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Original Research Article

Ligustrum vulgare leaves and fruit extracts induce apoptosis of human leukemia cells

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Abstract

Background and purpose. *Cytotoxic effects of* Ligustrum vulgare *leaves on HeLa cervical tumor cells suggested that* Ligustrum vulgare *extracts should be investigated as potential anticancer agents. Therefore, we examined a potential antileukemic activity of methanolic extracts of* Ligustrum vulgare *leaves and fruit extracts on two types of leukemia cells, MOLT-4 and JVM-13, lymphocytes isolated from the blood of 33 chronic lymphocytic leukemia (CLL) patients and on mononuclear leukocytes isolated from the blood of 18 healthy individuals.*

Material and methods. *The cytotoxicity of examined extracts was measured by MTT assay and LDH activity test. The antiapoptotic potential of tested extracts was measured by Annexin V/7AAD flowcytometric assay.*

Results. *The results showed that both extracts exhibited a moderate cytotoxic effect on all three types of leukemia cells. The* Ligustrum vulgare *leaf extract was the most effective on MOLT-4 cells, the fruit extract on JVM-13 cells and both extracts were equally effective on CLL cells. In addition, none of the tested extracts was toxic to healthy mononuclear cells. Both extracts acted by inducing apoptosis of leukemic cells.*

Conclusion. Ligustrum vulgare *extracts exhibit significant antileukemic potential and should be further investigated.*

INTRODUCTION

Natural extracts are a rich source of ingredients that have numerous applications in tumor therapy. In addition, a large number of natural ingredients provide basic molecules, which can be modified and used to improve therapy. Over 70% of the substances used in the treatment of tumors are either substances of natural origin or substances obtained by modification of natural substances *(1)*. Since less than 15% of higher plants have been systematically tested, the examination of natural substances as chemotherapeutics deserves increased attention and the application of multidisciplinary scientific research *(1, 2)*.

Ligustrum vulgare (LV) is the Latin name of a plant that is also known as wild privet, European privet or common privet *(2)*. It is a shrubby, deciduous plant, up to 5 meters high, very widespread in Central Europe and Southern Europe, Southwestern Asia and North Africa *(2-4)*. It is a common plant in oak belt forest communities. It is sometimes planted in parks and yards as a hedge. The plant, and especially the fruit of the plant, is considered to be poisonous, especially for children *(5)*.

Nowadays in the folk medicine of the Balkans, the leaves of LV are used in the treatment of soft tissue inflammation, and in the folk med-

icine of Cyprus and southern Italy, the leaves of LV are considered to have anti-inflammatory, antioxidant and antirheumatic effects, and are used accordingly *(4, 6, 7)*.

Application of LV in traditional medicine, as well as later scientific research indicating the cytotoxic effect of *Ligustrum vulgare* leaves on HeLa cervical tumor cells, suggest that *Ligustrum vulgare* extracts should be investigated as potential anticancer agents *(8)*. Various *in vitro* experiments with the extract of the leaves of this plant show extensive pharmacological potential due to the presence of flavonoids, phenylpropanoids and terpenoids *(9-11)*.

Chronic lymphocytic leukemia (CLL) is a disease characterized by the progressive accumulation of morphologically mature, but functionally immature lymphocytes in various tissues and organs. Since such lymphocytes cannot survive *in vitro* for a long time because they are subject to spontaneous apoptosis, to date, no cell line has been developed that corresponds to this disease *(12)*. Those few available cell lines derived from CLL patients (CD5– MEC1 and MEC2, PCL12, OSU-CLL and MDA-BM5) may represent EBV+ B-lymphoblastoid cells rather than bonafide B-CLL cells *(13-16)*. Therefore, there are very few preclinical studies examining the activity of various bioactive substances of natural or synthetic origin on CLL lymphocytes.

The main aim of this study was to examine the potential cytotoxicity of methanolic extracts of *Ligustrum vulgare* on two types of leukemia cells, MOLT-4 and JVM-13, lymphocytes isolated from the blood of CLL patients and on mononuclear leukocytes isolated from the blood of healthy individuals (control).

MATERIAL AND METHODS

The study was designed as an experimental study on the material of human origin *in vitro*. Two leukemic cell lines MOLT-4 and JVM-13, lymphocytes isolated from blood samples of CLL patients treated at the Clinic for Hematology of the Clinical Center Kragujevac, as well as peripheral blood mononuclear cells (PBMCs) obtained from the blood samples of healthy subjects in Service for the supply of blood and blood products KC Kragujevac, were used for the study. All chemicals were obtained from Sigma-Aldrich unless stated otherwise.

Cell lines

Two human leukemia cell lines were used in the study, namely the acute lymphoblastic leukemia cell line MOLT-4 (ATCC® CRL-1582 ™) and the prolymphocytic leukemia cell line JVM-13 (ATCC® CRL-3003). All cells were maintained at 37°C in an atmosphere containing 5% CO_2 in a complete cell culture medium consisting of RPMI-1640 with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS).

The examined population

The study included adult subjects of both sexes, who had a confirmed diagnosis of chronic lymphocytic leukemia based on clinical, laboratory and immunophenotypic parameters, who were treated at the Clinic of Hematology KC Kragujevac and who agreed to participate in the study following the Declaration of Helsinki *(17)*. Immunophenotypic parameters determined for the diagnosis of CLL were in accordance with the revised guidelines of the NCI Working Group *(18)*. The control group consisted of healthy subjects of similar gender and age structure, who did not suffer from diseases or conditions that could in any way affect the value of the examined parameters.

Sampling

The subjects of the experimental group, of which there were 33, were recruited in the study from the total population of patients with chronic lymphocytic leukemia who were treated at the Clinic for Hematology of the Clinical Center Kragujevac. Inclusion was carried out successively, according to the principle of random selection (without previously determining randomization) until a sufficient number of respondents was reached according to the calculation of the study sample size.

Based on the characteristics of the subjects of the experimental group, the control group of healthy people was selected after a medical examination, from a cohort of healthy voluntary blood donors (same-sex and age ± 2.5 years, for the appropriate person from the experimental group) and consisted of 18 subjects. Blood samples from the control group were obtained from the Blood and Blood Products Service of the Clinical Center Kragujevac. The inclusion of healthy subjects was also carried out according to the principle of random selection until the day when the total number of subjects was reached, and according to the calculation of the size of the study sample.

The study did not include subjects who at the time of the study had positive humoral markers of an inflammatory syndrome, disease or positive history of medication or substances (corticosteroids, cyclosporine A, alcohol, etc.) that could affect the values of the tested parameters.

All respondents were first asked for written informed consent. After obtaining consent, anamnestic data were collected, after which a blood sample was taken from the subjects. Mononuclear leukocytes were first isolated from the blood samples of the experimental subjects as well as the control group subjects according to the Boyum method *(19)*, and then the lymphocytes were separated from the monocytes according to the method described by Kennedy and Reynolds *(20)*.

Isolation of lymphocytes (CLL cells) and mononuclear leukocytes (PBMNCs)

In the morning, 10 mL of blood was collected from the study participants in tubes coated with anticoagulant

(Terumo®, Silicone coated EDTA) and CLL lymphocytes and mononuclear leukocytes were isolated from blood samples of CLL patients and healthy subjects, respectively. Afterwards, the blood was centrifuged at room temperature for 10 minutes at a rate of 400xg. Serum was then collected from the top of the tubes and later added to the medium (RPMI-1640) in which the cells were resuspended. From the contact layer of plasma and cells, a "white carpet" (a layer of white blood cells) was collected and transferred to tubes to which 3 mL of Histopaque 1077 (Sigma-Aldrich) had already been added. Centrifugation of these tubes for 25 minutes at 800xg separated mononuclear cells from polymorphonuclear leukocytes and erythrocytes *(19)*. Mononuclear leukocytes located in the layer between plasma and Histopaque 1077 were collected and washed three times in RPMI-1640 medium and resuspended in the complete medium which, in this case, consisted of RPMI-1640 with 2mM L-glutamine and 10% of autologous serum. Separation of lymphocytes from mononuclear leukocytes was performed due to the ability of monocytes to adhere to the plastic (bottom of the Petri dishes) previously coated with autologous serum *(20)*. Incubation of the mononuclear leukocyte suspension at 37°C in an atmosphere with 5% CO_2 for 1 h resulted in monocyte adhesion to Petri dishes, and the lymphocytes remained in suspension. Lymphocytes were aspirated, counted and resuspended in complete RPMI-1640.

Preparation of the extracts

Methanolic extracts of the leaves and fruits of the plant *Ligustrum vulgare* were obtained in the following manner. After collection, the leaves were dried and the fruits were used fresh, immediately after harvesting. The leaves and the fruit of LV were transferred to bottles with dark glass, and subsequently, 200 mL of methanol was added. After 24 hours, the extracts were filtered through Whatman No. filter paper. 1. The supernatants were dried in a vacuum at a 40°C using a rotary evaporator. The obtained extracts were transferred to sterile tubes and stored in a refrigerator at 4°C. Immediately before the experiments, the extracts were dissolved in dimethyl sulfoxide and diluted in the medium.

MTT assay

We investigated cytotoxicity of LV leaves and fruit extracts in concentrations from 10 μg/mL to 500 μg/mL (10 μg/mL, 25 μg/mL, 100 μg/mL, 250 μg/mL and 500 μg/mL). Both extracts were first dissolved in dimethyl sulfoxide (DMSO) and then diluted in the complete cell culture medium so that the final concentration of DMSO in the medium was never greater than 0.5% (*v/v*) *(21)*.

The viability of examined cells was determined after 24 and 48 hours of incubation with tested substances or in complete medium alone (control).

The 50μL of cells' suspension was added in microtiter plates and 50μL of the appropriate concentration of tested compounds was supplemented in each well. After 24 or 48 hours, another 100 μL of MTT working solution was added to each well and the cells were incubated for up to 4 hours at 37°C in an atmosphere with 5% CO_2. The plates were then centrifuged for 5 minutes at 400xg, the supernatant was carefully extracted, and 150 μL of DMSO was added to each well. The plate was then placed on a shaker for 30 minutes and then the absorbance at 595nm was evaluated on Elisa reader (Zenyth 3100, Anthos Labtec Instruments). Absorbance values of treated cells were compared with the absorbance values of untreated cells and based on that, the decrease (or increase) of cells' viability due to the action of examined extracts was calculated.

The cytotoxicity was calculated by the formula: Cytotoxicity (%) = 100%-((absorbance treated well–blank)/(mean absorbance control well–blank]) × 100) *(21)*.

IC_{50} values were calculated as we previously described *(21)*.

Lactate dehydrogenase activity test (LDH test)

The LDH test is a cytotoxicity test. If LDH activity is measured in the cell supernatant after 30 minutes to 4 hours from the moment the test substance is added, the LDH test shows the degree of cellular necrosis. If the length of treatment with the test substance is increased to 24 or 48 hours, the death of those cells that have entered the process of apoptosis is also detected. Examination of the change in LDH activity in the supernatant of treated cells in comparison to untreated cells after 48 hours of cultivation will detect concentrations in which bioactive substances have a cytotoxic effect on cells regardless of the dominant type of cell death induced by the test substances. The LDH test was performed using Lactate Dehydrogenase Activity Assay Kit (MAK066) by Sigma-Aldrich.

After placing the cells in the microtiter plate in the same manner as in the MTT test, the plate was incubated for 48 hours at 37°C in an atmosphere with 5% CO_2. After incubation, the plate was centrifuged for 5 minutes at 400xg, and the supernatant was transferred from each well to a new microtiter plate *(22)*.

Measurement of lactate dehydrogenase (LDH) activity from the lymphocyte supernatant 48 hours after treatment with the test substances was performed on an Olympus AU400 analyzer. The principle of the method is based on the fact that the enzyme LDH catalyzes the reduction of pyruvate to lactate at neutral pH. This reaction was coupled with the oxidation of NADH to NAD+. The decrease in the amount of NADH is proportional to the decrease in absorbance measured at 340 nm and is directly proportional to the enzymatic activity in the sample. The measured values of enzyme activity were compared with the activity of lactate dehydrogenase of untreated cells and based on that, the increase (or decrease) of LDH activity due to the action of bioactive substances was calculated.

ANNEXIN V-FITC apoptotic test

The effect of bioactive substances on the apoptosis of leukemic lymphocytes was examined using the ANNEXIN V-FITC apoptotic test. Annexin V-FITC/7-AAD (Annexin V– fluorescein isothiocyanate (FITC)/7-amino-actinomycin D (7-AAD) Apoptosis Kit, BD Biosciences) was used to determine the percentage of apoptotic cells after 48 hours of incubation *(23)*.

In the early phase of apoptosis, the integrity of the cell membrane is preserved, but the asymmetry of the membrane phospholipids is lost. Namely, phosphatidylserine is a negatively charged phospholipid that is usually found on the inside of the plasma membrane and which in the early phase of apoptosis is exposed to the outside of the cell membrane. Annexin V is a protein that can bind to calcium and phospholipids and binds to phosphatidylserine. In the late phase of apoptosis and necrosis, there is a loss of cell membrane integrity allowing viable dyes, such as FITC (fluorescein isothiocyanate) and 7-AAD (7-amino-actinomycin D), to bind to the DNA molecule. The kit of reagents used in our experiments contains 7-AAD that binds directly to DNA molecules. Therefore, Annexin V (-)/ 7-AAD (-) cells are assumed to be viable, Annexin V (+)/7-AAD (-) cells are in early apoptosis, Annexin V (+) / 7-AAD (+) cells are in the late phase of apoptosis and that the necrotic cells are Annexin V (-) / 7-AAD (+) *(24)*.

After treatment with bioactive substances, the cells were washed in PBS and resuspended in tubes with ice-cold binding buffer to a final cell concentration of $1x10^6$ / mL. Afterwards, 10 µl of Annexin-V-FITC and 20 µl of 7-AAD were added to 100 µl of cell suspension. The cells were then incubated for 15 minutes in the dark. The contents of the tubes were then resuspended in 400 µl of binding buffer and analyzed on a flow cytometer (The Cytomics FC500 Series, Beckman Coulter) to 20,000 events. Percentages of cells in early and late apoptosis and necrosis were determined using CXP Cytometer and Flowing software v2.5.

Table 1. *IC50 values after 48h* Ligustrum vulgare *leaves (LL) and fruit (LF) extracts treatment of human acute lymphoblastic leukemia cells MOLT-4, prolymphocytic leukemia cells JVM-13, chronic lymphocytic leukemia cells and peripheral blood mononuclear cells PBMCs.* *n.a. - not applicable

IC50 48h [µg/mL]	MOLT-4	JVM-13	CLL cells	PBMCs
LL	360±26	472±31	678±38	n.a.
LF	685±48	326±28	661±44	n.a.

Statistical analysis

All values were expressed as mean ± standard deviation (SD). Each experiment was performed in triplicate and conducted on every sample. Commercial SPSS version 20.0 for Windows was used for statistical analysis. The distributions of data were evaluated for normality using the Shapiro-Wilk test. Statistical evaluation was performed by Student's t-test for paired observations, or one-way ANOVA depending on data distribution. *P* values less than 0.05 were considered significant.

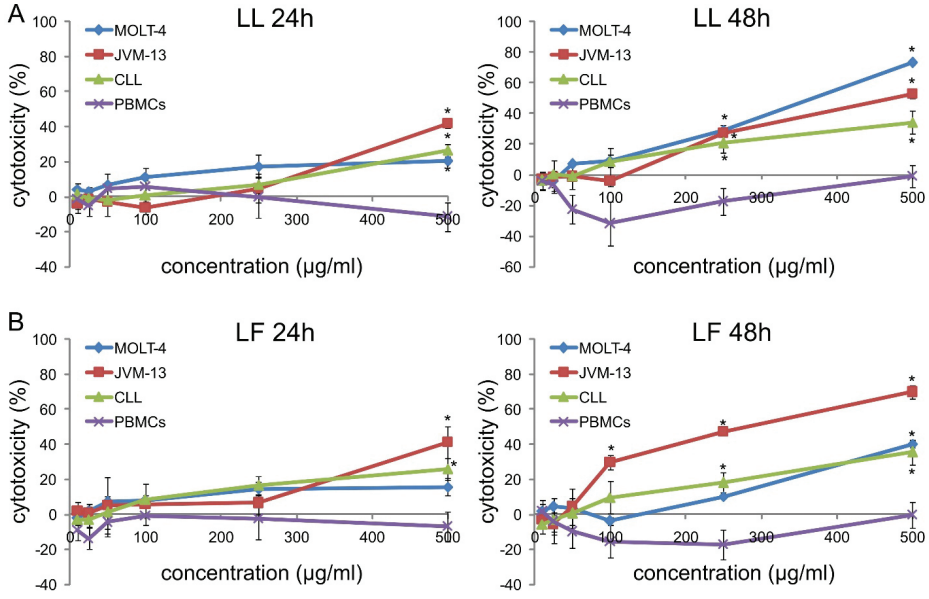


Figure 1. Antileukemic properties of *Ligustrum vulgare* methanolic extracts. *Cytotoxicity of Ligustrum vulgare leaves (LL) and fruit (LF) extracts against human acute lymphoblastic leukemia cells MOLT-4 (blue), prolymphocytic leukemia cells JVM-13 (red), chronic lymphocytic leukemia cells (green) and non-leukemic, peripheral blood mononuclear cells PBMCs (purple) after 24 and 48 hours. All values were expressed as mean ± standard deviation (SD). *p<0.05 compared to the untreated cells*

RESULTS

The results of the MTT test showed that both extracts exhibited a moderate cytotoxic effect on all three types of leukemia cells. After 24 hours, the most sensitive to the effect of the extracts were JVM-13 cells, where at high concentrations of extracts of 500 μg/mL the cytotoxicity of LL extract was 41.95 ± 2.72%, and the cytotoxicity of LF extract was 41.26 ± 9.09%. However, both extracts showed IC_{50} values> 500 μg/mL after 24 hours of incubation. Only after 48 hours of incubation of cells with LL and LF extracts, a stronger cytotoxic effect was manifested. The strongest cytotoxic effect after 48 hours of incubation, LL extract showed on MOLT-4 cells, with an IC_{50} value of 360 ± 26 μg/mL, slightly less potent cytotoxicity was displayed on JVM-13 cells with an IC_{50} of 472 ± 31 μg/mL and the least cytotoxicity of LL extract was displayed on CLL cells with an IC_{50} of 678 ± 38 μg/mL (Table 1, $p<0.05$).

On the other hand, LF extract showed the strongest cytotoxic effect after 48 hours of incubation on JVM-13 cells, with the IC_{50} value of 326 ± 28 μg/mL, and almost twice weaker effect on MOLT-4 and CLL cells with IC_{50} values of 685 ± 48 μg/mL and 661 ± 44 μg / mL, respectively (Table 1, $p<0.05$).

However, it should be emphasized that both extracts in the tested concentrations did not have a cytotoxic effect on PBMCs, ie on the population of non-cancerous cells neither after 24 hours nor after 48 hours of incubation in the tested concentrations (Figure 1, $p>0.05$).

To confirm the obtained results, the next step was to examine the cytotoxicity of LL and LF extracts using the LDH test. The results showed that both extracts display dose-dependent cytotoxicity to CLL cells, ie, with increasing concentration of extracts, the value of lactate dehydrogenase released activity in the supernatant of treated cells increases (figure 2, $p<0.05$).

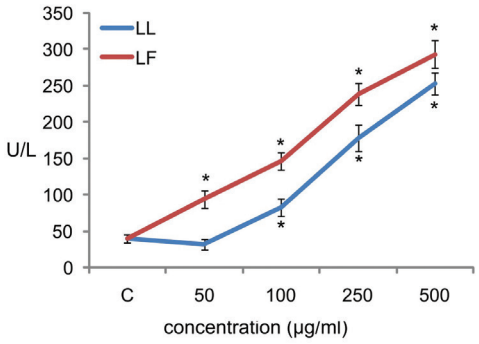
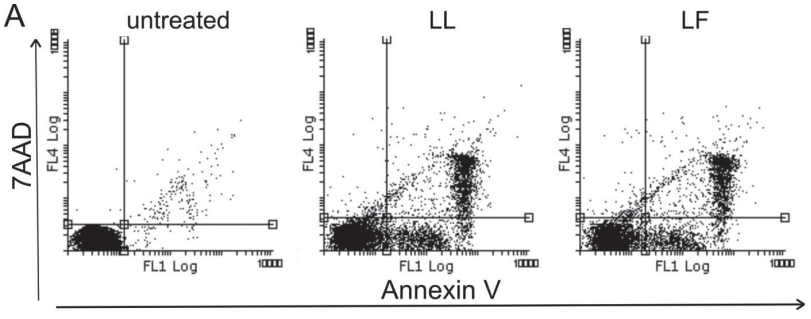


Figure 2. *Increase of LDH activity in chronic lymphocytic leukemia cells as a consequence of* Ligustrum vulgare *leaves (LL) and fruit (LF) extracts 48h treatment. U/l-Units per litre; All values were expressed as mean ± standard deviation (SD).* *p<0.05 compared to control

Since both tested extracts showed a cytotoxic effect on leukemic cells and did not have a cytotoxic effect on healthy cells, the next step in our study was to examine the mechanism of action of these complexes on leukemic cells (CLL). The results obtained by Annexin V/7AAD staining showed that the dominant type of cell death which leads to a decrease in the viability of treated tumor cells is apoptosis ($p<0.05$). The highest percentage of non-viable CLL cells is found in the stage of early (25.2 ± 9.1% and 20.7 ± 6.6%, for LL and LF extract, respectively) or late apoptosis (18 ± 6.8% and 20.8 ± 4.3%, for LL and LF extract, in that order). Only a small percentage of cells (4.2 ± 2.1% and 3.8 ± 1.7%, for LL and LF extract, respectively) were necrotic after exposure to IC_{50} values of LL and LF extracts (Figure 3).



B

	untreated	LL	LF
V	95.1±1.9	55.9±4.7	57.2±7.4
EA	0.8±0.6	25.2±9.1	20.7±6.6
LA	0.7±0.5	18±6.8	20.8±4.3
N	4.3±1.8	4.2±2.1	3.8±1.7

Figure 3. *Flow cytometric analysis of Annexin V-FITC/7-AAD stained CLL cells after 48 h treatment with IC50 of* Ligustrum vulgare *leaves (LL) and fruit (LF) extracts. Representative dot plots (A) and the percentages of viable (V-lower left quadrant), early apoptotic (EA-lower right quadrant), late apoptotic or secondary apoptotic (LA-upper right quadrant) and necrotic cells (N-upper left quadrant) in untreated and treated CLL cells. (B). All values were expressed as mean ± standard deviation (SD).*

DISCUSSION

Plants are an inexhaustible source of new compounds that can be used as medicines *(25)*. There is a wide number of medicinal plants, plant-derived products or isolated compounds, mainly polyphenols, with significant anticancer activity *(26)*. Their anticancer properties are mediated through various mechanisms including interaction with DNA repair systems, stimulation of immune system, antiproliferative activity on cancer cells, induction of apoptosis, alteration to the metabolism of anticancer drug and impact on progressive stages of carcinogenesis by suppression of angiogenesis and metastasis *(27-30)*.

Ligustrum vulgare is a plant that has been used for years in folk medicine in southern Europe *(31, 32)*. However, its antitumor properties have been very little studied. Despite the synthesis of a large number of chemical compounds such as metal complexes (Pt, Pd, Au, Cu, Zn, Ru, etc), only a small number of these compounds are further tested in vivo, and/or tested for these compounds in clinical studies. One of the reasons is that these compounds are very often too toxic for the body and are poorly tolerated *(33)*. Therefore, it may be necessary to change the strategy in testing substances with potential antitumor activity and to test not those substances that show the strongest antitumor activity *in vitro*, but those substances that are not toxic to non-tumor cells.

Extracts of the plant *Ligustrum vulgare* have not yet been sufficiently tested (34). To our knowledge, only two studies examined the cytotoxic effect of methanol extract of the *Ligustrum vulgare* leaves and fruit against cancer cells *(31, 32)*. The results showed that both leaf extract and fruit extract showed a dose- and time-dependent cytotoxic effect on HCT116 colorectal cancer cells with IC_{50} values of 28.2 ± 0.76 and 47.4 ± 3.54 μg/mL for leaf and fruit extract in that order. Compared to the results of our study where IC_{50} values for leukemia cells are between 300 and 600 μg/mL, these are about 10-15 times better results. Leaf and fruit extracts at 10-15 times lower concentrations reduce the viability of colorectal cancer cells compared to leukemic cells by 50%. The main reason is the fact that leukemic cells are generally more resistant to chemotherapeutics than HCT116 cells and that in CLL cells, the main pathophysiological mechanism of disease development is inhibition of apoptosis, and inhibition of excessive cell proliferative activity *(35)*. However, the main result of our research is that these extracts do not influence the viability of mononuclear cells, ie. healthy, non-tumor cells. What Curcic et al. have shown is that these extracts in combination with palladium complexes act even more effectively on tumor cells, so they could be used as adjuvants in the treatment of cancer *(31)*.

The leaf extract was shown to be most effective on MOLT-4 cells, while the fruit extract was most effective on JVM13 cells. Both extracts acted similarly to CLL cells.

The basic mechanism of action of these extracts is the induction of apoptosis. Namely, the results of our research showed that the dominant type of cell death, which leads to a decrease in the viability of the tested cells, is apoptosis. These results are consistent with the results of other, similar studies *(8, 31)*.

CONCLUSION

We must emphasize that the leaf extract was the most effective against the proliferation of MOLT-4 cells and the fruit extract against JVM-13 cells and that both extracts were equally effective on CLL cells. But above all, none of the tested extracts was toxic to healthy mononuclear cells. Both extracts acted by inducing apoptosis of leukemic cells. Although the antileukemic potential of these substances is relatively moderate, it should not be overlooked that in combination with other chemotherapeutics, these compounds might be more effective in the treatment of leukemias, especially considering that *Ligustrum vulgare* extracts do not have toxic effects on healthy cells.

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