ABSTRACT

MOORE, ERIN MADISON. Combined Therapeutic Potential of Spotted Wintergreen and Botanical Oils in Dermatology. (Under the direction of Dr. Slavko Komarnytsky).

The incidence of skin microbial infections is on continued rise, exaggerated by the increase in global antibiotic resistance and spread, that lead to higher medical costs, extended hospital stays, and increased mortality. Discovering new classes of antimicrobial agents to address this problem is a major challenge. The costs and time associated with large antimicrobial screening efforts cause research programs to reduce numbers, frequency, or sample sizes, thus resulting in diminishing throughputs and outcomes. In this study, we used a low-cost Mobile Discovery approach for nondestructive in situ screening and reporting of antimicrobial activity of North Carolina flora and identified spotted wintergreen (*Chimaphila maculata* (L.) Pursh, Ericaceae) as a primary hit. Preclinical testing in skin fibroblast and macrophage cell cultures identified a group of low polarity quinones and phenolic glucosides that are responsible for the antimicrobial and anti-inflammatory properties of the plant. We next reviewed botanical oils used in traditional and cosmetic applications and identified whole spectrum hemp oil (*Cannabis sativa* L., Cannabaceae) as an ideal carrier oil for application of wintergreen-based treatments to the skin. Hemp oil contained polyunsaturated fatty acids (PUFA) in a desired omega-6 to omega-3 ratio of 4:1 (including 20% alpha-linoleic acid, 3% gamma-linoleic acid, and 1% stearidonic acid) that promoted in vitro skin cell metabolism and migration into the damaged area. As whole spectrum hemp oil contains compounds known to interact with the endocannabinoid system such as cannabidiol (CBD), we also developed a classroom simulation case study as a teaching technique to educate students, practitioners, and general public on health promoting and possible over-added toxicity effects of CBD-rich hemp oil in skin care.

Combined Therapeutic Potential of Spotted Wintergreen and Botanical Oils in Dermatology

by
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DEDICATION

I dedicate this work to my parents and grandparents.

BIOGRAPHY

Erin Moore was born on October 3rd, 1997 and grew up in Westfield, NC. She received her Bachelors of Science in Biology at Catawba College in 2018. She then decided to work towards her Master's degree in Food Science at North Carolina State University under the direction of Dr. Slavko Komarnytsky in the Plants for Human Health Institute, Kannapolis, NC and Food, Bioprocessing, and Nutrition Sciences, Raleigh, NC.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: THE BIOACTIVITY AND TOXICTY OF BOTANICAL OILS FOR SKIN CARE

Abstract

Botanical oils are increasingly used in traditional and cosmetic applications, but also as dietary supplements and pharmaceutical-grade enhancers for transdermal drug delivery. The focus of this review is to evaluate the existing knowledge regarding the differential biological and toxicological effects of major bioactive constituents of plant oils, and to correlate them to the compositional changes in their fatty acid profiles. Multiple skin diseases that result in depletion or disturbance of skin lipids may require tailored mixtures of several botanical oils to simultaneously maintain natural skin-barrier function, promote repair and regeneration of wounded tissues, and achieve corrective modulation of immune disorders. Furthermore, as bioactive constituents of botanical oils enter the human body by oral or topical application and often accumulate in measurable blood concentrations, there is a need for monitoring their hazardous effects to reduce the possible over-added toxicity and promote maximal normal tissue sparing.

Introduction

Botanical oils are lipids or fats derived from one or more plant parts and can be broadly classified into fixed (vegetable) and essential (volatile) oils (Johnson, 1961). Four agricultural crops (oil palm, soybeans, rapeseed and sunflower) serve as major sources of fixed oil for nutritional applications (Dyer et al., 2008). These oils are a combination of saturated (no double bonds), monounsaturated (1 double bond), and polyunsaturated (2 or more double bonds) fatty acids of varying carbon chain length, attached to a glycerol molecule. Natural mono- (MUFA) and poly- (PUFA) unsaturated oils contain double bonds in less thermodynamically stable *cis* configuration that is prone to oxidative deterioration. As such, unsaturated oils can be

industrially processed to remove (saturate) double bonds by partial hydrogenation. This process, however, introduces *trans* configuration into the fatty acids that has detrimental health effects (Ascherio, 2006). The degree of fatty acid saturation defines the fluidity, molecular packing, lipoxidative damage, and integrity of cell membranes (Pamplona, 2008).

Essential oils are volatile and aromatic complexes of terpene, terpenoid, or phenylpropene origins that evaporate when exposed to heat. The characteristic flavor and aroma that they impart can directly attract pollinators, repel herbivores, and protect plants from biotic and abiotic environmental stresses (Unsicker et al., 2009). As different plants have evolved in diverse ecogeographical areas and developed unique repellent or bactericidal properties, the chemical constituents of essential oils show a much greater structural variety when compared to fixed oils (Bakkali et al., 2008). A single essential oil typically consists of 20-80 constituents of various concentrations, with 2-4 primary structures that largely define its physiochemical and biological properties (Islam et al., 2016). Terpenes (or hydrocarbons) consist of different number of isoprene units such as monoterpenes (C10), sesquiterpenes (C15), and diterpenes (C20), and represent over 90% of essential oil constituents in most plants (Tongnuanchan and Benjakul, 2014). The remaining components of essential oils are broadly identified within the groups of oxygenated (esters, aldehydes, ketones, alcohols, phenols, and oxides), aromatic, and sulfur-containing compounds (Dhifi et al., 2016).

As complex bioactive constituents in fixed and essential oils coevolved to mediate plant-animal interactions, they likely contain functional, biologically relevant chemical spaces and pharmacophores that were selected to interact with animal and human cell targets (Sharifi-Rad et al., 2017). Botanical oils are widely used to prevent or ameliorate human diseases, especially in the form of topical applications to promote skin health, heal injuries and burns, decrease scarring, improve cosmetic outcomes, reduce social stigmatizing, and promote wellbeing

(Vaughn et al., 2018). Plant oils can rapidly penetrate through the lipid structures of the skin and interact with the cell membrane proteins to induce their conformational modifications (Herman and Herman, 2015). Their unique physiochemical properties are utilized for natural enhancement of skin penetration during transdermal drug delivery (Edris, 2007).

This review therefore summarizes recent evidence on utility of fixed and essential botanical oils for topical skin care, including maintenance of natural skin-barrier function, repair and regeneration of wounded tissues, and modulation of immune skin disorders. As bioactive constituents of fixed and essential oils achieve measurable blood concentrations following the oral absorption, skin penetration, or inhalation routes, there is a need for monitoring hazardous effects of these bioactive and their metabolites to reduce the possible over-added toxicity and promote maximal normal tissue sparing.

Structure and function of the skin

Skin functions to maintain temperature and hydration, while protecting the body from environmental injuries and microbial infections. Damaged skin allows entry of chemical irritants, microorganisms, and allergens that promote and amplify skin inflammatory and immune responses (Chen and DiPietro, 2017).

Skin consists of two major structural layers, the epidermis with embedded sebaceous glands, hair follicles, and sweat glands (epithelium) and the dermis (a mixture of loose and dense connective tissues). While the epidermis consists predominantly of continuously replenished and shed, terminally differentiated keratinocytes, dermis is populated by a variety of cell types including fibroblasts, immune cells, and white adipocytes surrounded by the fibrillar collagen. Basement membrane comprising type IV collagen and laminin separates these two layers and serves as an anchor for dermal papillae and the smooth muscles that control the hair follicle (Watt and Fujiwara, 2011). The epithelial layer is also colonized by neural crest-derived

melanocytes responsible for melanin production (skin color and UV protection) and dendritic immune antigen-presenting cells (monocyte-derived Langerhans and thymus-derived T cells) that respond to injury or infection with the production of proinflammatory cytokines, thus maintaining the innate immune response of the skin (Ohteki and Koyasu, 2001).

Upper epidermis (*stratum corneum*) also contains keratohyalin protein granules and lamellar lipid bodies that prevent water loss. The major proteins of *stratum corneum* are type I (acidic) and type II (basic) keratins, filaggrin (proteolytically cleaved to release the amino acids as a moisturizing factor), loricrin and involucrin (cross-linking factors), and small proline rich proteins (SPRs) (Wickett and Visscher, 2006). Main components of lamellar lipid bodies are ceramides (sphingolipids linked to long-chain fatty acids, 50%), cholesterol (25%), and free fatty acids (cleaved from keratinocyte membrane phospholipids, 15%) that maintain the acidic skin surface pH of 4.0-5.5 and the diversity of skin microbiome (Elias and Choi, 2005). Cosmetic-grade glycerin (a natural component of triglyceride lipids) and petroleum-distilled mineral oil (Baby Oil as branded by Johnson & Johnson) or petrolatum (Vaseline as branded by Unilever) are effective skin-conditioning agents that increase hydration and improve elasticity of the epidermis (Rawlings and Lombard, 2012).

The dermis contains stromal cells, with fibroblasts making up the major cell type. Structural cells of the peripheral nervous, immune, vascular, and lymph systems either reside or temporarily migrate through the skin (Rognoni and Watt, 2018). Upper papillary (proliferative) and lower reticular (secretory) dermal layers are separated by the vascular plexus. Fibroblast residing in each layer are epigenetically modified to either proliferate or secrete extracellular matrix (ECM) (Collins et al., 2011). Type I, III and V collagens are the most abundant fibrillary ECM proteins. Additional fibril-associated collagens connect collagen I and III fibrils with decorin and perlecan proteoglycans (Reed and Iozzo, 2002). The elastic fiber network and

glycosaminoglycans such as hyaluronic acid allow for functional interaction and capture of water to generate osmotic pressure responsible for skin turgor (Juhlin, 1997). As the dermis is critical in maintenance of healthy and repair of wounded skin by means of functional and nutritional support of the epidermis (Waller and Maibach, 2006), it often serves as a primary target for therapeutic and cosmetic interventions targeting collagen and elastin production, or cellular responses within the dermal tissue (Badenhorst et al., 2014).

Lipids of healthy skin

Skin surface lipids derived from the epidermis and sebaceous glands are found in decreasing order on scalp > face > back > chest > abdomen > arms > legs > palms and soles. The latter do not contain sebaceous glands but receive small amounts of carryover lipids from other areas of the body (Downing and Strauss, 1974). Human sebaceous glands are a unique source of wax esters and squalene (**Table 1.1**). The composition of human skin lipids also differs from that of other mammals by higher content of triacylglycerols and free fatty acids (Cheng and Russell, 2004). Fatty acids naturally present in human *stratum corneum* are mostly saturated docosanoic acid 22:0, lignoceric acid 24:0, and hexacosanoic acid 26:0 that are often branched, methylated, and/or hydroxylated, although smaller quantities of oleic acid 18:1(n-9) and linoleic acid 18:2(n-6) have been also reported (Bouwstra and Honeywell-Nguyen, 2002; Vicanová et al., 1997). The perceived oiliness of the skin, however, does not depend on total surface lipids nor the proportion of free fatty acids, but rather correlates with the larger ratios of unsaturated fatty acids and wax esters in the sebum.

As the most abundant saturated fatty acid in human sebum, palmitic acid 16:0 is metabolized by both fatty acid desaturase 2 (FADS2) and stearoyl-CoA (SCD) to sapienic acid 16:1(n-10) and sebaleic acid 18:2(n-10). Desaturation of stearic acid 18:0 by the SCD enzyme also results in accumulation of oleic acid 18:1(n-9) in human sebum (Park et al., 2016). Small

amounts of two essential fatty acids, linoleic acid 18:2(n-6) and α-linolenic acid 18:3(n-3), as well as a conditionally essential arachidonic acid 20:4(n-6) that becomes essential if a deficiency in linoleic acid develops, are also found in human sebum (**Table 1.2**).

The extracutaneous traffic of lipids into the epidermis plays a significant role in permeability barrier formation. Dietary fatty acids (Reynolds et al., 1978), sterols (Bhattacharyya et al., 1983), and glucosylceramides (Tsuji et al., 2006) traffic through the extracutaneous tissues to contribute to the epidermal lipid pool. Antimicrobial lauric acid 12:0 and sapienic acid 16:1(n-10), antifungal caprylic acid 8:0 and capric acid 10:0, and antioxidant vitamin E are delivered to the skin surface to naturally reduce oxidative damage and provide basic antimicrobial defenses (Fischer et al., 2014). The epidermis also lacks d6 and d5 desaturase activity and imports arachidonic acid 20:4(n-6) from the extra epidermal sites (Chapkin and Ziboh, 1984). Finally, essential fatty acids critical for the efficient structure and function of the skin must be also delivered from the diet and incorporated into ceramides (Kendall et al., 2017) (**Table 1.3**). Deficiency of essential fatty acids leads to scaliness of the skin and an increased water consumption, mainly due to disruption of the water permeability barrier and an increase in trans-epidermal water loss (Basnayake and Sinclair, 1956). Linoleic acid 18:2(n-6) is also selectively targeted for β-oxidation by the sebaceous cells as a unique energy source for their function (Pappas et al., 2002), while application of nicotinamide (Tanno et al., 2000) and L-lactic acid (Rawlings et al., 1996) produce similar effects.

Lipids in skin diseases and wound healing

External damage by physical (mechanical injury, UV-irradiation, heat, excessive moisture, pressure, or friction), chemical (solvents, irritants, or allergens) or microbial assaults (bacteria, fungi, or viruses) results in injuries in the form of wounds, burns, calluses, or scars. Dry, cracked, or fissured skin is often presented with major changes in its lipid profile, resulting

in excessive water loss and direct exposure to allergens and microbes that further irritate and inflame skin.

Injured skin heals in four overlapping stages that include hemostasis (blood clot formation), inflammation (infiltration of immune cells), proliferation (angiogenesis, granulation, epithelialization, and extracellular matrix (ECM) remodeling by proliferating and migrating fibroblasts and keratinocytes), and maturation (wound contraction and resolution of inflammation) of skin layers. When these processes are disturbed due to an underlying genetic or clinical disorder, the healing pathology results in an ulcerative chronic wound, a hypertrophic scar, or a keloid (Eming et al., 2014). Proinflammatory cytokines and lipid mediators synthesized and released by neutrophils at the site of the wound must be tightly regulated and resolved, otherwise leading to persistent inflammation and uncontrolled proliferation and collagen secretion by skin cells. This pathology directly interferes with contraction of the wound that comprises of proliferation and migration of keratinocytes into the wounded area, and differentiation of fibroblasts into myofibroblasts (Leoni et al., 2015). While some organisms are fully capable of repairing and regenerating injured tissues, in humans this process occurs only in fetal skin and is partially preserved in the gut epithelium and hematopoietic system (Lorenz et al., 1992).

Direct supplementation or replacement of skin lipids can be explored in the prevention or treatment of skin pathologies. Lipid-based barrier repair creams like EpiCeram® by Promius Pharma (ceramides, cholesterol, and free fatty acids, 3:1:1), Lipobase by Astellas Pharma (sorbitan oleate, carnauba wax, ceramide 3, oleic acid, palmitic acid, and cholesterol), CeraVe® by L'Oréal (ceramides 6II, 3, 1, phytosphingosine, hyaluronic acid), Triceram® by Dentaurum (lanolin, ceramides, soybean sterol, linoleic acid, hyaluronic acid), Atopiclair™ by Alliance Pharma (glycyrrhetinic acid 2%, hyaluronic acid, grapevine extract, telmesteine, shea butter),

and MimyX™ by Stiefel Laboratories (a cannabinoid agonist N-palmitoylethanolamine, olive oil, palm glycerides, vegetable oil, squalene) have been cleared for marketing by the Food and Drug Administration (FDA) as 510(k) medical devices with no defined active ingredients. The synthetic sebum mixture consisting of 45% triglycerides, 25% wax monoesters (jojoba oil), 17% fatty acids, and 12% squalene has been also proposed (Wertz, 2009). Additional mixtures of unsaturated fatty acids that modulate skin proliferative and immune responses may also promote wound closure by direct effects on skin inflammation and permeability (Cardoso et al., 2004).

Majority of skin diseases also present with variation or depletion of the major skin lipids as reported for atopic dermatitis (reduced ceramides and C20-26 fatty acids), psoriasis (reduced ceramides), type 2 Gaucher disease (increased glucosylceramides), acne vulgaris (reduced sphingolipids), atopic eczema (increased short-chain ceramides), and aged dry skin (reduced ceramides) (Sahle et al., 2015). Pathological microbial infection of the skin also alters skin lipid profiles as shown for *Propionibacterium* infections observed in acne (Saint-Leger et al., 1986) and *Pityrosporum* folliculitis infections associated with seborrheic dermatitis (Bergbrant et al., 1991). Stress and other physiological factors often exacerbate skin conditions and healing processes by changes in neurohormone and steroid hormone levels that directly affect blood flow, metabolic and immune status of the skin, and function of hair follicles (Hunter et al., 2015).

Botanical oils for topical skin care

Inexpensive and readily available botanical oils are routinely used for topical skin applications. They may enhance skin function by forming a physical barrier, supplying fatty acids to different skin layers, activating peroxisome proliferator-activated receptor-α (PPAR-α) signaling, or decreasing cutaneous inflammation (De Luca and Valacchi, 2010). Chemical diversity found in botanical oils leads to a variety of pharmacological activities and modes of

action depending on quantities and proportions of individual chemical constituents in these complex mixtures. In general, it appears high linoleic acid 18:2(n-6) containing botanical oils (i.e., sunflower) are more beneficial to skin health (Hanley et al., 1998) when compared to the high oleic acid 18:1(n-9) counterparts (Jiang et al., 2000). Therefore, different ratios of individual fatty acids present in botanical oils often result in opposite, either beneficial or detrimental, effects on epidermal barrier function and comedogenicity and merit detailed inquiry (Darmstadt et al., 2002).

Saturated fatty acids

Fixed oils of botanical origin (vegetable oils) are complex mixtures of triacylglycerols (fatty acid esters of glycerol) with some minor components such as tocopherols, phytosterols, and polyphenols that are either cold-pressed (virgin oils), solvent extracted from oil-containing plant parts, or mechanically separated from the aqueous phase after crushing. Dietary vegetable oils are also for the most part refined to produce a bland, stable oil for consumption. Saturated fatty acids present in fixed oils do not contain double bonds and are found mostly as white solids under normal conditions.

Caprylic acid 8:0 and capric acid 10:0

These saturated fatty acids are found in high quantities in goat milk, but also present as minor constituents of coconut oil and palm kernel oil. Large quantities of these fatty acids are only found in seed oils of several species of the genus *Cuphea*, while capric acid also dominates the fatty acid profile of elm *Ulmus americana* seed oil. Known for their antimicrobial properties (Valipe et al., 2011), both molecules play predominantly formulation roles in cosmetic applications by decreasing the melting point, lowering viscosity, providing efficient solvent, oxidative resistance, emollient, and conditioning properties to skin products (Chaudhuri and Bojanowski, 2017).

Lauric acid 12:0 and myristic acid 14:0

Lauric acid 12:0, as a component of triglycerides, is a major fatty acid present in coconut, palm, laurel, babassu, murumuru, and ucuhuba oil and butter. Lauric acid 12:0 reacted with sodium hydroxide produces laurate salts that form the basis for soap production. Among these plant oils, laurel fruit oil is unique due to its low saturation ratio (42-45%) compared to the other oils in this group (80-90%). Additionally, nutmeg and ucuhuba butters have a unique saturated fatty acid profile that is dominated by myristic acid 14:0 (**Table 1.4**). Both lauric acid 12:0 and myristic acid 14:0 showed a moderate degree of bacteriostatic properties, with the former one also being bactericidal with a minimal bactericidal concentration (MBC) range of 7-375 µg/ml (Fischer et al., 2012). Lauric acid 12:0 is also reaching the skin surface naturally as a part of the outward sebum flow that is dominated by the palmitoleic acid 16:1(n-7) isoforms (Weitkamp et al., 1947), and exerts moderate inhibitory effects on the growth of skin bacteria associated with inflammatory acne at the MIC range of 1-4 µg/ml (Nakatsuji et al., 2009). Among these plant oils, only coconut (Strunk et al., 2018) and palm kernel (Chiabi et al., 2011) oils have been studied clinically for skin care, especially that of neonates. Even though these oils were noted as good emollients that prevent transdermal water loss and increase skin moisture, controversy subsists about other beneficial effects associated with their use. As topically applied botanical oils penetrate largely only into the upper layers of the epidermis (Patzelt et al., 2012), their application occludes the skin surface and leads to break outs on most skin types, other than very dry skin, due to their high comedogenic properties.

Palmitic acid 16:0 and stearic acid 18:0

Palmitic acid 16:0 and stearic acid 18:0 are the most common saturated fatty acid esters in animal fat, often rendered as semisolid tallows used in a variety of traditional foods (shortenings, pemmican), salves, and ointments for irritated and inflamed skin conditions. All

animal tallows are dominated by oleic acid 18:1(n-9) (from 70% in bear to from 26% in sheep fat), followed by palmitic acid 16:0 (from 28% in beef to 7% in bear fat) and stearic acid 18:0 (from 30% in goat to 3% in bear fat). Their saturation ratios vary from 69:29:2 in goat tallow, to 42:46:6 in pig lard, to 12:70:9 in bear tallow. Among botanical oils, palm pulp, coffee beans, sea buckthorn fruits, and cocoa beans contain naturally high amounts of palmitic acid 16:0. Cocoa beans oil also contains high levels of stearic acid 18:0, similar to kokum, sal, mango, and shea butters that is responsible for their semisolid appearance and consistency (**Table 1.4**). In human sebaceous glands, exogenously supplied palmitic acid 16:0 is desaturated at an unusual C6 position to produce sapienic acid 16:1(n-10), and both molecules can undergo elongation to stearic acid 18:0 and sebaleic acid 18:2(n-10), respectively. Both palmitic acid 16:0 and stearic acid 18:0 are preferred over palmitoleic acid 16:1(n-7) and oleic acid 18:1(n-9) for incorporation into wax esters, while linoleic acid 18:2(n-6) is the only fatty acid metabolized into two carbon precursors via β-oxidation in the skin (Pappas et al., 2002). The excess of palmitic acid 16:0 inhibits metabolism of linoleic acid 18:2(n-6) and α-linolenic acid 18:3(n-3) into the respective elongation products dihomo-γ-linolenic acid 20:3(n-6) and eicosatetraenoic acid 20:4(n-3), with the former one being a direct precursor of anti-inflammatory eicosanoids (Park et al., 2016).

Arachidic acid 20:0, behenic acid 22:0, and lignoceric acid 24:0

Long chain saturated fatty acids with carbon chain length over C20 are present in skin in small quantities (**Tables 1.2 and 1.3**). In plants, moderate amounts of arachidic acid 20:0 can be found in rambutan, kusum, cupuacu and peanut oils. Likewise, behenic acid 22:0 can be found in ben (moringa) and peanut oils, while lignoceric acid 24:0, a byproduct of plant lignin biosynthesis, can be found in wood tar and in minor quantities in peanut oil (**Table 1.4**). Despite their low bioavailability, these fatty acids are cholesterol-raising agents in humans (Cater and

Denke, 2001), and therefore are used in topical skin and hair applications mostly for their lubricant and moisturizing properties.

Unsaturated fatty acids

Unsaturated fatty acid esters present themselves as colorless liquids under normal conditions and are classified into monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. They are further distinguished accordingly to the number and location of the double *cis* bonds in relationship to the carboxyl terminus as omega- (n-, delta-) 12, 11, 9, 7, 6, 5 and 3 fats. Two fatty acids, linoleic acid18:2(n-6) and linolenic acid18:3(n-3) are essential and must be obtained from the dietary sources. Additionally, many of plant secondary metabolites found in small amounts in unsaturated botanical oils have complimentary anti-inflammatory, antimicrobial, and antioxidant properties that can be also utilized manage clinical manifestations associated with skin damage or an immune disorder (Elshafie and Camele, 2017).

Oleic 18:1(n-9) and petroselinic 18:1(n-12) acids

Oleic acid 18:1(n-9) is the most common botanical MUFA that is classified as omega-9. It is also the most common fatty acid of human adipose tissues (47-52%), followed by palmitic (22-25%), linoleic (11-13%), stearic (4-8%), palmitoleic (4-8%), and myristic (2-3%) acids (Kokatnur et al., 1979). Oleic acid triglycerides comprise the majority of botanical oils, including carrot seed, pataua, *Camellia* tea seed, papaya seed, marula, hazelnut, moringa, buriti, almond, plum, apricot, and peach kernels, olive, pistachio, canola, macadamia nut, avocado, peanut, mango seed, pecan, neem seed, argan, jatropha, cupuacu, sesame, oat, brazil nut, and rice bran, to name a few (**Table 1.4**). High oleic (70-80%) cultivars of sunflower, safflower, and canola have been also developed as a primary source of dietary vegetable oils. Due to presence of a *cis* double bond, addition of oleic acid 18:1(n-9) to lipid bilayers increases instability and progressive structural loss (Akinshina et al., 2016). As such, oleic acid-based infusions,

adjuvants, micelles, and vesicles are often used to penetrate the epithelium and enhance the topical delivery of drugs (Zakir et al., 2010).

Oleic acid 18:1(n-9) is toxic to keratinocytes when applied directly to the cells, and this effect is decreased in the presence of the functional epithelium barrier of the skin. Mild visible skin irritation and increased traffic of the inflammatory cells, combined with increased IL-1α and other cytokine production, have been also observed (Boelsma et al., 1996). Similarly, almond and avocado oils showed moderate skin and eye irritation when tested as pure oils and 2-10% aqueous emulsions in a subchronic 90-day repeated dose study in rabbit (Guillot et al., 1979). While dietary intake of olive oil has several beneficial effects on metabolic and skin health (Owen et al., 2000), its topical application is less effective due to its sensitizing or irritant effects (Kränke et al., 1997), and can be largely attributed to its polyphenol and squalene content. The beneficial effects of avocado oil on collagen metabolism (Werman et al., 1991), argan oil on cardiovascular health (Monfalouti et al., 2010), and oat lipids on keratinocyte differentiation (Chon et al., 2015) are also likely mediated by secondary bioactive compounds found in these oils.

Petroselinic acid is a naturally occurring positional isomer of oleic acid that belongs to omega-12 fats. Found in parsley (*Petroselinum crispum*), coriander (*Coriandrum sativum*), and geranium (*Geranium sanguineum*) seed oils, it exhibits the disruptive tendencies in the lipid structures of the stratum corneum similar to those of oleic acid (Takeuchi et al., 1998).

Gondoic 20:1(n-9), erucic 22:1(n-9), and nervonic 24:1(n-9) acids

These monounsaturated long chain fatty acids are omega-9 elongation products of oleic acid 18:1(n-9) found in small quantities in some botanical oils. Camelina seed oil is the major source of gondoic acid 20:1(n-9), while jojoba and mustard oils are the major sources of erucic acid 22:1(n-9) and nervonic acid 24:1(n-9) (**Table 1.4**). Similar to oleic acid, these molecules

disrupt skin lipid layers and enhance permeation of drugs (Morimoto et al., 1996). Dietary supplementation with omega-9 fats can be also protective against metabolic risk factors associated with cardiovascular disease (Gillingham et al., 2011). In rare instances when β-oxidation of long chain saturated fatty acids is genetically disrupted, supplementation with long chain monenoic acid fatty acids can be beneficial to slow down demyelination (Sargent et al., 1994). However, dietary supplementation with erucic acid 22:1(n-9) induces alopecia and scaly skin lesions similar to those observed in essential fatty acids deficiency, suggesting that this fatty acid may interfere with dermal metabolism of essential fatty acids (Hulan et al., 1976).

Myristoleic 14:1(n-5), palmitoleic 16:1(n-7) and paullinic 20:1(n-7) acids

These fatty acids are desaturation omega-5 and omega-7 products of the respective saturated fatty acids. Less common in nature, these fatty acids are found in small quantities in butters and animal fats. Among plant sources, nutmeg and saw palmetto oils contain measurable levels of myristoleic acid 14:1(n-5), while sea buckthorn and macadamia nut oils are a good source of palmitoleic acid 16:1(n-7) (**Table 1.4**). Paullinic acid 20:1(n-7) is rather uncommon, but it can be found in large quantities (40%) in guarana *Paullinia elegans* seed oil, alongside another uncommon cis-vaccenic acid 18:1(n-7) (20%) (Spitzer, 1995). While generally regarded as anti-inflammatory, especially in the case of palmitoleic acid 16:1(n-7), their direct application to metabolic and immune health remains unclear (de Souza et al., 2018). Direct effects of palmitoleic-rich sea buckthorn oil (Hwang et al., 2012) or palmitoleic acid 16:1(n-7) alone (Weimann et al., 2018) on wound healing and skin aging was confirmed in the animal models, and sea buckthorn fruit oil (but not seed oil) was shown to improve symptoms of atopic dermatitis following oral supplementation with 5 g oil daily for 4 months to 49 subjects as a part of a placebo-controlled, double-blind clinical study (Yang et al., 1999).

Linoleic acid 18:2(n-6)

Linoleic acid 18:2(n-6) is the most common botanical PUFA that is classified as an essential omega-6 fat. Large quantities of linoleic acid are found in evening primrose, safflower, sunflower, passion fruit seed, poppy seed, grape seed, watermelon seed, walnut, black cumin seed, hemp, raspberry seed, cotton seed, corn, soybean, pumpkin seed, rosehip, black currant seed, borage, cranberry seed, and sea buckthorn seed oils (**Table 1.4**). In humans, it serves as a starting point for biosynthesis of long-chain PUFAs, such as γ-linolenic acid 18:3(n-6), dihomo-γ-linolenic acid 20:3(n-6), and arachidonic acid 20:4(n-6), the latter being the major precursor to prostaglandins, leukotrienes, and endocannabinoids. Linoleic acid 18:2(n-6) is also selectively targeted for β-oxidation in the sebaceous gland to synthesize squalene and wax esters. Low levels of linoleic acid 18:2(n-6) also impair the epidermal barrier function and increase permeability of comedonal wall (Cunliffe et al., 2004). Consequently, sunflower oil high in linoleic acid 18:2(n-6) better preserves lipid integrity, does not cause erythema, and improves skin hydration in contrast to application of olive oil (Danby et al., 2013). However, many randomized controlled trials performed with evening primrose (Bamford et al., 2013) or borage (Foster et al., 2010) oils showed only minor to no beneficial effects on skin health outcomes, suggesting that minor components of linoleic-rich oils or increased proportion of oleic acid in these and other high linoleic oils could be partially responsible for these observations.

In this regard, hemp oil stands out among the linoleic-rich oils by having relatively low oleic 18:1(n-9) and high α-linolenic 18:3(n-3) content. Dietary hempseed oil improved skin dryness, itchiness, and decreased dermal medication use in a 20-week randomized crossover clinical study with atopic dermatitis patients (Callaway et al., 2005). Furthermore, a non-psychotropic phytocannabinoid, cannabidiol, naturally present in hemp oil, showed some

evidence of sebostatic actions like decreasing lipolysis, keratinocyte differentiation, and immune skin cell activation (Oláh et al., 2014).

γ-Linolenic 18:3(n-6), dihomo-γ-linolenic 20:3(n-6), arachidonic 20:4(n-6), and calendic 18:3(n-6) acids

γ-Linolenic acid 18:3(n-6) is found in several botanical oils including borage, black currant, and evening primrose (**Table 1.4**). Although generally considered anti-inflammatory, its clinical effectiveness for rheumatoid arthritis and skin conditions is questionable (Sergeant et al., 2016). Upon absorption, γ-linolenic acid is rapidly converted to dihomo-γ-linolenic acid 20:3(n-6) and stored in cellular glycerolipids of the immune (neutrophils) and skin cells, while it is converted both to dihomo-γ-linolenic acid and pro-inflammatory arachidonic acid 20:4(n-6) in liver and systemic circulation. Combination of γ-linolenic acid supplementation with omega-3 fatty acid such as DHA or EPA, however, seem to preferentially inhibit conversion of γ-linolenic acid to arachidonic acid (Barham et al., 2000) and improve immune outcomes in part by modulating the ratios of series 2 versus series 1 prostaglandins (**Figure 1.1**). This effect can be achieved directly by a combined supplementation of γ-linolenic botanical oils (such as borage) and omega-3 enriched botanical oils such as hemp or echium (Lee et al., 2014). Arachidonic acid is also a major source of oxidized bioactive lipid mediators that stimulate proliferation, migration, and homing of cells in the wound bed and promote early stages of wound healing (Dhall et al., 2015).

Calendic acid 18:3(n-6) is a positional isomer of linoleic acid found in significant quantities in seeds of pot marigold (Dulf et al., 2013). Similar to other conjugated linolenic acids, supplementation with calendic acid improved metabolic risk factors (Chardigny et al., 2003), however its relationship to skin health has not been established.

α-Linolenic 18:3(n-3) and punicic 18:3(n-5) acids

α-Linolenic acid is an essential omega-3 fatty acid found in multiple seed oils, such as chia, perilla, flax, sacha inchi, camelina, sea buckthorn, cranberry, rosehip, black currant, raspberry and hemp (**Table 1.4**). This acid is a minor physiological component of cellular and mitochondrial membranes that regulates cell signaling and transport across the lipid bilayers. Both α-linolenic acid and linoleic acids reduce UV-associated damage and hyperpigmentation of the skin (Barham et al., 2000). Dietary α-linolenic acid can be slowly metabolized into eicosapentaenoic acid 20:5n-3 and docosahexaenoic acid 22:6n-3, however with the efficiency of only a few percent (Burdge and Calder, 2005). The bulk of α-linolenic acid metabolites, however, is used to synthesize anti-inflammatory series of prostaglandins and leukotrienes.

A topical formulation containing 4% chia seed oil applied for 8 weeks improved skin hydration and subjective itching in subjects with pruritus and xerosis (Jeong et al., 2010). A randomized, double-blind 12-week supplementation with flax seed oil increased smoothness and hydration of the skin, while reducing skin irritation and scaling following the nicotinate challenge (Neukam et al., 2011). Two months supplementation with black currant seed oil also enhanced the skin immune response and reduced prostaglandin E(2) production (Wu et al., 1999). Sacha inchi and rosehip oils high in α-linolenic acid showed no toxicity towards keratinocytes and moderate inhibition of *S. aureus* adherence in cell culture (Gonzalez-Aspajo et al., 2015) and promoted wound healing promoted wound healing by improving scarring outcome and macrophage phenotypes transition (Lei et al., 2018). Punicic acid, a conjugated positional isomer of α-linolenic acid at *cis*-9, *trans*-11, and *cis*-13 positions found in high amounts in pomegranate seed oil, also enhanced transdermal delivery of resveratrol (Liu et al., 2018).

Docosahexaenoic 22:6(n-3), eicosapentaenoic 20:5(n-3), and stearidonic 18:4(n-3), acids

Long chain PUFA such as docosahexaenoic acid (DHA) 22:6(n-3), and eicosapentaenoic acid (EPA) 20:5(n-3) found in abundance in marine fish and krill oils are produced by various types of marine algae and accumulate in these animals as a part of their alga-based diet. As various alga have a different capacity to synthesize DHA or EPA, the compositions of marine oils vary considerably upon fish species, diet, body part, extraction method employed, and typically lack significant amounts of long chain PUFA when derived from freshwater sources (Mohanty et al., 2016). Their various metabolites are essential in the regulation of inflammation and immune outcomes, similar to their precursors α-linolenic and stearidonic acids (McCusker and Grant-Kels, 2010). While DHA is predominantly found in the brain and retina, EPA shows direct competition with arachidonic acid metabolic pathways, thus reducing pro-inflammatory eicosanoids levels and suppressing inflammation (Lorente-Cebrián et al., 2015).

In the skin, both DHA and EPA reduce UVB damage and Interleukin 8 (IL-8) secretion (Storey et al., 2005), and this effect can be achieved by topical application of 10 μmol DHA by upregulation of Nrf2 and heme oxygenase-1 signaling (Yum et al., 2017). A larger dose of DHA (30 μM) was also effective to promote wound healing (Arantes et al., 2016), although another study suggested a following order of potency in modulating skin wound healing: omega-9 > omega-6 > omega-3 (Cardoso et al., 2004).

Stearidonic acid 18:4(n-3) is a metabolic precursor to DHA and EPA that can be found in certain botanical oils like hemp, black currant, echium, and spirulina (**Table 1.4**). While only 5-20% and 1-9% of α-linolenic acid is converted to EPA and DHA in humans, it is estimated that stearidonic acid is much more efficient substrate (17-40%) for such a conversion (Whelan, 2009). Its direct relationship to skin health has not been established.

Essential oils

Botanical essential oils are complex mixtures of highly volatile substances extracted by water, steam, and dry distillation, or cold pressed from *Citrus* fruits. While the major constituents of essential oils are generally mono- and sesquiterpenes (Friedrich, 1976), many plants also accumulate volatile components of terpenoid and phenylpropanoid origin (Badenhorst et al., 2014). The factors that determine essential oil composition, however, vary considerably depending on plant chemotype, geographical and seasonal variation, as well as methods of extraction and storage (Hussain et al., 2008). Their predominantly hydrophobic nature allows them to partition into lipids of the cell membranes, thus modulating the structure and affecting receptors and downstream molecular signaling cascades. Oxygenated essential oils that contain mainly phenol and aldehyde derivatives are generally characterized by higher levels of biological activity (Dhifi et al., 2016).

Terpene hydrocarbons include β-myrcene (bay laurel, cardamom, hemp, hops, parsley, thyme), α- and β-ocimene (allspice, basil, bay laurel, bergamot, hemp, lavender, mint, parsley, pepper), terpinolene or δ-terpinene (apple, citrus, cumin, juniper, lilac, mint, nutmeg, pine, rosemary, sage, tea tree), α- and β-phellandrene (angelica, balsam fir, dill, eucalyptus, fennel, lavender, mint, parsley, pine), D-and L-limonene (citrus, mint), camphene (cypress, fir, ginger, lavender, nutmeg, sage, valerian), sabinene (carrot, juniper, spruce), α- and β-farnesene (apple, gardenia, perilla), A- and D-germacrene (red deadnettle), α- and β-selinene (celery), valencene (citrus), α- and β-caryophyllene (cloves, hemp, hops, rosemary). Evaporation of essential oils can also lead to isolation of complex resins or galipots identified as turpentine, a fluid composed of terpene mixtures dominated by α- and β-pinene (hemp, pine, sage), and rosin (abietic acid).

Oxygenated fragrant terpenes or terpenoids further include citronellal (kaffir lime), R- and S-linalool (basil, birch, cinnamon, citrus, coriander, lavender, mint, rosewood), R- and S-

carvone (caraway, dill, spearmint), thymol (oregano, thyme), fenchone (fennel, wormwood), α- and β-thujone (cypress, mint, oregano, sage, thuja, wormwood), nerolidol (ginger, jasmine, lavender, lemon grass, neroli, tea tree), farnesol (chamomile, ginger, hops, turmeric), bisabolene (lemon, oregano), borneol (camphor, lavender, rosemary, wormwood), carvacrol (oregano, thyme), eucalyptol (eucalyptus).

Phenylpropenes or benzene derivative components of essential oils are less common and include anethole (anis, fennel), estragole (basil, tarragon), myristicin (anise, dill, nutmeg), eugenol (bay laurel, basil, cinnamon, clove, nutmeg), guaiacol (coffee, wood), vanillin (vanilla).

Overall biological activity of essential oils often cannot be attributed to a single major constituent, while inactive compounds might influence absorption and diffusion of bioactives in the mixture (Aziz et al., 2018). As such, the high cost of essential oils and relatively low concentrations of their bioactive constituents limit their direct use in skin care. Instead, they are often mixed with carrier botanical oils or applied in the form of aromatherapy (Orchard and van Vuuren, 2017).

Potential toxicities associated with botanical oils

Botanical oils contain complex mixtures of both saturated and unsaturated fatty acids, typically esterified in the form of triglycerides, that act as powerful lipophilic solvents to selectively extract and accumulate nonpolar secondary metabolites produced by the source plants. Although data is limited, the most efficient solvent-like molecules in the fixed (oleic and linoleic acids) and essential (limonene) botanical oils are likely of low toxicity. However, these compounds may cause mild skin irritation, while their oxidation products may produce dermal sensitization in humans (DeWitt and Bebarta, 2004). The potentially detrimental effects may also vary with dose, form (water or fat soluble), and site of application (sebaceous gland, hair

follicles), which will define absorption rates and accumulation of the bioactive compounds in various layers of the skin and systemic circulation.

Most of botanical oils are generally well tolerated in adults, with occasional allergic skin reactions occurring, and are "generally recognized as safe" (GRAS) as a food (dietary supplement) by the U.S. Food and Drug Administration. This designation, however, does not require the manufacturer to prove the safety and efficacy of the product prior to marketing. Botanical oils produced by different manufacturers may also contain different ingredients that do not match the actual ingredients, or their amounts listed on the label. As such, botanical oils are considered as alternative (complimentary) strategies used to supplement the perceived failures and side effects of conventional medicines.

Site of application

Regional permeability of the human body is not uniform and is typically ranked as follows: scrotum > face/scalp > trunk/extremities > palm/sole > nail. Within those regions, further variations in stratum corneum thickness, the number of sebaceous glands, and hydration status can all affect absorption and metabolism of botanical oils and their bioactive components. Understanding the parameters that affect the permeability of the skin barrier is essential for achieving correct dosing and adherence regimens with a goal of reaching therapeutic targets within the local skin environment (ointment or cream) or systemic uptake via dermal capillary beds (Prausnitz and Langer, 2008). The use of botanical oils as vehicles for therapeutic drug delivery provides a wide variety of choices between achieving optimal drug potency and therapeutic effectiveness, as well as the risk of over-added toxicity, as the same drug may appear in different potency classes when formulated in different vehicles or applied to different target site (Williams and Barry, 2004).

Neonatal skin sensitization

Infant skin is susceptible to dryness and irritation from external factors, including topical skin care products not formulated for the infant's skin (Kuller, 2016). Topical products with adverse effects on skin barrier function, however, carry a potential to develop atopic dermatitis or eczema (Danby et al., 2013). The practice of recommending and using topical oils for the prevention or treatment of baby dry skin or for massage, including the increased societal interest in natural interventions, often ignores the fact that specific topical oils may have an adverse effect on skin barrier function (Cooke et al., 2011). While oils with the lowest oleic acid content provide a lower risk of irritant contact dermatitis (Kuller, 2016), sunflower-based oils may also may retard postnatal skin barrier maturation in infants (Kanti et al., 2014). Skin ointments containing components of food origin also carry the risk of possible percutaneous sensitization to food proteins that may promote development of contact dermatitis and persistent eczema, as it was shown for almond oil (Guillet and Guillet, 2000).

Secondary metabolites and biological reactive intermediates

While most botanical oils can be considered safe, a few contain compounds that can be converted to biological reactive intermediates, causing toxicity (Llana-Ruiz-Cabello et al., 2015). Although health promoting effects of secondary metabolites coextracted into the botanical oils may be beneficial, they may also have potential toxic effects and local higher levels of exposure due to topical application. For example, rosemary oil has been demonstrated to induce lipid and protein oxidation at high doses (Estévez and Cava, 2006). High doses of the monoterpenoid phenols, carvacrol and thymol, increase the levels of malondialdehyde, resulting in membrane damage, and 8-hydroxy-deoxyguanosine, causing cell DNA damage (Ozkan and Erdogan, 2012). Eugenol present in clove oil can be oxidized to phenoxyl radicals that induce reactive oxygen species-mediated apoptosis in human cells (Yoo et al., 2005). Borage plant parts contain

pyrrolizidine alkaloids that are toxic to the liver and lungs, and maybe coextracted into borage seed oil (Low Dog, 2009). Raw botanical oil materials often originate from different sources and storage timeframes, complicating comparisons of bioactive ingredients and lack of potentially toxic contaminants in them.

Genotoxicity and photosensitivity

Some of secondary metabolites coextracted with botanical oils may form genotoxic DNA adducts or activate detoxification enzymes, as it was shown for safrole and quinones in sassafras oil or epoxides found in pennyroyal oil (Dietz and Bolton, 2011). Other botanical oils and their constituents may exhibit a dual genotoxic and antigenotoxic effect, as it was shown for β-caryophyllene (Di Sotto et al., 2010). Adverse cutaneous responses to the combined action of the botanical oil or its bioactive constituent and UV radiation may cause phototoxic reactions that result in sunburn, edema, hyperpigmentation, photoaging and cancer (Gould et al., 1995). Some of these effects, however, may be beneficial in alleviating multiple symptoms of psoriasis, vitiligo, and cutaneous T-cell lymphoma (Bark et al., 2010).

Overdose in pregnant women and children

In rare instances, some commercially marketed hemp seed oils could lead to mild cannabinoid poisoning in children (Chinello et al., 2017) and pregnant women (Yang et al., 2017). While food-grade strains of hemp must contain less than 0.3% THC by weight (whole plant), hemp seeds or stems used to produce hemp oil may become contaminated by THC-rich trichomes of hemp flowers and thus acquire THC (Yang et al., 2017). Due to the polymorphic nature of cytochrome P450 enzymes that can be further affected by age, liver impairment, or potential drug interactions, people consuming hemp products may gradually accumulate THC due to its slow metabolism or relatively long half-life in the body, resulting in potentially higher concentrations (Watanabe et al., 2007).

Conclusions

Both topical and dietary interventions with botanical oils may produce different functional outcomes according to their phytochemical composition and the pathophysiological state of the target tissue. The depletion or disturbance of any of the skin lipid classes results in a rapid disruption of skin integrity and leads to a variety of structural (barrier), physiological (repair and regeneration), and pathological (inflammation) changes that allow further entry of microbial and chemical irritants and deterioration of the affected, aged or diseased skin. Replenishment of those lipids by direct replacement or enhancement of their *in-situ* production with botanical oils may restore skin function and reduce pathophysiological symptoms associated with the disease.

Among inexpensive, widely available oils, sunflower oil high in the omega-6 linoleic acid, and flax or hemp oil enriched with the omega-3 α-linolenic acid, offer an attractive combination of enhanced metabolic and reduced inflammatory and comedogenic effects. On the other hand, application of olive oil with high oleic acid content is warranted when deep transdermal penetration is desired, and the target skin site can be further sealed off with the application of highly saturated coconut or shea butters. The presence of dissolved bioactive secondary metabolites that target a specific health outcome will further substantiate the use of a particular plant source within the group of botanical oils with similar physiochemical properties.

To become established in clinical settings, the required mixtures and doses should be individually determined in randomized controlled trials that simultaneously monitor for hazardous effects of botanical oil supplementation to reduce the possible over-added toxicity associated with the interventions.

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Table 1.1. Lipid class composition of various skin sites (% total lipid).

Lipid class[1]	Human skin surface					Other mammals	
	Sebum	**Scalp**	**Face**	**Forehead**	**Back**	**Mouse**	**Sheep**
Basic analysis							
Squalene	12	12-14	12	12	12-16	--	--
Wax esters	26	21-23	23	26	22-23	5	10
Fatty acids (total)	58	56-65	65	62	61-66	6	--
Extended analysis							
Squalene	12	12-13	12	12	11-16	--	--
Wax esters	26	20-22	23	25	22	5	10
Triglycerides (bound)	--	29-32	35	43	**43-46**	6	--
Fatty acids (free)	--	**29-33**	**27**	16	16	--	--
Sterol esters	3	3	3	2	3	10	46
Sterols	2	2	1	1	1-2	13	12
Diesters	--	--	--	--	--	**65**	**21**
Hydrocarbons	--	1	1	--	1-2	--	--

[1] Adapted with modifications from (Downing and Strauss, 1974).

Table 1.2. Fatty acid composition from various body sites (%).

Fatty acid	Scalp[1]	Sole[2]		Forearm[3]			Erythrocyte[4]	Plasma[5]
	Surface	Live	SC	N	PU	PI	Membrane	Lipids (µmol/L)
Lauric	0.1-1.9	*t*-0.1	0.2	nr	nr	nr	--	--
Myristic	4-8	1.4-2.5	1.1-1.9	1.1	1.5	0.8	--	16.2-325.7
Palmitic	**18-29**	40.6-48.6	24.6-25.1	14	13.9	12	**23-26**	285.4-4064.5
Palmitoleic	--	--	--	2.3	2.6	3.9	--	27.7-555.9
Sapienic		--	--				nr	--
Stearic	2-5	33.9-34.8	18.6-19.3	11.1	10.9	10.1	**14-21**	110.2-1013.7
Oleic	**11-19**	86.2-86.0	83.6-80.0	15.1	13.7	16.8	**14-19**	178.7-3210.5
Linoleic	1-2	96.7	96.1-96.7	21.5	21	15.7	11-12	279.7-4970.5
α-Linolenic	--	--	--	nr	nr	nr	0.1-0.3	--
Arachidic	0.2-1.8	1.0-1.2	3.9-4.8	1.6	1.7	1.8	--	*t*-29.8
Mead	--	--	--	1.5	1.4	1.4	1-2	--
Arachidonic	--	--	--	6.2	6.5	5.0	**14-16**	42.7-882.8
EPA	--	--	--	nr	nr	nr	1	4.4-215.4
Behenic	0.1-1.2	1.2-2.9	7.5-7.8	2.7	2.9	1.4	--	*t*-39.0
DPA	--	--	--	nr	nr	nr	2-3	*t*-88.5
DHA	--	--	--	nr	nr	nr	4-7	7.2-237.5
Lignoceric	0.3-1.2	1.0-3.7		10	10.5	4.2	--	*t*-15.7

Adapted with modifications from: [1] scalp skin surface (Koch et al., 1982); [2] sole skin epidermis (Ansari et al., 1970); [3] normal (N), uninvolved (PU), and involved (PI) psoriatic forearm skin (Chapkin et al., 1986); [4] erythrocyte membrane (Akinyemi et al., 2017); [5] plasma lipids (Abdelmagid et al., 2015).

Table 1.3. Compositions of skin bound fatty acids by various lipid class (%).

Fatty acid[1]	Lipid number	Free	Ester-linked		Long-chain bases		Amide-linked		Cholesteryl esters
			Cer 1	Cer 6I	Cer 2	Cer 3	Cer 2	Cer 6I	
Myristic	14:0	0.8	2	--	--	--	0.2	--	--
Myristoleic	14:1	--	2.3	--	--	--	--	--	--
Palmitic	16:0	7.4	**18.0**	**30.2**	0.6	3.1	3.6	1.7	9.9
Palmitoleic	16:1(n-7)	0.7	4.8	--	0.7	--	--	--	3.0
Margaric	17:0	0.8	1.2	--	2.1	8.0	0.4	1.4	1.1
Stearic	18:0	9.1	9.1	4.8	11.7	12.8	4.4	9.9	4.6
Oleic	18:1(n-9)	5.7	11.6	--	**35.6**	--	--	--	**68.2**
Linoleic	18:2(n-6)	1.4	**20.7**	--	--	--	--	--	--
Nonadecylic	19:0	1.1	0.1	--	1.6	16.0	1.0	1.7	--
Arachidic	20:0	5.9	3.5	3.6	6.5	14.4	3.8	1.6	6.6
Heneicosylic	21:0	1.9	0.2	3.1	20.7	10.1	1.2	1.4	1.1
Behenic	22:0	15.3	4.8	3.3	1.5	21.4	8.7	1.7	1.3
Tricosylic	23:0	6.2	--	--	--	2.1	5.7	1.3	1.0
Lignoceric	24:0	**26.9**	**8.4**	**20.2**	10.5	5.1	**30.4**	**27.9**	1.4
Pentacosylic	25:0	5	1.8	6.3	--	2.1	7.8	11.3	1.1
Cerotic	26:0	8.5	4.1	18.5	--	4.9	**18.3**	**34.8**	1.1

[1] Adapted with modifications from (Wertz et al., 1987).

Table 1.4. Fatty acid composition of botanical oils sorted by their major constituents.

Common name and part used ***Latin name***	Coconut kernel *Cocos nucifera*	Palm kernel *Elaeis guineensis*	Laurel fruit *Laurus nobilis*	Babassu seed *Attalea speciosa*	Murumuru seed *Astrocaryum murumuru*	Nutmeg nut *Myristica fragrans*
Saturation ratio	93:6:2	82:16:3	48:37:15	80:17:3	90:7:3	88:8:2
% Major fatty acid composition	Lauric 48% Myristic 19% Palmitic 9% Caprylic 8%	Lauric 46% Myristic 18% Oleic 16% Palmitic 8%	Lauric 43% Oleic 37% Linoleic 15% Palmitic 5%	Lauric 34% Myristic 19% Oleic 17% Palmitic 11%	Lauric 49% Myristic 30% Palmitic 7% Oleic 7%	Myristic 79% Oleic 7% Palmitic 6% Lauric 2%

Common name and part used ***Latin name***	Ucuhuba seed *Virola surinamensis*	Palm pulp *Elaeis guineensis*	Coffee bean *Coffea arabica*	Buckthorn fruit *Hippophae rhamnoides*	Kokum seed *Garcinia indica*	Sal fruit *Shorea robusta*
Saturation ratio	93:4:1	50:39:11	44:7:49	28:47:25	61:38:1	64:37:1
% Major fatty acid composition	Myristic 71% Lauric 16% Palmitic 4% Oleic 4%	Palmitic 44% Oleic 39% Linoleic 10% Stearic 4%	Linoleic 48% Palmitic 33% Stearic 7% Oleic 7%	Palmitic 27% Palmitoleic 25% Linoleic 16% Oleic 15%	Stearic 59% Oleic 38% Palmitic 2% Linoleic 1%	Stearic 48% Oleic 37% Arachidic 8% Palmitic 7%

Common name and part used ***Latin name***	Mango seed *Mangifera indica*	Shea nut *Vitellaria paradoxa*	Cupuacu bean *Theobroma grandiflorum*	Cocoa bean *Theobroma cacao*	Kusum seed *Schleichera oleosa*	Tallow fat *Sevum*
Saturation ratio	49:45:3	45:48:6	45:48:6	61:35:3	35:62:1	58:36:3
% Major fatty acid composition	Oleic 45% Stearic 42% Palmitic 7% Linoleic 3%	Oleic 48% Stearic 40% Palmitic 5% Linoleic 6%	Oleic 39% Stearic 38% Palmitic 11% Arachidic 8%	Oleic 35% Stearic 33% Palmitic 28% Linoleic 3%	Oleic 43% Arachidic 21% Gadoleic 15% Palmitic 8%	Oleic 47% Palmitic 26% Stearic 14% Myristic 3%

Table 1.4 (continued).

Common name and part used ***Latin name***	Rambutan seed *Nephelium lappaceum*	Peanut bean *Arachis hypogaea*	Ben seed *Moringa oleifera*	Rice bran *Orysa sativa*	Brazil nut *Bertholletia excelsa*	Oat seed *Avena sativa*
Saturation ratio	40:57:1	11:56:26	14:71:2	26:38:34	24:39:36	17:40:39
% Major fatty acid composition	Oleic 51% Stearic 15% Arachidic 15% Palmitic 8%	Oleic 56% Linoleic 26% Palmitic 8% Stearic 3%	Oleic 66% Palmitic 9% Behenic 4% Stearic 7%	Oleic 38% Linoleic 34% Palmitic 22% Stearic 3%	Oleic 39% Linoleic 36% Palmitic 13% Stearic 11%	Oleic 40% Linoleic 39% Palmitic 15% Stearic 2%

Common name and part used ***Latin name***	Sesame seed *Sesamum indicum*	Jatropha seed *Jatropha curcas*	Argan fruit *Argania spinosa*	Neem seed *Azadirachta indica*	Pecan nut *Carya illinoinensis*	Avocado seed *Persea americana*
Saturation ratio	15:40:43	16:44:34	15:46:34	39:46:12	9:50:39	22:58:12
% Major fatty acid composition	Linoleic 43% Oleic 40% Palmitic 10% Stearic 5%	Oleic 44% Linoleic 34% Palmitic 9% Stearic 7%	Oleic 46% Linoleic 34% Palmitic 14% Linolenic 1%	Oleic 46% Palmitic 21% Stearic 16% Linoleic 12%	Oleic 50% Linoleic 39% Palmitic 7% Linolenic 2%	Oleic 58% Palmitic 20% Linoleic 12% Palmitoleic 8%

Common name and part used ***Latin name***	Macadamia nut *Macadamia Integrifolia*	Canola seed *Brassica napus*	Pistachio nut *Pistacia vera*	Peach kernel *Prunus persica*	Apricot kernel *Prunus armeniaca*	Plum kernel *Prunus domestica*
Saturation ratio	14:79:2	6:61:21	12:63:25	8:65:25	6:66:27	3:68:23
% Major fatty acid composition	Oleic 59% Palmitoleic 19% Palmitic 9% Stearic 5%	Oleic 61% Linoleic 21% Linolenic 9% Palmitic 4%	Oleic 63% Linoleic 25% Palmitic 11%	Oleic 65% Linoleic 25% Palmitic 6% Stearic 2%	Oleic 66% Linoleic 27% Palmitic 6%	Oleic 68% Linoleic 23% Palmitic 3%

Table 1.4 (continued).

Common name and part used ***Latin name***	Olive fruit *Olea europaea*	Almond nut *Prunus dulcis*	Buriti fruit *Mauritia flexiosa*	Hazel nut *Corylus avellana*	Marula fruit *Sclerocarya birrea*	Papaya seed *Carica papaya*
Saturation ratio	17:69:12	7:71:18	19:71:7	8:75:10	18:75:4	18:76:3
% Major fatty acid composition	Oleic 69% Palmitic 14% Linoleic 12% Stearic 3%	Oleic 71% Linoleic 18% Palmitic 7%	Oleic 71% Palmitic 17% Linoleic 7% Stearic 2%	Oleic 75% Linoleic 10% Palmitic 5% Stearic 3%	Oleic 75% Palmitic 11% Stearic 7% Linoleic 4%	Oleic 76% Palmitic 13% Stearic 5% Linoleic 3%

Common name and part used ***Latin name***	Tea seed *Camellia sinensis*	Pataua fruit *Oenocarpus bataua*	Carrot seed *Daucus carota*	Camelina seed *Camelina sativa*	Jojoba seed *Simmondsia chinensis*	Mustard seed *Brassica napus*
Saturation ratio	11:77:8	17:78:3	4:80:13	8:77:10	8:92:0	12:60:21
% Major fatty acid composition	Oleic 77% Palmitic 9% Linoleic 8% Stearic 2%	Oleic 78% Palmitic 13% Stearic 4% Linoleic 3%	Oleic 80% Linoleic 13% Palmitic 4%	Gondoic 33% Oleic 14% Linolenic 10% Linoleic 9%	Gadoleic 75% Erucic 15% Oleic 10% Nervonic 3%	Erucic 42% Linoleic 15% Oleic 12% Linolenic 6%

Common name and part used ***Latin name***	Buckthorn seed *Hippophae rhamnoides*	Cranberry seed *Vaccinium macrocarpon*	Borage seed *Borago officinalis*	Black currant seed *Ribes nigrum*	Rosehip fruit *Rosa canina*	Pumpkin seed *Cucurbita pepo*
Saturation ratio	10:24:66	8:23:69	14:20:66	8:13:76	6:12:82	19:33:48
% Major fatty acid composition	Linoleic 36% Linolenic 28% Oleic 24% Palmitic 7%	Linoleic 37% Linolenic 32% Oleic 23% Palmitic 6%	Linoleic 43% gLinolenic 24% Oleic 20% Palmitic 10%	Linoleic 46% Linolenic 29% gLinolenic 19% Oleic 13%	Linoleic 46% Linolenic 31% Oleic 12% Palmitic 4%	Linoleic 50% Oleic 33% Palmitic 11% Stearic 8%

Table 1.4 (continued).

Common name and part used ***Latin name***	Soybean seed *Glycine max*	Corn seed *Zea mays*	Cotton seed *Gossypium arboreum*	Raspberry seed *Rubus idaeus*	Hemp seed *Cannabis sativa*	Black cumin seed *Nigella sativa*
Saturation ratio	18:75:4	14:32:51	26:18:52	3:13:81	8:12:57	16:22:60
% Major fatty acid composition	Linoleic 50% Oleic 24% Palmitic 11% Linolenic 8%	Linoleic 51% Oleic 32% Palmitic 12% Stearic 2%	Linoleic 52% Oleic 18% Palmitic 13% Stearic 12%	Linoleic 55% Linolenic 26% Oleic 13% Palmitic 3%	Linoleic 57% Linolenic 21% Oleic 12% Palmitic 6%	Linoleic 60% Oleic 22% Palmitic 13% Stearic 3%

Common name and part used ***Latin name***	Walnut seed *Juglans Regia*	Watermelon seed *Citrullus lanatus*	Grape seed *Vitis vinifera*	Poppy seed *Papaver somniferum*	Passion seed *Passiflora edulis*	Sunflower seed *Helianthus annuus*
Saturation ratio	9:18:60	21:18:60	12:20:68	12:17:69	13:15:70	11:16:70
% Major fatty acid composition	Linoleic 60% Oleic 18% Palmitic 7% Stearic 2%	Linoleic 60% Oleic 18% Palmitic 11% Stearic 10%	Linoleic 68% Oleic 20% Palmitic 8% Stearic 4%	Linoleic 69% Oleic 17% Palmitic 10% Stearic 2%	Linoleic 70% Oleic 15% Palmitic 10% Stearic 3%	Linoleic 70% Oleic 16% Palmitic 7% Stearic 4%

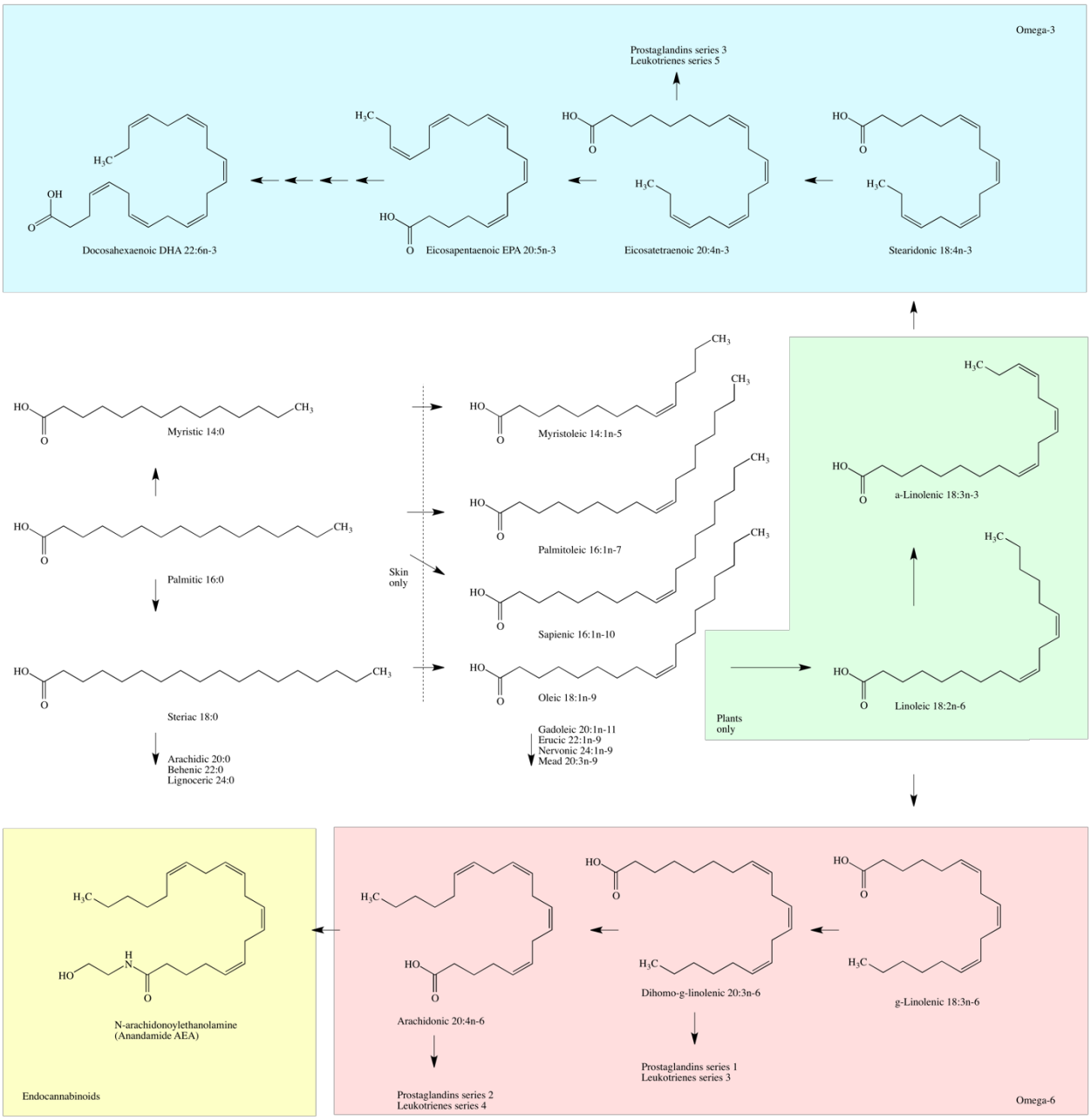

Figure 1.1 Schematic biosynthesis of MUFA and PUFA fatty acids.

CHAPTER 2: COMBINATION OF SPOTTED WINTERGREEN LIPIDS WITH HEMP OIL IMPROVES MICROBIAL AND IMMUNE SKIN HEALTH OUTCOMES

Abstract

Mobile Discovery program provides a low-cost approach to explore chemical diversity of local ecosystems and discover its potential to improve human health. As a part of this program, we identified spotted wintergreen (*Chimaphila maculata* (L.) Pursh, Pyloraceae), a native Southern Appalachians plant of cultural significance to the Eastern Band of Cherokee people, as a botanical source of lipophilic bioactives with potent antimicrobial and anti-inflammatory properties. Preclinical testing in skin and macrophage cell cultures showed high regenerative potential of spotted wintergreen preparations. When combined with hemp oil (*Cannabis sativa* L., Cannabaceae) as a carrier botanical oil and a distinctive source of essential polyunsaturated fatty acids critical to support skin function and β-oxidation, spotted wintergreen lipid fraction promoted migration and scratch wound closure in cell culture. Based on this data, we proposed botanical-infused hemp oils as a novel group of topical skin care formulations potentially effective at alleviating cutaneous infections and disorders associated with abnormal skin regeneration.

Introduction

Spotted wintergreen (*Chimaphila maculata* (L.) Pursh, Pyloraceae) is a native Southern Appalachians plant of cultural significance to the Eastern Band of Cherokee Indians. The plant is a perennial evergreen listed in several botanical collections as diuretic, tonic, antirheumatic and antiseptic with an extensive record of prior human use including *Extractum Chemaphilae Fluidum* in the US Pharmacopeia and *Decoctum Chimaphilae* in the Eclectic Materia Medica (Millspaugh, 1974). This species is still used in Appalachia for its diuretic, tonic and astringent properties, including skin eruptions (Jacobs and Burlage, 1958). The herb is also used in preparation of *tesgüino* fermented corn beverage by the Tarahumara Indians of Sierra Madre in Mexico (Ulloa et al., 1974). A related northern species, pipsissewa (*Chimaphila umbellata* (L.) Barton) is used by Wasco Chinook Indians in Oregon and the First Nations Peoples in Canada to heal irritated eyes, blisters, fungal and tubercular infections (Galván et al., 2008). The leaves extract is used in formulations (CAS #89997-56-8) and flavorings (FEMA GRAS #2914).

Fixed (vegetable) botanical oils are esters of glycerin with fatty acids of varying carbon chain length and saturation. Mono- (MUFAs) and poly- (PUFAs) unsaturated fatty acids define the fluidity, molecular packing, and integrity of cell membranes (Pamplona, 2008), however they contain double bonds in less thermodynamically stable *cis* configuration that is prone to lipoxidative deterioration. Botanical oils are widely used to prevent or ameliorate human diseases, especially in the form of topical applications to promote skin health, heal injuries and burns, enhance transdermal drug delivery, decrease scarring, improve cosmetic outcomes and wellbeing, and reduce social stigmatizing (Vaughn et al., 2018). Oils containing saturated fatty acids are used in topical skin and hair applications for their lubricant, moisturizing, emollient, and antimicrobial properties, however they have high comedogenic activity (Patzelt et al., 2012). Unsaturated fatty acid esters, in particular those of omega-3, omega-6, and omega-9 groups, can

be used to manage clinical manifestations associated with skin damage or immune disorders due to their permeation and anti-inflammatory properties (Elshafie and Camele, 2017). In general, it appears that high linoleic acid 18:2(n-6) containing botanical oils are more beneficial to skin health (Hanley et al., 1998) when compared to the high oleic acid 18:1(n-9) counterparts that cause skin irritation (Jiang et al., 2000). Additionally, skin imports arachidonic acid 20:4(n-6) together with linoleic 18:2(n-6) and α-linolenic 18:3(n-3) acids from the extra-epidermal sites (Chapkin and Ziboh, 1984). Linoleic acid 18:2(n-6) is also selectively targeted for β-oxidation by the sebaceous skin cells as a unique energy source for their function (Pappas et al., 2002). Different ratios of individual fatty acids present in botanical oils may therefore result in either beneficial or detrimental effects on epidermal barrier function and comedogenicity, and merit detailed investigation for skin care (Darmstadt et al., 2002).

Botanical oils also contain various secondary plant metabolites that may be partially responsible for their health promoting properties. While dietary intake of olive oil promotes metabolic health, its topical benefits are largely attributed to polyphenol and squalene content (Kränke et al., 1997). The beneficial effects of avocado oil on collagen metabolism (Werman et al., 1991), argan oil on cardiovascular health (Monfalouti et al., 2010), and oat lipids on keratinocyte differentiation (Chon et al., 2015) are also likely mediated by secondary bioactive compounds found in these oils. These "carrier" properties of botanical oils make them valuable products for dissolving and delivering bioactive plant constituents to the target skin layers.

Forest botanicals used for flavorings, cosmetics, and in manufacturing of medicinal and nutritional supplement products represent a continuing economic opportunity for wildcrafting and small specialty manufacturers. In this study, we report the antimicrobial and wound healing activity of lipophilic constituents from spotted wintergreen. In addition, we describe effects of individual fatty acids on the anti-inflammatory and wound closing cellular responses that

promote wound tissue homeostasis and healing, and the use of botanical oils with distinct fatty acid profiles as carrier oils that work synergistically with spotted wintergreen to facilitate the beneficial skin health outcomes.

Materials and methods

Chemicals

All chemicals used in this study were American Chemical Society (ACS) grade and purchased from Fischer or VWR unless specified otherwise. Botanical oils from palm kernel, avocado, olive, soybean and sunflower origin were purchased as reference analytical standards from Sigma (St. Louis, MO). Virgin hempseed oil (unrefined, cold-pressed) was purchased from Sweet Essentials (Winston-Salem, NC).

Mobile Discovery of antimicrobial leads

Mobile Discovery program was designed as a participatory science approach to sample chemical diversity from local natural environments and to promote global health discovery in search of new antimicrobial agents. Academic and industrial research scientists, educators, students and general public participants requested a Mobile Discovery kit, performed the assay, and uploaded the screening results to the Mobile Discovery database curated at the Plants for Human Health Institute, Kannapolis, NC (http://mobilediscovery.org). Mobile Discovery assays were performed *in situ* on the day of collection by testing 50-100 mg of fresh plant tissue against bacteria present in the participant's saliva according to the instructions provided with the kit. Antimicrobial activity of the samples was visually scored as 3 (strong, bacterial growth of 0-25%), 2 (moderate, bacterial growth of 25-50%), 1 (weak, bacterial growth of 50-75%), and 0 (no activity, bacterial growth of 75-100% relative to saliva-only controls).

Collection and extraction of high scoring hits

Spotted wintergreen (*Chimaphila maculata* (L.) Pursh, Pyloraceae) (SWG) was identified as a high scoring hit during the Antibiotic Resistance and Drug Discovery laboratory class at the Catawba College, Salisbury, NC in 2017. The plants were subsequently collected in the Uwharrie National Forest, Uwharrie, NC during the summer and fall of 2018. Plant material (300 g FW) was freeze dried (Labconco Freezone 18, Kansas City, MO), ground to powder (IKA A11 mill, Wilmington, NC), and 50 g were sequentially extracted two times (1:10, w:v) with hexane (SWG1, 0.82 g), diethyl ether (SWG2, 1.94 g), chloroform (SWG3, 0.61 g), ethyl acetate (SWG4, 2.82 g), methanol (SWG5, 6.36 g), and water (SWG6, 6.95 g). Combined fractions were evaporated to dryness at temperatures not exceeding 40 °C (Buchi Rotavapor R210, Flawil, Switzerland) and stored at -80 °C. Unless otherwise specified, extracts and fractions use were dissolved in dimethyl sulfoxide (DMSO) as 1000x stocks and stored at −20 °C until use for *in vitro* testing.

Antimicrobial testing under laboratory conditions

Gram-positive *Staphylococcus aureus* ATCC 25923 and Gram-negative *Escherichia coli* ATCC 47093 (Manassas, VA) were grown under the aerobic conditions on the surface of lysogeny broth (LB) agar and transferred to LB broth for overnight cultivation (37 °C, 100 rpm, New Brunswick Innova 43 incubator, Enfield, CT). Disk diffusion assay was then used to determine the antibacterial activities of high scoring plants in the laboratory setting. For each 100 mm Petri/LB agar plate surface spread with the appropriate bacterial culture, 6 filter paper disks (6 mm) were imprinted with 10 µL of vehicle (DMSO, negative control) or three concentrations of the test article, tested in duplicate. Antibiotics tetracycline and ampicillin (5 µg per disk) were used as reference drugs for Gram-positive and Gram-negative bacteria, respectively. After an

overnight incubation at 37 °C, the diameter of the inhibition zones was measured with a digital caliper in mm.

Anticancer activity

Human HT-29 colorectal adenocarcinoma cells of epithelial origin (ATCC HTB-38, Manassas, VA) were routinely passaged every 3–4 days and maintained in high glucose Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA), and 1% penicillin-streptomycin (Fisher Scientific, Pittsburg, PA) at 37 °C and 5% CO2. Cells were seeded in 96-well plates ($1x10^4$ cells/well) for 24 h and treated with test articles at designated concentrations for 72 h. Next, the media was removed and living cells were fixed with 10% trichloroacetic acid for 1 hour at 4 °C. Plates were then washed 4 times with cold phosphate buffer saline (PBS) dried overnight. Cell viability was quantified using sulforhodamine B (SRB) test (Skehan et al., 1990) by absorbance read at 510 nm on a Synergy H1 microplate spectrophotometer (BioTek, Sunnyvale, CA). Briefly, cells were stained with 100 µl of 0.056% SRB in 1% acetic acid for 30 min and washed with 1% acetic acid 4 times, and bound SRB was extracted with 200 µl of 10 mM Trizma for 30 min. For every experiment, one positive control (paclitaxel at 0.5 µM) and one negative control (DMSO at a final concentration of 0.1%) were included. Three replicates were made for both the treatments and the controls.

Anti-inflammatory activity

Mouse macrophage cell line RAW 264.7 (ATCC TIB-71, Manassas, VA) was maintained in high glucose DMEM containing 10% FBS and 1% penicillin-streptomycin, at 37 °C and 5% CO_2. Cells were seeded in 24-well plates ($5x10^5$ cells/well) 24 h prior to treatment. Cells were treated simultaneously with test articles at designated concentrations and elicited with lipopolysaccharide (LPS) at 1 µg/ml for 8 h. For every experiment, one positive control

(dexamethasone at 10 μM) and one negative control (DMSO at a final concentration of 0.1%) were included. Three replicates were made for both the treatments and the controls.

Scratch wound healing activity

Mouse NIH/3T3 fibroblasts (ATCC CRL-1658, Manassas, VA) maintained in high glucose DMEM containing 10% FBS and 1% penicillin-streptomycin, at 37 °C and 5% CO2. Cells were seeded into 24-well plates at a concentration of $3x10^5$ cells/well and cultured to a nearly confluent cell monolayer. A linear wound was generated in the monolayer with a sterile 100 μL plastic pipette tip. Any cellular debris was removed by washing with phosphate buffer saline. DMEM medium with vehicle (0.1% glycerol), FBS (20%, positive control), or various concentrations of the test articles were added to set of 3 wells per dose and incubated for 24 h. Cells were visualized with 10% methylene blue for 5 minutes. Three representative images from each well of the scratched areas under each condition were taken to estimate the relative width of the scratch wound area at 0 and 24 h past treatment. The data was analyzed using ImageJ software by calculating the percentage of scratch wound closure relative to control.

RNA extraction and cDNA synthesis

The total RNA was isolated from cells using TRIzol reagent (Life Technologies) following the manufacturer's instructions. RNA was quantified using the Synergy H1/Take 3 spectrophotometer (BioTek). The cDNAs were made on an ABI GeneAMP 9700 thermal cycler using 2 μg of RNA for each sample using a high-capacity cDNA Reverse Transcription kit (Life Technologies).

Quantitative PCR analysis

cDNAs were amplified in duplicate by real-time quantitative polymerase chain reaction (PCR) using SYBR green PCR Master Mix (Life Technologies). To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using Primer

Express version 2.0 software (Applied Biosystems, Foster City, CA, USA) as follows: β-actin, forward primer 5′-AAC CGT GAA AAG ATG ACC CAG AT-3′, reverse primer 5′-CAC AGC CTG GAT GGC TAC GT-3′; COX2, forward primer 5′-TGG TGC CTG GTC TGA TGA TG-3′, reverse primer 5′-GTG GTA ACC GCT CAG GTG TTG-3′; iNOS, forward primer 5′-CCC TCC TGA TCT TGT GTT GGA-3′, reverse primer 5′-TCA ACC CGA GCT CCT GGA A-3′; IL6, forward primer 5′-TAG TCC TTC CTA CCC CAA TTT CC-3′, reverse primer 5′-TTG GTC CTT AGC CAC TCC TTC-3′; IL1β, forward primer 5′-CAA CCA ACA AGT GAT ATT CTC CAT G-3′, reverse primer 5′-GAT CCA CAC TCT CCA GCT GCA-3′. qPCR amplifications were performed on an ABI 7500 Fast real-time PCR (Life Technologies) using 1 cycle at 50 °C for 2 min and 1 cycle of 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The melting curve was completed with 1 cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. mRNA expression was analyzed using the ΔΔCT method and normalized with respect to the expression of the β-actin housekeeping genes.

Measurement of cellular bioenergetics

Fibroblasts were seeded in 24-well XF assay plate (50,000 cells per well) overnight and were subjected to real-time measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), using Agilent Seahorse XF24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA, USA) after treatment with vehicle or test article for 24 h. Cells were transferred to 500 µL of XF assay medium (DMEM without NaHCO3, 10 mM glucose, 2 mM pyruvate, pH 7.4), and equilibrated in a non-CO_2 incubator at 37 °C for 1 h. Basal OCR and ECAR rates were determined by averaging the last 9 basal measurements. For mitochondrial stress tests, the mitochondrial complex inhibitors were next injected sequentially in the following order oligomycin (1 µM), FCCP (0.75 µM), antimycin A/rotenone (1 µM each), and 3 readings were taken after each inhibitor.

Statistical analysis

Statistical analyses were performed using Prism 7.0 (GraphPad Software, San Diego, CA). Values were reported as means ± SEM. Data was analyzed by a one-way ANOVA with treatment as a factor. Post hoc analyses of differences between individual experimental groups were made using Dunnett's multiple-comparison tests. Significance was set at $p < 0.05$.

Results

Identification of a high scoring antimicrobial hit

An exemplary 24-well Mobile Discovery (MD) plate used in the antimicrobial screening program during the Antibiotic Resistance and Drug Discovery laboratory class at the Catawba College. Each MD plate contained blank wells (A1-A2), saliva-only control wells (A3-A4), and a set of 20 fresh plant samples collected by each student (wells A5-D6). Among 280 plant samples screened, spotted wintergreen (*Chimaphila maculata* (L.) Pursh, Pyloraceae) (SWG) was identified as a high scoring hit as shown in the well B3 (**Figure 2.1A**) with an antimicrobial score of 0 on a 0-3 semiquantitative bioactivity scale (**Figure 2.1B**).

Fractionation and biological activity

We subsequently collected a 300 g FW batch of spotted wintergreen plants growing in the Uwharrie National Forest and fractionated the dried powdered sample into 6 fractions of increasing polarity (**Figure 2.2A**). Antiproliferative effects of the spotted wintergreen fractions were evaluated by quantifying HT-29 colorectal cancer cell growth following ta 72 h exposure. Lipophilic diethyl ether (SWG2) and chloroform (SWG3) fractions tested at 50 µg/ml showed strong (78.6%) and moderate (37.9%) anticancer activity that compared favorably to the reference drug Taxol used at 0.5 µM (83.1%) (**Figure 2.2B**). Lipophilic fractions SWG1-3 also showed a significant inhibition of the LPS-induced inflammation in macrophages as evident from 38.7%, 95.1%, and 92.2% reduction in nitric oxide production, respectively (**Figure 2.2C**).

The observed anti-inflammatory effects mediated by nitric oxide reduction were dose dependent for both fractions SWG2 and SWG3 in the range of 0.15-50 µg/ml (**Figure 2.3A**). This biological activity was mediated by decreased expression of cycloouygenase-2 (Cox2), inducible nitric oxide synthase (iNos), interleukin-1 Beta (IL1B), and interleukin 6 (IL6) genes that drive key regulatory signaling networks responsible for progression of the inflammatory response (**Figure 2.3B-E**).

Antimicrobial disk diffusion and MIC determination

Antimicrobial effects of spotted wintergreen crude extract were confirmed by disk diffusion assay to validate the Mobile Discovery screening approach. Moderate antimicrobial activity against gram-positive *S. aureus* (**Figure 2.4A**) and weak activity against gram-negative *E. coli* (**Figure 2.4B**) were observed for the crude SWG extract. When tested in broth microdilution assays, the crude SWG extract showed an IC_{50} of 1.71 µg/ml for *S. aureus* and 12.81 µg/ml for *E. coli*. A reference antibiotic ampicillin showed the respective IC_{50} of 0.19 µg/ml and 0.36 µg/ml when tested under the identical conditions (**Figure 2.4C-D**). The bulk of SWG antimicrobial activity was fractionated into lipophilic fractions SWG1-3 (**Figure 2.4E-F**), while the hydrophilic fractions SWG4-6 had little activity (not shown). All lipophilic fractions showed strong antibacterial effect in the potency order of SWG3 (chloroform) > SWG2 (diethyl ether) > SWG1 (hexane), with the highest IC_{50} achieved by SWG3, 1.34 and 5.03 µg/ml, respectively. The crude SWG extract also showed minimum inhibitory concentrations (MICs), the lowest concentrations that inhibited the visible growth of a microorganism after overnight incubation, in the range of 7.52-9.61 µg/ml (data not shown).

The choice of carrier oil

Due to lipophilic nature of the SWG2-3 bioactive fractions isolated from the spotted wintergreen extract, we next explored a series of botanical (vegetable) oils to determine the

optimal carrier oil for formulation and topical delivery of the bioactive substances. Six oils in the order of decreased saturation (**Figure 2.5A**) were tested for their ability to promote migration into a scratch wound area and enhance metabolism in 3T3 fibroblasts. Stock oils were dissolved at 44 µg/ml (50 µL/ml) in DMEM containing 0.1% glycerol and applied to scratched 3T3 fibroblast cultures for 24 h. Among 6 oils tested, highly saturated oils (palm kernel) and monounsaturated high oleic oils (avocado, olive) failed to promote fibroblast migration into the scratch wound area (**Figure 2.5B**). Addition of high polyunsaturated oils (soybean, hemp, and sunflower) to cultured fibroblasts increased cell migration in the range of 20-40% over vehicle control. This effect was most prominent with the use of hemp oil and seemed to correlate with the apparent ratio of omega-6 to omega-3 fatty acids in the order of potency hemp oil (4:1) > soybean (7:1) > sunflower (60:1). To further test this observation, we prepared two-compound mixtures of major omega-6 (linoleic acid) and omega-3 (α-linolenic acid) fatty acids in different proportions and applied them to wounded fibroblasts. Indeed, mixtures in the range of 10:1 to 3:1 proportion of omega-6/omega-3 fatty acids significantly improved fibroblast migration into the wounded area (40-60% over the vehicle control), while 1:1 ratio as well as linoleic acid and α-linolenic acid alone showed much less efficacy in this assay (**Figure 2.5C**). Addition of 1% SWG crude extract to the carrier hemp oil showed similar efficacy at promoting fibroblast migration into the scratch wound area as the carrier oil alone, thus allowing us to extend lipophilic antimicrobial and anti-inflammatory properties of the spotted wintergreen extract to the hemp oil formulation without apparent detrimental effects on skin cells *in vitro* (**Figure 2.5D**).

Bioenergetic characterization of carrier botanical oils

Under basal conditions following a 24 h exposure of fibroblasts cells to the carrier botanical oils, all treatments showed comparable OCRs that were slightly elevated in hemp and

sunflower groups (data not shown). Increased mitochondrial function was also evident in these groups at the level of maximal OCR (FCCP uncoupling), especially for hemp oil. High oleic botanical oils generally decreased mitochondrial efficacy, and the same findings were noted for soybean oil. In the latter case, these effects could not be explained by the fatty acid profile of soybean oil alone and therefore could be associated with other metabolites found in soybean oil, for example the ones that were associated with its oestrogenic like activity (elSattar ElBaltran, 2001).

Discussion

Innovative approaches to conservation, management, and utilization of forest botanicals hold high promise to improve ethnic, cultural, and ecological resilience, as well as provide jobs and income opportunities to the rural communities. These strategies are deeply rooted in Southern Appalachians through the long history of wildcrafting plants (root digging) by both Native American nations, including the Eastern Band of Cherokee people, and the European settlers to the region. At the beginning of the 20th century, over 70% of country's crude drugs were sourced and processed in Appalachia (Manget, 2016). This can be attributed in part to Central and Southern Appalachian Mountains being one of the most biologically diverse regions (biodiversity hotspot that was never glaciated) in the continental US. The Great Smokey Mountains National Park alone holds more tree species than found in Europe, showcasing the broad diversity of flora in Appalachia (Lopez et al., 2008).

The modern Appalachian region experiences high rates of rural migration and loss of traditional food and foraging, both potentiated by declining health and income opportunities for the local communities. Mobile Discovery program was specifically developed to rebuild the mutually beneficial relationships between people and regional ecologies by providing an inexpensive screening tool to discover natural products with application to human health,

educate communities on practical uses of these products, and confirm the traditional knowledge preserved in the region by oral tradition and herbal texts (Wagner et al., 2017). This study reports on establishing the Mobile Discovery educational program at Catawba College that emphasizes ecologically sustainable, nondestructive screening of local flora to identify novel antimicrobial leads, engage student participants in STEM education, and conserve traditional knowledge in modern rural settings. Collectively, 14 students enrolled in the Antibiotic Resistance and Drug Discovery class assayed 280 plants samples and identified spotted wintergreen (*Chimaphila maculata* (L.) Pursh, Pyloraceae) as a potent antimicrobial lead. A related northern species *Chimaphila umbellata* (pipsissewa) contains several biologically active compounds, including 2,7-dimethyl-1,4-naphthoquinone (chimaphilin) and hydroquinone glycoside (arbutin). Traditional uses of pipsissewa include healing blisters, sore eyes, rheumatic conditions, urinary tract infections, as tonic, and when drank regularly, to cure cancers, as reviewed previously (Pengelly and Bennett, 2011). On the contrary, even though spotted wintergreen is a plant of cultural significance to the Eastern Band of Cherokee people, its traditional use is restricted to Central and Southern Appalachia (Jacobs and Burlage, 1958), and little is known about its phytochemical or pharmacological profile.

A variety of skin disorders including open wounds proceed through a coordinated set of inflammatory, migratory, and tissue remodeling healing stages before the pathological condition is resolved (Broughton et al., 2006). Local and systemic immune responses in the damaged skin tissues are generally driven by macrophages and neutrophils, and once excessive, lead to immune suppression and increased incidence of infection (Schäffer and Barbul, 1998). Our data suggested that lipophilic components of the crude spotted wintergreen extract showed an IC_{50} of 1.71 µg/ml against *S. aureus* and 12.81 µg/ml against *E. coli* microorganisms. Fractionation of this extract revealed several distinct subfractions of different polarity with higher IC_{50} values of

1.34 and 5.03 µg/ml, respectively. This suggested that spotted wintergreen extracts hold a high potential for topical control of microbial infections, and validated their traditional use to treat skin eruptions and eye infections (Jacobs and Burlage, 1958). While some of this activity could be likely attributed to chimaphilin as described in pipsissewa (Sheth et al., 1967) or wood nymph (Saxena et al., 1996), presence of strong antimicrobial activity in multiple lipophilic fractions indicated that other, yet unidentified bioactives may be biosynthesized by spotted wintergreen and warrant further investigation.

The antiproliferative effects of spotted wintergreen were localized to the lipophilic fraction 2, while its anti-inflammatory properties were associated with the lipophilic fractions 2 and 3. Previously it was shown that chimaphilin induced apoptosis in human breast cancer MCF-7 cells via a ROS-mediated mitochondrial pathway (Ma et al., 2014), but it remains unclear which spotted wintergreen compounds are responsible for its anti-inflammatory effects. Although several plant 1,4-naphotoquinones such as shikonin, plumbagin, or juglone have been implicated in downregulating central mediators of mammalian inflammation (Widhalm and Rhodes, 2016), these compounds have not been described in spotted wintergreen or pipsissewa.

The establishment of inflammatory response also includes fibroblast migration and skin tissue remodeling by production of extracellular matrix. These steps are critically regulated by metabolism of arachidonic acid mediators and endogenous cannabinoid receptor ligands (Rouzer and Marnett, 2011). Fatty acids, especially polyunsaturated fatty acids (PUFAs) in the form of phospholipids, are fundamental cell membrane components that modulate skin cell interaction and signaling in health and disease outcomes (Calder, 2001). Driven by the lipophilic nature of spotted wintergreen bioactives, in this study we attempted to identify a carrier oil of botanical origin that achieves two objectives: solubilizes hydrophobic plant secondary metabolites and directly modulates skin healing. The existing literature on the subject is rather controversial.

While some studies indicated that both omega-6 and omega-3 fatty acids promote wound healing (Ruthig and Meckling-Gill, 1999), other studies argued that the initial wound healing response benefited from linoleic acid and oleic acid, but was delayed by linolenic acid (Cardoso et al., 2004). This disagreement also extends to dietary interventions, where codfish oil promoted wound healing (Kietzmann, 1999) while menhaden fish oil slowed it down (Scardino et al., 1999). It is worth noting that only linoleic acid is selectively targeted for β-oxidation by the sebaceous skin cells as a unique energy source for their function (Pappas et al., 2002).

In an attempt to unravel this discrepancy, we tested six botanical oils of various degree of saturation in their ability to promote fibroblast migration and metabolism. Our data clearly suggested that saturated and high oleic oils are detrimental to these mechanisms, while high linoleic oils generally promoted fibroblast health, however this effect did not correlate with linoleic acid content of the oil alone. A double mixture analysis of linoleic and linolenic acids in different proportions revealed an optimal ratio in the range of 10:1 to 3:1 of omega-6/omega-3 fatty acids that significantly improved fibroblast migration into the wounded area, with hemp oil being closet match among the botanical oils used in this study. Indeed, spotted wintergreen bioactives could be successfully incorporated in the carrier hemp oil with no detrimental effects on fibroblast migration. A future effort to combine spotted wintergreen bioactives with full spectrum *Cannabis* oils may improve the topical efficacy of this combination in wound healing and skin health further by introducing hemp cannabinoids like cannabidiol (CBD) known to activate adenosine A2A receptors (Carrier et al., 2006) that, in turn, may accelerate wound healing (Montesinos et al., 1997).

Conclusions

In summary, our results revealed the beneficial role of lipophilic bioactives from spotted wintergreen on microbial, proliferative, and inflammatory components of the wound healing

process *in vitro*. Its combination with a carrier hemp oil that contains a near optimal ratio of linoleic and linolenic acids working together to promote healthy metabolism and migration of skin cells in to the scratch wound area, created a novel botanical intervention that can be tested in preclinical and clinical models to improve wound care and management of immune skin disorders. The presented data also provides initial guidelines on choice of botanical carrier oils in skin care or cosmetic applications. The fact that the initial lead discovery for this study was achieved by a Mobile Discovery-driven approach in the educational settings relevant to the biogeographical region and traditional knowledge of the Appalachia represents a continuing economic opportunity in the rural areas not suited to more familiar farm herbs.

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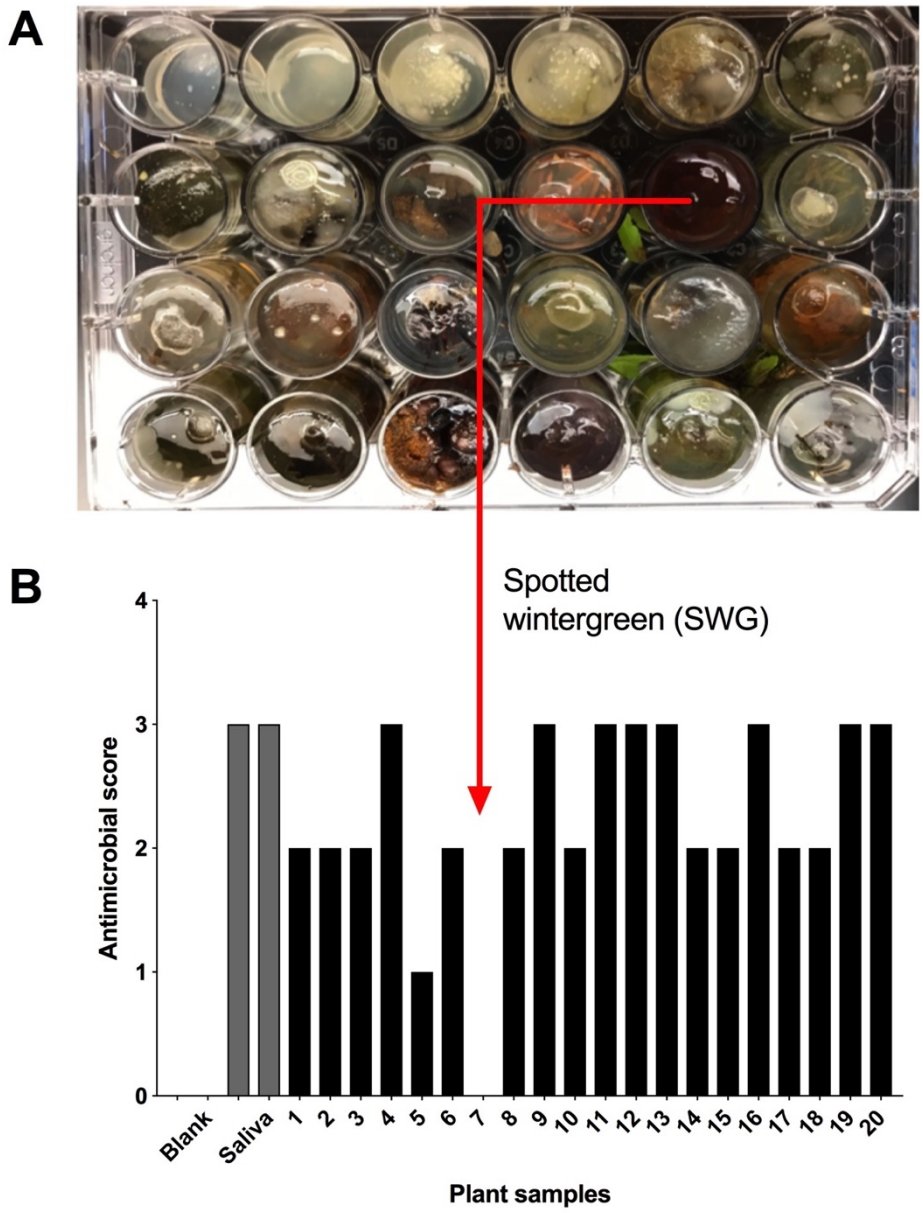

Figure 2.1. Mobile Discovery-based identification of lead antimicrobial plant. (A) Visual detection of inhibitory activity of spotted wintergreen sample on the growth of bacteria present in human saliva. (B) Semiquantitative score of 0 (high activity) assigned to the spotted wintergreen among 20 Appalachian plant samples tested on this plate.

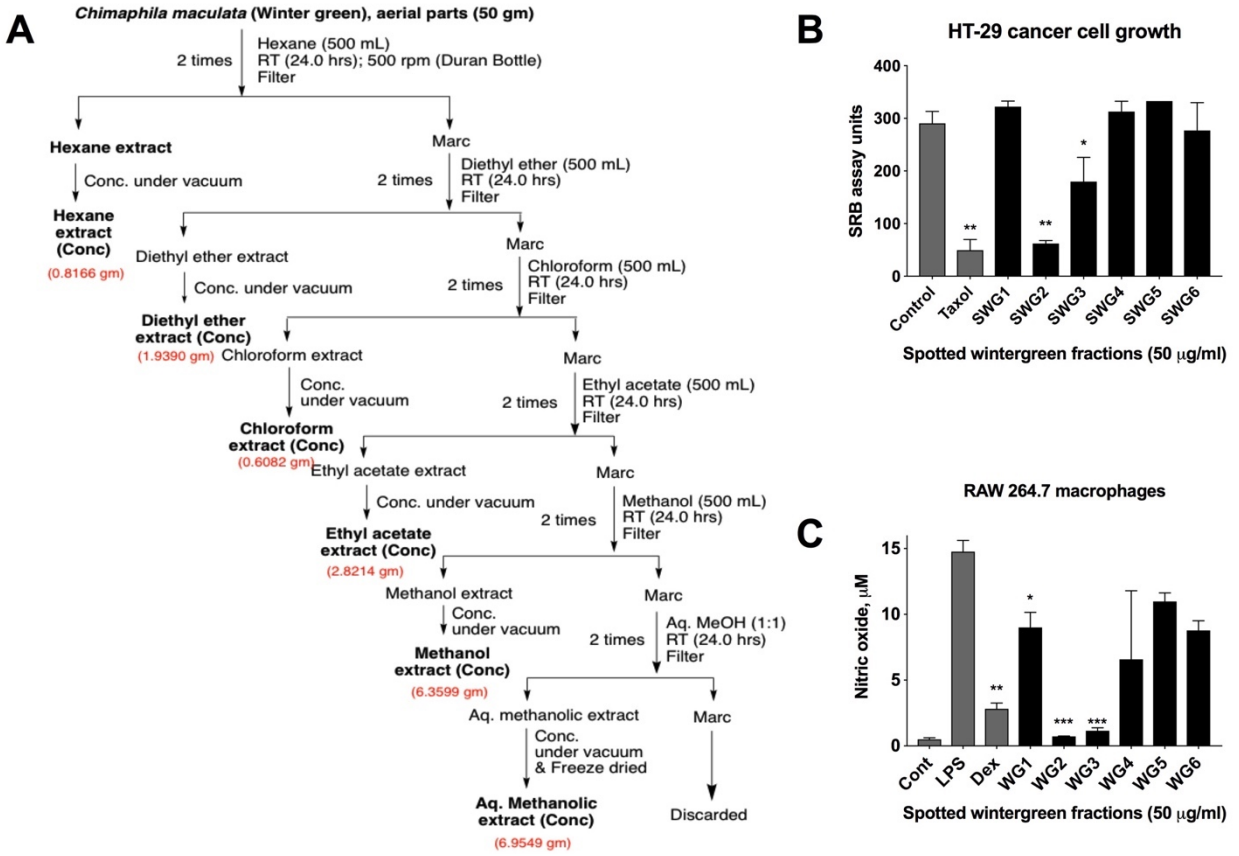

Figure 2.2. Schematic fractionation and biological activity of spotted wintergreen. (A) Dried plants were subsequently extracted with six solvents of increasing polarity. SWG fractions inhibited HT-29 cancer cell proliferation and (C) inflammatory nitric oxide production following the LPS stimulation of Raw 264.7 macrophages.

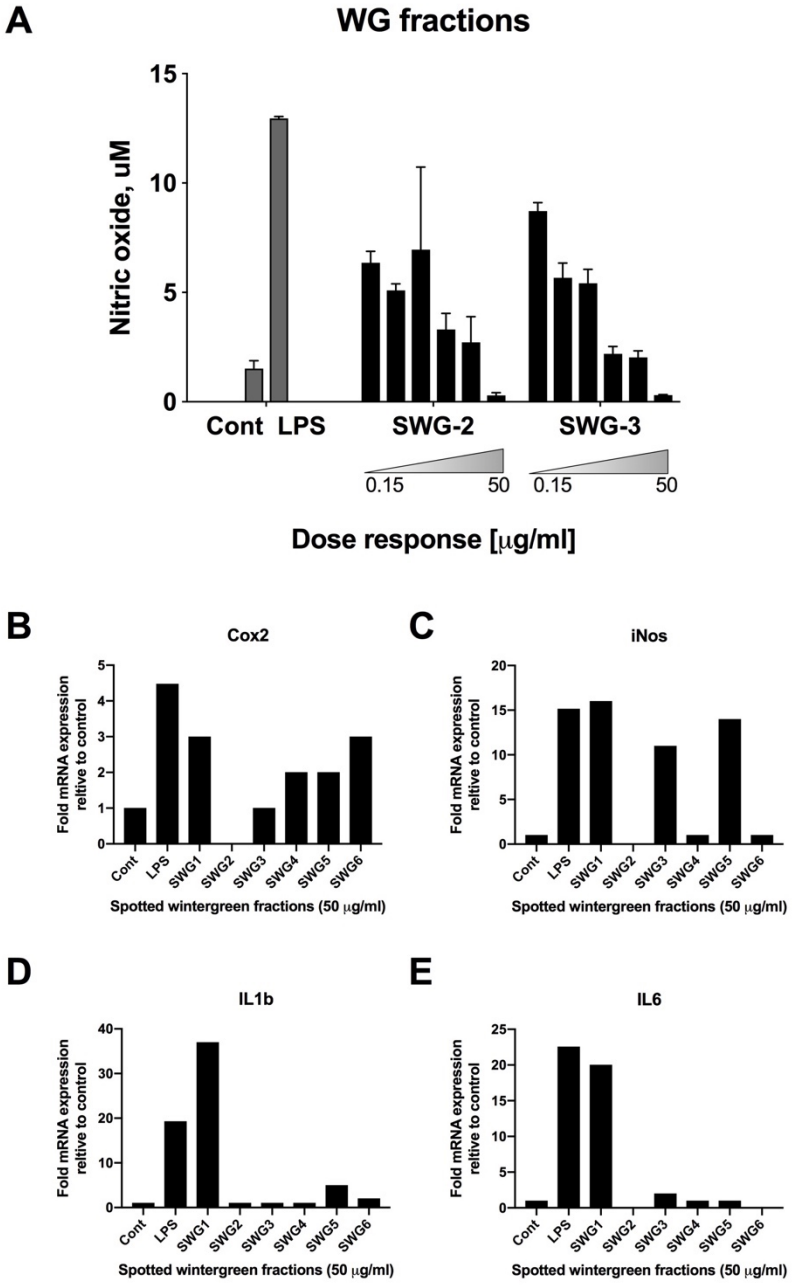

Figure 2.3. Anti-inflammatory effects of spotted wintergreen. (A) Lipophilic fractions 2 and 3 showed dose dependent inhibition of nitric oxide production in RAW 264.7 macrophages. Effects of SWG fractions on the target inflammatory genes cox2 (B), iNOS (C), IL-1b (D), and IL6 (E) implicated in initiation and propagation of the inflammatory response.

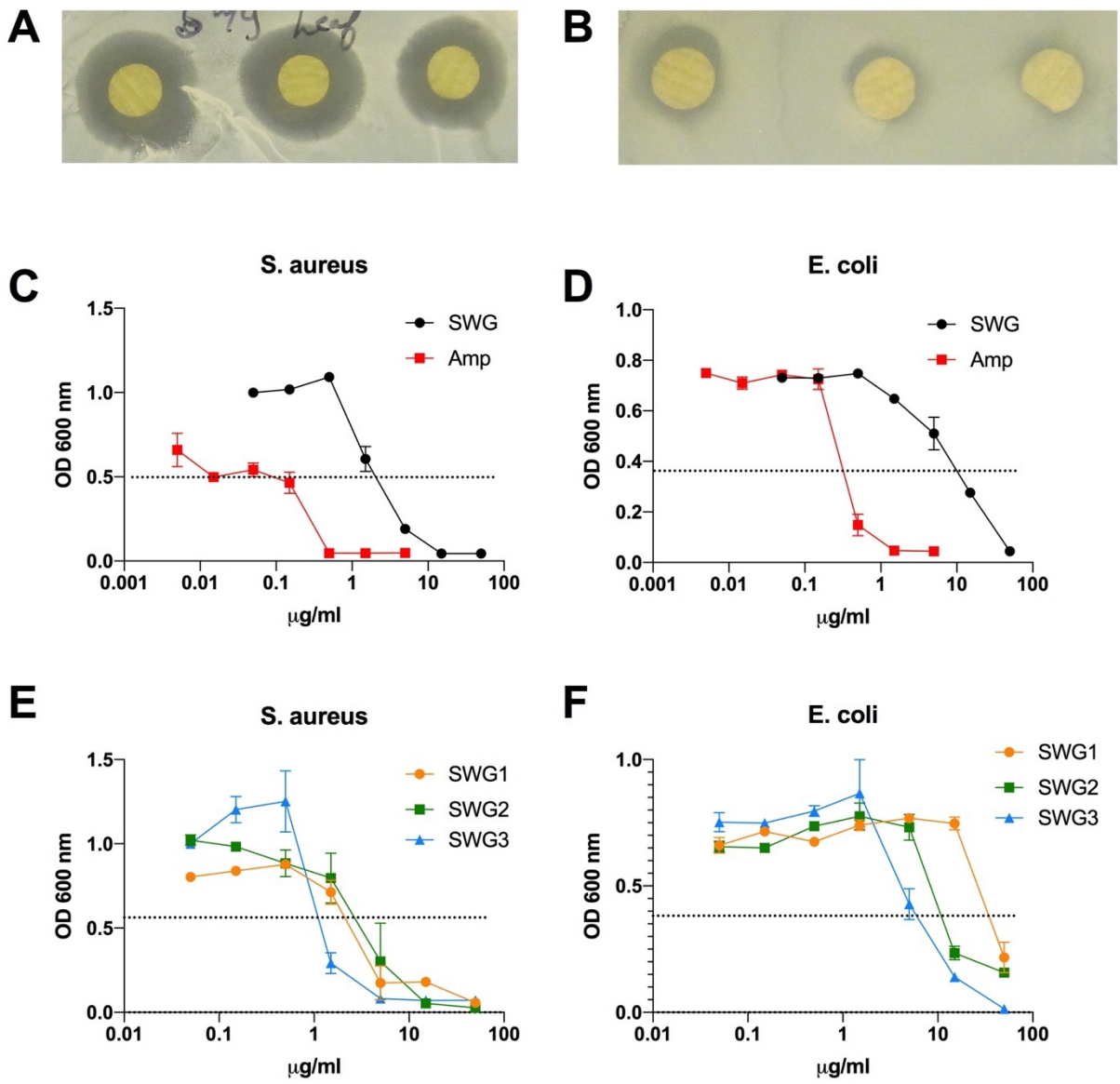

Figure 2.4. Antimicrobial effects of spotted wintergreen and its fractions. Disk diffusion assays against (A) gram-positive *S. aureus* and (B) gram-negative *E. coli* microorganisms. Growth inhibition curves of the spotted wintergreen crude methanolic extract (C-D) and bioactive fractions 1-3 (E-F) as compared to the reference antibiotic ampicillin.

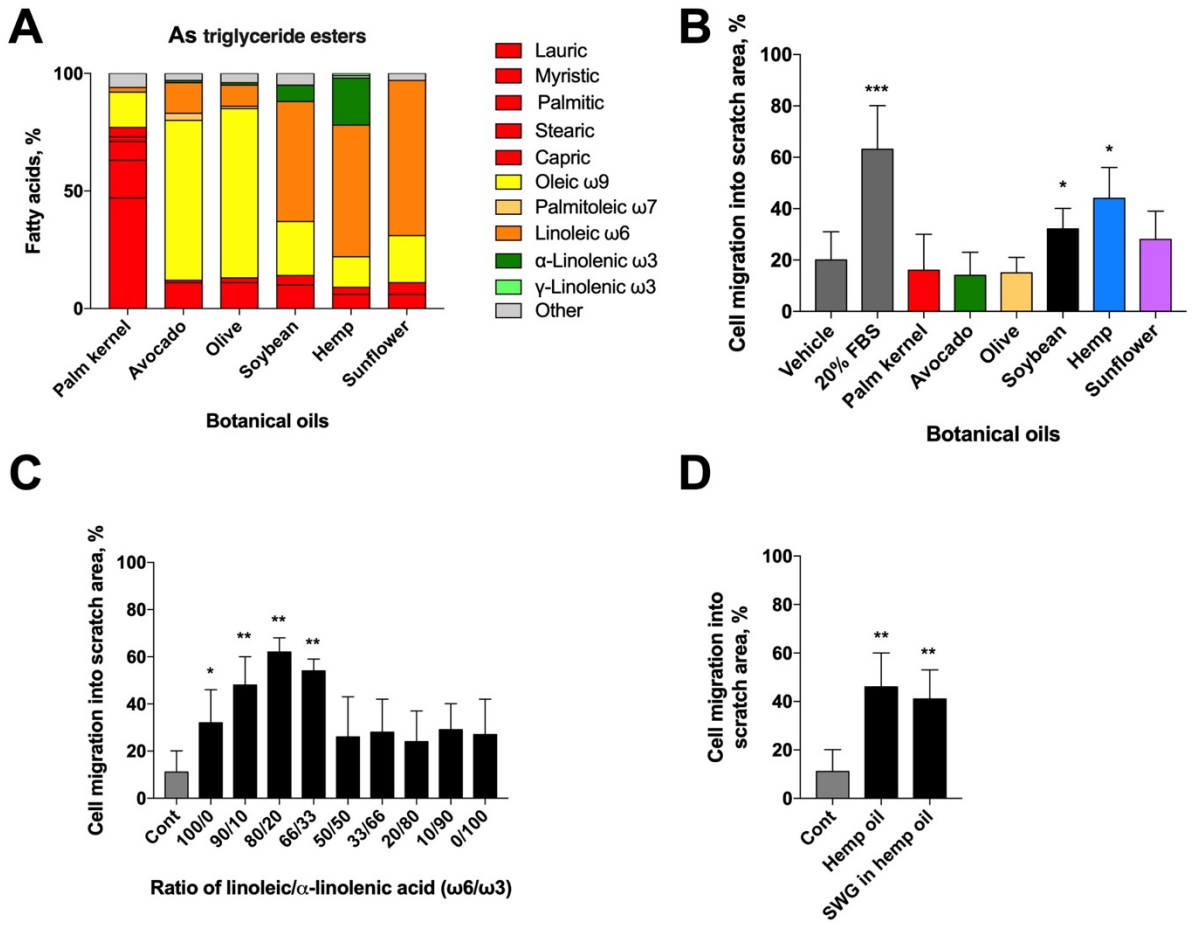

Figure 2.5. Use of hemp oil as botanical carrier oil for spotted wintergreen bioactives. (A) Fatty acid profile of six botanical (vegetable) oils used in this study. (B) Changes in migration into the scratch wound area of fibroblast cells in response to botanical oils of different saturation. (C) Mixtures of omega 6 (linoleic) and omega-3 (linolenic) acids and their effect on fibroblast migration *in vitro*. (D) Spotted wintergreen bioactives were incorporated into the carrier hemp oil at 1% with no detrimental effects on fibroblast migration. Values are means±SEM, *P<0.05 (n=3).

CHAPTER 3

CLASSROOM SIMULATION TO TEACH SAFE USE OF CBD-RICH HEMP OIL

Abstract

Classroom simulation as a method to teach evaluation of efficacy and safety of botanical oils for skin care applications can stimulate students to develop scientific reasoning and increase their knowledge by providing additional resources and materials that relate to differential bioactivity and potential overuse toxicity associated with their topical application to the skin. As a nonintoxicating, pharmacologically relevant constituent of hemp oil, cannabidiol (CBD) exerts a number of skin health promoting effects, including anti-inflammatory and neuroprotective activity. While CBD-rich hemp oil is widely touted as a miracle panacea to manage skin ailments, hemp tissues used to produce hemp oil may become contaminated by THC-rich trichomes of hemp flowers and thus acquire THC. In this study, we investigated the use of role play and scenarios to educate students about the therapeutic potential of CBD-rich hemp oil and promote active learning, critical understanding, teamwork, and leadership towards addressing the putative side effects associated with its use. The scenarios reproduced skin care situations in the beauty salon industry which allowed us to evaluate and discuss exposure to *Cannabis*-derived CBD and THC. Four additional fact sheets focused on dermatological, toxicological, neurological and psychiatric aspects of *Cannabis* use assisted with reflections and debriefing of the students. The strategy presented here can facilitate the teaching-learning process of students, and can be easily transferred and applied in other educational institutions for the safe and effective method to study application of botanical oils to skin care.

Introduction

The use of a classroom simulation as a teaching technique is widely encouraged due to its ability to promote active learning, critical understanding of the topic at hand, reasoning, decision making, teamwork and leadership. Considering the recent recognition of CBD-rich hemp oil in the general population, consumer industry, and medical environment, classroom simulation can be an effective form of teaching in undergraduate scientific and medicinal courses.

CBD-rich hemp oil is a complex botanical mixture originating from *Cannabis sativa* L. Studies have shown that students are unable to distinguish the derivatives of *Cannabis* (Atakan, 2012). Furthermore, students often associated four derivatives of *Cannabis* interchangeably: hemp, medical CBD, medical THC and marijuana. As a result, difficulties have arisen when understanding the usage and benefits of hemp vs. medical CBD vs. medical THC vs. marijuana. Not everyone who used the derivatives of cannabis were affected in the same way – a common misconception common among consumers (Atakan, 2012). There is a need for the creation of courses and/or stimulations focused on improving students understanding of this subject.

Marijuana term refers to *Cannabis* flower and leaf tissues that contain high amounts (~20%) of THC and low amounts (~2%) of CBD. Marijuana can be used both recreationally and medicinally. There are many negative insinuations associated with THC, many of which lead to psychological and psychotic disorders. On the other hand, hemp, also commonly referred to as industrial hemp, is a selected group *Cannabis* cultivar that contain high amounts (~15%) of CBD and less than 0.3% of THC (Brutlag & Hommerding, 2018). Uses of industrial hemp include: hemp oil, food, and clothing. Industrial hemp can be refined to create CBD-rich hemp oil. Understanding how CBD-rich hemp plants are agriculturally bred is a good way to differentiate CBD from THC and how it might affect the body. CBD-rich hemp oil can be extracted in three different ways: CO_2 method, oil method, and ethanol method.

Given this relationship, classroom simulation as a method to teach can stimulate students to understand and differentiate the various derivatives of *Cannabis sativa* L, to develop clinical application and increase the confidence of their knowledge on CBD-rich hemp oil specifically. The preparation of scenarios and role play is a crucial component for success of the simulation, and discussion of each role play among groups is important for educational purposes (Almeida et al., 2015). Based on the need to incorporate new methods in the teaching and learning environment on this subject, a case study classroom simulation has been developed for undergraduate students in scientific and medicinal courses.

Methods

This study reports the experience of creating and utilizing a case study for classroom simulation for undergraduate students; the simulation was developed as part of the activities of the undergraduate and graduate Food Chemistry and Bioprocessed Materials course at the North Carolina State University in 2018. The objective of the case study was to provide a template for undergraduate students in scientific or medicinal courses to develop scientific application and decision-making when assessing CBD-rich hemp oil. Those responsible for assessing the simulation were an MS student in a class setting with eight peer graduate students, and a professor with expertise in food science. The scenario was applied to undergraduate students who were in their third and fourth years in either food science, nutrition, or bioprocessing. This activity can be further explored in other scientific and medicinal courses.

For transfer and application of this simulation to other educational institutions, the facilitator of the activity will be responsible for providing key information during the scenario to guide students in scientific reasoning and subsequently, overseeing a questionnaire. The participants of the case study will be divided into groups and assigned a health professional involved in the study: dermatologist, toxicologist, neurologist or psychiatrist. One person in each

group will then take on the role of the health professional. This allows students to actively participate, make choices, receive feedback, and refine their understanding of the concept while adding dramatization to make the experience seem more real (Cogo et al., 2017). Subsequently, students will be given an additional role and asked to determine the efficacy of using CBD-rich hemp oil in the following health-related outcomes: psoriasis, epilepsy, psychosis, and pregnancy. Among the expected results from the classroom simulation, we focused on active learning, reasoning among groups, post-simulation discussion and decision-making on the use of CBD-rich hemp oil.

Results

In the scenario of using of CBD-rich hemp oil in a tanning bed, the objective was to evaluate what happened to the individual – Stevie. The student/group(s) had to investigate the symptoms the individual experienced, identify the factor that caused the symptoms and discuss the results. Students used the fact sheets provided to identify the relationship among the symptoms and CBD-rich hemp oil, if any. Subsequently, students were asked to evaluate an additional factor and determine the therapeutic potential of using CBD-rich hemp oil in each instance. During the debriefing the participants had the opportunity to reflect on their potential actions, skills, decision-making and the possible treatments that should be used in that case.

Case Study Setup Dialogue: Is using 'CBD rich hemp oil' right for Stevie?

A local tanning salon in Winston-Salem. It's around 1:00 in the afternoon. The tanning salon owner stocks the shelves with new products. A young woman enters the salon.

STEVIE
Hello! I'd like to lay in the level three tanning bed for twelve minutes.

SAMANTHA
Hi Stevie, good to see you again! I turned bed ten on for you. Would you be interested in trying our new product, CBD-rich hemp oil? By using hemp oil, it will reduce the chance of getting dry skin due

to UV exposure.

STEVIE
I have noticed my skin is dry each time I leave the
Salon …. let's give it a try.

While lying in the tanning bed, Stevie begins to feel weird, as if she was suffocating. Her skin also began to itch. She realized she was experiencing an anxiety attack and was having an allergic reaction. Stevie ends her tanning session and rushes out of the tanning salon.

SAMANTHA
…. Stevie, are you okay? screamed Samantha
as Stevie sprinted out the door.

When Stevie got home, she was reluctant to tell her parents. Her parents already frowned upon the use of tanning bed and she felt dumb listening to the tanning salon owner without knowing the side effects of CBD-rich hemp oil. She decided to do research on her own using the Internet – Stevie wanted to find out exactly what CBD-rich hemp oil consisted of.

CBD is a compound known as cannabinoid found in *Cannabis sativa* L (Scalzo & Rivera-Sepulveda, 2018). Humans produce endogenous cannabinoids that bind to cannabinoid receptors. CBD produces effects in the body by attaching to two receptors, CB1 and CB2. Nerve cells within the brain express CB1 receptors that deal with movement, pain, emotions, mood, thought processing, and memories. CB2 receptors are located in the immune system and are linked to inflammation and pain (Bonaccorso et al., 2018). Potential benefits of CBD hemp oil include: natural alternative to managing pain, inflammation, and epilepsy.

During her research, Stevie noticed that all articles on CBD hemp oil were recently published, indicating its recent recognition in the general population and medical environment. Frustrated and unable to explain what had happened to her, Stevie turned to her family for advice and guidance. Stevie was sure that her family members would be able to help her due to knowledge in their fields of study. Stevie's family members include: Tracy (mother and dermatologist), Ivy (aunt and toxicologist), Jack (father and neurologist) and James (uncle and psychiatrist).

TRACY
I have told you over and over Stevie that
using a tanning bed is detrimental to your skin,
now you tell me that you used CBD-rich hemp oil as
a lotion while in the bed? Unreal!

IVY
Interesting…. injected aunt Ivy. Stevie, has your
skin been itching? What other symptoms have
you been experiencing? Tell me everything.
I cannot wait to further research this.

JACK

Stevie, CBD can amplify feelings of anxiousness and paranoia…. have you previously felt anxious or paranoid prior to this incident? Maybe CBD altered the activity of CB1 receptor….

JAMES

Exactly my thoughts. Stevie, I think its time we schedule a session.

Case Study Analysis and Role Play

The case study was organized in the form of four discussion groups that focused on different dermatological, neurological, psychiatric and toxicological aspects of using CBD-rich hemp oil for skin health applications. Subsequently, each group was assigned an additional factor that should be taken into consideration. The additional factor correlated with the family members field of study. The study will follow the outline as described in **Chart 4.1.**

Table 3.1. Outline for group discussion and role play.

Time	Action expected from participants	Role Play
0-9 min	Break into four groups.	Each group will take on the role of one of the family members. Each group will be given a fact sheet summarizing the positive and negative effects of CBD rich hemp oil in relation to their field of study. Choose a group leader.
10-19 min	Read over fact sheets.	Discuss fact sheet, health conditions and answer the questions. Do not share any information with the other groups (i.e. family members).
20-24 min	Read over additional factor that correlates with the family members field of study.	Discuss additional factor among your group.
25-34 min	Discussion.	Discuss whether Stevie should use CBD rich hemp oil going forward and whether CBD rich hemp oil is appropriate to use with the 'additional factors.' Try to convince the entire group that your viewpoint on CBD rich hemp oil is the most reasonable. Come to an agreement with justification.
35-50 min	Reconvene as a class.	Group leader will present your groups viewpoint on Stevie's situation and the additional factor.

Background information on four additional factors

Group One: Psoriasis

Stevie's sister, Sydney, uses CBD-rich hemp oil on her skin for the treatment of psoriasis. Having noticed similar scaly plaques appearing on her own skin, Stevie thinks it might be beneficial to use CBD-rich hemp oil for treatment as well.

Group Two: Epilepsy

Your one-year-old cousin suffers from a rare epileptic seizure known as Dravet syndrome (DS). The medications used for treatment are often times ineffective when controlling the episodes. Dravet syndrome is a genetic condition that appears early in life involving frequent, fever-related seizures (Gaston & Szaflarski., 2018). CBD (Epidiolex) was recently approved by the FDA for controlling DS episodes; however, there are age restrictions for use of CBD-rich hemp oil on individuals with DS. Is using CBD-rich hemp oil an effective form of treatment?

Group Three: Psychosis

CBD-rich hemp oil can reduce psychotic, anxiety and withdrawal symptoms through the modification of neurotransmitter signaling and functional cerebral changes (Mandolini et al., 2018).

Group Four: Pregnancy

You are a three-week pregnant female with no history of any health conditions. You have been told that marijuana use during pregnancy can cause impaired fetal development of neurons. In rare instances, some commercially marketed hemp oils could lead to mild cannabinoid poisoning in children and pregnant women (Yang et al., 2017). If using CBD-rich hemp oil while pregnant, will there be similar adverse effects?

Role-Play Fact Sheet for Tracy (Dermatologist)

In the human body, skin is one of the largest organs. Skin has two distinct layers – the epidermis and dermis. The epidermis consists of tightly packed keratinocyte layers and serves as a protective barrier against bacterial contact and environmental interactions. Resident immune cells also help to maintain this protective barrier (Eagleston et al., 2018). The dermis is composed of collagen, adaptable fibers, and an assortment of extracellular matrix proteins (Chelliah et al., 2018). Dermal layer also contains blood and lymphatic vessels, nerve endings, fibroblasts and a mixture of immune cells (Hashim et al., 2017). Overstimulation of immune cells in the skin can promote excess cellular permeation and apoptosis, resulting in inflamed skin and autoimmune disorders.

Autoimmune disorders of the skin often lead to secondary complications such as open wounds/cracks in the skin and bacterial infections. Until recently, steroids have been the most effective form of treatment, but their long term use ultimately damages the skin. The prevalence of topical cannabinoids, specifically CBD rich hemp oil, has risen sharply in clinical dermatology. CBD rich hemp oil offers a safe and natural means to manage atopic dermatitis (AD), psoriasis, acne vulgaris and skin cancer (Hashim et al., 2017).

Atopic dermatitis, commonly referred to as eczema, is the itchy inflammation of the skin. The main symptom is a rash that appears on the arms and behind the knees. Recent studies have suggested that atopic dermatitis can be managed by cannabinoids and topical agonists of cannabinoid receptors (Hashim et al., 2017). Psoriasis is an inflammatory disease characterized by raised scaly skin, erythema, and hypergenesis (Chelliah et al., 2018). The course of disease is often unclear. CB2 receptor agonist reduced recruitment of mast cells and a decreased blood concentration of histamine suggesting that CBD can be a useful topical agent for skin conditions related to mast cell activation (Hashim et al., 2017).

Acne vulgaris is a condition that occurs when skin cells plug hair follicles. The hair follicles are blocked by oil and dead cells (Eagleston et al., 2018). A single randomized clinical study reported the effects of 3% cannabidiol infused cream on acne vulgaris (Ali et al., 2015). When applied twice a day for 8 weeks to infected areas, the CBD cream significantly decreased the acne present in comparison to a control group.

Abnormal growth of skin cells can lead to skin cancer. There are three common types: squamous cell carcinoma, basal cell carcinoma, and melanoma. Squamous cell carcinoma is caused by the uncontrollable growth of aberrant squamous cells (Parekh & Seykora, 2017). Basal cell carcinoma begins in the basal cells. Basal cells function to produce new skin cells and lie underneath squamous cells (Pellegrini et al., 2017). Melanoma is the most aggressive and serious type of skin cancer (Ko. 2017). Melanoma, basal, and squamous cell carcinoma express cannabinoid receptors, CB1 and CB2. CB2 plays a greater role in inducing apoptosis of cancer cells than CB1. CB2 decreases the expression of endothelial growth factor, inhibits melanoma progression, and reduces cancerous cells ability to metastasize (Eagleston et al., 2018).

Table 3.2. Debriefing – Dermatologist fact sheet.

Questions to discuss among Tracy's group	
1	Is it possible to experience adverse symptoms if using CBD-rich hemp oil for the first time on your skin?
2	Is CBD-rich hemp oil anti-microbial?
3	Which type of skin cancer, squamous, basal or melanoma is CBD-rich hemp oil most effective in treating?

Role-Play Fact Sheet for Ivy (Toxicologist)

Over the past decade, the legality of marijuana, both recreationally and medically has changed dramatically across North America, specifically in the United States and Canada. Some states allow for the recreational use of cannabis whereas others strictly limit cannabis for medicinal purposes (Brutlag & Hommerding, 2018). Recently, Canada passed a law to allow both recreational/medicinal use of cannabis. The legality of transporting cannabis across state borders for medicinal and research purposes has recently changed in the United States.

There are four different forms of cannabis: marijuana, hemp, medical CBD and medical THC. Of the four, medical CBD is the only one that has no psychoactive properties. The THC concentration present in marijuana is 15% whereas the concentration of CBD is less than 0.3% (Brutlag & Hommerding, 2018). If asked to take a drug test while using CBD, whether orally or topically, there would be no trace amounts of THC present in the urine. In relation to its constituents and physiological properties, cannabinoids are complex plants that can have opposing effects. Cannabinoids chemical profile and pharmacology have yet to be fully understood. Containing oxygen and aromatic hydrocarbon compounds, cannabinoids compose 70 of the 400 constituents in *Cannabis sativa* L (Inci et al., 2017).

Three recent reviews assessed the safety and efficacy of CBD. One study concluded that CBD-rich hemp oil does not alter food intake, affect heart rate, blood pressure, and body temperature even with repeated use at doses as high as 1,500 mg a day (Iffland & Grotenhermen, 2017). However, in vitro and animal studies on the effects of CBD hemp extract suggest that at high doses of cannabinoids, adverse toxicological effects occurred (Marx et al., 2018). Another observation is the that “plant-based” and “purified” CBD-rich hemp oil can be used at different doses. Individuals using a plant-based extracts of CBD reported using a significantly lower dose in comparison to purified CBD (Pamplona, da Silva, Coan., 2018).

When using CBD-rich hemp oil, adverse effects can occur. Individuals report experiencing adverse effects when using purified CBD-rich hemp oil instead of plant-based extracts. The most common reported adverse effects include: appetite changes, sleepiness, gastrointestinal discomforts, diarrhea, weight changes, fatigue, and nausea (Pamplona, da Silva, Coan., 2018). When paired with other drugs, further adverse effects occurred, specifically when used on individuals with epilepsy. Direct CBD toxicity should not be associated with adverse effects, but drug interaction. Further studies are needed to assess the toxicological effects of CBD extract.

Table 3.3. Debriefing – Toxicologist fact sheet.

Questions to discuss among Ivy's group	
1	At what dose are cannabinoids considered toxic? Does this apply to both animals and humans?
2	What percentage of CBD is found within CBD-rich hemp oil?

Role-Play Fact Sheet for Jack (Neurologist)

CBD produces neurological effects in the body by modulating CB1 cannabinoid receptors. CB1 cannabinoid receptors are located in the motor system, cortex, limbic system, amygdala and hippocampus. CB1 receptors augment the release of presynaptic dopamine (Bonaccorso et al., 2018). Recent studies suggest CBD hemp oil as an effective solution for treating multiple neurological diseases such as epilepsy, Alzheimer's disease and Parkinson's disease.

Epilepsy is a neurological disorder characterized by episodes of sensory disturbance, episodes of unconsciousness, unusual electrical activity in the brain, resulting in epileptic seizures (Gaston & Szaflarski, 2018). Although there is no cure for epilepsy, medications,

surgery, devices, or dietary changes can offer some relief. In two specific epileptic conditions, Lennox-Gastaut syndrome (LGS) and Dravet syndrome (DS), medications are often times ineffective (McCoy, 2018). After reviewing the safety and efficacy of CBD-rich hemp oil for treating epilepsy, the FDA approved the use of CBD (GW Pharmaceutical's Epidiolex) for both Lennox-Gastaut syndrome and Dravet syndrome (McCoy et al., 2018). Use of Epidiolex for individuals with epilepsy helps to alleviate pain associated with seizures and can reduce further neurodegenerative conditions associated with epilepsy (Pamplona, da Silva, Coan., 2018).

Alzheimer's Disease (AD) is a neurodegenerative disease. The accumulation of beta-amyloid plaques and tau tangles destroy neuron connections within the brain (Watt & Karl, 2017). Damages in cortex and hippocampus results in impaired memories. Currently, there are no forms of treatment that stop or reverse impairments caused by AD. However, CBD has been investigated as a potential treatment option for AD. Research suggests that CBD was able to prevent the development of social recognition deficit in individuals with Alzheimer's disease. CBD could help individuals with Alzheimer's to recognize familiar faces (Watt & Karl, 2017). This is the first evidence that suggests CBDs ability to slow the detrimental progression of Alzheimer's disease.

Parkinson's disease is a central nervous system (CNS) disorder that affects movement. Symptoms are gradual, starting with a slight tremor in one hand (Crippa, 2018). Although tremors are a common symptom, stiffness or slow movement can also occur (Ganos, 2018). CBD may help to improve motor impairment in individuals with Parkinson's disease. CBD can also target psychotic symptoms of individuals with Parkinson's disease (Crippa, 2018). CBD modulates cell fate regulatory pathways such as autophagy for neuronal survival in neurodegenerative experimental models, suggesting the potential benefit of CBD treatment for psychiatric/cognitive symptoms associated with neurodegeneration.

Table 3.4. Debriefing – Neurologist fact sheet.

Questions to discuss among Jack's group	
1	At what age is it safe to use Epidiolex on children with Dravet syndrome? Is the dosage body weight dependent?
2	How does CBD-rich hemp oil help individuals with epilepsy?
3	During what stage of Alzheimer's Disease can CBD-rich hemp oil help individuals recognize faces?
4	What psychotic symptoms can CBD-rich hemp oil target in individuals with Parkinson's disease?

Role-Play Fact Sheet for James (Psychiatrist)

Fear and anxiety are adaptive responses essential to managing threats to survival. The autonomic nervous system works to adapt and respond to your immediate environment. During high emotions, the sympathetic nervous system produces a 'fight or flight' response (Richards, 2017). During feelings of relaxation, the parasympathetic nervous system is activated, lowering your heart rate and blood pressure (Richards, 2017). This necessary and adaptive system is responsible for feelings of anxiety. Marijuana and THC infused products have been known to trigger or amplify feelings of anxiousness and paranoia. However, CBD rich hemp oil is gaining major traction in the medical world, as people praise its ability to reduce anxiety in people with certain related disorders.

Generalized Anxiety Disorder (GAD) is defined as the persistent and excessive worry that interferes with daily activities. Areas in the brain involved with emotional processing and heightened amygdala response activation are often associated with anxiety disorders, such as general anxiety disorder (Noel, 2018). Acting as an agonist to 5-HT_{1A} receptor and $GABA_A$

receptor, CBD rich hemp oil decreases heart rate and blood pressure, ultimately reducing the anxiety-related behavior associated with GAD (Resstel et al., 2009).

Stress and experiencing a traumatic event lead to the development of post-traumatic stress disorder (PTSD). Individuals diagnosed with PTSD may feel anxious or scared even when they are not in danger (Blessing et al., 2015). The systemic administration of CBD rich hemp oil reduces the acute increase in heart rate and blood pressure induced by stress. These anxiolytic effects depend upon CB1 receptor activation (Bonaccorso et al., 2018). CB1 receptors in the brain deal with movement, pain, emotions, mood, thought process, and memories.

Social anxiety disorder is an intense fear of being judged, evaluated negatively, or rejected in a social environment. When validating the effects of CBD on social anxiety disorder, using a Simulated Public Speaking Test (SPST) predominates because the fear of public speaking is a prime example of the illness. It is suggested that CBD's anxiolytic action may be mediated by the 5-HT_{1A} receptors (Noel, 2018).

Table 3.5. Debriefing – Psychiatrist fact sheet.

Questions to discuss among James group	
1	At what dose of cannabidiol is needed to produce the desired anxiolytic effect? Would this dose differ among various anxiety-related conditions?
2	How long would CBD rich hemp oil have to be administered before there is a decrease in anxiety-related conditions?
3	If paired with other medications, will CBD rich hemp oil cause an adverse effect?

Final Dialogue: Possible Explanation of Toxic Effects

JAMES
Stevie, where is the bottle of CBD-rich hemp oil you purchased and used from the tanning salon?

STEVIE
It's in my room, let me go grab it.

Stevie exits the room and returns moments later with the bottle of CBD-rich hemp oil. Tracy, Jack, Ivy and James inspect the bottle closely, specifically the ingredient label.

IVY
Ahh, I see now! Stevie, you were misinformed by the tanning salon owner. It looks as if this CBD-rich hemp oil is infused with THC as well. Although CBD-rich hemp oil is anxiolytic, if coupled with THC, adverse effects can occur.

JAMES
Your mental state and the environment you're in when using THC can greatly affect how you might react. Perhaps laying in the tanning bed coupled with THC heightened your feelings, emotions and mood.

JACK
Stevie, I have to agree with your aunt and uncle. I believe using this product is the underlying cause of your anxiety attack and feelings of claustrophobia.

Discussion

Classroom simulation is an effective form of teaching. Using this method allows students to engage in critical thinking, work as a team and develop leadership skills. The construction and use of a classroom simulation are valuable approaches that must be based on scientific reasoning, clinical evidence, reviewed by peer graduate students and undergo a series of trial runs. Thus, the construction and formatting of a case study used in classroom simulation must by paired with feedback from students, peers, facilitators and professors. A carefully formatted case study and stimulation combination enhances learning through processing, adaptation, and discussion (Almeida et al., 2015).

In practice, students are able to enhance their understanding of CBD-rich hemp oil and its application to human health. The facilitators must be familiar with the topics discussed in the

case study before contributing to the classroom simulation. During the simulation, the facilitators must be available to answer questions and help guide the students towards the desired goal. Therefore, formatting the case study to reach the intended goal is imperative, including adding dramatization to the dialogues to make the experience seem more real and clearly defining the objectives. The use of role play, fact sheets and additional factors for each group to take into consideration comprised the success of the case study and simulation. Subsequently, discussing each groups viewpoint as a class allows for collaboration, debate and in some cases, concession.

The classroom simulation and case study demonstrate the various therapeutic potentials of CBD-rich hemp oil. Although CBD-rich hemp oil is a newly discovered phenomenon and remains a Schedule I controlled substance, treatments using CBD-rich hemp oil are effective and safe in relation to atopic dermatitis, psoriasis, acne vulgaris, skin cancer, epilepsy, Alzheimer's disease, Parkinson's disease, generalized anxiety disorder, post-traumatic stress disorder, and social anxiety disorder.

CBD-rich hemp oil can act as an anti-inflammatory and anti-bacterial agent, two crucial events that can prevent mast cell activation in individuals with psoriasis and atopic dermatitis (Hashim et al., 2017). Also corresponding to inflammation, acne vulgaris can be treated with CBD-rich hemp oil; the production of sweat by the sebaceous glands is lowered, leading to a reduced accumulation of acne (Ali et al., 2015). The spread of skin cancer can be prevented by use of topical CBD-rich hemp oil; cancer cells undergo induced apoptosis and are unable to metastasize (Eagleston et al., 2018). In epileptic conditions, CBD-rich hemp oil can put individuals at ease during seizures and help to lower neurodegeneration associated with epilepsy (Pamplona, da Silva, Coan., 2018). The majority of the individuals used in each study obtained substantial benefits from the use of CBD-rich hemp oil and if adverse effects were to occur, they were mild and minimal.

Conclusion

The construction of scientific simulation scenarios focused on the evaluation of botanical oils and their application to skin health allowed the students, as well as the teachers, to develop scientific reasoning more accurately, thus, promoting safer skin care. The participants could live situations close to real ones in a controlled classroom environment, thus, being able to perform tasks and procedures without causing harm to consumers. Given the growing number of consumers with interest in hemp-and marijuana-based products, and the availability of new botanical extracts for management of skin ailments, there is a critical need for courses and teaching strategies that effectively promote learning, assist in the preparation of students and professionals, and ensure the cost-effectiveness when choosing the treatment.

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