



UNIVERSITÀ DEGLI STUDI DI SALERNO



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***Hairy root culture as source of novel  
plant-derived active compounds with  
applications in Cosmetics***

Settore scientifico disciplinare di afferenza: *AGR/07*

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*"Science is organized knowledge.  
Wisdom is organized life"*

*Immanuel Kant*



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**Abstract**

Hyperpigmentation is the process by which an excess of melanin is produced by the skin. Typically, hyperpigmentation occurs as a result of stress, damage or prolonged inflammation of the skin. The most common cause is sun damage, though hyperpigmentation is often a consequence of inflammation following acne, eczema, psoriasis, dermatitis etc. Hyperpigmentation may also occur in the skin due to hormonal changes in the body typically associated with pregnancy or the taking of oral contraception. Beside this medical aspects, the global skin depigmenting product market has been forecast to reach a value of \$19.8 billion by 2018, driven by the growing desire for light-coloured skin among both men and women primarily from the Asian, African and Middle East regions. Although products do exist that can actually bleach the skin, these products contain dangerous or toxic ingredients (such as hydroquinone and mercury) and are banned in most countries.

Blocking or reducing the accumulation of melanin in the skin can be obtained either by switching off one or more components of the pathway that go from the receptor activation to the enzymatic inhibition of melanin formation catalyzed by the tyrosinase. For this purpose, several antimelanogenic reagents have been developed and discovered nowadays. However, only a few of these inhibitors have been introduced and used due to their problems in cytotoxicity (affecting the cell growth and survival), selectivity, solubility and stability.

The present project was aimed at identifying new total plant extracts exerting beneficial effects in skin care, with special emphasis on the development of novel plant-derived actives with hypopigmenting effects. Experimental activities were carried out in collaboration with Arterra Bioscience S.r.l, in the frame of the programme "Dottorato di Ricerca in Azienda", funded by European Commission and Regione Campania (POR Campania FSE 2007-2013). Arterra is an Italian research-based Biotech company mostly involved in developing new plant-derived extracts to be used as active ingredients with cosmetic application.

Hairy root cultures of three different plant species (*Cichorium intybus*, *Brassica rapa* subsp. *pekinensis* and *Helianthus annuus*) were generated. Hairy roots of *Brassica rapa* subsp. *pekinensis* were selected for further studies on the base of a preliminary screening for anti-oxidant activity of a total crude ethanol extract and a sugar/peptides mixture derived from cell walls, coupled to an active growth.

Crude ethanol extract and a sugar/peptides mixture derived from cell wall of *Brassica rapa* subsp. *pekinensis* hairy roots were tested in murine melanoma cells (B16-F1) and human epidermal melanocytes isolated from lightly pigmented adult skin (HEMa-LP), by using a panel of *in vitro* and *in vivo* biological assays to assess their role in modulating melanogenesis. Both extracts at different concentrations demonstrated to inhibit the cellular tyrosinase, a key enzyme in melanin production, and to reduce melanin content in murine melanoma cells. In addition, the sugar/peptides mixture of *Brassica rapa* subsp. *pekinensis* hairy roots significantly inhibited the levels of cyclic adenosine monophosphate (cAMP), an important second messenger within melanogenesis signalling pathway. Furthermore, the same extract significantly decreased the expression of microphthalmia-associated transcription factor (MITF) and its promoter activity of about 30%, analyzed by *in vitro* reporter (*luc+*)-assay. Altogether these data indicates that the sugar/peptides mixture isolated from cell wall of *Brassica rapa* subsp. *pekinensis* hairy roots might exert its inhibitory effect on melanogenesis through the downregulation of MITF transcription.

## ***Abstract***

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Furthermore, *Brassica rapa* subsp. *pekinensis* ethanol extract was able to enhance the expression levels of important genes encoding for proteins involved into extracellular matrix (ECM) assembly.

Finally, a competitive industrial production hairy-root based platform was developed by *Brassica rapa* subsp. *pekinensis* hairy root biomass scaling-up and improved extraction procedures.

Overall, these results, under pending patent application, will contribute to introduce product and process innovations at Arterra Bioscience s.r.l, for the identification of new and safer plant-derived melanogenesis inhibitors. In general, the developed industrial production platform will be also extended to the screening of actives from other plant species and to the release of novel plant-derived products in different segments of the cosmetic market.

# **Introduction**



## 1. Introduction

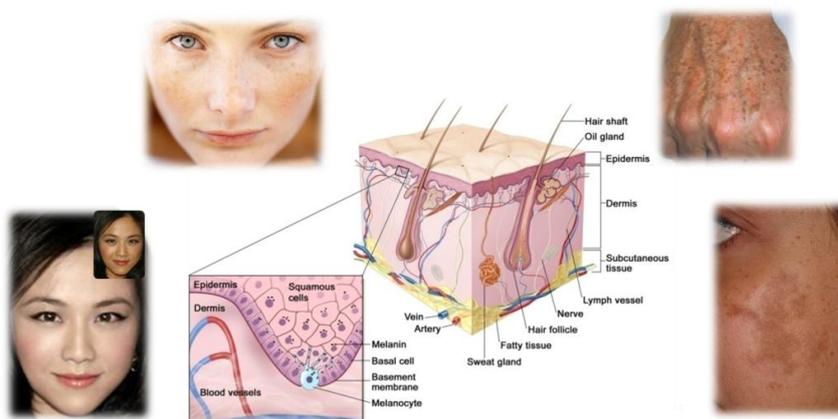
In the increasingly ageing population with significant cumulative sun exposure, the control of hyperpigmentation and depigmentation is not only a significant clinical, but also a cosmetic challenge (Ortonne *et al.*, 2008). In fact, as reported by Łopaciuk and Łoboda (2013), the global market for skin lighteners is projected to reach \$19.8 billion by 2018, driven by the clear preference for bright skin emerged especially from market analyses in East Asian societies.

According to GIA's research (Gandr  International Aesthetics), fairness products account for over 45% of the Asian skin care market and are continuing to grow at double-digit rates annually, especially in that part of the world where the concept of a bright skin is related to old beauty beliefs and models.

Asian habits of making more clear one's skin are directly related to the desire to achieve higher social status, indeed a person's skin color has been historically a clear indicator of economic and social status, insomuch as there was a tight correlation between wealth and skin color. It was easy to distinguish farm laborers darkened by working in the sun from the Chinese upper class able to stay indoors and avoid sun damage. The historic correlation between economic status and skin color naturally lead to the development of a variety of treatments in order to correct an "undesirable" dark skin tone (Ortonne *et al.*, 2008).

On the other hand, skin lightening represents a relatively smaller market in the West when compared to Asia. However, hyperpigmentation occurs as a result of sun damage or prolonged inflammation of the skin following acne, eczema, psoriasis, dermatitis etc (Callender *et al.*, 2011). Hyperpigmentation may also occur in the skin due to hormonal changes in the body and dyspigmentation is also viewed as a prominent marker of facial skin ageing (Matts *et al.*, 2007) (see Fig.1).

Therefore, for all these reasons, there is an increasing demand of novel safe and effective plant-derived agents that could modulate skin pigmentation.



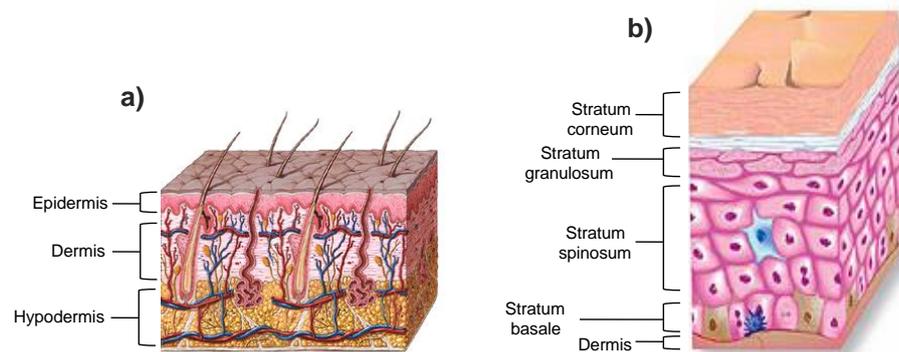
**Figure 1. Skin appearance and disorders.** Melanin pigment is produced by melanocytes in the epidermal layer of the skin. Beyond the high demand for a bright skin aspect, emerged from the Asian market, depigmenting cosmetic products are used to correct several blemishes ranging from solar freckles to post-inflammation spots (<sup> </sup>2008 - Terese Winslow).

### 1.1 The skin

The skin, through its layers represented by the epidermis, the dermis and the hypodermis, plays an extremely important role providing a physical barrier against mechanical, microbial and chemical factors that may affect the physiological status of the whole body (Ortonne *et al.*, 2008) (**Fig.2a**). In addition, to these functions, the skin also acts as an immune network and, through its pigments, provides a unique defense system against UV radiation (UV-R) (Callender *et al.*, 2011). Thus, melanocytes transfer melanosomes through their dendrites to keratinocytes, where they form the melanin caps that reduce UV-induced DNA damage in human epidermis.

The **epidermis** represents the outermost, stratified epithelium devoid of blood or nerve supplies of about 5-100  $\mu\text{m}$  thickness (Costin *et al.*, 2007), and it is characterized by several distinct cell populations. In particular, keratinocytes and melanocytes are the main constituents, of which the first comprise about 95% of the epidermis, while melanocytes represent a very minor population of cells specialized in the synthesis and distribution of the pigmented biopolymer melanin. As shown in **Fig. 2b**, the cells are arranged in four layers:

- *Stratum basale* is a single layer of cells attached to a non cellular basement membrane, that separates the epidermis from the dermis, and consists mostly of basal keratinocytes, which have stem cell-like properties, and at least two different types of neural crest-derived cells: Merkel cells and melanocytes;
- *Stratum spinosum* contains irregular polyhedral keratinocytes with some limited capacity for cell division. Also found here are Langerhans' cells, which represent the antigen-presenting cells of the skin and play a vital role in immunological reactions such as allergic contact dermatitis;
- *Stratum granulosum* or granular layer contains non dividing keratinocytes migrating from the underlying *stratum spinosum* and producing keratohyalin granules, a protein that increase in size and number as the cell nuclei gradually degenerate and the cells die;
- *Stratum corneum* represents the outermost layer of epidermis and it contains dead but biochemically active cells called corneocytes. The keratinocytes continue to differentiate as they move from the innermost layer to the *stratum corneum*, resulting in cornified cells that contain abundant filamentous keratin. These cornified cells provide a barrier against the physical and chemical agents present in the environment that may adversely affect the body. More specifically, the epidermal barrier functions to reduce trans-epidermal water loss from within and to prevent invasion by infectious agents and noxious substances from without (Matts *et al.*, 2007).



**Figure 2. Structure of the skin.** a) Anatomy of the skin, showing the epidermis, dermis and subcutaneous tissue. b) Morphological characteristics of the four different layers within the epidermis.

The **dermis** is a thin layer of connective tissue and fibroblasts, localized between the epidermis and subcutaneous tissues, which houses the neural, vascular, lymphatic, and secretory apparatus of the skin. It is composed of three major types of cells: macrophages, adipocytes and fibroblasts; the latter one represent the main cell type and is required for synthesis and degradation of the extracellular matrix (ECM) (Ortonne *et al.*, 2008), a complex structure composed of highly organized collagen, elastic and reticular fibers. Furthermore, in this layer are localized multifunctional cells of the immune system that are able to trigger allergic reactions by secreting bioactive mediators such as histamine.

In the dermis, **collagen** provides the skin with tensile strength and tissue integrity, whereas **elastin** provides elasticity and plasticity. Besides collagen and elastic fibers, the dermis contains the extrafibrillar matrix composed of a complex mixture of proteoglycans, glycoproteins, water and hyaluronic acid. Glycoproteins, such as laminins, fibronectin, etc., are involved in cell adhesion, cell migration, and cell-cell communication, which are extremely important processes taking place in the skin (Costin *et al.*, 2007).

The innermost layer of the skin is represented by **hypodermis**, the thickest part immediately above dermis, and attached to the latter by collagen fibers. It is composed in the majority of specialized cells grouped together in lobules separated by connective tissue and known as adipocytes. The hypodermis acts as an energy reserve given that fats contained in the adipocytes can be put back into circulation during intense efforts or when there is a “lack of calories”, and at least it is involved in thermoregulation thanks to the insulating capacity of fat.

Whilst the hypodermis is distributed over the whole body, the anatomical diffusion it seems to be clearly related to sexual characteristic: in fact, it basically accumulates over the abdomen and shoulders in men, and around the thighs and hips in women.

## 1.2 Melanocytes: origin and development

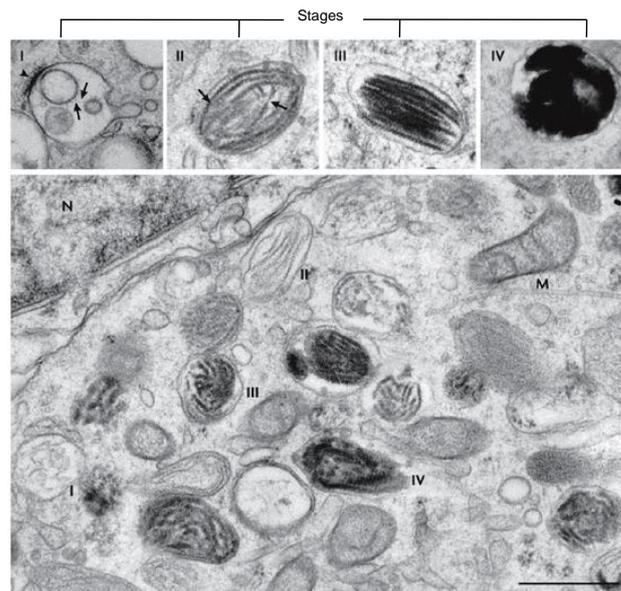
**Melanin** biosynthesis is a complex pathway that appears in highly specialized cells, called **melanocytes**, within membrane-bound organelles referred to as melanosomes (Van Den Bossche *et al.*, 2006). Pigment globules containing multiple melanosomes are released via various dendrites from melanocytes to surrounding keratinocytes, where they play a critical role in photoprotection.

The anatomical relationship between keratinocytes and melanocytes is known as “the epidermal melanin unit” and it has been estimated that each melanocyte is in contact with about 40 keratinocytes in the basal and suprabasal layers (Joshi *et al.*, 2007).

Several important steps must occur for the synthesis and distribution of melanin, starting from the development of melanocyte precursor cells (melanoblasts) and their migration from the neural crest to peripheral sites.

Melanoblasts derive from the neural crest beginning in the second month of human embryonic life and migrate throughout the mesenchyme of the developing embryo. They reach specific target sites, mainly the dermis, epidermis, and hair follicles. Once melanoblasts have reached their final destinations, they differentiate into melanocytes, which at about the sixth month of fetal life are already established at epidermal-dermal junction sites (Ortonne *et al.*, 2008).

Dermal melanocytes gradually decrease in number during gestation and virtually disappear by birth, whereas epidermal melanocytes established at the epidermal-dermal junction continue to proliferate and start to produce melanin. Once established *in situ*, melanocytes start producing melanosomes, highly organized elliptic membrane-bound organelles in which melanin synthesis takes place (Costin *et al.*, 2007).



**Figure 3. Morphological characteristics of melanosomes at their different stages.**

Melanosomes and their precursors can be classified into four stages of development based on morphology (Source: Howard *et al.*, 2003).

As shown in **Fig. 3**, classical electron microscopy studies of skin melanocytes allow to classify melanosomes into four maturation stages (I–IV) determined on the basis of the arrangement, quantity, quality of the melanin produced and by their structure (Tobin, 2006). Nascent melanosomes are assembled in the perinuclear region near the Golgi stacks, where they receive all enzymatic and structural proteins required for melanogenesis process.

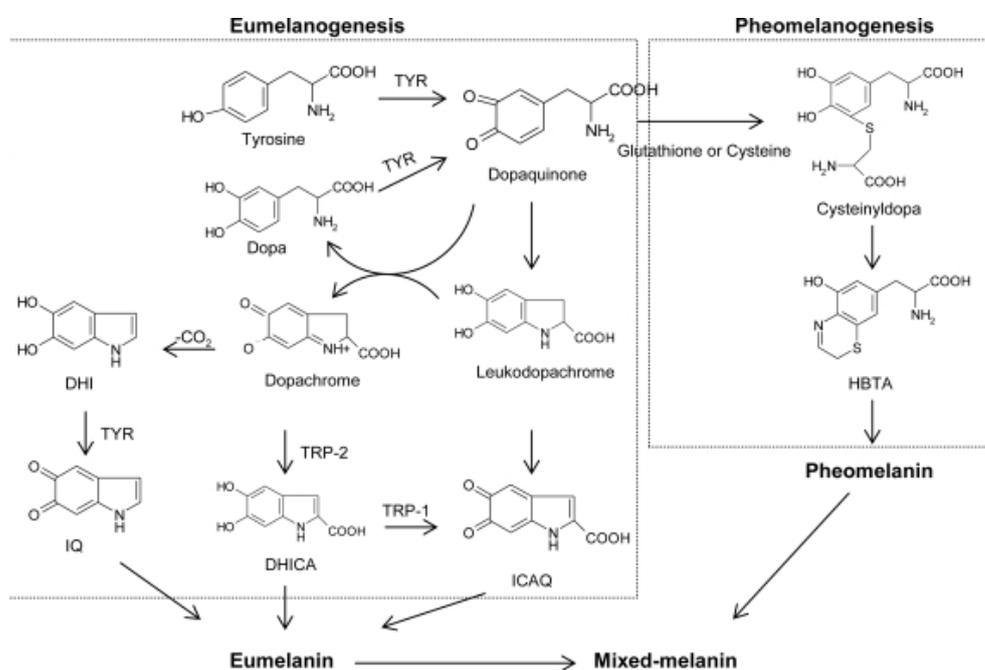
Stage I pre-melanosomes are non pigmented vacuoles that are derived from the endosomal system and lacking tyrosinase activity. These acquire characteristic internal striations and important structural proteins, determining passage to elongated organelles, known as stage II melanosomes. At this stage, melanosomes exhibit minimal deposition of pigment and contain the enzyme tyrosinase. Melanin pigment is deposited onto the striations (stage III), eventually giving rise to mature, fully melanised stage IV melanosomes with minimal tyrosinase activity.

The developmental stages detailed above refer mainly to eumelanosomes (containing black-brown pigments); however, they are quite similar to pheomelanosomes (containing yellow-reddish melanin), the only difference being that the latter remain round and are not fibrillar during maturation (Elias, 2005).

### 1.3 Melanogenesis pathway and extracellular matrix (ECM) organization

Within melanosomes, through a combination of both chemical and enzymatic reactions, melanogenesis occurs leading to the synthesis of the pigment **melanin**.

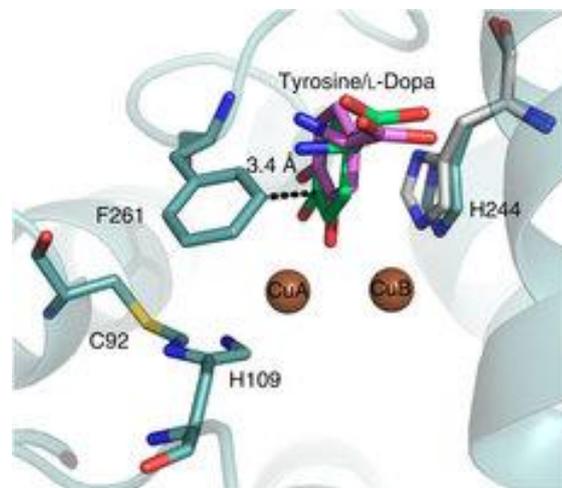
The biosynthetic pathway of melanogenesis has been elucidated and, as shown in **Fig. 4**, two types of melanin are synthesized within melanosomes: **eumelanin** and **pheomelanin** (Chang, 2009). The first step of melanogenesis is initiated by the key enzyme **tyrosinase**, which catalyzes tyrosine oxidation to dopa-quinone. This first step is the only rate-limiting step in melanin synthesis because the rest of the reaction sequence can proceed spontaneously at a physiological pH value (Slominski *et al.*, 2004). After dopaquinone formation by tyrosinase, the compound is converted to dopa and dopa-chrome through auto-oxidation. Dopa is also the substrate of tyrosinase and it is oxidized to dopaquinone again by the enzyme. Finally, the black pigment eumelanin is formed through a series of oxidation reactions from dihydroxyindole (DHI) and dihydroxyindole-2-carboxylic acid (DHICA), which are the reaction products from dopachrome. In the presence of cysteine or glutathione, dopaquinone is also converted to cysteinyl-dopa or glutathionyl-dopa, and the red-yellowish pheomelanin is subsequently formed (Chang, 2009).



**Figure 4. Biosynthetic pathway of melanin.** [TYR, tyrosinase; TRP; tyrosinase related protein; dopa, 3,4 dihydroxyphenylalanine; DHICA, 5,6 dihydroxyindole 2 carboxylic acid; DHI, 5,6 dihydroxyindole; ICAQ, indole 2 carboxylic acid 5,6 quinone; IQ, indole 5,6 quinone; HBTA, 5 hydroxy 1,4 benzothiazinylalanine]. (Source: Chang, 2009).

Even though the three enzymes tyrosinase, tyrosinase-related protein 1 and 2 (TRP1 and TRP2) are basically involved in the melanogenesis pathway, only tyrosinase is absolutely necessary for melanogenesis, due to its key role in the process. The enzyme is a glycoprotein located in the membrane of the melanosomes, with an inner melanosomal domain that contains the catalytic region, followed by a short transmembrane domain and a cytoplasmic domain. The well-known feature observed in tyrosinase is a conserved central copper-binding domain, which contains strictly conserved amino acid residues, including three histidines (**Fig. 5**). Only melanocytic cells produce tyrosinase, then following its synthesis and subsequent processing in the endoplasmic reticulum (ER) and Golgi, it is trafficked to melanosomes, wherein the pigment melanin is synthesized as before described (Chang, 2012).

Tyrosinase catalyzes the oxidations of both monophenols (monophenolase activity) and *o*-diphenols (diphenolase activity) into reactive *o*-quinones (Chang, 2009).



**Figure 5. Active site of the key enzyme tyrosinase.** An unambiguous picture of the interaction between tyrosine/L-Dopa and a model of the dicopper active site of tyrosinase is reported. Source: <http://www.uni-kiel.de/>

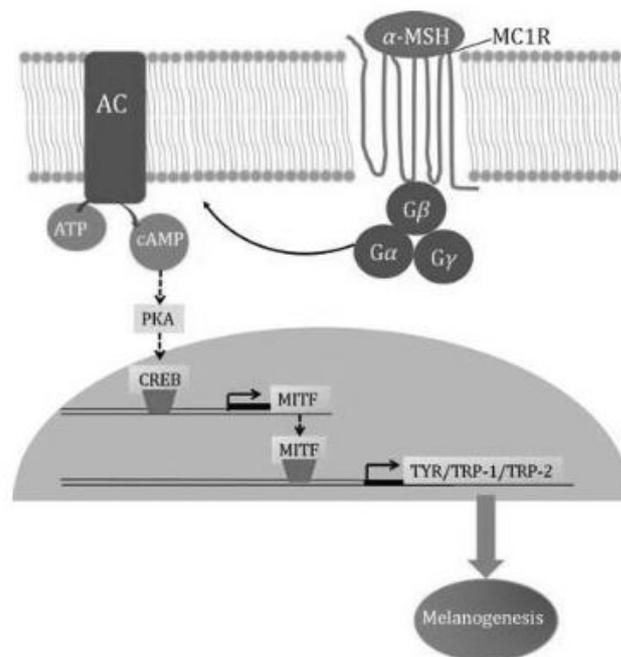
In mammals it is possible to control melanogenesis pathway at different levels, each one very complex (Chang, 2009). As described before, during their development, melanocytes are initially derived from the neural crest and migrate throughout the embryo to the target sites. The first level of melanogenesis regulation is exactly represented by genetic control of migration patterns. Melanin synthesis is also regulated at the cellular level controlling formation of melanosomes, which can be produced in varying numbers, sizes and densities depending on pigment content. Finally, it is possible to regulate melanogenesis at the subcellular level, where the gene expression encoded by the melanogenesis-related enzymes (including tyrosinase, TRP1 and TRP2) is regulated by intracellular pathways. These signal pathways are initiated by a variety of interleukins, interferons, growth factors, and prostaglandins, which determine not only the quantity but also the quality of the

## Introduction

synthesized melanin. The hormones provide the complex signals that respond to UV exposure or other environmental stimulations (Chang, 2012).

Signal pathways involve microphthalmia-associated transcription factor (MITF), which is a transcription factor with the structural domain of basic helix-loop-helix leucine zipper. MITF can regulate melanogenesis via binding to the M box of a promoter region and, subsequently, regulating the gene expression of tyrosinase, TRP-1, and TRP-2 (Gillbro and Olsson, 2011), but it is also involved in the differentiation, proliferation and survival of melanocytes.

As shown in **Fig. 6**, the up-regulation of MITF activity induces the expression of the melanogenesis-related enzymes, thus producing a final effect of enhanced melanogenesis process. In contrast, the down-regulation of MITF activity depresses the expression of the related enzymes, thereby inhibiting melanogenesis.



**Figure 6. The melanocortin signalling pathway.**  $\alpha$ -MSH binds to and activates the Gs protein-coupled MC1R. The G proteins transmit signals from MC1R to AC which, in turn, catalyses the conversion of cytoplasmatic ATP to cAMP. Increased levels of cAMP act as a second messenger to activate PKA. Phosphorylated CREBs then induce the expression of genes containing CRE consensus sequences in their promoters, such as the transcription factor MITF. The transcription factor MITF binds to the promoter of the pigimentary genes tyrosinase TRP-1 and TRP-2 (DCT).

(Source: Gillbro JM and Olsson MJ, 2011)

Alpha melanocyte-stimulating hormone ( $\alpha$ -MSH), a peptide derived from proopiomelanocortin (POMC), regulates melanogenesis via a cyclic adenosine monophosphate (cAMP)-dependent pathway (Lee and Noh, 2013).

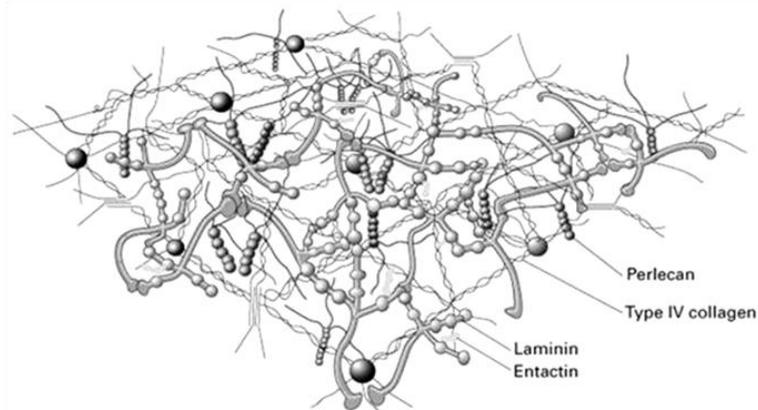
When binding to its receptor, melanocortin receptor 1 (MC1R), on the membrane of melanocytes, the hormone activates adenylatecyclase (AC) to produce cAMP as an intracellular second message via a G-protein-coupled receptor (GPCR)-type activation (**Fig. 6**).

The second messenger cAMP activates protein kinase A (PKA), which then activates the gene expression of MITF via phosphorylation of the cAMP response element-binding protein (CREB). Finally, MITF efficiently activates the melanogenesis-related enzymes and stimulates melanin synthesis. The binding of  $\alpha$ -MSH to MC1R is able to trigger melanogenesis up to a 100-fold increase (Lee and Noh, 2013).

Photo-damaged skin is characterized by several irregular pigmentation states including hyperpigmented lesions, commonly referred to as “*age spots*”. These UV-induced lesions are the result of uneven distribution or local loss of melanocytes, and modified keratinocyte-melanocyte interaction. As investigated by Seiberg *et al.* (2011), besides the possibility to interfere with the melanin production, a bright and uniform skin aspect is also the result of a correct organization of the extracellular matrix (ECM) components. The epidermal extracellular matrix is a basement membrane, whereas the dermal ECM is a complex aggregate of distinct collagenous and non-collagenous components; for this reason optimal quantities and delicate interactions of these components are necessary to maintain normal stability and physiologic properties of skin (Watt *et al.*, 2011).

The predominant extracellular matrix component of the dermis and a variety of other human tissues is collagen; in particular type IV collagen fibers are thought to act as a scaffold, which allows interactions with other non collagenous components, such as laminin, nidogen, and heparan sulfate proteoglycan (Halper and Kjaer, 2014) (**see Fig. 7**).

Furthermore, healthy skin also contains a number of other structural elements, including collagen fibers and fibroblasts located in the basement membrane, a thin fibrous tissue that separates the epidermis from the underlying dermis, lending strength to the skin. These structures can positively interact with dermal ECM components in conferring a stable and uniform distribution of the different top cells, included melanocytes responsible for melanin production.



**Figure 7. Molecular structure of basal lamina.** The basal lamina is formed by specific interactions between the proteins type IV collagen, laminin, and entactins plus the proteoglycan, perlecan. (Source: <http://lcelula.udl.es/>)

### 1.4 Melanogenesis inhibitors: from synthetic to natural sources

#### 1.4.1 Synthetic melanogenesis inhibitors

Melanin has been mainly reported to have a photoprotective function in human skin, although excess of pigment production or abnormal distribution can cause irregular hyperpigmentation of the skin, a serious esthetic problem in human beings.

Exposure to certain drugs and chemicals as well as the existence of certain disease states, such as melasma and age spots, can result in hyperpigmentation and to date there is an increasing demand for new molecules in order to prevent dyspigmentations. This has prompted screening of novel melanogenesis regulators from natural sources, which are usually more attractive for consumers compared to chemically synthesized compounds. A number of inhibitors from drugs and simple chemicals (e.g. hydroxylamines) have been reported to date, and summarized below:

**Captopril** [(2S)-N-(3-mercapto-2-methylpropionyl)-L-proline], even though is a drug widely used in the treatment of hypertension and heart failure, it showed irreversible non-competitive inhibition on the monophenolase activity of mushroom tyrosinase, while exhibiting irreversible competitive inhibition for the diphenolase activity of tyrosinase (Espin *et al.*, 2001).

Captopril is known as a copper chelator and it mainly exerts its inhibitory effect by chelating copper ions at the active site of tyrosinase (Espin *et al.*, 2001). In addition, the inhibitory process may involve a disulfide interchange reaction between captopril and cysteine-rich domains at the active site of the enzyme.

Some other skin-depigmenting drugs also have an inhibitory effect on tyrosinase activity, as reported for penicillamine and the antithyroid drug **methimazole** (Chan *et al.*, 2014). Methimazole (1-methyl-2-mercaptoimidazole) inhibited both the mono- and diphenolase activities of mushroom tyrosinase, which was a mixed-type inhibitor.

Methimazole represents a mixed-type inhibitor of mushroom tyrosinase activity: it conjugates *o*-quinones, thereby causing apparent inhibition in pigmented product formation, and chelates copper at the active site of the enzyme (Chan *et al.*, 2014).

Among all the inhibitors assayed to date, **tropolone** (2-hydroxy-2,4,6-cycloheptatriene) is one of the most potent tyrosinase inhibitors. It is structurally analogous to *o*-diphenolic substrates of tyrosinase, as well as an effective copper chelator (Ismaya *et al.*, 2011), and it showed slow-binding inhibition on key enzyme only binding the oxy form.

**Hydroquinone** (1,4-dihydroquinone) was one of the most widely prescribed depigmenting agents and it has been the conventional standard for treating hyperpigmentation for more than 45 years. Hydroquinone interacts with tyrosinase by binding histidines at the active site of the enzyme, resulting in reduction of skin pigmentation in general, in melanoses, but also on affected skin of vitiligo patients to reduce overall pigmentation (Jow and Hantash, 2014).

Additionally, hydroquinone induced generation of reactive oxygen species leading to the oxidative damage of membrane lipids and proteins such as tyrosinase.

Hydroquinone is also thought to inhibit pigmentation by depleting glutathione, reducing DNA and RNA synthesis with concomitant melanosome degradation and melanocyte damage (Draelos, 2007).

### 1.4.2 Depigmenting agents from microorganisms

There are also other non-quinone-related agents with tyrosinase-inhibiting activities such as **kojic acid** (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one). Kojic acid is a naturally occurring hydrophilic fungal metabolite obtained from *Aspergillus* and *Penicillium* species (Yamada *et al.*, 2014). The activity of kojic acid is believed to arise from chelating copper atoms in the active site of tyrosinase as well as suppressing the tautomerization of dopachrome to DHICA.

Although kojic acid is a popular treatment for melasma, it can cause contact dermatitis, sensitization and erythema (Suzuki *et al.*, 2012).

**Azelaic acid** (1,7-heptanedicarboxylic acid) is a saturated dicarboxylic acid, found naturally in wheat, rye, and barley. It is a natural substance that is produced by *Pityrosporum ovale*, a yeast strain (Saha, 2012). It is used as a treatment for acne, rosacea, skin pigmentation, freckles, nevi and senile lentiginos.

The compound is able to bind amino- and carboxyl- groups and may prevent the interaction of tyrosine in the active site of tyrosinase, thus functioning as a competitive inhibitor (Picardo and Ottaviani, 2014).

### 1.4.3 Botanical extracts as topical agents for skin depigmentation

**Arbutin** is a derivative of hydroquinone, used for skin-lightening purposes, which is found in cranberries, blueberries, wheat and pears (Durkee *et al.*, 2006).

This aquinone is used as an effective treatment of hyperpigmentary disorders and displays less melanocyte cytotoxicity than hydroquinone. As for hydroquinone, arbutin inhibits melanogenesis by competitively and reversibly binding tyrosinase, without influencing the mRNA transcription of tyrosinase. The milder effect of arbutin, compared to its mother compound hydroquinone, could be attributed to the glycoside form where the glycosidic bond needs to be cleaved prior affecting tyrosinase (Sugimoto *et al.*, 2004). The synthetically produced derivate of arbutin, deoxyarbutin, has been shown to be effective and safer skin-lightening agent.

Hu *et al.* (2009) compared the effects of hydroquinone, arbutin and deoxyarbutin reporting that all three compounds had similar inhibitory effects on tyrosinase activity; the protein expression of tyrosinase was not affected by arbutin or hydroquinone, whereas an effect on the protein level was caused by deoxyarbutin.

Also, less melanocyte cytotoxicity was associated to deoxyarbutin compared to the two other quinones. In a human clinical trial, topical treatment with deoxyarbutin for 12 weeks resulted in a significant reduction in overall skin lightness and improvement in solar freckles in a population of light-skinned or dark-skinned individuals, respectively (Boissy *et al.*, 2005).

**Aloesin**, a compound isolated from the aloe plant, has been proven to competitively inhibit tyrosinase from human, mushroom, and murine sources. Studies have shown that aloesin inhibit in a dose-dependent manner tyrosine hydroxylase and DOPA oxidase activities of tyrosinase from normal human

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melanocyte cell lysates. The topical application of aloesin on UV-irradiated (210 mJ) human volar forearm (four times a day for 15 days) showed pigmentation suppression in a dose-dependent manner (Jones *et al.*, 2002).

Aloesin, along with arbutin, was observed to synergistically inhibit melanin production by combined mechanisms of noncompetitive and competitive inhibitions of tyrosinase activity (Wu *et al.*, 2012).

Licorice extracts have several active compounds that may stimulate or suppress melanogenesis, and **glabridin** represents the main ingredient in the hydrophobic fraction of licorice extract. It inhibits tyrosinase activity in cultured B16 murine melanoma cells, at concentrations from 0.1 to 1.0 mg ml<sup>-1</sup>, without affecting DNA synthesis. Other active compounds, such as glabrene, isoliquiritigenin licuraside, isoliquiritin, and licochalcone A, isolated from licorice extracts, were also shown to inhibit tyrosinase activity (Damle, 2014).

Chang *et al.* (2011) evaluated ethanol extracts from twigs and root bark of *Morus alba* for their capacity to inhibit tyrosinase activity. The active component **Mulberroside F**, showed inhibitory effects on tyrosinase activity and on melanin formation in murine melanoma cells. This compound also exhibited superoxide scavenging activity that is involved in the protection against auto-oxidation (Katsube *et al.*, 2006), suggesting a role for *Morus alba* extract as a component of lightening formulations to be used for cosmetic application.

**Polyphenols** are a class of compound that have antioxidant capacity and are found widely within plants. The inhibition of melanogenesis was observed with many types of polyphenolic plant extracts (Arung *et al.*, 2007). Proanthocyanidins or procyanidins, classified as polyphenols, are present in red wine and cranberry juice; grape seeds are another especially rich source. The antioxidative and anti-melanogenic activities of proanthocyanidins from different sources, such as peanut skin (Tatsuno *et al.*, 2012) or *Polyalthia longifolia* leaves (Chen *et al.*, 2014), were found to be much stronger than the activity of vitamin C or E.

Ellagic acid is another natural polyphenol that is widely found in fruits such as raspberries, strawberries, cranberries, walnuts, pecans, pomegranates, and other plant foods. The extract of the rinds of pomegranate contains 90% ellagic acid and showed strong *in vitro* anti-melanogenic activity against mushroom tyrosinase; the mechanism of action seems to be suppression of melanocytes proliferation coupled to the inhibition of tyrosinase (Usta *et al.*, 2013).

### 1.5 Plant tissue cultures as innovative source of active compounds for cosmetic applications

The use of plants as medicines was traditionally a medical practice in all the passed civilizations, and still now natural products, in particular plant metabolites, are extensively used for their therapeutic applications. It was evaluated that in the last 20 years 35% of the new chemical entities were natural products or derived from them, with another 25% created around a pharmacophore from a natural product (Talano *et al.*, 2012). These data confirm that in the early years of this century, plants have been considered economically important as pharmaceuticals and essential for human health.

Natural products have played an important role in lead discovery, mainly in the areas of oncology, cardiovascular and metabolic diseases, and as just described, the discovery of different melanogenesis inhibitors from synthetic to natural sources, was deeply investigated in the recent years. In addition, the isolation of new active metabolites from plants seems to represent the preferential way to achieve a sure and clean final product for customers. However, there are still some limitations in using several of these compounds in cosmetic formulations for the presence of different side effects.

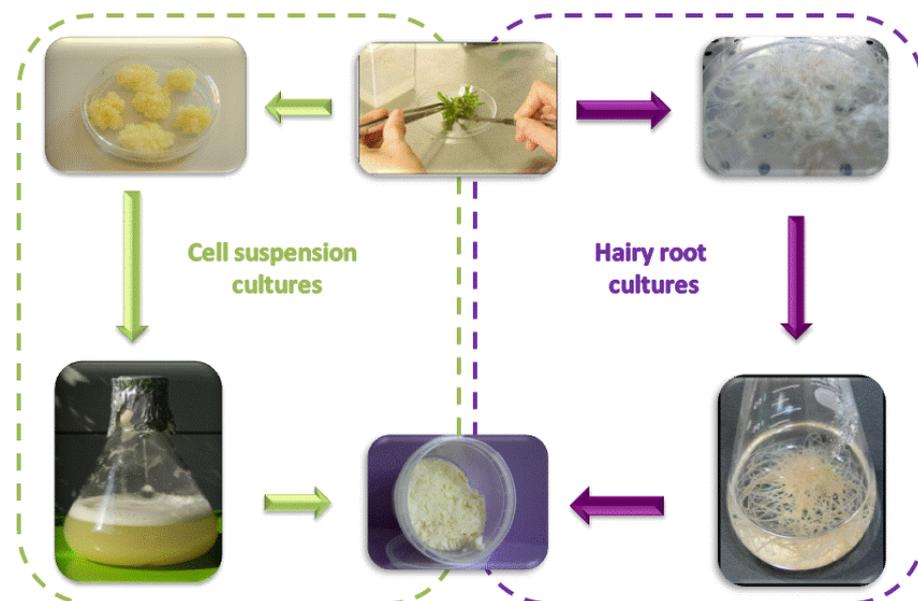
One example is represented by synthetic or plant-derived hydroquinone, indeed its golden days seem to have come to an end as this potent skin-lightening agent can lead to permanent loss of melanocytes. As a consequence of its oxidative damage of membrane lipids, it has been observed an irreversible loss of inherited skin color. In addition, it was recognized that this substance is transported rapidly from the epidermis into the vascular system and is detoxified within the liver into inert compounds (Tse, 2010). The European Committee banned hydroquinone because of the risks of side effects, such as permanent depigmentation and exogenous ochronosis following long-term use (24th Dir 2000/6/EC).

Nowadays, the volume and the range of phytochemicals used by modern society as nutrients, biopesticides and especially as cosmetic additives are continuously expanding, and these high demands are driving research efforts to develop new ways to produce plant-derived metabolites.

As a consequence, plant cell and tissue culture techniques (**Fig. 8**) are valuable alternative systems for producing innovative metabolites, especially because through this systems is possible to cultivate plant cells, tissue and organs in sterile conditions totally independently from geographical and climatic factors, so several technologies based on them were developed (Murthy *et al.*, 2014). In particular, plant cell cultures were largely used for the preparation of many extracts, included in skin care products, as they are rich in antioxidants and other plant beneficial compounds (Barbulova *et al.*, 2010; Bimonte *et al.*, 2011; Tito *et al.*, 2011). Thanks to their totipotency, plant cells grown as liquid suspension cultures can be used as "biofactories" for the production of commercially interesting secondary metabolites, which are in many cases synthesized in low amount in plant tissues.

Despite several advantages of cell suspension cultures, they are characterized by some limitations related to their undifferentiated state, which means lack of stability in producing some specific compounds.

The exploitation of transformed root cultures, also known as "hairy roots", by analogy with the natural plant disease, which causes massive production of adventitious roots, represents a relatively novel approach to *in vitro* plant biotechnology and has received increasing attention in recent years (Talano *et al.*, 2012).



**Figure 8. In vitro plant tissue cultures techniques.** Plant cell cultures have been valuable source of extracts with cosmetic application. Because of some limitations of the latter system, hairy root cultures can represent an innovative and alternative production platform.

Although the mechanism of *Agrobacterium rhizogenes*-mediated genetic transformation has been known for more than 35 years, the enormous capacity of transformed root cultures was largely neglected for most of that time. However, in the mid-1980s, hints of the biosynthetic potential of hairy roots were obtained through a series of investigations, focusing mainly on their alkaloids production (Payne *et al.*, 1987).

During the last years research in this field entered an exponential phase and, up to date, more than 2500 papers on hairy roots have been published (source: *SCOPUS database*). The published studies highlighted several advantages of transformed root cultures, including their relatively fast growth rates in hormone-free media, genetic and biochemical stability and capacity for their synthesis of metabolites (Sevon and Oksman-Caldentey, 2002).

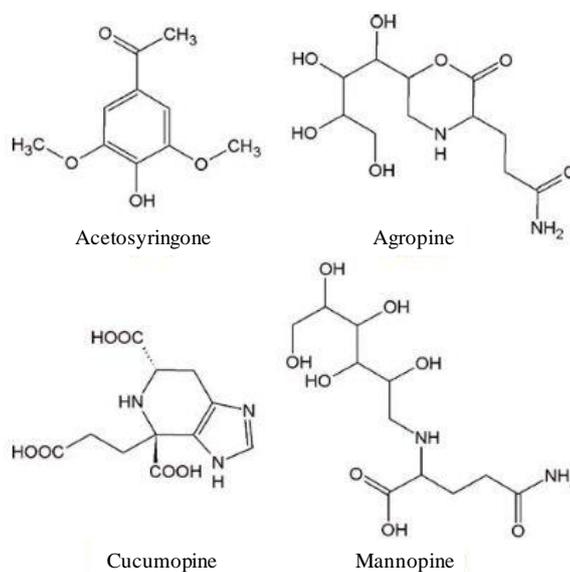
In addition, recent developments in bioreactor systems provide ways in which it could be possible to scale up hairy root cultivation from small-scale systems to large-scale industrial processes (Guillon *et al.*, 2006). Furthermore, growing interest in hairy root systems from private companies (e.g. ROOTec bioactives GmbH, Basel, Switzerland) should facilitate the transfer of knowledge from academic laboratories to the pharmaceutical, food and especially cosmetic companies, as in the specific case of Arterra Bioscience S.r.l.

### 1.6 Hairy root cultures: background and establishment

Two plant diseases known as crown-gall and hairy root caused significant losses in vineyards at the beginning of the last century, and several investigations were initiated to elucidate their nature and the infection mechanisms involved. Several years later, the molecular mechanisms underlying these diseases were elucidated and shown to be based on the natural genetic engineering abilities of phytopathogenic soil bacteria of the genus *Agrobacterium* (Rhizobiaceae family) (Hooykaas, 2015).

When various plants, and in particular dicotyledon species, are wounded in natural ecosystems or fields, they produce simple phenolics, such as acetosyringone. This substance induces the plasmid-localized virulence genes (*vir*), which are responsible for transferring the T-DNA fragments to the plant cells from the *Ti*-plasmid (tumor inducing) in the case of *Agrobacterium tumefaciens* or *Ri*-plasmid (root inducing), in the case of *Agrobacterium rhizogenes*.

Genes of the transferred DNA fragment mediate the formation of neoplastic crown gall tumors and hairy root tissues, which produce and secrete into the soil molecules reported as opines (**Fig. 9**), further used by *Agrobacterium* as nutrients (Georgiev *et al.*, 2007).



**Figure 9. Structure of the most common opines.** Chemical structure of acetosyringone, a phenolic compound released by plants in response to wounding, necessary to activate the *vir* genes of *Agrobacterium* spp. It is also reported the structure of some opines, unusual amino acids produced by *Agrobacterium*-infected plants, and used by the bacteria as nitrogen source. (Source: Georgiev *et al.* 2007).

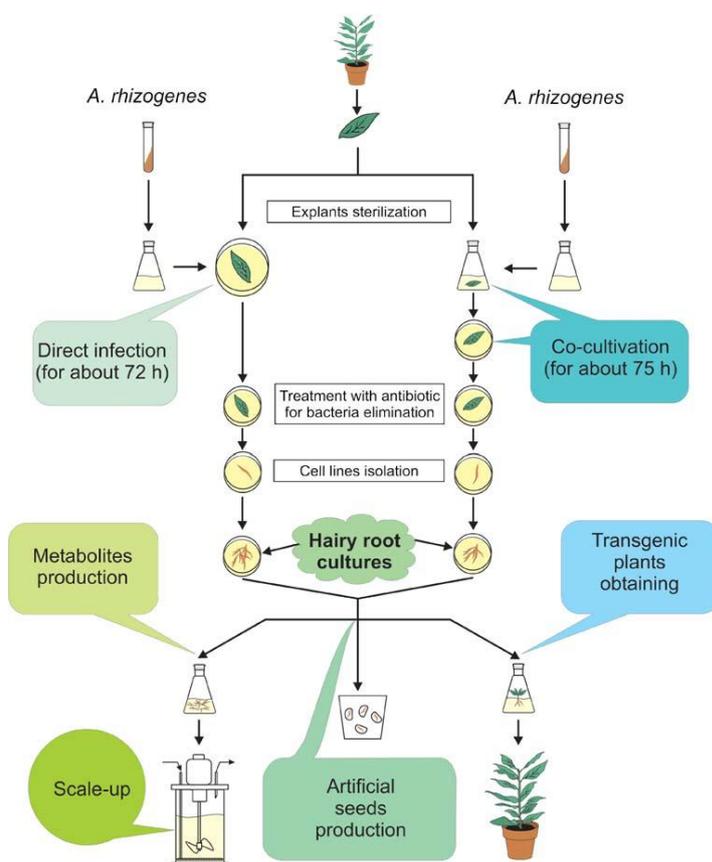
Most *Agrobacterium* strains contain only one T-DNA, but those carrying agropine type *Ri*-plasmids transfer two independent T-DNAs, denoted  $T_L$ -DNA and  $T_R$ -DNA. Both  $T_L$ -DNA and  $T_R$ -DNA are transferred and integrated independently into the host

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plant genome, but the transfer of  $T_L$ -DNA is essential for induction of the hairy root syndrome, while the transfer of  $T_R$ -DNA does not provoke onset of transformed roots from cultured explants (Tempe and Casse-Delbart, 2012).

$T_R$ -DNA contains two genes, *iaaM* and *iaaH*, responsible for the biosynthesis of auxins and the genes responsible for the synthesis of mannopine (*mas1'* and *mas2'*) and agropine (*ags*).  $T_R$ -DNA carries 18 open reading frames (ORF), four of which are essential for hairy root formation; ORF10, ORF11, ORF12 and ORF15 correspond to the genes *rolA*, *rolB*, *rolC* and *rolD*, respectively. The *rolB* gene is absolutely essential for the induction of hairy roots, and even if expressed alone, it can induce significant hairy root production (Bulgakov *et al.*, 2011).

The exploitation of this natural phenomenon represents a strategy in biotechnological research in order to generate transformed root cultures. As shown in **Fig. 10**, the procedure used to induce hairy roots includes direct infection or co-cultivation of wounded explants from plant with suspensions of *Agrobacterium rhizogenes* in aseptic conditions (Sevon and Oksman-Caldentey, 2002).



**Figure 10.** Flow chart for the induction of transformed hairy root cultures. (Source: Georgiev *et al.*, 2007).

Wounded explants can be infected with *Agrobacterium* strains either by direct inoculation with bacterial suspensions and incubation on a solid medium or by co-

cultivation in liquid media. In either case, the infected explants have to be subsequently transferred to a solid medium with cefotaxime or penicillin derivatives, usually about 72h later, to eliminate the bacteria (Rahman *et al.*, 2004).

The transformed roots usually appear after 1–5 weeks, and are able to grow in a profusely branched manner, with abundant lateral branches, on Murashige and Skoog or modified Gamborg's B5 hormone-free media (Pavlov *et al.*, 2003).

Successful genetic transformation can be demonstrated in either of two ways: directly by detecting T-DNA or indirectly by measuring opines, even if the first method is preferred as in some cases opine production is not stable. To detect T-DNA, either polymerase chain reactions or Southern blot hybridizations can be used (Palazon *et al.*, 2003; Le Flem-Bonhomme *et al.*, 2004). Then, after a short period of adaptation, the transformed root cultures can then be used to produce metabolites, transgenic plants or artificial seeds (Ono and Tian, 2011).

Many factors influence a successful transformation of the mother plant tissue and hairy root induction, including the species, age and type of plant tissue, the type of *Agrobacterium* strain and the density of the bacterial suspensions (Rai *et al.*, 2012). Moreover, a number of chemicals, such as acetosyringone, may also promote these processes.

The biosynthetic potential of transformed root cultures was neglected for years, and most of investigations were performed on them trying to clarify the mechanisms underlying hairy root syndrome. However, during the early 2010s, there were several investigations on their production of biologically active substances, especially alkaloids (Zhao *et al.*, 2013), which revealed the biosynthetic capacity of the transformed root systems. It is approved that hairy root cultures have several attractive features including relatively fast growth rates compared to normal roots and high genetic stability compared to undifferentiated cultures; thanks to these advantages they receive more attention as biological matrices for producing valuable metabolites (Zhou *et al.*, 2011). Furthermore, growth regulators are not required for their cultivation, which is an important consideration, as some hormones as 2,4-dichlorophenoxyacetic acid are toxic, so their presence in many end products is unacceptable.

Root-based cultures technologies become popular sustainable production platforms generating specific secondary metabolites. Hairy roots technologies advantages include biochemical stability, ability to produce the metabolites found in the mother plants, and the opportunity to discover new chemical entities. In addition, use of hairy root systems is advantageous for the production of a number of secondary metabolites that are synthesized in plant roots and then accumulated in aerial parts of the plant, as such metabolites are accumulated at very low amounts, or not at all, in undifferentiated or shoot cultures (Georgiev *et al.*, 2012).

One of the aspects of transformed root cultures that has attracted intense commercial interest is their potential for protein production (Woods *et al.*, 2008), but in addition to proteins and well-known standard metabolites of the mother plant, hairy roots are also potential sources of new, natural compounds.

For example, Abbott *et al.* (2010) and Leea *et al.* (2013) isolated resveratrol, which is known to possess strong anti-oxidant activities from *Arachis hypogaea* and *Scutellaria baicalensis* cultures, respectively. Hairy root induction was also attempted in *Artemisia annua* for enhancing the production of the secondary metabolite artemisinin, a powerful antimalarial drug (Shaneeja *et al.*, 2014).

Thus, hairy root cultures offer possibilities for isolating new compounds with specific applications and also cosmetic value.

### 1.7 Bioreactors for large-scale cultivation of hairy roots

Hairy roots are so recognized to have rapid growth rate, high product yield, simple medium requirements and culture stability. Despite that, one of the most important limitations for the commercial exploitation of this alternative culture system is the development of technologies for large-scale culture. A variety of reactor configurations have been investigated to cultivate hairy roots, including stirred tank reactor, bubble column reactor, airlift reactor, trickle bed reactor and nutrient mist reactors (Mishra and Ranjan, 2008).

In recent years, researchers have focused on mist reactors because they present several advantages over liquid phase reactors including the ability to manipulate the gas composition, to control secondary metabolite production and to allow effective gas exchange in a densely growing biomass. Roots growing in the highly aerated environment of a mist reactor do not exhibit O<sub>2</sub> limitation and stress. In mist reactor, the plant organ culture is dispersed in an air phase by immobilizing on the mesh containing support and the liquid medium is introduced into the reactor as a mist of small droplets by an ultrasonic transducer. In this way is possible to control the growth rate eliminating the need for mechanical agitation and thereby reducing the damaging shear (Stiles and Liu, 2013).

It would be difficult to select the best bioreactor for scaling-up hairy root cultures, in fact for a successful cultivation, whatever type of bioreactor is used, several factor should be contemplated, including the morphology, physiology and high stress sensitivity of transformed roots (Curtis, 2000). However, the bioreactor cultivation of hairy roots was considered in different reviews (Srivastava, 2007; Sivakumar, 2006; Eibl *et al.*, 2009) and several recent advances were displayed; one such advance is the improvement of disposable bioreactor systems, known as CultiBags (**Fig. 11**).



**Figure 11.** The BIostat<sup>®</sup> CultiBag RM Optical provides full process automation with sophisticated feedback control. It consists of a rocker and a digital control unit. The control tower is connected to the rocking unit for monitoring and controlling the culture, including pO<sub>2</sub>, pH, agitation and temperature in batch and fed batch mode operation.

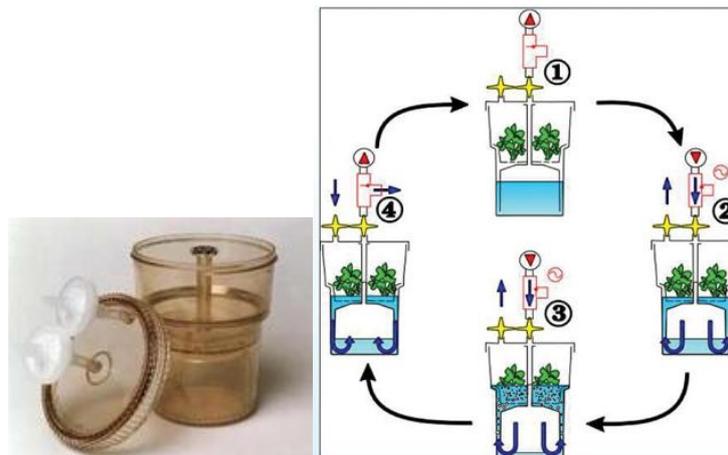
The working principle of these systems is based on rocking motion agitation, which first of all reduces shearing stress levels. Utilization of plastic disposable bags

minimizes time-consuming sterilization and cleaning procedures and facilitates the fulfillment of GMP requirements. Moreover, the feedback control loops using single use sensors for important parameters (pH, temperature, pO<sub>2</sub>), bring new levels of confidence to process development and drastically reduce risks of cross contamination.

As an example, ginsenoside production from hairy roots of *Panax ginseng* using wave-based systems was evaluated in detail (Palazon *et al.*, 2003) and best results in term of biomass generation were obtained in 2-l bags compared to shaken flask. Furthermore, Ritala *et al.* (2014) evaluated *Nicotiana tabacum* hairy roots for the production of geraniol using wave-mixed bioreactors. Nowadays, large-scale wave systems with capacities up to 600 l are commercially available by Wave Biotech AG<sup>®</sup>, Switzerland.

The alternative system of temporary immersion bioreactors (RITA<sup>®</sup>), developed at the beginning for plant *in vitro* propagation, was also used for cultivating hairy root cultures from *Beta vulgaris* (Pavlov *et al.*, 2003). These bioreactors are characterized by their advantages in reducing and solving a problem so-called hyperhydration, beyond lower consumable and labor costs (**Fig. 12**).

Furthermore, temporary immersion bioreactors make possible to use small amount of culture media, and as the daily cumulative duration of flooding can vary from minutes to several hours, they offer an attractive and innovative scale-up system for hairy root cultures (Eibl *et al.*, 2009).



**Figure 12.** Temporary immersion system (RITA<sup>®</sup>) developed by CIRAD (France) to combine aeration and the positive effects of liquid medium culture in the same machine. TIBs provide programmable partial or total contact between the plant material and the liquid medium (flooding process - steps from 1 to 4).

Source: <http://www.cirad.fr/en>

### 1.8 Identification of new plant-derived skin depigmenting agents

As already reported, plants are an attractive source for new skin depigmenting actives, also because consumers appreciate more plant-derived compounds than synthetic compounds added in the cosmetic formulations.

With the aim to identify new plant sources, we focused our attention on three species belonging to Asteraceae and Brassicaceae families, reported for their *in vivo* content of interesting compounds, as shown in **Fig. 13**.



**Figure 13.** Selected plant species for the exploitation of hairy root cultures. *Brassica rapa subsp. pekinensis* (Brassicaceae), *Cichorium intybus* and *Helianthus annuus* (Asteraceae), were selected for their *in vivo* content of interesting compounds.

*Helianthus annuus* (Asteraceae), an annual species of **sunflower** grown as a crop for its edible oil and edible fruits, was selected. As reported by Aboki *et al.* (2012) *Helianthus annuus* seed oils, consisting primarily of triglycerides of linoleic and oleic acids, are used in cosmetics and personal care products for the formulation of a wide variety of product types, including bath products, makeup, cleansing products, depilatories, hair conditioners, and shampoos. Furthermore, Kamal (2013) determined allelochemicals in roots of sunflower using thin layer chromatography for alkaloids and spectrophotometry for phenols and flavonoids, reporting high accumulation of these molecules in *in vivo* root system. All these compounds represent interesting targets, especially flavonols, because of their competitive activity against tyrosinase that was found to come from their ability to chelate copper in the active site, via their 3-hydroxy-4-keto moiety (Kim and Uyama, 2005).

Another interesting plant species is *Cichorium intybus* (Asteraceae), the common **chicory**, is a somewhat woody, perennial herbaceous plant usually with bright blue flowers. Around 1990 it was found that dried chicory root extracts contain up to 90% inulin, a polysaccharide similar to starch. Inulin is mainly found in the plant family Asteraceae as a storage carbohydrate, it is used as a sweetener in the food industry but is also gaining popularity as a source of soluble dietary fiber and functional food (Madrigal and Sangronis, 2007), which may help humans with weight loss.

Beside these nutritional effects, chicory roots were also found to contain between twelve new compounds also azelaic acid, a dicarboxylic acid used for treatment of skin pigmentation including melasma and post inflammatory hyperpigmentation, particularly in those with darker skin types (He *et al.* 2002). As a tyrosinase inhibitor, azelaic acid reduces synthesis of melanin and it has been recommended as an alternative to hydroquinone (Grimes, 2011).

The family Brassicaceae consists of 350 genera and about 3,500 species, and includes several genera. The genus *Brassica* is the most important one within the tribe Brassicaceae, which includes some crops and species of great worldwide economic importance such as **Chinese cabbage** (*Brassica rapa* subsp. *pekinensis*).

At present, it is known that crops from *B. rapa* species contain a high amount of phenolic compounds and as result, they are an appreciable source of polyphenols, especially flavonoids. The main polyphenols identified in *B. rapa* vegetables are acylated mono-, di-, tri- and tetraglucosides of quercetin, kaempferol and isorhamnetin as well as esters of hydroxycinnamic acids with malic acid, glycosides, and quinic acid (Harbaum *et al.*, 2008)

In a recent study, the phenolic profiles of fifteen *B. rapa* crops, including *B. rapa* subsp. *pekinensis*, were reported by Lin and Harnley (2010). The major phenolic compounds identified were kaempferol 3-O-sophoroside-7-O-glucoside derivatives, isorhamnetin 3-O-glucoside-7-O-glucoside, hydroxycinnamoyl gentiobioses, hydroxycinnamoylmalic acids and hydroxycinnamoylquinic acids, together with the presence in the roots of ferulic acid and sinapic acid derivatives.

Many of these phenolic compounds are known to have potent antioxidant activity, and a number of naturally occurring melanogenic inhibitors contain a phenolic structure (Kim *et al.*, 2005b; Alam *et al.*, 2011).



**Aims of the project**



## **2. Aims of the project**

The principal aim of the present PhD project was the implementation of an industrial production platform based on hairy root cultures as source for the extraction of novel plant-derived extracts with hypopigmenting effects. Three different plants (*Brassica rapa* subsp. *pekinensis*, *Cichorium intybus* and *Helianthus annuus*) were selected, on the base of the previous experimental evidences of their activities in cosmetics.

The experimental activities were planned and developed through a collaborative project between the Department of Pharmacy (University of Salerno), Arterra Bioscience S.r.l and VTT, Technical Research Centre of Finland.

Arterra is an Italian research-based Biotech company mostly involved in developing new plant-derived extracts to be used as active ingredients with cosmetic application. VTT represents the largest multidisciplinary research organization in Northern Europe that provides high end technology solutions and innovation services in plant tissue culture.

The specific objectives of the project are summarized below:

- Generation of hairy root cultures of the selected plant species by transformation with *Agrobacterium rhizogenes* and determination of growth kinetics;
- Preparation of crude total ethanol extracts and sugar/peptides mixtures from hairy roots and preliminary anti-oxidant profiling of the extracts;
- Characterization of the anti-melanogenesis activities of the plant extracts by a panel of biological assays (melanin content, tyrosinase activity, intracellular cAMP levels and MITF gene expression) and definition of the involvement in enhancing expression of important genes encoding proteins involved into extracellular matrix (ECM) assembly;
- Setting up a reporter assay system for measuring the MITF promoter activity, as a rapid screening method of new plant-derived skin depigmenting agents, based on a luciferase reporter vector;
- Implementation of a hairy root-based industrial platform for the extraction of new plant-derived active ingredients, coupled to high-performance extraction procedures and cryopreservation methods of root cultures for long-term storage at ultra-low temperatures.



## **Materials and methods**



### 3. Materials and methods

#### 3.1 Plant material and *A. rhizogenes* strains

Seeds of Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), chicory (*Cichorium intybus*) and sunflower (*Helianthus annuus*) were obtained from HorizonHerbs (Oregon, USA). They were surface sterilized in 70% ethanol v/v for 2 min, followed by 15 min in 2% hypochlorite containing few drops of Tween 20.

Finally, the seeds were rinsed several times with sterile water, dried using sterile papers, and transferred aseptically to solid Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands), containing 3% w/v sucrose and 7 g l<sup>-1</sup> agar type A (Sigma-Aldrich Italia, Milan, Italy). The medium pH was adjusted using KOH to 5.8 before autoclaving. Small plastic jars with seeds were incubated at 24 °C under day-night illumination regime (photoperiod 16:8). After about one month, first cotyledons were obtained and cut into sections (ca. 25 mm<sup>2</sup>) ready to be infected through *Agrobacterium rhizogenes*.

Different *A. rhizogenes* strains, many of which have been used to produce hairy roots from medicinal plants, were used to infect plant materials: agropine strain (ATCC15834) induce agropine, mannopine and agropinic acid production while the mannopine strain (8196) induce the production of one single opine.

The *A. rhizogenes* strains 8196 and A4RS, derived from the wild strain A4 modified for the resistance to rifampicin and spectinomycin antibiotics, were grown on MYA medium (5 g l<sup>-1</sup> yeast extract, 0.5 g l<sup>-1</sup> casein hydrolisate, 8 g l<sup>-1</sup> mannitol, 2 g l<sup>-1</sup> MgSO<sub>4</sub>, 5 g l<sup>-1</sup> NaCl and to prepare plates bacterial agar to 1.5% was added, pH 6.6) (Petit and Tempé, 1978). On the other hand, the strain ATCC 15834, was grown on YEB medium (5 g l<sup>-1</sup> beef extract, 1 g l<sup>-1</sup> yeast extract, 5 g l<sup>-1</sup> peptone, 5 g l<sup>-1</sup> sucrose, 0.5 g l<sup>-1</sup> MgCl<sub>2</sub> and to prepare plates add bacterial agar to 1.5%, pH 7.0) (Vervliet *et al.*, 1975).

#### 3.2 Co-cultivation and induction of hairy roots

The leaf explants of *Brassica rapa* subsp. *pekinensis*, *Cichorium intybus* and *Helianthus annuus*, respectively, were co-cultivated with *Agrobacterium rhizogenes* ATCC15834, 8196 and A4RS strain to induce hairy roots. *Agrobacterium* cultures were maintained by subculturing into a 100 x 15 mm culture tubes, containing 5 ml of respective medium (MYA or YEB) and grown for 72 hours in the dark at 28 °C.

For co-cultivation, a minimum of 25 wounded explants were immersed in the bacterial broth culture of OD<sub>600</sub> ~ 0.3 - 0.5 and swirled for 20 min. 50-150 µM acetosyringone (Sigma-Aldrich, Milan, Italy) was incorporated in the co-cultivation medium, then the explants were blotted to remove excess of bacterial inoculum.

Liquid media without bacteria was applied to the explants as a control. All the explants were cultured on sterilized Petri plates comprising solid modified half strength B5 medium (1.55 g l<sup>-1</sup> B5 Gamborg including vitamins, 7 g l<sup>-1</sup> plant agar type A, pH 5.7) without phytohormones, and supplemented 30 g l<sup>-1</sup> with sucrose and 250 mg l<sup>-1</sup> myo-inositol. The medium was solidified with 0.7% (w/v) agar, the pH adjusted to 5.7 ± 0.2 and autoclaved at 121 °C for 15 min. Bacteria were incubated in the dark at 27 °C for 72 hours, and then transferred to fresh modified B5 medium, containing 0.5 g l<sup>-1</sup> cefotaxime for every 15 days, to eliminate agrobacteria.

Infected leaf explants were cultured on hormone free medium and about after 5 weeks hairy roots differentiated at the site of bacterial infections. The efficiency of transformation was determined as follows:

## Materials and methods

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$$\% \text{ transformation frequency} = \frac{\text{number of explants inducing hairy roots}}{\text{total number of explants infected}} \times 100$$

### 3.3 Detection of Ri T-DNA integration

Transformation and integration of *Ri* T-DNA into hairy root genome was confirmed by PCR analysis. The bacteria-free roots grown in B5 modified medium were removed from plates, dried on sterile filter paper and quickly frozen at – 80 °C. Thereafter, genomic DNA from putative transformed was extracted, using GenElute DNA extraction kit (Sigma-Aldrich, Milan, Italy). The *rolB* gene was amplified using the following primer set (fw- 5'-TGTGTGATGCCGCAAGCAAT-3' e rv 5'-TCTATCTCGCGAGAAGATGC-3'), to yield an amplicon with expected size of 290bp. The PCR mixture (25 µl) contained 50 ng of DNA prepared from hairy roots respectively as the template, 1X PCR buffer, 25 pmoles of each primer, 2.5 mM of dNTPs and 1 unit of Taq DNA polymerase (Fermentas). PCR for *rolB* was carried out by amplifying with initial denaturation at 94 °C for 5 min followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C with a final extension of 72 °C for 10 min using a thermal cycler (Mastercycler ep Gradient S, Eppendorf). PCR analysis was performed using *virD2* (301 bp) gene to ascertain the absence of residual contaminating *Agrobacterium* in the hairy roots.

The PCR conditions were same as those used for amplifying the *rolB* gene. The primers for *virD2* amplification were as follows: FW5'-GAATATCTATCCCGGAAGGG-3' and RV 5'-GACGATTGACGACGACATGC-3'.

The PCR products obtained were loaded on 1.5% agarose gel, stained with ethidium bromide, and the amplification bands were observed and documented using the Geliance 200 Imaging system (Perkin Elmer).

### 3.4 Hairy root growth

#### 3.4.1 Small-scale cultivation

The bacteria free hairy roots were transferred to fresh medium at 1-month intervals, and maintained in a dark condition at 25-26 °C. Approximately 50 mg actively growing hairy roots were inoculated in 2L conical Erlenmeyer flasks, containing 50 ml of hormone-free liquid medium consisting of modified half-strength B5 (Gamborg *et al.*, 1968) medium, as mentioned before.

Freshly inoculated hairy root lines were started from 12 independent samples for each plant species, and maintained on a orbital shaker at 50 rpm and 25 ± 2 °C temperature, under dark condition. Roots were harvested after of period of 30 days, verified for micro-organism contamination (37°C O/N) and frozen at - 80°C, until processed for extracts preparation.

#### 3.4.2 BIOSTAT® CultiBag RM 20 system

A wave-mixed bioreactor, the BIOSTAT® CultiBag RM 20 (Sartorius) was used to cultivate hairy roots in a batch mode. The experiment was initiated using a

disposable 2D cultivation bag (basic screw cap of 10 liters) and by inoculating modified Gamborg's B5 medium (half-strength B5M) with 1 g FW l<sup>-1</sup> of 7-day-old viable hairy root material, grown on solid medium.

The culture conditions were maintained at constant values during the experiment, and six feedings steps were introduced by replacing 20% (v/v) of the initial culture volume of modified Gamborg's B5 liquid medium. For sampling, 20 ml of the culture supernatant was taken with a 50 ml Luer lock syringe (Braun Medical Inc., Bethlehm, PA, USA) connected to the pre-assembled sampling port.

After inoculation, and between days four and five, conductivity and pH were monitored offline (MC226 and Five Easy, Mettler Toledo, Greifensee, Switzerland). The experiment lasted 32 days and final fresh weight was determined by filtering the biomass 3 min using a reusable filtration unit (Corning, NY, USA). For final dry weight determination, the filtered and frozen biomass was lyophilized using the ALPHA 2-4 freeze dryer (Martin Christ GmbH, Osterode am Harz, Germany). Specific process parameters and features are summarized in **Table 4**.

**Table 4.** Main process parameters and features of the single-use bag bioreactor.

<i>Bioreactor</i>	<i>Parameters and specific features</i>	
BIOSTAT® CultiBag RM 20 (Sartorius Stedim Biotech)	Cultivation bag Platform Rocking rate Rocking angle Aeration Temperature Sensors Feeding Light	CultiBag RM 10L basic screw cap Basic for CultiBag RM 10L 8 rpm 6° 0.20 l min <sup>-1</sup> 25 °C None Day 5, 10, 15, 20, 25 and 30 Darkness

### 3.4.3 RITA® Temporary immersion bioreactor (TIB)

Two different amounts of fresh root tips (250 mg and 500 mg, respectively) were cultivated in triplicates using RITA® TIB vessels (VIT-ROPIC, Saint-Mathieu-de-Trévières, France), available at VTT Technical Centre of Finland, which were sterilized by autoclaving at 114 °C for 25 min prior to use. Each vessel contained 200 ml of modified Gamborg's B5 liquid medium, as proposed by Teisson and Alvard (1995).

TIB cultures were established with the following immersion frequency: 15 min flooding/ 3 hours stand-by periods. The flow rate of inlet air was about 40 l h<sup>-1</sup> for each RITA® vessel. Four weeks after the onset of experiment, plant material was harvested and filtered for 3 min using a reusable filtration unit (Corning, NY, USA). For final dry weight determination, the filtered and frozen biomass was lyophilized using the ALPHA 2-4 freeze dryer (Martin Christ GmbH, Osterode am Harz, Germany).

### **3.5 Extraction methods and preliminary assays**

#### **3.5.1 Sugar and cell wall peptides mixture preparation**

In order to obtain a mixture of cell wall peptides and sugars (Pep), about 100 g of harvested hairy roots were grinded, when still partially frozen, in PBS 1X pH 7.2 and 1% Triton (1:2 w/v) in a mortar with a pestle or by mechanical grinder. The homogenized product was filtered through a cloth filter and the soluble fraction was collected in a sterile becker placed in ice to obtain a pellet, that was processed as follows. To get rid of pectins and starch, the pellet was dissolved in 2 mM Na<sub>2</sub>EDTA (1:2 w/v; stock 0.5M) and boiled at 100 °C for 20 min, then the solution was cooled down in ice.

The insoluble fraction was filtered, washed extensively in distilled water and then boiled with 2 volumes of 0.1N HCl (pH 1.0) for 60 min at 100 °C to hydrolyze sugars. The solution was cooled in ice and the obtained cell wall proteins were digested with 1 mg ml<sup>-1</sup> pepsin (Sigma-Aldrich, Milan, Italy) overnight at 37 °C. At this step, no purification was needed and pepsin was added to the acid treated solution directly. After digestion, soluble fraction was recovered by centrifugation at 8000/10000 rpm and at 4 °C for 15 min and pH adjusted to 6.0.

The supernatant was analyzed for absence of micro-organism contamination (37 °C for 48 hours on plates with Letheen agar and in 5 ml tubes with liquid Letheen), and then was frozen in dry ice and lyophilized until powder was obtained.

#### **3.5.2 Total ethanol extract preparation**

In order to obtain a total ethanol extract (EtOH), harvested roots were mechanically grinded into pure grade 96% EtOH (1:4 w/v) and left to macerate in stirring condition for 4 hours. The homogenized product was filtered through a cloth filter and the soluble fraction was collected, centrifuged at 8000-10000 rpm at 4 °C for 15 min, then its volume concentrated 100X using a rotary vacuum evaporator (IKA® RV8, IKA® - Werke GmbH & Co. KG, Germany).

#### **3.5.3 Accelerated solvent extraction**

Accelerated solvent extraction was performed using a Dionex model ASE® 200 equipped with a solvent controller (Dionex Corp, Sunnyvale, CA). One circular cellulose filter 1 cm in diameter (Dionex Co.) was placed at each end of the standard stainless steel extraction cell (33 ml). Thermo Scientific Dionex ASE Prep DE (diatomaceous earth) in a 1:1 ratio was used for the moisture removal under accelerated solvent extraction conditions.

A "sequential extraction" was performed, and the residue of the previous extraction was used as the feed for the next extraction. The extraction temperature was 70 °C for *n*-hexane and ethyl acetate, 80 °C for ethanol and 120 °C for water. The operation pressure was also monitored to be 1500 psi.

For each solvent the duration of static extraction time (10 min) was then followed by 1 min of pre-heating and 5 min equilibration. Following the extraction, the thimble was flushed with solvent (60%) and purged with nitrogen. The solvent was collected in 50 ml vials with Teflon septa. Subsequently, each extraction cell containing the

same sample went through another identical extraction cycle and solvent was collected in the same vial. The total extract volume (50 ml) was transferred into 100 ml pear-shaped flasks and concentrated to about 500  $\mu$ l using a rotary vacuum evaporator (Hei-Vap Heidolph; Essen, Germany) at 35 °C or evaporated to dryness at 45 °C using a gentle stream of nitrogen.

### 3.5.4 Cryopreservation

Root-tips (about 5 mm) were excised from hairy roots grown in liquid culture. All manipulations were performed in liquid half-strength B5 medium in order to avoid desiccation of the fragile root apices. Cultures were selected, normally after a 7-10 day sub-culture interval, dense and senescent cultures were not selected.

Root-tips were cryoprotected for 1 hour in 10% (v/v) spectroscopic grade dimethyl sulphoxide (DMSO, Sigma-Aldrich, Milan, Italy) made up in liquid half-strength B5 medium. Then two different freezing methods were investigated. (a) Ultra-rapid freezing in which the root-tips were dispensed in 2 ml cryovials with 500  $\mu$ l of cryoprotectant (25 °C). The vials were placed into CoolCell boxes at - 80 °C for about 30 minutes and then directly immersed into liquid nitrogen (- 196 °C).

(b) Controlled cooling in which the root-tips were dispensed in 2 ml cryovials with 500  $\mu$ l of cryoprotectant (25 °C). The vials were then placed into a PLANER Kryo 560 series programmable freezer unit and subjected to a cooling/freezing programme as follow:

- Start temperature 0 °C
- -1.00 °C/min to -17 °C
- -30 °C/min to -30 °C
- +20 °C/min to -19 °C
- -1 °C/min to -35 °C

For thawing the vials were immersed for 2-3 minutes in a water bath maintained at 45°C. Roots were then recovered on solid half-strength B5 medium and viability was assessed using fluorescein diacetate vital staining.

### 3.5.5 Skin cell cultures

Murine fibroblasts (NIH-3T3) and immortal human keratinocytes (HaCaT) were grown in DMEM containing 10% FCS or FBS, in a 95% air, 5% CO<sub>2</sub> humidified atmosphere at 37 °C. Melanoma cells (B16-F1) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) modified to contain 2 mM L-glutamine, 4500 mg l<sup>-1</sup> glucose, 1 mM sodium pyruvate, 1500 mg l<sup>-1</sup> sodium bicarbonate and 10% FBS, incubated at 37 °C, under 5% CO<sub>2</sub> atmosphere.

Human epidermal melanocytes isolated from lightly pigmented adult skin (HEMa-LP) were maintained in Medium 254 (Gibco, Italy), supplemented with Human Melanocyte Growth Supplement-2 (HMGS-2) in the absence of antibiotics and antimycotics, and incubated in a 95% air, 5% CO<sub>2</sub> humidified atmosphere at 37 °C.

### 3.5.6 Cell vitality assay (MTT assay)

Immortal human keratinocytes (HaCaT) or melanoma cells (B16-F1) were seeded at density of  $1.3 \times 10^4$  in 96-well plates, grown for 8 hours and treated for 48-72 hours with different concentrations of the two plant extracts. After treatments, cells were washed with PBS and incubated with  $100 \mu\text{l well}^{-1}$  of "reaction buffer" containing: 10 mM Hepes, 1.3 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgSO}_4$ , 5 mM glucose and  $0.5 \text{ mg ml}^{-1}$  of colorimetric substrate MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide] in PBS buffer at pH 7.4, according to the method described by Mosmann (1983). After 3 hours at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$  atmosphere, cells were solubilized by adding  $100 \mu\text{l}$  of "solubilization solution" (10% Triton X100, 0.1 N HCl in isopropanol), and the plate was incubated for 4 hours at room temperature. The number of viable cells is directly proportional to the level of the formazan product created. The developed color was then quantified at 595 nm by a Multiwell Spectrophotometer (ELISA reader).

### 3.5.7 ORAC assay

The Oxygen Radical Absorbance Capacity (ORAC) assay measures the total anti-oxidant power of a compound or extract and is based on the ability to inhibit the oxidation of a fluorophore, generally fluorescein, by a potent oxidant, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). Twenty-five  $\mu\text{l}$  of the extract dilutions in phosphate buffer 75 mM, pH 7.4, (at concentration of 0.01%) was aliquoted into 96-well plate and  $150 \mu\text{l}$  of fluorescein solution (8.5 nM in phosphate buffer) was added to each sample. After incubation at  $37^\circ\text{C}$  for 15 min,  $25 \mu\text{l}$  of AAPH solution (153 mM in phosphate buffer) was pipetted into each well, and the progress of the reaction was monitored at 535 nm (excitation at 485 nm), using a fluorescence multi-well reader. The fluorescence was measured every minute for 70 min.

Anti-oxidant power of the mixture was calculated according to the method described by Huang *et al* (2002). The net area under the curve (AUC) of the samples and standards (represented by different dilutions of Trolox) was calculated. The standard curve was obtained by plotting Trolox concentrations against the average AUC (Area Under Curve) of three measurements for each concentration of plant extract. Net AUC was obtained by subtracting the AUC of the blank from that of the sample or the standard. ORAC values of the samples were expressed as  $\mu\text{mole}$  of Trolox equivalents per g of extract.

### 3.5.8 Comet assay

NIH-3T3 cells were plated at a density of  $1.5 \times 10^5$  per well in 6-well plates. After 16 h incubation with the peptide/sugar mixture or ethanol extract of *Brassica rapa* subsp. *pekinensis* hairy roots, cells were treated with  $175 \mu\text{M}$   $\text{H}_2\text{O}_2$  for 2.5 h and detached from the plate using  $1 \text{ ml well}^{-1}$  of non-enzymatic solution (Sigma-Aldrich, Milan, Italy). Cells were transferred to eppendorf tubes, centrifuged at 1.5 k and washed once with PBS1X. The cell pellet was resuspended in  $10\text{-}20 \mu\text{l}$  of PBS, depending on the amount of cells recovered. Eighty–ninety  $\mu\text{l}$  volume of Low Melting Point Agarose (LMPA) 0.5% in PBS and equilibrated at  $37^\circ\text{C}$  was added to each tube, and the solution immediately dropped onto a Normal Melting Agarose (NMA) pre-coated slide. Coverslip slides were placed on the top, without squeezing the cells, and the slides were put on a tray at  $4^\circ\text{C}$  until the agarose layer hardens (10-15 min). Coverslips were then gently slid off without scraping the agarose layer

containing the cells. The slides were placed in cold Lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma Base pH 8.5, 1% Triton X100) for at least 2 hours at 4 °C.

Slides were gently removed from Lysis solution and placed in an electrophoresis tank, filled with cold Electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). Slides were left in alkaline buffer for 10 min to allow DNA unwinding and the expression of alkali-labile damage, and then power supply was turned on to 24 V. Slides were electrophoresed for 20 min and at the end placed in cold Neutralization buffer (0.4 M Tris-HCl, pH 7.5) for at least 10 min. After drying the slides, cells were stained with a 10 µg ml<sup>-1</sup> solution of ethidium bromide, covered with a coverslip and scored at a fluorescence microscope.

### **3.6 Determination of depigmenting activity in B16-F1 and HEMa-LP cells**

#### **3.6.1 Melanin content analysis**

Melanoma cells (B16-F1) were seeded at density of  $3 \times 10^3$  in 96-well plates in a modified Dulbecco's Modified Eagle's Medium (DMEM), containing 2 mM L-glutamine, 4500 mg l<sup>-1</sup> glucose, 1 mM sodium pyruvate and 1500 mg l<sup>-1</sup> sodium bicarbonate, and incubated for 20 hours at 37 °C under 5% CO<sub>2</sub> atmosphere.

Then, the cells were treated with the different plant extracts in presence of 50 µM IBMX for 72 hours. At the end of the treatments, the cells were washed with PBS, and lysed in 50 µl of 1N NaOH at 70°C for 20 min. The absorbance, measured at 492 nm, is proportional to the melanin content. Then, 2 µl of each sample was used to measure their total protein content by the Bradford reagent.

#### **3.6.2 Tyrosinase activity assay**

Melanoma cells (B16-F1) were seeded at density of  $1-4 \times 10^4$  cells into 6-well plates (DMEM medium). After 20 hours of incubation at 37 °C under 5% CO<sub>2</sub> atmosphere, they were treated with the plant extracts in absence of 50 µM IBMX and incubated for 72 hours under the same conditions.

Human epidermal melanocytes isolated from lightly pigmented adult skin (HEMa-LP) were seeded at density of  $1.5 \times 10^5$  cells in 6-well plates using Medium 254 (Gibco, Life Technologies, Milan, Italy), supplemented with Human Melanocyte Growth Supplement-2 (HMGS-2, Invitrogen) in the absence of antibiotics and antimycotics.

After 20 hours, they were treated with plant extracts for 24 hours. In both cases, after the incubation, the cells were washed with PBS and collected in 100 µl of lysis buffer (50 mM phosphate buffer pH 6.8, 1% Triton, 0.1 mM PMSF). The total protein content of each lysate was measured by the Bradford reagent and then 50 µg of proteins were incubated with 2 mM of L-DOPA (L-3,4-dihydroxyphenylalanine) at room temperature. Absorbance at 490 nm was measured every 10 minutes for 1 hour for tyrosinase activity evaluation, since the oxidation of the substrate is directly proportional to the activity of the enzyme.

### 3.6.3 Semi-quantitative RT-PCR

B16-F1 cells ( $5 \times 10^4$ ), HDF and HaCaT cells ( $1.5 \times 10^5$ ) were seeded and grown in 6-well plates. After 20 hours, the cells were incubated for 6 hours with plant extracts at 37 °C under 5% CO<sub>2</sub> atmosphere. Then, cells were collected and total RNA was extracted with the GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich, Milan, Italy), according to the manufacturer's instructions, and treated with DNase I at 37 °C for 30 min, to eliminate any contaminating genomic DNA. The first strand cDNA was synthesized from 1 µg using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA).

RT-PCR was performed using gene specific primers and the Quantum RNA™ 18S internal standard (Ambion, Foster City, CA, USA), according to manufacturer's instructions. The QuantumRNA™ kit contains primers to amplify 18S rRNA along with competitors that reduce the amplified 18S rRNA product within the range to allow it to be used as endogenous standard. The genes of interest were amplified with the the primers, reported in **Table 5**:

**Table 5.** List of primers used for RT-PCR.

Name	Forward primer (5'→3')	Reverse primer (5'→3')	Tm primers (forward/reverse) °C
MITF	CAGGTAAAGCAGTACCTTC	CAGTGCTCTTGCTTCAGACT	49/51
LAMA3	ATGTGATGATTGCGACAGC	AGCACGAAGGTCACTGAGT	49/51
LAMB3	AGACCTATGATGCGGACCT	GAAGACATCTCCAGCCTCA	51/51
LAMC2	ATCAACAGGTGAGCTATGG	CAATCTCCTGTGTCTGGAT	49/49
Col-IV	TGTGACCAGGCATAGTCA	GAGCCAAAAGCTGTAAGC	48/48
LOX	GTCCATGTACAACCTGAG	TCCTGTGTAGCGAATGTC	48/48
SpO	ATGAGGGCCTGGATCTTCTTTC	CGCAGTTCTGCTTCTCAGT	54/53

The amplification reactions were performed with the following scheme: 2 min at 94 °C followed by 35 cycles of 94 °C for 30 sec, annealing temperature (specific for each gene) for 30 sec, and 72 °C for 30-60 sec, with a 10 min final extension at 72 °C. The PCR products obtained were loaded on 1.5% agarose gel, and the amplification bands were visualized and quantified with the Geliance 200 Imaging system (Perkin Elmer, Shelton, CT, USA). The amplification bands corresponding to the genes analyzed were normalized to the amplification band corresponding to the 18S. The values obtained were finally converted into percentage values, by considering the measure of the untreated controls as 100%.

### 3.6.4 cAMP intracellular levels

B16-F1 cells ( $5 \times 10^4$ ) were seeded in 6-well plates and cultivated for 16 hours at 37 °C under 5% CO<sub>2</sub> atmosphere. Then, cells were washed with PBS 1X and detached from the plate with 1 ml of non-enzymatic dissociation solution (Sigma-Aldrich, Milan, Italy). Cells were washed in PBS 1X and resuspended in stimulation buffer (0.1% BSA + 0.5% IBMX in PBS 1X), containing Alexa 647-labeled anti-cAMP antibody (1:100 dilution). Then, 12,000 cells in 6 µL volume were aliquoted in 384

well plates, and 6  $\mu$ L solution of our extracts to test in stimulation buffer were added to each well. In parallel, a standard curve for cAMP was prepared: cAMP with known concentrations were dissolved in stimulation buffer in presence of anti-cAMP antibody labeled with Alexa 647. The plates were incubated for 1 hour at room temperature. Finally 12  $\mu$ L of detection mix: streptavidin labeled with Eu-W8044 (Eu-SA), diluted 1:2200 and biotin cAMP (b-cAMP), diluted 1:625, were added.

After 2 hours, the plate was read by the multi-well reader EnVision (Perkin Elmer, Shelton, CT, USA), exciting the samples at 320 nm or 340 nm and recording the fluorescence at 615 nm and 665 nm.

Light pulse at 340 nm excites the Europium-chelate of the Eu-SA/b-cAMP tracer. The energy emitted from the Eu-chelate is transferred to the Alexa Fluor<sup>®</sup> 647 labelled anti-cAMP antibodies bound to the tracer, generating a TR-FRET signal at 665 nm. Residual energy from the Eu-chelate will produce light at 615 nm. cAMP present in the samples competes with the tracer for antibody binding sites and causes signal reduction.

### 3.6.5 MITF promoter:luciferase construct and cell transfection

The human microphthalmia-associated transcription factor promoter [Gene ID 4286, Homo sapiens chr. 3, MITF] was inspected using Genomatix<sup>®</sup> software tools (Genomatix Software GmbH; Munich, Germany), searching for transcription factor binding sites. PCR amplification was performed to isolate a fragment (572bp) of the MITF gene upstream region, including TATA box, CRE (cAMP response elements) sequences and promoter region, using the following primers: MITFpFw 5'-GCAAGAAGAGGCTGTTGAACTT-3' and MITFpRv 5'-ATCCAGTGAGAGACGGTAGACT-3'.

The PCR product was then cloned into pCR2.1 TOPO<sup>®</sup> (Invitrogen) and afterwards was sequenced (CEINGE Biotecnologie Avanzate s.c.a.r.l) using specific oligonucleotides M13 Fw and M13 Rv. The fragment resulting from *KpnI* and *XhoI* digestion (738 bp) was cloned into the multiple cloning region of the pGI3-basic vector (Promega) to produce the plasmid pGI3bMITF:Luc.

For transient transfections, B16F1 cells ( $1.5 \times 10^4$ ) were seeded in 96-well plates and cultivated for 24 hours at 37 °C under 5% CO<sub>2</sub> atmosphere, in DMEM modified medium containing 10% FBS. After incubation, each well received 100  $\mu$ L of mixture containing 150 ng of plasmid DNA and 750ng of LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) in a DMEM modified medium without serum.

After 5 hours at 37 °C under 5% CO<sub>2</sub> atmosphere, cells were incubated with 10  $\mu$ M forskolin and two different concentrations of plant extracts for 24 hours.

The day after, the medium was removed and cells were washed using a buffer solution (PBS 1X, 0.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>) and incubated for 5 min in 10 mg ml<sup>-1</sup> of luciferase substrate (Steady-Glo luciferase substrate, Promega, Madison, WI) to allow sufficient cell lysis, then luminescence was measured with a luminometer Victor3 (PerkinElmer, Shelton, CT, USA).

### Statistical analyses

Unless indicated otherwise, all data are expressed as the mean  $\pm$  standard deviation (SD) from triplicate experiments. One-way ANOVA test was used for multiple comparisons using Microsoft Excel program. A value of  $p < 0.05$  was considered significant.



**Results**

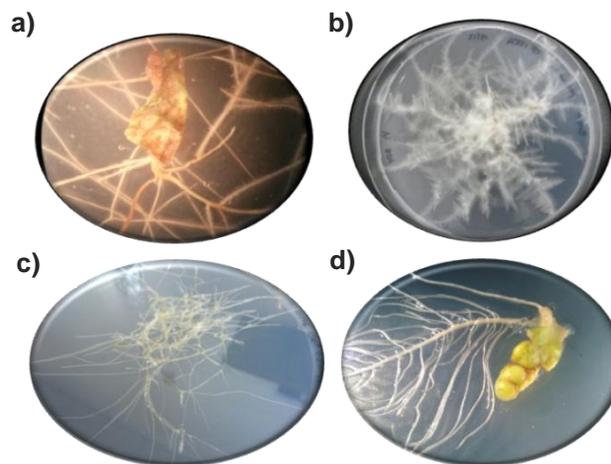


## 4. Results

### 4.1 Generation of hairy roots and molecular characterization

To induce hairy roots from leaf explants of *Cichorium intybus* (Ci), *Brassica rapa* subsp. *pekinensis* (Bp) and *Helianthus annuus* (Ha), three different strains of *Agrobacterium rhizogenes* were tested for their ability to differentiate hairy roots (A4RS, ATCC15834 and 8196). After 72 hours of co-culture, for removing *A. rhizogenes*, explants tissue were transferred to an agar-solidified half strength B5 medium containing 250 mg l<sup>-1</sup> cefotaxime. Three days after co-cultivation with the different strains, each species was susceptible to infection by a specific strain. Manual wounding resulted in more than 50% transformation frequency of the leaf explants of *C. intybus* and *H. annuus* twenty days after inoculation with *A. rhizogenes* 8196 and A4RS strain, respectively, in presence of 100 µM acetosyringone, instead *A. rhizogenes* ATCC15834 strain infected more than 70% of the leaf explants of *B. rapa* subsp. *pekinensis* thirty days after inoculation. The infection and wounding method showed unique pattern of hairy root induction with varying percentage of transformation frequency. Infection of leaf explants by manual wounding resulted in induction of hairy roots originating from the mid-vein region (Fig. 14a-c).

Hairy root initials emerged only from wound sites on leaf within 15 days after inoculation, and after 20 days, hairy roots of *B. rapa* subsp. *pekinensis* and *C. intybus* began to grow more rapidly (Fig. 14b-d).



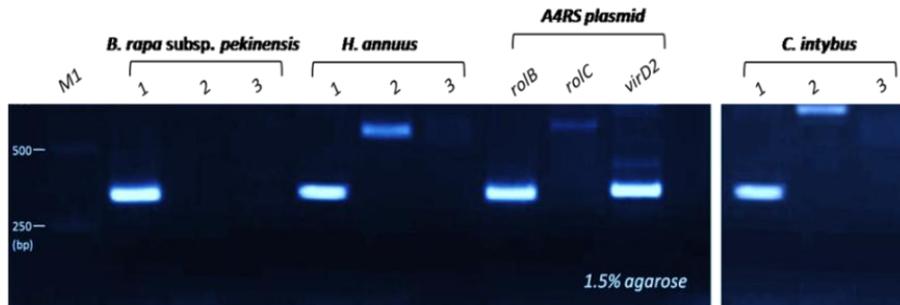
**Figure 14.** Induction of genetically modified hairy roots by manual wounding and co-cultivation with different strains of *A. rhizogenes*. a-b) Induction of *Brassica rapa* subsp. *pekinensis* hairy roots by co-cultivation with ATCC15834 strain. c) Induction of *Cichorium intybus* transformed roots by co-cultivation with *A. rhizogenes* 8196 and (d) of *Helianthus annuus* using A4RS strain.

Persisting *A. rhizogenes* contamination was eliminated by frequent subcultures on medium containing 125 mg l<sup>-1</sup> cefotaxime and after repeated transfer to fresh medium for three months, bacteria-free root tips were transferred to half strength B5 liquid culture medium.

## Results

Culturing of the hairy root samples in LB medium did not show any bacterial contamination, indicating the absence of live *Agrobacterium rhizogenes*.

The transgenic nature of hairy roots was confirmed by PCR using *rolB* specific primers in bacteria free hairy roots genomic DNA. A 290bp *rolB* expected size fragment was obtained only in hairy roots and absent in normal roots; furthermore PCR with *virD2* (301 bp) primers revealed the absence of the gene into plant genomic DNA (**Fig. 15**); plasmid DNA of A4RS strain was used as positive control.



**Figure 15.** PCR amplification of *rolB* and *virD2* genes in transformed roots of three different plant species. Lanes M1: 1kb ladder; 1: *rolB* gene detection (290 bp fragment) in transformed roots; 2: *rolB* gene detection (untransformed roots); 3: *virD2* gene detection (301 bp fragment). The PCR products were loaded on 1.5% agarose gel and the amplification bands were observed and documented using the Geliance 200 Imaging system (Perkin Elmer).

### 4.2 Sugar/peptides mixture and ethanol extract preparation

In order to obtain the sugar/peptides mixture from plant hairy root material, cell wall preparations from liquid cultures of *Cichorium intybus*, *Brassica rapa* subsp. *pekinensis* and *Helianthus annuus* were prepared, using a procedure described by Apone *et al.* (2009).

After isolation of the cell walls from the total homogenate, they were extensively washed to remove contaminating cytoplasmic components and to ensure that only proteins and glycoproteins covalently attached to the cell walls contributed to the mixture of peptides and carbohydrates obtained by chemical and enzymatic treatment of the cell wall preparation. Harvested frozen hairy roots of *Brassica rapa* subsp. *pekinensis* were processed to obtain  $707 \pm 20.5$  mg of powder extract  $100 \text{ g}^{-1}$  of material (FW), instead from hairy roots of *Cichorium intybus* and *Helianthus annuus*,  $880 \pm 12.5$  mg  $100 \text{ g}^{-1}$  FW and  $620 \pm 20.2$  mg  $100 \text{ g}^{-1}$  FW were obtained, respectively.

Crude ethanol extracts were obtained by mechanically grinding of harvested hairy roots in ethanol (EtOH) pure grade ~96% (v/v) in the ratio 1:4 (w/v), which were left to macerate in stirring condition for 4 hours. The homogenized product was then filtered and the soluble fraction was collected and concentrated hundred times (100X) using a rotary vacuum evaporator, to get rid of excess solvent.

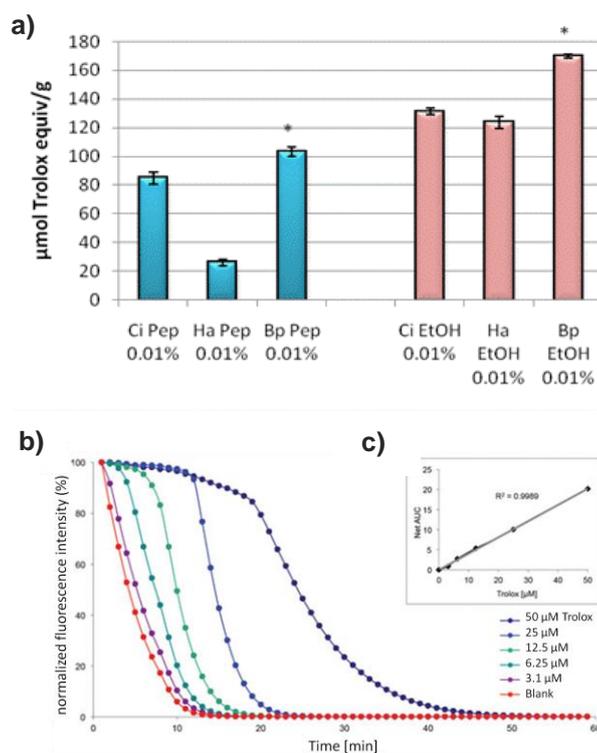
From 50 g (FW) of each species,  $2 \pm 0.2$  ml of liquid extract were obtained and used for biological assays.

### 4.3 Preliminary anti-oxidant characterization

It is well-known that antioxidant agents are capable of scavenging the ROS generated in the skin and can inhibit the stimulation of melanogenesis (Masaki, 2010). Therefore, a series of preliminary assays were conducted to measure the total anti-oxidant power of the plant extracts both *in vitro* and in cultured skin-derived NIH-3T3 cells.

Antioxidant activity was evaluated by Oxygen Radical Absorbance Capacity (ORAC) assay. Dilutions of the ethanol extracts and sugar/peptide mixtures were mixed with fluorescein, the reaction started by the addition of AAPH and the values of reducing capacity were expressed as  $\mu\text{mol}$  of Trolox  $\text{g}^{-1}$ . The total reducing capacity of the mixtures were calculated from the results shown in **Fig. 16**.

The highest values were obtained for *Brassica rapa* subsp. *pekinensis* hairy roots: it was  $1.03 \times 10^2 \mu\text{mol}$  of Trolox per gram of peptide/sugar extract and  $1.72 \times 10^2 \mu\text{mol}$  of Trolox per gram of ethanol extract, values comparable to those of other anti-oxidant compounds or extracts used in cosmetics (Yildiz *et al.*, 2008; Huang *et al.*, 2002).



**Figure 16.** Total anti-oxidant power of sugar/peptides mixtures and ethanol extracts by ORAC assay. A concentration of the extracts (expressed as %, w/v) was analyzed for its anti-oxidant power by the Oxygen Radical Absorbing Capacity (ORAC) assay. **a)** The values reported in the graph are averages of three independent measures, derived from 3 different experiments, and the error bars represent standard deviations (\* $p < 0.5$ ). **b)** Signal curves measured with different Trolox concentrations (results normalized to initial fluorescence signals after AAPH addition). **c)** Linearity of Trolox standard curve.

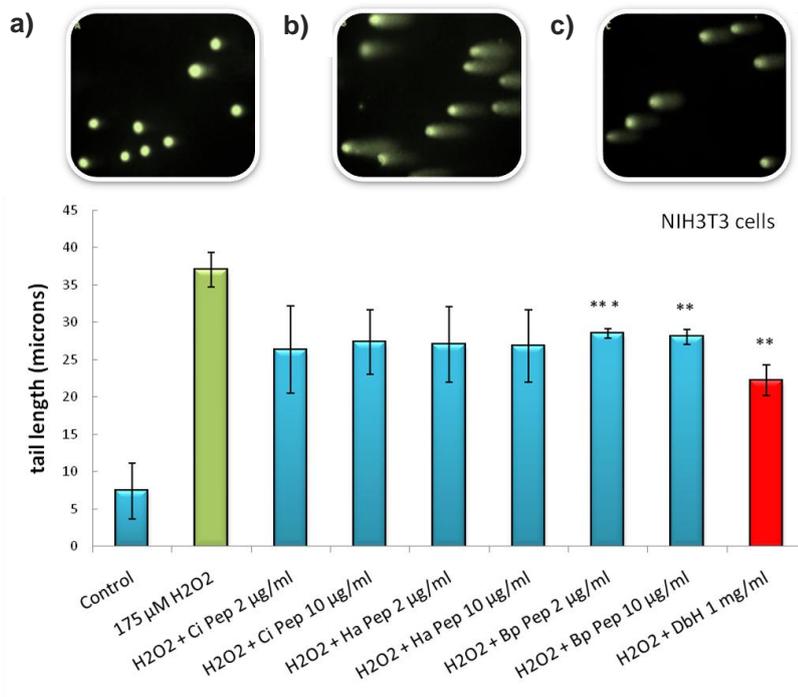
## Results

Before performing additional cellular assays, the IC<sub>50</sub> of the sugar/peptides mixture of *Brassica rapa* subsp. *pekinensis* was determined, in both NIH-3T3 fibroblasts and HaCaT keratinocytes.

Viability of cells was not affected by treatments with the sugar/peptides mixture at a concentration of 2 µg ml<sup>-1</sup> and 10 µg ml<sup>-1</sup> and ethanol extract at a concentration of 100 µg ml<sup>-1</sup> and 300 µg ml<sup>-1</sup> (data not shown).

The protective effect against stressing agent, such as H<sub>2</sub>O<sub>2</sub>, of the sugar/peptides mixture on cell genomic DNA was tested by the “single cell electrophoresis assay” or Comet assay. This assay is based on the direct measure of DNA fragmentation, an index of the entirety of DNA damages produced by the oxidative burst.

As shown in **Fig. 17**, the treatment with the lowest concentration of sugar/peptides mixture of *Brassica rapa* subsp. *pekinensis* produced a clear protection of genomic DNA from damages caused by H<sub>2</sub>O<sub>2</sub>.



**Figure 17. Comet assay.** Pictures show the damages to cell nuclear DNA produced by 175 µM H<sub>2</sub>O<sub>2</sub> treatment (**b-c**) compared to an untreated control (**a**). Cells in the panel **c** were previously incubated with 2 µg ml<sup>-1</sup> of *Brassica* sugar/peptides mixture for 16 h. Each value reported in the graph was calculated in microns as average of 100 different measures (\*\*p<0.01; \*\*\*p<0.001).

The length of the nucleus comet, index of DNA fragmentation, is significantly reduced in the cells treated with the sugar/peptides mixture compared to the samples treated with only H<sub>2</sub>O<sub>2</sub>. A sample of cells was treated with another plant cell product (DbH-extract), developed by Arterra Bioscience S.r.l., known to contain compounds with protective effects on the cells from DNA-induced damages (Bimonte *et al.*, 2014). This product is a total hydrosoluble *Dolichos biflorus* cell

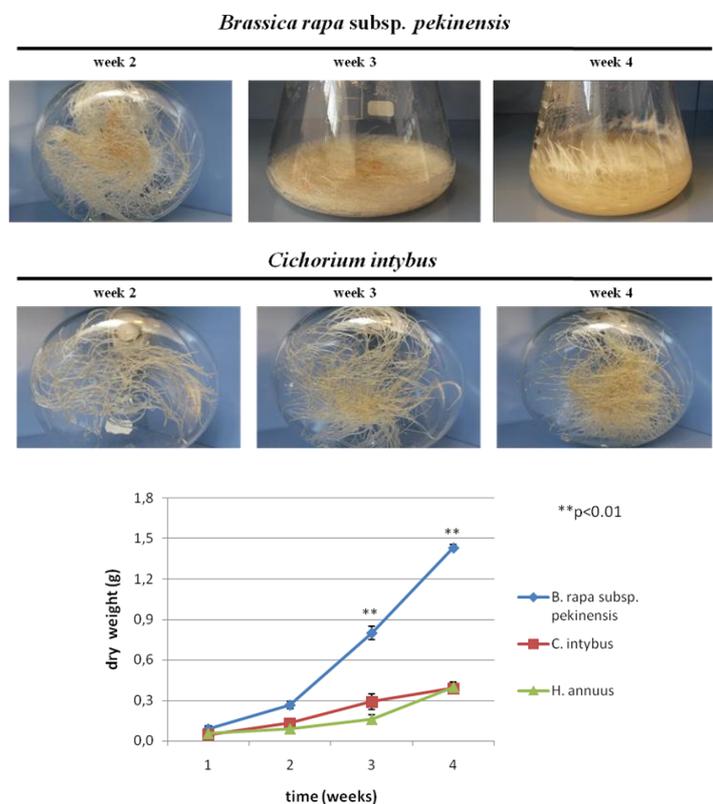
culture extract at a stock concentration of  $0.1 \text{ g ml}^{-1}$ , and already used as active ingredient in cosmetic formulations (INCI name: Dolichos Biflorus Callus Extract/ Glycerin/ Water, CAS no. 93348-15-3/56-81-5/7732-18-5). In the assay, the stock was diluted in water 100 times (0.1% final, corresponding to  $1 \text{ mg ml}^{-1}$ ).

The graph reported in **Fig. 17** shows that the sugar/peptides mixture of *Brassica rapa* subsp. *pekinensis* significantly decreased the tail length compared to the other extracts ( $23 \pm 1.2 \%$ ), when used in a concentration of  $2 \mu\text{g ml}^{-1}$ .

#### 4.4 Small scale cultivation and growth rate kinetics

Plant biomass is a critical step toward the development of an industrial platform aimed to a reliable, constant and standardized extraction of plant-derived active ingredients. Growth kinetics and final biomass of the three plant species, were determined in a small-scale and orbitally shaken system and Erlenmeyer flasks.

Fresh and dry weights of samples were determined every week and, as shown in **Fig. 18**, hairy roots of *Brassica rapa* subsp. *pekinensis* exhibited a greater biomass production after a 30 day-culture period. The growth rate for the hairy root cultures of *Cichorium intybus* and *Helianthus annuus* was  $1.1 \pm 0.35 \text{ g l}^{-1}\text{d}^{-1}$  and  $1.1 \pm 0.23 \text{ g l}^{-1}\text{d}^{-1}$ , while the growth rate of the hairy root cultures of *Brassica rapa* subsp. *pekinensis* was  $2.9 \pm 0.14 \text{ g l}^{-1}\text{d}^{-1}$ .



**Figure 18.** Growth rate kinetics of *B. rapa pekinensis*, *H. annuus* and *C. intybus* hairy roots. The pictures show the biomass production during 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> week. Values of dry weights reported in the graph, were calculated in grams as average of 3 independent experiments at different end-points and the error bars represent standard deviations (\*\* $p < 0.01$ ).

## Results

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On the basis of these promising preliminary results on total anti-oxidant activity and growth rate, it was decided to focus our attention on *Brassica rapa* subsp. *pekinensis* hairy roots, to develop different extraction procedures and to better characterize the biological activities of these extracts as new plant-derived products with potential applications as depigmenting agents.

### 4.5 Inhibitory effects of hairy root extracts on melanin content and tyrosinase activity

To investigate whether the sugar/peptides mixture and crude ethanol extract from hairy roots of *Brassica rapa* subsp. *pekinensis* exerted cytotoxic effects, they were tested in B16-F1 murine melanoma cells, that provide an excellent cell line for examining the melanin content and tyrosinase activity after treatments with different plant-derived compounds. B16-F1 cells were treated with different concentrations of the sugar/peptides mixture and crude ethanol extract for three days.

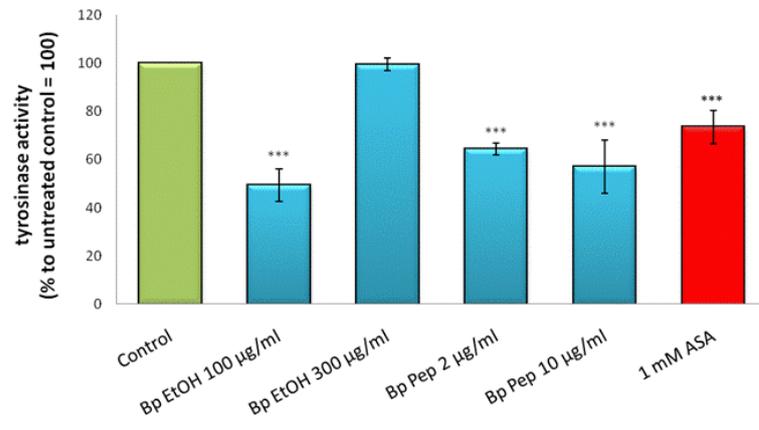
Viability of B16-F1 cells, measured via MTT assay, was not different from control cells (treated only with water and ethanol), when incubate with the sugar/peptides mixture at a concentration of  $2 \mu\text{g ml}^{-1}$  and  $10 \mu\text{g ml}^{-1}$  and ethanol extract at a concentration of  $100 \mu\text{g ml}^{-1}$  and  $300 \mu\text{g ml}^{-1}$  (data not shown).

To characterize the effects of both extracts, melanin content was measured in treated and un-treated B16-F1 cells, while tyrosinase activity was determined in HEMA-LP (human epidermal melanocytes) and B16-F1 cells.

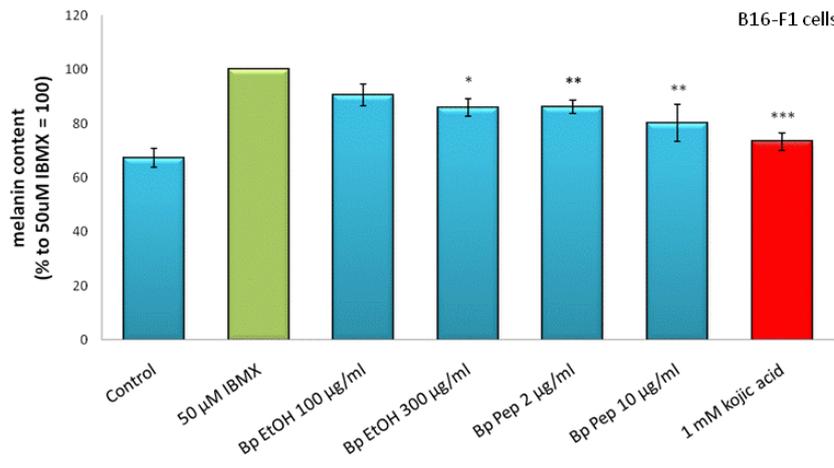
Kojic acid, ASA (acetylsalicylic acid) and ascorbic acid were used as positive controls, on the basis of their known inhibitory effects on melanin synthesis. The levels of melanin in B16-F1 cells and tyrosinase activity in both B16-F1 and HEMA-LP cells were significantly reduced as a result of different treatments.

As shown in **Fig. 19**, the concentrations of  $10 \mu\text{g ml}^{-1}$  and  $100 \mu\text{g ml}^{-1}$ , respectively of sugar/peptides mixture and ethanol extract caused a  $38 \pm 2.5\%$  and  $51 \pm 6.7\%$  inhibition of tyrosinase activity in B16-F1, suggesting an inhibitory activity of both the extracts, even higher than that of the positive control acetylsalicylic acid ( $26.5 \pm 6.8\%$  inhibition). However, ethanol extract did not exert any significant inhibitory effect on tyrosinase activity in B16-F1, when used in a higher dose of  $300 \mu\text{g ml}^{-1}$ . This might be explained by the nature of our extracts, given that they are not purified molecules but mixtures of compounds, it could be possible that some other components cancel the inhibitory effects exerted at lower concentrations, when extracts are used at higher doses.

All the extracts at both concentrations caused a slight decrease of melanin synthesis (15% - 20%) as shown in **Fig. 20**, and melanin was significantly reduced in a dose dependent manner after treatments in the presence of IBMX (to raise cAMP levels and enhance melanogenesis).



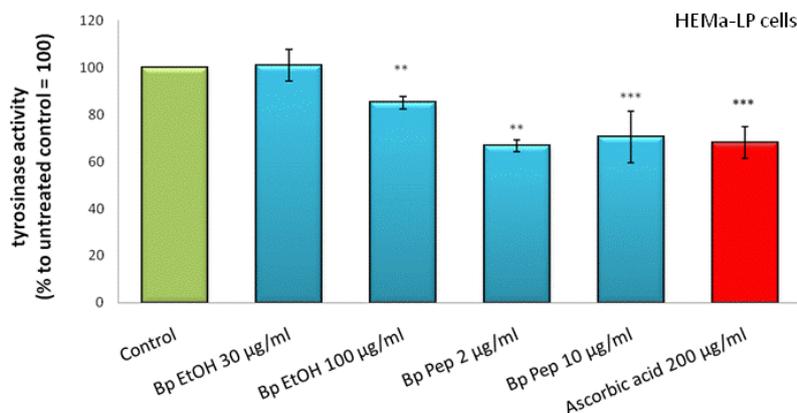
**Figure 19. Tyrosinase activity in B16-F1 cells.** Cells were treated with different concentrations of sugar/peptides mixture and ethanol extract of *Brassica* hairy roots, and with acetylsalicylic acid. After treatments of 72 h, 50 µg of each sample were incubated with L-DOPA. The values are mean of independent measures obtained from 3 different experiments, and expressed as percentage to the untreated control, arbitrarily set as 100% (\*\*\*p<0.001).



**Figure 20. Melanin content in B16-F1 cells.** Cells were treated with different amounts of sugar/peptides mixture and ethanol extract of *Brassica* hairy roots, and with 1 mM kojic acid. After treatments of 72 h in presence of 50 µM IBMX, samples were analyzed as described in section 3.6.1. The values are mean of independent measures obtained from 3 different experiments, and expressed as percentage to 50 µM IBMX control, arbitrarily set as 100% (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001).

## Results

In addition, sugar/peptides mixture at the same concentrations also decreased tyrosinase activity by  $29.6 \pm 3.1\%$  in HEMa-LP (**Fig. 21**), comparable to the reduction exerted by the positive control ascorbic acid used at a concentration 20 times higher ( $10 \mu\text{g ml}^{-1}$  versus  $200 \mu\text{g ml}^{-1}$ ), whereas for ethanol extracts it was necessary to decrease 3 times the concentrations to avoid cytotoxic effects on cells.

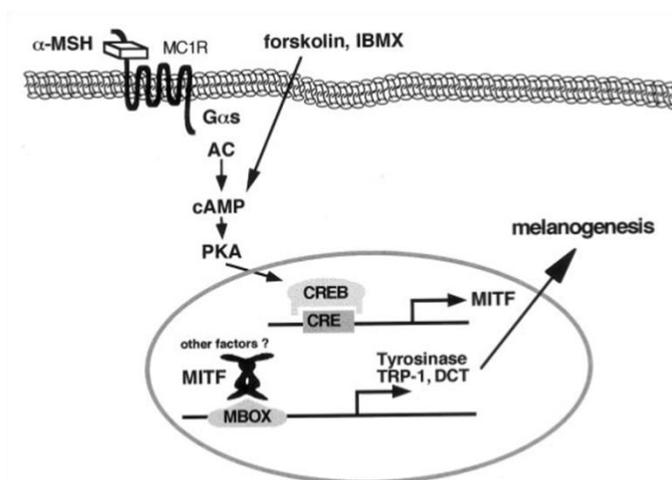


**Figure 21. Tyrosinase activity in HEMa-LP cells.** Cells were treated with different amounts of sugar/peptides mixture and ethanol extract of *Brassica* hairy roots, and with  $200 \mu\text{g ml}^{-1}$  ascorbic acid. After treatments of 24 h,  $50 \mu\text{g}$  of each sample were incubated with L-DOPA as described in section 3.6.2. The values are mean of independent measures obtained from 2 different experiments, and expressed as percentage to the untreated control, arbitrarily set as 100% (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

Altogether these findings suggest that both extracts, but especially the sugar/peptides mixture, from hairy roots of *Brassica rapa* subsp. *pekinensis* are able to regulate the tyrosinase activity and, subsequently, reduce melanin synthesis in melanoma cells and human epidermal melanocytes, with a final hypopigmenting effect.

#### 4.6 Inhibitory effects on intracellular cAMP levels

Melanogenesis is known to be controlled by a signalling pathway, which is regulated not only at the level of proteins. As shown in **Fig. 22**, cyclic adenosine monophosphate (cAMP), an important second messenger which mediates many different physiological responses, is also involved into melanogenesis pathway.

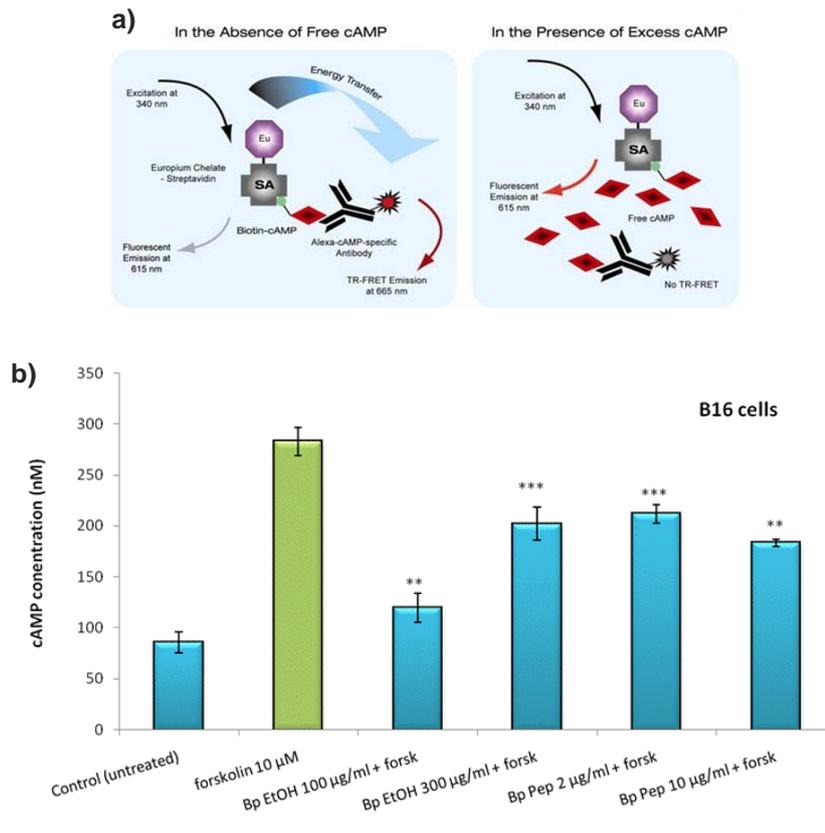


**Figure 22.** The melanogenesis pathway.  $\alpha$ -MSH binds to and activates the Gs protein-coupled MC1R. The G proteins transmit signals from MC1R to AC which catalyses the conversion of ATP to cAMP. Increased levels of cAMP act as a second messenger to activate PKA. Phosphorylated CREBs then induce the expression of genes as the transcription factor MITF. (Source: Bentley et al., 1994).

The LANCE<sup>®</sup> cAMP assay was used to test potential effects of *Brassica rapa* subsp. *pekinensis* extract on cAMP level. As shown in **Fig. 23a**, this assay is a homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay designed to measure cAMP produced upon modulation of adenylyl cyclase activity by GPCRs.

B16-F1 cells were incubated with both extracts for 1 hour in presence of 10  $\mu$ M forskolin (to raise cAMP levels) and as shown in **Fig. 23b**, treatments with 10  $\mu$ g ml<sup>-1</sup> of peptide/sugar mixture from hairy roots of *Brassica rapa* subsp. *pekinensis* and 100  $\mu$ g ml<sup>-1</sup> of ethanol extract produced a reduction of cAMP levels of 35.8  $\pm$  1.2% and 45  $\pm$  6.7%, respectively.

## Results



**Figure 23. Intracellular content of cAMP.** **a)** LANCE cAMP assay principle. **b)** cAMP intracellular levels in B16-F1 cells after incubation for 1h with different concentrations of *Brassica* root extracts (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

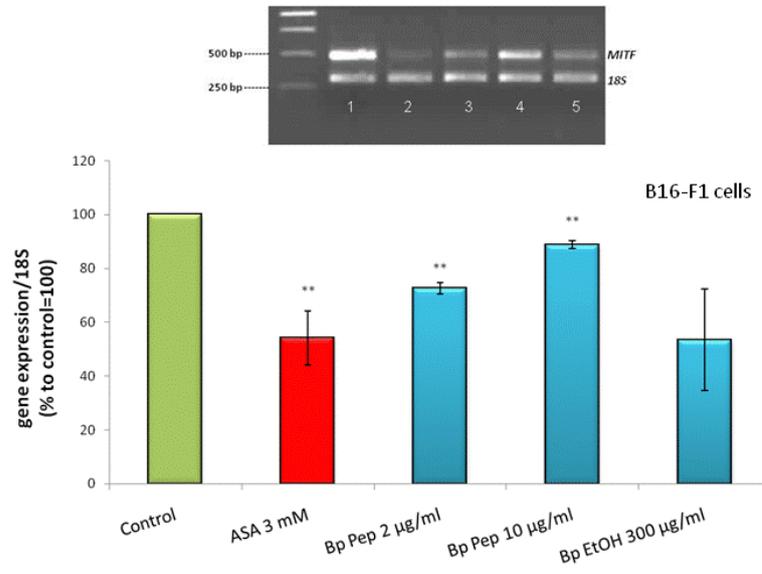
### 4.7 MITF gene expression and promoter activity by a luciferase-reporter assay system

It is well known that MITF (microphthalmia-associated transcription factor) is implicated in melanocytes development and in the regulation of melanogenesis. MITF is thought to bind to the M-box promoter elements of tyrosinase, tyrosinase-related protein-1 and dopachrome tautomerase/tyrosinase related protein-2 and transactivate these genes, resulting in increased pigmentation (Bentley *et al.*, 1994) (Fig. 22).

As MITF controls tyrosinase protein levels, and since a decreased MITF gene expression may be associated to a reduced level of MITF protein, extracts from *Brassica rapa* subsp. *pekinensis* were tested for their potential effects on MITF gene expression.

Semi-quantitative RT-PCR analysis from RNA extracted from B16-F1 treated and untreated with plant extracts, using MITF-specific primers, revealed a significant suppression of MITF gene expression. Incubation for 6 hours with 2  $\mu\text{g ml}^{-1}$  of

peptide/sugar mixture and  $300 \mu\text{g ml}^{-1}$  of ethanol extract, produced a  $25 \pm 5.7\%$  and  $46.5 \pm 18.8\%$  reduction of MITF gene expression, respectively, as shown in **Fig. 24**.



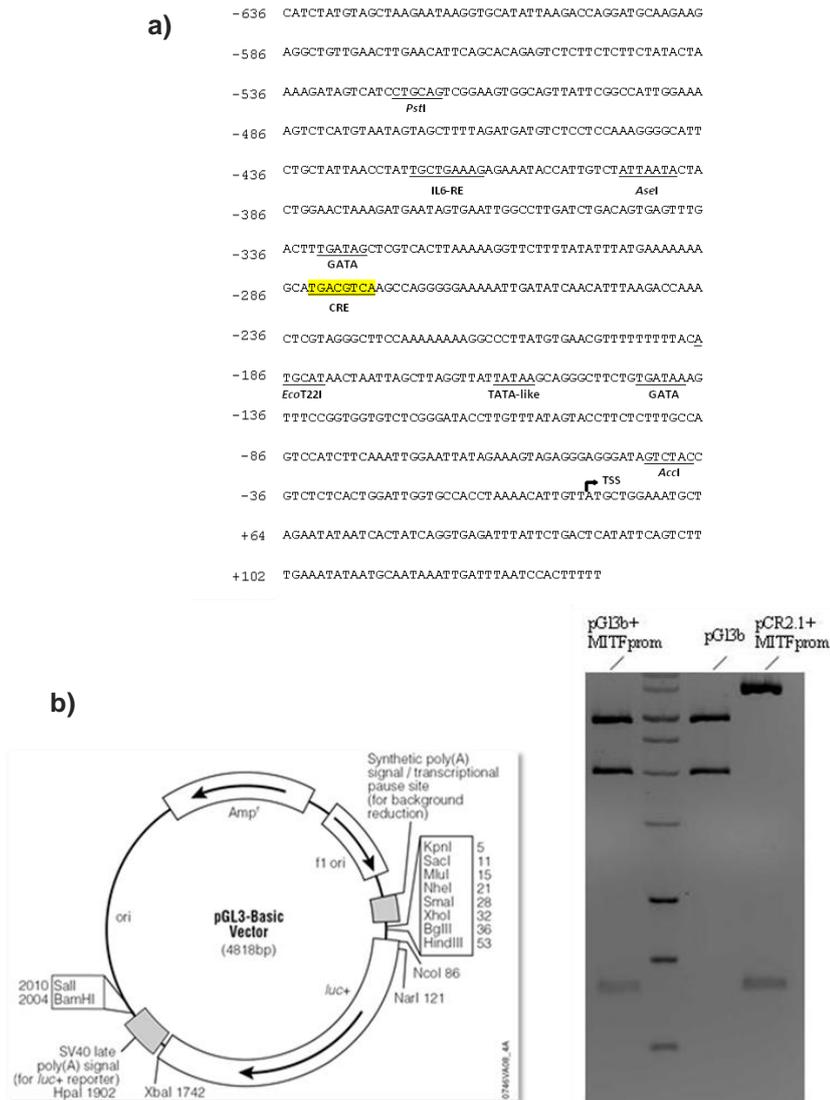
**Figure 24.** RT-PCR analysis of genes expression in B16-F1. Cells were treated with different amounts of peptide/sugar mixture and ethanol extract of *Brassica* hairy roots. The picture show the amplification bands of the gene MITF. The values obtained were normalized to the reference standard 18S and reported in the graph below the pictures expressed as percentage to the control, set as 100%. Lane 1, control; lane 2, 3 mM ASA; lane 3,  $2 \mu\text{g ml}^{-1}$  Bp Pep; lane 4,  $10 \mu\text{g ml}^{-1}$  Bp Pep; and lane 5,  $300 \mu\text{g ml}^{-1}$  Bp EtOH (\*\* $p < 0.01$ ).

To further confirm whether or not extracts from *Brassica rapa* subsp. *pekinensis* hairy roots may modulate and inhibit the transcriptional activity of the MITF, a luciferase-reporter vector driven by MITF promoter was generated.

The region of the human MITF gene from -636bp to + 102bp was analyzed, using the software Genomatix<sup>®</sup>, and a CRE (cAMP Responsive Element), consensus sequence (TGACGTCA) was located in the region upstream the TSS (**Fig. 25**).

By using specific primers, a 572 bp fragment was amplified from genomic DNA extracted from human epidermal melanocytes cells and then cloned into the expression vector pCR2.1 TOPO<sup>®</sup>. Subsequently, a 738 bp fragment was generated through double enzymatic digestion with *KpnI* and *XhoI* of the shuttle vector and inserted upstream of the firefly luciferase gene in a *pGL3basic* vector (Promega).

## Results

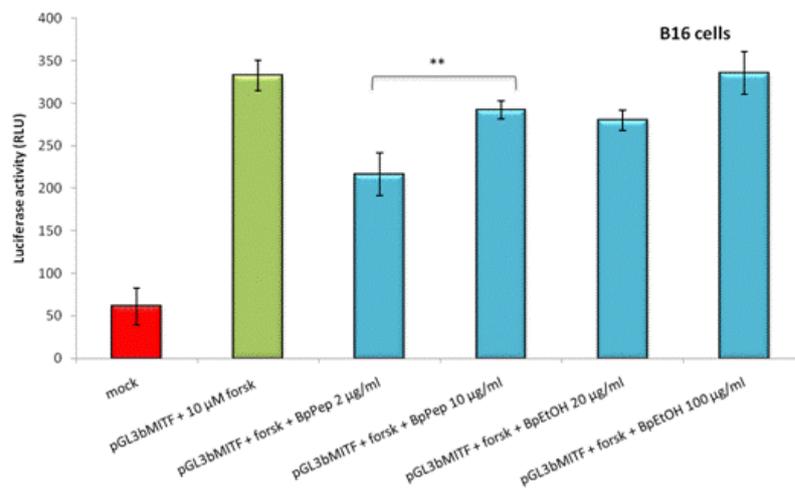


**Figure 25. Nucleotidic sequence of human MITF promoter** (from -636bp to + 102bp). **a)** Consensus sequences are indicated, including also a CRE box. **b)** A 738 bp fragment, generated through double enzymatic digestion with *KpnI* and *XhoI* of the shuttle vector, was inserted up-stream of the firefly luciferase gene in a pGL3basic vector (Promega).

To evaluate the effects of plant extracts on MITF promoter activity, murine B16-F1 melanoma cells were transiently transfected with the human MITF promoter-luciferase construct. Validation of the vector with 10  $\mu$ M forskolin, a known cAMP elevating agent, revealed an induction of MITF promoter activity ( $545 \pm 5.7\%$ ) compared with the untreated control. Therefore, forskolin was used as a positive control in the experiments.

As shown in **Fig. 26**, there was a significantly reduction ( $35 \pm 9.8\%$ ) of MITF promoter activity after 24 hours of incubation with the lowest concentration of peptide/sugar mixture ( $2 \mu\text{g ml}^{-1}$ ) from the hairy roots of *Brassica rapa* subsp. *pekinensis* and with the concentration of  $20 \mu\text{g ml}^{-1}$  ( $18 \pm 3.4\%$  reduction), confirming the inhibitory effects seen on MITF gene transcription.

This luciferase reporter vector is a valuable tool for rapid screening of large collection of plant extracts and will contribute to further optimize the industrial platform of production and screening of new plant-derived skin de-pigmenting actives we are currently developing at Arterra Bioscience S.r.l



**Figure 26. MITF promoter activity in B16-F1.** Cells were transfected with the MITF prom:luciferase construct and the promoter activity was monitored after incubation for 24h with different concentrations of *Brassica* root extracts and 10  $\mu\text{M}$  forskolin (\*\* $p < 0.01$ ).

## Results

### 4.8 Effects of *B. rapa* subsp. *pekinensis* extracts on the extracellular matrix (ECM) assembly

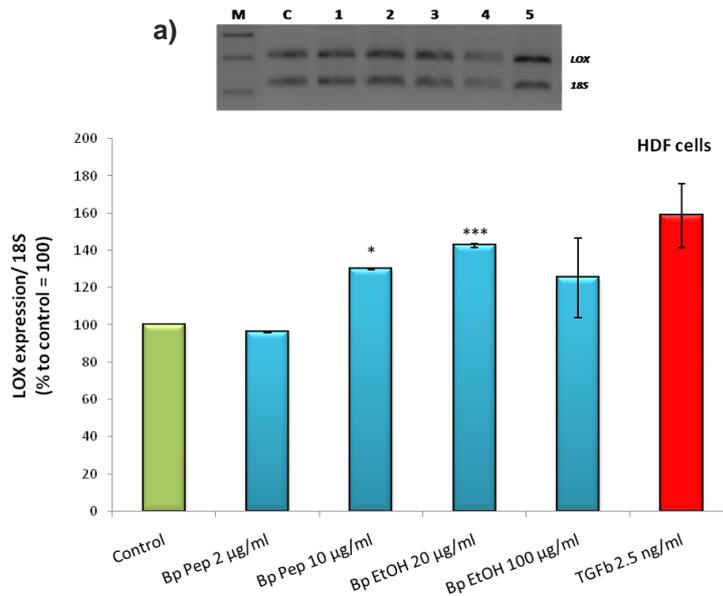
A clear, bright and uniform aspect of the skin is also due to a correct organization of the dermal extracellular matrix (ECM). ECM is composed of an interlocking mesh of fibrous proteins (especially collagen and elastin) and glycosaminoglycans (GAGs) and it may serve many functions, such as providing support, segregating tissues from one another, and regulating intercellular communication.

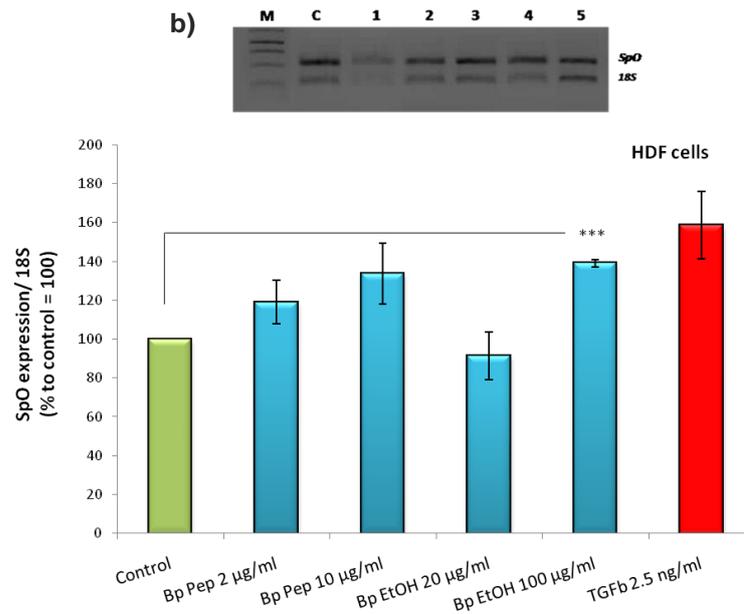
For these reasons, sugar/peptides mixture and ethanol extract of *Brassica rapa* subsp. *pekinensis* roots were also tested for their potential effect on the expression of genes involved in the ECM assembly.

Lysyl oxidase (LOX), also known as protein-lysine 6-oxidase, is a protein encoded by the *LOX* gene, responsible of cross-linking collagen and elastin, which is essential for stabilization of collagen fibrils and for the integrity and elasticity of skin. After 6 hours of treatment, HDF cells (human dermal fibroblast) were collected and the expression of *LOX* gene analyzed by RT-PCR. As shown in **Fig. 27a**, the treatment with the ethanol extract (20  $\mu\text{g ml}^{-1}$ ) induced a significant increase in LOX expression ( $40 \pm 2.3\%$ ,  $***p < 0.001$ ) comparable to that exerted by positive control treated with 2.5  $\text{ng ml}^{-1}$  TGF $\beta$ .

Secreted Protein Acidic and Rich in Cysteine (SPARC, osteonectin) is a highly conserved matricellular protein, encoded by the *SpO* gene, and a critical factor in the formation of collagenous ECM (Bradshaw, 2009). SPARC has at least two roles in collagen fibril assembly – one that modulates interaction of collagen with the cell surface and another that regulates collagen incorporation to fibrils.

In parallel experiments, the expression of *SpO* was analyzed by RT-PCR in HDF cells treated with different concentrations of sugar/peptides mixture and ethanol extract. As shown in **Fig. 27b**, the treatment with the ethanol extract (100  $\mu\text{g ml}^{-1}$ ) produced a significant increase in *SpO* expression ( $39 \pm 1.8\%$ ,  $***p < 0.001$ ) comparable to that exerted by the positive control ( $57 \pm 17.2\%$ ,  $*p < 0.05$ ).





**Figure 27. RT-PCR analysis of gene expression in HDF.** Cells were treated with different amounts of peptide/sugar mixture and ethanol extract of *Brassica* hairy roots. The pictures show the amplification bands of the gene *LOX* (a) and *SpO* (b). The values obtained were normalized to the reference standard 18S and reported in the graphs below the pictures expressed as percentage to the untreated control, set as 100%. Lane M: 1 kb ladder; lane C: untreated control; lane 1: 2 µg ml<sup>-1</sup> Bp Pep; lane 2: 10 µg ml<sup>-1</sup> Bp Pep; lane 3: 20 µg ml<sup>-1</sup> Bp EtOH; lane 4: 100 µg ml<sup>-1</sup> Bp EtOH; and lane 5: 2.5 ng ml<sup>-1</sup> TGFβ (\*p<0.05; \*\*\*p<0.001).

#### 4.9 Effects of hairy root extracts on basal lamina assembly

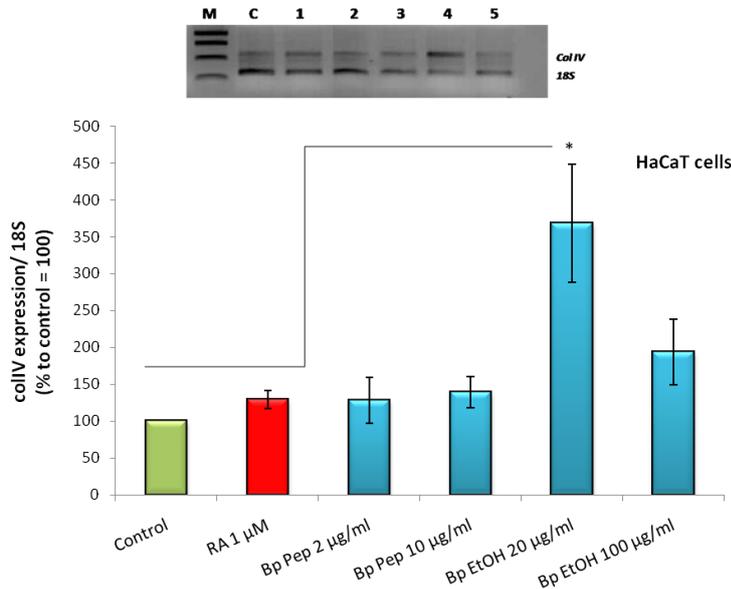
Many plant extracts used in cosmetics, besides their role as depigmenting products and enhancers of ECM proteins expression, have also been studied for their effects in inducing the synthesis of new basal lamina proteins (Knott *et al.*, 2008). These structures can positively interact with ECM components in conferring a stable and uniform distribution of the different top cells, included melanocytes responsible for melanin production. Of all the common components of the skin basement membrane, namely, laminin, proteoglycans, entactin, nidogen, fibronectin, type VII collagen, as well as collagen IV, the latter is the predominant type. Type IV collagen occurs uniquely in basement membranes and is responsible for their structural integrity, mechanical stability and for mediating their attachment to other components of the extracellular matrix and to cell membranes.

Therefore, we also analyzed whether the two different extracts from *Brassica rapa* subsp. *pekinensis* hairy roots had any effect also on the collagen type IV and laminins gene expression.

To evaluate the production of new collagen by cultured immortal human keratinocytes (HaCaT), cells were incubated with 2 different concentrations of the mixtures, as well as with 1 µM retinoic acid as control, and then collagen type IV gene (*ColIV*) expression was analyzed.

## Results

As reported in **Fig. 28**, only the ethanol extract of *Brassica rapa* subsp. *pekinensis* hairy roots produced a significant effect of increasing collagen IV gene expression: this increase was about  $268 \pm 60\%$  ( $*p < 0.05$ ) in the samples treated with  $20 \mu\text{g ml}^{-1}$  of mixture, significantly higher than that produced by retinoic acid.

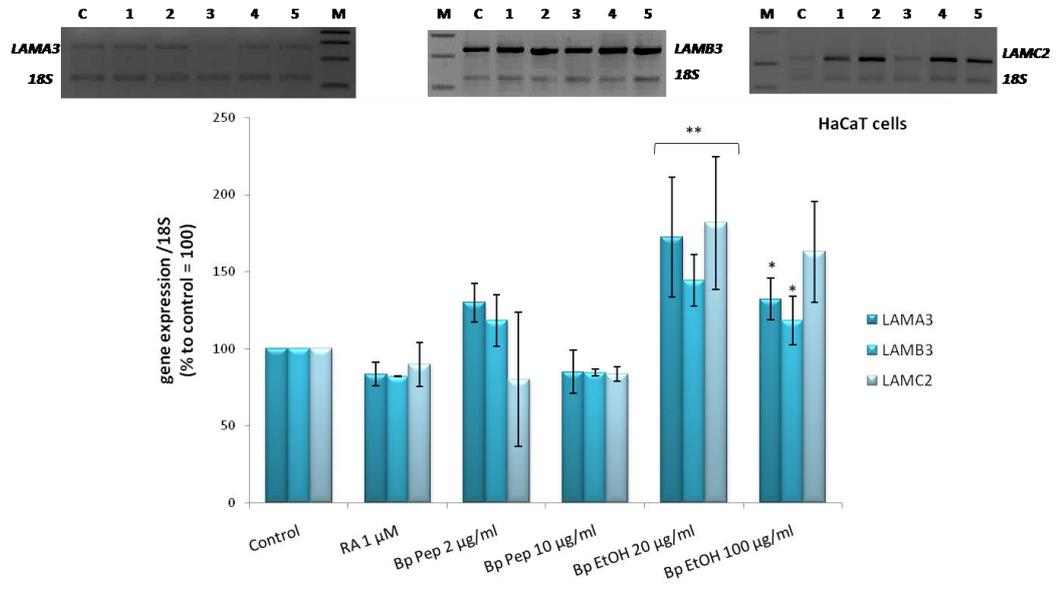


**Figure 28.** CollIV gene expression levels in cells treated with the two extracts of *Brassica* hairy roots. HaCaT cells were treated and incubated for 6 hours with the indicated concentrations, then RNA was extracted and the expression level of CollIV was measured by RT-PCR. Lane M: 1 kb ladder; lane C: untreated control; lane 1: 1  $\mu\text{M}$  retinoic acid; lane 2: 2  $\mu\text{g ml}^{-1}$  Bp Pep; lane 3: 10  $\mu\text{g ml}^{-1}$  Bp Pep; lane 4: 20  $\mu\text{g ml}^{-1}$  Bp EtOH; lane 5: 100  $\mu\text{g ml}^{-1}$  Bp EtOH ( $*p < 0.05$ ).

Laminin 5 ( $\alpha 3\beta 3\gamma 2$ ) is a heterotrimeric protein that plays an essential role in epidermal adhesion (Carter *et al.*, 1991). In skin, laminin 5 is localized to the interface of the *lamina lucida* and the *lamina densa* where it is thought to form a critical link between hemidesmosome-anchoring filament complexes, the *lamina densa*, and underlying anchoring fibrils.

In another series of experiments, the potential laminin 5 induction following the treatment with both peptide/sugar mixture and ethanol extract was studied by analyzing the expression of laminin 5 subunits genes (*LAMA3*, *LAMB3*, *LAMC2*). Also in this case, exclusively the treatment with ethanol extracts exerted a significant induction of genes expression in HaCaT cells: this increase was about 30%, 20% and 60% ( $**p < 0.01$ ), for *LAMA3*, *LAMB3* and *LAMC2* respectively, in the samples treated with  $20 \mu\text{g ml}^{-1}$  of extract (**Fig. 29**).

This results suggest that the root-derived ethanol extracts from *Brassica rapa* subsp. *pekinensis*, besides its positive effect on the expression of *LOX* and *SpO* genes, encoding proteins responsible for extracellular matrix assembly, has also an effect of inducing genes for new collagen and laminin synthesis, which may confer a final bright and uniform aspect to the skin.



**Figure 29. LAMA3, LAMB3 and LAMC2 genes expression levels.** HaCaT cells were treated with different concentrations of two extracts of *Brassica* hairy roots. Cells were treated and then RNA was extracted and the expression levels were measured by RT-PCR. Lane M: 1 kb ladder; lane C: untreated control; lane 1: 1 μM retinoic acid; lane 2: 2 μg ml<sup>-1</sup> Bp Pep; lane 3: 10 μg ml<sup>-1</sup> Bp Pep; lane 4: 20 μg ml<sup>-1</sup> Bp EtOH; lane 5: 100 μg ml<sup>-1</sup> Bp EtOH (\*p<0.05; \*\*p<0.01).

## Results

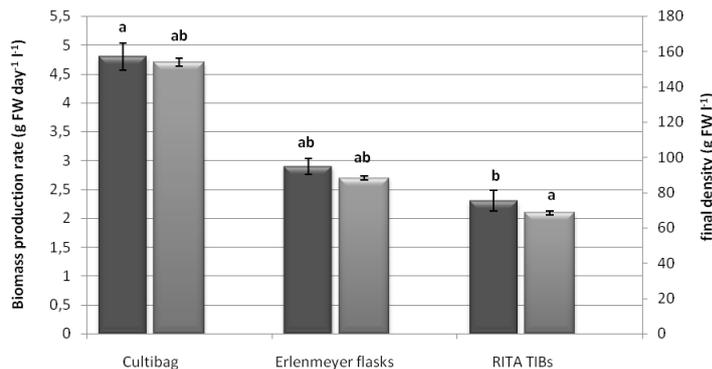
### 4.10 Toward a scale-up production of hairy root culture

Production in temporary immersion bioreactors (TIB – RITA<sup>®</sup>) and in wave-mixed bags (CultiBag), were investigated in collaboration with the VTT, Technical Research Centre of Finland.

The bioreactor scale-up studies were carried out using the best-performing hairy root clone of *Brassica rapa* subsp. *pekinensis*. The main challenge was the heterogenic morphology of hairy roots, as well as their irregular growth and their higher shear stress sensitivity.

Three different growth systems for hairy growth were compared in terms of final biomass and two parameters, pH and conductivity, were monitored offline. All the experiments were performed in triplicate and the final biomass productivity rate was  $4.8 \pm 0.23 \text{ g l}^{-1} \text{ d}^{-1}$  (FW) in the wave-mixed bioreactor and  $2.3 \pm 0.18 \text{ g l}^{-1} \text{ d}^{-1}$  (FW) in the TIB. Scale-up experiments demonstrated the successful transfer from basic culture in shaken flasks to wave-mixed bag where the final density of culture was ca. 1.7-fold higher than in the first case, combined with less manipulations and as a consequence with less contamination events.

As shown in **Fig. 30**, there were significant differences in biomass growth when the hairy roots were cultivated in wave-mixed bag compared to the growth in TIB (Temporary Immersion Bioreactors) or in orbitally-shaken flasks. There was a slight difference in term of biomass productivity when hairy roots were cultured in TIBs or in flasks (ca. 1.2-fold higher than the temporary immersion system), although TIB system could be improved to take advantage of less manipulations and contaminations.

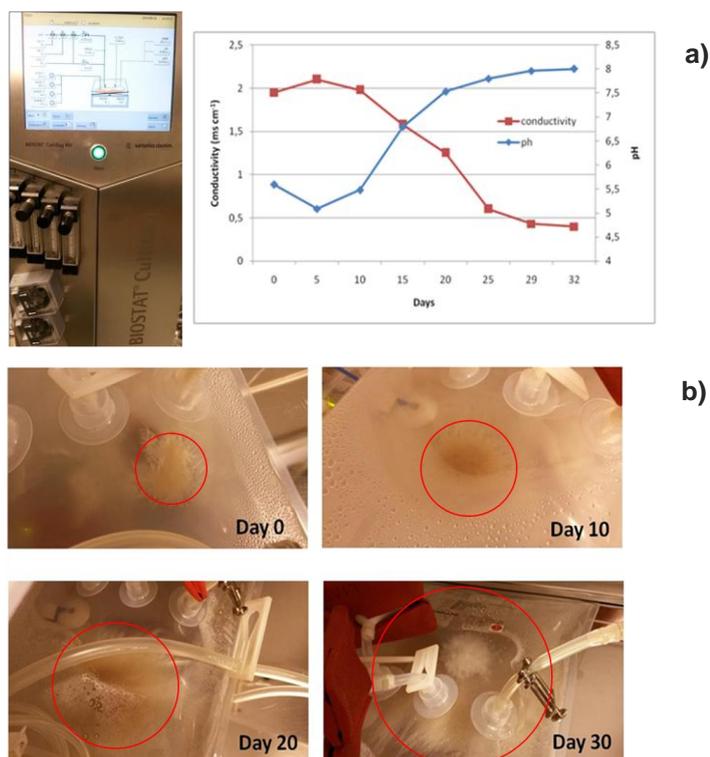


**Figure 30.** Biomass production rate and final density in *Brassica rapa* subsp. *pekinensis* hairy root (HR) lines. Presented values are means  $\pm$  SD of three replicates, after one month of cultivation in 3 different systems. Within each parameter values with the same letter are not significantly different at the  $p \leq 0.01$  level according to the LSD test.

As shown in **Table 1**, the highest final density and biomass production rates were achieved in wave-mixed bioreactor, followed by flasks and TIB. The culture bag contained a viable, white and hairy biomass with a core of thick brown roots as shown in **Fig. 31**.

**Table 1.** Specific density and biomass productivity rate of *Brassica rapa* subsp. *pekinensis* hairy roots in different culture systems.

	Initial density	Final FW	Final DW	Specific growth rate	Doubling time
<b>CultiBag</b>	1 g l <sup>-1</sup>	147.6 g	8.35 g	0.157 d <sup>-1</sup>	4.4 d
<b>Erlenmeyer flask</b>	1 g l <sup>-1</sup>	48.5 g	1.43 g	0.149 d <sup>-1</sup>	4.6 d
<b>TIB</b>	1.25 g l <sup>-1</sup>	13.7 g	0.57 g	0.141 d <sup>-1</sup>	4.9 d



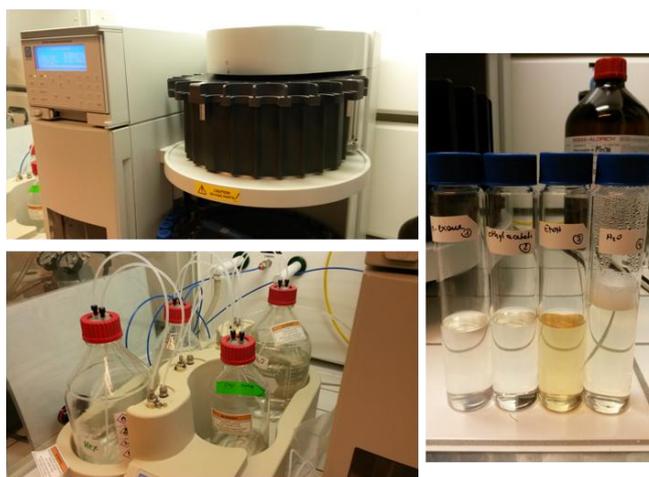
**Figure 31.** Scale-up of *Brassica rapa* subsp. *pekinensis* hairy roots production process in a CultiBag 10L. a) Online and offline measurement of medium culture parameters. b) Morphology of cultured hairy roots at different stages.

## Results

### 4.11 Optimized extraction by accelerated solvent extraction (ASE)

As reported previously, crude ethanol extract from hairy roots of *Brassica rapa* subsp. *pekinensis* exerted very promising anti-melanogenesis activities and involvement into skin ECM assembly.

To further investigate new crude extracts from this hairy root culture, it was possible at VTT to generate solvent partitioned fractions of *Brassica rapa* subsp. *pekinensis* hairy roots, through a relatively new technique, known as pressurized liquid extraction or accelerated solvent extraction (ASE). Whereas crude plant extracts contain compounds of different polarities, a sequential extraction procedure using *n*-hexane, ethyl acetate, ethanol and water was performed (**Fig. 32**).



**Figure 32.** Accelerated solvent extraction (ASE) system. Representative samples of *Brassica rapa* subsp. *pekinensis* extracted in 4 different solvents.

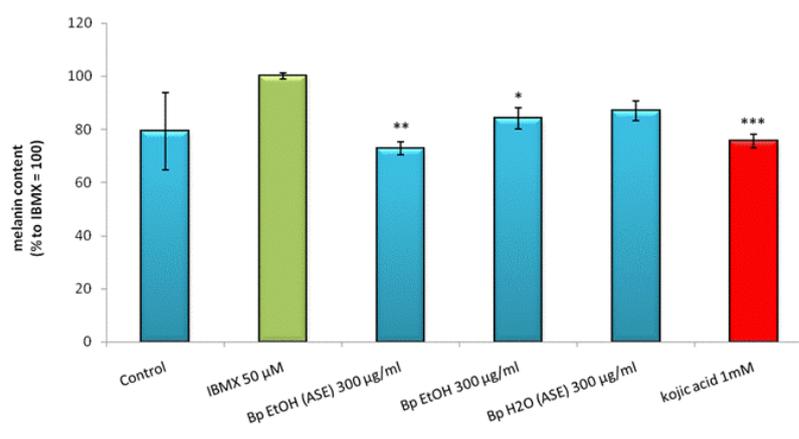
In this case freeze-dried material was used as substrate for extractions, and different parameters were observed and compared to those of standard procedure performed at Arterra Bioscience S.r.l., reported in **Table 2** (representative data of one experiment).

**Table 2.** Comparison between classic extraction and ASE procedure applied to *Brassica* samples. Only results of ethanol extractions are here shown.

	Arterra Bioscience	VTT (Finland)
Initial FW	50 g	50 g
EtOH used	200 ml	50 ml
Final DW	380 mg	1128 mg
Extraction time	~ 4 hours	~ 30 minutes

All the fractions were then collected and tested on B16-F1 cells in order to perform a preliminary analysis to measure melanin content after 3-day-treatments.

As shown in **Fig. 33**, ASE ethanol fraction from hairy roots of *Brassica rapa* subsp. *pekinensis* at the concentration of 300  $\mu\text{g ml}^{-1}$  decreased melanin content of about  $28.2 \pm 2.5\%$  against control cells stimulated with 50  $\mu\text{M}$  IBMX (\*\* $p < 0.01$ ). In addition, this new extract was also more effective than the classic EtOH extract used at the same concentration ( $16 \pm 3.9\%$  of reduction, \* $p < 0.05$ ). A non-significant reduction was observed after treatments with water fraction, while both *n*-hexane and ethyl acetate fractions have shown cytotoxic effects.



**Figure 33. Melanin content determination in B16-F1 cells.** Each fraction was analysed in triplicate in presence of 50  $\mu\text{M}$  IBMX. Bars represent the mean values  $\pm$  SD. ASE ethanol fraction of *Brassica* hairy roots has significantly (\*\* $p < 0.01$ , student's T test) reduced melanin content in murine melanoma cells compared to IBMX and the Et-OH extract obtained with the procedure available at Arterra srl.

#### 4.12 Cryopreservation

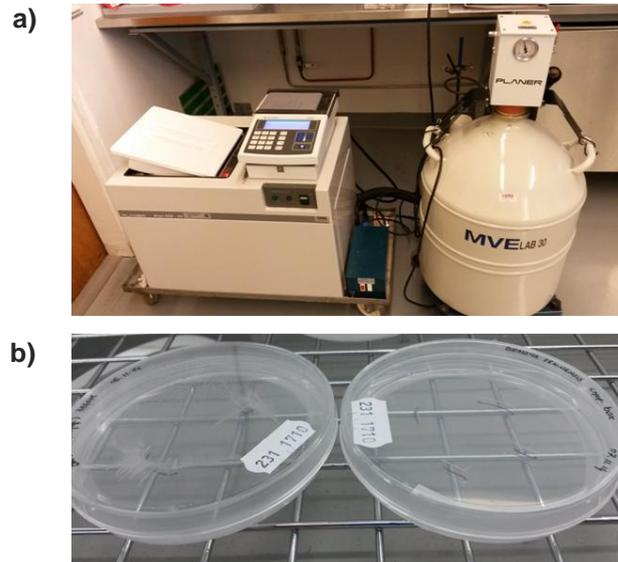
Cryopreservation is an increasingly important aspect of plant biotechnology, since, in the future it will be necessary to establish safe repositories for patented cultures of commercial interest.

At VTT, it was possible to investigate the effects of storage at ultra-low temperatures ( $-196\text{ }^{\circ}\text{C}$ ) on the subsequent growth of recovered *Brassica rapa* subsp. *pekinensis* hairy roots. Since DMSO has been reported to be toxic under certain conditions (Kartha, 1985), it was important to test its effects on the hairy root cultures. Such protective dehydration is an advantage when freezing vacuolated cells, but it can also lead to the toxic concentration of solutes in highly cytoplasmic meristematic tissues, such as root and shoot-tip meristems. For this reason *Brassica rapa* subsp. *pekinensis* roots were not pre-treated with sorbitol before protecting them with DMSO.

All experiments were based on replicate recovery plates containing 4-6 root-tips. For the assessment of short-term recovery, viability was assessed using fluorescein diacetate vital staining. Then, the cultures on solid medium were microscopically examined after 3-7 days for signs of early proliferation.

## Results

After 15 days the conversion of surviving root-tips to roots was monitored. As shown in **Fig. 34b**, the growth of root-tips with 10% (v/v) DMSO was unaffected by the cryoprotectant. However, as clearly shown in and **Table 3** there was no active proliferation of hairy root-tips recovered from liquid nitrogen storage, after different cooling programmes described in section 3.5.4. These unsatisfactory results pointed at the need to investigate, in the future, new procedures and treatments for plant cell and organ cultures cryopreservation.



**Figure 34. Cryopreservation of hairy root-tips.** a) Programmable freezer unit (PLANER Kryo 560 series). b) Active proliferation of *Brassica rapa* subsp. *pekinensis* hairy root-tips (left side) after protection with 10% DMSO, compared to 15-day-old recovered samples (right side) after cooling programme.

**Table 3. The effects of pre-growth, cryoprotection, rapid freezing, and post-freeze recovery** on the survival of root-tips excised from hairy roots of *Brassica rapa* subsp. *pekinensis*.

	Treatments		
	Control	DMSO	Frozen
Recovery media	SS	SS	SS
Viability (FDA test) (%)	100 (5)	100 (5)	74 ± 13.6 (7)
Proliferation (%)	100 (5)	100 (5)	2.3 ± 1.2 (7)
Conversion to roots (%)	100 (5)	100 (5)	None (15)

Values in parentheses are the number of days post-treatment.  
SS = semi-solid medium; error values = s.ds

**Discussion**



## 5. Discussion

Hyperpigmentation is the process by which an excess of melanin is produced by the skin. Typically, hyperpigmentation occurs as a result of stress, damage or prolonged inflammation of the skin. The most common cause is sun damage, though hyperpigmentation is often a consequence of inflammation following acne, eczema, psoriasis, dermatitis etc. Hyperpigmentation may also occur in the skin due to hormonal changes in the body typically associated with pregnancy or the taking of oral contraception. Beside these medical aspects, the global skin depigmenting product market has been forecast to reach a value of \$19.8 billion by 2018, driven by the growing desire for light-coloured skin among both men and women primarily from the Asian, African and Middle East regions. Although products do exist that can actually bleach the skin, these products contain dangerous or toxic ingredients (such as hydroquinone and mercury) and are banned in most countries.

Blocking or reducing the accumulation of melanin in the skin can be obtained either by switching off one or more components of the pathway that goes from the receptor activation to the enzymatic inhibition of melanin formation catalyzed by the tyrosinase. For this purpose, several anti-melanogenic reagents have been developed and discovered nowadays. However, only a few of these inhibitors have been introduced and used due to their problems in cytotoxicity (affecting the cell growth and survival), selectivity, solubility and stability.

Through a collaborative project between Arterra Bioscience S.r.l, Department of Pharmacy, University of Salerno and VTT (Finland), an industrial platform for the production and the extraction of ingredients from Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) hairy roots, to be used as active ingredients in cosmetic products as regulators of skin pigmentation and *basal lamina* assembly, was developed.

Previous studies at Arterra S.r.l have led to a patented extraction procedure (WO 2007104489) of an amino acid and peptide rich mixture obtained from plant cell cultures of *Nicotiana sylvestris*, containing small peptides and amino acids significantly effective as anti-ageing ingredients. In particular, proline, can activate different signaling pathways in human skin cells, leading to the up-regulation of specific ageing-associated genes and making cells more resistant to stress factors Apone *et al.* (2009). Additional studies from other groups have also demonstrated a beneficial effect of protein extracts and synthetic peptides on wound healing (Bentley *et al.*, 1990) and on the production of new collagen fibers (Katayama *et al.*, 1993).

Crude ethanol extract and a peptide/sugar mixture derived from cell wall from hairy roots of this species were tested in murine melanoma cells (B16-F1) and human epidermal melanocytes isolated from lightly pigmented adult skin (HEMa-LP) by using a panel of *in vitro* and *in vivo* biological assays to assess their role in modulating melanin content and tyrosinase activity, or their influence on cAMP levels and MITF (Microphthalmia Associated Transcription Factor) gene expression.

To our knowledge, no previous results are available on the effects of a sugar/peptide mixture from hairy roots of *Brassica rapa* subsp. *pekinensis* (BpPep), on melanogenesis. Our results have shown that this extract exerted a significant inhibitory effect against tyrosinase activity in murine melanoma cells (B16-F1), when used at concentration in the range of 2 to 10  $\mu\text{g ml}^{-1}$ , with a maximum reduction of  $51 \pm 6.7\%$ , compared to untreated cells. Furthermore, the same extract at the same concentration, reduces melanin content of about 20.5% in B16-F1, compared to IBMX-treated cells.

As reported by Eller *et al.* (2000) in B16-F1 cells treated with IBMX, increased melanogenesis is accompanied by increasing tyrosinase activity, protein and mRNA expression, which is similar to the delayed tanning response that occurs after UV

## Discussion

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radiation. Therefore, in this study we present evidence that BpPep extract effectively inhibited IBMX and forskolin-induced melanogenesis in B16-F1 cells, by inhibiting the enzymatic activity of tyrosinase.

In addition, when B16-F1 cells were treated with forskolin, an activator of adenylate cyclase, intracellular cAMP contents were significantly increased. However, the presence of sugar/peptide mixture of *Brassica rapa* susp. *pekinensis* hairy roots significantly inhibited the levels of cAMP, that is consistent with results observed after IBMX stimulation. Taken together, these results suggest that the mechanism of BpPep action could occur via a cAMP-dependent pathway.

Additional results have indicated that BpPep extract could be involved in a deep regulation of melanogenesis through the MITF-pathway. It is well known that protein kinase A (PKA), which is activated by various stimuli in melanocytes, induces the cAMP response element binding protein, which binds to the cAMP response element (CRE sequences) present in the promoter of the MITF gene. The increase in MITF expression then induces up-regulation of the tyrosinase gene family, which leads to increased melanin synthesis (Levy *et al.*, 2006). We have demonstrated that treatments with BpPep extract significantly decreased the expression of MITF mRNA and promoter activity of about 30%, suggesting that a sugar/peptide mixture isolated from cell wall of *Brassica rapa* subsp. *pekinensis* hairy roots exerted its inhibitory effect through the downregulation of MITF transcription.

Furthermore, an *in vitro* reporter assay (luc+) for an efficient and rapid screening of new plant-derived anti-melanogenesis compounds was also developed.

Different studies have accumulated considerable evidence that crude extracts from roots or whole plant thanks to their content in terpenoids, phenylpropanoids and methoxylated flavonoids, secondary metabolites involved in enhancing the natural defense response in the plant, have different and even contradictory roles on melanin production (Karim *et al.*, 2014; Pouillot *et al.*, 2011). Purified compounds, as calycosin from *Astragalus membranaceus* and hagin A from *Lespedeza cyrtobotrya* are two examples of root flavonoids produced which have been studied for their hypopigmentary effect in melanocytes (Kim *et al.*, 2009). Other examples are provided by the terpenoids glabridin, isoliquiritigenin, and glabrene, present in high amount in the licorice roots, which strongly inhibit the activity of *in vitro* recombinant tyrosinase (Nerya *et al.*, 2003).

As reported by Chan *et al.* (2011) *Sargassum polycystum* ethanolic extract from the whole plant and its fractions had little or no inhibitory effect on mushroom tyrosinase activity ( $12 \pm 1.2\%$  reduction). The authors observed that the ethanolic extract tested at concentration of  $100 \mu\text{g ml}^{-1}$  on cellular tyrosinase showed significant inhibition of enzyme activity ( $35 \pm 2.3\%$  reduction). Moreover, Choi (2013) reported that the methanolic extract of *Cyrtomium fortunei* J. Smith roots appeared to inhibit tyrosinase activity ( $34 \pm 4.3\%$  reduction) and melanin production ( $20.6 \pm 6.0\%$  reduction) in melanoma cells treated with  $100 \mu\text{g ml}^{-1}$  of the crude extract. Lv *et al.* (2007) observed that ethanolic extract of *Radix angelicae dahuricae* effectively inhibited isobutylmethylxanthine-induced melanogenesis in B16 melanoma cells, through inhibition of tyrosinase activity when used at dose of  $30 \mu\text{g ml}^{-1}$  (about 40% reduction).

In all the above-mentioned studies, total extracts from roots or whole plants were used in a dose range much higher than  $2\text{-}10 \mu\text{g ml}^{-1}$ , concentrations that we have demonstrated to be active in these studies. Thus, our findings prove that the peptide/sugar mixture from hairy roots of *Brassica rapa* subsp. *pekinensis* is able to regulate melanogenesis at very low concentrations, compared to other natural extracts reported in literature, and with less cytotoxic side effects that could limit their cosmetic application.

In addition to these findings, in the present study we have also evaluated the *in vitro* effects of *Brassica rapa* subsp. *pekinensis* hairy root extracts in modulating the gene expression of important genes involved in the extracellular matrix (ECM) and basal lamina assembly. In fact, several plant extracts used in cosmetics, besides their role as depigmenting products, have also been studied for their capacity to produce a final effect of bright and uniform skin. As reported by Seiberg (2011), non-denatured soybean extracts, their formulations and their products, are safe and effective in improving numerous skin care parameters. The multiple benefits of non-denatured soybean extracts for skin care range from skin lightening, extracellular matrix enhancement, to a protective effect from photodamage.

Despite both extracts from hairy roots of *Brassica rapa* subsp. *pekinensis* were assayed to confirm their involvement in these additional activities, our results provide evidence that only ethanol extract interfere positively with gene expression of lysyl oxidase (LOX gene) responsible of cross-linking of collagen and elastin, enhancing by 40% its expression in human dermal fibroblast (HDF cells), after treatments with  $20 \mu\text{g ml}^{-1}$  of the ethanol extract.

The same effects were observed in HaCaT cells, where the ethanol extract used at the same dose enhanced the gene expression of collagen type IV and laminins, both responsible for structural integrity of basement membranes, mechanical stability and for mediating their attachment to other components of the extracellular matrix.

The obtained effects in both cell lines could be related to the involvement of ethanol extract also in suppressing cAMP levels, as we have previously proved. In fact, as described by Schiller *et al.* (2010), increased intracellular levels of cAMP induced by artificial stimulators, as forskolin, negatively regulate the expression of different investigated ECM genes.

Altogether these findings suggest that both ethanol and sugar/peptide mixtures of *Brassica rapa* subsp. *pekinensis* hairy roots could synergically act in cosmetic formulations to achieve a final depigmenting and brightening effect.

Since safety remains the primary consideration for hypo-pigmenting agents, especially those used in cosmetic products, extracts derived from hairy roots of *Brassica rapa* subsp. *pekinensis* could replace efficiently the most widely employed molecules in the worldwide market (i.e arbutin, azelaic acid, kojic acid) that have some dangerous cytotoxic side effects that limit the range of their application.

Beyond biological characterization through different assays aimed to clarify the potential application in cosmetics of Chinese cabbage hairy root extracts, we also developed an efficient production process of *Brassica rapa* subsp. *pekinensis* hairy roots using disposable bioreactors (CultiBag and RITA<sup>®</sup>), and a time-saving extraction procedure to improve extraction. It has been possible also to compare the above-mentioned methods for scaling-up *in vitro* hairy root cultures with the classic method involving orbitally shaken flasks, in order to develop a competitive and innovative production platform with industrial application.

The key challenges toward commercial exploitation of new plant-derived actives are represented by the possibility to scale-up the culture biomass, to reduce heterogenic morphology of hairy roots, as well as their irregular growth and their shear stress sensitivity in bioreactors.

Currently, there are few examples of scaled-up plant tissue culture to replace the production of a specific compound earlier extracted from plant material. A benchtop-scale process of *Nicotiana tabacum* L. cv. Petit Havana SR1 hairy roots was developed in a 20-l wave-mixed bioreactor yielding hundreds of grams of biomass and milligram quantities of geraniol per cultivation bag (Ritala *et al.*, 2014) The performance and ginsenoside production of *Panax ginseng* hairy roots in 2-l wave bioreactors have been studied in detail by Palazon *et al.* (2003). The results showed

## Discussion

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that both biomass accumulation and ginsenoside production were significantly higher in 2-l CultiBag than in shaken flasks. Large-scale wave systems with capacity up to 600 l are now commercially available (source: Wave Biotech AG<sup>®</sup>, Tagelswangen, Switzerland).

Concerning our studies, through the use of wave-mixed bags (CultiBag) it has been possible to take advantages from remote and sensitive control of several important parameters such as temperature, rocking angle, pH, conductivity and pO<sub>2</sub>. All these improvements resulted in a massive biomass production of *Brassica rapa* subsp. *pekinensis* hairy roots about 1.7-fold higher than that obtained in shaken flask system (4.8 g l<sup>-1</sup> d<sup>-1</sup> versus 2.9 g l<sup>-1</sup> d<sup>-1</sup>).

Furthermore, temporary immersion systems (RITA<sup>®</sup>) have also been described as an alternative method for cultivating hairy root cultures from *Beta vulgaris* (Pavlov, 2003) and for secoiridoid glycosides production by *Centaurium maritimum* hairy root cultures. Although the RITA<sup>®</sup> systems have been developed for plant *in vitro* propagation, their advantages make them attractive because seem to be able to solve hyperhydration problem associated with the bioreactor cultivation of transformed root cultures.

In parallel, the results achieved from comparison between temporary immersion bioreactors (RITA<sup>®</sup>) and shaken flasks proved that there was a slight difference in term of biomass productivity when hairy roots were cultured in bioreactors (2.3 g l<sup>-1</sup> d<sup>-1</sup> versus 2.9 g l<sup>-1</sup> d<sup>-1</sup>). Even though a lower productivity, the temporary immersion system can offer some advantages related to low cost of bioreactors, reduced consumption of culture media, less manipulation of fast growing biomass and, as a consequence, lower incidence of transversal contaminations.

A relatively new technique, known as pressurized liquid extraction or accelerated solvent extraction (ASE), has been also tested here as an improved exhaustive extraction method that requires only small volumes of solvents and permits faster and thorough extraction of compounds. In our experiments, the effects of changing temperature and pressure were investigated on the extraction efficiency, because it is known that a certain pressure is needed to maintain the solvents as liquid above their boiling points and allow compounds that are blocked in pores to be more rapidly extracted than it is possible at room temperature and atmospheric pressure.

We established that using this innovative extraction procedure it is possible to drastically reduce solvent (EtOH) volumes by 4-fold compared to the standard EtOH extraction procedure. In addition, extraction time was significantly reduced from 4 hours to 30 minutes and an increase of about 3-fold in extraction yields was obtained, starting from the same fresh weight of hairy roots of *Brassica rapa* subsp. *pekinensis*.

The above-mentioned results, together with the previous evidences in term of biomass generation, provided the basis for setting up a competitive industrial production platform to be applied to hairy root cultures of our interest.

Above all, results gained from the present PhD project are of crucial importance for Arterra Bioscience S.r.l in order to introduce crucial technical innovations in the pipeline of developing and commercializing plant-derived active ingredients for skin care market.

## **Conclusions**



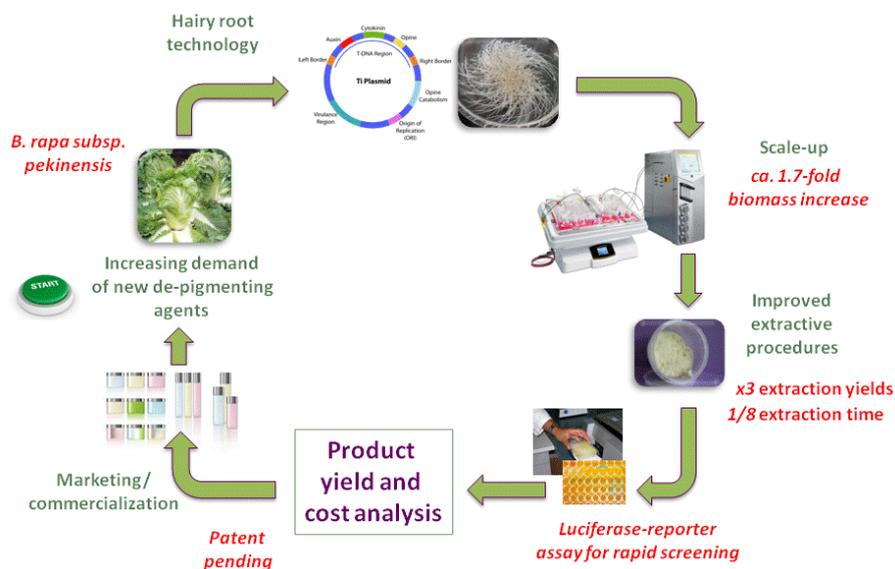
## 6. Conclusions

The present project was aimed at identifying new plant-derived extracts exerting beneficial effects in skin care, with special emphasis on the development of novel plant-derived actives with hypopigmenting effects.

The main results achieved, summarized in **Fig. 35**, were:

- generation of a stable and actively growing *Brassica rapa* subsp. *pekinensis* hairy root culture;
- detailed characterization of crude ethanol extracts and sugar/peptides mixtures of *Brassica rapa* subsp. *pekinensis* hairy roots, for their activity in regulating melanogenesis pathway and skin extracellular matrix (ECM) components assembly, through a panel of complementary biological assays;
- development of a rapid screening of new melanogenesis inhibitors, by a luciferase-reporter assay system, measuring the MITF-promoter activation;
- implementation of a competitive industrial hairy-root based production platform, by biomass scaling-up and improved extraction procedures.

Overall, these results, under pending patent application, will contribute to introduce product and process innovations at Arterra Bioscience s.r.l., for the identification of new and safer plant-derived melanogenesis inhibitors. In general, the developed industrial production platform will be also extended to the screening of actives from other plant species and to the release of novel plant-derived products in different segments of the cosmetic market.



**Figure 35.** Flow chart of the hairy root-based innovative platform for plant-derived anti-melanogenesis active ingredients production.



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