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Research Article

Mechanism of action of essential oil-antibiotic combinations on bacteria involved in foodborne toxin infections

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Abstract

Background. Bacterial resistance to antibiotics threatens public health in Burkina Faso. This calls for exploring alternative solutions alongside traditional methods.

Objectives. To evaluate the efficacy of combined essential oils-antibiotic against antibiotic-resistant pathogens causing toxic infections in Burkina Faso.

Methods. The methodological approach involved testing the combined effects of essential oils from two plants, *Hyptis suaveolens* and *Laggera aurita* with two antibiotics: amoxicillin plus clavulanic acid, and colistin, on pathogenic bacteria. The antibacterial properties of the essential oils were confirmed through MIC and MBC tests. The antibacterial mechanism was examined on bacteria such as *Staphylococcus aureus* ATCC 2523, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* 0366 V, *Bacillus cereus* 0998 V and *Yersinia enterocolitica* 0938 V.

The mechanism of action of the essential oil-antibiotic combination was examined by quantifying the released DNA.

Results. The main results showed MIC values ranging from 9.57 mg/mL to 38.28 mg/mL for *Hyptis suaveolens* and from 1.69 mg/mL to 64.92 mg/mL for *Laggera aurita*. Of the 20 combinations essential oils-antibiotics tested on bacteria, 70% showed total synergy, 15% showed partial synergy and no synergy respectively.

The DNA release rate was 93.76%, indicating a mechanism involving simultaneous damage to the peptidoglycan and plasma membrane.

Conclusion. Essential oils and antibiotics combination could constitute an excellent mean against bacterial resistance to antibiotics.

Keywords: Antibacterial mechanism; Antibiotics; Essential oils; *Hyptis suaveolens*; *Laggera aurita*; Combination action.

1. INTRODUCTION

The worldwide surge in antibiotic resistance, driven by the rapid spread of innovative resistance pathways, is progressively underming clinical efficacy of a global scale [1]. Pathogenic bacteria pose risks of food poisoning that must be addressed [2]. Indeed, these bacteria can contaminate various common food products *via* diverse transmission routes, notably through dairy products such as milk [3], eggs [4,5], market garden products, meat products, and fish [6].

According to the World Health Organization (WHO), antimicrobial resistance (AMR) is one of the leading public health concerns [7]. The WHO estimates that nearly 50.000 people die worldwide every day from infectious diseases caused by bacteria and fungi, with financial burden reaching 380 million euros [8].

In recent years, antibiotic-resistant pathogens is one of the major public health issues. In 2019, the ANSSEAT (National Agency for Environmental, Food, Occupational Health and Safety), on biological products (vomitus,

diarrheal stools, etc.) has isolated resistant bacterial strains [9]. Colistin resistance prevalence in Africa has been estimated at 1.2 % and 1.5 % for *K. pneumoniae* and *E. cloacae*, respectively [10]. Like other countries, Burkina Faso is facing an alarming frequency of antibiotic resistance. Indeed, Ki Ba *et al*, Nadembega *et al* [9,11] reported that 32 % of *S. aureus* strains were methicillin-resistant, 98.3% of *E. coli* and 94.7 % of *K. pneumoniae* were resistant to amoxicillin + clavulanic acid, and 36.44 % of *E. coli* and 26.3 % of *K. pneumoniae* were resistant to third-generation cephalosporins. The resistance rate of beta-lactamase-producing Gram-negative bacteria was 35 %.

Nearly 30 % of antibiotic prescriptions are considered to have little to no clinical efficacy [12]. To address this declining effectiveness, researchers has explored plant-based alternatives to combat bacterial resistance. In this context, the use of essential oils as antimicrobial agents has garnered significant attention due to their broad spectrum of inhibitory effects against various bacterial strains [13].

Conventional antibiotic combinations designed to combat antimicrobial resistance are currently reaching their limits due to the emergence of multidrug-resistant bacteria. Nevertheless, the integration of essential oils in conjunction with antibiotics appears to enhance the efficacy of the latter, thereby mitigating therapeutic failures [14,15].

Consequently, numerous studies have investigated the synergistic use of essential oils in combination with antibiotics to counteract antimicrobial resistance [8,16-18]. However, in Burkina Faso, few studies have addressed the combined effects of essential oils and antibiotics or their underlying mechanisms.

Antibiotics function by inhibiting the biosynthesis of nucleic acids, with their primary targets being the bacterial cell wall and ribosomes. This high degree of specificity, coupled with the exceptional adaptive capacity of bacteria, represents one of the inherent limitations of antibiotic therapy [13].

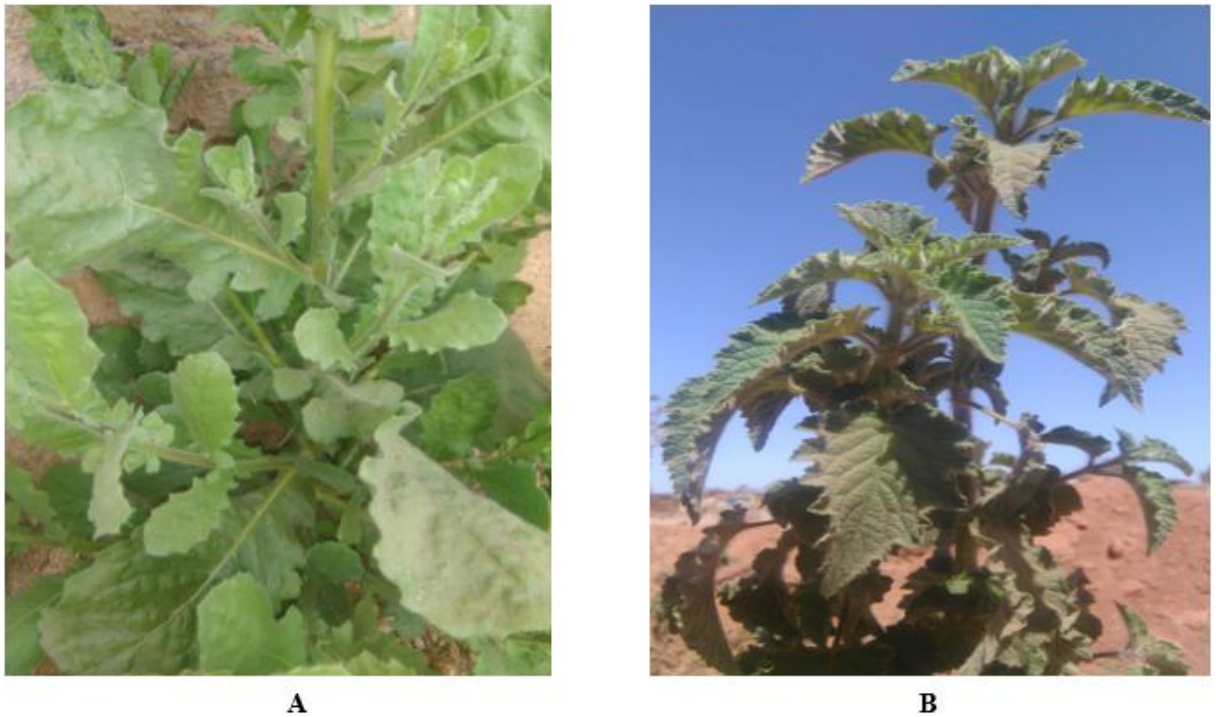
Conversely, plants have evolved their own internal mechanisms for biotic control, including defense against microbial infections. These processes favor the synthesis of a diverse array of weakly active molecules, which reduces selective pressure and hinders the development of resistance [19]. Notably, *Hyptis suaveolens* and *Laggera aurita* are widely distributed throughout tropical and subtropical regions. In Burkina Faso, these species are employed in traditional medicine [20-24]. Several studies have highlighted the antibacterial and antifungal properties of essential oils derived from *H. suaveolens* and *L. aurita* [20,25-27] underscoring the necessity of exploring their potential in combination with commonly used antibiotics.

The objective of this study is to evaluate the efficacy of the combined effects of conventional antibiotics and essential oils against antibiotic-resistant bacteria.

2. MATERIALS AND METHODS

2.1. Essential oils

A botanist of the National Center for Scientific and Technology Research (CNRST) in Ouagadougou identified the leaves of *H. suaveolens* and *L. aurita*. The voucher specimens of *L. aurita* and *H. suaveolens* are n° 152A preserved in the National Herbarium of Burkina Faso (HNBU) and n° 110 preserved in the herbarium of University Joseph Ki-Zerbo at the Laboratory of Ecology and plant Biology (LaBEV) respectively.



A B

Figure 1: Leaves of *L. aurita* (A) and *H. suaveolens* (B)

2.2. Antibiotics

Amoxicillin + clavulanic acid (Glaxo SmithKline Pharmaceuticals Ltd) and colistin (Coli-4800 ws) obtained from a pharmacy in Burkina Faso were used for this study.

The different bacterial targets of inhibition (wall bacteria, cytoplasmic membrane) and availability in local pharmacy led to the choice of these antibiotics in our study.

2.3. Bacterial strains

The following bacteria were used as test strains : *Staphylococcus aureus* ATCC 2523, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027 provided by the American Type Culture Collection (ATCC), *Enterococcus faecalis* 0366 V, *Bacillus cereus* 0998 V, and *Yersinia enterocolitica* 0938 V provided by the Food and Nutritional Biological Sciences Research Center (CRSBAN) at University Joseph Ki-Zerbo in Ouagadougou.

The food-borne diseases caused by these bacteria led to the choice of these pathogens for our study.

2.4. Plants leaf sample collection and essential oil extraction

H. suaveolens and *L. aurita* plants were harvested in Ouagadougou, specifically in the « Bassinko » (12°25'30.96'' N, 1°40'21.78'' O), lowlands and near the dam of « Tanghin » (12°23'40.11'' N, 1°32'18.10'' O). Figure 2 shows the location of the harvesting areas.

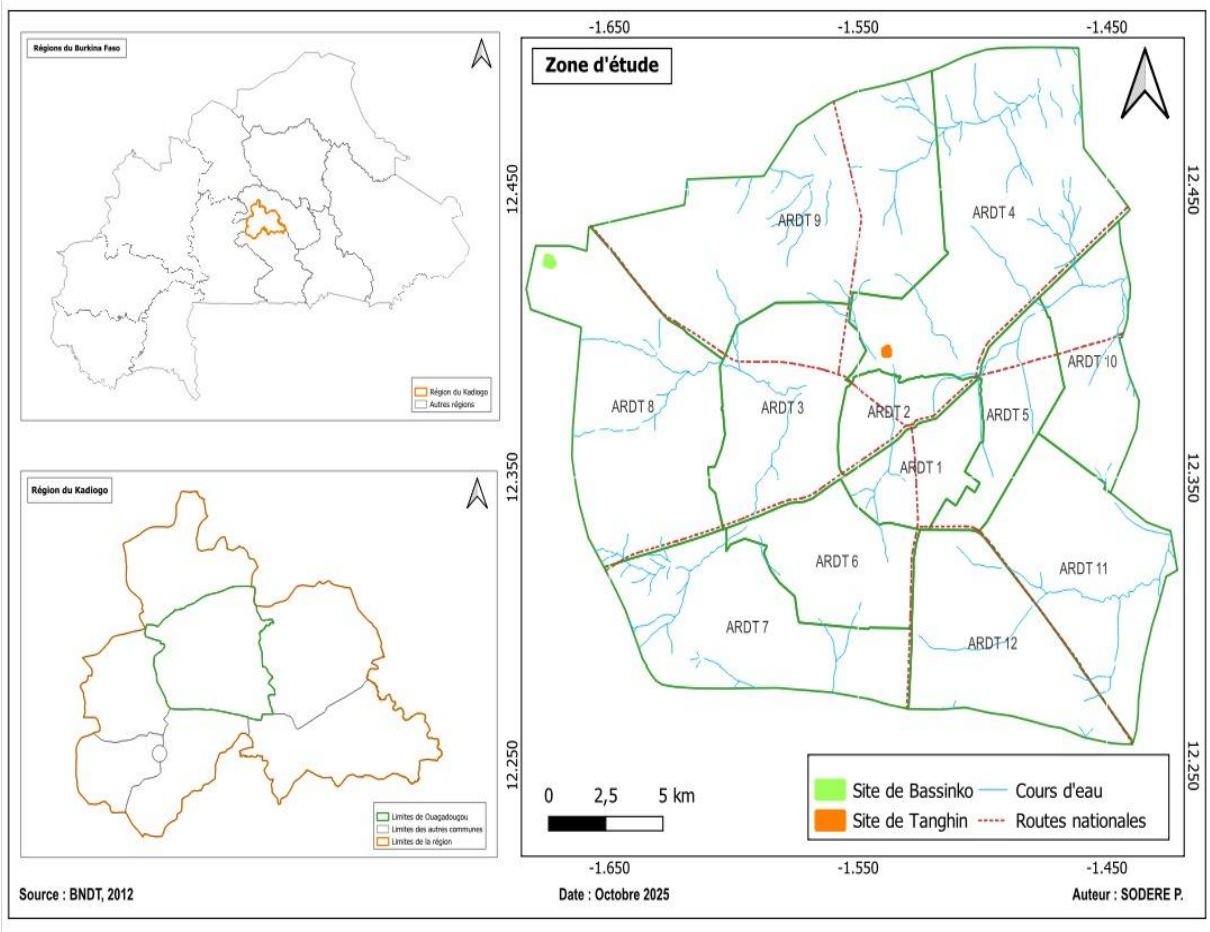


Figure 2: Geographical location of the harvest zones

The plants were transported to Institute for Research in Applied Sciences and Technologies (IRSAT) in « Kossodo/ Ouagadougou » where leaves were removed, washed two times with clean water to remove adhering debris. In the shaded area the leaves were air dried at approximately 37 °C. Essential oils were extracted by hydrodistillation using a modified Clevenger-type apparatus following the protocol described by Bayala [28]. Briefly, 10 kg of dried leaves were immersed in a 100-liter still. The mixture was brought to a boil for three hours, and the resulting essential oil-rich vapors were condensed and collected. The organic phase was separated *via* decantation. The essential oils characterized by densities of 0.7657 g/mL for *L. aurita* and 0.6493 g/mL for *H. suaveolens*, were stored in amber bottles wrapped in aluminum foil at 4 °C shielded from UV radiation. Extraction yield was expressed as a percentage (%) and the density in g/mL.

Yield (%) = (Mass of essential oil / Mass of dried leaves) x 100

Density (g/mL) = Mass of essential oil / Volume of essential oil

L. aurita and *H. suaveolens* concentrations of 0.7657 g/mL and 0.6493 g/mL, respectively, were obtained and

placed in bottles wrapped with aluminum foil and stored at 4°C in a refrigerator away from ultraviolet rays.

2.5. Bacterial strains culture conditions

All bacterial were cultured in nutritional broth media (India, HiMedia. Laboratories Pvt. Limited) for 24 h. After incubation of 24 h, the bacteria were washed according to Rhayour [17]. The 24h bacterial culture was centrifuged at 400 rpm for 25 min; the resulting supernatant was discarded, and the bacterial pellet was resuspended in a volume of Phosphate Buffered Saline (PBS). The resulting bacterial suspension is hereafter referred to as the washed bacterial suspension. The optical density was read at 625 nm using a spectrophotometer, and the final inoculum was adjusted to 1.5 x 10^6 CFU/mL according to the standard 0.5 Mac Farland suspension.

2.6. Preparation of antibiotics and essential oils concentrations

Antibiotic solutions were prepared by dissolving 500 mg of each antibiotic in 1 mL of sterile tween 80. The mixture was transferred to a sterile flask containing 199 mL of sterile distilled water to obtain a concentration of 2.5 mg/mL, in accordance with CLSI (29) guidelines**.**

2.7. MIC and MBC Assays

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of the essential oils and antibiotics were determined *via* the broth microdilution method CLSI [29]**.** Mueller-Hinton broth (MHB) (India, HiMedia. Laboratories Pvt. Limited) supplemented with tween 80 was utilized. A volume of 190 µL was added to first column, and 100 µL to the subsequent wells of 96-well microplate. Ten serials twofold dilutions of each antibacterial agent (*L. aurita, H.* suaveolens, amoxicillin + clavulanic acid or colistin) were performed from the essential oils and antibiotic concentrations previously prepared to obtain final concentrations ranging from 76.56 to 0.07 mg/mL for *H. suaveolens*, from 64.92 to 0.06 mg/mL for *L. aurita* and from 125 to 0.06 µg/mL for each antibiotic. The wells of the broth without antimicrobials served as controls. The 96-well microplates were incubated at 37 °C for 24 h and turbidity or growth was observed. The lowest concentration with no turbidity among the test-wells was recorded as the MIC [30]**.**

The MBC was considered as the lowest antimicrobial concentration that killed 99.9% of the bacterial inocula after 24 h of incubation at 37 °C [31]. MBC values were determined by subculturing the contents of test-wells without turbidity or no visible growth from MIC determinations to neutral sterile Mueller–Hinton agar plates.

The antibacterial activity was assessed by determining the MIC and the MBC of antibiotics. and essential oil. The bacterial susceptibility to antibiotics was determined via the critical concentrations [32] listed in Table 1. All tests were performed independently in triplicate.

Table 1: Susceptibility of bacteria to antibiotics

Antibiotics	Critical concentrations (µg/mL)
Amoxicillin + clavulanic acid	4-16
Colistin	2-8

2.8. Synergistic effects of the checkerboard method

Synergistic interactions were evaluated using the checkerboard method [33] specifically on resistant strains. Serial twofold dilutions of essential oils were prepared horizontally, while antibiotics dilutions were prepared vertically. The Fractional Inhibitory Concentration (FIC) and the Fractional Inhibitory Concentration Index (FICI) were calculated as follows:

FIC of essential oil = MIC of essential oil in the combination/MIC of essential oil alone; FIC of antibiotic = MIC of antibiotic in the combination/MIC of antibiotic alone.

FICI = FIC of essential oil + FIC of antibiotic as described by Schelz *et al.* [34]**.**

Synergy (FICI < 0.5); addition (0.5 ≤ FICI ≤ 1); indifference (1 < FICI ≤ 4) and antagonism (FICI > 4). All tests were performed independently in triplicate.

2.9. Loss of 260 nm-absorbing material

The release of the cytosolic material absorbing at 260 nm at different times of treatment (0h, 12h, and 24 h) were quantified as described by Rhayour [17] using Epoch Biotek spectrophotometer. For each bacterial strain (1.5 x 10^6 CFU/mL), tube containing PBS and an untreated suspension of washed bacteria were performed as control. Tube containing PBS, the washed bacterial suspension, and the antibiotic MIC and tube containing PBS, the washed bacterial suspension, and the synergistic MICs of the essential oil-antibiotic combinations were performed as samples. Tubes were incubated at 37 °C under constant agitation at 200 rpm. At specific time intervals (t = 0, 12h, and 24 h), a 200 µL aliquot was withdrawn from each tube and transferred into a 96-well microplate. Absorbance was subsequently measured at 260 nm. Distilled water served as the blank for the measurements. All assays were performed in triplicate.

2.10. Quantification of DNA

The concentration of DNA released into the supernatant was quantified using a BioDrop Cambridge CB4 OFJ spectrophotometer. Following 24 h of incubation under the conditions described above, control and samples were centrifuged at 8000 rpm for 2 min. The resulting supernatant was analyzed to determine DNA content [35]. The control was read as blank. All tests were performed independently in triplicate.

2.11. Statistical Analysis of Data

Analysis of variance (ANOVA) was used to compare variable means. Fisher's Least Significant Difference (LSD) test was used for pairwise multiple comparisons of

means where there was a significant difference between all means. The difference between means was considered significant when the p-value was less than 0.05. XLSTAT version 7.5.2 2016 software was used for statistical analysis.

3. RESULTS AND DISCUSSION

3.1. Yield of essential oils

L. aurita and *H. suaveolens* essential oils yields and density are listed in Table 2.

Table 2: Yields and densities of essential oils

Essential oils	Yield (%)	Density (g/mL)
L. aurita	0.20± 0,01	0.7657± 0,01
H. suaveolens	0.23± 0,01	0.6493± 0,01

The essential oil yield obtained from the leaves of *L. aurita via* hydrodistillation was 0.20 %. This value was lower than the 0.3 % reported by Samaté [36] in Burkina Faso, whose samples were harvested during the flowering period. Conversely, the present yield was higher than the 0.008 % recorded by Kabera *et al.* [37] for the same species, yet remained comparable to the findings of Mihin *et al.* [27] in Burkina Faso, who reported a yield of 0.22 %.

For *H. suaveolens*, the essential oil yield was 0.23 %. This result aligns closely with yields reported by Mihin *et al.* [27] in Burkina Faso (0.21 %), Ngom *et al.* [38] in Senegal (0.22%), and Adjou and Soumanou [39] in Benin (0.23 %). However, it was lower than the yield of 0.34 % observed by Goly *et al.* [25]. When compared with the literature, the analysis of essential oil yields for *L. aurita* and *H. suaveolens* reveals both similarities and discrepancies, regardless of whether the studies were conducted in Burkina Faso or other West African countries such as Benin and Senegal. Indeed, factors such as the duration and degree of drying, the date and geographical location of harvest, and the extraction technique employed have a significant impact on both the yield and the chemical profile of essential oils [27,38,40-42].

3.2. MIC/MBC Values of Antibiotics

The results from the broth microdilution tests for the *in vitro* antibacterial activity of the antibiotics are presented in Table 3. The MIC of amoxicillin + clavulanic acid ranged from 06 µg/mL to 29.66 µg/mL, while for colistin it ranged from 0.15 µg/mL to 5 µg/mL.

Regarding the MBC, values ranged from 24 µg/mL to 96 µg/mL for amoxicillin + clavulanic acid and from 24 µg/mL to 60 µg/mL for colistin.

The lowest MIC (0.15 µg/mL) was obtained with colistin on *S. aureus* and the highest MIC (29.66 µg/mL) was obtained with amoxicillin + clavulanic acid on *P. aeruginosa*.

The lowest MBC (24 µg/mL) was obtained with amoxicillin + clavulanic acid and colistin on *E. faecalis* and *Y. enterocolitica* the highest MBC (96 µg/mL) was obtained with amoxicillin + clavulanic acid on *P. aeruginosa*.

Amoxicillin + clavulanic acid and colistin revealed 6 bactericidal effects and 6 bacteriostatic effects respectively.

The resistance rates for amoxicillin + clavulanic acid and colistin were 66.66 % and 83.33 %, respectively (Table 3). These findings corroborate the patterns of antimicrobial resistance previously observed in Burkina Faso. Notably, the resistance rate for colistin was higher than that of amoxicillin + clavulanic acid. The elevated resistance to colistin compared to amoxicillin + clavulanic acid may be explained by the relative inaccessibility of the cytoplasmic membrane compared to the bacterial cell wall [43]**.** Furthermore, this high prevalence of colistin resistance could be linked to its extensive use in veterinary medicine**.** Additionally, reduced antibiotic permeability in certain bacterial strains may contribute to high colistin resistance. Efflux pumps, which actively export antibiotics out of the cell though this mechanism is infrequently observed [44] further hinder colistin's access to its primary target: the plasma membrane [45].

The MIC for *P. aeruginosa* was significantly higher (ANOVA, $P < 0.05$) than those of other pathogenic strains. High MIC values for *P. aeruginosa* have also been reported by Gang-Joon *et al*. [46]. Our findings align with those of Lambert *et al.* [47] who reported elevated MIC for *P. aeruginosa* across 40 bacterial strains. The pronounced resistance of *P. aeruginosa* to most antibacterial agents is due to its possession of nearly all known enzymatic and mutational mechanisms of bacterial resistance [48].

Table 3 : Antibacterial activity of antibiotics

Minimum Inhibitory Concentration (µg/ml)					**MBC/MIC ratio and Capacity**	
	Amoxicillin + clavulanic acid		**Colistin**		**Amoxicillin + clavulanic acid**	**Colistin**
Bacterial strains						
	MIC	MBC	MIC	MBC		
***E. faecalis* 0366V**	04±0.00^{d} (S)	24±0.00^{c}	3.75±0.00^{b} (R)	24±0.00^{c}	4*	6**

***Y. enterocolitica* 0938V**	04±0.00[d] (S)	24±0.00[c]	3.75±0.00[b] (R)	24±0.00[c]	4*	6**
***B. cereus* 0998V**	13±1.00[c] (R)	48±0.57[b]	3.33±1.40[c] (R)	30±0.57[b]	4*	9**
***E. coli* ATCC 25922**	23.33±0.57[b] (R)	48±0.00[b]	5±0.00[a] (R)	30±0.00[b]	2*	6**
***P. aeruginosa* ATCC 9027**	29.66±0.57[a] (R)	96±0.00[a]	5±0.00[a] (R)	60±0.00[a]	3*	12**
***S. aureus* ATCC 2523**	24±0.00[b] (R)	48±0.00[b]	0.15±0.06[d] (S)	30±0.00[b]	2*	200**

Notes: The values represent the means of three trials ± standard deviations. Values in the same column with the same superscript letters (a, b, c, d) are not significantly different ($p<0.05$). Values with superscript (*) Bactericidal effects (**) Bacteriostatic effects. (S): Sensitivity, (R): Resistance.

3.3. MIC and MBC Values of essential oil determination

The MIC of *H. suaveolens* essential oil ranged from 9.57 mg/mL (against *P. aeruginosa* and *B. cereus*) to 38.28 mg/mL (against *E. faecalis*, *Y. enterocolitica*, *E. coli*, and *S. aureus*). Its MBC ranged from 38.28 mg/mL for *S. aureus* and *B. cereus* to 76.56 mg/mL for *E. coli* and *P. aeruginosa* (Table 4).

For *L. aurita* essential oil, the MIC ranged from 1.69 mg/mL against *S. aureus* to 64.92 mg/mL against *E. coli*. The MBC values ranged from 30 mg/mL against *E. coli* to 64.92 mg/mL against *E. faecalis* and *Y. enterocolitica* (Table 4). Both *H. suaveolens* and *L. aurita* essential oils exhibited five bactericidal effects and one bacteriostatic effect each.

The antibacterial activity of *H. suaveolens* which is characterized by high concentrations of beta-caryophyllene, 1,8-cineole, sabinene, and beta-pinene (comprising monoterpenes, sesquiterpenes, terpenoids, and sterols) likely functions by damaging bacterial cell structures and stimulating the release of cellular potassium ions, a process that proves lethal to the microorganisms [49,50,51]. In contrast, the antibacterial potency of *L. aurita* can be attributed to its high content of terpenoid compounds such as carvacrol, thymol, and eugenol, alcohols like linalool, and aldehydes such as cinnamaldehyde [36].

Statistical analysis (ANOVA, $P < 0.05$) revealed that the antibacterial activity of *L. aurita* was significantly more pronounced than that of *H. suaveolens*. This difference may be explained by the distinct chemical profiles of the two essential oils. Specifically, *H. suaveolens* essential oil contains fewer oxygenated terpenic compounds [52,53]. It is widely recognized that essential oils rich in these specific molecules tend to exhibit the highest antimicrobial efficacy [27,54,55].

The most extensively studied essential oils with potent antibacterial properties are those predominantly composed of oxygenated compounds such as eugenol, thymol, and carvacrol. Therefore, the richness of *L. aurita* in these constituents likely accounts for its superior antibacterial performance [56,57].

Table 4: Antibacterial activity of essential oils

Minimum Inhibitory Concentration (mg/ml)					**MBC/MIC ratio and Capacity**	
	Hyptis suaveolens		***Laggera aurita***		***Hyptis suaveolens***	***Laggera aurita***
Bacterial strains						
	MIC	MBC	MIC	MBC		
***E. faecalis* 0366V**	38.28±0.00[a]	76.25±0.00[a]	36.46±0.00[b]	64.92±0.00[a]	2*	2*
***Y. enterocolitica* 0938V**	38.28±0.00[a]	76.25±0.00[a]	36.46±0.00[b]	64.92±0.00[a]	2*	2*
***B. cereus* 0998V**	9.57±0.00[c]	38.28±0.00[b]	8.11±0.00[c]	32.46±0.00[b]	4*	4*
***E. coli* ATCC 25922**	38.28±0.00[a]	76.56±0.00[a]	64.92±0.00[a]	30±0.00[c]	2*	2*
***P. aeruginosa* ATCC 9027**	9.57±0.00[c]	76.56±0.00[a]	8.11±0.00[c]	32.46±0.00[b]	8**	4*
***S. aureus* ATCC 2523**	38.28±0.00[a]	38.28±0.00[b]	1.69±0.00[d]	32.46±0.00[b]	4*	19**

Notes: The values represent the means of three trials ± standard deviations. Values in the same column with the same superscript letters (a, b, c) are not significantly different ($p<0.05$). Values with superscript (*) Bactericidal effects. (**) bacteriostatic effects.

3.4. Synergistic effects of essential oils and antibiotics

The FIC indices for the essential oils of *H. suaveolens* and *L. aurita* plus antibiotics against pathogen strains was used to determine synergistic MIC of essential oils and antibiotics.

Out of 20 combinations tested on bacterial strains, 14 Synergistic effects were observed between the tested agents (Table 5).

L. aurita exhibited a higher number of synergistic combinations compared to *H. suaveolens*, further corroborating its superior antibacterial potency noted previously. The antibacterial efficacy of both colistin and amoxicillin + clavulanic acid against antibiotic-resistant bacteria was significantly enhanced when combined with *L. aurita*. This essential oil demonstrated the highest frequency of synergism with both antibiotics, a phenomenon likely attributable to its high concentration of terpenic compounds [36]. Notably, the combination of amoxicillin + clavulanic acid with *L. aurita* rendered *S. aureus* susceptible to the antibiotic, despite its initial resistance. A similar synergistic interaction was reported by Gallucci *et al.* [58] when combining carvone with penicillin against *S. aureus*.

Most combinations demonstrated synergistic effects against *P. aeruginosa*, a strain widely recognized as one of the most resistant to antimicrobial agents [47].

Oussalah *et al.* [59] reported that monoterpenes particularly the phenolic components of essential oils induce damage to the outer membrane of bacteria. This leads to increased membrane permeability to protons and potassium ions, depletion of intracellular ATP reserves, disruption of the proton motive force, and denaturation of intracellular proteins [47,60]. Similarly, amoxicillin + clavulanic acid inhibits bacterial cell wall synthesis by inactivating key enzymes involved in peptidoglycan assembly [61]. Thus, the observed synergies may be explained by a complementary mechanism of action between the essential oils and the antibiotics.

It should be noted that not all combinations resulted in synergistic effects. This may be due to the fact that essential oil constituents interact differently depending on the specific antibiotic and the target bacterial species [62]; Consequently, such combinations must undergo rigorous in vitro testing before their potential application in clinical treatment.

Table 5: Synergistic effects of essential oils and different antibiotics against pathogens.

Bacterial strains	Antibacterial agents	MICc	MICa	$\frac{MICa}{MIC_C}$	IFCI	Combination effects
***E. faecalis* 0366V**	Colistin	3.75±0.00	0.93±0.57	0.24	0.49	Synergy
	Hyptis suaveolens	38.28±0.00	9.57±1.00	0.25		
	Colistin	3.75±0.00	0.46±0.57	0.12	0.34	Synergy
	Lagerra aurita	36.46±0.00	8.11±1.00	0.22		
***S. aureus* ATCC 2523**	Amoxiclav	24.00±0.00	3.75±1.00	0.15	0.27	Synergy
	Hyptis suaveolens	38.28±0.00	4.78±0.57	0.12		
	Amoxiclav	24.00±0.00	3.75±1.00	0.15	0.44	Synergy
	Lagerra aurita	1.69±0.00	0.50±0.57	0.20		
	Colistin	0.15±0.06	0.12±1.00	0.80	0.93	Addition
	Hyptis suaveolens	38.28±0.00	4.78±0.00	0.13		
	Colistin	0.15±0.06	0.12±0.00	0.80	1.00	Addition
	Lagerra aurita	1.69±0.00	0.34±1.00	0.20		
***B. cereus* 0998V**	Amoxiclav	13.00±1.00	3.75±0.00	0.29	0.78	Addition
	Hyptis suaveolens	9.57±0.00	4.78±1.00	0.49		
	Amoxiclav	13.00±1.00	0.93±1.00	0.07	0.32	Synergy
	Lagerra aurita	8.11±0.00	2.02±0.57	0.25		
	Colistin	3.33±1.40	3.75±1.00	1.12	3.12	Indifference
	Hyptis suaveolens	9.57±0.00	19.14±1.00	2.00		
	Colistin	3.33±1.40	0.58±1.00	0.17	0.41	Synergy
	Lagerra aurita	8.11±0.00	2.02±0.57	0.24		
***E. coli* ATCC 25922**	Amoxiclav	23.33±0.57	3.75±0.00	0.16	0.28	Synergy
	Hyptis suaveolens	38.28±0.00	4.78±1.00	0.12		
	Amoxiclav	23.33±0.57	3.75±0.57	0.16	0.28	Synergy
	Lagerra aurita	64.92±0.00	8.11±1.00	0.12		
	Colistin	5.00±0.00	9.37±0.00	1.87	3.87	Indifference
	Hyptis suaveolens	38.28±0.00	19.14±0.57	0.50		

	Colistin	5.00±0.00	9.37±1.00	1.87	2.12	Indifference
	Lagerra aurita	64.92±0.00	16.23±1.00	0.25		
***Y. enterocolitica* 0938V**	Colistin	3.75±0.00	0.78±0.00	0.20	0.22	Synergy
	Lagerra aurita	36.46±0.00	1.01±0.00	0.02		
	Colistine	3.75±0.00	1.56±0.57	0.41	0.43	Synergy
	Hyptis suaveolens	38.28±0.00	2.39±0.57	0.06		
***P. aeruginos a* ATCC 9027**	Amoxiclav	29.66±0.57	3.75±0.57	0.12	0.37	Synergy
	Hyptis suaveolens	9.57±0.00	2.39±0.00	0.25		
	Amoxiclav	29.66±0.57	1.87±1.00	0.06	0.31	Synergy
	Lagerra aurita	8.11±0.00	2.02±0.00	0.25		
	Colistin	5.00±0.00	0.78±0.57	0.16	0.41	Synergy
	Hyptis suaveolens	9.57±0.00	2.39±1.00	0.25		
	Colistin	5.00±0.00	1.25±0.00	0.25	0.37	Synergy
	Lagerra aurita	8.11±0.00	1.01±0.00	0.12		

Notes: The values represent the means of the three trials ± standard deviations. **Abbreviations**: MICc, MIC of essential oils or antibiotics tested in combination; MICa, MIC of essential oils or antibiotics tested alone; FICI, Fractional Inhibitory Concentration Index.

3.5. Loss of 260 nm-absorbing material

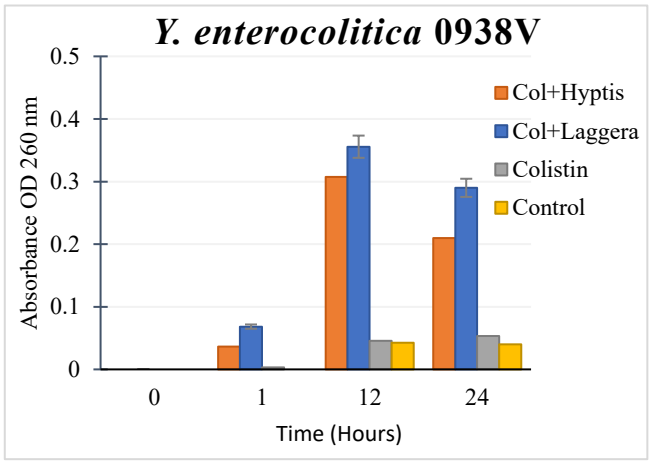
To elucidate the mechanism of action underlying the synergism between essential oils and antibiotics, the leakage of intracellular components was evaluated. This investigation sought to determine whether such combinations compromise the structural integrity and permeability of bacterial membranes. Given that intracellular components release occurs almost instantaneously upon plasma membrane rupture, their exudation was quantified *via* spectrophotometric monitoring at 260 nm.

The mean absorbance of released material following treatment of resistant bacteria with synergistic combinations was markedly higher (0.297) than that observed for antibiotics alone (0.064) or the control (0.038). Kinetic analysis (Figure 3) revealed an initial phase of rapid leakage during the first hour of treatment accounting for 58% of the total 24-hour release followed by a more gradual, sustained efflux. These observations align with findings by Phitaktim *et al* and Li *et al.* [63,64] who reported comparable kinetics when combining *Garcinia mangostana* L essential oil, which shares a similar chemical profile with *L. aurita* and *H. suaveolens*, with oxacillin. Absorbance values for the synergistic combinations were significantly elevated compared to the control (ANOVA, $p < 0.05$), suggesting that 260 nm-absorbing material release was directly induced by the combined agents rather than occurring as a secondary consequence of cell wall weakening and subsequent osmotic lysis [65]. Furthermore, the release of absorbing material was significantly more pronounced in Gram-positive bacteria than in Gram-negative bacteria (ANOVA, $p < 0.05$). This disparity is likely due to the less complex cell wall structure of Gram-positive species [18,66]. However, no significant difference (ANOVA, $p > 0.05$) was observed between the various synergistic combinations themselves regarding the volume of cytoplasmic leakage.

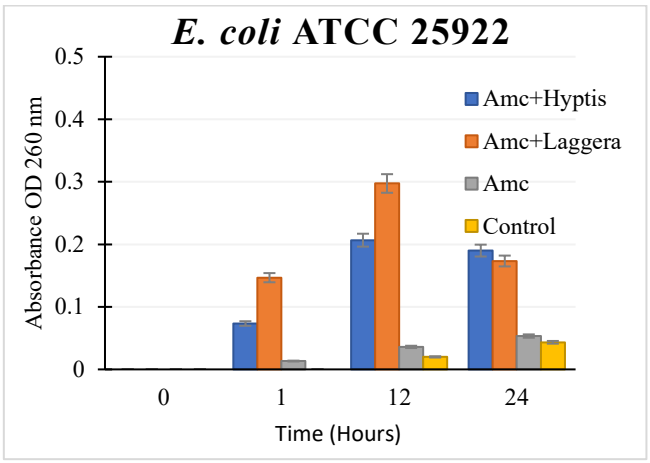
These results indicate that while intracellular components release depends on the bacterial wall structure, this release varies slightly regardless of the type of essential antibiotic combinations. The substantial loss of membrane integrity and increased permeability likely facilitate the restoration of susceptibility in previously resistant strains. These findings further suggest that *L. aurita* and *H. suaveolens* essential oils may inhibit beta-lactamase activity or interfere with lipopolysaccharide (LPS) charge modifications, both pivotal resistance mechanisms. Similar effects were observed by Eumkeb and Chukrathok [67] where the combination of ceftazidime and galangin caused ultrastructural damage and restored ceftazidime sensitivity in *S. aureus*.

As the mechanisms of colistin and amoxicillin + clavulanic acid are well characterized [68], these data strongly suggest that the bacterial cell envelope remains the primary target for *L. aurita* and *H. suaveolens* essential oils, consistent with other oils of similar chemical composition [64,65,69,70,71].

The combination of these oils with antibiotics may result in the independent binding of each agent to the cell membrane, leading to lysis, increased permeability, and subsequent genomic degradation and cell death. Alternatively, these agents may form complexes that subsequently target the membrane. Combinations of *L. aurita* or *H. suaveolens* with colistin and amoxicillin + clavulanic acid consistently triggered the release of intracellular contents, mirroring mechanisms observed when similar oils were combined with polymyxin B and piperacillin. [72,73]. In conclusion, the combination of *L. aurita* and *H. suaveolens* with colistin and amoxicillin + clavulanic acid possesses the capacity to induce membrane disruption and cytoplasmic leakage [74,75]. Given the heterogeneous nature of these essential oils, it is improbable that a single component or mechanism accounts for the observed antimicrobial efficacy. Consequently, further research is warranted to fully elucidate these complex pathways.



(A)



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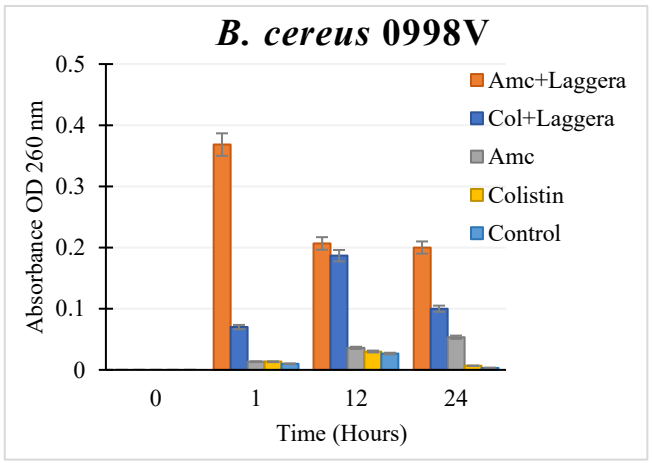

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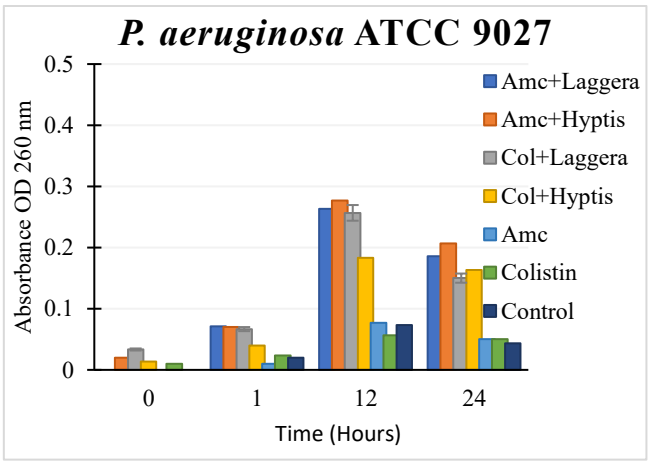

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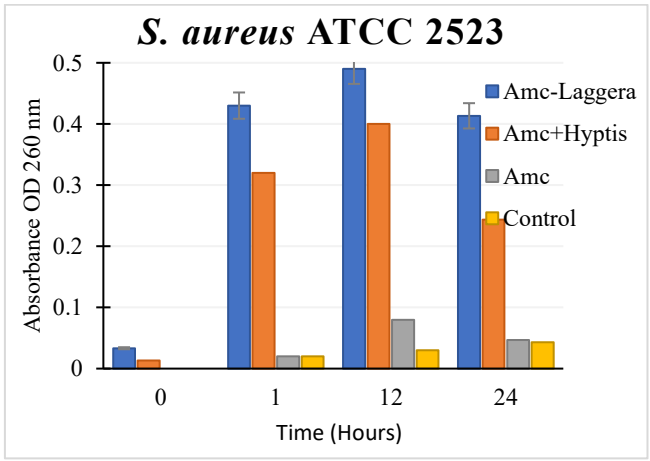

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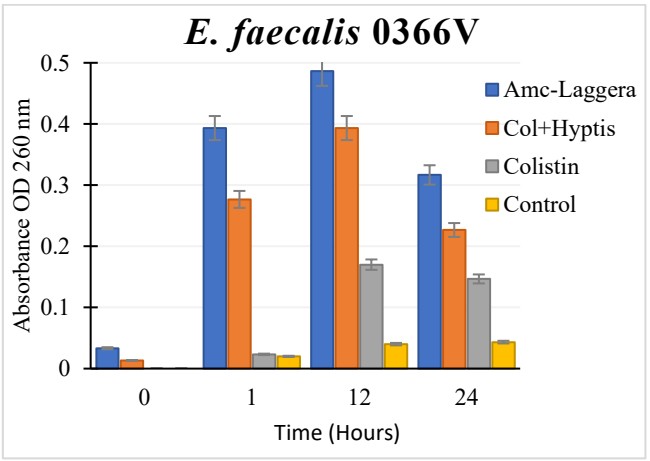

(F)

Figure 3: Loss of 260 nm-absorbing material from the controls and from (A). *Y.enterolitica*, (B). *E. coli*, (C). *B. cereus*, (D). *P. aeruginosa* (E) *E. faecalis* (F). *S. aureus* treated with Col+ Hyptis= Colistin + *Hyptis suaveolens*, Col+Laggera= Colistin + *Laggera aurita*, Amc+Hyptis= Amoxicillin + clavulanic acid + *Hyptis suaveolens*, Amc+Laggera = Amoxicillin + clavulanic acid + *Laggera auita*, Amc= Amoxicillin + clavulanic acid, Values represent the mean of three trials; vertical bars indicate standard errors.

3.6. Quantification of DNA released by treated bacteria

Figure 4 illustrates the quantity of DNA released by bacteria.

The essential oil-antibiotic combinations tested against the bacterial strains resulted in a mean DNA release of approximately 102.28 µg/mL. In contrast, bacteria only treated with antibiotics exhibited a mean release of 32.04 µg/mL. While the control group showed a minimal release of 10.59 µg/mL. The highest concentration of released DNA (270 µg/mL) was recorded during the treatment of *S. aureus* with the combination of amoxicillin + clavulanic acid and *L. aurita*. The lowest release among the treated groups (44 µg/mL) occurred during the treatment of *P. aeruginosa* with the combination of amoxicillin + clavulanic acid and *H. suaveolens*.

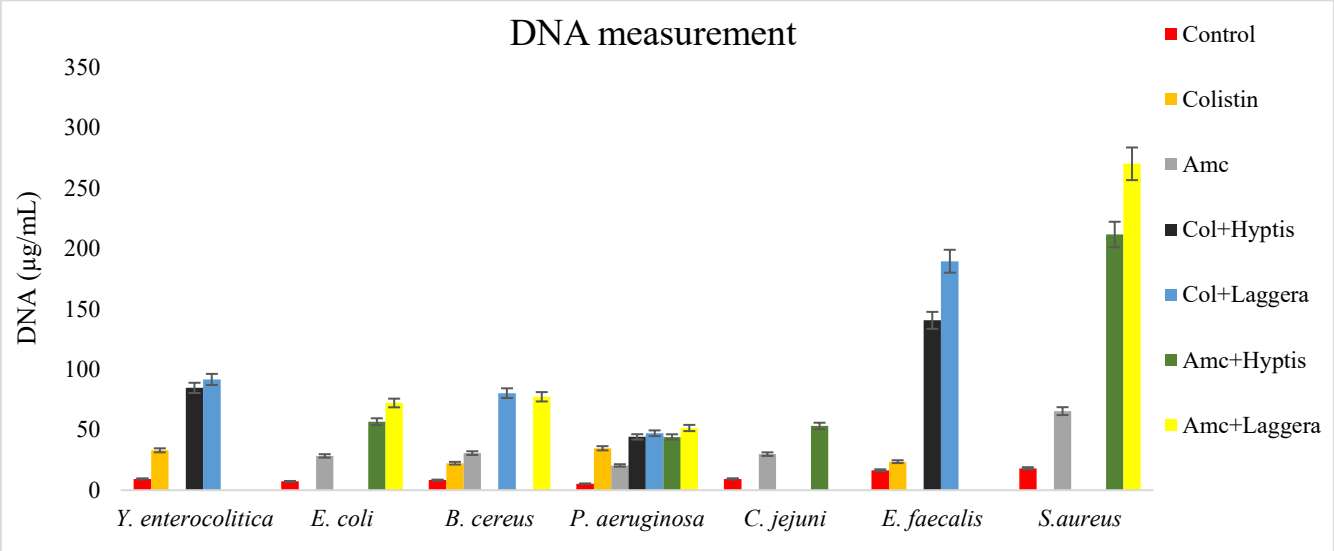

Figure 4: DNA release from antibiotic-resistant strains treated for 24 hours with Colistin, Amc= Amoxicillin + clavulanic acid, Col+Hyptis= Colistin + *Hyptis suaveolens,* Col+Laggera = Colistin + *Laggera aurita*, Amc+Hyptis = Amoxicillin + clavulanique acid + *Hyptis suaveolens*, Amc+Laggera = Amoxicillin + clavulanique acid + *Laggera aurita* compared to control. Values represent the mean of three trials; vertical bars indicate standard errors.

The amount of DNA released by treated bacteria was significantly higher than that of the untreated control (Table 6), though release rates varied by strain. The maximum DNA release rate (93.29 %) was obtained with the amoxicillin + clavulanic acid and *L. aurita* combination against *S. aureus*. The lowest rate among synergistic treatments (89.62 %) was observed with colistin and *L. aurita* against *B. cereus*.

Table 6: Amount of DNA released by bacteria treated with synergistic combinations compared to untreated bacteria.

Bacterial strains	DNA (µg/mL)		
	Control	Bacteria treated with synergistic MIC	% of DNA release
***Y. enterocolitica* 0938V**	9.33^{c}±0.02	91.66^{c}±0.04	89.82^{cd}±0.02
***E. coli ATCC* 25922**	7.26^{e}±0.01	72.16^{e}±0.01	89.93^{c}±0.02
***B. cereus* 0998V**	8.33^{d}±0.00	80.3^{d}±0.01	89.62^{d}±0.01
***P. aeruginosa* ATCC 9027**	5.33^{f}±0.01	51.46^{g}±0.03	89.64^{d}±0.03
***E. faecalis* 0366V**	16.43^{b}±0.02	189.46^{b}±0.00	91.32^{b}±0.02
***S. aureus* ATCC 2523**	18.10^{a}±0.03	270.1^{a}±0.00	93.29^{a}±0.05

Statistical analysis (ANOVA, $P < 0.05$) revealed a significant difference between the DNA quantities released by resistant bacteria treated with antibiotics alone versus those treated with synergistic combinations. This discrepancy is likely attributable to initial antibiotic resistance mechanisms [17]. These results align with those of Rhayour [17] who noted that *E. coli* treated with bactericidal doses of polymyxine B showed no detectable nucleic acid bands, unlike those treated with essential oils.

While Ghaly [76] observed similar trends testing seven essential oils, their reported maximum release rate was

lower (46.94 %) than the rates observed in this study. Consistent with Fawzy [77].

DNA release from treated bacteria significantly (ANOVA, $P < 0.05$) exceeded that of the controls. Furthermore, a significant difference (ANOVA, $P < 0.05$) was observed between Gram-positive and Gram-negative bacteria; the former exhibited higher DNA release rates (Table 7), confirming that Gram-positive strains are more susceptible to antibacterial agents [27,78,79].

A DNA release rate of 93.76 % suggests a mechanism involving the simultaneous disruption of the peptidoglycan layer and the plasma membrane. UV spectroscopy quantification confirms that synergistic combinations successfully damaged the structural integrity of *Y. enterocolitica*, *E. coli*, *B. cereus*, *P. aeruginosa*, *E. faecalis*, and *S. aureus*, leading to substantial DNA loss. These results validate the efficacy of essential oil-antibiotic combinations against multidrug-resistant bacteria.

This study highlights the potential of *L. aurita* and *H. suaveolens* essential oils to enhance antibiotic efficacy. These findings also provide a theoretical basis for developing simplified bacterial lysis methods for DNA/RNA extraction that bypass the need for specific enzymes (e.g., lysozymes, lysostaphin) or denaturing detergents like SDS or Triton X100 [80].

4. CONCLUSION

In conclusion, the findings of this study demonstrate that the essential oils of *Laggera aurita* and *Hyptis suaveolens* possess not only intrinsic antibacterial activity against multidrug-resistant bacteria but also potent synergistic properties when combined with conventional antibiotics. The synergistic interaction between these essential oils and beta-lactams or glycopeptides likely involves dual mechanisms of action: the physical disruption of the bacterial cell wall and plasma membrane, alongside the potential inactivation of beta-lactamase activity or the inhibition of charge modifications in the lipopolysaccharide (LPS) layer.

This antimicrobial mechanism induced a bactericidal effect against strains previously resistant to both amoxicillin + clavulanic acid and colistin. Most of these effective essential oil-antibiotic combinations enhanced the antibacterial potency of the tested antibiotics to varying degrees. Collectively, these results provide a theoretical framework for future *in vivo* studies, which may ultimately lead to the development of novel antimicrobial agents formulated from *L. aurita* and *H. suaveolens* essential oils in combination with antibiotics. Such advancements could significantly improve the efficacy of existing clinical treatments in the fight against multidrug-resistant pathogenic bacteria. However, further research is required to identify the specific bioactive compounds responsible for these effects within the complex oil-antibiotic mixtures and to fully elucidate the molecular interactions at play.

Finally, to address the challenges associated with the low solubility and chemical instability of essential oils, future research should focus on encapsulation or nano-emulsion technologies. These delivery systems are essential to optimize bioavailability, ensure targeted release, and minimize potential cytotoxicity.

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