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Review

Membrane Disruption Properties of Essential Oils—A Double-Edged Sword?

Polly Soo Xi Yap [1,2], Khatijah Yusoff [3], Swee-Hua Erin Lim [4], Chou-Min Chong [1,*] and Kok-Song Lai [4,*]

1 Aquatic Animal Health and Therapeutics Laboratory, Institute of Bioscience, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia; polly.yap@monash.edu
2 Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, Darul Ehsan 47500, Selangor, Malaysia
3 Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia; kyusoff@upm.edu.my
4 Health Sciences Division, Abu Dhabi Women's College, Higher Colleges of Technology, Abu Dhabi 41012, United Arab Emirates; lerin@hct.ac.ae
* Correspondence: choumin@upm.edu.my (C.-M.C.); lkoksong@hct.ac.ae (K.-S.L.)

Abstract: The emerging literature has suggested essential oils (EOs) as new possible weapons to fight antimicrobial resistance due to their inherent antimicrobial properties. However, the potential pharmaceutical use of EOs is confronted by several limitations, including being non-specific in terms of drug targeting, possessing a high cytotoxicity as well as posing a high risk for causing skin irritation. Furthermore, some EOs have been demonstrated to adversely affect the cellular lipid profiles and permeability of the cell membrane, which may result in undesirable outcomes for the cells. Nevertheless, owing to their naturally complex compositions, EOs still hold undiscovered potential to mitigate antimicrobial resistance, as an alternative to existing antibiotics. To address the issue of overuse in antibiotics for crops which have led to the growing threat of antimicrobial resistance globally, EOs have also been proposed as potential biopesticides. Since the perceived advantages of antimicrobial attributes in EOs remain largely unexplored, this review aims to provide a discourse into its current practical usefulness in the agricultural setting. Finally, updated bioengineering techniques with emphasis of the biopesticide potential of EOs as a means to alleviate antimicrobial resistance will be included.

Keywords: essential oils; antimicrobial; cell membrane; membrane permeability; biopesticides; encapsulation; polymeric nanoparticle

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1. Introduction

Essential oils (EOs) are highly concentrated and complex mixtures of chemical components produced by aromatic plants in the form of secondary metabolites. EOs are usually obtained by various extraction methods, such as steam distillation, hydro-distillation and supercritical carbon dioxide [1]. EO components are typically volatile and hydrophobic; these attributes are representative of the unique physiochemical properties for a particular EO as a key marker of the oil quality and its characteristics. Various in vitro and in vivo studies have documented the biological benefits of EOs and their active compounds, such as possessing antimicrobial, antioxidant, anti-tumour and anti-inflammatory properties [2,3]. In addition to the rapid emergence of the antibiotic resistance crisis worldwide, there is a clear urgency for the development of new antimicrobial compounds; EOs have also been favourably viewed in this regard due to their promising antimicrobial properties and therapeutic potential [4,5]. The mechanisms of action of the EOs and/or their components against bacteria have also been widely explored. O'Bryan et al. (2015) [6] compiled a wealth of information on various antibacterial targets of EOs against the bacterial cell; these included the bacterial cell wall or membrane [7], cellular respiration processes [8,9] and quorum sensing mechanisms [10,11].

Some compounds in EOs have been observed to be toxic to the cells. In fact, among the major problems encountered in the pharmaceutical applications of EOs is their toxicity to mammalian cells, despite this occurrence being documented relatively poorly. Evidence has shown that the lipophilic character of EO compounds and interactions with hydrophobic parts of the cell play a crucial role in the mechanism of toxicity. Thus, experimental data on the toxic effects of the lipophilic EOs on microorganisms in general will be valuable in postulating a general toxicity pathway on other biological membranes in the system.

In this review, the dual nature of EOs, particularly of the antibacterial versus biological membrane toxicity of EOs, will be elucidated. A practical outlook of the antimicrobial applications of EOs and their safety concerns as a complement to the growing literature on the mechanism of action of EOs and their components will be presented. Furthermore, the aspect of bioavailability of the usually poorly soluble hydrophobic compounds with respect to the in situ application of bioengineering approaches will be included.

2. Membrane Disruption Activities of EOs

While diverse antimicrobial mechanisms have been described, the bacterial cell wall and membrane have frequently been reported to be the first target of EOs [7,10,12,13]. Toxic effects on membrane integrity and function are generally used to explain the antibacterial activity of EOs, as attributed by the hydrophobic nature of EOs [6]. Studies have indicated that the mechanism of action of the EOs is not isolated but connected to a series of events that involve both external envelopes of the cell and the cytoplasm [12]. Both Gram-positive and Gram-negative bacteria possess peptidoglycans in their cell wall, which are essential for maintaining the cell shape and providing structural integrity. The bacterial plasma membrane is composed of a phospholipid bilayer and plays a general role in cellular function as a permeability barrier for molecules in and out of the cell. Unlike the Gram-positive bacteria, the Gram-negative bacteria possess a lipid-rich outer membrane and thin peptidoglycan. Among the major components of the outer membrane of Gram-negative bacteria is the lipopolysaccharide (LPS); the assembly of these lipids and porins forms a selective barrier to protect the bacteria from several antibiotics, detergents and dyes that would otherwise damage the inner membrane. Various studies have demonstrated that the outer membrane of Gram-negative bacteria is an effective and evolving antibiotic barrier [14,15] (Figure 1), which makes the outer membrane-targeting antibiotic, colistin, inevitably the treatment of last resort in response to infections caused by extensively multidrug-resistant Gram-negative pathogens. EOs, however, provide a promising alternative as they are not only limited to a certain class of bacteria, but they have also been shown to have potent effects on both Gram-positive and Gram-negative bacteria.

Quantitative indicators of increased membrane permeability leading to the ultimate loss of cell viability include measurements of potassium leakage [8,17], protein leakage [13], leakage of genetic materials (DNA and RNA) [13,18] and membrane potential [7,10]. High phenolic contents of EOs, such as the presence of carvacrol, eugenol and thymol, were found to be the major drivers for the disruption of the cytoplasmic membrane [19], leading to the passive flux of protons and other ions [20]. *Origanum compactum* EO was found to exert an effect on the membrane integrity of both Gram-positive and Gram-negative bacteria, through the quantification of RNA, DNA and protein released from the cytoplasm [13]. In another study, although finger citron (*Citrus medica L. var. sarcodactylis*) EO generally showed a better bactericidal effect on Gram-positive bacteria than Gram-negative, a similar trend of cell permeability disruption was observed for these two bacteria [21]. This is intriguing because Gram-negative bacteria were reported to be generally more resistant to EOs and other hydrophobic antibiotics compared to Gram-positive bacteria [12,22]. Membrane potential refers to the extracellular and intracellular potential difference of the bacteria, and it plays a crucial role in bacterial metabolism. The decrease in membrane potential may be attributed to the structural damage of the cell membrane [23]. Measuring the bacterial membrane potential can be achieved by determining the ratio between fluorescence dye intensities inside and outside the cell [23,24]. The membrane potential-

sensitive dye, 3,3′-dipropylthiacarbocyanide iodide (DiSC35) [25–27] and bis-oxonol [9] are commonly used to stain the depolarised bacterial cells after exposure to EOs. Nevertheless, it is worth noting that altered membrane depolarisation may not always lead to cell death, but rather, it can be conditional on the degree of alteration or whether the functionality of the cell was affected. For instance, thymol was found to be able to induce membrane alteration through integration with polar head-groups of the lipid bilayer; however, at low concentrations, the adapted membrane lipid compositions can still regulate and maintain membrane fluidity and function [12,28]. A previous study has suggested that the alteration of zeta potential could be correlated to increased membrane permeability and eventually be linked to decreased cell viability [29]. Other studies have also reported on an altered zeta potential in *Escherichia coli* after exposure to lavender EO [10], cinnamon bark EO [11], citronellal, carveol and carvone [30]; these have been substantiated by other membrane disruption tests, including potassium leakage assay and SEM observation. Additionally, Yang et al. [7] reported reduced zeta potential on carbapenemase producing *Klebsiella pneumoniae* after treatment with lavender EO alone and lavender EO in combination with meropenem. Zeta potential measurement showed that lavender EO increased the overall surface charge of the bacterial cells, which is associated to the loss of LPS [7].

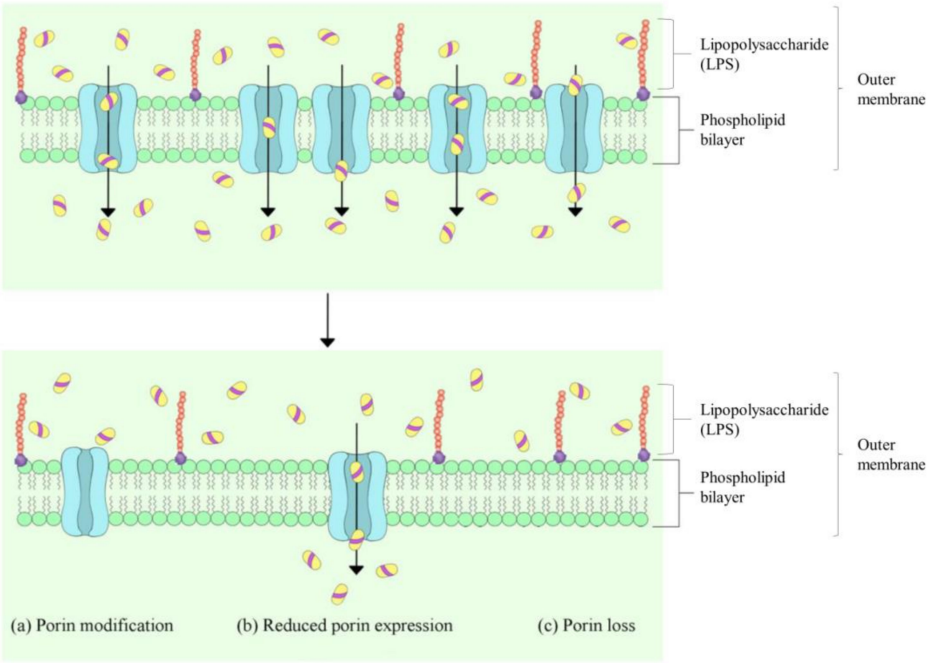

Figure 1. Generalised mechanisms for reduced porin-mediated outer membrane permeability in Gram-negative bacteria. Adapted from Yang et al. (2018) [16].

EOs contain many highly conjugated cyclic hydrocarbons, such as aromatic rings and terpenes; these structures are toxic to microorganisms [6,31]. The accumulation of hydrocarbon molecules results in the swelling of the membrane bilayer, leading to cellular content leakage as a direct effect [20], and can also be qualitatively assessed by electron microscopy. Scanning electron microscopy (SEM) enables the observation on the ultrastructure of the microorganism, while transmission electron microscopy (TEM) reveals the plasma membranous structures of a cell. Burt and Reinders (2003) demonstrated morphological alterations and loss of contents of *Escherichia coli* O157:H7 cells by SEM observation following the treatment of oregano EO, which is rich in carvacrol and thymol [32]. Tyagi and Malik (2012) studied the morphological and ultrastructural alterations of lemon grass oil and its vapour against *E. coli* and showed that the oil-treated cells appeared aggregated, shrunken and partially deformed under SEM observation, whereas TEM photomicrographs revealed uneven cell wall thickness as compared to the control [33]. Lemon grass vapour-treated

cells showed the most extensive internal damage and abnormalities compared to lemon grass in liquid phase under TEM inspection. Subsequent atomic force microscopy (AFM) analysis confirmed that the effect of volatile phase of this EO on the bacterial cell wall and/or membrane was more prominent than its liquid phase [33]. Such an observation could be attributed to a more efficient partition into membrane structures of bacteria in the gaseous phase, thereby requiring lower effective bactericidal concentrations to minimise the effects on organoleptic properties [34]. Di Pasqua et al. (2007) evaluated the structure and lipid profile alterations of both Gram-positive and Gram-negative bacteria after exposure to various EO constituents by fatty acid analysis and SEM examination [35]. Although the study showed varying levels of membrane disruption of tested compounds on different bacteria, the mechanism of action indicates a direct interference on the membrane lipid biosynthesis pathway [35]. Similar ultrastructural alterations were also observed through the accumulation and increase in electron density of lipid droplets in EO-treated parasitic cells via TEM [36,37].

3. Toxicity of EOs and Components on the Biological Membranes

The hydrophobicity of EOs and/or their components enables the hydrophobic molecules to preferentially reside in the lipids of the cell membrane and mitochondria, disrupting the structure and rendering cell permeability to be compromised, eventually leading to the leakage of cellular contents [20]. Emerging evidence has revealed that EO antimicrobial activities are attributed to the incorporation of exogenous fatty acids (the EOs) in altering the cell membrane's phospholipid profile [12,38,39]. While phospholipids are the common membrane constituents found in bacteria, plants and mammals, this commonality, however, raises concerns that this particular EO mechanism may be rendered unspecific, as EOs could also exert the same type of toxic effects on any biological membranes.

Lavender (*Lavandula angustifolia*) EO has been demonstrated to work synergistically with meropenem against the carbapenemase-producing *K. pneumoniae* at a minimal inhibitory concentration (MIC) of 0.63% *v/v* [7]. The effective dose may be high, considering that lavender EO was also found to be cytotoxic to human endothelial and fibroblast cells in vitro at a concentration of 0.25% *v/v*, in which membrane toxicity was proposed as the potential mechanism [40]. Furthermore, *L. angustifolia* EO also exhibited considerable cytotoxic effects on the normal human lung fibroblast cell line, at an IC50 of 75 µg/mL [41]. Despite these cytotoxicity activities, studies have found that the direct application of lavender EO on human skin and inhalation requires 100% of its concentration for pain relief [42,43]. Studies on the membrane toxicity of EO should not be limited only to in vitro cell culture. Instead, more wholesome information can be obtained from in vivo or even scaffolded lab-manufactured tissues. Freshly excised human full-thickness abdominal skin has been used to explore the potential use of *Backhousia citriodora* EO for topical application, by applying histopathological assessment to evaluate its epidermal cell damage [44]. High toxicological potential owing to the presence of monoterpenes as membrane permeability agents has also been observed in dermatological testing [45]. EOs including tea tree, eucalyptus, clove and sage, whereby the main constituents of these consist of terpenoids, are known for their skin irritation properties and stimulating allergic reactions [46,47]. While it is evident that certain EOs exert cell membrane disruption activities, with the growing literature on their potential biological applications, only a few studies have focused on the EOs' effects with respect to both target and non-target organisms. Table 1 summarises EOs and the components which have been previously shown to demonstrate membrane toxicity. Future studies need to focus on the in situ testing of the EO membrane toxicity on the non-target organism in the biological system of application. For instance, freshwater fish has been tested as a non-target organism alongside testing the mosquito larvicide potential of the EO of *Zanthoxylum monophyllum* leaf [48].

Table 1. Essential oils (Eos) and components with membrane toxicity.

EOs/Component	Mechanism	References
Thymol	Alteration of membrane fatty acids profiles. Perturbation of the lipid fractions in plasma membrane. Perturbation of the membrane lipid fractions. Reduction in the intracellular ATP.	[35] [49] [50] [51]
Eugenol	Alteration of membrane fatty acids profiles.	[35]
Carvacrol	Reducing membrane potential. Alteration of membrane fatty acids profiles. Reduction in the intracellular ATP. Perturbation of the membrane lipid fractions.	[26] [35] [51] [50]
Cymene	Destabilisation of cell membrane. Reduction in membrane potential. Perturbation of the membrane lipid fractions.	[26] [50]
Limonene Cinnamaldehyde	Alteration of membrane fatty acids profiles.	[35]
γ-terpinene	Perturbation of the membrane lipid fractions.	[50]
Menthol Linalyl acetate	Perturbation of the lipid fractions in plasma membrane.	[49]
Farnesol Nerolidol Plaunotol	Membrane disruption leading to potassium ions leakage.	[52]
Lavandula angustifolia	Induction of oxidative stress on outer membrane.	[7]
Amomum villosum Lour	Interference in bacterial hydrophobicity, protein synthesis and biofilm formation.	[18]
Citrus medica L. var. sarcodactylis	Alteration of membrane morphology.	[21]
Robinia pseudoacacia	Increased release of the extracellular ATP.	[53]
Cinnamomum verum J. Presl	Induction of oxidative stress on outer membrane.	[54]
Origanum compactum	Disruption of cell membrane integrity.	[13]
Enteromorpha linza L.	Enzymatic degradation of bacterial intracellular enzymes. Deposition in the cytosol.	[55]
Melaleuca alternifolia	Induction of potassium ions leakage.	[8]
Origanum vulgare L. *Rosmarinus officinalis L.*	Alteration of cell wall structure.	[56]

Within the framework of antimicrobial discovery, EOs have been viewed as promising antibiotic alternatives because the complex mixture of the oil constituents suggests various antimicrobial activities, which may help in preventing the development of resistance after repeated antibiotic exposure. Nevertheless, for drug development, target identification is crucial to reduce the probability of late-stage attrition. This is because non-specific actions of the drug molecules would be compounded into pronounced toxicity, creating more challenges in follow-up studies [57]. While some approaches may utilise the isolated EO compound as a test candidate, direct interaction with a single target, however, may lead to a presumptive conclusion that the phenotypic changes are attributed by a single compound. This hypothesis may be oversimplified. This is because the EO-induced membrane effects observed in bacterial cells could represent a cascade effect on multiple targets [12]. Furthermore, the chemical composition of EOs is highly volatile, and this subjects various compounds within EOs to natural fluctuations. Factors such as plant condition, growth stage, climate as well as the harvest time could play a role in these seemingly minor, but significant differences [58]. For instance, terpenoids are generally

found to be volatile and thermolabile, thereby enhancing sensitivity to oxidation during processing and storage [59]. These properties add to the challenge for the actual quantitative and qualitative evaluations of the EO composition. It is worth noting that EOs which have been shown to have generalised effects on the bacterial membrane lipids [35] may also interact with other "off-target" lipids, for, e.g., the biological membrane of the non-target organism. Thus, the ensuing challenges are to identify compounds specific to the pathogen, with an acceptable toxicity profile on other biological membranes in the applied biological system and demonstrated safety in humans. Ideally, the compound should have an effective concentration which is high enough to exert bactericidal activity, but low enough at the same time to enable the adaptation of the membranous lipid to the exogenous lipid. Furthermore, EOs that show anti-quorum sensing ability, which are targeting only bacteria, can also be considered. Lavender EO was demonstrated to exhibit a significant anti-quorum sensing effect at 0.01% *v/v* [10], which is a concentration much lower than the reported membrane disruptive and cytotoxic concentrations [7,40]; thus, establishing safe concentrations prior to any applications of EO biologically is imperative.

While the challenges for EOs and/or their constituents in drug discovery remain, perhaps the most attractive aspect of exploiting the antimicrobial potential of EOs is replacing antimicrobial pesticides with biopesticides. The increase overall antibiotic use in crops to curb plant pathogens has further exacerbated the phenomenon of antimicrobial resistance worldwide [60]. Although EOs are generally viewed as safe for the environment, their inevitable impact on non-target organisms, such as beneficial soil microbes, crops and humans, should be carefully considered before largescale introduction. Phytocytotoxicity and cytotoxic activities of EOs associated with the presence of terpenoids as major constituents have been reported, leading to membrane phospholipids inhibiting shoot and root growth [61]. Thus, the phytotoxicity of EOs warrants serious attention, especially when developing products for agricultural and environmental applications. Additionally, other major in situ challenges of EOs as biopesticides include the loss of efficiency on site due to their high volatility nature [62]. This highlights the need for efficient stabilisation processes in delivery, such as encapsulation and nanoencapsulation.

4. Bioengineering Strategies for In Situ Applications of EOs as Biopesticides

Pesticide is a generic term, which can be broadly applied to various chemicals, including insecticides, herbicides and fungicides. Commonly used antibiotics in plant agriculture include oxytetracycline and streptomycin [63]. The misuse and overuse of antimicrobial pesticides have further intensified the crisis of antimicrobial resistance in non-clinical environments. A recent report by Malagon-Rojas et al. (2020) draws attention to the effects of these chemical substances on microorganisms [64]. The extended use of pesticides has caused the development of a cross-resistance phenomenon [65], because the approach of developing chemicals aimed at eradicating all pathogens has introduced rapid microbial evolution under strong selective pressures. The cross-resistant mechanism has been associated with a plasmid-mediated resistance via an unspecific organophosphorous hydrolase, which also degrades various classes of antibiotics, including cephalosporins, aminoglycosides and tetracyclines [66]. Thus, EOs with antimicrobial and insecticidal properties will pose a reduced risk of resistance development in microbes to function as biopesticides in mitigating the public health threat of antimicrobial resistance.

Despite the growing literature on the great prospects for EOs as antimicrobial alternatives, most studies carried out have only focused on screening the efficacy of EOs against one or more target organisms in vitro, and only a few practical results have been devised to deal with the effect of EOs on non-target organisms [62]. In this section, we discuss the potential use of bioengineering strategies to achieve this missing link towards the field applications of EOs as potential antimicrobial pesticide alternatives.

Due to the high volatility of EOs, a high concentration is often required to ensure the final achievement of desired efficiency in the field application. Adversely, a higher concentration also leads to a higher risk of toxicity concerning the non-target organisms.

Thus, ideally, bioengineering solutions should be designed to solve two main issues of EO formulation on site: (1) the low persistence of efficacy towards target organisms, and (2) membrane disruptive activities on non-target biological membranes leading to acute toxicity. It is hoped that biopesticidal compounds may be applied at lower concentrations than those that are acutely toxic to other biological membranes in the system, thereby lowering their negative impacts on the balance of the ecosystem. EO encapsulation techniques have received great attention to address the short half-lives of EOs in the final delivery system. Encapsulation technologies to entrap or coat bioactive agents within a carrier material of choice vary depending on the application, for instance, targeted release in response to external conditions (e.g., pH and temperature) [58,62]. Although a wide range of natural or synthetic polymers has been applied in the encapsulating matrix for food and pharmaceutical applications, relatively few inexpensive and efficient methods have been studied for the application of EOs as biopesticides [62]. Chenni et al. (2020) recently reported on using a solvent-free microwave extraction method for basil oil, followed by encapsulation with maltodextrins/acacia gum as a carrier for direct insecticidal activity [67]. While gum arabic (acacia gum) has been proven to be an effective encapsulating carrier [68], the addition of maltodextrins, which are widely available from maize, wheat and potato starch, offer an effective complement to gum arabic [69], suggesting that this could be a cost-efficient option needed for formulating EOs as biopesticides. Although maltodextrin is widely applied in spray drying technology for the food industry, the encapsulation of the secondary metabolites by this technique should be treated with precaution, as high temperatures may exert a great impact on the physiochemical properties of the bioactive molecules [68]. The introduction of nanoparticle formulation in the development of biopesticides has made the utilisation of EO at low concentrations with the controlled release of biomolecules possible. Lemongrass and clove EOs encapsulated into mesoporous silica nanoparticles as a controlled release formulation was found to be effective against wheat take-all disease. The results indicated that the antifungal effects of both encapsulated EOs increased by up to three times [70]. Similarly, the antifungal activities of these EOs have been indicated to exert membrane disruption effects, but investigations into the interaction between these EO-mesoporous silica composites and fungi, as well as the plant ecological system, remain limited [70]. Nevertheless, silica in the formulation can be broken down into by-products of silicic acid [70]; silicic acid reduces mineral toxicities and acts as a biostimulant for cellular regulation and plant growth [71,72]. Thus, silica may be able to offset the membrane toxicities of EOs on non-target biological membranes.

Recently, polymeric nanoparticle preparation to encapsulate EO for enhanced stability, controlled delivery, bioavailability and efficacy has attracted great attention. Compared to the nanoparticles in the matrix, the polymerisation of the nanoparticles improved the stability by forming a vesicular system in which the bioactive compound is confined to an inner core surrounded by a polymeric membrane [73]. Furthermore, the enhanced controlled-release properties acquired from polymeric nanoparticles favour the amplification of the insecticidal effects of EOs at lower concentrations [74]. Among the agriculture applications, *Zanthoxylum rhoifolium* L. EO encapsulated in poly-ε-caprolactone nanospheres exerted improved efficacy compared to the EO tested in natura against whitefly using tomato as the host plant [74]. In an attempt to develop a hypothesis of the mechanism of action behind the insecticidal activity of this EO, it was found that the synthesis of terpenoids was improved when the plants were infected by this pest [75]. Additionally, a study involving Rosmarinus officinalis EO loaded into poly-ε-caprolactone nanospheres showed enhanced fumigant and contact toxicity against the red flour beetle as compared to the non-formulated EO [76]. It can be concluded that the enhanced biological effects of EO in polymeric nanoencapsulation are attributed to the increased surface area of the nanoparticles and the controlled release of EO. Taban et al. reported the increased herbicidal activity of nanoencapsulated savory EO against amaranth (as a weed) while having a mild effect on tomato (as a crop) as compared to the non-nano and non-crosslinker EO emulsion [77]. While the majority of the biological activities of EOs are still attributed to their membrane permeability ability,

technical and knowledge gaps exist in addressing the non-specific membrane toxicities of the EOs. Vokou and Liotiri simulated the interaction between soil microbes and EOs with soil samples, and found evidence of the biodegradation of EOs being used as a carbon and energy by the soil microorganisms [78]. Nevertheless, actual interactions between EOs and soil microorganisms, especially the beneficial microbes, have yet to be elucidated. Furthermore, very few investigators have documented the duration, frequency as well as the effects of the long-term exposure, bioaccumulation or biomagnification of EOs in terms of ecotoxicology [79].

5. Conclusions

Growing attention on the promising biological activities of EOs has driven research to focus on developing new bioengineering approaches to the stability and bioactivity of these bioactive molecules. With the growing literature of membrane disruption activities in various types of EOs, we raise concerns on the toxicity of EOs regarding their use for application in in situ biological systems, thereby referencing this double-edged sword. The mechanisms of action and effects of sublethal concentrations on target and non-target biological membranes of the organisms should be fully clarified for the better alignment of bioengineering technique development in this field for the near future.

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