CMJ Original Research March 2016, Volume: 38, Number: 1

Cumhuriyet Medical Journal 6-19

http://dx.doi.org/10.7197/cmj.v38i1.5000165142

Anticancer and antiangiogenic effects of methanol extracts of *Lonicera caprifolium* L. on C6 rat glioma cells

C6 sıçan glioma hücreleri üzerinde Lonicera caprifolium L. metanol ekstraktlarının anti-kanser ve antianjiyogenik etkileri

Nergiz Hacer Turgut[1], Ahmet Altun[2], Haki Kara[3], Bektaş Tepe[4], Merve Ergül[1], Mustafa Ergül[5], Necati Barış Tuncel[6], Neşe Yılmaz Tuncel[6]

[1]Department of Pharmacology, Cumhuriyet University School of Pharmacy, TR-58140, Sivas.
[2]Department of Pharmacology, Cumhuriyet University School of Medicine, TR-58140 Sivas.
[3]Department of Pharmacology and Toxicology, Cumhuriyet University School of Veterinary Medicine, TR-58140, Sivas.
[4]Department of Molecular Biology and Genetics, Kilis University School of Science and Literature, TR-79000 Kilis.
[5]Department of Biochemistry, Cumhuriyet University School of Pharmacy, TR-58140, Sivas.
[6]Department of Food Engineering, Canakkale University School of Engineering, TR-17000, Canakkale.
Corresponding author: Nergiz Hacer Turgut, Department of Pharmacology, Cumhuriyet University School of Pharmacy, TR-58140 Sivas.
E-mail: nergizht@yahoo.com
Received/Accepted: January 29, 2016 / February 12, 2016
Conflict of interest: There is not a conflict of interest.

SUMMARY

Objective: Gliomas are brain tumors with high morbidity and mortality. For the treatment of gliomas, it is important to develop new and powerful treatments that could complement existing clinical treatment. *Lonicera caprifolium* L. (*L. caprifolium*) has various uses in herbal traditional medicine. This study was conducted to determine the phenolic acid levels and DNA damage protection potential of *L. caprifolium* extract, and to explore the antitumor effect of the extract by investigating its toxicity on C6 rat glioma cell lines and normal L929 mouse fibroblast cell lines. We also aimed to investigate the antiangiogenic potential of the extract.

Method: Phenolic acid content was determined by HPLC analysis. DNA damage protection potential was evaluated on pBR322 plasmid DNA. The effect of extracts on the proliferation of cancer cells was evaluated by XTT assay. Antiangiogenic effect was determined with Chorioallantoic membrane model.

Results: The extract was found rich in vanillic acid (273.003 µg/g); while the amount of chlorogenic acid was almost at negligible level (0.028 µg/g). 0.005-0.05 mg / ml extract protected against the hazardous effects of UV and H2O2 in all DNA bands. The presence of the extract significantly reduced C6 cell proliferation compared to control ($p<0.05$). The extract had antiproliferative effect with a half maximum inhibition of concentration (IC_{50}) value of 0.45 mg/ml. *L. caprifolium* extract in 10^{-6}, 10^{-5} and 10^{-4} M concentrations caused antiangiogenic effect. Antiangiogenic scores of *L. caprifolium* were 0.6, 0.73 and 1.6, respectively.

Conclusions: These results show that *L. caprifolium* has potential cytotoxic and antiangiogenic effect on C6 rat glioma cells and that the phenolic acid content of the plant may partially influence these activities.

Keywords: Glioma, Lonicera caprifolium, angiogenesis, cytotoxic effect, phenolic acid

ÖZET

Amaç: Gliomolar yüksek morbitite ve mortalite oranı olan beyin tümörleridir. Gliomaların tedavisi için, mevcut klinik tedaviye tamamlayıcı olabilecek yeni ve güçlü tedavi çözümleri geliştirmek önemlidir. *Lonicera caprifolium* L.'nin (*L. caprifolium*) bitkisel geleneksel tıpta çeşitli kullanımları vardır. Bu çalışma, *Lonicera caprifolium* L. (Bahçe Hanımelisi) ekstraktının fenolik asit düzeylerini ve DNA hasarı koruma potansiyelini belirlemek ve C6 sıçan glioma hücre hatları ve normal L929 fare fibroblast hücre hatları üzerindeki toksisitesinin inceleyerek *Lonicera caprifolium* ekstraktının anti-tümör etkisini araştırmak için gerçekleştirilmiştir. Bu çalışmada ayrıca ekstraktın anti-anjiyogenik potansiyelinin araştırılması amaçlanmıştır.

Yöntem: Ekstraktların fenolik asit içeriği, HPLC analizi ile tespit edildi. DNA hasarı koruma potansiyeli pBR322 plazmid DNA üzerinde değerlendirildi. Ekstraktın kanser hücrelerinin proliferasyonu üzerindeki etkisi XTT testi ile değerlendirildi. Anti-anjiyogenik etki koryoallonterik membran modeli ile tespit edildi.

Bulgular: Ekstrakt vanillik asitten (273,003 µg/g), zengin bulundu; klorogenik asit miktarı hemen hemen göz ardı edilebilir seviyede (0,028 µg/g) tespit edildi. 0.005-0.05 mg/ml aralığındaki ekstrakt, tüm DNA bantlarını UV ve H_2O_2, nin tehlikeli etkisine karşı korudu. Kontrol grubu ile karşılaştırıldığında ekstraktın varlığı önemli ölçüde C6 hücre çoğalmasını azalttı ($p<0.05$). Ekstraktın, 0.45 mg / ml yarı maximum inhibisyon konsantrasyon (IC50) değerinde antiproliferatif etkisi gözlendi. *Lonicera caprifolium* ekstraktı 10^{-6}, 10^{-5} ve 10^{-4} M konsantrasyonlarda anti-anjiyogenik etkiye neden oldu. *Lonicera caprifolium* ekstraktının anti-anjiyogenik skorları sırasıyla 0.6, 0.73 ve 1.6 idi.

Sonuç: Bu sonuçlar, *Lonicera caprifolium'un* C6 sıçan glioma hücreleri üzerinde potansiyel sitotoksik ve anti-anjiogenik etkisi olduğunu göstermektedir ve bu aktivitelerde bitki fenolik asit içeriğinin, kısmen, etkisi olabilir.

Anahtar sözcükler : Glioma; Lonicera caprifolium; Anjiogenez; Sitotoksik etki; Fenolik asit

INTRODUCTION

Gliomas are the most common malignant brain tumor with high morbidity and mortality[1]. These tumors arise from glia cells in the central nervous system and because of their infiltrative nature they surround the brain tissue which usually makes surgical removal impossible[2]. Adjuvant therapies like radiotherapy and chemotherapy are generally needed to control the disease but there is often therapeutic resistance[3]. Also for disease control, therapeutic agents against specific targets such as epidermal growth factor receptor (EGFR) inhibitors, monoclonal antibody (bevacizumab) and vascular endothelial growth factor inhibitors are used[4, 5]. Despite treatment, gliomas recur early and the median survival is still low for patients with malignant gliomas[6]. Therefore for the treatment of malignant gliomas, it is important to identify new and potent therapeutic solutions which may be a useful supplementary to the current clinical therapy.

Lonicera caprifolium L. (*L. caprifolium*), goat-leaf honeysuckle is a species of flowering plants belonging to Caprifoliaceae family. It is native to Europe but grown around the world. It is a deciduous climber growing up to 8 meters with oval, blue-green leaves, trumpet shaped cream-colored flowers followed by yellow to red berries, appearing in midsummer. There is essential oil of Lonicera in the content of many herbal medicinal and cosmetic preparations. Flower, seeds and leaves of honeysuckle were used in herbal traditional medicine to treat bacterial and viral infections, inflammation and also used for antioxidant activities[7, 8]. There are pharmacological studies that have used plant extracts or essential oil to approve these properties[9-11]. However the effects of *L. caprifolium* on DNA damage, cancer cell proliferation and angiogenesis are not yet known.

Phenolic acids and their analogs are widely distributed in many vegetables and fruits and they are a class of compounds with many pharmacological functions[12-15]. Reactive oxygen species have a wide variety of pathological effects such as carcinogenesis, DNA damage and cell degeneration related to aging. It has been suggested in many in vitro and in vivo studies that dietary phenolic acids could protect and also inhibit the oxidative stress by scavenging free radicals and exposing antioxidant capacity[16-19]. The inhibition of carcinogenesis by phenolic compounds has revealed oncoprotective properties beneficial for the use of nutraceuticals in cancer therapy.

The body immune system can be enhanced by the phenolics to destroy cancer cells. Phenolics can also inhibit the development of angiogenesis that is necessary for tumor growth and also decrease adhesiveness and invasiveness of cancer cells. The possible mechanisms for the oncoprotective effect of these phenolics are multi-faceted and include antioxidant effects, steroid receptor binding, direct interaction with intracellular elements, aryl hydrocarbon receptor binding and regulation of growth factor-receptor interactions and cell signaling cascades[14, 20, 21]. The potential value of natural compounds has leaded to search compounds with strong antioxidant capacity but low cytotoxicity.

To the best of our knowledge there has been no study that has investigated the anticancer effect of *L. caprifolium* on glioma cells. Therefore the objective of the present study was to determine the phenolic acid levels and DNA damage protection potential of *L. caprifolium* extract and to explore the antitumor effect of *L. caprifolium* extract by investigating its effects on cell growth pattern and their toxicity on C6 rat glioma cell lines and normal mouse fibroblast cell lines. Additionally we also aimed to investigate the antiangiogenic potential of *L. caprifolium* extract.

MATERIALS AND METHODS

Plant material and sample preparation

The aerial parts of *L. caprifolium* were collected from southeast region of Turkey (Batman center, 550 m) in June 2012. Plant material was scientifically identified by a senior taxonomist, Dr. Erol Dönmez, from Department of Biology, Faculty of Science, Cumhuriyet University, Sivas-TURKEY. The collected material was dried in an environment with no direct sunlight and good air flow and grounded by a blender. The air-dried and grounded samples were extracted by using a method described by Sokmen et al.[22],. The sample, weighing about 100 g was extracted in a Soxhlet apparatus with methanol (MeOH) at 60 ºC for 6 h. Because of the polar characteristics of phenolic acids, the extract was further fractionated with chloroform and distilled water. Water sub-fraction was frozen and eventually, it was lyophilized and kept in the dark at +4 ºC until tested.

Determination of phenolic acid levels of extracts

The analysis of phenolic acids was employed according to the method described by Ozturk et al.[23] with a slight modification using an Agilent HPLC series 1200 (Agilent, Waldbronn, Germany). The separation of gallic (GA), protocathechuic (protoCA), p-hydroxy benzoic (p-hydBA), vanillic (VA), caffeic (CA), chlorogenic (ChA), syringic (SA), p-coumaric (p-COU), ferulic (FA), o-coumaric (o-COU), rosemarinic (RMA) and trans-cinnamic (tr-CIN) acids was performed on an Agilent Zorbax Eclipse XDB-C18 column (150 mm, 4.6 mm i.d., 5 µm particle size). The chromatographic conditions were: flow rate 1 mL / min, sample injection volume of 5 µL, operation temperature of 23 ºC, UV detection at 280 nm and mobile phase A (methanol : water : formic acid (10 : 88 : 2, (v/v)) and B (methanol : water : formic acid (90 : 8 : 2, (v/v)). A gradient program was used as follows: 100% A; 0 - 20 min, changed to 80% A; 25 - 50 min, to %50 A; 50 - 54 min, followed by isocratic elution of 50% A; 54 - 64 min, 0% A; and 64 - 70 min, %100 A. The results were evaluated with regard to the areas of the peaks and their retention times. Quantitation was based on calibration curves built for each of the compounds identified in the samples.

Determination of DNA damage protection potential of extracts

DNA damage protection potential of the extract was evaluated on pBR322 plasmid DNA (vivantis). Plasmid DNA was oxidized with H_2O_2 + UV treatment in the presence of extracts and checked on 1% agarose gels according to Russo et al.[24] after some modifications. In brief, the experiments were performed in a volume of 10 µl in a microfuge tube containing 3 µlpBR322 plasmid DNA (172 ng/µl), 1 µl of 30% H_2O_2, and 5 µl of extract in the concentrations of 0.005, 0.01, 0.02, 0.04 and 0.05 mg/ml, respectively. The reactions were initiated by UV irradiation and

continued for 5 min on the surface of a UV transilluminator (DNR-IS) with an intensity of 8000 µW/cm^2 at 302 nm at room temperature. After irradiation, the reaction mixture (10 µl) along with gel loading dye (6×) was loaded on a 1% agarose gel for electrophoresis. Untreated pBR322 plasmid DNA was used as a control in each run of gel electrophoresis along with partially treated plasmid, i.e. only UV or only H_2O_2 treatment. Gels were stained with EtBr and photographed with the Gel documentation system (DNR-IS, MiniBIS Pro).

Cell lines and reagents

Rat glioma (C6) cell lines and mouse fibroblast (L929) cell lines were supplied from ATCC (American Type Culture Collection, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and sterile phosphate buffer saline (PBS) were purchased from PAA Ltd. (France). Trypsin-EDTA was purchased from Biological Industries Ltd. (Haemek, Israel). RPMI-1640 without phenol red and L-glutamine–penicillin–streptomycin solution were purchased from Sigma-Aldrich (Steinheim am Albuch, Germany). XTT reagent (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) was purchased from Roche Diagnostic.

In vitro cytotoxicity assay

Cell culture

The cytotoxicity of the *L. caprifolium* was tested against, C6 rat glioma cell lines and L929 mouse fibroblast cell lines. The cells, which were adherent cell lines and grow as monolayers, were routinely cultured in low glucose Dulbecco's modified Eagle medium(DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin in 25cm^2 polystyrene flasks and maintained in a humidified incubator containing 5% CO_2 at 37 °C. Growth and morphology were monitored and cells were passaged when they had reached 90% confluence.

Cell proliferation assay

In order to determine *L. caprifolium* toxicity on C6 cancer cells and L929 fibroblast cells, the Roche Cell proliferation Kit II (Roche, Mannheim, Germany) was used. This method is as sensitive as radioactive assay with a significantly lower inter- and intra-tester variability[25]. Initially, the cancer cells were seeded at a density of 1x10^4 cells per well in a 96-well flat bottom plate in 100 µl of culture medium and was allowed to adhere for 24h incubation in incubator before treatment. Then these cells were administrated with *L. caprifolium* at six different concentrations ranging from 2 mg/ml to 0, 0625 mg/ml at 24h. After incubation, the medium was removed and wells were washed twice with 200 µl phosphate buffered saline (PBS). At the end of these periods, for determination of living cells, 100 µl RPMI and 50 µl XTT labeling mixture were added to each well and incubated again for 4h at 37 °C. After this incubation period, the absorbance of the samples was measured using micro plate (ELISA) reader at 450 nm against the control (the same cells without any treatment). The cell viability was expressed in % related to control (100% of viability).

Angiogenesis assay using chorioallantoic membrane (CAM) assay

Preparation of the pellets

L. caprifolium extract were prepared and mixed with agarose in order to form pellet. The agarose (Merck, Damstadt, Germany) is added to distilled water to obtain a 2.5% (w/v) solution. This solution is put into the autoclave in 121 °C and under 1 atmospheric pressure to provide dissolution and sterilization. Then, it is let to be cooled in a sterile container up to 37 °C. The extract used in the study is added at this stage. Appropriate volumes of solutions were used to achieve three different concentrations of *L. caprifolium* extract (100 nM, 10 nM and 1 nM per 10 µl pellet). Approximately one hundred pellets for each study set are used. Thus, approximately 1 ml of combined agar and drug solution (10 µl×100=1 ml) was prepared initially for *L. caprifolium* extract. The extract solutions with 10 nM and 1 nM concentrations were

prepared by diluting these initial mixtures ten folds with the agarose solution again. Using a micropipette, 10 µl drops of this mixed solution were placed on previously sterilized, vertical, cylindrical stainless steel rods which were 5 mm in diameters to obtain circular pellets with the same diameter. Then the pellets were let to be solidified at room temperature in a sterile setting.

Chicken chorioallantoic membrane (CAM) assay

Ross 308 strain fertilized hens' eggs were obtained from Yemsel Poultry Company (Kayseri, Turkey).The work described has been carried out in accordance with EU Directive 2010/63/EU for animal experiments and the study protocol was approved by the Cumhuriyet University Animal Ethics Committee. The fertilized hens' eggs were incubated in horizontal position with environmental conditions of 37.5 °C temperature and 80% relative humidity. On the fifth day of the incubation period, 5 ml of albumen was taken through the eggshell with a syringe [Figure 1a] and a shell piece of 2–3 cm in diameter was removed from the contrary side of the eggs. Normal development of the CAM was verified [Figure 1b] and malformed or dead embryos were excluded. The windows on the egg shells were sealed with gelatin and thereafter, the eggs were incubated for 72 more hours to have CAM reaching 2 cm in diameter. Subsequently (on day 8), the seal was removed and the pellets were placed on the chorioallantoic membrane of each egg [Figure 1c]. The seal was placed again and the eggs were then incubated for 24 hours. The angiogenesis level was evaluated after that period. For each concentration of drugs, twenty eggs were used. As the negative control group, pellets containing just agar were utilized. As the positive control group, pellets containing bevacizumab, FDA approved antiangiogenic agent, was used. All the tests were duplicated. The eggs in which the pellets caused inflammation and embryo toxicity were excluded.

Angiogenesis scoring

The inhibitory effects of the drugs were determined with a stereoscopic microscope according to the scoring system used in a number of studies[26, 27]. In this system, the change in the density of the capillaries around the pellet and the extent of the effect are assessed [Figure 1d]. For each subject initial scoring was evaluated as follows:

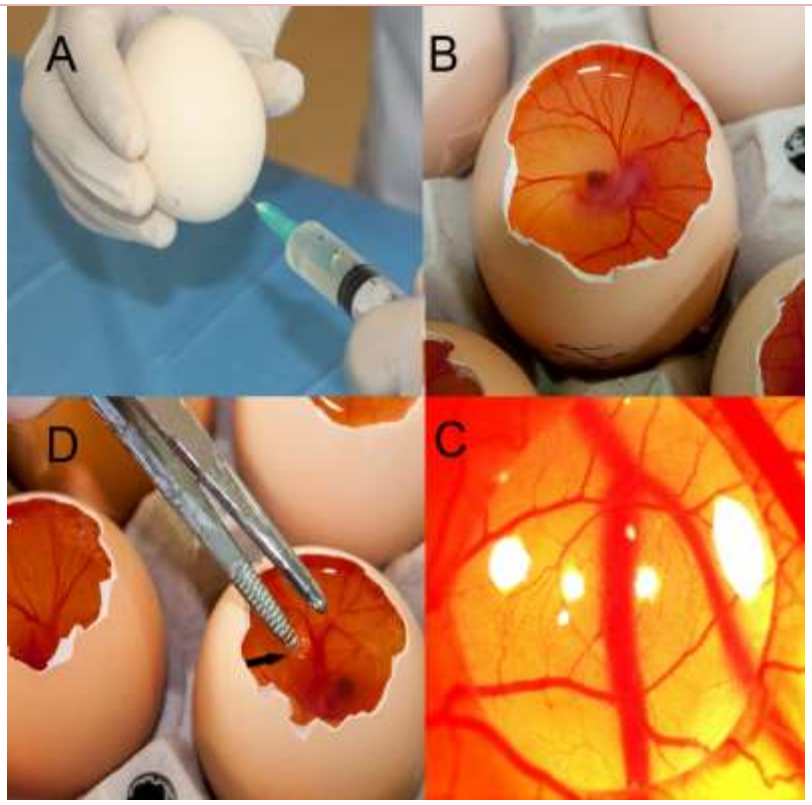

Figure 1- (A) Albumen is removed with a syringe; (B) The appearance of the chorioallantoic membrane (CAM) through a window on the egg Shell (x8); (C) The placment of the pellet on the CAM(x8); (D) Inhibition of the capillaries on the CAM (score:1) by the *Lonicera caprifolium* L. extract (x8), 247 x 47mm (300 x 300 DPI).

Score 0: indicated the absence of any demonstrable antiangiogenic effect (normal embryo and no difference in surrounding capillaries).

Score 0.5: represented a very weak antiangiogenic effect (no capillary-free area but an area with reduced density of capillaries which is not larger than the pellet area).

Score 1: a weak moderate antiangiogenic effect (a small capillary-free area or a small area with significantly decreased density of capillaries; less than double the size of the pellet is involved).

Score 2: a strong antiangiogenic effect (a capillary free area around the pellet which is equal to or more than double the size of the pellet itself).

The equation used for the determination of the average score was as follows: Average score= (Number of eggs (Score 2) ×2 + Egg number (Score 1) ×1] / [Total number of eggs (Score 0, 1, and 2)]. According to this scoring system, a score of < 0.5 meant that there was no antiangiogenic effect; a score of 0.5 to 1 indicated a weak antiangiogenic effect, and a score of >1 implied a strong antiangiogenic effect.

Statistical analysis

The cytotoxicity results expressed as mean ± standard error (SEM) of three replicates were analyzed statistically by using one-way analysis of variance (ANOVA) at 95% confidence levels for multiple comparisons. Tukey test has been used as post-hoc test. P values less than or equal to 0.05 were considered to be statistically significant. The scores of angiogenesis were compared with Kruskal-Wallis ANOVA test and Mann-Whitney U test. A p value of less than 0.05 was considered as statistically significant.

RESULTS

Phenolic acid levels of extracts

In this study, amounts of gallic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, chlorogenic, syringic, p-coumaric, ferulic, o-coumaric, rosmarinic and trans-cinnamic acids were quantified in methanol extracts of *L. caprifolium.* Methanol extract obtained from the plant was also further fractionated with equal amounts of chloroform and distilled water. By this way, non-polar phytochemicals were eliminated from the target extract. Polar phytochemicals brought together within the water fraction was lyophilized and stored at 4°C until tested and analyzed. In *L. caprifolium* extract, chlorogenic acid and vanillic acid was determined. The amount of vanillic acid was remarkable (273.003 µg/g) in the *L. caprifolium* extract; however, the amount of chlorogenic acid was negligible (0.028 µg/g) [Figure 2].

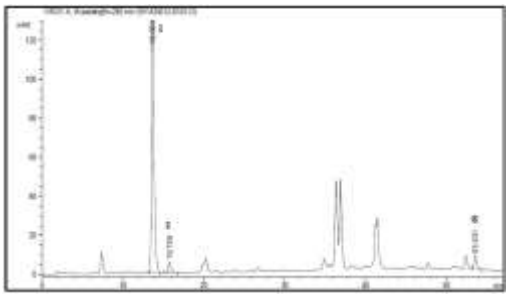

Figure 2- HPLC chromatogram showing phenolic acids available in *Lonicera caprifolium* L. [1: Vanillic acid, Rt (minute): 13.664, Source of confirmation: UV, 2: chlorogenic acid, Rt (minute); 15.759, IS: Internal Standard, Rt (minute): 53.531].

DNA damage protection potential of extracts

Figure 3 shows the electrophoretic pattern of DNA after UV-photolysis of H_2O_2 in the absence and presence of the aqueous extract of *L. caprifolium*. DNA derived from pBR322 plasmid showed two bands on agarose gel electrophoresis (column 1) the faster moving band corresponded to the native form of supercoiled circular DNA (scDNA) and the slower moving band corresponded to the open circular form (ocDNA). The UV irradiation of DNA in the presence of H_2O_2 (column 3) resulted in the cleavage of ocDNA to a faint linear DNA and smears on the agarose gel, indicating that the $OH^{\cdot}$ generated from UV-photolysis of H_2O_2 produced DNA strand scission.

The addition of 0.005, 0.01, 0.02, 0.04and 0.05 mg/ml *L. caprifolium* extract showed a significant DNA damage protection potential [Figure 3; columns 5, 6, 7, 8 and 9].

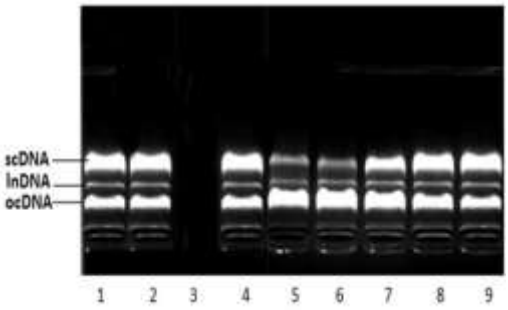

Figure 3- Electrophoretic pattern of pBR322 plasmid DNA after treatment with UV and H_2O_2 in the presence of *Lonicera caprifolium* aqueous extract. The addition of 0.005, 0.01, 0.02, 0.04 and 0.05 mg/ml *Lonicera caprifolium* extract showed a significant DNA damage protection potential on columns 5, 6, 7, 8 and 9.

Column 1: plasmid DNA (3 µl) + dH_2O (6 µl), **Column 2:** plasmid DNA (3 µl) + dH_2O (6 µl) + UV, **Column 3:** plasmid DNA (3 µl) + dH_2O (6 µl) + UV + H_2O_2 (1 µl), **Column 4:** plasmid DNA (3 µl) + dH_2O (6 µl) + H_2O_2 (1 µl), **Column 5:** plasmid DNA (3 µl) + aqueous extract (0.005 mg/ml) (5 µl) + UV + H_2O_2 (1 µl), **Column 6:** plasmid DNA (3 µl) + aqueous extract (0.01 mg/ml) (5 µl) + UV + H_2O_2 (1 µl), **Column 7:** plasmid DNA (3 µl) + aqueous extract (0.02 mg/ml) (5 µl) + UV + H_2O_2 (1 µl), **Column 8:** plasmid DNA (3 µl) + aqueous extract (0.04 mg/ml) (5 µl) + UV + H_2O_2 (1 µl), **Column 9:** plasmid DNA (3 µl) + aqueous extract (0.05 mg/ml) (5 µl) + UV + H_2O_2 (1 µl).

In vitro cytotoxicity of L. caprifolium extract

Cytotoxicity of the extract was tested both on C6 cell lines and L929 cell lines by XTT assay. In the presence of extract at 0.125, 0.25, 0.5, 1 and 2 mg/ml concentrations reduced significantly C6 cell proliferation ($p<0.05$) when compared with the control group. The value of IC_{50} of *L. cap-*

rifolium in C6 cell lines was found as 0.45 mg/ml. Results are shown in Figure 4 and Table 1. In the presence of extract at 1mg/ml concentration increased significantly L929 cell proliferation ($p<0.05$) when compared with the control group. Results are shown in Figure 5 and Table 2.

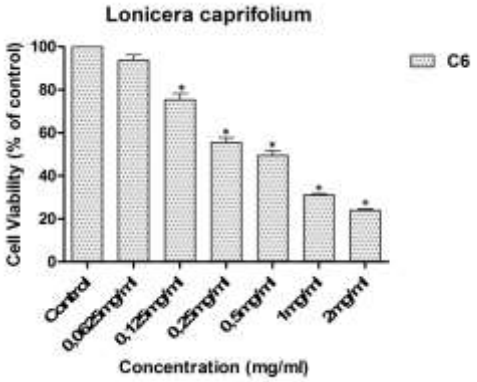

Figure 4- Antitumor activity of different concentrations of *Lonicera caprifolium* on C6 cell line as estimated by XTT assay (24h). * p< 0.05 versus all groups.

Table 1- Anticancer activity of *Lonicera caprifolium* as % cell viability after 24h

Control	100
0.0625 (mg/ml)	93,77 ± 2,43
0.125 (mg/ml)	75,36 ± 2,92
0.25 (mg/ml)	55,52 ± 2,17
0.5 (mg/ml)	49,43 ± 2,16
1 (mg/ml)	31,09 ± 0,54
2 (mg/ml)	23,97 ± 0,63

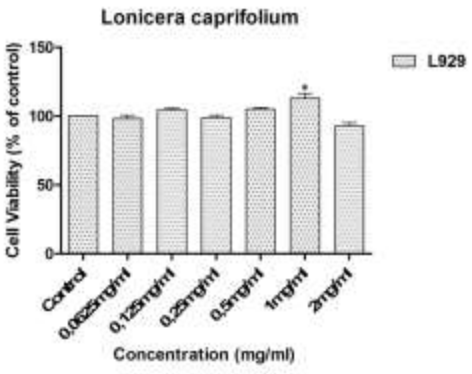

Figure 5- In vitro cytotoxicity of different concentrations of *Lonicera caprifolium* on L929 cell line as estimated by XTT assay (24h). * p< 0.05 versus all groups.

Table 2- In vitro cytotoxicity of *Lonicera caprifolium* as % cell viability after 24h

Control	100
0.0625 (mg/ml)	98,07 ± 2,48
0.125 (mg/ml)	104,42 ± 1,74
0.25 (mg/ml)	98,87 ± 1,77
0.5 (mg/ml)	105,01 ± 0,99
1 (mg/ml)	112,80 ± 3,60
2 (mg/ml)	92,97 ± 2,24

Determining antiangiogenic effects of L. caprifolium extract

The eggs on which a 10 µl agarose pellet with no plant extract was installed demonstrated no significant antiangiogenic effect (average antiangiogenic score=0.1). All the study extracts demonstrated some antiangiogenic effect compared to the negative control ($p<0.05$) (Data not shown). Figure 6 shows the antiangiogenic scores of *L. caprifolium* extract in 10^{-6}, 10^{-5} and 10^{-4} M concentrations. *L. caprifolium* extract in 10^{-6}, 10^{-5} and 10^{-4} M concentrations caused antiangiogenic effect. Antiangiogenic scores of *L. caprifolium* were 0.6, 0.73 and 1.6, respectively. These scores show that *L. caprifolium* caused concentration dependent antiangiogenic effect on CAM. As shown in the scatter graph, the antiangiogenic score with the 10^{-4} M of *L. caprifolium* extract was significantly higher than 10^{-6} M concentration ($p<0.05$) [Figure 6].

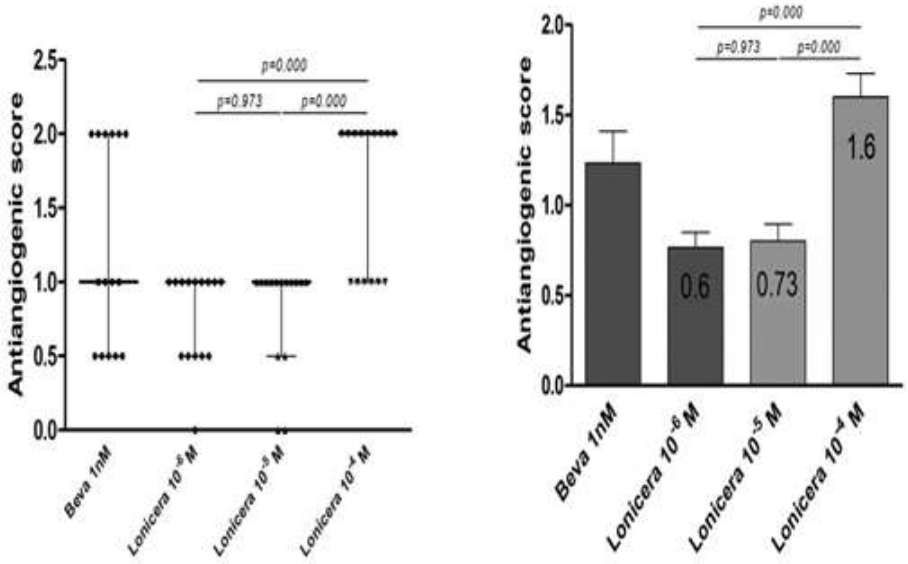

Figure 6- The antiangiogenic scores of *Lonicera caprifolium* extracts within the concentrations of 10^{-6}, 10^{-5}, 10^{-4} M. Beva (bevacizumab) is used as a positive control.

DISCUSSION

Malignant gliomas represent the most common and devastating types of primary brain tumors with an annual incidence of 5.26 per 100 000 population[2]. They have poor prognosis and also negative repercussions on cognitive functions and quality of life. The World Health Organization classifies the gliomas as astrocytomas, oligodendrogliomas, ependymomas and oligo-astrocytomas (mixed gliomas)[28]. Astrocytomas are also divided as follows: grade 1, pilocytic; grade 2, diffuse; grade 3, anaplastic; and grade 4, glioblastoma multiforme (GBM). Glioblastomas account for 60-70% of all gliomas. Gliomas are seen in all age groups, but they are lot more common in the sixth through eight decades of life and the disease is more common among men when compared to women. Current treatment is difficult both because of the invasiveness of the glioma into other brain tissue and high recurrence rates[29] [29].

Treatment options are expanding and improving for this disease, but due to the limited understanding of the biology of the disease there has not been any major increase in the survival rate of patients[30]. The first step of the treatment includes surgical resection which is followed by radiotherapy and chemotherapy. However because of tumor location or widespread of disease in brain, surgery is not indicated for a majority of patients. Also because of invasion of cells radiotherapy can miss many of the tumor cells. Gene therapy including virotherapy, the use of stem cells and exosomes are novel developing adjuvant therapies for this cancer[31,32].

In this study, we determined the phenolic acid levels and DNA damage protection potential of *L. caprifolium* extracts in addition to its antitumor effect by investigating on cell growth pattern and toxicity on C6 rat glioma cell lines and normal mouse fibroblast cell lines. Additionally we determined the antiangiogenic potential of *L. caprifolium* extract. To the best of our knowledge, DNA damage protecting potential, cytotoxic and antiangiogenic effect of *L. caprifolium* has not been previously reported.

There is great interest to natural products because of their potential anticancer activities. Phenolic compounds in natural com-

pounds can inhibit carcinogenesis by destroying cancer cells and inhibiting angiogenesis. These compounds can also decrease invasiveness of cancer cells[20]. In this study, gallic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, chlorogenic, syringic, p-coumaric, ferulic, o-coumaric, rosmarinic and trans-cinnamic acids were investigated in methanol extracts of, *L. caprifolium*. According to HPLC analysis, vanillic acid (273.003 µg/g) and chlorogenic acids (0.028 µg/g) were determined at a total amount of 273.031µg/g in *L. caprifolium* extract. The amount of vanillic acid was remarkable however, the amount of chlorogenic acid was negligible. Vanillic acid (4-hydroxy-3-methoxybenzoic acid), an oxidized form of vanillin, is a benzoic acid derivative from edible plants and fruits. It is a medicinal compound which is widely used in traditional medicine for ulcer, fever, hypertension, inflammation and cancer. Vanillic acid is a strong antioxidant because of the presence of the carboxyl group[33]. Previously the cardioprotective effect of vanillic acid via free radical scavenging, antioxidant and anti-inflammatory properties has been reported in isoproterenol induced myocardial infarcted rats[34]. It has been shown that vanillc acid has antihypertensive activity in L-NAME induced hypertensive rats[35] and by suppressing the activation of caspase-1 and nuclear factor-kappa B (NF-κB) vanillic acid has been shown to inhibit inflammatory mediators[36]. It has been reported that vanillic acid shows chemopreventive effect in experimentally induced carcinogenesis rat model[37]. Durant and Karran have reported that vanillic acid is an inhibitor of non-homologous DNA end-joining which is a major pathway of double strand break repair in human cells and they have also reported that vanillic acid increases the sensivity of cancer cells to cisplatin[38]. Chlorogenic acid (3-O-caffeoylquinic acid), which is formed from caffeic and quinic acids is one of the major phenolic components seen in tobacco leaves and coffee seeds. Chlorogenic acid has multipharmacological properties such as antimicrobial[39], anti-inflammatory effects[40, 41] and cardioprotective effects[42, 43]. It is a potent antioxidant, widespread in fruits and vegetables. It has been suggested that chlorogenic acid inhibits lipid peroxidation and DNA damage by scavenging free radicals and increasing the resistance of low-density lipoprotein (LDL)[44]. Chlorogenic acid has been reported to prevent carcinogenesis by reducing DNA damage[45, 46] and also by inhibiting microsomal glucose-6-phosphate translocase (G6PT) and antagonizing, growth factors induced calcium mobilization and extracellular signal-regulated kinases (ERK)[47]. In this study, DNA damage protection potential of the extracts was determined. The addition of 0.005- 0.01- 0.02- 0.04- 0.05 mg/ml *L. caprifolium* extract showed a significant protection to the damage of all DNA bands. These results show that *L. caprifolium* extract has DNA damage protection potential at a significant level.

Cytotoxic drugs are still the backbone of cancer treatment, but these drugs are limited because of a narrow therapeutic index, significant toxicities and frequently acquired resistance. Chemotherapy, as it is applied systemically is limited by diffusion through the tumor of brain cancer. If patients are asymptomatic, the common practice is to carry over with a DNA alkylating agent temozolamide with close radiographic follow-up. Temozolamide kills cancer cells by forming O^6-methylguanine in DNA that miss-pair with thymine during the next DNA replication cycle. If patients are highly symptomatic; surgery, corticosteroids or alternative treatments such as using an antiangiogenic agent bevacizumab are considered[29]. It is crucial developing agents which can eliminate only premalignant and malignant cells without harming normal healthy central nervous system tissue. Therefore in this study, cytotoxic effect of *L. caprifolium* extract was both investigated on C6 rat glioma cell lines and on L929 normal mouse fibroblast cell lines. In our study *L. caprifolium* extracts demonstrated action in inhibiting C6 cell growth with a 0.45 mg/ml IC50 value. The same extract showed no inhibition on L929 cell growth and additionally in the presence of extract at 1mg/ml concentration it significantly increased L929 cell proliferation. These results indicate the toxic effect of *L. caprifolium* extract on

tumoral cells but not on normal cells.

Malignant gliomas are highly vascular tumors. Angiogenesis and its key regulator, vascular endothelial growth factor (VEGF), are important therapeutic targets in treatment of gliomas[48]. Bevacizumab is a humanized VEGF monoclonal antibody which targets VEGF[49]. As VEGF is a promotor of vascular permeability, bevacizumab reduces peritumoral edema and this allows reduction in the corticosteroid dose used for symptom relief or control. Two randomized trials has demonstrated that addition of bevacizumab increases progression free survival but not overall survival[50, 51]. As solid tumors like malignant gliomas need the generation of new blood vessels in order to develop, novel therapies are based on antiangiogenic strategies. In this study the effect of *L. caprifolium* extract on angiogenesis was determined with Chorioallantoic membrane model. *L. caprifolium* extract in 10^{-6}, 10^{-5} and 10^{-4} M concentrations caused antiangiogenic effect. Antiangiogenic scores of *L. caprifolium* were 0.6, 0.73 and 1.6, respectively. These scores show that *L. caprifolium* caused concentration dependent antiangiogenic effect on CAM.

CONCLUSIONS

In conclusion, gliomas are intelligent tumors which remain one of the most challenging cancers to treat as they achieve to escape from known radiation and chemotherapy treatment methods. There are novel promising therapies that are under evaluation including trials of alternative antiangiogenic agents, targeted agents, signal transduction pathways, gene therapy, immunotherapy, radiolabeled drugs and a lot more. Additionally in order to overcome the blood brain barrier which restricts entry of the drugs into brain tumors targeting drug delivery systems, as nanocarriers are an alternative approach for treating gliomas. Our study demonstrated that *L. caprifolium* extract has antiproliferative and antiangiogenic effects on C6 rat glioma cells and this study also showed that this extract has a DNA damage protection potential at a significant level on pBR322 plasmid DNA. Additionally we demonstrated that *L. caprifolium* extract has no inhibitory effect on normal L929 cell growth, indicating the toxic effect of *L. caprifolium* extract on tumoral cells but not on normal cells. We think that the vanillic acid, a natural phenolic acid with multi pharmacological properties, detected in the *L. caprifolium* extracts has an important role in these effects. Additional research with more detailed work is required to completely understand the molecular mechanisms behind the positive effect of *L. caprifolium* extract on glioma cells.

Acknowledgement

This study was supported by Cumhuriyet University Scientific Research Project V-012 (CUBAP, Sivas, Turkey). The authors would like to thank to Dr. Erol DONMEZ from Department of Biology, School of Science, Cumhuriyet University, Sivas-TURKEY for his kind help for the identification of plant material.

REFERENCES

1. Teng CC, Sze CI, Liao WC. Induction of apoptosis of malignant gliomas cells by a prenylated chalcone. Pharm Biol 2014; 52: 471-8.
2. Dolecek TA, Propp JM, Stroup NE, Kruchko C. CBTRUS statistical report: primarybrain and central nervous system tumors diagnosed in the United States in 2005-2009. Neuro Oncol 2012; 14: 1-49.
3. Laks DR, Visnyei K, Kornblum HI. Brain tumor stem cells as therapeutic targets in models of glioma. Yonsei Med J 2010; 51: 633-40.
4. Bhattacharya D, Singh MK, Chaudhuri S, Acharya S, Basu AK, Chaudhuri S. T11TS impedes glioma angiogenesis by inhibiting VEGF signaling and pro-survival PI3K/Akt/eNOS pathway with concomitant upregulation of PTEN in brain endothelial cells, J Neurooncol 2013; 113: 13-25.
5. Grant R, Kolb L, Moliterno J. Molecular and genetic pathways in gliomas: the future of personalized therapeutics. CNS Oncol 2014; 3: 123-36.

6. Sarin H. Recent progress towards development of effective systemic chemotherapy for the treatment of malignant brain tumors. J Transl Med 2009; 7: 77.
7. Xiang Z, Ning Z. Scavenging and antioxidant properties of compound derived from chlorogenic acid in South-China honey suckle. Food Sci- and Technol 2008; 41:1189-203.
8. Bubulica MV, Anghel I, Grumezescu AM, Saviuc C, Anghel GA, Chifiriuc MC, Gheorghe I, Lazar V, Popescu A. In vitro evaluation of bactericidal and antibiofilm activity of Lonicera tatarica and Viburnum opulus plant extracts on staphylococcus strains. Farmacia 2012; 60: 80-91.
9. Han J, Lv QY, Jin SY, Zhang TT, Jin SX, Li XY, Yuan HL. Comparison of anti-bacterial activity of three types of di-O-caffeoylquinic acids in Lonicera japonica flowers based on microcalorimetry. Chin J Nat Med 2014; 12: 108-13.
10. Kim SJ, Yoon SJ, Kim YM, Hong SW, Yeon SH, Choe KI, Lee SM. HS-23, Lonicera japonica extract, attenuates septic injury by suppressing toll-like receptor 4 signaling. J Ethnopharmacol 2014; 155: 256-66.
11. Sochor J, Jurikova T, Pohanka M, Skutkova H, Baron M, Tomaskova L, Balla S, Klejdus B, Pokluda R, Mlcek J, Trojakova Z, Saloun J. Evaluation of antioxidant activity, polyphenolic compounds, amino acids and mineral elements of representative genotypes of Lonicera edulis. Molecules 2014; 19: 6504-23.
12. Erdem MG, Cinkilic N, Vatan O, Yilmaz D, Bagdas D, Bilaloglu R. Genotoxic and anti-genotoxic effects of vanillic acid against mitomycin C-induced genomic damage in human lymphocytes in vitro. Asian Pac J Cancer Prev 2012; 13: 4993-8.
13. Soory M. Nutritional antioxidants and their applications in cardiometabolic diseases, Infect Disord Drug Target 2012; 12: 388-401.
14. Weng CJ, Yen GC. Chemopreventive effects of dietary phytochemicals against cancer invasion and metastasis: phenolic acids, monophenol, polyphenol, and their derivatives. Cancer Treat Rev 2012; 38: 76-87.
15. Habibi E, Shokrzadeh M, Chabra A, Naghshvar F, Keshavarz-Maleki R, Ahmadi A. Protective effects of Origanum vulgare ethanol extract against cyclophosphamide-induced liver toxicity in mice. Pharm Biol 2015; 53: 10-5.
16. Husein AI, Ali-Shtayeh MS, Jondi WJ, Zatar NA, Abu-Reidah IM, Jamous RM. In vitro antioxidant and antitumor activities of six selected plants used in the Traditional Arabic Palestinian herbal medicine. Pharm Biol 2014; 52: 1249-55.
17. Flanigan PM, Niemeyer ED. Effect of cultivar on phenolic levels, anthocyanin composition, and antioxidant properties in purple basil (Ocimum basilicum L.). Food Chem 2014; 164: 518-26.
18. Kolodziejczyk-Czepas J, Nowak P, Kowalska I, Stochmal A. Biological activity of clovers – Free radical scavenging ability and antioxidant action of six Trifolium species. Pharm Biol 2011; 52: 1308-14.
19. Taheri S, Abdullah TL, Karimi E, Oskoueian E, Ebrahimi M. Antioxidant Capacities and Total Phenolic Contents Enhancement with Acute Gamma Irradiation in Curcuma alismatifolia (Zingiberaceae) Leaves. Int J Mol Sci 2014; 15: 13077-90.
20. Wahle KW, Brown I, Rotondo D, Heys SD. Plant phenolics in the prevention and treatment of cancer. Adv Exp Med Biol 2010; 698: 36-51.
21. Mahassni SH, Al-Reemi RM. Cytotoxic effect of an aqueous extract of *Lepidium sativum* L. seeds on human breast cancer cells. Indian Journal of Traditional Knowledge 2013; 12: 605-614.
22. Sokmen A, Jones BM, Erturk M. The in vitro antibacterial activity of Turkish plants. J Ethnopharmacol 1999; 67: 79-86.
23. Ozturk N, Tuncel M & Tuncel NB. Determination of phenolic acids by a modified HPLC: Its application to various plant materials. J Liq Chromatogr Relat Technol 2007; 30: 587–96.
24. Russo A, Acquaviva R, Campisi A, Sorrenti V, Di Giacomo C, Virgata G,

Barcellona ML, Vanella A. Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors. Cell Biol Toxicol 2000; 16: 91-8.
25. Jost LM, Kirkwood JM, Whiteside TL. Improved short- and long term XTT-based colorimetric cellular cytotoxicity assay for melanoma and other tumor cells. J immunol methods 1992; 147: 153-65.
26. Bürgermeister J, Paper DH, Vogl H, Linhardt RJ, Franz G. LAPSvS1, a (1- ->3) – beta galactan sulfate and its effect on angiogenesis in vitro and in vivo. CarbohydrRes 2002; 337: 1459-66.
27. Demirci B, Dadandi MY, Paper DH, Franz G, Başer KH. Chemical composition of the essential oil of Phlomis linearis Boiss & Bal., and biological effects on CAM assay: a safety evaluation. Z. Naturforsch C 2003; 58: 826-29.
28. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 2007; 114: 97-109.
29. Omuro A, De Angelis LM. Glioblastoma and other malignant gliomas: a clinical review. JAMA 2013; 310: 1842-50.
30. Chaichana KL, Zadnik P, Weingart JD, Olivi A, Gallia GL, Blakeley J, Lim M, Brem H, Quiñones-Hinojosa A. Multiple resections for patients with glioblastoma: prolonging survival. J Neurosurg 2013; 118: 812-20.
31. Kroeger KM, Muhammad AK, Baker GJ, Assi H, Wibowo MK, Xiong W, Yagiz K, Candolfi M, Lowenstein PR, Castro MG. Gene therapy and virotherapy: novel therapeutic approaches for brain tumors. Discov Med 2010; 10: 293-304.
32. Guo Y, Wang G, Gao WW, Cheng SW, Wang R, Ju SM, Cao HL, Tian HL. Induction of apoptosis in glioma cells and up regulation of Fas expression using the human interferon-β gene. Asian Pac J Cancer Prev 2012; 13: 2837-40.
33. Chou TH, Ding HY, Hung WJ, Liang CH. Antioxidative characteristics and inhibition of alpha-melanocyte-stimulating hormone-stimulated melanogenesis of vanillin and vanillic acid from Origanum vulgare. Exp Dermatol 2010; 19: 742-50.
34. Stanely Mainzen Prince P, Rajakumar S, Dhanasekar K. Protective effects of vanillic acid on electrocardiogram, lipid peroxidation, antioxidants, proinflammatory markers and histopathology in isoproterenol induced cardiotoxic rats. Eur J Pharmacol 2011; 668: 233-40.
35. Kumar S, Prahalathan P, Raja B. Antihypertensive and antioxidant potential of vanillic acid, a phenolic compound in L-NAME-induced hypertensive rats: a dose-dependence study. Redox Rep 2011; 16: 208-15.
36. Kim MC, Kim SJ, Kim DS, Jeon YD, Park SJ, Lee HS, Um JY, Hong SH. Vanillic acid inhibits inflammatory mediators by suppressing NF-κB in lipopolysaccharide-stimulated mouse peritoneal macrophages. Immunopharmacol Immunotoxicol 2011; 33: 525-32.
37. Tsuda H, Uehara N, Iwahori Y, Asamoto M, Iigo M, Nagao M, Matsumoto K, Ito M, Hirono I. Chemopreventive effects of beta-carotene, alpha-tocopherol and five naturally occurring antioxidants on initiation of hepatocarcinogenesis by 2-amino-3-methylimidazo[4,5-f]quinoline in the rat. Jpn J Cancer Res 1994; 85: 1214-9.
38. Durant S, Karran P. Vanillins--a novel family of DNA-PK inhibitors. Nucleic Acids Res 2003; 31: 5501-12.
39. Karunanidhi A, Thomas R, van Belkum A, Neela V. In vitro antibacterial and antibiofilm activities of chlorogenic acid against clinical isolates of Stenotrophomonas maltophilia including the trimetoprim/sulfamethoxazole resistants train, Biomed Res Int 2013; 392058.
40. Mekhora C, Muangnoi C, Chingsuwanrote P, Dawilai S, Svasti S, Chasri K, Tuntipopipat S. Eryngium foetidum suppresses inflammatory mediators produced by macrophages. Asian Pac J Cancer Prev 2012; 13: 653-64.
41. Hwang SJ, Kim YW, Park Y, Lee HJ, Kim KW. Anti-inflammatory effects of chlorogenic acid in lipopolysaccharide-stimulated RAW 264.7cells. Inflamm Res 2014; 63: 81-90.

42. Suzuki A, Yamamoto N, Jokura H, Yamamoto M, Fujii A, Tokimitsu I, Saito I. Chlorogenic acid attenuates hypertension and improves endothelial function in spontaneously hypertensive rats, J Hypertens 2006; 24: 1065-73.
43. Kanno Y, Watanabe R, Zempo H, Ogawa M, Suzuki J, Isobe M. Chlorogenic acid attenuates ventricular remodeling after myocardial infarction in mice. Int Heart J 2013; 54: 176-80.
44. Wan CW, Wong CN, Pin WK, Wong MH, Kwok CY, Chan RY, Yu PH, Chan SW. Chlorogenic acid exhibits cholesterol lowering and fatty liver attenuating properties by up-regulating the gene expression of PPAR-α in hypercholesterolemic rats induced with a high-cholesterol diet. Phytother Res 2013; 27: 545-51.
45. Rakshit S, Mandal L, Pal BC, Bagchi J, Biswas N, Chaudhuri J, Chowdhury AA, Manna A, Chaudhuri U, Konar A, Mukherjee T, Jaisankar P, Bandyopadhyay S. Involvement of ROS in chlorogenic acid-induced apoptosis of Bcr-Abl+ CML cells. Biochem Pharmacol 2010; 80: 1662-75.
46. Ooi KL, Muhammad TS, Tan ML, Sulaiman SF. Cytotoxic, apoptotic and anti-α-glucosidase activities of 3,4-di-O-caffeoyl quinic acid, an antioxidant isolated from the polyphenolic-rich extract of Elephantopus mollis Kunth. J Ethno pharmacol 2011; 135: 685-95.
47. Belkaid A, Currie JC, Desgagnés J, Annabi B. The chemopreventive properties of chlorogenic acid reveal a potential new role for the microsomal glucose-6-phosphate translocase in brain tumor progression. Cancer Cell Int 2006; 6: 7.
48. Arrillaga-Romany I, Reardon DA, Wen PY. Current status of antiangiogenic therapies for glioblastomas. Expert Opin Investig Drugs 2014; 23: 199-210.
49. Zhu LM, Zhao YZ, Ju HX, Liu LY, Chen L, Liu BX, Xu Q, Luo C, Ying JE, Yang YS, Zhong HJ. Efficacy and safety of bevacizumab in Chinese patients with metastatic colorectal cancer. Asian Pac J Cancer Prev 2014; 15: 6559-64.
50. Wick W, Cloughesy TF, Nishikawa R, Mason W, Saran F, Henricksson R, Hilton M, Kerloeguen Y, Chino OL. Tumor response based on adapted Macdonald criteria and assessment of pseudoprogression (PsPD) in the phase III AVAglio trial of bevacizumab (Bv) plus temozolomide (T) plus radiotherapy (RT) in newly diagnosed glioblastoma (GBM). Journal of Clinical Oncology 2013; 31: 2002.
51. Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA, Colman H, Chakravarti A, Pugh S, Won M, Jeraj R, Brown PD, Jaeckle KA, Schiff D, Stieber VW, Brachman DG, Werner-Wasik M, Tremont-Lukats IW, Sulman EP, Aldape KD, Curran WJ Jr, Mehta MP. A randomized trial of bevacizumab for newly diagnosed glioblastoma. N Engl J Med 2014; 370: 699-708.