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Original Article

Comparative Antibacterial and Antibiofilm Potential of *Syzygium aromaticum* and *Melaleuca alternifolia* Methanolic Extracts Against Antibiotic-Resistant Clinical *Escherichia coli* Isolates

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Abstract:

Multidrug resistance and biofilm formation are challenging the treatment of *Escherichia coli* infections, and plant extracts are being investigated as complementary antimicrobial agents. **Methods:** The methanolic extracts of clove, Syzygium aromaticum, and tea tree, Melaleuca alternifolia, were compared with 4 clinical isolates of E. coli, 3 from Al-Ramadi Teaching Hospital and 1 from Heet General Hospital. The antibiotic susceptibility testing was performed by the Kirby-Bauer disk diffusion method (ceftriaxone, ciprofloxacin, amikacin, ceftazidime). The antibacterial activity was evaluated using the agar well diffusion method; biofilm production was evaluated using the Congo red agar (CRA), and the effect on biofilm production was evaluated using the crystal violet microtiter plate (CV-MP) method read at 450 nm. **Results:** Ciprofloxacin was the most active antibiotic (zone up to 48 mm), while most other zones were in the resistant zone (Heet isolate). Inhibition zones of 15- 28 mm were produced in the case of tea tree extract and 9- 13 mm in the case of clove extract. All isolates produced dark brown to black colonies, which indicates the production of biofilm on CRA. In the microtiter assay, the wells treated with extract were stained lighter than the untreated wells, and the OD450 readings were in the range of 0.045 – 0.419. **Conclusions:** In these conditions, tea tree extract produced larger inhibition zones, and both extracts were effective at inhibiting crystal violet staining. Due to the limited number of isolates, lack of replicate statistics, and lack of a methanol-matched control in the biofilm assay, the results are preliminary and require further evaluation by MIC/MBC and sub-inhibitory antibiofilm and phytochemical evaluation.

Keywords: *Escherichia coli*; biofilm; *Syzygium aromaticum*; *Melaleuca alternifolia*; plant extracts; antimicrobial resistance

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1. INTRODUCTION

Antimicrobial resistance (AMR) is considered to be one of the greatest challenges to global public health. *Escherichia coli* bacteria are an important cause of urinary tract infection, bloodstream infection, wound infection, and multidrug-resistant (MDR) strains spread through horizontal transmission of resistance genes on plasmids and transposons [1,2]. Biofilm formation exacerbates resistance: the bacteria in the biofilm are resistant to antibiotics and host defences at concentrations much higher than those needed against planktonic bacteria, and favour persistent infections where the bacteria are device-associated [3–5].

Plant-derived products are also appealing alternatives to antibiotic use due to their structurally diverse

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secondary metabolites, which can often target multiple targets such as membrane integrity, efflux and quorum sensing [6-9]. Clove buds contain the phenylpropanoid eugenol, in addition to eugenyl acetate, β-caryophyllene, flavonoids, and tannins, which typically account for 70–85% of the essential oil produced from the bud [10,11]. The dominant compound in tea tree (Melaleuca alternifolia, Myrtaceae) leaf oil is terpinen-4-ol (30–40%), followed by γ-terpinene, α-terpinene and 1,8-cineole [12,13]. Both plants are known to be active against resistant bacteria [14-16].

However, most published reports are focused on the essential oils or isolated compounds and direct comparison of clove and tea tree preparations against clinical E. coli, which include the growth inhibition and biofilm endpoints is limited [17,18]. The goals of this study were therefore to (i) characterize the antibiotic susceptibility of four clinical E. coli isolates, (ii) compare the antibacterial activity of the methanolic extracts of S. aromaticum and M. alternifolia, and (iii) assess whether the isolates produce biofilms and if these extracts affect biofilm formation.

2. MATERIALS AND METHODS

2.1 Plant extracts

Clove buds and tea tree material were ground to a fine powder. Twenty-five grams of each powder were suspended in 250 mL methanol in a glass beaker sealed with plastic film and stirred continuously on a magnetic stirrer, without heating, for three days. The mixture was filtered through several layers of sterile gauze and then through Whatman filter paper. The filtrate was poured into glass Petri dishes, and the solvent was evaporated in an oven overnight; the dry residue was scraped off with a sterile spatula and stored in sterile Eppendorf tubes [19]. For testing, 0.080 g of dry extract was dissolved in 1 mL methanol (80 mg/mL) and vortexed.

2.2 Bacterial isolates

Four clinical *E. coli* isolates were used: three from Al-Ramadi Teaching Hospital (designated 1, 18 and 20) and one from Heet General Hospital (H). The isolates, previously cultured and maintained on MacConkey agar, were subcultured on fresh MacConkey agar to confirm purity and reactivated on nutrient agar 24 h before each experiment.

2.3 Antibiotic susceptibility

Susceptibility was tested by the Kirby–Bauer disk diffusion method on Mueller–Hinton agar, one plate per isolate [20]. Well-isolated colonies were suspended in 2 mL sterile distilled water and vortexed, and the suspension was swabbed evenly over the agar. Discs of ceftriaxone (CRO, 10 µg), ciprofloxacin (CIP, 10 µg), amikacin (AK, 10 and 30 µg), and ceftazidime (CAZ, 30 µg) were applied with sterile forceps. Plates were sealed with Parafilm and incubated at 37 °C for 24 h, and inhibition zones were measured in mm. Zones were interpreted indicatively against CLSI M100 breakpoints [21]; an isolate non-susceptible to at least one agent in three or more antimicrobial categories was considered MDR [22].

2.4 Agar well diffusion

Mueller–Hinton plates were inoculated as above, and three wells were punched with a sterile cork borer. Wells received 60 µL of clove extract, tea tree extract, or sterile distilled water (control). Plates were sealed, incubated at 37 °C for 24 h, and the diameter of each inhibition zone was measured in mm.

2.5 Biofilm detection on Congo red agar

Nutrient agar was autoclaved and cooled to about 45 °C; 0.02 g Congo red and 9 g glucose were added and mixed, and the medium was poured into sterile Petri dishes. Each isolate was streaked on a separate plate and incubated at 37 °C for 24 h. Black or dark colonies were taken as evidence of exopolysaccharide-producing (biofilm-forming) isolates [23].

2.6 Microtiter plate biofilm assay

Brain heart infusion (BHI) medium with 0.25% glucose was autoclaved and cooled. For each isolate, twelve wells of a sterile 96-well plate were allocated:

six for clove and six for tea tree, of which three contained the extract and three served as extract-free controls. Treated wells received 150 µL BHI, 30 µL extract solution, and 20 µL bacterial suspension; control wells received BHI and bacterial suspension only. After 24 h at 37 °C, the wells were emptied, washed gently with normal saline, inverted on sterile gauze to dry, stained with 20 µL crystal violet for 20 min, washed, and dried. Bound dye was solubilised with 200 µL of 5% isopropanol in distilled water, and the optical density (OD) was read at 450 nm with an ELISA microplate reader [24]. No statistical analysis was applied; zones and readings are reported as measured.

3. RESULTS AND DISCUSSION

3.1 Extraction

Methanolic extraction yielded dry crude extracts from both plants (Fig. 1). The clove extract was dark brown with a strong clove-like odour, whereas the tea tree extract was greenish-brown. Methanol is a polar solvent that recovers phenolics, flavonoids, and tannins, and the antibacterial activity of clove is generally ascribed to eugenol [10,25]. The chemical composition of the present extracts was not determined, so such attributions are inferred from the literature rather than demonstrated.

3.2 Antibiotic susceptibility

Susceptibility varied among isolates (Fig. 2, Table 1). Ciprofloxacin was the most active agent: the Heet isolate gave a 48 mm zone, isolate 18 gave 21 mm, and isolate 1 gave 12 mm, whereas isolate 20 was resistant (0 mm). Ceftriaxone zones were small (7–12 mm; isolate 18, 0 mm); amikacin gave 9–14 mm depending on disc content and isolate, and ceftazidime was ineffective against most isolates, the exception being isolate 20 (14 mm).

Applying CLSI M100 breakpoints indicatively, every reported zone except ciprofloxacin against the Heet isolate falls in the resistant range. Isolates 1, 18 and 20 therefore appear to be resistant to cephalosporins, aminoglycosides and fluoroquinolones and to meet the MDR definition [22], while the Heet isolate retained ciprofloxacin susceptibility. This pattern agrees with the widespread resistance of clinical *E. coli* to β-lactams, aminoglycosides and fluoroquinolones [1,2,26]. The disc contents used for ceftriaxone and ciprofloxacin (10 µg) differ from the standard CLSI discs (30 µg and 5 µg), so the categories should be regarded as indicative.

3.3 Antibacterial activity of the extracts

Both extracts inhibited all four isolates in the agar well diffusion assay, but to different degrees (Fig. 3, Table 2). Tea tree extract was the more active, with zones of 15–28 mm: isolate 20 (28 mm), isolate 1 (27 mm), isolate 18 (22 mm), and the Heet isolate (15 mm). Clove extract produced moderate zones of 9–13 mm.



Figure 1. Plant extracts. (a) Methanolic extraction of clove (left flask) and tea tree (right flask). (b) Dried crude extracts stored in sterile tubes.

Table 1. Antibiotic susceptibility of the four *E. coli* isolates (disk diffusion).

Antibiotic	Disc (µg)	Zone (mm)	Indicative CLSI category
CIP	10	H 48; 18: 21; 1: 12; 20: 0	S (H); R (others)
CRO	10	7–12; isolate 18: 0	R
AK	10, 30	9–14	R
CAZ	30	0 (most); isolate 20: 14	R

CIP, ciprofloxacin; CRO, ceftriaxone; AK, amikacin; CAZ, ceftazidime; S, susceptible; R, resistant

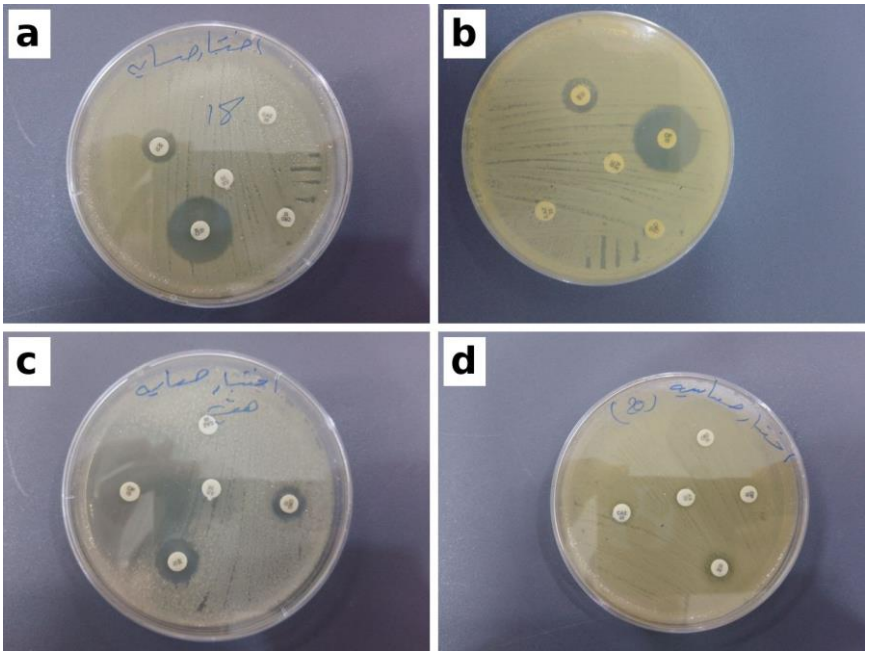


Figure 2. Antibiotic susceptibility testing on Mueller–Hinton agar. (a–d) Plates of the four *E. coli* isolates with antibiotic discs.

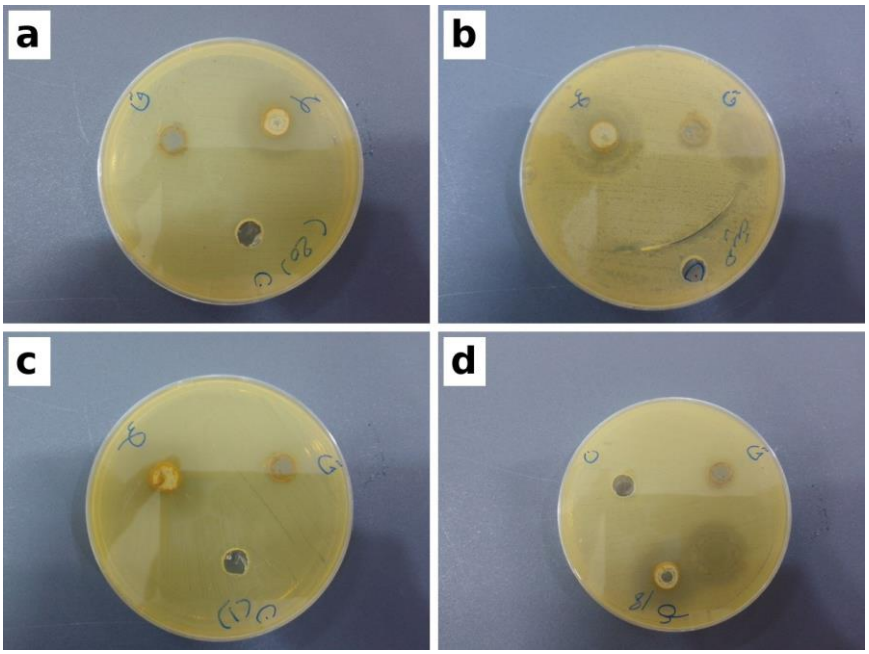


Figure 3. Agar well diffusion. (a–d) Inhibition zones around wells containing clove extract, tea tree extract, and the distilled-water control for the four *E. coli* isolates.

The largest tea tree zones were recorded for isolates 20 and 1, which had the smallest ciprofloxacin zones (0 and 12 mm), and the smallest tea tree zone for the only ciprofloxacin-susceptible isolate (H). With four isolates, this cannot be tested statistically, but it suggests that extract activity was not reduced by the antibiotic-resistance phenotype, consistent with the multitarget, membrane-active mechanisms described for terpenoids and phenolics [6,16]. Comparable activity of clove preparations against MDR uropathogenic *E. coli* has been reported [15]. Zone diameters should nevertheless be compared with caution: they depend on diffusion of each extract through agar, which is poorer for lipophilic constituents, and on the well diameter [27].

3.4 Biofilm production on Congo red agar

All isolates produced dark brown to black colonies on CRA after 24 h (Fig. 4), indicating exopolysaccharide production and biofilm-forming capacity; some isolates were distinctly black and others dark brown, suggesting differences in the level of production. In *E. coli*, Congo red binding is largely attributed to curli fimbriae and cellulose [3]. CRA is, however, a qualitative screening test whose agreement with quantitative assays varies [28], which is why the microtiter assay was added. Biofilm formation may enhance survival and contribute to persistent infection by protecting cells from antimicrobials and host defences [4].

3.5 Effect of the extracts on biofilm formation

After crystal violet staining (Fig. 5), control wells containing only medium and bacteria were visibly dark, indicating strong biofilm formation, while wells treated with clove extract were lighter. Tea tree-treated wells were also lighter than the controls, although the reduction looked slightly less pronounced than with clove. Colour intensity differed between isolates, indicating isolate-dependent biofilm capacity. These observations are visual; they are supported by the raw reader values in Table 3, which ranged from 0.045 to 0.419. Most wells gave low readings (<0.12), and nine of the 96 wells read ≥0.17, five of them adjacent (row D, columns 8–12).

Table 2. Inhibition zones of the methanolic extracts (80 mg/mL; 60 µL per well).

Extract	Range (mm)	Zones by isolate (mm)
Tea tree	15–28	20: 28; 1: 27; 18: 22; H: 15
Clove	9–13	See Fig. 3

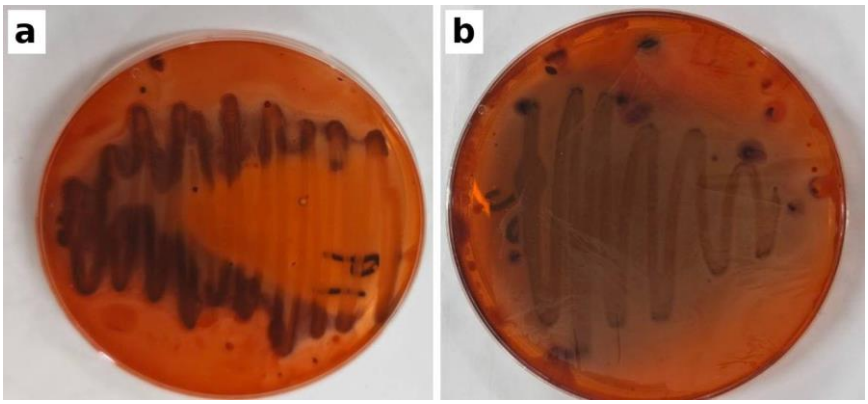


Figure 4. Biofilm-producing *E. coli* isolates on Congo red agar (a, b): dark brown to black colonies.

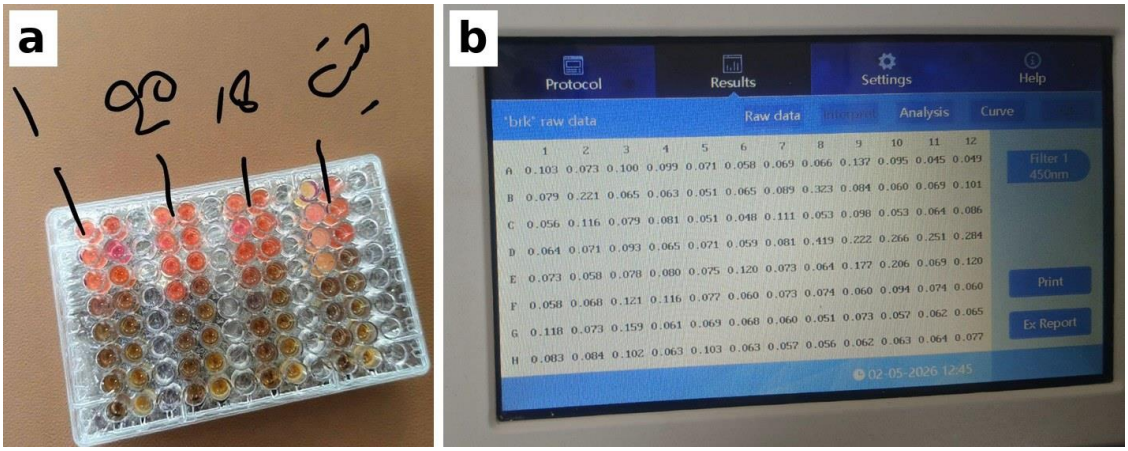


Figure 5. Crystal violet microtiter plate assay. (a) Plate after staining: biofilm-containing wells are dark, wells with reduced biofilm are light orange or pink; numbers above the plate indicate the isolates. (b) Raw OD readings displayed by the microplate reader (filter 450 nm).

Table 3. Raw OD_{450} readings of the 96-well microtiter plate (rows A–H, columns 1–12) transcribed from the microplate-reader display. Values ≥ 0.17 are in bold.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.103	0.073	0.100	0.099	0.071	0.058	0.069	0.066	0.137	0.095	0.045	0.049
B	0.079	**0.221**	0.065	0.063	0.051	0.065	0.089	**0.323**	0.084	0.060	0.069	0.101
C	0.056	0.116	0.079	0.081	0.051	0.048	0.111	0.053	0.098	0.053	0.064	0.086
D	0.064	0.071	0.093	0.065	0.071	0.059	0.081	**0.419**	**0.222**	**0.266**	**0.251**	**0.284**
E	0.073	0.058	0.078	0.080	0.075	0.120	0.073	0.064	**0.177**	**0.206**	0.069	0.120
F	0.058	0.068	0.121	0.116	0.077	0.060	0.073	0.074	0.060	0.094	0.074	0.060
G	0.118	0.073	0.159	0.061	0.069	0.068	0.060	0.051	0.073	0.057	0.062	0.065
H	0.083	0.084	0.102	0.063	0.103	0.063	0.057	0.056	0.062	0.063	0.064	0.077

The reduced staining in extract-treated wells is consistent with reports that plant phenolics and terpenoids interfere with adhesion, matrix production, and quorum sensing [9,29]. Two features of the design, however, prevent this from being read as a specific antibiofilm effect (Section 3.6): the extracts also inhibited planktonic growth (Section 3.3), and the treated wells received the extract dissolved in methanol while the controls did not. Crystal violet absorbance is conventionally read at 570–600 nm [24]; readings at 450 nm will underestimate biofilm mass, which may explain the low overall values.

3.6 Limitations

(i) Only four isolates were studied, and replicate numbers, means, standard deviations, and statistical tests are not reported. (ii) Treated wells contained 30 µL of methanolic extract in 200 µL (about 15% v/v methanol), whereas control wells contained none, and the well-diffusion control was water; solvent-matched controls are needed. (iii) The botanical authentication, source, part used, and yield of the plant material were not recorded, and the extracts were not chemically profiled; tea tree activity in the literature derives mainly from the essential oil rather than a methanolic extract. (iv) Inocula were prepared from colonies in distilled water without McFarland standardisation. (v) Species identification beyond growth on MacConkey agar and the cork-borer diameter was not documented. (vi) Biofilm was read at 450 nm without a blank-based cut-off, and no sub-inhibitory (sub-MIC) concentrations were tested, so antibiofilm activity cannot be separated from growth inhibition.

4. CONCLUSIONS

Under these experimental conditions: (1) all four clinical *E. coli* isolates produced biofilm on Congo red agar; (2) the isolates were largely resistant to the

antibiotics tested, with ciprofloxacin the most active and three isolates appearing MDR; (3) both extracts inhibited all isolates, with tea tree extract giving larger zones (15–28 mm) than clove extract (9–13 mm); and (4) both extracts reduced crystal violet staining relative to controls, apparently more so with clove. These results are preliminary. Future work should determine MIC and MBC values by broth microdilution, test antibiofilm activity at sub-MIC concentrations with methanol- or DMSO-matched controls, use replicates with statistical analysis and absorbance at 570–600 nm, characterise the extracts by GC–MS or HPLC, examine synergy with antibiotics, and assess cytotoxicity.

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