.05-1000 ug/ml) of N.oleander extracts in culture medium for 48 h and cytotoxicity index was assessed by MTT assay. Means \pm SD are shown. * $p < 0.05$, ** $p < 0.01$.

RESULTS

Cytotoxic effects of No extracts

The present study has demonstrated that ethanol extracts of leaf, stem and root parts obtained from No have cytotoxic effects on the leukemia cells (HL60, K562). As shown in Figure 1 we have found cytotoxicity index of 66.22 % in leaf extracts, 57.82 % in root extracts and 58.10 % in stem extracts on K562 cells and 69.33 %, 66.50 %, 62.81 % respectively on HL60 in highest doses. We have seen that leaf, stem and root extracts in both cell series have cytotoxic effect according to LC_{50} value calculated from dose-response curves (Figure 1) and but LC_{50} value of the stem extracts are higher (Figure 2). The difference between the tissues is caused by the fact that the chemicals included in No are spreaded in different amounts in plant tissues.

P-gp role in No cytotoxicity

Secondly, we assessed K562 cells having the lowest P-gp expression (18%) as sensitive and having the highest P-gp expression (70%) as resistant according to P-gp expression after were measured Pgp levels of K562 cells and sublines. Later, we incubated with the leaf, stem and root extracts of 500 µg/ml one night and were measured to P-gp levels for understanding whether cytotoxic effect have a relation with P-gp or not. Table 1 shows levels of P-gp before and after incubation. Figure 3 and 4 show also distribution of P-gp positive sensitive and resistance cells in Flowcytometry images. After incubation, a reduction P-gp levels was seen both of sensitive and resistant cells in changing ratio.

DISCUSSION

This study aimed to investigate the effects of No extracts on the leukemia cells and its P-gp relationship in this effect. Intensively, effects on the heart of No extracts have been studied in search of importance of the cardiac glycosides. It has been concluded that this effect occurs when the Na^+,K^+ -ATPase pump is inhibited (McConkey et al., 2000; Manna et al., 2001; Ni et al., 2002). The research about the anticancer effects of the plant, however, has been still continued. There is lack of literature information about the effects of No extracts on in vitro leukaemia cells in vitro¹. In cancer treatment, it is known that the cells are resistant against the drugs. One of the reasons of the resistance is P-gp that pumps the toxic material out of the cell. Although there is a few studies related that No is cytotoxic and anticancer (McConkey et al., 2000; Manna et al., 2001; Ni et al., 2002) it has not been done any study explaining the relation between these effects and P-gp therefore this study was designed.

AnvirzelTM, a water-soluble extract that was obtained from No plant was used in traditional medicine and patented by Dr. Huseyin Ziya Ozel, a Turkish physician who began to experiments with these extracts in 1966. Z. Ozel had initiated of folk traditions and treated patients with advanced and inoperable cancer since 1970s¹. Now, phase I studies have been just started and is still going on (Mekhail et al., 2001). Ozelle Pharmaceuticals, Inc., was founded to further explore and develop the potential of this preparation which was trade-marked under the name AnvirzelTM. This extract has been prepared by dissolving plant in hot-water, and non-toxic dose¹.

Table 1. Levels of P-gp before and after incubation with leaf, stem and root extracts of N.oleander in K 562 cells.

		Sensitive K562 P-gp < 20%	Resistant K562 P-gp > 20%
Levels of P-gp before incubation		18	70
Levels of P-gp after incubation	Leaf extract	13	66
	Steam extract	10	48
	Root extract	16	49

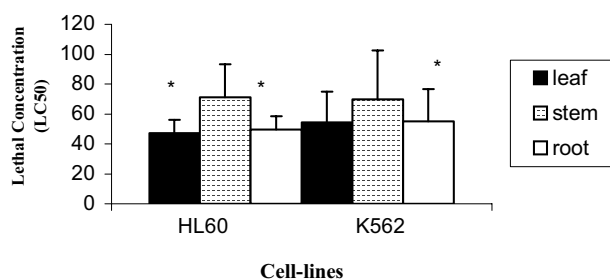


Figure 2. The differences of lethal concentration (LD50 = concentration killing 50 % of cells). LD 50 was changed in HL 60 and K 562 cells treated with leaf, stem and root extracts of N.oleander. Means± SD are shown. *p< 0.05 , **p< 0.01.

But we had first consumed plant tissues with oil-ether and, later with ethanol in order to gather in the extract the compound which dissolves both in water and oil while getting the extracts from No. Anvirzel contains hydrophylic compounds (glikosides, acids, etc.) which can be dissolved in only water, but our extract dissolved oil-ether and ethanol comprise also

lipophylic compounds (aglicons and the others) other than hydrophylic ones. Therefore, both of them have different effects in cancer cells.

Recently, it was published few studies about cytotoxic effect of No exerting its effect through oleandrin in mainly. McConkey et al.(2000) have shown that oleandrin has 50 times more cytotoxic

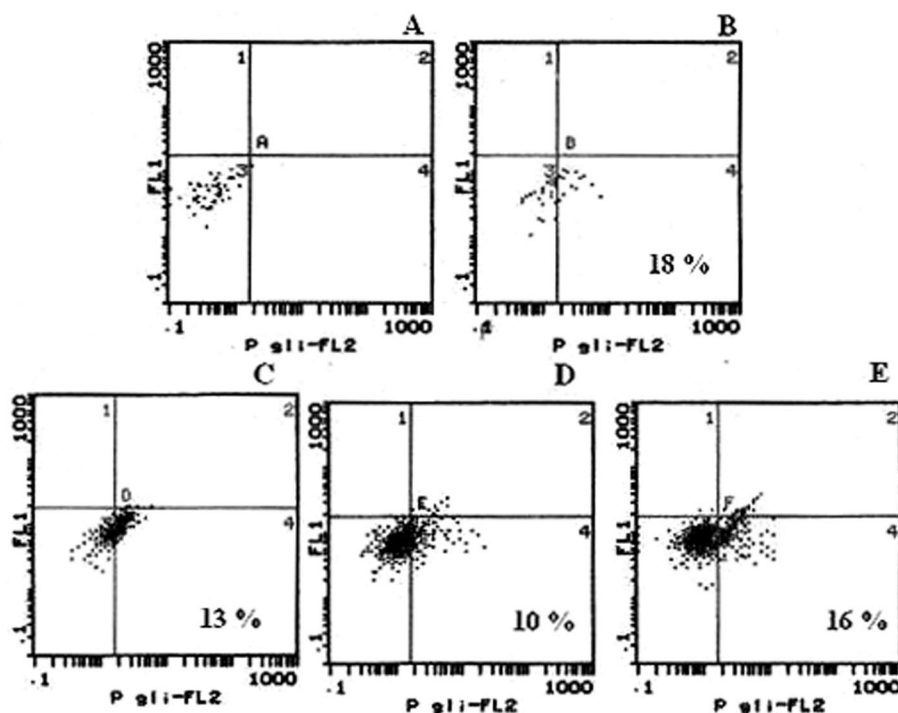


Figure 3. Dot plots are shown sensitive K562 cells of incubated N.oleander extracts and not incubated (A-E). In plate A are given K562 cells unstained with phycoerythrin-conjugated P-gp antibody (PE-Pgp) as a negative control. In plate B are given the cells stained ratio of 18 % with PE-Pgp but not incubated N.oleander extracts (positive control). In other plates shown the cells incubated with leaf, stem and root extracts and stained with PE-Pgp antibody and exhibited 13 % (C), 10 % (D) and 16 % (E) positivity, respectively.

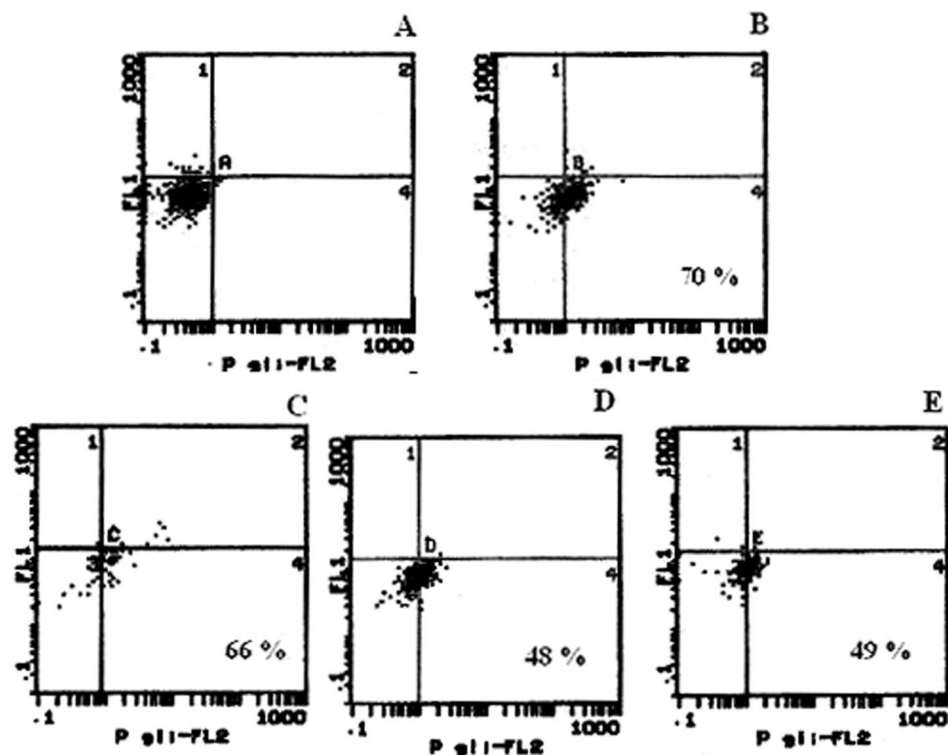


Figure 4. Dot plots are shown resistant K562 cells of incubated N.oleander extracts and not incubated (A-E). In plate A are given K562 cells unstained with phycoerythrin-conjugated P-gp antibody (PE-Pgp) as a negative control. In plate B are given the cells stained ratio of 70 % with PE-Pgp but not incubated N.oleander extracts (positive control). In other plates shown the cells incubated with leaf, stem and root extracts and stained with PE-Pgp antibody and exhibited 66 % (C), 48 % (D) and 49 % (E) positivity, respectively.

effect than Anvirzel on PC prostate cell series and subclons. Newman and et al. (Newman and et al., 2001) have analyzed the cytotoxic effects of the oleandrin, oleandrigenin, ouabain included Anvirzel on human BRO melanoma and mouse melanoma B16 cells. LC_{50} value of Anvirzel was not same in the cells of different origins (in human BRO melanoma cell: $1.6 \pm 0.6 \mu\text{g/ml}$; in mouse melanoma B16 cell: $2424 \pm 219 \mu\text{g/ml}$). The lethal concentrations in the human and mouse melanoma cells defined above vary between 1.6 - 2424 $\mu\text{g/ml}$ (Newman and et al., 2001). However, according to our results showing extracts which may be more effective, the LC_{50} values of No extracts vary between 47.16 - 71 $\mu\text{g/ml}$ in human based leukemia cell series (HL60, K562) in Figure 2. It is possible that these differences in the lethal concentrations may be caused by the way extracts are prepared, by the origins of the cancer cells, and even by the differences between the species. In other study, Pathak et al. (Pathak et al., 2001) have analyzed the cytotoxic effect of Anvirzel

and oleandrin on the human and dog tumour cells. They have revealed that the cell killing potential of oleandrin is higher than that of Anvirzel. The results of these studies, it is thought that the main toxic effect in the extracts may be produce by oleandrin.

There have been some studies to explain how No extracts (Anvirzel) have anti tumour effect. In recently study showed that oleandrin produces apoptosis in prostate tumor cells and that this effect is mediated through inhibition of Na^+ , K^+ -ATPase with resulting increased intracellular calcium (McConkey et al., 2000). Smith et al. (2001) have demonstrated that anvirzel, like oleandrin, inhibited FGF-2 export in vitro from PC3 (51.9 %) and DU145 (30.8 %) prostate cancer cells. FGF-2, a regulatory peptide secreted from cells, is involved in a variety of biological processes including cell differentiation, cell growth and migration, angiogenesis, and tumor formation. Manna et al. (2001) have studied the effects of oleandrin and Anvirzel on cell signal mechanisms. $\text{NF-}\kappa\text{B}$ is a eukaryotic transcription factor that is critically

involved in regulating the expression of specific genes that participate in inflammation, apoptosis and cell proliferation. It is known that the agents suppressing the nuclear factor- $\kappa\beta$ (NF- $\kappa\beta$) and AP-1 activity play a big role in cell proliferation inhibiting the tumorigenesis and inflammation. In this study, it was shown that oleandrin and Anvirzel blocked NF- $\kappa\beta$ in various cells induced by TNF α . It has been seen that NF- $\kappa\beta$ activation improves the proliferation by blocking the apoptosis, and provides resistance against the chemotherapeutic agents. It is demonstrated that blocking the NF- $\kappa\beta$ is not by Na⁺, K⁺-ATPase pump (Manna et al., 2001; Streenivasan et al., 2003). These results make us think about the fact that No extracts have cytotoxic effect by apoptosis. But there is No study about the role of P-gp in this cytotoxicity in spite of above mentioned data. P-gp, a membran pump of importance in efflux of toxic substances should not be thought not to possess a possible role in cytotoxicity, because it was shown that digoxin efflux is mediated by P-gp in the small intestine and nephron in recent studies (Cavet et al., 1996; Johansson et al., 2001; Pauli-Magnus et al., 2001; Mikkaichi et al., 2004)

As a result, our study indicated that there is a direct or indirect relation between No extracts and P-gp. But, it is not obvious how this relation is occurred, yet. Moreover, No extracts may be much more cytotoxic by inhibiting P-gp pump. But, it has not been overlooked that the other proteins like MRP,LRP, BCRP responsible from transport may be effective in membran. Our study is an original one in terms of showing the P-gp role and the cytotoxic effects of No extract on in vitro leukaemia cells.

CONCLUSIONS

If it is taken into consideration that No extracts and effective cytotoxic chemical compounds (oleandrin etc.) may be used in cancer treatment in the future, our study is important especially in resistant cases in terms of the fact that it interacts with P-gp pump. As our findings are supported with the studies which will have more detailed in vitro biochemical and molecular basis, there may be a hope for the clinical practices.

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