SQUALENE – NATURAL RESOURCES AND APPLICATIONS

IOANA POPA[1], NARCISA ELENA BĂBEANU[2*], SULTANA NIȚĂ[3], OVIDIU POPA[4]

[1]PhD student at Faculty of Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Bucharest
[2]PhD, Professor, Faculty of Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Bucharest
[3]PhD, Head of Analytical Department at National Institute for Chemical Pharmaceutical Research and Development
[4]PhD, Professor at Faculty of Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Bucharest
** corresponding author: narcisa.babeanu@gmail.com*

Abstract

Squalene is a natural dehydrotriterpenic hydrocarbon ($C_{30}H_{50}$) with six double bonds, an intermediate for the biosynthesis of phytosterol/cholesterol in plants/animals and humans, widespread in animal and vegetal kingdom. We have reviewed the most important natural resources for the purified squalene separation. Because of its unique properties, squalene has multiple applications in different fields of human activity and the most significant are presented.

Rezumat

Scualenul este o hidrocarbură naturală cu formula $C_{30}H_{50}$, cu șase duble legături în moleculă, intermediar în biosinteza fitosterolului/colesterolului în organismul vegetal/ animal, larg răspândită în regnul vegetal și animal. Autorii au realizat o cercetare a datelor din literatura de specialitate privind cele mai importante surse naturale pentru obținerea scualenului. Proprietățile sale unice au determinat o largă gamă de utilizări în diferite domenii ale activității umane, dintre care sunt prezentate cele mai importante în viziunea autorilor.

Keywords: Squalene, shark liver oil, olive oil, amaranth, antioxidant.

Introduction

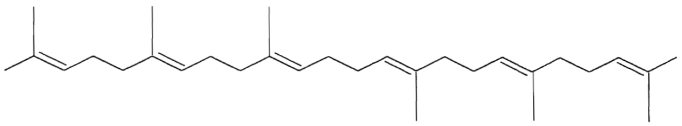
Squalene is a triterpene with the formula $C_{30}H_{50,}$ an intermediate for the biosynthesis of phytosterol or cholesterol in plants, animals and humans, widespread in animal and vegetal kingdom.

Squalene was discovered in 1906 by the Japanese researcher dr. Mitsumaru Tsujimoto, expert in oils and fats at Tokio Industrial Testing Station. He separated the unsaponifiable fraction from the shark liver oil ``*kuroko-zame*'' and discovered the existence of a highly unsaturated hydrocarbon [89].

Although he did not isolate at that time the substance, he obtained the bromine addition compound, as a white powder, which decomposed at 155°C and had the following composition: 26.93% C, 3.94% H, and 69.28% Br. He proposed for this compound the formula $C_{10}H_{18}Br_4$. Ten years later, Tsujimoto succeeded to obtain by fractional vacuum distillation of the oil from the liver of two deep sea shark *specia*, an unsaturated hydrocarbon, with the composition $C_{30}H_{50}$, which he named "Squalene" [90]. The name came from the denomination of the sharks' family: *Squalidae*.

Almost in the same period, independently, Chapman [14] also separated an unsaturated hydrocarbon with the composition 87.75% C, 12.25% H and the molecular weight of 375, from the liver of two sharks (*Centrophorus grunulosus* and *Scymnus lichia*) from Portugal. Chapman proposed for this compound the name of "spinacene", as both sharks were from the family *Spinacidae* or *Squalidae*. This remained even today one of the medical label of the compound, together with "supraene", or the latin name of "*squalene exogeno oleum*".

Subsequent research [34, 35, 32, 42] confirmed the mentioned chemical formula proposed by Tsujimoto, squalene having the following structural formula:



(*E*)-2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene and the chemical and physical properties [28]:

Table I

Chemical and physical properties of Squalene [28]

Properties	Value
Molecular weight	410.7 g/mol
Melting point	-75°C
Refractive index	1.499
Viscosity at 25°C	12 cP
Density	0.858 g/mL
Boiling point at 25°C	285°C
Flash point	110°C
Iodine number	381g/ 100g
Infrared peaks	2728, 1668, 1446, 1380, 1150, 1180, 964, 835 cm^{-1}
Surface tension	~ 32 mN/m

Natural sources for squalene

Animal sources

The greatest concentration of squalene in the living world is met in the liver of certain species of fish, especially sharks living in sea at depth under 400 m. In the case of deep sea sharks, the liver is the main organ for lipids' storage, being in the same time an energy source and means for adjusting the buoyancy. In their case, the unsaponifiable matter represents 50 – 80% of the liver, the great majority thereof being squalene.

Shark liver oil represents the richest natural source of squalene. A limitative reason in the use of this natural source for squalene is represented by the presence in the environmental sea of different persistent organic pollutants (POPs), like PCB (polychlorinated biphenyl), PBDE (polybrominated diphenylether), organochlorine pesticides, polycyclic aromatic hydrocarbons, dioxin, heavy metals, which can still be found in the purified squalene, together with the concern for the preservation of marine life.

Recent studies [91] show that the POP level in the Atlantic Ocean grown salmon is under the limit established by World Health Organization and the European Authority for Food Safety.

Anyway, the squalene extracted from the fish liver oil can't be used in cosmetic or pharmaceutical applications because, despite the deodorisation treatment, it still has a persistent unpleasant smell and even, undesired impurities. Pharmaceutical industry has very rigorous manufacturing standards what cannot be easily accomplished by the squalene of shark origin. Sharks may be infected by pathogens that are also infectious for humans or which may produce substances dangerous for humans.

For a long period of time, shark liver oil was considered the only industrial scale source for squalene. Even after the olive oil became known as the second largest natural source for squalene, shark liver oil remained the most important source for squalene and its hydrogenated derivative, squalane, for the following economic reasons: the production of squalene from shark liver oil is less complex and more cheaper than from olive oil. Anyway, the intensive fishing of these sharks put in danger the existence of these species, many of them close to extinction, as their reproductive cycle is quite long and the growth is slow. That is why Europe drastically reduced lately the fishing quotas for these shark species.

For all these reasons there is today a great interest in finding new natural sources for squalene, especially vegetal ones. It was shown that squalene is widespread in the animal kingdom, being a precursor of

cholesterol. In human body squalene is synthesized by the liver and is secreted in large quantities by the sebaceous glands. It is transported in blood by the small and very small density lipoproteins [72]. It is interesting to notice that squalene represents 12% of the lipids secreted by the sebaceous glands [81,68].The squalene concentration in human skin lipids is of about 500 μg/g, and in the adipose tissue, 300 μg/g [88].

Vegetal sources

Squalene was also identified in many vegetal oils in different concentrations. Vegetal origin squalene is highly appreciated in cosmetic and pharmaceutical industry due to some remarkable properties: easy incorporation in cosmetic emulsions as effect of its light consistency and excellent spreadability, non-greasy texture, high stability, but also due to the fact the plant origin squalene is odourless and colourless, without substances harmful to humans, opposite to the squalene extracted from shark liver oil.

Chronologically, the first plant oil in which it was found was the olive oil [85]. More recently, Frega et al. [24] determined the squalene concentration in olive oil – 564 mg/100 g, soybean oil – 9.9 mg/100 g, grape seed oil – 14.1 mg/100 g, hazelnuts oil – 27.9 mg/100 g, peanuts oil – 27.4 mg/100 g, corn oil – 27.4 mg/100 g.

Most vegetal oils are obtained by mechanical pressing or by chemical extraction with organic solvents, like hexane. Oil refining is necessary in almost all the cases, except the extra virgin oils. Refining aims the removal of undesired substances with unfavourable effects on the oils' taste, smell, aspect or stability (phospholipids, diacylglycerols, free fatty acids, pigments, oxidation products). In literature it is reported the influence of different refining processes on the concentration of squalene in olive oil: the decrease due to physical refining was 13.0%, discolouring – 7.0%, deodorisation – 15.6% [63]. Due to the small concentration of squalene in vegetable oils, this relatively low decrease could become relevant for the final productivity of the process.

The chemical composition of the olive oil reveals a saponifiable fraction which represents 98-99% of the total weight, and the unsaponifiable fraction this accounts for 0.5-2% of the total weight. This last one contains several precious components, as squalene, β-carotene, tocopherols. By nowadays technology, it isn't economic to separate these valuable constituents directly from the vegetal oils, as the concentration is too small. The by-products obtained in the process of oils' refining, like the oil deodorizer distillate, contain 15 to 30% unsaponifiable fraction, with a concentration of up to 80% squalene, depending on several factors.

Serious research studies on the squalene concentration in the olive oil have showed that this hydrocarbon represents over 50% of the unsaponifiable fraction, and up to 90% of the total hydrocarbons content thereof [50, 67]. The squalene concentration in olive oil varies between 200 and 7,500 mg/kg of oil, depending on the cultivar [54] and the geographical cultivation area [6]. Experiments made on two olive tree cultivar types from Tunisia showed a difference of about 10% in the squalene concentration, but this difference can reach even a bigger value of about 24%, as it was proved in the experiments made on six olive tree cultivar types from Italy [54, 74].

Samaniego-Sanchez et al. have determined a concentration range of 50-100 μg/mL for squalene in the virgin olive oil, obtained by cold extraction, and noticed the differences between three harvests between 2001 and 2004, pointing out a content of squalene of 5012.7 mg/kg in 2001-2002, and a maximum of 6502.7 mg/kg content of squalene in 2003-2004 [76]. These findings are in principle consistent with data obtained by other researchers [94], who studied the squalene content in olive trees, cultivated in Northern Italy, in two years 2000 and 2004. They found a difference of about 10% between the squalene content in olive oil from the two years, considered to be due to the different pedo-climatic conditions in the mentioned years.

Boskou mentioned in his book that the squalene concentration could reach in Greek olive oils, a value of 5.1 – 9.6 g/L [11]. Anyway, the final concentration is significantly influenced by the methods of extraction and oil refining [3, 55]. Commercially available methods for squalene extraction from olive oil yields to a vegetal squalene with around 97.5% purity, while squalene obtained from shark liver oil can reach 99.9% purity [28].

Olive oil deodorization distillate (OODD) is an important natural source for squalene [22]. The squalene concentration in the unsaponifiable fraction thereof reaches 77% (wt/wt) or a concentration of 284 g/kg, as it was reported following the experiments conducted on a selection of olive oils from Italy [50]. The need for squalene from vegetal sources is not satisfied only by the olive oil and its by-products from the distillation, extensive researches being conducted all over the world to identify new valuable vegetal sources, new methods to obtain squalene, less complicated and cheaper.

Palm oil contains 250-540 mg/L squalene, larger concentrations (2,400-13,500 mg/L) being found in palm oil deodorizer distillate (PODD) [84]. If the refining is made by physical methods, the distillate resulted from deacidification and deodorization contains about 1% squalene [70].

Another valuable vegetal source of squalene is the soybean oil deodorizer distillate (SODD), a by-product obtained in the deodorization step of soybean oil refining. Depending on the soybean origin, SODD could be a precious source of active substances, as tocopherols, phytosterols, free fatty acids or squalene, the total concentration of valuable products could reach up to 32% (wt/wt) [31, 43, 59]. Except the soybean origin, an important factor influencing the content of valuable compounds is the refining process parameters, like temperature or pressure.

The greatest concentration of squalene is met in the vegetal kingdom in the oil extracted from *Amaranthus sp*. They has a pseudo grain having seeds and leafs with a great content of oil for a cereal, but significantly lower than oily grains, like soybean. The lipids are mainly contained in the coat-embryo fraction of the seed, representing about 25% of its total weight. Results of a research project an *Amaranthus* as an alternative crop in Central Europe, [30] published comparative data for the concentration of squalene in amaranth oil (7-11% , wt/wt) and olive oil (1%, wt/wt). Some of the chemical and physical properties of the amaranth oil are presented in Table II.

Table II

Physical and Chemical properties of amaranth oil [92]

Properties	Value
Index of refraction	1.4762
Acid number (mg KOH/g)	1.3
Non-fat mixtures %	1.1
Iodine number I_2/100g	86.74
Moisture and volatile matter (%)	0.067
Density (g/cm^3)	0.921

The oil content in the *Amaranthus* seeds varies as a function of species and pedo-climatic conditions. The environmental conditions as height, the solar light length over the day, the precipitation regime, could influence the seed size and this parameter being correlated with the chemical composition of the seed, will affect consequently, the squalene concentration. He and Corke reported in a study analysing 104 genotypes from 30 *Amaranthus* species, an oil content in seeds between 1.9% – 8.7% (wt/wt) and a squalene concentration in oil, ranging from traces to 7.3%, with a media of 4.2% (wt/wt) [33]. The lipid content in mature leaves was 1.08 to 1.63% and the squalene concentration in the lipids extracted from leaves varied from 0 to 0.77% (0.26% media).

Detailed studies regarding the oil composition of *Amaranthus cruentus* originated from Congo-Brazzaville, revealed a squalene

concentration of 4.16 g/kg of seeds, the oil content of the seeds between 11-14% (wt/wt), with 5-7.21% unsaponifiable fraction in the oil [19, 51].

A synthetic review of the different vegetal sources for squalene, as presented in the studied literature, is presented in Table III.

Table III

Vegetal sources of squalene

No.	Vegetable source/origin country	Concentration mg Kg^{-1}	Observation	Reference
1	Pumpkin oil Soybean oil Sunflower oil Corn oil *Amaranthus* seed oil Olive oil distillate Soybean oil distillate Palm fatty acids distillate	2,600-3,500 30-200 0-190 100-170 20,000-80,000 100,000-300,000 18,000-35,000 2,000-13,000		[62] 2011
2	Virgin olive oil/Spain	800-12,000	250 samples of Spanish virgin olive oil	[50] 1994
3	Crude palm oil Palm fatty acids distillate	200-500 12,000-18,000		[26] 1985
4	Virgin olive oil/Tunisia	2,317 2,627	Jemry-Bouchouka olive cultivar Chemlali-Tataouin olive cultivar	[74] 2012
5	Virgin olive oil/Italy	3,852 3,917 4,479	Mean value of 2 Gentile (Larino) olive cultivars Mean value of 2 Gentile (Colletorto) olive cultivars Mean value of 2 Coratina olive cultivars	[54] 1998
6	Virgin olive oil/Greece	2,192 – 4,677 1,718 – 4,992 2,037 – 4,657	6 samples, determined by RP-HPLC, after fractional crystallization 6 samples, determined by RP-HPLC after saponification 6 samples, determined by RP-HPLC after SPE	[29] 2007
7	Virgin olive oil/Croatia	6,683	Northern Adriatic region	[25] 2001

8	Virgin olive oil/Greece	3,740/4,382 3,873/4,455	Mean of 4 values obtained at different harvesting times (Nov/Dec/Dec/Jan)in 2000/2004 from non organic cultivars Mean of 4 values obtained at different harvesting times (Nov/Dec/Dec/Jan)in 2000/2004 from organic cultivars	[2] 2011
9	Soyabean oil deodorizer distillate (SODD)/Taiwan	18,300	Average value of 3 determinations	[31] 2008
10	Soyabean oil deodorizer distillate (SODD)/USA	55,000	Determination with GC-FID, after silylation	[20] 2007
11	Palm fatty acid distillate (PFAD)/Colombia	10,300	Determination with GC-FID, after molecular distillation	[70] 2007
12	Crude palm oil	250 - 750		[15] 2004
13	Olive oil deodorizer distillate (OODD)/Italy	280,000	Determined by GLC, after separation by TLC	[10] 1993
14	Rice bran oil	3,200		[75] 1991
15	Crude palm oil/Malaysia Refined, bleached, deodorized palm oil/Malaysia	433±3 415±5	Rapid GC technique	[61] 2005
16	*A.caudatus* 713/Nigeria *A.cruetus*/Nigeria	34,800 33,500	Concentration in oil obtained from seeds	[4] 1989
17	*A.cruentus*/USA	69,600	Concentration in oil obtained from seeds	[52] 1987
18	*A.hybridus*/Nigeria *A.hypochondriacus*/Nigeria	24,000 26,300	Concentration in oil obtained from seeds	[4] 1989
19	104 genotypes of 30 species of *Amaranthus* from several countries	10,400-69,800	Concentration in oil obtained from seeds, separation by TLC, determined by GLC	[33] 2003
20	Brasil nut Pecan nut Pine nut Pistachio nut Cashew nut	1,377.8 151.7 39.5 91.7 89.4	Concentration in oil, determined by HPLC (column Supelcosil LC-18-DB, mobile phase MeOH:H_2O = 99:1, flow rate 1.2 mL/min, column	[1] 2009

			temperature maintained at 25°C), λ 215 nm	
21	Rosaceae seed oils/Germany *Alchemilla caucasica* *Cotoneaster simonsii*	0.2 2.9	Concentration in oil extracted from seeds ; determined by HPLC coupled with spectrophotometer	[57] 2014
22	*Amaranthus mantegazzianus* Moscow region/ Tatarstan/ Voroneg region/ Sankt Petersburg region (light-painted seeds)	5.8/5.3 5.0/4.7	Concentration (% of lipid fraction of seeds)	[92] 2013
23	*Amaranthus paniculathus* L./ Kazakhstan (black seeds)	7.0	Concentration (% of lipid fraction of seeds)	[92] 2013
24	*Amaranthus cruentus* L./ Kazakhstan (light-painted seeds)	8.0	Concentration (% of lipid fraction of seeds)	[92] 2013

RP-HPLC = Reverse Phase High Performance Liquid Chromatography; SPE = Solid Phase Extraction; GC-FID = Gas Chromatograph with Flame Ionization Detector; GLC = Gas Liquid Chromatography; TLC = Thin Layer Chromatography; GC = Gas Chromatography; LC-18-DB = Liquid Chromatography C18 Column; MeOH = Metanol

Microbial sources

In recent years the microbial biosynthesis of squalene became a promising alternative source. Although the microorganisms don't accumulate as much squalene as shark liver or some plants, they grow very fast and in controlled conditions. There are several experimental reports related to the separation of squalene from microorganisms: yeast, especially *Saccharomyces* [41], *Torulaspora delbrueckii* [7], bacteria as *Pseudomonas* [93], algae *Euglena* [44], microalgae as *Traustochytrium* [41], *Schizochytrium mangrovei* [40], or *Botryococcus braunii* [5].

After experiments with wild-type strains of *Saccharomyces cerevisiae,* Mantzouridou et al. [53] concluded that there are many factors with impact on the increase of squalene yield and bioprocess selectivity, including yeast strain, aeration strategy, inoculum size; using an optimised control strategy for the bioprocess parameters, a concentration of 1.6 mg squalene/g dry biomass was achieved, values more competitive as previously reported, but too low for commercial exploitation.

The cultivation of *Torulaspora delbrueckii* in limited oxygen conditions yield to 0.24 g squalene/kg biomass. After 48 h of anaerobic fermentation, the best yield of squalene was obtained in the case of 5% inoculum. The decrease in squalene content observed after 72 h was attributed to the utilization of squalene as a carbon source by the yeast cells. The glucose medium supplemented with yeast extract and peptone, was further anaerobically cultivated for 24 hours. The squalene obtained in the

cultivation of *Torulaspora delbruecki* was further separated by extraction with supercritical CO_2, at 60ºC and 250-255 barr pressure, at a CO_2 constant flow of 0.2 L/min. It was obtained a maximum yield of 11.12 μg squalene /g dried biomass.

Chang et al.[13] isolated a new yeast strain from sea environmental, which produces a higher concentration of squalene and more polyunsaturated fatty acids. They succeeded to characterize it by genetic analysis as belonging to the genus *Pseudozyma sp*. They established for it the optimal conditions for maximum squalene production, i.e. 40 g/L glucose and a glucose/yeast extract ratio of 4.5. In these conditions *Pseudozyma* sp. JCC207 succeeded to produce 5.20 g/L biomass and 340.52 mg/L squalene. For these reasons, *Pseudozyma* sp. JCC207 was considered a good candidate for the commercial production of squalene. The advantages of producing squalene by yeast fermentation consist in the possibility to carry out the process at large scale, with a high yield in order to obtain squalene of high purity in controlled conditions.

Microalgae, typically found in fresh water and marine systems, are another microbial source for the production of squalene. By the cultivation of microalgae *Aurantiochytrium mangrovei* FB3 in the conditions of squalenepoxydase inhibition by addition of terbinafine, Jiang et al [40] obtained 0.53 g squalene/kg biomass, corresponding to a yield of 2.90 mg squalene/L cultivation media. In the patent WO 2012/159979 [69] it is described a process for obtaining squalene from microalgae of the family *Traustochytrid sp.*, at an optimised temperature between 28 and 32^{O}C, in a medium enriched with B12, B1 or B6 vitamins. In these conditions, the squalene yield was between 2 and 12 g for 100g dried biomass. It has to be mentioned that the industrial biomass of yeast *Saccharomyces uvarum* (bottom fermentation) contains 1.34 g squalene/100 g dry biomass [9].

Applications

Squalene comes into attention of the scientific world due to the finding that the beneficial effects of some natural products on the health and the wellbeing of humans are due to its action. It is now known that squalene is the main component of the shark liver oil. From ancient times, fishermen all over the world benefited the important properties of the oil, extracted from the liver of sharks living beneath 1,000 m. It was used to improve or to cure a wide range of conditions [86]. In Sweden and Norway fishermen traditionally used this oil to cure wounds or the various conditions of the respiratory tract.

On the other hand, the other major traditional natural source for squalene, the olive oil, came into attention of the scientific community due to the healthy properties of an olive oil based diet. The epidemiological evidence of a lower incidence of CHD (cardiovascular heart disease) and

certain cancers in the Mediterranean area, stimulated researches on the potentially protective action of the olive oil's minor constituents [45].

Comparative studies were made regarding the incidence of certain cancers in Mediterranean countries like Greece, Spain or Italy, where the olive oil is a constant part of the daily diet and in the Scandinavian countries and USA. While in Mediterranean area the daily uptake of squalene (from olive oil) reaches 200–400 mg, in US the average daily intake of squalene is about 30 mg [80]. The incidence of breast cancer in Greece is 65% lower than in USA [87]. Analysing statistical health studies from Greece, Spain and Italy comparative with USA, Newmark [64] suggested that this protective effect of olive oil could be related to its high concentration of squalene.

Physical and chemical properties of squalene and in the same time, the more profound understanding of its *in vivo* actions ensure multiple fields of applications in different sectors of human activity: food, cosmetics, pharmaceutics, medical prevention and treatment.

- *Food*

A squalene daily dose (up to 85% absorbtion) from food has been related to many health benefits. Today there are on the market several squalene formulations as nutraceuticals. In the olive oil industry a certain level of the squalene concentration in oil is a mark of the *extravirgin* olive oil and improves the oil stability against frying much better than the synthetic antioxidants (Table IV) [88].

- *Cosmetics*

In the 1950's it was discovered that squalene is an important component of human sebum, fact that justifies its role in the physiology of skin: it was demonstrated its role in skin hydratation, repairing the damaged skin, rejuvenating the ageing skin. The emollient and hydration properties of Squalene and also its biocompatibility with skin, makes squalene an important component in cosmetical formulations (moisturizing creams, makeup, lipstick, nail and hair products) [39]. It is considered one of the greatest natural emollients, being rapidly and efficiently absorbed into the skin, restoring its natural suppleness and flexibility, without back oily residues. To its wide application in cosmetics also contribute its odourless and colourless, high spreadability, light consistency, non-greasy texture, rapid transdermal absorbtion, antibacterial properties. All these characteristics make it an excellent skin protector, being used in healing eczema, damaged hair, anti-aging and wrinkle protection. Squalene appears to play an essential role in protecting skin from free radical oxidative damage. Squalene is not very susceptible to peroxidation and it acts at the skin level as a quencher of singlet oxygen, protecting by this mechanism the

skin surface from lipid peroxidation due to exposure to UV light. Kohno et al. [47] showed in their studies that the rate constant of quenching of singlet oxygen by squalene is much higher than those of other lipids in human skin and is comparable to that of 3,5-di-t-butyl-4-hydroxytoluene (BHT). They also stated that it seems to be unlikely to appear the chain reaction of lipid peroxidation in human skin when proper levels of squalene are present (Table IV).

Table IV

Squalene applications in cosmetics

Ref	Procedure	Aim	Results
[66] 1996	60 male Wistar rats were used in 3 equal member groups; on their back skin was applied hot water to cause deep dermal burns-28mm diameter. Right side was treated, the left one was control. 1st group: squalene cooled at -40°C, applied 5 times, 0.1g/each time, at 10s; 2nd group: same procedure with squalane at -40°C; 3rd group: distilled water at 20°C, same procedure	To provide a therapeutic coolant based on squalene/ squalane for local treatment of burns	An eosinophilic layer observed in the upper dermis over a relative wide area in the control group; such destructive changes were mild on the treated groups.
[83] 2001	38 healthy volunteers, 19 with `Japanese`skin, 19 with`Caucasian`skin were clinical observed after the application to a surface of ~50mm^2 on the left sub-cappilary region of the skin, of the tested composition; the contact was maintained for 48h. The indice of cutaneous tolerance express the mean of the sum of the quantified effects from each volunteer (erythema, edema, vesicles, skin dryness, skin roughness, reflectivity of the skin)	To obtain a composition comprising an oil phase (solvent can be squalene), an aqueous phase, one emulsifying agent of the oil in water type, in the form of an auto-invertible latex, to be used in different cosmetic formulation with better cutaneous tolerance	The results show in a surprising manner that squalene potentializes the polymeric cutaneous tolerance of the inverse latex
[77] 2010	Human volunteers tested in a blind trial hair shampoo, comprising 0.05% Amaranth oil or 0.5% Amaranth oil + 0.3% Polyquatenium-10 vs. shampoos with only 0.3% Polyquatenium-10 or Polyquatenium-10 + 0.5% almond oil; contact time was 1 – 45 minutes, than the shampoo was removed	There were tested the hair properties after washing, as shine and combing properties; results were quantified from 1- very bed to 5- very good	The results for shampoos containing Amaranth oil were between 3.75 and 4.25 comparative with 3.25 to 3.80 for the other shampoos.

Due to its antibacterial properties, squalene in admixture is used for preparing a cooling formulation for the local treatment of burns [66]. Squalene has a melting point low enough to allow the cooling composition to remain liquid, even at temperatures between -10ºC and -60ºC, unlike the ordinary oily topical drugs.

- *Pharmaceutics*

Squalene is frequently used in the preparation of stable emulsions as either the main ingredient or the secondary oil. An important application of squalene emulsion is as an adjuvant for vaccine delivery. An immunological adjuvant is a substance employed to increase or to modulate the immune response against an antigen. An ideal adjuvant would increase the potency of the immune response while remaining nontoxic and safe for the host [58].

The first used as adjuvants in human vaccines were aluminum hydroxide and aluminum phosphate, but they did not show sufficient activity when used with various antigens as typhoid vaccine, influenza virus hemagglutinin. Various reports [8, 36] showed that mineral adjuvants were not safe, causing a variety of pathologies. Squalene-in-water emulsions stabilized with polysorbate 80 proved to be a solution to these problems.

Squalene was used as an adjuvant in vaccines, stimulating the immune response and increasing the patient's response to vaccine. It is added to lipid emulsions as drug carrier in vaccine applications [23]. Lipid emulsions are interesting as drug delivery systems because they easily incorporate drugs with poor solubility in the dispersal phase. By using lipid emulsions as drug carriers, it is avoided the direct contact between the active substance and human body fluid or tissue and by this avoiding the possible side effects. The experiments conducted by Kim et. al on a mouse model demonstrated that a squalene emulsion has the most potent transfection activity and proved the least cytoxicity after the intravenous administration [47].

An influenza vaccine (FLUAD, Chiron, Italy) using a squalene emulsion (10 mg/dose) was approved in Europe in 1997. Several patents [37, 38] revealed that by adding different substances (squalene 10%, lecithin 1% and Tween 80) to vaccine formulations, they become more effective in inducing high antibody titters than squalene emulsions stabilized only with polysorbate 80. The most used nowadays adjuvant including squalene is MF59 which belongs to Novartis, as patented compound. It comprises squalene together with two surfactants Tween 80 and Span 85, as an oil-in-water microemulsion. It is used as adjuvant in several vaccines against hepatitis B and C, *herpes simplex* virus, influenza virus. It was demonstrated [17] that the use of MF59 for the vaccine delivery is safe as no anti-squalene antibodies were produced and no enhancement of pre-existing anti-squalene

antibodies titters was observed. Futhermore [78] suggests that MF59, after injection, increases the immune response causing a considerable influx of phagocytic cells into the vaccination site. It is the first oil-in-water influenza vaccine commercially used [28]. Data published by World Health Organization in 2008 showed that squalene was present in over 22 million flu vaccines distributed to patients in Europe since 1997 and no adverse effects were reported [97].

- *Medical prevention and treatment*

Squalene, an isoprenoid natural compound, has a similar structure to β-carotene and it is an intermediate metabolite in the cholesterol synthesis in humans. About 60% of the dietary squalene is absorbed and then transported from the small intestine in serum in association with chylomicrons (the largest and the least dense of the lipoproteins). The combination of squalene with low density lipoproteins are distributed by blood in human tissues, the majority of the absorbed squalene being found in the skin, as the major component of the skin surface lipids.

Antioxidant activity of squalene

Squalene is a highly unsaturated isoprenoid hydrocarbon, containing 6 double bonds. Due to this double bond structure this isoprenoid action as a strong anti-oxidant and natural antibiotic. Also as a consequence of its biochemical structure, it is extremely reactive, and transforms into the oxidized form. The unsaturated carbons of squalene bind hydrogen ions from water and release 3 unbound oxygen molecules, providing the saturated form squalane, according to the reaction:

$$C_{30}H_{50} + 6\ H_2O \longrightarrow C_{30}H_{62} + 3\ O_2$$

Due to this reaction, the oxygen reaches the cells, the cellular metabolism is intensified, and the function of certain organs like liver and kidney is enhanced [46]. Squalene is not particularly susceptible to peroxidation and it is stable against peroxide radical's attack, that's why a protective effect of skin exposed to UV radiation is obtained when appropriate levels of squalene are present in the skin [95].

Squalene *versus* hypercholesteromia and Coronary Heart Disease (CHD)

The incidence of CHD is very high today due also to the modern life style. The studies performed in the Mediterranean area suggested that a diet reach in olive oil reduces the incidence of CHD and cholesterol conditions, and squalene was proposed as the main factor responsive of these effects.

Only a few reports are available regarding the effects of a diet containing squalene administrated to humans [12, 82].

The results of a clinical trial [12], conducted on elderly pacients suffering from hypercholesteremia showed, by contrary, a decrease in total cholesterol, LDL cholesterol and TAG (triacylglicerol) levels and an increase of HDL cholesterol. Other reports [56] showed that an amaranth oil diet, known for its high concentrations of squalene, produced health benefits by decreasing headaches, weakness and fatigue. An issue still in dispute today is the fact that a daily diet containing amaranth oil reduces the serum cholesterol due to the function of squalene, the important constituent of amaranth oil. Shin et al. [79] performed some experiments on rats with amaranth grain, oil and squalene in order to study their hypocholesterolemic effect. They observed a different effect of squalene from amaranth source *versus* squalene from shark origin: the vegetal squalene proved a hypolipidemic action in blood and liver and an increase of cholesterol excretion in fecals, effects that were not observed when shark squalene was administrated.

According to these reports, following a suplimentation of squalene of about 850-900 mg to the daily diet, no higher levels of cholesterol in serum were reported, although the concentration of squalene in serum raised up to about 17 times. The proposed explanation was the important growth of cholesterol elimination in feces which compensates the increased rate of cholesterol biosynthesis. Experiments on animals suggested the protective effect of squalene against CHD, explained by its effect to inhibit the isoprenaline-induced lipid peroxidation [21].

Anticancer and cytoprotective activity of squalene

It is already known in the scientific medical community that cancer chemotherapy damages the normal healthy tissues, leading even to organ toxicity and by these, limiting the anticancer drugs dosage and worse, the treatment failure. In many of the cases drug medication and radiation therapy also produce free radicals responsive of the mentioned toxic effects. Squalene already proved to be effective as an antioxidant. The common antioxidants used today in cancer therapy seem to have serious side effects – a reason for which the natural antioxidant squalene to be experimentally tested and it proved to be a well-tolerated, non-toxic, important cytoprotective agent [16].

The primary application of squalene in cancer therapy seems to be as a potentiating agent for the chimiotherapic drugs. There are reports about the good results obtained by testing on animal models of the squalene in combination with anti-tumor agents as ACNU (3-[(4-amino-2-methyl-5-

pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea) against lymphocytic leukemia [96], or as bleomycin [60]. So far, no experimental trials on humans have been reported in order to confirm data obtained on animal models. Experimental data existing up to now could indicate that squalene is implicated on the biochemical way by which anti-cancer drugs action. It seems that squalene may stop the tumour cells' development, or to prevent some forms of chemically-induced cancer and even to produce regression of exisiting tumors in some cases. The suggested mechanisms by which squalene could inhibit tumor formation imply either - its inhibitory effect on the catalytic activity of β-hydroxy-β-methylglutaryl-CoA (HMG CoA) reductase and subsequent inhibition of farnesylation of Ras oncoproteins; or – modulation of the biosynthesis and functional activity of the enzymes implicated in xenobiotic metabolism; or – its action as a free radical scavenger [72].

A literature survey for the squalene applications in medicine, with experimental preclinical and clinical data is presented in Table V.

Table V

Squalene (SQ) applications in medicine

Ref.	Treatment procedure	Aim	Results/observation
Hypocholestorolemic action			
[80] 2004	-rats fed for 4 weeks with a diet comprising 1% cholesterol and Amaranth oil (amaranth group)/ SQ from Amaranth; -rats fed for 7 days with cholesterol diet and salt (control) and a diet containing amaranth SQ and shark oil SQ	-examination of the hypocholestorolemic effect of amaranth oil and SQ -comparison of amaranth SQ to shark oil SQ activity	-decrease of total blood and liver cholesterol levels and triacylglycerol conc.; increase in fecal and bile acid cholesterol excretion for amaranth group. -amaranth SQ showed hypolipidemic effect in blood and liver, increase in fecal and bile acid cholesterol excretion, effects not obseerved for shark oil SQ
[49] 2006	-rats fed for 28 days with a diet containing 5% Amaranth oil	- study of the hypocholesterolemic effect of Amaranth oil	-15% decrease of total cholesterol and 22% decrease of non-high density lipoprotein cholesterol vs. control; it was not observed increase of cholesterol level in rat liver
[27] 2006	-125 human patients with ischemic heart disease and hyperlipoproteinimia were provided a diet supplented with 100/200/400/600 mg SQ/day for 3 months	- investigate the diet influence on: -immune status of pacients -dynamic of lipid profile and fatty acid	-the consumtion of 200-400 mg SQ/day produces the greatest improvement of the immune status -the antiatherosclerotic diet with 600 mg SQ/day produces

		composition of erytrocytes	positive changes in serum cholesterol, TG level and fatty acid composition of erytrocyte membrane
[56] 2007	125 human patients with CHD, hypertension and obesity, with a hyposodium, antiatherogenic diet (HAD) received for 3 weeks: -40 (control)-only HAD -25 (1st test group)-100mg SQ/day (3 mL Amaranth oil) -20 (2nd test group)-200mg SQ/day (6 mL Amaramth oil) -20 (3rd test group)-400mg SQ/day (12 mL Amaramth oil) -20 (4th test group)-600mg SQ/day (18 mL Amaramth oil)	Study of the influence of a diet with Amaranth oil (SQ) on total cholesterol, triglycerides, LDL and VLDL levels, humoral immunity at patients with CHD, hypertension and obesity	-systolic arterial pressure decreases by 18%, 19%, 21%, 20% and 18%, respectively for 1st, 2nd, 3rd, 4th and control groups; -weight loss was similar for test and control groups; -total cholesterol decreases by 19%, 14%, 17%, 20% and 12%, respectively for 1st, 2nd, 3rd, 4th and control groups; -tryglicerides in blood serum decrease by 8%, 14%, 21%, 36% and 16%, respectively for 1st, 2nd, 3rd, 4th and control groups; -LDL decreases by 23%, 19%, 23%, 25% and 12%, respectively for 1st, 2nd, 3rd, 4th and control groups; -VLDL decreases by 7%, 18%, 21%, 37% and 16%, respectively for 1st, 2nd, 3rd, 4th and control groups. Throughout the clinical trials no single case of intolerance, dispepsia, allergic reactions was noticed. The best effects of inclusion of Amaranth oil in HAD was observed at a dose of 18mL (600mg SQ)/day
[12] 1996	-20 weeks clinical double-blind placebo controlled trial on 102 elderly people suffering from high cholesterol levels. They received 10 mg pravastatin and/or 860 mg SQ daily either separatley or in combination	Determination of the effectiveness and safety of SQ alone and in combination with pravastatin in lowering cholesterol levels	Both SQ and pravastatin reduced levels of total cholesterol and LDL cholesterol and increase HDL cholesterol levels; although pravastatin was more effective than SQ when administrated separately, in the combination was even more efficacious
Anticancer action			
[18] 1996	Mice injected with 7,12-dimethyl	Study of chemopreventive effect of SQ -suppresing	The incidence of tumors in mice injected with SQ only-27%, with Roidex-20% vs.

	benz[a]-antracene (chemically induced cancer) were treated with 5% SQ or with Roidex formulation (SQ, vitamines A and E, mineral oil and aloe vera) or only with mineral oil	tumor cells' growth	33% in case of mineral oil; Roidex administration caused 33% regression of tumors vs. 3% case of mineral oil administration. Results indicate SQ seems to have a significant contribution to inhibiting tumor growth, possibly independent of the other components of the formulation
[71] 1998	1% SQ diet was orally administrated to rats with early prenoplastic lesions in colon carcinogenesi (induced by azo-xymethane) over 10 weeks	Proving the chemopreventive effect against colon cancer	1% SQ diet resulted in 45% inhibition of colon cancer compared to control group
[73] 1982	Test rats were administrated a 3 weeks diet with 8% SQ; control rats were administrated the same period a diet with 8% paraffine	Investigate SQ action in detoxification of xenobiotics	After SQ diet the amount of HCB excreted with feces was 3 times higher and the half time for HCB elimination decreased from 110 days in control group to 34-38 days in test group; SQ being a nonpolar substance seems to have higher affinity for un-ionized drugs

Conclusions

Squalene is a natural substance belonging to the terpenoid family, widespread in the nature, with various applications in many human life areas. We have reviewed the existent literature data about the natural sources for squalene, with emphasis on the reachest natural sources, like shark liver oil, OODD, SODD, amaranth oil, and also the newest experimental studies performed on micoorganisms. Due its unique properties, squalene has diverse applications, especially in nutrition, health and cosmetics.

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