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Research article

Disruption of bacterial redox homeostasis by *Tanacetum argyrophyllum* essential oil in antibiotic-sensitive and resistant *Escherichia coli*

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Abstract: The emergence of antibiotic-resistant bacteria highlights the need for novel natural antimicrobial agents. This study aimed to evaluate the chemical composition, antimicrobial activity, and redox-modulatory effects of *Tanacetum argyrophyllum* essential oil (EO) against *Escherichia coli* wild-type (*E. coli* K12) and kanamycin-resistant (*E. coli* pARG-25) strains. EO extraction yielded 0.1% (v/w), with eucalyptol (35%), camphor (24%), and camphene (17%) as major constituents, thus defining a eucalyptol–camphor chemotype. The minimum inhibitory concentrations were 100 µL/mL for both strains. Biophysical analyses revealed that EO exposure delayed the decline in the extracellular oxidation-reduction potential (ORP) and altered the pH dynamics, thus reflecting interference with bacterial kinetics, metabolic activity, and organic acid production. Biochemical assays indicated strain-specific oxidative stress responses: malondialdehyde formation increased by ~50% in the resistant strain, accompanied by substantial upregulation of superoxide dismutase and catalase activities, whereas the wild-type strain showed moderate changes. These data suggest that *T. argyrophyllum* EO disrupts bacterial redox homeostasis, likely via the modulation of antioxidant defenses, thus resulting in reduced viability and enhanced oxidative stress in resistant cells.

The findings provide some influence modes into EO-mediated bacteriostatic effects and support its potential as a natural agent that targets kanamycin-resistant bacteria through redox-dependent pathways.

Keywords: Armenian flora; prooxidant activity; SOD; catalase; ORP; redox status

Abbreviations: AI: antioxidant index; AMR: antimicrobial resistance; BCA: bicinchoninic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EO: Essential oil; GC-MS: Gas Chromatography Mass Spectrometry; GPx: glutathione peroxidase; HPLC: High-performance liquid chromatography; MDA: malondialdehyde; MIC: minimum inhibitory concentration; NIST: National Institute of Standards and Technology; ORP: Oxidation–reduction potential; ROS: reactive oxygen species; RRI: Relative Retention Index; SD: Standard deviation; SOD: Superoxide dismutase; TBA: Thiobarbituric Acid; TBARS: Thiobarbituric Acid Reactive Substances; WT: wild type

1. Introduction

Compounds of plant origins have been used for the treatment and prevention of various diseases since ancient times, and are still being used today.

The accelerating spread of antimicrobial resistance (AMR) among Gram-negative bacteria represents a major global health concern and necessitates the identification of alternative bioactive agents with distinct and multifactorial mechanisms of action. Plant-derived essential oils (EOs) have attracted considerable attention due to their capacity to simultaneously target multiple cellular processes, including membrane integrity, metabolic pathways, redox homeostasis, etc. [1–3]. Such multimodal activity may reduce the likelihood of resistance development compared with single-target antibiotics. However, many endemic or geographically restricted aromatic species remain insufficiently investigated with respect to their phytochemical composition and mechanisms of antimicrobial activity.

Tanacetum argyrophyllum (C. Koch) Tzvel., an Asteraceae family aromatic plant, is known for its biological effects: *T. argyrophyllum* extract shows inhibitory activity against acetylcholinesterase and butyrylcholinesterase, thus suggesting potential applications in treating Alzheimer's disease. Additionally, it displays α-glycosidase and α-amylase inhibition, thus indicating potential anti-diabetic effect [4]. Moreover, there is some information concerning the anticancer, anticoagulant, anthelmintic, insecticidal, antiulcer, antioxidant, phytotoxic, antifungal, and antibacterial activities of this plant [4–6]. However, the antimicrobial properties of *T. argyrophyllum* have yet to be comprehensively characterized.

It is known that plant origin metabolites can act as oxidative stress promoters in bacterial cells, thereby influencing their metabolism and viability [7–9]. Plant-derived secondary metabolites are increasingly recognized, not only as antimicrobial agents, but also as modulators of bacterial redox homeostasis. Numerous phytochemicals act as pro-oxidative stress inducers in bacterial cells, thereby influencing cellular metabolism, membrane function, and overall viability. Although many plant metabolites are traditionally classified as antioxidants in eukaryotic systems, in prokaryotes, they may exert the opposite effect by promoting the intracellular accumulation of reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH) [10–12]. Excessed ROS generation disrupts the bacterial redox balance, thus leading to the oxidative modification of proteins, lipid peroxidation, DNA damage, and the impairment of essential metabolic pathways.

One major mechanism that underlies phytochemical-induced oxidative stress involves the perturbation of the cytoplasmic membrane and respiratory chain. Lipophilic compounds such as phenolics, monoterpenoids, and phenylpropanoids readily partition into bacterial membranes, thus altering lipid packing, membrane permeability, and electron transport chain dynamics [13,14]. This

disruption enhances electron leakage toward molecular oxygen, thereby stimulating superoxide formation and downstream ROS production. In addition, membrane destabilization compromises the proton motive force and ATP synthesis, thus imposing further bioenergetic stress on bacterial cells [15].

Additionally, plantg metabolites may directly interfere with the bacterial antioxidant defense systems. In many Gram-negative bacteria, oxidative stress responses are regulated by transcriptional systems such as OxyR and SoxRS, which coordinate the expression of catalases, peroxidases, superoxide dismutases, and thiol-dependent redox enzymes [16]. Certain phytochemicals can either impair these regulatory networks or inhibit antioxidant enzymes, thereby limiting the bacterial capacity to detoxify ROS and amplifying oxidative damage. Furthermore, redox-active plant compounds can participate in metal-dependent Fenton reactions, thereby promoting hydroxyl radical formation and causing extensive macromolecular damage [11,12].

Beyond generalized oxidative injury, ROS accumulation influences central metabolic processes. The oxidative modification of respiratory enzymes, dehydrogenases, and ATP synthase perturbs metabolic fluxes and disrupts redox cofactors such as $NADH/NAD^+$, thus ultimately leading to metabolic collapse [17]. Oxidative DNA lesions and strand breaks impair replication and transcription, while protein carbonylation affects the enzyme structure and catalytic activity. When ROS generation exceeds the buffering capacity of bacterial antioxidant systems, cells undergo the irreversible loss of viability [18].

Therefore, based on the above-described information, this study aims to provide some insight into the antimicrobial and oxidative stress–modulating properties of *T. argyrophyllum* EO and to contribute to the evidence-based evaluation of this plant as a potential source of bioactive compounds which target antibiotic-resistant bacteria.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich Co. Ltd. (Taufkirchen, Germany) and VWR International (Pennsylvania, USA).

2.2. Plant material and essential oil extraction

Tanacetum argyrophyllum (C. KOCH) TZVEL plant was harvested from the Vayots Dzor Province of the Republic of Armenia (Jermuk) at 2080 m above sea level, in May-June, during the early flowering period. The plant was identified by Dr. Armen Sahakyan (Mesrop Mashtots Institute of Ancient Manuscripts, Matenadaran, Armenia). The plant collection and use were in accordance with all the relevant guidelines. EO was extracted from dried aerial parts of the plant by Clevenger-type apparatus as described before [19]. The extracted EO was stored in a dark and cool place (8–10 ºC). The number of independent extractions reached 4−5.

2.3. Determination of EO chemical composition

The chemical composition of the *T. argyrophyllum* EO was determined by a Gas Chromatography Mass Spectrometry analysis (GC-MS), as described before [6,19].

2.4. DPPH assay

The free radical scavenging ability of the EO was tested using an ethanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) [19]. The concentration range of 100–0.001 μL/mL of *T. argyrophyllum* EO was tested. Catechin was used as a standard. The sample solution contained 125 μL (1 mM) DPPH, 375 μL ethanol, and 500 μL of the test solution (EO or catechin with different concentrations). In the control sample, the test solution was replaced by ethanol. The absorbance was measured at the wavelength of 517 nm. The radical scavenging activity was calculated using the following formula:

Radical scavenging activity (%) = Ac – As/Ac × 100,

where Ac is the absorbance of the control (DPPH without the addition of test solution), and As is the absorbance of the sample.

The IC50 denotes the concentration of investigated samples required to decrease the DPPH absorbance at 517 nm by 50%.

2.5. Broth dilution assay, investigated strains and growth conditions

The minimal inhibitory concentration (MIC) of *T. argyrophyllum* EO was determined using the broth microdilution method in 96-well plates following standard protocols (using EO different concentrations ranging from 3.125 to 200 $\mu L mL^{-1}$, diluted in 96% ethanol) [17]. The test panel included *Escherichia coli* K-12 (as a reference strain) and kanamycin-resistant *E. coli* pARG-25 strains. The microbial strains were purchased from the Depository center of the Scientific and Production Center "Armbiotechnology" of the National Academy of Sciences of Armenia. The latter carries a high-copy-cloning plasmid which contains a ColE1-type (pMB1-derived; pBR322/pUC) origin of replication and a KanR cassette, thus providing resistance to kanamycin (plasmid size: 6.341 kb). Cells from one colony were transferred to the liquid medium and cultivated overnight at 37 °C. Then, the fresh MP liquid nutrient medium (peptone –20 gL^{-1}, glucose –2 gL^{-1}, NaCl –5 gL^{-1}, K_2HPO_4 –2 gL^{-1}, pH = 7.5) was inoculated with bacteria and incubated for 18–22 hours in aerobic conditions (by shaking with 150 rpm) to use in further experiments. To exclude any potential solvent effects, control experiments were performed using the same final concentrations of ethanol as in EO-treated samples.

2.6. Growth kinetics of E. coli K12 *and E. coli pARG-25 strains under the influence of T. argyrophyllum essential oil*

The growth kinetics assay was employed to understand the pattern of bacterial growth under the influence of *T. argyrophyllum* EO. After the incubation in MP liquid nutrient medium, bacterial growth curves were determined by measuring the turbidity of samples that contain bacteria at 565 ± 15 nm every 30 min using a densitometer (DEN-1B, BIOSAN, Latvia), and the data is presented in McFarland units (MF) [20].

The μ was calculated by the following formula:

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1},$$

where
lnN_2-lnN_1 represents the logarithmic difference of doubled optical reading,
and t_2-t_1 represents the difference between doubling time.
The cell doubling time was determined by the ln2/μ equation [6].

2.7. Determination of SOD activity

The determination of the total SOD activity was carried out by the Beauchamp and Fridovich method (1971) [21].

The EO was applied at 10 μLmL^{-1} in experiments (as sub-inhibitory concentration) to gain the proper quantity of bacterial biomass. A concentrated stock solution of the EO, which was prepared in ethanol (final concentration of ethanol is under 1% and does not disturb the experiments), was added at a volume to 100 mL of liquid nutrient medium that had been pre-inoculated with the bacterial culture. Samples were incubated for 18–22 h at 37 °C on a shaker (150 rpm). The microbial suspension was centrifuged (15 min at 3500 xg), and the supernatant was decanted. The biomass was suspended in a 50 mM phospate buffer, pH = 7.4. In order to break the bacterial cell wall, a BRANSON ultrasonic disintegrator and a 1/4" Tapered Microtip were used (Pulse; J 00: 00: 50sec (on); 00: 00: 60sec (off); 12W, total (on)–2min). Then, the cell lisate was centrifuged at 3500 x g for 10 min and the supernatant was collected and used for enzyme determination assays. The protein concentration of the cell lysate was determined by the bicinchoninic acid (BCA) assay (Bio-Rad Laboratories) [22]. The SOD activity was determined as U/ mg protein and expressed as % of the control.

2.8. Determination of catalase activity

Cell lysate preparation and protein content determination were performed as described above. The determination of the catalase activity was carried out using the spectrophotometric measurement of the dynamics of the hydrogen peroxide concentration at 240 nm using an Agilent Cary 60 UV-Vis spectrophotometer. During the measurements, the temperature was maintained at 37 °C using the Agilent Cary Single Cell Peltier accessory (California, USA). The enzyme kinetics method was used with the following parameters: measurement at 240 nm; duration: 5 min. The reaction mixture contained 12 mM hydrogen peroxide dissolved in 50 mM phosphate buffer (37 °C, pH = 7.4). During the measurement, a quartz cuvette (d = 10 mm, V = 3.5 mL) contained 2.99 mL of the reaction mixture and 10 μL of lysate. The change in absorbance was calculated in 1 minute (slope) using software tools. The catalase activity was calculated using the following formula:

$$Specific\ catalase\ activity = \frac{\Delta \mathrm{Abs}_{240\mathrm{nm}} \cdot \mathrm{min}^{-1}}{43.2} \times \mathrm{N} \times \frac{1}{[protein] mg \cdot l^{-1}},$$

where N is the dilution factor. Data were expressed as % of the control.

2.9. TBARS assay

The determination of the antioxidant capacity of *T. argyrophyllum* EO was carried out by studying

its ability to inhibit malonaldehyde synthesis in bacterial cells, and α-tocopherol was used as positive standard [9].

20% trichloroacetic acid (TCA) was used to precipitate the proteins. The samples were thoroughly mixed and incubated under cold conditions for 10 min. The mixtures were centrifuged at 8000 rpm for 10 min. The resulting supernatants were carefully transferred to new 2 mL microcentrifuge tubes. A control sample containing an equal volume of distilled water instead of cell lysate was prepared in parallel.

200 µL of a thiobarbituric acid (TBA) solution and 200 µL of 0.25 N HCl were sequentially added to each tube, and the samples were thoroughly vortexed. The tubes were tightly sealed and incubated in a water bath at 95 °C for 30 min.

After the incubation, the samples were rapidly cooled on ice for 10 min. An aliquot of 200 µL from each reaction mixture was transferred into a 96-well microplate, and the absorbance was measured at 532 nm using a SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany).

The test solution contained the cell lysate of EO-treated bacteria, and the control solution had the lysate without treatment with EO.

The quantity of malondialdehyde (MDA) was calculated by the following formula:

$C = D \times \varepsilon/A$,

where C is the MDA quantity (nM min mg^{-1}), the extinction coefficient (ε) is 1.56 x 105 M^{-1} cm^{-1}, D represents the absorbance, and A represents the protein quantity (mg/ml).

Data were expressed as the antioxidant index (AI), which is the value based on the percentage of lipid peroxidation inhibition against the control. The following formula was used:

$AI\ (\%) = (C_c - C_s)/C_c \times 100$,

where C_c is the MDA concentration in control tubes, and C_s represents the MDA concentration in the test tubes (nM $(min\ mg)^{-1}$).

2.10. Determination of oxidation–reduction potential ORP (mV) and extracellular pH

The extracellular pH (pH_{out}) was determined using the Milwaukee MW150 MAX 3-in-1 pH/OPR/Temp. Bench Meter (Szeged, Hungary) with a selective pH electrode (HI1131, Hanna Instruments, Portugal), and the ORP was measured using an ORP electrode (HI3131B, Hanna Instruments, Portugal).

The external or medium pH (pH_{out}) and redox potential (ORP, mV) were measured during aerobic growth for up to 24 hours [23].

2.11. Data processing

The experimental data represent the mean ± standard deviation (SD) from at least three biologically independent experiments implemented in at least three technical replicates. The statistical significance was evaluated using a grouped two-way ANOVA with GraphPad Prism, version 8.0.3 (GraphPad Software, San Diego, CA, USA). The software is available at the following link: https://www.graphpad.com. Differences were considered statistically significant at $p < 0.05$, unless stated otherwise. Variability between biological replicates was assessed by comparing independently cultured and treated samples, thus ensuring that reported effects reflect reproducible biological

responses rather than technical variation. Graphical data visualization was performed using GraphPad Prism and Microsoft Excel 2010.

3. Results and discussion

The EO yield of *T. argyrophyllum* (aerial parts) generally ranges from 0.78% to 1.03% (v/w) on a dried mass basis [24]. The yield can significantly vary depending on the growing place, soil conditions, weather, subspecies, harvest time, plant parts used, etc. [25,26]. In our experiments, the *T. argyrophyllum* EO yield reached 0.1 %, v/w, which may reflect environmental factors such as humidity, as well as differences in the plant material and extraction conditions. While a reduced yield may influence the relative proportion of minor constituents, the dominant chemotype identified here is generally consistent with previous reports. Nevertheless, variability in minor compounds may affect reproducibility and should be considered in future studies. From a practical perspective, the low yield may limit economic feasibility for large-scale applications, thus highlighting the need for further optimization of the cultivation and extraction parameters.

The environmental conditions and harvesting time also affect the chemical composition of the EO. Our results from the quantitative and qualitative analyses of *T. argyrophyllum* EO showed the presence of 16 chemical constituents, thus determining its eucalyptol–camphor chemotype. The quantity of the main component eucalyptol (1,8-cineole) reaches 35.0% of the total content, followed by camphor (24.0%) and camphene (17.0%). The identified eucalyptol–camphor chemotype is consistent with reports for several Tanacetum species and other aromatic plants rich in oxygenated monoterpenes. EO dominated by eucalyptol and camphor are known to exhibit both antimicrobial and redox-modulating activities, including pro-oxidative effects under certain conditions [27]. Therefore, the oxidative stress observed in this study is not entirely unique, but likely reflects a common property of eucalyptol-rich oils. However, the magnitude and strain-specific nature of the response may be influenced by the full composition of the oil, including minor constituents and potential synergistic interactions among the 16 identified compounds. This highlights that, while the general behavior aligns with similar chemotypes, the specific biological effects are shaped by the complete chemical profile.

The broth dilution assay revealed that the MIC values of *T. argyrophyllum* EO were 100 μL/mL against the tested *E. coli* strains, including both the wild-type (K-12) and kanamycin-resistant (pARG-25) variants. The investigated EO inhibitory effect against the kanamycin-resistant *E. coli* pARG-25 strain indicates its potential as a natural antimicrobial agent that targets drug-resistant pathogens. Our previous studies suggested that *T. argyrophyllum* EO reduced the bacterial viability, prolonged the lag phase, and suppressed some bioenergetic processes, including proton fluxes and ATPase activities [6].

For further elucidation of any possible influence factors, some biophysical and biochemical parameters of the antioxidant defense system of the bacteria were investigated under the treatment of the *T. argyrophyllum* EO.

In chemical-based tests, *T. argyrophyllum* EO showed a remarkable radical scavenging ability: the DPPH assay showed an IC_{50} value of 19.4 ± 2.5 μL/mL. This relatively low IC_{50} value indicates that this EO possesses a pronounced ability to donate hydrogen atoms or electrons to stabilize free radicals. Such activity is likely associated with the presence of phenolic and oxygenated terpenoid constituents [19,28]. This property may suggest the context-dependent redox behavior of the EO depending on the biological system and environmental conditions. The DPPH assay reflects the ability of EO constituents to act as hydrogen- or electron-donating radical scavengers in a simplified, cell-

free chemical system. However, in bacterial cells, these same compounds may exert pro-oxidant effects through multiple mechanisms, including membrane perturbation, the disruption of electron transport processes, and redox regulation, thus ultimately leading to intracellular ROS accumulation.

MDA is produced both as a result of oxidation and as a breakdown of fatty acids and lipids; this metabolite could serve as a marker of the level of lipid peroxidation processes in food, cells, tissues, and organisms [9,29].

According to our investigations, the MDA formation under the treatment of *T. argyrophyllum* EO was increased by 49.88% in *E. coli* pARG-25 (AI value was 50.22 ± 0.71 %). This parameter was not changed in the control strain (Figure 1). The positive control (α-tocopherol) decreases MDA formation by 91.1 ± 1.9 %.

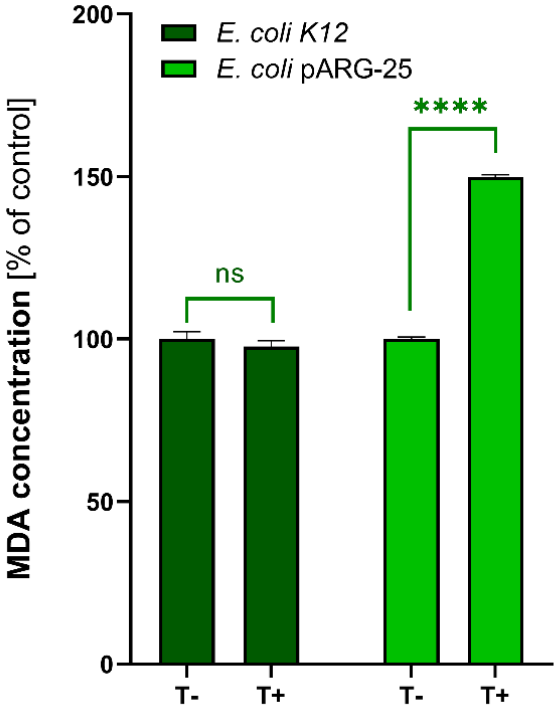

Figure 1. The synthesis of MDA in *E. coli* K12 and *E. coli* pARG-25 cells treated by *T. argyrophyllum* EO (T - are the control cells without treatment, T + -cells treated with *T. argyrophyllum* EO). The results are means ± SD of three independent experiments implemented in triplicate (****$p < 0.0001$, ns – non significant).

The obtained data could indicate the pro-oxidative nature of the acting metabolites of this EO in kanamycin-resistant *E. coli* pARG-25 strain. In order to ensure this proposed statement, the influence of EO on microbial antioxidant defense system enzymes was investigated. Upon a vast number of non-enzymatic defense mechanisms in bacterial cells, the enzymatic system is engaged in the quenching of ROS, among which SOD and catalase play pivotal roles [30,31]. Therefore, in addition to other indicators, the changes in the activity of SOD and catalase in bacteria could speak about the particular redox status of the cells. The principal enzymatic components of the cellular antioxidant defense system include catalase, glutathione peroxidase (GPx), and SOD. Among these, SOD represents the

first line of defense in all aerobic organisms [32]. Therefore, assessing alterations in SOD and the catalase activity in cells exposed to *T. argyrophyllum* EO may provide valuable insights into the underlying mechanisms of action of this EO.

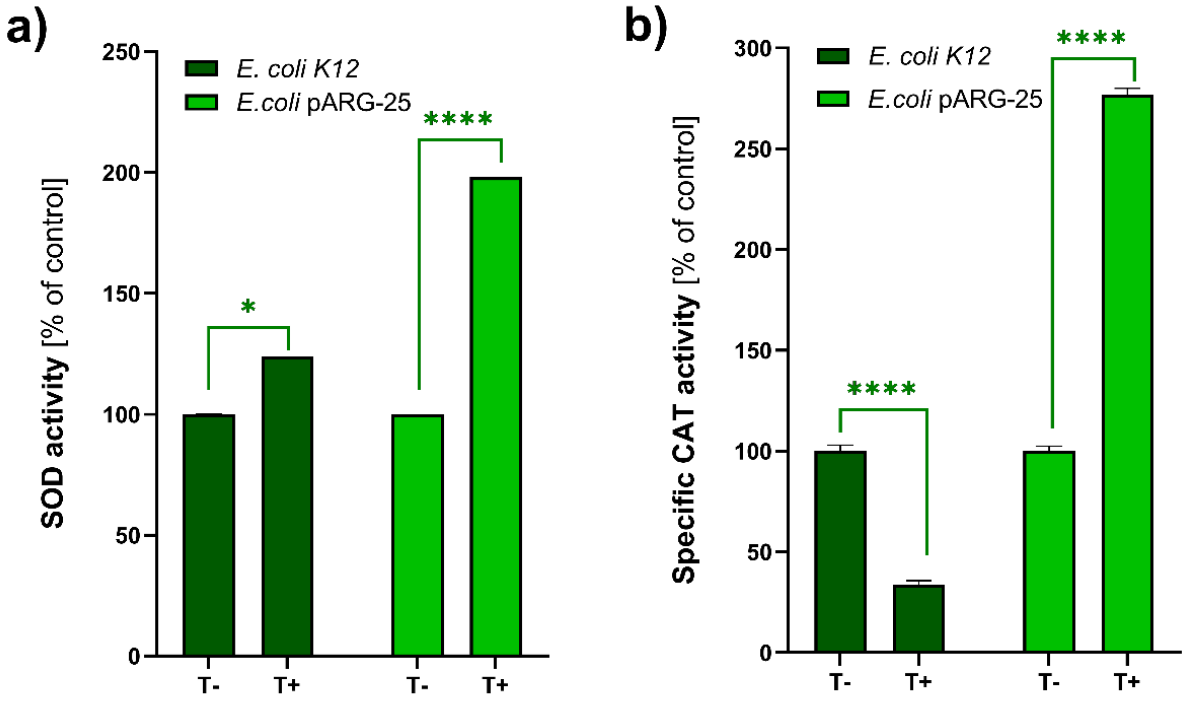

Figure 2. The activity of SOD (a) and catalase (b) in *E. coli* K12 and *E. coli* pARG-25 under the treatment with *T. argyrophyllum* EO (10 μLmL^{-1}). T - are the control cells without treatment, and T + are cells treated with *T. argyrophyllum* EO. The results are means ± SD of three independent experiments implemented in triplicate (*$p < 0.05$; **** $p < 0.0001$).

In *E. coli* K12 cells, the activity of SOD increased by 24% under the treatment of 10 μLmL^{-1} *T. argyrophyllum* EO; meanwhile, we observed the 98% increase of this parameter in the kanamycin-resistant *E. coli* pARG-25 strain (Figure 2a). In case of catalase, we have some decrease in the control strain (by around 33.6%); however, this enzyme activity increased in kanamycin-resistant strain by 177%, which can speak about the evidence of oxidative stress in these cells treated by the investigated EO (Figure 2b).

The observed moderate increase in SOD activity in *E. coli* K12 suggests a compensatory response to superoxide overproduction induced by *T. argyrophyllum* EO. However, the markedly stronger induction observed in the kanamycin-resistant *E. coli* pARG-25 strain (98%) indicates a substantially enhanced oxidative challenge for resistant cells [33]. Similarly, the decrease in catalase activity in the control strain, contrasted with a 177% elevation in the resistant strain, implies differential redox adaptation mechanisms, possibly reflecting altered metabolic reprogramming associated with antibiotic resistance. These findings could support the notion that the EO exerts a more pronounced pro-oxidative effect in the kanamycin-resistant strain. The opposing changes in catalase activity

between the two strains suggest a strain-specific redox adaptation to EO exposure. As it is suggested, the marked increase in catalase activity in *E. coli* pARG-25 may reflect a compensatory response to elevated oxidative stress, potentially linked to the metabolic burden imposed by the plasmid and its impact on the cellular redox balance. In contrast, the reduction of catalase activity in the wild-type strain may indicate either enzyme inhibition or an alternative stress response strategy. These findings highlight that EO-induced oxidative stress responses depend on the physiological and genetic background of the bacteria. However, as direct measurements of intracellular ROS and gene expression were not performed, these mechanisms remain speculative and warrant further investigation. The observed increases in SOD, catalase, and MDA levels, particularly in the resistant strain, do not by themselves establish a causal link to direct ROS generation, nor do they distinguish between primary oxidative effects and secondary stress responses. The measured parameters reflect integrated cellular responses, which may arise from multiple sources, including metabolic imbalance, membrane perturbation, or signaling cascades.

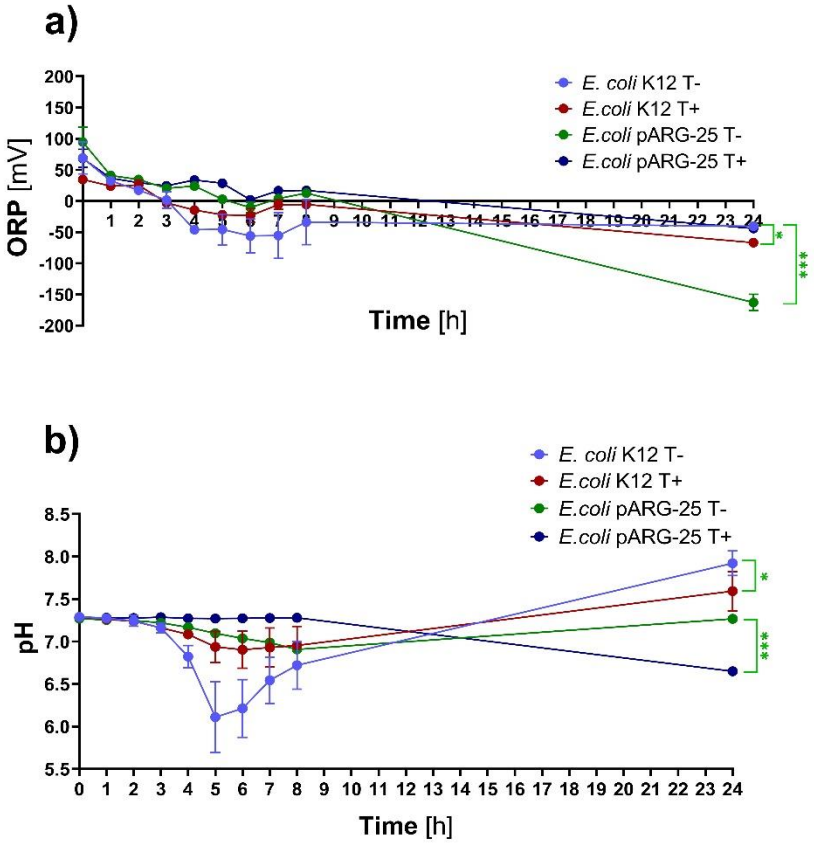

Figure 3. ORP (mV) (a) and pH value (b) changes during the growth of *E. coli* K12 and *E. coli* pARG-25 strains. For more information, see Materials and methods. T- are the control cells without treatment, and T + are the cells treated with *T. argyrophyllum* EO. The results are means ± SD of three independent experiments implemented in triplicate.

During aerobic cultivation for up to 24 h, both *E. coli* strains demonstrated dynamic changes in ORP. In control cultures, without *T. argyrophyllum* EO treatment, a noticeable decrease in ORP was

observed between the 4th and 7th hours of cultivation in both strains ($P < 0.05$). The lowest ORP values observed to be −55.3 mV and −15.1 mV in *E. coli* K12 and *E. coli* pARG-25 strains, respectively (Figure 3a). This decline possibly reflects active metabolic processes associated with exponential growth, during which increased cellular respiration and metabolic activity contribute to alterations in the redox state of the medium. These findings may indicate that antibiotic resistance in this particular case is accompanied by measurable shifts in redox homeostasis, which can be sensitively tracked through ORP dynamics during bacterial growth.

In contrast, EO-treated cultures exhibited a delayed ORP decline, with the decrease becoming apparent only from approximately the 6th hour of cultivation (Figure 3a). This delay may indicate that EO exposure interferes with early metabolic processes, thereby potentially affecting membrane-associated respiratory activities and electron transport processes, which were observed during our experiments and previously published [6], thereby altering the redox dynamics of the bacterial culture during the initial stages of growth. In this case, the lowest ORP values were −23.4 mV and −1.7 mV in *E. coli* K12 and *E. coli* pARG-25 strains, respectively (Figure 3a).

At the end of the cultivation period, the difference in ORP values between EO-treated and untreated *E. coli* K12 culture was approximately 15 mV. In contrast, the kanamycin-resistant *E. coli* strain exhibited a markedly greater difference, reaching 124.8 mV. These data could indirectly confirm the evidence of the bacteriostatic nature of *T. argyrophyllum* EO, which we observed in our previous investigations [6], as a decrease of the ORP value at the end of cultivation could be evidence of working compensatory mechanisms in the investigated bacteria, thereby protecting them from the oxidative stress. Moreover, this is in line with the data of the changes in activity of antioxidant enzymes in the investigated bacterial strains (Figure 2).

The changes in extracellular pH strictly reflects the changes in ORP values in both tested bacterial cultures, thus indicating the accumulation tendency of organic acids in accordance to their metabolic activity level (Figure 3b).

The ORP dynamics broadly reflected the growth kinetics of both treated and untreated bacterial cultures. In the control conditions, the progressive decline in ORP corresponded to active bacterial growth and metabolic activity. In contrast, EO treatment was associated with a delayed and attenuated ORP decrease, consistent with suppressed growth. This observation is supported by the reduction in growth parameters, as indicated by decreased MF units in both strains under EO exposure (Figure 3 and 4). According to our previous results, EO treatment reduced the specific growth rates of *E. coli* K-12 and *E. coli* pARG-25 by 1.6-fold and 1.5-fold, respectively. In addition, the EO significantly prolonged the lag phase, thereby increasing its duration by 63% in *E. coli* K-12 and by 52% in the antibiotic-resistant *E. coli* pARG-25 strain [6]. Together, these findings suggest that the altered ORP profiles are linked to an inhibited metabolic activity rather than representing a direct measure of specific redox pathways.

The obtained data, in complex with our previously published data [6], create a platform that insists on the redox-dependent mode of influence of the investigated EO on bacterial cells, thereby highlighting the reduction of viability in both tested *E. coli* strains. In addition, in our previous investigations, we demonstrated a synergistic interaction between this EO and the antibiotic kanamycin, with a fractional inhibitory concentration index (FICI) of approximately 0.5, which is indicative of true synergy [6]. Such interaction suggests that the EO enhances the antibiotic efficacy, thereby potentially modulating the membrane permeability or interfering with resistance-associated cellular processes. This property substantially increases the therapeutic relevance of the EO, thus highlighting

its potential as an antimicrobial agent.

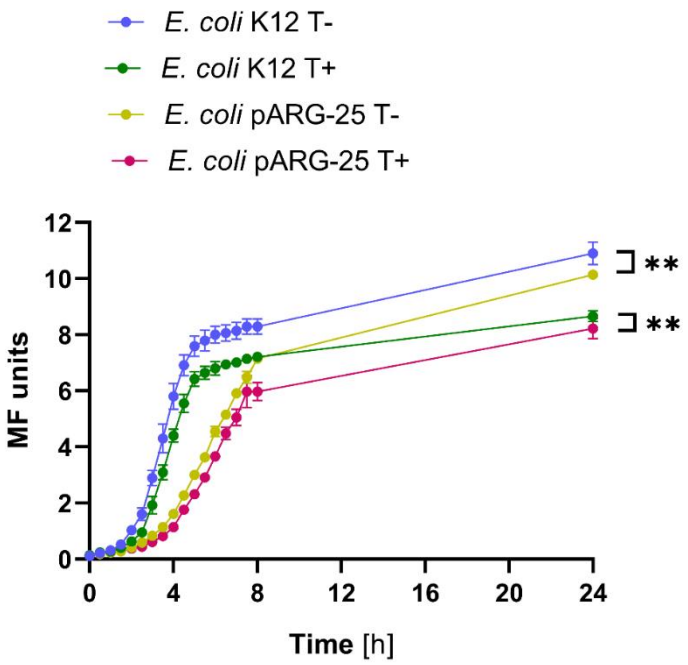

Figure 4. The growth curves of *E. coli* K12 and kanamycin resistant *E. coli* pARG-25 under the treatment with *T. argyrophyllum* EO. T- are the control cells without treatment, and T+ are cells treated with *T. argyrophyllum* EO. The results are means ± SD of three independent experiments implemented at least in triplicate.

Future studies should incorporate more direct approaches, such as intracellular ROS quantification, the assessment of redox-sensitive reporters, and measurements of glutathione redox balance. These additions could support the clarification of the scope of our conclusions and outline clear directions to strengthen the mechanistic interpretation in subsequent work.

4. Conclusions

This study demonstrated that *T. argyrophyllum* EO exhibits antibacterial activity against both wild-type (*E. coli* K12) and kanamycin-resistant (*E. coli* pARG-25) strains, with MIC values of 100 μL mL^{-1}. A chemical analysis revealed a eucalyptol–camphor chemotype, with eucalyptol, camphor, and camphene as the dominant constituents. Exposure to the EO was associated with alterations in redox-related parameters, including delayed changes in the extracellular oxidation–reduction potential, shifts in the extracellular pH, and increased markers of oxidative stress, particularly in the resistant strain. These effects suggest that the EO may influence the bacterial physiology through disruption of redox homeostasis and membrane-associated processes.

However, while the results provide initial insight into potential redox-associated antibacterial effects, further studies that incorporate direct measurements of intracellular reactive oxygen species and membrane integrity are required to clarify the underlying mechanisms and to assess the practical relevance of this EO, particularly in the context of drug-resistant bacteria. This highlights its potential

as a natural antimicrobial agent capable of targeting bacteria during the development of strategies for topical treatments, food preservation, or surface disinfection.

Use of generative-AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declared no conflict of interest.

Author contributions

All authors contributed to the conception and design of study. LM and ST carried out the investigations and analyzed the data. NS and KT directed the experiments, corrected, and edited the manuscript. All authors read and approved the final manuscript and have participated sufficiently in the work and agreed to be accountable for all aspects of the work and provided approval of the final submitted manuscript.

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