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Characterization of the effects of thymol derivatives on colorectal cancer spheroids

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Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies with a high mortality rate. In the last few years, attention has been focused on substances of natural origin with anticancer activity. One such substance is thymol and its derivatives, which have been shown to have an antitumor effect also against CRC cells. In our study, we focused on determining the biological and antibacterial effects of thymol and thymol derivatives. Analyses were performed on a 3D model of human colon carcinoma cell lines (HCT-116 and HT-29) – spheroids. The cytotoxic (MTT assay) and genotoxic effect (comet assay) of thymol and derivatives: acetic acid thymol ester and thymol ß-D-glucoside were determined. ROS levels (ROS-Glo™ H2O2 Assay) and total antioxidant status (Randox TAS Assay) were also monitored. Last but not least, we also detected the effect of the derivatives using a disk diffusion assay and determined the number of colonies on the plates on selected bacteria such as *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Lactobacillus brevis*, *Lactobacillus pentosus* and *Weizmannia coagulans*. The derivatives did not show a significant inhibitory effect on the growth of LAB bacteria (lactic acid bacteria) in contrast to thymol. Overall, thymol derivatives are cytotoxic, genotoxic and increase ROS levels. Among the derivatives tested, acetic acid thymol ester (IC50 ~ 0.2 µg/ml) was more effective. The second derivative tested (thymol β-D-glucoside) was effective at higher concentrations than thymol. Our research confirmed that thymol derivatives have a toxic effect on the 3D model of intestinal tumor cells, while they do not have a toxic effect on selected intestinal bacteria. Thus, they could bring new significance to the prevention or treatment of CRC.

Key words: spheroid; thymol; acetic acid thymol ester; thymol ß-D-glucoside; colorectal cancer

Thymol (2-isopropyl-5-methylphenol) is a natural monoterpene phenol with a characteristic odor. Thymol has been isolated from many plant species, for example: *Origanum L.*, *Thymus V.*, *Satureja L.*, *Carum copticum L.* It occurs mainly in plants of the *Lamiaceae* family, especially in the species *Thymus vulgaris*. This plant occurs naturally in the Mediterranean region, where it was widely used for its medicinal effects. It is often used in the preparation of dishes in both fresh and dried form [1].

Thymol is registered as a safe substance and is also used as a food additive. It is also used in the cosmetic or pharmacological industry in the treatment of inflammation in the oral cavity. Its effect against cardiovascular, neurological, rheumatological, gastrointestinal, and metabolic diseases has also been confirmed [2]. A study in rats also suggested its positive effect on epilepsy and against convulsions, by the mechanism of blocking sodium channels and modulating GABA receptors [3]. Thymol is also used in the prevention of inflammatory bowel diseases. In untreated cases, colon cancer can develop [4].

The studies published so far also point to its anti-cancer effect. In tumor cell lines, thymol has the ability to induce oxidative stress, mitochondrial dysfunction, and apoptosis, inhibits their proliferation, angiogenesis, or migration [2, 5]. A 2020 study on HCT-116 tumor cell lines reported inhibiting activation of the Wnt/β catenin signaling pathway after thymol treatment. The effect of thymol on slowing the growth and metastasis of CRC *in vivo* was also confirmed [6]. Thymol also inhibits the migration of HT-29 tumor cells by acting on the PI3K/AKT and ERK signaling pathways [7]. Its effect was also monitored, for example, on SKOV-3 ovarian cancer cells [8], HeLa cervical cancer cells, MCF-7 breast cancer cells [9], or Neuro-2a neuroblastoma [10]. Not only thymol but also the extract from *Thymus vulgaris* acts against tumor cells. Specifically, the cytotoxic effect of this extract was observed in HepG2 cells [11]. The main disadvantages of thymol are

primarily its physico-chemical and pharmacokinetic properties (solubility, absorption, bioavailability, etc.) [2].

Thymol also affects the intestinal microbiome; it inhibits the growth of lactic acid bacteria after 72 h of treatment most effectively (together with carvacrol) among several essential oils evaluated. Bacteria such as *L. plantarum*, *L. buchneri*, *L. citrovorum* were tested *in vitro* [12]. Other research has focused on the action of thymol *in vivo*. Thymol was administered into the diet of weaned pigs and the microbial diversity of the small intestine was monitored. [13]. A reduced incidence of bacteria was not observed [14]. Studies also suggest the cytotoxicity of thymol (or thymol-containing essential oils) and its inhibitory effect on breast tumor cell spheroids such as MDA-MB-231, 4T1, or TC1 [15, 16].

The beneficial effects of thymol on human health are well known for many years. However, due to its low solubility, its use is limited. Therefore, new thymol derivatives have been synthesized [17], which are expected to be more efficient due to better cell penetration. In this report, we determined the cytotoxic and genotoxic effects, ROS generation, and total antioxidant status of thymol and its hydrophilic derivatives in an *in vitro* model using 3D colorectal cell models (HCT-116, HT-29). In addition, the studied substances were also screened for antibacterial properties against LAB bacterial strains, namely: *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Lactobacillus brevis*, *Lactobacillus pentosus,* and the Gram-positive bacterium *Weizmannia coagulans*.

Materials and methods

Cell culture. CRC cell lines HCT-116 and HT-29 were obtained from the American Type Culture Collection (USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) in low glucose (1 g/l) with added 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Thermo Fisher Scientific, USA). The cells were placed in an incubator at 5% CO_2 and 37 °C. Media and chemicals used for cell cultivation were purchased from Gibco BRL (Paisley, UK).

Bacterial strains. All bacterial strains used were obtained from the collection of microorganisms of the Food Research Institute in Bratislava.

Chemicals. Thymol, used as a standard, was obtained from Sigma-Aldrich (St. Louis, MO, USA). New thymol derivatives [acetic acid thymol ester (DT1) and thymol β-D-glucoside (DT2)] were synthesized at the Institute of Chemistry of the Faculty of Natural Sciences of Comenius University, Bratislava, Slovakia [17].

Formation of spheroids. Two methods based on the same principle were used for the formation of spheroids. In the first case, the cells were seeded in a 96-well ULA microplate. The cells were placed in an incubator at 37 °C and 5% CO_2. Cells formed compact spheroids after 5 d of cultivation. In the second case, the hanging drop method was used according to the available protocol from Foty [16].

Determination of cytotoxicity (MTT assay). The cytotoxicity of studied substances was determined by MTT assay (3-[4,5-dimethyl-thiazolyl]-2,5-diphenyltetrazolium bromide). Briefly, 1×10^6 cells were seeded in 96-well ULA microplates and cultured in a complete DMEM medium. Then, thymol (0–600 μg/ml), DT1 (0–0.4 μg/ml), or DT2 (0–3,000 μg/ml) were added and the cells were incubated at 37 °C in a 5% CO_2 atmosphere for 24 hours. The samples were then centrifuged for 5 min at 151×g and washed with phosphate-buffered saline (PBS). Subsequently, the samples were incubated with 1 mg/ml MTT for 4 h. Then the samples were centrifuged again, MTT was removed, and formazan crystals were dissolved for 40 min with dimethyl sulfoxide. Subsequently, each sample was mixed by pipetting. The absorbance at 540 nm was measured using an xMark microplate spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the background absorbance at 690 nm was subtracted.

Determination of genotoxicity (comet assay, SCGE). Genotoxicity was determined by the alkaline comet assay (single-cell gel electrophoresis, SCGE), which allows the detection of DNA breaks [18, 19]. Cells were seeded on Petri dishes according to the hanging drop method [20] and treated with the test substance for 24 h. Concentrations from 30 to 140 μg/ml were used for thymol, from 0.04 to 0.2 μg/ml for DT1, and from 500 to 2,500 μg/ml for DT2. A lysis solution consisting of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tri-HCl (pH 10), and 1% Triton X-100 was prepared. The samples were placed in this chilled solution for 1 h in a fridge. After lysis, an electrophoretic solution was prepared in the following composition: 300 mM NaOH, 1 mM Na_2EDTA, pH>13. The samples were allowed to unwind in the electrophoretic apparatus for 30 min in the dark at 4 °C and the electrophoresis itself was carried out in the cold for 20 min at 19 V and 300 mA. The samples were neutralized in neutralizing solution for 2×10 min (0.4 M Tris-HCl, pH 7.4) and fixed in ethanol for 5 min. Staining of the slides was carried out by applying 5 g/ml ethidium bromide. Slides were examined with a Zeiss Imager Z2 fluorescence microscope using computer-assisted image analysis (Metafer 3.6, MetaSystems GmbH, Altlussheim, Germany). The percentage of DNA in the tail was used as a parameter to measure DNA damage (DNA strand breaks). Five hundred comets were evaluated for each sample in one cycle of electrophoresis.

Determination of ROS production. The oxidative stress was analyzed using the ROS-Glo™ H_2O_2 Assay (Promega, Madison, WI, USA). The ROS-Glo™ H_2O_2 Assay is a bioluminescent assay that measures the level of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), directly in cell culture or in defined enzyme reactions. Briefly, cells were seeded by the hanging drop method and cultured in a complete DMEM medium. The studied thymol (30–140 μg/ml), DT1 (0.04–0.2 μg/ml), and DT2 (500–2500 μg/ml) were then added, and the cells were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. H_2O_2 substrate solution was added for 6 hours to

generate a luciferin precursor. The addition of ROS-Glo™ Detection Solution (20 min) converts the precursor to luciferin and provides Ultra-Glo™ Recombinant Luciferase to produce a light signal that is proportional to the level of H_2O_2 present in the sample. The relative luminescence was measured.

Determination of the total antioxidant status (TAS). Randox TAS Assay kit (Randox Laboratories, UK) was used to determine the total antioxidant status. ABTS$^{\bullet}$ (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation ABTS$^{\bullet+}$ with a blue-green color. Cells were seeded on a Petri dish using the hanging drop method and treated for 24 h. The following concentration range was used: thymol 30–140 µg/ml, DT1 0.04–0.2 µg/ml, and DT2 500–2,000 µg/ml. R2 solution (phosphate-buffered saline 80 mM, pH 7.4, metmyoglobin 6.1 µM, ABTS 610 µM) was added to the samples. Absorbance at a wavelength of 600 nm was measured using an xMark microplate spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After that, R3 solution (hydrogen peroxide 250 µM, phosphate-buffered saline 80 mM, pH 7.4) was added to the samples and after 3 min of exposure, the absorbance of the samples was measured again. Antioxidants in the sample cause suppression of this color production to a degree, which is proportional to their concentration.

Evaluation of antibacterial activity. The antibacterial effect of thymol and derivatives was analyzed by disc diffusion test [21]. The effect on four lactic acid bacteria-LAB (*Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Lactobacillus brevis*, *Lactobacillus pentosus*) was monitored and the Gram-positive bacterium *W. coagulans* was also selected. Nutrient medium for *W. coagulans* and De Man, Rogosa and Sharpe for LAB bacteria were used for cultivation. The culture was carried out anaerobically at 37 °C for 24 h. Cell cultures were diluted and inoculated onto nutrient media. Subsequently, the test substances were added and the diameter of the inhibition zone around the discs was evaluated after 24 h of treatment. Bacterial survival, specifically the number of colonies forming units in 1 ml of sample, was also evaluated.

Statistical analysis. The results represent a mean from 3 to 5 experiments ± standard deviation (SD). The differences between defined groups were tested for statistical significance using Student's t-test (*p<0.05; **p<0.01; ***p<0.001).

Results

Determination of cytotoxicity (MTT Assay). The cytotoxic effects of thymol derivatives on HT-29 and HCT-116 CRC spheroids were determined using the MTT assay. Cells in the form of spheroids were treated with the studied substance for 24 h and changes in cell viability were noted. The results are presented in Figures 1A–1C. After 24 h, a decrease in cell viability was observed in direct dependence on the applied concentration of the substance-derivative. IC_{50} values (median inhibitory concentrations that cause approximately 50% cell death) were determined for both cell lines tested.

For thymol, the IC_{50} value was determined to be ~112 µg/ml for the HT-29 cell line and ~150 µg/ml for HCT-116 (Figure 1A). The DT1 derivative was more effective, showing cytotoxicity at much lower concentrations, namely ~0.18 µg/ml for both cell lines (Figure 1B). The second of the analyzed DT2 derivatives showed high IC_{50} values, for cell line HT-29 ~1,340 µg/ml and for HCT-116 up to ~2,510 µg/ml (Figure 1C). Based on these results,

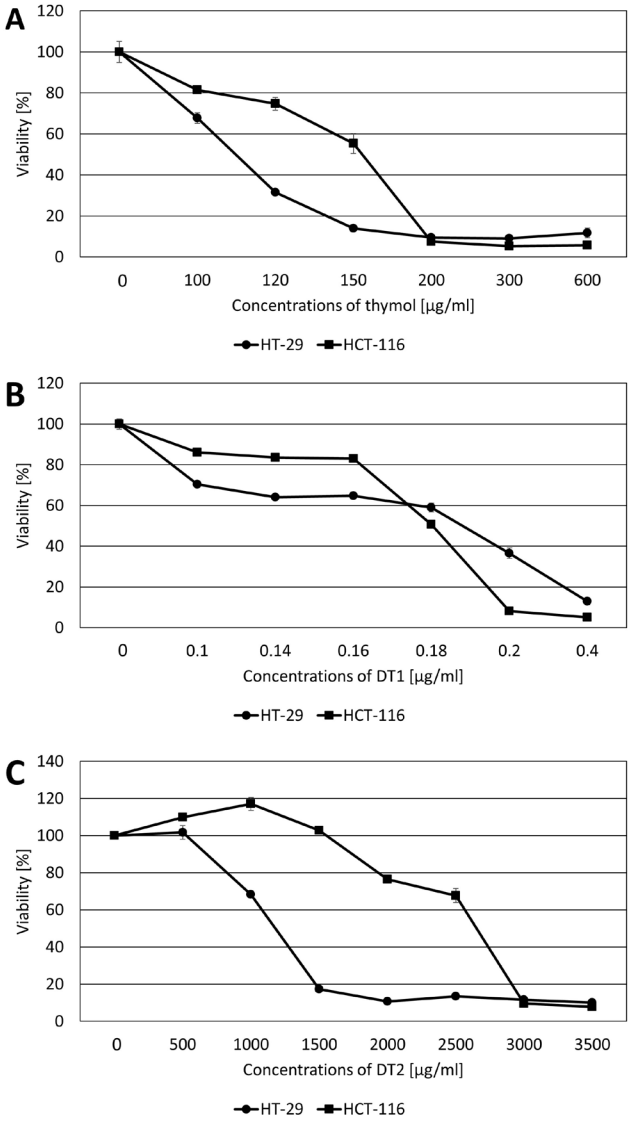

Figure 1. Cytotoxic effect of thymol (A), acetic acid thymol ester (B), and thymol β-D-glucoside (C) on the spheroids of HT-29 and HCT-116 tumor cell lines after 24 h of treatment. Data represent means ± SD of three independent experiments.

we further focused on determining the genotoxic effect of thymol derivatives.

Determination of genotoxicity (comet assay, SCGE). DNA chain breaks were determined by the comet assay method. Spheroids of HT-29 and HCT-116 cell lines were treated with thymol and derivatives (DT1 and DT2).

Evaluation of the results showed that the standard substance – thymol did not induce DNA damage even at the highest tested concentration of 140 µg/ml (Figure 2A) in studied spheroids of HT-29 and HCT-116 cells. Compared with the control, the DT1 derivative also induced a non-significant enough increase in DNA damage in both cell lines (Figure 2B). The highest concentration tested was at 0.2 µg/ml. A more significant increase was observed for the DT2 derivative from a concentration of 1,500 µg/ml in the HCT-116 cell line and from a value of 2,500 µg/ml (Figure 2C).

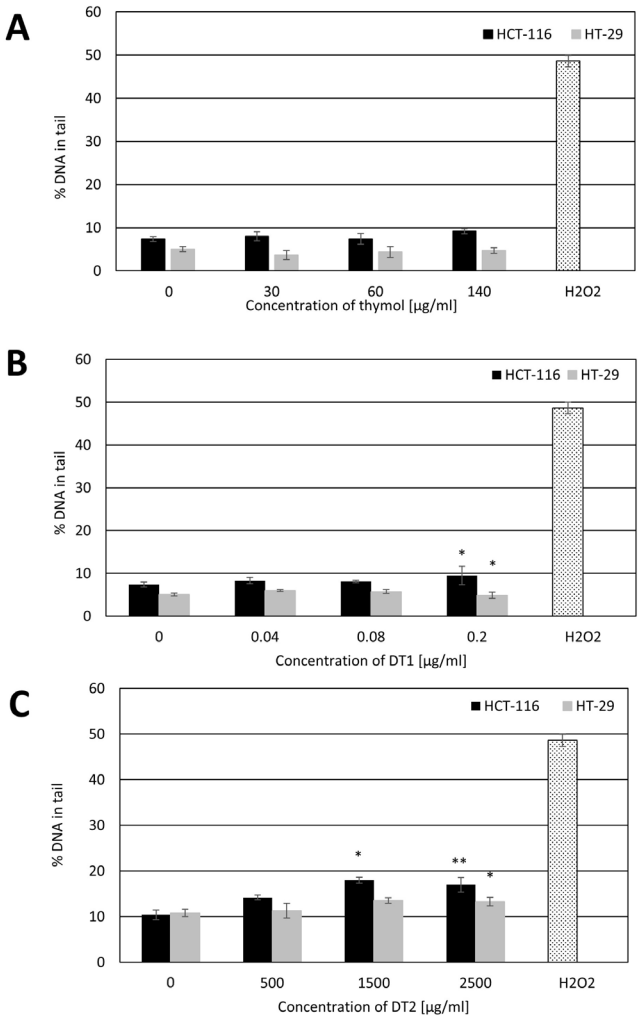

Figure 2. Genotoxic effect of thymol (A), acetic acid thymol ester (B), and thymol β-D-glucoside on the spheroids of HT-29 and HCT-116 cell lines after 24 h of treatment with selected concentrations. Data represent means ± SD of three independent experiments. *p<0.05; **p<0.01; indicate statistically significant differences compared to the control (Student's t-test). As a positive control is used H_2O_2 (concentration 300 µmol/l).

Determination of ROS production. ROS production was analyzed using the ROS-Glo™ H_2O_2 Assay. Three substances with different concentration ranges (30–140 µg/ml for thymol, 0.04–0.2 µg/ml for DT1, and 500–2,500 µg/ml for DT2) were evaluated. In the case of HCT-116 cells (Figure 3), a significant increase in ROS production was observed for the DT1 (from 0.08 to 0.2 µg/ml) and DT2 derivative (1,500 and 2,500 µg/ml). Thymol did not show such a significant increase. Similar results were observed for the HT-29 cell line (Figure 4) and the increase in ROS levels was at the highest concentration tested for both derivatives (DT1 0.2 µg/ml and DT2 1,300 µg/ml).

Determination of total antioxidant status. Total antioxidant status was analysed by Randox TAS Assay using ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]). In the case of the HCT-116 cell line (Table 1), all test substances showed a significant increase in TAS compared to the negative control. However, thymol at a concentration of 140 µg/ml proved to be the most effective. Derivatives DT1 and DT2 were the most effective at the highest concentrations applied. However, the HT-29 cell line did not respond to the presence of thymol and derivatives with elevated TAS levels in either case, and values ranged from 1.02 to 1.14 mmol/prot. These values were at the level of the negative control.

Determination of antibacterial activity (Disk diffusion test). Antibacterial activity was determined by the disc diffusion test, which is standardly used. Concentrations in the range from 0.1 to 3,000 µg/ml were applied. Four strains of lactic acid bacteria were tested: *Lacticaseibacillus rhamnosus, Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*,

Table 1. Total antioxidant status (TAS) in HCT-116 and HT-29 cells exposed to thymol, DT1 (acetic acid thymol ester), and DT2 (thymol β-D-glucoside) for 24 h.

Studied substances	Dose [µg/ml]	TAS [mmol/prot]	
		HT-29	HCT-116
Control (-)	–	1.02±0.05	1.57±0.07
Control (+)	–	8.20±0.07	4.86±0.18
Thymol	30	1.05±0.05	2.27±0.12**
	60	1.14±0.09	2.53±0.08***
	140	1.11±0.09	3.75±0.11***
DT1	0.04	1.02±0.05	1.32±0.09
	0.08	1.07±0.17	1.53±0.05
	0.2	1.05±0.13	1.81±0.08**
DT2	500	1.12±0.03	1.66±0.1
	800	1.04±0.07	–
	1,300	1.10±0.01	–
	1,500	–	1.67±0.08
	2,000	–	1.72±0.11*

Notes: Control (–) untreated cells; control (+) ascorbic acid; data represent means ± SD of three independent experiments; *p<0.05; **p<0.01; ***p<0.001 indicate statistically significant differences compared to the untreated control cells (Student's t-test)

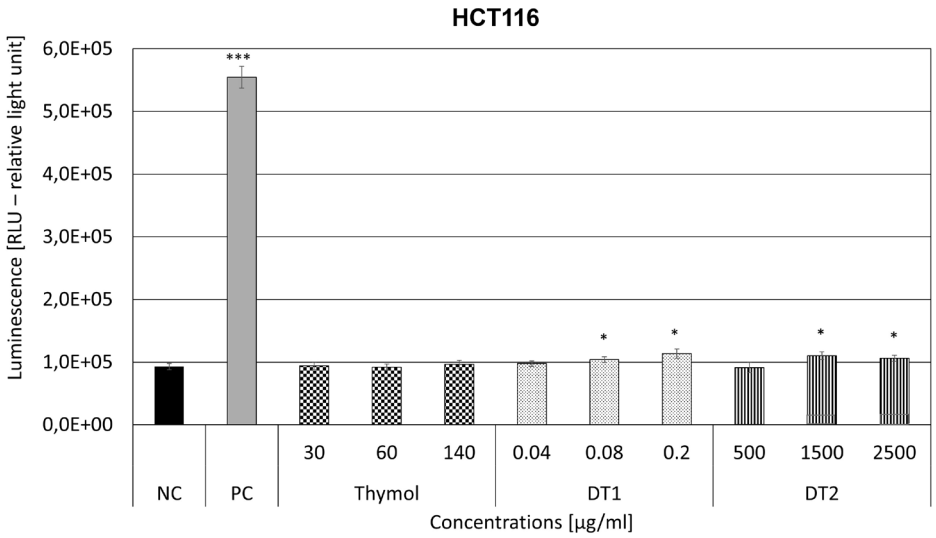

Figure 3. Effect of thymol, acetic acid thymol ester (DT1), and β-D-glucoside of thymol (DT2) on HCT-116 spheroids for ROS production after 24 h treatment. Data represent means ± SD of three independent experiments. *p<0.05; **p<0.01; ***p<0.001 indicate statistically significant differences compared to the negative control (Student's t-test). Abbreviations: NC-negative control; PC-positive control (menadion 50 µmol/l)

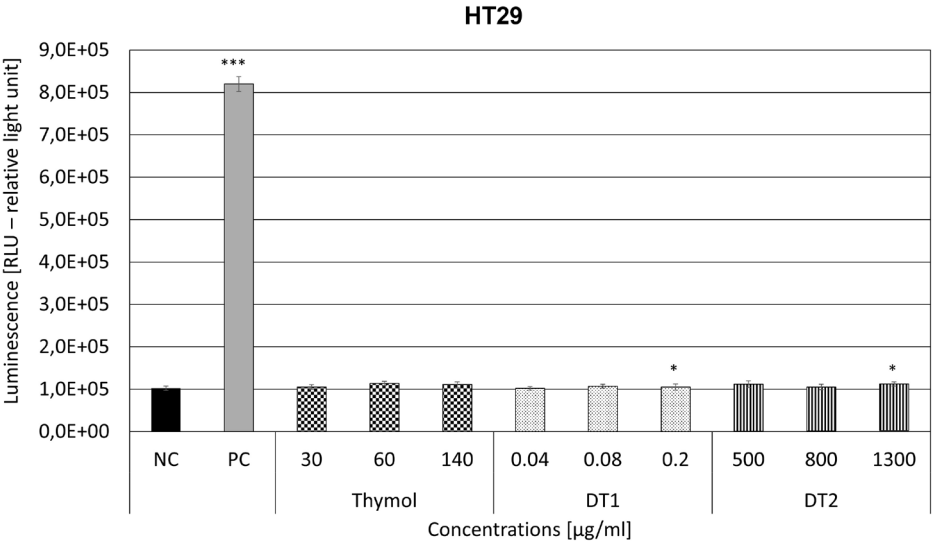

Figure 4. Effect of thymol, acetic acid thymol ester (DT1), and β-D-glucoside of thymol (DT2) on HT-29 spheroids for ROS production after 24 h treatment. Data represent means ± SD of three independent experiments. *p<0.05; **p<0.01; ***p<0.001 indicate statistically significant differences compared to the negative control (Student's t-test). Abbreviations: NC- negative control PC- positive control (menadion 50 µmol/l)

Lactiplantibacillus pentosus, *Lactobacillus brevis,* and one strain of the Gram-positive bacterium *Weizmannia coagulans*. In the case of the DT2 derivative, no growth inhibition was observed for any of the strains tested (Table 2). Thymol and DT1 inhibited the growth of all bacteria at the concentration tested, with a much larger diameter zone of inhibition for thymol. In the case of survival in MRS (HiMedia) and NB liquid medium (VWR Chemicals), inhibition was observed only in the case of thymol (Table 3).

Discussion

Many scientific studies have confirmed the therapeutic effect of thymol on various types of cancer cells [22–24]. In our study, we aimed to determine the effect of thymol and its derivatives on the spheroids of CRC tumor cells, as the incidence of this disease is steadily increasing. The solubility of thymol in water is low, hence its penetration into the cell and its application in practice are limited [2]. Thymol has

Table 2. Effect of thymol and its derivatives on lactic acid bacteria and *W. coagulans* determined by the disc method.

Strains on MRS and NA solid media	Thymol 150 µg/ml [mm]	DT1 0.1 µg/ml [mm]	DT2 3,000 µg/ml [mm]
Lactiplantibacillus plantarum	25±0.76	8±0.76	<0.2
Lacticaseibacillus rhamnosus	20±1.27	6±1.50	0.5±0.50
Lactiplantibacillus pentosus	23±0.76	6±0.76	<0.2
Lacticaseibacillus paracasei	24±1.04	9±0.76	<0.2
Lactobacillus brevis	26±1.00	8±0.50	0.5±0.50
Weizmannia coagulans	21±0.76	6±1.30	<0.2

Note: data represent means ± SD of three independent experiments

the ability to inhibit the growth of *S. aureus* and *E. coli*, the principle being thought to be interaction with the plasma membrane. This may result in a change in membrane permeability and penetration of thymol into the internal environment of the cell [25]. Therefore, studied thymol derivatives have been synthesized [17], which are expected to be more efficient due to better cell penetration. Effects such as cytotoxicity, genotoxicity, total antioxidant activity, ROS production, and last but not least, we focused on the antibacterial effect of thymol and thymol derivatives were determined.

We determined the cytotoxic effect on the spheroids of CRC cells using the MTT assay. All three substances tested showed a toxic effect, and in the case of thymol, this effect has been described in several studies. Jamali et al. in 2018 [24] investigated the effect of essential oils such as thymol, carvacrol, and p-cymene on MDA-MB-231 and MCF-7 breast tumor cells. A cytotoxic effect was found for both 2D and 3D cultures. For thymol, the IC_{50} value was determined to be 56 µg/ml (MDA-MB-231 line) and 47 µg/ml (MCF-7 line) after 24 h for 2D cultures. For spheroids, higher concentrations were required, namely 149 µg/ml (MDA-MB-231 line) and 134.5 µg/ml (MCF-7 line). Thymol also induced apoptosis via ROS in MDA-MB-231 cells, caspase 3 activity was increased, and changes in the cell cycle were also observed. In another study, the same effect was observed on 4T1 tumor cells [15]. An essential oil with a significant presence of thymol and carvacrol was tested. There were significantly higher levels of ROS in treated cells confirming our result. MTT test results were less significant for spheroids. The essential oil-induced cytotoxicity after 24 h at an IC_{50} concentration of 47.3 µg/ml. In the case of spheroids, the IC_{50} was 130.4 µg/ml. In the case of healthy L929 cells, thymol was not toxic. The thymol derivative DT1 tested by us proved to act much more effectively.

Thymol did not show a genotoxic effect on CRC cells in the comet assay, but some increase was observed for the derivatives. In the case of DT1, we observed some increase at a concentration of 0.2 µg/ml for both cell lines, however, this derivative did not show genotoxic damage. Similarly, in the case of the DT2 derivative, the highest concentration tested was 2,500 µg/ml, which was also the most potent. The HCT-116 cell line showed a more significant genotoxic effect than the HT-29 line. Our results are in agreement with existing studies that have shown that thymol does not induce genotoxic damage in colorectal cancer cells at 24 h exposure [17, 26]. Concentrations ranging from 0.001625 to 60 µg/ml were tested.

Our previous work [17] also focused on thymol derivatives: thymol acetic acid ester and thymol β-D-glucoside, where we observed and confirmed the biological effect of these derivatives on colorectal cancer cells. The analysis was performed in a 2D cell model. One of the derivatives, namely acetic acid thymol ester, was much more potent and at lower concentrations than thymol (~0.08 µg/ml) in most of the assays. Both thymol derivatives showed cytotoxic and genotoxic effects. We observed an increase in the percentage of DNA in the comet tail in both DT1 and DT2 in HT-29 and HCT-116 cell lines. Significant increases for DT1 were observed at concentrations as low as 0.06 µg/ml and for DT2 as low as 1,000 µg/ml. The derivatives also effectively inhibited the cell proliferation rate and increased ROS production was also observed. These results provided us with a good basis for further analyses using the 3D models. In the case of spheroids, it is expected that higher concentrations will be required to observe the same biological effect as in the 2D model.

We used the ROS-Glo™ H_2O_2 Assay to analyze and measure ROS levels. ROS results showed significant increases after the application of DT1 (0.08 and 0.2 µg/ml) and DT2 (1,500 and 2,500 µg/ml). Thus, thymol derivatives can potentially

Table 3. Effect of thymol and its derivatives on the survival of lactic acid bacteria and *W. coagulans*.

Strains	Control [log10 CFU/ml]	Thymol 150 µg/ml [log10 CFU/ml]	DT1 0.1µg/ml [log10 CFU/ml]	DT2 3,000 µg/ml [log10 CFU/ml]
Lactiplantibacillus plantarum	6.70 ± 0.12	0	5.85 ± 0.11	6.36 ± 0.15
Lacticaseibacillus rhamnosus	7.18 ± 0.08	0	5.90 ± 0.06	6.74 ± 0.05
Lactiplantibacillus pentosus	6.45 ± 0.06	0	6.11 ± 0.09	5.36 ± 0.08
Lacticaseibacillus paracasei	6.78 ± 0.09	0	5.76 ± 0.07	6.40 ± 0.06
Lactobacillus brevis	6.74 ± 0.15	0	6.04 ± 0.12	6.18 ± 0.11
Weizmannia coagulans	5.30 ± 0.11	0	5.59 ± 0.13	5.20 ± 0.12

Notes: bacterial levels were determined by plate counts on MRS or nutrient agar plates; 0=no cells-this concentration inhibited growth; data represent means ± SD of three independent experiments

act as a drug. Several studies have shown that thymol can inhibit ROS levels in T24, SW780, and J82 bladder tumor cells [27] or A549 lung tumor cells [28] and thus exert antitumor effects. This study tested the effect of thymol on T24, SW780, and J82 bladder tumor cells. However, the changes in ROS production after thymol treatment are likely dependent on the cell line used, the concentration or time of thymol treatment, and the method applied for ROS determination. After ROS determination, TAS values were also measured. The HCT-116 tumor cell line showed a significant increase after thymol treatment, and also the highest concentrations of thymol derivatives relative to the negative control. Altinas et al. [29] also demonstrated an increase in TAS after affecting HepG2 tumor cells with thymol after 24 h treatment. According to our results, the HCT-116 cell line demonstrated both an increase in ROS and an increase in TAS at the high concentrations tested for thymol derivatives. These increases may be a response to oxidative stress of the cells after treatment with the aforementioned substances when oxidation-reduction events may be impaired [30]. These results may indicate that the treatment of cells with thymol caused an increase in ROS levels and a concomitant increase in TAS. The increase in TAS was a consequence and response of the cells to the disturbance of the balance by the increase in ROS.

The antibacterial analysis shows that thymol exhibits this property against all micro-organisms tested at a concentration of 150 µg/ml. The hydrophilic derivatives tested showed only minimal or no inhibitory activity against the selected microorganisms. In the case of the DT1 derivative, the concentration tested was 0.1 µg/ml, and in the case of DT2 3,000 µg/ml.

These concentrations were already toxic to the studied spheroids. The antibacterial activity of thymol or thyme has been reported by several studies, confirming our results. Laurel et al. [31] monitored the inhibitory activity of both thymol and thyme on nine LAB strains (*Pediococcus acidilactici*, *Pediococcus damnosus*, *Leuconostoc citrovorum*, *Leuconostoc mesenteroides*, *Lactobacillus buchneri*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus fructivorans,* and *Lactobacillus plantarum*) for a treatment period of 72 h. Thyme oil inhibited the growth of all microorganisms at concentrations ranging from 2,000 to 1,000 µg/ml. Thymol was more effective when concentrations in the range of 1,000 to 500 µg/ml were sufficient to apply. Similar results were published in another study [32] where thymol inhibited the growth of *Lactobacillus acidophilus*, *Lactobacillus reuteri,* and *Lactobacillus salivarius* at a concentration of 1,500 µg/ml. In addition, inhibition of pathogenic bacteria such as *Salmonella typhimurium* or *Clostridium perfringens* was also demonstrated. The study also investigated the effect of thymol and carvacrol on chickens infected with *Clostridium perfringens*. The bacterium caused lesions, which were reduced by the application of thymol and carvacrol to the chickens' feed.

This study brings new original results regarding the more effective internalization of a newly synthesized hydrophilic thymol derivative into colorectal cancer cells. Biological activity was evaluated in more complex *in vitro* models such as 3D spheroids that will show relevant information than a 2D model. In this study, the derivative of thymol acetic acid thymol ester shows toxic effects and produces ROS at much lower concentrations than thymol. This confirmed the hypothesis that the hydrophilic properties of the derivatives have the potential to act more effectively and have the potential to be applied in the treatment of colorectal cancer. In the case of the second of the tested derivatives-thymol β-D-glucoside, relatively high concentrations were applied. From this point of view, it is a rather uninteresting derivative for potentially further and more detailed analyses. However, we feel that it may be valuable knowledge in the context of targeted modification of the chemical structure for the synthesis of much more potent substances in the future.

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