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Article

Physicochemical, Antioxidant and Antimicrobial Properties of Three Medicinal Plants from the Western Part of the Rhodope Mountains, Bulgaria

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Abstract: The present study investigated the physicochemical, antioxidant and antimicrobial properties of three medicinal plants – thyme, cotton thistle and hawthorn fruit – from the Western Rhodope Mountains, Bulgaria. The first stage determined the physicochemical characteristics (moisture, ash, carbohydrates, proteins and vitamin C) of the three herbs. The second stage ascertained four types of extracts (aqueous, oil, methanolic and ethanolic), from which the total phenolic content, presence of phenolic compounds (flavonoids and phenolic acids), antioxidant activity and their antimicrobial properties. Thyme was characterized by the highest ash, proteins and vitamin C contents. The hawthorn fruit showed the highest moisture and carbohydrate contents. The 70% ethanolic extracts of the three herbs exhibited the highest levels of phenolics and consequently a pronounced antioxidant activity, compared to the other three types of extracts. The flavonoid quercetin-3-ß-glucoside was with the highest concentration in the thyme and hawthorn fruit, while myricetin dominated in the cotton thistle. The phenolic acid content analysis showed a prevalence of rosmaric acid in thyme, whereas chlorogenic acid was detected in the highest concentration in hawthorn fruit and cotton thistle. High antimicrobial potential of the ethanolic and methanolic extracts was observed.

Keywords: antioxidants; herbs; phenolic compounds; extracts; antimicrobial activity

1. Introduction

In recent years, numerous scientific studies have revealed that the consumption of plant foods, including wild herbs and fruits, is of great importance for preventing and treating different diseases, as well as for increasing the vitality and restoring good health due to their high nutritional value and healing properties [1-4]. This fact has been confirmed scientifically showing that medicinal plants are valuable source of vitamins, fibres and polyphenolic compounds presented in their tissues. Polyphenols are known to be compounds with antioxidant potential, but they also determine the colour, flavour, biological activities and health benefits of the medicinal plants [5-7].

Some recent studies have shown that polyphenols in foods of plant origin play protective role against the development of cardiovascular disease, diabetes, osteoporosis, neurological degenerative disorders and cancer [8]. Plant crops, particularly the wild ones, are the major sources of natural antioxidants [6, 9, 10]. Many biologically active compounds possessing antioxidant activity (phenolic compounds, carotenoids, anthocyanins, tocopherols and others) can be found in some rarely used indigenous plant sources, such as dogwood, mountain ash, sea buckthorn, rosehip, elderberry, bilberry, mulberry, hyssop, thyme and hawthorn, whose consumption increased in the last years due

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to their pleasant taste and health beneficial properties. The antioxidants, vitamins and minerals that they contain have been suggested to exert many positive effects on human health [11, 12].

Bulgaria is located in South Eastern Europe, which in the influence of certain climatic factors is divided into five climatic zones (temperate continental, transitional continental, continental Mediterranean, Black Sea and mountainous), each of them is characterized by a great soil and botanical diversity. According to some recent reports, more than 700 species (approximately 20% of the Bulgarian flora) are recognized as herbs, and most of them are wild plants growing in the mountainous regions of the country [13].

The Rhodope Mountains or the Rhodopes are the largest mountain range in Bulgaria, located in the southern part of the country. The Rhodopes are divided into two parts: Western (average altitude of 1098 m) and Eastern (average altitude of 329 m). In the higher altitude regions of the Western Rhodopes prevails the mountainous climate, and in the lower altitude regions – transitional continental climate, while the southern parts are characterized by Mediterranean climate. A study of Zahariev et al. [14] revealed that the medicinal plants in the Rhodopes include 714 species, belonging to 393 genera and 101 families. Most of this area is designated as a protected area of the European ecological network NATURA 2000 for the protection of rare and threatened habitats, plants and animals. The medicinal plants were discovered mainly in grasslands and meadows, but there is a risk of extinction due to negative changes in the environment. The most common medicinal plants in the Rhodope Mountains belong to the families *Asteraceae*, *Lamiaceae*, *Rosaceae*, *Amrillydaceae*, *Crassulaceae*, *Plantaginaceae*, *Oleaceae* and *Solanaceae* [15]. Although Bulgaria is the second largest European exporter and one of the world's leading exporters of herbs, the information about the botanical diversity, the phytochemical composition and the biological properties of the wild plants growing in mountainous regions, in particular the Rhodope Mountains, is still very limited [13].

Hawthorn (*Crataegus monogyna*) is a plant of the *Rosaceae* family, widely used in medicine and in the culinary practice. The fruits of hawthorn have been found to contain vitamin C, sugars, organic acids, phenols, flavonoids and anthocyanins that contribute to its red, purple or blue colour [16-19]. The hawthorn fruits are rich in minerals – Na, K, Ca, P, Al [20], Mg, Cu, Zn and Fe [21]. Therefore, they are used for prevention and treatment of cardiovascular diseases, cancer, diabetes, asthma and nephritis, as well as for improving memory [22]. According to Tadic et al. [23], extracts of dried hawthorn (*Crataegus monogyna* Jacq., *C. oxyacantha* L. and *C. laevigata*) can be used as an anti-inflammatory, gastroprotective and antimicrobial agent. Kostić et al. [24] found that acetone extracts of dried hawthorn (*C. oxyacantha* L.) demonstrated antioxidant activity. The same finding was confirmed by Ziouche et al. [25], who stated that hawthorn leaves and fruits are rich in polyphenolic compounds (rutin, quercetin and isoquercetin) and exhibit significant antioxidant activity.

The genus *Thymus* belongs to the *Lamiaceae* family, which includes about 350 aromatic perennial species, indigenous to the Mediterranean region. The most popular species cultivated for culinary, medicinal and ornamental purposes is *Thymus vulgaris* or common thyme. Among the 21 *Thymus* species occurring in Bulgaria are *Thymus serpyllum* (wild thyme) and *Thymus callieri* Borbás ex Velen, which have been found in different natural localities of Bulgaria. In the Rhodope Mountains, *T. callieri* Borbás ex Velen has been discovered in two locations near the town of Dospat at an altitude of about 1200 m [26, 27]. Studies by Djenane et al. [28] and Hyldgaard et al. [29] observed that thyme contains polyphenols (flavonoids), organic acids, vitamin C, pigments, minerals (Ca, Fe, Mn) and dietary fibres [27]. Thyme contains about 0.15-1.5% essential oil, the amount of which varies depending on the species, and up to 5% tannins and resinoid substances. The essential oil of thyme is extracted from the whole plant and contains aromatic and volatile compounds (thymol, carvacrol, quercol, α-terpineol, L-borneol, L-cymol, L- and D-pinene, γ-terpene, caryophyllene, linalool) known to possess antimicrobial and antioxidant properties [30, 31].

Cotton thistle (*Onopordum acanthium* L.) is a biennial spiny herb of the *Asteraceae* family, widely spread in the world (Europe, Asia, America and Australia), including all over Bulgaria, and growing mainly on dry and rocky places [32]. The leaves of cotton thistle contain tannins, the sesquiterpene lactone arctiopicrin, saponins and alkaloids. The flower baskets contain the polysaccharide inulin and an array of phytochemical compounds including polyphenols, phenolic acids, flavonoids,

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acetylene, triterpenes [33], fatty acids (oleic, linolic, linolenic, stearic, palmitic, pentadecanoic and erucic) and phytosterols [34, 35], which contribute to their antioxidant, anti-inflammatory, antimicrobial, diuretic, cardioprotective and wound-healing properties.

2. Materials and Methods

2.1. Materials

2.1.1. Plant material

The current study used three medicinal plants – thyme, cotton thistle and hawthorn fruit. The herbs were harvested in the Western part of Rhodope Mountains, Bulgaria, in the period June–August 2022, and identified according to the Herbarium Academiae Scientiarum Bulgariae (Table 1).

Table 1. Origin of the three Bulgarian medicinal plants.

Plant	Region	District	GPS coordinates	Altitude, m
Thyme (*Thymus callieri* Borbás ex Velen.)	Near Dospat	Smolyan	41°66′N 24°16′E	1214
Cotton thistle (*Onopordum acanthium* L.)	Dospat, Chillii locality	Smolyan	41°66′N 24°16′E	1207
Hawthorn (*Crataegus monogyna* Jacq.)	Satovcha, Aspen locality	Blagoevgrad	41°63′N 24°51′E	1134

2.1.2. Plant extracts

The dried herbs were finely ground using a blender. Aqueous (infusion-type) extracts were obtained by pouring 10 g of the ground dry material with boiling distilled water at a hydromodulus of 1:20. The extraction was carried out for 30 min. After filtration, the extracts were stored at 4°C for no more than 24 h until analysis. Oil extracts were obtained by pouring 10 g of the ground dry material with refined sunflower oil (Biser, Biser Oliva AD, Bulgaria), and then heated at 80°C for 24-48 h under constant stirring. Methanolic and ethanolic extracts were obtained through maceration of 1 g of the ground dry material with 15 mL of methanol / 70% ethanol (Sigma-Aldrich, Merck, Germany), after that the samples were stirred by vortex (V-1, Biosan, Latvia) for 10-15 s and left at room temperature for 48 h, in darkness. The obtained extracts were filtered through filter paper, and then stored at 4°C for further analyses. Before use, the ethanol was vacuum evaporated and the ethanolic extracts were diluted in methanol (which is not known to possess antimicrobial activity against the used test microorganisms).

2.1.3. Test microorganisms

Twenty microorganisms from the collection of the Department of Microbiology at the University of Food Technologies, Plovdiv, Bulgaria were selected for the antimicrobial activity screening. They included six Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Bacillus amyloliquefaciens* 4BCL-YT, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* NBIMCC 8632, *Enterococcus faecalis* ATCC 19433, *Micrococcus luteus* 2YC-YT), six Gram-negative bacteria (*Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* NBIMCC 1672, *Klebsiella pneumonia* ATCC 13883, *Escherichia coli* ATCC 25922,

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Proteus vulgaris ATCC 6380, *Pseudomonas aeruginosa* ATCC 9027), two yeasts (*Candida albicans* NBIMCC 74, *Saccharomyces cerevisiae* ATCC 9763) and six fungi *Aspergillus niger* ATCC 1015, *Aspergillus flavus*, *Penicillium chrysogenum*, *Rhizopus* sp., *Mucor* sp.– plant isolates, *Fusarium moniliforme* ATCC 38932).

B. subtilis, B. amyloliquefaciens and M. luteus were cultured on LBG agar at 30°C for 24 h, while S. aureus, L. monocytogenes, L. innocua, E. faecalis, E. faecium, S. enteritidis, Klebsiella sp., E. coli, P. vulgaris and P. aeruginosa were cultured on LBG agar at 37°C for 24 h. The yeasts C. albicans was cultured on MEA at 37°C, while S. cerevisiae was cultured on MEA at 30°C for 24 h. The fungi A. niger, A. flavus, Penicillium sp., Rhizopus sp., and F. moniliforme were grown on MEA at 30°C for 7 days or until sporulation.

2.1.4. Culture media

Luria-Bertani agar medium with glucose (LBG agar). LBG agar was used for the cultivation of test bacteria. A quantity of 50 g of LBG-solid substance mixture was dissolved in 1 L of deionized water, pH 7.5 ± 0.2.

Malt extract agar (MEA). MEA was used for the cultivation of test yeasts and fungi. A quantity of 50 g of the MEA-solid substance mixture was dissolved in 1 L of deionized water, pH 5.4 ± 0.2.

Both culture media were prepared in accordance to the manufacturer's (Scharlab SL, Spain) instructions and autoclaved at 121°C for 20 min before use.

2.2. Methods

2.2.1. Physicochemical analyses

Physicochemical characteristics of the herbs investigated in the study – moisture content, ash content, carbohydrates, proteins and vitamin C contents –were determined according to the following Bulgarian State Standards (Table 2).

Table 2. Standards for determination of the physicochemical characteristics.

Parameter	Standard
Moisture	BSS ISO 939:2021 [36]
Ash	BSS ISO 928:2004 [37]
Carbohydrates	BSS 7169:1989 [38]
Proteins	BSS 15438:1982 [39]
Vitamin C	BSS 11812:1991[40]

2.2.2. Total phenolic content

The total phenolic content (TPC) was assessed using the method of Ivanov et al. [41]. The reaction mixture was prepared with 1 mL of Folin-Ciocalteu reagent (Sigma-Aldrich, Merck), 0.8 mL of 7.5% sodium carbonate (Sigma-Aldrich, Merck) and 0.2 mL of the tested plant extract. The samples were left at room temperature for 20 min (in darkness). The absorbance was measured spectrophotometrically (Camspec M107, Spectronic-Camspec Ltd., UK) at 765 nm against a blank (distilled water). The results were presented as mg equivalent of gallic acid (mg GAE)/g.

2.2.3. Antioxidant activity

DPPH radical scavenging assay. The reaction mixture was prepared with 2.85 mL of DPPH reagent (2,2-diphenyl-1-picrylhydrazyl) and 0.15 mL of the tested plant extract. The samples were incubated at 37°C for 15 min, and then the absorbance was measured at 517 nm against a blank (methanol). The antioxidant activity was expressed as μM Trolox equivalents (TE)/g of dry weight (dw) [41].

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ORAC (Oxygen radical absorbance capacity) assay. ORAC was determined by the method described by Teneva et al. [42]. The reaction mixture was prepared with 170 µL of fluorescein disodium salt (FL), 20 µL of 2,2′-Azobis (2-amidinopropane) dihydrochloride and 10 µL of the tested plant extract. The FL solution and tested extract were incubated at 37 °C for 20 min in a microplate reader, then the AAPH (dissolved in a phosphate buffer with pH 7.4 at 37 °C) was added, and additionally incubated for 30 s. The fluorescence readings were taken at the end of each cycle after shaking. As a blank, 10 µL of a phosphate buffer was used. The standard curve was built using Trolox solutions. ORAC was measured using a plate reader FLUOstar OPTIMA (BMG Labtech, Germany) at 485/520 nm. The results were expressed as µmol Trolox equivalents (µmol TE)/g of dw.

HORAC (Hydroxyl Radical Averting Capacity) assay. HORAC was assessed by the method of Teneva et al. [42]. The FL solution (170 µL) and the tested extract (10 µL) were incubated at 37 °C for 10 min in the microplate reader. Next, 10 µL of hydrogen peroxide and 10 µL of Co (II) solution (15.7 mg of $CoF_2 \bullet 4H_2O$ and 20 mg of picolinic acid dissolved in 20 mL of distilled water) were added. The initial fluorescence was measured, after which the readings were taken every minute after shaking. As a blank, a phosphate buffer (pH = 7.4) was used. The standard curve was built using solutions of 100, 200, 600, 800 and 1000 µM gallic acid in a phosphate buffer. HORAC values were measured using a FLUOstar OPTIMA plate reader (BMG Labtech, Germany) at 485/520 nm. The results were expressed as µmol gallic acid equivalents (µmol GAE)/g of dw.

2.2.4. HPLC analysis of phenolic compounds

The phenolic compounds of the three investigated herbs were determined according to the method previously described by Teneva et al. [42] using a Nexera-i LC-2040C Plus UHPLC system (Shimadzu, Japan), equipped with a UV detector and a binary pump at 280 nm with sample injection volume of 20 µL. The separation of phenolic compound was performed on an Agilent TC-C18 column (5 µm, 4.6 mm × 250 mm) at 25 °C as the mobile phases included 0.5% acetic acid (A) and 100% acetonitrile (B) at a flow rate of 0.8 mL/min. The phenolic compounds were identified by comparing the retention times of unknown analytes with analytical grade standards. The results were expressed as mg/100 g of dw.

2.2.5. Antimicrobial activity assay

The antimicrobial activity of plant extracts was determined by the agar well diffusion method [43]. In the first stage of the experiment, bacterial, yeasts and fungal inocula were prepared. A bacterial counting chamber Thoma (Poly-Optik, Germany) was used for determination of the viable cells and fungal spore counts, after that their final concentrations for inoculation were adjusted to 10^8 cfu/mL (for bacterial/yeasts cells) and to 10^5 cfu/mL (for fungal spores). In the second stage, preliminarily melted and tempered at 45-48°C LBG/MEA were inoculated with the bacterial/yeasts/fungal inocula, the inoculated media were transferred in quantity of 18 mL in sterile Petri dishes (d = 90 mm) (Gosselin™, France) and allowed to harden for 1-2 h. The extracts were pipetted in duplicates of 60 µL into preliminarily prepared wells (d = 6 mm) in the agar media. The Petri dishes were incubated at identical conditions according to the type of the test microorganism.

After incubation for 24/48 h, the antimicrobial activity of the extracts was determined by measuring the diameter of the inhibition zones (IZ) around the agar wells. Sensitive were considered microorganisms with diameter of IZ of 18 mm or more; moderately sensitive were those in which the diameter of IZ was between 12 and 18 mm; resistant were those in which the IZ were up to 12 mm or completely missing.

2.2.6. Statistical analysis

Data from triplicate experiments (for the antimicrobial activity – in duplicates) were processed with MS Office Excel 2010 software using statistical functions to determine the mean value, standard deviation (±SD) and maximum estimation error at significance level $p \leq 0.05$.

3. Results

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The results from the physicochemical analyses of the three investigated herbs are presented in Table 3.

Table 3. Physicochemical characteristics of thyme, cotton thistle and hawthorn fruit.

Parameter	Thyme	Cotton thistle	Hawthorn fruit
Moisture, %	8.15±0.25	7.53±0.24	8.50±0.25
Ash, %	6.62±0.01	4.91±0.01	2.62±0.00
Carbohydrates, %	3.20±0.22	0.60±0.06	4.20±0.22
Proteins, %	11.30±0.73	8.33±0.55	2.95±0.26
Vitamin C, mg/100g	571.00±2.02	407.00±1.35	430.00±1.57

The results show that thyme, cotton thistle and hawthorn fruit had similar values of the moisture content. Regarding the ash content, the thyme showed the highest value (6.62 %) followed by cotton thistle and hawthorn fruit. The hawthorn fruit exhibited the highest content of carbohydrates (4.2 %) followed by thyme and cotton thistle. From the three studied herbs, thyme showed the highest protein content (11.3 %), while the lowest value was determined for the hawthorn fruit. The highest vitamin C content was found in thyme (571 mg/100 g dw) followed by hawthorn fruit and cotton thistle.

3.2. Polyphenolic content and antioxidant activity

As seen from the results presented in Table 4 and Table 5, the 70% ethanolic extracts of the three herbs demonstrated the highest total polyphenolic content (TPC) and antioxidant activity (determined by the DPPH, ORAC and HORAC methods) in comparison with the aqueous, oil and methanolic extracts, which exhibited significantly lower values. From the three investigated herbs, thyme extracts (aqueous, oil, methanolic and ethanolic) were distinguished with the highest content of total phenols and antioxidant activity, except the oil extract examined by the DPPH assay. Consequently, the type of solvent has a significant influence on the activity of the extract as the organic solvents exhibit better extraction of biologically active substances.

Table 4. Polyphenolic content and antioxidant activity of aqueous and oil extracts of thyme, cotton thistle and hawthorn fruit.

Plant extract	Polyphenols, mg GAE/g		Antioxidant activity					
			DPPH, µmol TE/g		ORAC, µmol TE/g		HORAC, µmol GAE/g	
	Oil	Aqueous	Oil	Aqueous	Oil	Aqueous	Oil	Aqueous
Thyme	27.20 ±0.61	8.20 ±0.13	35.93 ±0.45	15.66 ±0.21	1483.08 ±10.3	29.63 ±1.11	426.72 ±6.91	n.d.
Cotton thistle	6.20 ±0.05	5.30 ±0.02	5.26 ±0.03	19.93 ±0.18	241.05 ±0.43	11.41 ±0.56	48.93 ±2.74	n.d.
Hawthorn fruit	12.80 ±0.14	3.70 ±0.00	11.28 ±0.15	21.80 ±0.23	469.51 ±0.81	10.30 ±0.52	173.05 ±6.22	n.d.

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Table 5. Polyphenolic content and antioxidant activity of methanolic and ethanolic extracts of thyme, cotton thistle and hawthorn fruit.

Plant extract	Polyphenols, mg GAE/g		Antioxidant activity					
			DPPH, μmol TE/g		ORAC, μmol TE/g		HORAC, μmol GAE/g	
	MeOH	70% EtOH	MeOH	70% EtOH	MeOH	70% EtOH	MeOH	70% EtOH
Thyme	31.70 ±0.75	310.00 ±4.25	320.75 ±3.39	397.48 ±2.98	458.98 ±3.14	3221.95 ±41.92	170.19 ±6.09	961.21 ±2.36
Cotton thistle	14.90 ±0.31	168.00 ±2.05	4.67 ±0.04	45.00 ±1.12	117.49 ±1.33	1742.49 ±33.80	42.13 ±0.82	716.37 ±6.75
Hawthorn fruit	9.20 ±0.08	13.60 ±0.11	2.09 ±0.01	38.99 ±0.89	177.13 ±5.99	1891.73 ±12.67	65.24 ±7.06	669.83 ±1.39

3.3. Flavonoids and phenolic acid contents

Flavonoids are the polyphenolic compounds most abundant and widely distributed in plants. The results from the high performance liquid chromatographic analysis (HPLC) of the flavonoid content of the three investigated herbs are presented in Table 6.

The flavonoid content of thyme was characterized by the highest amount of quercetin-3-β-glucoside (374.5 mg/100 g), followed by luteolin (73.8 mg/100 g), while apigenin and kaempferol were detected in significantly lower concentrations. The flavonoid content of cotton thistle was characterized by the highest amount of myricetin, followed by apigenin and kaempferol. In contrast, only quercetin-3-β-glucoside and low amount of myricetin were presented in the flavonoid content of the hawthorn fruit.

Regarding the phenolic acid content of the three plants determined by HPLC method (Table 7), thyme was distinguished by the highest presence of rosmaric acid (995 mg/100 g) and significantly lower concentration of caffeic acid. Phenolic acid content of cotton thistle was presented by chlorogenic acid (324 mg/100 g) and lower amounts of neochlorogenic acid and gallic acid. Phenolic acid content of the hawthorn fruit included chlorogenic acid, neochlorogenic acid and caffeic acid.

Table 6. Flavonoid content of thyme, cotton thistle and hawthorn fruit determined by HPLC.

70% EtOH extracts	Quercetin-3-β-glucoside, mg/100g	Myricetin, mg/100g	Kaempferol, mg/100g	Apigenin, mg/100g	Luteolin, mg/100g
Thyme	374.5 ± 4.0	-	16.1 ± 0.1	16.4 ± 0.4	73.8 ± 0.2
Cotton thistle	-	152.3 ± 0.3	42.2 ± 0.1	85.1 ± 0.2	-
Hawthorn fruit	48.5 ± 0.1	10.9 ± 0.2	-	-	-

Table 7. Phenolic acid content of thyme, cotton thistle and hawthorn fruit determined by HPLC analysis.

70% EtOH extracts	Neochlorogenic acid, mg/100g	Chlorogenic acid, mg/100g	Gallic acid, mg/100g	Rosmaric acid, mg/100g	Caffeic acid, mg/100g
Thyme	-	-	-	995.0± 0.6	26.0 ± 1.6

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Cotton thistle	19.2± 0.1	324.0± 4.4	5.8± 0.1	-	-
Hawthorn fruit	12.3± 0.5	27.7± 0.1	-	-	9.4 ± 0.1

3.4. Antimicrobial activity

As seen from the results in Table 8, the ethanolic extracts of the three studied herbs exhibited the highest antimicrobial activity in comparison with the methanolic, aqueous and oil extracts. The aqueous extracts exhibited limited antimicrobial potential, while the oil extracts had no antimicrobial activity against the used test microorganisms.

The aqueous extract from thyme demonstrated the highest inhibitory activity against *M. luteus* 2YC-YT, moderate activity against *L. monocytogenes* NBIMCC 8632, *S. enteritidis* ATCC 13076 and low inhibitory activity against *S. aureus* ATCC 25923, and fungi *P. chrysogenum* and *Rhizopus* sp. The aqueous extract from hawthorn fruit showed moderate inhibitory effect on *L. monocytogenes* NBIMCC 8632, *E. faecalis* ATCC 29212, *M. luteus* 2YC-YT and fungi *Rhizopus* sp., and weak inhibitory effect on *B. subtilis* ATCC 6633, *P. aeruginosa* ATCC 9027, *E. coli* ATCC 25922 and fungus *P. chrysogenum*. The aqueous extract from cotton thistle demonstrated the highest inhibitory activity against *M. luteus* 2YC-YT, moderate activity against *L. monocytogenes* NBIMCC 8632, *E. faecalis* ATCC 29212, *S. enteritidis* ATCC 13076 and fungus *P. chrysogenum*, while inhibitory effect on the fungus *Rhizopus* sp. was weak.

The methanolic extract of thyme possessed moderate inhibitory effect on *B. subtilis* ATCC 6633, *S. enteritidis* ATCC 13076, *S. typhimurium* NBIMCC 1672, *K. pneumonia* ATCC 13883, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027 and *P. vulgaris* ATCC 6380.

The antimicrobial activity against *B. amyloliquefaciens* 4BCL-YT, *S. aureus* ATCC 25923, *L. monocytogenes* NBIMCC 8632, *E. faecalis* ATCC 29212, *M. luteus* 2YC-YT, yeasts *C. albicans* NBIMCC 74, *S. cerevisiae* ATCC 9763, fungi *A. niger* ATCC 1015, *P.chrysogenum*, *Rhizopus* sp. and *F. moniliforme* ATCC 38932 was low. The methanolic extract from hawthorn fruit exhibited moderate inhibitory effect only on *K. pneumonia* ATCC 13883, while the inhibitory effect on *B. subtilis* ATCC 6633, *B. amyloliquefaciens* 4BCL-YT, *M. luteus* 2YC-YT, *S. typhimurium* NBIMCC 1672, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *P. vulgaris* ATCC 6380, yeasts *C. albicans* NBIMCC 74 and fungus *P. chrysogenum* was weak. The methanolic extract from cotton thistle showed moderate inhibitory effect on *P. aeruginosa* ATCC 9027 and *P. vulgaris* ATCC 6380. The antimicrobial activity against *B. amyloliquefaciens* 4BCL-YT, *B. subtilis* ATCC 6633, *M. luteus* 2YC-YT, *S. typhimurium* NBIMCC 1672, *K. pneumonia* ATCC 13883, *E. coli* ATCC 25922, yeasts *C. albicans* NBIMCC 74 and fungus *P. chrysogenum* was low.

The ethanolic extract of thyme possessed high antimicrobial activity against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, moderate activity against *B. subtilis* ATCC 6633, *B. amyloliquefaciens* 4BCL-YT, *L. monocytogenes* NBIMCC 8632, *E. faecalis* ATCC 29212, *M. luteus* 2YC-YT, *S. enteritidis* ATCC 13076, *K. pneumonia* ATCC 13883, *P. vulgaris* ATCC 6380 and *P. aeruginosa* ATCC 9027, and weak antimicrobial activity against *S. typhimurium* NBIMCC 1672, yeasts *S. cerevisiae* ATCC 9763, and fungi *P. chrysogenum*, *Rhizopus* sp. and *F. moniliforme* ATCC 38932. The ethanolic extract from hawthorn fruit showed moderate inhibitory effect on *B. subtilis* ATCC 6633, *L. monocytogenes* NBIMCC 8632, *M. luteus* 2YC-YT, *P. aeruginosa* ATCC 9027 and *E. coli* ATCC 25922, and weak inhibitory effect on *B. amyloliquefaciens* 4BCL-YT, *E. faecalis* ATCC 29212, *S. enteritidis* ATCC 13076, *S. typhimurium* NBIMCC 1672, *K. pneumoniae*ATCC 13883 and *P. vulgaris* ATCC 6380.

The ethanolic extract from cotton thistle demonstrated strong inhibitory effect on *B. subtilis* ATCC 6633, *M. luteus* 2YC-YT and *E. coli* ATCC 25922, moderate inhibitory effect on *S. aureus* ATCC 25923, *L. monocytogenes* NBIMCC 8632, *E. faecalis* ATCC 29212, *S. enteritidis* ATCC 13076, *S. typhimurium* NBIMCC 1672, *P. aeruginosa* ATCC 9027 and *K. pneumonia* ATCC 13883. The antimicrobial activity against *P. vulgaris* ATCC 6380, yeasts *C. albicans* NBIMCC 74, *S. cerevisiae* ATCC 9763, and fungi *A. niger* ATCC 1015, *P. chrysogenum*, *Rhizopus* sp. and *F. moniliforme* ATCC 38932 was low.

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Table 8. Antimicrobial activity of thyme, cotton thistle and hawthorn fruit extracts.

Test microorganism	Inhibition zones (IZ), mm											
	Aqueous			Oil			MeOH			70% EtOH		
	T*	HF**	CT***	T*	HF**	CT***	T*	HF**	CT***	T*	HF**	CT***
***B. subtilis* ATCC 6633**	-	10	-	-	-	-	12	10	11	14	14	20
***B. amyloliquefaciens* 4BCL-YT**	-	-	-	-	-	-	10	8	10	13	9	-
***S. aureus* ATCC 25923**	10	-	-	-	-	-	9	-	-	18	-	17
***L. monocytogenes* NBIMCC 8632**	13	13	13	-	-	-	9	-	-	14	12	13
***E. faecalis* ATCC 29212**	-	13	13	-	-	-	8	-	-	12	10	13
***M. luteus* 2YC-YT**	20	15	22	-	-	-	10	9	9	17	16	16
***S. enteritidis* ATCC 13076**	12	-	12	-	-	-	12	-	-	13	10	13
***S. typhimurium* NBIMCC1672**	-	-	-	-	-	-	12	10	11	11	11	11
***K. pneumoniae* ATCC 13883**	-	-	-	-	-	-	14	14	10	14	11	10
***E. coli* ATCC 25922**	-	8	-	-	-	-	12	10	10	18	15	18
***P. vulgaris* ATCC 6380**	-	-	-	-	-	-	13	12	12	12	11	8
***P. aeruginosa* ATCC 9027**	-	8	-	-	-	-	13	10	12	17	15	16

*T – Thyme extract; **HF - Hawthorn fruit extract; ***CT - Cotton thistle extract; d_{well} = 6 mm.

Table 8. Cont.

Test microorganism	Inhibition zones (IZ), mm											
	Aqueous			Oil			MeOH			70% EtOH		
	T*	HF**	CT***	T*	HF**	CT***	T*	HF**	CT***	T*	HF**	CT***
***C. albicans* NBIMCC 74**	-	-	-	-	-	-	8	8	8	-	-	8
***S. cerevisiae* ATCC 9763**	-	-	-	-	-	-	8	-	-	8	-	8
***A. niger* ATCC 1015**	-	-	-	-	-	-	8	-	-	-	-	8

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A. flavus	-	-	-	-	-	-	-	-	-	-	-	-
P. chrysogenum	10	11	15	-	-	-	10	10	8	8	-	8
***Rhizopus* sp.**	11	12	10	-	-	-	8	-	-	8	-	8
***F. moniliforme* ATCC 38932**	-	-	-	-	-	-	8	-	-	8	-	8
***Mucor* sp.**	-	-	-	-	-	-	-	-	-	-	-	-

*T – Thyme extract; **HF - Hawthorn fruit extract; ***CT - Cotton thistle extract; d_{well} = 6 mm.

4. Discussion

4.1. Physicochemical characteristics

Similar physicochemical characteristics of thyme (*Thymus vulgaris* Linne) were reported by Balladin and Headley [44], who stated that the moisture content of thyme dried at different temperature regimes varied between 10% and 12.5%, while ash content varied between 1.5% and 2.26%, whose values were lower than our results. In contrast to our data, Shahar et al. [45] determined higher protein (21.39%) and carbohydrates (11.85%) amounts, but lower ash content (2.73%) and vitamin C concentration (43.8 mg/100 g dw) of thyme (*Thymus serpyllum* L.) from Kargil Ladakh district, India.

The results from the analyses of cotton thistle (*Onopordum acanthium* L.) revealed that the main phytochemical compounds are alkaloids, saponins, flavonoids, triterpenes, sesquiterpen lactones, sterols, lipids, nitrogen-containing compounds, phenolic acids, coumarins, inulin, sugars, proteins, oil and fatty acids [46, 47]. The protein content (14.9%) of *O. acanthium* L. originating from Turkey was higher in comparison with our results (8.33%) [47]. Similar to our results were previously reported by Petkova et al. [48], who investigated *O. acanthium* L. from Bulgaria and determined moisture content of 7.9% and ash content of 5.8%.

Mironeasa et al. [49] investigated the physicochemical characteristics of fresh wild hawthorn fruits and determined moisture content of 69.14%, ash content of 1.75%, total carbohydrates of 24.81% and protein content of 3.5%. The ash and protein contents were in agreement with our results; however, the fresh fruits examined by the same authors showed significantly higher moisture and carbohydrates contents. Özcan et al. [50] obtained similar results for fresh wild hawthorn fruits from Turkey with moisture content of 64.26%, ash content of 2.28% and crude protein content of 2.48%. Yanar et al. [51] reported that vitamin C values in three Turkish wild hawthorn genotypes varied between 1.3 and 6 mg/100 g fw, which was significantly lower compared to our results for dried fruits (430 mg/100 g dw). The same authors detected sucrose, glucose and fructose as main sugar compounds in hawthorn fruits, whose values varied between 2.48 and 10.42 g/100 g of fresh weight (fw).

4.2. Polyphenolic content and antioxidant activity

Lower than our results for the total phenolic content of thyme (*T. vulgaris*) from Egypt were obtained by Roby et al. [52], who determined values of 8.1, 7.3, 6.15 and 4.75 mg GAE/g dw for the methanolic, ethanolic, diethyl ether and hexane extracts, respectively. According to Köksal et al. [53], the TPC of an aqueous and ethanolic extracts of dried thyme (*T. vulgaris*) from Turkey were 256 µg GAE/mg and 158 µg GAE/mg, respectively. A study on the antioxidant potential and the bioactive compounds of another *Thyme* species (*T. serpyllum* L.) from the Ladakh plateau, India, revealed that the TPC amounted to 86.6 mg GAE/g dw, which was higher than our results for aqueous, oil and methanolic thyme extracts, but lower than the ethanolic one. The authors stated that antioxidant activity of the aqueous thyme extract showed a value of 48.58 µg/mL determined by the DPPH method [45]. Sarfaraz et al. [54] reported TPC values of 35.73 mg GAE/g dw of a methanolic extract obtained from Iranian *T. vulgaris*, which is close to our result for methanolic extract (31.7 mg GAE/g dw).

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Georgieva et al. [55] investigated five medicinal plants from the Western Rhodopes, Bulgaria, and determined that the total phenolic content (TPC) of 70% ethanolic extracts of *T. callieri* varied in the range of 20.32 mg GAE/g to 86.19 mg GAE/g for a frozen and a dried herb, respectively. Similar to our results were obtained for the TPC of 70% ethanolic extracts of *C. monogyna* fruit, whose values were between 13.19 mg GAE/g and 26.88 mg GAE/g for a frozen and a dried herb, respectively. Regarding the antioxidant activity, the values for *T. callieri* were 121.30 and 218.97 mM TE/g (for a frozen and a dried herb), while for *C. monogyna* fruit were 85.43 and 176.23 mM TE/g (for a frozen and a dried herb) determined by the DPPH method.

Total phenolic content and antioxidant activity of cotton thistle (*O. acanthium* L.) from Bulgaria were previously examined by Petkova et al. [48]. The authors determined TPC values of 4.06 mg GAE/g dw for the aqueous thistle extract, which is comparable with our result. In contrast, the antioxidant activity of the aqueous thistle extract determined by the DPPH method (22.73 mM TE/g), which was higher than our result.

Parzhanova et al. [56] investigated the total phenolic content, total flavonoids and antioxidant potential of cotton thistle (*O. acanthium* L.) from the Rhodope Mountains, Bulgaria. The two ethanolic extracts (50% and 70%) of flowering heads of cotton thistle demonstrated higher TPC (50 mg GAE/g dry extract) in comparison with the aqueous extract. However, the results from the antioxidant activity test demonstrated that 70% ethanolic extract possessed the highest antiradical value (298.3 mM TE/g) evaluated by the DPPH assay, while the water extract exhibited the highest value by the FRAP assay. In the same research, the authors reported that the 50% ethanolic extract of flowering heads of cotton thistle originating from the Rhodope Mountains demonstrated the highest TFC (110 mg QE/g dry extract) compared to the water and 70% ethanolic extract of the same plant.

4.3. Flavonoids and phenolic acid contents

Georgieva et al. [55] determined that the total flavonoid content (TFC) of 70% ethanolic extracts of *T. callieri* from the Western Rhodopes, Bulgaria varied in the range of 7.47 mg QE/g to 26.43 mg QE/g for a frozen and a dried herb, respectively, while the TFC values of 70% ethanolic extracts of *C. monogyna* fruit from the same geographic location were between 2.27 mg QE/g and 2.89 mg QE/g for a frozen and a dried herb, respectively. In the same research, the authors evaluated the impact of ethanol concentration on the extraction of biologically active compounds, and concluded that the highest extraction yield was reached with 70% ethanol (maximal values of TPC, TFC and antioxidant activity by DPPH and FRAP methods for the frozen and dried herbs, respectively), which is in compliance with our results.

Roby et al. [52] investigated by HPLC the phenolic content of a methanolic extract of thyme (*T. vulgaris*) from Egypt and identified quinic acid, p-coumaric acid, caffeic acid, rosmaric acid, ferulic acid, carnosic acid, caffeic and ferulic acid derivatives, quercetin-7-o-glucoside, methyl rosmarenate, apigenin, naringnin and luteolin-7-o-rutinose. The HPLC analysis of Iranian thyme extract performed by Sarfaraz et al. (2023) [54] demonstrated the presence of gallic acid (5.5 mg/100 g dw), epicatechin (2.3 mg/100 g dw), caffeic acid (11.8 mg/100 g dw), luteolin-7-o-glucoside (0.8 mg/100 g dw), p-coumaric acid (2.9 mg/100 g dw), ferulic acid (25.6 mg/100 g dw), rosmarinic acid (87.4 mg/100 g dw), salvianolic acid (90 mg/100 g dw), cinnamic acid (32.3 mg/100 g dw) as major phenolic acids, as well as apigenin (12.2 mg/100 g dw), naringenin (0.6 mg/100 g dw) and kaempferol (1.1 mg/100 g dw) as the major favonoid compounds. In comparison, the results for *T. callieri* showed that in our samples gallic acid was not detected, while caffeic acid, rosmarinic acid, apigenin and kaempferol were found in higher amounts.

Yanar et al. [51] analyzed wild hawthorn fruits (*C. monogyna*) belonging to three different genotypes, and found the following phenolic compounds varying in different amounts: gallic acid (0.32 - 1.61 mg/100g fw), catechin (15.1 - 143.82 mg/100g fw), epicatechin (1.32 - 35.09 mg/100g fw), rutin (6.62 - 21.82 mg/100g fw), epigallocatechin gallate (0.75 - 79.5 mg/100g fw) and caffeic acid (0.01 - 1.03 mg/100g fw). Our results for hawthorn fruit showed that gallic acid was not detected, while the amount of caffeic acid was higher (9.4 mg/100g dw). Other authors have also detected chlorogenic acid in hawthorn fruits [19, 57], as well as caffeic acid and ellagic acid [58]. The antioxidant activity

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of the hawthorn fruits determined by two methods showed values of 280.7 μmol TE/g (by ORAC) and 107.5 μmol GAE/g (by HORAC) [58]. Khokhlova et al. [59] identified caffeic acid, chlorogenic acid, oleanolic acid, ursolic acid, hyperoside, vitexin, vitexin 2-rhamnoside, vitexin 2-O-rhamnoside, rutin and naringenin in hawthorn fruit. In contrast to our results, which showed the presence only of quercetin-3-β-glucoside and myricetin, Keser et al. [60] identified the flavonoids rutin, apigenin, myricetin, quercetin, naringenin and kaempferol.

4.4. Antimicrobial activity

Al-Juraifani [61] evaluated four plants from Saudi Arabia and found that ethanolic-water extract from thyme (*T. vulgaris*) exhibited the highest antimicrobial activity against *Streptococcus* sp., *S. aureus*, *Vibrio tubiashii*, *M. luteus* ATCC 9341, *Cellulosimicrobiumcellulans*, *B. cereus*, *Legionella pneumophila* and two fungal species –*A. flavus* and *Fusarium oxysporum*, as the inhibitory activity increased with heightened concentrations of the plants' extracts. Mokhtari et al. [62] observed high antimicrobial activity of a methanolic extract of thyme (*T. vulgaris*) against *B. cereus* with an inhibition zone (IZ) in diameter of 24.87 mm, *S. aureus* (IZ = 23.56 mm), *E. coli* (IZ = 18.43 mm) and *S. typhimurium* (IZ = 17.11 mm), and concluded that the investigated extract could be successfully applied as an antibacterial agent. According to the same authors, the antibacterial activity of the thyme is due to the presence of the biologically active compounds thymol, cinnamic acid and carvacrol, which increase the permeability of the cell membrane, inhibit the bacterial enzymes and disrupt the synthesis of the cell structural components or the genetic substance, especially in the Gram-positive bacteria.

A study performed by Tadić et al. [23] on the antimicrobial activity of an ethanolic extract of hawthorn fruit at concentration of 10 mg/ml showed that the extract had strong inhibitory effect on *Micrococcus flavus* and *B. subtilis*, moderate inhibitory effect on *L. monocytogenes*, *Streptococcus epidermidis*, *M. luteus*, *P. aeruginosa* and weak effect on *E. coli*, *S. aureus* and yeasts *C. albicans*. Yiğit et al. [63] examined aqueous and methanolic extracts of hawthorn fruit and leaves against clinical isolates of human pathogenic strains (*Enterobacter aerogenes*, *S. aureus, E. coli*, *P. aeruginosa* and yeasts *C. albicans*, *Candida glabrata* and *Candida parapisilosis*) and stated that both extracts possessed limited antimicrobial activity. Methanolic extract of hawthorn fruit exhibited low inhibitory activity against *S. aureus*, while aqueous hawthorn fruit extract had no inhibitory activity against the tested microorganisms. Pugna et al. [64] found that inner layers of hawthorn fruit possessed high inhibitory activity against *E.coli* ATCC 25992 (IZ = 40 mm) and *P. aeruginosa* ATCC 27852 (IZ = 30 mm), but did not inhibit the pathogen *S. aureus* ATCC 25923.

Few studies about the antimicrobial activity of *O. acanthium* have been previously reported. Zare et al. [65] determined that methanolic extract from cotton thistle seeds displayed significant antibacterial activity against *Staphylococcus epidermidis* (IZ = 18.66 mm) and *M. luteus* (IZ = 21 mm), but had no inhibitory effect on *E.coli*, *S. aureus* and *K. pneumoniae*. Móricz et al. [66] studied the antimicrobial effect of *O. acanthium* L. leaf extract and identified, by HPLC analysis, the three compounds responsible for its antibacterial activity – linoleic, linolenic acid and germacranolide sesquiterpene lactone (onopordopicrin).

5. Conclusions

Based on the estimations of the present research, we can conclude that the three herbs – thyme, hawthorn fruit and cotton thistle from the Western Rhodope Mountains, Bulgaria– represent an excellent source of nutrients and compounds with promising antioxidant activity (biological potential factors), which can be used in the food industry as food ingredients and food enhancers, or in the pharmaceutical industry as active components of different medical formulations. Regarding the antimicrobial potential of the investigated herb extracts, they can find successful practical application in biopreservation and improvement of the shelf life of various food products.

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