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Evaluation of Natural Essential Oils as Sustainable Disinfectants for the Control of Microbial Contaminants *in Vitro* Plant Tissue Culture

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ABSTRACT

The current investigation assessed the impact of five natural essential oils on the *in vitro* sterilization effectiveness of different explants. The different explants were subjected to three concentrations (1%, 2%, 3% v/v) and three exposure times (1, 2, and 3 min). In addition, the sterilization rates were assessed after 14 days. Results explained that sterilization effectiveness was clearly affected by both oil type and treatment factors. At 1% concentration, eucalyptus and neem attained the highest early sterilization in *Bougainvillea* (83.3% and 76.0%, respectively), though peppermint and clove confirmed lower activity (56.0% and 66.7%, respectively). The results indicated that increasing the exposure time to 3 min usually increased sterilization rates, with clove, eucalyptus, peppermint, thyme, and neem oils succeeding in 89 to 100% sterilization through species but caused loss of viability. At 2% concentration and 2 min exposure, most oils efficiently sterilized explants while maintaining tissue viability; for example, *A. julibrissin* explants reached 83.3 to 100% sterilization without death, whereas 3% concentration often caused explant death, mainly in *Bougainvillea* and *R. chinensis minima*. Thyme and neem oils reliably confirmed the highest and most reliable activity, attaining 100% sterilization through concentrations and exposure times in *R. chinensis minima* and *A. julibrissin*, emphasizing their broad-spectrum antimicrobial potential. Clove required greater concentrations to reach similar efficacy due to its eugenol content, whereas eucalyptus and peppermint performed moderately well at lower concentrations. Based on these results, a recommended sterilization protocol for *in vitro* culture means using 2% essential oil concentration with 2-minute exposure time.

Keywords: Tissue culture, Essential oils, *In vitro,* Sterilization

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INTRODUCTION

More than 60% of currently available pharmaceuticals originate directly or indirectly from plant and animal sources, highlighting the indispensable role of natural products in modern medicine [1]. Nature-derived compounds remain fundamental to drug discovery pipelines and industrial biotechnology, constituting a major platform for biotechnological production. Nevertheless, the biosynthesis and recovery of these metabolites are highly dependent on environmental and cultivation conditions, necessitating their production across diverse growth systems. Recently, increasing attention has been directed toward both volatile and fixed natural oils because of their capacity to modulate biological processes and enhance the in vitro biosynthesis of valuable secondary metabolites [2]. Essential oils are volatile aromatic liquids synthesized by plants and are primarily composed of secondary metabolites that play critical roles in plant defense. These compounds exhibit inherent antimicrobial properties, contributing significantly to resistance against pathogenic microorganisms [3]. The growing application of essential oils as natural food preservatives reflects rising consumer concerns regarding synthetic additives. In parallel, the global burden of food-borne diseases underscores the urgent need for safer preservation strategies. Extensive experimental evidence confirms the broad-spectrum antibacterial activity of essential oils. Although recent investigations have elucidated certain mechanisms of action, the majority of molecular targets and pathways influenced by essential oil constituents remain incompletely characterized. Importantly, essential oils possess both antimicrobial and antioxidant activities, which can influence metabolic fluxes and compromise cellular membrane integrity [4, 5]. The biological effects of essential oils are highly concentration- dependent. Elevated levels may inhibit growth or interfere with biosynthetic pathways, whereas lower concentrations can induce defense-related responses and enhance stress tolerance. Consequently, precise evaluation of both exposure concentration and treatment duration is critical in in vitro experimental systems to achieve optimal biological outcomes [6].
It is volatile and aromatic according to [7]. Material that has been removed from plants by methods such as flowers and leaves, bark and roots, whole plants themselves, seeds, peel, or branches can yield this chemical compound, which naturally occurs in things. Other herbs containing essential oils have been usable for medicine, cosmetic products, and perfumery for centuries. They also play a crucial role in viticulture and other agricultural activities [8]. Moreover, commercial production uses over 300 different types of essential oils. Some fragrances and flavorings (Burt, 2004) can be based on natural oils. These too are made up of terpenes, phenols, and quite similar chemicals. Our group discovered bioactive substances of a more useful type, such as this one from

the group we call antigens, that can enhance the production of useful natural products through defense-related signal pathways like salicylate and jasmonate. This was only confirmed once again when we found that several groups containing thymol, eugenol, and carvacrol - the active ingredients in aromatic oils are easily available for commercial use and add to cell membrane permeability, cause release of reactive oxygen species (ROS), and so on. They also raise production genes that are concerned not only with alkaloids but also fixed oils, phenolic compounds, and terpenoids in ways never known before [3, 9]. Furthermore, the significant enhancement of relevant pharmaceutical metabolite synthesis seen during many in vitro system responses to natural oils is clearly a green way of maximizing bioreactor productivity.

Essential oils (EOs) are deemed to be secondary metabolites and essential for plant defense as they often control antimicrobial properties by using secondary metabolites [10], which were first estimated using EOs vapors by De la Croix in 1881 [11]. In addition, EOs and their components have been confirmed to have antiparasitic [12], insecticidal [13], antiviral [14], fungicide [15], and antioxidant [16] properties. Additionally, they also function as growth enhancers for [17, 18].

Plant tissue culture is a technique that focuses on the aseptic cultivation of different plant cells, tissues, or organs under constant controlled conditions for purposes of rapid multiplication, genetic stability, and conservation of plant genetic resources [19-21]. The major challenge in the sterilization of the explant is over-sterilization or under-sterilization. Over-sterilization damages the explant tissue. Under-sterilization, on the other hand, fails to remove contaminant microbial growth. It results in loss of microculture [22]. The use of chemical disinfectants, specially designed for plant tissue cultures, can have adverse effects like toxicity on plant tissue and impart physiological stress and harmful by-product generation, which restricts or hinders plant growth and reduces culture success [23]. Natural oils, like clove, neem, eucalyptus, thyme, and peppermint oils, have impressive antimicrobial properties that are less toxic to humans and the environment [24]. The oils serve as a safer option for plant tissue culture sterilization treatments.

It was hypothesized that essential oils exhibit concentration- and time-dependent antimicrobial activity, and that moderate treatments, particularly thyme and neem oils, provide effective sterilization without causing phytotoxic effects. The objective of this study was to evaluate the effectiveness of selected essential oils (clove, eucalyptus, peppermint, thyme, and neem) as natural sterilizing agents in in vitro plant tissue culture, and to determine suitable concentrations and exposure times that achieve high sterilization efficiency while maintaining explant viability in four ornamental species.

MATERIALS AND METHODS

Plant Extracts Collection

During March to May 2025, fresh apical buds and shoot tips were collected from four ornamental plants, namely bougainvillea (*Bougainvillea glabra Choisy*), baby rose (*Rosa chinensis* Jacq. var. minima), cypress (*Cupressus sempervirens* L.), and silk tree (*Albizia julibrissin* Durazz.), from the Research Gardens of the College of Agriculture, University of Kirkuk (35°28'11.0"N 44°23'08.0" E). Explants were carefully excised via a sterile scalpel under field conditions, with an equal length of 1.5 ± 0.1 cm, and directly transferred to the laboratory in sterile, humidified containers. Before any treatment, explants were completely washed under running tap water for 20 min with gentle rubbing to remove the dust and surface debris, adding two drops of dishwashing liquid as a surfactant. After washing, all explants underwent quick initial surface sterilization against fungus and bacteria with 70% ethanol for 30 s under a sterile laminar airflow cabinet (Class II Biosafety Cabinet). Murashige and Skoog (MS) basal medium was prepared by dissolving commercial MS powder in distilled water, supplemented with 30 g/L sucrose and solidified with 7 g.L agar. The pH of the medium was adjusted to 5.7 ± 0.1 prior to autoclaving at 121 °C and 1.1 kg/cm² for 20 min. Cultures were maintained under cool white, fluorescent light with an intensity of approximately 40 μmol/m²/s¹ and a photoperiod of 16 h light/8 h dark at 25 ± 1 °C.

Essential Oils

During this study, five pure essential oils with certificates of analysis (98.6% purity) were used from Bahar Factory, a producer of essential oils, certified to, ISO 22000:2018 (731124D). These essential oils have the following active compounds: clove oil (*Syzygium aromaticum*) eugenol (85.2%), eucalyptus oil (*Eucalyptus globulus*) cineole (78.6%), peppermint oil (*Mentha × piperita*) menthol (42.1%), thyme oil (*Thymus vulgaris*) thymol (45.8%), and neem oil (*Azadirachta indica*) azadirachtin (1.2%). To examine the effects of these oils without external interference, the solutions were prepared without the addiction of alcohol. The solutions were prepared at 1%, 2%, and 3% (v/v) concentrations by diluting the pure essential oils in an aqueous solution containing 0.1% Tween 80 according to the World Health Organization standards. Solutions were used within 2 hours of preparation to maintain efficacy, and fresh solutions were prepared daily. As a positive control, 2% sodium hypochlorite solution was used for 15 min.

Experimental Design

A Completely Randomized Design (CRD) was used. In total, the experiment included 4 plant species × 5 essential oils × 3 concentrations × 3 exposure times, plus positive and negative controls, resulting in a total of 190 treatment combinations, with 10 replicates per treatment (5 explants per replicate). 100 ml of each oil solution was placed in sterile Petri dishes, and explants were fully dipped with gentle agitation to ensure uniform coverage. After the designated time, the explants were immediately transferred to new Petri dishes and washed 3 times with sterile distilled water for 2 minutes/wash to remove all the oil residues. Following the final wash, explants were cultured in vessels containing hormone free basal Murashige and Skoog (MS) medium, pH 5.7, and placed in a growth chamber at 25 ± 1 °C with a 16hour light/8hour dark photoperiod. The samples were examined daily for 14 days, and the final evaluation was recorded on day 14. The successful sterilization rate was determined by the visible absence of microbial growth on the explants and the surrounding culture medium during the 14-day observation period.

Statistical Analysis

Data was analyzed using SAS software (version 9.4), and two-way ANOVA was applied to analyze the factor effects. When significant differences were found ($P < 0.05$), Duncan's multiple range test was performed for pairwise comparisons of the means. Results are presented as mean ± standard error (Mean ± SE), with ten replicates per treatment, ensuring adequate statistical power (>0.8). Levene's tests confirmed that the data met the homogeneity assumptions required for ANOVA.

RESULTS AND DISCUSSION

The data in Table 1 shows the activity of essential oils types, concentrations, and exposure time on the sterilization rate of *Bougainvillea* spp. explants. The data showed that after 1m, the essential oils of eucalyptus and neem recorded the highest activity at 1% concentration (83.3% and 76.0%, respectively), and peppermint essential oil displayed the lowest value (56.0%). In addition, all essential oils showed a regular rise in activity when the exposure time was extended to 3 min. Results indicated that at the 3m exposure time, clove, eucalyptus, peppermint, thyme, and neem achieved values ranging from 89% to 100%. Eucalyptus, thyme, and neem oils kept activity levels over 86% at all exposure times and got complete activity (100%) at 3 m, but all the explants died when the essential oils concentration was increased to 3%/3m compared with 1 and 2 concentrations and exposure time, the explants were alive. At the highest concentration (3%), eucalyptus, thyme, peppermint, and neem showed the greatest effects with death in all plant extracts as shown in Table 1. Clove oil reached 96 to 100% activity depending on exposure time, while eucalyptus, thyme, peppermint, and neem oils exhibited 100% activity at almost all time periods at the highest concentration (3%). The main conclusion of this data is that the biological activity of the essential oils was significantly affected with concentration and exposure time [25]. 2% concentration and 2 min exposure time recorded the useful by consideration of the sterilization rate on Bougainvillea Spp. Explants.

Table 1 Effects of different essential oil, concentrations, and exposure time on sterilization rate on *Bougainvillea* spp. explants

Essential oil	Concentration (%)	Exposure time		
		1 min	2 min	3 min
Clove	1%	66.7 ± 1.2 b	70.0 ± 1.0 b	93.3 ± 0.8 a
	2%	66.7 ± 1.2 b	70.0 ± 1.0 b	93.3 ± 0.8 a
	3%	96.0 ± 0.0 a	99.0 ± 0.0 a	100.0 ± 0.0 b
Eucalyptus	1%	83.3 ± 0.9 a	86.7 ± 0.8 a	96.7 ± 0.5 a
	2%	85.3 ± 0.9 a	86.7 ± 0.8 a	96.7 ± 0.5 a
	3%	100.0 ± 0.0 b	100.0 ± 0.0 b	100.0 ± 0.0 a
Peppermint	1%	56.0 ± 0.0 a	60.2 ± 0.0 a	88.0 ± 0.0 a
	2%	67.0 ± 0.0 a	73.1 ± 0.0 a	87.5 ± 0.0 a
	3%	94.5 ± 0.0 b	100.0 ± 0.0 b	100.0 ± 0.0 b
Thyme	1%	66.8 ± 0.0 c	70.0 ± 0.0 c	89.5 ± 0.0 a
	2%	89.5 ± 0.0 b	88.6 ± 0.0 b	97.0 ± 0.0 b
	3%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 ab
Neem	1%	76.0 ± 0.0 c	80.0 ± 0.0 b	100.0 ± 0.0 a
	2%	88.0 ± 0.0 b	96.0 ± 0.0 a	100.0 ± 0.0 a
	3%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a

* Data is expressed as mean ± standard error (SE). Within each exposure time, values carrying different superscript letters differ significantly ($p < 0.05$), indicating the influence of oil type, concentration, and exposure duration on activity.

Data in Table 2 showed that *A. julibrissin* explants sterilization response to the essential oils type, concentration, and time of exposure to essential oils. The analysis of variance varied significantly for all the treatments. Results recorded that at 1% concentration, clove oil demonstrated comparatively modest sterilization rates ranging from 44.4 to 51.1%; however, at 2% clove oil concentration, the effect was increased, exclusively after 3 minutes, and the sterilization achieved 83.3%. While with 3% clove oil and 3 minutes of exposure, achieve 100% sterilization is achieved at all exposure times with death on the *A. julibrissin* explant. In the country with the lowest concentrations and exposure time 1 and 2, the explant was alive. On the other hand, Eucalyptus oil showed uniform response at all oil concentrations, sustaining sterilizing levels from 83.3% to 90.0%, with no visible variations between concentrations at any exposure time (Table 2). Sterilization percentage ranging from 94.4% at 1 m to 100% at 3 m. While peppermint oil established strong effectiveness based on the oil concentration and exposure time. The results increased from 88–90% at 1% concentration to 98–100% at greater concentrations and longer exposure times. Thyme oil also showed outstanding sterilizing performance. In addition, neem oil succeeded complete sterilization (100%) at both 2% and 3% concentrations through all exposure times; it only showed moderate sterilizing at 1% concentration (77–94%). The results concluded that peppermint, thyme, and neem oils offer the greatest and most reliable sterilization rates. Statistical analysis revealed substantial differences among treatments, indicating that both concentration and exposure time influenced sterilization effectiveness.

Table 2 Effects of different essential oil, concentrations, and exposure time on sterilization rate on *A. julibrissin* explants

Essential oil	Concentration (%)	Exposure time		
		1 min	2 min	3 min
Clove	1%	44.4 ± 1.5 c	47.8 ± 1.3 c	51.1 ± 1.0 b
	2%	55.6 ± 1.2 b	60.0 ± 0.9 b	83.3 ± 0.7 a
	3%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
Eucalyptus	1%	83.3 ± 0.9 a	86.7 ± 0.8 a	90.0 ± 0.7 a
	2%	83.3 ± 0.9 a	86.7 ± 0.8 a	90.0 ± 0.7 a
	3%	83.3 ± 0.9 a	86.7 ± 0.8 a	90.0 ± 0.7 a
Peppermint	1%	94.4 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
	2%	94.4 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
	3%	94.4 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
Thyme	1%	88.0 ± 0.0 b	90.0 ± 0.0 b	100.0 ± 0.0 a
	2%	91.0 ± 0.0 a	96.0 ± 0.0 a	100.0 ± 0.0 a
	3%	92.0 ± 0.0 a	98.0 ± 0.0 a	100.0 ± 0.0 a
Neem	1%	77.0 ± 0.0 b	87.0 ± 0.0 b	94.0 ± 0.0 b
	2%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
	3%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a

* Data is expressed as mean ± standard error (SE). Within each exposure time, values carrying different superscript letters differ significantly ($p < 0.05$), indicating the influence of oil type, concentration, and exposure duration on activity.

The sterilization response of *Cupressus sempervirens* explants was effectively affected by essential oil type, concentration, and exposure time, as found in Table 3. Clove oil established a clear improvement with increasing concentration, with sterilization increasing from 50.0–83.3% at 1% concentration to 83.3–100.0% at 2%, and accomplishing complete sterilization (100%) at all exposure times when applied at 3%. In addition, Eucalyptus oil consistently maintained high sterilization effectiveness, ranging from 94.4% to 100.0% through all oil concentrations and exposure times, with no significant differences between treatments. Data in Table 3 for Peppermint oil showed similarly powerful activity, making sterilization rates 94.0% and 100.0%, and attaining complete sterilization at 3 m for all oil concentrations. While Thyme oil was effective, rising from 88 to 100% at 1% concentration to whole sterilization at 3%, mainly at extended exposure time. Data in Table 3 for Neem oil confirmed a moderate impact at 1% concentration (65.0–95.0%), but sterilization increased substantially with high concentrations, reaching 98.0% at 2% and completing whole sterilization (100%) at 3% for all exposure times. The results indicated significant differences, which confirmed that sterilization performance varied between concentration and exposure time with eucalyptus, peppermint, and thyme. The high-concentration clove and neem oils presented the most stable and effective sterilization of *C. sempervirens* explants.

Table 3 Effects of different essential oil, concentrations, and exposure time on sterilization rate on *Cupressus sempervirens* explants

Essential oil	Concentration (%)	Exposure time 1 min	2 min	3 min
Clove	1%	50.0 ± 1.4 c	66.7 ± 1.1 b	83.3 ± 0.8 a
	2%	83.3 ± 0.8 a	90.0 ± 0.7 a	100.0 ± 0.0 a
	3%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
Eucalyptus	1%	94.4 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
	2%	94.4 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
	3%	94.4 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
Peppermint	1%	94.0 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
	2%	96.4 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
	3%	98.4 ± 0.6 a	98.8 ± 0.4 a	100.0 ± 0.0 a
Thyme	1%	88.0 ± 0.0 c	90.0 ± 0.0 b	100.0 ± 0.0 a
	2%	90.0 ± 0.0 b	95.0 ± 0.0 a	100.0 ± 0.0 a
	3%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
Neem	1%	65.0 ± 0.0 b	70.0 ± 0.0 b	95.0 ± 0.0 b
	2%	66.0 ± 0.0 b	72.0 ± 0.0 b	98.0 ± 0.0 a
	3%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a

* Data is expressed as mean ± standard error (SE). Within each exposure time, values carrying different superscript letters differ significantly ($p < 0.05$), indicating the influence of oil type, concentration, and exposure duration on activity.

The data exhibited in Table 4 showed that the sterilization efficiency of *Rosa chinensis minima* explants varies significantly based on the essential oil type, concentration, and exposure time. The results in Table 4 indicated that Clove oil displayed that at the concentration of 1%, the sterilization ratio varied from 38.9% to 61.1%, whereas increasing the concentration to 2% clearly improved sterilization to 66.7–90.0%, while the complete sterilization (100%) was only reached at the highest concentration of 3%, regardless of exposure time, demonstrating that clove oil requires higher doses to reach full antimicrobial potential. At Table 4, Eucalyptus oil determined consistently high efficiency through all concentrations, getting 94.4–100% sterilization compared with the lowest concentration. A similar relationship was viewed for peppermint oil, which explained stable sterilization rates (88.9–95.6%) through concentrations and exposure times, reflecting reasonable but reliable antimicrobial activity. Finally, the Thyme and neem oils were the most effective treatments in this study, succeeding in 100% sterilization at all concentrations and exposure periods. Their complete and uniform action proposes the presence of strong bioactive constituents capable of removing a broad range of surface contaminants without requiring prolonged coverage or enhanced concentration. These results highlight thyme and neem oils as superior natural sterilizing agents for *in vitro* culture applications.

Table 4 Effects of different essential oil, concentrations, and exposure time on sterilization rate on *Rosa chinensis minima* explants

Essential Oil	Concentration (%)	Exposure time 1 min	2 min	3 min
Clove	1%	38.9 ± 1.6 d	44.4 ± 1.4 c	61.1 ± 1.1 b
	2%	66.7 ± 0.9 b	77.8 ± 0.8 a	90.0 ± 0.7 a
	3%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
Eucalyptus	1%	94.4 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
	2%	94.4 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
	3%	94.4 ± 0.6 a	97.8 ± 0.4 a	100.0 ± 0.0 a
Peppermint	1%	88.9 ± 0.7 a	92.2 ± 0.6 a	95.6 ± 0.5 a
	2%	88.9 ± 0.7 a	92.2 ± 0.6 a	95.6 ± 0.5 a
	3%	88.9 ± 0.7 a	92.2 ± 0.6 a	95.6 ± 0.5 a
Thyme	1%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
	2%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
	3%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
Neem	1%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
	2%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
	3%	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a

* Data is expressed as mean ± standard error (SE). Within each exposure time, values carrying different superscript letters differ significantly ($p < 0.05$), indicating the influence of oil type, concentration, and exposure duration on activity.

The current study shows that the sterilization efficiency of plant explants is clearly influenced by essential oil type, concentration, and exposure time. Through all tested species (*Bougainvillea spp.*, *A. julibrissin*, *Cupressus sempervirens*, and *Rosa chinensis minima*), the essential oils for instance, thyme, neem, eucalyptus, peppermint, and clove displayed variable antimicrobial activities based on concentration and exposure time, and these finding agree with previous reports of [11, 26] who showed the properties of plant essential oils as antimicrobial for different subjects.

Mainly, the lower concentrations (1%) of the present essential oils showed balanced sterilization activity. Eucalyptus and neem oils indicated the highest early effectiveness in *Bougainvillea spp.* (83.3% and 76.0%, respectively), while peppermint displayed the lowest activity (56.0%). Likewise, clove oil showed low activity in *A. julibrissin* (44–51%) and *Rosa chinensis minima* (38.9–61.1%) at 1%, representing a dose-dependent behavior typical of eugenol-rich oils, which need high concentrations to use their full antimicrobial potential [3]. Delaying exposure time always improved sterilization rates through all species, reaching 89–100% after 3min for most oils, indicating the role of essential oil, which induced microbial deactivation [27]. The results indicated that increasing the essential oil concentration to 2% normally increased sterilization rates without causing significant damage to explants (death). For example, 2% clove oil increased the sterilization rate in *A. julibrissin* from 51% to 83.3% at 3m., while eucalyptus, thyme, and neem provided high activity (>86%) throughout species and exposure times. While at 3% concentration, approximately all oils succeeded in sterilization (100%) in all plant extracts; in some plants, such as *Bougainvillea Spp,* the explants did not survive, highlighting the phytotoxic potential of high essential oil concentrations [28]. These outcomes underscore the weight of balancing antimicrobial efficacy with tissue viability.

Among the assessed oils, thyme and neem always presented the brightest and most reliable sterilizing activity throughout all species and conditions. Eucalyptus and peppermint oils also worked well, getting high sterilization levels even at lower concentrations and shorter exposure times. The greater performance of thyme and neem oils can be attributed to their bioactive constituents, thymol, carvacrol, and azadirachtin, which have been described as disrupting microbial cell membranes and preventing metabolic pathways and offering broad-spectrum antimicrobial properties [29-31].

The results of the present study both confirm and extend previous findings on essential oil antimicrobial activity in plant tissue culture. Consistent with Burt (2004) and Bakkali *et al*. (2008), thyme and neem oils exhibited the highest and most reliable sterilization across all tested plant species, confirming their broad-spectrum antimicrobial potential. Similarly, the dose-dependent behavior of clove oil, which required higher concentrations to achieve full sterilization, aligns with the observations of Hyldgaard *et al.* (2012), highlighting that eugenol-rich oils need sufficient concentration to be fully effective. Our findings also corroborate Lambert *et al.* (2001), as prolonging exposure time from 1 to 3 minutes consistently improved sterilization rates across all oils and plant species. However, unlike some previous studies, we observed phytotoxic effects at the highest concentration (3%) in Bougainvillea spp., which caused explant death, emphasizing the importance of balancing antimicrobial efficacy with tissue viability, as noted by Kasavi *et al.* (2012). Additionally, the consistent high activity of eucalyptus and peppermint oils, even at lower concentrations, provides a nuanced comparison with earlier reports [29-32], confirming that chemical composition and plant

species susceptibility critically influence effectiveness. Overall, our results not only validate previous knowledge on essential oils but also provide specific guidance on optimal concentration (2%) and exposure time (2 minutes) for routine in vitro sterilization, highlighting a practical balance between antimicrobial activity and explant survival.

CONCLUSION

This study demonstrates that essential oils are a viable natural alternative to conventional chemical sterilants, with thyme and neem oils being the most effective for explant sterilization. The findings provide practical guidance for optimizing sterilization protocols in plant tissue culture while minimizing phytotoxicity. Medium concentrations (2%) combined with moderate exposure times (2 minutes) offer an optimal balance between effective sterilization and explant survival, making these conditions suitable for routine in vitro culture applications.

Supplementary Materials

No Supplementary Materials.

Author Contributions

S.A.W., A.M.N., H.K.H., J.K.O. methodology, writing—original draft preparation, S.A.W., A.M.N., H.K.H., N.R.A. writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

All research procedures were conducted according to the ethical principles of the University of Kirkuk, with ethical approval number KUEthics2024Plant03C01L001 obtained from the College Research Committee.

Informed Consent Statement

No Informed Consent Statement.

Data Availability Statement

No Data Availability Statement.

Conflicts of Interest

The authors declare no conflict of interest.

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