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Evaluation of the antimicrobial, antioxidant and anticancer activity of *Teucrium polium* extracts and application of its nanoparticles as food preservative in some dairy products

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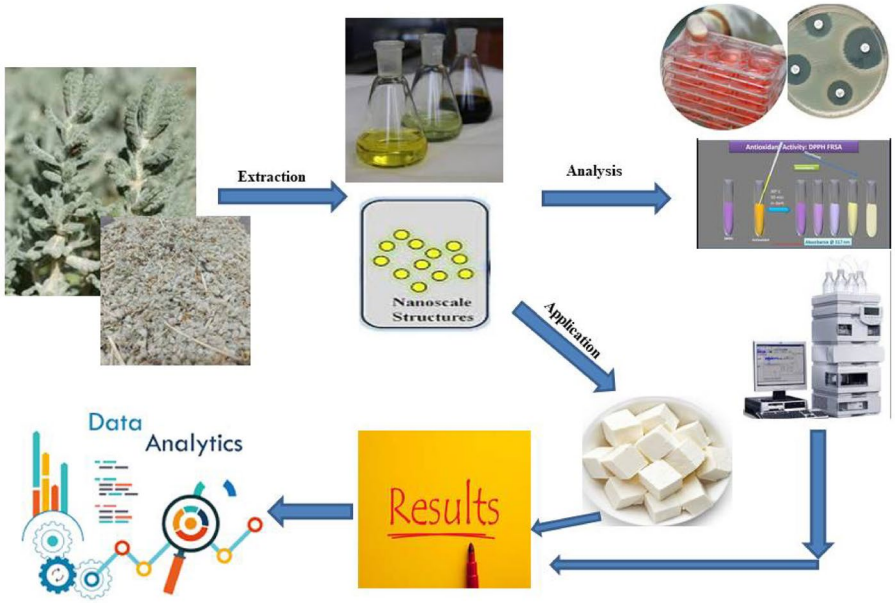
Abstract

Teucrium polium is a natural herbal and it is consumed as herbal tea. The current study's objective is to assess *T. polium* extracts' antibacterial properties and its potential preservative activity of its nanoparticles in some dairy products. Ethanol, methanol, chloroform and ethyl acetate extracts were obtained and tested for its antimicrobial, antioxidants, anticancer and polyphenols were determined. An ethyl acetate extract nanoparticle was obtained and nanoparticles were characterized. The preservative effect of *T. polium* ethyl acetate nanoparticles in soft white cheese as a dairy model was evaluated. Ethyl acetate extracts and its nano form were the only extracts exhibited antimicrobial activity against all tested bacteria, fungi and yeast. However, ethanol and methanol extracts exhibits activity against *Candida albicans*, Gram Positive and Gram negative bacteria except *E. coli and Salmonella enterica* but not against fungi. No antibacterial activity was demonstrated by the chloroform extract. Methanol extract recorded the highest antioxidant activity with 58 µg/ml followed by ethanol and ethyl acetate extracts. The three extracts found to contains polyphenols compounds include Protocatechuic acid, *p*-hydroxybenzoic acid, Syringic acid, Vanillic acid, Rutin, Quercetin and Kaempferol. The potency of extracts toward breast cancer cell lines was relatively weak. In-Situ application of *T. polium* ethyl acetate extract Nano-formulated in soft white cheese as a food model extract significantly reduced numbers of total bacterial count as well as mold count by 1.15 and 2.78 CFU/ml respectively after three weeks of storage the thing which indicates the preservative effect of *T. polium* extracts.

Keywords Teucrium polium, Antimicrobial activity, Soft cheese, Antioxidant, Anticancer, Solvent extracts

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Graphical abstract



1 Introduction

As a seasonally dimorphic plant, *Teucrium polium* appears quite differently in the winter and summer [1]. The stereomediterranean region is home to the perennial shrub species *T. polium* L., commonly referred to as golden germander, which belongs to the Lamiaceae family. Since more than 2,000 years ago, this plant and others within the same genus have been utilized in green therapy. Neo-clerodane-diterpenoids and flavonoids are isolated from *T. polium* [2]. *T. polium* is primarily used in traditional medicine to enhance mental function. However, in a mouse model of scopolamine-induced amnesia, it was recently reported that *T. polium* extract possesses anti-amnestic qualities. *T. polium* may therefore be a useful herbal substitute for improving memory, and further research into its potential for treating cognitive impairments will be beneficial [3]. Saleh et al. [4], reported that all of 27 and 28 compounds were identified from the essential oils (EOs) of *T. polium* extracted by hydrodistillation (HD) and microwave- assisted extraction (MAE) respectively. The hydro-distilled EO was mainly represented (57.68%) by Oxygenated sesquiterpenes. On the other hand, the MAE method gave sesquiterpene hydrocarbons by (54.48%) [4]. the aerial parts of *T. polium* have an antiseptic properties and antioxidants [5] and anti-inflammatory [6]. *Teucrium polium* is well known for its antipyretic, diuretic, antispasmodic, antihypertensive and antidiabetic properties [7]. The essential oil from *T. polium* exhibited moderate antibacterial and antifungal activity [8]. The Turkish T. polium extracts exhibit strong anti-Staphylococcus aureus activity but no anti- *E. coli* activity [9]. The ethyl acetate, n-butanol and chloroform extract of *T. Polium* exhibited low antifungal and antibacterial activity [10]. The antimicrobial activity of plant extracts is related to the presence of various bioactive secondary metabolites such as phenolic compounds, alkaloids and terpenoids, so antioxidant analysis for ethanol, methanol, chloroform and ethyl acetate extracts were conducted in our study. Essential oils have garnered increased interest as possible alternatives to synthetic

molecules used in food preservation and insecticides against phytopathogenic microorganisms. Numerous pharmacological properties; hypoglycemic, hepatoprotective, hypolipidemic, antioxidant, anti-inflammatory, broad-spectrum antimicrobial activities were reported for different extracts of *T. polium* [11]. Additionally, *T. Polium* has the ability to inhibit *Pseudomonas aeruginosa* PAO1 motility in a concentration dependent manner [12]. Terpenes, terpenoids, as well as flavonoids; the main isolated compound from *T. polium* could have a considerable application in food preservation [11]. The main gap to be solved in this study was to evaluate the antibacterial, antifungal activity, anticancer of ethanol; methanol, chloroform and ethyl acetate extract against some pathogens and studying shelf-life extension of some dairy products through controlling the growth of accidently incident fungi using nanoparticles form of *T. polium* extract.

2 Materials and methods

2.1 Plant materials

Teucrium polium L. was taxonomically identified using voucher number 903 from the Herbarium of National Research Center after it was taken from Wadi El Arbean in St. Catherine, South Sinai.

Ethics approval and agreement to participate None of the writers have conducted any experiments with humans or animals in this work. Local and national regulations were followed when collecting the plants and plant materials for our investigation.

2.2 Extract Preparation

The aerial parts of *T. polium* were collected during spring (May - June) from Sinai, St. Catherine, Wadi El Arbaen, dried for 10 days in the shadow at room temperature and then ground to a fine powder. Twenty-five grams of powder were extracted by ethanol, methanol, chloroform and ethyl acetate solvent (1:10) then shaking overnight and filtration to collect the supernatant [13]. Solvents were then removed by evaporation and the dry films were kept refrigerated until subsequent use.

3 Determination of antimicrobial activity *T. polium* extracts

3.1 Tested microorganisms

Eight bacterial strains were used to evaluate the antibacterial activity of the obtained solvent extracts of *T. polium*. Four Gram positive; *Bacillus cereus* EMCC 1080, *Staphylococcus aureus* ATCC 13565, *Listeria monocytogenes* and *Streptococcus mutans* (kindly provided from collection of dairy microbiological lab, NRC, Egypt [14] and Four Gram negative bacteria; *Escherichia coli*, *Escherichia coli* O157-H7 ATCC 51659, *Salmonella enterica* and *Pseudomonas auruginosa* NRRLB- 272 and *Candida albicans* ATCC 10231 as yeast. The strains were grown on nutrient agar dishes at 37 °C for 24 h and kept in a refrigerator at 4 °C until use. The antifungal activity was evaluated using four fungal strains; including *Aspergillus flavus* NRRL 3357, *A. ochraceus* ITAL 14, *A. niger* ATCC-16888, and *Fusarium verticillioides* ITEM 10027 grown on Potato Dextrose Agar (PDA) media at 28 °C for 5 days.

The antibacterial assay conducted using bacterial isolates grown in tryptic soy broth tubes for 4 h (with turbidity of 0.5 McFarland standards). Antibacterial activity via disc diffusion (inhibition zones diameters in mm) was assayed on nutrient agar plates and incubation for 18 h at 35 °C following the British Society for Antimicrobial

Chemotherapy (BSAC) guidelines [15]. Likewise, from fresh fungal spore suspension in sterile tween 80 water solution antifungal activity was carried out onto Potato Dextrose Agar (PDA) medium, after incubation for 48 h. at 30 °C. Clotrimazole (10 µg/disc) and Amoxicillin/Clavulanic acid (30 µg/disc) were respectively considered as antifungal and antibacterial positive controls.

4 Determination of the minimal inhibitory concentrations (MICs)

MICs were carried out by poisoned agar [16]. Extracts were introduced to the molten agar to achieve final concentrations that ranged from 0.5 mg/ml to 50 mg/ ml. Poisoned agar plates were inoculated (spot) with 1 µl of 10^7 CFU/ml of microbial suspension. Plates were incubated and results were measured as the lowest concentration, at which the growth disappeared.

5 In vitro antioxidant activity assay of *T. polium* extracts

5.1 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$)- free radical scavenging assay

The antioxidant activity was evaluated using DPPH assay as follows:

Different concentrations (50 to 200 µg/ml) of the various extracts were added to DPPH• disolving in methanol (0.0039%) [17]. The absorbance at 517 nm was measured following a 30-minute incubation period at room temperature. The following formula (Eq. 1) was used to determine the percentage inhibition of DPPH.

$$\text{Radical Scavenging Activity } \% = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100 \tag{1}$$

where Ac is the DPPH solution's absorbance and As is the sample's absorbance. A graph showing the % inhibition against extract concentration was used to calculate the IC_{50}. As a standard, butylated hydroxytoluene BHT was employed at varying concentrations (0–100 µg/ml).

5.2 Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) $ABTS^{\cdot+}$ free radical scavenging assay

Each extract was added to $ABTS^{\cdot+}$ solution at varying quantities (50–200 µg/ml), incubated for 10 min at 37 °C in the dark, and measured for absorbance at 734 nm [18]. The following formula (Eq. 2) was used to determine the inhibition of $ABTS^{\cdot+}$ radical scavenging activity:

$$\% \text{ Inhibition } = \frac{\text{A0} - \text{A1}}{\text{A0}} \times 100 \tag{2}$$

Where A0 is the control reaction's $ABTS^{\bullet+}$ absorbance, and A1 is the sample's $ABTS^{\cdot+}$ absorbance. The effective concentration at which the $ABTS^{\cdot+}$ radical was 50% scavenged was identified as the IC_{50} value.

6 Polyphenols analysis of *T. polium* extracts using HPLC

T. polium extracts in ethanol, methanol, and ethyl acetate were redissolved in 2 milliliters of methanol, filtered through a 0.22 µm filter, and then subjected to HPLC analysis. The HPLC analysis was carried out using a liquid chromatograph from Agilent Technologies' 1260 series, which is outfitted with a diode-array detector and an auto sampler, in accordance with the methodology outlined by Kim et al., [19]. An analytical column with

a C18 guard column (Phenomenex, Torrance, CA) and an Eclipse XDB-C18 (150 × 4.6 µm; 5 µm). As the mobile phase, acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B) were employed. The flow rate was set at 0.8 ml/min for a total run time of 60 min, injection volume was 50 µl and peaks were observed simultaneously at 280 and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively as well as 360 nm for flavonoids.

7 Nanoparticles Preparation of Ethyl acetate extract of *T. polium*

Synthesize *Teucrium polium*-NPs using freeze drying technique with some modifications [20]. Briefly, dissolving 0.3 g of *T. polium* ethyl acetate extract in 5 ml distaled water and 1 ml tween 80, then add it dropwise to 10 ml d.w containing 2 g of gum Arabic with melding agitation for 72 h at room temperature. The previous mixture was sonicated for 20 min using DAIGGER ULTRA-SONIC Model GEX 750, USA. The final product freeze-dried at -55 °C for 24 h, and stored at -20 °C in sealed container until use.

7.1 Physical characterization of nanoparticles

7.1.1 Fourier transform infrared spectroscopy (FTIR)

FTIR was used to evaluated the association degree between substances of *T. polium* ethyl acetate extract and gum arabic as a polymerm, the resolution was 4 cm^{-1} and the scale range was 4000 –400 cm^{-1}.

7.1.2 Zetasizer

Zetasizer was used to evalute the zeta potential values, polydispersity index (PDI) and size of particles (Nano- ZS, Malvern instruments Ltd.,UK). The *T. polium* nanoparticles were measured by dispersing the sample in distilled water, followed by brief sonication to obtain a homogeneous suspension [21].

7.1.3 Transmission electron microscope (TEM)

The structural and morphological features of the prepared *Teucrium polium* -NP were analyzed using a high-resolution transmission electron microscope (HR-TEM, JEM-2100, JEOL Ltd., Tokyo, Japan). A suspension of the sample was ultrasonicated for 5 min in an ultrasonic bath (Crest Ultrasonics Corp., New Jersey, USA) to achieve good particle dispersion. A few drops of the dispersed solution were placed onto a carbon-coated copper grid and allowed to air-dry. To enhance image contrast, 1% phosphotungstic acid solution was added dropwise to the grid, followed by drying at room temperature. The dried grids were subsequently examined under the HR-TEM to obtain detailed microstructural images.

8 Assessment of the invitro Anti-Cancer effect of *T. polium* extracts

8.1 Cell culture

Breast cancer cell lines; MDA-MB-231 and MCF-7 (VACSERA, Egyptian) were cultured in high glucose, L-glutamine RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma Aldrich Chemical Co., St. Louis, MO, USA). Cells were maintained humidified under 5% CO_2 at 37 °C with 1:4 ratio refreshment every 3 days.

8.2 Cell viability assay

Skehan et al. used the Sulphorhodamine-B (SRB) technique to determine the cytotoxicity of various extracts [22]. To create a final stock solution of 25 mM, *T. polium* extracts were dissolved in DMSO. *T. polium* extracts were prepared at the final tested concentration using serum-free medium. In order to create the DMSO vehicle control, the maximum amount of DMSO utilized to manufacture the tested *T. polium* extracts was added to medium, ensuring that the final DMSO concentration did not surpass 0.2%. In a 96-well plate, MDA-MB-231 and MCF-7 cells (4 × 103 cells/well) were planted. Following a 24 h. period, adherent cells were exposed to progressively higher concentrations of various *T. polium* extracts (15.6 to 500 µg/ml). Following a 48 h. incubation period with the extracts, cells were fixed for one hour at 4 °C using 10% trichloroacetic acid, stained for 30 min using 0.4% (w/v) SRB, and then rinsed with 1% acetic acid. A microplate reader (TecanSunriseTM, Germany) was used to measure the absorbance at 570 nm after 10 mM Tris solution at pH 10.5 had liberated the SRB-bounded cells. The dosage response curve fitting model (Graph Pad, Prism software) was used to determine the extracts' IC_{50} values.

9 In-Situ application of *Teucrium* extract Nano-formulated in soft white cheese as a food model

According to Mehaia's description, two batches of soft white cheese (2% NaCl) were produced using traditional methods [23]. One batch was treated with Nano-EA extract (3 mg/ml) before rennet was added, and the other batch was kept as a control.

Microbiological evaluation of safety and quality was performed. Aerobic mesophilic bacterial count [24, 25], *Escherichia coli* [26], detection of *Salmonella* was assayed according to American Public Health Association (APHA) [24], , Food and Drug Administration (FDA) [25], , while Molds and yeasts were enumerated as recommended by Galloway and Burgess [27], .

10 Statistical analysis

Statistical significance was determined using Minitab 18. The means were determined by analysis of variance test (ANOVA, One Way analysis) ($p < 0.05$). Fisher's LSD (Least Significant Difference) Method ($\alpha = 0.05$) was applied to compare significant differences between treatments.

11 Results and discussion

11.1 Antimicrobial activity of different extracts of *T. polium*

Data presented in Table 1 illustrate the antimicrobial activity of *T. polium* different extracts against some pathogenic bacteria, fungi and yeast. The obtained results revealed that ethyl acetate extract exhibit an effective antimicrobial activity toward all tested microorganisms. The most sensitive organism toward ethyl acetate extract was *C. albicans* with inhibition zone diameter 37.67 mm followed by *Pseudomonas aeruginosa* with 26.33 mm. The least sensitive organism toward ethyl acetate extract was *A. flavus* and *A. ochraceus* with 8.67 mm each. Also ethyl acetate nano form (EA-Nano) showed remarkable activity against tested organisms. Gram positive bacteria were the most susceptible organisms to it with inhibition zones ranged from 24.5 mm to 27.0 mm for *B.cereus* and *S. mutans* respectively. Ethyl acetate extract and EA- Nano form were the only

Table 1 Effect of *T. polium* extracts on the different pathogenic microorganisms (Inhibition zone diameter (mm)

Indicator organisms		Inhibition zone diameter (mm)					
		Ethyl Acetate	Ethanol	Methanol	Oil	EA- Nano	C+
Bacteria	*B. cereus*	22.50 ± 0.29 ^{B}	16.17 ± 0.17 ^{C}	12.33 ± 0.34 ^{D}	8.67 ± 0.34 ^{E}	24.5 ± 0.18 ^{A}	22.00 ± 1.18 ^{B}
	S. aureus	22.33 ± 0.34 ^{B}	15.00 ± 0.00 ^{C}	13.83 ± 0.61 ^{D}	9.00 ± 0.00 ^{E}	25.0 ± 0.18 ^{A}	15.50 ± 0.29 ^{C}
	L. monocytogenes	24.50 ± 0.51 ^{A}	12.67 ± 0.68 ^{C}	15.17 ± 0.17 ^{B}	0.00 ± 0.00 ^{D}	24.5 ± 0.18 ^{A}	25.67 ± 0.34 ^{A}
	S. mutans	25.33 ± 0.34 ^{B}	14.33 ± 0.34 ^{D}	10.67 ± 0.34 ^{F}	11.67 ± 0.34 ^{E}	27.0 ± 0.18 ^{A}	16.33 ± 0.34 ^{C}
	E. coli	23.50 ± 0.29 ^{A}	ND	ND	ND	22.5 ± 0.18 ^{B}	22.17 ± 0.17 ^{B}
	E. coli O157: H7	23.33 ± 0.34 ^{A}	8.33 ± 0.34 ^{C}	11.00 ± 0.59 ^{B}	ND	23.00 ± 0.18 ^{A}	11.33 ± 0.34 ^{B}
	S. enterica	24.17 ± 0.17 ^{A}	ND	ND	ND	23.0 ± 0.18 ^{B}	21.17 ± 0.17 ^{C}
	P. aeruginosa	26.33 ± 0.34 ^{A}	8.33 ± 0.34 ^{E}	9.33 ± 0.34 ^{D}	ND	24.00 ± 0.18 ^{B}	10.33 ± 0.34 ^{C}
Molds	*F. verticillioides.*	17.33 ± 0.34 ^{A}	8.17 ± 0.17 ^{C}	ND	ND	9.00 ± 0.21 ^{B}	ND
	A. flavus	10.33 ± 0.34 ^{A}	ND	ND	ND	7.00 ± 0.21 ^{C}	9.17 ± 0.17 ^{B}
	A. ochraceous	14.33 ± 0.34 ^{A}	ND	ND	ND	7.00 ± 0.21 ^{C}	9.17 ± 0.17 ^{B}
	A. niger	15.00 ± 0.59 ^{A}	ND	ND	ND	7.00 ± 0.21 ^{C}	10.17 ± 0.17 ^{B}
Yeasts	*S. cerevisiae*	19.33 ± 0.19 ^{A}	ND	ND	ND	15.00 ± 0.21 ^{B}	10.45 ± 0.13 ^{C}
	C. albicans	37.67 ± 0.34 ^{A}	22.17 ± 0.34 ^{B}	22.67 ± 0.68 ^{B}	12.33 ± 0.34 ^{C}	11.00 ± 0.21 ^{D}	8.17 ± 0.17 ^{E}

ND Not detected, *Mean* ± *SE* sharing a letter within the same raw means no significance

C+ Clotrimazole (10 µg/disc) and Amoxicillin/Clavulanic acid (30 µg/disc) were respectively considered as antifungal and antibacterial positive controls

two effective extracts against all tested microorganisms. Ethanol and methanol extract of *T. polium* also exhibited notable activity against tested bacteria except *E. coli* and *S. enterica*. *C. albicans* also was the most sensitive organism toward ethanol and methanol extract with 19.33 and 19.67 mm respectively. The *T. polium* essential oil showed the lowest inhibition zones against tested bacteria with zone diameters ranged from zero to 12.33 mm while chloroform extract did not show any antimicrobial activity against any of tested organisms. *T. polium* has been used in folk medicine as antifungal, antibacterial, antipyretic and antispasmodic. Polyphenolic compounds, flavonoids, monoterpenes alkaloids and essential oils were identified as reported by Rahmouni et al. [28], . El Atki et al. [29], studied the antibacterial activity of *T. polium* subspecies against six bacterial (*S. aureus*, *K. pneumonia*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Citrobacter koseri and Escherichia coli* and recorded that the tested EOs showed a wide antibacterial activity against the six tested strains with inhibition ranged from 8.0 to 23 mm. the

Table 2 Estimated values of minimal inhibitory concentration of *T. polium* extracts for tested microorganisms

Tested Microorganisms		Estimated MIC values (mg/ml)			
		EA	Nano EA	Ethanol	Methanol
Bacteria	*B. cereus*	3.13 ± 0.00^{A}	2.50 ± 0.00^{B}	3.13 ± 0.00^{A}	3.13 ± 0.00^{A}
	S. aureus	12.5 ± 0.00^{A}	10.00 ± 0.00^{B}	12.5 ± 0.00^{A}	6.25 ± 0.00^{C}
	L. monocytogenes	12.5 ± 0.00^{A}	10.00 ± 0.00^{B}	12.5 ± 0.00^{A}	12.5 ± 0.00^{A}
	S. mutans	12.5 ± 0.00^{A}	10.00 ± 0.00^{B}	12.5 ± 0.00^{A}	12.5 ± 0.00^{A}
	E. coli	50.0 ± 0.00^{A}	10.00 ± 0.00^{D}	25.0 ± 0.00^{B}	12.5 ± 0.00^{C}
	E. coli O157: H7	12.5 ± 0.00^{A}	10.00 ± 0.00^{B}	12.5 ± 0.00^{A}	12.5 ± 0.00^{A}
	S. enterica	50.0 ± 0.00^{A}	10.00 ± 0.00^{D}	25.0 ± 0.00^{B}	12.5 ± 0.00^{C}
	P. aeruginosa	12.5 ± 0.00^{A}	10.00 ± 0.00^{B}	12.5 ± 0.00^{A}	12.5 ± 0.00^{A}
Fungi	*F. verticillioides.*	25.0 ± 0.00^{A}	15.0 ± 0.00^{B}	25.0 ± 0.00^{A}	12.5 ± 0.00^{C}
	A. flavus	25.0 ± 0.00^{A}	15.0 ± 0.00^{B}	25.0 ± 0.00^{A}	25.0 ± 0.00^{A}
	A. ochraceous	25.0 ± 0.00^{A}	15.0 ± 0.00^{B}	25.0 ± 0.00^{A}	25.0 ± 0.00^{A}
	A. niger	12.5 ± 0.00^{B}	7.5 ± 0.00^{C}	25.0 ± 0.00^{A}	12.5 ± 0.00^{B}
Yeast	*C. albicans*	0.50 ± 0.00^{A}	0.50 ± 0.00^{A}	0.50 ± 0.00^{A}	0.50 ± 0.00^{A}
	S. cerrevisiae	1.00 ± 0.00^{A}	0.50 ± 0.00^{B}	0.50 ± 0.00^{B}	0.50 ± 0.00^{B}

Mean ± SE sharing a letter within the same raw means no significance

Table 3 Antioxidant capacity (IC_{50}) of different extracts of *T. polium* using DPPH˙ and ABTS˙⁺ assays

Tested Extracts	DPPH (µg/ml)	ABTS˙⁺(µg/ml)
ET.OH	$92^{c} \pm 5.89$	$100^{cd} \pm 0.33$
ME.OH	$58^{c} \pm 1.83$	$107^{cd} \pm 5.14$
ET.AC	$93^{c} \pm 3.22$	$135^{c} \pm 1.98$
ET.AC Nano Form	$832^{b} \pm 4.01$	$362^{b} \pm 14.6$
Gum Arabic	$2268^{a} \pm 15.9$	$1108^{a} \pm 101.85$
BHT	$5.03^{c} \pm 0.72$	$5.03^{d} \pm 0.72$
LSD	159.12	102.8

Data are expressed as the mean ± SD. Means with different letters (at the same Colum) are significantly different at $p \leq 0.05$

strength of antimicrobial activity varied depending on the species of microorganisms, plant species and extract type [30]. There are many differences and variability of EOs of *T. polium* collected from three different localities in Algeria which may be attributed to different environmental and genetic factors. These differences mirrored in its chemical and antimicrobial results as concluded by Lograda et al. [31], .

The estimated MIC concentrations of *T. polium* extracts with high antimicrobial activity as mentioned above in Table 1 were illustrated in Table 2. Nano form of ethyl acetate extract (Nano-EA) showed the lowest MIC concentrations which ranged from 0.5 mg/ml for *C. albicans* and *S. cerevisiae* to 15 mg/ml for tested fungi *A. flavus*, *A. ochraceous* and *Fusarium spp.* Methanol and ethyl acetate extracts also showed low MIC values against tested microorganisms. The obtained data showed that tested yeasts ware the most susceptible organisms toward *T. polium* extracts followed by Gram-positive and Gram- negative bacteria. The tested fungi exhibit some degree of resistance.

11.2 Antioxidant potential of different extracts from *T. polium* using DPPH˙ and ABTS˙⁺ radical scavenging assays

T. polium is considered a natural herbal and it is used as herbal tea. The hydrophilicity of *T. polium* makes it desirable for potential drugs [32]. The antioxidant potential of various extracts of *T. polium* using DPPH˙ and ABTS˙⁺ assays compared to synthetic antioxidant (BHT) are shown in Table 3. The results illustrated that the methanol extract

of *T. polium* has the highest antioxidant activity compared to other extractions with IC_{50} of 58 µg/ml for DPPH and 107 µg/ml for $ABTS^{\cdot+}$. This could be dependent on the high amount of total phenols in methanolic extract. Numerous investigations demonstrated a strong linear relationship between total phenolic content and antioxidant capacity [33–35]. Conversely, the findings indicated no significant difference between the ethanol and ethyl acetate extracts, with IC_{50} values for DPPH and $ABTS^{\cdot+}$ ranging from 92 to 93 µg/ml and 100 to 135 µg/ml, respectively. The nano form of ethyl acetate extract showed the lowest antioxidant activity with IC_{50} of 832 µg/ml for DPPH and 362 µg/ml for $ABTS^{\cdot+}$ follwed by gum arabic as a polymer with IC_{50} of 2268 µg/ml for DPPH and 1108 µg/ml for $ABTS^{\cdot+}$ compared to BHT as synthetic antioxidant (5 µg/ml). The methanolic extract of *T. polium* demonstrated relatively higher antioxidant activity, which may be attributed to its specific phenolic composition. HPLC analysis revealed that this extract contained notable levels of gentisic acid, catechin, ferulic acid, rutin, and rosmarinic acid compared to the other extracts. These compounds are well documented for their strong radical scavenging properties; for example, rutin and catechin exhibit significant DPPH and $ABTS^{\cdot+}$ scavenging effects due to their hydroxyl substitutions and ability to donate hydrogen atoms, while rosmarinic and ferulic acids are effective chain breaking antioxidants that stabilize free radicals through resonance [36, 37]. The higher abundance of these compounds in the methanol extract is consistent with its lower IC_{50} values in both DPPH and $ABTS^{\cdot+}$ assays compared to the ethanol and ethyl acetate extracts. In contrast, although the ethanol extract and ehteyl acetate also contained bioactive compounds such as ferulic acid, and rosmarinic acid, their relative abundance was lower, which may explain its comparatively moderate antioxidant performance. Therefore, the results suggest that not only the total phenolic content, but also the qualitative composition and relative concentration of individual phenolics, play a decisive role in determining the antioxidant potential of *T. polium* extracts.

11.3 Phenolic profile of *T. polium* extracts analyzed using HPLC

Table 4 illustrates the identified phenolic compounds in different extract of *T. polium* as detected by HPLC. The obtained data indicate that the tested extracts (methanol, ethanol and ethyl acetate) extract contains high amount of total phenol the thing which illustrates its antioxidant activity. The detected concentrations of phenolic compounds varying from one extract to another. About 19 compounds found in methanol extract and 17, 16 compounds found in ethanol and ethyl acetate respectively. Gentisic acid and Catechin were found in methanol extract with 4.57 and 50.31 µg/g respectively while missed in ethanol and ethyl acetate extracts. Protocatechuic acid, *p*-hydroxybenzoic acid, Syringic acid, Vanillic acid, Rutin, Quercetin and Kaempferol were the main phenolic compounds found in tested extracts with notable concentrations. Boutefaha et al. [38], reported the presence of nine, eight and eleven compounds in methanolic, ethanolic and aqueous extracts respectively. Predominance of ellagic acid, catechin, coumaric acid and quercetin was noticed in methanolic extract. However, all of chlorogenic acid, pyrocatechol and 4`0.7-Dihydroxyiso flavone did not exist in methanolic nor ethanolic extracts. Also; catechin was completely absent in ethanolic and aqueous extracts. These obtained results referred to *T. polium* leaves as a good source of natural antioxidant agents like polyphenols such as naringenin and quercetin. Variations in phenolic content

Table 4 Phenolic profile of *T. polium* extracts as detected by HPLC

Compounds	Phenolic compounds Concentrations (µg/g)		
	Me-OH	E-OH	EA-OH
Gallic acid	0.00	0.00	0.00
Protocatechuic acid	178.86	45.77	358.56
Gentisic acid	4.57	0.00	0.00
p-hydroxybenzoic acid	341.71	588.22	794.19
Catechin	50.31	0.00	0.00
Chlorogenic acid	97.91	72.47	24.29
Caffeic acid	12.61	36.25	120.55
Syringic acid	366.66	251.15	740.42
Vanillic acid	197.64	207.63	326.04
Ferulic acid	107.74	35.45	64.58
Sinapic acid	20.55	6.43	0.00
Rutin	1632.36	535.93	536.46
p-coumaric acid	144.30	373.33	102.89
Apigenin-7-glucoside	17.48	23.85	67.63
Rosmarinic acid	87.17	25.80	51.99
Cinnamic acid	15.69	10.75	21.31
Quercetin	144.42	243.10	269.21
Apigenin	46.96	188.03	135.49
Kaempferol	2146.07	6372.50	5358.27
Chrysin	6.60	29.06	19.91

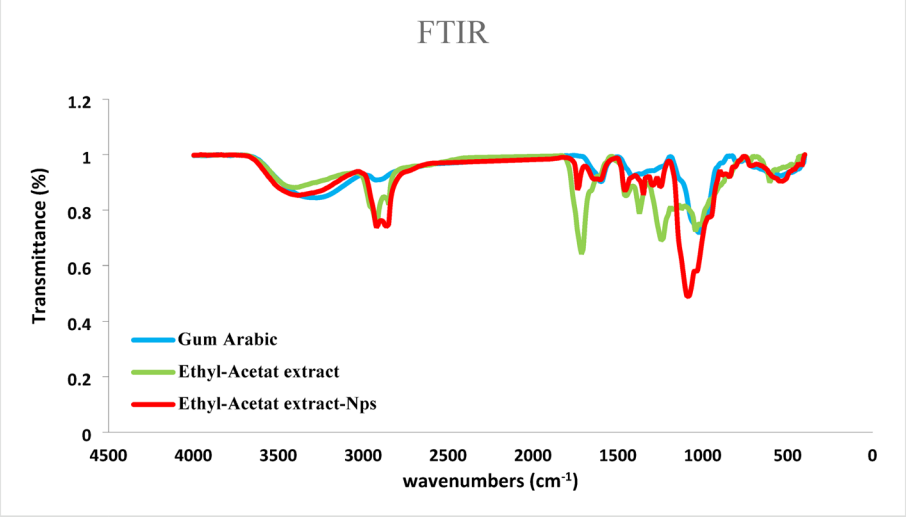


Fig. 1 Fourier-transform infrared spectroscopy (FTIR) spectra of gum Arabic, ethyl acetate extract of *T. polium* and ethyl acetate extract -Nps

among the three extracts may be attributed to the degree of compound solubility in particular solvent [39–41].

12 Physical characterization of *T. polium* Nps

12.1 FTIR of Ethyl acetate extract of *T. polium* –Nps

FTIR technique used to estimate the linkage level between the compounds and nanomaterial. FTIR spectrum of gum Arabic, ethyl acetate extract of *T. polium* and its nanoform are shown in Figure 1. The blue spectrum demonstrated gum Arabic as polymer which distinct peaks at 3277 cm^{-1} assigned to -OH group, 2925 cm^{-1} assigned -CH_2,

1598 cm^{-1} assigned to -C-O, 1412 cm^{-1} assigned to –CH, 1020 cm^{-1} assigned to C–O–C and 775 cm^{-1}, 599 assigned to –C–H linkage [42]. The green spectrum demonstrated ethyl acetate extract of *T. polium* which showed strong absorption bands at 3406 cm^{-1} corresponds to -OH group, 2917 cm^{-1} indicated to -CH_2, 2849 cm^{-1} indicated to the stretching vibrations -CH_2, 1711 cm^{-1} correspond to C = O, 1455 cm^{-1} assigned to C = C,1373 cm^{-1} indicated to N-O, 1239 cm^{-1} indicated to C–N, 1177 cm^{-1} indicated to C–O stretching vibrations, 1124 cm^{-1} indicated to C-N and 719 cm^{-1} assigned to cis = C–H out-of-plane bending. The red spectrum showed that the overlap between ethyl acetate extract and gum Arabic with new beaks appeared in nanoform, may be due to a chemical reaction of ethyl acetate extract with another ingredient [43]. The FTIR spectra provide important insights into the molecular interactions occurring during the formation of *T. polium* ethyl acetate extract–gum Arabic nanoparticles. In the spectrum of gum Arabic, the characteristic broad peak at 3277 cm^{-1} corresponds to the stretching vibration of hydroxyl groups, which are abundant in polysaccharide backbones. These groups are known to act as hydrogen-bond donors and can participate in stabilizing the nanoparticle surface. Similarly, the bands at 2925 cm^{-1} (–CH_2 stretching) and 1020 cm^{-1} (C–O–C) reflect the polysaccharide skeleton, which serves as a stabilizing matrix and capping agent [44]. The ethyl acetate extract of *T. polium* displayed distinct functional groups including hydroxyl (3406 cm^{-1}), carbonyl (1711 cm^{-1}), aromatic C = C (1455 cm^{-1}), and amine-related vibrations (1239–1124 cm^{-1}), confirming the presence of diverse phytoconstituents such as flavonoids, terpenoids, and alkaloids. These biomolecules are rich in reactive moieties capable of reducing and capping nanoparticles. Upon nanoparticle formation, the FTIR spectrum of the nanoform exhibited distinct alterations compared to the spectra of the individual components, confirming substantial molecular interactions underlying nanoparticle synthesis. Specifically, the observed shift and broadening of the –OH stretching band, accompanied by the emergence of additional absorption peaks, strongly indicate the establishment of extensive hydrogen bonding networks and potential covalent linkages between the phytochemical constituents of *T. polium* extract and the gum Arabic matrix. The significant decrease in intensity of the carbonyl stretching band (around 1711 cm^{-1}) further suggests the direct involvement of carbonyl functionalities either in the reduction of metal ions or in stabilizing the nanoparticle surface via coordination or condensation reactions [45, 46]. Moreover, the spectral modifications within the C–O–C and C–N stretching regions point toward the possible formation of ester, ether, or amide-type bonds, implying that the phytochemicals are not simply physically adsorbed but rather chemically integrated into the polymeric network. Such interactions likely result in the formation of a crosslinked polymeric shell surrounding the nanoparticle core, providing steric and electrostatic stabilization and thereby preventing agglomeration. Functionally, these chemical linkages are anticipated to enhance colloidal stability, modulate the release kinetics of the encapsulated bio actives, and protect the phytochemicals from environmental degradation. Consequently, the FTIR findings collectively demonstrate that nanoparticle synthesis induces strong and multifaceted intermolecular interactions hydrogen bonding, electrostatic attraction, and potential covalent crosslinking between the plant-derived metabolites and gum Arabic. These interactions not only explain the improved physicochemical stability of the nanoform but also suggest enhanced solubility, sustained release, and superior biological efficacy of the encapsulated phytochemicals [47].

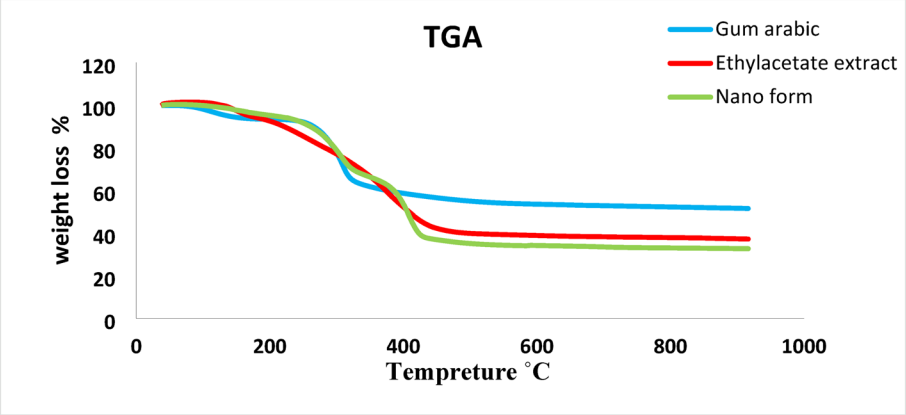


Fig. 2 TGA for gum Arabic, ethyl acetate extract of *T. polium* and its nanoform

Table 5 The particle size and polydispersity index (PDI) of *T. polium* Ethyl acetate extract-Nps

	Ethyl acetate extract -nps
Particle size (n.m.)	7.73 ± 1.20
PDI	0.58

12.2 Thermo gravimetric analysis (TGA)

The thermal stability of gum Arabic, ethyl acetate extract of *T. polium* and its nanoform were shown in figure 2. The thermal stability of samples was assessed in air heated at 10 °C /min by using TGA. The results presented the weight loss of gum arabic about 10% appeared at 295 °C, while the weight loss up to 39% in the range of 826 to 916 °C [48]. The ethyl acetate extract of *T. polium* appeared about 10% (weight loss) at 273 °C, while the weight loss up to 39% in the range of 400 to 404 °C. When converted the ethyl acetate extract to nanoform the weight loss was in the same range of native ethyl acetate extract (10% at 294 and 39% at 404). The TGA results demonstrated that gum Arabic decomposed in multiple steps, with initial weight loss near 295 °C due to dehydration and major degradation of its polysaccharide backbone at higher temperatures, consistent with reported gum-based polymers. The *T. polium* extract degraded earlier (273 °C), reflecting the lower thermal stability of phytochemicals such as flavonoids and terpenoids. After nanoparticle formation, the onset of degradation shifted slightly higher (294 °C), indicating that gum Arabic stabilized the extract through intermolecular interactions, as observed in other polymer phytochemical nanocarriers. This enhanced stability suggests that encapsulation improves the functional integrity and applicability of the nanoform [49].

12.3 Particle size distribution

Table 5 showed the particle size (nm) and Polydispersity index (PDI) of ethyl acetate extract -nps of *T. polium*, the results demonstrated the particle size was less than 100 nm (7.73 ± 1.20 nm), confirming their nanoscale nature, which is desirable as smaller nanoparticles typically exhibit higher surface area, better solubility, and improved cellular uptake of bioactive compounds [50]. Polydispersity index (PDI) was 0.58 indicates a moderately broad but acceptable size distribution. Although values < 0.3 are considered highly monodisperse, plant-mediated nanoparticles often show higher PDI due to the diverse nature of phytochemicals, yet remain stable and functionally effective [51].

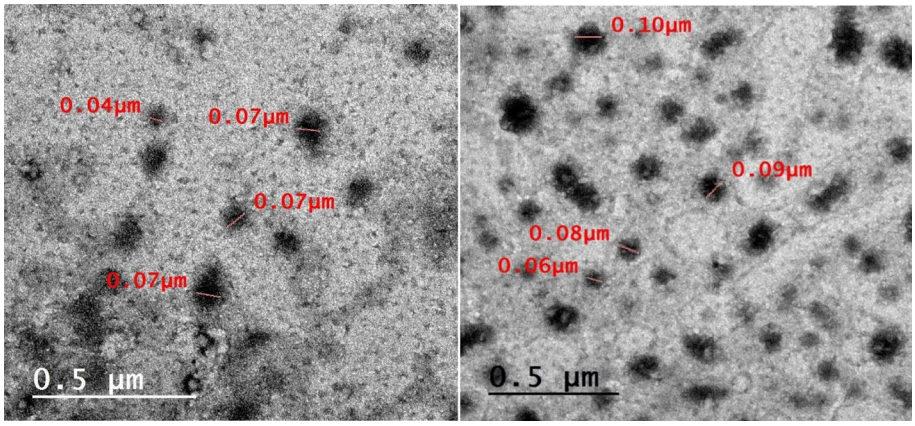


Fig. 3 TEM micrograph of *T. polium* ethyl acetate extract nanoparticles

Table 6 Anticancer activity of *T. polium* extracts against breast cancer cell lines MDA-MB231 and MCF-7

Tested Concentrations	Tested Extract of T. polium					
	METHANOL		ETHANOL		ETHYL ACETATE	
	MDA	MCF-7	MDA	MCF-7	MDA	MCF-7
0	1.00 ± 0.01	1.00 ± 0.06	1.00 ± 0.01	1.00 ± 0.06	1.00 ± 0.01	1.00 ± 0.06
15.6	0.93 ± 0.02[b]	0.92 ± 0.02	0.99 ± 0.03	0.93 ± 0.12	0.98 ± 0.06	0.88 ± 0.04
31.2	0.92 ± 0.02[b]	0.89 ± 0.04	0.99 ± 0.02	0.94 ± 0.02	0.98 ± 0.06	0.93 ± 0.06
62.5	0.78 ± 0.05[d]	0.79 ± 0.02	0.94 ± 0.03[a]	0.89 ± 0.03[c]	0.95 ± 0.04	0.86 ± 0.08[a]
125	0.47 ± 0.02[d]	0.44 ± 0.02	0.72 ± 0.04[d]	0.66 ± 0.03[d]	0.55 ± 0.02[d]	0.53 ± 0.03[d]
250	0.41 ± 0.02[d]	0.38 ± 0.01	0.56 ± 0.02[d]	0.53 ± 0.03[d]	0.51 ± 0.02[d]	0.54 ± 0.11[d]
500	0.39 ± 0.0[d]	0.43 ± 0.08	0.47 ± 0.02[d]	0.45 ±0.04[d]	0.46 ± 0.03[d]	0.46 ± 0.01[d]
IC50	117 µg/ml	112 µg/ml	357 µg/ml	312 µg/ml	283 µg/ml	402 µg/ml

All experiments were performed in triplicate, and data are expressed as mean ± standard deviation (SD). Statistical significance was determined by comparing different concentrations of *T. polium* extracts to the control (untreated). The results are denoted as follows: a indicates a significant difference at $p \leq 0.05$, b indicates $p \leq 0.01$, c indicates $p \leq 0.001$, and d indicates $p \leq 0.0001$. The IC_{50} values represent the concentration of extract required to inhibit cell viability by 50%

12.4 Transmission electron microscopy (TEM)

Transmission electron microscopy analysis revealed that the synthesized particles were circular, but sometimes amorphous in nature with irregular and diffuse boundaries, which is characteristic of amorphous materials Figure 3. Measurements from the TEM images indicated that the particle sizes ranged from approximately 40 to 100 nm average of 72.5 nm ± 18.32, with a relatively uniform distribution across the observed field. These observations confirm the successful formation of nanosized, amorphous particles with consistent morphology.

13 Anticancer activity of *Teucrium polium* extracts

Teucrium Polium extracts were tested for possible inhibitory effects against the growth of breast cancer cell lines MDA-MB-231 and MCF-7 Table 6; Figure 4. The IC_{50} values obtained from the study varied from 117 µg/ml to 402 µg/ml. The values didn't meet the criteria for significance by the American Cancer Institute, which is IC_{50} < 30 µg/ml for crude extracts [52]. This indicates that the potency of the extracts was relatively weak. These findings align with previous findings by Nikodijević et al. [53], which also stated a weak but good potent effect of *T. polium* extracts on the MDA cell line. The study by Nematollahi-Mahani [54], also revealed a weak response of MCF-7 breast cancer cells

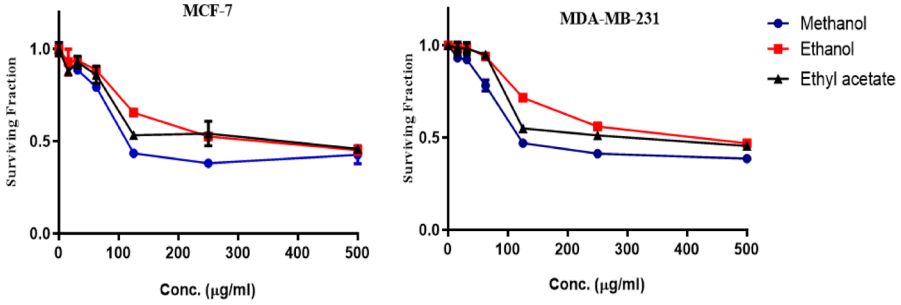


Fig. 4 Effect of *T. polium* extracts against breast cancer cell lines MDA-MB 231 and MCF7

to the ethanolic extract of *T. polium*, with an IC_{50} value of 140 µg/ml. This supports the observation of varying and relatively higher IC_{50} values, particularly with ethanolic extracts of *T. polium* on MCF7 breast cancer cells [54]. Methanol extracts demonstrated the highest potency in both cell lines. The obtained findings of antioxidant and phytochemical analyses supported these findings demonstrated significant bioactive compounds, including flavonoids such as Kaempferol, Rutin, and the polyphenolic compound Ferulic Acid, with the methanolic extract containing the highest concentration. These compounds showed potential in providing antioxidant and anticancer effects. A limitation of the present study is that the cytotoxic activity of the tested crude extracts did not reach the screening threshold commonly used for crude botanical extracts. The United States National Cancer Institute (NCI) and many screening studies use an $IC_{50} \lesssim$ 30 µg/ml as an initial benchmark for promising crude extract [55].

Our extracts therefore show only weak to moderate cytotoxicity by that criterion; similar findings have been reported widely for crude extracts, where IC_{50} values vary substantially depending on solvent, cell line, exposure time, and extract complexity [56]. Importantly, crude extracts are complex mixtures in which active constituents may be present at low concentration; bioassay-guided fractionation frequently concentrates those actives and yields fractions or pure compounds with substantially improved potency [57]. Finally, even extracts with modest IC_{50} values can be valuable starting points for isolation of selective agents or for identification of novel mechanisms. Therefore, while we acknowledge the limited potency of the crude extracts in the present screening, we consider these results a valid starting point for fractionation, selectivity testing, and mechanistic studies.

Kaempferol, recognized by its anti-inflammatory and antioxidant properties, could potentially interfere with cancer-related processes. Rutin, which is abundant in "EA-OH," may have anticancer attributes, but this could be influenced by concentration and context [58]. Ferulic Acid, which is prevalent in "Me-OH," also exhibits antioxidant and anticancer potential [59].

14 In-Situ application of *T. polium* Ethyl acetate extract Nano-formulated in soft white cheese as a food model

The In-Situ experiment supplied soft white cheese (2% NaCl) that was supplemented with 3 mg/ml of the nano-formulated EA *T. polium* extract. Results of microbiological analyses were reported in Table 7. Estimating the quality of cheese, the initial aerobic mesophilic bacteria (AMB) is the same in the untreated and treated samples ($P < 0.05$). Then, populations of AMB developed significantly to reach 3.39 log CFU after 1 week,

Table 7 Microbiological profile of soft white cheese as a food model after treatment with *T. polium* Ethyl acetate Nano-formulated extract

Microbiological analyses	Microbial counts (CFU/g)							
	Zero time	Control			Zero time	Treated		
		1 week	2 weeks	3 weeks		1 week	2 weeks	3 weeks
Aerobic mesophilic count	2.73 ± 0.03^{F}	3.39 ± 0.05^{CD}	4.01 ± 0.08^{B}	4.58 ± 0.02^{A}	2.75 ± 0.03^{F}	3.01 ± 0.04^{E}	3.24 ± 0.09^{D}	3.43 ± 0.11^{C}
Enterobacteriaceae	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Coliforms count	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Salmonella	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Escherichia coli	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Mold and yeast	< 10	1.80 ± 0.10^{C}	2.83 ± 0.09^{B}	3.78 ± 0.13^{A}	< 10	1.00 ± 0.00^{D}	1.00 ± 0.00^{D}	1.00 ± 0.00^{D}

ND Not detected, *Mean* ± *SE* sharing a letter within the same raw means no significance

4.01, and 4.58 log CFU after 2 weeks and 3 weeks respectively without treatment. Supplementation of the extract significantly reduced numbers till 3.01, 3.24, and 3.43 log CFU/g ($P < 0.05$) at the end of 1week, 2 weeks, and 3 weeks respectively. Likewise, mold and yeast numbers increased significantly to 1.8, 2.83, 3.78 log CFU/g in the batch without treatment. Within the treated samples, there was no considerable change ($P < 0.05$).

Previous studies [60] proved that plant-based ethyl acetate extracts possess a good antifungal effect in food and non-food systems [61]. Moreover, chromatographic analyses attributed this preservative activity to their content of phenolic compounds. Other studies showed that nano-formulation of such extracts gives additional activity toward fungal concerns. Additionally, this is a good step on the way of sustainable food production [62].

15 Conclusion

This study highlights *Teucrium polium* as a potentially useful natural source of bioactive substances for food preservation. Significant amounts of polyphenols were discovered in the extracts, which also showed antibacterial and antioxidant properties. Among the tested fractions, the ethyl acetate extract and its nano-form exhibited the broadest antimicrobial spectrum, effectively inhibiting all tested microorganisms, particularly fungi. Incorporating the ethyl acetate nanoparticle extract into white soft cheese significantly reduced bacterial, mold, and yeast counts, suggesting its ability to extend shelf life and maintain product quality. These findings support the potential of *T. polium* ethyl acetate nanoparticles as a natural, safe, and effective preservative for dairy products, and warrant further studies on its safety, sensory impact, and large-scale application in the food industry.

Author contributions

S. H. S., A. A. G. and H. A. A., Study designing, chromatographic, antioxidant, nano formation and characterization analysis, data handling and interpretation, writing – review & editing the manuscript,. M. F. E. ***,*** manufactured the soft cheese, microbiological analysis, and participated in study designing, data handling, writing and revising the manuscript, E. G. A. and H. E., studying Anticancer activity , data handling and interpretation, writing – review & editing the manuscript. S. H. S., final revision of manuscript, journal format and served as a corresponding author.

Funding

Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). The authors declare that no funds, grants or support was received from any organization for this research as herewith submitted.

Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

None of the writers have conducted any experiments with humans or animals in this work. Local and national regulations were followed when collecting the plants and plant materials for our investigation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 24 April 2025 / Accepted: 3 December 2025

Published online: 23 December 2025

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