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Natural Cooling Agent: Utilizing Menthol as a Paraben-Free Preservative in Herbal Cosmetics

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Abstract

The quest for natural and sustainable alternatives in cosmetic products has intensified, driving the search for effective natural preservatives. Menthol, derived from mint plants, is being recognized for its cooling properties and potential as a paraben-free preservative. This study investigates menthol's efficacy as a preservative in herbal cosmetics, addressing consumer concerns about synthetic preservatives while ensuring product safety and longevity. This research examines menthol's antimicrobial effects on common cosmetic contaminants such as Staphylococcus aureus, Escherichia coli, and Candida albicans. Various microbiological assays were conducted to assess and compare menthol's inhibitory effects against these microorganisms with those of traditional paraben-based preservatives. Additionally, the study evaluated the physical and chemical stability of herbal cosmetic formulations, including creams, lotions, and balms, over a six-month period under controlled conditions. The findings reveal that menthol exhibits significant antimicrobial activity, effectively inhibiting the growth of bacteria and fungi at concentrations suitable for cosmetic use. Moreover, formulations containing menthol demonstrated stability and preservation comparable to those using conventional preservatives, without affecting the sensory qualities or therapeutic benefits of the herbal ingredients. In conclusion, menthol emerges as a promising natural preservative for herbal cosmetics, offering a paraben-free solution that aligns with consumer preferences for safer, more natural products. Future research should aim to optimize menthol concentrations and explore its synergistic effects with other natural preservatives to enhance efficacy and broaden its application in the cosmetic industry.

Keywords: Menthol, natural preservative, stability study, herbal cosmetics, natural stabilizers

INTRODUCTION

Microbial growth is a common concern in cosmetics and personal care products due to various sources of contamination such as raw materials, packaging, and manufacturing processes that may not maintain adequate sterility. Additionally, these products are often exposed to temperature fluctuations, which can promote the growth of microorganisms, especially when stored at higher temperatures than recommended. Once opened, these products become even more susceptible to contamination through repeated contact with non-sterile surfaces, such as fingers and skin, leading to discoloration, odors, and degradation of active ingredients. To ensure product stability and safety, preservatives are essential to prevent spoilage and protect consumers from infections caused by harmful microorganisms. In Europe, strict regulations dictate the selection and use of preservatives from an approved list, with each cosmetic formulation requiring a tailored preservative system based on its specific needs, including the type and dosage of preservative used. Individual care and cosmetic substances are required to stop microbial spreads since their shelf life prevents deterioration and

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effective health danger for people. Preservatives are needed in reaching this by preventing the growth of deadly microbes. In Europe, strict regulations govern the use of preservatives in cosmetics, requiring manufacturers to select from approved preservatives listed in Annex V of Regulation (EC). Each cosmetic formulation must have a customized preservative system tailored to its specific requirements, including selecting the appropriate type and dosage of preservative. Synthetic preservatives such as p-hydroxybenzoic acid, phenoxyethanol, and imidazolidinyl urea have been widely used in cosmetics for their affordability, broad-spectrum activity against bacteria and fungi, compatibility with other ingredients, and minimal interference with fragrance and color. However, its long-term use has increased awareness among the public for its potential health dangers. Many synthetic preservatives are petroleum-based and can cause skin irritations or promote infections, especially on damaged or sensitive skin areas. Parabens, one of the most used synthetic preservatives, have faced significant scrutiny. They have been linked to mimicking estrogen, increasing breast cancer incidence, and influencing the development of malignant melanoma. Finally, there is developing pressure on cosmetic developers to create natural alternatives. Natural preservatives offer both health benefits and a marketing advantage. They are perceived as healthier alternatives by consumers and can be marketed as "preservative-free" products. Additionally, the discovery and use of natural ingredients with preservative properties provide an opportunity for cosmetic companies to align with consumer preferences for natural and sustainable products. In response to the controversies surrounding synthetic preservatives, cosmetic industries are increasingly turning to innovative natural ingredients. This shift not only addresses consumer concerns about health and safety but also allows companies to differentiate themselves in the market and appeal to consumers seeking more natural beauty solutions [1].

In present years, researchers have aimed to look up natural stabilizers to decrease the nurture of bacteria and fungi in food, forwarded by enhancing public awareness of the strong negative health results of chemical stabilizers. Natural stabilizers are procured from different biological living beings such as plants, animals, bacteria, algae, and fungi. These belong to microbial-related stabilizers (e.g., bacteriocin), plant-related stabilizers (e.g., thyme essential oil, tea polyphenols, rosemary extract), and animal-related stabilizers (e.g., chitosan from crab or shrimp shells), all of which have described antimicrobial or antioxidant action. Compounds were synthesized by algae and fungi, such as mushrooms, also had oaths as strong non-commercial stabilizer for food.

One investigation discovered novel compounds of citric, ascorbic, and lactic acid, at 800 mg/kg each, necessary for storing hake, megrim, and angler for antimicrobial effects. Additionally, sodium salts of low molecular mass organic acids like acetic, citric, and lactic acids have been utilized to control microbial development, promote sensory activities, and increase the expiry date of fish. Mixtures of citric acid and potassium sorbate have proved actions in destroying microbial generation and balancing low levels of volatile base nitrogen in fish storage.

Other investigations have explained that emerging salmon slices in aqueous phase of sodium lactate, sodium acetate, and sodium citrate can effectively decrease spoilage microbes, prevent lipid oxidation, and increase shelf life during cold storage. Organic acids and salt, with their pleasant taste and tenderizing actions, are normal for marinating fish for stabilization actions. For example, frozen sardine fillets mixed in a solution of 14% sodium chloride and 7% acetic acid in barrels for 22 days at 4°C exhibited extended preservation.

Essential oils (EOs), complex mixtures of volatile organic compounds produced by plants, are increasingly used as natural preservatives due to their antimicrobial and antioxidant properties. Some EOs have been proven effective against foodborne harmfull pyrogens; namely, *S. Typhimurium*, *E. coli O157: H7*, *Campylobacter*, *L. monocytogenes*, and *S. aureus.* The effectiveness of EOs were affected by differences in features such as chemical structure, concentration, matching antimicrobial actions with target microbes, reacting with the food channel, and useful procedures.

Plant extracts offer broad application prospects in fish preservation, primarily due to their antimicrobial activities. These functions are characterized by polyphenols that adsorb to the bacterial outer layer known as membrane, leading to membrane breakage and subsequent lysis of cellular substances. Additionally, plant extracts exhibit antifungal, antioxidant, and antimutagenic properties, as well as the ability to inhibit lipid oxidation in food. When specialized in-vitro researchers have evaluated the antimicrobial actions of plant extracts, few investigations have goal on fish preservation, as the actions of plant extracts is ordinarily not as recognized as that of chemical stabilizers in fish. This may be due to the constituents of flavonoids in the form of glycosides in plant crude extracts, where the sugar quantity inhibits activities against some food-carrying pyrogens.

Natural wood smoke, produced by controlled wood smoldering without oxygen or at reduced oxygen levels, also possesses antimicrobial properties. Different classes of wood smoke, incorporating redwood, black walnut, hickory, birch, and cherry, have been determined for their antimicrobial activities against bacteria such as *A. hydrophila* and *S. aureus*. Smoke treatment can enhance the redness of fish muscle and stabilize it during frozen storage. However, wood smoke contains harmful compounds such as polycyclic aromatic hydrocarbons (PAHs), leading to the development of liquid smoke preparations since the 1970s. Liquid smoke can be added as a surface additive or an inactive constituent during processing to decrease or remove food-borne pyrogens and plays a needy smoky flavor to substances. The pieces of research have shown that the antimicrobial action of liquid smoke can be improved by vacuum packaging, EOs, and sodium chloride.

Algae and mushrooms, as natural sources of bioactive compounds, offer a wide range of biological activities, including antimicrobial and antioxidant properties. The major bioactive ingredients in algae and mushrooms, such as proteins, antioxidants, polyunsaturated fatty acids, and polysaccharides, have demonstrated antimicrobial activities. Algal polysaccharides and sulfated polysaccharides may act on glycoprotein receptors on bacterial cell surfaces, increasing membrane permeability and causing protein leakage and DNA binding. Similarly, the antimicrobial activities of mushrooms are attributed to various secondary metabolites, including gallic acids, phenols, volatile compounds, and free fatty acids.

- *Saponins:* Saponins, natural glycoside compounds found in certain plants, have demonstrated broad-spectrum antimicrobial and antifungal activities. Their antifungal activity involves interaction with cytoplasmic membrane sterols, particularly ergosterol, leading to pore formation and loss of membrane integrity, ultimately resulting in cell death.
- *Flavonoids:* Flavonoids, naturally discovered in different plant parts, having broad-spectrum antimicrobial actions by synthesizing materials with extracellular and soluble proteins, as well as with bacterial membranes. The hydroxyl groups at specific sites on the aromatic rings of flavonoids enhance their antimicrobial activity, while methylation of active hydroxyl groups generally decreases activity. Hydrophobic replacements like prenyl classes, alkylamino chains, alkyl chains, and nitrogen or oxygen-containing heterocyclic species complexically increase action. Further research is needed to explore their potential use as food preservatives as consumers increasingly seek natural alternatives.
- *Animal-derived compounds:* Several animal-derived antimicrobial compounds are used for fish preservation, including chitosan from shellfish, lactoperoxidase, lactoferrin from milk, and lysozymes from eggs. However, one major challenge with animal-derived antimicrobials is the allergen risk associated with their sources, which often include allergen-containing foods such as shellfish, milk, and eggs.
- *Chitosan:* Chitosan, a polycation biopolymer naturally found in arthropod exoskeletons, has excellent inhibitory effects on various microorganisms, including bacteria and fungi. Its antimicrobial action is influenced by factors such as chitosan type, degree of polymerization, substrate composition, and environmental conditions. Chitosan also discovers utilization in the shape of edible biopolymer-constituting films for increasing the expiry of fish by removing oxygen, moisture, solute transports, and aromas.

- *Lysozyme:* Lysozyme, discovered in mammalian milk and poultry eggs, shows antimicrobial action by lysing the β(1→4) bond between N-acetylglucosamine and N-acetyl-wall acid in the cell wall peptidoglycan of Gram-positive bacteria. Its antimicrobial spectrum can be expanded by destabilizing the outer membrane of Gram-negative bacteria using compounds such as nisin or EDTA. Combination treatments with lysozyme have been shown to effectively inhibit microbial growth and extend the shelf life of fish products.
- *Lactoferrin:* Lactoferrin, an 80 kDa whey glycoprotein, exhibits antimicrobial activity by depriving microbes of iron and through its cationic patches on the protein surface. It has been reported to have bactericidal effects against various bacteria, although its efficacy depends on its iron-binding state. Lactoferrin has also been studied for its immune-boosting effects in fish, but its use in fish preservation requires further investigation.
- *Lactoperoxidase:* Lactoperoxidase, a group of the peroxidase chain, shows antimicrobial action by creating oxidizing substances such as hypo thiocyanic acid and hypothiocyanite, which decrease microbial germination by oxidizing sulfhydryl classes of microbial enzymes and proteins. It is influential against Gram-negative bacteria, which are natural spoilage microbes in refrigerated fish. Lactoperoxidase reactions have been shown to increase the shelf life of fish substances, particularly when used in mixture with other natural stabilizers or procedures [2].

Public demand for high-quality seafood that retains its sensory and nutritional properties during processing and storage is increasing. However, seafood quality declines due to enzymatic, microbiological, and chemical reactions postharvest. To counter this, synthetic preservatives are used, though concerns about their health risks have prompted the search for natural alternatives. Natural stabilizers, including plant extracts, chitosan, bacteriocins, and EOs, have shown strong antioxidant and antimicrobial properties, making them effective and non-toxic alternatives in seafood preservation. EOs disrupt microbial cell membranes, leading to cell death and preventing contamination by harmful bacteria like *Salmonella*, *E. coli*, and Listeria [3]. The rise in urbanization and income has driven increased consumption of animal-origin foods, with global meat consumption expected to grow by 2028, particularly in developing countries. Meat, as a key protein source, is highly susceptible to contamination, spoilage, and foodborne illnesses. Concerns over the health risks of synthetic preservatives have led to a preference for natural antimicrobials, derived from renewable sources, to improve food safety and quality. EOs have shown strong antimicrobial effects in various meat products like pork and sausages. Other natural preservatives, including bacteriocins, organic acids, plant extracts, and chitosan, have demonstrated efficacy in extending shelf life, reducing lipid oxidation, and preserving color. These natural solutions may be more effective when used in combination with each other or alongside synthetic preservatives and specific packaging methods [4, 5].

In recent food sanitation regulations, food complexes are regulated by the Food Code. Naturally derived preservatives like benzoic acid and propionic acid can be naturally occurring or produced as metabolites during manufacturing processes such as fermentation. To observe the levels of naturally formed benzoic acid and propionic acid, a total of 145 samples were evaluated, incorporating berries (prune, cranberry), functional foods (propolis liquid, ginseng product), vinegars (vinegar-based drink, vinegar beverage, vinegar), and salted and pickled products (olive, pickled cucumber, salted/pickled product), using HPLC-PDA and GC-FID.

The results showed that benzoic acid was detected and identified in 144 samples, while propionic acid was detected in 64 samples. The amount of benzoic acid ranged from $4.1{\sim}478.4;ppm$ in cranberry, $49.7{\sim}491;ppm$ in propolis liquid, and $2.5{\sim}10.2;ppm$ in ginseng, with very small quantities in other tested samples. Propionic acid levels ranged from $179.8{\sim}951.9;ppm$ (average 553.6 ppm) in vinegar (persimmon vinegar 100%), which was the highest among fermented foods, $13.7{\sim}247.0;ppm$ in propolis liquid, $2.0{\sim}180.7;ppm$ in vinegar-based drink, and $1.6{\sim}76.6;ppm$ in olive. Vinegar beverage and pickled cucumber consisted of 24 and 18 ppm

of propionic acid, accordingly, while it was not screened in prune, cranberry, ginseng, and pickled/salted substances [6].

Microbial contamination is a common concern for products, including cosmetics that contain water and various organic or inorganic compounds. Effective preservation systems, whether intrinsic or extrinsic, are essential to protect these products from microbial degradation both while sealed and during use. Despite the generally excellent safety record of personal care products in recent years, microbial contamination remains a potential risk, albeit rare, leading to infections.

Investigations have identified various general microbes found in cosmetics, such as *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Burkholderia cepacia*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Enterobacter gergoviae*, and *Serratia marcescens*, as well as other bacteria, fungi, and yeasts. While the skin and mucous membranes provide some protection against microorganisms, their presence in cosmetic products can increase the risk of infection.

Microbial spreads were formed during manufacturing (primary) or during consumer application (secondary spread). Figure 1 illustrates the causes, consequences, and prevention methods for both types of contamination. To mitigate these risks, it is crucial to identify and monitor all potential sources of contamination throughout the production and usage lifecycle. This involves inspecting and controlling raw materials, ensuring proper manufacturing processes, monitoring the delivery of the final product, and educating consumers on safe product usage [7].

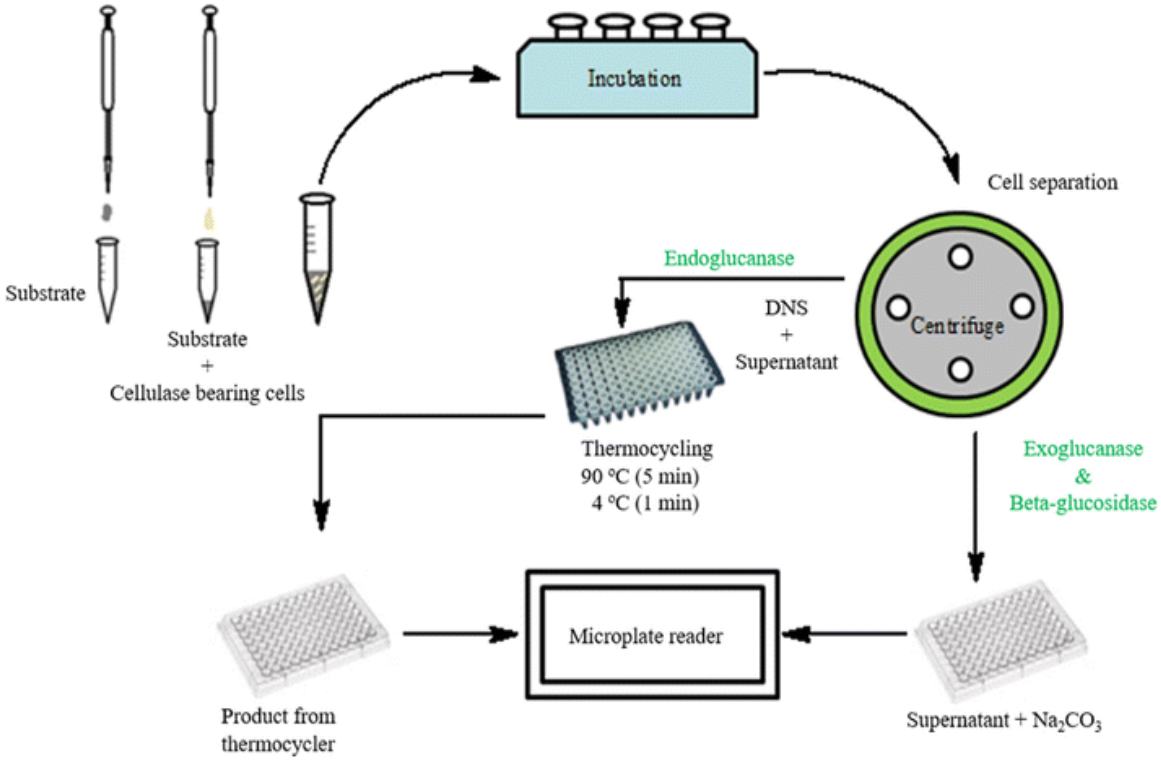

Figure 1. Enzyme activity assays [26].

Artificial stabilizers, though commonly regarded as safe, can have negative and potentially life-threatening actions. Nitrates, for instance, are converted to nitrites upon ingestion, which can react with hemoglobin, leading to methemoglobin formation. This product can create damage to consciousness and even death, especially in infants. Additionally, nitrites in the stomach can produce nitrosamines, known carcinogens. Increased levels of nitrates in food have been linked to higher mortality rates from Alzheimer's, Parkinson's, and Type 2 diabetes.

Consumption of food containing monosodium glutamate (MSG) can lead to symptoms such as headache, sweating, skin redness, nausea, and weakness. Sulfite-containing preservatives may trigger severe allergic reactions and worsen asthma symptoms.

Toxic chemicals like parabens, often used in combination with methylchloroisothiazolinone and methylisothiazolinone, have been associated with neurological damage in animal studies and are potent irritants and allergens. Pregnant women's use of these chemicals may adversely affect fetal brain development.

Formaldehyde and related compounds like DMDM hydantoin, diazolidinyl urea, and imidazolidinyl urea are potent irritants for the skin, eyes, and lungs. High levels of exposure can also cause DNA damage to sperm.

Research suggests that certain food additives found in many children's foods and drinks can lead to temper tantrums and disruptive behavior [8].

The cosmetic industry is responding to consumers' preferences for minimizing preservatives by developing preservative-free or self-preserving cosmetics, utilizing plant-based raw materials instead. This investigation focused to get the better antimicrobial action of extracts (*Matricaria chamomilla*, *Aloe vera*, *Calendula officinalis)* and EOs (*Lavandula officinalis*, *Melaleuca alternifolia*, *Cinnamomum zeylanicum*) with methylparaben.

Extracts (2.5%) and EOs (2.5%) were examined alongside methylparaben (0.4%) against *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *S. aureus* ATCC 29213, and *C. albicans* ATCC 14053. EOs exhibited greater inhibitory activity against the tested microorganism strains compared to extracts and methylparaben. Across the tested microorganism strains, all extracts and EOs showed antimicrobial activity 0.8–1.7 and 1–3.5 times stronger than methylparaben, respectively.

These findings suggest that the tested extracts and EOs could serve as alternatives to methylparaben, ensuring microbiological purity of cosmetics during use [9].

MATERIALS AND METHOD

Preservative

Natural preservatives have been developed as alternatives to synthetic ones in various industries such as food and agriculture. Multi-component nanoemulsions (MCNs) adding a blend of *Eucalyptus globulus* and *Mentha piperita* EOs, different chemical and mechanical situations with ultrasonication, were evaluated for preservation role of *E. globulus* and *M. piperita* EOs on MCN stability with potent antibacterial properties are potential candidates for natural preservatives which formulated a phospholipid-based multi-component nanoemulsion (PNE) with extended shelf life, the stability of the emulsions was monitored for up to 730 days, with stable formulations further studied for approximately two years, zeta potential measurements confirmed anionic surface charge, while dynamic light scattering and transmission light scattering revealed droplet sizes ranging between 93.40 and 100.00 nm, a homogeneous formula remained stable for over 730 days, with ultrasonication treatment and 1% v/v of food-grade castor oil as a co-emulsifier being critical for stability, the minimum inhibitory concentration (MIC) values against *E. coli*, *S. aureus*, and *S. enteritidis* were found to be (31, 8, and 63) μg/ml, respectively. Compared to the original mixture of EOs, there was a significant reduction in MIC values by factors of 32, 250, and 15 against *E. coli*, *S. aureus*, and *S. enteritidis*, respectively. Mass transfer followed a case-I transfer (Fick law diffusion) as determined by the Koresmeyer–Peppas kinetic model ($R^2 = 0.88$). In conclusion, MCN could serve as promising natural preservatives for applications in the food, agriculture, and cosmetic industries [10].

Menthol, also known as mint camphor, is a cyclic monoterpene alcohol found as a major constituent in the EOs of *Mentha canadensis* L. (cornmint) and *M. piperita* L. (peppermint). Alongside menthone, isomenthone, and other substances, menthol gives the cooling minty flavor and smell to plants, especially those in the *Mentha* genus. From the beginning, plants were the sole origin of menthol and had been obtained for medicinal focuses in Japan for centuries before the compound was separated and characterized (Lawrence, 2013) [11]. The Dutch botanist Gambius first isolated menthol as a crystalline principle in 1771. Generally, when referring to menthol, the l- or (−)-menthol is implied. Commercially, this compound is the most significant natural isolate [12–22]. Approximately 30,000–32,000 metric tonnes of menthol are received yearly, producing it one of the most necessary flavoring chemical ingredients after vanilla and citrus (Ruskin et al., 2007; Etzold et al., 2009) [23, 24].

Menthol is prominently used in many tobacco products, first as an additive in the 1920s (Ruskin et al., 2007; Etzold et al., 2009) [23, 24]. Even non-mentholated cigarette brands may contain low levels of menthol, with approximately one-quarter of cigarettes sold estimated to contain it (Ruskin et al., 2007) [23]. Smokers in the United States often prefer mentholated cigarettes due to the perceived "coolness" of the smoke (Werley et al., 2007) [25]. Additionally, menthol (CAS No. 2216-51-5, EINECS No. 218-690-9, FEMA No. 2665) is widely used as an ingredient in various consumer products, including pharmaceuticals, cosmetics, pesticides, candies, chewing gum, liqueurs, toothpaste, shampoos, and soaps, for its cooling and/or flavor-enhancing properties.

Menthol is a principal EO ingredient of a small number of aromatic plants identified for expressing various biological actions such as antimicrobial, anticancer, and anti-inflammatory effects. These plants are also applied as insect repellents or fumigants.

Chemically, menthol (C10H20O, mol. wt. 156.27) is a cyclic monoterpene with three asymmetric carbon atoms, obtaining as four pairs of optical isomers: (+)- and (−)-isomenthol, (+)- and (−)-menthol, (+)- and (−)-neomenthol, and (+)- and (−)-neoisomenthol. The predominant structure found in nature is (−)-menthol (l-menthol), having greater cooling actions than other menthol isomers. Menthol is a white or colorless, flaky, crystalline product in solid at room temperature, with a density of 0.890 kg/dm3 (25°C) and a melting point of 41–44°C based on its clarity. It is not completely soluble in water (435.5 mg/l at 25°C) but easily soluble in alcohol, diethyl ether, or chloroform. Like many terpene alcohols, menthol does not absorb UV light well in the 290–320 nm range but absorbs below 290 nm with peak absorption at 220 nm.

Menthol, like other monoterpenes, is primarily derived from aromatic plants and acts as a chemical messenger with diverse functions along with other organic compounds of EOs. It is obtained from cornmint oil through steam distillation, with cornmint oil typically containing 55%–85% menthol. Natural menthol is preferred due to the influence of contaminants on the scent of synthetic l-menthol during the crystallization process. Menthol is biosynthesized in plants through an 8-step pathway from primary metabolism. The main supply of menthol is obtained naturally, with approximately 19,000 tonnes produced in 2007, primarily from *M. canadensis*. Only around 6300 tonnes were produced synthetically (Etzold et al., 2009), with companies like Symrise (Germany), Takasago (Japan), and to a lesser extent, Camphor & Allied (India) being major producers. Recently, BASF has also begun producing synthetic menthol (Lawrence, 2013) [11]. Various routes have been described for the industrial synthesis of (−)-menthol, including synthesis from (−)-β-pinene, (+)-limonene, (+)-citronellal, (+)-pulegone, (−)-piperitone, thymol, β-phellandrene, δ-3-carene, citral, or m-cresol as precursors.

Increased consumer concern regarding the various side effects of synthetic preservatives and the development of antibiotic-resistant strains has led to a growing interest in natural compounds with

antimicrobial properties in the food and pharmaceutical industries. EOs, volatile aromatic liquids derived from various parts of plants, such as leaves and flowers, have been extensively utilized in cosmetic industries to produce soaps, perfumes, and toiletries due to their aromatic properties. Numerous studies have confirmed the broad bioactivity of EOs, including antibacterial, antiviral, anti-inflammatory, antifungal, antimutagenic, anticarcinogenic, and antioxidant activities. These oils may give natural antimicrobial solutions, decreasing the reliance on synthetic stabilizers. However, EOs are prone to oxidation due to their unsaturated carbon chains, are highly volatile, and have low-aqueous solubility, limiting their direct application in aqueous-based food products like beverages.

Menthol, a monocyclic monoterpene alcohol found naturally in peppermint or other mint oils, is widely used as a flavoring agent in toothpaste, hygiene products, chewing gum, etc. Even though its recognized antimicrobial and antifungal characteristics, its high volatility, instability, insolubility, and speedy crystallization in aqueous mediums pose major difficulties for its use and shelf life. To solve these problems, microencapsulation is an easy technique. Encapsulation methods majorly save natural substances from chemical reactions and unwanted interactions with other food substances, grow solubility, inhibit migration, and store stability during procuring and preservation. In food engineering, nanoencapsulation techniques are used to protect bioactive compounds like vitamins, `antioxidants, proteins, and lipids, resulting in functional foods with enhanced stability and functionality.

Lipid-based nanoparticles, such as nanostructured lipid carriers (NLCs), interact with various bacterial and fungal cell types, after that menthol-loaded NLCs were prepared to enhance the solubility, stability, and antimicrobial efficacy of menthol for potential use as a preservative in the food industry [12].

The growing consumer demand for natural and organic products, coupled with concerns over the harmful effects of artificial antioxidants, has prompted researchers to explore edible herbs and plants as sources of safe and effective antioxidants for the food industry. Like other oil-water emulsions, dairy products are prone to oxidative and hydrolytic rancidity, primarily due to the reaction of oxygen with lipids. Synthetic ingredients such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butyl hydroquinone (TBHQ) are commonly utilized to stop fat and oil rancidity, as well as oxidative degeneration in dairy substances.

A research study evaluated the effectiveness of antioxidants from natural sources in controlling lipid deterioration through oxidation. Mint, beet, and ginger were found to possess potent antioxidant properties comparable to synthetic antioxidants like TBHQ, BHT, and BHA. Blending mint or ginger with beet resulted in even better retardation of lipid oxidation compared to beet alone. Natural antioxidants offer the advantage of being free from undesirable health effects, making them advisable for adoption by the dairy industry to enhance the health benefits of their products.

Consumer rights groups are increasingly advocating for the replacement of chemical preservatives with “natural-green” alternatives to extend the shelf life of food products. Many herbs and spices contain EOs with antimicrobial properties that can help maintain food quality by delaying the growth of foodborne microorganisms. Peppermint essential oil, for example, demonstrated significant inhibitory effects on *S. aureus* and *Salmonella enteritidis* in nutrient broth, even at low concentrations. This suggests mentha as a potential as a promising food stabilizer [13].

The World Health Organization (WHO) declares that an important part of the world’s wide area believes in traditional medicine for essential healthcare, further strongly revealing the need of opening the uses of aromatic plants in curing infectious disorders. Various studies have demonstrated the in vitro and in vivo biological activity of plant extracts and EOs, showing their potential benefits. This has led to a justification for more focused research on the antimicrobial action of these plant derivatives. Menthol, a naturally occurring cyclic terpene alcohol found in various *Mentha* species,

has demonstrated appreciable antibacterial effects against several bacteria, including *Enterobacter aerogenes, Clostridium sporogenes, Klebsiella pneumonia, Salmonella pullorum, P. aeruginosa, S. aureus, Comamonas terrigena, and Streptococcus faecalis*. Gram-negative bacilli constitute over 10% of the indigenous bacterial flora and are therefore ubiquitous. Changes in humidity and temperature, along with declining counts of Gram-positive organisms, contribute to the rapid proliferation of Gram-negative bacilli, leading to various clinical infections. Peppermint leaf and stem extracts were evaluated for their antibacterial effects against 11 different species of Gram-negative bacilli, showing significant variability in inhibition. Subsequent studies by Saeed et al. investigated the effects of various peppermint formulations against the same strains of Gram-negative bacilli. EO showed the highest activity, followed by peppermint juice, while decoction and aqueous infusion had negligible effects.

The ethnomedicinal relevance of pennyroyal (*Mentha pulegium*) for its antiseptic properties was confirmed through screening the EO against various microbes, particularly Gram-positive bacteria. Significant antimicrobial efficacy was observed, suggesting its potential therapeutic application as an antibiotic alternative. Further research by Bupesh et al. evaluated the anti-pathogenic effects of peppermint leaf extracts against several bacterial strains, confirming their potency in eliminating pathogens. Giardiasis, caused by *Giardia lamblia*, is typically treated with medications like furazolidone and metronidazole, which can cause side effects and resistance development. Vidal et al. investigated the effects of peppermint extracts on *G. lamblia* trophozoites, demonstrating significant antigiardial activity with minimal cytotoxic effects on intestinal cells. *Helicobacter pylori* is the main causative organism for peptic ulcers and chronic gastritis, with conventional antibiotic treatment facing challenges due to drug resistance and side effects. Several studies have explored the anti-*H. pylori* effects of medicinal herbs, with aqueous extracts of *M. piperita* showing promising inhibitory effects. Peppermint oil also showed significant virucidal and inhibitory activities against herpes simplex virus types 1 and 2, suggesting its potential therapeutic use against these viruses. Each ingredient sourced from mint, such as carvone, have described potential antimicrobial action against a broad spectrum of pathogenic bacteria and fungi. Given the wealth of compounds with antimicrobial properties in plants, screening programs for antifungal activity may lead to the discovery of novel antifungal constituents. Ethanol extracts and oils from various medicinal plants, including peppermint and *Mentha arvensis*, have shown appreciable anti-*C. albicans* activity, highlighting their potential as natural antifungal agents. These findings underscore the importance of exploring plant-derived compounds as alternatives to traditional antibiotics and antifungals, paving the way for the development of effective and safer treatments for microbial diseases [13].

Extraction and Isolation Process of Menthol

Steam Distillation

Steam distillation is a naturally used procedure for extracting EOs due to its market price. This method involves distillation at low temperatures, allowing for the separation of non-volatile substances and substances that are immiscible in water below their boiling points. Mint oil was extracted using the steam distillation method, specifically in a 2-L steam distillation apparatus. The EOs of *M. arvensis* were collected when the first drop of oil appeared in the Florentine flask, marking the start of each distillation. The mint oil was processed directly at the completion of each distillation after switching off the electric power. The substances of corn mint oils were weighed on an analytical balance, and the oil quantity (yield) was obtained as grams of oil per 100 g of dried Japanese corn mint shoots. The term "content" refers to the amount of EO in biomass per 100 g, while "concentration" indicates the percentage of individual constituents present in the mint oil. The complete outcome of various components was determined based on the yield of EO and the concentration of each component in the oil. The yield of menthol was found to be 74 to 79%, and the extraction temperature ranged from 35 to 40ºC.

Hydrodistillation is a widely used method for extracting EOs from plant materials. In this process, the herb material is placed in a still, and steam is applied directly for heating. Alternatively, the herb

material can be soaked in warm water, or a perforated plate can be used to suspend the herb material above the water level in the still. The steam generated carries the volatile components of the plant material, which then pass through a condenser. The condensate, containing both water and EO, is collected in a Florentine flask where they separate from each other. The water can be recycled back to the still or used as fragrant water. Some researchers have utilized hydrodistillation for the extraction of mint oil. The leaf samples are first dried at 30 ºC in a hot air oven to a constant weight. The dried leaves of *M. arvensis* are then crushed, and 100 g of this crushed sample is subjected to hydrodistillation for 3 hours using a Clevenger-type apparatus. The distilled *M. arvensis* EO is then desiccated over anhydrous sodium sulfate. After desiccation, the oil is filtered, and the obtained EO is stored at -4 ºC until analysis. During summer, the yield of EO from M. arvensis was found to be 17.0 g/kg. The principal ingredients in the EOs of M. arvensis in summer and winter were menthol (78.90% and 81.30%, respectively) and isomenthone (6.35% and 6.19%, respectively).

Microwave-assisted extraction has become crucial in the research and development of extracting compounds from medicinal plants. This technique relies on dipole rotation and ionic conduction to generate heat, with the efficiency depending on the dielectric properties of the herb material. The process involves the absorption of energy by the internal water in the plant due to microwaves, leading to increased pressure within the plant material. This pressure results the cell structure to break, permitting the solvent to enter the cell plasma.

This method is employed for extracting mint oil. Microwave-assisted extraction was done based on a face-centered central composite experimental charts with two steps and two times at the main point. Individual investigation used 2 g of mint powder with 60 ml of an ethanol-hexane solvent combinations. Ethanol and hexane were the primary reagents used for the extraction experiments. The extracted material was vacuum filtered using Whatman 1 filter paper and rinsed with the solvent mixture. The oil was isolated from the solvent by vaporizing with a rotary evaporator in a heating bath set at 66ºC and 55 rpm.

The utilization of a compound of ethanol and hexane as solvents gave in increased yields of extracted components such as menthone, menthofuran, and menthol.

Isolation of Menthol

Fractional Distillation

Menthol, typically found in *M. arvensis* EO along with menthone, poses a challenge for isolation due to their similar physical properties. Fractional distillation is considered an effective technique for isolating menthol from mint oil. In a study, fractionation was conducted under a pressure of 50 mmHg to distill off xenthone and lower boiling components. Subsequently, steam distillation separated menthol from the non-volatile residue left behind. The purification of menthol involved repeated maceration in boiling water until the product met the desired United States Pharmacopeia (U.S.P.) standard melting point.

Fractional distillation was performed in a batch column with approximately 50 theoretical plates at a pressure of 20 mm. The fractionally distilled mint oil contained 65.2% menthol, 12.5% menthone, and 1.8% methyl esters. The distillation process was stopped, yielding a product known as menthone-free-oil, which solidified at room temperature and accounted for almost 70% of the total charge. This product consisted of about 89% menthol and 6.5% unstable components.

The steam distillation without having menthone oil under atmospheric pressure was carried out until all thermolabile substances disappeared. The condensate is isolated into two layers, and upon cooling, the menthol layer condensed into a crystalline mass. After drying, the melting point of menthol was in the range of 29°C–35°C, not yet reaching the U.S.P. standard. The crude menthol yield was approximately 88% of the menthone-free-oil.

To purify the crude menthol, it was absorbed with water to remove impurities. Around 10 g of crude menthol were warmed with 100 ml of water, and the mixture was stirred continuously to ensure intimate contact between the layers. The menthol layer was solidified, separated from the solution, and then melted at 38°C–40.5°C upon drying. The process was repeated, resulting in needle-like crystals of pure menthol with a melting point within the U.S.P. specifications (41.6°C–42.7°C). Approximately 92% of the U.S.P. standard menthol was obtained from the crude menthol. Despite some loss of menthol with impurities in the water solution, about 85% of the total menthol present in the mint oil was isolated.

Chromatographic Adsorption

The ingredients of mint oils, although complex, is mainly made by constituents such as menthol, menthone, menthyl esters, and terpene hydrocarbons. *M. arvensis* EO typically contains about 60%–85% menthol. Chromatographic adsorption is a method used to isolate menthol and menthone from mint oil on a commercial scale.

In this method, corn mint oil is solubilized in a non-polar solvent and spread over an adsorbent. The adsorbed substance is then cleaned with eluants to isolate menthone and menthol into different portions. These components can be further isolated from the solvents by various methods, such as solvent evaporation.

Adsorbents commonly used in chromatographic adsorption include fuller's earth, activated magnesia, activated charcoal, and activated alumina. Eluants are selected to achieve rapid and complete separation of components.

In a study, corn mint oil containing approximately 65.2% menthol, 12.5% menthone, and 1.8% methyl esters was used as raw material. Activated carbon was used as an adsorbent, packed into a glass column. The mint oil, refined by steam distillation, was dissolved in a hydrocarbon solvent and adsorbed onto the column. The column was then developed with a volume mixture of hydrocarbon solvent.

After receiving the portions, three separate portions were gained: terpenes and methyl esters were cleared out with water first, preceded by the menthone fraction, and at last, the menthol fraction. The menthol gained melted at 41ºC–43ºC. The yield of menthol and menthone was 95% and 90%, respectively [18].

Like traditional synthetic preservatives, natural antimicrobial ingredients can also be categorized into general classes based on their origin and function. Cosmetic chemists today have several options for natural antimicrobial ingredients, including:

- Plant/herbal extracts;
- Organic acids;
- Enzyme/substrate systems;
- Antimicrobial peptides [14].

Mechanism of Action of Menthol as Preservative

The mechanism of action of menthol as a preservative involves its antimicrobial properties, which inhibit the growth and proliferation of microorganisms responsible for food spoilage.

Disruption of Cell Membrane Integrity

Menthol breaks microbial cell membranes by interacting with the lipid bilayer. This destabilization and permeabilization lead to leakage of cellular contents, loss of cellular homeostasis, and ultimately cell death.

Interference with Cellular Processes

Menthol interferes with essential cellular processes for microbial survival and growth. It may disrupt enzyme activity, protein synthesis, or DNA replication, thereby inhibiting microbial proliferation

- *pH regulation*: Menthol affects the pH balance within microbial cells, creating an unfavorable environment for growth and survival.
- *Antioxidant activity*: Menthol exhibits antioxidant properties, scavenging free radicals, and inhibiting lipid oxidation, which helps preserve the quality of food products.
- *Anti-biofilm activity*: Menthol inhibits the formation of microbial biofilms, preventing microbial attachment and growth on food surfaces. Overall, menthol acts as a multifunctional preservative by targeting various aspects of microbial physiology and metabolism, leading to the inhibition of microbial growth and extending the shelf life of food products.

Antibacterial Assays

Preparation of Experimental Samples

- To develop herbal cosmetic formulations with various concentrations of menthol.
- To ensure consistency of other components across samples to isolate menthol's effect.

Bacterial Culture Preparation

- To choose bacterial strains commonly found on the skin or associated with skin infections.
- To culture the bacteria in suitable growth media under optimal conditions.

Antibacterial Assays

Disk Diffusion Method

- To inoculate agar plates with bacterial cultures.
- To place paper disks containing different menthol concentrations on the plates.
- To Incubate and measure zones of inhibition.

Broth Microdilution Method

- To prepare serial dilutions of menthol in broth media.
- To inoculate each dilution with bacterial strains.
- To investigate the MIC of menthol.

Control Groups

- To include control groups lacking menthol to compare effectiveness.

Data Collection

- To record the diameter of inhibition zones in the disk diffusion method.
- To note MIC values in the broth microdilution method.

Statistical Analysis

- To conduct statistical tests to assess results' significance.
- To compare antibacterial activity among different menthol concentrations and control groups.

Interpretation

- To interpret results, to consider menthol's effectiveness as an antibacterial agent.
- To discuss trends, strain differences, and implications for herbal cosmetics use.
- To summarize findings on menthol's antibacterial activity in herbal cosmetics.
- To offer recommendations for further research or practical applications.
- To ensure proper controls and replication for accuracy.
- To adhere to safety precautions when handling bacteria and chemicals.

- To verify menthol's stability in cosmetic formulations over time.
- To consider testing formulations on human skin to confirm efficacy and assess skin reactions.

Two American Type Culture Collection (ATCC) optimized strains were used: *S. aureus* ATCC 6538P and *E. coli* ATCC 15221. The MIC of the products was determined following the M7-A5 procedure was set up by the National Committee for Clinical Laboratory Standards [18]. MICs for the bacterial strains were determined using a microdilution method on 96-well culture plates with Mueller–Hinton broth (Becton-Dickinson, Milan, Italy). Eight twofold dilutions of the samples were prepared, beginning with a concentration of 0.5% (w/v) (5% Tween 20). All preparations were sterilized using a 0.22-µm filter. The wells were growing with a microorganism suspension at a density of 105 cells/ml and incubated at 37°C for 24 hours. After incubation, the plates were studied to evaluate the MICs. Correct blanks were prepared concurrently, and samples were tested in three times [15].

Antibacterial activity of menthol and thymol was assessed using the bacterial broth dilution method (modified from Stock, 1987). Concentrations ranging from 300 to 3100 mg/ml of thymol and menthol were tested against bacterial isolates obtained from women with genital infections [16].

The antimicrobial activity of EOs from both herbs was evaluated against 68 isolates of *A. actinomycetemcomitans* using the disc diffusion method. Initial screening tests were done using undiluted oil, and MIC and minimum bactericidal concentrations (MBC) were evaluated through microbroth dilution processes.

Disc Diffusion Process

The disc diffusion process was tested on cation-adjusted Mueller–Hinton agar (MHA) plates supplemented with 0.6% yeast extract. A 48-hour culture of *A. actinomycetemcomitans*, grown in enriched brain heart infusion (BHI) broth, was adjusted to match the opacity of a McFarland 0.5 standard (1.5×10^8 colony forming units/ml) and swabbed over the agar plate.

Sterile discs of 6-mm diameter were impregnated with one milliliter of undiluted EO from *M. piperita* (peppermint), *M. arvensis* (wild mint or corn mint). These discs were placed on the enriched MHA plates, and the zones of inhibition were measured in millimeters after incubation in a candle jar (5%–10% CO2) at 37°C for 48 hours. A disc containing DMSO (dimethyl sulfoxide) as a diluent was included as a negative control.

The disc diffusion tests were observed in three times, and the mean value of the zone of inhibition in millimeters was calculated. Additionally, a doxycycline disc (30 µg), the drug of choice for *A. actinomycetemcomitans*, was used as a reference antimicrobial compound. *A. actinomycetemcomitans* ATCC 29522 was also tested alongside the clinical strains.

Determination of MIC and MBC of EOs

The microbroth dilution procedure was done to evaluate MIC according to Clinical and Laboratory Standards Institute (CLSI) methods. Thirty strains of *A. actinomycetemcomitans* were used for the study. All tests were performed on cation adjusted Mueller–Hinton broth enriched with 2.5% sterile sheep blood. Briefly, chain doubling dilutions of the extract were made in a 96-well microtiter plate ranging from 100 to 0.78 µl/ml. Finally, from a 48-hour culture, diluted 100 µl (106 cfu/ml) of bacterial suspension was added to each well except broth control. The final concentrations of the EO were doubled in each well after addition of equal amounts of the test strains. Each well had a complete volume of 200 µl.The plates were lined loosely with plastic film to confirm that the bacteria did not accept dehydration during incubation. The test was done in triplicates, and they were incubated at 37°C for 24–48 hours in a candle jar. The strongest dilution which revealed the dearth of practical turbidity was regarded as MIC. The MBC was known as the weakest strength of the extract

at which the incubated microbes was fully destroyed. This resulted by plating out the test bacteria into enriched BHI agar from the MIC wells which had no visible growth. The method was done in triplicates [17].

Determination of the Disruption of Cell Membrane Integrity Caused by Menthol

- *Fluorescent dyes:* Fluorescent dyes such as propidium iodide or SYTOX Green can be used. These dyes penetrate cells with compromised membranes, binding to nucleic acids and producing a fluorescent signal. Increased dye uptake indicates cell membrane disruption.
- *Electron microscopy:* Transmission electron microscopy (TEM) or scanning electron microscopy (SEM) can observe alterations in cell morphology done by menthol-induced membrane lysis. Direct observation of membrane damage, such as irregularities or ruptures, is possible.
- *Flow cytometry:* Flow cytometry can quantify cell membrane integrity by measuring the uptake of fluorescent dyes or the loss of membrane integrity markers such as annexin V or 7-AAD.
- *Release of intracellular components:* Measurement of the release of intracellular components such as ions, proteins, or enzymes into the extracellular environment can indicate membrane damage. For example, the release of lactate dehydrogenase (LDH) can be estimated by enzymatic procedures.
- *Cell viability assays:* Cell viability assays such as the MTT assay or the resazurin assay indirectly assess membrane integrity by measuring metabolic activity. A decrease in metabolic activity indicates cell membrane damage and compromised cell viability.
- *Patch-clamp technique:* This technique involves measuring ion currents across the cell membrane using a patch-clamp electrode. Changes in ion conductance can indicate alterations in membrane integrity.
- *Membrane potential assays:* Membrane potential-sensitive dyes such as DiBAC4(3) or JC-1 can assess changes in membrane potential resulting from membrane disruption.

Each of these methods provides insight into the disruption of cell membrane integrity caused by menthol and can be selected based on the specific requirements of the study and available resources.

Menthol's Interference with Essential Cellular Processes for Microbial Survival and Growth Can be Determined Through Various Methods (Figures 1–8)

- *Enzyme activity assays:* Specific enzymatic assays can be used to assess the effect of menthol on enzyme activity. Changes in enzyme activity levels or kinetics can indicate interference with essential cellular processes (Figure 1).
- *Protein synthesis inhibition:* Techniques such as western blotting or metabolic labeling with radiolabeled amino acids can evaluate menthol's impact on protein synthesis. Decreased protein synthesis rates suggest interference with cellular processes (Figures 2 and 3).
- *DNA replication inhibition:* Methods such as DNA polymerase assays or DNA replication assays can examine the effect of menthol on DNA replication. Reduced DNA replication rates or inhibition of DNA polymerase activity may indicate interference with cellular processes.
- *Gene expression estimation:* Methodologies like estimative real-time PCR or RNA sequencing can evaluate menthol's influence on gene expression. Alterations in the expression steps of genes included in essential cellular procedures can conclude interference by menthol (Figure 5).
- *Cellular morphology:* Microscopic examination of menthol-treated microbial cells can reveal alterations in cellular morphology, such as changes in shape, size, or structural integrity. These changes may result from interference with cellular processes (Figure 6).
- *Metabolic profiling:* Metabolic-profiling techniques such as metabolomics can assess changes in metabolite levels in menthol-treated microbial cells. Disruption of cellular processes may lead to alterations in metabolic pathways and metabolite profiles.

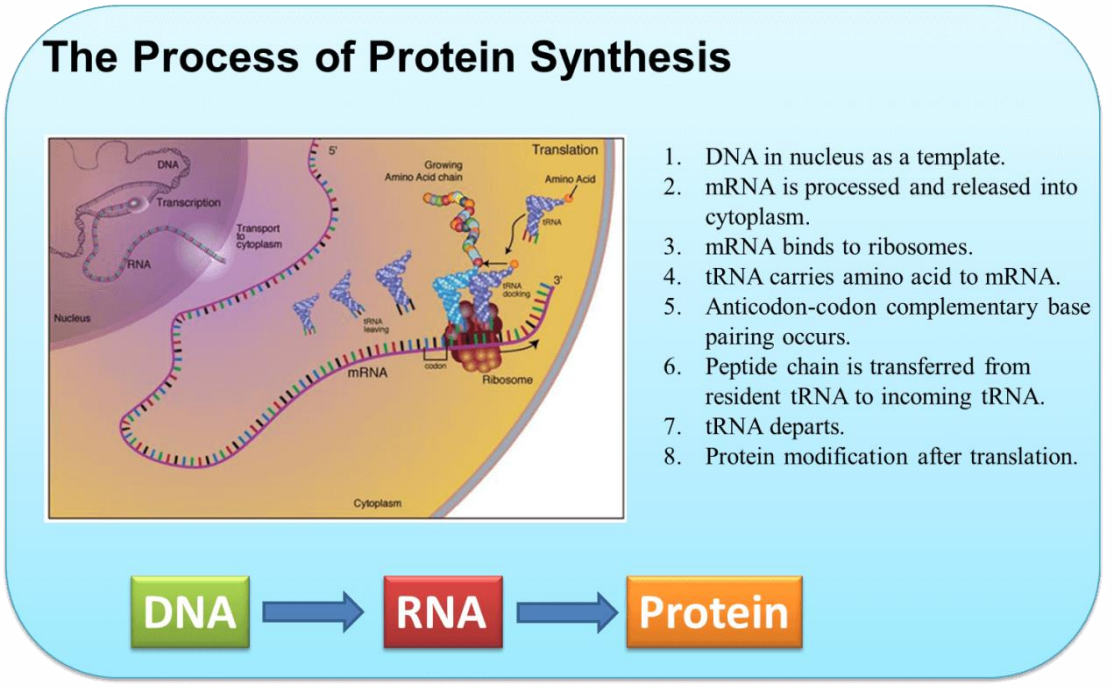

Figure 2. Protein synthesis [protein synthesis [illustration]. Creative Biostructure. 2024. Available at https://www.creative-biostructure.com/protein-synthesis-451.html].

Cell viability assays: Cell viability assays, such as the MTT assay or the resazurin assay, can indirectly assess menthol's impact on cellular processes by measuring changes in cell viability and metabolic activity.

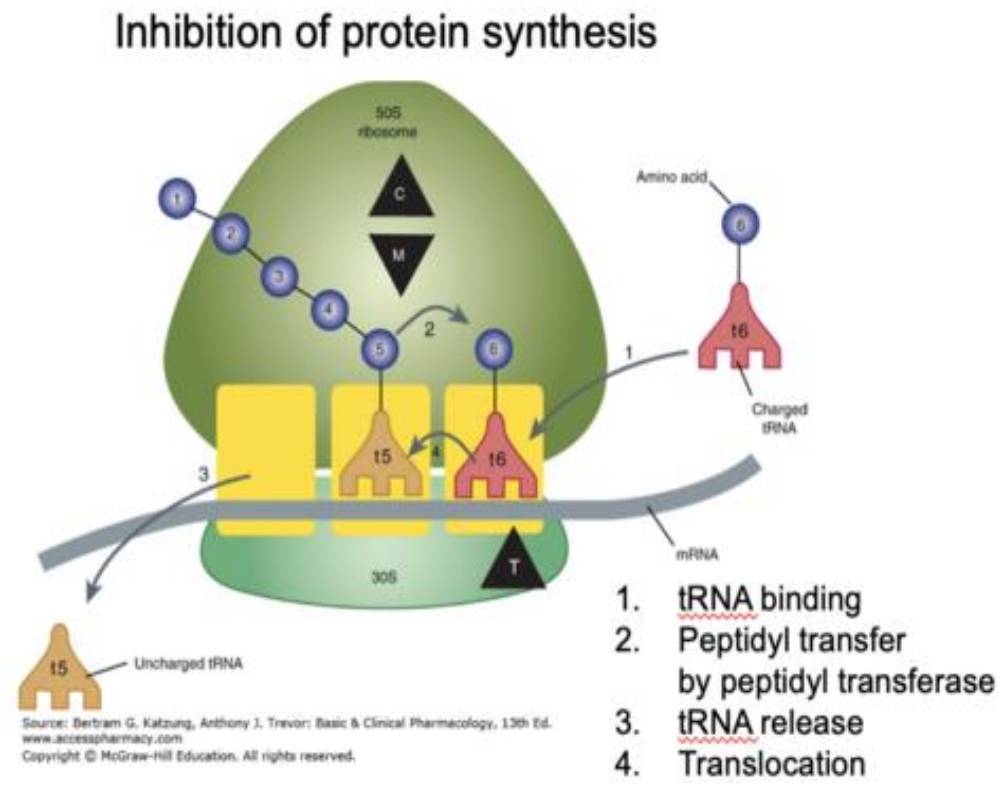

Figure 3. Protein synthesis inhibition. [9–10 protein synthesis inhibitors [illustration]. Available at https://quizlet.com/558554893/9-10-protein-synthesis-inhibitors-flash-cards/].

To Determine How Menthol Affects pH Regulation Within Microbial Cells, Various Methods Can Be Employed

- *pH measurement:* Direct measurement of intracellular pH using pH-sensitive fluorescent dyes such as BCECF or SNARF can indicate changes caused by menthol. Decreases or increases in pH levels can suggest alterations in pH regulation mechanisms.
- *Proton flux assays:* Proton flux assays involve measuring the movement of protons across the cell membrane. Menthol-induced changes in proton flux can indicate disruptions in pH regulation mechanisms.
- *ATPase activity assays:* ATPases are enzymes included in regulating intracellular pH by pumping protons into the cell. Menthol's effect on ATPase activity can be assessed using enzymatic assays. Inhibition of ATPase activity may lead to pH dysregulation.
- *pH-sensitive fluorescent proteins:* pH-sensitive fluorescent proteins such as pHluorin can be expressed in microbial cells to monitor intracellular pH changes in real-time. Menthol-treated cells can be observed under fluorescence microscopy to detect alterations in pH.
- *Metabolic profiling:* Metabolomic analysis can reveal changes in metabolite profiles related to pH regulation. Alterations in metabolites involved in buffering systems or pH homeostasis can indicate the impact of menthol on cellular pH.
- *Cellular respiration assays:* Measurement of oxygen consumption or carbon dioxide production can indirectly assess changes in intracellular pH due to alterations in cellular respiration caused by menthol.
- *Growth curves:* Monitoring microbial growth in the presence of menthol under different pH conditions can provide insights into its effect on pH regulation. A decrease in growth rate or viability at specific pH levels may indicate pH dysregulation by menthol.

Antioxidant Activity

Peppermint EO has been reported to have significant antioxidant activity. Singh et al. evaluated its DPPH free radical scavenging activity, with inhibition percentages ranging from 70% to 93%. Wu et al. also demonstrated the Fe^{3+} reducing activity of peppermint EO and its ability to scavenge radicals in vivo using CAA (Cellular Antioxidant Assay) with the IPEC-J2 (porcine intestinal epithelial cell line) cell line. The main chemical constituents responsible for its antioxidant activity include menthol, L-menthol, eucalyptol, and neo-menthol. It is valued stated that few pieces of research with the radical scavenging action of peppermint EOs with the presence of menthol and menthone, which contain the hydroxyl radical (-OH).

The antioxidant assay of menthol can be determined through various methods. One naturally applied procedure is the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging analysis.

- *Preparation of menthol solution:* To prepare different concentrations of menthol solutions in suitable solvents such as ethanol or DMSO.
- *Preparation of DPPH solution:* To prepare a DPPH solution in ethanol at a concentration of typically 0.1 mM.
- *Reaction mixture:* To mix equal volumes of the prepared menthol solutions and the DPPH solution in a 96-well microplate.
- *Incubation:* To incubate the microplate in the dark at room temperature for about 30 minutes to allow the reaction between menthol and DPPH to occur.
- *Measurement of absorbance:* After incubation, to measure the absorbance of the reaction mixture at a suitable wavelength (e.g., 515 nm) using a microplate reader.
- *Calculation of antioxidant activity:* To calculate the percentage inhibition of DPPH radical by menthol using the formula:

$$\text{Percentage inhibition} = (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100\%$$

A_{control} = The absorbance of the control (without menthol).
A_{sample} = The absorbance of the sample (with menthol).

- *Determination of IC50:* Calculate the concentration of menthol required to scavenge 50% of the DPPH radicals, known as the IC50 value. This can be evaluated by making a graph of percentage inhibition against the concentration of menthol and interpreting the concentration at 50% inhibition.

Other assays such as the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay or FRAP (Ferric Reducing Antioxidant Power) assay can also be used to determine the antioxidant activity of menthol.

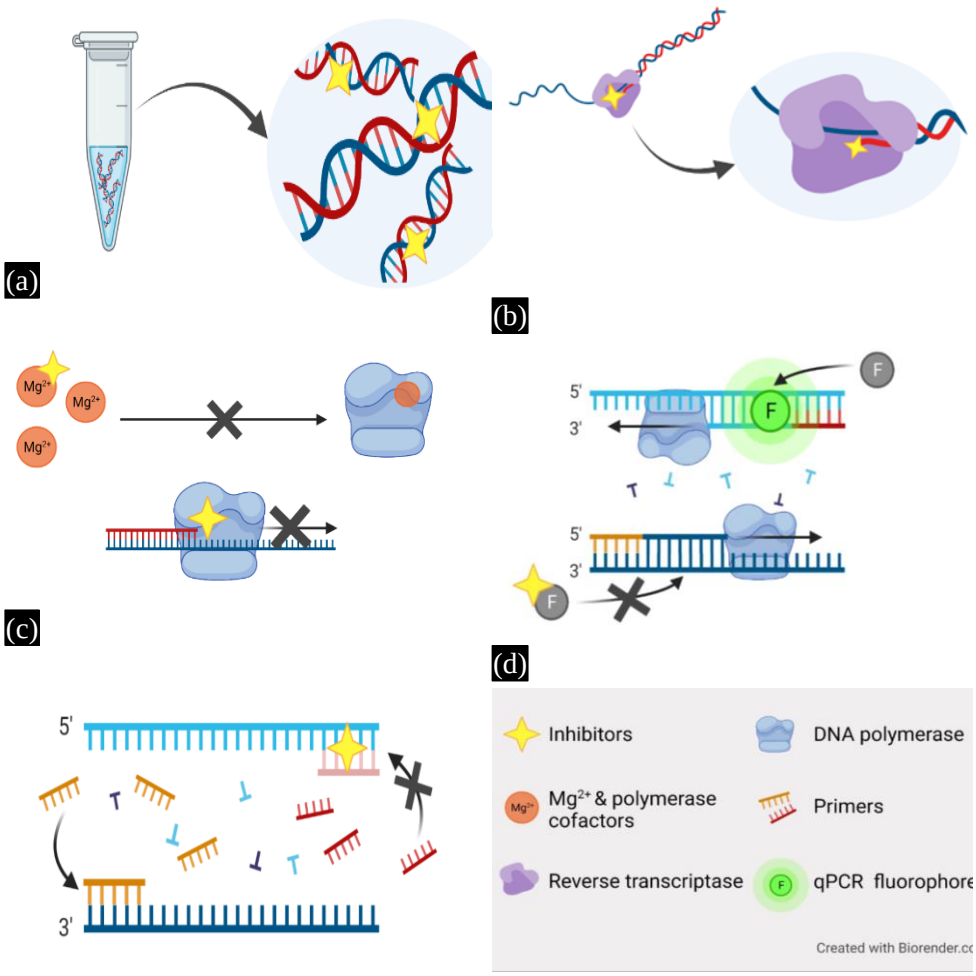

Figure 4. Representation of the attack points of PCR inhibitors during sample preparation and PCR procedure. (a) Inhibitors can interact with both double-stranded and single-stranded nucleic acids, leading to co-precipitation during the purification process. (b) Certain substances decrease the availability of Mg^{2+} and other cofactors necessary for polymerase activity, thereby disrupting DNA function. (c) Inhibitors can obstruct the annealing of primers. (d) Some agents can attach to reverse transcriptase or directly to the DNA strand, resulting in termination of the chain. (e) Inhibitors may hinder the binding of fluorophores to the newly synthesized DNA. Available at https://www.bioecho.com/blog/inhibit-inhibition-pcr-inhibition-and-how-to-prevent-it].

Determination of Menthol's Anti-Biofilm Activity

- *Crystal violet assay:* Menthol-treated and untreated biofilms are stained with crystal violet to quantify biomass. The optical density of stained biofilms is measured to determine the extent of biofilm inhibition.

- *Live/dead staining:* Fluorescent dyes like SYTO 9 and propidium iodide are used to visualize viable and non-viable cells within biofilms. An inhibition in the number of viable cells shows anti-biofilm action.
- *CFU count:* Biofilm substances are inoculated on agar plates to determine the number of viable cells. A decrease in CFUs indicates anti-biofilm activity.
- *EPS extraction and quantification:* Menthol's effect on extracellular polymeric substances (EPS) is assessed by quantifying EPS production using methods such as the phenol-sulfuric acid assay or the Bradford assay.
- *SEM:* SEM permits observation of biofilm figure and morphology. Changes in biofilm architecture, such as disruption or reduction in thickness, indicate anti-biofilm activity.
- *Confocal laser scanning microscopy (CLSM):* CLSM provides three-dimensional images of biofilms to assess biofilm viability and structure. Reduction in biofilm thickness and viability indicates anti-biofilm activity.

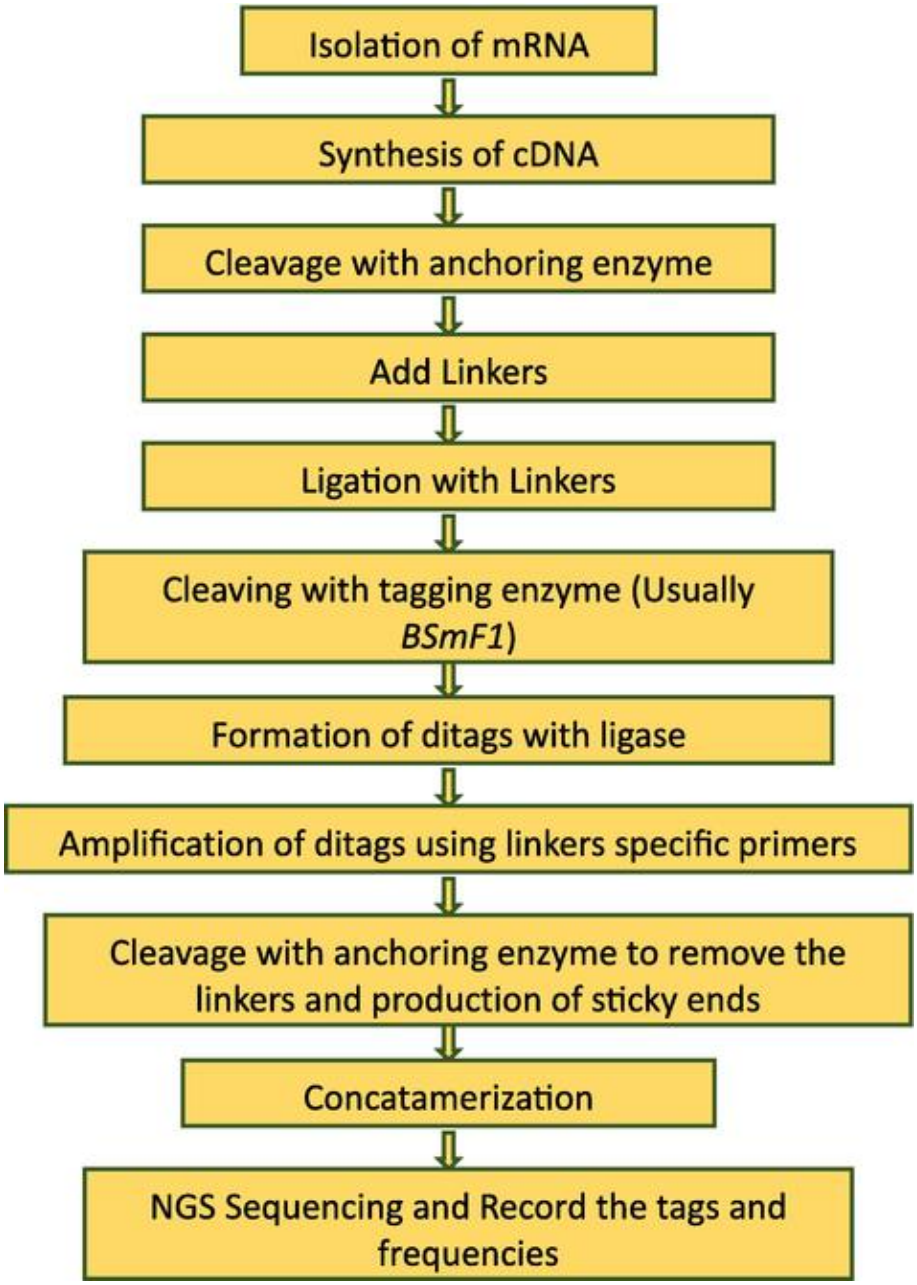

Figure 5. Serial analysis of gene expression [27].

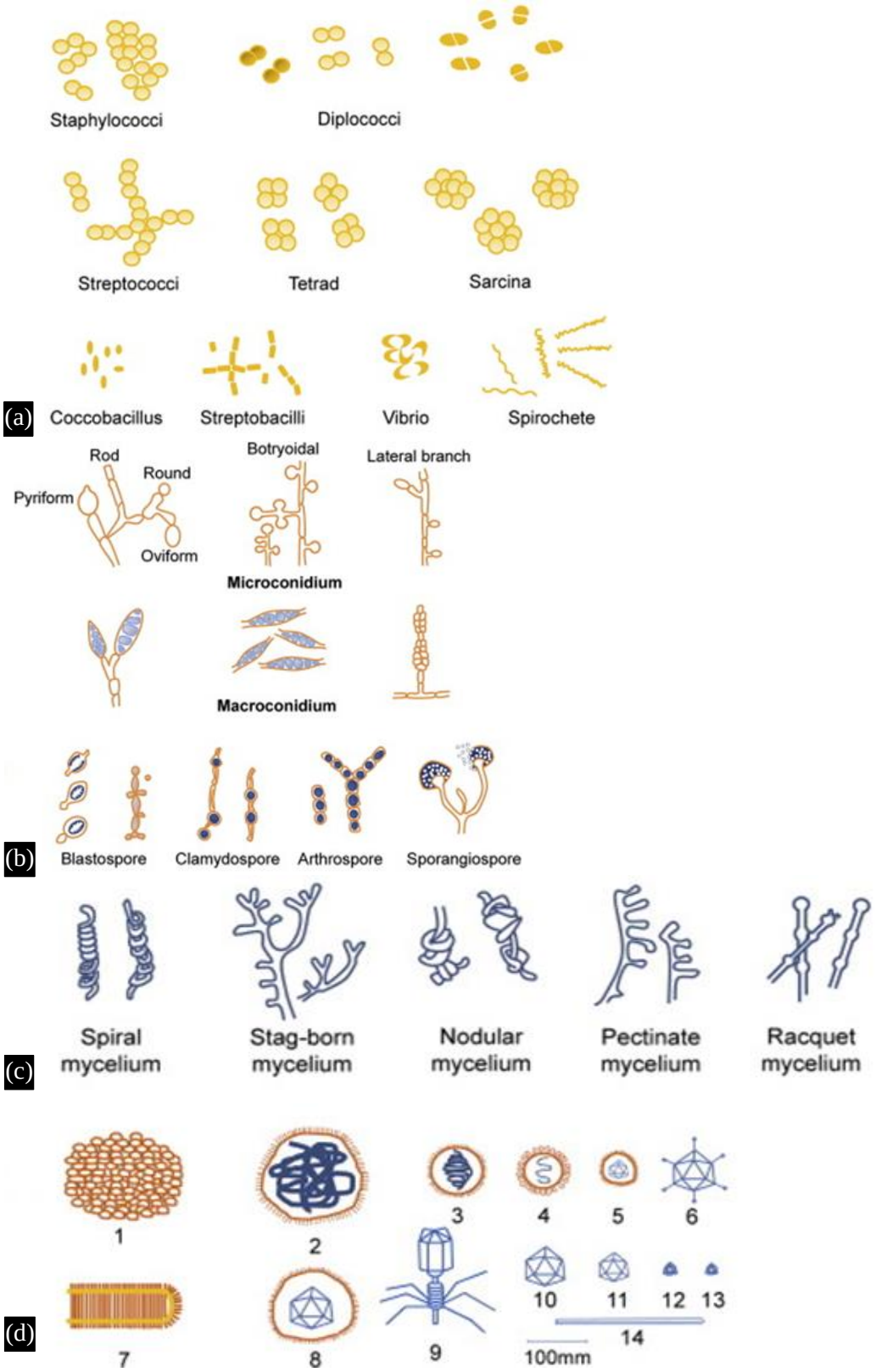

Figure 6. Microbial morphology. (a) Basic shape of bacteria. (b) Fungal spores. (c) Fungal hyphae. (d) Morphology and structure of viruses: 1. Poxvirus, 2. Paramyxovirus, 3. Orthomyxovirus, 4. Coronavirus, 5. Togaviridae, 6. Adenovirus, 7. Bullet-shaped virus, 8. Herpes virus, 9. T2 bacteriophage, 10. Reovirus, 11. Papovavirus, 12. Picornavirus, 13. Picodnavirus, 14. Tobacco mosaic virus [28].

Figure 7. Fungal metabolomics workflows typically involve several steps: serial inoculation of cultures, sample collection and homogenization, metabolite extraction using a solvent, and subsequent solvent evaporation before analysis. In contrast, the microscale workflow simplifies the process by enabling arrayed inoculation and on-chip metabolite extraction, eliminating the need for culture collection and homogenization. This micrometabolomics platform not only streamlines the procedure but also significantly reduces solvent usage by approximately 1000 times, thereby decreasing evaporation time and speeding up the workflow [29].

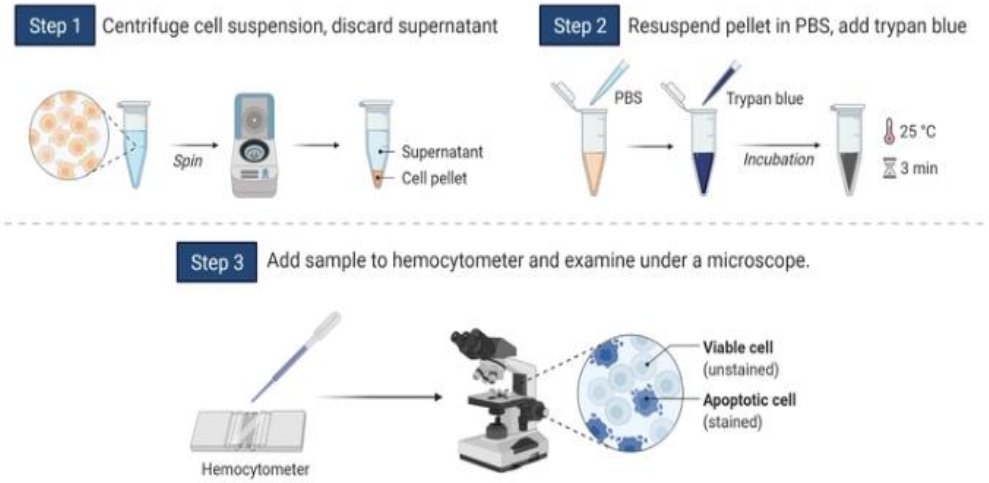

Figure 8. Trypan blue dye exclusion assay Bioquest. 2024. Available at https://www.aatbio.com/catalog/cell-viability-assays].

RESULTS AND DISCUSSIONS

The investigation attached to antimicrobial action of three monoterpenes: linalyl acetate, (+)menthol, and thymol, against gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria. The results presented in Table 1 indicate that all three monoterpenes exhibit inhibitory effects on the growth of both bacterial strains. However, *S. aureus* appears to be more susceptible to the compounds compared to *E. coli*. The MICs reveal that thymol and, to a slightly lesser extent, (+)menthol, exhibit higher toxicity against *S. aureus* compared to linalyl acetate. Conversely, (+)menthol demonstrates the highest toxicity against *E. coli* [15].

Kifer et al. (2016) reported a higher antimicrobial activity of thymol compared to menthol against Staphylococcus, as indicated by the MIC [30]. Badawy et al. (2019) found MIC values of 400 mg/l for menthol and 135 mg/l for thymol against *S. aureus*. Karpanen et al. (2008) confirmed thymol's antimicrobial activity against *S. epidermidis*, while Walsh et al. (2019) reported a 25.67% inhibition ratio of *S. epidermidis* by thymol [31, 32].

The antimicrobial action of menthol and thymol is attributed to their ability to alter the permeability of cytoplasmic membranes, causing leakage of intracellular materials and bacterial cell elimination (Kifer et al., 2016) [30]. Trombetta et al. (2005) demonstrated that terpenes and phenols like thymol and menthol can bind to lipids in the plasma membrane, changing membrane porosity due to their lipophilic nature and water solubility, allowing them to penetrate the plasma membrane and bind to it. This contributes to their activity in inhibiting bacterial growth, as they contain hydroxyl groups that disrupt oxidative phosphorylation [15].

Kwon et al. (2019) noted thymol's inhibitory effect on environmental and clinical *S. aureus* with an MIC of 512 mg/ml. Cristani et al. (2005) [15] suggested that the activity of thymol and menthol is related to their ability to enter the cell and interact with intracellular components. The activity of monoterpene compounds to their ability to disrupt the cell wall, increase plasma membrane permeability, inhibit respiration, alter ion transfer, reduce proton motive force, and decrease ATP synthesis [16].

Gulluce et al. conducted a study to assess the antioxidant activities of both the EO and methanol extract of *M. Longifolia* [33]. Their findings showed that the methanol extract exhibited superior antioxidant activity compared to the EO, particularly in assays, such as the inhibition of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the β-carotene/linoleic acid systems. Other studies have attributed the better antioxidant effect of methanol extract to its higher content of phenolic compounds, which are known to correlate positively with antioxidant activity. The IC50 value for *M. longifolia*, determined by the ABTS assay, was found to be 476.3 ± 11.7. Apigenin derivatives have also been identified as antioxidative molecules in some studies. In another investigation, the methanolic extracts of *M. longifolia* demonstrated significant antioxidant activity (79%) as determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity assay. Berselli et al. demonstrated the efficacy of a 1-hour preincubation with a characterized *M. longifolia* extract in protecting human keratinocytes from chemically induced oxidative stress. Additionally, a mixture of extracts from four plants, including *M. longifolia*, showed noteworthy antioxidant properties at low concentrations. Monoterpene ketones, specifically menthone and isomenthone, were identified as the most potent scavenging complexes present in the EOs of *M. longifolia* in another study. The antioxidant effect of the plant varies based on the extraction method used. For instance, extracts from naturally dried herbs exhibited the highest antioxidant activity due to their higher phenol and flavonoid content, while extracts from herbs dried in a laboratory oven showed lower antioxidant activity. Hydro-alcoholic extracts of *M. longifolia* described good antioxidant actions in many in vitro assay methodologies, including DPPH radical-scavenging, nitric oxide-scavenging, Fe^{2+} chelating ability, linoleic acid model, and scavenging hydrogen peroxide. In yet another study, *M. longifolia* extract exhibited the strongest activity as an ABTS scavenger compared to other assays. The high-antioxidant activity observed in tissues of many *M. longifolia* variants native to Israel was attributed in part to the presence of rosmarinic acid (RA). In conclusion, these findings show the diverse antioxidant actions of *M. longifolia* and underscore the importance of the extraction method in determining its antioxidant activity [20].

Oliveira et al. investigated the EO from *Mentha pulegium* collected during different seasons and found significant variations in its composition. In the spring, they detected 6.62% borneol and 61.43% pulegone, while in the winter, they found 8.87% borneol and 28.40% pulegone [10]. This study evaluated the effect of seasonality, with a 54% inhibition in the concentration of pulegone during the winter season. Pulegone works as the preactivator for the synthesis of stereoisomers of menthone, leading to an inhibition in the pulegone quantity of the EOs. These stereoisomers synthesize acetylated menthyl, neoisomenthyl, and isomenthyl compounds. The concentration of pulegone (61.43%) noticed in the spring aligns with the results of the present work.

Ait-Ouazzou et al. also observed pulegone as the principal constituent (69.8%) in the EO from *M. pulegium*, consecutively by piperitenone (3.1%), isopulegone (1.8%), and cis-piperitone epoxide

(1.7%) [12]. They consistently researched pulegone in all their works on the EOs from *M. pulegium*. Difference in the concentration of EOs can be added to factors such as area, period of collection, and natural stress. Therefore, it's common to observe differences in the concentrations of constituents when working with EOs [21–25].

CONCLUSION

The study's findings underscore the promising potential of *M. Piperita* EO due to its diverse range of chemical compounds exhibiting antioxidant, antimicrobial, and cytotoxic activities. This information aligns with the traditional use of this species by communities and in literature. Moreover, considering the reasonable and beneficial use of these plants for preventing and treating various human diseases, the research contributes to leveraging and incorporating products from Brazilian biodiversity. This is particularly significant for isolated regions where primary care health centers may lack access to certain drugs.

In conclusion, menthol offers significant potential as a natural preservative, thanks to its broad-spectrum antimicrobial properties derived from *M. piperita* (peppermint). Its effectiveness against bacteria and fungi makes it a promising candidate for various industries, including food, cosmetics, and pharmaceuticals.

The use of menthol as a natural preservative provides several advantages, including its natural origin, broad antimicrobial spectrum, and relatively low toxicity compared to synthetic alternatives. Moreover, it aligns with consumer preferences for clean-label and natural products, contributing to sustainability efforts and reducing reliance on synthetic chemicals. Nevertheless, further research is necessary to optimize its application, including determining the most effective concentration, compatibility with different product formulations, and its stability over time. Additionally, addressing regulatory considerations and ensuring consumer acceptance are important factors when incorporating menthol into products as a preservative. Overall, menthol holds promise as a natural preservative, offering a sustainable and effective alternative to synthetic options while leveraging the beneficial properties of *M. piperita*. Its utilization can contribute to the development of safer and more environmentally friendly products across various industries.

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