

## Research Article

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## Synergistic Anti-*Borreliae* Efficacy of a Composition of Naturally-occurring Compounds: an *In vitro* Study

Goc A\*, Niedzwiecki A\* and Rath M

Department of Infectious Diseases, Dr. Rath Research Institute, USA

**\*Correspondences:** Aleksandra Niedzwiecki, Department of Infectious Diseases, Dr. Rath Research Institute BV, 5941 OpticalCt, San Jose, 95138 CA, USA, Tel: 408-567-5050; Fax: 408-748-1726; E-mail: a.niedz@drath.com  
Anna Goc, Department of Infectious Diseases, Dr. Rath Research Institute BV, 5941 OpticalCt, San Jose, 95138 CA, USA, Tel: 408-588-7108; Fax: 408-748-1726; E-mail: a.goc@drath.com

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### Abstract

**Background:** *Borrelia* sp, which is a pathogenic agent of Lyme diseases in mammals, has become an increasing problem worldwide due to the emergence of persistence. In this study we investigated whether a defined composition of naturally occurring substances could display a broad and synergistic action *in vitro* against both active and persistent forms of *Borrelia* spp.

**Methods:** A formulation of six plant-derived compounds combined at their 1/32-1/2 MIC values was tested *in vitro* against two species of *Borrelia* recognized as causative agents of Lyme disease in North America and Europe.

**Results:** The results showed that a composition of baicalein, luteolin, rosmarinic acid, monolaurin, cis-2-decenoic acid, and iodine at their 1/8 MIC values has significant synergistic effect against the active and persisting latent forms. This composition revealed anti-oxidative properties affecting *Borrelia's* membrane but not DNA. Finally, we observed its inhibitory effect on the release of IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6 by human CD14<sup>+</sup> monocytes stimulated with live *Borrelia* sp.

**Conclusion:** These results suggest that such a formulation of compounds might be considered and further explored for its significant pleotropic anti-*Borreliae* efficacy. Additional *in vivo* and human studies are warranted to validate this possibility.

**Keywords:** *Borrelia* spp, Persisters, Phytochemicals, Redox, Cytokines

**Abbreviations:** AAPH: 5 mM 2,2'-Azobis(2-methylpropionamide); ARP: Aldehydes Reactive Probe; 10-CDA: Cis-2-decenoic acid; CV: Crystal Violet; Cep: Cefoperazone; Dap: Daptomycin; Dox: Doxycycline; LD: Lyme Disease; LPS: Lipopolysaccharide; MIC: Minimal Inhibitory Concentration; MBC: Minimal Biocidal Concentration; MDA: Malondialdehyde; ORP: Oxidation/Reduction Potential; PI: Propidium Iodide; pH: The test mixture; PTLDS: Post-treatment Lyme Disease Syndrome

### Introduction

Lyme Disease (LD) is a multi-systemic bacterial infection that has become the most frequent vector-borne disease in North America and Europe, and it is a growing health concern worldwide [1]. It is caused by bacteria from genus *Borrelia*, which is a tick-transmitted micro-aerophilic and invasive group of pathogens and

includes species such as *Borrelia burgdorferi* sensu stricto and *Borrelia mayonii* (both recognized to cause LD in North America) as well as *Borrelia afzelii* and *Borrelia garinii* (predominantly causing LD in Europe) [2,3]. In the presence of challenging conditions, *Borrelia* sp. can transform from its spiral-shaped form into pleomorphic dormant forms such as knob-shaped/rounded-looking structures (i.e., round bodies/cysts, L-forms/spheroplasts,

and granular structures) and bio film-like aggregates [4-8]. The presence of these atypical forms has been demonstrated *in vitro* and in humans and is suggested as one of the possible factors that may attribute to the persistence of this illness [9-13]. However, the reason as well as the mechanisms explaining why the persistent symptoms of this infection promulgate in patients after the recommended treatment period with prescribed antibiotics awaits clarification, and highlights the need for either new or improved treatment options [14,15].

The commonly prescribed drugs for LD patients are limited to a major pharmacological class of antibiotics belonging to beta lactams and tetracycline's, and include doxycycline, amoxicillin, and cefuroxime axetil [1]. Their single administration *in vitro* showed to be effective against the active form of *Borrelia* sp. but not against their pleomorphic persistent forms, including bio film-like aggregates [16-19]. Instead, antibiotics such as doxycycline, penicillin, and amoxicillin have been shown experimentally to induce transformation of spirochetes into the latent cystic forms [16-20]. In addition, the continued use of antibiotics as monotherapy has limitations since their long-term effectiveness has not been confirmed and instead may involve toxic side effects [21-24]. However, recent *in vitro* studies demonstrate that a triple combination of antibiotics such as doxycycline/daptomycin/cefoperazone can be considered as an option since it also showed to be effective against persistent forms of *Borrelia burgdorferi* [25]. Moreover, two triple combinations of antibiotics such as artemisinin/cefoperazone/doxycycline and sulfachlorpyridazine/daptomycin/doxycycline revealed to be effective against amoxicillin-induced round bodies [26]. Further human studies, however, are needed to confirm these findings.

The positive outcome of any therapy highly depends on adequate treatment, and therapies based on non-synthetic agents have been increasingly considered as one of the alternatives [27-29]. In particular, polyphenols have been intensively examined and measured as potential new anti-infectious agents [28,29]. A variety of them have shown *in vitro* activity against a plethora of bacteria and fungi species including *Borrelia* sp. [30-33]. In addition, many of them demonstrated anti-inflammatory and immune-modulating characteristics that have relevance in anti-LD therapies, and substantial progress has been made in understanding the mechanisms of their antimicrobial action [13,34,35]. These include affecting the bacterial membrane resulting in the leakage of intracellular constituents and/or upsetting crucial

molecules responsible for maintaining basic bacterial physiology [36]. In addition, research has been conducted to understand how persisting cells can be eliminated and was focused either on eradicating them or by "forcing" them to revert back to an active state. Eradication of persistent forms has been accomplished by their exposure to biocidal agents, reactive species, and weak electrical streams, whereas the approach to transform persisters back to their active form include the use of metabolic stimuli in order to increase the metabolism and/or the ability of drugs to permeate the membranes of these forms [36-38].

A synergistically or additively composed combination of naturally occurring compounds with antimicrobial activity and immune-modulatory properties could be one such LD treatment option. This approach takes advantage of exploiting various structural and/or metabolic interactions allowing for attacking multiple targets at once and maximizing the biological efficacy. In addition, if properly designed, it could potentially reduce the cost and side effects of the application. Our recent study revealed that polyphenols such as baicalein, luteolin, rosmarinic acid, fatty acids (monolaurin and cis-2-decenoic acid), and iodine are potent against *Borrelia* spp. [32]. In addition, we showed that the combinations of baicalein with luteolin as well as monolaurin with cis-2-decenoic acid displayed synergistic anti-spirochete and additive anti-biofilm effects. Moreover, baicalein and luteolin when combined with rosmarinic acid or iodine produced additive bacteriostatic and bactericidal effects against spirochetes and persisters, respectively [39].

In this study, we tested the effects of the mixture composed of these six natural agents (i.e., baicalein MIC = 150 µg/ml, luteolin MIC = 125 µg/ml, rosmarinic acid MIC = 150 µg/ml, monolaurin MIC = 100 µg/ml, cis-2-decenoic acid MIC = 125 µg/ml, and iodine MIC = 5 µg/ml) utilizing a "golden standard" method of direct counting and dark field microscopy, against all pleomorphic forms of *Borrelia burgdorferi*. s. and *Borrelia garinii* [16,17,25,26,32,40,41]. We evaluated the anti-*Borreliae* effect of the defined composition of phytochemicals (at its concentration ranging from 20.5-328 µg/ml as well as a recently developed high throughput screening method assessing viability of logarithmic phase (adequate to phase rich in spirochetes) and stationary phase (adequate to phase rich in persisters). Additionally, we tested the composition quantitatively and qualitatively on the form of the most resistant biofilm-like aggregates. Finally, we explored the potential mode of action of this composition and its anti-

inflammatory properties.

## Materials and Methods

### Test compounds

The compounds such as baicalein, cis-2-decenoic acid (10-CDA), doxycycline, daptomycin, and cefoperazone with the purity between 90-98% according to the manufacturer, were obtained from Sigma Aldrich (St. Louis, MO). Luteolin and rosmarinic acid, with the purity between 97-99% according to the manufacturer, were purchased from Tocris Bioscience (Bristol, United Kingdom). Organic kelp with standardized iodine content (i.e., 150 µg/ml as 100% daily allowance) purchased from World Organic Ltd. (New Zealand), Monolaurin (Lauricidin®), as a pure sn-1 monolaurin (glycerol monolaurate) derived from coconut oil, was obtained from Med-Chem Laboratories, Inc. (Goodyear, AZ).

### Preparation of test mixture for susceptibility testing

A stock solution (50-100 mg/ml) of each compound (depending on solubility of the substance) was prepared by suspending individual test compounds in absolute ethanol and sterilized by 0.22 µm syringe filtration. All stock solutions were stored in aluminum foil-wrapped tubes at -20°C. Due to the bactericidal effect of a high percentage of ethanol, its added amount to the growth medium was kept below 0.4% (v/v). The appropriate amount of each stock solution (150 µg/ml of baicalein, 125 µg/ml of luteolin, 150 µg/ml of rosmarinic acid, 100 µg/ml of monolaurin, 125 µg/ml of 10-CDA, and 5 µg/ml of iodine; i.e., each of these six agents combined at its MIC value in a 1:1 ratio altogether) was then added to 1.8 ml sterile screw-cap test tubes or 24-well plates containing 1 ml of BSK-H complete medium at their 1/32-1/2 MIC concentration that equalized to 20.5-378 µg/ml of the test mixture.

### Test microorganisms

Two *Borrelia* species such as *Borrelia burgdorferi* sensu stricto and *Borrelia garinii* were tested in their three morphological forms: spirochetes, knob/rounded-shaped persisters, and biofilm-like aggregates. Low passage isolates of the B31 strain of *Borrelia burgdorferi* and the CIP103362 strain of *Borrelia garinii* were obtained from the American Type Culture Collection (Manassas, VA). The B31 strain is an isolate from *Ixodes dammini*, whereas the CIP103362 strain is an isolate from *Ixodes ricinus*. The stocks of both species were cultured in commonly used conditions, i.e., Barbour-Stoner-Kelly H (BSK-H)

medium supplemented with 6% rabbit serum (Sigma, St. Louis, MO) without antibiotics at 33°C with 5% CO<sub>2</sub> in 15 ml polypropylene sterile screw-cap test tubes with or without gentle shaking.

### Preparation of test microorganisms for susceptibility testing

Both *Borrelia* sp. were prepared for testing according to Sapi et al. [16]. Briefly, the strains were activated from original cryobank vials and inoculated into 10 ml BSK-H complete medium, and maintained at 33°C. Generation of homogeneous logarithmic culture (i.e., having only spirochetes/active form) of tested *Borrelia* sp. were obtained by maintaining inoculum in a shaking incubator at 33°C and 250 rpm, where there was no biofilm-like aggregates formation for 2-3 days. Generation of stationary culture (i.e., rich in knob/rounded-shaped cells/persistent forms) of tested *Borrelia* sp. was obtained by maintaining inoculum in an incubator at 33°C for 7-8 days. Generation of biofilm-like aggregates of tested *Borrelia* sp. was attained by incubation of inoculums in four-well chambers (BD Biosciences, Sparks, MD) coated with collagen Type I from rat tail for at least one week without shaking.

### Evaluation of the anti-*Borreliae* effects of test mixture against active and persistent forms of *Borrelia* spp

Anti-*Borreliae* efficacy of the test mixture was assessed using direct counting and/or spectrofluorometry according to previously reported methodology [16,25,26,40,41]. Briefly, 1.8 ml sterile screw-cap test tubes or 24-well plates containing 1 ml BSK-H medium, supplemented with the test mixture, were inoculated with  $2 \times 10^6$  -  $1 \times 10^7$  cells/ml (in logarithmic phase, i.e., 2-3 days culture that was composed mostly of typical motile spirochetes or stationary phase, i.e., 7-8 days culture that was enriched in knob/rounded-shaped persisters). Long-term sub-culture study was performed by transferring 50 µl of the stationary phase of *Borrelia* sp. culture treated with the test mixture to 1 ml of fresh agent-free BSK-H medium. Control wells were treated with ethanol (0.1-0.4%), a triple combination of doxycycline/daptomycin/cefoperazone (30 µg/ml, 10 µg/ml each) or 82 µg/ml of doxycycline alone. The tubes/plates were then incubated at 33°C with 5% CO<sub>2</sub> and monitored at regular intervals for up to 72 h except for sub-culturing where the samples were incubated for 14 days. Assessment was done using a bacterial Petroff-Hausser counting chamber with dark field microscopy and LIVE/DEAD® BacLight™ Bacterial

Viability staining with fluorescent microscopy and/or SYBR Green I/PI staining with spectro fluoroscopy (Nikon, Eclipse E600). The excitation wavelength was set at 485 nm and the absorbance wavelength at 535 nm. All experiments were done in triplicate.

### **Evaluation of anti-*Borrelia* effects of the test mixture against biofilm-like aggregates of *Borrelia* spp**

Qualitative and quantitative anti-*Borrelia* effect of the test mixture against biofilm was assessed in four-well chambers coated with collagen Type I from rat tail according to Sapi et al. [8,16,41]. Briefly,  $1 \times 10^7$  cells/ml was inoculated into each sterile chamber containing 1 ml BSK-H medium, and incubated for up to one week at 33°C with 5% CO<sub>2</sub>, followed by 72 h of incubation with the test mixture. Control wells were treated with ethanol (0.1-0.4%), and a triple combination of doxycycline/daptomycin/cefoperazone (30 µg/ml, 10 µg/ml each) or 82 µg/ml of doxycycline alone. Eradication effect was evaluated by applying the commonly used crystal violet (CV) staining method [8,16]. All wells were fixed with 500 µl of cold methanol-formalin (1:1) for 30 min. and stained with 1 ml of CV (0.1%) for 10 min. The biofilms were carefully washed three times with 1 x PBS (phosphate-buffered saline), and 1 ml of methanol was added to each well to extract a dye, and the amount was assessed at an optical density of 595 nm using a spectrophotometer (Molecular Device, Spectra Max 340) for quantitative results. Earlier studies in our laboratory have documented a lack of antifungal carryover using this procedure [32]. For quantitative assessment, all wells were fixed with 500 µl of cold formalin acetic acid mixture for 20 min., followed by staining with 200 µl of 2 x BacLight staining mixture for 15 min. in the dark, according to the manufacturer's recommendation. Pictures were immediately taken from untreated and treated mounted slides using a fluorescence microscope (Nikon, Eclipse E600). All experiments were done in triplicate.

### **Evaluation of redox potency of the test mixture**

Oxidation/reduction potential (ORP) was performed by inoculating  $1 \times 10^6$  cells/ml (logarithmic phase) and  $1 \times 10^7$  cells/ml (stationary phase), respectively, into 1.8 ml sterile screw-cap test tubes containing 1 ml BSK-H medium, supplemented with the test mixture. The tubes were then incubated at 33°C with 5% CO<sub>2</sub> and the ORP (mV) was measured at 0 h and 72 h using a Micro Oxidation-Reduction Measurement System with a suitable ORP-146S micro combination redox electrode with built in reference electrode from LAZAR Research Laboratories, J Nutri Bio, 5(1): 350-363 (2019)

Inc. (Netmerize Innovations, Inc., LA, CA). All experiments were done in triplicate.

### **Evaluation of the membrane and lipid damage by the test mixture**

The membrane permeabilization was performed as previously described by measuring a release of UV-absorbing material using a UV-VIS spectrophotometer (Molecular Device, Spectra Max 340) [42,43]. Briefly,  $1 \times 10^6$  cells/ml (in logarithmic phase) and  $1 \times 10^7$  cells/ml (in stationary phase), respectively, were inoculated into 1.8 ml sterile screw-cap test tubes containing 1 ml BSK-H complete medium, supplemented with the test mixture. The tubes were then incubated at 33°C with 5% CO<sub>2</sub>. After 72 h treatment, samples (1.0 ml) were filtered through a sterile nitrate cellulose membrane (0.22 µm) and OD<sub>260</sub> value of the supernatant was measured followed by calculation of percentage of the extracellular UV-absorbing materials released by the cells. The lipid damage was assessed as previously reported [43]. Briefly, *Borrelia* spp.  $1 \times 10^6$  cells/ml (logarithmic phase) and  $1 \times 10^7$  cells/ml (stationary phase), respectively, were inoculated into 1.8 ml sterile screw-cap test tubes containing 1 ml BSK-H complete medium, supplemented with the test mixture. After the incubation, cells were harvested by centrifugation (1000 g, 5 min, 4°C) and washed with HN buffer (20 mM NaCl, 50 mM HEPES, pH 7.6). Next, all samples were subjected to Lipid Peroxidation (MDA) ELISA assay kit (Abcam, Cambridge, MA) by generating MDA-TBA adducts and measuring the output immediately at OD<sub>532</sub>, according to manufacturer's protocol. The amount of MDA in the sample was calculated by using a standard curve. As a positive control, 5 mM 2,2'-azobis (2-methylpropionamidine) (AAPH) was used. All experiments were done in triplicate.

### **Evaluation of DNA damage**

DNA damage was determined using the DNA Damage Quantification Colorimetric Assay kit (Oxford Biomedical Research, Oxford, MI) according to the manufacturer's protocol. Briefly,  $1 \times 10^6$  cells/ml (logarithmic phase) and  $1 \times 10^7$  cells/ml (stationary phase), respectively, were inoculated into 1.8 ml sterile screw-cap test tubes containing 1 ml BSK-H complete medium, supplemented with the test mixture. The tubes were then incubated at 33°C with 5% CO<sub>2</sub>. Next, 0.5 µg/ml of DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI) and was mixed with an equal volume of 10 mM biotinylated aldehydes reactive probe (ARP) reagent and incubated for 1 h at 37°C. The DNA-ARP product was precipitated, washed three times with 70%

ethanol, and re-suspended in Tris-EDTA buffer to final concentration of 0.5 µg/ml. The DNA-ARP product was left overnight at 37°C for binding to the wells of a 96-well microplate. All wells were then washed again four times with TPBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>3</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5% Tween 20, pH 7.4). In the meantime, the HRP-streptavidin conjugate was diluted to 0.5 µg/ml in assay buffer (0.15 M NaCl, 10 mM Na<sub>3</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM KCl, 5 mg/ml BSA, 0.1% Tween, pH 7.5), and 100 µl of it was added to each well followed by incubation for 1 h at room temperature. After incubation, the wells were washed four times with TPBS, 100 µl of substrate was added to each well, and incubated again for 1 h at 37°C. In the end, the reaction was quenched with 100 µl of 1 M sulphuric acid and the reaction was monitored at 450 nm. The number of aldehydes reactive probe (DNA base lesions) per 10<sup>5</sup> bp DNA was determined using a standard curve. As a positive control, DNA from *E. coli* (American Type Culture Collection, Manassas, VA) treated with 100 µM H<sub>2</sub>O<sub>2</sub> for 30 min. was used. All experiments were done in triplicate.

### Evaluation of the pro-inflammatory cytokines and chemokines release

Pro-inflammatory cytokines and chemokines release was assessed using the Multi-Analyte Profiler ELISA array assay kit (SA Biosciences, Germantown, MD) according to the manufacturer's protocol. Briefly, 2 × 10<sup>6</sup> of human CD14<sup>+</sup> monocytes (ATCC, Manassas, VA) were plated in 6-well plates and allowed to settle for 2-4 h, followed by incubation with *Borrelia* spp. (monocytes: bacteria ratio = 1:10) for 12 h at 37°C in 5% CO<sub>2</sub> atmosphere, with or without test mixture. After the incubation period, all supernatants were collected and subjected to ELISA assay according to manufacturer's protocol. As a positive control, LPS-stimulated CD14<sup>+</sup> monocytes were used. All experiments were done in triplicate.

### MTT assay

Cell number was assessed using MTT assay according to manufacturer's protocol. Briefly, human CD14<sup>+</sup> monocytes were plated in 48-well plates at 1 × 10<sup>5</sup> cells per well in the RPMI containing 10% FBS. After 2-4 h, the medium was replaced with the same medium supplemented with 82 µg/ml test mixture. After 12 h of treatment, cell viability was measured at 570 nm, using an ELISA reader (Molecular Device, Spectra Max 340). The whole experiment was repeated four times.

### Statistical analysis

All data are presented as means ± SD (n = 3). The J Nutri Bio, 5(1): 350-363 (2019)

ANOVA and/or Student's two-tailed t test was used to determine statistically significant differences set at 0.05 levels. Statistical analysis was performed using GraphPad software. Adopted COLBY equation

$$E = A + B + C + D + E + F \left[ \frac{(A \cdot B) + (A \cdot C) + (A \cdot D) + (A \cdot E) + (A \cdot F) + (B \cdot C) + (B \cdot D) + (B \cdot E) + (B \cdot F) + (C \cdot D) + (C \cdot E) + (C \cdot F) + (D \cdot E) + (D \cdot F) + (E \cdot F)}{100} + \frac{[A \cdot B \cdot C \cdot D \cdot E \cdot F]}{100^{6-1}} \right]$$

was applied to calculate synergistic cooperation of the compound in the test mixture as described [44], where E=expected value (calculated value using COLBY equation) and A,B,C,D,E,F = observed values (values obtained experimentally). Additional validating statistical analysis was performed at the Department of Statistics at UC Davis, CA.

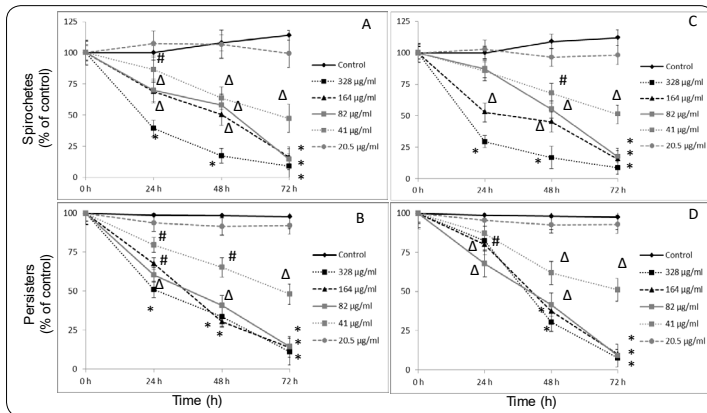
## Results

### Screening of different concentrations of the test mixture using direct counting

The effect of various concentrations (ranging from 20.5 µg/ml to 328 µg/ml) of the test mixture composed of baicalein, luteolin, rosmarinic acid, monolaurin, 10-CDA, and iodine against spirochetes and persisters of *Borrelia burgdorferi* s. and *Borrelia garinii* are presented in figure 1. The results show time- and dose-dependent anti-*Borreliae* effects against active and persistent forms. No differences were seen in the susceptibility between the two tested species of *Borrelia*. Using this method of testing we found that the most effective concentrations of the composition were between 82 µg/ml to 328 µg/ml corresponding to 1/8-1/2 of the MIC value for each phytochemical which allowed for reducing the presence of both active and persistent forms about 90%, respectively. COLBY-based statistical evaluation, the recommended approach in the research setting studied here, indicated a synergistic cooperation of the plant-based compounds against all pleomorphic forms of both tested *Borrelia* sp (Table 1).

Antagonism; observed value - % of obtained from performed experiment inhibition/eradication, expected value - % of inhibition/eradication calculated using COLBY equation, S-synergy as described [44]. A-observed value of the test mixture; observed values of individual compounds in the test mixture: baicalein-15.3, luteolin-16.8, rosmarinic acid-12.5, monolaurin-8.9, 10-CDA-7.9, iodine-5.6; b-observed value of the test mixture; observed values of individual compounds in the test mixture: baicalein-18.2, luteolin-17.3, rosmarinic acid-15.7, monolaurin-10.2, 10-CDA-11.3, iodine-8.2; c-observed value of the test mixture; observed values of individual compounds in the test mixture: baicalein-19.1,

luteolin-14.2, rosmarinic acid-13.9, monolaurin-12.7, 10-CDA-10.8, iodine-11.5; d-observed value of the test mixture; observed values of individual compounds in the test mixture: baicalein-18.9, luteolin-15.8, rosmarinic acid-16.3, monolaurin-14.5, 10-CDA-11.4, iodine-10.9; e-observed value of the test mixture; observed values of individual compounds in the test mixture: baicalein-10.6, luteolin-10.2, rosmarinic acid-10.4, monolaurin-9.1, 10-CDA-9.2, iodine-4.1; f-observed value of the test mixture; observed values of individual compounds in the test mixture: baicalein-10.1, luteolin-10.5, rosmarinic acid-9.2, monolaurin-8.7, 10-CDA-8.5, iodine-4.2.



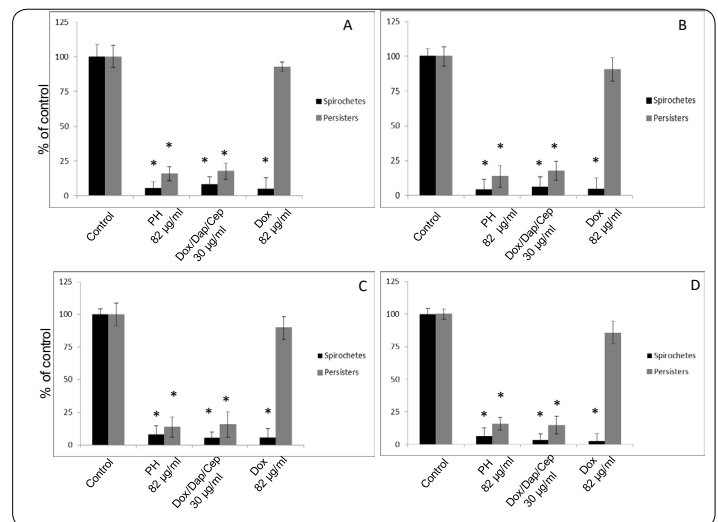
**Figure 1:** Kinetic evaluation of anti-*Borreliae* effects of different concentrations of the test mixture.

Dose-dependent efficacy of the test mixture against spirochetes and persisters of *Borrelia burgdorferi* (A and B) and *Borrelia garinii* (C and D) was monitored up to 72 h using direct counting and dark field microscope; #  $p \leq 0.05$ ,  $\Delta p \leq 0.01$ , \*  $p \leq 0.001$ .

### Anti-*Borreliae* efficacy of the test mixture against logarithmic and stationary phase of *Borrelia* spp

Treatment of spirochetes and persisters of *Borrelia burgdorferi* s. and *Borrelia garinii* with 82 µg/ml (i.e., baicalein 18.8 µg/ml, luteolin 15.6 µg/ml, rosmarinic acid 18.8 µg/ml, monolaurin 12.5 µg/ml, 10-CDA 15.6 µg/ml, iodine 0.625 µg/ml) of the test mixture using SYBR Green I/PI staining (Figure 2A and 2C) and SYTO9/PI staining (Figure 2B and 2D), caused about 90% reduction in viability of spirochetes and persisters. This examination confirmed our preliminary screening results obtained by direct counting with a dark field microscope. There were no observed significant differences in efficacy assessment between both tested *Borrelia* spp. Additional confirmation of effectiveness of the test mixture on persistent cells was evaluated using a 14-day sub-culturing experiment in fresh agent-free BSK-H medium to detect any regrowth to motile spirochetes. This experiment showed

~ 12% ( $< 1.5 \times 10^5$  cells/ml) repopulation rate of viable cells compared to control with no observed significant differences between the two tested *Borrelia* spp. (Figure 3). The efficacy of 30 µg/ml of triple combination of antibiotics (doxycycline/daptomycin/cefoperazone, 10 µg/ml each) showed ~ 90% susceptibility rate and ~ 10% ( $< 1.5 \times 10^5$  cells/ml) repopulation rate, whereas 82 µg/ml of doxycycline revealed to be effective against spirochetes but not persisters with ~ 50% repopulation rate, which corroborated previously reported results [25,41].

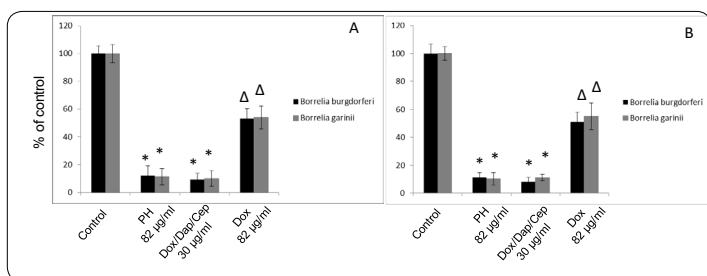


**Figure 2:** Evaluation of the anti-*Borreliae* effect of the test mixture against spirochetes and persisters of *Borrelia burgdorferi* (A, B) and *Borrelia garinii* (C, D). Efficacy was determined after 72 h by SYBR Green I/PI assay using spectrofluoroscopy (A, C) and direct counting of live and dead forms stained with SYTO9/PI using fluorescence microscopy (B, D); Control- 0.1-ethanol, pH- the test mixture of phytochemicals, Dox/Dap/Cep- doxycycline/daptomycin/cefoperazone; \*  $p \leq 0.001$ .

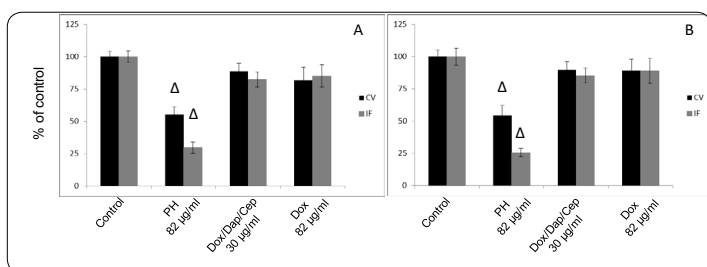
### Anti-*Borreliae* efficacy of the test mixture against biofilm-like aggregates

The efficacy of the composition of phytochemicals against biofilm-like aggregates of *Borrelia burgdorferi* s. and *Borrelia garinii* are presented in figure 4. Performed examination showed that, compared to control, treatment with 82 µg/ml (i.e., baicalein 18.8 µg/ml, luteolin 15.6 µg/ml, rosmarinic acid 18.8 µg/ml, monolaurin 12.5 µg/ml, 10-CDA 15.6 µg/ml, iodine 0.625 µg/ml) of test mixture reduced biofilm-like aggregates formed by the two tested *Borrelia* sp. ~ 45-55%, as assessed by crystal violet quantitative staining. An additional qualitative experiment, in which SYBR Green I/PI dye was used, revealed that ~ 70% of remaining biofilm-like aggregates

were composed of mainly dead cells. Triple combination of antibiotics (doxycycline/daptomycin/cefoperazone) administrated at 30 µg/ml showed to eradicate biofilm-like aggregates in ~ 12% and ~ 14% of the remaining biomass was composed of mainly dead cells. Doxycycline administrated at 82 µg/ml revealed to eradicate biofilm-like aggregates ~ 10% and ~ 12% of the remaining biomass was composed of mainly dead cells. Our results corroborate previously reported results [41]. There were no significant differences in the efficacy of the test mixture between both tested *Borrelia* sp.



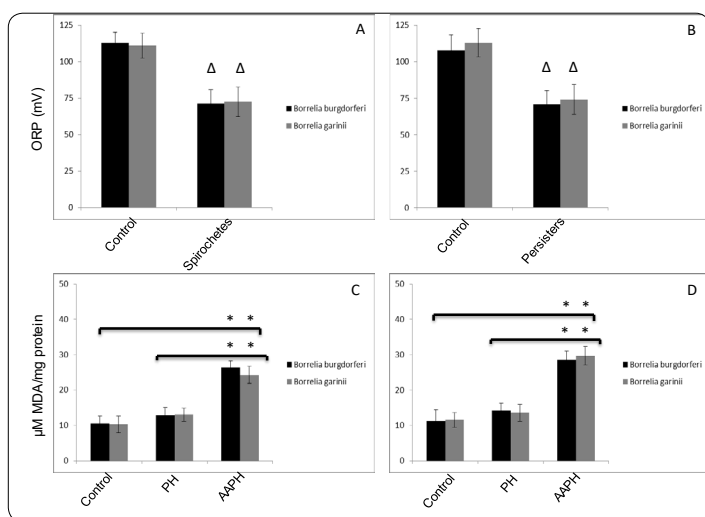
**Figure 3:** Estimation of repopulated spirochete form of *Borrelia burgdorferi* and *Borrelia garinii*. *Borrelia* spp. were treated with the test mixture for 72 h and transferred to fresh tubes containing medium only. After 14 days of sub-culturing, the presence of typical motile spirochetes were determined by SYBR Green I/PI assay using spectrofluoroscopies (A) and direct counting of live and dead forms stained with SYTO9/PI using fluorescence microscopy (B); Control- 0.1% ethanol, pH- the test mixture of phytochemicals, Dox/Dap/Cep -doxycycline/daptomycin/cefoperazone;  $\Delta$   $p \leq 0.01$ , \*  $p \leq 0.001$ .



**Figure 4:** Susceptibility of *Borrelia burgdorferi* (A) and *Borrelia garinii* (B) biofilm-like aggregates to the test mixture. Biofilm-like aggregates were treated with the test mixture for 72 h. Next, the percentage of eradication of biofilm mass was determined by crystal violet (CV) and SYTO9/PI (IF) staining method; Control- 0.1% ethanol, pH- the test mixture of phytochemicals, Dox/Dap/Cep-doxycycline/daptomycin/cefoperazone;  $\Delta$   $p \leq 0.01$ .

## Redox potency of the test mixture

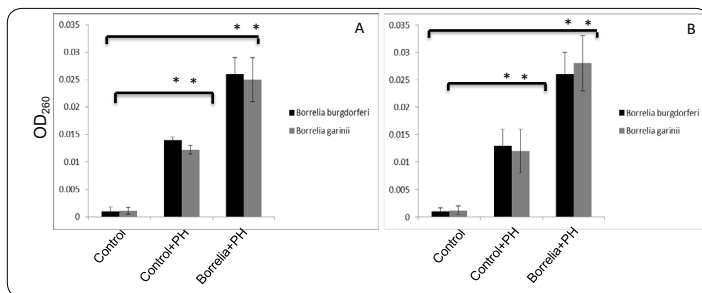
Oxidation/reduction potential as a measure of the tendency of an agent to acquire electrons, performed with the test mixture against active and persistent forms of *Borrelia* spp. is presented in figure 5A and 5B. An approximately 35% decrease of ORP value was observed ( $p < 0.01$ ) when the bacterial cells were treated with the 82 µg/ml of the test mixture compared to control. There were no observed significant differences between the two studied *Borrelia* spp. Analysis of MDA (malondialdehyde) production, as a marker of lipids peroxidation, a stable end-product upon oxidative agent exposure, is presented in figure 5C and 5D. The results confirmed no oxidative damage caused by the test mixture as opposed to AAPH agent, a free radical generator, the addition of which caused a significant ~ 2.0-2.5 fold increase of MDA amount compared to control ( $p < 0.001$ ). Untreated (control) and treated with the test mixture, spirochetes (logarithmic phase) and persisters (stationary phase) contained between 10-15 µM of MDA per mg of protein compared to cells treated with AAPH 25-30 µM of MDA per mg of protein. There were no observed significant differences in assessments between spirochetes and persisters in both tested *Borrelia* sp.



**Figure 5:** Redox potency of the test mixture against spirochetes and persisters of *Borrelia burgdorferi* and *Borrelia garinii*. *Borrelia* spp. were treated with the test mixture and after 72 h the redox potency was determined by redox micro-electrode system (A and B) and MDA generation using spectrophotometry (C and D); Control - 0.1% ethanol, pH- 82 µg/ml test mixture of phytochemicals, AAPH - 5 mM 2,2'-azobis(2-methylpropionamide);  $\Delta$   $p \leq 0.01$ , \*  $p \leq 0.001$ .

## Membrane status after treatment with the test mixture

The cellular permeability measured by UV-absorbing release materials is demonstrated in figure 6 and showed to be significantly ( $p < 0001$ ) increased after treatment of spirochetes (logarithmic phase) and persisters (stationary phase) of both *Borrelia* spp. with the test mixture at 82  $\mu\text{g}/\text{ml}$  concentration, respectively, compared to control (containing test mixture at 82  $\mu\text{g}/\text{ml}$  concentration without bacteria), a  $\sim 1.5$ -2.0 times increase was noticed. There were no observed significant differences in valuations between spirochetes and persisters in both tested *Borrelia* sp.

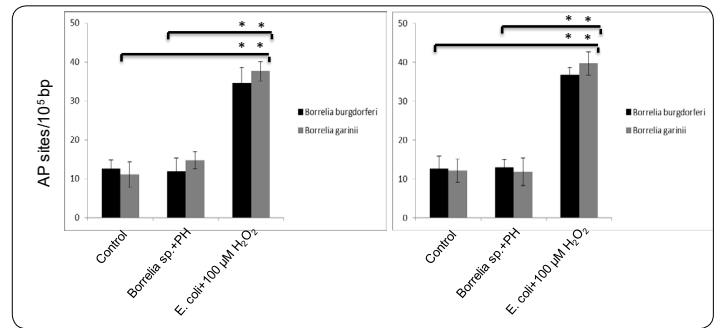


**Figure 6:** The presence of 260 nm absorbing materials.

*Borrelia* spp. were treated with the test mixture and after 72 h the presence of 260 nm absorbing materials in supernatants of spirochetes (A) and persisters (B) of *Borrelia burgdorferi* and *Borrelia garinii* was determined; Control- 0.1% ethanol, Control +pH - no *Borrelia* sp. +pH, pH- 82  $\mu\text{g}/\text{ml}$  test mixture of phytochemicals;  $*p \leq 0.001$ .

## Evaluation of DNA damage after treatment with the test mixture

The effect of 82  $\mu\text{g}/\text{ml}$  of the test mixture on DNA of both studied *Borrelia* sp. was determined based on assessment of AP sites (DNA base lesions) as previously reported [42,43]. The results that are presented in figure 7 show that the numbers of AP sites per  $10^5$  bp DNA from *Borrelia* sp. treated with test mixture is equal to control and significantly ( $p < 0001$ ) increased in DNA samples from *E. coli* treated with 100  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  that was used here as a positive control as reported previously [43]. This indicates that the addition of the test mixture did not increase the number of DNA base lesions and did not cause DNA damage. There were no observed significant differences in evaluations between spirochetes and persisters in both tested *Borrelia* sp.



**Figure 7:** Effect of the test mixture on DNA damage.

*Borrelia* spp. were treated with the test mixture and after 72 h DNA damage, as the number of aldehydes reactive probe (DNA base lesions) per  $10^5$  bp DNA using standard curve, of spirochetes (A) and persisters (B) of *Borrelia burgdorferi* and *Borrelia garinii* was determined; Control- no *Borrelia* sp. +pH, pH - 82  $\mu\text{g}/\text{ml}$  test mixture of phytochemicals;  $*p \leq 0.001$ .

## Pro-inflammatory cytokines and chemokine release status after treatment of the test mixture

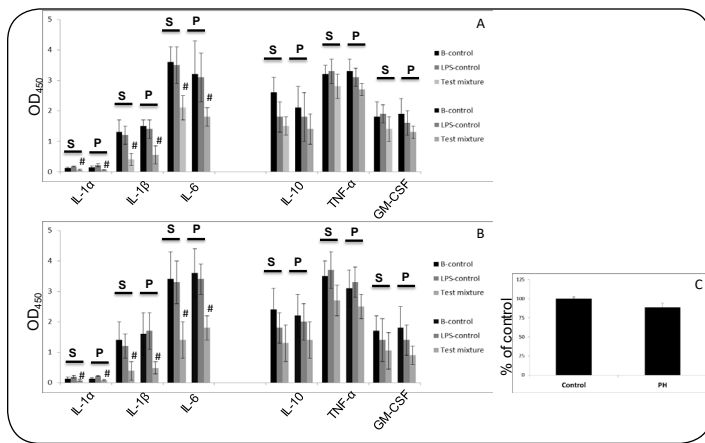
The effect of 16 h post-treatment with the test mixture on the release of 12 different interleukins and chemokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, INF $\gamma$ , TNF $\alpha$ , GM-CSF) by human CD14<sup>+</sup> monocytes stimulated with studied *Borrelia* spp. is presented in figure 8A and 8B. The results show that compared to control (CD14<sup>+</sup> monocytes stimulated with *Borrelia* sp.), the 82  $\mu\text{g}/\text{ml}$  of the test mixture significantly ( $p < 0.05$ ) inhibited the secretion of IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6. A decreasing tendency was noticed for IL-10, TNF $\alpha$ , and GM-CSF, and no differences were observed in the status of IL-2, IL-4, IL-8, IL-12, IL-17, and INF $\gamma$ . A similar trend was observed for LPS-stimulated CD14<sup>+</sup> monocytes. There were no significant differences in measurements between spirochetes and persisters between both tested *Borrelia* sp. In addition, MTT assay revealed statistically not significant with  $\sim 10\%$  mortality of CD14<sup>+</sup> monocytes incubated with the 82  $\mu\text{g}/\text{ml}$  of test mixture (Figure8C).

## Discussion

Growing evidence of persistency of *Borrelia* spp. in mammals and the resultant health concerns underlie the need for the development of more comprehensive treatments, but what causes it and why the phenomena of persisting symptoms in patients with late LD or PTLDS exists is still debatable [14,45-48]. Previously we reported that particular phytochemicals such as polyphenols (baicalein, luteolin, and rosmarinic acid),



fatty acids (monolaurin, 10-CDA), and iodine express *in vitro* antibacterial efficacy when applied individually and in dual combinations against the *Borrelia* spp. that are recognized as causative pathogens of LD in the USA and Europe [32,39].



**Figure 8:** Cytokine and chemokine release status from human CD14<sup>+</sup> monocytes after 12 h post-treatment. B-control - cells stimulated with *Borrelia burgdorferi* (A) or *Borrelia garinii* (B) at cells: bacteria 1:10 ratio. (C) Viability of CD14<sup>+</sup> monocytes after 12 h post-treatment with test mixture assessed by MTT assay; LPS-control-cells stimulated with 100 ng/ml LPS, Test mixtures – cells stimulated with *Borrelia* sp. at 1:10 ratio and treated with 82 µg/ml test mixture of phytochemicals, S - spirochetes, P- persisters, pH- 82 µg/ml test mixture of phytochemicals; #  $p \leq 0.05$ .

In the present *in vitro* study we took another step towards increasing the efficacy and expanding the pleotropic mechanistic effects of these selected compounds against *Borrelia* spp. We combined all of them at their 1/32-1/2 MIC values and tested them against active and persistent latent forms of *Borrelia burgdorferi* s. and *Borrelia garinii* as the test mixture and checked its anti-*Borreliae* and anti-inflammatory features. This examination indicated that such a defined composition of the plant-derived active substances has significant antimicrobial efficacy against active and latent forms of tested *Borrelia* spp.

The preliminary testing was assessed by direct counting and dark field microscopy, and used for evaluation of different concentrations of phytochemicals in the mixture. The results showed dose- and time-dependent killing effects against spirochetes and persisters of tested *Borrelia* spp. in the range of 82-328 µg/ml in the mixture. Moreover, the test mixture displayed statistically significant synergism against all pleomorphic forms of *Borrelia burgdorferi* s. and *Borrelia garinii*. By using high throughput spectrofluoroscropy supported by direct counting and fluorescence microscopy, we confirmed that exposure of tested *Borrelia* spp. to 82 µg/ml (i.e., baicalein 18.8 µg/ml, luteolin 15.6 µg/ml, rosmarinic acid 18.8 µg/ml, monolaurin 12.5 µg/ml, 10-CDA 15.6 µg/ml, iodine 0.625 µg/ml) concentration of this phytochemical mixture lead to decreased viability of both active and persistent forms [16,25,26,40,41]. Similarly, as recently reported, a 30 µg/ml triple combination of antibiotics (daptomycin, doxycycline, and cefoperazone) was effective against spirochetes and persisters, in contrast to 82 µg/ml doxycycline acting only against spirochetes. The sub-culturing study where we tried to regrow the active forms of *Borrelia* spp. from their persistent forms resulted in the presence of ~ 12% of regrown viable cells. The triple combination of the antibiotics showed to have ~ 10% regrowth rates, and doxycycline ~ 50% regrowth rates. Consistent data were obtained when two methodologies, i.e., a quantitative method developed by Feng et al. as well as one reported by Sapi, *et al.*, were utilized [16,40]. This result corroborates the studies of other research groups that observed the same pattern [17,25,41]. In addition, reported lower and higher concentrations of doxycycline than the one used here also do not exhibit efficacy against latent forms of *Borrelia* sp. but only typical motile spirochetes [16,17,25,26,32,41]. Efficacy against persistent forms was achieved only when the combination of the three different antibiotics was applied, however, this treatment was still ineffective against biofilm-like aggregates of *Borrelia* sp. [25,41]. Our composition revealed ~ 45-55% efficacy in eliminating *Borrelia*'s aggregates with ~ 70% killing effect. Biofilm-like aggregates is the most difficult structure to eliminate due

**Table 1:** Synergistic effect of the test mixture against pleomorphic forms of *Borrelia* spp.

Morphological Form	<i>Borrelia burgdorferi</i> s.s.				<i>Borrelia garinii</i>			
	Observed Value	Expected Value	P value	Type of Cooperation	Observed Value	Expected Value	P value	Type of Cooperation
Spirochetes	95.6a	51.2	<0.001	S	96.8b	58.3	<0.001	S
Persisters	90.1c	58.8	<0.001	S	89.9d	61.4	<0.001	S
Biofilm-like aggregates	55.2e	43.1	<0.05	S	50.6f	41.5	<0.05	S

to its protective function, thus its development by *Borrelia* limits penetration, accessibility, and action of a natural or synthetic agent. Alternatively, compounds and their derivatives that do not induce but target latent persistent forms might improve prospects for a combination treatment to combat the bacteria causing Lyme disease.

Toward this, the exact mechanisms underlying the pleotropic anti-*Borreliae* effects of the tested combination of phytochemicals merit further investigation. However, our study sheds new light on this subject by demonstrating low redox potency of this mixture, which could explain a lack of observed lipid peroxidation, and increased release of intracellular constituents. It was shown that antioxidant polyphenols such as baicalein and luteolin affect the structure and possibly permeabilized the membrane of bacteria, allowing higher concentrations of these compounds to access cytoplasmic space affecting structures and function of proteins and nucleic acids [49-52]. Rosmarinic acid, a well-known antioxidant, also demonstrated similar activity [53,54]. Likewise, fatty acids being inert but hydrophobic agents can resemble action of detergents by solubilizing the lipids and phospholipids causing the membrane's disintegration and disruption [55-58]. The loss of osmotic protection may lead to disturbed cell division and death. On the other hand, iodine, which is known as pro-oxidant, can rapidly penetrate into microorganisms and attack, in particular, the free-sulfur amino acids, nucleotides, and fatty acids. This action culminates in cell death but also destabilizes membrane fatty acids by reacting with unsaturated carbon bonds, and interacts preferentially with the proteins of the cytoplasm membrane [36,58]. However, we did not observe a damaging effect of our composition of phytochemicals on *Borrelia's* DNA, which further points to proteins as its primary target, although determining the effect on lipids still cannot be excluded. It appears that pro-oxidant and anti-oxidant properties of individual components in the mixture shifted cumulatively towards more anti-oxidative efficacy. The anti-oxidative property of this composition could be considered as beneficial, especially in view of the reported data about the increased oxidative stress in Lyme patients leading to depolarization of the mitochondrial membrane, disruption of homeostasis, and a release of pro-inflammatory cytokines [59]. The basis for observed efficacy of the test mixture towards biofilm-like aggregates is still elusive. However, it may be suggested by some hydrophobicity changes on their bacterial membrane and increased penetration by the action of 10-CDA that was shown to facilitate dispersal of biofilms by inducing cell transition from a biofilm to a

planktonic (free-swimming) phenotype [37,60].

Finally, the multiple anti-*Borreliae* efficacy of this novel composition of phytochemicals also includes its anti-inflammatory effect. With our test mixture, we were able to significantly inhibit the release of pro-inflammatory cytokines IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6, and observed a reducing release trend with IL-10, TNF- $\alpha$ , and GM-CSF by human CD14<sup>+</sup> monocytes. These cells were shown to be the first among innate immune cells to be activated in LD, which is attributed to the interaction of the membrane lipoproteins of *Borrelia* sp. with CD14 and/or TLR1/2 on the surface of monocyclic cells, and as being a main source of pro-inflammatory responses upon *Borrelia's* stimulation [61-63]. Whether or not it is beneficial in a human host warrants additional study.

## Conclusion

Collectively, our combination of six phytochemicals was effective against pleomorphic forms of *Borrelia burgdorferi* s. and *Borrelia garinii* and showed anti-inflammatory properties, which demonstrate its potential for the management of LD infection. Although the effective doses of individual compounds in this anti-*Borreliae* combination were mineralized through their synergistic interactions, the possibility exists that this synergy might exert some toxicity against human cells. Nonetheless, the data presented here, demonstrate that our approach may represent a novel valuable direction or adjuvant option for antimicrobial chemotherapy. Further steps are in progress to demonstrate the efficacy of this combination *in vivo* and in human studies.

## Declarations

### Ethics approval and consent to participate

Not applicable. The article does not include any animal or human data or tissue.

### Consent for publication

Not applicable. The article does not include a clinical study.

### Availability of data and material

All data are fully available from the corresponding author(s) on reasonable request.

### Competing interests

Dr. Niedzwiecki is a member of the Dr. Rath Health Foundation and receives no revenues from it. The founding sponsors had no role in the study design, performance, data collection and analysis, decision to

publish, or preparation/writing of the manuscript. No conflict of interest declared.

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## Authors' contributions

A.G. conceived, designed, and performed the experiments, analyzed data, wrote the paper and had primary responsibility for final content; A.N. and M.R. conceived, wrote the paper and had primary responsibility for final content. All authors read and approved the final manuscript.

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