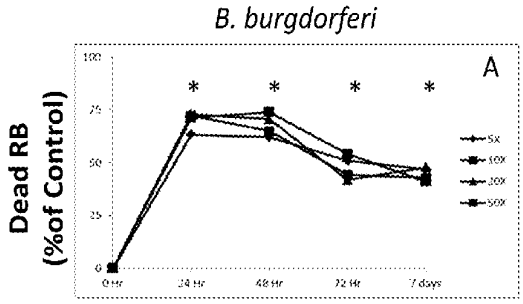


FIGURE 12 A

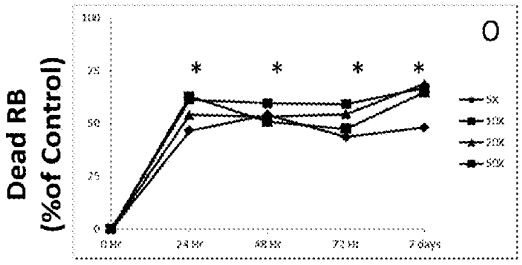


FIGURE 12 B

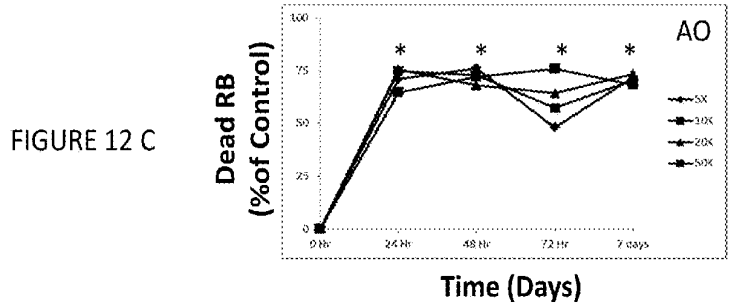


FIGURE 12 C

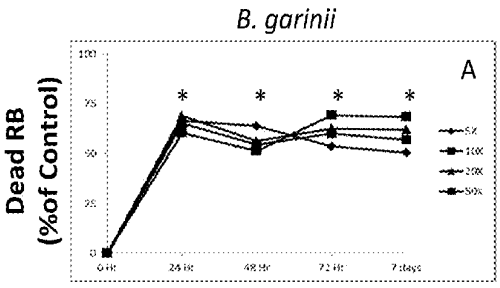


FIGURE 13 A

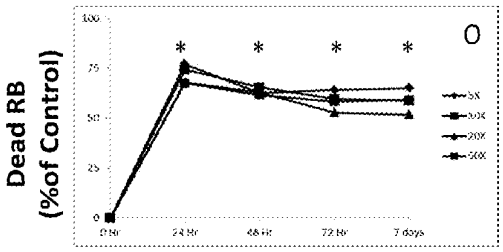


FIGURE 13 B

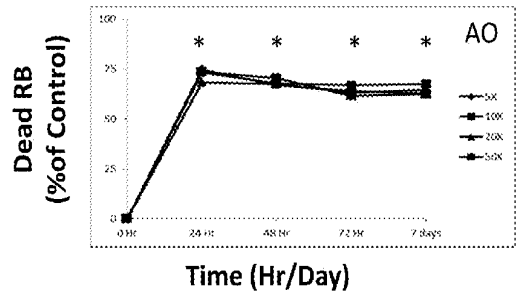


FIGURE 13 C

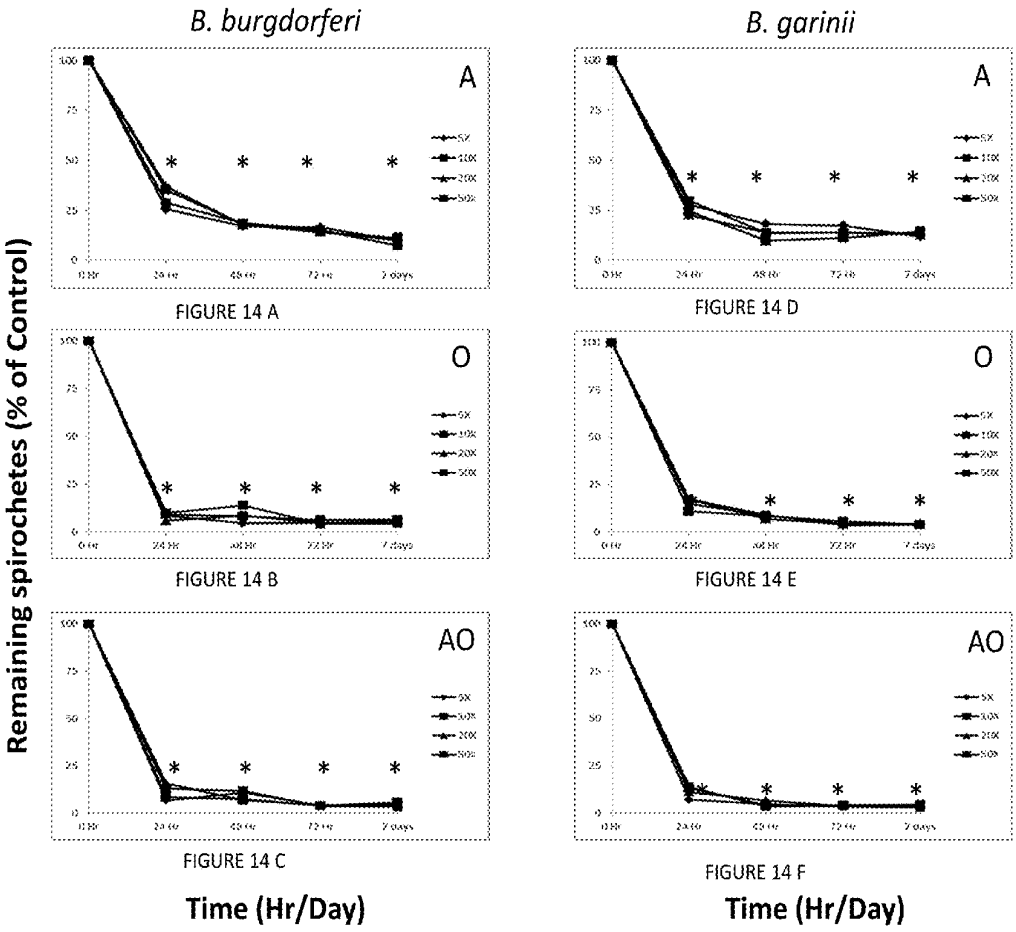


FIGURE 14 A

FIGURE 14 D

FIGURE 14 B

FIGURE 14 E

FIGURE 14 C

FIGURE 14 F

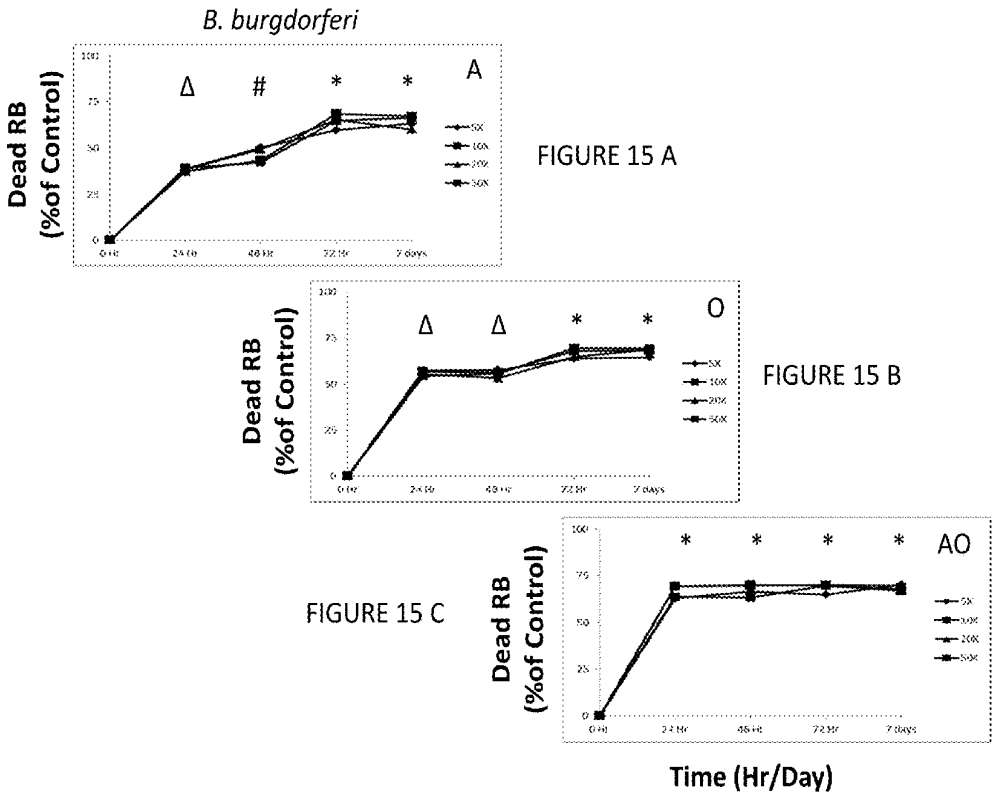


FIGURE 15 A

FIGURE 15 B

FIGURE 15 C

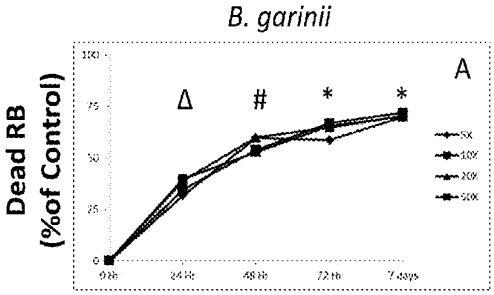


FIGURE 16 A

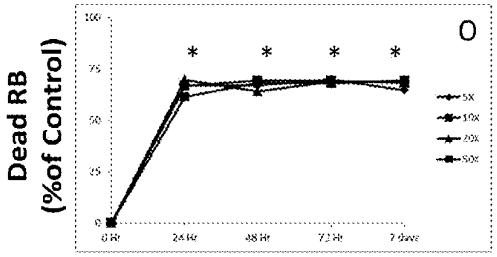
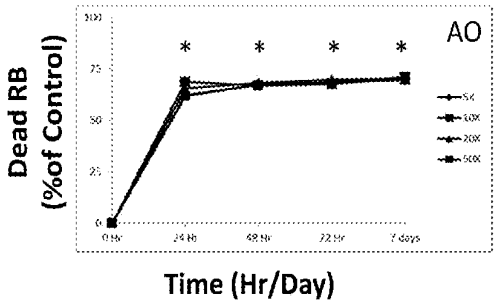


FIGURE 16 B

FIGURE 16 C



NOVEL COMPOSITION METHOD OF USING THE SAME FOR THE TREATMENT OF LYME DISEASE

FIELD OF TECHNOLOGY

[0001] This disclosure relates generally to a novel composition and the use of novel composition to treat Lyme disease.

BACKGROUND

[0002] Lyme disease is the most common tick-borne illness in the world today. *Borrelia burgdorferi* and *Borrelia garinii* are bacteria transmitted by ticks and cause Lyme disease. *Borrelia garinii* has only been found in ticks in Eurasia, while *Borrelia burgdorferi* is found in North America and Eurasia. The Center for Disease Control (CDC) states that there may be more than 300000 cases and as high as one million cases per year in United States alone (Stricker et al. 2014). Stricker (2014) further states that Lyme disease is approaching an epidemic proportion and the monotherapy of using antibiotics may not be sufficient. Stricher et al. (2011) states that chronic Lyme disease involves latent forms such as rounded bodies and biofilm formation. This publication further emphasizes that there is a need to develop new drugs to target these novel infectious processes. Development of agents other than antibiotics that would not cause bacterial resistance, but effectively target spirochetes, rounded forms and biofilms may also provide valuable insight into the treatment of other chronic infections, not only Lyme disease. There is a need to find more effective drugs for use as a treatment of Lyme disease.

SUMMARY

[0003] The current invention discloses a method of making and using a novel composition of vitamins and other plant-derived bioactive compounds (phytobiologicals) and using the same to treat Lyme disease. In one embodiment, several compounds in permutation and combination were made and tested for bactericidal and bacteriostatic effect against *Borrelia burgdorferi* and *Borrelia garinii*. In another embodiment, the individual compounds were, but not limited to, Vitamin D3, Vitamin C, L-lysin, Tranexamic acid, Quercetin 3D, Hydroxytyrosol, Fulvic acid, Teasel Root Extract, Cis-2 decenoic acid, Serrapeptase, Trimesic acid, Aminocaproic acid, Defferoxamine, Ellagic acid, Oregano oil, Oleuropein, Apigenin, Luteolin, Kelp (Kelp (Iodine)), Rottlerin, Grape seed extract (OPC), Malvidin, Piceatannol, *Aronia*, Myricetin, Rosmarinic acid, Kaempherol, Baicalein, Monolaurin, E-viniferin, Olein, Fucoinad, Nordihydroguaiaretic acid, Morin, Fisetin and Vitamin B complex.

[0004] In one embodiment, several Mixes were made and tested to find the optimum Mix having the best bactericidal and bacteriostatic effect on specific species of bacteria that caused Lyme disease. The Mixes were as following:

Mix A: Vitamin D3, Cis-2-decenoic acid, Kelp (Iodine), Monolaurin, Luteolin, Rosmarinic acid.

Mix A1: Vitamin D3, Cis-2-decenoic acid, Kelp (Iodine), Monolaurin, Serrapeptase, Luteolin, Rosmarinic acid.

Mix B: Hydroxytyroslo, Morin, Oenin, E-Viniferin, Baicalein.

[0005] Mix C: Vitamin D3, Cis-2-decenoic acid, Kelp (Iodine), Monolaurin, Serrapeptase, Luteolin, Rosmarinic acid, Hydroxytyrosol, Morin, Oenin, E-Viniferin, Baicalein.

Mix D: Vitamin D3, Cis-2-decenoic acid, Kelp (Iodine), Monolaurin, Luteolin, Hydroxytyrosol.

Mix E: Vitamin D3, Vitamin C, Cis-2-decenoic acid, Kelp (Iodine), Monolaurin, Luteolin, Hydroxytyrosol.

Mix F: Vitamin D3, Cis-2-decenoic acid, Kelp (Iodine), Monolaurin, Luteolin, Hydroxytyrosol, Rosmarinic acid.

Mix G: Vitamin D3, Vitamin C, Cis-2-decenoic acid, Kelp (Iodine), Monolaurin, Luteolin, Hydroxytyrosol, Rosmarinic acid.

Mix H: Vitamin D3, Vitamin C, Cis-2-decenoic acid, Kelp (Iodine), Monolaurin, Luteolin, Hydroxytyrosol, Rosmarinic acid, Baicalein.

Mix I: Vitamin D3, Vitamin C, Cis-2-decenoic acid, Kelp (Iodine), Monolaurin, Luteolin, Hydroxytyrosol, Rosmarinic acid, Baicalein, Oenin.

Mix J: Vitamin B complex, Vitamin C, Cis-2-decenoic acid, Monolaurin.

Mix K: Vitamin B complex, Vitamin C, Kelp (Iodine), Monolaurin, Luteolin.

Mix L: Vitamin B complex, Vitamin C, Kelp (Iodine), Monolaurin, Oenin.

Mix M: Vitamin B complex, Vitamin C, Kelp (Iodine), Monolaurin, Rosmarinic acid.

[0006] Mix N: Vitamin B complex, Vitamin C, Kelp (Iodine), Monolaurin, Baicalein.

Mix O: Vitamin B complex, Vitamin C, Kelp (Iodine), Monolaurin, Rosmarinic acid, Baicalein.

Mix P: Vitamin B complex, Vitamin C, Hydroxytyrosol, Monolaurin, Rosmarinic acid, Baicalein, Luteolin.

Mix AO: Vitamin D3, Cis-2-decenoic acid, Kelp (Iodine), Monolaurin, Luteolin, Rosmarinic acid, Vitamin B complex, Vitamin C and Baicalein.

[0007] In one embodiment, bacteria that cause Lyme disease were selected. The selected species were *Borrelia burgdorferi* and *Borrelia garinii*. In another embodiment, these bacteria were grown individually as a biofilms to test the efficacy of the Mix A-P. In another embodiment, series of studies were performed to test the efficacy of the Mixes for bactericidal and bacteriostatic effects at their different concentrations.

[0008] In one embodiment, the best Mixes were used over a period of time to determine as a repeated treatment method in a short and long term resistance study for these Mixes to prevent the growth of the bacteria.

[0009] In one embodiment, three morphological forms of *B. burgdorferi* and *B. garinii* (spirochetes, rounded bodies, and biofilm) were subjected to testing and analysis. Since *Borrelia* sp. are aero-tolerant anaerobes thus they were cultured stationary in the presence of 5% CO_2 in tightly screw-caped tubes.

[0010] In another embodiment, the first set of experiments was focused on establishing what compound/mixture will inhibit growth of *B. burgdorferi* and *B. garinii*. In another embodiment, a set of experiments was intended to check what compound/mixture will express the bactericidal effect on *B. burgdorferi* and *B. garinii*. In another embodiment, a set of experiments was design to evaluate what compound/mixture will reveal bacteriostatic and/or bactericidal effect against biofilms of *B. burgdorferi* and *B. garinii*. In another embodiment, a set of experiments was performed to establish the minimal concentration of compound/mixture that expresses the bacteriostatic and/or bactericidal effect on *B. burgdorferi* and *B. garinii*. As another embodiment, a set of experiments was carried out to check whether tested mixtures and at what

concentrations will cause *B. burgdorferi* and *B. garinii* to become resistant after short and long-period of undergone treatment.

[0011] In one embodiment, a novel composition is being proposed for the treatment of Lyme disease. A treatment method with a novel compound for treating all three forms of the bacteria (once infected is found in the blood stream) is being treated simultaneously in the cells. In one embodiment, a superior treatment effect is observed either alone or in conjunction with regular antibiotic is shown. In one embodiment, a kit and a pharmaceutical composition is also disclosed with Mix A to P, AO.

[0012] In one embodiment, a method, comprises of making a mixture at a specific concentration using at least one of a vitamin, a small chain fatty acid signaling molecule, an essential amino acid, a Lauric acid derivative, a flavonoid, a phenol, a plant extract, and a mineral; and treating of a Lyme disease caused by a specific bacteria using the mixture and preventing progression of the disease from the acute stage to the chronic stage, wherein the preventing progression of the Lyme disease is the advancement of the Lyme disease from spirochete stage to a rounded bodies and further to the formation of a biofilm.

[0013] The composition, method, and treatment disclosed herein may be implemented in any means for achieving various aspects, and may be executed in a form suitable for the mammal.

BRIEF DESCRIPTION OF DRAWINGS

[0014] Example embodiments are illustrated by way of example and not limitation in the Figures of the accompanying drawings, in which like references indicate similar elements and in which:

[0015] FIG. **1** A shows susceptibility of the spirochetes of *B. burgdorferi* (left panel) and FIG. **1** B *B. garinii* (right panel) to the most effective concentration of the chosen three most effective mixtures.

[0016] FIG. **2** A shows susceptibility of the spirochetes of *B. burgdorferi* (left panel) and FIG. **2** B *B. garinii* (right panel) to doxycycline.

[0017] FIG. **3**A shows susceptibility of the rounded forms of *B. burgdorferi* (left panel) and FIG. **3**B *B. garinii* (right panel) to the most effective concentration of the chosen three most effective mixtures.

[0018] FIG. **4** A shows susceptibility of the rounded forms of *B. burgdorferi* (upper panel) and FIG. **4** B *B. garinii* (lower panel) to doxycycline.

[0019] FIGS. **5** A, B, C, D and E shows analysis of two *Borrelia* species on biofilm and their treatment effect with Mix's as therapy.

[0020] FIGS. **6** A, B, C, D, and E shows analysis of two *Borrelia* species on biofilm and their treatment effect with Mixes's as prevention.

[0021] FIGS. **7** A and B shows analysis of two *Borrelia* species on biofilm and their treatment effect with doxycycline.

[0022] FIGS. **8** A, B, C, D, E and F shows analysis of spirochetes of two *Borrelia* species treated with different concentrations of the chosen three most effective mixtures.

[0023] FIGS. **9** A, B and C shows susceptibility of the rounded forms of *B. burgdorferi* to the different concentrations of the chosen three most effective mixtures.

[0024] FIGS. **10** A, B and C shows susceptibility of the rounded forms of *B. garinii* to the different concentrations of the chosen three most effective mixtures.

[0025] FIGS. **11** A, B, C,D, E and F shows susceptibility of the spirochetes of *B. burgdorferi* (left panel) and *B. garinii* (right panel) treated with different concentrations of the chosen three most effective mixtures for a short term resistance.

[0026] FIGS. **12** A, B and C shows susceptibility of the rounded forms of *B. burgdorferi* treated with different concentrations of the chosen three most effective mixtures for a short term resistance.

[0027] FIGS. **13** A, B and C shows susceptibility of the rounded forms of *B. garinii* to the different concentrations of the chosen three most effective mixtures for a short term resistance.

[0028] FIGS. **14** A, B, C,D, E and F shows susceptibility of the spirochetes of *B. burgdorferi* (left panel) and *B. garinii* (right panel) treated with different concentrations of the chosen three most effective mixtures for a long term resistance.

[0029] FIGS. **15** A, B and C shows susceptibility of the rounded forms of *B. burgdorferi* treated with different concentrations for a long term resistance.

[0030] FIGS. **16** A, B and C shows susceptibility of the rounded forms of *B. garinii* to the different concentrations of the chosen three most effective mixtures at different concentration for a long term resistance.

[0031] Other features of the present embodiments will be apparent from the accompanying drawings and from the detailed description that follows.

DETAILED DESCRIPTION

[0032] In the instant disclosure a novel composition of natural and synthetic compounds were tested and used for bactericidal and bacteriostatic effect on three morphological forms of *B. burgdorferi* and *B. garinii* (spirochetes, rounded bodies, and biofilm) as a treatment for Lyme disease. Since *Borrelia* sp. are aero-tolerant anaerobes thus they were cultured stationary in the presence of 5% CO_2 in tightly screw-caped tubes. The first set of experiments was focused on establishing what compound/mixture will inhibit growth of *B. burgdorferi* and *B. garinii*. Second set of experiments was intended to check what compound/mixture will express the bactericidal effect on *B. burgdorferi* and *B. garinii*. The third set of experiments was design to evaluate what compound/mixture will reveal bacteriostatic and/or bactericidal effect against biofilms of *B. burgdorferi* and *B. garinii*. The fourth set of experiments was performed to establish the minimal concentration of compound/mixture that expresses the bacteriostatic and/or bactericidal effect on *B. burgdorferi* and *B. garinii*. The last set of experiments was carried out to check whether tested mixtures and at what concentrations will cause *B. burgdorferi* and *B. garinii* to become resistant after short and long-period of undergone treatment. The accompanying tables and figures show the results of various sets of experiments and prove that the composition and the method of treatment is very effective in short and long term for all forms of *B. burgdorferi* and *B. garinii*.

Materials and Methods

[0033] Test Compounds:

[0034] The following compounds, with the purity between 90%-98% according to the manufacturer, were obtained from Sigma (St. Louis, Mo.): Vitamin D3, Vitamin C, L-Lysin,

Tranexamic acid, Quercetin 3D, Deferoxamine, Fucoidan, c-Viniferin, Ellagic acid, Hydroxytyrosol, Baicalein, cis-2-decenoic acid, Morin, Oenin, Oleuropein, Nordihydroguaiaretic acid, Myricetin, Malvidin, and as a positive control antibiotic doxycycline (100 mg/ml stock suspension in absolute ethanol) since it is use as a standard therapeutic treatment for patients with Lyme disease. The following compounds, with the purity between 97%-99% according to the manufacturer, were purchased from Tocris Bioscience (Bristol, United Kingdom): Rosmarinic acid, Kaempferol, Piceatannol, Rottlerin, Grape seed extract (OPC), Luteolin, Fisetin, Apigenin. Other reagents used in this study were: organic kelp with standardized Kelp (Iodine) content (i.e. 150 μg/ml as a 100 of % Daily Value) purchased from World Organic Ltd., New Zealand), monolaurin (Lauricidin®) bought from Med-Chem Laboratories, Inc., Goodyear, Ariz. as a pure sn-1 monolaurin (glycerol monolaurate) derived from coconut oil, serrapeptase (SerraEzyme 80,000 Iu™) obtained from Good Health Naturally, USA with unspecified by manufacture's purity, vitamin B-complex "100" purchased from Solgar, Inc., NJ with unspecified by manufacture's purity, fulvic acid from Nano Health Solution Inc. Charlston N.C. with unspecified by manufacture's purity, organic teasel root extract in 45-55% of ethanol from Woodland Essence, Cold Brook, N.Y. with unspecified by manufacture's purity, aminocaprionic acid from Selleckchem.com Houston Tex. with unspecified by manufacture's purity, and oregano oil (with standardized i.e. 70% of carvacrol content) from VitaCost, Lexington, N.C. with unspecified by manufacture's purity.

[0035] Preparation of Test Compounds for Susceptibility Testing.

[0036] A stock solution of 50-100 mg/ml for solid compounds (depending on solubility of each substance) was prepared by suspending each of the test compounds in absolute ethanol. All stock solutions were stored in aluminum foil-wrapped tubes at −20° C. Since a high percentage of ethanol could be bactericidal, the amount of ethanol added to the growth medium was kept as low as possible in order to minimize the potential effect on growth of *Borrelia* sp. A preliminary experiment was carried out to determine the maximum percentage of ethanol which could be applied without growth inhibition of *Borrelia* sp. and was found to be 0.5% (vol/vol) (data not shown). The final concentration of ethanol present in the growth medium was kept below 0.4% (vol/vol). Each stock solution was serially diluted in 10% ethanol, and appropriate amount of each dilution was then added to 1.5 ml sterile screw-cap test tubes containing 1 ml of BSH complete medium to yield final concentrations of 100-1000 μg/ml for solid compounds. For doxycycline, as a positive control, the final used concentration was 250 μg/ml. As a negative control ethanol at 0.1-0.4% (vol/vol) was applied.

[0037] Test microorganisms. Two *Borrelia* species i.e. *Borrelia burgdorferi* and *Borrelia garinii* were tested in this study. Low passage isolates of the B31 strain of *B. burgdorferi* and CIP103362 strain of *B. garinii* were obtained from the American Type Culture Collection (Manassas, Va.). The stocks of both species were cultured in commonly used conditions, i.e. medium such as Barbour-Stoner-Kelly H (BSK-H) supplemented with 6% rabbit serum (Sigma, St Louis, Mo.) without antibiotics at 33° C. with 5% CO_2, in sterile screw-cap 15 mL polypropylene tubes with or without gentile shaking depends on type of experiment. B31 strain is an isolate from *Ixodes dammini* whereas CIP103362 strain is an isolate from *Ixodes ricinus*. Both strains are well known human pathogenic factors of Lyme disease. So far, *Borrelia* burgdoferi has been found in ticks from North America and Eurasia, while *Borrelia garinii* only in ticks in Eurasia.

[0038] Preparation of Test Microorganisms for Susceptibility Testing.

[0039] The strains of *B. burgdorferi* and *B. garinii* were prepared for testing as described by Sapi et al. Infection and Drug Resistance 2011. Briefly, the strains were activated from original cryobank vials and inoculated into 10 ml BKS-H compete medium, and maintained at 33° C. For generation of homogeneous cultures (i.e. having only spirochete form) of *B. burgdorferi* or *B. garinii*, spirochetes were inoculated and maintained in a shaking incubator at 33° C. and 250 rpm, where there is no biofilm formation (Sapi et al. Infection and Drug Resistance 2011 supported by own observation). For generation of biofilm-like colonies of *B. burgdorferi* or *B. garinii*, spirochetes were inoculated in four-well chambers (BD Biosciences, Sparks, Md.) coated with rat-tail collagen type I and incubated for 1 week without shaking.

[0040] Bacteriostatic Assessment of Test Compounds on Test Microorganisms.

[0041] To determine what compound/mixture will inhibit visible growth of *B. burgdorferi* and *B. garinii*, a new and accepted micro-dilution method was used according to Sapi et al. Infection and Drug Resistance 2011. Briefly, sterile 1.5 ml test tightly caps screwed tubes containing 1 ml BSK-H medium, supplemented with the test compound of interest were inoculated with 2×10^6 CFU/ml of the homogenous bacterial suspension. The tubes were then incubated at 33° C. and growth was monitored at regular intervals for up to 72 h. The whole experiment was repeated three times for each strain and each concentration. Control cultures were treated with ethanol (i.e. 0.1-0.4 vol/vol) alone. For doxycycline, the final used concentration was 250 μg/ml. Cell growth was assessed by a bacterial Petroff-Hausser counting chamber after the 0 hour, 24 hrs, 48 hrs, 72 hrs and 7 days of incubation using dark field microscopy (direct cell counting), as a standard procedure.

[0042] Evaluation of Bactericidal Effect of Test Compounds on Test Microorganisms.

[0043] To determine what compound/mixture will have the bactericidal effect a fluorescence method was used according to Sapi et al. Infection and Drug Resistance 2011. Briefly, 2×10^6 CFU/ml of the homogenous bacterial suspension was inoculated into each sterile 1.5 ml test tightly caps screwed tubes containing 1 ml BSK-H medium, supplemented with the test compound/mixture of interest. Control cultures were treated with ethanol (i.e. 0.1-0.4 vol/vol) alone. For doxycycline, as a positive control, the final used concentration was 250 μg/ml. The tubes were then incubated at 33° C. and viability was monitored at regular intervals for up to 72 h. The whole experiment was repeated three times for each strain and each concentration. The susceptibility of spirochetes and round body forms to the test compound was then assessed after 24 h, 48 h, 72 h and 7 days by LIVE/DEAD® BacLight™ Bacterial Viability Assay using fluorescent microscopy, were the ratio of live (green) and dead (red) *B. burgdorferi* and *B. garinii* morphological forms were calculated.

[0044] Valuation of Test Compounds on Bacterial Biofilm.

[0045] Qualitative effect of the test compounds against biofilm-like colonies of *B. burgdorferi* and *B. garinii* was evaluated using commonly used and well accepted crystal violet (CV) staining method, according to protocol provided by Sapi et al. Infection and Drug Resistance 2011. Briefly, for