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Natural Products as Antibacterial Agents — Antibacterial Potential and Safety of Post-distillation and Waste Material from *Thymus vulgaris* L., Lamiaceae

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Additional information is available at the end of the chapter

Abstract

Medicinal plants have a long tradition of use in folk and conventional medicine. In recent years numerous studies confirm various bioactivities of natural products, among them antibacterial activity. Natural antibacterial agents such are essential oils and isolated compounds now represent a notable source for pharmaceutical and food industry and are widely used in cosmetology. They meet standards of 'green consumerism' together with excellent antibacterial activity. Aromatic plants such is *Thymus vulgaris* L. are the major sources of essential oils. Thyme essential oil, as well as dominant compounds thymol and carvacrol are generally recognised as safe and have been registered by European Commission for use as flavouring agents in foodstuffs. However, essential oil is present in very low amount (0,8-2,6%) in thyme leaves. Thus, the majority of plant material remains unused after the isolation. Nowadays, the biological potential of various plant waste materials are in focus of numerous studies. These investigations also include the antimicrobial activity considering the fact that waste material extracts represent the valuable source of different phenolic compounds. Regarding all this, the aim of the present study was to determine antibacterial potential of chemically characterised extracts obtained from waste material remaining after the preparation of drug (stems) and isolation of thyme essential oil (deodorised leaves, postdistillation decoction) on selected bacterial strains. Also, in order to determine safety of waste extracts their cytotoxicity was investigated. All extracts were prepared with maceration using 45% or 75% ethanol

(EtOH) for 24 h at room temperature (1:10 w/v). Total phenolic compounds and flavonoids were determined spectrophotometrically. Extracts were chemically characterized by HPLC/DAD analysis. Antibacerial testing was done with broth dilution method against several bacterial strains (*Staphylococcus aureus, Bacillus cereus, Salmonella infantis, Escherichia coli* and *Campylobacter jejuni*). Cytotoxicity and cytoprotection studies were performed by XTT assay. Result of HPLC analysis showed that investigated extracts, especially those obtained from deodorised leaves represent a valuable source of rosmarinic acid and luteolin 7-O-glucuronide. Antibacterial testing indicated that all waste material extracts, except the extract T2, possess similar or even stronger bacteriostatic activity than T1. No cytotoxicity nor cytoprotection were determined. In conclusion, results of this study confirmed antibacterial potential investigated thyme extracts. High concentrations of rosmarinic acid and luteolin 7-O-glucuronide, which both have numerous pharmacological activities, were determined. This indicates that thyme postdistillation waste material extracts could be used for isolation of dominant compounds or as addities in pharmaceutical and food industry.

Keywords: aromatic plants, thyme, postdistillation waste, antibacterial potential, cytotoxicity

1. Introduction

Plants have been used as food, spice, and medicine since ancient time. Much before the discovery of the existence of microorganisms, plants were an integrated part of the traditional medicine of many communities used for the treatment of various infectious diseases. Likewise, the use of plants as spices, not only affected the organoleptic properties of food, but it was also one of the ways for its preservation [1]. Today, modern consumers demand food that will be minimally processed and contain, as much as possible, additives of natural origin that unlike synthetic ones, will be safe and ecologically acceptable. Also, the approach in raising livestock and production of food of plant origin is shifting towards the use of traditional methods in protection against pests and diseases [2]. In addition, the irrational use of antibiotics not only in human and veterinary medicine, but also to promote growth in agriculture, has led to more frequent occurrence of resistance to conventional antibiotics of some pathogenic microorganisms [3, 4, 5] such are *Escherichia coli, Staphylococcus aureus, Enterococcus* spp., *Salmonella* spp., and *Campylobacter* spp. [6, 7, 8, 9]. Moreover, it is important to bear in mind the potential synergistic action of different natural products and conventional antibiotics in order to affect the emerging resistance [10, 11, 12].

Aromatic plants such as *Thymus vulgaris* L. (common thyme) and many other representatives of the family Lamiaceae have a long tradition of use in both folk and conventional medicine and in the pharmaceutical industry [13]. Common thyme is indigenous to Europe, especially the Mediterranean region, and is extensively cultivated worldwide. It is a small, bushy herb,

with small, elliptical, greenish-grey, and shortly-stalked leaves. Thyme has a characteristic odour of thymol and is used as a culinary herb [14]. The active principle is the essential oil (*Thymi aetheroleum*), with thymol (25%–50%) and carvacrol (3%–10%) as dominant compounds (Table 1) (Figure 1) [15].

| Compounds	Percentage (%)	References
Monoterpene Hydrocarbons		
α-Terpinene	1.25–3.17	17
p-Cimene	0.8–16.13	16, 17
γ-Terpinene	7.3–1.87	12, 16, 17
Oxygenated Monoterpenes		
1, 8-Cineole	0.8–2.17	16, 17
Linalool	2.03–6.8	12, 16, 17
Menthone	2.2	16
Borneol	2.6–8.9	12, 16
Neomenthol	2.8	16
Terpinen-4-ol	1–2.63	16, 17
Aromatic Oxygenated Monoterpenes		
Carvacrol methyl ether	3.9	12
Thymol	35.51–47.9	12, 16, 17
Carvacrol	4.43–12	
Sesquiterpene Hydrocarbons		
trans-Caryophyllene	1.13–5.1	12, 17
β-Cubebene	2.4	16
Oxygenated Sesquiterpenes		
Caryophyllene oxide	0.33–4.6	12, 17
Aliphatic compounds		
1-Octen-3-ol	2.8	12

Table 1. Dominant compounds of *Thymus vulgaris* essential oil

The percentage of the major constituents varies greatly, depending on numerous complex factors, both endogenous and exogenous such are: chemotype, ontogenesis, geographic and climatic conditions, methods used for plant material processing, and essential oil isolation [18]. Also, similar to other medicinal plants, the drug could be adulterated or replaced with other representatives of the genus *Thymus*, such as *T. serpyllum*, *T. marschallianus*, *T. pannonicus*, etc., that affect the differences in chemical composition and biological activities [19, 20]. Neverthe-less, a minimum 40% of the thyme essential oil is required to be aromatic oxygenated mono-terpenes [15].

	Thyme essential oil (µl/mL)	Thymol (µg/mL)	Carvacrol (µg/mL)	References
Staphylococcus aureus	0.2–2.5	250–2000	125–280	12, 21, 22
Salmonella sp.	0.45–20	250–3755	125–375	3, 12, 21
Escherichia coli	0.45–1.25	250–2000	125–560	12, 21, 22, 23
Bacillus cereus	1	250–560	125–140	12, 21

Table 2. Minimal inhibitory concentrations of thyme essential oil, thymol, and carvacrol on selected bacterial strains

Thyme essential oil, thymol, and carvacrol are generally recognised as safe (GRAS status) and have been registered by the European Commission for use as flavouring agents in foods [24]. GRAS is a rigorous process that relies on common knowledge and expert consensus about the safety of the substance for its intended use. Well-conducted toxicology studies are one of the factors that are used to assess the safety of various natural products, for example, plant extracts or essential oils that are composed of a mixture of tens or hundreds of compounds [25].
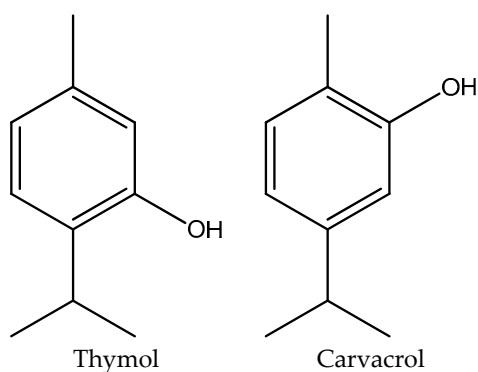

Figure 1. Chemical structures of dominant monoterpenes in thyme essential oil

In recent years, numerous studies confirm various bioactivities of thyme essential oil and isolated dominant monoterpenes, among them antibacterial and antifungal activity, together with the inhibition of mycotoxin production (Table 2) [3, 12, 16, 21, 23, 26, 27, 28]. Generally, most of the essential oils used in the pharmaceutical, cosmetic, and food industries possess strong antioxidant activity and represent a notable source of natural additives for different products [12, 29, 30]. What is more important is that they meet standards of 'green consumerism' [21]. However, the essential oil is present in very low amounts (0.8%–2.6%) in thyme leaves. Thus, the majority of plant material remains unused after the isolation.

On the other hand, thyme leaves are found to be a rich source of various phenolic compounds, particularly phenolic acids, flavonoids, and other secondary metabolites that are expected to contribute to its biological activities (Table 3) [14].

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Figure 5) widely found in the plant kingdom and presumably accumulated as a defense compound. In the

Lamiaceae family, the occurrence is mainly restricted to the subfamily Nepetoideae [31] and it is identified as one of the active components of several aromatic medicinal plants (e.g., *Salvia officinalis*, *Mentha* x *piperita*, *Lavandula officinalis*, *Ocimum basilicum*, *Origanum vulgare*, *Origanum majorana*, *Thymus vulgaris*, *Melissa officinalis*, *Rosmarinus officinalis*) [32]. For rosmarinic acid numerous and notable biological activities are confirmed in different *in vitro* and *in vivo* examinations. It is of pharmaceutical importance because of its non-specific complement activation and inhibition of the biosynthesis of leukotriens (leading to an anti-inflammatory effect), as well as its antimicrobial and especially antiviral activity [14, 33]. Additionally, it exhibits a very strong antioxidant effect; it is an inhibitor of acetyl and butyryl cholinesterase and neuroprotector that propose its use in the prevention and symptomatic treatment of Alzheimer's disease. However, it should be considered that bioavailability of rosmarinic acid in the brain is very unlikely. Also, in some test systems rosmarinic acid expresses anticarcinogenic activity [34, 35, 36].
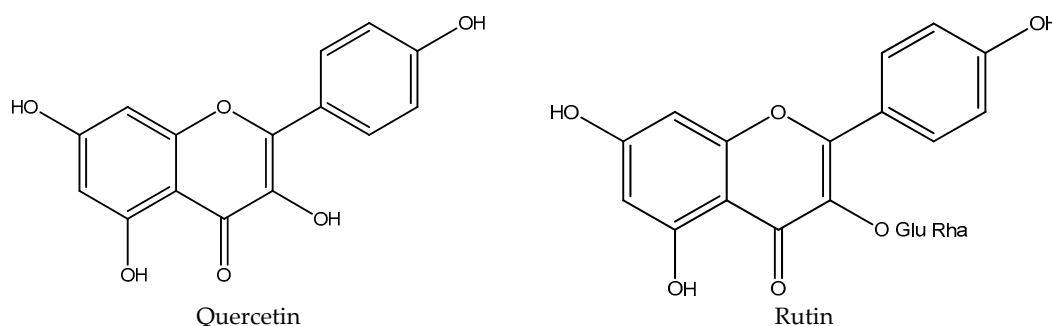
Figure 2. Chemical structures of flavonoids in investigated thyme extracts (Glu = glucose, Rha = rhamnose)

Extraction procedure	Identified compounds	References
Butanol-soluble fraction	Acetophenone glycosides (Androsin, Picein, glycosides of 4-hydroxyacetophenone derivatives)	[37]
Methanol, reflux, residue was extracted with distilled water at 100°C for 2 h	Acidic polysaccharide	[38]
Acetone using a Polytron homogenizer for 1 min	Caffeic acid, Luteolin, Rosmarinic acid, Hispidulin	[39]
CH_2Cl_2 soluble fraction	5,4`-Dihydroxy-6,7,8-trimethoxyflavone, 5,3`,4`-Trihydroxy-7-methoxyflavone, 5,4`-Dihydroxy-6,7,3`-trimethoxyflavone, 5,4`-Dihydroxy-6,7,8,3`-tetramethoxyflavone	[40]
Extracted at 20°C with 1L of EtOH-H_2O-HOAc (80:19:1) under N_2 for 5 days; further fractionation	Rosmarinic acid, 3`-O-(8``-Z-Caffeoyl) rosmarinic acid, Eriodictyol, Taxifolin, Luteoline-7-O-glucuronide	[41]

Extraction procedure	Identified compounds	References
Methanol; 1 day at room temperature	biphenyl compounds (e.g. 3,4,3`,4`-tetrahydroxy-5,5`-diisopropyl-2,2`-dimethylbiphenyl)	[42]
Deodorised aqueous extracts	Total phenolics (mg/g gallic acid equivalents)	[43]
Methanol extract	Monoterpene glucosides (p-Cymenol-9-O-β-glucoside, 2- and 5-O-β-glucosides of thymoquinol, angelicoidenol-O-β-glucoside); Arbutin	[44]
Ethanol/water (30:70, v/v) with the aid of sonication for 10 min	Rosmarinic acid, Caffeic acid	[45]
methanol extract	compounds related to hydroxyjasmone (e.g. 5-hydroxyjasmone-5-O-ß-glucoside) and simple phenol glucosides (3-Hydroxy-4-methoxyphenethyl-3-O-ß-glucoside, eugenol-O-ß-glucoside, syringin)	[46]
Methanol, 20 min, 60°C in a water bath with shaking	Luteolin-glucoside, Rosmarinic acid, Eriodictyol, Luteolin, Apigenin	[47]
Methanol, 6 h, Soxhlet apparatus	Oleanolic acid, Ursolic acid	[48]
aqueous extracts (boiling water), 30 min	Luteolin-7-O-glucuronide, Lithospermic acid, Rosmarinic acid, Caffeic acid	[49]
Aquous, aq. methanolic, methanolic (1:100 or 1:200); infusion, hot methanolic, usage of ultrasonic bath for 15 or 30 min	Arbutin, Hydroquinone, Naringenin, Naringenin-7-O-ß-glucoside, Narirutin, Eriodictyol, Eriodictyol-7-O-ß-glucoside, Eriocitrin, Hesperidin, Luteolin, Luteolin-7-O-ß-glucuronide, Luteolin-7-O-ß-rutinoside, Rosmarinic acid, Methyl rosmarinate, Caffeic acid	[50]
95% ethanol at 60°C for 2 h with stirring	Total phenolics (mg/g sinapic acid equivalents)	[51]
Liquid nitrogen powdered samples were extracted with acidified methanol	Caffeic acid, Rosmarinic acid	[52]

Table 3. Comparison of literature data on identified compounds in *Thymus vulgaris* leaves

Chlorogenic acid (5-O-caffeoylquinic acid) is a natural antioxidant that is produced in various plants as a response to pathogen microorganisms, different mechanical impairment, or excessive exposure to light (Figure 5) [53]. Due to these characteristics, chlorogenic acid found its use in pharmaceutical and food industries, as well as in cosmetology [54]. Gallic and ferulic acids are also proven antioxidant agents (Figure 3) [45].

Furthermore, many beneficial properties have been identified for flavonoids (e.g., quecetin and rutin as the most widely distributed in nature) (Figure 2), and one of the most popularly cited property is their antioxidant activity. Other actions that are proposed to contribute to their biological effects include chelating metal ions, stimulating phase II detoxifying enzyme activity, and inhibiting proliferation and inducing apoptosis. Also, for some of the flavonoids
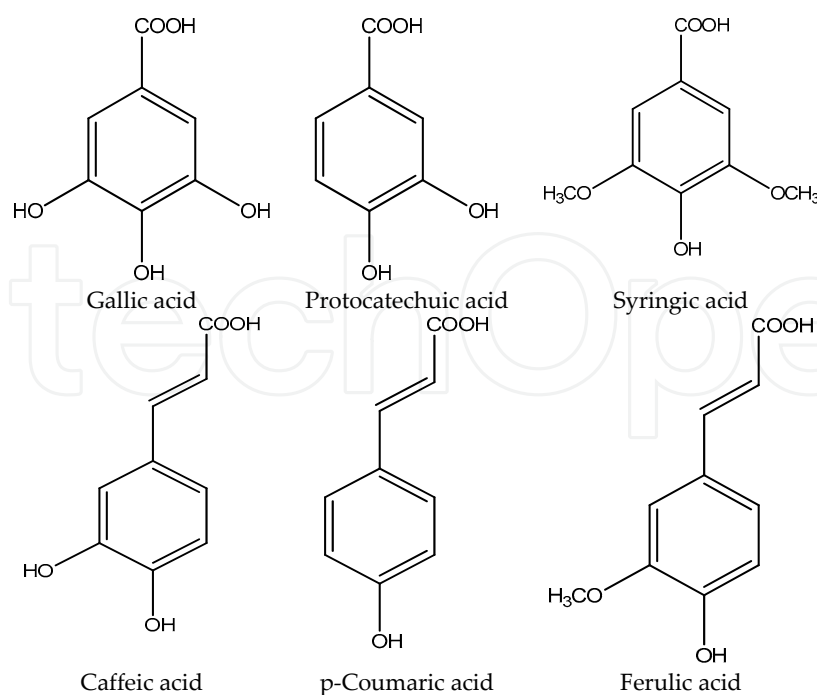
Figure 3. Chemical structures of hydroxybenzoic and hydroxycinnamic acids present in thyme extracts

where anti-inflammatory activity has been confirmed, they decrease the vascular cell adhesion and molecule expression, increase endothelial nitric oxide synthase activity, as well as inhibit platelet aggregation [55].

Specifically, it is determined that luteolin (3',4',5,7-tetrahydroxyflavone) exhibit notable antioxidant, antimicrobial, and anti-inflammatory activities [56]. Also, there is some evidence that apigenin and luteolin act as antimutagenic and anticarcinogenic agents (Figure 4) [57].
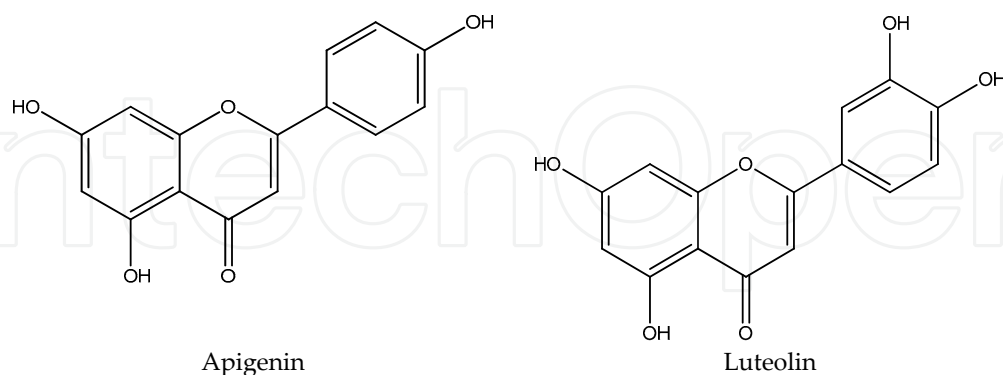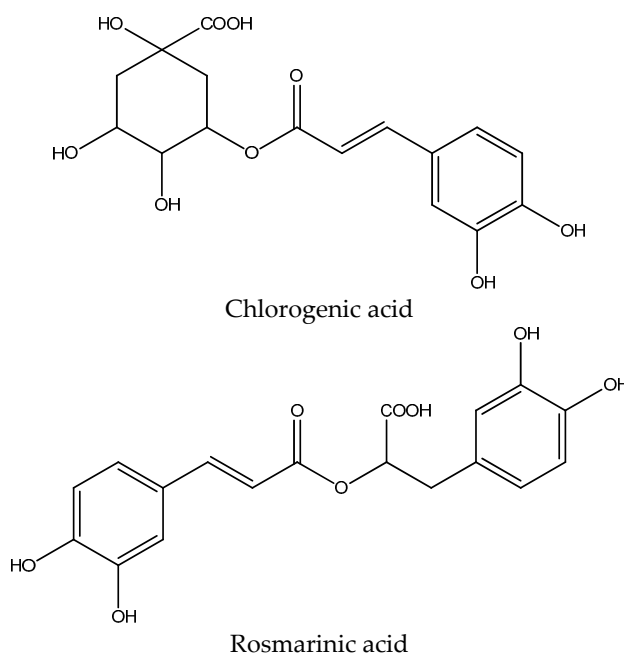

Figure 4. Chemical structures of dominant flavones in investigated thyme extracts

Since various plant extracts and isolated natural compounds are widely used and represent one of the major approaches in alternative and complementary medicine that are also accepted by conventional medicine, their quality, efficacy, and safety are of the utmost importance [58]. Safety issues are especially significant due to increasing body of evidence on side effects and

interactions between herbal remedies or/and food or conventional drugs. These interactions sometimes can be very serious. They may lead to increased or lack of action of conventional drugs and caution must be taken in the application of plant extracts [59].

Nowadays, the biological potential of various plant waste materials is the focus of numerous studies. This trend includes not only the examination of aromatic plants [43, 60, 61, 62], but also different fruits and plant products such as wine, olives, beetroot, tomato, garlic, and pomegranate [63, 64, 65, 66]. These investigations also include their antimicrobial activity, considering the fact that waste material extracts represent the valuable source of different phenolic compounds. These studies are important, both in terms of economy and ecology, in order to elucidate the way to exploit post-distillation waste material of aromatic plants and other plant waste materials more efficiently.

Chlorogenic acid

Rosmarinic acid

Figure 5. Chemical structures of dominant caffeic acid oligomers in thyme extracts

The aim of the present study was to determine the antibacterial potential of chemically well-characterised extracts obtained from waste material that remain after the preparation of the drug *Thymi folium* (stems) and the isolation of thyme essential oil (deodorised leaves and post-distillation decoction) on selected bacterial strains. Also, in order to determine the safety of the waste extracts, their cytotoxicity was investigated.

2. Methods

2.1. Plant material and extract preparation

In July 2013, aboveground parts of thyme (*Thymus vulgaris* L., Lamiaceae) were collected, just before flowering, in Padej, the Vojvodina province, Republic of Serbia. Voucher specimen of

collected plants (no. ThV-15/10) [67] was confirmed and deposited at the Herbarium of the Laboratory of Pharmacognosy, Department of Pharmacy, Faculty of Medicine, University of Novi Sad. Leaves were separated from the previously air-dried stems and stored separately at a cool and dry place for extract preparation. Standard thyme leaves extract [15, 68] was prepared by maceration procedure in 45% ethanol (EtOH) as a solvent for 24 h (1:10 w/v, 10 g dried leaves) at room temperature (T1). Essential oil was isolated using hydrodistillation technique [69]. The waste material, remaining after isolation, was filtered and obtained decoction was used to prepare post-distillation extract (T2). Deodorised leaves were air dried and macerated with 45% (T3) and 75% (T4) EtOH for 24 h. Dried stems were grinded (sieve 0.75) and macerated with 45% EtOH for 24 h to obtain the extract T5.

After the maceration, extracts were collected, filtered, and evaporated to dryness under vacuum. The quantities of dry extracts were determined gravimetrically. Residues were dissolved in water to make 10% (w/v) stock solutions for further investigation. For the high-performance liquid chromatography (HPLC analysis), residues were dissolved in methanol, 1% formic acid mixture (50:50 v/v) to make 2% (w/v) stock solutions.

2.2. Chemical composition

2.2.1. Determination of total phenolic compounds and total flavonoid content

The amount of total phenolic compounds in investigated extracts was determined spectro-photometrically with Folin-Ciocalteu (FC) reagent [70]. The concentration of total phenolic compounds was expressed in mg of gallic acid equivalents (GAE) per g of dry extract (d.e.), using a standard curve of gallic acid (concentration range 0.08–0.24 mg/mL). Total flavonoid content was also determined spectrophotometrically using a method based on the formation of a flavonoid-aluminium complex with an absorptivity maximum at 430 nm [71]. Flavonoid content was expressed in mg of quercetin equivalents (QE) per g of dry extract, using a standard curve of quercetin (concentration range 10–100 μg/mL). All measurements were done in triplicate.

2.2.2. HPLC/DAD analysis

Investigated thyme leaves extracts are rich in a wide range of phenolic compounds [50]. Therefore, a method that allows simultaneous detection of various phenolics in a single run was developed. This decreases the time necessary for analysis and reduces the analysis costs [72]. HPLC analysis was performed using a liquid chromatograph (Agilent 1200 series), equipped with diode array detector (DAD) and Eclipse XDB-C18, 1.8 μm, 4.6×50 mm column, at a flow-rate of 1 mL/min. Solvent gradient was performed by varying the proportion of solvent A (methanol) to solvent B (1% formic acid in water (v/v)) [73]. The total running time and post-running time were 45 and 10 min, respectively. The column temperature was 30ºC. The injected volume of samples and standards was 5 μL and it was done automatically using an autosampler. The spectra were acquired in the range of 210–400 nm and chromatograms plotted at 280, 330, and 350 nm with a bandwidth of 4 nm, and with reference wavelength/bandwidth of 500/100 nm. Quantification of selected phenolics: gallic acid, protocatechuic acid, caffeic acid, chlorogenic acid, syringic acid, *p*-coumaric acid, ferulic acid, rutin, rosmarinic acid,

quercetin, and apigenin (Figure 6) were done using the calibration curve of standard compounds.
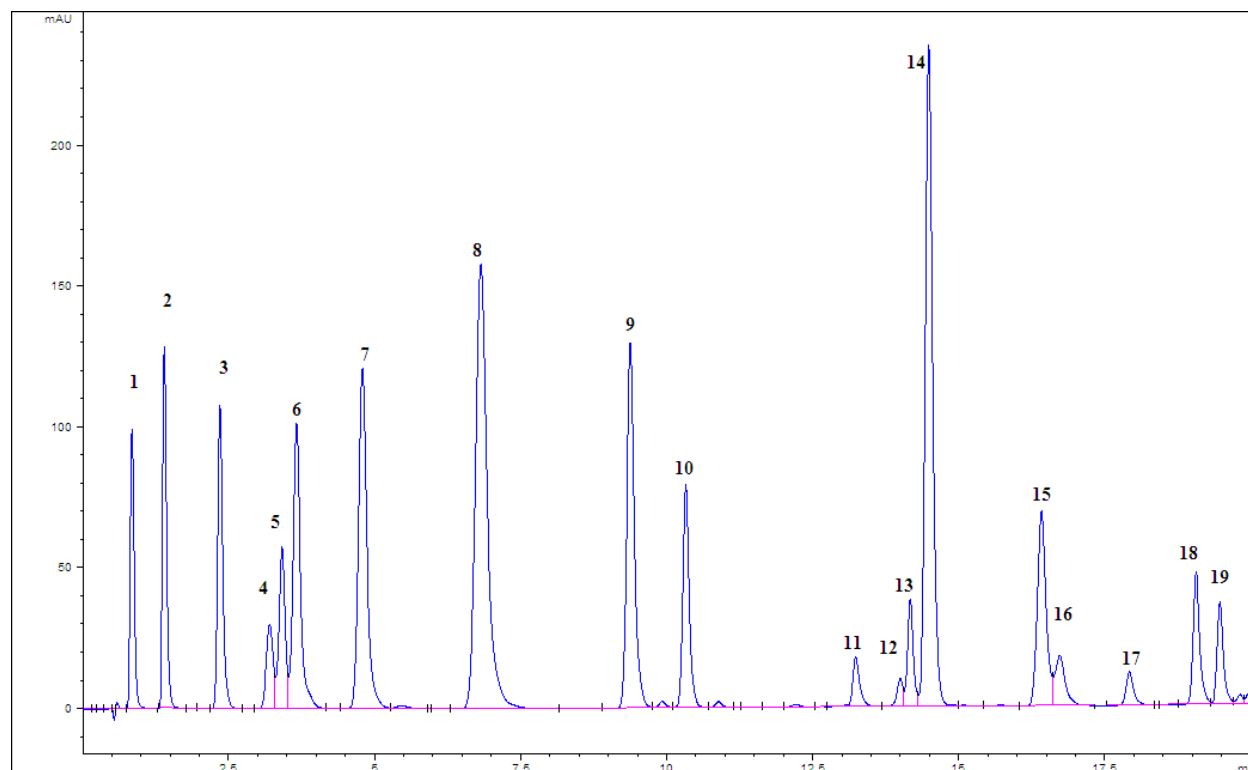
Figure 6. The chromatogram of the mixture of standards at 280nm used for chemical analysis of investigated thyme extracts. The order of elution of compounds was: 1-gallic acid, 2-protocatechuic acid, 3-*p*-hydroxybenzoic acid, 4-caffeic acid, 5-vanillic acid, 6-chlorogenic acid, 7-syringic acid, 8-*p*-coumaric acid, 9-ferulic acid, 10-sinapic acid, 11-rutin, 12-myricetin, 13-rosmarinic acid, 14-cinnamic acid, 15-quercetin, 16-naringenin, 17-luteolin 7-O-glucuronide, 18-kaemferol, 19-apigenin

2.3. Antibacterial testing

2.3.1. Bacterial strains and growth conditions

Selected bacterial strains, namely *Bacillus cereus* WSBC 10530 (clinical isolate), *Staphylococcus aureus* ATCC 25923 (clinical isolate), *Salmonella* Infantis ŽMJ 106 (poultry meat isolate), *Escherichia coli* O157:H7 ŽM 370 (clinical isolate), and *Campylobacter jejuni* NCTC 11168) were used for antibacterial testing. All bacterial strains were stored at –80°C. *B. cereus*, *S. aureus*, *S. Infantis* and *E. coli* were grown on Mueller-Hinton agar (MHA, Oxoid) at 37°C for 24 h. *C. jejuni* was grown on Columbia blood agar (Oxoid, Basingstoke, UK) at 42°C for 24 h under microaerobic conditions. For the antimicrobial activity assays, cultures were suspended in Mueller-Hinton broth (MHB, Oxoid) to 10^5–10^6 CFU/mL.

2.3.2. Broth microdilution method

Antimicrobial test systems should ideally be simple, reproducible, rapid, and inexpensive [74]. Several methods are currently available to determine antimicobial activity of different plant

extracts or isolated pure compounds. Since they are not based on the same principle, the obtained results are influenced not only by the selected method, but also by the microorganism used, as well as by the extraction procedure (polar or non-polar extracts, essential oils, etc.) or the solubility of the tested compound [75]. In order to avoid overemphasizing the usefulness of various plant extracts or isolated compounds as antimicrobials, applied methods, both for extraction and antimicrobial testing, should be standardised to obtain comparable and reproducible results, prior to their *in vivo* application [76]. Broth macro- or microdilution methods for Minimum Inhibitory Concentration (MIC) determination are recommended by the Clinical and Laboratory Standards Institute (CLSI) [77]. In our study, micromethod, as it was less expensive and easier, was used to yield reproducible results. On the other hand, bacterial growth could be assessed visually, spectrophotometrically, or by using bioluminescence of fluorimetry measurements. However, all of the proposed methods have its shortcomings [78] and in this context colorimetric methods could represent an alternative approach, using tetrazolium salts as indicators. Bacteria convert them to coloured formazan derivatives that can be quantified, but difficulties may arise because of autofluorescence, salt reduction, and the antioxidant properties of plant products, especially for XTT (2,3-Bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), TTC (2,3,5-triphenyl tetrazolium chloride), and resazurin. This makes them less convenient indicators for MIC assay. Therefore, INT (2-[4-iodophenyl]-3-[4-dinitrophenyl]-5-phenyltetrazolium chloride) was more suitable and was used in our survey. Change of colour occurs due to the reduction process as INT acts as an artificial terminal electron acceptor in respiration. Nevertheless, tetrazolium salts are not appropriate for microaerophilic campylobacters since they indicate the respiratory activity. Therefore, we used broth microdillution method with ATP measurement as rapid and accurate tools [74].

For the broth microdilution test, 50 µL of each bacterial suspension in suitable growth medium was added to the wells of a sterile 96-well microtitre plate already containing 50 µL of two-fold serially diluted standardised (T1) and post-distillation waste thyme extracts (T2, T3, T4, and T5) in proper growth medium. The final volume in each well was 100 µL. Control wells were prepared with culture medium, bacterial suspension only, plant extracts only, and ethanol in amounts corresponding to the highest quantity present. The contents of each well were mixed on a microplate shaker (Eppendorf, Hamburg, Germany) at 800 rpm for 1 min prior to incubation for 24 h in the cultivation conditions described above. The MIC was the lowest concentration where no viability was observed after 24 h on the basis of metabolic activity. To indicate respiratory activity, the presence of colour was determined after adding 10 µL/well of INT (Sigma) dissolved in water (INT 2 mg/mL) and incubated under appropriate cultivation conditions for 30 min in the dark [79]. To determine the ATP activity, the bioluminescence signal was measured by a Microplate Reader (Tecan, Mannedorf/Zurich, Switzerland) after adding 100 µL/well of BacTiter-Glo™ reagent (Promega, Madison, USA) after 5 min of the incubation in the dark [80]. Positive controls were wells with a bacterial suspension in an appropriate growth medium and a bacterial suspension in an appropriate growth medium with ethanol in amounts corresponding to the highest quantity present in the broth microdilution assay. Negative controls were wells with growth medium and plant extract. All measurements of MIC values were repeated in triplicate. Results are expressed in mg of total phenolics (TP).

2.4. Cytotoxicity studies

2.4.1. Cell culture

Human MRC-5 lung fibroblasts were grown in Minimum Essential Medium (MEM, Gibco, Invitrogen, Austria) supplemented with 2 mM of L-glutamine (Sigma, MO, USA), 10% of heat-inactivated fetal bovine serum (FBS, PAA laboratories, Austria), 100 units/mL of Penicillin (PAA laboratories), and 100 μg/mL of Streptomycin (PAA laboratories) (1% Pen/Strep). Cells were kept in a humidified 5% CO_2 atmosphere at 37°C and passaged at 90% confluence. Passages P26 and P27 were used for the assays.

2.4.2. XTT assay for the determination of cytotoxicity

An automated microculture tetrazolium assay (MTA) has long been used for the *in vitro* assessment of drug effects on cell growth. The use of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reagent requires dimethyl sulfoxide solubilization MTT-formazan generated by cellular reduction. This is not only laborious, but also may risk exposure of laboratory personnel to large quantities of potentially hazardous solutions in dimethyl sulfoxide (DMSO). Furthermore, frequent DMSO exposure also produces deleterious effects upon some laboratory equipment. Therefore, to allow the investigation of a simplified MTA and to address potential problems associated with solvent handling, a series of new tetrazolium salts have been developed which, upon metabolic reduction by viable cells, yield aqueous-soluble formazans [81]. One of the possible alternatives was found to be XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-S-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide), which is metabolically reduced in viable cells to a water soluble formazan product. This allows reproducible estimates of drug sensitivity in a variety of human and other tumor cell lines. However, it is also applicable for the cytotoxicity studies of various natural products, such are plant extracts or isolated natural compounds.

Cell proliferation kit II (XTT) (Cat. No. 11 465 015 001) was obtained from Roche Diagnostics (Mannheim, Germany). Aliquots (100 μL) of 1×10^5 cells/mL were seeded into 96 well plates (flat bottom) and grown for 24 h before the extracts were added. Afterwards, cells were incubated for 4 h or 24 h with investigated thyme extracts (20 μg/mL) before XTT solution was added. The XTT solution consisted of a XTT labeling reagent and an electron-coupling reagent and was always freshly prepared. XTT is a yellow tetrazolium salt and cleaved by metabolic active cells into an orange, water-soluble formazan dye. Since these color changes only occur in viable cells, it can be directly quantified using a scanning multiwell spectrophotometer [81]. Numbers of viable cells were determined with the following formula and expressed as percentage of control [82]:

Absorbance of treated cells/Absorbance of untreated cells x 100

Control cells represent vehicle-treated cells. Vehicle was 0.5% of DMSO (final concentration), which did not affect the cells as shown by control experiments.

2.4.3. Cytoprotection assay

Aliquots (100 μL) of 1×10^5 cells/mL were seeded into 96 well plates (flat bottom) and grown for 24 h before the extracts (20 μg/mL) were added for 4 h or 24 h. Afterwards, the medium with the extracts was removed and replaced by 100 μl of a H_2O_2 solution (50 μM, 100 μM, 200 μM or 500 μM) in MEM medium and further incubated for 2 h or 24 h. To finally determine the cell viability, XTT solution was added and analyzed as described above.

2.5. Statistical analysis

The data were reported as mean values ± standard deviation (SD) and processed using Microsoft Office Excel for Windows v. 2007.

3. Results

3.1. The amount of dry extract

The amounts of dry extracts obtained by maceration of thyme leaves (T1), deodorised thyme leaves (T3 and T4), and thyme stems (T5) and post-distillation extract remaining after the evaporation of thyme decoction (T2) are shown in Table 4. Generally, the amount of dry extract T1 is in accordance with previously published data, considering the fact that numerous factors could influence the extraction yield [47, 83]. When the thyme waste extracts were analyzed, the highest amount was obtained in T2 [43]. The difference in the extraction yield between T1 and T2 may be due to the mere extraction process since T2 is obtained after hydrodistillation of thyme leaves that favours extraction of polar substances, but also present carbohydrates genuine to the leaves or generated after hydrolysis of present glycosides. Deodorised leaves and stems yield a smaller amount of dry extracts when compared to T1.

3.2. Total phenolic compounds and flavonoid content

Spectrophotometric methods used are simple and reproducible for rough screening of total phenolic compounds and total flavonoids before detailed chemical analysis. The results are presented in Table 4. Total phenolic compounds in examined thyme leaves and stem extracts varied from 35.69 (T2) to 62.45 (T4) mg GAE/g d.e., while total flavonoids were in the 11.31 (T5)–52.89 (T3) mg QE/g d.e. range. Interestingly, deodorised leaves extracts (T3 and T4) have a relatively high content of total phenolic compounds and total flavonoids when compared to T1. The extract T2, for which the highest amount of dry extract was determined, is poor in total phenolic compounds and total flavonoids when compared to T1, but also to T3 and T4 [43, 51]. However, when absolute amounts (in plant material) are considered, T2 may also represent a valuable source of phenolic compounds and flavonoids. Stem extract (T5) have relatively high total phenolics, while are poor in total flavonoids.

Type of extract	% dry extract (g d.e./100 g of plant material)	Total phenolic compounds (mg GAE/g d.e.)	Total flavonoid content (μg QE/g d.e.)
T1	9.31±0.05	49.71±0.02	33.88±0.014
T2	37.19±0.1	35.69±0.019	22.79±0.005
T3	3.45±0.03	58.23±0.019	52.89±0.012
T4	3.85±0.04	62.45±0.006	50.09±0.006
T5	5.39±0.05	45.95±0.03	11.31±0.005

Table 4. The amount of dry extract, total phenolic compounds, and total flavonoid content

3.3. HPLC analysis

The qualitative-quantitative data from the chromatographic analyses are presented in Table 5, with representative chromatograms for T1 and T3 (Figure 7). The following components: gallic, protocatechuic, caffeic, chlorogenic, syringic, p-coumaric, ferulic, and rosmarinic acid, as well as rutin, quercetin, luteolin 7-O-glucuronide andapigenin were identified and quantified within the investigated samples.

Type of extract c (mg/g d.e.)	T1	T2	T3	T4	T5
Gallic acid	2.46	2.58	1.76	0.77	4.99
Protocatechuic acid	0.23	0.22	0.27	0.37	0.22
Caffeic acid	0.35	0.23	0.13	0.33	0.53
Chlorogenic acid	2.06	2.02	3.77	4.75	7.75
Syringic acid	0.23	0.42	0.3	0.36	0.25
p-Coumaric acid	0.06	0.04	0.05	0.06	0.16
Ferulic acid	1.05	0.89	1.56	1.9	5.77
Rutin	0.4	0.12	2.4	3.68	0.32
Rosmarinic acid	**62.22**	**60.49**	**86.01**	**105.08**	**52.36**
Quercetin	0.35	0.04	2.2	3.98	0.36
Luteolin 7-O-glucuronide	**42.20**	**34.44**	**81.24**	**87.11**	**11.01**
Apigenin	1.81	0.79	3.36	4.45	0.27

Table 5. Results of HPLC-DAD analysis of thyme leaves and post-distillation waste extracts

Regarding the hydroxybenzoic, hydroxycinnamic acids, and caffeic acid oligomers, rosmarinic acid was identified as the major component in all the investigated thyme extracts and was present within the range of 52.36 (T5) to 105.08 (T4) mg/g d.e, which is similar to the previously published data for officially prepared thyme extracts (T1) [50]. Also, gallic, ferulic, and chlorogenic acids were present in considerable amounts. In general, 75% EtOH extract of the deodorised leaves (T4) abundant in rosmarinic, chlorogenic, and ferulic acid, whilst 45% EtOH contained higher amounts of gallic acid. In the flavonoid fraction, luteolin 7-O-glucuronide was the dominant and the second major identified component in all investigated extracts (from 11.01 (T5) to 87.11 (T4) mg/g d.e.). Similarly to the total phenolic content, the highest amounts of almost all quantified components, especially the dominant ones were determined in

deodorised leaves extracts (T3 and T4). Also, although the stem is confirmed to be relatively poor in phenolic compounds, chlorogenic and ferulic acids are present in considerable amounts.
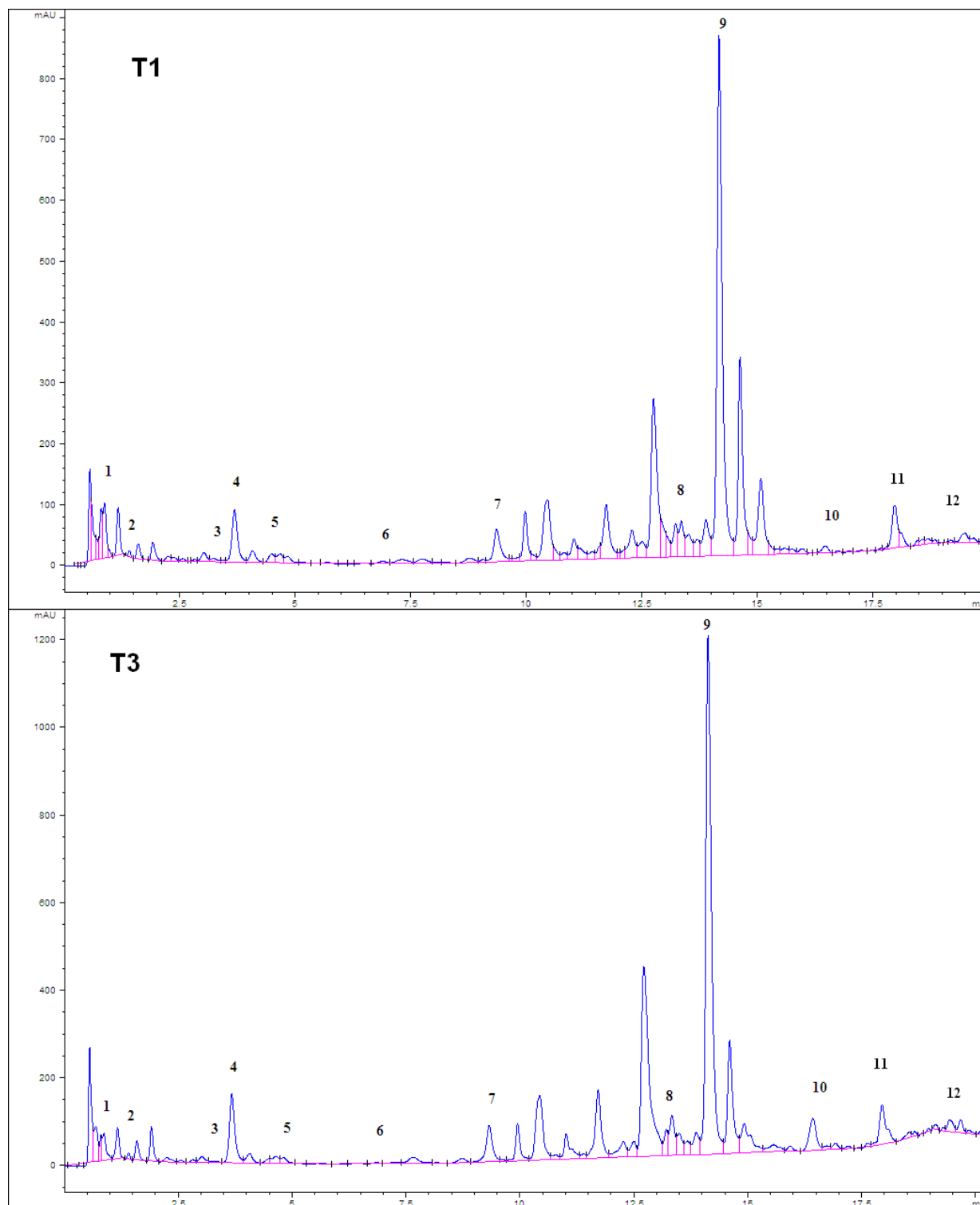
Figure 7. Chromatograms of examined thyme leaves extracts (T1 and T3) at 280 nm: 1-gallic acid, 2-protocatechuic acid, 3-caffeic acid, 4- chlorogenic acid, 5-syringic acid, 6-*p*-coumaric acid, 7- ferulic acid, 8-rutin, 9-rosmarinic acid, 10-quecetin, 11-luteolin 7-O-glucuronide, 12-apigenin

3.4. Antibacterial testing

Numerous studies have confirmed the use of herbs and spices as anti-infective agents [84]. Among them, different essential oils and phenolic compounds, or plant extracts rich in phenolic compounds stand out for their antibacterial activity [85, 86]. This is particularly important, given that phenolic compounds possess a strong antioxidant potential. This joint action is significant for their usage in pharmaceutical and food industries, but also in all other fields where it is important to prevent both oxidation and contamination of certain products [87].

All investigated post-distillation thyme waste extracts exhibited relatively strong antimicrobial potential, against all tested bacterial strains, especially deodorised leaves extracts (T3 and T4) (Table 6) [88]. Also, thyme stem extract (T5) expressed notable antibacterial action. What is more important, by comparing these results with the data obtained for butylhydroxytoluene (BHT), often used as a synthetic additive, antibacterial potential of thyme post-distillation waste material extracts becomes even more evident. Also, interestingly, all investigated thyme extracts showed lower minimal inhibitory concentration on all investigated bacterial strains when compared to isolated rosmarinic acid. This may indicate that phenolic compounds possess a synergistic antibacterial effect, which is important in terms of emerging occurrence of resistance.

	Staphylococcus aureus MIC (mg TP/mL)	*Salmonella liverpool* MIC (mg TP/mL)	*Escherichia coli* MIC (mg TP/mL)	*Bacillus cereus* MIC (mg TP/mL)
T1	0.09	0.28	0.14	0.14
T2	0.14	0.39	0.56	1.55
T3	**0.04**	**0.12**	**0.12**	**0.03**
T4	**0.06**	**0.12**	**0.12**	**0.03**
T5	0.09	0.13	0.19	0.06
BHT (mg/mL)	0.55	0.55	0.55	0.28
Rosmarinic acid*	5	2.5	2.5	5

* reference [74]

Table 6. Minimal inhibitory concentrations of investigated thyme extracts on selected bacterial strains

Despite many recent technological advances in the food industry, which have contributed to increased safety in the food supply chain, we are still facing high incidence of foodborne illnesses. In 2011 alone, there were 69,553 human cases originating from foodborne zoonotic outbreaks in the EU [89]. Ninety-three of them were fatal. In the same year, *Campylobacter*, the most prevalent foodborne zoonotic agent, which is especially a problem in the production and processing of meat, caused 220 of the 209 registered, mostly sporadic human illnesses. It is assumed that the real burden is even much higher [4].

Two selected extracts, T3 prepared from deodorised leaves and T1 as an officially prepared one, were tested against *Campylobacter jejuni*. Both T1 and T3 expressed relatively strong antibacterial potential. The activity of deodorised extract T3 in gram negative *C. jejuni* was

comparable to that in gram positive *S. aureus* and *B. cereus*. On the other hand, standard thyme extract T1 expressed substantially stronger activity in *C. jejuni* compared to all other tested species, suggesting that the phenolic compounds present in standard extract T1 may contribute more to the antimicrobial activity in *C. jejuni* than in other species. Furthermore, obtained MIC values of extracts were lower in comparison with rosmarinic acid, indicating the importance of synergistic effects of different compounds present in investigated extracts that can contribute to the antimicrobial activity through different mechanisms. They can interfere with bacterial membrane and thereby increase the cell leakage or act indirectly antimicrobial by facilitating the influx of antimicrobial phenolic compounds [21]. In this, as well as the economical aspect, the use of plant extracts might be more sensible than the use of pure compounds. However, obtained MIC values were much higher than those of selected antibiotics (Table 7).

|	*Campylobacter jejuni*	References
T1	0.05	[88]
T3	0.06	[88]
Rosmarinic acid	1.25	[74]
Ciprofloxacin	0.25	[4]
Erythromycin	0.25	[4]

Table 7. Minimal inhibitory concentrations of selected thyme extracts (mg TP/mL), rosmarinic acid (mg/mL) and selected antibiotics (µg/mL) on *Camphylobacter jejuni*

3.5. Cytotoxicity studies

Cancer is a leading cause of death worldwide and sustained focus is on the discovery and development of newer and better-tolerated anti-cancer drugs, especially from plants. About 50% of all anti-cancer drugs approved internationally are either natural products or natural product mimics. They were developed on the basis of the knowledge obtained from small or macromolecules existing in nature [90].

The major challenges associated with currently available anti-cancer agents include selectivity, toxicity, resistance, and development of a secondary malignancy. These drawbacks have motivated the search for newer, more efficacious, and better tolerated anti-tumor drugs, with natural products, especially plants, offering an inexhaustible reservoir for new drug discovery and development. Also, molecular targeted agents are currently being studied in all treatment settings including that of chemoprevention, which is defined as the use of natural or synthetic nonessential dietary agents to interrupt the process of carcinogenesis and to prevent or delay tumor growth [91].

In vitro cytotoxicity studies are the first step in selecting from the vast number of still unexamined plant extracts or isolated compounds. Also, these studies can provide a valuable insight in the safety of different natural products. This is especially important for waste plant material, in our case for pos-tdistillation waste material of thyme leaves, if their possible future application in pharmaceutical or food industry should be considered.
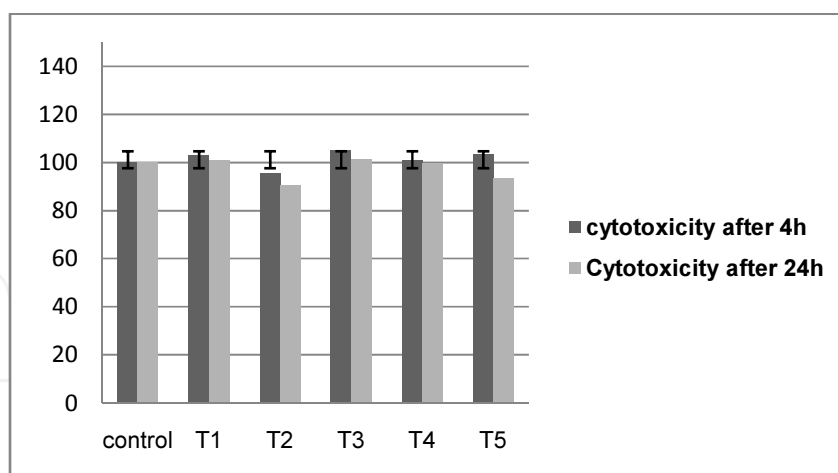
Figure 8. Metabolically active cells (% of control) in XTT assay after post-distillation thyme extracts application
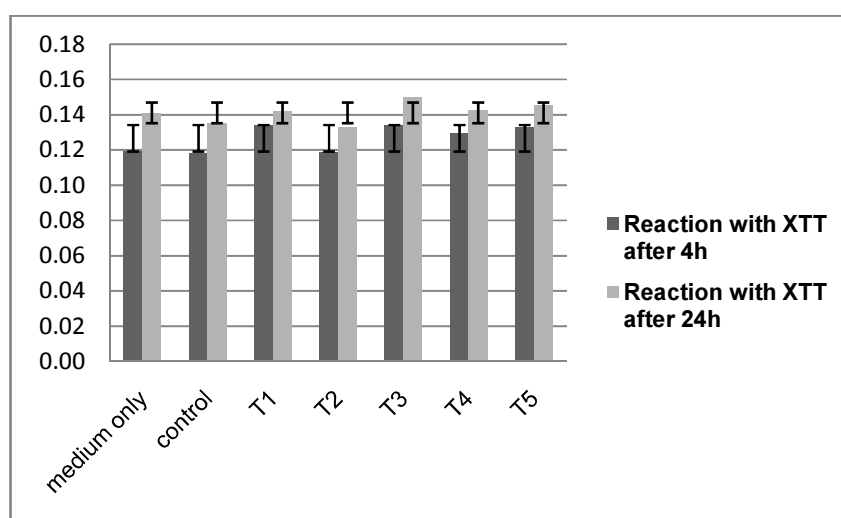

Figure 9. Absorbance values (490–650 nm) of extracts mixed with XTT solution in culture medium

The effects of investigated post-distillation thyme waste extracts against human MRC-5 lung fibroblasts are presented in Figures 8–15. Cytotoxicity of the extracts, a possible reaction with the XTT solvent and a potential cytoprotective activity were evaluated. As shown in Figure 8, none of the investigated extracts at concentrations of 20 μg/ml exhibited cytotoxicity after 4 h or 24 h of incubation. To exclude a direct reaction of the extracts with the XTT solvent and, therefore, false-negative results, extracts and XTT solution were mixed in culture medium and incubated for 4 h or 24 h. There were no significant differences in the absorbance values of medium and XTT alone compared to DMSO (control) or extracts (20 μg/ml) in medium (Figure 9) that reveals that the extracts did not interact with the XTT solution.

Finally, a potential cytoprotective effect of the extracts was investigated. Cells were incubated with the extracts for 4 h or 24 h and cytoprotection was evaluated by adding different concentrations of H_2O_2, which is a well-known cell damaging agent causing oxidative stress.
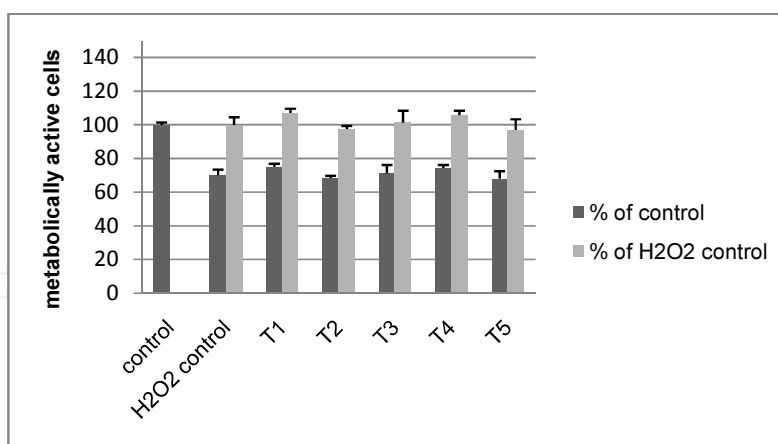
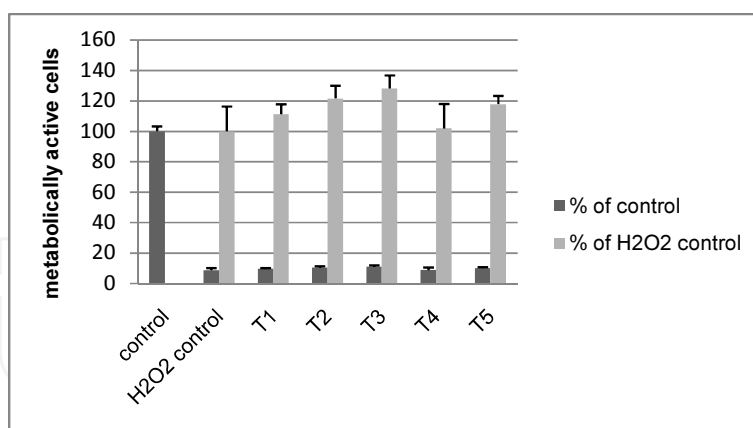**Figure 10.** Cells incubated with the extracts for 24 h, medium replaced by 50 μM H_2O_2 and further incubated for 4 h (XTT added after 2h and read out after 2h)
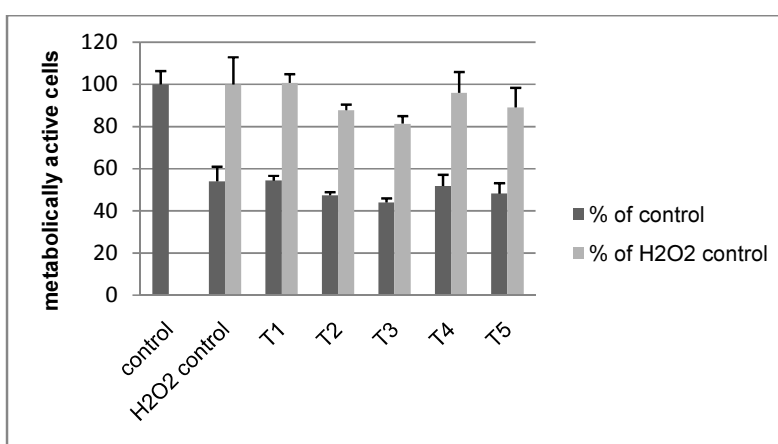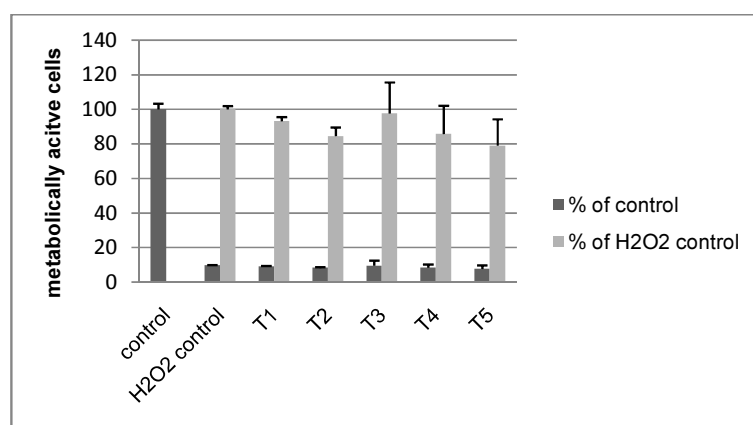

Figure 11. Cells incubated with the extracts for 24 h, medium replaced by 100 μM H_2O_2 and further incubated for 4h (XTT added after 2 h and read out after 2 h)
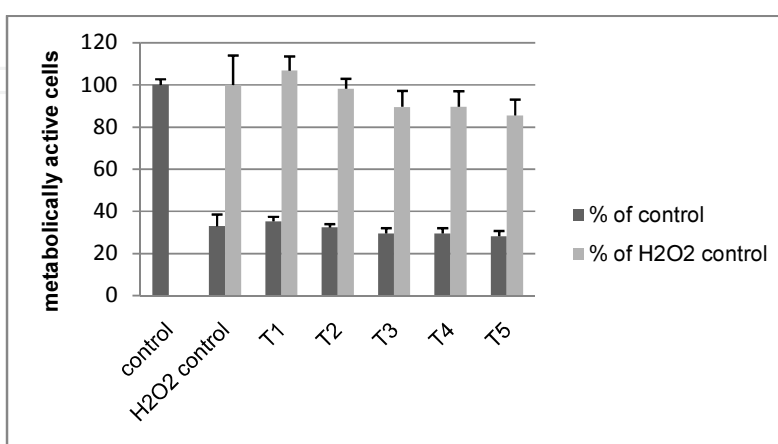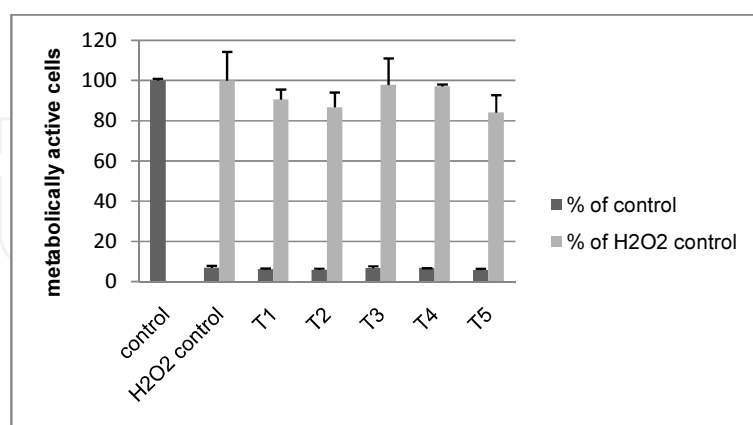

Figure 12. Cells incubated with the extracts for 24 h, medium replaced by 200 μM H_2O_2 and further incubated for 4h (XTT added after 2 h and read out after 2 h)

Figure 13. Cells incubated with the extracts for 24 h, medium replaced by 500 μM H₂O₂ and further incubated for 4 h (XTT added after 2 h and read out after 2 h)

Figure 14. Cells incubated with the extracts for 4 h, medium replaced by 500 μM H₂O₂ and further incubated for 4 h (XTT added after 2 h and read out after 2 h)

Figure 15. Cells incubated with the extracts for 4 h, medium replaced by 500 μM H₂O₂ and further incubated for 24 h (XTT added after 24 h and read out after 2 h)

The effects were compared to vehicle-treated (DMSO) cells (control) and to H₂O₂ treated cells (H₂O₂ control). As shown in Figures 10–15, none of the investigated extracts exhibited notable cytoprotective activity.

4. Further research

Results of microbiological tests indicate the possibility to use thyme postdistillation waste extracts primarily in the food or pharmaceutical industry as natural additives, regarding the relatively strong antibacterial activity on investigated bacterial strains.

Also, examined extracts represent a valuable source of phenolcarboxylic acids and flavonoids, which have numerous biological effects. Therefore thyme post-distillation waste material could be used for the isolation of rosmarinic acid and luteolin 7-O-glucuronide, as dominant compounds.

However, only limited data are presently available on the antimicrobial activity of plant extract in food systems and this indicates continuation of our study and the need for further work. Additionally, there is growing evidence on synergistic action of different plant products (extracts, essential oils, isolated compounds) with each other or with conventional antibiotics used in human and veterinary medicine [92]. Besides, it would be very interesting to collect more data on the efficiency of both thyme essential oil and investigated thyme extracts as a dietary supplementation in animal feed on protective or prebiotic effects on the gut microflora. Also, it is important to test their influence on antioxidant and antimicrobial stability and shelf life of meat and meat products [93, 94, 95].

Furthermore, the results of our study recorded neither cytotoxicity, nor cytoprotection of examined extracts. Yet, detailed in vivo toxicology analyses are required in order to confirm the safety of usage of thyme postdistillation waste material.

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References

[1] Rios JL, Recio MC. Medicinal plants and antimicrobial activity. Journal of Ethnopharmacology 2005;100 80–84.

[2] Reverter M, Bontemps N, Lecchini D, Banaigs B, Sasal P. Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. Aquaculture 2014;433 50–61.

[3] Palaniappan K, Holley RA. Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. International Journal of Food Microbiology 2010;140 164–168.

[4] Kovač J, Gavarić N, Bucar F, Smole Možina S. Antimicrobial and resistance modulatory activity of *Alpinia katsumadai* seed phenolic extract, essential oil and post-distillation extract. Food Technology and Biotechnology, 2014;52(2) 248–254.

[5] Singer RS, Williams-Nguyen J. Human health impacts of antibiotic use in agriculture: A push for improved causal inference. Current Opinions in Microbiology 2014;19 1–8.

[6] Machado TB, Pinto AV, Pinto MC, Leal IC, Silva MG, Amaral AC, Kuster RM, Netto-dos Santos KR. *In vitro* activity of Brazilian medicinal plants, naturally occurring naphthoquinones and their analogues, against methicilin-resistant *Staphylococcus aureus*. International Journal of Antimicrobial Agents 2003;21 279–284.

[7] Voravuthikunchai S, Lortheeranuwat A, Jeeju W, Sririrak T, Phongpaichit S, Supawita T. Effective medicinal plants against enterohaemorrhagic *Escherichia coli* O157:H7. Journal of Ethnopharmacology 2004;94 49–54.

[8] Fukai T, Oku Y, Hano Y, Terada S. Antimicrobial activites of hydrophilic 2-arylbenzofurans and an isoflavone against vancomycin-resistant enterococci and methicilin-resistant *Staphylococcus aureus*. Planta Medica 2004;70 685–687.

[9] Rani P, Khullar N. Antimicrobial evaluation of some medicinal plants for their anti-enteris potential against multi-drug resistant *Salmonella typhi*. Phytotherapy Research 2004;18 670–673.

[10] Shibata H, Kondo K, Katsuyama R, Kawazoe K, Sato Y, Murakami K, Takaishi Y, Arakaki N, Higuti T. Alkyl gallates, intensifiers of beta-lactam susceptibility in methicilin-resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 2005;49 549–555.

[11] Garcia-Garcia R, Lopez-Malo A, Palou E. Bactericidal action of binary and ternary mixtures of carvacrol, thymol, and eugenol against *Listeria innocua*. Journal of Food Science 2011;76 95–100.

[12] Gavaric N, Smole Možina S, Kladar N, Božin B. Chemical profile, antioxidant and antibacterial activity of thyme and oregano essential oils, thymol and carvacrol and

their possible synergism. Journal of Essential Oil Bearing Plants 2015;doi: 10.1080/0972060X.2014.971069.

[13] Mimica-Dukic N, Bozin N. Essential oils from Lamiaceae species as promising anti-oxidant and antimicrobial agents. Natural Product Communications 2007;2(4) 445–452.

[14] Heinrich M, Barnes J, Gibbons S, Williamson EM. Fundamentals of Pharmacognosy and Phytotherapy. Edinburgh, London, New York, Oxford, Philadelphia, St Louis, Sydney, Toronto: Churchill Livingstone; 2004.

[15] *European Pharmacopoea*. 6th edition. Strasbourgh: European Directorate for the Quality of Medicines & Health Care, Council of Europe; 2007.

[16] Božin B, Mimica-Dukić N, Simin N, Anačkov G. Characterization of the volatile com-position of essential oils of some Lamiaceae spices and the antimicrobial and antioxi-dant activities of the entire oils. Journal of Agricultural and Food Chemistry 2006;54 1822–1828.

[17] Pirbalouti AG, Hashemi M, Ghahfarokhi FT. Essential oil and chemical compositions of wild and cultivated *Thymus daenensis* Celak and *Thymus vulgaris* L. Industrial Crops and Products 2013;48 43–48.

[18] Bozin B, Mimica-Dukic N, Anackov G, Zlatkovic B, Igic R. Variability of Content and Composition of *Mentha aquatica* L. (Lamiaceae) Essential Oil in Different Phenophas-es. Journal of Essential Oil Bearing Plants 2006a;9(3) 223–229.

[19] Bozin B, Mimica-Dukic N, Bogavac M, Suvajdzic L, Simin N, Samojlik I, Couladis M. Chemical composition, antioxidant and antibacterial properties of Achillea collina Becker ex Heimerl s.l. and A. pannonica Scheele essential oils. Molecules 2008;13(9) 2058–2068.

[20] Nabavi SM, Marchese A, Izadi M, Curti V, Daglia M, Nabavi SF. Plants belonging to the genus Thymus as antibacterial agents: From farm to pharmacy. Food Chemistry 2015;173 339-347.

[21] Burt S. Essential oils: Their antibacterial properties and potential applications in foods-a review. International Journal of Food Microbiology 2008;94 223–253.

[22] Tepe B, Deferera D, Sokmen M, Polissiou M, Sokmen A. *In vitro* antimicrobial and antioxidant activities of the essential oils and various extracts of *Thymus eigii* M. Zoh-ary et P. H. Davis. Journal of Agricultural and Food Chemistry 2004;52 1132–1137.

[23] Rivas L, McDonnell MJ, Burgess CM, O'Brien M, Navarro-Villa A, Fanning S, Duffy G. Inhibition of verocytotoxigenic Escherichia coli in model broth and rumen sys-tems by carvacrol and thymol. International Journal of Food Microbiology 2010;139 70–8.

[24] FDA. US Food and Drug Administration. www.cfsan.fda.gov/~dms/eafus.html. 2010.

[25] Booth N, Kruger C, Wallace Hayes A, Clemens R. An innovative approach to the safety evaluation of natural products: Cranberry leaf aqueous extracts as a case study. Food and Chemical Toxicology 2012;50 3150–3165.

[26] Jeršek B, Anđić M, Markov K, Lakić N, Božin B, Smole Možina S. Antifungal effect of essential oils on *Aspergillus westerdijkiae*, *A. ochraceus*, *A. flavus* and *Penicillium nordicum*. 6th Central European Congress on Food, Novi Sad, Serbia: May 2012.

[27] Božin B, Lakić N, Srđenović Čonić B, Kladar N, Orčić D, Mimica-Dukić N. Antioxidant and antimicrobial properties of a new chemotype of woodland sage (*Salvia nemorosa* L. subsp. *nemorosa*, Lamiaceae) essential oil. Biologica Serbica 2012;1–2(34) 51–60.

[28] Calo JR, Crandall PG, O'Bryan CA, Ricke SC, Essential oils as antimicrobials in food systems-a review. Food Control 2015;doi:10.1016/j.foodcont.2014.12.040.

[29] Bozin B, Mimica-Dukic N, Samojlik I, Jovin E. Antimicrobial and antioxidant properties of Rosemary and Sage (Rosmarinus officinalis L. and Salvia officinalis L., Lamiaceae) essential oils. Journal of Agricultural and Food Chemistry 2007;55(19) 7879–7885.

[30] Samojlik I, Lakić N, Mimica-Dukić N, Đaković-Švajcer K, Božin B. Antioxidant and Hepatoprotective Potential of Essential Oils of Coriander (*Coriandrum sativum* L.) and Caraway (*Carum carvi* L.) (Apiaceae). Journal of Agricultural and Food Chemistry 2010;58 8848–8853.

[31] Takhtajan A. Flowering Plants. Berlin: Springer Verlag; 2009.

[32] Petersen M, Abdullah Y, Benner J, Eberle D, Gehlen K, Hücherig S, Janiak V, Kim KH, Sander M, Weitzel C, Wolters S. Evolution of rosmarinic acid biosynthesis. Phytochemistry. 2009,70(15–16) 1663–1679.

[33] Nolkemper S, Reichling J, Stintzing FC, Carle R, Schnitzler P. Antiviral effect of aqueous extracts from species of the Lamiaceae family against Herpes simplex virus type 1 and type 2 in vitro. Planta Medica 2006;72(15) 1378–1382.

[34] Akhondzadeh, S. Abbasi, SH. Herbal medicine in treatment of Alzheimer's disease. American Journal of Alzheimer's Disease and Other Dementias 2006;21 113–118.

[35] Lee HJ, Cho H, Park E, Kim S, Lee S, Kim C, Kim DK, Kim S, Chun HS. Rosmarinic acid protects human dopaminergic neuronal cells against hydrogen peroxide-induced apoptosis. Toxicology 2008;250 109–115.

[36] Dastmalchi K, Ollilainen V, Lackman P, Boije af Gennäs G, Damien Dorman, HJ, Järvinen P, Yli-Kauhaluoma J, Hiltunen R. Acetylcholinesterase inhibitory guided fractionation of *Melissa officinalis* L. Bioorganic & Medicinal Chemistry 2009;17 867–871.

[37] Wang H, Kikuzaki H, Lin CC, Kahyaoglu A, Huang MT, Nakatani N. Acetophenone glycosides from thyme (*Thymus vulagris* L.). Journal of Agricultural and Food Chemistry 1999;47 1911–1914.

[38] Chun H, Jun WJ, Shin DH, Hong BS, Cho HY, Yang HC. Purification and characterization of anti-complementary polysaccharide from leaves of *Thymus vulgaris* L. Chemical and Pharmaceutical Bulletin 2001;49 762–764.

[39] Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs. Journal of Agricultural and Food Chemistry 2001;49(11) 5165–5170.

[40] Miura K, Kikuzaki H, Nakatani N. Antioxidant activity of chemical components from sage (*Salvia officinali*L.) and thyme (*Thymus vulgaris* L.) measured by the oil stability index method. Journal of Agricultural and Food Chemistry 2002;50 1845–1851.

[41] Depkevicius A, Van Beek TA, Lelyveld GP, Van Veldhuizen A, De Groot A, Linssen JPH, Venskutonis R. Isolation and structure elucidation of radical scavengers from *Thymus vulgaris* leaves. Journal of Natural Products 2002;65 892–896.

[42] Okazaki K, Kawazoe K, Takaishi Y. Human platelet aggregation inhibitors from thyme (*Thymus vulgaris* L.). Phytotherapy Research 2002;16 398–399.

[43] Dorman HJD, Peltoketo A, Hiltunen R, Tikkanen MJ. Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. Food Chemistry 2003;83 255–262.

[44] Tagashira M, Ohtake Y. New monoterpene glucosides from aerial parts of thyme (*Thymus vulgaris* L.). Planta Medica 1998;64 555–558.

[45] Wang H, Provan GJ, Halliwell K. Determination of rosmarinic acid and caffeic acid in aromatic herbs by HPLC. Food Chemistry 2004;87 307–311.

[46] Kitajima J, Ishikawa T, Urabe A. A new hydroxyjasmone glucoside and its related compounds from the leaf of thyme. Chemical and Pharmaceutical Bulletin 2004;52 1013–1014.

[47] Kosar M, Dorman HJD, Hiltunen R. Effect of acid treatment on the phytochemical and antioxidant characteristics of extracts from selected Lamiaceae species. Food Chemistry 2005;91 522–533.

[48] Janicsak G, Mathe I, Miklossay-Vari V, Blunden G. Comparative studies of the rosmarinic and caffeic acid contents of Lamiaceae species. Biochemical Systematics and Ecology 1999;27 733–738.

[49] Fecka I, Raj D, Krauze-Baranowska M. Quantitative determination of four water-soluble compounds in herbal drugs from Lamiaceae using different chromatographic techniques. Chromatographia 2007;66 87–93.

[50] Fecka I, Turek S. Determination of polyphenolic compounds in commercial herbal drugs and spices from Lamiaceae: Thyme, wild thyme and sweet marjoram by chromatographic techniques. Food Chemistry 2008;108 1039–1053.

[51] Amarowicz R, Zegarska Z, Rafalowski R, Pegg RB, Karamac M, Kosinska A. Antioxidant activity and free radical-scavenging capacity of ethanolic extracts of thyme, oregano and marjoram. European Journal of Lipid Science and Technology 2009;111 1111–1117.

[52] Lee J. Caffeic acid derivatives in dried Lamiaceae and *Echinacea purpurea* products. Journal of Functional Foods 2010;2 158–162.

[53] Farah A, Danangelo CM. Phenolic compounds in coffee. Brazilian Journal of Plant Physiology 2006;18 23–36.

[54] Marques V, Farah A. Chlorogenic acids and related compounds in medicinal plants and infusions. Food Chemistry 2009;113 1370–376.

[55] Dewick P. Medicinal natural Products. John Wiley & Sons; 2002.

[56] Kobayashi R, Itou T, Hanaya K, Shoji M, Hada N, Sugai T.Chemo-enzymatic transformation of naturally abundant naringin to luteolin, a flavonoid with various biological effects. Journal of Molecular Catalysis B: Enzymatic 2013;92 14–18.

[57] Lin J, Chen Y, Lee Y, Rolis Hou C, Chen F, Yang D. Antioxidant, anti-proliferative and cyclooxygenase-2 inhibitory activities of ethenolic extracts from lemon balm (*Melissa officinalis* L.) leaves. LWT-Food Science and Technology 2012;49 1–7.

[58] Samojlik I, Mijatović V, Gavarić N, Krstin S, Božin B. Consumers' attitude towards the use and safety of herbal medicines and herbal dietary supplements in Serbia. International Journal of Clinical Pharmacy 2013;35 835-840.

[59] Williamson E, Driver S, Baxter K, editors. Stockley's herbal medicines interactions. London: Pharmaceutical Press; 2009.

[60] Božin B, Lakić N, Mišan A, Smole Možina S. Phenolic profile, antioxidant and antimicrobial activity of oregano (*Origanum vulgare* L., Lamiaceae) postdistillation waste extracts, 6th Central European Congress on Food, Novi Sad, Serbia: May 2012.

[61] Peev G, Penchev P, Peshev D, Angelov G. Solvent extraction of rosmarinic acid from lemon balm and concentration of extracts by nanofiltration: Effect of plant pre-treatment by supercritical carbon dioxide. Chemical Engineering Research and Design 2011;89 2236–2243.

[62] Dzamic A, Sokovic M, Novakovic M, Jadranin M, Ristic M, Tesevic V, Marin P. Composition, antifungal and antioxidant properties of *Hyssopus officinalis* L. subsp. *pilifer* (Pant.) Murb.Essential oil and deodorized extracts. Industrial Crops and Products 2013;51 401–407.

[63] Cioffi G, Pesca MS, De Caprariis P, Braca A, Severino L, De Tommasi N. Phenolic compounds in olive oil and olive pomace from Cilento (Campenia, Italy) and their antioxidant activity. Food Chemistry 2010;121(1) 105–111.

[64] Sagdic O, Ozturk I, Ozkan G, Yetim H, Ekici L, Yilmaz MT. RP-HPLC-DAD analysis of phenolic compounds in pomace extracts from five grape cultivars: Evaluation of their antioxidant, antiradical and antifungal activities in orange and apple juices. Food Chemistry 2011;126 1749–1758.

[65] Vulic J, Canadanović-Brunet J, Cetković G, Tumbas V, Djilas S, Cetojević-Simin D, Canadanović V. Antioxidant and cell growth activities of beet root pomace extracts. Journal of Functional Foods 2012;4 670–678.

[66] Kallel F, Driss D, Chaari F, Belghith L, Bouaziz F, Ghorbel R, Chaabouni SE. Garlic (*Allium sativum* L.) husk waste as a potential source of phenolic compounds: Influence of extracting solvents on its antimicrobial andantioxidant properties. Industrial Crops and Products 2014;62 34–41.

[67] Tutin TG, Heywood HV, Burges NA, Valentine DH, Walters SM, Webb DA. Diapensiaceae to Myoporaceae. In Flora Euroapea, vol. 3. Cambridge, United Kingdom: Cambridge University Press; 2001.

[68] European Medicines Agency. *Community Herbal Monograph on Thymus vulgaris L. and Thymus zygis L., herba.* http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/herbal/medicines/herbal_med_000029.jsp&mid=WC0b01ac058001fa1d (Accessed 15 December 2014).

[69] *European Pharmacopeia*. 2002. 4th edition. Strasbourg Cedex: Council of Europe, Vol. 2.8.12: 183–184.

[70] Lakic N, Mimica-Dukic N, Isak J, Bozin B. Antioxidant properties of *Galium verum* L. (Rubiaceae) extracts. Central European Journal of Biology 2010;5(3) 331–337.

[71] Jia Z, Tang M, Wu J. The determination of flavonoids content in mulbery and scavenging effect on superoxide radicals. Food Chemistry 1999;64 555–599.

[72] Božin B, Kladar N, Grujić N, Anačkov G, Samojlik I, Gavarić N, Srđenović Čonić B. Impact of origin and biological source on chemical composition, anticholinesterase and antioxidant properties of some St. John' Wort species (*Hypericum spp.*, Hypericaceae) from Central Balkans. Molecules 2013;18 11733–11750.

[73] Misan A, Mimica-Dukic N, Mandic A, Sakac M, Milovanovic I, Sedej I. Development of a rapid resolution HPLC method for the separation and determination of 17 phenolic compounds in crude plant extracts. Central European Journal of Chemistry 2011;9(1) 133–142.

[74] Klančnik A, Piskernik S, Jeršek B, SmoleMožina S. Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. Journal of Microbiological Methods 2010;81 121–126.

[75] Valgas C, de Souza SM, Smânia EFA, Smânia JA. Screening methods to determine antibacterial activity of natural products. Brazilian Journal of Microbiology 2007;38 369–380.

[76] Das K, Tiwari RKS, Shrivastava DK. Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. Journal of Medicinal Plants Research 2010; 4(2) 104–111.

[77] CLSI (NCCLS) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved Standard 23. National Committee for Clinical Laboratory Standards, Wayne, PA, USA; 2003.

[78] Grare M, Fontanay S, Cornil C, Finance C, Duval ER. Tetrazolium salts for MIC determination in microplates: Why? Which salt to select? How? Journal of Microbiological Methods 2008;75 156–159.

[79] Ellof JN. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 1998;64 711–713.

[80] Klančnik A, Guzej B, HadolinKolnar M, Abramovič H, SmoleMožina S. *In vitro* antimicrobial and antioxidant activity of commercial rosemary extract formulations. Journal of Food Protection 2009;72 1744–1752.

[81] Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity using human and other tumor cell lines. Cancer Research 1988;48 4827–4833.

[82] Rinner B, Kretschmer N, Knausz H, Mayer A, Boechzelt H, Hao XJ, Heubl G, Efferth T, Schaider H, Bauer R. A petrol ether extract of the roots of *Onosmapaniculatum* induces cell death in a caspase dependent manner. Journal of Ethnopharmacology 2010;129 182–188.

[83] Herodež SS, Hadolin M, Škerget M, Knez Ž. Solvent extraction study of antioxidants from balm (*Melissa officinalis* L.) leaves. Food Chemistry 2003;80(2) 275–282.

[84] Negi PS. Plant extracts for the control of bacterial growth: Efficacy, stability, and safety for food application. International Journal of Food Microbiology 2012;156 7–17.

[85] Mimica-Dukic N, Bozin B. *Mentha* L. Species (Lamiaceae) as promising sources of bioactive secondary metabolites. Current Pharmaceutical Design 2008;14(29) 3141–3150.

[86] Sohn HY, Son KH, Kwon GS, Kang SS. Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: *Morus alba* L., *Morus mongolica* Schneider, *Broussnetia papyrifera* (L.) Vent., *Sophora flavescens* Ait and *Echinosophora koreensis* Nakai. Phytomedicine 2004;11 666–672.

[87] Moure A, Cruz JM, Franco D, Domínguez JM, Sinieiro J, Domínguez H, Núñez MJ, Parajó JC. Natural antioxidants from residual sources. Food Chemistry 2001;72 145–171.

[88] Lakic N. Postdistillation waste of aromatic plants as a potential source for pharmaceutical industry. PhD thesis. University of Novi Sad; 2013.

[89] EFSA, The European Union report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011, EFSA J. 11 (2013) 3129.

[90] Kaur K, Kumar V, Sharma AK, Gupta GK. Isoxazoline containing natural products as anticancer agents: A review. European Journal of Medicinal Chemistry 2014;77 121–133.

[91] Garamukulya Y, Abou-Elella F, Wamunyokoli F, Aei-Shemy H. Phytochemical screening, anti-oxidant activity and in vitro anticancer potential of ethanolic and water leaves extracts of Annona muricata (Graviola) Asian Pacific Journal of Tropical Medicine 2014;7(suppl 1) S355–S363.

[92] Smole Možina S, Kurinčič M, Klančnik A, Mavri A. *Campylobacter* and its multi-resistance in the food chain. Trends in Food Science & Technology 2011;22 91–98.

[93] Voljč M, Levart A., Žgur S, Salobir J. The effect of α-tocopherol, sweet chestnut wood extract and their combination on oxidative stress in vivo and the oxidative stability of meat in broilers. British Poultry Science 2013;54(1) 144–156.

[94] Dalle Zotte A, Cullere M, Sartori A, Szendrő Z, Kovàcs M, Giaccone V, Dal Bosco A. Dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation to growing rabbits: Effects on raw and cooked meat quality, nutrient true retention and oxidative stability. Meat Science 2014;98(2) 94–103.

[95] Dal Bosco A, Gerencsér Zs, Szendrő Zs, Mugnai C, Cullere M, Kovàcs M, Ruggeri S, Mattioli S, Castellini C, Dalle Zotte A. Effect of dietary supplementation of Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) on rabbit meat appearance, oxidative stability and fatty acid profile during retail display. Meat Science 2014;96(1) 114–119.