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In Vitro Potent Anticancer, Antifungal, and Antioxidant Efficacy of Walnut (*Juglans regia* L.) Genotypes

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Abstract: Walnuts are one of the healthiest foods in the world because they are one of the best sources of beneficial nutrients, minerals, and antioxidants. They also contain key sources of energy. Despite several traditional uses, the leaves of *Juglans regia* L. have received little attention regarding phytochemical and pharmacological potential. Thus, the current study intended to find the walnut genotypes with the greatest antioxidant, anticancer, and antifungal activity. The total polyphenolic, flavonoid, and flavanol contents of leaves from 14 walnut genotypes were determined. Genotypes that accumulate flavonoid/flavonol contents (99.8–111.93 mg/g quercetin equivalent (QE) and 101.67–111.83 mg/g QE) showed significantly higher ferric reducing antioxidant potential (FRAP) activity (128.2–148.1 μM Fe^{2+}/g dry weight (DW)] than other genotypes. Maximum divergence in the quercetin content (0.8–1.23 mg/g) of walnut genotypes was obtained by cluster analysis. The active component, quercetin, was measured using RP-HPLC. Moreover, the extracts were investigated for antifungal and anticancer assays. We report the significant antifungal potential of walnut leaf genotypes against *Candida glabrata*, *Candida albicans*, and *Candida tropicalis*, with 57.7–93.6%, 26.8–51.5%, and 26.8–51.5% inhibition, respectively. The most significant antiproliferative effect was shown by *Opex Culchry*, which exhibited 9.4% cell viability at a concentration of 25 μL (0.75 mg) against lung (A549) cell lines. *Chenovo* exhibited 2.9, 6.2, and 2.2% cell viability, *Opex Culchry* exhibited 2, 1.5, and 2.4% cell viability, and *Sulieman* showed 7.6, 0.9, and 7% cell viability against the colon (HCT116) cell lines. The results showed that walnut leaves possess enormous potential as antioxidants, and as anticancer and antifungal agents.

Keywords: walnut; bioactive compounds; phenolics; flavonoids; flavanols; HPLC; anticancer; antifungal; antioxidant

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1. Introduction

Plants have been considered an essential source of medicine since ancient times, and are used in traditional systems of medicine, such as folk medicines [1]. One such therapeutic plant is *Juglans regia* L. (Juglandaceae). The word *Juglans*, from Latin, means "the acorn of Jupiter", while *regia* refers to royalty. Its origin is ancient Persia. The most valuable nut crop is usually grown as a nutrient-rich fruit. Walnut, belonging to the genus *Juglans* of the Juglandaceae family, consists of approximately 40 species and comprises both monoecious

and heterodichogamous characterizations, favouring outcrossing. Walnuts are highly concentrated with essential life components, such as proteins, fats, nutrients, and minerals. In addition, they are a rich source of phytoconstituents, such as phenolics, flavonoids, sterols, and other polyphenolic compounds [2]. Food polyphenols/flavonoids are believed to have powerful antioxidant effects that prevent and treat pathological conditions or diseases caused by reactive oxygen species (ROS) [3]. All parts of the plant are significant: bark, leaves, shells, kernels, husks, flowers, seeds, oil, and nuts. Among these, walnut leaves are highly effective in acting as an alternative therapy, as it is utilized for astringent, depurative, antidiarrheal, antimicrobial, keratolytic, hypoglycaemic, anthelmintic, and carminative purposes, as well as for treating cold, sinusitis, and stomach ache [4]. Furthermore, walnut leaves have antihypertensive, antimicrobial, hypoglycaemic, and antioxidative effects, as reported by different researchers [5,6]. The characteristic compound contained in the *Juglans* species, Juglone (5-hydroxy-1,4-naphthoquinone), has also been reported to be present in fresh walnut leaves [6]. Juglone, a substance that is especially important for treating cancer, has been found to have special significance in this area because of its ability to resist cancer cell proliferation, induce apoptosis in cancer cells, induce autophagy, inhibit the migration and invasion of cancer cells, etc. [7]. Walnut leaves have traditionally been used to treat skin inflammation, hemorrhoidal symptoms, ulcers, venous insufficiency, hyperhidrosis, and diarrhoea. They have antioxidant, antiseptic, antibacterial, astringent, and chemopreventive properties, as well as anthelmintic and depurative effects. Often utilized for treating itching, chronic dysentery, frostbite, and itching, the leaves of the Kashmir Himalaya were also used as mosquito repellents and lice killers; the fruits were utilized as aphrodisiacs, as well as to treat constipation and rheumatism; the oil was applied to treat dandruff and muscular pain, as well as to improve vision and memory; while the roots were used to prevent hair loss and tooth decay, and to treat wounds [8]. Walnut leaves are rich sources of flavonoids, which are considered essential components in the human diet. Flavonoids play a significant role in regulating the immune system and enhancing anticancer potential. Moreover, many pharmacological properties occur in flavonoids, such as anti-tumour, antihepatotoxic, antimicrobial, antiviral, central vascular, and anti-inflammatory effects [9]. One subclass of flavonoids, flavonol, with its major component quercetin, is universally rich in nutritional value, often used as a representative component of flavonoids for its defensive potential. It has exclusively diversified natural potential, comprising compelling anti-diabetic, anti-tumour, antioxidant, and antiviral efficacy [10]. Despite many traditional claims, the leaves of *Juglans regia* L. are less explored in terms of phytochemical and pharmacological potential. Considering all of these facts, the present investigation aimed to assess the phytochemical composition, as well as the efficacy of the antioxidative, antifungal, and antiproliferative properties of walnut (*Juglans regia* L.) leaf genotypes.

2. Material and Methods

2.1. Plant Material

The collection of leaves from *Juglans regia* L. was conducted at the experimental field gene bank of ICAR-Central Institute of Temperate Horticulture, Rangreth Srinagar (Jammu and Kashmir), India, with collected specimens representing 14 walnut genotypes (*Tuttle*, *Nugget*, *Franquette*, *Sel 1*, *Suleiman*, *Sel 4*, *Payne*, *Hamdan*, *Sel 3*, *Serr*, *Sel 2*, *DW Sel 5*, *Opex Culchry* and *Chenovo*). The genotypes were authenticated by Prof. Akhtar H Malik, Curator; Dept. of Taxonomy, University of Kashmir, Hazratbal Srinagar, under Voucher specimen No.2647-(KASH) Herbarium, Centre for Biodiversity and Taxonomy, University of Kashmir, 16-04-2018, CITH, Srinagar, Kashmir.

2.2. Fungal Strains Used

Candida Albicans (ATCC 24433), Candida Glabarata (ATCC 2001), Candida Tropicalis (ATCC 750).

2.3. Cell Lines

The Human Lung Cancer Cell Line A549 and Colon Cancer Cell Line HCT-116 were obtained from NCCS, Pune, India.

2.4. Extraction

Properly dried and finely powdered leaves were taken for extraction preparation. 2 g of each walnut leaf was ground using a mortar and pestle in 33 mL of methanol, and then, using a magnetic stirrer, it was stirred for 1 h and left throughout the night. The supernatant was collected after centrifugation of the sample for 15 min at 4000 rpm, and then the filtrate was obtained through a Whatman filter paper. The semisolid slurry extract was obtained after vaporizing the solvent using a rotary evaporator. In order to avoid extra sample dilapidation, each extract was freshly prepared.

2.5. Determination of Total Polyphenolic, Flavonoid, and Flavonol Content

The modified Folin–Ciocalteau method was used to determine the total phenol content of methanolic leaf extracts of walnut [11,12], which was measured as gallic acid equivalent in mg/g. Total flavonoids in the walnut leaf extract were determined, and measured as quercetin equivalent in mgQE/g, and, thus, total flavonoid concentration was determined. Total flavonol determination was measured as quercetin equivalent (mg/g) as a standard compound.

2.6. Antioxidant Activity

The Benzie and Strain method, with minor alterations [13], was utilized for the FRAP assay. Thereafter, using the method of Rather et al., with slight modifications, a DPPH free radical scavenging assay was done [14].

2.7. Quantification of Quercetin

2.7.1. Sample and Standard Preparation for HPLC

In HPLC-grade methanol, a 100-ppm solution of leaf sample extracts was prepared. Per calibration, the standard was prepared in HPLC-grade methanol as 100–500 ppm quercetin. First, 0.2 µm syringe filters were used to filter samples, and then injection of filtered samples of about 10 µL into an HPLC combined with a PDA detector was performed.

2.7.2. HPLC Analysis

Shimadzu HPLC (Kyoto, Japan) was used to carry out the HPLC analysis, and it was equipped with quaternary pumps, a 20 µL loop injection valve, and a degasser. The separation was performed by injecting 10 µL of the sample, with 10 min duration at the flow rate of 1.1 mL min^{-1}. Each sample was analysed three times. Quercetin, with a retention time of 2.699 min, was detected at 320 nm, and the Standard from Sigma Aldrich, USA, was used. Chromatographic separations were performed on C18 (250 mm × 4.6 mm), in a 5 µm column in an isocratic mode, using a solvent system comprised of acetonitrile (20%), water (20%) and methanol (60%). The membrane filter (0.45 µm) (Millipore, Bedford, MA, USA) was used to filtrate the mobile phase, and then the filtrate was ultrasonicated for 40 min. A Shimadzu software, i.e., Class WP software (version 6.1), was used to control instruments, data processing, and acquisition. At a particular retention time versus concentration, quantitative determinations were made for the peak areas of standards, and expressed in mg/g of walnut leaves.

2.8. Preparation of Medium for the Growth of Candida Cells

The mixture of 5 g of YEPD (yeast extract peptone dextrose) powder and 2.5 g of agar type I in 100 mL distilled water was prepared, and it was autoclaved at 121 °C for 15 min.

Antifungal activity by disc diffusion method: The antifungal activity was performed by the disc diffusion method, following the standard CLSI (Clinical and Laboratory Stan-

dards Institute) protocol [5]. Candida cells were inoculated in YEPD broth and were kept overnight at 30 °C for incubation, shaking at 200 rpm. Thereafter, the harvested cells were suspended in sterile saline (0.9% NaCl), and the cell density (OD_{600} = 0.1) was set up to 10^6 cells/mL). 1 mL of this suspension was then poured into YEPD-agar plates. In order to evaluate the antifungal activity of walnut leaf extracts, 2 mg (10 µL/disc) of each extract, at the final concentration of 200 mg/mL, was impregnated on three sterile 6 mm paper discs (Whatman paper no. 3). Another paper disc was impregnated with 5 µL (4 mg/mL) of fluconazole placed on agar plates. Thereafter, the containers were incubated (18–24 h) at 30 °C. After 24 h of incubation, the inhibition zones (diameter) around each disk were measured and recorded as the mean diameter (mm). As a positive control, 5 µL (4 mg/mL) fluconazole was used. Triplicate tests were performed, and the results were obtained as the mean average.

2.9. Preparation of Extracts for Cell Culture

The three samples, one indigenous (Suleiman) and two exotic ones (Opex Culchry and Chenovo), showing the maximum free radical scavenging activity and phenolic content, were further used for cell culture experiments. A 0.22 µm membrane filter (Millipore, Bedford, MA, USA) was used to filter the extracts, and the stock solutions, at a concentration of 250 µL (30 mg/mL), were prepared.

2.9.1. Cell Culture

In a humidified atmosphere of 5% CO_2 at 37 °C, the DMEM (Dulbecco's Modified Eagle Medium) was added with 1% penicillin–streptomycin and 10% foetal bovine serum; using this medium, the human cell lines lung A549 and colon HCT116 were grown in monolayer until the cells attained a confluency of 80 to 90%. The medium was changed continuously. The counted cells (haemocytometer) were seeded in a 96-well plate at a concentration of 1×10^5 cells.

2.9.2. MTT Assay

An MTT assay, with slight modifications [15], was employed to determine the antiproliferative activity of walnut leaf extracts on human cancer cells (lung A549 and colon HCT116). The assay is based on the principle of colorimetry that measures the reduction of yellow MTT (3-(4, 5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide) to MTT-formazan (3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyl formazan). In viable cells, by the action of mitochondrial enzyme succinate dehydrogenase, MTT, i.e., yellow water-soluble dye, is reduced to a dark purple insoluble product, i.e., MTT-formazan. This product is made soluble by adding an organic solvent, such as isopropanol, and is measured spectrophotometrically. The assay was carried out by seeding cancer cells (A549 and colon HCT116) into 96-well plates (1×10^5 cells) and treating them with seven different concentrations of the methanolic extracts. The positive control was methanol (500 µL), and the negative control was the sample of untreated cells (only with DMEM). After the drug treatment, the samples were incubated for 24 h. Next, the media were removed, and then 50 µL (5 mg/mL) of MTT solution was added to each well; wells were then incubated for up to 2–3 h. at 37 °C. After removing this solution, 50 µL of DMSO (Dimethyl sulfoxide) was added to the wells, and then the absorbance was measured at 570 nm, using 495 nm as a reference in a plate reader spectrophotometer. Finally, the triplicate assay was carried out. Cell viability was calculated as the percentage of absorbance compared to the control.

$$\% \text{ Cell Viability} = (\text{Absorbance of sample}/\text{Absorbance of control}) \times 100.$$

2.10. Statistical Analysis

Various statistical tests were performed on the data, including cluster analysis and correlation, in order to determine the best genotypes for DPPH and FRAP assays. Duncan's test and one-way analysis of variance (ANOVA) were used. Correlation analysis was also

performed by using Pearson's test. All statistical analysis was done using SPSS 13 (SPSS Inc., Chicago, IL, USA) Software.

3. Results

3.1. Phytochemical Determinations

3.1.1. Total Polyphenolic Content (TPC)

The phenolic composition of the 14 walnut leaf extracts ranged from 2.024 mg/g GAE (*Nugget*) to 13.23 mg/g GAE (*Opex Culchry*), with an average of 9.09 mg/g GAE. Depending upon the phenolic content present, walnut genotypes were differentiated into three types. One category represents five genotypes (*Sel 3*, *Tuttle*, *Sel-5*, *Serr* and *Opex Culchry*), which exhibited higher phenolic content values (12.5–13.23 mg/g GAE). The second category represents four genotypes (*Suleiman*, *Sel-1*, *Chenovo*, and *Hamdan*) exhibiting moderate values (8.39–10.62 mg/g GAE) of phenolic content. The third category represents five genotypes (*Nugget*, *Franquette*, *Sel-2*, *Sel-4* and *Payne*) exhibiting lower values (2.02–7.63 mg/g GAE) of phenolic content. (Table 1).

Table 1. Comparative estimation of phenols, flavonoids, and antioxidative potential of 14 walnut genotypes.

S.NO.	Genotype	Phenol (mg/g GAE)	Flavonol (mg/g QE)	Flavonoid (mg/g QE)	Quercetin (mg/g)	DPPH (%Inhibition)	FRAP µM Fe^{2+}/g DW
1	*Tuttle*	10.13 ^{d} ± 0.12	111.5 ^{g} ± 0.61	110 ^{f} ± 0.22	0.82 ^{a} ± 0.11	45.27 ^{b} ± 0.18	148.1 ^{f} ± 0.17
2	*Nugget*	2.02 ^{a} ± 0.00	111.83 ^{g} ± 0.20	111.93 ^{g} ± 0.24	0.81 ^{a} ± 0.06	65.37 ^{d} ± 0.19	145.3 ^{bcd} ± 0.28
3	*Franquette*	7.13 ^{bc} ± 0.12	108.67 ^{def} ± 0.41	108.67 ^{ef} ± 0.33	0.86 ^{b} ± 0.02	45.37 ^{a} ± 0.35	145.2 ^{bc} ± 0.34
4	*Sel-2*	7.55 ^{bc} ± 0.24	110.33 ^{fg} ± 0.28	104.10 ^{b} ± 0.22	0.87 ^{b} ± 0.02	71.47 ^{ef} ± 0.13	144.9 ^{b} ± 0.03
5	*Suleiman*	8.39 ^{c} ± 0.10	109.50 ^{efg} ± 0.35	107.00 ^{d} ± 0.22	0.89 ^{bc} ± 0.04	75.50 ^{f} ± 0.27	146.7 ^{cdef} ± 0.0
6	*Sel-4*	6.47 ^{b} ± 0.10	109.50 ^{efg} ± 0.13	106.50 ^{cd} ± 0.11	0.93 ^{cd} ± 0.02	68.87 ^{de} ± 0.07	147.3 ^{ef} ± 0.19
7	*Payne*	7.63 ^{bc} ± 0.12	106.33 ^{bcd} ± 0.20	104.83 ^{b} ± 0.17	0.96 ^{d} ± 0.10	67.77 ^{de} ± 0.25	146.2 ^{bcde} ± 0.7
8	*Hamdan*	8.42 ^{c} ± 0.11	109.63 ^{efg} ± 0.15	107.57 ^{de} ± 0.11	0.85 ^{b} ± 0.05	58.67 ^{c} ± 0.11	146.8 ^{def} ± 0.12
9	*Sel-3*	10.62 ^{d} ± 0.12	105.33 ^{b} ± 0.28	105.00 ^{bc} ± 0.22	0.93 ^{cd} ± 0.03	48.20 ^{b} ± 0.22	145.6 ^{bcd} ± 0.29
10	*Serr*	12.57 ^{e} ± 0.18	105.67 ^{bc} ± 0.43	103.37 ^{b} ± 0.22	0.99 ^{d} ± 0.07	65.37 ^{d} ± 0.15	147.8 ^{f} ± 0.07
11	*Sel-1*	10.49 ^{d} ± 0.11	101.67 ^{a} ± 0.41	99.80 ^{a} ± 0.06	0.86 ^{b} ± 0.06	54.50 ^{c} ± 0.16	145.8 ^{bcd} ± 0.09
12	*Sel-5*	12.05 ^{e} ± 0.32	107.67 ^{bcde} ± 0.4	105.00 ^{bc} ± 0.22	1.03 ^{d} ± 0.04	43.30 ^{ab} ± 0.16	145.4 ^{bcd} ± 0.15
13	*Opexculchry*	13.23 ^{e} ± 0.20	107.93 ^{cdef} ± 0.2	104.67 ^{b} ± 0.13	1.20 ^{e} ± 0.00	74.80 ^{f} ± 0.19	144.6 ^{b} ± 0.22
14	*Chenovo*	10.60 ^{d} ± 0.12	101.67 ^{a} ± 0.41	100.13 ^{a} ± 0.05	1.23 ^{e} ± 0.10	72.47 ^{ef} ± 0.25	128.2 ^{a} ± 0.29

Means followed by the same letter within the columns are not significantly different (p = 0.05) using Duncan's multiple range test.

3.1.2. Total Flavonoid and Flavonol Content Determination

The total flavonoid and flavonol contents of the walnut leaf extract ranged from 99.80 (*sel 1*) to 111.93 (*Nugget*) mg/g quercetin equivalent and 101.67 (*sel 1*) to 111.83 (*Nugget*) mg/g QE, respectively, with averages of 105.6 mg/g QE and107.6 mg/g QE, respectively, across 14 genotypes (Table 1). As per estimated observations in the flavonoid content, the genotypes of walnut were distinguished into three categories: one category comprised four genotypes (*Tuttle*, *Nugget*, *Franquette*, and *Hamdan*) displaying higher values (107.57–111.93 mg/g quercetin equivalent) of flavonoid content; a second category comprised eight genotypes (*Sel 2*, *Suleiman*, *Sel 4*, *Payne*, *Sel 3*, *Serr*, *Sel 5*, and *Opex Culchry*) exhibiting moderate values (103.37–107 mg/g QE) of flavonoid content; and the third category comprised two genotypes (*Sel 1* and *Chenovo*) demonstrating lower values (99.8–100.13 mg/g QE) of flavonoid content. Based on flavonol content, walnut genotypes were distinguished into three categories: one category comprised six genotypes (*Tuttle*, *Nugget*, *Sel 2*, *Suleiman*, *Sel 4*, and *Hamdan*) with higher values (109.50–111.83 mg/g quercetin equivalent) of flavonol content; a second category comprised six genotypes (*Franquette*, *Payne*, *Sel 3*, *Serr*, *Sel 5*, and *Opex Culchry*) exhibiting moderate

values (105.33–108.67 mg/g QE); and the third category comprised two genotypes (*Sel 1* and *Chenovo*), displaying lower values (101.67 mg/g QE) of flavonol content.

3.2. Quantification of Quercetin in Walnut Leaves by RP-HPLC

Among the 14 genotypes of walnut leaf extract, the total quercetin content ranged from 0.81 mg/g (*Nugget*) to 1.23 mg/g (*Chenovo*) with an average of 0.94 mg/g (Table 1 and Figure 1). Maximum quercetin contents of 0.96, 0.99, 1.03, 1.20, and 1.23 mg/g were displayed by *Payne*, *Serr*, *Sel5*, *Opex Culchry*, and *Chenovo* respectively.

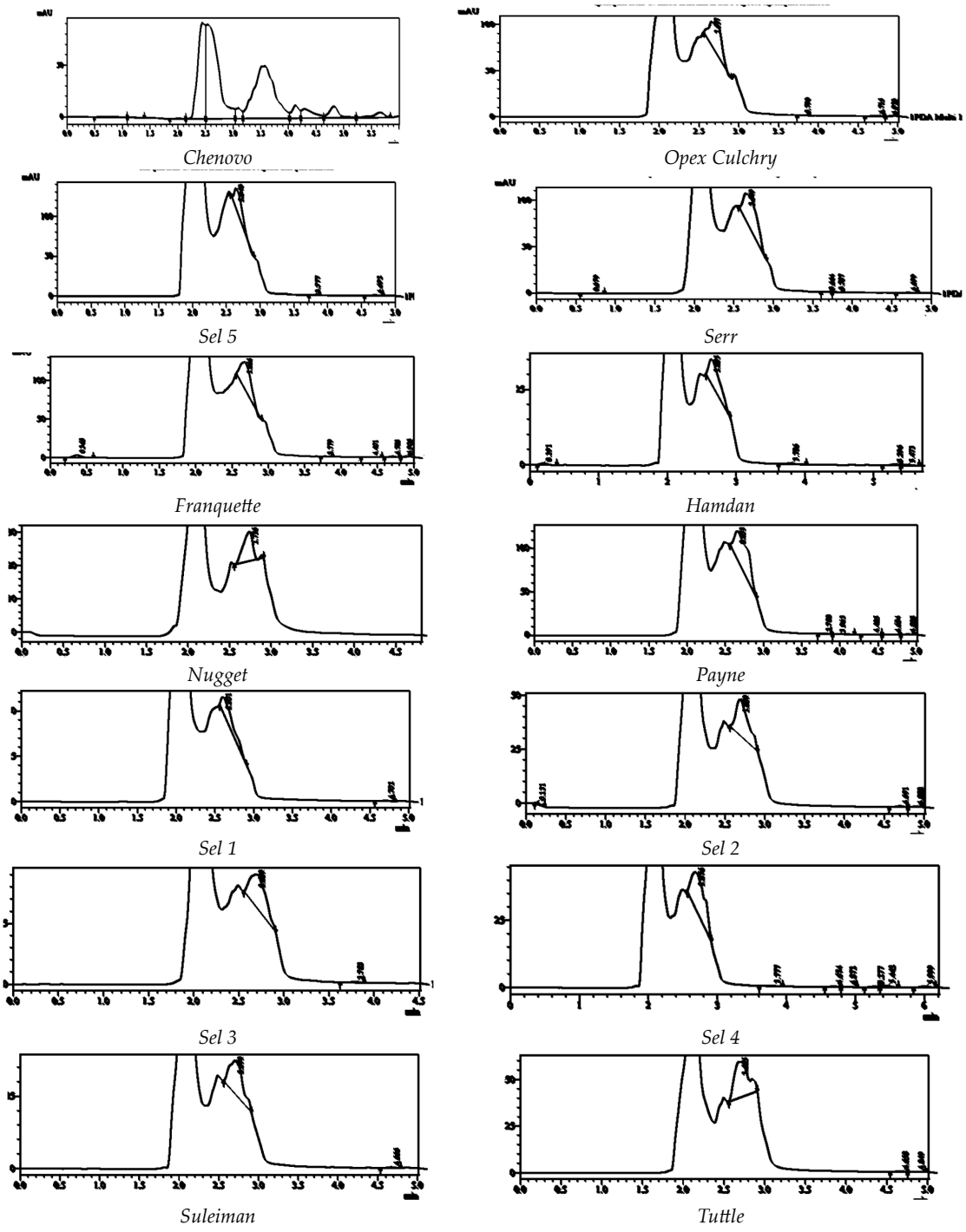


Figure 1. RP-HPLC chromatograms of 14 walnut genotypes.

3.3. Determination of Antioxidant Potential of Walnut

3.3.1. Antioxidant Activity (DPPH Free Radical Scavenging Activity)

Antioxidant activity (DPPH free radical scavenging activity): The percentage of scavenging potential was found to be higher (75.50) in *Suleiman* and lower (43.30) in *Sel-5*, which is considerably more significant than the reference standard (ascorbic acid). Based on the assessed percent inhibition (PI), walnut genotypes were grouped into three classifications: one category was represented by four genotypes (*Sel 2*, *Suleiman*, *Chenovo*, and *Opex Culchry*) with a higher range (71.47–75.50%) of antioxidative potential; a second category was represented by four genotypes (*Nugget*, *Serr*, *Sel 4*, and *Payne*) with moderate antioxidative potential (65.37–67.77%); and the third category was represented by six genotypes (*Sel 5*, *Tuttle*, *Franquette*, *Sel 3*, *Sel 4*, and *Hamdan*) with a lower range (43.30–58.67%) of antioxidative potential.

3.3.2. Ferric Reducing Antioxidant Potential (FRAP) Assay

FRAP values ranged from 128.2 μM Fe^{2+}/g DW (dry weight) in *Chenovo* to 148.1 μM Fe^{2+}/g DW in *Tuttle* (Table 1). Based on the estimation of FRAP values, walnut genotypes were grouped into three categories: one category was represented by six genotypes (*Tuttle*, *Suleiman*, *Sel 4*, *Payne*, *Hamdan*, and *Serr*) exhibiting higher values (146.2–148.1 Mm Fe^{2+}/g DW) of antioxidative potential; a second category was represented by five genotypes (*Franquette*, *Nugget*, *Sel 3*, *Sel 5*, and *Sel 1*) exhibiting moderate values (145.2–145.8 μM Fe^{2+}/g DW) of FRAP; and the third category was represented by three genotypes (*Chenovo*, *Opex Culchry*, and *Sel 2*) showing lower values (128.2–144.9 μM Fe^{2+}/g DW) of FRAP.

FRAP values ranged from 128.2 μM Fe^{2+}/g DW (dry weight) in *Chenovo* to 148.1 μM Fe^{2+}/g DW in *Tuttle* (Table 1). Based on the estimation of FRAP values, walnut genotypes were grouped into three categories: one category was represented by six genotypes (*Tuttle*, *Suleiman*, *Sel 4*, *Payne*, *Hamdan*, and *Serr*) exhibiting higher values (146.2–148.1 Mm Fe^{2+}/g DW) of antioxidative potential; a second category was represented by five genotypes (*Franquette, Nugget, Sel 3, Sel 5*, and *Sel 1*) exhibiting moderate values (145.2–145.8 μM Fe^{2+}/g DW) of FRAP; and the third category was represented by three genotypes (*Chenovo*, *Opex Culchry*, and *Sel 2*) showing lower values (128.2–144.9 μM Fe^{2+}/g DW) of FRAP.

3.4. Antifungal Activity against Candida Species

3.4.1. Antifungal Activity against Candida Albicans

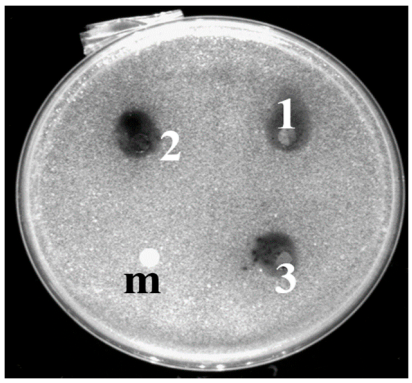
The diameter of inhibition zones ranged from 6.5 mm (*Sel 3*) to 13.2 mm (Hamdan), with an average diameter of 10.35 mm, obtained across different extracts. Si genotypes (Franquette, Serr Nugget, Tuttle, Payne, and Hamdan) exhibited maximum inhibition zones ranging from 11 mm to 13.2 mm. Seven genotypes (*Sel 5*, *Opex Culchry*, *Chenovo*, *Sel 2*, *Suleiman*, *Sel 1*, and *Sel 4*) exhibited moderate inhibition zones ranging from 9.2 mm to 10.3 mm; one genotype (*Sel 3*) exhibited the minimum (6.5 mm) inhibition zone. The standard antifungal drug fluconazole was active against the Candida strain, showing a zone of inhibition with the range of 24.2–25.6 mm and demonstrating strong antifungal activity against Candida albicans (Table 2 and Figure 2).

3.4.2. Antifungal Activity against CANDIDA Glabrata

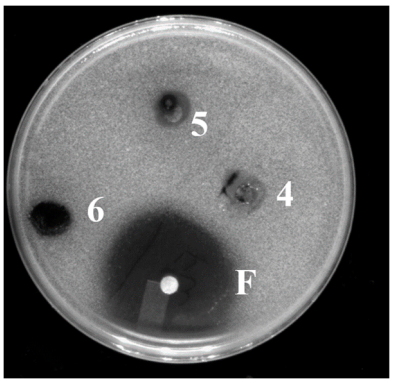
The diameter of inhibition zones ranged from 5.2 mm (*Hamdan*) to 10.5 mm (*Ser*), with an average diameter of 8.8 mm. Four genotypes (*Franquette*, *Suleiman*, *Serr*, and *Sel 1*) exhibited maximum zones of inhibition, ranging from 10.1 to 10.5 mm; four genotypes (*Tuttle*, *Nugget*, *Sel 5*, and *Opex Culchry*) exhibited moderate zones of inhibition, ranging from 9.1 to 9.6 m; and six genotypes (*Sel 2*, *Sel 4*, *Payne*, *Hamdan*, *Sel 3*, and *Chenovo*) exhibited minimum zones of inhibition, ranging from 5.2 to 8.5 mm. The standard antifungal drug fluconazole, used as a positive control, showed an 11.1–11.5 mm inhibition zone (Table 3 and Figure 3).

Table 2. The inhibition zone diameters of methanolic extracts of *Juglans regia* L. leaf extracts against *Candida albicans*.

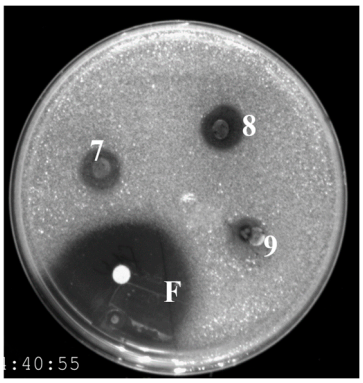
Average Diameters of the Inhibition Zones (mm) of *Candida Albicans*		
Extracts	**Inhibition Zones with Extracts**	**Inhibition Zones with Fluconazole**
Tuttle	11.3	25.4
Nugget	11.2	25.5
Franquette	11.0	25.2
Sel 2	9.6	25.1
Suleiman	10.2	25.0
Sel 4	10.3	24.3
Payne	12.4	25.4
Hamdan	13.2	25.6
Sel 3	6.5	24.2
Serr	11.1	25.6
Sel 1	10.2	25.2
Sel 5	9.3	24.7
Opex Culchry	9.2	24.9
Chenovo	9.4	25.0
Average	**10.35**	**25.08**



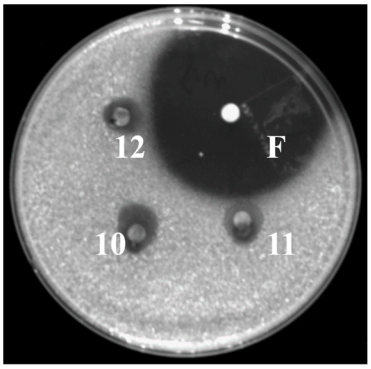
***1. Tuttle, 2. Nugget, 3. Franquette,* Methanol.**



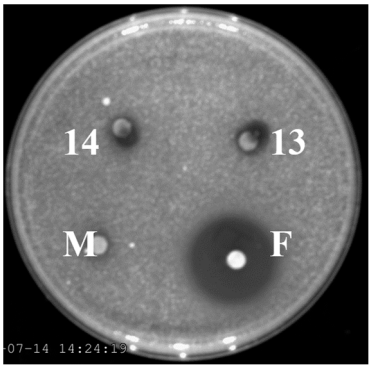
***4. Sel-2, 5. Suleiman, 6. Sel-4,* Fluconazole.**



***7. Serr, 8. Hamdan, 9. Sel-3,* Fluconazole**



***10. Payne, 11. Sel 1, 12. Sel-5,* Fluconazole**

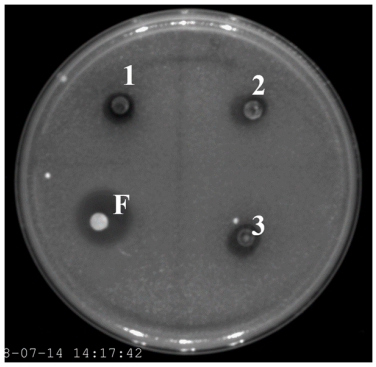


***13. Opex Culchry, 14. Chenovo,* Methanol, Fluconazole.**

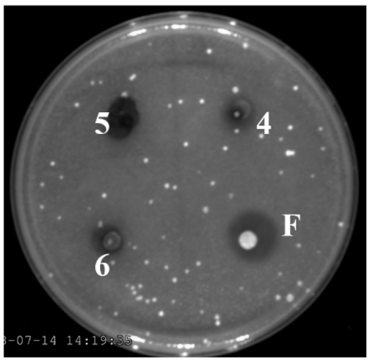
Figure 2. Antifungal activity of *Juglans regia* L. leaf extracts against *Candida albicans*.

Table 3. The inhibition zone diameters of methanolic extracts of *Juglans regia* L. leaf extracts against *Candida glabrata*.

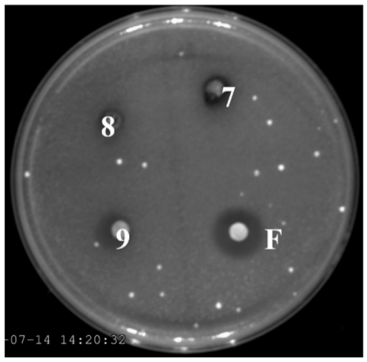
Average Diameters of the Inhibition Zones (mm) of *Candida Glabrata.*		
Extracts	**Inhibition Zones with Extracts**	**Inhibition Zones with Fluconazole**
Tuttle	9.2	11.1
Nugget	9.1	10.0
Franquette	10.3	12.2
Sel 2	8.5	11.2
Suleiman	10.1	12.1
Sel 4	8.4	10.2
Payne	7.5	10.5
Hamdan	5.2	9.0
Sel 3	7.3	10.7
Serr	10.5	12.2
Sel 1	10.4	11.1
Sel 5	9.6	10.9
Opex Culchry	9.1	11.5
Chenovo	8.2	12.3
Average	8.81	11.07



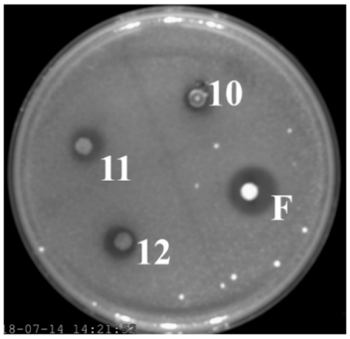
1.Tuttle, 2. Nugget, 3. Franquette, **Fluconazole**



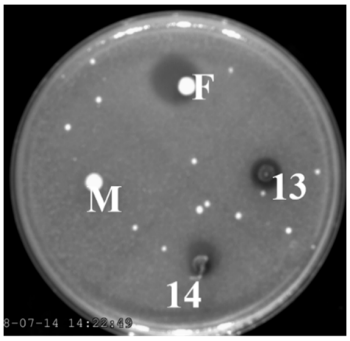
4.Sel 2, 5. Suleiman,6. Sel 4, **Fluconazole**



7.Payne, 8. Hamdan, 9. Sel 3, **Fluconazole**



10.Serr, 11. Sel 1, 12. Sel 5, **Fluconazole**



13.Opex Culchry, 14. Chenovo, **Fluconazole, Methanol**

Figure 3. Antifungal activity of *Juglans regia* L. leaf extracts against *Candida glabrata*.

3.4.3. Antifungal Activity against *Candida tropicalis*

The diameter of inhibition zones ranges from 6.1 mm (Franquette) to 8.6 mm (Serr), with an average diameter of 7.3 mm. Ten genotypes (*Nugget*, *Sel 2*, *Suleiman*, *Sel 4*, *Payne*, *Hamdan*, *Sel 3*, *Serr*, Opex *Culchry*, and *Chenovo*) exhibited maximum inhibition zones ranging from 7.0 to 8.6 mm, and four genotypes (*Tuttle*, *Franquette*, *Sel 1*, and *Sel 5*) showed 6.1–6.5 mm zones of inhibition. The standard antifungal drug fluconazole was used as

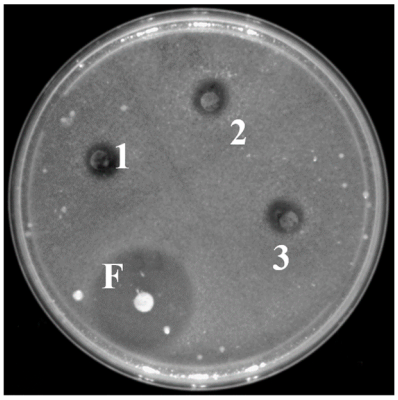
the positive control, and the zone of inhibition ranged from 19.0 to 21.2 mm (Table 4 and Figure 4).

Table 4. The inhibition zone diameters of methanolic extracts of *Juglans regia* L. leaf extracts against *Candida tropicalis*.

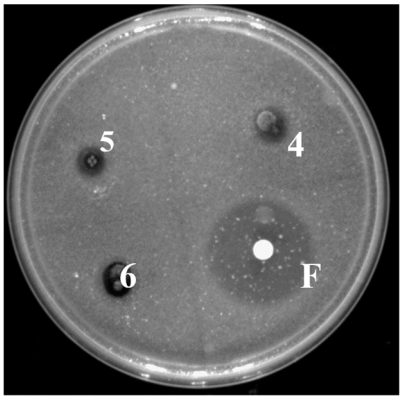
Average Diameters of the Inhibition Zones (mm) of *Candida tropicalis*		
Extracts	**Inhibition Zones with Extracts**	**Inhibition Zones with Fluconazole**
Tuttle	6.5	20.4
Nugget	7.2	19.9
Franquette	6.1	21.1
Sel 2	7.7	20.2
Suleiman	7.3	19.8
Sel 4	7.5	20.2
Payne	8.2	19.5
Hamdan	8.1	19.0
Sel 3	7.4	21.2
Serr	8.6	20.5
Sel 1	6.5	20.8
Sel 5	6.3	19.7
Opex Culchry	7.0	19.0
Chenovo	7.9	20.0
Average	7.31	20.09

3.4.4. Relative Percentage Inhibition Concerning Positive Control (Fluconazole)

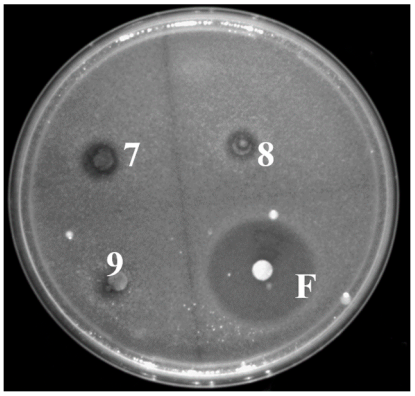
The percentage of inhibition of fourteen walnut genotypes concerning fluconazole against *Candida albicans* ranges from 26.8 (*Sel 3*) to 51.5% (*Hamdan*), with an average of 40.34% inhibition, so among all genotypes, *Sel 3* has the lowest antifungal potential, and *Hamdan* has the highest antifungal potential. The percentage of inhibition against *Candida glabrata* ranged from 57.7 (*Hamdan*) to 93.6% (*Sel 1*), with an average of 79.27% inhibition. Therefore, *Sel 1* has the highest antifungal potential, and *Hamdan* has moderate antifungal activity against *Candida glabrata*. The percentage of inhibition against *Candida tropicalis* ranged from 28.9 (*Franquette*) to 42.6% (*Hamdan*); therefore, Franquette has the lowest, and Hamdan has the highest, antifungal potential against *Candida tropicalis*. All walnut genotypes have comparatively less antifungal activity against *Candida tropicalis* than against *Candida glabrata* and *Candida albicans*. However, the genotypes demonstrate the highest antifungal potential against *Candida glabrata* (Table 5).



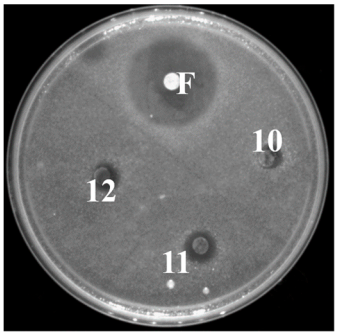
***1. Tuttle, 2. Nugget, 3. Franquette,* Fluconazole**



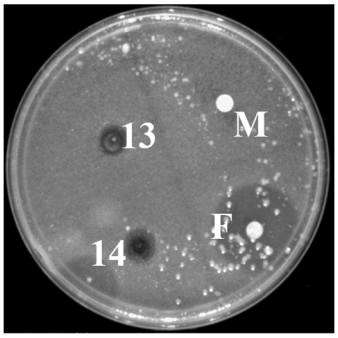
***4. Sel 2, 5. Suleiman, 6. Sel 4,* Fluconazole**



***7. Payne, 8. Hamdan, 9. Sel 3,* Fluconazole**



***10. Sel1, 11. Serr, 12. Sel 5,* Fluconazole**



***13. Opex Culchry, 14. Chenovo,* Fluconazole, Methanol**

Figure 4. Antifungal activity of *Juglans regia* L. leaf extracts against *Candida tropicalis*.

Table 5. Relative percentage inhibition concerning positive control (Fluconazole).

Extracts	*Candida Albicans*	*Candida Glabrata*	*Candida Tropicalis*
	%Inhibition	%Inhibition	%Inhibition
Tuttle	44.4	82.2	31.8
Nugget	43.9	91.1	36.1
Franquette	43.1	84.4	28.9
Sel 2	27.4	75.8	38.1
Suleiman	40.8	83.4	36.8
Sel 4	42.3	82.3	37.1
Payne	48.8	71.4	42.0
Hamdan	51.5	57.7	42.6
Sel 3	26.8	68.2	34.9
Serr	43.3	86.0	41.9
Sel 1	40.4	93.6	31.2
Sel 5	37.6	88.0	32.9
Opex Culchry	36.9	79.1	36.8
Chenovo	37.6	66.6	39.1
Average	40.34	79.27	36.44

3.5. Antiproliferative Activity of Walnut Leaf Extracts on Human Cancer Cell Lines Using MTT Assay

3.5.1. Antiproliferative Effect against Lung (A549) Cell Lines

Our results indicated that *Chenovo*, at seven different concentrations—100 µL (3 mg), 50 µL (1.5 mg), 25 µL (0.75 mg), 12.5 µL (0.375 mg), 6.25 µL (0.1875 mg), 3.125 µL (0.09375 mg), and 1.575 µL (0.04725 mg)—showed 46.4, 20.2, 19.4, 32, 55.8, 51.3, and 35.7% cell viability, respectively. Similarly, *Opex Culchry*, at the same concentrations, showed 44.6, 32.7, 9.4, 17.9, 40.3, 44.3, and 19.9% cell viability, and *Sulieman* showed 37.3, 28.1, 10.9, 27.3, 46.4, 50.8, and 22.1% cell viability, demonstrating overall significance [** $p = 0.003$]. Among these three extracts, *Opex Culchry* exhibited 9.4% cell viability at the concentration of 25 µL (0.75 mg) [** $p = 0.008$], followed by *Suleiman* and *Chenovo*, which showed 10.9% and 19.4% cell viability, respectively. Therefore, it has a strong antiproliferative effect against lung A549 cell lines (Figure 5).

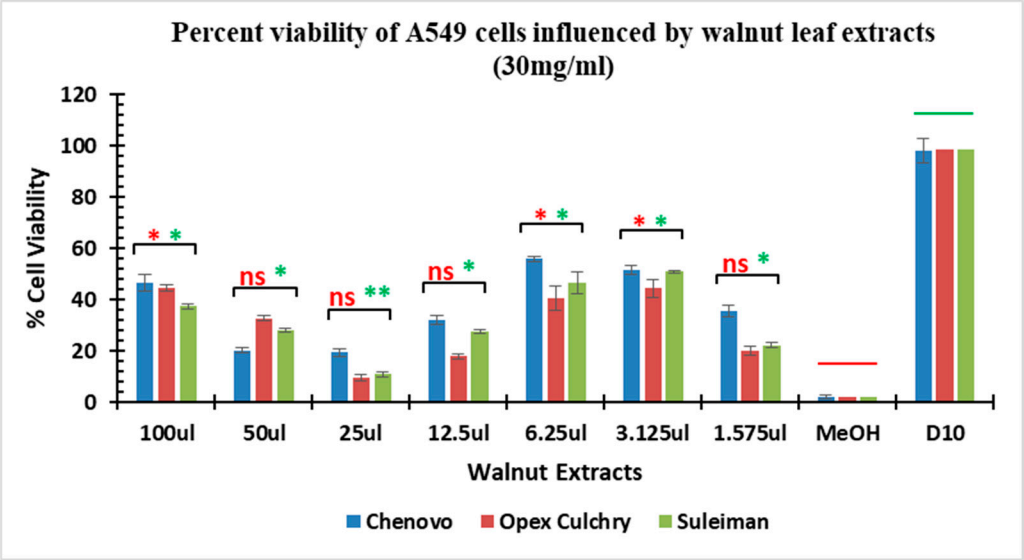


Figure 5. Antiproliferative activity of methanolic extracts (*Chenovo*, *Opex Culchry*, and *Suleiman*) against cancer cell line (lung A549); they were overall statistically significant [** $p = 0.003$] when compared to both MeOH and D10 controls. Using ANOVA analysis, data are represented as mean ± SD of results obtained from three independent experiments. * represents non-significant.

3.5.2. Antiproliferative Effect against Colon (HCT116) Cell Lines

At concentrations of 100 µL (3 mg), 50 µL (1.5 mg), 25 µL (0.75 mg), 12.5 µL (0.375 mg), 6.25 µL (0.1875 mg), 3.125 µL (0.09375 mg), and 1.575 µL (0.04725 mg), *Chenovo* showed 2.9, 6.2, 2.2, 20.2, 20.2, 57.8, 54.6, and 45.9% cell viability, respectively. Similarly, *Opex Culchry*, at these concentrations, showed 2, 1.5, 2.4, 3.9, 33.3, 46.1, and 33.3% cell viability, and *Sulieman* showed 7.6, 0.9, 7, 21.9, 46.3, 58.2 and 38.4% cell viability, demonstrating overall significance [** $p = 0.001$]. All three extracts showed a strong proliferative effect at the concentrations of 25 µL (0.75 mg) [**** $p < 0.0001$], 50 µL (1.5 mg) [**** $p < 0.0001$], and 100 µL (3 mg) [**** $p < 0.0001$], with *Chenovo* exhibiting 2.9, 6.2, and 2.2% cell viability; *Opex Culchry* exhibiting 2, 1.5, and 2.4% cell viability; and *Sulieman* exhibiting 7.6, 0.9, and 7% cell viability, respectively (Figure 6).

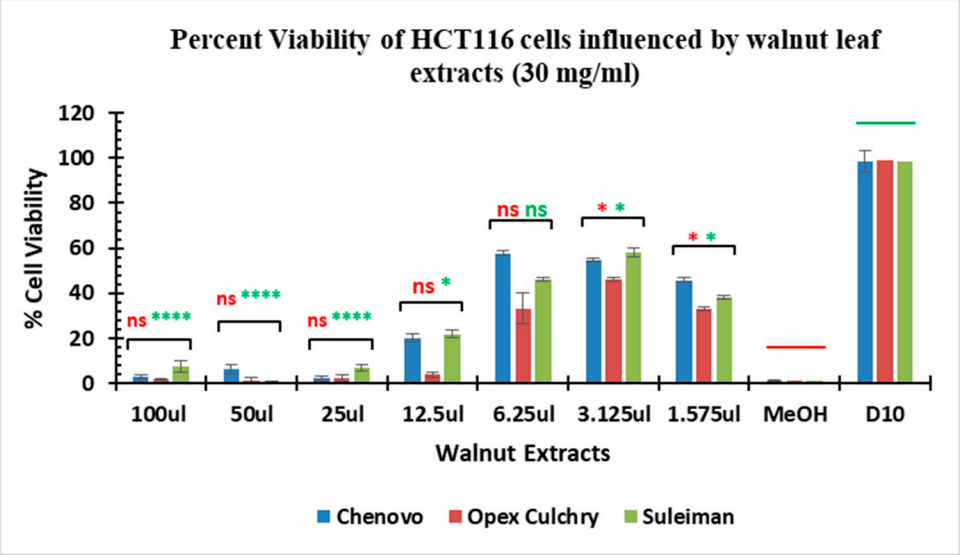


Figure 6. Antiproliferative activity of methanolic extracts (*Chenovo*, *Opex Culchry*, and *Suleiman*) against cancer cell line (colon HCT116); they were overall statistically significant [** p = 0.001] when compared to both MeOH and D10 controls. Using ANOVA analysis, data are represented as mean ± SD of results obtained from three independent experiments. * represents non-significant.

The results indicated that walnut leaf extracts show a considerable decrease in cell viability as the concentration increases. Thus, it can be declared that Walnut leaves consist of an adequate concentration of antioxidants and chemotherapeutic agents that can be used as anticancer agents.

3.5.3. Statistical Analysis

Cluster Analysis

Cluster I is represented by the eight genotypes (*Sel 4*, *Payne*, *Sel 2*, *Suleiman*, *Opex Culchry*, *Serr*, *Nugget*, and *Hamdan*) demonstrating high DPPH potential, with an average of 68.48%, while cluster II is represented by the five genotypes (*Tuttle*, *Franquette*, *Sel 5*, *Sel 3*, and *Sel 1*) demonstrating low DPPH potential, with an average of 47.33%. One genotype (*Chenovo*) is not included in a cluster (72.47%). Cluster I and cluster II are represented by thirteen walnut genotypes showing high FRAP potential, with averages of 146.2 and 146.02 µM Fe^{2+}/g DW, respectively. One genotype, *Chenovo*, is not included in a cluster (128.2 µM Fe^{2+}/g DW). Cluster II is represented by five walnut genotypes (*Tuttle*, *Franquette*, *Sel 5*, *Sel 3*, and *Sel 1*) with high phenolic content, with an average of 9.98 mg/g GAE (Gallic acid equivalent). In comparison, as cluster I is represented by eight genotypes with low phenolic content, with an average of 8.29 mg/g GAE, while *Chenovo* is not included in a cluster (10.60 mg/gGAE). Cluster I and cluster II are represented by eight genotypes with high flavonoid and flavonol content, with averages of 106.25, 105.69, 108.84, and 106.97 mg/gQE, and exhibiting high FRAP activity. Cluster I is represented by eight genotypes containing quercetin, with an average of 0.94 and 0.9 mg/g; *Chenovo* is not included in a cluster (1.23 mg/g) (Figure 7).

Correlation Studies

Correlation studies reveal that antioxidant assay FRAP was positively correlated (r = 0.549) with flavonol and flavonoid (r = 0.483). FRAP was negatively correlated with phenols (r = −0.125) and quercetin (r = −0.662). Another antioxidative assay, i.e., DPPH, was negatively correlated with all other parameters (Table 6). Phenols were found to be positively correlated with quercetin (r = 0.598), but negatively correlated with flavonol (r = 0.522) and flavonoid (r = −0.606).

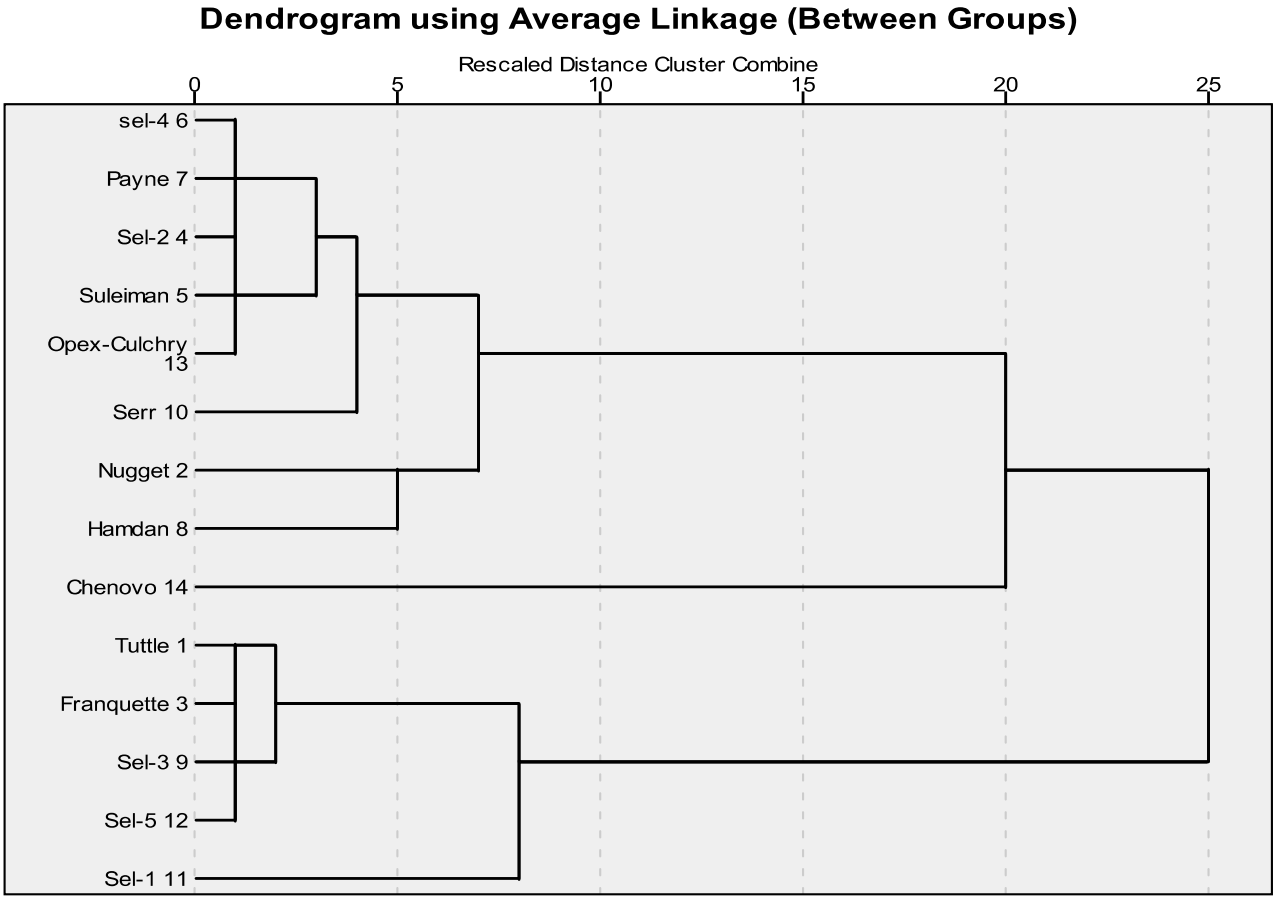


Figure 7. Grouping of fourteen walnut genotypes based on metabolic profiling.

Table 6. Correlation among total phenols, flavonoids, and antioxidative assays of 14 walnut genotypes.

Correlation Matrix							
		DPPH	**FRAP**	**Phenols**	**Flavonol**	**Flavonoid**	**Quercetin**
Correlation	DPPH		−0.280	−0.141	−0.015	−0.226	0.377
	FRAP			−0.125	0.542	0.483	−0.662
	phenols				−0.522	−0.606	0.598
	flavonol					0.884	−0.522
	flavonoid						−0.572
	quercetin						
Sig. (1-tailed)	DPPH		0.166	0.315	0.479	0.218	0.092
	FRAP			0.335	0.023	0.040	0.005
	phenols				0.028	0.011	0.012
	flavonol					0.000	0.028
	flavonoid						0.016
	quercetin						

Principal Component Analysis

Multivariate analysis done through Principal Component Analysis (PCA) revealed that 87.73% variance is contributed by the first three components, where component 1 contributed 54.89% variance, and components 2 and 3 contributed 21.27% and 11.57%

variance, respectively. Eigen values for the first, second, and third components were 3.293, 1.276, and 0.694, respectively. Therefore, the total variance was depicted by six principal components (Table 7).

Table 7. Multivariate analysis performed through Principal Component Analysis.

Total Variance Explained						
Component	**Initial Eigenvalues**			**Extraction Sums of Squared Loadings**		
	Total	**% of Variance**	**Cumulative %**	**Total**	**% of Variance**	**Cumulative %**
1	3.293	54.891	54.891	3.293	54.891	54.891
2	1.276	21.270	76.161	1.276	21.270	76.161
3	0.694	11.570	87.731			
4	0.574	9.564	97.296			
5	0.108	1.800	99.096			
6	0.054	0.904	100.000			
Extraction Method: Principal Component Analysis.						

4. Discussion

Walnuts are rich in bioactive molecules, which are expressed in different parts of the plant, and beneficial to the treatment of several chronic diseases, such as cancer [16]. Walnuts contain a wide assortment of antioxidant and anti-inflammatory bioactive components that may be hostile to carcinogenic properties [16]. Polyphenolic compounds, such as gamma-tocopherol, phytosterols, ellagic acid, and omega-3 fatty acids, reduce the risk of chronic oxidative stress and inhibit inflammatory properties, resulting in decreased cancer progression. Walnuts contain bioactive polyphenols that modulate cancer cells' signalling pathways, such as P53, NF-B, MAPK, and PI3K/AKT. Inhibition of glucose uptake in cancerous cells is one of the properties of several of these compounds, e.g., quercetin, rutin, and myricetin [17]. A lower occurrence of colon, breast, and prostate cancer was related to walnut consumption [18–21].

We report the significant antifungal potential of walnut leaf genotypes against *Candida glabrata*, *Candida albicans*, and *Candida tropicalis*, with 57.7–93.6%, 26.8–51.5%, and 26.8–51.5% inhibition, respectively. The most significant antiproliferative effect was shown by *Opex Culchry*, which exhibited 9.4% cell viability at the concentration of 25 µL (0.75 mg) against lung (A549) cell lines. In addition, *Chenovo*, *Suleiman*, and *Opex Culchry* showed a strong antiproliferative effect against colon (HCT116) cancer cell lines at the concentrations of 25 µL (0.75 mg), 50 µL (1.5 mg), and 100 µL (3 mg).

Carvalho et al. (2010) reported that methanolic seed extracts had the highest total phenolic content (mean value of 116.22 ± 3.76 mg of GAE/g) compared to the leaf methanolic, i.e., 94.39 ± 5.63 mg of GAE/g of extract. Shah et al. (2018) reported a higher total phenolic content (37.61–46.47 mg/g GAE) in walnut leaf extracts. They also reported lower flavonoid content, recording a range from 5.52 (SKUA0023) to 28.48 (KB1) mg/g quercetin equivalent, and flavonol content, recording a range from 4.11 (SKUA23) to 21.76 (KB1) mg/g QE in methanolic extracts of walnut genotypes, which are lower than our reports. The principal chemical compound of the flavonoid class present in walnut leaves is quercetin, an essential part of the human diet. Shah et al. [20] reported the genotypes with regard to their minimum quercetin contents, i.e., CITH W16 and CPB4, with 2.86 and 2.91 mg/100 g, respectively, and the genotypes SKUA20 SULAIMAN, BRUS10, KUA24 and BB2S, with maximum quercetin contents of 5.23, 5.67, 5.67, 5.78, and 5.11 mg/100 g, respectively. On the contrary, in our findings, the leaf extracts of fourteen walnut genotypes presented total quercetin contents that ranged from 0.81 mg/g (*Nugget*) to 1.23 mg/g (*Chenovo*), with an average of 0.94 mg/g. Maximum quercetin contents of 0.96, 0.99, 1.03, 1.20, and 1.23 mg/g were displayed by *Payne*, *Serr*, *Sel 5*, *Opex Culchry*, and *Chenovo*, respectively.

In our results, cluster analysis revealed flavonoid- and flavonol-rich walnut genotypes, cluster I and cluster II, exhibit high DPPH and FRAP activity (Figure 1), and correlation studies showed a positive correlation (r = 0.549) of FRAP with flavonol and flavonoid (r = 0.483). FRAP was negatively correlated (r = −0.125) with phenols and quercetin (r = −0.662). DPPH was negatively correlated with all other parameters. Phenols were found to be positively correlated (r = 0.598) with quercetin, but negatively correlated with flavonol (r = −0.522) and flavonoid (r = −0.606) contents. Multivariate analysis done through Principal Component Analysis (PCA) revealed that 87.73% of the variance was contributed by the first three components, where component 1 contributed 54.89% variance, and components 2 and 3 contributed 21.27% and 11.57% variance, respectively. Eigenvalues for the first, second, and third components were 3.293, 1.276, and 0,.694, respectively. The advantage of using the DPPH assay in determining the antioxidant activity is the presence of more stable DPPH radicals compared to other hydroxyl radicals. As the radical accepts an electron from an antioxidant molecule, it loses its purple colour [14]. Our studies revealed an antioxidative potential of 75.50% (*Suleiman*) through DPPH assay, which is higher than the results of Shah et al. [20], who reported about 73.50% scavenging potential in walnut leaf extract of the NDPB1 genotype. Shah et al. (2015) reported that the methanolic leaf extract showed the highest DPPH potential, with an EC50 value of 0.199 ± 0.023 mg/mL, while an aqueous leaf extract showed less potential, with an EC50 value of 2.991 ± 0.740. Carvalho et al. (2010) reported that all of the methanolic extracts showed strong concentration-dependent antioxidant activity. Using various in vitro assays, the essential oil obtained from *Juglans regia* leaves was evaluated and found to have prominent antioxidant potential. In vivo assays have also been performed in rat models of oxidative stress carbon tetra chloride-induced liver damage, in which the liver was shown to be protected in the animals by feeding them walnut leaf extract for four weeks [4]. By scavenging free radicals and ROS, or inhibiting proinflammatory signalling pathways, walnuts, and their gut microbiota-derived metabolites, possess antioxidant and anti-inflammatory properties. Cancer cells may be inhibited from growing and surviving if walnut consumption is increased [22]. The production of inflammatory mediators, such as leukotrienes, was downregulated by extracts of different walnut matrices [23]. Chronic diseases are also treated with walnut components, which reduce inflammation and oxidative stress [24]. The 14 indigenous and exotic walnut genotypes analysed in our study have excellent antioxidant potential, as they contain a high number of phytochemicals, such as phenols and flavonoids, and it was confirmed in our studies that walnut leaves collected in early May contain high phenol and flavonoid contents, which provide excellent antioxidant potential. As methanol is a polar solvent, the extracts prepared using this solvent exhibited higher phenolic and flavonoid fractions, so it can be considered that methanol is the best solvent for phenolic extraction.

Management of fungal infections has become difficult because species such as *Candida albicans*, and other related pathogens, resist antifungal drugs. Therefore, natural plant products have been screened for antifungal properties [25,26]. The parts of the *Juglans regia* (walnut) tree, including the shells, barks, leaves, husks, seeds, and kernels, have been used for their remarkable medicinal properties. Noumi et al. (2010) reported the antifungal potential of diluted acetone, methanol, and ethyl acetate extracts of *Juglans regia* L. against some *candida* species. It was found that all of the leaf extracts were active against *Candida* strains. Still, the methanolic leaf extract was most active against *C. albicans*. At the same time, as in our findings, *Candida glabrata* was comparatively more sensitive to methanolic leaf extracts than *Candida albicans* and *Candida tropicalis*. Sytykiewicz et al. (2014) reported that ethyl acetate and hydrolysed methanolic preparations are the least effective at inhibiting fungal growth; methanolic extracts of walnut leaves show the strongest antifungal activity [27]. By comparing the antifungal activity of different walnut varieties against various pathogenic fungi, it has been found that, in addition to the important constituent of walnut, i.e., juglone, other phenolic compounds have also been proven to demonstrate potent antifungal activity [28]. Currently, oncologists are widely researching plant-based drugs with anti-

cancer potential; this can be correlated to the phytochemicals present in these medicinal plants [29–32]. Quercetin, one of the vital compounds of the flavonoid class, which was determined in our studies by HPLC, has been reported to have prominent antiproliferative potential against stomach and breast cancer cell lines [12]. Genovese et al. (2020) reported that various anti-apoptotic molecules induce cancer cell death, including epigallocatechin, epigallocatechin gallate, p-coumaric acid hexoside, quercetin 3-O-rhamnoside, and quercetin 3-O-glucoside [32]. The incidence of various types of cancers, including stomach, lung, pharynx, oesophageal, pancreatic, oral cavity, and colon cancers, can be reduced by the regular intake of nuts [11]. Walnuts have antioxidant and chemotherapeutic properties because of the presence of phytochemicals, such as polyphenols, flavonoids, phytosterols, gallic acid, ellagic acid, and other antioxidants such as vitamin C, provitamin A, and vitamin E. Polyphenols are the compounds that block the cancer agents by inhibiting the initiation and progression of carcinogenesis [11] Carvalho et al. (2010) reported the antiproliferative activity of methanolic walnut leaf, green husk, and seed extracts against human renal (A-498 and 769-P) and colon (caco-2) cancer cell lines, and it was revealed that methanolic leaf extracts showed the greatest concentration-dependent cell growth inhibition, followed by green husk and then seed. Our results indicated the concentration-dependent growth inhibition of human cancer cell lines lung A549 and colon HCT116. Shah et al. (2018) also reported the concentration-dependent growth inhibition of human cancer cell lines, such as HBL-100, U2OS, THP-1, and IMR-32. As the extracts' concentration increased, cell viability decreased [30]. It was reported that chloroform and ethyl extracts of walnut leaf against human cancer cell lines (769-P renal and Caco2 colon) revealed an elevated rate of antiproliferative competence than walnut seeds. In contrast, as in our findings, walnut methanolic leaf extracts showed higher antiproliferative activity against human cancer cell lines lung A549 and colon HCT116. It has been reported that Persian walnut protein hydrolysates have effective antioxidant and antiproliferative potential against the colon (H-29) and human breast (MDA-MB231) cell lines [31]. Several researchers have reported that walnut has diverse antiproliferative potential against cancer cells, such as Caco-2 colon and HepG2 liver cancer cells [11]. Salimi et al. [33] also reported that cancer cell lines, including breast adenocarcinoma, human oral cancer, and colon adenocarcinoma, are sensitive against chloroform leaf extracts of walnut.

5. Conclusions

Based on our findings, walnut leaf methanolic extracts exhibit high levels of phenols, flavonoids, and quercetin, which have excellent antioxidant properties. It has also been discovered that premature walnut leaves contain many therapeutically active compounds. Furthermore, the safety, efficacy, and dosage of phytoconstituents should be determined by performing various clinical investigations. Further research should be carried out for structural analysis of the phytoconstituents, and the molecular-level mechanisms should be examined. The antifungal and antiproliferative potential of 14 walnut genotypes was deciphered and, thus, provided basic information about the genotypes which can potentially be utilized for pharmaceutical properties. The leaf extracts from walnut genotypes can be further improved through high proliferation and concentration methods for developing potential drugs. This work needs to be elucidated in detail using in vivo animal models. The genotypes identified in the present study can be further evaluated using biochemical, molecular, immunological, and pharmacological studies in order to determine the essential compounds responsible for antiproliferative and antifungal activities.

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References

1. Sharma, P.; Gomathi, D.; Ravikumar, G.; Kalaiselvi, M.; Chandrasekaran, U. Phytochemical screening and high-performance thin layer chromatography fingerprinting analysis of green hull of *Juglans regia* (walnut). *Elect. Med. J.* **2014**, *2*, 235–239. [CrossRef]
2. Delaviz, H.; Mohammadi, J.; Ghalamfarsa, G.; Mohammadi, B.; Farhadi, N. A Review Study on Phytochemistry and Pharmacology Applications of *Juglans regia* Plant. *Pharmacogn. Rev.* **2017**, *11*, 145–152. [CrossRef]
3. Rudrapal, M.; Khairnar, S.J.; Khan, J.; Dukhyil, A.B.; Ansari, M.A.; Alomary, M.N.; Alshabrmi, F.M.; Palai, S.; Deb, P.K.; Devi, R. Dietary Polyphenols and Their Role in Oxidative Stress-Induced Human Diseases: Insights Into Protective Effects, Antioxidant Potentials and Mechanism(s) of Action. *Front. Pharmacol.* **2022**, *13*, 806470. [CrossRef] [PubMed]
4. Panth, N.; Paudel, K.R.; Karki, R. Phytochemical profile and biological activity of *Juglans regia*. *J. Integr. Med.* **2016**, *14*, 359–373. [CrossRef] [PubMed]
5. Noumi, E.; Snoussi, M.; Hajlaoui, H.; Valentin, E.; Bakhrouf, A. Antifungal properties of Salvadora persica and *Juglans regia* L. extracts against oral Candida strains. *Eur. J. Clin. Microbiol. Infect. Dis.* **2010**, *29*, 81–88. [CrossRef]
6. Zhao, M.H.; Jiang, Z.T.; Liu, T.; Li, R. Flavonoids in *Juglans regia* L. leaves and evaluation of in vitro antioxidant activity via intracellular and chemical methods. *Sci. World J.* **2014**, *2014*, 6. [CrossRef]
7. Tang, Y.T.; Li, Y.; Chu, P.; Ma, X.D.; Tang, Z.Y.; Sun, Z.L. Molecular biological mechanism of action in cancer therapies: Juglone and its derivatives, the future of development. *Biomed. Pharmacother.* **2022**, *148*, 112785. [CrossRef]
8. Al-Snafi, A.E. Traditional uses of Iraqi medicinal plants. *IOSR J. Pharm.* **2018**, *8*, 32–96.
9. Amaral, J.S.; Valentão, P.; Andrade, P.B.; Martins, R.C.; Seabra, R.M. Do cultivar, geographical location and crop season influence the phenolic profile of walnut leaves. *Molecules* **2008**, *13*, 1321–1332. [CrossRef]
10. Spencer, J.P. Flavonoids: Modulators of brain function. *Br. J. Nutr.* **2008**, *99*, 60–77. [CrossRef]
11. Shah, T.I.; Sharma, E.; Shah, G.A. Anti-proliferative, cytotoxicity and antioxidant activity of *Juglans regia* extract. *Am. J. Cancer Prev.* **2015**, *3*, 45–50.
12. Shah, U.N.; Mir, J.I.; Ahmed, N.; Jan, S.; Fazili, K.M. Bioefficacy potential of different walnut *Juglans regia* L. *J. Food Sci. Genotypes Technol.* **2018**, *55*, 605–618. [CrossRef]
13. Rahimipanah, M.; Hamedi, M.; Mirzapour, M. Antioxidant activity and phenolic contents of Persian walnut (*Juglans regia* L.) green husk extract. *Afr. J. Food Sci. Technol.* **2010**, *1*, 105–111.
14. Rather, M.A.; Dar, B.A.; Dar, M.Y.; Wani, B.A.; Shah, W.A.; Bhat, B.A.; Ganai, B.A.; Bhat, K.A.; Anand, R.; Qurishi, M.A. Chemical composition, antioxidant and antibacterial activities of the leaf essential oil of *Juglans regia* L. and its constituents. *Phytomedicine* **2012**, *19*, 1185–1190. [CrossRef]
15. Van Meerloo, J.; Kaspers, G.J.; Cloos, J. Cell sensitivity assays: The MTT assay. *Methods Mol. Biol.* **2011**, *731*, 237–245. [CrossRef] [PubMed]
16. Catanzaro, E.; Greco, G.; Potenza, L.; Calcabrini, C.; Fimognari, C. Natural products to fight cancer: A focus on *Juglans regia*. *Toxins* **2018**, *10*, 469. [CrossRef]
17. Choi, S.W.; Choi, J.; Kim, J.; Kim, Y.; Friso, S. Walnut substantially alters the DNA methylation profile in colon cancer stem cells. *FASEB J.* **2016**, *30*, 912–915.
18. Mateș, L.; Rusu, M.E.; Popa, D.-S. Phytochemicals and Biological Activities of Walnut Septum: A Systematic Review. *Antioxidants* **2023**, *12*, 604. [CrossRef]
19. Al-Mahmood, A.A.; Shu, L.; Kim, H.; Ramirez, C.; Pung, D.; Guo, Y.; Li, W.; Kong, A.N.T. Prostate cancer and chemoprevention by natural dietary phytochemicals. *J. Chin. Pharm. Sci.* **2016**, *25*, 633–650.
20. Perugu, S.; Vemula, R.; Rao, M. Walnut pedunculagin a probable serm for breast cancer treatment. *Int. J. Pharm. Pharm. Sci.* **2014**, *7*, 233–235.
21. Hardman, W.E.; Primerano, D.A.; Legenza, M.T.; Morgan, J.; Fan, J.; Denvir, J. Dietary walnut altered gene expressions related to tumor growth, survival, and metastasis in breast cancer patients: A pilot clinical trial. *Nutr. Res.* **2019**, *66*, 82–94. [CrossRef] [PubMed]
22. Bourais, I.; Elmarrkechy, S.; Taha, D.; Badaoui, B.; Mourabit, Y.; Salhi, N.; Alshahrani, M.M.; Al Awadh, A.A.; Bouyahya, A.; Goh, K.W.; et al. Comparative Investigation of Chemical Constituents of Kernels, Leaves, Husk, and Bark of *Juglans regia* L.; Using HPLC-DAD-ESI-MS/MS Analysis and Evaluation of Their Antioxidant, Antidiabetic, and Anti-Inflammatory Activities. *Molecules* **2022**, *27*, 8989. [CrossRef] [PubMed]
23. Chauhan, A.; Chauhan, V. Beneficial Effects of Walnuts on Cognition and Brain Health. *Nutrients* **2020**, *12*, 550. [CrossRef] [PubMed]
24. Prasad, R.; Shah, A.H.; Dhamgaye, S. Mechanism of Drug Resistance in Fungi and Their Significance in Biofilms. *Antibiofilm Agents* **2014**, *8*, 45–65.

25. Prasad, R.; Shah, A.H.; Rawal, M.K. Antifungals: Mechanism of Action and Drug Resistance. *Yeast Membr. Transp. Adv. Exp. Med. Biol.* **2016**, *892*, 327–349.
26. Sytykiewicz, H.; Chrzanowski, G.; Czerniewicz, P.; Leszczyński, B.; Sprawka, I.; Krzyzanowski, R.; Matok, H. Antifungal activity of *Juglans regia* (L.) leaf extracts against candida albicans isolates. *Pol. J. Environ. Stud.* **2015**, *24*, 1339–1348. [CrossRef]
27. Wianowska, D.; Garbaczewska, S.; Cieniecka-Roslonkiewicz, A.; Dawidowicz, A.; Jankowska, A. Comparison of antifungal activity of extracts from different *Juglans regia* cultivars and juglone. *Microb. Pathog.* **2016**, *100*, 263–267. [CrossRef]
28. Timothy, J.; Bradbury, K.E.; Aurora, P.-C.; Key, T.J.; Bradbury, K.E.; Perez-Cornago, A.; Sinha, R.; Tsilidis, K.K.; Tsugane, S. Diet, nutrition, and cancer risk: What do we know and what is the way forward? *Analysis* **2020**, *2020*, 368.
29. Negi, A.S.; Luqman, S.; Srivastava, S.; Krishna, V.; Gupta, N.; Darokar, M.P. Antiproliferative and antioxidant activities of *Juglans regia* fruit extracts. *Pharm. Biol.* **2011**, *49*, 669–673. [CrossRef]
30. Raheleh Jahanbani, S. Mahmood Ghaffari Maryam Salami Kourosh Vahdati Houri Sepehri Nazanin Namazi Sarvestani Nader Sheibani Ali Akbar Moosavi-Movahedi. Antioxidant and Anticancer Activities of Walnut (*Juglans regia* L.) Protein Hydrolysates Using Different Proteases. *Plant Foods Hum. Nutr.* **2016**, *71*, 402–409. [CrossRef]
31. Yang, J.; Liu, R.H.; Halim, L. Antioxidant and antiproliferative activities of common edible nut seeds. *LWT-Food Sci. Technol.* **2009**, *42*, 1–8. [CrossRef]
32. Genovese, C.; Cambria, M.T.; D'angeli, F.; Addamo, A.P.; Malfa, G.A.; Siracusa, L.; Salmeri, M. The double effect of walnut septum extract (*Juglans regia* L.) counteracts A172 glioblastoma cell survival and bacterial growth. *Int. J. Oncol.* **2020**, *57*, 1129–1144. [CrossRef] [PubMed]
33. Salimi, M.; Majd, A.; Sepahdar, Z.; Azadmanesh, K.; Irian, S.; Ardestaniyan, M.H.; Hedayati, M.H.; Rastkari, N. Cytotoxicity effects of various *Juglans regia* (walnut) leaf extracts in human cancer cell lines. *Pharm. Biol.* **2012**, *50*, 1416–1422. [CrossRef] [PubMed]