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ORIGINAL RESEARCH PAPER

DETERMINATION OF POLYPHENOLS CONTENT, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF *NIGELLA SATIVA* L. SEED PHENOLIC EXTRACTS

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Abstract: In this work, we studied the phenolic extracts of the seeds of *Nigella sativa* L., from the point of view of the chemical composition and the antioxidant activity. The extraction of polyphenols was carried out using two different solvent systems, namely methanol: water 7: 3 (v / v) and acetone: water 7: 3 (v / v). The qualitative and quantitative analysis of methanolic and acetonic extracts by HPLC (High Performance Liquid Chromatography) revealed the presence of gallic acid, apigenin, naringenin, rutin, quercetin and kaempferol. The quantification of total phenols by Folin-Ciocalteu colorimetric method and flavonoids by the $AlCl_3$ method gave higher values with the methanolic extracts compared to the acetonic extract. The total polyphenol content of the methanolic extract 1.3714 ± 0.0315 mg/g (mg GAE / g dw) is more elucidated than the content in acetone extract 0.5962 ± 0.0046 mg/g (mg GAE/g dw). The evaluation of the antioxidant power of the phenolic extracts was made by two chemical tests namely the DPPH test and the β-carotene bleaching test and we finished this work by studying the antibacterial and antifungal activity of these phenolic extracts.

Keywords: *antibacterial activity, antifungal activity, antioxidant activity, flavonoids, HPLC, polyphenol*

INTRODUCTION

The use of cereals or plants for their healing properties was created, answered and transmitted in the oldest known civilization. It is one of the manifestations of man's immemorial effort to understand and use the nature, thus responding to one of his oldest anxieties, which stems from sickness and suffering [1, 2]. *Nigella sativa* is one of the most popular plants around the world. The seed of *N. sativa* is traditionally used in food as well as "medicine" [3]. The fixed oils of Nigella seeds have many pharmacological properties and are considered as antioxidant, anti-inflammatory, immunomodulatory, antitumor, antidiabetic agent and it plays a significant role in the cardiovascular and gastrointestinal systems [4]. During the last twenty years, many researchers have been interested in *Nigella sativa*. Most indications claimed in traditional medicine have been confirmed and other properties have been added. We focused our research on the search for biologically active molecules in *Nigella sativa* seeds, studying their phenolic fraction.

MATERIAL AND METHODS

Chemicals

All solvents and reagents were of the highest purity required for each application. Hexane, acetone, methanol, ethyl acetate, orthophosphoric acid, aluminum chloride, ammonium sulfate, sodium sulfate, vitamin E, linoleic acid, radical 1,1-diphenyl-2-picrylhydrazyl (DPPH, 98 %), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and standards (gallic acid, catechin, vanillic acid, epicatechin, coumaric acid, apigenin, rutin, quercetin, kaempferol) were purchased from Sigma-Aldrich (SARL Prochima-Sigma, Tlemcen, Algeria). β-carotene and Tween 20 were purchased from Flucka (Buchs, Germany). The bacterial strains are batches of ATCC (American Type Culture Collection, USA).

Plant materials

Seeds of *Nigella sativa*, obtained from Saudi Arabia markets, constitute the part of the plant used in this study. The seeds were ground in a blender in order to obtain a powder.

Preparation of extracts

Having extracted the fixed oils from the seeds by Soxhlet, we left the cakes to dry. The dried cake was then milled thoroughly by an electric mill (NutriMill, Germany) and then 5 g of the dried material were macerated in an acetone : water 70 : 30 (v/v) and methanol : water 7 : 3 (v/v) solvent, for 72 hours in the dark and at room temperature. After filtration, the acetone was evaporated from the filtrate under reduced pressure at 40 °C. A volume of 2 mL of ammonium sulfate (20 %) and 2 mL of orthophosphoric acid (2 %) are added to the aqueous phase. The polyphenols were extracted with ethyl acetate in a ratio of aqueous phase: ethyl acetate 1 : 1 (v/v) [5]. The extraction is repeated three times. The organic phase is dried by adding a sufficient

amount of anhydrous sodium sulfate. The solvent is evaporated to dryness using a rotary evaporator (Büchi Rotavapor R-200, Germany). The residue is taken up in 10 mL of methanol and stored in a cool place (4 °C).

Determination of total phenolic content

The total phenol was assayed according to the method of Singleton and Ross (1965) with the Folin-Ciocalteu reagent [6]. A volume of 100 µL of each extract (diluted in methanol) was added to 500 µL of Folin-Ciocalteu reagent (10 %). The solutions were mixed for 1 minute and then 2 mL of the sodium carbonate solution (2 %) were added. The mixture is incubated for 30 minutes in the dark, at room temperature. The absorbance of all the extracts was measured by the spectrophotometer (OPTIZEN 2120UV, Korea) at 760 nm.

Quantification of flavonoids content

The method of aluminum chloride is used to quantify the flavonoids in our extracts. Using catechin as standard, the flavonoid contents are expressed in milligrams of quercetin equivalents (mg QE) per gram of dry matter [7]. 1 mL volume of each extract and standard is dissolved in methanol with suitable dilutions then added to an equal volume of an $AlCl_3$ solution (2 % in methanol). The mixture was stirred vigorously and spectrophotometry reading was carried out at 430 nm after 15 minutes of incubation.

Dosage of polyphenol by HPLC

The qualitative and quantitative analysis of the phenolic compound of the acetonic and methanolic extracts of *Nigella sativa* seeds were carried out on an analytical HPLC system Varian Pro Star model 230 (Varian Associates, Walnut Creek, California, USA) equipped with a ternary pump (model Q2 Pro Star 230). The HPLC separation of the active compounds was carried out on C-18 reverse phase HPLC column (Zorbax, 30 cm × 4.6 mm, particle size 5 µm). Elution was performed using a binary system consisting of solvent A (acetonitrile) and solvent B (2 % glacial acetic acid solution (*p*H = 2.6)) provided by Sigma-Aldrich (Germany). The gradient program was used as follows: 0-5 min: 5 % A and 95 % B, 25-30 min: 35 % A and 65 % B, 35-45 min: 70 % A and 30 % B with a flow rate of 0.9 mL/min. The injection volume was 20 µL and the detection was carried out at 280 and 360 nm [8]. The identification of the peaks was confirmed by comparing their retention times with pure standards and quantified by comparing the peak areas in the chromatograms of the samples with those of the standards (gallic acid, catechin, vanillic acid, epicatechin, coumaric acid, apigenin, rutin, quercetin, kaempferol). All measurements were done in triplicate, expressing the result as mean values ± standard deviation of micrograms of phenolic compound / g of seed meal of *Nigella sativa*.

Antioxidant activity

β -carotene bleaching test

This method consists of measuring at 470 nm, the discoloration of β-carotene resulting from its oxidation by the decomposition products of linoleic acid. The dispersion of linoleic acid and β-carotene in the aqueous phase has been prepared using Tween 20. The oxidation of linoleic acid is catalyzed by heat (50 °C). The addition of pure antioxidants or in the form of plant extracts induces a delay in the kinetics of decolorization of β-carotene [9]. The study of the antioxidant activity by the β-carotene decolorization method was carried out by the experimental protocol described by Ozsoy *et al.* [10]. To prepare the emulsion of β-carotene, 2 mg of the latter are dissolved in 10 mL of chloroform, then 1 mL of this solution is mixed with 40 mg of purified linoleic acid and 400 mg of Tween 20. Then, the chloroform is evaporated under reduced pressure by a rotary evaporator, and the obtained residue is taken up in 50 mL of distilled water. Tubes containing 3 mL of this emulsion are prepared, for which 50 µL of methanolic extracts or reference antioxidant (BHA) at different concentrations are added. The mixture is well shaken, and the reading of the absorbance at 470 nm is immediately done at t_0 against a blank which contains the emulsion without the β-carotene. The covered tubes are placed in a water bath set at 50 °C, and the reading of the absorbance is performed every 15 minutes for 120 minutes. A negative control is carried out in parallel, comprising 3 mL of the β-carotene emulsion and 50 µL of methanol. The results obtained are expressed in terms of percent inhibition of the β-carotene decolorization using the following formula [10]:

$$\%\, I = \left(1 - \frac{A_0 - A_{120}}{A_{c0} - A_{c120}}\right) \times 100 \qquad (1)$$

where: A_0 is the absorbance of the sample at t_0, A_{120} is the absorbance of the sample after 120 minutes incubation, A_{c0} is the absorbance of the negative control at t_0 and A_{c120} is the absorbance of the negative control after 120 minutes incubation.

DPPH free radical scavenging assay

This test is carried out on all methanolic extracts. An aliquot of 100 µL of the sample at different concentrations is added to 1.9 mL of the DPPH solution (0.033 $g \cdot L^{-1}$). A negative control was prepared by mixing 100 µL of methanol with 1.9 mL of the DPPH solution. The reading of the absorbance is made against a white at 517 nm after 30 min incubation in the dark and at room temperature. We used the BHA and the BHT as a reference; the assay is repeated 3 times. The results are expressed as percent inhibition I % [11]:

$$IP\,\% = \frac{Abs_0 - Abs_E}{Abs_0} \times 100 \qquad (2)$$

where *IP %* is inhibition power; Abs_0 is the absorbance of the control and Abs_E is the DPPH absorbance in the presence of the test compound at different concentrations after 30 min incubation.

Study of antibacterial and antifungal activity

The microbial strains used are *Escherichia coli* ATCC 10536, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, yeast *Candida albicans* ATCC 10231.

Aromatogram method

The aromatogram is based on a technique used in medical bacteriology, called antibiogram. It has the advantage of being very flexible in the choice of products to be tested and to apply to a large number of bacterial species [12].

We use 9 mm diameter filter paper discs impregnated with methanolic and acetonic extract. We deposit these discs on the surface of an agar medium seeded on the surface with a bacterial suspension. The incubation is done in an oven (Memmert, Germany) at 37 °C for 24 hours for bacteria and at 25 °C for 48 hours for yeasts [13]. The absence of microbial growth results in a translucent halo around the disc whose diameter is measured and expressed in millimeters. The control box presents a disc not soaked by the extract. Another sample box, without disk, is seeded under the conditions of the experiment. It tells us about the homogeneity of the bacterial carpet.

Statistical analysis

All results obtained were expressed as mean ± SEM. Statistical analyzes were performed by one-way ANOVA, followed by Tukey multiple comparisons. A difference was considered statistically significant when $p < 0.05$. Excel Microsoft software is used for data processing.

RESULTS AND DISCUSSION

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The quantification of polyphenols and flavonoids in the various extracts of cakes seeds of *Nigella sativa* was made using colorimetric methods (Folin-Ciocalteu reagent and aluminum chloride). A calibration curve was established with gallic acid as standard at different concentrations for polyphenols and catechin for flavonoids. The results were expressed in mg of gallic acid equivalent (GAE) / g and catechin equivalent (mg EC / g) of extract on dry weight (dw). All measurements were taken in triplicate and mean values were calculated. The results of determination of the polyphenols and flavonoids of *Nigella sativa* seed cake in the case of methanolic and acetonic extracts are summarized in Table 1.

Table 1. *Polyphenol and flavonoid content in methanolic extract and acetone extracts of seed cake from Nigella sativa L.*

Extracts	TPC mg/g (mg GAE/g dw)	TFC (mg QE /g dw)
Methanol/water (70/30)	1.3714 ± 0.0315	0.4418 ± 0.0157
Acetone/water (70/30)	0.5962 ± 0.0046	0.2746 ± 0.0053

According to these results, note that the methanolic extract represents the highest value. It is in the range of 1.3714 ± 0.0315 mg GAE / g, in the case of acetone extracts the value of the amount of the polyphenols is 0.5962 ± 0.0046 mg GAE / g. This difference is due to the polarity of the solvent [14]. The same remark was recorded for the flavonoid content, as the high value 0.4418 ± 0.0157 mg EC / g corresponds to the solvent system methanol : water 7 : 3 (v/v) in the case of the solvent extract acetone : water 70 : 30 (v/v) system is 0.2746 ± 0.0053.

HPLC analyses

We realized the qualitative and quantitative analysis of polyphenols from the methanolic and acetonic extracts of the cake by HPLC. The chromatograms of the two extracts have the same profile (Figure 1 and Figure 2), with a difference in concentration of constituents. Comparison of standard retention times with those recorded in chromatograms, allowed us to identify different compounds (phenolic acids and flavonoids).

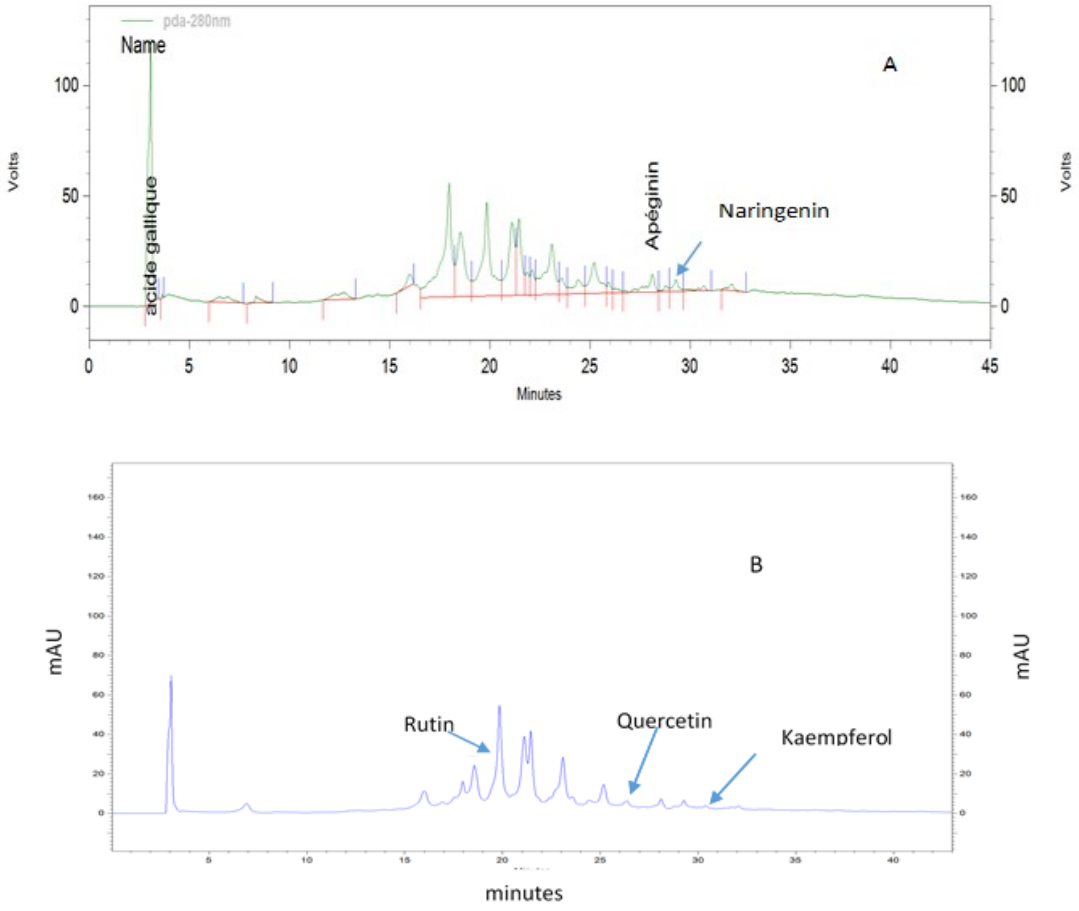

Figure 1. *Chromatograms of the analysis of the phenolic compounds of methanolic extract (methanol:water 70 : 30 (v:v)) at 280 nm (A) and 360 nm (B)*

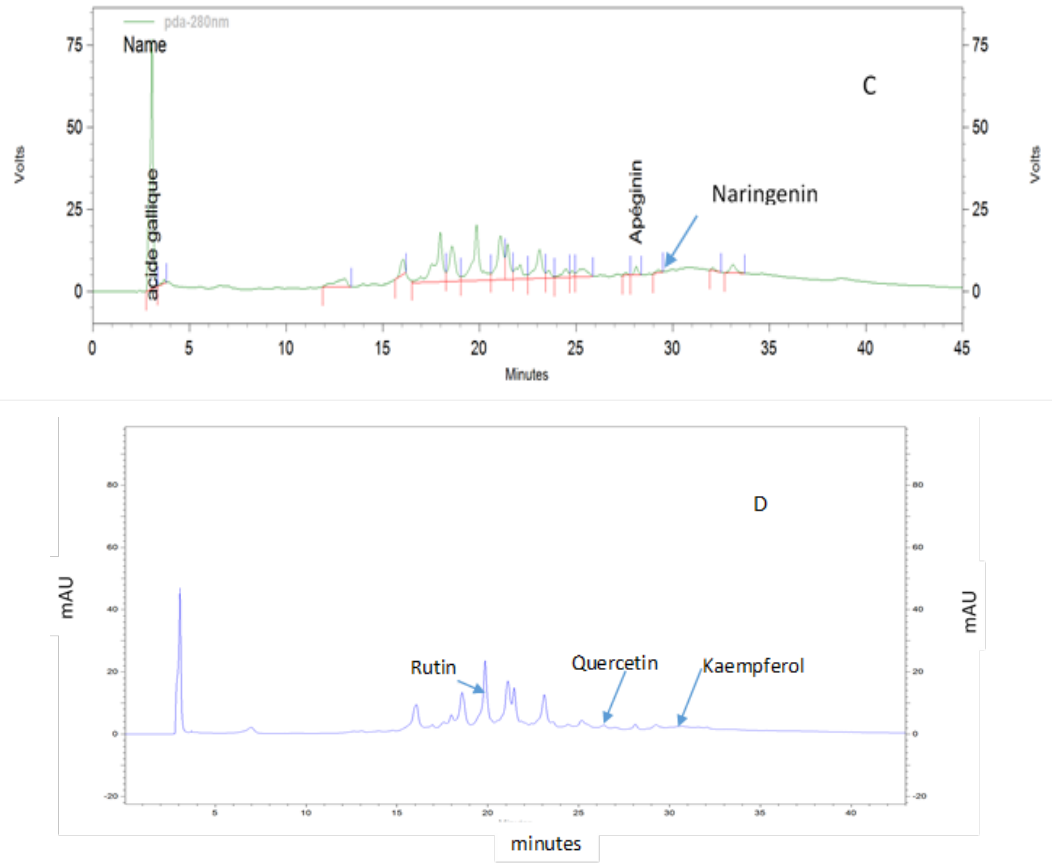

Figure 2. *Chromatograms of the analysis of the phenolic compounds of acetonic extract (acetone:water 70 : 30 (v:v)) at 280 nm (C) and 360 nm (D)*

The results obtained (Table 2) show the presence of important compounds such as gallic acid, kaempferol, rutin, apigenin, naringenin and quercetin, and the absence of catechin, vanillic acid, epicatechin and coumaric acid in both extracts.

Table 2. *Phenolic acids and flavonoids quantified in the methanolic and acetonic extracts of seed meal of Nigella sativa L.*

Compound	Methanolic extracts (methanol : water 70:30 (v/v))		Acetonic extracts (acetone : water 70:30 (v/v))	
	content [µg·mL^{-1}]	content [µg·g^{-1} dw]	content [µg·mL^{-1}]	content [µg·g^{-1} dw]
Gallic acid	69.64 ± 2.94	139.28 ± 5.88	11.94 ± 1.45	23.88 ± 1.90
Catechin	-	-	-	-
Vanillic acid	-	-	-	-
Epicatechin	-	-	-	-
Coumaric acid	-	-	-	-
Apigenin	15.06 ± 1.98	139.28 ± 5.88	10.27 ± 1.35	20.45 ± 2.70
Naringenin	0.84 ± 0.08	1.68 ± 0.16	0.40 ± 0.06	0.80 ± 0.12
Rutin	120.21 ± 4.52	240.42 ± 9.04	22.38 ± 3.23	44.76 ± 6.46
Quercetin	3.30 ± 0.89	6.60 ± 1.74	0.30 ± 0.02	0.60 ± 0.04
Kaempferol	2.82 ± 0.53	5.64 ± 1.06	0.36 ± 0.03	0.76 ± 0.06

From these results it is found that the major compounds in the various extracts are gallic acid (69.64 ± 2.94 µg·mL^{-1} for methanolic extract and 11.94 ± 1.45 µg·mL^{-1} for acetone extract) and rutin (120.21 ± 4.52 µg·mL^{-1} for methanolic extract and 22.38 ± 3.23 µg·mL^{-1} for the acetonic extract). These two compounds are also potent antioxidants and demonstrated various health benefits by exhibiting antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory actions [15].

Antioxidant activity

Phenolic compounds represent the majority of the antioxidant activity in plants, in which the antioxidant properties are mainly due to their redox potential, which allows them to act as reducing agents, hydrogen donors, metal chelators and singlet oxygen quenchers [16].
In this study we realized two chemical tests, the β-carotene bleaching test and the DPPH free radical scavenging test. The IC_{50} value is defined as the concentration of extract that inhibits 50 % β-carotene degradation and DPPH radical scavenging. This value is calculated by modeling the percent inhibition as a function of the extract concentration using the exponential equation, lower value of IC_{50} indicating higher antioxidant activity [16].
The IC_{50} values determined in mg·mL^{-1} expressing the effective concentration of the various antioxidant extracts of *Nigella sativa* seeds necessary to trap and reduce 50 % moles of DPPH dissolved in ethanol are reported in Table 3.

Table 3. *Antioxidant activities of methanolic and acetonic extracts and standard by both methods*

Extracts	IC_{50} [mg·mL^{-1}]	
	β-carotene bleaching assay	**DPPH radical scavenging**
Methanol : water 7:3 (v/v)	0.0711 ± 0.0013	0.0734 ± 0.0001
Acetone : water 7:3 (v/v)	0.2448 ± 0.0063	0.1202 ± 0.0084
BHT	0.0005 ± 0.00001	0.0013 ± 0.0002
BHA	0.0073 ± 0.0004	0.0011 ± 0.0004

From the results obtained, the methanolic extracts showed moderate antiradical activity (IC_{50} = 0.0734 ± 0.0001 mg·mL^{-1}) whereas the acetonic extract revealed a low antioxidant activity with a value of IC_{50} = 0.1202 ± 0.0084 mg·mL^{-1}. This activity remains less than that of reference antioxidants: BHT (IC_{50} = 0.0013 ± 0.0002 mg·mL^{-1}) and BHA (IC_{50} = 0.1202 ± 0.0084 mg·mL^{-1}). The results of the antioxidant activity show that the seed cakes of *Nigella sativa* contain a multitude of active ingredients that can act simultaneously against a certain number of pathologies.
The kinetics of β-carotene bleaching (Figure 3) clearly shows that the two extracts have a significant effect; they have the capacity to trap free radicals derived from linoleic acid. Methanolic extract is more active (IC_{50} = 0.0711 ± 0.0013 mg·mL^{-1}) than acetone extract (IC_{50} = 0.2448 ± 0.0063 mg·mL^{-1}) and we also find that both extracts are less active than the reference antioxidants (BHA) (IC_{50} = 0.0073 ± 0.0004 mg·mL^{-1}) and (BHT) (IC_{50} = 0.0005 ± 0.00001 mg·mL^{-1}). A positive correlation is recorded between the polyphenol content of our extracts and the β-carotene bleaching test.

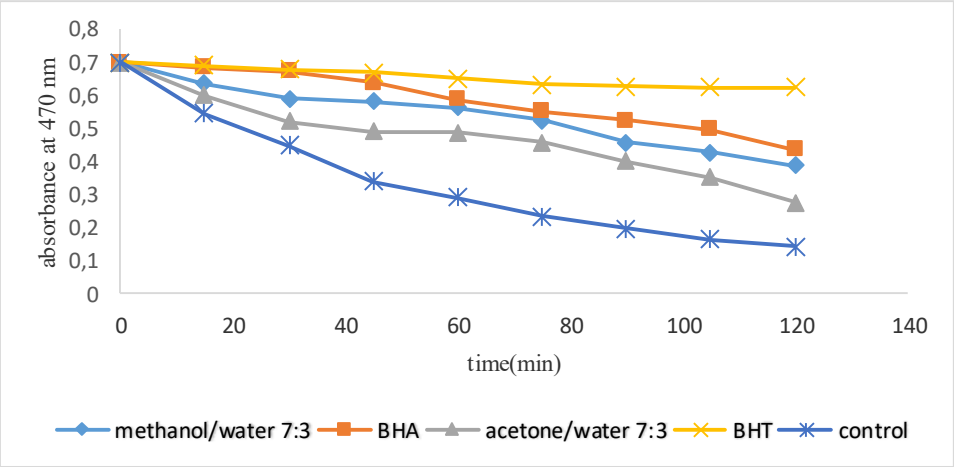

***Figure 3**. Kinetics of β-carotene bleaching at 470 nm in the absence and presence of methanolic and acetonic extracts of seed cake from Nigella sativa L.*

Antibacterial activity

The results obtained are shown in Table 4.

***Table 4.** The zone of sensitivity of the bacteria to the methanol extracts expressed in mm*

Strains		Extracts		Reference
		methanol : water (7 : 3) (v/v)	acetone : water (7 : 3) (v/v)	
		Inhibition diameter, d [mm]		
Gram -	*Escherichia coli*	10.50 ± 0.50 (+)	6.00 ± 0.20 (-)	ATCC 10536
Gram -	*Bacillus subtilis*	15.5 ± 0.30 (++)	14.00 ± 0.50 (+)	ATCC 6633
Gram +	*Staphylococcus aureus*	12.00 ± 0.70 (+)	6.50 ± 0.50 (-)	ATCC 6538
Yeast	*Candida albicans*	13.00 ± 0.50 (+)	10.10 ± 0.30 (+)	ATCC 10231

The (+) and (-) values represent the degree of sensitivity of the strains: no sensitive or resistant (-): diameter ≤ 9 mm; average sensitivity (+): 9 mm < diameter < 14 mm; strong sensibility (++): 15 mm < diameter < 19 mm. All the results obtained were expressed as mean ± SEM.

The two methanolic and acetonic extracts have almost the same level of activity against yeast (*Candida albicans* ATCC 10231) the sensitivity diameter for the two extracts equal respectively (13.00 ± 0.50 mm) and (10.10 ± 0.30 mm). In contrast, in the case of bacterial strains, the methanolic extract reveals a considerable effect against the bacterial strains studied. With high activity against *Bacillus subtilis* (Gram -) and moderate activity against *Escherichia coli* (Gram -) and *Staphylococcus aureus* (Gram +). The acetonic extract has strong activity against *Bacillus subtilis* (Gram -) and weak against *Escherichia coli* (Gram -) and *Staphylococcus aureus* (Gram +). This difference in sensitivity is due to the concentration of polyphenols in the extracts, the nature of the molecules present in our extracts and the nature of the outer wall of the studied bacteria. The bacterial wall of *Escherichia coli*, for example, is very rich in lipopolysaccharides (LPS) which prevent hydrophobic molecules from crossing the membrane [17].

CONCLUSIONS

This study covers an important part of the secondary metabolite. *Nigella sativa* L. is a plant widely diffused in traditional medicine. The results of this study revealed the presence of important phenolic compounds such as (gallic acid, rutin). The chemical tests of the antioxidant activity (DPPH radical scavenging, β-carotene bleaching) have allowed us to conclude that despite the small amount of polyphenols found in seed cake of *Nigella sativa* L. they have a good activity. The antibacterial activity of the studied fraction correlates with the polyphenol content.

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