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Investigation on effects of walnut essential oil against glutamate toxicity on cortex neuron and LN405 cancer cell lines, diabetes, and some microorganisms

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ABSTRACT

In this study, leaf essential oil effects on glutamate toxicity model formed in cortex neurons and LN405 cell cultures were investigated. Antidiabetic activity was evaluated by α-amylase and α-glucosidase inhibitions. MIC was used for antimicrobial activity. Seven groups were examined with MTT. Glutamate 10-5 mM in cortex showed 62% viability whereas oil viability did not increase in a dose-dependent manner and the highest viability rate was observed. There are four types of glandular trichomes in leaf anatomy of walnut. The oil exhibited half as much α-glucosidase inhibitory activity with an IC50 value of 8105 µg/mL, compared to positive control acarbose (IC50 = 4762 µg/mL). MIC of oil was determined to be 625 µg/mL against E. coli, C. albicans, and C. parapsilosis, whereas S. aureus exhibited a MIC of 1250 µg/mL. Major compounds of oil were found as β-pinene (17.6%), α-pinene (11.3%), β-eudesmol (8.6%), and caryophyllene oxide (6.2%).

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Walnut; juglans regia; LN405; glutamate; antidiabetic; antimicrobial

Introduction

Cancer occurs as a result of impaired cell division in cell populations or a malignant transformation in mutated cell lines. Among all malignant tumour cells, Glioblastoma multiforme (GBM) is the most aggressive brain tumour of the central nervous system, with an incidence in 100,000 adults worldwide each year. Despite all scientific advances, radiotherapy, chemotherapy, immunotherapy and surgical resection treatments used against GBMs remain insufficient. For this reason, scientists have recently turned to various herbal research to find new treatments (1–5).

Juglans regia L. genus, which has many species and is popularly called walnut, is a tree that grows in temperate regions, spread over large areas such as Asia, America and Europe. Leaves, green walnuts, bark, kernels and seeds are frequently used in the pharmaceutical and cosmetic industry (4). Terpenoids such as pinene and eudesmol are abundant in essential oils obtained from walnut leaves. α-pinene, a reactive alkene molecule, is the most common among the natural terpenes in the plant World (6). Recent studies have shown that pinenes have antimicrobial and antioxidant properties as well as a cancer treatment. The anticancer effect of α-pinenes; is done by important mechanisms such as cell cycle change and induction of apoptosis (6,7). However, it shows a cell protective effect because it causes a decrease in anti-inflammatory markers in normal cells. β-Eudesmol is a terpenoid derivative found in excess after pinenes. Plants with high levels of β-eudesmol (Atractylodes lancea) are used in local medicine; It is used in colds, fever, sore throat, and digestive disorders (8). In anticancer studies, scientists have proven that β-eudesmol has antiangiogenic and anticancer effects (9).

Walnut trees also shed a significant amount of leaves. These leaves are used in traditional medicine for the treatment of venous insufficiency, haemorrhoids, antidiarrheal, anthelmintic and microbial infections. Since it is an oily plant, it is very rich in fat-soluble substances. Anticancer, antibacterial and antifungal effects have been demonstrated in many studies with the essential oils obtained (10,11).

Diabetes mellitus (DM) is a metabolic disease characterized by chronic hyperglycemia, micro and macrovascular diseases, caused by impaired insulin secretion

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and/or insulin activation, causing disturbances in carbohydrate, fat and protein metabolism in the body. After a long period of metabolic disorders, specific complications of diabetes can occur and atherosclerosis accelerates (12).

Medicinal plants have been widely used in the treatment of diseases for hundreds of years. Recently, the acceptance of traditional medicine as an alternative form of health care and increasing resistance to existing antibiotics has led researchers to investigate the antimicrobial activities of these plants. Obtaining and evaluating pure and active ingredients from essential oils of medicinal and aromatic plants is scientifically and economically important. It is seen in the literature that these essential oils have high antimicrobial and antifungal activities. In addition, the pharmacological properties of these oils are examined and used in wide areas such as medicine, cosmetics and industry (13).

In this study, the effects of walnut leaf essential oil were investigated by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Total Antioxidant Capacity (TAC) Total Oxidant Status (TOS) methods on primary neuron culture and glioblastoma cell lines (LN-405 cancer cell line) and also essential oil analyzes were made via gas chromatography and gas chromatography- flame ionization gas chromatography (GC-FID) and gas chromatography/mass spectrometry (GC-MS). In addition, α-amylase and α-glucosidase enzyme inhibition and antimicrobial activities of essential oil were evaluated. Also, microscopic analysis of the leaf was investigated.

Materials and methods

Chemicals and reagent

MTT, neurobasal medium (NBM), Dulbecco modified Eagles medium (DMEM), dimethyl sulfoxide (DMSO), phosphate buffer solution (PBS), Trypsin – EDTA (% 0.25), Fetal bovine serum (FBS) and Penicillin/streptomycin were purchased from Sigma® Co. (St. Louis, MO, USA).

Plant material

The author Fatma Yesilyurt collected the leaves of *Juglans regia* L. from Senyurt village, Tortum in Erzurum in May 2018 and the plant was identified by Songul Karakaya. The leaves of the plant were dried in a shaded place away from the sun and moisture. The voucher specimen is conserved at the Herbarium of Ataturk University, Faculty of Pharmacy (AUEF 1362). The crushed dried leaves (500 g) of *J. regia* were put to hydrodistillation for 3 h through a Clevenger-type apparatus in compliance with the method advised at the European Pharmacopoeia. Attained oil was dried over anhydrous sodium sulfate and preserved in sealed vials at + 4°C temperature in the dark till analysed. Essential oil % yields and colour were 0.2% and dark yellow, respectively.

Microscopic analysis

For anatomical investigations, sections were made manually from the leaf of Juglans regia in 70% alcohol. The sections were prepared with Sartur (14) reagent. Images prepared with these reagents were registered with a Zeiss 51,425 camera attached to a light microscope (Zeiss 415,500–1800–000, Carl Zeiss Microscopy, GmbH Konigsallee 9–21, 37081 Gottingen GERMANY).

GC-MS and GC analyses of essential oil

GC/MS and GC analyses methods for essential oil isolation were actualised in proportion to Karakaya et al., 2016 (15). The GC-MS analysis was established with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was utilised with helium as carrier gas (0.8 ml/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass ionization was obtained at 70 eV. Mass range was from *m/z* 35 to 450. The GC analysis was established utilizing an Agilent 6890N GC system. FID detector temperature was 300°C. To obtain the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms. Identification of the essential oil components were established by comparison of their relative retention times with those of authentic specimens or by comparison of their relative retention index (RRI) to series of *n*-alkanes.

Cell cultures

All cell lines were obtained from Ataturk University, in the Department of Medical Pharmacology (Erzurum, Turkey). Briefly, the cells were dissolved and centrifuged at 1200 rpm for 5 min. The cells were seeded in 24 well plate in an intensity of 1×10^5 cells by fresh medium (antibiotic 1%, B27 2%, FBS 10% and Neurobasal medium for cortex neurons), (antibiotic

1%, FBS 15% and DMEM for LN405) and than incubate in 5% CO_2 and 37°C) (16,17).

Walnut Leaf Essential Oil (WLEO) Administration

After exposing the cells to 10^{-5} µM glutamate for 20 min, walnut oil essential oil was added to the dark cells at a concentration of 10^{-1}-10^{-5} and left for 24 hours. Only cell medium was added to the control group, while cell medium+DMSO was used in the positive control group.

MTT assay

At the end of the experiment, 10 µL MTT solution was added to each well for 24 h. These plates were incubated in an incubator (37°C, 5% CO_2) for 4 h. Then, 100 µL of Dimethyl sulfoxide (DMSO) solution was joined into all of the wells to solve formazan crystals. The cell viability of (%) was tested at 570 nm by Multiskan ™ GO Microplate spectrophotometer reader.

TAC-TOS assay

In total oxidant status (TOS) and total antioxidant capacity (TAC) analysis, by measuring spectrophotometry evaluation is performed. The cell media were collected when the experiments were completed. According to the manufacturing procedure, TAC (Trolox Equiv/mmol L^{-1}) at 660 nm and TOS (H_2O_2 Equiv/mmol L^{-1}) absorbance were evaluated at 530 nm was evaluated.

α-amylase and α-glucosidase inhibitory activities

α-glucosidase inhibitory assay

The α-glucosidase inhibitory effect was established accordingly (18) with some small changes as described in advance (19). Entire samples (20 µL), an enzyme solution (10 µL, 1 Unite/mL) and potassium phosphate buffer (50 µL, 50 mM, pH 6.9) were admixtured in the plate. The admixture was incubated at 37°C temperature for 5 min. Then p-nitrophenyl-α-D-glucopyranoside as substrate (20 µL, 3 mM) was annexed for starting of reaction and the admixture was incubated at 37°C temperature for 30 min. Afterwards, the incubation, 0.1 M sodium carbonate (50 µL) was annexed to whole wells to eliminate the reaction. The whole solution was got in a buffer system. Acarbose was utilised as a positive control. The sum of released p-nitrophenol was surveyed utilizing a 96-well microplate reader at 405 nm. Every test for whole specimens was performed in triplicate. Percentile inhibition of Entire samples was figured out utilising the equation below:

$$\text{Inhibition}(\%) = \left(1 - A_{\text{specimen}}/A_{\text{control}}\right) \times 100$$

α-amylase inhibitory assay

The α-amylase inhibitory effect was established accordingly (20) with some small changes as described in advance (19). Entire samples (100 mL) and 1% starch solution (100 mL) in 20 mM sodium phosphate buffer (pH 6.9 within 6 mM sodium chloride) were incubated at 25°C temperature for 10 min in a 24-well microplate. Afterwards, incubation, 100 µL α-amylase solution (0.5 mg/mL) was annexed to every well and the reaction admixtures were incubated at 25°C temperature for 10 min. Afterwards, incubation, dinitrosalicylic acid colour reagent (200 µL) was annexed to stop the reaction. The microplate was that incubated in a boiling water bath for 5 min and refrigerated to room temperature. It was gone 50 µL from every well and then was annexed to a 96-well microplate. The reaction admixture was diluted after annexing 200 µL distilled water and absorbance was calculated at 540 nm. Acarbose was utilized as a positive control. Every test for the whole specimen was carried out in triplicate. Percentile inhibition of entire samples was calculated utilising the equation below:

$$\text{Inhibition}(\%) = \left(1 - A_{\text{sample}}/A_{\text{control}}\right) \times 100$$

Antimicrobial activity (µg/mL)

In vitro antimicrobial activity was assessed against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10,231 and *C. parapsilosis* ATCC 22,019 by microdilution methods. The microorganisms were purchased from Microbiologics (San Diego, CA). The standard drugs moxifloxacin and fluconazole (64–0.125 µg/mL) were prepared within water and dimethyl sulfoxide (DMSO). The walnut essential oil was prepared with a final concentration range of 2500 to 4.88 µg/mL. The antimicrobial activity was determined using a slight modification of microdilution methods for aerobic microorganisms (*M*-7-A7) and fungi (*M*-27-A3) published by the Clinical Laboratory Standards Institute (CLSI). The standard cultures were kept at-85°C. The cultures were cultivated into Petri plates containing Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA) and incubated at 37°C for 24 h. At the end of the incubation, it was taken from the single colonies that developed on the growth medium and

transferred to the tubes with Mueller Hinton Broth (MHB) (RPMI medium for *Candida* species) and incubated again at 37°C for 24 h. After 18–24 h of incubation, the cultures were prepared according to McFarland No: 0.5 tube (10^8 cfu/mL for bacteria, 10^6 cfu/mL for yeast culture). Microbial growth was determined by adding 20 µL of resazurin of 0.01% with minor modifications of CLSI standards. It was incubated for another 1–2 h at 37°C for colouration. Moxifloxacin and fluconazole were used as positive control and DMSO alone as a negative control. At the end of incubation, active bacterial cells reduce the resazurin (blue) to the resorufin (pink). The essential oil was evaluated for antimicrobial activity against *Candida*, Gram (+) and Gram (-) bacteria strains. The experiments were carried out in triplicates and results were calculated as mean ± SD.

Statistically analysis

The statistical analysis was done by employing SPSS 22.0 windows software and using one-way ANOVA and Tukey's HSD methods. Values $P < 0.05$ were considered statistically significant.

Results and discussion

After the essential oil components obtained from walnut leaves were determined by GC-MS, they were tested on cortex neurons and LN-405 cancer cell lines. After the cells were treated with WLEO, MTT, TAC, and TOS tests were performed. In addition, the antimicrobial and antidiabetic activities of oil were evaluated. Microscopic analysis of leaf was also investigated.

Microscopic analysis

The accumulation of essential oils in plants is usually limited to specialised secretory structures, namely the glandular trichomes, which are multicellular epidermal glands found in several families that secrete terpene in an extracellular space at the apex of the trichomes. Besides, the storage of terpenoids in these structures can be utilized to limit the risk of toxicity to the plant itself. The morphology of these structures varies with irrigation conditions and the toxicity of the intracellular ingredients and may vary with the phenology of the plant. The glands of aromatic plants come in several shapes and sizes to provide a specific function. The variation between the gland types includes some aspects such as structure, mode of secretion, and timing of secretion (21). Where available, the oil glandular trichomes are the principal sites of essential oil biosynthesis, and plants lacking such specialised structures can only synthesize and accumulate trace amounts of monoterpenes (22). To this end, this article contains several microscopic observations of glandular trichomes and their combinations with the composition of oil and classification.

The anatomy of the leaves of *Juglans regia* was investigated previously (23). There are types 1 (unicellular stalked, multicellular-headed glandular trichome), 2 (unicellular-headed glandular trichome), 3 (multicellular-stalked, multicellular-headed glandular trichome), and 4 (unicellular-stalked, unicellular-headed glandular trichome) glandular trichomes in leaflet midrib cross-section, leaflet upper and lower superficial sections, petiole of leaflet cross-section, and leaf midrib cross-section anatomies of *J. regia* (SOM-Figures S1–5). The lower number of glandular trichomes was at the upper and lower superficial sections. In addition, the leaf glandular trichomes are defined by a greater presence of monoterpene hydrocarbons (31.9%) and oxygenated sesquiterpenes (26%). The findings we obtained as a result of the anatomical studies on the leaves are compatible with the properties given by Baldemir and Güvenç (2007) for *J. regia* leaves. In our study, there have been found four types of trichomes but in the mentioned study, there were two types.

GC-FID and GC-MS analyses of essential oil

The colour of the essential oil of *Juglans regia* was dark yellow and the essential oil % yield was 0.2 w/v, %. A total of 37 compounds finding 85.6% of the oil were defined in the leaves of *J. regia* essential oil. β-Pinene (17.6%), α-pinene (11.3%), β-eudesmol (8.6%), and caryophyllene oxide (6.2%) were the primal constituents of the leaf essential oil. Monoterpene hydrocarbons (31.9%), oxygenated sesquiterpenes (26%), and oxygenated monoterpenes (15.8%) were in the essential oils one of the dominating group of compounds otherwise, sesquiterpenes hydrocarbons (9.4%) was the lowest components of the essential oils (Table 1). Previous studies on the *Juglans regia* revealed that the main volatile compounds of the essential oil of the leaf were β-pinene (30.5%), α-pinene (15.1%), β-caryophyllene (15.5%), and germacrene D (14.4%) (7). It was reported that the primal components of the leaf of *J. regia* were eugenol (27.5%), germacrene D (21.4%) methyl salicylate (16.2%), and (E)-β-farnesene (8.2%) (24). (E)-caryophyllene (1.4–47.9%), β-pinene (4.5–39.5%), germacrene D (5.0–23.3%), α-pinene (1.5–18.1%), α-humulene (1.1–11.8%) were determined as major compounds of leaves essential oil of *J. regia* (25). Caryophyllene oxide (16.9–27.4%), β-caryophyllene (4.0–22.5%), germacrene (1.2–9.4%), and β- Pinene (2.8–9.5%). were found to be primal compounds of leaves oil of *J. regia* (26). Organic

Table 1. The Composition of the Essential Oils of *Juglans Regia*.

KI[a]	RRI[b]	Compound	%	IM
1008–1039[c]	1032	α-Pinene	11.3	RRI, MS
1085–1130[c]	1118	β-Pinene	17.6	RRI, MS
1098–1140[c]	1132	Sabinene	0.9	RRI, MS
1140–1175[c]	1174	Myrcene	0.3	RRI, MS
1178–1219[d]	1203	Limonene	1.5	RRI, MS
1013–1039[c]	1213	1,8-Cineole	2.4	RRI, MS
1246–1291[d]	1280	p-Cymene	0.3	RRI, MS
1477–1511[c]	1499	α-Campholene aldehyde	0.5	MS
1496–1546[c]	1535	β-Bourbonene	1.1	MS
1545–1590[c]	1586	Pinocarvone	1.9	RRI, MS
1545–1601[c]	1601	Nopinone	0.3	MS
1564–1630[c]	1611	Terpinen-4-ol	0.4	RRI, MS
1570–1685[c]	1612	β-Caryophyllene	1.7	RRI, MS
1597–1648[c]	1648	Myrtenal	2.6	MS
1627–1668[c]	1668	(Z)-β-Farnesene	3.7	MS
1643–1671[c]	1670	trans-Pinocarveol	3.8	MS
1665–1691[c]	1683	trans-Verbenol	1.8	MS
1659–1724[c]	1706	α-Terpineol	0.4	RRI, MS
1696–1735[c]	1725	Verbenone	0.1	MS
1676–1726[c]	1726	Germacrene D	1.5	MS
1693–1740[c]	1733	Neryl acetate	0.9	RRI, MS
1699–1751[c]	1751	Carvone	0.1	RRI, MS
1743–1788[c]	1786	ar-Curcumene	1.1	MS
1727–1809[c]	1798	Methyl salicylate	0.7	RRI, MS
1743–1808[c]	1804	Myrtenol	1.4	MS
1782–1836[e]	1830	β-Damascone	0.5	MS
1805–1850[c]	1845	trans-Carveol	0.4	MS
1820–1873[c]	1868	(E)-Geranyl acetone	0.4	MS
1936–2023[c]	2008	Caryophyllene oxide	6.2	RRI, MS
2016–2043[c]	2037	Salvial-4 (14)-en-1-one	0.1	MS
1995–2055[c]	2050	(E)-Nerolidol	0.5	MS
2003–2071[c]	2071	Humulene epoxide-II	0.1	MS
2156[d]	2200	α-Guaiol	1.4	MS
2145–2205[e]	2204	Eremoligenol	3.7	MS
2193[d]	2210	Hinesol	3.1	MS
2186–2250[c]	2250	α-Eudesmol	2.3	MS
2196–2272[c]	2257	β-Eudesmol	8.6	MS
		Monoterpene Hydrocarbons	31.9	
		Oxygenated Monoterpenes	15.8	
		Sesquiterpene Hydrocarbons	9.4	
		Oxygenated Sesquiterpenes	26	
		Others	2.5	
		Total	85.6	

[a]KI from literature (46[c], 47[d], 48[e],), [b]RRI: Relative retention indices calculated against *n*-alkanes; % calculated from FID data; t: Trace (<0.1 %) IM: Identification method based on the relative retention indices (RRI) of authentic compounds on the HP Innowax column; MS, identified based on computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.

solvent extraction, cold pressing and hot pressing were used for obtaining walnut oils. A total of 92 volatile substances, including trans-2-heptenal (21.00%–28%.61%), n-hexanal (15.09%–20.72%) and cis-2-octene (9.77–16.28%) were detected in 11 walnut oil specimens (27). The biggest concerns regarding walnut quality are kernel oxidation sensitivity and pellicle darkening. It was found that pentanal, 2-methylpropanal, (E)-2-octenal, 1-octenal-3 in nuclei -ol, benzaldehyde (E,E)-2,4-nonadienal hexanal, 3-octanone, octanal, (E)-2-pentenal, hexanoic acid (Z)-2-penten-1-ol, and hexanol were sufficient to distinguish oxidation levels and as oxidative markers in walnuts (28). The major frequent components in walnut seeds were β-pinene (33.6%)and α-pinene (31.4%); that are reactive hydrocarbons (29). Walnut leaves were collected from two different locations (Adana and Ankara) in Turkey. Thymol (23.1%) was the main compound of Adana essential oil whereas caryophyllene oxide (33.8%) was found as the major compound of Ankara oil by GC/MS and GC-FID analyses (30). Essential oils of fresh and dried walnut leaves were analysed via GC and GC/MS. 46 and 42 compounds representing 89.29% and 96.38% of the fresh and dried oils were identified respectively. The composition of the 5 main compounds such as (E)-caryophyllene, germacrene D, α-zingiberene, δ-cadinene and (E)-β-farnesene was found to considerably rise after shade drying (31). Walnut oil includes over 85% unsaturated fatty acids, that are with ease oxidized along storage. The qualitative composition of essential oxidation compounds was evaluated via the SPME/GC-MS combination. 18 compounds, containing aldehydes, alcohols and acids were determined and 2-octenal, hexanal, 2-heptenal, 1-octen-3-ol, hexanoic

acid and nonanal were found as the major products composed along oxidation (32). As with most other studies, the major frequent components in walnut essential oils were β-pinene and α-pinene; which are reactive hydrocarbons.

MTT assay

Cortex neuron culture and viability of LN-405 cells were measured by the MTT test. According to our results, the viability was defined as 100% in the control groups and the other groups were rated accordingly. When the positive control group and the control group were compared in cortex neuron cells, there was no difference between them. Glutamate control 10^{-5}μM and WLEO 10^{-1} concentration had the lowest viability rates compared to the control group with values of 67% and 67.5%, respectively (**$P < 0.001$ When the WLEO 10^{-2} concentration was compared with the control group, the viability rate was 77% (*$P < 0.05$). The highest vitality rate in walnut leaf essential oil was seen at WLEO 10^{-4} and WLEO 10^{-5} concentrations.

MTT results obtained in LN405 cell line are presented in SOM-Figure S7. In the study, no significant difference was found between the control group and the positive control group. When the glutamate control 10–5 μM and the control group were compared, the survival rate in the glutamate group was 82% (*$P < 0.05$). WLEO 10^{-1}, 10^{-2}, and 10^{-3} concentrations were the lowest when compared to the control group, with 70%, 73% and 78% viability, respectively (**$P < 0.001$).

TAC assay

Our TAC test results of cortex neuron culture and LN-405 cells are shown in SOM-Figures S8 and S4 based on Trolox equiv/mmol L^{-1}. According to the results of this test, antioxidant levels in the cortex neuron cells were found as 6.15 in the control group and 5.78 in the positive control group, no significant difference was found when these values were compared. Glutamate control 10^{-5} mM and WLEO 10^{-1} concentrations had the lowest antioxidant levels compared to the control group, with values of 3.66 and 3.49, respectively (**$P < 0.001$). When the WLEO 10^{-2} concentration was compared with the control group, the antioxidant level was found to be 4.36 (*$P < 0.05$). The highest antioxidant level in walnut leaf essential oil was observed in WLEO 10^{-3}, 10^{-4} and 10^{-5} concentrations.

Trolox equiv. obtained in the LN405 cell line. The level/mmol L^{-1} is given in SOM-Figure S9. According to the results of this test, antioxidant levels of the control group, positive control group and glutamate control group were determined as 10^{-5} mM, respectively, 5.15, 4.98 and 4.15, no significant difference was found when these values were compared. WLEO 10^{-1} concentration had the lowest antioxidant level with a value of 2.24 compared to the control group (**$P < 0.001$). When the WLEO 10^{-2} concentration was compared with the control group, the antioxidant level was found to be 2.79 (*$P < 0.05$). Antioxidant levels in cancer cells were found to be highest at WLEO 10^{-3}, 10^{-4} and 10^{-5} concentrations, with values of 3.65, 3.81 and 4.26, respectively.

TOS assay

Our TOS test results of cortex neuron culture and LN-405 cells H_2O_2 equiv/mmol L^{-1} values are shown in SOM-Figures S10 and S11. The level of H_2O_2 equiv/mmol L^{-1} in cortex neuron cells is given in SOM-Figure S10. According to the results of this test, the control group had an oxidant level of 2.62 and the positive control group had an oxidant level of 2.87, and when these values were compared, there was no significant difference between them. Glutamate control 10^{-5} μM and WLEO 10^{-1} concentration had the highest oxidant levels compared to the control group, with values of 4.99 and 4.80, respectively (**$P < 0.001$). When the WLEO 10^{-2} concentration was compared with the control group, the oxidant level was found to be 3.94 (*$P < 0.05$). The lowest oxidant levels in WLEO 10^{-3}, 10^{-4} and 10^{-5} concentrations (groups) in cortex neuron cells were found as 3.75, 3.42 and 3.10, respectively.

The level of H_2O_2 equiv/mmol L^{-1} obtained in the LN-405 cell line is given in SOM-Figure S11 According to the results of this test, the control group, positive control group and glutamate control group had 10^{-5} μM antioxidant levels of 2.27, 2.52 and 2.68, respectively, and when these values were compared, there was no significant difference between them. Compared to the control group, WLEO 10^{-1}, 10^{-2} and 10^{-3} concentrations in cancer cells had the highest oxidant levels with values of 5.14, 4.98 and 4.52, respectively (**$P < 0.001$). When the WLEO 10^{-4} concentration was compared with the control group, the oxidant level was found to be 3.67 (*$P < 0.05$). When the WLEO 10^{-5} concentration was compared with the control group, the oxidant level was found to be 3.40.

In this study, the essential oil obtained from the walnut leaf was analyzed by the GC-MS method. Then, cortex neuron and LN-405 GBM cancers, which reached

85% density in 96 plates, were exposed to fat concentrations for 24 h. After our study period was completed, it was measured by MTT, TAS and TOS tests.

Walnut fruit is a plant that contains a high amount of phytochemicals (33,34) In addition, the fruit of the walnut contains high amounts of omega-3 and omega-6 fatty acids. These omega-3 fatty acids are known to have neuroprotective and antioxidant effects (35). Miraliakbari et al. reported that components of the walnut tree such as wood, root, fruit and leaf show antioxidant activity (36).

According to our MTT results, the concentration of WLEO 10^{-3} showed a neuroprotective effect in cortex neurons in Supplemental online material (SOM)-Figure S6 and also induced apoptosis in cancer cells in SOM-Figure S7 (7,8,36,37). There are studies examining the antimicrobial, antioxidant and anticancer properties of walnut leaves. In this way, we think that the substances found in the essential oil of the leaf may also be neuroprotective.

In the studies of Kamalak et al., glutamate toxicity has an important place in neuron cell degeneration (38). As the toxicity increases, positive ions enter the cell and cause degeneration. According to the studies of Wenzel et al., antioxidant substances were eliminated by extraction from walnut leaves and black walnut shells and their effects were examined (39). In the TAS results of cortex neurons in SOM-Figure S8 and cancer cells in SOM-Figure S9, the antioxidant level decreased as the dose increased. In our study, a high level of viability was observed in neuron cells as a result of TAS of WLEO 10^{-3} concentration. In this way, it has been observed that the essential oil of walnut oil is neuroprotective. On the other hand, apoptosis was observed at the same concentration in cancer cells.

α-amylase and α-glucosidase inhibitory activities

The essential oil exhibited half as much α-glucosidase inhibitory activity with half maximal inhibitory concentration value (IC_{50}) of 8105 ± 411 µg/mL, compared to the positive control acarbose (IC_{50} = 4762 ± 100 µg/mL). No α-amylase inhibitory activity of essential oil had been observed when compared to that of acarbose (IC_{50} = 464 ± 46 µM). The results of antidiabetic activity was given in Table 2. To our knowledge, it was the first study to evaluate α-glucosidase and α-amylase inhibitory activities of the essential oil of leaves. According to the literature, the antidiabetic effect of the leaves of walnut had been evaluated in many *in vitro*, *in vivo*, and clinical studies.

Table 2. The Results of Antidiabetic Activity of *Juglans Regia*.

Concentration (µg/mL)	Essential oil	Acarbose
	α-Glucosidase Inhibition (%)	
5000	30.32	62.88
1000	7.36	31.58
750	4.10	20.81
500	3.12	16.39
250	0.71	7.91
100	0.11	6.51
50	−0.34	3.76
	α-Amylase Inhibition (%)	
750	−16.21	72.09
500	−48.38	48.64
250	−27.19	36.09
100	−14.38	25.25

In one study, the efficacy of 70% ethanolic leaf extract on hyperglycemia and lipid profiles was investigated in type II diabetic patients (n = 61). Treatment with the capsule containing 100 mg of extract twice daily for 3 months improved lipid profile and glycemic control without any adverse effects (40).

In another study, the *in vitro* antidiabetic, *in vivo* antihyperglycemic effects of 60% methanolic extract of leaves were evaluated. In the *in vivo* study, dry powder of leaves (25, 50 and 100 mg/kg) was given orally, twice daily to streptozocin-induced diabetic rats for 28 days. At the end of the *in vivo* study, it was shown that administration of leaf extract concluded in weight gain, glycaemic control, and reversal of dyslipidaemia (41). The antidiabetic effect of the leaf can be attributed to the essential oil.

Antimicrobial activity (µg/mL)

The antimicrobial activity of the walnut essential oil was studied against *E. coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *C. albicans* ATCC 10,231 and *C. parapsilosis* ATCC 22,019. The walnut essential oil minimum inhibitory concentration (MIC) values were observed between 625 and 1250 µg/mL against the strains. The minimum inhibitory concentrations were determined as MIC = 1250 µg/mL for *E. coli* ATCC 8739, *C. albicans* ATCC 10,231 and *C. parapsilosis* ATCC 22,019.

Table 3. The Antimicrobial (MICs) Results of Essential Oil of *J. Regia*.

Essential oil and standards	*E. coli* ATCC 8739	*S. aureus* ATCC 6538	*C. albicans* ATCC 10,231	*C. parapsilosis* ATCC 22,019
The walnut essential oil	625	1250	625	625
Moxifloaxacine	0.25 >	0.25 >	0.25>	-
Fluconazole	-	-	64	2.0

The antimicrobial (MICs) results of essential oil of *J. regia* are given in Table 3. Previous works screened different walnut leaf extracts for their antimicrobial activities against many kinds of microorganisms (42–44). In a study, *Rather et al.* reported that the essential oil from the leaves of *Juglans regia L.* had antimicrobial activity. The essential oil was evaluated against a group significant Gram-positive, *Staphylococcus aureus, Staphylococcus epidermidis* MTCC-435, *Bacillus subtilis* MTCC-441, Gram-negative bacteria *Proteus vulgaris* MTCC-321, *Pseudomonas aeruginosa* MTCC-1688, *Escherichia coli, Klebsiella pneumonia, Salmonella typhi* and *Shigella dyssenteriae. B. subtilis* MTCC-441, *S. epidermidis* MTCC-435 and *S. aureus* were found the most sensitive bacterial strains to the essential oil. Contrary to our results, *S. aureus* was found to be more effective with MIC = 15.62 µg/mL in this study (8). In vitro antimicrobial effects of essential oils of *J. regia* was assessed by utilizing disc diffusion methods against *Salmonella enterica* serovar typhimirium ATCC 14,028 and *Staphylococcus aureus* subsp. *aureus* ATCC 25,923, *Yersinia pseudotuberculosis* ATCC 911 and *Bacillus cereus* 702 ROMA, *Enterobacter aerogenes* CCM 2531, *Bacillus subtilis* IMG 22 *and Proteus* vulgaris FMC. The best resistance zone was against *P. vulgaris* and *Staphylococcus aureus* subsp. *aureus* with 17 mm diameter whereas the lowest resistance zone was seen against *Y. pseudotuberculosis*, 6 mm diameter (45–48).

Conclusion

In this study, the essential oil of the leaf of Juglans regia was evaluated for antioxidant, anticancer, antidiabetic and antimicrobial activities. Natural products have gained significant acclaim as an alternative and/or complementary health care approach with extensive pharmaceutical and biological properties. High sugar levels in the blood and tissues cause bacteria to grow and infections to develop faster. The major components the of oil were found β-pinene (17.6%) and α-pinene (11.3%). MIC value of oil was found to a MIC = 625 µg/mL against *E. coli, C. albicans* and *C. parapsilosis*. Glutamate 10–5 mM in the cortex showed 62% viability whereas oil viability did not increase in a dose-dependent manner and the highest viability rate was observed. Glutamate 10–5 mM has a 77% viability in LN405 cancer cells. There are four types of glandular trichomes in leaf anatomy.

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