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Anti-proliferative effects of alcoholic and aqueous extract of Ginkgo biloba green leaves on MCF-7 cell line

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Abstract**:** ***Breast cancer is the most frequently diagnosed cancer and the first leading cause of cancer death in females worldwide. Ginkgo biloba, the ginkgo tree or Maidenhair tree is the oldest living tree, with a long history of use in traditional Chinese medicines. Antioxidative effect of ginkgo extract was demonstrated but there have been very limited studies on the anti-proliferative activity of Ginkgo biloba extract. The aim of this study is to evaluate anti-proliferative effects of alcoholic and aqueous ginkgo extracts on human breast cancer cell line (MCF-7). MTT assay were performed using AGE and AQGE concentration of 5, 25,250,500,1000,2500 ,5000µg/ml in time intervals of 24, 48 and 72 h. Our results showed the highest concentration (5000 µg/ml) of AGE and AQGE had an anti-proliferative effects at in 24, 48 and 72 hours of incubation(AGE IC50 24h =2143.3±235.88 µg/ml, r^2=0.912, AQGE IC50 24h =2470±352.69 µg/ml, r^2=0.904). AGE and AQGE showed a significant cytotoxicity effect in compare to control group in MCF-7 cell line. Anti-proliferative effect of Ginkgo extract revealed this fact that AGE and AQGE could have cell cytotoxicity characteristics beside its cytoprotection mechanisms.***

Keywords: Ginkgo biloba; Alcoholic ginkgo extract; Aqueous ginkgo extract; MTT assay.

1. Introduction

Cancer is a leading cause of cancer related death worldwide, accounting for 8.2 million deaths in 2012. Lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year. It is expected that annual cancer cases will rise from 14 million in 2012 to 22 within the next two decades. [1]

Drugs used for the treatment of cancer are not effective and serious side effects are the main problem in chemotherapeutics of cancers. This has forced scientists to find more effective drugs with less toxicity.

Herbal medicines are generally low in cost, plentiful, and show very little toxicity or side effects in clinical practice. Traditional Chinese medicine-based herbal medicines have gained increasing acceptance worldwide in recent years and are being pursued by pharmaceutical companies as rich resources for drug discovery. [2]

G*inkgo biloba*, the ginkgo tree or Maidenhair tree is the oldest living tree, with a long history of use in traditional Chinese medicine. In recent years, its leaf extracts have been widely sold as phytomedicine in Europe and as a dietary supplement worldwide. Effects of *Ginkgo biloba* extracts have been postulated to include improvement of memory, increased blood circulation, as well as beneficial effects to sufferers of Alzheimer's disease. The most unique components of the extracts are the terpene trilactones, that is, ginkgolides and bilobalide. [3]

Recent studies conducted with various molecular, cellular and whole animal models have revealed that leaf extracts of *Ginkgo biloba* may have anticancer (chemopreventive) properties that are related to their antioxidant, anti-angiogenic and gene-regulatory actions.[4]

However, there have been very limited studies on the anti-proliferative activity of *Ginkgo biloba* extract.

The aim of this study is to evaluate the role of alcoholic and aqueous extract of ginkgo biloba green leaves on human breast cancer cell line (MCF-7).

2. Materials

Human breast cancer cell line (MCF-7) was purchased from Pasteur institute of Tehran-Iran with C135 NCBI number. Other necessary materials were obtained with details below:

MTT (dimethylthiazol diphenyl tetrazolium Bromide) from Sigma-Aldrich company with Lot No 123k74156 & EC No 32-608-9. Fetal bovine serum (FBS), TRYPSIN-EDTA (1x), Culture medium RPMI 1640, Dulbecco's PBS (1x) with Ca&Mg (sterile), Penicillin/Streptomycin (100x), all from PAA (Austria).

Plant Material

Ginkgo biloba leaves were collected from *Noshahr Botanical Garden, Mazandaran,Iran*. Green leaves were harvested in December 2012. A voucher specimen was deposited in the herbarium of the Faculty of Pharmacy. Botanical specification was adapted and confirmed by Noshahr Botanical Garden. The leaves were dried completely in the shade for 5 days and were triturated in a mechanical mill. The powder kept in tightened protected containers before use.

3. Methods

Extract preparation:

Aqueous extract: Dry powder of leaves (200g) were extracted with soxhlet extractor.The powder were placed in filter paper , then put this package in B part of glass soxhlet equipment and extracted with 250 ml diluted water for 4 hours. After the extraction procedure, the solvent were evaporated by Rotary Evaporator at 35–40 °C. Finally the extract was freeze dried for 24 hours to provide extract powder.

Alcoholic extract: using Maceration , 300 grams of dried powder leaves were placed in a stoppered Container with the solvent (200 ml ethanol 70%) and allowed to stand at room temperature for a period of 7 days with frequent agitation until the soluble matter had dissolved. The mixture then was strained, the marc (the damp solid material) was pressed, and the combined liquids were clarified by filtration with filter paper and the solvent were evaporated with Rotary evaporator at 35–40 °C. Finally the extract was freeze dried for 24 hours to provide extract powder. [5],[6]

Concentrations preparation:

In order to prepare stock solution (the most concentrated solution), 0.025 grams of extract powder dissolved in minimum amount of culture medium. A filtration was done using a 0.22 micron filter. Then diluted with complete culture medium to 5 ml volume.

Other concentrations (2500, 1000,500,250,25,5 µg/ml) were obtained by dilution of the stock. The solvent was complete culture medium (RPMI with 10% FBS). All processes were performed in sterile condition.

In order to increase the preciseness and reduce errors during this process, concentrations were obtained in greater volumes than usual.

Culture of cells

Breast cancer cell line MCF-7 was obtained from Pasteur institute (Tehran, Iran). Cell line was routinely grown in RPMI-1640 medium containing 10% FBS, 1% antibiotic(Pen/Strep) in 25 cm^2 plastic flasks (Nest biotechnology, China) at 37 °C in a 5% CO_2 humidified atmosphere. Cells were passaged at 70–80% confluence, about twice a week by trypsinization. For MTT experiment, the MCF-7 cells were seeded in 96 multiwell plates (Nest biotechnology, China) at 10^5 cell/well in 200 µl of complete culture medium and cells were allowed to attach for 24 h. After this time the culture medium was removed and each concentration of ginkgo biloba, within a range from 5 to 5000 µg/ml, was tested in triplicate wells. The positive controls (Etoposide 10 to 100 µmolar) and negative controls (cells without extract) were included on each plate. The cells were then incubated for various periods of time (24, 48 & 72 h). [7]

a) MTT colorimetric assay

After 24, 48 and 72 hours of incubation, Cells were then labelled with 20µl of MTT solution (5 mg/ml in PBS) for 4 h and resulting formazan was solubilized with DMSO (200 µl). Absorbance was measured at 490 to 630 nm in a plate reader (ELISA Reader Biotek instruments, E1x800, USA).

Then the half maximal inhibitory concentration, IC50 was calculated by the prism software Ver.6 (prism ver.6, GraphPad company, USA).

4. Results

Following the use of absorbance, concentrations and calculation IC50s, the effect of extracts were evaluated. Alcoholic extract at 2500 and 5000 µg/ml was cytotoxic and resulted in reduction of cell viability less than 50%.(IC50=2143.3±235.88 µg/ml , r^2=0.912) (Figure 1).

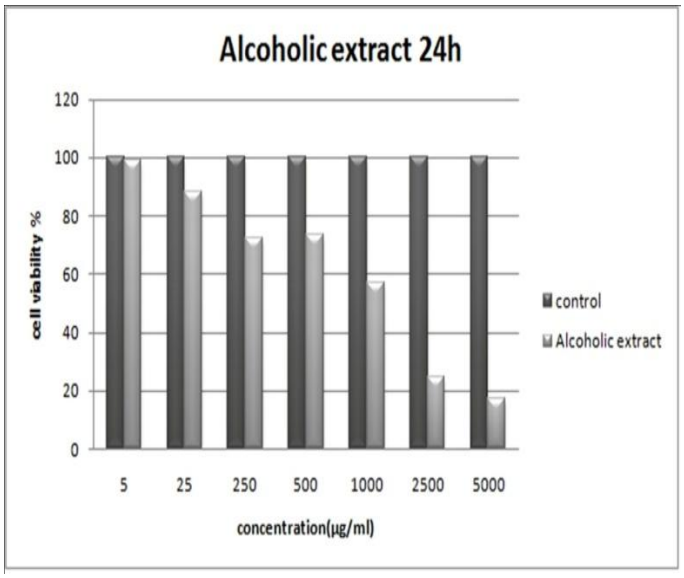

Figure1.MCF-7 cell viability % affected by alcoholic extract for 24 hours.

Aqueous extract was cytotoxic at both concentrations above and IC50, was calculated with a little changes, 2470±352.69 µg/ml. (r2=0.904) (Figure 2).Figure 3 is showing comparison between cell viability of alcoholic and aqueous extract of ginkgo biloba. A comparison of calculated IC50 for extracts and positive control (Etoposide), showed significant differences. (Figure 4)

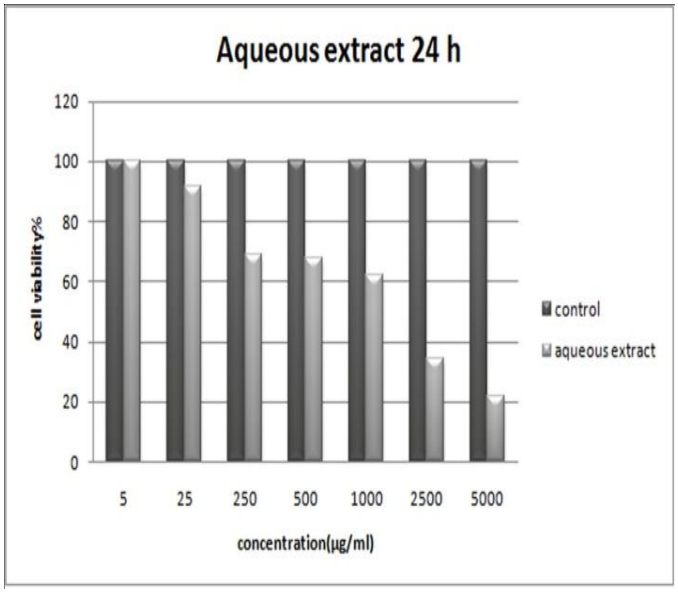

Figure2. MCF-7 cell viability % affected by aqueous extract for 24 hours.

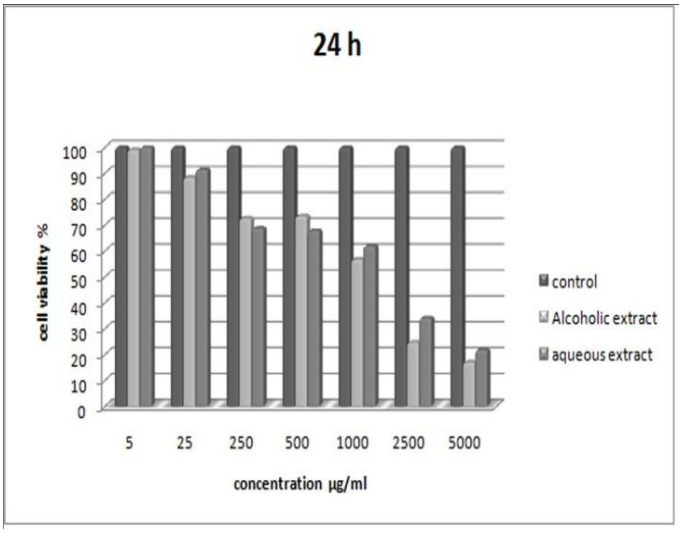

Figure3.Comparison of alcoholic & aqueous extract cell viability% on MCF-7 cell line.

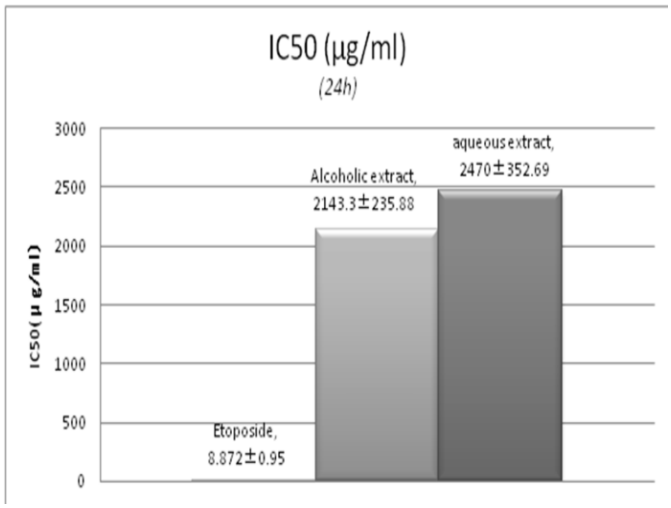

Figure 4.Comparison of extracts and Etoposide IC50s.

In 48 h incubation, cytotoxicity was seen only at 5000 µg/ml of alcoholic extract and other concentrations, resulted in increase of cell viability up to 50% and IC50 was not obtained (Figure 5).

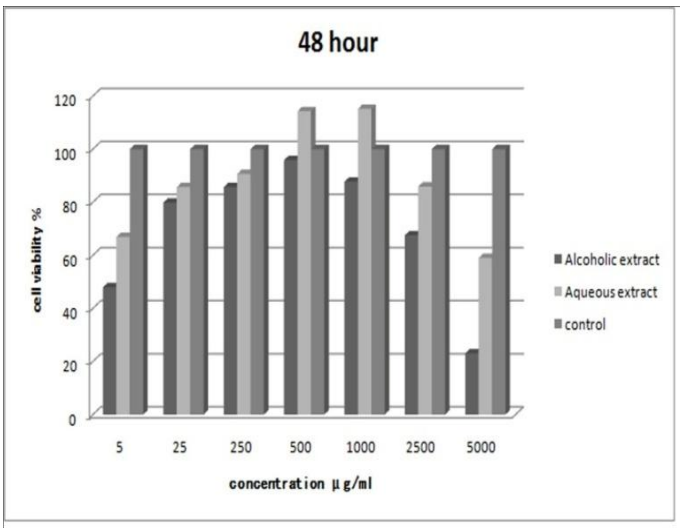

Figure5. MCF-7 cell viability % affected by alcoholic and aqueous extract for 48 hours.

Also in 72 h incubation, cytotoxicity was seen in both alcoholic and aqueous extract at the highest concentration (2500 & 5000 µg/ml). But at other concentrations, increasing of cell viability up to 50%, was observed (Figure 6).

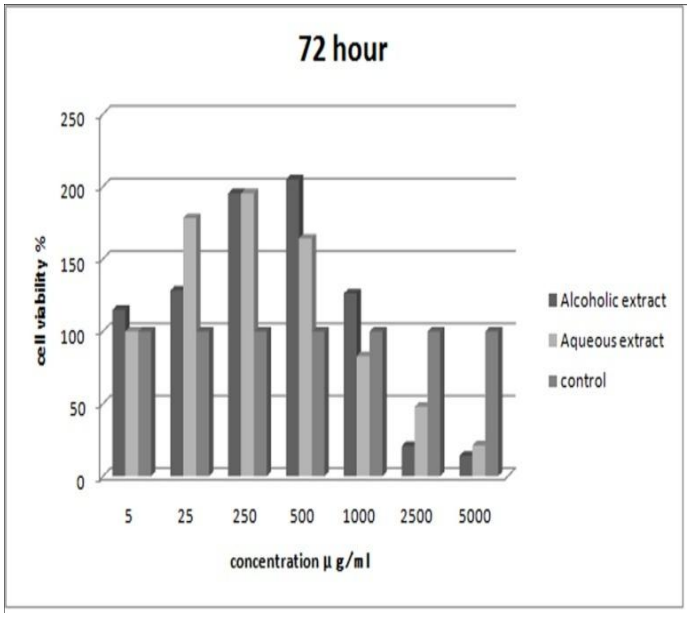

Figure6. MCF-7 cell viability % affected by alcoholic and aqueous extract for 72 hours.

5. Discussion

Cytotoxicity is the quality of being toxic to cells. Cells exposed to a cytotoxic compound can respond in a number of ways. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis; they can stop growing and dividing; or they can activate a genetic program of controlled cell death, termed apoptosis. There are many ways to measure cytotoxicity, but most involve assessment of cell membrane integrity. Membrane integrity can be evaluated by using vital dyes (such as trypan blue or propidium iodide), by protease biomarkers, with MTT or MTS redox potential assays, or by measuring ATP content.

The MTT assay is a colorimetric assay for assessing cell viability. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color.

The aim of this study was to investigate the effect of two kinds of ginkgo biloba extract against human breast cancer cell line (MCF-7) whether inhibition or stimulation activity on cells viability.

The antioxidant and associated anti-lipoperoxidative effects of Ginkgo extracts appear to involve both their flavonoid and terpenoid constituents. The anti-angiogenic activity of the extracts may involve their antioxidant activity and their ability to inhibit both inducible and endothelial forms of nitric oxide synthase. [4]

A finding has suggested that EGb may have beneficial chemopreventive roles against hepatocarcinogenesis through

their antioxidant, antiangiogenic and antigenotoxic activities. [8]

Standard *Ginkgo biloba* extract, EGb761 (commercial name), contains 22 - 27%flavonoids (ginkgo-flavone glycosides) and 5-7%terpenoids (ginkgolides and bilobalides), which are the most important active substances in the extract. The most important flavonoids are glycosides of kaempferol, quercetin, and isorhamnetin with glucose or rhamnose. [9]

Ginkgo Biloba extract at low concentration (10, 20 µg/ml) has showed poor inhibitory effect on human breast cancer cell line MDA-MB-231. [10]

Chao (2004) studied the effect of Ginkgo biloba extract (EGb 761) on cell proliferation and cytotoxicity in human hepatocellular carcinoma (HCC) cells. They reported that EGb 761 (50-1 000 mg/L) significantly suppressed cell proliferation and increased LDH release in HepG2 and Hep3B cells compared with the control group. [9]

The results of a study conducted by (1996) showed that Ginkgo biloba extract protected biomembranes from oxidative injury by decreasing intracellular LDH leakage from Pulmonary artery endothelial cells. MTT assay showed that GBE minimized loss of cell viability induced by oxidative injury. [11]

For as much as our tests were done in 24, 48 and 72 hours incubation, they demonstrate that the highest concentration (5000 µg/ml) of ginkgo leaves extract (both alcoholic and aqueous) is cytotoxic and has inhibitory effect on cell proliferation.

If we assume that antioxidative effect of ginkgo extracts is depends on terpenoid compounds (ginkgolides and bilobalides) [4] , results were observed at less than 5000 µg/ml in 48 and 72 hours mtt assay can be attributed to the antioxidative effect of terpene trilactones that are exist in extracts.

Although, to achieve a certain results, and detection of pro-apoptosis activity of Ginkgo extracts, further studies should be done to quantified the molecular events of studied cells.

In conclusion, Ginkgo biloba aqueous and alcoholic extracts showed a significant cytotoxicity effect in compare to control group in MCF-7 cell line. Anti-proliferative effect of Ginkgo extract revealed this fact that Ginkgo biloba aqueous and alcoholic extracts could have cell cytotoxicity characteristics beside its cytoprotection mechanisms.

6. Conflict Of Interest

No conflict of interest to declare.

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