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Assessment of the biological activity of thyme essential oil in the presence of the classic antibiotic tetracycline

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Abstract

The present study was purported to assess the activities: (i) antibacterial and synergistic against three types of both gram-positive and gram-negative bacteria that are susceptible to drug resistance and (ii) cytotoxic and synergistic on colorectal adenocarcinoma cells, of thyme essential oil in combination with tetracycline. Antibacterial and synergistic properties were determined by disk diffusion method and cytotoxic activity by quantifying viable cells by using MTT assay. Thyme essential oil has antibacterial activity against both gram-positive and gram-negative bacteria, especially on *S. aureus* and *K. pneumoniae* at the highest concentration tested, having also a synergistic effect when combined with tetracycline. Essential oil-treated cells showed a dose-dependent reduction in colorectal adenocarcinoma cell viability, while combination with tetracycline leads to a significantly attenuated decrease in viability.

Keywords: thyme, tetracycline, antimicrobial, cytotoxic, colorectal cells

1. Introduction

Antibiotics are considered one of the most successful discoveries in the history of medicine [1,2]. For thousands of years, because nothing was known about infections and their prevention, antibiotics or vaccines, people were helpless in the face of huge waves of epidemics, such as cholera, smallpox, plague, typhoid fever, malaria, tuberculosis, leprosy, syphilis. The period between 1940 and 1960 was the Golden Age of antibiotic discovery [1]. During this period, natural antibiotics isolated from actinomycetes were discovered (aminoglycosides, tetracyclines, amphenicols, macrolides, glycopeptides, ansamycins, lincosamides, streptogramins and cycloserine), from fungal origin (penicillins, cephalosporins) and synthetic antibiotics (e.g. sulfones, nitrofurans, quinolones, azoles, phenazines, ethambutol, thioamides). The vast majority of these antibiotics are still in clinical use, but due to increased antimicrobial resistance their therapeutic efficacy has decreased [3].

Considering that the development of antibiotic resistance is largely attributed to their overuse, inadequate prescription and suboptimal dosing, it is necessary to take certain measures to re-evaluate and optimize the current dose of antibiotics. Improving the prescription of antibiotics is directly correlated with well-established criteria, based on the relationship between concentration-dose, beneficial effects-side effects [4].

Natural products offer extraordinary chemical diversity with a wide variety of biological effects, and thus have been the most promising sources for drug discovery and development [5]. Plant extracts, isolated plant compounds and other natural substances are a rich potential source of active molecules that can destroy or attenuate the action of pathogens [6,7]. Medieval societies have used a multitude of natural substances to treat health issues that are currently diagnosed as microbial infections, and there has been multiple research on the likely effectiveness of these treatments [8].

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To meet the challenges of antibiotic resistance, a key approach is to stimulate the discovery of bioactive substances at an early stage. Complementing the pharmacological actions of classical antibiotics with substances of natural origin is intensively studied to achieve efficiency in the clinic, correlated with a real goal of developing a new generation of chemotherapeutic drugs derived from natural sources, taking into account their unmatched chemical diversity.

The resistance developed by Gram-positive and Gram-negative bacteria to several drugs made them difficult to treat and / or even untreatable with currently available antibiotics [9]. Moreover, it is of major importance to identify new targets and also new classes of antibiotics that can deal with drug-resistant bacterial pathogens. This requires basic research to discover new gaps and to develop new approaches to antibiotics.

The aim of the present research was to evaluate a combination of thyme essential oil (TEo) and tetracycline (Tcyc) in terms of: (i) antimicrobial activity against Gram-positive (*Bacilus cereus*, *Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and (ii) cytotoxic activity on human colorectal adenocarcinoma cells (HT-29).

2. Materials and Method

2.1.Essential oil extraction

Aerial parts of thyme were received from Agricultural Technologies Department from Faculty of Agriculture Timisoara. 200 g plant material was dried and subjected to hydro distillation for four hours in a Clevenger-type device following methods described in the literature [10]. The final mass resulted was dried and stored at -20˚C until further determinations and evaluations.

2.2. Chemical composition evaluation by GC-MS

The predominant chemical composition of thyme essential oil was determined by utilizing a GS/MS system (Shimadzu gas-chromatograph couplet with mass spectrometer QP2010 Plus) equipped with a 30 m length DB-Wax capillary column (0.32 mm x 1 µm). Some of the parameters set were: (1) carrier gas helium, (2) flow rate 1 mL/min, (3) start temperature 40 °C, increased with a rate of 5 °C/min until reached 250 °C and hold for 5 min, (4) injector temperature 250 °C, (5) ion source temperature 220 °C, (6) injection volume 1 µL. Individual compounds were calculated as percentage based on GC peaks and LRI (linear retention indices) were determined as described in the literature [11].

2.3.Antioxidant potential

The DPPH assay was used to estimate the radical-scavenging ability of the different dilutions of thyme essential oil. Shortly: (1) 0.5 mL of sample was diluted with 2.5 mL ethanol 50%, (2) 0.5 mL of DPPH 1 mM was added, (3) absorbance was measured at 516 nm, (4) ascorbic acid was used as positive control while negative control was distilled water. Antioxidant activity expressed as percentage was calculated according to the formula presented in literature [12].

2.4.Antimicrobial activity analysis

Essential oil of thyme in combination with tetracycline was evaluated for antimicrobial potential against Gram-positive and Gram-negative bacteria (strains detailed in table 1) acquired from American Type Culture Collection (Manassas, USA). Experiments were conducted in specific Petri plates by using a 0.001 dilution of the fresh bacterial strains culture and an inoculum equivalent to a 0.5 McFarland standard, incubation at 37 °C for 24-48h. Water was used as negative control and gentamicin disks as positive control.

Table 1. Bacterial strains utilized in the present study in order to evaluate the activity of thyme essential oil combined with tetracycline

Type	Strain	American Type Culture Collection Code
+	*B. cereus*	11778
+	*S. aureus*	25923
+	*S. pyogenes*	19615
-	*E. coli*	25922
-	*K. pneumoniae*	700603
-	*P. aeruginosa*	27853

2.5.Cell culture and viability assessment

The cell line used in this study was human colorectal adenocarcinoma (HT-29) obtained from American Type cell Collection (frozen sample) and preserved in liquid nitrogen until the cultivation for conducting experiments. McCoy's 5a modified medium purchased from ATCC was necessary for cell culture (on standard conditions, 37 °C and 5% CO_2), supplemented with 10% FBS (fetal bovine serum) and 1% of antibiotic mixture.

In brief, 10,000 cells were seeded in 96-well plates and after a ~90% confluence was reached, cells were treated with five concentrations of TEo (5, 7.5, 10, 25 and 50 µL/mL) or Tcyc (1, 2.5, 5, 7.5 and 10 µg/mL) and three concentrations of TEo and Tcyc mixture (10, 25, or 50 µL/mL with 10 µg/mL). After 48 hours of stimulation, 10 µL/well of MTT solution was added, followed by three hours of incubation. Number of viable cells was determined by MTT test according to the protocol presented in the literature [13].

3. Results and discussions

Following the GC-MS analysis, 16 known chemical constituents were identified in the essential oil of thyme and are presented in table 2. The highest percentages were as follows: thymol (~35%), *p*-cimen (~29%), γ-terpinen (~17%), (+)-4-Carene (~3%), α-pinene (~2%), borneol (~3%), and caryophyllene (~2%). The data obtained are in accordance with those described in the literature regarding predominant compounds [14,15]. Certainly these compounds show percentage variations depending on the geographical area and climatic conditions [16].

Table 1. Chemical composition quantification by GC-MS of thyme essential oil

No.	Compounds	LRI [17]	Molecular formula	Concentration (%)
1	Cyclohexane	729	C_6H_{12}	0.694
2	*α*-Pinene	1010	$C_{10}H_{16}$	2.358
3	Camphene	1046	$C_{10}H_{16}$	0.725
4	β-Myrcene	1155	$C_{10}H_{16}$	1.634
5	(+)-*4*-Carene	1149	$C_{10}H_{16}$	3.062
6	γ-Terpinene	1223	$C_{10}H_{16}$	16.869
7	*o*-Cymene	1298	$C_{10}H_{14}$	1.117
8	*p*-Cymene	1283	$C_{10}H_{14}$	28.792
9	Borneol	1690	$C_{10}H_{18}O$	2.546
10	Terpinen-4-ol	1606	$C_{10}H_{18}O$	0.921
11	*α*-Terpineol	1718	$C_{10}H_{18}O$	0.696
12	Thymol	2153	$C_{10}H_{14}O$	35.420
13	Caryophyllene	1562	$C_{15}H_{24}$	2.304
14	Humulene	1649	$C_{15}H_{24}$	0.491
15	(−)-Spathulenol	2121	$C_{15}H_{24}O$	0.345
16	Caryophyllene oxide	1975	$C_{15}H_{24}O$	1.225
				99.199

In the figure 1 is presented the antioxidant activity of thyme essential oil tested at three concentrations. Samples showed a moderate antioxidant activity compared to that of ascorbic acid, the values being directly proportional to the concentration. Therefore, at the highest concentration the average antioxidant activity is around 71%, at the first dilution it decreases to the value of 41% and at the lowest concentration it is around 30% (figure 1).

Bacteria susceptibility to the thyme essential oil and its combination with tetracycline, determined by disk diffusion method, pointed out that TEo shows increased dose-dependent activity on all strains tested except *P. aeruginosa*, with the highest inhibitory effects produced inhibition zones of 20-41 mm diameter.

Concerning antimicrobial activity, the diameter inhibition zone expressed in mm (as the average of two determinations in report to the effectiveness of the positive control) are presented in figures 2 and 3. The maximum zone of inhibition revealed by thyme essential oil was at the maximum concentration tested (50 µL/mL) against *S. aureus* (~35 mm, figure 2) and K. pneumoniae (~40 mm, figure 3) while the combination with tetracycline led to a slight increase in diameter, *S. aureus* (~38 mm) and *K. pneumoniae* (~41 mm).

It is well known that thyme essential oil rich in carvacrol and thymol has strong antimicrobial effects [18]. In the present case, more than half of its content have thymol and cymene.

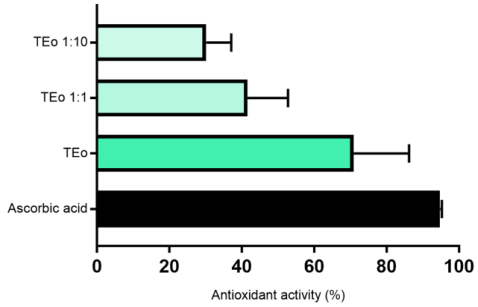

Figure 1. Antioxidant activity of thyme essential oil evaluated by DPPH method and expressed as percentage

The HT-29 cell line was selected due to its use mainly in studies related to the transport of drugs and food compounds but also to study the intestinal immune response to bacteria (survival, adhesion, invasion of microorganisms) [19]. In order to determine the cytotoxic effect of thyme essential oil and tetracycline, were tested different concentrations on human colorectal adenocarcinoma cell line - HT-29. Cell viability was assessed by using the MTT assay at 48-hour intervals. In the case of tetracycline, a slight decrease in cell viability was observed only at the highest concentration tested (10 µg/mL) as can be seen in the figure 4.

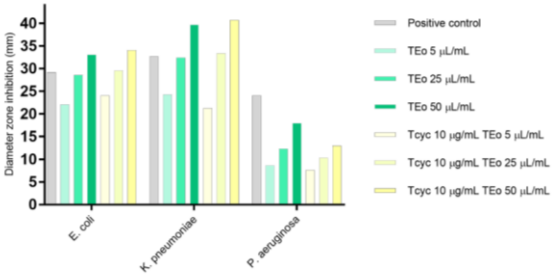

Figure 2. Antimicrobial activity of thyme essential oil and tetracycline mixture as inhibition zones (mm) on gram-positive bacterial strains

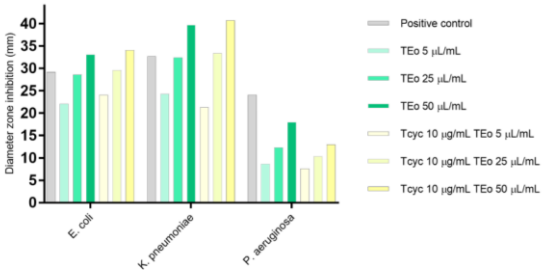

Figure 3. Antimicrobial activity of thyme essential oil and tetracycline mixture as inhibition zones (mm) on gram-negative bacterial strains

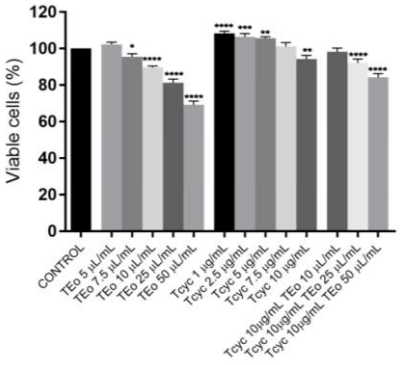

Figure 4. Percentage of human colorectal adenocarcinoma viable cells (HT-29) assessed *in vitro* after stimulation with thyme essential oil, tetracycline and their combination at different concentrations, by MTT assay. One-way ANOVA analysis, followed by Tukey's post-test were employed in order to establish statistical differences (*p < 0.1, **p < 0.01, ***p < 0.001 and ****p < 0.0001).

In contrast, thyme essential oil exerts a decrease in cell viability by more than 30 percent at the highest concentration tested (50 µL/mL). Regarding the evaluation of the highest concentration of tetracycline and three different concentration of thyme essential oil on the behaviour of cells the following values were obtained: ~98% (Tcyc 10 µg/mL + TEo 10 µL/mL), ~92% (Tcyc 10 µg/mL + TEo 25 µL/mL) and ~84% (Tcyc 10 µg/mL TEo 50 µL/mL).

4.Conclusion

The current research study revealed that thyme essential oil manifests a series of biological properties quantified by an increased antioxidant activity, a broad-spectrum antibacterial activity against both gram-positive and gram-negative bacteria and a good cytotoxic potential. The association between the biological product and the classic antibiotic tetracycline has shown the preservation of its cytotoxic properties and the increase of antimicrobial activity against *S. aureus*, *E. coli* and *K. pneumoniae*.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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