Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 3 Spl Issue III 2014: 186-190

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

Full Length Article

Study of cytotoxic effects of Ethanolic extract of *Eucalyptus camaldulensis* leaf on the cells k562 of human chronic Myelogenous leukemia (CML) under *in Vitro* conditions

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ABSTRACT
Chronic Myeloid Leukemia (CML) is a common and malignant clonal disorder of basic hematopoietic cells which results in increase of myeloid cells, erythroid cells and platelets in the peripheral blood and hyperplasia in bone marrow. Eucalyptus has many health benefits and antioxidant properties. The research evaluates the cytotoxic effects of ethanolic extract of Eucalyptus camaldulensis leaves on the cells K562 as a chronic myeloid leukemia.After culturing and proliferation of K562 cells, these cells were exposed to various ethanolic extract of Eucalyptus by 12.5, 25, 50, 100 µg/ml and were incubated for 24, 48 and 72 hours to determine antioxidant effect of ethanolic extract of Eucalyptus leaf . The MTT assay was used to assess the cytotoxicity of the extract after incubation. The results of MTT assay test showed that, the compounds existing in ethanolic extract of Eucalyptuscamaldulensis leaf have time dependent and dose independent cytotoxic effects on K562 cells.The ethanolic extractof Eucaleptusleaf has efficient antioxidant compounds and can treat some diseases such as cancer by inhibiting free radicals. The use of Eucalyptus with acceptable doses of antioxidant can be recommended asinhibitors of the growth of tumor cells.
Key Words: K562 cell line, Eucalyptus, MTT assay, Leukemia

Received 17.03.2014 Revised 19.05.2014 Accepted 15.06. 2014

INTRODUCTION

Leukemia is a kind of cancer which begins from blood-forming tissue. Leukemia means white blood. This disease disrupts the process of reproduction, hematopoiesis and normal immune of the body. The accumulation of cancer cells outside the bone marrow causes to form tumors in vital organs of the body such as the brain or enlargement of lymph nodes, spleen, liver, abnormal performance of the body's vital organs.Various types of leukemia form about 15% of total human population cancers and is known as the fifth common cancer in the world. Numerous factors such as age, gender, race, blood group, radiation and H. pylori-infected mothers, affect the incidence of leukemia. Cancers of hematopoietic tissues of the body including bone marrow and lymphatic system are generated by white blood cells and lymph. White blood cells usually grow as controlled and regular if the body needs; but, leukemia hampers this process and makes the growth blood cells out of control [1, 2]. In this disease, philadelphia chromosome is created by a reciprocal translocation between the long arms of two chromosomes No. 9 and No. 22. During this movement, part 3 of the ABL gene on chromosome 34q9 is transferred to section 5 of the BCR gene and a BCR-ABL Fusion Gene is created which generates an mRNA during transcription [3].

On the other hand, plant compounds have been used for several centuries to treat cancers. The investigations show that, over 1400 plant genus have been used to treat cancers. Some tests have been conducted on the plants used in traditional medicine for treating cancer, and found antitumor compounds in them [4]. Eucalyptus camaldulensis is a plant of Myrtaceae family and has been generated by Pacific huge and fast growing trees spreading around the world, and vast forests have been created in India, and Morocco [5]. There are some chemical compounds such as valeric aldehyde, small amount of ethylic alcohol, acetate, a crystallization substance, etc. in most of eucalyptus species. 60-80% of the essence is cineole which is the most important and main material forming Eucalyptus essence [6, 7, 8]. Eucalyptus is highly used in traditional treatment due to its biological activities such as antioxidant, antibacterial and antivirus traits [9]. Eucalyptus eaf is the only used part of eucalyptus in terms of treatment. Middle-aged

leaves are more appropriate which have vasoconstrictor, refrigerant and disinfectant effects. Moreover, Eucalyptus leaves contain eucalyptol (cineol), tryneol,sesquiterpene alcohols, aliphatic aldehyde, flavonoids, phenols, alcohols isoamyl and terpenes [10]. Since, the effects of cytotoxic of this species on K526 line has not been yet investigated, this study has attempted to investigate the effect of ethanolicextract of Eucalyptus on K562 line using ANOVA test.

MATERIALS AND METHODS

This study was conducted in Azad University of Falavarjan in 2012. Eucalyptus camaldulensis leaves were collected in the late November 2012, after the sample correspondence and identification of the species withherbarium specimens of Agriculture and Natural Resources Research Center of Isfahan with identification code 15257, the samples were washed by distilled water and were dried under appropriate conditions. The leaf of dried plant was powdered in laboratory by a sterile mechanical grinding. 50 g of the powder with 300 ml of 98% alcohol was thrown in a suitable and dark container and was shaken for 48 hours. After extracting, the extracts were thrown in a sterile petri dish and were kept in a fully dark place to become as the form of wax or honey. The obtained extract was kept in a refrigerator until being used.

In this study, four different concentrations of the extracts (12.5, 25, 50. 100 µg/ml) were needed so, it was acted to provide these concentrations as below:

First, 0.01 gr of the extracts was weighted and then was dissolved in 1 ml of DMSO and was reached to the volume of 10 ml by PBS. The achieved material was considered as Stock. Thr considered concentrations were provided through Serial dilution method using deionized distilled water. Three replications were considered for each concentration. K562 cell line was provide from Pasteur Institute of Iran and was cultured inRPMI -1640 with10% FBS and incubated underconditions of 5% CO2, 37 ^{0}C, and then growth curve of the cells was produced. Cell counting was done by neobar lam after several times of cell culture and cells placement in logarithmic growth phase. Live cells percentage was determined using Trypan blue test; so that, the color 0.4% Trypan blue was produced and added to the cells. By color infiltration, dead cells with blue color were detectable. MTT was used to evaluate cytotoxic effects of the extracts. This method is Applicable even when the cell metabolically active but proliferation is not occurred and has a high accuracy and precision. Also, measurement of a much number of samples is possible due to the use of 96-unit micro plate. Therefore, K562 cell line was incubated in RPMI -1640 with 10% FBS and incubated under conditions of 5% CO2, 37 ^{0}C to determine the amount of extract cytotoxicity of Eucalyptus leaf; then, they were treated by the prepared concentrations of ethanolic extract of Eucalyptus leaves and were incubated for 24, 48, 72 hours under 37 ^{0}C and 5% CO_2. After passing the incubation period, its cytotoxicity was investigated by adding MTT. In this regard, $20\ \mu l$ of MTT solution to all units.Then, the 96-unit plate was kept in incubator for 3 hours under 37 ^{0}C and 5% CO_2. Then, $180\ \mu l$ of DMSO was added to each unit containing precipitated Formazan crystals. After that, light absorption was investigated using ELISA Stat-fax 2100 in wavelength of 540 nm. SPSS software (version 19, 20) and t-test were used to analyze the achieved results, and $p<0.05$ was considered as significance criterion.

RESULTS

The results achieved from ANOVA test and treating the cells by ethanolic extract of Eucalyptus leaf in the studied times showed that, viability percentage was decreased in 72 h incubation compared to 24 and 48 h incubation; so, this duration is the best to reduce viability percentage of K562 cells. Also, the best concentration in destroying the cancer cells was 50 µg/ml which had the highest cytotoxic effect compared to the other three concentration. However, al the studied concentrations almost equally affected the cancer cells. But, an accurate look on the numbers can conclude that, the concentration 50 µg/ml has had the highest cytotoxic effect on K562 cell line. The amount of this concentration is significantly different to the negative control group ($p<0.001$).

DISCUSSION AND CONCLUSION

Investigating the anticancer effects of plants and producing the effective drugs for treatment of cancer from them is an important research subject throughout the world which is being conducted in various dimensions [11]. In this research, inhibiting effect of ethanoic extract of Eucalyptus leaf on the growth of tumor cells has been investigated. The results of MTT assay stated that, the concentration does not any special trend in the amount of the cells' viability. In other words, viability control is not dependent on dose. However, it has shown higher cytotoxic effects by over the time and depends on the time. According to the results, there is a suitable relationship between the number and amount of light absorption in K562 cell line. According to Adebula et al. (1999), Takasaki et al. (2000) and Sidjiko et al. (2004), Eucalyptus

has been used to treat some disease such as influenza, tonsillitis, dysentery and skin diseases, and its leaf has anti-cancer, anti-inflammatory, analgesic, antioxidant, lowering blood sugar, anti-Malaria, anti-fungal and anti-viral properties [12, 13, 14]. Ashur (2008) studied the effect of essence and oil of Eucalyptus on the cells of HEPG2 and MCF7 lines and proposed anticancer anti-fungal and anti-bacterial properties. He stated that, cytotoxic variations of the cells are increased over the time. He studied the concentrations with low dilution [15]. In the present study, higher doses of the extract were used and acceptable results were achieved. However, cytotoxic effects of Eucalyptus' extract on viability of the cells of K562 is independent to dose. In a study conducted by Dahlbuskardin et al. (2012), the cytotoxic effects of Eucalyptus essence and the role of terpenes on tumor cells were investigated. In this study which was done on the cell lines NB4t ,HL60 ,Hela ,A549 ,HEPG2 ,MCF7t, K562 using Eucalyptus essence by MTT assay, the percentage of inhibition was announced by 50%. In this study, both young and old leaves were used and investigated for the treatment durations (24, 48, 72 hours). Cytotoxic effects were proved for some cell lines such as K562. So that, the best effect of essence was reported by 72 hours (16). The results of these scientists were the same as our research which showed that, the duration of 72 hours is the most effective time for the effect of Eucalyptus pant on this cell line. Of course, the consumption dose in our research was 59 µg/ml which shows that, Eucalyptus hydro-alcoholic extract in our research has had higher and effective antioxidant cases to eliminate cancer cells of K562. Also, it can be mentioned that, anticancer effects of Eucalyptus in our study is probably due to having terpene compounds. Eucalyptus also can have anticancer effect in the live creatures. A study was conducted on te effects of Eucalyptus on Swiss albino mice. In the mentioned study, 20-25 gr adult mice were used in six groups. Eucalyptus treatment were applied in concentrations 25, 6, 5, 12, 25, 50 and 100 mg/mlfor 5 days. Finally, the increase of concentrations caused to reduce tumor weight. The Eucalyptus prevented the cancer cells growth by interfering in G2 phase [17]. The cause of cancer cells inhibition in our research is to make disorder in G2 phase by Eucalyptus. In mammals, there are two main routes to apoptosis including lateral pathway which is by mediation ofthe cell surface receptors.In this way,causing death ligands create a band withdeath receptor such as Fas which is a transmembrane protein, and these receptors become closed and by activating thecaspase 8, apoptosis signaling pathway is stimulated and then, in a cascade mechanism, the other caspases including caspase 3, 6, 7, also are stimulated and cause cellular death. The other one is the main pathway in which, cytochrome C is released by mitochondrial membrane depolarization and makes a band with caspase 9 and Apaf-1 and then, the signaling pathway of cellular death is stimulated and by activating caspase 3, cellular death occurs. This pathway is adjusted by BCL proteins family. Generally, some factors such as caspases and protein kinase B (PKB), protein AKT and PI3Kinase are involved in regulation of apoptosis signaling pathway [18]. Probably, the compounds existing in ethanolic extract of Eucalyptus leaf causes morphological variations and inter-nucleosomal DNA fragmentation and apoptotic cell death which is due to activation of caspase 8 and 3 and release of cytochrome C. TCN is one of the most important flavonoid derivatives of Eucalyptus that has ability of anti-inflammatory and anti-cancer activity. In a study onTCN effects on human liver cancer cells, the results showed that, TCN causes to increase ROS level and reduce glutathione and results in planned cell death and induction of cascade of mitochondrial and apoptosis pathways. TCN activity leads to proliferation of cancer cells and causes a break in DNA. TCN causes induction of apoptotic death through caspase 8 activity. These studies probably represent mechanisms and trend of apoptosis action in ethanolic extract of Eucalyptus which is very important in inhibiting trend. Considering that, most of Eucalyptus types have cytotoxic properties, their inhibitory effect is a natural characteristic and they are very effective and inhibition apoptosis trend in tumors due to having an appropriate amount of terpenes, tannins, phenols and flavonoids [19]. According to Amakora et al. (2002), Eucalyptus has a variety of phloroglucinol and tannins which have antioxidant biological activities. In many flavonoids, their strong antioxidant properties are due to the reaction of phenolic hydroxyl groups [20]. Diverse biological activities of flavonoids and other phenolic compounds including antioxidant, antimicrobial and anti-inflammatoryeffects have been reported in many studies. Also it has been proved that, the origin of many medicinal and treatment material is due to secondary metabolism in plants; and phenolic compounds with antioxidant and medicinal properties are considered as secondary metabolites in plants. Studies have shown that, the risk of cancer with consumption of foods enrichedby polyphenols is inversely related. Many studies indicate that, flavonoids have anti-cancer effects. The flavonoids cause apoptosis in cancer cells by different ways includinginhibition of topoisomerase I and II, the reduction of reactive oxygen species, heat shock protein expression, release of cytochrome C and activation of caspase 9 and 3, increased expression of apoptosis-stimulating proteins, increased expression of nuclear transcription factor and activation of endonucleases, and inhibition of protein MCL-1. Also, we have in continue that, flavonoids prevent proliferation of cancer cells by inhibiting cyclooxygenase, lipo-oxygenase and xanthine-oxidase enzymes. It should be noted that, activities of these enzymes are increased in tumor

cells. Several experiments state inhibiting effect of flavonoids on ornithine decarboxylase stimulated by the tumor which causes to reduce polyamines and inhibition of protein synthesis. Flavonoids also inhibit conducting signals pathway including PTK, PKC and P13Kinase, all of those have a significant role in proliferation of cancer cells process. Flavonoids are able to stop cell division cycle in cancer cells through inhibiting the G1, G2/M, CDKs phases which is the important regulator of cell cycle. There are many evidences about the existence of a correlation among the increase of flavonoids and cancer risk reduction [21]. In the present study, the flavonoids existing in Eucalyptus has caused to generate apoptosisin the discussed cancer cells. It can be mentioned that, the existing flavonoids has caused viable reduction of the cancer cells through the mentioned pathways. Some other cases also could be mentioned including: the interval 1996-1997 of group studies indicate a 70% risk reduction in cancers of the oral cavity, pharynx, larynx and trachea resulted from flavonoidsconsumption. The effect of more than 30 types of flavonoid on two lines of colon cancer with Ht-29 and Caco-2 indicated that, most of these compounds have decreasing effect on proliferation of these cancer cells [22]. In this study it was found that, Eucalyptus ethanolic extracts with concentration of 50 µg/ml as non-dose-dependent and time-dependent have acceptable cytotoxic effects on the cell line K562, and so, as it was mentioned, reduction of free radicals in cancer cells and inducing apoptosis in them is a mechanism of actions of various compounds such as flavonoids and phenols. According to the mentioned contents, the amount of flavonoid compounds is an important cause of the studied extracts' effectiveness. Also it should be mentioned that, the extract is able to extract an appropriate amount of flavonoid and phenolic compounds but, the cause of increasing inhibiting effect of extracts by decreasing the concentration may be extraction of some compounds which have reduced cytotoxic effect of the flavonoids which has led to reduction of its cytotoxic impact by increasing the concentration of this extract. Of course, the extract was able to extract various useful compounds due to the existence of water which had been used to dilute the extract; but, not all the extracted compounds have necessarily have cytotoxic impact.

ACKNOWLEDGEMENT

At the end of this study, I appreciate the masters who helped us by their valuable experiences. I wish their success in all scientific areas.

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