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Enhanced Antibacterial Efficacy of *Cymbopogon winterianus* Jowitt in Combination with Carvacrol

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Abstract

The continuous increase of antibiotic resistance and the lag in development of novel antibiotics are two important challenges in treatment of infectious diseases. Essential oils (EOs) and their combinations are promising alternatives for treatment of bacterial infections. *Cymbopogon winterianus* Jowitt EO, recognized by the European Medicines Agency for its sleep-enhancing and anxiety-relieving properties, also possesses sedative qualities, as noted in the German Commission E monograph, and aids in treating sleep disorders. The aim of the study was to investigate antibacterial activities of the *C.winterianus* EO and carvacrol combination. Commercial *C. winterianus* EO and carvacrol were tested for antibacterial activity against the American Type Culture Collection quality control strains *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Klebsiella pneumoniae* using the broth microdilution method. Ciprofloxacin served as the standard antimicrobial agent. Checkerboard assay was used to evaluate the combined effects of *C. winterianus* EO and carvacrol.

The results showed that *C. winterianus* EO exhibited moderate antibacterial activity against both Gram positive and Gram negative bacteria. The interaction between *C. winterianus* EO and carvacrol was additive against all tested bacteria. The minimum inhibitory concentration of carvacrol decreased by 2- to 8-fold when the carvacrol combined with *C. winterianus* EO, and carvacrol also enhanced the antibacterial activity of EO by 2- to 16-fold.

Keywords

Antibacterial, carvacrol, combination, *Cymbopogon winterianus*, essential oil, synergy.

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INTRODUCTION

Cymbopogon winterianus Jowitt is a member of the Poaceae family and is grown in South and Central America. The main constituents of *C. winterianus* essential oil (EO) which is known as Java citronella oil are geraniol, citronellol, and citronellal. These compounds are widely utilized in soap, fragrance, cosmetic, and flavoring industries worldwide (Wany et al., 2013). Citronella oil has diuretic, febrifuge, antimicrobial, and antispasmodic properties (Simic et al., 2008). There are many products that contain the oil which are used as biopesticides (Munda and Lal, 2020). In trade, the EO is divided into two categories: Java type citronella oil, which is derived from *C. winterianus* (superior type), and Ceylon type citronella oil, which is derived from *Cymbopogon nardus* (inferior type). Both oils that were medicinally used in the ancient and modern eras consist of different secondary metabolites such as terpenoids and alkaloids (Wany et al., 2013). Tropical or subtropical parts of Asia, Africa, and America are known as plant cultivation areas. Taiwan, Guatemala, Honduras, Malaya, Brazil, Ceylon, India, Equador, Madagascar, Mexico, and the West Indies remain as the largest producers for Java citronella oil (Shasany et al., 2000).

Carvacrol ($C_{10}H_{14}O$), known as 2-methyl-5-[1-methylethyl] phenol, is a phenolic monoterpenoid and a derivative of cymene that naturally is found in EOs (Imran et al., 2022). Commercial carvacrol is produced using biotechnological and chemical processes. Carvacrol is a lipophilic substance with a density of 0.976 g/ml at ambient temperature (25 °C); it is soluble in ethanol, acetone, and diethyl ether but insoluble in water (Yadav et al., 2009). Numerous biological activities of carvacrol including antiviral (Sánchez et al., 2015), antibacterial, antifungal (Nostro et al., 2004), antioxidant (Milos et al., 2012), and anticarcinogenic (Ozkan and Erdogan, 2011) effects. Although agar dilution, well or disk diffusion methods are used for the detection of antibacterial activities of natural products, broth microdilution technique remains as the golden standard method.

Combination studies are increasingly attracting attention in the scientific area. When drugs are tested in combinations, they can exhibit varying pharmacodynamic interactions such as synergy, additivity, antagonism, or indifference. Checkerboard assay in a 96-well microplate provides an effective assessment of the combined effects of natural products (Berenbaum, 1978; Doern et al., 2010). In the checkerboard test, two samples are tested in double serial dilutions, and the concentration of each drug is tested

individually and together. Therefore, it is possible to ascertain the impact of each individual medication, but also most importantly, the impact created by their combination (Berenbaum, 1978). Checkerboard analysis is utilized to measure synergy and determine how the combined effects of samples compared to their individual effects on potency.

In this study, it was aimed to investigate the antibacterial activity of *C. winterianus* and its interaction with carvacrol.

MATERIALS AND METHODS

American Type Culture Collection (ATCC) strains of *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 700603 were included in the study. All bacteria were deposited at the Faculty of Pharmacy, Eastern Mediterranean University, at – 85 °C and were refreshed prior to the assays.

Commercial *C. winterianus* EO was kindly supplied by Doallin Ltd., Istanbul. Ciprofloxacin and carvacrol were obtained from Sigma Aldrich (Germany). The properties of *C. winterianus* EO are given in Table 1.

Table 1: The properties of *C. winterianus* essential oil (Technical Data from Doalinn- Orlife Global Ltd.).

Organoleptic Properties	
Appearance	Clear mobile liquid
Color	Pale yellow to brownish
Odor	Citronellal-like, lemon, grassy
Physico-Chemical Properties	
Density at 20 ºC	0.880-0.895
Refractive index at 20 ºC	1.4650-1.4750
Flash point	94
Rotatory power	-5 / 1
Main Components (>0.5% by GCMS)	
Sulcatone	0.727
Citronellal	26.059
Linalool	0.908
Neoisopulegol	0.747
Caryophyllene	3.006
Citronellyl acetate	1.480
Neral	8.215
Geranial	10.907
Geranyl acetate	1.304
Delta cadinene	0.788
Citronellol	15.294
Geraniol	26.335

Antibacterial Activities and Checkerboard Assays

Broth microdilution method was used to investigate antibacterial activities of *C. winterianus* EO and carvacrol as suggested by Clinical and Laboratory Standards Institute (CLSI) with minor modifications (CLSI, 2023).

Stock solutions at the concentration of 1024 mg/mL were prepared using dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany). These solutions were then diluted using Mueller Hinton broth (MHB), adding Tween 80 when necessary. Two-fold serial dilutions of the compound and the EO were prepared in 96-well microplates. Each well was filled with 100 µL of the dilution and 10 µL of each bacterium. The final concentrations of the DMSO in the well was ≤3%. Each bacterium were included in the wells at the final concertation of 5 x 10^5 cfu/mL. Additionally, wells containing 3% DMSO with the bacterial inoculum and those with the compound or EO alone served as positive and negative controls, respectively. Ciprofloxacin was included as the reference antibacterial agent.

The microplates were incubated at 37 °C under an aerobic environment for 16 to 20 hours. The minimum inhibitory concentrations (MICs) of the compound and EO were identified as the lowest concentrations that visually inhibited bacterial growth. MIC was further confirmed by addition of 10 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) at the concentration of 5 mg/mL.

The antimicrobial activity of binary combinations of *C. winterianus* EO and carvacrol was assessed using checkerboard assay following CLSI guidelines with slight modifications (CLSI, 2023). The compounds and EOs were prepared at concentrations eight times greater than their MICs. Two-fold decreasing serial dilutions of the compound were combined with two-fold increasing serial dilutions of the EO, each at 50 µL. The first rows and columns of the plate included only either the compound or the EO. Finally, 10 µL of bacterial suspension at the concentration of 5 x 10^6 cfu/mL was added to each well.

Fractional inhibitory concentration index (FICI) was calculated by adding the FICs of the compound and the EO. The FIC for the compound was determined as the ratio of its MIC in combination to its MIC when tested alone; while the FIC for the EO was the ratio of its MIC in combination to its MIC alone

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FICI was calculated using the formula below:

$$\text{FICI} = \frac{\text{MIC } carvacrol \text{ (in the combination)}}{\text{MIC } carvacrol \text{ (alone)}} + \frac{\text{MIC } EO \text{ (in the combination)}}{\text{MIC } EO \text{ (alone)}}$$

The interaction between carvacrol and the EO was classified as; synergistic when FICI ≤ 0.5, additive when 0.5 < FICI < 1, indifferent when 1 ≤ FICI ≤ 4, and antagonistic when FICI > 4 (Berenbaum, 1978). All of the experiments were performed in triplicates.

RESULTS

Antibacterial Activities of *C. winterianus* EO and Carvacrol

The MICs of the EO of *C. winterianus* against *E. coli, S. aureus,* and *E. faecalis* were 8 mg/ml whereas the MIC was 4 mg/ml against *K. pneumoniae*. The MIC of carvacrol was 0.25 mg/ml against all of the strains tested. *C. winterianus* EO and carvacrol had lower antibacterial activity than ciprofloxacin (Table 2).

Table 2: MICs (mg/mL) of carvacrol and *C. winterianus* essential oil.

Species	*C. winterianus* (mg/ml)	Carvacrol (mg/ml)	Ciprofloxacin (mg/L)
E. coli	8	0.25	0.008
K. pneumoniae	4	0.25	0.015
S. aureus	8	0.25	0.125
E. faecalis	8	0.25	0.5

Antibacterial Interactions between *C. winterianus* EO and Carvacrol

In the present study, the interaction between *C. winterianus* and carvacrol was found to be additive against the Gram positive and Gram negative bacteria tested. Synergistic, indifference and antagonistic interactions were not detected.

Combinations tested against the bacteria and the FICI results of the best combinations are given in Table 3.

Table 3: FICI of *C. winterianus* essential oil and carvacrol combinations.

Species	Concentrations for the combination (mg/mL)		FICI (Interaction)*
	C. winterianus	Carvacrol	>0.5
E. coli	1	0.125	0.625 (A)
K. pneumoniae	0.25	0.125	0.562 (A)
S. aureus	2	0.125	0.75 (A)
E. faecalis	4	0.03	0.62 (A)

*A: Additive.

When *C. winterianus* oil and carvacrol were individually tested against the bacteria, it was observed that they were effective at relatively high concentrations, contrary to their combinations. The activity of EO against *K. pneumoniae*, *E. coli*, *S. aureus*, and *E. faecalis* was increased with carvacrol addition by 16-, 8-, 4-, and 2-fold, respectively. In addition, *C. winterianus* increased the efficacy of carvacrol 2-fold against *E. coli*, *K. pneumoniae*, *S. aureus* and 8-fold against *E. faecalis* (Table 3).

DISCUSSION

In a previous study, EO of *C. winterianus* showed antibacterial activity against *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Salmonella* Typhimurium, and *C. albicans* (Verma et al., 2020). In another study, *C. winterianus* EO was reported to reveal moderate antibacterial activity against *Bacillus cereus*, *Micrococcus luteus*, and *S. aureus* but less activity against Gram negative bacteria. The MIC of *C. winterianus* against *S. aureus* was 2 µL/mL. MICs against Gram negative bacteria including *E. coli*, *Proteus mirabilis*, and *Salmonella* Enteriditis were 4-6 µL/mL (Simic et al., 2008). The activity of EO of *C. winterianus* against fifteen strains of *C. albicans* was determined by MIC, minimum fungicidal concentration (MFC) and time-kill methods. The oil showed antifungal activity against *C. albicans*. MIC of the oil ranged from 78 to 625 µg/mL, whereas MFC was in between 312 and 1250 µg/mL (Oliveira et al., 2011). It was also noticed that geraniol, one of the main components of *C. winterianus* EO, presented many pharmacological properties including antibacterial and antifungal activity (Lira et al., 2020). Combination studies using geraniol and different EOs have been carried out. The most commonly used technique in these studies was checkerboard assay. Combinations of geraniol and several standard antibiotics such as norfloxacin and chloramphenicol were found to be synergistic for majority of the strains tested. Antagonism was reported with tetracycline (Lira et al., 2020). However, the combination studies are limited. Collectively, findings of the present study were promising against *S. aureus*, *E. faecalis*, *E. coli* and *K. pneumoniae*. Combinations of both EO and carvacrol additively increased the antibacterial activity of one another.

Consequently, the additive effect of EO and carvacrol appears promising, potentially offering new strategies against the tested

bacteria. Nevertheless, additional *in vivo* studies are necessary to explore the effectiveness of combinations of EOs with antibiotics as well as to assess different formulations.

CONCLUSION

As a result, the combined use of EOs and carvacrol is promising. Since the oral use of EOs is limited, studies on externally used products containing these combinations can be planned in the future.

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