Evaluation of phenolic profile, enzyme inhibitory and antimicrobial activities of *Nigella sativa* L. seed extracts

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ABSTRACT

Black cumin (*Nigella sativa* L. [*N.sativa*]) seed extracts demonstrated numerous beneficial biological effects including, among others, antidiabetic, anticancer, immunomodulatory, antimicrobial, anti-inflammatory, antihypertensive, and antioxidant activity. To better understand the phytochemical composition of *N. sativa* seeds, methanol seed extracts were analyzed for phenolic acid and flavonoid content. Furthermore, we tested *N. sativa* methanol, *n*-hexane, and aqueous seed extracts for their inhibitory activity against butyrylcholinesterase (BChE) and catalase (CAT) as well as for antimicrobial activity against several bacterial and a yeast strains. The phenolic content of *N. sativa* was analyzed using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). The inhibition of BChE was assessed by modified Ellman's method, and the inhibition of CAT was determined by monitoring hydrogen peroxide consumption. The extracts were tested against *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella enterica*, and *Escherichia coli* using the agar diffusion method. The UHPLC-MS/MS method allowed the identification and quantification of 23 phenolic compounds within 15 minutes. The major components found in *N. sativa* seed extract were sinapinic acid (7.22 ± 0.73 μg/mg) as a phenolic acid and kaempferol (11.74 ± 0.92 μg/mg) as a flavonoid. All extracts showed inhibitory activity against BChE, with methanol seed extract demonstrating the highest inhibitory activity (inhibitory concentration 50% [IC_{50}] 79.11 ± 6.06 μg/ml). The methanol seed extract also showed strong inhibitory activity against CAT with an IC_{50} value of 6.61 ± 0.27 μg/ml. Finally, the methanol extract exhibited considerable inhibitory activity against the tested microbial strains. Overall, this is the first study to investigate the ability of black cumin seed extracts to inhibit CAT. Our results indicate that *N. sativa* seed can be considered as an effective inhibitor of CAT activity.

KEY WORDS: *Nigella sativa*; UHPLC/MS-MS; phenolic compounds; butyrylcholinesterase inhibition; catalase inhibition; antimicrobial potential; black cumin

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INTRODUCTION

Plants are natural producers of chemical compounds, many of which are used to promote health and fight diseases, and are marketed as food or herbal medicines [1]. Bioactive compounds commonly found in plants have been shown to have health benefits mainly because they inhibit oxidative damage and may consequently prevent inflammatory processes [2], aging, and development of neurodegenerative diseases [3]. Among various medicinal plants, *Nigella sativa* L. (*N. sativa*; family Ranunculaceae) has been historically known as a miracle herb. Recent studies revealed a wide spectrum of pharmacologically active components in *N. sativa*. It is an annual plant, commonly known as black cumin, and is native to the Middle East, North Africa, and Western Asia, but also cultivated in many countries worldwide. The seeds are used as a spice for food, especially bakery products and cheese, and in the preparation of traditional sweet dishes. Black cumin has long been used as a natural medicine for the treatment of many acute, as well as chronic conditions, acting as diuretic, diaphoretic, stomachic, and liver tonic [4]. *N. sativa*

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seed extract has several beneficial biological effects including antidiabetic, anticancer, immunomodulatory, analgesic, antimicrobial, anti-inflammatory, spasmolytic, bronchodilatory, hepatoprotective, antihypertensive, renal protective, and antioxidant [5,6]. Due to its wide range of medical uses, the plant has undergone extensive phytochemical studies, and a number of different compounds have been isolated. The seeds of black cumin contain a yellowish fixed oil, proteins, amino acids, reducing sugars, alkaloids, organic acids, tannins, fats, crude fibers, minerals, and vitamins [7-9]. Among the most important active compounds are thymoquinone, thymohydroquinone, dithymoquinone, *p*-cymene, and *trans*-anethol [10,11]. Studies have shown that the biological activity of *N. sativa* seeds is mainly attributed to its essential oil component, thymoquinone [12]. A phytochemical analysis indicated that polyphenols can be found in different parts of *N. sativa*. Phenolic acids were found in the highest concentration, and vanillic acid represented the major compound in the mixture of phenolic acids [13].

The aim of this study was to identify and quantify other phenolics in *N. sativa* methanol seed extracts by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Furthermore, we tested *N. sativa* methanol, n-hexane, and aqueous seed extracts for their inhibitory activity against butyrylcholinesterase (BChE) and catalase (CAT) as well as for antimicrobial activity against several bacterial and a yeast strains.

MATERIALS AND METHODS

Plant materials

N. sativa seed was purchased from a herbal store in Sarajevo, Bosnia and Herzegovina and identified by a plant expert (S.Đ.) and pharmacognosist (K.D.). A description of black cumin seed includes oblong and ovoid, dull black seed with 3-4 edges that are 2-3.5 mm long. The reticular surface is finely granular. The odor is strongly aromatic when the seed is crushed, resembling the odor of anise or nutmeg. The taste is slightly bitter at first, then spicy and somewhat pungent. The macroscopic and microscopic authentication was performed according to the Ayurvedic Pharmacopoeia of India. Due to the characteristic morphology, odor and taste of the drug, a microscopic examination was considered unnecessary. On the other hand, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are important in the identification of pharmacologically active quinines and other substances in the black cumin seed extract or seed oil [14]. A voucher specimen (Sofic 004/2017) was deposited at the herbarium of the Department of Biology, Faculty of Science, University of Sarajevo, Bosnia and Herzegovina. The seeds were grounded into fine powder and used for analysis.

Chemicals

Acetonitrile, apigenin, butyrylthiocholine iodide (minimum 99%), caffeic acid, CAT from bovine liver (EC 1.11.1.6), chlorogenic acid, chrysin, 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB, minimum 99%) ferulic acid, formic acid, galangin, gallic acid monohydrate, hydrogen peroxide (30%), 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 5-hydroxyferulic acid, kaempferol, myricetin, naringenin, *p*-coumaric acid, pinocembrin, protocatechuic acid, quercetin dihydrate, rosmarinic acid, salicylic acid, sinapinic acid, syringic acid, *trans*-cinnamic acid, and vanillic acid were of the highest purity available and purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Ascorbic acid and diethyl ether were obtained from Merck KgaA (Darmstadt, Germany). BChE from equine serum (EC 3.1.1.8) was purchased from Biochemica (Billingham, UK), hydrochloric acid, methanol (minimum 99.5%, pro analysis [p.a.]), and sodium hydroxide (minimum 99%) was obtained from Semikem (Sarajevo, Bosnia and Herzegovina), while sodium dihydrogen phosphate and sodium hydrogen phosphate were from Carlo Erba (Milano, Italy). *Bacillus subtilis* ATCC® 6633™, *Staphylococcus aureus* ATCC® 33591™, *Salmonella enterica* subsp. *enterica serovar abony* NCTC® 6017™, *Escherichia coli* ATCC® 8739™, and *Candida albicans* ATCC® 10231™ were purchased from MicroBioLogics (St. Cloud, Minnesota, USA). Ampicillin, gentamicin, nystatin, and oxacillin, as well as Mueller-Hinton agar and Sabouraud dextrose broth were purchased from HiMedia Laboratories (Mumbai, India).

UHPLC-MS/MS analysis

UHPLC-MS/MS instrumentation and analytical conditions

UHPLC-MS/MS analyses were carried out using Shimadzu Ultra Performance LCMS 8050 system (Japan) with a triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) source operating in negative mode. Lab Solutions software version 5.2 (Shimadzu Corporation, Japan) was used for the instrument control, data acquisition as well as processing. The sample solutions were injected into a reversed phase column (BEH C8, 1.7 μm, 2.1 mm × 150 mm, Waters, Milford, MA, USA) with appropriate pre-columns. The column was maintained at 40°C. The mobile phase consisted of the mixture of aqueous solutions of 10 mM formic acid (Solvent A) and acetonitrile (Solvent B) at a flow rate of 0.25 ml/minutes. The linear gradient and isocratic flows of the mobile phase were slightly modified according to the method of Gruz et al. [15], i.e. 5% B for 0.8 minutes, 5-10% B for next 0.4 minutes, isocratic 10% B for 0.70 minutes, 10-15% B for next 0.5 minutes, isocratic 15% B for 1.30 minutes, 15-21% B for 1.30 minutes, isocratic 21% B for 1.20 minutes, 21-27% B for next 0.50 minutes, then 27-50% B for 3.30 minutes, 50-100% B

for 2.00 minutes, isocratic 100% B for 1.00 minutes, and 100-5% B over 5 minutes. At the end of the program, the column was equilibrated under the initial conditions for 2.70 minutes. The pressure ranged from 45 to 50 MPa during the chromatographic run. The effluent was introduced into an electrospray source (interface temperature 300°C, heat block temperature 400°C, and capillary voltage 3.0 kV). Argon was used as collision gas and nitrogen as nebulizing gas. The interface between the LC and the MS detector, in this case, was carried out with ESI. After precursor ion full scan in the negative-ion mode (i.e. $[M-H]^-$), the product ions were determined using MS/MS. To achieve a high specificity in addition to the high sensitivity, we utilized an analysis in multiple reaction monitoring (MRM) mode.

Preparation of calibration standards for UHPLC-MS/MS analysis

Standard solutions of 23 target compounds were first prepared in methanol at 1 mM concentrations, and solutions were gradually diluted in the mobile phase to the working concentrations that ranged from 0.01 to 50 μM.

Each solution contained 15 phenolic acids, (caffeic, chlorogenic, *trans*-cinnamic, ferulic, gallic, 3-hydroxybenzoic, 4-hydroxybenzoic, 5-hydroxyferulic, *p*-coumaric, protocatechuic, rosmarinic, salicylic, sinapinic, syringic, *trans*-cinnamic, and vanillic acid) and 8 flavonoids (apigenin, chrysin, galangin, kaempferol, myricetin, naringenin, pinocembrin, and quercetin).

Sample preparation for UHPLC/MS-MS analysis

The analysis of phenolic compounds by UHPLC-MS/MS method was done with extracts prepared from plant material according to slightly modified methods [16,17]. Plant material (100 mg) was homogenized and sonificated for 15 minutes with 800 μl of 80% methanol (Figure 1). After centrifugation for 10 minutes at 17,000 g and collection of the supernatant, the extraction was repeated with the new amount of 80% methanol. The supernatants were collected and evaporated to the dryness, while the residue was kept for isolation of non-soluble phenolics (NS-P4). The volume of 500 μl of 0.1 M HCl was added to the supernatants, and free phenolics (NS-P1) were extracted using 2 × 1 ml of diethyl ether, then the ether was evaporated to the dryness, and the dry samples were stored at 4°C until analysis. The volume of 1 ml of 1 M NaOH containing 0.5% ascorbic acid was added into the remaining acidic layer, and the reaction mixture was incubated for 3 hours at 25°C. Next, a new amount of 200 μl of concentrated hydrochloric acid was added, phenolic acids hydrolyzed from corresponding esters were extracted using 2 × 1 ml of diethyl ether, and the ether was evaporated to the dryness. Solid samples were stored at 4°C until analysis (NS-P2). Phenolic glycosides (NS-P3) were hydrolyzed from the remaining acidic layer for 1 hour at 50°C. The hydrolyzed phenolics were extracted using 2 × 1 ml of diethyl ether, the ether was evaporated to the dryness, and the samples were stored at 4°C until analysis. The volume of 1 ml of 1 M NaOH was added into the residue from the initial methanol extraction, and the reaction mixture was incubated for 3 hours at 25°C. The volume of 200 μl of concentrated hydrochloric acid was added, and the non-soluble fraction of phenolics was extracted using 2 × 1 ml of diethyl ether, next the ether was evaporated to the dryness and the obtained dry samples were stored at 4°C until analysis (NS-P4).

All samples were dissolved into the mobile phase (5% acetonitrile in 10 mM formic acid solution), sonicated for 15 minutes, and centrifuged for 10 minutes at 17,000 g prior to the analysis. Using this isolation method, it is possible to separate plant phenolics into four classes, i.e. free, esters, glycosides, and phenolics non-soluble in 80% MeOH, which was used as the extraction solvent. As the result, all phenolic compounds are present in free form, which is the most convenient form for their reversed-phase chromatographic separation, due to the solubility in the mobile phase and faster separation through the column [15,17].

Analysis of *N. sativa* seed extracts bioactivity

To evaluate the bioactivity of *N. sativa* seed extracts, the inhibition of BChE and CAT was assayed, as well as the antimicrobial activity of the seed extracts.

Sample preparation

The compounds were extracted from the seeds (15 g) with the following three methods: 1) extraction under reflux for 1 hour with methanol (NS-RM) and in an aqueous solution (NS-RW); 2) extraction with methanol (NS-SM) and *n*-hexane (NS-SH) for 4 hours in a Soxhlet apparatus; and 3) extraction in an ultrasonic bath for 2 hours at room temperature with methanol (NS-UM) and *n*-hexane (NS-UH). The solvents (methanol and *n*-hexane) were evaporated at low temperature under reduced pressure in a rotary evaporator. These extracts were used to determine the inhibitory activity of black cumin seed against BChE and CAT, as well as its antimicrobial activity.

Inhibitory activity against BChE

The inhibition of BChE activity was determined by a slightly modified Ellman's method [18]. In this method, thiocholine that is produced by BChE (EC 3.1.1.8) from butyrylthiocholine iodide reacts with Ellman's reagent, DTNB, to produce a yellow thio-nitrobenzoate anion. The progress of the hydrolysis was measured spectrophotometrically at 412 nm using a spectrophotometer Multiskan GO (Thermo Fisher Scientific, Waltham, MA, USA). The stock solutions of BChE

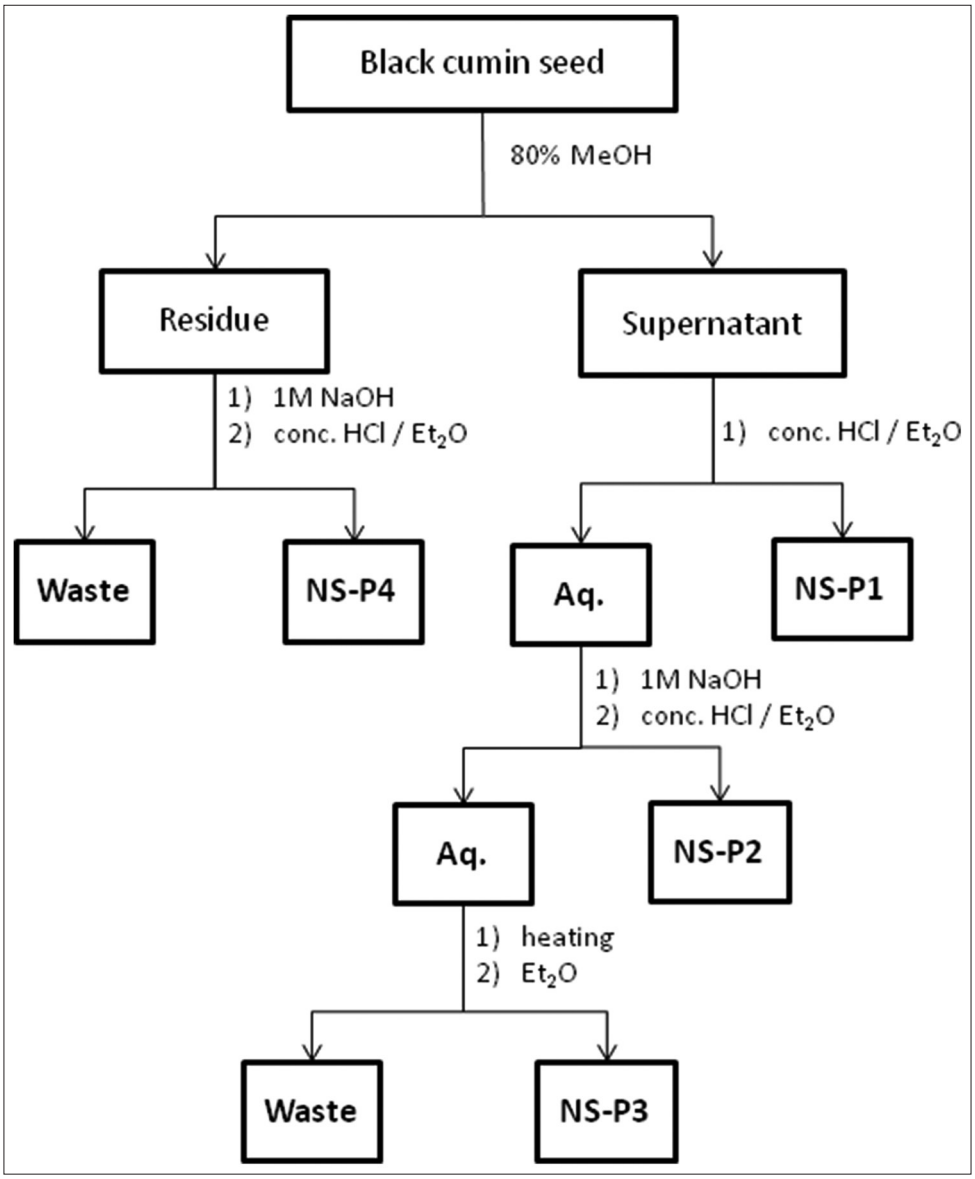

FIGURE 1. Preparation of *Nigella sativa* seed extracts for ultra-high performance liquid chromatography tandem mass spectrometry method. Using this isolation method, it is possible to separate plant phenolics into four classes, i.e., free phenolics (NS-P1), esters (NS-P2), phenolic glycosides (NS-P3), and phenolics non-soluble in 80% MeOH (NS-P4), which was used as the extraction solvent. All phenolic compounds are present in free form, which is the most convenient form for their reversed-phase chromatographic separation, due to the solubility in the mobile phase and faster separation through the column.

and butyrylthiocholine iodide were prepared in phosphate buffer (pH 8.0); Ellman's reagent was prepared in 0.1 M phosphate buffer (pH 7.0). In brief, a pre-incubation volume of 280 µl 0.1 M phosphate buffer (pH 8.0) included various concentrations of the test sample (50 µl), 80 µl of DTNB (6.6 mM), and 25 µl of BChE (0.66 U/ml). The mixture was incubated for 5 minutes at 25°C with continuous shaking. Following the pre-incubation, 25 µl of the substrate (15.4 mM) was added. The color was measured in a microplate reader. All reactions were performed in triplicate. The inhibitory activity against BChE and inhibition percentage of the test samples were determined. The percent inhibition (I) was calculated using the formula:

$$I(\%)=\frac{A_{control}-A_{sample}}{A_{control}}\times 100$$

The inhibitory concentration 50% (IC_{50}) was determined by log-probit analysis.

Inhibitory activity against CAT

The inhibition of CAT activity (EC 1.11.1.6) was determined by measuring hydrogen peroxide consumption as a decrease in absorbance at 240 nm, according to the method of Aebi [19]. The reaction mixture contained 1 ml of 20 mM H_2O_2, 0.9 ml phosphate buffer (pH 7.0), 100 µl of test samples of different concentration, and 50 µl of CAT from bovine liver. The samples were incubated for 2 minutes at room temperature, and the absorbance of the sample was spectrophotometrically monitored for 60 seconds at 240 nm using a Lambda 25 UV/VIS spectrophotometer (PerkinElmer, Shelton, CT, USA). Changes in the absorbance were considered to be

proportional to the breakdown of H_2O_2. The inhibitory activities against CAT and inhibition percentage of the test samples were determined. The percent inhibition (I) was calculated using the formula:

$$I(\%)=\frac{A_{control}-A_{sample}}{A_{control}}\times 100$$

The IC_{50} value was determined by log-probit analysis.

Antimicrobial activity

The antimicrobial activity of black cumin seed was tested using the disc diffusion test, with two Gram-positive (*B. subtilis* ATCC® 6633™ and *S. aureus* ATCC® 33591™), two Gram-negative bacteria (*S. enterica* subsp. *enterica serovar abony* NCTC® 6017™ and *E. coli* ATCC® 8739™), and one fungal strain (*C. albicans* ATCC® 10231™) [20]. The bacterial strains were cultured in Mueller-Hinton broth, while the fungal strain was cultured in Sabouraud broth. The broth media was incubated for 24 hours at 37°C. Sterile filter paper discs, 5 mm in diameter, were saturated with 25 µl of the extracts and placed on the inoculated plates; these plates were then incubated at 37°C for 24 hours. The diameter of the inhibition zones (in mm) was considered as a measure of the antimicrobial activity. Oxacillin, gentamicin, ampicillin, and nystatin were used as positive reference standards to determine the sensitivity of the strains and isolate. A disc with dimethyl sulfoxide (DMSO) was used as a negative control.

RESULTS

Yields of *N. sativa* seed extracts

The yields of extracts obtained with the three different extraction methods are shown in Table 1. In general, the yields of total extractable compounds decreased with increasing polarity of the solvent in the order of *n*-hexane, methanol, and aqueous solution. The extraction from seeds using Soxhlet method gave the highest yield, followed by 18.73% yield with *n*-hexane and 18.32% yield with methanol. This may be explained by the high ability of *n*-hexane to extract some of the lipid components in seeds as well as by the longer extraction period (i.e. 4 hours) when compared to the other extraction methods. The small yield of extracts obtained using the ultrasound bath, compared with the relevant literature, is probably due to the difference in temperature used for the extraction [21].

TABLE 1. Yields of *N. sativa* seed extracts with different extraction methods

Extraction methods	Extraction period (minutes)	Solvent	Sample	Yield (%)
Reflux	60	Water	NS-RW	4.73
		Methanol	NS-RM	5.28
Soxhlet	240	*n*-Hexane	NS-SH	18.73
		Methanol	NS-SM	18.32
Ultrasonic	120	n-Hexane	NS-UH	2.22
		Methanol	NS-UM	2.42

NS: Non-soluble; *N. sativa*: *Nigella sativa*

Identification of phenolic compounds using UHPLC-MS/MS

Fifteen phenolic acid and eight flavonoid standards were used for the identification and quantification of phenolic compounds. The information on standards is given in Table 2. Seven phenolic acids and flavonoids were determined in four different fractions. The results showed that all four fractions contained ferulic acid, sinapinic acid was detected in three fractions (NS-P2, NS-P3, and NS-P4), and salicylic acid was also found in three fractions [NS-P1, NS-P2, and NS-P4] (Table 3). These compounds have been identified according to their retention time (RT) and the mother ions from the mass spectra in negative mode (MRM). The structure of major phenolic compounds found in *N. sativa* seed extracts is given in Figure 2. The results presented in Table 3 indicate that the majority of phenolic acids in the seeds of *N. sativa* are actually ester-bound phenolics (bound to the cell walls), followed by the corresponding esters and glycosides. It has been reported that phenolic acids in free forms are rarely present in plants, whereas the majority of phenolic acids are present in a bound form [22].

Inhibitory activity against BChE

The inhibitory activity and capacity of different *N. sativa* seed extracts against BChE decreased as follows: methanol extracts > aqueous extracts > *n*-hexane extracts (Table 4). The samples with the highest inhibitory activity against BChE were NS-UM (IC_{50} = 79.11 ± 6.06 µg/ml) and NS-RW (IC_{50} = 536.71 ± 9.12 µg/ml).

Inhibitory activity against CAT

The heme-containing enzyme CAT, which catalyzes hydrogen peroxide conversion to water and dioxygen, is considered to be one of the major antioxidant enzymes in biological systems. Hydrogen peroxide can be converted to highly damaging species such as hydroxyl radicals via the Fenton reaction or by peroxidase activity or it can be efficiently detoxified by CAT. We monitored CAT activity and H_2O_2 degradation spectroscopically in the absence and presence of the isolated extracts. The spectra showed a continuous decrease of absorbance at 240 nm, over 60 seconds, which could be explained by the decomposition of the substrate, H_2O_2. The inhibitory activity and capacity against CAT were the highest with the methanol extracts (Table 4). The range of inhibitory activity was from 6.61 ± 0.27 µg/ml for sample NS-SM to 181.97 ± 5.65 µg/ml for sample NS-SH. The inhibitory activity of different *N. sativa* seed extracts against CAT was as follows:

TABLE 2. Chromatographic parameters for phenolic acid and flavonoid standards

Compound	RT (min)	Equation	R^2	LOQ (pmol/inj)
Gallic acid	3.081±0.061	y=(74190.5±1925.2) x–(10309.3±1762.2)	0.9943±0.0020	1.0
Chlorogenic acid	5.187±0.027	y=(446884.7±10548.2) x–(309651.1±31807.2)	0.9327±0.0146	5.0
4-Hydroxybenzoic acid	5.578±0.048	y=(521105.7±9307.3) x–(137474.0±42229.1)	0.9874±0.0056	1.0
Caffeic acid	6.027±0.039	y=(867587.8±21526.8) x–(674042.5±137390.5)	0.9573±0.0297	5.0
Vanillic acid	6.194±0.036	y=(37334.6±1042.2) x–(2807.4±445.4)	0.9982±0.0007	5.0
5-Hydroxyferulic acid	6.207±0.054	y=(468939.4±5339.1) x–(125378.7±1166.2)	0.9963±0.0001	1.0
Protocatechuic acid	6.327±0.042	y=(636007.1±25370.7) x+(28490.5±5993.9)	0.9981±0.0011	1.0
Syringic acid	6.360±0.045	y=(90591.7±185.6) x–(2851.5±865.5)	0.9998±0.0001	5.0
3-Hydroxybenzoic acid	6.565±0.063	y=(56506.0±5925.6) x–589.3±13.5)	0.9991±0.0002	1.0
p-Coumaric acid	7.274±0.045	y=(853903.0±14026.9) x+(14433.7±3509.7)	0.9998±0.0001	1.0
Sinapinic acid	7.473±0.038	y=(73456.0±4512.9) x+(24466.5±1062.2)	0.9655±0.0214	1.0
Ferulic acid	7.801±0.038	y=(217233.5±3073.6) x–(9367.4±996.4)	0.9998±0.0002	5.0
Myricetin	8.469±0.041	y=(478904.4±6854.3) x+(62878.7±17297.3)	0.9884±0.0069	1.0
Rosmarinic acid	8.536±0.087	y=(850735.9±46762.6) x+(123704.3±16772.0)	0.9701±0.0045	1.0
Salicylic acid	9.093±0.045	y=(934225.8±56356.9) x+(71237.3±5861.8)	0.9909±0.0087	1.0
Quercetin	9.387±0.028	y=(1394700.2±366670.4) x+(157048.0±10221.9)	0.9947±0.0055	1.0
trans-Cinnamic acid	10.054±0.116	y=(7163.5±387.4) x+(3012.8±136.6)	0.9940±0.0029	10.0
Apigenin	10.063±0.024	y=(539432.0±29159.0) x+(137881.6±32869.0)	0.9955±0.0041	5.0
Naringenin	10.119±0.034	y=(152935.4±4592.0) x+(51285.2±9465.8)	0.9732±0.0225	5.0
Kaempferol	10.169±0.041	y=(19806.2±120.3) x+(6186.1±240.6)	0.9934±0.0033	5.0
Chrysin	11.430±0.001	y=(70169.6±11514.3) x+(38697.1±972.3)	0.9647±0.0401	5.0
Pinocembrin	11.534±0.139	y=(313792.3±75256.5) x+(7455.7±619.4)	0.9999±0.0002	1.0
Galangin	11.790±0.158	y=(145758.6±30824.0) x+(13863.0±589.4)	0.9996±0.0005	1.0

LOQ: Limit of quantification; RT: Retention time

TABLE 3. Phenolic compound content (μg/mg) in four different fractions

Standard/sample	NS-P1 free	NS-P2 esters	NS-P3 glycosides	NS-P4 ester-bound
5-Hydroxyferulic acid				0.308±0.004
3-Hydroxybenzoic acid	0.441±0.004			0.451±0.007
Ferulic acid	0.308±0.026	3.883±0.373	1.941±0.156	2.098±0.053
Sinapinic acid		7.222±0.729	1.638±0.139	4.418±0.164
Salicylic acid	0.755±0.029	0.150±0.014		0.095±0.030
Quercetin			1.373±0.087	
Kaempferol			11.745±0.919	

NS: Non-soluble

NS-SM>NS-UM>NS-RW>NS-RM>NS-UH>NS-SH. The extracts obtained with polar solvents showed stronger inhibitory activity against CAT compared to the nonpolar extracts.

Evaluation of antimicrobial activity

The *N. sativa* seed extracts did not show any antimicrobial activity against *B. subtilis* and *C. albicans* strains. Furthermore, NS-RW and NS-UH samples showed no antimicrobial activity against all tested bacteria and fungus. The results presented in Table 5 indicate that the methanol extracts possess some inhibitory potential against the tested microbial strains compared to the water extracts, but this activity was still low compared to the antibiotics.

DISCUSSION

The UHPLC-MS/MS method used in this study for the identification of phenolic compounds in *N. sativa* seed extracts showed better performance compared to other chromatographic methods (i.e. gas chromatography [GC] and HPLC). In the case of GC, the analytes must be derivatized into corresponding esters prior to the analysis, due to their very low volatility [23]. The use of capillary electrophoresis [24] and countercurrent chromatography [25] has also been reported. However, MS detector seems to be a very powerful tool due to its high sensitivity, as well as the fact that complete chromatographic separation is not always necessary before the detection of analytes [15,22]. A relatively novel chromatographic technique is UHPLC, which in comparison with HPLC, gives an improved resolution, shorter RT and higher sensitivity [26].

The metabolism of phenolic compounds in biological systems is a very complex and carefully controlled process. The phenolic content varies considerably between different organisms, tissues, developmental stages, and in relation to environmental conditions [27]. Two families of phenolic acids are commonly found in plants: derivatives of benzoic acid and

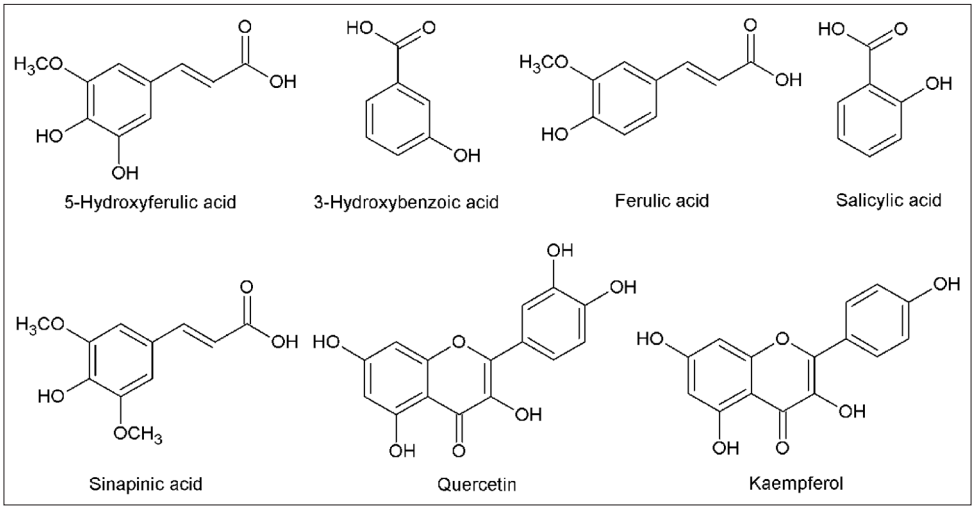

FIGURE 2. Structure of major phenolic compounds in *Nigella sativa (N. sativa)* seed extracts. All four fractions contained ferulic acid, sinapinic acid was detected in three fractions (NS-P2, NS-P3, and NS-P4), and salicylic acid was also found in three fractions (NS-P1, NS-P2, and NS-P4). The majority of phenolic acids in the seeds of *N. sativa* are actually ester-bound phenolics (bound to the cell walls), followed by the corresponding esters and glycosides.

TABLE 4. Inhibitory activity of different *N. sativa* seed extracts against BChE and CAT enzymes

Sample	NS-RW	NS-RM	NS-SH	NS-SM	NS-UH	NS-UM
IC_{50} –BChE µg/ml	536.71±9.12	702.25±37.12	706.12±49.05	783.67±75.06	902.35±9.01	79.11±6.06
IC_{50} –CAT µg/ml	28.84±1.01	60.26±4.40	181.97±5.65	6.61±0.27	104.71±11.09	19.95±2.11

IC_{50}: Inhibitory concentration 50%; BChE: Butyrylcholinesterase; CAT: Catalase; NS: Non-soluble; *N. sativa: Nigella sativa*

TABLE 5. Antibacterial activity of *N. sativa* seed extracts

Sample	*S. aureus* subsp. *aureus* ATCC® 6538™	*S. enterica* subsp. *enterica serovar abony* NCTC® 6017TM	*E. coli* ATCC® 8739™
NS-RM	7	NA	6
NS-SH	NA	7	6
NS-SM	13	NA	NA
NS-UM	13	NA	NA
Oxacillin	27	17	16
Gentamicin	25	15	15
Ampicillin	0	7	0

*The diameters of the inhibition zones were measured in millimeters. NA: Not active; *S. aureus: Staphylococcus aureus; S. enterica: Salmonella enterica; E. coli: Escherichia coli; N. sativa: Nigella sativa*

derivatives of cinnamic acid. Both types of phenolic acids, as well as flavonoids, usually occur in conjugated forms, mostly as esters and glycosides which are linked either to structural components of the plant (i.e. cellulose, proteins, and lignin), larger polyphenols (flavonoids), smaller organic molecules (e.g. glucose, quinic, maleic, or tartaric acid), or other natural products (e.g. terpenes) [28,29].

The major compounds identified in this study were ferulic and sinapinic acid as phenolic acid esters and quercetin and kaempferol as flavonoid glycosides. Only a few published studies investigated phenolic compounds from *N. sativa*. For example, Toma et al. [30] identified the following compounds in the seed of *N. sativa*: quercitrin, quercetin, and kaempferol as major phenolic compounds and two phenolic acids, *p*-coumaric and ferulic acid. Various compounds, such as gallic, *p*-dihydroxybenzoic, chlorogenic, vanillic, *p*-coumaric, *trans*-cinnamic, and *trans*-2- hydroxycinnamic acid have been detected in black cumin shoots and roots. Interestingly, vanillic acid was detected in both plant parts, contributing about 66% of the total mass that was isolated [13], while methanol or aqueous fraction of black cumin seed cake contained hydroxybenzoic, syringic and *p*-coumaric acids, with a high concentration of *p*-coumaric acid in both of these two fractions [31].

The second part of our study was focused on the biological activity of *N. sativa* seed extracts by determining their inhibitory activity against BChE and CAT, as well as their antimicrobial activity. All living organisms have endogenous defense systems against oxidative damage. There are two main antioxidant defense mechanisms: the first is antioxidant defense with enzymes such as superoxide dismutase and CAT while the second is antioxidant defense with non-enzymatic components, such as polyphenols, ascorbic acid, and carotenoids [32]. Oxidative stress is a process in which the physiological balance between pro-oxidants and antioxidants is disrupted in favor of the former, leading to a potential

damage to the organism. Reactive oxygen species production can induce DNA damage, protein carbonylation, and lipid peroxidation, and initiate the development of a number of pathologies such as cardiovascular disease, cancer, and Alzheimer's disease. Cholinesterase (ChE) plays an important role in the central nervous system. Inhibitors of ChE, such as galantamine and donepezil, are frequently used in pharmacotherapy. These approved drugs are limited in use due to their adverse side-effects such as gastrointestinal disturbance and bioavailability problems. BChE has recently been a focus of research, because the concentration of BChE does not change, or is even up-regulated in the brain of patients suffering from Alzheimer's disease. In the late stages of Alzheimer's disease, BChE represents the predominant ChE in the brain. New studies have been heavily focused on the BChE catalytic mechanism and its control that might, in turn, lead to the control or at least a delay in the progression of Alzheimer's disease [33]. Thymoquinone, the main constituent of *N. sativa,* might be the major component responsible for the inhibitory effect against BChE. Earlier research showed that isolated thymoquinone exhibits strong anti BChE activity [34]. However, our results showed that the investigated *N. sativa* seed extracts did not possess significant anti ChE activity, except the methanol extract (NS-UM) [35]. The similar results are also found in the literature [36]. On the other hand, for the first time, we demonstrated *in vitro* inhibitory activity of *N. sativa* seed extracts against CAT. Hydrogen peroxide that is decomposed by CAT is a very reactive molecule and plays a role in the pathologies of many diseases. The damaging effect of hydrogen peroxide depends on the formation of hydroxyl or hydroxyl-like free radicals by the Fenton reaction. Radicals can also act as signaling molecules. Intercellular signaling of cancer cells by free radicals can be restored when hydrogen peroxide is supplied. The inhibition of CAT activity by black cumin seed extracts results in the increase of oxidative stress that triggers apoptosis and eventually leads to the cell death. These phenomena can be therapeutically used for the cancer prevention. The combined effect of all the major activities of *N. sativa* seed, such as antioxidant, pro-oxidant, and enzyme inhibitory effect leads to cell-specific cell growth suppression. Collectively, these results explain the biochemical basis of specific apoptosis induction in cancer cells and can be used in the development of pharmacological preparations and their application as anticancer drugs [37].

The results of antimicrobial activity test indicated a low antimicrobial activity of the *N. sativa* seed extracts against the tested bacterial strains, with a slightly higher activity against the Gram-positive compared to Gram-negative bacteria. The difference in the sensitivity against Gram-positive and Gram-negative bacteria might be attributed to the differences in the morphological constitution of these microorganisms. Gram-negative bacteria have an outer phospholipid membrane, almost impermeable to lipophilic compounds [38]. The absence of this barrier in Gram-positivebacteria allows direct contact of the hydrophobic constituents with the phospholipid bilayer of the cell membrane, causing either an increase of ion permeability and leakage of vital intracellular constituents, or impairment of the bacterial enzyme systems [39].

CONCLUSION

Overall, sinapinic acid and kaempferol were the two major phenolic components in the *N. sativa* seed extracts detected by UHPLC-MS/MS method. Our results indicated that *N. sativa* seed extracts can inhibit BChE activity, but the inhibition is relatively low. Furthermore, the extracts exhibited some antimicrobial activity against the tested microorganisms. In addition, this is the first study showing that black cumin seed extracts can efficiently inhibit CAT activity. This inhibitory mechanism of black cumin seed extracts can be potentially used for the design and development of new and efficient CAT inhibitors.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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