Topical Formulation Comprising Fatty Acid Extract from Cod-liver Oil: Development, Evaluation and Stability Studies

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MS thesis in Pharmaceutical Sciences

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May 2016

Þróun útvortis lyfs sem inniheldur fitusýrur unnar úr þorskalýsi

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Meistararitgerð í lyfjavísindum

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Lyfjafræðideild

Heilbrigðisvísindasvið Háskóla Íslands

Maí 2016

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Printing: Háskólaprent
Reykjavik, Iceland 2016

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Ágrip

Tilgangur rannsóknarinnar var að þróa lyfjaform úr fiskiolíu sem væri rík af omega-3 fríum fitusýrum, s.s. EPA og DHA til staðbundinnar notkunar á húð.

Sýnt hefur verið fram á með ótal rannsóknum og það almennt viðurkennt, að dagleg inntaka þorskalýsis og annarra fiskiolía hefur jákvæð áhrif á heilsufar fólks og því ljóst að líffræðileg virkni þeirra er hefur ekki verið rannsökuð til fulls.Samkvæmt in vitro rannsóknum eru fríar fitusýrur (20% (v / v)) mjög öflugar gegn gram-jákvæðum bakteríum á borð við *Staphylococcus aureus, Enterococcus faecalis, Streptococcus pyogenes* og *Streptococcus pneumoniae* og með því að vinna fjölómettaðar fitusýrur (PUFA) úr þorskalýsi eða öðrum fiskiolíum er möguleiki að þróa gagnlegt, öruggt og náttúrulegt sýklalyf.

Algengt er að nota ýmis afbrigði af náttúrulegu og tilbúnu vaxi í snyrtivörur, lyf og matvæli til að bæta áferð og útlit vörunnar. Frá lyfjafræðilegu sjónarmiði, er notkun á vaxi viðurkennt til að bæta útlit, uppbyggingu og seigjustig húðlyfja auk þess sem vax getur lengt geymslutíma.

Í þessari rannsókn voru þróuð smyrsl þar sem virka efnið er fríar fitusýrur ríkar af omega-3 fitusýrum. Fríar fitusýrur hafa bakteríu-, veiru- og sveppadrepandi áhrif auk þess sem þekkt eru bólgueyðandi áhrif omega-3 fitusýra. Hjálparefni sem notuð voru eru ýmsar tegundir af náttúrulegu vaxi.

Áhrif mismunandi tegunda vaxs s.s. carnauba, ozokerite, laurel, býflugnavax, hrísgrjónaklíð, candelilla og sellulósa vax, í styrkleika frá 1- 5% (w/w), voru metin m.t.t. áferðar, þéttleika og stöðugleika.

Niðurstöður hvað varðar áferð og skynjun (sensory profile) voru mjög mismunandi háð vaxi og styrkleika. Ástæðan var rakin til eðli vaxins og keðjusamsetningar. Sellulósavax gaf bestan árangur en laurelvax, býflugnavax og hrísgrjónaklíðisvax gáfu einnig mjög góða áferð, þ.e. svipaða skynjun (sensory profile) og viðeigandi seigjustig.

Abstract

The current study was designed to develop a pharmaceutical formulation that contained fish oil extract rich in free omega 3 FAs such as EPA and DHA for topical use. Although the health benefits of cod-liver oil and other fish oils taken as a dietary supplement have been vastly acknowledged and exploited, it is clear that their use could be further extended to cover other biological properties. Based on *in vitro* evaluation, 20% (v/v) fish oil extract was found to be highly potent against gram-positive bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptoccoccus pyogenes* and *Streptoccoccus pneumonia*. Therefore, free PUFAs from cod-liver oil or other fish oils could be useful as safe and natural antibacterial agents.

Today, varieties of natural and synthetic waxes are used in numerous cosmetic, pharmaceutical and food applications for texture enhancement and benefits in sensory profile. Seen from a pharmacological aspect, waxes are recognized as a potential for enhancing aesthetics and maximizing therapeutic benefits on topical formulation by viscosity building and prolonging the retention time.

In this study, ointment compositions containing FFAs as active antibacterial agents were prepared by using various natural waxes and characterized. The effects of different waxes, such as carnauba, ozokerite, laurel, beeswax, rice bran, candelilla and microcrystalline wax, in the concentration range 1 to 5% (w/w) on the ointment texture, consistency and stability were evaluated. The results showed significant variations in texture and sensory profile. This was attributed to the wax nature and chain composition. Microcristalline wax gave the best results but laurel wax, beeswax and rice bran wax also gave excellent texturing, similar sensory profile and well-balanced rheological properties.

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Abbreviations

AA	Arachidonic acid
ALA	Alfa linolenic acid
API	Active pharmaceutical ingredient (i.e. the drug)
AV	Anisidine value
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DGLA	Dihomo gamma linolenic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ETA	Eicosatetraenoic acid
FA	Fatty acids
GLA	Gamma linolenic acid
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acids
LX	Lipoxins
LOX	Lipoxygenase
LT	Leukotrienes
MIC	Minimum inhibitory concentration
MDA	Malondialdehyde
4-HNE	4-hydroxy-2-nonenal
NSAID	Non-steroidal anti-inflammatory drug
PG	Prostaglandins
PV	Peroxide value
PUFA	Polyunsaturated fatty acids
SDA	Stearidonic acid
SC	*Stratum corneum*
TX	Thromboxa

1. Introduction

Omega-3 fatty acids have historically been part of our diet long before their nutritional and medical benefits were known and investigated. However, over the last years and decades the medical interest in fatty acids has grown significantly since the awareness of the importance and benefits of fatty acids has considerably increased.

Presently, polyunsaturated fatty acids (PUFAs) are attracting attention as potential effective medical compounds for various pharmaceutical formulations due to their powerful antimicrobial and anti-inflammatory properties. Considering PUFAs are known to possess powerful antibacterial and anti- inflammatory properties, a potential ground exists for their application in topical pharmaceutical formulations. Additional benefits of using PUFAs as antimicrobial agents are that these compounds are natural and safe. Despite the benefits of synthetic compounds, fish oils rich in PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may successfully replace the synthetic compounds. It is hypothesized that the utilization of fish oil is a potentially great active pharmaceutical ingredients for novel effective pharmaceutical formulations, especially in pharmaceutical products for prevention and treatment of skin diseases.

1.1 Fatty Acids

1.1.1 Chemistry of Fatty Acids

Chemically, fatty acids (FAs) belong to the class of lipids. As a lipid constituent, FAs occur in phospholipids that are essential structural elements of cell membranes, where they are involved in formation and function of permeability barrier of cells. In nature, FAs can either exist in unesterified form as free fatty acids (FFAs), or esterified to glycerol in natural fats and oils. Depending on the number of fatty acids esterified to glycerol, mono-, di- or triglycerides are formed, also referred to as neutral fats or lipid. In biological systems, FAs can be released enzymatically from lipids and become FFAs, which are further metabolized to various bioactive compounds involved in important biological functions [1, 2]. FAs are aliphatic carboxylic acid

with a various lengths of hydrocarbon chain and a terminal carboxyl group (COOH) that is known to give an acid property. Usually, FAs contain an even number of carbon atoms, predominately between 14 and 24, and can be functionally divided into short (C16), medium chain (C18), or long-chain (>C18) fatty acids based on the length of hydrocarbon chain. FAs can also be classified based on the degree of unsaturation as saturated, monounsaturated or polyunsaturated FAs. Saturated FAs contain as their name implies only single bonds, monounsaturated have only one double bond and when FAs have more than one double bond in the hydrocarbon chain they are considered to be polyunsaturated. The configuration of the double bond in an unsaturated fatty acid can be either in *cis* or *trans* form depending upon the orientation of the radicals around the axis of the double bond. In most natural unsaturated fatty acids, the double bounds are dominantly in a *cis* configuration and their presence determines the packing in to the biological membrane. In addition, double bonds introduce a twist in the hydrocarbon chain that influences the structure and physical properties of the fatty acid molecule. For example, one or more of *cis* double bonds interpose the tight packaging, resulting in flexible, fluid states at room temperature, whereas saturate fatty acids are closely packed forming rigid arrays, making them solid at room temperature [3, 4]. There exist over 1000 FAs with different chain lengths, configuration and type of unsaturation, but 20 or less are considered as commercial important, and are exploited in food and industrial use [5].

FAs can be named in at least three manners. An initial is the trivial or common name, for example for FAs having 18 carbon chains with no double bond, the name is stearic acid. The corresponding systematic name, also known as IUPAC, is octadecanoic derived from the name of its parent hydrocarbon (i.e octadecane) by substituting the final e with an oic. In the series of C18 FAs, FAs with one double bond is called octadecenoic acid, with two double bonds octadecadienoic acid, and with three double bonds is octadecatrienoic acid. As systematic names are massive and complex, there are shorthanded names (i.e. C18:0, 18:1, 18:2, 18:3) where the first number shows how many carbon atoms are in the fatty acid molecule, and the number after the colon shows the number of double bonds. There is also an alternative system of naming, that is only used for PUFAs, known as omega (ω) or n signifying the position of the first double bound in the hydrocarbon chain counting from the distal end of the chain [3].

1.1.2 Essential Fatty Acids

In the human body, there are two key families of essential fatty acids (EFAs): omega-3 fatty acids and omega-6 fatty acids. Both omega-3 and omega-6 fatty acids referred to as EFAs because they are necessary for human health, but the body cannot produce them, and must therefore be obtained from exogenous sources. However, not all FAs are considered as EFAs. Some PUFAs can be synthetized by humans [6].

Table1. The systematic and trivial names of PUFAs encountered most often, together with their shorthand designation.

PUFAs	Common name	Shorthand	Systematic name
Omega-3	α-Linolenic acid	18:3 n-3	9,12,15-octadecatrienoic acid
	Eicosatetraenoic acid	20:4 n-3	8,11,14,17-eicosatetraenoic acid
	Eicosapentaenoic acid	20:5 n-3	5,8,11,14,17-eicosapentaenoic acid
	Docosahexaenoic acid	22:6 n-3	4,7,10,13,16,19-docosaxexaenoic acid
Omega-6	Linoleic acid	18:2 n-6	9,12-octadecadienoic acid
	γ-Linolenic acid	18:3 n-6	6,9,12-octadecatrienoic acid
	Dihomo-γ-linolenic acid	20:3 n-6	8,11,14 -eicosatrienoic acid
	Arachidonic acid	20:4 n-6	5,8,11,14 -eicosatetraenoic acid

Two known key exceptions are linoleic acid (LA) and alpha-linolenic (ALA) acid (Fig.1). These two FAs cannot be synthesized in the human body and are designated as "truly" essential. From LA (18:2 n-6) can be produced all other LC-PUFAs (long chain polyunsaturated fatty acids) in omega-6 family, and from ALA (18:3 n-3) all LC-PUFAs in omega-3 family. The human body is not able to produce those FAs because humans totally lack the converting enzyme (desaturase) for inserting double bounds. Unlike humans, plants are able to synthetize *de novo* and interconvert ω-3 and in ω-6 fatty acids family since they are known to possess the required enzyme –desaturase. The other LC-PUFAs in omega-3 and omega-6 families are not necessary considered essential because the human body can produce them modestly through series of

desaturation and elongation reactions possessing required enzymes for lengthening the hydrocarbon chain [7].

Fig. 1. Structure of linoleic acid and α-linolenic acid: the two essential fatty acids which are biosynthetic precursors of omega-6 and omega- 3 families, respectively.

Although, in humans, the long-chain omega-3 fatty acids, EPA and DHA (Fig.2) can be synthesized from ALA, due to low conversion efficiency it is recommended to obtain EPA and DHA via diet or supplementation. For instance, fish is known as an excellent natural source since it contains high amount of omega-3 LC- PUFAs, EPA and DHA. Fish conversion of EPA and, particularly, DHA is relatively efficient, evidencing that fish have required enzymes to convert ALA to DHA and EPA such as Δ5-desaturase and Δ6-desaturase. It also suggests that the dietary fish supplementation represents an efficient alternative to decrease human essential fatty acid deficiency [7-9].

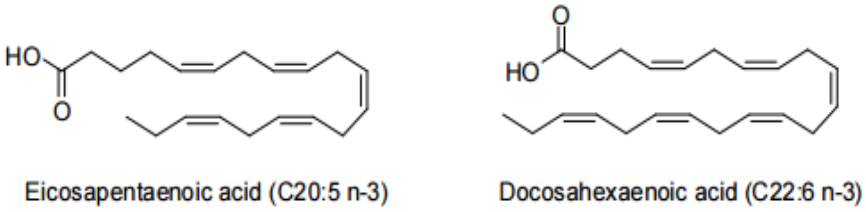


Fig.2. Structures of EPA (5,8,11,14,17-eicosapentaenoic acid) and DHA (4,7,10,13,16,19-docosaxexaenoic acid).

1.1.2.1 Metabolism of EFAs in the skin

The skin is an organ that displays a highly active lipid metabolism. The level and replenishment of FAs in the epidermis is vital for normal epidermal function. The skin possesses enzyme

systems able to metabolize and interconvert a variety of lipids. For example, saturated fatty acids, monoglycerides, cholesterol, and ceramides can be synthesized and modified in the skin [10]. Contrary to other lipids, the EFAs must be obtained from external sources since the skin is unable to produce long-chain metabolites such as GLA, AA, EPA and DHA.

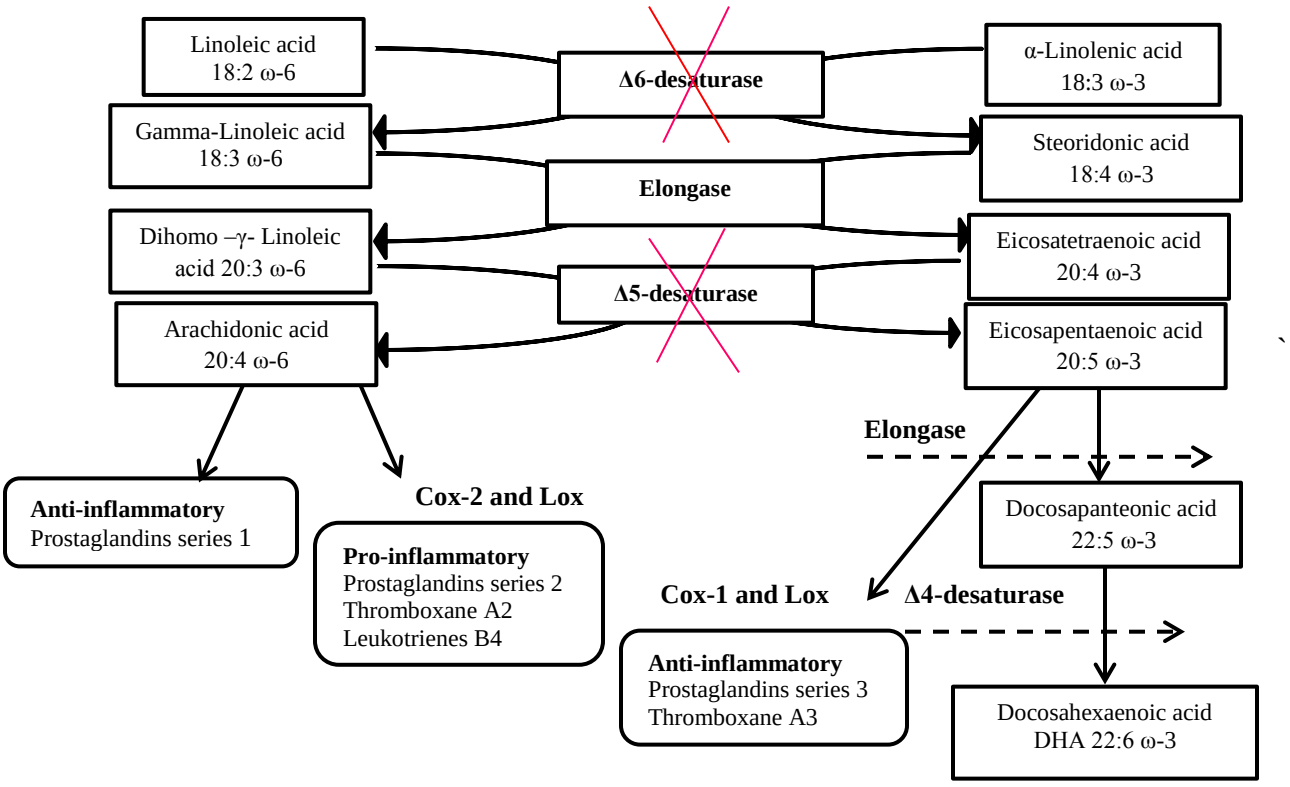


Fig.3. Simplified flow chart represents metabolic pathway of elongation and desaturation of fatty acids in the skin, ALA conversion to DHA and LA to AA by a series of enzymes. Elongase increases the number of carbon atoms, Δ5- and Δ6-desaturase increase the unsaturation by adding more double bonds. Based on [9-14]

As illustrated in (Fig.3), the shorter chain EFAs, ALA, is the initial biosynthetic precursor for *in vivo* synthesis of the longer omega-3 family of fatty acids and LA is the initial biosynthetic precursor for the omega-6 family [7, 9, 14, 15]. The metabolic formation of LC-PUFAs involves a sequence of chain elongation and desaturation steps. The desaturation reactions are catalyzed by two enzymes: Δ5- and Δ6-desaturase, wherein the Δ6-desaturase catalyzes conversion of ALA (18:3n-3) to SDA (18:4 n-3) and the Δ5-desaturase catalyzes conversion of ETA (20:4n-3)

to EPA (20:5n-3). The elongase enzyme catalyzes the conversion of EPA (20:5n-3) to DHA (22:5n-3) which represents the major product of this pathway and a key structural component of membrane structure. The same enzymes catalyze equivalent elongation and desaturation in omega- 6 pathway. Consequently, arachidonic acid (AA; 20:4n-6) and the C20 PUFAs such as dihomo-gamma-linolenic acid (DGLA; 20:3n-6) and EPA (20:5n-3) are synthetized and act as precursors to various bioactive eicosanoids that are formed by the action of specific enzymes called cyclooxygenase (COX) and lipoxygenase (LOX) [11, 15-17].

In contrast to other tissues, wherein converting LA to γ-linolenic acid (GLA) and AA, and converting ALA to EPA are present, human epidermis is lacking Δ5- and Δ6-desaturase which are required enzymes that insert double bound to the hydrocarbon chain. Hence, these two series of reactions have not been detected in human epidermis. Because of skin inability to synthetize these long-chain bioactive metabolites, GLA, AA, EPA, and DHA are also considered essential nutrients for the skin. The potential consequences of EFAs deficiency as well as disturbance of metabolic pathway are affected skin function leading to disruption of the permeability barrier and increased transdermal loss [12, 13, 15]. The skin lipid deficiency may be intervened either by diet [18] or by topical application of oil rich in EFAs [19, 20]. Fish oil supplementation could provide the skin with a direct source of the omega-3 series of FAs bypassing the need to convert ALA to EPA and DHA. Dietary supplementation might influence the fatty acid composition of the epidermis, and incorporate into membrane phospholipids. As part of cell membranes, EPA and DHA have the ability to affect membrane function by altering permeability, fluidity, lipid phase properties and metabolic parameters in the cells [21]. However, the effectiveness of diet rich in omega-3 has been open to question. The conversion of ALA is relatively poor because of the high amount of ALA directed toward b-oxidation before being delivered to peripheral tissues. Because of the limited conversation, topical application could be an efficient route of delivery [16, 22]. Clinical evidence for the efficient topical application was reported by Darmstadt, G.L in a study in which LA-rich oils are employed in the treatment of essential fatty acids deficiency in preterm infants and chronic fat malnutrition [20].

1.1.2.2 PUFAs and skin inflammation

Fatty acids are not only structural compounds of cell membrane; they are also fundamentally involved in various biological processes in human body. Omega-6 and omega-3 PUFAs, as outlined above, are biological precursors of potent lipid mediators, collectively termed eicosanoids. Although, eicosanoids are released in small quantity by all cutaneous cell types, they are key players in numerous physiological and pharmacological functions [23-25]. It is believed that eicosanoids are involved in the modulation of inflammatory and immune processes including blood platelet aggregation, respond to injury and repair of cell membranes. Therefore, they deserve particular interest. Moreover, eicosanoids are thought to be responsible for inflammatory skin disease such as psoriasis and atopic dermatitis because of their biological effects on the skin and their presence in greater amount in inflamed skin [14, 26].

Eicosanoids represent a large class of biologically active lipid mediators, signaling molecules that includes prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), mono- and polyhydroxy fatty acids, and lipoxins (LX). The pathophysiological role of eicosanoids in inflammatory skin disease is multiply and fascinatingly complex. It is known that different eicosanoids oppose each other in their actions, some eicosanoids act as pro-inflammatory, and some act as anti-inflammatory. However, both pro-inflammatory and anti-inflammatory eicosanoids are subsequently derived from a common precursor, AA, by two metabolic pathways induced by COX (COX1 and COX2) and LOX (Fig.4). These enzymes (by adding more hydrogen double bounds and extending the carbon chain) are able to make less saturated and longer fatty acid chains. As result, their chemical properties are changed, allowing them to perform different physiological functions from their parent [23, 27]. The COX pathway through which AA is enzymatically cyclized and oxygenated consists of two related catalytic steps: a formation of prostaglandin G2 (PGG2) and a peroxidase induced reduction of PGG2 to unstable PGH, which is rapidly converted into prostacyclin (PGI2), and thromboxane A2. Products of the COX pathway are important mediators of inflammation. The prostaglandins modulate immune function via the lymphocyte. PGE2 has been recognized as most important eicosanoid that predominates in some vascular pathologies, and possibly involved in mutagenesis, and cancer

promotion [28]. Thromboxane is a potent stimulator of thrombus formation and linked with coronary artery disease and myocardial infarction. This pathway, especially COX2 is the major target of numerous non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors which have potent anti-inflammatory and anti-thrombotic action, but minimum gastrointestinal toxicity [29].

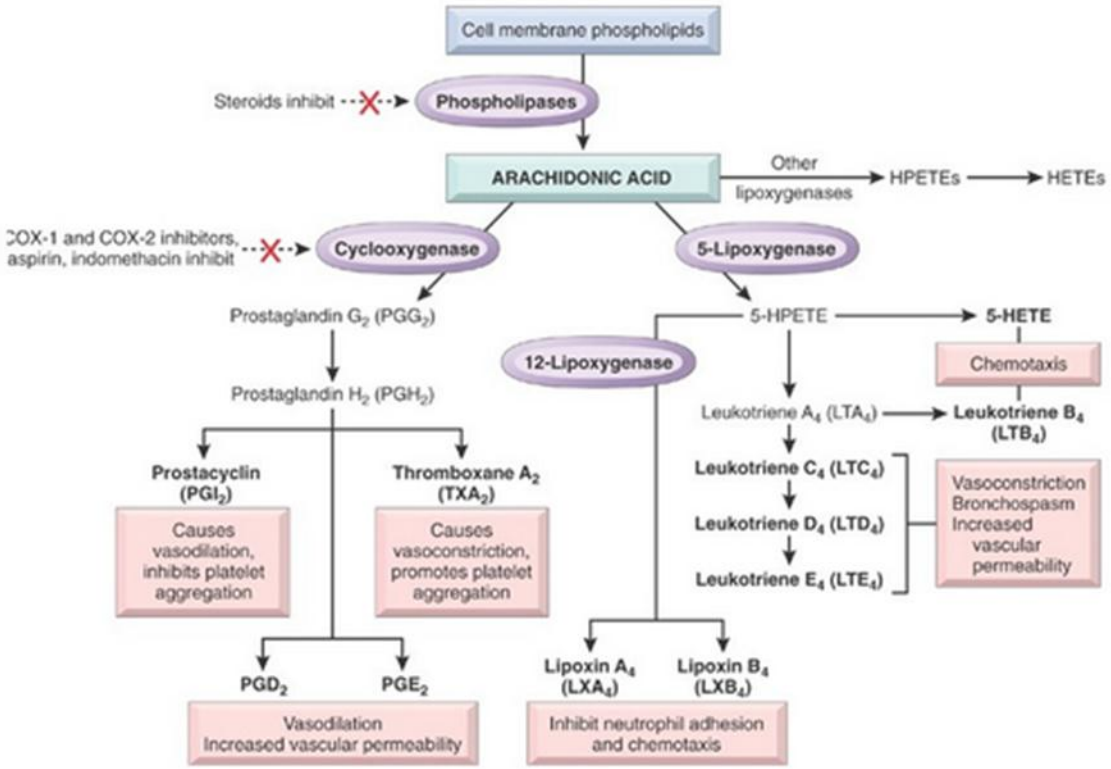


Fig.4. Diagram representing arachidonic acid metabolic pathways and its role in inflammation. Arachidonic acid released from phospholipids by the action of the enzyme phospholipase A2 can be metabolized by either cyclooxygenase 1 and 2, or by 5-lipoxygenase [30].

The second pathway i.e. lipoxygenase pathway, is synthesis of various hydroperoxy acids by the action of lipoxygenase enzymes. The 5-LOX is especially important as it catalyzes the production of pro-inflammatory leucotrienes, which are best known for their role in inflammatory diseases like asthma, inflammatory bowel disease and arthritis [31-33]. In addition, *in vivo*, leukotriene B4 has been demonstrated to induce skin inflammation. For example, one

study reported that topical application of microgram amounts of leukotriene B4 to normal skin leads to visible and persistent inflammatory reactions accompanied by significant histopathologic changes within the epidermis lasting several days [34].

Omega-3 PUFAs have anti-inflammatory properties, generally attributed to their ability to inhibit AA metabolism, and consequently block the formation of the inflammatory mediators (eicosanoids and cytokines). EPA and AA compete for the same enzyme (i.e Δ5- desaturase) to eventually be converted into anti-inflammatory prostaglandins (PGE3) or into pro-inflammatory prostaglandins (PGE2). Findings suggest that an increased intake of EPA lowers AA levels and favors anti-inflammatory prostaglandins leading to a new balance of eicosanoids. In a diet high in omega-3 fatty acids, most of the Δ5- desaturase will be used in the omega-3 pathway. Thus, DGLA ends up being converted into the anti-inflammatory PG1 and inflammation is therefore decreased [27, 35]. This explains, however, that fish oil as a natural source of anti-inflammatory omega-3 FAs could be used as an equally significant non-steroidal anti-inflammatory drug (NSAID). Fish oil appears to be a safe alternative with fewer side effects in effective therapy for various acute and chronic inflammatory diseases [36]. A nine- months double-blind, placebo-controlled randomized study in patients with rheumatoid arthritis has demonstrated that a daily intake of 10 g of cod liver oil significantly reduces the daily NSAID requirement by more than a third. Therefore, fish oil may lower the NSAID intake in rheumatoid arthritis patients and reduce the gastrointestinal adverse events associated with these drugs [37].

1.1.3 Antibacterial and antiviral effects of PUFAs

Apart from their primary role as a source of energy, lipids can also act as an antibacterial agent, mainly targeting the bacterial cell membranes. Previous studies have demonstrated that naturally occurring lipids such as fatty alcohols, FFAs and monoglycerides possess antibacterial qualities [38, 39]. Kabara and coworkers studied the relationship between antimicrobial activities of various FAs, their derivatives and their structure. In a summary, they concluded that structural properties of FAs, such as carbon chain length, unsaturation, hydroxide radical, esterification,

and functional groups can been directly linked to their antibacterial activities. Further studies of the relationship between FAs and their antibacterial properties showed that the inhibitory effect of FAs decreased with their esterification and increased with their unsaturation [39]. Several studies on the antiviral and antibacterial effects on PUFAs have shown that PUFAs are effective both *in vitro* and *in vivo*. For example, it has been shown that unsaturated fatty acids are highly active against enveloped viruses [40]. In another study the antiviral effect of FFAs was reported and the antiviral effects of various PUFAs compared. Evidence proved that FAs extract from cod liver oil results in a significant reduction of viral HSV-1 activity. 1% FAs extract caused a 50,000 fold or greater ($\geq$ 4.7 log10) reduction of viral infectivity in 10 minutes [41].

Table.2. Representative bioactivities of various polyunsaturated fatty acids.

Activity	Fatty Acids	Reference (s)
Antibacterial (Gram-positive)	LA; C18:2 n-6 GLA; C18:3 n-6 DGLA; C20:3n-6 EPA; C20:5 n-3 DHA; C22:6 n-3	[42],[43] ,[44],[45]
Antibacterial (Gram negative)	ALA; C18:3 n-3 SDA; C18:4 n-3 EPA; C20:5 n-3 DHA; C22:6 n-3 LA; C18:2 n-6 GLA; C18:3 n-6 AA; C20:4 n-6	[46]
Antiviral	DHA; C22:6n-3 EPA; C20:5 n-3	[41],[47]
Antifungal	SDA; 18:4 n-3, EPA; 20:5 n-3 DPA; 22:5 n-3 DHA; 22:6 n-3 LA; C18:2 n-6 ALA; C18:3 n-3	[48],[49]

Similar, PUFAs exhibit antibacterial activities against Gram- positive and Gram- negative bacteria [41, 42]. In particular, various LC- PUFAs, including EPA (C20:5n-3) [43, 44], DHA (C22:6n-3) [44], GLA (C18:3n-6) [45] and DGLA (C20:3n-6) [44] have been shown to exert highly potent activity against Gram-positive bacteria [46, 47], but activities against Gram-

negative have been also reported [48]. Extended investigations on relationship between FAs structure and their antimicrobial activity found that the antibacterial activity of FAs is correlated with their shape and structure. By increasing the unsaturation through the addition of *cis* double bonds, it is theorized that FAs are more effective because the *cis* bonds in unsaturated FAs cause a kink in the fatty acids chain that keeps them from packing uniformly and tightly in the membrane. Thus, medium- and LC-PUFAs make the membranes more fluid and permeable. In parallel, medium- and long-chain saturated FAs lack a kinked structure which makes it easier to pack their molecules tightly in a stable repeating array or crystalline lattice resulting in reduced permeability [38, 49].

In addition, PUFAs not only possess antibacterial and antiviral activity but also antifungal activity. Presumably, the best known is 10-undecenoic acid, monounsaturated FAs used as active pharmaceutical ingredient in many topical formulations used against fungal skin infections. The antifungal activities of many fatty acids as well as the possible mechanisms of antifungal activity have been reported by Desbois and Pohl in separated studies [50, 51].

In recent years, there has been a dramatic increase in the demand of natural antibacterial agents as a result of the growing resistance to conventional antibiotics. Furthermore, treating some bacterial infections with synthetic antibacterial agents can cause the release of more toxins and may worsen disease outcome. Therefore, researchers are now looking to nature with an enthusiasm for other ways of fighting infection. In spite of modern trends towards the development of natural antimicrobial agents, the overall contribution of fish oil can be high because it displays a high antibacterial activity of FAs against Gram-positive and Gram-negative bacteria, fungi and mycobacteria at a variety of pathogen entry sites, including the skin and mucosa-associated lymphoid tissue lymphoma [52, 53]. Eventually, it is realized that fish oil as a source of antibacterial compounds could be successfully utilized in various biotechnological applications, especially in formulation of safe and natural antimicrobial medicinal products [53, 54].

1.1.3.1 Mechanism of antimicrobial action of fatty acids

To date, the antibacterial mechanisms of FAs have not been resolved, and this continues to be a subject of considerable research effort. Although, the exact mode of action of fatty acid still remains unclear, it is thought that the cellular membrane is the primary target. The mechanism of antibacterial interaction of FAs has been extensively investigated, and different models describing the correlation have been proposed. Beside the cellular membrane disruption, there are other likely mechanisms at play, as reported by Desbois that may be involved in the bacterial growth inhibition and cause the death of bacteria [55]. According to his study, the multiple mechanism of FAs antibacterial activity may involve enzyme inhibition, impairment of nutrient uptake, cell lysis and the generation of toxic peroxidation and autoxidation product. Likewise, the mechanism by which FFAs inhibit bacterial growth or eventually kill the bacteria depends on fatty acids structure, the target bacterium, and the sites that the FFAs penetrate.

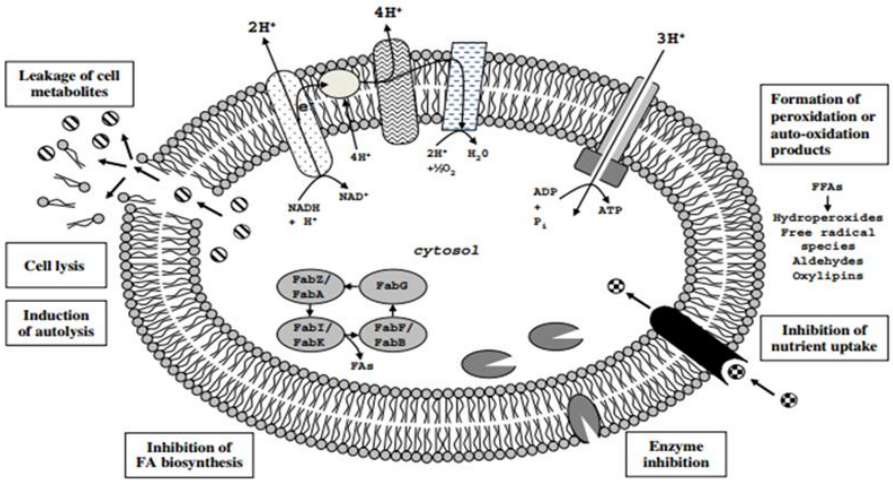


Fig.5. An illustration of possible mechanisms of antibacterial action of FFAs. Adopted from [55]

In general, Gram-positive bacteria are less resistant against FAs than Gram negative. The difference in susceptibility has been linked to different outer cell wall structures, which serves as a barrier and protect the cell membrane. The ability to penetrate cellular membranes is

influenced by the different structure of the much simpler cell wall of Gram-positive bacteria and the outer membrane of Gram-negative bacteria that acts as a selective barrier. The Gram-negative envelope is mainly composed of protein, lipid, and peptidoglycan with predominate lipopolysaccharide on the outer surface instead of the typical glycerophospholipid found in most biological membranes. In contrast to the glycerophospholipid that contains only two fatty acid residue, the lipopolysaccharide contains varying polysaccharide chains length with six or seven covalently linked FAs chains. Also, all the FAs in lipopolysaccharide are saturated which significantly reduces the fluidity [38, 56]. Other important factors relating to the different susceptibility include surface hydrophobicity and net surface charge that dictate the adherence to surfaces. Generally, high cell surface hydrophobicity appeared to increase tendency of microorganisms to adhesion, stimulating the organisms to adapt a protective biofilm which offers a protection against the host immune system and antibiotics. Similarly, positively charged surfaces increase the microbial adherence and thereby enhance the interaction between microorganisms and host cells. However, the negative electrostatic charge of bacteria and most mammalian cells is unsuitable for adhesive interactions because it creates repulsive forces between the cells and thus disables bacterial cells to adhere and attach to surfaces [57, 58].

Up to present time, exactly how various external factors (e.g., as how pH and temperature alter the antibacterial activities) has not been fully understood. It is uncertain whether changes in FFAs solubility caused by alteration of the external conditions influences bacterial susceptibility and affects membrane functions. Despite the many studies on the principal activity of FFAs in the defense of organisms against bacterial threats, the knowledge of this subject is still limited. The mechanisms and varying potency are far from a complete understanding. It is likely that the mechanism of action of FAs is a multifactorial combination of various effects. For instance, alteration of pH could either change the ionization state of fatty acid or cause variation in the inferential tension to such extent that exhibit transition behavior and consequently interact with cell membranes. Changes in temperature may also modify surfactant properties of the FAs leading to altered solubility and increased membrane permeability, and eventually facilitating their absorption as a result of straighten surfactant properties with a raising temperature [55, 59-61].

Beside the strong antibacterial properties, FFAs also possess antifungal properties. Thormar investigated extensively the antiviral and antifungal effect of FA, and found that FA affect the viral envelope, causing leakage and complete disintegration of the envelope. Further, it has been documented that FAs kill fungi by disintegration of the plasma membranes of tissue culture cells resulting in cell lysis and death [40, 62].

1.2 Fish oil

Omega-3 FAs are found in a wide variety of plants such as flaxseed, walnuts or canola oil, as well as in most fish families such as mackerel, salmon, herring, tuna and cod. The composition of ω-3 fatty acids in fish oils differs from that of plants. In comparison to oil of plant origin, fish oil is rich in the preformed LC-PUFAs (e.g., EPA, DHA), and delivers these omega-3 FAs in the form of highly bioavailable triglycerides, although other types of lipids such as wax esters or phospholipids may be present. Plant-based oils contain the short-chain omega-3 FA, ALA, which has to be converted to EPA and DHA in the body. Being rich in EPA and DHA, fish is considered to be an excellent natural source of omega-3 fatty acids [8]. Fish oils have been found to contain a variety of saturated, monounsaturated and PUFAs. Among the saturated fatty acids those with a medium chain length (C14:0, C16:0) dominate, although there is a difference in fatty acid composition among groups of fish. The hydrocarbon chain lengths of the unsaturated acids range generally from C14 to C22 but predominant FAs include monounsaturated (C16:1-n-7, C18:1n-9), and polyunsaturated (C20:5n-3, C22:6n-3)[63].

The fatty acids composition and their amount mainly depend on fish diet, but other factors may also influence fatty acid profile such as their age, reproductive status, seasons and geographic location. The fatty acid profile may differ particularly among fish species with high content of long chain-monounsaturates such as herring, mackerel, and cod. Fish oil may also contain vitamins A and D, and the natural antioxidant vitamin E (α-tocopherol) that can also contribute to the overall positive effect of the fish oil on health. Vitamin E composition and concentration of individual vitamins present in fish oil varies widely. For example, cod-liver oil contains higher

amount of vitamin E than typical fish oil and more than 90% of its vitamin E is as α-tocopherol [63, 64].

Atlantic cod (Gadus morhua) (Fig.6) is a popular species to extract oil from. Fish oil is derived from either the whole body or isolated parts including muscles, blubber or liver. Cod-liver oil is extracted from the liver and contains predominantly triglycerides, free fatty acids, diglycerides, but also small amount of monoglycerides, sterols and sterol esters.

Fig.6. Atlantic cod (*Gadus morhua*)

Industrialized fish oils, including cod-liver oil, are mainly produced in two steps: oil extraction and refining. Each of these steps can be modified resulting in a variety of alternatives. Extraction of oil goes through few stages, and normally starts with heating. In a steam cooker, livers are heated to separate water and oil from protein and the result is floating liver oil on the top separated from other liquids by centrifuge. After extraction, the oil is very lightly filtered to remove particles of liver tissue. The crude cod-liver oil contains minor amount of water and a variety of unwanted substances such as pigments, FFAs, glycerides, phospholipids, sterols and toxic heavy metals as well. The high amount of FFAs and water soluble compounds are eliminated by neutralization or de-acidification with sodium hydroxide. Further, in the refinery, cod liver oil is bleached and deodorized to remove volatile components and secondary oxidation product responsible for the fishy odor. The realized cod liver oil is stable, pale colored with a slight but pleasant fishy flavor [64, 65].

1.2.1 Fish oil and oxidation

Fish oil is particularly susceptible to rancidity because of its high content of omega-3 PUFAs. Their large number of double bonds makes them highly susceptible to oxidation, as an increase

in the number of double bonds in PUFAs also means that there are more available sites where the oxidation reaction can occur. Thus, polyunsaturated fats are more prone to oxidation than monounsaturated FAs [66]. Lipid oxidation is generally triggered by heat, light, oxygen or enzymatic activity [67].

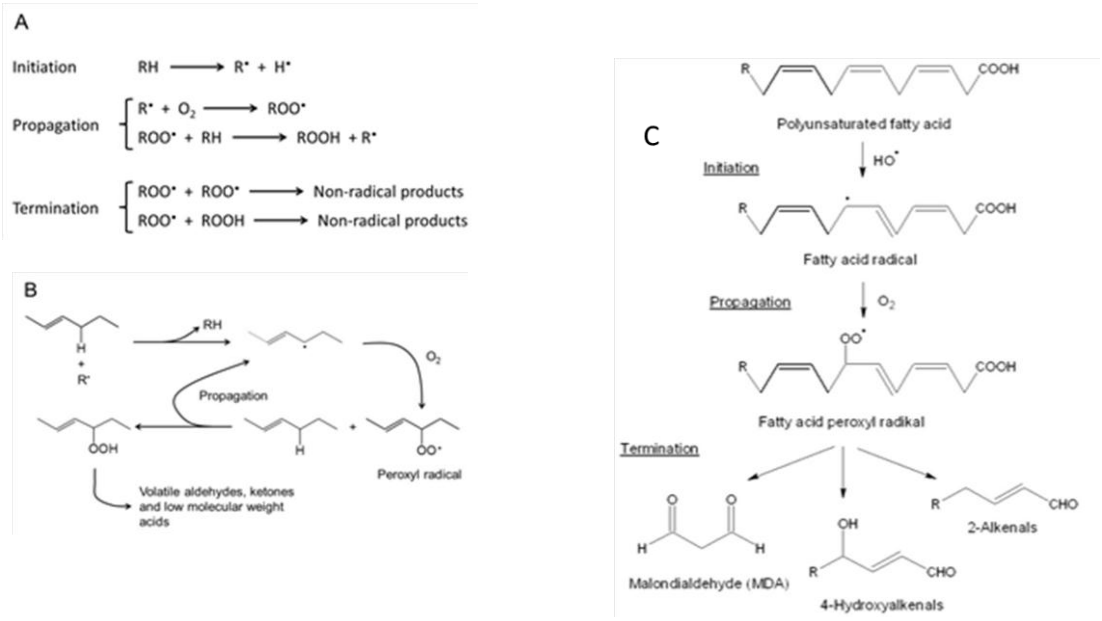


Figure 7. Illustration of autoxidation (A) and rancidification of unsaturated fat (B) [68]. Formation of harmful lipid peroxides malondialdehyde and 4-hydroxy-2-nonenal (C) [69].

Lipid peroxidation is a complex process that causes lipid deterioration via a free- radical chain mechanism. It occurs in three stages: initiation, propagation and termination. The initiation starts when molecular oxygen combines with unsaturated fatty acids, yielding highly reactive and unstable hydroperoxides and peroxyl free radicals. In the propagation phase, the unstable by-products of the first stage start a free radical lipid peroxidation chain reaction abstracting hydrogen from other lipid releasing a new peroxy radical that reacts with unsaturated FAs and produce breakdown products including aldehydes and ketones. The reaction enters to its third stage called termination when two free radicals conjugate each other to terminate the chain or in the presence of a chain-breaking antioxidant. Lipid oxidation generates a sequence of breakdown products, starting with primary oxidation products (peroxides, dienes, FFAs) then secondary

products (aldehydes and ketones). Of these final products, aldehydes are considered the most important breakdown products because they are responsible for development of rancid off-flavors and overall organoleptic changes. As the lipid breakdown progresses, the unpleasant odor and flavor progressively become more intense and unacceptable [68-70].

Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative rancidity in many quality control labs. The quantification of lipid peroxidation is commonly performed by using two basic laboratory tests: peroxide value (PV) for primary oxidation products and anisidine value (AV) for secondary oxidation products. These two parameters are frequently used together to estimate the total oxidation value (Totox) which is considered to be most important indicator of the overall rancidity or quality of oil. [63]. While a variety of analytical techniques are available for accurate quantification of various lipid breakdown products, subjective sensory evaluations may also be helpful and simple strategy to detect rancidity. In fact, taste and smell as indicators of oil quality are part of everyday life because the process of oxidation leads to sensory changes that people often associate with rancidity [72].

Lipid peroxidation is not only responsible for oxidative deterioration of lipids, but also for damage to tissues *in vivo*, where it may be involved in pathogenesis of Alzheimer's disease, cancer, inflammation, atherosclerosis, and aging. The harmful effects are considered to be caused by formation of aldehydes. Of particular interest are malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), which are generated by decomposition of arachidonic acid and LC-PUFAs. Both, MDA and 4-HNE are generally accepted markers for assessing oxidative stress *in vivo.* For a long time, MDA was considered as the most important lipid peroxidation metabolite. However, MDA was found to be chemically stable and less toxic than 4-HNE. Moreover, 4-HNE has been pointed out as a key biomarker for oxidative stress and important player for mediating a number of signaling pathways. With the advances in analytical science, many methods have been developed for determinations of marker metabolites, which are usually performed in blood, red blood cells or plasma [70, 71].

Oxidation is a normal process that happens with all fats and oils that contain PUFAs and fish oil is not an exception. All fish oils, even the finest ones, will get rancid eventually. Fish oil contains natural antioxidants but their amount decreased during processing. As the antioxidant content decreases, stability becomes more and more important as a factor in the determination of fish oil quality. Since there is less antioxidant present in refined oil some means must be employed to enhance stabilization and protection of the final product. Numerous techniques are employed to protect fish oil against oxidation. Most often, fish oil is partially hydrogenated which involves an addition of hydrogen to unsaturated bonds, therefore, substantially increasing the oxidative stability and altering the melting behavior without affecting its nutritional value [67, 72]. Further, a variety of natural and synthetic antioxidants alone or in mixture has been used to obtain and maintain oxidative stability of fish oil. Among the most extensively used natural antioxidants in food, cosmetic and pharmaceutical products are flavonoids, polyphenols, ascorbic acid (vitamin C) and tocopherols (vitamin E). Synthetic antioxidants include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and ethoxyquin. The effectiveness of natural antioxidants is limited, so synthetic antioxidants are used when less expensive and more effective antioxidants are preferred. Leading by common perception that natural antioxidants are better and harmless there has been an increasing trend to replace or at least reduce the use of synthetic antioxidants with natural ones. Another way to protect the fish oil against oxidation is encapsulation using different techniques such as spray drying, complex coacervation and freeze-drying. Finally, modified atmosphere packaging can also be an efficient method of preventing lipid oxidation [66, 67].

1.2.2 Fish oil and health benefits

Numerous data exists regarding the health benefits of fish oil for chronic diseases. Various studies have linked the beneficial health effects of fish oil to its omega 3-PUFA. In fact, EPA and DHA have been recognized as very important FAs but they are often deficient in modern diets. Due to the opposing effects of omega-3 and omega-6 fatty acids, a healthy diet should contain a balanced omega-6: omega-3 ratio. Ideally, the ratio of omega-6 to omega-3 fatty acids

should be 1:1. However, western societies are generally known to consume heavily processed food that have excessive amounts of omega-6 FAs and a very high ratio of omega-6: omega- 3.

The fatty acid imbalance, and insufficient levels of omega 3 have been associated with increased risk of many chronic diseases by altering lipid metabolism and inducing inflammatory signaling pathways [73].

The health benefits of omega-3 PUFAs have been recognized in many fields. But, most of the researches have been done in the cardiovascular field. Especially strong is the research supporting EPA and DHA in lowering the body's production of triglycerides that can lead to coronary artery disease, heart disease, and stroke [7, 74]. Today, pharmaceutical formulations of DHA and EPA, like Lovaza™, Vascepa™, and Epanova™ have been approved by FDA for the treatment of adults with hypertriglyceridemia. Vascepa™ contains only EPA while Lovaza™ and Epanova™ contain a combination of DHA and EPA in esterified and free from, respectively[75].

Furthermore, numerous studies have reported the ability of fish oil to elicit pharmacological responses such as antihypertensive [76], antioxidant [77], anti-depression [78], anti-aging [79], anti-arthritis [37, 80] and anticancer effects [58]. Dietary omega-3 oil taken regularly has been shown to be capable of reducing photo immunosuppression which affects the body's ability to fight skin cancer. To investigate the chemoprotective effects of omega-3 oil, a double-blind, randomized controlled study among 79 volunteers at age 22–60 was performed in the United Kingdom. Each participant consumed 5 g of n−3 PUFAs (70% EPA and 10% DHA) or a placebo daily for 12 week exposed to special light equivalently for 8, 15 and 30 minutes. This study showed a decreased immunosuppression of approximately 50% in individuals who took the n-3 PUFAs oil and those exposed to 8 and 15 minutes sunlight compared to placebo group. The 30 minutes group showed no significant influence of n-3 PUFAs supplementation [81]. In addition, current research on DHA has demonstrated that DHA is essential for the proper development of the infant's brain because as brain growths, large amounts of DHA are included in the biosynthesis of neural membranes. DHA is also essential for maintaining normal brain structure and function in adults. Namely, DHA is capable of enhancing the synaptic activities in neuronal

cells and reducing the risk of age related neurodegenerative diseases. The intake of DHA has been associated with lowering the chances of experiencing Alzheimer dieses, hyperactivity, schizophrenia, and depression. Furthermore, DHA reduces the triglycerides and increases the presence of the good cholesterol (known as HDL) in the blood, resulting in decreased risk of coronary artery diseases [82-84]. In addition to improving brain and heart function, there is evidence that DHA can reduce the symptoms of cancer and of inflammatory diseases such as rheumatoid arthritis, ulcerative colitis and Crohn's disease [82, 85].

However, the modern research continues to investigate and acknowledge possible beneficial effects of fish oil, including cod liver oil. For example, very interesting results have been obtained with cod liver oil as a penetration enhancer in transdermal drug delivery and this effect was associated with the high content of unsaturated FAs. Research published in the journal Pharmazie concluded that FAs extract from cod liver oil can be used as an effective pharmaceutical excipient that enhance drug delivery through skin and oral mucosa [86]. Furthermore, a double-blinded study showed that suppositories containing relatively large amounts of FA extract from cod-liver oil are highly effective in therapy for constipation. In the study, rectal treatment with FAs extract from cod-liver oil led to a significant increase in bowel movement causing defecation without irritation or any toxic effects [87]. In addition, topically applied cod liver ointment significantly accelerated wound epithelialization and vascularization in a study using the hairless mouse ear wound model [88].

1.3 Topical and transdermal delivery

1.3.1 Skin structure and function

Skin is the largest organ of the human body with a surface of about 1.8 m^2 and represents an extremely complex organ capable of continuous renewal. Central physiological function of the skin is to provide a protective barrier against mechanical, physical, and thermal trauma imposed by external agents. Skin also serves as a barrier that prevents pathogenic microbes and hazardous chemicals from entering the body, and it also regulates body temperature, secreting fluids and

water loss. Most of the defensive functions are linked structurally and biochemically to the lipid structure, composition and hydrophobic character of *stratum corneum* (SC) [89, 90]. In addition, skin is largely involved in UV induced synthesis of vitamin D [91].

The skin consists of two major structural layers: epidermis and dermis of connective tissue (Fig.8). The relatively thin (50–100 μm), and tough, outermost layer of the skin is known as the epidermis, which is predominately composed of cells (i.e., keratinocytes) that are stacked on top of each other, forming differing sub-layers [92]. At upper surface of the epidermis, the old keratinocytes are constantly being shed as dead cells. Continually, keratinocytes originated from the basal layers of the epidermis divide producing new keratinocytes to replace the old keratinocytes as they move towards the surface of epidermis. During the simultaneous epidermal differentiation and maturation, structural proteins and lipids undergo modification mediated by various enzymes. Consequently, keratinocytes become flat anucleated cells, known as corneocytes or horny cells, which develop the SC.

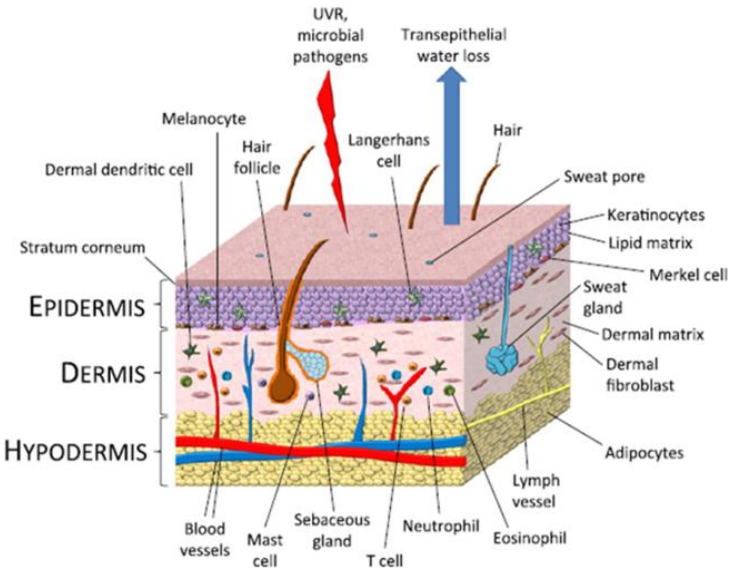


Fig.8 Schematic illustration represents the cross –section of the skin, and the complex structure of the three main layers: epidermis, dermis and subcutaneous layers (hypodermis).

Each corneocyte is surrounded by a thick cornified envelope architected by enzymatically cross-linked insoluble protein and covalently attached lipids, and is filled with water-retaining keratin

protein filaments. In addition, cornecytes are embedded to a multiple lipid organized lamellae that fill the extracellular spaces between the corneocytes forming a very densely packed structure that provides structural integrity against mechanical trauma. Moreover, these two components : corneocytes containing amorphous protein that represent a hydrophilic component and lamellae containing neutral lipids responsible for control of water transport though the skin, are absolutely necessary for normal physical, water-retaining function and overall permeability of epidermis [87, 91-94]. It is believed that disturbance of lipid metabolic pathway as for example the incomplete differentiation consequences hyperproliferative disease of the skin such as psoriasis [95]. Besides keratinocytes, Merkel, Melanocytes and Langerhans cells are other equivalently important cells found in the epidermis, which have special functions. Merkel cells act as sensory receptors for light and touch sensation, Langerhans cells are bone marrow derived, and essential elements in the organism's defense system, and Melanocytes that donate pigment to the skin and protect it from UV light [89].

The outermost layer of the epidermis is morphologically divided into four separate layers from inside to outside: *stratum basale* or *stratum germinativum, stratum spinosum, stratum granulosum* and *stratum corneum* [87, 88]. Due to absent blood supply, the transport of nutrition and waste in epidermis is performed by diffusion through the basement membrane separating epidermis from dermis.

Located between epidermis and subcutaneous layer, the dermis is a thick layer (1-3 mm) full with blood vessels, lymphatic vessels and nerves. Generally, the dermis is thicker than the epidermis, but less dense. The key components of the dermis are fibroblasts that synthesize collagen, together with smaller amount of elastic fibers and glycosaminoglycans, macrophages and immunocompetentmast cells. Collagen is the main compound of the dermis, and is responsible for skin’s strength and toughness. Having loosely arranged elastin fibers, dermis provides elasticity, flexibility to the skin, and cushions the body from stress and strain. Thus, the primary role of the dermis is a mechanical support for the epidermis, providing integrity and flexibility to skin. Sweat glands and hair follicles, together with sebaceous glands, known as skin appendages, are also situated in the dermis [89, 92, 93].

Finally, the most internal layer of our skin is the hypodermis, which attaches the skin to underlying tissues and organs. The thickness of hypodermis varies according to the anatomical region. It is composed of loose connective tissue and fat that protects the body and maintains its temperature. The fat gives the body energy when the intake of nutrients is insufficient or simply compromises the body‘s heat loss [89].

1.3.1.1 Lipid composition in stratum corneum.

The structure of SC and its permeability function are closely intertwined. In fact, lipids distributed throughout the SC play the main role in the barrier function of the skin. Consequently, an understanding of the lipid composition of SC is fundamental for understanding skin permeability as well as for the development of effective topical or transdermal drugs. The SC permeability is mainly determined by the unique mixture of lipids, composed of ceramides, FFAs and cholesterol, which all play a role in maintaining skin’s ability to retain moisture and prevent permeation across the skin.

Ceramides are dominantly present with 50% of SC lipids by weight. In human SC, ceramides are generated either from *de novo* synthesis or hydrolyze of sphingomyelin [94, 95] Structurally very heterogeneous, ceramides are composed of sphingoid base linked to a fatty acid via amide bond. Until now, in human epidermis four sphingoid moieties have been observed i.e sphingosine, dihydrosphingsine, phytosphingosine and 6-hydrosysphingosine. There are three types of FAs active in ceramides synthesis: α -hydroxy fatty acid, ω -hydroxy fatty acid and non-hydroxylated fatty acids, with a varying chain length between C16-C30 [92, 96]. The ceramides of particular interest for SC permeability function is named CER1 composed of a ω-hydroxy fatty acid chain linked to esterified linoleic acid [97]. Moreover, linoleic acid has been identified as the most significant FAs involved in establishing and maintaining the epidermal water barrier [98]. In addition, ceramides are not only critical for helping skin retain water but they also help repair the skin’s natural barrier property and regulate cells. There is increasing evidence that skin disorders like psoriasis and atopic dermatitis reduce or alter the production of ceramides. It has

been observed that skin affected by atopic dermatitis is poor in ceramides, whereas psoriatic skin is imbalanced in its composition [90, 99, 100]. Therefore, natural and synthetic ceramides are now commonly added into cosmetics and other skin care preparations in order to prevent moisture loss and help restore the skin's lipid barrier [101]. For example, topically applied linoleic acid proved to be effective therapeutic agents for the treatment of skin disorders and for the maintenance of healthy skin [98, 102]

Within epidermis, quantitatively less than ceramides, FFAs are the other important compounds comprising 10% of total epidermal lipids. Like ceramides, FFAs also exhibit a high variability in the carbon chain length and saturation of the chemical bonds. The most abundant of the FFAs associated with the lamellar lipids are the saturated FFAs with C22 and C24 chain length.

Although present in a small amount, (< 5 %), cholesterol is another fundamental lipid compound in SC [92]. In human epidermis, cholesterol determines the physical properties of the membranes, and also increases and stimulates the desquamation of SC acting as a protease inhibitor [103].

1.3.1.2 Lipid organization in stratum corneum

As noted previously, the permeability barrier function of the SC is strongly determined by the physical properties and molecular structure of lamellar bodies in the intercellular spaces. Therefore, an extensive amount of research has been based upon lipid organization towards explanation of arrangement in the lamellar domains. With the advanced techniques such as X-ray diffraction, nuclear magnetic resonance (NMR) and Fourier Transform Infrared Spectroscopy (FTIR), new insights in molecular structure of the intercellular lipids in human SC have been accomplished. Studies with electron microscopy, small-angle X-ray diffraction (SAXD) and neutron diffraction of isolated SC showed the presence of lateral lipid organization within the lipid matrix. Furthermore, SAXD measurements on human SC disclosed the presence of two lamellar phases, the short-periodicity phase (SPP) and long-periodicity phase (LPP) with a repeating distance of approximately 6 nm and 13 nm, respectively [92, 104, 105].

Over the years, different models have been proposed to describe and explain the structural organization of the human SC lipid bilayers. For instance, the “sandwich model” was introduced by Bouwstra et al. by which two broad lipid layers of about 5nm each with a crystalline structure is separated by a narrow central lipid layer of about 3 nm with fluid domain. It also has been reported that CER and cholesterol are essential for the formation of the two lamellar phases, while FFAs impacts the formation of an orthorhombic lateral packing where the chains are packed in a very tight crystalline array. Additionally, studies showed that the continuous lamellar organization as well as the tight packing of the lipids within these lamellae seems important for both the integrity of the permeability barrier and SC cohesion [104, 106].

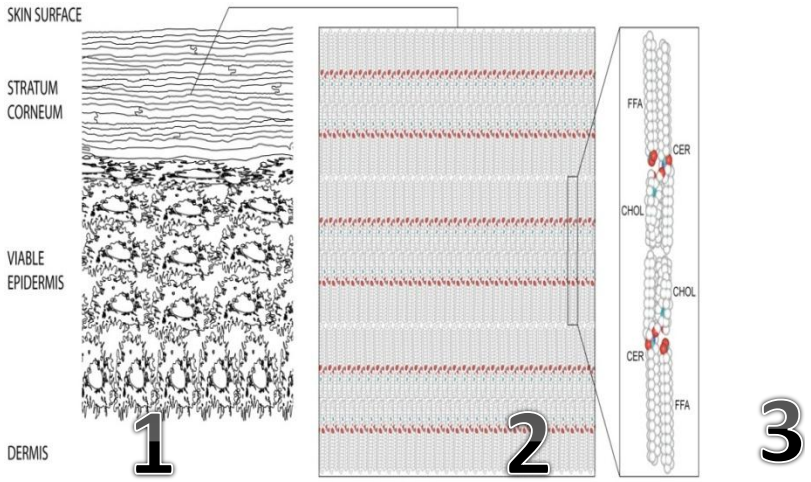


Fig. 9. Schematic diagram of the epidermis lipids presented in three parts. The structure of epidermis is shown in part 1and two-compartment model of SC composed of highly organized lamellar phases (the lipophilic part) and corneocytes (the hydrophilic part) in part 2. Part.3 shows the SC‘s unique composition of repeating molecules of CERs along with FFAs and cholesterol. http://www.skin-care-forum.basf.com/

Among other interesting models are Norlén’s “single gel-phase model” that describes the lipid structure as a single lamellar gel phase [107], the asymmetric bilayer model proposed by McIntos [108] and the Forslind´s domain mosaic model [109]. Despite many efforts to explain the lipid organization of the SC, a complete understanding has not yet been achieved. It must be mentioned that each model has its limitations, some general and some specifically relevant to different SC composition among species and selected instrumental techniques of analyzes [104].

1.3.2 Dermal drug delivery

Regardless of where the location of the drug receptor is located (i.e. in the skin or within the body) any drug applied on the skin surface must pass through the outer part of the epidermis, the *stratum corneum*. Drugs mainly go through SC by intercellular or transcellular routes. The intercellular pathway involves drug permeation through the dense lipid space between cornfield cells and this route is considered as the major route for lipophilic drugs. In transcellular route, the hydrophilic and charged molecules first diffuse through keratin fillings and then must across the intercellular lipids. For this reason, the transport of hydrophilic dugs is especially difficult. However, there is an alternative way for transport of drugs molecules through hair follicles or sweet glands bypassing the SC intact molecules, named as transappendageal way. As the diffusional resistance is lowest in sweat glands and hair follicles, this route, also known as shunt route, could be an option for the effective delivery of drugs. However, this route has been generally neglected because hair follicles and sweat glands only cover 0.1% of the skin [110-112].

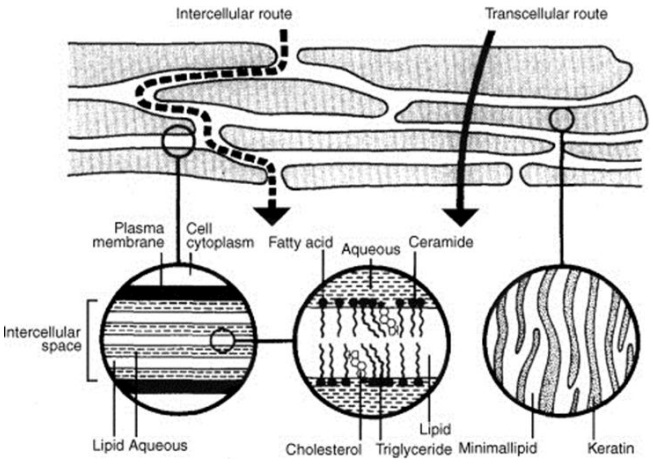


Fig.10 Simplified illustration of the SC lipid organization and the major routes drug penetration are also illustrated : transcellular and intercellular pathways. The diffusion is however much more complicated and the drug has to go through many hindrances to reach the dermis [111].

Although passive diffusion is still the most important route for drug absorption, lately, the interest for follicular delivery route has considerably increased since it is known that the follicles enter to dermis, and some even extend deep into the hypodermis. With the

complete disintegration of the glandular cells, a secretion (i.e., sebum) is formed which releases a mixture of short chain fatty acids involved in fungal and bacterial growth. Most likely, the sebum is secreted when the hair grows and then discharged via ducts into the upper part of the follicular canal. The lipophilic molecules penetrate through this region around the follicle rich in neutral non-polar lipids [113, 114]. Follicular penetration has been studied incorporating stripping, staining, laser scanning microscopy and other techniques [115, 116]. Different fluorescent dyes including curcumin have been used to examine the depth of penetration into the follicular canals and subcutaneous area that acts as a drug reservoir [116].

The rate at which drugs are transported across SC can be determined by Fick's Law of diffusion**:**

$$\frac{\boldsymbol{dm}}{\boldsymbol{dt}} = \frac{\boldsymbol{D\Delta C}}{\boldsymbol{X}}\boldsymbol{K}$$

Where dm/dt is the diffusion flux across SC, D is the diffusion coefficient, ΔC is the drug concentration gradient per unit length across the SC, K is the partition coefficient of the drug between skin and formulation medium and x is the thickness of the SC [111].

Due to the excellent barrier properties of the SC layer of the skin, numerous techniques, chemical and physical, have been developed to enhance the drug delivery across the skin. Chemical approach is based on using various chemical penetration agents that interacts with the skin compounds, disrupting lipid bilayers and promoting the drug flux. The most well-known are sulfoxides, pyrrolidones, fatty acids, essential oils, terpenes, terpenoids, Azone®, oxazolidinones and urea which differ by the mode of action. In general, many of these enhancers appear to be effective, though their employment has been limited due to their unknown mechanism and toxicity [117-119] .

Innovative physical technologies also have been employed for enhancing the drug delivery. Such technologies include iontophoresis, electroporation, ultrasound [120] and microneedles [121]. More recently, a great attention has been paid to the use of liposomes in enhancing the transdermal drug delivery. Liposomes have been acknowledged to possess a great potential in transdermal drug delivery as penetration enhancers in follicular delivery [122]. Cyclodextrins are a new class of chemical agents that act as penetration enhancers. Their effect on drug delivery through biological membranes and application in ophthalmic drug delivery has been widely investigated by Loftsson et al. [123, 124].

1.3.3 Topical formulations

The knowledge of skin, especially SC organization and its function has increased considerably over the last decades explaining both the routes by which drugs penetrate into and through the skin and composition of topical formulations that can efficiently deliver drugs. As a consequence, skin is seen as an ideal site for the delivery of drug substances for both local and systemic effects. Topical formulations have meanwhile become a popular method for treating skin dieses [125]. Topical pharmaceutical systems comprise numerous products, which are presented in the forms of ointments, creams, lotions, gels, powders and pastes. The most common commercial dermatological formulations are ointments and creams [126, 127]. When applied, topical formulations tend to treat a pathological condition or affect the whole body after being absorbed into the systematic circulation. For a systematic effect, the most common product is a transdermal patch, which continuously delivers a predetermined dose of medication over a certain period of time.
Avoidance of first pass metabolism and fluctuation in drug level, suitability for self-medication, avoidance of the risks and inconveniences of intravenous therapy, no drug deactivation by gastro-intestinal enzymes and a relatively large area of application are some of the main advantages of topical dosage form. Although, there is number of benefits, disadvantages are also associated with topical dosage forms, such as: skin irritation,

possibility of allergenic reactions, suitability only for potent drugs, drugs of smaller particle size and molecular weight to absorb through the skin.

When formulating topical formulations, a large number of aspects have to be considered. Formulation development is typically a lengthy process and it often begins with the identification of the active ingredient to be used to provide a desired effect. Based on the physicochemical properties of the drug and intended targeted area a rational strategy can be developed to create the vehicle. An improper choice of vehicle can impact a degree of drug solubility in various excipients, drug release efficiency, compatibility with potential excipients, poor patient compliance and product degradation and instability. Another point worth highlighting is that a simple change in properties, such as pH, viscosity, the relative amounts of oil, water, surfactants, stabilizers, particle size, or the method of preparation, can often influence skin absorption and clinical efficacy as well as product stability. Additionally, suitable manufacturing process and packaging are necessarily required. An appropriate manufacturing process and quality testing help ensure that pharmaceutical formulation meet the appropriate quality attributes for the intended topical administration [112, 127-129].

1.3.3.1 Semisolid dosage forms

Semisolid dosage forms are dermatological preparations intended to apply externally on the skin to produce local or systemic effect. Some examples are ointments, creams, pastes and gels. Ointments are defined as semi-solid, homogenous, viscous pharmaceutical preparations usually intended for external use to the skin or mucous membrane. Furthermore, two forms of ointment are generally recognized: non-medicated or medicated. Non-medicated are generally used to protect the skin, and prevent moisture evaporation acting as occlusive barrier. In contrast, medicated ointments contain one or more medicaments dispersed or dissolved in the base, and they are designed to maximize the penetration of the medicine into the skin. Ointment contains a base, also known as vehicle or carrier for the medicament. Generally, ointment bases are classified into four classes:

hydrocarbon bases, absorption bases, water-removable bases, and water-soluble bases [127]. The most important hydrocarbon bases are petrolatum, paraffin and mineral oil. While several bases can be used, petrolatum is commonly used base in topical pharmaceutical formulations and is found in majority of ointments. It is shown to provide a protective film on the skin, and often functions as a strong occlusive agent. Among hydrocarbon basses, there is also a variety of naturally occurring waxes having different physicochemical properties depending on the source of wax material [112, 129].

When selecting a suitable ointment base, the factors such as the effect desired, nature of the drug to be incorporated, drug-base compatibility and the stability of the ointment are to be of great importance. It is highly advantageous if the base does possess other desired properties such as: smooth texture, non-irritation, physically and chemically stability, compatibility with the skin and with the incorporated medicaments and it also should be of such a consistency that it spreads and softens easily when applied to the skin. In addition, some bases, although resist microbiological contamination may require an addition of preservatives because of their high contain of water. Often, antioxidants are also added to the formulation, especially when oxidative deterioration is expected [129, 130].

1.3.3.2 Waxes

The chemical composition of waxes is versatile and oftentimes indeterminable, but in general, it is a mixture of esters of fatty acids with long chain monohydric alcohols and high content of hydrocarbons making them highly hydrophobic. Physical properties of waxes correlate the nature and length of the hydrocarbon chain as well as wide distribution of molecular weights. For example, an increased chain length, or a high molecular weight results in higher melting point. Furthermore, waxes that contain predominantly straight-chain hydrocarbons are characterized by a clearly defined crystal structure compared to waxes composed of branched chains that exist in poorly defined crystalline or amorphous compounds.

With regard to physical characteristics, waxes rather have irregular profile and polymer-like properties, and can be described as thermoplastic materials as being solid at ambient temperature but become liquids at higher temperatures. The chain lengths in waxes are too long to allow good crystallization, so it is almost impossible to form regular array as in high polymers [131, 132]. Synthetic waxes have been developed from vegetable oils and naturally occurring waxes by a process of hydrogenation and catalytic splitting [133]. Although, synthetic waxes are chemically closely related to the naturally occurring waxes and more homogenous than natural waxes, they however have not been completely accepted as pharmaceutical excipients, and usually are regarded considerably inferior to the natural waxes as consumers believe that anything natural is more safe and devoid of side effects [134]. The properties of both natural and synthetic waxes results from the chemical composition, which ultimately determine if a particular wax is a right choice for a given application. Many of the physicochemical properties of waxes are quite similar. For example, due to low thermal conductivity waxes are difficult to be subjected on uniform heating and must undergo heating for long time enough to achieve thorough softening throughout material. Moreover, waxes have elastic properties; they melt without decomposition and tend to re-solidify unchanged. In addition, waxes are very resistant to moisture, oxidization and microbial attack. They, however, exhibit dissimilarities in thermal contractions on solidifying and cooling which is important in determining the thermal properties including dimensional changes [132].

As essentially natural and nontoxic materials, waxes are widely used in numerous cosmetic, pharmaceutical and food applications. In pharmaceutical formulations, waxes are effectively utilized as base compounds or excipients (additives) for imparting, improving or modifying specific product properties such as dimensional stability, resistance to flow or viscosity and consistency. Distinguished by good lipophilic properties, waxes find applications in semisolid preparations like ointments, creams, lotions, and suppositories. Waxes can also act as release retardant dugs and as such have been successfully used in sustained release oral and topical delivery mechanisms for active ingredients [135, 136].

2. Aims

The overall aim of this project was to investigate the antibacterial effect exerted by fish oil extract obtained by hydrolyzes of omega-3 PUFAs and design a suitable topical dosage form containing the fatty acid extract. The specific aims of this study were to:

I. Determine the antibacterial properties of free fatty acid extract

II. To develop topical preparation of free fatty acid extract using wax as a suitable lipophilic base

- Evaluate the physicochemical properties of the ointment in regard to the influence of selected waxes.
- To investigate the stability of ointment that contains high amount of FFAs without addition of antioxidant.

3. Materials, Equipment and Methods

3.1 Materials

3.1.1 Materials used in ointment compositions

Fish oil extract

FFAs extract was made by hydrolysis from omega-3 fish-liver oil, which has an active monograph in the current European Pharmacopoeia (Fish oil, rich in omega-3 acids 07/2012:1912). The fish oil used in this study was supplied from Lýsi ltd (Iceland). It contains a high amount of omega -3 polyunsaturated fatty acids, and has a pale yellow color and distinct fishy odor. Fatty acid profile of the fish oil is shown in Table3.

Table 3. The fatty acid composition of cod-liver extract [86]

Fatty acid		Amount (%)
Name	Shorthand	
Saturated fatty acids		
Myristic acid	14:0	3.6
Palmatic acid	16:0	10.4
Stearic acid	18:0	2.6
Monounsaturated fatty acids		
Palmitoleic acid	16:1 n-7	6.5
cis-Vaccenic acid	18:1 n-7	4.4
Oleic acid	18:1 n-9	16.2
Gondoic acid	20:1 n-9	9.4
Gadoleic acid	20:1 n-11	1.6
Erucic acid	22: n-9	0.6
Cetoleic acid	22:1 n-11	7.8
Polyunsaturated fatty acids		
Linoleic acid	18:2 n-6	1.5
Moroctic acid	18:4 n-3	2.4
Eicosapentaenoic acid (EPA)	20:5 n-3	9.3
Docosahexaenoic acid (DHA)	20:6 n-3	11.9

Waxes

The waxes used in this study were natural, derived from various sources and have a melting range 50 – 90°C depending on the chemical composition of the wax. Following table details the descriptions of waxes used primarily as carriers in semi-solid dosage forms but also enhance their thickness and consistency due to viscoelastic properties of waxes.

Table 4.Waxes together with their general properties and sources

Wax	Manufacturer Supplier	Country	Melting Point°C	Origin	Description	Wax Appearance
Carnauba	Strahl&Pitch	USA	80-86	Plant	Yellow flakes, faint odor	
Ozokerite	Strahl&Pitch	USA	68-71	Mineral	White pastilles, odorless	
Laurel	Strahl&Pitch	USA	38-46	Plant	Cakes, green, slight characteristic odor	
Beeswax	Making cosmetics	USA	61-68	Insect	White flakes, none to faint honey-like odor	
Candelilla	Strahl&Pitch	USA	69-73	Plant	Yellow flakes, none to slight – characteristic	
Rice Bran Wax	Strahl&Pitch	USA	79-82	Plant	Yellow pastilles, none to slight – characteristic	
Microcrystalline	Making cosmetics	USA	70-74	Mineral	Off white solid, odorless	

Vaseline used in all formulations was purchased from Duchefa Farma (Netherlands). Together with waxes, Vaseline is used as an occlusive agent to increase the moisture level in skin by providing a physical barrier to epidermal water loss.

Coconut butter was purchased from Fagron (UK). It works as emollient in the ointment formulation and keeps the skin moist and soft when applied to the skin.

Lemon oil and lavender oil were used as excipients to affect the odor resulting in more pleasant and attractive smell of the ointment. In fact the fishy smell comes from the omega-3 FAs which are present in high amount in cod-liver oil. Both oils were purchased from Now Foods (USA).

3.1.2 Reagents

Potassium iodide (KI)	Sigma Aldrich (Germany)
Sodium thiosulfate ($Na_2S_2O_3$)	Sigma Aldrich (Germany)
Starch	Sigma Aldrich (Germany)

3.1.3 Materials that used for *in-vitro* antibacterial activity testing

Staphylococcus aureus (ATCC 29213)

Pseudomonas aeruginosa (ATCC 27853)

Escherichia coli (ATCC 25922)

Enterococcus faecalis (ATCC 2921)

*Streptoccoccus pyogenes (*ATCC 19615)

Streptoccoccus pneumonia (ATCC 6305, ATCC 49169)

Bacterial strains and Mueller-Hinton agar were purchased from Oxoid (UK)

Petri dishes	Sigma Aldrich (Germany)
Ethanol	Sigma Aldrich (Germany)

3.2 Equipment:

Water bath	Kottermann (UK)
Analytical scale	Ohaus Galaxy 160(Switzerland)
Microscope Olympus BH-2	Olympus (Japan)
Camera Olympus C-5050-200M	Olympus (Japan)
PH-200 Waterproof pH Meter	HM Digital, Inc.(USA)
Magnetic stirrer hot plate	Stuart scientific (UK)

Standard weights
Burette
Beakers
Graduated cylinders
Spoons, glass,
Aluminum tubes

3.3 Methods

3.3.1 Preparations of ointment

In this study, the ointments were prepared by the fusion method using seven types of waxes bases at three different concentrations (Table.4) and constant concentration of FFAs. FFAs extract forms the active pharmaceutical ingredient and is a natural product derived from omega-3 cod-liver oil. Despite the FFAs being susceptible to oxidation the ointment does not contain an antioxidant. The excipients used to form the ointment base were selected based on their compatibility with the free fatty acids. During the study, FFAs were kept at a constant temperature in a refrigerator at 4°C in dark, brown bottles to limit temperature influence and light exposure. The bottle containing FFAs was heated gently on a water bath at 50°C until the entire content has melted. Simultaneously, wax was completely melted under stirring. Care was taken that the water bath temperature was not far above the melting point of the wax. Otherwise deterioration of the lipid can be expected. When wax base has melted, both Vaseline and coconut oil were added. The entire mixture was allowed to remain on the hot plate until liquefied. FFAs

extract was added to the mixture slowly under gentle stirring. Then, lavender oil and lemon oil were added and mixed together with Vaseline, coconut oil, waxes and FFAs. Following liquefaction, the ointment was poured into 15 ml aluminum tubes before the ointment solidified. Filled tubes were sealed right after the ointment had reached room temperature to prevent the ointment from being contaminated by the environment. Sealing was done mechanically using tube-folding plier. The ointment achieved its final consistency and texture approximately 15-30 minutes after they had been compounded.

3.3.2 Evaluation of *in vitro* antibacterial efficacy of FFAs

In essence, the key to this project is the antibacterial nature of the FFAs. In order to pharmacologically rationale the employment of FFAs a preliminary screening study was conducted. The microorganisms used for our screening studies were those frequently encountered in clinical microbiological specimens including *Staphylococcus aureus (S. aureus) Enterococcus faecalis (E. faecalis), Streptococcus pyogenes (S. pyogenes), Streptococcus pneumoniae (S. pneumoniae)* and *Pseudomonas aeruginosa (P. aeruginosa), Escherichia coli (E.coli)* representing Gram- positive and Gram-negative pathogens, respectively. Antibacterial activities of these common pathogens were evaluated by agar dilution susceptibility testing method, in accordance with the procedures according to the CLSI standard for methods for dilution antimicrobial tests for bacteria that grow aerobically [137]. Double dilutions of FFAs extract of cod-liver oil were prepared. 1 ml of each included 381 µl of FFAs, 200µl ethanol and 419 µl of sterile distilled water that was added to 19 ml of melted Mueller-Hinton agar, mixed and poured in sterile petri dishes to obtain the final concentrations of 1-16.384 µg/ml. For the MIC testing of streptococci the agar was supplemented with 5% sheep blood. Bacterial suspensions were made to include approximately 10^7 CFU/ml, to give the final number of 10^4 CFU in the 1µl that was placed on the agars that were incubated at 37°C for 18-24 hours. The results were recorded as MIC (µg/ml). The MIC determinations were done two times for each strain and dilution. Various concentrations of ethanol (20%, 40%, 60%, 80%, and 100%) without

FFAs extract was used to evaluate the effect of the ethanol. Mueller Hinton agar without alcohol and FAs extract was used for growth control.

3.3.3 Determination of peroxide value (PV)

A modified method of Wheeler described in ISO 3960:2007 was used, in which lipid peroxide is reacted with potassium iodide (KI) to liberate iodine, which is then titrated with a standard solution of sodium thiosulfate ($Na_2S_2O_3$). The KI solution was prepared by dissolving 7 g of KI in 5 ml of distilled water. This solution expires after 2 weeks, it is best if the solution is freshly prepared. The starch indicator is prepared by dissolving 1g of potato starch dissolved in 100 ml of water and boiled for 3 minutes. Sample of 1 g with ± 0.001 g accuracy was weighed and melted in a 100 ml flask, mixed with 10 ml of solvent mixture (glacial acetic acid: chloroform volume ratio 3:2), and 0.2 ml saturated KI solution was added under stirring. After exactly one minute stirring, the reaction was stopped with 20 ml distilled water, and the liberated iodine was titrated with 0.01M $Na_2S_2O_3$, using a starch indicator. This amount of liberated iodide represents the peroxide content, allowing its exact determination. The reaction between liberated purple iodine and $Na_2S_2O_3$ to form colorless NaI is represented by the following equation:

$$2\ Na_2S_2O_3 + I_2 \rightarrow Na_2S_4O_6 + 2NaI$$

Purple Colorless

PV is defined as the amount of peroxides oxygen per 1 kg fat (mmolO2/kg) and the equation used to calculate PV is:

$$\text{PV}\ (mmol/kg) = \frac{(V1 - Vo)f \times 5}{m}$$

Where, V1 is volume of 0.01mol/l $Na_2S_2O_3$, Vo is volume of the blank solution of 0.01mol/l $Na_2S_2O_3$, *m* is mass of the sample in grams and f is factor for 0.01mol/l sodium thiosulphate equivalent to 1.

3.3.4 Measurements of pH

In the present study, a simple method was adopted for measuring pH [138]. The pH was measured using a pH meter, which was calibrated before each use with standard buffer solutions at pH 4, 7 and 9.The pH of ointment formulations were determined by extraction method, in which 2.5 grams of ointment was suspended in 50 ml distilled water in a 100 ml beaker, heated on water bath at about 70ºC for 10 minutes, and cooled down to room temperature. The suspension was vigorously stirred for 10 minutes at magnetic stirrer to prompt extraction before phase separation resulting in supernatant extract. At least three samples were measured for each formulation and the mean values reported.

3.3.5 Physical evaluation of ointment

The ointments were evaluated for their physical properties such as spreadability and extrudability, pH as well as their appearance. Spreadability and extrudability studies were carried out in triplicate and average values are reported. The prepared ointment formulations were inspected visually for their color, consistency, and odor by smelling.

3.3.5.1 Spreadability measurement

Spreadability is commonly measured by the sample (i.e., cream, ointment) spread diameter or area using a known weight for a predetermined time duration. The lower the viscosity of ointment sample, the lower the surface tension and the more the ointment is easily spread and absorbed into the skin [139].

Spreadability test in the present study was performed by employing a simple method: 1.0 g of the ointment was placed in a 10 mm diameter circle on a glass plate. After being covered with another glass plate, the sample was pressed with fixed 500 g weight. Spreadability was

determined as a difference in diameter values before and after 30 sec on a millimeter scale placed under the lower glass plate.

3.3.5.2 Tube Extrudability:

In the present study, a simple method was adapted for evaluating ointment formulation for extrudability determined in terms of weight in grams required to extrude a 0.5 cm of ribbon of ointment in 5 seconds [140]. More quantity extruded means better extrudability. The slightly elevated temperature of the freshly prepared ointment may result in somewhat different consistency. Extrudability measurements were preformed 48 h after the ointment had achieved equilibrium at room temperature 22±0.5 ºC. All formulations under present study were filled in a clean, aluminum collapsible tube with a nozzle tip of 5 mm opening and apply the pressure on tube by the help of finger. Tube extrudability was then determined by measuring the amount of ointment extruded from the tube when pressure was applied on tube.

3.3.6 Sensory evaluation

Sensory parameters (i.e. color, odor and texture) of ointments were compared and evaluated at different storage conditions that is, 40°C ± 2°C, 75% RH ± 5% RH for 3 months and under ambient condition for 6 months. A standardized quantity of 1g of ointment was applied over the dorsal surface of the left hand and sensory properties descriptively graded using terms as follows:

Excellent: smooth and homogeneous texture, light brown color, distinctive fishy odor.

Satisfactory: smooth texture, non-consistency, light brown color, distinctive fishy odor.

Unsatisfactory: rough texture, visible solid particles, non- consistency, dark brown color, rancid fishy odor.

3.3.7 Microscopic evaluation

Sample preparation in the case of microscopic evaluation was rather simple. It was performed on fresh samples by squeezing a small amount of ointment on a microscope slide, placing another slide to cover the sample and the observing the sample with Olympus BH-2 microscope. The sample was observed manually to determine the optimum magnification for the measurement. The 40x magnification was chosen for the ointment and sample images were taken with professional camera Olympus C-5050-200M.

3.3.8 Stability study

Ideally, stability testing should be performed over 2 years under real- time conditions, in which various physical characteristics should be monitored e.g. microbiology, pH, viscosity, appearance, color and odor. However, an alternative approach (i.e. accelerated study) may be used as a means of predicting shelf-life up to 2 years. Both, short and long-term stability testing were performed according to the International Conference on Harmonization (ICH) when odor, color, texture, and PV were monitored [141]. Aluminum tubes filled with ointment were kept at room temperature with no access of oxygen, during 6 months period. All the developed formulations were also subjected to accelerated stability at testing at 40 ± 2°C, 75% RH ± 5% RH for 3 months. For 3 month accelerated study, samples are tested at 1, 2 and 3 months.

4. Results and Discussion

This section provides results and a general discussion of the experimental work. It covers the development, evaluation and stability testing of the various ointments composed of FFAs extract

from cod liver oil and different waxes. The first part covers determination of the FFAs extract antibacterial properties against selected strains of Gram-negative and Gram-positive bacteria. The development of the ointment was based on pre-experimental troubleshooting and examination of possible antimicrobial properties of FFAs extract. Actually, *in vitro* evaluation presented here was chosen because the model is an indicator of antimicrobial properties on which employment of cod-liver oil extract is based. Certainly, if scientific evidence indicates antibacterial activity in the screening testing, other practical points are raised such as that the active compound (i.e. FFAs) must be delivered to the site of action by suitable and stable topical formulation which design and evaluation are further described in this thesis.

4.1 *In vitro* evaluation of antibacterial efficiency of FFAs extract

The first step in the present study was to investigate the antibacterial properties of FFAs extract obtained from cod -liver containing high amounts of n-3 poly unsaturated fatty acids, shown in Table 3. The FFAs extract was subjected to antibacterial efficiency testing choosing represented samples of both Gram-positive and Gram-negative bacteria.
Experimental results in this study revealed that FFAs extract possess antibacterial activity, and was found to have an overall higher activity against Gram-positive bacteria than Gram-negative bacteria. As determined by the agar dilution method, FFAs extract showed strong antibacterial activity against all four tested gram positive bacteria: *Staphylococcus aureus, Enterococcus faecalis, Streptoccoccus pyogenes* and *Streptoccoccus pneumonia***.** Against *S. pneumonia*, FAs extract was most effective (MIC =128µg/ml), but least effective against S. aurues (MIC =512µg/ml). However, the extract did not appear to have a desirable antibacterial activity against *Pseudomonas aeruginosa* and *Escherichia coli* in this study (MIC >16 µg/ml).

These results agree with previous studies where PUFAs were shown to be more potent against Gram-positive bacteria than Gram-negative bacterial [39, 40]. This may be due to the different cell wall nature of gram-positive and Gram-negative bacteria. Cell wall membranes are known for their protective function to the inner part of cell. The difference in susceptibility is closely

related to different outer cell wall structure, which has also been reported in previous studies [38, 142]. MIC determinations were done twice for each strain and identical results were observed for all antimicrobial agents and for all strains except slight differences were observed for *S. pyogenes* and *S. pneumonia*. MIC values are shown in Table 5.

Table 5. MIC concentration

Mueller-Hinton agar plate number	Culture	MIC- 1 testing µg/ml	MIC-2 testing µg/ml
ATCC 25922	*Escherichia coli*	>16.384	>16.384
ATCC 29212	*Enterococcus faecalis*	256	256
ATCC 29213	*Staphylococcus aureus*	512	512
ATCC 27853	*Pseudomonas aeruginosa*	>16.384	>16.384
Mueller-Hinton agar with 5% blood			
ATCC 19615	*Streptoccoccus pyogenes*	512	256
ATCC 49169	*Streptoccoccus pneumonia*	256	128
ATCC 6305	*Streptoccoccus pneumonia*	256	256
ATCC 27853	*Pseudomonas aeruginosa*	16.384	16.384

Controls				
Ethanol conc.v/v (%)	*Enterococcus faecalis* ATCC 29212	*Staphylococcus aureus* ATCC 29213	*Pseudomonas aeruginosa* ATCC 27853	*Escherichia Coli* ATCC 25922
Pure medium	Good growth	Good growth	Good growth	Good growth
100	Little grow	Little grow	Absent grow	Little grow
80	Less grow	Less growth	Little grow	Less
60	Regressive effects of ethanol			
40				
20	Good growth	Good growth	Good growth	Good growth

Gram-negative bacteria include *E. coli, P. aeruginosa,*

Gram-positive bacteria include *S. aureus, E. faecalis, S.pyogenes, S.pneumonia*

In all control tests, ethanol effect on the growth of *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* was observed down to 20% (v/v) solution. All four bacterial cultures demonstrated an inhibitory response to pure ethanol control but also the effect of ethanol was regressive and very little in 40% (v/v) solution.

It is worthwhile to note that these measurements were done with diluted 20% (v/v) FFAs extract, and measurements of FFAs activity in higher concentration are probably not possible with same agar dilution method because of the effect of precipitation/accumulation of FFAs extract and hemolysis in agar that could influence the results. Precipitation/accumulation of FFAs was visible on agar surface but gradually decreased to 128 µg/ml in both Mueller-Hinton agar with and without blood. Perception/accumulation was more visible in Mueller-Hinton agar with blood and moreover showed signs of hemolysis and it was also gradually decreased to 512 µg/ml.

4.2 Ointment design

Once the antibacterial activity of the FAs extract had been verified, next step was a study undertaken to develop a semi-solid topical formulation or, more precisely an ointment that can be used safely for the treatment of some bacterial infections. Considering that various ingredients are available for topical formulation design, such as moisturizers, emulsifiers, humectants, antioxidants and emollients, the ointment formulation was based on a principle of simplicity**.** In other words, when it comes to terms of topical formulation design, sometimes short ingredient list is definitely better than a long one. However, this makes the simple concept of formulation design very pertinent in achieving the desired qualities such as: smooth and free from grittiness texture, easy application, uniform distribution of active compound through the base, compounds compatibility, adequate retention, and stable formulation that melts or softens when placed in contact with the body [143].

Twenty one formulations were prepared by the fusion method, in which FFAs extract (40% w/w) was combined with a melted wax base (60% w/w) and evaluated for physicochemical parameters and stability. All the compositions are summarized in Table.6. Generally, the fusion method is employed when compounds are stable at fusion temperature, and when paraffin, wax and other high molecular hydrocarbons form the ointment base [144].

To assess which wax is most suitable for ointment formulation, seven waxes of different origin were selected and tested. Each of selected wax was used at a concentration of 1%, 3% and 5% (w/w) and changes in the ointments' physical properties regarding consistency, homogeneity and stability were tested.

Table 6. Compositions of ointments tested.

Formulation code	**Compound (%)**					
	FFAs extract	Vaseline	Coconut butter	Wax base	Lavender oil	Lemon oil
C1, O1, L1, B1, Cd1, Rb1, M1	40	43.5	14	1	1	0.5
C2, O2, L2, B2, Cd1, Rb2, M2	40	43.5	12	3	1	0.5
C3, O3, L3, B3, Cd3, Rb3, M3	40	43.5	10	5	1	0.5

Compositions of ointments tested. Wax base is carnauba (C1,C2,C3), ozokerite (O1,O2,O3), laurel (L1,L2,L3), bees (B1, B2,B3), candelilla (Cd1,Cd2,Cd3), rice bran (Rb1,Rb2,Rb3) and microcrystalline (M1,M2,M3). Each ointment formulation tested (C, O, L, B, Cd, Rb and M) contained 1, 3 or 5% (w/w) wax, respectively.

FFAs extract is the active ingredient obtained from fish-liver oil by hydrolyzes of omega 3 PUFAs, which FAs profile ingredient is shown in section.4.1. Coconut oil is an emollient, Vaseline is a humectant that generates an occlusive effect and lavender oil and lemon oil were added in an attempt to reduce fish like odor. Although, PUFAs are acknowledged as being susceptible to oxidation as discussed earlier, none of the ointment formulations tested contain antioxidants. The oxidation of the PUFAs was prevented by protecting the ointments from light and oxygen in airtight aluminum tubes.

4.3 Choice of ointment base

Without question, selection of an appropriate additive is critical when comes to formulation and many factors should be considered before selecting an appropriate additive (excipient). Some

factors are of aesthetic nature such as appearance, odor, color and firmness of the formulation which significantly influence the patient acceptability. Other important factors include the physicochemical nature of the vehicle in which the active pharmaceutical ingredient (i.e. the drug) remains stable, drug- excipient interactions and the overall chemical and physical stability of the topical formulation. Sometimes, it is necessary to use several different excipients to obtain vehicle of desired physicochemical properties. The main reason is that it may be impossible for any single excipient to possess ideal characteristics, but if combined with other excipients could enhance aesthetics as well as maximizing therapeutic value of a topical formulation [145]. Attractive physical properties of waxes make them very interesting for the cosmetic and pharmaceutical industry. Various waxes are frequently used as lipophilic excipients for preparing topical ointments, which often differ in their chemical composition, molecular weight, hardness and melting point. Due to their distinct physicochemical properties, their presence in ointment impacts the formulation performance influencing the ointment texture and hardness, and contributing to an extended melting period [129, 146].

In present study, all waxes were selected predominantly by their ability to influence the consistency, texture, retention time and as well as their compatibility with other lipid based ingredients. Our aim was on one hand to perform an extensive study on possibility of use of waxes as ointment excipients, and on the other hand to examine how the quantity of incorporated wax influenced the ointment rheological characteristics. This explains why different waxes as excipients at three levels (1%, 3% and 5%) were selected and their effect on ointment formulation evaluated with regard to homogeneity, spreadability, consistency and thickness. Waxes used were shown in section 3.1.1.

Including waxes in the ointments did not present any difficulties, and the entire process went smoothly. All waxes used in this study were inviolate, non-hazardous with a slight characteristic odor. Vaseline is a widely known occlusive agent which enhances penetration of the active ingredient, and thus should improve the therapeutic efficacy of the product. To avoid a heavy feeling on skin that occlusive agents like Vaseline can leave, they often are combined with other ingredients like emollients to improve consumer appeal [112]. Furthermore, our experience is, that use of Vaseline alone as an excipient where prolonged drug retention is desirable, is not

likely to result in acceptable formulation. Waxes were chosen as viscosity-building excipients to improve the ointments' performance and give them optimal rheological profiles. The combination of Vaseline and waxes produced pharmaceutically acceptable ointments that were less greasy than pure Vaseline and that were retained on the skin for a sufficiently long time to allow for extended drug delivery.

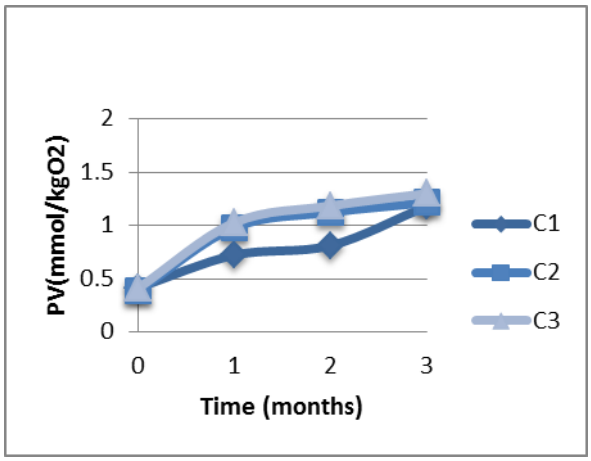
4.4 Changes in the peroxide value of ointment

Polynsaturated fatty acids (PUFAs) like omega- 3 are highly liable to be oxidized at ambient oxygen and light . Peroxide value (PV) is among the most commonly used paremeter in oil industry for stability evaluation of unsaturated lipids. By mesuring PV, the content of of primary oxidation products, especially hydroperoxides can be determined. The peroxide value is regarded as a quality criterion for judging the rancidity and stability of oils. The lower the figure, the better oil quality of [147]. PV was determined for both fresh and stored ointments and changes recorded. Full data is preseneted in Appendix A.

The content of primary lipid oxidation products measured as PV of the FFAs extract was in the range 1.073–1.804 mmol/kg at the end of 3 months storage and 0.824-1.298 mmol/kg at the end of 6 months storage at room temperature.

As can be seen in Figure 11(a-g) there was a rapid increase in peroxide value in all formualtions the first month of storage at 40º C. A possible explanation could be presence of oxygen in the headspace of the sealed tubes. After that, PV in almost all formaultion tested (except O2) increased slightly over time under both, real (i.e. at room temperature) and accelarated conditions (i.e. at 40°C). The results showed that there were no statistically significant differences in the PV values between the different formulations. In the case of Cd1, C2, Cd3 and M1, M2, M3 the PV was almost identical as well as in C1, C2 and L1, L2.Unlike other members of the lipid family, waxes have been found to be resistant to degradation and do not catalyze oxydation of PUFAs [131]. This fact suggested that waxes are not expected to shorten the shelf-life of the PUFAs ointments. Indeed, no correlation between PV and presence of wax was found, and the increased concetration of wax did not alter the PV of any of the ointment formulations tested. Therefore, it

can be concluded that oxidation of the PUFAs occurred under both real and accelerated storage conditions, but it was independent of ointment composition.



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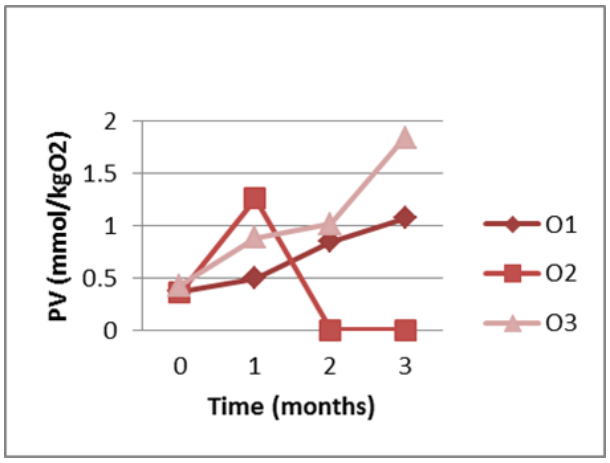

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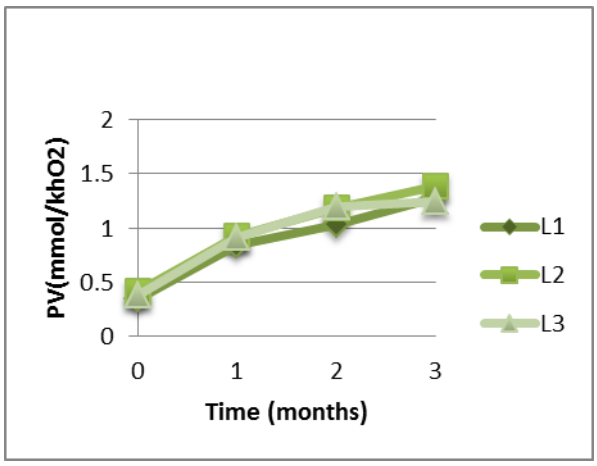

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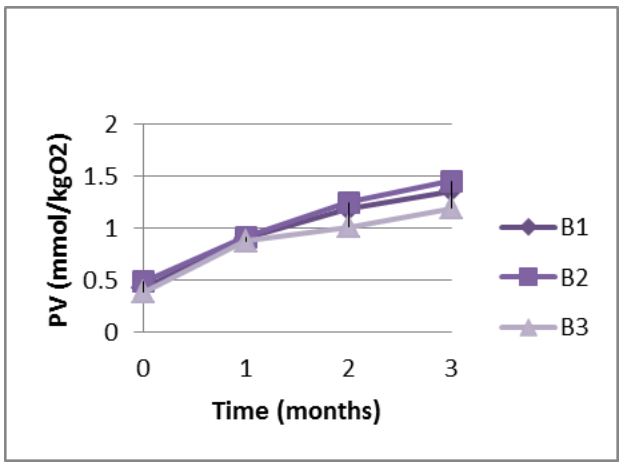

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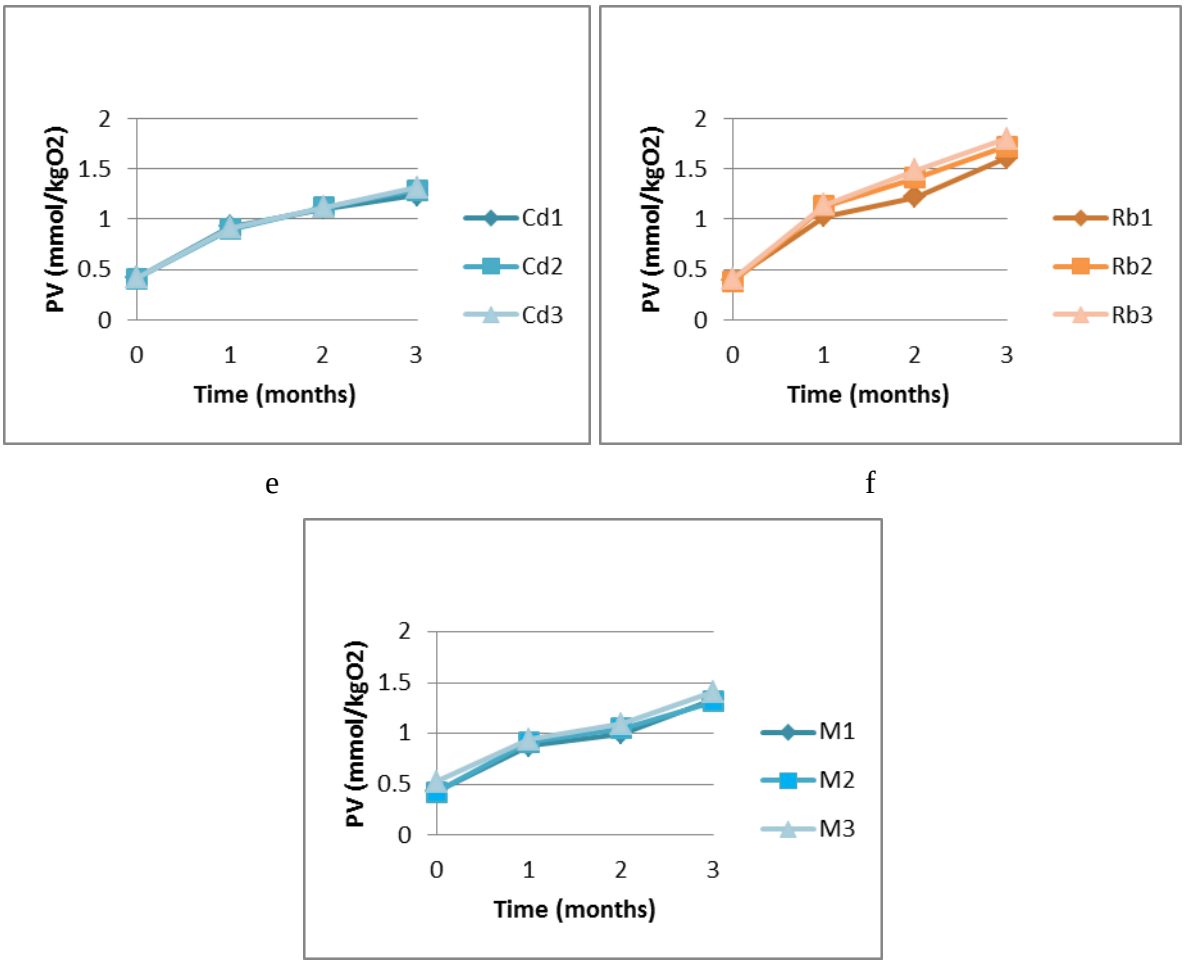

Figure 11. (a-g) Lipid oxidation over accelerated tested period of time as measured by peroxide value

A significant difference was observed only in formulations containing ozokerite wax as indicated by a high variation in formulation O2 as shown in Fig.11b. Surprisingly, O2 formulation (contains 3% ozekirite wax and 40% FFAs) demonstrated highly unpredictable results (PV=0), but only under the accelerated storage conditions (i.e. at 40°C). As can be noticed, PV value in both, O1 (PV=1.246) and O3 (PV=1.120) was not significantly higher than in other formulations over the 6 months storage at room temperature. A probable reason for this unexpected PV of O2 formulation could be human factor such as, human error, technical skills to complete titration experiments, or luck of thorough and meticulous sample preparation. Another reason might be a

possible secondary oxidation resulting in zero PV. When lipids oxidize, PV decreases. Besides the mentioned factors, the increased oxidation in this study could be result of the age of fish oil extract, storage conditions and improper temperature when ointment was prepared and inadequate tube sealing. By increasing the temperature and time exposure, it is possible that fish oil had been affected and lead to increased oxidation. These results are in contrast with other two formulations containing ozokerite wax, O1 and O2. For example, O3 (5% ozokerite wax) compared O2 (3% ozokerite wax) required longer period of heating to melt the entire amount of wax at same temperature, but no increased oxidation was observed. Other explanation could be that aluminum tubes were not sealed properly so the oxygen access and heat applied could have increased the oxidation.

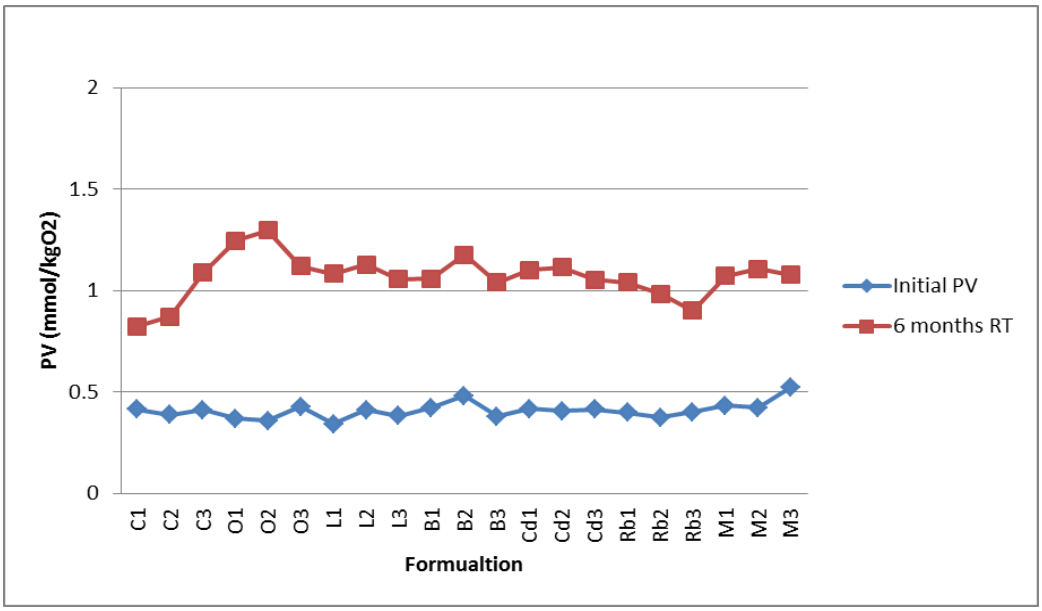

Figure 12. Lipid oxidation over real time tested period as measured by peroxide value.

It should be mentioned that PV method has its own limitations because it is very sensitive method, and can only be used for evaluation on of oxidative stability in the early stages of the oxidation process. Therefore, anisidine test might be a better indicator of the extent of lipid oxidation since is based on measurements of secondary oxidation products [131]. Additionally, it is also possible to calculate Totox (i.e. the total oxidation value) as stated above. The Totox

provides a better estimation of overall oxidative deterioration, but these two parameters (i.e. anisidine test and Totox) were out of the scope of this project**.** However, PV of all formulations was within the range which is considered to be acceptable for fish oils for human consumption. According to the European Pharmacopeia, PV in refined fish oils used for dietary supplements should not exceed 5mmol/kg. It is most likely that when PV reaches 10mmol/kg fish oil cannot be used because of rancidity and does not meet the quality standards [148]. But, it also must be considered that this formulation was not intended for oral dosage form, but a topical. While it is very unlikely that peroxide changes are a risk to the patient, the appearance and odor change is of special importance for the patient acceptance, and may determine the product acceptance as well as the shelf-life of a pharmaceutical product.

4.5. pH determination

The normal pH of the skin is slightly acidic or in the range of pH 4-6. Skin can tolerate variations in this range, but extremes of pH in either direction, acidic and basic, can cause irritation or redness at the site of application. Therefore, topical formulations that have different pH than skin can cause skin irritation, especially in people with tender skin [149]. Those products can disrupt the lipid barrier of the skin and can also weaken the skin's natural defenses to pathogens and environmental influences. It is important to mention that the relative success or failure of topical product, among other determining factors, depends also on its appropriate pH balance, that it does not interfere with skin pH [150].

The results of pH measurements, with the exception of L1, L2 and L3 formulations were not surprising. As can be seen from results shown in Fig.13 the pH was found to be between 3.83 to 5.44 and for almost all ointment by increasing the wax concentration from 1 to 5 % resulted in slight pH decrease with the exception of L1, L2, and L3 wherein pH slightly increased. In fact, pH increased when laurel wax concentration was increased from 1% to 3%, but almost no changes were found when laurel concentration was increased from 3% to 5%. Of all the ointments, the lowest pH was observed in L1 (1% laurel wax), and the highest in C1 (1% carnauba wax). Initially, slightly acidic pH values were expected, largely due to high amount of

FFAs (40%) in each formulation. This can be because FAs are weak acids with dissociation constant pKa value of about 4.8 that increases slightly with increasing chain length. Acidic pH values are most likely associated with the physical state on FAs that is probably to be found in the interface between the lipid and water. Fatty acids do not dissolve in water, instead they have ability to extend out dissociated polar carboxyl groups into the surrounding water forming bilayers, and changing surface pH [3, 4, 151].

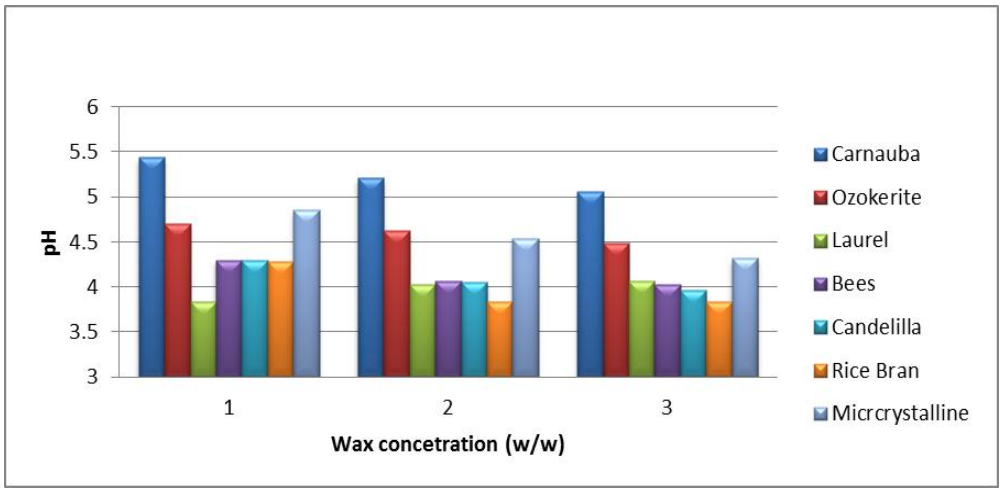

Figure 13. The graph shows the average experimental pH values on the ointments from at least 3 replicates

A possible explanation for the changes in pH influenced by the choice of wax lies in the versatile wax composition that as discussed above tends to vary much depending on the compositional balance of hydrocarbons and fatty acids esters they contain. Moreover, the pH differences may be due simply to differences in the fatty acid content. These findings suggested that nature of wax may have had an important influence on the expected safe pH level for ointment application. When wax concentration increased, there was no dramatic change in pH. However, it may be concluded that the pH range of nearly all ointments was in acceptable limits and skin can tolerate variation in the indicated pH range by ointment compositions. pH of L1 (3.83± 0.03), B3 (3.96 ± 0.01),Cd2 (3.84 ± 0.02), Cd3 (3.83 ± 0.02) was slightly below the 4. Even though that is only weakly acidic it still might lead to a slight irritation. Being aware of possible skin irritation, ointments with pH lower than 4 (L1, B3, Cd2 and Cd3) were experienced by applying a small

amount of ointment on 1cm^2 area of the skin and observed for about 15 minutes to see if a reaction develops such as redness, itching or pain in the area of application. It is worth noting that this test procedure maybe is not best but may indicate sensitivity and tend to produce quick results. However, skin irritation was not observed that suggest that all formulations were dermally safe. Furthermore, our findings correlated with the recommendation of pKa value for drug to be used between 4 and 8 to avoid feel of itching or skin irritation [128].

4.6 Influence of ointment base on spreadability

Spreadability is one of the most important physical characteristic of semi-solid dosage forms that can affect patient/consumer acceptability and greatly impacts the delivery of the correct dose. This is of particular concern especially when potent drug needs to be delivered. In practical terms, a reduced dose would not deliver the desired effect, and an excessive dose may also cause undesirable side effects. Spreadability, in principle, correlates with the rheological properties and presents a simple and accurate approach to understand the thickness, viscosity and firmness of formulations [152, 153].

In efforts to develop an easily deliverable formulation, it was necessary to analyze the influence of the compositional parameters on the ointment's spreadability and consistency. In other words, understanding the ointmment's rheological profile empower us to select the best compounds leading to development of efficient formulation. Various waxes such as beeswax, carnauba wax, ozokerite wax, microcrystalline wax, candelilla wax, laurel wax , and rice bran wax were tested in combination with Vaseline to create an acceptable pharmaceutical ointment. It was attempted to prepare ointment only from FFAs extract, Vaseline and coconut butter, but the composition containing as much as 43.5 % Vaseline was too soft and melted immediately after applying. A major limitation of the topical route of administration is the lack of retention of the dosage form at the site of application. At the levels employed, coconut butter contributed to emolliating properties, but it did not give the optimal ointment thickness and expected prolonged retention. The majority of the waxes used in this study were able to improve the thickness and influence

resistance to flow, and it was therefore of interest to determine what factors and to what extent different waxes were effective.

Spreadability measurements are presented in Fig 14 and showed the highest spreadability of formulations B1, B2, and B3 and the lowest spreadability of C1, C2, C3 and O1, O2 and O3. No statistically significant difference was found between formulations L1, L2, L3 and M1, M2, M3. The formulations Cd1, Cd2, Cd3 and Rb1, Rb2, Rb3 showed good spreadability, wherein were graded not as fluid as B1, B2, B3 and not as stiff as C2, C3, O2, O3.
The spreading values of these formulations were in the following order: B1 > B2 > B3 > L1 > M1 > L2 > Rb1 > M2 > C1 > O1 > L3 > Cd1> Rb2 > M3> Cd3 > Cd2 > Rb3 >O2 > C2 > C3 > O3.

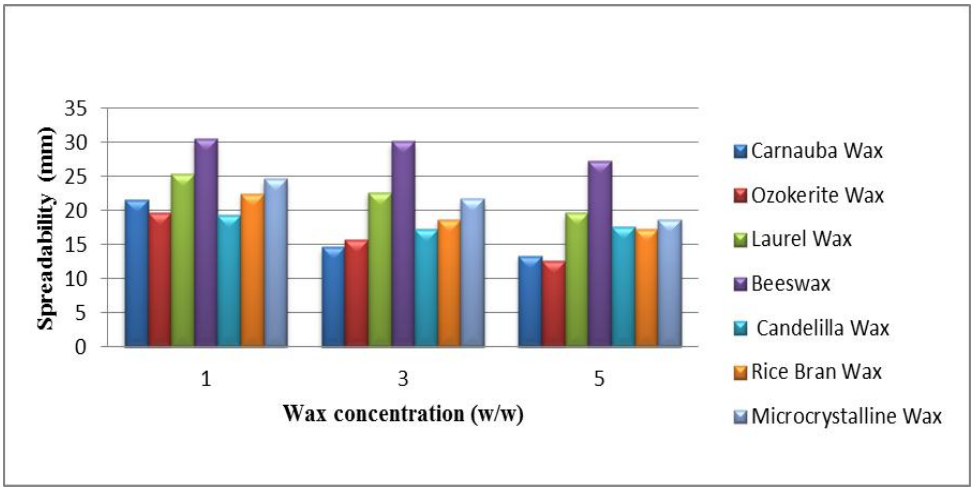

Figure 14. Spreadability average comparison within and among all waxes tested.

Ointment' spreadability was also found to be inversely proportional to wax concentration. As the wax amount gradually increased, ointments were thickened and in all cases increased wax concentration from 1 to 5% led to lower spreadability, indicating that correlation exists between both the wax content and with the resulting spreadability. Further, the presence of any of selected waxes increased the viscosity which directly and adversely influences otintment'spreadability. To ensure optimal and homogeneous spreadability, and thus provide augmented drug delivery, the wax selection and its amount must be carefully considered.

4.7 Influence of ointment base on tube extrudability

Extrudability measures how easily a formulation can be squeeze from the container. Tube extrudability indicates the flow behavior of the product and may be used as a tool to predict a viscosity of the product. Although this approach is not perfectly accurate and perhaps too simplistic, the ointment's extrudability can reflect the ointment´s viscosity. In fact, it is very common results for extrudability to estimate viscosity. Of course, better measuring techniques are available to quantify viscosity, but we decided to simplify the measurements assuming that extrudability, in principle, correlates with ointment flow properties and viscosity in general [154, 155]. We believe that an instrument as viscometer would have given more precise numerical statement, but the estimation result for extrudability can be representative along with its uncertainty.

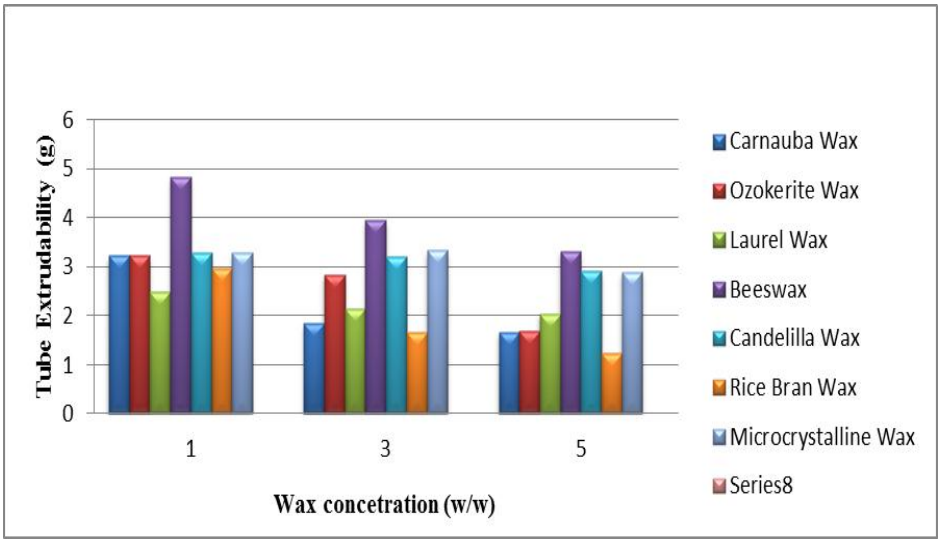

Figure 15. Tube extrudability average comparison within and among all waxes tested

The extrudability values of these formulations were in the following order: Rb3 > C3 =Rb2 > O3 > C2 > L3 > L2 > L1 > O2 > M3 > Cd3 > Rb1> O1=C1 > M1=Cd1 > B3 > Rb3 >M2 > B2 > B1.

It was found that how much squeeze force was applied determines the output. If a small squeeze force was applied, a small amount was extruded. It was tempting to apply same size of the force to all formulations in the same amount of time. However, inability to apply a highly accurate force led to relative figures that seemed efficient to conclude that there was a linear regression between the amount of wax and extrudability. The increase in wax concentration resulted in the decrease in extrudability. In nearly all cases increased wax concentration from 1 to 5% led to considerably decrease in tube extrudability except in formulations Cd1, Cd2, Cd3, and M1, M2, M3, and L2, L3 wherein decrease in extrudability was less pronounced. It can be concluded that between 1 and 5% wax content, ointments formulated with carnauba wax are the hardest, while ointments formulated with bees wax are less viscous.

The rheological performance of ointments is likely to be explained with the rheological profile of waxes that can be explained by the nature of the fatty acid moieties of the waxes. For example, carnauba wax is the hardest, easily recognized by highest melting natural wax, is composed mainly of esters of saturated very long-chain fatty acid (C24 and C28) and straight-chained primary alcohol (C32 and C34). In contrast, beeswax is a mixture of wax esters of C16 and Cl8 carboxylic acids and C30 and C32 straight-chained alcohol, and unsaturated hydrocarbons from C21 up to C35 with one or two double bonds [156]. Thus, addition of carnauba wax to the ointment vehicle gave lowest spreadability and extrudability while adding beeswax gave highest spreadability and extrudability.

As in the case of ointment‘spreadability, ointment’s extrudability was dramatically affected by wax presence and its amount in the ointment. In conclusion, waxes offered increased viscosity and enhanced retention of drug, which in turn results in the effective treatment of dermatological diseases.

4.8 Sensory evaluation

When topical formulation is being developed, sensory perceptions on texture, consistency and homogeneity are factors that need to be considered. These factors may directly determine overall

patient experience and acceptance and can be extremely challenging. But we surely have to bear in mind that the results from sensory evaluation are far from objective. Certainly, when ointment is applied, differences in color are possible with subjective visual assessment. Same fact refers to odor assessment. Thus, improved accuracy, precision and larger panel study appeared to be highly recommended to ensure consistent observations. From the presented results given in appendix B, it was concluded that all the formulated ointment showed overall good appearance. The physical appearance of the ointments was light brown in general, which was the color they got from the fish oil extract. Regardless the wax presence and content, only minor changes in brown color were observed within ointment .As far as the odor concern, all formulations had strong distinctive smell of fish odor.

However, the main focus was on the ointment texture. The present study showed significant difference in consistency and homogeneity when comparing different waxes. Most likely, as already noted above, the wax nature and chemical composition greatly impact the ointment texture. The overall best results were obtained when we used beeswax, rice bran, laurel and microcrystalline wax as a base. Our results showed that all ointments composed of these four waxes had homogenous texture and free of particles during the period of study. Overall cosmetic acceptability was rated satisfactory for C1, C2, and Cd1 and Cd2 .But, an immediately altered texture was seen with use of wax at concentrations as high as 5% for C3 and Cd3, which were graded as unsatisfactory. B1, B2 and B3 as well as L1, L2 and L3 had an advantage of providing an additional glossiness appearance without having a negative impact on homogeneity and greasiness. The use of ozokerite affected the sensory profile differently. Initially, O1 and O2 showed an excellent sensory profile, but after 1 month under accelerated conditions the texture worsened resulting in gritty texture. O3 formulation was considered to be of satisfactory grade during the whole study period. Compared to other formulations that had rough, gritty texture (carenuba, ozokerite), ointments that contain candelilla wax differ by possessing large visible particles distributed throughout glossy, homogenous surrounded base. Although, some of ointments had rather satisfactory parameters, visible phase separation was not observed. However, it was found that particles detected in unsatisfactory formulations melted within few seconds when applied on the skin surface. Thus, this problem may demand an administration of

homogenizer that actually reduces the size of particles and distribute it evenly throughout the other constituents in the formulation.

4.9 Microscopic evolution

The results of sensory evaluation showed different texture, consistency and homogeneity of the ointment compositions. These differences were also confirmed by observing the microstructure of the ointments described by the particle's shape and size.

The first assumption made when the ointments were formulated was that the dispersion of the solid wax portion depended principally on their melting range. It was assumed that if wax melting point was significantly higher than other compounds in ointments, solidification would rapidly occur resulting in non-homogenous structure. Melted FFAs have low viscosity and can stay in liquid state for a long period, so wax incorporation was performed at temperature far below solidification point except in the case of laurel wax. Thus, melting points of waxes are of limited usefulness and the reverse process (i.e. solidification) attracted our attention. Apart of melting and solidification profile of the composition, many other factors also can influence the ointment's non-homogeneous texture including variations in preparing techniques and crystallization conditions such as cooling rate. In particular, the solidification behaviors of waxes and their influence on homogeneity and consistency of the finished products are of primary concern because except type of wax used, all the ointment components were identical as well as the method of preparation.

Solidification behavior of waxes can range from the relatively simple crystallization to very well organized crystalline arrangements. During the solidification, the temperature decreases and crystal structures in solid-wax deposits are produced. Formation of crystal structures depends on temperature change and purity of compounds. So when the temperature drops rapidly or impurities are present, crystal malformation may occur. The major reason for the variation on wax solidification properties is the diversity of wax molecular structure. Also, the chemical composition, that is different, especially regarding chain lengths of fatty acids and alcohols, affects the phase behavior of the waxes leading to the solidification differences. For example,

microcrystalline wax is of mineral origin and is mainly composed of straight-chain-saturated hydrocarbon chains that crystalize in small fine plates or needles [157, 158].

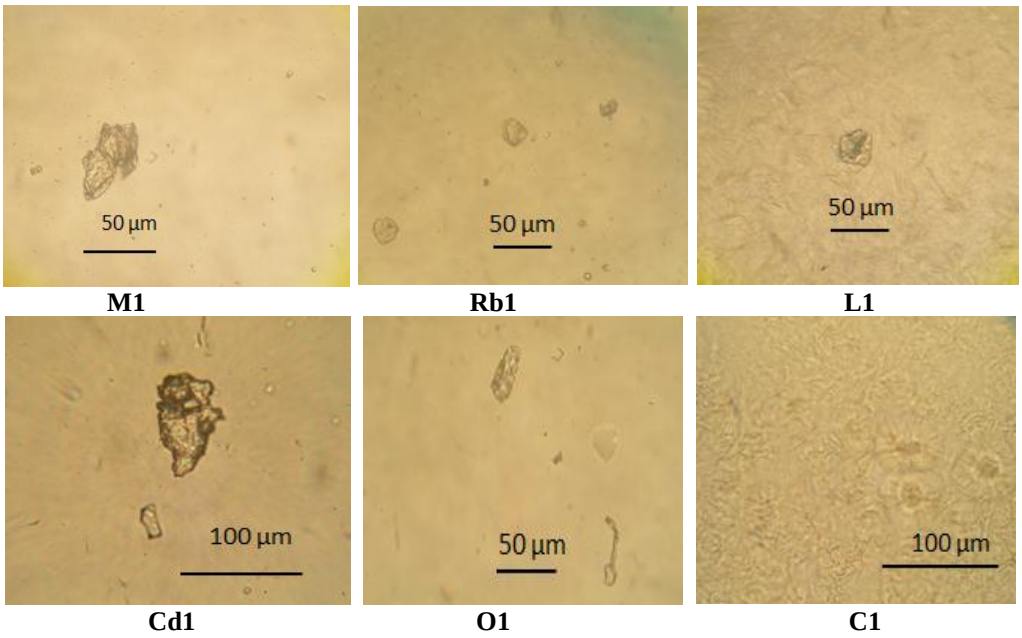

Fig.16. The microstructure of M1, Rb1, L1, Cd1, O1 and C1 formulations evidenced differences in particle‘s size and shape. It was observed small crystals of microcrystalline, rice bran, laurel, carnauba and ozokerite that are suspended in ointment compared to the candelilla wax at the same concentration

Initially, only best textured formulations were selected for microscopic analyses .But later, when ointment containing candelilla wax displayed relatively interesting texture the focus was extended to include the other five formulations, C1 ,O1, L1, Rb1, M1 and of course Cd1. Analyzing the images in Fig.16, it became apparent that the overall shape of the crystals was very similar in the case of Rb1, L1 and C1 (medium-high sphericity) but sphericity was not observed in the case of M1, Cd1 and O1. All ointments, except the ointment containing candelilla wax showed good morphological characters with a particle size of approximately 50µm in diameter. Cd1ointment showed an unusual morphological character in having much larger crystals, eventually clusters. It was found that the particle size was almost twice bigger (aprox.100µm or less) as compared to others. Clusters are likely present in M1, but rather smaller size than C1. The solid particles in Cd1 originally precipitated at the surface broke when a slide

cover was placed to cover the sample. However, it can be concluded that the shape and size of the particles formed make the dispersion processes of waxes more complex than it first appeared, and likely to be associated with very complex properties of waxes that evidently depend on their compositional profile. On the other hand, thermal fluctuation or disturbance during the crystal-growth process may alter the crystal formation. For example, rapid cooling may lead to loss of wax solubility resulting in wax participation. In our study, all compositions solidified spontaneously at ambient temperature, so the temperature reduction can be disregarded as a reasonable factor for uneven dispersion or precipitation. Therefore, wax nature determined by fatty acids moieties may be considered be the most influential in exhibiting different wax dispersion which adversely influences the precipitation of formulation Cd1.

4.10 Stability studies

Once an active pharmaceutical ingredient (API) has been selected and pharmaceutical formulation has been developed, the next step in product development is to check API stability within the ointment formulation. The product stability is important to ensure that the efficacy and safety of pharmaceutical products is maintained throughout its shelf-life. This can be accomplished by evaluation a set of data such as peroxide value, spreadability, consistency, microbial growth and sensory profile throughout the expected shelf-life [155]. It should be pointed out that not all factors were investigated here, and our main focus was the possible lipid oxidation due to the high PUFAs content of the ointment. Therefore, lipid peroxides and sensory data were emphasized. Due to the effective product protection of the packaging, especially against oxidation as discussed above, antioxidants were not used in the product.

Since omega-3 PUFAs are chemically unstable, fish oils rapidly oxidize during storage catalyzed by heat or light. As a result of chemical degradation, loss of potency may be detected and unpleasant odor might be observed [67]. Nevertheless, peroxide value (PV) assay was effective way to estimate lipid oxidation and estimating shelf-life when used together with other tests, especially in the presence of sensory evaluation and other tests. While odor and color again should have no great impact on overall potency, they still can be disturbing to patients and as

such can be restrictive factors for product acceptance. Another factor that might attribute to the patients use of the product is its texture profile. Clearly, there is a difference in sensory experiences acceptable to people. Inhomogeneous and gritty texture may not result in a change in pharmacological activity, but it can give unpleasant sensations. Thus, to reach comfortable and pleasant experience, special attention has to be given to the texture and overall sensory properties of the formulation.

The results of PV assay indicated that formulation O2 was oxidized, however, neither odor nor color change was observed. All formulations had strong distinctive smell of fish odor, but other smells or smell of rancid fish oil was not detected. It should be noted that smell was subjectively evaluate by the evaluator. A slight change in color in the formulations at 45°C and “bleeding“ after 90 days was visually observed. The phenomenon bleeding was attributed to the storage at high temperature. This is a well-known change in consistency where oils sweat or bleed in blends of oils and waxes that usually appear on the top of filled tube, and is frequently referred to as ointments main stability problem [155].

The data in appendix C represents how stable the ointments were during 6 months at ambient temperature and for 3 months at accelerated conditions (40°C). It has to be emphasized that all ointments containing microcrystalline wax, rice bran wax, laurel wax and beeswax were stable during 6 months storage at room temperature and displayed no change in the texture or smell of oxidized FFAs. The results from the short accelerated aging study showed also that these formulations passed the testing displaying both physical and chemical stability. A deviation from initial sensory parameters was observed in the case of C1, C3, O1, O2, Cd1 and Cd2 after 6 months ambient conditions as well as accelerated aging. According to the results obtained from short-time stability study, C1, C2, O1, O2, Cd1, Cd2 and all formulations containing rice bran wax , laurel wax and bees wax were slightly different in homogeneity than the original samples. However, besides the textural and sensory changes, phase separation was not observed.

5. Conclusion

In the current trend for development of natural and safe anti-bacterial compounds with minimal side effects, fish oil has proved to be an effective antibacterial agent. The *in vitro* study of fish oil extract showed that it has significant antibacterial activity against Gram- positive bacteria such as *S. aureus, E. faecalis, S.pyogenes* and *S.pneumonia*.

The ointments were shown to have a great potential as an effective and safe way to administer PUFAs for topical antimicrobial therapy. Based on this study, it may be concluded that waxes had significant effect on the ointments' rheological properties and could be successfully employed as effective lipophilic excipients for topical product such as ointments. In our studies we used seven types of waxes. It appeared that presence of wax in the ointments tested had a thickening and aesthetic effect on the ointment composition. Significant differences in rheological properties were observed among waxes, showing that the viscosity is dependent not only on the amount of wax, but also of its chemical nature. Of the waxes that were evaluated, microcrystalline wax appeared superior to the other waxes tested. Potentially, rice bran wax, laurel wax and beeswax could also be used as suitable excipients for ointments even though they influenced differently the rheological and sensory profile of ointments.

Further studies are recommended to be carried out in two steps:

I. The employment of instruments is required to confirm the existing results from rheological properties. Although, rheological and sensory profiling may be simplified and to some extent predictive, instrumental measurements are needed that gave accurate and reproducible results.

II. Extend the testing to include combinations of different waxes. It would be interesting to see how a combination of waxes influences the rheological and sensory profiling. Perhaps, two or more different waxes in combination can influence the rheological properties and result in a topical formulation with better sensory and therapeutic profile.

6. Acknowledgement

First of all, I would like to express my sincere gratitude to my supervisor Prof. Þorsteinn Loftsson for the continuous support of my MSc study and for his patience, motivation, and immense knowledge.

This thesis would not have been possible unless Prof. Martha Ásdis Hjálmarsdóttir, Head of Department, Faculty of Medicine, contributed with all the helpful information, involvement and some experimental details for the project.

I also want to thank Guðrun Marta Ásgrimsdóttir, MSc.Pharm, for her valuable assistance, as well as her support and advice.

I am especially thankful to Þór Sigfússon, PhD, Founder and Head of Sjávarklasinn, for his motivation and encouragement.

At last, I must thank my family and friends for their support during my studies, especially to my husband and my two wonderful daughters for always being there for me. This accomplishment would not have been possible without their support, patience and love.

Thank you all.

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Appendix A

PV measurements (mmol O_2/kg)

Formulation	Initial			1month			2 months 40 ºC/75%RH			3months			6 months RT		
	ml	g	PV	ml	g	PV	Ml	g	PV	Ml	G	PV	ml	g	PV
C1	0.09	1.08	**0.416**	0.15	1.07	**0.723**	0.16	1.02	**0.812**	0.24	1.02	**1.183**	0.17	1.04	**0.824**
C2	0.12	1.04	**0.388**	0.20	1.00	**0.982**	0.23	1.04	**1.124**	0.26	1.07	**1.215**	0.1	1.06	**0.871**
C3	0.08	1.05	**0.412**	0.21	1.03	**1.027**	0.23	1.01	**1.185**	0.26	1.00	**1.300**	0.22	1.01	**1.089**
O1	0.08	1.08	**0.370**	0.1	1.01	**0.495**	0.17	1.03	**0.848**	0.22	1.04	**1.073**	0.24	1.00	**1.246**
O2	0.07	1.01	**0.360**	0.26	1.03	**1.261**	0	1.05	**0**	0	1.08	**0**	0.27	1.04	**1.298**
O3	0.09	1.05	**0.428**	0.18	1.02	**0.882**	0.1	1.03	**1.019**	0.38	1.03	**1.844**	0.22	1.01	**1.120**
L1	0.12	1.02	**0.344**	0.17	1.01	**0.844**	0.21	1.07	**1.028**	0.26	1.02	**1.284**	0.22	1.04	**1.082**
L2	0.09	1.05	**0.412**	0.18	1.02	**0.927**	0.24	1.02	**1.180**	0.28	1.03	**1.382**	0.23	1.02	**1.128**
L3	0.22	1.04	**0.382**	0.18	1.02	**0.912**	0.24	1.04	**1.200**	0.25	1.02	**1.246**	0.21	1.02	**1.055**
B1	0.08	1.05	**0.422**	0.18	1.03	**0.904**	0.24	1.04	**1.183**	0.27	1.00	**1.355**	0.21	1.02	**1.058**
B2	0.10	1.00	**0.482**	0.18	1.00	**0.912**	0.25	1.01	**1.245**	0.30	1.03	**1.456**	0.24	1.02	**1.176**
B3	0.07	1.02	**0.380**	0.17	1.02	**0.876**	0.20	1.02	**1.008**	0.25	1.05	**1.190**	0.21	1.01	**1.039**
Cd1	0.08	1.03	**0.418**	0.18	1.02	**0.927**	0.22	1.01	**1.102**	0.25	1.01	**1.238**	0.22	1.01	**1.102**
Cd2	0.08	1.00	**0.408**	0.19	1.08	**0.902**	0.23	1.06	**1.118**	0.26	1.01	**1.293**	0.22	1.02	**1.114**
Cd3	0.08	1.01	**0.416**	0.18	1.01	**0.914**	0.23	1.03	**1.120**	0.27	1.05	**1.315**	0.21	1.01	**1.054**
Rb1	0.08	1.02	**0.398**	0.21	1.03	**1.026**	0.24	1.01	**1.220**	0.33	1.04	**1.612**	0.21	1.03	**1.039**
Rb2	0.07	1.02	**0.376**	0.23	1.02	**1.128**	0.29	1.04	**1.412**	0.35	1.02	**1.726**	0.19	1.00	**0.983**
Rb3	0.08	1.04	**0.402**	0.22	1.00	**1.145**	0.30	1.04	**1.490**	0.36	1.01	**1.804**	0.18	1.00	**0.902**
M1	0.09	1.04	**0.433**	0.18	1.05	**0.880**	0.20	1.00	**1.002**	0.27	1.03	**1.337**	0.22	1.03	**1.073**
M2	0.08	1.02	**0.424**	0.18	1.01	**0.917**	0.21	1.02	**1.048**	0.27	1.02	**1.316**	0.23	1.05	**1.108**
M3	0.10	1.05	**0.524**	0.19	1.02	**0.944**	0.22	1.03	**1.092**	0.28	1.02	**1.412**	0.22	1.04	**1.078**

Appendix B

Full data on pH, spreadability and extrudability measurements

Formulation Code	pH	Spreadability (mm ± SD)	Tube extrudability (g ± SD)
C1	5.44 ± 0.04	21.5 ± 0.87	3.23 ± 0.19
C2	5.21 ± 0.01	14.66 ± 0.20	1.84 ± 0.05
C3	5.06 ± 0.05	13.33 ± 0.57	1.67 ± 0.10
O1	4.7± 0.03	19.66 ± 0.57	3.23 ± 0.39
O2	4.6 2± 0.07	15.66 ± 0.2	2.84 ± 0.62
O3	4.48 ± 0.02	12.66 ± 1.5	1.69 ± 0.53
L1	3.83 ± 0.03	25.33 ± 2.51	2.50 ± 0.12
L2	4.03 ± 0.005	22.66 ± 0.57	2.15 ± 0.07
L3	4.06 ± 0.05	19.66 ± 1.52	2.04 ± 0.09
B1	4.30 ± 0.03	30.5 ± 1.32	4.84 ± 0.79
B2	4.05 ± 0.05	30.13 ± 0.28	3.95 ± 0.49
B3	3.96 ± 0.01	27.33 ± 0.57	3.32 ± 0.07
Cd1	4.28 ± 0.04	19.33 ± 1.15	3.30 ± 0.26
Cd2	3.84 ± 0.02	17.33 ± 0.57	3.20 ± 0.1
Cd3	3.83 ± 0.02	17.66 ± 0.57	2.92 ± 0.36
Rb1	4.85 ± 0.016	22.5 ± 0.5	2.97 ± 0.67
Rb2	4.54 ± 0.026	18.66 ± 0.57	1.67 ± 0.34
Rb3	4.32 ± 0.018	17.33 ± 1.52	1.24 ± 0.16
M1	4.24 ± 0.016	24.66 ± 6.65	3.30 ± 0.26
M2	4.21 ± 0.008	21.66 ± 0.57	3.33 ± 0.14
M3	4.09 ± 0.016	18.6 6 ± 2.88	2.90 ± 0.13

Appendix C

Sensory evaluation on ointments

Formulation	Initial	1 month	2 months 40°C	3 months	6 months RT
C1	**	**	*	*	*
C2	**	*	*	*	**
C3	*	*	*	*	*
O1	***	***	**	**	**
O2	***	***	**	**	**
O3	**	**	**	**	**
L1	***	***	***	**	***
L2	***	***	***	**	***
L3	***	***	***	**	***
B1	***	***	***	**	***
B2	***	***	***	**	***
B3	***	***	***	**	***
Cd1	**	*	*	*	*
Cd2	**	*	*	*	*
Cd3	*	*	*	*	*
Rb1	***	***	***	**	***
Rb2	***	***	***	**	***
Rb3	***	***	***	**	***
M1	***	***	***	***	***
M2	***	***	***	***	***
M3	***	***	***	***	***

***Excellent ** Satisfactory * Unsatisfactory

Appendix D

QUALITY CONTROL LABORATORY
Rannsóknarstofa

LYSI

CERTIFICATE OF ANALYSIS
Efnagreiningarvottorð

Customer:	Lipid Pharmaceuticals ehf.
Description:	91-002 Free Fatty Acids
Sample no:	1821-2015

RESULTS OF ANALYSIS

Free fatty acids (%):	97	(method SKL 5-20)

Reykjavík, 29/09/2015

Einar Lúthersson
Einar Lúthersson
Lysi hf. laboratory

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Appendix E

Journal of Drug Delivery Science and Technology xxx (2016) 1–5

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Review article

Fatty acids from marine lipids: Biological activity, formulation and stability

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ARTICLE INFO

Article history:
Received 23 November 2015
Received in revised form
7 March 2016
Accepted 8 March 2016
Available online xxx

Keywords:
Polyunsaturated fatty acids
Marine lipids
Formulation
Stability

ABSTRACT

Cod-liver oil from Atlantic cod (*Gadus Morrhua* L.) and other fish oils are the main source of ω-3 polyunsaturated fatty acids (n-3 PUFAs) such as EPA and DHA that are known to possess numerous of health benefits and, thus, fish oils are commonly used as dietary supplements. Free unsaturated fatty acids (FAs) extracted from fish oils do also possess wide variety of biological effects. Free FAs extracted from cod-liver oil have been shown to possess antibacterial, antifungal and antiviral effect when formulated as water-free ointments and to possess laxative effect when given rectally in suppositories. The free FAs from cod-liver oil can also be used as enabling pharmaceutical excipients that enhance drug permeation through skin and the oral mucosa. Although in some cases these natural free FAs are less potent than many synthetic drugs they are also less likely to cause toxic side effects and this allows for administration of larger doses. Most often the potency of a given biologically active agent is not important per se but rather the therapeutic index of the agent, that is to say the ratio of the dose that causes toxic side effects and the dose producing the desired therapeutic effect.

Contents

Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CD, cyclodextrin; PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; MUFA, mono-unsaturated fatty acid; PhEur, European Pharmacopoeia, 8th Edition; SE, standard error; SFA, saturated fatty acids.

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http://dx.doi.org/10.1016/j.jddst.2016.03.007
1773-2247/

1. Introduction

The health benefits of marine lipids containing polyunsaturated fatty acids (PUFAs), especially ω-3 polyunsaturated fatty acids (n-3 PUFAs), have been intensively studied over the past several decades. In general, *in vitro* experiments and studies in animals have consistently shown health benefits while studies in humans have

Please cite this article in press as: T. Loftsson, et al., Fatty acids from marine lipids: Biological activity, formulation and stability, Journal of Drug Delivery Science and Technology (2016), http://dx.doi.org/10.1016/j.jddst.2016.03.007

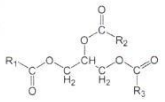
sometimes given mixed results [1–4]. Nevertheless, it is believed that PUFAs can have beneficial effects against wide variety of diseases including cardiovascular diseases [5,6], psychiatric disorders [7], age-related macular degeneration [8], cancer [9], arthritis [10], colitis [11] and pancreatitis [12], some of which are related to the anti-inflammatory effects of the long chain n-3 PUFAs [13–15]. But PUFAs are known to possess other biological effects such as antibacterial, antiviral and antifungal effects [16,17], and they have been shown to have laxative effect when given rectally [18], and to enhance drug permeation through skin [19] and mucosa [20]. These biological effects are frequently associated with specific PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The studies show that PUFAs can be used as enabling pharmaceutical excipients as well as active pharmaceutical ingredients. Following is a short review on the pharmaceutical applications of free fatty acid mixtures extracted from cod-liver oil, their composition, chemistry and biological activities.

2. Chemical stability and stabilization

Fatty acids (FAs) can exist in their free form and as esters in more complex lipids such as triglycerides, phospholipids and glycolipids as well as parts of complex structures such as lipoproteins and lipopolysaccharides. Fish oil mainly consists of mixed triglycerides (>90%) where three fatty acids, of which at least two are different, are bonded via ester linkage to a glycerol backbone. Other ingredients include other lipids (about 8%) such as monoglycerides, diglycerides and phospholipids, and unsaponifiable matter (1.5–2%) such as sterols, glyceryl ethers, fatty alcohols and vitamins A, D and E [19,21]. Cod-liver oil is purified fatty oil obtained from fresh livers of Atlantic cod (*Gadus Morrhua* L.). This is a pale yellowish clear liquid that mainly consists of mixed triglycerides of saturated and unsaturated fatty acids, including long-chain n-3 PUFAs such as EPA and DHA (Table 1). While vegetable oils such as corn oil may contain large amounts of linoleic acid (18:2 n-6) marine lipids such as cod-liver oil contain relatively high concentrations of EPA (20:5 n-3) and DHA (22:6 n-3) and are the major source of n-3 PUFAs. FA triglycerides are hydrolyzed in the small intestine to form free FAs and FA monoglycerides before being absorbed into the general blood circulation and reassembled again as triglycerides [22,23]. PUFAs such as EPA and DHA have somewhat higher oral bioavailability when given as triglycerides than when they are given as free FAs while ethyl esters of EPA and DHA, that are found in many nutritional supplements, have somewhat lower bioavailability [4].

Unsaturated FAs, and particularly PUFAs, are highly susceptible to oxidative degradation, especially in their free (i.e. unesterified) form. The main obstacle in the usage of unsaturated FA in pharmaceutical products is their chemical instability. Free and esterified unsaturated FAs undergo autoxidation, a complex oxidative degradation that proceeds through free radical chain reactions under formation of volatile secondary oxidation products. The radical chain process consists of three steps (Fig. 1) [24,25]. A free radical is formed in the initiation step, frequently through thermal or photochemical hemolytic cleavage of an R-H bond. Atmospheric oxygen is added to the free radical in the propagation step and the peroxyl radical formed extracts hydrogen atom from RH to form another $R^{\cdot}$ radical. In the termination step the chain reaction is broken when two free radicals react to form non-radical products. The oxidative rancidity of fat is a form of autoxidation that is catalyzed by oxygen, heat, light and metal ions. The rancidification gives a distinct rancid smell and teste due to formation of volatile aldehydes, ketones and low molecular weight acids. The susceptibility of FA to oxidation increases with increasing degree of

Table 1
Relative amount of fatty acids in cod-liver oil and some vegetable oils. The oils consist mainly of mixed triglycerides where three fatty acids, of which at least two are different, are bonded via ester linkage to a glycerol backbone..



Trivial name of fatty acid (and nomenclature)	PhEur range for cod-liver oil (%)	Cod-liver oil (%) [19]	Portuguese extra virgin olive oil (%) [50]	Corn oil (%) [51]	Fatty acid extract from co-liver oil (%) [19]
Saturated fatty acids (SFAs):					
Myristic acid (14:0)	2.0–6.0	3.6	–	–	3.6
Palmitic acid (16:0)	7.0–14.0	10.5	11.3	11.6	10.4
Stearic acid (18:0)	1.0–4.0	2.6	3.3	1.8	2.6
Mono-unsaturated fatty acids (MUFAs):					
Palmitoleic acid (16:1 n-7)	4.5–11.5	6.5	1.0	–	6.5
cis-Vaccenic acid (18:1 n-7)	2.0–7.0	4.4	–	–	4.4
Oleic acid (18:1 n-9)	12.0–21.0	16.3	76.6	25.2	16.2
Gadoleic acid (20:1 n-11)	1.0–5.5	1.5	–	–	1.6
Gondoic acid (20:1 n-9)	5.0–17.0	9.6	–	–	9.4
Erucic acid (22:1 n-9)	0–1.5	0.6	–	–	0.6
Cetoleic acid (22:1 n-11 (+13))	5.0–12.0	7.7	–	–	7.8
Poly-unsaturated fatty acids (PUFAs):					
Linoleic acid (18:2 n-6)	0.5–3.0	1.6	6.6	59.7	1.5
α-Linolenic acid (18:3 n-3)	0–2.0	Not determined[a]	0.6	0.8	Not determined[a]
Moroctic acid (18:4 n-3)	0.5–4.5	2.4	–	–	2.4
Eicosapentaenoic acid (EPA) (20:5 n-3)[b]	7.0–16.0	9.6	–	–	9.3
Docosahexaenoic acid (DHA) (22:6 n-3)[c]	6.0–18.0	12.5	–	–	11.9

[a] Not determined but cod-liver oil usually contains less than 1% α-linolenic acid.
[b] Timnodonic acid (PhEur).
[c] Cervonic acid (PhEur).

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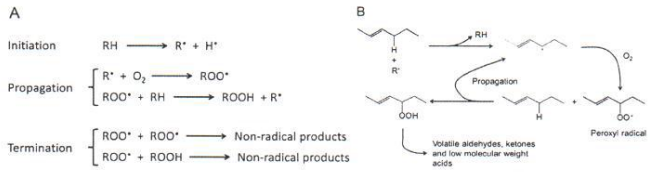

Fig. 1. Autoxidation (A) and rancidification of unsaturated fat (B) [25].

unsaturation and, thus, PUFAs such as EPA and DHA are more prone to oxidative rancidity than monounsaturated FAs. Autoxidation of marine lipids such as fish oil and free unsaturated FA can be suppressed by exclusion of oxygen or by addition of antioxidants. Storage under cool and dry conditions also improves the shelf-life of products containing marine lipids. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are sometimes used to prolong shelf-life of unsaturated fatty acids but generally natural preservatives are preferred. For example, mixtures of related natural tocopherols are used as antioxidants in vegetable oils and marine lipids. Pure fish oils like cod-liver oil are relatively stable towards oxidation when stored in closed containers. Oxygen does not dissolve well in the pure oil. However, fish oils and free unsaturated FA, especially PUFA such as EPA and DHA, can be very unstable in various pharmaceutical formulations where oxygen can gain access to the unsaturated compounds. Monographs for cod-liver oil are in 8th Edition of the European Pharmacopoeia (PhEur, monographs no. 1192 and 1193, and for farmed cod-liver oil 2398).

3. Cod-liver oil/cyclodextrin complexes

Cyclodextrins (CDs) are cyclic oligosaccharides containing six (αCD), seven (βCD) or eight (γCD) α-1,4 linked glucopyranose subunits formed by bacterial digestion of starch. They have a hydrophilic outer surface and a somewhat lipophilic central cavity [26,27]. CDs are able to form water-soluble inclusion complexes with many poorly soluble compounds by taking up some lipophilic moieties of the compounds into the central cavity. The inclusion complexes formed have tendency to self-assembly to form aggregates with diameters that range from few nm to couple of mm [26,28]. Complexes of soybean oil and αCD have been shown to form well-defined particles in aqueous solution with diameter between 1 and 2 mm without presence of organic solvents or surfactants [29,30]. The particles formed can be loaded with lipophilic poorly soluble drugs. Similarly cod-liver oil forms surface active CD complexes in aqueous solutions that self-assembly to form microparticles (or microbeads) with a hydrophilic CD shell and a lipophilic core (Fig. 2) [31]. The cod-liver oil/γCD microparticles, containing 40% (w/w) cod-liver oil, are somewhat robust and can be compressed into tablets. Formation of cod-liver oil/γCD microparticles delays oxidative degradation of cod-liver oil under aerobic conditions but does not increase its long-term stability [31]. Free FAs do not readily form FA/γCD microparticles by themselves but can be incorporated into the γCD microparticles in the presence of cod-liver oil. Tablets containing cod-liver oil/γCD microparticles, with or without free PUFAs, can be used as omega-3 supplements but their manufacturing cost will always be much higher than that of omega-3 softgel capsules.

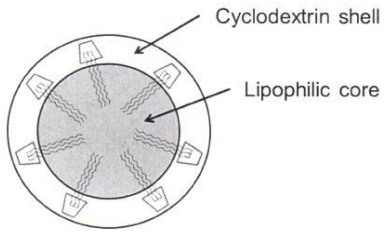

Fig. 2. Schematic drawing of a cod-liver oil/γCD complex particle showing its hydrophilic CD shell and a lipophilic core. The observed particles had somewhat irregular shape with diameter of about 15 μm. The average diameter of the lipophilic core was determined to be about 5 μm [31].

4. Fatty acids as penetration enhancers

The main barrier to dermal and transdermal drug permeation is the thin outermost layer of the skin, the stratum corneum. A drug must penetrate the stratum corneum to reach the viable skin layers and be absorbed into the systemic blood circulation. Clinical usefulness of a drug is frequently hampered by its inability to penetrate through this barrier. Stratum corneum is formed by several layers of dead keratinized cells. Free FAs are known to enhance transdermal drug permeation and have been used as penetration enhancers for a wide variety of drugs [32–35]. Mixture of free FAs was extracted from cod-liver oil and the FA composition of the extract obtained was shown to be almost identical to that of cod-liver oil itself (Table 1) [19]. The effect of the extract on hydrocortisone permeation through hairless mouse skin was tested *in vitro* and compared to the enhancing effects of various purified FAs (Table 2). Including small amount of the extract containing mixtures of saturated and unsaturated free FAs enhance drug permeation through the intact skin by penetrating into the skin barrier and temporary decreasing

Table 2
Flux of hydrocortisone through excised hairless mouse skin. The vehicle was a solution containing 1% (w/v) hydrocortisone and 1% (v/v) fatty acid or 1% (v/v) of the fatty acid extract in propylene glycol [19,36].

Fatty acid	Flux ± SE × 10^4(mg h^{-1} cm^{-2})	Ratio
None	2.52 ± 0.02	1.0
FA extract from cod-liver oil	62.6 ± 11.1	25
Myristic acid (14:0)	2.03 ± 0.21	0.8
Palmitic acid (16:0)	6.50 ± 1.72	2.6
Stearic acid (18:0)	1.60 ± 0.29	0.6
Palmitoleic acid (16:1 n-7)	1620 ± 170	640
cis-Vaccenic acid (18:1 n-7)	952 ± 76	380
Oleic acid (18:1 n-9)	624 ± 111	250
EPA (20:5 n-3)	1110 ± 264	440
DHA (22:6 n-3)	812 ± 220	320

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its barrier properties. The extract had similar effects on transdermal permeation of other drugs such as 17β-estradiol and nitroglycerine [19]. The penetration enhancing effect was shown to be associated with presence of unsaturated FAs in the extract. Neither the saturated FAs nor cod-liver oil itself (contains triglycerides of saturated and unsaturated FAs) were effective as penetration enhancers [36]. *In vitro* permeation studies through keratinized epithelial-free membrane of the hamster cheek pouch and *in vivo* studies in guinea pigs have shown that the FA extract from cod-liver oil also enhances buccal absorption of ergotamine tartrate [20,37]. The studies showed that the enhancing effect of each fatty acid was significantly lower than that of the FA extract containing mixture of FAs; the mixture was a more effective penetration enhancer than any of its free fatty acids [38].

5. Ointments possessing antibacterial and antiviral activity

It is well known that many FAs and their monoglycerides possess antimicrobial, antiviral and antifungal activities [39,40]. It has been shown that the FA extract from cod-liver oil has significant antibacterial activity while cod-liver oil itself, containing the same FAs in the same ratio in the form of triglycerides, does not affect bacterial growth (Fig. 3) [41]. Furthermore, it has been shown that both the FA extract from cod-liver oil and hydrophobic ointment containing the extract have a notable antiviral effect against herpes simplex virus type 1 (HSV-1) [42]. One per cent FA extract caused a 50,000 fold or greater (≥4.7 log10) reduction of viral infectivity in 10 min. Hydrophobic ointment containing 30% extract caused 1.5 million-fold (≥6.2 log10) reduction of viral infectivity. It is also known that FAs from cod-liver oil have anti-inflammatory effect [2,14]. Ointments containing high concentrations of FA extract from cod-liver oil are being tested against wide variety of symptoms in both humans and animals. Hydrophobic ointments are semi-solid drug formulations consisting of single lipid phase formed by, for example, liquid paraffins, vegetable oils, animal fats, synthetic glycerides, waxes and liquid polyalkylsiloxanes (see PhEur 04/2010:0132). In general, oxygen does not dissolve in hydrophobic ointments and hydrophobic ointments can only absorb small amounts of water. Free FA extract from cod-liver oil, as well as cod-liver oil itself, are stable towards oxidative degradation in hydrophobic ointment bases stored in tubes protected from light and oxygen.

6. PUFAs in suppositories

Constipation is a common problem worldwide with prevalence ranging from 0.7% to 29.6% in children and from 2% to 35% in adults [43]. Functional or chronic idiopathic constipation is the most common form [43]. Chronic constipation can cause hemorrhoids (i.e. swelling in the veins located in the anus and rectum) and anal fissures (i.e. tiny tears in the lining of the lower rectum). Thus, suppositories containing relatively large amounts of FA extract from cod-liver oil were tested as laxatives [18,44,45]. Laxatives can be classified according to their mechanism of action [46,47]. Bulk-forming agents such as dietary fibers absorb water increasing the stool volume, stool softeners like some anionic surfactants retain water and fats within the stool, lubricants like mineral oil make the stool slippery, hyperosmotic agents such as sorbitol retain water in the colon, and stimulants such as bisacodyl stimulate the bowel muscles. Bulk-forming agents and stool softeners act in the small intestine a colon while lubricants, hyperosmotic agents and stimulants act in the colon. After rectal administration the FA extract from cod-liver oil stimulated bowel movement causing defecation without causing diarrhea, mucus secretion or any prolonged effect after defecation [18]. Suppositories containing the FA extract were tested as a bowel-cleansing agent prior to flexible sigmoidoscopy and compared to docusate sodium and sorbitol rectal solution (Klyx®, Ferring, Denmark). The suppositories were shown to be as good as docusate sodium and sorbitol rectal solution in regard to providing view of the rectum [46]. Suppository bases can consist of hydrophobic excipients that melt at body temperature such as mixture of hard fat and beeswax (see PhEur 01/2008:1145). Free FA extract from cod-liver oil is stable towards oxidative degradation in hydrophobic suppository bases stored protected from light and oxygen.

7. Conclusions

Cod-liver oil and other fish oils are the main source of n-3 PUFAs such as EPA and DHA that are known to possess numerous of health benefits and, thus, fish oils are commonly used as dietary supplements. PUFAs have also been marketed as ethyl esters. For example, Omacor® that contains ethyl esters of n-3 PUFAs, predominantly EPA and DHA, and used to treat hypertriglyceridemia [48]. Free unsaturated FAs extracted from fish oils do also possess wide variety of biological effects. Free FAs extracted from cod-liver oil have been shown to possess antibacterial, antifungal and antiviral effect when

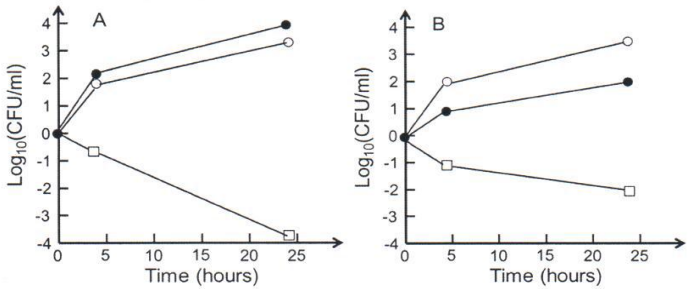

Fig. 3. The antibacterial effect of cod-liver oil (○) and fatty acid extract from cod-liver oil (□) against *Staphylococcus aureus* (A) and Streptococcus pyrogenesis (B). The concentration of both the cod-liver oil and the extract was 3.25 mg/ml. The solvent was ethanol (●) [41].

Please cite this article in press as: T. Loftsson, et al., Fatty acids from marine lipids: Biological activity, formulation and stability, Journal of Drug Delivery Science and Technology (2016), http://dx.doi.org/10.1016/j.jddst.2016.03.007

formulated as water-free ointments and to possess laxative effect when given rectally in suppositories. The free FAs from cod-liver oil can also be used as enabling pharmaceutical excipients that enhance drug permeation through skin and the oral mucosa. Furthermore, free PUFAs are known to possess anti-inflammatory effect as well as numerous other biological effects and have been developed into therapeutically useful drug products. Although in some cases these natural free FAs are less potent than many synthetic drugs they are also less likely to cause toxic side effects that allows for administration of larger doses. In many cases the potency of a given biologically active agent is not important per se, rather the therapeutic index of the agent, that is the ratio of the dose that causes toxic side effects and the dose producing the desired therapeutic effect [49].

Conflict of interest

OTO has served as a speaker, a consultant and an advisor for Lipid Pharmaceuticals. GMA is an employee of Lipid Pharmaceuticals. ES and TL own shares in Lipid Pharmaceuticals.

Lipid pharmaceuticals is performing clinical studies suppositories containing polyunsaturated fatty acids extracted from cod-liver oil.

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Please cite this article in press as: T. Loftsson, et al., Fatty acids from marine lipids: Biological activity, formulation and stability, Journal of Drug Delivery Science and Technology (2016), http://dx.doi.org/10.1016/j.jddst.2016.03.007

marine drugs

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Article

Topical formulation comprising fatty acid extract from cod-liver oil: Development, evaluation and stability studies

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Received: date; Accepted: date; Published: date

Academic Editor: name

Abstract: The purpose of this study was to develop a pharmaceutical formulation containing fatty acid extract rich in free omega- 3 fatty acids such as eicosapentaenoic acid and docosahexaenoic acid for topical use. Although, the health benefits of cod-liver oil and other fish oils taken orally as a dietary supplement have been acknowledged and exploited, it is clear that their use can be extended further to cover their antibacterial properties. In vitro evaluation showed that 20% (v/v) fatty acid extract show good activity against strains of the Gram-positive bacteria *Staphylococcus aureus, Enterococcus faecalis, Streptococcus pyogenes* and *Streptococcus pneumonia*. Therefore, free polyunsaturated fatty acids from cod- liver oil or other fish oils can be used as safe and natural antibacterial agents.

In this study, ointment compositions containing free fatty acids as active antibacterial agents were prepared by using various natural waxes and characterized. The effects of different waxes, such as carnauba wax, ozokerite wax, laurel wax, beeswax, rice bran wax, candelilla wax and microcrystalline wax, in the concentration range 1 to 5% (w/w) on the ointment texture, consistency and stability were evaluated. The results showed significant variations in texture, sensory and rheological profile. This was attributed to the wax nature and chain composition. Microcrystalline wax gave the best results but laurel wax, beeswax and rice bran wax exhibit excellent texturing, similar sensory profile and well-balanced rheological properties.

Keywords: PUFA: polyunsaturated fatty acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; FA: fatty acid; ointment

1. Introduction

The nutritional and medical benefits of fish oils that are rich in polyunsaturated fatty acids (PUFAs) have been extensively investigated. For example, numerous studies have confirmed that PUFAs

possess anti-inflammatory properties and, thus, regular fish oil consumption is thought to reduce the risk of cardiovascular dieses [1-3]. Fish-liver oil for human consumption is commonly extracted from Atlantic cod (*Gadus morhua*). The fatty acid composition of cod liver oil is shown in Table 1. It contains saturated, monounsaturated and various PUFAs, including both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [4]. Antibacterial, antiviral and antifungal effects have also been associated with PUFAs [5-9]. It has been reported that the double bound present in the PUFAs structure increases the fatty acids (FAs) potency and widens their antibacterial activity, while esterified FAs are less effective than free FAs [10]. Similarly, for cod- liver oil, it has been shown that extract of free FAs from cod liver oil are more potent than cod-liver oil containing the same FAs in the form of triglycerides [11]. Besides the strong antibacterial properties, free FAs also possess antiviral properties and the FA extract from cod-liver oil has been shown to cause a significant reduction of viral HSV-1 activity [12]. In addition, PUFAs have been used as penetration enhancers in transdermal and buccal drug formulations [13].To date, the mechanisms by which FAs act as antibacterial agents have not been resolved, and this continues to be a subject of some research effort. Although, the exact mode of bactericidal action of fatty acid still remains unclear, the cellular membrane is thought to be the primary target [14, 15].

Table 1. The fatty acid composition of fatty acid extract from cod-liver oil [13].

Fatty acid		Amount (%)
Name	Shorthand	
Saturated fatty acids		
Myristic acid	14:0	3.6
Palmitic acid	16:0	10.4
Stearic acid	18:0	2.6
Monounsaturated fatty acids		
Palmitoleic acid	16:1 n-7	6.5
cis-Vaccenic acid	18:1 n-7	4.4
Oleic acid	18:1 n-9	16.2
Gondoic acid	20:1 n-9	9.4
Gadoleic acid	20:1 n-11	1.6
Erucic acid	22: n-9	0.6
Cetoleic acid	22:1 n-11	7.8
Polyunsaturated fatty acids		
Linoleic acid	18:2 n-6	1.5
Moroctic acid	18:4 n-3	2.4
Eicosapentaenoic acid (EPA)	20:5 n-3	9.3
Docosahexaenoic acid (DHA)	20:6 n-3	11.9

Ointments are semi-solid, homogenous, viscous pharmaceutical preparations usually intended for external use such as on skin or mucosal membranes [16]. Waxes are frequently used as hydrophobic excipients in topical drug formulations to obtain desired viscosity and consistence [17]. Waxes are a large and complex group of lipophilic compounds that generally consist of mixtures of esters of fatty acids with long chain monohydric alcohols [18]. Today, a wide variety of natural and synthetic waxes are utilized in cosmetics and pharmaceutical products. Seen from a pharmacological aspect, waxes are recognized as a potential for enhancing aesthetic and maximizing therapeutic benefits of topical formulations by increasing the viscosity and prolonging the drug retention on the skin surface [19, 20]. The objective of this study is to evaluate the antibacterial properties of the fatty acid extract from cod liver oil and design a suitable pharmaceutical formulation for topical administration of the fatty acid extract. This article briefly describes the use of waxes as lipophilic excipients for ointments and how selected waxes influence the ointments' rheological and sensory properties.

2. Results

The compositions of the ointments tested are shown in Table 2. The final ointment formulation was obtained by adding 40% (w/w) fatty acid extract to a mixture of Vaseline, coconut butter, wax base, lavender oil and lemon oil.

Table 2. Composition (% w/w) of ointments.

Formulation code	Compound (% w/w)					
	Fatty acid extract	Vaseline	Coconut butter	Wax base	Lavender oil	Lemon oil
C1, O1, L1, B1, Cd1, Rb1, M1	40	43.5	14	1	1	0.5
C2, O2, L2, B2, Cd1, Rb2, M2	40	43.5	12	3	1	0.5
C3, O3, L3, B3, Cd3, Rb3, M3	40	43.5	10	5	1	0.5

Compositions of ointments tested. Wax base is carnauba (C1,C2,C3), ozokerite (O1,O2,O3), laurel (L1,L2,L3), bees (B1, B2,B3), candelilla (Cd1,Cd2,Cd3), rice bran (Rb1,Rb2,Rb3) and microcrystalline (M1,M2,M3). Each ointment formulation tested (C, O, L, B, Cd, Rb and M) contained 1, 3 or 5% (w/w) wax, respectively.

2.1. *In vitro* evaluation of antibacterial activity of fatty acid extract

The fatty acid extract from fish-liver oil was subjected to antibacterial efficiency testing using strains of representative species of both Gram-positive and Gram-negative bacteria. As determined by the agar dilution method, the extract showed antibacterial activity against all four tested Gram-positive strains: *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes* and *Streptococcus pneumonia*. However, the extract did not show antibacterial activity against the Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* in this study (MIC >16.384 μg /ml) (Table 3).

Table 3. Minimum inhibitory concentration (MIC) for the fatty acid extract.

Strains	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Streptococcus pneumonia*	*Enterococcus faecalis*	*Pseudomonas aeruginosa*	*Escherichia coli*
	ATCC 29213	ATCC 19615	ATCC 49169	ATCC 29212	ATCC 27853	ATCC 25922
MIC (μg/ml)	512	256	128	256	>16.384	>16.384

2.2. Evaluation of ointments

The ointments were evaluated for their physical properties such as their spreadability, extrudability and pH as well as their appearance. Spreadability, extrudability and pH studies were carried out in triplicate and average values are reported.

Table 4. Spreadability, extrudability and pH values of ointments

Formulation	Spreadability (mm ± SD)	Tube extrudability (g ± SD)	pH
C1	21.50 ± 0.87	3.23 ± 0.19	5.44 ± 0.04
C2	14.66 ± 0.20	1.84 ± 0.05	5.21 ± 0.01
C3	13.33 ± 0.57	1.67 ± 0.10	5.06 ± 0.05
O1	19.66 ± 0.57	3.23 ± 0.39	4.70± 0.03
O2	15.66 ± 0.2	2.84 ± 0.62	4.62± 0.07
O3	12.66 ± 1.5	1.69 ± 0.53	4.48 ± 0.02

L1	25.33 ± 2.51	2.50 ± 0.12	3.83 ± 0.03
L2	22.66 ± 0.57	2.15 ± 0.07	4.03 ± 0.005
L3	19.66 ± 1.52	2.04 ± 0.09	4.06 ± 0.05
B1	30.50 ± 1.32	4.84 ± 0.79	4.30 ± 0.03
B2	30.13 ± 0.28	3.95 ± 0.49	4.05 ± 0.05
B3	27.33 ± 0.57	3.32 ± 0.07	3.96 ± 0.01
Cd1	19.33 ± 1.15	3.30 ± 0.26	4.28 ± 0.04
Cd2	17.33 ± 0.57	3.20 ± 0.10	3.84 ± 0.02
Cd	17.66 ± 0.57	2.92 ± 0.36	3.83 ± 0.02
Rb1	22.50 ± 0.50	2.97 ± 0.67	4.85 ± 0.02
Rb2	18.66 ± 0.57	1.67 ± 0.34	4.54 ± 0.03
Rb3	17.33 ± 1.52	1.24 ± 0.16	4.32 ± 0.02
M1	24.66 ± 6.65	3.30 ± 0.26	4.24 ± 0.02
M2	21.66 ± 0.57	3.33 ± 0.14	4.21 ± 0.01
M3	18.66 ± 2.88	2.90 ± 0.13	4.09 ± 0.02

2.5.1. Spreadability measurements

Ointment spreadability was found to be inversely proportional to the wax concentration. As the wax amount was increased the ointments became more viscous and, consequently, increasing the wax concentration from 1 to 5% led to decreased spreadability. Spreadability measurements are presented in Table 4. Formulations B1, B2, and B3 exhibited the highest spreadability while formulations C1, C2, C3 and O1, O2 and O3 displayed the lowest spreadability. No statistically significant difference was found between formulations L1, L2, L3 and M1, M2, M3. Formulations Cd1, Cd2, Cd3 and Rb1, Rb2, Rb3 showed good spreadability and were not as fluid as B1, B2, B3 and not as stiff as C2, C3, O2, O3.

The spreading values of these formulations were in the following order: B1 > B2 > B3 > L1 > M1 > L2 > Rb1 > M2 > C1 > O1 > L3 > Cd1> Rb2 > M3> Cd3 > Cd2 > Rb3 >O2 > C2 > C3 > O3.

2.5.2. Extrudability measurements

Increasing the wax concentration resulted in the decreased extrudability. In nearly all cases increased wax concentration from 1 to 5% led to considerable decrease in tube extrudability except in formulations Cd1, Cd2, Cd3, and M1, M2, M3, and L2, L3 wherein decrease in extrudability with increasing wax concentration was less pronounced. Increasing concentration of rice bran wax and microcrystalline wax had a less dramatic but sill pronounced effect on extrudability. As can be seen in Table 4 ointments formulated with carnauba wax had the lowest extrudability, while ointments formulated with bees wax were less viscous and had highest extrudability.

The extrudability values of these formulations were in the following order: Rb3 > C3 =Rb2 > O3 > C2 > L3 > L2 > L1 > O2 > M3 > Cd3 > Rb1> O1=C1 > M1=Cd1 > B3 > Rb3 >M2 > B2 > B1.

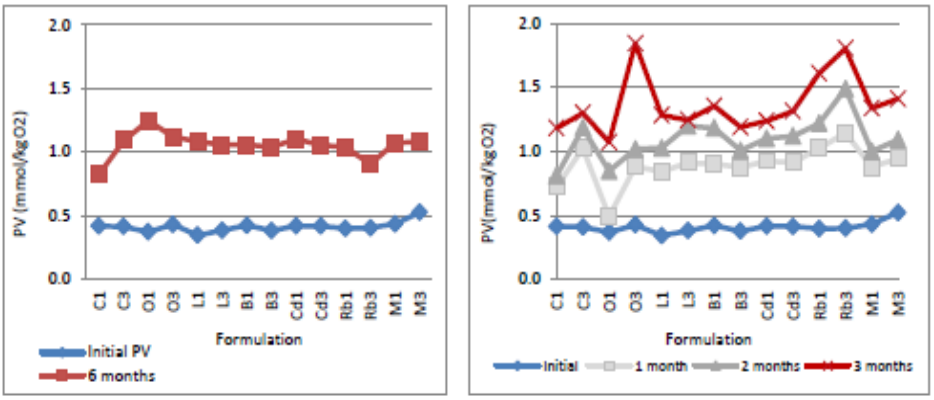
2.5.3. Determination of pH

As shown in Table 4, the pH of the ointments ranged from 3.83 to 5.44, and in general increasing the wax concentration from 1 to 5 % resulted in slight pH decrease with the exception of L1, L2, and L3 wherein pH slightly increased. In fact, pH increased when laurel wax concentration was increased from 1% to 3%, but almost no changes were found when laurel concentration was increased from 3% to 5%. pH of L1 (3.83± 0.03), B3 (3.96 ± 0.01),Cd2 (3.84 ± 0.02), Cd3 (3.83 ± 0.02) was slightly below 4. Of all the ointments tested the lowest pH was observed in L1 (contains 1% laurel wax), and the highest in C1 (contains 1% carnauba wax).

2.5.4. Determination of lipid oxidation

The results of peroxide value (PV) assay (Figure 1) represent how stable the PUFAs were during storage for 6 months at ambient temperature (i.e. 22-23°C) and for 3 months under accelerated conditions (i.e. 40°C). The content of primary lipid oxidation products measured as PV of the extract

was in the range 1.073 –1.804 mmol/kgO_2 at the end of 3 months accelerated testing and 0.824-1.298 mmol/kgO_2 at the end of 6 months storage at room temperature. In both cases no statistically significant differences in the PV values were observed when wax content was increased.



(a) (b)

Figure 1. Lipid oxidation measured as peroxide value: (a) real time tested period; (b) accelerated tested period.

2.5.5. Sensory evaluation

The physical appearance of the ointments was light brown, the color they got from the extract present in high quantity (40 w/w %) in all the ointments tested. Regardless of the type of wax used and its concentration, only minor changes in the brown color were observed. All formulations had strong distinctive smell of fish odor. The overall best results were obtained when beeswax, rice bran wax, laurel wax or microcrystalline wax were used in the ointment base. Overall cosmetic acceptability was rated satisfactory for C1, C2, and Cd1 and Cd2. Specifically, formulations containing beeswax as well as laurel wax had an advantage of providing an additional glossy appearance without having a negative impact on the homogeneity and greasiness. Compared to other unsatisfactory formulations that had rough, gritty texture such as ointments containing carnauba wax or ozokerite wax, ointments that contain candelilla wax differ by possessing large visible particles distributed throughout glossy, homogenous ointment base.

3. Discussion

3.1. Antibacterial activity of the fatty acid extract

In the present study, the antibacterial effect of the fatty acid extract obtained by hydrolysis of cod-liver oil was investigated and ointments containing the extract were prepared. The fatty acid extract showed antibacterial activity against all the strains of the Gram-positive bacteria tested and their MICs were similar, 128-512 μg/ml. The fatty acid extract did not show antibacterial activity against the Gram negative strains in the highest concentration measured. These results agree with previous studies where PUFAs were shown to be more potent against Gram-positive bacteria than Gram-negative bacteria [7, 10, 21]. A possible explanation for the difference in susceptibility lies in the difference in the cell wall structure of Gram-positive and Gram-negative bacteria [14, 22]. Some accumulation of the fatty acid extract was observed on the agar surface but it gradually decreased upon progressive dilution to 128μg/ml. The agar dilution method is probably not applicable for antibacterial measurements of free FAs at high concentrations due to accumulation of the fatty acid extract on the agar surface.

173 3.2. Ointment design and evaluation

174 During pharmaceutical formulation, selection of appropriate excipients and their amount is critical
175 and various factors have to be considered such as the formulation appearance, odor, color and
176 firmness, all of which can influence the patient acceptability. Other important factors include the
177 chemical stability of the active pharmaceutical ingredient (i.e. the drug), and the overall chemical
178 and physical stability of the formulation. Sometimes, it is necessary to use several different
179 excipients to obtain vehicle of desired physicochemical properties. The main reason is that it is
180 frequently impossible for a single excipient to produce an ideal vehicle (here an ointment base), but
181 a combination of several excipients frequently will give vehicles with enhanced aesthetics as well
182 as increase drug potency and bioavailability [23, 24]. Vaseline is most common lipophilic excipient
183 in dermal formulations and its usage is sometimes associated with its occlusive properties that can
184 enhance penetration of the active ingredient into skin and improve the therapeutic efficacy of the
185 product [17]. Our experience is, however, that use of Vaseline alone as an excipient where
186 prolonged drug retention is desirable, is not likely to result in acceptable formulation. Waxes were
187 chosen as viscosity-building excipients to improve the ointments' performance and give them
188 optimal rheological profiles. The combination of Vaseline and waxes produced pharmaceutically
189 acceptable ointments that were less greasy than pure Vaseline and that were retained on the skin
190 for a sufficiently long time to allow for extended drug delivery. The spreadability and extrudability
191 profiles were found to be satisfactory for a topical dosage form. Spreadability and tube
192 extrudability do, in principle, correlate with the rheological properties and present a simple and
193 accurate approach to understand the relationship between viscosity and the force needed to
194 squeeze the ointment out of a tube [25-28]. Both measurements showed the ointment tendency to
195 become stiffer upon incorporation of wax. The rheological effect can be explained by the nature of
196 the fatty acid moieties of the waxes. The greater the saturated fatty acid fraction of the wax
197 structure is the more viscous the ointments will be, and, conversely, the viscosity of the ointments
198 will decrease with increasing content of unsaturated fatty acids [29]. Spreadability and
199 extrudability values were influenced by both the amount and type of wax used.
200 In most cases the pH of the ointments was within acceptable pH range for dermal preparations (the
201 pH of the skin surface is between 4.2 and 5.6). The presence of wax led to increase in pH, except in
202 the case of laurel wax. No significant change in pH was observed when the wax concentration of
203 the ointments was increased. Acidic pH values were expected due to the high concentration (40%
204 w/w) of free FAs. FAs are weak acids with pKa values of about 4.8 that increases slightly with
205 increasing chain length [30]. In general, it is recommended that the pKa value for drug to be used is
206 between 4 and 8 to avoid itching or skin irritation [31]. Irritation or redness at the site of application
207 is frequently observed if the active ingredients have pH different from that of skin, especially in
208 people with tender skin [32, 33].
209 Besides the therapeutic efficacy, sensory acceptability also decides the marketing success of topical
210 formulation. Formulating product that possess both optimal efficacy and expected sensory
211 qualities can be extremely challenging [34]. Analysis of sensory profile is not a straight forward but
212 frequently it is related to the physiological properties of the product ascribed by the formulation's
213 homogeneity, consistency or firmness [35]. Factors that can influence the homogeneity of the
214 ointment include solidification profile of the composition during its preparation such as the cooling
215 rate, and variations in preparing techniques. Except type of wax used, all the ointment components
216 were identical as well as the method of preparation.

217 3.2. Stability studies

218 Fish oil is particularly susceptible to rancidity due to its high content of omega-3 PUFAs which are
219 highly prone to oxidation. Monitoring the PV of the ointments allowed us to determine the fatty
220 acid rancidification. As can be seen in Figure 1, no correlation between PV and presence of wax was
221 found, and the increased wax concentration did not alter the PV of any of the ointments tested. In
222 all cases, except in formulation O1, there was a rapid increase in peroxide value during the first

month of storage at 40° C. A possible explanation could be presence of oxygen in the headspace of the sealed tubes. After that, PV increased only slightly with time under both ambient and accelerated conditions (i.e. at 40°C). It was evident that oxidation of the PUFAs occurred under both ambient and accelerated storage conditions, but it was independent of ointment composition. However, there was very little lipid peroxidation, PVs in all of ointments were below the suggested limit for acceptability and quality for fish oils for human consumption. All formulations tested met the quality standards of the European Pharmacopeia [36].

Stability studies of ointments were further supported by the evaluation of sensory parameters (data not shown). All ointments containing microcrystalline wax, rice bran wax, laurel wax or beeswax were stable during the 6 months storage at room temperature and displayed no change in the texture, color or smell. A deviation from initial sensory parameters was observed in the case of C1, C3, O1, O2, Cd1 and Cd2 after 6 months storage under ambient conditions as well as at 40°C. A slight change in color in the formulations at 40°C and "bleeding"after 90 days was observed. The phenomenon bleeding was attributed to the storage at high temperature that in fact is a well-known change in consistency that typically appears on the top of filled tube and is frequently referred to as ointments main stability problem [28]. According to the results obtained from short-time stability study, C1, C2, O1, O2, Cd1, Cd2 and all formulations containing rice bran, laurel or beeswax were slightly different in homogeneity than the original samples. However, besides the textural and sensory changes, phase separation was not observed.

4. Materials and Methods

4.1. Antibacterial assay

The strains selected to use for the antibacterial assay were *Staphylococcus aureus* (ATCC2913), *Enterococcus faecalis* (ATCC29212), *Streptococcus pyogenes* (ATCC19615) and *Streptococcus pneumoniae* (ATCC49169) representing common Gram positive species, and *Pseudomonas aeruginosa* (ATCC27853), and *Escherichia coli* (ATCC 25922) representing Gram negative species. MIC determinations were performed using agar dilution according to the CLSI standard for methods for dilution antimicrobial tests for bacteria that grow aerobically [37]. Double dilutions of FAs extract of cod-liver oil were prepared. 1 ml of each included 381 µl of FAs, 200 µl ethanol and 419 µl of sterile distilled water that was added to 19 ml of melted Mueller-Hinton agar (Oxoid,UK), mixed and poured in sterile petri dishes to obtain the final concentrations of 1-16.384 µg/ml. For the MIC testings of streptococci the agar was supplemented with 5% sheep blood. Bacterial suspensions were made to include approximately 10^7CFU/ml, to give the final number of 10^4 CFU in the 1 µl that was placed on the agars that were incubated at 37°C for 18-24 hours. The MIC determinations were done two times for each strain and dilution. Various concentrations of ethanol (20%, 40%, 60%, 80%, and 100%) (Sigma Aldrich, Germany) without FAs extract was used to evaluate the effect of the ethanol. Mueller Hinton agar without alcohol and FAs extract was used for growth control.

4.2. Ointment preparation

The fatty acid extract was supplied by Lýsi ltd (Iceland).The method to determine the content and composition of FAs present in cod-liver oil and its extract has been previously described [13]. Vaseline was purchased from Duchefa Farma (Netherlands), carnauba wax, ozokerite wax, laurel wax, candelilla wax, rice bran wax were purchased from Strahl&Pitch (USA), and beeswax and microcrystalline wax were purchased from Making cosmetics (USA), coconut butter was purchased from Fagron (UK), lavender oil and lemon oil were purchased from Now Foods (USA).

Twenty one formulations were prepared by fusion method, in which the FAs extract (40% w/w) was combined with a melted wax base and evaluated for physicochemical parameters [38]. All the compositions are summarized in Table 2. Free FAs extract was heated gently on a water bath at

50°C until the entire content has melted. Simultaneously, wax was completely melted under stirring. When wax base had melted, both Vaseline and coconut oil were added. Free FAs extract was added slowly to the mixture under gentle stirring. Then, lavender oil and lemon oil were added and mixed together with Vaseline, coconut oil and waxes. The ointments were filled in airtight aluminum tubes in order to prevent access of light and oxygen.

4.3 Evaluation of ointments

4.3.1 Spreadability

A simple spreadability test was performed: 1.0 g of the ointment was placed in a 10 mm diameter circle on a glass plate. After being sandwiched with another glass plate, the sample was pressed with fixed 500 g weight [39]. Spreadability was determined as a difference in diameter values before and after 30 sec on a millimeter scale placed under the lower glass plate.

4.3.2. Extrudability

Tube extrudability was determined by measuring the amount of ointment extruded from the tube when a pressure was applied on the tube. The larger amount extruded the better extrudability. Extrudability was determined in terms of weight in grams required to extrude 0.5 cm of ribbon of ointment in 5 seconds [40].

4.3.3 pH determination

The pH of ointments were determined by an extraction method where 2.5 grams of ointment was suspended in 50 ml distilled water in a 100 ml beaker, heated in a water bath at about 70° C for 10 minutes, and then cooled to room temperature. The suspension was vigorously stirred for 10 minutes on magnetic stirrer to prompt extraction before phase separation resulting in supernatant extract [41]. The pH was measured using a PH-200 Waterproof pH Meter.

4.3.4. Sensory evaluation

Sensory parameters of ointments were evaluated at different storage conditions that is, 40°C ± 2°C, 75% RH ± 5% RH for 3 months and under ambient condition for 6 months. The colors, odors and textures of the ointments were evaluated and compared. A standardized quantity of 1g of ointment was applied over the dorsal surface of the left hand and sensory properties descriptively graded using terms as follows:

Excellent: smooth and homogeneous texture, light brown color, distinctive fishy odor.

Satisfactory: smooth texture, non-consistency, light brown color, distinctive fishy odor.

Unsatisfactory: rough texture, visible solid particles, non- consistency, dark brown color, rancid fishy odor.

4.3.6. Determination of peroxide value

A modified method of Wheeler described in ISO 3960:2007 was used where lipid peroxide is reacted with potassium iodide (KI) to liberate iodine that is then titrated with a standard solution of sodium thiosulfate ($Na_2S_2O_3$). The amount of liberated iodide represents the peroxide content. PV is defined as the amount of peroxides oxygen per 1 kg fat (mmol O_2/kg). Aluminum tubes were kept at room temperature with no access of oxygen, during 6 months period. All the developed formulations were also subjected to accelerated stability at testing at 40 ± 2°C, 75% RH ± 5% RH for 3 months. For 3 months accelerated study, PV was assessed at 1, 2 and 3 months.

5. Conclusions

In the current trend for development of natural and safe anti-bacterial compounds with minimal side effects, although not potent fatty acid extract from fish-liver oil has proved to be an effective antibacterial agent against Gram positive bacteria. As such, fish oil holds a great potential as an active pharmaceutical ingredient for novel effective pharmaceutical formulations, especially for prevention and treatment of skin diseases. The ointments were shown to have a great potential as an effective and safe way to administer PUFAs for topical antimicrobial therapy. In addition, it may be concluded that waxes could be used as suitable excipients for ointments creating compositions with appreciated rheological and sensory profile that perform safe and overall effective dermal therapy.

Author Contributions:

Author B.I. designed the experimental protocol, performed the measurements and wrote the manuscript. Author M.H. designed the antibacterial studies and analyzed the results. Author T.L. supervised the entire work.

Conflicts of Interest:

The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:
FAs: Fatty acids
PUFAs: Polyunsaturated fatty acid
PV: Peroxide value
MIC: Minimum inhibitory concentration

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