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Original Article

In vitro and *in vivo* antimicrobial activity of an active plant-based quadrocomplex for skin hygiene

[Actividad antimicrobiana *in vitro* e *in vivo* de un preparado vegetal activo para la higiene de la piel]

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Abstract

Context: The current epidemiological situation causes a new surge of interest to perspective antimicrobial formulations for proper skin hygiene.

Aims: To evaluate *in vitro* and *in vivo* the antimicrobial activity of a novel active quadrocomplex (QC) for skin hygiene based on *Melaleuca alternifolia* essential oil, eucalyptol, (-)-α-bisabolol and silver citrate. In addition, to analyze the phytochemical constituents by gas chromatography-mass spectrometry (GC-MS) and to assess the skin irritant potential after regular washing.

Methods: The phytochemical analysis was performed using GC-MS. The Minimum Inhibitory Concentrations (MICs) were assessed using a colony-counting method with resazurin. The type of pharmacological interaction was investigated using a modern checkerboard assay.

Results: The chemical composition exhibited 18 resolved phytochemicals with the highest concentrations for (-)-α-bisabolol (32.2%) and terpinen-4-ol (31.6%) through the GC-MS analysis. QC agents showed antimicrobial activity against Gram-positive and Gram-negative strains, with MIC values ranging from 1.25 to 40.00 mg/mL. The checkerboard assay demonstrated reduced MIC values for the combinations of QC agents against all reference strains. QC showed significant inhibition of *Staphylococcus aureus* growth with an average efficiency of 99.91% and *Candida albicans* 99.94 %. *In vivo*, the investigation of QC showed higher immediate and prolonged efficiency compared to base formulation ($p<0.05$). Dermatology evaluation indicated that QC added to the soap base did not affect the skin of hands during regular hand-washing and did not cause any negative effects on the skin.

Conclusions: QC exhibits a balanced performance between antimicrobial activity and biological safety and can be considered a promising bioactive composition for regular hand hygiene application. Therefore, additional investigations are needed to study the mechanism of antimicrobial activity.

Keywords: antimicrobial agents; drug synergism; Gram-negative pathogens; Gram-positive pathogens; phytochemicals; skin microbiota.

Resumen

Contexto: La situación epidemiológica actual genera un nuevo interés por formular perspectivas antimicrobianas para una correcta higiene de la piel.

Objetivos: Evaluar *in vitro* e *in vivo* la actividad antimicrobiana de un novedoso quadrocomplejo (QC) activo para la higiene de la piel a base de aceite esencial de *Melaleuca alternifolia*, eucaliptol, (-)-α-bisabolol y citrato de plata. Además de analizar los constituyentes fitoquímicos por cromatografía de gases-espectrometría de masas (GC-MS) y evaluar el potencial irritante de la piel después del lavado regular.

Métodos: El análisis fitoquímico se realizó mediante GC-MS. Las concentraciones inhibitorias mínimas (CIM) se evaluaron utilizando un método de recuento de colonias con resazurina. El tipo de interacción farmacológica se investigó utilizando un ensayo de tablero de ajedrez moderno.

Resultados: La composición química mostró 18 fitoquímicos resueltos con las concentraciones más altas para (-)-α-bisabolol (32,2%) y terpinen-4-ol (31,6%) mediante el análisis GC-MS. Los agentes de control de calidad mostraron una actividad antimicrobiana contra las cepas Gram-positivas y Gram-negativas analizadas con valores de CIM que variaron de 1,25 a 40,00 mg/mL. El ensayo de tablero de ajedrez demostró valores de MIC reducidos para las combinaciones de agentes de control de calidad frente a todas las cepas de referencia. QC mostró una inhibición significativa del crecimiento de *Staphylococcus aureus* con una eficiencia promedio del 99,91% y *Candida albicans* 99,94 %. *In vivo*, la investigación de QC mostró una mayor eficacia inmediata y prolongada en comparación con la formulación base ($p<0,05$). La evaluación dermatológica indicó que QC agregado a la base de jabón no afectó la piel de las manos durante el lavado regular de manos y no causó ningún efecto negativo en la piel.

Conclusiones: QC exhibe un rendimiento equilibrado entre la actividad antimicrobiana y la seguridad biológica y puede considerarse una composición bioactiva prometedora para la aplicación regular de higiene de manos. Por lo tanto, se necesitan investigaciones adicionales para estudiar el mecanismo de la actividad antimicrobiana.

Palabras Clave: agentes antimicrobianos; fitoquímicos; microbiota de la piel; patógenos Gram-negativo; patógenos Gram-positivo; sinergismo de fármacos.

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Abbreviations: ATCC: American Type Culture Collection; CFU: Colony-forming units; EUCAST: Expert rules and expected phenotypes; FDA: Food and Drug Administration; FICI: Fractional inhibitory concentration index; GRAE: Generally recognized as effective; GRAS: Generally recognized as safe; MIC: Minimum Inhibitory Concentration; OTC: Over-the-counter; QC: Quadrocomplex; SB: Soap base; SSC: Stabilized silver complex; TTO: Tea tree essential oil.

INTRODUCTION

The emergence and spread of infectious diseases occurred regularly throughout history. In the fight against viruses and pathogenic bacteria, both non-pharmaceutical and pharmaceutical measures have been developed to prevent and reduce their exposure. Nevertheless, it is difficult to keep control in the battle against some of the infections. Diseases show a wider spread due to the acquisition of drug resistance, tolerance of potentially dangerous carriers of the pathogens to chemicals, poor sanitation, land use, climate changes, and increased human mobility and travel (Cutler et al., 2010).

There are no approaches to guarantee people full protection, but proper hand hygiene remains the first line of human defense against many diseases that burden global health (Mathur, 2011; WHO, 2009). Therapy against a certain pathogen may not be available for everyone and may require complicated schemes with antimicrobial or antiviral agents affecting the target and the human organism. In this view, hand washing is the simplest, most cost-effective, safe, and most available method helping to save millions of lives worldwide with proven effectiveness (Aiello et al., 2008; Burton et al., 2011; WHO, 2009). Numerous hand-washing and hand-treatment formulations have been developed to understand the importance of hand hygiene in preventing and reducing the spread of diseases (Jing et al., 2020).

The efficiency of the hand sanitization procedure depends on the potency of disinfecting agents. In 2013 Food and Drug Administration (FDA) gave a list of eligible antiseptic agents used in the non-prescription - over-the-counter (OTC) personal hand wash (FDA, 2016). Nineteen active ingredients were listed in the report: quaternary ammonium compounds (benzalkonium chloride, benzethonium chloride), chloroxylenol, cloflucarban, fluorosalan, hexylresorcinol, iodine complex (ammonium ether sulfate and polyoxyethylene sorbitan monolaurate), iodine complex (phosphate ester of alkylaryloxy polyethylene glycol), nonylphenoxypoly (ethyleneoxy) ethanoliodine, poloxamer-iodine complex, povidone-iodine 5%–10%, undecoylium chloride iodine complex, methylbenzethonium chloride, phenol (less, equal to or greater than 1.5%), secondary amyltricresols, sodium oxychlorosene, triclocarban, and triclosan. Nevertheless, in view of questionable effectiveness and safety, most of these ingredients were not included in FDA's GRAE (generally recognized as effective) and GRAS (generally recognized as safe) list for use as components in consumer antiseptic wash (FDA, 2016).

Attempts to replace FDA-banned agents such as triclosan led to increased exposure to such alternatives as benzalkonium chloride, benzethonium chloride, chloroxylenol, and chlorhexidine. Simultaneously, the rising incidence of allergic and irritant contact dermatitis was reported (Rundle et al., 2019).

The exact mechanisms leading to sensitization (instead of tolerance) to hand hygiene products are still being discussed. First, hand hygiene products may alter skin barrier integrity and function. Prolonged hand exposure to water and other chemical agents may induce several pathophysiologic changes, such as epidermal barrier disruption, impairment of keratinocytes, the subsequent release of proinflammatory cytokines, activation of the skin immune system, and delayed-type hypersensitivity reactions (Beiu et al., 2020; Skowron et al., 2021). Reported irritants include iodophors, antimicrobial soaps based on chlorhexidine, chloroxylenol, triclosan, detergents, alcohol-based products, and other additives in hand cleansing products (Beiu et al., 2020; Bissett, 2007; Skowron et al., 2021). The use of detergent-based substances leads to the highest rates of dermatitis by reducing moisture in the stratum corneum and stripping away protective lipids, thereby making skin more vulnerable to irritation (Chiller et al., 2001; Rundle et al., 2020; Sherwani et al., 2018; Wolfe et al., 2016).

On the other hand, a properly working skin microbiome provides effective allergy defense and determines good skin functioning (Sherwani et al., 2018; Skowron et al., 2021). Skin serves as a barrier that protects us against harmful microorganisms, and at the same time, it hosts a wide array of beneficial bacteria such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococcus* spp., *Propionibacterium* spp. and *Corynebacterium* spp. (Chiller et al., 2001; Cogen et al., 2008). Dysbiosis due to the long-term use of topical antimicrobials or frequent hand washing may lead to numerous systemic disorders in skin immune barrier functioning (Jing et al., 2020; Skowron et al., 2021). It also alters the proportions of organisms compared to the healthy skin microbiome and may trigger the pathogenic potential of the commensals (Skowron et al., 2021). Nevertheless, the switch can occur when the normal skin flora is disrupted: *S. epidermidis*, which usually colonizes human skin without any negative health effects, can cause serious illness, including atopic dermatitis and, as proposed, inflammation (Bjerre et al., 2021; Cau et al., 2021; Otto, 2009).

Table 1. Components of quadrocomplex.

No.	Chemical	Purity	Origin	CAS number	Manufacturer
1	Essential oil of *M. alternifolia*	>99.0%	Leaves of *Melaleuca alternifolia* (tea tree)	68647-73-4	Bernardi Group, France
2	Eucalyptol	>99.5%	Leaves of *Eucalyptus spp*.	470-82-6	Wuxi Lotus Essence Co., LTD, China
3	(-)-α-Bisabolol	>93.0%	Leaves of *Eremanthus erythropappus*	23089-26-1	Merck KGaA, Germany
4	Stabilized silver complex	Silver content >0.22%	Synthetic, aqueous solution of silver citrate and citric acid	126-45-4	Laboratorios Argenol S.L., Spain

The current epidemiological situation, the pandemic outbreak, causes a new surge of interest in perspective antiseptic formulations for proper hand hygiene. However, because of the data above, we need a new generation of active agents: safe, effective, eco-friendly, exhibiting neither sensitizing nor irritancy potential, and skin-friendly – not disrupting the skin microbiome and/or balancing its diversity.

This study describes a novel active complex for skin hygiene based on natural antimicrobial and anti-irritant compounds – *Melaleuca alternifolia* essential oil, eucalyptol, (-)-α-bisabolol, and silver citrate.

The essential oil of *M. alternifolia* (tea tree) (TTO) has a heterogeneous composition and broad-spectrum antimicrobial activity (Carson et al., 2002). The main compounds of TTO are terpinene-4-ol, terpinolene, α-terpinene, and 1,8-cineole. TTO has been reported to exhibit antibacterial, antifungal, antiviral, and anti-protozoal activity (Banes-Marshall et al., 2001; Carson et al., 2002; 2006; Carson and Riley, 1993; Hammer et al., 1996; Mikus et al., 2000; Nenoff et al., 1996). Numerous studies support the evidence attributing anti-inflammatory activity to TTO *in vitro* and *in vivo* (Caldefie-Chezet et al., 2004; Hart et al., 2000; Koh et al., 2002; Pearce et al., 2005).

Eucalyptol – 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo[2.2.2]acetate) – is a bicyclic terpenoid that is a component of essential oils obtained from various plant species (Mączka et al., 2021). The compound is known for its antiseptic, antimicrobial properties, and anti-inflammatory activity (Juergens et al., 2004; 2020; Maczka et al., 2021; Yadav and Chandra, 2017). 1,8-cineole has been shown to have both antibiotic properties and the ability to block receptors that receive signals from various autoinducers, thus affecting quorum sensing (Sybiya Vasantha Packiavathy et al., 2012). According to previous studies, 1,8-cineole can enhance the antimicrobial effect of antiseptics (Li et al., 2014; Simsek and Duman, 2017).

a-(-)-Bisabolol is a monocyclic sesquiterpene alcohol. This active ingredient is commonly used in dermatological and cosmetic formulations due to its antibacterial, anti-irritant, and anti-inflammatory properties in combination with GRAS status (Kamatou and Viljoen, 2010).

Silver ions are effective against a broad range of microorganisms (Woo et al., 2008). Today, silver ions are used as antimicrobial agents in various medical and nonmedical applications.

This study investigates the combined antibacterial activity of the designed quadrocomplex on skin residential microorganism and evaluates skin irritant potential after hand washing using healthy volunteers, in addition to determining the content of phytochemicals in the quadrocomplex.

MATERIAL AND METHODS

Chemicals and plant materials

The compounds selected for the study are presented in Table 1. *Melaleuca alternifolia* leaf essential oil (*Myrtaceae*), eucalyptol, α-(-)-bisabolol, and stabilized silver complex (SSC) were purchased from Sigma-Aldrich (Sigma Chemical Co, St. Louis, MO, USA) and were dealt with by standard methods before use.

Bioactive soap formulations

QC was prepared by mixing four ingredients including 0.3% TTO, 0.15% eucalyptol, 0.10% (-)-α-bisabolol, and 0.01% SSC in specific ratio 30:15:10:1, respectively. Final hand-washing products were obtained by mixing the quadrocomplex with soap base (SB) ingredients in concentrations shown in Table 2. This specific ratio was chosen in accordance with the previous determination of MIC and FICI values for combinations (Tables 7 and 8), *in vitro* assessment of the antimicrobial activity of QC in soap base (Table 10), the accelerated stability of soap formulations after thirty days following Russian General Pharmacopoeia Monograph 1.1.0009.15 (https://docs.rucml.ru/feml/pharma/v13/vol1/#200). All formulations of soap with antibacterial quadrocomplex (2, 3, 4, 5, and 6) were tested for activity and efficiency, as shown in Table 3.

Table 2. Composition of soap base.

No.	Substance	CAS number	Function	Ingredient quantity, %
1	Aqua	7732-18-5	Solvent	83.28
2	Sodium coco-sulfate	97375-27-4	Anionic surfactant	6.00
3	Coco-glucoside	110615-47-9, 68515-73-1	Nonionic surfactant	3.00
4	Glycerin	56-81-5	Cosolvent, humectant	2.95
5	Cocamidopropyl betaine	61789-40-0	Amphoteric surfactant	2.60
6	Sunfloweroyl methylglucamide	1591782-99-8	Nonionic surfactant	1.00
7	Lactic acid	50-21-5, 79-33-4	Regulator of pH, humectant	0.40
8	Benzyl alcohol	100-51-6	Preservative	0.37
9	Sodium chloride	7647-14-5	Regulator of viscosity	0.12
10	Tetrasodium glutamate diacetate	51981-21-6	Complexing agent	0.09
11	Benzoic acid	65-85-0	Preservative	0.06
12	Mentha piperita oil	8006-90-4, 84082-70-2	Essential oil, Fragrance	0.05
13	Dehydroacetic acid	520-45-6	Preservative	0.04
14	Gossypium herbaceum seed extract	223749-08-4	Skin conditioning	0.01
15	Potassium sorbate	24634-61-5, 590-00-1	Preservative from extract	0.01
16	Sodium benzoate	532-32-1	Preservative from extract	0.01
17	Sodium hydroxide	1310-73-2	Regulator of pH	0.01

Table 3. Formulations with antibacterial quadrocomplex.

No.	Formulation	Composition	Ingredient quantity of quadrocomplex, %
1	Soap base (SB)	Aqua, sodium coco-sulfate, coco-glucoside, glycerin, cocamidopropyl betaine, sunfloweroyl methylglucamide, lactic acid, benzyl alcohol, sodium chloride, tetrasodium glutamate diacetate, benzoic acid, mentha piperita oil, dehydroacetic acid, gossypium herbaceum seed extract, potassium sorbate, sodium benzoate, sodium hydroxide.	0.0
2	SB + QC	Soap base with TTO, eucalyptol, (-)-α-bisabolol, SSC	0.2
3	SB + QC	Soap base with TTO, eucalyptol, (-)-α-bisabolol, SSC	0.4
4	SB + QC	Soap base with TTO, eucalyptol, (-)-α-bisabolol, SSC	0.6
5	SB + QC	Soap base with TTO, eucalyptol, (-)-α-bisabolol, SSC	0.8
6	SB + QC	Soap base with TTO, eucalyptol, (-)-α-bisabolol, SSC	1.0

TTO: Tea tree essential oil; SSC: Stabilized silver complex.

Gas chromatography-mass spectrometry analysis

Screening for the presence of various phytochemicals was performed for both TTO and the mixture of TTO, eucalyptol, and (-)-α-bisabolol in a ratio 30:15:10, respectively, using GC-MS analysis.

In a preliminary test, 50 µL of the TTO sample was dissolved in 1 mL chloroform and analyzed with an Agilent Technologies 6890N gas chromatograph coupled with a 7000 mass spectrometer (Agilent, Palo Alto, CA, USA). The system was equipped with an HP-5 MS low bleed capillary column [20 m × 0.18 mm i.d., 0.18 µm film thickness (Agilent Technologies, CA, USA)]. During the analysis, an injection volume of 1 µL was subjected to a splitless mode, with helium used as the carrier medium at a constant flow rate of 1.25 mL/min. The oven temperature was maintained at 80°C for 1 min, then programmed to increase by 10°C/min to 140°C and 20°C/min to 280°C. The obtained compound profiles were identified by comparing the corresponding reference retention indices and mass spectra in databases (NIST-11, Willey-08, Adams libraries), as well as by comparing their mass spectra and linear retention indices (LRI) with published data (Babushok et al., 2011; Linstrom and Mallard, 2001; NIST, 2017; Sparkman, 2005).

The analysis of the mixture composition was performed in the same procedure with some modifications for the best possible separation of the components (Marriott et al., 2001). The system used was equipped with an HP-5 MS low bleed capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness

(Agilent Technologies, CA, USA)). During the analysis, an injection volume of 1 µL was subjected to a splitless mode, with helium used as the carrier medium at a constant flow rate of 1.25 mL/min. The oven temperature was maintained at 70°C for 1 min, then programmed to increase by 10°C/min to 290°C and held at this temperature for 20 min. The compound profiles were identified by comparing the corresponding reference retention indices and mass spectra in databases (NIST-14 MS Library).

In vitro antimicrobial activity of compounds

Test microorganisms and growth conditions

The components were tested against several bacterial strains. All the test microorganisms were sourced from ATCC. In the *in vitro* laboratory tests, there were investigated strains of three Gram-positive bacteria *Bacillus cereus* ATCC 10702, *Staphylococcus epidermidis* ATCC 14990, and *Staphylococcus aureus* ATCC 6538-P or ATCC 29213, two Gram-negative bacteria *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 9027, Gram-positive to Gram-variable bacterium *Micrococcus luteus* 10240a, and fungi *Candida albicans* ATCC 10231. None of them were multidrug-resistant. The species of the microorganisms were grown at 37°C for 24 h for bacteria and 48 h for fungi under aerobic conditions in Mueller Hinton broth, tryptone soy agar (TSA; Graso, Poland) (Adamczak et al., 2020; CLSI, 2002).

In the *in vitro* tests in health care facility [Moscow Regional Research and Clinical Institute ("MONIKI")], the clinical isolates of *S. aureus* and *E. coli* were used for the evaluation of quadrocomplex antibacterial activity. The isolates were grown at 37°C for 24 h in Mueller Hinton broth and tryptone soy agar (TSA; Graso, Poland). *Staphylococcus epidermidis* and *Micrococcus luteus* isolated from the hands of volunteers were also tested in a susceptibility assay according to the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases (EUCAST-ESCMID, 2000).

Determination of antibacterial activity by agar diffusion test

The antibacterial activity of the substances of plant origin was performed as described previously (Al-Zereini, 2014) and according to the Laboratory Standards Institute guidelines (CLSI) by agar diffusion test. The Gram-positive *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922 were used as test bacterial strains. An aliquot of an overnight culture of the test bacterial strain in nutrient broth (Oxoid, UK) was seeded on Muller Hinton agar plates (Oxoid, UK) at a final cell density of 10^6 bacterial cells/mL. The test was performed in triplicate assays, and results were presented as means ± SD.

In the Agar diffusion test, 1 and 5 µL/disc of substances of plant origin (in DMSO 1:1) were applied on sterile 6 mm blank discs that were placed on the top of bacterial-seeded agar plates. Also, QC was obtained after mixing TTO, eucalyptol, (-)-α-bisabolol, and SSC in equal proportion 1:1:1:1 by weight for the determination type of antibacterial activity to be compared with pure substances (Prabuseenivasan et al., 2006). A volume of 5 µL DMSO/disc and benzalkonium chloride (5 µL/disc) were used as negative and positive controls, respectively. Antibacterial activity was determined by plotting the preparations, where there were no visible growths on agar plates with proper medium and incubation for 24 h (Balouiri et al., 2016).

Determination of the minimum inhibitory concentration (MIC)

The micro-broth dilution assay was used to determine minimum inhibitory concentrations (MICs) of the bioactive compounds against the bacterial strains tested. Experiments were performed in 96-well microtiter plates (96 Well EDGE Cell Culture Plates, Nest Scientific Biotechnology, Wuxi, China) according to the EUCAST rules for antimicrobial susceptibility testing (Leclercq et al., 2013). The solubility of bioactive compounds in aqueous systems is low; therefore, polysorbate 20 at 0.3% was used for solubilization.

The concentrations of the compounds used for MICs determination were 20, 10, 5, 2.5, and 1.25 mg/mL. Benzalkonium chloride from 16 to 1 µg/mL served as control. In brief, 100 µL of two-fold dilutions of the agents were mixed with 100 µL of inoculum for each strain 1.0×10^6 CFU/mL and incubated at 37°C for 24 h. The wells containing bacterial inoculum without any agents and wells filled with culture media served as positive and negative controls, respectively (Balouiri et al., 2016).

The MICs of the agents tested were determined as their lowest concentration, for which no visible bacterial growth could be observed using the resazurin cell viability method. For the assay, 200 µL of bacterial suspension was mixed with 10 µL of 0.01% resazurin. Measurements were performed after 5-min incubation. The experiment was performed twice with three replicates.

Table 4. Combinations of the bioactive agents tested.

No.	Agent A	Agent B
1	Stabilized silver complex	Essential oil of *M. alternifolia*
2	Stabilized silver complex	Eucalyptol
3	Stabilized silver complex	(-)-α-Bisabolol
4	Essential oil of *M. alternifolia*	(-)-α-Bisabolol
5	Essential oil of *M. alternifolia*	Eucalyptol
6	Eucalyptol	(-)-α-Bisabolol

Assessment of bioactive compound interaction using checkerboard assay

The effects of the combinations of the bioactive agents against reference strains were evaluated by a checkerboard assay, similarly to the MICs testing, in 96-well microtiter plates (96 Well EDGE Cell Culture Plates, Nest Scientific Biotechnology, Wuxi, China). Each plate contained serial dilutions of A and B agents in a checkerboard fashion (Leclercq et al., 2013). The evaluation was performed for the combinations exhibited in Table 4.

The starting concentration for every agent was 4 × MIC. The solubility of bioactive compounds in aqueous systems is low; therefore, polysorbate 20 at 0.3% was used for solubilization. The 96-well microtiter plates (96 Well EDGE Cell Culture Plates, Nest Scientific Biotechnology, Wuxi, China) were incubated at 37°C for 20 h. Cell viability was determined using the resazurin method (Bouhdid et al., 2009; Castilho et al., 2015). To assess the interaction of bioactive compounds, the fractional inhibitory concentration index (FICI) for each double combination was calculated using the following equation [1]:

$$FICI_{A/B} = \frac{MIC_{[A]\text{ combination with }[B]}}{MIC_{[A]}} + \frac{MIC_{[B]\text{ combination with }[A]}}{MIC_{[B]}} \quad [1]$$

The FICIs were counted from the concentrations in the first non-turbid well found in each row and column along the turbidity/non-turbidity interface, and the lowest FICI value was used to characterize the synergy. The estimated FICI values for combinations were interpreted as follows: synergistic effect, FICI ≤ 0.5; additive effect or indifference, 0.5 < FICI > 4.0; and antagonistic effect, FICI ≥ 4.0 (Den Hollander et al., 1998; Odds, 2003).

Antimicrobial susceptibility of laboratory strains in vitro

The colony-counting method assessed the antimicrobial susceptibility of laboratory strains in the presence and absence of QC. *In vitro* antimicrobial susceptibility testing was performed using the modified serial dilution technique (Sharafutdinov et al., 2017). The tubes containing 1.8 mL of each liquid soap, were inoculated with 0.2 mL of bacterial suspension (1 × 10^9 CFU/mL) of *E. coli*, *B. cereus*, *S. aureus*, *P. aeruginosa* and *C. albicans*. The stock solution of each soap was not diluted.

The mixtures were incubated for 30 min at 37°C. Then, 1 mL of each sample was transferred to a tube containing 9 mL of 0.9% saline solution. After 15 min incubation, the inoculum was serially diluted to 1 × 10^3 CFU/mL with sterile 0.9% saline solution. 0.1 mL of each tested microbial suspension was transferred onto the surface of the agar plates. The plates were incubated at 37°C, and the bacterial concentration (CFU/mL) was calculated after 24 h of incubation for bacteria and 48 h for fungi (Adamczak et al., 2020; CLSI, 2002). The experiments were carried out in triplicate.

Efficacy evaluation of the formulations in vitro using clinical isolates

The antimicrobial efficacy of formulations with QC or without QC against the clinical isolates of *S. aureus* and *E. coli* was assessed using two *in vitro* methods (Balouiri et al., 2016): (i) determining bacterial concentration by the colony-counting method, and (ii) determining cell viability by the resazurin cell viability method.

The colony-counting method was performed as described above with minor modifications (CLSI, 2012). After 10 min incubation, the prepared inoculum was serially diluted to 1 × 10^5 CFU/mL with sterile 0.9% saline solution. 0.1 mL of each tested microbial suspension was transferred onto the surface of Mueller-Hinton agar. The bacterial concentration (CFU/mL) was calculated after 24 h incubation at 37°C. The experiments were carried out in triplicate.

In the resazurin cell viability method (Bouhdid et al., 2009; Castilho et al., 2015), 50 µL of the bacterial suspension (1×10^6 CFU/mL) was placed into each well of black-opaque 96-well plates (96 Well EDGE Cell Culture Plates, Nest Scientific Biotechnology, Wuxi, China). A volume of 50 µL of test samples was added to each well. The samples were incubated at

37°C in the dark for 24 h. The ability of bacteria to reduce resazurin, added at a concentration of 0.02% into fluorescent resorufin, was measured by spectrofluorimetry using xMarkTM Microplate Spectrophotometer (Bio-Rad Laboratories, Inc., CA, USA). Resazurin exhibits an absorption peak at 610 nm, while resorufin at 575 nm. Absorbance at a wavelength of 610 nm was detected. Three independent experiments were performed.

Efficacy evaluation of formulations employing human volunteer subjects

The prospective randomized placebo-controlled clinical study was conducted in accordance with the ethical principles for medical research outlined in the Declaration of Helsinki 1964 and approved by the local Ethics Committee (protocol code №1-09-20, September 1st, 2020) for studies involving healthy volunteers. The clinical study was conducted by the Moscow Regional Research and Clinical Institute ("MONIKI"). A written informed consent was obtained from all participants involved in the clinical study.

Forty adult volunteers were recruited to participate in the test at random. Eligible volunteers had healthy skin and no cuts, wounds, or other disorders on their hands. During 21 day-study, all participants were asked to wash their hands with warm water and 1.5 mL of test soap for 30 s less frequently than four times a day. The test group of 20 volunteers used liquid soap with QC for home hand-washing. The control group consisted of 20 volunteers who washed their hands with liquid soap without QC.

Samples from the human volunteers were collected by hand-washing with a sterile swab for 30 s before soap testing, after 1, 2, and 3 h from the beginning of the experiment, on 7th and 21st days of soap use. The swab specimens were placed in Stuart transport media and transferred to the research laboratory within an hour. The specimens were inoculated onto selective differential agar medium, including meat-peptone agar, tryptone soya agar, Endo agar, and Sabouraud agar. Carbohydrate utilization tests were performed using Giss' media. The isolates were identified by standard microbiological techniques such as colony morphology, microscopic features, and biochemical properties (Ferone et al., 2020; Gilligan, 2013).

A volunteer survey was performed as a questionnaire that asked individuals about their opinion concerning the smell, consistency, cleaning ability, and feeling after hand-washing with test soaps. All participants were examined by a dermatologist on the presence of the signs of skin dryness, irritation, allergy-like and other skin negative reactions associated with hand-washing on the hands.

Statistical analysis

The experimental data were expressed as mean value ± standard deviation (SD) calculated from three parallel experiments. The statistical analysis was performed by one-way ANOVA using Microsoft Excel (version 2016). Statistical analysis involving the Student's t-test was implemented using Statistica software (version 9.0, StatSoft, Tulsa, OK). Differences described by $p \leq 0.05$ were considered statistically significant. The results are presented as mean ± SD.

RESULTS AND DISCUSSION

Ideal antimicrobial products for hand hygiene should not only protect consumers from potentially harmful organisms but also not cause any negative effects. We need to consider the inherent risks in both the chemical exposure health risks of consumers and the potential increase in antibiotic-resistant pathogens in the environment. As reported, prolonged use of antibacterial soaps could disrupt the normal skin flora, developing allergic reactions to the skin and harming body tissues. On the other hand, the overuse of antimicrobial soaps may lead to developing resistance to antimicrobial agents. Several studies have purported to show a relationship between the use of triclosan (or other antimicrobial soaps) and antibiotic resistance (Mwambete and Lyombe, 2011; White and McDermott, 2001). The selection of clinically resistant microbial strains due to the overuse of antimicrobial agents in consumer products, in turn, could exacerbate the problem and make the treatment of infections even more difficult (Mwambete and Lyombe, 2011).

As the picture is much more complex than finding a certain antibacterial agent for hand hygiene able to kill all microorganisms, and actually no agent is capable of killing all of them, the present research was carried out to develop a novel active quadrocomplex for hand hygiene based on natural antimicrobial and anti-irritant compounds – *M. alternifolia* essential oil, eucalyptol, (-)-α-bisabolol and silver citrate. The current study is focused on the identification of key QC phytochemicals, the evaluation of antimicrobial activity *in vitro* and *in vivo*, the investigation of the interaction between components, and the assessment of QC skin effects during regular hand-washing application.

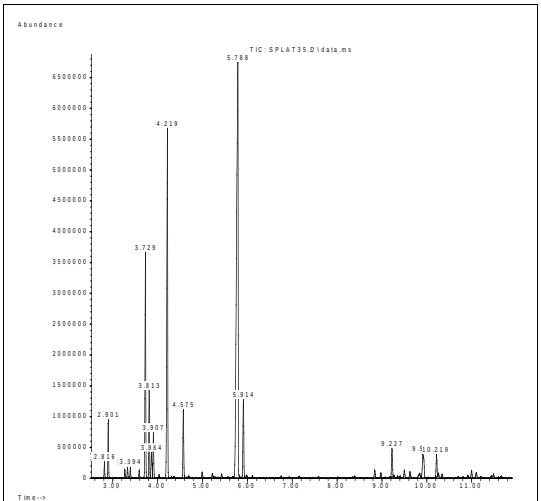

Figure 1. GC-MS chromatogram of TTO

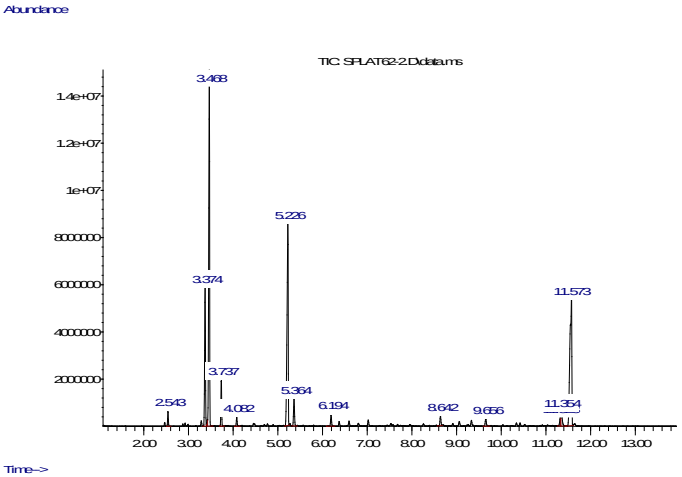

Figure 2. GC-MS chromatogram of QC.

Table 5. Gas Chromatography-Mass Spectrometry analysis of tea tree oil.

No.	Retention time	Compound
1	2.816	Thujene
2	2.901	α-Pinene
3	3.331	β-Pinene
4	3.394	Myrcene
5	3.729	α-Terpinene
6	3.813	o-Cymene
7	3.864	D-Limonene
8	3.907	Eucalyptol
9	4.219	γ-Terpinene
10	4.575	Terpinolene
11	5.788	Terpinen-4-ol
12	5.914	α-Terpineol
13	9.227	Aromadendrene
14	9.914	Ledene
15	10.219	δ-Cadinene

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of phytochemicals from TTO and QC

The GC–MS chromatogram of TTO, a key phytochemical of quadrocomplex, recorded 15 peaks corresponding to bioactive compounds, which were recognized by comparing its mass spectra along with their analogs reported in the NIST-11 and Willey-08 libraries (Fig. 1).

The major components present in TTO were thujene, α-pinene, β-pinene, myrcene, α-terpinene, o-cymene, D- limonene, eucalyptol, γ-terpinene, terpinolene, terpinen-4-ol, α-terpineol, aromadendrene, ledene, and δ-cadiene (Table 5).

The analysis of QC revealed an increase in the abundance of phytoconstituents in the mixture compared to TTO alone. During the GC-MS experiment, the structures of 18 phytocompounds were identified (Fig. 2).

The qualitative analysis confirmed the presence in the composition of unmodified (-)-α-bisabolol, eucalyptol and TTO constituents such as δ-cadiene, α-terpineol, terpinen-4-ol, γ-terpinene, D-limonene, α-pinene and β-pinene (Table 6). The monoterpenes α-phellandrene and β-phellandrene were not initially detected in the TTO sample but were recorded in QC in trace amounts not exceeding 0.3 %. The identified monoterpenes were derived from TTO. Both α- and β-phellandrene were previously referred to as the phytoconstituents of the TTO of various origins (Chambers et al., 2008; de Groot and Schmidt, 2016; Hausen, 2004).

Table 6. Gas Chromatography-Mass Spectrometry analysis of quadrocomplex compounds.

No.	Retention time	Compound	Peak area	Relative content, %
1	2.468	α-phellandrene	1506924	0.302036
2	2.543	α-pinene	7164038	1.435904
3	2.873	β-phellandrene	1069427	0.214348
4	2.924	β-pinene	1501752	0.301000
5	2.989	butanoic acid 3-methyl-1-ethenyl-1,5-dimethyl-hexenyl ester	799390	0.160224
6	3.286	4-carene	2874355	0.576113
7	3.374	D-limonene	78028680	15.639460
8	3.42	Eucalyptol	2507694	0.502623
9	3.737	γ-terpinene	24054795	4.821356
10	4.082	2-carene	5170407	1.036316
11	5.226	Terpinen-4-ol	157458670	31.559790
12	5.364	α-terpineol	17919777	3.591701
13	6.194	Trans-ascaridole glycol	7320769	1.467318
14	8.642	Alloaromadendrene	6214193	1.245525
15	9.656	δ-cadinene	5887001	1.179945
16	11.323	Trans-α-bisabolene	5209691	1.044190
17	11.354	Farnesol	12948962	2.595389
18	11.573	(-)-α-Bisabolol	161285230	32.32676

The mixture of sesquiterpene products – trans-α-bisabolene and farnesol – revealed by GC-MS was originated from (-)-α-bisabolol sample as derivative and by-product, respectively. Farnesol concentration was estimated to be 2.6%, trans-α-bisabolene 1.0% (Table 6).

Additionally, five novel derivatives were found to be new. Identified compounds were structurally related to eucalyptol and presumably generated in the combined mixture in the presence of (-)-α-bisabolol and some bioactive compounds of TTO. The new phytoconstituents in QC were recognized as butanoic acid 3-methyl-1-ethenyl-1,5-dimethyl-hexenyl ester, 4-carene, 2-carene, trans-ascaridole glycol, alloaromadendrene in small amounts.

Butanoic acid 3-methyl-1-ethenyl-1,5-dimethyl-hexenyl ester (syn: linalyl isovalerate), as a new derivative in QC complex, was previously identified in the chemical composition of *Ammi visnaga* L. essential oil (endemic, medicinal plant) demonstrated effectiveness against bacteria and moulds (Feirouz and Salima, 2014). The compound 4-carene, also identified amongst the main constituents of the Iranian *Cymbobogon olivieri* essential oil, has been implicated in the antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and yeast *C. albicans* (Mahboubi and Kazempour, 2012). Interestingly, alloaromadendrene was reported to have not only a higher antimicrobial activity than 1,8-cineole (eucalyptol), but can also enhance its activity (Mulyaningsih et al., 2010). The sesquiterpene aromadendrene bears a reactive exocyclic methylene group and a cyclopropane ring, which can alkylate proteins and thereby disturb the conformation of these compounds. Additionally, since the compound is highly lipophilic, it causes the disruption of cellular biomembranes (Sikkema et al., 1994; van Vuuren and Viljoen, 2007; Wink, 2008). It was shown that the combinations of aromadendrene and 1,8-cineole apparently exhibited synergistic and additive antimicrobial properties. The checkerboard assay demonstrated that combinations of 1,8-cineole and aromadendrene reduce the MIC in most cases in an additive way, whereas the time-kill assay indicates a synergistic effect. The antibacterial activity of the combination was estimated against methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus*, *B. subtilis*, and *Streptococcus pyogenes* (Mulyaningsih et al., 2010).

The quantitative analysis of QC phytochemicals showed the highest concentrations for (-)-α-bisabolol 32.3 %, terpinen-4-ol 31.6 %, and D-limonene 15.6 %. Overall, the content of 18 phytocompounds identified in QC is presented in Table 6, along with their retention time. A previous study corroborated that even minor components play a role in antibacterial activity, possibly by producing synergistic effects with other components (Burt, 2004). Thus, terpinen-4-ol is the major active component responsible for TTO's antibacterial efficacy, while the minor components in TTO also contributed to its efficacy (Lee et al., 2013).

A synergistic effect was previously reported for other QC components – a combination of D-limonene and 1,8-cineole (van Vuuren and Viljoen, 2007).

Antibacterial activity of QC compounds by agar diffusion test

The effect of each bioactive compound in QC on the growth of microbial reference strains *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *S. epidermidis* ATCC 14990 was estimated in an agar diffusion test. TTO showed anti-Gram-positive and anti-Gram-negative with predominant activity against *S. epidermidis* at both tested concentrations; it caused inhibition zones ranged from 17-22.3 mm at different concentrations. However, *S. aureus* was less susceptible at the highest applied concentration (i.e., 5 µL/disc) with an 8.6 mm inhibition zone. Eucalyptol was active only against *S. epidermidis* with inhibition zones ranged from 8-17.5 mm at the different concentrations. (-)-α-Bisabolol showed significant antibacterial activity against Gram-positive bacteria and had inhibition zones such as benzalkonium chloride, while *E. coli* was less susceptible to tested (-)-α-bisabolol than the positive control. SSC exhibited high antibacterial activities against all tested bacterial strains (17.5-24.3 mm at 5 µL/disc), with the Gram-negative *E. coli* being the least sensitive bacteria to the tested SCC. The Gram-negative bacterium *E. coli* was inhibited up to the maximum applied concentration in agar diffusion (5 µL/disc) (Table 7). In general, *S. epidermidis* was significantly more susceptible to tested single substances than *S. aureus* and *E. coli* due to another cell wall structure.

Novel QC showed the highest antibacterial activity against both Gram-positive and Gram-negative bacteria (29.5-35.7 mm at 5 µL/disc) as compared with benzalkonium chloride ($p<0.05$). The herein-reported antibacterial activities of QC could be attributed to the high content of oxygenated monoterpenes, mainly 1,8-cineole (eucalyptol), terpinene-4-ol, α-terpineol (Lee et al., 2013). Remarkably, the antibacterial activity of QC was ascribed to the combination of 1,8-cineole and other terpenes (Chao et al., 2008; Kon and Rai, 2012). In fact, 1,8-cineole influences the integrity of the cell membrane and causes alteration in the morphology of the bacterial cell wall (Carson et al., 2002). QC containing mainly aromatic phenols or aldehydes has been reported to exhibit substantial antimicrobial activity. Furthermore, the lower susceptibility of Gram-negative bacteria than Gram-positive bacteria to the single compound could be attributed to the presence of the outer membrane and the lipopolysaccharide barrier for the hydrophobic compounds that deteriorate their bioactivities. Thus, QC can be used as an active plant-based substance for the microflora regulation of the skin.

Determination of MIC for QC compounds

The effect of each bioactive agent in QC on the growth of microbial reference strains *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *S. epidermidis* ATCC 14990, and *M. luteus* ATCC 10240a, was estimated in a serial-dilution-based MIC method. According to the experimental data, MIC values of the agents for different strains varied from 1.25 to 40.00 mg/mL compared to benzalkonium chloride used as control (Table 8).

TTO and eucalyptol gave MIC values of 40 mg/mL against both *S. aureus* and *M. luteus*. The same results were obtained when eucalyptol and bisabolol were tested against *E. coli*, whereas higher sensitivity of the strain was observed for TTO – the MIC value was 5 mg/mL. Bisabolol inhibited the growth of *M. luteus* and *S. aureus* with MIC values of 20 mg/mL and 5 mg/mL, respectively. The lowest MIC was obtained with TTO, eucalyptol and bisabolol against *S. epidermidis* with 1.25, 2.50, and 1.25 mg/mL, respectively.

SSC presented a MIC value of 1.25 mg/mL against all microorganisms, thus showing a strong antibacterial activity on all strains studied regardless of the difference in cell wall composition between Gram-positive and Gram-negative bacteria.

Interaction between combined biologically active compounds

Once MIC values were determined for all components of QC, inhibitory concentrations of the combined agents were also evaluated in order to identify a type of interaction between them. As a means of control, the bacteria were exposed separately to the MIC of every single agent studied.

All combinations caused growth inhibition of reference strains *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *S. epidermidis* ATCC 14990, and *M. luteus* ATCC 10240a (Table 9). The FICI was calculated for each combination and interpreted as follows: synergistic effect, $FICI \leq 0.5$; additive effect or indifference, $0.5 < FICI > 4.0$; and antagonistic effect, $FICI \geq 4.0$ (Den Hollander et al., 1998; Odds, 2003). FICI was expressed as mean ± SD.

In this study, additive effect or indifference was revealed for the following combinations (Table 8). The FICI between 0.5 and 1 was interpreted as addition and between 1 and 4 as indifference (Bassolé and Juliani, 2012).

Table 7. Antibacterial activity of QC compounds against tested bacteria via agar diffusion test.

Bacteria strain[a]	Concentration (µL/disc)	Diameter of inhibition zone (mm)		
		S. aureus	*S. epidermidis*	*E. coli*
TTO	1	-	17.0 ± 2.7	8.5 ± 0.6
	5	8.6 ± 1.4	22.3 ± 3.3*	13.4 ± 2.3
Eucalyptol	1	-	8.3 ± 0.3	-
	5	5.5 ± 0.7	17.5 ± 1.2*	6.9 ± 0.9
(-)-α-Bisabolol	1	12.7 ± 1.5	18.5 ± 1.5	-
	5	21.5 ± 3.4	24.0 ± 3.8*	5.4 ± 0.2
Stabilized silver complex	1	16.0 ± 1.9	16.5 ± 1.4	12.0 ± 1.5
	5	24.3 ± 2.1*	23.2 ± 1.6*	17.5 ± 1.8
QC (mixture of compounds 1:1:1:1)	1	29.3 ± 2.3*	31.7 ± 2.1*	23.0 ± 3.3*
	5	35.7 ± 1.5*	33.1 ± 1.9*	29.5 ± 3.1*
Benzalkonium chloride (positive control)	5	21.5 ± 1.2	15.8 ± 2.1	15.3 ± 0.7

The data are expressed as means ± SD (n = 3). Benzalkonium chloride was used as the positive control. Asterisks indicated statistically significant differences in the inhibition zones due to the activity of QC compounds to the positive control ($p<0.05$). The mean inhibition zones with similar letters are not significantly different from each other based on the post hoc Tukey HSD test. [a]The bacteria were purchased from ATCC. ATCC: American Type Culture Collection; QC: Quadrocomplex; TTO: Tea tree essential oil.

Table 8. MIC of single QC components and control via broth-dilution method.

No.	Bacteria strain[b]	MIC (mg/mL)[a]				
		TTO	Eucalyptol	(-)-α-Bisabolol	Stabilized silver complex	Benzalkonium chloride
1	*S. aureus* ATCC 29213	40.00	40.00	5.00*	1.25**	5.00
2	*E. coli* ATCC 25922	5.00	40.00	40.0	1.25**	10.00
3	*S. epidermidis* ATCC 14990	1.25*	2.50	1.25*	1.25*	5.00
4	*M. luteus* ATCC 10240a	40.00	40.00	20.00	1.25**	5.00

The data are represented as the minimum value of three independent readings. Benzalkonium chloride was used as standard antimicrobial substance. All experiments were triplicated. [a]The MIC values were determined via colorimetric assay using resazurin after 24 h of treatment. [b]The bacteria were purchased from ATCC. *$p<0.05$, **$p<0.01$ (ANOVA). ATCC: American Type Culture Collection; MIC: Minimum inhibitory concentration; TTO: Tea tree essential oil.

Table 9. FICI values for combinations of QC.

FICI[a]	Bacteria strain[b]			
	S. aureus ATCC 29213	*E. coli* ATCC 25922	*S. epidermidis* ATCC 14990	*M. luteus* ATCC 10240a
SSC + TTO	1.142 ± 0.333	1.156 ± 0.099	1.063 ± 0.001	1.178 ± 0.303
SSC + eucalyptol	1.031 ± 0.001	1.031 ± 0.001	1.031 ± 0.001	1.031 ± 0.001
SSC + (-)-α-bisabolol	1.062 ± 0.001	1.031 ± 0.001	1.078 ± 0.049	1.031 ± 0.001
TTO + (-)-α-bisabolol	1.025 ± 0.013	1.953 ± 0.979	0.625 ± 0.261*	0.769 ± 0.261
TTO + eucalyptol	1.913 ± 0.276	0.726 ± 0.532	0.344 ± 0.125*	1.903 ± 0.306
Eucalyptol + (-)-α-bisabolol	3.052 ± 0.565	2.000 ± 0.001	1.500 ± 0.435	1.316 ± 0.472

The data are represented as mean ± SD (n = 3). [a]The FICI values were determined via checkerboard assay using resazurin after 24 h of treatment. [b]The bacteria were purchased from ATCC. *$p<0.05$ (ANOVA). ATCC: American Type Culture Collection; MIC: minimum inhibitory concentration; FICI: Fractional inhibitory concentration index; SSC: Stabilized silver complex; TTO: Tea tree essential oil.

(-)-α-Bisabolol and TTO against all reference strains showed FICI values ranged from 0.625 to 2.250. (-)-α-Bisabolol and SSC against all reference strains exhibited FICI values ranged from 1.031 to 1.125. SSC and eucalyptol against all reference strains demostrated FICI value of 1.031. SSC and TTO against all reference strains with FICI values ranged from 1.031 to 1.250. (-)-α-Bisabolol and eucalyptol against

M. luteus with a FICI value of 1.031. Eucalyptol and TTO against *S. aureus* and *M. luteus* with FICI value of 2.000. (-)-α-Bisabolol, in combination with the eucalyptol, exhibited an antagonistic effect against *S. aureus* exhibiting FICI values greater than 4.0 (Balouiri et al., 2016).

Clear synergistic activity was observed for eucalyptol and TTO against *S. epidermidis*, with FICI values for the combination varying from 0.344 to 0.625 with statistical deviations. Combination of eucalyptol and TTO had an additive antibacterial effect against *E. coli* with FICI value 0.726 ± 0.532. The interpretation was based on a recommendation for checkerboard assay (Balouiri et al., 2016; Bassolé and Juliani, 2012). The combined action of TTO components and 1,8-cineole with possible synergistic effects was indicated in previous studies (Chao et al., 2008; Kon et al., 2012). An example of this synergy is the interaction between 1,8-cineole and terpinene. The effect of 1,8-cineole itself is mild, but in combination with terpene 1,8-cineole can increase the permeability of the bacterial membrane, thus enhancing uptake of terpene and contributing its activity (Carson et al., 2002).

In vitro assessment of antimicrobial activity of QC

An *in vitro* comparative evaluation of the antimicrobial activity of hand-washing SB and formulations enhanced with QC at different concentrations (Table 10) was conducted using the colony-counting method. In the preliminary experiment, reference microbial strains namely *E. coli* ATCC 25922, *B. cereus* ATCC 10702, *S. aureus* ATCC 6538-P, *P. aeruginosa* ATCC 9027, and *C. albicans* ATCC 10231, were tested. According to the results obtained, QC addition in soap base inhibited the growth of the standard strain. Although the reduction in colony-forming units (CFU) appeared to depend on the concentration of QC and the microorganism tested.

It has been found that both QC and SB exhibited pronounced antifungal activity against *C. albicans,* resulting in the complete inactivation of the fungi after contact with various concentrations of the bioactive complex (Table 10). Also, SB and QC were active in experiments using *B. cereus* reference strain. No bacterial growth was found when QC was added at all concentrations tested (Table 10). SB formulation was equally potent against *B. cereus* strain.

QC demonstrated dose-dependent inhibition of *S. aureus* growth, with maximum inhibition achieved at 0.8% QC concentration ($p<0.05$). SB displayed pronounced antibacterial activity against *S. aureus* strain.

The evaluation of QC antimicrobial activity against *E. coli* and *P. aeruginosa* strains revealed a more complicated picture. The bioactive complex affected *E. coli* and *P. aeruginosa* growth at a certain concentration range (Table 10). The differential effect between SB and QC-containing formulations was observed only at 0.8% QC concentration against *E. coli* and only at 0.4% QC concentration against *P. aeruginosa* ($p<0.05$).

The detailed elucidation of antimicrobial activity of SB enhanced with QC using the colony-counting method revealed significant inhibition of *E. coli* growth with an average efficiency of 99.15% (equivalent to 2log10 CFU reduction) for 0.8% QC concentration, which is higher compared to SB alone ($p<0.05$). Similar results were obtained when SB + QC was applied against *S. aureus*: SB enhanced with QC was more active than SB in *S. aureus* growth inhibition with an efficiency of about 99.91% (equivalent to 3log10 CFU reduction) (Table 10).

Table 10. Antimicrobial activity of soap formulations using colony-counting method.

No.	Bacteria strain[b]	Log10 CFU[a]				
		SB	SB + 0.2% QC	SB + 0.4% QC	SB + 0.6% QC	SB + 0.8% QC
1	*S. aureus* ATCC 29213	<1	2.72 ± 0.16*	2.71 ± 0.19*	2.72 ± 0.26*	3.04 ± 0.41*
2	*E. coli* ATCC 25922	<1	<1	<1	1.18 ± 0.14	2.07 ± 0.35*
3	*P. aeruginosa* ATCC 9027	<1	<1	1.57 ± 0.11*	1.78 ± 0.35*	1.96 ± 0.29*
4	*B. cereus* ATCC 10702	2.00 ± 0.17	2.00 ± 0.23	2.01 ± 0.13	2.34 ± 0.31	2.25 ± 0.54
5	*C. albicans* ATCC 10231	3.21 ± 0.24	3.21 ± 0.22	3.21 ± 0.26	3.21 ± 0.41	3.21 ± 0.42

The data are represented as the minimum value of three independent readings (mean ± SD). [a]The Log10 CFU values were determined via counting the colony-forming units (CFUs). CFU was counted after a 24 h incubation (for bacteria) and 48 h incubation (for fungi) at 37 °C. [b]Bacteria were purchased from ATCC. *$p<0.05$ (ANOVA). ATCC: American Type Culture Collection; CFU: Colony-forming unit; SB: Soap base; QC: Quadrocomplex.

The paired t-test compared SB + QC and SB formulations and revealed significant inter-group differences concerning antimicrobial efficacy with the highest reduction of bacterial counts for SB-containing QC ($p<0.05$). The efficiency of SB + QC formulation in the reduction of viable microbial counts was estimated to be 99.94 % (equivalent to 3log10 CFU reduction) against *C. albicans*. The antimicrobial efficacy of different soap formulations was further evaluated against clinical skin isolates *S. aureus* and *E. coli*. Results obtained using the resazurin cell viability method showed that such QC components as SSC 0.01% and TTO 0.30% displayed bacteriostatic activity against Gram-negative bacteria *E. coli* and Gram-positive bacteria *S. aureus*, while SB without QC components was less effective against isolated potential skin pathogen *S. aureus* and *E. coli*. (-)-α-Bisabolol 0.10% and eucalyptol 0.15% as single active agents of soap formulations were found to be efficient in inhibiting the growth of selected potential pathogens.

Among the different formulations studied only QC composition – TTO 0.30% + (-)-α-bisabolol 0.10% + eucalyptol 0.15% + SSC 0.01% – exhibited bacteriostatic effect against both Gram-negative and Gram-positive bacteria *S. aureus* and *E. coli* isolated from skin (Table 10). A relative richness of QC composition allows it to demonstrate a complex antibacterial activity with minor phytoconstituents also contributed to its efficacy compared to single agents. In general features, the heterogeneous composition of TTO and the antimicrobial activities of many of its components predetermine the complicated mechanisms of TTO antimicrobial action, including the ability to inhibit respiration and increase membrane permeability in microbial cells (Cox et al., 2001). TTO destructive action results from inhibiting membrane-located metabolic events and losing chemiosmotic control (Cox et al., 2001). Eucalyptol (1,8-cineole) acts as a penetration enhancer. 1,8-cineole in combination with TTO components can increase the permeability of the bacterial membrane, thus enhancing the up-take of bioactive agents and contributing to overall activity (Carson et al., 2002; Chao et al., 2008; Kon et al., 2012). It is supposed that (-)-α-bisabolol induces apoptosis in fungi and bacteria by interacting with bacterial or fungal membranes (Lucca et al., 2011). Silver ions are effective against a broad range of microorganisms due to multiple mechanisms of action (Woo et al., 2008). The interaction of silver ions with enzymes and proteins plays an essential role in antimicrobial action. Silver ions cause the release of K^+ ions from bacteria; thus, the bacterial plasma or cytoplasmic membrane, which is associated with many important enzymes, is an important target site for silver ions. Silver ions cause a marked inhibition of bacterial growth, inhibit cell division and damage the cell envelope and the contents of bacteria, interact with nucleic acids (Woo et al., 2008).

The evaluation of the comparative efficiency of different formulations using the colony-counting method revealed a significant reduction of the colony-forming units of bacteria for soap formulation containing QC compared to other samples presented in Table 11 ($p<0.05$). CFU count is exhibited as mean ± SD.

Clinical evaluation of the effects of QC on skin hygiene

The high antimicrobial activity of QC is combined with careful hand-skin action. The effects of hand-washing liquid soap with QC were estimated during a 21-day comparative study between a test group of 20 volunteers, and a control group consisted of 20 volunteers. A questionnaire survey was performed to evaluate the level of the volunteers' satisfaction of regular home hand-washing with studied soap formulations. All participants reported relatively high satisfaction with antibacterial liquid soap with QC. According to the volunteer self-assessments, soap formulation containing QC has a pleasant scent and consistency, high cleaning ability, and delicate action on the skin during washing.

Dermatology evaluation indicated that QC added to the soap base to enhance its antibacterial action did not affect the skin of the hands during regular hand-washing. QC did not cause any signs of skin dryness, irritation, allergy-like, or other skin disorders associated with the hand-washing procedure.

The investigation of hand-washing with soap formulations on bacterial contamination of hands was performed as a long-term study. The paired t-test compared the pre-wash and post-wash bacterial counts for SB and SB containing QC and illustrated that each formulation reduced statistically significant CFU counts with SB + QC demonstrating higher efficiency ($p<0.05$).

Though the difference in the immediate effect between the groups was significant but not so pronounced as the dynamics of prolonged antibacterial action clearly revealed a significantly higher reduction in total CFU counts for SB + QC compared to the control (Table 12). Thus, QC can be used constantly for cleaning purposes as a bioactive complex with balanced antimicrobial activity.

Table 11. Comparative effect of different antimicrobial formulations on the growth of clinical isolates.

No.	Formulation	CFU count[a]	
		E. coli[b]	*S. aureus*[b]
1	SB without QC components	1000 ± 32	1250 ± 44
2	SB + TTO 0.30%	70 ± 11*	64 ± 10*
3	SB + SSC 0.01%	92 ± 26*	106 ± 23*
4	SB + (-)-α-bisabolol 0.10%	84 ± 20*	105 ± 28*
5	SB + eucalyptol 0.15%	90 ± 17*	109 ± 15*
6	SB + QC 0.46%	11 ± 6*	21 ± 12*
7	Control without SB and QC components	2080 ± 39	3340 ± 57

The data are represented as the minimum value of three independent readings (mean ± SD). [a]The CFU counts were determined via counting the colony-forming units (CFUs). CFU was counted after a 24 h incubation (for bacteria) at 37°C. [b]The bacteria were isolated from the hands of human volunteers. *p<0.05 (ANOVA). ATCC: American Type Culture Collection; CFU: Colony-forming unit; SB: Soap base; QC: Quadrocomplex.

Table 12. Effect of hand-washing formulations with and without QC on bacterial contamination of hands.

Testing period	CFU count[a]	
	Formulation SB	Formulation SB + QC
0 h (before soap application)	19.7 ± 11.06	32.3 ± 12.11
1 h	3.00 ± 3.18*	12.45 ± 10.16*
2 h	1.65 ± 2.13*	4.45 ± 5.01*
3 h	0.70 ± 1.17*	1.30 ± 2.41*
7th day	15.45 ± 5.98	6.75 ± 5.87*
21th day	14.35 ± 7.09	7.00 ± 4.92*

The data are represented as the minimum value of three independent readings (mean ± SD). [a]The CFU counts were determined via counting the colony-forming units (CFUs). CFU was counted after a 24 h incubation at 37°C. The bacteria were isolated from the hands of human volunteers. *p<0.05 (ANOVA). CFU: Colony-forming unit; SB: Soap base; QC: Quadrocomplex.

CONCLUSION

The novel described an active complex for hand hygiene based on natural antimicrobial and anti-irritant compounds – *M. alternifolia* leaf essential oil, eucalyptol, (-)-α-bisabolol and silver citrate – and exhibits a balanced performance between antimicrobial activity and biological safety. QC components display additive or synergistic activities against the most strains studied. In view of its favorable safety profile and no skin irritant potential, QC can be considered a promising bioactive composition for hand hygiene applications. Further studies should be carried out to study the underlying mechanism of antimicrobial activity for QC chemicals.

CONFLICT OF INTEREST

The funder Splat Global, LLC has a role in the funding, study design, collection, analysis, and interpretation of data, writing of the manuscript, and decision to publish the results. Filatov V is an employee of Splat Global LLC. The other authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Filatov VA	Kulyak OY	Kalenikova EI
Concepts or ideas	x		
Design	x		
Definition of intellectual content	x	x	x
Literature search	x		
Experimental studies	x		
Clinical trial	x		
Data acquisition	x		
Data analysis	x	x	x
Statistical analysis	x	x	x
Manuscript preparation	x	x	x
Manuscript editing	x	x	x
Manuscript review	x	x	x

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