

## Antibacterial Effect of Combination of Cinnamon Essential Oil and Thymol, Carvacrol, Eugenol, or Geraniol

### Abstract

Bacterial resistance to classic antibiotics is an alarming rate to put this into control with the use of natural products of plant derivatives. The objective of this study was to determine the phytochemical of cinnamon essential oil (EO) and to evaluate its antibacterial activity alone and in combination with some main components of EOs such as thymol, carvacrol, eugenol, or geraniol against three bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*). The phytochemical analysis of cinnamon EO was evaluated using gas chromatography-flame ionization detector and gas chromatography-mass spectrometer analysis. The antibacterial activity of tested compounds was determined by agar disk diffusion and minimum inhibitory concentration (MIC) assays. The checkerboard method was used to quantify the efficacy of cinnamon EO in combination with those compounds. The results showed that the major compound in the cinnamon EO was trans-cinnamaldehyde (91.01%). Cinnamon oil was the highest antibacterial activity with MIC of 0.005, 0.005, and 0.02 mg/mL against *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively. Synergistic activity was shown only against *S. aureus* by the combination of cinnamon EO and thymol. The additive effect was found against *E. coli* when cinnamon EO was combined with thymol or carvacrol, and against *S. aureus* when cinnamon EO was combined with carvacrol. However, the combination of EO and thymol or carvacrol showed an indifference action against *P. aeruginosa*. The combination of cinnamon EO with thymol or carvacrol can be used as an alternative therapeutic agent for medical application and as a natural preservative.

**Keywords:** Antibacterial activity, cinnamon, combination, gas chromatography-mass spectrometer analysis

### Introduction

Antibacterial resistance is widely known as a dangerous level in all parts of the world.<sup>[1]</sup> Overuse and misuse of antibiotics in clinical context are extensively considered as major pathways of promoting antibiotic resistance. Other nonclinical large-scale uses of antibiotics in aquaculture, livestock, and poultry farms have strongly contributed to antibiotic contaminations with development of pathogenic bacterial resistance.<sup>[2]</sup> The discovery of new antibacterial agents is mainly based on natural products that can be obtained from different sources, including plants, animals, algae, fungi, and bacteria, but there has been a growing interest in bioactive compounds provided by the plant as an alternative to the common antibiotic.<sup>[3]</sup> Essential oils (EOs) account for a source of very promising natural compounds for producing new antibacterial drugs. Numerous studies have reported a strong antibacterial

effect of EOs.<sup>[4-7]</sup> Among these oils, the potential antibacterial of cinnamon has been documented frequently.<sup>[8-11]</sup>

Furthermore, the combinations, either single EO or mixtures of purified main components (MCs), would assure the exhibition of the target bacteria to many chemical compounds and usually lead to better activity.<sup>[12]</sup> Combining different EO has been recently studied in a view to increasing their antibacterial effects without increasing their concentrations.<sup>[13-15]</sup> It was argued that the combined treatment with cinnamon and some plants EOs showed an additive effect against bacterial species as compared with their pure EOs.<sup>[15,16]</sup> The combination of cinnamon and mustard EOs possesses an additive effect against some food-borne bacteria.<sup>[15]</sup> In another study, cinnamon oil combined with thyme or clove displayed an additive antibacterial effect.<sup>[16]</sup> An important synergistic effect of some antibiotics and cinnamon against *Staphylococcus aureus* and *Escherichia coli* was proved.<sup>[17]</sup> On the

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contrary, the antibacterial propriety of the MCs of EOs, including thymol, carvacrol, and eugenol, largely studied, and it has been proved to be a strong antimicrobial.<sup>[18-20]</sup> Although the antibacterial mechanism of EOs and their constituents is not fully understood, recent studies have shown that constituents with a phenolic structure, such as thymol carvacrol and eugenol, have the greatest bactericidal activities, followed by aldehydes, ketones, alcohols, ethers, and hydrocarbons.<sup>[1,18,19]</sup> However, to the best of our knowledge, there are no available data about the antibacterial effect of cinnamon EO combined with the main monoterpenes of EOs such as thymol, carvacrol, eugenol, and geraniol, which are frequently absent in cinnamon EO. The aim of this study was to search the possible synergistic antibacterial effect of cinnamon EO associated with certain compounds such as thymol, carvacrol, eugenol, or geraniol against *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853.

## Materials and Methods

### Essential oil extraction

The barks of *Cinnamomum cassia* (cinnamon) were purchased from a local supermarket in Fez (Morocco). Identification was confirmed by professor Amina Bari, a botanist at the Department of Biological Sciences, Sidi Mohammed Ben Abdellah University, Morocco. A powder of 200 g cinnamon were hydro-distilled for 3 h with 700 mL of water using a modified Clevenger-type apparatus: the hydrosol was collected in a separating flask, so that the heavy oil was decanted at the bottom of the flask, whereas the water of the hydrosol was recycled into the flask containing the boiling powder plant. The obtained EO was stored at 4°C before analysis.

### Chemical analysis of the essential oils

#### Gas chromatography-flame ionization detector

The cinnamon EO was diluted in hexane and 1 µL of diluted EO was sampled for the gas chromatographic analysis. Trace gas chromatograph (GC) (ULTRA S/N 20062969, Thermo Fischer, Waltham, USA), the GC (TRACE GC-ULTRA, S/N 20062969, Thermo-Fischer) analysis equipped with flame ionisation detector (GC-FID), using an HP-5MS nonpolar fused silica capillary column (60 m × 0.32 mm, film thickness 0.25 µm). The operating conditions were as follows: oven temperature program from 50 °C (2 min) to 280 °C at 5 °C/min and the final temperature kept for 10 min; “split mode” ratio 1:20; carrier gas nitrogen, flow rate 1 mL/min; temperature of detector (flame ionization detector) and injector were fixed at 280 °C and 250 °C, respectively.

#### Gas chromatography-mass spectrometry analysis

The cinnamon EO was analyzed by a capillary GC (Thermo Fischer) directly coupled to the mass spectrometer system (model GC ULTRA S/N 20062969; Polaris QS/N 2107, Thermo-Fischer; Waltham, USA), involving an HP-5MS apolar fused silica capillary column (60 m × 0.32 mm, 0.25 µm film thickness). The operating condition of GC-MS oven

temperature was as follows: initial temperature 40°C for 2 min, programmed rate 2°C per min up to final temperature 260°C with isotherm for 10 min; injector temperature 250°C. The carrier gas was the helium with a flow rate of 1 mL/min. The EO sample was diluted in hexane. The injected specimen volume was 1 µL of diluted EO; systems were operated with a split ratio of 1:15. Ionisation of the sample components was performed in electron impact mode (EI, 70 eV). The ion source temperature was fixed to 200°C. The mass range from 40–650 amu, was scanned at a rate of 2.9 scans/s and Transfer line temperature was 300°C. The characterization of the components was determined by their retention indices (RI) relative to those homologous *n*-alkanes (C<sub>8</sub>–C<sub>20</sub>) series (Fluka, Buchs/sg, Switzerland) and by matching their recorded mass spectra with those stored in the database of spectrometer (NIST MS Library v. 2.0) and the bibliography.<sup>[21]</sup>

### Bacterial strains and inoculums standardization

In this study, the antibacterial activity of cinnamon oil, alone and in combination with some MCs of EOs such as thymol, carvacrol, eugenol, or geraniol (Sigma-Aldrich, St. Louis, MO), was tested against three bacterial strains: *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *P. aeruginosa* ATCC 27853, which was provided by the Pasteur Institute of Casablanca (Morocco). The inoculum suspension was obtained by taking colonies from 24-h cultures. The colonies were suspended in sterile 0.9% aqueous solution of NaCl. The density was adjusted to the turbidity of a 0.5 McFarland standard (1–5 × 10<sup>8</sup> CFU/mL).<sup>[20,21]</sup>

### Agar disk-diffusion assay

The agar disk-diffusion assay was determined in triplicate according to the Kirby–Bauer experiment<sup>[23]</sup>; the suspensions of microorganisms (1–5 10<sup>8</sup> CFU/mL) were flood inoculated on to the surface of Mueller–Hinton (MH) agar plates. Sterile filter disks of 6 mm diameter (Whatman Paper No. 3) were impregnated with 10 µg/disk of the compound and were put on to the surface of the inoculated MH agar. All plates were incubated for 18 h at 37°C. Antibacterial effect was evaluated by measuring the inhibition zones.

### Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was performed using a microdilution assay in 96-well microtiter plates according to the National Committee for Clinical Laboratory Standards (NCCLS).<sup>[24]</sup> In fact, different concentrations of cinnamon EO and MCs were prepared in a suspension containing 0.2% agar in sterile distilled water in order to disperse the compounds without adding solvent or detergent.<sup>[25]</sup> They were carried out by successive dilutions 1/2 ranging from 1.25 to 0.002 mg/mL for EO, thymol, and carvacrol and from 25 to 0.04 mg/mL for eugenol and geraniol. Bacterial suspensions were prepared in the same manner described previously and plated in 96-well plates at a density of 1–5 × 10<sup>6</sup> CFU/mL. Cinnamon EO or MCs were added at different concentrations at the corresponding wells in microtiter plates. Finally, all plates were incubated during 18 h at 37°C; bacterial

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proliferation was visually by adding to each well 20  $\mu\text{L}$  of 2,3,5-triphenyltetrazolium chloride (TTC) aqueous solution (1%), with additional incubation for 1 h. MIC was the lowest concentration that does not produce red color.<sup>[22]</sup>

### Checkerboard method

The evaluation of the interaction between cinnamon EO and MCs (thymol, carvacrol, eugenol, or geraniol) was carried out according to the method of Moody.<sup>[26]</sup> Briefly, ten concentrations of cinnamon EO and eight concentrations of the MCs were prepared in sterile tubes by dilutions 1/2. EO at decreasing concentrations, going from MIC  $\times$  4 to MIC/128, was introduced horizontally into 96-well microtiter plates. In the same manner, the MCs at decreasing concentrations, going from MIC  $\times$  4 to MIC/32, were introduced vertically. The final volume in each well was 200  $\mu\text{L}$  comprising 25  $\mu\text{L}$  of EO, 25  $\mu\text{L}$  of MC dilution, and 150  $\mu\text{L}$  of MH media containing  $1-5 \times 10^6$  CFU/mL of bacterial suspensions. All plates were then incubated during 18 h at 37 °C. The analysis of the combination was obtained by calculating the fraction inhibitory concentration index (FICI) using the following formula<sup>[26]</sup>:

$$\text{FIC index (FICI)} = \text{FIC}_A + \text{FIC}_B$$

$$\text{FIC}_A = \frac{\text{MIC of (A) in combination}}{\text{MIC of (A) alone}}$$

$$\text{FIC}_B = \frac{\text{MIC of (B) in combination}}{\text{MIC of (B) alone}}$$

where (A) is cinnamon EO and (B) is one of the MCs.

The FICI values were interpreted as follows: a synergistic effect when  $\text{FICI} \leq 0.5$ ; an additive effect when  $0.5 < \text{FICI} < 1$ ; an indifferent (no interaction) when  $1 < \text{FICI} < 4$ ; and an antagonistic effect when  $\text{FICI} > 4$ .

## Results

### Essential oil composition

The yield of the EO of cinnamon was 1.26% (v/w, dark yellow) calculated on a dry weight basis. The GC-MS analysis of EO is presented in Table 1. Eleven components were identified comprising 98.44% of the total amount. Trans-cinnamaldehyde was found as the single major component (91.01%). The other component's insignificant percent were as follows: cis-cinnamyle acetate (2.04%), linalool (1.3%), caryophyllene oxide (1.06%),  $\gamma$ -terpinene (1.03%), and  $\delta$ -cadinene (0.9%) [Table 1].

### Antibacterial activity

The antibacterial activity of the cinnamon EO, thymol, carvacrol, eugenol, and geraniol, tested against three bacterial strains (*E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *P. aeruginosa* ATCC 27853), is shown in Table 2. Cinnamon EO was the strongest antibacterial effect against all strains tested:

*E. coli*, *S. aureus*, and *P. aeruginosa*, with the MIC values of 0.005, 0.005, and 0.02 mg/mL, respectively. In addition, *E. coli* and *S. aureus* were sensitive to the thymol and carvacrol (MIC: 0.072 and 0.31 mg/mL, respectively) and were resistant to eugenol and geraniol. *Pseudomonas aeruginosa* was relatively resistant to all MCs tested.

### Combined effects of cinnamon essential oil and main components

Synergistic interactions between different plant extracts are the aim of the herbal formulation in folk medicine.<sup>[27]</sup> The interaction of plant with MCs of EOs is one of the novel ways to inhibit the resistance mechanisms of bacteria. In this study, the combined antibacterial effect of cinnamon oil was evaluated by the checkerboard method, in order to determine the fractional inhibitory concentration (FIC). Table 3 shows the cinnamon EO tested against three bacterial strains in combination with some MCs. FICI were calculated and interpreted as synergy, addition, indifference, or antagonism. As shown in Table 3, mixing MCs with cinnamon EO reduces the MICs, 2-fold for *P. aeruginosa* and 2–8-fold for *E. coli* and *S. aureus*. The synergistic activity was obtained only by the combination of cinnamon EO and thymol against *S. aureus*, with the FICI value of 0.5. In addition, the combination of cinnamon oil with thymol showed an additive effect against *E. coli* with the FICI of 0.75. An additive effect was also found when cinnamon oil was combined with carvacrol against *E. coli* and *S. aureus* with the FICI of 1 and 0.625, respectively, whereas the combination of cinnamon with thymol or carvacrol showed an indifferent effect against *P. aeruginosa*. However, the combination of cinnamon EO and eugenol or geraniol displayed an antagonism action against all bacteria tested [Table 3].

## Discussion

Eleven components were identified in EO of cinnamon bark. Trans-cinnamaldehyde was found as its single major component (91.01%). Several studies on the chemical composition of cinnamon EO were reported and showed that trans-cinnamaldehyde was the single major compound.<sup>[10,11,16]</sup> Simic *et al.*<sup>[28]</sup> reported

**Table 1: Chemical composition of cinnamon bark essential oil**

Compounds	Kovats index	Area (%)
$\alpha$ -Pinene	909	<0.1
$\gamma$ -Terpinene	924	1.03
Limonene	1027	0.2
1,8-Cineole	1031	<0.1
Linalool	1116	1.3
Trans-Cinnamaldehyde	1277	<b>91.01</b>
Cis-Cinnamyle acetate	1291	2.04
Caryophyllene oxide	1321	1.06
$\alpha$ -Copaene	1375	0.5
$\delta$ -Cadinene	1534	0.9
Benzyl benzoate	1660	0.4
Totale	–	98.44

**Table 2: Antibacterial activity of cinnamon EO, thymol, carvacrol, eugenol, and geraniol**

	<i>Escherichia coli</i> ATCC 25922		<i>Staphylococcus aureus</i> ATCC 25923		<i>Pseudomonas aeruginosa</i> ATCC 27853	
	ID	MIC	ID	MIC	ID	MIC
Cinnamon EO and main components						
EO	29.0 ± 0.7	0.005	40.0 ± 0.0	0.005	30.5 ± 1.0	0.02
Thymol	41.2 ± 0.0	0.078	42.3 ± 0.1	0.078	12.2 ± 0.3	0.04
Carvacrol	39.6 ± 0.3	0.156	38.6 ± 0.4	0.315	10.0 ± 0.0	0.625
Eugenol	14.0 ± 0.5	>5	16.1 ± 0.5	>5	8.0 ± 0.2	Nt
Geraniol	18.1 ± 0.2	>5	18.0 ± 1.0	>5	Ni	Nt

ID = inhibition zone diameter (mm), MIC = minimal inhibitory concentrations (mg/mL), Nt = not tested, Ni = no inhibition

Inhibition zone includes diameter of disk (6 mm)

Values of inhibition diameter are given as mean ± SD

**Table 3: FIC and FICI values of the combinations**

	<i>Escherichia coli</i> ATCC 25922				<i>Staphylococcus aureus</i> ATCC 25923				<i>Pseudomonas aeruginosa</i> ATCC 27853			
	MIC alone	MIC combined	FIC	FICI	MIC alone	MIC combined	FIC	FICI	MIC alone	MIC combined	FIC	FICI
EO+thymol												
EO	0.005	0.002	0.5	0.75(A)	0.005	0.001	0.25	0.5(S)	0.02	0.02	1	1.5(I)
Thymol	0.078	0.020	0.25		0.078	0.02	0.25		0.039	0.02	0.5	
EO+carvacrol												
EO	0.005	0.002	0.50	1.0(A)	0.005	0.002	0.5	0.625(A)	0.02	0.039	2	2.5(I)
Carvacrol	0.156	0.078	0.50		0.312	0.039	0.125		0.625	0.313	0.5	
EO+eugenol												
EO	0.005	0.02	4.00	4.12(At)	0.005	0.020	4.00	4.063(At)	0.02	0.08	4	6 (At)
Eugenol	25	3.125	0.12		25.000	1.563	0.063		200	100	2	
EO+geraniol												
EO	0.005	0.020	4.00	4.12(At)	0.005	0.020	4.000	4.125 (At)	Nt	Nt	-	-
Geraniol	12.5	1.563	0.12		12.500	1.563	0.125					

EO = cinnamon essential oils, S = synergy, Ad = addition, I = indifference, At = antagonism, Nt = not tested

trans-cinnamaldehyde (62.8%) and cinnamaldehyde propylene (5.5%) as the major compounds from cinnamon (*C. zeylanicum*) volatile oil. Marongino *et al.*<sup>[29]</sup> found that the MCs of cinnamon (*C. zeylanicum*) were trans-cinnamaldehyde (77.1%), trans-β-caryophyllene (6.0%), and α-terpineol (4.4%).

Concerning the antibacterial effect, our results are higher than that described by Clemente *et al.*,<sup>[15]</sup> who found that cinnamon EO inhibits the growth of *E. coli*, *S. aureus*, and *P. aeruginosa* at MIC values of 0.4, 0.4, and 0.2 mg/mL, respectively. They are in accordance with the data reported by Gallucci *et al.*<sup>[30]</sup> *Escherichia coli* and *S. aureus* are sensitive to thymol and carvacrol (MIC: 15.07, 7.53, 7.62, and 3.82 mg/mL, respectively), and resistant to eugenol and geraniol (MIC > 20 mg/mL). Pei *et al.*<sup>[31]</sup> revealed that *E. coli* growth was inhibited by thymol, carvacrol, and eugenol at MIC values of 1.6, 0.4, and 0.4 mg/mL, respectively. In a study conducted by Miladi *et al.*,<sup>[32]</sup> carvacrol had better activity against *S. aureus* (MIC: 64 mg/mL) and thymol and eugenol at MIC values of 256 mg/mL. In addition, the sensitivity of *P. aeruginosa* to cinnamon EO is probably attributed to the combined effects of several compounds constituting this EO, acting on various cell targets. Bouhdid *et al.*<sup>[33]</sup> showed that cinnamon EO damages the cell membrane of *P. aeruginosa*, which leads to cell death.

According to the previous studies, the high antibacterial activity observed with cinnamon EO may be because of the action of trans-cinnamaldehyde, which is considered as its single major compound.<sup>[9-11]</sup> It has been reported that trans-cinnamaldehyde possess the highest antimicrobial activity in comparison with other constituents of cinnamon oil.<sup>[12,20,34]</sup> Furthermore, thymol, carvacrol, eugenol, and geraniol are the main compounds of thyme, oregano, clove, and geranium plants, respectively. The antibacterial effect of their EOs has been reported.<sup>[35-36]</sup> Moreover, several authors show the antibacterial activity of various EOs plants including, thyme, oregano, lemon balm, basil, marjoram, and baccharis against the bacterial strains under consideration in this study.<sup>[13,14]</sup>

Previous studies have explored the antibacterial effect of cinnamon EO combinations. Clemente *et al.*<sup>[15]</sup> demonstrated that the combination of cinnamon with mustard EOs showed an additive effect against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and some other bacteria species. Lu *et al.*<sup>[16]</sup> used the checkerboard assay to determine the activity of cinnamon combined with thyme and clove EOs. Both cinnamon combinations displayed in most cases an additive or indifferent action against food-borne bacteria. These results were



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confirmed by this study, thus highlighting the effectiveness of cinnamon oil when combined with thymol or carvacrol. Moreover, eugenol and geraniol are the MCs of EOs from clove and pelargonium, respectively. The additive effects of these EOs have been reported.<sup>[16,37]</sup>

On the contrary, the mechanism that is responsible for the antimicrobial activity of cinnamon includes its chemical composition such as cinnamaldehyde,<sup>[8]</sup> which is an electronegative molecule that could interfere with the biological process in microorganism particularly nitrogen containing substances such as proteins and nucleic acids.<sup>[38]</sup> Furthermore, cinnamon EOs and their MCs have been reported to inhibit bacteria via Antiquorum sensing effects, inhibiting cell division, ATPase, biofilm formation membrane porine, and mobility; altering the lipid profile<sup>[8]</sup>; and thereby acting cell membrane producing lumps and autoaggregation.<sup>[15]</sup> Furthermore, thymol and carvacrol are phenolic compounds; their hydroxyl groups play a major role in their antibacterial activities.<sup>[39]</sup> They are able to alter the cell outer membrane<sup>[40]</sup> and combine with the charged groups of membrane through increasing its permeability.<sup>[41]</sup> Furthermore, carvacrol had ATPase inhibitory propriety, which causes dissipation of the motive force of the proton, and can subsequently inhibit other enzymes.<sup>[42]</sup> However, there are limited reports on the action mechanisms of a mixture of EOs and theirs purified components on bacteria. Nevertheless, it is possible to explain the synergistic or additive effects caused by the combination of cinnamon EO and thymol or carvacrol by the fact that the thymol or carvacrol could increase the cell membrane permeability, making it easier for cinnamon compounds to penetrate into the cell and combine with proteins and nucleic acids. In addition, some explanations for the mechanisms of antibacterial interaction that produce antagonism include the use of compounds that act on the same target of the microorganism, combinations of bactericidal and bacteriostatic agents, and chemical interactions between compounds.<sup>[43,44]</sup>

## Conclusion

This work has shown that cinnamon EO possesses a stronger antimicrobial effect than all main compounds tested against resistant bacteria. This effect could be because of the trans-cinnamaldehyde, which is considered as the major compound of this EO. The synergistic effect was shown only against *S. aureus* with the combination of cinnamon oil and thymol. In addition, we have shown that the combination of cinnamon EO with thymol or carvacrol and their synergistic or additive effects can be used as an alternative therapeutic agent for medical application, as a natural preservative and food additive.

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## Conflicts of interest

There are no conflicts of interest.

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