Journal of Food Measurement and Characterization (2022) 16:3568–3577
https://doi.org/10.1007/s11694-022-01455-6

ORIGINAL PAPER

Evaluation of the antioxidant properties and total phenolic content of a dairy product (yogurt) supplemented with *Thymus willdenowii* essential oil from Algeria

Karima Benguedouar[1] · Soumeya Bencharif Betina[1] · Ramazan Erenler[2] · Nusret Genç[2] · Mesut Gok[5] · Mohamed Sebti[3] · Nassim Madi[4] · Loubna Mekdade[4] · Lynda Gali[4] · Malika Barkat[1]

Received: 8 March 2022 / Accepted: 11 May 2022 / Published online: 7 June 2022
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

Abstract

The purpose of this study was to evaluate the antioxidant properties and total phenolic content of a dairy product (yogurt) supplemented with *Thymus willdenowii* (synonym: *Thymus hirtus)* essential oil. The evaluation of the antioxidant and antibacterial activities of the oil against food spoilage bacteria and lactic acid strains was also assessed. The chemical composition of the oil was obtained by GC/MS. The evaluation of the antioxidant activity of a fortified yogurt with this oil was assessed by Oxygen radical absorbance capacity test (ORAC). The main component of the oil was Linalool (89.55%). DPPH, and Ferric reducing power revealed a weak antioxidant activity ($IC_{50} > 1600$ µg/mL, > 800 µg/mL respectively) while a moderate activity was obtained with ABTS and CUPRAC tests (38.06 ± 0.30, 56.15 ± 1.07 µg/mL respectively). A high concentration of the oil is needed to delay the inhibition of the β-carotene (> 800 µg/mL). Thus, a strong antibacterial activity was observed against the pathogenic strains with the high inhibition diameter for *B.cereus* ATCC10876 (31.75 ± 1.50 mm) while a weak effect was observed against lactic acid bacteria. A significant decrease in the antioxidant potential of non-supplemented yogurt was observed from day 1 to 21 while there was no significant difference in ORAC of supplemented yogurt with the oil. The maximum of total phenolic content was observed in day 21 of cold storage (252.92 ± 3.01 µg GAE/mL of yogurt). The essential oil studied could be an effective natural additive to yogurt avoiding the use of synthetic preservatives.

Keywords *Thymus willdenowii* · Essential oil · Antibacterial activity · Antioxidant activity · Yogurt · Total phenolic content

Introduction

Several food products are naturally perishable and require various processing techniques to ensure a high quality and extend their shelf-life [1]. Aromatic and medicinal plants and their essential oils (Eos) have been widely used from long time in human history in order to preserve perishable foods, they have been used to mask unpleasant odors, add new flavors and aroma characteristics to prepared dishes [2]. In spite of their flavoring properties, they have been used in medicine regarding to their biological properties [3] including antioxidant, antibacterial and antifungal activities [4–6]. Eos are a mixture of secondary metabolites with wide activities, which can be mediated by single compounds or groups of compounds and these secondary metabolites have biological functions in the plant, such as protection against

✉ Malika Barkat
barkat.malika@umc.edu.dz

1 Biotechnology and food quality laboratory, Institute of Nutrition, Food and Agri-Food Technologies (INATAA), University of Mentouri Brothers - Constantine1, Constantine, Algeria

2 Laboratory of chemistry, Gaziosmanpasa University, Tokat, Turkey

3 Mohamed Seddik Benyahia University, Jijel, Algeria

4 Biotechnology research center, Constantine, Algeria

5 Science and Technology Application and Research Center, Siirt University, Siirt, Turkey

predators and microbial pathogens, as well as involvement in defense mechanisms against abiotic stress [7] or bacterial invasions [8]. According to available papers and patents, Eos seem to be a potential alternative to synthetic compounds, especially in the case of the resistance of pathogenic microorganisms [9]. Therefore, the interest for food processors and consumers in antibacterial preservatives from plant origin, such as Eos, is increasing and attracted many researchers to investigate the potential biological activities and production of safe food without synthetic compounds [10]. Among studies carried these last few years, the interest in preserving dairy products from food spoilage microorganisms by natural preservatives and elevating their antioxidant activities, have been observed [11]. The dairy industry represents the most diversified dairy sector worldwide and the most developed, leading to the innovation and creation of targeted dairy formulations [12]. In recent years, several studies focused on the importance of the substitution of synthetics preservatives, generally not safe for human health, by natural products in order to fight several diseases [8]. The Intake of antioxidants in the form of antioxidative supplements of foods rich in antioxidants may protect the body from oxidative stress and damage and milk is known to be rich in antioxidant components such as polyphenolic metabolites, sulfur containing aminoacids, vitamins A, E, enzyme systems [13]. In fact, *Thymus willdenowii* belonging to the *Lamiaceae* family is an important ornamental, medicinal, and aromatic plant which produces essential oils that are used in traditional and modern medicine and as a healthy additive for food enrichment due to its beneficial effects for prevention of certain diseases [14]. *Thymus* genus are generally propagated in the Mediterranean area [15, 16] as one of the important genus in some species in the *Lamiaceae* family including around 360 species of aromatic plants with evergreen leaves and geographically present in Asia, North Africa, and Europe [17]. It has already been reported in previous studies that the essential oil of *Thymus* plant has antioxidant and antimicrobial properties [17–19], and classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) [20]. The present work determinates the chemical composition of the essential oil of *Thymus willdenowii* (*Tw*Eo) and explore the, *in vitro*, antioxidant and antibacterial activities by namely DPPH, ABTS, CUPRAC, Ferric reducing power and β-carotene bleaching test for antioxidant tests and disc diffusion assay for antibacterial tests. The total phenolic content was assessed for the Eo and a supplemented yogurt with this oil. Therefore, the other objective was, in the same way, evaluating the antioxidant capacity of the yogurt supplemented with *Tw*Eo by Oxygen Radical Absorbance Capacity (ORAC) test. The objective is to determine whether the addition of the oil to the fermented milk has improved the antioxidant potential and total phenolic content of the final product and the oil. The incorporation of this oil in fermented milks aims to obtain a functional food with natural flavouring agents and preservative in order to procure beneficial effects for human health.

Material and methods

plant materials

The aerial parts of *Thymus willdenowii* (*Thymus hirtus*) was collected in Blida (Western region of Algiers, Algeria) during the flowering phase (May-August 2018). Dr. Mohamed Sebti (University of Jijel, Algeria) identified the plant and a Voucher was deposited at the herbarium of the Biotechnology and food quality laboratory in the Institute of the Nutrition, Food, and Agri-Food Technologies (INATAA), University of Mentouri Brothers - Constantine1, Algeria. (Voucher specimen No. Tw-L1/18).

Extraction of the essential oil (EO)

The essential oil was extracted from the dried leaves of the plant by hydrodistillation using a Clevenger type apparatus. Extraction was carried out for 3 h using 100 g of the dry matter soaked in distilled water (1000ml). Residual moisture in the resulting Eo was removed by adding anhydrous sodium sulfate (Na_2SO_4) and stored in amber vials at 4 °C for further analysis. The yield of extraction was calculated using the following equation:

Yield%= (Mass of Eo/Mass of dried plant)*100.

Gas Chromatography-Mass Spectrometry (GC/MS)

In the Science and Technology Application and Research Center, Siirt University, Turkey, the qualitative and quantitative analysis of the oil was carried out on a GC associated with a Thermo Scientific Trace 1310ve ISQ LT Single Quadrupole mass spectrometer. The separations were carried out in DB-Wax capillary columns (60 m 0.25 mm I.D., 0.25 mm, TG-WAX-MS). Helium was used as the carrier gas (1.2 ml/min CF) and the oven temperature was programmed as follows: the initial temperature was set at 60 °C and held for 6 min, then increased to 230 °C with a heating rate of 3 °C/min and maintained at this temperature for 30 min. The temperatures of the ion sources were set at 250 °C. The mass spectra were recorded over the mass range 50–550 amu. The sample volume was 10µL prepared in chloroform (1:15, v/v), and injected in split mode

(1: 5) [21, 22]. Compounds were identified referring to the libraries Wiley 7, NIST DEMO, WinMain, Wiley 9, Replib, Mainlib and Winrep.

Antioxidant activities of the oil

DPPH free radical scavenging activity of the EO

The capacity of *Tw*Eo to scavenge DPPH radicals was evaluated as previously described by Blois [23]. 160µL of a methanolic solution of DPPH (1 mM in methanol) was mixed in a microplate with 40 µL of *Tw*Eo at different concentrations ranging from 25 to 1600 µg/mL. The microplate was kept in the dark for 30 min and the absorbance was measured at 517 nm. The standards butylhydroxyanisole (BHA), butylhydroxytoluene (BHT) and α-tocopherol were tested in parallel as positive controls. The following equation was used to calculate the inhibition rate of the DPPH radical:

DPPH inhibition (%) = [(absorbance control - absorbance sample) / absorbance control)] × 100.

The concentration inducing a reduction in the absorbance of the initial solution of DPPH by 50% defined as IC_{50} was determined from the regression curve.

ABTS Radical Cation Scavenging Activity assay

The $ABTS^{\bullet+}$ trapping activity was determined according to Pellegrini et al. [24]. The $ABTS^{\bullet+}$ radical was generated by the reaction between 7 mM of ABTS in water and 2.45 mM of potassium persulfate and the mixture was stored in the dark at room temperature for 12-16 h. The $ABTS^{\bullet+}$ solution was then diluted with distilled water to obtain an absorbance of 0.7 ± 0.025 at 734 nm. In a 96-well microplate, 160 µL of $ABTS^{\bullet+}$ were added to 40 µL of each dilution of essential oil made in ethanol at final concentrations ranging from 25 to 1600 µg/ mL and left to react for 10 min. Before that, the absorbance is read at 734 nm. BHA, BHT and α-tocopherol were used as antioxidant standards for comparison. The results were given as 50% inhibition concentration IC_{50} (µg/mL).

Cupric reducing antioxidant capacity (CUPRAC) assay

The essential oil was assessed for its capacity to reduce copper ions following the cupric reducing antioxidant capacity assay as reported by Apak et al. [25]. In a 96-well microplate, 50 µL of copper (II) chloride solution (0.01 M), 60 µL of ammonium acetate (1 M) and 50 µL of neocuproine (0.0075 M) were mixed to 40 µL of the different concentrations (25 to 1600 µg/ mL) of the Eo or standards (BHA and BHT). The microplate was incubated for 1 h at room temperature, and the absorbance was then recorded at 450 nm. The results were given as $A_{0.5}$ (µg/mL) corresponding to the concentration indicating 0.50 absorbance.

Ferric reducing power assay

Reducing power was carried out using the method described by Oyaizu et al. [26] with some modifications. In a 96-well microplate, 10 µL of the sample at different concentrations (12.5–800 µg/mL) were mixed with 40 µL of sodium phosphate buffer (0.2 M, pH 6.7) and 50 µL potassium ferricyanide solution [$K3Fe(CN)_6$] (1%) and incubated for 20 min at 50 °C. After cooling to room temperature, 10µL ferric chloride ($FeCl_3$, 0.1%), 50µL trichloroacetic acid (10%), and 10µL of water were added to the reaction. The absorbance was immediately recorded at 700 nm. Trolox and ascorbic acid were used as antioxidant standards.

β-carotene bleaching assay

The β-carotene/linoleic acid bleaching assay was conducted according to the method reported by Marco et al. [27]. This method is based on the loss of the yellow color of β-carotene due to its reaction with radicals resulting from the linoleic acid oxidation in an emulsion. The β-carotene/linoleic acid emulsion was prepared by dissolving 0.5 mg of β-carotene in 1 mL of chloroform, to which were added 200 µL of Tween 40 and 25 µL of linoleic acid. The chloroform was therefore removed using a rotary evaporator. The obtained residue was dissolved in hydrogen peroxide (H_2O_2) to provide a yellowish solution, the absorbance of which was adjusted to 0.8 at 470 nm. Then, in a 96 well microplate, 160 µL of the emulsion was added to 40 µL of EO and reference antioxidants (BHA and BHT) at different concentrations. The absorbance at 470 nm was read at 0 min and after 120 min incubation at 50° C. The percent inhibition was calculated by the following equation:

$I\% = 1-((A_{(t=0)}-A_{(t=120)}/A0_{(t=0)}-A0_{(t=120)})*100$.

I%: percentage of inhibition.

$A_{(t=0)}$ and $A_{(t=10)}$ correspond to the absorbance of the reaction medium containing samples at t = 0 and 120 min respectively. $A0_{(t=0)}$ and $A0_{(t=120)}$ are of the absorbance of the negative control without samples at t = 0 and 120 min, respectively.

Antibacterial activity

Microbial strains

Escherichia coli ATCC25922, *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC10876, *Salmonella enteritidis* ATCC13076, *Pseudomonas aeruginosa* ATCC27853, *Lactobacillus rhamnosus* ATCC 53,103 were provided by the Biotechnology research center (Constantine, Algeria). *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Thermophilic yoghurt culture CHR Hansen – YoFlex YC-X16, freeze dried lactic culture for direct Vat) were gently provided by GIPLAIT Constantine, (Algeria) a dairy industry factory.

Disc diffusion method

The essential oil was preliminarily screened for antibacterial activity using the disc diffusion assay according to Rota et al. [19]. After growing in 10 mL Muller Hinton broth (MHB) medium for 18 h at 37 °C, the pathogenic strains suspensions were standardized to approximately 10^5CFU/mL with sterile saline solution (0.85%). Next, 100 µL of the adjusted suspensions were spread over Mueller-Hinton agar previously poured in petri dishes using a sterile cotton swab. A sterile filter paper disk (Whattman N°1. 6 mm diameter) containing 10 µL of the Eo was deposited on the inoculated agar. After incubation at 37 °C during 24 h, the antibacterial activity was expressed as the inhibition zone diameter expressed in mm.

The sensitivity of the three lactic acid bacteria (LAB) to the Eo was carried out following the same method described above using Man, Rogosa and Sharp (MRS broth) and (MRS agar) mediums for *L. bulgaricus* and *L. rhamnosus*. (M17 broth) and (M17 agar) were used for *S. thermophilus* at 37 °C for 48 h. The bacterial inoculums were standardized using MRS and M17 broths and the bacterial charge was adjusted to approximately 10^9 CFU/mL corresponding to a DO of 0.9 at 600 nm.

Determination of the Minimum Inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the EO

The minimum inhibitory concentration was determined using the microdilution method on a 96-well microplate [5, 28]. Twice serial dilutions ranging from 0.1 to 50 µL/mL were prepared in a sterile microplate using dimethyl sulfoxide (DMSO) as a solvent. Then, 160 µL of MHB or MRS or M17 inoculated with 20 µL of the standardized bacterial suspension were added to each well. Controls containing only the bacterial suspension in the corresponding broth or containing only the culture medium were carried out in parallel. The microplate was incubated at 37 °C for 24 h for the pathogenic bacteria and at 37 °C for 48 h for the LAB. The MIC corresponding to the lowest dilution at which no bacterial growth is observed (absence of cloudiness) was determined visually.

The minimum bactericidal concentration (MBC) was determined by transferring 5 µL from each well showing no growth to MH agar medium for pathogenic strains and to MRS or M17 agar for LAB as mentioned before, which will then be incubated at 37 °C for 24 h or 48 h. The MBC corresponds to the lowest dilution showing no bacterial growth visually determined by the absence of colonies.

Yogurt manufacture

Yogurts samples were manufactured in the Food Biotechnology department in the Biotechnology research center (Constantine, Algeria). A 14% (w/v) skim milk was gently provided by the local dairy industry GIPLAIT (Constantine, Algeria). 40 g/L of milk powder were added to the skim milk with 40 g/L of sugar and then heat treated at 95 °C for 15 min and cooled to 45 °C [29]. The starter culture (*Lactobacillus bulgaricus, Streptococcus thermophilus*) provided by the same industry were used to inoculate the milk following the manufacturer recommendations. At this stage, the *Tw*Eo was added to the mixture at the concentration of 25µL/L and mixed. Fermentation was carried out at 45 °C for 4 h. The mixture was finally cooled at 4 °C during the whole period of cold storage of 28 days.

Preparation of Water-Soluble extracts (WSE) of control and supplemented yogurt with (TEo)

In order to obtain the Water-soluble extracts, 10 mL yogurt samples (non-supplemented NSY and supplemented yogurt SY) were centrifuged at 5,000 × g at 4 °C for 20 min following by a filtration through a membrane filter (0.45 nm) and then used to measure the antioxidant activities and total phenolic content at days 1, 7, 21, 28 of cold storage at 4 °C [30].

Determination of total phenolic content of *Tw*Eo and Yogurt samples (NSY and SY)

The total polyphenol content was determined using the Folin-Ciocalteu reagent method [31] according to a microplate assay method described by Muller et al. [32]. Briefly, 20 µL of a 1 mg/mL of *Tw*Eo in Methanol or WSE were added to 100 µL of Folin Ciocalteu reagent (1:10) and 75 µL of Na_2CO_3 in a 96 well microplate. Then, the samples were incubated in the dark for 120 min at room temperature. A blanc was prepared by methanol or distilled water for Eo and WSE respectively. Finally, the absorbance was measured at 765 nm with a microplate reader. The concentration of total phenolics was calculated based on a standard curve of gallic acid. The results were expressed as µg of gallic acid equivalent (GAE) per mL of sample (µg GAE/mL).

Antioxidant activities of WSE of yogurt samples by Oxygen Radical Absorbance Capacity (ORAC)

Antioxidant activities of the SY and NSY extracts were examined for their peroxyl and hydroxyl radical scavenging capacities, using the oxygen radical absorbance capacity (ORAC) assay system [33–35]. A Microplate fluorescence reader (Perkin Elmer, Enspire) was used with the wavelength 485 nm excitation and 538 nm emission. The assay observes over time the decay of the fluorescein (FL) (3',6'-dihydroxy-3 H-spiro(2-benzofuran1,9'-xanthen)-3-one) (Sigma-Aldrich CASNumber: 518-47-8) (fluorescent probe). For this purpose, 25 µL of WSE of SY and NSY were placed into a black 96 well microplate. 25 µL of fluorescein (44,782 µM) were added and the mix was incubated at 37 °C for 20 min before adding 200 µL of AAPH (122,499mM) (2,2'-azo-bis (2-methylpropionamidinedihydrochloride) (Sigma-Aldrich CASNumber: 2997-92-4) as a peroxyl radical generator to initiate the reaction in a final volume of 250 µL. The fluorescence was recorded for 90 min. Trolox solutions (5–40 µM) were used for defining the standard curve. In this assay, the ability of *Tw*Eo to protect fluorescein from oxidation was monitored by its decay curve. ORAC values were calculated by using the areas under the curves (AUC). Results were expressed as micromoles of Trolox Equivalents (TE) per mL of yogurt sample.

Table 1 Essential oil composition of aerial parts of *Tw*Eo harvested in Blida, Algeria

	Compound	RT	RI_{exp}	RI_{lit}	Area%
1	Linalool L	15,55	1080	1082	**89.55**
2	Endo-Borneol	17.25	1145	1148	0.49
3	α- Terpineol	19.55	1170	1172	0.6
4	Linalyl acetate	22.73	1230	1232	**1.74**
5	Carvacrol	24.43	1268	1275	**5.6**
6	Caryophyllene	29.85	1422	1424	**2.01**
Total					99.99
Oxygenated monoterpenes : (No : 1, 2, 3, 5)					96.24
Sesquiterpenes : (No : 6)					2.01
Others : (No : 4)					1.74

Bold values indicate the major compounds essential oil

RI exp = Retention indices calculated from n-alkane series (C8-C20) in HP-5 capillary column,

RI lit = Retention indices of literatures

RT = Retention times

Statistical analysis

Data were expressed as means ± standard deviation of three samples repetitions. Statistical analysis was performed with GRAPHPAD Prism 6. Differences were tested for significance by using the ANOVA (analysis of variance) with a significance level of $p < 0.05$.

Results and discussion

Phytochemical study

The yield of the essential oil extracted from *T.willdenowii* harvested in Blida, Algeria, reached a value of 0.83%. This value is much greater than that obtained by Bnina et al. [36] (0.35%). However, 3% was the yield obtained for the same plant in a previous study [36]. The main compounds constituting the Eo identified by GC / MS are summarized in Table 1. Six components representing 99.99% of the composition of the Eo have been identified. The L-Linalool represented the dominant compound in the Eo (89.55%). In addition, Carvacrol, Caryophyllene and Linalyl acetate are present in the oil (5.62, 2.01, 1.74% respectively). Bnina et al. [36] found that α-pinene, camphor and 1,8-cineole are the dominant compounds of essential oil of *T.hirtus* collected in Monastir (Tunisia). Our result is consistent with the previous study reported by Miguel et al. [37] which also found that Linalool was the major compound in Portuguese thyme while thymol and carvacrol were present at low levels. Radi et al. [38] investigated *T.willdenowii* from Morocco and found oxygenated monoterpenes (32.81%) with the dominance of carvacrol (16.19%), geranyl acetate (8.35%), caryophyllene oxide (6.90%), camphor (5.99%), and (E)-caryophyllene (5.59%), However, recently, Zeghib et al. [15] investigated *Thymus Willdenowii Boiss. & Reut* (synonym: *Thymus hirtus*) growing in Bekira (Constantine, North-East, Algeria) and yielding 1,8-cineole (34.62%), camphor (18.55%), α-pinene (9, 46%) and camphene (5.38%) as the main components among 44 compounds representing 97.3% of the total oil. In addition, Guesmi et al. [39, 40] studies are in concordance with our findings

and reported the absence of thymol in *Thymus hirtus* sp. *algeriensis* growing in Tunisia. The variation in the results for the essential oil composition can be attributed to many factors including the stage of development of the plant, the part of the plant used, the extraction method and analysis conditions, and the location of the harvest [41, 42]. We can observe that the *Tw*Eo studied here is marked by the presence of (96.24%) of oxygenated monoterpenes.

Antibacterial activity

Table 2 summarizes the antibacterial activity of the *Tw*Eo harvested in Blida, (Algeria) and expressed in terms of zones of inhibition (Fig. 1), MIC and MBC. Among the bacteria tested, *B. cereus* was the most sensitive to the oil with an inhibition zone of 31.75 ± 1.50 mm. The *Tw*Eo exhibited also a powerful inhibitory effect against *S. enteritidis*. *E. coli*. and *P. aeruginosa* with inhibition zones of 27.75 ± 0.50 mm. 27.5 ± 0.58 mm and 26.5 ± 0.58 mm. respectively, while *S.aureus* presents an inhibition zone of 13.5 ± 0.58 mm which indicates that this bacteria is more resistant than the others. Eos are considered to be active when they induce an inhibition zone greater than or equal to 12 mm [43]. Our results are in concordance with the study of Guesmi et al. [43], where the antibacterial activity of essential oil of *T.hirtus* harvested in Tunisia revealed strong inhibitory activity on *E.coli* (35–44 mm). The effect of the essential oil on lactic bacteria was weak with inhibition zones ranging from 11.25 ± 0.5 to 17.5 ± 0.58 mm comparing to pathogenic bacteria. Bakirci [44] assessed some herbs (*Allium sp., Thymus sp., Anhriscus sp.* and *Ferule sp*.) on growth and acid production of thermophilic cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) and conclude that there was no effect on bacterial count. Factors determining antibacterial activity are the composition and functional groups present in active components and their synergistic effect [45]. It is commonly believed that the antibacterial activity of phenolic compounds within essential oils, namely carvacrol is attributed to the hydroxyl group contained on their structures. Lipid solubility of these phenolics allows interaction between the antimicrobial and the fatty acid dense microbial cell membrane [46].

Our results confirm what previous studies showed, oxygenated terpenoids such as alcoholic and phenolic terpenes have more antimicrobial activity than the other constituents [47]. The plant studied here presents the major compound of Linalool (89.55%) which is an acyclic terpene alcohol and 5.6% Carvacrol which is a phenolic terpenoid, together contribute to the disruption of the cell membrane increasing its fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration chain, and alteration of ion transport processes as explained by Gann et al. [46].

Table 2 Antibacterial activity of *Tw*Eo

	Inhibition zone (mm)	MIC (µL/mL)	MBC (µL/mL)
E. coli	27.5 ± 0.58	1.56	1.56
S. enteritidis	27.75 ± 0.50	> 0.1	0.1
S. aureus	13.5 ± 0.58	0.8	12.5
B. cereus	31.75 ± 1.50	> 0.1	12.5
P. aeruginosa	26.5 ± 0.58	> 0.1	1.56
L. bulgaricus	13.5 ± 0.58	12.5	50
S. thermophilus	11.25 ± 0.5	6.25	12.5
L. rhamnosus ATCC 53,103	17.5 ± 0.58	> 50	> 50

Despite their outer membrane, Gram – strains are the sensitive to the oil, this is probably due to the presence of porins in the membrane which allows the transport of macromolecules and the constituents of the Eo can penetrate outer the membrane by theses porin proteins and access to the periplasmic and cytoplasmic space [48]. In recent years, food industry developed a great interest in research on antimicrobial compounds, which guarantee food safety and microbiological quality and allows extending the shelf life of food products. However, MIC and MBCs of LAB are greater than those of pathogenic bacteria, we can conclude that there was no great bactericidal effect of *Tw*Eo on theses LAB which are used in the manufacture of dairy products.

Antioxidant activity of *Tw*Eo

The, *In vitro*, assessment of the antioxidant activity of the *Tw*Eo was conducted using five different methods. The results are reported as IC_{50} or Absorbance $_{0.5}$.

The results obtained showed that the oil exerted a weak scavenging effect against the radical DPPH translated by a high value of IC_{50} (1600 µg/mL) represented in Table 3. For all the concentration tested, the standards BHA, BHT and α-tocopherol were the most effectives. Radi et al. [38] found an IC_{50} of 6.78 ± 0.30 µg/mL concerning the same plant cultivated in Morocco. However, testing the Eo with ABTS test gave a moderate effect with an IC_{50} of 38.06 ± 0.30 µg/mL comparing to the standards BHA, BHT and α-tocopherol (1.29 ± 0.30. 1.81 ± 0.10 and 13.02 ± 5.17 respectively) showed in Table 4. The different behaviors of the Eo on DPPH and ABTS can be explained by the fact that the reaction of antioxidants with DPPH radical is slow and occurs over time unlike ABTS which is rapidly reduced [49].

The antioxidant action of the Eo was assessed also by the reduction of copper ions (Cu^{2+}) using the CUPRAC test. As shown in Table 5, a high absorbance (2.64 ± 0.10) was recorded with *Tw*Eo at 1600 µg/mL. The concentration of the oil which gave an absorbance of 0.5 is estimated to be 56.15 ± 1.07 µg/mL wich is greater than standards. On the other hand, the capacity of the oil to reduce iron ions

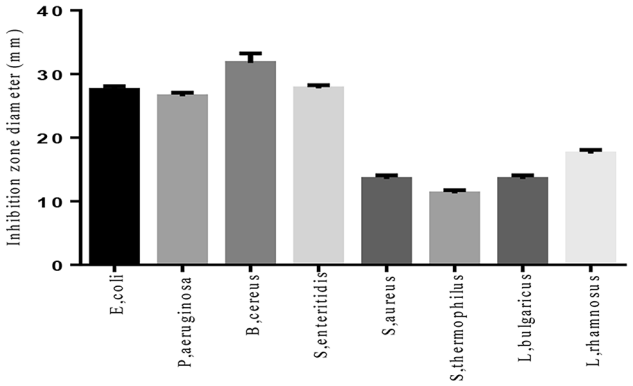


Fig. 1 Inhibition zones diameters in mm

was evaluated Table 6, the Eo at the high concentration used (800 µg/mL) showed a slight increase in absorbance (0.30 ± 0.01) but this result is considered as low comparing to the standards (5.25 ± 0.20 and 3.62 ± 0.29 for Trolox and ascorbic acid respectively). Inhibition of lipid peroxidation by *Tw*Eo was assessed by the nonenzymatic β-carotene bleaching test. The oxidation of linoleic acid leads to the generation of free radicals which can attack the β-carotene in the absence of antioxidants and undergoes a rapid discoloration (loss of orange color) resulting from the loss of its double bonds. *Tw*Eo had lower antioxidant activity (> 800 µg/mL) comparing to standards BHA and BHT (0.91 ± 0.01 and 1.05 ± 0.03) as mentioned in Table 7.

Overall, the antioxidant effect exhibited by the essential oil can be considered as weak and this may be related to its composition with Linalool as the main constituent, which is an acyclic terpene alcohol and previously reported to have low antioxidant activity [47]. In addition, it was reported a low antioxidant activity of *T. hirtus* sp. *algeriensis* Eo assessed using DPPH [15]. The attack of beta-carotene by

Table 3 DPPH free radical Scavenging effect of *Tw*Eo and the standards

DPPH								
T. willdenowii Eo (µg/mL)	25	50	100	200	400	800	1600	IC_{50} (µg/mL)
	na	na	na	0.30 ± 0.92	5.47 ± 1.53	12.00 ± 1.08	20.56 ± 0.53	> 1600
Standards	12.5	25	50	100	200	400	800	IC_{50} µg/mL
BHA	76.55 ± 0.48	79.89 ± 0.26	81.73 ± 0.10	84.18 ± 0.10	87.13 ± 0.17	89.36 ± 0.19	90.14 ± 0.00	6.14 ± 0.41
BHT	49.09 ± 0.76	72.63 ± 2.06	88.73 ± 0.89	94.00 ± 0.31	94.97 ± 0.08	95.38 ± 0.41	95.02 ± 0.23	12.99 ± 0.41
α-Tocopherol	37.21 ± 1.82	81.53 ± 1.51	89.23 ± 0.12	89.38 ± 0.19	89.45 ± 0.22	89.99 ± 0.23	89.52 ± 0.33	13.02 ± 5.17

Table 4 ABTS free radical Scavenging effect of *Tw*Eo and the standards

ABTS								
T. willdenowii Eo (µg/mL)	25	50	100	200	400	800	1600	IC_{50} (µg/mL)
	37.12 ± 1.43	62.69 ± 0.83	88.26 ± 0.19	91.44 ± 0.44	91.88 ± 0.25	87.65 ± 0.00	81.89 ± 1.43	38.06 ± 0.30
Standards	12.5	25	50	100	200	400	800	*IC_{50}µg/mL*
BHT	69.21 ± 0.40	78.23 ± 1.34	88.12 ± 1.28	88.76 ± 3.07	90.85 ± 1.74	90.95 ± 0.51	96.68 ± 0.39	1.29 ± 0.30
BHA	92.83 ± 1.42	94.68 ± 0.42	94.95 ± 0.90	95.32 ± 0.25	95.59 ± 0.47	95.83 ± 0.15	95.86 ± 0.10	1.81 ± 0.10
α-Tocopherol	37.21 ± 1.82	81.53 ± 1.51	89.23 ± 0.12	89.38 ± 0.19	89.45 ± 0.22	89.99 ± 0.23	89.52 ± 0.33	13.02 ± 5.17

Table 5 Cupric reducing antioxidant capacity (CUPRAC) of *Tw*Eo and the standards

CUPRAC								
T. willdenowii Eo (µg/mL)	25	50	100	200	400	800	1600	$A_{0.50}$ (µg/mL)
	0.32 ± 0.01	0.38 ± 0.11	0.46 ± 0.03	0.76 ± 0.02	1.22 ± 0.06	1.91 ± 0.15	2.64 ± 0.10	56.15 ± 1.07
Standards	12.5	25	50	100	200	400	800	$A_{0.5}$(µg/mL)
BHA	1.2 ± 0.5	1.5 ± 0.1	3.14 ± 0.46	3.58 ± 0.42	3.35 ± 0.20	3.77 ± 0.19	3.92 ± 0.13	5.35 ± 0.71
BHT	1.41 ± 0.03	2.22 ± 0.05	2.42 ± 0.02	2.50 ± 0.01	2.56 ± 0.05	2.86 ± 0.07	3.38 ± 0.13	8.97 ± 3.94

Table 6 Reducing power of *Tw*Eo and the standards

Reducing power								
T. willdenowii Eo (µg/mL)	12.5	25	50	100	200	400	800	$A_{0.5}$ (µg/mL)
	0.08 ± 0.00	0.09 ± 0.00	0.1 ± 0.01	0.13 ± 0.01	0.19 ± 0.03	0.22 ± 0.01	0.30 ± 0.01	> 800
Standards (µg/mL)	0.0976	0.195	0.390	0.781	1.562	3.125	6.25	$A_{0.5}$ (µg/mL)
Trolox	0.07 ± 0.00	0.08 ± 0.00	0.09 ± 0.01	0.13 ± 0.00	0.19 ± 0.02	0.28 ± 0.05	0.60 ± 0.04	5.25 ± 0.20
Ascorbic acid	0.07 ± 0.00	0.09 ± 0.01	0.12 ± 0.01	0.17 ± 0.01	0.25 ± 0.02	0.47 ± 0.03	0.79 ± 0.09	3.62 ± 0.29

Table 7 Beta carotene/ linoleic acid bleaching test of *Tw*Eoand the standards

Extracts	β-carotene linoleic acid bleaching assay							
	12.5 µg	25 µg	50 µg	100 µg	200 µg	400 µg	800 µg	IC_{50} µg/mL
*T. willdenowii Eo (*µg/mL)	na	na	na	na	37.75 ± 1.84	44.96 ± 4.23	49.31 ± 8.35	> 800
BHT	88.29 ± 0.10	91.70 ± 0.36	93.65 ± 0.30	93.68 ± 0.46	94.49 ± 0.07	94.88 ± 0.10	95.58 ± 0.19	0.91 ± 0.01
BHA	93.48 ± 0.44	95.52 ± 0.33	96.34 ± 0.55	97.56 ± 0.19	97.64 ± 2.22	97.85 ± 0.32	99.66 ± 0.52	1.05 ± 0.03

free radicals was delayed by *TwEo* but in a week capacity. This result is in concordance with Zeghib et al. [15], the same plant exhibited a weak antioxidant capacity which may support our findings.

Total phenolic content of *Tw*Eo and supplemented yogurt

The total phenolic content (TPC) assay showed a gradually increase in TPC in supplemented yogurt ($p<0.0001$) from day 1 to day 21 and reached the maximum at day 21 (252.92 ± 3.01 µg GAE/mL of yogurt) (Fig. 2). A slight drop occurred from day 21 until day 28. Furthermore, the Eo presented the highest value of total phenolic content (407.65 ± 13.08 µg GAE/mg of Eo). The presence of the phenol monoterpenoid Carvacrol in *Tw*Eo may be related to the obtained results in this study. Moreover, metabolic pathways of biosynthesis or degradation of phenolic compounds by LAB have not been completely described [50].

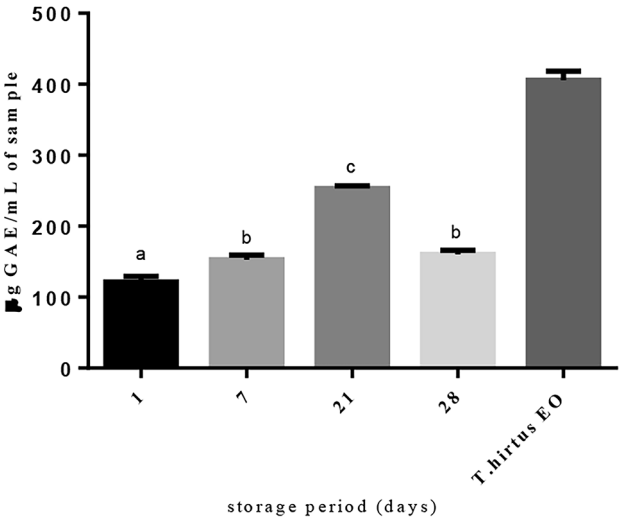


Fig. 2 Total phenolic content of *Tw*Eo and Supplemented yogurt SY at different days of storage (1, 7, 21, 28)

Antioxidant activity of supplemented yogurt with ORAC assay

It is known that the best way to extend the shelf life of the milk is by fermentation [51]. Hence, Antioxidant activity in yogurt is essential for enhancing the shelf life and inhibiting the oxidation reactions in the human body [52]. However, in this study the antioxidant capacity of the SY and NSY yogurts with *Tw*Eo was assessed by ORAC test and showed

Table 8 Oxygen radical absorbance capacity of NSY and SY yogurts

	ORAC (µM Trolox Equivalents (TE) per mL of yogurt)	
Storage day	NSY	SY
1	221.10 ± 6.49[a]	195.48 ± 18.50 [a]
7	160.52 ± 10.48 [a]	162.34 ± 22.63[b]
21	157.66 ± 15.41 [a]	188.79 ± 12.99 [b]
28	193.66 ± 1.47 [a]	197.10 ± 5.78 [c]

NYS: non supplemented yogurt with *Tw*Eo

SY: supplemented yogurt

Values are given as means ± standard deviation (SD)

Different values with different superscripts in the same column are significantly different

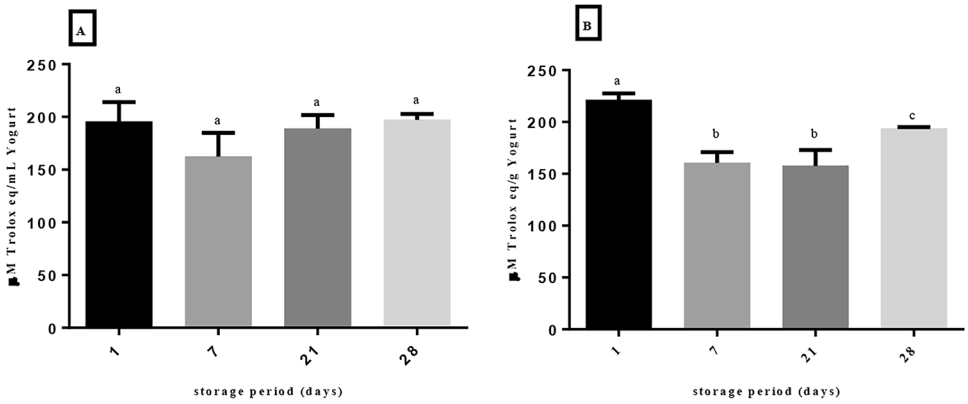


Fig. 3 Antioxidant capacity of yogurt samples with ORAC test A.ORAC of supplemented yogurt with (*Tw*Eo) (SY) B.ORAC of non-supplemented (NSY)

in Table 8. Our results conclude that there was no significant difference in ORAC values from day 1 to 28 ($p<0.05$) for SY (195.48 ± 18.50, 162.34 ± 22.63, 188.79 ± 12.99, 197.10 ± 5.78 µM Trolox Equivalents (TE) per mL of yogurt respectively). For the NSY there was a highly significant decrease between day 1 and the other days 7, 21, 28 ($p<0.0001$) as showed in Fig. 3. The ORAC assay is based on H atom transfer mechanisms and measures the oxidative degradation of a fluorescent molecule after being mixed with free radical generators [53]. In this study, the loss of antioxidant capacity in NSY may be explained by the lack of added antioxidants compounds present in the essential oil that constitute probably a good protection against a lipid peroxidation as described in previous studies [54, 55].

Conclusion

The present work assessed the chemical composition, antibacterial and antioxidant activity of *Thymus willdenowii* essential oil and the evaluation of total phenolic content and antioxidant potential of a supplemented yogurt with the oil. According to the analyzed data, we can conclude that the oil exhibited a strong antibacterial activity against pathogenic bacteria which is an important aspect in agri-food industries, this antibacterial potential is in relation with total phenolic content of this oil studied also in this work. Moreover, the definition of the MICs of lactic acid bacteria allows us to fix the limits of the acceptable doses to incorporate into yogurt following the MICs. These findings express an encouraging way to substitute synthetic molecules or preservatives used in dairy industries. This study allows us also to define the antioxidant potential of a supplemented fermented milk with this oil and this supplementation didn't change the antioxidant potential and stabilized it over the 28 days of cold storage.

References

1. A. Hassoun, M. Carpena, M.A. Prieto, J. Simal-Gandara, F. Özogul, Y. Özogul, ÖE. Çoban, M. Guðjónsdóttir, F.J. Barba, F.J. Marti-Quijal, A.R. Jambrak, N. Maltar-Strmečki, J.G. Kljusurić, J.M. Regenstein, Antioxidants **9**, 882 (2020). https://doi.org/10.3390/antiox9090882
2. K. Wińska, W. Mączka, J. Łyczko, M. Grabarczyk, A. Czubaszek, A. Szumny, Molecules **24**, 2130 (2019). https://doi.org/10.3390/molecules24112130
3. D.N. Reddy, Essential Oils Extracted from Medicinal Plants and Their Applications, in *Natural Bio-active Compounds*, ed. by M. Akhtar, M. Swamy, U. Sinniah (Springer, Singapore, 2019), https://doi.org/10.1007/978-981-13-7154-7_9
4. M. Kačániová, L. Galovičová, E. Ivanišová, N.L. Vukovic, J. Štefániková, V. Valková, P. Borotová, J. Žiarovská, M. Terentjeva, S. Felšöciová, E. Tvrdá, Foods **9**, 282 (2020). https://doi.org/10.3390/foods9030282
5. S. Prabuseenivasan, M. Jayakumar, S. Ignacimuthu, BMC Complement. Altern. Med. **6**, 39 (2006). https://doi.org/10.1186/1472-6882-6-39
6. M. Catauro, F. Bollino, E. Tranquillo, L. Sapio, M. Illiano, I. Caiafa, S. Naviglio, J. Essent. Oil Res. **29**, 461 (2017). https://doi.org/10.1080/10412905.2017.1351405
7. K. Böhme, J. Barros-Velázquez, P. Calo-Mata, S.P. Aubourg, in *Antimicrobial Compounds*, edited by T. G. Villa and P. Veiga-Crespo (Springer Berlin Heidelberg, Berlin, Heidelberg, 2014), pp. 51–81.https://doi.org/10.1007/978-3-642-40444-3_3
8. A.P. Mishra, H.P. Devkota, M. Nigam, C.O. Adetunji, N. Srivastava, S. Saklani, I. Shukla, L. Azmi, M.A. Shariati, H.D. Melo, Coutinho, A. Mousavi Khaneghah, LWT **133**, 110116 (2020). https://doi.org/10.1016/j.lwt.2020.110116
9. N.R. Menković, K.P. Šavikin, G.M. Zdunić, G. Gojgić-Cvijović, J. Essent. Oil Res. **21**, 89 (2009). https://doi.org/10.1080/10412905.2009.9700119
10. S. Roller, N. Ernest, J. Buckle, J. Altern. Complement. Med. **15**, 275 (2009). https://doi.org/10.1089/acm.2008.0268
11. F. Yangilar, P.O. Yildiz, J. Food Process. Preserv. **42**, e13332 (2018). https://doi.org/10.1111/jfpp.13332
12. H. Punia, J. Tokas, A. Malik, S. Sangwan, S. Baloda, N. Singh, S. Singh, A. Bhuker, P. Singh, S. Yashveer, S. Agarwal, V.S. Mor, Molecules **25**, 3328 (2020). https://doi.org/10.3390/molecules25153328
13. I.T. Khan, M. Nadeem, M. Imran, R. Ullah, M. Ajmal, M.H. Jaspal, Lipids Health Dis **18**, 41 (2019). https://doi.org/10.1186/s12944-019-0969-8
14. A. Sharifi-Rad, G. Sureda, M. Tenore, M. Daglia, M. Sharifi-Rad, R. Valussi, M. Tundis, M. Sharifi-Rad, A. Loizzo, R. Ademiluyi, S. Sharifi-Rad, Ayatollahi, M. Iriti, Molecules **22**, 70 (2017). https://doi.org/10.3390/molecules22010070
15. A. Zeghib, C.-A. Calliste, A. Simon, R. Charfeddine, M. Aouni, J.-L. Duroux, A. Kabouche, Z. Kabouche, Nat. Prod. Res. **35**, 845 (2021). https://doi.org/10.1080/14786419.2019.1602830
16. M. Ouknin, A. Romane, J. Costa, L. Majidi, Nat. Prod. Res. **33**, 2398 (2019). https://doi.org/10.1080/14786419.2018.1443089
17. D. Sarfaraz, M. Rahimmalek, G. Saeidi, Sci. Rep. **11**, 5019 (2021). https://doi.org/10.1038/s41598-021-84449-6
18. G. Vardar-Ünlü, F. Candan, A. Sökmen, D. Daferera, M. Polissiou, M. Sökmen, E. Dönmez, B. Tepe, J. Agric. Food Chem. **51**, 63 (2003). https://doi.org/10.1111/jfpp.13332
19. M.C. Rota, A. Herrera, R.M. Martínez, J.A. Sotomayor, M.J. Jordán, Food Control **19**, 681 (2008). https://doi.org/10.1016/j.foodcont.2007.07.007
20. CFR - Code of FederaL ReguLations TitLe 21. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=182&showFR=1
21. D.J. Daferera, B.N. Ziogas, M.G. Polissiou, J. Agric. Food Chem. **48**, 2576 (2000). https://doi.org/10.1021/jf990835x
22. J. Safaei-Ghomi, A.H. Ebrahimabadi, Z. Djafari-Bidgoli, H. Batooli, Food Chem. **115**, 1524 (2009). https://doi.org/10.1016/j.foodchem.2009.01.051
23. M.S. Blois, Nature **181**, 1199 (1958). https://doi.org/10.1038/1811199a0
24. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Free Radic. Biol. Med. **26**, 1231 (1999). https://doi.org/10.1016/S0891-5849(98)00315-3
25. R. Apak, K. Güçlü, M. Özyürek, S.E. Karademir, J. Agric. Food Chem. **52**, 7970 (2004). https://doi.org/10.1021/jf048741x
26. M. Oyaizu, Jpn J. Nutr. Diet. **44**, 307 (1986). https://doi.org/10.5264/eiyogakuzashi.44.307
27. G.J. Marco, J. Am. Oil Chem. Soc. **45**, 594 (1968). https://doi.org/10.1007/BF02668958

28. A. Chebaibi, Z. Marouf, F. Rhazi-Filali, M. Fahim, A. Ed-Dra, Phytothérapie **14**, 355 (2016). https://doi.org/10.1007/s10298-015-0996-1
29. M. Michael, R.K. Phebus, K.A. Schmidt, Int. Dairy J. **20**, 665 (2010)
30. A. Perna, I. Intaglietta, A. Simonetti, E. Gambacorta, J. Dairy Sci. **97**, 6662 (2014). https://doi.org/10.3168/jds.2013-784
31. V.L. Singleton, J.A. Rossi, Am. J. Enol. Vitic **16**, 144 (1965)
32. L. Müller, S. Gnoyke, A.M. Popken, V. Böhm, LWT - Food Science and Technology **43**, 992 (2010). https://doi.org/10.1016/j.lwt.2010.02.004
33. M.E. Alañón, L. Castro-Vázquez, M.C. Díaz-Maroto, M.H. Gordon, M.S. Pérez-Coello, Food Chem. **128**, 997 (2011). https://doi.org/10.1016/j.foodchem.2011.04.005
34. N. Bernaert, D. De Paepe, C. Bouten, H. De Clercq, D. Stewart, E. Van Bockstaele, M. De Loose, B. Van Droogenbroeck, Food Chem. **134**, 669 (2012). https://doi.org/10.1016/j.foodchem.2012.02.159
35. Y. Rao. Fu, Y. Zhang. Guo, F. Chen, South Afr. J. Bot. **93**, 98 (2014). https://doi.org/10.1016/j.sajb.2014.04.003
36. E.B. Bnina, S. Hammami, M. Daami-Remadi, I. Cheraif, H. Hajjlaoui, H.B. Jannet, M.B. Said, Z. Mighri, J. Essent. Oil Res. **21**, 567 (2009). https://doi.org/10.1080/10412905.2009.9700247
37. M.G. Miguel, C. Gago, M.D. Antunes, C. Megías, I. Cortés-Giraldo, J. Vioque, A.S. Lima, A.C. Figueiredo, Evidence-Based Complementary and Alternative Medicine 2015, 1 (2015).https://doi.org/10.1155/2015/851721
38. F. Radi, M. Bouhrim, H. Mechchate, M. Al-zahrani, A. Qurtam, A. Aleissa, D. Aziz, N. Hadaq, Z. Touria, Plants **11**, 1 (2021). https://doi.org/10.3390/plants11010015
39. F. Guesmi, M. Ben Ferhat, M. Mejri, A. Landoulsi, Lipids Health Dis **13**, 114 (2014). https://doi.org/10.1186/1476-511X-13-114
40. F. Guesmi, S. Issam, H. Najla, A. Landoulsi, Annual Res. Rev. Biology 1 (2018).https://doi.org/10.9734/ARRB/2018/43583
41. T. Baj, I. Korona-Głowniak, R. Kowalski, A. Malm, Open Chem. **16**, 317 (2018). https://doi.org/10.1515/chem-2018-0032
42. S. Djebili, M. Taş, A. Bouguerra, S. Küçükaydın, C. Ozgur, M. Duru, M. Barkat, J. Food Meas. Charact. **16**, 987 (2021). https://doi.org/10.1515/chem-2018-003210.1007/s11694-021-01220-1
43. F. Guesmi, I. Saidi, H. Bouzenna, N. Hfaiedh, A. Landoulsi, South Afr. J. Bot. **127**, 234 (2019). https://doi.org/10.1016/j.sajb.2019.09.004
44. I. Bakirci, Nahrung **43**, 333 (1999).https://doi.org/10.1002/(SICI)1521-3803(19991001)43:5<333::AID-FOOD333>3.0.CO;2-T
45. A.C. Guimarães, L.M. Meireles, M.F. Lemos, M.C.C. Guimarães, D.C. Endringer, M. Fronza, R. Scherer, Molecules **24**, 2471 (2019). https://doi.org/10.3390/molecules24132471
46. L.D. Gann, A.F.T. Council, C.R. Hodges, Antimicrobial Activity of Essential Oils and Their Components Against Lactic Acid Bacteria (2013).https://trace.tennessee.edu/utk_gradthes/2604
47. H. Zengin, A. Baysal, Molecules **19**, 17773 (2014). https://doi.org/10.3390/molecules1911177733
48. H. Nikaido, Science **264**, 382 (1994). https://doi.org/10.1126/science.8153625
49. A. Emad. Shalaby, S.M.M. Shanab, NISCAIR-CSIR, India 556 (2013).https://www.researchgate.net/publication/287536387
50. H. Rodríguez, J.A. Curiel, J.M. Landete, B. de las Rivas, F.L. de Felipe, C. Gómez-Cordovés, J.M. Mancheño, R. Muñoz, Int. J. Food Microbiol. **132**, 79 (2009). https://doi.org/10.1016/j.ijfoodmicro.2009.03.025
51. T. Gjorgievski, M. Dimitrovska, and Shariati (2014), Journal of Hygienic Engineering and Design 615 (2014).https://www.researchgate.net/publication/286440609_DETERMINATION_OF_THE_ANTIOXIDANT_ACTIVITY_IN_YOGURT
52. A.B. Shori, J. Taibah Univ. Sci. **14**, 1000 (2020). https://doi.org/10.1080/16583655.2020.1798072
53. A. Fardet, E. Rock, Nutr. Res. Rev. **31**, 52 (2018). https://doi.org/10.1017/S0954422417000191
54. A. Citta, A. Folda, V. Scalcon, G. Scutari, A. Bindoli, M. Bellamio, E. Feller, M.P. Rigobello, Food Sci. Nutr. **5**, 1079 (2017). https://doi.org/10.1002/fsn3.493
55. W. Liao, W. Badri, A.H. Alhibshi, E. Dumas, S. Ghnimi, A. Gharsallaoui, A. Errachid, A. Elaissari, in *Black Cumin (Nigella Sativa) Seeds: Chemistry, Technology, Functionality, and Applications*, edited by M. Fawzy Ramadan (Springer International Publishing, Cham, 2021), pp. 433–455.http://link.springer.com/https://doi.org/10.1007/978-3-030-48798-0_28

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.