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Cytotoxic Effect of Aqueous and Alcoholic Extracts of Pterocarya fraxinifolia Leaves on K562 Cell Line

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Abstract

Background: Chronic myeloid leukemia (CML) is a malignant clonal disorder of hematopoietic stem cells which results in increase of myeloid cells, erythroid cells and platelets in the peripheral blood and hyperplasia in bone marrow. *Pterocarya fraxinifolia*; (Juglandaceae) is widely distributed in northern area of Iran. The research evaluates the cytotoxic effect of n-butanol fraction, aqueous, methanolic and ethanolic extracts of *P. fraxinifolia* leaves on K562 cell line as a model of chronic myeloid leukemia.

Materials and Methods: Leaves of *P. fraxinifolia* collected from Astara city and extraction using soxhlet method. K562 cells were cultured and treated with concentrations of extracts (12.5-400 µg/ml). Cytotoxicity of *P. fraxinifolia* extracts against K562 leukemia cells was estimated by the MTT test method. The absorbance was measured using an ELISA plate reader at 540 nm.

Results: Ethanolic extract showed the highest cytotoxic effect (IC_{50}=148.66±11.64 µg/ml) whereas n-butanol fraction extract were least cytotoxic effect (IC_{50}=248.97±6.71 µg/ml) among the extracts. Aqueous and methanol extracts showed the cytotoxic effect with the IC_{50}=183.14±4.71 and 226.02±6.08 µg/ml on K562 cell line. Both ethanolic and n-butanol fraction extracts exhibited a dose-dependent cytotoxic effect on K562 cell line.

Conclusion: Considering the cytotoxic effects aqueous and alcoholic extracts of *P. fraxinifolia* leaves on K562 cells, the plant can be considered as a potential candidate for further studies on CML treatment.

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Introduction

Chronic myelogenous leukemia (CML) is one the most known types of leukemia that causes in blood stem cells by a reciprocal translocation between Ab1 gene on chromosome 9 and Bcr gene on chromosome 22 [1]. The resulting Bcr-Abl oncogene of this reciprocal translocation encodes $P210^{Bcr-Ab1}$ protein whose continuous protein kinase activity leads to myeloid cells precursor uncontrolled proliferations and disorder in apoptosis. Different methods used for treatment of CML [2]. For example, the first line of treatment is using the tyrosine kinase inhibitor of imatinib mesylate that specifically inhibits Bcr-Abl, but using this drug causes side effects such as drug resistance [3]. Also the drugs applied to chemotherapy, despite that they lead to induce apoptosis and growth inhibition, their side effects and the resistance that cancer cells show to them considers as major problems of this method [4]. All these cases indicates the fact that human knowledge doesn't achieve to a proper and remarkable place in leukemia treatment yet, thus, efforts continue to find high potential, novel and effective compounds and drugs with no negative effects of chemotherapy drugs.

Medicinal plants, due to their availability and reduced side effects always have been considered as proper alternatives for synthetic drugs and especially interested by researchers in recent decades [5]. *Pterocarya fraxinifolia* (Lam.) Spach from Juglandaceae family is a wild tree that grows in north of Iran, its small populations also is observed in Lorestan and Ilam provinces, and in addition of Iran grows in Turkey and Caucasus [6]. Local people use its leaves as anesthesia drug to catch fish and its leaves and bark contains joglone [7]. The leaves of *P. fraxinifolia* also have antifungal and antibacterial properties [8]. Studies have been performed on different parts of this plant show the presence of phenol and flavonoid compounds [6, 9]. Studies also show the antioxidant effect of this plant [6].

Considering that very few studies have been conducted on *P. fraxinifolia* so far, especially no cytotoxicity studies, and also no definitive treatment offers for CML, thus, the purpose of current study is investigation of *P. fraxinifolia* aqueous and alcoholic extracts on cell line K562 as a CML model to assess its effectiveness in treatment of this type of leukemia.

Materials and Methods

This was an experimental study and has been conducted on chronic myeloid leukemia cells. The *P. fraxinifolia* leaves were collected in summer 2011 from Astara, and after its verification by herbarium expert of Islamic Azad University of Falavarjan, were air dried and then

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grounded. Extraction was done with 20 g of plant dry powder using methanol and ethanol 80% solvents and twice distilled water by a soxhlet extractor for 12 h [10]. Methanol and ethanol solvents and aqueous solvent were vaporized in temperature room and 40ºC dry heat, respectively and the resulting dried extract was kept in 4ºC. In order to preparation of n-butanol fraction extract, initially 10 g of leaf powder was soaked in 30 cc petroleum ether for 24 h and after separating liquid phase from plant, was extracted using methanol solvent for 12 h by soxhlet extractor. The resulting dried extract was mixed with 20 cc water and 20 cc n-butanol, was poured into decanter funnel to separate aqueous phase from n-butanol phase [11], then dried with 40ºC dry heat and the resulting dried extract was maintained in 4ºC. To prepare 12.5, 25, 50, 100, 150, 200, 250, 300, 350, 400 µg/ml concentrations of each extract, DMSO (Dimethyl-sulfoxide), (Sigma, Germany) and PBS (Phosphate Buffer Saline) were applied.

Cell line K562 was purchased from Pasteur Institute of Iran and transported to cell culture laboratory of Islamic Azad University of Falavarjan. Cells were cultured in RPMI-1640 (PAA- Austria) medium enriched with 10% fetal bovine serum (FBS), (Gibco-Scotland) and Streptomycin (50 µg/ml) and penicillin (50 IU/ml) antibiotics, in an incubator with 5% CO_2, 37ºC and humidity 95% [12]. After 5 or 6 times passages, cells went to logarithmic phase.

In order to investigate the leaf extracts effect of *P. fraxinifolia* on cell death rate, methyl thiazol tetrazolium (MTT) assay was used which is based on breaking tetrazolium yellow salt into purple insoluble crystals in pramosone water via metabolically mitochondrial activity of living cells [12].

To this purpose, 180 µl cell suspension contained 3×10^4 cells was poured into 96 wells of a microplate and incubated 24 h in mentioned conditions. Afterwards, 20 µl of different extract concentrations (12.5 to 400 µg/ml) were added to wells. The microplate was then incubated for 48 h and after this time, 180 µl culture medium of each well was exchanged with new medium and 20 µl MTT (Sigma-Germany) was added to each well and incubated for 3 h. Next, 180 µl of the old culture medium contained MTT was exchanged with 150 µl DMSO to dissolve pramosone crystals and absorbance was read in 540 nm by ELISA plate reader (Statefax-USA) [12].

Each concentration of sample was repeated in 3 separate experiments and in each experiment 4 replicates was used. The growth rate in negative control samples considered 100% and doxorubicin was used as positive control. Statistical analysis was carried out by SPSS-18 software and ANOVA test and consequently Tukey test were carried out. Charts were drawn by Microsoft Excel 2007.

Results

Investigating cytotoxic effect of methanol, ethanol, n-butanol fraction and aqueous extracts (Fig. 1) showed that all 4 extracts have cytotoxic effect on chronic myelogenous leukemia cells (K562). Ethanol and n-butanol fraction extracts showed their effect dose-dependent, but methanol and aqueous extracts effects increased by reducing concentration. So that, the most effective concentrations that killed more than half of the cells, was 150-400 µg/ml for ethanol extract, 12.5-250 µg/ml for aqueous extract, 12.5-200 µg/ml for methanol extract and 250-400 µg/ml for n-butanol fraction extract (Fig. 1).

According to table 1, ethanol extract had the highest cytotoxic effect among the other extracts and n-butanol fraction extract had the lowest effect, and aqueous and methanol extracts after ethanol extract had the highest effects respectively.

Discussion

The findings of current study, indicates the fact that ethanol and n-butanol fraction extracts of *P. fraxinifolia* leaves have cytotoxic effect on K562 cell line, as a model for chronic myelogenous leukemia, that is dose-dependent and the effect of aqueous and methanol extracts increased by reducing concentration. Also, the highest anticancer effect is for ethanol extract and aqueous, methanol and n-butanol fraction extracts have the next highest effects respectively.

Juglone is a naphthoquinone compound that is the most prominent compound in different organs of walnut especially in its leaves [13]. According to researches, juglone was separated by RP-HPLC method from different extracts of *P. fraxinifolia* leaves and bark such as methanol and aqueous. In this study, juglone level in 100 g of the plant dry leaves, in May, June, July, August and September, was determined 2.15, 2.74, 1.77, 1.12 and 0.34 g respectively [7]. In addition, this compound was separated by chromatography from methanol extract of *P. fraxinifolia* [8]. Different researches show the anticancer effect of juglone. Juglone has dose-dependent cytotoxic effect on cell line SGC-790 (stomach cancer). Bcl-2 expression reduces by increasing concentration of this compound and conversely, Bax expression increases. This leads to increasing mitochondrial transfer power and releasing cytochrome c and caspase 3 and 9 activation and apoptosis occurs [14]. The anticancer effect of juglone has been approved also on HL-60 (acute myelogenous leukemia) [15] and HCT-15 (Intestine carcinoma) cell lines [16]. This research determined that ethanol, aqueous, methanol and n-butanol fraction extracts have anticancer effect on K562 cell line. Thus, according to cytotoxic property proof of juglone on different cancer and considering the presence of this compound in *P. fraxinifolia* leaves, one of the reasons for the result of this study can be attributed to this compound. It should be noted that Girzu et al. reported that keeping juglone in methanol solution is not sustainable [17].

This research specified that methanol extract had less cytotoxic effect than ethanol and aqueous extracts and its fatal effect increased on K562 cells by reducing concentration.

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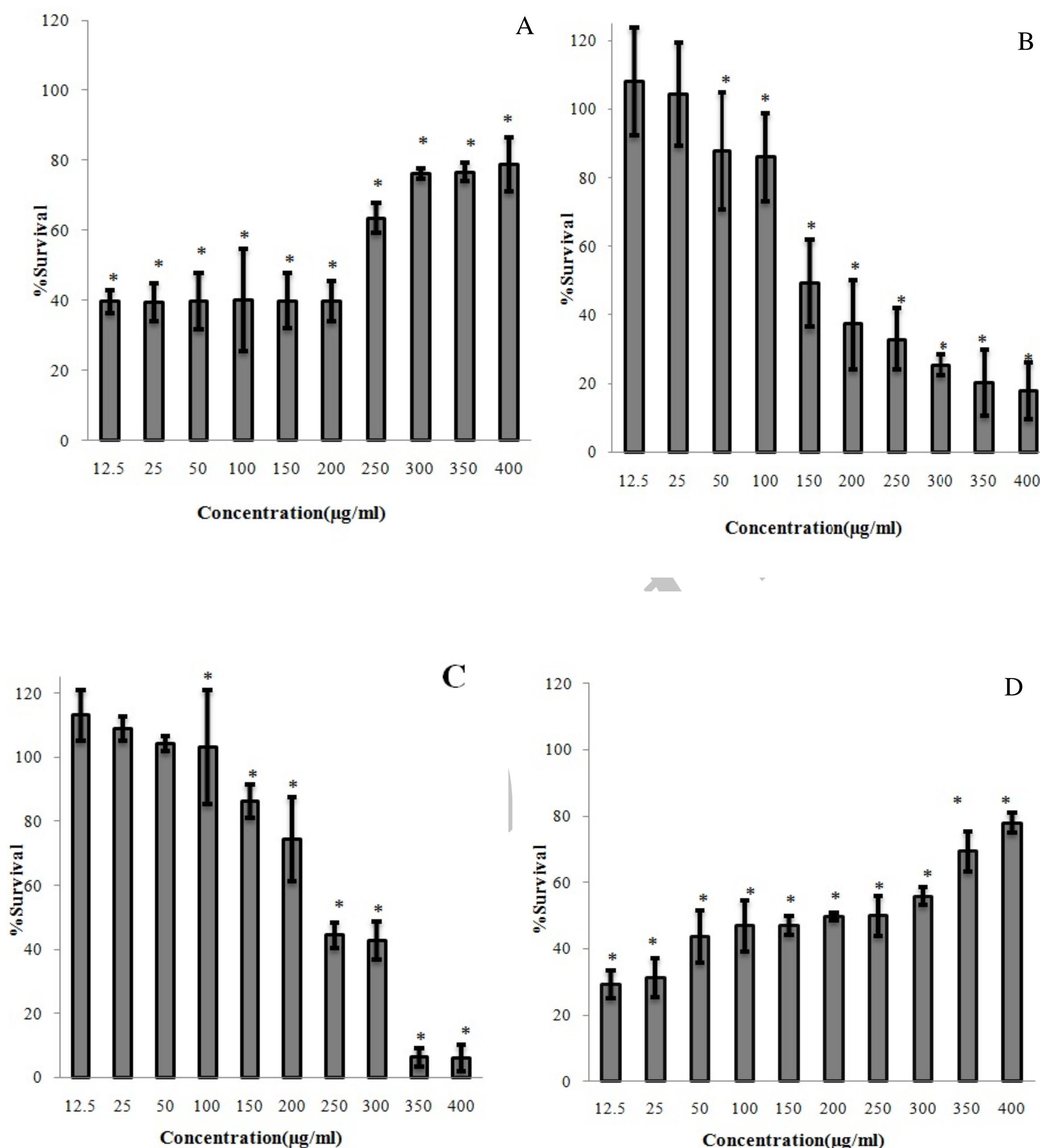

Figure 1. The cytotoxic effect of *P. fraxinifolia* leaves methanol (A), ethanol (B), n-butanol fraction (C) and aqueous (D) extracts on K562 cell line. N=12, * *p*<0.05

Table 1. IC_{50} level of *P. fraxinifolia* leaves extracts on K562 cell line

Extract	IC_{50} (µg/ml)
Ethanol	148.66±11.64
Aqueous	183.14±4.71
Methanol	226.02±6.08
N-butanol fraction	248.97±6.71

IC50 ± Standard deviation

The investigations showed that *P. fraxinifolia* leaves and bark have antioxidant effect and phenol and flavonoid compounds and these compounds level in leaf is significantly more than branch bark [6].

Diverse biologic activity of flavonoids and other phenolic compounds including their antioxidant effects, has been reported in many studies. It is also approved that the origin of many pharmaceutical and medical materials is due to secondary metabolism in plants that phenolic compounds with antioxidant and pharmaceutical properties are of these metabolites [18]. Flavonoids can cause apoptosis in cancer cells by different methods such as topoisomerase I and II inhibition, cascade caspases activation, inhibiting effective enzymes in cell proliferation like cyclooxygenase, lipooxigenase, xanin

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oxidase and ornithine decarboxylase and etc. There are various evidences that show a correlation between increasing flavonoid level and decreasing cancer risk [19]. For example, the effect of more than 30 types of flavonoids on two colon cancer cell lines including Ht-29 and Caco-2 has been shown that most of these compounds have decreasing effect on this cancer cells proliferation [20]. A flavonoid named quercetin has cytotoxic effect on K562 cells. This compound leads to reducing the regulation of C-myc and Ki-ras oncogenes and IP3 (Inositol-1, 4, 5-trisphosphate) rapid reduction in cells [21]. In another research, 28 flavonoids has been investigated on HL-60 cell line and revealed that 8 compounds of them have significant inhibitory effect on these cells proliferation [22]. Increasing the concentration of phenolic compounds directly increases the ability of different extracts to inhibit free radicals [19]. Al Tavel et al., indicated that n-butanol fraction extract and the flavonoid extracted of this fraction that has been obtained from *Rapistrum rugosum* aerial parts, have cytotoxic effect on HepG2 cell line (hepatic carcinoma) [23]. Also in another investigation, the cytotoxic effect of *Arctium lappa* L. fruit n-butanol fraction extract has been proved on HepG2 cell line [24]. In this research it was determined that ethanol and n-butanol fraction extracts have dose-dependent anticancer effect on K562 cell line. Therefore, it is likely that one of the reasons for this is more extracting of phenol and flavonoid compounds in higher concentrations of these two extracts. Of course, methanol and aqueous solvents can also extract these compounds because of their high polarity, but this research has shown that these two *P. fraxinifolia* leaf extracts are more effective in lower concentrations, therefore, the reason of this fact is probably that in addition to useful compounds like flavonoids and phenols, methanol and aqueous solvents extract other compounds that due to increasing of this ineffective substances, the effect of compounds like flavonoids and phenols is reduced in higher concentrations.

Considering the very few researches has already been carried out on this plant and in order to improve researching in this field, some implementations is essential such as extracting different types of secondary metabolites from this plant species and examining the effects of different plant parts extracted compounds on different cancers and understanding the cell death molecular pathways induced by them.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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