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A Study on Phytochemical Composition, Antioxidant, and Anti-Cancer Activities of *Gingko biloba* L.

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Abstract: The study aims to investigate the phytochemical composition, antioxidant potential, and anti-cancer activities of *Ginkgo biloba* L. leaf methanol extract. In this study, the phytochemical compounds, total phenolic and flavonoid contents of *G. biloba* leaf methanol extract were investigated. Additionally, antioxidant and anti-cancer activities [against HT-29 (human colon cancer line), HeLa (human cervical cancer line), and HEK-293 (human embryonic kidney cell line)] were assayed. The main phytochemical compounds were identified as gallic (0.94±0.01 mg/g), *p*-hydroxy benzoic (0.71±0.01 mg/g), and protocatechuic (0.60±0.02 mg/g) acids in *G. biloba* leaf methanol extract by HPLC. The total phenolic and total flavonoid contents were measured as 71.20±0.42 µg GAE/mg extract and 13.24±0.35 µg QE/mg extract, respectively. The high antioxidant activity was found in $ABTS^{\bullet+}$ assay (89.04±0.71%) while moderate antioxidant activity was observed in $DPPH^{\bullet}$ (43.31±0.75%), metal chelating (49.04±0.49%), CUPRAC (absorbance: 0.85±0.01), and phosphomolybdenum (absorbance: 1.16±0.02) assays at 400 µg/mL. The IC_{50} values of *G. biloba* leaf methanol extract on HT-29, HeLa, and HEK-293 cell lines were recorded as 406.70±1.55, 84.86±0.98, and >800 µg/mL, respectively. The present study features a new addition to the antioxidant and anti-cancer properties of the therapeutically valuable *G. biloba* with its phytochemical content.

Keywords: Maidenhair tree, methanol extract, HT-29 cell line, HeLa cell line, HPLC, HEK-293 cell line.

Gingko biloba L.'nın Fitokimyasal Bileşimi, Antioksidan ve Anti-Kanser Aktiviteleri Üzerine Bir Araştırma

Öz: Çalışma, *Ginkgo biloba* L. yaprak metanol ekstresinin fitokimyasal bileşimini, antioksidan potansiyelini ve anti-kanser aktivitesini araştırmayı amaçlamaktadır. Bu çalışmada, *G. biloba* yaprağından elde edilen metanol ekstresinin fitokimyasal bileşikleri, toplam fenolik ve flavonoid içerikleri incelenmiştir. Ayrıca, antioksidan ve anti-kanser [HT-29 (insan kolon kanseri hücre hattı), HeLa (insan rahim ağzı kanseri hücre hattı) ve HEK-293 (insan embriyonik böbrek hücre hattı)] aktiviteleri test edilmiştir. *G. biloba* yaprak metanol ekstresinin başlıca fitokimyasal bileşikleri HPLC ile gallik asit (0.94±0.01 mg/g), *p*-hidroksi benzoik asit (0.71±0.01 mg/g) ve protokateşik asit (0.60±0.02 mg/g) olarak belirlenmiştir. Toplam fenolik ve toplam flavonoid içerikleri sırasıyla 71.20±0.42 µg GAE/mg ekstre ve 13.24±0.35 µg QE/mg ekstre olarak ölçülmüştür. 400 µg/mL'de $ABTS^{\bullet+}$ yönteminde yüksek antioksidan aktivite (%89.04±0.71) belirlenirken, $DPPH^{\bullet}$ (%43.31±0.75), metal kelatlama (%49.04±0.49), CUPRAC (absorbans: 0.85±0.01) ve fosfomolibden (absorbans: 1.16±0.02) yöntemlerinde orta derecede antioksidan aktivite gözlenmiştir. *G. biloba* yaprak metanol ekstresinin HT-29, HeLa ve HEK-293 hücre hatlarındaki IC_{50} değerleri sırasıyla 406.70±1.55, 84.86±0.98 ve >800 µg/mL olarak kaydedilmiştir. Bu çalışma, fitokimyasal içeriği ile terapötik açıdan değerli *G. biloba*'nın antioksidan ve anti-kanser özelliklerine yeni bir katkı sunmaktadır.

Anahtar kelimeler: Mabet ağacı, Metanol ekstresi, HT-29 hücre hattı, HeLa hücre hattı, HPLC, HEK-293 hücre hattı.

1. Introduction

Nature is acknowledged as an immense source of bioactive compounds and products leading to diverse and unique medicinal properties and technological applications. Although natural products can be located in animals, plants, minerals, and microorganisms, many of them are based on plants (Les et al., 2021). The foundations in the field of medicinal use of plants were laid with the discovery of the similarity between the chemical compounds used in drug production and the plant active substances. While the use of herbal medicines was more common in the past; as a result of the development of chemical applications, this rate has decreased gradually. However, with the research and development of new therapeutic uses of plants in recent years, the demand for natural herbal products has increased. Due to the richness of the chemical structures of plants, their use for the development of new and highly effective drug formulations has also been one of the research areas of pharmacology. In developed and developing countries, 25% of prescription drugs consist of active ingredients derived from plants. Bioactive compounds that develop because of secondary metabolic activities of plants and cannot be consumed as food but have beneficial effects for human health are called 'phytochemicals'. The precursors of these substances, called secondary metabolites, are intermediates, mostly consisting of products of primary metabolism; it forms when the plant cell uses all available carbon for primary activities (Tiwari & Shukla, 2020). The most known phytochemical compounds are phenolic compounds (polyphenols), tannins, saponins, carotenoids, coumarins, tocopherols, terpenes, isothiocyanates, sulfites, terpenoids, alkaloids, flavonoids, phytosterols, phytoestrogens, and indoles and are accepted as

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micronutrients in our diets. These compounds act as antioxidant, anti-degenerative, anti-allergenic, anti-inflammatory, antimicrobial, antithrombotic (preventing blood clotting), anti-cancer, antiatherogen (preventing atherosclerosis), antiulcer, and vasodilator (blood vessel dilator) agents (Demir & Akpınar, 2020).

Cancer is still one of the most incurable diseases and the negative side effects of all approaches used for treatment today increase the tendency towards new searches of natural origin. The second most common cancer in women and the third most common cancer in men is colon cancer. More than 1.9 million new cases were identified in 2020. Colon cancer ranks as the second most common cause of cancer-based deaths and is estimated to be responsible for approximately 935.000 cancer deaths. It is one of the cancers with increasing incidence globally and accounts for 11% of all cancer diagnoses. It is estimated that the total number of deaths from colon cancer will increase by 60-70% by 2035. Cytotoxic drugs, surgery, chemotherapy, and radiotherapy are the typical treatment methods used in the treatment of colon cancer (Alzahrani et al., 2021; Hossain et al., 2022; Sawicki et al., 2021). Cervical cancer, the fourth most common female cancer after breast, lung, and colon cancer, is associated with 600.000 new cases and 340.000 deaths per year. Even more worrying is that about 83% of all new cases of cervical cancer and 88% of all deaths ensure in low- or low-income countries (Burmeister et al., 2022; Hull et al., 2020). Despite significant improvements in our understanding of cervical cancer as a potentially avoidable disease, it is worrying that there has not yet been great improvement in patient survival with chemotherapy, radiotherapy, and/or surgical interventions and thus, the disease burden remains high (Pang et al., 2022).

Ginkgo biloba L., which is a member of family of Ginkgoaceae and pronounced as Maidenhair tree, is a valuable source of new herbal medicines dominating a wide variety of bioactive compounds with proven therapeutic efficacy. *G. biloba* known as the living fossil of the plant kingdom is one of the oldest seed plants. This plant, which made its name in history as the first plant to grow after the explosion of the Hiroshima atomic bomb in Japan in 1946, was a plant that is little affected by chemical pollutants, microorganisms, environmental factors, and insects (Brondino et al., 2013; Tabassum et al., 2022). *G. biloba* has been used as a traditional medicinal plant for more than two thousand years in especially in China and other regions of the world and today it is preferred in Asia, Europe, Argentina, New Zealand, and North America. It has been emphasized that this plant is therapeutic for diseases such as tuberculosis, asthma, stomach discomfort, skin problems, hearing loss, bronchitis, irritability, thrombus formation, arteriosclerosis, diabetes mellitus, and ischemic heart treatment. It has been stated that *G. biloba*, with its components such as flavonoids, terpenoids, bioflavonoids and organic acids, leads to antioxidant, antibacterial, anti-cancer, anti-inflammatory, antilipidemic, antiradiation, neuroprotective, antiapoptotic, antitumor, antiviral, antiatherosclerosis, hepatoprotective, and antidiabetic activities. The best recognized health supplements with *G. biloba* available on the market are Nutricost *Ginkgo biloba*, Nature's Bounty *G. biloba*, VH Nutrition *G. biloba,* and GreeNatr Panax Ginseng *G. biloba* tablets (Barbalho et al., 2022; Tabassum et al., 2022).

In this study, *G. biloba* leaf methanol extract was investigated for total phenolic and total flavonoid contents, phytochemical compounds with HPLC, antioxidant, and anti-cancer [HT-29 (human colon cancer line), HeLa (human cervical cancer line), and HEK-293 (human embryonic kidney cell line)] activities.

2. Material and Methods

2.1. Plant material and extraction

Gingko biloba L. leaves were purchased from the local herbal market of Konya, Turkey. Powdered plant material (20 g) was macerated with methanol (250 mL) at room temperature overnight. The methanol was decanted and the residue was macerated three more days with the methanol. The pooled methanol was combined and filtered; then, the filtrate was concentrated under reduced pressure and the obtained methanol extract was stored at +4^{o}C until the analysis.

2.2. Phytochemical composition

Phytochemical composition of *G. biloba* leaf methanol extract was investigated by using HPLC (Agilent 1260 infinity series). An Ace Generix reverse phase C_{18} column (5 µm, 250 mm × 4.6 mm i.d) thermostatted at 30°C was used for the separation. 0.8 mL/min solvent flow rate and 10 µL sample injection volume was used. The mobile phases used were: 0.1% phosphoric acid in water (A) and acetonitrile (B). The elution gradient was as follows: 0-7' (0-17% B); 7-20' (17-15% B); 20-24' (15-20% B); 24-28' (20-25% B); 28-30' (25-30% B); 30-32' (30-40% B); 32-36' (40-50% B); 36-40' (50-70% B); 40-45' (70-17% B). Detection was applied by photodiode array detector (PDA) at 280 nm wavelength. Retention times and UV data were matched with commercial standards to identify the compounds. Three parallel analyses were practiced. The known concentrations (in the range of 0.0 and 1.0 ppm) of different standard compounds i.e. rutin, naringin, ascorbic acid, hesperidin, gallic acid, flavone, protocatechuic acid, catechin, *p*-hydroxy benzoic acid, vanillic acid, gentisic acid, *p*-coumaric acid, ferulic acid, *o*-coumaric acid, neohesperidin, resveratrol, quercetin, coumarin, *trans*-cinnamic acid, alizarin were injected and calibration curves were obtained for the quantitative analysis of the phytochemical compounds.

2.3. Total phenolic and total flavonoid contents

The total phenolic amount of the methanol extract was performed as reported by Slinkard & Singleton (1977). The results were given as µg gallic acid equivalents (GAE). Total flavonoid amount of the methanol extract was performed as reported by Park et al. (1997). The results were given as µg quercetin equivalents (QE).

2.4. Antioxidant activity

Five different assays comprising of DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging, phosphomolybdenum, cupric reducing antioxidant capacity (CUPRAC), and metal chelating assays were used to test antioxidant activity as mentioned by Çayan et al. (2019) and Prieto et al. (1999). Ascorbic acid, α-tocopherol, BHA, and EDTA were used as the standards. The results were presented as inhibition

percentage (%) and absorbance at 400 µg/mL concentration, IC_{50} and $A_{0.50}$ values.

2.5. Anti-cancer activity

The anti-cancer activity of the methanol extract against HT-29 (human colon cancer line), HeLa (human cervical cancer line), and HEK-293 (human embryonic kidney cell line) was studied using Alamar Blue experiment (Yılmaz, 2022). Cells kept frozen at -80°C were thawed in a 37°C water bath, centrifuged, then transferred to the growth medium. Subsequently, DMEM medium (10% FBS, 1% penicillin-streptomycin, 0.01% gentamicin) and RPMI medium (10% FBS, 1% penicillin-streptomycin, 0.01% gentamicin) in 5% CO_2 atmosphere at 37°C were used for the incubation of the cells. The medium for passage was removed after the active cells reached 80-90% occupancy and the cells were washed with PBS. Cell pellets obtained by removing cells from the surfaces according to the trypsinization method were diluted in the appropriate amount of medium and passaged in cell culture dishes containing new medium. Alamar Blue® experiment was used to test anti-cancer activity. Cells seeded in 96-well plates were incubated at 37°C and 5% CO_2. After removing the growth medium and adding extract, standard or control to each well, Alamar Blue® reagent was added 18 h later and incubated for 4 h. Absorbance was measured at 570 nm and 600 nm with a 96-well microplate reader. Cisplatin and doxorubicin were used as the standards. The results were presented as IC_{50} values. The percent viability was expressed as a percentage of control in the presence of tested samples.

2.6. Statistical analysis

All results were the average of three parallel sample measurements and presented as the mean ± S.E. (standard error). Student's *t* test was used to analyze significant differences and *p* values <0.05 were accepted as significant.

3. Results

The phytochemical compounds of *G. biloba* leaf methanol extract were investigated by HPLC. All screened and identified compounds are listed in Table 1. The chromatograms of the standards and *G. biloba* leaf methanol extract are shown in Figs. 1 and 2. The main phytochemical compounds were specified as gallic, *p*-hydroxy benzoic and protocatechuic acids among the twenty screened compounds with the levels of 0.94±0.01, 0.71±0.01, and 0.60±0.02 mg/g extract, respectively. Low amounts of quercetin (0.08±0.02 mg/g extract), naringin (0.05±0.01 mg/g extract), *trans*-cinnamic acid (0.04±0.01 mg/g extract), and coumarin (0.03±0.01 mg/g extract) were also detected.

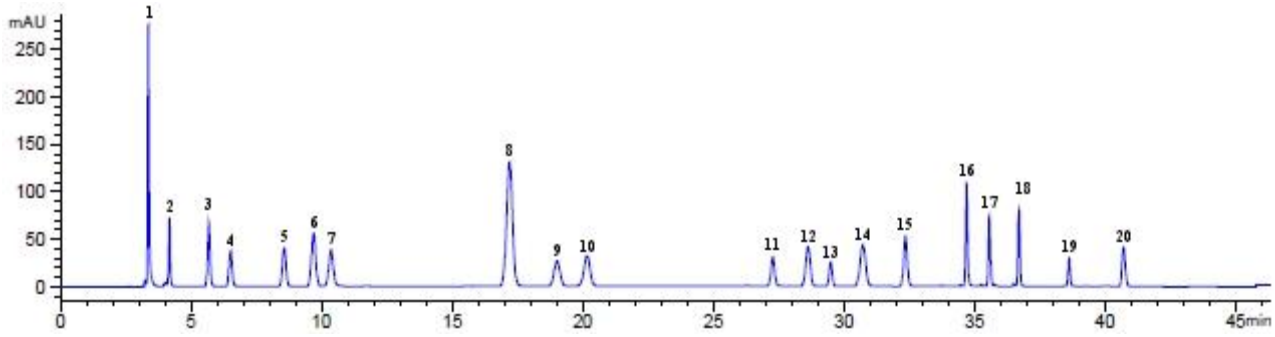

Figure 1. HPLC chromatogram of the standards

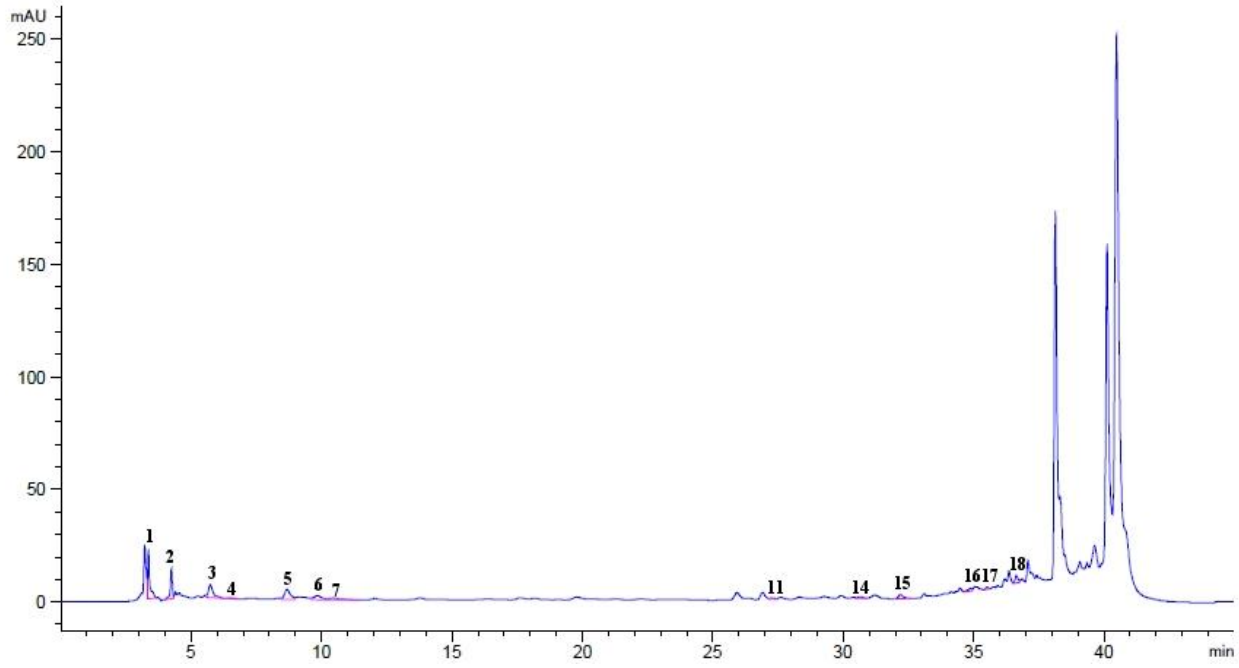

Figure 2. HPLC chromatogram of *G. biloba* leaf methanol extract

Table 1. Phytochemical compounds of *G. biloba* leaf methanol extract

Peak number	Compounds	Retention time (min)	Concentration (mg/g)
1	Ascorbic acid	3.372	0.19±0.01
2	Gallic acid	4.191	0.94±0.01
3	Protocatechuic acid	5.693	0.60±0.02
4	Catechin	6.518	0.17±0.01
5	*p*-Hydroxy benzoic acid	8.573	0.71±0.01
6	Vanillic acid	9.722	0.22±0.01
7	Gentisic acid	10.355	0.21±0.01
8	*p*-Coumaric acid	17.214	nd
9	Rutin	19.084	nd
10	Ferulic acid	20.223	nd
11	Naringin	27.374	0.05±0.01
12	*o*-Coumaric acid	28.686	nd
13	Neohesperidin	29.581	nd
14	Coumarin	30.805	0.03±0.01
15	Resveratrol	32.399	0.17±0.01
16	Quercetin	34.732	0.08±0.02
17	*trans*-Cinnamic acid	35.603	0.04±0.01
18	Hesperidin	36.702	0.25±0.01
19	Alizarin	38.661	nd
20	Flavone	40.769	nd

nd: Not detected.

Total phenolic and flavonoid contents of *G. biloba* leaf methanol extract were spectrophotometrically measured as 71.20±0.42 µg GAE/mg extract and 13.24±0.35 µg QE/mg extract, respectively.

Five different assays comprising of DPPH• and ABTS•+ scavenging, phosphomolybdenum, cupric reducing antioxidant capacity (CUPRAC), and metal chelating assays, were used to test the antioxidant activity of *G. biloba* leaf methanol extract and the results are presented in Table 2. The inhibition values of *G. biloba* leaf methanol extract in DPPH•, ABTS•+ and metal chelating assays were 43.31±0.75, 89.04±0.71, and 49.04±0.49%, respectively at 400 µg/mL. The absorbance of *G. biloba* leaf methanol extract in the CUPRAC and phosphomolybdenum assays were 0.85±0.01 and 1.16±0.02, respectively. Also, IC_{50} value of the extract was calculated as 173.78±0.78 for ABTS•+ assay, $A_{0.50}$ values as 191.77±0.36 µg/mL for CUPRAC assay and 93.26±0.15 µg/mL for phosphomolybdenum assay.

Table 2. Antioxidant activity of *G. biloba* leaf methanol extract[a]

	Antioxidant Activity									
	ABTS•+		DPPH•		CUPRAC		Phosphomolybdenum		Metal chelating	
	Inhibition[b]	IC_{50}[c]	Inhibition[b]	IC_{50}[c]	Absorbance[d]	$A_{0.50}$[e]	Absorbance[d]	$A_{0.50}$[e]	Inhibition[b]	IC_{50}[c]
G. biloba	89.04 ±0.71	173.78 ±0.78	43.31 ±0.75	>400	0.85 ±0.01	191.77 ±0.36	1.16 ±0.02	93.26 ±0.15	49.04 ±0.49	>400
α-Tocopherol[f]	91.86 ±0.12	38.51 ±0.54	87.44 ±0.09	37.20 ±0.41	1.81 ±0.10	66.72 ±0.81	NT[g]	NT[g]	NT[g]	NT[g]
BHA[f]	91.10 ±0.25	11.82 ±0.18	87.27 ±0.03	19.80 ±0.36	3.10 ±0.03	24.51 ±0.47	NT[g]	NT[g]	NT[g]	NT[g]
Ascorbic acid[f]	90.70 ±0.04	5.24 ±0.18	89.65 ±0.03	6.68 ±0.94	3.42 ±0.01	20.67 ±0.01	3.65 ±0.01	13.66 ±0.19	NT[g]	NT[g]
EDTA[f]	NT[g]	NT[g]	NT[g]	NT[g]	NT[g]	NT[g]	NT[g]	NT[g]	95.20 ±0.13	3.50 ±0.44

[a] Values represent the means ± S.E. of three parallel measurements ($p < 0.05$); [b] Inhibition (%) at 400 µg/mL concentration; [c] IC_{50} results are given as µg/mL; [d] Absorbance at 400 µg/mL concentration; [e] $A_{0.50}$ results are given as µg/mL; [f] Standards; [g] NT: not tested.

Anti-cancer activity of *G. biloba* leaf methanol extract on HT-29 (human colon cancer line), HeLa (human cervical cancer line), and HEK-293 (human embryonic kidney cell line) was studied using Alamar Blue experiment. The cell growth values (%) are presented in Fig. 3 and the IC_{50} values are given in Table 3. The cell growth value of *G. biloba* leaf methanol extract was found as 50.84±0.78% on HT-29, 3.70±0.42% on HeLa, and 91.50±1.98% on HEK-293 at 800 µg/mL. Furthermore, the IC_{50} values of *G. biloba* leaf methanol extract on HT-29,

HeLa, and HEK-293 were recorded as 406.70±1.55, 84.86±0.98, and > 800 µg/mL, respectively.

Table 3. Anti-cancer activity of *G. biloba* leaf methanol extract[a]

	HT-29[b]	HeLa[b]	HEK-293[b]
G. biloba	406.70±1.55	84.86±0.98	> 800
Doxorubicin[c]	15.56±0.96	19.78±0.02	NT[d]
Cisplatin[c]	14.75±0.87	31.02±0.05	NT[d]

[a] Values represent the means ± S.E. of three parallel measurements ($p < 0.05$); [b] IC_{50} results are given as µg/mL; [c] Standards; [d] NT: not tested.

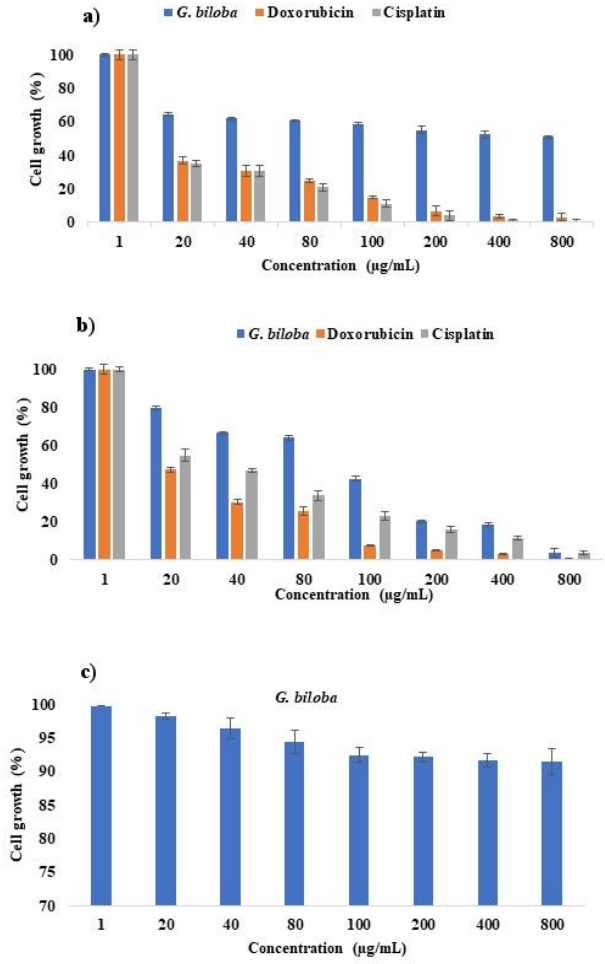

Figure 3. The cell growth values (%) of *G. biloba* leaf methanol extract a) HT-29 cell line b) HeLa cell line c) HEK-293 cell line

4. Discussion

Phytochemical compounds of *G. biloba* leaf methanol extract were investigated by HPLC. The main phytochemical compounds were specified as gallic, *p*-hydroxy benzoic and protocatechuic acids among the twenty screened compounds. All the phytochemical compounds identified in *G. biloba* leaf methanol extract were confirmed to have invaluable properties on health in prior reports. The studies have evidenced that gallic acid had cardioprotective, neuroprotective, antioxidant, and anti-cancer properties (Kiokias et al., 2020). The broad bioactive portfolio of protocatechuic acid was reported to consist of antioxidant, antiulcer, anti-cancer, antibacterial, antiaging, antiviral, antifibrotic, anti-inflammatory, analgesic, antiseptic, and antidiabetic activities (Kakkar & Bais, 2014). The well documented bioactivities of *p*-hydroxy benzoic acid were listed as antimutagenic, antimicrobial, antialgal, hypoglycemic, antioxidant, antiestrogenic, nematicidal, anti-platelet aggregating, anti-inflammatory, and antiviral (Manuja et al., 2013). The beneficial health effects of hesperidin, one of the main bioflavonoids of the food industry, involve antioxidant, anti-inflammatory, antibacterial, anti-cancer, and antimicrobial properties (Pyrzynska, 2022). Vanillic acid is a valuable phenolic acid that draws attention with its antimicrobial, antioxidant, anti-cancer, antidiabetic, anti-inflammatory, antiulcer, and antinociceptive effects beyond its flavoring feature (Malik et al., 2023). Gentisic acid is an important phenolic acid with beneficial therapeutic effects were presented as anti-inflammatory, antimicrobial, hepatoprotective, antigenotoxic, and neuroprotective, remarkably antioxidant activities in *in vivo* and *in vitro* studies (Abedi et al., 2020). Abbreviated list of medicinal values of ascorbic acid was reported to prevent from cancer, cardiac diseases, lead toxicity, ocular tissue diseases, and gout; treatment of cancer, diabetes, cognitive impairment, pulmonary function, and common cold (Jain, 2015). Catechin is a phytochemical compound known for its effectiveness as anti-inflammatory, antioxidant, anti-cancer, hepatoprotective, anti-diabetic, bactericidal, memory enhancer, anti-neuroprotective, and anti-arthritis (Baranwal et al., 2022). Resveratrol is a polyphenolic compound with health benefits associated with cardioprotective, antioxidant, neuroprotective, anti-cancer, anti-obesity, and antidiabetic activities (Zhang et al., 2021). Quercetin is accepted as a safe nutritional supplement that can be used as an antimicrobial, antidiabetic, anti-cancer, antioxidant, and anti-inflammatory agent in humans and animals (Azeem et al., 2023). The pharmacological activities of naringin were noted as antioxidant, anti-apoptotic, anti-inflammatory, anti-osteoporosis, and anti-ulcer activities in detailed investigations (Wang et al., 2023). Cinnamic acid and derivatives are used as anti-cancer, antioxidant, and antimicrobial drugs in *in vitro* studies (Ruwizhi & Aderibigbe, 2020). It has been reported that coumarin, which is found in many natural sources, is therapeutically antimicrobial, anti-inflammatory, anti-HIV, anticonvulsant, anti-cancer, and antioxidant active (Srikrishna et al., 2018). As parallel with our findings, *p*-hydroxy benzoic (0.043±0.0066, 0.0417±0.002, 0.1263±0.0084 mg/g extract), protocatechuic (0.2118±0.0025, 0.2324±0.0035, 0.235±0.046 mg/g extract), gallic (0.0078±0.0002, 0.0123±0.0014, 0.0212±0.0018 mg/g extract), and vanillic (0.002±0.00006, 0.0194±0.0002, 0.0445±0.0055 mg/g extract) acids were characterized in the methanol extract of cell, callus and leaf of *G. biloba* by RP-HPLC-UV (Szewczyk et al., 2021). In a study on the characterization of nine different *G. biloba* L. supplements in the form of paste, tablets, and liquid extracts formulated in mixture with bee products by UPLC-MS/MS, *p*-hydroxy benzoic (1.3-306.5 mg/kg) and protocatechuic (2.6-914.4 mg/kg) acids were presented as the most abundant components, similar to the results here (Akyildiz et al., 2021). Vanillic acid (12.65±0.09 mg/100 g), caffeic acid (44.90±1.17 mg/100 g), *p*-coumaric acid (14.25±0.78 mg/100 g), ferulic acid (9.68±0.59 mg/100 g), rutin (35.98±1.08 mg/100 g), narinigen (8.45±0.76 mg/100 g), quercetin (173.2±2.23 mg/100 g), and kaempferol (157.41±2.64 mg/100 g) were identified in *G. biloba* leaf methanol extract with HPLC-UV (El-Beltagi & Badawi, 2013). Zheng & Wang (2001) described

the presence of vanillic acid as 1.45±0.04 mg/100 g and caffeic acid as 39.8±2.31 mg/100 g in *G. biloba* leaf methanol extract by using HPLC-UV. In the study of Aybastier (2020), rutin (11.95±0.75, 8.75±0.06, 0.65±0.02 mg/g) and protocatechuic acid (2.28±0.11, 0.08±0.01, 0.17±0.01 mg/g) were the dominant phenolic compounds in the methanol extract of drug, food supplement and leaves of *G. biloba* by HPLC-UV. Morin (16, 81, 0 µg/g), quercetin (64, 322, 0 µg/g), myricetin (322, 569, 153 µg/g), kaempferol (295, 661, 235 µg/g), and isorhamnetin (154, 146, 123 µg/g) were detected in the water infusion, aqueous acetone, and ethanol extracts of *G. biloba* by HPLC-UV (Kobus et al., 2009). Since a single method to optimally obtain phytochemical compounds from natural sources has not been determined yet, researchers use different techniques in this way. For these reasons, different factors such as temperature, time, solvent, and pH affect the diversity of phytochemical compounds, even in the studies for the same species (Bach et al., 2019). The difference of the results obtained from the literature can be attributed to these effects.

Foodstuffs contain a wide variety of phytochemicals ranging from 500-25.000 and phenolic compounds, which construct about 500 of them, are the most common group of compounds due to their proportional relationship with health. The biological activities of phenolic compounds are related to their antioxidant, antimicrobial, anti-inflammatory, and antiviral properties. The phenolic compounds performance play an important role in retaining the balance between oxidants and antioxidants in the body. Studies have shown that dietary intake of phenolic compounds associated with oxidative stress can prevent diseases such as cancer, high cholesterol, coronary heart, cataracts, diabetes, cardiovascular diseases, and aging (Karabulut & Yemiş, 2019). Total phenolic and flavonoid contents of *G. biloba* leaf methanol extract were measured as 71.20±0.42 µg GAE/mg extract and 13.24±0.35 µg QE/mg extract, respectively. In an earlier study, total phenolic and flavonoid levels of *G. biloba* leaf methanol extract were 75.30±0.69 mg GAE/g extract and 84.59±1.43 mg QE/g extract (El-Beltagi & Badawi, 2013). Maltas et al. (2011) found the total phenolic amount of *G. biloba* leaf methanol extract as 76.0±5.2 mg GAE/g dry weight. Total phenolics and flavonoids were 14.13±0.53 mg GE/g and 71.33±0.34 mg RE/g in *G. biloba* leaf ethanol extract (Klomsakul et al., 2022). In a different study, total phenolic (37.71±0.04-129.5±5.30 mg GAE/g) and flavonoid (1.39±0.32-14.87±0.84 mg CE/g) contents of *G. biloba* leaf infusion, water, and methanol extracts were calculated (Perreira et al., 2013).

The roles of antioxidants in protecting against oxidative deterioration in foods and against pathological processes associated with oxidative stress in the body remains attractive to the scientists in the food and medical fields. Reliable and diverse antioxidant activity evaluation methods are needed to effectively investigate natural antioxidant reserves and to design new antioxidants (Munteanu & Apetrei, 2021). Antioxidant activity of *G. biloba* leaf methanol extract was tested by five different assays with this study. *G. biloba* leaf methanol extract showed inhibition at half of all standards in DPPH$^{\bullet}$ scavenging and metal chelating assays. *G. biloba* leaf methanol extract inhibited ABTS$^{\bullet+}$ radical to compete with all standards. Absorbance of *G. biloba* leaf methanol extract in CUPRAC assay was found as the half of α-tocopherol while for phosphomolybdenum was half of ascorbic acid. As a result of HPLC characterization of *G. biloba* leaf methanol extract, it was observed that the extract had high concentrations of gallic, protocatechuic, *p*-hydroxy benzoic, vanillic, and gentisic acids and hesperidin. It is also reported in the literature that these major compounds and other minor compounds identified by HPLC displayed antioxidant activity. Antioxidant activity of *G. biloba* leaf methanol extract could be associated with these valuable compounds described herein. Antioxidant activities of extracts of *G. biloba,* which is considered one of the most valuable members of the plant kingdom, have been investigated in previous studies by using many different methods. Pereira et al. (2013) investigated antioxidant activity of *G. biloba* leaf infusion, water, and methanol extracts and *G. biloba* methanol extract was found as the most antioxidant active according to DPPH$^{\bullet}$ scavenging, β-carotene bleaching, TBARS inhibition, reducing power assays with EC_{50} values of 0.74±0.04, 4.47±0.08, 0.13±0.01, 0.36±0.01 mg/mL, respectively. DPPH$^{\bullet}$ scavenging (75.86% at 300 µg/mL), ferrous ion chelating (32.2% at 200 µg/mL), and superoxide anion scavenging (62.31% at 300 µg/mL) activities of *G. biloba* leaf methanol extract were reported by El-Beltagi & Badawi (2013). ABTS$^{\bullet+}$ scavenging values of the methanol extract of drug, food supplement, and leaf of *G. biloba* were measured as 59.75±3.57, 15.88±0.43, and 41.06±1.88 mg trolox/g extract, respectively (Aybastier, 2020). IC_{50} value in DPPH$^{\bullet}$ assay was found as 162.07±9.5 µg/mL in *G. biloba* leaf methanol extract (Klomsakul et al., 2022). Kaur et al. (2012) extracted *G. biloba* leaf with 60% ethanol with five different extraction methods as maceration, Soxhlet, ultrasound assisted, the use of an orbital shaker, and microwave and tested antioxidant activities of all extracts utilizing of DPPH$^{\bullet}$ scavenging (48.2-93.4 mg trolox/g), ABTS$^{\bullet+}$ scavenging (54.7-99.8 mg trolox/g), and FRAP (18.1-41.7 mg trolox/g) assays. Li et al (2021) determined that the IC_{50} values of the ethanol extracts of *G. biloba* leaves from fourteen different regions varied between 0.131-0.461 mg/mL in ABTS$^{\bullet+}$ scavenging assay.

G. biloba leaf methanol extract showed moderate anti-cancer activity on HT-29 and significant anti-cancer activity on HeLa. Also, the methanol extract was recorded as not cytotoxic on HEK-293. Considering the negative interaction of cancer drugs used today with healthy cells, obtained results reveal the potential of *G. biloba* leaf methanol extract to be used in cancer treatment. Gallic acid was identified as the major compounds in *G. biloba* leaf methanol extract. You et al. (2010) reported that gallic acid caused death of HeLa cells via apoptosis and necrosis. Again, in a different study on gallic acid, it was stated that HT-29 proliferation was suppressed by inhibition of SRC and EGFR phosphorylation (Lin et al., 2021). Therefore, anti-cancer activity of *G. biloba* leaf methanol extract can be attributed to these effects of gallic acid on HeLa and HT-29 cell lines. Consistent with the results here, a commercially available *G. biloba* extract (EGb 761), whose content consists of 6% terpene and 24% ginkgo lavone glycoside lactones, exhibited ~ 60% cell viability at 320 mg/L after 72 h on HT-29. It was also determined that EGb 761

reduced the progression of HT-29 by enhanced caspase-3 activities, p53 upregulation, and BCL-2 genes downregulation (Chen et al., 2011). In the research of Shu et al. (2020), among eleven compounds isolated from *G. biloba* extract, only icariside B_6 was reported as moderate cytotoxic on HeLa with IC_{50} value of 58.95 µM. Ginkgetin isolated from *G. biloba* leaf inhibited HT-29 with 1.92±0.33 µM IC_{50} value in the study of Hu et al. (2019). The cell growth values of bilobol purified from *G. biloba* leaf were reported as ~ 40, ~ 20, ~ 10, and ~ 40% at 50 µg/mL against B16F10, 293, HCT116, BJAB colon cancers. It was shown that bilobol caused apoptotic cell death in a dose-dependent manner by increasing the expression of active caspase-3 and caspase-8 in HCT116 cells (Kim & Yim, 2022). One of the bioactive compounds from *G. biloba* was recorded as anti-cancer active on Siha (human cervical cancer lines) with a proliferation value of around 50% at 100 µM. It is also suggested that this compound induces cell cycle arrest and stimulates cell apoptosis by modulating the MAPK signaling pathway (Xu et al., 2020). Bilobetin (IC_{50}: 14.79±0.64 µM) and isoginkgetin (IC_{50}: 8.38 ± 0.63 µM) from *G. biloba* flower were found as anti-cancer active on HeLa by Li et al. (2019). It is also shown that ginkgolic acids from *G. biloba* inhibit the G0/G1 phase in human colon cancer and trigger intrinsic apoptosis and autophagy modulated by ROS production and suppress colon cancer cell proliferation (Liu et al., 2018).

5. Conclusion

Phytochemical compounds, total phenolic and total flavonoid contents, antioxidant, and anti-cancer (HT-29, HeLa and HEK-293) activities of *G. biloba* leaf methanol extract were investigated in this study. HPLC analysis led to the detection of phytochemical compounds with the predominance of gallic, *p*-hydroxy benzoic, and protocatechuic acids in *G. biloba* leaf methanol extract. The results demonstrated that the methanol extract exerted significant antioxidant and anti-cancer activities and had high amounts of total phenolic and flavonoid contents. The findings could be assumed as new additions to the bioactivities and phytochemical compounds of *G. biloba* species.

Ethics committee approval: Ethics committee approval is not required for this study.

Conflict of interest: The authors declare that there is no conflict of interest.

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