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Profiling of Phenolic Compounds, Antioxidant and Cytotoxic Properties of Turkish Black Cumin Seeds

Running Title: Biological Properties of Turkish Black Cumin Seeds

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ABSTRACT

This work investigated the effects of extraction processes on phenolic compound extractions and evaluated the antioxidant and cytotoxicity properties of *Nigella sativa* seed. The most abundant phenolic compound, epigallocatechin gallate (EgCg), was identified in free flavan-3-ol extract (127.85±4.73 µg g^{-1} $_{DWE}$), which exhibited considerable ferrous-ion chelation and cytotoxicity on HepG2 cancer cells. Epigallocatechin (EgC) was second and recognized in the bound flavan-3-ol extract (113.31±3.49 µg g^{-1} $_{DWE}$), which showed the largest 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging activity and ferric-ion reducing power, as well as the most cytotoxic on HepG2 (IC_{50} = 24.91±1.45 µg mL^{-1}). Caffeic, sinapic, and *p*-hydroxybenzoic acids were found in bound phenolic acid from basic-hydrolysis extract (BPBH), which had the highest hydroxyl radical ($^{\bullet}$OH) and nitric oxide radical (NO$^{\bullet}$) scavenging activity and appreciable cytotoxicity on HepG2. Ferulic and *p*-coumaric acids were detected in acid-hydrolysable phenolic acid extract (AHPA) and bound phenolic acid from acid-hydrolysis extract (BPAH), respectively. The two extracts demonstrated higher ferric-ion reducing antioxidant power (FRAP) values and were also cytotoxic to HeLa and HepG2 cell lines. To the best of our knowledge, EgC, EgCg and rosmarinic acid were identified in *N. sativa* seed for the first time. Our study indicates *N. sativa* seeds as a promising source of phenolic compounds with antioxidant and anticancer properties.

Keywords: Antioxidant activity, Cytotoxicity, Free radicals, *Nigella sativa*, Phenolic compounds.

INTRODUCTION

The efficient approach to suppressing free radicals which cause oxidative stress and cancer is using antioxidants. Antioxidants, generally categorized into endogenous and exogenous, are compounds that inhibit oxidation, a chemical reaction that can produce free radicals that may damage living cells (Souri *et al.*, 2022). Polyphenols such as flavonoids (e.g., flavones,

flavanones and flavonols) and phenolic acids (e.g., hydroxybenzoic acid, hydroxycinnamic acid and hydroxyphenyl acetic acid derivatives) are the most widely known natural exogenous antioxidants from plant species (Panche *et al.*, 2016). There is rapidly escalating interest concerning exogenous natural antioxidants of plant origin, especially in food and pharmaceutical industries due to their favorable properties, for example environmentally friendly, low cost, and less toxic nature (Gao *et al.*, 2023). Because of their significant antioxidant and anticancer properties, and their noticeable impact on the prevention of numerous oxidative stress-related diseases, phenolic acids and flavonoids have become a key focus of medical-related studies (Alrashidi *et al.*, 2022).

Nigella sativa (English: Black cumin) belongs to the genus *Nigella*, which has around twenty-two different species found mostly in the Middle East, Northern Africa, Northern-Southern Europe and Western Asia countries. Among these species, thirteen were found in wild form and cultivated in several places in Türkiye (Dönmez *et al.*, 2010). Black cumin is a potential therapeutic plant, and its bioactive components such as thymoquinone, flavonoid, and phenolic acids, as well as the total phenolics, flavonoids, and tannin contents, antioxidant and anticancer activities of seed extracts, have been investigated (Ahirwar and Ahirwar, 2020; Alrashidi *et al.*, 2022; Balyan and Ali, 2022; Kadam and Lele, 2017; Shafi *et al.*, 2009; Shahbazi *et al.*, 2022). *N. sativa* exhibits not only antioxidant and anticancer activities but also demonstrates antidiabetic (Dalli *et al.*, 2021a; Dalli *et al.*, 2022), antimicrobial (Dalli *et al.*, 2021b), and anti-inflammatory properties (Dalli *et al.*, 2022). The aim of this study was to extract phenolic compounds from black cumin seeds using various extraction procedures, investigate the antioxidant activity of the extracts, and evaluate the cytotoxicity the extracts on HepG2 and HeLa cell lines.

MATERIALS AND METHODS

Chemicals and Cell Lines Collection

Acetonitrile, diethyl ether, ethanol, ethyl acetate, methanol, and n-hexane were obtained from Sigma-Aldrich (St. Louis, Missouri, United States) at analytical and/or HPLC grade. Phenolic standards including epigallocatechin, epigallocatechin gallate, hesperidin, quercitrin, benzoic, caffeic, ferulic, rosmarinic, sinapic, vanillic, *p*-hydroxybenzoic, and *p*-coumaric acids were also acquired from Sigma-Aldrich (St. Louis, Missouri, United States). The HepG2 (HB-8065™) and HeLa (CCL-2™) cell lines were sourced from the American Type Culture Collection and cultured in Dulbecco's modified eagle medium from Grand Island, New York, United States.

Plant Material and Preparation

Commercially cultivated black cumin seeds were purchased from Gökçehan Baharatları Izmir/Türkiye. The lyophilized seeds were ground and subjected to n-hexane extraction (4 hours) in a Soxhlet. Flavonoid and phenolic acid extracts were made from the defatted sample.

Extraction of Flavonoids

Flavone extraction was performed inside orbital shaker at 25 °C 20 min using diethyl ether solvent (Figure 1). The filtered residue was re-extracted with diethyl ether (75 mL) two times for 10 min (Valentão *et al.*, 1999). The flavanones were extracted with 80% ethanol (120 mL) at 90 °C 2 hours, followed by centrifugation (Pellati *et al.*, 2004). The flavonols were extracted under reflux for 2 h using 200 mL 95% aqueous methanol and 60 mL 25% HCl (Olszewska, 2008). The flavan-3-ols were extracted using two approaches: The free flavan-3-ols were extracted with absolute methanol in an ultrasonic machine at 60 °C 2 hours. The resulting residue underwent acidic hydrolysis with 2.5 M HCl-methanol (4:1) in a water bath at 100 °C 2 hours, followed by liquid-liquid-extraction (LLE) threefold with 40 mL diethyl ether and fourfold with 40 mL ethyl acetate to generates bound flavan-3-ol extract (de Villiers *et al.*, 2004). The extracts were concentrated using a Buchi R-100 rotary evaporator under reduced pressure, and subjected to freeze-drying and lyophilization.

Extraction of Phenolic Acid

The extraction of phenolic acid began with 120 mL of an 80% methanol solution on a magnetic stirrer to generate crude methanolic extract (CME). The CME subjected to centrifugation, evaporation, and lyophilization (Kim *et al.*, 2006). The lyophilized CME was dissolved in 12 mL of acidified water (pH 2.0 using HCl) and underwent three rounds of LLE with 30 mL diethyl ether to yield the free phenolic acids extract (FPA). The watery portion (pH 7.0) was lyophilized, dissolved in 12 mL 2 M NaOH and adjusted to pH 2.0, and LLE as previously described to obtain alkaline-hydrolysable phenolic acid extract (BHPA) (Figure 2). After BHPA extraction, 12 mL 6 M HCl was added to the aqueous layer and heated at 95 °C 20 min before LLE to get acid-hydrolysable phenolic acid extract (AHPA).

The dry residue produced after an initial 80% methanol extraction was divided into two portions. One portion was subjected to hydrolysis using 60 mL 6 M HCl at 95 °C 60 min, followed by centrifugation, filtration, and the resulting supernatant was designated as bound phenolic acid from acid-hydrolysis extract (BPAH). The other part was hydrolyzed using 60 mL 2 M NaOH at room temperature for 4 h, followed by centrifugation, filtration, and the

resulting supernatant was designated as bound phenolic acid from basic-hydrolysis extract (BPBH). The BPAH and BPBH extracts were adjusted (pH 2.0) and underwent threefold of LLE using 60 mL of diethyl ether.

RP-HPLC-DAD Analysis

The lyophilized phenolic extracts were dissolved in methanol (1000 µg mL^{-1}) for HPLC and antioxidant activity evaluations. RP-HPLC-DAD analysis was conducted using an Agilent 1100 series instrument, employing a C18 reverse-phase column with the following specifications: a length of 150 mm, an inner diameter of 4.6 mm, and a particle size of 5 µm. The chromatogram was detected using a G1315B DAD at 25 °C, with an injection volume of 20 µL. Flavone chromatograms were detected at 350 nm employing mobile phases consisting of 5% formic acid (A) and HPLC-grade methanol (B), with a flow rate of 1 mL min^{-1}, a 35-minute run time, and a gradient program: 0–5 min at 50% B, 5–30 min at 60% B, and 30–35 min at 80% B (Valentão *et al.*, 1999). Flavanones were identified at 285 nm using 0.6% acetic acid (A) and HPLC-grade methanol (B), with a 0.4 mL min^{-1} flow rate, a 30-minute run time, and a gradient program: 0–5 min 20% B, 5–12 min 40% B, 12–30 min 60% B (Pellati *et al.*, 2004). Flavonols were identified at 254 nm using 0.5% orthophosphoric acid (A) and HPLC-grade methanol (B), with a 1 mL min^{-1} flow rate, a 30-minute run time, and a gradient program: 0–10 min 40% B, 10–23 min 60% B and 23–30 min 40% B (Olszewska, 2008).

Flavan-3-ols were identified at 280 nm using 2% acetic acid (A) and 70% acetonitrile (B), a flow rate of 1.2 mL min^{-1} for 33 min, and a gradient of 0–8 min 5% B, 8–10 min 15% B, 10–12 min 20% B, 12–20 min 25% B, 20–30 min 40% B, 30–31 min 80% B and 31–33 min 5% B (de Villiers *et al.*, 2004). Phenolic acids were identified at 280 nm for benzoic acid derivatives and 320 nm for cinnamic acid derivatives using HPLC-grade acetonitrile (A) and a 2% acetic acid (B) mobile phase, with a 1 mL min^{-1} flow rate, a 60-minute run time, and a gradient program: 0–30 minutes at 100% B, 30–50 minutes at 85% B, 50–55 minutes at 50% B, and 55–60 minutes at 0% B (Kim *et al.*, 2006). Identification of flavonoids and phenolic acids involved comparing their retention times and spectral features with established standards. These standards were prepared in methanol, injected into an HPLC system, and linear calibration curves were obtained by plotting peak areas against concentrations (Table 1).

Antioxidant Activity Assays

The antioxidant activities of the flavonoid and phenolic acid extracts were evaluated according to the methods in the literature. The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging

activity (Brand-Williams *et al.*, 1995), hydroxyl radical ($^{\bullet}OH$) scavenging activity by the deoxyribose method (Halliwell *et al.*, 1987), nitric oxide radical ($NO^{\bullet}$) scavenging activity following Griess reagent method (Rao, 1997), chelation power on ferrous-ions following Dinis et al. method (Dinis *et al.*, 1994), ferric-ion reducing power by $Fe^{3+}(CN^{-})_6$ reducing at pH close to neutral (Oyaizu, 1986), and ferric reducing antioxidant power (FRAP) by reducing of Fe^{3+}-TPTZ complex in an acidic medium (Thaipong *et al.*, 2006) were investigated.

MTT Assay

The study employed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to assess the cytotoxicity of phenolic extracts on HeLa and HepG2 cancer cell lines (Mosmann, 1983). Cells were seeded at a density of 1.0×10^5 cells mL^{-1} per well, treated with extract concentrations ranging from 20 to 80 µg mL^{-1} in 10% dimethyl sulfoxide, and incubated for either 24- or 48-hour period at 37 °C in a 5% CO_2 incubator. Control cells were treated with 10% dimethyl sulfoxide alone. Following incubation, each well received the addition of 20 µL of a 5 mg mL^{-1} MTT solution, followed by a 3.5-hour incubation period. Formazan crystals were solubilized with 100 µL of dimethyl sulfoxide per well, and absorbance was measured at 570 nm using a microplate reader. IC_{50} values were determined by correlating the percentage of inhibition with the corresponding extract concentration.

Statistical Analysis

All data are shown as the mean ± SD of three replicates from the same extract. IBM SPSS Statistics 25 was used for one-way ANOVA, and Tukey-multiple Kramer's comparisons test was employed to evaluate significant differences ($p < 0.05$).

RESULTS AND DISCUSSION

Phenolic Acid Profile

This study identified benzoic, rosmarinic, vanillic, ferulic, *p*-coumaric, sinapic, caffeic, and *p*-hydroxybenzoic acids in Turkish black cumin seed extracts (Table 2). The *p*-hydroxybenzoic acid was the most prominent phenolic acid determined in this study having been determined in significant amount in FPA (15.69±1.37 µg g^{-1} $_{DWE}$), BHPA (12.69±0.87 µg g^{-1} $_{DWE}$) and BPBH (20.44±1.17 µg g^{-1}_{DWE}) extracts. The current study determined *p*-coumaric, *p*-hydroxybenzoic, caffeic and sinapic acids in two bound phenolic acid extracts, BPAH and BPBH. Hydrolysis

with acid or base is commonly used for the cleavage of the ester bond of phenolic conjugates and hence release phenolic compounds from plant materials (Kim *et al.*, 2006).

In comparison to the literature, *p*-coumaric acid (4.01±0.03 µg g^{-1} $_{DW}$) and ferulic acid (25.53±0.05 µg g^{-1} $_{DW}$) were reported in an acid-hydrolyzed extract of Romanian black cumin seeds (Toma *et al.*, 2015). Topcagic *et al.* (2017) determined ferulic and sinapic acids in free phenolic extract, NaOH containing 0.5% ascorbic acid hydrolyzed phenolic extract, and bound phenolics: acid hydrolyzed extract. Bourgou *et al.* (2012) determined *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids in black cumin seeds collected in northeastern Tunisia. This corresponds to our findings because our study identified ferulic acid in FPA, BHPA and AHPA extracts, sinapic acid in BHPA extract, and *p*-coumaric acid in acid-hydrolyzed fractions of BPAH. Higher phenolic content is linked to antioxidants and anticancer activities, as well as the nutritional quality of plant foods, enhancing the plant's food and nutritional industrial applications.

Flavonoids Profile

As can be seen in Table 3, hesperidin, quercitrin, EgC and EgCg were determined in significant amounts in flavanone, flavonol, free flavan-3-ol and bound flavan-3-ol extracts, respectively. Quercitrin (4.63±0.01 µg $g^{-1}$$_{DW}$) was reported in 70% ethanolic extract of black cumin seed (Toma *et al.*, 2015). Based on in-depth engagement with the available literature, this work is the first to identify EgC and EgCg in black cumin seed extract. Flavonoids can function as antioxidants against reactive oxygen species (ROS), and as potent pro-oxidants in cancer cells. Hesperidin, quercitrin, EgC and EgCg have been shown to exert a wide variety of anticancer effects through apoptosis induction against human cancer cell lines. The antioxidant properties of these flavonoids is a structure-dependent and mainly due to the position and the total number of OH and methoxy groups (Pandey *et al.*, 2019). Flavonoids are responsible for the color of fruits and flowers, as well as acting as antibacterial agents and UV filters. They also aid in heat acclimation, cold tolerance, and drought resilience (Panche *et al.*, 2016). Flavonoids are increasingly gaining popularity as natural alternatives to synthetic preservatives in food due to their aroma qualities.

Radical Scavenging Activity

Free radicals are beneficial to living cells as they are required for several biochemical processes (Gulcin and Alwasel, 2022). However, excess generation of them is detrimental to health. Considering this, this research examined DPPH, OH, and NO radicals scavenging

activities. It was observed that the free radical scavenging activities of the extracts increased as the concentration rose (as shown in Table 4). The IC_{50} value of bound flavan-3-ol extract was different from other extracts, and this extract exhibited the largest DPPH$^{\bullet}$ scavenging activity (IC_{50} = 82.51±1.18 µg mL^{-1}). The HPLC-DAD assay revealed a significant amount of EgC in the bound flavan-3-ol extract. Black cumin seeds showed higher DPPH$^{\bullet}$ scavenging activity than previous studies (Ahirwar and Ahirwar, 2020; Kadam and Lele, 2017; Toma *et al.*, 2015).

Hydroxyl radical, a highly reactive member of ROS, can attack and damage the target structure, for example, lipids, proteins, and DNA (Halliwell *et al.*, 1987). All the extracts exhibited strong $^{\bullet}$OH scavenging activity with IC_{50} in a narrow range spanning from 8.81±0.07 to 13.52±0.20 µg mL^{-1} (Table 4). The extracts with the highest scavenging activity i.e., BPBH, flavanone and flavonol extracts showed no significant differences ($p > 0.05$) between their IC_{50} values. Black cumin seed was reported to scavenge $^{\bullet}$OH in the ranges of 200 to 1000 µg mL^{-1} (Balyan and Ali, 2022). HPLC analysis on BPBH fraction, extract with the largest $^{\bullet}$OH scavenging activity, revealed a significant amount of *p*-hydroxybenzoic, caffeic and sinapic acids. The chemical structure of these phenolic acids is believed to strongly account for their antioxidant activities that, in turn, link to certain anti-carcinogenic properties (Espíndola *et al.*, 2019).

The Griess test was utilized to determine the NO$^{\bullet}$ scavenging activity. BPBH extract showed the largest potent activity against NO$^{\bullet}$ (IC_{50} = 24.14±0.15 µg mL^{-1}) (Table 4). NO$^{\bullet}$ is a key cell signaling and regulatory molecule but at high concentrations, it can cause nitrosative stress and DNA lesion (Behl *et al.*, 2023). Phenolic extracts of black cumin seed showed a significant NO$^{\bullet}$ scavenging effect, therefore, the seed may be used to control the destructive effects and assist in interrupting the chain of chemical reactions started by the overproduction of NO radicals in the viable cells.

Ferrous Ion Chelating Property

Metal ions, for example, Fe, Cu and Zn, are essential for the continued execution of critical functions in living organisms, but high-level of them can lead to metal poisoning and ROS generation which can lead to oxidative stress that will subsequently cause oxidative damage to lipids, proteins and DNA (Gulcin and Alwasel, 2022). Fe^{2+} reacts with H_2O_2 to generate $^{\bullet}$OH via the Fenton reaction. Fe^{2+} chelation is crucial in avoiding ROS generation in living cells. Chelating agents can bind to toxic metal ions, forming intricate complexes that facilitate their easy excretion from the body, thereby eliminating them from both intracellular and extracellular spaces (Flora and Pachauri, 2010). Among all the extracts, flavone extract (IC_{50} = 43.99±1.29

μg mL^{-1}) was the best Fe^{2+} chelator (Table 4). Interestingly, no flavone was identified in the current study. The chromatogram peak observed in flavone extract could be for another compound that is not within the scope of the present study. Apigenin, chrysoeriol, diosmin, eupatorin and luteolin were the only flavone family utilized in this work.

Fe^{3+} Reducing Power Property

Ferric-ion reducing power assay depends on the reduction of potassium ferricyanide to potassium ferrocyanide by the sample and demonstrates the ability of an antioxidant, that may be found in the sample, to give up electrons at a pH close to neutral (de Melo *et al.*, 2022). All the extracts accomplished significant Fe^{3+}-reducing ability (Table 5). The high reduction potential shown by the bound flavan-3-ol fraction may be related to the EgC identified in the extract. The reduced Fe^{2+} could easily be captured through Fe^{2+} chelation. The reducing ability of black cumin seed extracts signifies it potential antioxidant property.

Fe^{3+}-TPTZ Reducing Property

The purpose of this assay was to examine the capacity of different extracts of black cumin seed to reduce the $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ complex to the $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ complex at lower pH (Benzie and Strain, 1999). All the extracts reduced $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ complex to $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ complex significantly (Table 5). The FRAP value of the extracts ranges from 30.77±0.76 to 210.48±1.50 μg vitamin-c-equivalent g^{-1} $_{DWE}$. The antioxidant molecules in two extracts with the greatest FRAP value were *p*-coumaric acid in BPAH and ferulic acid in AHPA. FRAP value of 1.85±0.2 mM Trolox equivalent was reported in black cumin seed (Kadam and Lele, 2017). Reducing compounds have the property to act as antioxidants that would scavenge ROS and prevent oxidative stress (Gulcin and Alwasel, 2022).

Cytotoxic Activity on HeLa and HepG2 Cancer Cells

Increasing concentrations of phenolic extracts induced increasing cytotoxic effects. Four phenolic extracts, namely free flavan-3-ol, FPA, AHPA and BPAH, exhibited strong cytotoxicity activity against HepG2 cell lines at 24 h after incubation (Figure 3). The effects may be attributed to phenolic compounds in the extracts. Moreover, treatment of HepG2 cell lines for up to 48 h (Figure 3) shows all the extracts to have strong cytotoxic effects (IC_{50} < 100 μg mL^{-1}). To be precise, the cytotoxic effects of bound flavan-3-ol, BPAH, FPA, flavonol and BPBH phenolic extracts were remarkable. Anticancer compounds EgC in bound flavan-3-ol, benzoic and *p*-hydroxybenzoic acids in FPA, quercitrin in flavonol, and sinapic and caffeic acids in BPBH were identified in the extracts. Supplementation of rats with caffeic acid was

shown to inhibit the growth of HepG2 cell lines. The anticancer activity of caffeic acid is associated with its pro-oxidant capacity through its ability to chelate metals and thus cause damage to the DNA of cancer cells by oxidation (Espíndola *et al.*, 2019). According to the established criterion by the American National Cancer Institute, the *in vitro* cytotoxicity activity assessment for the crude extracts of plant materials should have an IC_{50} of lower than 30 µg mL^{-1} (Suffness, 1990). The bound flavan-3-ol extract showed activity lower than this established limit (IC_{50} = 24.91±1.45 µg mL^{-1}), and BPAH extract has also been found to exhibit a strong IC_{50} value very close to the established limit (31.25±1.52 µg mL^{-1}).

AHPA extract (IC_{50} = 59.72±2.89 µg mL^{-1}) and BHPA extract (IC_{50} = 71.47±3.51 µg mL^{-1}) exhibited strong *in vitro* cytotoxic activity against HeLa cell lines at 24 h after incubation (Figure 4). The high cytotoxic efficiency observed in these extracts could be related to the phenolic compounds identified. For the 48-hour incubation period, bound flavan-3-ol, FPA and BHPA extracts showed a significant cytotoxicity effect on HeLa cell lines (IC_{50} < 100 µg mL^{-1}). EgC in bound flavan-3-ol extract, benzoic and *p*-hydroxybenzoic in FPA extract were determined in significant concentrations. These phenolic compounds have been shown to inhibit the growth of cancer cells (Anantharaju *et al.*, 2017). Elkady (2012) determined the IC_{50} values for aqueous and ethanolic extracts of Egyptian black cumin seeds as 75 and 100 µg mL^{-1} for 48 and 24 h after treatment, respectively. In another study, the IC_{50} values of methanolic, n-hexane and chloroform extracts obtained through Soxhlet extraction from the black cumin seed were 2.28 µg mL^{-1}, 2.20 µg mL^{-1} and 0.41 ng mL^{-1}, respectively (Shafi *et al.*, 2009). However, it should be noted that the authors prepared the extracts with 100% DMSO, while our study used only 10% DMSO. In a high concentration, DMSO is known to be toxic to cells. In one study the DMSO had found to have significant toxicity and inhibition of proliferation in four human cancer cell lines (Nguyen *et al.*, 2020).

CONCLUSIONS

In this study, four benzoic acid derivatives, four cinnamic acid derivatives, and four flavonoids were successfully determined in the commercially cultivated Turkish black cumin seed extracts obtained after different extraction procedures. The phenolic acids were determined more in extractable phenolic extracts than in the bound phenolic extracts. The three principal phenolic compounds detected were EgCg, EgC and *p*-hydroxybenzoic acid, which were found in free flavan-3-ol, bound flavan-3-ol, and BPBH extracts, respectively. The flavonoid and phenolic acid extracts exhibited significant DPPH, OH and NO radicals scavenging activity, ferrous-ion chelation, ferric-ion reducing power and FRAP. Additionally, the cytotoxic effect of extracts

was more on HepG2 than against HeLa with bound flavan-3-ol and BPAH extracts exhibiting impressive activity. Based on the data obtained in this study, *N. sativa* seed offers promising antioxidant and anticancer properties and these biological properties may be related to the flavonoid and phenolic acids identified.

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Table 1. Phenolics standards

Phenolic Compounds	λ (nm)	Linear equation	R^2
Hesperidin	285	y = 143.06x	0.9968
Quercitrin	254	y = 37.91x	0.998
Epigallocatechin	280	y = 2.2169x	0.9982
Epigallocatechin gallate	280	y = 18.728x	0.9992
Benzoic acid	280	y = 10.661x	0.9937
p-hydroxybenzoic acid	280	y = 36.063x	0.9964
Rosmarinic acid	280	y = 22.499x	0.9894
Vanillic acid	280	y = 43.058x	0.9999
Caffeic acid	320	y = 126.91x	0.9981
Ferulic acid	320	y = 127.37x	0.9914
Sinapic acid	320	y = 64.552x	0.9807
p-coumaric acid	320	y = 110.3x	0.9747

Table 2. Phenolic acids in the extracts (µg per gram dry weight of extract).

Phenolic acids	Extractable phenolic acid extracts			Bound phenolic acid extracts	
	FPA	BHPA	AHPA	BPAH	BPBH
Hydroxybenzoic acid derivatives (280 nm)					
Benzoic	3.42±0.05	[a]nd	nd	nd	nd
p-hydroxybenzoic	15.69±1.37	12.69±0.87	nd	nd	20.44±1.17
Rosmarinic	nd	2.83±0.05	nd	nd	nd
Vanillic	0.95±0.01	2.85±0.13	nd	nd	nd
Hydroxycinnamic acid derivatives (320 nm)					
Caffeic	nd	nd	nd	nd	2.45±0.14
Ferulic	0.78±0.01	3.19±0.22	1.71±0.02	nd	nd
Sinapic	nd	1.03±0.05	nd	nd	3.20±0.28
p-coumaric	nd	nd	nd	7.59±0.53	nd

[a]nd: not detected/trace.

Table 3. Flavonoids in the extracts (µg g^{-1} $_{DWE}$).

Extracts	Compound	Amount
Flavone	[a]nd	
Flavanone	Hesperidin	2.92±0.14
Flavonol	Quercitrin	1.10±0.10
Free flavan-3-ol	EgCg	127.85±4.73
Bound flavan-3-ol	EgC	113.31±3.49

[a]nd: not detected/trace.

Table 4. Antioxidant activity by DPPH, OH, and NO radicals scavenging, and ferrous ion chelating property.

Extracts	DPPH• scavenging	•OH scavenging	NO• scavenging	Fe^{2+} chelation
	IC_{50} (µg mL^{-1})			
Flavone	144.27±4.08[a]	10.14±0.36[a]	60.29±0.72[a]	43.99±1.29[a]
Flavanone	111.28±1.26[b]	9.07±0.14[b]	32.06±0.56[b]	49.79±1.23[b]
Flavonol	117.36±1.25[b]	9.59±0.12[b]	45.56±0.56[c]	110.21±1.06[c]
Free flavan-3-ol	114.57±1.38[b]	10.93±0.19[d]	35.32±0.51[b]	46.80±0.57[b]
Bound flavan-3-ol	82.51±1.18[e]	11.22±0.17[d]	34.31±0.43[b]	72.19±1.29[e]
FPA	145.21±1.81[a]	11.08±0.13[d]	64.57±1.56[f]	47.72±0.69[b]
BHPA	150.59±1.66[a]	11.56±0.15[d]	80.40±1.21[g]	47.53±0.49[b]
AHPA	100.02±1.74[h]	13.52±0.20[h]	69.27±1.25[h]	54.61±0.73[h]
BPAH	98.14±1.27[h]	13.44±0.16[h]	46.68±0.56[c]	57.03±1.00[h]
BPBH	127.94±1.18[k]	8.81±0.07[b]	24.14±0.15[k]	46.80±0.70[b]

Data followed by a different superscript letter in the same column represent a significant difference at $p < 0.05$.

Table 5. Antioxidant activity by Fe^{3+} reducing and Fe^{3+}-TPTZ reducing.

Extracts	Fe^{3+} reducing power	Fe^{3+}-TPTZ reducing power
	µg vitamin-c-equivalent g^{-1} $_{DWE}$	
Flavone	52.90±0.43[a]	30.77±0.76[a]
Flavanone	95.79±1.33[b]	117.83±1.36[b]
Flavonol	86.99±1.44[c]	47.42±1.11[c]
Free flavan-3-ol	83.78±1.31[d]	111.07±1.59[d]
Bound flavan-3-ol	230.05±2.63[e]	175.15±2.14[e]
FPA	92.20±1.30[b]	64.00±1.29[f]
BHPA	88.60±1.41[c]	74.90±1.35[g]
AHPA	111.49±1.30[h]	200.39±1.64[h]
BPAH	173.59±1.45[i]	210.48±1.50[i]
BPBH	123.49±1.37[l]	114.08±0.95[d]

Data followed by a different superscript letter in the same column represent a significant difference at $p < 0.05$.

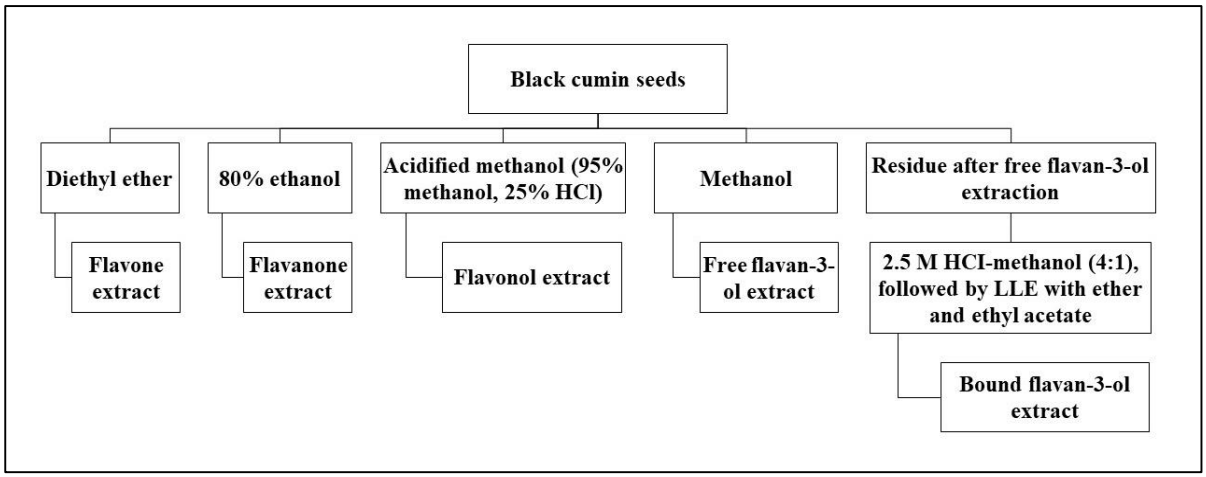

Figure 1. Flow diagram illustrating the extraction procedures for flavonoids.

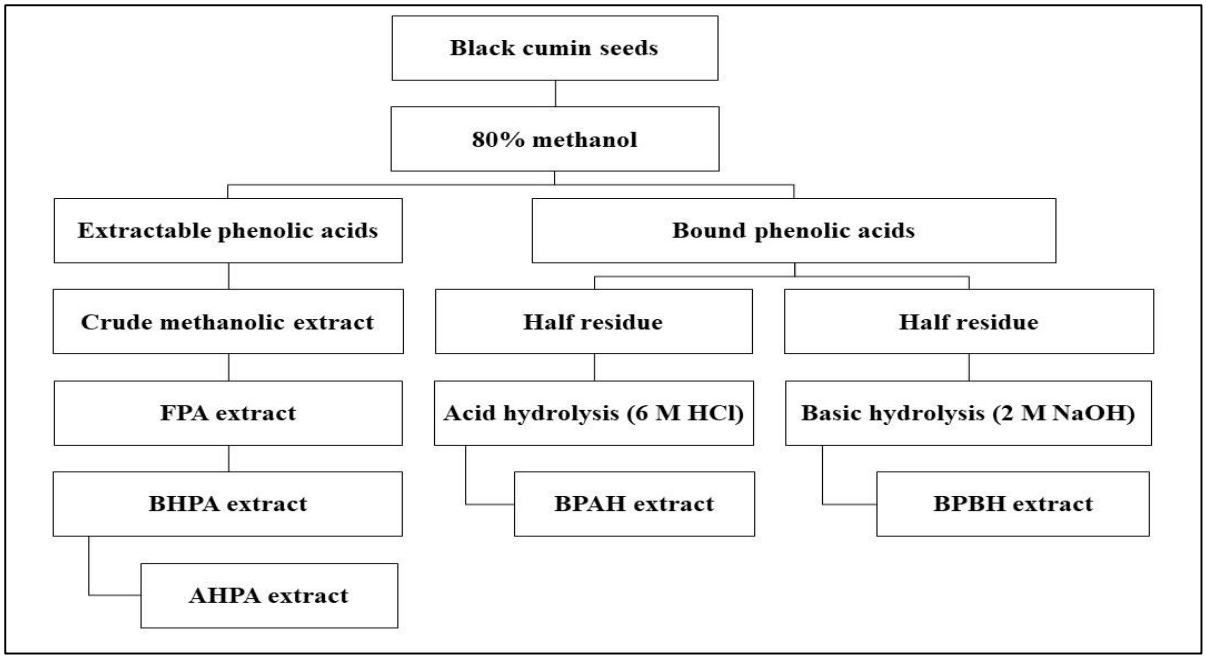

Figure 2. Flow diagram illustrating the extraction procedures for phenolic acids.

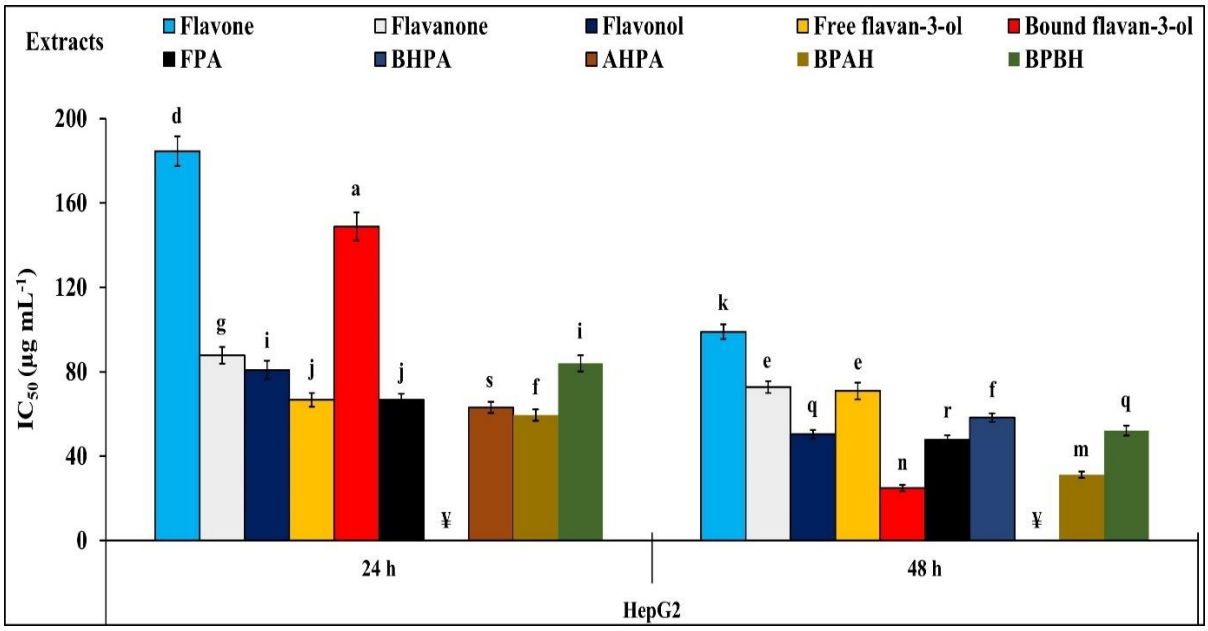

Figure 3. Cytotoxic effects of extracts on HepG2 cell lines. Bars labeled with dissimilar superscript letters are deemed statistically significant ($p < 0.05$). IC_{50} values equal to or exceeding 200 µg mL^{-1} are excluded and denoted as "¥."

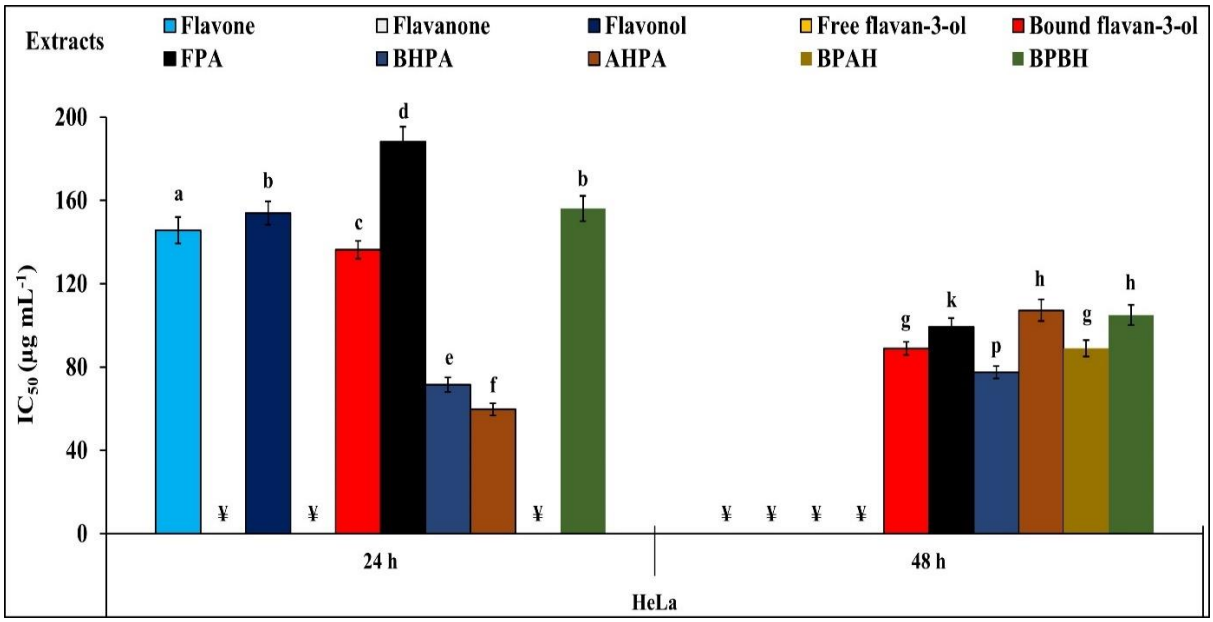

Figure 4. Cytotoxic effects of extracts on HeLa cell lines. Bars labeled with dissimilar superscript letters are deemed statistically significant ($p < 0.05$). IC_{50} values equal to or exceeding 200 µg mL^{-1} are excluded and denoted as "¥."