Journal of Physical Chemistry and Functional Materials

Home Page of Journal: https://dergipark.org.tr/jphcfum

Investigation of Phytochemical Contents and Anticancer, Antioxidant, Antimicrobial Activities of *Cucurbita pepo* Leaves

Lara Saeed [a,b], Mehmet Mürşit Temüz[b], Serhat Keser[c], Şule İnci[d], Lütfiye Kadıoğlu Dalkılıç[e], Arzu Karatepe[f]

[a] *Department of Chemistry, Faculty of Science, Koya University, Koya, Iraq*

[b] *Department of Chemistry, Faculty of Science, Firat University, Elazig, Türkiye*

[c] *Department of Chemical Technology, EOSB Vocational School, Firat University, Elazig, Türkiye*

[d] *Medical Laboratory Techniques Program, Department of Medical Services and Techniques, Health Services Vocational School, Istanbul Rumeli University, Istanbul, Türkiye*

[e] *Department of Biology, Faculty of Science, Firat University, Elazig, Türkiye*

[f] *Department of Chemistry, Faculty of Science, Bingol University, Bingol, Türkiye*

**Corresponding author: E-mail: serhatkeser@gmail.com*

ABSTRACT

Ethanol and acetone extracts of *Cucurbita pepo* leaves were *in vitro* analyzed to determine the vitamin contents, antioxidant, anticancer, and antimicrobial activities. According to HPLC analyses, the leaves contained slight amounts of vitamin C, A, and E. The analysis for antioxidant activity revealed that both ethanol and acetone extracts exhibited high activity values against $ABTS^{+\bullet}$, $OH^{\bullet}$, and $DPPH^{\bullet}$ radicals. The total phenols and flavonoid assay revealed high values for the extracts. The MTT test showed that both ethanol and acetone extracts exhibited cytotoxic activity toward human breast cancer cells (MCF-7). But no significant activities observed against hepatocellular carcinoma cells (HepG-2). The antimicrobial activity assay revealed activity of the extracts towards selected Gram's positive and Gram's negative bacteria. These results have provided scientific motivation to use *Cucurbita pepo* leaves as a medicinal plant to treat oxidative and inflammatory-related diseases.

ARTICLE INFO

***Keywords*:**

Cucurbita pepo
Antioxidant
Anticancer
Antimicrobial
Antiradical

Received: 2024-05-27
Accepted: 2024-07-03
ISSN: 2651-3080
DOI: 10.54565/jphcfum.1490712

1. Introduction

Cucurbita pepo are phytochemicals that are affluent in antioxidants and have many health benefits [1]. They prevent and help reduce many diseases by being rich in vitamins and minerals, besides antioxidant and anti-inflammatory properties [2]. *Cucurbita pepo* contain water, protein, fat, carbohydrate, fibre, calcium, iron, potassium, sodium, magnesium, phosphorus, zinc, vitamin C, vitamin B (B_1, B_2, B_3, B_6, B_9), β-carotene, vitamin E, vitamin K_1, saturated fatty acids, as well as polyunsaturated fatty acids [3]. In addition to phenolic compounds, vitamins, minerals, alkaloids, saponins, phytates, and tannins that are responsible for the antioxidant and antimicrobial activity [4-6]. Antioxidants are compounds that inhibit, decrease, or repair damage caused by free radicals. Free radicals are ions or molecules with an unpaired electron; they either donate these unpaired electrons or receive electrons from other molecules leading to reactions that cause oxidative stress (imbalance between the free radicals produced and the antioxidants that neutralize them) and diseases. Free radicals either produce endogenously during cellular

metabolism, or come from external causes such as X-ray radiation, etc. [7,8]. The mechanism of antioxidants defense against free radicals includes prevention of free radicals and their derivatives activity, repairing and disrupting oxidative reactions, as well as inactivation of free radicals' yields [9].

Antioxidants are divided into natural antioxidants, that either exist within the human body (endogenous) such as superoxide dismutase, etc. or can be consumed from dietary sources (exogenous) such as vitamins, etc. [10,11]. Synthetic antioxidants such as cerium oxide nanoparticles, etc. [11,12]. The antioxidants that exist in *Cucurbita pepo* leaves are: vitamin C (which hinders free radicals, preserves against oxidative damage, etc.) [13,14], β-carotene (which scavenges free radicals and inhibits lipid peroxidation as well as reducing ischemic heart disease etc.) [15,16], vitamin E (which prevent cell wall damage caused by free radicals, and regulate specific enzymes that aid the antioxidant mechanism, etc.) [17], vitamin K_1 (which inhibits cell death that results from oxidative stress, inhibit lipid peroxidation, as well as having anti-inflammatory activity, and reducing type 1 diabetes, etc.) [18-21], zinc (which takes part in the antioxidant mechanism and reduces oxidative stress, as well as protecting against genetic mutations, etc.) [22], phenolic acids (that are strong antioxidants, have antitumor properties, and inhibit lipid peroxidation, etc.) [23-25], tannins (that reduce free radicals, as well as having antibacterial, cardioprotective, antitumor, and anti-inflammatory properties, etc.) [26-28], flavonoids (that are potent antioxidants, prevent lipid peroxidation, as well as having anticancer, hepatoprotective, anti-inflammatory, and antibacterial properties, etc.) [29,30], alkaloids (that have important antioxidant activities, as well as antimicrobial, anti-human immunodeficiency, antimalarial, and antitumor activities, etc.) [31,32], saponins (that have antioxidant activities, antitumor, antibiotic, and fungicidal properties, etc.) [33-35], and finally, phytates (that are antioxidants that prevent lipid peroxidation, vital signaling molecules, as well as having antitumor and anticancer properties, etc.) [36-38].

In this study aims to determine various biological activities of *Cucurbita pepo* leaves to declare the advantages of these leaves, particularly as a source of antioxidants, for supplying unique nutrients antioxidants whether as food or for medicinal purposes as antioxidants.

2. Materials and methods

The green leaves of the *Cucurbita pepo* plant were collected from Elazig, Turkey. The leaves were dried in the shade and at room temperature. The dried leaves were grounded to make a fine powder using an electric hand blender. To determine the biological activities of the green leaves of *Cucurbita pepo*, plant samples were extracted with ethanol and acetone and analyzed with different methods.

2.1. Preparation of the extracts

Sample extraction was performed according to the procedure followed by Pekdemir et al. [39]. Accordingly, for the ethanol extract, 20 g of leaf powder sample was weighed on a precision balance and 200 mL of ethanol was added to it. The solution was sonicated for 10 minutes in an ultrasonic homogenizer. The samples were then shaken in a shaker for 18 hours. The resulting solution was filtered with Whatman No:1 filter paper. The solvent in the filtrate was removed by evaporator. The obtained extract was stored at -20 °C in the refrigerator until the determination of antioxidant and antimicrobial activity. To obtain the acetone extract, acetone was used as the solvent, and the same method was applied.

2.2. Antioxidant assays

2.2.1. ABTS radical scavenging activity

The ABTS radical scavenging activity was determined by spectrophotometric analysis following Re et al. [40] procedure. The cation radical $ABTS^{\bullet+}$ was initiated by the reaction of 2 mM ABTS with 2.45 mM potassium persulfate $K_2S_2O_8$ and kept in the dark for 2 hours at room temperature. Later the $ABTS^{\bullet+}$ solution was diluted with 0.1 M sodium phosphate buffer (pH=7.4) to attain an absorbance of 1.520±0.025 at 734 nm. Then 1 mL of $ABTS^{\bullet+}$ solution was added to 3 mL (500 µg/mL of the prepared acetone and ethanol sample extracts), and the absorbance was measured with laboratory UV–visible spectrophotometer at 734 nm after a half of an hour.

2.2.2. Hydroxyl radical ($OH^{\bullet}$) scavenging activity

The capacity of the extracts to hinder non-site-specific hydroxyl radical-mediated peroxidation was measured following Halliwell et al. [41] procedures. 3.6 mM of 2-deoxy-D-ribose in 20 mM (pH=7.4) KH_2PO_4–KOH buffer, along with 200 µL of 100 µM $FeCl_3$, and 200 µL of 104 mM EDTA, as well as 100 µL of 1.0 mM H_2O_2 and finally 100 µL of 1.0 mM aqueous ascorbic acid was added to 500 µL of our acetone and ethanol extracts. Then the mixture was mixed using laboratory Clifton™ Cyclone Vortex Mixer, and incubated at 37 °C in laboratory incubator for one hour. Later 1 mL of 2.8% TCA and 1 mL of 1.0% TBA was added to the samples and mixed again with laboratory Clifton™ Cyclone Vortex Mixer, and placed in water bath at 50°C for 30

minutes. The range of 2-deoxyribose oxidation was valued by the solution absorbance at 532 nm.

2.2.3. DPPH radical scavenging activity

Free radical scavenging activity of ethanol and acetone extracts was performed using the previously reported method [42]. 4 mL of a previously prepared solution of 25 mg/L DPPH was mixed with 500 µg/mL of samples. The mixture was stored at room temperature for 30 minutes in the dark. The absorbance of the mixtures was measured at 517 nm with laboratory UV–visible spectrophotometer. Butylated hydroxytoluene (BHT) was used as a positive control for all the antiradical tests [43-46].

2.3. Determination of Phytochemical Contents

2.3.1. Determination of Total Phenolic Content

Total phenolic content was determined according to Slinkard and Singleton [47] procedures using Folin-Ciocalteu reagent. Gallic acid was used as a standard phenolic compound. 1 mL of samples with a concentration of 500 ppm was taken and diluted with 46 mL of distilled water. Then, 1 mL of Folin-Ciocalteu reagent was added and mixed. After three minutes, 3 mL of 2% Na_2CO_3 was added and the mixture was shaken randomly for two hours. Absorbance measurement was made with a UV spectrophotometer at 760 nm. Total phenolic content (TPC) was determined as milligram gallic acid equivalents per gram of dry extract.

2.3.2. Determination of Total Flavonoids Content

The determination of the total flavonoid content was accomplished following Kim et al. [48] procedures. Catechine was used as a reference standard. To our 0.5 mL (500 µg/mL) extracts, 4 mL of distilled water was added, and then 0.3 mL of 5% sodium nitrite solution was added, as well as 0.3 mL of 10% aluminum chloride solution, the test tubes were incubated in for 5 minutes at room temperature. Later the mixture was mixed using laboratory Clifton™ Cyclone Vortex Mixer and the absorbance was measured at 510 nm. Total flavonoid content was given as µg catechin equivalent per gram dry weight of the extract.

2.3.3. Determination of Proanthocyanidin Content

The determination of proanthocyanidin content was carried out following Amaeze et al. [49] procedures. 0.5 mL of (500 µg/mL) extract was mixed with 1.5 mL of 4% vanillin-methanol solution along with 0.75 mL of concentrated hydrochloric acid, then the mixture was let to stand for 15 min, later the absorbance was measured at 500 nm. Catechin was used as the standard for the total proanthocyanidin content. Total proanthocyanidin contents was expressed as µg catechin equivalent/g extract.

2.4. Vitamin Content Determination

2.4.1. Materials

The high-performance liquid chromatography (HPLC) was used for vitamin analysis of microorganisms treated with plant extract. HPLC system consisted of CTO-10AS VP column oven, LC-20AD pumps, DGU-20A5 degassers, SIL 20A auto sampler, SPD-M20A DAD system. Communication model: Model CBM-20A which connects the other apparatus together, LC Solution.

2.4.2. Applied cells

In the study, *Saccharomyces cerevisiae* yeast cells were used to determine the vitamin analysis. *Saccharomyces cerevisiae*, belonging to the fungal kingdom, is single-celled yeast containing high levels of vitamins and used as a model for the molecular response to oxidative stress to determine antioxidant activity [50]. The dry sample of *Saccharomyces cerevisiae* was kept at 4 °C throughout the experiment. Cultivation of the yeast was done at the microbiology laboratory: the yeast was imbued in Difco™ Malt extract broth for one day at 25 °C. Later, the yeast culture was immunized into YEDP (1 g yeast in 100 mL extract, 2 g Bacto™ Peptone, 2 g glucose, 2 g agar) and adjusted to a standard density at a rate of 1% (10^3 CFU per mL for the yeast) using DEN-1 McFarland densitometer, then incubated at 25 °C for 2 days. 2 mL from *Saccharomyces cerevisiae* (at density of 10^3 CFU per mL) samples was added to 50 µL and 100 µL from the prepared samples extract and incubated for one day.

2.4.3. Determination of MDA and Vitamin C

Malondialdehyde (MDA) and vitamin C levels were determined using the procedure of Karatepe [51] procedure. First of all, the liquid treated samples were agitated using a syringe to keep the medium in a mixed form. Then 1 mL of 0.5 M $HClO_4$ was added to our samples extracts to precipitate proteins and release MDA bound to amino groups and other amino compounds, along with 0.5 mL distilled water and mixed using Clifton™ Cyclone Vortex Mixer for few minutes. After that the samples were centrifuged using centrifuge machine for 5 min. The supernatant was obtained and 20 µL of it was injected into HPLC instrument. The mobile phase was 17.5% methanol, 85.5% (30 M potassium phosphate buffer, pH=3.6) (v/v).

2.4.4. Determination of Vitamin E and β-carotene

The liquid soluble vitamins of the extract's samples were analyzed following Catignani and Bieri [52] procedure. To 100 µL of the treated extracts samples, 200 µL of ethanol along with 99:1 H_2SO_4 and 100 µL distilled water were added in a test tube for proteins precipitation, then the samples were mixed with laboratory Clifton™ Cyclone

Vortex Mixer, and later centrifuged at 4500 rpm for 5 min. After centrifugation 100 µL of n-hexane along with 0.05% BHT were added to the mixture, so that to extract the lipid soluble vitamins in the samples. The mixture was mixed and centrifuged again. Then the hexane layer was obtained and transferred to another test tube. Another 100 µL of n-hexane was added to the sample residue, mixed, centrifuged and added to the previous hexane layer. Then the hexane layer was evaporated by nitrogen gas, and to test tube residues 50 µL methanol, 25 µL acetonitrile, 25 µL chloroform was added. Finally, 20 µL from the test tube mixture was injected into the HPLC instrument. The mobile phase was (47:42:11) (v/v) methanol/ acetonitrile/chloroform.

2.5. Statistical analysis

Statistical analysis was made using SPSS 22.0 for Windows software. The statistical significance was at $p<0.05$, and the results were expressed as mean ± S.D. Independent sample T-test was used for evaluation of mean values.

2.6. Anticancer determination

The human breast cancer (MCF-7) and hepatocellular carcinoma (HepG-2) cell lines used in the study were obtained from Ankara University Biotechnology Institute. Experimental studies were carried out at Firat University Faculty of Medicine.

2.6.1. Cell culture

The anticancer activity of *Cucurbita pepo* leaves extracts was tested through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method. Human breast cancer and hepatocellular carcinoma cells were developed in 25 cm^2 flasks in Dulbecco's modification of Eagle medium (1% L-glutamine, 10% fetal bovin serum, and 1% Penicillin-Streptomycin) medium, at 37°C beneath 5% CO_2 atmosphere condition. After the development of the cells in the flasks and covering the flask surface the medium was removed from the cells and the cells were washed with sterile phosphate-buffered saline solution, then 1 mL of trypsin-EDTA was added to the flasks and incubated for 2 min at 37°C in an oven with 5% CO_2. Later, the cells were separated from the surface, 5 mL of the medium was added to inactivate Trypsin-EDTA and the cells were occupied from the flask and centrifuged at 1200 rpm for 5 min, the supernatant was discarded and the pellet was dissolved in 10 mL of the medium. After that 100 µL from the cells in the medium was embedded in 96-well plates, while the first row was embedded with the medium alone as a blank. The plates were incubated for one day at 37 °C in an oven with 5% CO_2. 2.5 ng/mL doxorubicin was used as positive control while the medium is used as negative control. After incubation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test was applied [53,54].

2.6.2. MTT test

MTT test is used to measure cell feasibility, viability, and cytotoxicity. The test is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, a salt that can pass through the cell membrane of lively cells mitochondria and receiving electrons inside the cell and changing into water soluble formazan crystals [55,56]. 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution was added to the stock prepared in sterile phosphate buffer (pH=7.2) cells in well plates, and incubated for 4 hours at 37 °C with 5% CO_2 in the dark. Later, the medium was removed and the salt crystals were dissolved with 100 µL DMSO. 100 µL of the salt solution was added to 96-well plates and kept in the dark for 10 min. Later the absorbance of the salt solution was determined at 450 nm with a plate reader machine. The average of the control wells absorbance was obtained and accepted as 100% viable cells. The gained absorbance from the *Cucurbita pepo* leaves extracts wells was proportional to the control absorbance and the percent of viability was calculated [53]. The measured absorbance values were compared with the control and the data were transported to diagrams.

2.6.3. Analysis

The absorbance values were quantified with Enzyme Linked Immunosorbent Assay (ELISA) plate reader at 450 nm and the average of the absorbance values was quantified and after comparing with the control the percentage was calculated.

2.7. Antimicrobial activity

The effects of ethanol and acetone extracts against bacteria and fungi were evaluated according to the disc diffusion method [57]. The antimicrobial activity of the extracts was evaluated against:

A/ Bacteria strains: *Escherichia coli* (ATCC 25322), *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 25923), *Bacillus megaterium* (DSM 32) were cultivated in Difco™ nutrient broth medium, for one day at 35 °C. (Nutrient broths are biological media used for providing nutrition requirements of bacteria).

B/ Yeast strain: *Candida albicans* (FMC 17) was imbued in Difco™ Malt extract broth for two days at 25 °C. The cultivated cells of bacteria and yeast were imbued into Mueller-Hinton agar together with Sabouraud dextrose agar and well mixed and then adjusted to standard density at a rate of 1% (10^5 CFU per mL for bacteria, and 10^3

CFU per mL for yeast) using DEN-1 McFarland densitometer. Then 25 mL of the cells were poured into sterilized petri dishes (diameter=6 mm) with homogenous spreading until the agar was hardened. Oxoid™ antimicrobial discs (diameter=6 mm) were soaked with 100 µL of ethanol extract and 100 µL of acetone extract respectively and were placed on the hardened agar and stored in the laboratory incubator for two hours at 4 °C to permit the extracts dispersion into the agar media. Later, the petri dishes with the bacterial strains were incubated in a laboratory incubator at 37 °C for one day. In contrast, the petri dishes with the yeast strain were incubated at 25 °C for two days. Streptomycin sulfate 10 µg/disc was used as a control disk for bacteria, while Nystatin 10 µg/disc was used as a control disk for yeast, and dimethyl sulfoxide (DMSO) was used as a negative control. On the next day, the zone inhibitions were measured in millimeters.

3. Results and discussion

3.1. The antioxidants activities

The values of ABTS, OH as well as DPPH radical scavenging activity are given in Table 1. Accordingly, it was observed that ethanol extract and acetone extract showed high radical scavenging activity. The best efficacy when comparing to BHT was the ethanol extract, due to its higher content of phytochemicals indicating radical scavenging trends (Fig.1). The percentage of ABTS, OH, along with DPPH radicals are indicated by the equation:

% Scavenging Activity = $[(A_0 - A_1)/A_0] \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract's samples.

The total flavonoids in the ethanol extract of *Cucurbita pepo* leaves were 5527.40 µg CE/g extract. While the total flavonoids in the acetone extract was 6031.40 µg CE/g extract. The total phenolic content in the ethanol extract of *Cucurbita pepo* leaves was 55.14 mg GAE/g extract. While the total phenolic content in the acetone extract was 62.51 GAE/g extract. As can be seen in Table 2.

Table 1. Radical scavenging activities (%)

Samples	$ABTS^{+\bullet}$	$DPPH^{\bullet}$	$OH^{\bullet}$
Acetone extract	91.18	68.94	60.70
Ethanol extract	92.43	79.10	67.94
BHT	97.89	96.53	95.37

Table 2. Total flavonoids, total phenolics and total proanthocyanidins concentration values

Samples	Total flavonoids (µg CE/g)	Total phenolics (µg CE/g)	Total proanthocyanidins (µg CE/g)
Acetone extract	6013.40	1289.67	62.51
Ethanol extract	5527.40	151.22	55.14

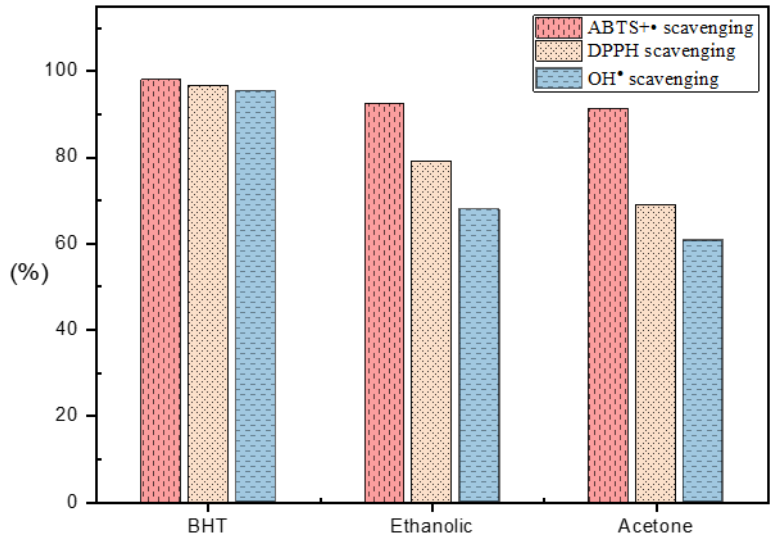

Fig.1. Graphical representation of ABTS, DPPH and OH radical scavenging activities

3.2. Vitamin Content Determination

The HPLC chromatograms for MDA with 7.179 retention time, vitamins C with 4.099 retention time, vitamin E with 3.922 retention time, β-carotene with 2.342 retention time, were like the followings in Table 3.

Table 3 The mean values of MDA, vitamins C, E, β-carotene for the extracts at $p < 0.05$.

Samples	MDA (ppm)	Vitamin C (ppm)	β-carotene (ppm)	Vitamin E (ppm)
Control	23.00±0.26	0.82±0.02	0.08±0.009	0.79±0.06
Acetone extract (50 µL)	22.67±1.2	0.79±0.05	0.06±0.011	0.75±0.05
Acetone extract (100 µL)	21.34±0.88	0.82±0.04	0.08±0.001	0.84±0.06
Ethanol extract (50 µL)	21.83±1.26	0.82±0.04	0.09±0.009	0.86±0.08
Ethanol extract (100 µL)	23.55±2.02	0.83±0.05	0.09±0.008	0.88±0.08

The malondialdehyde is an indicator of lipid peroxidation because MDA is one of the fatty acid lipid peroxidation products [58]. The concentration of MDA in the extracts samples is almost the same as the concentration of the MDA in the control, which indicates that vitamin antioxidants in the extracts had no effect on inhibiting MDA formation. Our sample extracts' vitamin C concentration was equal to or slightly lower than the control value. The concentration of β-carotene in the acetone extract samples was similar to or slightly lower than the control concentration, whereas the concentration of β-carotene in the ethanol extract samples was slightly higher than the control concentration, β-carotene concentration with increasing extract sample concentration. The vitamin E concentration in the 50 µL acetone extract sample was lower than the control concentration, while the 100 µL acetone extract sample was higher than the control concentration. The levels of vitamin E in the ethanol and acetone extracts were higher than the control. Vitamin E concentration with increasing extract sample concentration. The vitamins values (especially vitamin C) are less than they were expected and this can be due to immoderate loss of the vitamins content throughout proceeding the analytical procedures, like drying and storage methods, common errors that result throughout sampling strategies, as well as analysis duration [59,60].

3.3. Anticancer Activity

The phytochemicals in *Cucurbita pepo* leaves are regarded

as anticancer sources, as mentioned in the introduction section. The MTT test was used to determine the effect of our acetone and ethanol extracts on the cancer cells viability. The extract samples showed no significant effect fighting against the hepatocellular carcinoma, as can be seen in Figure 2 and in Table 4. The acetone extracts (5, 2.5, 1.25 ppm) had no effect on decreasing the cancer cells viability, but the 10 ppm acetone extract had 2.5% less cancer cell viability than the negative control, indicating that the activity of the extract sample against the cancer cells increase with the concentration of the extract. The same situation happened with the (5, 2.5, 1.25 ppm) ethanol extracts, while the 10 ppm extract had 6.2% less cancer cells viability than the negative control.

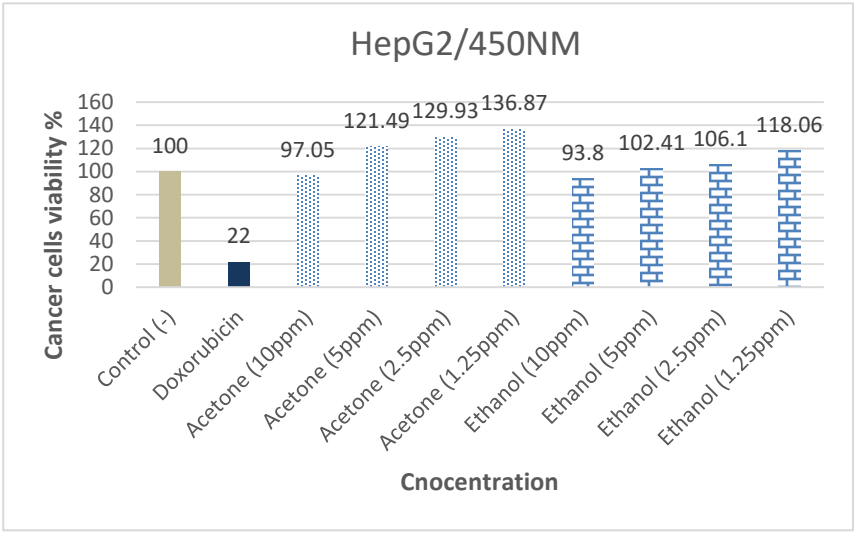

Fig. 2. The effect of the acetone and ethanol extracts on hepatocellular carcinoma.

While the extract samples showed significant effects against human breast cancer as can be seen in Figure 3.

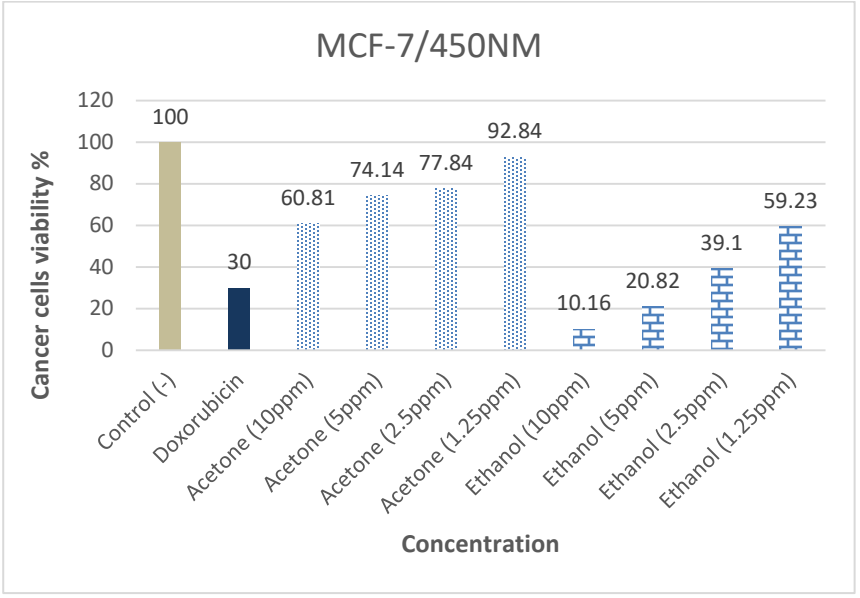

Fig. 3. The effect of acetone and ethanol extracts on hepatocellular carcinoma.

The acetone extract at a concentration of 10 ppm had 39.19% less cancer cells viability than the negative control, the acetone extract at a concentration of 5 ppm had 25.86% less cancer cell viability than the negative control, the acetone extract at a concentration of 2.5 ppm had 22.16% less cancer cells viability than the negative control, besides the acetone extract at a concentration of 1.25 ppm that had 7.16% less cancer cells viability than

the negative control. It shows that the extract sample activity increases with increasing the extract concentration. The ethanol extract at a concentration of 10 ppm had 19.84 less cancer cells viability than the Doxorubicin (a breast cancer therapy), the ethanol extract at a concentration of 5 ppm had 9.18 less cancer cells viability than Doxorubicin, the ethanol extract at a concentration of 2.5 ppm had 60.9% less cancer cells viability than the negative control, while the ethanol extract at a concentration of 1.25 ppm had 40.77% less cells viability than the negative control. Once more, it shows that the extract sample activity increases with increasing the extract concentration. The ethanol sample extract appears to be a potent anti-breast cancer activity. It is also understood that the phytochemicals responsible for their anti-cancer activities are more soluble in ethanol solvent than acetone solvent. As can be seen in Table 4.

Table 4. The effect of acetone and ethanol extracts samples on hepatocellular carcinoma and human breast cancer.

Samples	Concentration	Cell lines (Viability %)	
		MCF-7 cell line	HepG-2 cell line
Acetone extract	**10 ppm**	60.81	97.05
	5 ppm	74.14	121.49
	2.5 ppm	77.84	129.93
	1.25 ppm	92.84	136.87
Ethanol extract	**10 ppm**	10.16	93.8
	5 ppm	20.82	102.41
	2.5 ppm	39.1	106.1
	1.25 ppm	59.23	118.06

3.4. Antimicrobial effect

Both acetone and ethanol extracts of the leaves were effective against the fungus and bacteria, except for the ethanol extract against the two gram-negative bacteria *E. coli* and *K. pneumonia*, as can be seen in Figure 4-Figure 8 as well as Table 5. The diameter of the inhibition zone of the leaves extracts was measured in millimeters, and (-) refers to no inhibition zone.

Table 5. The leaves extracts inhibition zone diameters.

Samples	*E. coli*	*K. pneumoniae*	*B. megaterium*	*S. aureus*	*C. albicans*
Acetone	10 mm	5 mm	9 mm	8 mm	10 mm
Ethanol	-	-	10 mm	10 mm	10 mm
Control	19 mm	20 mm	25 mm	19 mm	24 mm

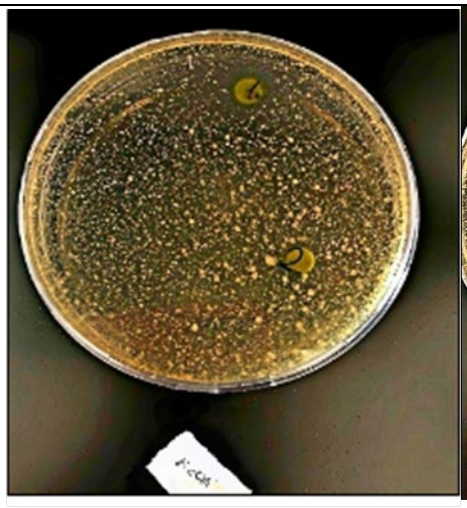

Figure 4. The anti-microbial effect of acetone (1) and ethanol (2) extracts on *E. coli* bacteria.

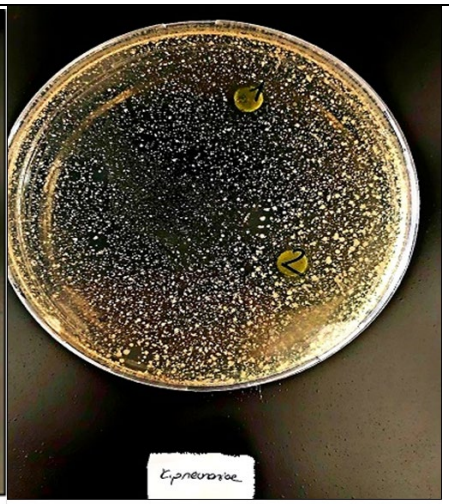

Figure 5. The anti-microbial effect of acetone (1) and ethanol (2) extracts on *K. pneumonia* bacteria.

Figure 6. The anti-microbial effect of acetone (1) and ethanol (2) extracts on *B. megaterium* bacteria.

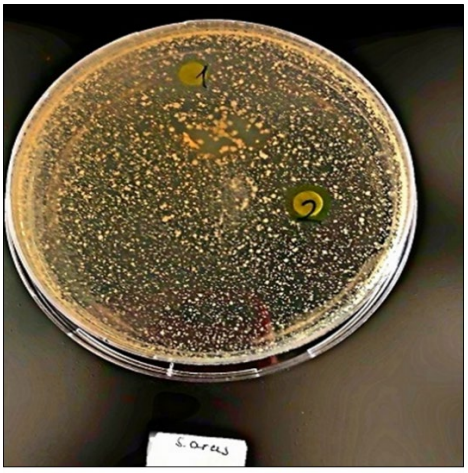

Figure 7. The anti-microbial effect of acetone (1) and ethanol (2) extracts on *S. aureus* bacteria.

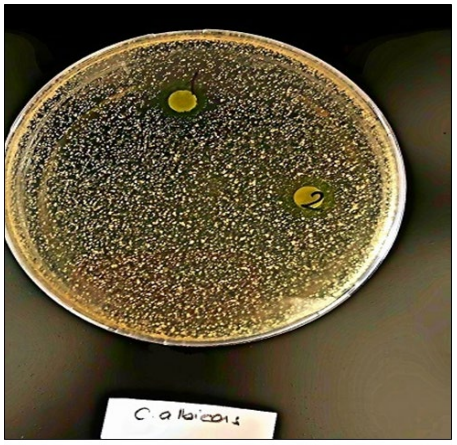

Figure 8. The anti-microbial effect of acetone (1) and ethanol (2) extracts on *C. albicans* bacteria.

The acetone extract was effective against *E. coli* bacteria, while the ethanol extract showed no effect; the reason why acetone extract affected *E. coli* while ethanol extract had no effect on *E. coli* can be related to that the phenolic compounds that exist in the leaves are more soluble in the acetone solvent than in the ethanol solvent [61]. The

higher concentration of these compounds in the acetone extract may result in higher antimicrobial resistance. While the reason why the ethanol extract was effective against the gram-positive bacteria and had no effect against the gram-negative ones is due to the gram-negative bacteria outer membrane that has lipopolysaccharide that shields the thin peptidoglycan inner wall, and so the outer membrane is a barrier to the antibacterial constituents, contrasted to the gram-positive bacteria in which the absence of the outer layer and a single membrane structure cause less resistance [62].

The same case happened with the gram-negative *K. pneumoniae* bacteria, as the ethanol extract showed no effect against *K. pneumoniae* bacteria, while the acetone extract showed resistance only around one side of the disk with a diameter of 10 mm, so we divided it by two and wrote 5 mm because it was from one side of the disk.

Both extracts were effective against *B. megaterium* bacteria, with 9 mm zone inhibition diameter from the acetone, and 10 mm diameter from the ethanol extract. The same case happened with *S. aureus* bacteria; the acetone extract zone inhibition diameter was 9 mm, while the ethanol zone inhibition diameter was 10 mm. The last recording was against *C. albicans* fungus, both extracts had a 10 mm zone inhibition diameter.

Some published leaves extract antimicrobial activity articles, agree with our results: Dar et al. [63] recorded that ethanol extract had no effect on *S. aureus* bacteria. While Fakoya et al. [64] agree with our results at similar concentrations of the ethanol extract, but they also recorded increasing the activity when increasing the ethanol extract concentration, and this quite makes sense because *Cucurbita pepo* leaves extracts are effective against bacteria growth or replication but are dependent on the concentration of the plant extract.

And some partially agree with our results: Dissanayake et al. [5] recorded that the acetone extract had no effect on *E. coli* bacteria, the same as our results. Still, the extract also had no effect on *S. aureus* bacteria, while our results showed an effect on *S. aureus* bacteria. As well Oboh et al. [65] reported inhibition against *E. coli* bacteria with a 4 mm zone diameter, while the other results agree with our result. The difference in the obtained results may be attributable to differences in the microbial strains, the imbuement methods, the bacterial imbuement size, the medium of growing, incubation circumstances, as well as the methods of preparing the extracts [66,67].

The antimicrobial activity of the samples can be calculated according to the equation:

$$\text{Antimicrobial activity (\%)} = \frac{Dc - Ds}{Dc} \times 100$$

Where Dc = inhibition diameter of the control, Ds= inhibition diameter of the sample for the acetone extract:

- *E. coli*: Antimicrobial activity= 47.36%
- *K. pneumonia*: Antimicrobial activity= 75%
- *B. megaterium*: Antimicrobial activity= 64%
- *S. aureus*: Antimicrobial activity= 57.8%
- *C. albicans*: Antimicrobial activity= 58.3%

For the ethanol extract:

- *E. coli*: Antimicrobial activity= zero (No inhibition)
- *K. pneumonia*: Antimicrobial activity=zero (No inhibition)
- *B. megaterium*: Antimicrobial activity= 60%
- *S. aureus*: Antimicrobial activity= 47.36%
- *C. albicans*: Antimicrobial activity= 58.3% [68].

4. Conclusion

Through our experiments, we could prove that *Cucurbita pepo* leaves indeed contain significant phytochemicals, and we look forward to valuating these leaves and not to discard them. As they can be used as herbal medicines or folklore medicine, orderly intakes of this vegetable have the ability to reduce or inhibit several diseases, as mentioned in the introduction part.

Our experimental results showed that the *Cucurbita pepo* leaves' acetone and ethanol extracts had radical scavenging activities against $ABTS^{+\bullet}$, $OH^{\bullet}$, and $DPPH^{\bullet}$ radicals, besides our experimental results also showed that the leaves extracts had a high total flavonoid, total phenolic content.

Our experimental results for the leaves extracts content of vitamins had only trace amounts of β-carotene and vitamin E, but had no significant vitamin C content. There was no antioxidant effect from vitamins (C, E and β-carotene) of the extract's samples treated with *Saccharomyces cerevisiae* yeast and against MDA. Our experimental results revealed that the acetone and ethanol leaves extracts samples had no significant effect on hepatocellular carcinoma, while the acetone leaves extract decreased breast cancer effects, and the ethanol extract showed a higher effect decreasing human breast cancer than Doxorubicin which is a breast cancer therapy. And the extracts samples activity increased with increasing the concentration of the extract's samples.

Our experimental results also showed that *Cucurbita pepo* leaves extracts have antibacterial and fungicidal properties, these properties are due to the saponins, tannins, flavonoids, and alkaloids contents of *Cucurbita pepo* leaves that are mentioned in the introduction part. However, for the best results, our thesis recommends using high concentration plant extracts because the properties mentioned above are dose-dependent, as we have earlier mentioned in the discussion part.

Furthermore, our experiments showed that the same

extracts differently affect different types of bacteria: the ethanol extract was effective against the gram-positive bacteria but showed no effect gram-negative bacteria due to the dissimilarity in their cell wall structure of the bacteria as we have mentioned in the discussion part. Acetone is a better solvent than ethanol for determining the antibacterial activity of the two gram-negative *E. coli* and *K. pneumonia.* And both extracts showed similar effects against the gram-positive bacteria.

Acknowledgments

This study was produced from the master thesis of Lara Saeed from Firat University, Institute of Sciences, Department of Chemistry. Lara Saeed would like to thank Dr. M.E. Pekdemir for his guidance, assistance, and support.

References

[1] Zeng, G., Wu, W., and Huang, W. (2019). Evaluation of nutritional and other functional qualitiesas well as dietary safety of pumpkin leaves, Journal of Food Safety and Food Quality, 70, 23-29.

[2] Njeme, C. Goduka, N.I., George, G. (2014). Indigenous leafy vegetables (imifino, morogo, muhuro) in South Africa: A rich and unexplored source of nutrients and antioxidants, African Journal of Biotechnology, 13, 1933-1942.

[3] Grubben, G.J.H. (2004). Plant Resources of Tropical Africa, Vegetables, PROTA Foundation. p.118-275.

[4] Ko, J.Y., Ko, M.O., Kim, D.S., Lim, S. (2016). Enhanced production of phenolic compounds from pumpkin leaves by subcritical water hydrolysis, Preventive Nutrition and Food Science, 21, 132-137.

[5] Dissanayake, H., Deraniyagala, S., Hettiarachchi, C., Thiripuranathar, G. (2018). The study of antioxidant and antibacterial properties of skin, seeds and leaves of the Sri Lankan variety of pumpkin, IOSR Journal of Pharmacy, 8, 43-48.

[6] Kulczyński, B., Gramza-Michałowska, A., Królczyk, J.B. (2020). Optimization of extraction conditions for the antioxidant potential of different pumpkin varieties (*Cucurbita maxima*), Sustainability, 12, 1305.

[7] Collin, F. (2019). Chemical basis of reactive oxygen species reactivity and involvement in neurodegenerative diseases, International Journal of Molecular Sciences, 20, 2407.

[8] Rao, P.S., Kalva, S., Yerramilli, A., Mamidi, S. (2011). Free radicals and tissue damage: role of antioxidants, Free Radicals and Antioxidants, 1, 2-7.

[9] Ruiz-Núñez, B., Pruimboom, L., Dijck-Brouwer, D.A., Muskiet, F.A. (2013). Lifestyle and nutritional imbalances associated with Western diseases: causes and consequences of chronic systemic low-grade inflammation in an evolutionary context, Journal of Nutritional Biochemistry, 24, 1183-1201.

[10] Zhao, Y., Wang, Y., Li, Y., Santschi, P., Quigg, A. (2017). Response of photosynthesis and the antioxidant defense system of two microalgal species (*Alexandrium minutum* and *Dunaliella salina*) to the toxicity of BDE-47, Marine Pollution Bulletin, 124, 459-469.

[11] Rosenow, P., Jakob, P., Tonner, R. (2016). Electron–vibron coupling at metal-organic interfaces in theory and experiment, Journal of Physical Chemistry Letters, 7, 1422-1427.

[12] Hariri, M., Maghsoudi, Z., Darvishi, L., Askari, G., Hajishafiee, M., Ghasemi, S., Khorvash, F., Iraj, B., Ghiasvand, R. (2013). Antioxidants intake is negatively correlated with risk of stroke in Iran, International of Preventive Medicine, 4, S248-S289.

[13] Halliwell, B., Gutteridge, J.M.C., (1989). Free Radicals in Biology and Medicine, Clarendon Press., pp. 121-197.

[14] EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies). Turck, D., Bresson, J.L., Burlingame, B., Dean, T., Fairweather-Tait, S., Heinonen, M., Hirsch-Ernst, K.I., Mangelsdorf, I., McArdle, H.J., Naska, A., Neuhauser-Berthold, M., Nowicka, G., Pentieva, K., Sanz, Y., Sjodin, A., Stern, M., Tome, D., Van Loveren, H., Vinceti, M., Willatts, P., Martin, A., Strain, J.J., Heng, L., Valtuena-Martinez, S., Siani. A. (2018). Guidance for the scientific requirements for health claims related to antioxidants, oxidative damage and cardiovascular health (Revision 1). EFSA Journal, 16, 5136, 21.

[15] Brubaker, R.F., Bourne, W.M., Bachman, L.A., McLaren, J.W. (2000). Ascorbic acid content of human corneal epithelium, Investigative Ophthalmology & Visual Science, 41, 1681-1683.

[16] Ojima, F., Sakamoto, H., Ishiguro, Y., Terao, J. (1993). Consumption of carotenoids in photosensitized oxidation of human plasma and plasma low-density lipoprotein, Free Radical Biology and Medicine, 15, 377-384.

[17] Gaziano, J.M., Manson, J.E., Ridker, P.M., Buring, J.E., Hennekens, C.H. (1990). Beta carotene therapy for chronic stable angina, Circulation, 82.

[18] Traber, M.G., Atkinson, J. (2007). Vitamin E, antioxidant and nothing more, Free Radical Biology and Medicine, 43, 4-15.

[19] Li, J., Wang, H., Rosenberg, P.A. (2009). Vitamin K prevents oxidative cell death by inhibiting the activation of 12-lipoxygenase in developing oligodendrocytes, Journal of Neuroscience Research, 87, 1997-2005.

[20] Ohyashiki, T., Yabunaka, Y., Matsui, K. (1991). Antioxidant effect of vitamin K homologues on ascorbic acid/$Fe^{(2+)}$-induced lipid peroxidation of lecithin liposomes, Chemical and Pharmaceutical Bulletin, 39,

976-979.
[21] Ohsaki, Y., Shirakawa, H., Miura, A., Giriwono, P.E., Sato, S., Ohashi, A., Iribe, M., Goto, T., Komai, M. (2010). Vitamin K suppresses the lipopolysaccharide-induced expression of inflammatory cytokines in cultured macrophage-like cells via the inhibition of the activation of nuclear factor κB through the repression of IKKα/β phosphorylation, Journal of Nutritional Biochemistry, 21, 1120-1126.
[22] Varsha, M.K.N.S., Thiagarajan, R., Manikandan, R., Dhanasekaran, G. (2015). Vitamin K1 alleviates streptozotocin-induced type 1 diabetes by mitigating free radical stress, as well as inhibiting NF-κB activation and iNOS expression in rat pancreas, Nutrition, 31, 214-222.
[23] Cruz, J.B.F., Soares, H.F. (2011). Uma revisão sobre o zinco, Ensaios e Ciência: Ciências Biológicas, Agrárias e da Saúde, 15, 207-222.
[24] Laghari, A.H., Memon, S., Nelofar, A., Khan, K.M., Yasmin, A. (2011). Determination of free phenolic acids and antioxidant activity of methanolic extracts obtained from fruits and leaves of *Chenopodium album*, Food Chemistry, 126, 1850-1855.
[25] Espíndola, K.M.M., Ferreira, R.G., Narvaez, L.E.M., Silva Rosario, A.C.R., da Silva, A.H.M., Silva, A.G.B., Vieira, A.P.O., Monteiro, M.C. (2019). Chemical and pharmacological aspects of caffeic acid and its activity in hepatocarcinoma, Frontiers in Oncology, 9, 541.
[26] Siquet, C., Paiva-Martins, F., Lima, J.L., Reis, S., Borges, F. (2006). Antioxidant profile of dihydroxy-and trihydroxyphenolic acids-A structure–activity relationship study, Free Radical Research, 40, 433-442.
[27] Magalhães, L., Ramos, I., Reis, S., Segundo, M. (2014). Antioxidant profile of commercial oenological tannins determined by multiple chemical assays, Australian Journal of Grape and Wine Research, 20, 72-79.
[28] Gülçin, İ., Huyut, Z., Elmastaş, M., Aboul-Enein, H.Y. (2010). Radical scavenging and antioxidant activity of tannic acid, Arabian Journal of Chemistry, 3, 43-53.
[29] Anderson, R.J., Bendell, D.J., Groundwater, P.W. (2004). Organic Spectroscopic Analysis, Royal Society of Chemistry.
[30] Kalinovski, H.O., Berger, S., Braun, S. (1988). Carbon-13 NMR Spectroscopy. John Wiley & Sons.
[31] Pihlaja, K., Kleinpeter, E. (1994). Carbon-13 NMR Chemical Shifts in Structural and Stereochemical Analysis.
[32] Tauc, J., Menth, A. (1972). States in the gap, Journal of Non-Crystalline Solids, 8–10, 569-585.
[33] Singh, D., Chaudhuri, P.K. (2018). Structural characteristics, bioavailability and cardioprotective potential of saponins, Integrative Medicine Research, 7, 33-34.
[34] Man, S., Gao, W., Zhang, Y., Huang, L., Liu, C. (2010). Chemical study and medical application of saponins as anti-cancer agents, Fitoterapia, 81, 703-714.
[35] Sparg, S.G., Light, M.E., Van Staden, J. (2004). Biological activities and distribution of plant saponins, Journal of Ethnopharmacology, 94, 219-243.
[36] Hawkins, P.T., Poyner, D.R., Jackson, T.R., Letcher, A.J., Lander, D.A., Irvine, R.F. (1993). Inhibition of iron-catalysed hydroxyl radical formation by inositol polyphosphates: a possible physiological function for myo-inositol hexakisphosphate, Biochemical Journal, 294, 929-934.
[37] Lee, S., Kim, M.-G., Ahn, H., Kim, S. (2020). Inositol pyrophosphates: signaling molecules with pleiotropic actions in mammals, Molecules, 25, 2208.
[38] Shamsuddin, A.M., Yang, G.Y., Vucenik, I. (1996). Novel anti-cancer functions of IP6: growth inhibition and differentiation of human mammary cancer cell lines *in vitro*, Anticancer Research, 16, 3287-3292.
[39] Pekdemir, M.E., Pekdemir, S., İnci, Ş., Kırbağ, S., Çiftci, M. (2021). Thermal, magnetic properties and antimicrobial effects of magnetic iron oxide nanoparticles treated with *Polygonum cognatum*, Iranian Journal of Science and Technology, Transactions A: Science, 45, 1579-1586.
[40] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radical Biology and Medicine, 26, 1231-1237.
[41] Halliwell, B., Gutteridge, J.M., Aruoma, O.I. (1987). The deoxyribose method: a simple “test-tube” assay for determination of rate constants for reactions of hydroxyl radicals, Analytical Biochemistry, 165, 215-219.
[42] Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity, LWT-Food science and Technology, 28, 25-30.
[43] Keser, S. (2014). Antiradical activities and phytochemical compounds of firethorn (*Pyracantha coccinea*) fruit extracts. Natural Product Research, 28, 1789–1794.
[44] Keser, S., Keser, F., Kaygili, O., Tekin, S., Demir, E., Turkoglu, I., Turkoglu, S., Parlak, A.E., Yilmaz, O., Karatepe, M., Sandal, S., Kirbag, S. (2018). Phytochemical compounds and antiradical, antimicrobial, and cytotoxic activities of the extracts from *Hypericum scabrum* L. Flowers. Natural Product Research, 34, 714–719.
[45] Keser, S., Keser, F., Karatepe, M., Kaygili, O., Tekin, S., Turkoglu, I., Demir, E., Yilmaz, O., Kirbag, S., Sandal, S. (2019). Bioactive contents, *in vitro* antiradical, antimicrobial and cytotoxic properties of rhubarb (*Rheum ribes* L.) extracts. Natural Product Research, 34, 3353–3357.

[46] Keser, S., Kak, O. (2021). *In vitro* antimicrobial, antiradical, anticancer evaluation, and phytochemical contents of endemic *Scorzonera semicana* DC. Journal of Food Processing and Preservation, 45, e15971.

[47] Slinkard, K. Singleton, V.L. (1977). Total phenol analysis: automation and comparison with manual methods, American Journal of Enology and Viticulture, 28, 49-55.

[48] Kim, D.O., Chun, O.K., Kim, Y.J., Moon, H.Y., Lee, C.Y. (2003). Quantification of polyphenolics and their antioxidant capacity in fresh plums, Journal of Agricultural and Food Chemistry, 51, 6509-6515.

[49] Amaeze, O., Ayoola, G., Sofidiya, M., Adepoju-Bello, A., Adegoke, A., Coker, H. (2011). Evaluation of antioxidant activity of *Tetracarpidium conophorum* (Müll. Arg) Hutch & Dalziel leaves, Oxidative Medicine and Cellular Longevity, 2011, 976701.

[50] Offiong, O.E., Martelli, S. (1997). Stereochemistry and antitumour activity of platinum metal complexes of 2-acetylpyridine thiosemicarbazones, Transition Metal Chemistry, 22, 263-269.

[51] Karatepe, M. (2004). Simultaneous determination of ascorbic acid and free malondialdehyde in human serum by HPLC-UV, LC-GC North America, 22, 362-365.

[52] Catignani, G.L, Bieri, J.G. (1983). Simultaneous determination of retinol and alpha-tocopherol in serum or plasma by liquid chromatography, Clinical Chemistry, 29, 708-712.

[53] Dalkilic, S., Dalkilic, L.K., İnci, S., Korkmaz, İ., Kirbag, S. (2021). Investigation of cytotoxic effect of black mulberry (*Morus nigra* L.) fruit, Indian Journal of Traditional Knowledge, 20, 54-58.

[54] Van Meerloo, J., Kaspers, G.J.L, Cloos, J. (2011). Cell sensitivity assays: the MTT assay, Methods in Molecular Biology, 731, 237-245.

[55] Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, Journal of Immunological Methods, 65, 55-63.

[56] Gerlier, D., Thomasset, N. (1986). Use of MTT colorimetric assay to measure cell activation, Journal of Immunological Methods, 94, 57-63.

[57] Erecevit Sönmez, P., Kırbağ, S., İnci, Ş. (2019). Antifungal and antibacterial effect of dodder (*Cuscuta campestris*) used for hepatitis treatment of mothers and newborn infants in province Mardin in Turkey, Yuzuncu Yil University Journal of Agricultural Sciences, 29, 722-730.

[58] Ayala, A., Muñoz, M.F., Argüelles, S. (2014). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal, Oxidative Medicine and Cellular Longevity, 2014, 360438.

[59] Van Jaarsveld, P., Faber, M., Van Heerden, I., Wenhold, F., van Rensburg, W.J., Van Averbeke, W. (2014). Nutrient content of eight African leafy vegetables and their potential contribution to dietary reference intakes, Journal of Food Composition and Analysis, 33, 77-84.

[60] Dafam, D.G., Agunu, A., Dénou, A., Christopher, D., Kagaru, T.L.O., Ajima, U., Damos, J.N., Okwori, V.A. (2020) Determination of the ascorbic acid content, mineral and heavy metal levels of some common leafy vegetables of Jos Plateau State (North Central Nigeria), International Journal of Biosciences, 16, 389-396.

[61] Zuorro, A., Iannone, A., Lavecchia, R. (2019). Water–organic solvent extraction of phenolic antioxidants from brewers' spent grains, Processes, 7, 126.

[62] Felhi, S., Daoud, A., Hajlaoui, H., Mnafgui, K., Gharsallah, N., Kadri, A. (2017). Solvent extraction effects on phytochemical constituents profiles, antioxidant and antimicrobial activities and functional group analysis of *Ecballium elaterium* seeds and peels fruits, Food Science and Technology, 37, 483-492.

[63] Dar, P., Farman, M., Dar, A., Khan, Z., Munir, R., Rasheed, A., Waqas, U. (2017). Evaluation of antioxidant potential and comparative analysis of antimicrobial activity of various extracts of *Cucurbita pepo* L. leaves, Journal of Agricultural Science and Food Technology, 3,103-109.

[64] Fakoya, S., Aderoboye, O.Y., Olusola, S.E. (2019). Phytobiotics effects of pawpaw (*Carica papaya*) leaves and fluted pumpkin (*Telferia ocidentalis*) leaves extracts against certain aquatic pathogens, Medicinal and Aromatic Plants, 8, 1000328.

[65] Oboh, G., Nwanna, E., Elusiyan, C. (2006). Antioxidant and antimicrobial properties of *Telfairia occidentalis* (fluted pumpkin) leaf extracts, Journal of Pharmacology and Toxicology, 1, 167-175.

[66] Nwanna, E.E., Oboh, G. (2007). Antioxidant and hepatoprotective properties of polyphenol extracts from *Telfairia occidentalis* (fluted pumpkin) leaves on acetaminophen induced liver damage, Pakistan Journal of Biological Sciences, 10, 2682-2687.

[67] Yang, E.C., Hsieh, Y.Y., Chuang, L.Y. (2021). Comparison of the phytochemical composition and antibacterial activities of the various extracts from leaves and twigs of *Illicium verum*, Molecules, 26, 3909.

[68] Balouiri, M., Sadiki, M., Ibnsouda, S.K. (2016). Methods for *in vitro* evaluating antimicrobial activity: a review, Journal of Pharmaceutical Analysis, 6, 71-79.