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Flax Engineering for Biomedical Application

Magdalena Czemplik[1], Aleksandra Boba[1], Kamil Kostyn[1],
Anna Kulma[1], Agnieszka Mituła[1], Monika Sztajnert[1],
Magdalena Wróbel- Kwiatkowska[2], Magdalena Żuk[1],
Jan Szopa[1] and Katarzyna Skórkowska- Telichowska[3]
[1]Faculty of Biotechnology, Wrocław University
[2] Department of Pharmaceutical Biology and Botany,
Medical University in Wrocław
[3]IVth Military Hospital in Wroclaw
[4]Linum Foundation, Wroclaw
Poland

1. Introduction

Flax (Linum usitatissimum) is an important crop plant that is widely distributed in the Mediterranean and temperate climate zones. It has great significance for industry as a valuable source of oil and fibres. A unique feature of flax is the possibility of whole plant exploitation with almost no waste products. For this reason, flax has quite significant potential for biotechnological application. To increase the valuable qualities of flax products, the flax genome has been genetically modified, with the specific aims to improve the plant's pathogen resistance, taste and nutritional properties, and to produce pharmaceuticals and other compounds. In this chapter, we describe the plant characteristics that show the biochemical and industrial importance of flax oil and fibres and their various possible applications and the relevant genetic modifications.

Since ancient times, flax has been known to be a source of oil and fibres, and it has been cultivated as a dual-purpose plant for a long time. Nowadays, it is a multi-purpose plant and its exploitation is not restricted to the production of linen fibre and oil. Actually, whole plant exploitation is possible, which justifies the name given to it by Linnaeus: *L. usitatissimum*, meaning "useful flax". There is a wide range of possible applications of flax (Fig.1). The long fibres are used in the textile industry, and the short fibres in paper production, isolation materials and biocomposite production. The wooden shives released during flax scutching can serve as an energy source. Flax seeds also have many important applications, and due to its high nutritional value, it is used in the food, pharmaceutical and health care industries. The seedcake, which is rich in antioxidants, is used in the pharmaceutical and cosmetic industries.

The development of molecular biology emerged as an important tool for the genetic modification of plants, and enabled the improvement of many different features of wild type plants. These modifications broadened the range of practical applications for flax, making the plant more valuable and more significant for the innovative biotechnological industry.
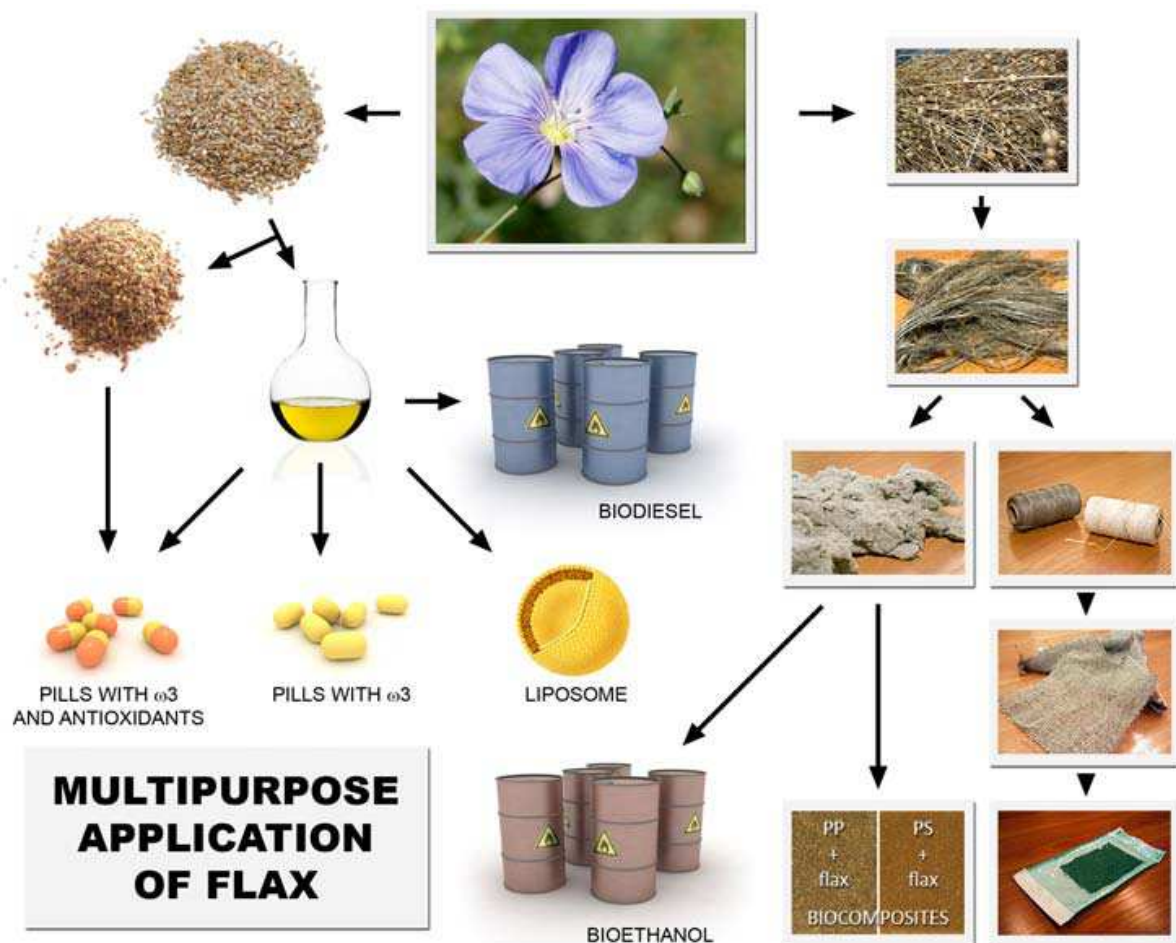
Fig. 1. The multipurpose application of flax

Flax is a good source of unsaturated fatty acids, dietary fibre and another nutrients. It is composed mainly of fat (41%), protein (20%) and dietary fibre (28%). The contents may vary depending on genetics, environment, seed processing and the analysis method. *Linum usitatissimum* is the best-known species with a high concentration of α-linolenic acid (ALA). Polyunsaturated fatty acids compose about 73% of the total fatty acid content. Flax proteins are rich in arginine, aspartic acid and glutamic acid. *Linum usitatissimum* is characterized with a high content of polysaccharidic mucilage. It confers from 6 to 8% of the dry weight. The acidic polysaccharide consists of L-rhamnose, L-galactose and D-galacturonic acid and the neutral polysaccharides L-arabinose, D-xylose and D-galactose. The amino acid composition of flax indicates that the most abundant are glutamic acid, aspartic acid and arginine. Moreover a series of cyclic polypeptides, which contains between eight and ten amino acids, have been identified in *Linum usitatissimum*. Some of them exhibit immunosuppressive activity. Phytochemicals that have been identified in flax mainly consist of lignans, isoprenoids, phenolic acids, flavonoids and cyanogenic glucosides. All these compounds, apart from cyanogenic glucosides are known to have antioxidant properties or inhibitory activity against carcinogen induced tumors.

2. Flax fibre quality improvement and its biomedical application

Flax fibres have many useful applications. They are flexible, lustrous and soft. Moreover, flax fibres are stronger than cotton but less elastic. They absorb humidity and are allergen-

free. These properties make flax fibres useful in the textile industry but they are also used in the manufacture of car-door panels, plant pots and retaining mats. Recently, some research has been carried out to improve the quality of flax fibres and make them suitable for the biomedical industry. Innovative flax fibre-containing products have been developed with potential applications in the medical field. The main strategy was to make use of genetically modified flax fibres with unusual and unique properties.

2.1 Fibre quality improvement

Flax is a great source of fibre. Plant fibres are divided into three groups: the phloem stem fibres (phloem stem fibres or xylem stem fibres) of dicotyledonous plants; the leaf fibres of monocotyledonous plants; and the seed and fruit fibres (Ilvessalo-Pfaffli, 1995). Flax fibres belong to the first group.

The flax stem is 70% composed of cellulose. These hollow tubes grow together as bundles and are held by complex carbohydrates such as pectins, gums and waxes. These function as a plant support. The fibre separation process from the non-fibre tissues is called retting (Antonov *et al.*, 2007). Retting is mainly the enzymatic action of polygalacturonase, which degradates the pectin polymers of the middle lamella into soluble galacturonic acid. This process is mainly carried out by plant pathogens like filamentous fungi. To obtain high-quality fibres, the proper degree of retting is necessary (Zhang *et al.*, 2000). The efficiency of retting depends on the method used, but traditional dew retting is still the most widely performed method. In this method, the flax stalks are left in the field after the harvesting of the flax seeds, and the soil microorganisms digest the cell matrix polysaccharides. The dew retting method is weather dependent, which makes it uncontrollable.

The chemical composition of the flax stems can affect the degree of retting. Fibres which have more lignins need a longer period of retting. However, a longer exposure to fungal and bacterial enzymes decomposes the cellulose and weakens the fibres. One solution to this problem is to harvest the flax before seed maturity when the level of stem lignification is still low. Another solution is to genetically manipulate the flax, yielding an improved retting process. It is known that the pectin and hemicellulose contents of the fibres influence the fibre processing. A new technology to modify the biosynthesis and degradation of pectins with beneficial consequences for the flax fibre properties has been recently developed. The flax plant was transformed with *Aspergillus aculeatus* genes coding for polygalacturonase (PGI) or rhamnogalacturonase (RHA), which are the enzymes required to break down pectin of the flax fibres. The transformants were characterized by an increased enzyme activity and a significant reduction of pectin content. The reduction in pectin content was in the range of 56–68% for both the PGI and RHA plants. These results correlated with the retting efficiency, which was more than 2-fold higher in the transgenic flax than in the control plants (Musialak *et al.*, 2007). Interestingly, the overexpression of the enzymes did not affect the fibre composition. No changes in the lignin or cellulose contents was observed in comparison to the control. Similarly, the levels of soluble sugars and starch were at the same levels as in the non-transformed flax. As the biochemical parameters of the cell wall components remained similar to those for the control plants and the fibre quality did not change, it is suggested that these modifications might be important for industrial and medical application.

Another strategy of improving flax fibre quality was reducing the level of lignin synthesis. Lignins are complex polymers of three aromatic alcohols: coniferyl, sinapyl and p-coumaryl (Amthor, 2003), and cinnamyl alcohol dehydrogenase (CAD) is an enzyme that catalyses the

biosynthesis of lignin monomers (hydroxycinnamoylalcohols) from the corresponding aldehydes. CAD is the specific marker of lignification (Barakat *et al.*, 2009). Flax fibres comprise 3-5% lignins, and they are mainly responsible for mechanical resistance. They create a physical barrier against pathogen infection, and are highly accumulated and deposited in response to pathogen attack (Tobias *et al.*, 2005). The accumulation of lignins negatively influences the retting efficiency. To overcome this problem, transgenic flax with a silenced *cad* gene was created. In the generated plants, the CAD enzyme activity was 20-40% lower, and the lignin level decreased by up to 40 % (Wróbel- Kwiatkowska *et al.*, 2006). Moreover, this modification influenced the composition of the cell wall. The pectin content and the hemicellulose content was significantly lower. Decreasing the level of the above-mentioned compounds facilitated the retting process. Furthermore, the mechanical properties of the modified fibres were strongly improved, as the ratio of cellulose to lignin increased. Cellulose is the fibrous component of the cell wall, and the hemicellulose, pectins and lignins are the matrix components. An increased proportion of the fibrous to matrix components is the reason for the improvements in the mechanical properties of the stem. This data indicates that *via* genetic modification, it is possible to improve the mechanical properties of flax fibres and make them more useful for further application.

2.2 Biodegradable flax biocomposite material as a new medical polymer

Composites are materials made of matrix reinforced with natural fibres, and the term biocomposites is used for composites employed in bioengineering (Ramakrishna *et al.*, 2004). The development of biocomposites is attractive because the properties of two or more types of material can be combined, which influences the properties of the composites. Most medical devices used in medicine and made of a single material, such as a polymer, metal or ceramic, are too flexible or too weak or too stiff to host tissues, and some may also be sensitive to corrosion or cause allergic reactions (e.g. nickel and chromium). All these disadvantages led to the development of composite materials for medical use. Nowadays, composites are used in numerous biomedical applications: sutures, cardiovascular patches, wound dressings, regeneration devices and tissue engineering scaffolds (Misra *et al.*, 2006). Many composites have poor biomechanical properties and poor bioactivities. The composites containing biodegradable polymers can be divided into two groups: one based on natural polymers (e.g. starch, alginate, silk) containing as reinforcement natural fibres (lignocellulosic or cellulosic fibres); and the second based on synthetic polymers (polylactic acid PLA, polyglicolic acid PGA, poly-ε-caprolactone PCL) (Rezwan *et al.*, 2006). Natural fibres replace glass, ceramics or carbon fibres (Bax and Mussig, 2008). In the last decade biocomposites were used by the automotive industry for door panels, seat backs, headrests and package trays among other things (Fig. 2), (Suddel *et al.*, 2003; Bledzki *et al.*, 2006).

Biocomposites have favourable biomechanical properties and they can also have bioactive properties, for example antioxidative and bacteriostatic action. The main problem in preparing biocomposites is often the poor adhesion between the matrix and the fibres used as reinforcement. This influences the mechanical properties of the composites and remains a significant disadvantage. Better contact between the fibres and the matrix also enhances the hydrophobicity of the composite. The possible solution to this problem might be the production of a biocomposite containing transgenic flax fibres enriched with hydrophobic and thermoplastic poly-β-hydroxybutyrate (PHB). This non-toxic and water-insoluble compound displays chemical and physical properties similar to polypropylene. PHB is a biodegradable, ecologically friendly compound, and may be an alternative to conventional

Fig. 2. Applications of biocomposites in industrial products

plastics used as the matrix component of composites, particularly those reinforced with fibres of natural origin (Peijs, 2002). PHB was discovered in the bacterium *Bacillus megaterium* and is found in other species of bacteria, including Alcaligenes, Azotobacter, Bacillus, Nocardia, Pseudomonas and Rhizobium. It is synthesized in a three-step reaction catalysed by β-ketothiolase (phbA), acetoacetyl-CoA reductase (phbB) and by PHB synthase (phbC) (Fig. 3), (Steinbuchel and Fuchtenbusch, 1998). In bacteria, PHB serves as a source of carbon and energy.

Isolating PHB from bacteria is expensive, so producing PHB in plants could be a promising method. Transgenic flax plants with overexpression of the three genes encoding PHB synthesis have been generated, and shown to be useful for biomedical applications. The stem-specific 14-3-3 promoter was used for the transformation. Three genes coding for β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase were derived from *R. eutropha*. The generated plants (named M plants) exhibited a PHB content of up to 4.62 μg/gFW (Wróbel *et al.*, 2004). The electron-lucent granules of PHB were detected in the stroma of the plastids in the M plants. Moreover, the PHB synthesis affected the shape and size of the chloroplasts: the diameter of chloroplast increased, and they were characterized by a more oval shape. The accumulation of PHB resulted in changes in the stem's mechanical properties. These properties were measured using Young's modulus. This parameter was two-fold higher in the M plants, which indicates that transgenic fibres enriched with PHB have a higher average resistance to tensile loads and better elastic properties. The fibre composition of the M plants was examined using the infrared (IR) spectra method.
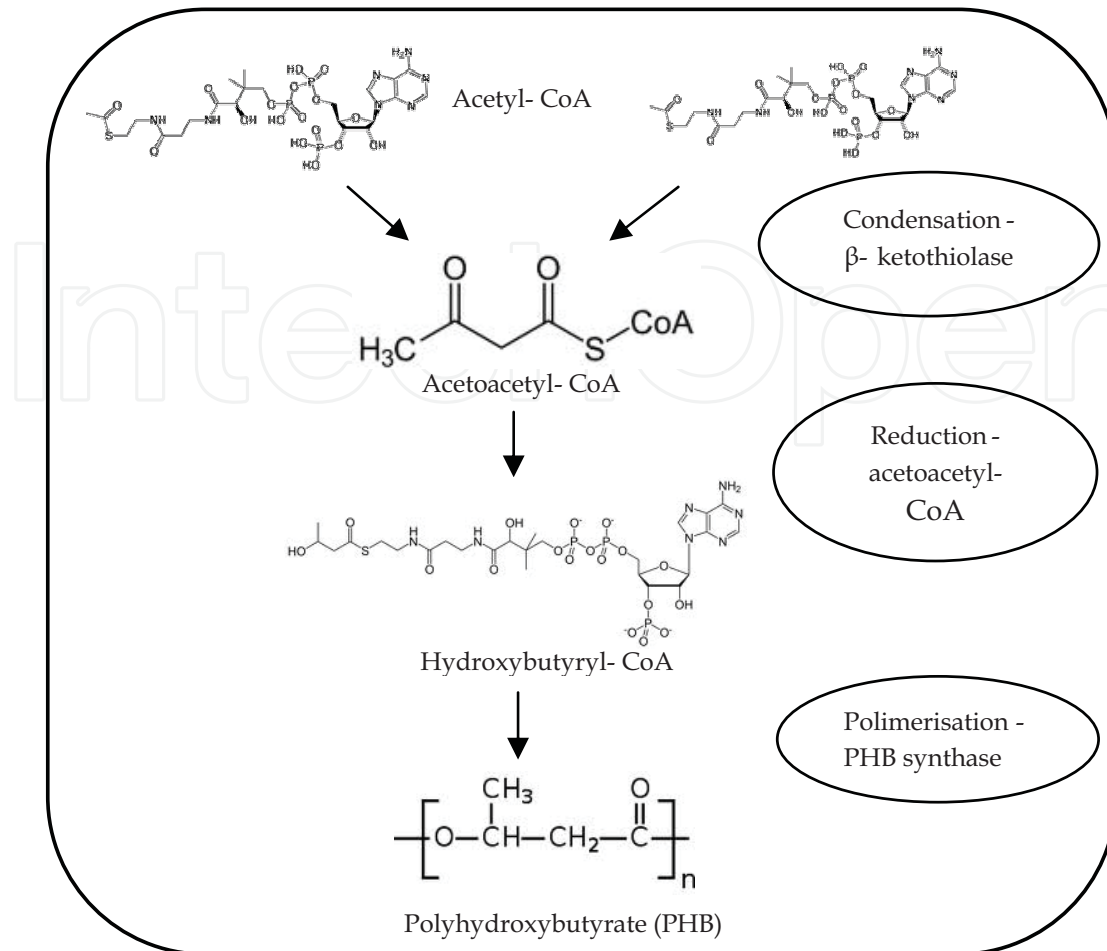
Fig. 3. Polyhydroxybutyrate (PHB) synthesis pathway

The greater structural disorder of the M fibres resulted from the formation of celluloses with an amorphous structure and from the shortening of the cellulose chain lengths. What is more, the M plants exhibited stronger coupling between the elementary fibres, which made them more stable (Wróbel- Kwiatkowska et al., 2009). Introducing PHB to flax fibres yielded the commercially utilized bast fibres. Fibres derived from those plants can be particularly used as the reinforcement in biocomposites (Fig.4).

First of all, the PHB in those fibres improves the adhesion between the fibres and the matrix, and secondly, such fibres remain a great source of phenolic acids, which possess antioxidative properties that are especially important when the fibres are used for medical purposes. Previous studies showed that composites of transgenic flax fibres enriched with PHB and polypropylene do not promote platelet aggregations in contrast to pure polypropylene (Szopa *et al.*, 2009). It was also noticed that those transgenic fibres have bacteriostatic properties (data not published).

A new generation of entirely biodegradable composites were made of polylactic acid (PLA) and alternatively of poly-ε-caprolactone (PCL) enriched with bioplastic flax fibres. Determining the level of platelet aggregations on the surface of the prepared composites and the level of colonization of bacteria (*E. coli*) to their surfaces showed the composites' anti-aggregational and bacteriostatic properties. The new composites also exhibited improved biomechanical properties in comparison to membranes made of pure PLA or PCL, and good *in vitro* biocompatibility, even though the cell viability of mouse fibroblast cells treated with

Fig. 4. Biodegradable flax composites made of polypropylene (PP) enriched with different content of bioplastic flax fibres (M plants). The control composite is made of polypropylene with no addition of flax fibres

these composites was slightly reduced and the amount of dead cells also slightly increased when compared to untreated cells (Gredes et al., 2010). It was also shown that implanting the tested biocomposites based on PLA and transgenic flax fibres into rat skeletal muscle had no influence on the gene expression of the most analysed genes, i.e. vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and growth differentiation factor 8 (GDF8) (Gredes et al., 2010). The used implants composed of transgenic plastic fibres in a PLA matrix showed better biocompatibility than pure PLA or PHB implants, and they did not have any negative effect on muscle function and gene expression. Thus, the new biocomposites created with bioplastic flax fibres might be considered as a new material for tissue engineering and other branches of medicine.

2.3 The new bandage based on transgenic flax products

The number of patients with serious ulcer wounds is still increasing. This is a consequence of chronic diseases such as diabetes, obesity and atherosclerosis. An ulcer that is considered chronic, or non-healing, is one which takes more than eight weeks to heal despite optimal local and general treatment. Wound healing is a complex and dynamic process, divided into three overlapping stage: cleaning, proliferation, and wound constriction and cicatrisation. The complex treatment of ulcers mainly consists of wound diagnostics, casual treatment directed at the primary disease, the exclusion of other factors that inhibit healing processes, and general and local treatment (Abbade & Lastória, 2005). There are many factors that can influence wound healing, and using a proper wound dressing is among them. In recent years, many different specialized wound dressings were developed, such as hydroxycellulose, hydrocolloid, polyuretic-foam dressing, alginans, hydrogel dressing and dressing containing silver (Jones et al., 2006; Skórkowska-Telichowska et al., 2009). The

purpose of a dressing is to isolate the wound, keep it at an optimal moisture level, remove excess exudates, help clean the wound of debris and necrotic tissue, help combat microbiological infection when necessary, and stimulate tissue regeneration.

One recently proposed wound dressing is made from genetically modified flax fabric (Fig.5). Flax has been used since ancient times for the production of linen cloth widely used for making clothes that are especially useful in humid climates due to certain flax fibre properties, the effects of which are called "wicking". This refers to their capillary action, which channels moisture away from the body (Muir & Westcott, 2003). This makes flax an attractive material for wound dressing, as it may be useful in keeping the wound at the optimal moisture level. Additionally, the loose weave of flax tissue enables wound purification from various contaminations.
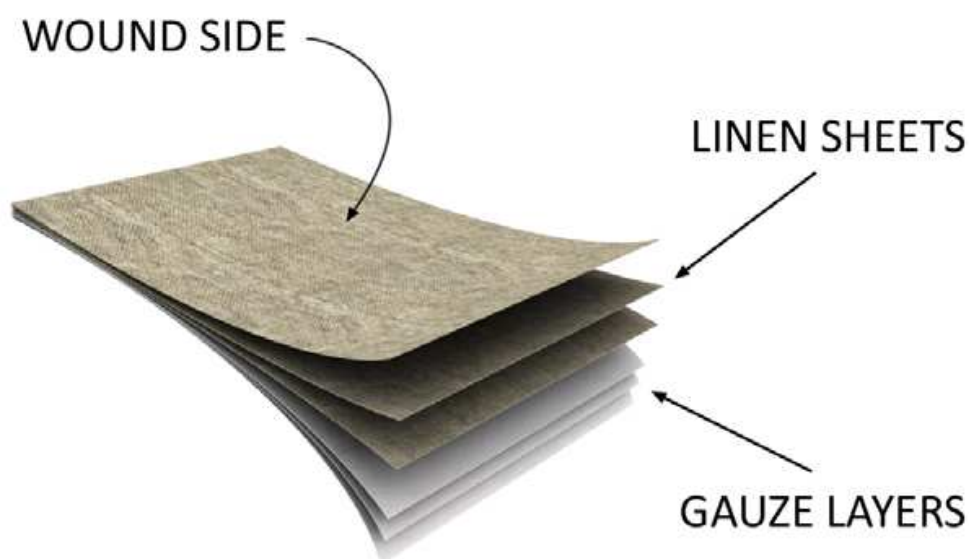

Fig. 5. Scheme of flax dressing

The biotechnological engineering was used to improve the flax fibres for medicinal use. After it was suggested that reactive oxygen species are responsible for chronic wound pathogenesis and anti-healing processes because they reduce the cell proliferation capacity (Chen et al., 2004; Wall et al., 2008), the use of flax fibres enriched in antioxidants in making of a wound dressing was proposed. Oxidative stress causes damage to cellular macromolecules; the deregulation of key proteins involved in DNA replication, the cell cycle, and cellular resistance to such stress; and the promotion of wound fibroblast apoptosis. Antioxidant-rich flax plants called W92 were created using three genes controlling the synthesis of plant secondary metabolites from the phenylopropanoid pathway: chalcone synthase, chalcone isomerase, and dihydroflavonol reductase. The introduction of those genes resulted in the accumulation of various antioxidative compounds like flavonoids, phenolic acids and lignans in the plant seeds and fibres (Lorenc-Kukuła et al., 2005). The antioxidant properties of these compounds might have a great significance for wound healing, because they inhibit enzymatic and non-enzymatic peroxidation. Flavonoids and phenolic acids also exhibit anti-inflammatory, antihistamine, antiviral and vasodilatory properties. It is suggested that the high level of phenylopropanoid compounds strengthens wound tissue defenses against biotic and abiotic stresses (Afaq et al., 2007; Chiang et al., 2005; Bae et al., 2009). The simultaneous use of

fibres, oil emulsion and seedcake extract from genetically modified flax plants promoted the healing of chronic skin ulcerations (Skórkowska-Telichowska et al., 2010). In the pilot clinical trial, 30 patients with chronic skin ulceration (having lasted from 2 to 23 years) were treated with a linen dressing for 12 weeks. The treatment was divided into three phases: the dry phase with use of a highly hygroscopic linen dressing aimed at drying and cleaning the wounds; the second stage with linen dressing wetted with an oil emulsion derived from transgenic flax, with the aim to supply the wounds with polyunsaturated fatty acids (PUFA) and antioxidants; and the third stage with flax bandages wetted with seedcake extract rich in lignans, which are anti-inflammatory and cell proliferation promoting compounds. Such treatment effectively reduced the wound exudates in almost 67% of the subjects. Moreover, 93% of the patients exhibited a decrease in the fibrin level, which is one of the steps in wound healing. An important and objective parameter that was assessed was the wound size, and it emerged that in 80% of the patients, the ulcer size was reduced, of which 23% were totally cured (Fig.6). Interestingly and importantly, the bandage diminished the pain accompanying chronic venous ulceration as reported by patients.

It is believed that the effectiveness of a flax wound dressing is thanks to:

- the hygroscopic properties of the flax fibres providing an optimal moisture level and absorbing the excess of exudates;
- the phenolic acid and flavonoid content in the flax fibres reducing the inflammation in the wound;
- the unsaturated fatty acids which reinforce the integrity of the plasma membranes of the fibroblasts
- the presence of lignans stimulating fibroblast proliferation;
- the protection of the wound from mechanical irritation;
- the wound being kept clean and insulated from necrotic elements and contamination that move beyond the dressing surface.

Recently this new bandage, which is called Flax Aid, has become certified and is now offered by the Linum Foundation (www.leczenielnem.pl).

There is a proposal to replace the linen dressings from W92 plants with dressings from M plants (bioplastic fibres) because the induction of cell proliferation is very important for wound healing. For this reason, polyhydroxybutyrate (PHB) has received particular interest. Upon contact with body fluids, the polymer degrades to release D,L-β-hydroxybutyrate, promoting the proliferation of cells in high-density cultures by preventing apoptotic cell death (Cheng et al., 2006) making it an attractive candidate for tissue engineering applications, especially those requiring the regeneration of large numbers of cells as described in the previous section. The fabric, made from plants enriched in polyhydroxybutyrate (M plants), was tested for suitability as a wound dressing. In this case, only the first stage (flax fabric wetted with isotonic solution) was investigated and only the wound size was considered, and the results were compared to those obtained with the W92 type dressing. During the four weeks of flax dressing treatment (twenty cases with chronic ulcerations of venous origin), the majority of the subjects treated with M flax bandages showed a statistically significant reduction in wound size. The average reduction in the ulcer size within this group was 28% (6 cm2), which is a far better result than that (7.78%) obtained for dressings prepared from the fibres of W92 plants. Thus, it is suggested that the fabric from the fibres of M plants is more effective in wound healing than that from W92 plants. It should be pointed out that both transgenic fabrics inhibit the growth of pathogenic bacteria and fungi in *in vitro* studies and are more effective than control and cotton fibres.
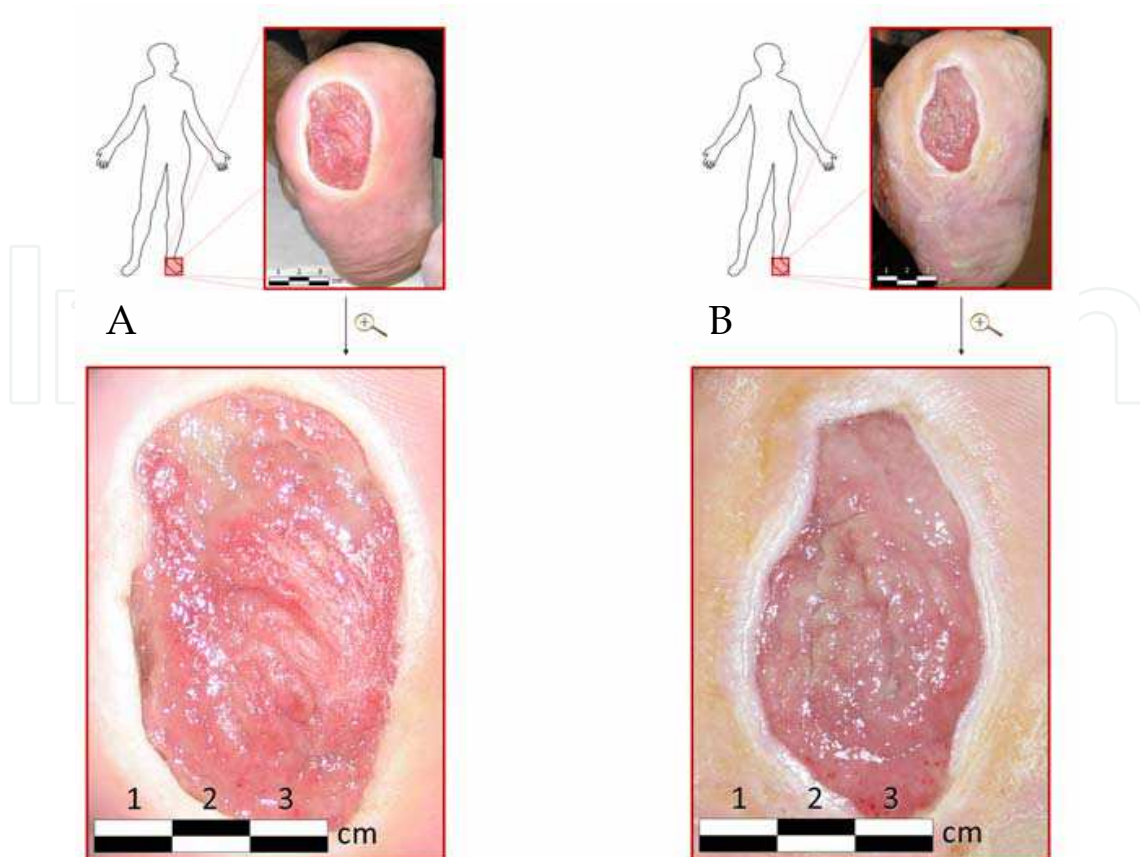
Fig. 6. Foot ulcer before (A) and after (B) twelve- week treatment with flax dressing

The antimicrobial activity of a flax fabric dressing is of great interest, as from clinical practice it is known that quite often long-term wounds can become infected. Very often this is caused by a antibiotic-resistant bacterial and fungal strains, so new ways to combat microbiological infections are needed. These results show that linen dressings obtained from GM flax have beneficial effects on wound healing and can be used as an innovative flax biotechnological product.

3. Transgenic flax as a source of bioactive compounds designated for therapy and health-promoting actions

Linseed oil has commercial value as a component of adhesives, paints and varnishes, plasticizers, inks and linoleum. It is also a precursor of nylon and composite materials. That said, the most important aspect for humans is the dietary properties of flax seeds. Linseed oil is one of the richest sources of α-linolenic acid (ALA), with a content of 44–57%; it also contains 15–29% linoleic acid and 13–29% oleic acid (Muir and Westcott, 2003). As the human body cannot produce ALA, which belongs to the ω-3 family, it is an essential fatty acid in our diet. Therefore, linseed oil plays an important role in the food, health care and pharmaceutical industries. The unsaturated fatty acids of linseed oil have a positive influence on the metabolism and peristalsis. They can lower the total and LDL cholesterol and glucose levels in the blood (Pellizzon et al., 2007).

The market value of linseed oil is limited by the lability of polyunsaturated fatty acids (PUFA), which are susceptible to peroxidation. Only a few cultivars of the flax with low

PUFA contents are suitable for commercial use as an edible oil. The rancidity and the development of an undesirable odor in linseed oil is mainly caused by the interaction of reactive oxygen species (ROS) with PUFA. Lipid peroxidation degrades unsaturated fatty acids and leads to the accumulation of dangerous products. ROS are known to be associated with aging, inflammation, carcinogenesis and atherosclerosis (Choi et al., 2002). For this reason, the inactivation and elimination of ROS might be very beneficial not only in industrial but also in nutritional applications. To avoid oxidation, linseed oil is supplemented with vitamin E and stored in low temperature in dark bottles. However, these methods are not satisfactory. A promising strategy might be the genetic manipulation of flax to improve the oil quality and the stability of the PUFA, and thus the natural supplementation of protective antioxidant compounds in flax seeds. There are a few interesting approaches in which various antioxidant compounds were synthesized in transgenic plants. The goal of this research was to improve flax seed quality by increasing the level and stability of unsaturated fatty acids and antioxidant compounds *via* genetic engineering. The modifications were directed toward improving the beneficial effects of flax seeds.

3.1 Manipulation of the flax genes of the phenylopropanoid pathway

Breeding plants which are able to produce pharmaceuticals and other valuable compounds is one major reason for the genetic modification of plants. The phenylopropanoid pathway seems to be one of the most important metabolic pathways due to its involvement in the synthesis of a large range of natural products in plants, including lignans, lignin, flavonoids and anthocyanins. All of these compounds act as antioxidants, chelators of divalent cations, photoreceptors, and visual attractors. The pathway of flavonoid synthesis begins with chalcone synthesis catalysed by chalcone synthase (CHS) and flavonon and flavonol synthesis catalysed by chalcone isomerase (CHI), leading to flavan production in a reaction catalysed by dihydroflavonol reductase (DFR) (Fig.7). Flavan is direct precursor of anthocyanidins and proantocyanidins. The last step in flavonoid biosynthesis is their glycosylation by glycosyl transferase. The phenylopropanoid synthesis pathway is the source of different compounds of great biomedical application like phenolic acids, lignans catecholamines and lignins.

Many phenolic compounds (lignans, phenolic acids) have been identified in both the green parts and the seeds of flax plants, which is the irrefutable proof of the presence of an active phenylopropanoid pathway in this plant. The enzymes of the phenylpropanoid pathway are appropriate targets for genetic manipulation, and the modulation of their levels alters the content of secondary metabolites. There are two alternative ways to increase the phenolic compound contents in plants: increase the expression of the key enzymes of synthesis; and overexpress the enzymes regulating stability of these compounds.

The first strategy was used to create flax plants overexpressing three genes of the phenylopropanoid synthesis pathway: chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR). Such plants were characterized by a significant increase in the levels of flavonoids in the seeds, fibres and in the green parts of the plant (Lorenc-Kukuła et al., 2005). The major differences in the antioxidant levels were observed in the phenolic acid content, and more importantly for human consummates, flax seeds obtained from transgenic plants are rich in the strong antioxidants secoisolariciresinol diglucoside (SDG), kaempferol and quercetin. The results obtained for these plants are presented in Figure 8.
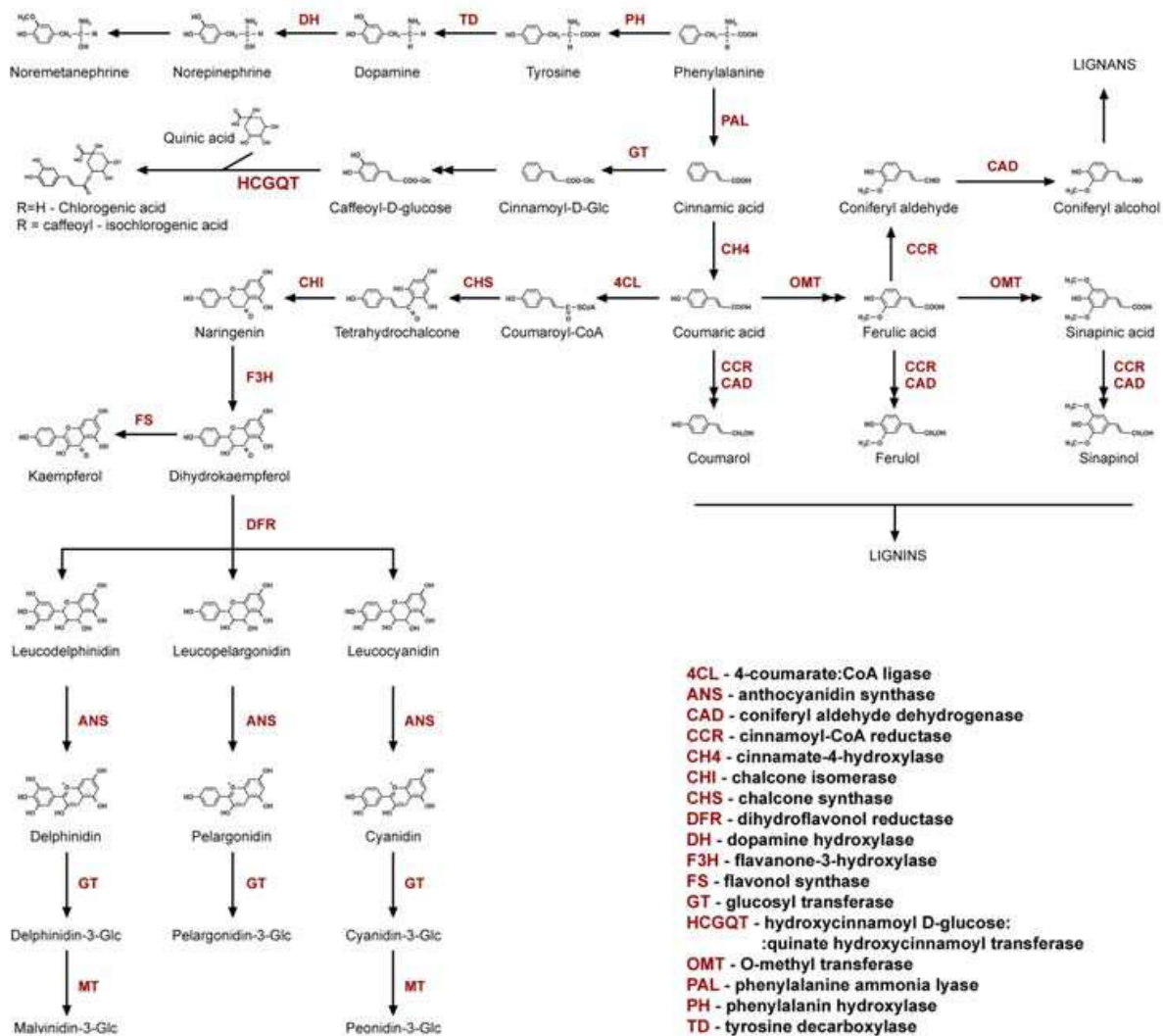
Fig. 7. The phenylopropanoid pathway

A similar effect was obtained for flax plants with overproduction of glycosyltransferase (the second strategy), which resulted in higher accumulation of proanthocyanin, lignans and phenolic acids compared to the control (Lorenc-Kukuła et al., 2009) (Fig 8). Linseed oil is one of the richest sources of unsaturated fatty acids, especially α-linolenic acid (ALA). Unfortunately, this product is unstable during long storage, because of the tendency to oxidation of fatty acids. Both modifications resulted in unsaturated fatty acid accumulation in the seeds. This observation suggests the involvement of polyphenol glycosides in the protection of unsaturated fatty acids against oxidation, which improves the oil.

The genetic engineering approach could involve the overproduction of various natural antioxidants within the flax grains. In addition to preventing fat rancidity, antioxidants such as flavonoids might also have a beneficial effect on human health and such plants are a very good source of compounds for biomedical applications. Recently, there has been a growing interest in the probiotic properties of flax and in its beneficial effects on coronary heart disease, some types of cancer, and neurological and hormonal disorders (Saffron, 2008; Huang & Ziboch, 2001; Simopoulos, 2002). Flax seeds are the richest source of the plant lignan secoisolariciresinol diglucoside (SDG), which can be metabolized by the colonic microflora to yield the mammalian lignans, enterodiol (ED) and enterolactone (EL)
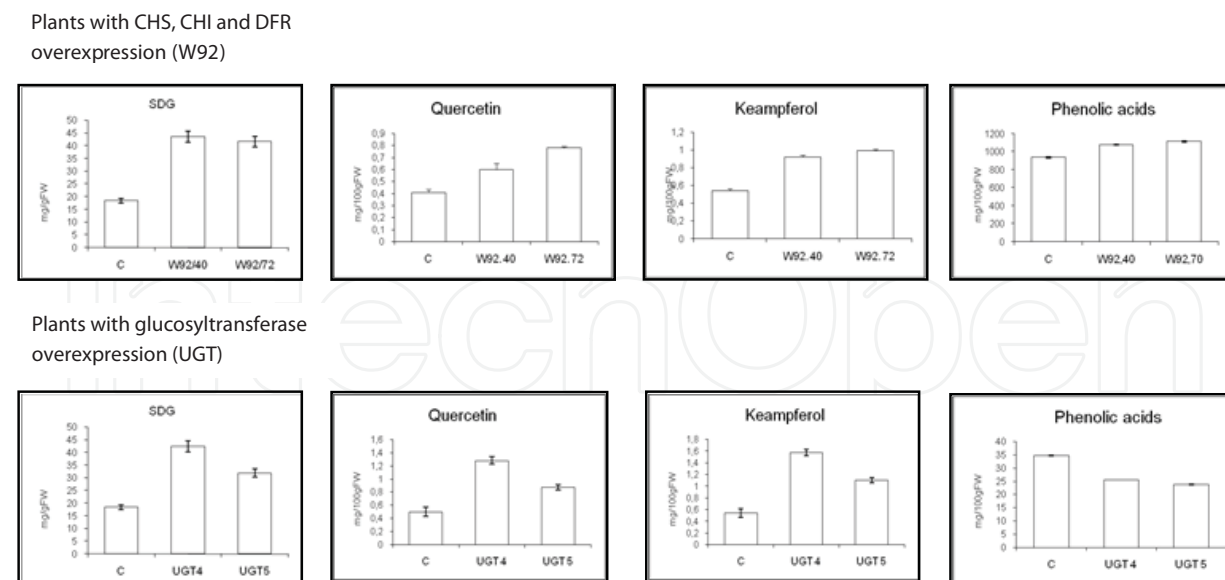
Plants with CHS, CHI and DFR
overexpression (W92)

Plants with glucosyltransferase
overexpression (UGT)

Fig. 8. The contents of compounds with potential biomedical applications in transgenic plants overexpressing genes from the phenylopropanoid pathway

Phytoestrogens such as lignans, which exhibit weak estrogenic and antiestrogenic properties in a tissue-specific manner, have potential in the prevention and treatment of breast cancer (Adlercreutz, H. & Herman, 2002). Flax seeds and their lignans have been reported to inhibit chemically induced mammary tumorigenesis at the preinitiation, early, and late promotion stages of carcinogenesis in rats (Thompson et al., 1996), and the growth and metastasis of human breast cancer in nude mice (Chen et al., 2002). The last published evidence also suggests that a dose of at least 500 mg SDG/day for approximately 8 weeks is enough to observe positive effects on cardiovascular risk factors in human patients. Flax seeds and their lignan extracts appear to be safe for most adult populations (Adolphe et al., 2010).

It is indicated that a diet rich in flavonoid compounds is strongly correlated with a lower risk of cancer (Ross and Kasum 2010). The compound with an especially higher anti-cancer action is quercetin, particularly in synergic action with keampferol (Brusselmans et al., 2005). In both prostate and breast cancer cells, a remarkable dose-response parallelism was observed between the flavonoid-induced inhibition of fatty acid synthesis, the inhibition of cell growth, and the induction of apoptosis. The dose which caused a reduction of the cancer cell proliferation to 19% is 100 µM quercetin; 100 µM kaempferol caused a reduction to 32% (Brusselmans *et al.*, 2005).

3.2 Flax with elevated levels of terpenoids

Terpenoids, also called isoprenoids, are group of about 40000 compounds. The group contains both primary and secondary metabolites of great significance for the growth and development of plants. Gibberellic acid, abscisic acid (phytohormons), carotenoids, chlorophyll, and ubiquinone can be distinguished among the primary metabolites (Aharoni *et al.*, 2008). The classification of terpenoids is based on the number of 5-carbon isoprene units, which compose the backbone of the molecule. Hemi-, mono-, sesqui-, di-, tri- and tetraterpenes correspond to 5-, 10-, 15-, 20-, 30- and 40-carbon terpenoids (Saremi and Arora, 2009). In plants, the isoprene units are produced from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which may originate from different pathways (Fig. 9).

The mevalonate pathway occurs in the cytosol, and was discovered first. IPP synthesis begins with transformation of 2 molecules of acetyl-CoA to 3-Hydroxy-3-Methyl-Glutaryl-CoA (HMG-CoA), which is subsequently converted by HMG-CoA reductase to mevalonate. The mevalonate undergoes phosphorylation to mevalonyl phosphate (catalysed by mevalonyl kinase), and then to mevalonyl diphosphate (catalysed by mevalonyl phosphate kinase). Isopentenyl pyrophosphate (IPP) is formed *via* the action of mevalonyl phosphate decarboxylase. This is next transformed by isopentyl isomerase to dimethylallyl pyrophosphate (DMAPP) (Siemieniuk & Skrzydlewska, 2005). It is suggested that this pathway is the source for 15- and 30-carbon terpenoids (squalene, sterols, brassinosteroids). The second, non-mevalonate pathway was discovered 10 years ago, and some of its steps are not fully known. It takes place in the plastids and is responsible for the synthesis of 10-, 20- and 40-carbon terpenoids (gibberellins, carotenoids, chlorophyll, tocopherol). The substrates are pyruvate and glyceraldehyde 3-phosphate, which are converted by DXS synthase to 1-deoxy-D-xylulose 5-phosphate (DXP). DXP is then transformed by DXR reductase into methylerytritol phosphate (MEP). The final step is the reduction of MEP to isopentenyl phosphate (IPP) (Cheng et al., 2007).
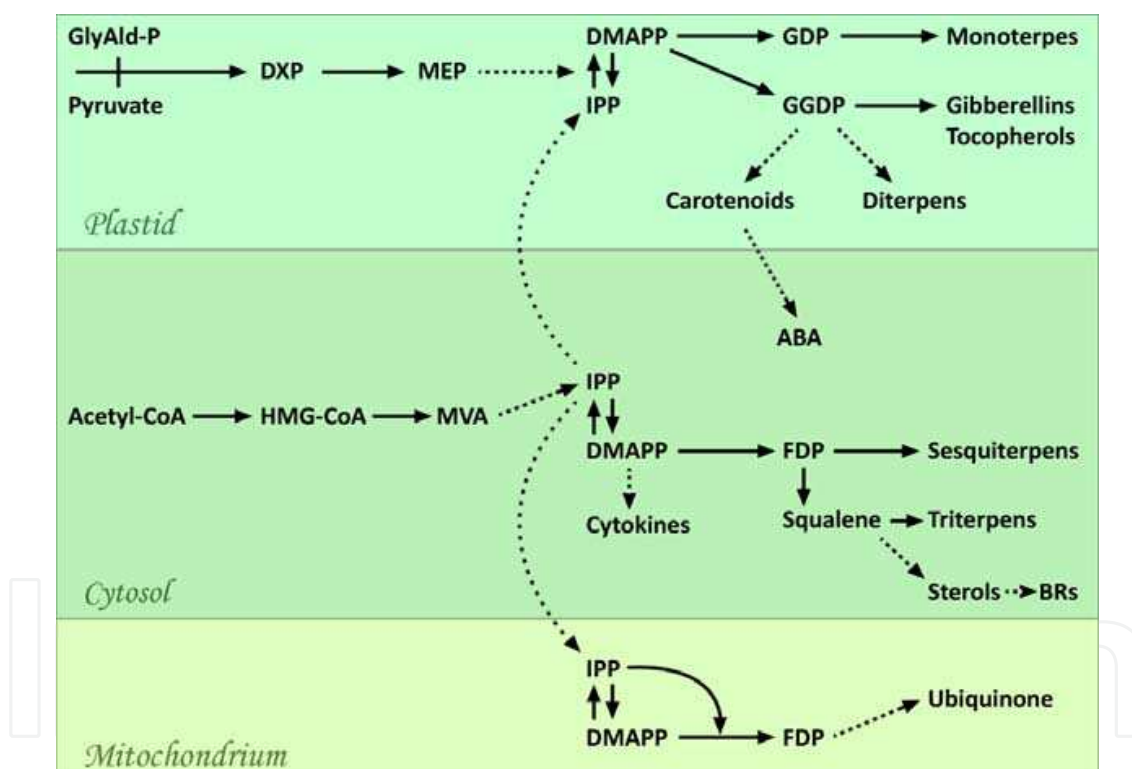

Fig. 9. The pathway of terpenoid biosynthesis

In the second step of terpenoid synthesis, 5-carbon molecule IPP and DMAPP undergo condensation due to the activity of prenyltransferases. The combination of one IPP and one DMAPP molecule yields geranyl diphosphate (in a reaction catalysed by GDP synthase), which is a precursor for monoterpenes. Two IPP molecules associated with DMAPP are transformed to farnesyl diphosphate (catalysed by FDP synthase), which gives a 15-carbon precursor of sesquiterpenes and triterpenes. Three molecules of IPP connected to DMAPP give geranylgeranyl diphosphate (catalysed by GGDP synthase), which is a precursor of the 20- and 40-carbon compounds. The last step of terpenoid synthesis comprises the

transformation of prenyl diphosphates (GDP, FDP and GGDP) by the appropriate terpenoid synthases (e.g. squalene synthase) (Cheng et al., 2007).

In plants, terpenoids play mainly protective roles against various environmental stresses. Many of them possess antioxidative properties (carotenoids, tocopherols), bacterio- and mycostatic properties (menthol, camphor), and recently, anti-neoplastic and anti-inflammatory properties are frequently reported (squalene, tocopherols, carotenoids, taxol) (Peltier et al., 2006). The ability to quench free radicals produced both during normal cell functioning, as well as under stress conditions, also contributes to the inhibition of many free radical-based diseases, including neoplasms (Grassmann, 2005; Gershenzon and Dudareva, 2007). The above-mentioned properties of terpenoids make them attractive dietary supplements.

Stress, pollution, lack of movement and bad nutritional habits cause many diseases, including neoplasms. Pharmacies offer a plethora of vitamin preparations, but they are mainly composed of substances obtained *via* chemical synthesis. The newest results indicate that the assimilability of compounds obtained from natural sources is much higher. Therefore, instead of "swallowing" more pills, nutritionists advise the consumption of products rich in natural bioactive compounds. Without a doubt, linseed oil is such a product. It constitutes the main source of omega-3 and omega-6 fatty acids. A drawback of this oil is its lability, caused by the presence of highly unsaturated fatty acids. Enriching it in natural substances increases its stability (tocopherol, carotenoids) and improves its health-oriented properties (squalene), expanding its potential for use in prophylaxis against many diseases, including diseases of the circulatory system and eyesight. This may make flax more competitive in relation to imported oils. An innovative product with a broad spectrum of action will result from such modifications.

Carotenoids are a group of over 700 compounds of yellow or orange colour. The majority are tetraterpenes consisting of 8 isoprene units. The molecule consists of 40 carbon atoms. The human organism is unable to synthesize carotenoids, and thus must intake them with nutrients. Studies prove that a diet rich in these compounds, provided they are of natural origin, lowers the risk of several diseases, particularly neoplasms and eyesight diseases. The anti-cancer character of carotenoids is based on different mechanisms, such as their antioxidative activity, strengthening of the immunological system, stimulation of gap junction formation in intracellular communication, induction of detoxification enzymes, and inhibition of cell proliferation. The anti-cancer activity of carotenoids was mainly observed and studied in the case of lung cancer (β-carotene), prostate cancer (lycopene), gullet and larynx cancer (lycopene), stomach cancer (lycopene), and leukemia (β-carotene). Carotenoids are mainly accumulated in the testicles, epididymides and liver (mostly β-carotene and lycopene). Without a doubt, the best-known benefit of carotenoid intake is the fact that about 50% of them can be converted into vitamin A, and the most prominent pro-vitamin activity is shown by β-carotene. Vitamin A is transformed to retinen, which is an indispensable component of the retinal pigments, and which conditions normal sight. Intake of nourishment containing those carotenoids protects the retina from degradation and diseases connected with age (Johnson, 2002).

Carotenoids are insoluble in water, but they dissolve very well in fats. The production of flax plants with an elevated carotenoid content was recently described (Fujisawa *et al.*, 2008). Flax was successfully transformed with the phytoene synthase gene (*crtB*) derived from the soil bacterium *Pantoea ananatis* under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The genetically modified flax produced orange seeds with a high

accumulation of phytoene, α- and β-carotene. The carotenoid amounts in the seeds reached 63.4 to 156.3 μg/g of fresh weight, which was a 7.8 to 18.6-fold increase in comparison to non-transformed plants (Fujisawa *et al.*, 2008). It is suggested that the flux of phytoene synthesis from geranylgeranyl diphosphate was first promoted by the expressed crtB gene product, and then the phytoene was decomposed to the downstream metabolites as α- and β-carotene and lutein. It is expected that oil obtained from the seeds of those plants will contain an elevated pool of carotenoids. This will improve the stability and quality of the oil, rich in highly unsaturated fatty acids. *In vitro* studies showed that the addition of β-carotene to oils protects unsaturated fatty acids from oxidation. The oil is characterized with an elevated amount of vitamin A and lutein, and is a potential innovative product.

Squalene is a triterpene obtained via the mevalonate pathway. It is also a precursor for cholesterol biosynthesis (Reddy and Couvreur, 2009). For commercial purposes, it is acquired mainly from shark liver oil, though there are also plants rich in this compound, e.g. amaranth seeds, rice bran, wheat germs and olives. Its direct anti-tumour activity is based on inhibiting the catalytic activity of β-HMGCoA. Although it is a weak inhibitor of tumour-cell proliferation, it contributes directly or indirectly to cancer treatment by strengthening the influence of antigens, which bind to it. Thus, it is widely used in vaccines and medicines (Kelly, 1999). Squalene diminishes the side effects of chemotherapy (Reddy and Couvreur, 2009). In addition, it normalizes the level of cholesterol in blood.

Introducing a gene of lycopene β-cyclase from *Arabidopsis thaliana* to flax caused homological repression and lowered carotenoid content. At the same time, it redirected the substrates to produce the remaining terpenoids. The plants generated in this way turned out to be more resistant to *Fusarium* infection (unpublished data), which is an additional argument for the cultivation of this transgenic type.

Through the repression of carotenoid synthesis, flax with an elevated squalene content was obtained (Table 1). The seeds of the transformed flax (L plants) are richer in squalene (data not published). An important aspect of this modification is not only that plants rich in a health-oriented compound are obtained, but also that a new strategy can be proposed for the modification of the metabolism in a preferred direction *via* repression of a pathway or pathways which are not desired. This strategy is justified by the fact that repression of a gene in plants is much easier than its activation, as only a short DNA fragment (100-200 bp) homologous to the endogenous gene is required.

Terpenoid	Linola	L9	L18
Squalene	100%	80%	192%
Tocopherol alpha	100%	136%	217%
Tocopherol beta	100%	170%	818%
Gibberellic acid GA3	100%	85%	182%
Menthol	100%	156%	141%
β-carotene	100%	62,5%	50%
lutein	100%	98%	80%

Table 1. Percentage level of different terpenoids in flax plants with repression of lycopene beta cyclase gene (named L)

3.3 Modified flax oil as a precious source of unsaturated fatty acids

An increasing coefficient of morbidity of civilization diseases such as obesity, atherosclerosis, heart disease and hypertension was observed with the development of industry and the economy. The Western diet is characterized by high intakes of animal fat, saturated acids, omega-6 acids, and *trans*-fatty acids, and this contributes to civilization diseases. The consumption of flax seeds is suggested to be beneficial for human health. Flax seeds have about 40% fat in dry weight (Łukaszewicz et al., 2004). Linseed oil consists of about 16% linoleic acid and 54% linolenic acid, and is the richest source of these polyunsaturated fatty acids (PUFA), (Table 2).

Fatty acids	Olive oil	Rapeseed oil	Soybean oil	Sunflower oil	Corn oil	Grapeseed oil	Linseed oil
14:0	0	0	0.11	0.08	0	0	0
16:0	11.46	4.68	10.62	6.66	10.1	6.79	5.06
16:0	0.96	0	0.09	0.08	0	0.10	0
18:0	2.20	2.36	3.76	4.27	1.6	3.63	3.73
18 : 1 n-9	68.76	57.14	21.67	24.2	31.4	17.8	19.68
18 : 1 n-7	0	3.40	1.61	0.58	0	0	0.68
18 : 2 n-6	10.51	21.16	55.07	63.65	56.3	65.9	16.21
18 : 3 n-3	0.67	11.25	6.89	0.19	0.4	0.38	54.52

Table 2. The fatty acid composition of seed oils (%) in different plant oils
(http://www.biuletynfarmacji.wum.edu.pl/0501Jelinska/0Jelinska.html)

PUFA are divided into two main groups: omega-6 and omega-3. Their precursors are linoleic (LA) and α- linolenic acids (ALA), respectively. LA and ALA are numbered among essential fatty acids because they cannot be synthesized in mammals. LA and ALA delivered in food play an important role in the correct development and functioning of the human body. They are structural components of the cell membranes and substrates in the biosynthesis pathway of arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and their eicosanoids (Fig. 10). Biosynthesis of omega-6 and omega-3 fatty acids is catalyzed by the same enzymes. Through the conversion by desaturase and elongase enzymes, LA is transformed to arachidonic acid (AA), and ALA to eicosapentaenoic acid (EPA), and then to docosahexaenoic acid (DHA). Supplementing the diet with ALA promotes the omega-3 pathway, leading to the inhibition of synthesis of arachidonic acid and eicosanoids derived from AA, which stimulate inflammation (Muir and Westcott, 2003).

Numerous epidemiological and experimental studies have shown the positive effects of omega-3 fatty acids on the reduction in risk of cardiovascular disease, stroke and atherosclerosis. They revealed anti-cancer and anti-inflammatory activities (Saravanan et al., 2010).

PUFAs, especially α- linolenic acid, are susceptible to oxidation, which causes a change in taste and odour, and causes problems with long-term storage. A mutation in the *FAD3* genes, responsible for the conversion of linoleic acid to linolenic acid in flax, was achieved in order to minimize the oxidation processes. This led to a new type of flax seeds, described as the solin type (low α- linolenic acid content), which are suitable for the production of edible oil (Rowland, 1991). The mutation induced with methane sulphonate (EMS) resulted in a

stable low linolenic acid mutant. The low linolenic character is controlled by recessive alleles at two independent loci, which are the result of a double mutation.
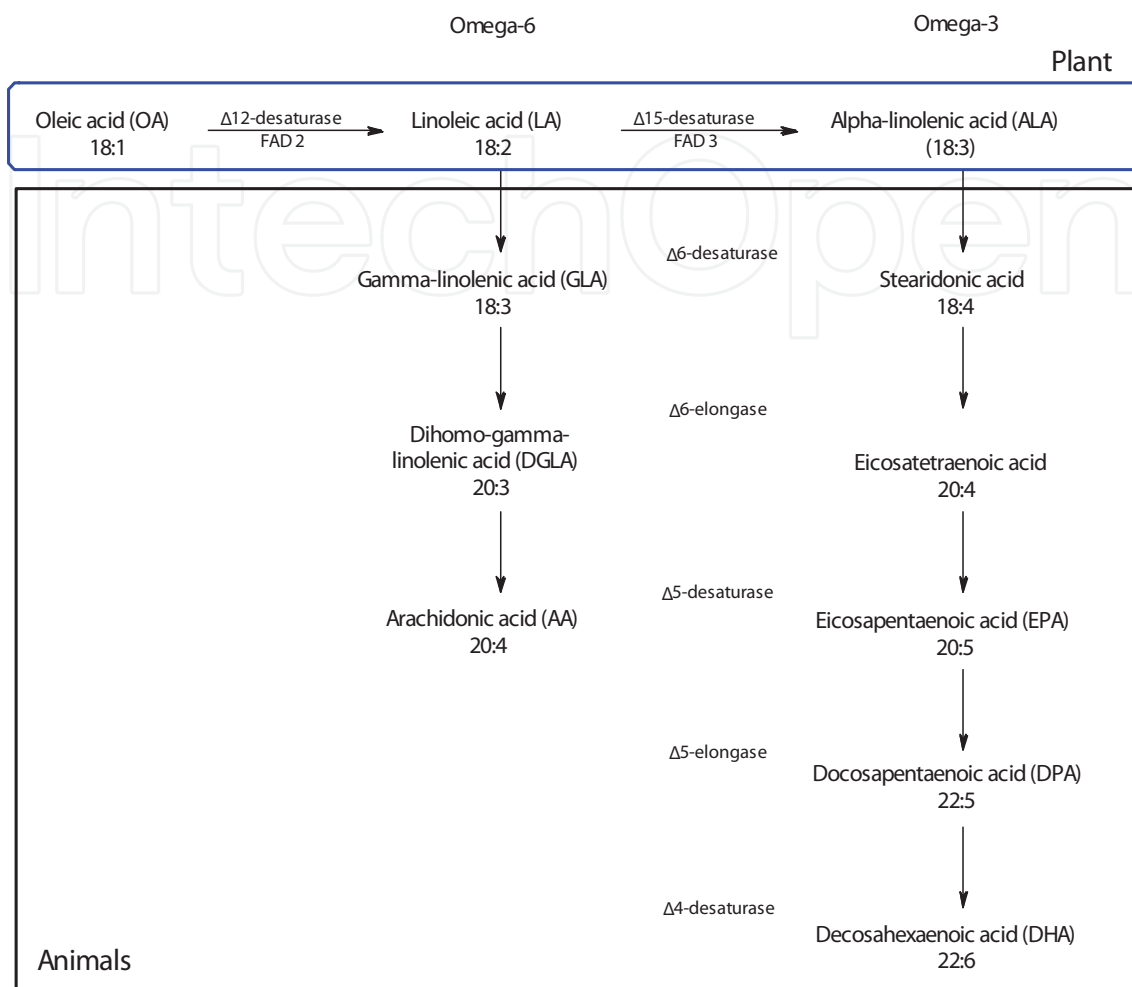

Fig. 10. The metabolic pathway of polyunsatured fatty acids. Linoleic acid and alpha-linolenic acid are the respective parents for the omega-6 and omega-3 fatty acids. They are synthesized in flax seeds by desaturation catalysed by a set of enzymes coded by the *FAD2* and *FAD3* genes

During the cold extraction of oil most of the natural antioxidants are lost. It is desirable to find a technology of oil production with improved oxidative protection. One possibility is oil supplementation with natural antioxidants. This supplementation was performed on flax seeds *via* various transgenic modifications. One of these modification was transformation with the *Solanum sogarandinum*-derived gene (designated SsGT1) coding for glycosyltransferase (UGT) under a seed-specific napin promoter. UGTs are large group of enzymes found in all living organisms which catalyse the transfer of a nucleotide diphosphate-activated monosaccharide unit (glucose, rhamnose, galactose, xylose, rutinose, and neohesperidose) to an acceptor molecule. Plant UGTs play a role in developmental and metabolic homeostasis, and also take part in detoxification processes. It is known that the glycosylation of low molecular weight compounds such as flavonoids makes the molecules more stable, increases their solubility in the vacuole, and can enhance the pathogen resistance of plants. Flax plants overexpressing SsGT1 accumulated higher levels of

glucosylated flavonoids in the seeds, mainly the kaempferol and quercetin glycoside (Lorenc-Kukuła et al., 2009). Overproduction of SsGT1 in transgenic flax (named UGT plants) also resulted in proanthocyanin, phenolic acid and unsaturated fatty acid accumulation in the seeds. The linseed oil of UGT plants was examined *via* gas chromatography, which revealed an increase in unsaturated 18:1 (11-14%), 18:2 (9.5-24.7%), and 18:3 (23.7-54.5%) fatty acids. The antioxidant compounds are hydrophilic in nature. They accumulated in seeds, partially cross to the oil phase. Moreover, their level changes during the oil purification processes and remains the highest if cold-oil pressing is used. Various hydrophilic and hydrophobic compounds can stabilize linseed oil depending on their concentration, structure and proportions. The antioxidant compounds found in UGT plants were able to improve oil storage. It was observed that the rate of conjugated diene and aldehyde formation in the heated oils from transgenic seeds was lower than in the oil of the control plants (data not published). A similar positive effect on oil storage was achieved in flax oil derived from plants overexpressing three genes of the flavonoid route: *chs*, *chi* and *dfr*. As described in section 2.1 (Manipulation of flax genes of phenylopropanoid pathway), W92 transgenic flax was characterised by an elevated level of flavonoids and by an increased antioxidant capacity of the seeds (Lorenc-Kukuła et al., 2007). The accumulation of antioxidants in W92 plants had a positive effect on oil storage (Prescha et al., 2008).

Among the fatty acids that belong to the omega-3 group, EPA and DHA have beneficial effects on health. These PUFAs mostly occur in fish oil, but a drop in the quantity, and the contamination of this source has been continuously observed. A diet rich in ALA elevates erythrocyte EPA and DPA concentration in humans (Barceló-Coblijn et al., 2008). Another alternative was the expression of enzymes, such as Δ6-desaturase, Δ6-elongase, and Δ5-desaturase, which participate in biosynthesis of very-long-chain polyunsaturated fatty acids in flax. In spite of the high level of accumulation of C18 desaturated fatty acids, these products were channeled to triacyloglycerols, which led to a lack of substrates for Δ6-elongase. Only 5% C20 polyunsaturated fatty acids were obtained (Abbadi et al., 2004).

The oxidative stability of linseed oil is still the main goal to achieve. The high production and accumulation of very long-chain polyunsaturated fatty acids in linseed oil, especially EPA and DHA, is the next challenge.

Variation in oil lipid composition of the different flax cultivars is presented in Table 3.

It is worth mentioning the geometric and positional isomers of linoleic acid, the conjugated linoleic acids (CLA). The main sources of CLA are dairy products and the fat tissue of ruminant animals. Studies indicate that CLA has anti-cancer, anti-atherosclerotic, anti-diabetic, anti-inflammatory, and anti-obesity activities (Wahle et al., 2004). Linseed oil is still underestimated as a valuable source of compounds that are beneficial for human health. The wide range of action of polyunsaturated fatty acids make linseed oil a desirable and helpful product in medical treatment.

3.4 New anti-inflammatory and analgesic compounds derived from flax

The most recent studies on flax products' properties have revealed many health-beneficial actions in aspects of different diseases. One of the latest investigated activities is the anti-inflammatory nature of flax plant and product extracts. This aspect of the flax plant studies was based on promising results of flax bandage use in the healing of chronic wounds of different etiologies. The direct reasons for chronic ulcerations are always connected to the inflammation state of the organism, so inflammation-related processes are very important in wound healing.

Cultivar/fatty acid	16:0	18:0	18:1	18:2	18:3	20:0
Voroezski	6.11	4.6	22.0	14.9	52.2	0.24
Jenny	6.91	5.7	19.7	14.2	52.1	0.36
La Estanzuela AR	6.28	4.1	17.7	14.1	57.5	0.46
Opal	5.43	3.73	18.2	15.3	56.1	1.22
Szafir	5.19	3.28	14.1	11.9	65.4	0.11
Abby	5.99	3.4	13.3	18.2	58.9	0.11
Linola	6.28	1.91	13.5	74.5	3.27	0.28
W92.40	6.03	3.37	15.94	70.92	2.25	0.14
W92.86	5.85	4.74	21.98	41.71	24.54	0.18
UGT/NAP	6.30	3.85	17.96	69.07	1.43	0.12

Table 3. Variation in oil lipid composition of the different flax cultivars (Łukaszewicz et al, 2004; Lorenc-Kukuła et al,2004; Lorenc-Kukuła et al, 2009)

There are many groups of compounds that can be partially responsible for flax bandage anti-inflammatory action, for example the previously mentioned groups of antioxidants, directly regulating the oxidation processes connected to inflammation. Among the different types of molecules, we chose the terpenophenols for further investigation, as some very interesting ones have recently been found in flax extracts. The new compound identification is based on UPLC retention time, UV spectra and GC-MS analysis in comparison to some terpenophenolic standards. The identification is still of a preliminary nature, but all the methods used confirm the presence of cannabinoid-like compounds in the examined extracts (results not published). Cannabinoids have never previously been discovered in any plants other than cannabis plants, so these results are very intriguing and interesting. Cannabinoids are a group of terpenophenolics that accumulate in considerable amounts in the glandular trichomes of *Cannabis sativa* (Cannabaceae). In cannabis plants, the biosynthesis of these terpenoids involves a common isoprenoid building block, isopentenyl diphosphate, while the biosynthesis of the phenyl part of the cannabinoid, olivetolic acid is connected to the polyketide pathway. Further alkylation of olivetolic acid with geranyl pyrophosphate leads to the formation of cannabigerolic acid (CBGA), the central precursor of various cannabinoids like tetrahydrocannabinol (THC), cannabidiol (CBD) or cannabichromene (CBC) (Sirikantaramas et al., 2007). The synthesis pathway of cannabinoids in cannabis plants is presented in Figure 11. All the precursors for the biosynthesis are present at relatively high levels in flax plants, so a similar pathway of synthesis is possible. Investigations of the activity of such a biosynthesis pathway are in progress, but there is still no evidence of its existence in flax.

The special properties of cannabinoids have been known and exploited for thousands of years for medical purposes, mainly as anti-inflammatory and pain- realising agents. There are many studies nowadays providing evidence for the biological activity of these compounds in pain relief and healing of many kinds of diseases, including neuropatic pain, multiple sclerosis, Alzheimer's disease, atherosclerosis, rheumatoid arthritis, asthma, many allergies and other inflammatory diseases including AIDS (Pacher et al., 2006). The inflammation state plays a crucial role in all the mentioned health issues and cannabinoids have been proven to have an influence on this process. Activation of specific cannabinoid receptors (CB1 and/or CB2) and the downstream signaling cascade inhibits the production
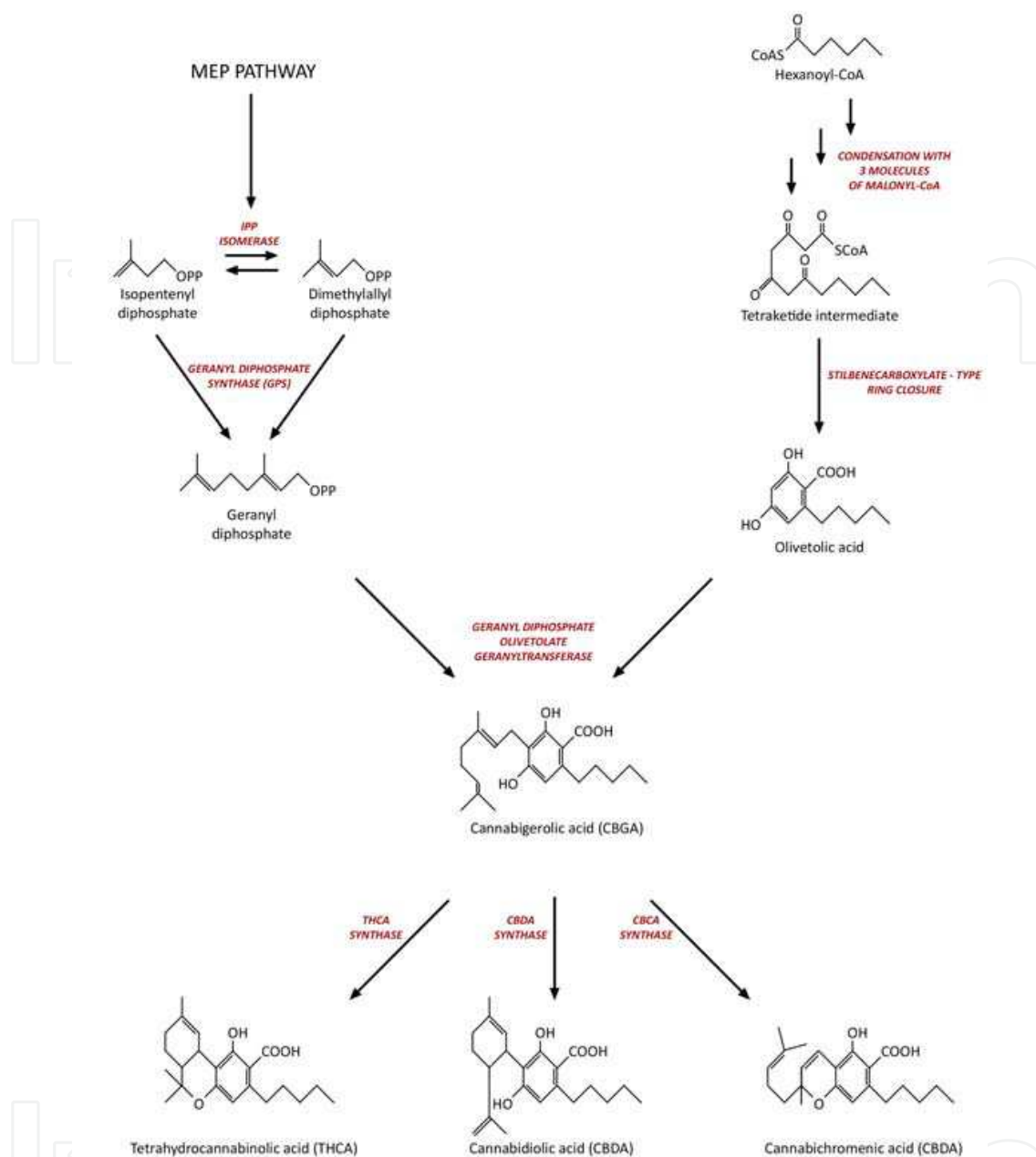
Fig. 11. The synthesis pathway of cannabinoids in cannabis plants

of cytokines and activates anti-inflammatory protein expression (Klein et al., 2000). The process of inflammation inhibition is presented in Fig. 12. This system, as the main target in new flax application, was the focus of examinations of the biological activity of the flax extracts containing cannabinoid-like compounds.

Real-Time PCR expression measurements of common cytokines level in both mouse and human fibroblast cell line experiments showed the inhibition of pro-inflammatory gene expression after flax cannabinoid-like extract treatments (results not published). Biological activity assays of the newly discovered compounds still need to be confirmed in many aspects, but the first results are very promising.

The existence, nature, biosynthesis pathway and activity of cannabinoids in flax requires much further investigation, but the discovered compounds give great new possibilities for

flax product applications. The experiments in this field will give the opportunity for manipulations of the type and level of the compounds produced, which would be of great significance in the medical application of the natural non-psychoactive anti-inflammatory and pain-relieving products derived from flax.
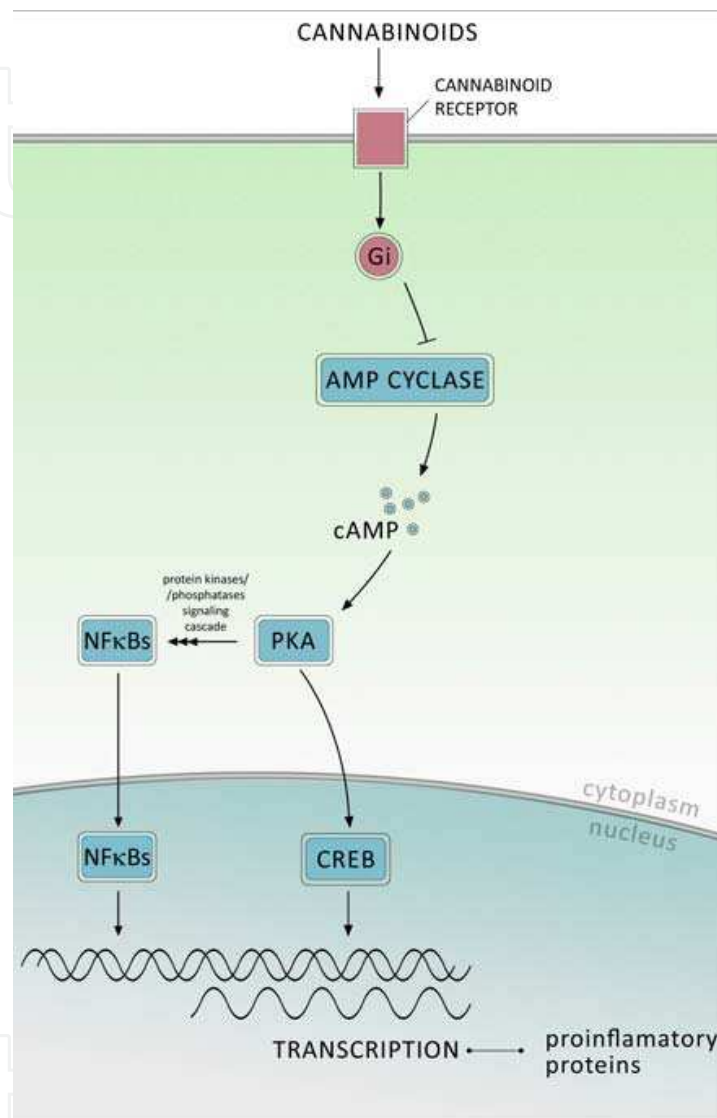

Fig. 12. The process of inflammation inhibition by cannabinoids through CB receptor

4. Conclusion

The interest in the medicinal, nutraceutical and industrial value of flax is still growing. The utility of this crop plant is not only restricted to edible oil and textile fibre production. Genetic manipulation enabled its use to be broadened in many fields, but mainly in medicine and human health. The research is not only focused on unsaturated fatty acids but also on precious antioxidants such as phenylopropanoids, lignans and terpenoids. Many new transgenic lines of flax characterized by better properties of fibre and oil have been generated. Genetic engineering methods allow the manipulation of the desired genes to improve the traits of flax. As the transformation protocol of flax is well established and the

results are very promising, it is clear that genetically modified flax has a future in medical applications.

5. Acknowledgment

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