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Developing Highly Diluted Alcoholic Sanitizers Loaded with Eugenol and Cinnamaldehyde as Antibacterial Boosters for Controlling Some Pathogenic Bacteria

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Abstract

The authors aimed at developing novel diluted hydro-alcoholic sanitizer containing significantly lower content of alcohol. Antibacterial boosters like eugenol and cinnamaldehyde were used in the developed formula to compensate for the low alcohol content. The maximum water content and the solubility (miscibility) of eugenol and cinnamaldehyde in the diluted alcoholic formula was investigated using the Gibbs' phase diagrams. The broth dilution assay was used to evaluate the growth inhibition of the developed sanitizing formula against *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* and *Pseudomonas aeruginosa*. Results indicated that a homogenous highly diluted hydro-alcoholic formula can be achieved using only 22.5% alcohol (isopropanol), 75% water and up to 2.5% eugenol. On the other hand, cinnamaldehyde showed different attitude, where the maximum dilution of alcohol that can be reached is 37.5% with 60 % water and up to 2.5% cinnamaldehyde. Evaluation of the antibacterial activity of the two formulas showed complete inhibition of the growth of the tested pathogens, where the count after treatment was zero cfu/ml. This antibacterial activity was similar to that of commercial hydro-alcoholic sanitizer that contains 70% alcohol. The study represents an insight towards an applicable alcoholic sanitizer characterized by significantly lower content of alcohol without compromising efficiency.

Keywords: Alcoholic sanitizers; pathogens; growth inhibition; alcohol reduction; eugenol; cinnamaldehyde; antibacterial.

1. Introduction

Covid-19 pandemic draw the attention to the necessity of using sanitizers for instant decontamination of hands and surfaces in order to prevent the spreading of the disease. The use of sanitizers should not be restricted to the seasons of viral pandemic but it should also be considered seriously at all occasions for controlling infectious diseases caused by pathogenic microorganisms. Alcohol-based sanitizing products (e.g., hydro-alcoholic solutions, gels and creams) are the most popular setting which are used for that purpose [1-3]. It is applied on personal levels in gathering places like hospitals, supermarkets, teller and vending machines. For instance, alcoholic hand rubs play important role in controlling global hospital acquired infections and transmission of some multidrug resistance bacteria [3-5]. Alcohol-based sanitizers are also used efficiently in disinfection of gloved hands of the medical staff in hospitals [6]. In addition, some medical check-up devices like stethoscopes and other equipment are also decontaminated using alcohol-based sanitizers [7,8]. All these successful decontamination applications are due to the efficiency of alcohol-based sanitizers against pathogenic bacteria which shows less resistance to alcohol compared to their high resistance toward antibiotics [9].

Ethanol and isopropanol are the common types of short chain alcohols which are used at 85%-60% (commonly 70% v/v) dilution in distilled water for hand sanitization purpose [10,11]. Interestingly, the inclusion of water molecules at 30% - 40% v/v helps to optimize the hydrophobicity-hydrophilicity balance of the ethanol, allowing better penetration of the chemical entity into and/or through the bacterial cell membrane [12]. At 70% (v/v), alcoholic sanitizers were found to kill up to 99.99 microbial cell [13]. This hydro-alcoholic solution can cause perturbation in the microbial cell wall membrane leading to altered permeability and proteins denaturation, which ultimately lead to microbial cell death [14]. It is worth noting that alcohol-based sanitizers may also contain small amount of other adjuvant antimicrobial agents like chlorhexidine (4%) or triclosan in order to increase the persistent residual bactericidal activity of the alcoholic sanitizer [15].

Despite that bright picture of alcohol-based sanitizers in decontamination, the overuse of alcohol lead to immergence of alcohol-tolerant microorganisms [16,17], like for instance *Enterococcus faecium*, *Bacillus cereus*

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and *Enterobacter cloacae*. That tolerance is developed from the change in the fatty acid composition of the microbial cells wall, especially the increase of unsaturated fat ratio [18]. On one hand, alcohol tolerance is considered to be an advantage in the bio-fuel production in which the microorganism that ferment carbohydrate into ethanol will be able to survive even at high ethanol accumulation [19]. However, on the other hand, ethanol tolerance is obviously representing a challenge in the field of disinfection and decontamination because that will blow up the whole sanitization process by making it ineffective. The mechanisms and tools used for understanding how the microbial resistance to alcohol is developed are fully discussed on molecular and genetic levels elsewhere [19]. Beside the challenge of bacterial adaptation to alcohols, sanitizing products based on alcohols are also represent environmental hazard and health risk to human health due to toxicity upon ingestion, especially for children under 12 yeas [20].

As a result, alternatives to alcohol-based sanitizers including benzalkonium chloride [21] and quaternary ammonium disinfectants [22] were investigated. In addition to these synthetic sanitizers, herbal disinfection was also appeared as another alternative to alcohol-based sanitizers. The antimicrobial active principles of these herbs include volatile oils (essential oils) and non-volatile principles like flavonoid and polyphenols [23]. Essential oils in particular are promising natural sanitizing agents due to their content of antimicrobial active volatile phenols, aldehydes and other terpenic compounds [24,25]. Water-based formulas of essential oils like nanoemulsions [26] and microemulsions [27] were developed for controlling antibiotic resistant pathogenic bacteria. These water-based and alcohol-free colloidal systems seems to be a potential alternative to alcohol-based sanitizers. However, some emulsifiers like the polysorbates (Tween family), which are indispensable for the formulation of these emulsions can interfere with, and deactivate the essential oils and their individual components. That was verified by our research group [28,29] and also reported previously by other investigators [30,31]. That could be due to the encapsulation of essentials oils in the inner hydrophobic core of the surfactant aggregates which shield its effect from the surrounding microorganisms.

Based on the above-mentioned advantages and liabilities of alcoholic sanitizers and other sanitizing formulas, the authors in the current study planned to develop a novel highly diluted hydro-alcoholic sanitizer for controlling the growth of some pathogenic bacteria. The formula is fortified with some volatile antibacterial boosters to compensate for the low alcohol content. We expect that such diluted alcoholic sanitizer can slow down alcohol depletion from the stock, cut the overuse of alcohol in sanitizers which develop alcohol-tolerant bacterial strains, decrease alcohol emission to the environment to limit the greenhouse effect. In addition to lowering the risk of alcohol toxicity upon incidental ingestion by children.

In our first study of this trend [32] the authors investigated the potentials of carvacrol, which is a volatile phenolic compound derived from oregano essential oil, for boosting the antibacterial activity of diluted (45%) alcoholic sanitizer. In the current study, we continue this endeavour by developing significantly highly diluted (<30%) alcoholic sanitizer fortified with two volatile compounds namely eugenol and cinnamaldehyde as antibacterial boosters. These two compounds are the major volatile constituents of clove and cinnamon essential oils, respectively, which give these oils their potent antimicrobial activity [33]. They also increase the effectiveness of β-lactam antibiotics against multi-drug resistant *Acinetobacter baumannii* [34]. Different formulation and antibacterial assessments related to the purpose of this study is adopted in order to support the objective.

2. Materials and methods

2.1 Chemicals and pathogenic microorganisms strain collection

Eugenol (CAS No. 97-53-0) and cinnamaldehyde (CAS No. 104-55-2) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, USA). Absolute isopropanol (99.9%) was obtained from Fischer Scientific (Leicestershire, UK). Tryptone soy agar (TSA) (Oxoid, CMO 131, Oxoid limited, Hampshire, England). Local equipment available at the authors' lab were used for the distillation and sterilization of water which was used to prepare the different concentrations of the hydro-alcoholic sanitizer.

Four pathogenic bacteria which were used in the current investigation provided from the culture collections of the Microbiological Department National Research Centre (NRC) Dokki, Giza, Egypt. These include one strain of Gram-positive *Listeria monocytogenes* (ATCC 35152) and three strains of Gram-negative bacteria *Salmonella typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 27325) and *Pseudomonas aeruginosa* ATCC 9027.

2.2. Investigation of the maximum alcohol dilution and solubility (miscibility) of eugenol and cinnamaldehyde in the hydro-alcoholic sanitizer

That was studied by constructing the Gibbs' triangle phase diagram. The procedure was described in part (I) of our previous relevant investigation [32]. The method is originally adopted from the work of Garti et al. [35] with the replacement of the surfactant in their study with alcohol (isopropanol) in our investigation. In details, each oil phase (eugenol and cinnamaldehyde) was mixed (separately) with absolute isopropanol in glass vials at different weight ratios including 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1, respectively. Then, each vial was titrated

with predetermined percentages of distilled water starting from 10% up to 99% of the weight of the three components (oil, isopropanol and water), followed by vortex for 1 minute. After the titration of the first batch of water (10%) the vials were left for at least 30 minutes at room temperature to equilibrate. Then, the next batch of water (20%) was titrated, and so on till reaching 99% water content. The percentile (composition by weight) of each of the oil phase, isopropanol, and water was calculated and then represented as points on the Gibbs' triangle phase diagram. Then, all points were connected to form a border line which separate the phase diagram into two main solubility (miscibility) zones. The first one lies on the right-hand side of the border line at which the three components of the hydro-alcoholic sanitizer are perfectly miscible in a homogenous formula (green zone, Fig. 1). On the other hand, the left-hand side of the border line represents a non-homogeneous composition where the three components of the hydro-alcoholic sanitizer are not miscible, so it looks cloudy or hazy (Fig. 1). Each phase diagram (corresponding to eugenol and cinnamaldehyde) was constructed twice in order to be sure of the position of the border line and the solubility regions of each oil phase in the different dilutions of the hydro-alcoholic solutions.

2.3. Antibacterial evaluation

The antibacterial activity of the diluted hydro-alcoholic sanitizers was evaluated against four pathogenic bacterial strains using the broth dilution assay. The content of the antibacterial boosters namely eugenol, cinnamaldehyde and their mixture (at 1:1 weight ratio) was fixed at 1.5% of the total sanitizer. The broth dilution assay was conducted as follows:

Each antibacterial booster (0.15 ml) was dissolved in absolute isopropanol (2.85 ml) in sterile glass test tubes. Then, 6.0 ml of sterile liquid broth media (LBM) was added to that mixture followed by 1.0 ml (which contain 10^6 cfu/ml) suspension in water of each of the four bacterial strains, separately. The final concentration of the ingredients in each test tube was 70.0% total aqueous phase, 28.50% isopropanol, and 1.5% of each of the tested antibacterial booster.

Three control solutions (2 negative and 1 positive) were prepared without any of the antibacterial boosters and subjected to the same evaluation.

The first control represented the standard commercial alcoholic sanitizer with 70.0% isopropanol and 30% water. That was prepared by mixing 7.0 ml absolute isopropanol with 2.0 ml LBM and 1.0 ml aqueous bacterial suspension.

The second control represented the developed diluted alcoholic sanitizer (without the antibacterial booster) which contains only 30% isopropanol and 70% water. That was prepared by mixing 3.0 ml absolute isopropanol with 6.0 ml (LBM) and 1.0 ml aqueous bacterial suspension.

Finally, the third control contains 100% water which was prepared by mixing 9.0 ml LBM and 1.0 ml aqueous bacterial suspension.

All formulas and controls were incubated for 24h at 37°C, then 1.0 ml of each test tube was subculture in tryptone soy agar and incubated for another 24h at 37°C. After that, all bacterial colonies were counted, and the results were reported as mean bacterial count (log cfu/ml) ± SD of two replicates for each treatment.

2.4. Statistical analysis

All experiments and analysis were done in duplicate. Data was statistically analyzed using GLM procedure of SAS [36] software (Version 9.2). Level of significance between treatments was determined by Duncan test. Probability of <0.05 was considered as significantly different.

3. Results

3.1. Investigation of the maximum alcohol dilution and solubility (miscibility) of eugenol and cinnamaldehyde in the hydro-alcoholic sanitizer

Data from the phase diagrams (Fig. 1a,b) shows the maximum amount of water which can be used to dilute an alcoholic sanitizer containing absolute isopropanol combined with eugenol or cinnamaldehyde as antibacterial boosters. Figure (1a) indicates that the maximum percentage of water that can be used to formulate the diluted hydro-alcoholic formula is 75% (Max 75). At that high dilution, up to 2.5% eugenol and only 22.5% isopropanol can be incorporated homogenously to form a homogenous and transparent sanitizing formula (Fig 1a, right photo).

Increasing alcohol dilution by adding more than 75% water or incorporation of more than 2.5% eugenol into that hydro-alcoholic formula leads to disturbance of the miscibility of the components and shift the equilibrium outside the green area into the non-homogeneous composition. That is manifested visually by the change of the appearance of the formula from clear transparent into cloudy-hazy dispersion (Fig. 1a, left photo). That is due to separation of eugenol droplets from the formula indicating a physically unstable state. Consequently, it becomes unsuitable for efficient sanitizing application.

On the other hand, in the case of cinnamaldehyde (Fig. 1b), the maximum percentage of water that can be used to make the diluted hydro-alcoholic formula is 60% (Max 60). This amount can mix perfectly with 37.5% isopropanol containing up to 2.5% cinnamaldehyde.

As a summary of the results, a homogenous 22.5% and 37.5% diluted hydro-alcoholic sanitizers containing up to 2.5% eugenol or cinnamaldehyde, respectively, as antibacterial boosters to compensate for the low alcohol content, can be formulated.

3.2. Antibacterial activity

Table (1) indicate that a diluted hydro-alcoholic solution containing 70% water, 28.5% isopropanol and fortified with 1.5% eugenol, cinnamaldehyde or their mixture showed complete inhibition of the growth of the four tested pathogenic bacteria. That growth inhibition was found to be equivalent to standard commercial hydro-alcoholic sanitizer containing 70% isopropanol (control 1). On the other hand, diluted alcoholic formula containing 30% isopropanol and 70% water without incorporation of eugenol or cinnamaldehyde (control 2) showed weak effect on the growth of the tested pathogenic bacteria where the count ranged between 6.1-7.7 log cfu/ml. In the absence of any content of alcohol or antimicrobial booster in the formula (control 3), which is just an aqueous phase, the pathogens were proliferated freely reaching their maximum count of 9.4 log cfu/ml (control 3).

4. Discussion

4.1. Maximum alcohol dilution and miscibility of eugenol and cinnamaldehyde

Short chain alcohols like ethanol and isopropanol which are used for sanitization settings are perfectly miscible with water (via hydrogen bonding) at all proportions. Therefore, dilution of absolute alcohols with water to make 70% commercial hydro-alcoholic sanitizers should not represent a technological problem.

In the current investigation the authors aimed at reducing alcohol content in sanitizers by applying more dilution with water. In order to compensate for alcohol dilution below 70%, the alcoholic sanitizers were fortification with some antibacterial boosters which are found in essential oils of aromatic plants, like eugenol and cinnamaldehyde. These oily components are perfectly miscible with the alcohol (isopropanol) but practically immiscible with water Therefore, to design a diluted hydro-alcoholic sanitizer containing a hydrophobic oily component, the maximum solubility (miscibility) of each one of these components in different dilutions of the hydro-alcoholic formula must be considered.

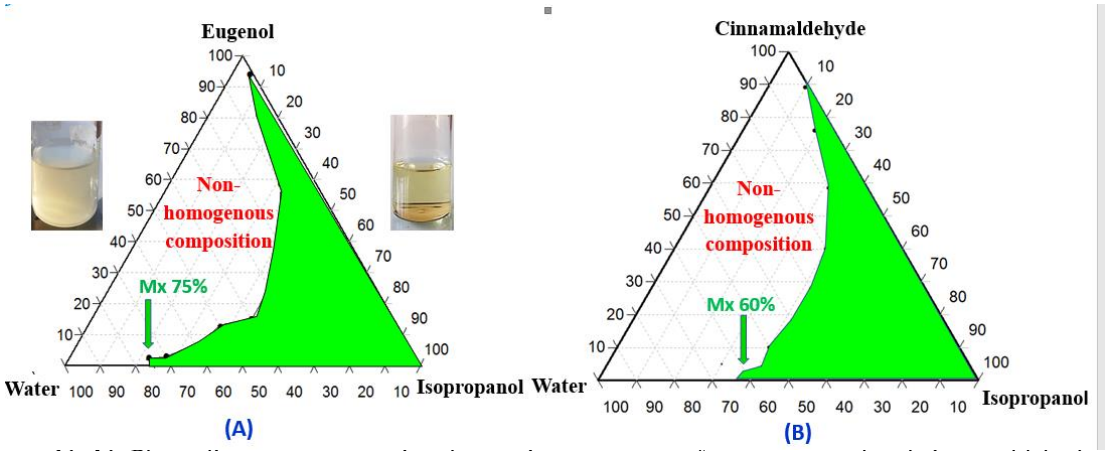

Figure 1(a,b). Phase diagrams representing the maximum amount of water, eugenol and cinnamaldehyde that can be incorporated in different dilutions of hydro-isopropanol formulas

Table 1. Antibacterial evaluation of diluted (28.5%) hydro-isopropanol sanitizer containing 70% water and fortified with eugenol, cinnamaldehyde and their mixture as antibacterial boosters.

	Antibacterial boosters and controls					
	Eugenol (1.5 %)	Cinnamaldehyde (1.5%)	Mixture* (1.5%)	Control (1)	Control (2)	Control (3)
Bacterial strain	Bacterial count (log cfu/ml)					
L. monocytogenes	0.0^{C}	0.0^{C}	0.0^{C}	0.0^{C}	$6.16^{B} \pm 0.26$	$9.30^{A} \pm 0.44$
S. typhimurium	0.0^{C}	0.0^{C}	0.0^{C}	0.0^{C}	$6.12^{B} \pm 0.15$	$9.25^{A} \pm 0.12$
E. coli	0.0^{C}	0.0^{C}	0.0^{C}	0.0^{C}	$6.41^{B} \pm 0.18$	$9.32^{A} \pm 0.36$
P. aeruginosa	0.0^{C}	0.0^{C}	0.0^{C}	0.0^{C}	$7.7^{B} \pm 0.49$	$9.45^{A} \pm 0.94$

*: Mixture of eugenol and cinnamaldehyde at 1:1 weight ratio
Control (1): 70% isopropanol, 30% water (standard alcoholic sanitizer).
Control (2): 30% isopropanol, 70% water.
Control (3): 100% water.
Data is represented as mean ±SD of duplicates of each treatment
All percentages are weight %.
Means with different superscripted letters are significantly different at $p<0.05$.

This task was already fulfilled in the results section by investigation of their solubility behaviours using the phase diagrams (Fig. 1a,b). From the results section it could be concluded that the maximum water dilution of the alcoholic sanitizer depends on the type of the antibacterial booster which is used to compensate for the reduction of alcohol content.

For instance, in case of eugenol containing sanitizer, 22.5% diluted alcohol with 75% water can be formulated to carry up to 2.5 eugenol in a homogenous formula. When eugenol is replaced with the same percentage of cinnamaldehyde (2.5%), more alcohol (37.5%) and less water (60%) are needed to formulate the homogenous formula (data extracted from the phase diagram Fig1b). The reason behind this finding is the difference in the inherent water solubility of eugenol and cinnamaldehyde which was found to be (2.46g/L) and (1.35 g/L) at 25°C, respectively [37,38].

It is worth noting that, the extent of alcohol dilution in case of sanitizers containing eugenol and cinnamaldehyde is considered to be higher than that of carvacrol which we previously investigated [32] for the same purpose. That is also due to the low inherent water solubility of carvacrol (0.330g/L) compared to eugenol (2.46g/L) and cinnamaldehyde (1.35 g/L).

4.2 Antimicrobial activity

In the current section the authors investigated the antibacterial activity of these diluted alcoholic sanitizers in comparison with a standard commercial 70% alcoholic sanitizer. For this purpose, two diluted alcoholic formulas containing 70 wt% water, 28.5wt% isopropanol and 1.5 wt% of eugenol, cinnamaldehyde and their mixture (at equal weight) were evaluated. The chosen amount of eugenol and cinnamaldehyde (at 1.5%) is based on the well known powerful antibacterial activity of these components which made the authors to run the evaluation at relatively low percentage as 1.5% instead of 2.5% which is the maximum load at high water dilution (see results section).

Table (1) indicate that a diluted (28.5%) hydro isopropanol sanitizer containing 70% water and 1.5% eugenol, cinnamaldehyde and their mixture (at equal weight) can completely inhibit the growth of the four tested pathogenic bacteria equivalent to that of the standard 70% alcoholic sanitizer (control 1).

We would like to draw the attention of the reader that the four tested bacteria fall under the food and/or water borne pathogenic category. The contamination cycle of these pathogens can also involve hard surfaces like food cutting boards, kitchen and bathroom floors, hospital floor and medical examination setting. That makes the developed diluted alcoholic sanitizer part of the antibacterial controlling regime.

The relatively high growth inhibitory activity of cinnamaldehyde, eugenol and their mixture against the tested pathogens can be attributed to the mechanism by which each individual component can exert its activity. That is generally include destruction of cell membrane, depletion of proton motive force and down regulation of some gene related to membrane proteins formation. For example, regarding cinnamaldehyde, the mechanisms which were proposed related all to interaction of this compound with the cell membrane of the pathogens which leads to perturbation and disperse of the proton motive force [39,40]. Inhibition of some biosynthetic enzymes like amino acid decarboxylase is another mechanism by which cinnamaldehyde can perform its antimicrobial activity [41].

Regarding eugenol, this compound inhibits bacterial growth on the genetic level by down regulation of important membrane proteins like (yidC) [42]. In addition, eugenol has the ability to inhibit the FtsZ protein which is encoded by the ftsZ gene leading to the disruption of bacterial cell division [43]. Eugenol can also act against some bacterial enzymes like ATPase and others [44], beside alteration of the cell membranes which affect transport of ions and ATP of different pathogens [40].

The question now is that, can the developed formula containing eugenol, cinnamaldehyde or their mixture be used to decontaminate any objects including hands or other parts of the skin or it should be restricted only to hard surfaces and medical equipment? We raise this question due to some suspected skin irritation or allergic reactions associated with eugenol [45] and cinnamaldehyde [46,47]. Surprisingly, despite this fact, these two compounds are approved as perfumery materials that are used in products applied directly on the skin as in fragrances, deodorants, creams and other relevant cosmetic products [48]. In addition, eugenol and cinnamaldehyde are already used in some topical application in dermatological drugs to treat fungal infections [49]. The essential oils that bear large quantities (>80%) of eugenol and cinnamaldehyde are also used as skin penetration enhancers to facilitate transdermal delivery of drugs [50]. Interestingly, despite its skin irritation properties, eugenol showed promising activity in treatment of dermatitis especially after formulation in polymeric nanocarriers [51]. Similarly, cinnamaldehyde was topically applied on the skin to enhance wound healing from infections of pathogenic bacteria like P. aeruginosa [52].

From the above mentioned cons and prose of applying eugenol and cinnamaldehyde directly on the skin via different topical pharmaceutical products, we can conclude that the dermal side effects of these components depend on the concentration used. In our study we get complete eradication of the four tested pathogenic bacteria at 1.5% of both compounds. Therefore, a complementary dermatological study is required in order to verify the safety of the developed formula on the skin.

In this concern we would like to remind the reader that even the commercial 70% standard hydro alcoholic sanitizer can also induce some side effects on the skin manifested as skin dehydration and flaking beside other skin disorders [53]. However, this issue was overcome by fortifying the 70% commercial alcohol formula with some humectant and emollients like aloe vera and glycerine. These additives may potentially perform the same protective effect to alleviate the skin reaction (if any) toward eugenol and cinnamaldehyde. However, that should be verified practically in a future dermatological complementary study.

Conclusion

A highly diluted hydro-alcoholic sanitizing formula with minimum content of alcohol, as 22.5%, and 37.5% was developed carrying eugenol and cinnamaldehyde, respectively, as antimicrobial boosters. Both dilute alcoholic formulas were able to completely inhibit the growth of four pathogenic bacteria as efficient as standard 70% alcoholic sanitizer. The diluted alcoholic formula can slow down alcohol depletion from the stock, cut the overuse of alcohol in sanitizers which develop alcohol-tolerant bacteria, decrease alcohol emission to the environment and lower the risk of alcohol toxicity upon incidental ingestion. The formulas can be considered for controlling the growth of some pathogenic bacteria on hard surfaces which represent part of pathogenic bacterial contamination cycle. Application as hand sanitizer is under investigation through a separate dermatological study to ensure skin safety.

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Author's contributions

We hereby confirm that the authors H. Shabaan and S. Abdelhamid were responsible for the antimicrobial evaluations and the third author A. Edris was responsible for the formulation process and designing the phase diagrams. The three authors contributed equally in the conceptualization of this study, data curation, writing and revising of the article to put it in the final legible format.

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Data Availability Statement

Available from the corresponding author Amr Edris

Conflict of interests

The authors declare that they have no competing interests of any kind.

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