Egypt. J. Chem. **Vol. 64,** No. 5 pp. 2493 - 2498 (2021)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Boosting the Antimicrobial Activity of Highly Diluted Aqueous Alcoholic Sanitizers by Fortification with Essential Oil Components: I- Carvacrol

Hamdy A. Shaaban[a], Amr E. Edris[a,*], Asmaa A. Ramadan[b]

[a]*Aroma & Flavor Chemistry Department, National Research Center, Dokki, Cairo, Egypt*

[b]*Nutrition & Food Science Department, National Research Center, Dokki, Cairo, Egypt*

Abstract

The current study aims at developing alcoholic-based sanitizing formula against some pathogenic bacteria based on highly diluted (< 70.0%) aqueous isopropanol solution which is fortified with small amount of an essential oil component like carvacrol. This phenolic compound can act as antimicrobial booster for the compensation of alcohol reduction in the sanitizer. The solubility behaviour of carvacrol in the aqueous alcoholic solution containing different ratios of isopropanol and water was investigated using the Gibbs' phase diagram. Based on that, a selected formula containing 1.0% carvacrol dissolved in the highest possible dilution of isopropanol (45.0%) was chosen for testing its antimicrobial activity against some gram positive and negative pathogenic bacteria in comparison to the standard 70.0% alcohol formula without carvacrol. Results indicated that a 45.0% aqueous isopropanol solution can hold up to 5.0% carvacrol at maximum in a physically stable and homogenous sanitizing formula. Antimicrobial evaluation of the developed diluted aqueous isopropanol fortified with 1.0% carvacrol showed the same inhibition against the tested pathogenic bacteria as the standard 70.0% alcohol sanitizer without carvacrol. In conclusion, 45.0% diluted isopropanol fortified with a minimum of 1.0% up to 5.0% carvacrol (in case of intensive sanitization) can possibly be used as sanitizer for protection against some pathogenic bacteria. The practical significance of this study is the production of diluted alcoholic sanitizing formula for decontamination of surfaces against some pathogenic bacteria with the advantage of increasing alcohol dilution in order to spare the absolute alcohol reserve for more quantitative production of the sanitizer.

Key words: Alcoholic sanitizers; alcohol reduction; essential oils; pathogenic bacteria; carvacrol.

1. Introduction

Pathogenic microorganisms generally exist in the environment and can transfer to the human body through food, water or air causing serious health and economic problems. Sanitizers and disinfectants are considered to be a chemical approach to protect against these environmental pathogens which can adhere to different objects. That includes hard surfaces and human skin especially the hands. Sanitizers and disinfectants usually belong to different chemical classes including organic acids, aldehydes, peroxides, phenols, halogens, quaternary ammonium salts, iodophors and biguanides [1,2].

In addition to these decontaminating chemicals**,** short chain alcohols like ethanol and isopropanol can also be used in sanitization due to their wide spectrum antimicrobial activity against pathogenic bacteria, fungi and viruses [3]. Short chain alcohols perform their sanitization/disinfection activities by interaction with microbial cell wall causing denaturation of proteins and alteration of cell metabolism leading to cell lyses and death [4,5]. Therefore ethanol and isopropanol are commonly used in decontamination and sanitization of hands and some medical equipments at hospitals like stethoscopes [6,7]. Nowadays many alcohol-based hand sanitizers are available in the market to protect against different bacterial and other microbial pathogens especially the new comer, COVID-19 virus [8]. This recent crisis dictates frequent use of alcohol-based sanitizers at shorter time intervals

*Corresponding author e-mail: amr_edris@hotmail.com and amredris07@gmail.com.
Receive Date: 14 January 2021, Revise Date: 26 January 2021, Accept Date: 07 February 2021
DOI: 10.21608/EJCHEM.2021.57361.3249

during the day. As a result, the demand on ethanol and isopropanol is severely increased to a degree that creates a shortage of these alcohols in the markets. One of the approaches to relief that issue lies in the additional production of these alcohols in order to compensate for the rabid depletion of the existing reserve. However, that can be out of reach to many societies which have not enough facilities or resources to increase their alcohol production.

Another possible approach which is proposed for the first time in the current investigation is to develop sanitizing formula based on highly diluted aqueous alcoholic solution (< 70%) which is fortified with small amount of certain essential oil component to act as a booster for compensation. Therefore, the available reserve of pure alcohols can be spared and last for a longer period of time to serve more population compared to the standard 70% alcohol formula which relatively depletes the alcohol reserve faster.

Carvacrol is chosen in the current investigation as a phenolic sanitizer that can boost the antimicrobial activity of the proposed diluted alcohol-based formula. This compound exists as a major constituent in renewable natural resources like the essential oil of aromatic plants which belong to genus *Origanum* [9]. Its antimicrobial activity against pathogenic bacteria is well studied and proved efficiency in sanitization, disinfection, food preservation, and even wound healing [9-12].

Based on that, the current study investigated the solubility behaviour of carvacrol in different diluted isopropanol solutions. That can consequently help in determining the highest alcohol dilutions that can accommodate the maximum amount of carvacrol in a homogenous sanitizing formula. Then, the chosen formula will be evaluated for antimicrobial activity against some pathogenic bacteria in comparison to the standard 70% isopropanol solution in order to evaluate the efficiency of the developed diluted alcoholic sanitizer that contains carvacrol. Justification of using isopropanol as model alcohol instead of ethanol will be also discussed.

2. Experimental

Materials and microbial stains

Carvacrol (98%) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Absolute isopropanol was obtained from Fischer Scientific (Leicestershire, UK). Water used to prepare the different alcohol dilutions was distilled and sterilized using the local equipments available at the authors' lab. The microorganisms (pathogenic bacteria) which were used in the current investigation were provided from the culture collections of the Microbiological Department National Research Center (NRC) Dokki, Giza, Egypt. These include one strain of Gram-positive *Listeria monocytogenes* (ATCC 35152) and two strains of Gram-negative bacteria *Salmonella typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 27325).

Solubility behaviour of carvacrol and construction of the phase diagram

The phase diagram of a three-component system composed of carvacrol, isopropanol and water was constructed as previously reported [13] with the substitution of the surfactant in the reference with isopropanol in the current study. In details, carvacrol was mixed with absolute isopropanol in different glass vials at predetermined volume ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9, respectively. Each vial containing carvacrol/isopropanol was titrated with distilled water at percentages starting from 10% to 99% of the volume of the three components, followed by vortexing for 1 minute. The vials were left for at least 30 minutes at room temperature to equilibrate between the titration of each batch of water. The percentile (by volume) of carvacrol, isopropanol, and water corresponding to each volume ratio of the three components were calculated and then represented as points on the Gibbs' triangle phase diagram. Then, a border line passing through all these points was drawn to separate the phase diagram into two main solubility zones. The first one lies on the right side of the boarder line at which the three components are perfectly mixed in a homogenous isotropic phase. On the other hand, the left zone of the border line represents a cloudy or hazy heterogeneous phase where the three components are not perfectly mixed.
The phase diagram was constructed twice in order to be sure of the position of the boarder line and the solubility regions of carvacrol in the diluted isopropanol.

Antimicrobial Evaluation against some pathogenic bacteria

The agar well diffusion method [14] was employed for the determination of antibacterial activities. In details: 0.1 ml of the diluted inoculums (10^7 CFU/ml) of test pathogen was spread on tryptone soy agar (TSA) plates. Wells of 5 mm diameter were punched into the agar medium and filled with 50 µl of the tested formula containing carvacrol. The same was repeated using 70%

standard isopropanol solution. Plates, which were prepared in triplicates, were incubated at 37ºC for 24h before recording the diameter (mm) of the inhibition zones of each tested formula, which was taken as a measure for the antibacterial activity.

Statistical analysis

Results obtained from evaluating the antimicrobial activities of the carvacrol-containing formula and 70% isopropanol are expressed as mean inhibition zone diameter (mm) ±SD. Data was analyzed using SPSS software (Version 22). Mann Whitney's non-parametric test was used to compare means of each formula for the three strains of the pathogenic bacteria. Means with p values less than or equal to 0.05 were considered significantly different.

3. Results and Discussion

The theme of the current investigation is to design and evaluate an antibacterial sanitizing formula that contains highly diluted alcohol (less than 70%), without compromising the efficiency. This trend is deemed by the authors to be beneficial in slowing down the attrition of pure alcohol reserve, especially these days where the world is witnessing the pandemic of Covid -19 virus. Due to that crisis, the demand on alcohol reached its climax to make alcohol-based sanitizers which creates a fast attrition in supply. However, such a highly diluted alcoholic formula will axiomatically be less effective against pathogenic microorganisms compared to the 70% alcoholic formula which is the standard that is imposed by WHO and CDCP [15,16]. Therefore, the authors planned to compensate the deficiency of alcohol in the developed formula by fortification with trace amounts (1.0%) of one of the plant-based volatile phenolic compounds like carvacrol. The choice of this compound is based on its versatility as a natural antimicrobial agent as was previously reviewed in the introduction section.

The challenge that is expected to confront the development of the proposed formula is: first, how dilution that isopropanol can reach in order to be able to hold the maximum amount of carvacrol in a stable homogenous sanitizing formula? Second, what will be the antibacterial efficiency of the proposed diluted alcoholic formula that contains only 1.0% carvacrol compared to that of the standard 70% alcohol? Therefore, the study subjected these issues to investigation by constructing a phase diagram which represents the solubility behaviour of the three components carvacrol, isopropanol and water (Fig. 1), followed by an *in-vitro* antimicrobial activity evaluation.

Alcohol dilution and solubility behaviour of carvacrol on the phase diagram

The reader should take into consideration that both water and isopropanol (in absence of carvacrol) are perfectly soluble into one another at any proportions. However, the presence of carvacrol as a third ingredient in the formula will change the solubility behaviour of the three components because carvacrol is generally immiscible (or scarcely soluble) in water. Its reported solubility value is 0.125g/100 ml water, which is equivalent to 0.01M [17]. However, to our knowledge there is no available solubility data of carvacrol in different dilutions of aqueous isopropanol solutions. Therefore, the authors have to investigate that subject practically by plotting a Gibbs' triangle phase diagram of the three components carvacrol, isopropanol and water (Fig. 1), which all together comprise the proposed sanitizing formula.

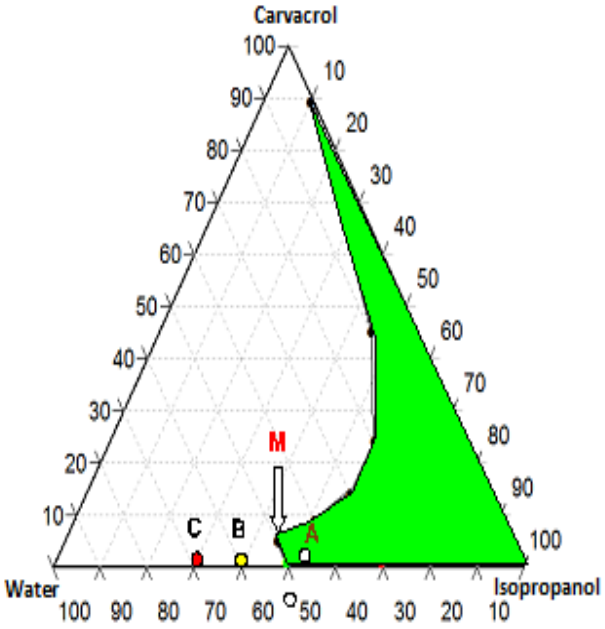

Fig. 1. Phase diagram representing the solubility behaviour of carvacrol in different diluted isopropanol solution.

Results obtained from that figure indicate that there are two solubility zones that can be distinguished on the phase diagram, the first is the green zone which represents an isotropic homogenous phase where the three components of the formula can exist together in perfectly soluble state. Beside that, there is a second solubility region (white zone) on the left boarder of the first one which represents a heterogeneous phase where the three components are not soluble and appear as cloudy or

separated phases. The reader can infer from Fig. (1) that the maximum possible alcohol dilution in the presence of carvacrol is limited to 45.0% isopropanol, 50.0% water, and up to 5.0% carvacrol (refer to the arrow "M" which points to that composition on the phase diagram). This composition can lead to the formation of a homogenous carvacrol-containing diluted isopropanol formula (45.0%) where all three ingredients are perfectly soluble to form one stable homogeneous phase. On the other hand, if isopropanol is further diluted below 45.0%, it can no longer hold 5.0% carvacrol (or even less) due to the hydrophobicity of this compound, and the whole formula will be shifted outside of the green zone indicating a heterogeneous cloudy or separated formula (refer to Fig 1). This finding indicates that 45.0% is the maximum dilution of isopropanol that can be reached in the presence of up to 5.0% carvacrol.

In-vitro antimicrobial evaluation of the diluted alcoholic solution containing carvacrol

Before interpreting the results of antimicrobial activity in Table (1), we would like to indicate that isopropanol was chosen in the current study as a model of sanitizing alcohol instead of ethanol due to its higher germicidal activity which was indicated in early studies [18] as well as more recent studies [6]. In addition, isopropanol at 70% showed the same bacterial count reduction as 0.5% chlorohexidene after immediate post-wash hand effect indicating high antibacterial activity [19]. Moreover, isopropanol is also used for the preservation of some cosmetics against the growth of spoilage and pathogenic bacteria [20], which could make this study also useful in this concern.

The antimicrobial activity of the reduced-alcohol formula containing 45% isopropanol, 50% water, and 5.0% carvacrol (as revealed from the phase diagram), is supposed to be evaluated. However, based on our previous experience with the potent antibacterial activity of carvacrol against pathogenic bacteria [13] we can affirm that 5.0% is too high for running an agar diffusion evaluation. Therefore, the evaluation was conducted at only 1.0% carvacrol incorporated in 49% isopropanol and 50% water. This composition was represented on the phase diagram as point "A" which lies inside the green zone (Fig. 1) where the three components are mixed homogenously. The reader can notice that we did not used 45% isopropanol (although it is valid to give a homogenous formula up to 5.0% carvacrol) only because the percentile of the three components represented on the phase diagram should be 100%.

From Table (1) we can conclude that 49% isopropanol fortified with 1.0% carvacrol has the same antibacterial activity as the standard 70% alcoholic solution. Moreover, in case of *S. typhimurium* the antimicrobial activity of the formula exceeded that of the 70% alcohol. This high antimicrobial activity of the diluted alcoholic formula is due to the effect of carvacrol which is a well known antimicrobial active compound. The activity originates from the hydrophobicity of this compound which is expressed as the octanol/water partition coefficient (*Log P*) [21]. This value is a measure for the ability of carvacrol to partition into the cytoplasmic membrane, which is the primary site of the toxic action of any antimicrobial compound [22]. Log *P* for carvacrol was reported to be 3.52 [21] which is considered hydrophobic enough for partitioning into bacterial outer membrane. That will lead ultimately to decreases the membrane's integrity and increase the proton passive flux across the membrane, leading to cell death.
The same authors [21] also indicated that carvacrol can be more effective against pathogenic bacteria than other plant-based volatile phenolics like eugenol. That is due to the high hydrophobicity of carvacrol (log *P* 3.52) compared to that of eugenol (log *P* = 2.73), which illustrate the importance of hydrophobicity for demonstrating higher antimicrobial activity.

Beside hydrophobicity, there is another important factor that must be considered when taking about the distinctive antibacterial activity of carvacrol. This factor is the unique chemical structure of carvacrol in which the pair of electrons on the hydroxyl group of carvacrol is conjugated with the 6 π electrons of the benzene ring making delocalized electron's effect [23]. This effect is important for the antimicrobial activity of phenolic compounds, such as carvacrol, thymol and phenol. Therefore, this compound acts as proton exchanger, thereby reducing proton gradient across the cytoplasmic membrane. The resulting collapse of the proton motive force and depletion of the ATP pool lead eventually to cell death [23]. On the other hand, Veldhuizen *et al.* [24] concluded that the hydroxyl group of carvacrol is not essential for activity but

Table 1
Antibacterial activity of diluted alcohol formula fortified with carvacrol.

Pathogenic bacteria	Inhibition zone (mm)		p-value
	Diluted isopropanol fortified with carvacrol*	Standard 70% isopropanol	
Escherichia coli	13.83±1.6	14.67±0.82	0.394
Listeria monocytogenes	18.33±1.21	19.17±1.17	0.31
Salmonella typhimurium	16.83±1.17	13.67±1.75	0.004**

* 49% isopropanol, 50% water and 1.0% carvacrol

** Means are significantly different for each formula at $p \leq 0.05$.

Results are expressed as mean ± S.D

does have special features that add to the antimicrobial mode of action of carvacrol.

The previously mentioned results encouraged us to evaluate the distinctive antimicrobial activity of 1.0% carvacrol in much highly diluted isopropanol solution such as 40.0% (60.0% water) or even 30.0% (70.0% water) in order to fulfil the claimed title of this investigation and to spare more absolute alcohol. However, from the formulation point of view using the phase diagram, the composition of these two highly diluted formulas (40.0%, 30.0% isopropanol and 1.0% carvacrol) lies outside the green zone (Fig.1 points "B" and "C", respectively). This means that these two formulas cannot hold 1.0% carvacrol in a homogenous formula due to the high hydrophobic nature of this compound.

Conclusion

Volatile phenolic compound like carvacrol at 1.0% can compensate for increased isopropanol dilution to 45% in an alcoholic sanitizing formula which was designed against some pathogenic bacteria. Such formula showed the same antimicrobial activity as the standard 70% alcoholic sanitizer with the advantage of sparing much absolute alcohol that can be used for making more sanitizers to the market. The described carvacrol-containing formula can be used for surface sanitization or hand sanitization after studying its skin sensitization safety.

Conflicts of interest

There are no conflicts to declare.

Formatting of funding sources

No funding source to declare

4. References

[1] Itiki, R. and Chowdhury, P., Fast deployment of COVID-19 disinfectant from common ethanol of gas stations in Brazil. *Health Policy and Technology* **9**, 384-390 (2020). doi: 10.1016/j.hlpt.2020.07.002

[2] Dvorak, G., Disinfection 101. Center for Food Security and Public Health. Iowa Stat University, (2008).

[3] Morton, E., Alcohols. In Book of Disinfection, Sterilization, and Preservation (3rd edition). eds. Bloch, S. S. pp. 225–239. Philadelphia: Lea & Febiger. (1983).

[4] Golin , A., Choi, D. and Ghahary, A., Hand sanitizers: A review of ingredients, mechanisms of action, modes of delivery, and efficacy against coronaviruses. *American Journal of Infection Control* **48**, 1062−1067 (2020). doi: 10.1016/j.ajic.2020.06.182

[5] Larson, L. and Morton, E., Alcohols. In Book of Disinfection, sterilization, and preservation (4th edition) eds. Block, S.S. pp. 191–203. Philadelphia: eds. Lea & Febiger (1991).

[6] Jokar, A. and Mohebbi, Z., Comparing the efficacy of alcohol isopropyl and ethanol on the reduction of contamination of medical check-up devices in children ward and neonatal intensive care unit (NICU). *International Research Journal of Pharmacy and Pharmacology* **1**, 075-078 (2011).

[7] Lecat, P., Cropp, E., McCord, G. and Haller, N., Ethanol-based cleanser versus isopropyl alcohol to decontaminate stethoscopes. *American Journal of Infection Control.* **37**, 241-243 (2009). doi:org/10.1016/j.ajic.2008.08.006

[8] Berardi, A., Perinelli, D., Merchant, H., Bisharat, L., Basheti, I., Bonacucina, G., Cespi, M. and Palmieri, G., Hand sanitizers amid CoViD-19: A critical review of alcohol-based products on the market and formulation approaches to respond to increasing demand. *International Journal of Pharmaceutics* **584**,119431 (2020). doi:org/10.1016/j.ijpharm.2020.119431

[9] Moeini, A., Pedram, P., Makvandi, P., Malinconico, M. and d'Ayala, G., Wound healing and antimicrobial effect of active secondary metabolites in chitosan-based wound dressings: A review. *Carbohydrate Polymers* **233**, 115839 (2020). doi:org/10.1016/j.carbpol.2020.115839

[10] Liu, F., Jin, P., Sun, Z., Du, D., Wang, D., Zhao, T. and Doyle, M., Carvacrol oil inhibits biofilm formation and exopolysaccharide production of *Enterobacter cloacae*. *Food Control* **119**, 10747 (2021). doi:org/10.1016/j.foodcont.2020.107473.

[11] Mira, M., Permana, A., Tekko, I., McCarthy, H. Ahmed, N. Rehman, A. and Donnelly, R., Microneedle liquid injection system assisted delivery of infection responsive nanoparticles: A promising approach for enhanced site-specific delivery of carvacrol against polymicrobial biofilms-infected wounds. *International Journal of Pharmaceutics* **587**, 119643 (2020). doi:org/10.1016/j.ijpharm.2020.119643.

[12] Porter, J., Morey, A. and Monu E., Antimicrobial efficacy of white mustard essential oil and carvacrol against *Salmonella* in refrigerated ground chicken. *Poultary Science* **99**, 5091-5095 (2020). doi:org/10.1016/j.psj.2020.06.027.

[13] Shaaban, H. and Edris, A., Factors affecting the phase behavior and antimicrobial activity of carvacrol microemulsions. *Journal of Oleo Science* **64**, 393-404 (2015). doi:10.5650/jos.ess14194

[14] Perez, C., Pauli, M. and Bazerque, P., An antibiotic assay by the well agar method. *Acta Biologiae et Medicine Experimentalis* **15**, 113–115 (1990).

[15] WHO, World Health Organization, Infection prevention and control during health care when novel coronavirus (nCoV) infection is suspected, Interim guidance 19 March; 2020, Geneva, Switzerland. (2020). https://www.who.int/publications/i/item/10665-331495.

[16] CDCP, Centers for Disease Control and Prevention, Statement for Healthcare Personnel on Hand Hygiene during the Response to the International Emergence of COVID-19; 2020, USA (2020). https://core.ac.uk/download/pdf/289148633.

[17] Yalkowsk, H. and Dannenfelser, M., Pubchem, National Library of Medicine. Available on the web at https://pubchem.ncbi.nlm.nih.gov/compound/Carvacrol (1992).

[18] Coulthard, C. and Skyes, G., Germicidal effect of alcohol. *Pharmaceutical Journal* **137**, 79–81 (1936).

[19] Aly, R. and Maibach, I., Comparative study on the antimicrobial effect of 0.5% chlorhexidine gluconate and 70% isopropyl alcohol on the normal flora of hands. *Applied and Environmental Microbiology* **37**, 610-613 (1979).

[20] Lens, C., Malet, G. and Cupferman, S., Antimicrobial activity of Butyl acetate, Ethyl acetate and Isopropyl alcohol on undesirable microorganisms in cosmetic products. *International Journal of Cosmetic Science* **38**, 476–480 (2016). doi: 10.1111/ics.12314.

[21] Ben Arfa, A., Combes, S., Preziosi-Belloy, L., Gontard, N. and Chalier, P., Antimicrobial activity of carvacrol related to its chemical Structure. *Letters in Applied Microbiology* **43**, 149–154 (2006). doi:10.1111/j.1472-765X.2006.01938.x.

[22] Sikkema, J., De Bont, J. and Poolman, B., Mechanisms of membrane toxicity of hydrocarbons. *Microbiology Reviews* **59**, 201–222 (1995).

[23] Ultee, A., Bennik, J. and Moezelaar, R., The phenolic hydroxyl group of carvacrol is essential for action against the food borne pathogen Bacillus cereus. *Applied Environmental Microbiology* **68**, 1561–1568 (2002). doi: 10.1128/AEM.68.4.1561-1568.2002

[24] Veldhuizen, E., Bokhoven, J., Zweijtzer, C., Burt, S. and Haagsman, H., Structural requirements for the antimicrobial activity of carvacrol. *Journal of Agriculture and Food Chemistry* **54**, 1874-1879 (2006). doi: 10.1021/jf052564y.